Nos. 19-2255, -2285

IN THE

United States Court of Appeals for the Federal Circuit

BIO-RAD LABORATORIES, INC., THE UNIVERSITY OF CHICAGO,
Plaintiffs-Appellees,

v.

10X GENOMICS INC.,
Defendant-Appellant.

On Appeal from the United States District Court
for the District of Delaware
No. 1:15-cv-00152-RGA, Hon. Richard G. Andrews

OPENING BRIEF AND ADDENDUM OF 10X GENOMICS INC.

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U.S. Patent No. 8,889,083: Claim 1

1. A microfluidic system comprising:

a non-fluorinated microchannel;

a carrier fluid comprising a fluorinated oil and a fluorinated surfactant comprising a hydrophilic head group in the microchannel;

at least one plug comprising an aqueous plug-fluid in the microchannel and substantially encased by the carrier-fluid, wherein the fluorinated surfactant is present at a concentration such that surface tension at the plug-fluid/microchannel wall interface is higher than surface tension at the plug-fluid/carrier fluid interface.

U.S. Patent No. 8,329,407: Claim 1

1. A method for conducting a reaction in plugs in a microfluidic system, comprising the steps of:

providing the microfluidic system comprising at least two channels having at least one junction;

continuously flowing an aqueous fluid containing at least one biological molecule and at least one reagent for conducting the reaction between the biological molecule and the at least one reagent through a first channel of the at least two channels;

continuously flowing a carrier fluid immiscible with the aqueous fluid through the second channel of the at least two channels;

forming at least one plug of the aqueous fluid containing the at least one biological molecule and the at least one reagent by partitioning the aqueous fluid with the flowing immiscible carrier fluid at the junction of the at least two channels, the plug being substantially surrounded by the immiscible carrier fluid flowing through the channel, wherein the at least one plug comprises at least one biological molecule and the at least one reagent for conducting the reaction with the at least one biological molecule; and

providing conditions suitable for the reaction in the at least one plug involving the at least one biological molecule and the at least one reagent to form a reaction product.

FORM 9. Certificate of Interest **Form 9**
Rev. 10/17

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

Bio-Rad Laboratories, Inc. **v.** 10X Genomics, Inc.

Case No. 19-2255, 19-2285

CERTIFICATE OF INTEREST

Counsel for the:
☐ (petitioner) ■ (appellant) ☐ (respondent) ☐ (appellee) ☐ (amicus) ☐ (name of party)

10X Genomics, Inc.

certifies the following (use "None" if applicable; use extra sheets if necessary):

1. Full Name of Party Represented by me	2. Name of Real Party in interest (Please only include any real party in interest NOT identified in Question 3) represented by me is:	3. Parent corporations and publicly held companies that own 10% or more of stock in the party
10X Genomics, Inc.	None	None

4. The names of all law firms and the partners or associates that appeared for the party or amicus now represented by me in the trial court or agency or are expected to appear in this court **(and who have not or will not enter an appearance in this case)** are:

Ashby & Geddes: Steven J. Balick, Andrew Colin Mayo
Irell & Manella LLP: David I. Gindler, Andrei Iancu, Lindsay A. Kelly, Lauren N. Drake, Elizabeth C. Tuan, Michael H. Strub, Dennis Courtney
Paul, Weiss, Rifkind, Wharton & Garrison LLP: Nicholas Groombridge, David J. Ball, Jennifer H. Wu, Josephine Young, Jennifer R. Deneault, Simone Park
Richards, Layton & Finger, PA: Frederick L. Cottrell, III, Alexandra Ewing, Jason James Rawnsley
Tensegrity Law Group LLP: Daniel Radke

FORM 9. Certificate of Interest **Form 9**
Rev. 10/17

5. The title and number of any case known to counsel to be pending in this or any other court or agency that will directly affect or be directly affected by this court's decision in the pending appeal. *See* Fed. Cir. R. 47. 4(a)(5) and 47.5(b). (The parties should attach continuation pages as necessary).

None.

10/18/2019
Date

/s/ E. Joshua Rosenkranz
Signature of counsel

Please Note: All questions must be answered

E. Joshua Rosenkranz
Printed name of counsel

cc: Counsel of Record

Reset Fields

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STATEMENT OF RELATED CASES

No appeal in or from the same civil action was previously before this or any other appellate court.

Counsel is not aware of any case pending in this or any other court or agency that will directly affect or be directly affected by this Court's decision in the pending appeal.

INTRODUCTION

Defendant-Appellant 10x Genomics Inc. has pioneered a revolution in modern biology, called "single-cell technology." This technology gives scientists unprecedented visibility into genes and genetic activity *in individual cells*. *Science* magazine named it the 2018 "Breakthrough of the Year." Researchers are using 10x's diverse suite of products to make fundamental discoveries in cancer and immunology. In just the four-and-a-half years after the launch of 10x's first product, 500+ peer-reviewed scientific articles had attributed discoveries to 10x products. No other company offers the same quality and breadth of single-cell products as 10x.

Certainly not Plaintiff-Appellee Bio-Rad Laboratories, Inc. It started trying to develop a single-cell product around the same time as 10x. Its efforts largely failed. Bio-Rad's single-cell product simply does not meet researchers' needs.

Bio-Rad's product failed in the lab and the marketplace, so Bio-Rad resorted to the courts. Bio-Rad bought itself a patent portfolio and an existing lawsuit to try to shut 10x down. The patents have nothing to do with single-cell technology, and do not even address the many

knotty problems with making that technology a success. Yet, Bio-Rad secured an infringement verdict, a whopping 15% royalty, and an injunction—each marred by legal error. This Court should reverse the verdict and vacate the injunction.

JURISDICTIONAL STATEMENT

The district court had jurisdiction under 28 U.S.C. §§ 1331 and 1338. The court entered a permanent injunction on August 14 and final judgment on August 15, 2019. Appx39-40; Appx41-47. 10x timely filed a notice of appeal on August 16, 2019. Appx31134-31135; *see also* Appx31101-31102. This Court has jurisdiction under 28 U.S.C. §§ 1292(c)(1) and 1295(a)(1).

STATEMENT OF THE ISSUES

1. Is Bio-Rad barred as a matter of law from asserting infringement under the doctrine of equivalents where it amended its claims to exclude the very terrain it now accuses, and where the equivalent (consisting of a fluorinated material) vitiates a claim limitation (requiring a "non-fluorinated" one)?

2. Did Bio-Rad fail to prove literal infringement as a matter of law, where another limitation of the same patent requires an "interface"

between two surfaces and Bio-Rad concedes those two surfaces never touch in 10x's products?

3. Did the district court err in construing a preamble as non-limiting, where the body of the claim references the preamble as an antecedent, the specification repeatedly recites the limitation emphasized by the preamble, and the examiner added the language in question to the preamble?

4. Where a damages expert relies on licenses that are not comparable to the hypothetical negotiation and fails to apportion damages to the value of the patented technology, must the damages award be vacated?

5. Where Bio-Rad never claimed that its products competed with four of 10x's five product lines, and its products do not meaningfully compete on the fifth, did the district court abuse its discretion in enjoining all five lines?

STATEMENT OF THE CASE

Single-Cell Technology Is Revolutionizing Life-Sciences Research

10x is leading the single-cell arena, which gives scientists unprecedented visibility into genetic activity at the level of an

individual cell. 10x's tools enable researchers to access and analyze enormous volumes of information at a cell-by-cell resolution never before possible. These tools are yielding great strides in our understanding of how diseases like cancer and autoimmune disorders develop—and how they can be cured. Appx28502-28511; Appx28885-28915; *see* Dkt. 9.4-9.8.[1] This technology promises to uncover transformational biological discoveries.

The basics are familiar: Cells are the building blocks of all organisms. The human body contains 40 trillion cells. Appx30198. Each cell houses a copy of our DNA, the genetic code consisting of about three billion base pairs (A-T and G-C). Appx15717; Appx30159. Different portions of that DNA can be translated into strands of RNA, containing instructions for building proteins. Appx30520-30521. Proteins are the body's machinery, ranging from enzymes to antibodies to structural components of bone and muscles. Appx30520-30521. Each cell in an organism typically contains the same DNA. Appx30162-30163. But cells behave differently depending on differences in gene

[1] "Dkt. __" refers to the docket in Fed. Cir. No. 19-2285.

expression (which genes are "turned on"). Appx30162-30163; Appx30520-30521. Cells can also behave differently when their DNA mutates—making those cells different from other cells in the body. Appx15719-15721. These factors dictate whether a cell develops into a skin cell or a liver cell, or whether a cell is healthy or diseased. Appx15719-15721; Appx30162-30163.

Before 10x developed its single-cell technology, scientists could manually isolate an individual cell and test its responses to various stimuli, Appx28903-28905; Dkt. 9-7(¶9, ¶11), or they could extract and analyze genetic material or proteins in bulk from thousands of cells, Appx16106; Appx16143-16144. What they could not do was simultaneously analyze and compare the DNA, RNA, and proteins within vast numbers of individual cells. Appx30159-30160.

Single-cell technology changed that. It can be used, for example, to study which genes are expressed in cancer cells versus normal cells, to determine whether cancer cells contain certain mutations that respond to specific treatments, or to detect when a cancer cell develops a new mutation. Appx30148-30150. 10x's products even enabled scientists to discover a previously unknown type of lung cell believed to

be responsible for cystic fibrosis, leading to hopes for treating this deadly disease. Appx30199-30200. Scientists around the globe use 10x products to study cancer cells, Appx28886-28888; Appx28892; Appx28897; Appx28902-28911; primary human blood cells, Appx28890; and the genetic underpinnings of inherited and infectious diseases, Appx28894-28895; Appx28913-28915. The record is replete with world-renowned scientists' testimonials that 10x's products fuel discoveries that no other product on the market can achieve. Appx28849-28860; Appx28863-28866; Appx28877-28915. These are the sorts of discoveries that led *Science* magazine to name single-cell technology the 2018 "Breakthrough of the Year." Elizabeth Pennisi, *Development Cell by Cell*, Science, Dec. 2018, https://vis.sciencemag.org/breakthrough2018/finalists/#cell-development.

10x Is Founded By Scientists Devoted To Innovation

10x's founders—Drs. Ben Hindson, Kevin Ness, and Serge Saxonov—are serial life-sciences entrepreneurs. They founded a company called QuantaLife, which formed in 2008. Appx30150-30151. QuantaLife did not develop a single-cell product. It developed a product that performs an improved version of a chemical reaction called

polymerase chain reaction ("PCR"). Appx30151. PCR is a Nobel Prize-winning reaction developed in the 1980s. Appx30576. PCR makes many copies of a specified segment of DNA—for example, a particular cancer mutation—to make it easier to detect. Appx16104-16105; Appx29497-29498. QuantaLife's product, called droplet digital PCR ("ddPCR"), separated strands of DNA into tiny droplets of water, and then amplified the DNA to obtain highly accurate measurements. Appx29578-29579; Appx32795.

Bio-Rad saw ddPCR as a "big opportunity" and bought QuantaLife in 2011. Appx29570-29571. Bio-Rad is a decades-old behemoth that sells 9,000+ different life-sciences products. Appx29564-29565; Appx66. Upon the acquisition, the QuantaLife team joined Bio-Rad. Appx29570-29571. The QuantaLife scientists helped Bio-Rad integrate and market ddPCR. Appx29578; Appx30153-30154. To this day, that product remains a "great business" for Bio-Rad, to the tune of hundreds of millions of dollars. Appx29576-29579.

Despite ddPCR's success, this was not a happy marriage. QuantaLife's founders were eager to continue developing other innovations. Appx30154-30155. Bio-Rad was not supportive.

Appx30153-30155. So the founders left Bio-Rad in 2012. Appx30156-30158.

10x Develops Groundbreaking Research Tools To Unlock Cells

The QuantaLife founders formed a new company, 10x. Appx30156-30158. There, they developed a series of transformative inventions that are incorporated into the five product lines at issue in this litigation. None of them involves PCR in droplets. Appx30254. Indeed, they are the technological opposites: ddPCR's purpose is "to make all the droplets the same," but 10x's goal "is to make every reaction different." Appx30254. 10x's specialized reactions are "what enable[] the applications that [10x's] customers … and the rest of the world are really excited about." Appx30254.

10x's first product line, now called "Linked-Reads," applied a new approach to sequencing long chains of DNA. Appx28849; Appx30158-30159. 10x's next four products—called the "Single Cell" products—allow scientists to study the contents of hundreds of thousands of individual cells simultaneously. Appx28849-28852. As described more fully below, each product line "provides fundamentally different biological information." Appx28848-28852.

Each product line runs on the same piece of hardware, a "controller" that can cost upwards of $100,000. Appx30615; Appx33743-33744. And each relies on a specialized "microfluidic chip" on which the sample is separated into individual cells (or strands of DNA in Linked-Reads). Appx30167. The single-use chips are about 4" x 2" and look like this:

Appx33353; *see* Appx33745. Etched into each chip is a network of "microfluidic channels," each about the width of a human hair, through which cells and fluids will flow. Appx30167-30168. For each product line, 10x also developed specialized reagents—also single-use—including customized enzymes and chemicals. Appx28848-28849; Appx30175-30177; Appx30181-30186. A researcher loads the sample

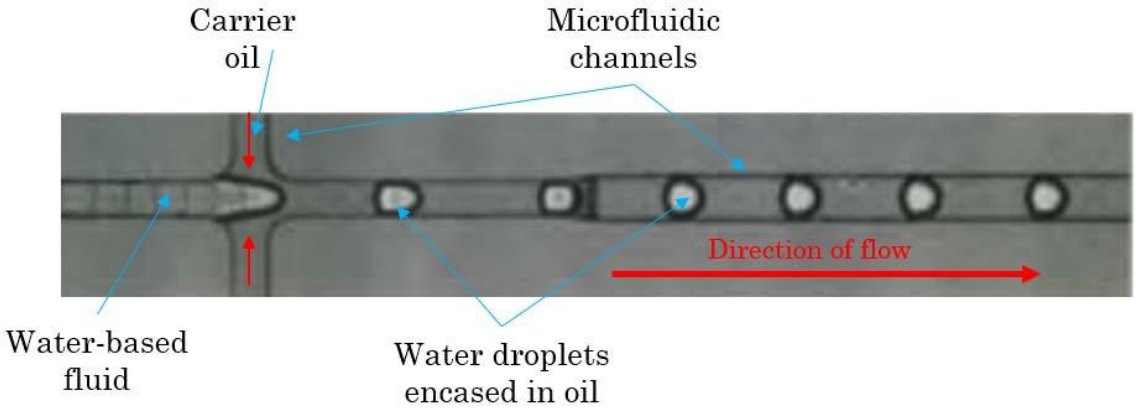
and the reagents onto the chip, Appx30167, and then loads the chip into the controller as shown below:

Appx15657 (labels added).

To study a biological sample at a single-cell level, the first step is to place each cell into its own container or "partition." Appx30162-30163. Each partition is like a tiny test tube designed to hold a single cell and the reagents used to analyze the cell. There are several ways to partition samples. A researcher can use droplets, microwells, or microcapsules, or even manually isolate cells. Appx15661-15667; Appx29650-29651. 10x's accused products use droplets—tiny volumes of water suspended in oil.

These droplets are like the droplets of vinegar that form in an oil-based salad dressing—only much tinier and more uniform. Appx29489;

Appx29568. Each droplet is about 100 picoliters of liquid (100 trillionths of a liter) that travels in a carrier liquid made of oil. Appx15245-15246. In the accused products, the droplets form at the junctions of the channels of the microfluidic chip, where a stream of water-based fluid meets a stream of oil-based fluid, as shown in this highly magnified photo:



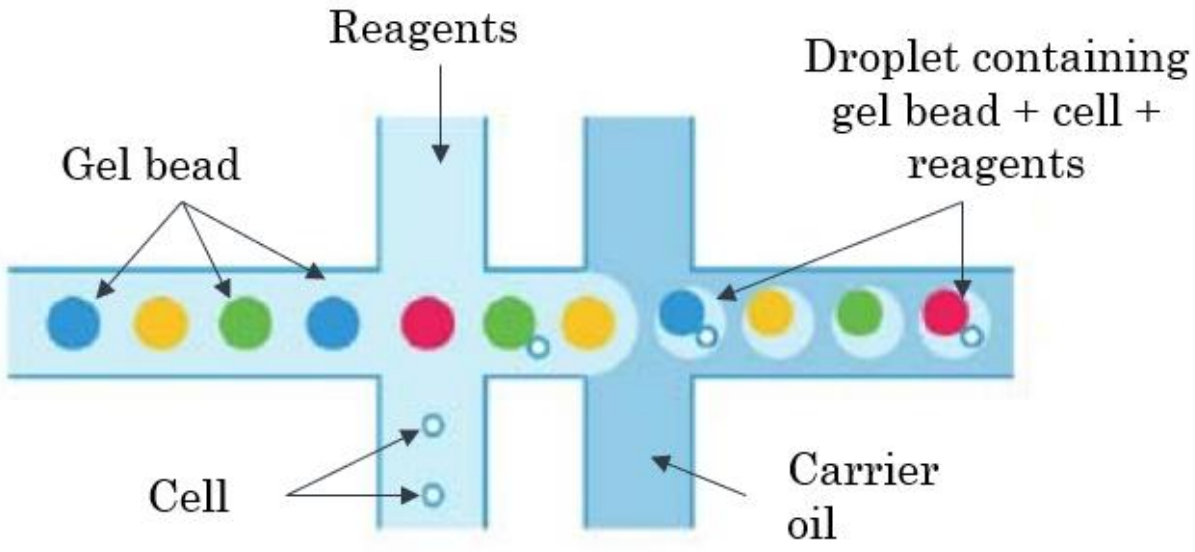
Appx15173-15174 (annotated). A tiny volume of water is pinched off into a droplet and carried along by the oil. Appx30420; Appx30429. The controller regulates this process, pushing the water-based reagents and the carrier oil through the chip to form droplets. Appx30167-30168.

The mechanics of droplet formation are not new; it was known more than 10 years before 10x was founded and before the asserted patents were filed. *Infra* 20-21; Appx254 1:40-43; Appx29695-29696; Appx30412. 10x achieved success by devising an innovative system

that can simultaneously analyze the contents of hundreds of thousands of individual cells. Appx30254.

A key to 10x's success is distinguishing one droplet from another, and one cell from another, using a proprietary microscopic "gel bead." Appx30161; Appx30174-30177. They are dissolvable polyacrylamide beads containing a tiny genetic "barcode." Appx30169; Appx30177; Appx30583. A barcode is a short strand of synthetic DNA with a unique sequence. Appx30161. Each bead contains millions of copies of its own unique barcode. Appx30161.

As illustrated below, 10x's instrument pairs a unique barcoded gel bead (colored balls) with a single cell (small blue circles) and mixes them with the requisite reagents (light blue fluid). It then harnesses the old droplet technology to separate each bead/cell/reagent combination into its own droplet by merging that stream into a stream of carrier oil:

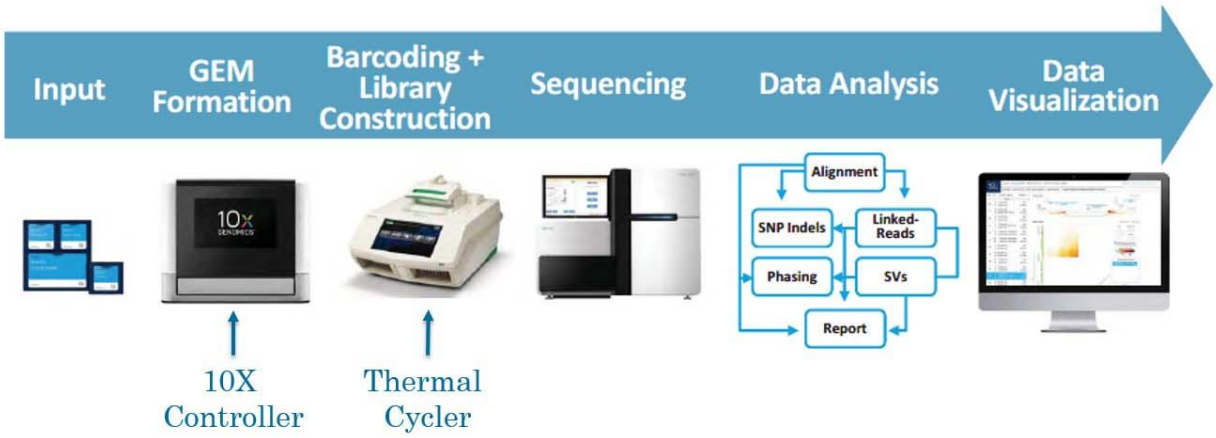


Appx15470 (labels added).

After the controller creates the droplets containing a gel bead, cell, and reagents, a researcher removes the droplets from the microfluidic chip and places them into a standard laboratory instrument called a thermal cycler. Appx30169-30170. At this point, each cell is still intact and the reagents have not started interacting with the cell. Appx30170. The thermal cycler is a highly accurate, rapid hotplate. Appx30170. As the thermal cycler heats the droplets, the gel bead dissolves (releasing the DNA barcodes), and 10x's specialized reagents break down the cell. Appx30170. Then, through 10x's proprietary chemistry, the unique barcode from the bead attaches to the genetic material in the cell (DNA

or RNA, depending on the specific product)—adding a label to the cell's genetic material. Appx30170. Throughout this process, the droplets must stay intact to prevent the barcodes in different droplets from mixing. Appx30182. Once all the genetic material is labeled with a barcode, the droplets can be broken.

Then the researcher uses another piece of standard lab equipment, a DNA sequencer, to "read" both the barcode and each cell's genetic material. Appx30170-30171. Finally, 10x's proprietary software helps scientists understand the "ton[s] of data" from the sequencer. Appx30171-30174. This schematic shows 10x's workflow:



Appx28924 (labels underneath added).

Adapting the pre-existing microfluidic technology to this single-cell approach was "a really, really hard challenge." Appx30373. 10x invented "whole new classes of chemistry" and "mathematic …

algorithms," Appx30373, while working out of a founder's garage, Appx30369-30370. Here are some of the problems 10x solved:

1. How to reliably pair a single bead with a single cell, without creating "doublets"—two cells paired with one bead, which "confus[es]" the data. Appx30174-30175; *see also* Appx28926-28927; Appx30178-30181; Appx30195-30196.

2. How to conduct the pairing without wasting too many cells, which often come from "precious" small samples, such as a biopsy from a tumor in a patient's breast. Appx30175; Appx30179; Appx28886-28888.

3. How to prevent these solid materials—beads and cells—from clogging the chip. Appx30178; Appx30490-30491.

4. How to improve special chemicals—called surfactants—to keep the droplets from merging when removed from the chip, the way vinegar droplets in salad dressing constantly break apart, merge together, and form different droplets. Appx30175; Appx30181-30185; Appx30524-30525.

5. How to develop computer software that could process and visually present the huge volume of data its single-cell technology produces, a challenge that required "some of the best minds" in "firmware, mechanical engineering software[,] [and] [c]omputational biology." Appx30373; *see* Appx30171.

To date, 10x has devoted over $250 million just to crack these technological puzzles. Dkt. 9-4(¶3).

Each of 10x's product lines uses the same basic workflow but is designed to analyze different aspects of a cell, Appx28849-28850; Appx30164, as follows:

- ***Single Cell 3' Gene Expression***: analyzes cells' RNA to determine which genes are turned on and off. Appx28851-28852; Appx28855-28857.
- ***Single Cell V(D)J***: analyzes immune receptor genes. Appx28849-28850; Appx28853.
- ***Single Cell ATAC-seq***: analyzes how a cell regulates gene expression. Appx28850-28851; Appx28853-28854.
- ***Single Cell CNV***: measures mutations between cells and across the genome. Appx28851; Appx28854-28855.
- ***Linked-Reads:*** uses barcodes to label individual chains of DNA (~65,000 base-pairs). Appx15466-15468; Appx28849.

There was no evidence that any other company offers products that perform the functions of any of 10x's product lines, except one: Single Cell 3′. About 10 other companies offer products that perform some of the same functions as that one product line. Among them are Fluidigm, Takara, and Becton Dickinson. Appx30192; Appx35327.

After Losing In The Lab And The Marketplace, Bio-Rad Wages A Litigation War Against 10x

While 10x was solving all these technological challenges and releasing product after innovative product to scientific acclaim, Bio-Rad was spinning its wheels. The juxtaposition is stark. In 2012, the same year 10x was founded, Bio-Rad began a project to adapt known microfluidic technology to single-cell analysis. Appx16136; Appx30157-30158.

It took Bio-Rad five years to launch the resulting product as "ddSEQ." Appx30191. It was hardly worth the wait. ddSEQ was an abysmal failure. Appx29645-29647; Appx30522-30523; *see* Appx24738-24739 (fewer than 75 instruments sold). It performed the same functions as just one of 10x's products (Single Cell 3′), and researchers publicly panned it as having "very serious issues" making it "unsuitable," "completely inadequate," and "inferior to the point that it is unusable" for their work. Appx28885-28888; Appx28891-28892; 28896-28897.

Indeed, the undisputed record is that 10x's technology is far superior, Appx28931:

- 10x can isolate almost 70 times more cells than Bio-Rad in a single run. Appx30194-30195.
- 10x wastes far less of a precious sample—with an overall yield of useable data from 65% of cells versus Bio-Rad's 5-10%. Appx30195; Appx30569-30570.
- 10x creates far fewer "doublets"—less than 1% of droplets in 10x's products will have two cells, compared to more than 5% for Bio-Rad. Appx30195-30196.

By Bio-Rad's own admission, 10x's single-cell technology is "much, much more successful," Appx29652, and it performs "substantially better" on a key metric that "customers care about," Appx29643. Bio-

Rad does not dispute that none of its products even purported to perform the functions of 10x's other four product lines. Yet, Bio-Rad blamed 10x for its own technological and marketplace failures and launched a litigation war against 10x.

In this litigation campaign, Bio-Rad has cast blame in the form of two inconsistent themes. First, it claims that 10x's founders sabotaged Bio-Rad's product development by leaving the unhappy marriage and applying their expertise to a new venture. Bio-Rad unsuccessfully leveled those allegations in an earlier litigation, asserting that 10x's founders had breached their employment agreements with Bio-Rad and misappropriated Bio-Rad's trade secrets. *See Bio-Rad Labs., Inc. v. 10X Techs., Inc.*, No. MSC-14-01751 (Cal. Super. Ct. Sept. 22, 2014). An arbitrator flatly rejected Bio-Rad's contract claims, *see* Appx28989-29072, and Bio-Rad then dismissed the related trade secrets claim, *see* No. MSC-14-01751 (Cal. Super. Ct. June 13, 2017) (order dismissing case).

Bio-Rad's second theme was that 10x beat it in the marketplace only "because 10x had a really, really big head start, frankly we felt using *our technology*." Appx29581 (emphasis added). This theme is

even more unfounded—as is evident from a glance at the timeline below (at 24). When Bio-Rad and 10x embarked on their respective single-cell ventures, *neither* had rights to the patents that Bio-Rad now asserts. Bio-Rad did not have these patent rights during the entire five-year period (2012-2017) in which it was developing its only single-cell product, ddSEQ. *Supra* 17. Bio-Rad did not have these rights when 10x introduced its Linked-Reads product in 2015, or its first single-cell product in 2016.

Bio-Rad first acquired these patent rights in early 2017. It acquired them by purchasing a failed start-up called RainDance. Appx29573-29574. It was RainDance that filed this lawsuit against 10x in early 2015. Appx482-501. Bio-Rad bought RainDance, taking over as plaintiff. Appx9942-9943; Appx29620.

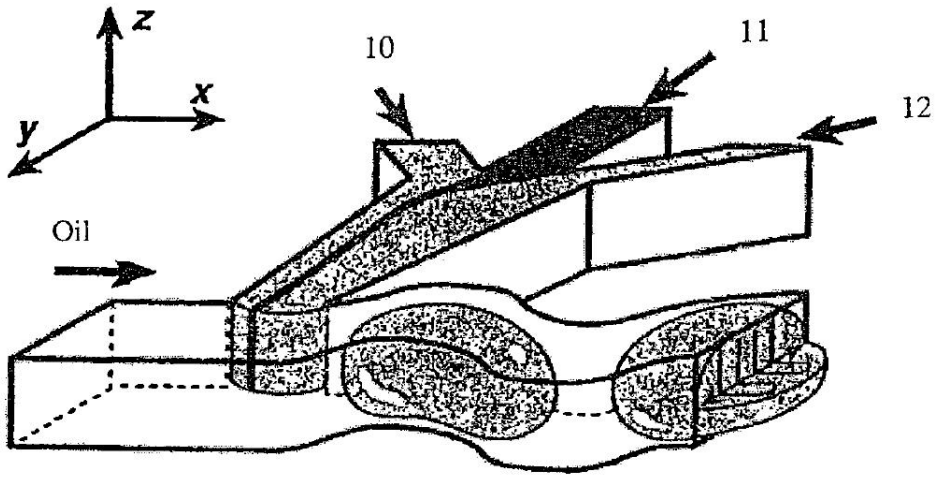
The Asserted Patents Concern Details Of Specific Components In A Microfluidic System

RainDance had an exclusive license to the patents asserted here: a family of patents issued to Dr. Rustem Ismagilov and assigned to the University of Chicago (co-plaintiff here). Appx31847-31863. The complaint asserted six patents, three of which ultimately went to the jury: U.S. Patent Nos. 8,889,083; 8,304,193; and 8,329,407 (collectively,

"the Ismagilov patents"). Appx371-379. The Ismagilov patents claim systems and methods for forming droplets (which they call "plugs") within microchannels on a microfluidic chip.[2] They contemplate using those droplets for performing generic chemical reactions. They do not teach anything about single-cell analysis.

Figure 10A illustrates the basics: three fluids containing reagents (10, 11, 12) are introduced into a channel containing carrier oil, and the mixture forms plugs (the jellybeans):

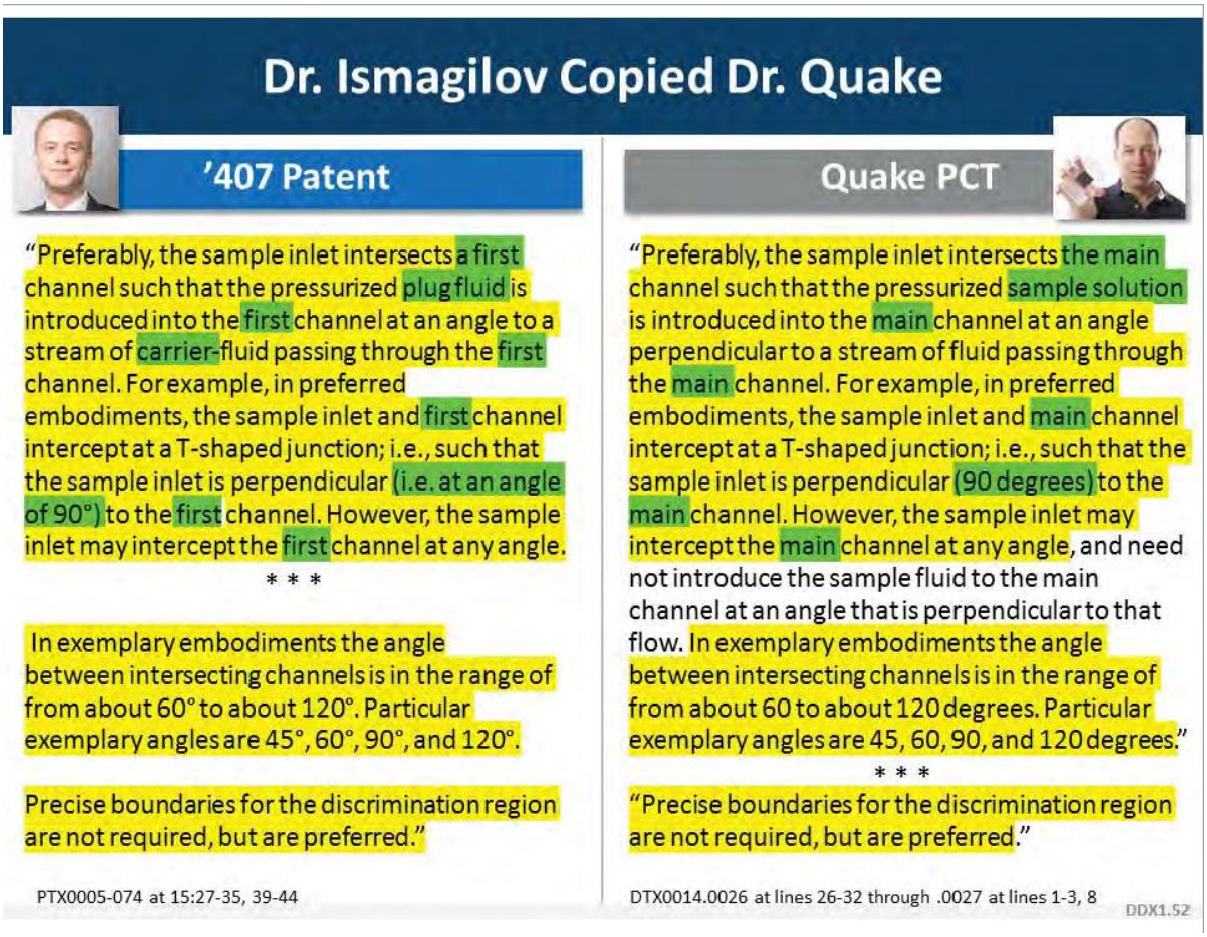
FIG. 10A



Appx202 (Fig. 10A); Appx266 26:55-63; Appx29665-29666. The prior art already taught how to make droplets by introducing water-based

[2] The Ismagilov patents largely share the same specification; where there is no meaningful difference, we cite to the '407 patent.

fluids into a stream of carrier oil. Appx254 1:40-43; Appx29695-29696. Indeed, Dr. Ismagilov copied huge swaths of his specification—well beyond background information—almost verbatim from a prior art patent by Dr. Stephen Quake, including passages on droplet formation, surfactants, and analysis of droplets. Appx25247-25249; Appx25269-25359 (comparison of Ismagilov provisional to Quake); Appx25489-25594 (comparison of issued '407 patent to Quake); Appx29759-29760. Here is a representative example of the copying:



Appx28975.

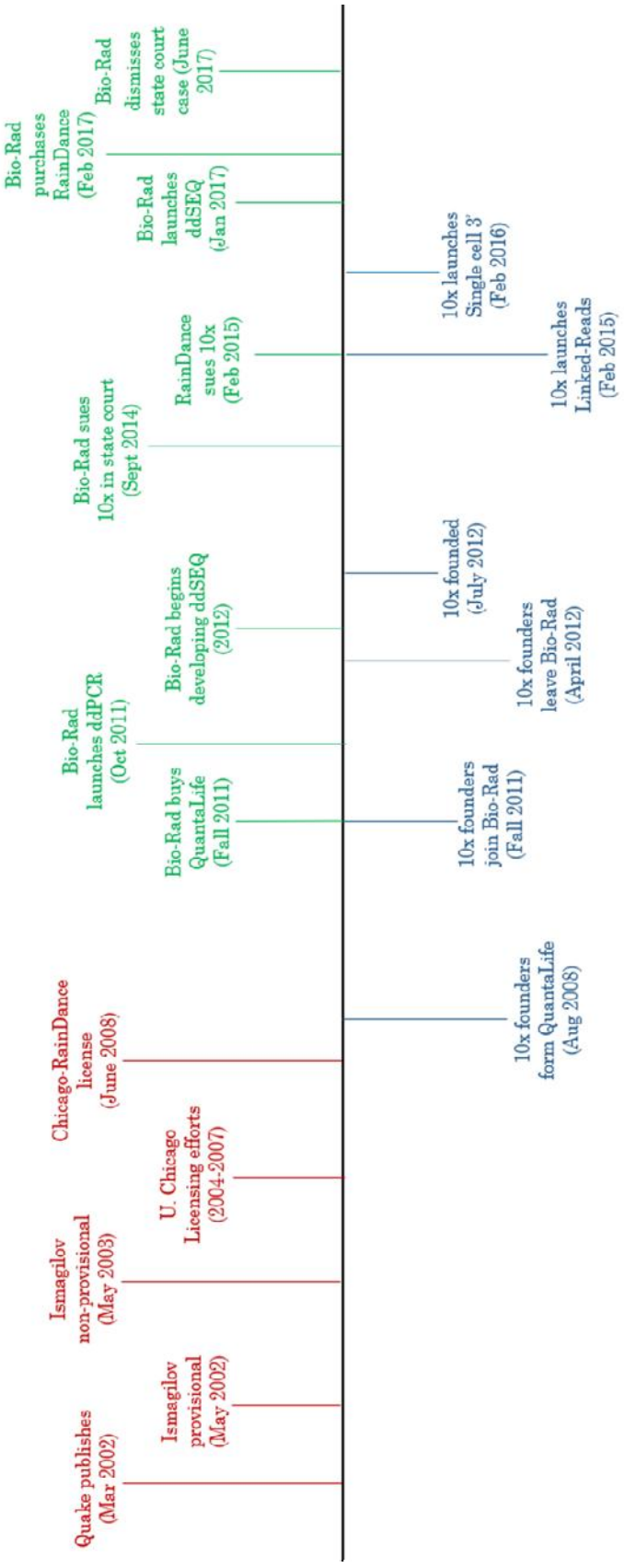
The Ismagilov patents purport to advance the art only by tweaking details of the chip relating to how the droplets flow through the microchannels without leaching reagents. Toward that end, they focus on the chemical composition of each system component and how they interact with each other. *See, e.g.*, Appx259 12:53-61; Appx264 21:7-34; Appx271-272 36:31-37:32. They also describe specific geometries for the microfluidic channels to better mix the fluids within a droplet. *See, e.g.*, Appx196-197; Appx204 (Figs. 5-6, 11); Appx259 12:31-52.

The '083 patent claims microfluidic systems and methods of conducting reactions within them; the claims, like the specification, are focused on details of chip chemistry. The asserted claims recite a microfluidic system with a "non-fluorinated" microchannel; a fluorinated, oil-based carrier fluid; and a water-based plug. Appx369 73:11-21. They also specify that surface tension between the plug and the carrier fluid must be less than the surface tension between the plug and the microchannel. Appx369 73:11-21.

The '407 and '193 patents, meanwhile, claim methods of conducting reactions in the plugs in the microfluidic system. In the '407 patent, the asserted claims recite conducting any sort of "biological" reaction, while the '193 patent claims recite "autocatalytic" reactions. *Compare* Appx292 78:58-62 *with* Appx187 78:12-15. That is the only difference between these two patents relevant to this appeal.

The asserted Ismagilov patents and their ancestors issued between 2006 and 2014. Appx31750; Appx294. The private sector was unimpressed. The University of Chicago contacted at least 19 companies about licensing the patents. Appx29782-29783; Appx35181. Bio-Rad was among them; the University's licensing representative personally met with Bio-Rad about the patents in 2010. Appx30034-30035; Appx32345-32346. Bio-Rad, and almost everyone else, flatly rejected a license. Appx30035. In 2008, RainDance took an exclusive license to the patents at rates of 1% for instruments and 3% for chips and reagents. Appx30021-30023. As of trial, the University had collected less than $2 million from licensing the patents. Appx30039.

This timeline shows the history of the Ismagilov patents, Bio-Rad, and 10x, as relevant to this case:


Quake publishes (Mar 2002)
Ismagilov provisional (May 2002)
Ismagilov non-provisional (May 2003)
U. Chicago Licensing efforts (2004-2007)
Chicago-RainDance license (June 2008)
10x founders form QuantaLife (Aug 2008)
Bio-Rad buys QuantaLife (Fall 2011)
Bio-Rad launches ddPCR (Oct 2011)
10x founders join Bio-Rad (Fall 2011)
Bio-Rad begins developing ddSEQ (2012)
10x founded (July 2012)
10x founders leave Bio-Rad (April 2012)
Bio-Rad sues 10x in state court (Sept 2014)
RainDance sues 10x (Feb 2015)
10x launches Linked-Reads (Feb 2015)
10x launches Single cell 3' (Feb 2016)
Bio-Rad launches ddSEQ (Jan 2017)
Bio-Rad purchases RainDance (Feb 2017)
Bio-Rad dismisses state court case (June 2017)

The Jury Finds Infringement And The District Court Permanently Enjoins 10x's Accused Products

The jury in this case ruled that 10x infringed the three patents and rejected validity challenges. It awarded plaintiffs their full requested damages—$23.9 million, representing 15% royalties on all accused products, whether a $125,000 instrument or a single-use reagent. Appx378; Appx30092. The court denied 10x's renewed motion for judgment as a matter of law or new trial. Appx29414-29447.

After trial, Bio-Rad moved for a permanent injunction. Appx28424-28425. Bio-Rad sought to enjoin all sales of 10x's accused products, with no exceptions—even though Bio-Rad did not claim to compete with 10x on four out of five product lines and (for reasons discussed above, at 16-17) does not compete meaningfully on the fifth. Appx28437-28438. Over a dozen researchers and one of the world's leading genetic research institutions, the Broad Institute, urged the court not to enjoin 10x's products. Appx28502-28511; Appx28877-28915.

The court granted an injunction that prohibits the sale of all of 10x's accused products, with a carve-out to allow scientists to purchase chips and reagents for pre-injunction instruments. The court

acknowledged that Bio-Rad did not purport to offer a competing product for four out of five 10x products, but held this was not "an appropriate distinction for purposes of the injunction." Appx68.

10x timely appealed both the final judgment and the permanent injunction to this Court. Appx31134-31135. The Court partially stayed the permanent injunction pending appeal. Fed. Cir. No. 19-2255, Dkt. 23.

SUMMARY OF ARGUMENT

I. The '083 patent claims a microfluidic system with a "non-fluorinated microchannel." Starting in 2017, 10x added a fluorinated polymer to all of its accused chips, making the microchannels fluorinated. Appx22264. The district court impermissibly allowed the jury to find infringement under the doctrine of equivalents. Appx373. Bio-Rad was barred from asserting infringement under the doctrine of equivalents for two independent reasons: prosecution history estoppel and claim vitiation.

A separate limitation of the '083 patent requires measuring the "surface tension at the plug-fluid/microchannel wall interface." This means that the droplets must touch the wall of the microfluidic channel.

10x's products are carefully designed so that this never happens. Bio-Rad's expert candidly admitted that in 10x's products "the droplet doesn't touch the channel wall." Appx29834. It is therefore impossible for 10x's products to infringe the claim. *Southwall Techs., Inc. v Cardinal IG Co.*, 54 F.3d 1570, 1575 (Fed. Cir. 1995).

II. The district court erred in construing the two remaining patents. The preamble of the '407 patent claims "a method for conducting a reaction in plugs in a microfluidic system." The preamble of the '193 patent is nearly the same: "A method for conducting an autocatalytic reaction in plugs in a microfluidic system." The claims were amended to include that limitation during prosecution. The phrases "reaction" and "microfluidic system" provide antecedent basis for the same terms in the body of the claims. And the specification repeatedly emphasizes that reactions take place on the microfluidic chip and within the microfluidic system. All of the "guideposts" this Court looks to support finding the preambles limiting. *Catalina Mktg. Int'l, Inc. v. Coolsavings.com, Inc.*, 289 F.3d 801, 808 (Fed. Cir. 2004).

Under the correct construction of the claims, 10x's products do not infringe the '407 or '193 patents as a matter of law. All of 10x's

biological and autocatalytic "reactions" occur while its droplets are in a thermal cycler—not in 10x's instrument or microfluidic chip. And that thermal cycler is not part of 10x's microfluidic system.

III. Bio-Rad's damages expert cherry-picked three licenses out of the dozens produced in this case as "comparable." The only unifying feature was that his selected licenses included the highest rates: 15% for "competitors." None of those licensed patents involved single-cell technology, or even droplet technology. None was comparable to the hypothetical negotiation, and the trial testimony did not account for the technological and economic differences between the licenses and the hypothetical negotiation.

Bio-Rad's damages theory also did not apportion the reasonable royalty to the value of the patented technology, which is required whenever the accused technology does not make up the whole of the accused product. Bio-Rad lifted the rates in the "comparable" licenses wholesale, without any adjustment for 10x's contributions to the accused products. Bio-Rad's expert did not provide anything close to the "reliable and tangible" evidence required to support the damages award. *Commonwealth Sci. & Indus. Research Org. v. Cisco Sys., Inc.*,

809 F.3d 1295, 1130 (Fed. Cir. 2015) (*CSIRO*) (quoting *Garretson v. Clark*, 111 U.S. 120, 121 (1884)).

IV. The injunction—entered against all five 10x product lines—should be vacated. The injunction does not prevent any irreparable harm to Bio-Rad. For four of 10x's product lines, Bio-Rad has never even argued that it offers a competing or substitute product. For the fifth, Bio-Rad presented no evidence that 10x's sales were causing Bio-Rad's ddSEQ product to suffer. ddSEQ's failures are related to its poor performance, not 10x's success. Bio-Rad's other effort to establish irreparable harm—10x's "head start" in the market—is belied by the facts. Bio-Rad's showing on the other *eBay* factors was no better.

STANDARD OF REVIEW

This Court reviews claim construction and the availability of the doctrine of equivalents de novo. *Catalina Mktg.*, 289 F.3d at 807; *Warner-Jenkinson Co. v. Hilton Davis Chem. Co.*, 520 U.S. 17, 39 n.8 (1997). The admissibility of expert testimony is reviewed for abuse of discretion. *In re Paoli R.R. Yard PCB Litig.*, 35 F.3d 717, 749 (3d Cir. 1994). The denial of judgment as a matter of law is reviewed de novo, and the denial of a new trial is reviewed for abuse of discretion.

McKenna v. City of Phila., 582 F.3d 447, 460 (3d Cir. 2009). The grant of an injunction is reviewed for abuse of discretion. *Presidio Components, Inc. v. Am. Tech. Ceramics Corp.*, 875 F.3d 1369, 1383 (Fed. Cir. 2017).

ARGUMENT

I. 10x Is Entitled To Judgment Of Non-Infringement Of The '083 Patent As A Matter Of Law.

The '083 patent claims are clear and specific about the chemistry and mechanics of the microfluidic chip. Claim 1 recites:

> 1. A microfluidic system comprising:
>
> a *non-fluorinated microchannel*;
>
> a carrier fluid comprising a fluorinated oil and a fluorinated surfactant comprising a hydrophilic head group in the microchannel;
>
> at least one plug comprising an aqueous plug-fluid in the microchannel and substantially encased by the carrier-fluid, wherein the fluorinated surfactant is present at a concentration such that *surface tension at the plug-fluid/microchannel wall interface is higher than surface tension at the plug-fluid/carrier fluid interface.*

Appx369 73:11-21 (italics added). 10x's accused products do not meet either of the italicized limitations as a matter of law.

A. 10x's chips with fluorinated microchannels cannot be equivalent to the "non-fluorinated microchannel" required by the '083 patent.

The "boundaries" of the patentee's monopoly "should be clear"; "[a] patent holder should know what he owns, and the public should know what he does not." *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co.*, 535 U.S. 722, 730-31 (2002). The inventors here assured the public that they owned only a microfluidic system with "a non-fluorinated microchannel." Appx369 73:12 (claim 1). And it was clear to the public exactly why they drew that boundary: They added the "non-fluorinated" limitation during prosecution to overcome prior art that disclosed microchannels that *were* fluorinated. Appx16640-16641; *see* Appx8512 (Quake 19:9-17); Appx29516.

The microchannels on the more recent version of the accused chips contain fluorine. In 2017, 10x started manufacturing the accused chips from a material made of 0.02% polyvinylidene fluoride, a fluorinated polymer known as "Kynar." Appx16526-16527; Appx29820; Appx29899; Appx30542-30543. Because 10x manufactured these chips from a fluorinated substance, the microchannels etched onto the chip are also fluorinated. Appx30243.

Naturally, the jury found that these chips do not literally infringe the "non-fluorinated microchannel" limitation. Appx373. The jury nonetheless found infringement under the doctrine of equivalents. Appx373. But "[t]he doctrine of equivalents is not a license to ignore claim limitations," and the district court should never have given the jury that option. *Dolly, Inc. v. Spalding & Evenflo Cos.*, 16 F.3d 394, 398 (Fed. Cir. 1994); *see Duncan Parking Techs., Inc. v. IPS Grp., Inc.*, 914 F.3d 1347, 1362 (Fed. Cir. 2019). Here, two independent legal principles implicating those critical functions barred Bio-Rad's theory of equivalence: prosecution history estoppel (§ I.A.1) and claim vitiation (§ I.A.2).

1. Prosecution history estoppel precludes Bio-Rad's equivalence theory.

Prosecution history estoppel "prevent[s] a patentee from using the doctrine of equivalents to recapture subject matter surrendered from the literal scope of a claim during prosecution." *Amgen Inc. v. Coherus BioSciences Inc.*, 931 F.3d 1154, 1159 (Fed. Cir. 2019) (quotation marks omitted). It keeps the doctrine of equivalents "tied to its underlying purpose": If the unamended claims covered the alleged equivalent, "the

patentee cannot assert that he lacked the words to describe the subject matter in question." *Festo*, 535 U.S. at 734.

Here, we know the inventors had the words. They started with a generic "microchannel" and then inserted the "non-fluorinated" modifier as "a narrowing amendment" to overcome the prior art. *Id.* at 736. Specifically, the examiner rejected the claims as obvious over a combination based on the prior-art Quake reference, which likewise disclosed microchannels in a microfluidic system that forms droplets. Appx16625-16632; *see* Appx8483-8485 (Quake Figs. 4a-6); Appx8493-8494 (Quake Figs. 14-15); Appx8495-8497 (Quake Figs. 16A-17C); Appx8502 (Quake Fig. 22); Appx8504-8505 (Quake cols. 3-6); Appx8512 (Quake 19:42-46); Appx8514 (Quake 24:22-27); Appx8529-8530 (Quake 54:15-55:47).

The inventors responded by distinguishing Quake based on fluorination. Appx16640-16641. Quake described fluorinating the microchannel by using either "a coating … intrinsic to the material from which the device is manufactured," such as Teflon, or by "appl[ying] [a coating] after the structural aspects of the channels have been microfabricated." Appx8512 (Quake 19:9-17); Appx29516. In amending

their claim to require a "non-fluorinated microchannel," the inventors explained that this would make the channel walls "chemically different" from the "*fluorinated* surfactant" and "*fluorinated* oil" that make up the carrier fluid. Appx16635; Appx16640 (emphasis added).

With this amendment, the inventors presumptively surrendered "all territory between the original claim limitation and the amended claim limitation." *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co.*, 344 F.3d 1359, 1367 (Fed. Cir. 2003) (en banc). The inventors "chose to emphasize" that their microchannels were "non-fluorinated" and thus "limited [themselves] to exclude any" fluorinated microchannel. *Wang Labs., Inc. v. Toshiba Corp.*, 993 F.2d 858, 868 (Fed. Cir. 1993).

The district court correctly held that it is "clear … that the presumption of total surrender applies." Appx22266. It erred, however, in applying the exception for a narrowing amendment that is merely "tangential" to the alleged equivalent. Appx22266; Appx29421-29422. "[A]n amendment made to avoid prior art that contains the equivalent in question is not tangential; it is central to allowance of the claim." *Festo*, 344 F.3d at 1369. The Quake patent unambiguously contained

the accused equivalent: It specifically disclosed microchannels made from a material with "suitable surface properties" such as Teflon, a fluorinated polymer. Appx8512 (Quake 19:9-17); *see* Appx29516.

Moreover, the amendment to "non-fluorinated" microchannels put this case on a par with *Festo*, where this Court held that an amendment specifying that a sleeve must be "magnetizable" was not "tangential, or peripheral, to the accused equivalent of a *nonmagnetizable*" sleeve. *Festo*, 344 F.3d at 1371-72. There, as here, the patentee could not provide a "tangential" rationale for choosing to narrow the claims to the opposite of the equivalent. *Id.*

The district court could not avoid this law by recasting the inventors' disclaimer as covering only "'microchannel[s]' 'coated' with fluorine *for a purpose*," Appx22267 (emphasis added); *see* Appx29421-29422, and letting the jury resolve the disputed factual question of whether the fluorine here served a purpose. That is not what the inventors said. They simply told the examiner that Quake's reference to fluorinated channel walls "does not disclose or suggest the elements of the amended claims of a non-fluorinated microchannel." Appx16640-16641. What counts for public notice is what the inventors in fact gave

up, not their subjective intent or unclaimed "purpose." By rewriting their claim to encompass only "non-fluorinated" microchannels, they surrendered the right to expand their monopoly to cover microchannels containing fluorine—for whatever purpose. *E.g.*, *Wang Labs.*, 993 F.2d at 867-68 ("[w]hile a two-row construction may not read on the prior art," patentee amended claim to "'only in a single row'").

Because Bio-Rad failed to rebut the presumption of total surrender, "prosecution history estoppel bars [it] from relying on the doctrine of equivalents for the accused element"—thus precluding any inquiry into "whether the accused element is in fact equivalent to the limitation at issue … on the merits." *Festo*, 344 F.3d at 1367. The Court should accordingly direct judgment of non-infringement of the '083 patent for 10x's chips made with Kynar.

2. Claim vitiation independently bars Bio-Rad's equivalence theory.

Bio-Rad's theory of equivalence also runs headlong into a separate principle: The doctrine of equivalents "cannot be employed in a manner that wholly vitiates a claim limitation." *SciMed Life Sys., Inc. v. Advanced Cardiovascular Sys., Inc.*, 242 F.3d 1337, 1346-47 (Fed. Cir. 2001); *see also Duncan*, 914 F.3d at 1362. The vitiation principle

applies with particular force where, as here, a feature is "specifically excluded from the scope of the claims." *Athletic Alts., Inc. v. Prince Mfg., Inc.*, 73 F.3d 1573, 1582 (Fed. Cir. 1996).

Put simply, "[t]he *presence* of a feature in an accused device … cannot possibly be equivalent to the claimed *absence* of that feature, and no reasonable factfinder could conclude otherwise." *Moore U.S.A., Inc. v. Standard Register Co.*, 229 F.3d 1091, 1115 n.5 (Fed. Cir. 2000) (emphases added). This Court has accordingly rejected, as a matter of law, the following purported equivalents:

- "minority" (47.8%) and "majority" (50.0001%), *id.* at 1106;
- "mounted" and "unmounted," *Asyst Techs., Inc. v. Emtrak, Inc.*, 402 F.3d 1188, 1195 (Fed. Cir. 2005);
- "surfactant" and "non-surfactant," *Novartis Pharm. Corp. v. Abbott Labs.*, 375 F.3d 1328, 1339 (Fed. Cir. 2004);
- "metallic" and "non-metallic," *SciMed*, 242 F.3d at 1347; and
- "inert" and "reactive," *Eastman Kodak Co. v. Goodyear Tire & Rubber Co.*, 114 F.3d 1547, 1560-61 (Fed. Cir. 1997), *abrogated on other grounds by Cybor Corp. v. FAS Techs., Inc.*, 138 F.3d 1448 (Fed. Cir. 1998).

The same result is compelled for the pair of opposites here: "fluorinated" and "non-fluorinated." Inventors who carefully crafted language to claim a "non-fluorinated microchannel" obviously foresaw

the potential of a microchannel that *is* "fluorinated"—as we know to a certainty the inventors did here. They made "a clear and binding statement to the public that [fluorinated microchannels] are excluded from the protection of the patent," and Bio-Rad cannot now "recapture the excluded subject matter." *SciMed*, 242 F.3d at 1347.

The district court recognized this vitiation problem at summary judgment, holding that "Plaintiffs may not assert the '083 patent, which claims a 'non-fluorinated microchannel,' against a product containing a 'fluorinated microchannel.'" Appx22268. And during Bio-Rad's case-in-chief, the court repeated its concern, observing that "I might end up striking the doctrine of equivalence [sic] opinion," Appx29830, and "we're probably in" the specific exclusion "ballpark," Appx29889. But it reversed course at the end of trial, concluding that the vitiation doctrine was inapplicable because 10x's "addition of Kynar did not change how [10x's] microchannels [previously] worked." Appx29423.

The district court ignored this Court's numerous cases holding the doctrine legally unavailable for diametric opposites, even if a jury could find that the alleged equivalent functioned just like the asserted claim. This Court has held, for example, the doctrine of equivalents could not

capture an ingredient of an accused product that was a surfactant when the claim required a "*non-surfactant* lipophilic excipient," even though the patentee argued that the ingredient did not "'*function* as a surfactant'" in the accused product. *Novartis*, 375 F.3d at 1337-38. This Court dismissed that argument as irrelevant; the only question was whether the ingredient qualified as a "non-surfactant," which a surfactant does not. *Id.* at 1337-39. Likewise here, it is irrelevant whether, or how much, the Kynar in 10x's chips changes the function of the microchannels. The fact that those microchannels are indisputably "fluorinated" means they cannot be the equivalent of a "non-fluorinated microchannel."

In the face of all these cases about opposites, the district court invoked only dicta from *Deere & Co. v. Bush Hog, LLC*, 703 F.3d 1349 (Fed. Cir. 2012). *See* Appx29422. But *Deere* considered whether the phrase "into engagement with" encompassed engagement via indirect contact, and it emphasized that these were not "'binary'" opposites. 703 F.3d at 1355-56. Meanwhile, it reaffirmed that "courts properly refuse to apply the doctrine of equivalents 'where the accused device contain[s] the antithesis of the claimed structure.'" *Id.* at 1356 (citation omitted).

In short, "the public has a right to rely on [Bio-Rad's non-fluorinated claim] limit[] in conducting its business activities," and so Bio-Rad cannot now apply the doctrine of equivalents to "effectively remove such a limitation." *Sage Prods., Inc. v. Devon Indus., Inc.*, 126 F.3d 1420, 1425 (Fed. Cir. 1997).

B. All of 10x's products lack a "plug-fluid/microchannel wall interface" and therefore cannot infringe.

10x is entitled to judgment of non-infringement as a matter of law because none of its products satisfy the final claim limitation:

> wherein the fluorinated surfactant is present at a concentration such that surface tension at *the plug-fluid/microchannel wall interface* is higher than surface tension at the plug-fluid/carrier-fluid interface.

Appx369 73:16-21 (emphasis added). This limitation cannot be satisfied unless there *is* a "plug-fluid/microchannel wall interface"—which happens only when the plug-fluid touches the channel wall. Moreover, without that interface, there is no way to measure the relevant "surface tension."

It is undisputed that none of the accused products has a "plug-fluid/microchannel wall interface," precisely because the droplets in 10x's chips (unlike those claimed in the '083 patent) are "fully encased

by the carrier fluid." Appx29834; *compare* Appx369 73:17; Appx29913-29914. Bio-Rad's expert Dr. Samuel Sia admitted this explicitly: In the 10x products "the droplet doesn't touch the channel wall." Appx29834. The interface recited by the claim doesn't exist. Without it, 10x cannot infringe. *Southwall Techs.*, 54 F.3d at 1575 ("[t]o establish literal infringement, every limitation set forth in a claim must be found in an accused product, exactly").

The district court rewrote the claim language when it declined to "limit the claims so as to require direct contact" between the microchannel wall and the plug-fluid. Appx22268-22269. There cannot be an "interface" between two items unless they make "direct contact" with one another.

The court repeated the same mistake post-trial when it accepted Bio-Rad's argument that it proved infringement. Appx29423-29425. Bio-Rad's scientists merely placed a droplet on the back side of one of 10x's microfluidic chips and measured the surface tension there. Appx29835-29837. Obviously, that does not prove that in 10x's actual product, the *microchannel wall* forms the requisite interface with the plugs (having conceded the opposite). It does not matter what the

hypothetical surface tension between the droplet fluid and the microchannel *would be if* they did touch in *some other* product. The claims require a specific "interface," and the accused product has none.

The district court was not permitted to "rewrite claims," but only to "give effect to the terms chosen by the patentee." *K-2 Corp. v. Salomon S.A.*, 191 F.3d 1356, 1364 (Fed. Cir. 1999). Giving effect to the patentee's word choices, no reasonable jury could find infringement.

***

Vacating any part of the infringement finding under the '083 patent affects the judgment, independent of the other two asserted patents. The '083 patent's claims are the only asserted apparatus claims. If this Court reverses only as to this patent, it must at a minimum vacate any damages based on foreign sales (about 42% of the award) and exclude such sales from the injunction. *See Finjan, Inc. v. Blue Coat Sys., Inc.*, 879 F.3d 1299, 1302 (Fed. Cir. 2018).

II. 10x Is Entitled To Judgment Of Non-Infringement Of The '407 And '193 Patents As A Matter Of Law.

The remaining two asserted patents both claim methods for conducting particular types of "reactions"—"biological" ('407 patent) or "autocatalytic" ('193 patent). The preambles specify where the relevant

reaction occurs: "in plugs *in a microfluidic system*." The district court refused to enforce the requirement that the plugs be "in a microfluidic system" when the reaction takes place. The court erred in failing to give limiting effect to the second geographic aspect of the claims' preambles. § II.A. Under the correct claim construction, 10x is entitled to judgment of non-infringement as a matter of law. § II.B.

A. The district court erred in removing one of the two geographic limitations from the claims.

The '407 and '193 patents, continuations of the same parent application, share many similarities, starting with their titles, which contemplate "Reactions … in a Microfluidic System": specifically, "Method for Conducting *Reactions* Involving Biological Molecules in Plugs *in a Microfluidic System*" ('407), Appx188 (emphasis added), and "Method for Conducting An Autocatalytic *Reaction* in Plugs *in a Microfluidic System*" ('193), Appx83 (emphasis added). The claims, too, parallel each other and mirror the patents' titles. Claim 1 of the '407 patent recites:

> 1. A method for conducting a *reaction in plugs in a microfluidic system*, comprising the steps of:
>
> providing *the microfluidic system* comprising at least two channels having at least one junction;

> continuously flowing an aqueous fluid containing at least one biological molecule and at least one reagent for conducting *the reaction* between the biological molecule and the at least one reagent through a first channel of the at least two channels;
>
> continuously flowing a carrier fluid immiscible with the aqueous fluid through the second channel of the at least two channels;
>
> forming at least one plug of the aqueous fluid containing the at least one biological molecule and the at least one reagent by partitioning the aqueous fluid with the flowing immiscible carrier fluid at the junction of the at least two channels, the plug being substantially surrounded by the immiscible carrier fluid flowing through the channel, wherein the at least one plug comprises at least one biological molecule and the at least one reagent for conducting the reaction with the at least one biological molecule; and
>
> providing conditions suitable for the reaction in the at least one plug involving the at least one biological molecule and the at least one reagent to form a reaction product.

Appx292-293 78:54-79:12 (emphasis added). The district court described the '193 patent's lone independent claim as "identical except that the '193 patent specifies 'an autocatalytic reaction,'" rather than a biological one. Appx29426.[3]

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[3] The difference between the two sorts of reactions has no bearing on this issue of claim construction. For simplicity, we refer to the '407 patent, but the arguments apply equally to the '193 patent unless otherwise noted.

The preamble limits the claims because "the claim drafter ch[ose] to use *both* the preamble and the body to define the subject matter of the claimed invention." *Bell Commc'ns Research, Inc. v. Vitalink Commc'ns Corp.*, 55 F.3d 615, 620 (Fed. Cir. 1995). This Court has identified three "guideposts" for when preamble language is limiting, any one of which can be sufficient: (1) "dependence on a particular disputed preamble phrase for antecedent basis"; (2) when the preamble "recit[es] additional structure or steps" that are "underscored as important by the specification"; or (3) "clear reliance on the preamble during prosecution to distinguish the claimed invention from the prior art." *Catalina Mktg.*, 289 F.3d at 808. The preambles here exhibit the first two and an aspect of the third.

First, the preambles supply antecedent basis. The first limitation in the body of each claim recites "providing *the* microfluidic system." Appx292 78:56; Appx187 78:10 (emphasis added). Which "microfluidic system"? The one in the preamble—where the reaction occurs. The preamble is the antecedent to which this first limitation refers. Similarly, the second limitation of the '407 patent recites the step of flowing a reagent "for conducting *the* reaction" through a channel.

Appx292 78:58-62 (emphasis added). Which "reaction"? Again, the one in the preamble—which occurs in the microfluidic system.

In a long line of cases, this Court has held that the same device—using words like "the" or "said" to refer back to a preamble—communicates the drafter's intention to treat the preamble as limiting. *See Pacing Techs., LLC v. Garmin Int'l, Inc.*, 778 F.3d 1021, 1024 (Fed. Cir. 2015); *Bicon, Inc. v. Straumann Co.*, 441 F.3d 945, 952-53 (Fed. Cir. 2006); *NTP, Inc. v. Research in Motion, Ltd.*, 418 F.3d 1282, 1306 (Fed. Cir. 2005), *abrogated on other grounds by Zoltek Corp. v. United States*, 672 F.3d 1309 (Fed. Cir. 2012) (en banc); *Eaton Corp. v. Rockwell Int'l Corp.*, 323 F.3d 1332, 1339-40 (Fed. Cir. 2003).

This Court did exactly that in the one case the district court discussed, *TomTom, Inc. v. Adolph*, 790 F.3d 1315 (Fed. Cir. 2015). When a claim's body used the phrase "said mobile unit" and "the mobile unit" to refer back to the "mobile unit" mentioned in the preamble, this Court held that meant the "mobile unit" in question must have the particular attributes recited in the preamble. *Id.* at 1323; *see also Bell*, 55 F.3d at 621 (claim's use of "said packet" meant that the packet in question must have the particular attributes recited in the preamble);

Proveris Sci. Corp. v. Innovasystems, Inc., 739 F.3d 1367, 1373 (Fed. Cir. 2014) ("The phrase 'the image data' clearly derives antecedent basis from the 'image data' that is defined in greater detail in the preamble."). So too here, "*the* reaction" and "*the* microfluidic system" must be the ones the preamble describes, and therefore subject to the preamble's limitations.

Second, the specification's focus on the location of the reactions reinforces the same conclusion. Beyond the titles, which are themselves revealing, the abstracts describe the "present invention" as "methods of conducting reactions *within* [microfabricated] substrates"—i.e., in microfluidic chips. Appx188 (emphasis added). The patents also consistently specify that the reaction occurs "in" the microfluidic system. *See, e.g.,* Appx287-288 67:24-25, 67:60-62, 68:42-43.

The specification is also revealing on another dimension: This Court has found it telling when a specification describes an "inventive concept" that is not achieved unless the device operates in the manner described only in the preamble. *Proveris*, 739 F.3d at 1373; *Deere*, 703 F.3d at 1358. That is the case here—twice over. The specification notes two "advantage[s]" over the prior art: The invention enables the

researcher to "obtain information involving complex reactions at several times, simultaneously, *simply by observing the channels* at different distances from the point of origin." Appx272 38:51-54 (emphasis added). Likewise, "[t]he reaction time can be monitored *at various points along a channel*—each point will correspond to a different reaction or mixing time." Appx272 38:55-57 (emphasis added). By definition, any reaction that is monitored *in* the channels is happening on the chip, which means the reaction is occurring *in* the microfluidic system. There is no way to achieve those inventive objectives unless the reaction occurs "in a microfluidic system," as the preamble specifies.

The final guidepost, the prosecution history, supports the same conclusion. In the very office actions allowing these claims, the examiner amended the preambles to specify that the reactions are conducted "in plugs in a microfluidic system." Appx8625-8629; Appx8631-8635. The examiner also amended the titles of the patents to emphasize that the reactions occur "in plugs in a microfluidic system." Appx8626; Appx8632. It is telling that she considered the location of the reaction important enough to insert it prominently into the claims and titles of the patents.

Putting all these guideposts together, the preambles limit the claimed reactions to ones that take place when the plugs are "in the microfluidic system." The preambles are "'necessary to give life, meaning, and vitality' to the claim[s]." *Catalina Mktg.*, 289 F.3d at 808 (citation omitted).

The district court's rationale for reaching the opposite conclusion was neither consistent nor clear. On claim construction, 10x argued that the entire preamble (including "in a microfluidic system") is limiting; Bio-Rad argued that the preamble was not limiting at all. Appx7011-7016. The district court devised its own construction: "The entire preamble is not limiting," but it "is limiting only to the extent that it provides an antecedent basis." Appx8845-8846. The court later acknowledged being "puzzled" by its own construction. Appx29725. The court then issued a summary judgment ruling that was premised on the view that the preamble is limiting. Appx22262-22263. On the first day of trial, however, the court changed direction again. Appx29726.

Ultimately, the court prohibited 10x from arguing that reactions must take place in the microfluidic system. Appx29472-29473. It

explained its reasoning for the first time when it denied 10x's request for post-trial relief. Appx29427-29428. The court correctly concluded "[t]hat 'reaction' and 'microfluidic system' provide antecedent basis for the use of those terms in the body of the claim," but then held that "the portion of the preamble that states '*conducting* a reaction in plugs *in a* microfluidic system' is not limiting." Appx29428. The court's only explanation for the distinction was that "[n]othing in the body of the claims further limits the location of the reaction." Appx29426-29427.

By that logic, no preamble would ever be limiting. The district court failed to address any of the guideposts this Court has prescribed. Thus, for example, the court discussed this Court's opinion in *TomTom* at length. But it overlooked the crucial point, discussed above (at 46), that *TomTom* actually found a portion of a preamble limiting because the body of the claim directed the reader back to the preamble with words like "the" and "such," just as these claims do.

Instead, the only lesson the district court extracted from *TomTom* was that it is possible for one part of a preamble to be limiting even though another portion is not, when the two are "separate[]." 790 F.3d at 1323. There, the preamble contained two phrases that the parties

stipulated should be construed independently: "A method for [1] *generating and updating data* [2] *for use in a destination tracking system of at least one mobile unit* comprising …." *Id.* at 1318, 1323 (numbers added). This Court found the second phrase limiting (for the reasons discussed) even though the first phrase was not. *Id.* at 1323-24. But just because it is possible to give different effect to two "separate[]" phrases in a preamble does not mean that it's the norm. *Id.* at 1323. As important, *TomTom* does not authorize different treatment where, as here, the terms are not "separate." By its own account, the district court here carved up a single phrase as follows, giving limiting effect to the bolded terms but not to the italicized ones nested around them: "*conducting* a **reaction in plugs** *in a* **microfluidic system**." Appx29428. The district court's dissection of the preamble is not just "puzzl[ing]," Appx29725, but paradoxical.

B. As a matter of law, 10x does not infringe under the correct construction of the claims.

Because the district court's "incorrect claim construction … remove[d] from the jury a basis on which the jury could reasonably have reached a different verdict, the verdict should not stand." *Cardiac Pacemakers, Inc. v. St. Jude Med., Inc.*, 381 F.3d 1371, 1383 (Fed. Cir.

2004). Bio-Rad is not entitled to another trial, because the evidence does not support an infringement verdict under the correct claim construction. *See, e.g., Saffran v. Johnson & Johnson*, 712 F.3d 549, 563-64 (Fed. Cir. 2013) (entering judgment of non-infringement after reversing claim construction). Based on the undisputed evidence presented at trial, the biological/autocatalytic reactions occur in the thermal cycler, which is not part of 10x's microfluidic system. So as a matter of law, the claimed reactions do not occur "in a microfluidic system."

There is no dispute that 10x's "proprietary barcoding reactions," Appx15733—Landlord, PHASE, and GEM-RT——do not occur until after a researcher removes the droplets from the microchannel and places them in the thermal cycler. Appx29860; Appx30169-30171; *see* Appx29579 ("I think every droplet system that I'm aware of … moved to a thermal cycler to do their reactions.").

Bio-Rad has no evidence that any other relevant reaction occurs before the droplets are placed in the thermal cycler. Bio-Rad pointed to the dissolution of the gel beads as a "biological" (but not "autocatalytic") reaction. Appx29848. But the evidence Bio-Rad relied on to prove that

the reaction occurred in 10x's controller did not reflect "10x Genomics products in any way." Appx30376-30378; *see* Appx29926-29928. And, the dissolution of the polyacrylamide gel beads is *not* a biological reaction under the district court's construction. Appx8871; Appx29848; Appx30593; Appx30596-30597.

The record is also clear that the thermal cycler is not part of the microfluidic system. Bio-Rad's expert Dr. Sia testified that 10x's "microfluidic system" consists of a "microfluidic chip," "reagents that are loaded into the chip," and the controller "that is used to run that microfluidic chip." Appx29812; *see* Appx29814 ("[T]his is their product. It's introducing reagents into the microfluidic chip putting the chip into an instrument and that instrument runs the chip. This is a microfluidic system."); Appx29818; Appx30523.

Moreover, the thermal cycler is indisputably *not* a 10x product; they are "common" to "most labs," which purchase them separately from "a lot of different companies." Appx30170; *see* Appx30171-30172. The reactions therefore do not even occur in a 10x product, let alone within 10x's microfluidic system.

III. The Damages Award Should Be Vacated.

The jury awarded Bio-Rad its full asserted damages of $23.9 million, representing a 15% royalty on worldwide sales of all instruments, chips, and reagents. Appx29441. The award should be vacated, as based on evidence that was both inadmissible and insufficient.

In the hypothetical negotiation, 10x would have negotiated with RainDance and the University of Chicago. Appx30605-30606. Normally, the analysis would begin with "[a]ctual licenses to the patented technology," which are generally the most "highly probative as to what constitutes a reasonable royalty for those patent rights." *LaserDynamics, Inc. v. Quanta Comput., Inc.*, 694 F.3d 51, 79 (Fed. Cir. 2012). In keeping with this norm, 10x's expert, Dr. Ryan Sullivan, relied on the University of Chicago's license of the asserted patents to RainDance. Appx30665-30666; Appx31864-31881; Appx32713-32729. That was an *exclusive* license for a royalty of 1% on instruments and 3% on consumables (or less, with a royalty-stacking offset). Appx30667; Appx30672; Appx32717. It was one of 15 licenses with rates ranging from 0.25% to 3%. Appx16178-16194; Appx29161; Appx30609-30610.

Bio-Rad's damages expert, James Malackowski, swept away all 15 licenses, including the license to the asserted patents, and focused exclusively on three outliers. Appx30066-30068. In his opinion, RainDance and Chicago would have demanded, and 10x would have paid, a 15% royalty on all of 10x's sales, Appx30062-30064—15 times what Chicago accepted on instruments and 5 times what it accepted on consumables in its negotiation with RainDance.

10x moved to exclude Mr. Malackowski's opinion as relying on non-comparable licenses and for failing to apportion damages. Appx17010-17016 (*Daubert*). At first, the district court rejected Mr. Malackowski's methodology for lack of apportionment. Appx22361-22362. Mr. Malackowski then submitted a new report, relying on the same inputs and reaching the same 15% outcome with little further analysis. Appx22390-22392. Mr. Malackowski still did not apportion, but merely *asserted* that the patents covered by the three cherry-picked licenses represented a similar value to the licensed products as the asserted patents do to 10x's products. Appx22392; *see* Appx22864-22880. Nonetheless, the district court admitted his testimony. Appx25650-25653.

After the jury delivered its verdict, the district court held that one of Mr. Malackowski's three reference licenses was not comparable, yet upheld the verdict anyway. Appx29441-29446. The verdict should be vacated for that reason alone. It should also be vacated, whether on sufficiency or admissibility grounds, because: (A) the other two licenses were not comparable either; and (B) regardless, the award was not properly apportioned.

A. Bio-Rad's expert relied on licenses that were not comparable to the hypothetical negotiation.

Bio-Rad was permitted to rely on "[t]he rates paid by the licensee for the use of other patents comparable to the patent in suit," *Georgia-Pac. Corp. v. U.S. Plywood Corp.*, 318 F. Supp. 1116, 1120 (S.D.N.Y. 1970), but it "had the burden to prove that the licenses were sufficiently comparable to support the … damages award," *Lucent Techs., Inc. v. Gateway, Inc.*, 580 F.3d 1301, 1329 (Fed. Cir. 2009).

Instead of meeting that burden, Bio-Rad violated this Court's directions in two ways: First, it excluded the Chicago/RainDance license to the asserted patents in favor of three licenses that are not comparable (let alone more comparable than the license that included the asserted patents). *See ResQNet.com, Inc. v. Lansa, Inc.*, 594 F.3d

860, 870-73 (Fed. Cir. 2010) (rejecting expert's reliance on insufficiently comparable licenses to the exclusion of the one agreement to the asserted patents). Second, Bio-Rad cited to only the highest-value licenses while excluding the many other licenses, which "served no purpose other than to increase the reasonable royalty rate above rates more clearly linked to the economic demand for the claimed technology." *LaserDynamics*, 694 F.3d at 80 (internal citations omitted) (error to permit testimony to non-comparable licenses "to the exclusion of the many licenses expressly for the" asserted patent).

Mr. Malackowski's excuse for cherry-picking the outlier licenses was that patentees impose higher rates on "competitors," Appx30071-30076; *see* Appx17066, so any license between non-competitors (including the Chicago/RainDance license covering the patents-in-suit) was utterly irrelevant, Appx30064-30067. Never mind that the hypothetical licensors here—Chicago and RainDance—never actually competed with 10x. Appx29618; Appx30102-30103. And never mind that the licenses Mr. Malackowski chose—on different technologies in wildly different circumstances—do not come close to demonstrating a 15% norm for competitors on *this* technology in *this* case.

Applera/Bio-Rad. The first license is simple: The district court held that Bio-Rad failed to show it was comparable. So the jury should never have heard it. Appx29443-29445. Bio-Rad acceded to this license from Applera with a 15% royalty on sales of PCR instruments as Bio-Rad was on the verge of being held in contempt of an injunction. Appx29605-29606; Appx30613-30614; Appx32029-32065. The rate was so high because PCR is a Nobel Prize-winning invention that "launched the human genome project." Appx30578. As the district court explained, the license is not comparable because 10x's controller is not a PCR instrument and the asserted patents are not PCR patents. Appx29444-29445.

On this basis alone, this Court should at least order a new trial. The admission of improper testimony is not harmless unless "it is highly probable that the error[] did not affect the outcome of the case." *Hirst v. Inverness Hotel Corp.*, 544 F.3d 221, 228 (3d Cir. 2008). There can be no such confidence here, since this was one out of only three licenses adduced in support of the verdict, it is the only one that covers the sale of instruments, and it is the only license reflecting the single 15% rate the jury adopted, as distinguished from a range.

AppliedBio/QuantaLife. In the second license, QuantaLife agreed to pay AppliedBio 10-15% for certain *reagents* also for *PCR*. Appx30152-30153; Appx30617-30619; Appx32568-32630. Those are two critical distinctions.

First, the license does not require payments for instruments—or even chips. Appx30152-30153; Appx30616-30617. The reagents sold at around $2.00, yielding a royalty payment of 24 cents per unit. Appx30153; Appx30619. That 10-15% rate cannot be uncritically translated into a rate for instruments bearing price tags of $60,000 to $125,000, Appx30615, any more than a 15% royalty on a gallon of gas translates into the royalty rate on the price of the entire car. Mr. Malackowski made no adjustments for the difference—even though the most probative license in the record (the Chicago/RainDance license) applied different rates to instruments and consumables. Appx32717.

Second, the PCR focus was as disqualifying here as the district court found it to be for Applera/Bio-Rad: This license covered a PCR enzyme that "transformed the field" and "enabled modern molecular biology." Appx30577-30578; *see* Appx29597; Appx29894-29895; *supra* 7.

The district court reached a different result here based on the testimony of Bio-Rad's technical expert, Dr. Sia, that (1) the AppliedBio/QuantaLife license covered "reagents for doing … PCR in the droplets," Appx29895; and (2) the Ismagilov patents also "deal with the subject [of] trying to do PCR and trying to do it better using the droplet technologies," Appx29894; *see* Appx29445-29446. That is like saying a license to the recipe for Coca-Cola is comparable to a license to an improved bottling machine, because Coca-Cola is something you can bottle.

10x was not bargaining for a license to PCR (or anything like the prized Coca-Cola recipe), because 10x's accused products do not conduct PCR in droplets. Appx30254. And the asserted patents do not claim "reagents for doing the PCR"; they mention PCR—an already well known reaction—as an example of a reaction that can hypothetically be performed in a droplet, but teach no advancements to PCR technology. Appx276 45:30-33.

As with the Applera license, what one company would pay for PCR reagents that "enabled modern molecular biology," Appx30577, has no bearing on what 10x would pay for the asserted patents. As discussed

(at 20-22), the Ismagilov patents—mainly copied verbatim from another patent—do not improve PCR and were nothing close to transformative in microfluidics.

Mr. Malackowski, for his part, addressed none of this. He found the license comparable merely because it was "between competitors, comparable technology, non-exclusive." Appx30080-30081. Such "'superficial testimony' and the simple recitation of royalty numbers" simply does "not support the jury's award when no analysis is offered." *Whitserve, LLC v. Comput. Packages, Inc.*, 694 F.3d 10, 32 (Fed. Cir. 2012).

Caliper/RainDance. The third license was one Caliper granted to RainDance for a portfolio of 550+ patents. Appx29932-29933. This one, too, did not cover instruments, but only reagents and chips. The license had two rates: RainDance agreed to pay 2% for "non-screening" uses, which did not compete with Caliper, but 15% for "screening" uses that "directly and demonstrably impact sales of Caliper's products." Appx30076; Appx30620; Appx32631-32712. RainDance "never paid at a 15% rate" because it never competed with Caliper. Appx30620. The

license was not comparable along several of the same dimensions as the AppliedBio license—and more.

First, a license on consumables cannot be applied with no adjustments to a license on very expensive instruments. *Supra* 59.

Second, the technology was different. Caliper's technology involved some aspects of microfluidics, but "not droplets." Appx29892. The district court accepted Dr. Sia's opinion that the Caliper license was comparable because it "dealt with microfluidics and all sorts of ways to control fluids," which "is similar to … the subject matter" of the asserted patents. Appx29892; *see* Appx29442-29443. But a license to a portfolio of 550+ microfluidics patents is not "similar to" a license to three patents that largely share a specification and deal with discrete aspects of droplet generation and manipulation. *Supra* 20-22. Dr. Sia and Mr. Malackowski's testimony left the jury without the evidence needed to "adequately evaluat[e] the probative value of" the Caliper/RainDance license. *Lucent*, 580 F.3d at 1328 (rejecting reliance on IBM's portfolio-wide "personal computer" license as applied to a hypothetical negotiation to a specific feature of computer program); *see also LaserDynamics*, 694 F.3d at 80 (rejecting a comparison between

DVD-related licensing programs and the patent-in-suit despite the overlap in DVD focus, because "no evidence shows that [the licensing programs] even involve[d] a disc discrimination method.").

Third, the 15% rate in that license was pure fiction. *See Wordtech Sys., Inc v. Integrated Networks Sols., Inc.*, 609 F.3d 1308, 1320 (Fed. Cir. 2010). Mr. Malackowski seemed to think that RainDance's acceptance of a potential 15% competitor rate proved that any competitor would accept that same rate. Appx30079. But RainDance never exceeded the 2% rate, because it never competed with Caliper. Appx30620-30621. Even Bio-Rad recognized that Caliper "hadn't thought about droplets or what RainDance wanted to do." Appx29591. Indeed, when conducting due diligence on licenses needed for the ddPCR product, Bio-Rad determined that "what Caliper is doing … was in no way competing with anything [Bio-Rad] planned to do with the droplets." Appx29595.

If you run a profitable deli and lease out the adjacent storefront to a drycleaner for "$500 if used as a drycleaner or $50,000 if used as a deli," that may say something about your desire to prevent a competitor from entering the market, but it says nothing about the actual market

rate for delis. So too here, a price that no one expected RainDance ever to pay cannot support an opinion that actual competitors would pay 15% and therefore cannot support the jury's verdict. And, more importantly, it cannot support applying a prohibitively expensive rate—which kicked in only if sales "directly and demonstrably impact" the licensor—to 10x, which never sold a product that competes with RainDance's products. Appx30102-30103; Appx30620.

* * *

Bio-Rad did no more than "alleg[e] a loose or vague comparability between different technologies or licenses," which "does not suffice" to support the jury's verdict. *LaserDynamics*, 694 F.3d at 79. Telling the jury that three outlier rates—chosen over numerous others in the 0.25-3% range—reflect a universal 15% "competitor" rate does not "account for 'the technological and economic differences' between" the licenses and the hypothetical negotiation. *Wordtech*, 609 F.3d at 1320. Bio-Rad's expert opinion therefore cannot support the jury's verdict. *See id.*; *Ericsson, Inc. v. D-Link Sys., Inc.*, 773 F.3d 1201, 1227 (Fed. Cir. 2014). It was impermissible to let the jury fill in the gaps based on "speculative and unreliable evidence divorced from proof of economic harm linked to

the claimed invention." *ResQNet.com*, 594 F.3d at 868. For this reason alone, the damages award should be vacated.

B. Bio-Rad's expert did not apportion damages to the value of the patented technology.

Even if the reference licenses were comparable, the verdict cannot stand because Mr. Malackowski failed to apportion. Bio-Rad was required to "'give evidence tending to separate or apportion the defendant's profits and the patentee's damages between the patented feature and the unpatented features, and such evidence must be reliable and tangible, and not conjectural or speculative.'" *CSIRO*, 809 F.3d at 1130 (quoting *Garretson*, 111 U.S. at 121).

There is no dispute that 10x's products have numerous non-infringing features and components—and highly consequential ones at that. Appx30091; Appx30669-30671. That much is evident from the technological challenges 10x overcame, and all the ways in which its single-cell products outperform Bio-Rad's ddSEQ, even though both systems use droplets. *Supra* 12-15, 17-18.

Nevertheless, Mr. Malackowski opined that it was appropriate to take the 15% royalty in his cherry-picked reference licenses and slap it onto 10x's products, with no adjustment at all. Appx30074-30075;

Appx30091. He claimed that that this 15% rate was already apportioned because he had "looked at the relative value of the droplets technology that's at issue in this case versus the non-infringing contributions that 10x asserts it brings," and then compared that "general relationship" to the licensed technology and unlicensed features of the licensed product. Appx30074-30075. In other words, he purported to compare these two ratios:

$$\frac{Asserted\ patents}{\begin{matrix}Noninfringing\ features\\ of\ 10x's\ products\end{matrix}} \quad v. \quad \frac{Licensed\ patents}{\begin{matrix}Unlicensed\ features\\ of\ licensed\ products\end{matrix}}$$

He never provided any numerical values to support his analysis. Yet, coincidentally, the ratio on the right turned out to be the same in each of the three licenses he considered. And even more coincidentally, those three ratios all turned out to be the same as the one on the left. Only by supporting that quadruple coincidence could Mr. Malackowski apply the same royalty rate across all these products.

The first problem with Mr. Malackowski's analysis is that it does not apportion to the value of the patented technology as a matter of law. The starting point for apportionment must be the actual value of the

patented technology, not the value of other technology to other products. For example, in *CSIRO*, the district court relied on negotiations over a license *to the asserted patent* to set a royalty rate. This Court accepted that comparable license's "built in apportionment" as a "starting point" because the rate was "negotiated over the value of the asserted patent." 809 F.3d at 1303.

The second problem is that there is no evidence supporting any of Mr. Malackowski's coincidences. Mr. Malackowski's testimony lacks *any* evidence that he actually performed the analysis he claimed to have performed. He gave no actual numbers. He provided no facts that a jury could have used to confirm that he actually balanced the novel equation that he proposed. Neither he nor any other witness provided useful evidence showing the relative value of the licensed technology and unlicensed features in the reference licenses. So Bio-Rad had no proof supporting the righthand side of the formula. Without that analysis, there is no way to say that the lefthand side has been properly apportioned.

Mr. Malackowski is an accountant. Appx30059-30060. Because he lacks technical expertise, he could not—and did not purport to—

opine on the technical contributions of *any* patent. Appx30067-30068. Instead, he relied on Dr. Sia and Ms. Tumolo to draw conclusions about the technologies covered by the licenses. Appx30067-30068; Appx30082. But they did not fill in the gaps.

Bio-Rad/Applera. For the Bio-Rad/Applera license (which the district court found not comparable), Mr. Malackowski "pick[ed] an example number" to illustrate how he would undertake the apportionment analysis, and in the same breath concluded he was "good to go with the 15 percent." Appx30073-30075.

Caliper/RainDance. For the Caliper/RainDance license, Mr. Malackowski relied on Dr. Sia, who explicitly conceded he did not undertake the purported apportionment analysis:

> Q. And you did not … do any analysis of the licensed products for the RainDance license versus the licensed products in this case, the 10x products, you didn't do that in your report, did you?
>
> A. No, … that wasn't really part of the scope.

Appx29934. Ms. Tumolo, meanwhile, simply asserted that the Caliper patents were "a small part of the value of [RainDance's] products … in my mind." Appx29592. She never explained how a "small part of the value" could ever translate into a 15% royalty. (Perhaps because

RainDance *never paid* a 15% royalty. *Supra* 63-64.) Yet from that testimony and unadmitted "information that [he] reviewed in this case," Mr. Malackowski told the jury "it was comparable both at a technical economic and rights perspective." Appx30078-30079. He did not identify the unlicensed features of RainDance's product (the denominator in his formula). He did not say whether those unnamed features were important. So he had no basis for balancing the equation and concluding no adjustment to the (fictional) 15% rate was necessary.

AppliedBio/QuantaLife. As to the AppliedBio/QuantaLife license, Ms. Tumolo testified only that the licensed patents cover "sort of basic rights if you want to do PCR," Appx29597, while unspecified "software," "chemistry," and "emulsion work" was the "value in [her] mind" of QuantaLife's product, Appx29604-29605. Based on nothing but these sparse observations and Dr. Sia's testimony that the AppliedBio patents cover "some reagents that would help you to do PCR," Appx29894-29895, Mr. Malackowski testified:

> [L]ook at the technology [AppliedBio] brought to the table, the ddPCR technology[,] versus [what] QuantaLife was bringing to the table, which is the list of factors or technology that wasn't included in the license. And, again, I was able to confirm that that relative ratio would not be greater on the green circle than it was on the blue circle.

Appx30081. "[T]he list of factors or technology" and blue and green circles are nowhere in the record. They appeared on a demonstrative slide displayed during Mr. Malackowski's testimony. The district court instructed the jury that demonstratives are "just … an aid to whatever testimony you're listening to," unless admitted into evidence. Appx31369. Mr. Malackowski's slides were not moved into evidence. The jury therefore could not have relied on the slides in reaching its verdict, and they are not in the record on appeal. *See* Fed. R. App. P. 10(a); *Finisar Corp. v. DIRECTV Grp., Inc.*, 217 F. App'x 981 (Fed. Cir. 2007) ("Because the demonstrative exhibits were not filed with the district court, they are not part of the record on appeal.").

"When parties rely on demonstratives to present evidence or mathematical calculations to the jury, it is their burden to assure that the record captures the substance of the data so presented. [This court] can not guess at what the jury saw." *Whitserve*, 694 F.3d at 32 n.16. Bio-Rad failed to meet its burden to provide record evidence reflecting the substance of Mr. Malackowski's analysis. And Mr. Malackowski's abstract and conclusory testimony about blue and green circles cannot

provide the substantial apportionment evidence necessary to uphold the award.

* * *

When concluding his testimony and reciting the *Georgia-Pacific* factors, Mr. Malackowski simply asserted: "[I]n all of those 15-percent competitive licenses, you remember that pie chart analysis I did, each of those parties were also bringing the types of things that 10x is bringing." Appx30091. But his "pie chart analysis" is also not in evidence. And a vague reference to other licensees—to other patents, covering other products—bringing "the types of things that 10x is bringing" is not tangible evidence of anything, let alone of a reliable apportionment analysis.

Mr. Malackowski never stated what he determined to be the relative value of the licensed features to the licensed products—not for any one of the three licenses on which he relied. Nor did he ever state what he determined to be the relative value of the asserted patents to the accused 10x products. Instead, he underscored how hollow his testimony was by explaining, "I just pick[ed] an example number. If droplets were, say, a quarter of the value of what's being brought to the

table in the hypothetical negotiation, I need to be certain that the [licensed technology] is not half the value.… And so that's what I did." Appx30075.

Mr. Malackowski's vague testimony reflects a "complete lack of economic analysis to quantitatively support" the verdict. *LaserDynamics*, 694 F.3d at 69 (rejecting "vague qualitative notions of the relative importance" of the patented technology); *see ResQNet.com*, 594 F.3d at 873. It "amounts to nothing more than speculation," and "[w]ithout a more detailed analysis, the jury is simply left to speculate or adopt the expert's unsupported conclusory opinion." *Exmark Mfg. Co., v. Briggs & Stratton Power Prods. Grp., LLC*, 879 F.3d 1332, 1350 (Fed. Cir. 2018).

The district court, for its part, did not "scrutinize the evidence carefully to ensure that the 'substantial evidence' standard is satisfied," as it was required to do. *Lucent*, 580 F.3d at 1336. When 10x explained the deficiencies in Mr. Malackowski's testimony, Appx30284-30285, the court simply remarked that "the Federal Circuit will … be available to fix that." Appx30298.

And so it should.

IV. The Permanent Injunction Should Be Vacated.

"An injunction is a drastic and extraordinary remedy, which should not be granted as a matter of course." *Monsanto Co. v. Geertson Seed Farms*, 561 U.S. 139, 165 (2010). Bio-Rad was required to prove that it meets each of the four traditional equitable factors governing injunctions. *eBay Inc. v. MercExchange, L.L.C.*, 547 U.S. 388, 391 (2006). Bio-Rad wholly failed to justify its request for a permanent injunction, especially as to the four product lines on which Bio-Rad did not even purport to compete.

A. Bio-Rad did not show irreparable harm that cannot be compensated by monetary damages.

1. The district court premised the injunction on the notion that Bio-Rad and 10x are direct competitors and "[d]irect competition strongly suggests the potential for irreparable harm." Appx61. But one undisputed fact is fundamental to both the court's premise and any assessment of irreparable harm: Bio-Rad *did not even claim to compete* with four out of five of 10x's product lines. Appx28498-28500; Appx30196. Thus, despite one Bio-Rad executive's vague assertion that Bio-Rad "can fill that need" for 10x's products, Appx28499, Bio-Rad plainly could not replace sales for the four product lines for which it had

no substitute product. It was improper for the court to enjoin sales in the name of competition where there is not—and could not possibly be—any competition.

The only 10x product line with which Bio-Rad's ddSEQ even purported to compete is 10x's Single Cell 3′. Appx29619. But even here, there is no harm, because ddSEQ is so inferior that researchers do not want to use it. Appx28886-28915; Dkt. 9.5-9.8. Moreover, with respect to that one product line, Bio-Rad faces competition from at least 10 other competitors. Appx35327. That is one reason Bio-Rad had to withdraw its lost profits claim when the district court demanded an offer of proof; Bio-Rad could not prove that 10x caused it to lose even a single ddSEQ sale. Appx25238; Appx25242. The district court impermissibly ignored this clear evidence that enjoining 10x is unlikely to help Bio-Rad's competitive position. *See Apple Inc. v. Samsung Elecs. Co.*, 678 F.3d 1314, 1324 (Fed. Cir. 2012).

Instead, the court took Bio-Rad's attorney argument and cursory corporate declaration at face value. Appx28498-28501. But there was no *evidence* to substantiate the conclusory assertions about lost sales or market share, pricing pressures, increased marketing costs, or

reputational harm. Appx28498-28501. *Compare Robert Bosch LLC v. Pylon Mfg. Corp.*, 659 F.3d 1142, 1151 (Fed. Cir. 2011) (detailing "overwhelming evidence" of irreparable harm); *Broadcom Corp. v. Emulex Corp.*, 732 F.3d 1325, 1336-37 (Fed. Cir. 2013) (patentee provided "conclusive[]" evidence of "lost market share").

2. Without any evidence of meaningful competition between 10x's and Bio-Rad's specific products, the district court found 10x and Bio-Rad both participate in a broader market: "the market for products that perform genetic analysis on a droplet platform." Appx61.

This concept of "competition" is foreign to the law of injunctions. The notion of a "droplet product market" is like saying that microscopes and telescopes compete in an "optical instrument" market for tools that use lenses at opposite ends of movable cylinders. The applications are totally different: A telescope cannot help a microbiologist observe a cell, and a microscope cannot help an astronomer see Mars. Selling a microscope that infringes a patent on lenses cannot cause irreparable harm to a company that sells only telescopes.

So too here. Just because Bio-Rad and 10x both sell products that put a variety of items (chemicals, plus cells or genetic material) in

droplets does not mean they compete in the sense that is relevant to irreparable harm. 10x's sale of a CNV product, for example, could never inflict harm on Bio-Rad's completely separate and concededly non-competing ddPCR products. And Bio-Rad submitted zero evidence that 10x caused Bio-Rad to lose any ddPCR sales.

Relatedly, the court also justified the injunction on the ground that 10x was "captur[ing] and defin[ing] the market" and it was now necessary to help Bio-Rad overcome 10x's "strong market lead over Bio-Rad." Appx63-65. A "market lead" in what? Certainly not in launching a "product[] that perform[s] genetic analysis on a droplet platform," the market the district court considered relevant. Appx62. Thanks to 10x's founders and the QuantaLife technology they brought to Bio-Rad, Bio-Rad sold the ddPCR product—to great success—before 10x even existed. Thus, the court was plainly wrong in asserting that "10x's infringement coincided with the emergence of the droplet market." Appx64. QuantaLife launched its original droplet PCR product in 2011, four years before 10x's first product and five years before 10x's first single-cell product. If there really were some sort of relevant market for all products that use "droplets," then Bio-Rad should be enjoying all the

"head start" benefits it attributes to 10x. Nor did 10x have a "first to market position" in the single-cell market, as Bio-Rad claimed. Appx28499. The undisputed evidence was that Fluidigm was on the market with a single cell product *five years* before 10x's first single-cell product. Appx16135-16136; Appx30191.

Regardless, the court failed to explain how the prospective injunction would remedy any supposed market lead. By allowing scientists to continue buying consumables for existing instruments, the court acceded to allowing the "sticky" customer relationships to continue. The injunction Bio-Rad sought and received simply does not enable it to reclaim its supposedly "lost" market share with 10x's existing customers.

B. The balance of the hardships and public interest do not support an injunction.

Bio-Rad had an affirmative burden to show that the balance of the hardships and the public interest would not be disserved by an injunction. *eBay*, 547 U.S. at 391; *Amgen Inc. v. Sanofi*, 872 F.3d 1367, 1381 (Fed. Cir. 2017), *cert. denied*, 139 S. Ct. 787 (2019). Failing to sustain that burden means that the district court "may not issue an injunction," and this Court cannot affirm it. *Amgen*, 872 F.3d at 1381.

The district court acknowledged that 10x is a much smaller company and Bio-Rad is a Goliath, and the sole product the injunction could protect accounts for "only 0.2% of [Bio-Rad's] $2 billion in sales." Appx65-66. But the court found the balance of hardships "weighs in favor of granting injunctive relief, or, at minimum, is neutral," Appx65-66, based on (1) 10x's ability to sell a limited set of new, non-infringing products and (2) Bio-Rad's investments in "droplets" broadly. But the district court ignored that 10x *does not* have a new design for two of its product lines. Appx28862. And the district court carried forward its misunderstanding of the relevant market in finding Bio-Rad's "droplet" investments (in ddPCR) relevant to the injunction analysis. The balance weighs strongly against an injunction here.

On the public interest, the court accepted Bio-Rad's argument that "[i]t is generally in the public interest to uphold patent rights." Appx66. That is insufficient to justify an injunction. *ActiveVideo Networks, Inc. v. Verizon Commc'ns, Inc.*, 694 F.3d 1312, 1341 (Fed. Cir. 2012); *see eBay*, 547 U.S. at 392-93. The undisputed evidence was that scientists need 10x's products to do their research, and that Bio-Rad's (and the larger market) do not meet that need. Appx28857-28859, Appx28862-

28868. 10x should not be enjoined from providing the scientific community with the tools necessary to carry out life-saving research into the causes and cures for devastating human diseases. *See Cordis Corp. v. Bos. Sci. Corp.*, 99 F. App'x 928, 935 (Fed. Cir. 2004).

CONCLUSION

For the foregoing reasons, the Court should reverse the judgment of infringement and vacate the damages award and injunction or, at a minimum, remand for a new trial.

Respectfully submitted,

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October 18, 2019

ADDENDUM

UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

BIO-RAD LABORATORIES, INC. and THE UNIVERSITY OF CHICAGO Plaintiffs, v. 10X GENOMICS, INC. Defendant.	Civ. A. No. 15-152-RGA

FINAL JUDGMENT

This 15th day of August 2019, the Court having held a jury trial, and the jury having rendered a verdict, pursuant to Fed. R. Civ. P. 58(b)(2), IT IS HEREBY ORDERED that:

Judgment in the amount of $34,475,069 is entered for Plaintiffs Bio-Rad Laboratories, Inc. and The University of Chicago and against Defendant 10X Genomics, Inc. on the Second, Third, and Fifth Counts of the Third Amended Complaint. (D.I. 85). This includes the $23,930,718 verdict award, $8,341,368 in supplemental damages through the date of verdict, and $2,202,983 in interest through August 15, 2019. Judgment is further entered in the amount of $1,681 per day for Plaintiffs Bio-Rad Laboratories, Inc. and The University of Chicago and against Defendant 10X Genomics, Inc. in post-judgment interest that will accrue from this day forward until the monetary judgment is fully paid.

Judgment is entered for Plaintiffs Bio-Rad Laboratories, Inc. and The University of Chicago and against Defendant 10X Genomics, Inc. on the Third, Fourth, Fifth, Sixth, Ninth, and Tenth Counterclaims of 10X Genomics, Inc.'s Answer and Counterclaims to Plaintiffs' Third Amended Complaint. (D.I. 87).

All other claims and counterclaims are dismissed and the parties take nothing from them.

United States District Judge

UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

BIO-RAD LABORATORIES, INC. and THE UNIVERSITY OF CHICAGO Plaintiffs, v. 10X GENOMICS, INC. Defendant.	Civ. A. No. 15-152-RGA

[AMENDED ~~PROPOSED~~] PERMANENT INJUNCTION

WHEREAS the Court has found that defendant 10X Genomics, Inc. ("10X") has infringed claims 1 and 9 of plaintiffs Bio-Rad Laboratories, Inc. and The University of Chicago's (collectively, "Plaintiffs") U.S. Patent No. 8,889,083 (the "'083 Patent"), claims 6 and 8 of Plaintiffs' U.S. Patent No. 8,304,193 (the "'193 Patent"), and claims 1, 10, and 11 of Plaintiffs' U.S. Patent No. 8,329,407 (the "'407 Patent") (collectively, the "'083 Patent," "'193 Patent," and "'407 Patent" shall be referred to as the "Patents In-Suit");

WHEREAS, the Court has found that Plaintiffs will suffer irreparable harm if 10X continues its infringement, that monetary damages cannot adequately compensate Plaintiffs for this resulting irreparable harm, that the balance of equities weighs in favor of granting injunctive relief, or, at minimum, is neutral, and that public interest weighs in favor of granting a permanent injunction;

NOW THEREFORE, having considered the entire record in this action, the verdict of the jury, relevant orders of the Court, and the papers submitted by the parties, and good cause having been shown:

I. PROHIBITED ACTIVITIES – '083 AND '407 PATENTS

IT IS HEREBY ORDERED that, except in connection with the Permitted Activities provided in Section III, defendant 10X and any of its officers, agents, servants, employees, attorneys, and persons or entities in active concert or participation with them, who receive actual notice of this Permanent Injunction, are permanently enjoined and restrained from infringing, or inducing or contributing to, the infringement of claims 1 and 9 of the '083 Patent and claims 1, 10, and 11 of the '407 Patent (collectively, the "'083 and '407 Asserted Claims") from the Effective Date (which is fourteen (14) days from the date of this signed Permanent Injunction) until these Patents' expiration, by:

(a) using within the United States any product that infringes the '083 and '407 Asserted Claims, including without limitation the Chromium Genome/Exome, GemCode Long Read, Chromium Single Cell 3', or Chromium Single Cell V(D)J systems (collectively, the "'083 and '407 Accused Products"), and those no more than colorably different;

(b) actively inducing infringement of the '083 and '407 Asserted Claims by 10X's United States customers of the '083 and '407 Accused Products;

(c) contributing to infringement of the '083 and '407 Asserted Claims by selling within the United States the '083 and '407 Accused Products, products no more than colorably different, or their components where such components are especially made or especially adapted for use in an infringement of such patents, and are not a staple article or commodity of commerce suitable for substantial noninfringing use; and/or

(d) supplying from the United States for combination abroad any component especially made or especially adapted for use in claims 1 and 9 of the '083 Patent including the '083 Accused Products, products no more than colorably different, or their components where such components are not a staple article or commodity of commerce suitable for substantial noninfringing use.

None of the above prohibits 10X from making, using, or selling within the United States (or supplying from the United States) components of the '083 and '407 Accused Products for a non-infringing use.

II. PROHIBITED ACTIVITIES – '193 PATENT

IT IS FURTHER HEREBY ORDERED that, except in connection with the Permitted Activities provided in Section III, defendant 10X and any of its officers, agents, servants, employees, attorneys, and persons or entities in active concert or participation with them, who receive actual notice of this Permanent Injunction, are permanently enjoined and restrained from infringing, or inducing or contributing to, the infringement of claims 6 and 8 of the '193 Patent (collectively, the "'193 Asserted Claims") from the Effective Date until the expiration of the '193 Patent, by:

(a) using within the United States any product that infringes the '193 Asserted Claims, including without limitation the Chromium Genome/Exome and GemCode Long Read systems (collectively, the "'193 Accused Products") (collectively, the '083 and '407 Accused Products and '193 Accused Products shall be referred to as the "Enjoined Products"), and those no more than colorably different;

(b) actively inducing infringement of the '193 Asserted Claims by 10X's United States customers of the '193 Accused Products and those no more than colorably different; and/or

(c) contributing to infringement of the '193 Asserted Claims by selling within the United States the '193 Accused Products, products no more than colorably different, or their components where such components are especially made or especially adapted for use in an infringement of such patent and are not a staple article or commodity of commerce suitable for substantial noninfringing use.

None of the above prohibits 10X from making, using, or selling within the United States (or supplying from the United States) components of the '193 Accused Products for a non-infringing use.

III. HISTORICAL INSTALLED BASE

The Prohibited Activities of Sections I and II do not apply to consumables for use with the (i) the '083 and '407 Accused Products and components thereof, (ii) the '193 Accused Products and components thereof, and (iii) products not colorably different from those that are sold or in use before the Effective Date of this injunction (collectively, the "Historical Installed Base"), as set forth below. Without violating this Permanent Injunction, 10X (and any of its officers, agents, servants, employees, attorneys, customers, vendors, sales agents (including third party resellers and distributors), and persons or entities in active concert or participation with them) may also continue to support, service, repair, and replace under warranty[1] the Historical Installed Base.

[1] If 10X charges for a replacement under warranty, the revenue for that replacement will be subject to the 15% escrow deposit provisions below.

This authorization of the sale of consumables that would otherwise be prohibited under the Prohibited Activities in Sections I and II above for use with the Historical Installed Base ("Permitted Historical Installed Base Sales") is conditional on 10X depositing into an interest-bearing escrow account a 15% royalty on the net revenue 10X receives from the Permitted Historical Installed Base Sales until the expiration of the Patents In-Suit. These deposits shall be made within forty-five (45) days after March 31, June 30, September 30, or December 31 of a given calendar year. Plaintiffs shall have a right to a quarterly royalty report in which 10X shall identify the aggregate amount of Permitted Historical Installed Base Sales and how it performed its royalty calculation and an annual accounting audit. If Plaintiffs request an annual accounting audit, the audit will be conducted during regular business hours by an independent, third-party auditor and only for the purpose of verifying 10X's royalty statements and payments under this provision. The independent auditor shall be required to keep confidential all information received during any such inspection. Nothing in this injunction is an acknowledgement that 10X's actions do not violate other Bio-Rad rights.

The determination of the on-going royalty (if any) for the sales governed by this Section III (including the post-verdict, pre-injunction infringing sales) is SEVERED AND STAYED. The deposits required by this section do not prejudice the parties' ability to propose and pursue a different royalty rate before this court or on appeal or to argue that such royalties are not proper. If the royalty amount, rate, or base are altered or the reasonable royalty finding is otherwise vacated or modified on appeal and/or based on this Court's determination of the appropriate ongoing royalty following appeal, necessary refunds or supplements will be made including appropriate interest.

IV. FUTURE INSTRUMENT SALES

If, after the Effective Date, 10X sells instruments that are otherwise capable of operating with 10X consumables that have been found to infringe, 10X shall ensure that before such sale they have verifiably installed firmware on all such instruments to preclude them from use in an infringing way with such consumables or consumables not colorably different. Such firmware may be user-modifiable for upgrades provided by 10X but must not be user-modifiable in a way that would allow users to modify the firmware to permit such instruments to use in an infringing way consumables that have been found to infringe or consumables no more than colorably different. This provision (and this Permanent Injunction in general) is not an acknowledgment by Plaintiffs that any of 10X's activities do not violate Plaintiffs' other rights.

V. NOTICE

IT IS FURTHER ORDERED that, within five (5) business days from the Effective Date, 10X shall provide a copy of this Permanent Injunction to each customer, vendor, sales representatives (including third party resellers and distributors), employee and all other persons in active concert or participation with them as of the Effective Date.

IT IS FURTHER ORDERED that, within fourteen (14) days from the Effective Date, 10X shall file with the Court under seal and serve on all parties a notice stating the names and addresses of each party that it has notified in compliance with this section.

VI. CONTINUING JURISDICTION

The court specifically retains jurisdiction to enforce, modify, extend, or terminate this Permanent Injunction as the equities may require, upon a proper showing, and to adopt procedures for resolution of any dispute whether a product not specifically covered by this Permanent Injunction is more than colorably different from the adjudged infringing products.

IT IS SO ORDERED.

Dated: August 14, 2019

The Honorable Richard G. Andrews
United States District Judge

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

BIO-RAD LABORATORIES INC. and THE UNIVERSITY OF CHICAGO, Plaintiffs, v. 10X GENOMICS, INC., Defendant.	No. 15-cv-152-RGA

ORDER

For the reasons set forth in the accompanying memorandum opinion, **IT IS HEREBY ORDERED** that Defendant's motion for judgment as a matter of law under Federal Rule of Civil Procedure 50(b), new trial under Federal Rule of Civil Procedure 59, and remittitur (D.I. 509) is **DENIED**.

Entered this 3 day of July 2019.

United States District Judge

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

BIO-RAD LABORATORIES INC. and THE UNIVERSITY OF CHICAGO,

Plaintiffs,

v. No. 15-cv-152-RGA

10X GENOMICS, INC.,

Defendant.

ORDER

For the reasons set forth in the accompanying memorandum opinion, **IT IS HEREBY ORDERED** that Plaintiffs' post-trial motion (D.I. 512) is **GRANTED** with respect to the permanent injunction, supplemental damages, and pre- and post-judgment interest. The motion is **DENIED** with respect to the attorneys' fees and enhanced damages.

Within five days, the parties shall submit, consistent with the accompanying memorandum opinion:

(1) A proposed final judgment, wherein Plaintiffs are awarded:

(a) Prejudgment supplemental damages for the period from July 1 to November 13, 2018, based on a 15% royalty;

(b) Prejudgment interest at the prime rate, compounded quarterly, applied to the total prejudgment damages including supplemental damages; and

(c) Post-judgment interest at the Treasury bill rate as defined in 28 U.S.C. § 1961(a), compounded annually, applied to the total prejudgment damages including prejudgment interest.

(2) A revised proposed permanent injunction, wherein:

(a) Defendant is not required to provide notice to companies to which it "intends in the future to directly or indirectly sell" the enjoined products; and

(b) The effective date of the permanent injunction is two weeks from its entry.

Entered this 24 day of July 2019.

/s/ Richard G. Andrews
United States District Judge

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

BIO-RAD LABORATORIES INC. and THE UNIVERSITY OF CHICAGO, Plaintiffs, v. 10X GENOMICS, INC., Defendant.	No. 15-cv-152-RGA

MEMORANDUM OPINION

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Attorneys for Defendant.

July 24, 2019

ANDREWS, U.S. DISTRICT JUDGE:

Presently before the Court is Plaintiffs' post-trial motion (D.I. 512). Plaintiffs seek a permanent injunction, attorneys' fees, enhanced damages, supplemental damages, and pre- and post-judgment interest. (*Id.*). I have reviewed the parties' briefing and the related *amicus curiae* submission from the Broad Institute, Inc. (D.I. 513, 524, 536, 522). For the following reasons, Plaintiffs' motion is **GRANTED** with respect to the permanent injunction, supplemental damages, and pre- and post-judgment interest, and **DENIED** with respect to the attorneys' fees and enhanced damages.

I. BACKGROUND

On February 12, 2015, RainDance Technologies, Inc. and the University of Chicago filed suit against 10X Genomics, Inc. alleging infringement of several patents. On May 30, 2017, Bio-Rad Laboratories Inc. substituted for RainDance. (D.I. 180). I held a jury trial from November 5 to 13, 2018.[1] Only three patents remained at issue—U.S. Patent Nos. 8,889,083 ("the '083 patent"), 8,304,193 ("the '193 patent"), and 8,329,407 ("the '407 patent"). (*See* D.I. 499). The jury found all three patents valid and infringed, that the infringement was willful, and that Plaintiffs were entitled to $23,930,718 in damages. (D.I. 476).

II. PERMANENT INJUNCTION

Courts "may grant injunctions in accordance with the principles of equity to prevent the violation of any right secured by patent, on such terms as the court deems reasonable." 35 U.S.C. § 283. "According to well-established principles of equity, a plaintiff seeking a permanent injunction must satisfy a four-factor test before a court may grant such relief." *eBay Inc. v. MercExchange, LLC*, 547 U.S. 388, 391 (2006). "A plaintiff must demonstrate: (1) that it

[1] I cite to the trial transcript as "Tr."

has suffered an irreparable injury; (2) that remedies available at law, such as monetary damages, are inadequate to compensate for that injury; (3) that, considering the balance of hardships between the plaintiff and defendant, a remedy in equity is warranted; and (4) that the public interest would not be disserved by a permanent injunction." *Id.* "The essential attribute of a patent grant is that it provides a right to exclude competitors from infringing the patent." *Acumed LLC v. Stryker Corp.*, 551 F.3d 1323, 1328 (Fed. Cir. 2008).

For the following reasons, I find that Plaintiffs have satisfied the *eBay* factors in support of their proposed permanent injunction. (D.I. 513, Ex. A).

A. Irreparable Injury

Direct competition strongly suggests the potential for irreparable harm absent an injunction. *Presidio Components, Inc. v. Am. Tech. Ceramics Corp.*, 702 F.3d 1351, 1363 (Fed. Cir. 2012) (vacating denial of a permanent injunction based on finding no irreparable injury because the record showed "direct and substantial competition between the parties"); *see also Douglas Dynamics, LLC v. Buyers Prod. Co.*, 717 F.3d 1336, 1345 (Fed. Cir. 2013) ("Where two companies are in competition against one another, the patentee suffers the harm—often irreparable—of being forced to compete against products that incorporate and infringe its own patented inventions."). A patentee may establish irreparable harm by showing "that [the parties] were competitors and that [the patentee] lost market share while [the infringer] gained it." *See Broadcom Corp. v. Emulex Corp.*, 732 F.3d 1325, 1338 (Fed. Cir. 2013) (upholding the district court's grant of a permanent injunction).

Plaintiffs argue that Bio-Rad and 10X are competitors in "the market for products that perform genetic analysis on a droplet platform," and within that market, they are "undisputedly head-to-head competitors with their single-cell droplet products." (D.I. 513 at 6-7). At trial,

10X's counsel stated that Bio-Rad's ddSEQ product competes directly with 10X's single cell product. Tr. at 92:3-7, 1519:4-6. Ms. Tumolo, Bio-Rad's President of Life Sciences, agreed. *Id.* at 168:4-8.

10X argues that Bio-Rad is not a direct competitor. 10X has five accused product lines,[2] each of which allegedly "profiles different aspects of a sample and provides fundamentally different biological information, using different chemistries, data analysis, and visualization software." (D.I. 524 at 7). Of those five, 10X argues that the only one that may compete with Bio-Rad is the single cell product, but that customers view Bio-Rad's product as inferior. (*Id.* at 5, 7-8).

I find that 10X and Bio-Rad are direct competitors in the market for products that perform genetic analysis on a droplet platform. 10X admitted at trial that its single cell product competes directly with Bio-Rad's ddSEQ. Tr. at 92:3-7. Each of 10X's products are variants of the same infringing droplet process. (*See* D.I. 536 at 4; D.I. 476). Regardless, 10X's single cell product accounts for over 80% of 10X's sales. (*See* D.I. 536 at 4; PTX 1255). The fact that customers may prefer 10X's single cell product to ddSEQ does not negate the fact that the products are competing.

Plaintiffs argue that, as direct competitors, 10X is causing Bio-Rad lasting competitive harm by using infringing technology to gain a lead in the emerging droplet market and derail Bio-Rad's product roadmap. (D.I. 513 at 7). Ms. Tumolo testified that Bio-Rad "felt a lot of pressure to get [its single cell] product on the market because 10X had a really, really big head start, frankly we felt using our technology." Tr. at 130:3-6. Plaintiffs assert that the same head

[2] There are six accused products: Chromium Genome/Exome, Chromium Genome/Exome with Kynar, GemCode Long Read, Chromium Single Cell 3', Chromium Single Cell 3' with Kynar, and Chromium Single Cell V(D)J with Kynar. (D.I. 476). 10X does not state which products belong to which product line. (D.I. 524 at 7).

start has allowed 10X to collaborate with early adopters and key opinion leaders and develop "sticky" customer relationships. (D.I. 513 at 7-8). As a result, 10X has cultivated a market bias towards its single cell product and Bio-Rad has been forced to increase its marketing costs. (*Id.* at 8).

Plaintiffs have shown that they will suffer irreparable competitive harm absent an injunction. Plaintiffs are being forced to compete with 10X's products that incorporate and infringe their own patented inventions. *See Douglas Dynamics*, 717 F.3d at 1345. Based on those infringing products, 10X has established a strong market lead over Bio-Rad—10X has sold over 1000 of its single cell units, while Bio-Rad has sold less than 100. (D.I. 398, Ex. Z, at K-2, J-3, J-4). It seems likely that, absent an injunction, Bio-Rad will struggle to regain its lost market share and will continue to suffer associated harms such as increased marketing costs.

The party seeking an injunction must also show a causal nexus between the infringement and the harm. The infringing features do not need to be the "exclusive or predominant reason" that consumers buy the accused products, but there must be "'some connection' between the patented features and the demand for [the accused] products." *Apple Inc. v. Samsung Elecs. Co.*, 809 F.3d 633, 642 (Fed. Cir. 2015). That is, "the patented features impact consumers' decisions to purchase the accused devices." *Id.*

10X argues that Plaintiffs have failed to show a causal nexus because they have not proven that "simply using droplets—as opposed to other, non-patented features—drives demand for, or contributes to the success of, any 10X products." (D.I. 524 at 5). 10X applies the wrong standard. Plaintiffs need not show that the patented features drive demand, but just that they "impact consumers' decisions to purchase the accused devices." *Apple*, 809 F.3d at 642; *Genband US LLC v. Metaswitch Networks Corp.*, 861 F.3d 1378, 1384-85 (Fed. Cir. 2017). The

patented droplet technology is the foundation of 10X's droplet products. In fact, 10X tried and failed with other methods of partitioning such as capsules and wells before moving to droplets. Tr. at 953:1-954:13; (D.I. 513 at 10). There is clearly "some connection" between the patented technology and the demand for 10X's products. Therefore, Plaintiffs have shown that they will suffer irreparable harm from 10X's infringement absent injunctive relief.

B. Remedies Available at Law

Damages are inadequate to compensate for loss of market share. *Douglas Dynamics*, 717 F.3d at 1345 ("[M]ere damages will not compensate for a competitor's increasing share of the market, a market which Douglas competes in, and a market that Douglas has in part created with its investment in patented technology."); *E.I. DuPont de Nemours & Co. v. Unifrax I LLC*, 2017 WL 4004419, at *5 (D. Del. Sept. 12, 2017) ("Monetary damages are inadequate to compensate Plaintiff here because Plaintiff would be forced to compete against a rival gaining market share with Plaintiff's technology."), *aff'd*, 921 F.3d 1060 (Fed. Cir. 2019). The underlying concerns are particularly strong here because 10X's infringement coincided with the emergence of the droplet market, thus allowing 10X to capture and define the market with its infringing technology. *See Illumina, Inc. v. Qiagen, N.V.*, 207 F. Supp. 3d 1081, 1093 (N.D. Cal. 2016) (finding the patentee would suffer irreparable harm in part because, being at a "crucial inflection point in the development of the market," the infringer would be allowed to "capture and define the market with pirated technology").

10X argues that damages are adequate based on quantifiable licensing fees derived from Bio-Rad's internal documents. (D.I. 524 at 13). I disagree. Although Bio-Rad seems to have had some interest in licensing the asserted patents (DTX 1481 at 26), they were never actually licensed. *Cf. Nichia Corp. v. Everlight Americas, Inc.*, 855 F.3d 1328, 1343 (Fed. Cir. 2017)

("[T]he fact of the grant of previous licenses, the identity of the past licensees, the experience in the market since the licenses were granted, and the identity of the new infringer all may affect the district court's discretionary decision concerning whether a reasonable royalty from an infringer constitutes damages adequate to compensate for the infringement.") (quoting *Acumed LLC v. Stryker Corp.*, 551 F.3d 1323, 1328 (Fed. Cir. 2008)).

Therefore, I find damages inadequate to compensate for 10X's infringement.

C. Balance of Hardships

When assessing the balance of hardships, it is appropriate for courts to consider "the parties' sizes, products, and revenue sources." *i4i Ltd. P'ship v. Microsoft Corp.*, 598 F.3d 831, 862-63 (Fed. Cir. 2010), *aff'd*, 564 U.S. 91 (2011). Not relevant, however, are the expenses incurred in creating the infringing products and the consequences to the infringer of its infringement, such as the cost of redesigning the infringing products. *Id.* at 863.

An injunction would prevent 10X from selling any of its current products. (D.I. 524 at 14). 10X thus argues that an injunction would devastate the company, possibly causing it to go out of business. It is clear, however, that 10X has been pursuing a design-around for some time. At the September 5, 2018 discovery conference, 10X's counsel represented that the components of the new design were complete but the "full commercialized product" was not. (D.I. 365 at 20:25-21:11). In January 2019, 10X's Dr. Schnall-Levin testified that 10X intended to have its redesign on sale in April 2019 and that 10X was "confident that the chip will work as well" as the existing product. (D.I. 537, Ex. 4 at 19:19-22, 25:17-25). His only caveat about an April launch was that 10X might not have "all the training materials" and "quite as much rigor around having naive users try [the product] out." (*Id.* at 25:25-26:7). Thus, it now being July 2019, I would expect 10X to be nearly ready, if not ready, to bring its design-around to market.

On the other hand, Bio-Rad is undoubtedly a much larger operation. Bio-Rad is a multibillion-dollar company with over 9,000 products. (D.I. 524 at 14 (citing Bio-Rad 10-Q at 29)). Bio-Rad's ddSEQ product accounted for only 0.2% of its $2 billion in sales in 2017. (*Id.* (citing D.I. 398, Ex. Z at J-2)). Those revenues, however, are greatly outstripped by Bio-Rad's investments in its droplet business. Ms. Tumolo testified that Bio-Rad has spent over half a billion dollars to develop its droplet products, including acquisitions and $20 to $25 million a year on research and development. Tr. at 121:6-22, 125:23-126:5.

"[O]ne who elects to build a business on a product found to infringe cannot be heard to complain if an injunction against continuing infringement destroys the business so elected." *Windsurfing Int'l Inc. v. AMF, Inc.*, 782 F.2d 995, 1003 (Fed. Cir. 1986). The fact that 10X has gained commercial success from its infringing products and thus risks losing that success does not shield 10X from injunctive relief. *See i4i*, 598 F.3d at 863 (finding the defendant "not entitled to continue infringing simply because it successfully exploited its infringement"); *Broadcom Corp. v. Qualcomm Inc.*, 543 F.3d 683, 704 (Fed. Cir. 2008) (same). Regardless, given that 10X has a design-around that is complete or very close to complete, I do not think 10X is likely to be "devastated" if enjoined from selling its existing products. On the other hand, although Bio-Rad's ddSEQ currently accounts for only a fraction of Bio-Rad's revenues, Bio-Rad has invested substantial resources in developing its droplet business. Therefore, I find the balance of hardships weighs in favor of granting injunctive relief, or, at minimum, is neutral.

D. Public Interest

It is generally in the public interest to uphold patent rights. *Broadcom*, 543 F.3d at 704 (citing *Rite-Hite Corp. v. Kelley Co.*, 56 F.3d 1538, 1547 (Fed. Cir. 1995)). However, "[i]f a patentee's failure to practice a patented invention frustrates an important public need for the

invention, a court need not enjoin infringement of the patent. Accordingly, courts have in rare instances exercised their discretion to deny injunctive relief in order to protect the public interest." *Rite-Hite*, 56 F.3d at 1547 (internal citations omitted).

10X's main argument is that its customers, many of whom are in the middle of long-term studies, would lose valuable data and funding if forced to stop using their 10X systems and switch to new systems mid-study. (D.I. 524 at 15-17); *see also* D.I. 522, Ex. 1. That argument would be compelling if it were true. Plaintiffs have made clear that 10X's current customers will not be enjoined from using their installed systems so long as 10X pays the appropriate damages. (D.I. 536 at 1, 8-9). "By excluding users who purchased or licensed infringing [10X] products before the injunction's effective date, the injunction greatly minimizes adverse effects on the public." *See i4i*, 598 F.3d at 863 (upholding the district court's finding that the public interest favored injunctive relief). To extent that the public may be harmed because there are no current alternatives to 10X's products, both 10X and Bio-Rad have indicated that they will be releasing new products soon. As discussed, 10X's design-around is largely complete and expected to work as well as its existing products. *See supra* Section II.C. Bio-Rad has also asserted that it expects to release a new system this year "to leap-frog 10X in performance." (D.I. 513 at 8). Therefore, I find the public interest weighs in favor of granting injunctive relief.

E. Scope of the Permanent Injunction

1. Enjoined Products and Notice of Injunction

Plaintiffs request that 10X be enjoined from making, selling, offering to sell, using and importing the accused products and "those no more than colorably different," and from otherwise infringing the '083, '193, and '407 patents. (D.I. 513 at 12 & Ex. A). As discussed, Plaintiffs agree that 10X may continue to sell consumables, at a 15% royalty, for use with already sold

systems. (D.I. 536 at 9). Plaintiffs also include a notice provision requiring 10X to provide a copy of the injunction to all affiliates, including customers and "any company to which 10X intends in the future to directly or indirectly sell" the enjoined products. (D.I. 513, Ex. A § III).

10X asserts that Plaintiffs' proposed injunction is overbroad. First, 10X argues that its existing instruments and reagents, when combined with its new redesigned chips, will not infringe the asserted patents. Therefore, 10X should not be enjoined from making, using, or selling its existing instruments and reagents for use with the new chips. (D.I. 524 at 17-18). 10X essentially asks the Court to find that its new redesigned product does not infringe the asserted patents. An injunction has satisfactory scope if it prohibits infringement by the accused products and those that are not "colorably different." *United Constr. Prod., Inc. v. Tile Tech, Inc.*, 843 F.3d 1363, 1371 (Fed. Cir. 2016) (discussing Federal Rule of Civil Procedure 65(d)). Whether 10X's new product is "colorably different" is a separate legal issue that has yet to be addressed and which may never need to be addressed.

Second, 10X argues that it should be able to sell its products that do not compete against Bio-Rad's ddSEQ product. (D.I. 524 at 18). I do not think that is an appropriate distinction for purposes of the injunction. The parties are competitors in the droplet market, *see supra* Section II.A, and each of 10X's accused products uses the same infringing droplet system.

Third, 10X argues that Bio-Rad's notice provision is unduly burdensome and unnecessary. 10X argues that it should not be required to give notice to "its thousand customers" and companies to which it intends to sell enjoined products, because customers will not be able to practice the asserted patents once 10X stops selling its consumables (reagents and chips). (D.I. 524 at 18). 10X will not be enjoined, however, from continuing to sell consumables to customers with existing systems. Given that 10X almost certainly maintains customer lists, I do

not think it will be unduly burdensome for 10X to make reasonable efforts to provide its existing customers with notice. *See Power Integrations, Inc. v. Fairchild Semiconductor Int'l, Inc.*, 2008 WL 5210843, at *2 (D. Del. Dec. 12, 2008) (upholding the notice provision of a permanent injunction). However, I do not see why 10X should be required to provide notice to customers to which it "intends in the future" to sell the accused products. 10X will be enjoined from making any sales to new customers regardless of whether 10X had intended to make those sales or not.

2. Start Date

10X requests that if an injunction is entered, it be stayed pending appeal. (D.I. 524 at 19-20). Four factors guide the stay analysis: "(1) whether the stay applicant has made a strong showing that he is likely to succeed on the merits; (2) whether the applicant will be irreparably injured absent a stay; (3) whether issuance of the stay will substantially injure the other parties interested in the proceeding; and (4) where the public interest lies." *Standard Havens Prod., Inc. v. Gencor Indus., Inc.*, 897 F.2d 511, 512 (Fed. Cir. 1990) (quoting *Hilton v. Braunskill*, 481 U.S. 770, 776 (1987)). As discussed, Plaintiffs have shown that they will be irreparably injured absent an injunction and that the public interest lies in their favor. *See supra* Sections II.A, II.D. 10X argues that it is likely to succeed on the merits on appeal. (D.I. 524 at 19-20). I disagree. 10X merely repeats two arguments from its Rule 50(b) motion for judgment as a matter of law. (D.I. 510 at 1-9). I addressed those arguments in my opinion denying 10X's motion. (D.I. 559 at 7-10, 13-15).[3] Therefore, I find the factors weigh against a stay.

At minimum, 10X requests a nine-month sunset period to allow it to finish its design-around and for researchers to complete their ongoing experiments and transition to the new system. (D.I. 524 at 20-22). Again, because customers with existing systems will be allowed to

[3] Although 10X raises a reasonable argument under the doctrine of equivalents, *see infra* p. 17, I do not think it has made a "strong showing" of likely success on appeal.

continue to use those systems, the injunction does not need to account for ongoing experiments. As for the design-around, 10X stated in September 2018 that the design was complete, and in January 2019 that it intended for the new product to be on sale in April 2019. It is now July 2019. It would follow that 10X is ready, or nearly ready, to sell its design-around. *See supra* Section II.C. To allow 10X an additional nine months would be a windfall. Therefore, I do not think a sunset period is warranted.

Thus, I will deny the request for stay pending appeal. However, I will delay the effective date of the permanent injunction by two weeks from its entry in order to give the Court of Appeals an opportunity to consider any expedited appeal relating to the denial of the stay.

III. ATTORNEYS' FEES AND ENHANCED DAMAGES

A. Attorneys' Fees

"The court in exceptional cases may award reasonable attorney fees to the prevailing party." 35 U.S.C. § 285. "[A]n 'exceptional' case is simply one that stands out from others with respect to the substantive strength of a party's litigating position (considering both the governing law and the facts of the case) or the unreasonable manner in which the case was litigated. District courts may determine whether a case is 'exceptional' in the case-by-case exercise of their discretion, considering the totality of the circumstances." *Octane Fitness, LLC v. ICON Health & Fitness, Inc.*, 572 U.S. 545, 554 (2014). In assessing the totality of the circumstances, the Court may consider "frivolousness, motivation, objective unreasonableness (both in the factual and legal components of the case) and the need in particular circumstances to advance considerations of compensation and deterrence." *Id.* at 554 n.6. The party seeking fees must show that a case is exceptional by a preponderance of the evidence. *Id.* at 557-58.

Plaintiffs argue that this is an exceptional case because 10X willfully infringed, had unusually weak defenses, and engaged in substantial litigation misconduct. (D.I. 513 at 13-22). For the following reasons, I do not find this case to be exceptional.

1. Willful Infringement

Plaintiffs argue that the jury's willfulness finding favors awarding fees, particularly given 10X's "contrived and baseless" attempts to rebut willfulness. (D.I. 513 at 13-14).

Although willfulness is a factor relevant to an exceptional case determination, it is not dispositive. *See Octane Fitness*, 572 U.S. at 554 ("[T]here is no precise rule or formula for making [exceptional case] determinations, but instead equitable discretion should be exercised") (internal citation and quotation marks omitted); *Energy Heating, LLC v. Heat On-The-Fly, LLC*, 889 F.3d 1291, 1307 (Fed. Cir. 2018) (citing case law from 1986 requiring "an explanation of why the case was not exceptional in the face of an express finding of willful infringement").

Plaintiffs focus on testimony from Dr. Ness, co-founder and former Chief Technology Officer of 10X, which 10X presented as part of its rebuttal to willfulness. (D.I. 513 at 13-14). Dr. Ness initially stated that he formed the personal view that 10X's products, because of their high number of partitions, did not infringe the asserted patents. Tr. at 930:8-18. He later admitted that he did not have any belief as to whether the products infringed while at 10X. *Id.* at 934:22-935:5. Therefore, he could not testify as to 10X's state of mind to rebut Plaintiffs' allegation of willful infringement. Dr. Ness's testimony does not support awarding fees. At most, Plaintiffs have shown that the testimony was irrelevant to willfulness, an issue for which Plaintiffs bore the burden of proof. That does not make this an exceptional case.

2. Strength of Defenses

Plaintiffs assert that 10X relied on unsupportable invalidity and non-infringement defenses. Plaintiffs take a "kitchen sink" approach, arguing that none of the following invalidity theories were viable: (1) the '193 and '407 patents are anticipated by, or obvious in view of, the Quake reference, (2) the '193 and '407 patents are not enabled, and (3) the '083 patent is indefinite. Plaintiffs, however, did not move for summary judgment on any of 10X's invalidity positions presented at trial, which suggests that Plaintiffs did not always view those positions as frivolous. (D.I. 524 at 13); *see also Stragent, LLC v. Intel Corp.*, 2014 WL 6756304, at *5 (E.D. Tex. Aug. 6, 2014). Plaintiffs further assert that 10X did not have any legally cognizable non-infringement theories. I do not think Plaintiffs have shown that 10X's theories were frivolous, unreasonable, or brought in bad faith.

First, Plaintiffs address 10X's theory that the '193 and '407 patents are invalid in view of the Quake reference. Plaintiffs assert that the same theory was previously rejected by the Patent Office, which declined to institute an IPR on either patent and upheld the validity of both on *ex parte* reexamination. (D.I. 513 at 15; D.I. 26 at 2; D.I. 378). Although the Patent Office did consider invalidity arguments based on Quake, 10X asserts that its theories at trial relied on Quake in combination with other references not considered by the Patent Office. (D.I. 524 at 23). It was not unreasonable for 10X to argue invalidity based on those different combinations.

Plaintiffs also argue that 10X's expert, Dr. Chang, presented "half-baked" invalidity arguments and made no effort to show reasonable expectation of success in combining the prior art references. (D.I. 513 at 15). I disagree. Dr. Chang provided reasonable explanations for his theories and gave testimony relating to reasonable expectation of success. Tr. at 968:20-1017:8; *id.* at 983:20-984:14, 998:6-23, 999:1-20.

Second, Plaintiffs address 10X's argument that Dr. Ismagilov, an inventor of the asserted patents, copied portions of those patents from Quake. (D.I. 513 at 15-16). The Court allowed 10X to proceed with its copying theory for the limited purpose of impeaching Dr. Ismagilov's credibility as witness. (D.I. 429). Plaintiffs argue that 10X blatantly ignored the Court's order and attempted to use the copying as direct evidence of invalidity. During openings, 10X stated that the language in the asserted patents was "copied essentially verbatim" from Quake. Tr. 99:24-101:23. 10X failed to mention that the copying only related to Dr. Ismagilov's credibility, despite representing that it would be "very explicit." *Id.* at 7:20-24, 108:20-24. While questioning Dr. Ismagilov, however, 10X did make clear that it was addressing "the issue of credibility." *Id.* at 306:18-327:3, 328:6-330:4. The Court also followed 10X's questioning with a limiting instruction. *Id.* at 327:4-328:5 ("[A]ll this testimony has been for a very limited purpose and it has only to do with . . . evaluating Professor Ismagilov's credibility in his testimony."). Therefore, although close, I do not think 10X crossed the line with respect to its copying theory.

Third, Plaintiffs address 10X's enablement and indefiniteness theories. (D.I. 513 at 16-17). 10X argued that the '193 and '407 patents are not enabled because they fail to teach reactions outside the microfluidic chip and the necessary surfactants, and that the '083 patent is indefinite because the claimed surface tension relationship is impossible to measure. Plaintiffs rely on select citations from the trial record to argue that 10X's positions were frivolous or unreasonable. (D.I. 513 at 16-17). I do not find Plaintiffs' arguments persuasive. Regarding enablement, Plaintiffs also imply that 10X acted in bad faith by presenting evidence that the claimed inventions required "bushy" surfactants. (*Id.* at 16). Plaintiffs assert that the "bushy" language was not presented during discovery and that 10X used new images at trial depicting its

surfactants as "bushier" than in internal documents prepared before litigation. (D.I. 513 at 16). Plaintiffs do not argue that 10X failed to disclose their legal theory of lack of enablement of the necessary surfactants. (*Id.* at 16-17). I do not think the use of the term "bushy" and related demonstratives at trial indicates bad faith.

Fourth, Plaintiffs argue that 10X had no non-infringement defenses. (D.I. 513 at 17-18). 10X presented non-infringement arguments for every accused product and asserted patent. Tr. at 1526:24-1536:7.[4] Regardless, if true, the fact that 10X did not have a non-infringement theory for each of the twelve pairings between asserted patents and accused products does not make this an exceptional case.[5] 10X's primary defense was invalidity.

The theories that 10X did present were not unreasonable. I denied summary judgment of infringement of the '407 patent because there was a material dispute of fact as to "whether the thermal cycler is part of the 'microfluidic system.'" (D.I. 351 at 4). I reiterated that finding in my *Daubert* opinion (D.I. 361 at 5), and later denied Plaintiffs' Rule 50(a) motion, Tr. at 1338:6-24. I do not think an argument that survived multiple challenges should be considered meritless.

For the '193 patent, 10X argued that the Chromium Genome/Exome product did not meet the "autocatalytic reaction" limitation because the Landlord reaction was not "autocatalytic." Tr. at 1120:25-1121:3; (D.I. 510 at 9-10). Despite having found that 10X relied on an incorrect reading of my claim construction, I do not think 10X's view was unreasonable. (D.I. 559 at 12-13).

[4] Plaintiffs assert, without citation, that 10X had no defense for infringement of the '193 patent by the GemCode Long Read product. (D.I. 513 at 18). That does not appear to be true. *See* Tr. at 1533:2-1534:12 (arguing the accused products do not meet the microfluidic system limitation in the '193 patent).

[5] Six products were accused of infringing the '083 patent, two products were accused of infringing the '193 patent, and four products were accused of infringing the '407 patent. (D.I. 476).

For the '083 patent, 10X applied its indefiniteness theory to argue that the accused products did not meet the claimed surface tension relationship because that relationship could not be measured. Although I disagree, I do not think that argument was unreasonable. (*See* D.I. 559 at 10-11, 27). 10X also argued that the products with Kynar did not meet the "non-fluorinated microchannel" limitation. That theory, at least regarding literal infringement, clearly had merit as the jury found no literal infringement. (D.I. 476 at 3). Further, although 10X lost under the doctrine of equivalents, I noted on several occasions that 10X had raised legitimate concerns regarding vitiation of the "non-fluorinated" limitation. Tr. at 379:3-8, 438:1-11; (D.I. 559 at 7-10).

3. Litigation Misconduct

"[L]itigation misconduct and unprofessional behavior may suffice, by themselves, to make a case exceptional under § 285." *Monolithic Power Sys., Inc. v. O2 Micro Int'l Ltd.*, 726 F.3d 1359, 1366 (Fed. Cir. 2013). "[M]any forms of misconduct can support a district court's exceptional case finding, including . . . litigation misconduct; vexatious, unjustified, and otherwise bad faith litigation; a frivolous suit; or willful infringement." *Id.* Plaintiffs argue that 10X engaged in a pattern of misconduct including, violation of the protective order, pursuit of "baseless positions," and misrepresentations during trial. (D.I. 513 at 18-22).

It is undisputed that 10X violated the protective order. (D.I. 524 at 26). I already addressed the issue and determined that the violation was not done in bad faith. (D.I. 350 at 33:24-34:11).[6]

[6] Plaintiffs make much of the fact that at the hearing over four months before trial, 10X represented that the violating attorney played a key role in its damages case. (D.I. 350 at 20:24-21:9). As Plaintiffs note, that attorney did not visibly participate in the trial. What Plaintiffs do not mention is that 10X switched lead trial counsel between the hearing and the trial. Regardless, 10X asserts that the attorney remained involved in preparing damages arguments. (D.I. 524 at 26).

As for the "baseless positions," Plaintiffs cite to several instances where the Court ruled against 10X. (D.I. 513 at 21). I do not think those instances rise to the level of litigation misconduct. Both parties, in the name of zealous advocacy, made innumerable arguments over the course of this litigation, some which were undoubtedly weak. The fact that Plaintiffs were able to identify a handful of 10X's weaker arguments does not show that 10X engaged in a pattern of misconduct.

Lastly, Plaintiffs point to several statements made by 10X's counsel during trial that allegedly mispresented key facts. (D.I. 513 at 21-22). Only two statements, both during closing, could possibly be significant—(1) "there's evidence in the record specifically from PTX 333 at Page 66 that the [10X] chips come in from Germany," Tr. at 1532:4-6, and (2) "Quake expressly says the fluids used in the invention may contain additive or surfactants such as fluorinated oil," *id.* at 1541:3-6.

I already addressed the Germany statement at length. *Id.* at 1572:2-1587:20. Plaintiffs argue that page 66 of PTX 333 was not discussed at trial or in evidence and was excluded by the Court's order on motions *in limine*. (D.I. 513 at 21). PTX 333 appears to be a document from other unrelated actions, which was likely within the scope of my order granting Plaintiffs' motion *in limine* to exclude reference to those actions. Tr. at 1577:2-10, 1587:16-18; (D.I. 371 at 1). However, there seemed to be a great deal of confusion over whether page 66 specifically had been admitted into evidence and I determined that neither party had acted improperly. Tr. at 1572:2-1587:20.

Regarding the Quake statement, Plaintiffs argue that 10X mischaracterized the evidence because Quake never uses fluorinated oil as a carrier fluid, "which is a crucial distinction from the invention." (D.I. 513 at 21). 10X's expert, Dr. Chang, testified that Quake "mentions

fluorinated oil," but does not disclose its use as a carrier fluid. He opined, however, that it would have been obvious to a person of ordinary skill in the art to use it as a carrier fluid. Tr. at 981:12-18. I do not think 10X's statements in closing are inconsistent with the testimony from Dr. Chang or an intentional misrepresentation of the record.

Therefore, considering the totality of the circumstances, Plaintiffs have failed to meet their burden of showing that this is an exceptional case warranting attorneys' fees.

B. Enhanced Damages

"[T]he court may increase the damages up to three times the amount found or assessed." 35 U.S.C. § 284. Section 284 "provid[es] that punitive or increased damages could be recovered in a case of willful or bad-faith infringement." *Halo Elecs., Inc. v. Pulse Elecs., Inc.*, 136 S. Ct. 1923, 1930 (2016) (quotation marks omitted). "The *Halo* test merely requires the district court to consider the particular circumstances of the case to determine whether it is egregious." *Presidio Components, Inc. v. Am. Tech. Ceramics Corp.*, 875 F.3d 1369, 1382 (Fed. Cir. 2017).

Although not required, the court may consider the *Read* factors as part of its analysis. *Presidio*, 875 F.3d at 1382 (citing *Read Corp. v. Portec, Inc.*, 970 F.2d 816, 827 (Fed. Cir. 1992)). The *Read* factors include: (1) whether the infringer deliberately copied the ideas or design of another, (2) whether the infringer, when he knew of the other's patent protection, investigated the scope of the patent and formed a good-faith belief that it was invalid or that it was not infringed; (3) the infringer's behavior as a party to the litigation; (4) defendant's size and financial condition; (5) closeness of the case; (6) duration of defendant's misconduct; (7) remedial action by defendant; (8) defendant's motivation for harm; and (9) whether defendant attempted to conceal its misconduct. 970 F.2d at 827.

Plaintiffs do not address *Read* factors one or nine. (*See* D.I. 513 at 23). For factor one, 10X argues that testimony from Drs. Ness and Hindson[7] shows that 10X did not rely on the asserted patents to develop its products. (D.I. 524 at 28). In view of the jury's willful infringement verdict, which I upheld on JMOL, it is not clear to what extent 10X considered the asserted patents while developing its accused products. (*See* D.I. 559 at 22). However, it is clear that Plaintiffs have not identified any evidence of copying. For factor nine, it is undisputed that the accused products were freely available for purchase. (*Id.*). Thus, I find both factors one and nine weigh against enhanced damages.

For *Read* factor two, Plaintiffs argue that the jury found willful infringement and that 10X had no good-faith belief of non-infringement. (D.I. 513 at 23). I agree that, in view of the willful infringement verdict, 10X did not have a good-faith belief of non-infringement. (*See* D.I. 559 at 22). Thus, I find factor two weighs in favor of enhanced damages.

For *Read* factor three, Plaintiffs argue that 10X engaged in extensive litigation misconduct. (D.I. 513 at 23). As discussed, I do not think 10X's behavior rose to the level of misconduct. *See supra* Section III.A.3. Thus, I find factor three weighs against enhanced damages.

For *Read* factor four, Plaintiffs argue that 10X is the market leader and has made significant revenues from its infringing products. (D.I. 513 at 23). 10X admits that it has made tens of millions of dollars in yearly revenue but asserts that it has yet to turn a profit. (D.I. 524 at 29 (citing D.I. 246, Ex. 10 at C-1 (10X quarterly financials from 2015-2017))). Thus, I find factor four is neutral.

[7] Dr. Hindson is co-founder and Chief Scientific Officer of 10X. (D.I. 559 at 18).

For *Read* factor five, Plaintiffs argue that 10X presented weak defenses and that the jury quickly ruled in their favor.[7] (D.I. 513 at 23). As discussed, I do not think 10X's defenses were so weak as to be meritless. *See supra* Section III.A.2. In fact, the jury found in 10X's favor on literal infringement of the '083 patent for the products with Kynar. *Id.* Thus, I find factor five weighs against enhanced damages.

For *Read* factor six, Plaintiffs again argue that 10X engaged in litigation misconduct, which persisted throughout the case. (D.I. 513 at 23). Thus, for the same reasons as for factor three, I find factor six weighs against enhanced damages.

For *Read* factor seven, Plaintiffs argue that 10X has taken no post-trial remedial actions. (*Id.*). It is undisputed that 10X is actively working on a design-around product. *See supra* Section II.C. On the other hand, despite 10X's representations, nothing has been launched. Thus, I find factor seven is neutral.

For *Read* factor eight, Plaintiffs argue that the market for droplets is rapidly expanding and has the potential to be worth up to a billion dollars. (D.I. 513 at 23). I do not think evidence of market incentives is probative of motivation for harm warranting enhanced damages. "[T]he fact that the infringer acted pursuant to a financial motive does not distinguish this case from the garden-variety infringement case." *Idenix Pharm. LLC v. Gilead Scis., Inc.*, 271 F. Supp. 3d 694, 702 (D. Del. 2017), *appeal pending*, No. 18-1691 (Fed. Cir.) (citing *Sprint Commc'ns Co. L.P. v. Time Warner Cable, Inc.*, 2017 WL 978107, at *14 (D. Kan. Mar. 14, 2017)); *see also Nox Med. Ehf v. Natus Neurology Inc.*, 2018 WL 6427686, at *4 (D. Del. Dec. 7, 2018) (finding evidence of "harm incidental to direct business competition" did not show "motivation for harm"). Thus, I find factor eight is neutral.

[7] I do not think the length of jury deliberation is a meaningful metric.

On balance, the *Read* factors weigh against enhanced damages. Plaintiffs essentially repeat the arguments they made under § 285. Just as those arguments did not persuade me that this case is exceptional, they do not persuade me that the facts of this case are egregious.[8] Therefore, despite the jury's finding of willful infringement, I do not think enhanced damages are warranted.

IV. SUPPLEMENTAL DAMAGES AND INTEREST

Plaintiffs seek prejudgment supplemental damages and pre- and post-judgment interest. (D.I. 513 at 23-29). 10X only objects as to the interest rates that should apply. (D.I. 524 at 30).

The jury's damages award covered 10X sales through July 1, 2018. (*Id.* at 23). The supplemental damages account for the sales made from July 1, 2018 through the date of the judgment, November 13, 2018. (*Id.* at 24). The supplemental damages shall be calculated based on the jury's implied 15% royalty rate.[9] (D.I. 513 at 25; D.I. 515 ¶ 11).

Plaintiffs also seek interest on their damages. Prejudgment interest should be awarded "absent some justification for withholding such an award." *Gen. Motors Corp. v. Devex Corp.*, 461 U.S. 648, 657 (1983). The only dispute is over the interest rate and compounding period that should apply. Plaintiffs argue for the prime rate, compounded quarterly. (D.I. 513 at 25-28). 10X argues for the 1-year Treasury constant maturity rate, compounded annually. (D.I. 524 at 30; D.I. 527 ¶ 3). "A trial court is afforded wide latitude in the selection of interest rates and may award interest at or above the prime rate." *Uniroyal, Inc. v. Rudkin-Wiley Corp.*, 939 F.2d 1540, 1545 (Fed. Cir. 1991) (citations omitted). The decision to award compound interest is also within the trial court's discretion. *Rite-Hite Corp*, 56 F.3d at 1555 ("It has been recognized that

[8] I gave the jury a willfulness instruction that did not require any finding of "egregiousness" or the equivalent. (D.I. 470 at 29).

[9] The jury's lump sum damages award is based on Plaintiffs' proposed 15% rate. (D.I. 476); Tr. at 611:20-613:2.

an award of compound rather than simple interest assures that the patent owner is fully compensated.") (citation and quotation marks omitted).

This Court has noted that "the prime rate best compensate[s] a patentee for lost revenues during the period of infringement because the prime rate represents the cost of borrowing money, which is a better measure of the harm suffered as a result of the loss of the use of money over time." *Finjan Software, Ltd. v. Secure Computing Corp.*, 2009 WL 2524495, at *13 (D. Del. Aug. 18, 2009), *aff'd in part*, *rev'd in part on other grounds*, 626 F.3d 1197 (Fed. Cir. 2010) (citations and quotation marks omitted). As for the compounding period, the prior license agreements relied on by the parties' experts specify quarterly payments with interest compounded quarterly. (D.I. 513 at 28). Therefore, I find prejudgment interest should be calculated based on the prime rate, compounded quarterly.

Post-judgment interest is governed by 28 U.S.C. § 1961. Section 1961(a) provides, "Such interest shall be calculated from the date of the entry of the judgment, at a rate equal to the weekly average 1-year constant maturity Treasury yield, as published by the Board of Governors of the Federal Reserve System, for the calendar week preceding the date of the judgment."

Accordingly, the Court will award Plaintiffs: (1) prejudgment supplemental damages for the period from July 1 to November 13, 2018, based on a 15% royalty, (2) prejudgment interest at the prime rate, compounded quarterly, and (3) post-judgment interest at the Treasury bill rate as defined in § 1961(a), compounded annually. The prejudgment interest applies to the total prejudgment damages, including supplemental damages. The post-judgment interest applies to the total prejudgment damages plus prejudgment interest.

V. CONCLUSION

A separate order will be entered.

US008304193B2

(12) **United States Patent**
Ismagilov et al.

(10) **Patent No.: US 8,304,193 B2**
(45) **Date of Patent: Nov. 6, 2012**

(54) **METHOD FOR CONDUCTING AN AUTOCATALYTIC REACTION IN PLUGS IN A MICROFLUIDIC SYSTEM**

(75) Inventors: **Rustem F Ismagilov**, Chicago, IL (US); **Joshua David Tice**, Webster, NY (US); **Cory John Gerdts**, Chicago, IL (US); **Bo Zheng**, Chicago, IL (US)

(73) Assignee: **The University of Chicago**, Chicago, IL (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/024,155**

(22) Filed: **Feb. 9, 2011**

(65) **Prior Publication Data**

US 2011/0177609 A1 Jul. 21, 2011

Related U.S. Application Data

(63) Continuation of application No. 12/777,099, filed on May 10, 2010, which is a continuation of application No. 10/765,718, filed on Jan. 26, 2004, now Pat. No. 7,901,939, which is a continuation-in-part of application No. 10/434,970, filed on May 9, 2003, now Pat. No. 7,129,091.

(60) Provisional application No. 60/394,544, filed on Jul. 8, 2002, provisional application No. 60/379,927, filed on May 9, 2002.

(51) **Int. Cl.**
C12Q 1/68 (2006.01)

(52) **U.S. Cl.** **435/6.12**; 435/6.19; 436/535; 436/164; 436/172

(58) **Field of Classification Search** ..... None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,960,187	A	6/1976	Stock et al.	
4,853,336	A	8/1989	Saros et al.	
4,963,498	A	10/1990	Hillman et al.	
5,185,099	A	2/1993	Delpuech et al.	
5,739,036	A	4/1998	Parris	
5,872,010	A	2/1999	Karger et al.	
5,887,755	A	3/1999	Hood, III	
5,997,636	A	12/1999	Gamarnik et al.	117/70
6,118,849	A	9/2000	Tanimori et al.	
6,130,098	A	10/2000	Handique et al.	
6,140,053	A	10/2000	Köster	435/6
6,180,372	B1	1/2001	Franzen	435/91.1
6,296,673	B1	10/2001	Santarsiero et al.	
6,409,832	B2	6/2002	Weigl et al.	

(Continued)

FOREIGN PATENT DOCUMENTS

CN 2482070 3/2002

(Continued)

OTHER PUBLICATIONS

Beer et al. "On-Chip, Real-Time, Single-Copy Polymerase Chain Reaction in Picoliter Droplets", Anal. Chem., 2007, v. 79, pp. 8471-8475.*

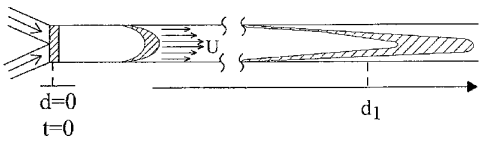
(Continued)

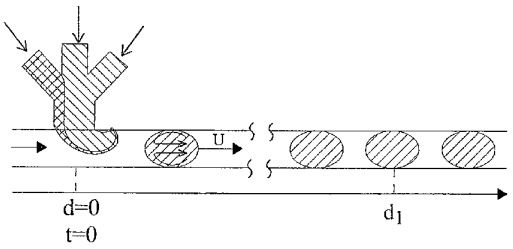
Primary Examiner — Yelena G Gakh
(74) *Attorney, Agent, or Firm* — Brinks Hofer Gilson & Lione

(57) **ABSTRACT**

The present invention provides microfabricated substrates and methods of conducting reactions within these substrates. The reactions occur in plugs transported in the flow of a carrier-fluid.

14 Claims, 63 Drawing Sheets





PLAINTIFFS' TRIAL EXHIBIT
PTX003
C.A. No. 15-152-RGA

RDTX00001630

PTX003-001

U.S. PATENT DOCUMENTS

6,569,631	B1	5/2003	Pantoliano et al.	
6,630,006	B2	10/2003	Santarsiero et al.	
6,656,267	B2	12/2003	Newman	
6,766,817	B2	7/2004	Silva	
6,790,328	B2	9/2004	Jacobson et al.	
6,797,056	B2	9/2004	David	
6,872,250	B2	3/2005	David et al.	
7,041,481	B2	5/2006	Anderson et al.	
7,129,091	B2	10/2006	Ismagilov et al.	
7,156,917	B2	1/2007	Moriyama et al.	
7,294,503	B2	11/2007	Quake et al.	
2001/0048900	A1	12/2001	Bardell et al.	
2002/0012971	A1	1/2002	Mehta	435/91.2
2002/0058332	A1	5/2002	Quake et al.	
2002/0067800	A1	6/2002	Newman et al.	
2003/0061687	A1	4/2003	Hansen et al.	
2003/0064414	A1	4/2003	Benecky et al.	
2003/0229376	A1	12/2003	Sandhu	
2004/0005582	A1	1/2004	Shipwash	
2004/0037813	A1	2/2004	Simpson et al.	
2004/0136497	A1	7/2004	Meldrum et al.	
2004/0181131	A1	9/2004	Maynard et al.	
2004/0224419	A1	11/2004	Zheng et al.	436/69
2004/0258203	A1	12/2004	Yamano et al.	
2005/0170431	A1	8/2005	Ibrahim et al.	
2006/0094119	A1	5/2006	Ismagilov et al.	
2006/0257893	A1*	11/2006	Takahashi et al.	435/6
2007/0026439	A1	2/2007	Faulstick et al.	
2009/0021728	A1	1/2009	Heinz et al.	
2009/0060797	A1	3/2009	Mathies et al.	
2010/0022414	A1	1/2010	Link et al.	

FOREIGN PATENT DOCUMENTS

EP	0 912 238	B1	10/2001
GB	2 097 692	A	11/1982
WO	WO 84/02000		5/1984
WO	WO 98/02237		1/1998
WO	WO 98/52691		11/1998
WO	WO 01/12327	A1	2/2001
WO	WO 01/64332	A1	9/2001
WO	WO 02/23163	A1	3/2002
WO	WO2004/038363		5/2003
WO	PCT/US2006/009175		3/2006

OTHER PUBLICATIONS

Gu et al. "Droplets Formation and Merging in Two-Phase Flow Microfluidics", Int. J. Mol. Sci., 2011, v. 12, pp. 2572-2597.*

Thorsen et al. "Dynamic Pattern Formation in a Vesicle-Generating Microfluidic Device", Phys. Rev. Lett., 2001, v. 86, No. 18, pp. 4163-4166.*

Atencia and Beebe "Controlled microfluidic interfaces", Nature, 2005, v. 437, No. 29, pp. 648-655.*

Shestopalov, I., Tice, J. D. and Ismagilov, R. F. Multi-step synthesis of nanoparticles performed on millisecond time scale in a microfluidic droplet-based system. Lab Chip, 2004, v. 4, pp. 316-321.*

Tanaka, H. et al., "*Ethanol Production from Starch by a Coimmobilized Mixed Culture System of Aspergillus awamori and Zymomonas mobilis*", Biotechnology and Bioengineering, vol. XXVII, 1986, pp. 1761-1768.

De-Bashan, L. E. et al., "*Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae Chlorella vulgaris coimmobilized in alginate beads with the microalgae growth-promoting bacterium Azospirillum brasilense*", Water Research 36 (2002), pp. 2941-2948.

Goodall, J. L. et al., "*Operation of Mixed-Culture Immobilized Cell Reactors for the Metabolism of Meta- and Para-Nitrobenzoate by Comamonas* Sp. JS46 *and Comamonas* Sp. JS47", Biotechnology and Bioengineering, vol. 59, No. 1, Jul. 5, 1998, pp. 21-27.

He, Mingyan et al., "*Selective Encapsulation of Single Cells and Subcellular Organelles into Picoliter- and Femtoliter-Volume Droplets*", Analytical Chemistry, vol. 77, No. 6, Mar. 15, 2005, pp. 1539-1544.

Huebner, A. et al., "*Quantitative detection of protein expression in single cells using droplet microfluidics*", Chem. Commun, 2007, pp. 1218-1220.

Seong, G.H. et al., "*Fabrication of Microchambers Defined by Photopolymerized Hydrogels and Weirs within Microfluidic Systems: Application to DNA Hybridization*", Analytical Chemistry, vol. 74, No. 14, Jul. 15, 2002, pp. 3372-3377.

Seong G.H. et al., "*Efficient Mixing and reactions within Microfluidic Channels Using Microbead-Supported Catalysts*", J. Am. Chem. Soc. 2002, 124, pp. 13360-13361.

Nisisako, T. et al., "*Formation of Droplets Using Branch Channels in a Microfluidic Circuit*", SICE 2002, Aug. 5-7, 2002, Osaka, pp. 1262-1264.

Chayen, N.E., "*Crystallization with oils: a new dimension in macromolecular crystal growth*", Journal of Crystal Growth 196 (1999), pp. 434-441.

Titomanlio et al., "*Capillary experiments of flow induced crystallization of HDPE*", AIChe Journal, 1990, v36, No. 1, pp. 13-18.

Garcia-Ruiz et al., "*Investigation on protein crystal growth by the gel acupuncture method*", Acta, Cryst., 1994, D50, 99. pp. 484-490.

Sugiura et al., "*Interfacial tension driven monodispersed droplet formation from microfabricated channel array*", Langmuir, 2001, v17, pp. 5562-5566.

Garcia-Ruiz et al. "*A super-saturation wave of protein crystallization*", J. Crystal Growth, 2001, v232, pp. 149-155.

Ng et al., "*Protein crystallization by capillary counter-diffusion for applied crystallographic structure determination*", J. Struct. Biol. 2003, v142, pp. 218-231.

Torkkeli et al., "*Droplet Manipulation on a Superhydrophobic Surface for Microchemical Analysis*", Transducers '01 Eurosensors XV, 11th International Conference on Solid-State Sensors and Actuators, Munich, Germany, Jun. 10-14, 2001, 4 pgs.

Anna, Shelley A. et al., "*Formation of Dispersions Using 'Flow Focusing' in Microchannels*", Applied Physics Letters, vol. 82, No. 3, 2003, pp. 364-366.

Auroux, Pierre-Alain et al., "*Micro Total Analysis Systems. 2. Analytical Standard Operations and Applications*", analytical Chemistry, vol. 74. No. 12, 2002, pp. 2637-2652.

Bico, Jose et al., "*Rise of Liquids and Bubbles in Angular Capillary tubes*", Journal of colloid and Interface Science, vol. 247, 202, pp. 162-166.

Bringer et al. "*Microfluidic System for Chemical Kinetics That Rely on Chaotic Mixing in Droplets*", Phil. Trans. R. Soc. Lond., pp. 1-18, 2004.

Burns, J. R. et al., "*The Intensification of Rapid Reactions in Multiphase systems Using Slug Flow in Capillaries*", Lab on a Chip, vol. 1, 2001, pp. 10-15.

Burns, Mark et al., "*An Integrated Nanoliter DNA Analysis Device*", Science, vol. 282, 1998, pp. 484-487.

Chan, Emory M. et al. "*Size-Controlled Growth of CdSe Nanocrystals in Microfluidic Reactors*", Nano Letters, vol. 3, No. 2, 2003, pp. 199-201.

Cho, Sung Kwon et al., "*Splitting a Liquid Droplet for Electrowetting-Based Microfluidics*", Proceedings of 2001 ASME International Mechanical Engineering Congress and Exposition, 2001, pp. 1-7.

Duffy, David C. et al., "*Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane)*", Analytical Chemistry, vol. 70, 1998, pp. 4974-4984.

Edel, Joshua B. et al., "*Microfluidic Routes to the Controlled Production of Nanoparticles*", Chemical Communications, 2002, pp. 1136-1137.

Eggers, Jens et al., "*Coalescence of Liquid Drops*", J. Fluid Mech., vol. 401, 1999, pp. 293-310.

Fowler, Jesse et al., "*Enhancement of Mixing by Droplet-Based Microfluidics*", 2002 Institute of electrical Engineers 15th International Conference on Micro Electro Mechanical Systems, 2002, pp. 97-100.

Gerdts et al., "*A Synthetic Reaction Network: Chemical Amplification Using Nonequilibrium Autocatalytic Reactions Coupled in Time*", J. Am. Chem. Soc., vol. 126, pp. 6327-6331, 2004.

Handique, K. et al., "*On-Chip Thermopneumatic Pressure for Discrete Drop Pumping*", Analytical Chemistry, vol. 73, 2001, pp. 1831-1838.

Hansen, Carl L. et al., "*A Robust and Scalable Microfluidic Metering Method That Allows Protein Crystal Growth by Free Interface Diffusion*", PNAS, vol. 99, No. 26, 2002, pp. 16531-16536.
Harries, N. et al., "A Numerical Model for Segmented Flow in a Microreactor", International Journal of Heat and Mass Transfer, vol. 46, 2003, pp. 3313-3322.
Hosokawa, Kazuo et al., "*Handling of Picoliter Liquid Samples in a Poly(dimethylsiloxane)-Based Microfluidic Device*", Analytical Chemistry, vol. 71, No. 20, 1999, pp. 4781-4785.
Ismagilov, "*Integrated Microfluidic Systems*", Agnew. Chem. Int. Ed., vol. 42, pp. 4130-4132.
Knight, James B., "*Hydrodynamic Focusing on a Silicon Chip: Mixing Nanoliters in Microseconds*", Physical Review Letters, vol. 80, No. 17, 1998, pp. 3863-3866.
Liu, Robin H. et al., "*Passive Mixing in a a Three-Dimensional Serpentine Microchannel*", Journal of Microelectromechanical Systems, vol. 9, No. 2, 2000, pp. 190-197.
McDonald, J. Cooper et al.,"*Fabrication of Microfluidic Systems in Poly(dimethylsiloxane)*" Electrophoresis, vol. 21, 2000, pp. 27-40.
Nisisako, Takasi et al. "*Droplet Formation in a Microchannel Network*", Lab on a Chip, vol. 2, 2002, pp. 24-26.
Pabit, Suzette A. et al., "*Laminar-Flow Fluid Mixer for Fast Fluorescence Kinetics Studies*", Biophysical Journal, vol. 83, 2002, pp. 2872-2878.
Peng, Shuangjiu et al., "*Controlled Production of Emulsions Using a Crossflow Membrane*", Particle & Particle Systems Characterization, vol. 15, 1998, pp. 21-25.
Reyes, Darwin R. et al., "*Micro Total Analysis Systems. 1. Introduction, Theory and Technology*", Analytic Chemistry, vol. 74, No. 12, 2002, pp. 2623-2636.
Shestopalov et al., *Multi-Step Synthesis of Nanoparticles Performed on Millisecond Time Scale in a Microfluidic Droplet-Based System*, The Royal Society of Chemistry, vol. 4, pp. 316-332, 2004.
Song et al., "*A Microfluidic System for Controlling Reaction Networks in Time*", Angew. Chem.. Int. Ed., vol. 42, No. 7, pp. 768-772, 2003.
Song et al., "*Experimental Test of Scaling of Mixing by Chaotic Advection in Droplets Moving Through Microfluidic Channels*", Applied Physics Letters, vol. 83, No. 22, pp. 4664-4666, 2003.
Song et al., "*Millisecond Kinetics on a Microfluidic Chip Using Nanoliters of Reagents*", J. Am. Chem. Soc., vol. 125, pp. 14613-14619, 2003.
Song, Helen et al., "*A Microfluidic System for Controlling Reaction Networks in Time*", Angew. Chem. Int. Ed., vol. 42, No. 7, 2003, pp. 768-772.
Stroock, Abraham D. et al., "*Chaotic Mixer for Microchannels*", Science, vol. 295, 2002, pp. 647-651.
Sugiura, Shinji et al., "*Effect of Channel Structure on Microchannel Emulsification*", Langmuir, vol. 18, 2002, pp. 5708-5712.
Takayama et al., "*Patterning Cells and Their Environments Using Multiple Lamina Fluid Flows in Capillary Networks*", Proc. Natl. Acad. Sci. USA, vol. 96, pp. 5545-5548, 1999.
Taniguchi, Tomohiro et al., "*Chemical Reactions in Microdroplets by Electrostatic Manipulation of Droplets in Liquid Media*", Lab on a Chip, Vo.. 2, 2002, pp. 19-23.
Thorsen et al., "*Microfluidic Large-Scale Integration*", Science, vol. 298, pp. 580-584, 2002.
Thorsen, Todd et al., "*Dynamic Pattern Formation in a Vesicle-Generating Microfluidic Device*", Physical Review Letters, vol. 86, No. 18, 2001, pp. 4163-4166.
Tice, "*Effects of Vicosity on Droplets and Mixing in Multiphase Microfluidics at Low Values of the Reynolds and the Capillary Numbers*", Langmuir, vol. 19, pp. 927-9133, 2003.
Tice et al., "*Formation of Droplets and Mixing in Multiphase Microfluids at Low Values of the Reynolds and the Capillary Numbers*", Langmuir, vol. 19, pp. 9127-9133, 2003.
Tokesh, Manabu et al., "*Continuous-Flow Chemical Processing on a Microchip by Combining Microunit Operations and a Multiphase Flow Network*", Analytical Chemistry, vol. 74, No. 7, 2002, pp. 1565-1571.
Umbanhowar, P.B. et al., "*PMonodisperse Emulsion Generation Via Drop Break Off in a Coflowing Stream*", Langmuir, vol. 16, 2000, pp. 347-351.
Wang, Hongzhi et al., "*Preparation of Titania Particles Utilizing the Insoluble Phase Interface in a Microchannel Reactor*", Chemical Communications, 2002, pp. 1462-1463.
Zhao, Bin et al., "*Control and Applications of Immiscible Liquids in Microchannels*", J. Am. Chem. Soc., vol. 124, 2002, pp. 5284-5285.
Zheng et al., "*A Droplet-Based, Composite PDMS/Glass Capillary Microfluidic System for Evaluating Protein Crystalization Conditions by Microbatch and Vapor-Diffusion Methods with On-Chip X-Ray Diffraction*", Angew. Chem., pp. 1-4, 2004.
Zheng et al., "*Screening of Protein Crystallization Conditions on a Microfluidic Chip Using Nanoliter-Size Droplets*", Journal of the American Chemical Society, vol. 125, No. 37, pp. 11170-11171, 2003.
Burns et al., "*Microfabricated structures for integrated DNA analysis*", Proc. Natl. Acad. Sci. USA, May 1996, vol. 93, pp. 5556-5561.
Pollack et al., "*Electrowetting-based actuation of droplets for integrated microfluidics*", Lab Chip, 2002, v. 2, pp. 96-101.
Vogelstein, et al., "*Digital PCR*", Proc. Natl. Acad. Sci. USA, Aug. 1999, vol. 96, pp. 9236-9241, Genetics.
Zheng et al., "*Formation of Droplets of Alternating Composition in Microfluidic Channels and Applications to Indexing of Concentrations in Droplet-Based Assays*", Anal. Chem., 2004, v. 76, pp. 4977-4982.

* cited by examiner

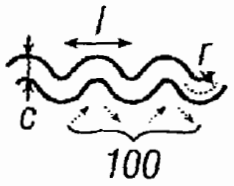


FIG. 1A

FIG. 1B-1

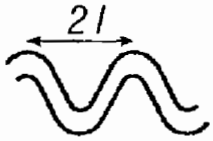


FIG. 1B-2

FIG. 1B-3

FIG. 1B-4

FIG. 1C-1

FIG. 1C-2

FIG. 1C-3

FIG. 1C-4

FIG. 2A-1

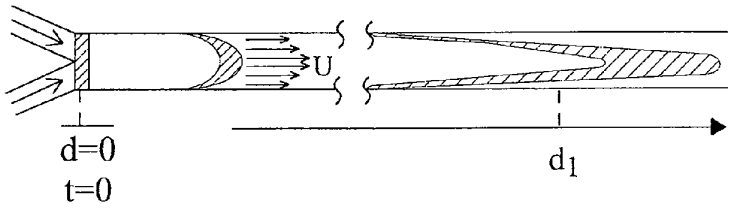


FIG. 2A-2

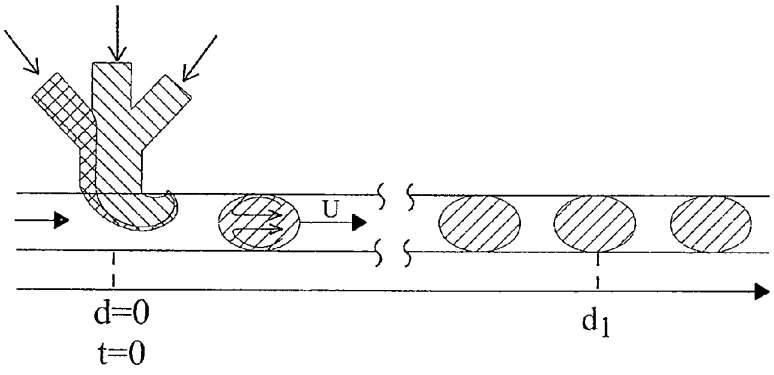
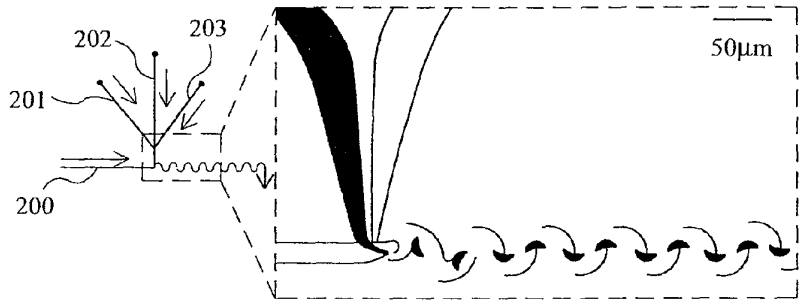


Fig. 2A

FIG. 2B-1



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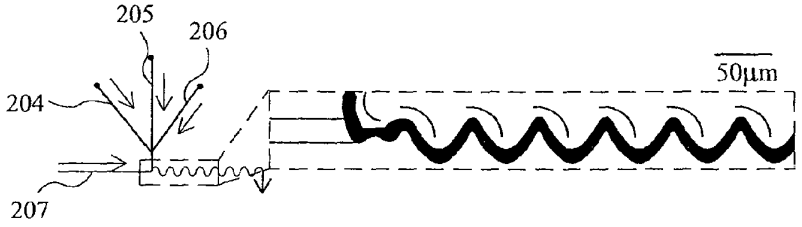


Fig. 2B

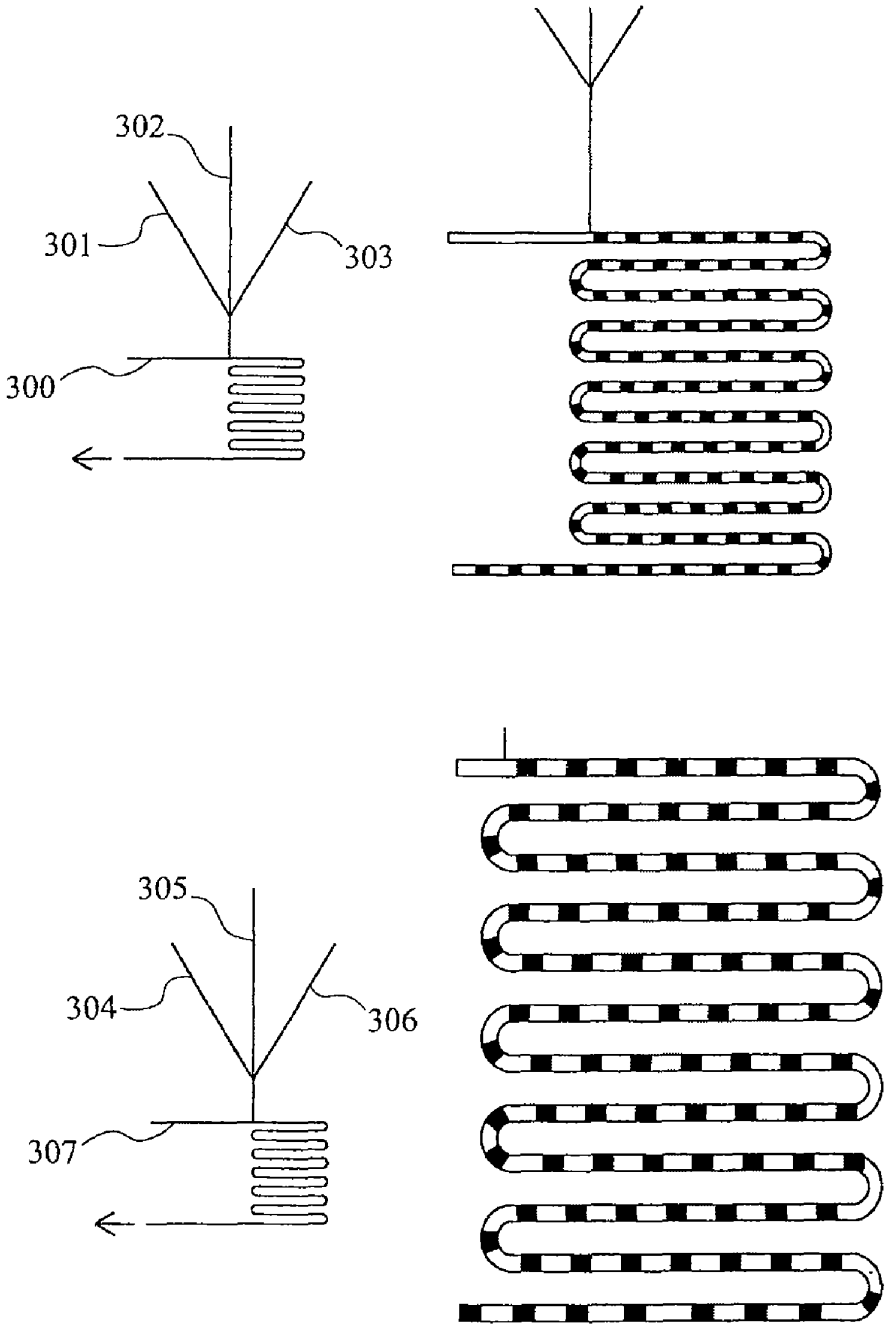


Fig. 3

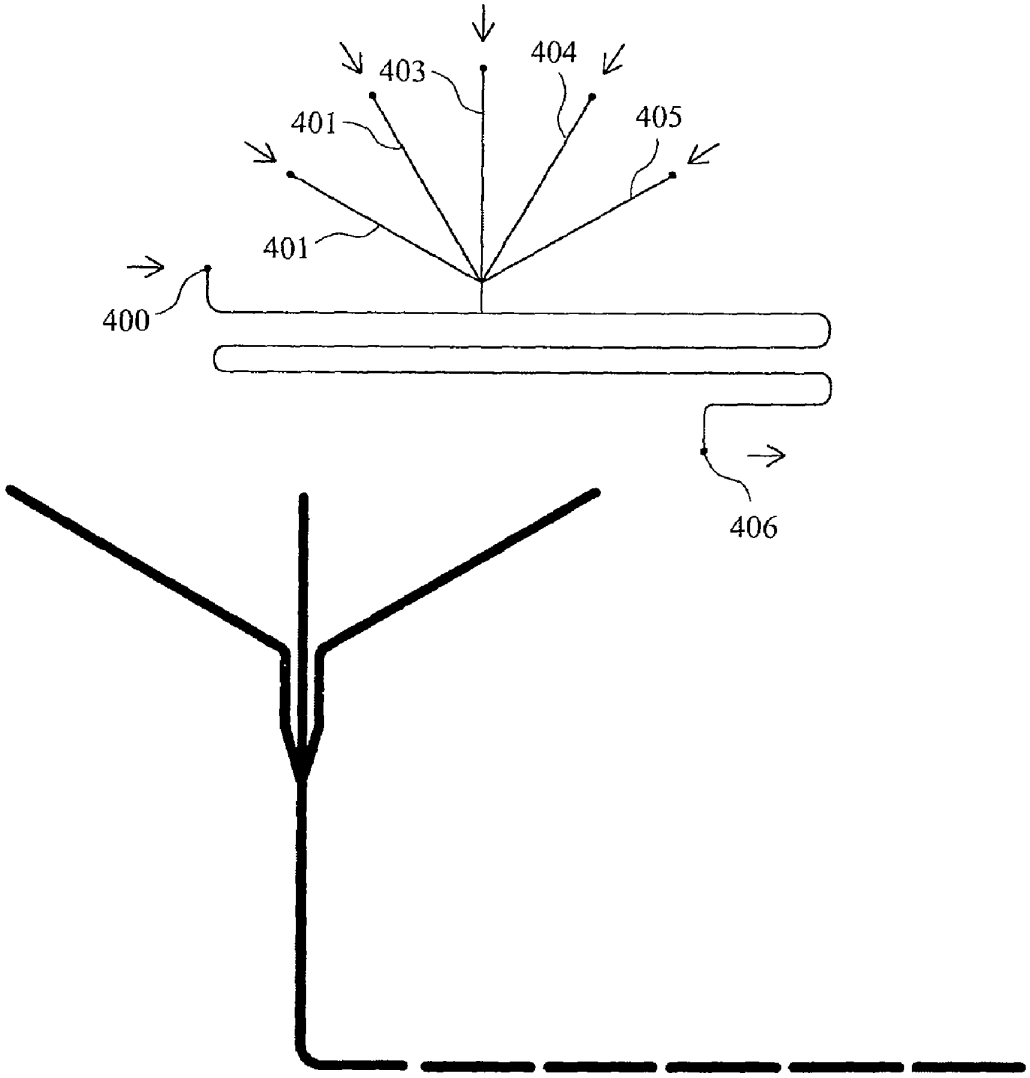


Fig. 4

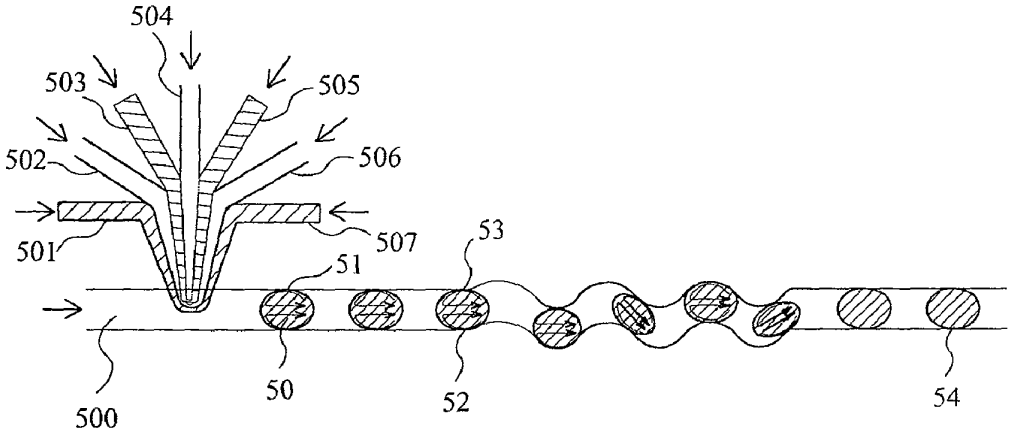


Fig. 5

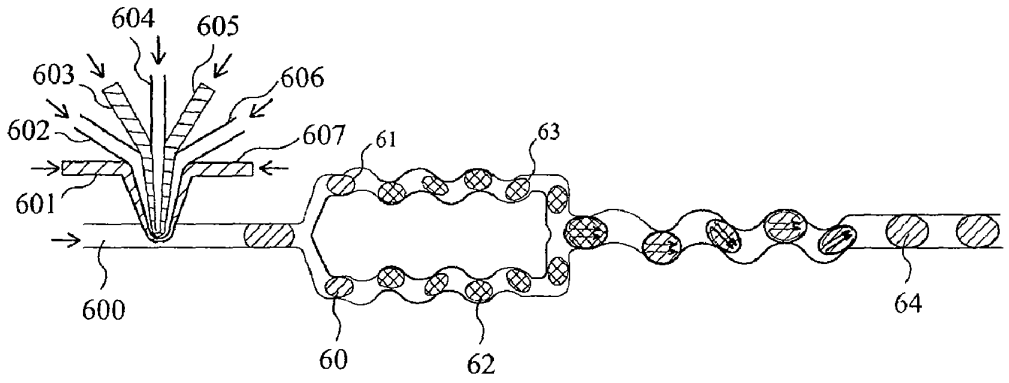


Fig. 6

FIG. 7A

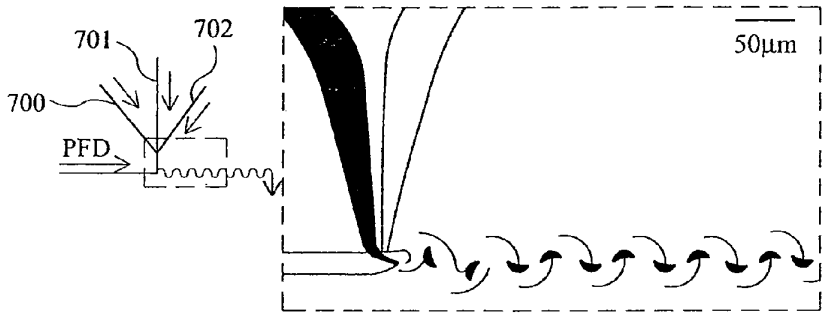


FIG. 7B

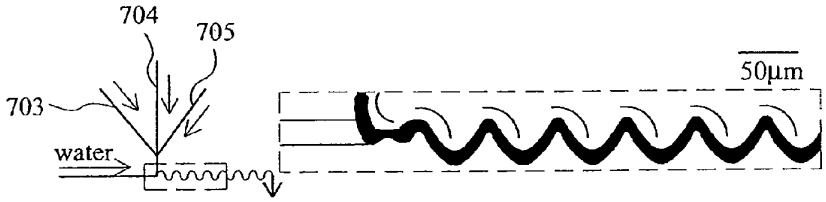


FIG. 7C

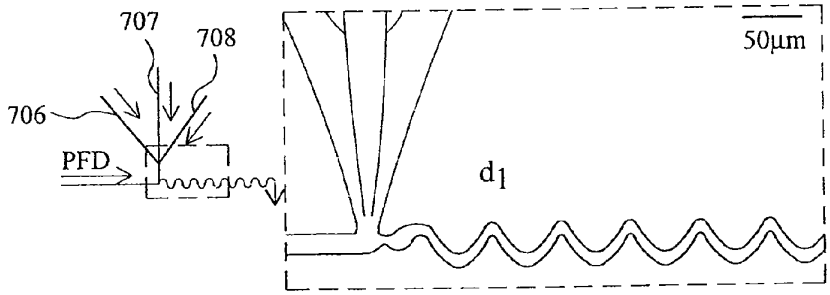


FIG. 7D

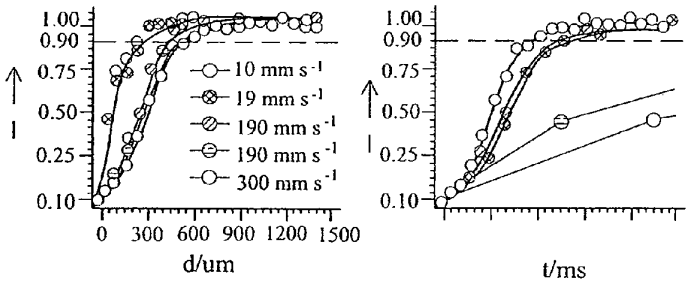


FIG. 7E

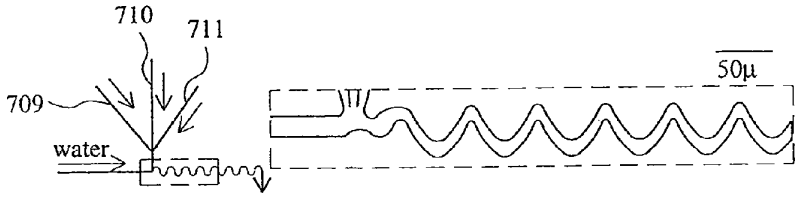


Fig. 7

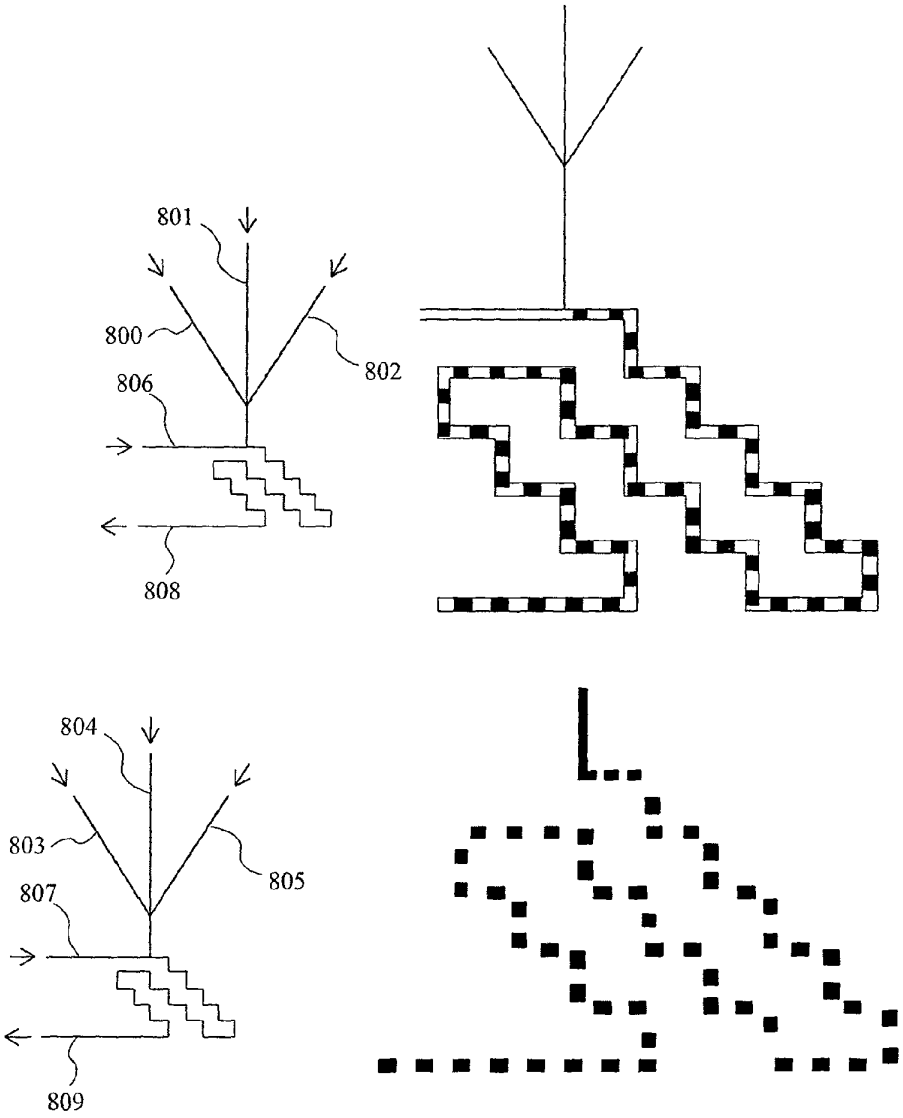


Fig. 8

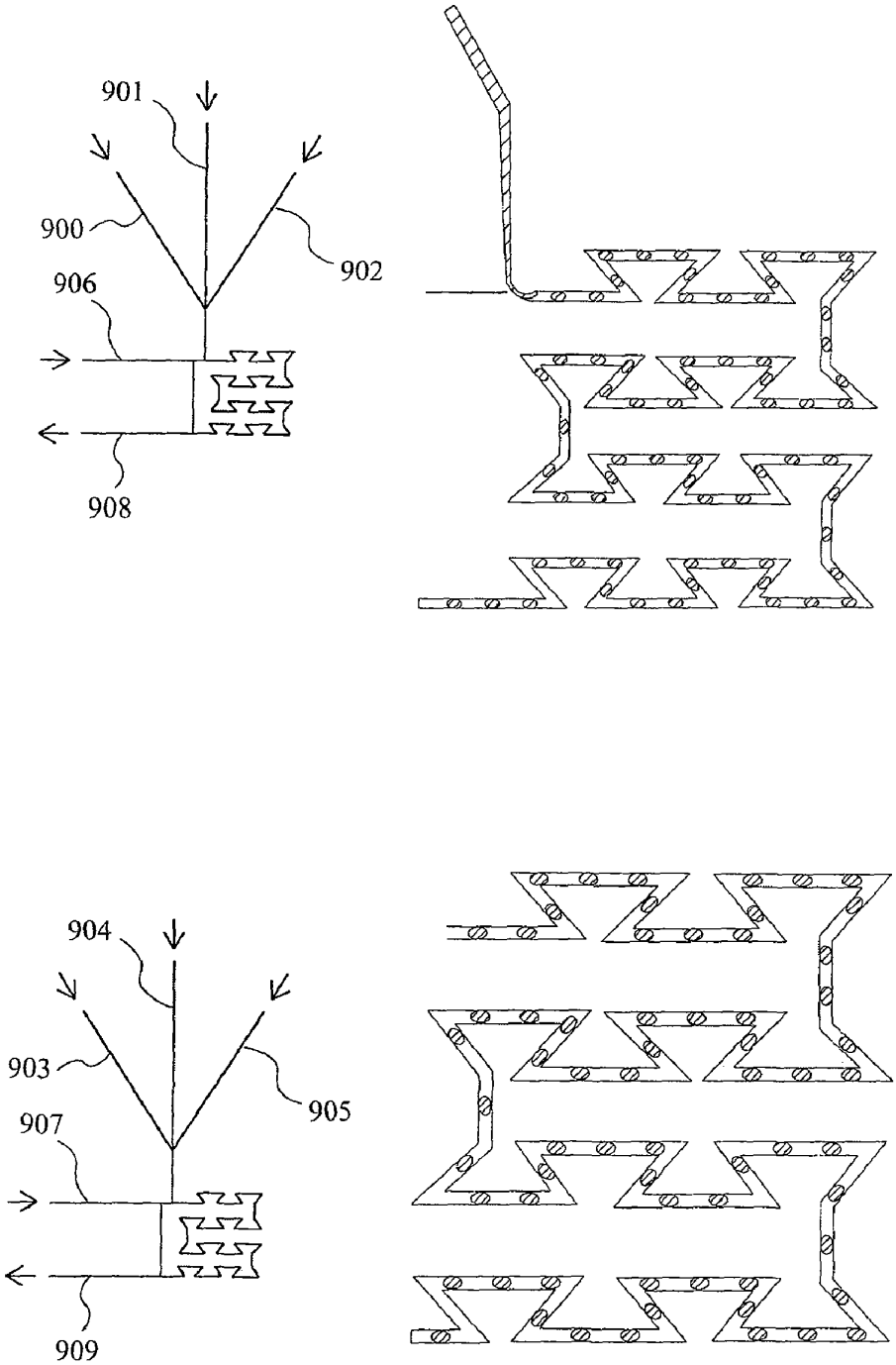


Fig. 9

FIG. 10A

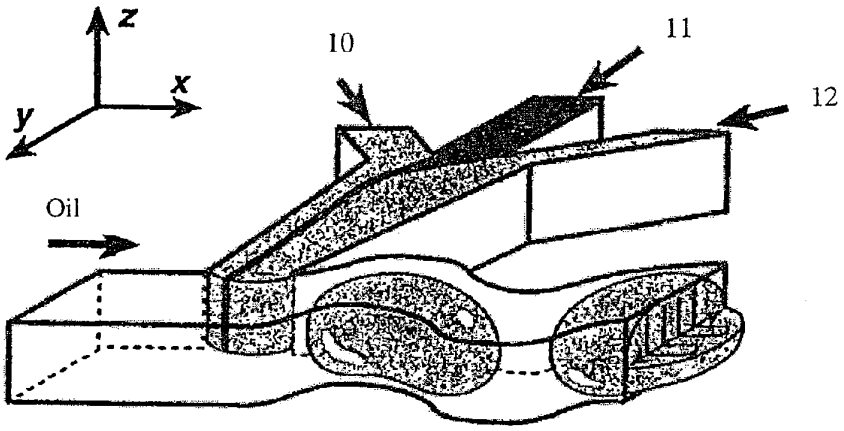


FIG. 10B

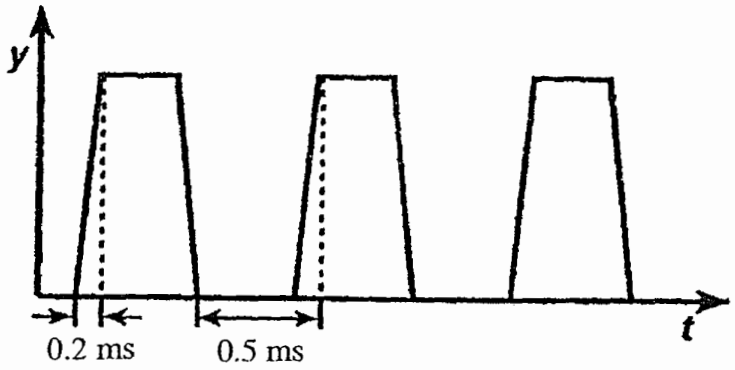


Fig. 10

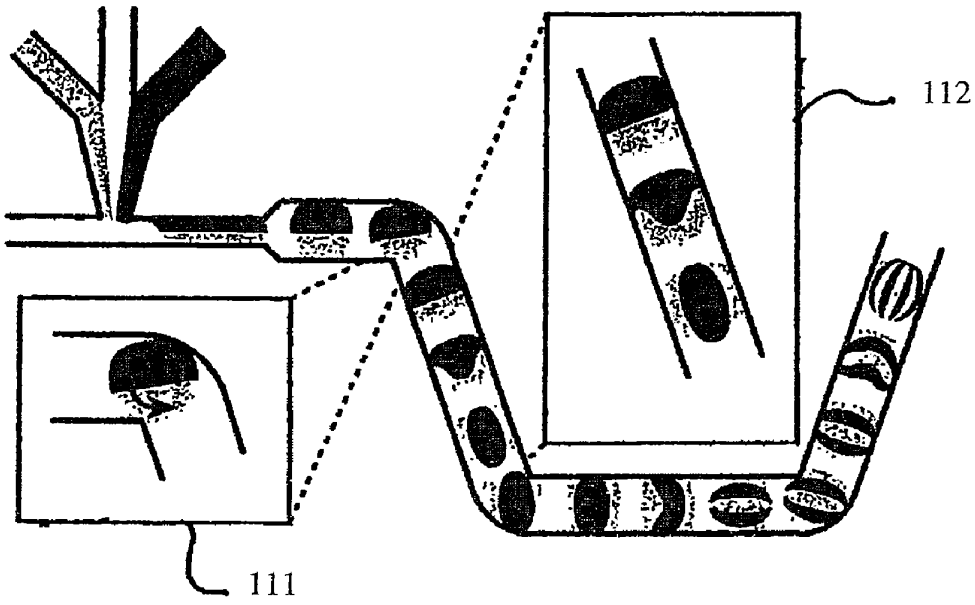


Fig. 11

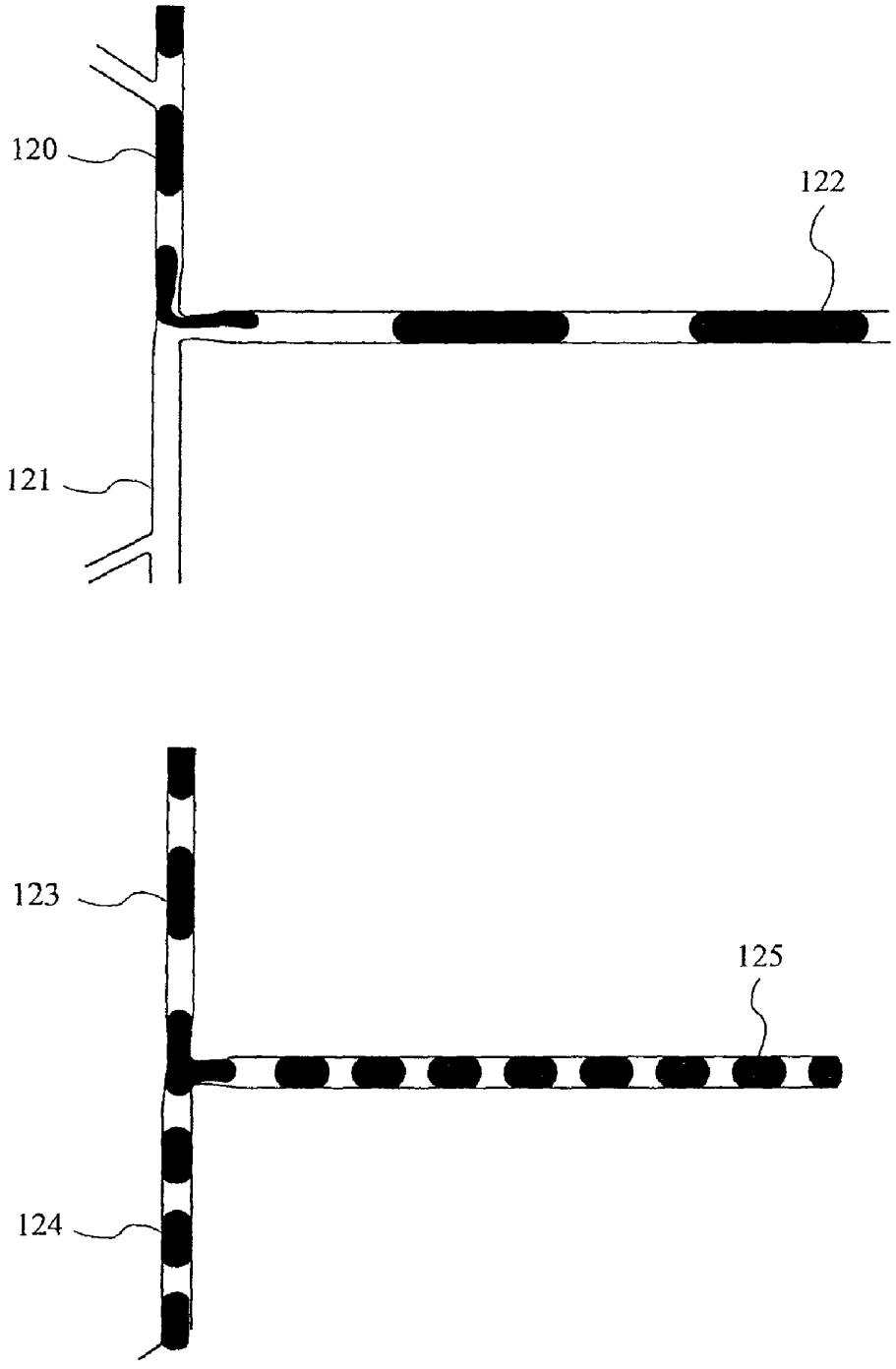


Fig. 12

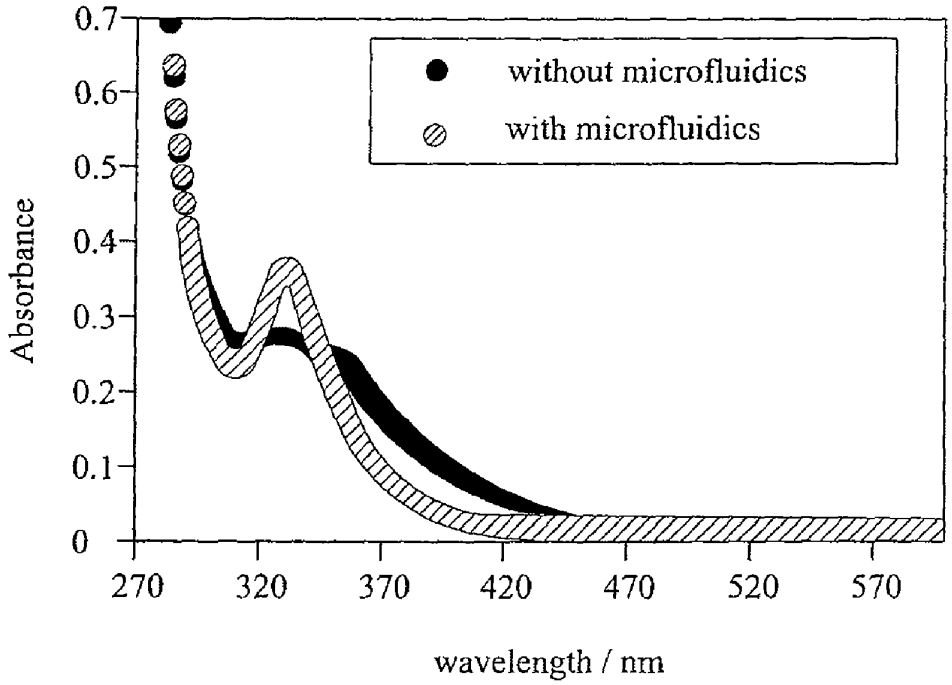

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
without microfluidics
with microfluidics
270
320
370
420
470
520
570
wavelength / nm

Fig. 13

FIG. 14A

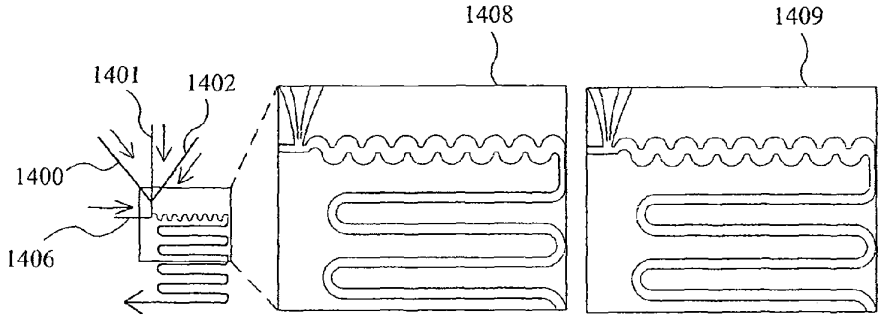


FIG. 14B

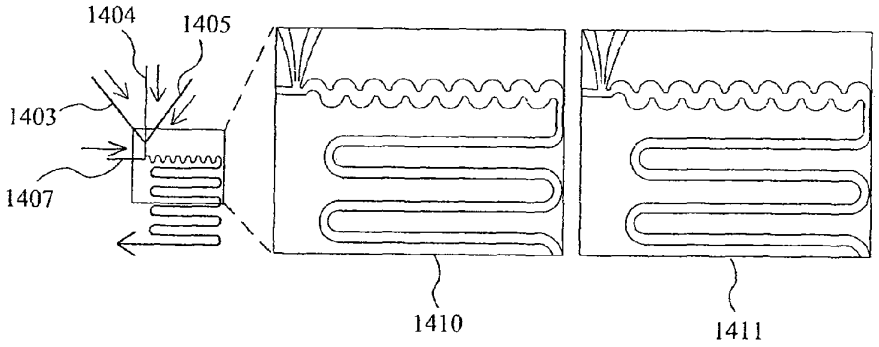


Fig. 14

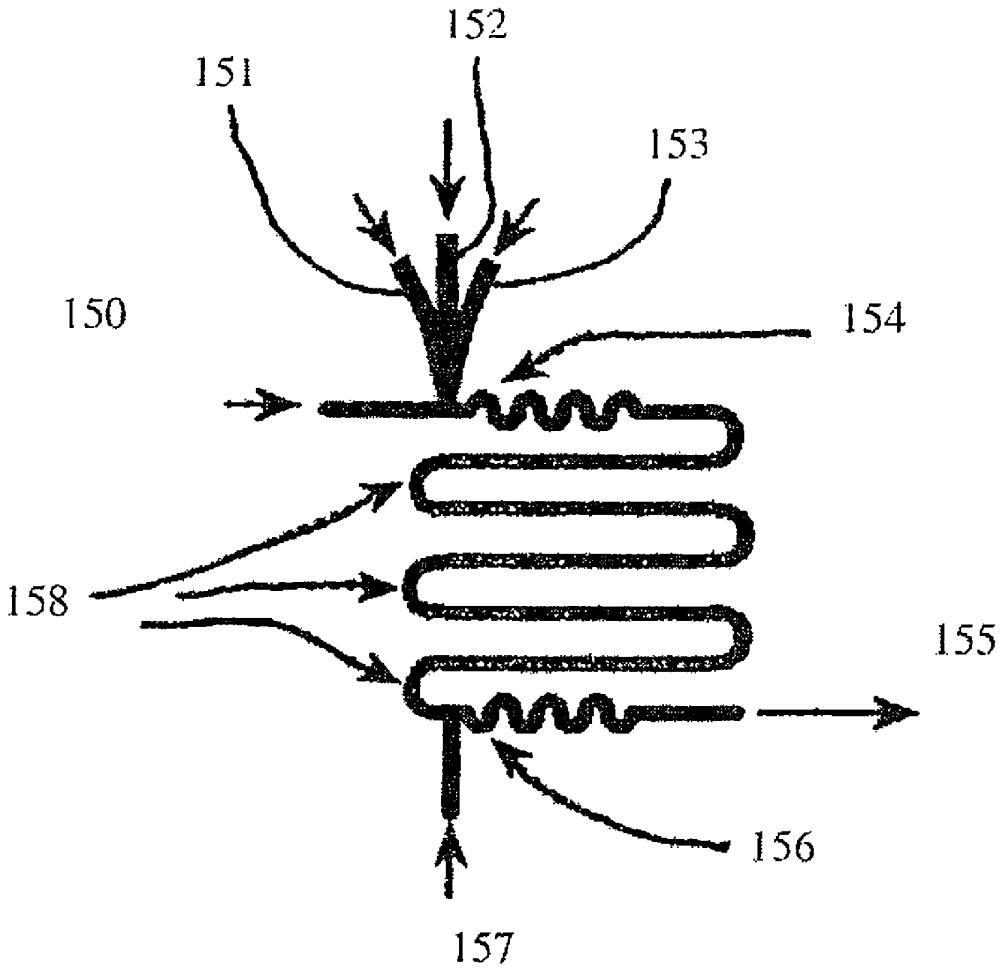


Fig. 15

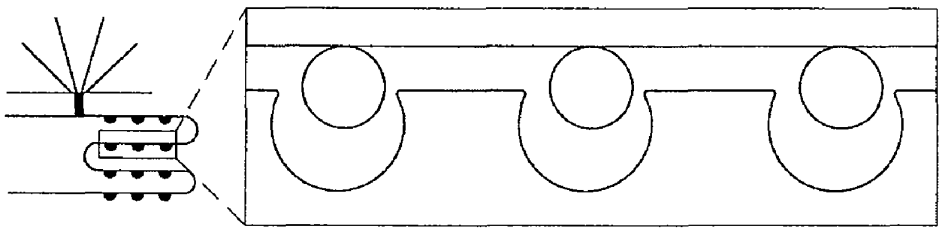


Fig. 16

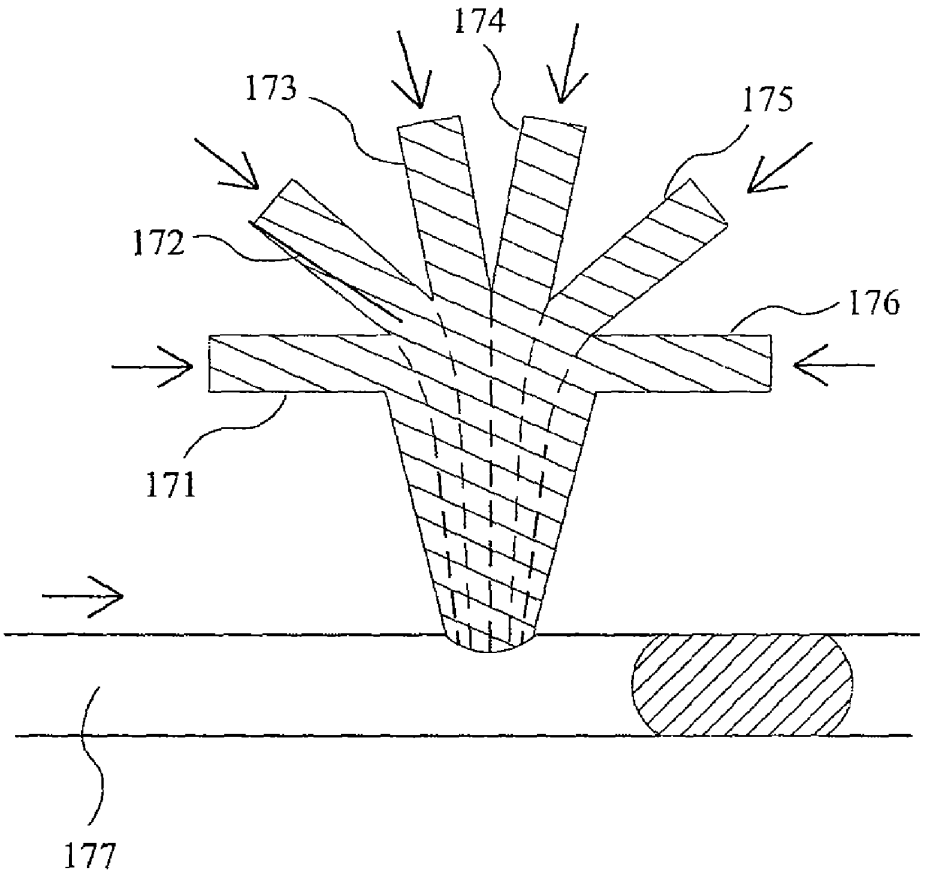


Fig. 17

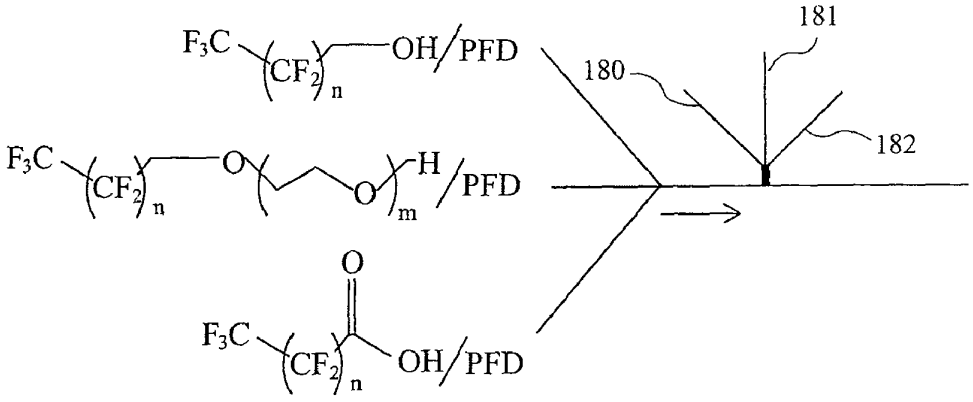


Fig. 18

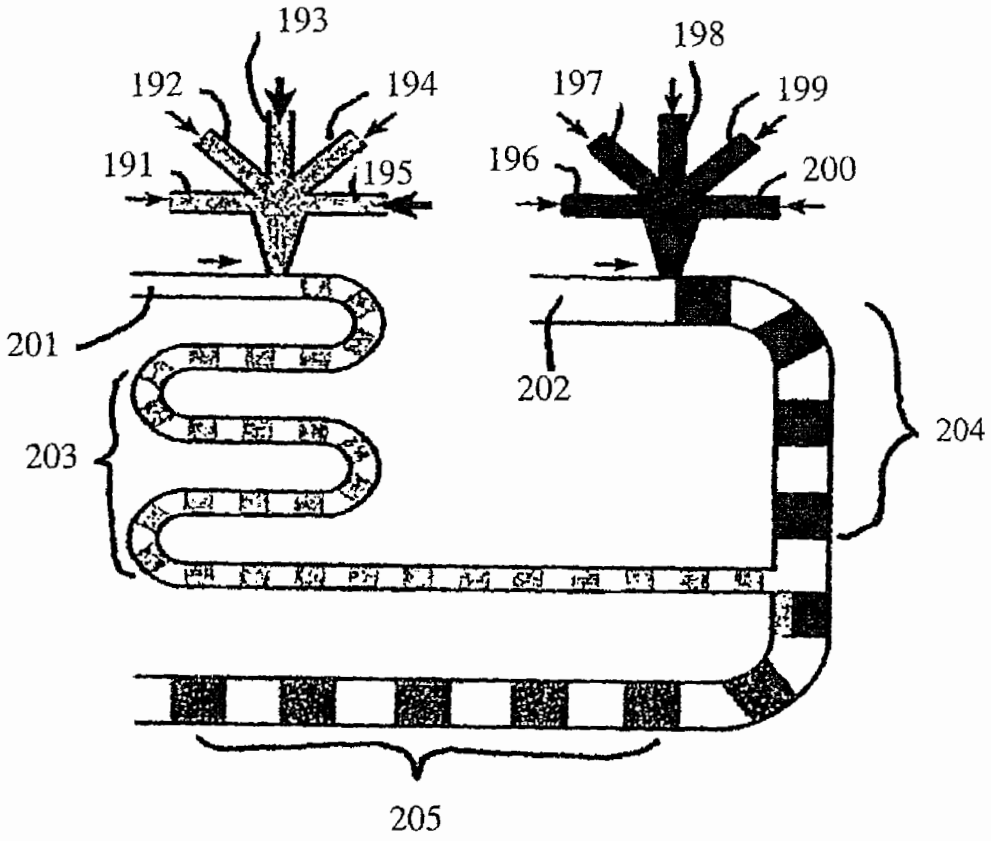


Fig. 19

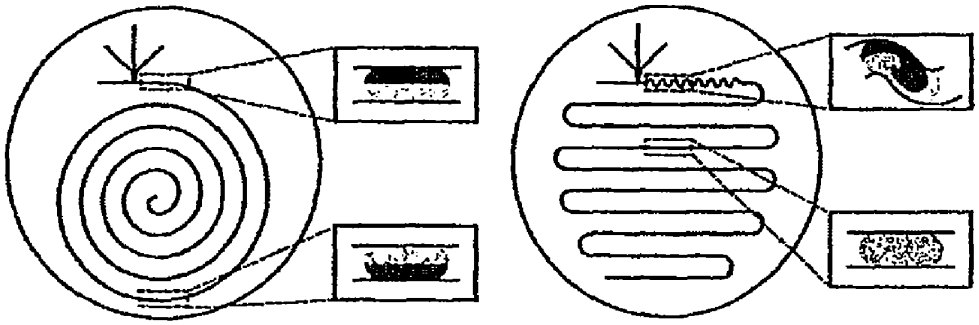


Fig. 20

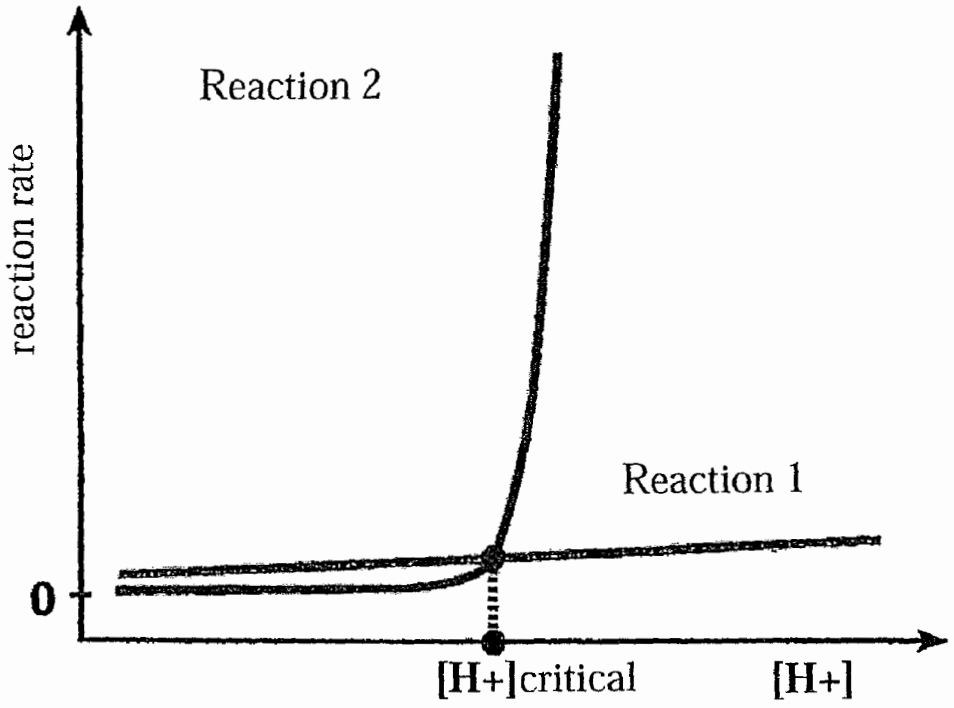


Fig. 21

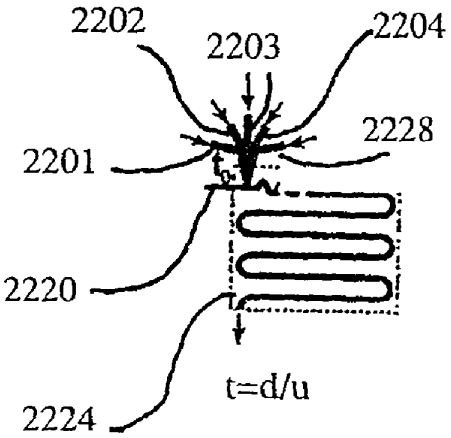


FIG. 22A

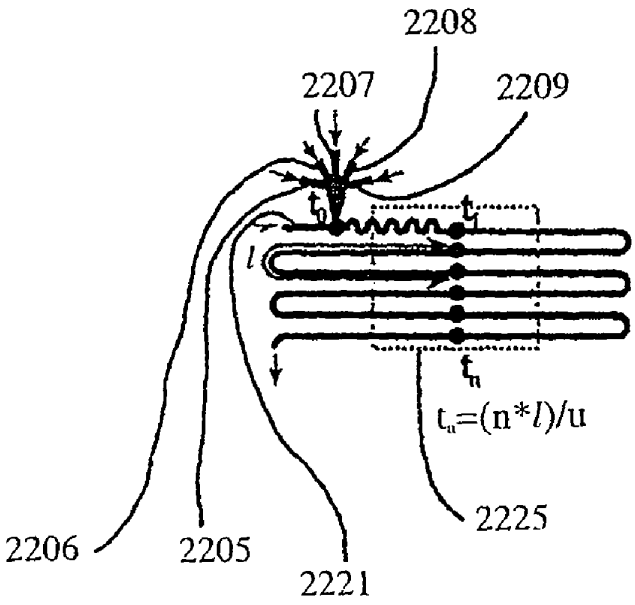


FIG. 22B

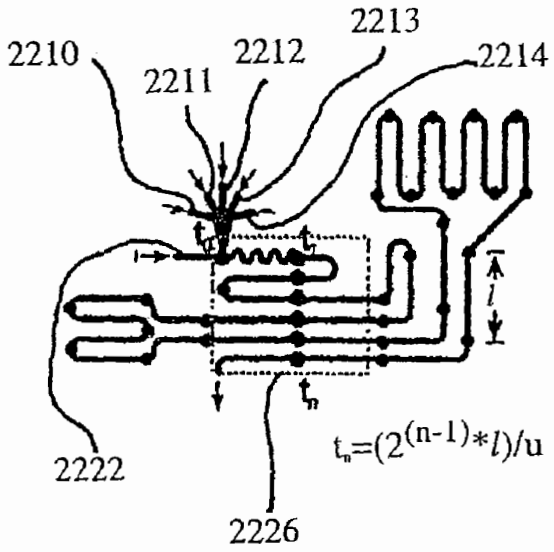


FIG. 22C

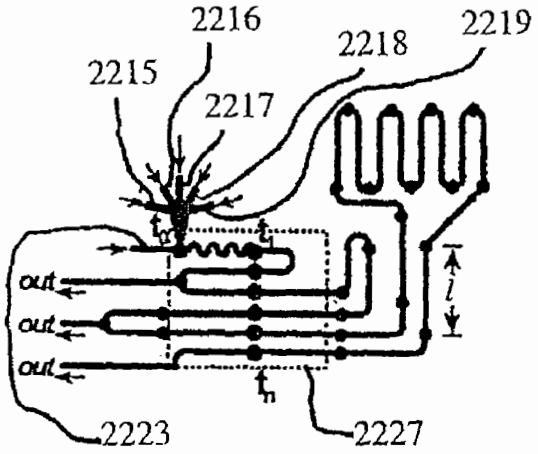
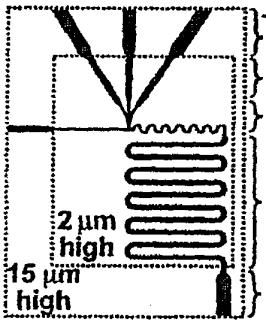


FIG. 22D



Size of channel (height x width)	ΔP [atm]	U [m/s]	length [μm]	time
15 μm x 50 μm	0.003	0.001	10000	
2 μm x 5 μm	0.4	0.08	200	
2 μm x 1 μm	0.45	0.4	10	25 μs → mix
2 μm x 3 μm	2.0	0.13	400	3000 μs = 3.0 ms → observe
15 μm x 50 μm	0.003	0.001	10000	

Fig. 23

FIG. 24A

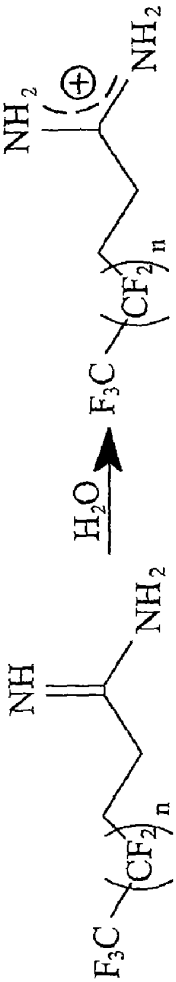


FIG. 24B

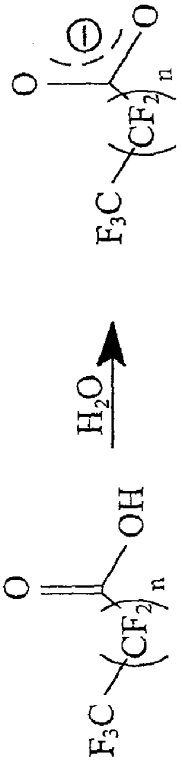
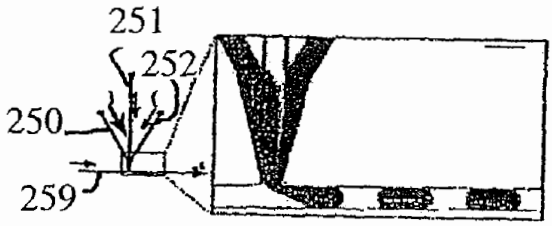


FIG. 24C

Fig. 24

FIG. 25A



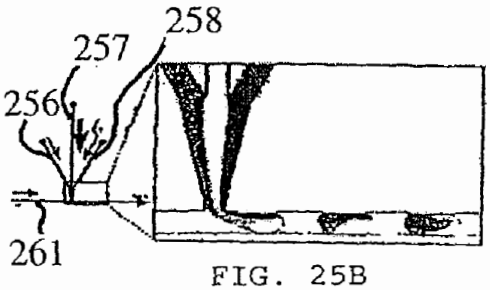


FIG. 25B

FIG. 25C

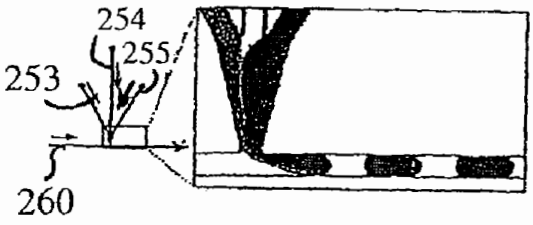


Fig. 25

FIG. 26A

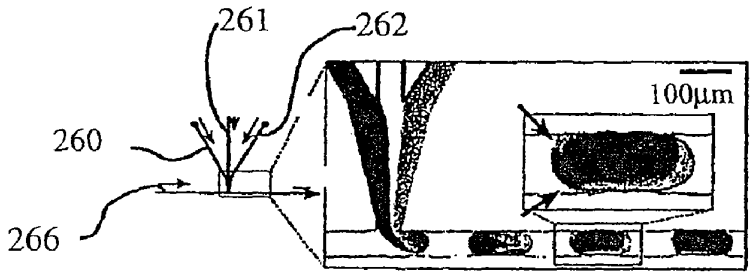


FIG. 26B

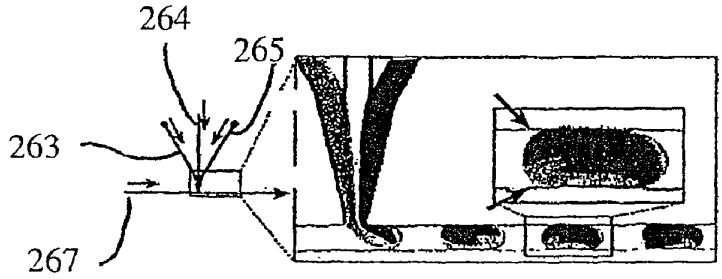


Fig. 26

FIG. 27A-1

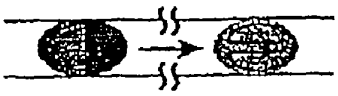
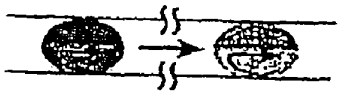


FIG. 27A-2



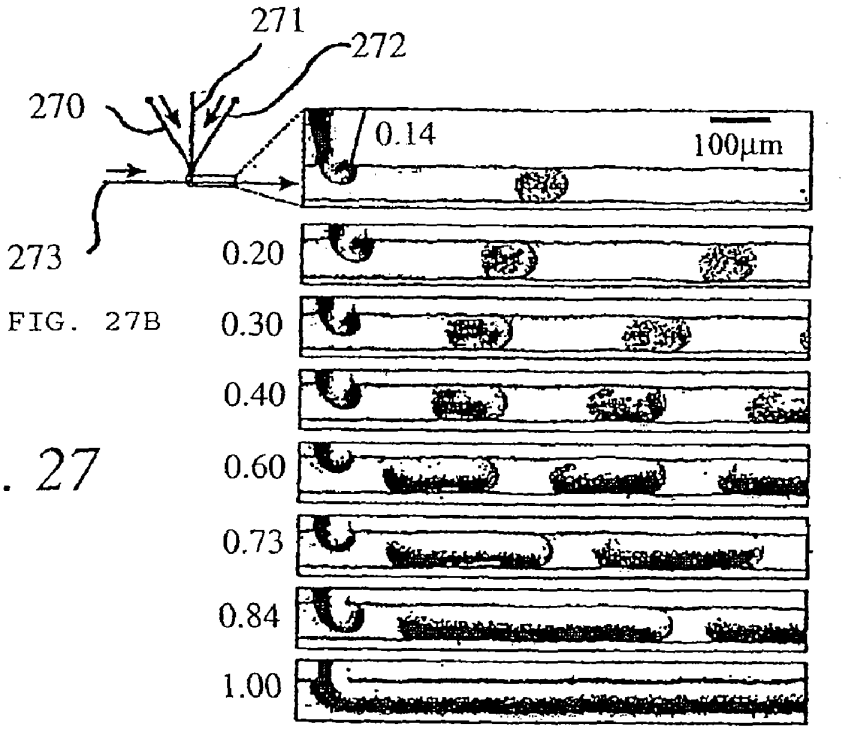


FIG. 27B

Fig. 27

FIG. 27C-1

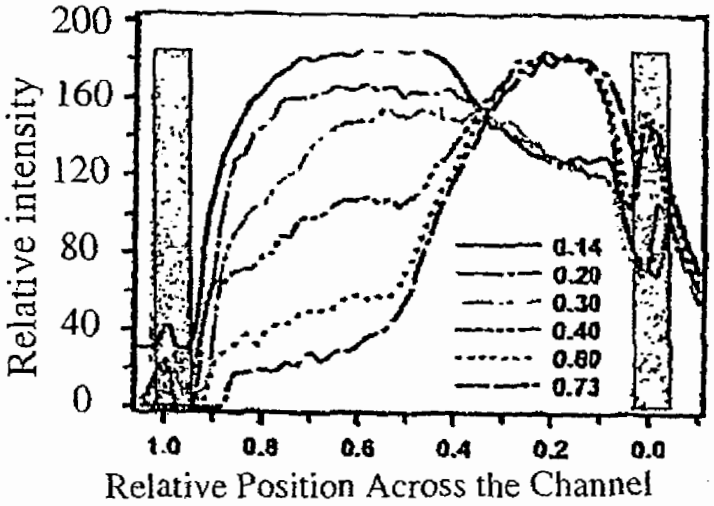
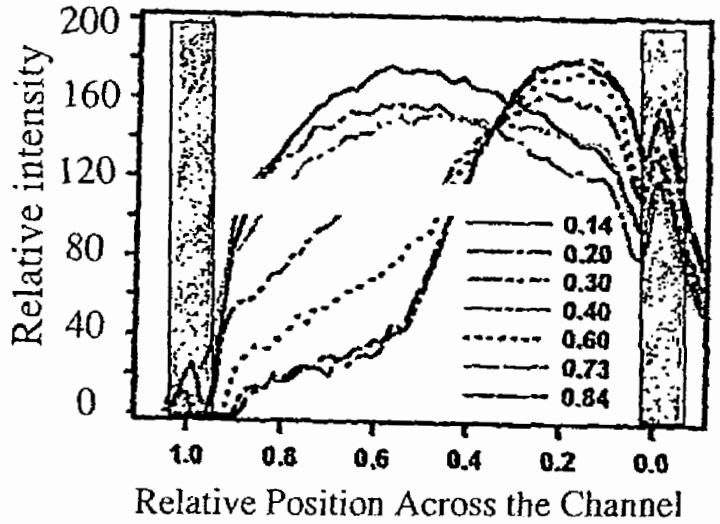


FIG. 27C-2



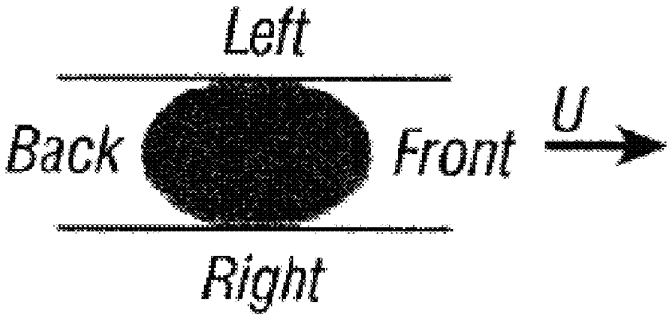


FIG. 28

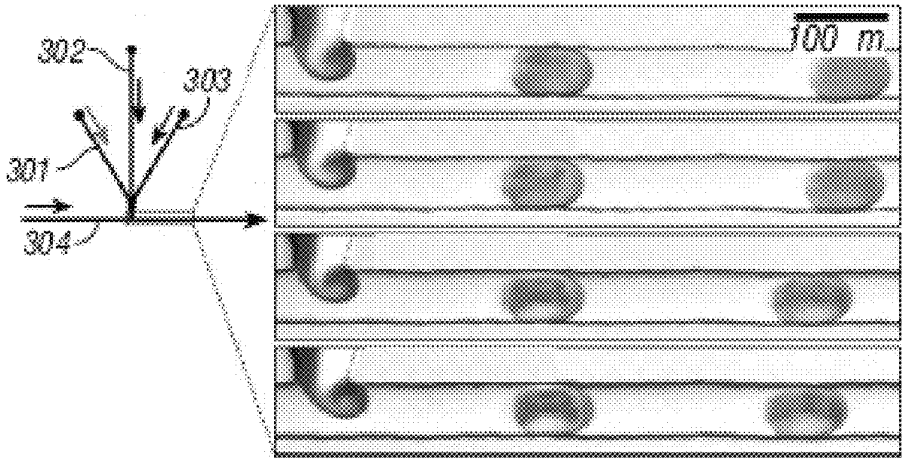


FIG. 30

FIG. 29A

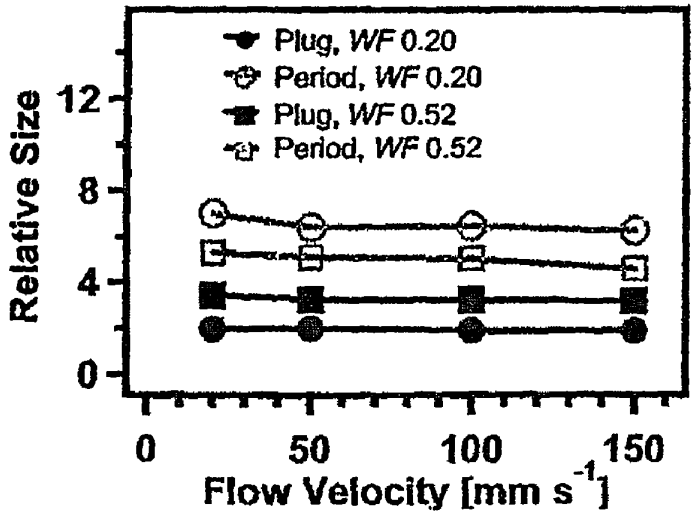


FIG. 29B

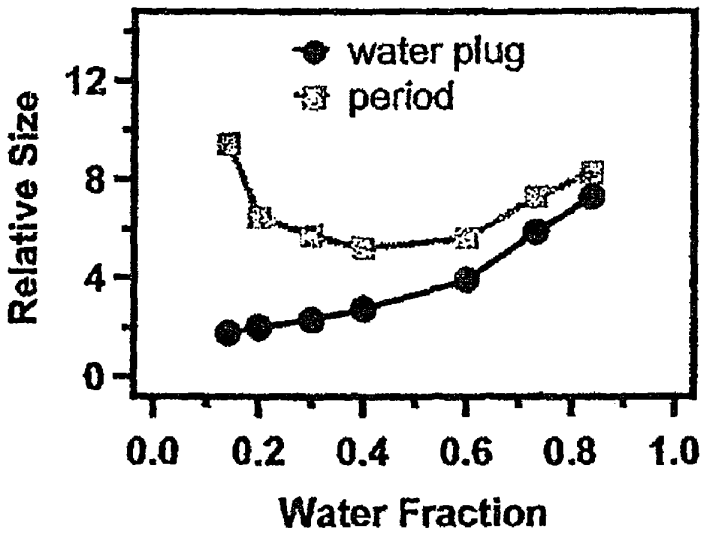


Fig. 29

FIG. 31A

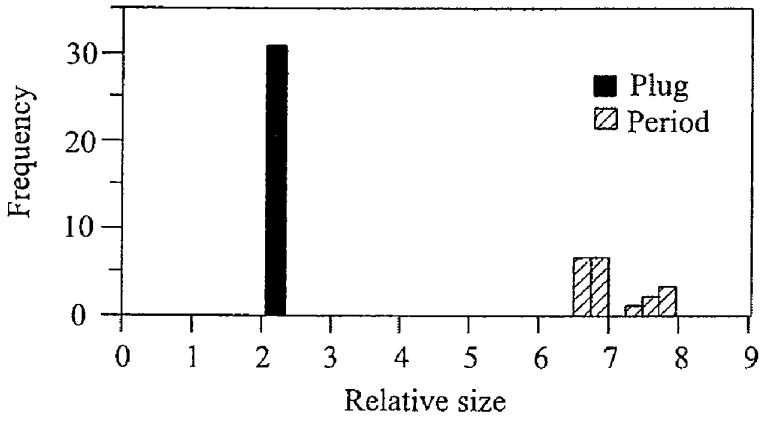


FIG. 31B

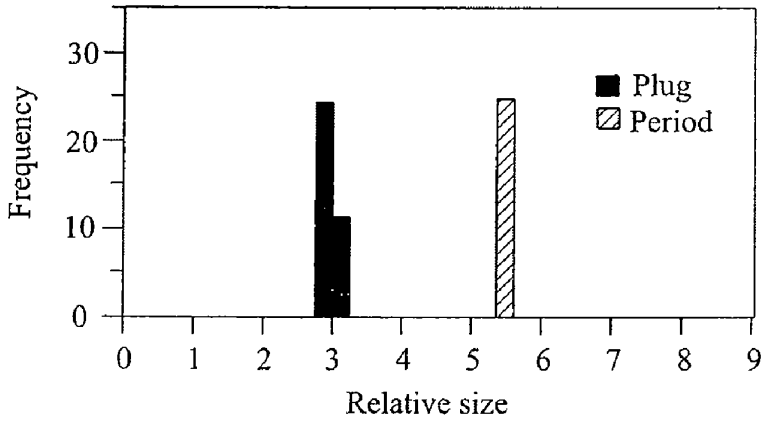


FIG. 31C

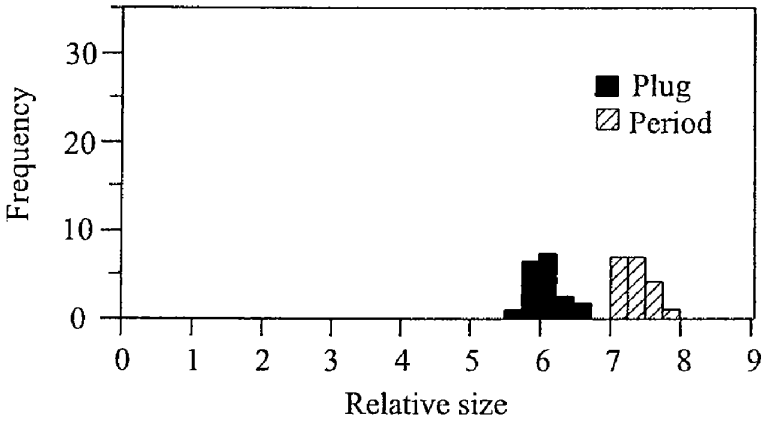


Fig. 31

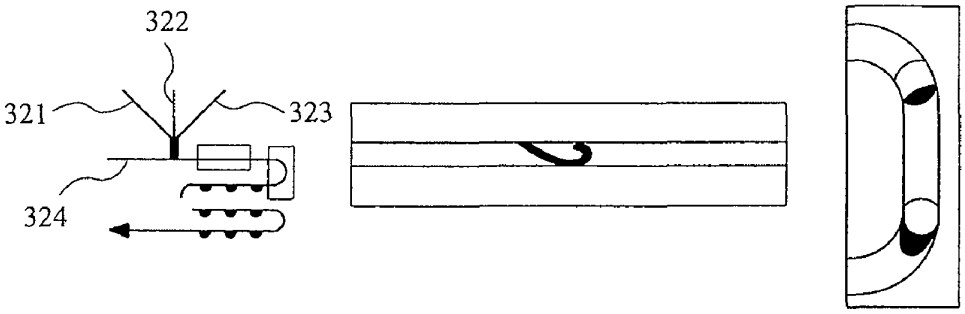


Fig. 32

FIG. 33A

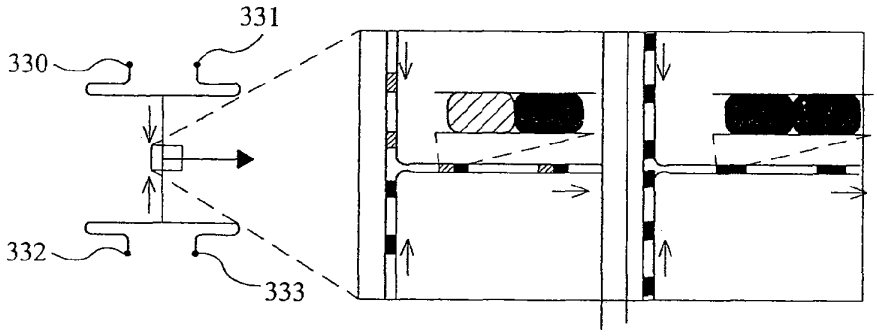


FIG. 33B

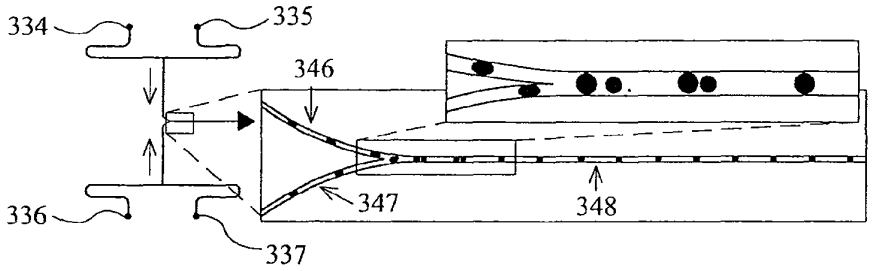


FIG. 33C

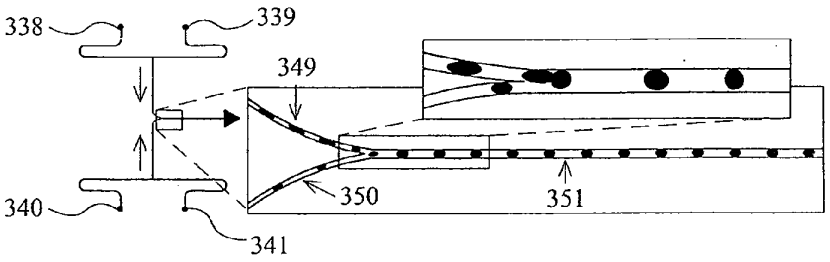


FIG. 33D

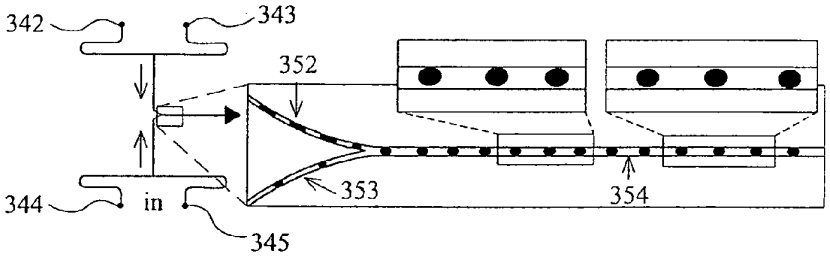


Fig. 33

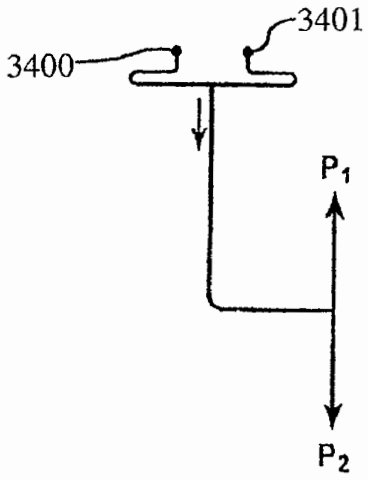


FIG. 34A

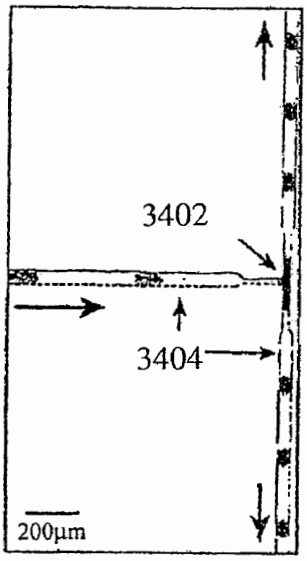


FIG. 34B

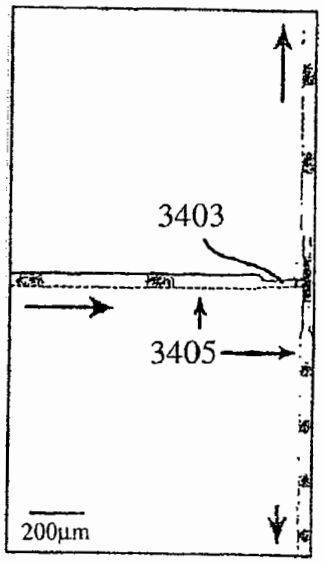


FIG. 34C

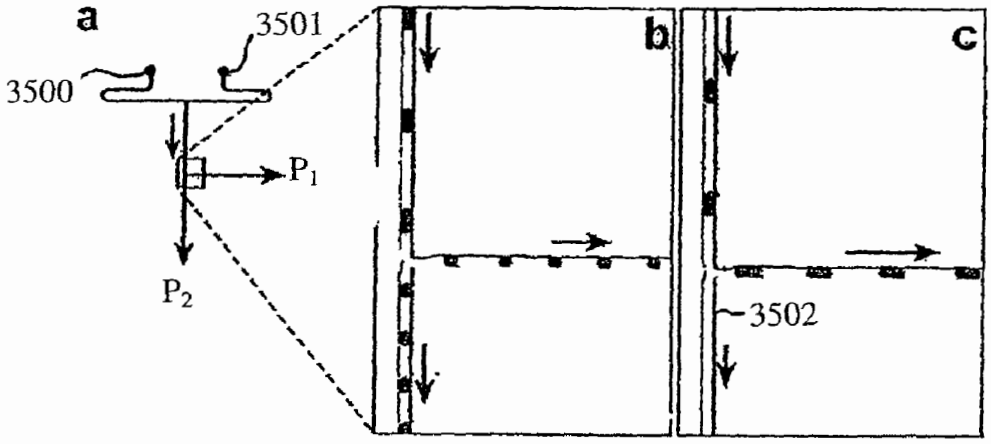


Fig. 35

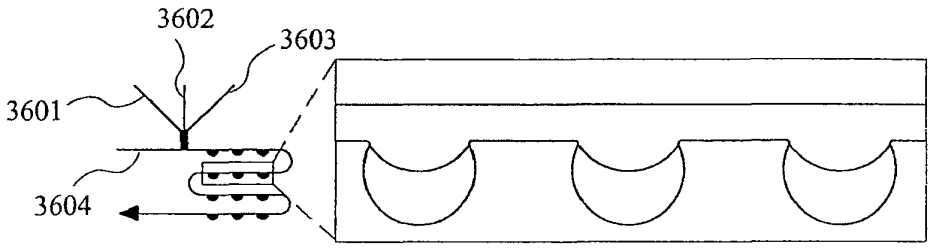


Fig. 36

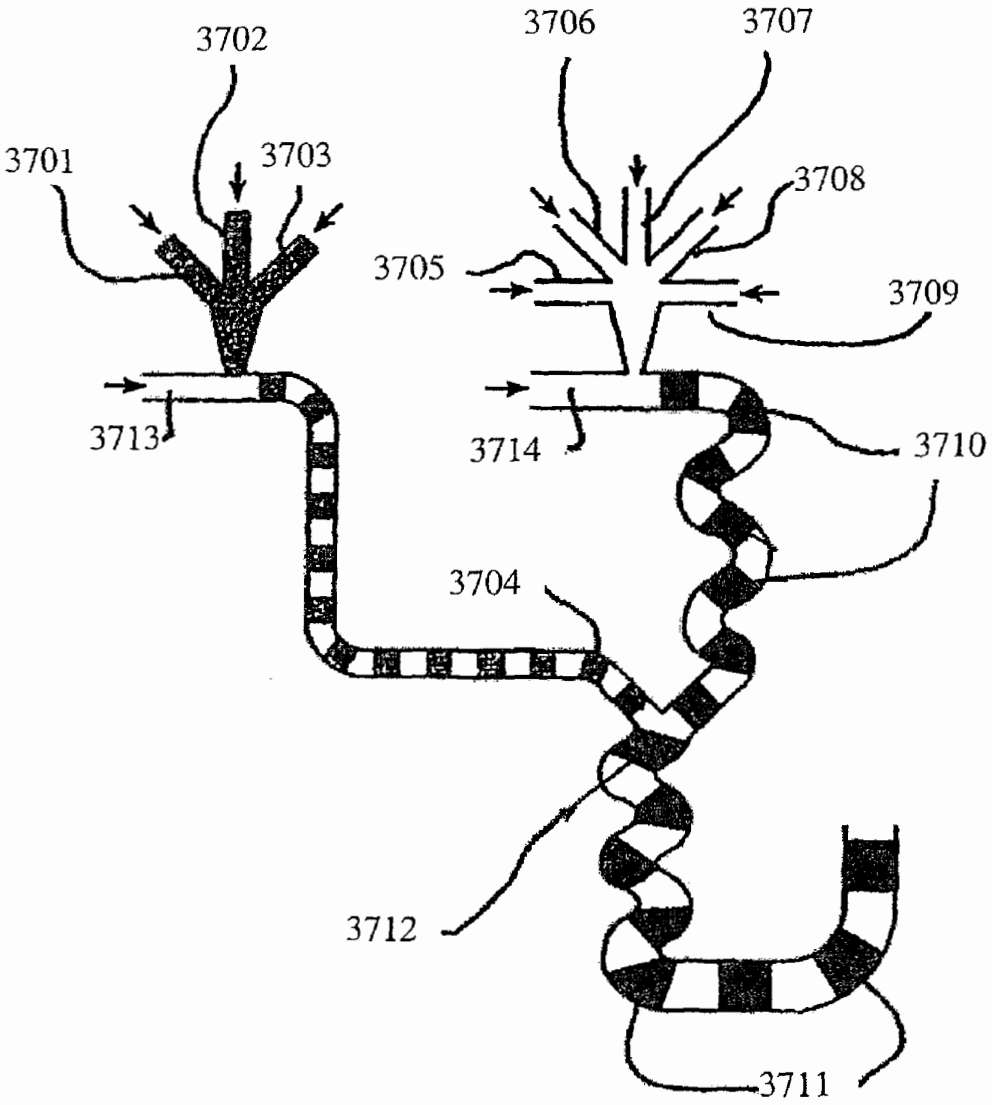

3702
3706
3707
3701
3703
3708
3705
3709
3713
3714
3710
3704
3712
3711

Fig. 37

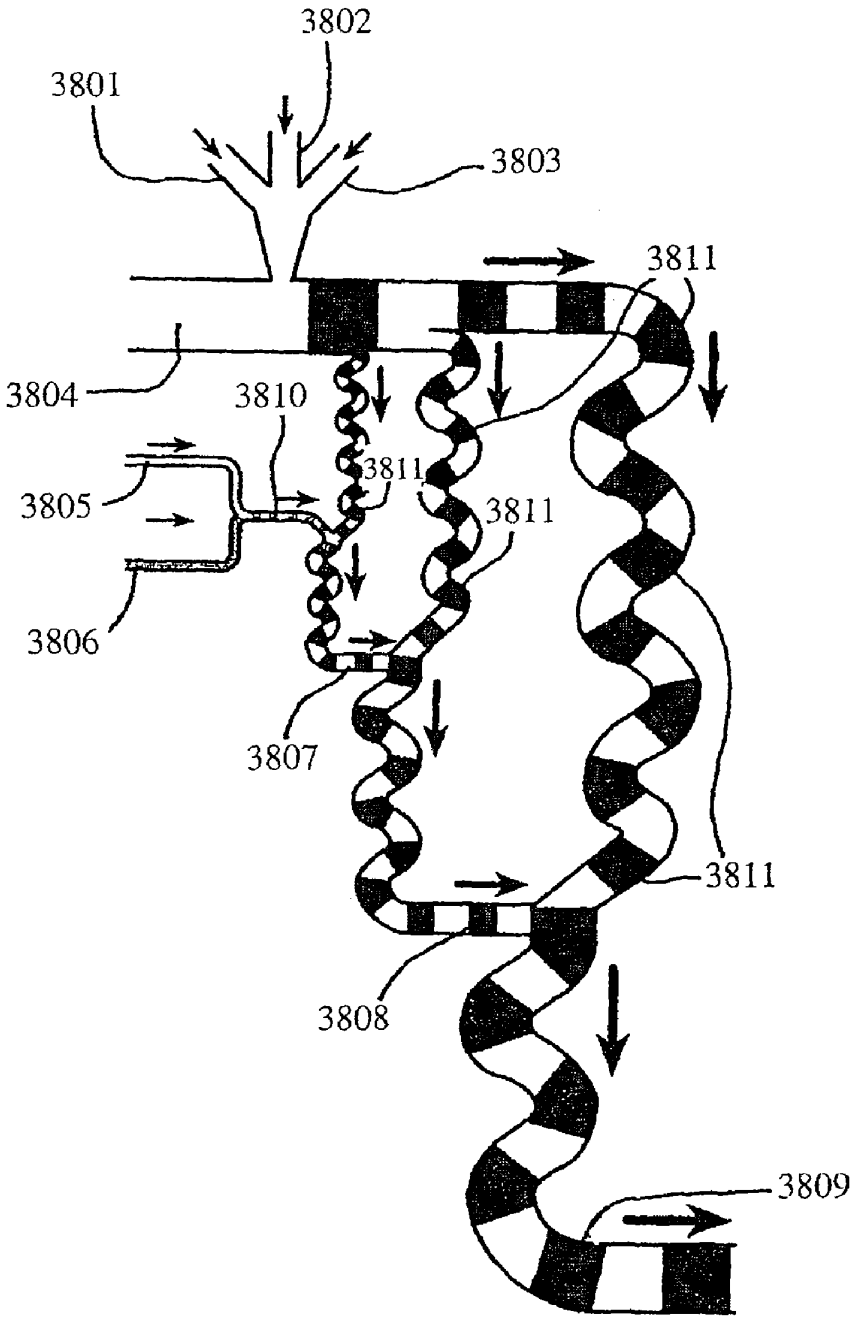

3802
3801
3803
3811
3804
3810
3811
3805
3811
3806
3807
3811
3808
3809

Fig. 38

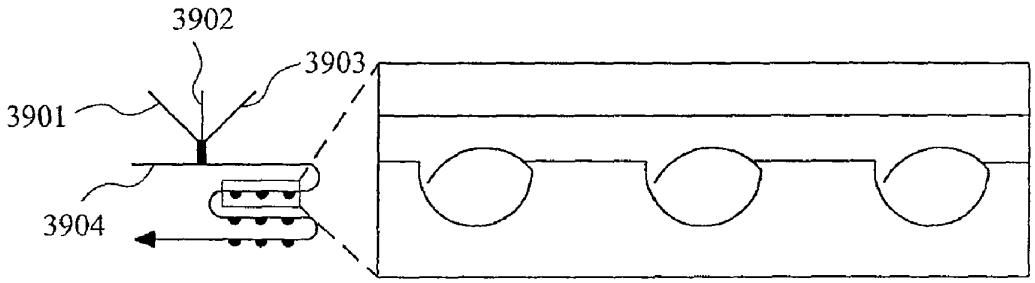


Fig. 39

FIG. 40

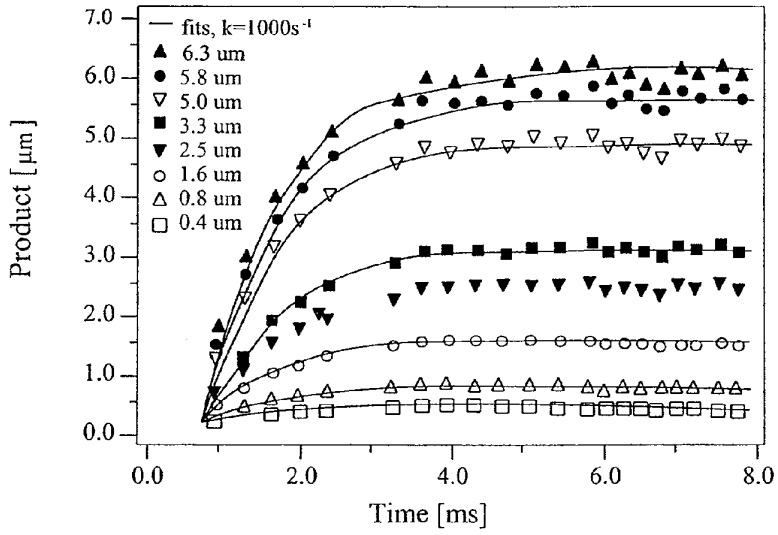

fits, k=1000s-1
6.3 um
5.8 um
5.0 um
3.3 um
2.5 um
1.6 um
0.8 um
0.4 um
Product [µm]
Time [ms]
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
0.0
2.0
4.0
6.0
8.0

FIG. 40A

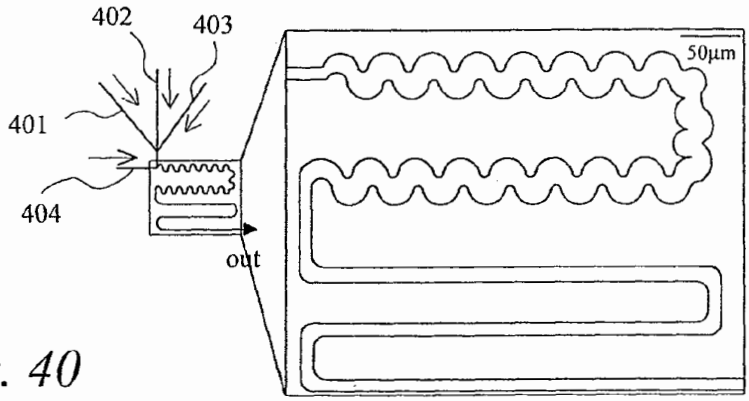
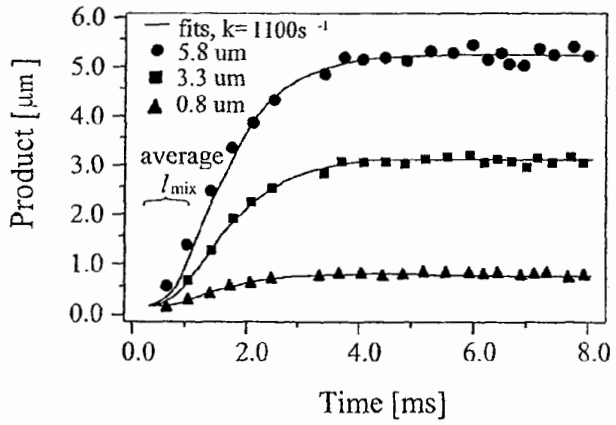


Fig. 40

FIG. 40B



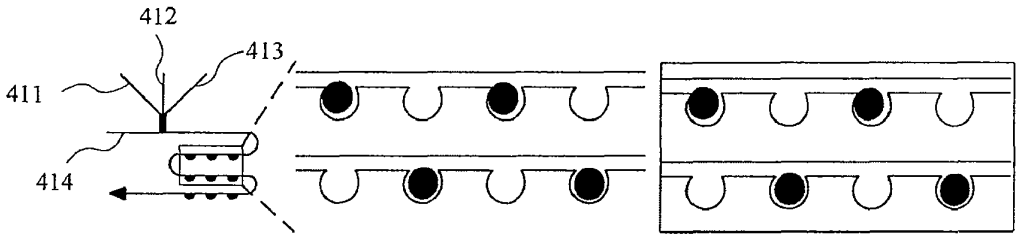


FIG. 41A FIG. 41B FIG. 41C

Fig. 41

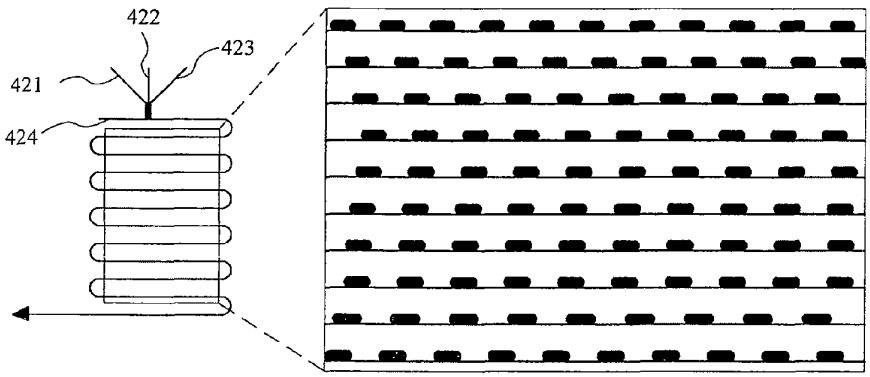


Fig. 42

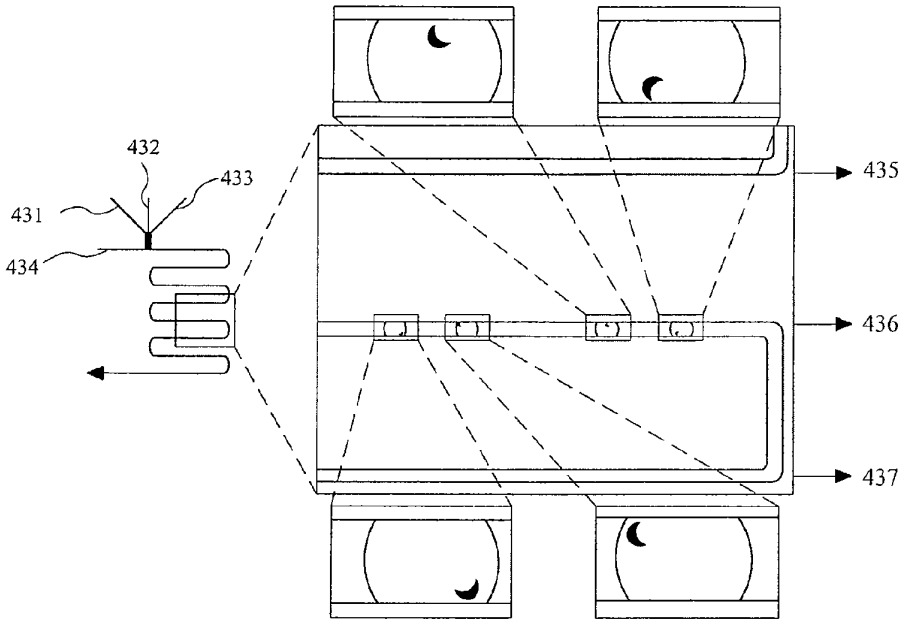


Fig. 43

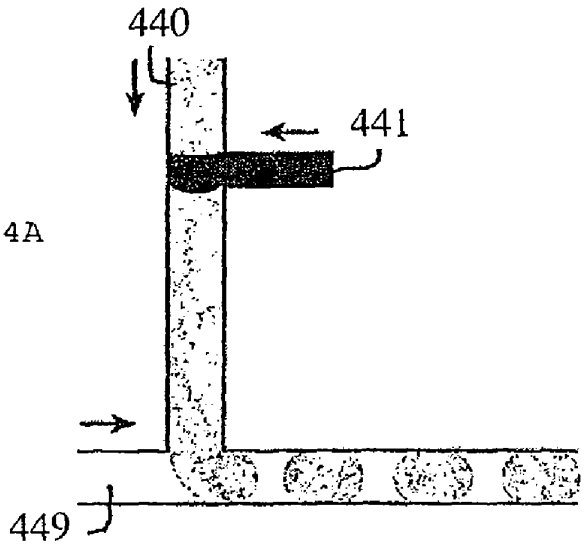


FIG. 44A

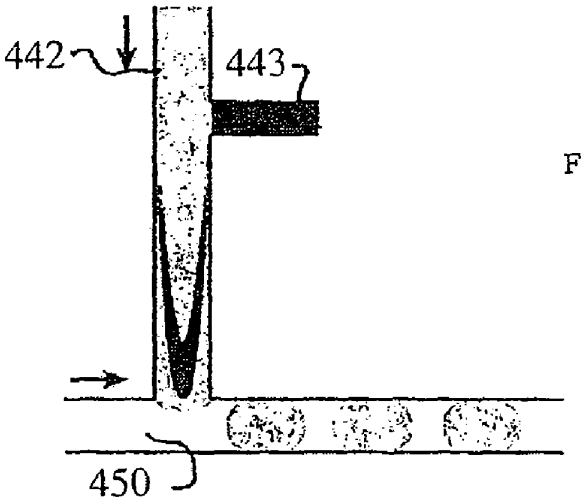


FIG. 44B

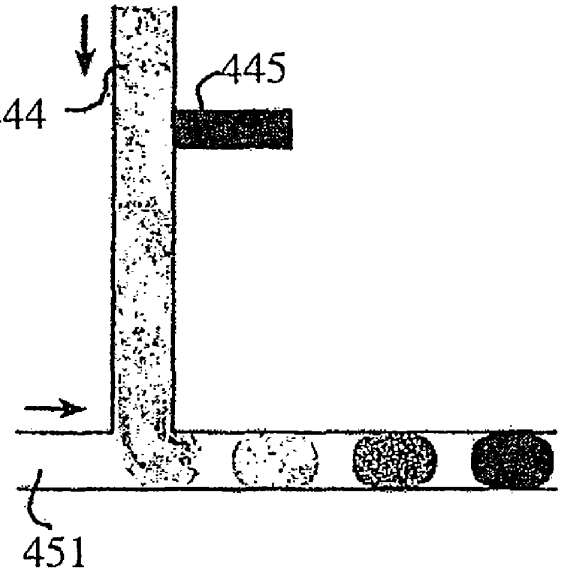


FIG. 44C

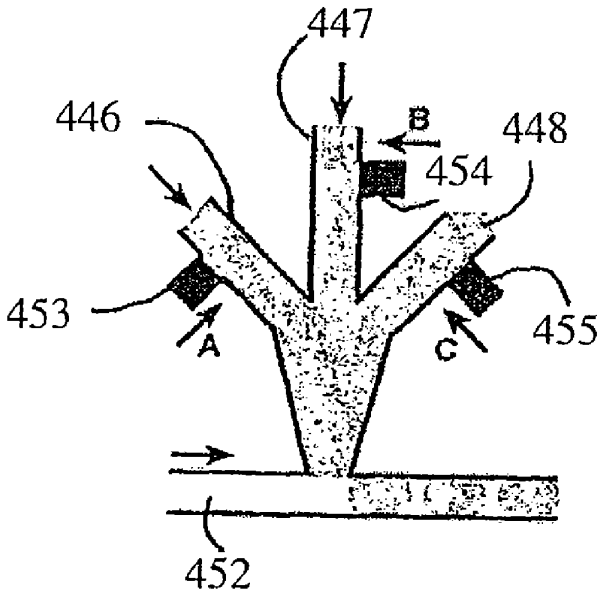
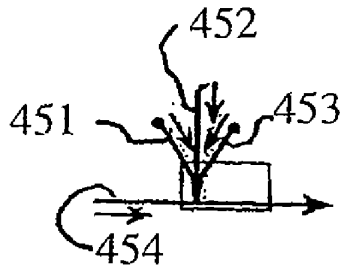


FIG. 44D

FIG. 45A



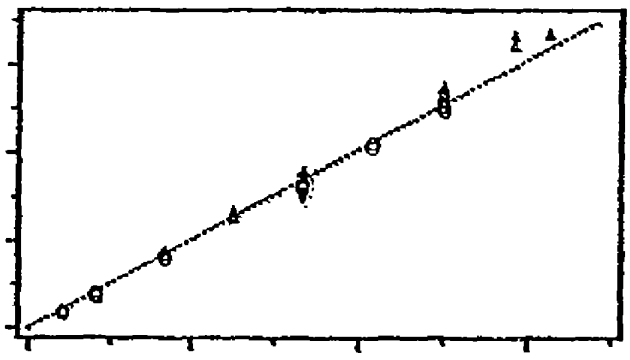


FIG. 45B

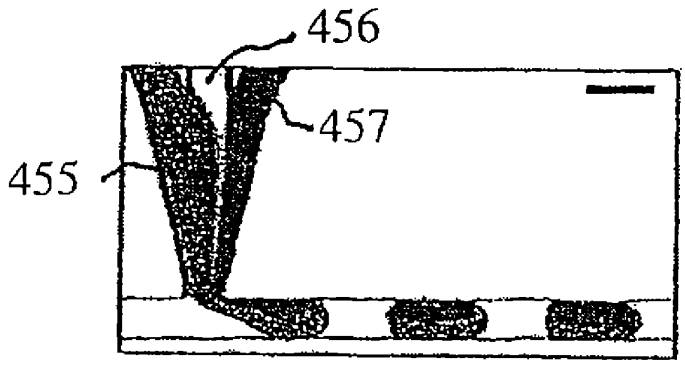


FIG. 45C

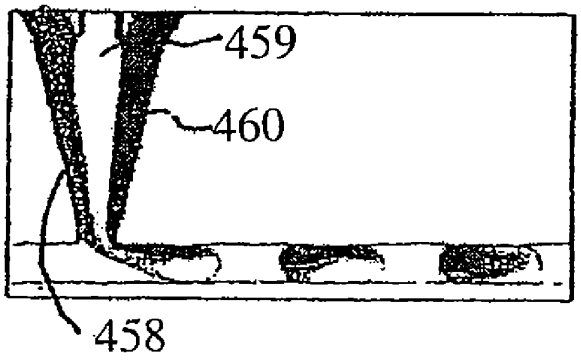


FIG. 45D

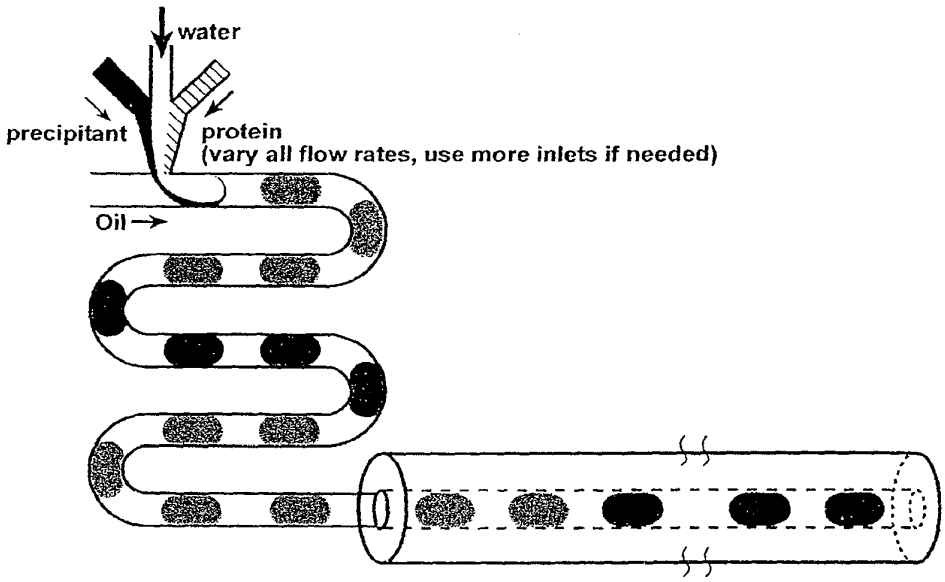

water
precipitant
protein
(vary all flow rates, use more inlets if needed)
Oil

FIGURE 46

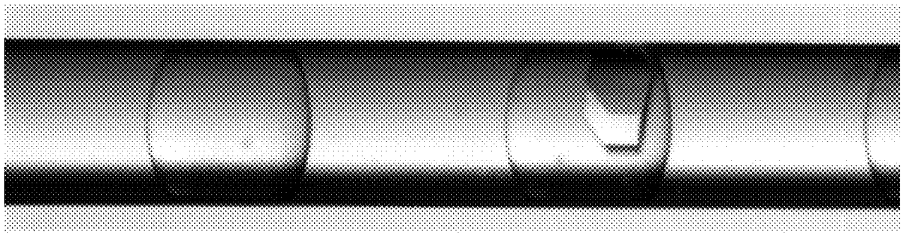


FIGURE 47A

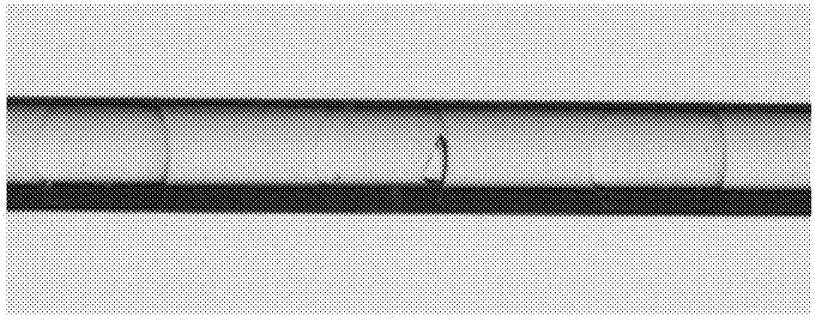


FIGURE 47B

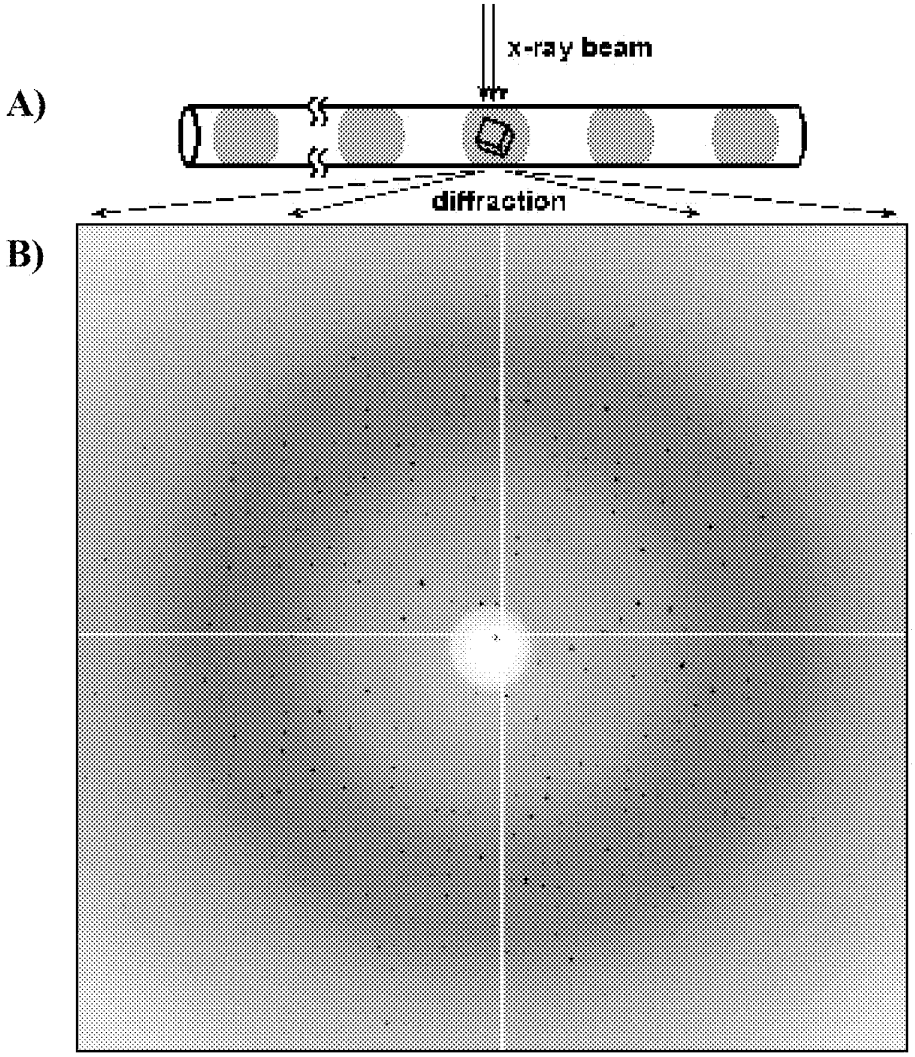


FIGURE 48

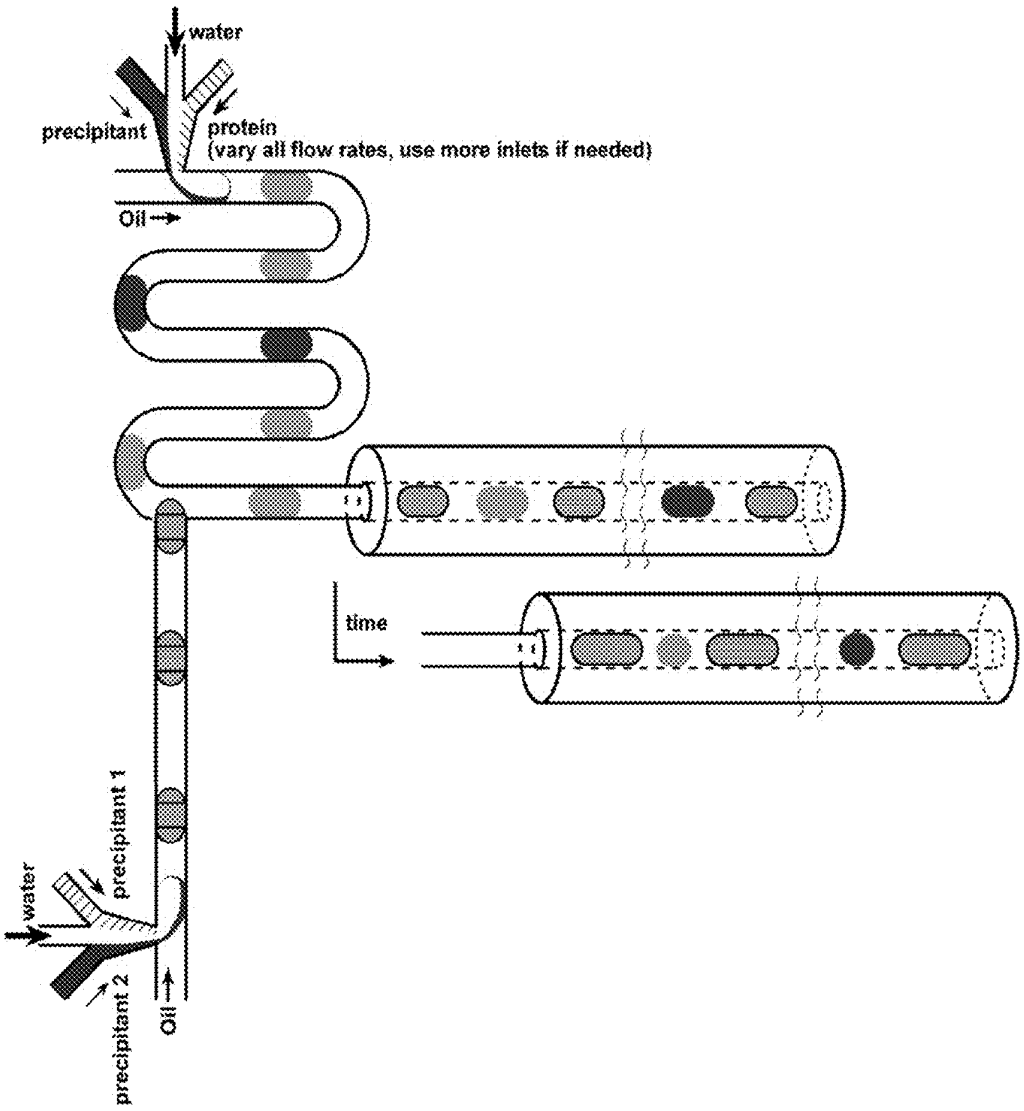

water
precipitant
protein
(vary all flow rates, use more inlets if needed)
Oil
time
water
precipitant 1
precipitant 2
Oil

FIGURE 49

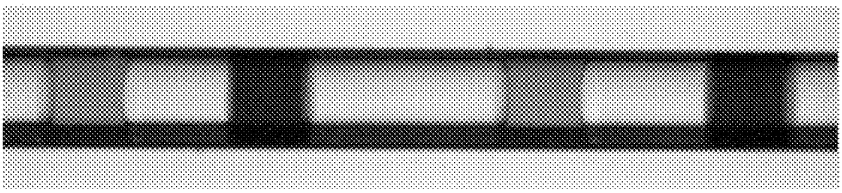


FIGURE 50A

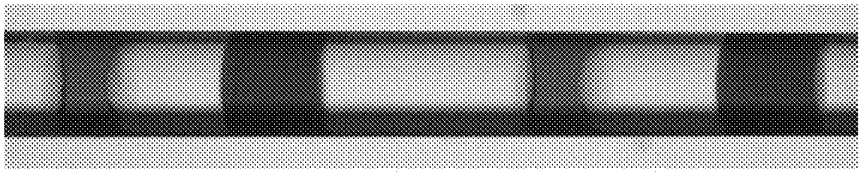


FIGURE 50B

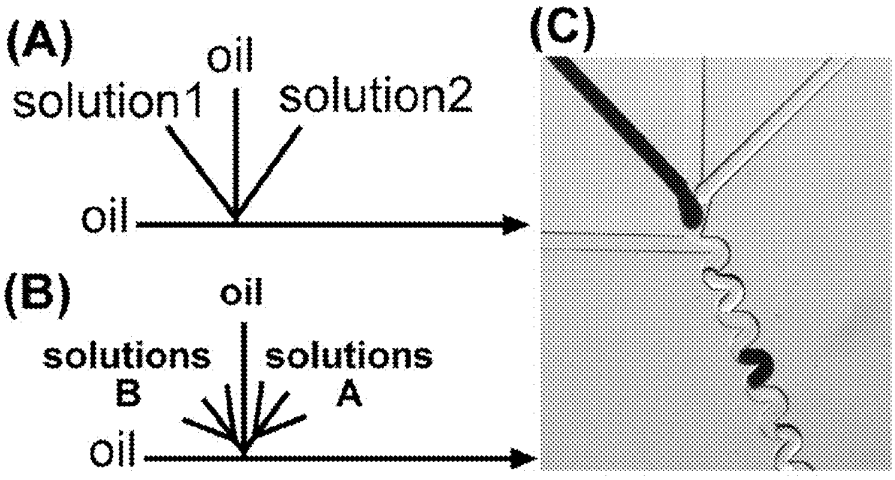

(A)
oil
solution1
solution2
oil
(B)
oil
solutions
B
solutions
A
oil
(C)

FIGURE 51

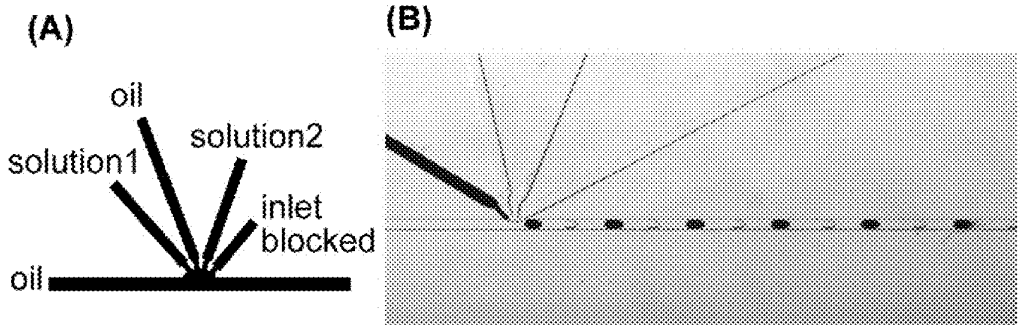


FIGURE 52

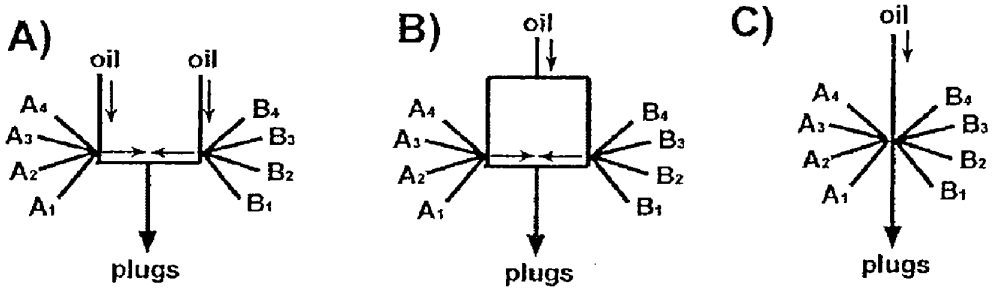

A)
oil
oil
A4
A3
A2
A1
B4
B3
B2
B1
plugs
B)
oil
A4
A3
A2
A1
B4
B3
B2
B1
plugs
C)
oil
A4
A3
A2
A1
B4
B3
B2
B1
plugs

FIGURE 53

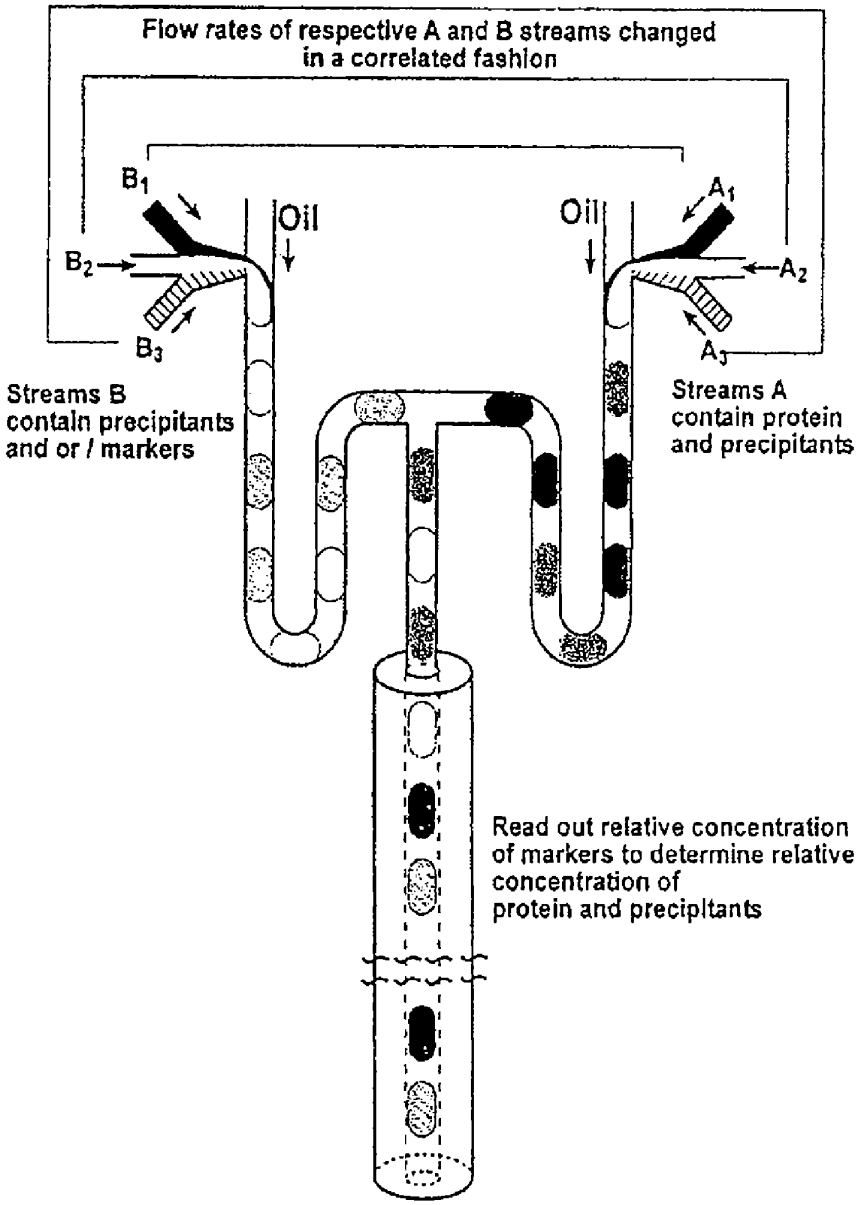

Flow rates of respective A and B streams changed in a correlated fashion
B1
B2
B3
Oil
Oil
A1
A2
A3
Streams B contain precipitants and or / markers
Streams A contain protein and precipitants
Read out relative concentration of markers to determine relative concentration of protein and precipitants

FIGURE 54A

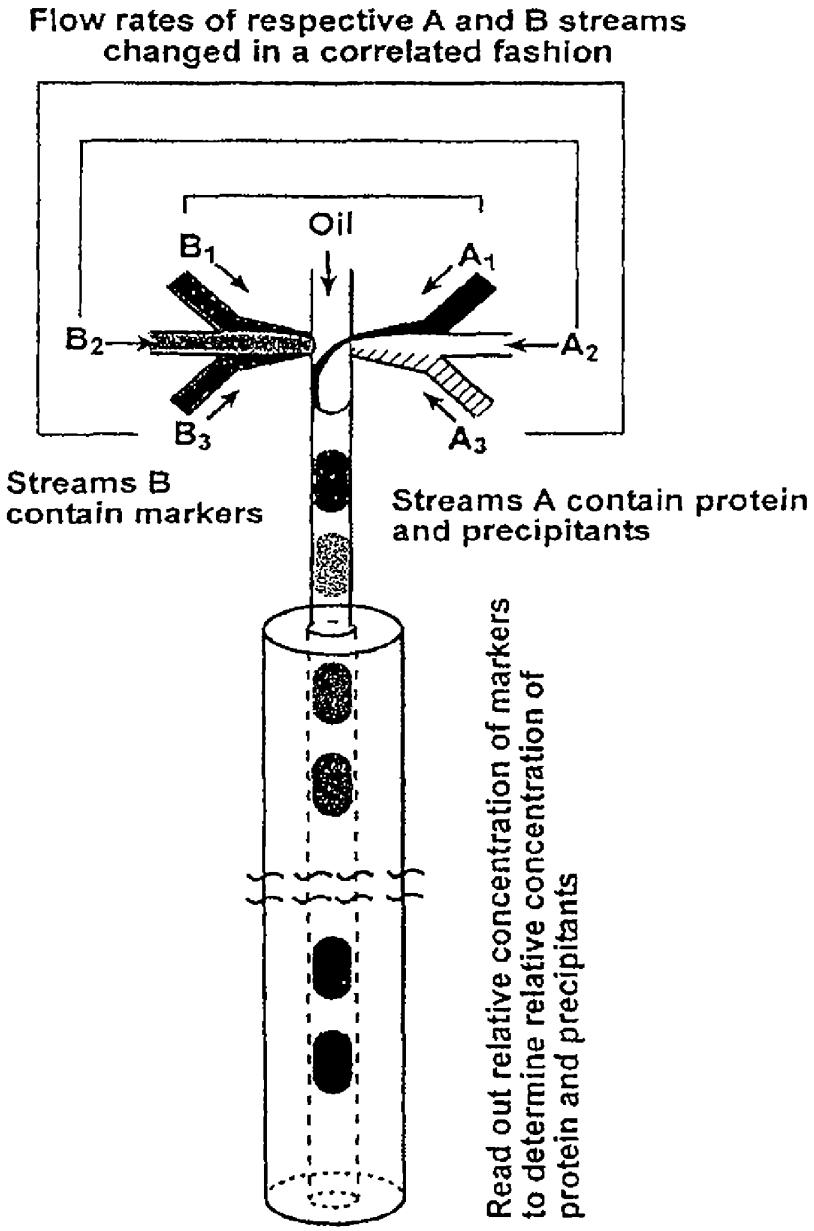

Flow rates of respective A and B streams changed in a correlated fashion
Oil
B1
A1
B2
A2
B3
A3
Streams B contain markers
Streams A contain protein and precipitants
Read out relative concentration of markers to determine relative concentration of protein and precipitants

FIGURE 54B

METHOD FOR CONDUCTING AN AUTOCATALYTIC REACTION IN PLUGS IN A MICROFLUIDIC SYSTEM

This application is a continuation of application Ser. No. 12/777,099, filed May 10, 2010, which is a continuation of application Ser. No. 10/765,718, filed Jan. 26, 2004, now U.S. Pat. No. 7,901,939, which is a continuation-in-part of application Ser. No. 10/434,970, filed May 9, 2003 now U.S. Pat. No. 7,129,091, which claims the benefit of U.S. Provisional Application No. 60/394,544, filed Jul. 8, 2002, and U.S. Provisional Application No. 60/379,927, filed May 9, 2002, all of which are incorporated herein by reference.

BACKGROUND

Nonlinear dynamics, in conjunction with microfluidics, play a central role in the design of the devices and the methods according to the invention. Microfluidics deals with the transport of fluids through networks of channels, typically having micrometer dimensions. Microfluidic systems (sometimes called labs-on-a-chip) find applications in microscale chemical and biological analysis (micro-total-analysis systems). The main advantages of microfluidic systems are high speed and low consumption of reagents. They are thus very promising for medical diagnostics and high-throughput screening. Highly parallel arrays of microfluidic systems are used for the synthesis of macroscopic quantities of chemical and biological compounds, e.g., the destruction of chemical warfare agents and pharmaceuticals synthesis. Their advantage is improved control over mass and heat transport.

Microfluidic systems generally require means of pumping fluids through the channels. In the two most common methods, the fluids are either driven by pressure or driven by electroosmotic flow (EOF). Flows driven by EOF are attractive because they can be easily controlled even in complicated networks. EOF-driven flows have flat, plug-like velocity profile, that is, the velocity of the fluid is the same near the walls and in the middle of the channel. Thus, if small volumes of multiple analytes are injected sequentially into a channel, these plugs are transported as non-overlapping plugs (low dispersion), in which case the dispersion comes mostly from the diffusion between plugs. A main disadvantage of EOF is that it is generated by the motion of the double layer at the charged surfaces of the channel walls. EOF can therefore be highly sensitive to surface contamination by charged impurities. This may not be an issue when using channels with negative surface charges in DNA analysis and manipulation because DNA is uniformly negatively charged and does not adsorb to the walls. However, this can be a serious limitation in applications that involve proteins that are often charged and tend to adsorb on charged surfaces. In addition, high voltages are often undesirable, or sources of high voltages such as portable analyzers may not be available.

Flows driven by pressure are typically significantly less sensitive to surface chemistry than EOF. The main disadvantage of pressure-driven flows is that they normally have a parabolic flow profile instead of the flat profile of EOF. Solutes in the middle of the channel move much faster (about twice the average velocity of the flow) than solutes near the walls of the channels. A parabolic velocity profile normally leads to high dispersion in pressure-driven flows; a plug of solute injected into a channel is immediately distorted and stretched along the channel. This distortion is somewhat reduced by solute transport via diffusion from the middle of the channel towards the walls and back. But the distortion is made worse by diffusion along the channel (the overall dispersion is known as Taylor dispersion).

Taylor dispersion broadens and dilutes sample plugs. Some of the sample is frequently left behind the plug as a tail. Overlap of these tails usually leads to cross-contamination of samples in different plugs. Thus, samples are often introduced into the channels individually, separated by buffer washes. On the other hand, interleaving samples with long buffer plugs, or washing the system with buffer between samples, reduces the throughput of the system.

In EOF, flow transport is essentially linear, that is, if two reactants are introduced into a plug and transported by EOF, their residence time (and reaction time) can be calculated simply by dividing the distance traveled in the channel by the velocity. This linear transport allows precise control of residence times through a proper adjustment of the channel lengths and flow rates. In contrast, dispersion in pressure-driven flow typically creates a broad range of residence times for a plug traveling in such flows, and this diminishes time control.

The issue of time control is important. Many chemical and biochemical processes occur on particular time scales, and measurement of reaction times can be indicative of concentrations of reagents or their reactivity. Stopped-flow type instruments are typically used to perform these measurements. These instruments rely on turbulent flow to mix the reagents and transport them with minimal dispersion. Turbulent flow normally occurs in tubes with large diameter and at high flow rates. Thus stopped-flow instruments tend to use large volumes of reagents (on the order of ml/s). A microfluidic analog of stopped-flow, which consumes smaller volumes of reagents (typically μL/min), could be useful as a scientific instrument, e.g., as a diagnostic instrument. So far, microfluidic devices have not be able to compete with stopped-flow type instruments because EOF is usually very slow (although with less dispersion) while pressure-driven flows suffer from dispersion.

In addition, mixing in microfluidic systems is often slow regardless of the method used to drive the fluid because flow is laminar in these systems (as opposed to turbulent in larger systems). Mixing in laminar flows relies on diffusion and is especially slow for larger molecules such as DNA and proteins.

In addition, particulates present handling difficulty in microfluidic systems. While suspensions of cells in aqueous buffers can be relatively easy to handle because cells are isodense with these buffers, particulates that are not isodense with the fluid tend to settle at the bottom of the channel, thus eventually blocking the channel. Therefore, samples for analysis often require filtration to remove particulates.

SUMMARY ACCORDING TO THE INVENTION

In one aspect, a method includes the steps of providing a microfluidic system comprising one or more channels, and providing within the one or more channels a carrier fluid comprising an oil and at least one plug substantially surrounded by the carrier fluid. The plug includes reagents sufficient for an autocatalytic reaction including a first species of molecule in a concentration such that the plug contains no more than a single molecule of the first species. The method further includes the step of conducting an autocatalytic reaction such that the single molecule is amplified.

In another aspect, the single molecule is a single biological molecule. The single biological molecule may be DNA or RNA and the autocatalytic reaction may be a polymerase-chain reaction.

In another aspect, the conducting step includes heating.

In a another aspect, the method further includes the step of providing a detector to detect, analyze, characterize, or monitor one or more properties of the autocatalytic reaction during and/or after it has occurred. The properties may include light emission. The reagents may include one or more fluorescent labels and the detector may detect emissions from the one or more fluorescent labels.

In another aspect, the method further includes the step of directing the plug to a first channel of the one or more channels when the detector detects, analyzes, characterizes, or monitors one or more of a first set of properties and directing the plug to a second channel of the one or more channels when the detector detects, analyzes, characterizes, or monitors one or more property not included in the first set.

In yet another aspect, the oil is fluorinated oil.

In a further aspect, the carrier fluid further includes a surfactant. The surfactant may be a fluorinated surfactant.

In another aspect, the at least one plug is a merged plug.

In yet another aspect, the one or more channels includes an expansion portion where the at least one plug is trapped for a period of time during or after the reaction.

In a further aspect, the at least one plug is substantially spherical in shape.

In another aspect, the method further includes the step of separating the at least one plug from the carrier fluid.

BRIEF DESCRIPTION OF THE DRAWINGS AND PHOTOGRAPHS

FIG. **1**A is a schematic diagram of a basic channel design that may be used to induce rapid mixing in plugs. FIG. **1**B(**1**)-(**4**) are schematic diagrams depicting a series of periodic variations of the basic channel design. FIG. **1**C(**1**)-(**4**) are schematic diagrams depicting a series of aperiodic combinations resulting from a sequence of alternating elements taken from a basic design element shown in FIG. **1**A and an element from the periodic variation series shown in FIGS. **10**B(**1**)-(**4**).

FIG. **2**A is a schematic diagram contrasting laminar flow transport and plug transport in a channel. FIG. **2**B(**1**) shows a photograph (right side, top portion) illustrating rapid mixing inside plugs moving through winding channels. FIG. **2**B(**2**) shows a photograph (right side, lower portion) showing that winding channels do not accelerate mixing in a laminar flow in the absence of PFD.

FIG. **3** shows photographs (right side) and schematic diagrams (left side) that depict a stream of plugs from an aqueous plug-fluid and an oil (carrier-fluid) in curved channels at flow rates of 0.5 μL/min and 1.0 μL/min.

FIG. **4** shows a photograph (lower portion) and a schematic diagram (upper portion) that illustrate plug formation through the injection of oil and multiple plug-fluids.

FIG. **5** is a schematic diagram that illustrates a two-step reaction in which plugs are formed through the injection of oil and multiple plug-fluids using a combination of different geometries for controlling reactions and mixing.

FIG. **6** is a schematic representation of part of a microfluidic network that uses multiple inlets and that allows for both splitting and merging of plugs. This schematic diagram shows two reactions that are conducted simultaneously. A third reaction (between the first two reaction mixtures) is conducted using precise time delay.

FIG. **7**(*a*)-(*b*) show microphotographs (10 μs exposure) illustrating rapid mixing inside plugs (a) and negligible mixing in a laminar flow (b) moving through winding channels at the same total flow velocity. FIG. **7**(*c*) shows a false-color microphotograph (2 s exposure, individual plugs are invisible) showing time-averaged fluorescence arising from rapid mixing inside plugs of solutions of Fluo-4 and $CaCl_2$. FIG. **7**(*d*) shows a plot of the relative normalized intensity (I) of fluorescence obtained from images such as shown in (c) as a function of distance (left) traveled by the plugs and of time required to travel that distance (right) at a given flow rate. FIG. **7**(*e*) shows a false-color microphotograph (2 s exposure) of the weak fluorescence arising from negligible mixing in a laminar flow of the solutions used in (c).

FIG. **8** shows photographs (right side) and schematics (left side) that illustrate fast mixing at flow rates of about 0.5 μL/min and about 1.0 μL/min using 90°-step channels.

FIG. **9** shows schematics (left side) and photographs (right side) illustrates fast mixing at flow rates of about 1.0 μL/min and about 0.5 μL/min using 135°-step channels.

FIG. **10***a*) is a schematic diagram depicting three-dimensional confocal visualization of chaotic flows in plugs. FIG. **10***b*) is a plot showing a sequence preferably used for visualization of a three-dimensional flow.

FIG. **11** shows a schematic diagram of a channel geometry designed to implement and visualize the baker's transformation of plugs flowing through microfluidic channels.

FIG. **12** shows photographs depicting the merging of plugs (top) and splitting of plugs (bottom) that flow in separate channels or channel branches that are perpendicular.

FIG. **13** shows UV-VIS spectra of CdS nanoparticles formed by rapid mixing in plugs (spectrum with a sharp absorption peak) and by conventional mixing of solutions.

FIG. **14** shows schematic diagrams (left side) and photographs (right side) that illustrate the synthesis of CdS nanoparticles in PDMS microfluidic channels in single-phase aqueous laminar flow (FIG. **14**A) and in aqueous plugs that are surrounded by water-immiscible perfluorodecaline (FIG. **14**B).

FIG. **15** shows schematic representations of the synthesis of CdS nanoparticles inside plugs.

FIG. **16** is a schematic illustration of a microfluidic device according to the invention that illustrates the trapping of plugs.

FIG. **17** is a schematic of a microfluidic method for forming plugs with variable compositions for protein crystallization.

FIG. **18** is a schematic illustration of a method for controlling heterogeneous nucleation by varying the surface chemistry at the interface of an aqueous plug-fluid and a carrier-fluid.

FIG. **19** is a schematic diagram that illustrates a method of separating nucleation and growth using a microfluidic network according to the present invention.

FIG. **20** show schematic diagrams that illustrate two methods that provide a precise and reproducible degree of control over mixing and that can be used to determine the effect of mixing on protein crystallization.

FIG. **21** is a reaction diagram illustrating an unstable point in the chlorite-thiosulfate reaction.

FIG. **22**A-D are schematic diagrams that show various examples of geometries of microfluidic channels according to the invention for obtaining kinetic information from single optical images.

FIG. **23** shows a schematic of a microfluidic network (left side) and a table of parameters for a network having channel heights of 15 and 2 μm.

FIG. **24** shows a reaction scheme that depicts examples of fluorinated surfactants that form monolayers that are: (a) resistant to protein adsorption; (b) positively charged; and (c) negatively charged. FIG. **24***b* shows a chemical structure of neutral surfactants charged by interactions with water by

protonation of an amine or a guanidinium group. FIG. **24***c* shows a chemical structure of neutral surfactants charged by interactions with water deprotonation of a carboxylic acid group.

FIG. **25** are schematic diagrams of microfluidic network (left side of a), b), and c)) that can be used for controlling the concentrations of aqueous solutions inside the plugs, as well as photographs (right side of a), b), and c)) showing the formation of plugs with different concentrations of the aqueous streams.

FIG. **26** are schematic diagrams of microfluidic network (left side of a) and b)) and photographs (right side of a) and b)) of the plug-forming region of the network in which the aqueous streams were dyed with red and green food dyes to show their flow patterns.

FIG. **27** are photographs and plots showing the effects of initial conditions on mixing by recirculating flow inside plugs moving through straight microchannels. FIG. **27***a***1**) is a schematic diagram showing that recirculating flow (shown by black arrows) efficiently mixed solutions of reagents that were initially localized in the front and back halves of the plug. FIG. **27***a***2**) is a schematic diagram showing that recirculating flow (shown by black arrows) did not efficiently mix solutions of reagents that were initially localized in the left and right halves of the plugs. FIG. **27***b*) shows a schematic diagram showing the inlet portions (left side) and photographs of images showing measurements of various periods and lengths of plugs. FIG. **27***c***1**) shows a graph of the relative optical intensity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs of varying lengths. FIG. **27***c***2**) is the same as FIG. **7***c***1**) except that each plug traverses a distance of 1.3 mm.

FIG. **28** is a schematic illustration of a plug showing the notation used to identify different regions of the plugs relative to the direction of motion.

FIG. **29***a*)-*b*) are plots of the periods and the lengths of plugs as a function of total flow velocity (FIG. **29***a*)) and water fraction (FIG. **29***b*)).

FIG. **30** shows photographs illustrating weak dependence of periods, length of plugs, and flow patterns inside plugs on total flow velocity.

FIG. **31** are plots showing the distribution of periods and lengths of plugs where the water fractions were 0.20, 0.40, and 0.73, respectively.

FIG. **32** shows photographs (middle and right side) that show that plug traps are not required for crystal formation in a microfluidic network, as well as a diagram of the microfluidic network (left side).

FIG. **33***a*-*d* (left side) are top views of microfluidic networks (left side) and photographs (right side) that comprise channels having either uniform or nonuniform dimension. FIG. **33***a* shows that merging of the plugs occurs infrequently in the T-shaped channel shown in the photographs. FIG. **33***b* illustrates plug merging occurring between plugs arriving at different times at the Y-shaped junction (magnified view shown). FIG. **33***c* depicts in-phase merging, i.e., plug merging upon simultaneous arrival of at least two plugs at a junction, of plugs of different sizes generated using different oil/water ratios at the two pairs of inlets. FIG. **33***d* illustrates defects (i.e., plugs that fail to undergo merging when they would normally merge under typical or ideal conditions) produced by fluctuations in the relative velocity of the two incoming streams of plugs.

FIG. **34***a*-*c* show a schematic diagram (a, left side) and photographs (b, c) each of which depicts a channel network viewed from the top. FIG. **34***a* is a schematic diagram of the channel network used in the experiment. FIG. **34***b* is a photograph showing the splitting of plugs into plugs of approximately one-half the size of the initial plugs. FIG. **34***c* is a photograph showing the asymmetric splitting of plugs which occurred when $P_1 < P_2$.

FIG. **35** shows a schematic diagram (a, left side) and photographs (b, c) that depicts the splitting of plugs using microfluidic networks without constrictions near the junction.

FIG. **36** shows a photograph (right side) of lysozyme crystals grown in water plugs in the wells of the microfluidic channel, as well as a diagram (left side) of the microfluidic network used in the crystallization.

FIG. **37** is a schematic diagram that depicts a microfluidic device according to the invention that can be used to amplify a small chemical signal using an autocatalytic (and possibly unstable) reaction mixture.

FIG. **38** is a schematic diagram that illustrates a method for a multi-stage chemical amplification which can be used to detect as few as a single molecule.

FIG. **39** shows a diagram (left side) of the microfluidic network and a photograph (right side) of water plugs attached to the PDMS wall.

FIG. **40** is a schematic representation (left side) of a microfluidic network used to measure kinetics data for the reaction of RNase A using a fluorogenic substrate (on-chip enzyme kinetics), and plots that shows the kinetic data for the reaction between RNase A and a fluorogenic substrate.

FIG. **41** shows a photograph (middle and right side) of the water droplet region of the microfluidic network (T stands for time), as well as a diagram of the microfluidic network (left side).

FIG. **42** shows a schematic diagram (left side) of a microfluidic network and a photograph (right side) of the ink plug region of the microfluidic network in which the gradients were formed by varying the flow rates.

FIG. **43** shows a schematic diagram (left side) of a microfluidic network and a photograph (right side) of lysozyme crystals formed in the microfluidic network using gradients.

FIG. **44** are schematic illustrations showing how an initial gradient may be created by injecting a discrete aqueous sample of a reagent B into a flowing stream of water.

FIG. **45***a*) shows a schematic of the microfluidic network used to demonstrate that on-chip dilutions can be accomplished by varying the flow rates of the reagents. The blue rectangle outlines the field of view for images shown in FIG. **45***c*)-*d*). FIG. **45***b*) shows a graph quantifying this dilution method by measuring fluorescence of a solution of fluorescein diluted in plugs in the microchannel.

FIG. **46** shows a microbatch protein crystallization analogue scheme using a with a substrate that includes capillary tubing.

FIG. **47***a*) shows a lysozyme crystal grown attached to a capillary tube wall.

FIG. **47***b*) shows a thaumatin crystal grown at the interface of protein solution and oil.

FIG. **48***a*) shows a schematic illustration of a process for direct screening of crystals in a capillary tube by x-ray diffraction.

FIG. **48***b*) shows an x-ray diffraction pattern from a thaumatin crystal grown inside a capillary tube using a microbatch analogue method (no evaporation).

FIG. **49** shows a vapor-diffusion protein crystallization analogue scheme with a substrate that includes capillary tubing.

FIG. **50***a*) shows vapor diffusion in droplets surrounded by FMS-121 inside a capillary right after the flow was stopped and the capillary was sealed.

FIG. **50***b*) shows vapor diffusion in droplets surrounded by FMS-121 inside a capillary 5 days after the flow was stopped and the capillary was sealed.

FIG. **51***a*) shows a schematic drawing of an experimental setup to form alternating droplets.

FIG. **51***b*) shows a schematic drawing of an experimental setup to form alternating droplets where instead of single solutions **1** and **2**, a set of multiple solutions A and B can be used in a similar system.

FIG. **51***c*) shows a microphotograph illustrating the formation of alternating NaCl—$Fe(SCN)_3$—NaCl droplets.

FIG. **52***a*) shows another example of generating alternating droplets from two different aqueous solutions.

FIG. **52***b*) shows a microphotograph illustrating the formation of alternating NaCl—$Fe(SCN)_3$—NaCl droplets.

FIG. **53***a-c*) shows several representative geometries in which alternating plugs may be formed.

FIG. **54***a-b*) illustrates two representative geometries for indexing a component in a plug using markers.

DETAILED DESCRIPTION ACCORDING TO THE INVENTION

The term "analysis" generally refers to a process or step involving physical, chemical, biochemical, or biological analysis that includes characterization, testing, measurement, optimization, separation, synthesis, addition, filtration, dissolution, or mixing.

The term "analysis unit" refers to a part of or a location in a substrate or channel wherein a chemical undergoes one or more types of analyses.

The term "capillary tube" refers to a hollow, tube-shaped structure with a bore. The cross-sections of the tube and bore can be round, square or rectangular. The corners of the tube or bore can also be rounded. The bore diameters can range in size from 1μ to several millimeters; the outer diameters can be between about 60 μm up to several millimeters. The tube can be made using any material suitable for x-ray diffraction analysis (e.g., silica, plastic, etc.), and can additionally include coatings (e.g. polyimide) suitable for use under variable (e.g, high) temperatures or for UV transparency.

The term "carrier-fluid" refers to a fluid that is immiscible with a plug-fluid. The carrier-fluid may comprise a substance having both polar and non-polar groups or moieties.

The term "channel" refers to a conduit that is typically enclosed, although it may be at least partially open, and that allows the passage through it of one or more types of substances or mixtures, which may be homogeneous or heterogeneous, including compounds, solvents, solutions, emulsions, or dispersions, any one of which may be in the solid, liquid, or gaseous phase. A channel can assume any form or shape such as tubular or cylindrical, a uniform or variable (e.g., tapered) diameter along its length, and one or more cross-sectional shapes along its length such as rectangular, circular, or triangular. A channel is typically made of a suitable material such as a polymer, metal, glass, composite, or other relatively inert materials. As used herein, the term "channel" includes microchannels that are of dimensions suitable for use in devices. A network of channels refers to a multiplicity of channels that are typically connected or in communication with each other. A channel may be connected to at least one other channel through another type of conduit such as a valve.

The term "chemical" refers to a substance, compound, mixture, solution, emulsion, dispersion, molecule, ion, dimer, macromolecule such as a polymer or protein, biomolecule, precipitate, crystal, chemical moiety or group, particle, nanoparticle, reagent, reaction product, solvent, or fluid any one of which may exist in the solid, liquid, or gaseous state, and which is typically the subject of an analysis.

The term "detection region" refers to a part of or a location in a substrate or channel wherein a chemical is identified, measured, or sorted based on a predetermined property or characteristic.

The term "device" refers to a device fabricated or manufactured using techniques such as wet or dry etching and/or conventional lithographic techniques or a micromachining technology such as soft lithography. As used herein, the term "devices" includes those that are called, known, or classified as microfabricated devices. A device according to the invention may have dimensions between about 0.3 cm to about 15 (for 6 inch wafer) cm per side and between about 1 micrometer to about 1 cm thick, but the dimensions of the device may also lie outside these ranges.

The term "discrimination region" refers to a part of or a location in a substrate or channel wherein the flow of a fluid can change direction to enter at least one other channel such as a branch channel.

The term "downstream" refers to a position relative to an initial position which is reached after the fluid flows past the initial point. In a circulating flow device, downstream refers to a position farther along the flow path of the fluid before it crosses the initial point again. "Upstream" refers to a point in the flow path of a fluid that the fluid reaches or passes before it reaches or passes a given initial point in a substrate or device.

The term "flow" means any movement of a solid or a fluid such as a liquid. For example, the movement of plug-fluid, carrier-fluid, or a plug in a substrate, or component of a substrate according to the invention, or in a substrate or component of a substrate involving a method according to the invention, e.g., through channels of a microfluidic substrate according to the invention, comprises a flow. The application of any force may be used to provide a flow, including without limitation: pressure, capillary action, electro-osmosis, electrophoresis, dielectrophoresis, optical tweezers, and combinations thereof, without regard for any particular theory or mechanism of action.

The term "immiscible" refers to the resistance to mixing of at least two phases or fluids under a given condition or set of conditions (e.g., temperature and/or pressure) such that the at least two phases or fluids persist or remain at least partially separated even after the phases have undergone some type of mechanical or physical agitation. Phases or fluids that are immiscible are typically physically and/or chemically discernible, or they may be separated at least to a certain extent.

The term "inlet port" refers to an area of a substrate that receives plug-fluids. The inlet port may contain an inlet channel, a well or reservoir, an opening, and other features that facilitate the entry of chemicals into the substrate. A substrate may contain more than one inlet port if desired. The inlet port can be in fluid communication with a channel or separated from the channel by a valve.

The term "nanoparticles" refers to atomic, molecular or macromolecular particles typically in the length scale of approximately 1-100 nanometer range. Typically, the novel and differentiating properties and functions of nanoparticles are observed or developed at a critical length scale of matter typically under 100 nm. Nanoparticles may be used in constructing nanoscale structures and they may be integrated into larger material components, systems and architectures. In some particular cases, the critical length scale for novel properties and phenomena involving nanoparticles may be under 1 nm (e.g., manipulation of atoms at approximately 0.1 nm) or

it may be larger than 100 nm (e.g., nanoparticle reinforced polymers have the unique feature at approximately 200-300 nm as a function of the local bridges or bonds between the nanoparticles and the polymer).

The term "nucleation composition" refers to a substance or mixture that includes one or more nuclei capable of growing into a crystal under conditions suitable for crystal formation. A nucleation composition may, for example, be induced to undergo crystallization by evaporation, changes in reagent concentration, adding a substance such as a precipitant, seeding with a solid material, mechanical agitation, or scratching of a surface in contact with the nucleation composition.

The term "outlet port" refers to an area of a substrate that collects or dispenses the plug-fluid, carrier-fluid, plugs or reaction product. A substrate may contain more than one outlet port if desired.

The term "particles" means any discrete form or unit of matter. The term "particle" or "particles" includes atoms, molecules, ions, dimers, polymers, or biomolecules.

The term "particulate" refers to a cluster or agglomeration of particles such as atoms, molecules, ions, dimers, polymers, or biomolecules. Particulates may comprise solid matter or be substantially solid, but they may also be porous or partially hollow. They may contain a liquid or gas. In addition, particulates may be homogeneous or heterogeneous, that is, they may comprise one or more substances or materials.

"Plugs" in accordance with the present invention are formed in a substrate when a stream of at least one plug-fluid is introduced into the flow of a carrier-fluid in which it is substantially immiscible. The flow of the fluids in the device is induced by a driving force or stimulus that arises, directly or indirectly, from the presence or application of, for example, pressure, radiation, heat, vibration, sound waves, an electric field, or a magnetic field. Plugs in accordance with the present invention may vary in size but when formed, their cross-section should be substantially similar to the cross-section of the channels in which they are formed. When plugs merge or get trapped inside plug traps, the cross-section of the plugs may change. For example, when a plug enters a wider channel, its cross-section typically increases.

Further, plugs in accordance with the present invention may vary in shape, and for example may be spherical or non-spherical. The shape of the plug may be independent of the shape of the channel (e.g., a plug may be a deformed sphere traveling in a rectangular channel). The plugs may be in the form of plugs comprising an aqueous plug-fluid containing one or more reagents and/or one or more products formed from a reaction of the reagents, wherein the aqueous plug-fluid is surrounded by a non-polar or hydrophobic fluid such as an oil. The plugs may also be in the form of plugs comprising mainly a non-polar or hydrophobic fluid which is surrounded by an aqueous fluid. The plugs may be encased by one or more layers of molecules that comprise both hydrophobic and hydrophilic groups or moieties. The term "plugs" also includes plugs comprising one or more smaller plugs, that is, plugs-within-plugs. The relative amounts of reagents and reaction products contained in the plugs at any given time depend on factors such as the extent of a reaction occurring within the plugs. Preferably, plugs contain a mixture of at least two plug fluids.

The term "plug-forming region" refers to a junction between an inlet port and the first channel of a substrate according to the invention. Preferably, the fluid introduced into the inlet port is "incompatible" (i.e., immiscible) with the fluid in the first channel so that plugs of the fluid formed in the plug-forming region are entrained into the stream of fluid from the first channel.

The term "plug-fluid" refers to a fluid wherein or using which a reaction or precipitation can occur. Typically, the plug-fluid contains a solvent and a reagent although in some embodiments at least one plug-fluid may not contain a reagent. The reagent may be soluble or insoluble in the solvent. The plug-fluid may contain a surfactant. At least two different plug-fluids are used in the present invention. When both plug-fluids contain reagents, the fluids are typically miscible, but can also be partially immiscible, so long as the reagents within each plug-fluid can react to form at least one product or intermediate.

The term "polymer" means any substance or compound that is composed of two or more building blocks ('mers') that are repetitively linked to each other. For example, a "dimer" is a compound in which two building blocks have been joined together. Polymers include both condensation and addition polymers. Typical examples of condensation polymers include polyamide, polyester, protein, wool, silk, polyurethane, cellulose, and polysiloxane. Examples of addition polymers are polyethylene, polyisobutylene, polyacrylonitrile, poly(vinyl chloride), and polystyrene. Other examples include polymers having enhanced electrical or optical properties (e.g., a nonlinear optical property) such as electroconductive or photorefractive polymers. Polymers include both linear and branched polymers.

The term "protein" generally refers to a set of amino acids linked together usually in a specific sequence. A protein can be either naturally-occurring or man-made. As used herein, the term "protein" includes amino acid sequences that have been modified to contain moieties or groups such as sugars, polymers, metalloorganic groups, fluorescent or light-emitting groups, moieties or groups that enhance or participate in a process such as intramolecular or intermolecular electron transfer, moieties or groups that facilitate or induce a protein into assuming a particular conformation or series of conformations, moieties or groups that hinder or inhibit a protein from assuming a particular conformation or series of conformations, moieties or groups that induce, enhance, or inhibit protein folding, or other moieties or groups that are incorporated into the amino acid sequence and that are intended to modify the sequence's chemical, biochemical, or biological properties. As used herein, a protein includes, but is not limited to, enzymes, structural elements, antibodies, hormones, electron carriers, and other macromolecules that are involved in processes such as cellular processes or activities. Proteins typically have up to four structural levels that include primary, secondary, tertiary, and quaternary structures.

The term "reaction" refers to a physical, chemical, biochemical, or biological transformation that involves at least one chemical, e.g., reactant, reagent, phase, carrier-fluid, or plug-fluid and that generally involves (in the case of chemical, biochemical, and biological transformations) the breaking or formation of one or more bonds such as covalent, noncovalent, van der Waals, hydrogen, or ionic bonds. The term includes typical chemical reactions such as synthesis reactions, neutralization reactions, decomposition reactions, displacement reactions, reduction-oxidation reactions, precipitation, crystallization, combustion reactions, and polymerization reactions, as well as covalent and noncovalent binding, phase change, color change, phase formation, crystallization, dissolution, light emission, changes of light absorption or emissive properties, temperature change or heat absorption or emission, conformational change, and folding or unfolding of a macromolecule such as a protein.

The term "reagent" refers to a component of a plug-fluid that undergoes or participates (e.g., by influencing the rate of a reaction or position of equilibrium) in at least one type of

reaction with one or more components of other plug-fluids or a reagent-containing carrier-fluid in the substrate to produce one or more reaction products or intermediates which may undergo a further reaction or series of reactions. A reagent contained in a plug-fluid may undergo a reaction in which a stimulus such as radiation, heat, temperature or pressure change, ultrasonic wave, or a catalyst induces a reaction to give rise to a transformation of the reagent to another reagent, intermediate, or product. A reagent may also undergo a reaction such as a phase change (e.g., precipitation) upon interaction with one or more components of other plug-fluids or a reagent-containing carrier-fluid.

The term "substrate" refers to a layer or piece of material from which devices or chips are prepared or manufactured. As used herein, the term "substrate" includes any substrate fabricated using any traditional or known microfabrication techniques. The term "substrate" also refers either to an entire device or chip or to a portion, area, or section of a device or chip which may or may not be removable or detachable from the main body of the device or chip. The substrate may be prepared from one or more materials such as glass, silicon, silicone elastomer, and polymers including, but not limited to, polypropylene or polyethylene.

The discussion below provides a detailed description of various devices and methods according to the invention for forming plugs, generating gradients in a series of plugs, varying the concentration of reagents inside plugs, rapid mixing in plugs, and scaling of mixing times. In particular, a detailed description of methods for merging, splitting and/or sorting plugs using channels, which form the bases for various applications ranging from the manufacture and analysis of various products to applications in electronics, medicine, diagnostics, and pharmaceuticals, to name a few, is discussed. Methods of detection and measurement of, among others, plugs and processes occurring within plugs are also described.

Among the various applications involving the devices and methods according to the invention are particle separation/sorting, synthesis, investigation of nonlinear and stochastic systems, nonlinear amplification using unstable autocatalytic mixtures, use of stochastic chemical systems for chemical amplification, kinetic measurements, time control of processes, increasing the dynamic range of kinetic measurements, ultrafast measurements, crystallization of proteins, and dynamic control of surface chemistry.

In addition, the devices and methods according to the invention offer a wide-range of other applications. For example, the devices and methods according to the invention provide for effective, rapid, and precise manipulation and monitoring of solutions or reactions over a range of time scales (e.g., from tens of microseconds, to hours or weeks in case of, for example, crystallization) and over a range of solution volumes (e.g., from femtoliters to hundreds of nanoliters).

In one aspect of the invention, the various devices and methods according to the invention are used to overcome one or more of the following problems involving microfluidics. First, the substantial dispersion of solutes in microfluidic channels increases reagent consumption and makes experiments or measurements over long time scales (e.g., minutes to hours) difficult to perform. Various devices and methods according to the invention are intended to overcome this problem by localizing reagents inside plugs that are encapsulated by an immiscible carrier-fluid.

Second, slow mixing of solutions renders experiments, tests, or reactions involving very short time scales (e.g., tens of milliseconds and below) either difficult or impossible to perform with existing technologies. In addition, turbulence-based mixing techniques prohibitively increase sample consumption. In accordance with the present invention, this problem is preferably addressed by conducting the mixing process inside plugs. Rather than relying on turbulence, the various devices and methods according to the invention preferably rely on chaotic advection to accelerate the mixing process. An advantage provided by chaotic advection is that it is expected to operate efficiently in both small and large channels.

Third, achieving control over the chemistry of internal surfaces of devices can be very important at small scales. Thus, being able to control surface chemistry in small devices for example is highly desirable. In accordance with the devices and methods according to the invention, the surface chemistry to which solutions are exposed is preferably controlled through a careful selection of surfactants that are preferably designed to assemble at the interface between the plugs and the immiscible fluid that surrounds them.

Devices and methods of the invention are also provided for use in traditional areas of microfluidics where, for example, miniaturization and speed are important. Thus, the devices and methods according to the invention may be used to develop various tools such as those for high-throughput chemical or biophysical measurements, chemical synthesis, particle formation, and protein crystallization. They may also be used in high-throughput screening, combinatorial synthesis, analysis, and diagnostics, either as a self-contained platform, or in combination with existing technologies particularly those that rely on the use of immiscible fluid flows.

Importantly, the devices of the invention can be adapted to work with automation and robotic technology. They may be used, for example, as a basis for ultra-high throughput automated systems for structural and functional characterization of biological molecules. Thus, the various devices and methods according to the invention provide rapid, economical, and accessible means of synthesis, analysis, and measurements in the fields of biology, chemistry, biophysics, bioengineering, and medicine (e.g., for diagnostics).

The devices and methods of the invention have numerous other possible applications. For example, chaotic mixing at low values of Reynolds number can be exploited as an important tool for controlling unstable chemical reactions. In addition, the systems and devices of the invention may be used for controlling and/or monitoring reactions that generate highly unstable (or explosive) intermediates. They can also be valuable for controlling or monitoring reactions or processes involving autocatalytic reactions. For example, pure hydrogen peroxide (H_2O_2) is an inexpensive and highly effective oxidant, but its autocatalytic decomposition often leads to explosions upon storage and handling. In the microfluidic systems of the invention, H_2O_2 is preferably generated in-situ, stabilized by the chaotic flow, and used to destroy chemical and biological warfare agents. Because the unstable mixtures in these systems are localized inside plugs formed in accordance with the invention, occasional autocatalytic decomposition in one or more plugs is kept localized within those plugs thereby preventing a catastrophic reaction involving the whole system. In addition, large arrays of microfluidic reactors may be operated in parallel to provide substantial throughput.

It is also possible to couple multiple autocatalytic reactions in a single network using the devices and methods according to the invention. For example, a sample plug could be split into many smaller plugs and forwarded to individual amplification cascades. Because the contents of the cascades' outflows exhibit patterns that correspond to the patterns of analytes present in these systems, these patterns could be analyzed using artificial neural network (ANN) (Jackson, R.

B. a. T. *Neural Computing: An Introduction*, Hilger, New York, 1991; Zornetzer et al., *An Introduction to Neural and Electronic Networks*, Academic Press, San Diego, Calif., 1990) algorithms. For example, patterns that arise in blood or saliva analysis may correspond to certain normal or abnormal (e.g., disease, fatigue, infection, poisoning) conditions involving, for example, human and animals.

Moreover, it may be possible to create intelligent microfluidic systems in accordance with the invention, where the nonlinear chemical reactions perform not only detection, but also analysis using ANN algorithms. For example, after amplification, the channels of the present invention typically will contain sufficient amounts of material to operate hydrogel-based valves (Liu et al., "Fabrication and characterization of hydrogel-based microvalves," *J. Microelectromech. Syst.* 2002, vol. 11, pp. 45-53; Yu et al., "Responsive biomimetic hydrogel valve for microfluidics," Appl. Phys. Lett. 2001, vol. 78, pp. 2589-2591; Beebe et al., "Functional hydrogel structures for autonomous flow control inside microfluidic channels," *Nature,* 2000, vol. 404, 588). These valves can be used to control flows inside the system as a function of the sample plug composition. Feedforward and even feedback (e.g., by using the hydrogel valves to control the flow of the input streams) networks may thus be created and used for analysis. Such nonlinear networks may be used not only to recognize patterns pre-programmed by the connectivity of the channels (Hjelmfelt et al., "Pattern-Recognition in Coupled Chemical Kinetic Systems," *Science,* 1993, 260, 335-337) but also to learn patterns by reconfiguring themselves (Jackson, R. B. a. T. *Neural Computing: An Introduction*, Hilger, New York, 1991; Zornetzer et al., *An Introduction to Neural and Electronic Networks*, Academic Press, San Diego, Calif., 1990). Such intelligent microfluidic devices could have unprecedented capabilities for fully autonomous detection, analysis, and signal processing, perhaps surpassing those of biological and current man-made systems.

The devices and methods of the invention are also useful in genomics and proteomics, which are used to identify thousands of new biomolecules that need to be characterized, or are available only in minute quantities. In particular, the success of genomics and proteomics has increased the demand for efficient, high-throughput mechanisms for protein crystallization. X-ray structure determination remains the predominant method of structural characterization of proteins. However, despite significant efforts to understand the process of crystallization, macromolecular crystallization largely remains an empirical field, with no general theory to guide a rational approach. As a result, empirical screening has remained the most widely used method for crystallizing proteins.

The following areas also provide applications of the devices and methods according to the invention. For example, a number of problems still beset high-throughput kinetics and protein crystallization. When it comes to determining protein structure and quantitatively ascertaining protein interactions, there are at least two technological challenges: (1) most robotic technology still only automate existing methods and are often too expensive for a small research laboratory; and (2) there remains the need for conceptually new methods that provide greater degree of control over the crystallization process. In addition, setting up and monitoring crystallization trials typically involve handling of sub-microliter volumes of fluids over periods ranging from seconds to days.

Thus, various devices and methods according to the present invention are designed to provide novel and efficient means for high-throughput crystallization of soluble and membrane proteins. In addition to being a simple and economical method of setting up thousands of crystallization trials in a matter of minutes, a system according to the invention will enable unique time control of processes such as the mixing and nucleation steps leading to crystallization. A system according to the present invention may also be used to control protein crystallization by controlling not only short time-scale events such as nucleation but also long time-scale events such as crystal growth.

Further, the devices and methods of the present invention may be used in high-throughput, kinetic, and biophysical measurements spanning the 10^{-5}-10^{7} second time regime. Preferably, the various devices and methods according to the present invention require only between about a few nanoliters to about a few microliters of each solution. Applications of such devices and methods include studies of enzyme kinetics and RNA folding, and nanoparticle characterization and synthesis, which are discussed in detail below.

Channels and Devices

In one aspect of the invention, a device is provided that includes one or more substrates comprising a first channel comprising an inlet separated from an outlet; optionally, one or more secondary channels (or branch channels) in fluid communication with the first channel, at least one carrier-fluid reservoir in fluid communication with the first channel, at least two plug-fluid reservoirs in fluid communication with the first channel, and a means for applying continuous pressure to a fluid within the substrate.

A device according to the invention preferably comprises at least one substrate.

A substrate may include one or more expansions or areas along a channel wherein plugs can be trapped. The substrates of the present invention may comprise an array of connected channels.

The device may have one or more outlet ports or inlet ports. Each of the outlet and inlet ports may also communicate with a well or reservoir. The inlet and outlet ports may be in fluid communication with the channels or reservoirs that they are connecting or may contain one or more valves. Fluid can be introduced into the channels via the inlet by any means. Typically, a syringe pump is used, wherein the flow rate of the fluid into the inlet can be controlled.

A plug-forming region generally comprises a junction between a plug-fluid inlet and a channel containing the carrier-fluid such that plugs form which are substantially similar in size to each other and which have cross-sections which are substantially similar in size to the cross-section of the channel in the plug-forming region. In one embodiment, the substrate may contain a plurality of plug-forming regions.

The different plug-forming regions may each be connected to the same or different channels of the substrate. Preferably, the sample inlet intersects a first channel such that the pressurized plug fluid is introduced into the first channel at an angle to a stream of carrier-fluid passing through the first channel. For example, in preferred embodiments, the sample inlet and first channel intercept at a T-shaped junction; i.e., such that the sample inlet is perpendicular (i.e. at an angle of 90°) to the first channel. However, the sample inlet may intercept the first channel at any angle.

A first channel may in turn communicate with two or more branch channels at another junction or "branch point", forming, for example, a T-shape or a Y-shape. Other shapes and channel geometries may be used as desired. In exemplary embodiments the angle between intersecting channels is in the range of from about 60° to about 120°. Particular exemplary angles are 45°, 60°, 90°, and 120°. Precise boundaries for the discrimination region are not required, but are preferred.

The first and branch channels of the present invention can, each independently, be straight or have one or more bends. The angle of a bend, relative to the substrate, can be greater than about 10°, preferably greater than about 135°, 180°, 270°, or 360°.

In one embodiment of the invention, a substrate comprises at least one inlet port in communication with a first channel at or near a plug-forming region, a detection region within or coincident with all or a portion of the first channel or plug-forming region, and a detector associated with the detection region. In certain embodiments the device may have two or more plug-forming regions. For example, embodiments are provided in which the analysis unit has a first inlet port in communication with the first channel at a first plug-forming region, a second inlet port in communication with the first channel at a second plug-forming region (preferably downstream from the first plug-forming region), and so forth.

In another embodiment, a substrate according to the invention may comprise a first channel through which a pressurized stream or flow of a carrier-fluid is passed, and two or more inlet channels which intersect the first channel at plug-forming regions and through which a pressurized stream or flow of plug fluids pass. Preferably, these inlet channels are parallel to each other and each intercept the first channel at a right angle. In specific embodiments wherein the plugs introduced through the different plug forming regions are mixed, the inlet channels are preferably close together along the first channel. For example, the first channel may have a diameter of 60 μm that tapers to 30 μm at or near the plug-forming regions. The inlet channels then also preferably have a diameter of about 30 μm and, in embodiments where plug mixing is preferred, are separated by a distance along the first channel approximately equal to the diameter of the inlet channel (i.e., about 30 μm).

In an embodiment according to the invention, the substrate also has a detection region along a channel. There may be a plurality of detection regions and detectors, working independently or together, e.g., to analyze one or more properties of a chemical such as a reagent.

A detection region is within, communicating, or coincident with a portion of a first channel at or downstream of the plug-forming region and, in sorting embodiments, at or upstream of the discrimination region or branch point. Precise boundaries for the detection region are not required, but are preferred.

A typical substrate according to the invention comprises a carrier-fluid inlet that is part of and feeds or communicates directly with a first channel, along with one or more plug fluid inlets in communication with the first channel at a plug-forming region situated downstream from the main inlet (each different plug-fluid inlet preferably communicates with the first channel at a different plug-forming region).

Plugs formed from different plug-fluids or solutions may be released in any order. For example, an aqueous solution containing a first plug-fluid may be released through a first inlet at a first plug-forming region. Subsequently, plugs of an aqueous second plug-fluid may be released through a second inlet at a second plug-forming region downstream of the first inlet.

Fabrication of Channels, Substrates, and Devices

The substrates and devices according to the invention are fabricated, for example by etching a silicon substrate, chip, or device using conventional photolithography techniques or micromachining technology, including soft lithography. The fabrication of microfluidic devices using polydimethylsiloxane has been previously described. These and other fabrication methods may be used to provide inexpensive miniaturized devices, and in the case of soft lithography, can provide robust devices having beneficial properties such as improved flexibility, stability, and mechanical strength. Preferably, when optical detection is employed, the invention also provides minimal light scatter from, for example, plugs, carrier-fluid, and substrate material. Devices according to the invention are relatively inexpensive and easy to set up.

Machining methods (e.g., micromachining methods) that may be used to fabricate channels, substrates, and devices according to the invention are well known in the art and include film deposition processes, such as spin coating and chemical vapor deposition, laser fabrication or photolithographic techniques, or etching methods, which may be performed either by wet chemical or plasma processes.

Channels may be molded onto optically transparent silicone rubber or polydimethylsiloxane (PDMS), preferably PDMS. This can be done, for example, by casting the channels from a mold by etching the negative image of these channels into the same type of crystalline silicon wafer used in semiconductor fabrication. The same or similar techniques for patterning semiconductor features can be used to form the pattern of the channels. In one method of channel fabrication, an uncured PDMS is poured onto the molds placed in the bottom of, for example, a Petri dish. To accelerate curing, the molds are preferably baked. After curing the PDMS, it is removed from on top of the mold and trimmed. Holes may be cut into the PDMS using, for example, a tool such as a cork borer or a syringe needle. Before use, the PDMS channels may be placed in a hot bath of HCl if it is desired to render the surface hydrophilic. The PDMS channels can then be placed onto a microscope cover slip (or any other suitable flat surface), which can be used to form the base/floor or top of the channels.

A substrate according to the invention is preferably fabricated from materials such as glass, polymers, silicon microchip, or silicone elastomers. The dimensions of the substrate may range, for example, between about 0.3 cm to about 7 cm per side and about 1 micron to about 1 cm in thickness, but other dimensions may be used.

A substrate can be fabricated with a fluid reservoir or well at the inlet port, which is typically in fluid communication with an inlet channel. A reservoir preferably facilitates introduction of fluids into the substrate and into the first channel. An inlet port may have an opening such as in the floor of the substrate to permit entry of the sample into the device. The inlet port may also contain a connector adapted to receive a suitable piece of tubing, such as Teflon® tubing, liquid chromatography or HPLC tubing, through which a fluid may be supplied. Such an arrangement facilitates introducing the fluid under positive pressure in order to achieve a desired pressure at the plug-forming region.

A substrate containing the fabricated flow channels and other components is preferably covered and sealed, preferably with a transparent cover, e.g., thin glass or quartz, although other clear or opaque cover materials may be used. Silicon is a preferred substrate material due to well-developed technology permitting its precise and efficient fabrication, but other materials may be used, including polymers such as polytetrafluoroethylenes. Analytical devices having channels, valves, and other elements can be designed and fabricated from various substrate materials. When external radiation sources or detectors are employed, the detection region is preferably covered with a clear cover material to allow optical access to the fluid flow. For example, anodic bonding of a silicon substrate to a PYREX® cover slip can be accomplished by washing both components in an aqueous H_2SO_4/

H_2O_2 bath, rinsing in water, and then, for example, heating to about 350° C. while applying a voltage of 450 V.

A variety of channels for sample flow and mixing can be fabricated on the substrate and can be positioned at any location on the substrate, chip, or device as the detection and discrimination or sorting points. Channels can also be designed into the substrate that place the fluid flow at different times/distances into a field of view of a detector. Channels can also be designed to merge or split fluid flows at precise times/distances.

A group of manifolds (a region consisting of several channels that lead to or from a common channel) can be included to facilitate the movement of plugs from different analysis units, through the plurality of branch channels and to the appropriate solution outlet. Manifolds are preferably fabricated into the substrate at different depth levels. Thus, devices according to the invention may have a plurality of analysis units that can collect the solution from associated branch channels of each unit into a manifold, which routes the flow of solution to an outlet. The outlet can be adapted for receiving, for example, a segment of tubing or a sample tube, such as a standard 1.5 ml centrifuge tube. Collection can also be done using micropipettes.

Methods of Forming Plugs

The various channels, substrates, and devices according to the invention are primarily used to form and manipulate plugs.

In a preferred embodiment, plug-fluids do not significantly mix at or before they are introduced into the first channel. The plug-fluids may form distinct laminar streams at or before the inlet. They may be separated by an additional fluid. Alternatively, they may be introduced into the carrier-fluid via inlets of differing size. The concentration of plug-fluids in the plugs may be adjusted by adjusting volumetric flow rates of the plug-fluids. Further, the diameters of the first channel and the branch channel(s) may differ.

FIG. **2**A is a schematic diagram contrasting laminar flow transport and plug transport in a channel. In the lower figure which depicts the transport of plugs, two aqueous reagents (marked in red and blue) form laminar streams that are separated by a "divider" aqueous stream. The three streams enter a channel with flowing oil, at which point plugs form and plug fluids mix. During plug transport, rapid mixing of the plug-fluids typically occurs within the plugs. In contrast, in laminar flow transport, fluid mixing occurs slowly, and with high dispersion, as shown in the upper figure. In the upper figure, the time t at a given point d_1 can be estimated from $t_1 \approx d_1/U$, where d1 is the distance from d=0 and U is the flow velocity. In the lower figure, the time t is given by $t_1=d_1/U$.

FIG. **2**B shows a photograph and a schematic diagram that depict mixing in water/oil plugs (upper schematic and photograph) and in laminar streams (lower schematic and photograph) comprising only aqueous plug-fluids. The oil (carrier-fluid in this case) is introduced into channel **200** of a substrate. Instead of oil, water is introduced into the corresponding channel **207** in the case of mixing using laminar streams. The three aqueous plug-fluids are introduced by inlet ports **201**, **202**, **203** into the carrier-fluid (and by inlet ports **204**, **205**, **206** in the case of laminar streams). A preferred scheme is one in which the aqueous plug-fluids initially coflow preferably along a short or minimal distance before coming in contact with the carrier-fluid. In a preferred embodiment, the distance traversed by the coflowing plug-fluids is approximately or substantially equal to the width of the channel.

The middle or second aqueous plug-fluid in the top figure may be plain water, buffer, solvent, or a different plug-fluid. The middle aqueous plug-fluid would preferably initially separate the two other aqueous plug-fluids before the aqueous fluids come into contact with the carrier-fluid. Thus, the intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous plug-fluids before they come in contact with the carrier-fluid. The plugs that form in the plug-forming region can continue along an unbranched channel, can split and enter a channel, can merge with plugs from another channel, or can exit the substrate through an exit port. It can be seen in FIG. **2** that, in the absence of an oil, the aqueous plug-fluids flow in laminar streams without significant mixing or with only partial mixing. In contrast, plug-fluids mix substantially or completely in the plugs.

FIG. **3** shows photographs and schematic diagrams that depict a stream of plugs from an aqueous plug-fluid and an oil (carrier-fluid) in curved channels at flow rates of 0.5 μL/min (top schematic diagram and photograph) and 1.0 μL/min (bottom schematic diagram and photograph). This scheme allows enhanced mixing of reagents in the elongated plugs flowing along a curved channel with smooth corners or curves. The carrier-fluid is introduced into an inlet port **300**, **307** of a substrate while the three aqueous plug-fluids are introduced in separate inlet ports **301**-**306**. As in FIG. **2**, a preferred scheme would be one in which the plug-fluids initially coflow preferably along a short or minimal distance before coming in contact with the carrier-fluid. In a preferred embodiment, the distance traversed by the coflowing plug-fluids (e.g., aqueous plug-fluids) is approximately or substantially equal to the width of the channel. The middle or second aqueous plug-fluid may comprise plain water, buffer, solvent, or a plug-fluid, and the middle aqueous plug-fluid preferably initially separates the two other aqueous plug-fluids before the aqueous plug-fluids come into contact with the carrier-fluid which, in this case, is an oil. Thus, the intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous plug-fluids before they come in contact with the oil (or carrier-fluid).

FIG. **4** shows a photograph and schematic diagram that illustrate plug formation through the injection of oil and multiple plug-fluids. Although FIG. **4** shows five separate plug-fluids, one may also separately introduce less than or more than five plug-fluids into the substrate. The reagents or solvents comprising the plug-fluids may be different or some of them may be identical or similar. As in FIG. **2**, the oil is introduced into an inlet port **400** of a substrate while the aqueous plug-fluid is introduced in separate inlet ports **401**-**405**. The water plugs then flow through exit **406**. A preferred scheme is one in which the aqueous plug-fluids would initially coflow preferably along a short or minimal distance before coming in contact with the oil. In a preferred embodiment, the distance traversed by the coflowing plug-fluids is approximately or substantially equal to the width of the channel. One or more of the aqueous plug-fluids may comprise plain water, buffer, solvent, or a plug-fluid, and at least one aqueous plug-fluid would preferably initially separate at least two other aqueous streams before the aqueous plug-fluid comes into contact with the oil. Thus, the at least one intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous streams before the aqueous streams come in contact with the oil. FIG. **5** shows a microfluidic network, which is similar to that shown in FIG. **4**, in which several reagents can be introduced into the multiple inlets. In addition, FIG. **5** shows a channel having a winding portion through which the plugs undergo mixing of the four reagents A, B, C, and D. As shown in FIG. **5**, the reagents A, B, C, and D are introduced into inlet ports **501**, **503**, **505**, and **507**, while aqueous streams are introduced

into inlet ports **502**, **504**, **506**. FIG. **5** shows plugs through the various stages of mixing, wherein mixture **50** corresponds to the initial A+B mixture, mixture **51** corresponds to the initial C+D mixture, mixture **52** corresponds to the mixed A+B mixture, mixture **53** corresponds to the mixed C+D mixture, and mixture **54** corresponds to the A+B+C+D mixture.

The formation of the plugs preferentially occurs at low values of the capillary number C.n., which is given by the equation

$$C.n.=U\mu/\gamma \quad \text{Eqn. (1)}$$

where U is the flow velocity, μ is the viscosity of the plug fluid or carrier-fluid, and γ is the surface tension at the water/surfactant interface.

The plugs may be formed using solvents of differing or substantially identical viscosities. Preferably, the conditions and parameters used in an experiment or reaction are such that the resulting capillary number lies in the range of about 0.001≦C.n.≦about 10. Preferably, the values of parameters such as viscosities and velocities are such that plugs can be formed reliably. Without wishing to be bound by theory, it is believed that as long as flow is not stopped, the C.n. is ≦about 0.2, and as long as the surface tension of the plug-fluid/carrier-fluid interface is lower than the surface tension of the solution/wall interface, plug formation will persist. The C.n. number is zero when flow is stopped.

In one embodiment, in which perfluorodecaline was used as the carrier-fluid and the plug-fluid was aqueous, it was found that this system can be operated at values of C.n. up to ~0.1 (at 300 mm s^{-1}). In this system, as the value of the C.n. increased above ~0.2, the formation of plugs became irregular. The viscosity of perfluorodecaline is 5.10×10^{-3} kg m^{-3} s^{-1}, the surface tension at the interface between the plugs and the carrier-fluid was 13×10^{-3} N m^{-1}.

The length of the plugs can be controlled such that their sizes can range from, for example, about 1 to 4 times a cross-sectional dimension (d, where d is a channel cross-sectional dimension) of a channel using techniques such as varying the ratio of the plug-fluids and carrier-fluids or varying the relative volumetric flow rates of the plug-fluid and carrier-fluid streams. Short plugs tend to form when the flow rate of the aqueous stream is lower than that of a carrier-fluid stream. Long plugs tend to form when the flow rate of the plug-fluid stream is higher than that of the carrier stream.

In one approximation, the volume of a plug is taken equal to about $2\times d^3$, where d is a cross-sectional dimension of a channel. Thus, the plugs can be formed in channels having cross-sectional areas of, for example, from 20×20 to 200×200 μm^2, which correspond to plug volumes of between about 16 picoliters (pL) to 16 nanoliters (nL). The size of channels may be increased to about 500 μm (corresponding to a volume of about 250 nL) or more. The channel size can be reduced to, for example, about 1 μm (corresponding to a volume of about 1 femtoliter). Larger plugs are particularly useful for certain applications such as protein crystallizations, while the smaller plugs are particularly useful in applications such as ultrafast kinetic measurements.

In one preferred embodiment, plugs conform to the size and shape of the channels while maintaining their respective volumes. Thus, as plugs move from a wider channel to a narrower channel they preferably become longer and thinner, and vice versa.

Plug-fluids may comprise a solvent and optionally, a reactant. Suitable solvents for use in the invention, such as those used in plug-fluids, include organic solvents, aqueous solvents, oils, or mixtures of the same or different types of solvents, e.g. methanol and ethanol, or methanol and water. The solvents according to the invention include polar and non-polar solvents, including those of intermediate polarity relative to polar and non-polar solvents. In a preferred embodiment, the solvent may be an aqueous buffer solution, such as ultrapure water (e.g., 18 MΩ resistivity, obtained, for example, by column chromatography), 10 mM Tris HCl, and 1 mM EDTA (TE) buffer, phosphate buffer saline or acetate buffer. Other solvents that are compatible with the reagents may also be used.

Suitable reactants for use in the invention include synthetic small molecules, biological molecules (i.e., proteins, DNA, RNA, carbohydrates, sugars, etc.), metals and metal ions, and the like.

The concentration of reagents in a plug can be varied. In one embodiment according to the invention, the reagent concentration may be adjusted to be dilute enough that most of the plugs contain no more than a single molecule or particle, with only a small statistical chance that a plug will contain two or more molecules or particles. In other embodiments, the reagent concentration in the plug-fluid is adjusted to concentrate enough that the amount of reaction product can be maximized.

Suitable carrier-fluids include oils, preferably fluorinated oils. Examples include viscous fluids, such as perfluorodecaline or perfluoroperhydrophenanthrene; nonviscous fluids such as perfluorohexane; and mixtures thereof (which are particularly useful for matching viscosities of the carrier-fluids and plug-fluids). Commercially available fluorinated compounds such as Fluorinert™ liquids (3M, St. Paul, Minn.) can also be used.

The carrier-fluid or plug-fluid, or both may contain additives, such as agents that reduce surface tensions (e.g., surfactants). Other agents that are soluble in a carrier-fluid relative to a plug-fluid can also be used when the presence of a surfactant in the plug fluid is not desirable. Surfactants may be used to facilitate the control and optimization of plug size, flow and uniformity. For example, surfactants can be used to reduce the shear force needed to extrude or inject plugs into an intersecting channel. Surfactants may affect plug volume or periodicity, or the rate or frequency at which plugs break off into an intersecting channel. In addition, surfactants can be used to control the wetting of the channel walls by fluids. In one embodiment according to the invention, at least one of the plug-fluids comprises at least one surfactant.

Preferred surfactants that may be used include, but are not limited to, surfactants such as those that are compatible with the carrier and plug-fluids. Exemplary surfactants include Tween™, Span™, and fluorinated surfactants (such as Zonyl™ (Dupont, Wilmington Del.)). For example, fluorinated surfactants, such as those with a hydrophilic head group, are preferred when the carrier-fluid is a fluorinated fluid and the plug-fluid is an aqueous solution.

However, some surfactants may be less preferable in certain applications. For instance, in those cases where aqueous plugs are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort biomaterials, a water soluble surfactant such as SDS may denature or inactivate the contents of the plug.

The carrier-fluid preferably wets the walls of the channels preferentially over the plugs. If this condition is satisfied, the plug typically does not come in contact with the walls of the channels, and instead remains separated from the walls by a thin layer of the carrier-fluid. Under this condition, the plugs remain stable and do not leave behind any residue as they are transported through the channels. The carrier-fluid's preferential wetting of the channel walls over the plug-fluid is achieved preferably by setting the surface tension by, for

example, a suitable choice of surfactant. Preferably, the surface tension at a plug fluid/channel wall interface (e.g., about 38 mN/m surface tension for a water/PDMS interface) is set higher than the surface tension at a plug fluid/carrier-fluid interface (e.g., about 13 mN/m for a water/carrier-fluid interface with a surfactant such as 10% 1H,1H,2H,2H-perfluorooctanol in perfluorodecaline as the carrier-fluid). If this condition is not satisfied, plugs tend to adhere to the channel walls and do not undergo smooth transport (e.g., in the absence of 1H,1H,2H,2H-perfluorooctanol the surface tension at the water/perfluorodecaline interface is about 55 mN/m, which is higher than the surface tension of the water/PDMS interface (e.g., about 38 mN/m)), and plugs adhere to the walls of the PDMS channels. Because the walls of the channels (PDMS, not fluorinated) and the carrier-fluid (fluorinated oil) are substantially different chemically, when a fluorinated surfactant is introduced, the surfactant reduces the surface tension at the oil-water interface preferentially over the wall-water interface. This allows the formation of plugs that do not stick to the channel walls.

The surface tension at an interface may be measured using what is known as a hanging drop method, although one may also use other methods. Preferably, the surface tension is sufficiently high to avoid destruction of the plugs by shear.

The plug-fluids and carrier-fluids may be introduced through one or more inlets. Specifically, fluids may be introduced into the substrate through pneumatically driven syringe reservoirs that contain either the plug-fluid or carrier-fluid. Plugs may be produced in the carrier-fluid stream by modifying the relative pressures such that the plug-fluids contact the carrier-fluid in the plug-forming regions then shear off into discrete plugs.

In the invention, plugs are formed by introducing the plug-fluid, at the plug-forming region, into the flow of carrier-fluid passing through the first channel. The force and direction of flow can be controlled by any desired method for controlling flow, for example, by a pressure differential, or by valve action. This permits the movement of the plugs into one or more desired branch channels or outlet ports.

In preferred embodiments according to the invention, one or more plugs are detected, analyzed, characterized, or sorted dynamically in a flow stream of microscopic dimensions based on the detection or measurement of a physical or chemical characteristic, marker, property, or tag.

The flow stream in the first channel is typically, but not necessarily continuous and may be stopped and started, reversed or changed in speed. Prior to sorting, a non-plug-fluid can be introduced into a sample inlet port (such as an inlet well or channel) and directed through the plug-forming region, e.g., by capillary action, to hydrate and prepare the device for use. Likewise, buffer or oil can also be introduced into a main inlet port that communicates directly with the first channel to purge the substrate (e.g., of "dead" air) and prepare it for use. If desired, the pressure can be adjusted or equalized, for example, by adding buffer or oil to an outlet port.

The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug-forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs. Periodicity and plug volume may also depend on channel diameter and/or the viscosity of the fluids.

Mixing in Plugs

FIG. **7**(*a*)-(*b*) show microphotographs (10 μs exposure) illustrating rapid mixing inside plugs (a) and negligible mixing in a laminar flow (b) moving through winding channels at the same total flow velocity. Aqueous streams were introduced into inlets **700**-**705** in FIGS. **7**(*a*)-(*b*). In FIGS. **7**(*c*) and **7**(*e*), Fluo-4 was introduced into inlets **706**, **709**, buffer was introduced into inlets **707**, **710**, and $CaCl_2$ was introduced into inlets **708**, **711**. FIG. **7**(*c*) shows a false-color microphotograph (2 s exposure, individual plugs are invisible) showing time-averaged fluorescence arising from rapid mixing inside plugs of solutions of Fluo-4 (54 μM) and $CaCl_2$ (70 μM) in aqueous sodium morpholine propanesulfonate buffer (20 μM, pH 7.2); this buffer was also used as the middle aqueous stream. FIG. **7**(*d*) shows a plot of the relative normalized intensity (I) of fluorescence obtained from images such as shown in (c) as a function of distance (left) traveled by the plugs and of time required to travel that distance (right) at a given flow rate. The total intensity across the width of the channel was measured. Total PFD/water volumetric flow rates (in μL min^{-1}) were 0.6:0.3, 1.0:0.6, 12.3:3.7, 10:6, and 20:6. FIG. **7**(*e*) shows a false-color microphotograph (2 s exposure) of the weak fluorescence arising from negligible mixing in a laminar flow of the solutions used in (c). All channels were 45 μm deep; inlet channels were 50 μm and winding channels 28 μm wide; Re~5.3 (water), ~2.0 (PFD).

FIG. **8** shows photographs and schematics that illustrate fast mixing at flow rates of about 0.5 μL/min (top schematic diagram and photograph) and about 1.0 μL/min (lower schematic diagram and photograph) using 90°-step channels while FIG. **9** illustrates fast mixing at flow rates of about 1.0 μL/min (top schematic diagram and photograph) and about 0.5 μL/min (lower schematic diagram and photograph) using 135°-step channels. Aqueous streams are introduced into inlets **800**-**805** in FIG. **8** (inlets **900**-**905** in FIG. **9**), while a carrier fluid is introduced into channels **806**, **807** (channels **906**, **907** in FIG. **9**). The plugs that form then flow through exits **808**, **809** (FIG. **8**) and exits **908**, **909** (FIG. **9**). As can be seen in FIG. **8** and FIG. **9**, the plugs are transported along multi-step channels, instead of channels with smooth curves (as opposed to channels with sharp corners). An advantage of these multi-step configurations of channels is that they may provide further enhanced mixing of the substances within the plugs.

Several approaches may be used to accelerate or improve mixing. These approaches may then be used to design channel geometries that allow control of mixing. Flow can be controlled by perturbing the flow inside a moving plug so that it differs from the symmetric flow inside a plug that moves through a straight channel. For example, flow perturbation can be accomplished by varying the geometry of a channel (e.g., by using winding channels), varying the composition of the plug fluid (e.g., varying the viscosities), varying the composition of the carrier-fluid (e.g., using several laminar streams of carrier-fluids that are different in viscosity or surface tension to form plugs; in this case, mixing is typically affected, and in some cases enhanced), and varying the patterns on the channel walls (e.g., hydrophilic and hydrophobic, or differentially charged, patches would interact with moving plugs and induce time-periodic flow inside them, which should enhance mixing).

Various channel designs can be implemented to enhance mixing in plugs. FIG. **1**A shows a schematic of a basic channel design, while FIG. **1**B shows a series of periodic variations of the basic channel design. FIG. **1**C shows a series of aperiodic combinations resulting from a sequence of alternating elements taken from a basic design element shown in FIG.

1A and an element from the periodic variation series shown in FIGS. 1B(1)-(4). When the effects of these periodic variations are visualized, aperiodic combinations of these periodic variations are preferably used to break the symmetries arising from periodic flows (see FIG. 1C). Here, the relevant parameters are channel width, period, radius of curvature, and sequence of turns based on the direction of the turns. The parameters of the basic design are defined such that c is the channel width, l is the period, and r is the radius of curvature. For the basic design, the sequence can be defined as (left, right, left, right), where left and right is relative to a centerline along the path taken by a plug in the channel.

FIGS. 1B(1)-4) show schematic diagrams of a series of periodic variations of the basic design. At least one variable parameter is preferably defined based on the parameters defined in FIG. 1*a*). In FIG. 1B(1), the channel width is c/2; in FIG. 1B(2), the period is 2 l; and in FIG. 1B(3), and the radius of curvature is 2 r. In FIG. 1B(4), the radius of curvature is r/2 and the sequence is (left, left, right, right).

FIGS. 1C(1)-(4) show a schematic diagram of a series of aperiodic combinations formed by combining the basic design element shown in FIG. 1A with an element from the series of periodic variations in FIG. 1B(1)-(4). In FIG. 1C(1), the alternating pattern of a period of the basic design shown in FIG. 1A (here denoted as "a") and a period of the channel in FIG. 1B(1) (here denoted as "b1") is given by a+b1+a+ . . . . In FIG. 1C(2), the aperiodic combination is given by a+b2+a. In the channel shown in FIG. 1C(3) (here denoted as "c3"), the aperiodic combination is given by a+c3+a. In the channel shown in FIG. 1C(4) (here denoted as "c4"), a (right, left) sequence is introduced with a kink in the pattern. A repeating (left, right) sequence would normally be observed. By adding this kink, the sequence becomes (left, right, left, right)+(right, left)+(left, right, left, right).

Another approach for accelerating mixing relies on rationally-designed chaotic flows on a microfluidic chip using what is known as the baker's transformation. Reorientation of the fluid is critical for achieving rapid mixing using the baker's transformation. The baker's transformation leads to an exponential decrease of the striation thickness (the distance over which mixing would have to occur by diffusion) of the two components via a sequence of stretching and folding operations. Typically, every stretch-fold pair reduces the striation thickness by a factor of 2, although this factor may have a different value. The striation thickness (ST) can be represented, in an ideal case, by Eqn. (2) below. Thus, in the ideal case, in a sequence of n stretch-fold-reorient operations, the striation thickness undergoes an exponential decrease given by

$$ST(t_n)=ST(t_0)\times 2^{-n} \quad \text{Eqn. (2)}$$

where $ST(t_n)$ represents the striation thickness at time t_n, $ST(t_0)$ represents the initial striation thickness at time t_0, and n is the number of stretch-fold-reorient operations.

In accordance with the invention, the baker's transformation is preferably implemented by creating channels composed of a sequence of straight regions and sharp turns. FIG. 11 shows a schematic diagram of a channel geometry designed to implement and visualize the baker's transformation of plugs flowing through microfluidic channels. Other designs could also be used. The angles at the channel bends and the lengths of the straight portions are chosen so as to obtain optimal mixing corresponding to the flow patterns shown. Different lengths of straight paths and different turns may be used depending on the particular application or reaction involved.

A plug traveling through every pair of straight part 112 and sharp-turn part 111 of the channel, which is equivalent to one period of a baker's transformation, will experience a series of reorientation, stretching and folding. In a straight part of the channel, a plug will experience the usual recirculating flow. At a sharp turn, a plug normally rolls and reorients due to the much higher pressure gradient across the sharp internal corner and also due to larger travel path along the outside wall. This method of mixing based on the baker's transformation is very efficient and is thus one of the preferred types of mixing. In particular, this type of mixing leads to a rapid reduction of the time required for reagent mixing via diffusion.

It is believed that plug formation can be maintained at about the same flow rate in channels of different sizes because the limit of a flow rate is typically set by the capillary number, C.n., which is independent of the channel size. At a fixed flow rate, the mixing time t_{mix} may decrease as the size of the channel (d) is reduced. First, it is assumed that it takes the same number n of stretch-fold-reorient cycles to mix reagents in both large and small channels. This assumption (e.g., for n~5) is in approximate agreement with previously measured mixing in d=55 and d=20 micrometer (μm) channels. Each cycle requires a plug to travel over a distance of approximately 2 lengths of the plug (approximately 3 d). Therefore, mixing time is expected to be approximately equal to the time it takes to travel 15 d, and will decrease linearly with the size of the channel, t_{mix}~d. A method that provides mixing in about 1 ms in 25-μm channels preferably provides mixing in about 40 μs in 1-μm channels. Achieving microsecond mixing times generally requires the use of small channels. High pressures are normally required to drive a flow through small channels.

Without wishing to be bound by theory, theoretical modeling indicates that the number of cycles it takes for mixing to occur in a channel with diameter d is given approximately by

$$n\times 2^{2n}\approx dU/D \quad \text{Eqn. (3)}$$

where n is the number of cycles, U is the flow velocity, D is the diffusion constant, one cycle is assumed to be equal to 6 d, and mixing occurs when convection and diffusion time scales are matched. The mixing time is primarily determined by the number of cycles. This result indicates that mixing will be accelerated more than just in direct proportion to the channel diameter. For example, when d decreases by a factor of 10, mixing time decreases by a factor of d×Log(d)=10×Log(10). With properly designed channels, mixing times in 1-μm channels can be limited to about 20 μs. Even at low flow rates or long channels (such as those involving protein crystallization), however, significant mixing can still occur. In addition, without being bound by theory, it is expected that increasing the flow rate U by a factor of 10 will decrease the mixing time by a factor of Log(U)/U=(Log (10))/10.

To visualize mixing in a channel according to the invention, a colored marker can be used in a single plug-fluid. The initial distribution of the marker in the plug has been observed to depend strongly on the details of plug formation. As the stationary aqueous plug was extruded into the flowing carrier-fluid, shearing interactions between the flow of the carrier-fluid and the plug-fluid induced an eddy that redistributed the solution of the marker to different regions of the plug. The formation of this eddy is referred to here as "twirling" (see FIG. 27*b*)). Twirling is not a high Reynolds number (R_e) phenomenon (see FIG. 30) since it was observed at substantially all values of R_e and at substantially all velocities. However, the flow pattern of this eddy appears to be slightly affected by the velocity.

Various characteristics and behavior of twirling were observed. Twirling redistributed the marker by transferring it from one side of the plug to the other, e.g., from the right to the left side of the plug. The most efficient mixing was observed when there was minimal fluctuations in intensity, i.e., when the marker was evenly distributed across the plug. While twirling was present during the formation of plugs of all lengths that were investigated, its significance to the mixing process appears to depend on the length of the plug. For example, the extent of twirling was observed to be significantly greater for short plugs than for long plugs. Twirling was also observed to affect only a small fraction of the long plugs and had a small effect on the distribution of the marker in the plugs. Moreover, twirling occurred only at the tip of the forming plug before the tip made contact with the right wall of the microchannel. Also, the amount of twirling in a plug was observed to be related to the amount of the carrier-fluid that flowed past the tip. The results of experiments involving twirling and its effect on mixing show that twirling is one of the most important factors, if not the most important factor, in determining the ideal conditions for mixing occurring within plugs moving through straight channels. By inducing twirling, one may stimulate mixing; by preventing twirling, one may suppress complete mixing. Suppressing mixing may be important in some of the reaction schemes, for example those shown in FIG. **5** and FIG. **6**. In these reaction schemes, selective mixing of reagents A with reagent B, and also reagent C with reagent D, can occur without mixing of all four reagents. Mixing of all four reagents occurs later as plugs move through, for example, the winding part of the channel. This approach allows several reactions to occur separated in time. In addition, suppressing mixing may be important when interfaces between plug fluids have to be created, for example interfaces required for some methods of protein crystallization (FIG. **20**).

The eddy at the tip of a developing plug may complicate visualization and analysis of mixing. This eddy is normally significant in short plugs, but only has a minor effect on long plugs. For applications involving visualization of mixing, the substrate is designed to include a narrow channel in the plug-forming region is designed such that narrow, elongated plugs form. Immediately downstream from the plug-forming region, the channel dimension is preferably expanded. In the expanded region of the channel(s), plugs will expand and become short and rounded under the force of surface tension; this preserves the distribution of the marker inside the plugs. This approach affords a relatively straightforward way of visualizing the mixing inside plugs of various sizes. Video microscopy may be used to observe the distribution of colored markers inside the drops. A confocal microscope may also be used to visualize the average three-dimensional distribution of a fluorescent marker. Visualization can be complemented or confirmed using a Ca^{2+}/Fluo-4^{-4} reaction. At millimolar concentrations, this reaction is expected to occur with a half-life of about 1 μs. Thus, it can be used to measure mixing that occurs on time scales of about 10 μs and longer.

The following discussion describes at least one method for three-dimensional visualization of flows in plugs. Visualization of chaotic transport in three-dimensions is a challenging task especially on a small scale. Predictions based on two-dimensional systems may be used to gain insight about plugs moving through a three-dimensional microfluidic channel. Experiments and simulations involving a two-dimensional system can aid in the design of channels that ensure chaotic flow in two-dimensional liquid plugs. Confocal microscopy has been used to quantify steady, continuous three-dimensional flows in channels. However, due to instrumental limitations of an optical apparatus such as a confocal microscope, it is possible that the flow cannot be visualized with sufficiently high-resolution to observe, for example, self-similar fractal structures characteristic of chaotic flow. Nonetheless, the overall dynamics of the flow may still be captured and the absence of non-chaotic islands confirmed. Preferably, the channels (periodic or aperiodic) used in the visualization process are fabricated using soft lithography in PDMS. A PDMS replica is preferably sealed using a thin glass cover slip to observe the flow using confocal microscopy.

In one experiment according to the invention, a series of line scans are used to obtain images of a three-dimensional distribution of fluorescent markers within the plugs. FIG. **10***a*) is a schematic diagram depicting a three-dimensional confocal visualization of chaotic flows in plugs. Plugs are preferably formed from three laminar streams. The middle stream **11** preferably contains fluorescent markers. Preferably, the middle stream **11** is injected into the channel system at a low volumetric flow rate. The volumetric flow rates of the two side streams **10**, **12** are preferably adjusted to position the marker stream in a desired section of the channel. Preferably, a confocal microscope such as a Carl Zeiss LSM 510 is used. The LSM 510 is capable of line scans at about 0.38 ms/512 pixel line or approximately 0.2 ms/100 pixel line. Fluorescent microspheres, preferably about 0.2 μm, and fluorescently labeled high-molecular weight polymers are preferably used to visualize the flow with minimal interference from diffusion. A channel such as one with 100 μm wide and 100 μm deep channel may be used. The line scan technique may be applied to various sequences such as one that has about 200-μm long plugs separated by about 800-μm long oil stream.

A beam is preferably fixed in the x and z-directions and scanned repeatedly back and forth along the y-direction. The movement of the plug in the x-direction preferably provides resolution along the x-direction. Line scan with 100 pixels across a 100 μm-wide channel will provide a resolution of about 1 μm/pixel in the y-direction. Approximately 200 line scans per plug are preferably used to give a resolution of about 1 μm/pixel in the x-direction. For a 200 μm plug moving at about 2000 μm/s, about 200 line scans are preferably obtained over a period of about (200 μm)/(2000 μm/s)=0.1 s, or about 0.5 ms per line.

The sequence shown in FIG. **10***b*) is preferably used for visualization of a three dimensional chaotic flow. Each line scan preferably takes about 0.2 ms with about 0.3 ms lag between the scans to allow the plug to move by about 500 μm. Some optical distortions may result during the approximately 0.2 ms scan as the plug is translated along the x-direction by about 0.2 μm. However, these distortions are believed to be comparable to the resolution of the method. For a given position along the x-direction, a series of line scans are preferably obtained for about 10 seconds for each point along the z-direction to obtain an x-y cross-sections of ten plugs. Scans along the z-direction are preferably taken in 1 μm increments to obtain a full three-dimensional image of the distribution of the fluorescent marker in the plug. This procedure is preferably repeated at different positions along the x-direction to provide information such as changes in the three-dimensional distribution of the fluorescent marker inside the plug as the plug moves along the channels.

In case of periodic perturbations, the fluorescent cross-sections of the plug in the y-z plane recovered from the above procedure represent Poincaré sections corresponding to the evolution of the initial thin sheet of dye. The twirling of the aqueous phase upon formation of the small plugs could distribute the dye excessively throughout the plug and could

make visualization less conclusive. This twirling is prevented preferably by designing a small neck in the plug-forming region, and then beginning the first turn in a downward direction. This approach has been successfully applied to flow visualization, and may be useful for conducting reactions.

Merging Plugs

The invention also provides a method of merging of plugs within a substrate (see upper portion of FIG. **12**). Plugs are formed as described above. Plugs containing different reagents can be formed by separately introducing different plug-fluids into a channel. The plugs containing different reagents may be substantially similar in viscosity or may differ. The plugs containing different reagents may be substantially similar in size or they may differ in size. Provided that the relative velocities of the plugs containing different reagents differ, the plugs will merge in the channels. The location of merging can be controlled in a variety of ways, for example by varying the location of plug-fluid inlet ports, by varying the location of channel junctions (if one of the plug forming fluids is introduced into a secondary channel), varying the size of the plugs, adjusting the speed at which different sets of plugs are transported varying the viscosity or surface tension of plugs having substantially the same size, etc.

As shown in FIG. **12** (top photograph), plugs may be merged by directing or allowing the plugs **120**, **121** to pass through a T-shaped channel or a T-shaped region of a channel. The resulting merged plugs **122** flow in separate channels or channel branches which may be perpendicular, as shown in FIG. **12**, or nonperpendicular (FIG. **33**). The merged plugs **122** may undergo further merging or undergo splitting, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more reactions or "treatments" such as one or more types of characterizations, measurements, detection, sorting, or analysis.

In one embodiment, large and small plugs flow along separate channels or channel branches towards a common channel where they merge. In a case where a large and a small plug do not converge at the same point at the same time, they eventually form a merged plug as the larger plug, which moves faster than the smaller plug, catches up with the small plug and merges with it. In the case where the larger and smaller plugs meet head on at the same point or region, they immediately combine to form a merged plug. The merged plugs may undergo splitting, described below, or further merging in other channels or channel regions, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more types of characterizations, measurements, detection, sorting, or analysis.

In another embodiment, plugs can be merged by controlling the arrival time of the plugs flowing in opposite directions towards a common point, area, or region of the channel so that each pair of plugs arrive at the common point, area, or region of the channel at around the same time to form a single plug.

In another embodiment, an arched, semi-circular, or circular channel provides a means for increasing the efficiency of plug merging. Thus, for example, a greater frequency of merging would occur within a more compact area or region of the substrate. Using this scheme, plugs flowing along separate channels towards a common channel may merge within a shorter distance or a shorter period of time because the arched, semi-circular, or circular channel or channel branch converts or assists in converting initially out-of-phase plug pairs to in-phase plug pairs. Specifically, the arched, semi-circular, or circular channel or channel branch would allow a lagging plug to catch up and merge with a plug ahead of it, thereby increasing the number of merged plugs in a given period or a given area or region of a substrate.

Splitting and/or Sorting Plugs

The present invention also provides a method for splitting of plugs within a substrate. Plugs can be split by passing a first portion of a plug into a second channel through an opening, wherein the second channel is downstream of where the plug is formed. Alternatively, plugs may be split at a "Y" intersection in a channel. In both embodiment, the initial plug splits into a first portion and a second portion and thereafter each portion passes into separate channel (or outlet). Either initially formed plugs can be split or, alternatively, merged plugs can be split. FIG. **6** shows a schematic diagram illustrating part of a microfluidic network that uses multiple inlets (inlets **601**, **603**, **605**, **607** for reagents A, B, C, and D; inlets **602**, **604**, **606** for aqueous streams) and that allows for both splitting and merging of plugs. This schematic diagram shows two reactions that are conducted simultaneously. A third reaction (between the first two reaction mixtures) is conducted using precise time delay. Plugs can be split before or after a reaction has occurred. In addition, FIG. **6** shows plugs at various stages of mixing from the initial mixture **60** (A+B) and initial mixture **61** (C+D) through the mixed solutions **62** (A+B), **63** (C+D), and the 4-component mixture **64** (A+B+C+D).

As shown in FIG. **12** (lower photograph), plugs may be split by directing or allowing the plugs **123**, **124** to pass through a T-shaped channel or a T-shaped region of a channel. In a preferred embodiment, the area or junction at which the plugs undergo splitting may be narrower or somewhat constricted relative to the diameter of the plugs a certain distance away from the junction. The resulting split plugs **125** flow in separate channels or channel branches which may be perpendicular, as shown in FIG. **12**, or nonperpendicular (FIG. **33**). The split **125** plugs may undergo merging or further splitting, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more reactions or "treatments" such as one or more types of characterizations, measurements, detection, sorting, or analysis.

In another embodiment, aqueous plugs can be split or sorted from an oil carrier fluid by using divergent hydrophilic and hydrophobic channels. The channels are rendered hydrophilic or hydrophobic by pretreating a channel or region of a channel such that a channel or channel surface becomes predominantly hydrophilic or hydrophobic. As discussed in more detail below, substrates with hydrophilic channel surfaces may be fabricated using methods such as rapid prototyping in polydimethylsiloxane. The channel surface can be rendered hydrophobic either by silanization or heat treatment. For example, (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.) vapor may be applied to the inlets of the substrate with dry nitrogen as a carrier gas to silanize the channel surface.

Once plugs have been split into separate channels, further reactions can be performed by merging the split plugs with other plugs containing further reactants.

Manipulation of plugs and reagents/products contained therein can also be accomplished in a fluid flow using methods or techniques such as dielectrophoresis. Dielectrophoresis is believed to produce movement of dielectric objects, which have no net charge, but have regions that are positively or negatively charged in relation to each other. Alternating, nonhomogeneous electric fields in the presence of plugs and/or particles, cause the plugs and/or particles to become electrically polarized and thus to experience dielectrophoretic forces. Depending on the dielectric polarizability of the particles and the suspending medium, dielectric particles will

move either toward the regions of high field strength or low field strength. Using conventional semiconductor technologies, electrodes can be fabricated onto a substrate to control the force fields in a micro fabricated device. Dielectrophoresis is particularly suitable for moving objects that are electrical conductors. The use of AC current is preferred, to prevent permanent alignment of ions. Megahertz frequencies are suitable to provide a net alignment, attractive force, and motion over relatively long distances.

Radiation pressure can also be used in the invention to deflect and move plugs and reagents/products contained therein with focused beams of light such as lasers. Flow can also be obtained and controlled by providing a thermal or pressure differential or gradient between one or more channels of a substrate or in a method according to the invention.

Preferably, both the fluid comprising the plugs and the carrier fluid have a relatively low Reynolds Number, for example 10^{-2}. The Reynolds Number represents an inverse relationship between the density and velocity of a fluid and its viscosity in a channel of given cross-sectional dimension. More viscous, less dense, slower moving fluids will have a lower Reynolds Number, and are easier to divert, stop, start, or reverse without turbulence. Because of the small sizes and slow velocities, fabricated fluid systems are often in a low Reynolds number regime (R_e<<1). In this regime, inertial effects, which cause turbulence and secondary flows, are negligible and viscous effects dominate the dynamics. These conditions are advantageous for analysis, and are provided by devices according to the invention. Accordingly the devices according to the invention are preferably operated at a Reynolds number of less than 100, typically less than 50, preferably less than 10, more preferably less than 5, most preferably less than 1.

Detection and Measurement

The systems of the present invention are well suited for performing optical measurements using an apparatus such as a standard microscope. For example, PDMS is transparent in the visible region. When it is used to construct a substrate, a glass or quartz cover slip can be used to cover or seal a PDMS network, thereby constructing a set of channels that can be characterized using visible, UV, or infrared light. Preferably, fluorescent measurements are performed, instead of absorption measurements, since the former has a higher sensitivity than the latter. When the plugs are being monitored by optical measurements, the refractive index of the carrier-fluid and the plug-fluids are preferably substantially similar, but they can be different in certain cases.

In a plug-based system according to the invention, the relative concentrations (or changes in concentrations) can be typically measured in a straightforward fashion. In some instances, the use of plugs to perform quantitative optical measurements of, for example, absolute concentrations is complicated by the presence of non-horizontal oil/water interfaces surrounding the plugs. These curved interfaces act as lenses, and may lead to losses of emitted light or optical distortions. Such distortions may adversely affect or prevent visual observation of growing protein crystals, for example. Exact modeling of these losses is usually difficult because of the complicated shape that this interface may adopt at the front and back of a plug moving in a non-trivial pressure gradient.

This problem can be overcome or minimized in accordance with the invention by using a technique such as refractive index matching. The losses and distortions depend on the difference between the refractive index (η_D) of the aqueous phase and the refractive index of the immiscible carrier-fluid. Preferably, the carrier-fluid used in an analysis have refractive indices that are substantially similar to those of water and aqueous buffers (TABLE 1), e.g., fluorinated oils having refractive indices near that of water close to the sodium D line at 589 nm.

Preferably, for applications involving detection or measurement, the carrier-fluids used are those having refractive indices that match those of commonly used aqueous solutions at the wavelengths used for observation. To calibrate a system for quantitative fluorescence measurements, the plugs preferably contain known concentrations of fluorescein. Preferably, the fluorescence originating from the plugs are measured and then compared with the fluorescence arising from the same solution of fluorescein in the channel in the absence of oil. It is believed that when the refractive indexes are matched, the intensity (I) of fluorescence arising from the plugs will be substantially similar or equal to the intensity of the fluorescence from the aqueous solutions after making adjustments for the fraction of the aqueous stream:

$$I_{plug}=I_{solution}*V_{water}/(V_{water}+V_{oil}) \quad \text{Eqn. (3)}$$

where V is the volumetric flow rate of the fluid streams. It is expected that smaller plugs with a higher proportion of curved interfaces will show larger deviations from ideal plug behavior, i.e., those smaller plugs will tend to cause greater optical distortion. If necessary, measurements are performed partly to determine the errors associated with refractive index mismatch. Information from these measurements is useful when unknown fluids are analyzed, or when a compromise between matching the refractive index and matching the viscosities of the two fluids is required.

TABLE 1

Physical properties of some fluids used in certain embodiments of the microfluidic devices.

Fluid	Refractive index, η_D	Viscosity, μ [mPa-s]
water	1.3330	1.00
aqueous PBS buffer, 1%	1.3343	1.02
aqueous PBS buffer, 10%	1.3460	1.25
perfluorohexane	1.251	0.66
perfluoro(methylcyclohexane)	1.30	1.56
perfluoro(1,3-dimethylcyclohexane)	1.2895	1.92
perfluorodecaline	1.314	5.10
perfluoroperhydrofluorene	1.3289	9.58
perfluoroperhydrophenanthrene	1.3348	28.4
perfluorotoluene	1.3680	N/A
hexafluorobenzene	1.3770	N/A

The detector can be any device or method for evaluating a physical characteristic of a fluid as it passes through the detection region. Examples of suitable detectors include CCD detectors. A preferred detector is an optical detector, such as a microscope, which may be coupled with a computer and/or other image processing or enhancement devices to process images or information produced by the microscope using known techniques. For example, molecules can be analyzed and/or sorted by size or molecular weight. Reactions can be monitored by measuring the concentration of a product produced or the concentration of a reactant remaining at a given time. Enzymes can be analyzed and/or sorted by the extent to which they catalyze a chemical reaction of an enzyme's substrate (conversely, an enzyme's substrate can be analyzed (e.g., sorted) based on the level of chemical reactivity catalyzed by an enzyme). Biological particles or molecules such as cells and virions can be sorted according to whether they contain or produce a particular protein, by using an optical

detector to examine each cell or virion for an optical indication of the presence or amount of that protein. A chemical itself may be detectable, for example by a characteristic fluorescence, or it may be labeled or associated with a tag that produces a detectable signal when, for example, a desired protein is present, or is present in at least a threshold amount.

Practically any characteristic of a chemical can be identified or measured using the techniques according to the invention, provided that the characteristic or characteristics of interest for analysis can be sufficiently identified and detected or measured to distinguish chemicals having the desired characteristic(s) from those which do not. For example, particulate size, hydrophobicity of the reagent versus carrier-fluids, etc. can be used as a basis for analyzing (e.g., by sorting) plug-fluids, reaction products or plugs.

In a preferred embodiment, the plugs are analyzed based on the intensity of a signal from an optically detectable group, moiety, or compound (referred to here as "tag") associated with them as they pass through a detection window or detection region in the device. Plugs having an amount or level of the tag at a selected threshold or within a selected range can be directed into a predetermined outlet or branch channel of the substrate. The tag signal may be collected by a microscope and measured by a detector such as a photomultiplier tube (PMT). A computer is preferably used to digitize the PMT signal and to control the flow through methods such as those based on valve action. Alternatively, the signal can be recorded or quantified as a measure of the tag and/or its corresponding characteristic or marker, e.g., for the purpose of evaluation and without necessarily proceeding to, for example, sort the plugs.

In one embodiment according to the invention, a detector such as a photodiode is larger in diameter than the width of the channel, forming a detection region that is longer (along the length of channel) than it is wide. The volume of such a detection region is approximately equal to the cross sectional area of the channel above the diode multiplied by the diameter of the diode.

To detect a chemical or tag, or to determine whether a chemical or tag has a desired characteristic, the detection region may include an apparatus (e.g., a light source such as a laser, laser diode, high intensity lamp such as mercury lamp) for stimulating a chemical or tag for that characteristic to, for example, emit measurable light energy. In embodiments where a lamp is used, the channels are preferably shielded from light in all regions except the detection region. In embodiments where a laser is used, the laser can be set to scan across a set of detection regions. In addition, laser diodes may be fabricated into the same substrate that contains the analysis units. Alternatively, laser diodes may be incorporated into a second substrate (i.e., a laser diode chip) that is placed adjacent to the analysis or sorter substrate such that the laser light from the diodes shines on the detection region(s).

In preferred embodiments, an integrated semiconductor laser and/or an integrated photodiode detector are included on the silicon wafer in the device according to the invention. This design provides the advantages of compactness and a shorter optical path for exciting and/or emitted radiation, thus minimizing, for example, optical distortion.

As each plug passes into the detection region, it may be examined for a characteristic or property, e.g., a corresponding signal produced by the plug, or the chemicals contained in the plugs, may be detected and measured to determine whether or not a given characteristic or property is present. The signal may correspond to a characteristic qualitatively or quantitatively. Typically, the amount of signal corresponds to the degree to which a characteristic is present. For example, the strength of the signal may indicate the size of a molecule, the amount of products(s) formed in a reaction, the amount of reactant(s) remaining, the potency or amount of an enzyme expressed by a cell, a positive or negative reaction such as binding or hybridization of one molecule to another, or a chemical reaction of a substrate catalyzed by an enzyme. In response to the signal, data can be collected and/or a flow control can be activated, for example, to direct a plug from one channel to another. Thus, for example, chemicals present in a plug at a detection region may be sorted into an appropriate branch channel according to a signal produced by the corresponding examination at a detection region. Optical detection of molecular characteristics or the tag associated with a characteristic or property that is chosen for sorting, for example, may be used. However, other detection techniques, for instance electrochemistry, or nuclear magnetic resonance, may also be employed.

In one embodiment according to the invention, a portion of a channel corresponds to an analysis unit or detection region and includes a detector such as a photodiode preferably located in the floor or base of the channel. The detection region preferably encompasses a receive field of the photodiode in the channel, which receive field has a circular shape. The volume of the detection region is preferably the same as, or substantially similar, to the volume of a cylinder with a diameter equal to the receive field of the photodiode and a height equal to the depth of the channel above the photodiode.

The signals from the photodiodes may be transmitted to a processor via one or more lines representing any form of electrical communication (including e.g. wires, conductive lines etched in the substrate, etc.). The processor preferably acts on the signals, for example by processing them into values for comparison with a predetermined set of values for analyzing the chemicals. In one embodiment, a value corresponds to an amount (e.g., intensity) of optically detectable signal emitted from a chemical which is indicative of a particular type or characteristic of a chemical giving rise to the signal. The processor preferably uses this information (i.e., the values) to control active elements in a discrimination region, for example to determine how to sort the chemicals (e.g., valve action).

When more than one detection region is used, detectors such as photodiodes in a laser diode substrate are preferably spaced apart relative to the spacing of the detection regions in the analysis unit. That is, for more accurate detection, the detectors are placed apart at the same spacing as the spacing of the detection region.

A processor can be integrated into the same substrate that contains at least one analysis unit, or it can be separate, e.g., an independent microchip connected to the analysis unit containing substrate via electronic leads that connect to the detection region(s) and/or to the discrimination region(s), such as by a photodiode. The processor can be a computer or microprocessor, and is typically connected to a data storage unit, such as computer memory, hard disk, or the like, and/or a data output unit, such as a display monitor, printer and/or plotter.

The types and numbers of chemicals based on the detection of, for example, a tag associated with or bound to the chemical passing through the detection region, can be calculated or determined, and the data obtained can be stored in the data storage unit. This information can then be further processed or routed to a data outlet unit for presentation, e.g. histograms representing, for example, levels of a protein, saccharide, or some other characteristic of a cell surface in the sample. The data can also be presented in real time as the sample flows through a channel.

If desired, a substrate may contain a plurality of analysis units, i.e., more than one detection region, and a plurality of branch channels that are in fluid communication with and that branch out from the discrimination regions. It will be appreciated that the position and fate of the reagents in the discrimination region can be monitored by additional detection regions installed, for example, immediately upstream of the discrimination region and/or within the branch channels immediately downstream of the branch point. The information obtained by the additional detection regions can be used by a processor to continuously revise estimates of the velocity of the reagents in the channels and to confirm that molecules, particles, and substances having a selected characteristic enter the desired branch channel.

In one embodiment, plugs are detected by running a continuous flow through a channel, taking a spatially resolved image with a CCD camera, and converting the relevant distance traversed by the plugs into time.

In another embodiment, plugs are detected following their exit through a channel point leading to a mass spectrometer (MS), e.g., an electrospray MS. In this embodiment, time-resolved information (e.g., mass spectrum) can be obtained when the flow rate and the distance traversed by the plugs are known. This embodiment is preferable when one wants to avoid using a label.

Varying the Concentration of Reagents Inside Plugs

The various devices and methods according to the invention allow the control and manipulation of plug composition and properties. For example, they allow the variation of reagent concentration inside plugs. In one aspect according to the invention, the concentrations of the reagents in the plugs are varied by changing the relative flow rates of the plug-fluids. This is possible in conventional systems, but is complicated by problems of slow mixing and dispersion. Methods according to the invention are convenient for simultaneously testing a large number of experimental conditions ("screening") because the concentrations can be changed within a single setup. Thus, for example, syringes do not have to be disconnected or reconnected, and the inlets of a system according to the invention do not have to be refilled when using the above technique for varying the reagent concentrations in plugs.

The concentration of aqueous solutions inside plugs can be varied by changing the flow rates of the plug-fluid streams (see FIG. **25**, discussed in detail in Example 11). In FIG. **25**, water is introduced into inlets **251-258** at various flow rates while perfluorodecaline flows through channels **259-261**. In aqueous laminar flows, the ratio of flow rates of laminar streams in a microfluidic channel may be varied from about 1000:1 and 1:1000, preferably 100:1 to 1:100, more preferably 1:20 to 20:1.

The actual relative concentrations may be quantified using a solution of known concentration of fluorescein. In this example, the intensity of a fluorescein stream can be used as a reference point to check for fluctuations of the intensity of the excitation lamp.

To illustrate an advantage offered by the invention over other techniques, consider the following example. The method(s) described in this example may be modified or incorporated for use in various types of applications, measurements, or experiments. Two or more reagents, such as reagents A, B, C, are to be screened for the effects of different concentrations of reagents on some process, and the conditions under which an inhibitor can terminate the reaction of the enzyme with a substrate at various enzyme and substrate concentrations is of interest. If A is an enzyme, B a substrate, and C an inhibitor, a substrate with 5 inlets such as A/water/B/water/C inlets can be used, and the flow rates at which A, B and C are pumped into the substrate can be varied. Preferably, the size of the plug is kept constant by keeping the total flow rate of all plug-fluids constant. Because different amounts of A, B, C are introduced, the concentrations of A, B, C in the plugs will vary. The concentrations of the starting solutions need not be changed and one can rapidly screen all combinations of concentrations, as long as an enzymatic reaction or other reactions being screened can be detected or monitored. Because the solutions are flowing and the transport is linear, one can determine not only the presence or absence of an interaction or reaction, but also measure the rate at which a reaction occurs. Thus, both qualitative and quantitative data can be obtained. In accordance with the invention, the substrate typically need not be cleaned between runs since most, if not all, reagents are contained inside the plugs and leave little or no residue.

To extend the range over which concentrations can be varied, one may use a combination of, say, reagents A, B, C, D, E and prepare a micromolar solution of A, a mM solution of B, and a M solution of C, and so on. This technique may be easier than controlling the flow rate over a factor of, say, more than 10^6. Using other known methods is likely to be more difficult in this particular example because changing the ratio of reagents inside the plug requires changing the size of the plugs, which makes merging complicated.

In another example, one may monitor RNA folding in a solution in the presence of different concentrations of Mg^{2+} and H^+. Previously, this was done using a stopped-flow technique, which is time consuming and requires a relatively large amount of RNA. Using a method according to the invention, an entire phase space can be covered in a relatively short period of time (e.g., approximately 15 minutes) using only μL/minute runs instead of the usual ml/shot runs.

These particular examples highlight the usefulness according to the invention in, for example, the study of protein/protein interaction mediation by small molecules, protein/RNA/DNA interaction mediation by small molecules, or binding events involving a protein and several small molecules. Other interactions involving several components at different concentrations may also be studied using the method according to the invention.

Generating Gradients in a Series of Plugs

In one aspect according to the invention, dispersion in a pressure-driven flow is used to generate a gradient in a continuous stream of plug-fluid. By forming plugs, the gradient is "fixed", i.e., the plugs stop the dispersion responsible for the formation of the gradient. Although the stream does not have to be aqueous, an aqueous stream is used as a non-limiting example below.

FIG. **44** illustrates how an initial gradient may be created by injecting a discrete aqueous sample of a reagent B into a flowing stream of water. In FIG. **44***a*), the water+B mixture flowed through channel **441**. Channels **443** and **445** contain substantially non-flowing water+B mixture. Water streams were introduced into inlets **440**, **442**, **444**, **446-448** while oil streams flowed through channels **449-452**. FIG. **44***d*) shows a multiple-inlet system through which reagents A, B, and C are introduced through inlets **453**, **454**, and **455**. A pressure-driven flow is allowed to disperse the reagent along the channel, thus creating a gradient of B along the channel. The gradient can be controlled by suitable adjustments or control of the channel dimensions, flow rates, injection volume, or frequency of sample or reagent addition in the case of multiple injections. This gradient is then "fixed" by the formation of plugs. Several of these channels are preferably combined into a single plug-forming region or section. In addition,

complex gradients with several components may be created by controlling the streams. This technique may be used for various types of analysis and synthesis. For example, this technique can be used to generate plugs for protein or lysozome crystallization. FIG. **42** shows an experiment involving the formation of gradients by varying the flow rates (the experimental details are described in Example 17). FIG. **43** illustrates the use of gradients to form lysozyme crystals (the experimental details are described in Example 18).

Formation and Isolation of Unstable Intermediates

The devices and methods according to the present invention may also be used for synthesizing and isolating unstable intermediates. The unstable intermediates that are formed using a device according to the invention are preferably made to undergo further reaction and/or analysis or directed to other parts of the device where they may undergo further reaction and/or analysis. In one aspect, at least two different plug-fluids, which together react to form an unstable intermediate, are used. As the unstable intermediates form along the flow path of the substrate, information regarding, for example, the reaction kinetics can be obtained. Such unstable intermediates can be further reacted with another reagent by merging plugs containing the unstable intermediate with another plug-fluid. Examples of unstable intermediates include, but are not limited to, free radicals, organic ions, living ionic polymer chains, living organometallic polymer chains, living free radical polymer chains, partially folded proteins or other macromolecules, strained molecules, crystallization nuclei, seeds for composite nanoparticles, etc.

One application of devices according to the invention that involves the formation of unstable intermediates is high-throughput, biomolecular structural characterization. It can be used in both a time-resolved mode and a non-time resolved mode. Unstable (and/or reactive) intermediates (for example hydroxyl radicals (OH)) can be generated in one microfluidic stream (for example using a known reaction of metal ions with peroxides). These reactive species can be injected into another stream containing biomolecules, to induce reaction with the biomolecules. The sites on the biomolecule where the reaction takes place correlate with how accessible the sites are. This can be used to identify the sites exposed to the solvent or buried in the interior of the biomolecule, or identify sites protected by another biomolecule bound to the first one. This method could be applied to understanding structure in a range of biological problems. Examples include but are not limited to protein folding, protein-protein interaction (protein footprinting), protein-RNA interaction, protein-DNA interactions, and formation of protein-protein complexes in the presence of a ligand or ligands (such as a small molecule or another biomolecule). Interfacing such a system to a mass-spectrometer may provide a powerful method of analysis.

Experiments involving complex chemical systems can also be performed in accordance with the invention. For example, several unstable intermediates can be prepared in separate plugs, such as partially folded forms of proteins or RNA. The reactivity of the unstable intermediates can then be investigated when, for example, the plugs merge.

Dynamic Control of Surface Chemistry

Control of surface chemistry is particularly important in microfluidic devices because the surface-to-volume ratio increases as the dimensions of the systems are reduced. In particular, surfaces that are generally inert to the adsorption of proteins and cells are invaluable in microfluidics. Polyethylene glycols (PEG) and oligoethylene glycols (OEG) are known to reduce non-specific adsorption of proteins on surfaces. Self-assembled monolayers of OEG-terminated alkane thiols on gold have been used as model substrates to demonstrate and carefully characterize resistance to protein adsorption. Surface chemistry to which the solutions are exposed can be controlled by creating self-assembled monolayers on surfaces of silicone or grafting PEG-containing polymers on PDMS and other materials used for fabrication of microfluidic devices. However, such surfaces may be difficult to mass-produce, and they may become unstable after fabrication, e.g., during storage or use.

In one aspect according to the invention, the reagents inside aqueous plugs are exposed to the carrier-fluid/plug-fluid interface, rather than to the device/plug-fluid interface. Using perfluorocarbons as carrier-fluids in surface studies are attractive because they are in some cases more biocompatible than hydrocarbons or silicones. This is exemplified by the use of emulsified perfluorocarbons as blood substitutes in humans during surgeries. Controlling and modifying surface chemistry to which the reagents are exposed can be achieved simply by introducing appropriate surfactants into the fluorinated PFD phase.

In addition, the use of surfactants can be advantageous in problems involving unwanted adsorption of substances or particles, for example, on the channel walls. Under certain circumstances or conditions, a reaction may occur in one or more channels or regions of the substrate that give rise to particulates that then adhere to the walls of the channels. When they collect in sufficient number, the adhering particulates may thus lead or contribute to channel clogging or constriction. Using methods according to the invention, such as using one or more suitable surfactants, would prevent or minimize adhesion or adsorption of unwanted substances or particles to the channel walls thereby eliminating or minimizing, for example, channel clogging or constriction.

Encapsulated particulates may be more effectively prevented from interfering with desired reactions in one or more channels of the substrate since the particulates would be prevented from directly coming into contact with reagents outside the plugs containing the particulates.

Fluorosurfactants terminated with OEG-groups have been shown to demonstrate biocompatibility in blood substitutes and other biomedical applications. Preferably, oil-soluble fluorosurfactants terminated with oligoethylene groups are used to create interfaces in the microfluidic devices in certain applications. Surfactants with well-defined composition may be synthesized. This is preferably followed by the characterization of the formation of aqueous plugs in the presence of those surfactants. Their inertness towards nonspecific protein adsorption will also be characterized. FIG. **24** shows examples of fluorinated surfactants that form monolayers that are: resistant to protein adsorption; positively charged; and negatively charged. For OEG-terminated surfactants, high values of n ($\geqq$16) are preferred for making these surfactants oil-soluble and preventing them from entering the aqueous phase. In FIG. **24**, compounds that have between about 3 to 6 EG units attached to a thiol are sufficient to prevent the adsorption of proteins to a monolayer of thiols on gold, and are thus preferred for inertness. In addition, surfactants that have been shown to be biocompatible in fluorocarbon blood substitutes may also be used as additives to fluorinated carrier fluids.

Applications: Kinetic Measurements and Assays

The devices and methods of the invention can be also used for performing experiments typically done in, for example, a microtiter plate where a few reagents are mixed at many concentrations and then monitored and/or analyzed. This can be done, for example, by forming plugs with variable composition, stopping the flow if needed, and then monitoring the plugs. The assays may be positionally encoded, that is, the

composition of the plug may be deduced from the position of the plug in the channel. The devices and methods of the invention may be used to perform high-throughput screening and assays useful, for example, in diagnostics and drug discovery. In particular, the devices and methods of the invention can be used to perform relatively fast kinetic measurements.

The ability to perform fast measurements has revolutionized the field of biological dynamics. Examples include studies of protein C folding and cytochrome C folding. These measurements are performed using fast kinetics instruments that rely on turbulence to mix solutions rapidly. To achieve turbulence, the channels and the flow rates normally have to be large, which require large sample volumes. Commercially available instruments for performing rapid kinetics studies can access times on the order of 1 ms. The improved on-chip version of a capillary glass-ball mixer gives a dead time of about 45 μs with a flow rate of more than about 0.35 mL/sec. The miniaturization of these existing methods is generally limited by the requirement of high flow rate to generate turbulence. Miniaturization afforded by devices and methods according to the invention is advantageous because it allows, for example, quantitative characterization, from genetic manipulation and tissue isolation, of a much wider range of biomolecules including those available only in minute quantities, e.g., microgram quantities. In addition, these new techniques and instruments afford a wide range of accessible time scales for measurements.

Time control is important in many chemical and biochemical processes. Typically, stopped-flow type instruments are used to measure reaction kinetics. These types of instruments typically rely on turbulent flow to mix the reagents and transport them while minimizing dispersion. Because turbulent flow occurs in tubes with relatively large diameters and at high flow rates, stopped-flow instruments tend to use large volumes of reagents (e.g., on the order of ml/s). A microfluidic analog of a stopped-flow instrument that consumes small volumes of reagents, e.g., on the order of μL/min, would be useful in various applications such as diagnostics. Thus far, microfluidic devices have not been able to compete with stopped-flow instruments because EOF is usually too slow (although it has less dispersion), and pressure-driven flows tend to suffer from dispersion. In addition, mixing is usually very slow in both systems.

Stopped-flow instruments typically have sub-millisecond mixing, and could be useful for experiments where such fast mixing is required. The devices and methods of the invention allow sub-millisecond measurements as well. In particular, the present invention can be advantageous for reactions that occur on a sub-second but slower than about 1 or about 10 millisecond (ms) time scale or where the primary concern is the solute volume required to perform a measurement.

Further, if a plug is generated with two reactive components, it can serve as a microreactor as the plug is transported down a channel. A plug's property, such as its optical property, can then be measured or monitored as a function of distance from a given point or region of a channel or substrate. When the plugs are transported at a constant flow rate, a reaction time can be directly determined from a given distance. To probe the composition of the plug as it exits a channel, the contents of the plugs may be injected into a mass spectrometer (e.g., an electrospray mass spectrometer) from an end of the channel. The time corresponding to the end of the channel may be varied by changing the flow rate. Multiple outlets may be designed along the channels to probe, for example, the plug contents using a mass spectrometer at multiple distance and time points.

An advantage of the devices and methods of the invention is that when plugs are formed continuously, intrinsically slow methods of observation can be used. For example, plugs flowing at a flow rate of about 10 cm/s through a distance of about 1 mm from a point of origin would be about 10 ms old. In this case, the invention is particularly advantageous because it allows the use of a relatively slow detection method to repeatedly perform a measurement of, for example, 10 ms-old plugs for virtually unlimited time. In contrast, to observe a reaction in a stopped-flow experiment at a time, say, between about 9 and 11 ms, one only has about 2 ms to take data. Moreover, the present invention allows one to obtain information involving complex reactions at several times, simultaneously, simply by observing the channels at different distances from the point of origin.

The reaction time can be monitored at various points along a channel—each point will correspond to a different reaction or mixing time. Given a constant fluid flow rate u, one may determine a reaction time corresponding to the various times $t_1, t_2, t_3, \ldots t_n$ along the channel. Thus, if the distance between each pair of points n and n−1, which correspond to time t_n and t_{n-1}, are the same for a given value of n, then the reaction time corresponding to point n along the channel may be calculated from t_n=nl/u. Thus, one can conveniently and repeatedly monitor a reaction at any given time t_n. In principle, the substrate of the present invention allows one to cover a greater time period for monitoring a reaction by simply extending the length of the channel that is to be monitored at a given flow rate or by decreasing the flow rate over a given channel distance (see, for example, FIG. **22**). In FIG. **22**, the following can be introduced into the following inlets: enzyme into inlets **2201**, **2205**, **2210**, **2215**; buffer into inlets **2202**, **2206**, **2211**, **2216**; substrate into inlets **2203**, **2207**, **2212**, **2217**; buffer into inlets **2204**, **2208**, **2213**, **2218**; inhibitor into inlets **2228**, **2209**, **2214**, **2219**. In FIG. **22**, a carrier fluid flows through the channel portions **2220**, **2221**, **2222**, **2223** from left to right. The channel portions enclosed by the dotted square **2224**, **2225**, **2226**, **2227** represent fields of view for the purpose of monitoring a reaction at various points along the channel.

The same principle applies to an alternate embodiment of the present invention, where the distance corresponding to a point n from a common point of origin along the channel differs from that corresponding to another channel by a power or multiples of 2. This can be seen more clearly from the following discussion. Given a constant fluid flow rate u, one may determine a reaction time corresponding to the various times $t_1, t_2, t_3, \ldots t_n$ along the channel. Thus, if the distance between each pair of points n and n−1, which correspond to time t_n and t_{n-1}, are the same for a given value of n, then the reaction time corresponding to point n along the channel may be calculated from t_n=nl/u. In a relatively more complex channel geometry such as the one shown in FIG. **22**(*c*), the corresponding equation is given by $t_n=2^{(n-1)}$l/u, which shows that the reaction times at various points n varies as a power or multiples of 2.

In one aspect, channels according to the invention are used that place into a field of view different regions that correspond to different time points of a reaction. The channels according to the invention allow various measurements such as those of a complete reaction profile, a series of linearly separated time points (such as those required for the determination of an initial reaction velocity in enzymology), and a series of exponentially separated time points (e.g., first-order kinetic measurements or other exponential analysis). Time scales in an image frame can be varied from microseconds to seconds by, for example, changing the total flow rate and channel length.

FIG. **22**A-D show various examples of geometries of microfluidic channels according to the invention for obtaining kinetic information from single optical images. The illustrated channel systems are suitable for studies such as measurements of enzyme kinetics in the presence of inhibitors. The device shown in FIG. **22**D has multiple outlets that can be closed or opened. In the device shown in FIG. **22**D, preferably only one outlet is open at a time. At the fastest flow rates, the top outlet is preferably open, providing reduced pressure for flow through a short fluid path l. As flow rates are reduced, other outlets are preferably opened to provide a longer path and a larger dynamic range for measurements at the same total pressure.

In FIG. **22**, n is the number of segments for a given channel length l traveled by the reaction mixture in time t_n (see p. 73, second full paragraph for a related discussion of reaction times and channel lengths). These systems allow the control of the ratio of reagents by varying the flow rates. The systems also allow a quick quantification of enzyme inhibition.

For example, ribonuclease A can be used with known inhibitors such as nucleoside complexes of vanadium and oxovanadium ions and other small molecules such as 5'-diphosphoadenosine 3'-phosphate and 5'-diphosphoadenosine 2'-phosphate. The kinetics may be characterized by obtaining data and making Lineweaver-Burk, Eadie-Hofstee, or Hanes-Wolfe plots in an experiment. The experiment can be accomplished using only a few microliters of the protein and inhibitor solutions. This capability is particularly useful for characterizing new proteins and inhibitors that are available in only minute quantities, e.g., microgram quantities.

Kinetic measurements of reactions producing a fluorescent signal can be performed according to the invention by analyzing a single image obtained using, for example, an optical microscope. Long exposures (i.e., about 2 seconds) have been used to measure fast (i.e., about 2 milliseconds) kinetics. This was possible because in a continuous flow system, time is simply equal to the distance divided by the flow rate. In the continuous flow regime in accordance with the invention, the accessible time scales can be as slow as about 400 seconds, which can be extended to days or weeks if the flow is substantially slowed down or stopped. Typically, the time scale depends on the length of the channel (e.g., up to about 1 meter on a 3-inch diameter chip) at a low flow rate of about 1 mm/s, which is generally limited by the stability of the syringe pumps, but may be improved using pressure pumping. The fastest time scale is typically limited by the mixing time, but it may be reduced to about 20 μs in the present invention. Mixing time is generally limited by two main factors: (1) the mixing distance (e.g., approximately 10-15 times the width of the channel); and (2) the flow rates (e.g., approximately 400 mm/s, depending on the capillary number and the pressure drop required to drive the flow). Mixing distance is normally almost independent of the flow rate. By using suitable designs of microfluidic channels, or networks of microfluidic channels, a wide range of kinetic experiments can be performed.

Reducing the channel size generally reduces the mixing time but it also increases the pressure required to drive a flow. The equation below describes the pressure drop, ΔP (in units of Pa), for a single-phase flow in a rectangular capillary:

$$\Delta P = 28.42 U \mu l / ab \qquad \text{Eqn. (9)}$$

where U (m/s) is the velocity of the flow, μ (kilogram/meter-second, kg m^{-1} s^{-1}) is the viscosity of the fluid, l (m) is the length of the capillary, a (m) is the height of the capillary, and b (m) is the width of the capillary. There is generally a physical limitation on how much pressure a microfluidic device can withstand, e.g., about 3 atm for PDMS and about 5 atm for glass and Si. This limitation becomes crucial for very small channels and restricts the total length of the channel and thus the dynamic range (the total distance through which this flow rate can be maintained at a maximum pressure divided by the mixing distance) of the measurement.

FIG. **23** depicts a microfluidic network according to the invention with channel heights of 15 and 2 μm. The channel design shown in FIG. **23** illustrates how a dynamic range of about 100 can be achieved by changing the cross-section of the channels. Under these conditions, mixing time in the winding channel is estimated to be about 25 μs and observation time in the serpentine channels are estimated to be about 3 ms.

As FIG. **23** shows, rapid mixing occurs in the 2 μm×1 μm (height×width) channels and measurements are taken in the 2 μm×3 μm) channels. The table in FIG. **23** shows the distribution of the pressure drop, flow velocity, and flow time as a function of the channel cross-section dimensions. A transition from a 1-μm wide to 3-μm wide channels should occur smoothly, with plugs maintaining their stability and decreasing their velocity when they move from a 20-μm wide into a 50-μm wide channel. Changing the width of the channel can be easily done and easily incorporated into a mask design. The height of the channel can be changed by, for example, using photoresist layers having two different heights that are sequentially spun on, for example, a silicon wafer. A two-step exposure method may then be used to obtain a microfluidic network having the desired cross-section dimensions.

In another example of the application of the devices and methods of the present invention, the folding of RNase P catalytic domain (P RNA C-domain) of *Bacillus subtilis* ribozyme can be investigated using channels according to the invention. RNA folding is an important problem that remains largely unsolved due to limitations in existing technology. Understanding the rate-limiting step in tertiary RNA folding is important in the design, modification, and elucidation of the evolutionary relationship of functional RNA structures.

The folding of P RNA C-domain is known to involve three populated species: unfolded (U), intermediate (I), and native (N, folded) states. Within the first millisecond, the native secondary structure and some of the tertiary structure would have already folded (the RNA is compacted to about 90% of the native dimension) but this time regime cannot be resolved using conventional techniques such as stopped-flow. Using channels and substrates according to the invention, the time-dependence of the P RNA folding kinetics upon the addition of Mg^{2+} can be studied.

Various types of assays (e.g., protein assays) known in the art, including absorbance assays, Lowry assays, Hartree-Lowry assays, Biuret assays, Bradford assays, BCA assays, etc., can be used, or suitably adapted for use, in conjunction with the devices and methods of the invention. Proteins in solution absorb ultraviolet light with absorbance maxima at about 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Absorbance assays offer several advantages. Absorbance assays are fast and convenient since no additional reagents or incubations are required. No protein standard need be prepared. The assay does not consume the protein and the relationship of absorbance to protein concentration is linear. Further, the assay can be performed using only a UV spectrophotometer.

The Lowry assay is an often-cited general use protein assay. It was the method of choice for accurate protein determination for cell fractions, chromatography fractions, enzyme preparations, and so on. The bicinchoninic acid

(BCA) assay is based on the same principle, but it can be done in one step. However, the modified Lowry is done entirely at room temperature. The Hartree version of the Lowry assay, a more recent modification that uses fewer reagents, improves the sensitivity with some proteins, is less likely to be incompatible with some salt solutions, provides a more linear response, and is less likely to become saturated.

In the Hartree-Lowry assay, the divalent copper ion forms a complex with peptide bonds under alkaline conditions in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. In addition to standard liquid handling supplies, the assay only requires a spectrophotometer with infrared lamp and filter. Glass or inexpensive polystyrene cuvettes may be used.

The Biuret assay is similar in principle to that of the Lowry, however it involves a single incubation of 20 minutes. In the Biuret assay, under alkaline conditions, substances containing two or more peptide bonds form a purple complex with copper salts in the reagent. The Biuret assay offer advantages in that there are very few interfering agents (ammonium salts being one such agent), and there were fewer reported deviations than with the Lowry or ultraviolet absorption methods. However, the Biuret consumes much more material. The Biuret is a good general protein assay for batches of material for which yield is not a problem. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed, with maximum transmission in the region of 450 nm. Glass or inexpensive polystyrene cuvettes may be used.

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis. Assay materials including color reagent, protein standard, and instruction booklet are available from Bio-Rad Corporation. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum (no special lamp or filter usually needed). Glass or polystyrene cuvettes may be used, but the color reagent stains both. Disposable cuvettes are recommended.

The bicinchoninic acid (BCA) assay is available in kit form from Pierce (Rockford, Ill.). This procedure is quite applicable to microtiter plate methods. The BCA is used for the same reasons the Lowry is used. The BCA assay is advantageous in that it requires a single step, and the color reagent is stable under alkaline conditions. BCA reduces divalent copper ion to the monovalent ion under alkaline conditions, as is accomplished by the Folin reagent in the Lowry assay. The advantage of BCA is that the reagent is fairly stable under alkaline condition, and can be included in the copper solution to allow a one step procedure. A molybdenum/tungsten blue product is produced as with the Lowry. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed with transmission set to 562 nm. Glass or inexpensive polystyrene cuvettes may be used.

The range of concentrations that can be measured using the above assays range from about 20 micrograms to 3 mg for absorbance at 280, between about 1-100 micrograms for absorbance at 205 nm, between about 2-100 micrograms for the Modified Lowry assay, between about 1-10 mg for the Biuret assay, between about 1-20 micrograms for the Bradford assay, and between about 0.2-50 micrograms for BCA assay. Many assays based on fluorescence or changes in fluorescence have been developed and could be performed using methods and devices of the invention.

A detailed description of various physical and chemical assays is provided in *Remington: The Science and Practice of Pharmacy*, A. R. Gennaro (ed.), Mack Publishing Company, chap. 29, "Analysis of Medicinals," pp. 437-490 (1995) and in references cited therein while chapter 30 of the same reference provides a detailed description of various biological assays. The assays described include titrimetric assays based on acid-base reactions, precipitation reactions, redox reactions, and complexation reactions, spectrometric methods, electrochemical methods, chromatographic methods, and other methods such as gasometric assays, assays involving volumetric measurements and measurements of optical rotation, specific gravity, and radioactivity. Other assays described include assays of enzyme-containing substances, proximate assays, alkaloidal drug assays, and biological tests such as pyrogen test, bacterial endotoxin test, depressor substances test, and biological reactivity tests (in-vivo and in-vitro).

In addition, *Remington: The Science and Practice of Pharmacy*, A. R. Gennaro (ed.), Mack Publishing Company, chap. 31, "Clinical Analysis," pp. 501-533 (1995) and references cited therein provide a detailed description of various methods of characterizations and quantitation of blood and other body fluids. In particular, the reference includes a detailed description of various tests and assays involving various body fluid components such as erythrocytes, hemoglobin, thrombocyte, reticulocytes, blood glucose, nonprotein nitrogen compounds, enzymes, electrolytes, blood-volume and erythropoeitic mechanisms, and blood coagulation.

Nonlinear and Stochastic Sensing

Stochastic behavior has been observed in many important chemical reactions, e.g., autocatalytic reactions such as inorganic chemical reactions, combustion and explosions, and in polymerization of sickle-cell hemoglobin that leads to sickle-cell anemia. Crystallization may also be considered an autocatalytic process. Several theoretical treatments of these reactions have been developed. These reactions tend to be highly sensitive to mixing.

Consider the extensively studied stochastic autocatalytic chemical reaction between $NaClO_2$ and $Na_2S_2O_3$ (chlorite-thiosulfate reaction). The mechanism of this reaction can be described by reactions (1) and (2),

$$4S_2O_3^{2-}+ClO_2^-+4H+\rightarrow 2S_4O_6^{2-}+2H_2O+Cl^- \underset{[H^+]}{\text{rate } (v)\alpha} \quad (1)$$

$$S_2O_3^{2-}+2ClO_2^-+H_2O\rightarrow 2SO_4^{2-}+2H^++2Cl^- \underset{[H^+]^2[Cl]}{\text{rate } (v)\alpha} \quad (2)$$

where $[H^+]$ stands for the concentration of H^+. At a slightly basic pH=7.5, the slow reaction (1) dominates and maintains a basic pH of the reaction mixture (since the rate of this reaction v is directly proportional $[H^+]$, this reaction consumes H^+ and is auto-inhibitory). Reaction (2) dominates at acidic pH (since the rate of this reaction varies in proportion to $[H^+]^2[Cl^-]$, this reaction produces both H^+ and Cl^- and is superautocatalytic). FIG. **21** shows the reaction diagram for two reactions corresponding to the curves **211**, **212**. The rates

of the two reactions (referred to here as reaction **211** and reaction **212**) are equal at an unstable critical point at a certain pH. The lifetime of the reaction mixtures of $NaClO_2$ and NaS_2O_3 at this critical point crucially depends on stirring. In the absence of stirring, stochastic fluctuations of $[H^+]$ in solution generate a localized increase in $[H^+]$. This increase in $[H^+]$ marginally increases the rate of reaction **212**, but it has a much stronger accelerating effect on reaction **211** because of the higher-order dependence on $[H^+]$ of this reaction. Therefore, in the region where local fluctuations increase local $[H^+]$, reaction **211** becomes dominant, and more H^+ is produced (which rapidly diffuses out of the region of the initial fluctuation). The initiated chemical wave then triggers the rapid reaction of the entire solution. Unstirred mixtures of $NaClO_2$ and NaS_2O_3 are stable only for a few seconds, and these fluctuations arise even in the presence of stirring.

FIG. **21** depicts a reaction diagram illustrating an unstable point in the chlorite-thiosulfate reaction. At $[H^+]$ values below the critical point, the slow reaction (1) dominates. At $[H^+]$ values above the critical point, the autocatalytic reaction (2) dominates. The reaction mixture at the $[H^+]$ value equal to the critical point is metastable in the absence of fluctuations. Under perfect mixing, the effects of small fluctuations average out and the system remains in a metastable state. Under imperfect mixing, fluctuations that reduce $[H^+]$ grow more slowly than those that increase $[H^+]$ due to the autocatalytic nature of reaction (2), and the reaction mixture thus rapidly becomes acidic.

It is known that chaotic flows should have a strong effect on diffusive transport within the fluid ("anomalous diffusion"). It is also known that chaotic dynamics can lead to non-Gaussian transport properties ("strange kinetics"). In one aspect according to the invention, these highly unstable mixtures are stabilized in the presence of chaotic mixing using channels according to the invention because this mixing can effectively suppress fluctuations. This invention can be used to understand the effects of mixing on the stochastic behavior of such systems, including for example, the chlorite thiosulfate system.

In a laminar flow, the flow profile in the middle of the channel is flat and there is virtually no convective mixing. Fluctuations involving $[H^+]$ that arise in the middle of the channel can grow and cause complete decomposition of the reaction mixture. Slow mixing reduces the probability of fluctuations in plugs moving through straight channels. When fluctuations that occur in the centers of vortices are not efficiently mixed away, one or more spontaneous reactions involving some of the plugs can take place. In the present invention, chaotic mixing in plugs moving through winding channels efficiently mix out fluctuations, and thus substantially fewer or no spontaneous reactions are expected to occur.

In a simple laminar flow, there is normally very little or no velocity gradient and substantially no mixing at the center of the channel. Thus, fluctuations that arise in the chlorite-thiosulfate reaction mixture prepared at the critical $[H^+]$ are able to grow and lead to rapid decomposition of the reaction mixture. Propagation of chemical fronts in autocatalytic reactions occurring in laminar flows has been described with numerical simulations, and back-propagation has been predicted (that is, a reaction front traveling upstream of the direction of the laminar flow). Using the method of the present invention, this back-propagation involving the reaction between $NaClO_2$ and Na S_2O_3 under laminar flow conditions was observed.

In accordance with the invention, chaotic flow within plugs that flow through winding channels suppresses fluctuations and gives rise to stable reaction mixtures. There exists, of course, a finite probability that fluctuations can arise even in a chaotically stirred plug. In one aspect according to the invention, the details of the evolution of these reactions are monitored using a high-speed digital camera. The plugs are preferably separated by the oil and are not in communication with each other, so the reaction of one plug will not affect the behavior of the neighboring plug. Statistics covering the behavior of thousands of plugs can be obtained quickly under substantially identical experimental conditions.

Whether a fluctuation would be able to trigger an autocatalytic reaction depends on factors such as the magnitude of a fluctuation and its lifetime. The lifetime of a fluctuation is typically limited by the mixing time in the system. In an unstirred solution, mixing is by diffusion and quite slow, and fluctuations may persist and lead to autocatalytic reactions. In a stirred solution, the lifetime of a fluctuation is relatively short, and only large fluctuations have sufficient time to cause an autocatalytic reaction.

Mixing time and the lifetime of fluctuations typically depend on the size of the plugs. As plug size decreases, mixing is accelerated and fluctuations are suppressed. However, very small plugs (e.g., about 1 μm^3 or 10^{-15} L) in a solution containing about 10^{-8} mole/liter concentration of H^+ (pH=8) will contain only a few H^+ ions per plug (about 10^{-23} moles or about $6H^+$ ions). When such small plugs are formed, the number of H^+ ions in them will have a Poisson distribution.

An important experimental challenge is to establish that the stochastic behavior in these systems is due mainly to internal fluctuations of concentrations. Other factors that may act as sources of noise and instability are: (1) temporal fluctuations in the flow rates of the incoming reagent streams, which can lead to the formation of plugs with varying amounts of reagents; (2) temperature fluctuations in solutions in a microfluidic device, which may arise due to, for example, illumination by a microscope; and (3) fluctuations due to impurities in carrier-fluids leading to variations in the surface properties of different plugs.

Microfluidic systems according to the invention may be used to probe various chemical and biochemical processes, such as those that show stochastic behavior in bulk due to their nonlinear kinetics. They can also be used in investigating processes that occur in systems with very small volumes (e.g., about 1 μm^3, which corresponds to the volume of a bacterial cell). In systems with very small volumes, even simple reactions are expected to exhibit stochastic behavior due to the small number of molecules localized in these volumes.

Autocatalytic reactions present an exciting opportunity for highly sensitive detection of minute amounts of autocatalysts. Several systems are known to operate on this principle, silver-halide photography being the most widely used. In silver-halide photography, the energy of photons of light is used to decompose an emulsion of silver halide AgX into nanometer-sized particles of metallic silver. A film that is embedded with the silver particles is then chemically amplified by the addition of a metastable mixture of a soluble silver(I) salt and a reducing agent (hydroquinone). Metallic silver particles catalyze reduction of silver(I) by hydroquinone, leading to the growth of the initial silver particles. Another example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.

However, a dilemma occurs when designing systems with very high sensitivity and amplification. To achieve a very highly sensitive amplification, the system typically has to be made very unstable. On the other hand, an unstable system is very sensitive to noise and has a very short lifetime. Also, in unstable systems, it is difficult to distinguish between spon-

taneous decomposition and a reaction caused by the analyte. In one aspect, microfluidic devices according to the invention, which allow chaotic mixing and compartmentalization, are used to overcome this problem.

To demonstrate the potential of microfluidic systems according to the present invention, a microfluidic system according to the invention is used to handle unstable mixtures. In one application, a microfluidic system according to the invention is preferably used to control a stochastic reaction between $NaClO_2$ and NaS_2O_3. In particular, this reaction is preferably used for a highly sensitive amplification process.

If a plug containing an unstable reaction mixture of $NaClO_2$ and NaS_2O_3 is merged with a small plug containing an amount of H^+ sufficient to bring the local concentration of H^+ above critical, a rapid autocatalytic reactions is generally triggered. This autocatalytic reaction typically leads to the production of large amounts of H^+. Thus, a weak chemical signal, e.g., a small amount of H^+, is rapidly amplified by an unstable reaction mixture. Thus, for example, this approach can be used to investigate biological reactions such as those that involve enzymes, in which small amounts of H^+ are produced.

The above autocatalytic system possesses several features that contribute to its novelty and usefulness. In one aspect, an unstable amplifying reaction mixture is prepared in-situ and is used within milliseconds before it has a chance to decompose. Preferably, the system is compartmentalized so a reaction that occurs in one compartment does not affect a reaction in another compartment. This compartmentalization allows thousands of independent experiments to be conducted in seconds using only minute quantities of samples. Importantly, chaotic mixing in the system reduces fluctuations and stabilizes the reaction mixture.

The applications of controlled autocatalytic amplification in accordance with the invention are not limited to the detection of protons or Co^{2+} ions. For example, the (Co(III)-5-Br-PAPS)/peroxomonosulfate oxidation reaction can also be used indirectly, for example, for a detection of small amounts of peroxidase, which can be used as a labeling enzyme bound to an antibody. The (Co(III)-5-Br-PAPS)/peroxomonosulfate oxidation reaction, which has been characterized analytically, involves the autocatalytic decomposition of violet bis[2-(5-bromo-pyridylazo)-5-(N-propyl-N-sulfopropyl-amino-phenolato] cobaltate, (Co(III)-5-Br-PAPS), upon oxidation with potassium peroxomonosulfate to produce colorless Co^{2+} ions, which serve as the autocatalyst (the order of autocatalysis has not been established for this reaction). (Endo et al., "Kinetic determination of trace cobalt(II) by visual autocatalytic indication," *Talanta,* 1998, vol. 47, pp. 349-353; Endo et al., "Autocatalytic decomposition of cobalt complexes as an indicator system for the determination of trace amounts of cobalt and effectors," *Analyst,* 1996, vol. 121, pp. 391-394.)

$$\text{Co(III)-[5-Br-PAPS]reduced}+HSO_5^- \rightarrow Co^{2+}+\text{[5-Br-PAPS]oxidized}+HSO^{4-}$$

Addition of small amounts of Co^{2+} to the violet mixture of the (Co(III)-5-Br-PAPS and peroxomonosulfate produces an abrupt loss of color to give a colorless solution. The time delay before this decomposition depends on the amount of the Co^{2+} added to the solution. This reaction has been used to detect concentrations of Co^{2+} as low as 1×10^{-10} mole/L. The reaction shows good selectivity in the presence of other ions (V(V), Cr(III), Cr(VI), Mn(II), Fe(II), Ni(II), Cu(II) and Zn(II)).

The devices and methods according to the invention may be applied to other autocatalytic reactions, some of which have been described in inorganic, organic and biological chemistry. Reactions of transition metal ions such as Cr(III) (B82) Mn^{2+} or colloidal MnO_2, and reactions of halides and oxohalides are often autocatalytic. Autocatalysis involving lanthanides (Eu^{2+}) and actinides (U^{4+}) has also been reported. All of these elements are potential targets for detection and monitoring in chemical waste, drinking water, or biological fluids. Intriguing possibilities arise from using asymmetric autocatalytic reactions to detect minute amounts of optically active, chiral impurities, such as biomolecules.

It is also possible to design new autocatalytic reactions. Autocatalysis is abundant in biology, and many enzymes are autocatalytic (e.g., caspases involved in programmed cell death, kinases involved in regulation and amplification, and other enzymes participating in metabolism, signal transduction, and blood coagulation. Emulsions of perfluorocarbons such as perfluorodecaline (PFD) are used as blood substitutes in humans during surgeries and should be compatible with a variety of biological molecules. Since the feasibility of quantitative measurements of enzyme kinetics has been demonstrated using plugs formed according to the invention, plugs formed according to the invention may also be applied to the detection of biological autocatalysts.

The devices and methods according to the present invention are not limited to the detection of the autocatalyst itself. For example, the labeling of an analyte using an autocatalyst is also within the scope of the present invention. Biomolecules are often labeled with metallic nanoparticles. Such metallic nanoparticles are highly effective autocatalysts for the reduction of metal ions to metals. Preferably, the systems and methods of the present invention are used in the visual detection of a single molecule of DNA, RNA, or protein labeled with nanoparticles via an autocatalytic pathway. In preliminary experiments in accordance with the invention, clean particle formation and transport within plugs were observed.

In addition, the generation of metal (e.g., copper, silver, gold, nickel) deposits and nanoparticles upon chemical reduction also proceed by an autocatalytic mechanism. These reactions are commonly used for electroless deposition of metals and should be useful for the detection of minute amounts of metallic particles. The presence of metallic particles in water can be indicative of the presence of operating mechanical devices. In one aspect according to the invention, devices and methods according to the invention are used to detect the presence of minute or trace quantities of metallic particles.

The devices in accordance with the present invention are simple in design, consume minute amounts of material, and robust. They do not require high voltage sources and can be operated, for example, using gravity or a pocket-sized source of compressed air. In one aspect, the systems according to the invention are used in portable and hand-held devices.

Autocatalytic reactions show a threshold response, that is, there is a very abrupt temporal change from unreacted mixture to reacted mixture. In the case where time is equal to distance, this abrupt transition over a short distance can be observed using the devices and methods of the invention. The time (and distance) is very sensitive to the initial concentration of the catalyst, and thus it should be easy to determine the concentration of the autocatalyst in the sample by noting how far the reaction system traveled before it reacted.

One example of an autocatalytic process is blood coagulation. It is very sensitive to flow and mixing, therefore experimenting with it in the absence of flow gives unreliable results or results that have little relevance to the real function of the coagulation cascade. A typical microfluidic system may be difficult to use with blood because once coagulation occurs, it

blocks the channel and stops the flow in the microfluidic device. In addition, coagulated blood serves as an autocatalyst; even small amounts of coagulated blood in the channels can make measurements unreliable.

These problems can be overcome using the devices of the present invention. Using plugs, autocatalytic reactions can be easily controlled, and the formation of solid clots would not be a problem because any solids formed will be transported inside the plugs out of the channel without blocking the channel and without leaving autocatalytic residue. In addition, flow inside plugs can be easily controlled and adjusted to resemble flow under physiological conditions.

To address the sensitivity of blood coagulation to surfaces (the cascade is normally initiated on the surface), microscopic beads containing immobilized tissue factor (the cascade initiator) on the surface may be added to one of the streams and transported inside the plugs. Also, surfactants may be used to control surface chemistry.

Thus, the devices and methods of the invention may be used, for example, to test how well the coagulation cascade functions (e.g., for hemophilia or the tendency to form thrombus) under realistic flow conditions. This test would be particularly valuable in diagnostics. Blood may be injected in one stream, and a known concentration of a molecule known to induce coagulation (e.g., factor VIIa) can be added through another stream prior to plug formation. At a given flow rate, normal blood would coagulate at a certain distance (which corresponds to a given time), which can be observed optically by light scattering or microscopy. Blood of hemophiliac patients would coagulate at a later time. This type of testing would be useful before surgical operations. In particular, this type of testing is important for successful child delivery, especially when hemophilia is suspected. Fetal testing may be performed since only minute amounts of blood are required by systems according to the invention. The blood may be injected directly from the patient or collected in the presence of anticoagulating agent (for example EDTA), and then reconstituted in the plug by adding Ca^{2+}. In some cases, the addition of Ca^{2+} may be sufficient to initiate the coagulation cascade.

The devices and methods of the invention may also be used to evaluate the efficacy of anticoagulating agents under realistic flow conditions. Plugs can be formed from normal blood (which may be used directly or reconstituted by adding Ca^{2+} or other agents), an agent known to induce coagulation, and an agent (or several agents that need to be compared) being tested as an anticoagulation agent. The concentrations of these agents can be varied by varying the flow rates. The distance at which coagulation occurs is noted, and the efficacy of various agents to prevent coagulation is compared. The effects of flow conditions and presence of various compounds in the system on the efficacy of anticoagulation agents can be investigated quickly. The same techniques may also be used to evaluate agents that cause, rather prevent, coagulation. These tests could be invaluable in evaluating drug candidates.

Synthesis

In accordance with the present invention, a method of conducting a reaction within a substrate is provided. The reaction is initiated by introducing two or more plug-fluids containing reactants into the substrate of the present invention.

In one aspect, the plug-fluids include a reagent and solvent such that mixing of the plug-fluids results in the formation of a reaction product. In another embodiment, one of the plug-fluids may be reagent free and simply contain fluid. In this embodiment, mixing of the plug-fluids will allow the concentration of the reagent in the plug to be manipulated.

The reaction can be initiated by forming plugs from each plug-fluid and subsequently merging these different plugs.

When plugs are merged to form merged plugs, the first and second set of plugs may be substantially similar or different in size. Further, the first and second set of plugs may have different relative velocities. In one embodiment, large arrays of microfluidic reactors are operated in parallel to provide substantial throughput.

The devices and methods of the invention can be used for synthesizing nanoparticles. Nanoparticles that are monodisperse are important as sensors and electronic components but are difficult to synthesize (Trindade et al., *Chem. Mat.* 2001, vol. 13, pp. 3843-3858). In one aspect, monodisperse nanoparticles of semiconductors and noble metals are synthesized under time control using channels according to the invention (Park et al, *J. Phys. Chem. B,* 2001, vol. 105, pp. 11630-11635.). Fast nucleation is preferably induced by rapid mixing, thereby allowing these nanoparticles to grow for a controlled period of time. Then their growth is preferably quickly terminated by passivating the surfaces of the particles with, for example, a thiol. Nanoparticles of different sizes are preferably obtained by varying the flow rate and therefore the growth time. In addition, devices according to the invention can be used to monitor the synthesis of nanoparticles, and thus obtain nanoparticles with the desired properties. For example, the nanoparticle formation may be monitored by measuring the changes in the color of luminescence or absorption of the nanoparticles. In addition, the growth of nanoparticles may be stopped by introducing a stream of quenching reagent at a certain position along the main channel.

Rapid millisecond mixing generated in channels according to the invention can help ensure the formation of smaller and much more monodisperse nanoparticles than nanoparticles synthesized by conventional mixing of solutions. FIG. **13** shows the UV-VIS spectra of CdS nanoparticles formed by rapid mixing in plugs (lighter shade spectrum with sharp absorption peak) and by conventional mixing of solutions (darker shade spectrum). The sharp absorption peak obtained for synthesis conducted in plugs indicates that the nanoparticles formed are highly monodisperse. In addition, the blue-shift (shift towards shorter wavelengths) of the absorption peak indicates that the particles formed are small.

FIG. **14**A-B illustrates the synthesis of CdS nanoparticles performed in PDMS microfluidic channels in single-phase aqueous laminar flow (FIG. **14**A) and in aqueous plugs that were surrounded by water-immiscible perfluorodecaline (FIG. **14**B). In FIGS. **14**A-B, Cd^{2+} was introduced into inlets **1400**, **1403**, aqueous stream was introduced into inlets **1401**, **1404**, and S^{2-} was introduced into inlets **1402**, **1405**. In FIG. **14**A, an aqueous stream flowed through channel **1406** while in FIG. **14**B, oil flowed through channel **1407**. FIG. **14**A shows portions of the channels **1408** and **1410** at time t=6 minutes and portions of the channels **1409**, **1411** at time t=30 minutes. It can be seen in FIG. **14**A that when laminar flow is used in the synthesis, large amounts of CdS precipitate form on the channel walls. When plugs were used for the synthesis, all CdS formed inside the plugs, and no surface contamination was observed. FIG. **15** illustrates a technique for the synthesis of CdS nanoparticles, which is discussed in detail in Example 13 below.

The following methods according to the invention can be used in synthesis involving nanoparticles:

(a) using self-assembled monolayers to nucleate nanoparticles with crystal structures not accessible under homogeneous nucleation conditions (e.g., controlling polymorphism by controlling the surface at which nucleation takes place).

(b) using merging of plugs to create core-shell nanoparticles with a range of core and shell sizes. In a stream of plugs of a first channel, small core nanoparticles such as CdSe particles can be synthesized in a matter of few milliseconds. The CdSe particles can then be used as seeds for mixing with solutions such as those containing Zn^{+2} and S^{-2}. The CdSe particles, acting as seeds for the formation of ZnS, thus allow the formation of CdSe(core)/ZnS(shell) nanoparticles. Core-shell particles with more than two layers may be obtained by simply repeating the merging process more than once.

(c) using merging of plugs to create composite nanoparticles. For example, small nanoparticles of CdSe and ZnS can be formed using streams of plugs from two separate channels. Merging of these streams leads to aggregation of these particles to form larger nanoparticles containing CdSe/ZnS composite. The composite nanoparticles that contain only a few of the original nanoparticles can be made non-centrosymmetric, which may have interesting photophysical properties.

(d) using the devices and methods according to the invention to synthesize medically important nanoparticles, such as encapsulated drugs and composite drugs.

(e) combinatorial synthesis of core-shell particles and other complex systems. For example, the luminescence of CdSe/ZnS particles may be monitored and the conditions adjusted to produce particles with various core and shell sizes, various doping impurities in the core and shell, and various ligand composition on the surface of the particles. These can be conducted in real time using a device according to the invention. The entire process can also be automated.

The devices and methods according to the present invention may also be used for synthesizing polymers. Since the invention allows precise control of the timing of a polymerization reaction, one or more properties of a polymer such as molecular weight, polydispersity and blockiness can be readily controlled or adjusted. In addition, use of the substrate of the present invention allows the user to precisely form block copolymers by merging plugs within a device, since the path length of the channel will correspond to a specific duration of the polymerization reaction. Similarly, a living polymer chain can be terminated with a specific end group to yield polymers with a discrete subset of molecular weights.

In addition, combinatorial libraries of drug candidates may be synthesized using similar approaches. The library may be encoded using the position of plugs in a channel. Plugs of variable composition may be created by varying flow rates. Combination of synthesis of the library may be combined with screening and assays performed on the same microfluidic chip according to the present invention. In some embodiments, merging, splitting and sorting of plugs may be used during synthesis, assays, etc.

All of the above synthesis methods of the present invention can be used to form macroscopic quantities of one or more reaction products by running multiple reactions in parallel.

Particle Separation/Sorting Using Plugs

The flow within the moving plugs can be used for separation of polymers and particles. Plugs can be used for separation by first using flow within a moving plug to establish a distribution of the polymers or particles inside the plug (for example, an excess of the polymer inside the front, back, right or left side of the plug) and then using splitting to separate and isolate the part of the plug containing higher concentration of the polymers or particles. When two polymers or particles are present inside the plug and establish different distributions, slitting can be used to separate the polymers or particles. This approach may be useful, for example, in achieving on a microfluidic chip any of, but not limited to, the following: separation, purification, concentration, membrane-less dialysis, and filtration.

Crystallization

The devices and methods of the invention allow fast, inexpensive miniaturization of existing crystallization methods and other methods that can be adapted into, for example, novel protein screening and crystallization techniques. The crystallization methods according to the invention may be applied to various drugs, materials, small molecules, macromolecules, colloidal and nanoparticles, or any of their combinations. Many relevant protein structures remain undetermined due to their resistance to crystallization. Also, many interesting proteins are only available in microgram quantities. Thus, a screening process must permit the use of small amounts protein for analysis. Current crystallization screening technologies generally determine the ideal conditions for protein crystallization on a milligram scale. Devices and methods according to the invention improve current bench-top methodology available to single users, and enables higher throughput automated systems with improved speed, sample economy, and entirely new methods of controlling crystallization.

A microfluidic system according to the invention can be applied to the crystallization of small molecules or macromolecules and their complexes.

For example, systems and methods in accordance with the present invention may include but are not limited to: (1) biological macromolecules (cytosolic proteins, extracellular proteins, membrane proteins, DNA, RNA, and complex combinations thereof); (2) pre- and post-translationally modified biological molecules (including but not limited to, phosphorylated, sulfolated, glycosylated, ubiquitinated, etc. proteins, as well as halogenated, abasic, alkylated, etc. nucleic acids); (3) deliberately derivatized macromolecules, such as heavy-atom labeled DNAs, RNAs, and proteins (and complexes thereof), selenomethionine-labeled proteins and nucleic acids (and complexes thereof), halogenated DNAs, RNAs, and proteins (and complexes thereof); (4) whole viruses or large cellular particles (such as the ribosome, replisome, spliceosome, tubulin filaments, actin filaments, chromosomes, etc.); (5) small-molecule compounds such as drugs, lead compounds, ligands, salts, and organic or metallo-organic compounds; (6) small-molecule/biological macromolecule complexes (e.g., drug/protein complexes, enzyme/substrate complexes, enzyme/product complexes, enzyme/regulator complexes, enzyme/inhibitor complexes, and combinations thereof); (7) colloidal particles; and (8) nanoparticles.

Preferably, a general crystallization technique according to the present invention involves two primary screening steps: a crude screen of crystallization parameters using relatively small channels with a large number of small plugs, and a fine screen using larger channels and larger plugs to obtain diffraction-quality crystals. For example, ten crude screens performed using channels with a $(50\ \mu m)^2$ cross-sectional dimension and with more or less one thousand 150-picoliter (pL) plugs corresponding to 10 mg/mL final concentration of a protein (10,000 trials total) will typically require about 1.5 μL of solution, produce crystals up to about $(10\ \mu m)^3$ in size, and will consume approximately 15 μg of protein. Up to 300 or more of such plugs can be formed in about 1 second in these microfluidic networks. A fine screen around optimal conditions in $(500\ \mu m)^2$ channels is expected to use more or less 50 plugs. Another ~5 μL of solution and another 50 μg of the protein are expected to be consumed. This can produce crystals up to $(100\ \mu m)^3$ in size. Approximately 30 plugs can be formed about every second or so. The throughput of the

system will generally be determined by the rate of plug formation, and may be limited by how rapidly the flow rates can be varied. Pressure control methods that operate at frequencies of 100 Hz are available and may be applied to PDMS microfluidic networks (Unger et al., "Monolithic fabricated valves and pumps by multilayer soft lithography," *Science* 2000, vol. 288, pp. 113-116).

Crystal properties such as appearance, size, optical quality, and diffractive properties may be characterized and measured under different conditions. For example, a Raxis IIc X-ray detector mounted on a Rigaku RU 200 rotating anode X-ray generator, which is equipped with double focusing mirrors and an MSC cryosystem, may be used for at least some of the characterizations and measurements. A synchrotron beam may be useful for characterization of small crystals. Also, these devices and methods may be used to build microfluidic systems according to the invention that are compatible with structural studies using x-ray beams.

A significant problem involving current crystallization approaches is determining the conditions for forming crystals with optimal diffractive properties. Normally crystals have to be grown, isolated, mounted, and their diffractive properties determined using an x-ray generator or a synchrotron. Microfluidic systems with thin, non-scattering walls would be desirable for determining the diffractive properties of crystals inside a microfluidic system. Preferably, crystallization is carried out inside this system using methods according to the invention, which are described herein. The crystals are exposed to x-ray beams either to determine their structure or diffractive properties (the screening mode). For example, a PDMS membrane defining two side walls of the channels could be sandwiched between two very thin glass plates (defining the top and bottom walls of the channels) that do not significantly scatter X-rays. Thus, the devices of the invention offer a further advantage in that structural characterization could be conducted while the sample is inside the microfluidic device. Thus, the sample can be characterized without the need to take out the sample, e.g., crystal, from the device.

The present system enables higher throughput automated systems with improved speed, sample economy, and entirely new methods of controlling crystallization. Microfluidic versions of microbatch, vapor phase diffusion and FID techniques may be carried out using the present invention, as described below, or using a combination of these techniques or other techniques. In addition, the nucleation and growth phases may be carried out in discrete steps through merging plugs, as described herein.

Screening for protein crystallization can involve varying a number of parameters. During crystallization screening, a large number of chemical compounds may be employed. These compounds include salts, small and large molecular weight organic compounds, buffers, ligands, small-molecule agents, detergents, peptides, crosslinking agents, and derivatizing agents. Together, these chemicals can be used to vary the ionic strength, pH, solute concentration, and target concentration in the plug, and can even be used to modify the target. The desired concentration of these chemicals to achieve crystallization is variable, and can range from nanomolar to molar concentrations.

A typical crystallization mix may contain a set of fixed, but empirically-determined, types and concentrations of precipitation agent, buffers, salts, and other chemical additives (e.g., metal ions, salts, small molecular chemical additives, cryoprotectants, etc.). Water is a key solvent in many crystallization trials of biological targets, as many of these molecules may require hydration to stay active and folded. Precipitation agents act to push targets from a soluble to insoluble state, and may work by volume exclusion, changing the dielectric constant of the solvent, charge shielding, and molecular crowding. Precipitation agents compatible with the PDMS material of certain embodiments according to the invention include, but are not limited to, nonvolatile salts, high molecular weight polymers, polar solvents, aqueous solutions, high molecular weight alcohols, divalent metals.

Precipitation agents, which include large and small molecular weight organics, as well as certain salts, may be used from under 1% to upwards of 40% concentration, or from <0.5M to greater than 4M concentration. Water itself can act in a precipitating manner for samples that require a certain level of ionic strength to stay soluble. Many precipitation agents may also be mixed with one another to increase the chemical diversity of the crystallization screen. Devices according to the invention are readily compatible with a broad range of such compounds.

A nonexclusive list of salts that may be used as precipitation agents is as follows: tartrates (Li, Na, K, Na/K, NH_4); phosphates (Li, Na, K, Na/K, NH_4); acetates (Li, Na, K, Na/K, Mg, Ca, Zn, NH_4); formates (Li, Na, K, Na/K, Mg, NH_4); citrates (Li, Na, K, Na/K, NH_4); chlorides (Li, Na, K, Na/K, Mg, Ca, Zn, Mn, Cs, Rb, NH_4); sulfates (Li, Na, K, Na/K, NH_4); maleates (Li, Na, K, Na/K, NH_4); glutamates (Li, Na, K, Na/K, NH_4.

A nonexclusive list of organic materials that may be used as precipitation agents is as follows: PEG 400; PEG 1000; PEG 1500; PEG 2K; PEG 3350; PEG 4K; PEG 6K; PEG 8K; PEG 10K; PEG 20K; PEG-MME 550; PEG-MME 750; PEG-MME 2K; PEGMME 5K; PEG-DME 2K; dioxane; methanol; ethanol; 2-butanol; n-butanol; t-butanol; jeffamine m-600; isopropanol; 2-methyl-2,4-pentanediol; 1,6 hexanediol.

Solution pH can be varied by the inclusion of buffering agents; typical pH ranges for biological materials lie anywhere between values of 3 and 10.5 and the concentration of buffer generally lies between 0.01 and 0.25 M. The microfluidics devices described in this document are readily compatible with a broad range of pH values, particularly those suited to biological targets.

A nonexclusive list of possible buffers that may be used according to the invention is as follows: Na-acetate; HEPES; Na-cacodylate; Na-citrate; Na-succinate; Na—K-phosphate; TRIS; TRIS-maleate; imidazole-maleate; bistrispropane; CAPSO, CHAPS, MES, and imidazole.

Additives are small molecules that affect the solubility and/or activity behavior of the target. Such compounds can speed up crystallization screening or produce alternate crystal forms or polymorphs of the target. Additives can take nearly any conceivable form of chemical, but are typically mono and polyvalent salts (inorganic or organic), enzyme ligands (substrates, products, allosteric effectors), chemical crosslinking agents, detergents and/or lipids, heavy metals, organometallic compounds, trace amounts of precipitating agents, and small molecular weight organics.

The following is a nonexclusive list of additives that may be used in accordance with the invention: 2-butanol; DMSO; hexanediol; ethanol; methanol; isopropanol; sodium fluoride; potassium fluoride; ammonium fluoride; lithium chloride anhydrous; magnesium chloride hexahydrate; sodium chloride; calcium chloride dihydrate; potassium chloride; ammonium chloride; sodium iodide; potassium iodide; ammonium iodide; sodium thiocyanate; potassium thiocyanate; lithium nitrate; magnesium nitrate hexahydrate; sodium nitrate; potassium nitrate; ammonium nitrate; magnesium formate; sodium formate; potassium formate; ammonium formate; lithium acetate dihydrate; magnesium acetate tetrahydrate;

zinc acetate dihydrate; sodium acetate trihydrate; calcium acetate hydrate; potassium acetate; ammonium acetate; lithium sulfate monohydrate; magnesium sulfate heptahydrate; sodium sulfate decahydrate; potassium sulfate; ammonium sulfate; di-sodium tartrate dihydrate; potassium sodium tartrate tetrahydrate; di-ammonium tartrate; sodium dihydrogen phosphate monohydrate; di-sodium hydrogen phosphate dihydrate; potassium dihydrogen phosphate; di-potassium hydrogen phosphate; ammonium dihydrogen phosphate; di-ammonium hydrogen phosphate; tri-lithium citrate tetrahydrate; tri-sodium citrate dihydrate; tri-potassium citrate monohydrate; diammonium hydrogen citrate; barium chloride; cadmium chloride dihydrate; cobaltous chloride dihydrate; cupric chloride dihydrate; strontium chloride hexahydrate; yttrium chloride hexahydrate; ethylene glycol; Glycerol anhydrous; 1,6 hexanediol; MPD; polyethylene glycol 400; trimethylamine HCl; guanidine HCl; urea; 1,2,3-heptanetriol; benzamidine HCl; dioxane; ethanol; iso-propanol; methanol; sodium iodide; L-cysteine; EDTA sodium salt; NAD; ATP disodium salt; D(+)-glucose monohydrate; D(+)-sucrose; xylitol; spermidine; spermine tetra-HCl; 6-aminocaproic acid; 1,5-diaminopentane diHCl; 1,6-diaminohexane; 1,8-diaminooctane; glycine; glycyl-glycyl-glycine; hexaminecobalt trichloride; taurine; betaine monohydrate; polyvinylpyrrolidone K15; non-detergent sulfo-betaine 195; non-detergent sulfo-betaine 201; phenol; DMSO; dextran sulfate sodium salt; Jeffamine M-600; 2,5 Hexanediol; (+/−)-1,3 butanediol; polypropylene glycol P400; 1,4 butanediol; tert-butanol; 1,3 propanediol; acetonitrile; gamma butyrolactone; propanol; ethyl acetate; acetone; dichloromethane; n-butanol; 2,2,2 trifluoroethanol; DTT; TCEP; nonaethylene glycol monododecyl ether, nonaethylene glycol monolauryl ether; polyoxyethylene (9) ether; octaethylene glycol monododecyl ether, octaethylene glycol monolauryl ether; polyoxyethylene (8) lauryl ether; Dodecyl-β-D-maltopyranoside; Lauric acid sucrose ester; Cyclohexyl-pentyl-β-D-maltoside; Nonaethylene glycol octylphenol ether; Cetyltrimethylammonium bromide; N,N-bis(3-D-gluconamidopropyl)-deoxycholamine; Decyl-β-D-maltopyranoside; Lauryldimethylamine oxide; Cyclohexyl-pentyl-β-D-maltoside; n-Dodecylsulfobetaine, 3-(Dodecyldimethylanimonio)propane-1-sulfonate; Nonyl-β-D-glucopyranoside; Octyl-β-D-thioglucopyranoside, OSG; N,N-Dimethyldecylamine-β-oxide; Methyl 0-(N-heptylcarbamoyl)-α-D-glucopyranoside; Sucrose monocaproylate; n-Octanoyl-β-D-fructofuranosyl-α-D-glucopyranoside; Heptyl-β-D-thioglucopyranoside; Octyl-β-D-glucopyranoside, OG; Cyclohexyl-propyl-β-D-maltoside; Cyclohexylbutanoyl-N-hydroxyethylglucamide; n-decylsulfobetaine, 3-(Decyldimethylammonio)propane-Isulfonate; Octanoyl-N-methylglucamide, OMEGA; Hexyl-β-D-glucopyranoside; Brij 35; Brij 58; Triton X-114; Triton X-305; Triton X-405; Tween 20; Tween 80; polyoxyethylene(6)decyl ether; polyoxyethylene(9)decyl ether; polyoxyethylene(10) dodecyl ether; polyoxyethylene(8)tridecyl ether; Decanoyl-N-hydroxyethylglucamide; Pentaethylene glycol monooctyl ether; 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; 3-[(3-Cholamidopropyl)-dimethylammonio] hydroxy-1-propane sulfonate; Cyclohexylpentanoyl-N-hydroxyethylglucamide; Nonanoyl-N-hydroxyethyglucamide; Cyclohexylpropanol-N-hydroxyethylglucamide; Octanoyl-N-hydroxyethylglucamide; Cyclohexylethanoyl-N-hydroxyethylglucamide; Benzyldimethyldodecyl ammonium bromide; n-Hexadecyl-β-D-maltopyranoside; n-Tetradecyl-β-D-maltopyranoside; n-Tridecyl-β-D-maltopyranoside; Dodecylpoly(ethyleneglycoether); n-Tetradecyl-N,N-dimethyl ammonio-1-propanesulfonate; n-Undecyl-β-D-maltopyranoside; n-Decyl D-thiomaltopyranoside; n-dodecylphosphocholine; α-D-glucopyranoside, β-D-fructofuranosyl monodecanoate, sucrose mono-caprate; 1-s-Nonyl-β-D-thioglucopyranoside; n-Nonyl-β-D-thiomaltoyranoside; N-Dodecyl-N,N-(dimethlammonio) butyrate; n-Nonyl-β-D-maltopyranoside; Cyclohexyl-butyl D-maltoside; n-Octyl-β-D-thiomaltopyranoside; n-Decylphosphocholine; n-Nonylphosphocholine; Nonanoyl-N-methylglucamide; 1-s-Heptyl-β-D-thioglucopyranoside; n-Octylphosphocholine; Cyclohexyl-ethyl D-maltoside; n-Octyl-N,N-dimethyl ammonio-1-propanesulfonate; Cyclohexyl-methyl-β-D-maltoside.

Cryosolvents are agents that stabilize a target crystal to flash-cooling in a cryogen such as liquid nitrogen, liquid propane, liquid ethane, or gaseous nitrogen or helium (all at approximately 100-120° K) such that crystal becomes embedded in a vitreous glass rather than ice. Any number of salts or small molecular weight organic compounds can be used as a cryoprotectant, and typical ones include but are not limited to: MPD, PEG-400 (as well as both PEG derivatives and higher molecular-weight PEG compounds), glycerol, sugars (xylitol, sorbitol, erythritol, sucrose, glucose, etc.), ethylene glycol, alcohols (both short- and long chain, both volatile and nonvolatile), LiOAc, LiCl, $LiCHO_2$, $LiNO_3$, Li_2SO_4, $Mg(OAc)_2$, NaCl, $NaCHO_2$, $NaNO_3$, etc. Again, materials from which microfluidics devices in accordance with the present invention are fabricated may be compatible with a range of such compounds.

Many of these chemicals can be obtained in predefined screening kits from a variety of vendors, including but not limited to Hampton Research of Laguna Niguel, Calif., Emerald Biostructures of Bainbridge Island, Wash., and Jena Bio-Science of Jena, Germany, that allow the researcher to perform both sparse matrix and grid screening experiments. Sparse matrix screens attempt to randomly sample as much of precipitant, buffer, and additive chemical space as possible with as few conditions as possible. Grid screens typically consist of systematic variations of two or three parameters against one another (e.g., precipitant concentration vs. pH). Both types of screens have been employed with success in crystallization trials, and the majority of chemicals and chemical combinations used in these screens are compatible with the chip design and matrices in accordance with embodiments of the present invention. Moreover, current and future designs of microfluidic devices may enable flexible combinatorial screening of an array of different chemicals against a particular target or set of targets, a process that is difficult with either robotic or hand screening. This latter aspect is particularly important for optimizing initial successes generated by first-pass screens.

In addition to chemical variability, a host of other parameters can be varied during crystallization screening. Such parameters include but are not limited to: (1) volume of crystallization trial; (2) ratio of target solution to crystallization solution; (3) target concentration; (4) cocrystallization of the target with a secondary small or macromolecule; (5) hydration; (6) incubation time; (7) temperature; (8) pressure; (9) contact surfaces; (10) modifications to target molecules; and (11) gravity.

Although the discussion below refers to proteins, the particular devices or methods described can also be used or suitably adapted for the crystallization of other types of samples such as those mentioned above (e.g., small molecules, other macromolecules, nanoparticles, colloidal particles, etc.). In one aspect of the present invention, protein crystallization is conducted using miniaturized microbatch conditions. The process consists of two steps. First, plugs are

preferably formed wherein the concentrations of the protein, precipitant, and additive are adjusted by varying the relative flow rates of these solutions. This step corresponds to a screening step. Once the optimal concentrations have been found, the flow rates can then be kept constant at the optimal conditions. In this step, plugs are preferably transported through the channel as they form. Second, the flow is preferably stopped once the desired number of plugs are formed. The plugs are then preferably allowed to incubate. In some embodiments according to the invention the flow may be continued, rather than stopped. In those embodiments, the flow is maintained sufficiently slow and the channels are made sufficiently long that plugs spend sufficient time in the channels for crystallization to occur (from tens of minutes to weeks, but may be faster or slower).

In one aspect, upon formation of the plugs, they are trapped using expansions in the channels. The expansions act as dead volume elements while the plugs are being formed in the presence of flow. Thus, the expansions do not interfere with the flow of the plugs through the channel. Once the flow is stopped, surface tension drives plugs into the expansions where surface tension is minimized. The expansions may be, but are not limited to, oval, round, square, rectangular, or star-shaped. In particular, a star-shaped expansion may prevent adherence of the plug or of a crystal to the walls of the expansion. The ratio of the size of the expansion opening to the width of the channel may be varied based on empirical results for a particular set of conditions. FIG. **16** is a schematic illustration of a microfluidic device according to the invention that illustrates the trapping of plugs. In experiments, plugs were sustained in perfluorodecaline inside a channel for one day, and did not appear to change during that time (a refractive index mismatch between the fluorinated and aqueous phase was introduced to aid in visualization of plugs).

The method described above allows a high degree of control over protein and precipitant concentrations. It also allows a high degree of control over a range of time scales through the control of plug size and composition. FIG. **17** shows a schematic of a microfluidic method for forming plugs with variable compositions for protein crystallization. Continuously varied flow rates of the incoming streams are preferably used to form plugs with various concentrations of the protein, precipitation agents, and additives. In FIG. **17**, for example, the following can be introduced into the various inlets: buffers into inlets **171**, **172**; PEG into inlet **173**; salt into inlet **174**; solvent into inlet **175**; and protein into inlet **176**. These various solutions can enter a channel **177** through which a carrier fluid such as perfluorodecaline flows. For example, a 1-meter long channel with a 200×80 μm^2 cross section can be used to form approximately two hundred 6 nL (nanoliter) plugs. If each plug contains enough protein to form a 40-μm^3 crystal, 200 trials will consume only about 1.2 μL of approximately 10 mg/mL protein solution (12 μg of protein). About one minute may be sufficient to form plugs in these trials.

In another aspect according to the invention, after plugs are formed as described above for the microbatch system, slow evaporation through a very thin PDMS membrane (or another membrane with slight water permeability) is preferably used for added control over the crystallization process. A slow decrease in the volume of the plug during evaporation is expected to produce a trajectory of the solution through the crystallization phase space similar to that in a vapor diffusion experiment. Hence, this method, in addition to microbatch methods, can be used to miniaturize and optimize vapor diffusion methods.

In the vapor diffusion method, a drop containing protein, stabilizing buffers, precipitants, and/or crystallization agents is allowed to equilibrate in a closed system with a much larger reservoir. The reservoir usually contains the same chemicals minus the protein but at an over all higher concentration so that water preferentially evaporates from the drop. If conditions are right, this will produce a gradual increase in protein concentration such that a few crystals may form.

Vapor diffusion can be performed in several ways. The one most often used is called Hanging Drop Technique. The drop is placed on a glass coverslip, which is then inverted and used to seal a small reservoir in a Linbro Plate. After a period of several hours to weeks, microscopic crystals may form and continue to grow. The other set up is known as Sitting Drop. In this method a drop (usually >10 uL) is placed in a depression in either a Micro Bridge in a Linbro Plate or a glass plate and again placed in a closed system to equilibrate with a much larger reservoir. One usually uses the sitting drop technique if the drop has very low surface tension, making it hard to turn upside down or if the drops need to be larger than 20 uL. Also, in some cases, crystals will grow better using one technique or the other.

In another embodiment, the plugs are preferably formed and transported such that excessive mixing of the protein with the precipitation agent is minimized or prevented. For example, gentle mixing using spiral channels may be used to achieve this and also to create interfaces between the protein and the precipitation agent. Alternatively, combining two streams of plugs in a T-junction without merging may be used to create plugs that diffuse and combine without significant mixing to establish a free interface after the flow is stopped. Diffusion of the proteins and precipitates through the interface induces crystallization. This is an analogue of the Free-Interface Diffusion method. It may be performed under either the microbatch or vapor diffusion conditions as described above.

Preferably, the spacing between plugs can be increased or the oil composition changed to reduce plug-plug diffusion. For example, a spacing of about 2.5 mm in paraffin oil can be used, which has been shown to be an effective barrier to aqueous diffusion in crystallization trials.

Visually identifying small crystals inside plugs with curved surfaces can be a challenge when performing microbatch experiments. In an aspect according to the invention, a method based on matching the refractive indices of carrier-fluid with that of the plug fluid to enhance visualization is used. Microscopic detection is preferably performed by using shallow channels and by matching the refractive indices of carrier-fluid mixtures to those of the aqueous solutions.

In addition, at least three other novel methods of controlling protein crystallization are described below: (1) using surface chemistry to effect nucleation of protein crystals; (2) using different mixing methods to effect crystallization; and (3) performing protein crystals seeding by separating nucleation and growth phases in space.

Control of nucleation is one of the difficult steps in protein crystallization. Heterogeneous nucleation is statistically a more favorable process than its solution-phase counterpart. Ideal surfaces for heterogeneous nucleation have complementary electrostatic maps with respect to their macromolecular counterparts. Critical nuclei are more stable on such surfaces than in solution. Further, the degree of supersaturation required for heterogeneous nucleation is much less than that required for the formation of solution-phase nuclei. Surfaces such as silicon, crystalline minerals, epoxide surfaces, polystyrene beads, and hair are known to influence the efficiency of protein crystallization. Few studies have been done,

but promising results have been shown for protein crystallization at the methyl, imidazole, hydroxyl, and carboxylic acid termini of self-assembled monolayers on gold. Using self-assembled monolayers, proteins were crystallized over a broader range of crystallization conditions and at faster rates than when using the traditional silanized glass.

FIG. **18** is a schematic illustration of a method for controlling heterogeneous nucleation by varying the surface chemistry at the interface of an aqueous plug-fluid and a carrier-fluid. In FIG. **18**, plugs are formed in the presence of several solutions of surfactants that possess different functional groups (left side of the diagram). The right side of FIG. **18** shows the aqueous phase region in which a precipitant, solvent, and protein may be introduced into inlets **180**, **181**, and **182**, respectively. The composition of the surfactant monolayer is preferably controlled by varying the flow rates. In another application of the method illustrated in FIG. **18**, the surface chemistry can be varied continuously. The manipulation and control of the surface chemistry can be used for screening, assays, crystallizations, and other applications where surface chemistry is important.

In one aspect of the invention, heterogeneous nucleation of proteins is controlled by forming aqueous plugs in a carrier-fluid, preferably containing fluoro-soluble surfactants if the carrier-fluid is a fluorocarbon. Varying the relative flow rates of the surfactant solutions may generate a wide variety of liquid-liquid interface conditions that can lead to the formation of mixed monolayers or mixed phase-separated monolayers. Preferably, several surfactants are used to control the heterogeneous nucleation of protein crystals. Ethylene-glycol monolayers are preferably used to reduce heterogeneous nucleation, and monolayers with electrostatic properties complementary to those of the protein are preferably used to enhance heterogeneous nucleation. These methods for controlling heterogeneous nucleation are designed to induce or enhance the formation of crystals that are normally difficult to obtain. These methods may also be used to induce or enhance the formation of different crystal polymorphs that are relatively more stable or better ordered.

As mentioned above, control of nucleation is highly desired in an advanced crystallization screen. One method that can be used to achieve control of nucleation involves the transfer of nucleating crystals from one concentration to another via dilution. This method, which has been applied in macroscopic systems primarily to vapor diffusion, was intended to allow decoupling of the nucleation and growth phases. This method is difficult to perform using traditional methods of crystallization because nucleation occurs long before the appearance of microcrystals.

FIG. **19** illustrates a method of separating nucleation and growth using a microfluidic network according to the present invention using proteins as a non-limiting example. The left side of FIG. **19** shows plugs that are formed preferably using high concentrations of protein and precipitant. In FIG. **19**, the following can be introduced into the various inlets shown: buffer into inlets **191**, **196**; PEG into inlets **192**, **197**; precipitant into inlets **193**, **198**; solvent into inlets **194**, **199**; and protein into inlets **195**, **200**. Oil flows through the channels **201**, **202** from left to right. The portions **203**, **204**, and **205** of the channel correspond to regions where fast nucleation occurs (**203**), no nucleation occurs (**204**), and where crystal growth occurs (**205**). The concentrations used are those that correspond to the nucleating region in the phase diagram. Nucleation occurs as the plugs move through the channel to the junction over a certain period. Preferably, these plugs are then merged with plugs containing a protein solution at a point corresponding to a metastable (growth, rather than nucleation) region (right side of FIG. **19**). This step ends nucleation and promotes crystal growth. When the combined channel has been filled with merged plugs, the flow is preferably stopped and the nuclei allowed to grow to produce crystals.

Nucleation time can be varied by varying the flow rate along the nucleation channel. The nucleus is preferably used as a seed crystal for a larger plug with solution concentrations that correspond to a metastable region. Existing data indicate the formation of nuclei within less than about 5 minutes.

Fluid mixing is believed to exert an important effect in crystal nucleation and growth. Methods according to the invention are provided that allow a precise and reproducible degree of control over mixing. FIG. **20** illustrates two of these methods. A method of mixing preferably places the solution into a nucleation zone of the phase diagram without causing precipitation. Preferably, gentle mixing (FIG. **20**, left side) is used to achieve this by preventing, reducing, or minimizing contact between concentrated solutions of the protein and precipitant. Alternatively, rapid mixing (FIG. **20**, right side) is used to achieve this by allowing passage through the precipitation zone sufficiently quickly to cause nucleation but not precipitation. The two methods used as examples involve the use of spiraling channels for gentle mixing and serpentine channels for rapid mixing.

The two methods in accordance with the invention depicted in FIG. **20** can be used to determine the effect of mixing on protein crystallization. In addition, the various methods for controlling mixing described previously (e.g., slow mixing in straight channels, chaotic mixing in non-straight channels, or mixing in which twirling may or may not occur) can be applied to crystallization, among other things.

After obtaining the crystals using any of the above described techniques, the crystals may be removed from the microfluidic device for structure determination. In other systems, the fragile and gelatinous nature of protein crystals makes crystal collection difficult. For example, removing protein crystals from solid surfaces can damage them to the point of uselessness. The present invention offers a solution to this problem by nucleating and growing crystals in liquid environments. In an aspect according to the invention, a thin wetting layer of a carrier-fluid covered with a surfactant is used to enable or facilitate the separation of a growing crystal from a solid surface. When the crystals form, they may be separated from the PDMS layer by using a thin layer of a carrier-fluid.

In one aspect, a microfluidic device of the present system can include further include capillary tubing suitable for collecting plugs ("the capillary device"; FIG. **46**). The tubing is preferably composed of a material that prevents uncontrolled evaporation of solutions (such as water) through its wall. Further, use of the capillary tubing can enable direct screening of crystals by x-ray diffraction analysis or other spectrophotometric detection/analysis means employing e.g., optical or infrared detection. Plugs in the capillary tubing have been found to be stable and did not show signs of evaporation over several months, even in the absence of humidity control. Therefore, the capillary device can be incubated for a much longer time than all-PDMS microfluidic chips. Water diffusion can be controlled by varying the starting salt concentration differences as well the distance between plugs. Production of crystals directly inside the capillary tubes can facilitate on-chip diffraction without having to move the crystal around.

Upon formation of plugs in the PDMS portion and their transfer into capillary tubing, the flow rates are stopped, the capillary tubing is disconnected from the PDMS portion and

the ends are sealed by capillary wax. The capillary tubing may be incubated under suitable crystallization conditions (e.g, temperature etc.) until crystals form inside the plugs. Formation of crystals can be monitored using optical detection and/or x-ray diffraction methods. Crystals grown at the fluid-fluid interface can be easily removed from the capillary by gentle flow, or by breaking the capillary and wicking the liquid out. Upon formation of suitable crystals, the capillaries are frozen and structures are directly determined from inside the capillary using e.g., synchrotron radiation. Because this method obviates the problem of handling and mounting crystals and because it can facilitate the determination of structure directly from within the capillary, it may be especially suitable for high-throughput, fully automated crystallization.

The plugs in the capillary tubing can be stable in both hydrophilic (e.g., treated with by chromic acid) or hydrophobic (e.g., silanized) capillaries for over a month, even if the capillary is placed vertically for over three days.

The use of x-ray capillary tubing for protein crystallization can also be applied to a controlled vapor diffusion process which lends itself to direct monitoring and structural determination of protein crystals in the capillary tubing (FIG. **49**). In this modified vapor-diffusion process an array of plugs is generated in the channel portion of a capillary device (as described above) where the protein and precipitant plugs alternate with plugs containing a high concentration of precipitant. Syringe pumps attached to the capillary device cause the plugs to flow into suitable x-ray capillary tubing. At the conclusion of the experiment, the flow is stopped, the capillary is disconnected from the PDMS portion and the ends are sealed with capillary wax. The x-ray capillary is incubated under optimal conditions until crystals form inside the plugs.

The use of carrier fluid (oil) permeable to water causes the water from the plugs to diffuse through from the oil from the plugs that are low in osmolarity into plugs that are higher in osmolarity, thereby increasing the concentration of the protein and precipitants in the plugs for crystallization. The rate of water transfer from the plugs and the amount of water transferred between the two types of plugs may be controlled by using oils having different water permeabilities, by changing the size or distance between plugs or by altering the precipitant concentrations between the different types of plugs (i.e., changing the difference in osmolarity between the different plug types). All of these parameters can be conveniently altered by changing the relative flow rates of the aqueous and carrier-fluid (oil) solutions. Poly-3,3,3-trifluoropropylmethylsiloxane (FMS-121) can be a suitable carrier-oil fluid for this procedure.

One scheme for generating alternating plugs by vapor diffusion involves attaching four different syringes to a PDMS device, each syringe associated with a syringe pump for introducing each of aqueous solutions A, B into respective aqueous inlet channels and for introducing each of carrier oil fluids C, D into respective oil inlet channels. The aqueous solutions can be the same or different. Multiple, distinct aqueous solutions can also be co-introduced together in one or both of the two aqueous channels. In principle, the same oil or different oils may be used in the two oil inlets. In either case, one oil inlet channel is parallel to the main channel; the other oil inlet channel is vertical to the main channel and is positioned between the two aqueous inlet channels to separate the two aqueous streams into alternating plugs.

Importantly, the flow rates of solutions A and B may be changed in a correlated fashion. Thus, when the flow rate of solution A_1 is increased and solution A_2 is decreased, the flow rate of solutions B_1 is also increased and solution B_2 is also decreased. This can allow one to maintain a constant difference in osmolarity between the plugs of stream A and stream B to ensure that transfer from all plugs A to all plugs B occurs at a constant rate. Moreover, if the flow rates of the corresponding A and B streams are changed in a correlated fashion, the composition of plugs B will reflect the composition of plugs A thereby allowing one to incorporate markers into the B stream plugs to serve as a code for the plugs in the A stream. Thus, if the two types of plugs are made in a correlated way, one type of droplet may be used for crystallization, while the other type of droplet is used for indexing provided it contains a label conferring a read out with respect to crystallization. In other words, absorption/fluorescent dyes or x-ray scattering/absorbing materials can be incorporated in markers in the B streams to facilitate optical density quantification or x-ray diffraction analysis to provide a read out of relative protein and precipitant concentrations in the A streams. This approach can provide a powerful means for optimizing crystallization conditions for subsequent scale-up experiments.

The use of markers may be performed using an oil that is impermeable to water (as in a microbatch procedure) to prevent transfer of water or any other material between the A plugs and B plugs. Alternatively, the B plugs may additionally incorporate a high concentration of dehydration agents (salt, other precipitants) in conjunction with a water-permeable oil as described above. In this way, the B plugs can serve both as markers for the A plugs and as sinks for excess water. Oils that are selectively permeable to materials other than water may also be used to induce transfer of other materials between the plugs and through the oil.

Alternating plugs may be generated using a range of channel geometries. The plugs may also alternate in patterns other than A:B:A:B. For example, other patterns (such as A:A:A:B:A:A:A:B, etc) may be obtained where transfer of water from A plugs adjacent to B plugs is faster than transfer of water from the middle A plug. This can create conditions favorable for creating multiple, different sets of crystallization conditions. The alternating droplet systems may be extended to more than two types of plugs alternating in the same channel or capillary (for example, A plugs with the crystallization solutions, B plugs with the dehydrating agents, and C plugs with markers or with a cryoprotectant).

The above described capillary systems are not limited to protein crystallization—other types of crystallizations and experiments may be performed. For example, the vapor diffusion/alternating droplet approach can be extended to e.g., a process for concentrating materials (such as protein). Such a process would be effected through diffusion of water plugs that are relatively low in osmolarity into plugs having a higher osmolarity. It should be noted, however, that solution materials in the different plug types do not have to be aqueous in nature, but can be in the form of solvents also. Alternatively, the A and B plugs do not have to be in solution at all, but can instead be in the form of emulsions or suspensions.

It will be clear to one skilled in the art that while the above techniques are described in detail for the crystallization of proteins, techniques similar to the ones described above may also be used for the crystallization of other substances, including other biomolecules or synthetic chemicals. In addition, the devices and methods according to the invention may be used to perform co-crystallization. For example, a crystal comprising more than one chemical may be obtained, for example, through the use of at least one stream of protein, a stream of precipitant, and optionally, a stream comprising a third chemical such as an inhibitor, another protein, DNA, etc. One may then vary the conditions to determine those that are optimal for forming a co-crystal.

Particle Separation/Sorting Using Plugs

The flow within the moving plugs can be used for separation of polymers and particles. Plugs can be used for separation by first using flow within a moving plug to establish a distribution of the polymers or particles inside the plug (for example, an excess of the polymer inside the front, back, right

or left side of the plug) and then using splitting to separate and isolate the part of the plug containing higher concentration of the polymers or particles. When two polymers or particles are present inside the plug and establish different distributions, splitting can be used to separate the polymers or particles.

The invention is further described below, by way of the following examples. It will be appreciated by persons of ordinary skill in the art that this example is one of many embodiments and is merely illustrative. In particular, the device and method described in this example (including the channel architectures, valves, switching and flow control devices and methods) may be readily adapted, e.g., used in conjunction with one or more devices or methods, so that plugs may be analyzed, characterized, monitored, and/or sorted as desired by a user.

EXAMPLE

Example 1

Fabrication of Microfluidic Devices and a General Experimental Procedure

Microfluidic devices with hydrophilic channel surfaces were fabricated using rapid prototyping in polydimethylsiloxane. The channel surfaces were rendered hydrophobic either by silanization or heat treatment. To silanize the surfaces of channels, (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.) vapor was applied to the inlets of a device with dry nitrogen as a carrier gas at around 40-60 mm Hg above about 1 atm pressure. Vacuum was simultaneously applied to the outlet of the device at about 650 mm Hg below atmospheric pressure. The silane vapor was applied for a period of between about 1-3 hours. To treat the channels using heat, a device was placed in an oven at approximately 120° C. for about three hours. Alternatively, a device can be heated in a Panasonic "The Genius" 1300 Watt microwave oven at power set to "10" for about ten minutes.

Oils and aqueous solutions were pumped through devices using a kdScientific syringe pump (Model 200) or Harvard Apparatus PhD 2000 pump. Hamilton Company GASTIGHT syringes were used (10-250 μl) and Hamilton Company 30 gauge Teflon® needles were used to attach the syringes to the devices. Oils and aqueous solutions were pumped through devices at volumetric flow rates ranging from about 0.10 μL/min to about 10.0 μL/min.

Aqueous solutions were colored using Crayola Original Formula Markers or Ferroin Indicator (0.025 M, Fisher Scientific). Oils that were used included perfluorodecaline (mixture of cis and trans, 95%, Acros Organics), perfluoroperhydrophenanthrene (tech., Alfa-Aesar), or 1H,1H,2H,2H-perfluorooctanol (98%, Alfa-Aesar). The experiments were typically performed using 10:1 mixtures of perfluorodecaline and 1H,1H,2H,2H-perfluorooctanol.

The experiments were monitored using a Lica MZFLIII stereoscope with Fostec (Schott-Fostec, LLC) Modulamps. Photographs of the experiments were taken with a Spot Insight Color Camera, Model # 3.2.0 (Diagnostic Instruments, Inc.). Spot Application version 3.4.0.0 was used to take the photographs with the camera.

Example 2

Varying the Concentration of Aqueous Solutions in Plugs

The left side of each of FIGS. **25**A-C shows a schematic diagram of the microfluidic network and the experimental conditions. The right side of each of FIGS. **25**A-C shows microphotographs illustrating the formation of plugs using different concentrations of the aqueous streams. Aqueous solutions of food dyes (red/dark and green/light) and water constituted the three streams. The volumetric flow rates of the three solutions (given in μL/min) are indicated. The dark stream is more viscous than the light stream. Therefore, the dark (more viscous) stream moves (measured in mm/s) more slowly and occupies a larger fraction of the channel at a given volumetric flow rate.

FIG. **45***a*) shows a schematic of the microfluidic network used to demonstrate that on-chip dilutions can be accomplished by varying the flow rates of the reagents. In FIG. **45***a*), the reagents are introduced through inlets **451**, **453** while the dilution buffer is introduced through inlet **452**. An oil stream flows through channel **454**. The blue rectangle outlines the field of view for images shown in FIG. **45***c*)-*d*). FIG. **45***b*) shows a graph quantifying this dilution method by measuring fluorescence of a solution of fluorescein diluted in plugs in the microchannel. Data are shown for 80 experiments in which fluorescein was flowed through one of the three inlets, where $C_{measured}$ and $C_{theoretical}$ [μM] are measured and expected fluorescein concentration. FIG. **45**(*c*) shows photographs illustrating this dilution method with streams of food dyes **455**, **456**, **457** having flow rates of 45 nL/s, 10 nL/s, and 10 nL/s, respectively. FIG. **45**(*d*) shows photographs illustrating this dilution method with streams of food dyes **458**, **459**, **460** having flow rates of 10 nL/s, 45 nL/s, and 10 nL/s, respectively. Carrier fluid was flowed at 60 nL/s.

Example 3

Networks of microchannels with rectangular cross-sections were fabricated using rapid prototyping in PDMS. The PDMS used was Dow Corning Sylgard Brand 184 Silicone Elastomer, and devices were sealed using a Plasma Prep II (SPI Supplies). The surfaces of the devices were rendered hydrophobic by baking the devices at 120° C. for 2-4 hours.

In FIG. **26**, the red aqueous streams were McCormick® red food coloring (water, propylene glycol, FD&C Red 40 and 3, propylparaben), the green aqueous streams were McCormick® green food coloring (water, propylene glycol, FD&C yellow 5, FD&C blue 1, propylparaben) diluted 1:1 with water, and the colorless streams were water. PFD used was a 10:1 mixture of perfluorodecaline (mixture of cis and trans, 95%, Acros Organics):1H,1H,2H,2H-perfluorooctanol (Acros Organics). The red aqueous streams were introduced in inlet **260**, **265** while the green aqueous streams were introduced in inlets **262**, **263** in FIG. **26***b*). The colorless aqueous stream was introduced in inlets **261**, **264**. The dark shadings of the streams and plug are due mainly from the red dye while the lighter shadings are due mainly from the green dye.

Aqueous solutions were pumped using 100 μL Hamilton Gastight syringes (1700 series, TLL) or 50 μL SGE gastight syringes. PFD was pumped using 1 mL Hamilton Gastight syringes (1700 series, TLL). The syringes were attached to microfluidic devices by means of Hamilton Teflon needles (30 gauge, 1 hub). Syringe pumps from Harvard Apparatus (PHD 2000 Infusion pumps; specially-ordered bronze bushings were attached to the driving mechanism to stabilize pumping) were used to infuse the aqueous solutions and PFD.

Microphotographs were taken with a Leica MZ12.5 stereomicroscope and a SPOT Insight Color digital camera (Model #3.2.0, Diagnostic Instruments, Inc.). SPOT Advanced software (version 3.4.0 for Windows, Diagnostic Instruments, Inc.) was used to collect the images. Lighting was provided from a Machine Vision Strobe X-Strobe X1200

(20 Hz, 12 μF, 600V, Perkin Elmer Optoelectronics). To obtain an image, the shutter of the camera was opened for 1 second and the strobe light was flashed once with the duration of the flash being about 10 μs.

Images were analyzed using NIH Image software, Image J. Image J was used to measure periods and lengths of plugs from microphotographs such as shown in FIG. **27***b*). Periods corresponded to the distance from the center of one plug to the center of an adjacent plug, and the length of a plug was the distance from the extreme front to the extreme back of the plug (see FIG. **28** for the definitions of front and back). Measurements were initially made in pixels, but could be converted to absolute measurements by comparing them to a measurement in pixels of the 50 μm width of the channel.

To make measurements of the optical intensity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs, microphotographs were converted from RGB to CMYK color mode in Adobe Photoshop 6.0. Using the same program, the yellow color channels of the microphotographs were then isolated and converted to grayscale images, and the intensities of the grayscale images were inverted. The yellow color channel was chosen to reduce the intensity of bright reflections at the extremities of the plugs and at the interface between the plugs and the channel. Following the work done in Photoshop, regions of plugs containing high concentrations of $Fe(SCN)_x^{(3-x)+}$ complexes appeared white while regions of low concentration appeared black. Using Image J, the intensity was measured across a thin, rectangular region of the plug, located halfway between the front and back of the plug (white dashed lines in FIG. **27***a***1**)). The camera used to take the microphotographs of the system was not capable of making linear measurements of optical density. Therefore, the measurements of intensity were not quantitative. Several of the plots of intensity versus relative position across the channel (FIG. **27***c*) were shifted vertically by less than 50 units of intensity to adjust for non-uniform illuminations of different parts of the images. These adjustments were justified because it was the shape of the distribution that was of interest, rather than the absolute concentration.

FIG. **29***a*)-*b*) shows plots of the sizes of periods and sizes of plugs as a function of total flow velocity (FIG. **29***a*)) and water fraction (wf) (FIG. **29***b*)). Values of capillary number (C.n.) were 0.0014, 0.0036, 0.0072 and 0.011, while values of the Reynolds number (R_e) were 1.24, 3.10, 6.21, and 9.31, each of the C.n. and R_e value corresponding to a set of data points with water fractions (wf) 0.20, 0.52, 0.52, and 0.20 (the data points from top to bottom in FIG. **29**A)). In turn, each of these sets of data points corresponds to a particular flow velocity as shown in FIG. **29***a*). Plugs in FIG. **29***b*) travel at about 50 millimeter/second (mm/s). All measurements of length and size are relative to the width of the channels (50 μm).

FIG. **30** shows microphotographs illustrating weak dependence of periods, length of plugs, and flow patterns inside plugs on total flow velocity. The left side of FIG. **30** shows a diagram of the microfluidic network. Here, the same solutions were used as in the experiment corresponding to FIG. **27**. The $Fe(SCN)_x^{(3-x)+}$ solution was introduced into inlet **301** while the colorless aqueous streams were introduced into inlets **302**, **303**. The same carrier fluid as used in the FIG. **27** experiment was flowed into channel **304**. The right side of FIG. **30** shows microphotographs of plugs formed at the same water fraction (0.20), but at different total flow velocities (20, 50, 100, 150 mm/s from top to bottom). Capillary numbers were 0.0014, 0.0036, 0.0072, and 0.011, respectively, from top to bottom. Corresponding Reynolds numbers were 1.24, 3.10, 6.21, and 9.31.

FIG. **31**A-C are plots showing the distribution of periods and lengths of plugs where the water fractions were 0.20, 0.40, and 0.73, respectively. The total flow velocity was about 50 mm/s, C.n.=0.0036, R_e=3.10 in all cases.

FIG. **27** shows the effects of initial conditions on mixing by recirculating flow inside plugs moving through straight microchannels. FIG. **27***a***1**) shows that recirculating flow (shown by black arrows) efficiently mixed solutions of reagents that were initially localized in the front and back halves of the plug. Notations of front, back, left, and right are the same as that in FIG. **28**. FIG. **27***a***2**) shows that recirculating flow (shown by black arrows) did not efficiently mix solutions of reagents that were initially localized in the left and right halves of the plugs. The left side of FIG. **27***b*) shows a schematic diagram of the microfluidic network. The two colorless aqueous streams were introduced into inlets **271**, **272** while a carrier fluid in the form of perfluorodecaline flowed through channel **273**. These solutions did not perturb the flow patterns inside plugs.

The right side of FIG. **27***b*) shows microphotographs of plugs of various lengths near the plug-forming region of the microfluidic network for water fractions of from 0.14 up to 1.00. FIG. **27***c***1**) shows a graph of the relative optical intensity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs of varying lengths. The intensities were measured from left (x=1.0) to right (x=0.0) across the width of a plug (shown by white dashed lines in FIG. **27***a***1**)-*a***2**)) after the plug had traveled 4.4 times its length through the straight microchannel. The gray shaded areas indicate the walls of the microchannel. FIG. **27***c***2**) is the same as FIG. **27***c***1**) except that each plug had traversed a distance of 1.3 mm. The d/l of each water fraction (wf) were 15.2 (wf 0.14), 13.3 (wf 0.20), 11.7 (wf 0.30), 9.7 (wf 0.40), 6.8 (wf 0.60), 4.6 (wf 0.73), and 2.7 (wf 0.84), where d is the distance traveled by the plug and l is the length of the plug.

Example 4

Merging of Plugs

Experiments were conducted to investigate the merging of plugs using different channel junctions (T- or Y-shaped), cross-sections, and flow rates (see FIG. **33***a*-*d*). The figures on the left side of FIGS. **33***a*-*d* show top views of microfluidic networks that comprise channels having either uniform or nonuniform dimension (e.g., the same or different channel diameters). The corresponding figures on the right are microphotographs that include a magnified view of two plug streams (from the two separate channels portions of which form the branches of the Y-shaped junction) that merges into a common channel.

In FIG. **33***a*, the oil-to-water volumetric ratio was 4:1 in each pair of oil and water inlets. The oil streams were introduced into inlets **330**, **332**, while the aqueous streams were introduced into inlets **331**, **333**. The flow rates of the combined oil/water stream past the junction where the oil and water meet was 8.6 mm/s. The channels, which were rectangular, had dimensions of 50 (width)×50 (height) μm^2. As shown in FIG. **33***a*, plugs that flow in uniform-sized channels typically merged only when they simultaneously arrived at the T-junction. Thus, plug merging in these channels occur infrequently. In addition, lagging plugs were typically not able to catch up with leading plugs along the common channel.

FIG. **33***b* illustrates plug merging occurring between plugs arriving at different times at the Y-shaped junction (magnified view shown). The oil streams were introduced into inlets **334**, **336**, while the aqueous streams were introduced into inlets

335, 337. In FIG. 33*b*, the flow rates for the combined oil/water fluid past the junction where the oil and water meet were 6.9 mm/s for channel **346** (the 50×50 μm^2 channel) and 8.6 mm/s for channel **347** (the 25×50 μm^2 channel). The oil-to-water volumetric ratio was 4:1 in each pair of oil and water inlets. The two channels (the branch channels) merged into a common channel **348** that had a 100×50 μm^2 cross-section. As shown in the figure, the larger plugs from the bigger channel are able to merge with the smaller plugs from the narrower channel even when they do not arrive at the junction at the same time. This is because lagging larger plugs are able to catch up with the leading smaller plugs once the plugs are in the common channel.

FIG. 33*c* depicts in-phase merging (i.e., plug merging upon simultaneous arrival of at least two plugs at a junction) of plugs of different sizes generated using different oil/water ratios at the two pairs of inlets. The oil streams were introduced into inlets **338**, **340**, while the aqueous streams were introduced into inlets **339**, **341**. The flow rate corresponding to the fluid stream through channel **349** resulting from a 1:1 oil-to-water volumetric ratio was 4.0 mm/s, while that through channel **350** corresponding to the 4:1 oil-to-water volumetric ratio was 6.9 mm/s. Each branch channel of the Y-shaped portion of the network (magnified view shown) had a dimension of 50×50 μm while the common channel **351** (the channel to which the branch channels merge) was 125×50 μm^2.

FIG. 33*d* illustrates defects (i.e., plugs that fail to undergo merging when they would normally merge under typical or ideal conditions) produced by fluctuations in the relative velocity of the two incoming streams of plugs. The oil streams were introduced into inlets **342**, **344**, while the aqueous streams were introduced into inlets **343**, **345**. In this experiment, the flow rate corresponding to the fluid stream through channel **352** resulting from a 1:1 oil-to-water volumetric ratio was 4.0 mm/s, while that through channel **353** corresponding to the 4:1 oil-to-water volumetric ratio was 6.9 mm/s. Each branch channel that formed one of the two branches of the Y-shaped intersection (magnified view shown) was 50×50 μm^2 while the common channel **354** (the channel to which the two branch channels merge) is 125×50 μm^2.

Example 5

Splitting Plugs Using a Constricted Junction

The splitting of plugs was investigated using a channel network with a constricted junction. In this case, the plugs split and flowed past the junction into two separate branch channels (in this case, branch channels are the channels to which a junction branches out) that are at a 180°-angle to each other (see FIGS. 34*a-c* each of which show a channel network viewed from the top). In these experiments, the outlet pressures, P_1 and P_2, past the constricted junction were varied such that either $P_1 \approx P_2$ (FIG. 34*b*) or $P_1 < P_2$ (FIG. 34*c*). Here, the relative pressures were varied by adjusting the relative heights of the channels that were under pressures P_1 and P_2. Since longer plugs tend to split more reliably, this branching point (or junction) was made narrower than the channel to elongate the plugs. FIG. 34*a* shows a schematic diagram of the channel network used in the experiment. The oil and water were introduced into inlets **3400** and **3401**, respectively. The oil-to-water ratio was 4:1 while the flow rate past the junction where the oil and water meet was 4.3 mm/s.

FIG. 34*b* is a microphotograph showing the splitting of plugs into plugs of approximately one-half the size of the initial plugs. The channels **3404**, which were rectangular, had a cross-section that measured 50×50 μm^2. The constricted section of the channel **3402** right next to the branching point measured 25×50 μm^2. The outlet pressures, P_1 and P_2, were about the same in both branch channels. Here, the plugs split into plugs of approximately the same sizes.

FIG. 34*c* is a microphotograph showing the asymmetric splitting of plugs (i.e., the splitting of plugs into plugs of different sizes or lengths) which occurred when $P_1 < P_2$. The microphotograph shows that larger plugs (somewhat rectangular in shape) flowed along the channel with the lower pressure P_1, while smaller plugs (spherical in shape) flowed along the channel with the higher pressure P_2. As in FIG. 34*b*, each of the channel **3405** cross-section measured 50×50 μm^2. The constricted section of the channel **3403** at the junction measured 25×50 μm^2.

Example 6

Splitting Plugs without Using a Constricted Junction

The splitting of plugs was investigated using a channel network without a constriction such as the one shown in FIGS. 35*b-c*. The channel network used was similar to that shown in FIG. 34(*a*) except that here the plugs split and flowed past the junction in two separate channels at a 90°-angle to each other (the plug flow being represented by arrows). The oil and aqueous streams (4:1 oil:aqueous stream ratio) were introduced into inlets **3500** and **3501**, respectively. An oil-only stream flowed through channel **3502**. All channels had a cross-section of 50×50 μm^2. The flow rate used was 4.3 mm/s. FIGS. 35*a-c*, which represent top views of a channel network, show that plugs behave differently compared to the plugs in Example 3 when they flow past a junction in the absence of a channel constriction, such as a constriction shown in FIGS. 35*b-c*. As FIG. 35*c* shows, when $P_1 < P_2$, the plugs remained intact after passing through the junction. Further, the plugs traveled along the channel that had the lower pressure (P_1 in FIG. 35*c*) while the intervening oil stream split at the junction. The splitting of the oil stream at the junction gives rise to a shorter separation between plugs flowing along the channel with pressure P_1 compared to the separation between plugs in the channel upstream of the branching point or junction.

Example 7

Monitoring Autocatalytic Reactions Using a Microfluidic System

FIG. 37 illustrates the design of an experiment involving chemical amplification in microfluidic devices according to the invention that involves an investigation of a stochastic autocatalytic reaction. This example illustrates how the devices of the present invention can be used to study the acid-sensitive autocatalytic reaction between $NaClO_2$ and NaS_2O_3. On the left side of the microfluidic network, a three-channel inlet introduces an aqueous stream through channel **3702**, an ester through channel **3701**, and an esterase through channel **3703**. Oil flowed through channels **3713**, **3714**. The reaction between ester and esterase yield plugs **3704** that contain a small amount of acid. On the right side of the microfluidic network, the five-channel inlet introduces $NaClO_2$ through inlet **3705**, an aqueous stream through inlet **3706**, a pH indicator through inlet **3707**, a second aqueous stream through inlet **3708**, and NaS_2O_3 through channel **3709**. A carrier fluid flows through channels **3713**, **3714**. Unstirred mixtures of $NaClO_2$ and NaS_2O_3 are highly

unstable and even a slight concentration fluctuation within that mixture leads to rapid decomposition. Thus, the plugs **3710** containing $NaClO_2/NaS_2O_3$ mixture must not only be quickly mixed but also promptly used after formation. In this proposed experiment, the curvy channels promote chaotic mixing. When a slightly acidic plug of the ester-esterase reaction is merged with a plug of an unstable $NaClO_2/NaS_2O_3$ mixture at the contact region **3712**, an autocatalytic reaction will generally be triggered. Upon rapid mixing of these two plugs, the resulting plugs **3711** become strongly acidic. The pH indicator introduced in the five-channel inlet is used to visualize this entire amplification process.

Example 8

Using Chemical Reactions as Highly Sensitive Autoamplifying Detection Elements in Microfluidic Devices

In one aspect according to the invention, a sequential amplification using controlled autocatalytic systems is used to amplify samples that contain single molecules of autocatalysts into samples containing a sufficiently high concentration of an autocatalyst such that the amplified autocatalyst can be detected with the naked eye can be detected with the naked eye. Although systems displaying stochastic behavior are expected to display high sensitivity and amplification, various autocatalytic systems can be used in accordance with the invention. A sequential amplification using the microfluidic devices according to the invention can be illustrated using a reaction that has been characterized analytically: the autocatalytic decomposition of violet bis[2-(5-bromo-pyridylazo)-5-(N-propyl-N-sulfopropyl-amino-phenolato] cobaltate, (Co(III)-5-Br-PAPS), upon oxidation with potassium peroxomonosulfate to produce colorless Co^{2+} ions. Here, the Co^{2+} ions serve as the autocatalyst (the order of autocatalysis, m, has not been established for this reaction).

$$\text{Co(III)-[5-Br-PAPS]}_{\text{reduced}}+HSO_5^- \rightarrow Co^{2+}+\text{[5-Br-PAPS]}_{\text{oxidized}}+HSO_4^- \quad (3)$$

Addition of small amounts of Co^{2+} to the violet mixture of (Co(III)-5-Br-PAPS and peroxomonosulfate produces an abrupt loss of color to give a colorless solution. The time delay before this decomposition depends on the amount of the Co^{2+} added to the solution. This reaction has been used to detect concentrations of Co^{2+} as low as about 1×10^{-10} mole/L. The reaction shows good selectivity in the presence of other ions (V(V), Cr(III), Cr(VI), Mn(II), Fe(II), Ni(II), Cu(II) and Zn(II)).

To use this reaction for amplification, a microfluidic network as shown in FIG. **38** is preferably used. An unstable solution of $\text{Co(III)-[5-Br-PAPS]}_{reduced}$ and peroxomonosulfate at pH=7 buffer in large plugs are preferably formed in a channel. These large plugs are preferably split in accordance with the invention into three different sizes of plugs. Preferably, the plug sizes are $(1\ \mu m)^3=10^{-15}$ L in the first channel; $(10\ \mu m)^3=10^{-12}$ L in the second channel; and $(100\ \mu m)^3=10^{-9}$ L in the third channel. A three-step photolithography is preferably used in the fabrication of masters for these microfluidic channels.

Example 9

Multi-Stage Chemical Amplification in Microfluidic Devices for Single Molecule Detection

FIG. **38** illustrates a method for a multi-stage chemical amplification for single molecule detection using microfluidic devices according to the invention. This example illustrates the use of an autocatalytic reaction between Co(III)-5-Br-PAPS (introduced through inlet **3803**) and $KHSO_5$ (introduced through inlet **3801**) in a pH=7 buffer (introduced through inlet **3802**) that is autocatalyzed by Co^{2+} ions. Oil streams are allowed to flow through channels **3804**, **3805**. This reaction mixture (contained in plugs **3811**) is unstable and decomposes rapidly (shown in red) when small amounts of Co^{2+} **3810** are added. Thus, this reaction mixture is preferably mixed quickly and used immediately. The reaction mixture is preferably transported through the network in $(1\ \mu m)^3$, $(10\ \mu m)^3$, $(100\ \mu m)^3$ size plugs. On the left side of the microfluidic network, the approximately 1 μm^3 plugs of the sample to be analyzed form at a junction of two channels (shown in green). The merging of plugs containing Co^{2+} ions and plugs containing the reaction mixture results in a rapid autocatalytic reaction. By using an amplification cascade in which larger and larger plugs of the reaction mixture are used for amplification, each Co^{2+} ion in a plug can be amplified to about 10^{10} Co^{2+} ions per plug. The result of amplification is visually detectable.

The $(10\ \mu m)^3$ plugs are preferably merged with larger $(100\ \mu m)^3$ plugs in the third channel to give approximately 4×10^{-8} mole/L solution of Co^{2+} ions. Autocatalytic decomposition in the approximately 10^{-9} L plugs will produce plugs **3809** with about 2.4×10^{10} Co^{2+} ions (4×10^{-5} mole/L). The flow rates in this system are preferably controlled carefully to control the time that plugs spend in each branch. The time provided for amplification is preferably long enough to allow amplification to substantially reach completion, but short enough to prevent or minimize slow decomposition.

Using different plug sizes is advantageous when merging plugs. Plugs with a size of about $(1\ \mu m)^3$ are preferably formed by flowing a sample containing about 3×10^{-9} mole/L Co^{2+} through channel **3806**. This reaction can be used to detect Co^{2+} at this, or lower, concentration (Endo et al., "Kinetic determination of trace cobalt(II) by visual autocatalytic indication," *Talanta,* 1998, vol. 47, pp. 349-353; Endo et al., "Autocatalytic decomposition of cobalt complexes as an indicator system for the determination of trace amounts of cobalt and effectors," *Analyst,* 1996, vol. 121, pp. 391-394). These plugs have a corresponding volume of about 10^{-15} L and carry just a few cobalt ions, on average about 1.8 ions per plug (corresponding to a Poisson distribution). These plugs **3810** are preferably merged with the $(1\ \mu m)^3$ plugs **3811** containing the Co(III)-5-Br-PAPS/peroxomonosulfate mixture (about 4×10^{-5} mole/L).

Upon autocatalytic decomposition of the complex, the number of Co^{2+} ions in the merged plug **3807** will increase by a factor of between about 10^4 to 1.2×10^4 Co^{2+} ions (2×10^{-5} mole/L in 2 μm^3). These plugs **3807** are preferably merged with the $(10\ \mu m)^3$ plugs **3811** containing the unstable mixture (about 4×10^{-5} mole/L). The concentration of Co^{2+} ions in these approximately 10^{-12} L plugs is preferably about 2×10^{-8} mole/L, which is sufficient to induce autocatalytic decomposition. The number of Co^{2+} ions will increase by a factor of between about 10^3 to about 2.4×10^7 ions/plug in plugs **3808**. The starting solution is dark violet ($\epsilon=9.8\times10^4$ L $mol^{-1}cm^{-1}$ for Co(III)-5-Br-PAPS). Channels are preferably designed to create an optical path through at least ten consecutive 100 μm plugs. These plugs will provide an approximately 1-mm long optical path, with absorbance of the starting 4×10^{-5} mole/L solution of about 0.4. This absorbance can be detected by an on-chip photodetector or with the naked eye. If Co^{2+} is present in the sample solution, an autocatalytic cascade will result in the disappearance of the color of the reaction mixture.

At low concentrations of Co^{2+} in the sample, the system may show stochastic behavior, that is, not every Co^{2+} ion would give rise to a decomposition cascade. However, the attractive feature of this system is that thousands of tests can be carried out in a matter of seconds, and statistics and averaging can be performed. Preferably, a sequence of controlled autocatalytic amplification reactions leads to a visual detection of single ions.

Example 10

Enzyme Kinetics

A microfluidic chip according to the invention was used to measure millisecond single-turnover kinetics of ribonuclease A (RNase A; EC 3.1.27.5), a well-studied enzyme. Submicroliter sample consumption makes the microfluidic chip especially attractive for performing such measurements because they require high concentrations of both the enzyme and the substrate, with the enzyme used in large excess.

The kinetic measurements were performed by monitoring the steady-state fluorescence arising from the cleavage of a fluorogenic substrate by RNase A as the reaction mixture flowed down the channel (see FIG. **40**(*a*)). In FIG. **40**, a substrate, buffer, and RNase A were introduced into inlets **401**, **401**, and **403**, respectively. A carrier fluid flowed through channel **404**. The amount of the product at a given reaction time t [s] was calculated from the intensity of fluorescence at the corresponding distance point d [m] (t=d/U where U=0.43 m/s is the velocity of the flow). The channels were designed to wind so that rapid chaotic mixing was induced, and were designed to fit within the field of view of the microscope so that the entire reaction profile could be measured in one spatially resolved image. Selwyn's test (Duggleby, R. G., *Enzyme Kinetics and Mechanisms, Pt D*; Academic Press: San Diego, 1995, vol. 249, pp. 61-90; Selwyn, M. J. *Biochim. Biophys. Acta,* 1965, vol. 105, pp. 193-195) was successfully performed in this system to establish that there were no factors leading to product inhibition or RNase A denaturation.

The flow rate of the stock solution of 150 μM of RNase A was kept constant to maintain 50 μM of RNase A within the plugs. By varying the flow rates of the buffer and substrate (see FIG. **45**), progress curves were obtained for eight different substrate concentrations. For $[E]_o >> [S]_o$, the simple reaction equation is $[P]_t = [S]_o(1-\mathrm{Exp}(-kt))$, where $[E]_o$ is the initial enzyme concentration, $[S]_o$ is the initial substrate concentration, $[P]_t$ is the time-dependent product concentration and k [s^{-1}] is the single-turnover rate constant. To obtain a more accurate fit to the data, the time delay Δt_n required to mix a fraction of the reaction mixture f_n was accounted for.

An attractive feature of the microfluidic system used is that the reaction mixture can be observed at time t=0 (there is no dead-time). This feature was used to determine Δt_n and f_n in this device by obtaining a mixing curve using fluo-4/Ca^{2+} system as previously described (Song et al., *Angew. Chem. Int. Ed.* 2002, vol. 42, pp.

$$[P]_t = \sum_n f_n[S]_0(1 - \mathrm{Exp}(-k(t - \Delta t_n)))$$

768-772), and correcting for differences in diffusion constants (Stroock et al., *Science,* 2002, vol. 295, pp. 647-651). All eight progress curves gave a good fit with the same rate constant of 1100±250 s^{-1}. The simpler theoretical fits gave indistinguishable rate constants. These results are in agreement with previous studies, where cleavage rates of oligonucleotides by ribonucleases were shown to be ~10^3 s^{-1}.

Thus, this example demonstrates that millisecond kinetics with millisecond resolution can be performed rapidly and economically using a microchannel chip according to the invention. Each fluorescence image was acquired for 2 s, and required less than 70 nL of the reagent solutions. These experiments with stopped-flow would require at least several hundreds of microliters of solutions. Volumes of about 2 μL are sufficient for ~25 kinetic experiments over a range of concentrations. Fabrication of these devices in PDMS is straightforward (McDonald, et al., *Accounts Chem. Res.* 2002, vol. 35, pp. 491-499) and no specialized equipment except for a standard microscope with a CCD camera is needed to run the experiments. This system could serve as an inexpensive and economical complement to stopped-flow methods for a broad range of kinetic experiments in chemistry and biochemistry.

Example 11

Kinetics of RNA Folding

The systems and methods of the present invention are preferably used to conduct kinetic measurements of, for example, folding in the time range from tens of microseconds to hundreds of seconds. The systems and methods according to the invention allow kinetic measurements using only small amounts of sample so that the folding of hundreds of different RNA mutants can be measured and the effect of mutation on folding established. In one aspect according to the invention, the kinetics of RNA folding is preferably measured by adding Mg^{2+} to solutions of previously synthesized unfolded RNA labeled with FRET pairs in different positions. In accordance with the invention, the concentrations of Mg^{2+} are preferably varied in the 0.04 to 0.4 μM range by varying the flow rates (see, for example, FIGS. **25**(*a*)-(*c*)) to rapidly determine the folding kinetics over a range of conditions. The ability to integrate the signal over many seconds using the steady-flow microfluidic devices according to the invention can further improve sensitivity.

As shown in FIGS. **25**(*a*)-(*c*), the concentrations of aqueous solutions inside the plugs can be controlled by changing the flow rates of the aqueous streams. In FIGS. **25**(*a*)-(*c*), aqueous streams were introduced into inlets **251**-**258** wherein flow rates of about 0.6 μL/min for the two aqueous streams and 2.7 μL/min was used for the third stream. The stream with the 2.7 μL/min volumetric flow rate was introduced in the left, middle, and right inlet in FIGS. **25**(*a*)-(*c*), respectively. A carrier fluid in the form of perfluorodecaline was introduced into channel **259**, **260**, **261**. The corresponding photographs on each of the right side of FIGS. **25**(*a*)-(*c*) illustrate the formation of plugs with different concentrations of the aqueous streams. The various shadings inside the streams and plugs arise from the use of aqueous solutions of food dyes (red/dark and green/light), which allowed visualization, and water were used as the three streams, the darker shading arising mainly from the red dye color while the lighter shading arising mainly from the green dye color. The dark stream is more viscous than the light stream, therefore it moves slower (in mm/s) and occupies a larger fraction of the channel at a given volumetric flow rate (in μL/min).

Example 12

Nanoparticle Experiments with and without Plugs

FIG. **15** illustrates a technique for the synthesis of CdS nanoparticles **155**. In one experiment, nanoparticles were

formed in a microfluidic network. The channels of the microfluidic device had 50 μm×50 μm cross-sections. A fluorinated carrier-fluid (10:1 v/v mixture of perfluorohexane and 1H,1H,2H,2H-perfluorooctanol) was flowed through the main channel at 15 μm min^{-1}. An aqueous solution, pH=11.4, of 0.80 mM $CdCl_2$ and 0.80 mM 3-mercaptopropionic acid was flowed through the left-most inlet channel **151** at 8 μL min^{-1}. An aqueous solution of 0.80 mM polyphosphates $Na(PO_3)_n$ was flowed through the central inlet channel **152** at 8 μL min^{-1}, and an aqueous solution of 0.96 mM Na_2S was flowed through the right-most inlet channel **153** at 8 μL min^{-1}. To terminate the growth of nanoparticles, an aqueous solution of 26.2 mM 3-mercaptopropionic acid, pH=12.1, was flowed through the bottom inlet of the device **157** at 24 μM min^{-1}. FIG. **15** shows various regions or points along the channel corresponding to regions or points where nucleation **154**, growth **158**, and termination **156** occurs. Based on the UV-VIS spectrum, substantially monodisperse nanoparticles formed in this experiment.

Nanoparticles were also formed without microfluidics. Solutions of $CdCl_2$, polyphosphates, Na_2S, and 3-mercaptopropionic acid, identical to those used in the microfluidics experiment, were used. 0.5 mL of the solution of $CdCl_2$ and 3-mercaptopropionic acid, 0.5 mL of polyphosphates solution, and 0.5 mL of Na_2S solution were combined in a cuvette, and the cuvette was shaken by hand. Immediately after mixing, 1.5 mL of 26.2 mM 3-mercaptopropionic acid was added to the reaction mixture to terminate the reaction, and the cuvette was again shaken by hand. Based on the UV-VIS spectrum, substantially polydisperse nanoparticles formed in this experiment.

Example 13

Crystallization

Networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The PDMS was purchased from Dow Corning Sylgard Brand 184 Silicone Elastomer. The PDMS devices were sealed after plasma oxidation treatment in Plasma Prep II (SPI Supplies). The devices were rendered hydrophobic by baking the devices at 120° C. for 2-4 hours. Microphotographs were taken with a Leica MZ12.5 stereomicroscope and a SPOT Insight color digital camera (Model#3.2.0, Diagnostic Instruments, Inc.). Lighting was provided from a Machine Vision Strobe X-strobe X1200 (20 Hz, 12 μF, 600V, Perkin Elmer Optoelectronics). To obtain an image, the shutter of the camera was opened for 1 second and the strobe light was flashed once with the duration of approximately 10 μs.

Aqueous solutions were pumped using 10 μl or 50 μl Hamilton Gastight syringes (1700 series). Carrier-fluid was pumped using 50 μl Hamilton Gastight syringes (1700 series). The syringes were attached to microfluidic devices by means of Teflon tubing (Weico Wire & Cable Inc., 30 gauge). Syringe pumps from Harvard Apparatus (PHD 2000) were used to inject the liquids into microchannels.

A. Microbatch Crystallization in a Microfluidic Channel

Microbatch crystallization conditions can be achieved. This experiment shows that size of plugs can be maintained and evaporation of water prevented. In this case, the PDMS device has been soaked in water overnight before the experiment in order to saturate PDMS with water. The device was kept under water during the experiment. During the experiment, the flow rates of carrier-fluid and NaCl solution were 2.7 μL/min and 1.0 μL/min, respectively. The flow was stopped by cutting off the Teflon tubing of both carrier-fluid and NaCl solution.

FIG. **16** shows a schematic illustration of a microfluidic device according to the invention and a microphotograph of plugs of 1M aqueous NaCl sustained in oil. The carrier-fluid is perfluorodecaline with 2% 1H,1H,2H,2H-perfluorooctanol. Inside a microchannel, plugs showed no appreciable change in size.

B. Vapor Diffusion Crystallization in Microchannels: Controlling Evaporation of Water from Plugs

This experiment shows that evaporation of water from plugs can be controlled by soaking devices in water for shorter amounts of time or not soaking at all. The rate of evaporation can be also controlled by the thickness of PDMS used in the fabrication of the device. Evaporation rate can be increased by keeping the device in a solution of salt or other substances instead of keeping the device in pure water.

The plug traps are separated by narrow regions that help force the plugs into the traps.

In this experiment, a composite glass/PDMS device was used. PDMS layer had microchannel and a microscopy slide (Fisher, 35x50-1) was used as the substrate. Both the glass slide and the PDMS were treated in plasma cleaner (Harrick) then sealed. The device was made hydrophobic by first baking the device at 120° C. for 2-4 hours then silanizing it by (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.).

During the experiment, a flow of carrier-fluid at 1.0 μL/min was established, then flow of aqueous solution was established at a total rate of 0.9 μL/min. Plug formation was observed inside the microchannel. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **41** shows a microphotograph (middle and right side) of the water plugs region of the microfluidic network. FIG. **41**(*b*)-(*c*) show the plugs at time t=0 and t=2 hours, respectively. Red aqueous solution is 50% waterman red ink in 0.5 M NaCl solution. Ink streams were then introduced into inlets **411**, **412**, **413**. An oil stream flowed through channel **414**. The carrier-fluid is FC-3283 (3M Fluorinert Liquid) with 2% 1H,1H,2H,2H-perfluorodecanol. This photograph demonstrates that the evaporation of water through PDMS can be controlled, and thus the concentration of the contents inside the drops can be increased (this is equivalent to microbatch crystallization). FIG. **41**(*a*) shows a diagram of the microfluidic network.

C. Controlling Shape and Attachment of Water Plugs

During the experiment, a flow of carrier fluid at 1.0 μL/min was established, then flow of aqueous solution was established at a total rate of 2.1 μL/min. Plug formation was observed inside the microchannel. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **39** shows a diagram (left side) of a microfluidic network according to the invention. Aqueous streams were introduced into inlets **3901**, **3902**, **3903** while an oil stream flowed through channel **3904**. FIG. **39** also shows a microphotograph (right side) of the water plug region of the microfluidic network. This image shows water plugs attached to the PDMS wall. This attachment occurs when low concentrations of surfactant, or less-effective surfactants are used. In this case 1H,1H,2H,2H-perfluorooctanol is less effective than 1H,1H,2H,2H-perfluorodecanol. In this experiment the oil is

FC-3283 (3M Fluorinert Liquid) with 2% 1H,1H,2H,2H-perfluorooctanol as the surfactant.

D. Examples of Protein Crystallization

During the experiment, a flow of oil at 1.0 μL/min was established. Then the flow of water was established at 0.1 μL/min. Finally flows of lysozyme and precipitant were established at 0.2 μL/min. Plug formation was observed inside the microchannel. The flow of water was reduced to zero after the flow inside the channel became stable. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **36** depicts lysozyme crystals grown in water plugs in the wells of the microfluidic channel. Lysozyme crystals started to appear inside aqueous plugs both inside and outside plug traps in approximately 10 minutes. The image of the three crystals in FIG. **36** was taken 1 hour after the flow was stopped. Lysozyme crystals appear colored because they were observed under polarized light. This is common for protein crystals.

The left side of FIG. **36** is a diagram of a microfluidic network according to the invention while the right side is microphotograph of the crystals formed in plugs in the microfluidic network. A precipitant, lysozyme, and water were introduced into inlets **3601**, **3602**, and **3603**, respectively. Oil was flowed through channel **3604**. The lysozyme solution contains 100 mg/ml lysozyme in 0.05 M sodium acetate (pH 4.7); the precipitant solution contains 30% w/v PEG (M.W. 5000), 1.0 M NaCl and 0.05 M sodium acetate (pH 4.7); The carrier-fluid is FC-3283 (3M Fluorinert Liquid) with 10% 1H,1H,2H,2H-perfluoro-octanol. The microchannel device was soaked in FC-3283/H_2O for one hour before experiment.

FIG. **32** shows that plug traps are not required for formation of crystals in a microfluidic network. FIG. **32** shows a diagram (left side) of the microfluidic network. A precipitant was introduced into inlet **321**, lysozyme was introduced into inlet **322**, and an aqueous stream was introduced into inlet **323**. Oil was flowed through channel **324**. FIG. **32** also shows microphotographs (middle and right side) of lysozyme crystals grown inside the microfluidic channel. The experimental condition is same as in FIG. **36**.

Example 14

Oil-Soluble Surfactants for Charged Surfaces

In accordance with the invention, neutral surfactants that are soluble in perfluorinated phases are preferably used to create positively and negatively-charged interfaces. To create charged surfaces, neutral surfactants that can be charged by interactions with water, e.g., by protonation of an amine or a guanidinium group (FIG. **24**B), or deprotonation of a carboxylic acid group (FIG. **24**C), are preferably used. Preferably, charged surfaces are used to repel, immobilize, or stabilize charged biomolecules. Negatively charged surfaces are useful for handling DNA and RNA without surface adsorption. Preferably, both negatively and positively-charged surfaces are used to control the nucleation of protein crystals. Many neutral fluorinated surfactants with acidic and basic groups ($RfC(O)OH$, $Rf(CH_2)_2NH_2$, $Rf(CH_2)_2C(NH)NH_2$) are available commercially (Lancaster, Fluorochem, Aldrich).

To synthesize oligoethylene-glycol terminated surfactants, a modification and improvement of a procedure based on the synthesis of perfluoro non-ionic surfactants is preferably used. In one aspect, the synthesis relies on the higher acidity of the fluorinated alcohol to prevent the polycondensation of the oligoethylene glycol. The modified synthesis uses a selective benzylation of one of the alcohol groups of oligoethylene glycol, followed by activation of the other alcohol group as a tosylate. A Williamson condensation is then performed under phase transfer conditions followed by a final deprotection step via catalytic hydrogenation using palladium on charcoal.

Example 15

Formation of Plugs in the Presence of Fluorinated Surfactants and Surface Tension

The surface tension of the oil/water interface has to be sufficiently high in order to maintain a low value of capillary number, C.n. The fluorosurfactant/water interfaces for water-insoluble fluorosurfactants have not been characterized, but these surfactants are predicted to reduce surface tension similar to that observed in a system involving Span on hexane/water interface (about 20 mN/m). The surface tensions of the aqueous/fluorous interfaces are preferably measured in the presence of fluorosurfactants using the hanging drop method. A video microscopy apparatus specifically constructed for performing these measurements has been used to successfully characterize interfaces. FIG. **24** illustrates the synthesis of fluorinated surfactants containing perfluoroalkyl chains and an oligoethylene glycol head group.

Example 16

Forming Gradients by Varying Flow Rates

FIG. **42** shows an experiment involving the formation of gradients by varying the flow rates. In this experiment, networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The width and height of the channel were both 50 μm. 10% 1H,1H,2H,2H-perfluorodecanol in perfluoroperhydrophenanthrene was used as oil. Red aqueous solution prepared from 50% waterman red ink in 0.5 M NaCl solution was introduced into inlet **421**. The oil flowed through channel **424** at 0.5 μl/min. Aqueous streams were introduced into inlets **422**, **423**. To generate the gradient of ink in the channel, the total water flow rate was gradually increased from 0.03 μl/min to 0.23 μl/min in 20 seconds at a ramp rate of 0.01 μl/min per second. At the same time, ink flow rate was gradually decreased from 0.25 μl/min to 0.05 μl/min in 20 seconds at a ramp rate of −0.01 μl/min per second. The total flow rate was constant at 0.28 μl/min. The established gradient of ink concentration inside the plugs can be clearly seen from FIG. **42**: the plugs further from the inlet are darker since they were formed at a higher ink flow rate.

Example 17

Lysozome Crystallization Using Gradients

FIG. **43** illustrates an experiment involving the formation of lysozome crystals using gradients. The channel regions **435**, **437** correspond to channel regions with very low precipitant concentration while channel region **436** corresponds to optimal range of precipitant concentration. In this experiment, networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The width of the channel was 150 μm and the height was 100 μm. 10% 1H,1H,2H,2H-perfluorodecanol in perfluoroperhydrophenanthrene was used as oil.

During the experiment, a flow of oil through channel **434** at 1.0 μl/min was established. Then the flow of water introduced through inlet **432** was established at 0.2 μl/min. The flows of lysozyme introduced through inlet **431** and precipitant introduced through inlet **433** were established at 0.2 μl/min. Plugs formed inside the channel. To create the gradient, water flow rate was first gradually decreased from 0.35 μl/min to 0.05 μl/min over 45 seconds at a ramp rate of (−0.01 μl/min per 1.5 seconds), then increased back to 0.35 μl/min in 45 seconds at a ramp rate of (0.01 μl/min per 1.5 seconds). At the same time, precipitant flow rate was gradually increased from 0.05 μl/min to 0.35 μl/min in 45 seconds at a ramp rate of (0.01 μl/min per 1.5 seconds), then decreased to 0.05 μl/min in 45 seconds at a ramp rate of (−0.01 μl/min per 1.5 seconds). The flow was stopped by pulling out the inlet tubing immediately after water and precipitant flow rates returned to the starting values. The plugs created in this way contained constant concentration of the protein but variable concentration of the precipitant: the concentration of the precipitant was lowest in the beginning and the end of the channel, and it peaked in the middle of the channel (the center row). Only the plugs in the middle of the channel have the optimal concentration of precipitant for lysozyme crystallization, as confirmed by observing lysozyme crystals inside plugs in the center row. Visualization was performed under polarized light. Preferably, all flow rates would be varied, not just the precipitant and water.

Example 18

Lysozyme Crystallization in Capillaries Using the Microbatch Analogue Method

To grow lysozyme crystal inside plugs within capillaries, a 10 μl Hamilton syringe was filled with 100 mg/ml lysozyme in 0.05 M NaAc buffer (pH4.7) and another 10 μl Hamilton syringe was filled with 30% (w/v) MPEG 5000 with 2.0 M NaCl in 0.05 M NaAc buffer (pH4.7) as precipitant. A 50 μl Hamilton syringe filled with PFP (10% PFO) was the oil supply. All three syringes were attached to the PDMS/capillary device and driven by Harvard Apparatus syringe pumps (PHD2000). The capillary has an inner diameter of 0.18 mm and outer diameter of 0.20 mm. Oil flow rate was 1.0 μl/min and both lysozyme and precipitant solution were at 0.3 μl/min. The channel was filled with oil first. Protein and precipitant streams converged immediately before entering the channel to form plugs. After the capillary (Hampton Research) was filled with the plugs containing lysozyme, the flows were stopped. The capillary was disconnected from the PDMS device, sealed with wax and stored in an incubator (18° C.). A lysozyme crystal appeared within an hour and was stable for at least 14 days without change of size or shape (FIG. **47**A).

Example 19

Thaumatin Crystallization in Capillaries Using the Microbatch Analogue Method

Experiment 1. A 10 μl Hamilton syringe was filled with 50 mg/ml thaumatin in 0.1 M ADA buffer (pH 6.5) and another 10 μl Hamilton syringe was filled with 1.5 M NaK Tatrate in 0.1 M HEPES (pH 7.0). A 50 μl Hamilton syringe filled with PFP (10% PFO) was the oil supply. All three syringes were attached to the PDMS/capillary device and driven by Harvard Apparatus syringe pumps (PHD2000). The capillary has an inner diameter of 0.18 mm and outer diameter of 0.20 mm. Oil flow rate was 1.0 μl/min and both thaumatin and precipitant solution were at 0.3 μl/min. The channel was filled with oil first. Protein and precipitant streams were mixed immediately before entering the channel to form plugs. After the capillary (Hampton Research) was filled with protein plugs, the flows were stopped. The capillary was cut from the PDMS device, sealed by wax and stored in an incubator (18° C.). The thaumatin crystal appeared in 2-3 days and was stable for at least 45 days without size or shape change (FIG. **47**B). Some thaumatin crystals grew at the interface of protein solution and oil, while others appeared to attach to the capillary wall.

Experiment 2. Thaumatin crystals were grown inside a capillary tube using 50 mg/mL thaumatin in 0.1M pH 6.5 ADA buffer and a precipitant solution of 1M Na/K tartrate in a 0.1M pH 7.5 HEPES buffer. Protein and precipitant solutions were mixed in a 1.4:1 protein:precipitant ratio. A fluorinated carrier fluid was a saturated solution of FSN surfactant in FC3283. The capillary was incubated at 18 degrees C. Tetragonal crystals appeared within 5 days (FIG. **48**A, B). X-ray diffraction was performed at BioCARS station 14BM-C at the Advanced Photon Source at Argonne National Laboratory. Beam wavelength was 0.9 A. The final length of a single crystal was estimated at 100-150 microns.

Capillaries were cut to the appropriate length without disturbing crystal-containing plugs, resealed using capillary waz, and mounted on clay-tipped cryoloop holders at a distance of 12+/−5 mm from base to crystal. The holder was placed on the x-ray goniometer. Crystals were centered on the beam. Snapshots were taken using 10 second (thaumatin) exposures. Distance from sample to detector was 150 mm. Diffraction to better than 2.2 A was obtained.

Example 20

Vapor Diffusion Protein Crystallization in Capillaries by an Alternating Droplet System

The principle of transferring water inside a capillary from one set of plugs to another set of plugs is illustrated in FIG. **50**. Briefly, a 10 μl Hamilton syringe was filled with 0.01 $Fe(SCN)_3$ and another 10 μl Hamilton syringe was filled with 0.1 M $Fe(SCN)_3$ with 2.5 M KNO_3. Two 50 μl Hamilton syringes were filled with FMS-121 (Gelest, Inc) (saturated with PFO), which provided the oil supply. All four syringes were attached to the PDMS/capillary device and driven by Harvard Apparatus syringe pumps (PHD2000). The capillary has an inner diameter of 0.18 mm and outer diameter of 0.20 mm. One of the oil inlet channels was between the two aqueous inlets channels to separate the two aqueous streams when forming the alternating plugs. This oil inlet channel was vertical to the main channel and had a flow rate of 2.0 μl/min. The other oil inlet channel had a flow rate of 1.0 μl/min and was parallel to the main channel. Both of the aqueous solutions had a flow rate of 0.5 μl/min. After establishing alternating aqueous droplet streams in the capillary, the flows were stopped, and the capillary was disconnected from the PDMS device, sealed with wax and stored in an incubator at 18° C. The size and color change of the plugs were monitored with a Leica microscope (MZ125) having a color CCD camera (SPOT Insight, Diagnostic Instruments, Inc.).

Following the stoppage of flow and sealing of the capillary tube, plugs containing 0.01 M $Fe(SCN)_3$ in water were yellow, while those containing 0.1 M $Fe(SCN)_3$ and 2.5 M KNO_3 in water were red (FIG. **50**A). However, FIG. **50**B shows that after 5 days, the yellow plugs were reduced in size and were more concentrated, while the red plugs increased in size and were more diluted. This demonstration reflects vapor diffusion conditions in the capillary tube that are predicted to

facilitate protein crystallization. This technique can be further adapted to other applications requiring concentration of reagents, such as proteins.

Alternating plugs from two different aqueous solutions may be generated in accordance with several representative geometries as set forth in FIG. **51**. In principle, the same oil or different oils may be used in the two oil inlets. One scheme for generating alternating plugs from two different aqueous solutions is depicted in FIG. **51**A. In this case, one 10 μl Hamilton syringe was filled with 0.1 $Fe(SCN)_3$, another with 1.5 M NaCl. Two 50 μl Hamilton syringes filled with PFP (with 10% PFO) provided the oil supply. All four syringes were attached to the PDMS device and driven by Harvard Apparatus syringe pumps (PHD2000). Alternatively, multiple solutions can be co-introduced together in each of the two aqueous channels as depicted in FIG. **51**B. In each of these two cases one of the oil inlet channels was between the two aqueous inlet channels. This oil inlet channel was used to separate the two aqueous streams into alternating plugs and was vertical to the main channel, having a flow rate of 2.0 μl/min. The other oil inlet channel was parallel to the main channel and had a flow rate of 1.0 μl/min. Each of the two aqueous solutions had flow rates of 0.5 μl/min. Alternating plugs were found to form in the channel (FIG. **51**C).

FIG. **52** illustrates another example of generating alternating plugs from two different aqueous solutions. In this case, one 10 μl Hamilton syringe was filled with 0.1 $Fe(SCN)_3$, the other with 1.5 M NaCl. Two 50 μl Hamilton syringes filled with FMS-121 (saturated with PFO) provided the oil supply. All four syringes were attached to the device and driven by Harvard Apparatus syringe pumps (PHD2000). One of the oil inlet channels was between the two aqueous inlet channels and was used to separate the two aqueous streams prior to formation of alternating plugs (FIG. **52**A). This oil inlet channel was vertical to the main channel and had a flow rate of 1.5 μl/min. The other oil stream had a flow rate of 1.5 μl/min and was parallel to the main channel. Each of the two aqueous solutions had flow rates of 0.5 μl/min. Alternating plugs were found to form in the channel (FIG. **52**B).

Other geometries that can support the formation of alternating plugs are depicted in FIG. **53**. Importantly, the flow rates of solutions A and B may be changed in a correlated fashion (FIG. **54**). Thus, when the flow rate of solution A_1 is increased and solution A_2 is decreased, the flow rate of solutions B_1 is also increased and solution B_2 is also decreased. This principle, depicted in FIG. **54**, is useful for maintaining a constant difference in salt concentration between the plugs of stream A and stream B to ensure that transfer from all plugs A to all plugs B occurs at a constant rate.

FIG. **54** provides a schematic illustration of a device for preparing plugs of varying protein concentrations where the flow rates of the A and B streams change in a correlated fashion. In this example, A_1 through A_3 are for protein solution, buffer and precipitants, such as PEG or salts. Highly concentrated salt solutions are injected through B_1~B_3. The flow rate ratio of inlet A_1 to that of B_i (i=1~3) is maintained constant. Therefore all of the protein plugs will shrink at a rate similar to the salt plugs.

FIG. **54** shows that if the flow rates of corresponding A and B streams are changed in a correlative fashion, the composition of plugs B will reflect the composition of plugs A. Therefore, one can incorporate markers into the B stream plugs to serve as a code for the plugs in the A stream. In other words, absorption/fluorescent dyes or x-ray scattering/absorbing materials can be incorporated in markers in the B streams to facilitate optical or x-ray-mediated quantification so as to provide a read out of relative protein and precipitant concentrations in the A streams. This approach can provide a powerful means for optimizing crystallization conditions for subsequent scale-up experiments.

We claim:

1. A method for conducting an autocatalytic reaction in plugs in a microfluidic system, comprising the steps of:

providing the microfluidic system comprising at least two channels having at least one junction;

flowing an aqueous fluid containing at least one substrate molecule and reagents for conducting an autocatalytic reaction through a first channel of the at least two channels;

flowing an oil through the second channel of the at least two channels;

forming at least one plug of the aqueous fluid containing the at least one substrate molecule and reagents by partitioning the aqueous fluid with the flowing oil at the junction of the at least two channels, the plug being substantially surrounded by an oil flowing through the channel, wherein the at least one plug comprises at least one substrate molecule and reagents for conducting an autocatalytic reaction with the at least one substrate molecule; and

providing conditions suitable for the autocatalytic reaction in the at least one plug such that the at least one substrate molecule is amplified.

2. The method of claim **1**, wherein the at least one substrate molecule is a single biological molecule.

3. The method of claim **2**, wherein the at least one substrate molecule is DNA and the autocatalytic reaction is a polymerase-chain reaction.

4. The method of claim **1**, wherein the providing step includes heating.

5. The method of claim **1**, further comprising the step of providing a detector to detect, analyze, characterize, or monitor one or more properties of the autocatalytic reaction during and/or after it has occurred.

6. The method of claim **1**, wherein the oil is fluorinated oil.

7. The method of claim **1**, wherein the carrier fluid further comprises a surfactant.

8. The method of claim **7**, wherein the surfactant is fluorinated surfactant.

9. The method of claim **1**, wherein the at least one plug is a merged plug.

10. The method of claim **1**, further comprising trapping the at least one plug for a period of time during or after the reaction in an expansion portion in the one or more channels.

11. The method of claim **1**, wherein the at least one plug is substantially spherical in shape.

12. The method of claim **1**, further comprising a mixing step, wherein the mixing step occurs via a special design of the at least one channel of the at least two channels below the junction.

13. The method of claim **12**, wherein the special design of the at least one channel comprises periodic or aperiodic turns and relevant parameters.

14. The method of claim **13**, wherein the relevant parameters are selected from the group comprising channel width, period, radius of curvature, and sequence of turns based on the direction of the turns.

* * * * *

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Ismagilov et al.

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(45) **Date of Patent: *Dec. 11, 2012**

(54) **METHOD FOR CONDUCTING REACTIONS INVOLVING BIOLOGICAL MOLECULES IN PLUGS IN A MICROFLUIDIC SYSTEM**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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Related U.S. Application Data

(63) Continuation of application No. 12/777,099, filed on May 10, 2010, which is a continuation of application No. 10/765,718, filed on Jan. 26, 2004, now Pat. No. 7,901,939, which is a continuation-in-part of application No. 10/434,970, filed on May 9, 2003, now Pat. No. 7,129,091.

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(51) **Int. Cl.**
C12Q 1/68 (2006.01)

(52) **U.S. Cl.** **435/6.12**; 435/6.19; 436/535; 436/164; 436/172

(58) **Field of Classification Search** ..... None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,960,187	A	6/1976	Stock et al.	
4,853,336	A	8/1989	Saros et al.	
4,963,498	A	10/1990	Hillman et al.	
5,185,099	A	2/1993	Delpuech et al.	
5,739,036	A	4/1998	Parris	
5,872,010	A	2/1999	Karger et al.	
5,887,755	A	3/1999	Hood, III	
5,997,636	A	12/1999	Gamarnik et al.	117/70
6,118,849	A	9/2000	Tanimori et al.	
6,130,098	A	10/2000	Handique et al.	
6,140,053	A	10/2000	Köster	435/6

(Continued)

FOREIGN PATENT DOCUMENTS

CN 2482070 3/2002

(Continued)

OTHER PUBLICATIONS

Pollack et al. "Electrowetting-based actuation of droplets for integrated microfluidics†", Lab Chip, 2002, v. 2, pp. 96-101.*

(Continued)

Primary Examiner — Yelena G Gakh
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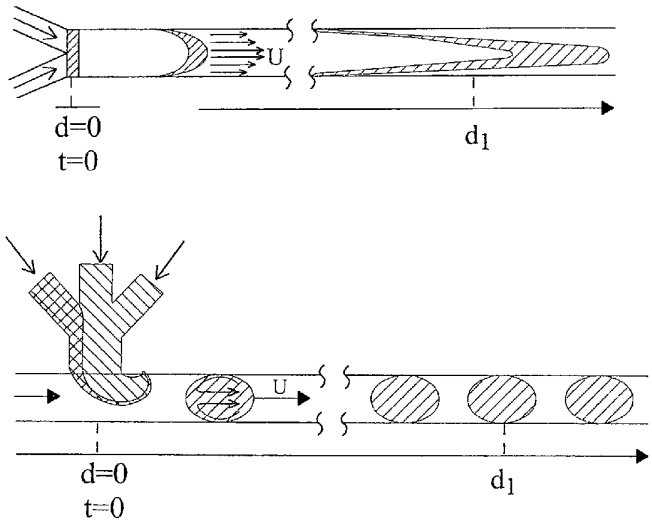
(57) **ABSTRACT**

The present invention provides microfabricated substrates and methods of conducting reactions within these substrates. The reactions occur in plugs transported in the flow of a carrier-fluid.

13 Claims, 63 Drawing Sheets



U.S. PATENT DOCUMENTS

6,180,372	B1	1/2001	Franzen	435/91.1
6,296,673	B1	10/2001	Santarsiero et al.	
6,409,832	B2	6/2002	Weigl et al.	
6,569,631	B1	5/2003	Pantoliano et al.	
6,630,006	B2	10/2003	Santarsiero et al.	
6,656,267	B2	12/2003	Newman	
6,766,817	B2	7/2004	Silva	
6,790,328	B2	9/2004	Jacobson et al.	
6,797,056	B2	9/2004	David	
6,872,250	B2	3/2005	David et al.	
7,041,481	B2	5/2006	Anderson et al.	
7,129,091	B2	10/2006	Ismagilov et al.	
7,156,917	B2	1/2007	Moriyama et al.	
7,294,503	B2	11/2007	Quake et al.	
2001/0048900	A1	12/2001	Bardell et al.	
2002/0008029	A1	1/2002	Williams et al.	204/453
2002/0012971	A1	1/2002	Mehta	435/91.2
2002/0017464	A1	2/2002	Parce et al.	204/601
2002/0022261	A1	2/2002	Anderson et al.	435/287.2
2002/0058332	A1	5/2002	Quake et al.	
2002/0067800	A1	6/2002	Newman et al.	
2003/0061687	A1	4/2003	Hansen et al.	
2003/0064414	A1	4/2003	Benecky et al.	
2003/0229376	A1	12/2003	Sandhu	
2004/0005582	A1	1/2004	Shipwash	
2004/0037813	A1	2/2004	Simpson et al.	
2004/0136497	A1	7/2004	Meldrum et al.	
2004/0181131	A1	9/2004	Maynard et al.	
2004/0224419	A1	11/2004	Zheng et al.	436/69
2004/0258203	A1	12/2004	Yamano et al.	
2005/0170431	A1	8/2005	Ibrahim et al.	
2006/0094119	A1	5/2006	Ismagilov et al.	
2007/0026439	A1	2/2007	Faulstick et al.	
2009/0021728	A1	1/2009	Heinz et al.	
2009/0060797	A1	3/2009	Mathies et al.	
2010/0022414	A1	1/2010	Link et al.	

FOREIGN PATENT DOCUMENTS

EP	0 816 837		1/1998
EP	0 912 238	B1	10/2001
GB	2 097 692	A	11/1982
WO	WO 84/02000		5/1984
WO	WO 97/29508		8/1997
WO	WO 98/02237		1/1998
WO	WO 98/52691		11/1998
WO	WO 00/21666		4/2000
WO	WO 01/12327	A1	2/2001
WO	WO 01/64332	A1	9/2001
WO	WO 01/77683	A1	10/2001
WO	WO 02/12856	A1	2/2002
WO	WO 02/23163	A1	3/2002
WO	WO 02/25243	A1	3/2002
WO	WO 03/044221	A1	5/2003
WO	WO2004/038363		5/2004
WO	PCT/US06/09175		3/2006

OTHER PUBLICATIONS

Burns et al. "Microfabricated structures for integrated DNA analysis", Proc. Natl. Acad. Sci. USA, May 1996, vol. 93, pp. 5556-5561.*

Gu et al. "Droplets Formation and Merging in Two-Phase Flow Microfluidics", Int. J. Mol. Sci., 2011, v. 12, pp. 2572-2597.*

Thorsen et al. "Dynamic Pattern Formation in a Vesicle-Generating Microfluidic Device", Phys. Rev. Lett., 2001, v. 86, No. 18, pp. 4163-4166.*

Atencia and Beebe "Controlled microfluidic interfaces", Nature, 2005, v. 437, No. 29, pp. 648-655.*

Shestopalov, I., Tice, J. D. and Ismagilov, R. F. Multi-step synthesis of nanoparticles performed on millisecond time scale in a microfluidic droplet-based system. Lab Chip, 2004, v. 4, pp. 316-321.*

Tanaka, H. et al., "*Ethanol Production from Starch by a Coimmobilized Mixed Culture System of Aspergillus awamori and Zymomonas mobilis*", Biotechnology and Bioengineering, vol. XXVII, 1986, pp. 1761-1768.

De-Bashan, L. E. et al., "*Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae Chlorella vulgaris coimmobilized in alginate beads with the microalgae growth-promoting bacterium Azospirillum brasilense*", Water Research 36 (2002), pp. 2941-2948.

Goodall, J. L. et al., "*Operation of Mixed-Culture Immobilized Cell Reactors for the Metabolism of Meta- and Para-Nitrobenzoate by Comamonas Sp. JS46 and Comamonas Sp. J547*", Biotechnology and Bioengineering, vol. 59, No. 1, Jul. 5, 1998, pp. 21-27.

He, Mingyan et al., "*Selective Encapsulation of Single Cells and Subcellular Organelles into Picoliter- and Femtoliter-Volume Droplets*", Analytical Chemistry, vol. 77, No. 6, Mar. 15, 2005, pp. 1539-1544.

Huebner, A. et al., "*Quantitative detection of protein expression in single cells using droplet microfluidics*", Chem. Commun, 2007, pp. 1218-1220.

Seong, G.H. et al., "*Fabrication of Microchambers Defined by Photopolymerized Hydrogels and Weirs within Microfluidic Systems: Application to DNA Hybridization*", Analytical Chemistry, vol. 74, No. 14, Jul. 15, 2002, pp. 3372-3377.

Seong G.H. et al., "*Efficient Mixing and reactions within Microfluidic Channels Using Microbead-Supported Catalysts*", J. Am. Chem. Soc. 2002, 124, pp. 13360-13361.

Nisisako, T. et al., "*Formation of Droplets Using Branch Channels in a Microfluidic Circuit*", SICE 2002, Aug. 5-7, 2002, Osaka, pp. 1262-1264.

Chayen, N.E., "*Crystallization with oils: a new dimension in macromolecular crystal growth*", Journal of Crystal Growth 196 (1999), pp. 434-441.

Titomanlio et al., "*Capillary experiments of flow induced crystallization of HDPE*", AIChe Journal, 1990, v36, No. 1, pp. 13-18.

Garcia-Ruiz et al., "*Investigation on protein crystal growth by the gel acupuncture method*", Acta, Cryst., 1994, D50, 99. pp. 484-490.

Sugiura et al., "*Interfacial tension driven monodispersed droplet formation from microfabricated channel array*", Langmuir, 2001, v17, pp. 5562-5566.

Garcia-Ruiz et al. "*A super-saturation wave of protein crystallization*", J. Crystal Growth, 2001, v232, pp. 149-155.

Ng et al., "*Protein crystallization by capillary counter-diffusion for applied crystallographic structure determination*", J. Struct. Biol. 2003, v142, pp. 218-231.

Torkkeli et al., "*Droplet Manipulation on a Superhydrophobic Surface for Microchemical Analysis*", Transducers '01 Eurosensors XV, 11th International Conference on Solid-State Sensors and Actuators, Munich, Germany, Jun. 10-14, 2001, 4 pgs.

Anna, Shelley A. et al., "*Formation of Dispersions Using 'Flow Focusing' in Microchannels*", Applied Physics Letters, vol. 82, No. 3, 2003, pp. 364-366.

Auroux, Pierre-Alain et al., "*Micro Total Analysis Systems. 2. Analytical Standard Operations and Applications*", analytical Chemistry, vol. 74. No. 12, 2002, pp. 2637-2652.

Bico, Jose et al., "*Rise of Liquids and Bubbles in Angular Capillary tubes*", Journal of colloid and Interface Science, vol. 247, 202, pp. 162-166.

Bringer et al. "*Microfluidic System for Chemical Kinetics That Rely on Chaotic Mixing in Droplets*", Phil. Trans. R. Soc. Lond., pp. 1-18, 2004.

Burns, J. R. et al., "*The Intensification of Rapid Reactions in Multiphase systems Using Slug Flow in Capillaries*", Lab on a Chip, vol. 1, 2001, pp. 10-15.

Burns, Mark et al., "*An Integrated Nanoliter DNA Analysis Device*", Science, vol. 282, 1998, pp. 484-487.

Chan, Emory M. et al. "*Size-Controlled Growth of CdSe Nanocrystals in Microfluidic Reactors*", Nano Letters, vol. 3, No. 2, 2003, pp. 199-201.

Cho, Sung Kwon et al., "*Splitting a Liquid Droplet for Electrowetting-Based Microfluidics*", Proceedings of 2001 ASME International Mechanical Engineering Congress and Exposition, 2001, pp. 1-7.

Duffy, David C. et al., "*Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane)*", Analytical Chemistry, vol. 70, 1998, pp. 4974-4984.

Edel, Joshua B. et al., "*Microfluidic Routes to the Controlled Production of Nanoparticles*", Chemical Communications, 2002, pp. 1136-1137.

Eggers, Jens et al., "*Coalescence of Liquid Drops*", J. Fluid Mech., vol. 401, 1999, pp. 293-310.
Fowler, Jesse et al., "*Enhancement of Mixing by Droplet-Based Microfluidics*", 2002 Institute of electrical Engineers 15th International Conference on Micro Electro Mechanical Systems, 2002, pp. 97-100.
Gerdts et al., "*A Synthetic Reaction Network: Chemical Amplification Using Nonequilibrium Autocatalytic Reactions Coupled in Time*", J. Am. Chem. Soc., vol. 126, pp. 6327-6331, 2004.
Handique, K. et al., "*On-Chip Thermopneumatic Pressure for Discrete Drop Pumping*", Analytical Chemistry, vol. 73, 2001, pp. 1831-1838.
Hansen, Carl L. et al., "*A Robust and Scalable Microfluidic Metering Method That Allows Protein Crystal Growth by Free Interface Diffusion*", PNAS, vol. 99, No. 26, 2002, pp. 16531-16536.
Harries, N. et al., "*A Numerical Model for Segmented Flow in a Microreactor*", International Journal of Heat and Mass Transfer, vol. 46, 2003, pp. 3313-3322.
Hosokawa, Kazuo et al., "*Handling of Picoliter Liquid Samples in a Poly(dimethylsiloxane)-Based Microfluidic Device*", Analytical Chemistry, vol. 71, No. 20, 1999, pp. 4781-4785.
Ismagilov, "*Integrated Microfluidic Systems*", Agnew. Chem. Int. Ed., vol. 42, pp. 4130-4132.
Knight, James B., "*Hydrodynamic Focusing on a Silicon Chip: Mixing Nanoliters in Microseconds*", Physical Review Letters, vol. 80, No. 17, 1998, pp. 3863-3866.
Liu, Robin H. et al., "*Passive Mixing in a a Three-Dimensional Serpentine Microchannel*", Journal of Microelectromechanical Systems, vol. 9, No. 2, 2000, pp. 190-197.
McDonald, J. Cooper et al.,"*Fabrication of Microfluidic Systems in Poly(dimethylsiloxane)*" Electrophoresis, vol. 21, 2000, pp. 27-40.
Nisisako, Takasi et al. "*Droplet Formation in a Microchannel Network*", Lab on a Chip, vol. 2, 2002, pp. 24-26.
Pabit, Suzette A. et al., "*Laminar-Flow Fluid Mixer for Fast Fluorescence Kinetics Studies*", Biophysical Journal, vol. 83, 2002, pp. 2872-2878.
Peng, Shuangjiu et al., "*Controlled Production of Emulsions Using a Crossflow Membrane*", Particle & Particle Systems Characterization, vol. 15, 1998, pp. 21-25.
Reyes, Darwin R. et al., "*Micro Total Analysis Systems. 1. Introduction, Theory and Technology*", Analytic Chemistry, vol. 74, No. 12, 2002, pp. 2623-2636.
Shestopalov et al., *Multi-Step Synthesis of Nanoparticles Performed on Millisecond Time Scale in a Microfluidic Droplet-Based System*, The Royal Society of Chemistry, vol. 4, pp. 316-332, 2004.
Song et al., "*A Microfluidic System for Controlling Reaction Networks in Time*", Angew. Chem.. Int. Ed., vol. 42, No. 7, pp. 768-772, 2003.
Song et al., "*Experimental Test of Scaling of Mixing by Chaotic Advection in Droplets Moving Through Microfluidic Channels*", Applied Physics Letters, vol. 83, No. 22, pp. 4664-4666, 2003.
Song et al., "*Millisecond Kinetics on a Microfluidic Chip Using Nanoliters of Reagents*", J. Am. Chem. Soc., vol. 125, pp. 14613-14619, 2003.
Song, Helen et al., "*A Microfluidic System for Controlling Reaction Networks in Time*", Angew. Chem. Int. Ed., vol. 42, No. 7, 2003, pp. 768-772.
Stroock, Abraham D. et al., "*Chaotic Mixer for Microchannels*", Science, vol. 295, 2002, pp. 647-651.
Sugiura, Shinji et al., "*Effect of Channel Structure on Microchannel Emulsification*", Langmuir, vol. 18, 2002, pp. 5708-5712.
Takayama et al., "*Patterning Cells and Their Environments Using Multiple Lamina Fluid Flows in Capillary Networks*", Proc. Natl. Acad. Sci. USA, vol. 96, pp. 5545-5548, 1999.
Taniguchi, Tomohiro et al., "*Chemical Reactions in Microdroplets by Electrostatic Manipulation of Droplets in Liquid Media*", Lab on a Chip, Vo.. 2, 2002, pp. 19-23.
Thorsen et al., "*Microfluidic Large-Scale Integration*", Science, vol. 298, pp. 580-584, 2002.
Thorsen, Todd et al., "*Dynamic Pattern Formation in a Vesicle-Generating Microfluidic Device*", Physical Review Letters, vol. 86, No. 18, 2001, pp. 4163-4166.
Tice, "*Effects of Vicosity on Droplets and Mixing in Multiphase Microfluidics at Low Values of the Reynolds and the Capillary Numbers*", Langmuir, vol. 19, pp. 927-9133, 2003.
Tice et al., "*Formation of Droplets and Mixing in Multiphase Microfluids at Low Values of the Reynolds and the Capillary Numbers*", Langmuir, vol. 19, pp. 9127-9133, 2003.
Tokesh, Manabu et al., "*Continuous-Flow Chemical Processing on a Microchip by Combining Microunit Operations and a Multiphase Flow Network*", Analytical Chemistry, vol. 74, No. 7, 2002, pp. 1565-1571.
Umbanhowar, P.B. et al., "*PMonodisperse Emulsion Generation Via Drop Break Off in a Coflowing Stream*", Langmuir, vol. 16, 2000, pp. 347-351.
Wang, Hongzhi et al., "*Preparation of Titania Particles Utilizing the Insoluble Phase Interface in a Microchannel Reactor*", Chemical Communications, 2002, pp. 1462-1463.
Zhao, Bin et al., "*Control and Applications of Immiscible Liquids in Microchannels*", J. Am. Chem. Soc., vol. 124, 2002, pp. 5284-5285.
Zheng et al., "*A Droplet-Based, Composite PDMS/Glass Capillary Microfluidic System for Evaluating Protein Crystalization Conditions by Microbatch and Vapor-Diffusion Methods with On-Chip X-Ray Diffraction*", Angew. Chem., pp. 1-4, 2004.
Zheng et al., "*Screening of Protein Crystallization Conditions on a Microfluidic Chip Using Nanoliter-Size Droplets*", Journal of the American Chemical Society, vol. 125, No. 37, pp. 11170-11171, 2003.
Beer et al., "*On-Chip, Real-Time, Single-Copy Polymerase Chain Reaction in Picoliter Droplets*", Anal. Chem., 2007, v. 79, pp. 847-8475.
Vogelstein, et al., "*Digital PCR*", Proc. Natl. Acad. Sci. USA, Aug. 1999, vol. 96, pp. 9236-9241, Genetics.
Zheng et al., "*Formation of Droplets of Alternating Composition in Microfluidic Channels and Applications to Indexing of Concentrations in Droplet-Based Assays*", Anal. Chem., 2004, v. 76, pp. 4977-4982.

* cited by examiner

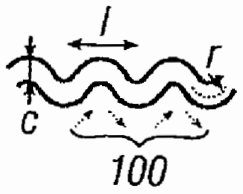


FIG. 1A

FIG. 1B-1

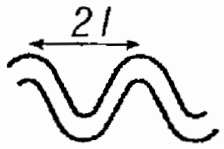


FIG. 1B-2

FIG. 1B-3

FIG. 1B-4

FIG. 1C-1

FIG. 1C-2

FIG. 1C-3

FIG. 1C-4

FIG. 2A-1

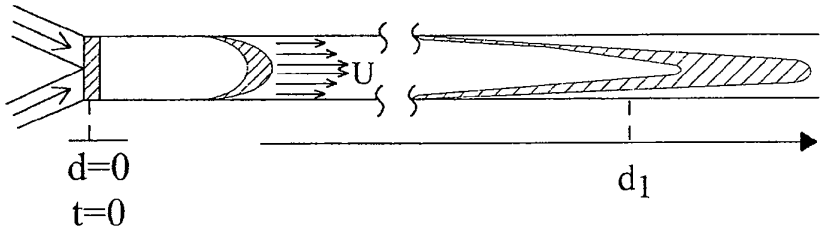


FIG. 2A-2

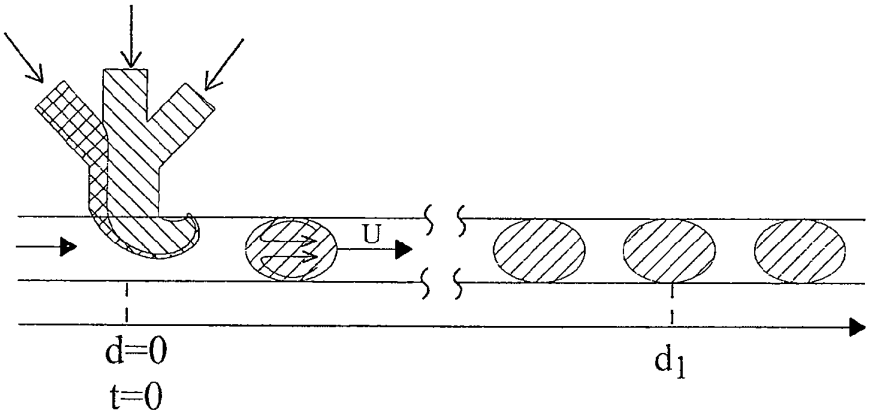


Fig. 2A

FIG. 2B-1

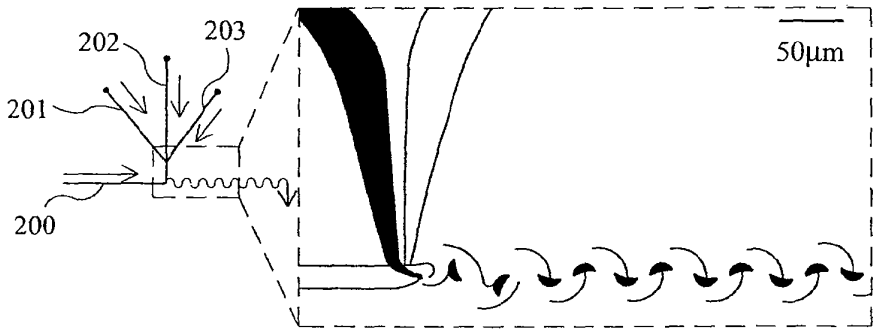


FIG. 2B-2

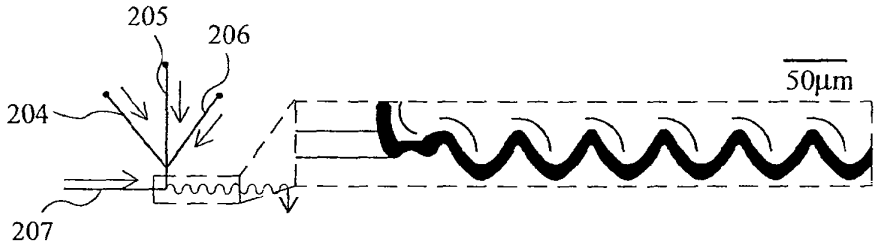


Fig. 2B

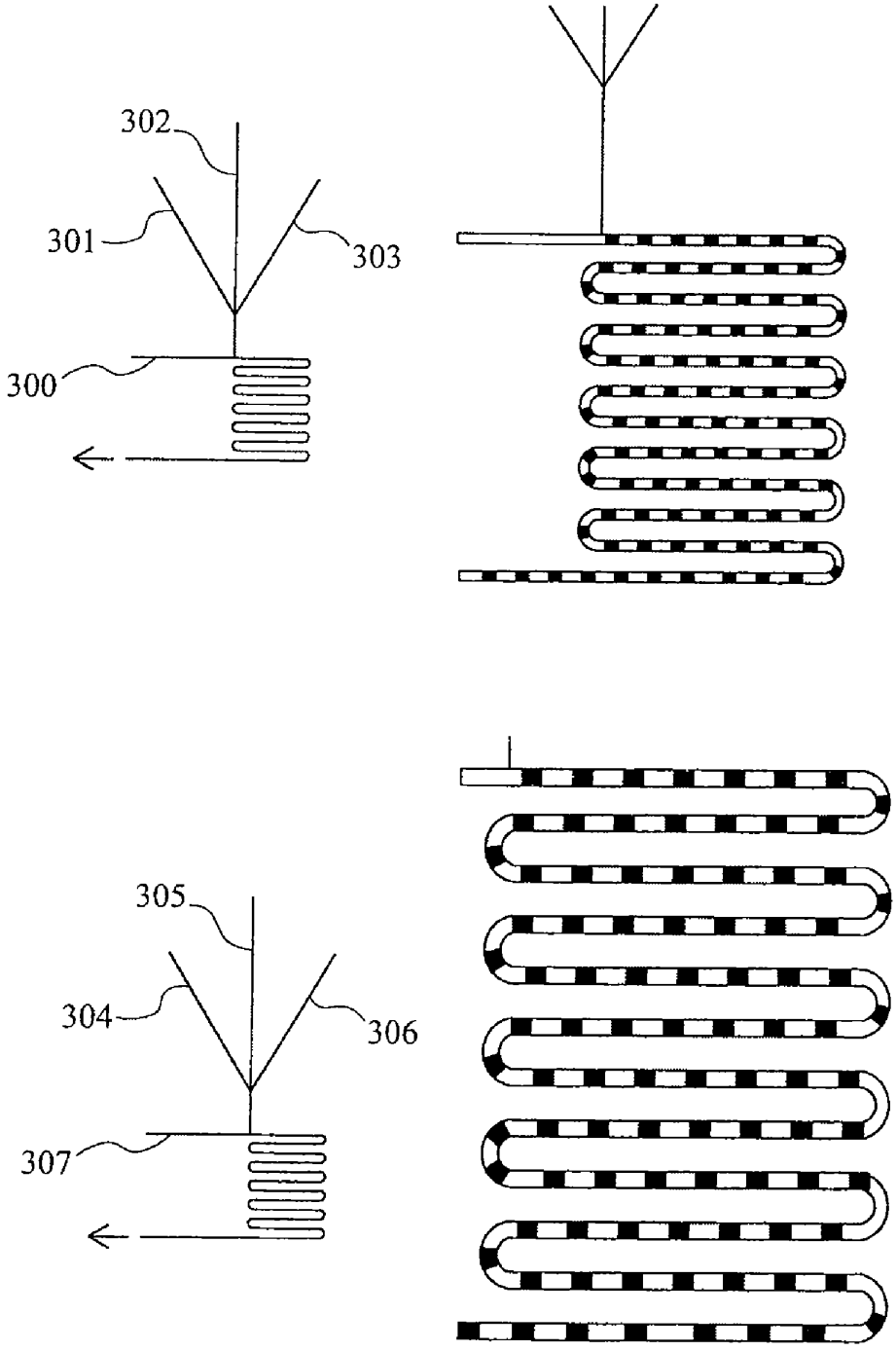


Fig. 3

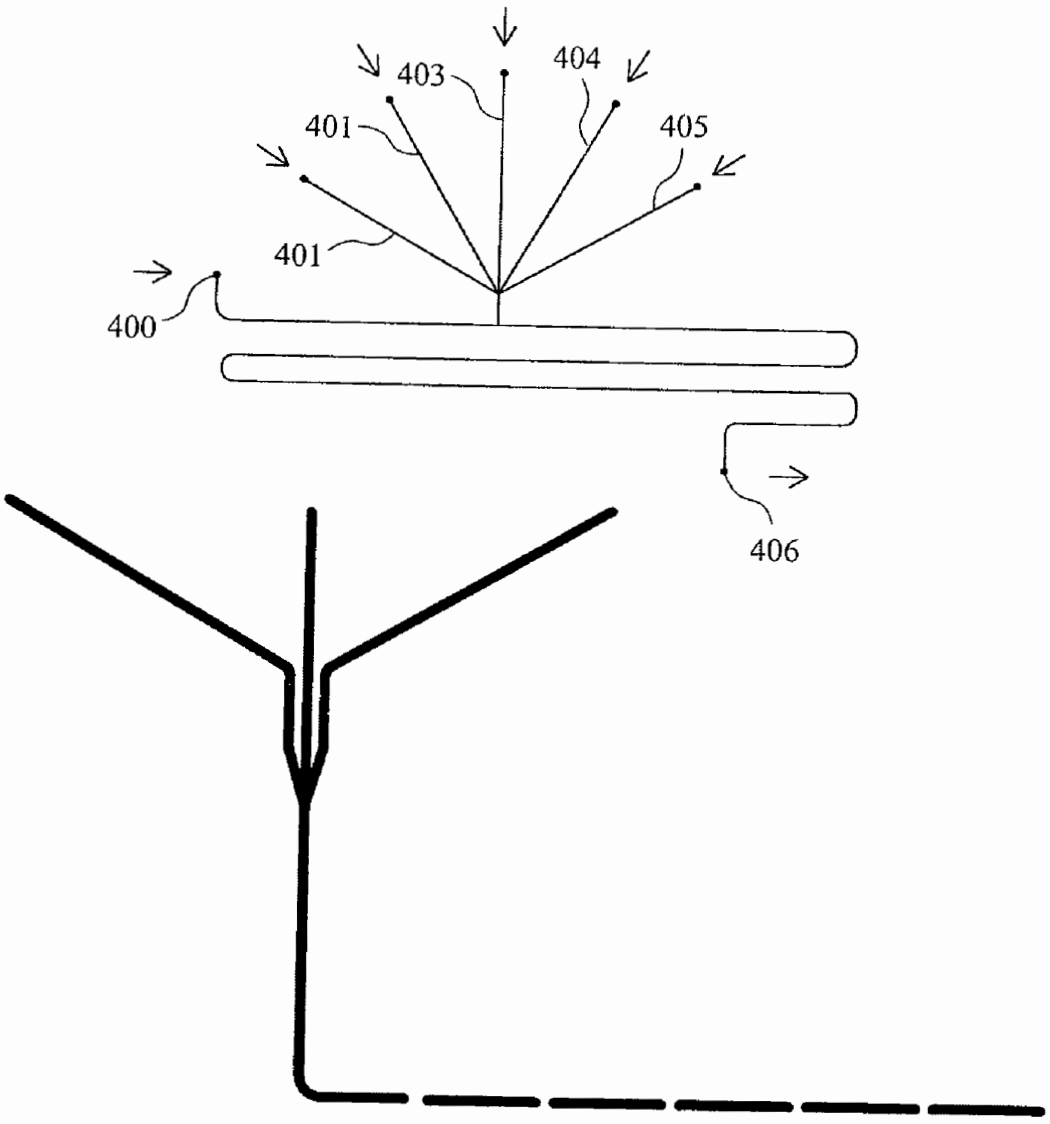


Fig. 4

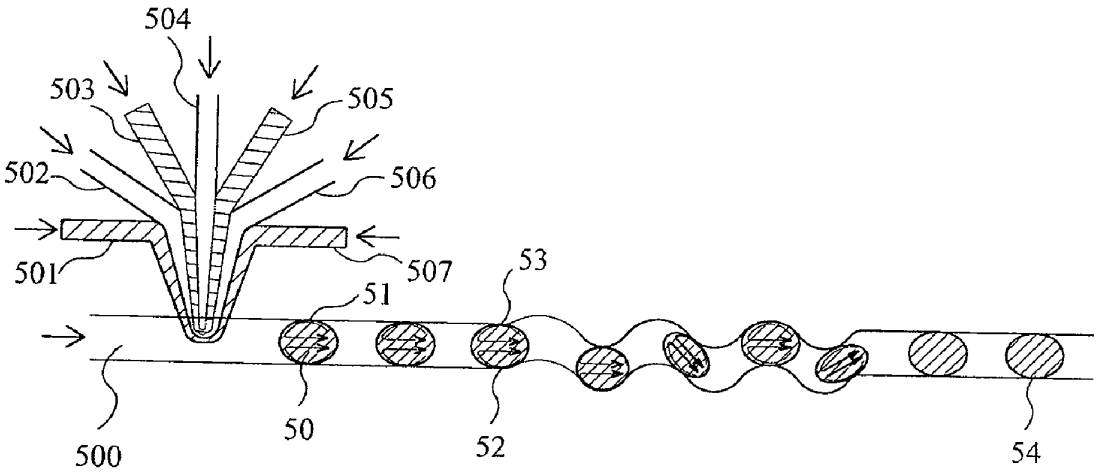


Fig. 5

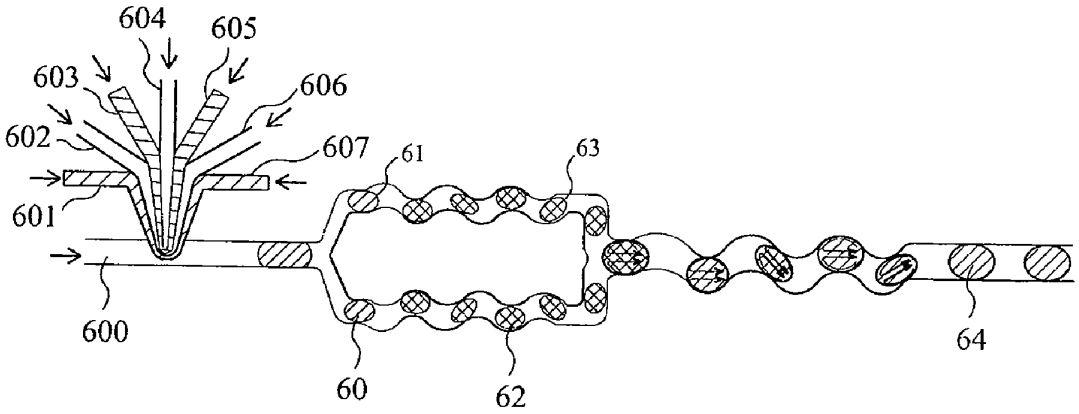


Fig. 6

FIG. 7A

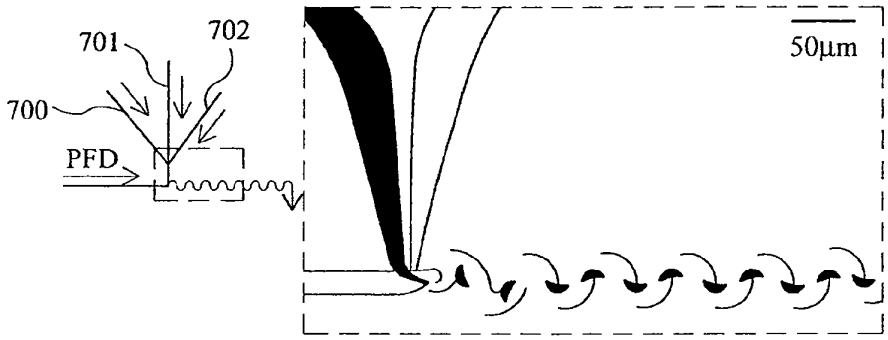


FIG. 7B

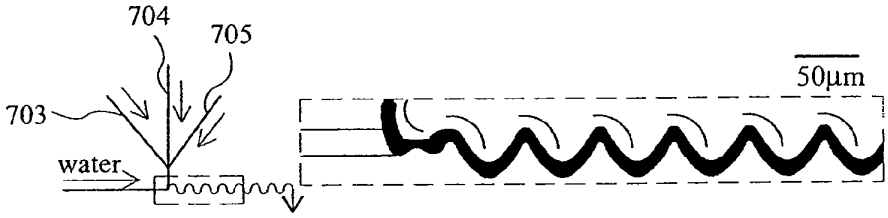


FIG. 7C

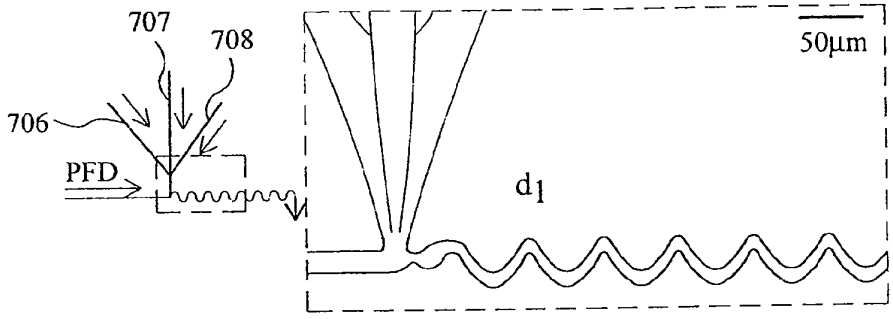


FIG. 7D

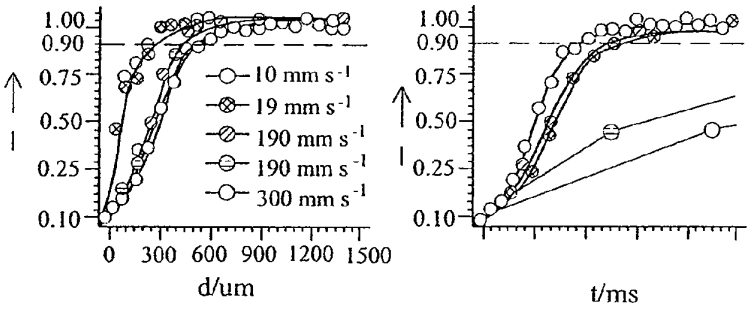


FIG. 7E

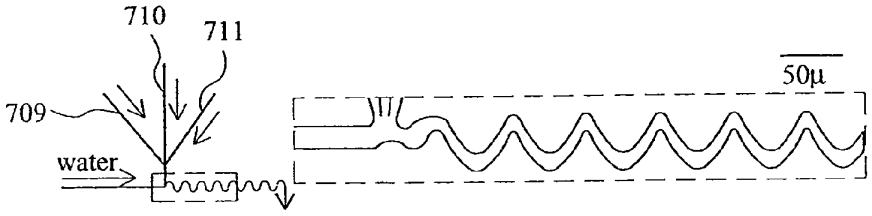


Fig. 7

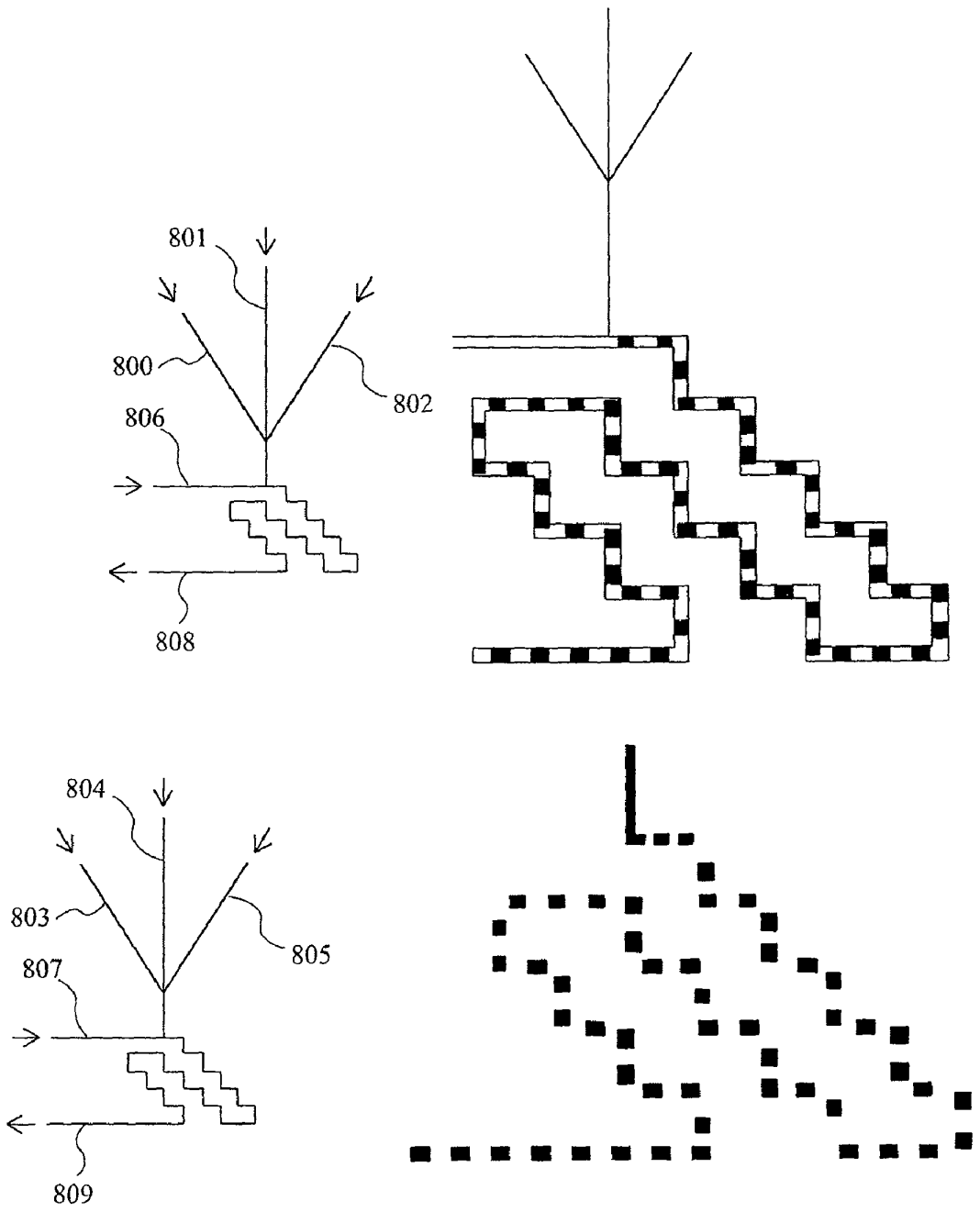


Fig. 8

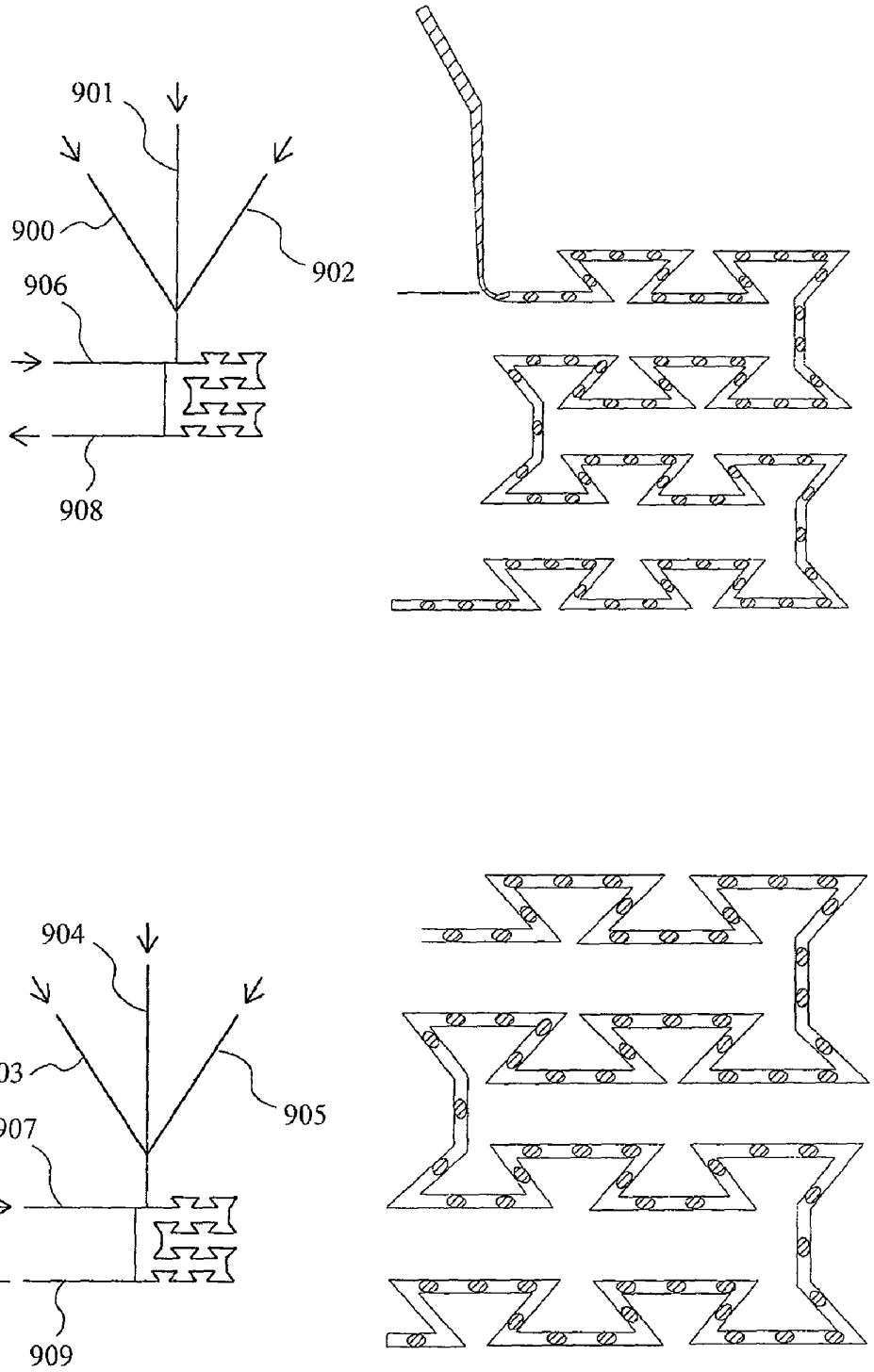


Fig. 9

FIG. 10A

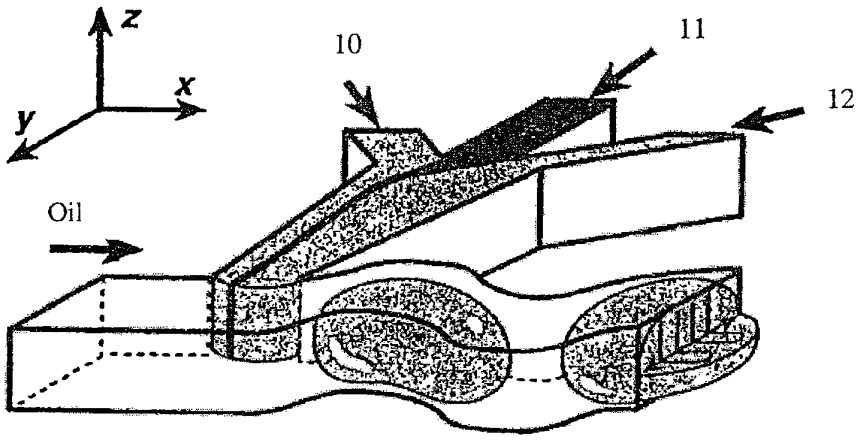


FIG. 10B

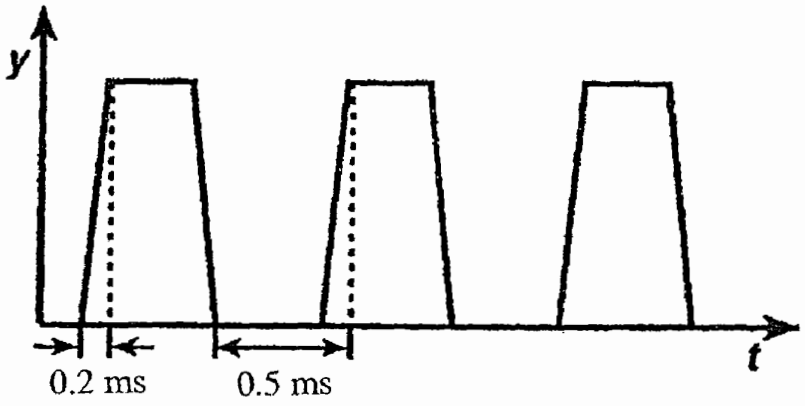


Fig. 10

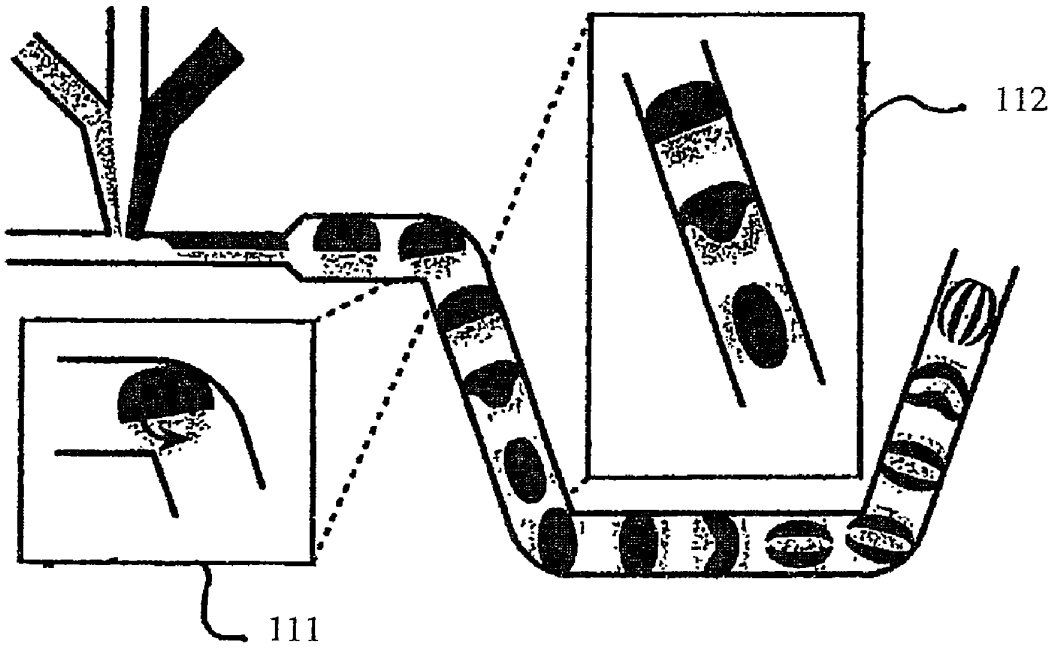


Fig. 11

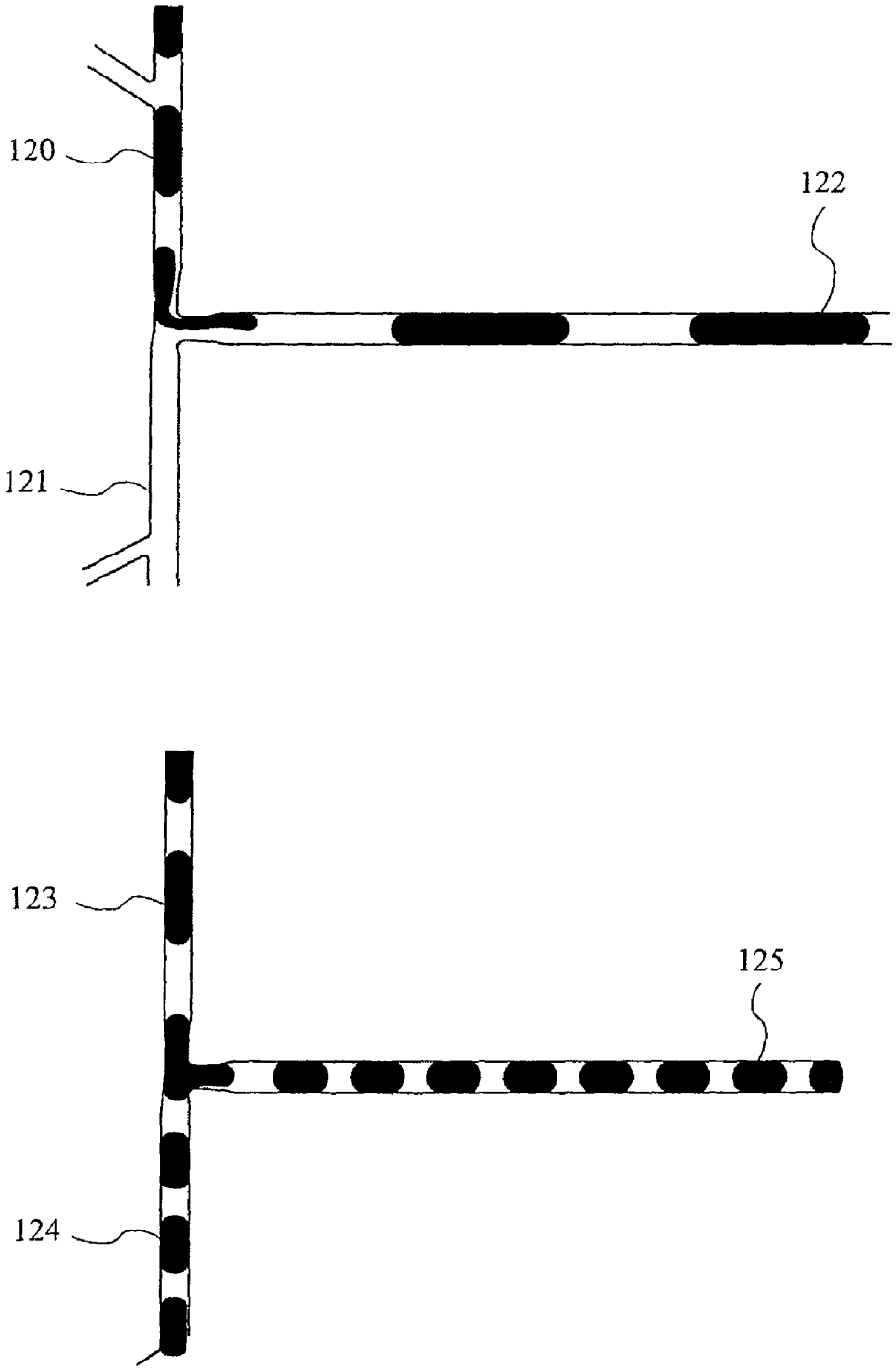


Fig. 12

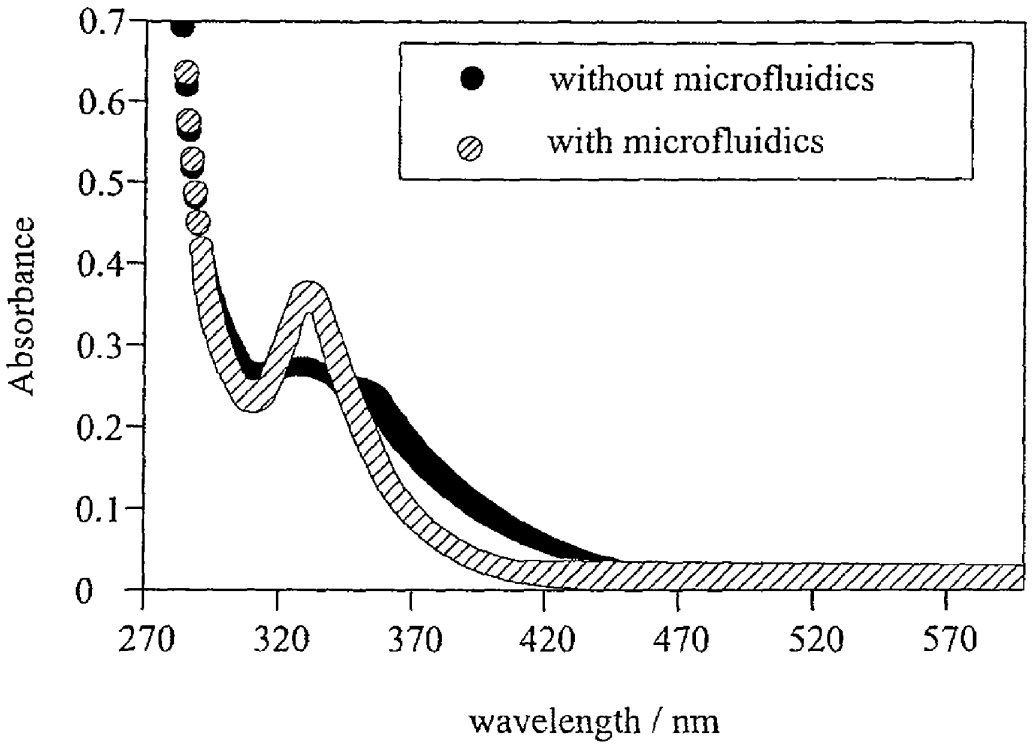

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
without microfluidics
with microfluidics
270
320
370
420
470
520
570
wavelength / nm

Fig. 13

FIG. 14A

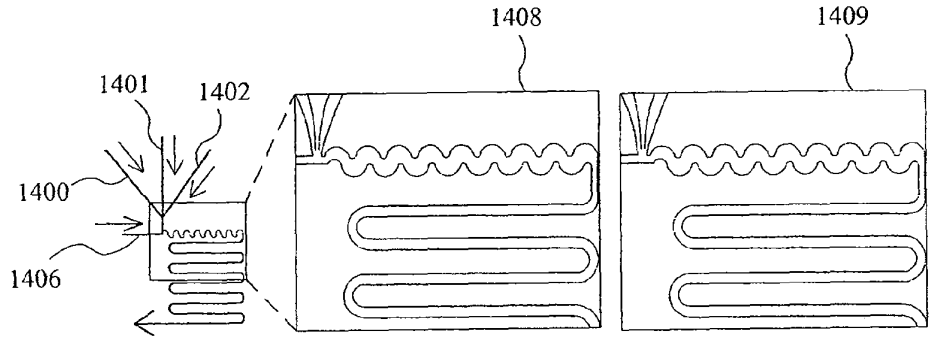


FIG. 14B

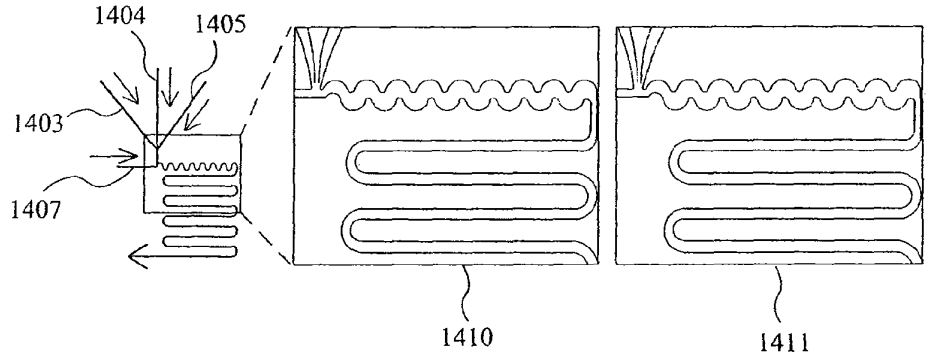


Fig. 14

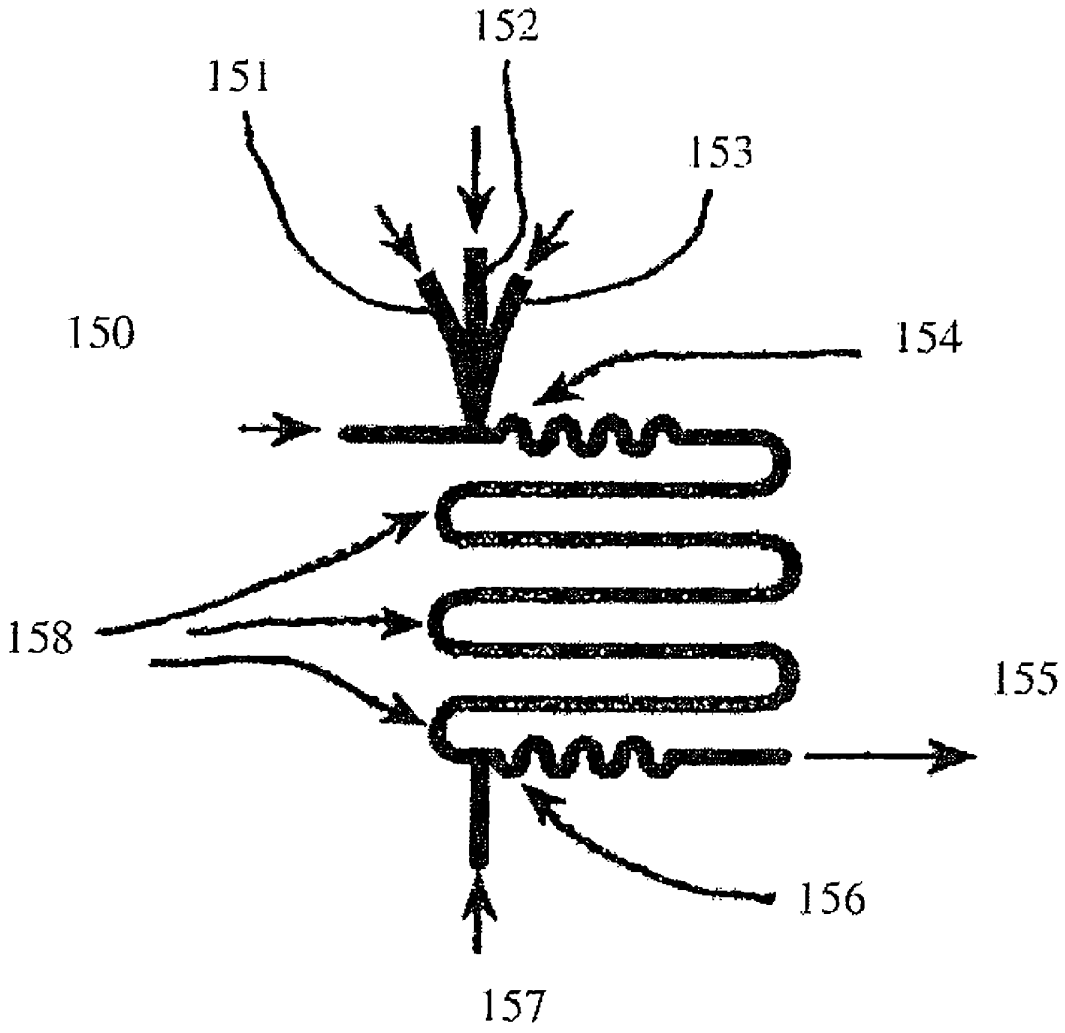


Fig. 15

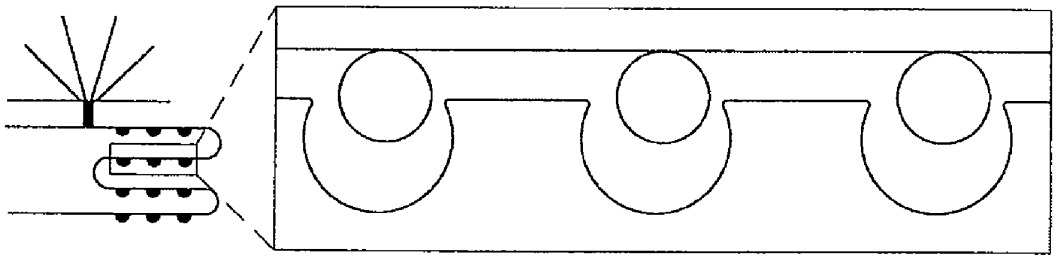


Fig. 16

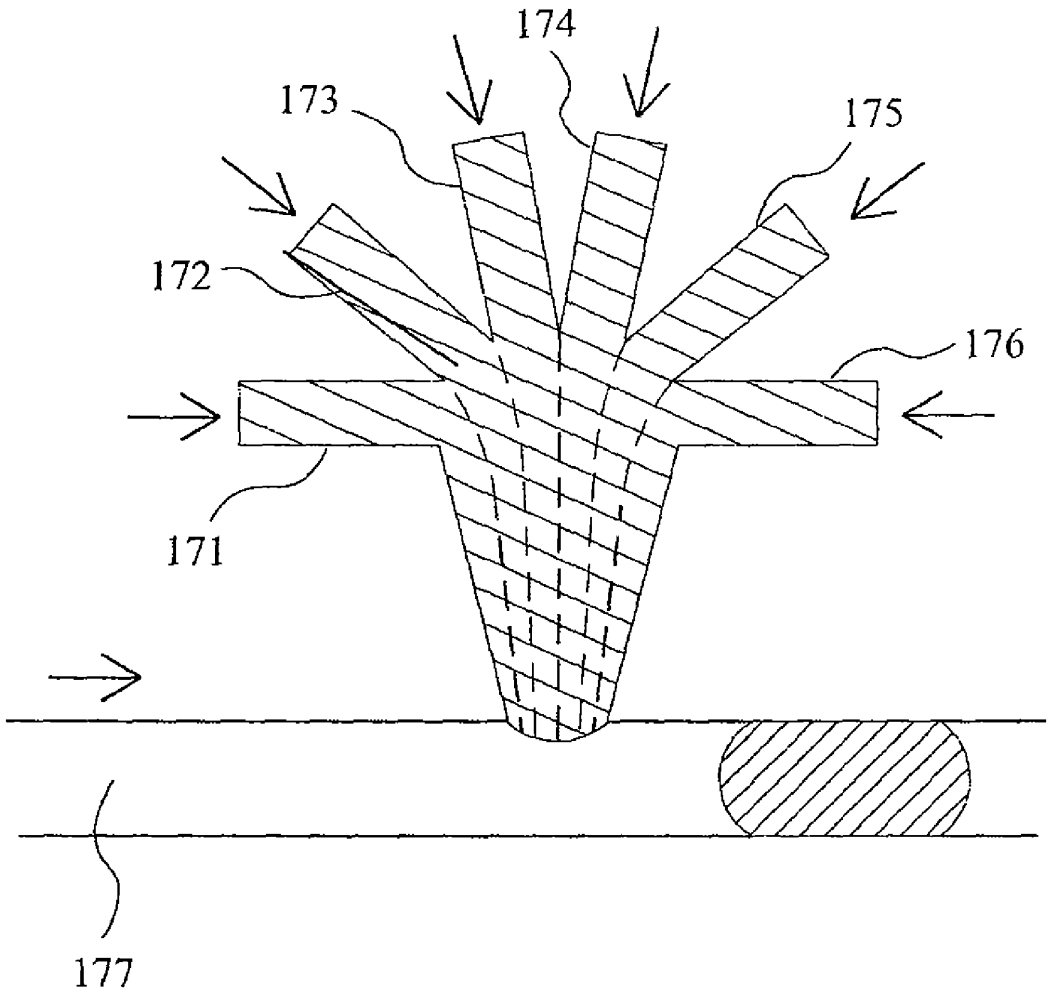


Fig. 17

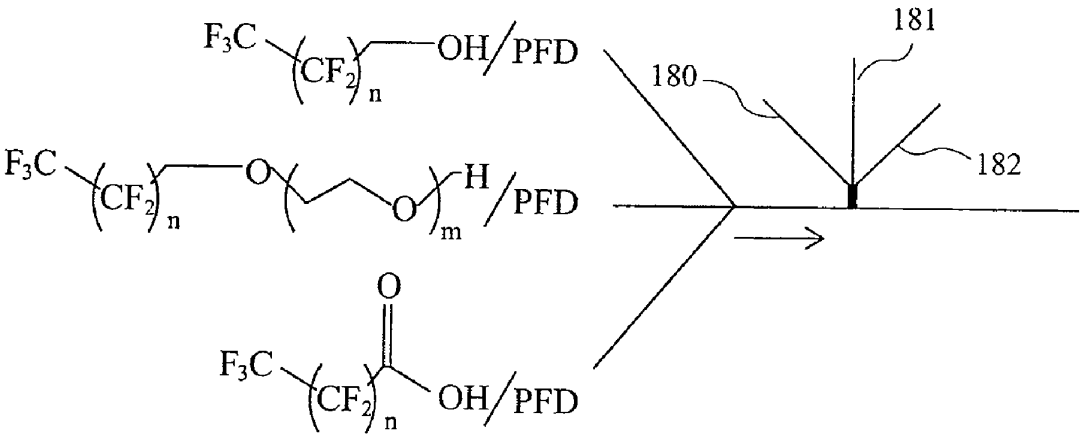


Fig. 18

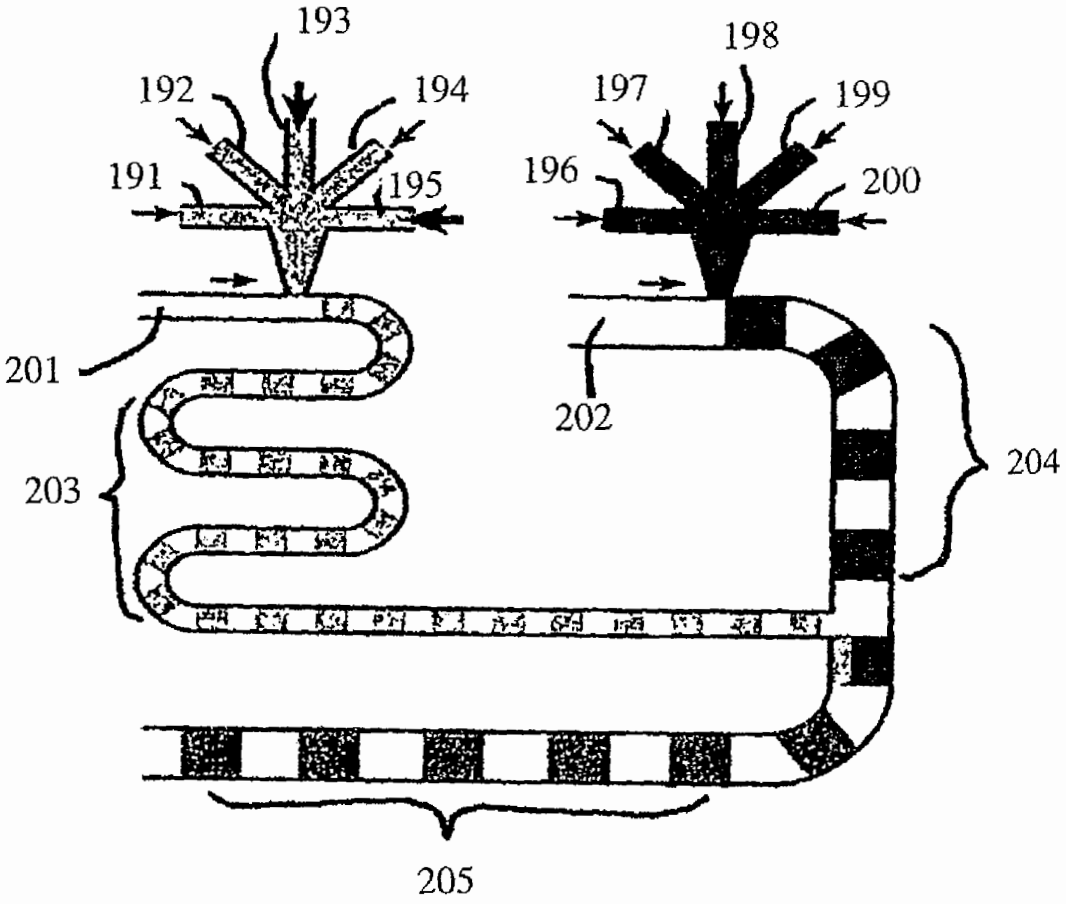


Fig. 19

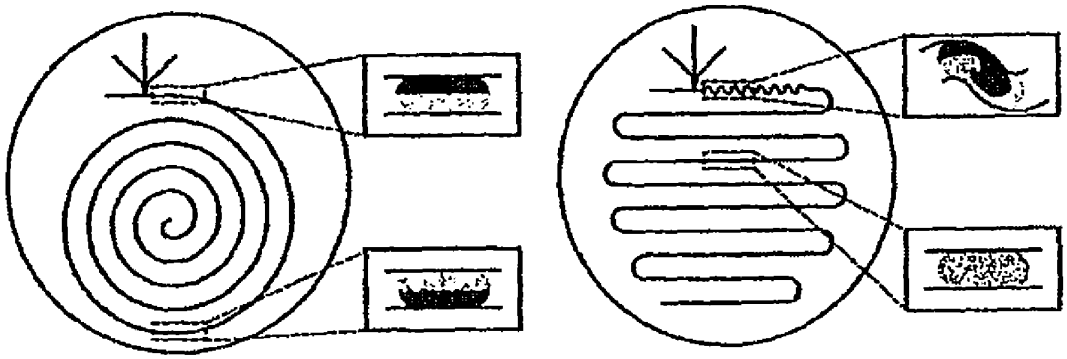


Fig. 20

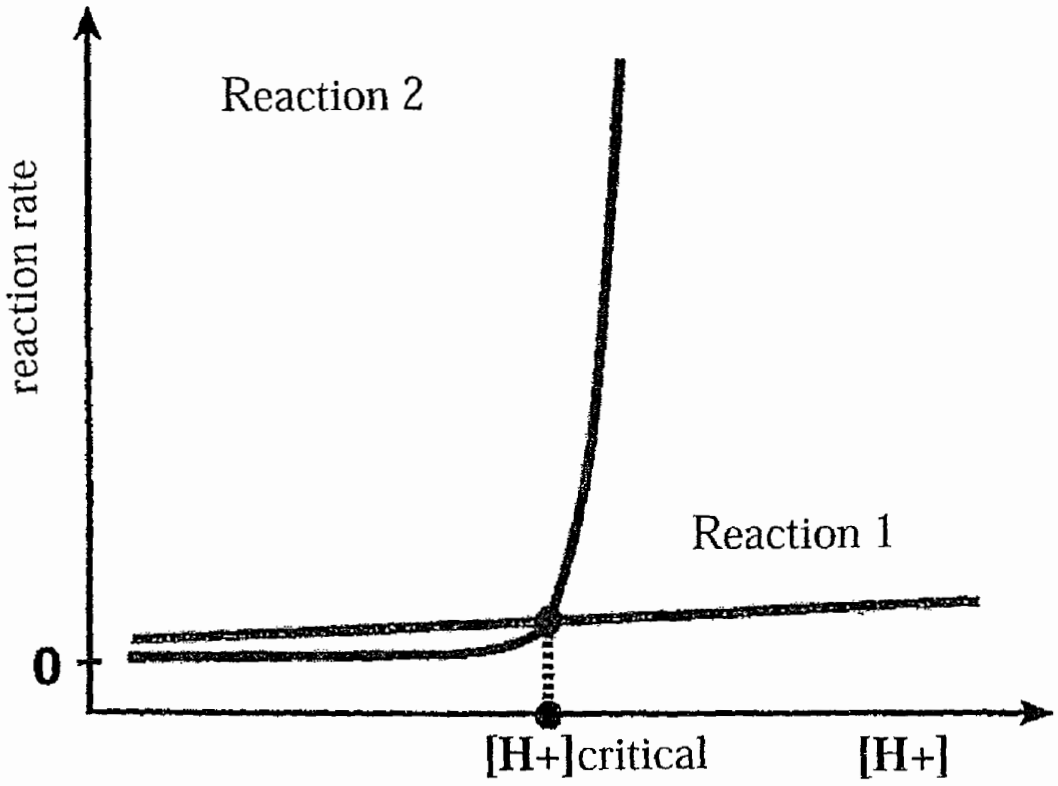


Fig. 21

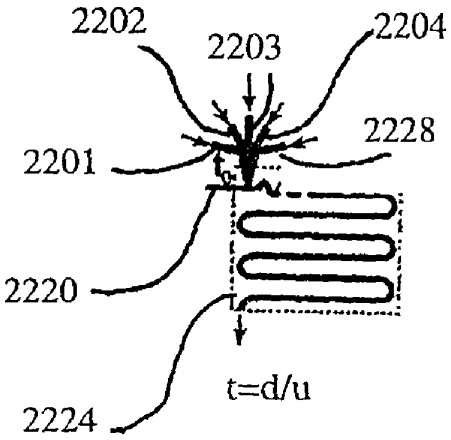


FIG. 22A

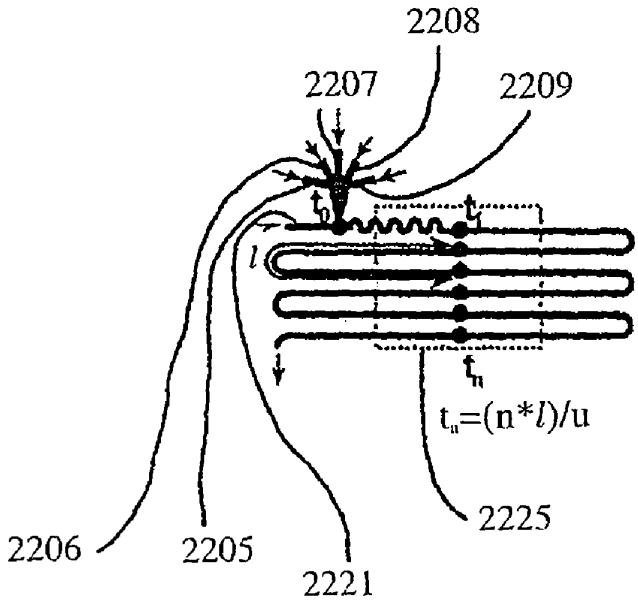


FIG. 22B

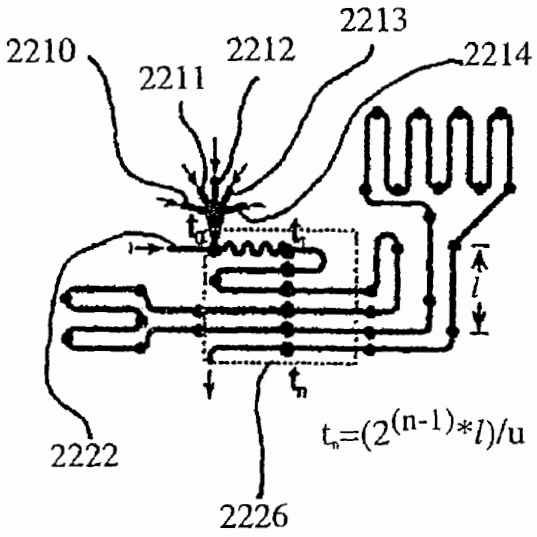


FIG. 22C

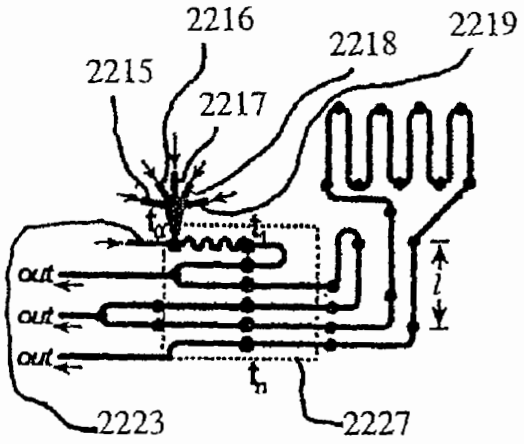
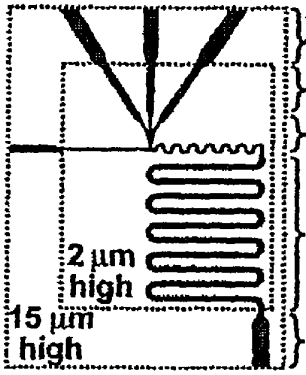


FIG. 22D



Size of channel (height x width)	ΔP [atm]	U [m/s]	length [μm]	time
15 μm x 50 μm	0.003	0.001	10000	
2 μm x 5 μm	0.4	0.08	200	
2 μm x 1 μm	0.45	0.4	10	25 μs → mix
2 μm x 3 μm	2.0	0.13	400	3000 μs = 3.0 ms → observe
15 μm x 50 μm	0.003	0.001	10000	

Fig. 23

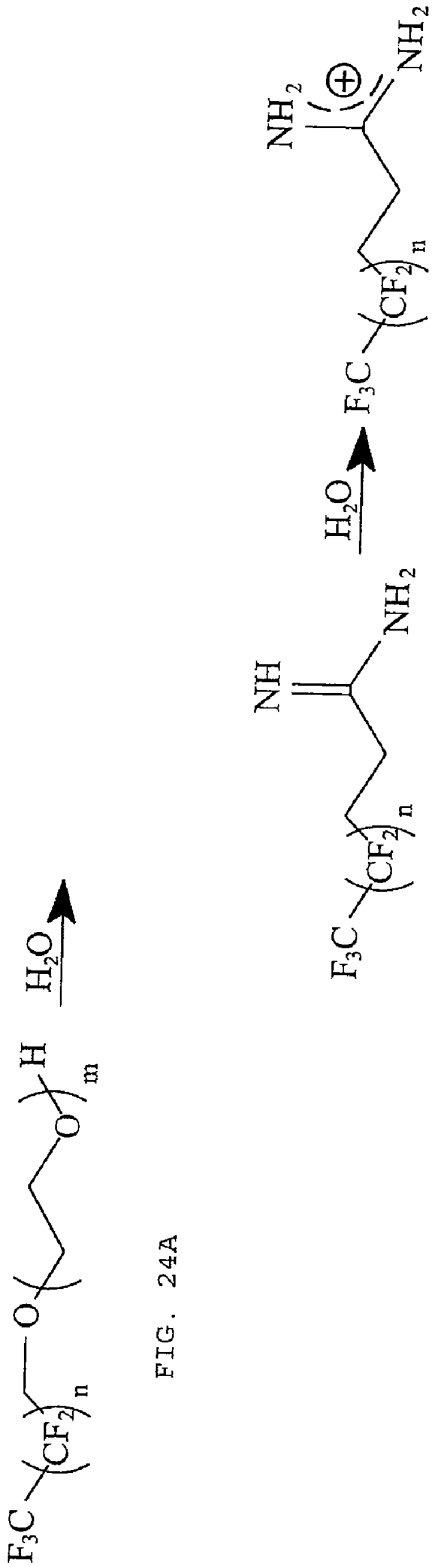


FIG. 24A

FIG. 24B

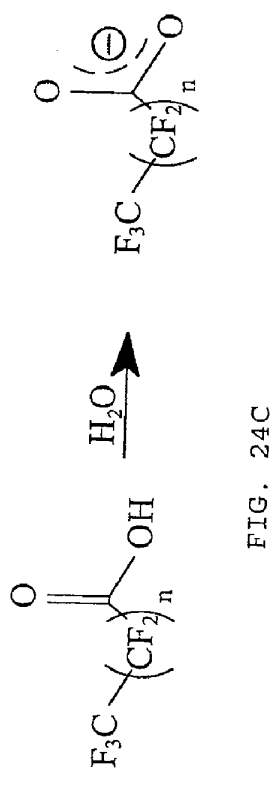
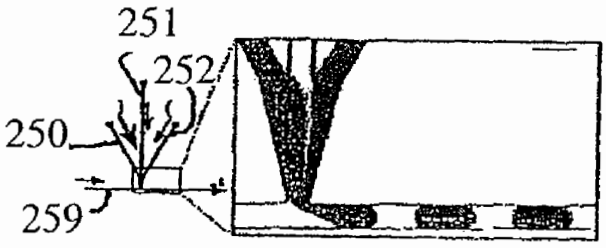


FIG. 24C

Fig. 24

FIG. 25A



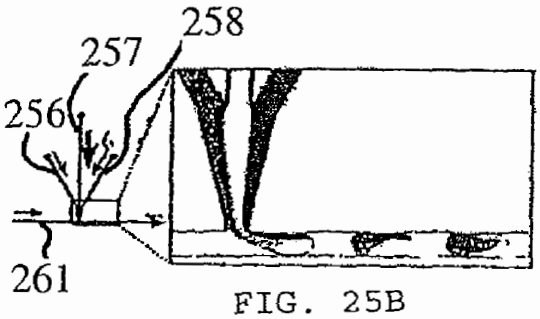


FIG. 25B

FIG. 25C

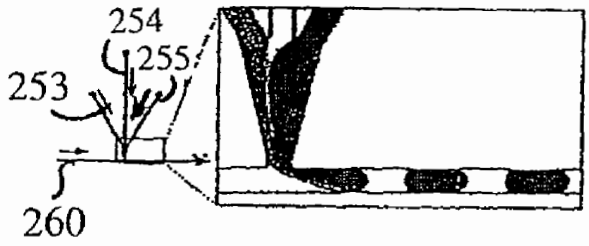


Fig. 25

FIG. 26A

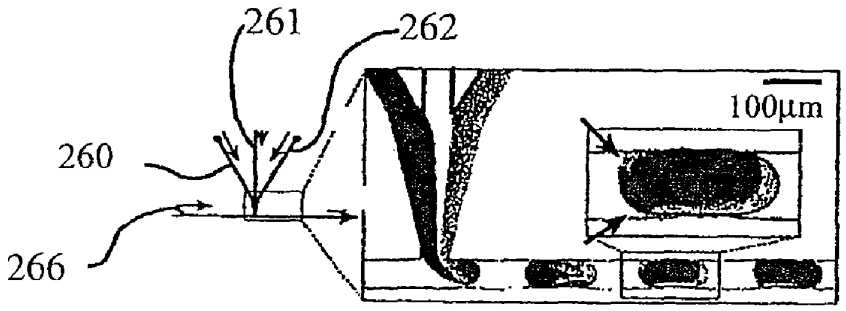


FIG. 26B

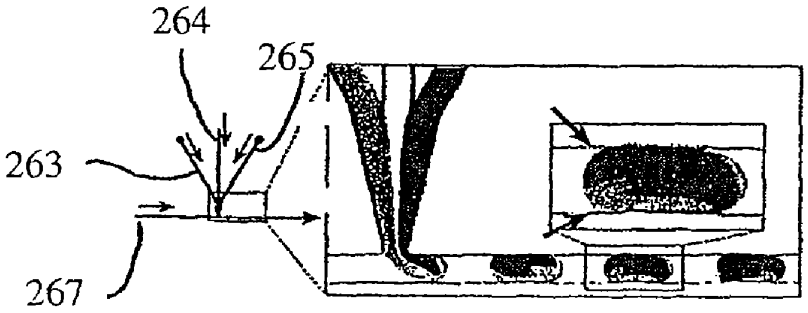


Fig. 26

FIG. 27A-1

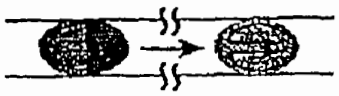
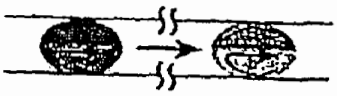


FIG. 27A-2



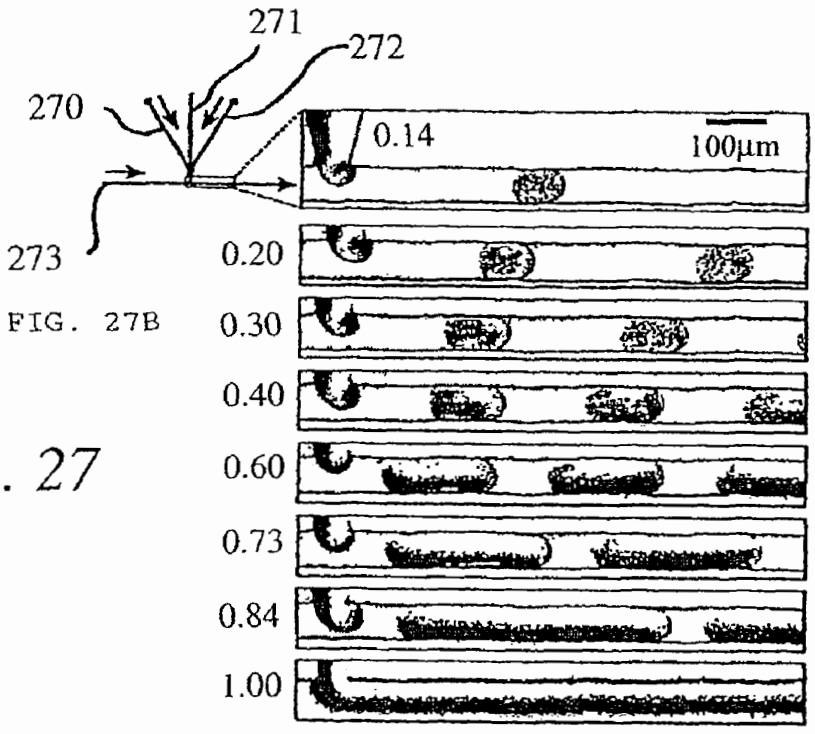


FIG. 27B

Fig. 27

FIG. 27C-1

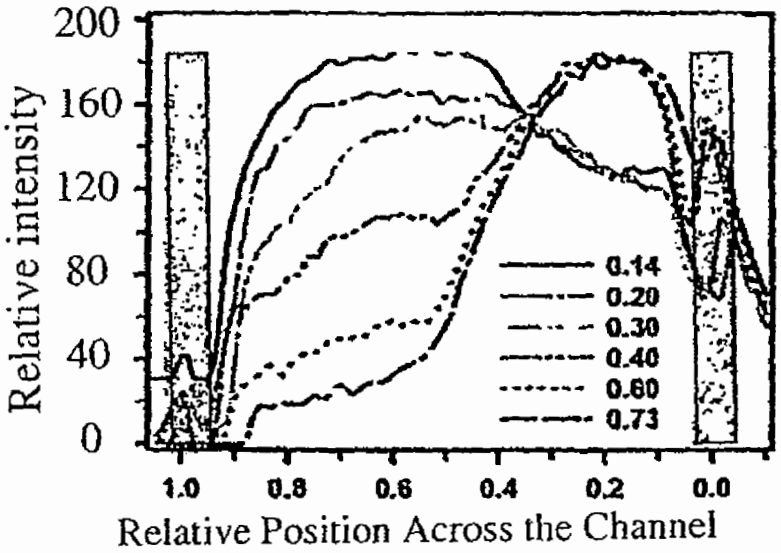
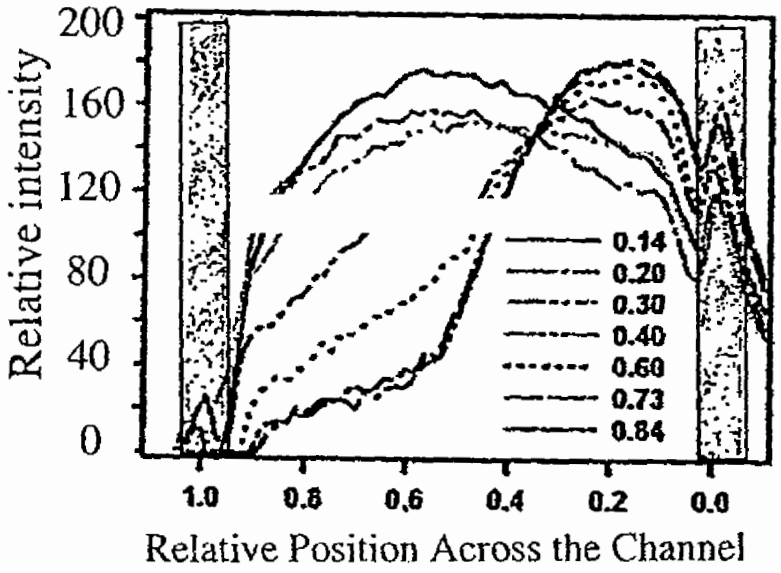


FIG. 27C-2



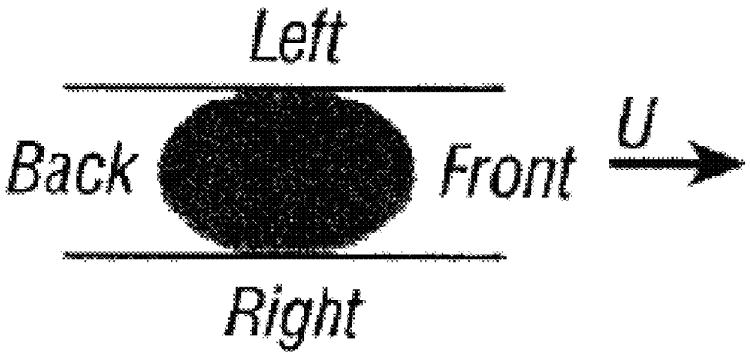


FIG. 28

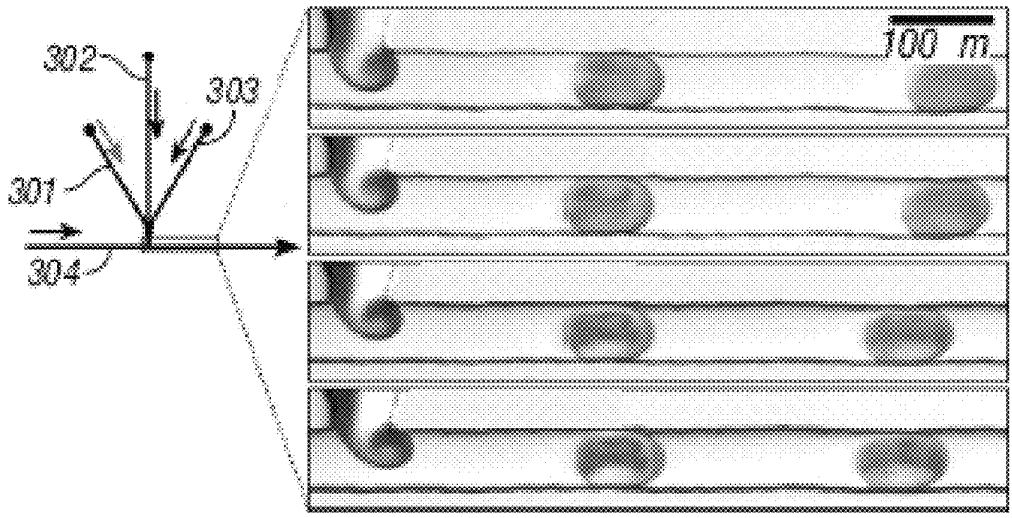


FIG. 30

FIG. 29A

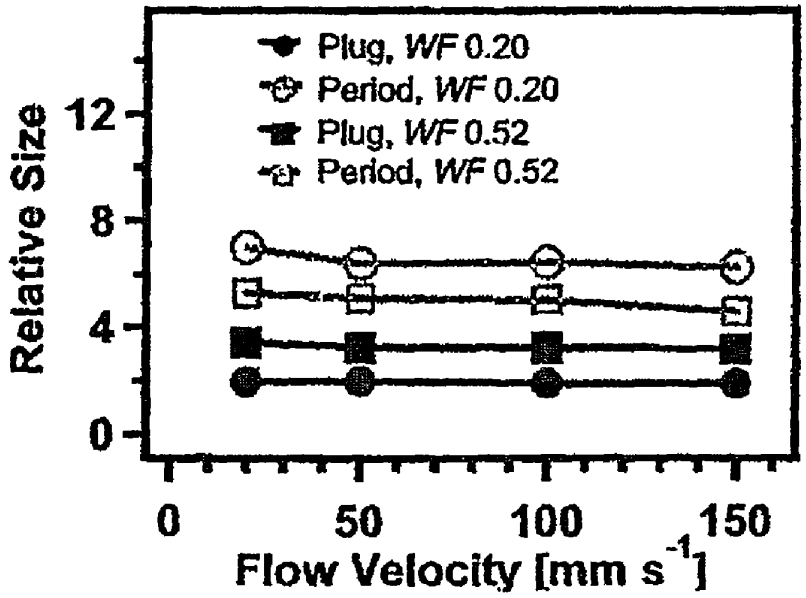


FIG. 29B

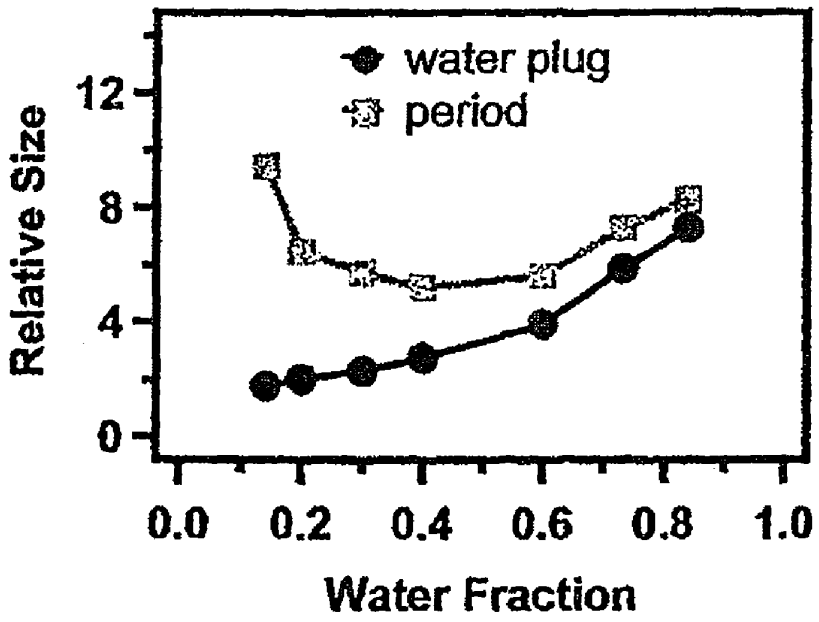


Fig. 29

FIG. 31A

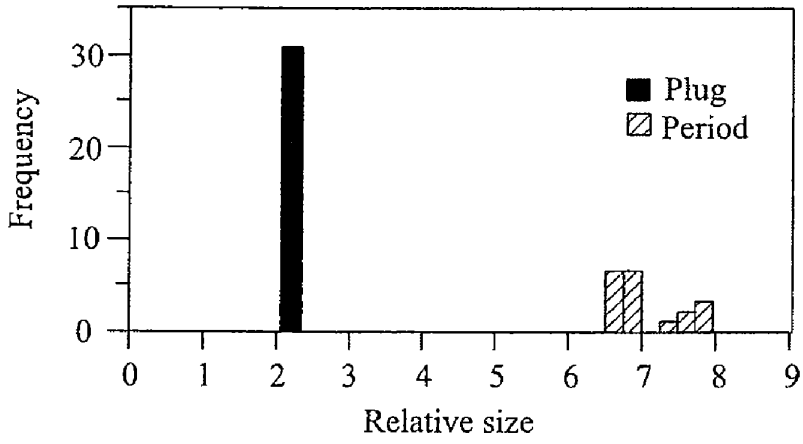


FIG. 31B

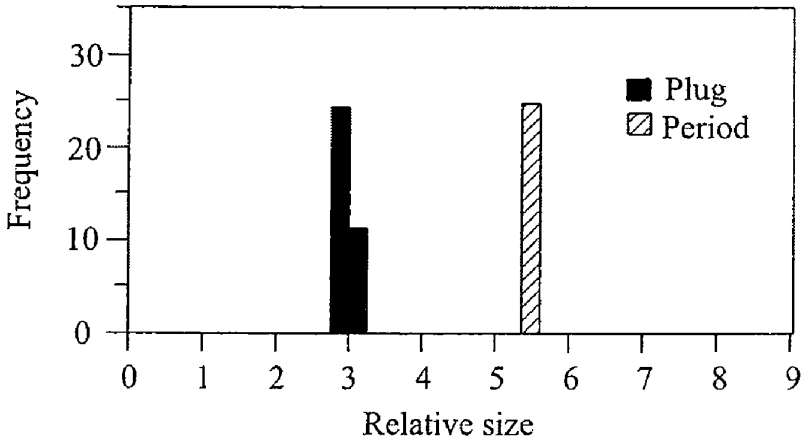


FIG. 31C

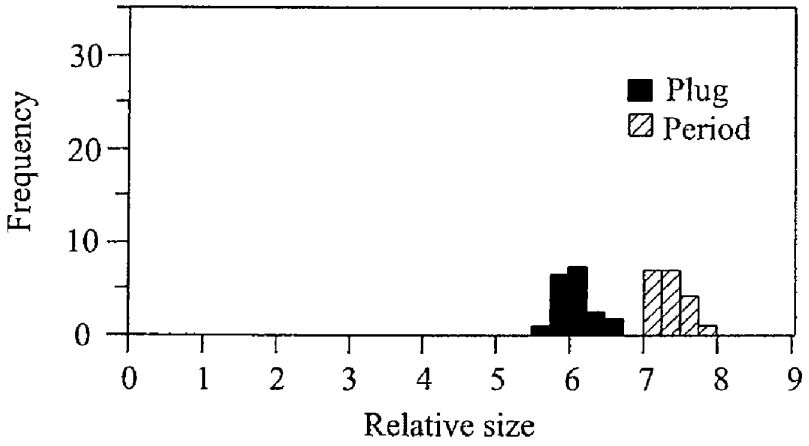


Fig. 31

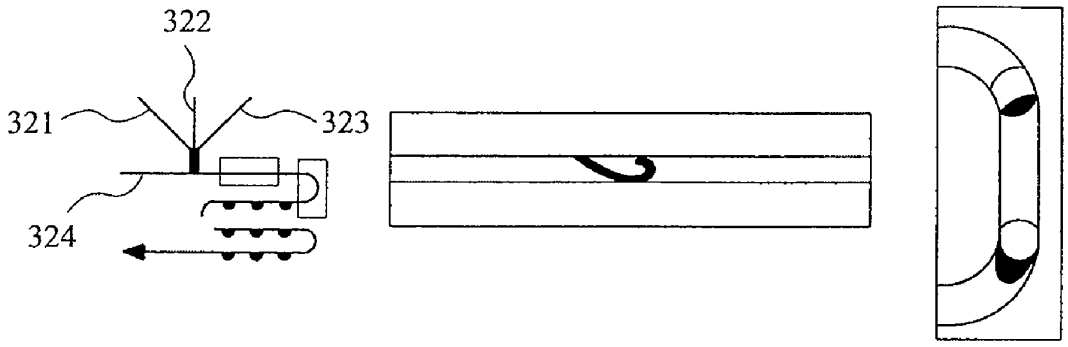


Fig. 32

FIG. 33A

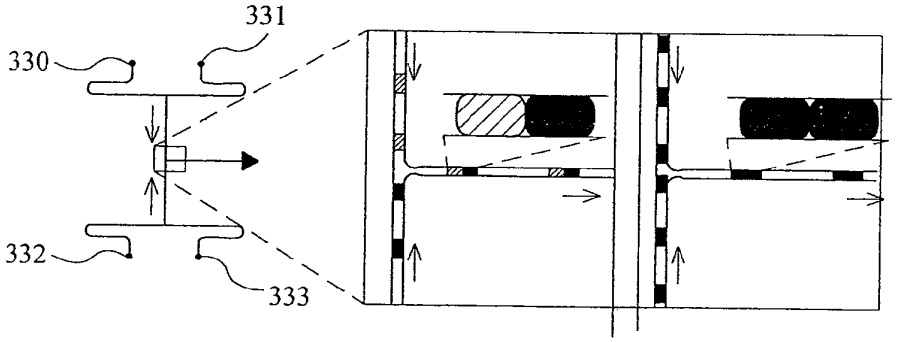


FIG. 33B

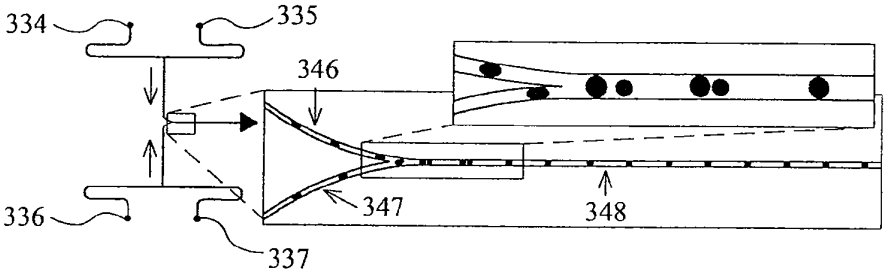


FIG. 33C

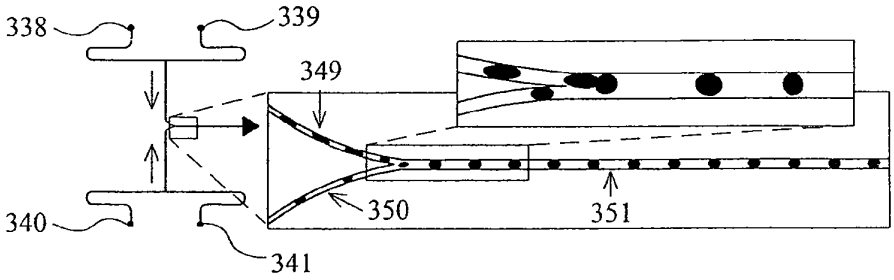


FIG. 33D

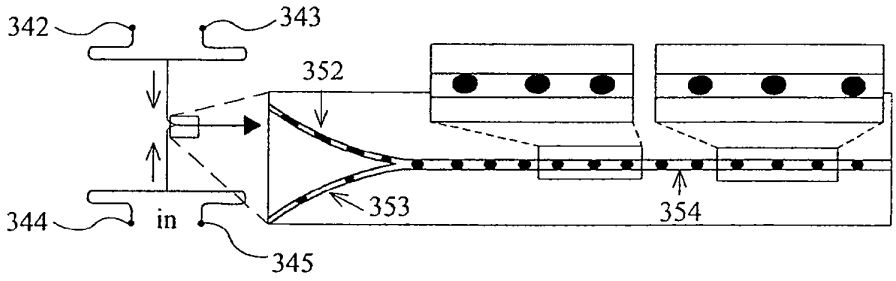


Fig. 33

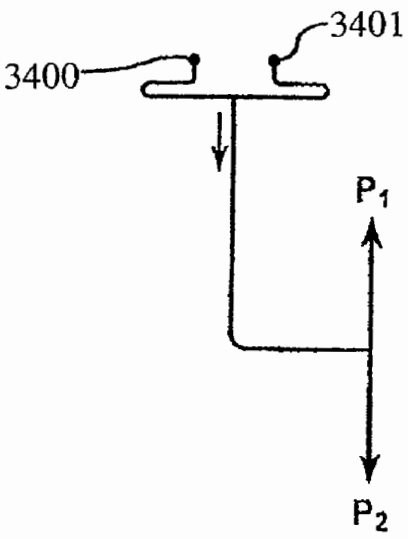


FIG. 34A

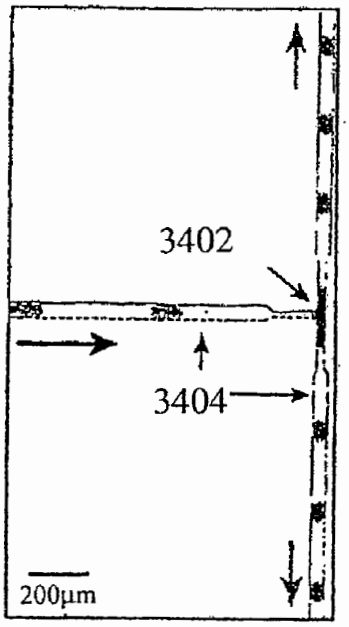


FIG. 34B

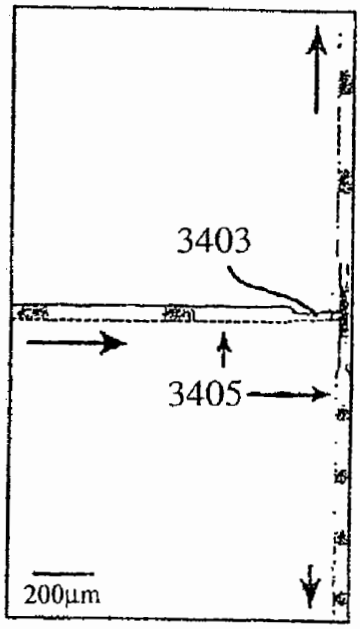


FIG. 34C

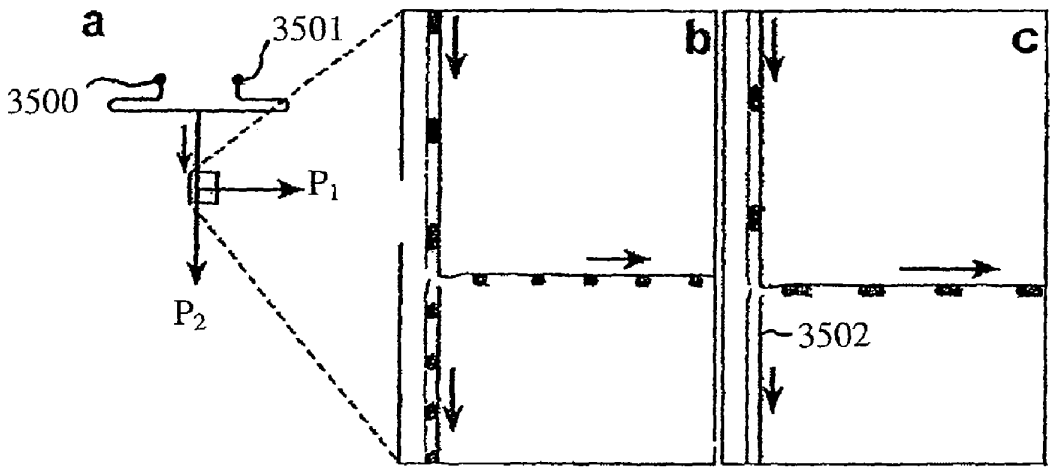


Fig. 35

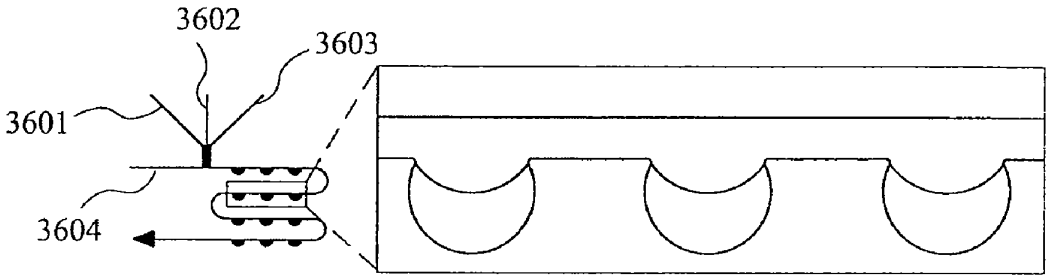


Fig. 36

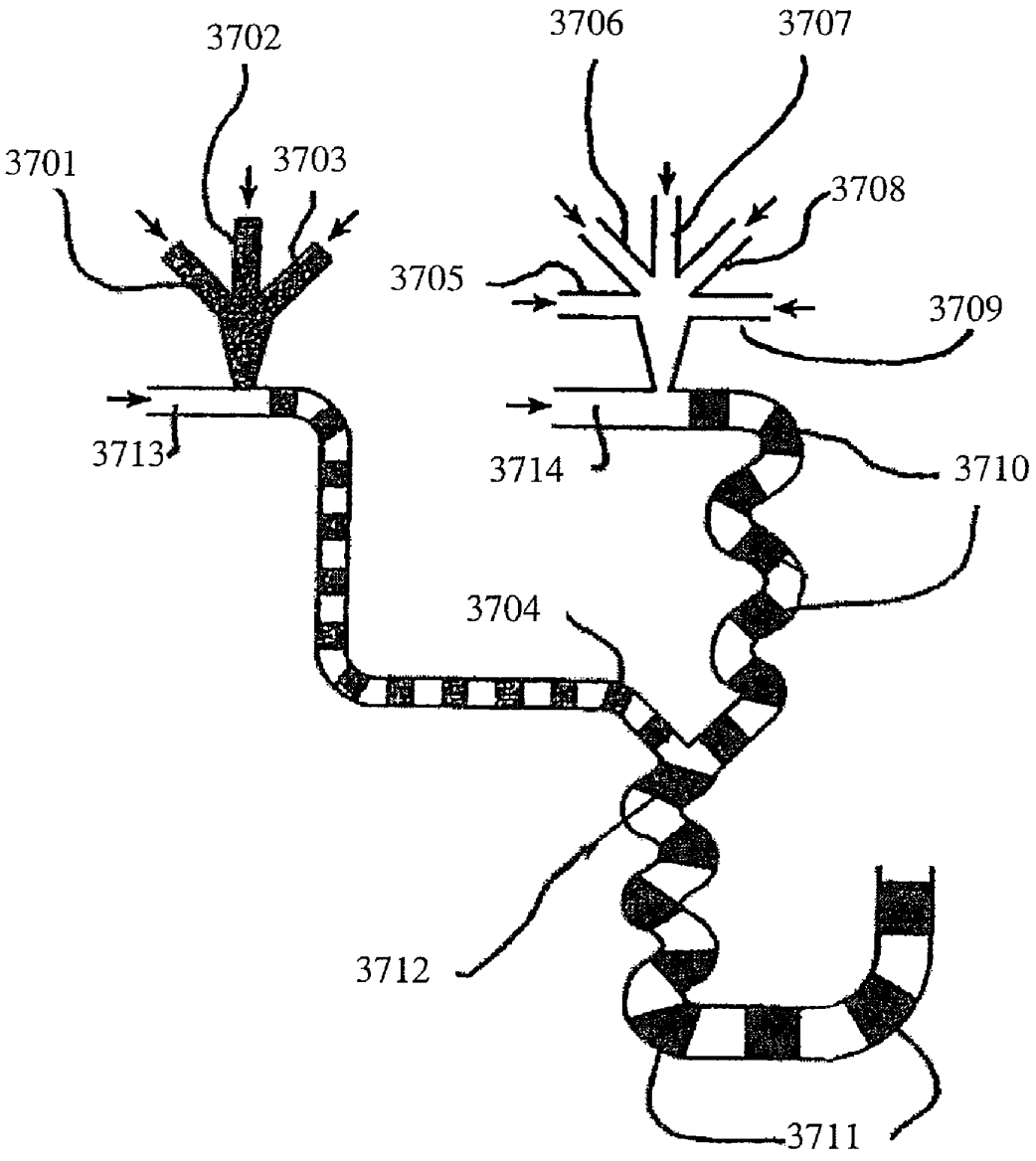

3702
3706
3707
3701
3703
3708
3705
3709
3713
3714
3710
3704
3712
3711

Fig. 37

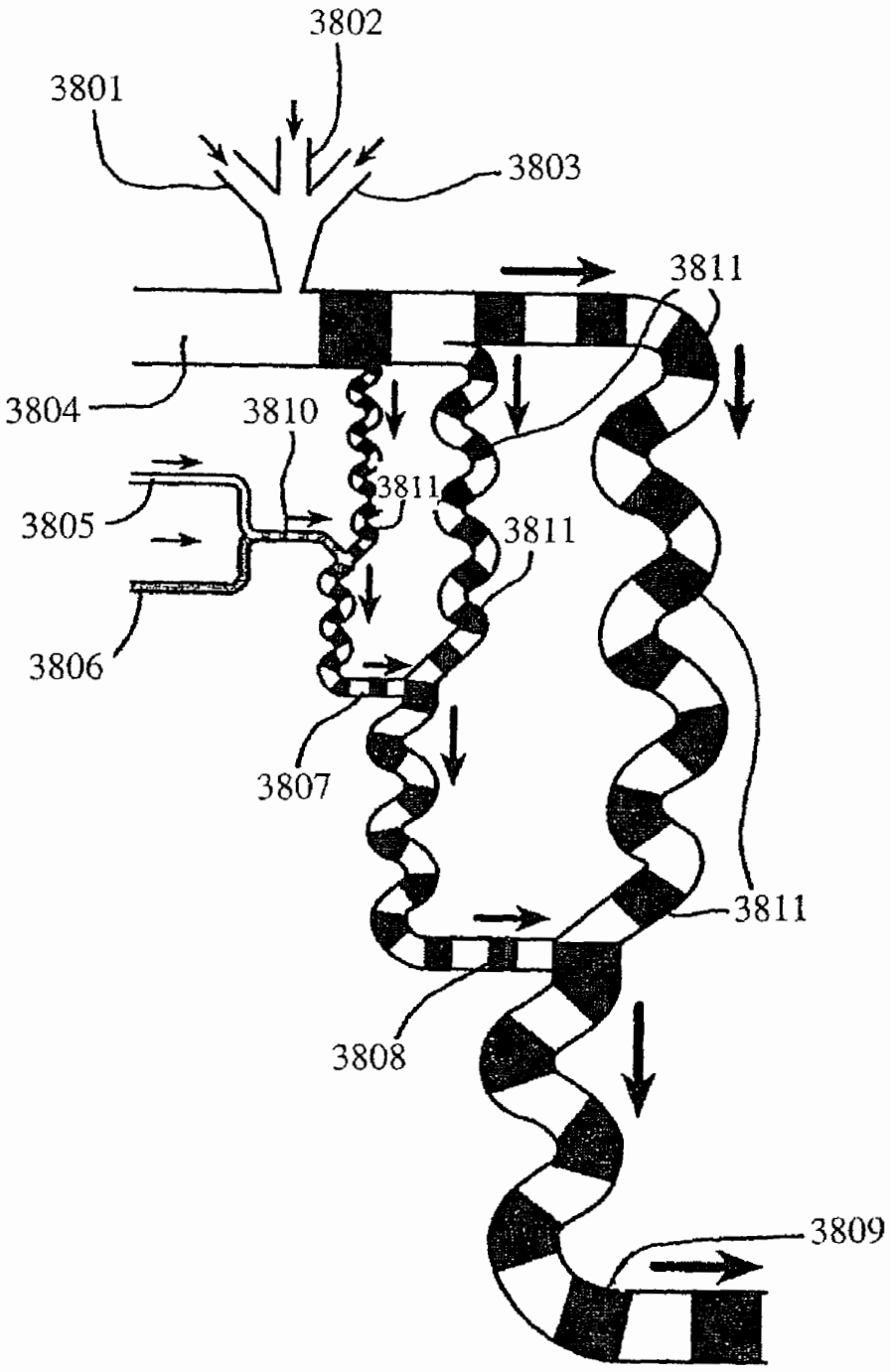

3802
3801
3803
3811
3804
3810
3811
3805
3811
3806
3807
3811
3808
3809

Fig. 38

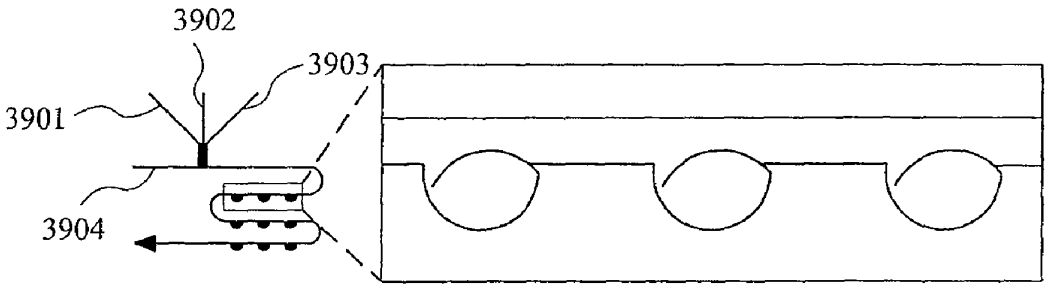


Fig. 39

FIG. 40

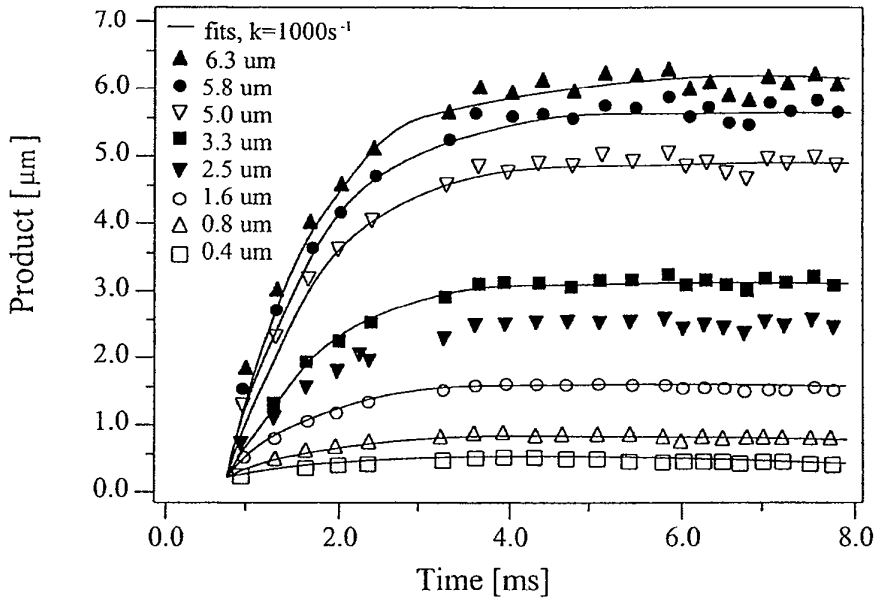

fits, k=1000s-1
6.3 um
5.8 um
5.0 um
3.3 um
2.5 um
1.6 um
0.8 um
0.4 um
Product [μm]
Time [ms]
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
0.0
2.0
4.0
6.0
8.0

FIG. 40A

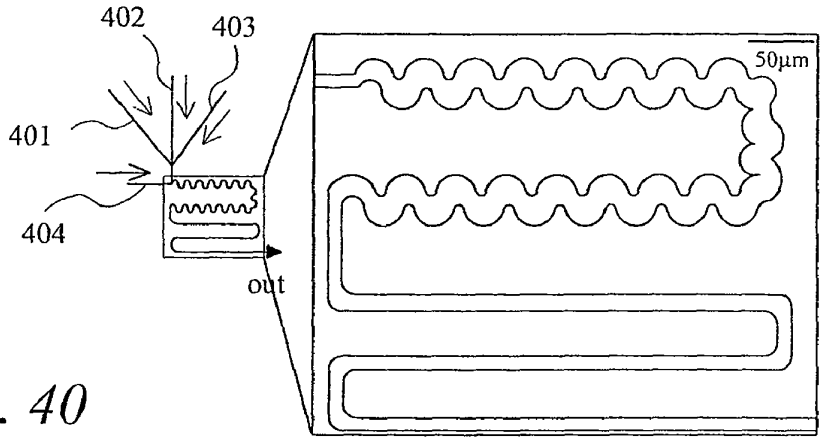
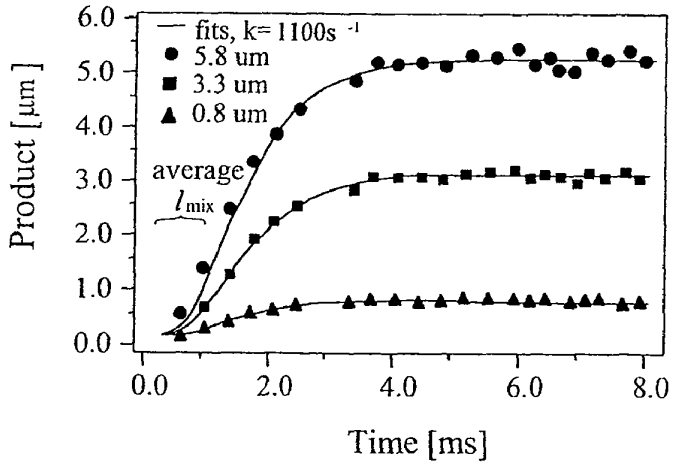


Fig. 40

FIG. 40B



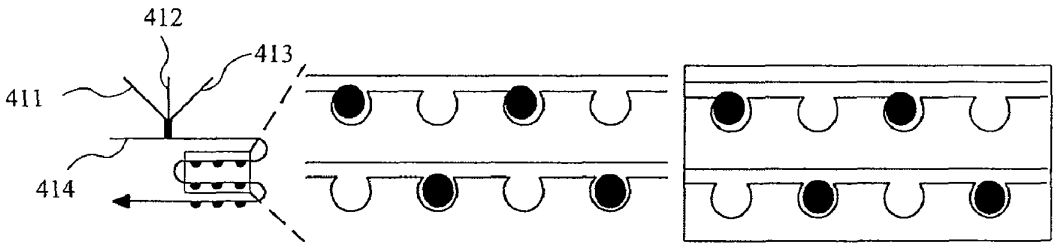


FIG. 41A FIG. 41B FIG. 41C

Fig. 41

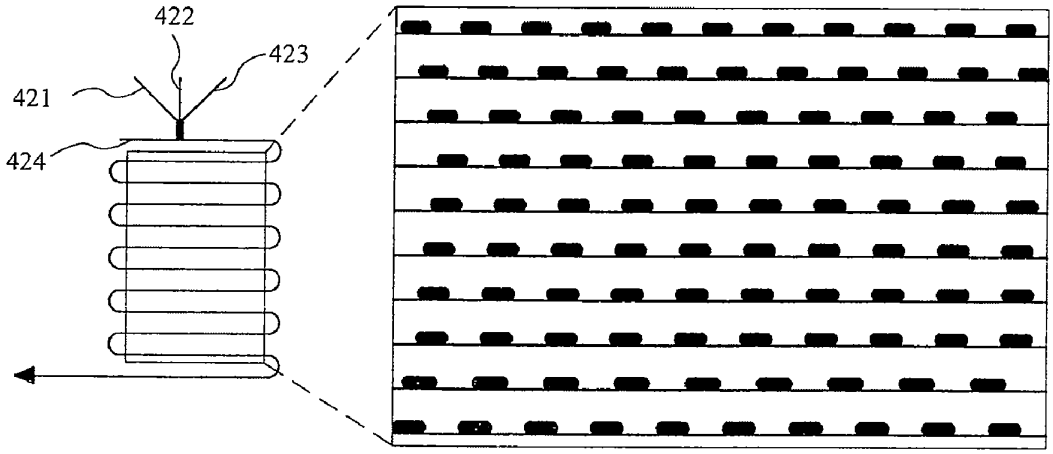


Fig. 42

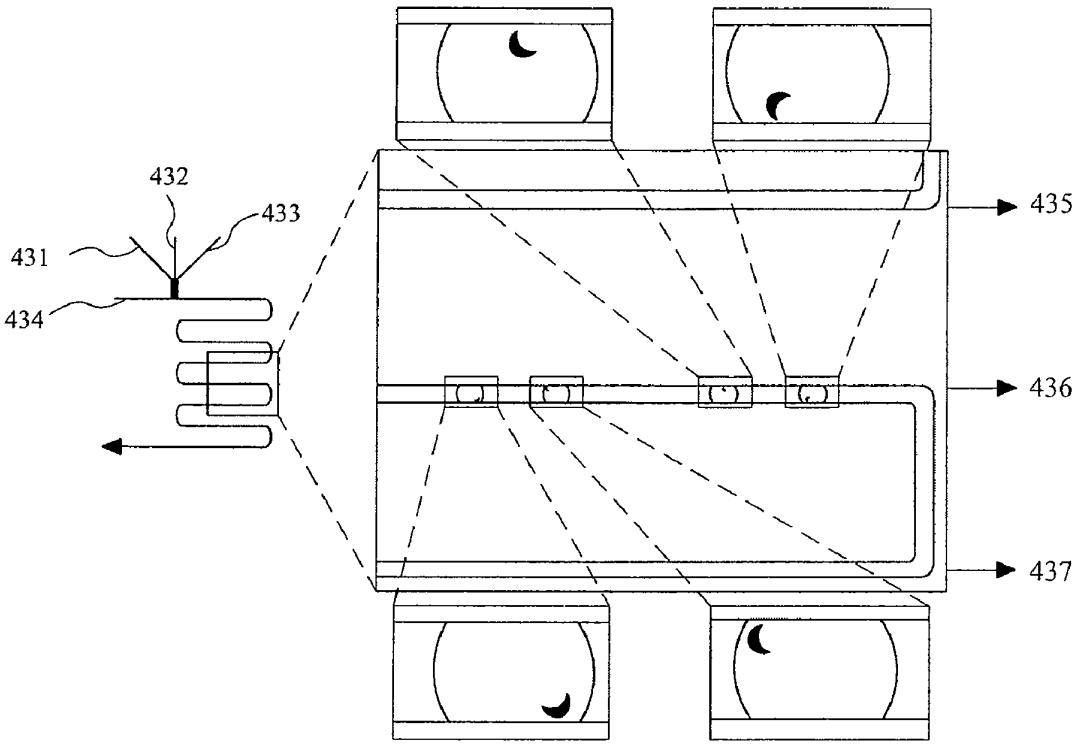


Fig. 43

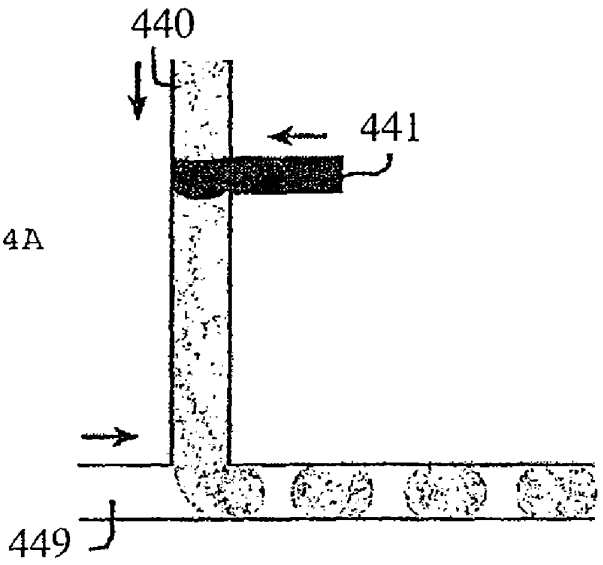


FIG. 44A

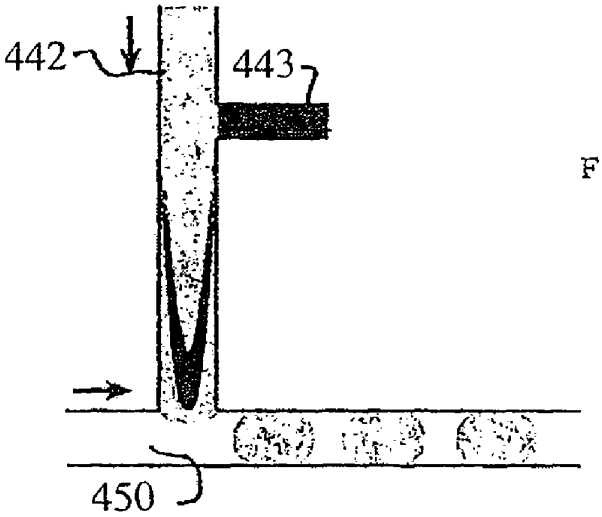


FIG. 44B

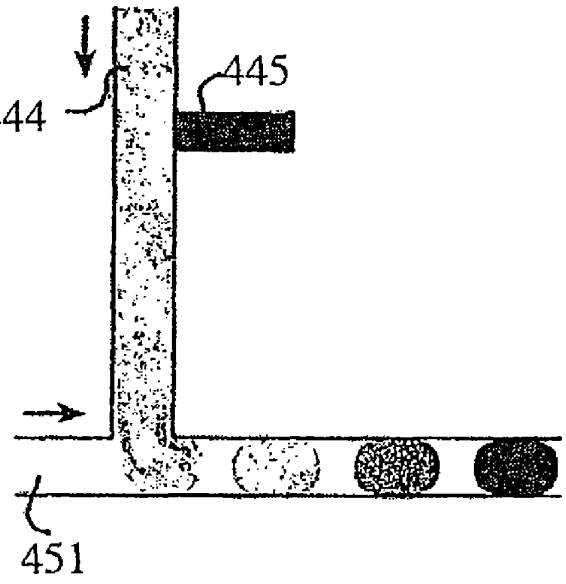


FIG. 44C

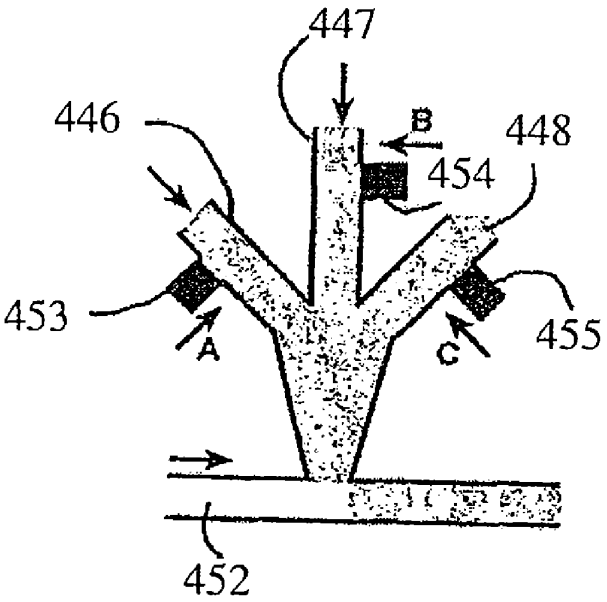
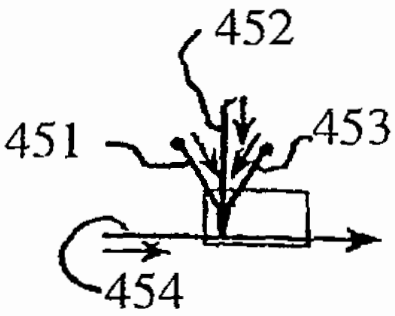


FIG. 44D

FIG. 45A



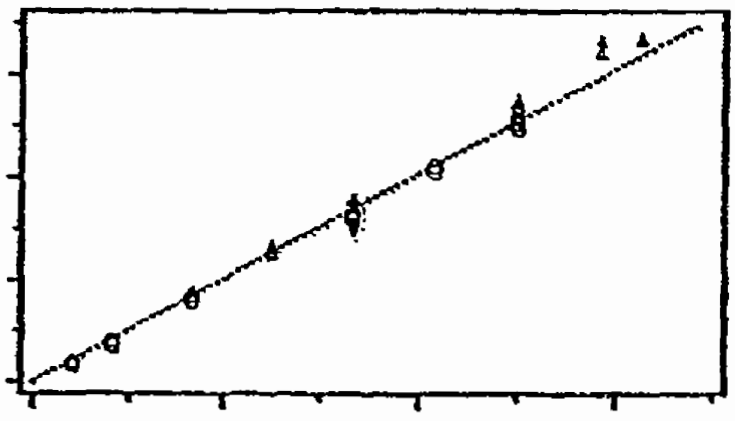


FIG. 45B

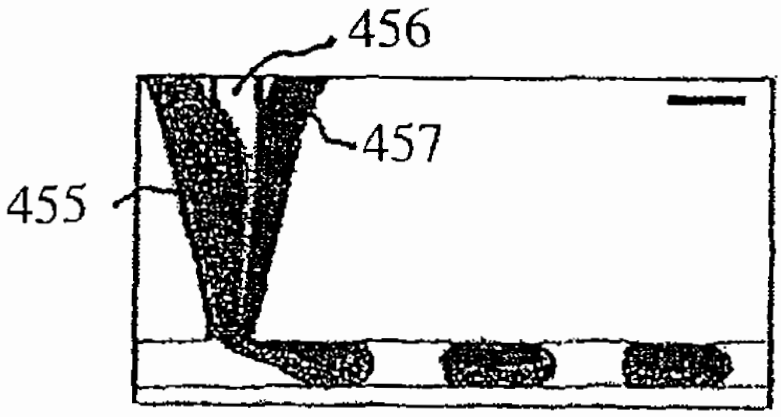


FIG. 45C

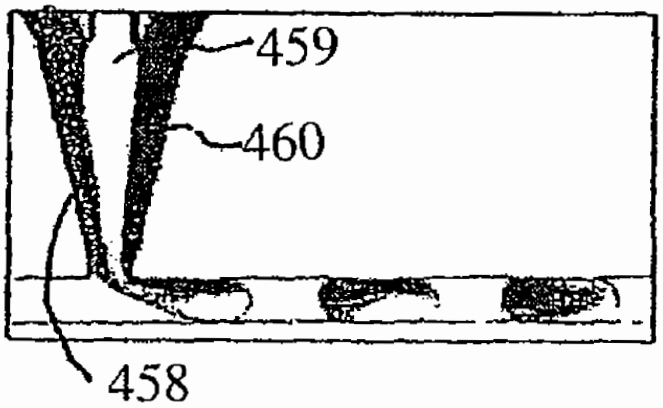


FIG. 45D

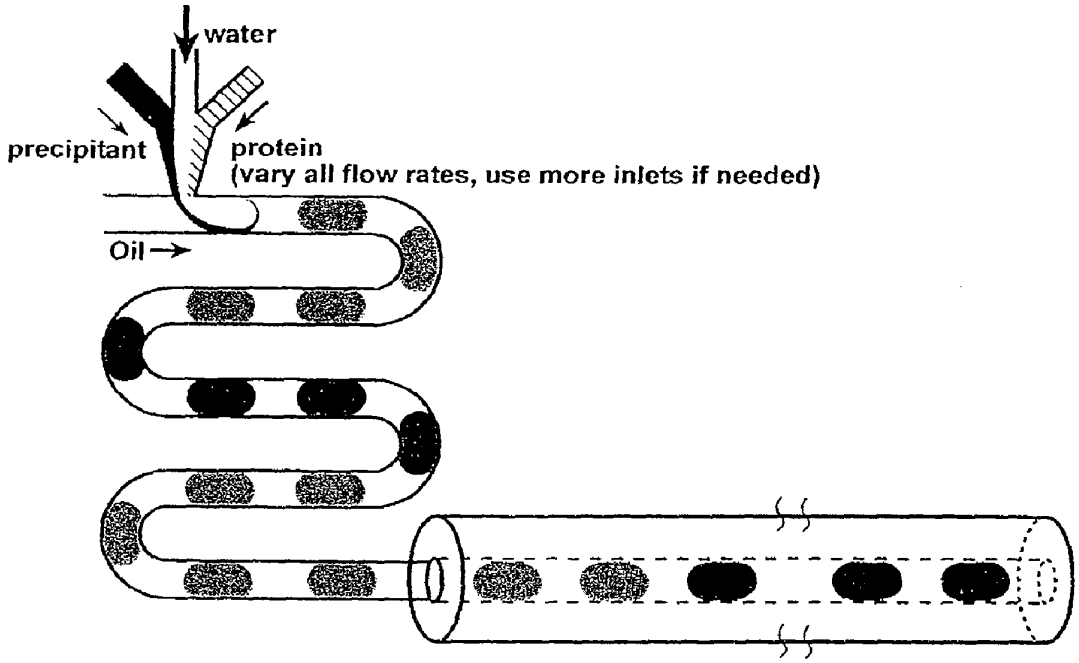

water
precipitant
protein
(vary all flow rates, use more inlets if needed)
Oil

FIGURE 46

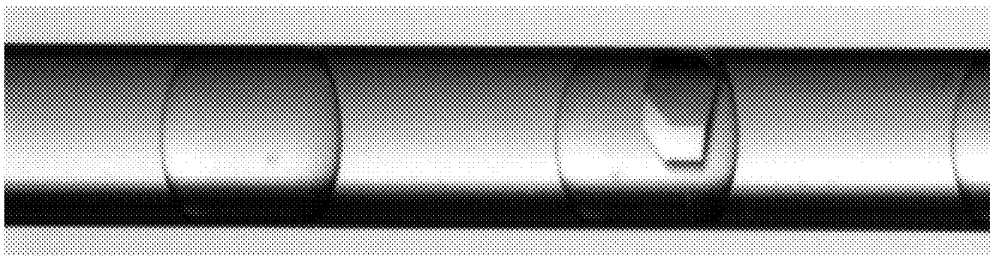


FIGURE 47A

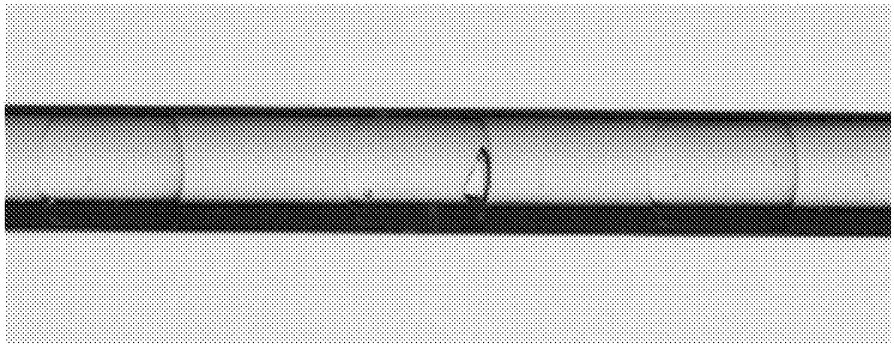


FIGURE 47B

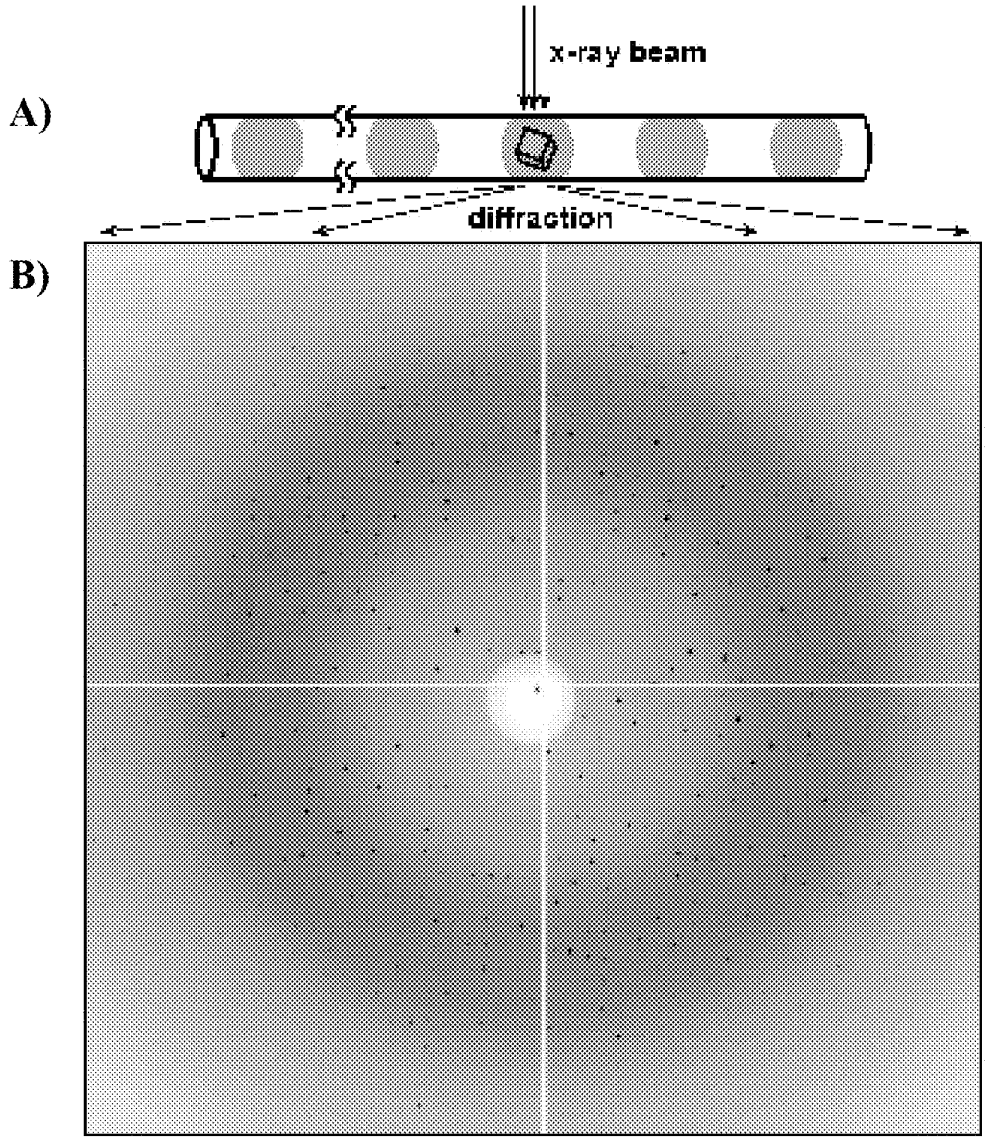


FIGURE 48

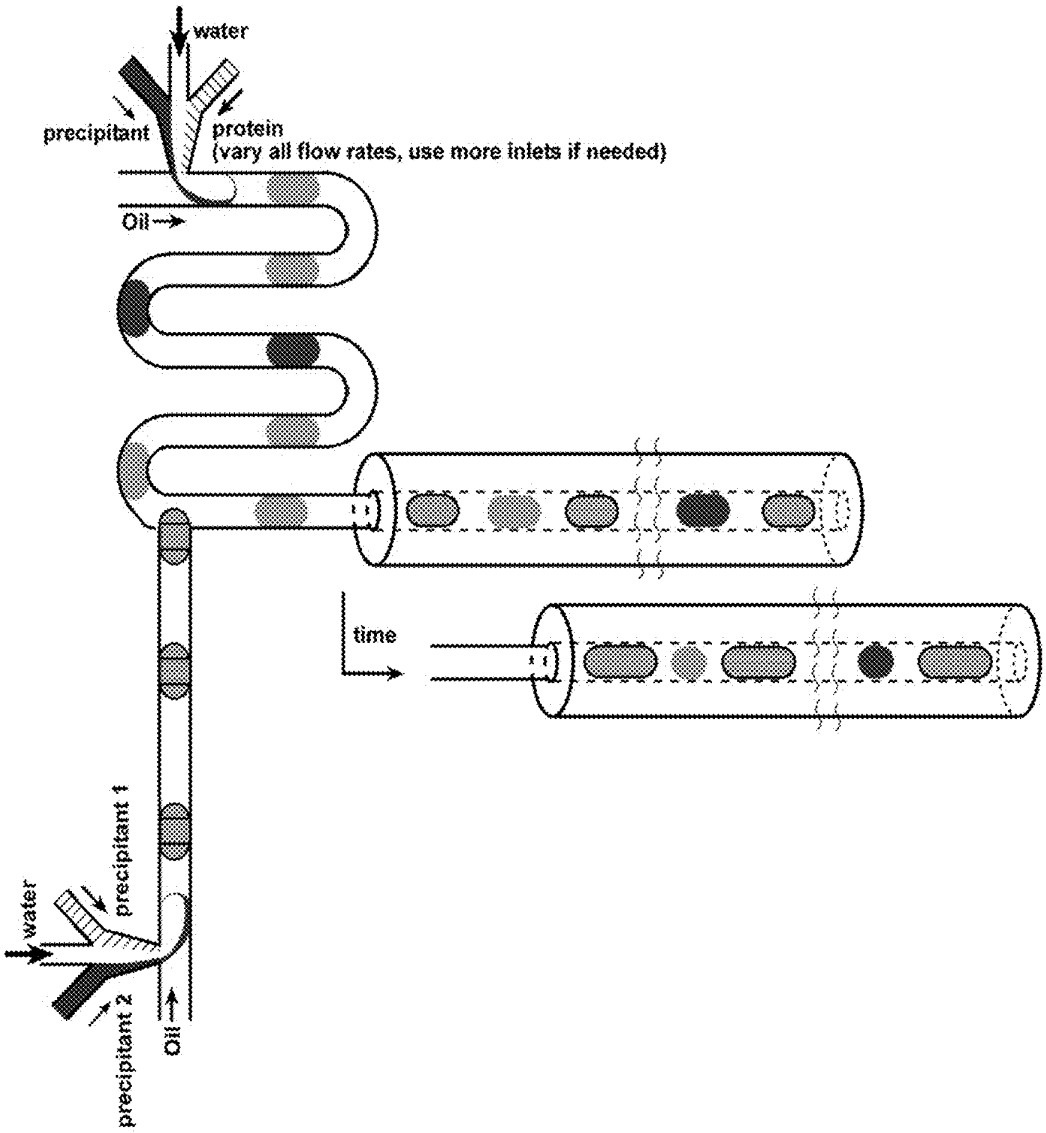

water
precipitant
protein
(vary all flow rates, use more inlets if needed)
Oil
time
water
precipitant 1
precipitant 2
Oil

FIGURE 49

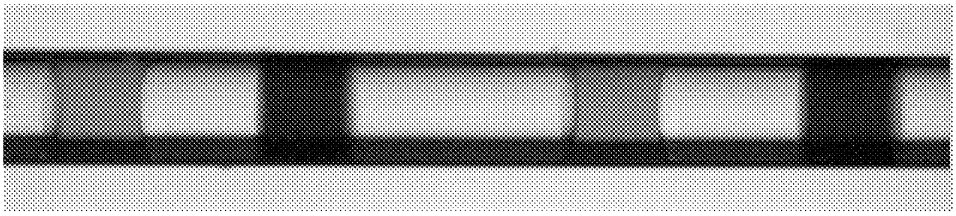


FIGURE 50A

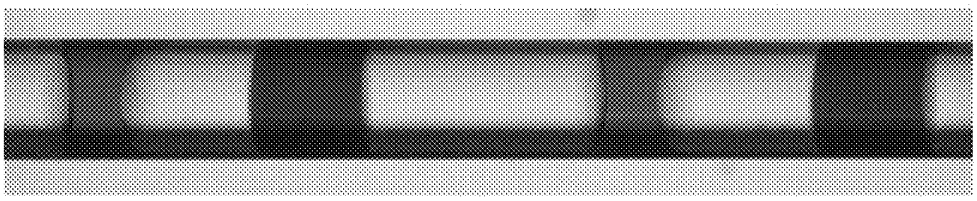


FIGURE 50B

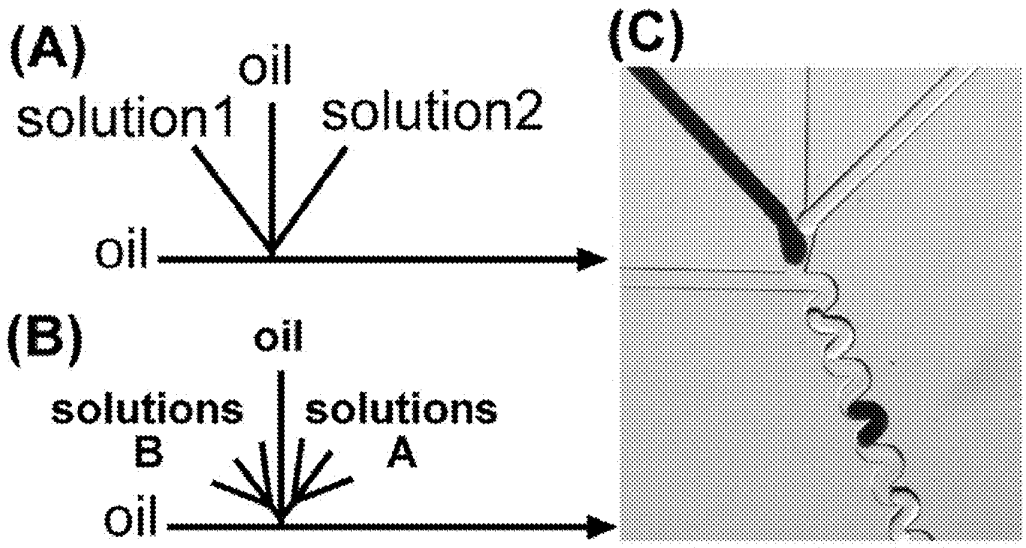

(A)
oil
solution1
solution2
oil
(B)
oil
solutions
B
solutions
A
oil
(C)

FIGURE 51

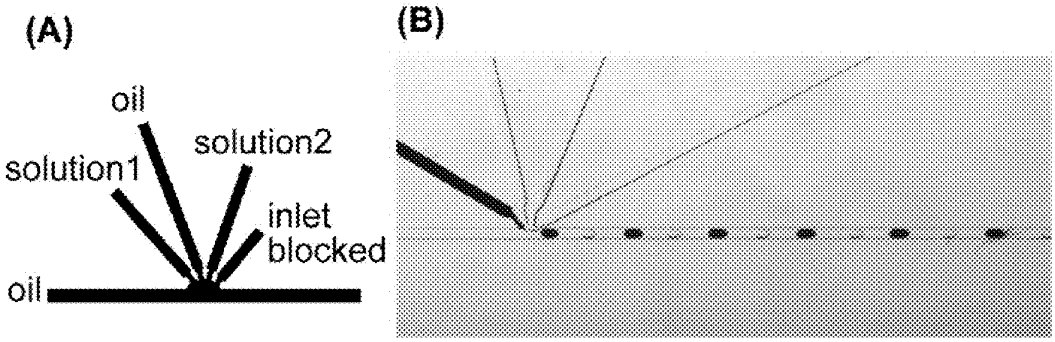


FIGURE 52

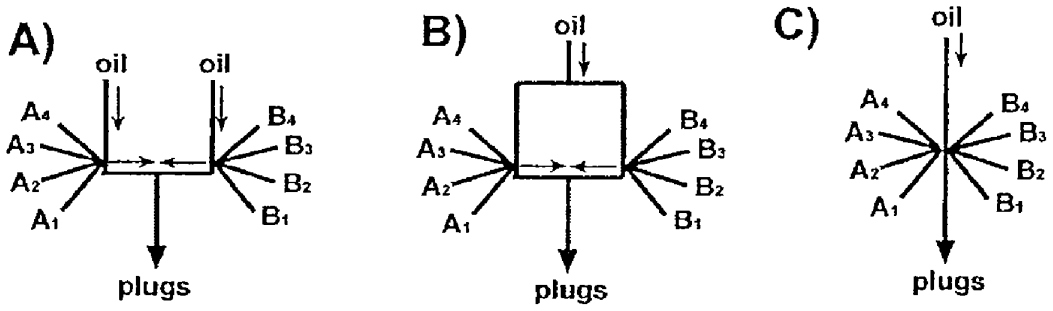

A)
oil
oil
A4
A3
A2
A1
B4
B3
B2
B1
plugs
B)
oil
A4
A3
A2
A1
B4
B3
B2
B1
plugs
C)
oil
A4
A3
A2
A1
B4
B3
B2
B1
plugs

FIGURE 53

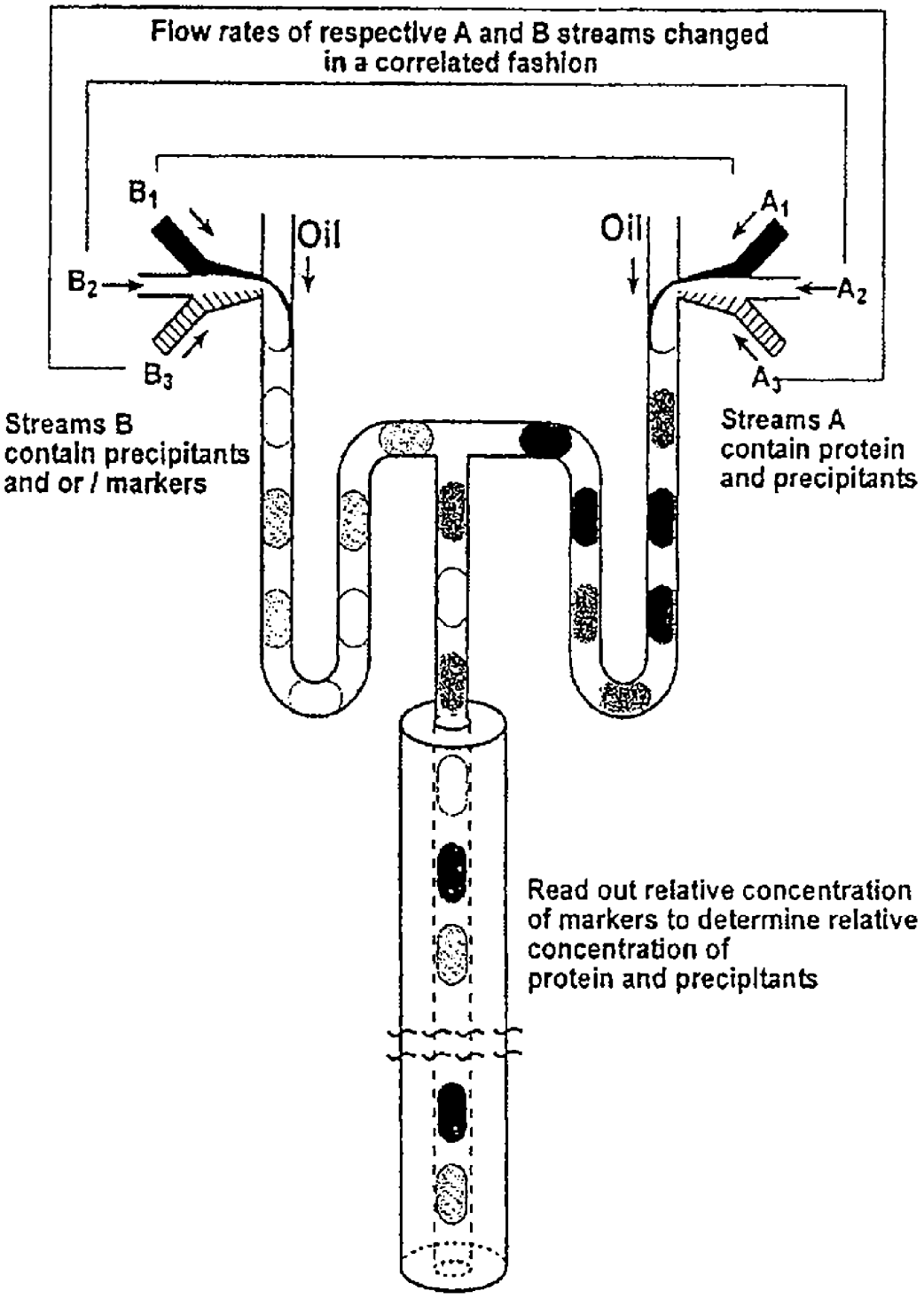

Flow rates of respective A and B streams changed in a correlated fashion
B1
B2
B3
Oil
Oil
A1
A2
A3
Streams B contain precipitants and or / markers
Streams A contain protein and precipitants
Read out relative concentration of markers to determine relative concentration of protein and precipitants

FIGURE 54A

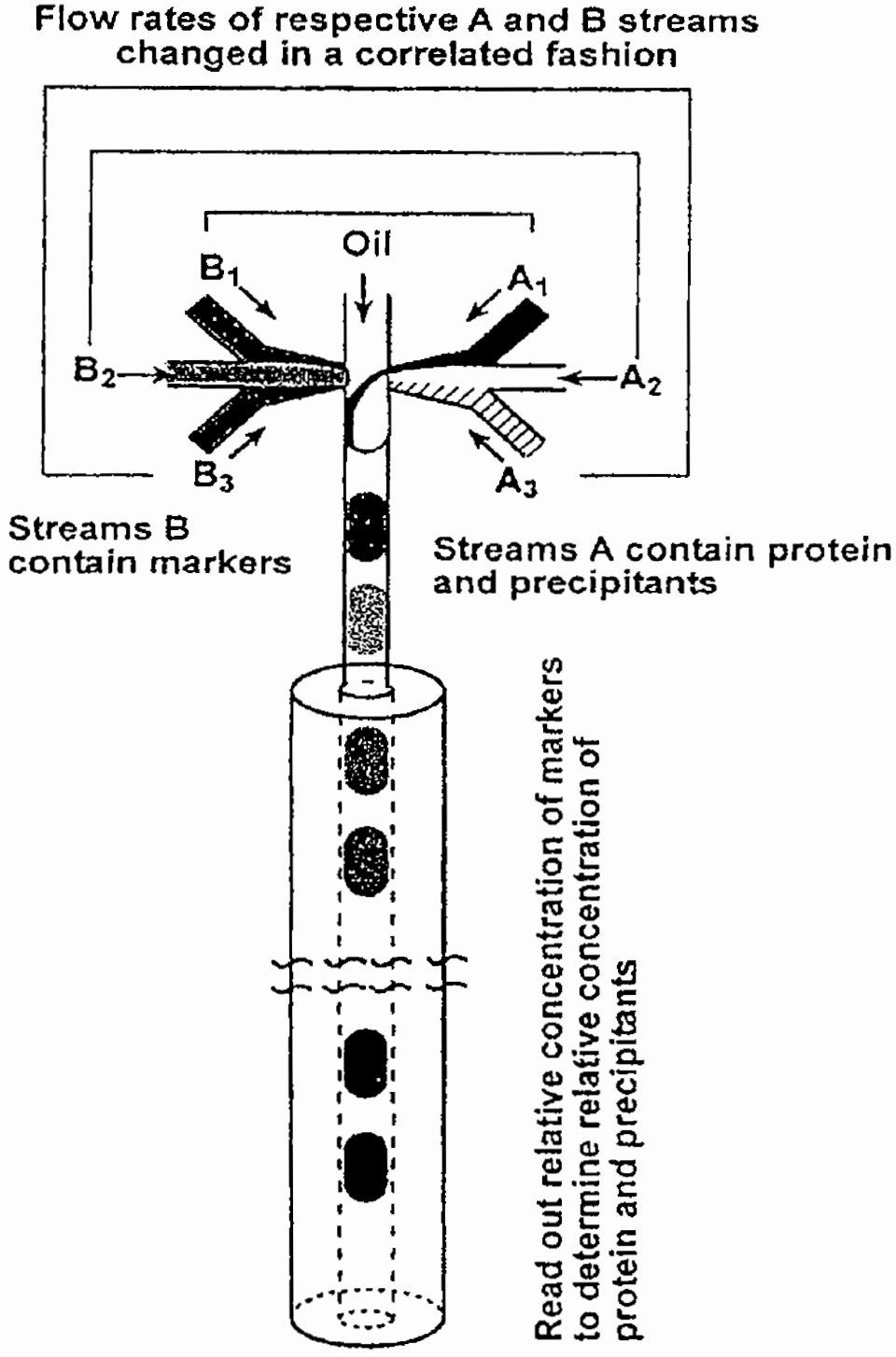

Flow rates of respective A and B streams changed in a correlated fashion
Oil
B1
A1
B2
A2
B3
A3
Streams B contain markers
Streams A contain protein and precipitants
Read out relative concentration of markers to determine relative concentration of protein and precipitants

FIGURE 54B

METHOD FOR CONDUCTING REACTIONS INVOLVING BIOLOGICAL MOLECULES IN PLUGS IN A MICROFLUIDIC SYSTEM

This application is a continuation of application Ser. No. 12/777,099, filed May 10, 2010, which is a continuation of application Ser. No. 10/765,718, filed Jan. 26, 2004 now U.S. Pat. 7,901,939, which is a continuation-in-part of application Ser. No. 10/434,970, filed May 9, 2003 now U.S. Pat. 7,129,091, which claims the benefit of U.S. Provisional Application No. 60/394,544, filed Jul. 8, 2002, and U.S. Provisional Application No. 60/379,927, filed May 9, 2002, all of which are incorporated herein by reference.

BACKGROUND

Nonlinear dynamics, in conjunction with microfluidics, play a central role in the design of the devices and the methods according to the invention. Microfluidics deals with the transport of fluids through networks of channels, typically having micrometer dimensions. Microfluidic systems (sometimes called labs-on-a-chip) find applications in microscale chemical and biological analysis (micro-total-analysis systems). The main advantages of microfluidic systems are high speed and low consumption of reagents. They are thus very promising for medical diagnostics and high-throughput screening. Highly parallel arrays of microfluidic systems are used for the synthesis of macroscopic quantities of chemical and biological compounds, e.g., the destruction of chemical warfare agents and pharmaceuticals synthesis. Their advantage is improved control over mass and heat transport.

Microfluidic systems generally require means of pumping fluids through the channels. In the two most common methods, the fluids are either driven by pressure or driven by electroosmotic flow (EOF). Flows driven by EOF are attractive because they can be easily controlled even in complicated networks. EOF-driven flows have flat, plug-like velocity profile, that is, the velocity of the fluid is the same near the walls and in the middle of the channel. Thus, if small volumes of multiple analytes are injected sequentially into a channel, these plugs are transported as non-overlapping plugs (low dispersion), in which case the dispersion comes mostly from the diffusion between plugs. A main disadvantage of EOF is that it is generated by the motion of the double layer at the charged surfaces of the channel walls. EOF can therefore be highly sensitive to surface contamination by charged impurities. This may not be an issue when using channels with negative surface charges in DNA analysis and manipulation because DNA is uniformly negatively charged and does not adsorb to the walls. However, this can be a serious limitation in applications that involve proteins that are often charged and tend to adsorb on charged surfaces. In addition, high voltages are often undesirable, or sources of high voltages such as portable analyzers may not be available.

Flows driven by pressure are typically significantly less sensitive to surface chemistry than EOF. The main disadvantage of pressure-driven flows is that they normally have a parabolic flow profile instead of the flat profile of EOF. Solutes in the middle of the channel move much faster (about twice the average velocity of the flow) than solutes near the walls of the channels. A parabolic velocity profile normally leads to high dispersion in pressure-driven flows; a plug of solute injected into a channel is immediately distorted and stretched along the channel. This distortion is somewhat reduced by solute transport via diffusion from the middle of the channel towards the walls and back. But the distortion is made worse by diffusion along the channel (the overall dispersion is known as Taylor dispersion).

Taylor dispersion broadens and dilutes sample plugs. Some of the sample is frequently left behind the plug as a tail. Overlap of these tails usually leads to cross-contamination of samples in different plugs. Thus, samples are often introduced into the channels individually, separated by buffer washes. On the other hand, interleaving samples with long buffer plugs, or washing the system with buffer between samples, reduces the throughput of the system.

In EOF, flow transport is essentially linear, that is, if two reactants are introduced into a plug and transported by EOF, their residence time (and reaction time) can be calculated simply by dividing the distance traveled in the channel by the velocity. This linear transport allows precise control of residence times through a proper adjustment of the channel lengths and flow rates. In contrast, dispersion in pressure-driven flow typically creates a broad range of residence times for a plug traveling in such flows, and this diminishes time control.

The issue of time control is important. Many chemical and biochemical processes occur on particular time scales, and measurement of reaction times can be indicative of concentrations of reagents or their reactivity. Stopped-flow type instruments are typically used to perform these measurements. These instruments rely on turbulent flow to mix the reagents and transport them with minimal dispersion. Turbulent flow normally occurs in tubes with large diameter and at high flow rates. Thus stopped-flow instruments tend to use large volumes of reagents (on the order of ml/s). A microfluidic analog of stopped-flow, which consumes smaller volumes of reagents (typically μL/min), could be useful as a scientific instrument, e.g., as a diagnostic instrument. So far, microfluidic devices have not be able to compete with stopped-flow type instruments because EOF is usually very slow (although with less dispersion) while pressure-driven flows suffer from dispersion.

In addition, mixing in microfluidic systems is often slow regardless of the method used to drive the fluid because flow is laminar in these systems (as opposed to turbulent in larger systems). Mixing in laminar flows relies on diffusion and is especially slow for larger molecules such as DNA and proteins.

In addition, particulates present handling difficulty in microfluidic systems. While suspensions of cells in aqueous buffers can be relatively easy to handle because cells are isodense with these buffers, particulates that are not isodense with the fluid tend to settle at the bottom of the channel, thus eventually blocking the channel. Therefore, samples for analysis often require filtration to remove particulates.

SUMMARY ACCORDING TO THE INVENTION

In one aspect, a method includes the steps of providing a microfluidic system comprising one or more channels, and providing within the one or more channels a carrier fluid, a first plug, and a second plug. The first and second plugs each are immiscible with the carrier fluid. The first plug moves at a first velocity and the second plug moves at a second velocity faster than the first velocity when they are within a common channel of the one or more channels such that a distance between the first and second plugs decreases over time. The method further includes the step of merging the first and second plugs into a merged plug when the second plug catches up to the first plug.

In another aspect, the first plug, the second plug, the carrier fluid, or any combination thereof includes a surfactant. The surfactant may include a fluorinated surfactant.

In another aspect, the first plug and the second plug are surrounded by the carrier fluid within the common channel.

In another aspect, the merging step further includes the step of providing an electric field to manipulate the first plug, the second plug, or both.

In yet another aspect, the first plug includes at least a first reactant or first solvent, the second plug includes at least a second reactant or second solvent, and the merged plug includes the first reactant or first solvent and the second reactant or second solvent. The first reactant may be different than the second reactant, and the first solvent may be different than the second solvent, or both.

In another aspect, the method further includes the step of mixing the first reactant or first solvent and the second reactant or second solvent within the merged plug. The mixing step may be accelerated by varying an electric charge of at least a portion of the common channel.

In another aspect, after a reaction time, the merged plug further includes an intermediate of a reaction between the first and second reactants, a reaction product of a reaction between the first and second reactants or either of them with the intermediate, or any combination thereof.

In another aspect, the method includes the step of providing a detector to detect, monitor, or analyze one or more properties of the first plug, the second plug, the first reactant, the second reactant, an intermediate of a reaction between the first and second reactants, a reaction product of a reaction between the first and second reactants or either of them with the intermediate, or any combination thereof.

In yet another aspect, the method further includes the step of providing within the one or more channels a plurality of third plugs that are substantially similar in size to the first plug and comprise the first reactant or first solvent, and providing within the one or more channels a plurality of fourth plugs that are substantially similar in size to the second plug and comprise the second reactant or second solvent, wherein the third and fourth plugs are arranged in the common channel in an alternating order of third plug, fourth plug, third plug, fourth plug, and so on. Each of the first and third plugs may be substantially the same first size, each of the second and fourth plugs may be substantially the same second size, and the second size may be different than the first size.

In another aspect, the first plug, the second plug, or both are substantially spherical in shape.

In yet another aspect, the first plug has a first size and the second plug has a second size that is different than the first size at the time of the merging step.

In a further aspect, the first plug, the second plug, or both include at least one reagent for an autocatalytic reaction. The autocatalytic reaction may be a polymerase-chain reaction.

In another aspect, the first plug, the second plug, or both include at least one of a cell, a virion, a protein, an enzyme, a carbohydrate, a sugar, a DNA and an RNA, or a portion thereof.

In another aspect, the first plug, the second plug, or both include only a single one of a cell, a virion, a protein, an enzyme, a carbohydrate, a sugar, a DNA and an RNA, or a portion thereof.

In yet another aspect, the method further includes the step of splitting the merged plug into at least a first part and a second part. The method may also include the step of providing that, at a time after splitting, the first part and the second part flow in different channels of the one or more channels.

In a further aspect, the method includes separating the merged plug from the carrier fluid.

BRIEF DESCRIPTION OF THE DRAWINGS AND PHOTOGRAPHS

FIG. **1**A is a schematic diagram of a basic channel design that may be used to induce rapid mixing in plugs. FIG. **1**B(**1**)-(**4**) are schematic diagrams depicting a series of periodic variations of the basic channel design. FIG. **1**C(**1**)-(**4**) are schematic diagrams depicting a series of aperiodic combinations resulting from a sequence of alternating elements taken from a basic design element shown in FIG. **1**A and an element from the periodic variation series shown in FIGS. **10**B(**1**)-(**4**).

FIG. **2**A is a schematic diagram contrasting laminar flow transport and plug transport in a channel. FIG. **2**B(**1**) shows a photograph (right side, top portion) illustrating rapid mixing inside plugs moving through winding channels. FIG. **2**B(**2**) shows a photograph (right side, lower portion) showing that winding channels do not accelerate mixing in a laminar flow in the absence of PFD.

FIG. **3** shows photographs (right side) and schematic diagrams (left side) that depict a stream of plugs from an aqueous plug-fluid and an oil (carrier-fluid) in curved channels at flow rates of 0.5 μL/min and 1.0 μL/min.

FIG. **4** shows a photograph (lower portion) and a schematic diagram (upper portion) that illustrate plug formation through the injection of oil and multiple plug-fluids.

FIG. **5** is a schematic diagram that illustrates a two-step reaction in which plugs are formed through the injection of oil and multiple plug-fluids using a combination of different geometries for controlling reactions and mixing.

FIG. **6** is a schematic representation of part of a microfluidic network that uses multiple inlets and that allows for both splitting and merging of plugs. This schematic diagram shows two reactions that are conducted simultaneously. A third reaction (between the first two reaction mixtures) is conducted using precise time delay.

FIG. **7**(*a*)-(*b*) show microphotographs (10 μs exposure) illustrating rapid mixing inside plugs (a) and negligible mixing in a laminar flow (b) moving through winding channels at the same total flow velocity. FIG. **7**(*c*) shows a false-color microphotograph (2 s exposure, individual plugs are invisible) showing time-averaged fluorescence arising from rapid mixing inside plugs of solutions of Fluo-4 and $CaCl_2$. FIG. **7**(*d*) shows a plot of the relative normalized intensity (I) of fluorescence obtained from images such as shown in (c) as a function of distance (left) traveled by the plugs and of time required to travel that distance (right) at a given flow rate. FIG. **7**(*e*) shows a false-color microphotograph (2 s exposure) of the weak fluorescence arising from negligible mixing in a laminar flow of the solutions used in (c).

FIG. **8** shows photographs (right side) and schematics (left side) that illustrate fast mixing at flow rates of about 0.5 μL/min and about 1.0 μL/min using 90°-step channels.

FIG. **9** shows schematics (left side) and photographs (right side) illustrates fast mixing at flow rates of about 1.0 μL/min and about 0.5 μL/min using 135°-step channels.

FIG. **10***a*) is a schematic diagram depicting three-dimensional confocal visualization of chaotic flows in plugs. FIG. **10***b*) is a plot showing a sequence preferably used for visualization of a three-dimensional flow.

FIG. **11** shows a schematic diagram of a channel geometry designed to implement and visualize the baker's transformation of plugs flowing through microfluidic channels.

FIG. **12** shows photographs depicting the merging of plugs (top) and splitting of plugs (bottom) that flow in separate channels or channel branches that are perpendicular.

FIG. **13** shows UV-VIS spectra of CdS nanoparticles formed by rapid mixing in plugs (spectrum with a sharp absorption peak) and by conventional mixing of solutions.

FIG. **14** shows schematic diagrams (left side) and photographs (right side) that illustrate the synthesis of CdS nanoparticles in PDMS microfluidic channels in single-phase aqueous laminar flow (FIG. **14**A) and in aqueous plugs that are surrounded by water-immiscible perfluorodecaline (FIG. **14**B).

FIG. **15** shows schematic representations of the synthesis of CdS nanoparticles inside plugs.

FIG. **16** is a schematic illustration of a microfluidic device according to the invention that illustrates the trapping of plugs.

FIG. **17** is a schematic of a microfluidic method for forming plugs with variable compositions for protein crystallization.

FIG. **18** is a schematic illustration of a method for controlling heterogeneous nucleation by varying the surface chemistry at the interface of an aqueous plug-fluid and a carrier-fluid.

FIG. **19** is a schematic diagram that illustrates a method of separating nucleation and growth using a microfluidic network according to the present invention.

FIG. **20** show schematic diagrams that illustrate two methods that provide a precise and reproducible degree of control over mixing and that can be used to determine the effect of mixing on protein crystallization.

FIG. **21** is a reaction diagram illustrating an unstable point in the chlorite-thiosulfate reaction.

FIG. **22**A-D are schematic diagrams that show various examples of geometries of microfluidic channels according to the invention for obtaining kinetic information from single optical images.

FIG. **23** shows a schematic of a microfluidic network (left side) and a table of parameters for a network having channel heights of 15 and 2 μm.

FIG. **24** shows a reaction scheme that depicts examples of fluorinated surfactants that form monolayers that are: (a) resistant to protein adsorption; (b) positively charged; and (c) negatively charged. FIG. **24***b* shows a chemical structure of neutral surfactants charged by interactions with water by protonation of an amine or a guanidinium group. FIG. **24***c* shows a chemical structure of neutral surfactants charged by interactions with water deprotonation of a carboxylic acid group.

FIG. **25** are schematic diagrams of microfluidic network (left side of a), b), and c)) that can be used for controlling the concentrations of aqueous solutions inside the plugs, as well as photographs (right side of a), b), and c)) showing the formation of plugs with different concentrations of the aqueous streams.

FIG. **26** are schematic diagrams of microfluidic network (left side of a) and b)) and photographs (right side of a) and b)) of the plug-forming region of the network in which the aqueous streams were dyed with red and green food dyes to show their flow patterns.

FIG. **27** are photographs and plots showing the effects of initial conditions on mixing by recirculating flow inside plugs moving through straight microchannels. FIG. **27***a***1**) is a schematic diagram showing that recirculating flow (shown by black arrows) efficiently mixed solutions of reagents that were initially localized in the front and back halves of the plug. FIG. **27***a***2**) is a schematic diagram showing that recirculating flow (shown by black arrows) did not efficiently mix solutions of reagents that were initially localized in the left and right halves of the plugs. FIG. **27***b*) shows a schematic diagram showing the inlet portions (left side) and photographs of images showing measurements of various periods and lengths of plugs. FIG. **27***c***1**) shows a graph of the relative optical intensity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs of varying lengths. FIG. **27***c***2**) is the same as FIG. **7***c***1**) except that each plug traverses a distance of 1.3 mm.

FIG. **28** is a schematic illustration of a plug showing the notation used to identify different regions of the plugs relative to the direction of motion.

FIG. **29***a*)-*b*) are plots of the periods and the lengths of plugs as a function of total flow velocity (FIG. **29***a*)) and water fraction (FIG. **29***b*)).

FIG. **30** shows photographs illustrating weak dependence of periods, length of plugs, and flow patterns inside plugs on total flow velocity.

FIG. **31** are plots showing the distribution of periods and lengths of plugs where the water fractions were 0.20, 0.40, and 0.73, respectively.

FIG. **32** shows photographs (middle and right side) that show that plug traps are not required for crystal formation in a microfluidic network, as well as a diagram of the microfluidic network (left side).

FIG. **33***a-d* (left side) are top views of microfluidic networks (left side) and photographs (right side) that comprise channels having either uniform or nonuniform dimension. FIG. **33***a* shows that merging of the plugs occurs infrequently in the T-shaped channel shown in the photographs. FIG. **33***b* illustrates plug merging occurring between plugs arriving at different times at the Y-shaped junction (magnified view shown). FIG. **33***c* depicts in-phase merging, i.e., plug merging upon simultaneous arrival of at least two plugs at a junction, of plugs of different sizes generated using different oil/water ratios at the two pairs of inlets. FIG. **33***d* illustrates defects (i.e., plugs that fail to undergo merging when they would normally merge under typical or ideal conditions) produced by fluctuations in the relative velocity of the two incoming streams of plugs.

FIG. **34***a-c* show a schematic diagram (a, left side) and photographs (b, c) each of which depicts a channel network viewed from the top. FIG. **34***a* is a schematic diagram of the channel network used in the experiment. FIG. **34***b* is a photograph showing the splitting of plugs into plugs of approximately one-half the size of the initial plugs. FIG. **34***c* is a photograph showing the asymmetric splitting of plugs which occurred when $P_1<P_2$.

FIG. **35** shows a schematic diagram (a, left side) and photographs (b, c) that depicts the splitting of plugs using microfluidic networks without constrictions near the junction.

FIG. **36** shows a photograph (right side) of lysozyme crystals grown in water plugs in the wells of the microfluidic channel, as well as a diagram (left side) of the microfluidic network used in the crystallization.

FIG. **37** is a schematic diagram that depicts a microfluidic device according to the invention that can be used to amplify a small chemical signal using an autocatalytic (and possibly unstable) reaction mixture.

FIG. **38** is a schematic diagram that illustrates a method for a multi-stage chemical amplification which can be used to detect as few as a single molecule.

FIG. **39** shows a diagram (left side) of the microfluidic network and a photograph (right side) of water plugs attached to the PDMS wall.

FIG. **40** is a schematic representation (left side) of a microfluidic network used to measure kinetics data for the

reaction of RNase A using a fluorogenic substrate (on-chip enzyme kinetics), and plots that shows the kinetic data for the reaction between RNase A and a fluorogenic substrate.

FIG. **41** shows a photograph (middle and right side) of the water droplet region of the microfluidic network (T stands for time), as well as a diagram of the microfluidic network (left side).

FIG. **42** shows a schematic diagram (left side) of a microfluidic network and a photograph (right side) of the ink plug region of the microfluidic network in which the gradients were formed by varying the flow rates.

FIG. **43** shows a schematic diagram (left side) of a microfluidic network and a photograph (right side) of lysozyme crystals formed in the microfluidic network using gradients.

FIG. **44** are schematic illustrations showing how an initial gradient may be created by injecting a discrete aqueous sample of a reagent B into a flowing stream of water.

FIG. **45***a*) shows a schematic of the microfluidic network used to demonstrate that on-chip dilutions can be accomplished by varying the flow rates of the reagents. The blue rectangle outlines the field of view for images shown in FIG. **45***c*)-*d*). FIG. **45***b*) shows a graph quantifying this dilution method by measuring fluorescence of a solution of fluorescein diluted in plugs in the microchannel.

FIG. **46** shows a microbatch protein crystallization analogue scheme using a with a substrate that includes capillary tubing.

FIG. **47***a*) shows a lysozyme crystal grown attached to a capillary tube wall.

FIG. **47***b*) shows a thaumatin crystal grown at the interface of protein solution and oil.

FIG. **48***a*) shows a schematic illustration of a process for direct screening of crystals in a capillary tube by x-ray diffraction.

FIG. **48***b*) shows an x-ray diffraction pattern from a thaumatin crystal grown inside a capillary tube using a microbatch analogue method (no evaporation).

FIG. **49** shows a vapor-diffusion protein crystallization analogue scheme with a substrate that includes capillary tubing.

FIG. **50***a*) shows vapor diffusion in droplets surrounded by FMS-121 inside a capillary right after the flow was stopped and the capillary was sealed.

FIG. **50***b*) shows vapor diffusion in droplets surrounded by FMS-121 inside a capillary 5 days after the flow was stopped and the capillary was sealed.

FIG. **51***a*) shows a schematic drawing of an experimental setup to form alternating droplets.

FIG. **51***b*) shows a schematic drawing of an experimental setup to form alternating droplets where instead of single solutions **1** and **2**, a set of multiple solutions A and B can be used in a similar system.

FIG. **51***c*) shows a microphotograph illustrating the formation of alternating NaCl—$Fe(SCN)_3$—NaCl droplets.

FIG. **52***a*) shows another example of generating alternating droplets from two different aqueous solutions.

FIG. **52***b*) shows a microphotograph illustrating the formation of alternating NaCl—$Fe(SCN)_3$—NaCl droplets.

FIG. **53***a-c*) shows several representative geometries in which alternating plugs may be formed.

FIG. **54***a-b*) illustrates two representative geometries for indexing a component in a plug using markers.

DETAILED DESCRIPTION ACCORDING TO THE INVENTION

The term "analysis" generally refers to a process or step involving physical, chemical, biochemical, or biological analysis that includes characterization, testing, measurement, optimization, separation, synthesis, addition, filtration, dissolution, or mixing.

The term "analysis unit" refers to a part of or a location in a substrate or channel wherein a chemical undergoes one or more types of analyses.

The term "capillary tube" refers to a hollow, tube-shaped structure with a bore. The cross-sections of the tube and bore can be round, square or rectangular. The corners of the tube or bore can also be rounded. The bore diameters can range in size from 1μ to several millimeters; the outer diameters can be between about 60 μm up to several millimeters. The tube can be made using any material suitable for x-ray diffraction analysis (e.g., silica, plastic, etc.), and can additionally include coatings (e.g. polyimide) suitable for use under variable (e.g, high) temperatures or for UV transparency.

The term "carrier-fluid" refers to a fluid that is immiscible with a plug-fluid. The carrier-fluid may comprise a substance having both polar and non-polar groups or moieties.

The term "channel" refers to a conduit that is typically enclosed, although it may be at least partially open, and that allows the passage through it of one or more types of substances or mixtures, which may be homogeneous or heterogeneous, including compounds, solvents, solutions, emulsions, or dispersions, any one of which may be in the solid, liquid, or gaseous phase. A channel can assume any form or shape such as tubular or cylindrical, a uniform or variable (e.g., tapered) diameter along its length, and one or more cross-sectional shapes along its length such as rectangular, circular, or triangular. A channel is typically made of a suitable material such as a polymer, metal, glass, composite, or other relatively inert materials. As used herein, the term "channel" includes microchannels that are of dimensions suitable for use in devices. A network of channels refers to a multiplicity of channels that are typically connected or in communication with each other. A channel may be connected to at least one other channel through another type of conduit such as a valve.

The term "chemical" refers to a substance, compound, mixture, solution, emulsion, dispersion, molecule, ion, dimer, macromolecule such as a polymer or protein, biomolecule, precipitate, crystal, chemical moiety or group, particle, nanoparticle, reagent, reaction product, solvent, or fluid any one of which may exist in the solid, liquid, or gaseous state, and which is typically the subject of an analysis.

The term "detection region" refers to a part of or a location in a substrate or channel wherein a chemical is identified, measured, or sorted based on a predetermined property or characteristic.

The term "device" refers to a device fabricated or manufactured using techniques such as wet or dry etching and/or conventional lithographic techniques or a micromachining technology such as soft lithography. As used herein, the term "devices" includes those that are called, known, or classified as microfabricated devices. A device according to the invention may have dimensions between about 0.3 cm to about 15 (for 6 inch wafer) cm per side and between about 1 micrometer to about 1 cm thick, but the dimensions of the device may also lie outside these ranges.

The term "discrimination region" refers to a part of or a location in a substrate or channel wherein the flow of a fluid can change direction to enter at least one other channel such as a branch channel.

The term "downstream" refers to a position relative to an initial position which is reached after the fluid flows past the initial point. In a circulating flow device, downstream refers to a position farther along the flow path of the fluid before it

crosses the initial point again. "Upstream" refers to a point in the flow path of a fluid that the fluid reaches or passes before it reaches or passes a given initial point in a substrate or device.

The term "flow" means any movement of a solid or a fluid such as a liquid. For example, the movement of plug-fluid, carrier-fluid, or a plug in a substrate, or component of a substrate according to the invention, or in a substrate or component of a substrate involving a method according to the invention, e.g., through channels of a microfluidic substrate according to the invention, comprises a flow. The application of any force may be used to provide a flow, including without limitation: pressure, capillary action, electro-osmosis, electrophoresis, dielectrophoresis, optical tweezers, and combinations thereof, without regard for any particular theory or mechanism of action.

The term "immiscible" refers to the resistance to mixing of at least two phases or fluids under a given condition or set of conditions (e.g., temperature and/or pressure) such that the at least two phases or fluids persist or remain at least partially separated even after the phases have undergone some type of mechanical or physical agitation. Phases or fluids that are immiscible are typically physically and/or chemically discernible, or they may be separated at least to a certain extent.

The term "inlet port" refers to an area of a substrate that receives plug-fluids. The inlet port may contain an inlet channel, a well or reservoir, an opening, and other features that facilitate the entry of chemicals into the substrate. A substrate may contain more than one inlet port if desired. The inlet port can be in fluid communication with a channel or separated from the channel by a valve.

The term "nanoparticles" refers to atomic, molecular or macromolecular particles typically in the length scale of approximately 1-100 nanometer range. Typically, the novel and differentiating properties and functions of nanoparticles are observed or developed at a critical length scale of matter typically under 100 nm. Nanoparticles may be used in constructing nanoscale structures and they may be integrated into larger material components, systems and architectures. In some particular cases, the critical length scale for novel properties and phenomena involving nanoparticles may be under 1 nm (e.g., manipulation of atoms at approximately 0.1 nm) or it may be larger than 100 nm (e.g., nanoparticle reinforced polymers have the unique feature at approximately 200-300 nm as a function of the local bridges or bonds between the nanoparticles and the polymer).

The term "nucleation composition" refers to a substance or mixture that includes one or more nuclei capable of growing into a crystal under conditions suitable for crystal formation. A nucleation composition may, for example, be induced to undergo crystallization by evaporation, changes in reagent concentration, adding a substance such as a precipitant, seeding with a solid material, mechanical agitation, or scratching of a surface in contact with the nucleation composition.

The term "outlet port" refers to an area of a substrate that collects or dispenses the plug-fluid, carrier-fluid, plugs or reaction product. A substrate may contain more than one outlet port if desired.

The term "particles" means any discrete form or unit of matter. The term "particle" or "particles" includes atoms, molecules, ions, dimers, polymers, or biomolecules.

The term "particulate" refers to a cluster or agglomeration of particles such as atoms, molecules, ions, dimers, polymers, or biomolecules. Particulates may comprise solid matter or be substantially solid, but they may also be porous or partially hollow. They may contain a liquid or gas. In addition, particulates may be homogeneous or heterogeneous, that is, they may comprise one or more substances or materials.

"Plugs" in accordance with the present invention are formed in a substrate when a stream of at least one plug-fluid is introduced into the flow of a carrier-fluid in which it is substantially immiscible. The flow of the fluids in the device is induced by a driving force or stimulus that arises, directly or indirectly, from the presence or application of, for example, pressure, radiation, heat, vibration, sound waves, an electric field, or a magnetic field. Plugs in accordance with the present invention may vary in size but when formed, their cross-section should be substantially similar to the cross-section of the channels in which they are formed. When plugs merge or get trapped inside plug traps, the cross-section of the plugs may change. For example, when a plug enters a wider channel, its cross-section typically increases.

Further, plugs in accordance with the present invention may vary in shape, and for example may be spherical or non-spherical. The shape of the plug may be independent of the shape of the channel (e.g., a plug may be a deformed sphere traveling in a rectangular channel). The plugs may be in the form of plugs comprising an aqueous plug-fluid containing one or more reagents and/or one or more products formed from a reaction of the reagents, wherein the aqueous plug-fluid is surrounded by a non-polar or hydrophobic fluid such as an oil. The plugs may also be in the form of plugs comprising mainly a non-polar or hydrophobic fluid which is surrounded by an aqueous fluid. The plugs may be encased by one or more layers of molecules that comprise both hydrophobic and hydrophilic groups or moieties. The term "plugs" also includes plugs comprising one or more smaller plugs, that is, plugs-within-plugs. The relative amounts of reagents and reaction products contained in the plugs at any given time depend on factors such as the extent of a reaction occurring within the plugs. Preferably, plugs contain a mixture of at least two plug fluids.

The term "plug-forming region" refers to a junction between an inlet port and the first channel of a substrate according to the invention. Preferably, the fluid introduced into the inlet port is "incompatible" (i.e., immiscible) with the fluid in the first channel so that plugs of the fluid formed in the plug-forming region are entrained into the stream of fluid from the first channel.

The term "plug-fluid" refers to a fluid wherein or using which a reaction or precipitation can occur. Typically, the plug-fluid contains a solvent and a reagent although in some embodiments at least one plug-fluid may not contain a reagent. The reagent may be soluble or insoluble in the solvent. The plug-fluid may contain a surfactant. At least two different plug-fluids are used in the present invention. When both plug-fluids contain reagents, the fluids are typically miscible, but can also be partially immiscible, so long as the reagents within each plug-fluid can react to form at least one product or intermediate.

The term "polymer" means any substance or compound that is composed of two or more building blocks ('mers') that are repetitively linked to each other. For example, a "dimer" is a compound in which two building blocks have been joined together. Polymers include both condensation and addition polymers. Typical examples of condensation polymers include polyamide, polyester, protein, wool, silk, polyurethane, cellulose, and polysiloxane. Examples of addition polymers are polyethylene, polyisobutylene, polyacrylonitrile, poly(vinyl chloride), and polystyrene. Other examples include polymers having enhanced electrical or optical properties (e.g., a nonlinear optical property) such as electrocon-

ductive or photorefractive polymers. Polymers include both linear and branched polymers.

The term "protein" generally refers to a set of amino acids linked together usually in a specific sequence. A protein can be either naturally-occurring or man-made. As used herein, the term "protein" includes amino acid sequences that have been modified to contain moieties or groups such as sugars, polymers, metalloorganic groups, fluorescent or light-emitting groups, moieties or groups that enhance or participate in a process such as intramolecular or intermolecular electron transfer, moieties or groups that facilitate or induce a protein into assuming a particular conformation or series of conformations, moieties or groups that hinder or inhibit a protein from assuming a particular conformation or series of conformations, moieties or groups that induce, enhance, or inhibit protein folding, or other moieties or groups that are incorporated into the amino acid sequence and that are intended to modify the sequence's chemical, biochemical, or biological properties. As used herein, a protein includes, but is not limited to, enzymes, structural elements, antibodies, hormones, electron carriers, and other macromolecules that are involved in processes such as cellular processes or activities. Proteins typically have up to four structural levels that include primary, secondary, tertiary, and quaternary structures.

The term "reaction" refers to a physical, chemical, biochemical, or biological transformation that involves at least one chemical, e.g., reactant, reagent, phase, carrier-fluid, or plug-fluid and that generally involves (in the case of chemical, biochemical, and biological transformations) the breaking or formation of one or more bonds such as covalent, noncovalent, van der Waals, hydrogen, or ionic bonds. The term includes typical chemical reactions such as synthesis reactions, neutralization reactions, decomposition reactions, displacement reactions, reduction-oxidation reactions, precipitation, crystallization, combustion reactions, and polymerization reactions, as well as covalent and noncovalent binding, phase change, color change, phase formation, crystallization, dissolution, light emission, changes of light absorption or emissive properties, temperature change or heat absorption or emission, conformational change, and folding or unfolding of a macromolecule such as a protein.

The term "reagent" refers to a component of a plug-fluid that undergoes or participates (e.g., by influencing the rate of a reaction or position of equilibrium) in at least one type of reaction with one or more components of other plug-fluids or a reagent-containing carrier-fluid in the substrate to produce one or more reaction products or intermediates which may undergo a further reaction or series of reactions. A reagent contained in a plug-fluid may undergo a reaction in which a stimulus such as radiation, heat, temperature or pressure change, ultrasonic wave, or a catalyst induces a reaction to give rise to a transformation of the reagent to another reagent, intermediate, or product. A reagent may also undergo a reaction such as a phase change (e.g., precipitation) upon interaction with one or more components of other plug-fluids or a reagent-containing carrier-fluid.

The term "substrate" refers to a layer or piece of material from which devices or chips are prepared or manufactured. As used herein, the term "substrate" includes any substrate fabricated using any traditional or known microfabrication techniques. The term "substrate" also refers either to an entire device or chip or to a portion, area, or section of a device or chip which may or may not be removable or detachable from the main body of the device or chip. The substrate may be prepared from one or more materials such as glass, silicon, silicone elastomer, and polymers including, but not limited to, polypropylene or polyethylene.

The discussion below provides a detailed description of various devices and methods according to the invention for forming plugs, generating gradients in a series of plugs, varying the concentration of reagents inside plugs, rapid mixing in plugs, and scaling of mixing times. In particular, a detailed description of methods for merging, splitting and/or sorting plugs using channels, which form the bases for various applications ranging from the manufacture and analysis of various products to applications in electronics, medicine, diagnostics, and pharmaceuticals, to name a few, is discussed. Methods of detection and measurement of, among others, plugs and processes occurring within plugs are also described.

Among the various applications involving the devices and methods according to the invention are particle separation/sorting, synthesis, investigation of nonlinear and stochastic systems, nonlinear amplification using unstable autocatalytic mixtures, use of stochastic chemical systems for chemical amplification, kinetic measurements, time control of processes, increasing the dynamic range of kinetic measurements, ultrafast measurements, crystallization of proteins, and dynamic control of surface chemistry.

In addition, the devices and methods according to the invention offer a wide-range of other applications. For example, the devices and methods according to the invention provide for effective, rapid, and precise manipulation and monitoring of solutions or reactions over a range of time scales (e.g., from tens of microseconds, to hours or weeks in case of, for example, crystallization) and over a range of solution volumes (e.g., from femtoliters to hundreds of nanoliters).

In one aspect of the invention, the various devices and methods according to the invention are used to overcome one or more of the following problems involving microfluidics. First, the substantial dispersion of solutes in microfluidic channels increases reagent consumption and makes experiments or measurements over long time scales (e.g., minutes to hours) difficult to perform. Various devices and methods according to the invention are intended to overcome this problem by localizing reagents inside plugs that are encapsulated by an immiscible carrier-fluid.

Second, slow mixing of solutions renders experiments, tests, or reactions involving very short time scales (e.g., tens of milliseconds and below) either difficult or impossible to perform with existing technologies. In addition, turbulence-based mixing techniques prohibitively increase sample consumption. In accordance with the present invention, this problem is preferably addressed by conducting the mixing process inside plugs. Rather than relying on turbulence, the various devices and methods according to the invention preferably rely on chaotic advection to accelerate the mixing process. An advantage provided by chaotic advection is that it is expected to operate efficiently in both small and large channels.

Third, achieving control over the chemistry of internal surfaces of devices can be very important at small scales. Thus, being able to control surface chemistry in small devices for example is highly desirable. In accordance with the devices and methods according to the invention, the surface chemistry to which solutions are exposed is preferably controlled through a careful selection of surfactants that are preferably designed to assemble at the interface between the plugs and the immiscible fluid that surrounds them.

Devices and methods of the invention are also provided for use in traditional areas of microfluidics where, for example, miniaturization and speed are important. Thus, the devices and methods according to the invention may be used to develop various tools such as those for high-throughput chemical or biophysical measurements, chemical synthesis,

particle formation, and protein crystallization. They may also be used in high-throughput screening, combinatorial synthesis, analysis, and diagnostics, either as a self-contained platform, or in combination with existing technologies particularly those that rely on the use of immiscible fluid flows.

Importantly, the devices of the invention can be adapted to work with automation and robotic technology. They may be used, for example, as a basis for ultra-high throughput automated systems for structural and functional characterization of biological molecules. Thus, the various devices and methods according to the invention provide rapid, economical, and accessible means of synthesis, analysis, and measurements in the fields of biology, chemistry, biophysics, bioengineering, and medicine (e.g., for diagnostics).

The devices and methods of the invention have numerous other possible applications. For example, chaotic mixing at low values of Reynolds number can be exploited as an important tool for controlling unstable chemical reactions. In addition, the systems and devices of the invention may be used for controlling and/or monitoring reactions that generate highly unstable (or explosive) intermediates. They can also be valuable for controlling or monitoring reactions or processes involving autocatalytic reactions. For example, pure hydrogen peroxide (H_2O_2) is an inexpensive and highly effective oxidant, but its autocatalytic decomposition often leads to explosions upon storage and handling. In the microfluidic systems of the invention, H_2O_2 is preferably generated in-situ, stabilized by the chaotic flow, and used to destroy chemical and biological warfare agents. Because the unstable mixtures in these systems are localized inside plugs formed in accordance with the invention, occasional autocatalytic decomposition in one or more plugs is kept localized within those plugs thereby preventing a catastrophic reaction involving the whole system. In addition, large arrays of microfluidic reactors may be operated in parallel to provide substantial throughput.

It is also possible to couple multiple autocatalytic reactions in a single network using the devices and methods according to the invention. For example, a sample plug could be split into many smaller plugs and forwarded to individual amplification cascades. Because the contents of the cascades' outflows exhibit patterns that correspond to the patterns of analytes present in these systems, these patterns could be analyzed using artificial neural network (ANN) (Jackson, R. B. a. T. *Neural Computing: An Introduction*, Hilger, N.Y., 1991; Zornetzer et al., *An Introduction to Neural and Electronic Networks*, Academic Press, San Diego, Calif., 1990) algorithms. For example, patterns that arise in blood or saliva analysis may correspond to certain normal or abnormal (e.g., disease, fatigue, infection, poisoning) conditions involving, for example, human and animals.

Moreover, it may be possible to create intelligent microfluidic systems in accordance with the invention, where the nonlinear chemical reactions perform not only detection, but also analysis using ANN algorithms. For example, after amplification, the channels of the present invention typically will contain sufficient amounts of material to operate hydrogel-based valves (Liu et al., "Fabrication and characterization of hydrogel-based microvalves," *J. Microelectromech. Syst.* 2002, vol. 11, pp. 45-53; Yu et al., "Responsive biomimetic hydrogel valve for microfluidics," Appl. Phys. Lett. 2001, vol. 78, pp. 2589-2591; Beebe et al., "Functional hydrogel structures for autonomous flow control inside microfluidic channels," *Nature,* 2000, vol. 404, 588). These valves can be used to control flows inside the system as a function of the sample plug composition. Feedforward and even feedback (e.g., by using the hydrogel valves to control the flow of the input streams) networks may thus be created and used for analysis. Such nonlinear networks may be used not only to recognize patterns pre-programmed by the connectivity of the channels (Hjelmfelt et al., "Pattern-Recognition in Coupled Chemical Kinetic Systems," Science, 1993, 260, 335-337) but also to learn patterns by reconfiguring themselves (Jackson, R. B. a. T. *Neural Computing: An Introduction*, Hilger, N.Y., 1991; Zornetzer et al., *An Introduction to Neural and Electronic Networks*, Academic Press, San Diego, Calif., 1990). Such intelligent microfluidic devices could have unprecedented capabilities for fully autonomous detection, analysis, and signal processing, perhaps surpassing those of biological and current man-made systems.

The devices and methods of the invention are also useful in genomics and proteomics, which are used to identify thousands of new biomolecules that need to be characterized, or are available only in minute quantities. In particular, the success of genomics and proteomics has increased the demand for efficient, high-throughput mechanisms for protein crystallization. X-ray structure determination remains the predominant method of structural characterization of proteins. However, despite significant efforts to understand the process of crystallization, macromolecular crystallization largely remains an empirical field, with no general theory to guide a rational approach. As a result, empirical screening has remained the most widely used method for crystallizing proteins.

The following areas also provide applications of the devices and methods according to the invention. For example, a number of problems still beset high-throughput kinetics and protein crystallization. When it comes to determining protein structure and quantitatively ascertaining protein interactions, there are at least two technological challenges: (1) most robotic technology still only automate existing methods and are often too expensive for a small research laboratory; and (2) there remains the need for conceptually new methods that provide greater degree of control over the crystallization process. In addition, setting up and monitoring crystallization trials typically involve handling of sub-microliter volumes of fluids over periods ranging from seconds to days.

Thus, various devices and methods according to the present invention are designed to provide novel and efficient means for high-throughput crystallization of soluble and membrane proteins. In addition to being a simple and economical method of setting up thousands of crystallization trials in a matter of minutes, a system according to the invention will enable unique time control of processes such as the mixing and nucleation steps leading to crystallization. A system according to the present invention may also be used to control protein crystallization by controlling not only short time-scale events such as nucleation but also long time-scale events such as crystal growth.

Further, the devices and methods of the present invention may be used in high-throughput, kinetic, and biophysical measurements spanning the 10^{-5}-10^{7} second time regime. Preferably, the various devices and methods according to the present invention require only between about a few nanoliters to about a few microliters of each solution. Applications of such devices and methods include studies of enzyme kinetics and RNA folding, and nanoparticle characterization and synthesis, which are discussed in detail below.

Channels and Devices

In one aspect of the invention, a device is provided that includes one or more substrates comprising a first channel comprising an inlet separated from an outlet; optionally, one or more secondary channels (or branch channels) in fluid communication with the first channel, at least one carrier-

fluid reservoir in fluid communication with the first channel, at least two plug-fluid reservoirs in fluid communication with the first channel, and a means for applying continuous pressure to a fluid within the substrate.

A device according to the invention preferably comprises at least one substrate.

A substrate may include one or more expansions or areas along a channel wherein plugs can be trapped. The substrates of the present invention may comprise an array of connected channels.

The device may have one or more outlet ports or inlet ports. Each of the outlet and inlet ports may also communicate with a well or reservoir. The inlet and outlet ports may be in fluid communication with the channels or reservoirs that they are connecting or may contain one or more valves. Fluid can be introduced into the channels via the inlet by any means. Typically, a syringe pump is used, wherein the flow rate of the fluid into the inlet can be controlled.

A plug-forming region generally comprises a junction between a plug-fluid inlet and a channel containing the carrier-fluid such that plugs form which are substantially similar in size to each other and which have cross-sections which are substantially similar in size to the cross-section of the channel in the plug-forming region. In one embodiment, the substrate may contain a plurality of plug-forming regions.

The different plug-forming regions may each be connected to the same or different channels of the substrate. Preferably, the sample inlet intersects a first channel such that the pressurized plug fluid is introduced into the first channel at an angle to a stream of carrier-fluid passing through the first channel. For example, in preferred embodiments, the sample inlet and first channel intercept at a T-shaped junction; i.e., such that the sample inlet is perpendicular (i.e. at an angle of 90°) to the first channel. However, the sample inlet may intercept the first channel at any angle.

A first channel may in turn communicate with two or more branch channels at another junction or "branch point", forming, for example, a T-shape or a Y-shape. Other shapes and channel geometries may be used as desired. In exemplary embodiments the angle between intersecting channels is in the range of from about 60° to about 120°. Particular exemplary angles are 45°, 60°, 90°, and 120°. Precise boundaries for the discrimination region are not required, but are preferred.

The first and branch channels of the present invention can, each independently, be straight or have one or more bends. The angle of a bend, relative to the substrate, can be greater than about 10°, preferably greater than about 135°, 180°, 270°, or 360°.

In one embodiment of the invention, a substrate comprises at least one inlet port in communication with a first channel at or near a plug-forming region, a detection region within or coincident with all or a portion of the first channel or plug-forming region, and a detector associated with the detection region. In certain embodiments the device may have two or more plug-forming regions. For example, embodiments are provided in which the analysis unit has a first inlet port in communication with the first channel at a first plug-forming region, a second inlet port in communication with the first channel at a second plug-forming region (preferably downstream from the first plug-forming region), and so forth.

In another embodiment, a substrate according to the invention may comprise a first channel through which a pressurized stream or flow of a carrier-fluid is passed, and two or more inlet channels which intersect the first channel at plug-forming regions and through which a pressurized stream or flow of plug fluids pass. Preferably, these inlet channels are parallel to each other and each intercept the first channel at a right angle. In specific embodiments wherein the plugs introduced through the different plug forming regions are mixed, the inlet channels are preferably close together along the first channel. For example, the first channel may have a diameter of 60 μm that tapers to 30 μm at or near the plug-forming regions. The inlet channels then also preferably have a diameter of about 30 μm and, in embodiments where plug mixing is preferred, are separated by a distance along the first channel approximately equal to the diameter of the inlet channel (i.e., about 30 μm).

In an embodiment according to the invention, the substrate also has a detection region along a channel. There may be a plurality of detection regions and detectors, working independently or together, e.g., to analyze one or more properties of a chemical such as a reagent.

A detection region is within, communicating, or coincident with a portion of a first channel at or downstream of the plug-forming region and, in sorting embodiments, at or upstream of the discrimination region or branch point. Precise boundaries for the detection region are not required, but are preferred.

A typical substrate according to the invention comprises a carrier-fluid inlet that is part of and feeds or communicates directly with a first channel, along with one or more plug fluid inlets in communication with the first channel at a plug-forming region situated downstream from the main inlet (each different plug-fluid inlet preferably communicates with the first channel at a different plug-forming region).

Plugs formed from different plug-fluids or solutions may be released in any order. For example, an aqueous solution containing a first plug-fluid may be released through a first inlet at a first plug-forming region. Subsequently, plugs of an aqueous second plug-fluid may be released through a second inlet at a second plug-forming region downstream of the first inlet.

Fabrication of Channels, Substrates, and Devices

The substrates and devices according to the invention are fabricated, for example by etching a silicon substrate, chip, or device using conventional photolithography techniques or micromachining technology, including soft lithography. The fabrication of microfluidic devices using polydimethylsiloxane has been previously described. These and other fabrication methods may be used to provide inexpensive miniaturized devices, and in the case of soft lithography, can provide robust devices having beneficial properties such as improved flexibility, stability, and mechanical strength. Preferably, when optical detection is employed, the invention also provides minimal light scatter from, for example, plugs, carrier-fluid, and substrate material. Devices according to the invention are relatively inexpensive and easy to set up.

Machining methods (e.g., micromachining methods) that may be used to fabricate channels, substrates, and devices according to the invention are well known in the art and include film deposition processes, such as spin coating and chemical vapor deposition, laser fabrication or photolithographic techniques, or etching methods, which may be performed either by wet chemical or plasma processes.

Channels may be molded onto optically transparent silicone rubber or polydimethylsiloxane (PDMS), preferably PDMS. This can be done, for example, by casting the channels from a mold by etching the negative image of these channels into the same type of crystalline silicon wafer used in semiconductor fabrication. The same or similar techniques for patterning semiconductor features can be used to form the pattern of the channels. In one method of channel fabrication, an uncured PDMS is poured onto the molds placed in the

bottom of, for example, a Petri dish. To accelerate curing, the molds are preferably baked. After curing the PDMS, it is removed from on top of the mold and trimmed. Holes may be cut into the PDMS using, for example, a tool such as a cork borer or a syringe needle. Before use, the PDMS channels may be placed in a hot bath of HCl if it is desired to render the surface hydrophilic. The PDMS channels can then be placed onto a microscope cover slip (or any other suitable flat surface), which can be used to form the base/floor or top of the channels.

A substrate according to the invention is preferably fabricated from materials such as glass, polymers, silicon microchip, or silicone elastomers. The dimensions of the substrate may range, for example, between about 0.3 cm to about 7 cm per side and about 1 micron to about 1 cm in thickness, but other dimensions may be used.

A substrate can be fabricated with a fluid reservoir or well at the inlet port, which is typically in fluid communication with an inlet channel. A reservoir preferably facilitates introduction of fluids into the substrate and into the first channel. An inlet port may have an opening such as in the floor of the substrate to permit entry of the sample into the device. The inlet port may also contain a connector adapted to receive a suitable piece of tubing, such as Teflon® tubing, liquid chromatography or HPLC tubing, through which a fluid may be supplied. Such an arrangement facilitates introducing the fluid under positive pressure in order to achieve a desired pressure at the plug-forming region.

A substrate containing the fabricated flow channels and other components is preferably covered and sealed, preferably with a transparent cover, e.g., thin glass or quartz, although other clear or opaque cover materials may be used. Silicon is a preferred substrate material due to well-developed technology permitting its precise and efficient fabrication, but other materials may be used, including polymers such as polytetrafluoroethylenes. Analytical devices having channels, valves, and other elements can be designed and fabricated from various substrate materials. When external radiation sources or detectors are employed, the detection region is preferably covered with a clear cover material to allow optical access to the fluid flow. For example, anodic bonding of a silicon substrate to a PYREX® cover slip can be accomplished by washing both components in an aqueous H_2SO_4/H_2O_2 bath, rinsing in water, and then, for example, heating to about 350° C. while applying a voltage of 450 V.

A variety of channels for sample flow and mixing can be fabricated on the substrate and can be positioned at any location on the substrate, chip, or device as the detection and discrimination or sorting points. Channels can also be designed into the substrate that place the fluid flow at different times/distances into a field of view of a detector. Channels can also be designed to merge or split fluid flows at precise times/distances.

A group of manifolds (a region consisting of several channels that lead to or from a common channel) can be included to facilitate the movement of plugs from different analysis units, through the plurality of branch channels and to the appropriate solution outlet. Manifolds are preferably fabricated into the substrate at different depth levels. Thus, devices according to the invention may have a plurality of analysis units that can collect the solution from associated branch channels of each unit into a manifold, which routes the flow of solution to an outlet. The outlet can be adapted for receiving, for example, a segment of tubing or a sample tube, such as a standard 1.5 ml centrifuge tube. Collection can also be done using micropipettes.

Methods of Forming Plugs

The various channels, substrates, and devices according to the invention are primarily used to form and manipulate plugs.

In a preferred embodiment, plug-fluids do not significantly mix at or before they are introduced into the first channel. The plug-fluids may form distinct laminar streams at or before the inlet. They may be separated by an additional fluid. Alternatively, they may be introduced into the carrier-fluid via inlets of differing size. The concentration of plug-fluids in the plugs may be adjusted by adjusting volumetric flow rates of the plug-fluids. Further, the diameters of the first channel and the branch channel(s) may differ.

FIG. **2**A is a schematic diagram contrasting laminar flow transport and plug transport in a channel. In the lower figure which depicts the transport of plugs, two aqueous reagents (marked in red and blue) form laminar streams that are separated by a "divider" aqueous stream. The three streams enter a channel with flowing oil, at which point plugs form and plug fluids mix. During plug transport, rapid mixing of the plug-fluids typically occurs within the plugs. In contrast, in laminar flow transport, fluid mixing occurs slowly, and with high dispersion, as shown in the upper figure. In the upper figure, the time t at a given point d_1 can be estimated from $t_1 \approx d_1/U$, where d1 is the distance from d=0 and U is the flow velocity. In the lower figure, the time t is given by $t_1 = d_1/U$.

FIG. **2**B shows a photograph and a schematic diagram that depict mixing in water/oil plugs (upper schematic and photograph) and in laminar streams (lower schematic and photograph) comprising only aqueous plug-fluids. The oil (carrier-fluid in this case) is introduced into channel **200** of a substrate. Instead of oil, water is introduced into the corresponding channel **207** in the case of mixing using laminar streams. The three aqueous plug-fluids are introduced by inlet ports **201**, **202**, **203** into the carrier-fluid (and by inlet ports **204**, **205**, **206** in the case of laminar streams). A preferred scheme is one in which the aqueous plug-fluids initially coflow preferably along a short or minimal distance before coming in contact with the carrier-fluid. In a preferred embodiment, the distance traversed by the coflowing plug-fluids is approximately or substantially equal to the width of the channel.

The middle or second aqueous plug-fluid in the top figure may be plain water, buffer, solvent, or a different plug-fluid. The middle aqueous plug-fluid would preferably initially separate the two other aqueous plug-fluids before the aqueous fluids come into contact with the carrier-fluid. Thus, the intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous plug-fluids before they come in contact with the carrier-fluid. The plugs that form in the plug-forming region can continue along an unbranched channel, can split and enter a channel, can merge with plugs from another channel, or can exit the substrate through an exit port. It can be seen in FIG. **2** that, in the absence of an oil, the aqueous plug-fluids flow in laminar streams without significant mixing or with only partial mixing. In contrast, plug-fluids mix substantially or completely in the plugs.

FIG. **3** shows photographs and schematic diagrams that depict a stream of plugs from an aqueous plug-fluid and an oil (carrier-fluid) in curved channels at flow rates of 0.5 μL/min (top schematic diagram and photograph) and 1.0 μL/min (bottom schematic diagram and photograph). This scheme allows enhanced mixing of reagents in the elongated plugs flowing along a curved channel with smooth corners or curves. The carrier-fluid is introduced into an inlet port **300**, **307** of a substrate while the three aqueous plug-fluids are introduced in separate inlet ports **301**-**306**. As in FIG. **2**, a preferred scheme would be one in which the plug-fluids ini-

tially coflow preferably along a short or minimal distance before coming in contact with the carrier-fluid. In a preferred embodiment, the distance traversed by the coflowing plug-fluids (e.g., aqueous plug-fluids) is approximately or substantially equal to the width of the channel. The middle or second aqueous plug-fluid may comprise plain water, buffer, solvent, or a plug-fluid, and the middle aqueous plug-fluid preferably initially separates the two other aqueous plug-fluids before the aqueous plug-fluids come into contact with the carrier-fluid which, in this case, is an oil. Thus, the intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous plug-fluids before they come in contact with the oil (or carrier-fluid).

FIG. **4** shows a photograph and schematic diagram that illustrate plug formation through the injection of oil and multiple plug-fluids. Although FIG. **4** shows five separate plug-fluids, one may also separately introduce less than or more than five plug-fluids into the substrate. The reagents or solvents comprising the plug-fluids may be different or some of them may be identical or similar. As in FIG. **2**, the oil is introduced into an inlet port **400** of a substrate while the aqueous plug-fluid is introduced in separate inlet ports **401-405**. The water plugs then flow through exit **406**. A preferred scheme is one in which the aqueous plug-fluids would initially coflow preferably along a short or minimal distance before coming in contact with the oil. In a preferred embodiment, the distance traversed by the coflowing plug-fluids is approximately or substantially equal to the width of the channel. One or more of the aqueous plug-fluids may comprise plain water, buffer, solvent, or a plug-fluid, and at least one aqueous plug-fluid would preferably initially separate at least two other aqueous streams before the aqueous plug-fluid comes into contact with the oil. Thus, the at least one intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous streams before the aqueous streams come in contact with the oil. FIG. **5** shows a microfluidic network, which is similar to that shown in FIG. **4**, in which several reagents can be introduced into the multiple inlets. In addition, FIG. **5** shows a channel having a winding portion through which the plugs undergo mixing of the four reagents A, B, C, and D. As shown in FIG. **5**, the reagents A, B, C, and D are introduced into inlet ports **501**, **503**, **505**, and **507**, while aqueous streams are introduced into inlet ports **502**, **504**, **506**. FIG. **5** shows plugs through the various stages of mixing, wherein mixture **50** corresponds to the initial A+B mixture, mixture **51** corresponds to the initial C+D mixture, mixture **52** corresponds to the mixed A+B mixture, mixture **53** corresponds to the mixed C+D mixture, and mixture **54** corresponds to the A+B+C+D mixture.

The formation of the plugs preferentially occurs at low values of the capillary number C.n., which is given by the equation

$$\text{C.n.}=U\mu/\gamma \qquad \text{Eqn. (1)}$$

where U is the flow velocity, μ is the viscosity of the plug fluid or carrier-fluid, and γ is the surface tension at the water/surfactant interface.

The plugs may be formed using solvents of differing or substantially identical viscosities. Preferably, the conditions and parameters used in an experiment or reaction are such that the resulting capillary number lies in the range of about 0.001≦C.n.≦about 10. Preferably, the values of parameters such as viscosities and velocities are such that plugs can be formed reliably. Without wishing to be bound by theory, it is believed that as long as flow is not stopped, the C.n. is ≦about 0.2, and as long as the surface tension of the plug-fluid/carrier-fluid interface is lower than the surface tension of the solution/wall interface, plug formation will persist. The C.n. number is zero when flow is stopped.

In one embodiment, in which perfluorodecaline was used as the carrier-fluid and the plug-fluid was aqueous, it was found that this system can be operated at values of C.n. up to ~0.1 (at 300 mm s^{-1}). In this system, as the value of the C.n. increased above ~0.2, the formation of plugs became irregular. The viscosity of perfluorodecaline is 5.10×10^{-3} kg m^{-3} s^{-1}, the surface tension at the interface between the plugs and the carrier-fluid was 13×10^{-3} N m^{-1}.

The length of the plugs can be controlled such that their sizes can range from, for example, about 1 to 4 times a cross-sectional dimension (d, where d is a channel cross-sectional dimension) of a channel using techniques such as varying the ratio of the plug-fluids and carrier-fluids or varying the relative volumetric flow rates of the plug-fluid and carrier-fluid streams. Short plugs tend to form when the flow rate of the aqueous stream is lower than that of a carrier-fluid stream. Long plugs tend to form when the flow rate of the plug-fluid stream is higher than that of the carrier stream.

In one approximation, the volume of a plug is taken equal to about $2\times d^3$, where d is a cross-sectional dimension of a channel. Thus, the plugs can be formed in channels having cross-sectional areas of, for example, from 20×20 to 200×200 μm^2, which correspond to plug volumes of between about 16 picoliters (pL) to 16 nanoliters (nL). The size of channels may be increased to about 500 μm (corresponding to a volume of about 250 nL) or more. The channel size can be reduced to, for example, about 1 μm (corresponding to a volume of about 1 femtoliter). Larger plugs are particularly useful for certain applications such as protein crystallizations, while the smaller plugs are particularly useful in applications such as ultrafast kinetic measurements.

In one preferred embodiment, plugs conform to the size and shape of the channels while maintaining their respective volumes. Thus, as plugs move from a wider channel to a narrower channel they preferably become longer and thinner, and vice versa.

Plug-fluids may comprise a solvent and optionally, a reactant. Suitable solvents for use in the invention, such as those used in plug-fluids, include organic solvents, aqueous solvents, oils, or mixtures of the same or different types of solvents, e.g. methanol and ethanol, or methanol and water. The solvents according to the invention include polar and non-polar solvents, including those of intermediate polarity relative to polar and non-polar solvents. In a preferred embodiment, the solvent may be an aqueous buffer solution, such as ultrapure water (e.g., 18 MΩ resistivity, obtained, for example, by column chromatography), 10 mM Tris HCl, and 1 mM EDTA (TE) buffer, phosphate buffer saline or acetate buffer. Other solvents that are compatible with the reagents may also be used.

Suitable reactants for use in the invention include synthetic small molecules, biological molecules (i.e., proteins, DNA, RNA, carbohydrates, sugars, etc.), metals and metal ions, and the like.

The concentration of reagents in a plug can be varied. In one embodiment according to the invention, the reagent concentration may be adjusted to be dilute enough that most of the plugs contain no more than a single molecule or particle, with only a small statistical chance that a plug will contain two or more molecules or particles. In other embodiments, the reagent concentration in the plug-fluid is adjusted to concentrate enough that the amount of reaction product can be maximized.

Suitable carrier-fluids include oils, preferably fluorinated oils. Examples include viscous fluids, such as perfluorodeca-

line or perfluoroperhydrophenanthrene; nonviscous fluids such as perfluorohexane; and mixtures thereof (which are particularly useful for matching viscosities of the carrier-fluids and plug-fluids). Commercially available fluorinated compounds such as Fluorinert™ liquids (3M, St. Paul, Minn.) can also be used.

The carrier-fluid or plug-fluid, or both may contain additives, such as agents that reduce surface tensions (e.g., surfactants). Other agents that are soluble in a carrier-fluid relative to a plug-fluid can also be used when the presence of a surfactant in the plug fluid is not desirable. Surfactants may be used to facilitate the control and optimization of plug size, flow and uniformity. For example, surfactants can be used to reduce the shear force needed to extrude or inject plugs into an intersecting channel. Surfactants may affect plug volume or periodicity, or the rate or frequency at which plugs break off into an intersecting channel. In addition, surfactants can be used to control the wetting of the channel walls by fluids. In one embodiment according to the invention, at least one of the plug-fluids comprises at least one surfactant.

Preferred surfactants that may be used include, but are not limited to, surfactants such as those that are compatible with the carrier and plug-fluids. Exemplary surfactants include Tween™, Span™, and fluorinated surfactants (such as Zonyl™ (Dupont, Wilmington Del.)). For example, fluorinated surfactants, such as those with a hydrophilic head group, are preferred when the carrier-fluid is a fluorinated fluid and the plug-fluid is an aqueous solution.

However, some surfactants may be less preferable in certain applications. For instance, in those cases where aqueous plugs are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort biomaterials, a water soluble surfactant such as SDS may denature or inactivate the contents of the plug.

The carrier-fluid preferably wets the walls of the channels preferentially over the plugs. If this condition is satisfied, the plug typically does not come in contact with the walls of the channels, and instead remains separated from the walls by a thin layer of the carrier-fluid. Under this condition, the plugs remain stable and do not leave behind any residue as they are transported through the channels. The carrier-fluid's preferential wetting of the channel walls over the plug-fluid is achieved preferably by setting the surface tension by, for example, a suitable choice of surfactant. Preferably, the surface tension at a plug fluid/channel wall interface (e.g., about 38 mN/m surface tension for a water/PDMS interface) is set higher than the surface tension at a plug fluid/carrier-fluid interface (e.g., about 13 mN/m for a water/carrier-fluid interface with a surfactant such as 10% 1H, 1H,2H,2H-perfluorooctanol in perfluorodecaline as the carrier-fluid). If this condition is not satisfied, plugs tend to adhere to the channel walls and do not undergo smooth transport (e.g., in the absence of 1H,1H,2H,2H-perfluorooctanol the surface tension at the water/perfluorodecaline interface is about 55 mN/m, which is higher than the surface tension of the water/PDMS interface (e.g., about 38 mN/m)), and plugs adhere to the walls of the PDMS channels. Because the walls of the channels (PDMS, not fluorinated) and the carrier-fluid (fluorinated oil) are substantially different chemically, when a fluorinated surfactant is introduced, the surfactant reduces the surface tension at the oil-water interface preferentially over the wall-water interface. This allows the formation of plugs that do not stick to the channel walls.

The surface tension at an interface may be measured using what is known as a hanging drop method, although one may also use other methods. Preferably, the surface tension is sufficiently high to avoid destruction of the plugs by shear.

The plug-fluids and carrier-fluids may be introduced through one or more inlets. Specifically, fluids may be introduced into the substrate through pneumatically driven syringe reservoirs that contain either the plug-fluid or carrier-fluid. Plugs may be produced in the carrier-fluid stream by modifying the relative pressures such that the plug-fluids contact the carrier-fluid in the plug-forming regions then shear off into discrete plugs.

In the invention, plugs are formed by introducing the plug-fluid, at the plug-forming region, into the flow of carrier-fluid passing through the first channel. The force and direction of flow can be controlled by any desired method for controlling flow, for example, by a pressure differential, or by valve action. This permits the movement of the plugs into one or more desired branch channels or outlet ports.

In preferred embodiments according to the invention, one or more plugs are detected, analyzed, characterized, or sorted dynamically in a flow stream of microscopic dimensions based on the detection or measurement of a physical or chemical characteristic, marker, property, or tag.

The flow stream in the first channel is typically, but not necessarily continuous and may be stopped and started, reversed or changed in speed. Prior to sorting, a non-plug-fluid can be introduced into a sample inlet port (such as an inlet well or channel) and directed through the plug-forming region, e.g., by capillary action, to hydrate and prepare the device for use. Likewise, buffer or oil can also be introduced into a main inlet port that communicates directly with the first channel to purge the substrate (e.g., of "dead" air) and prepare it for use. If desired, the pressure can be adjusted or equalized, for example, by adding buffer or oil to an outlet port.

The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug-forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs. Periodicity and plug volume may also depend on channel diameter and/or the viscosity of the fluids.

Mixing in Plugs

FIG. **7**(*a*)-(*b*) show microphotographs (10 μs exposure) illustrating rapid mixing inside plugs (a) and negligible mixing in a laminar flow (b) moving through winding channels at the same total flow velocity. Aqueous streams were introduced into inlets **700**-**705** in FIGS. **7**(*a*)-(*b*). In FIGS. **7**(*c*) and **7**(*e*), Fluo-4 was introduced into inlets **706**, **709**, buffer was introduced into inlets **707**, **710**, and $CaCl_2$ was introduced into inlets **708**, **711**. FIG. **7**(*c*) shows a false-color microphotograph (2 s exposure, individual plugs are invisible) showing time-averaged fluorescence arising from rapid mixing inside plugs of solutions of Fluo-4 (54 μM) and $CaCl_2$ (70 μM) in aqueous sodium morpholine propanesulfonate buffer (20 μM, pH 7.2); this buffer was also used as the middle aqueous stream. FIG. **7**(*d*) shows a plot of the relative normalized intensity (I) of fluorescence obtained from images such as shown in (c) as a function of distance (left) traveled by the plugs and of time required to travel that distance (right) at a given flow rate. The total intensity across the width of the channel was measured. Total PFD/water volumetric flow rates (in $\mu L\ min^{-1}$) were 0.6:0.3, 1.0:0.6, 12.3:3.7, 10:6, and 20:6. FIG. **7**(*e*) shows a false-color microphotograph (2 s exposure) of the weak fluorescence arising from negligible mixing in a laminar flow of the solutions used in (c). All

channels were 45 μm deep; inlet channels were 50 μm and winding channels 28 μm wide; Re~5.3 (water), ~2.0 (PFD).

FIG. **8** shows photographs and schematics that illustrate fast mixing at flow rates of about 0.5 μL/min (top schematic diagram and photograph) and about 1.0 μL/min (lower schematic diagram and photograph) using 90°-step channels while FIG. **9** illustrates fast mixing at flow rates of about 1.0 μL/min (top schematic diagram and photograph) and about 0.5 μL/min (lower schematic diagram and photograph) using 135°-step channels. Aqueous streams are introduced into inlets **800**-**805** in FIG. **8** (inlets **900**-**905** in FIG. **9**), while a carrier fluid is introduced into channels **806**, **807** (channels **906**, **907** in FIG. **9**). The plugs that form then flow through exits **808**, **809** (FIG. **8**) and exits **908**, **909** (FIG. **9**). As can be seen in FIG. **8** and FIG. **9**, the plugs are transported along multi-step channels, instead of channels with smooth curves (as opposed to channels with sharp corners). An advantage of these multi-step configurations of channels is that they may provide further enhanced mixing of the substances within the plugs.

Several approaches may be used to accelerate or improve mixing. These approaches may then be used to design channel geometries that allow control of mixing. Flow can be controlled by perturbing the flow inside a moving plug so that it differs from the symmetric flow inside a plug that moves through a straight channel. For example, flow perturbation can be accomplished by varying the geometry of a channel (e.g., by using winding channels), varying the composition of the plug fluid (e.g., varying the viscosities), varying the composition of the carrier-fluid (e.g., using several laminar streams of carrier-fluids that are different in viscosity or surface tension to form plugs; in this case, mixing is typically affected, and in some cases enhanced), and varying the patterns on the channel walls (e.g., hydrophilic and hydrophobic, or differentially charged, patches would interact with moving plugs and induce time-periodic flow inside them, which should enhance mixing).

Various channel designs can be implemented to enhance mixing in plugs. FIG. **1**A shows a schematic of a basic channel design, while FIG. **1**B shows a series of periodic variations of the basic channel design. FIG. **1**C shows a series of aperiodic combinations resulting from a sequence of alternating elements taken from a basic design element shown in FIG. **1**A and an element from the periodic variation series shown in FIGS. **1**B(**1**)-(**4**). When the effects of these periodic variations are visualized, aperiodic combinations of these periodic variations are preferably used to break the symmetries arising from periodic flows (see FIG. **1**C). Here, the relevant parameters are channel width, period, radius of curvature, and sequence of turns based on the direction of the turns. The parameters of the basic design are defined such that c is the channel width, l is the period, and r is the radius of curvature. For the basic design, the sequence can be defined as (left, right, left, right), where left and right is relative to a centerline along the path taken by a plug in the channel.

FIGS. **1**B(**1**)-**4**) show schematic diagrams of a series of periodic variations of the basic design. At least one variable parameter is preferably defined based on the parameters defined in FIG. **1***a*). In FIG. **1**B(**1**), the channel width is c/2; in FIG. **1**B(**2**), the period is 2 l; and in FIG. **1**B(**3**), and the radius of curvature is 2r. In FIG. **1**B(**4**), the radius of curvature is r/2 and the sequence is (left, left, right, right).

FIGS. **1**C(**1**)-(**4**) show a schematic diagram of a series of aperiodic combinations formed by combining the basic design element shown in FIG. **1**A with an element from the series of periodic variations in FIG. **1**B(**1**)-(**4**). In FIG. **1**C(**1**), the alternating pattern of a period of the basic design shown in FIG. **1**A (here denoted as "a") and a period of the channel in FIG. **1**B(**1**) (here denoted as "b**1**") is given by a+b**1**+a+ . . . . In FIG. **1**C(**2**), the aperiodic combination is given by a+b**2**+a. In the channel shown in FIG. **1**C(**3**) (here denoted as "c**3**"), the aperiodic combination is given by a+c**3**+a. In the channel shown in FIG. **1**C(**4**) (here denoted as "c**4**"), a (right, left) sequence is introduced with a kink in the pattern. A repeating (left, right) sequence would normally be observed. By adding this kink, the sequence becomes (left, right, left, right)+(right, left)+(left, right, left, right).

Another approach for accelerating mixing relies on rationally-designed chaotic flows on a microfluidic chip using what is known as the baker's transformation. Reorientation of the fluid is critical for achieving rapid mixing using the baker's transformation. The baker's transformation leads to an exponential decrease of the striation thickness (the distance over which mixing would have to occur by diffusion) of the two components via a sequence of stretching and folding operations. Typically, every stretch-fold pair reduces the striation thickness by a factor of 2, although this factor may have a different value. The striation thickness (ST) can be represented, in an ideal case, by Eqn. (2) below. Thus, in the ideal case, in a sequence of n stretch-fold-reorient operations, the striation thickness undergoes an exponential decrease given by

$$ST(t_n)=ST(t_0)\times 2^{-n} \qquad \text{Eqn. (2)}$$

where $ST(t_n)$ represents the striation thickness at time t_n, $ST(t_0)$ represents the initial striation thickness at time t_0, and n is the number of stretch-fold-reorient operations.

In accordance with the invention, the baker's transformation is preferably implemented by creating channels composed of a sequence of straight regions and sharp turns. FIG. **11** shows a schematic diagram of a channel geometry designed to implement and visualize the baker's transformation of plugs flowing through microfluidic channels. Other designs could also be used. The angles at the channel bends and the lengths of the straight portions are chosen so as to obtain optimal mixing corresponding to the flow patterns shown. Different lengths of straight paths and different turns may be used depending on the particular application or reaction involved.

A plug traveling through every pair of straight part **112** and sharp-turn part **111** of the channel, which is equivalent to one period of a baker's transformation, will experience a series of reorientation, stretching and folding. In a straight part of the channel, a plug will experience the usual recirculating flow. At a sharp turn, a plug normally rolls and reorients due to the much higher pressure gradient across the sharp internal corner and also due to larger travel path along the outside wall. This method of mixing based on the baker's transformation is very efficient and is thus one of the preferred types of mixing. In particular, this type of mixing leads to a rapid reduction of the time required for reagent mixing via diffusion.

It is believed that plug formation can be maintained at about the same flow rate in channels of different sizes because the limit of a flow rate is typically set by the capillary number, C.n., which is independent of the channel size. At a fixed flow rate, the mixing time t_{mix} may decrease as the size of the channel (d) is reduced. First, it is assumed that it takes the same number n of stretch-fold-reorient cycles to mix reagents in both large and small channels. This assumption (e.g., for n~5) is in approximate agreement with previously measured mixing in d=55 and d=20 micrometer (μm) channels. Each cycle requires a plug to travel over a distance of approximately 2 lengths of the plug (approximately 3d). Therefore, mixing time is expected to be approximately equal to the time

it takes to travel 15d, and will decrease linearly with the size of the channel, t_{mix}~d. A method that provides mixing in about 1 ms in 25-μm channels preferably provides mixing in about 40 μs in 1-μm channels. Achieving microsecond mixing times generally requires the use of small channels. High pressures are normally required to drive a flow through small channels.

Without wishing to be bound by theory, theoretical modeling indicates that the number of cycles it takes for mixing to occur in a channel with diameter d is given approximately by

$$n \times 2^{2n} \approx dU/D \quad \text{Eqn. (3)}$$

where n is the number of cycles, U is the flow velocity, D is the diffusion constant, one cycle is assumed to be equal to 6d, and mixing occurs when convection and diffusion time scales are matched. The mixing time is primarily determined by the number of cycles. This result indicates that mixing will be accelerated more than just in direct proportion to the channel diameter. For example, when d decreases by a factor of 10, mixing time decreases by a factor of d×Log(d)=10×Log(10). With properly designed channels, mixing times in 1-μm channels can be limited to about 20 μs. Even at low flow rates or long channels (such as those involving protein crystallization), however, significant mixing can still occur. In addition, without being bound by theory, it is expected that increasing the flow rate U by a factor of 10 will decrease the mixing time by a factor of Log(U)/U=(Log (10))/10.

To visualize mixing in a channel according to the invention, a colored marker can be used in a single plug-fluid. The initial distribution of the marker in the plug has been observed to depend strongly on the details of plug formation. As the stationary aqueous plug was extruded into the flowing carrier-fluid, shearing interactions between the flow of the carrier-fluid and the plug-fluid induced an eddy that redistributed the solution of the marker to different regions of the plug. The formation of this eddy is referred to here as "twirling" (see FIG. **27***b*)). Twirling is not a high Reynolds number (R_e) phenomenon (see FIG. **30**) since it was observed at substantially all values of R_e and at substantially all velocities. However, the flow pattern of this eddy appears to be slightly affected by the velocity.

Various characteristics and behavior of twirling were observed. Twirling redistributed the marker by transferring it from one side of the plug to the other, e.g., from the right to the left side of the plug. The most efficient mixing was observed when there was minimal fluctuations in intensity, i.e., when the marker was evenly distributed across the plug. While twirling was present during the formation of plugs of all lengths that were investigated, its significance to the mixing process appears to depend on the length of the plug. For example, the extent of twirling was observed to be significantly greater for short plugs than for long plugs. Twirling was also observed to affect only a small fraction of the long plugs and had a small effect on the distribution of the marker in the plugs. Moreover, twirling occurred only at the tip of the forming plug before the tip made contact with the right wall of the microchannel. Also, the amount of twirling in a plug was observed to be related to the amount of the carrier-fluid that flowed past the tip. The results of experiments involving twirling and its effect on mixing show that twirling is one of the most important factors, if not the most important factor, in determining the ideal conditions for mixing occurring within plugs moving through straight channels. By inducing twirling, one may stimulate mixing; by preventing twirling, one may suppress complete mixing. Suppressing mixing may be important in some of the reaction schemes, for example those shown in FIG. **5** and FIG. **6**. In these reaction schemes, selective mixing of reagents A with reagent B, and also reagent C with reagent D, can occur without mixing of all four reagents. Mixing of all four reagents occurs later as plugs move through, for example, the winding part of the channel. This approach allows several reactions to occur separated in time. In addition, suppressing mixing may be important when interfaces between plug fluids have to be created, for example interfaces required for some methods of protein crystallization (FIG. **20**).

The eddy at the tip of a developing plug may complicate visualization and analysis of mixing. This eddy is normally significant in short plugs, but only has a minor effect on long plugs. For applications involving visualization of mixing, the substrate is designed to include a narrow channel in the plug-forming region is designed such that narrow, elongated plugs form. Immediately downstream from the plug-forming region, the channel dimension is preferably expanded. In the expanded region of the channel(s), plugs will expand and become short and rounded under the force of surface tension; this preserves the distribution of the marker inside the plugs. This approach affords a relatively straightforward way of visualizing the mixing inside plugs of various sizes. Video microscopy may be used to observe the distribution of colored markers inside the drops. A confocal microscope may also be used to visualize the average three-dimensional distribution of a fluorescent marker. Visualization can be complemented or confirmed using a Ca^{2+}/Fluo-4^{-4} reaction. At millimolar concentrations, this reaction is expected to occur with a half-life of about 1 μs. Thus, it can be used to measure mixing that occurs on time scales of about 10 μs and longer.

The following discussion describes at least one method for three-dimensional visualization of flows in plugs. Visualization of chaotic transport in three-dimensions is a challenging task especially on a small scale. Predictions based on two-dimensional systems may be used to gain insight about plugs moving through a three-dimensional microfluidic channel. Experiments and simulations involving a two-dimensional system can aid in the design of channels that ensure chaotic flow in two-dimensional liquid plugs. Confocal microscopy has been used to quantify steady, continuous three-dimensional flows in channels. However, due to instrumental limitations of an optical apparatus such as a confocal microscope, it is possible that the flow cannot be visualized with sufficiently high-resolution to observe, for example, self-similar fractal structures characteristic of chaotic flow. Nonetheless, the overall dynamics of the flow may still be captured and the absence of non-chaotic islands confirmed. Preferably, the channels (periodic or aperiodic) used in the visualization process are fabricated using soft lithography in PDMS. A PDMS replica is preferably sealed using a thin glass cover slip to observe the flow using confocal microscopy.

In one experiment according to the invention, a series of line scans are used to obtain images of a three-dimensional distribution of fluorescent markers within the plugs. FIG. **10***a*) is a schematic diagram depicting a three-dimensional confocal visualization of chaotic flows in plugs. Plugs are preferably formed from three laminar streams. The middle stream **11** preferably contains fluorescent markers. Preferably, the middle stream **11** is injected into the channel system at a low volumetric flow rate. The volumetric flow rates of the two side streams **10**, **12** are preferably adjusted to position the marker stream in a desired section of the channel. Preferably, a confocal microscope such as a Carl Zeiss LSM 510 is used. The LSM 510 is capable of line scans at about 0.38 ms/512 pixel line or approximately 0.2 ms/100 pixel line. Fluorescent microspheres, preferably about 0.2 μm, and fluorescently

labeled high-molecular weight polymers are preferably used to visualize the flow with minimal interference from diffusion. A channel such as one with 100 μm wide and 100 μm deep channel may be used. The line scan technique may be applied to various sequences such as one that has about 200-μm long plugs separated by about 800-μm long oil stream.

A beam is preferably fixed in the x and z-directions and scanned repeatedly back and forth along the y-direction. The movement of the plug in the x-direction preferably provides resolution along the x-direction. Line scan with 100 pixels across a 100 μm-wide channel will provide a resolution of about 1 μm/pixel in the y-direction. Approximately 200 line scans per plug are preferably used to give a resolution of about 1 μm/pixel in the x-direction. For a 200 μm plug moving at about 2000 μm/s, about 200 line scans are preferably obtained over a period of about (200 μm)/(2000 μm/s)=0.1 s, or about 0.5 ms per line.

The sequence shown in FIG. **10***b*) is preferably used for visualization of a three dimensional chaotic flow. Each line scan preferably takes about 0.2 ms with about 0.3 ms lag between the scans to allow the plug to move by about 500 μm. Some optical distortions may result during the approximately 0.2 ms scan as the plug is translated along the x-direction by about 0.2 μm. However, these distortions are believed to be comparable to the resolution of the method. For a given position along the x-direction, a series of line scans are preferably obtained for about 10 seconds for each point along the z-direction to obtain an x-y cross-sections of ten plugs. Scans along the z-direction are preferably taken in 1 μm increments to obtain a full three-dimensional image of the distribution of the fluorescent marker in the plug. This procedure is preferably repeated at different positions along the x-direction to provide information such as changes in the three-dimensional distribution of the fluorescent marker inside the plug as the plug moves along the channels.

In case of periodic perturbations, the fluorescent cross-sections of the plug in the y-z plane recovered from the above procedure represent Poincaré sections corresponding to the evolution of the initial thin sheet of dye. The twirling of the aqueous phase upon formation of the small plugs could distribute the dye excessively throughout the plug and could make visualization less conclusive. This twirling is prevented preferably by designing a small neck in the plug-forming region, and then beginning the first turn in a downward direction. This approach has been successfully applied to flow visualization, and may be useful for conducting reactions.

Merging Plugs

The invention also provides a method of merging of plugs within a substrate (see upper portion of FIG. **12**). Plugs are formed as described above. Plugs containing different reagents can be formed by separately introducing different plug-fluids into a channel. The plugs containing different reagents may be substantially similar in viscosity or may differ. The plugs containing different reagents may be substantially similar in size or they may differ in size. Provided that the relative velocities of the plugs containing different reagents differ, the plugs will merge in the channels. The location of merging can be controlled in a variety of ways, for example by varying the location of plug-fluid inlet ports, by varying the location of channel junctions (if one of the plug forming fluids is introduced into a secondary channel), varying the size of the plugs, adjusting the speed at which different sets of plugs are transported varying the viscosity or surface tension of plugs having substantially the same size, etc.

As shown in FIG. **12** (top photograph), plugs may be merged by directing or allowing the plugs **120**, **121** to pass through a T-shaped channel or a T-shaped region of a channel. The resulting merged plugs **122** flow in separate channels or channel branches which may be perpendicular, as shown in FIG. **12**, or nonperpendicular (FIG. **33**). The merged plugs **122** may undergo further merging or undergo splitting, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more reactions or "treatments" such as one or more types of characterizations, measurements, detection, sorting, or analysis.

In one embodiment, large and small plugs flow along separate channels or channel branches towards a common channel where they merge. In a case where a large and a small plug do not converge at the same point at the same time, they eventually form a merged plug as the larger plug, which moves faster than the smaller plug, catches up with the small plug and merges with it. In the case where the larger and smaller plugs meet head on at the same point or region, they immediately combine to form a merged plug. The merged plugs may undergo splitting, described below, or further merging in other channels or channel regions, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more types of characterizations, measurements, detection, sorting, or analysis.

In another embodiment, plugs can be merged by controlling the arrival time of the plugs flowing in opposite directions towards a common point, area, or region of the channel so that each pair of plugs arrive at the common point, area, or region of the channel at around the same time to form a single plug.

In another embodiment, an arched, semi-circular, or circular channel provides a means for increasing the efficiency of plug merging. Thus, for example, a greater frequency of merging would occur within a more compact area or region of the substrate. Using this scheme, plugs flowing along separate channels towards a common channel may merge within a shorter distance or a shorter period of time because the arched, semi-circular, or circular channel or channel branch converts or assists in converting initially out-of-phase plug pairs to in-phase plug pairs. Specifically, the arched, semi-circular, or circular channel or channel branch would allow a lagging plug to catch up and merge with a plug ahead of it, thereby increasing the number of merged plugs in a given period or a given area or region of a substrate.

Splitting and/or Sorting Plugs

The present invention also provides a method for splitting of plugs within a substrate. Plugs can be split by passing a first portion of a plug into a second channel through an opening, wherein the second channel is downstream of where the plug is formed. Alternatively, plugs may be split at a "Y" intersection in a channel. In both embodiment, the initial plug splits into a first portion and a second portion and thereafter each portion passes into separate channel (or outlet). Either initially formed plugs can be split or, alternatively, merged plugs can be split. FIG. **6** shows a schematic diagram illustrating part of a microfluidic network that uses multiple inlets (inlets **601**, **603**, **605**, **607** for reagents A, B, C, and D; inlets **602**, **604**, **606** for aqueous streams) and that allows for both splitting and merging of plugs. This schematic diagram shows two reactions that are conducted simultaneously. A third reaction (between the first two reaction mixtures) is conducted using precise time delay. Plugs can be split before or after a reaction has occurred. In addition, FIG. **6** shows plugs at various stages of mixing from the initial mixture **60** (A+B) and initial mixture **61** (C+D) through the mixed solutions **62** (A+B), **63** (C+D), and the 4-component mixture **64** (A+B+C+D).

As shown in FIG. **12** (lower photograph), plugs may be split by directing or allowing the plugs **123**, **124** to pass through a T-shaped channel or a T-shaped region of a channel.

In a preferred embodiment, the area or junction at which the plugs undergo splitting may be narrower or somewhat constricted relative to the diameter of the plugs a certain distance away from the junction. The resulting split plugs **125** flow in separate channels or channel branches which may be perpendicular, as shown in FIG. **12**, or nonperpendicular (FIG. **33**). The split **125** plugs may undergo merging or further splitting, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more reactions or "treatments" such as one or more types of characterizations, measurements, detection, sorting, or analysis.

In another embodiment, aqueous plugs can be split or sorted from an oil carrier fluid by using divergent hydrophilic and hydrophobic channels. The channels are rendered hydrophilic or hydrophobic by pretreating a channel or region of a channel such that a channel or channel surface becomes predominantly hydrophilic or hydrophobic. As discussed in more detail below, substrates with hydrophilic channel surfaces may be fabricated using methods such as rapid prototyping in polydimethylsiloxane. The channel surface can be rendered hydrophobic either by silanization or heat treatment. For example, (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.) vapor may be applied to the inlets of the substrate with dry nitrogen as a carrier gas to silanize the channel surface.

Once plugs have been split into separate channels, further reactions can be performed by merging the split plugs with other plugs containing further reactants.

Manipulation of plugs and reagents/products contained therein can also be accomplished in a fluid flow using methods or techniques such as dielectrophoresis. Dielectrophoresis is believed to produce movement of dielectric objects, which have no net charge, but have regions that are positively or negatively charged in relation to each other. Alternating, nonhomogeneous electric fields in the presence of plugs and/or particles, cause the plugs and/or particles to become electrically polarized and thus to experience dielectrophoretic forces. Depending on the dielectric polarizability of the particles and the suspending medium, dielectric particles will move either toward the regions of high field strength or low field strength. Using conventional semiconductor technologies, electrodes can be fabricated onto a substrate to control the force fields in a micro fabricated device. Dielectrophoresis is particularly suitable for moving objects that are electrical conductors. The use of AC current is preferred, to prevent permanent alignment of ions. Megahertz frequencies are suitable to provide a net alignment, attractive force, and motion over relatively long distances.

Radiation pressure can also be used in the invention to deflect and move plugs and reagents/products contained therein with focused beams of light such as lasers. Flow can also be obtained and controlled by providing a thermal or pressure differential or gradient between one or more channels of a substrate or in a method according to the invention.

Preferably, both the fluid comprising the plugs and the carrier fluid have a relatively low Reynolds Number, for example 10^{-2}. The Reynolds Number represents an inverse relationship between the density and velocity of a fluid and its viscosity in a channel of given cross-sectional dimension. More viscous, less dense, slower moving fluids will have a lower Reynolds Number, and are easier to divert, stop, start, or reverse without turbulence. Because of the small sizes and slow velocities, fabricated fluid systems are often in a low Reynolds number regime (R_e<<1). In this regime, inertial effects, which cause turbulence and secondary flows, are negligible and viscous effects dominate the dynamics. These conditions are advantageous for analysis, and are provided by devices according to the invention. Accordingly the devices according to the invention are preferably operated at a Reynolds number of less than 100, typically less than 50, preferably less than 10, more preferably less than 5, most preferably less than 1.

Detection and Measurement

The systems of the present invention are well suited for performing optical measurements using an apparatus such as a standard microscope. For example, PDMS is transparent in the visible region. When it is used to construct a substrate, a glass or quartz cover slip can be used to cover or seal a PDMS network, thereby constructing a set of channels that can be characterized using visible, UV, or infrared light. Preferably, fluorescent measurements are performed, instead of absorption measurements, since the former has a higher sensitivity than the latter. When the plugs are being monitored by optical measurements, the refractive index of the carrier-fluid and the plug-fluids are preferably substantially similar, but they can be different in certain cases.

In a plug-based system according to the invention, the relative concentrations (or changes in concentrations) can be typically measured in a straightforward fashion. In some instances, the use of plugs to perform quantitative optical measurements of, for example, absolute concentrations is complicated by the presence of non-horizontal oil/water interfaces surrounding the plugs. These curved interfaces act as lenses, and may lead to losses of emitted light or optical distortions. Such distortions may adversely affect or prevent visual observation of growing protein crystals, for example. Exact modeling of these losses is usually difficult because of the complicated shape that this interface may adopt at the front and back of a plug moving in a non-trivial pressure gradient.

This problem can be overcome or minimized in accordance with the invention by using a technique such as refractive index matching. The losses and distortions depend on the difference between the refractive index (η_D) of the aqueous phase and the refractive index of the immiscible carrier-fluid. Preferably, the carrier-fluid used in an analysis have refractive indices that are substantially similar to those of water and aqueous buffers (TABLE 1), e.g., fluorinated oils having refractive indices near that of water close to the sodium D line at 589 nm.

Preferably, for applications involving detection or measurement, the carrier-fluids used are those having refractive indices that match those of commonly used aqueous solutions at the wavelengths used for observation. To calibrate a system for quantitative fluorescence measurements, the plugs preferably contain known concentrations of fluorescein. Preferably, the fluorescence originating from the plugs are measured and then compared with the fluorescence arising from the same solution of fluorescein in the channel in the absence of oil. It is believed that when the refractive indexes are matched, the intensity (I) of fluorescence arising from the plugs will be substantially similar or equal to the intensity of the fluorescence from the aqueous solutions after making adjustments for the fraction of the aqueous stream:

$$I_{plug}=I_{solution}*V_{water}/(V_{water}+V_{oil}) \quad \text{Eqn. (3)}$$

where V is the volumetric flow rate of the fluid streams. It is expected that smaller plugs with a higher proportion of curved interfaces will show larger deviations from ideal plug behavior, i.e., those smaller plugs will tend to cause greater optical distortion. If necessary, measurements are performed partly to determine the errors associated with refractive index mismatch. Information from these measurements is useful

when unknown fluids are analyzed, or when a compromise between matching the refractive index and matching the viscosities of the two fluids is required.

TABLE 1

Physical properties of some fluids used in certain embodiments of the microfluidic devices.

Fluid	Refractive index, η_D	Viscosity, μ [mPa-s]
water	1.3330	1.00
aqueous PBS buffer, 1%	1.3343	1.02
aqueous PBS buffer, 10%	1.3460	1.25
perfluorohexane	1.251	0.66
perfluoro(methylcyclohexane)	1.30	1.56
perfluoro(1,3-dimethylcyclohexane)	1.2895	1.92
perfluorodecaline	1.314	5.10
perfluoroperhydrofluorene	1.3289	9.58
perfluoroperhydrophenanthrene	1.3348	28.4
perfluorotoluene	1.3680	N/A
hexafluorobenzene	1.3770	N/A

The detector can be any device or method for evaluating a physical characteristic of a fluid as it passes through the detection region. Examples of suitable detectors include CCD detectors. A preferred detector is an optical detector, such as a microscope, which may be coupled with a computer and/or other image processing or enhancement devices to process images or information produced by the microscope using known techniques. For example, molecules can be analyzed and/or sorted by size or molecular weight. Reactions can be monitored by measuring the concentration of a product produced or the concentration of a reactant remaining at a given time. Enzymes can be analyzed and/or sorted by the extent to which they catalyze a chemical reaction of an enzyme's substrate (conversely, an enzyme's substrate can be analyzed (e.g., sorted) based on the level of chemical reactivity catalyzed by an enzyme). Biological particles or molecules such as cells and virions can be sorted according to whether they contain or produce a particular protein, by using an optical detector to examine each cell or virion for an optical indication of the presence or amount of that protein. A chemical itself may be detectable, for example by a characteristic fluorescence, or it may be labeled or associated with a tag that produces a detectable signal when, for example, a desired protein is present, or is present in at least a threshold amount.

Practically any characteristic of a chemical can be identified or measured using the techniques according to the invention, provided that the characteristic or characteristics of interest for analysis can be sufficiently identified and detected or measured to distinguish chemicals having the desired characteristic(s) from those which do not. For example, particulate size, hydrophobicity of the reagent versus carrier-fluids, etc. can be used as a basis for analyzing (e.g., by sorting) plug-fluids, reaction products or plugs.

In a preferred embodiment, the plugs are analyzed based on the intensity of a signal from an optically detectable group, moiety, or compound (referred to here as "tag") associated with them as they pass through a detection window or detection region in the device. Plugs having an amount or level of the tag at a selected threshold or within a selected range can be directed into a predetermined outlet or branch channel of the substrate. The tag signal may be collected by a microscope and measured by a detector such as a photomultiplier tube (PMT). A computer is preferably used to digitize the PMT signal and to control the flow through methods such as those based on valve action. Alternatively, the signal can be recorded or quantified as a measure of the tag and/or its corresponding characteristic or marker, e.g., for the purpose of evaluation and without necessarily proceeding to, for example, sort the plugs.

In one embodiment according to the invention, a detector such as a photodiode is larger in diameter than the width of the channel, forming a detection region that is longer (along the length of channel) than it is wide. The volume of such a detection region is approximately equal to the cross sectional area of the channel above the diode multiplied by the diameter of the diode.

To detect a chemical or tag, or to determine whether a chemical or tag has a desired characteristic, the detection region may include an apparatus (e.g., a light source such as a laser, laser diode, high intensity lamp such as mercury lamp) for stimulating a chemical or tag for that characteristic to, for example, emit measurable light energy. In embodiments where a lamp is used, the channels are preferably shielded from light in all regions except the detection region. In embodiments where a laser is used, the laser can be set to scan across a set of detection regions. In addition, laser diodes may be fabricated into the same substrate that contains the analysis units. Alternatively, laser diodes may be incorporated into a second substrate (i.e., a laser diode chip) that is placed adjacent to the analysis or sorter substrate such that the laser light from the diodes shines on the detection region(s).

In preferred embodiments, an integrated semiconductor laser and/or an integrated photodiode detector are included on the silicon wafer in the device according to the invention. This design provides the advantages of compactness and a shorter optical path for exciting and/or emitted radiation, thus minimizing, for example, optical distortion.

As each plug passes into the detection region, it may be examined for a characteristic or property, e.g., a corresponding signal produced by the plug, or the chemicals contained in the plugs, may be detected and measured to determine whether or not a given characteristic or property is present. The signal may correspond to a characteristic qualitatively or quantitatively. Typically, the amount of signal corresponds to the degree to which a characteristic is present. For example, the strength of the signal may indicate the size of a molecule, the amount of products(s) formed in a reaction, the amount of reactant(s) remaining, the potency or amount of an enzyme expressed by a cell, a positive or negative reaction such as binding or hybridization of one molecule to another, or a chemical reaction of a substrate catalyzed by an enzyme. In response to the signal, data can be collected and/or a flow control can be activated, for example, to direct a plug from one channel to another. Thus, for example, chemicals present in a plug at a detection region may be sorted into an appropriate branch channel according to a signal produced by the corresponding examination at a detection region. Optical detection of molecular characteristics or the tag associated with a characteristic or property that is chosen for sorting, for example, may be used. However, other detection techniques, for instance electrochemistry, or nuclear magnetic resonance, may also be employed.

In one embodiment according to the invention, a portion of a channel corresponds to an analysis unit or detection region and includes a detector such as a photodiode preferably located in the floor or base of the channel. The detection region preferably encompasses a receive field of the photodiode in the channel, which receive field has a circular shape. The volume of the detection region is preferably the same as, or substantially similar, to the volume of a cylinder with a diameter equal to the receive field of the photodiode and a height equal to the depth of the channel above the photodiode.

The signals from the photodiodes may be transmitted to a processor via one or more lines representing any form of electrical communication (including e.g. wires, conductive lines etched in the substrate, etc.). The processor preferably acts on the signals, for example by processing them into values for comparison with a predetermined set of values for analyzing the chemicals. In one embodiment, a value corresponds to an amount (e.g., intensity) of optically detectable signal emitted from a chemical which is indicative of a particular type or characteristic of a chemical giving rise to the signal. The processor preferably uses this information (i.e., the values) to control active elements in a discrimination region, for example to determine how to sort the chemicals (e.g., valve action).

When more than one detection region is used, detectors such as photodiodes in a laser diode substrate are preferably spaced apart relative to the spacing of the detection regions in the analysis unit. That is, for more accurate detection, the detectors are placed apart at the same spacing as the spacing of the detection region.

A processor can be integrated into the same substrate that contains at least one analysis unit, or it can be separate, e.g., an independent microchip connected to the analysis unit containing substrate via electronic leads that connect to the detection region(s) and/or to the discrimination region(s), such as by a photodiode. The processor can be a computer or microprocessor, and is typically connected to a data storage unit, such as computer memory, hard disk, or the like, and/or a data output unit, such as a display monitor, printer and/or plotter.

The types and numbers of chemicals based on the detection of, for example, a tag associated with or bound to the chemical passing through the detection region, can be calculated or determined, and the data obtained can be stored in the data storage unit. This information can then be further processed or routed to a data outlet unit for presentation, e.g. histograms representing, for example, levels of a protein, saccharide, or some other characteristic of a cell surface in the sample. The data can also be presented in real time as the sample flows through a channel.

If desired, a substrate may contain a plurality of analysis units, i.e., more than one detection region, and a plurality of branch channels that are in fluid communication with and that branch out from the discrimination regions. It will be appreciated that the position and fate of the reagents in the discrimination region can be monitored by additional detection regions installed, for example, immediately upstream of the discrimination region and/or within the branch channels immediately downstream of the branch point. The information obtained by the additional detection regions can be used by a processor to continuously revise estimates of the velocity of the reagents in the channels and to confirm that molecules, particles, and substances having a selected characteristic enter the desired branch channel.

In one embodiment, plugs are detected by running a continuous flow through a channel, taking a spatially resolved image with a CCD camera, and converting the relevant distance traversed by the plugs into time.

In another embodiment, plugs are detected following their exit through a channel point leading to a mass spectrometer (MS), e.g., an electrospray MS. In this embodiment, time-resolved information (e.g., mass spectrum) can be obtained when the flow rate and the distance traversed by the plugs are known. This embodiment is preferable when one wants to avoid using a label.

Varying the Concentration of Reagents Inside Plugs

The various devices and methods according to the invention allow the control and manipulation of plug composition and properties. For example, they allow the variation of reagent concentration inside plugs. In one aspect according to the invention, the concentrations of the reagents in the plugs are varied by changing the relative flow rates of the plug-fluids. This is possible in conventional systems, but is complicated by problems of slow mixing and dispersion. Methods according to the invention are convenient for simultaneously testing a large number of experimental conditions ("screening") because the concentrations can be changed within a single setup. Thus, for example, syringes do not have to be disconnected or reconnected, and the inlets of a system according to the invention do not have to be refilled when using the above technique for varying the reagent concentrations in plugs.

The concentration of aqueous solutions inside plugs can be varied by changing the flow rates of the plug-fluid streams (see FIG. **25**, discussed in detail in Example 11). In FIG. **25**, water is introduced into inlets **251-258** at various flow rates while perfluorodecaline flows through channels **259-261**. In aqueous laminar flows, the ratio of flow rates of laminar streams in a microfluidic channel may be varied from about 1000:1 and 1:1000, preferably 100:1 to 1:100, more preferably 1:20 to 20:1.

The actual relative concentrations may be quantified using a solution of known concentration of fluorescein. In this example, the intensity of a fluorescein stream can be used as a reference point to check for fluctuations of the intensity of the excitation lamp.

To illustrate an advantage offered by the invention over other techniques, consider the following example. The method(s) described in this example may be modified or incorporated for use in various types of applications, measurements, or experiments. Two or more reagents, such as reagents A, B, C, are to be screened for the effects of different concentrations of reagents on some process, and the conditions under which an inhibitor can terminate the reaction of the enzyme with a substrate at various enzyme and substrate concentrations is of interest. If A is an enzyme, B a substrate, and C an inhibitor, a substrate with 5 inlets such as A/water/B/water/C inlets can be used, and the flow rates at which A, B and C are pumped into the substrate can be varied. Preferably, the size of the plug is kept constant by keeping the total flow rate of all plug-fluids constant. Because different amounts of A, B, C are introduced, the concentrations of A, B, C in the plugs will vary. The concentrations of the starting solutions need not be changed and one can rapidly screen all combinations of concentrations, as long as an enzymatic reaction or other reactions being screened can be detected or monitored. Because the solutions are flowing and the transport is linear, one can determine not only the presence or absence of an interaction or reaction, but also measure the rate at which a reaction occurs. Thus, both qualitative and quantitative data can be obtained. In accordance with the invention, the substrate typically need not be cleaned between runs since most, if not all, reagents are contained inside the plugs and leave little or no residue.

To extend the range over which concentrations can be varied, one may use a combination of, say, reagents A, B, C, D, E and prepare a micromolar solution of A, a mM solution of B, and a M solution of C, and so on. This technique may be easier than controlling the flow rate over a factor of, say, more than 10^6. Using other known methods is likely to be more difficult in this particular example because changing the ratio of reagents inside the plug requires changing the size of the plugs, which makes merging complicated.

In another example, one may monitor RNA folding in a solution in the presence of different concentrations of Mg^{2+}

and H^+. Previously, this was done using a stopped-flow technique, which is time consuming and requires a relatively large amount of RNA. Using a method according to the invention, an entire phase space can be covered in a relatively short period of time (e.g., approximately 15 minutes) using only μL/minute runs instead of the usual ml/shot runs.

These particular examples highlight the usefulness according to the invention in, for example, the study of protein/protein interaction mediation by small molecules, protein/RNA/DNA interaction mediation by small molecules, or binding events involving a protein and several small molecules. Other interactions involving several components at different concentrations may also be studied using the method according to the invention.

Generating Gradients in a Series of Plugs

In one aspect according to the invention, dispersion in a pressure-driven flow is used to generate a gradient in a continuous stream of plug-fluid. By forming plugs, the gradient is "fixed", i.e., the plugs stop the dispersion responsible for the formation of the gradient. Although the stream does not have to be aqueous, an aqueous stream is used as a non-limiting example below.

FIG. **44** illustrates how an initial gradient may be created by injecting a discrete aqueous sample of a reagent B into a flowing stream of water. In FIG. **44***a*), the water+B mixture flowed through channel **441**. Channels **443** and **445** contain substantially non-flowing water+B mixture. Water streams were introduced into inlets **440**, **442**, **444**, **446-448** while oil streams flowed through channels **449-452**. FIG. **44***d*) shows a multiple-inlet system through which reagents A, B, and C are introduced through inlets **453**, **454**, and **455**. A pressure-driven flow is allowed to disperse the reagent along the channel, thus creating a gradient of B along the channel. The gradient can be controlled by suitable adjustments or control of the channel dimensions, flow rates, injection volume, or frequency of sample or reagent addition in the case of multiple injections. This gradient is then "fixed" by the formation of plugs. Several of these channels are preferably combined into a single plug-forming region or section. In addition, complex gradients with several components may be created by controlling the streams. This technique may be used for various types of analysis and synthesis. For example, this technique can be used to generate plugs for protein or lysozome crystallization. FIG. **42** shows an experiment involving the formation of gradients by varying the flow rates (the experimental details are described in Example 17). FIG. **43** illustrates the use of gradients to form lysozyme crystals (the experimental details are described in Example 18).

Formation and Isolation of Unstable Intermediates

The devices and methods according to the present invention may also be used for synthesizing and isolating unstable intermediates. The unstable intermediates that are formed using a device according to the invention are preferably made to undergo further reaction and/or analysis or directed to other parts of the device where they may undergo further reaction and/or analysis. In one aspect, at least two different plug-fluids, which together react to form an unstable intermediate, are used. As the unstable intermediates form along the flow path of the substrate, information regarding, for example, the reaction kinetics can be obtained. Such unstable intermediates can be further reacted with another reagent by merging plugs containing the unstable intermediate with another plug-fluid. Examples of unstable intermediates include, but are not limited to, free radicals, organic ions, living ionic polymer chains, living organometallic polymer chains, living free radical polymer chains, partially folded proteins or other macromolecules, strained molecules, crystallization nuclei, seeds for composite nanoparticles, etc.

One application of devices according to the invention that involves the formation of unstable intermediates is high-throughput, biomolecular structural characterization. It can be used in both a time-resolved mode and a non-time resolved mode. Unstable (and/or reactive) intermediates (for example hydroxyl radicals (OH)) can be generated in one microfluidic stream (for example using a known reaction of metal ions with peroxides). These reactive species can be injected into another stream containing biomolecules, to induce reaction with the biomolecules. The sites on the biomolecule where the reaction takes place correlate with how accessible the sites are. This can be used to identify the sites exposed to the solvent or buried in the interior of the biomolecule, or identify sites protected by another biomolecule bound to the first one. This method could be applied to understanding structure in a range of biological problems. Examples include but are not limited to protein folding, protein-protein interaction (protein footprinting), protein-RNA interaction, protein-DNA interactions, and formation of protein-protein complexes in the presence of a ligand or ligands (such as a small molecule or another biomolecule). Interfacing such a system to a mass-spectrometer may provide a powerful method of analysis.

Experiments involving complex chemical systems can also be performed in accordance with the invention. For example, several unstable intermediates can be prepared in separate plugs, such as partially folded forms of proteins or RNA. The reactivity of the unstable intermediates can then be investigated when, for example, the plugs merge.

Dynamic Control of Surface Chemistry

Control of surface chemistry is particularly important in microfluidic devices because the surface-to-volume ratio increases as the dimensions of the systems are reduced. In particular, surfaces that are generally inert to the adsorption of proteins and cells are invaluable in microfluidics. Polyethylene glycols (PEG) and oligoethylene glycols (OEG) are known to reduce non-specific adsorption of proteins on surfaces. Self-assembled monolayers of OEG-terminated alkane thiols on gold have been used as model substrates to demonstrate and carefully characterize resistance to protein adsorption. Surface chemistry to which the solutions are exposed can be controlled by creating self-assembled monolayers on surfaces of silicone or grafting PEG-containing polymers on PDMS and other materials used for fabrication of microfluidic devices. However, such surfaces may be difficult to mass-produce, and they may become unstable after fabrication, e.g., during storage or use.

In one aspect according to the invention, the reagents inside aqueous plugs are exposed to the carrier-fluid/plug-fluid interface, rather than to the device/plug-fluid interface. Using perfluorocarbons as carrier-fluids in surface studies are attractive because they are in some cases more biocompatible than hydrocarbons or silicones. This is exemplified by the use of emulsified perfluorocarbons as blood substitutes in humans during surgeries. Controlling and modifying surface chemistry to which the reagents are exposed can be achieved simply by introducing appropriate surfactants into the fluorinated PFD phase.

In addition, the use of surfactants can be advantageous in problems involving unwanted adsorption of substances or particles, for example, on the channel walls. Under certain circumstances or conditions, a reaction may occur in one or more channels or regions of the substrate that give rise to particulates that then adhere to the walls of the channels. When they collect in sufficient number, the adhering particulates may thus lead or contribute to channel clogging or

constriction. Using methods according to the invention, such as using one or more suitable surfactants, would prevent or minimize adhesion or adsorption of unwanted substances or particles to the channel walls thereby eliminating or minimizing, for example, channel clogging or constriction.

Encapsulated particulates may be more effectively prevented from interfering with desired reactions in one or more channels of the substrate since the particulates would be prevented from directly coming into contact with reagents outside the plugs containing the particulates.

Fluorosurfactants terminated with OEG-groups have been shown to demonstrate biocompatibility in blood substitutes and other biomedical applications. Preferably, oil-soluble fluorosurfactants terminated with oligoethylene groups are used to create interfaces in the microfluidic devices in certain applications. Surfactants with well-defined composition may be synthesized. This is preferably followed by the characterization of the formation of aqueous plugs in the presence of those surfactants. Their inertness towards nonspecific protein adsorption will also be characterized. FIG. **24** shows examples of fluorinated surfactants that form monolayers that are: resistant to protein adsorption; positively charged; and negatively charged. For OEG-terminated surfactants, high values of n ($\geqq$16) are preferred for making these surfactants oil-soluble and preventing them from entering the aqueous phase. In FIG. **24**, compounds that have between about 3 to 6 EG units attached to a thiol are sufficient to prevent the adsorption of proteins to a monolayer of thiols on gold, and are thus preferred for inertness. In addition, surfactants that have been shown to be biocompatible in fluorocarbon blood substitutes may also be used as additives to fluorinated carrier fluids.

Applications: Kinetic Measurements and Assays

The devices and methods of the invention can be also used for performing experiments typically done in, for example, a microtiter plate where a few reagents are mixed at many concentrations and then monitored and/or analyzed. This can be done, for example, by forming plugs with variable composition, stopping the flow if needed, and then monitoring the plugs. The assays may be positionally encoded, that is, the composition of the plug may be deduced from the position of the plug in the channel. The devices and methods of the invention may be used to perform high-throughput screening and assays useful, for example, in diagnostics and drug discovery. In particular, the devices and methods of the invention can be used to perform relatively fast kinetic measurements.

The ability to perform fast measurements has revolutionized the field of biological dynamics. Examples include studies of protein C folding and cytochrome C folding. These measurements are performed using fast kinetics instruments that rely on turbulence to mix solutions rapidly. To achieve turbulence, the channels and the flow rates normally have to be large, which require large sample volumes. Commercially available instruments for performing rapid kinetics studies can access times on the order of 1 ms. The improved on-chip version of a capillary glass-ball mixer gives a dead time of about 45 μs with a flow rate of more than about 0.35 mL/sec. The miniaturization of these existing methods is generally limited by the requirement of high flow rate to generate turbulence. Miniaturization afforded by devices and methods according to the invention is advantageous because it allows, for example, quantitative characterization, from genetic manipulation and tissue isolation, of a much wider range of biomolecules including those available only in minute quantities, e.g., microgram quantities. In addition, these new techniques and instruments afford a wide range of accessible time scales for measurements.

Time control is important in many chemical and biochemical processes. Typically, stopped-flow type instruments are used to measure reaction kinetics. These types of instruments typically rely on turbulent flow to mix the reagents and transport them while minimizing dispersion. Because turbulent flow occurs in tubes with relatively large diameters and at high flow rates, stopped-flow instruments tend to use large volumes of reagents (e.g., on the order of ml/s). A microfluidic analog of a stopped-flow instrument that consumes small volumes of reagents, e.g., on the order of μL/min, would be useful in various applications such as diagnostics. Thus far, microfluidic devices have not been able to compete with stopped-flow instruments because EOF is usually too slow (although it has less dispersion), and pressure-driven flows tend to suffer from dispersion. In addition, mixing is usually very slow in both systems.

Stopped-flow instruments typically have sub-millisecond mixing, and could be useful for experiments where such fast mixing is required. The devices and methods of the invention allow sub-millisecond measurements as well. In particular, the present invention can be advantageous for reactions that occur on a sub-second but slower than about 1 or about 10 millisecond (ms) time scale or where the primary concern is the solute volume required to perform a measurement.

Further, if a plug is generated with two reactive components, it can serve as a microreactor as the plug is transported down a channel. A plug's property, such as its optical property, can then be measured or monitored as a function of distance from a given point or region of a channel or substrate. When the plugs are transported at a constant flow rate, a reaction time can be directly determined from a given distance. To probe the composition of the plug as it exits a channel, the contents of the plugs may be injected into a mass spectrometer (e.g., an electrospray mass spectrometer) from an end of the channel. The time corresponding to the end of the channel may be varied by changing the flow rate. Multiple outlets may be designed along the channels to probe, for example, the plug contents using a mass spectrometer at multiple distance and time points.

An advantage of the devices and methods of the invention is that when plugs are formed continuously, intrinsically slow methods of observation can be used. For example, plugs flowing at a flow rate of about 10 cm/s through a distance of about 1 mm from a point of origin would be about 10 ms old. In this case, the invention is particularly advantageous because it allows the use of a relatively slow detection method to repeatedly perform a measurement of, for example, 10 ms-old plugs for virtually unlimited time. In contrast, to observe a reaction in a stopped-flow experiment at a time, say, between about 9 and 11 ms, one only has about 2 ms to take data. Moreover, the present invention allows one to obtain information involving complex reactions at several times, simultaneously, simply by observing the channels at different distances from the point of origin.

The reaction time can be monitored at various points along a channel—each point will correspond to a different reaction or mixing time. Given a constant fluid flow rate u, one may determine a reaction time corresponding to the various times $t_1, t_2, t_3, \ldots t_n$ along the channel. Thus, if the distance between each pair of points n and n−1, which correspond to time t_n and t_{n-1}, are the same for a given value of n, then the reaction time corresponding to point n along the channel may be calculated from t_n=nl/u. Thus, one can conveniently and repeatedly monitor a reaction at any given time t_n. In principle, the substrate of the present invention allows one to cover a greater time period for monitoring a reaction by simply extending the length of the channel that is to be monitored at a given flow

rate or by decreasing the flow rate over a given channel distance (see, for example, FIG. **22**). In FIG. **22**, the following can be introduced into the following inlets: enzyme into inlets **2201**, **2205**, **2210**, **2215**; buffer into inlets **2202**, **2206**, **2211**, **2216**; substrate into inlets **2203**, **2207**, **2212**, **2217**; buffer into inlets **2204**, **2208**, **2213**, **2218**; inhibitor into inlets **2228**, **2209**, **2214**, **2219**. In FIG. **22**, a carrier fluid flows through the channel portions **2220**, **2221**, **2222**, **2223** from left to right. The channel portions enclosed by the dotted square **2224**, **2225**, **2226**, **2227** represent fields of view for the purpose of monitoring a reaction at various points along the channel.

The same principle applies to an alternate embodiment of the present invention, where the distance corresponding to a point n from a common point of origin along the channel differs from that corresponding to another channel by a power or multiples of 2. This can be seen more clearly from the following discussion. Given a constant fluid flow rate u, one may determine a reaction time corresponding to the various times t_1, t_2, t_3, . . . t_n along the channel. Thus, if the distance between each pair of points n and n−1, which correspond to time t_n and t_{n-1}, are the same for a given value of n, then the reaction time corresponding to point n along the channel may be calculated from t_n=nl/u. In a relatively more complex channel geometry such as the one shown in FIG. **22**(*c*), the corresponding equation is given by $t_n=2^{(n-1)}$l/u, which shows that the reaction times at various points n varies as a power or multiples of 2.

In one aspect, channels according to the invention are used that place into a field of view different regions that correspond to different time points of a reaction. The channels according to the invention allow various measurements such as those of a complete reaction profile, a series of linearly separated time points (such as those required for the determination of an initial reaction velocity in enzymology), and a series of exponentially separated time points (e.g., first-order kinetic measurements or other exponential analysis). Time scales in an image frame can be varied from microseconds to seconds by, for example, changing the total flow rate and channel length.

FIG. **22**A-D show various examples of geometries of microfluidic channels according to the invention for obtaining kinetic information from single optical images. The illustrated channel systems are suitable for studies such as measurements of enzyme kinetics in the presence of inhibitors. The device shown in FIG. **22**D has multiple outlets that can be closed or opened. In the device shown in FIG. **22**D, preferably only one outlet is open at a time. At the fastest flow rates, the top outlet is preferably open, providing reduced pressure for flow through a short fluid path l. As flow rates are reduced, other outlets are preferably opened to provide a longer path and a larger dynamic range for measurements at the same total pressure.

In FIG. **22**, n is the number of segments for a given channel length l traveled by the reaction mixture in time t_n (see p. 73, second full paragraph for a related discussion of reaction times and channel lengths). These systems allow the control of the ratio of reagents by varying the flow rates. The systems also allow a quick quantification of enzyme inhibition.

For example, ribonuclease A can be used with known inhibitors such as nucleoside complexes of vanadium and oxovanadium ions and other small molecules such as 5'-diphosphoadenosine 3'-phosphate and 5'-diphosphoadenosine 2'-phosphate. The kinetics may be characterized by obtaining data and making Lineweaver-Burk, Eadie-Hofstee, or Hanes-Wolfe plots in an experiment. The experiment can be accomplished using only a few microliters of the protein and inhibitor solutions. This capability is particularly useful for characterizing new proteins and inhibitors that are available in only minute quantities, e.g., microgram quantities.

Kinetic measurements of reactions producing a fluorescent signal can be performed according to the invention by analyzing a single image obtained using, for example, an optical microscope. Long exposures (i.e., about 2 seconds) have been used to measure fast (i.e., about 2 milliseconds) kinetics. This was possible because in a continuous flow system, time is simply equal to the distance divided by the flow rate. In the continuous flow regime in accordance with the invention, the accessible time scales can be as slow as about 400 seconds, which can be extended to days or weeks if the flow is substantially slowed down or stopped. Typically, the time scale depends on the length of the channel (e.g., up to about 1 meter on a 3-inch diameter chip) at a low flow rate of about 1 mm/s, which is generally limited by the stability of the syringe pumps, but may be improved using pressure pumping. The fastest time scale is typically limited by the mixing time, but it may be reduced to about 20 μs in the present invention. Mixing time is generally limited by two main factors: (1) the mixing distance (e.g., approximately 10-15 times the width of the channel); and (2) the flow rates (e.g., approximately 400 mm/s, depending on the capillary number and the pressure drop required to drive the flow). Mixing distance is normally almost independent of the flow rate. By using suitable designs of microfluidic channels, or networks of microfluidic channels, a wide range of kinetic experiments can be performed.

Reducing the channel size generally reduces the mixing time but it also increases the pressure required to drive a flow. The equation below describes the pressure drop, ΔP (in units of Pa), for a single-phase flow in a rectangular capillary:

$$\Delta P=28.42U\mu l/ab \qquad \text{Eqn. (9)}$$

where U (m/s) is the velocity of the flow, μ (kilogram/meter-second, kg $m^{-1}s^{-1}$) is the viscosity of the fluid, l (m) is the length of the capillary, a (m) is the height of the capillary, and b (m) is the width of the capillary. There is generally a physical limitation on how much pressure a microfluidic device can withstand, e.g., about 3 atm for PDMS and about 5 atm for glass and Si. This limitation becomes crucial for very small channels and restricts the total length of the channel and thus the dynamic range (the total distance through which this flow rate can be maintained at a maximum pressure divided by the mixing distance) of the measurement.

FIG. **23** depicts a microfluidic network according to the invention with channel heights of 15 and 2 μm. The channel design shown in FIG. **23** illustrates how a dynamic range of about 100 can be achieved by changing the cross-section of the channels. Under these conditions, mixing time in the winding channel is estimated to be about 25 μs and observation time in the serpentine channels are estimated to be about 3 ms.

As FIG. **23** shows, rapid mixing occurs in the 2 μm×1 μm (height×width) channels and measurements are taken in the 2 μm×3 μm) channels. The table in FIG. **23** shows the distribution of the pressure drop, flow velocity, and flow time as a function of the channel cross-section dimensions. A transition from a 1-μm wide to 3-μm wide channels should occur smoothly, with plugs maintaining their stability and decreasing their velocity when they move from a 20-μm wide into a 50-μm wide channel. Changing the width of the channel can be easily done and easily incorporated into a mask design. The height of the channel can be changed by, for example, using photoresist layers having two different heights that are sequentially spun on, for example, a silicon wafer. A two-step exposure method may then be used to obtain a microfluidic network having the desired cross-section dimensions.

In another example of the application of the devices and methods of the present invention, the folding of RNase P catalytic domain (P RNA C-domain) of *Bacillus subtilis* ribozyme can be investigated using channels according to the invention. RNA folding is an important problem that remains largely unsolved due to limitations in existing technology. Understanding the rate-limiting step in tertiary RNA folding is important in the design, modification, and elucidation of the evolutionary relationship of functional RNA structures.

The folding of P RNA C-domain is known to involve three populated species: unfolded (U), intermediate (I), and native (N, folded) states. Within the first millisecond, the native secondary structure and some of the tertiary structure would have already folded (the RNA is compacted to about 90% of the native dimension) but this time regime cannot be resolved using conventional techniques such as stopped-flow. Using channels and substrates according to the invention, the time-dependence of the P RNA folding kinetics upon the addition of Mg^{2+} can be studied.

Various types of assays (e.g., protein assays) known in the art, including absorbance assays, Lowry assays, Hartree-Lowry assays, Biuret assays, Bradford assays, BCA assays, etc., can be used, or suitably adapted for use, in conjunction with the devices and methods of the invention. Proteins in solution absorb ultraviolet light with absorbance maxima at about 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Absorbance assays offer several advantages. Absorbance assays are fast and convenient since no additional reagents or incubations are required. No protein standard need be prepared. The assay does not consume the protein and the relationship of absorbance to protein concentration is linear. Further, the assay can be performed using only a UV spectrophotometer.

The Lowry assay is an often-cited general use protein assay. It was the method of choice for accurate protein determination for cell fractions, chromatography fractions, enzyme preparations, and so on. The bicinchoninic acid (BCA) assay is based on the same principle, but it can be done in one step. However, the modified Lowry is done entirely at room temperature. The Hartree version of the Lowry assay, a more recent modification that uses fewer reagents, improves the sensitivity with some proteins, is less likely to be incompatible with some salt solutions, provides a more linear response, and is less likely to become saturated.

In the Hartree-Lowry assay, the divalent copper ion forms a complex with peptide bonds under alkaline conditions in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. In addition to standard liquid handling supplies, the assay only requires a spectrophotometer with infrared lamp and filter. Glass or inexpensive polystyrene cuvettes may be used.

The Biuret assay is similar in principle to that of the Lowry, however it involves a single incubation of 20 minutes. In the Biuret assay, under alkaline conditions, substances containing two or more peptide bonds form a purple complex with copper salts in the reagent. The Biuret assay offer advantages in that there are very few interfering agents (ammonium salts being one such agent), and there were fewer reported deviations than with the Lowry or ultraviolet absorption methods. However, the Biuret consumes much more material. The Biuret is a good general protein assay for batches of material for which yield is not a problem. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed, with maximum transmission in the region of 450 nm. Glass or inexpensive polystyrene cuvettes may be used.

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis. Assay materials including color reagent, protein standard, and instruction booklet are available from Bio-Rad Corporation. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum (no special lamp or filter usually needed). Glass or polystyrene cuvettes may be used, but the color reagent stains both. Disposable cuvettes are recommended.

The bicinchoninic acid (BCA) assay is available in kit form from Pierce (Rockford, Ill.). This procedure is quite applicable to microtiter plate methods. The BCA is used for the same reasons the Lowry is used. The BCA assay is advantageous in that it requires a single step, and the color reagent is stable under alkaline conditions. BCA reduces divalent copper ion to the monovalent ion under alkaline conditions, as is accomplished by the Folin reagent in the Lowry assay. The advantage of BCA is that the reagent is fairly stable under alkaline condition, and can be included in the copper solution to allow a one step procedure. A molybdenum/tungsten blue product is produced as with the Lowry. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed with transmission set to 562 nm. Glass or inexpensive polystyrene cuvettes may be used.

The range of concentrations that can be measured using the above assays range from about 20 micrograms to 3 mg for absorbance at 280, between about 1-100 micrograms for absorbance at 205 nm, between about 2-100 micrograms for the Modified Lowry assay, between about 1-10 mg for the Biuret assay, between about 1-20 micrograms for the Bradford assay, and between about 0.2-50 micrograms for BCA assay. Many assays based on fluorescence or changes in fluorescence have been developed and could be performed using methods and devices of the invention.

A detailed description of various physical and chemical assays is provided in *Remington: The Science and Practice of Pharmacy*, A. R. Gennaro (ed.), Mack Publishing Company, chap. 29, "Analysis of Medicinals," pp. 437-490 (1995) and in references cited therein while chapter 30 of the same reference provides a detailed description of various biological assays. The assays described include titrimetric assays based on acid-base reactions, precipitation reactions, redox reactions, and complexation reactions, spectrometric methods, electrochemical methods, chromatographic methods, and other methods such as gasometric assays, assays involving volumetric measurements and measurements of optical rotation, specific gravity, and radioactivity. Other assays described include assays of enzyme-containing substances, proximate assays, alkaloidal drug assays, and biological tests such as pyrogen test, bacterial endotoxin test, depressor substances test, and biological reactivity tests (in-vivo and in-vitro).

In addition, *Remington: The Science and Practice of Pharmacy*, A. R. Gennaro (ed.), Mack Publishing Company, chap. 31, "Clinical Analysis," pp. 501-533 (1995) and references cited therein provide a detailed description of various methods of characterizations and quantitation of blood and other body fluids. In particular, the reference includes a detailed description of various tests and assays involving various body fluid components such as erythrocytes, hemoglobin, thrombocyte, reticulocytes, blood glucose, nonprotein nitrogen compounds, enzymes, electrolytes, blood-volume and erythropoeitic mechanisms, and blood coagulation.

Nonlinear and Stochastic Sensing

Stochastic behavior has been observed in many important chemical reactions, e.g., autocatalytic reactions such as inorganic chemical reactions, combustion and explosions, and in polymerization of sickle-cell hemoglobin that leads to sickle-cell anemia. Crystallization may also be considered an autocatalytic process. Several theoretical treatments of these reactions have been developed. These reactions tend to be highly sensitive to mixing.

Consider the extensively studied stochastic autocatalytic chemical reaction between $NaClO_2$ and $Na_2S_2O_3$ (chlorite-thiosulfate reaction). The mechanism of this reaction can be described by reactions (1) and (2),

$$4S_2O_3^{2-}+ClO_2^-+4H+\rightarrow 2S_4O_6^{2-}+2H_2O+Cl^- \quad \text{rate } (\nu)\alpha \underset{[H^+]}{} \tag{1}$$

$$S_2O_3^{2-}+2ClO_2^-+H_2O\rightarrow 2SO_4^{2-}+2H^++2Cl^- \quad \text{rate } (\nu)\alpha \underset{[H^+]^2[Cl]}{} \tag{2}$$

where $[H^+]$ stands for the concentration of H^+. At a slightly basic pH=7.5, the slow reaction (1) dominates and maintains a basic pH of the reaction mixture (since the rate of this reaction ν is directly proportional $[H^+]$, this reaction consumes H^+ and is auto-inhibitory). Reaction (2) dominates at acidic pH (since the rate of this reaction varies in proportion to $[H^+]^2[Cl^-]$, this reaction produces both H^+ and Cl^- and is superautocatalytic). FIG. **21** shows the reaction diagram for two reactions corresponding to the curves **211**, **212**. The rates of the two reactions (referred to here as reaction **211** and reaction **212**) are equal at an unstable critical point at a certain pH. The lifetime of the reaction mixtures of $NaClO_2$ and NaS_2O_3 at this critical point crucially depends on stirring. In the absence of stirring, stochastic fluctuations of $[H^+]$ in solution generate a localized increase in $[H^+]$. This increase in $[H^+]$ marginally increases the rate of reaction **212**, but it has a much stronger accelerating effect on reaction **211** because of the higher-order dependence on $[H^+]$ of this reaction. Therefore, in the region where local fluctuations increase local $[H^+]$, reaction **211** becomes dominant, and more H^+ is produced (which rapidly diffuses out of the region of the initial fluctuation). The initiated chemical wave then triggers the rapid reaction of the entire solution. Unstirred mixtures of $NaClO_2$ and NaS_2O_3 are stable only for a few seconds, and these fluctuations arise even in the presence of stirring.

FIG. **21** depicts a reaction diagram illustrating an unstable point in the chlorite-thiosulfate reaction. At $[H^+]$ values below the critical point, the slow reaction (1) dominates. At $[H^+]$ values above the critical point, the autocatalytic reaction (2) dominates. The reaction mixture at the $[H^+]$ value equal to the critical point is metastable in the absence of fluctuations. Under perfect mixing, the effects of small fluctuations average out and the system remains in a metastable state. Under imperfect mixing, fluctuations that reduce $[H^+]$ grow more slowly than those that increase $[H^+]$ due to the autocatalytic nature of reaction (2), and the reaction mixture thus rapidly becomes acidic.

It is known that chaotic flows should have a strong effect on diffusive transport within the fluid ("anomalous diffusion"). It is also known that chaotic dynamics can lead to non-Gaussian transport properties ("strange kinetics"). In one aspect according to the invention, these highly unstable mixtures are stabilized in the presence of chaotic mixing using channels according to the invention because this mixing can effectively suppress fluctuations. This invention can be used to understand the effects of mixing on the stochastic behavior of such systems, including for example, the chlorite thiosulfate system.

In a laminar flow, the flow profile in the middle of the channel is flat and there is virtually no convective mixing. Fluctuations involving $[H^+]$ that arise in the middle of the channel can grow and cause complete decomposition of the reaction mixture. Slow mixing reduces the probability of fluctuations in plugs moving through straight channels. When fluctuations that occur in the centers of vortices are not efficiently mixed away, one or more spontaneous reactions involving some of the plugs can take place. In the present invention, chaotic mixing in plugs moving through winding channels efficiently mix out fluctuations, and thus substantially fewer or no spontaneous reactions are expected to occur.

In a simple laminar flow, there is normally very little or no velocity gradient and substantially no mixing at the center of the channel. Thus, fluctuations that arise in the chlorite-thiosulfate reaction mixture prepared at the critical $[H^+]$ are able to grow and lead to rapid decomposition of the reaction mixture. Propagation of chemical fronts in autocatalytic reactions occurring in laminar flows has been described with numerical simulations, and back-propagation has been predicted (that is, a reaction front traveling upstream of the direction of the laminar flow). Using the method of the present invention, this back-propagation involving the reaction between $NaClO_2$ and $Na\ S_2O_3$ under laminar flow conditions was observed.

In accordance with the invention, chaotic flow within plugs that flow through winding channels suppresses fluctuations and gives rise to stable reaction mixtures. There exists, of course, a finite probability that fluctuations can arise even in a chaotically stirred plug. In one aspect according to the invention, the details of the evolution of these reactions are monitored using a high-speed digital camera. The plugs are preferably separated by the oil and are not in communication with each other, so the reaction of one plug will not affect the behavior of the neighboring plug. Statistics covering the behavior of thousands of plugs can be obtained quickly under substantially identical experimental conditions.

Whether a fluctuation would be able to trigger an autocatalytic reaction depends on factors such as the magnitude of a fluctuation and its lifetime. The lifetime of a fluctuation is typically limited by the mixing time in the system. In an unstirred solution, mixing is by diffusion and quite slow, and fluctuations may persist and lead to autocatalytic reactions. In a stirred solution, the lifetime of a fluctuation is relatively short, and only large fluctuations have sufficient time to cause an autocatalytic reaction.

Mixing time and the lifetime of fluctuations typically depend on the size of the plugs. As plug size decreases, mixing is accelerated and fluctuations are suppressed. However, very small plugs (e.g., about 1 μm^3 or 10^{-15} L) in a solution containing about 10^{-8} mole/liter concentration of H^+ (pH=8) will contain only a few H^+ ions per plug (about 10^{-23} moles or about $6H^+$ ions). When such small plugs are formed, the number of H^+ ions in them will have a Poisson distribution.

An important experimental challenge is to establish that the stochastic behavior in these systems is due mainly to

internal fluctuations of concentrations. Other factors that may act as sources of noise and instability are: (1) temporal fluctuations in the flow rates of the incoming reagent streams, which can lead to the formation of plugs with varying amounts of reagents; (2) temperature fluctuations in solutions in a microfluidic device, which may arise due to, for example, illumination by a microscope; and (3) fluctuations due to impurities in carrier-fluids leading to variations in the surface properties of different plugs.

Microfluidic systems according to the invention may be used to probe various chemical and biochemical processes, such as those that show stochastic behavior in bulk due to their nonlinear kinetics. They can also be used in investigating processes that occur in systems with very small volumes (e.g., about 1 μm^3, which corresponds to the volume of a bacterial cell). In systems with very small volumes, even simple reactions are expected to exhibit stochastic behavior due to the small number of molecules localized in these volumes.

Autocatalytic reactions present an exciting opportunity for highly sensitive detection of minute amounts of autocatalysts. Several systems are known to operate on this principle, silver-halide photography being the most widely used. In silver-halide photography, the energy of photons of light is used to decompose an emulsion of silver halide AgX into nanometer-sized particles of metallic silver. A film that is embedded with the silver particles is then chemically amplified by the addition of a metastable mixture of a soluble silver(I) salt and a reducing agent (hydroquinone). Metallic silver particles catalyze reduction of silver(I) by hydroquinone, leading to the growth of the initial silver particles. Another example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.

However, a dilemma occurs when designing systems with very high sensitivity and amplification. To achieve a very highly sensitive amplification, the system typically has to be made very unstable. On the other hand, an unstable system is very sensitive to noise and has a very short lifetime. Also, in unstable systems, it is difficult to distinguish between spontaneous decomposition and a reaction caused by the analyte. In one aspect, microfluidic devices according to the invention, which allow chaotic mixing and compartmentalization, are used to overcome this problem.

To demonstrate the potential of microfluidic systems according to the present invention, a microfluidic system according to the invention is used to handle unstable mixtures. In one application, a microfluidic system according to the invention is preferably used to control a stochastic reaction between $NaClO_2$ and NaS_2O_3. In particular, this reaction is preferably used for a highly sensitive amplification process.

If a plug containing an unstable reaction mixture of $NaClO_2$ and NaS_2O_3 is merged with a small plug containing an amount of H^+ sufficient to bring the local concentration of H^+ above critical, a rapid autocatalytic reactions is generally triggered. This autocatalytic reaction typically leads to the production of large amounts of H^+. Thus, a weak chemical signal, e.g., a small amount of H^+, is rapidly amplified by an unstable reaction mixture. Thus, for example, this approach can be used to investigate biological reactions such as those that involve enzymes, in which small amounts of H^+ are produced.

The above autocatalytic system possesses several features that contribute to its novelty and usefulness. In one aspect, an unstable amplifying reaction mixture is prepared in-situ and is used within milliseconds before it has a chance to decompose. Preferably, the system is compartmentalized so a reaction that occurs in one compartment does not affect a reaction in another compartment. This compartmentalization allows thousands of independent experiments to be conducted in seconds using only minute quantities of samples. Importantly, chaotic mixing in the system reduces fluctuations and stabilizes the reaction mixture.

The applications of controlled autocatalytic amplification in accordance with the invention are not limited to the detection of protons or Co^{2+} ions. For example, the (Co(III)-5-Br-PAPS)/peroxomonosulfate oxidation reaction can also be used indirectly, for example, for a detection of small amounts of peroxidase, which can be used as a labeling enzyme bound to an antibody. The (Co(III)-5-Br-PAPS)/peroxomonosulfate oxidation reaction, which has been characterized analytically, involves the autocatalytic decomposition of violet bis[2-(5-bromo-pyridylazo)-5-(N-propyl-N-sulfopropyl-amino-phenolato] cobaltate, (Co(III)-5-Br-PAPS), upon oxidation with potassium peroxomonosulfate to produce colorless Co^{2+} ions, which serve as the autocatalyst (the order of autocatalysis has not been established for this reaction). (Endo et al., "Kinetic determination of trace cobalt(II) by visual autocatalytic indication," *Talanta,* 1998, vol. 47, pp. 349-353; Endo et al., "Autocatalytic decomposition of cobalt complexes as an indicator system for the determination of trace amounts of cobalt and effectors," *Analyst,* 1996, vol. 121, pp. 391-394.)

$$\text{Co(III)-[5-Br-PAPS]reduced}+HSO_5^- \rightarrow Co^{2+}+\text{[5-Br-PAPS]oxidized}+HSO^{4-}$$

Addition of small amounts of Co^{2+} to the violet mixture of the (Co(III)-5-Br-PAPS and peroxomonosulfate produces an abrupt loss of color to give a colorless solution. The time delay before this decomposition depends on the amount of the Co^{2+} added to the solution. This reaction has been used to detect concentrations of Co^{2+} as low as 1×10^{-10} mole/L. The reaction shows good selectivity in the presence of other ions (V(V), Cr(III), Cr(VI), Mn(II), Fe(II), Ni(II), Cu(II) and Zn(II)).

The devices and methods according to the invention may be applied to other autocatalytic reactions, some of which have been described in inorganic, organic and biological chemistry. Reactions of transition metal ions such as Cr(III) (B82) Mn^{2+} or colloidal MnO_2, and reactions of halides and oxohalides are often autocatalytic. Autocatalysis involving lanthanides (Eu^{2+}) and actinides (U^{4+}) has also been reported. All of these elements are potential targets for detection and monitoring in chemical waste, drinking water, or biological fluids. Intriguing possibilities arise from using asymmetric autocatalytic reactions to detect minute amounts of optically active, chiral impurities, such as biomolecules.

It is also possible to design new autocatalytic reactions. Autocatalysis is abundant in biology, and many enzymes are autocatalytic (e.g., caspases involved in programmed cell death, kinases involved in regulation and amplification, and other enzymes participating in metabolism, signal transduction, and blood coagulation. Emulsions of perfluorocarbons such as perfluorodecaline (PFD) are used as blood substitutes in humans during surgeries and should be compatible with a variety of biological molecules. Since the feasibility of quantitative measurements of enzyme kinetics has been demonstrated using plugs formed according to the invention, plugs formed according to the invention may also be applied to the detection of biological autocatalysts.

The devices and methods according to the present invention are not limited to the detection of the autocatalyst itself. For example, the labeling of an analyte using an autocatalyst is also within the scope of the present invention. Biomolecules are often labeled with metallic nanoparticles. Such metallic nanoparticles are highly effective autocatalysts for

the reduction of metal ions to metals. Preferably, the systems and methods of the present invention are used in the visual detection of a single molecule of DNA, RNA, or protein labeled with nanoparticles via an autocatalytic pathway. In preliminary experiments in accordance with the invention, clean particle formation and transport within plugs were observed.

In addition, the generation of metal (e.g., copper, silver, gold, nickel) deposits and nanoparticles upon chemical reduction also proceed by an autocatalytic mechanism. These reactions are commonly used for electroless deposition of metals and should be useful for the detection of minute amounts of metallic particles. The presence of metallic particles in water can be indicative of the presence of operating mechanical devices. In one aspect according to the invention, devices and methods according to the invention are used to detect the presence of minute or trace quantities of metallic particles.

The devices in accordance with the present invention are simple in design, consume minute amounts of material, and robust. They do not require high voltage sources and can be operated, for example, using gravity or a pocket-sized source of compressed air. In one aspect, the systems according to the invention are used in portable and hand-held devices.

Autocatalytic reactions show a threshold response, that is, there is a very abrupt temporal change from unreacted mixture to reacted mixture. In the case where time is equal to distance, this abrupt transition over a short distance can be observed using the devices and methods of the invention. The time (and distance) is very sensitive to the initial concentration of the catalyst, and thus it should be easy to determine the concentration of the autocatalyst in the sample by noting how far the reaction system traveled before it reacted.

One example of an autocatalytic process is blood coagulation. It is very sensitive to flow and mixing, therefore experimenting with it in the absence of flow gives unreliable results or results that have little relevance to the real function of the coagulation cascade. A typical microfluidic system may be difficult to use with blood because once coagulation occurs, it blocks the channel and stops the flow in the microfluidic device. In addition, coagulated blood serves as an autocatalyst; even small amounts of coagulated blood in the channels can make measurements unreliable.

These problems can be overcome using the devices of the present invention. Using plugs, autocatalytic reactions can be easily controlled, and the formation of solid clots would not be a problem because any solids formed will be transported inside the plugs out of the channel without blocking the channel and without leaving autocatalytic residue. In addition, flow inside plugs can be easily controlled and adjusted to resemble flow under physiological conditions.

To address the sensitivity of blood coagulation to surfaces (the cascade is normally initiated on the surface), microscopic beads containing immobilized tissue factor (the cascade initiator) on the surface may be added to one of the streams and transported inside the plugs. Also, surfactants may be used to control surface chemistry.

Thus, the devices and methods of the invention may be used, for example, to test how well the coagulation cascade functions (e.g., for hemophilia or the tendency to form thrombus) under realistic flow conditions. This test would be particularly valuable in diagnostics. Blood may be injected in one stream, and a known concentration of a molecule known to induce coagulation (e.g., factor VIIa) can be added through another stream prior to plug formation. At a given flow rate, normal blood would coagulate at a certain distance (which corresponds to a given time), which can be observed optically by light scattering or microscopy. Blood of hemophiliac patients would coagulate at a later time. This type of testing would be useful before surgical operations. In particular, this type of testing is important for successful child delivery, especially when hemophilia is suspected. Fetal testing may be performed since only minute amounts of blood are required by systems according to the invention. The blood may be injected directly from the patient or collected in the presence of anticoagulating agent (for example EDTA), and then reconstituted in the plug by adding Ca^{2+}. In some cases, the addition of Ca^{2+} may be sufficient to initiate the coagulation cascade.

The devices and methods of the invention may also be used to evaluate the efficacy of anticoagulating agents under realistic flow conditions. Plugs can be formed from normal blood (which may be used directly or reconstituted by adding Ca^{2+} or other agents), an agent known to induce coagulation, and an agent (or several agents that need to be compared) being tested as an anticoagulation agent. The concentrations of these agents can be varied by varying the flow rates. The distance at which coagulation occurs is noted, and the efficacy of various agents to prevent coagulation is compared. The effects of flow conditions and presence of various compounds in the system on the efficacy of anticoagulation agents can be investigated quickly. The same techniques may also be used to evaluate agents that cause, rather prevent, coagulation. These tests could be invaluable in evaluating drug candidates.

Synthesis

In accordance with the present invention, a method of conducting a reaction within a substrate is provided. The reaction is initiated by introducing two or more plug-fluids containing reactants into the substrate of the present invention.

In one aspect, the plug-fluids include a reagent and solvent such that mixing of the plug-fluids results in the formation of a reaction product. In another embodiment, one of the plug-fluids may be reagent free and simply contain fluid. In this embodiment, mixing of the plug-fluids will allow the concentration of the reagent in the plug to be manipulated.

The reaction can be initiated by forming plugs from each plug-fluid and subsequently merging these different plugs.

When plugs are merged to form merged plugs, the first and second set of plugs may be substantially similar or different in size. Further, the first and second set of plugs may have different relative velocities. In one embodiment, large arrays of microfluidic reactors are operated in parallel to provide substantial throughput.

The devices and methods of the invention can be used for synthesizing nanoparticles. Nanoparticles that are monodisperse are important as sensors and electronic components but are difficult to synthesize (Trindade et al., *Chem. Mat.* 2001, vol. 13, pp. 3843-3858). In one aspect, monodisperse nanoparticles of semiconductors and noble metals are synthesized under time control using channels according to the invention (Park et al, *J. Phys. Chem. B,* 2001, vol. 105, pp. 11630-11635). Fast nucleation is preferably induced by rapid mixing, thereby allowing these nanoparticles to grow for a controlled period of time. Then their growth is preferably quickly terminated by passivating the surfaces of the particles with, for example, a thiol. Nanoparticles of different sizes are preferably obtained by varying the flow rate and therefore the growth time. In addition, devices according to the invention can be used to monitor the synthesis of nanoparticles, and thus obtain nanoparticles with the desired properties. For example, the nanoparticle formation may be monitored by measuring the changes in the color of luminescence or absorption of the nanoparticles. In addition, the growth of

nanoparticles may be stopped by introducing a stream of quenching reagent at a certain position along the main channel.

Rapid millisecond mixing generated in channels according to the invention can help ensure the formation of smaller and much more monodisperse nanoparticles than nanoparticles synthesized by conventional mixing of solutions. FIG. **13** shows the UV-VIS spectra of CdS nanoparticles formed by rapid mixing in plugs (lighter shade spectrum with sharp absorption peak) and by conventional mixing of solutions (darker shade spectrum). The sharp absorption peak obtained for synthesis conducted in plugs indicates that the nanoparticles formed are highly monodisperse. In addition, the blue-shift (shift towards shorter wavelengths) of the absorption peak indicates that the particles formed are small.

FIG. **14**A-B illustrates the synthesis of CdS nanoparticles performed in PDMS microfluidic channels in single-phase aqueous laminar flow (FIG. **14**A) and in aqueous plugs that were surrounded by water-immiscible perfluorodecaline (FIG. **14**B). In FIGS. **14**A-B, Cd^{2+} was introduced into inlets **1400**, **1403**, aqueous stream was introduced into inlets **1401**, **1404**, and S^{2-} was introduced into inlets **1402**, **1405**. In FIG. **14**A, an aqueous stream flowed through channel **1406** while in FIG. **14**B, oil flowed through channel **1407**. FIG. **14**A shows portions of the channels **1408** and **1410** at time t=6 minutes and portions of the channels **1409**, **1411** at time t=30 minutes. It can be seen in FIG. **14**A that when laminar flow is used in the synthesis, large amounts of CdS precipitate form on the channel walls. When plugs were used for the synthesis, all CdS formed inside the plugs, and no surface contamination was observed. FIG. **15** illustrates a technique for the synthesis of CdS nanoparticles, which is discussed in detail in Example 13 below.

The following methods according to the invention can be used in synthesis involving nanoparticles:

(a) using self-assembled monolayers to nucleate nanoparticles with crystal structures not accessible under homogeneous nucleation conditions (e.g., controlling polymorphism by controlling the surface at which nucleation takes place).

(b) using merging of plugs to create core-shell nanoparticles with a range of core and shell sizes. In a stream of plugs of a first channel, small core nanoparticles such as CdSe particles can be synthesized in a matter of few milliseconds. The CdSe particles can then be used as seeds for mixing with solutions such as those containing Zn^{+2} and S^{-2}. The CdSe particles, acting as seeds for the formation of ZnS, thus allow the formation of CdSe(core)/ZnS(shell) nanoparticles. Core-shell particles with more than two layers may be obtained by simply repeating the merging process more than once.

(c) using merging of plugs to create composite nanoparticles. For example, small nanoparticles of CdSe and ZnS can be formed using streams of plugs from two separate channels. Merging of these streams leads to aggregation of these particles to form larger nanoparticles containing CdSe/ZnS composite. The composite nanoparticles that contain only a few of the original nanoparticles can be made non-centrosymmetric, which may have interesting photophysical properties.

(d) using the devices and methods according to the invention to synthesize medically important nanoparticles, such as encapsulated drugs and composite drugs.

(e) combinatorial synthesis of core-shell particles and other complex systems. For example, the luminescence of CdSe/ZnS particles may be monitored and the conditions adjusted to produce particles with various core and shell sizes, various doping impurities in the core and shell, and various ligand composition on the surface of the particles. These can be conducted in real time using a device according to the invention. The entire process can also be automated.

The devices and methods according to the present invention may also be used for synthesizing polymers. Since the invention allows precise control of the timing of a polymerization reaction, one or more properties of a polymer such as molecular weight, polydispersity and blockiness can be readily controlled or adjusted. In addition, use of the substrate of the present invention allows the user to precisely form block copolymers by merging plugs within a device, since the path length of the channel will correspond to a specific duration of the polymerization reaction. Similarly, a living polymer chain can be terminated with a specific end group to yield polymers with a discrete subset of molecular weights.

In addition, combinatorial libraries of drug candidates may be synthesized using similar approaches. The library may be encoded using the position of plugs in a channel. Plugs of variable composition may be created by varying flow rates. Combination of synthesis of the library may be combined with screening and assays performed on the same microfluidic chip according to the present invention. In some embodiments, merging, splitting and sorting of plugs may be used during synthesis, assays, etc.

All of the above synthesis methods of the present invention can be used to form macroscopic quantities of one or more reaction products by running multiple reactions in parallel.

Particle Separation/Sorting Using Plugs

The flow within the moving plugs can be used for separation of polymers and particles. Plugs can be used for separation by first using flow within a moving plug to establish a distribution of the polymers or particles inside the plug (for example, an excess of the polymer inside the front, back, right or left side of the plug) and then using splitting to separate and isolate the part of the plug containing higher concentration of the polymers or particles. When two polymers or particles are present inside the plug and establish different distributions, slitting can be used to separate the polymers or particles. This approach may be useful, for example, in achieving on a microfluidic chip any of, but not limited to, the following: separation, purification, concentration, membrane-less dialysis, and filtration.

Crystallization

The devices and methods of the invention allow fast, inexpensive miniaturization of existing crystallization methods and other methods that can be adapted into, for example, novel protein screening and crystallization techniques. The crystallization methods according to the invention may be applied to various drugs, materials, small molecules, macromolecules, colloidal and nanoparticles, or any of their combinations. Many relevant protein structures remain undetermined due to their resistance to crystallization. Also, many interesting proteins are only available in microgram quantities. Thus, a screening process must permit the use of small amounts protein for analysis. Current crystallization screening technologies generally determine the ideal conditions for protein crystallization on a milligram scale. Devices and methods according to the invention improve current bench-top methodology available to single users, and enables higher throughput automated systems with improved speed, sample economy, and entirely new methods of controlling crystallization.

A microfluidic system according to the invention can be applied to the crystallization of small molecules or macromolecules and their complexes.

For example, systems and methods in accordance with the present invention may include but are not limited to: (1) biological macromolecules (cytosolic proteins, extracellular

proteins, membrane proteins, DNA, RNA, and complex combinations thereof); (2) pre- and post-translationally modified biological molecules (including but not limited to, phosphorylated, sulfolated, glycosylated, ubiquitinated, etc. proteins, as well as halogenated, abasic, alkylated, etc. nucleic acids); (3) deliberately derivatized macromolecules, such as heavy-atom labeled DNAs, RNAs, and proteins (and complexes thereof), selenomethionine-labeled proteins and nucleic acids (and complexes thereof), halogenated DNAs, RNAs, and proteins (and complexes thereof); (4) whole viruses or large cellular particles (such as the ribosome, replisome, spliceosome, tubulin filaments, actin filaments, chromosomes, etc.); (5) small-molecule compounds such as drugs, lead compounds, ligands, salts, and organic or metallo-organic compounds; (6) small-molecule/biological macromolecule complexes (e.g., drug/protein complexes, enzyme/substrate complexes, enzyme/product complexes, enzyme/regulator complexes, enzyme/inhibitor complexes, and combinations thereof); (7) colloidal particles; and (8) nanoparticles.

Preferably, a general crystallization technique according to the present invention involves two primary screening steps: a crude screen of crystallization parameters using relatively small channels with a large number of small plugs, and a fine screen using larger channels and larger plugs to obtain diffraction-quality crystals. For example, ten crude screens performed using channels with a $(50\ \mu m)^2$ cross-sectional dimension and with more or less one thousand 150-picoliter (pL) plugs corresponding to 10 mg/mL final concentration of a protein (10,000 trials total) will typically require about 1.5 μL of solution, produce crystals up to about $(10\ \mu m)^3$ in size, and will consume approximately 15 μg of protein. Up to 300 or more of such plugs can be formed in about 1 second in these microfluidic networks. A fine screen around optimal conditions in $(500\ \mu m)^2$ channels is expected to use more or less 50 plugs. Another ~5 μL of solution and another 50 μg of the protein are expected to be consumed. This can produce crystals up to $(100\ \mu m)^3$ in size. Approximately 30 plugs can be formed about every second or so. The throughput of the system will generally be determined by the rate of plug formation, and may be limited by how rapidly the flow rates can be varied. Pressure control methods that operate at frequencies of 100 Hz are available and may be applied to PDMS microfluidic networks (Unger et al., "Monolithic fabricated valves and pumps by multilayer soft lithography," *Science* 2000, vol. 288, pp. 113-116).

Crystal properties such as appearance, size, optical quality, and diffractive properties may be characterized and measured under different conditions. For example, a Raxis IIc X-ray detector mounted on a Rigaku RU 200 rotating anode X-ray generator, which is equipped with double focusing mirrors and an MSC cryosystem, may be used for at least some of the characterizations and measurements. A synchrotron beam may be useful for characterization of small crystals. Also, these devices and methods may be used to build microfluidic systems according to the invention that are compatible with structural studies using x-ray beams.

A significant problem involving current crystallization approaches is determining the conditions for forming crystals with optimal diffractive properties. Normally crystals have to be grown, isolated, mounted, and their diffractive properties determined using an x-ray generator or a synchrotron. Microfluidic systems with thin, non-scattering walls would be desirable for determining the diffractive properties of crystals inside a microfluidic system. Preferably, crystallization is carried out inside this system using methods according to the invention, which are described herein. The crystals are exposed to x-ray beams either to determine their structure or diffractive properties (the screening mode). For example, a PDMS membrane defining two side walls of the channels could be sandwiched between two very thin glass plates (defining the top and bottom walls of the channels) that do not significantly scatter X-rays. Thus, the devices of the invention offer a further advantage in that structural characterization could be conducted while the sample is inside the microfluidic device. Thus, the sample can be characterized without the need to take out the sample, e.g., crystal, from the device.

The present system enables higher throughput automated systems with improved speed, sample economy, and entirely new methods of controlling crystallization. Microfluidic versions of microbatch, vapor phase diffusion and FID techniques may be carried out using the present invention, as described below, or using a combination of these techniques or other techniques. In addition, the nucleation and growth phases may be carried out in discrete steps through merging plugs, as described herein.

Screening for protein crystallization can involve varying a number of parameters. During crystallization screening, a large number of chemical compounds may be employed. These compounds include salts, small and large molecular weight organic compounds, buffers, ligands, small-molecule agents, detergents, peptides, crosslinking agents, and derivatizing agents. Together, these chemicals can be used to vary the ionic strength, pH, solute concentration, and target concentration in the plug, and can even be used to modify the target. The desired concentration of these chemicals to achieve crystallization is variable, and can range from nanomolar to molar concentrations.

A typical crystallization mix may contain a set of fixed, but empirically-determined, types and concentrations of precipitation agent, buffers, salts, and other chemical additives (e.g., metal ions, salts, small molecular chemical additives, cryoprotectants, etc.). Water is a key solvent in many crystallization trials of biological targets, as many of these molecules may require hydration to stay active and folded. Precipitation agents act to push targets from a soluble to insoluble state, and may work by volume exclusion, changing the dielectric constant of the solvent, charge shielding, and molecular crowding. Precipitation agents compatible with the PDMS material of certain embodiments according to the invention include, but are not limited to, nonvolatile salts, high molecular weight polymers, polar solvents, aqueous solutions, high molecular weight alcohols, divalent metals.

Precipitation agents, which include large and small molecular weight organics, as well as certain salts, may be used from under 1% to upwards of 40% concentration, or from <0.5M to greater than 4M concentration. Water itself can act in a precipitating manner for samples that require a certain level of ionic strength to stay soluble. Many precipitation agents may also be mixed with one another to increase the chemical diversity of the crystallization screen. Devices according to the invention are readily compatible with a broad range of such compounds.

A nonexclusive list of salts that may be used as precipitation agents is as follows: tartrates (Li, Na, K, Na/K, NH_4); phosphates (Li, Na, K, Na/K, NH_4); acetates (Li, Na, K, Na/K, Mg, Ca, Zn, NH_4); formates (Li, Na, K, Na/K, Mg, NH_4); citrates (Li, Na, K, Na/K, NH_4); chlorides (Li, Na, K, Na/K, Mg, Ca, Zn, Mn, Cs, Rb, NH_4); sulfates (Li, Na, K, Na/K, NH_4); maleates (Li, Na, K, Na/K, NH_4); glutamates (Li, Na, K, Na/K, NH_4.

A nonexclusive list of organic materials that may be used as precipitation agents is as follows: PEG 400; PEG 1000; PEG 1500; PEG 2K; PEG 3350; PEG 4K; PEG 6K; PEG 8K; PEG 10K; PEG 20K; PEG-MME 550; PEG-MME 750; PEG-

MME 2K; PEGMME 5K; PEG-DME 2K; dioxane; methanol; ethanol; 2-butanol; n-butanol; t-butanol; jeffamine m-600; isopropanol; 2-methyl-2,4-pentanediol; 1,6 hexanediol.

Solution pH can be varied by the inclusion of buffering agents; typical pH ranges for biological materials lie anywhere between values of 3 and 10.5 and the concentration of buffer generally lies between 0.01 and 0.25 M. The microfluidics devices described in this document are readily compatible with a broad range of pH values, particularly those suited to biological targets.

A nonexclusive list of possible buffers that may be used according to the invention is as follows: Na-acetate; HEPES; Na-cacodylate; Na-citrate; Na-succinate; Na—K-phosphate; TRIS; TRIS-maleate; imidazole-maleate; bistrispropane; CAPSO, CHAPS, MES, and imidazole.

Additives are small molecules that affect the solubility and/or activity behavior of the target. Such compounds can speed up crystallization screening or produce alternate crystal forms or polymorphs of the target. Additives can take nearly any conceivable form of chemical, but are typically mono and polyvalent salts (inorganic or organic), enzyme ligands (substrates, products, allosteric effectors), chemical crosslinking agents, detergents and/or lipids, heavy metals, organometallic compounds, trace amounts of precipitating agents, and small molecular weight organics.

The following is a nonexclusive list of additives that may be used in accordance with the invention: 2-butanol; DMSO; hexanediol; ethanol; methanol; isopropanol; sodium fluoride; potassium fluoride; ammonium fluoride; lithium chloride anhydrous; magnesium chloride hexahydrate; sodium chloride; calcium chloride dihydrate; potassium chloride; ammonium chloride; sodium iodide; potassium iodide; ammonium iodide; sodium thiocyanate; potassium thiocyanate; lithium nitrate; magnesium nitrate hexahydrate; sodium nitrate; potassium nitrate; ammonium nitrate; magnesium formate; sodium formate; potassium formate; ammonium formate; lithium acetate dihydrate; magnesium acetate tetrahydrate; zinc acetate dihydrate; sodium acetate trihydrate; calcium acetate hydrate; potassium acetate; ammonium acetate; lithium sulfate monohydrate; magnesium sulfate heptahydrate; sodium sulfate decahydrate; potassium sulfate; ammonium sulfate; di-sodium tartrate dihydrate; potassium sodium tartrate tetrahydrate; di-ammonium tartrate; sodium dihydrogen phosphate monohydrate; di-sodium hydrogen phosphate dihydrate; potassium dihydrogen phosphate; di-potassium hydrogen phosphate; ammonium dihydrogen phosphate; di-ammonium hydrogen phosphate; tri-lithium citrate tetrahydrate; tri-sodium citrate dihydrate; tri-potassium citrate monohydrate; diammonium hydrogen citrate; barium chloride; cadmium chloride dihydrate; cobaltous chloride dihydrate; cupric chloride dihydrate; strontium chloride hexahydrate; yttrium chloride hexahydrate; ethylene glycol; Glycerol anhydrous; 1,6 hexanediol; MPD; polyethylene glycol 400; trimethylamine HCl; guanidine HCl; urea; 1,2,3-heptanetriol; benzamidine HCl; dioxane; ethanol; iso-propanol; methanol; sodium iodide; L-cysteine; EDTA sodium salt; NAD; ATP disodium salt; D(+)-glucose monohydrate; D(+)-sucrose; xylitol; spermidine; spermine tetra-HCl; 6-aminocaproic acid; 1,5-diaminopentane diHCl; 1,6-diaminohexane; 1,8-diaminooctane; glycine; glycyl-glycyl-glycine; hexaminecobalt trichloride; taurine; betaine monohydrate; polyvinylpyrrolidone K5; non-detergent sulfo-betaine 195; non-detergent sulfo-betaine 201; phenol; DMSO; dextran sulfate sodium salt; Jeffamine M-600; 2,5 Hexanediol; (+/−)-1,3 butanediol; polypropylene glycol P400; 1,4 butanediol; tert-butanol; 1,3 propanediol; acetonitrile; gamma butyrolactone; propanol; ethyl acetate; acetone; dichloromethane; n-butanol; 2,2,2 trifluoroethanol; DTT; TCEP; nonaethylene glycol monododecyl ether, nonaethylene glycol monolauryl ether; polyoxyethylene (9) ether; octaethylene glycol monododecyl ether, octaethylene glycol monolauryl ether; polyoxyethylene (8) lauryl ether; Dodecyl-β-D-maltopyranoside; Lauric acid sucrose ester; Cyclohexyl-pentyl-β-D-maltoside; Nonaethylene glycol octylphenol ether; Cetyltrimethylammonium bromide; N,N-bis(3-D-gluconamidopropyl)-deoxycholamine; Decyl-β-D-maltopyranoside; Lauryldimethylamine oxide; Cyclohexyl-pentyl-β-D-maltoside; n-Dodecylsulfobetaine, 3-(Dodecyldimethylanimonio)propane-1-sulfonate; Nonyl-β-D-glucopyranoside; Octyl-β-D-thioglucopyranoside, OSG; N,N-Dimethyldecylamine-β-oxide; Methyl 0-(N-heptylcarbamoyl)-α-D-glucopyranoside; Sucrose monocaproylate; n-Octanoyl-β-D-fructofuranosyl-α-D-glucopyranoside; Heptyl-β-D-thioglucopyranoside; Octyl-β-D-glucopyranoside, OG; Cyclohexyl-propyl-β-D-maltoside; Cyclohexylbutanoyl-N-hydroxyethylglucamide; n-decylsulfobetaine, 3-(Decyldimethylammonio)propane-Isulfonate; Octanoyl-N-methylglucamide, OMEGA; Hexyl-β-D-glucopyranoside; Brij 35; Brij 58; Triton X-114; Triton X-305; Triton X-405; Tween 20; Tween 80; polyoxyethylene (6)decyl ether; polyoxyethylene(9)decyl ether; polyoxyethylene(10)dodecyl ether; polyoxyethylene(8)tridecyl ether; Decanoyl-N-hydroxyethylglucamide; Pentaethylene glycol monooctyl ether; 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; 3-[(3-Cholamidopropyl)-dimethylammonio]hydroxy-1-propane sulfonate; Cyclohexylpentanoyl-N-hydroxyethylglucamide; Nonanoyl-N-hydroxyethyglucamide; Cyclohexylpropanol-N-hydroxyethylglucamide; Octanoyl-N-hydroxyethylglucamide; Cyclohexylethanoyl-N-hydroxyethylglucamide; Benzyldimethyldodecyl ammonium bromide; n-Hexadecyl-β-D-maltopyranoside; n-Tetradecyl-β-D-maltopyranoside; n-Tridecyl-β-D-maltopyranoside; Dodecylpoly(ethyleneglycoether); n-Tetradecyl-N,N-dimethyl ammonio-1-propanesulfonate; n-Undecyl-β-D-maltopyranoside; n-Decyl D-thiomaltopyranoside; n-dodecylphosphocholine; α-D-glucopyranoside, β-D-fructofuranosyl monodecanoate, sucrose mono-caprate; 1-s-Nonyl-β-D-thioglucopyranoside; n-Nonyl-β-D-thiomaltoyranoside; N-Dodecyl-N,N-(dimethlammonio)butyrate; n-Nonyl-β-D-maltopyranoside; Cyclohexyl-butyl D-maltoside; n-Octyl-β-D-thiomaltopyranoside; n-Decylphosphocholine; n-Nonylphosphocholine; Nonanoyl-N-methylglucamide; 1-s-Heptyl-β-D-thioglucopyranoside; n-Octylphosphocholine; Cyclohexyl-ethyl D-maltoside; n-Octyl-N,N-dimethyl ammonio-1-propanesulfonate; Cyclohexyl-methyl-β-D-maltoside.

Cryosolvents are agents that stabilize a target crystal to flash-cooling in a cryogen such as liquid nitrogen, liquid propane, liquid ethane, or gaseous nitrogen or helium (all at approximately 100-120° K) such that crystal becomes embedded in a vitreous glass rather than ice. Any number of salts or small molecular weight organic compounds can be used as a cryoprotectant, and typical ones include but are not limited to: MPD, PEG-400 (as well as both PEG derivatives and higher molecular-weight PEG compounds), glycerol, sugars (xylitol, sorbitol, erythritol, sucrose, glucose, etc.), ethylene glycol, alcohols (both short- and long chain, both volatile and nonvolatile), LiOAc, LiCl, $LiCHO_2$, $LiNO_3$, Li_2SO_4, $Mg(OAc)_2$, NaCl, $NaCHO_2$, $NaNO_3$, etc. Again, materials from which microfluidics devices in accordance with the present invention are fabricated may be compatible with a range of such compounds.

Many of these chemicals can be obtained in predefined screening kits from a variety of vendors, including but not limited to Hampton Research of Laguna Niguel, Calif., Emerald Biostructures of Bainbridge Island, Wash., and Jena Bioscience of Jena, Germany, that allow the researcher to perform both sparse matrix and grid screening experiments. Sparse matrix screens attempt to randomly sample as much of precipitant, buffer, and additive chemical space as possible with as few conditions as possible. Grid screens typically consist of systematic variations of two or three parameters against one another (e.g., precipitant concentration vs. pH). Both types of screens have been employed with success in crystallization trials, and the majority of chemicals and chemical combinations used in these screens are compatible with the chip design and matrices in accordance with embodiments of the present invention. Moreover, current and future designs of microfluidic devices may enable flexible combinatorial screening of an array of different chemicals against a particular target or set of targets, a process that is difficult with either robotic or hand screening. This latter aspect is particularly important for optimizing initial successes generated by first-pass screens.

In addition to chemical variability, a host of other parameters can be varied during crystallization screening. Such parameters include but are not limited to: (1) volume of crystallization trial; (2) ratio of target solution to crystallization solution; (3) target concentration; (4) cocrystallization of the target with a secondary small or macromolecule; (5) hydration; (6) incubation time; (7) temperature; (8) pressure; (9) contact surfaces; (10) modifications to target molecules; and (11) gravity.

Although the discussion below refers to proteins, the particular devices or methods described can also be used or suitably adapted for the crystallization of other types of samples such as those mentioned above (e.g., small molecules, other macromolecules, nanoparticles, colloidal particles, etc.). In one aspect of the present invention, protein crystallization is conducted using miniaturized microbatch conditions. The process consists of two steps. First, plugs are preferably formed wherein the concentrations of the protein, precipitant, and additive are adjusted by varying the relative flow rates of these solutions. This step corresponds to a screening step. Once the optimal concentrations have been found, the flow rates can then be kept constant at the optimal conditions. In this step, plugs are preferably transported through the channel as they form. Second, the flow is preferably stopped once the desired number of plugs are formed. The plugs are then preferably allowed to incubate. In some embodiments according to the invention the flow may be continued, rather than stopped. In those embodiments, the flow is maintained sufficiently slow and the channels are made sufficiently long that plugs spend sufficient time in the channels for crystallization to occur (from tens of minutes to weeks, but may be faster or slower).

In one aspect, upon formation of the plugs, they are trapped using expansions in the channels. The expansions act as dead volume elements while the plugs are being formed in the presence of flow. Thus, the expansions do not interfere with the flow of the plugs through the channel. Once the flow is stopped, surface tension drives plugs into the expansions where surface tension is minimized. The expansions may be, but are not limited to, oval, round, square, rectangular, or star-shaped. In particular, a star-shaped expansion may prevent adherence of the plug or of a crystal to the walls of the expansion. The ratio of the size of the expansion opening to the width of the channel may be varied based on empirical results for a particular set of conditions. FIG. **16** is a schematic illustration of a microfluidic device according to the invention that illustrates the trapping of plugs. In experiments, plugs were sustained in perfluorodecaline inside a channel for one day, and did not appear to change during that time (a refractive index mismatch between the fluorinated and aqueous phase was introduced to aid in visualization of plugs).

The method described above allows a high degree of control over protein and precipitant concentrations. It also allows a high degree of control over a range of time scales through the control of plug size and composition. FIG. **17** shows a schematic of a microfluidic method for forming plugs with variable compositions for protein crystallization. Continuously varied flow rates of the incoming streams are preferably used to form plugs with various concentrations of the protein, precipitation agents, and additives. In FIG. **17**, for example, the following can be introduced into the various inlets: buffers into inlets **171**, **172**; PEG into inlet **173**; salt into inlet **174**; solvent into inlet **175**; and protein into inlet **176**. These various solutions can enter a channel **177** through which a carrier fluid such as perfluorodecaline flows. For example, a 1-meter long channel with a $200\times80\ \mu m^2$ cross section can be used to form approximately two hundred 6 nL (nanoliter) plugs. If each plug contains enough protein to form a 40-μm^3 crystal, 200 trials will consume only about 1.2 μL of approximately 10 mg/mL protein solution (12 μg of protein). About one minute may be sufficient to form plugs in these trials.

In another aspect according to the invention, after plugs are formed as described above for the microbatch system, slow evaporation through a very thin PDMS membrane (or another membrane with slight water permeability) is preferably used for added control over the crystallization process. A slow decrease in the volume of the plug during evaporation is expected to produce a trajectory of the solution through the crystallization phase space similar to that in a vapor diffusion experiment. Hence, this method, in addition to microbatch methods, can be used to miniaturize and optimize vapor diffusion methods.

In the vapor diffusion method, a drop containing protein, stabilizing buffers, precipitants, and/or crystallization agents is allowed to equilibrate in a closed system with a much larger reservoir. The reservoir usually contains the same chemicals minus the protein but at an over all higher concentration so that water preferentially evaporates from the drop. If conditions are right, this will produce a gradual increase in protein concentration such that a few crystals may form.

Vapor diffusion can be performed in several ways. The one most often used is called Hanging Drop Technique. The drop is placed on a glass coverslip, which is then inverted and used to seal a small reservoir in a Linbro Plate. After a period of several hours to weeks, microscopic crystals may form and continue to grow. The other set up is known as Sitting Drop. In this method a drop (usually >10 uL) is placed in a depression in either a Micro Bridge in a Linbro Plate or a glass plate and again placed in a closed system to equilibrate with a much larger reservoir. One usually uses the sitting drop technique if the drop has very low surface tension, making it hard to turn upside down or if the drops need to be larger than 20 uL. Also, in some cases, crystals will grow better using one technique or the other.

In another embodiment, the plugs are preferably formed and transported such that excessive mixing of the protein with the precipitation agent is minimized or prevented. For example, gentle mixing using spiral channels may be used to achieve this and also to create interfaces between the protein and the precipitation agent. Alternatively, combining two streams of plugs in a T-junction without merging may be used

to create plugs that diffuse and combine without significant mixing to establish a free interface after the flow is stopped. Diffusion of the proteins and precipitates through the interface induces crystallization. This is an analogue of the Free-Interface Diffusion method. It may be performed under either the microbatch or vapor diffusion conditions as described above.

Preferably, the spacing between plugs can be increased or the oil composition changed to reduce plug-plug diffusion. For example, a spacing of about 2.5 mm in paraffin oil can be used, which has been shown to be an effective barrier to aqueous diffusion in crystallization trials.

Visually identifying small crystals inside plugs with curved surfaces can be a challenge when performing microbatch experiments. In an aspect according to the invention, a method based on matching the refractive indices of carrier-fluid with that of the plug fluid to enhance visualization is used. Microscopic detection is preferably performed by using shallow channels and by matching the refractive indices of carrier-fluid mixtures to those of the aqueous solutions.

In addition, at least three other novel methods of controlling protein crystallization are described below: (1) using surface chemistry to effect nucleation of protein crystals; (2) using different mixing methods to effect crystallization; and (3) performing protein crystals seeding by separating nucleation and growth phases in space.

Control of nucleation is one of the difficult steps in protein crystallization. Heterogeneous nucleation is statistically a more favorable process than its solution-phase counterpart. Ideal surfaces for heterogeneous nucleation have complementary electrostatic maps with respect to their macromolecular counterparts. Critical nuclei are more stable on such surfaces than in solution. Further, the degree of supersaturation required for heterogeneous nucleation is much less than that required for the formation of solution-phase nuclei. Surfaces such as silicon, crystalline minerals, epoxide surfaces, polystyrene beads, and hair are known to influence the efficiency of protein crystallization. Few studies have been done, but promising results have been shown for protein crystallization at the methyl, imidazole, hydroxyl, and carboxylic acid termini of self-assembled monolayers on gold. Using self-assembled monolayers, proteins were crystallized over a broader range of crystallization conditions and at faster rates than when using the traditional silanized glass.

FIG. **18** is a schematic illustration of a method for controlling heterogeneous nucleation by varying the surface chemistry at the interface of an aqueous plug-fluid and a carrier-fluid. In FIG. **18**, plugs are formed in the presence of several solutions of surfactants that possess different functional groups (left side of the diagram). The right side of FIG. **18** shows the aqueous phase region in which a precipitant, solvent, and protein may be introduced into inlets **180**, **181**, and **182**, respectively. The composition of the surfactant monolayer is preferably controlled by varying the flow rates. In another application of the method illustrated in FIG. **18**, the surface chemistry can be varied continuously. The manipulation and control of the surface chemistry can be used for screening, assays, crystallizations, and other applications where surface chemistry is important.

In one aspect of the invention, heterogeneous nucleation of proteins is controlled by forming aqueous plugs in a carrier-fluid, preferably containing fluoro-soluble surfactants if the carrier-fluid is a fluorocarbon. Varying the relative flow rates of the surfactant solutions may generate a wide variety of liquid-liquid interface conditions that can lead to the formation of mixed monolayers or mixed phase-separated monolayers. Preferably, several surfactants are used to control the heterogeneous nucleation of protein crystals. Ethylene-glycol monolayers are preferably used to reduce heterogeneous nucleation, and monolayers with electrostatic properties complementary to those of the protein are preferably used to enhance heterogeneous nucleation. These methods for controlling heterogeneous nucleation are designed to induce or enhance the formation of crystals that are normally difficult to obtain. These methods may also be used to induce or enhance the formation of different crystal polymorphs that are relatively more stable or better ordered.

As mentioned above, control of nucleation is highly desired in an advanced crystallization screen. One method that can be used to achieve control of nucleation involves the transfer of nucleating crystals from one concentration to another via dilution. This method, which has been applied in macroscopic systems primarily to vapor diffusion, was intended to allow decoupling of the nucleation and growth phases. This method is difficult to perform using traditional methods of crystallization because nucleation occurs long before the appearance of microcrystals.

FIG. **19** illustrates a method of separating nucleation and growth using a microfluidic network according to the present invention using proteins as a non-limiting example. The left side of FIG. **19** shows plugs that are formed preferably using high concentrations of protein and precipitant. In FIG. **19**, the following can be introduced into the various inlets shown: buffer into inlets **191**, **196**; PEG into inlets **192**, **197**; precipitant into inlets **193**, **198**; solvent into inlets **194**, **199**; and protein into inlets **195**, **200**. Oil flows through the channels **201**, **202** from left to right. The portions **203**, **204**, and **205** of the channel correspond to regions where fast nucleation occurs (**203**), no nucleation occurs (**204**), and where crystal growth occurs (**205**). The concentrations used are those that correspond to the nucleating region in the phase diagram. Nucleation occurs as the plugs move through the channel to the junction over a certain period. Preferably, these plugs are then merged with plugs containing a protein solution at a point corresponding to a metastable (growth, rather than nucleation) region (right side of FIG. **19**). This step ends nucleation and promotes crystal growth. When the combined channel has been filled with merged plugs, the flow is preferably stopped and the nuclei allowed to grow to produce crystals.

Nucleation time can be varied by varying the flow rate along the nucleation channel. The nucleus is preferably used as a seed crystal for a larger plug with solution concentrations that correspond to a metastable region. Existing data indicate the formation of nuclei within less than about 5 minutes.

Fluid mixing is believed to exert an important effect in crystal nucleation and growth. Methods according to the invention are provided that allow a precise and reproducible degree of control over mixing. FIG. **20** illustrates two of these methods. A method of mixing preferably places the solution into a nucleation zone of the phase diagram without causing precipitation. Preferably, gentle mixing (FIG. **20**, left side) is used to achieve this by preventing, reducing, or minimizing contact between concentrated solutions of the protein and precipitant. Alternatively, rapid mixing (FIG. **20**, right side) is used to achieve this by allowing passage through the precipitation zone sufficiently quickly to cause nucleation but not precipitation. The two methods used as examples involve the use of spiraling channels for gentle mixing and serpentine channels for rapid mixing.

The two methods in accordance with the invention depicted in FIG. **20** can be used to determine the effect of mixing on protein crystallization. In addition, the various methods for controlling mixing described previously (e.g.,

slow mixing in straight channels, chaotic mixing in non-straight channels, or mixing in which twirling may or may not occur) can be applied to crystallization, among other things.

After obtaining the crystals using any of the above described techniques, the crystals may be removed from the microfluidic device for structure determination. In other systems, the fragile and gelatinous nature of protein crystals makes crystal collection difficult. For example, removing protein crystals from solid surfaces can damage them to the point of uselessness. The present invention offers a solution to this problem by nucleating and growing crystals in liquid environments. In an aspect according to the invention, a thin wetting layer of a carrier-fluid covered with a surfactant is used to enable or facilitate the separation of a growing crystal from a solid surface. When the crystals form, they may be separated from the PDMS layer by using a thin layer of a carrier-fluid.

In one aspect, a microfluidic device of the present system can include further include capillary tubing suitable for collecting plugs ("the capillary device"; FIG. **46**). The tubing is preferably composed of a material that prevents uncontrolled evaporation of solutions (such as water) through its wall. Further, use of the capillary tubing can enable direct screening of crystals by x-ray diffraction analysis or other spectrophotometric detection/analysis means employing e.g., optical or infrared detection. Plugs in the capillary tubing have been found to be stable and did not show signs of evaporation over several months, even in the absence of humidity control. Therefore, the capillary device can be incubated for a much longer time than all-PDMS microfluidic chips. Water diffusion can be controlled by varying the starting salt concentration differences as well the distance between plugs. Production of crystals directly inside the capillary tubes can facilitate on-chip diffraction without having to move the crystal around.

Upon formation of plugs in the PDMS portion and their transfer into capillary tubing, the flow rates are stopped, the capillary tubing is disconnected from the PDMS portion and the ends are sealed by capillary wax. The capillary tubing may be incubated under suitable crystallization conditions (e.g, temperature etc.) until crystals form inside the plugs. Formation of crystals can be monitored using optical detection and/or x-ray diffraction methods. Crystals grown at the fluid-fluid interface can be easily removed from the capillary by gentle flow, or by breaking the capillary and wicking the liquid out. Upon formation of suitable crystals, the capillaries are frozen and structures are directly determined from inside the capillary using e.g., synchrotron radiation. Because this method obviates the problem of handling and mounting crystals and because it can facilitate the determination of structure directly from within the capillary, it may be especially suitable for high-throughput, fully automated crystallization.

The plugs in the capillary tubing can be stable in both hydrophilic (e.g., treated with by chromic acid) or hydrophobic (e.g., silanized) capillaries for over a month, even if the capillary is placed vertically for over three days.

The use of x-ray capillary tubing for protein crystallization can also be applied to a controlled vapor diffusion process which lends itself to direct monitoring and structural determination of protein crystals in the capillary tubing (FIG. **49**). In this modified vapor-diffusion process an array of plugs is generated in the channel portion of a capillary device (as described above) where the protein and precipitant plugs alternate with plugs containing a high concentration of precipitant. Syringe pumps attached to the capillary device cause the plugs to flow into suitable x-ray capillary tubing. At the conclusion of the experiment, the flow is stopped, the capillary is disconnected from the PDMS portion and the ends are sealed with capillary wax. The x-ray capillary is incubated under optimal conditions until crystals form inside the plugs.

The use of carrier fluid (oil) permeable to water causes the water from the plugs to diffuse through from the oil from the plugs that are low in osmolarity into plugs that are higher in osmolarity, thereby increasing the concentration of the protein and precipitants in the plugs for crystallization. The rate of water transfer from the plugs and the amount of water transferred between the two types of plugs may be controlled by using oils having different water permeabilities, by changing the size or distance between plugs or by altering the precipitant concentrations between the different types of plugs (i.e., changing the difference in osmolarity between the different plug types). All of these parameters can be conveniently altered by changing the relative flow rates of the aqueous and carrier-fluid (oil) solutions. Poly-3,3,3-trifluoropropylmethylsiloxane (FMS-121) can be a suitable carrier-oil fluid for this procedure.

One scheme for generating alternating plugs by vapor diffusion involves attaching four different syringes to a PDMS device, each syringe associated with a syringe pump for introducing each of aqueous solutions A, B into respective aqueous inlet channels and for introducing each of carrier oil fluids C, D into respective oil inlet channels. The aqueous solutions can be the same or different. Multiple, distinct aqueous solutions can also be co-introduced together in one or both of the two aqueous channels. In principle, the same oil or different oils may be used in the two oil inlets. In either case, one oil inlet channel is parallel to the main channel; the other oil inlet channel is vertical to the main channel and is positioned between the two aqueous inlet channels to separate the two aqueous streams into alternating plugs.

Importantly, the flow rates of solutions A and B may be changed in a correlated fashion. Thus, when the flow rate of solution A_1 is increased and solution A_2 is decreased, the flow rate of solutions B_1 is also increased and solution B_2 is also decreased. This can allow one to maintain a constant difference in osmolarity between the plugs of stream A and stream B to ensure that transfer from all plugs A to all plugs B occurs at a constant rate. Moreover, if the flow rates of the corresponding A and B streams are changed in a correlated fashion, the composition of plugs B will reflect the composition of plugs A thereby allowing one to incorporate markers into the B stream plugs to serve as a code for the plugs in the A stream. Thus, if the two types of plugs are made in a correlated way, one type of droplet may be used for crystallization, while the other type of droplet is used for indexing provided it contains a label conferring a read out with respect to crystallization. In other words, absorption/fluorescent dyes or x-ray scattering/absorbing materials can be incorporated in markers in the B streams to facilitate optical density quantification or x-ray diffraction analysis to provide a read out of relative protein and precipitant concentrations in the A streams. This approach can provide a powerful means for optimizing crystallization conditions for subsequent scale-up experiments.

The use of markers may be performed using an oil that is impermeable to water (as in a microbatch procedure) to prevent transfer of water or any other material between the A plugs and B plugs. Alternatively, the B plugs may additionally incorporate a high concentration of dehydration agents (salt, other precipitants) in conjunction with a water-permeable oil as described above. In this way, the B plugs can serve both as markers for the A plugs and as sinks for excess water. Oils that are selectively permeable to materials other than water may also be used to induce transfer of other materials between the plugs and through the oil.

Alternating plugs may be generated using a range of channel geometries. The plugs may also alternate in patterns other than A:B:A:B. For example, other patterns (such as A:A:A:B:A:A:A:B, etc) may be obtained where transfer of water from A plugs adjacent to B plugs is faster than transfer of water from the middle A plug. This can create conditions favorable for creating multiple, different sets of crystallization conditions. The alternating droplet systems may be extended to more than two types of plugs alternating in the same channel or capillary (for example, A plugs with the crystallization solutions, B plugs with the dehydrating agents, and C plugs with markers or with a cryoprotectant).

The above described capillary systems are not limited to protein crystallization—other types of crystallizations and experiments may be performed. For example, the vapor diffusion/alternating droplet approach can be extended to e.g., a process for concentrating materials (such as protein). Such a process would be effected through diffusion of water plugs that are relatively low in osmolarity into plugs having a higher osmolarity. It should be noted, however, that solution materials in the different plug types do not have to be aqueous in nature, but can be in the form of solvents also. Alternatively, the A and B plugs do not have to be in solution at all, but can instead be in the form of emulsions or suspensions.

It will be clear to one skilled in the art that while the above techniques are described in detail for the crystallization of proteins, techniques similar to the ones described above may also be used for the crystallization of other substances, including other biomolecules or synthetic chemicals. In addition, the devices and methods according to the invention may be used to perform co-crystallization. For example, a crystal comprising more than one chemical may be obtained, for example, through the use of at least one stream of protein, a stream of precipitant, and optionally, a stream comprising a third chemical such as an inhibitor, another protein, DNA, etc. One may then vary the conditions to determine those that are optimal for forming a co-crystal.

Particle Separation/Sorting Using Plugs

The flow within the moving plugs can be used for separation of polymers and particles. Plugs can be used for separation by first using flow within a moving plug to establish a distribution of the polymers or particles inside the plug (for example, an excess of the polymer inside the front, back, right or left side of the plug) and then using splitting to separate and isolate the part of the plug containing higher concentration of the polymers or particles. When two polymers or particles are present inside the plug and establish different distributions, splitting can be used to separate the polymers or particles.

The invention is further described below, by way of the following examples. It will be appreciated by persons of ordinary skill in the art that this example is one of many embodiments and is merely illustrative. In particular, the device and method described in this example (including the channel architectures, valves, switching and flow control devices and methods) may be readily adapted, e.g., used in conjunction with one or more devices or methods, so that plugs may be analyzed, characterized, monitored, and/or sorted as desired by a user.

EXAMPLE

Example 1

Fabrication of Microfluidic Devices and a General Experimental Procedure

Microfluidic devices with hydrophilic channel surfaces were fabricated using rapid prototyping in polydimethylsiloxane. The channel surfaces were rendered hydrophobic either by silanization or heat treatment. To silanize the surfaces of channels, (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.) vapor was applied to the inlets of a device with dry nitrogen as a carrier gas at around 40-60 mm Hg above about 1 atm pressure. Vacuum was simultaneously applied to the outlet of the device at about 650 mm Hg below atmospheric pressure. The silane vapor was applied for a period of between about 1-3 hours. To treat the channels using heat, a device was placed in an oven at approximately 120° C. for about three hours. Alternatively, a device can be heated in a Panasonic "The Genius" 1300 Watt microwave oven at power set to "10" for about ten minutes.

Oils and aqueous solutions were pumped through devices using a kdScientific syringe pump (Model 200) or Harvard Apparatus PhD 2000 pump. Hamilton Company GASTIGHT syringes were used (10-250 μl) and Hamilton Company 30 gauge Teflon® needles were used to attach the syringes to the devices. Oils and aqueous solutions were pumped through devices at volumetric flow rates ranging from about 0.10 μL/min to about 10.0 μL/min.

Aqueous solutions were colored using Crayola Original Formula Markers or Ferroin Indicator (0.025 M, Fisher Scientific). Oils that were used included perfluorodecaline (mixture of cis and trans, 95%, Acros Organics), perfluoroperhydrophenanthrene (tech., Alfa-Aesar), or 1H,1H,2H,2H-perfluorooctanol (98%, Alfa-Aesar). The experiments were typically performed using 10:1 mixtures of perfluorodecaline and 1H,1H,2H,2H-perfluorooctanol.

The experiments were monitored using a Lica MZFLIII stereoscope with Fostec (Schott-Fostec, LLC) Modulamps. Photographs of the experiments were taken with a Spot Insight Color Camera, Model #3.2.0 (Diagnostic Instruments, Inc.). Spot Application version 3.4.0.0 was used to take the photographs with the camera.

Example 2

Varying the Concentration of Aqueous Solutions in Plugs

The left side of each of FIGS. **25**A-C shows a schematic diagram of the microfluidic network and the experimental conditions. The right side of each of FIGS. **25**A-C shows microphotographs illustrating the formation of plugs using different concentrations of the aqueous streams. Aqueous solutions of food dyes (red/dark and green/light) and water constituted the three streams. The volumetric flow rates of the three solutions (given in μL/min) are indicated. The dark stream is more viscous than the light stream. Therefore, the dark (more viscous) stream moves (measured in mm/s) more slowly and occupies a larger fraction of the channel at a given volumetric flow rate.

FIG. **45***a*) shows a schematic of the microfluidic network used to demonstrate that on-chip dilutions can be accomplished by varying the flow rates of the reagents. In FIG. **45***a*), the reagents are introduced through inlets **451**, **453** while the dilution buffer is introduced through inlet **452**. An oil stream flows through channel **454**. The blue rectangle outlines the field of view for images shown in FIG. **45***c*)-*d*). FIG. **45***b*) shows a graph quantifying this dilution method by measuring fluorescence of a solution of fluorescein diluted in plugs in the microchannel. Data are shown for 80 experiments in which fluorescein was flowed through one of the three inlets, where $C_{measured}$ and $C_{theoretical}$ [μM] are measured and expected fluorescein concentration. FIG. **45**(*c*) shows photographs

illustrating this dilution method with streams of food dyes **455**, **456**, **457** having flow rates of 45 nL/s, 10 nL/s, and 10 nL/s, respectively. FIG. **45**(*d*) shows photographs illustrating this dilution method with streams of food dyes **458**, **459**, **460** having flow rates of 10 nL/s, 45 nL/s, and 10 nL/s, respectively. Carrier fluid was flowed at 60 nL/s.

Example 3

Networks of microchannels with rectangular cross-sections were fabricated using rapid prototyping in PDMS. The PDMS used was Dow Corning Sylgard Brand 184 Silicone Elastomer, and devices were sealed using a Plasma Prep II (SPI Supplies). The surfaces of the devices were rendered hydrophobic by baking the devices at 120° C. for 2-4 hours.

In FIG. **26**, the red aqueous streams were McCormick® red food coloring (water, propylene glycol, FD&C Red 40 and 3, propylparaben), the green aqueous streams were McCormick® green food coloring (water, propylene glycol, FD&C yellow 5, FD&C blue 1, propylparaben) diluted 1:1 with water, and the colorless streams were water. PFD used was a 10:1 mixture of perfluorodecaline (mixture of cis and trans, 95%, Acros Organics):1H,1H,2H,2H-perfluorooctanol (Acros Organics). The red aqueous streams were introduced in inlet **260**, **265** while the green aqueous streams were introduced in inlets **262**, **263** in FIG. **26***b*). The colorless aqueous stream was introduced in inlets **261**, **264**. The dark shadings of the streams and plug are due mainly from the red dye while the lighter shadings are due mainly from the green dye.

Aqueous solutions were pumped using 100 μL Hamilton Gastight syringes (1700 series, TLL) or 50 μL SGE gastight syringes. PFD was pumped using 1 mL Hamilton Gastight syringes (1700 series, TLL). The syringes were attached to microfluidic devices by means of Hamilton Teflon needles (30 gauge, 1 hub). Syringe pumps from Harvard Apparatus (PHD 2000 Infusion pumps; specially-ordered bronze bushings were attached to the driving mechanism to stabilize pumping) were used to infuse the aqueous solutions and PFD.

Microphotographs were taken with a Leica MZ12.5 stereomicroscope and a SPOT Insight Color digital camera (Model #3.2.0, Diagnostic Instruments, Inc.). SPOT Advanced software (version 3.4.0 for Windows, Diagnostic Instruments, Inc.) was used to collect the images. Lighting was provided from a Machine Vision Strobe X-Strobe X1200 (20 Hz, 12 μF, 600V, Perkin Elmer Optoelectronics). To obtain an image, the shutter of the camera was opened for 1 second and the strobe light was flashed once with the duration of the flash being about 10 μs.

Images were analyzed using NIH Image software, Image J. Image J was used to measure periods and lengths of plugs from microphotographs such as shown in FIG. **27***b*). Periods corresponded to the distance from the center of one plug to the center of an adjacent plug, and the length of a plug was the distance from the extreme front to the extreme back of the plug (see FIG. **28** for the definitions of front and back). Measurements were initially made in pixels, but could be converted to absolute measurements by comparing them to a measurement in pixels of the 50 μm width of the channel.

To make measurements of the optical intensity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs, microphotographs were converted from RGB to CMYK color mode in Adobe Photoshop 6.0. Using the same program, the yellow color channels of the microphotographs were then isolated and converted to grayscale images, and the intensities of the grayscale images were inverted. The yellow color channel was chosen to reduce the intensity of bright reflections at the extremities of the plugs and at the interface between the plugs and the channel. Following the work done in Photoshop, regions of plugs containing high concentrations of $Fe(SCN)_x^{(3-x)+}$ complexes appeared white while regions of low concentration appeared black. Using Image J, the intensity was measured across a thin, rectangular region of the plug, located halfway between the front and back of the plug (white dashed lines in FIG. **27***a***1**)). The camera used to take the microphotographs of the system was not capable of making linear measurements of optical density. Therefore, the measurements of intensity were not quantitative. Several of the plots of intensity versus relative position across the channel (FIG. **27***c*) were shifted vertically by less than 50 units of intensity to adjust for non-uniform illuminations of different parts of the images. These adjustments were justified because it was the shape of the distribution that was of interest, rather than the absolute concentration.

FIG. **29***a*)-*b*) shows plots of the sizes of periods and sizes of plugs as a function of total flow velocity (FIG. **29***a*)) and water fraction (wf) (FIG. **29***b*)). Values of capillary number (C.n.) were 0.0014, 0.0036, 0.0072 and 0.011, while values of the Reynolds number (R_e) were 1.24, 3.10, 6.21, and 9.31, each of the C.n. and R_e value corresponding to a set of data points with water fractions (wf) 0.20, 0.52, 0.52, and 0.20 (the data points from top to bottom in FIG. **29**A)). In turn, each of these sets of data points corresponds to a particular flow velocity as shown in FIG. **29***a*). Plugs in FIG. **29***b*) travel at about 50 millimeter/second (mm/s). All measurements of length and size are relative to the width of the channels (50 μm).

FIG. **30** shows microphotographs illustrating weak dependence of periods, length of plugs, and flow patterns inside plugs on total flow velocity. The left side of FIG. **30** shows a diagram of the microfluidic network. Here, the same solutions were used as in the experiment corresponding to FIG. **27**. The $Fe(SCN)_x^{(3-x)+}$ solution was introduced into inlet **301** while the colorless aqueous streams were introduced into inlets **302**, **303**. The same carrier fluid as used in the FIG. **27** experiment was flowed into channel **304**. The right side of FIG. **30** shows microphotographs of plugs formed at the same water fraction (0.20), but at different total flow velocities (20, 50, 100, 150 mm/s from top to bottom). Capillary numbers were 0.0014, 0.0036, 0.0072, and 0.011, respectively, from top to bottom. Corresponding Reynolds numbers were 1.24, 3.10, 6.21, and 9.31.

FIG. **31**A-C are plots showing the distribution of periods and lengths of plugs where the water fractions were 0.20, 0.40, and 0.73, respectively. The total flow velocity was about 50 mm/s, C.n.=0.0036, R_e=3.10 in all cases.

FIG. **27** shows the effects of initial conditions on mixing by recirculating flow inside plugs moving through straight microchannels. FIG. **27***a***1**) shows that recirculating flow (shown by black arrows) efficiently mixed solutions of reagents that were initially localized in the front and back halves of the plug. Notations of front, back, left, and right are the same as that in FIG. **28**. FIG. **27***a***2**) shows that recirculating flow (shown by black arrows) did not efficiently mix solutions of reagents that were initially localized in the left and right halves of the plugs. The left side of FIG. **27***b*) shows a schematic diagram of the microfluidic network. The two colorless aqueous streams were introduced into inlets **271**, **272** while a carrier fluid in the form of perfluorodecaline flowed through channel **273**. These solutions did not perturb the flow patterns inside plugs.

The right side of FIG. **27***b*) shows microphotographs of plugs of various lengths near the plug-forming region of the microfluidic network for water fractions of from 0.14 up to 1.00. FIG. **27***c***1**) shows a graph of the relative optical inten-

sity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs of varying lengths. The intensities were measured from left (x=1.0) to right (x=0.0) across the width of a plug (shown by white dashed lines in FIG. **27***a***1**)-*a***2**)) after the plug had traveled 4.4 times its length through the straight microchannel. The gray shaded areas indicate the walls of the microchannel. FIG. **27***c***2**) is the same as FIG. **27***c***1**) except that each plug had traversed a distance of 1.3 mm. The d/l of each water fraction (wf) were 15.2 (wf 0.14), 13.3 (wf 0.20), 11.7 (wf 0.30), 9.7 (wf 0.40), 6.8 (wf 0.60), 4.6 (wf 0.73), and 2.7 (wf 0.84), where d is the distance traveled by the plug and l is the length of the plug.

Example 4

Merging of Plugs

Experiments were conducted to investigate the merging of plugs using different channel junctions (T- or Y-shaped), cross-sections, and flow rates (see FIG. **33***a-d*). The figures on the left side of FIGS. **33***a-d* show top views of microfluidic networks that comprise channels having either uniform or nonuniform dimension (e.g., the same or different channel diameters). The corresponding figures on the right are microphotographs that include a magnified view of two plug streams (from the two separate channels portions of which form the branches of the Y-shaped junction) that merges into a common channel.

In FIG. **33***a*, the oil-to-water volumetric ratio was 4:1 in each pair of oil and water inlets. The oil streams were introduced into inlets **330**, **332**, while the aqueous streams were introduced into inlets **331**, **333**. The flow rates of the combined oil/water stream past the junction where the oil and water meet was 8.6 mm/s. The channels, which were rectangular, had dimensions of 50 (width)×50 (height) μm^2. As shown in FIG. **33***a*, plugs that flow in uniform-sized channels typically merged only when they simultaneously arrived at the T-junction. Thus, plug merging in these channels occur infrequently. In addition, lagging plugs were typically not able to catch up with leading plugs along the common channel.

FIG. **33***b* illustrates plug merging occurring between plugs arriving at different times at the Y-shaped junction (magnified view shown). The oil streams were introduced into inlets **334**, **336**, while the aqueous streams were introduced into inlets **335**, **337**. In FIG. **33***b*, the flow rates for the combined oil/water fluid past the junction where the oil and water meet were 6.9 mm/s for channel **346** (the 50×50 μm^2 channel) and 8.6 mm/s for channel **347** (the 25×50 μm^2 channel). The oil-to-water volumetric ratio was 4:1 in each pair of oil and water inlets. The two channels (the branch channels) merged into a common channel **348** that had a 100×50 μm^2 cross-section. As shown in the figure, the larger plugs from the bigger channel are able to merge with the smaller plugs from the narrower channel even when they do not arrive at the junction at the same time. This is because lagging larger plugs are able to catch up with the leading smaller plugs once the plugs are in the common channel.

FIG. **33***c* depicts in-phase merging (i.e., plug merging upon simultaneous arrival of at least two plugs at a junction) of plugs of different sizes generated using different oil/water ratios at the two pairs of inlets. The oil streams were introduced into inlets **338**, **340**, while the aqueous streams were introduced into inlets **339**, **341**. The flow rate corresponding to the fluid stream through channel **349** resulting from a 1:1 oil-to-water volumetric ratio was 4.0 mm/s, while that through channel **350** corresponding to the 4:1 oil-to-water volumetric ratio was 6.9 mm/s. Each branch channel of the Y-shaped portion of the network (magnified view shown) had a dimension of 50×50 μm while the common channel **351** (the channel to which the branch channels merge) was 125×50 μm^2.

FIG. **33***d* illustrates defects (i.e., plugs that fail to undergo merging when they would normally merge under typical or ideal conditions) produced by fluctuations in the relative velocity of the two incoming streams of plugs. The oil streams were introduced into inlets **342**, **344**, while the aqueous streams were introduced into inlets **343**, **345**. In this experiment, the flow rate corresponding to the fluid stream through channel **352** resulting from a 1:1 oil-to-water volumetric ratio was 4.0 mm/s, while that through channel **353** corresponding to the 4:1 oil-to-water volumetric ratio was 6.9 mm/s. Each branch channel that formed one of the two branches of the Y-shaped intersection (magnified view shown) was 50×50 μm^2 while the common channel **354** (the channel to which the two branch channels merge) is 125×50 μm^2.

Example 5

Splitting Plugs Using a Constricted Junction

The splitting of plugs was investigated using a channel network with a constricted junction. In this case, the plugs split and flowed past the junction into two separate branch channels (in this case, branch channels are the channels to which a junction branches out) that are at a 180°-angle to each other (see FIGS. **34***a-c* each of which show a channel network viewed from the top). In these experiments, the outlet pressures, P_1 and P_2, past the constricted junction were varied such that either $P_1 \approx P_2$ (FIG. **34***b*) or $P_1 < P_2$ (FIG. **34***c*). Here, the relative pressures were varied by adjusting the relative heights of the channels that were under pressures P_1 and P_2. Since longer plugs tend to split more reliably, this branching point (or junction) was made narrower than the channel to elongate the plugs. FIG. **34***a* shows a schematic diagram of the channel network used in the experiment. The oil and water were introduced into inlets **3400** and **3401**, respectively. The oil-to-water ratio was 4:1 while the flow rate past the junction where the oil and water meet was 4.3 mm/s.

FIG. **34***b* is a microphotograph showing the splitting of plugs into plugs of approximately one-half the size of the initial plugs. The channels **3404**, which were rectangular, had a cross-section that measured 50×50 μm^2. The constricted section of the channel **3402** right next to the branching point measured 25×50 μm^2. The outlet pressures, P_1 and P_2, were about the same in both branch channels. Here, the plugs split into plugs of approximately the same sizes.

FIG. **34***c* is a microphotograph showing the asymmetric splitting of plugs (i.e., the splitting of plugs into plugs of different sizes or lengths) which occurred when $P_1 < P_2$. The microphotograph shows that larger plugs (somewhat rectangular in shape) flowed along the channel with the lower pressure P_1, while smaller plugs (spherical in shape) flowed along the channel with the higher pressure P_2. As in FIG. **34***b*, each of the channel **3405** cross-section measured 50×50 μm^2. The constricted section of the channel **3403** at the junction measured 25×50 μm^2.

Example 6

Splitting Plugs without Using a Constricted Junction

The splitting of plugs was investigated using a channel network without a constriction such as the one shown in FIGS. **35***b-c*. The channel network used was similar to that

shown in FIG. **34**(*a*) except that here the plugs split and flowed past the junction in two separate channels at a 90°-angle to each other (the plug flow being represented by arrows). The oil and aqueous streams (4:1 oil:aqueous stream ratio) were introduced into inlets **3500** and **3501**, respectively. An oil-only stream flowed through channel **3502**. All channels had a cross-section of $50\times50\ \mu m^2$. The flow rate used was 4.3 mm/s. FIGS. **35***a-c*, which represent top views of a channel network, show that plugs behave differently compared to the plugs in Example 3 when they flow past a junction in the absence of a channel constriction, such as a constriction shown in FIGS. **35***b-c*. As FIG. **35***c* shows, when $P_1<P_2$, the plugs remained intact after passing through the junction. Further, the plugs traveled along the channel that had the lower pressure (P_1 in FIG. **35***c*) while the intervening oil stream split at the junction. The splitting of the oil stream at the junction gives rise to a shorter separation between plugs flowing along the channel with pressure P_1 compared to the separation between plugs in the channel upstream of the branching point or junction.

Example 7

Monitoring Autocatalytic Reactions Using a Microfluidic System

FIG. **37** illustrates the design of an experiment involving chemical amplification in microfluidic devices according to the invention that involves an investigation of a stochastic autocatalytic reaction. This example illustrates how the devices of the present invention can be used to study the acid-sensitive autocatalytic reaction between $NaClO_2$ and NaS_2O_3. On the left side of the microfluidic network, a three-channel inlet introduces an aqueous stream through channel **3702**, an ester through channel **3701**, and an esterase through channel **3703**. Oil flowed through channels **3713**, **3714**. The reaction between ester and esterase yield plugs **3704** that contain a small amount of acid. On the right side of the microfluidic network, the five-channel inlet introduces $NaClO_2$ through inlet **3705**, an aqueous stream through inlet **3706**, a pH indicator through inlet **3707**, a second aqueous stream through inlet **3708**, and NaS_2O_3 through channel **3709**. A carrier fluid flows through channels **3713**, **3714**. Unstirred mixtures of $NaClO_2$ and NaS_2O_3 are highly unstable and even a slight concentration fluctuation within that mixture leads to rapid decomposition. Thus, the plugs **3710** containing $NaClO_2/NaS_2O_3$ mixture must not only be quickly mixed but also promptly used after formation. In this proposed experiment, the curvy channels promote chaotic mixing. When a slightly acidic plug of the ester-esterase reaction is merged with a plug of an unstable $NaClO_2/NaS_2O_3$ mixture at the contact region **3712**, an autocatalytic reaction will generally be triggered. Upon rapid mixing of these two plugs, the resulting plugs **3711** become strongly acidic. The pH indicator introduced in the five-channel inlet is used to visualize this entire amplification process.

Example 8

Using Chemical Reactions as Highly Sensitive Autoamplifying Detection Elements in Microfluidic Devices

In one aspect according to the invention, a sequential amplification using controlled autocatalytic systems is used to amplify samples that contain single molecules of autocatalysts into samples containing a sufficiently high concentration of an autocatalyst such that the amplified autocatalyst can be detected with the naked eye can be detected with the naked eye. Although systems displaying stochastic behavior are expected to display high sensitivity and amplification, various autocatalytic systems can be used in accordance with the invention. A sequential amplification using the microfluidic devices according to the invention can be illustrated using a reaction that has been characterized analytically: the autocatalytic decomposition of violet bis[2-(5-bromo-pyridylazo)-5-(N-propyl-N-sulfopropyl-amino-phenolato] cobaltate, (Co(III)-5-Br-PAPS), upon oxidation with potassium peroxomonosulfate to produce colorless Co^{2+} ions. Here, the Co^{2+} ions serve as the autocatalyst (the order of autocatalysis, m, has not been established for this reaction).

$$\text{Co(III)-[5-Br-PAPS]reduced}+HSO_5^- \rightarrow Co^{2+}+\text{[5-Br-PAPS]oxidized}+HSO_4^- \quad (3)$$

Addition of small amounts of Co^{2+} to the violet mixture of (Co(III)-5-Br-PAPS and peroxomonosulfate produces an abrupt loss of color to give a colorless solution. The time delay before this decomposition depends on the amount of the Co^{2+} added to the solution. This reaction has been used to detect concentrations of Co^{2+} as low as about 1×10^{-10} mole/L. The reaction shows good selectivity in the presence of other ions (V(V), Cr(III), Cr(VI), Mn(II), Fe(II), Ni(II), Cu(II) and Zn(II)).

To use this reaction for amplification, a microfluidic network as shown in FIG. **38** is preferably used. An unstable solution of $Co(III)\text{-}[5\text{-}Br\text{-}PAPS]_{reduced}$ and peroxomonosulfate at pH=7 buffer in large plugs are preferably formed in a channel. These large plugs are preferably split in accordance with the invention into three different sizes of plugs. Preferably, the plug sizes are $(1\ \mu m)^3=10^{-15}$ L in the first channel; $(10\ \mu m)^3=10^{-12}$ L in the second channel; and $(100\ \mu m)^3=10^{-9}$ L in the third channel. A three-step photolithography is preferably used in the fabrication of masters for these microfluidic channels.

Example 9

Multi-Stage Chemical Amplification in Microfluidic Devices for Single Molecule Detection

FIG. **38** illustrates a method for a multi-stage chemical amplification for single molecule detection using microfluidic devices according to the invention. This example illustrates the use of an autocatalytic reaction between Co(III)-5-Br-PAPS (introduced through inlet **3803**) and $KHSO_5$ (introduced through inlet **3801**) in a pH=7 buffer (introduced through inlet **3802**) that is autocatalyzed by Co^{2+} ions. Oil streams are allowed to flow through channels **3804**, **3805**. This reaction mixture (contained in plugs **3811**) is unstable and decomposes rapidly (shown in red) when small amounts of Co^{2+} **3810** are added. Thus, this reaction mixture is preferably mixed quickly and used immediately. The reaction mixture is preferably transported through the network in $(1\ \mu m)^3$, $(10\ \mu m)^3$, $(100\ \mu m)^3$ size plugs. On the left side of the microfluidic network, the approximately 1 μm^3 plugs of the sample to be analyzed form at a junction of two channels (shown in green). The merging of plugs containing Co^{2+} ions and plugs containing the reaction mixture results in a rapid autocatalytic reaction. By using an amplification cascade in which larger and larger plugs of the reaction mixture are used for amplification, each Co^{2+} ion in a plug can be amplified to about 10^{10} Co^{2+} ions per plug. The result of amplification is visually detectable.

The $(10\ \mu m)^3$ plugs are preferably merged with larger $(100\ \mu m)^3$ plugs in the third channel to give approximately 4×10^{-8} mole/L solution of Co^{2+} ions. Autocatalytic decomposition in the approximately 10^{-9} L plugs will produce plugs **3809** with about 2.4×10^{10} Co^{2+} ions (4×10^{-5} mole/L). The flow rates in this system are preferably controlled carefully to control the time that plugs spend in each branch. The time provided for amplification is preferably long enough to allow amplification to substantially reach completion, but short enough to prevent or minimize slow decomposition.

Using different plug sizes is advantageous when merging plugs. Plugs with a size of about $(1\ \mu m)^3$ are preferably formed by flowing a sample containing about 3×10^{-9} mole/L Co^{2+} through channel **3806**. This reaction can be used to detect Co^{2+} at this, or lower, concentration (Endo et al., "Kinetic determination of trace cobalt(II) by visual autocatalytic indication," *Talanta,* 1998, vol. 47, pp. 349-353; Endo et al., "Autocatalytic decomposition of cobalt complexes as an indicator system for the determination of trace amounts of cobalt and effectors," *Analyst,* 1996, vol. 121, pp. 391-394). These plugs have a corresponding volume of about 10^{-15} L and carry just a few cobalt ions, on average about 1.8 ions per plug (corresponding to a Poisson distribution). These plugs **3810** are preferably merged with the $(1\ \mu m)^3$ plugs **3811** containing the Co(III)-5-Br-PAPS/peroxomonosulfate mixture (about 4×10^{-5} mole/L).

Upon autocatalytic decomposition of the complex, the number of Co^{2+} ions in the merged plug **3807** will increase by a factor of between about 10^4 to 1.2×10^4 Co^{2+} ions (2×10^{-5} mole/L in 2 μm^3). These plugs **3807** are preferably merged with the $(10\ \mu m)^3$ plugs **3811** containing the unstable mixture (about 4×10^{-5} mole/L). The concentration of Co^{2+} ions in these approximately 10^{-12} L plugs is preferably about 2×10^{-8} mole/L, which is sufficient to induce autocatalytic decomposition. The number of Co^{2+} ions will increase by a factor of between about 10^3 to about 2.4×10^7 ions/plug in plugs **3808**. The starting solution is dark violet ($\in=9.8\times10^4$ L $mol^{-1}cm^{-1}$ for Co(III)-5-Br-PAPS). Channels are preferably designed to create an optical path through at least ten consecutive 100 μm plugs. These plugs will provide an approximately 1-mm long optical path, with absorbance of the starting 4×10^{-5} mole/L solution of about 0.4. This absorbance can be detected by an on-chip photodetector or with the naked eye. If Co^{2+} is present in the sample solution, an autocatalytic cascade will result in the disappearance of the color of the reaction mixture.

At low concentrations of Co^{2+} in the sample, the system may show stochastic behavior, that is, not every Co^{2+} ion would give rise to a decomposition cascade. However, the attractive feature of this system is that thousands of tests can be carried out in a matter of seconds, and statistics and averaging can be performed. Preferably, a sequence of controlled autocatalytic amplification reactions leads to a visual detection of single ions.

Example 10

Enzyme Kinetics

A microfluidic chip according to the invention was used to measure millisecond single-turnover kinetics of ribonuclease A (RNase A; EC 3.1.27.5), a well-studied enzyme. Submicroliter sample consumption makes the microfluidic chip especially attractive for performing such measurements because they require high concentrations of both the enzyme and the substrate, with the enzyme used in large excess.

The kinetic measurements were performed by monitoring the steady-state fluorescence arising from the cleavage of a fluorogenic substrate by RNase A as the reaction mixture flowed down the channel (see FIG. **40**(*a*)). In FIG. **40**, a substrate, buffer, and RNase A were introduced into inlets **401**, **401**, and **403**, respectively. A carrier fluid flowed through channel **404**. The amount of the product at a given reaction time t [s] was calculated from the intensity of fluorescence at the corresponding distance point d [m] (t=d/U where U=0.43 m/s is the velocity of the flow). The channels were designed to wind so that rapid chaotic mixing was induced, and were designed to fit within the field of view of the microscope so that the entire reaction profile could be measured in one spatially resolved image. Selwyn's test (Duggleby, R. G., *Enzyme Kinetics and Mechanisms, Pt D*; Academic Press: San Diego, 1995, vol. 249, pp. 61-90; Selwyn, M. J. *Biochim. Biophys. Acta,* 1965, vol. 105, pp. 193-195) was successfully performed in this system to establish that there were no factors leading to product inhibition or RNase A denaturation.

The flow rate of the stock solution of 150 μM of RNase A was kept constant to maintain 50 μM of RNase A within the plugs. By varying the flow rates of the buffer and substrate (see FIG. **45**), progress curves were obtained for eight different substrate concentrations. For $[E]_o>>[S]_o$, the simple reaction equation is $[P]_t=[S]_o(1-Exp(-kt))$, where $[E]_o$ is the initial enzyme concentration, $[S]_o$ is the initial substrate concentration, $[P]_t$ is the time-dependent product concentration and k [s^{-1}] is the single-turnover rate constant. To obtain a more accurate fit to the data, the time delay Δt_n required to mix a fraction of the reaction mixture f_n was accounted for.

An attractive feature of the microfluidic system used is that the reaction mixture can be observed at time t=0 (there is no dead-time). This feature was used to determine Δt_n and f_n in this device by obtaining a mixing curve using fluo-4/Ca^{2+} system as previously described (Song et al., *Angew. Chem. Int. Ed.* 2002, vol. 42, pp. 768-772), and correcting for differences in diffusion constants (Stroock et al., *Science,* 2002, vol. 295, pp. 647-651). All eight progress curves gave a good fit with the same rate constant of 1100±250 s^{-1}. The simpler theoretical fits gave indistinguishable rate constants. These results are in agreement with previous studies, where cleavage rates of oligonucleotides by ribonucleases were shown to be ~10^3 s^{-1}.

Thus, this example demonstrates that millisecond kinetics with millisecond resolution can be performed rapidly and economically using a microchannel chip

$$[P]_t = \sum_n f_n[S]_0(1 - \mathrm{Exp}(-k(t - \Delta t_n)))$$

according to the invention. Each fluorescence image was acquired for 2 s, and required less than 70 nL of the reagent solutions. These experiments with stopped-flow would require at least several hundreds of microliters of solutions. Volumes of about 2 μL are sufficient for ~25 kinetic experiments over a range of concentrations. Fabrication of these devices in PDMS is straightforward (McDonald, et al., *Accounts Chem. Res.* 2002, vol. 35, pp. 491-499) and no specialized equipment except for a standard microscope with a CCD camera is needed to run the experiments. This system could serve as an inexpensive and economical complement to stopped-flow methods for a broad range of kinetic experiments in chemistry and biochemistry.

Example 11

Kinetics of RNA Folding

The systems and methods of the present invention are preferably used to conduct kinetic measurements of, for example, folding in the time range from tens of microseconds to hundreds of seconds. The systems and methods according to the invention allow kinetic measurements using only small amounts of sample so that the folding of hundreds of different RNA mutants can be measured and the effect of mutation on folding established. In one aspect according to the invention, the kinetics of RNA folding is preferably measured by adding Mg^{2+} to solutions of previously synthesized unfolded RNA labeled with FRET pairs in different positions. In accordance with the invention, the concentrations of Mg^{2+} are preferably varied in the 0.04 to 0.4 μM range by varying the flow rates (see, for example, FIGS. **25***a*)-*c*)) to rapidly determine the folding kinetics over a range of conditions. The ability to integrate the signal over many seconds using the steady-flow microfluidic devices according to the invention can further improve sensitivity.

As shown in FIGS. **25***a*)-*c*), the concentrations of aqueous solutions inside the plugs can be controlled by changing the flow rates of the aqueous streams. In FIGS. **25***a*)-*c*), aqueous streams were introduced into inlets **251**-**258** wherein flow rates of about 0.6 μL/min for the two aqueous streams and 2.7 μL/min was used for the third stream. The stream with the 2.7 μL/min volumetric flow rate was introduced in the left, middle, and right inlet in FIGS. **25***a*)-*c*), respectively. A carrier fluid in the form of perfluorodecaline was introduced into channel **259**, **260**, **261**. The corresponding photographs on each of the right side of FIGS. **25***a*)-*c*) illustrate the formation of plugs with different concentrations of the aqueous streams. The various shadings inside the streams and plugs arise from the use of aqueous solutions of food dyes (red/dark and green/light), which allowed visualization, and water were used as the three streams, the darker shading arising mainly from the red dye color while the lighter shading arising mainly from the green dye color. The dark stream is more viscous than the light stream, therefore it moves slower (in mm/s) and occupies a larger fraction of the channel at a given volumetric flow rate (in μL/min).

Example 12

Nanoparticle Experiments with and without Plugs

FIG. **15** illustrates a technique for the synthesis of CdS nanoparticles **155**. In one experiment, nanoparticles were formed in a microfluidic network. The channels of the microfluidic device had 50 μm×50 μm cross-sections. A fluorinated carrier-fluid (10:1 v/v mixture of perfluorohexane and 1H,1H,2H,2H-perfluorooctanol) was flowed through the main channel at 15 μm min^{-1}. An aqueous solution, pH=11.4, of 0.80 mM $CdCl_2$ and 0.80 mM 3-mercaptopropionic acid was flowed through the left-most inlet channel **151** at 8 μL min^{-1}. An aqueous solution of 0.80 mM polyphosphates $Na(PO_3)_n$ was flowed through the central inlet channel **152** at 8 μL min^{-1}, and an aqueous solution of 0.96 mM Na_2S was flowed through the right-most inlet channel **153** at 8 μL min^{-1}. To terminate the growth of nanoparticles, an aqueous solution of 26.2 mM 3-mercaptopropionic acid, pH=12.1, was flowed through the bottom inlet of the device **157** at 24 μM min^{-1}. FIG. **15** shows various regions or points along the channel corresponding to regions or points where nucleation **154**, growth **158**, and termination **156** occurs. Based on the UV-VIS spectrum, substantially monodisperse nanoparticles formed in this experiment.

Nanoparticles were also formed without microfluidics. Solutions of $CdCl_2$, polyphosphates, Na_2S, and 3-mercaptopropionic acid, identical to those used in the microfluidics experiment, were used. 0.5 mL of the solution of $CdCl_2$ and 3-mercaptopropionic acid, 0.5 mL of polyphosphates solution, and 0.5 mL of Na_2S solution were combined in a cuvette, and the cuvette was shaken by hand. Immediately after mixing, 1.5 mL of 26.2 mM 3-mercaptopropionic acid was added to the reaction mixture to terminate the reaction, and the cuvette was again shaken by hand. Based on the UV-VIS spectrum, substantially polydisperse nanoparticles formed in this experiment.

Example 13

Crystallization

Networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The PDMS was purchased from Dow Corning Sylgard Brand 184 Silicone Elastomer. The PDMS devices were sealed after plasma oxidation treatment in Plasma Prep II (SPI Supplies). The devices were rendered hydrophobic by baking the devices at 120° C. for 2-4 hours. Microphotographs were taken with a Leica MZ12.5 stereomicroscope and a SPOT Insight color digital camera (Model#3.2.0, Diagnostic Instruments, Inc.). Lighting was provided from a Machine Vision Strobe X-strobe X1200 (20 Hz, 12 μF, 600V, Perkin Elmer Optoelectronics). To obtain an image, the shutter of the camera was opened for 1 second and the strobe light was flashed once with the duration of approximately 10 μs.

Aqueous solutions were pumped using 10 μl or 50 μl Hamilton Gastight syringes (1700 series). Carrier-fluid was pumped using 50 μl Hamilton Gastight syringes (1700 series). The syringes were attached to microfluidic devices by means of Teflon tubing (Weico Wire & Cable Inc., 30 gauge). Syringe pumps from Harvard Apparatus (PHD 2000) were used to inject the liquids into microchannels.

A. Microbatch Crystallization in a Microfluidic Channel

Microbatch crystallization conditions can be achieved. This experiment shows that size of plugs can be maintained and evaporation of water prevented. In this case, the PDMS device has been soaked in water overnight before the experiment in order to saturate PDMS with water. The device was kept under water during the experiment. During the experiment, the flow rates of carrier-fluid and NaCl solution were 2.7 μL/min and 1.0 μL/min, respectively. The flow was stopped by cutting off the Teflon tubing of both carrier-fluid and NaCl solution.

FIG. **16** shows a schematic illustration of a microfluidic device according to the invention and a microphotograph of plugs of 1M aqueous NaCl sustained in oil. The carrier-fluid is perfluorodecaline with 2% 1H,1H,2H,2H-perfluorooctanol. Inside a microchannel, plugs showed no appreciable change in size.

B. Vapor Diffusion Crystallization in Microchannels: Controlling Evaporation of Water from Plugs

This experiment shows that evaporation of water from plugs can be controlled by soaking devices in water for shorter amounts of time or not soaking at all. The rate of evaporation can be also controlled by the thickness of PDMS used in the fabrication of the device. Evaporation rate can be increased by keeping the device in a solution of salt or other substances instead of keeping the device in pure water.

The plug traps are separated by narrow regions that help force the plugs into the traps.

In this experiment, a composite glass/PDMS device was used. PDMS layer had microchannel and a microscopy slide (Fisher, 35×50-1) was used as the substrate. Both the glass slide and the PDMS were treated in plasma cleaner (Harrick) then sealed. The device was made hydrophobic by first baking the device at 120° C. for 2-4 hours then silanizing it by (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.).

During the experiment, a flow of carrier-fluid at 1.0 μL/min was established, then flow of aqueous solution was established at a total rate of 0.9 μL/min. Plug formation was observed inside the microchannel. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **41** shows a microphotograph (middle and right side) of the water plugs region of the microfluidic network. FIG. **41**(*b*)-(*c*) show the plugs at time t=0 and t=2 hours, respectively. Red aqueous solution is 50% waterman red ink in 0.5 M NaCl solution. Ink streams were then introduced into inlets **411**, **412**, **413**. An oil stream flowed through channel **414**. The carrier-fluid is FC-3283 (3M Fluorinert Liquid) with 2% 1H,1H,2H,2H-perfluorodecanol. This photograph demonstrates that the evaporation of water through PDMS can be controlled, and thus the concentration of the contents inside the drops can be increased (this is equivalent to microbatch crystallization). FIG. **41**(*a*) shows a diagram of the microfluidic network.

C. Controlling Shape and Attachment of Water Plugs

During the experiment, a flow of carrier fluid at 1.0 μL/min was established, then flow of aqueous solution was established at a total rate of 2.1 μL/min. Plug formation was observed inside the microchannel. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **39** shows a diagram (left side) of a microfluidic network according to the invention. Aqueous streams were introduced into inlets **3901**, **3902**, **3903** while an oil stream flowed through channel **3904**. FIG. **39** also shows a microphotograph (right side) of the water plug region of the microfluidic network. This image shows water plugs attached to the PDMS wall. This attachment occurs when low concentrations of surfactant, or less-effective surfactants are used. In this case 1H,1H,2H,2H-perfluorooctanol is less effective than 1H,1H,2H,2H-perfluorodecanol. In this experiment the oil is FC-3283 (3M Fluorinert Liquid) with 2% 1H,1H,2H,2H-perfluorooctanol as the surfactant.

D. Examples of Protein Crystallization

During the experiment, a flow of oil at 1.0 μL/min was established. Then the flow of water was established at 0.1 μL/min. Finally flows of lysozyme and precipitant were established at 0.2 μL/min. Plug formation was observed inside the microchannel. The flow of water was reduced to zero after the flow inside the channel became stable. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **36** depicts lysozyme crystals grown in water plugs in the wells of the microfluidic channel. Lysozyme crystals started to appear inside aqueous plugs both inside and outside plug traps in approximately 10 minutes. The image of the three crystals in FIG. **36** was taken 1 hour after the flow was stopped. Lysozyme crystals appear colored because they were observed under polarized light. This is common for protein crystals.

The left side of FIG. **36** is a diagram of a microfluidic network according to the invention while the right side is microphotograph of the crystals formed in plugs in the microfluidic network. A precipitant, lysozyme, and water were introduced into inlets **3601**, **3602**, and **3603**, respectively. Oil was flowed through channel **3604**. The lysozyme solution contains 100 mg/ml lysozyme in 0.05 M sodium acetate (pH 4.7); the precipitant solution contains 30% w/v PEG (M.W. 5000), 1.0 M NaCl and 0.05 M sodium acetate (pH 4.7); The carrier-fluid is FC-3283 (3M Fluorinert Liquid) with 10% 1H,1H,2H,2H-perfluoro-octanol. The microchannel device was soaked in FC-3283/H_2O for one hour before experiment.

FIG. **32** shows that plug traps are not required for formation of crystals in a microfluidic network. FIG. **32** shows a diagram (left side) of the microfluidic network. A precipitant was introduced into inlet **321**, lysozyme was introduced into inlet **322**, and an aqueous stream was introduced into inlet **323**. Oil was flowed through channel **324**. FIG. **32** also shows microphotographs (middle and right side) of lysozyme crystals grown inside the microfluidic channel. The experimental condition is same as in FIG. **36**.

Example 14

Oil-soluble Surfactants for Charged Surfaces

In accordance with the invention, neutral surfactants that are soluble in perfluorinated phases are preferably used to create positively and negatively-charged interfaces. To create charged surfaces, neutral surfactants that can be charged by interactions with water, e.g., by protonation of an amine or a guanidinium group (FIG. **24**B), or deprotonation of a carboxylic acid group (FIG. **24**C), are preferably used. Preferably, charged surfaces are used to repel, immobilize, or stabilize charged biomolecules. Negatively charged surfaces are useful for handling DNA and RNA without surface adsorption. Preferably, both negatively and positively-charged surfaces are used to control the nucleation of protein crystals. Many neutral fluorinated surfactants with acidic and basic groups ($RfC(O)OH$, $Rf(CH_2)_2NH_2$, $Rf(CH_2)_2C(NH)NH_2$) are available commercially (Lancaster, Fluorochem, Aldrich).

To synthesize oligoethylene-glycol terminated surfactants, a modification and improvement of a procedure based on the synthesis of perfluoro non-ionic surfactants is preferably used. In one aspect, the synthesis relies on the higher acidity of the fluorinated alcohol to prevent the polycondensation of the oligoethylene glycol. The modified synthesis uses a selective benzylation of one of the alcohol groups of oligoethylene glycol, followed by activation of the other alcohol group as a tosylate. A Williamson condensation is then performed under phase transfer conditions followed by a final deprotection step via catalytic hydrogenation using palladium on charcoal.

Example 15

Formation of Plugs in the Presence of Fluorinated Surfactants and Surface Tension

The surface tension of the oil/water interface has to be sufficiently high in order to maintain a low value of capillary number, C.n. The fluorosurfactant/water interfaces for water-insoluble fluorosurfactants have not been characterized, but

these surfactants are predicted to reduce surface tension similar to that observed in a system involving Span on hexane/water interface (about 20 mN/m). The surface tensions of the aqueous/fluorous interfaces are preferably measured in the presence of fluorosurfactants using the hanging drop method. A video microscopy apparatus specifically constructed for performing these measurements has been used to successfully characterize interfaces. FIG. **24** illustrates the synthesis of fluorinated surfactants containing perfluoroalkyl chains and an oligoethylene glycol head group.

Example 16

Forming Gradients by Varying Flow Rates

FIG. **42** shows an experiment involving the formation of gradients by varying the flow rates. In this experiment, networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The width and height of the channel were both 50 μm. 10% 1H,1H,2H,2H-perfluorodecanol in perfluoroperhydrophenanthrene was used as oil. Red aqueous solution prepared from 50% waterman red ink in 0.5 M NaCl solution was introduced into inlet **421**. The oil flowed through channel **424** at 0.5 μl/min. Aqueous streams were introduced into inlets **422**, **423**. To generate the gradient of ink in the channel, the total water flow rate was gradually increased from 0.03 μl/min to 0.23 μl/min in 20 seconds at a ramp rate of 0.01 μl/min per second. At the same time, ink flow rate was gradually decreased from 0.25 μl/min to 0.05 μl/min in 20 seconds at a ramp rate of −0.01 μl/min per second. The total flow rate was constant at 0.28 μl/min. The established gradient of ink concentration inside the plugs can be clearly seen from FIG. **42**: the plugs further from the inlet are darker since they were formed at a higher ink flow rate.

Example 17

Lysozome Crystallization Using Gradients

FIG. **43** illustrates an experiment involving the formation of lysozome crystals using gradients. The channel regions **435**, **437** correspond to channel regions with very low precipitant concentration while channel region **436** corresponds to optimal range of precipitant concentration. In this experiment, networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The width of the channel was 150 μm and the height was 100 μm. 10% 1H,1H,2H,2H-perfluorodecanol in perfluoroperhydrophenanthrene was used as oil.

During the experiment, a flow of oil through channel **434** at 1.0 μl/min was established. Then the flow of water introduced through inlet **432** was established at 0.2 μl/min. The flows of lysozyme introduced through inlet **431** and precipitant introduced through inlet **433** were established at 0.2 μl/min. Plugs formed inside the channel. To create the gradient, water flow rate was first gradually decreased from 0.35 μl/min to 0.05 μl/min over 45 seconds at a ramp rate of (−0.01 μl/min per 1.5 seconds), then increased back to 0.35 μl/min in 45 seconds at a ramp rate of (0.01 μl/min per 1.5 seconds). At the same time, precipitant flow rate was gradually increased from 0.05 μl/min to 0.35 μl/min in 45 seconds at a ramp rate of (0.01 μl/min per 1.5 seconds), then decreased to 0.05 μl/min in 45 seconds at a ramp rate of (−0.01 μl/min per 1.5 seconds). The flow was stopped by pulling out the inlet tubing immediately after water and precipitant flow rates returned to the starting values. The plugs created in this way contained constant concentration of the protein but variable concentration of the precipitant: the concentration of the precipitant was lowest in the beginning and the end of the channel, and it peaked in the middle of the channel (the center row). Only the plugs in the middle of the channel have the optimal concentration of precipitant for lysozyme crystallization, as confirmed by observing lysozyme crystals inside plugs in the center row. Visualization was performed under polarized light. Preferably, all flow rates would be varied, not just the precipitant and water.

Example 18

Lysozyme Crystallization in Capillaries Using the Microbatch Analogue Method

To grow lysozyme crystal inside plugs within capillaries, a 10 μl Hamilton syringe was filled with 100 mg/ml lysozyme in 0.05 M NaAc buffer (pH4.7) and another 10 μl Hamilton syringe was filled with 30% (w/v) MPEG 5000 with 2.0 M NaCl in 0.05 M NaAc buffer (pH4.7) as precipitant. A 50 μl Hamilton syringe filled with PFP (10% PFO) was the oil supply. All three syringes were attached to the PDMS/capillary device and driven by Harvard Apparatus syringe pumps (PHD2000). The capillary has an inner diameter of 0.18 mm and outer diameter of 0.20 mm. Oil flow rate was 1.0 μl/min and both lysozyme and precipitant solution were at 0.3 μl/min. The channel was filled with oil first. Protein and precipitant streams converged immediately before entering the channel to form plugs. After the capillary (Hampton Research) was filled with the plugs containing lysozyme, the flows were stopped. The capillary was disconnected from the PDMS device, sealed with wax and stored in an incubator (18° C.). A lysozyme crystal appeared within an hour and was stable for at least 14 days without change of size or shape (FIG. **47**A).

Example 19

Thaumatin Crystallization in Capillaries Using the Microbatch Analogue Method

Experiment 1. A 10 μl Hamilton syringe was filled with 50 mg/ml thaumatin in 0.1 M ADA buffer (pH 6.5) and another 10 μl Hamilton syringe was filled with 1.5 M NaK Tatrate in 0.1 M HEPES (pH 7.0). A 50 μl Hamilton syringe filled with PFP (10% PFO) was the oil supply. All three syringes were attached to the PDMS/capillary device and driven by Harvard Apparatus syringe pumps (PHD2000). The capillary has an inner diameter of 0.18 mm and outer diameter of 0.20 mm. Oil flow rate was 1.0 μl/min and both thaumatin and precipitant solution were at 0.3 μl/min. The channel was filled with oil first. Protein and precipitant streams were mixed immediately before entering the channel to form plugs. After the capillary (Hampton Research) was filled with protein plugs, the flows were stopped. The capillary was cut from the PDMS device, sealed by wax and stored in an incubator (18° C.). The thaumatin crystal appeared in 2-3 days and was stable for at least 45 days without size or shape change (FIG. **47**B). Some thaumatin crystals grew at the interface of protein solution and oil, while others appeared to attach to the capillary wall.

Experiment 2. Thaumatin crystals were grown inside a capillary tube using 50 mg/mL thaumatin in 0.1 M pH 6.5 ADA buffer and a precipitant solution of 1 M Na/K tartrate in a 0.1M pH 7.5 HEPES buffer. Protein and precipitant solutions were mixed in a 1.4:1 protein:precipitant ratio. A fluorinated carrier fluid was a saturated solution of FSN surfactant in FC3283. The capillary was incubated at 18 degrees C. Tetragonal crystals appeared within 5 days (FIGS. **48**A, B).

X-ray diffraction was performed at BioCARS station 14BM-C at the Advanced Photon Source at Argonne National Laboratory. Beam wavelength was 0.9 A. The final length of a single crystal was estimated at 100-150 microns.

Capillaries were cut to the appropriate length without disturbing crystal-containing plugs, resealed using capillary waz, and mounted on clay-tipped cryoloop holders at a distance of 12+/−5 mm from base to crystal. The holder was placed on the x-ray goniometer. Crystals were centered on the beam. Snapshots were taken using 10 second (thaumatin) exposures. Distance from sample to detector was 150 mm. Diffraction to better than 2.2 A was obtained.

Example 20

Vapor Diffusion Protein Crystallization in Capillaries by an Alternating Droplet System

The principle of transferring water inside a capillary from one set of plugs to another set of plugs is illustrated in FIG. **50**. Briefly, a 10 μl Hamilton syringe was filled with 0.01 $Fe(SCN)_3$ and another 10 μl Hamilton syringe was filled with 0.1 M $Fe(SCN)_3$ with 2.5 M KNO_3. Two 50 μl Hamilton syringes were filled with FMS-121 (Gelest, Inc) (saturated with PFO), which provided the oil supply. All four syringes were attached to the PDMS/capillary device and driven by Harvard Apparatus syringe pumps (PHD2000). The capillary has an inner diameter of 0.18 mm and outer diameter of 0.20 mm. One of the oil inlet channels was between the two aqueous inlets channels to separate the two aqueous streams when forming the alternating plugs. This oil inlet channel was vertical to the main channel and had a flow rate of 2.0 μl/min. The other oil inlet channel had a flow rate of 1.0 μl/min and was parallel to the main channel. Both of the aqueous solutions had a flow rate of 0.5 μl/min. After establishing alternating aqueous droplet streams in the capillary, the flows were stopped, and the capillary was disconnected from the PDMS device, sealed with wax and stored in an incubator at 18° C. The size and color change of the plugs were monitored with a Leica microscope (MZ125) having a color CCD camera (SPOT Insight, Diagnostic Instruments, Inc.).

Following the stoppage of flow and sealing of the capillary tube, plugs containing 0.01 M $Fe(SCN)_3$ in water were yellow, while those containing 0.1 M $Fe(SCN)_3$ and 2.5 M KNO_3 in water were red (FIG. **50**A). However, FIG. **50**B shows that after 5 days, the yellow plugs were reduced in size and were more concentrated, while the red plugs increased in size and were more diluted. This demonstration reflects vapor diffusion conditions in the capillary tube that are predicted to facilitate protein crystallization. This technique can be further adapted to other applications requiring concentration of reagents, such as proteins.

Alternating plugs from two different aqueous solutions may be generated in accordance with several representative geometries as set forth in FIG. **51**. In principle, the same oil or different oils may be used in the two oil inlets. One scheme for generating alternating plugs from two different aqueous solutions is depicted in FIG. **51**A. In this case, one 10 μl Hamilton syringe was filled with 0.1 $Fe(SCN)_3$, another with 1.5 M NaCl. Two 50 μl Hamilton syringes filled with PFP (with 10% PFO) provided the oil supply. All four syringes were attached to the PDMS device and driven by Harvard Apparatus syringe pumps (PHD2000). Alternatively, multiple solutions can be co-introduced together in each of the two aqueous channels as depicted in FIG. **51**B. In each of these two cases one of the oil inlet channels was between the two aqueous inlet channels. This oil inlet channel was used to separate the two aqueous streams into alternating plugs and was vertical to the main channel, having a flow rate of 2.0 μl/min. The other oil inlet channel was parallel to the main channel and had a flow rate of 1.0 μl/min. Each of the two aqueous solutions had flow rates of 0.5 μl/min. Alternating plugs were found to form in the channel (FIG. **51**C).

FIG. **52** illustrates another example of generating alternating plugs from two different aqueous solutions. In this case, one 10 μl Hamilton syringe was filled with 0.1 $Fe(SCN)_3$, the other with 1.5 M NaCl. Two 50 μl Hamilton syringes filled with FMS-121 (saturated with PFO) provided the oil supply. All four syringes were attached to the device and driven by Harvard Apparatus syringe pumps (PHD2000). One of the oil inlet channels was between the two aqueous inlet channels and was used to separate the two aqueous streams prior to formation of alternating plugs (FIG. **52**A). This oil inlet channel was vertical to the main channel and had a flow rate of 1.5 μl/min. The other oil stream had a flow rate of 1.5 μl/min and was parallel to the main channel. Each of the two aqueous solutions had flow rates of 0.5 μl/min. Alternating plugs were found to form in the channel (FIG. **52**B).

Other geometries that can support the formation of alternating plugs are depicted in FIG. **53**. Importantly, the flow rates of solutions A and B may be changed in a correlated fashion (FIG. **54**). Thus, when the flow rate of solution A_1 is increased and solution A_2 is decreased, the flow rate of solutions B_1 is also increased and solution B_2 is also decreased. This principle, depicted in FIG. **54**, is useful for maintaining a constant difference in salt concentration between the plugs of stream A and stream B to ensure that transfer from all plugs A to all plugs B occurs at a constant rate.

FIG. **54** provides a schematic illustration of a device for preparing plugs of varying protein concentrations where the flow rates of the A and B streams change in a correlated fashion. In this example, A_1 through A_3 are for protein solution, buffer and precipitants, such as PEG or salts. Highly concentrated salt solutions are injected through B_1~B_3. The flow rate ratio of inlet A_1 to that of B_i (i=1~3) is maintained constant. Therefore all of the protein plugs will shrink at a rate similar to the salt plugs.

FIG. **54** shows that if the flow rates of corresponding A and B streams are changed in a correlative fashion, the composition of plugs B will reflect the composition of plugs A. Therefore, one can incorporate markers into the B stream plugs to serve as a code for the plugs in the A stream. In other words, absorption/fluorescent dyes or x-ray scattering/absorbing materials can be incorporated in markers in the B streams to facilitate optical or x-ray-mediated quantification so as to provide a read out of relative protein and precipitant concentrations in the A streams. This approach can provide a powerful means for optimizing crystallization conditions for subsequent scale-up experiments.

We claim:

1. A method for conducting a reaction in plugs in a microfluidic system, comprising the steps of:

providing the microfluidic system comprising at least two channels having at least one junction;

continuously flowing an aqueous fluid containing at least one biological molecule and at least one reagent for conducting the reaction between the biological molecule and the at least one reagent through a first channel of the at least two channels;

continuously flowing a carrier fluid immiscible with the aqueous fluid through the second channel of the at least two channels;

forming at least one plug of the aqueous fluid containing the at least one biological molecule and the at least one

reagent by partitioning the aqueous fluid with the flowing immiscible carrier fluid at the junction of the at least two channels, the plug being substantially surrounded by the immiscible carrier fluid flowing through the channel, wherein the at least one plug comprises at least one biological molecule and the at least one reagent for conducting the reaction with the at least one biological molecule; and

providing conditions suitable for the reaction in the at least one plug involving the at least one biological molecule and the at least one reagent to form a reaction product.

2. The method according to claim **1**, wherein the at least one biological molecule is DNA or RNA.

3. The method according to claim **2**, wherein the reaction is an autocatalytic reaction.

4. The method according to claim **2**, wherein the reaction is a polymerase chain reaction.

5. The method according to claim **1**, wherein the reaction is an enzymatic reaction.

6. The method according to claim **1**, further comprising detecting the reaction product.

7. The method according to claim **6**, wherein detecting is optically detecting.

8. The method according to claim **1**, wherein the immiscible carrier fluid is an oil.

9. The method according to claim **8**, wherein the oil comprises a surfactant.

10. The method according to claim **9**, wherein the surfactant is a fluorosurfactant.

11. The method according to claim **8**, wherein the oil is a fluorinated oil.

12. The method of claim **1**, wherein the step of forming at least one plug comprises a plurality of plugs some of which contain at least one biological molecule and others are free of biological molecules.

13. The method according to claim **1**, wherein the providing step includes heating.

* * * * *

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(54) **DEVICE AND METHOD FOR PRESSURE-DRIVEN PLUG TRANSPORT AND REACTION**

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CPC ***G01N 35/08*** (2013.01); *B01L 2300/0883* (2013.01); *G01N 2015/1409* (2013.01); *G01N 15/1484* (2013.01); *B01J 2219/00725* (2013.01); *B01J 2219/00722* (2013.01); *B01J 2219/00783* (2013.01); *B01J 2219/00736* (2013.01); ***B01F 5/0647*** (2013.01); *B82Y 30/00* (2013.01); *B01J 2219/00599* (2013.01); ***B01J 19/0046*** (2013.01); *B01L 2400/0454* (2013.01); *B01J 2219/00975* (2013.01); ***B01L 3/502784*** (2013.01); ***G01N 35/085*** (2013.01); *B01J 2219/00869* (2013.01); *B01J 2219/00576* (2013.01); *B01L 2200/0668* (2013.01); ***B01L 3/502746*** (2013.01); *B01J 2219/00837* (2013.01); *B01J 2219/00756* (2013.01); ***B01F 13/0071*** (2013.01); ***B01F 5/0646*** (2013.01); *B01J 2219/0086* (2013.01); *B01L 2400/0448* (2013.01); *B01L 2300/0867* (2013.01); ***B01J 19/0093*** (2013.01); *B01L 2400/0487* (2013.01); *B01J 2219/00891* (2013.01); *B01J 2219/00585* (2013.01); *B01J 2219/00286* (2013.01); *B01L 2400/0424* (2013.01); *B01J 2219/00889* (2013.01); *B01J 2219/00977* (2013.01)

USPC **422/502**; 422/503; 422/504; 436/180

(58) **Field of Classification Search**

USPC 422/68.1, 73, 100, 101, 102, 103, 502, 422/503, 504, 547, 551, 552; 436/63, 164, 436/166, 174, 176, 177, 178, 179, 180

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,960,187 A	6/1976	Stock et al.
4,853,336 A	8/1989	Saros et al.

(Continued)

FOREIGN PATENT DOCUMENTS

CN	2482070	3/2002
EP	0 816 837	1/1998

(Continued)

OTHER PUBLICATIONS

Anna, Shelley A. et al., "Formation of Dispersions Using 'Flow Focusing' in Microchannels", *Applied Physics Letters*, vol. 82, No. 3, 2003, pp. 364-366.

(Continued)

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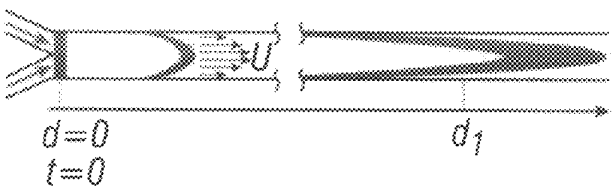
(57) **ABSTRACT**

The present invention provides microfabricated substrates and methods of conducting reactions within these substrates. The reactions occur in plugs transported in the flow of a carrier-fluid.

31 Claims, 36 Drawing Sheets



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(56) **References Cited**

U.S. PATENT DOCUMENTS

4,963,498	A	10/1990	Hillman et al.	
5,185,099	A	2/1993	Delpuech et al.	
5,739,036	A	4/1998	Parris	
5,872,010	A	2/1999	Karger et al.	
5,887,755	A	3/1999	Hood, III	
5,997,636	A	12/1999	Gamarnik et al.	117/70
6,130,098	A	10/2000	Handique et al.	
6,140,053	A	10/2000	Köster	435/6
6,180,372	B1	1/2001	Franzen	435/91.1
6,296,673	B1	10/2001	Santarsiero et al.	
6,409,832	B2	6/2002	Weigl et al.	
6,432,290	B1	8/2002	Harrison et al.	
6,524,456	B1	2/2003	Ramsey et al.	
6,569,631	B1	5/2003	Pantoliano et al.	
6,630,006	B2	10/2003	Santarsiero et al.	
6,790,328	B2	9/2004	Jacobson et al.	
6,797,056	B2	9/2004	David	
6,872,250	B2	3/2005	David et al.	
7,129,091	B2 *	10/2006	Ismagilov et al.	436/34
7,294,503	B2 *	11/2007	Quake et al.	435/288.5
7,655,470	B2 *	2/2010	Ismagilov et al.	436/55
2001/0036669	A1	11/2001	Jedrzejewski et al.	
2001/0048900	A1	12/2001	Bardell et al.	
2002/0008029	A1	1/2002	Williams et al.	204/453
2002/0012971	A1	1/2002	Mehta	435/91.2
2002/0017464	A1	2/2002	Parce et al.	204/601
2002/0022261	A1	2/2002	Anderson et al.	435/287.2
2002/0058332	A1	5/2002	Quake et al.	
2002/0160518	A1	10/2002	Hayenga et al.	
2003/0061687	A1	4/2003	Hansen et al.	
2003/0064414	A1	4/2003	Benecky et al.	
2003/0075446	A1	4/2003	Culbertson et al.	
2003/0089605	A1	5/2003	Timperman	
2003/0229376	A1	12/2003	Sandhu	
2003/0230486	A1	12/2003	Chien et al.	
2004/0005582	A1	1/2004	Shipwash	
2004/0037813	A1	2/2004	Simpson et al.	
2004/0181131	A1	9/2004	Maynard et al.	
2004/0188254	A1	9/2004	Spaid	
2004/0224419	A1	11/2004	Zheng et al.	436/69
2006/0094119	A1	5/2006	Ismagilov et al.	
2007/0026439	A1	2/2007	Faulstick et al.	
2009/0021728	A1	1/2009	Heinz et al.	
2009/0060797	A1	3/2009	Mathies et al.	
2010/0022414	A1	1/2010	Link et al.	

FOREIGN PATENT DOCUMENTS

EP	0 912 238	B1	10/2001
GB	2097692	A	11/1982
WO	WO 84/02000		5/1984
WO	WO 97/29508		8/1997
WO	WO 98/02237	A	1/1998
WO	WO 98/52691		11/1998
WO	WO 00/21666		4/2000
WO	WO 01/12327	A1	2/2001
WO	WO 01/64332	A1	9/2001
WO	WO 01/77683	A1	10/2001
WO	WO 02/12856	A1	2/2002
WO	WO 02/23163	A1	3/2002
WO	WO 02/25243	A1	3/2002
WO	WO 03/044221	A1	5/2003
WO	WO2004/038363		5/2004
WO	WO 2007/021343	A2	2/2007

OTHER PUBLICATIONS

Auroux, Pierre-Alain et al., "Micro Total Analysis Systems. 2. Analytical Standard Operations and Applications", *Analytical Chemistry*, vol. 74, No. 12, 2002, pp. 2637-2652.

Bico, Jose et al., "Rise of Liquids and Bubbles in Angular Capillary Tubes", *Journal of Colloid and Interface Science*, vol. 247, pp. 162-166, 2002.

Bico, Jose et al., "Self-Propelling Slugs", *J. Fluid Mech.*, vol. 467, pp. 101-127, 2002.

Bringer et al., "Microfluidic Systems for Chemical Kinetics That Rely on Chaotic Mixing in Droplets", *Phil. Trans. R. Soc. Lond.*, pp. 1-18, 2004.

Burns, J.R. et al., "The Intensification of Rapid Reactions in Multiphase Systems Using Slug Flow in Capillaries", *Lab on a Chip*, vol. 1, pp. 10-15, 2001.

Burns, Mark et al., "An Integrated Nanoliter DNA Analysis Device", *Science*, vol. 282, pp. 484-487, 1998.

Chan, Emory M. et al., "Size-Controlled Growth of CdSe Nanocrystals in Microfluidic Reactors", *Nano Letters*, vol. 3, No. 2, pp. 199-201, 2003.

Cho, Sung Kwon et al., "Splitting a Liquid Droplet for Electrowetting-Based Microfluidics", *Proceedings of 2001 ASME International Mechanical Engineering Congress and Exposition*, pp. 1-7, 2001.

Duff, David C. et al., "Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane)", *Analytical Chemistry*, vol. 70, pp. 4974-4984, 1998.

Edel, Joshua B. et al., "Microfluidic Routes to the Controlled Production of Nanoparticles", *Chemical Communications*, pp. 1136-1137, 2002.

Eggers, Jens et al., "Coalescence of Liquid Drops", *J. Fluid Mech.*, vol. 401, pp. 293-310, 1999.

Fowler, Jesse et al., "Enhancement of Mixing by Droplet-Based Microfluids", *2002 Institute of Electrical Engineers 15th International Conference on Micro Electro Mechanical Systems*, pp. 97-100, 2002.

Gerdts et al., "A Synthetic Reaction Network: Chemical Amplification Using Nonequilibrium Autocatalytic Reactions Coupled in Time", *J. Am. Chem. Soc.*, vol. 126, pp. 6327-6331, 2004.

Handique, K et al., "On-Chip Thermopneumatic Pressure for Discrete Drop Pumping", *Analytical Chemistry*, vol. 73, pp. 1831-1838, 2001.

Hansen, Carl L. et al., "A Robust and Scalable Microfluidic Metering Method That Allows Protein Crystal Growth by Free Interface Diffusion", *PNAS*, vol. 99, No. 26, pp. 16531-16536, 2002.

Harries, N. et al., "A Numerical Model for Segmented Flow in a Microreactor", *International Journal of Heat and Mass Transfer*, vol. 46, pp. 3313-3322, 2003.

Hosokawa, Kazuo et al., "Handling of Picoliter Liquid Samples in a Poly(dimethylsiloxane)- Based Microfluidic Device", *Analytical Chemistry*, vol. 71, No. 20, pp. 4781-4785, 1999.

Ismagilov, "Integrated Microfluidic Systems", *Angew. Chem. Int. Ed.*, vol. 42, pp. 4130-4132, 2003.

Knight, James B., "Hydrodynamic Focusing on a Silicon Chip: Mixing Nanoliters in Microseconds", *Physical Review Letters*, vol. 9, No. 2, pp. 190-197, 2000.

Liu, Robin H. et al., "Passive Mixing in a Three-Dimensional Serpentine Microchannel", *Journal of Microelectromechanical Systems*, vol. 9, No. 2, pp. 190-197, 2000.

McDonald J. Cooper et al., "Fabrication of Microfluidic Systems in Poly(dimethylsiloxane)", *Electrophoresis*, vol. 21, pp. 27-40, 2000.

Nisisako, Takasi et al., "Droplet Formation in a Microchannel Network", *Lab on a Chip*, vol. 2, pp. 24-26, 2002.

Nisisako, Takasi et al., "Formation of Droplets Using Branch Channels in a Microfluidic Circuit", *Sice*, pp. 1262-1264, Aug. 5-7, 2002.

Pabit, Suzette A. et al., "Laminar-Flow Fluid Mixer for Fast Fluorescence Kinetics Studies", *Biophysical Journal*, vol. 83, pp. 2872-2878, 2002.

Peng, Shuangjiu et al., "Controlled Production of Emulsions Using a Crossflow Membrane", *Particle & Particle Systems Characterization*, vol. 15, pp. 21-25, 1998.

Reyes, Darwin R. et al., "Micro Total Analysis Systems. 1. Introduction, Theory and Technology", *Analytic Chemistry*, vol. 74, No. 12, pp. 2623-2636, 2002.

Seong, Gi Hun et al., "Efficient Mixing and Reactions Within Microfluidic *Channels Using Microbead-Supported Catalysts", JACS Communications*, pp. 13360-13361, Published on the Web Oct. 17, 2002.

Seong, Gi Hun et al., "Fabrication of Microchambers Defined by Photopolymerized Hydrogels and Weirs Within Microfluidic Systems: Application to DNA Hybridization", *Analytical Chemistry*, vol. 74, No. 14, pp. 3372-3377, Jul. 15, 2002.

(56) **References Cited**

OTHER PUBLICATIONS

Shestopalov et al., "Multi-Step Synthesis of Nanoparticles Performed on Millisecond Time Scale in a Microfluidic Droplet-Based System", *The Royal Society of Chemistry*, vol. 4, pp. 316-321, 2004.

Song et al., "A Microfluidic System for Controlling Reaction Networks in Time", *Angew. Chem. Int. Ed.*, vol. 42, No. 7, pp. 768-772, 2003.

Song et al., "Experimental Test of Scaling of Mixing by Chaotic Advection in Droplets Moving Through Microfluidic Channels", *Applied Physics Letters*, vol. 83, No. 22, pp. 4664-4666, 2003.

Song et al., "Millisecond Kinetics on a Microfluidic Chip Using Nanoliters of Reagents", *J. Am. Chem. Soc.*, vol. 125, pp. 14613-14619, 2003.

Song, Helen et al., "A Microfluidic System for Controlling Reaction Networks in Time", *Angew. Chem. Int. Ed.*, vol. 42, No. 7, pp. 768-772, 2003.

Stroock, Abraham D. et al., "Chaotic Mixer for Microchannels", *Science*, vol. 295, pp. 647-651, 2002.

Sugiura, Shinji et al., "Effect of Channel Structure on Microchannel Emulsification", *Langmuir*, vol. 18, pp. 5708-5712, 2002.

Takayama et al., Patterning Cells and Their Environments Using Multiple Laminar Fluid Flows in Capillary Networks, *Proc. Natl. Acad. Sci. USA*, vol. 96, pp. 5545-5548, 1999.

Taniguchi, Tomohiro et al., "Chemical Reactions in Microdroplets by Electrostatic Manipulation of Droplets in Liquid Media", *Lab on a Chip*, vol. 2, pp. 19-23, 2002.

Thorsen et al., "Microfluidic Large-Scale Integration", *Science*, vol. 298, pp. 580-584, 2002.

Thorsen, Todd et al., "Dynamic Pattern Formation in a Vesicle-Generating Microfluidic Device", *Physical Review Letters*, vol. 86, No. 18, pp. 4163-4166, 2001.

Tice et al. "Formation of Droplets and Mixing in Multiphase Microfluidics at Low Values of the Reynolds and the Capillary Numbers", *Langmuir*, vol. 19, pp. 9127-9133, 2003.

Tice, "Effects of Viscosity on Droplet Formation and Mixing in Microfluidic Channels", *Analytica Chimica Acta*, vol. 507, pp. 73-77, 2004.

Tokeshi, Manabu et al., "Continuous-Flow Chemical Processing on a Microchip by Combining Microunit Operations and Multiphase Flow Network", *Analytical Chemistry*, vol. 74, No. 7, pp. 1565-1571, 2002.

Umbanhowar, P.B. et al., "Monodisperse Emulsion Generation Via Drop Break Off in a Coflowing Stream", *Langmuir*, vol. 16, pp. 347-351, 2000.

Wang, Hongzhi et al., "Preparation of Titania Particles Utilizing the Insoluble Phase Interface in a Microchannel Reactor", *Chemical Communications*, pp. 1462-1463, 2002.

Zhao, Bin et al., "Control and Applications of Immiscible Liquids in Microchannels", *J. Am. Chem. Soc.*, vol. 124, pp. 5284-5285, 2002.

Zheng et al., "A Droplet-Based, Composite PDMS/Glass Capillary Microfluidic System for Evaluating Protein Crystallization Conditions by Microbatch and Vapor-Diffusion Methods with On-Chip X-Ray Diffraction", *Angew. Chem.*, pp. 1-4, 2004.

Zheng et al., "Screening of Protein Crystallization Conditions on a Microfluidic Chip Using Nanoliter-Size Droplets", *Journal of the American Chemical Society*, vol. 125, No. 37, pp. 11170-11171, 2003.

Chayen, "Crystallization with oils: a new dimension in macromolecular crystal growth" *Journal of Crystal Growth 196* (1999), pp. 434-441.

Torkkeli A. et al., "Droplet Manipulation on a Superhydrophobic Surface for Microchemical Analysis", *11th International Conference on Solid-State Sensors and Actuators. Digest of Technical Papers*, Munich, Jun. 10-14, 2001; vol. 2, pp. 1150-1153.

Tanaka, H. et al., "*Ethanol Production from Starch by a Coimmobilized Mixed Culture System of Aspergillus awamori and Zymomonas mobilis*", Biotechnology and Bioengineering, vol. XXVII, 1986, pp. 1761-1768.

De-Bashan, L. E. et al., "*Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae Chlorella vulgaris coimmobilized in alginate beads with the microalgae growth-promoting bacterium Azospirillum brasilense*", Water Research 36 (2002), pp. 2941-2948.

Goodall, J. L. et al., "*Operation of Mixed-Culture Immobilized Cell Reactors for the Metabolism of Meta- and Para-Nitrobenzoate by Comamonas Sp. JS46 and Comamonas Sp. JS47*", Biotechnology and Bioengineering, vol. 59, No. 1, Jul. 5, 1998, pp. 21-27.

He, Mingyan et al., "*Selective Encapsulation of Single Cells and Subcellular Organelles into Picoliter- and Femtoliter-Volume Droplets*", Analytical Chemistry, vol. 77, No. 6, Mar. 15, 2005, pp. 1539-1544.

Huebner, A. et al., "*Quantitative detection of protein expression in single cells using droplet microfluidics*", Chem. Commun, 2007, pp. 1218-1220.

Seong, G.H. et al., "*Fabrication of Microchambers Defined by Photopolymerized Hydrogels and Weirs within Microfluidic Systems: Application to DNA Hybridization*", Analytical Chemistry, vol. 74, No. 14, Jul. 15, 2002, pp. 3372-3377.

Seong G.H. et al., "*Efficient Mixing and reactions within Microfluidic Channels Using Microbead-Supported Catalysts*", J. Am. Chem. Soc. 2002, 124, pp. 13360-13361.

Nisisako, T. et al., "*Formation of Droplets Using Branch Channels in a Microfluidic Circuit*", SICE 2002, Aug. 5-7, 2002, Osaka, pp. 1262-1264.

Chayen, N.E., "Crystallization with oils: a new dimension in macromolecular crystal growth", Journal of Crystal Growth 196 (1999), pp. 434-441.

Titomanlio et al., "*Capillary experiments of flow induced crystallization of HDPE*", AlChe Journal, 1990, v36, No. 1, pp. 13-18.

Garcia-Ruiz et al., "*Investigation on protein crystal growth by the gel acupuncture method*", Acta, Cryst., 1994, D50, 99. pp. 484-490.

Sugiura et al., "*Interfacial tension driven monodispersed droplet formation from microfabricated channel array*", Langmuir, 2001, v17, pp. 5562-5566.

Garcia-Ruiz et al. "*A super-saturation wave of protein crystallization*", J. Crystal Growth, 2001, v232, pp. 149-155.

Ng et al., "*Protein crystallization by capillary counter-diffusion for applied crystallographic structure determination*", J. Struct. Biol. 2003, v142, pp. 218-231.

Beer et al., "*On-Chip, Real-Time, Single-Copy Polymerase Chain Reaction in Picoliter Droplets*", Anal. Chem., 2007, v. 79, pp. 8478475.

Burns et al., "*Microfabricated structures for integrated DNA analysis*", Proc. Natl. Acad. Sci. USA, May 1996, vol. 93, pp. 5556-5561.

Pollack et al., "*Electrowetting-based actuation of droplets for integrated microfluidics*", Lab Chip, 2002, v. 2, pp. 96-101.

Vogelstein, et al., "*Digital PCR*", Proc. Natl. Acad. Sci. USA, Aug. 1999, vol. 96, pp. 9236-9241, Genetics.

Zheng et al., "*Formation of Droplets of Alternating Composition in Microfluidic Channels and Applications to Indexing of Concentrations in Droplet-Based Assays*", Anal. Chem., 2004, v. 76, pp. 4977-4982.

Atencia, Javier, et al. "*Controlled Microfluidic Interfaces*", Nature, 2005, vol. 437, No. 29, pp. 648-655.

Gu, Hao, et al., "*Droplets Formation and Merging in Two-Phase Flow Microfluidics*", Int. J. Mol. Sci., 2011, vol. 12, pp. 2572-2597.

Shestopalov, Ilya, et al., "*Multi-Step Synthesis of Nanoparticles Performed on Millisecond Time Scale in a Microfluidic Droplet-Based System*", Lab-Chip, 2004, vol. 4, pp. 316-321.

Thorsen, Todd, et al., "*Dynamic Pattern Formation in a Vesicle-Generating Microfluidic Device*", Phys. Rev. Lett., 2001, vol. 86, No. 18, pp. 4163-4166.

Kalinina, Olga, et al., "*Nanoliter Scale PCR with TaqMan Detection*," Nucleic Acids Research, vol. 25, No. 10, (1997), pp. 1999-2004.

* cited by examiner

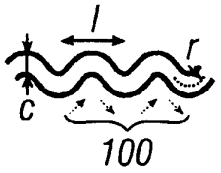

FIG. 1A

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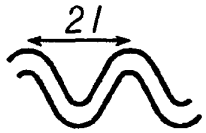


FIG. 1B-2

FIG. 1B-3

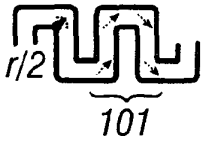


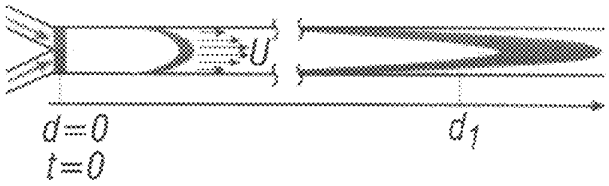
FIG. 1B-4

FIG. 1C-1

FIG. 1C-2

FIG. 1C-3

FIG. 1C-4



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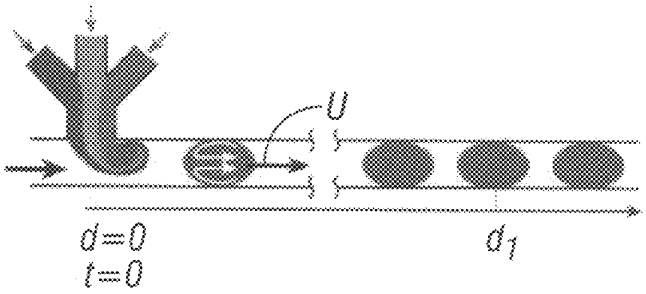

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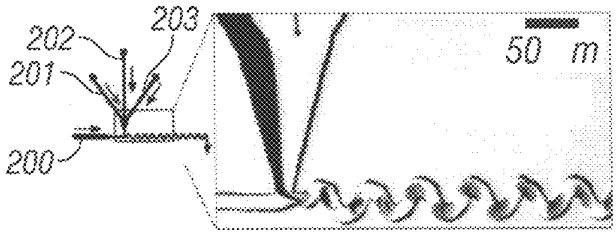

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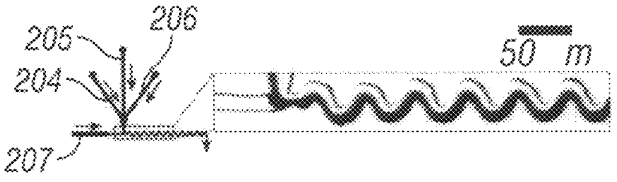

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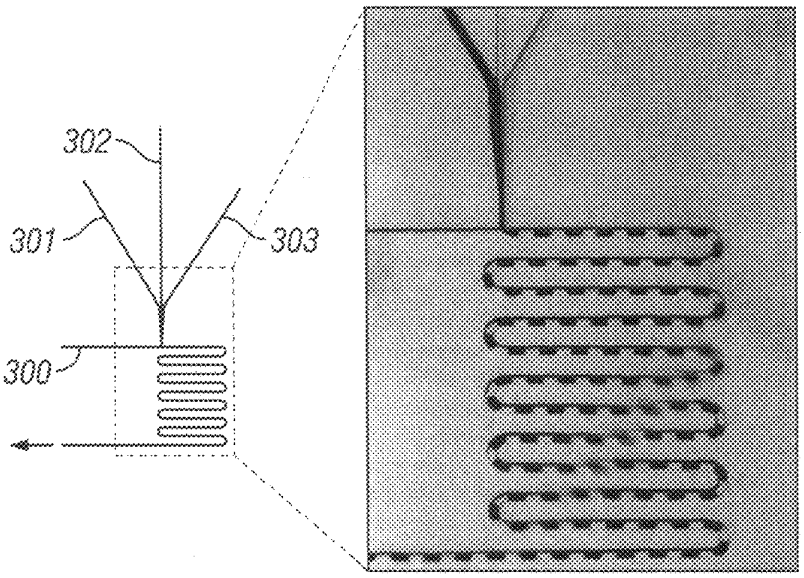

FIG. 3A

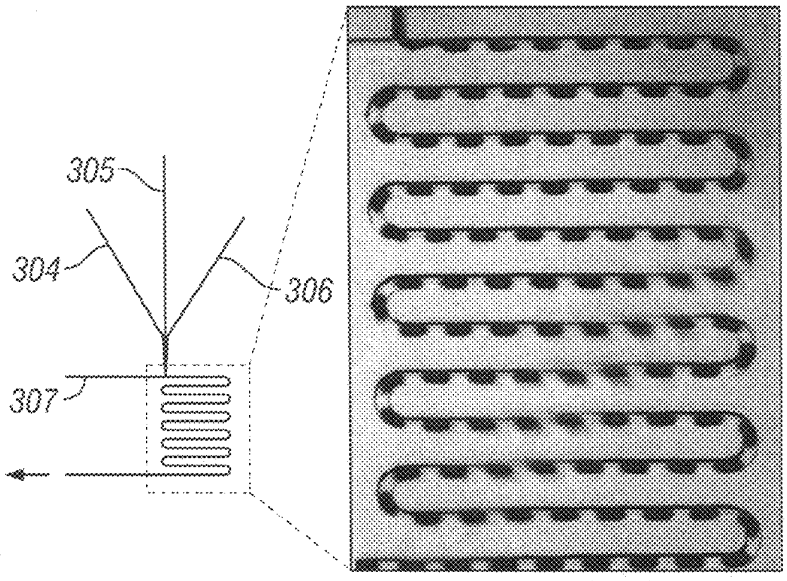

FIG. 3B

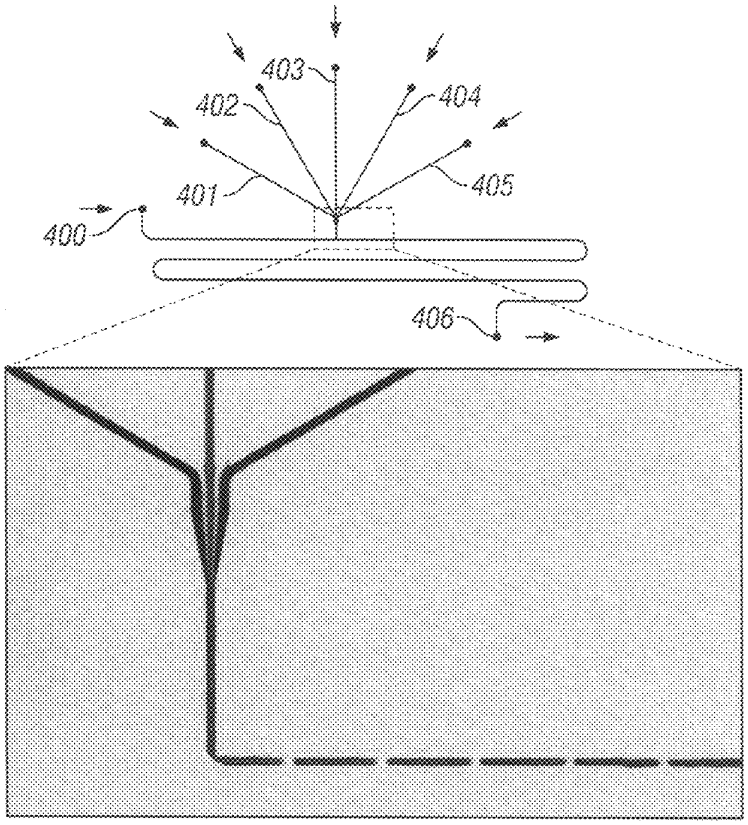

FIG. 4

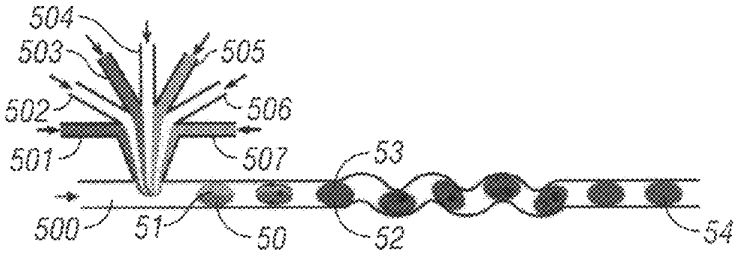
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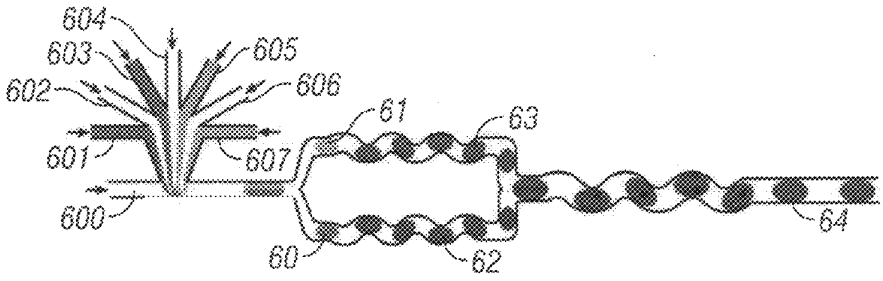
FIG. 5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62

FIG. 6

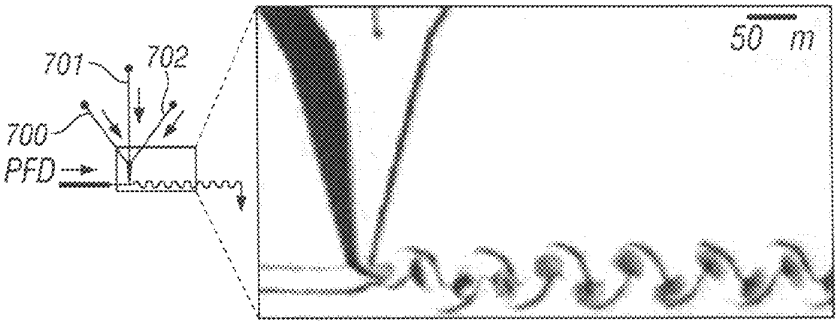


FIG. 7A

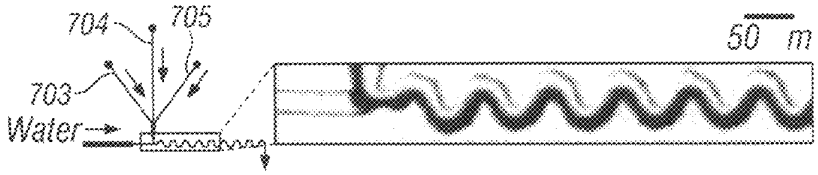


FIG. 7B

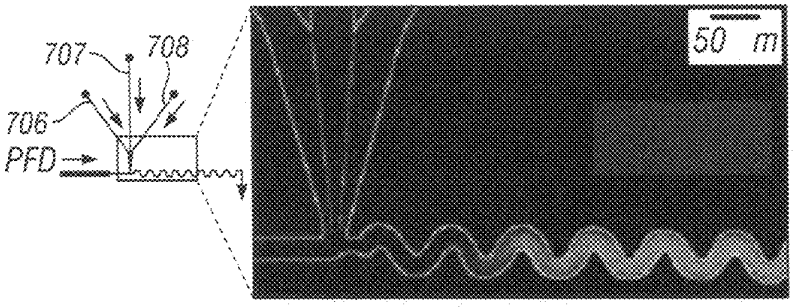


FIG. 7C

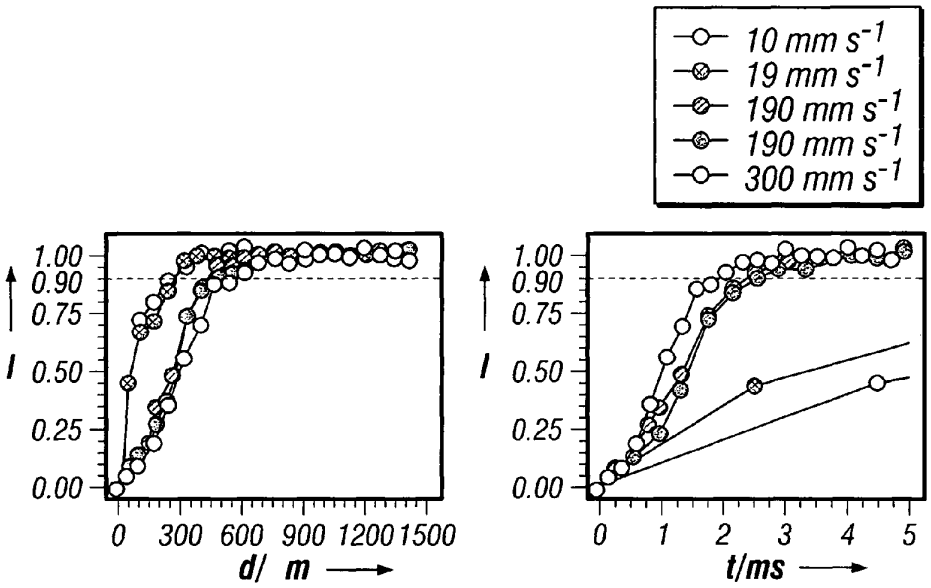


FIG. 7D(i)

FIG. 7D(ii)

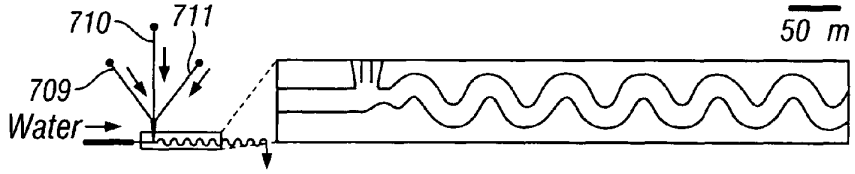


FIG. 7E

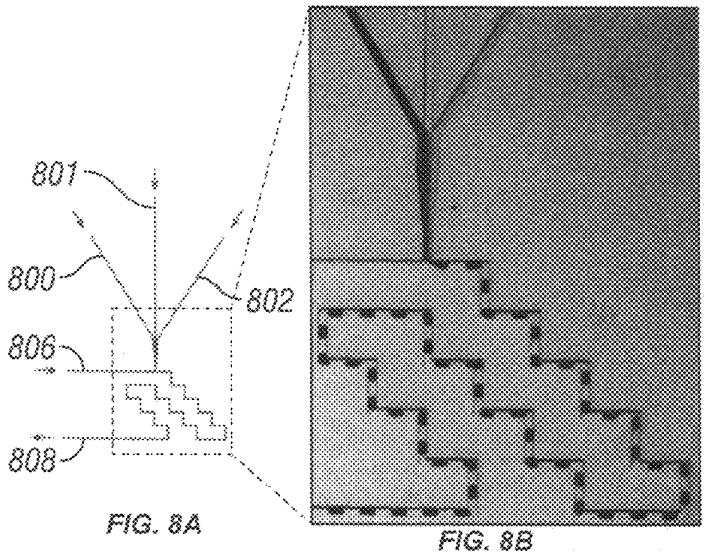


FIG. 8A

FIG. 8B

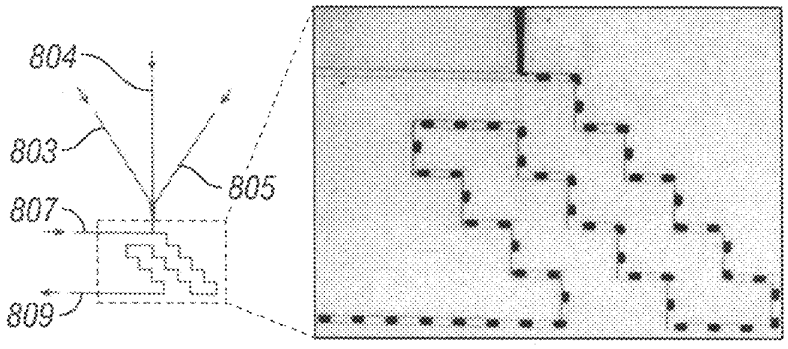


FIG. 8C

FIG. 8D

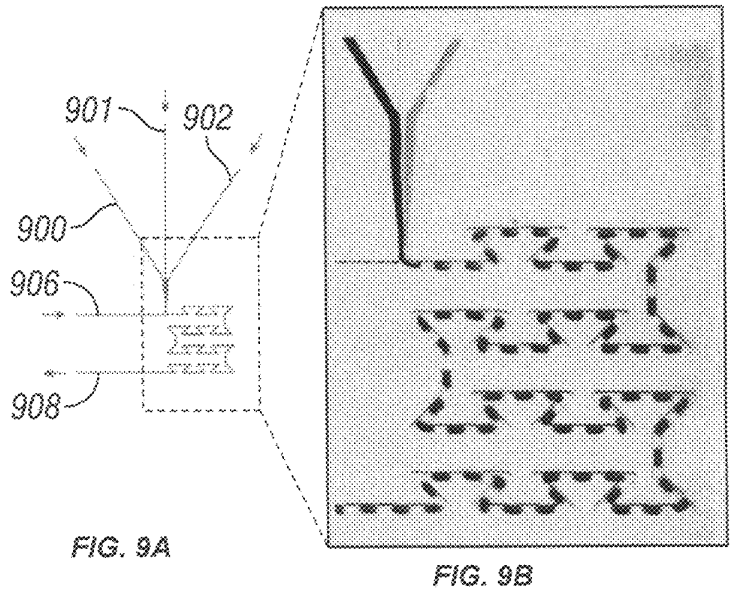


FIG. 9A

FIG. 9B

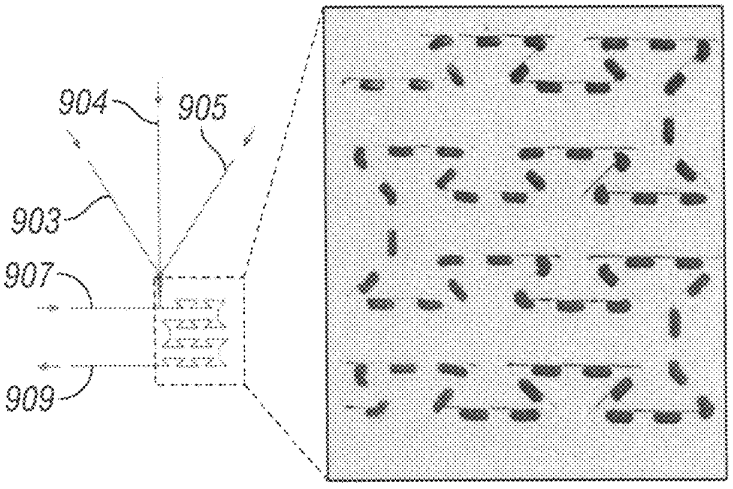


FIG. 9C

FIG. 9D

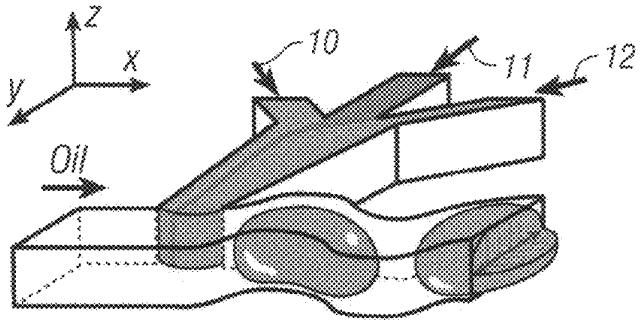


FIG. 10A

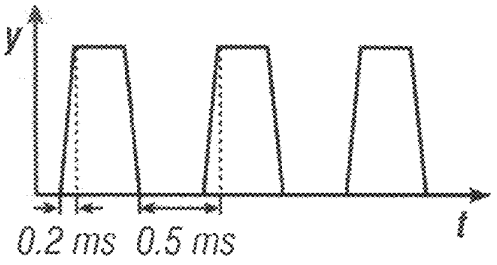


FIG. 10B

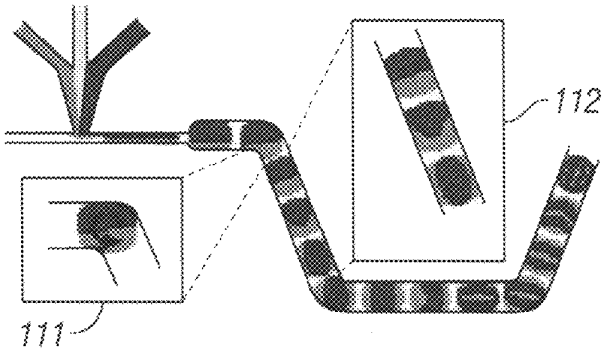


FIG. 11

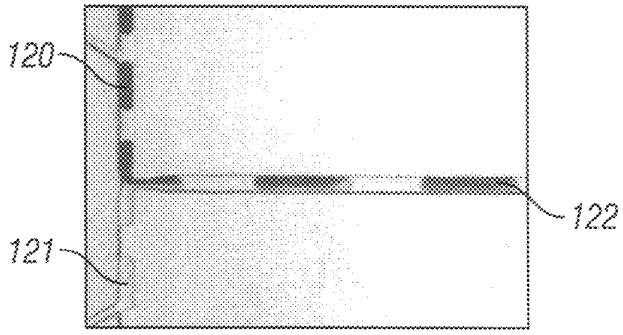


FIG. 12A

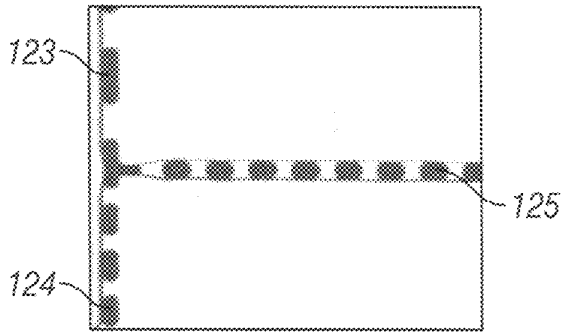


FIG. 12B

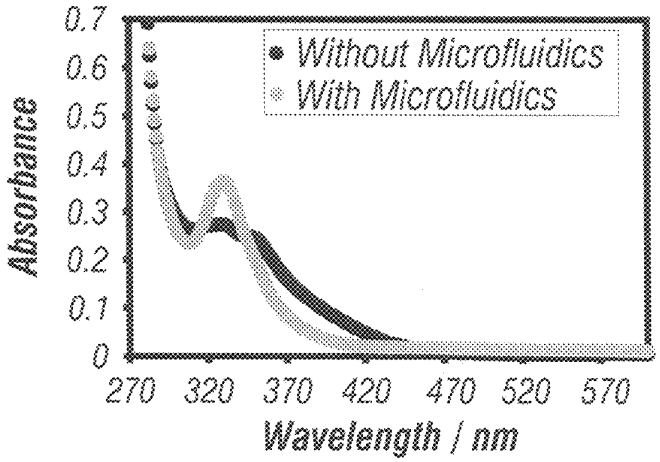


FIG. 13

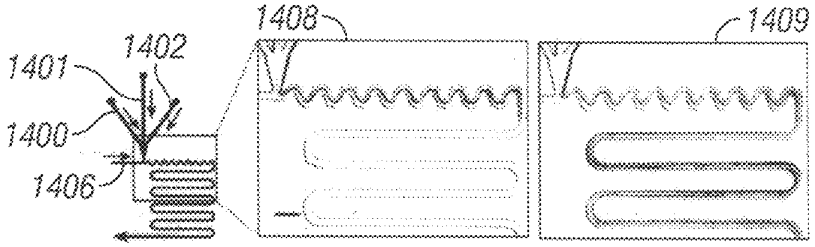


FIG. 14A(i) FIG. 14A(ii) FIG. 14A(iii)

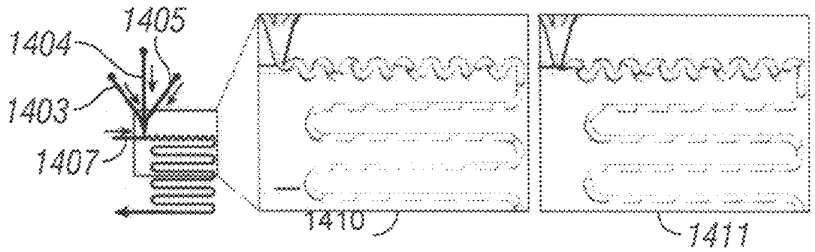


FIG. 14B(i) FIG. 14B(ii) FIG. 14B(iii)

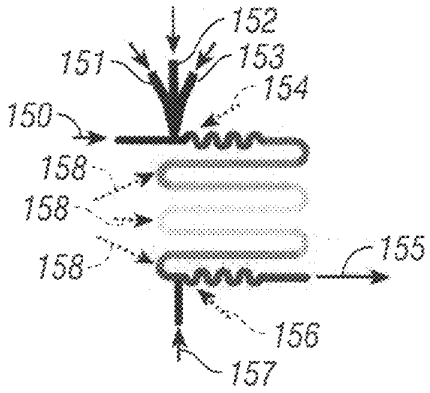


FIG. 15

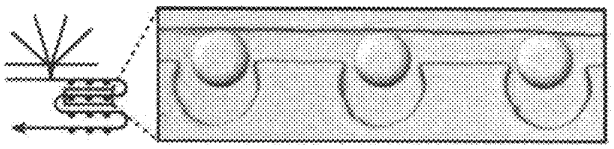


FIG. 16

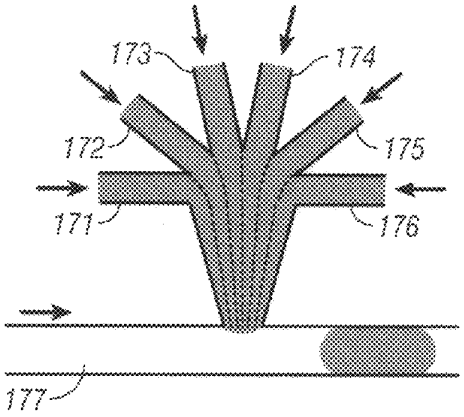


FIG. 17

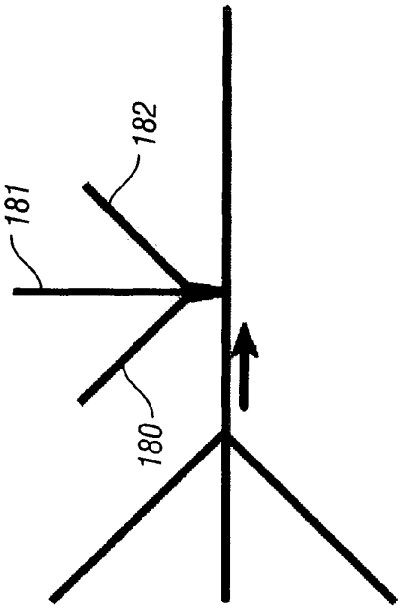


FIG. 18B

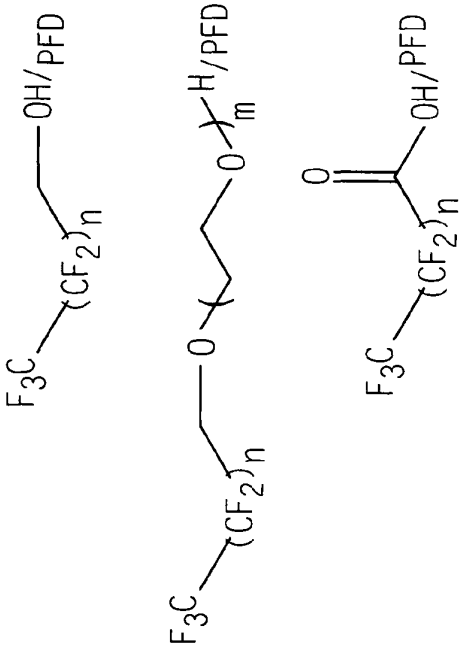


FIG. 18A

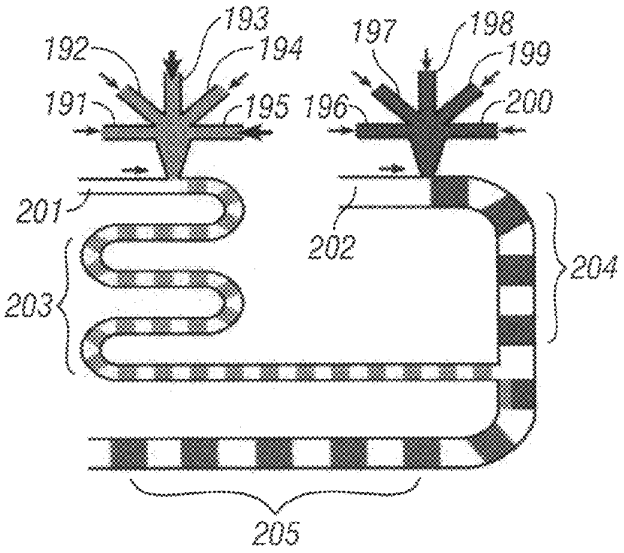


FIG. 19

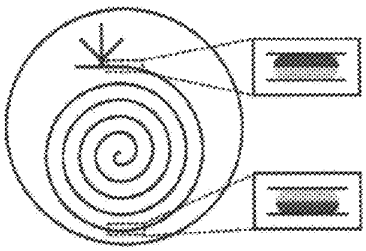


FIG. 20A

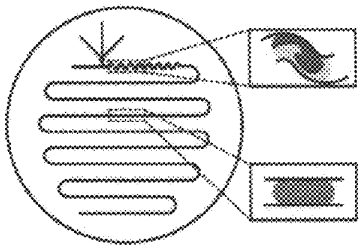


FIG. 20B

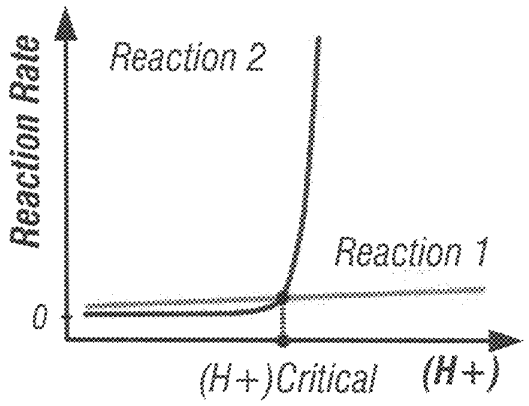


FIG. 21

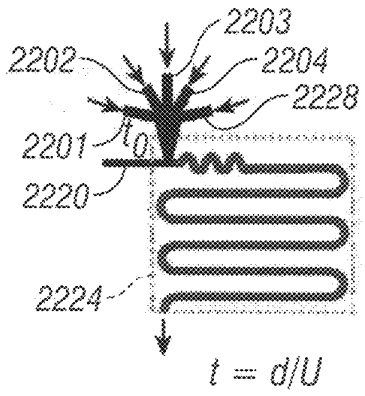
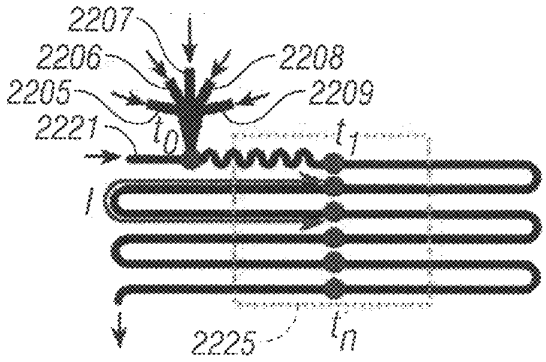
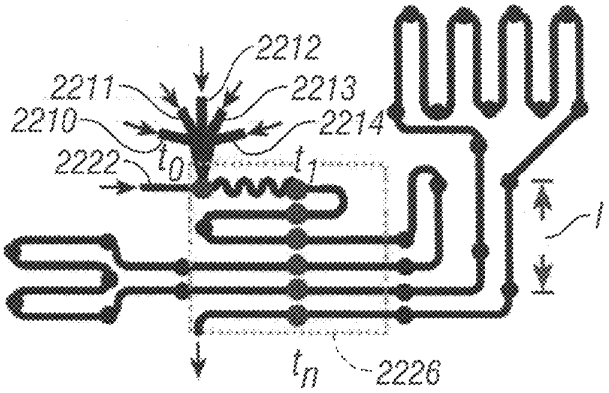


FIG. 22A



$t_n = (n*l)/U$

FIG. 22B



$t_n = (2^{(n-1)}*l)/U$

FIG. 22C

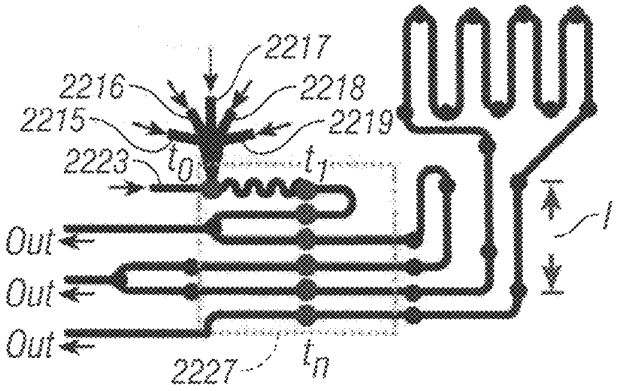
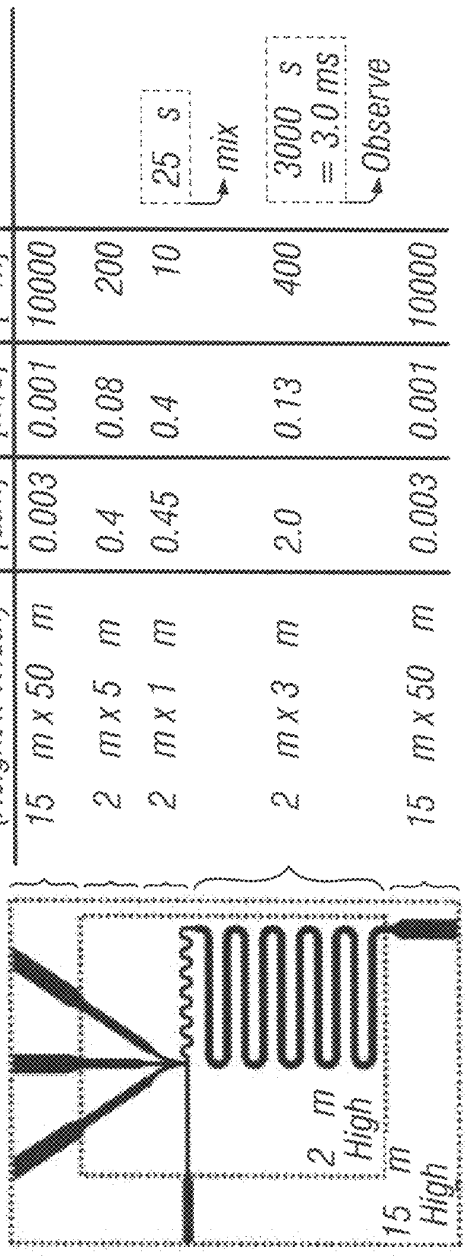


FIG. 22D



Size of Channel (Height x Width)	P [atm]	U [m/s]	Length [m]	Time
15 m x 50 m	0.003	0.001	10000	
2 m x 5 m	0.4	0.08	200	
2 m x 1 m	0.45	0.4	10	25 s → mix
2 m x 3 m	2.0	0.13	400	3000 s = 3.0 ms → Observe
15 m x 50 m	0.003	0.001	10000	

FIG. 23

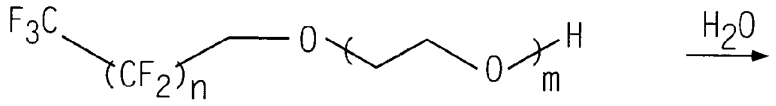


FIG. 24A

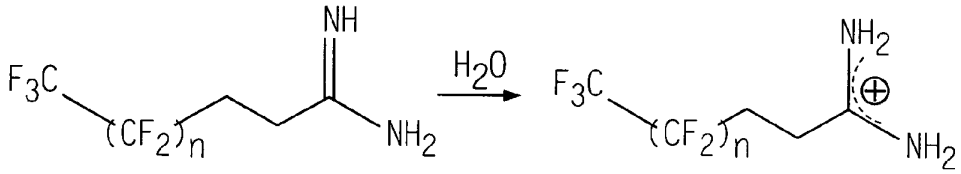


FIG. 24B

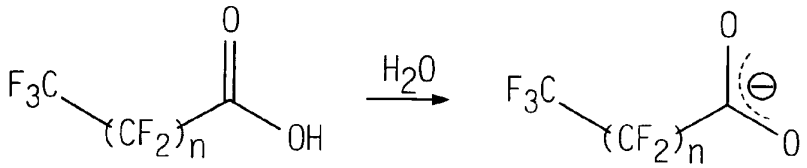


FIG. 24C

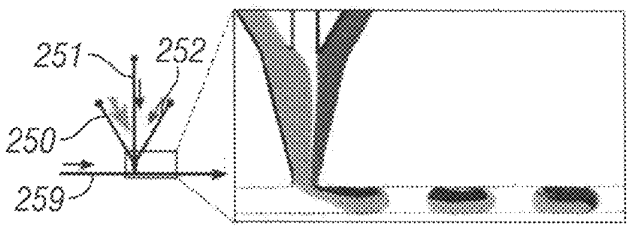


FIG. 25A

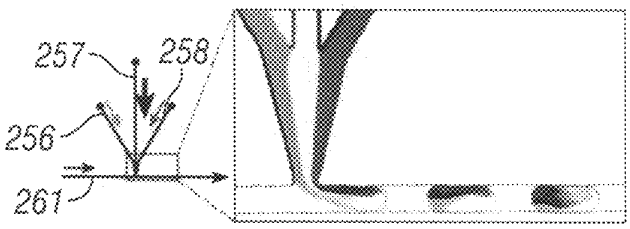


FIG. 25B

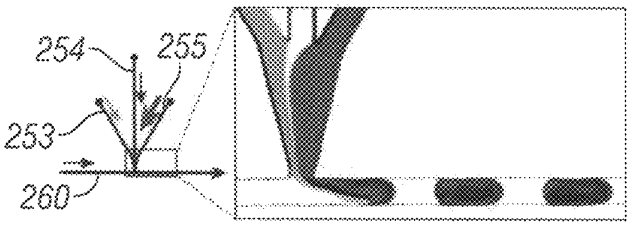


FIG. 25C

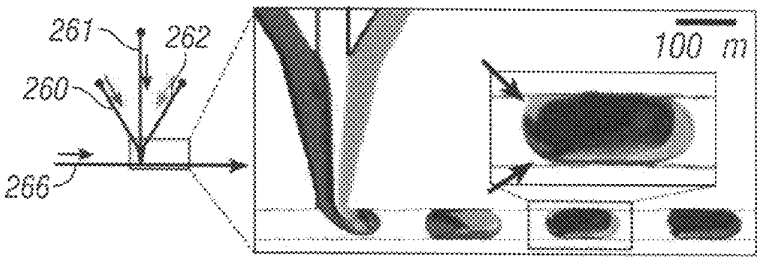


FIG. 26A

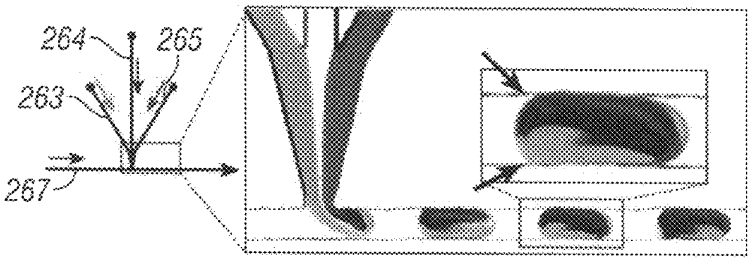


FIG. 26B

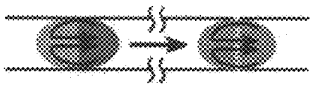


FIG. 27A-1

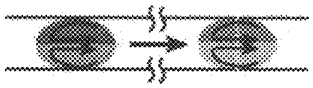


FIG. 27A-2

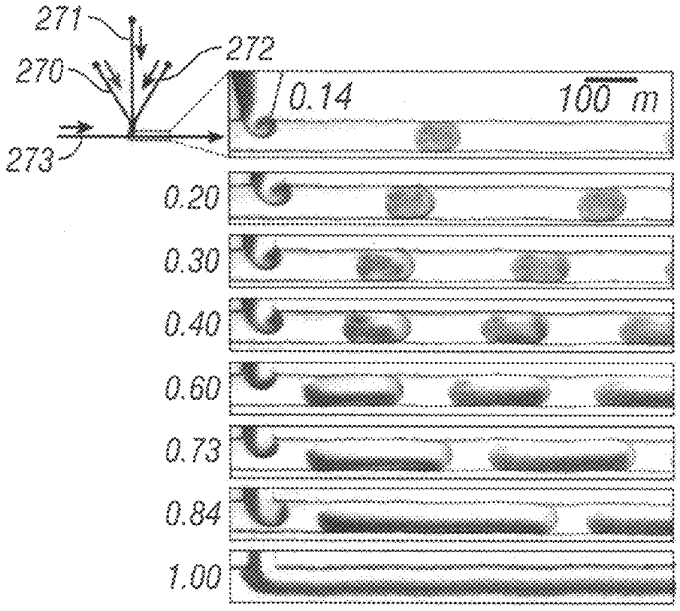


FIG. 27B

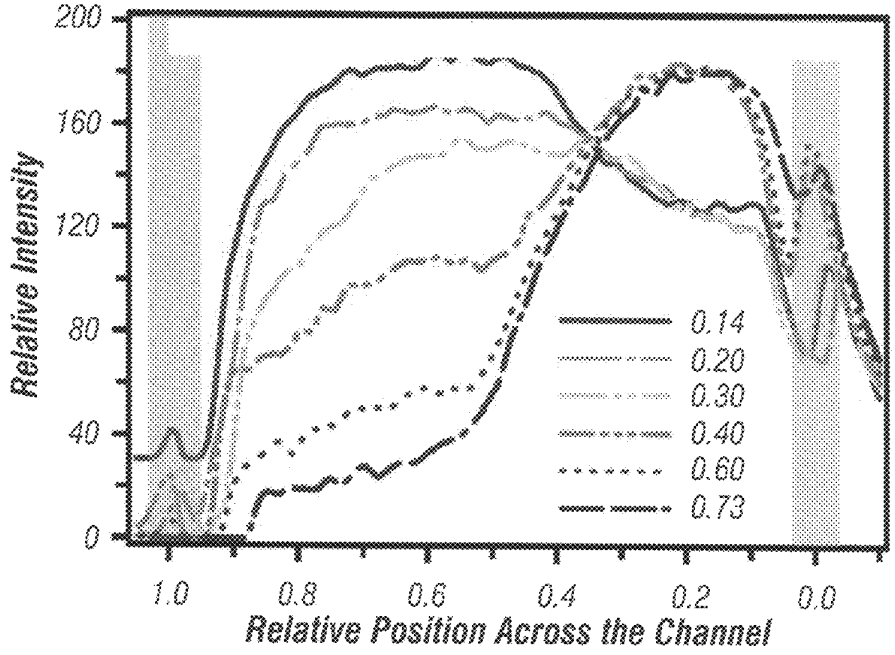


FIG. 27C-1

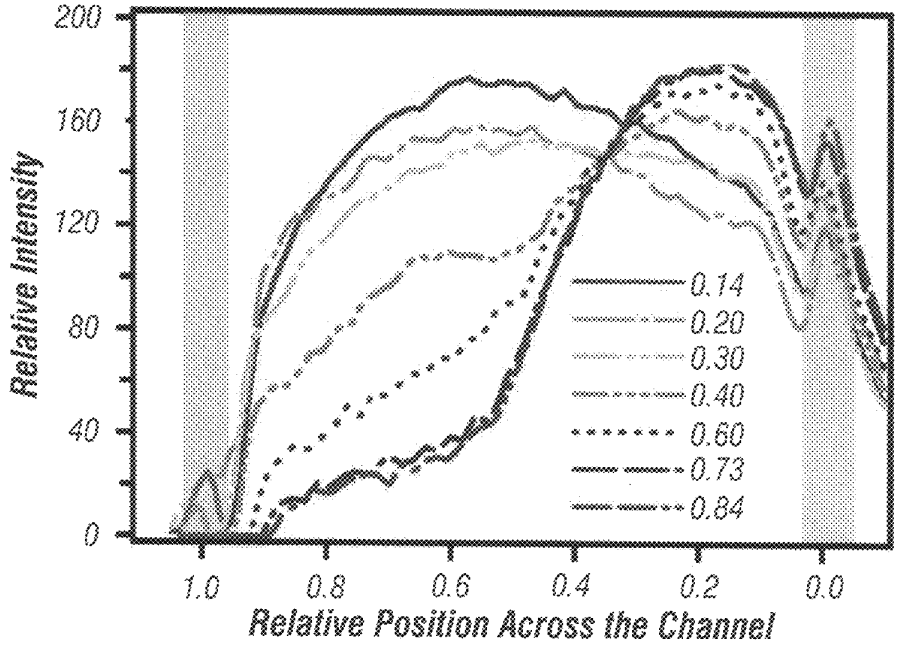


FIG. 27C-2

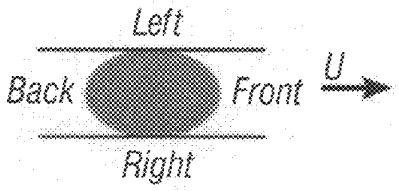


FIG. 28

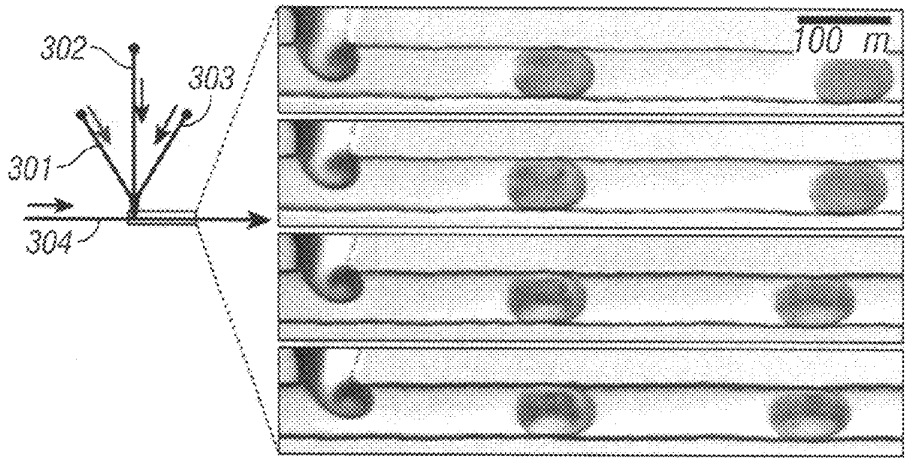


FIG. 30

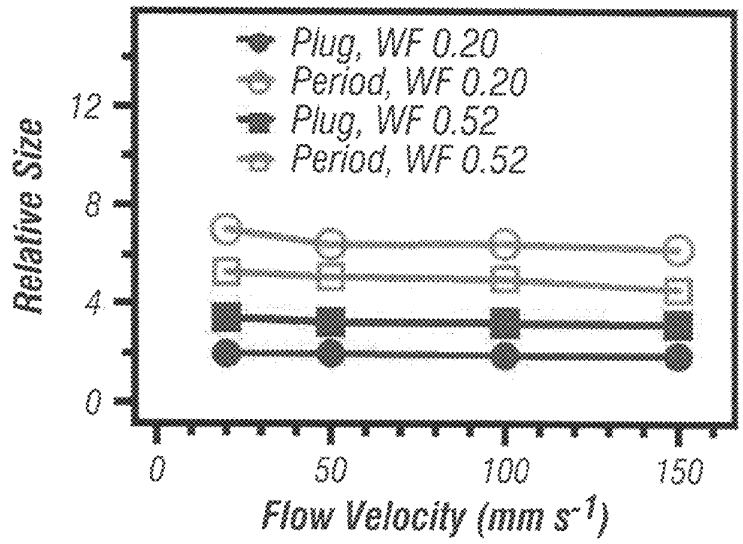


FIG. 29A

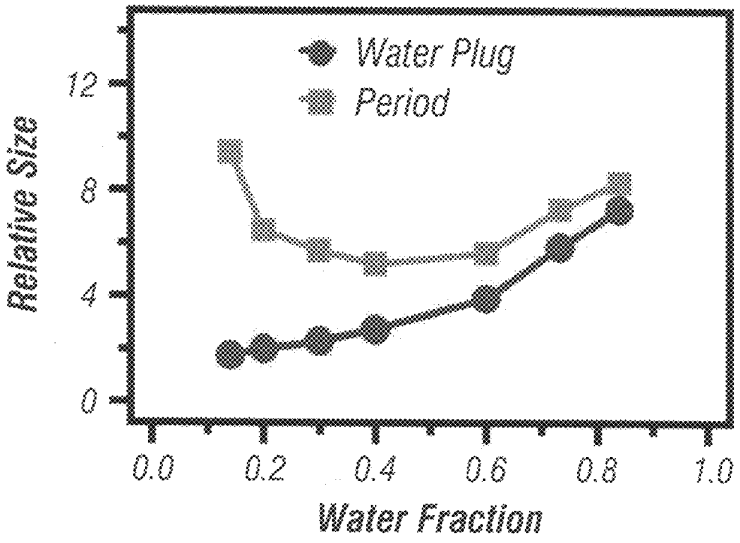


FIG. 29B

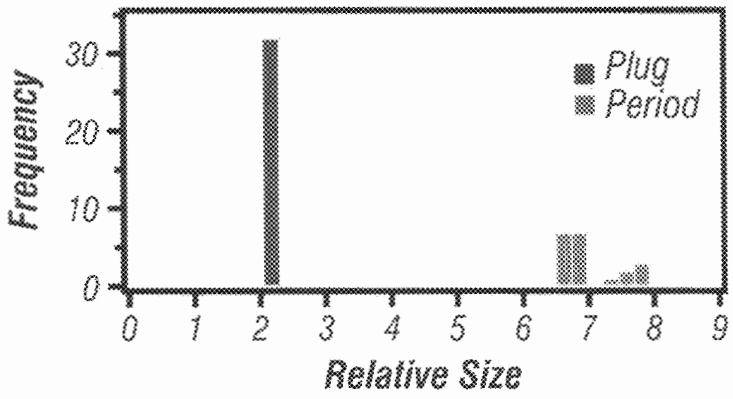


FIG. 31A

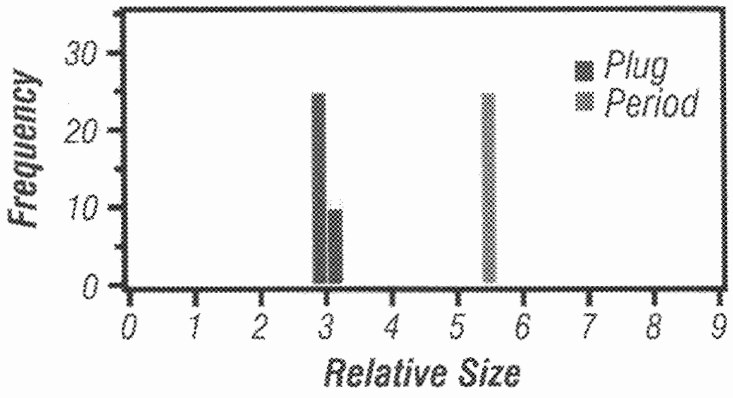


FIG. 31B

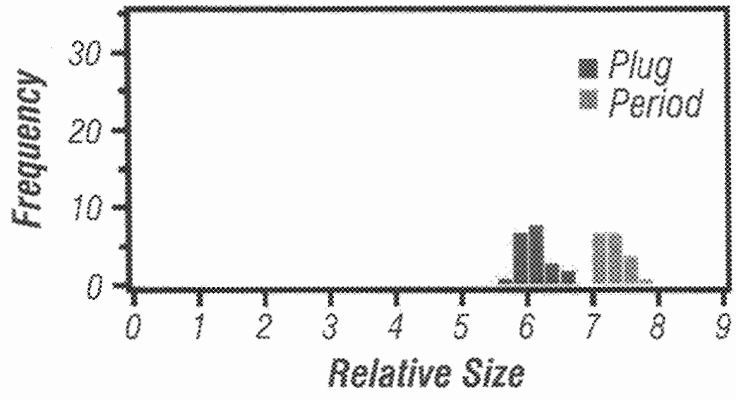


FIG. 31C

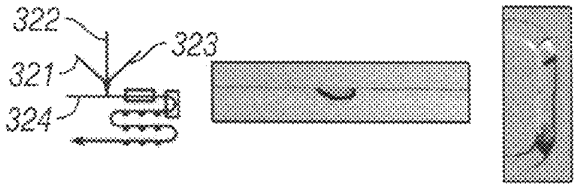


FIG. 32

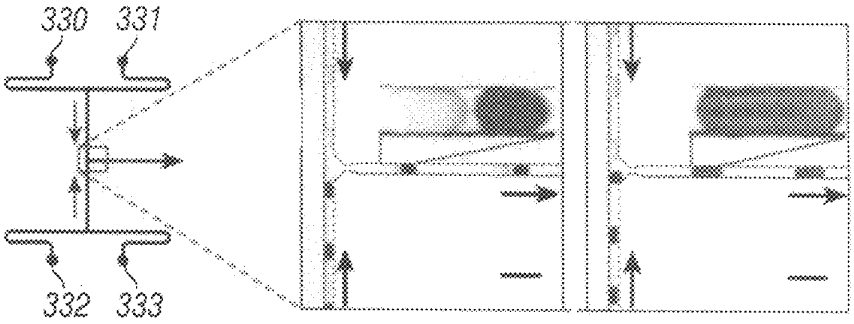


FIG. 33A

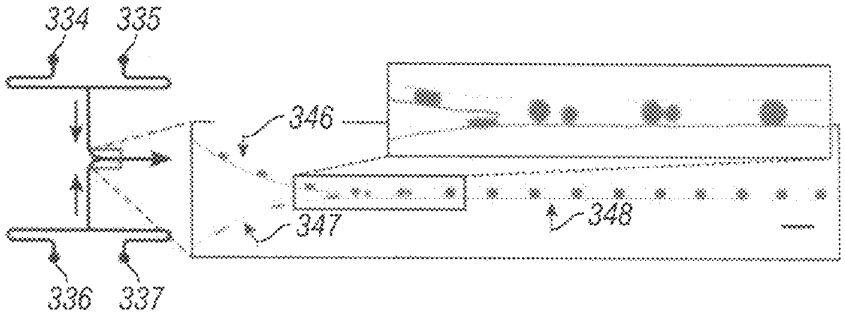


FIG. 33B

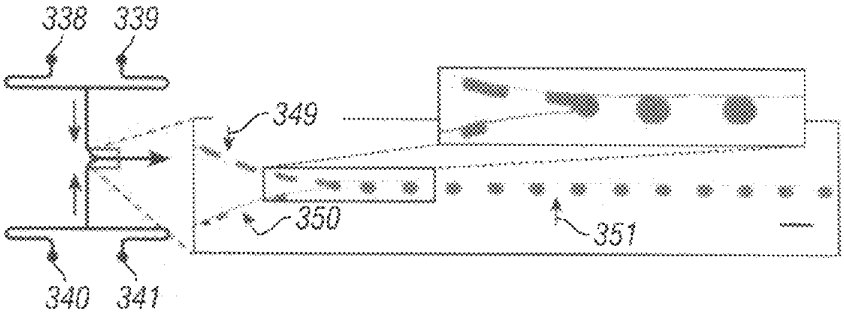


FIG. 33C

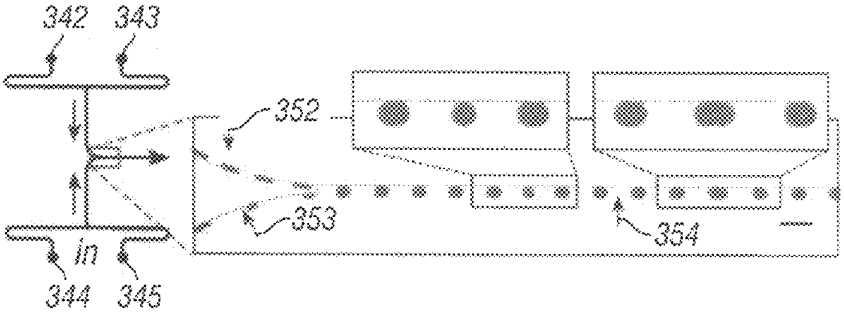


FIG. 33D

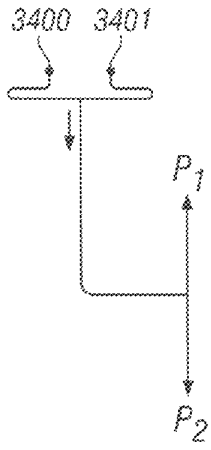


FIG. 34A

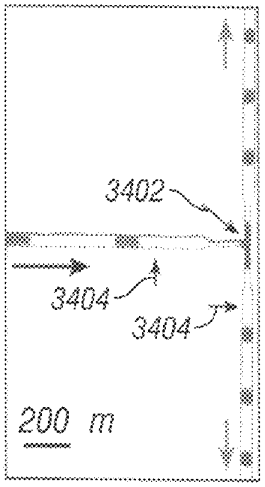


FIG. 34B

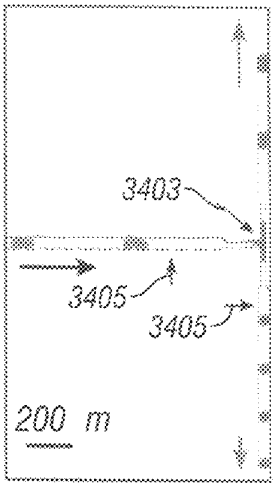


FIG. 34C

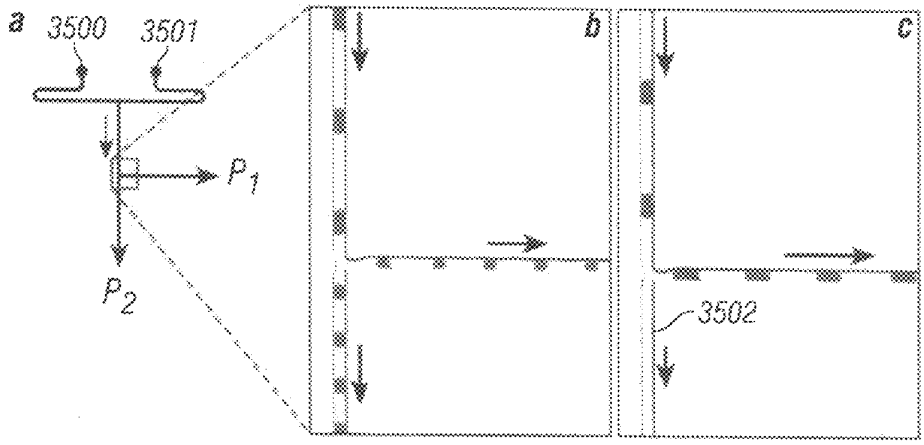


FIG. 35

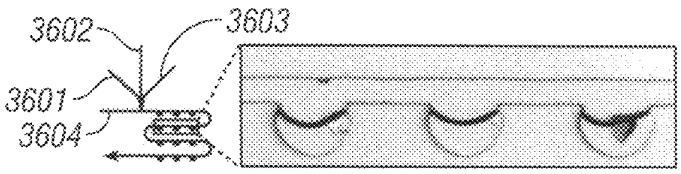
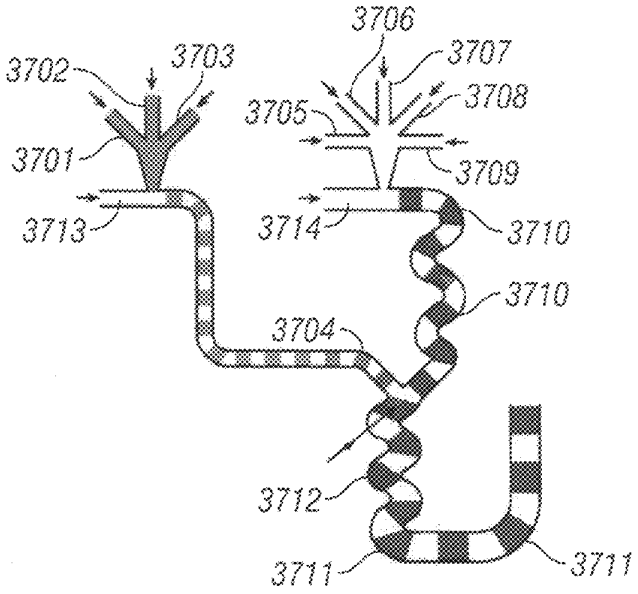

3602
3603
3601
3604

FIG. 36


3706
3702
3703
3707
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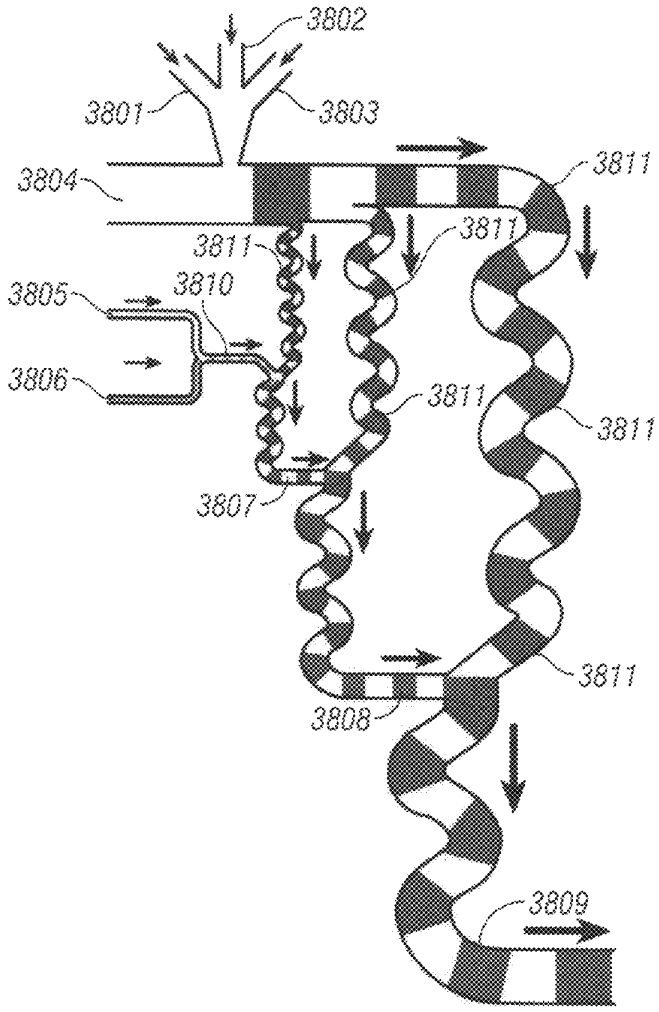
3704
3712
3711
3711

FIG. 37


3802
3801
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3811
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3808
3809

FIG. 38

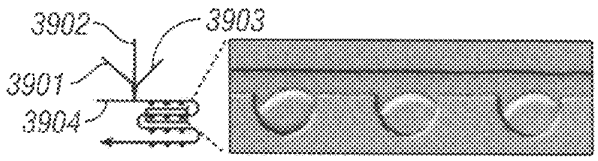
3902
3903
3901
3904

FIG. 39

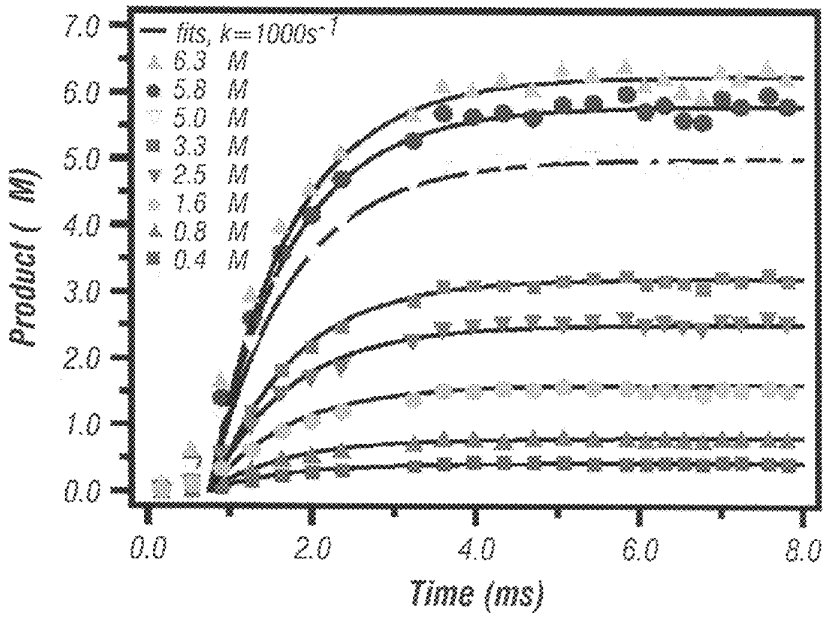
fits, k=1000s-1
6.3 M
5.8 M
5.0 M
3.3 M
2.5 M
1.6 M
0.8 M
0.4 M
Product (M)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.0
2.0
4.0
6.0
8.0
Time (ms)

FIG. 40

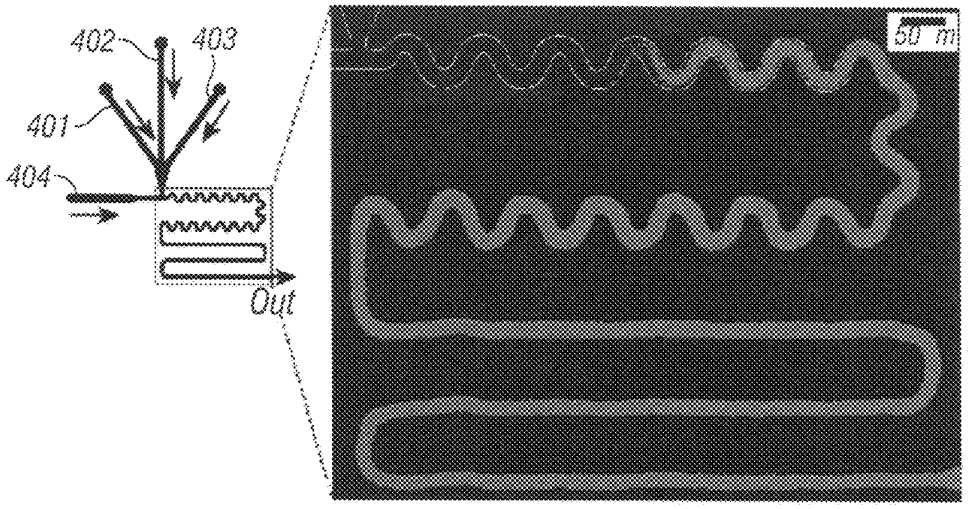

FIG. 40A

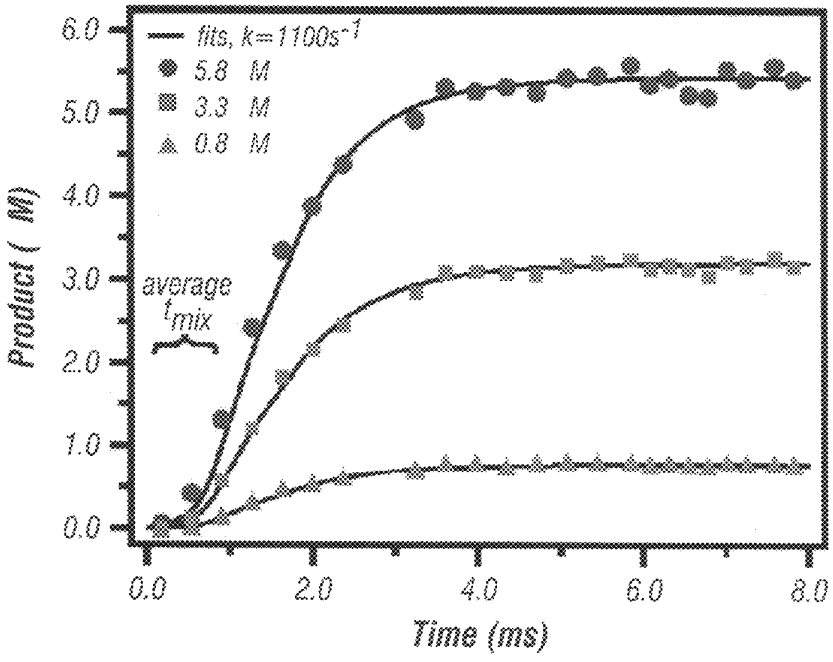

FIG. 40B

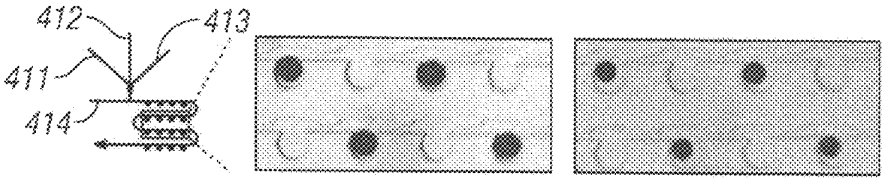

FIG. 41A FIG. 41B FIG. 41C

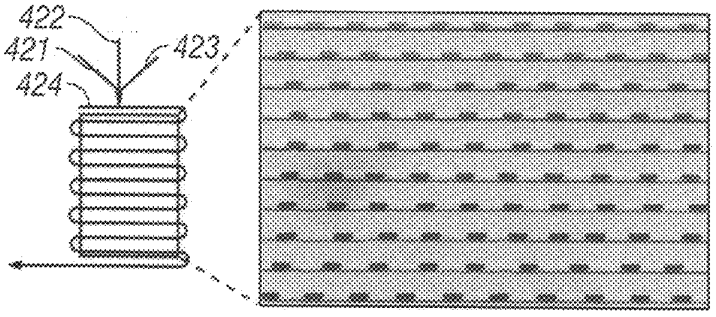

FIG. 42

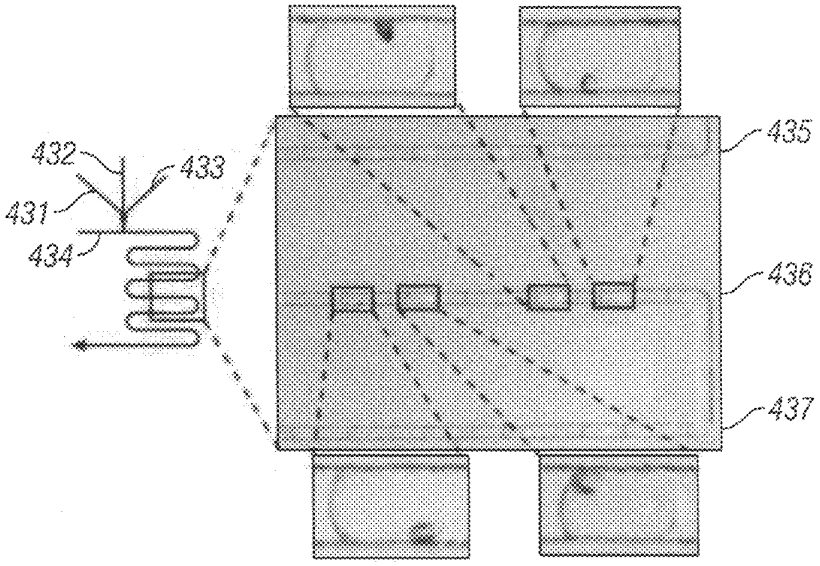

FIG. 43

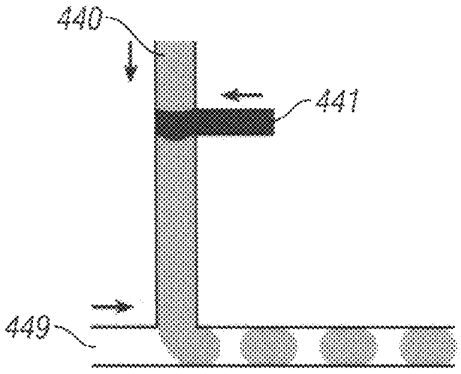

FIG. 44A

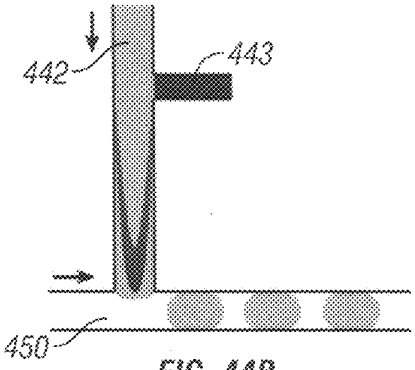

FIG. 44B

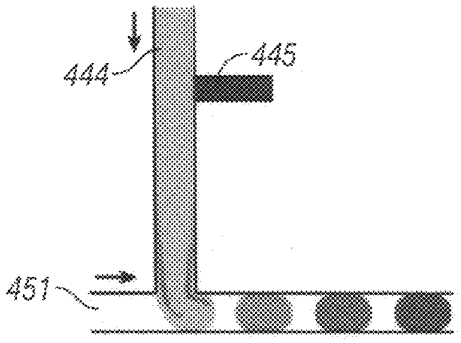

FIG. 44C

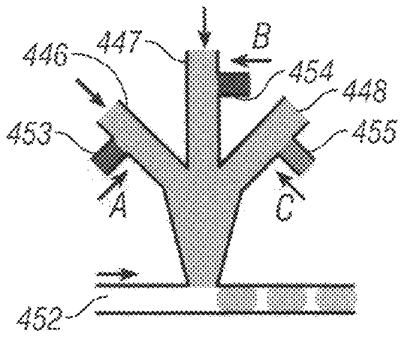

FIG. 44D

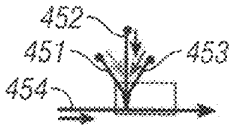

FIG. 45A

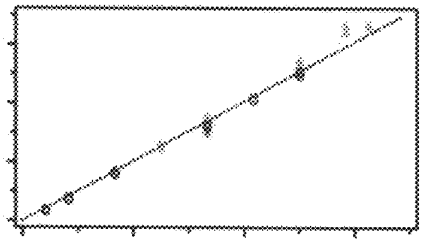

FIG. 45B

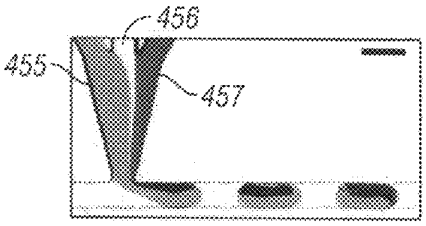

FIG. 45C

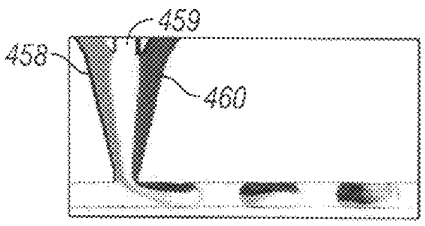

FIG. 45D

DEVICE AND METHOD FOR PRESSURE-DRIVEN PLUG TRANSPORT AND REACTION

This application is a divisional of U.S. application Ser. No. 10/434,970, filed on May 9, 2003 (issued as U.S. Pat. No. 7,129,091), which claimed priority to U.S. Provisional Application No. 60/379,927, filed on May 9, 2002 and which also claimed priority to U.S. Provisional Application No. 60/394,544 filed on Jul. 8, 2002, the entirety of which are incorporated herein by reference.

BACKGROUND

Nonlinear dynamics, in conjunction with microfluidics, play a central role in the design of the devices and the methods according to the invention. Microfluidics deals with the transport of fluids through networks of channels, typically having micrometer dimensions. Microfluidic systems (sometimes called labs-on-a-chip) find applications in microscale chemical and biological analysis (micro-total-analysis systems). The main advantages of microfluidic systems are high speed and low consumption of reagents. They are thus very promising for medical diagnostics and high-throughput screening. Highly parallel arrays of microfluidic systems are used for the synthesis of macroscopic quantities of chemical and biological compounds, e.g., the destruction of chemical warfare agents and pharmaceuticals synthesis. Their advantage is improved control over mass and heat transport.

Microfluidic systems generally require means of pumping fluids through the channels. In the two most common methods, the fluids are either driven by pressure or driven by electroosmotic flow (EOF). Flows driven by EOF are attractive because they can be easily controlled even in complicated networks. EOF-driven flows have flat, plug-like velocity profile, that is, the velocity of the fluid is the same near the walls and in the middle of the channel. Thus, if small volumes of multiple analytes are injected sequentially into a channel, these plugs are transported as non-overlapping plugs (low dispersion), in which case the dispersion comes mostly from the diffusion between plugs. A main disadvantage of EOF is that it is generated by the motion of the double layer at the charged surfaces of the channel walls. EOF can therefore be highly sensitive to surface contamination by charged impurities. This may not be an issue when using channels with negative surface charges in DNA analysis and manipulation because DNA is uniformly negatively charged and does not adsorb to the walls. However, this can be a serious limitation in applications that involve proteins that are often charged and tend to adsorb on charged surfaces. In addition, high voltages are often undesirable, or sources of high voltages such as portable analyzers may not be available.

Flows driven by pressure are typically significantly less sensitive to surface chemistry than EOF. The main disadvantage of pressure-driven flows is that they normally have a parabolic flow profile instead of the flat profile of EOF. Solutes in the middle of the channel move much faster (about twice the average velocity of the flow) than solutes near the walls of the channels. A parabolic velocity profile normally leads to high dispersion in pressure-driven flows; a plug of solute injected into a channel is immediately distorted and stretched along the channel. This distortion is somewhat reduced by solute transport via diffusion from the middle of the channel towards the walls and back. But the distortion is made worse by diffusion along the channel (the overall dispersion is known as Taylor dispersion).

Taylor dispersion broadens and dilutes sample plugs. Some of the sample is frequently left behind the plug as a tail. Overlap of these tails usually leads to cross-contamination of samples in different plugs. Thus, samples are often introduced into the channels individually, separated by buffer washes. On the other hand, interleaving samples with long buffer plugs, or washing the system with buffer between samples, reduces the throughput of the system.

In EOF, flow transport is essentially linear, that is, if two reactants are introduced into a plug and transported by EOF, their residence time (and reaction time) can be calculated simply by dividing the distance traveled in the channel by the velocity. This linear transport allows precise control of residence times through a proper adjustment of the channel lengths and flow rates. In contrast, dispersion in pressure-driven flow typically creates a broad range of residence times for a plug traveling in such flows, and this diminishes time control.

The issue of time control is important. Many chemical and biochemical processes occur on particular time scales, and measurement of reaction times can be indicative of concentrations of reagents or their reactivity. Stopped-flow type instruments are typically used to perform these measurements. These instruments rely on turbulent flow to mix the reagents and transport them with minimal dispersion. Turbulent flow normally occurs in tubes with large diameter and at high flow rates. Thus stopped-flow instruments tend to use large volumes of reagents (on the order of ml/s). A microfluidic analog of stopped-flow, which consumes smaller volumes of reagents (typically μL/min), could be useful as a scientific instrument, e.g., as a diagnostic instrument. So far, microfluidic devices have not be able to compete with stopped-flow type instruments because EOF is usually very slow (although with less dispersion) while pressure-driven flows suffer from dispersion.

In addition, mixing in microfluidic systems is often slow regardless of the method used to drive the fluid because flow is laminar in these systems (as opposed to turbulent in larger systems). Mixing in laminar flows relies on diffusion and is especially slow for larger molecules such as DNA and proteins.

In addition, particulates present handling difficulty in microfluidic systems. While suspensions of cells in aqueous buffers can be relatively easy to handle because cells are isodense with these buffers, particulates that are not isodense with the fluid tend to settle at the bottom of the channel, thus eventually blocking the channel. Therefore, samples for analysis often require filtration to remove particulates.

SUMMARY ACCORDING TO THE INVENTION

In accordance with the invention, a method of conducting a reaction within a substrate is provided that comprises introducing a carrier-fluid into a first channel of the substrate; introducing at least two different plug-fluids into the first channel; and applying pressure to the first channel to induce a fluid flow in the substrate to form substantially identical plugs comprising a mixture of plug-fluids. The plug-fluids are immiscible with the carrier-fluid. During plug formation, the cross-section of the plug is substantially similar to the cross-section of the first channel, so that the plug is substantially in contact with all walls of the first channel. After plug formation, the cross-section of the plug may be smaller than the cross-section of the channel. A thin layer of carrier-fluid typically exists between the wall of the channel and the plug, although in some cases this layer disappears. In general, each plug is substantially similar in size when initially formed in

the channel. In addition, the capillary number of the plug in the channel is low, typically less than 1, preferably ≤ about 0.2, more preferably ≤ about 0.1.

When plugs are formed from more than one plug-fluid, the fluids are rapidly mixed. Mixing inside plugs is further enhanced when the channels are not straight (i.e., when chaotic flows are generated). Aperiodic channel designs are preferred to induce rapid mixing within plugs. In other cases, mixing can be slowed down or controlled such as by using winding channels, varying the fluid viscosities, varying the plug-fluid composition, and twirling, which can also be controlled.

The device of the present invention can be used to merge one or more plug fluids. The plug-fluids are introduced either through a single inlet or from multiple inlets. When the plug-fluids are introduced through a single inlet, they are preferably mixed just upstream of the inlet, so that substantial mixing does not occur prior to introduction into the first channel. When plugs fluids are introduced through multiple inlets, one or more physical properties (such as the viscosity, plug dimensions, surface tension at the interface between the plug fluids and the carrier-fluid, or the surface tension at the interface between the plug fluids and the walls of the channel) of the plug-fluids are adjusted so that plugs composed of different plug-fluids merge into a series of plugs prior to the outlet (that is, a series of plugs are formed which are composed of a mixture of plug-fluids). Alternatively, the plug-fluids can be introduced into separate channels to form plugs composed of single plug-fluids. These channels are then merged into a single merged channel. The continuous fluid flow within the substrate forms merged plugs in the single merged channel.

The device of the present invention can be used to split plugs into two or more channels.

Using the above devices and techniques, a variety of reactions can be conducted, including polymerizations, crystallizations (including small molecule and proteins), nanoparticle synthesis, formation of unstable intermediates, enzyme-catalyzed reactions and assays, protein-protein binding, etc. More than one reaction can be conducted, either simultaneously or sequentially.

Further, the present invention also provides a device comprising one or more substrates in accordance with the present invention.

The devices and methods according to the invention include various non-limiting embodiments or modifications several of which are discussed in details below.

BRIEF DESCRIPTION OF THE DRAWINGS AND PHOTOGRAPHS

FIG. **1**A is a schematic diagram of a basic channel design that may be used to induce rapid mixing in plugs. FIG. **1**B(**1**)-(**4**) are schematic diagrams depicting a series of periodic variations of the basic channel design. FIG. **1**C(**1**)-(**4**) are schematic diagrams depicting a series of aperiodic combinations resulting from a sequence of alternating elements taken from a basic design element shown in FIG. **1**A and an element from the periodic variation series shown in FIGS. **1**B(**1**)-(**4**).

FIG. **2**A is a schematic diagram contrasting laminar flow transport and plug transport in a channel. FIG. **2**B(**1**) shows a photograph (right side, top portion) illustrating rapid mixing inside plugs moving through winding channels. FIG. **2**B(**2**) shows a photograph (right side, lower portion) showing that winding channels do not accelerate mixing in a laminar flow in the absence of PFD.

FIG. **3** shows photographs (right side) and schematic diagrams (left side) that depict a stream of plugs from an aqueous plug-fluid and an oil (carrier-fluid) in curved channels at flow rates of 0.5 μL/min (FIG. **3**(*a*)) and 1.0 μL/min (FIG. **3***b*)).

FIG. **4** shows a photograph (lower portion) and a schematic diagram (upper portion) that illustrate plug formation through the injection of oil and multiple plug-fluids.

FIG. **5** is a schematic diagram that illustrates a two-step reaction in which plugs are formed through the injection of oil and multiple plug-fluids using a combination of different geometries for controlling reactions and mixing.

FIG. **6** is a schematic representation of part of a microfluidic network that uses multiple inlets and that allows for both splitting and merging of plugs. This schematic diagram shows two reactions that are conducted simultaneously. A third reaction (between the first two reaction mixtures) is conducted using precise time delay.

FIG. **7**(*a*)-(*b*) show microphotographs (10 μs exposure) illustrating rapid mixing inside plugs (a) and negligible mixing in a laminar flow (b) moving through winding channels at the same total flow velocity. FIG. **7**(*c*) shows a false-color microphotograph (2 s exposure, individual plugs are invisible) showing time-averaged fluorescence arising from rapid mixing inside plugs of solutions of Fluo-4 and $CaCl_2$. FIG. **7**(*d*) shows a plot of the relative normalized intensity (I) of fluorescence obtained from images such as shown in (c) as a function of distance (FIG. **7**(*d*)(i)) traveled by the plugs and of time required to travel that distance (FIG. **7**(*d*)(ii)) at a given flow rate. FIG. **7**(*e*) shows a false-color microphotograph (2 s exposure) of the weak fluorescence arising from negligible mixing in a laminar flow of the solutions used in (c).

FIG. **8** shows photographs (FIGS. **8**(*b*) and (*d*)) and schematics (FIGS. **8**(*a*) and (*c*)) that illustrate fast mixing at flow rates of about 0.5 μL/min (FIGS. **8**(*a*) and (*b*)) and about 1.0 μL/min (FIGS. **8**(*c*) and (*d*)) using 90°-step channels.

FIG. **9** shows schematics (FIGS. **9**(*a*) and **9**(*c*)) and photographs (FIGS. **9**(*b*) and (**9***d*)) illustrates fast mixing at flow rates of about 1.0 μL/min (FIGS. **9**(*a*) and **9**(*b*)) and about 0.5 μL/min (FIGS. **9**(*c*) and **9**(*d*)) using 135°-step channels.

FIG. **10**(*a*) is a schematic diagram depicting three-dimensional confocal visualization of chaotic flows in plugs. FIG. **10**(*b*) is a plot showing a sequence preferably used for visualization of a three-dimensional flow.

FIG. **11** shows a schematic diagram of a channel geometry designed to implement and visualize the baker's transformation of plugs flowing through microfluidic channels.

FIG. **12** shows photographs depicting the merging of plugs (FIG. **12**(*a*)) and splitting of plugs (FIG. **12**(*b*)) that flow in separate channels or channel branches that are perpendicular.

FIG. **13** shows UV-VIS spectra of CdS nanoparticles formed by rapid mixing in plugs (spectrum with a sharp absorption peak) and by conventional mixing of solutions.

FIG. **14** shows schematic diagrams (FIGS. **14**(*a*)(i) and **14**(*b*)(i)) and photographs (FIGS. **14**(*a*)(ii), **14**(*a*)(iii), **14**(*b*)(ii) and **14**(*b*)(iii)) that illustrate the synthesis of CdS nanoparticles in PDMS microfluidic channels in single-phase aqueous laminar flow (FIG. **14**(*a*)) and in aqueous plugs that are surrounded by water-immiscible perfluorodecaline (FIG. **14**(*b*)).

FIG. **15** shows schematic representations of the synthesis of CdS nanoparticles inside plugs.

FIG. **16** is a schematic illustration of a microfluidic device according to the invention that illustrates the trapping of plugs.

FIG. **17** is a schematic of a microfluidic method for forming plugs with variable compositions for protein crystallization.

FIGS. **18** (*a*) and (*b*) are schematic illustrations of a method for controlling heterogeneous nucleation by varying the surface chemistry at the interface of an aqueous plug-fluid and a carrier-fluid.

FIG. **19** is a schematic diagram that illustrates a method of separating nucleation and growth using a microfluidic network according to the present invention.

FIGS. **20** (*a*) and (*b*) show schematic diagrams that illustrate two methods that provide a precise and reproducible degree of control over mixing and that can be used to determine the effect of mixing on protein crystallization.

FIG. **21** is a reaction diagram illustrating an unstable point in the chlorite-thiosulfate reaction.

FIG. **22**A-D are schematic diagrams that show various examples of geometries of microfluidic channels according to the invention for obtaining kinetic information from single optical images.

FIG. **23** shows a schematic of a microfluidic network (left side) and a table of parameters for a network having channel heights of 15 and 2 μm.

FIG. **24** shows a reaction scheme that depicts examples of fluorinated surfactants that form monolayers that are: (a) resistant to protein adsorption; (b) positively charged; and (c) negatively charged. FIG. **24***b* shows a chemical structure of neutral surfactants charged by interactions with water by protonation of an amine or a guanidinium group. FIG. **24***c* shows a chemical structure of neutral surfactants charged by interactions with water deprotonation of a carboxylic acid group.

FIG. **25** are schematic diagrams of microfluidic network (left side of a), b), and c)) that can be used for controlling the concentrations of aqueous solutions inside the plugs, as well as photographs (right side of a), b), and c)) showing the formation of plugs with different concentrations of the aqueous streams.

FIG. **26** are schematic diagrams of microfluidic network (left side of a) and b)) and photographs (right side of a) and b)) of the plug-forming region of the network in which the aqueous streams were dyed with red and green food dyes to show their flow patterns.

FIG. **27** are photographs and plots showing the effects of initial conditions on mixing by recirculating flow inside plugs moving through straight microchannels. FIG. **27***a***1**) is a schematic diagram showing that recirculating flow (shown by black arrows) efficiently mixed solutions of reagents that were initially localized in the front and back halves of the plug. FIG. **27***a***2**) is a schematic diagram showing that recirculating flow (shown by black arrows) did not efficiently mix solutions of reagents that were initially localized in the left and right halves of the plugs. FIG. **27***b*) shows a schematic diagram showing the inlet portions (left side) and photographs of images showing measurements of various periods and lengths of plugs. FIG. **27***c***1**) shows a graph of the relative optical intensity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs of varying lengths. FIG. **27***c***2**) is the same as FIG. **7***c***1**) except that each plug traverses a distance of 1.3 mm.

FIG. **28** is a schematic illustration of a plug showing the notation used to identify different regions of the plugs relative to the direction of motion.

FIG. **29***a*)-*b*) are plots of the periods and the lengths of plugs as a function of total flow velocity (FIG. **29***a*)) and water fraction (FIG. **29***b*)).

FIG. **30** shows photographs illustrating weak dependence of periods, length of plugs, and flow patterns inside plugs on total flow velocity.

FIG. **31** are plots showing the distribution of periods and lengths of plugs where the water fractions were 0.20, 0.40, and 0.73, respectively.

FIG. **32** shows photographs (middle and right side) that show that plug traps are not required for crystal formation in a microfluidic network, as well as a diagram of the microfluidic network (left side).

FIG. **33***a-d* (left side) are top views of microfluidic networks (left side) and photographs (right side) that comprise channels having either uniform or nonuniform dimension. FIG. **33***a* shows that merging of the plugs occurs infrequently in the T-shaped channel shown in the photographs. FIG. **33***b* illustrates plug merging occurring between plugs arriving at different times at the Y-shaped junction (magnified view shown). FIG. **33***c* depicts in-phase merging, i.e., plug merging upon simultaneous arrival of at least two plugs at a junction, of plugs of different sizes generated using different oil/water ratios at the two pairs of inlets. FIG. **33***d* illustrates defects (i.e., plugs that fail to undergo merging when they would normally merge under typical or ideal conditions) produced by fluctuations in the relative velocity of the two incoming streams of plugs.

FIG. **34***a-c* show a schematic diagram (a, left side) and photographs (b, c) each of which depicts a channel network viewed from the top. FIG. **34***a* is a schematic diagram of the channel network used in the experiment. FIG. **34***b* is a photograph showing the splitting of plugs into plugs of approximately one-half the size of the initial plugs. FIG. **34***c* is a photograph showing the asymmetric splitting of plugs which occurred when $P_1 < P_2$.

FIG. **35** shows a schematic diagram (a, left side) and photographs (b, c) that depicts the splitting of plugs using microfluidic networks without constrictions near the junction.

FIG. **36** shows a photograph (right side) of lysozyme crystals grown in water plugs in the wells of the microfluidic channel, as well as a diagram (left side) of the microfluidic network used in the crystallization.

FIG. **37** is a schematic diagram that depicts a microfluidic device according to the invention that can be used to amplify a small chemical signal using an autocatalytic (and possibly unstable) reaction mixture.

FIG. **38** is a schematic diagram that illustrates a method for a multi-stage chemical amplification which can be used to detect as few as a single molecule.

FIG. **39** shows a diagram (left side) of the microfluidic network and a photograph (right side) of water plugs attached to the PDMS wall.

FIG. **40**A is a schematic representation (left side) of a microfluidic network used to measure kinetics data for the reaction of RNase A using a fluorogenic substrate (on-chip enzyme kinetics), and FIG. **40** and FIG. **40**B are plots that shows the kinetic data for the reaction between RNase A and a fluorogenic substrate.

FIG. **41** shows a photograph (middle and right side) of the water droplet region of the microfluidic network (T stands for time), as well as a diagram of the microfluidic network (left side).

FIG. **42** shows a schematic diagram (left side) of a microfluidic network and a photograph (right side) of the ink plug region of the microfluidic network in which the gradients were formed by varying the flow rates.

FIG. **43** shows a schematic diagram (left side) of a microfluidic network and a photograph (right side) of lysozyme crystals formed in the microfluidic network using gradients.

FIG. **44** are schematic illustrations showing how an initial gradient may be created by injecting a discrete aqueous sample of a reagent B into a flowing stream of water.

FIG. **45***a*) shows a schematic of the microfluidic network used to demonstrate that on-chip dilutions can be accomplished by varying the flow rates of the reagents. The blue rectangle outlines the field of view for images shown in FIG. **45***c*)-*d*). FIG. **45***b*) shows a graph quantifying this dilution method by measuring fluorescence of a solution of fluorescein diluted in plugs in the microchannel.

DETAILED DESCRIPTION ACCORDING TO THE INVENTION

The term "analysis" generally refers to a process or step involving physical, chemical, biochemical, or biological analysis that includes characterization, testing, measurement, optimization, separation, synthesis, addition, filtration, dissolution, or mixing.

The term "analysis unit" refers to a part of or a location in a substrate or channel wherein a chemical undergoes one or more types of analyses.

The term "carrier-fluid" refers to a fluid that is immiscible with a plug-fluid. The carrier-fluid may comprise a substance having both polar and non-polar groups or moieties.

The term "channel" refers to a conduit that is typically enclosed, although it may be at least partially open, and that allows the passage through it of one or more types of substances or mixtures, which may be homogeneous or heterogeneous, including compounds, solvents, solutions, emulsions, or dispersions, any one of which may be in the solid, liquid, or gaseous phase. A channel can assume any form or shape such as tubular or cylindrical, a uniform or variable (e.g., tapered) diameter along its length, and one or more cross-sectional shapes along its length such as rectangular, circular, or triangular. A channel is typically made of a suitable material such as a polymer, metal, glass, composite, or other relatively inert materials. As used herein, the term "channel" includes microchannels that are of dimensions suitable for use in devices. A network of channels refers to a multiplicity of channels that are typically connected or in communication with each other. A channel may be connected to at least one other channel through another type of conduit such as a valve.

The term "chemical" refers to a substance, compound, mixture, solution, emulsion, dispersion, molecule, ion, dimer, macromolecule such as a polymer or protein, biomolecule, precipitate, crystal, chemical moiety or group, particle, nanoparticle, reagent, reaction product, solvent, or fluid any one of which may exist in the solid, liquid, or gaseous state, and which is typically the subject of an analysis.

The term "detection region" refers to a part of or a location in a substrate or channel wherein a chemical is identified, measured, or sorted based on a predetermined property or characteristic.

The term "device" refers to a device fabricated or manufactured using techniques such as wet or dry etching and/or conventional lithographic techniques or a micromachining technology such as soft lithography. As used herein, the term "devices" includes those that are called, known, or classified as microfabricated devices. A device according to the invention may have dimensions between about 0.3 cm to about 15 (for 6 inch wafer) cm per side and between about 1 micrometer to about 1 cm thick, but the dimensions of the device may also lie outside these ranges.

The term "discrimination region" refers to a part of or a location in a substrate or channel wherein the flow of a fluid can change direction to enter at least one other channel such as a branch channel.

The term "downstream" refers to a position relative to an initial position which is reached after the fluid flows past the initial point. In a circulating flow device, downstream refers to a position farther along the flow path of the fluid before it crosses the initial point again. "Upstream" refers to a point in the flow path of a fluid that the fluid reaches or passes before it reaches or passes a given initial point in a substrate or device.

The term "flow" means any movement of a solid or a fluid such as a liquid. For example, the movement of plug-fluid, carrier-fluid, or a plug in a substrate, or component of a substrate according to the invention, or in a substrate or component of a substrate involving a method according to the invention, e.g., through channels of a microfluidic substrate according to the invention, comprises a flow. The application of any force may be used to provide a flow, including without limitation: pressure, capillary action, electro-osmosis, electrophoresis, dielectrophoresis, optical tweezers, and combinations thereof, without regard for any particular theory or mechanism of action.

The term "immiscible" refers to the resistance to mixing of at least two phases or fluids under a given condition or set of conditions (e.g., temperature and/or pressure) such that the at least two phases or fluids persist or remain at least partially separated even after the phases have undergone some type of mechanical or physical agitation. Phases or fluids that are immiscible are typically physically and/or chemically discernible, or they may be separated at least to a certain extent.

The term "inlet port" refers to an area of a substrate that receives plug-fluids. The inlet port may contain an inlet channel, a well or reservoir, an opening, and other features that facilitate the entry of chemicals into the substrate. A substrate may contain more than one inlet port if desired. The inlet port can be in fluid communication with a channel or separated from the channel by a valve.

The term "nanoparticles" refers to atomic, molecular or macromolecular particles typically in the length scale of approximately 1-100 nanometer range. Typically, the novel and differentiating properties and functions of nanoparticles are observed or developed at a critical length scale of matter typically under 100 nm. Nanoparticles may be used in constructing nanoscale structures and they may be integrated into larger material components, systems and architectures. In some particular cases, the critical length scale for novel properties and phenomena involving nanoparticles may be under 1 nm (e.g., manipulation of atoms at approximately 0.1 nm) or it may be larger than 100 nm (e.g., nanoparticle reinforced polymers have the unique feature at approximately 200-300 nm as a function of the local bridges or bonds between the nanoparticles and the polymer).

The term "nucleation composition" refers to a substance or mixture that includes one or more nuclei capable of growing into a crystal under conditions suitable for crystal formation. A nucleation composition may, for example, be induced to undergo crystallization by evaporation, changes in reagent concentration, adding a substance such as a precipitant, seeding with a solid material, mechanical agitation, or scratching of a surface in contact with the nucleation composition.

The term "outlet port" refers to an area of a substrate that collects or dispenses the plug-fluid, carrier-fluid, plugs or reaction product. A substrate may contain more than one outlet port if desired.

The term "particles" means any discrete form or unit of matter. The term "particle" or "particles" includes atoms, molecules, ions, dimers, polymers, or biomolecules.

The term "particulate" refers to a cluster or agglomeration of particles such as atoms, molecules, ions, dimers, polymers,

or biomolecules. Particulates may comprise solid matter or be substantially solid, but they may also be porous or partially hollow. They may contain a liquid or gas. In addition, particulates may be homogeneous or heterogeneous, that is, they may comprise one or more substances or materials.

"Plugs" in accordance with the present invention are formed in a substrate when a stream of at least one plug-fluid is introduced into the flow of a carrier-fluid in which it is substantially immiscible. The flow of the fluids in the device is induced by a driving force or stimulus that arises, directly or indirectly, from the presence or application of, for example, pressure, radiation, heat, vibration, sound waves, an electric field, or a magnetic field. Plugs in accordance with the present invention may vary in size but when formed, their cross-section should be substantially similar to the cross-section of the channels in which they are formed. When plugs merge or get trapped inside plug traps, the cross-section of the plugs may change. For example, when a plug enters a wider channel, its cross-section typically increases.

Further, plugs in accordance with the present invention may vary in shape, and for example may be spherical or non-spherical. The shape of the plug may be independent of the shape of the channel (e.g., a plug may be a deformed sphere traveling in a rectangular channel). The plugs may be in the form of plugs comprising an aqueous plug-fluid containing one or more reagents and/or one or more products formed from a reaction of the reagents, wherein the aqueous plug-fluid is surrounded by a non-polar or hydrophobic fluid such as an oil. The plugs may also be in the form of plugs comprising mainly a non-polar or hydrophobic fluid which is surrounded by an aqueous fluid. The plugs may be encased by one or more layers of molecules that comprise both hydrophobic and hydrophilic groups or moieties. The term "plugs" also includes plugs comprising one or more smaller plugs, that is, plugs-within-plugs. The relative amounts of reagents and reaction products contained in the plugs at any given time depend on factors such as the extent of a reaction occurring within the plugs. Preferably, plugs contain a mixture of at least two plug fluids.

The term "plug-forming region" refers to a junction between an inlet port and the first channel of a substrate according to the invention. Preferably, the fluid introduced into the inlet port is "incompatible" (i.e., immiscible) with the fluid in the first channel so that plugs of the fluid formed in the plug-forming region are entrained into the stream of fluid from the first channel.

The term "plug-fluid" refers to a fluid wherein or using which a reaction or precipitation can occur. Typically, the plug-fluid contains a solvent and a reagent although in some embodiments at least one plug-fluid may not contain a reagent. The reagent may be soluble or insoluble in the solvent. The plug-fluid may contain a surfactant. At least two different plug-fluids are used in the present invention. When both plug-fluids contain reagents, the fluids are typically miscible, but can also be partially immiscible, so long as the reagents within each plug-fluid can react to form at least one product or intermediate.

The term "polymer" means any substance or compound that is composed of two or more building blocks ('mers') that are repetitively linked to each other. For example, a "dimer" is a compound in which two building blocks have been joined together. Polymers include both condensation and addition polymers. Typical examples of condensation polymers include polyamide, polyester, protein, wool, silk, polyurethane, cellulose, and polysiloxane. Examples of addition polymers are polyethylene, polyisobutylene, polyacrylonitrile, poly(vinyl chloride), and polystyrene. Other examples include polymers having enhanced electrical or optical properties (e.g., a nonlinear optical property) such as electroconductive or photorefractive polymers. Polymers include both linear and branched polymers.

The term "protein" generally refers to a set of amino acids linked together usually in a specific sequence. A protein can be either naturally-occurring or man-made. As used herein, the term "protein" includes amino acid sequences that have been modified to contain moieties or groups such as sugars, polymers, metalloorganic groups, fluorescent or light-emitting groups, moieties or groups that enhance or participate in a process such as intramolecular or intermolecular electron transfer, moieties or groups that facilitate or induce a protein into assuming a particular conformation or series of conformations, moieties or groups that hinder or inhibit a protein from assuming a particular conformation or series of conformations, moieties or groups that induce, enhance, or inhibit protein folding, or other moieties or groups that are incorporated into the amino acid sequence and that are intended to modify the sequence's chemical, biochemical, or biological properties. As used herein, a protein includes, but is not limited to, enzymes, structural elements, antibodies, hormones, electron carriers, and other macromolecules that are involved in processes such as cellular processes or activities. Proteins typically have up to four structural levels that include primary, secondary, tertiary, and quaternary structures.

The term "reaction" refers to a physical, chemical, biochemical, or biological transformation that involves at least one chemical, e.g., reactant, reagent, phase, carrier-fluid, or plug-fluid and that generally involves (in the case of chemical, biochemical, and biological transformations) the breaking or formation of one or more bonds such as covalent, noncovalent, van der Waals, hydrogen, or ionic bonds. The term includes typical chemical reactions such as synthesis reactions, neutralization reactions, decomposition reactions, displacement reactions, reduction-oxidation reactions, precipitation, crystallization, combustion reactions, and polymerization reactions, as well as covalent and noncovalent binding, phase change, color change, phase formation, crystallization, dissolution, light emission, changes of light absorption or emissive properties, temperature change or heat absorption or emission, conformational change, and folding or unfolding of a macromolecule such as a protein.

The term "reagent" refers to a component of a plug-fluid that undergoes or participates (e.g., by influencing the rate of a reaction or position of equilibrium) in at least one type of reaction with one or more components of other plug-fluids or a reagent-containing carrier-fluid in the substrate to produce one or more reaction products or intermediates which may undergo a further reaction or series of reactions.

A reagent contained in a plug-fluid may undergo a reaction in which a stimulus such as radiation, heat, temperature or pressure change, ultrasonic wave, or a catalyst induces a reaction to give rise to a transformation of the reagent to another reagent, intermediate, or product. A reagent may also undergo a reaction such as a phase change (e.g., precipitation) upon interaction with one or more components of other plug-fluids or a reagent-containing carrier-fluid.

The term "substrate" refers to a layer or piece of material from which devices or chips are prepared or manufactured. As used herein, the term "substrate" includes any substrate fabricated using any traditional or known microfabrication techniques. The term "substrate" also refers either to an entire device or chip or to a portion, area, or section of a device or chip which may or may not be removable or detachable from the main body of the device or chip. The substrate may be prepared from one or more materials such as glass, silicon,

silicone elastomer, and polymers including, but not limited to, polypropylene or polyethylene.

The discussion below provides a detailed description of various devices and methods according to the invention for forming plugs, generating gradients in a series of plugs, varying the concentration of reagents inside plugs, rapid mixing in plugs, and scaling of mixing times. In particular, a detailed description of methods for merging, splitting and/or sorting plugs using channels, which form the bases for various applications ranging from the manufacture and analysis of various products to applications in electronics, medicine, diagnostics, and pharmaceuticals, to name a few, is discussed. Methods of detection and measurement of, among others, plugs and processes occurring within plugs are also described.

Among the various applications involving the devices and methods according to the invention are particle separation/sorting, synthesis, investigation of nonlinear and stochastic systems, nonlinear amplification using unstable autocatalytic mixtures, use of stochastic chemical systems for chemical amplification, kinetic measurements, time control of processes, increasing the dynamic range of kinetic measurements, ultrafast measurements, crystallization of proteins, and dynamic control of surface chemistry.

In addition, the devices and methods according to the invention offer a wide-range of other applications. For example, the devices and methods according to the invention provide for effective, rapid, and precise manipulation and monitoring of solutions or reactions over a range of time scales (e.g., from tens of microseconds, to hours or weeks in case of, for example, crystallization) and over a range of solution volumes (e.g., from femtoliters to hundreds of nanoliters).

In one aspect of the invention, the various devices and methods according to the invention are used to overcome one or more of the following problems involving microfluidics. First, the substantial dispersion of solutes in microfluidic channels increases reagent consumption and makes experiments or measurements over long time scales (e.g., minutes to hours) difficult to perform. Various devices and methods according to the invention are intended to overcome this problem by localizing reagents inside plugs that are encapsulated by an immiscible carrier-fluid.

Second, slow mixing of solutions renders experiments, tests, or reactions involving very short time scales (e.g., tens of milliseconds and below) either difficult or impossible to perform with existing technologies. In addition, turbulence-based mixing techniques prohibitively increase sample consumption. In accordance with the present invention, this problem is preferably addressed by conducting the mixing process inside plugs. Rather than relying on turbulence, the various devices and methods according to the invention preferably rely on chaotic advection to accelerate the mixing process. An advantage provided by chaotic advection is that it is expected to operate efficiently in both small and large channels.

Third, achieving control over the chemistry of internal surfaces of devices can be very important at small scales. Thus, being able to control surface chemistry in small devices for example is highly desirable. In accordance with the devices and methods according to the invention, the surface chemistry to which solutions are exposed is preferably controlled through a careful selection of surfactants that are preferably designed to assemble at the interface between the plugs and the immiscible fluid that surrounds them.

Devices and methods of the invention are also provided for use in traditional areas of microfluidics where, for example, miniaturization and speed are important. Thus, the devices and methods according to the invention may be used to develop various tools such as those for high-throughput chemical or biophysical measurements, chemical synthesis, particle formation, and protein crystallization. They may also be used in high-throughput screening, combinatorial synthesis, analysis, and diagnostics, either as a self-contained platform, or in combination with existing technologies particularly those that rely on the use of immiscible fluid flows.

Importantly, the devices of the invention can be adapted to work with automation and robotic technology. They may be used, for example, as a basis for ultra-high throughput automated systems for structural and functional characterization of biological molecules. Thus, the various devices and methods according to the invention provide rapid, economical, and accessible means of synthesis, analysis, and measurements in the fields of biology, chemistry, biophysics, bioengineering, and medicine (e.g., for diagnostics).

The devices and methods of the invention have numerous other possible applications. For example, chaotic mixing at low values of Reynolds number can be exploited as an important tool for controlling unstable chemical reactions. In addition, the systems and devices of the invention may be used for controlling and/or monitoring reactions that generate highly unstable (or explosive) intermediates. They can also be valuable for controlling or monitoring reactions or processes involving autocatalytic reactions. For example, pure hydrogen peroxide (H_2O_2) is an inexpensive and highly effective oxidant, but its autocatalytic decomposition often leads to explosions upon storage and handling. In the microfluidic systems of the invention, H_2O_2 is preferably generated in-situ, stabilized by the chaotic flow, and used to destroy chemical and biological warfare agents. Because the unstable mixtures in these systems are localized inside plugs formed in accordance with the invention, occasional autocatalytic decomposition in one or more plugs is kept localized within those plugs thereby preventing a catastrophic reaction involving the whole system. In addition, large arrays of microfluidic reactors may be operated in parallel to provide substantial throughput.

It is also possible to couple multiple autocatalytic reactions in a single network using the devices and methods according to the invention. For example, a sample plug could be split into many smaller plugs and forwarded to individual amplification cascades. Because the contents of the cascades' outflows exhibit patterns that correspond to the patterns of analytes present in these systems, these patterns could be analyzed using artificial neural network (ANN) (Jackson, R. B. a. T. *Neural Computing: An Introduction*, Hilger, New York, 1991; Zornetzer et al., *An Introduction to Neural and Electronic Networks*, Academic Press, San Diego, Calif., 1990.) algorithms. For example, patterns that arise in blood or saliva analysis may correspond to certain normal or abnormal (e.g., disease, fatigue, infection, poisoning) conditions involving, for example, human and animals.

Moreover, it may be possible to create intelligent microfluidic systems in accordance with the invention, where the nonlinear chemical reactions perform not only detection, but also analysis using ANN algorithms. For example, after amplification, the channels of the present invention typically will contain sufficient amounts of material to operate hydrogel-based valves (Liu et al., "Fabrication and characterization of hydrogel-based microvalves," *J. Microelectromech. Syst.* 2002, vol. 1, pp. 45-53; Yu et al., "Responsive biomimetic hydrogel valve for microfluidics," Appl. Phys. Lett. 2001, vol. 78, pp. 2589-2591; Beebe et al., "Functional hydrogel structures for autonomous flow control inside microfluidic channels," *Nature,* 2000, vol. 404, 588.). These valves can be used to control flows inside the system as a function of the sample

plug composition. Feedforward and even feedback (e.g., by using the hydrogel valves to control the flow of the input streams) networks may thus be created and used for analysis. Such nonlinear networks may be used not only to recognize patterns pre-programmed by the connectivity of the channels (Hjelmfelt et al., "Pattern-Recognition in Coupled Chemical Kinetic Systems," *Science,* 1993, 260, 335-337.) but also to learn patterns by reconfiguring themselves (Jackson, R. B. a. T. *Neural Computing: An Introduction*, Hilger, New York, 1991; Zornetzer et al., *An Introduction to Neural and Electronic Networks*, Academic Press, San Diego, Calif., 1990.). Such intelligent microfluidic devices could have unprecedented capabilities for fully autonomous detection, analysis, and signal processing, perhaps surpassing those of biological and current man-made systems.

The devices and methods of the invention are also useful in genomics and proteomics, which are used to identify thousands of new biomolecules that need to be characterized, or are available only in minute quantities. In particular, the success of genomics and proteomics has increased the demand for efficient, high-throughput mechanisms for protein crystallization. X-ray structure determination remains the predominant method of structural characterization of proteins. However, despite significant efforts to understand the process of crystallization, macromolecular crystallization largely remains an empirical field, with no general theory to guide a rational approach. As a result, empirical screening has remained the most widely used method for crystallizing proteins.

The following areas also provide applications of the devices and methods according to the invention. For example, a number of problems still beset high-throughput kinetics and protein crystallization. When it comes to determining protein structure and quantitatively ascertaining protein interactions, there are at least two technological challenges: (1) most robotic technology still only automate existing methods and are often too expensive for a small research laboratory; and (2) there remains the need for conceptually new methods that provide greater degree of control over the crystallization process. In addition, setting up and monitoring crystallization trials typically involve handling of sub-microliter volumes of fluids over periods ranging from seconds to days.

Thus, various devices and methods according to the present invention are designed to provide novel and efficient means for high-throughput crystallization of soluble and membrane proteins. In addition to being a simple and economical method of setting up thousands of crystallization trials in a matter of minutes, a system according to the invention will enable unique time control of processes such as the mixing and nucleation steps leading to crystallization. A system according to the present invention may also be used to control protein crystallization by controlling not only short time-scale events such as nucleation but also long time-scale events such as crystal growth.

Further, the devices and methods of the present invention may be used in high-throughput, kinetic, and biophysical measurements spanning the 10^{-5}-10^{7} second time regime. Preferably, the various devices and methods according to the present invention require only between about a few nanoliters to about a few microliters of each solution. Applications of such devices and methods include studies of enzyme kinetics and RNA folding, and nanoparticle characterization and synthesis, which are discussed in detail below.

Channels and Devices

In one aspect of the invention, a device is provided that includes one or more substrates comprising a first channel comprising an inlet separated from an outlet; optionally, one or more secondary channels (or branch channels) in fluid communication with the first channel, at least one carrier-fluid reservoir in fluid communication with the first channel, at least two plug-fluid reservoirs in fluid communication with the first channel, and a means for applying continuous pressure to a fluid within the substrate.

A device according to the invention preferably comprises at least one substrate.

A substrate may include one or more expansions or areas along a channel wherein plugs can be trapped. The substrates of the present invention may comprise an array of connected channels.

The device may have one or more outlet ports or inlet ports. Each of the outlet and inlet ports may also communicate with a well or reservoir. The inlet and outlet ports may be in fluid communication with the channels or reservoirs that they are connecting or may contain one or more valves. Fluid can be introduced into the channels via the inlet by any means. Typically, a syringe pump is used, wherein the flow rate of the fluid into the inlet can be controlled.

A plug-forming region generally comprises a junction between a plug-fluid inlet and a channel containing the carrier-fluid such that plugs form which are substantially similar in size to each other and which have cross-sections which are substantially similar in size to the cross-section of the channel in the plug-forming region. In one embodiment, the substrate may contain a plurality of plug-forming regions.

The different plug-forming regions may each be connected to the same or different channels of the substrate. Preferably, the sample inlet intersects a first channel such that the pressurized plug fluid is introduced into the first channel at an angle to a stream of carrier-fluid passing through the first channel. For example, in preferred embodiments, the sample inlet and first channel intercept at a T-shaped junction; i.e., such that the sample inlet is perpendicular (i.e. at an angle of 90°) to the first channel. However, the sample inlet may intercept the first channel at any angle.

A first channel may in turn communicate with two or more branch channels at another junction or "branch point", forming, for example, a T-shape or a Y-shape. Other shapes and channel geometries may be used as desired. In exemplary embodiments the angle between intersecting channels is in the range of from about 60° to about 120°. Particular exemplary angles are 45°, 60°, 90°, and 120°. Precise boundaries for the discrimination region are not required, but are preferred.

The first and branch channels of the present invention can, each independently, be straight or have one or more bends. The angle of a bend, relative to the substrate, can be greater than about 10°, preferably greater than about 135°, 180°, 270°, or 360°.

In one embodiment of the invention, a substrate comprises at least one inlet port in communication with a first channel at or near a plug-forming region, a detection region within or coincident with all or a portion of the first channel or plug-forming region, and a detector associated with the detection region. In certain embodiments the device may have two or more plug-forming regions. For example, embodiments are provided in which the analysis unit has a first inlet port in communication with the first channel at a first plug-forming region, a second inlet port in communication with the first channel at a second plug-forming region (preferably downstream from the first plug-forming region), and so forth.

In another embodiment, a substrate according to the invention may comprise a first channel through which a pressurized stream or flow of a carrier-fluid is passed, and two or more inlet channels which intersect the first channel at plug-form-

ing regions and through which a pressurized stream or flow of plug fluids pass. Preferably, these inlet channels are parallel to each other and each intercept the first channel at a right angle. In specific embodiments wherein the plugs introduced through the different plug forming regions are mixed, the inlet channels are preferably close together along the first channel. For example, the first channel may have a diameter of 60 μm that tapers to 30 μm at or near the plug-forming regions. The inlet channels then also preferably have a diameter of about 30 μm and, in embodiments where plug mixing is preferred, are separated by a distance along the first channel approximately equal to the diameter of the inlet channel (i.e., about 30 μm).

In an embodiment according to the invention, the substrate also has a detection region along a channel. There may be a plurality of detection regions and detectors, working independently or together, e.g., to analyze one or more properties of a chemical such as a reagent.

A detection region is within, communicating, or coincident with a portion of a first channel at or downstream of the plug-forming region and, in sorting embodiments, at or upstream of the discrimination region or branch point. Precise boundaries for the detection region are not required, but are preferred.

A typical substrate according to the invention comprises a carrier-fluid inlet that is part of and feeds or communicates directly with a first channel, along with one or more plug fluid inlets in communication with the first channel at a plug-forming region situated downstream from the main inlet (each different plug-fluid inlet preferably communicates with the first channel at a different plug-forming region).

Plugs formed from different plug-fluids or solutions may be released in any order. For example, an aqueous solution containing a first plug-fluid may be released through a first inlet at a first plug-forming region. Subsequently, plugs of an aqueous second plug-fluid may be released through a second inlet at a second plug-forming region downstream of the first inlet.

Fabrication of Channels, Substrates, and Devices

The substrates and devices according to the invention are fabricated, for example by etching a silicon substrate, chip, or device using conventional photolithography techniques or micromachining technology, including soft lithography. The fabrication of microfluidic devices using polydimethylsiloxane has been previously described. These and other fabrication methods may be used to provide inexpensive miniaturized devices, and in the case of soft lithography, can provide robust devices having beneficial properties such as improved flexibility, stability, and mechanical strength. Preferably, when optical detection is employed, the invention also provides minimal light scatter from, for example, plugs, carrier-fluid, and substrate material. Devices according to the invention are relatively inexpensive and easy to set up.

Machining methods (e.g., micromachining methods) that may be used to fabricate channels, substrates, and devices according to the invention are well known in the art and include film deposition processes, such as spin coating and chemical vapor deposition, laser fabrication or photolithographic techniques, or etching methods, which maybe performed either by wet chemical or plasma processes.

Channels may be molded onto optically transparent silicone rubber or polydimethylsiloxane (PDMS), preferably PDMS. This can be done, for example, by casting the channels from a mold by etching the negative image of these channels into the same type of crystalline silicon wafer used in semiconductor fabrication. The same or similar techniques for patterning semiconductor features can be used to form the pattern of the channels. In one method of channel fabrication, an uncured PDMS is poured onto the molds placed in the bottom of, for example, a Petri dish. To accelerate curing, the molds are preferably baked. After curing the PDMS, it is removed from on top of the mold and trimmed. Holes may be cut into the PDMS using, for example, a tool such as a cork borer or a syringe needle. Before use, the PDMS channels may be placed in a hot bath of HCl if it is desired to render the surface hydrophilic. The PDMS channels can then be placed onto a microscope cover slip (or any other suitable flat surface), which can be used to form the base/floor or top of the channels.

A substrate according to the invention is preferably fabricated from materials such as glass, polymers, silicon microchip, or silicone elastomers. The dimensions of the substrate may range, for example, between about 0.3 cm to about 7 cm per side and about 1 micron to about 1 cm in thickness, but other dimensions may be used.

A substrate can be fabricated with a fluid reservoir or well at the inlet port, which is typically in fluid communication with an inlet channel. A reservoir preferably facilitates introduction of fluids into the substrate and into the first channel. An inlet port may have an opening such as in the floor of the substrate to permit entry of the sample into the device. The inlet port may also contain a connector adapted to receive a suitable piece of tubing, such as Teflon® tubing, liquid chromatography or HPLC tubing, through which a fluid may be supplied. Such an arrangement facilitates introducing the fluid under positive pressure in order to achieve a desired pressure at the plug-forming region.

A substrate containing the fabricated flow channels and other components is preferably covered and sealed, preferably with a transparent cover, e.g., thin glass or quartz, although other clear or opaque cover materials may be used. Silicon is a preferred substrate material due to well-developed technology permitting its precise and efficient fabrication, but other materials may be used, including polymers such as polytetrafluoroethylenes. Analytical devices having channels, valves, and other elements can be designed and fabricated from various substrate materials. When external radiation sources or detectors are employed, the detection region is preferably covered with a clear cover material to allow optical access to the fluid flow. For example, anodic bonding of a silicon substrate to a PYREX® cover slip can be accomplished by washing both components in an aqueous H_2SO_4/H_2O_2 bath, rinsing in water, and then, for example, heating to about 350° C. while applying a voltage of 450 V.

A variety of channels for sample flow and mixing can be fabricated on the substrate and can be positioned at any location on the substrate, chip, or device as the detection and discrimination or sorting points. Channels can also be designed into the substrate that place the fluid flow at different times/distances into a field of view of a detector. Channels can also be designed to merge or split fluid flows at precise times/distances.

A group of manifolds (a region consisting of several channels that lead to or from a common channel) can be included to facilitate the movement of plugs from different analysis units, through the plurality of branch channels and to the appropriate solution outlet. Manifolds are preferably fabricated into the substrate at different depth levels. Thus, devices according to the invention may have a plurality of analysis units that can collect the solution from associated branch channels of each unit into a manifold, which routes the flow of solution to an outlet. The outlet can be adapted for receiving,

for example, a segment of tubing or a sample tube, such as a standard 1.5 ml centrifuge tube. Collection can also be done using micropipettes.

Methods of Forming Plugs

The various channels, substrates, and devices according to the invention are primarily used to form and manipulate plugs.

In a preferred embodiment, plug-fluids do not significantly mix at or before they are introduced into the first channel. The plug-fluids may form distinct laminar streams at or before the inlet. They may be separated by an additional fluid. Alternatively, they may be introduced into the carrier-fluid via inlets of differing size. The concentration of plug-fluids in the plugs may be adjusted by adjusting volumetric flow rates of the plug-fluids. Further, the diameters of the first channel and the branch channel(s) may differ.

FIG. **2**A is a schematic diagram contrasting laminar flow transport and plug transport in a channel. In the lower figure which depicts the transport of plugs, two aqueous reagents (marked in red and blue) form laminar streams that are separated by a "divider" aqueous stream. The three streams enter a channel with flowing oil, at which point plugs form and plug fluids mix. During plug transport, rapid mixing of the plug-fluids typically occurs within the plugs. In contrast, in laminar flow transport, fluid mixing occurs slowly, and with high dispersion, as shown in the upper figure. In the upper figure, the time t at a given point d_1 can be estimated from $t_1 \approx d_1/U$, where dl is the distance from d=0 and U is the flow velocity. In the lower figure, the time t is given by $t_1 = d_1/U$.

FIG. **2**B shows a photograph and a schematic diagram that depict mixing in water/oil plugs (upper schematic and photograph) and in laminar streams (lower schematic and photograph) comprising only aqueous plug-fluids. The oil (carrier-fluid in this case) is introduced into channel **200** of a substrate. Instead of oil, water is introduced into the corresponding channel **207** in the case of mixing using laminar streams. The three aqueous plug-fluids are introduced by inlet ports **201**, **202**, **203** into the carrier-fluid (and by inlet ports **204**, **205**, **206** in the case of laminar streams). A preferred scheme is one in which the aqueous plug-fluids initially coflow preferably along a short or minimal distance before coming in contact with the carrier-fluid. In a preferred embodiment, the distance traversed by the coflowing plug-fluids is approximately or substantially equal to the width of the channel.

The middle or second aqueous plug-fluid in the top figure may be plain water, buffer, solvent, or a different plug-fluid. The middle aqueous plug-fluid would preferably initially separate the two other aqueous plug-fluids before the aqueous fluids come into contact with the carrier-fluid. Thus, the intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous plug-fluids before they come in contact with the carrier-fluid. The plugs that form in the plug-forming region can continue along an unbranched channel, can split and enter a channel, can merge with plugs from another channel, or can exit the substrate through an exit port. It can be seen in FIG. **2** that, in the absence of an oil, the aqueous plug-fluids flow in laminar streams without significant mixing or with only partial mixing. In contrast, plug-fluids mix substantially or completely in the plugs.

FIG. **3** shows photographs and schematic diagrams that depict a stream of plugs from an aqueous plug-fluid and an oil (carrier-fluid) in curved channels at flow rates of 0.5 μL/min (top schematic diagram and photograph) and 1.0 μL/min (bottom schematic diagram and photograph). This scheme allows enhanced mixing of reagents in the elongated plugs flowing along a curved channel with smooth corners or curves. The carrier-fluid is introduced into an inlet port **300**, **307** of a substrate while the three aqueous plug-fluids are introduced in separate inlet ports **301**-**306**. As in FIG. **2**, a preferred scheme would be one in which the plug-fluids initially coflow preferably along a short or minimal distance before coming in contact with the carrier-fluid. In a preferred embodiment, the distance traversed by the coflowing plug-fluids (e.g., aqueous plug-fluids) is approximately or substantially equal to the width of the channel. The middle or second aqueous plug-fluid may comprise plain water, buffer, solvent, or a plug-fluid, and the middle aqueous plug-fluid preferably initially separates the two other aqueous plug-fluids before the aqueous plug-fluids come into contact with the carrier-fluid which, in this case, is an oil. Thus, the intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous plug-fluids before they come in contact with the oil (or carrier-fluid).

FIG. **4** shows a photograph and schematic diagram that illustrate plug formation through the injection of oil and multiple plug-fluids. Although FIG. **4** shows five separate plug-fluids, one may also separately introduce less than or more than five plug-fluids into the substrate. The reagents or solvents comprising the plug-fluids may be different or some of them may be identical or similar. As in FIG. **2**, the oil is introduced into an inlet port **400** of a substrate while the aqueous plug-fluid is introduced in separate inlet ports **401**-**405**. The water plugs then flow through exit **406**. A preferred scheme is one in which the aqueous plug-fluids would initially coflow preferably along a short or minimal distance before coming in contact with the oil. In a preferred embodiment, the distance traversed by the coflowing plug-fluids is approximately or substantially equal to the width of the channel. One or more of the aqueous plug-fluids may comprise plain water, buffer, solvent, or a plug-fluid, and at least one aqueous plug-fluid would preferably initially separate at least two other aqueous streams before the aqueous plug-fluid comes into contact with the oil. Thus, the at least one intervening aqueous plug-fluid would prevent, delay, or minimize the reaction or mixing of the two outer aqueous streams before the aqueous streams come in contact with the oil. FIG. **5** shows a microfluidic network, which is similar to that shown in FIG. **4**, in which several reagents can be introduced into the multiple inlets. In addition, FIG. **5** shows a channel having a winding portion through which the plugs undergo mixing of the four reagents A, B, C, and D. As shown in FIG. **5**, the reagents A, B, C, and D are introduced into inlet ports **501**, **503**, **505**, and **507**, while aqueous streams are introduced into inlet ports **502**, **504**, **506**. FIG. **5** shows plugs through the various stages of mixing, wherein mixture **50** corresponds to the initial A+B mixture, mixture **51** corresponds to the initial C+D mixture, mixture **52** corresponds to the mixed A+B mixture, mixture **53** corresponds to the mixed C+D mixture, and mixture **54** corresponds to the A+B+C+D mixture.

The formation of the plugs preferentially occurs at low values of the capillary number C.n., which is given by the equation

$$C.n. = U\mu/\gamma \qquad \text{Eqn. (1)}$$

where U is the flow velocity, It is the viscosity of the plug fluid or carrier-fluid, and γis the surface tension at the water/surfactant interface.

The plugs may be formed using solvents of differing or substantially identical viscosities. Preferably, the conditions and parameters used in an experiment or reaction are such that the resulting capillary number lies in the range of about $0.001 \leq C.n. \leq$ about 10. Preferably, the values of parameters such as viscosities and velocities are such that plugs can be

formed reliably. Without wishing to be bound by theory, it is believed that as long as flow is not stopped, the C.n. is ≤ about 0.2, and as long as the surface tension of the plug-fluid/carrier-fluid interface is lower than the surface tension of the solution/wall interface, plug formation will persist. The C.n. number is zero when flow is stopped.

In one embodiment, in which perfluorodecaline was used as the carrier-fluid and the plug-fluid was aqueous, it was found that this system can be operated at values of C.n. up to ~0.1 (at 300 mm s^{-1}). In this system, as the value of the C.n. increased above 0.2, the formation of plugs became irregular. The viscosity of perfluorodecaline is 5.10×10^{-3} kg m^{-3} s^{-1}, the surface tension at the interface between the plugs and the carrier-fluid was 13×10^{-3} N m^{-1}.

The length of the plugs can be controlled such that their sizes can range from, for example, about 1 to 4 times a cross-sectional dimension (d, where d is a channel cross-sectional dimension) of a channel using techniques such as varying the ratio of the plug-fluids and carrier-fluids or varying the relative volumetric flow rates of the plug-fluid and carrier-fluid streams. Short plugs tend to form when the flow rate of the aqueous stream is lower than that of a carrier-fluid stream. Long plugs tend to form when the flow rate of the plug-fluid stream is higher than that of the carrier stream.

In one approximation, the volume of a plug is taken equal to about $2\times d^3$, where d is a cross-sectional dimension of a channel. Thus, the plugs can be formed in channels having cross-sectional areas of, for example, from 20×20 to 200×200 μm^2, which correspond to plug volumes of between about 16 picoliters (pL) to 16 nanoliters (nL). The size of channels may be increased to about 500 μm (corresponding to a volume of about 250 nL) or more. The channel size can be reduced to, for example, about 1 μm (corresponding to a volume of about 1 femtoliter). Larger plugs are particularly useful for certain applications such as protein crystallizations, while the smaller plugs are particularly useful in applications such as ultrafast kinetic measurements.

In one preferred embodiment, plugs conform to the size and shape of the channels while maintaining their respective volumes. Thus, as plugs move from a wider channel to a narrower channel they preferably become longer and thinner, and vice versa.

Plug-fluids may comprise a solvent and optionally, a reactant. Suitable solvents for use in the invention, such as those used in plug-fluids, include organic solvents, aqueous solvents, oils, or mixtures of the same or different types of solvents, e.g. methanol and ethanol, or methanol and water. The solvents according to the invention include polar and non-polar solvents, including those of intermediate polarity relative to polar and non-polar solvents. In a preferred embodiment, the solvent may be an aqueous buffer solution, such as ultrapure water (e.g., 18 MΩ resistivity, obtained, for example, by column chromatography), 10 mM Tris HCl, and 1 mM EDTA (TE) buffer, phosphate buffer saline or acetate buffer. Other solvents that are compatible with the reagents may also be used.

Suitable reactants for use in the invention include synthetic small molecules, biological molecules (i.e., proteins, DNA, RNA, carbohydrates, sugars, etc.), metals and metal ions, and the like.

The concentration of reagents in a plug can be varied. In one embodiment according to the invention, the reagent concentration may be adjusted to be dilute enough that most of the plugs contain no more than a single molecule or particle, with only a small statistical chance that a plug will contain two or more molecules or particles. In other embodiments, the reagent concentration in the plug-fluid is adjusted to concentrate enough that the amount of reaction product can be maximized.

Suitable carrier-fluids include oils, preferably fluorinated oils. Examples include viscous fluids, such as perfluorodecaline or perfluoroperhydrophenanthrene; nonviscous fluids such as perfluorohexane; and mixtures thereof (which are particularly useful for matching viscosities of the carrier-fluids and plug-fluids). Commercially available fluorinated compounds such as Fluorinert™ liquids (3M, St. Paul, Minn.) can also be used.

The carrier-fluid or plug-fluid, or both may contain additives, such as agents that reduce surface tensions (e.g., surfactants). Other agents that are soluble in a carrier-fluid relative to a plug-fluid can also be used when the presence of a surfactant in the plug fluid is not desirable. Surfactants may be used to facilitate the control and optimization of plug size, flow and uniformity. For example, surfactants can be used to reduce the shear force needed to extrude or inject plugs into an intersecting channel. Surfactants may affect plug volume or periodicity, or the rate or frequency at which plugs break off into an intersecting channel. In addition, surfactants can be used to control the wetting of the channel walls by fluids. In one embodiment according to the invention, at least one of the plug-fluids comprises at least one surfactant.

Preferred surfactants that may be used include, but are not limited to, surfactants such as those that are compatible with the carrier and plug-fluids. Exemplary surfactants include Tween™, Span™, and fluorinated surfactants (such as Zonyl™ (Dupont, Wilmington Del.)). For example, fluorinated surfactants, such as those with a hydrophilic head group, are preferred when the carrier-fluid is a fluorinated fluid and the plug-fluid is an aqueous solution.

However, some surfactants may be less preferable in certain applications. For instance, in those cases where aqueous plugs are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort biomaterials, a water soluble surfactant such as SDS may denature or inactivate the contents of the plug.

The carrier-fluid preferably wets the walls of the channels preferentially over the plugs. If this condition is satisfied, the plug typically does not come in contact with the walls of the channels, and instead remains separated from the walls by a thin layer of the carrier-fluid. Under this condition, the plugs remain stable and do not leave behind any residue as they are transported through the channels. The carrier-fluid's preferential wetting of the channel walls over the plug-fluid is achieved preferably by setting the surface tension by, for example, a suitable choice of surfactant. Preferably, the surface tension at a plug fluid/channel wall interface (e.g., about 38 mN/m surface tension for a water/PDMS interface) is set higher than the surface tension at a plug fluid/carrier-fluid interface (e.g., about 13 mN/m for a water/carrier-fluid interface with a surfactant such as 10% 1H,1H,2H,2H-perfluorooctanol in perfluorodecaline as the carrier-fluid). If this condition is not satisfied, plugs tend to adhere to the channel walls and do not undergo smooth transport (e.g., in the absence of 1H,1H,2H,2H-perfluorooctanol the surface tension at the water/perfluorodecaline interface is about 55 mN/m, which is higher than the surface tension of the water/PDMS interface (e.g., about 38 mN/m)), and plugs adhere to the walls of the PDMS channels. Because the walls of the channels (PDMS, not fluorinated) and the carrier-fluid (fluorinated oil) are substantially different chemically, when a fluorinated surfactant is introduced, the surfactant reduces the surface tension at the oil-water interface preferentially over

the wall-water interface. This allows the formation of plugs that do not stick to the channel walls.

The surface tension at an interface may be measured using what is known as a hanging drop method, although one may also use other methods. Preferably, the surface tension is sufficiently high to avoid destruction of the plugs by shear.

The plug-fluids and carrier-fluids may be introduced through one or more inlets. Specifically, fluids may be introduced into the substrate through pneumatically driven syringe reservoirs that contain either the plug-fluid or carrier-fluid. Plugs may be produced in the carrier-fluid stream by modifying the relative pressures such that the plug-fluids contact the carrier-fluid in the plug-forming regions then shear off into discrete plugs.

In the invention, plugs are formed by introducing the plug-fluid, at the plug-forming region, into the flow of carrier-fluid passing through the first channel. The force and direction of flow can be controlled by any desired method for controlling flow, for example, by a pressure differential, or by valve action. This permits the movement of the plugs into one or more desired branch channels or outlet ports.

In preferred embodiments according to the invention, one or more plugs are detected, analyzed, characterized, or sorted dynamically in a flow stream of microscopic dimensions based on the detection or measurement of a physical or chemical characteristic, marker, property, or tag.

The flow stream in the first channel is typically, but not necessarily continuous and may be stopped and started, reversed or changed in speed. Prior to sorting, a non-plug-fluid can be introduced into a sample inlet port (such as an inlet well or channel) and directed through the plug-forming region, e.g., by capillary action, to hydrate and prepare the device for use. Likewise, buffer or oil can also be introduced into a main inlet port that communicates directly with the first channel to purge the substrate (e.g., of "dead" air) and prepare it for use. If desired, the pressure can be adjusted or equalized, for example, by adding buffer or oil to an outlet port.

The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug-forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs. Periodicity and plug volume may also depend on channel diameter and/or the viscosity of the fluids.

Mixing in Plugs

FIG. **7** (*a*)-(*b*) show microphotographs (10 μs exposure) illustrating rapid mixing inside plugs (a) and negligible mixing in a laminar flow (b) moving through winding channels at the same total flow velocity. Aqueous streams were introduced into inlets **700**-**705** in FIGS. **7**(*a*)-(*b*). In FIGS. **7**(*c*) and **7**(*e*), Fluo-4 was introduced into inlets **706**, **709**, buffer was introduced into inlets **707**, **710**, and $CaCl_2$ was introduced into inlets **708**, **711**. FIG. **7**(*c*) shows a false-color microphotograph (2 s exposure, individual plugs are invisible) showing time-averaged fluorescence arising from rapid mixing inside plugs of solutions of Fluo-4 (54 μM) and $CaCl_2$ (70 μM) in aqueous sodium morpholine propanesulfonate buffer (20 μM, pH 7.2); this buffer was also used as the middle aqueous stream. FIG. **7**(*d*) shows a plot of the relative normalized intensity (I) of fluorescence obtained from images such as shown in (c) as a function of distance (left) traveled by the plugs and of time required to travel that distance (right) at a given flow rate. The total intensity across the width of the channel was measured. Total PFD/water volumetric flow rates (in μL min^{-1}) were 0.6:0.3, 1.0:0.6, 12.3:3.7, 10:6, and 20:6. FIG. **7**(*e*) shows a false-color microphotograph (2 s exposure) of the weak fluorescence arising from negligible mixing in a laminar flow of the solutions used in (c). All channels were 45 μm deep; inlet channels were 50 μm and winding channels 28 μm wide; Re~5.3 (water), ~2.0 (PFD).

FIG. **8** shows photographs and schematics that illustrate fast mixing at flow rates of about 0.5 μL/min (top schematic diagram and photograph) and about 1.0 μL/min (lower schematic diagram and photograph) using 90°-step channels while FIG. **9** illustrates fast mixing at flow rates of about 1.0 μL/min (top schematic diagram and photograph) and about 0.5 μL/min (lower schematic diagram and photograph) using 135°-step channels. Aqueous streams are introduced into inlets **800**-**805** in FIG. **8** (inlets **900**-**905** in FIG. **9**), while a carrier fluid is introduced into channels **806**, **807** (channels **906**, **907** in FIG. **9**). The plugs that form then flow through exits **808**, **809** (FIG. **8**) and exits **908**, **909** (FIG. **9**). As can be seen in FIG. **8** and FIG. **9**, the plugs are transported along multi-step channels, instead of channels with smooth curves (as opposed to channels with sharp corners). An advantage of these multi-step configurations of channels is that they may provide further enhanced mixing of the substances within the plugs.

Several approaches may be used to accelerate or improve mixing. These approaches may then be used to design channel geometries that allow control of mixing. Flow can be controlled by perturbing the flow inside a moving plug so that it differs from the symmetric flow inside a plug that moves through a straight channel. For example, flow perturbation can be accomplished by varying the geometry of a channel (e.g., by using winding channels), varying the composition of the plug fluid (e.g., varying the viscosities), varying the composition of the carrier-fluid (e.g., using several laminar streams of carrier-fluids that are different in viscosity or surface tension to form plugs; in this case, mixing is typically affected, and in some cases enhanced), and varying the patterns on the channel walls (e.g., hydrophilic and hydrophobic, or differentially charged, patches would interact with moving plugs and induce time-periodic flow inside them, which should enhance mixing).

Various channel designs can be implemented to enhance mixing in plugs. FIG. **1**A shows a schematic of a basic channel design, while FIG. **1**B shows a series of periodic variations of the basic channel design. FIG. **1**C shows a series of aperiodic combinations resulting from a sequence of alternating elements taken from a basic design element shown in FIG. **1**A and an element from the periodic variation series shown in FIGS. **1**B(**1**)-(**4**). When the effects of these periodic variations are visualized, aperiodic combinations of these periodic variations are preferably used to break the symmetries arising from periodic flows (see FIG. **1**C). Here, the relevant parameters are channel width, period, radius of curvature, and sequence of turns based on the direction of the turns. The parameters of the basic design are defined such that c is the channel width, l is the period, and r is the radius of curvature. For the basic design, the sequence can be defined as (left, right, left, right), where left and right is relative to a centerline along the path taken by a plug in the channel.

FIGS. **1**B(**1**)-**4**) show schematic diagrams of a series of periodic variations of the basic design. At least one variable parameter is preferably defined based on the parameters defined in FIG. **1***a*). In FIG. **1**B(**1**), the channel width is c/2; in FIG. **1**B(**2**), the period is 2 l; and in FIG. **1**B(**3**), and the radius

of curvature is 2r. In FIG. **1**B(**4**), the radius of curvature is r/2 and the sequence is (left, left, right, right).

FIGS. **1**C(**1**)-(**4**) show a schematic diagram of a series of aperiodic combinations formed by combining the basic design element shown in FIG. **1**A with an element from the series of periodic variations in FIG. **1**B(**1**)-(**4**). In FIG. **1**C(**1**), the alternating pattern of a period of the basic design shown in FIG. **1**A (here denoted as "a") and a period of the channel in FIG. **1**B(**1**) (here denoted as "b**1**") is given by a+b**1**+a+ . . . In FIG. **1**C(**2**), the aperiodic combination is given by a+b**2**+a. In the channel shown in FIG. **1**C(**3**) (here denoted as "c**3**"), the aperiodic combination is given by a+c**3**+a. In the channel shown in FIG. **1**C(**4**) (here denoted as "c**4**"), a (right, left) sequence is introduced with a kink in the pattern. A repeating (left, right) sequence would normally be observed. By adding this kink, the sequence becomes (left, right, left, right)+(right, left)+(left, right, left, right).

Another approach for accelerating mixing relies on rationally-designed chaotic flows on a microfluidic chip using what is known as the baker's transformation. Reorientation of the fluid is critical for achieving rapid mixing using the baker's transformation. The baker's transformation leads to an exponential decrease of the striation thickness (the distance over which mixing would have to occur by diffusion) of the two components via a sequence of stretching and folding operations. Typically, every stretch-fold pair reduces the striation thickness by a factor of 2, although this factor may have a different value. The striation thickness (ST) can be represented, in an ideal case, by Eqn. (2) below. Thus, in the ideal case, in a sequence of n stretch-fold-reorient operations, the striation thickness undergoes an exponential decrease given by

$$ST(t_n)=ST(t_0)\times 2^{-n} \quad \text{Eqn. (2)}$$

where $ST(t_n)$ represents the striation thickness at time t_n, $ST(t_0)$ represents the initial striation thickness at time t_0, and n is the number of stretch-fold-reorient operations.

In accordance with the invention, the baker's transformation is preferably implemented by creating channels composed of a sequence of straight regions and sharp turns. FIG. **11** shows a schematic diagram of a channel geometry designed to implement and visualize the baker's transformation of plugs flowing through microfluidic channels. Other designs could also be used. The angles at the channel bends and the lengths of the straight portions are chosen so as to obtain optimal mixing corresponding to the flow patterns shown. Different lengths of straight paths and different turns may be used depending on the particular application or reaction involved.

A plug traveling through every pair of straight part **112** and sharp-turn part **111** of the channel, which is equivalent to one period of a baker's transformation, will experience a series of reorientation, stretching and folding. In a straight part of the channel, a plug will experience the usual recirculating flow. At a sharp turn, a plug normally rolls and reorients due to the much higher pressure gradient across the sharp internal corner and also due to larger travel path along the outside wall. This method of mixing based on the baker's transformation is very efficient and is thus one of the preferred types of mixing. In particular, this type of mixing leads to a rapid reduction of the time required for reagent mixing via diffusion.

It is believed that plug formation can be maintained at about the same flow rate in channels of different sizes because the limit of a flow rate is typically set by the capillary number, C.n., which is independent of the channel size. At a fixed flow rate, the mixing time t_{mix} may decrease as the size of the channel (d) is reduced. First, it is assumed that it takes the same number n of stretch-fold-reorient cycles to mix reagents in both large and small channels. This assumption (e.g., for n~5) is in approximate agreement with previously measured mixing in d=55 and d=20 micrometer (μm) channels. Each cycle requires a plug to travel over a distance of approximately 2 lengths of the plug (approximately 3d). Therefore, mixing time is expected to be approximately equal to the time it takes to travel **15***d*, and will decrease linearly with the size of the channel, t_{mix}~d. A method that provides mixing in about 1 ms in 25-μm channels preferably provides mixing in about 40 μs in 1-μm channels. Achieving microsecond mixing times generally requires the use of small channels. High pressures are normally required to drive a flow through small channels.

Without wishing to be bound by theory, theoretical modeling indicates that the number of cycles it takes for mixing to occur in a channel with diameter d is given approximately by

$$n\times 2^{2n}\approx dU/D \quad \text{Eqn. (3)}$$

where n is the number of cycles, U is the flow velocity, D is the diffusion constant, one cycle is assumed to be equal to 6d, and mixing occurs when convection and diffusion time scales are matched. The mixing time is primarily determined by the number of cycles. This result indicates that mixing will be accelerated more than just in direct proportion to the channel diameter. For example, when d decreases by a factor of 10, mixing time decreases by a factor of d×Log(d)=10×Log(10). With properly designed channels, mixing times in 1-μm channels can be limited to about 20 μs. Even at low flow rates or long channels (such as those involving protein crystallization), however, significant mixing can still occur. In addition, without being bound by theory, it is expected that increasing the flow rate U by a factor of 10 will decrease the mixing time by a factor of Log(U)/U=(Log (10))/10.

To visualize mixing in a channel according to the invention, a colored marker can be used in a single plug-fluid. The initial distribution of the marker in the plug has been observed to depend strongly on the details of plug formation. As the stationary aqueous plug was extruded into the flowing carrier-fluid, shearing interactions between the flow of the carrier-fluid and the plug-fluid induced an eddy that redistributed the solution of the marker to different regions of the plug. The formation of this eddy is referred to here as "twirling" (see FIG. **27***b*)). Twirling is not a high Reynolds number (R_e) phenomenon (see FIG. **30**) since it was observed at substantially all values of R_e and at substantially all velocities. However, the flow pattern of this eddy appears to be slightly affected by the velocity.

Various characteristics and behavior of twirling were observed. Twirling redistributed the marker by transferring it from one side of the plug to the other, e.g., from the right to the left side of the plug. The most efficient mixing was observed when there was minimal fluctuations in intensity, i.e., when the marker was evenly distributed across the plug. While twirling was present during the formation of plugs of all lengths that were investigated, its significance to the mixing process appears to depend on the length of the plug. For example, the extent of twirling was observed to be significantly greater for short plugs than for long plugs. Twirling was also observed to affect only a small fraction of the long plugs and had a small effect on the distribution of the marker in the plugs. Moreover, twirling occurred only at the tip of the forming plug before the tip made contact with the right wall of the microchannel. Also, the amount of twirling in a plug was observed to be related to the amount of the carrier-fluid that flowed past the tip. The results of experiments involving twirling and its effect on mixing show that twirling is one of

the most important factors, if not the most important factor, in determining the ideal conditions for mixing occurring within plugs moving through straight channels. By inducing twirling, one may stimulate mixing; by preventing twirling, one may suppress complete mixing. Suppressing mixing may be important in some of the reaction schemes, for example those shown in FIG. **5** and FIG. **6**. In these reaction schemes, selective mixing of reagents A with reagent B, and also reagent C with reagent D, can occur without mixing of all four reagents. Mixing of all four reagents occurs later as plugs move through, for example, the winding part of the channel. This approach allows several reactions to occur separated in time. In addition, suppressing mixing may be important when interfaces between plug fluids have to be created, for example interfaces required for some methods of protein crystallization (FIG. **20**).

The eddy at the tip of a developing plug may complicate visualization and analysis of mixing. This eddy is normally significant in short plugs, but only has a minor effect on long plugs. For applications involving visualization of mixing, the substrate is designed to include a narrow channel in the plug-forming region is designed such that narrow, elongated plugs form. Immediately downstream from the plug-forming region, the channel dimension is preferably expanded. In the expanded region of the channel(s), plugs will expand and become short and rounded under the force of surface tension; this preserves the distribution of the marker inside the plugs. This approach affords a relatively straightforward way of visualizing the mixing inside plugs of various sizes. Video microscopy may be used to observe the distribution of colored markers inside the drops. A confocal microscope may also be used to visualize the average three-dimensional distribution of a fluorescent marker. Visualization can be complemented or confirmed using a Ca^{2+}/Fluo-4^{-4} reaction. At millimolar concentrations, this reaction is expected to occur with a half-life of about 1 μs. Thus, it can be used to measure mixing that occurs on time scales of about 10 μs and longer.

The following discussion describes at least one method for three-dimensional visualization of flows in plugs. Visualization of chaotic transport in three-dimensions is a challenging task especially on a small scale. Predictions based on two-dimensional systems may be used to gain insight about plugs moving through a three-dimensional microfluidic channel. Experiments and simulations involving a two-dimensional system can aid in the design of channels that ensure chaotic flow in two-dimensional liquid plugs. Confocal microscopy has been used to quantify steady, continuous three-dimensional flows in channels. However, due to instrumental limitations of an optical apparatus such as a confocal microscope, it is possible that the flow cannot be visualized with sufficiently high-resolution to observe, for example, self-similar fractal structures characteristic of chaotic flow. Nonetheless, the overall dynamics of the flow may still be captured and the absence of non-chaotic islands confirmed. Preferably, the channels (periodic or aperiodic) used in the visualization process are fabricated using soft lithography in PDMS. A PDMS replica is preferably sealed using a thin glass cover slip to observe the flow using confocal microscopy.

In one experiment according to the invention, a series of line scans are used to obtain images of a three-dimensional distribution of fluorescent markers within the plugs. FIG. **10***a*) is a schematic diagram depicting a three-dimensional confocal visualization of chaotic flows in plugs. Plugs are preferably formed from three laminar streams. The middle stream **11** preferably contains fluorescent markers. Preferably, the middle stream **11** is injected into the channel system at a low volumetric flow rate. The volumetric flow rates of the two side streams **10**, **12** are preferably adjusted to position the marker stream in a desired section of the channel. Preferably, a confocal microscope such as a Carl Zeiss LSM 510 is used. The LSM 510 is capable of line scans at about 0.38 ms/512 pixel line or approximately 0.2 ms/100 pixel line. Fluorescent microspheres, preferably about 0.2 μm, and fluorescently labeled high-molecular weight polymers are preferably used to visualize the flow with minimal interference from diffusion. A channel such as one with 100 μm wide and 100 μm deep channel may be used. The line scan technique may be applied to various sequences such as one that has about 200-μm long plugs separated by about 800-μm long oil stream.

A beam is preferably fixed in the x and z-directions and scanned repeatedly back and forth along the y-direction. The movement of the plug in the x-direction preferably provides resolution along the x-direction. Line scan with 100 pixels across a 100 μm-wide channel will provide a resolution of about 1 μm/pixel in the y-direction. Approximately 200 line scans per plug are preferably used to give a resolution of about 1 μm/pixel in the x-direction. For a 200 μm plug moving at about 2000 μm/s, about 200 line scans are preferably obtained over a period of about (200 μm)/(2000 μm/s)=0.1 s, or about 0.5 ms per line.

The sequence shown in FIG. **10***b*) is preferably used for visualization of a three dimensional chaotic flow. Each line scan preferably takes about 0.2 ms with about 0.3 ms lag between the scans to allow the plug to move by about 500 μm. Some optical distortions may result during the approximately 0.2 ms scan as the plug is translated along the x-direction by about 0.2 μm. However, these distortions are believed to be comparable to the resolution of the method. For a given position along the x-direction, a series of line scans are preferably obtained for about 10 seconds for each point along the z-direction to obtain an x-y cross-sections of ten plugs. Scans along the z-direction are preferably taken in 1 μm increments to obtain a full three-dimensional image of the distribution of the fluorescent marker in the plug. This procedure is preferably repeated at different positions along the x-direction to provide information such as changes in the three-dimensional distribution of the fluorescent marker inside the plug as the plug moves along the channels.

In case of periodic perturbations, the fluorescent cross-sections of the plug in the y-z plane recovered from the above procedure represent Poincaré sections corresponding to the evolution of the initial thin sheet of dye. The twirling of the aqueous phase upon formation of the small plugs could distribute the dye excessively throughout the plug and could make visualization less conclusive. This twirling is prevented preferably by designing a small neck in the plug-forming region, and then beginning the first turn in a downward direction. This approach has been successfully applied to flow visualization, and may be useful for conducting reactions.

Merging Plugs

The invention also provides a method of merging of plugs within a substrate (see upper portion of FIG. **12**). Plugs are formed as described above. Plugs containing different reagents can be formed by separately introducing different plug-fluids into a channel. The plugs containing different reagents may be substantially similar in viscosity or may differ. The plugs containing different reagents may be substantially similar in size or they may differ in size. Provided that the relative velocities of the plugs containing different reagents differ, the plugs will merge in the channels. The location of merging can be controlled in a variety of ways, for example by varying the location of plug-fluid inlet ports, by varying the location of channel junctions (if one of the plug

forming fluids is introduced into a secondary channel), varying the size of the plugs, adjusting the speed at which different sets of plugs are transported varying the viscosity or surface tension of plugs having substantially the same size, etc.

As shown in FIG. **12** (top photograph), plugs may be merged by directing or allowing the plugs **120**, **121** to pass through a T-shaped channel or a T-shaped region of a channel. The resulting merged plugs **122** flow in separate channels or channel branches which may be perpendicular, as shown in FIG. **12**, or nonperpendicular (FIG. **33**). The merged plugs **122** may undergo further merging or undergo splitting, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more reactions or "treatments" such as one or more types of characterizations, measurements, detection, sorting, or analysis.

In one embodiment, large and small plugs flow along separate channels or channel branches towards a common channel where they merge. In a case where a large and a small plug do not converge at the same point at the same time, they eventually form a merged plug as the larger plug, which moves faster than the smaller plug, catches up with the small plug and merges with it. In the case where the larger and smaller plugs meet head on at the same point or region, they immediately combine to form a merged plug. The merged plugs may undergo splitting, described below, or further merging in other channels or channel regions, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more types of characterizations, measurements, detection, sorting, or analysis.

In another embodiment, plugs can be merged by controlling the arrival time of the plugs flowing in opposite directions towards a common point, area, or region of the channel so that each pair of plugs arrive at the common point, area, or region of the channel at around the same time to form a single plug.

In another embodiment, an arched, semi-circular, or circular channel provides a means for increasing the efficiency of plug merging. Thus, for example, a greater frequency of merging would occur within a more compact area or region of the substrate. Using this scheme, plugs flowing along separate channels towards a common channel may merge within a shorter distance or a shorter period of time because the arched, semi-circular, or circular channel or channel branch converts or assists in converting initially out-of-phase plug pairs to in-phase plug pairs. Specifically, the arched, semi-circular, or circular channel or channel branch would allow a lagging plug to catch up and merge with a plug ahead of it, thereby increasing the number of merged plugs in a given period or a given area or region of a substrate.

Splitting and/or Sorting Plugs

The present invention also provides a method for splitting of plugs within a substrate. Plugs can be split by passing a first portion of a plug into a second channel through an opening, wherein the second channel is downstream of where the plug is formed. Alternatively, plugs may be split at a "Y" intersection in a channel. In both embodiment, the initial plug splits into a first portion and a second portion and thereafter each portion passes into separate channel (or outlet). Either initially formed plugs can be split or, alternatively, merged plugs can be split. FIG. **6** shows a schematic diagram illustrating part of a microfluidic network that uses multiple inlets (inlets **601**, **603**, **605**, **607** for reagents A, B, C, and D; inlets **602**, **604**, **606** for aqueous streams) and that allows for both splitting and merging of plugs. This schematic diagram shows two reactions that are conducted simultaneously. A third reaction (between the first two reaction mixtures) is conducted using precise time delay. Plugs can be split before or after a reaction has occurred. In addition, FIG. **6** shows plugs at various stages of mixing from the initial mixture **60** (A+B) and initial mixture **61** (C+D) through the mixed solutions **62** (A+B), **63** (C+D), and the 4-component mixture **64** (A+B+C+D).

As shown in FIG. **12** (lower photograph), plugs may be split by directing or allowing the plugs **123**, **124** to pass through a T-shaped channel or a T-shaped region of a channel. In a preferred embodiment, the area or junction at which the plugs undergo splitting may be narrower or somewhat constricted relative to the diameter of the plugs a certain distance away from the junction. The resulting split plugs **125** flow in separate channels or channel branches which may be perpendicular, as shown in FIG. **12**, or nonperpendicular (FIG. **33**). The split **125** plugs may undergo merging or further splitting, or they may be directed to other channels, channel branches, area, or region of the substrate where they may undergo one or more reactions or "treatments" such as one or more types of characterizations, measurements, detection, sorting, or analysis.

In another embodiment, aqueous plugs can be split or sorted from an oil carrier fluid by using divergent hydrophilic and hydrophobic channels. The channels are rendered hydrophilic or hydrophobic by pretreating a channel or region of a channel such that a channel or channel surface becomes predominantly hydrophilic or hydrophobic. As discussed in more detail below, substrates with hydrophilic channel surfaces may be fabricated using methods such as rapid prototyping in polydimethylsiloxane. The channel surface can be rendered hydrophobic either by silanization or heat treatment. For example, (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.) vapor may be applied to the inlets of the substrate with dry nitrogen as a carrier gas to silanize the channel surface.

Once plugs have been split into separate channels, further reactions can be performed by merging the split plugs with other plugs containing further reactants.

Manipulation of plugs and reagents/products contained therein can also be accomplished in a fluid flow using methods or techniques such as dielectrophoresis. Dielectrophoresis is believed to produce movement of dielectric objects, which have no net charge, but have regions that are positively or negatively charged in relation to each other. Alternating, nonhomogeneous electric fields in the presence of plugs and/or particles, cause the plugs and/or particles to become electrically polarized and thus to experience dielectrophoretic forces. Depending on the dielectric polarizability of the particles and the suspending medium, dielectric particles will move either toward the regions of high field strength or low field strength. Using conventional semiconductor technologies, electrodes can be fabricated onto a substrate to control the force fields in a micro fabricated device. Dielectrophoresis is particularly suitable for moving objects that are electrical conductors. The use of AC current is preferred, to prevent permanent alignment of ions. Megahertz frequencies are suitable to provide a net alignment, attractive force, and motion over relatively long distances.

Radiation pressure can also be used in the invention to deflect and move plugs and reagents/products contained therein with focused beams of light such as lasers. Flow can also be obtained and controlled by providing a thermal or pressure differential or gradient between one or more channels of a substrate or in a method according to the invention.

Preferably, both the fluid comprising the plugs and the carrier fluid have a relatively low Reynolds Number, for example 10^{-2}. The Reynolds Number represents an inverse relationship between the density and velocity of a fluid and its viscosity in a channel of given cross-sectional dimension.

More viscous, less dense, slower moving fluids will have a lower Reynolds Number, and are easier to divert, stop, start, or reverse without turbulence. Because of the small sizes and slow velocities, fabricated fluid systems are often in a low Reynolds number regime (R_e<<1). In this regime, inertial effects, which cause turbulence and secondary flows, are negligible and viscous effects dominate the dynamics. These conditions are advantageous for analysis, and are provided by devices according to the invention. Accordingly the devices according to the invention are preferably operated at a Reynolds number of less than 100, typically less than 50, preferably less than 10, more preferably less than 5, most preferably less than 1.

Detection and Measurement

The systems of the present invention are well suited for performing optical measurements using an apparatus such as a standard microscope. For example, PDMS is transparent in the visible region. When it is used to construct a substrate, a glass or quartz cover slip can be used to cover or seal a PDMS network, thereby constructing a set of channels that can be characterized using visible, UV, or infrared light. Preferably, fluorescent measurements are performed, instead of absorption measurements, since the former has a higher sensitivity than the latter. When the plugs are being monitored by optical measurements, the refractive index of the carrier-fluid and the plug-fluids are preferably substantially similar, but they can be different in certain cases.

In a plug-based system according to the invention, the relative concentrations (or changes in concentrations) can be typically measured in a straightforward fashion. In some instances, the use of plugs to perform quantitative optical measurements of, for example, absolute concentrations is complicated by the presence of non-horizontal oil/water interfaces surrounding the plugs. These curved interfaces act as lenses, and may lead to losses of emitted light or optical distortions. Such distortions may adversely affect or prevent visual observation of growing protein crystals, for example. Exact modeling of these losses is usually difficult because of the complicated shape that this interface may adopt at the front and back of a plug moving in a non-trivial pressure gradient.

This problem can be overcome or minimized in accordance with the invention by using a technique such as refractive index matching. The losses and distortions depend on the difference between the refractive index (ηD) of the aqueous phase and the refractive index of the immiscible carrier-fluid. Preferably, the carrier-fluid used in an analysis have refractive indices that are substantially similar to those of water and aqueous buffers (TABLE 1), e.g., fluorinated oils having refractive indices near that of water close to the sodium D line at 589 nm.

Preferably, for applications involving detection or measurement, the carrier-fluids used are those having refractive indices that match those of commonly used aqueous solutions at the wavelengths used for observation. To calibrate a system for quantitative fluorescence measurements, the plugs preferably contain known concentrations of fluorescein. Preferably, the fluorescence originating from the plugs are measured and then compared with the fluorescence arising from the same solution of fluorescein in the channel in the absence of oil. It is believed that when the refractive indexes are matched, the intensity (I) of fluorescence arising from the plugs will be substantially similar or equal to the intensity of the fluorescence from the aqueous solutions after making adjustments for the fraction of the aqueous stream:

$$I_{plug}=I_{solution}*V_{water}/(V_{water}+V_{oil}) \quad \text{Eqn. (3)}$$

where V is the volumetric flow rate of the fluid streams. It is expected that smaller plugs with a higher proportion of curved interfaces will show larger deviations from ideal plug behavior, i.e., those smaller plugs will tend to cause greater optical distortion. If necessary, measurements are performed partly to determine the errors associated with refractive index mismatch. Information from these measurements is useful when unknown fluids are analyzed, or when a compromise between matching the refractive index and matching the viscosities of the two fluids is required.

TABLE 1

Physical properties of some fluids used in certain embodiments of the microfluidic devices.

Fluid	Refractive index, η_D	Viscosity, μ[mPa- s]
water	1.3330	1.00
aqueous PBS buffer, 1%	1.3343	1.02
aqueous PBS buffer, 10%	1.3460	1.25
perfluorohexane	1.251	0.66
perfluoro(methylcyclohexane)	1.30	1.56
perfluoro(1,3-dimethylcyclohexane)	1.2895	1.92
perfluorodecaline	1.314	5.10
perfluoroperhydrofluorene	1.3289	9.58
perfluoroperhydrophenanthrene	1.3348	28.4
perfluorotoluene	1.3680	N/A
hexafluorobenzene	1.3770	N/A

The detector can be any device or method for evaluating a physical characteristic of a fluid as it passes through the detection region. Examples of suitable detectors include CCD detectors. A preferred detector is an optical detector, such as a microscope, which may be coupled with a computer and/or other image processing or enhancement devices to process images or information produced by the microscope using known techniques. For example, molecules can be analyzed and/or sorted by size or molecular weight. Reactions can be monitored by measuring the concentration of a product produced or the concentration of a reactant remaining at a given time. Enzymes can be analyzed and/or sorted by the extent to which they catalyze a chemical reaction of an enzyme's substrate (conversely, an enzyme's substrate can be analyzed (e.g., sorted) based on the level of chemical reactivity catalyzed by an enzyme). Biological particles or molecules such as cells and virions can be sorted according to whether they contain or produce a particular protein, by using an optical detector to examine each cell or virion for an optical indication of the presence or amount of that protein. A chemical itself may be detectable, for example by a characteristic fluorescence, or it may be labeled or associated with a tag that produces a detectable signal when, for example, a desired protein is present, or is present in at least a threshold amount.

Practically any characteristic of a chemical can be identified or measured using the techniques according to the invention, provided that the characteristic or characteristics of interest for analysis can be sufficiently identified and detected or measured to distinguish chemicals having the desired characteristic(s) from those which do not. For example, particulate size, hydrophobicity of the reagent versus carrier-fluids, etc. can be used as a basis for analyzing (e.g., by sorting) plug-fluids, reaction products or plugs.

In a preferred embodiment, the plugs are analyzed based on the intensity of a signal from an optically detectable group, moiety, or compound (referred to here as "tag") associated with them as they pass through a detection window or detection region in the device. Plugs having an amount or level of the tag at a selected threshold or within a selected range can be

directed into a predetermined outlet or branch channel of the substrate. The tag signal may be collected by a microscope and measured by a detector such as a photomultiplier tube (PMT). A computer is preferably used to digitize the PMT signal and to control the flow through methods such as those based on valve action. Alternatively, the signal can be recorded or quantified as a measure of the tag and/or its corresponding characteristic or marker, e.g., for the purpose of evaluation and without necessarily proceeding to, for example, sort the plugs.

In one embodiment according to the invention, a detector such as a photodiode is larger in diameter than the width of the channel, forming a detection region that is longer (along the length of channel) than it is wide. The volume of such a detection region is approximately equal to the cross sectional area of the channel above the diode multiplied by the diameter of the diode.

To detect a chemical or tag, or to determine whether a chemical or tag has a desired characteristic, the detection region may include an apparatus (e.g., a light source such as a laser, laser diode, high intensity lamp such as mercury lamp) for stimulating a chemical or tag for that characteristic to, for example, emit measurable light energy. In embodiments where a lamp is used, the channels are preferably shielded from light in all regions except the detection region. In embodiments where a laser is used, the laser can be set to scan across a set of detection regions. In addition, laser diodes may be fabricated into the same substrate that contains the analysis units. Alternatively, laser diodes may be incorporated into a second substrate (i.e., a laser diode chip) that is placed adjacent to the analysis or sorter substrate such that the laser light from the diodes shines on the detection region(s).

In preferred embodiments, an integrated semiconductor laser and/or an integrated photodiode detector are included on the silicon wafer in the device according to the invention. This design provides the advantages of compactness and a shorter optical path for exciting and/or emitted radiation, thus minimizing, for example, optical distortion.

As each plug passes into the detection region, it may be examined for a characteristic or property, e.g., a corresponding signal produced by the plug, or the chemicals contained in the plugs, may be detected and measured to determine whether or not a given characteristic or property is present. The signal may correspond to a characteristic qualitatively or quantitatively. Typically, the amount of signal corresponds to the degree to which a characteristic is present. For example, the strength of the signal may indicate the size of a molecule, the amount of products(s) formed in a reaction, the amount of reactant(s) remaining, the potency or amount of an enzyme expressed by a cell, a positive or negative reaction such as binding or hybridization of one molecule to another, or a chemical reaction of a substrate catalyzed by an enzyme. In response to the signal, data can be collected and/or a flow control can be activated, for example, to direct a plug from one channel to another. Thus, for example, chemicals present in a plug at a detection region may be sorted into an appropriate branch channel according to a signal produced by the corresponding examination at a detection region. Optical detection of molecular characteristics or the tag associated with a characteristic or property that is chosen for sorting, for example, may be used. However, other detection techniques, for instance electrochemistry, or nuclear magnetic resonance, may also be employed.

In one embodiment according to the invention, a portion of a channel corresponds to an analysis unit or detection region and includes a detector such as a photodiode preferably located in the floor or base of the channel. The detection region preferably encompasses a receive field of the photodiode in the channel, which receive field has a circular shape. The volume of the detection region is preferably the same as, or substantially similar, to the volume of a cylinder with a diameter equal to the receive field of the photodiode and a height equal to the depth of the channel above the photodiode.

The signals from the photodiodes may be transmitted to a processor via one or more lines representing any form of electrical communication (including e.g. wires, conductive lines etched in the substrate, etc.). The processor preferably acts on the signals, for example by processing them into values for comparison with a predetermined set of values for analyzing the chemicals. In one embodiment, a value corresponds to an amount (e.g., intensity) of optically detectable signal emitted from a chemical which is indicative of a particular type or characteristic of a chemical giving rise to the signal. The processor preferably uses this information (i.e., the values) to control active elements in a discrimination region, for example to determine how to sort the chemicals (e.g., valve action).

When more than one detection region is used, detectors such as photodiodes in a laser diode substrate are preferably spaced apart relative to the spacing of the detection regions in the analysis unit. That is, for more accurate detection, the detectors are placed apart at the same spacing as the spacing of the detection region.

A processor can be integrated into the same substrate that contains at least one analysis unit, or it can be separate, e.g., an independent microchip connected to the analysis unit containing substrate via electronic leads that connect to the detection region(s) and/or to the discrimination region(s), such as by a photodiode. The processor can be a computer or microprocessor, and is typically connected to a data storage unit, such as computer memory, hard disk, or the like, and/or a data output unit, such as a display monitor, printer and/or plotter.

The types and numbers of chemicals based on the detection of, for example, a tag associated with or bound to the chemical passing through the detection region, can be calculated or determined, and the data obtained can be stored in the data storage unit. This information can then be further processed or routed to a data outlet unit for presentation, e.g. histograms representing, for example, levels of a protein, saccharide, or some other characteristic of a cell surface in the sample. The data can also be presented in real time as the sample flows through a channel.

If desired, a substrate may contain a plurality of analysis units, i.e., more than one detection region, and a plurality of branch channels that are in fluid communication with and that branch out from the discrimination regions. It will be appreciated that the position and fate of the reagents in the discrimination region can be monitored by additional detection regions installed, for example, immediately upstream of the discrimination region and/or within the branch channels immediately downstream of the branch point. The information obtained by the additional detection regions can be used by a processor to continuously revise estimates of the velocity of the reagents in the channels and to confirm that molecules, particles, and substances having a selected characteristic enter the desired branch channel.

In one embodiment, plugs are detected by running a continuous flow through a channel, taking a spatially resolved image with a CCD camera, and converting the relevant distance traversed by the plugs into time.

In another embodiment, plugs are detected following their exit through a channel point leading to a mass spectrometer (MS), e.g., an electrospray MS. In this embodiment, time-resolved information (e.g., mass spectrum) can be obtained

when the flow rate and the distance traversed by the plugs are known. This embodiment is preferable when one wants to avoid using a label.

Varying the Concentration of Reagents Inside Plugs

The various devices and methods according to the invention allow the control and manipulation of plug composition and properties. For example, they allow the variation of reagent concentration inside plugs. In one aspect according to the invention, the concentrations of the reagents in the plugs are varied by changing the relative flow rates of the plug-fluids. This is possible in conventional systems, but is complicated by problems of slow mixing and dispersion. Methods according to the invention are convenient for simultaneously testing a large number of experimental conditions ("screening") because the concentrations can be changed within a single setup. Thus, for example, syringes do not have to be disconnected or reconnected, and the inlets of a system according to the invention do not have to be refilled when using the above technique for varying the reagent concentrations in plugs.

The concentration of aqueous solutions inside plugs can be varied by changing the flow rates of the plug-fluid streams (see FIG. **25**, discussed in detail in Example 11). In FIG. **25**, water is introduced into inlets **251-258** at various flow rates while perfluorodecaline flows through channels **259-261**. In aqueous laminar flows, the ratio of flow rates of laminar streams in a microfluidic channel may be varied from about 1000:1 and 1:1000, preferably 100:1 to 1:100, more preferably 1:20 to 20:1.

The actual relative concentrations may be quantified using a solution of known concentration of fluorescein. In this example, the intensity of a fluorescein stream can be used as a reference point to check for fluctuations of the intensity of the excitation lamp.

To illustrate an advantage offered by the invention over other techniques, consider the following example. The method(s) described in this example may be modified or incorporated for use in various types of applications, measurements, or experiments. Two or more reagents, such as reagents A, B, C, are to be screened for the effects of different concentrations of reagents on some process, and the conditions under which an inhibitor can terminate the reaction of the enzyme with a substrate at various enzyme and substrate concentrations is of interest. If A is an enzyme, B a substrate, and C an inhibitor, a substrate with 5 inlets such as A/water/B/water/C inlets can be used, and the flow rates at which A, B and C are pumped into the substrate can be varied. Preferably, the size of the plug is kept constant by keeping the total flow rate of all plug-fluids constant. Because different amounts of A, B, C are introduced, the concentrations of A, B, C in the plugs will vary. The concentrations of the starting solutions need not be changed and one can rapidly screen all combinations of concentrations, as long as an enzymatic reaction or other reactions being screened can be detected or monitored. Because the solutions are flowing and the transport is linear, one can determine not only the presence or absence of an interaction or reaction, but also measure the rate at which a reaction occurs. Thus, both qualitative and quantitative data can be obtained. In accordance with the invention, the substrate typically need not be cleaned between runs since most, if not all, reagents are contained inside the plugs and leave little or no residue.

To extend the range over which concentrations can be varied, one may use a combination of, say, reagents A, B, C, D, E and prepare a micromolar solution of A, a mM solution of B, and a M solution of C, and so on. This technique may be easier than controlling the flow rate over a factor of, say, more than 10^6. Using other known methods is likely to be more difficult in this particular example because changing the ratio of reagents inside the plug requires changing the size of the plugs, which makes merging complicated.

In another example, one may monitor RNA folding in a solution in the presence of different concentrations of Mg^{2+} and H^+. Previously, this was done using a stopped-flow technique, which is time consuming and requires a relatively large amount of RNA. Using a method according to the invention, an entire phase space can be covered in a relatively short period of time (e.g., approximately 15 minutes) using only μL/minute runs instead of the usual ml/shot runs.

These particular examples highlight the usefulness according to the invention in, for example, the study of protein/protein interaction mediation by small molecules, protein/RNA/DNA interaction mediation by small molecules, or binding events involving a protein and several small molecules. Other interactions involving several components at different concentrations may also be studied using the method according to the invention.

Generating Gradients in a Series of Plugs

In one aspect according to the invention, dispersion in a pressure-driven flow is used to generate a gradient in a continuous stream of plug-fluid. By forming plugs, the gradient is "fixed", i.e., the plugs stop the dispersion responsible for the formation of the gradient. Although the stream does not have to be aqueous, an aqueous stream is used as a non-limiting example below.

FIG. **44** illustrates how an initial gradient may be created by injecting a discrete aqueous sample of a reagent B into a flowing stream of water. In FIG. **44***a*), the water+B mixture flowed through channel **441**. Channels **443** and **445** contain substantially non-flowing water+B mixture. Water streams were introduced into inlets **440**, **442**, **444**, **446-448** while oil streams flowed through channels **449-452**. FIG. **44***d*) shows a multiple-inlet system through which reagents A, B, and C are introduced through inlets **453**, **454**, and **455**. A pressure-driven flow is allowed to disperse the reagent along the channel, thus creating a gradient of B along the channel. The gradient can be controlled by suitable adjustments or control of the channel dimensions, flow rates, injection volume, or frequency of sample or reagent addition in the case of multiple injections. This gradient is then "fixed" by the formation of plugs. Several of these channels are preferably combined into a single plug-forming region or section. In addition, complex gradients with several components may be created by controlling the streams. This technique may be used for various types of analysis and synthesis. For example, this technique can be used to generate plugs for protein or lysozome crystallization. FIG. **42** shows an experiment involving the formation of gradients by varying the flow rates (the experimental details are described in Example 17). FIG. **43** illustrates the use of gradients to form lysozyme crystals (the experimental details are described in Example 18).

Formation and Isolation of Unstable Intermediates

The devices and methods according to the present invention may also be used for synthesizing and isolating unstable intermediates. The unstable intermediates that are formed using a device according to the invention are preferably made to undergo further reaction and/or analysis or directed to other parts of the device where they may undergo further reaction and/or analysis. In one aspect, at least two different plug-fluids, which together react to form an unstable intermediate, are used. As the unstable intermediates form along the flow path of the substrate, information regarding, for example, the reaction kinetics can be obtained. Such unstable intermediates can be further reacted with another reagent by merging

plugs containing the unstable intermediate with another plug-fluid. Examples of unstable intermediates include, but are not limited to, free radicals, organic ions, living ionic polymer chains, living organometallic polymer chains, living free radical polymer chains, partially folded proteins or other macromolecules, strained molecules, crystallization nuclei, seeds for composite nanoparticles, etc.

One application of devices according to the invention that involves the formation of unstable intermediates is high-throughput, biomolecular structural characterization. It can be used in both a time-resolved mode and a non-time resolved mode. Unstable (and/or reactive) intermediates (for example hydroxyl radicals (OH)) can be generated in one microfluidic stream (for example using a known reaction of metal ions with peroxides). These reactive species can be injected into another stream containing biomolecules, to induce reaction with the biomolecules. The sites on the biomolecule where the reaction takes place correlate with how accessible the sites are. This can be used to identify the sites exposed to the solvent or buried in the interior of the biomolecule, or identify sites protected by another biomolecule bound to the first one. This method could be applied to understanding structure in a range of biological problems. Examples include but are not limited to protein folding, protein-protein interaction (protein footprinting), protein-RNA interaction, protein-DNA interactions, and formation of protein-protein complexes in the presence of a ligand or ligands (such as a small molecule or another biomolecule). Interfacing such a system to a mass-spectrometer may provide a powerful method of analysis.

Experiments involving complex chemical systems can also be performed in accordance with the invention. For example, several unstable intermediates can be prepared in separate plugs, such as partially folded forms of proteins or RNA. The reactivity of the unstable intermediates can then be investigated when, for example, the plugs merge.

Dynamic Control of Surface Chemistry

Control of surface chemistry is particularly important in microfluidic devices because the surface-to-volume ratio increases as the dimensions of the systems are reduced. In particular, surfaces that are generally inert to the adsorption of proteins and cells are invaluable in microfluidics. Polyethylene glycols (PEG) and oligoethylene glycols (OEG) are known to reduce non-specific adsorption of proteins on surfaces. Self-assembled monolayers of OEG-terminated alkane thiols on gold have been used as model substrates to demonstrate and carefully characterize resistance to protein adsorption. Surface chemistry to which the solutions are exposed can be controlled by creating self-assembled monolayers on surfaces of silicone or grafting PEG-containing polymers on PDMS and other materials used for fabrication of microfluidic devices. However, such surfaces may be difficult to mass-produce, and they may become unstable after fabrication, e.g., during storage or use.

In one aspect according to the invention, the reagents inside aqueous plugs are exposed to the carrier-fluid/plug-fluid interface, rather than to the device/plug-fluid interface. Using perfluorocarbons as carrier-fluids in surface studies are attractive because they are in some cases more biocompatible than hydrocarbons or silicones. This is exemplified by the use of emulsified perfluorocarbons as blood substitutes in humans during surgeries. Controlling and modifying surface chemistry to which the reagents are exposed can be achieved simply by introducing appropriate surfactants into the fluorinated PFD phase.

In addition, the use of surfactants can be advantageous in problems involving unwanted adsorption of substances or particles, for example, on the channel walls. Under certain circumstances or conditions, a reaction may occur in one or more channels or regions of the substrate that give rise to particulates that then adhere to the walls of the channels. When they collect in sufficient number, the adhering particulates may thus lead or contribute to channel clogging or constriction. Using methods according to the invention, such as using one or more suitable surfactants, would prevent or minimize adhesion or adsorption of unwanted substances or particles to the channel walls thereby eliminating or minimizing, for example, channel clogging or constriction.

Encapsulated particulates may be more effectively prevented from interfering with desired reactions in one or more channels of the substrate since the particulates would be prevented from directly coming into contact with reagents outside the plugs containing the particulates.

Fluorosurfactants terminated with OEG-groups have been shown to demonstrate biocompatibility in blood substitutes and other biomedical applications. Preferably, oil-soluble fluorosurfactants terminated with oligoethylene groups are used to create interfaces in the microfluidic devices in certain applications. Surfactants with well-defined composition may be synthesized. This is preferably followed by the characterization of the formation of aqueous plugs in the presence of those surfactants. Their inertness towards nonspecific protein adsorption will also be characterized. FIG. **24** shows examples of fluorinated surfactants that form monolayers that are: resistant to protein adsorption; positively charged; and negatively charged. For OEG-terminated surfactants, high values of n (≥16) are preferred for making these surfactants oil-soluble and preventing them from entering the aqueous phase. In FIG. **24**, compounds that have between about 3 to 6 EG units attached to a thiol are sufficient to prevent the adsorption of proteins to a monolayer of thiols on gold, and are thus preferred for inertness. In addition, surfactants that have been shown to be biocompatible in fluorocarbon blood substitutes may also be used as additives to fluorinated carrier fluids.

Applications: Kinetic Measurements and Assays

The devices and methods of the invention can be also used for performing experiments typically done in, for example, a microtiter plate where a few reagents are mixed at many concentrations and then monitored and/or analyzed. This can be done, for example, by forming plugs with variable composition, stopping the flow if needed, and then monitoring the plugs. The assays may be positionally encoded, that is, the composition of the plug may be deduced from the position of the plug in the channel. The devices and methods of the invention may be used to perform high-throughput screening and assays useful, for example, in diagnostics and drug discovery. In particular, the devices and methods of the invention can be used to perform relatively fast kinetic measurements.

The ability to perform fast measurements has revolutionized the field of biological dynamics. Examples include studies of protein C folding and cytochrome C folding. These measurements are performed using fast kinetics instruments that rely on turbulence to mix solutions rapidly. To achieve turbulence, the channels and the flow rates normally have to be large, which require large sample volumes. Commercially available instruments for performing rapid kinetics studies can access times on the order of 1 ms. The improved on-chip version of a capillary glass-ball mixer gives a dead time of about 45 μs with a flow rate of more than about 0.35 mL/sec. The miniaturization of these existing methods is generally limited by the requirement of high flow rate to generate turbulence. Miniaturization afforded by devices and methods according to the invention is advantageous because it allows, for example, quantitative characterization, from genetic

manipulation and tissue isolation, of a much wider range of biomolecules including those available only in minute quantities, e.g., microgram quantities. In addition, these new techniques and instruments afford a wide range of accessible time scales for measurements.

Time control is important in many chemical and biochemical processes. Typically, stopped-flow type instruments are used to measure reaction kinetics. These types of instruments typically rely on turbulent flow to mix the reagents and transport them while minimizing dispersion. Because turbulent flow occurs in tubes with relatively large diameters and at high flow rates, stopped-flow instruments tend to use large volumes of reagents (e.g., on the order of ml/s). A microfluidic analog of a stopped-flow instrument that consumes small volumes of reagents, e.g., on the order of μL/min, would be useful in various applications such as diagnostics. Thus far, microfluidic devices have not been able to compete with stopped-flow instruments because EOF is usually too slow (although it has less dispersion), and pressure-driven flows tend to suffer from dispersion. In addition, mixing is usually very slow in both systems.

Stopped-flow instruments typically have sub-millisecond mixing, and could be useful for experiments where such fast mixing is required. The devices and methods of the invention allow sub-millisecond measurements as well. In particular, the present invention can be advantageous for reactions that occur on a sub-second but slower than about 1 or about 10 millisecond (ms) time scale or where the primary concern is the solute volume required to perform a measurement.

Further, if a plug is generated with two reactive components, it can serve as a microreactor as the plug is transported down a channel. A plug's property, such as its optical property, can then be measured or monitored as a function of distance from a given point or region of a channel or substrate. When the plugs are transported at a constant flow rate, a reaction time can be directly determined from a given distance. To probe the composition of the plug as it exits a channel, the contents of the plugs may be injected into a mass spectrometer (e.g., an electrospray mass spectrometer) from an end of the channel. The time corresponding to the end of the channel may be varied by changing the flow rate. Multiple outlets may be designed along the channels to probe, for example, the plug contents using a mass spectrometer at multiple distance and time points.

An advantage of the devices and methods of the invention is that when plugs are formed continuously, intrinsically slow methods of observation can be used. For example, plugs flowing at a flow rate of about 10 cm/s through a distance of about 1 mm from a point of origin would be about 10 ms old. In this case, the invention is particularly advantageous because it allows the use of a relatively slow detection method to repeatedly perform a measurement of, for example, 10 ms-old plugs for virtually unlimited time. In contrast, to observe a reaction in a stopped-flow experiment at a time, say, between about 9 and 11 ms, one only has about 2 ms to take data. Moreover, the present invention allows one to obtain information involving complex reactions at several times, simultaneously, simply by observing the channels at different distances from the point of origin.

The reaction time can be monitored at various points along a channel—each point will correspond to a different reaction or mixing time. Given a constant fluid flow rate u, one may determine a reaction time corresponding to the various times $t_1, t_2, t_3, \ldots t_n$ along the channel. Thus, if the distance between each pair of points n and n−1, which correspond to time t_n and t_{n-1}, are the same for a given value of n, then the reaction time corresponding to point n along the channel may be calculated from t_n=nl/u. Thus, one can conveniently and repeatedly monitor a reaction at any given time t_n. In principle, the substrate of the present invention allows one to cover a greater time period for monitoring a reaction by simply extending the length of the channel that is to be monitored at a given flow rate or by decreasing the flow rate over a given channel distance (see, for example, FIG. **22**). In FIG. **22**, the following can be introduced into the following inlets: enzyme into inlets **2201**, **2205**, **2210**, **2215**; buffer into inlets **2202**, **2206**, **2211**, **2216**; substrate into inlets **2203**, **2207**, **2212**, **2217**; buffer into inlets **2204**, **2208**, **2213**, **2218**; inhibitor into inlets **2228**, **2209**, **2214**, **2219**. In FIG. **22**, a carrier fluid flows through the channel portions **2220**, **2221**, **2222**, **2223** from left to right. The channel portions enclosed by the dotted square **2224**, **2225**, **2226**, **2227** represent fields of view for the purpose of monitoring a reaction at various points along the channel.

The same principle applies to an alternate embodiment of the present invention, where the distance corresponding to a point n from a common point of origin along the channel differs from that corresponding to another channel by a power or multiples of 2. This can be seen more clearly from the following discussion. Given a constant fluid flow rate u, one may determine a reaction time corresponding to the various times $t_1, t_2, t_3, \ldots t_n$ along the channel. Thus, if the distance between each pair of points n and n−1, which correspond to time t_n and t_{n-1}, are the same for a given value of n, then the reaction time corresponding to point n along the channel may be calculated from t_n=nl/u. In a relatively more complex channel geometry such as the one shown in FIG. **22**(*c*), the corresponding equation is given by $t_n=2^{(n-1)}$l/u, which shows that the reaction times at various points n varies as a power or multiples of 2.

In one aspect, channels according to the invention are used that place into a field of view different regions that correspond to different time points of a reaction. The channels according to the invention allow various measurements such as those of a complete reaction profile, a series of linearly separated time points (such as those required for the determination of an initial reaction velocity in enzymology), and a series of exponentially separated time points (e.g., first-order kinetic measurements or other exponential analysis). Time scales in an image frame can be varied from microseconds to seconds by, for example, changing the total flow rate and channel length.

FIG. **22**A-D show various examples of geometries of microfluidic channels according to the invention for obtaining kinetic information from single optical images. The illustrated channel systems are suitable for studies such as measurements of enzyme kinetics in the presence of inhibitors. The device shown in FIG. **22**D has multiple outlets that can be closed or opened. In the device shown in FIG. **22**D, preferably only one outlet is open at a time. At the fastest flow rates, the top outlet is preferably open, providing reduced pressure for flow through a short fluid path **1**. As flow rates are reduced, other outlets are preferably opened to provide a longer path and a larger dynamic range for measurements at the same total pressure.

In FIG. **22**, n is the number of segments for a given channel length l traveled by the reaction mixture in time t_n (see p. 73, second full paragraph for a related discussion of reaction times and channel lengths). These systems allow the control of the ratio of reagents by varying the flow rates. The systems also allow a quick quantification of enzyme inhibition.

For example, ribonuclease A can be used with known inhibitors such as nucleoside complexes of vanadium and oxovanadium ions and other small molecules such as 5'-diphosphoadenosine 3'-phosphate and 5'-diphosphoadenosine 2'-phosphate. The kinetics may be characterized by

obtaining data and making Lineweaver-Burk, Eadie-Hofstee, or Hanes-Wolfe plots in an experiment. The experiment can be accomplished using only a few microliters of the protein and inhibitor solutions. This capability is particularly useful for characterizing new proteins and inhibitors that are available in only minute quantities, e.g., microgram quantities.

Kinetic measurements of reactions producing a fluorescent signal can be performed according to the invention by analyzing a single image obtained using, for example, an optical microscope. Long exposures (i.e., about 2 seconds) have been used to measure fast (i.e., about 2 milliseconds) kinetics. This was possible because in a continuous flow system, time is simply equal to the distance divided by the flow rate. In the continuous flow regime in accordance with the invention, the accessible time scales can be as slow as about 400 seconds, which can be extended to days or weeks if the flow is substantially slowed down or stopped. Typically, the time scale depends on the length of the channel (e.g., up to about 1 meter on a 3-inch diameter chip) at a low flow rate of about 1 mm/s, which is generally limited by the stability of the syringe pumps, but may be improved using pressure pumping. The fastest time scale is typically limited by the mixing time, but it may be reduced to about 20 μs in the present invention. Mixing time is generally limited by two main factors: (1) the mixing distance (e.g., approximately 10-15 times the width of the channel); and (2) the flow rates (e.g., approximately 400 mm/s, depending on the capillary number and the pressure drop required to drive the flow). Mixing distance is normally almost independent of the flow rate. By using suitable designs of microfluidic channels, or networks of microfluidic channels, a wide range of kinetic experiments can be performed.

Reducing the channel size generally reduces the mixing time but it also increases the pressure required to drive a flow. The equation below describes the pressure drop, ΔP (in units of Pa), for a single-phase flow in a rectangular capillary:

$$\Delta P = 28.42 U \mu l / ab \qquad \text{Eqn. (9)}$$

where U (m/s) is the velocity of the flow, μ (kilogram/meter-second, kg m^{-1} s^{-1}) is the viscosity of the fluid, l (m) is the length of the capillary, a (m) is the height of the capillary, and b (m) is the width of the capillary. There is generally a physical limitation on how much pressure a microfluidic device can withstand, e.g., about 3 atm for PDMS and about 5 atm for glass and Si. This limitation becomes crucial for very small channels and restricts the total length of the channel and thus the dynamic range (the total distance through which this flow rate can be maintained at a maximum pressure divided by the mixing distance) of the measurement.

FIG. **23** depicts a microfluidic network according to the invention with channel heights of 15 and 2 μm. The channel design shown in FIG. **23** illustrates how a dynamic range of about 100 can be achieved by changing the cross-section of the channels. Under these conditions, mixing time in the winding channel is estimated to be about 25 μs and observation time in the serpentine channels are estimated to be about 3 ms.

As FIG. **23** shows, rapid mixing occurs in the 2 μm×1 μm (height×width) channels and measurements are taken in the 2 μm×3 μm) channels. The table in FIG. **23** shows the distribution of the pressure drop, flow velocity, and flow time as a function of the channel cross-section dimensions. A transition from a 1-μm wide to 3-μm wide channels should occur smoothly, with plugs maintaining their stability and decreasing their velocity when they move from a 20-μm wide into a 50-μm wide channel. Changing the width of the channel can be easily done and easily incorporated into a mask design. The height of the channel can be changed by, for example, using photoresist layers having two different heights that are sequentially spun on, for example, a silicon wafer. A two-step exposure method may then be used to obtain a microfluidic network having the desired cross-section dimensions.

In another example of the application of the devices and methods of the present invention, the folding of RNase P catalytic domain (P RNA C-domain) of *Bacillus subtilis* ribozyme can be investigated using channels according to the invention. RNA folding is an important problem that remains largely unsolved due to limitations in existing technology. Understanding the rate-limiting step in tertiary RNA folding is important in the design, modification, and elucidation of the evolutionary relationship of functional RNA structures.

The folding of P RNA C-domain is known to involve three populated species: unfolded (U), intermediate (I), and native (N, folded) states. Within the first millisecond, the native secondary structure and some of the tertiary structure would have already folded (the RNA is compacted to about 90% of the native dimension) but this time regime cannot be resolved using conventional techniques such as stopped-flow. Using channels and substrates according to the invention, the time-dependence of the P RNA folding kinetics upon the addition of Mg^{2+} can be studied.

Various types of assays (e.g., protein assays) known in the art, including absorbance assays, Lowry assays, Hartree-Lowry assays, Biuret assays, Bradford assays, BCA assays, etc., can be used, or suitably adapted for use, in conjunction with the devices and methods of the invention. Proteins in solution absorb ultraviolet light with absorbance maxima at about 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Absorbance assays offer several advantages. Absorbance assays are fast and convenient since no additional reagents or incubations are required. No protein standard need be prepared. The assay does not consume the protein and the relationship of absorbance to protein concentration is linear. Further, the assay can be performed using only a UV spectrophotometer.

The Lowry assay is an often-cited general use protein assay. It was the method of choice for accurate protein determination for cell fractions, chromatography fractions, enzyme preparations, and so on. The bicinchoninic acid (BCA) assay is based on the same principle, but it can be done in one step. However, the modified Lowry is done entirely at room temperature. The Hartree version of the Lowry assay, a more recent modification that uses fewer reagents, improves the sensitivity with some proteins, is less likely to be incompatible with some salt solutions, provides a more linear response, and is less likely to become saturated.

In the Hartree-Lowry assay, the divalent copper ion forms a complex with peptide bonds under alkaline conditions in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. In addition to standard liquid handling supplies, the assay only requires a spectrophotometer with infrared lamp and filter. Glass or inexpensive polystyrene cuvettes may be used.

The Biuret assay is similar in principle to that of the Lowry, however it involves a single incubation of 20 minutes. In the Biuret assay, under alkaline conditions, substances containing two or more peptide bonds form a purple complex with copper salts in the reagent. The Biuret assay offer advantages in that there are very few interfering agents (ammonium salts being one such agent), and there were fewer reported devia-

tions than with the Lowry or ultraviolet absorption methods. However, the Biuret consumes much more material. The Biuret is a good general protein assay for batches of material for which yield is not a problem. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed, with maximum transmission in the region of 450 nm. Glass or inexpensive polystyrene cuvettes may be used.

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis. Assay materials including color reagent, protein standard, and instruction booklet are available from Bio-Rad Corporation. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum (no special lamp or filter usually needed). Glass or polystyrene cuvettes may be used, but the color reagent stains both. Disposable cuvettes are recommended.

The bicinchoninic acid (BCA) assay is available in kit form from Pierce (Rockford, Ill.). This procedure is quite applicable to microtiter plate methods. The BCA is used for the same reasons the Lowry is used. The BCA assay is advantageous in that it requires a single step, and the color reagent is stable under alkaline conditions. BCA reduces divalent copper ion to the monovalent ion under alkaline conditions, as is accomplished by the Folin reagent in the Lowry assay. The advantage of BCA is that the reagent is fairly stable under alkaline condition, and can be included in the copper solution to allow a one step procedure. A molybdenum/tungsten blue product is produced as with the Lowry. In addition to standard liquid handling supplies, a visible light spectrophotometer is needed with transmission set to 562 nm. Glass or inexpensive polystyrene cuvettes may be used.

The range of concentrations that can be measured using the above assays range from about 20 micrograms to 3 mg for absorbance at 280, between about 1-100 micrograms for absorbance at 205 nm, between about 2-100 micrograms for the Modified Lowry assay, between about 1-10 mg for the Biuret assay, between about 1-20 micrograms for the Bradford assay, and between about 0.2-50 micrograms for BCA assay. Many assays based on fluorescence or changes in fluorescence have been developed and could be performed using methods and devices of the invention.

A detailed description of various physical and chemical assays is provided in *Remington: The Science and Practice of Pharmacy*, A. R. Gennaro (ed.), Mack Publishing Company, chap. 29, "Analysis of Medicinals," pp. 437-490 (1995) and in references cited therein while chapter 30 of the same reference provides a detailed description of various biological assays. The assays described include titrimetric assays based on acid-base reactions, precipitation reactions, redox reactions, and complexation reactions, spectrometric methods, electrochemical methods, chromatographic methods, and other methods such as gasometric assays, assays involving volumetric measurements and measurements of optical rotation, specific gravity, and radioactivity. Other assays described include assays of enzyme-containing substances, proximate assays, alkaloidal drug assays, and biological tests such as pyrogen test, bacterial endotoxin test, depressor substances test, and biological reactivity tests (in-vivo and in-vitro)

In addition, *Remington: The Science and Practice of Pharmacy*, A. R. Gennaro (ed.), Mack Publishing Company, chap. 31, "Clinical Analysis," pp. 501-533 (1995) and references cited therein provide a detailed description of various methods of characterizations and quantitation of blood and other body fluids. In particular, the reference includes a detailed description of various tests and assays involving various body fluid components such as erythrocytes, hemoglobin, thrombocyte, reticulocytes, blood glucose, nonprotein nitrogen compounds, enzymes, electrolytes, blood-volume and erythropoeitic mechanisms, and blood coagulation.

Nonlinear and Stochastic Sensing

Stochastic behavior has been observed in many important chemical reactions, e.g., autocatalytic reactions such as inorganic chemical reactions, combustion and explosions, and in polymerization of sickle-cell hemoglobin that leads to sickle-cell anemia. Crystallization may also be considered an autocatalytic process. Several theoretical treatments of these reactions have been developed. These reactions tend to be highly sensitive to mixing.

Consider the extensively studied stochastic autocatalytic chemical reaction between $NaClO_2$ and $Na_2S_2O_3$ (chlorite-thiosulfate reaction). The mechanism of this reaction can be described by reactions (1) and (2),

$$4S_2O_3^{2-}+ClO_2^-+4H+2S_4O_6^{2-}+2H_2O+Cl^- \text{ rate (v)} \quad \alpha[H^+] \tag{1}$$

$$S_2O_3^{2-}+2ClO_2^-+H_2O\rightarrow 2SO_4^{2-}+2H^++2Cl^- \text{ rate (v)} \quad \alpha[H^+]^2[Cl] \tag{2}$$

where $[H^+]$ stands for the concentration of H^+. At a slightly basic pH=7.5, the slow reaction (1) dominates and maintains a basic pH of the reaction mixture (since the rate of this reaction v is directly proportional $[H^+]$, this reaction consumes H^+ and is auto-inhibitory). Reaction (2) dominates at acidic pH (since the rate of this reaction varies in proportion to $[H^+]^2[Cl^-]$, this reaction produces both H^+ and Cl^- and is superautocatalytic). FIG. **21** shows the reaction diagram for two reactions corresponding to the curves **211**, **212**. The rates of the two reactions (referred to here as reaction **211** and reaction **212**) are equal at an unstable critical point at a certain pH. The lifetime of the reaction mixtures of $NaClO_2$ and NaS_2O_3 at this critical point crucially depends on stirring. In the absence of stirring, stochastic fluctuations of $[H^+]$ in solution generate a localized increase in $[H^+]$. This increase in $[H^+]$ marginally increases the rate of reaction **212**, but it has a much stronger accelerating effect on reaction **211** because of the higher-order dependence on $[H^+]$ of this reaction. Therefore, in the region where local fluctuations increase local $[H^+]$, reaction **211** becomes dominant, and more H^+ is produced (which rapidly diffuses out of the region of the initial fluctuation). The initiated chemical wave then triggers the rapid reaction of the entire solution. Unstirred mixtures of $NaClO_2$ and NaS_2O_3 are stable only for a few seconds, and these fluctuations arise even in the presence of stirring.

FIG. **21** depicts a reaction diagram illustrating an unstable point in the chlorite-thiosulfate reaction. At $[H^+]$ values below the critical point, the slow reaction (1) dominates. At $[H^+]$ values above the critical point, the autocatalytic reaction (2) dominates. The reaction mixture at the $[H^+]$ value equal to the critical point is metastable in the absence of fluctuations. Under perfect mixing, the effects of small fluctuations average out and the system remains in a metastable state. Under

imperfect mixing, fluctuations that reduce [H^+] grow more slowly than those that increase [H^+] due to the autocatalytic nature of reaction (2), and the reaction mixture thus rapidly becomes acidic.

It is known that chaotic flows should have a strong effect on diffusive transport within the fluid ("anomalous diffusion"). It is also known that chaotic dynamics can lead to non-Gaussian transport properties ("strange kinetics"). In one aspect according to the invention, these highly unstable mixtures are stabilized in the presence of chaotic mixing using channels according to the invention because this mixing can effectively suppress fluctuations. This invention can be used to understand the effects of mixing on the stochastic behavior of such systems, including for example, the chlorite thiosulfate system.

In a laminar flow, the flow profile in the middle of the channel is flat and there is virtually no convective mixing. Fluctuations involving [H^+] that arise in the middle of the channel can grow and cause complete decomposition of the reaction mixture. Slow mixing reduces the probability of fluctuations in plugs moving through straight channels. When fluctuations that occur in the centers of vortices are not efficiently mixed away, one or more spontaneous reactions involving some of the plugs can take place. In the present invention, chaotic mixing in plugs moving through winding channels efficiently mix out fluctuations, and thus substantially fewer or no spontaneous reactions are expected to occur.

In a simple laminar flow, there is normally very little or no velocity gradient and substantially no mixing at the center of the channel. Thus, fluctuations that arise in the chlorite-thiosulfate reaction mixture prepared at the critical [H^+] are able to grow and lead to rapid decomposition of the reaction mixture. Propagation of chemical fronts in autocatalytic reactions occurring in laminar flows has been described with numerical simulations, and back-propagation has been predicted (that is, a reaction front traveling upstream of the direction of the laminar flow). Using the method of the present invention, this back-propagation involving the reaction between $NaClO_2$ and NaS_2O_3 under laminar flow conditions was observed.

In accordance with the invention, chaotic flow within plugs that flow through winding channels suppresses fluctuations and gives rise to stable reaction mixtures. There exists, of course, a finite probability that fluctuations can arise even in a chaotically stirred plug. In one aspect according to the invention, the details of the evolution of these reactions are monitored using a high-speed digital camera. The plugs are preferably separated by the oil and are not in communication with each other, so the reaction of one plug will not affect the behavior of the neighboring plug. Statistics covering the behavior of thousands of plugs can be obtained quickly under substantially identical experimental conditions.

Whether a fluctuation would be able to trigger an autocatalytic reaction depends on factors such as the magnitude of a fluctuation and its lifetime. The lifetime of a fluctuation is typically limited by the mixing time in the system. In an unstirred solution, mixing is by diffusion and quite slow, and fluctuations may persist and lead to autocatalytic reactions. In a stirred solution, the lifetime of a fluctuation is relatively short, and only large fluctuations have sufficient time to cause an autocatalytic reaction.

Mixing time and the lifetime of fluctuations typically depend on the size of the plugs. As plug size decreases, mixing is accelerated and fluctuations are suppressed. However, very small plugs (e.g., about 1 μm^3 or 10^{-15} L) in a solution containing about 10^{-8} mole/liter concentration of H^+ (pH=8) will contain only a few H^+ ions per plug (about 10^{-23} moles or about 6 H^+ ions). When such small plugs are formed, the number of H^+ ions in them will have a Poisson distribution.

An important experimental challenge is to establish that the stochastic behavior in these systems is due mainly to internal fluctuations of concentrations. Other factors that may act as sources of noise and instability are: (1) temporal fluctuations in the flow rates of the incoming reagent streams, which can lead to the formation of plugs with varying amounts of reagents; (2) temperature fluctuations in solutions in a microfluidic device, which may arise due to, for example, illumination by a microscope; and (3) fluctuations due to impurities in carrier-fluids leading to variations in the surface properties of different plugs.

Microfluidic systems according to the invention may be used to probe various chemical and biochemical processes, such as those that show stochastic behavior in bulk due to their nonlinear kinetics. They can also be used in investigating processes that occur in systems with very small volumes (e.g., about 1 μm^3, which corresponds to the volume of a bacterial cell). In systems with very small volumes, even simple reactions are expected to exhibit stochastic behavior due to the small number of molecules localized in these volumes.

Autocatalytic reactions present an exciting opportunity for highly sensitive detection of minute amounts of autocatalysts. Several systems are known to operate on this principle, silver-halide photography being the most widely used. In silver-halide photography, the energy of photons of light is used to decompose an emulsion of silver halide AgX into nanometer-sized particles of metallic silver. A film that is embedded with the silver particles is then chemically amplified by the addition of a metastable mixture of a soluble silver(I) salt and a reducing agent (hydroquinone). Metallic silver particles catalyze reduction of silver(I) by hydroquinone, leading to the growth of the initial silver particles. Another example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.

However, a dilemma occurs when designing systems with very high sensitivity and amplification. To achieve a very highly sensitive amplification, the system typically has to be made very unstable. On the other hand, an unstable system is very sensitive to noise and has a very short lifetime. Also, in unstable systems, it is difficult to distinguish between spontaneous decomposition and a reaction caused by the analyte. In one aspect, microfluidic devices according to the invention, which allow chaotic mixing and compartmentalization, are used to overcome this problem.

To demonstrate the potential of microfluidic systems according to the present invention, a microfluidic system according to the invention is used to handle unstable mixtures. In one application, a microfluidic system according to the invention is preferably used to control a stochastic reaction between $NaClO_2$ and NaS_2O_3. In particular, this reaction is preferably used for a highly sensitive amplification process.

If a plug containing an unstable reaction mixture of $NaClO_2$ and NaS_2O_3 is merged with a small plug containing an amount of H^+ sufficient to bring the local concentration of H^+ above critical, a rapid autocatalytic reactions is generally triggered. This autocatalytic reaction typically leads to the production of large amounts of H^+. Thus, a weak chemical signal, e.g., a small amount of H^+, is rapidly amplified by an unstable reaction mixture. Thus, for example, this approach can be used to investigate biological reactions such as those that involve enzymes, in which small amounts of H^+ are produced.

The above autocatalytic system possesses several features that contribute to its novelty and usefulness. In one aspect, an unstable amplifying reaction mixture is prepared in-situ and is used within milliseconds before it has a chance to decompose. Preferably, the system is compartmentalized so a reaction that occurs in one compartment does not affect a reaction in another compartment. This compartmentalization allows thousands of independent experiments to be conducted in seconds using only minute quantities of samples. Importantly, chaotic mixing in the system reduces fluctuations and stabilizes the reaction mixture.

The applications of controlled autocatalytic amplification in accordance with the invention are not limited to the detection of protons or Co^{2+} ions. For example, the (Co(III)-5-Br-PAPS)/peroxomonosulfate oxidation reaction can also be used indirectly, for example, for a detection of small amounts of peroxidase, which can be used as a labeling enzyme bound to an antibody. The (Co(III)-5-Br-PAPS)/peroxomonosulfate oxidation reaction, which has been characterized analytically, involves the autocatalytic decomposition of violet bis[2-(5-bromo-pyridylazo)-5-(N-propyl-N-sulfopropyl-amino-phenolato]cobaltate, (Co(III)-5-Br-PAPS), upon oxidation with potassium peroxomonosulfate to produce colorless Co^{2+} ions, which serve as the autocatalyst (the order of autocatalysis has not been established for this reaction). (Endo et al., "Kinetic determination of trace cobalt(II) by visual autocatalytic indication," *Talanta,* 1998, vol. 47, pp. 349-353; Endo et al., "Autocatalytic decomposition of cobalt complexes as an indicator system for the determination of trace amounts of cobalt and effectors," *Analyst,* 1996, vol. 121, pp. 391-394.)

$$\text{Co(III)-[5-Br-PAPS]reduced}+HSO_5^- \rightarrow Co^{2+}+\text{[5-Br-PAPS]oxidized}+HSO^{4-}$$

Addition of small amounts of Co^{2+} to the violet mixture of the (Co(III)-5-Br-PAPS and peroxomonosulfate produces an abrupt loss of color to give a colorless solution. The time delay before this decomposition depends on the amount of the Co^{2+} added to the solution. This reaction has been used to detect concentrations of Co^{2+} as low as 1×10^{-10} mole/L. The reaction shows good selectivity in the presence of other ions (V(V), Cr(III), Cr(VI), Mn(II), Fe(II), Ni(II), Cu(II) and Zn(II)).

The devices and methods according to the invention may be applied to other autocatalytic reactions, some of which have been described in inorganic, organic and biological chemistry. Reactions of transition metal ions such as Cr(III) (B82) Mn^{2+} or colloidal MnO_2, and reactions of halides and oxohalides are often autocatalytic. Autocatalysis involving lanthanides (Eu^{2+}) and actinides (U^{4+}) has also been reported. All of these elements are potential targets for detection and monitoring in chemical waste, drinking water, or biological fluids. Intriguing possibilities arise from using asymmetric autocatalytic reactions to detect minute amounts of optically active, chiral impurities, such as biomolecules.

It is also possible to design new autocatalytic reactions. Autocatalysis is abundant in biology, and many enzymes are autocatalytic (e.g., caspases involved in programmed cell death, kinases involved in regulation and amplification, and other enzymes participating in metabolism, signal transduction, and blood coagulation. Emulsions of perfluorocarbons such as perfluorodecaline (PFD) are used as blood substitutes in humans during surgeries and should be compatible with a variety of biological molecules. Since the feasibility of quantitative measurements of enzyme kinetics has been demonstrated using plugs formed according to the invention, plugs formed according to the invention may also be applied to the detection of biological autocatalysts.

The devices and methods according to the present invention are not limited to the detection of the autocatalyst itself. For example, the labeling of an analyte using an autocatalyst is also within the scope of the present invention. Biomolecules are often labeled with metallic nanoparticles. Such metallic nanoparticles are highly effective autocatalysts for the reduction of metal ions to metals. Preferably, the systems and methods of the present invention are used in the visual detection of a single molecule of DNA, RNA, or protein labeled with nanoparticles via an autocatalytic pathway. In preliminary experiments in accordance with the invention, clean particle formation and transport within plugs were observed.

In addition, the generation of metal (e.g., copper, silver, gold, nickel) deposits and nanoparticles upon chemical reduction also proceed by an autocatalytic mechanism. These reactions are commonly used for electroless deposition of metals and should be useful for the detection of minute amounts of metallic particles. The presence of metallic particles in water can be indicative of the presence of operating mechanical devices. In one aspect according to the invention, devices and methods according to the invention are used to detect the presence of minute or trace quantities of metallic particles.

The devices in accordance with the present invention are simple in design, consume minute amounts of material, and robust. They do not require high voltage sources and can be operated, for example, using gravity or a pocket-sized source of compressed air. In one aspect, the systems according to the invention are used in portable and hand-held devices.

Autocatalytic reactions show a threshold response, that is, there is a very abrupt temporal change from unreacted mixture to reacted mixture. In the case where time is equal to distance, this abrupt transition over a short distance can be observed using the devices and methods of the invention. The time (and distance) is very sensitive to the initial concentration of the catalyst, and thus it should be easy to determine the concentration of the autocatalyst in the sample by noting how far the reaction system traveled before it reacted.

One example of an autocatalytic process is blood coagulation. It is very sensitive to flow and mixing, therefore experimenting with it in the absence of flow gives unreliable results or results that have little relevance to the real function of the coagulation cascade. A typical microfluidic system may be difficult to use with blood because once coagulation occurs, it blocks the channel and stops the flow in the microfluidic device. In addition, coagulated blood serves as an autocatalyst; even small amounts of coagulated blood in the channels can make measurements unreliable.

These problems can be overcome using the devices of the present invention. Using plugs, autocatalytic reactions can be easily controlled, and the formation of solid clots would not be a problem because any solids formed will be transported inside the plugs out of the channel without blocking the channel and without leaving autocatalytic residue. In addition, flow inside plugs can be easily controlled and adjusted to resemble flow under physiological conditions.

To address the sensitivity of blood coagulation to surfaces (the cascade is normally initiated on the surface), microscopic beads containing immobilized tissue factor (the cascade initiator) on the surface may be added to one of the streams and transported inside the plugs. Also, surfactants may be used to control surface chemistry.

Thus, the devices and methods of the invention may be used, for example, to test how well the coagulation cascade functions (e.g., for hemophilia or the tendency to form thrombus) under realistic flow conditions. This test would be par-

ticularly valuable in diagnostics. Blood may be injected in one stream, and a known concentration of a molecule known to induce coagulation (e.g., factor VIIa) can be added through another stream prior to plug formation. At a given flow rate, normal blood would coagulate at a certain distance (which corresponds to a given time), which can be observed optically by light scattering or microscopy. Blood of hemophiliac patients would coagulate at a later time. This type of testing would be useful before surgical operations. In particular, this type of testing is important for successful child delivery, especially when hemophilia is suspected. Fetal testing may be performed since only minute amounts of blood are required by systems according to the invention. The blood may be injected directly from the patient or collected in the presence of anticoagulating agent (for example EDTA), and then reconstituted in the plug by adding Ca^{2+}. In some cases, the addition of Ca^{2+} may be sufficient to initiate the coagulation cascade.

The devices and methods of the invention may also be used to evaluate the efficacy of anticoagulating agents under realistic flow conditions. Plugs can be formed from normal blood (which may be used directly or reconstituted by adding Ca^{2+} or other agents), an agent known to induce coagulation, and an agent (or several agents that need to be compared) being tested as an anticoagulation agent. The concentrations of these agents can be varied by varying the flow rates. The distance at which coagulation occurs is noted, and the efficacy of various agents to prevent coagulation is compared. The effects of flow conditions and presence of various compounds in the system on the efficacy of anticoagulation agents can be investigated quickly. The same techniques may also be used to evaluate agents that cause, rather prevent, coagulation. These tests could be invaluable in evaluating drug candidates.

Synthesis

In accordance with the present invention, a method of conducting a reaction within a substrate is provided. The reaction is initiated by introducing two or more plug-fluids containing reactants into the substrate of the present invention.

In one aspect, the plug-fluids include a reagent and solvent such that mixing of the plug-fluids results in the formation of a reaction product. In another embodiment, one of the plug-fluids may be reagent free and simply contain fluid. In this embodiment, mixing of the plug-fluids will allow the concentration of the reagent in the plug to be manipulated.

The reaction can be initiated by forming plugs from each plug-fluid and subsequently merging these different plugs.

When plugs are merged to form merged plugs, the first and second set of plugs may be substantially similar or different in size. Further, the first and second set of plugs may have different relative velocities. In one embodiment, large arrays of microfluidic reactors are operated in parallel to provide substantial throughput.

The devices and methods of the invention can be used for synthesizing nanoparticles. Nanoparticles that are monodisperse are important as sensors and electronic components but are difficult to synthesize (Trindade et al., *Chem. Mat.* 2001, vol. 13, pp. 3843-3858.). In one aspect, monodisperse nanoparticles of semiconductors and noble metals are synthesized under time control using channels according to the invention (Park et al, *J. Phys. Chem. B,* 2001, vol. 105, pp. 11630-11635.). Fast nucleation is preferably induced by rapid mixing, thereby allowing these nanoparticles to grow for a controlled period of time. Then their growth is preferably quickly terminated by passivating the surfaces of the particles with, for example, a thiol. Nanoparticles of different sizes are preferably obtained by varying the flow rate and therefore the growth time. In addition, devices according to the invention can be used to monitor the synthesis of nanoparticles, and thus obtain nanoparticles with the desired properties. For example, the nanoparticle formation may be monitored by measuring the changes in the color of luminescence or absorption of the nanoparticles. In addition, the growth of nanoparticles may be stopped by introducing a stream of quenching reagent at a certain position along the main channel.

Rapid millisecond mixing generated in channels according to the invention can help ensure the formation of smaller and much more monodisperse nanoparticles than nanoparticles synthesized by conventional mixing of solutions. FIG. **13** shows the UV-VIS spectra of CdS nanoparticles formed by rapid mixing in plugs (lighter shade spectrum with sharp absorption peak) and by conventional mixing of solutions (darker shade spectrum). The sharp absorption peak obtained for synthesis conducted in plugs indicates that the nanoparticles formed are highly monodisperse. In addition, the blue-shift (shift towards shorter wavelengths) of the absorption peak indicates that the particles formed are small.

FIG. **14**A-B illustrates the synthesis of CdS nanoparticles performed in PDMS microfluidic channels in single-phase aqueous laminar flow (FIG. **14**A) and in aqueous plugs that were surrounded by water-immiscible perfluorodecaline (FIG. **14**B). In FIGS. **14**A-B, Cd^{2+} was introduced into inlets **1400**, **1403**, aqueous stream was introduced into inlets **1401**, **1404**, and S^{2-} was introduced into inlets **1402**, **1405**. In FIG. **14**A, an aqueous stream flowed through channel **1406** while in FIG. **14**B, oil flowed through channel **1407**. FIG. **14**A shows portions of the channels **1408** and **1410** at time t=6 minutes and portions of the channels **1409**, **1411** at time t=30 minutes. It can be seen in FIG. **14**A that when laminar flow is used in the synthesis, large amounts of CdS precipitate form on the channel walls. When plugs were used for the synthesis, all CdS formed inside the plugs, and no surface contamination was observed. FIG. **15** illustrates a technique for the synthesis of CdS nanoparticles, which is discussed in detail in Example 13 below.

The following methods according to the invention can be used in synthesis involving nanoparticles:

(a) using self-assembled monolayers to nucleate nanoparticles with crystal structures not accessible under homogeneous nucleation conditions (e.g., controlling polymorphism by controlling the surface at which nucleation takes place).

(b) using merging of plugs to create core-shell nanoparticles with a range of core and shell sizes. In a stream of plugs of a first channel, small core nanoparticles such as CdSe particles can be synthesized in a matter of few milliseconds. The CdSe particles can then be used as seeds for mixing with solutions such as those containing Zn^{+2} and S^{-2}. The CdSe particles, acting as seeds for the formation of ZnS, thus allow the formation of CdSe(core)/ZnS(shell) nanoparticles. Core-shell particles with more than two layers may be obtained by simply repeating the merging process more than once.

(c) using merging of plugs to create composite nanoparticles. For example, small nanoparticles of CdSe and ZnS can be formed using streams of plugs from two separate channels. Merging of these streams leads to aggregation of these particles to form larger nanoparticles containing CdSe/ZnS composite. The composite nanoparticles that contain only a few of the original nanoparticles can be made non-centrosymmetric, which may have interesting photophysical properties.

(d) using the devices and methods according to the invention to synthesize medically important nanoparticles, such as encapsulated drugs and composite drugs.

(e) combinatorial synthesis of core-shell particles and other complex systems. For example, the luminescence of CdSe/ZnS particles may be monitored and the conditions adjusted to produce particles with various core and shell sizes, various doping impurities in the core and shell, and various ligand composition on the surface of the particles. These can be conducted in real time using a device according to the invention. The entire process can also be automated.

The devices and methods according to the present invention may also be used for synthesizing polymers. Since the invention allows precise control of the timing of a polymerization reaction, one or more properties of a polymer such as molecular weight, polydispersity and blockiness can be readily controlled or adjusted. In addition, use of the substrate of the present invention allows the user to precisely form block copolymers by merging plugs within a device, since the path length of the channel will correspond to a specific duration of the polymerization reaction. Similarly, a living polymer chain can be terminated with a specific end group to yield polymers with a discrete subset of molecular weights.

In addition, combinatorial libraries of drug candidates may be synthesized using similar approaches. The library may be encoded using the position of plugs in a channel. Plugs of variable composition may be created by varying flow rates. Combination of synthesis of the library may be combined with screening and assays performed on the same microfluidic chip according to the present invention. In some embodiments, merging, splitting and sorting of plugs may be used during synthesis, assays, etc.

All of the above synthesis methods of the present invention can be used to form macroscopic quantities of one or more reaction products by running multiple reactions in parallel.

Particle Separation/Sorting Using Plugs

The flow within the moving plugs can be used for separation of polymers and particles. Plugs can be used for separation by first using flow within a moving plug to establish a distribution of the polymers or particles inside the plug (for example, an excess of the polymer inside the front, back, right or left side of the plug) and then using splitting to separate and isolate the part of the plug containing higher concentration of the polymers or particles. When two polymers or particles are present inside the plug and establish different distributions, slitting can be used to separate the polymers or particles. This approach may be useful, for example, in achieving on a microfluidic chip any of, but not limited to, the following: separation, purification, concentration, membrane-less dialysis, and filtration.

Crystallization

The devices and methods of the invention allow fast, inexpensive miniaturization of existing crystallization methods and other methods that can be adapted into, for example, novel protein screening and crystallization techniques. The crystallization methods according to the invention may be applied to various drugs, materials, small molecules, macromolecules, colloidal and nanoparticles, or any of their combinations. Many relevant protein structures remain undetermined due to their resistance to crystallization. Also, many interesting proteins are only available in microgram quantities. Thus, a screening process must permit the use of small amounts protein for analysis. Current crystallization screening technologies generally determine the ideal conditions for protein crystallization on a milligram scale. Devices and methods according to the invention improve current benchtop methodology available to single users, and enables higher throughput automated systems with improved speed, sample economy, and entirely new methods of controlling crystallization.

A microfluidic system according to the invention can be applied to the crystallization of small molecules or macromolecules and their complexes.

For example, systems and methods in accordance with the present invention may include but are not limited to: (1) biological macromolecules (cytosolic proteins, extracellular proteins, membrane proteins, DNA, RNA, and complex combinations thereof); (2) pre- and post-translationally modified biological molecules (including but not limited to, phosphorylated, sulfolated, glycosylated, ubiquitinated, etc. proteins, as well as halogenated, abasic, alkylated, etc. nucleic acids); (3) deliberately derivatized macromolecules, such as heavy-atom labeled DNAs, RNAs, and proteins (and complexes thereof), selenomethionine-labeled proteins and nucleic acids (and complexes thereof), halogenated DNAs, RNAs, and proteins (and complexes thereof); (4) whole viruses or large cellular particles (such as the ribosome, replisome, spliceosome, tubulin filaments, actin filaments, chromosomes, etc.); (5) small-molecule compounds such as drugs, lead compounds, ligands, salts, and organic or metallo-organic compounds; (6) small-molecule/biological macromolecule complexes (e.g., drug/protein complexes, enzyme/substrate complexes, enzyme/product complexes, enzyme/regulator complexes, enzyme/inhibitor complexes, and combinations thereof); (7) colloidal particles; and (8) nanoparticles.

Preferably, a general crystallization technique according to the present invention involves two primary screening steps: a crude screen of crystallization parameters using relatively small channels with a large number of small plugs, and a fine screen using larger channels and larger plugs to obtain diffraction-quality crystals. For example, ten crude screens performed using channels with a $(50\ \mu m)^2$ cross-sectional dimension and with more or less one thousand 150-picoliter (pL) plugs corresponding to 10 mg/mL final concentration of a protein (10,000 trials total) will typically require about 1.5 μL of solution, produce crystals up to about $(10\ \mu m)^3$ in size, and will consume approximately 15 μg of protein. Up to 300 or more of such plugs can be formed in about 1 second in these microfluidic networks. A fine screen around optimal conditions in $(500\ \mu m)^2$ channels is expected to use more or less 50 plugs. Another ~5 μL of solution and another 50 μg of the protein are expected to be consumed. This can produce crystals up to $(100\ \mu m)^3$ in size. Approximately 30 plugs can be formed about every second or so. The throughput of the system will generally be determined by the rate of plug formation, and may be limited by how rapidly the flow rates can be varied. Pressure control methods that operate at frequencies of 100 Hz are available and may be applied to PDMS microfluidic networks (Unger et al., "Monolithic fabricated valves and pumps by multilayer soft lithography," *Science* 2000, vol. 288, pp. 113-116.).

Crystal properties such as appearance, size, optical quality, and diffractive properties may be characterized and measured under different conditions. For example, a Raxis IIc X-ray detector mounted on a Rigaku RU 200 rotating anode X-ray generator, which is equipped with double focusing mirrors and an MSC cryosystem, may be used for at least some of the characterizations and measurements. A synchrotron beam may be useful for characterization of small crystals. Also, these devices and methods may be used to build microfluidic systems according to the invention that are compatible with structural studies using x-ray beams.

A significant problem involving current crystallization approaches is determining the conditions for forming crystals with optimal diffractive properties. Normally crystals have to be grown, isolated, mounted, and their diffractive properties determined using an x-ray generator or a synchrotron.

Microfluidic systems with thin, non-scattering walls would be desirable for determining the diffractive properties of crystals inside a microfluidic system. Preferably, crystallization is carried out inside this system using methods according to the invention, which are described herein. The crystals are exposed to x-ray beams either to determine their structure or diffractive properties (the screening mode). For example, a PDMS membrane defining two side walls of the channels could be sandwiched between two very thin glass plates (defining the top and bottom walls of the channels) that do not significantly scatter X-rays. Thus, the devices of the invention offer a further advantage in that structural characterization could be conducted while the sample is inside the microfluidic device. Thus, the sample can be characterized without the need to take out the sample, e.g., crystal, from the device.

The present system enables higher throughput automated systems with improved speed, sample economy, and entirely new methods of controlling crystallization. Microfluidic versions of microbatch, vapor phase diffusion and FID techniques may be carried out using the present invention, as described below, or using a combination of these techniques or other techniques. In addition, the nucleation and growth phases may be carried out in discrete steps through merging plugs, as described herein.

Screening for protein crystallization involves varying a number of parameters. During crystallization screening, a large number of chemical compounds may be employed. These compounds include salts, small and large molecular weight organic compounds, buffers, ligands, small-molecule agents, detergents, peptides, crosslinking agents, and derivatizing agents. Together, these chemicals can be used to vary the ionic strength, pH, solute concentration, and target concentration in the plug, and can even be used to modify the target. The desired concentration of these chemicals to achieve crystallization is variable, and can range from nanomolar to molar concentrations.

A typical crystallization mix contains set of fixed, but empirically-determined, types and concentrations of precipitation agent, buffers, salts, and other chemical additives (e.g., metal ions, salts, small molecular chemical additives, cryoprotectants, etc.). Water is a key solvent in many crystallization trials of biological targets, as many of these molecules may require hydration to stay active and folded. Precipitation agents act to push targets from a soluble to insoluble state, and may work by volume exclusion, changing the dielectric constant of the solvent, charge shielding, and molecular crowding. Precipitation agents compatible with the PDMS material of certain embodiments according to the invention include, but are not limited to, nonvolatile salts, high molecular weight polymers, polar solvents, aqueous solutions, high molecular weight alcohols, divalent metals.

Precipitation agents, which include large and small molecular weight organics, as well as certain salts, may be used from under 1% to upwards of 40% concentration, or from <0.5M to greater than 4M concentration. Water itself can act in a precipitating manner for samples that require a certain level of ionic strength to stay soluble. Many precipitation agents may also be mixed with one another to increase the chemical diversity of the crystallization screen. Devices according to the invention are readily compatible with a broad range of such compounds.

A nonexclusive list of salts that may be used as precipitation agents is as follows: tartrates (Li, Na, K, Na/K, NH_4); phosphates (Li, Na, K, Na/K, NH_4); acetates (Li, Na, K, Na/K, Mg, Ca, Zn, NH_4); formates (Li, Na, K, Na/K, Mg, NH_4); citrates (Li, Na, K, Na/K, NH_4); chlorides (Li, Na, K, Na/K, Mg, Ca, Zn, Mn, Cs, Rb, NH_4); sulfates (Li, Na, K, Na/K, NH_4); maleates (Li, Na, K, Na/K, NH_4); glutamates (Li, Na, K, Na/K, NH_4.

A nonexclusive list of organic materials that may be used as precipitation agents is as follows: PEG 400; PEG 1000; PEG 1500; PEG 2K; PEG 3350; PEG 4K; PEG 6K; PEG 8K; PEG 10K; PEG 20K; PEG-MME 550; PEG-MME 750; PEG-MME 2K; PEGMME 5K; PEG-DME 2K; dioxane; methanol; ethanol; 2-butanol; n-butanol; t-butanol; jeffamine m-600; isopropanol; 2-methyl-2,4-pentanediol; 1,6 hexanediol.

Solution pH can be varied by the inclusion of buffering agents; typical pH ranges for biological materials lie anywhere between values of 3 and 10.5 and the concentration of buffer generally lies between 0.01 and 0.25 M. The microfluidics devices described in this document are readily compatible with a broad range of pH values, particularly those suited to biological targets.

A nonexclusive list of possible buffers that may be used according to the invention is as follows: Na-acetate; HEPES; Na-cacodylate; Na-citrate; Na-succinate; Na—K-phosphate; TRIS; TRIS-maleate; imidazole-maleate; bistrispropane; CAPSO, CHAPS, MES, and imidazole.

Additives are small molecules that affect the solubility and/or activity behavior of the target. Such compounds can speed up crystallization screening or produce alternate crystal forms or polymorphs of the target. Additives can take nearly any conceivable form of chemical, but are typically mono and polyvalent salts (inorganic or organic), enzyme ligands (substrates, products, allosteric effectors), chemical crosslinking agents, detergents and/or lipids, heavy metals, organometallic compounds, trace amounts of precipitating agents, and small molecular weight organics.

The following is a nonexclusive list of additives that may be used in accordance with the invention: 2-butanol; DMSO; hexanediol; ethanol; methanol; isopropanol; sodium fluoride; potassium fluoride; ammonium fluoride; lithium chloride anhydrous; magnesium chloride hexahydrate; sodium chloride; calcium chloride dihydrate; potassium chloride; ammonium chloride; sodium iodide; potassium iodide; ammonium iodide; sodium thiocyanate; potassium thiocyanate; lithium nitrate; magnesium nitrate hexahydrate; sodium nitrate; potassium nitrate; ammonium nitrate; magnesium formate; sodium formate; potassium formate; ammonium formate; lithium acetate dihydrate; magnesium acetate tetrahydrate; zinc acetate dihydrate; sodium acetate trihydrate; calcium acetate hydrate; potassium acetate; ammonium acetate; lithium sulfate monohydrate; magnesium sulfate heptahydrate; sodium sulfate decahydrate; potassium sulfate; ammonium sulfate; di-sodium tartrate dihydrate; potassium sodium tartrate tetrahydrate; di-ammonium tartrate; sodium dihydrogen phosphate monohydrate; di-sodium hydrogen phosphate dihydrate; potassium dihydrogen phosphate; di-potassium hydrogen phosphate; ammonium dihydrogen phosphate; di-ammonium hydrogen phosphate; tri-lithium citrate tetrahydrate; tri-sodium citrate dihydrate; tri-potassium citrate monohydrate; diammonium hydrogen citrate; barium chloride; cadmium chloride dihydrate; cobaltous chloride dihydrate; cupric chloride dihydrate; strontium chloride hexahydrate; yttrium chloride hexahydrate; ethylene glycol; Glycerol anhydrous; 1,6 hexanediol; MPD; polyethylene glycol 400; trimethylamine HCl; guanidine HCl; urea; 1,2,3-heptanetriol; benzamidine HCl; dioxane; ethanol; iso-propanol; methanol; sodium iodide; L-cysteine; EDTA sodium salt; NAD; ATP disodium salt; D(+)-glucose monohydrate; D(+)-sucrose; xylitol; spermidine; spermine tetra-HCl; 6-aminocaproic acid; 1,5-diaminopentane diHCl; 1,6-diami-

nohexane; 1,8-diaminooctane; glycine; glycyl-glycyl-glycine; hexaminecobalt trichloride; taurine; betaine monohydrate; polyvinylpyrrolidone K15; non-detergent sulfobetaine 195; non-detergent sulfo-betaine 201; phenol; DMSO; dextran sulfate sodium salt; Jeffamine M-600; 2,5 Hexanediol; (+/−)-1,3 butanediol; polypropylene glycol P400; 1,4 butanediol; tert-butanol; 1,3 propanediol; acetonitrile; gamma butyrolactone; propanol; ethyl acetate; acetone; dichloromethane; n-butanol; 2,2,2 trifluoroethanol; DTT; TCEP; nonaethylene glycol monododecyl ether, nonaethylene glycol monolauryl ether; polyoxyethylene (9) ether; octaethylene glycol monododecyl ether, octaethylene glycol monolauryl ether; polyoxyethylene (8) lauryl ether; Dodecyl-β-D-maltopyranoside; Lauric acid sucrose ester; Cyclohexyl-pentyl-β-D-maltoside; Nonaethylene glycol octylphenol ether; Cetyltrimethylammonium bromide; N,N-bis(3-D-gluconamidopropyl)-deoxycholamine; Decyl-β-D-maltopyranoside; Lauryldimethylamine oxide; Cyclohexyl-pentyl-β-D-maltoside; n-Dodecylsulfobetaine, 3-(Dodecyldimethylanimonio)propane-1-sulfonate; Nonyl-β-D-glucopyranoside; Octyl-β-D-thioglucopyranoside, OSG; N,N-Dimethyldecylamine-β-oxide; Methyl 0-(N-heptylcarbamoyl)-α-D-glucopyranoside; Sucrose monocaproylate; n-Octanoyl-β-D-fructofuranosyl-α-D-glucopyranoside; Heptyl-β-D-thioglucopyranoside; Octyl-β-D-glucopyranoside, OG; Cyclohexyl-propyl-β-D-maltoside; Cyclohexylbutanoyl-N-hydroxyethylglucamide; n-decylsulfobetaine, 3-(Decyldimethylammonio)propane-1sulfonate; Octanoyl-N-methylglucamide, OMEGA; Hexyl-β-D-glucopyranoside; Brij 35; Brij 58; Triton X-114; Triton X-305; Triton X-405; Tween 20; Tween 80; polyoxyethylene(6)decyl ether; polyoxyethylene(9)decyl ether; polyoxyethylene(10) dodecyl ether; polyoxyethylene(8)tridecyl ether; Decanoyl-N-hydroxyethylglucamide; Pentaethylene glycol monooctyl ether; 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; 3-[(3-Cholamidopropyl)-dimethylammonio] hydroxy-1-propane sulfonate; Cyclohexylpentanoyl-N-hydroxyethylglucamide; Nonanoyl-N-hydroxyethyglucamide; Cyclohexylpropanol-N-hydroxyethylglucamide; Octanoyl-N-hydroxyethylglucamide; Cyclohexylethanoyl-N-hydroxyethylglucamide; Benzyldimethyldodecyl ammonium bromide; n-Hexadecyl-β-D-maltopyranoside; n-Tetradecyl-β-D-maltopyranoside; n-Tridecyl-β-D-maltopyranoside; Dodecylpoly(ethyleneglycoether); n-Tetradecyl-N,N-dimethyl ammonio-1-propanesulfonate; n-Undecyl-β-D-maltopyranoside; n-Decyl D-thiomaltopyranoside; n-dodecylphosphocholine; α-D-glucopyranoside, β-D-fructofuranosyl monodecanoate, sucrose mono-caprate; 1-s-Nonyl-β-D-thioglucopyranoside; n-Nonyl-β-D-thiomaltoyranoside; N-Dodecyl-N,N-(dimethlammonio) butyrate; n-Nonyl-β-D-maltopyranoside; Cyclohexyl-butyl D-maltoside; n-Octyl-β-D-thiomaltopyranoside; n-Decylphosphocholine; n-Nonylphosphocholine; Nonanoyl-N-methylglucamide; 1-s-Heptyl-β-D-thioglucopyranoside; n-Octylphosphocholine; Cyclohexyl-ethyl D-maltoside; n-Octyl-N,N-dimethyl ammonio-1-propanesulfonate; Cyclohexyl-methyl-β-D-maltoside.

Cryosolvents are agents that stabilize a target crystal to flash-cooling in a cryogen such as liquid nitrogen, liquid propane, liquid ethane, or gaseous nitrogen or helium (all at approximately 100-120° K.) such that crystal becomes embedded in a vitreous glass rather than ice. Any number of salts or small molecular weight organic compounds can be used as a cryoprotectant, and typical ones include but are not limited to: MPD, PEG-400 (as well as both PEG derivatives and higher molecular-weight PEG compounds), glycerol, sugars (xylitol, sorbitol, erythritol, sucrose, glucose, etc.), ethylene glycol, alcohols (both short- and long chain, both volatile and nonvolatile), LiOAc, LiCl, $LiCHO_2$, $LiNO_3$, Li_2SO_4, $Mg(OAc)_2$, NaCl, $NaCHO_2$, $NaNO_3$, etc. Again, materials from which microfluidics devices in accordance with the present invention are fabricated may be compatible with a range of such compounds.

Many of these chemicals can be obtained in predefined screening kits from a variety of vendors, including but not limited to Hampton Research of Laguna Niguel, Calif., Emerald Biostructures of Bainbridge Island, Wash., and Jena Bioscience of Jena, Germany, that allow the researcher to perform both sparse matrix and grid screening experiments. Sparse matrix screens attempt to randomly sample as much of precipitant, buffer, and additive chemical space as possible with as few conditions as possible. Grid screens typically consist of systematic variations of two or three parameters against one another (e.g., precipitant concentration vs. pH). Both types of screens have been employed with success in crystallization trials, and the majority of chemicals and chemical combinations used in these screens are compatible with the chip design and matrices in accordance with embodiments of the present invention. Moreover, current and future designs of microfluidic devices may enable flexible combinatorial screening of an array of different chemicals against a particular target or set of targets, a process that is difficult with either robotic or hand screening. This latter aspect is particularly important for optimizing initial successes generated by first-pass screens.

In addition to chemical variability, a host of other parameters can be varied during crystallization screening. Such parameters include but are not limited to: (1) volume of crystallization trial; (2) ratio of target solution to crystallization solution; (3) target concentration; (4) cocrystallization of the target with a secondary small or macromolecule; (5) hydration; (6) incubation time; (7) temperature; (8) pressure; (9) contact surfaces; (10) modifications to target molecules; and (11) gravity.

Although the discussion below refers to proteins, the particular devices or methods described can also be used or suitably adapted for the crystallization of other types of samples such as those mentioned above (e.g., small molecules, other macromolecules, nanoparticles, colloidal particles, etc.). In one aspect of the present invention, protein crystallization is conducted using miniaturized microbatch conditions. The process consists of two steps. First, plugs are preferably formed wherein the concentrations of the protein, precipitant, and additive are adjusted by varying the relative flow rates of these solutions. This step corresponds to a screening step. Once the optimal concentrations have been found, the flow rates can then be kept constant at the optimal conditions. In this step, plugs are preferably transported through the channel as they form. Second, the flow is preferably stopped once the desired number of plugs are formed. The plugs are then preferably allowed to incubate. In some embodiments according to the invention the flow may be continued, rather than stopped. In those embodiments, the flow is maintained sufficiently slow and the channels are made sufficiently long that plugs spend sufficient time in the channels for crystallization to occur (from tens of minutes to weeks, but may be faster or slower).

In one aspect, upon formation of the plugs, they are trapped using expansions in the channels. The expansions act as dead volume elements while the plugs are being formed in the presence of flow. Thus, the expansions do not interfere with the flow of the plugs through the channel. Once the flow is stopped, surface tension drives plugs into the expansions where surface tension is minimized. The expansions may be,

but are not limited to, oval, round, square, rectangular, or star-shaped. In particular, a star-shaped expansion may prevent adherence of the plug or of a crystal to the walls of the expansion. The ratio of the size of the expansion opening to the width of the channel may be varied based on empirical results for a particular set of conditions. FIG. **16** is a schematic illustration of a microfluidic device according to the invention that illustrates the trapping of plugs. In experiments, plugs were sustained in perfluorodecaline inside a channel for one day, and did not appear to change during that time (a refractive index mismatch between the fluorinated and aqueous phase was introduced to aid in visualization of plugs).

The method described above allows a high degree of control over protein and precipitant concentrations. It also allows a high degree of control over a range of time scales through the control of plug size and composition. FIG. **17** shows a schematic of a microfluidic method for forming plugs with variable compositions for protein crystallization. Continuously varied flow rates of the incoming streams are preferably used to form plugs with various concentrations of the protein, precipitation agents, and additives. In FIG. **17**, for example, the following can be introduced into the various inlets: buffers into inlets **171**, **172**; PEG into inlet **173**; salt into inlet **174**; solvent into inlet **175**; and protein into inlet **176**. These various solutions can enter a channel **177** through which a carrier fluid such as perfluorodecaline flows. For example, a 1-meter long channel with a 200×80 μm cross section can be used to form approximately two hundred 6 nL (nanoliter) plugs. If each plug contains enough protein to form a 40-μm^3 crystal, 200 trials will consume only about 1.2 μL of approximately 10 mg/mL protein solution (12 μg of protein). About one minute may be sufficient to form plugs in these trials.

In another aspect according to the invention, after plugs are formed as described above for the microbatch system, slow evaporation through a very thin PDMS membrane (or another membrane with slight water permeability) is preferably used for added control over the crystallization process. A slow decrease in the volume of the plug during evaporation is expected to produce a trajectory of the solution through the crystallization phase space similar to that in a vapor diffusion experiment. Hence, this method, in addition to microbatch methods, can be used to miniaturize and optimize vapor diffusion methods.

In the vapor diffusion method, a drop containing protein, stabilizing buffers, precipitants, and/or crystallization agents is allowed to equilibrate in a closed system with a much larger reservoir. The reservoir usually contains the same chemicals minus the protein but at an over all higher concentration so that water preferentially evaporates from the drop. If conditions are right, this will produce a gradual increase in protein concentration such that a few crystals may form.

Vapor diffusion can be performed in two ways. The one most often used is called Hanging Drop Technique. The drop is placed on a glass coverslip, which is then inverted and used to seal a small reservoir in a Linbro Plate. After a period of several hours to weeks, microscopic crystals may form and continue to grow. The other set up is known as Sitting Drop. In this method a drop (usually >10 uL) is placed in a depression in either a Micro Bridge in a Linbro Plate or a glass plate and again placed in a closed system to equilibrate with a much larger reservoir. One usually uses the sitting drop technique if the drop has very low surface tension, making it hard to turn upside down or if the drops need to be larger than 20 uL. Also, in some cases, crystals will grow better using one technique or the other.

In another embodiment, the plugs are preferably formed and transported such that excessive mixing of the protein with the precipitation agent is minimized or prevented. For example, gentle mixing using spiral channels may be used to achieve this and also to create interfaces between the protein and the precipitation agent. Alternatively, combining two streams of plugs in a T-junction without merging may be used to create plugs that diffuse and combine without significant mixing to establish a free interface after the flow is stopped. Diffusion of the proteins and precipitates through the interface induces crystallization. This is an analogue of the Free-Interface Diffusion method. It may be performed under either the microbatch or vapor diffusion conditions as described above.

Preferably, the spacing between plugs can be increased or the oil composition changed to reduce plug-plug diffusion. For example, a spacing of about 2.5 mm in paraffin oil can be used, which has been shown to be an effective barrier to aqueous diffusion in crystallization trials.

Visually identifying small crystals inside plugs with curved surfaces can be a challenge when performing microbatch experiments. In an aspect according to the invention, a method based on matching the refractive indices of carrier-fluid with that of the plug fluid to enhance visualization is used. Microscopic detection is preferably performed by using shallow channels and by matching the refractive indices of carrier-fluid mixtures to those of the aqueous solutions.

In addition, at least three other novel methods of controlling protein crystallization are described below: (1) using surface chemistry to effect nucleation of protein crystals; (2) using different mixing methods to effect crystallization; and (3) performing protein crystals seeding by separating nucleation and growth phases in space.

Control of nucleation is one of the difficult steps in protein crystallization. Heterogeneous nucleation is statistically a more favorable process than its solution-phase counterpart. Ideal surfaces for heterogeneous nucleation have complementary electrostatic maps with respect to their macromolecular counterparts. Critical nuclei are more stable on such surfaces than in solution. Further, the degree of supersaturation required for heterogeneous nucleation is much less than that required for the formation of solution-phase nuclei. Surfaces such as silicon, crystalline minerals, epoxide surfaces, polystyrene beads, and hair are known to influence the efficiency of protein crystallization. Few studies have been done, but promising results have been shown for protein crystallization at the methyl, imidazole, hydroxyl, and carboxylic acid termini of self-assembled monolayers on gold. Using self-assembled monolayers, proteins were crystallized over a broader range of crystallization conditions and at faster rates than when using the traditional silanized glass.

FIG. **18** is a schematic illustration of a method for controlling heterogeneous nucleation by varying the surface chemistry at the interface of an aqueous plug-fluid and a carrier-fluid. In FIG. **18**, plugs are formed in the presence of several solutions of surfactants that possess different functional groups (left side of the diagram). The right side of FIG. **18** shows the aqueous phase region in which a precipitant, solvent, and protein may be introduced into inlets **180**, **181**, and **182**, respectively. The composition of the surfactant monolayer is preferably controlled by varying the flow rates. In another application of the method illustrated in FIG. **18**, the surface chemistry can be varied continuously. The manipulation and control of the surface chemistry can be used for screening, assays, crystallizations, and other applications where surface chemistry is important.

In one aspect of the invention, heterogeneous nucleation of proteins is controlled by forming aqueous plugs in a carrier-fluid, preferably containing fluoro-soluble surfactants if the carrier-fluid is a fluorocarbon. Varying the relative flow rates of the surfactant solutions may generate a wide variety of liquid-liquid interface conditions that can lead to the formation of mixed monolayers or mixed phase-separated monolayers. Preferably, several surfactants are used to control the heterogeneous nucleation of protein crystals. Ethylene-glycol monolayers are preferably used to reduce heterogeneous nucleation, and monolayers with electrostatic properties complementary to those of the protein are preferably used to enhance heterogeneous nucleation. These methods for controlling heterogeneous nucleation are designed to induce or enhance the formation of crystals that are normally difficult to obtain. These methods may also be used to induce or enhance the formation of different crystal polymorphs that are relatively more stable or better ordered.

As mentioned above, control of nucleation is highly desired in an advanced crystallization screen. One method that can be used to achieve control of nucleation involves the transfer of nucleating crystals from one concentration to another via dilution. This method, which has been applied in macroscopic systems primarily to vapor diffusion, was intended to allow decoupling of the nucleation and growth phases. This method is difficult to perform using traditional methods of crystallization because nucleation occurs long before the appearance of microcrystals.

FIG. **19** illustrates a method of separating nucleation and growth using a microfluidic network according to the present invention using proteins as a non-limiting example. The left side of FIG. **19** shows plugs that are formed preferably using high concentrations of protein and precipitant. In FIG. **19**, the following can be introduced into the various inlets shown: buffer into inlets **191**, **196**; PEG into inlets **192**, **197**; precipitant into inlets **193**, **198**; solvent into inlets **194**, **199**; and protein into inlets **195**, **200**. Oil flows through the channels **201**, **202** from left to right. The portions **203**, **204**, and **205** of the channel correspond to regions where fast nucleation occurs (**203**), no nucleation occurs (**204**), and where crystal growth occurs (**205**). The concentrations used are those that correspond to the nucleating region in the phase diagram. Nucleation occurs as the plugs move through the channel to the junction over a certain period. Preferably, these plugs are then merged with plugs containing a protein solution at a point corresponding to a metastable (growth, rather than nucleation) region (right side of FIG. **19**). This step ends nucleation and promotes crystal growth. When the combined channel has been filled with merged plugs, the flow is preferably stopped and the nuclei allowed to grow to produce crystals.

Nucleation time can be varied by varying the flow rate along the nucleation channel. The nucleus is preferably used as a seed crystal for a larger plug with solution concentrations that correspond to a metastable region. Existing data indicate the formation of nuclei within less than about 5 minutes.

Fluid mixing is believed to exert an important effect in crystal nucleation and growth. Methods according to the invention are provided that allow a precise and reproducible degree of control over mixing. FIG. **20** illustrates two of these methods. A method of mixing preferably places the solution into a nucleation zone of the phase diagram without causing precipitation. Preferably, gentle mixing (FIG. **20**, left side) is used to achieve this by preventing, reducing, or minimizing contact between concentrated solutions of the protein and precipitant. Alternatively, rapid mixing (FIG. **20**, right side) is used to achieve this by allowing passage through the precipitation zone sufficiently quickly to cause nucleation but not precipitation. The two methods used as examples involve the use of spiraling channels for gentle mixing and serpentine channels for rapid mixing.

The two methods in accordance with the invention depicted in FIG. **20** can be used to determine the effect of mixing on protein crystallization. In addition, the various methods for controlling mixing described previously (e.g., slow mixing in straight channels, chaotic mixing in non-straight channels, or mixing in which twirling may or may not occur) can be applied to crystallization, among other things.

After obtaining the crystals using any of the above described techniques, the crystals may be removed from the microfluidic device for structure determination. In other systems, the fragile and gelatinous nature of protein crystals makes crystal collection difficult. For example, removing protein crystals from solid surfaces can damage them to the point of uselessness. The present invention offers a solution to this problem by nucleating and growing crystals in liquid environments. In an aspect according to the invention, a thin wetting layer of a carrier-fluid covered with a surfactant is used to enable or facilitate the separation of a growing crystal from a solid surface. When the crystals form, they may be separated from the PDMS layer by using a thin layer of a carrier-fluid.

It will be clear to one skilled in the art that while the above techniques are described in detail for the crystallization of proteins, techniques similar to the ones described above may also be used for the crystallization of other substances, including other biomolecules or synthetic chemicals. In addition, the devices and methods according to the invention may be used to perform co-crystallization. For example, a crystal comprising more than one chemical may be obtained, for example, through the use of at least one stream of protein, a stream of precipitant, and optionally, a stream comprising a third chemical such as an inhibitor, another protein, DNA, etc. One may then vary the conditions to determine those that are optimal for forming a co-crystal.

Particle Separation/Sorting Using Plugs

The flow within the moving plugs can be used for separation of polymers and particles. Plugs can be used for separation by first using flow within a moving plug to establish a distribution of the polymers or particles inside the plug (for example, an excess of the polymer inside the front, back, right or left side of the plug) and then using splitting to separate and isolate the part of the plug containing higher concentration of the polymers or particles. When two polymers or particles are present inside the plug and establish different distributions, splitting can be used to separate the polymers or particles.

The invention is further described below, by way of the following examples. It will be appreciated by persons of ordinary skill in the art that this example is one of many embodiments and is merely illustrative. In particular, the device and method described in this example (including the channel architectures, valves, switching and flow control devices and methods) may be readily adapted, e.g., used in conjunction with one or more devices or methods, so that plugs may be analyzed, characterized, monitored, and/or sorted as desired by a user.

EXAMPLE

Example 1

Fabrication of Microfluidic Devices and a General Experimental Procedure

Microfluidic devices with hydrophilic channel surfaces were fabricated using rapid prototyping in polydimethylsi-

loxane. The channel surfaces were rendered hydrophobic either by silanization or heat treatment. To silanize the surfaces of channels, (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.) vapor was applied to the inlets of a device with dry nitrogen as a carrier gas at around 40-60 mm Hg above about 1 atm pressure. Vacuum was simultaneously applied to the outlet of the device at about 650 mm Hg below atmospheric pressure. The silane vapor was applied for a period of between about 1-3 hours. To treat the channels using heat, a device was placed in an oven at approximately 120° C. for about three hours. Alternatively, a device can be heated in a Panasonic "The Genius" 1300 Watt microwave oven at power set to "10" for about ten minutes.

Oils and aqueous solutions were pumped through devices using a kdScientific syringe pump (Model 200) or Harvard Apparatus PhD 2000 pump. Hamilton Company GASTIGHT syringes were used (10-250 μl) and Hamilton Company 30 gauge Teflon® needles were used to attach the syringes to the devices. Oils and aqueous solutions were pumped through devices at volumetric flow rates ranging from about 0.10 μL/min to about 10.0 μL/min.

Aqueous solutions were colored using Crayola Original Formula Markers or Ferroin Indicator (0.025 M, Fisher Scientific). Oils that were used included perfluorodecaline (mixture of cis and trans, 95%, Acros Organics), perfluoroperhydrophenanthrene (tech., Alfa-Aesar), or 1H,1H,2H,2H-perfluorooctanol (98%, Alfa-Aesar). The experiments were typically performed using 10:1 mixtures of perfluorodecaline and 1H,1H,2H,2H-perfluorooctanol.

The experiments were monitored using a Lica MZFLIII stereoscope with Fostec (Schott-Fostec, LLC) Modulamps. Photographs of the experiments were taken with a Spot Insight Color Camera, Model # 3.2.0 (Diagnostic Instruments, Inc.). Spot Application version 3.4.0.0 was used to take the photographs with the camera.

Example 2

Varying the Concentration of Aqueous Solutions in Plugs

The left side of each of FIGS. **25**A-C shows a schematic diagram of the microfluidic network and the experimental conditions. The right side of each of FIGS. **25**A-C shows microphotographs illustrating the formation of plugs using different concentrations of the aqueous streams. Aqueous solutions of food dyes (red/dark and green/light) and water constituted the three streams. The volumetric flow rates of the three solutions (given in μL/min) are indicated. The dark stream is more viscous than the light stream. Therefore, the dark (more viscous) stream moves (measured in mm/s) more slowly and occupies a larger fraction of the channel at a given volumetric flow rate.

FIG. **45***a*) shows a schematic of the microfluidic network used to demonstrate that on-chip dilutions can be accomplished by varying the flow rates of the reagents. In FIG. **45***a*), the reagents are introduced through inlets **451**, **453** while the dilution buffer is introduced through inlet **452**. An oil stream flows through channel **454**. The blue rectangle outlines the field of view for images shown in FIG. **45***c*)-*d*). FIG. **45***b*) shows a graph quantifying this dilution method by measuring fluorescence of a solution of fluorescein diluted in plugs in the microchannel. Data are shown for 80 experiments in which fluorescein was flowed through one of the three inlets, where $C_{measured}$ and $C_{theoretical}$ [μM] are measured and expected fluorescein concentration. FIG. **45**(*c*) shows photographs illustrating this dilution method with streams of food dyes **455**, **456**, **457** having flow rates of 45 nL/s, 10 nL/s, and 10 nL/s, respectively. FIG. **45**(*d*) shows photographs illustrating this dilution method with streams of food dyes **458**, **459**, **460** having flow rates of 10 nL/s, 45 nL/s, and 10 nL/s, respectively. Carrier fluid was flowed at 60 nL/s.

Example 3

Networks of microchannels with rectangular cross-sections were fabricated using rapid prototyping in PDMS. The PDMS used was Dow Corning Sylgard Brand 184 Silicone Elastomer, and devices were sealed using a Plasma Prep II (SPI Supplies). The surfaces of the devices were rendered hydrophobic by baking the devices at 120° C. for 2-4 hours.

In FIG. **26**, the red aqueous streams were McCormick® red food coloring (water, propylene glycol, FD&C Red 40 and 3, propylparaben), the green aqueous streams were McCormick® green food coloring (water, propylene glycol, FD&C yellow 5, FD&C blue 1, propylparaben) diluted 1:1 with water, and the colorless streams were water. PFD used was a 10:1 mixture of perfluorodecaline (mixture of cis and trans, 95%, Acros Organics):1H,1H,2H,2H-perfluorooctanol (Acros Organics). The red aqueous streams were introduced in inlet **260**, **265** while the green aqueous streams were introduced in inlets **262**, **263** in FIG. **26***b*). The colorless aqueous stream was introduced in inlets **261**, **264**. The dark shadings of the streams and plug are due mainly from the red dye while the lighter shadings are due mainly from the green dye.

Aqueous solutions were pumped using 100 μL Hamilton Gastight syringes (1700 series, TLL) or 50 μL SGE gastight syringes. PFD was pumped using 1 mL Hamilton Gastight syringes (1700 series, TLL). The syringes were attached to microfluidic devices by means of Hamilton Teflon needles (30 gauge, 1 hub). Syringe pumps from Harvard Apparatus (PHD 2000 Infusion pumps; specially-ordered bronze bushings were attached to the driving mechanism to stabilize pumping) were used to infuse the aqueous solutions and PFD.

Microphotographs were taken with a Leica MZ12.5 stereomicroscope and a SPOT Insight Color digital camera (Model #3.2.0, Diagnostic Instruments, Inc.). SPOT Advanced software (version 3.4.0 for Windows, Diagnostic Instruments, Inc.) was used to collect the images. Lighting was provided from a Machine Vision Strobe X-Strobe X1200 (20 Hz, 12 μF, 600V, Perkin Elmer Optoelectronics). To obtain an image, the shutter of the camera was opened for 1 second and the strobe light was flashed once with the duration of the flash being about 10 μs.

Images were analyzed using NIH Image software, Image J. Image J was used to measure periods and lengths of plugs from microphotographs such as shown in FIG. **27***b*). Periods corresponded to the distance from the center of one plug to the center of an adjacent plug, and the length of a plug was the distance from the extreme front to the extreme back of the plug (see FIG. **28** for the definitions of front and back). Measurements were initially made in pixels, but could be converted to absolute measurements by comparing them to a measurement in pixels of the 50 μm width of the channel.

To make measurements of the optical intensity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs, microphotographs were converted from RGB to CMYK color mode in Adobe Photoshop 6.0. Using the same program, the yellow color channels of the microphotographs were then isolated and converted to grayscale images, and the intensities of the grayscale images were inverted. The yellow color channel was chosen to reduce the intensity of bright reflections at the extremities of the plugs and at the interface between the plugs and the channel. Fol-

lowing the work done in Photoshop, regions of plugs containing high concentrations of $Fe(SCN)_x^{(3-x)+}$ complexes appeared white while regions of low concentration appeared black. Using Image J, the intensity was measured across a thin, rectangular region of the plug, located halfway between the front and back of the plug (white dashed lines in FIG. **27***a***1**)). The camera used to take the microphotographs of the system was not capable of making linear measurements of optical density. Therefore, the measurements of intensity were not quantitative. Several of the plots of intensity versus relative position across the channel (FIG. **27***c*) were shifted vertically by less than 50 units of intensity to adjust for non-uniform illuminations of different parts of the images. These adjustments were justified because it was the shape of the distribution that was of interest, rather than the absolute concentration.

FIG. **29***a*)-*b*) shows plots of the sizes of periods and sizes of plugs as a function of total flow velocity (FIG. **29***a*)) and water fraction (wf) (FIG. **29***b*)). Values of capillary number (C.n.) were 0.0014, 0.0036, 0.0072 and 0.011, while values of the Reynolds number (R_e) were 1.24, 3.10, 6.21, and 9.31, each of the C.n. and R_e value corresponding to a set of data points with water fractions (wf) 0.20, 0.52, 0.52, and 0.20 (the data points from top to bottom in FIG. **29**A)). In turn, each of these sets of data points corresponds to a particular flow velocity as shown in FIG. **29***a*). Plugs in FIG. **29***b*) travel at about 50 millimeter/second (mm/s). All measurements of length and size are relative to the width of the channels (50 μm).

FIG. **30** shows microphotographs illustrating weak dependence of periods, length of plugs, and flow patterns inside plugs on total flow velocity. The left side of FIG. **30** shows a diagram of the microfluidic network. Here, the same solutions were used as in the experiment corresponding to FIG. **27**. The $Fe(SCN)_x^{(3-x)+}$ solution was introduced into inlet **301** while the colorless aqueous streams were introduced into inlets **302**, **303**. The same carrier fluid as used in the FIG. **27** experiment was flowed into channel **304**. The right side of FIG. **30** shows microphotographs of plugs formed at the same water fraction (0.20), but at different total flow velocities (20, 50, 100, 150 mm/s from top to bottom). Capillary numbers were 0.0014, 0.0036, 0.0072, and 0.011, respectively, from top to bottom. Corresponding Reynolds numbers were 1.24, 3.10, 6.21, and 9.31.

FIG. **31**A-C are plots showing the distribution of periods and lengths of plugs where the water fractions were 0.20, 0.40, and 0.73, respectively. The total flow velocity was about 50 mm/s, C.n.=0.0036, R_e=3.10 in all cases.

FIG. **27** shows the effects of initial conditions on mixing by recirculating flow inside plugs moving through straight microchannels. FIG. **27***a***1**) shows that recirculating flow (shown by black arrows) efficiently mixed solutions of reagents that were initially localized in the front and back halves of the plug. Notations of front, back, left, and right are the same as that in FIG. **28**. FIG. **27***a***2**) shows that recirculating flow (shown by black arrows) did not efficiently mix solutions of reagents that were initially localized in the left and right halves of the plugs. The left side of FIG. **27***b*) shows a schematic diagram of the microfluidic network. The two colorless aqueous streams were introduced into inlets **271**, **272** while a carrier fluid in the form of perfluorodecaline flowed through channel **273**. These solutions did not perturb the flow patterns inside plugs.

The right side of FIG. **27***b*) shows microphotographs of plugs of various lengths near the plug-forming region of the microfluidic network for water fractions of from 0.14 up to 1.00. FIG. **27***c***1**) shows a graph of the relative optical intensity of $Fe(SCN)_x^{(3-x)+}$ complexes in plugs of varying lengths. The intensities were measured from left (x=1.0) to right (x=0.0) across the width of a plug (shown by white dashed lines in FIG. **27***a***1**)-*a***2**)) after the plug had traveled 4.4 times its length through the straight microchannel. The gray shaded areas indicate the walls of the microchannel. FIG. **27***c***2**) is the same as FIG. **27***c***1**) except that each plug had traversed a distance of 1.3 mm. The d/l of each water fraction (wf) were 15.2 (wf 0.14), 13.3 (wf 0.20), 11.7 (wf 0.30), 9.7 (wf 0.40), 6.8 (wf 0.60), 4.6 (wf 0.73), and 2.7 (wf 0.84), where d is the distance traveled by the plug and l is the length of the plug.

Example 4

Merging of Plugs

Experiments were conducted to investigate the merging of plugs using different channel junctions (T- or Y-shaped), cross-sections, and flow rates (see FIG. **33***a*-*d*). The figures on the left side of FIGS. **33***a*-*d* show top views of microfluidic networks that comprise channels having either uniform or nonuniform dimension (e.g., the same or different channel diameters). The corresponding figures on the right are microphotographs that include a magnified view of two plug streams (from the two separate channels portions of which form the branches of the Y-shaped junction) that merges into a common channel.

In FIG. **33***a*, the oil-to-water volumetric ratio was 4:1 in each pair of oil and water inlets. The oil streams were introduced into inlets **330**, **332**, while the aqueous streams were introduced into inlets **331**, **333**. The flow rates of the combined oil/water stream past the junction where the oil and water meet was 8.6 mm/s. The channels, which were rectangular, had dimensions of 50 (width)×50 (height) μm^2. As shown in FIG. **33***a*, plugs that flow in uniform-sized channels typically merged only when they simultaneously arrived at the T-junction. Thus, plug merging in these channels occur infrequently. In addition, lagging plugs were typically not able to catch up with leading plugs along the common channel.

FIG. **33***b* illustrates plug merging occurring between plugs arriving at different times at the Y-shaped junction (magnified view shown). The oil streams were introduced into inlets **334**, **336**, while the aqueous streams were introduced into inlets **335**, **337**. In FIG. **33***b*, the flow rates for the combined oil/water fluid past the junction where the oil and water meet were 6.9 mm/s for channel **346** (the 50×50 μm^2 channel) and 8.6 mm/s for channel **347** (the 25×50 μm^2 channel). The oil-to-water volumetric ratio was 4:1 in each pair of oil and water inlets. The two channels (the branch channels) merged into a common channel **348** that had a 100×50 μm^2 cross-section. As shown in the figure, the larger plugs from the bigger channel are able to merge with the smaller plugs from the narrower channel even when they do not arrive at the junction at the same time. This is because lagging larger plugs are able to catch up with the leading smaller plugs once the plugs are in the common channel.

FIG. **33***c* depicts in-phase merging (i.e., plug merging upon simultaneous arrival of at least two plugs at a junction) of plugs of different sizes generated using different oil/water ratios at the two pairs of inlets. The oil streams were introduced into inlets **338**, **340**, while the aqueous streams were introduced into inlets **339**, **341**. The flow rate corresponding to the fluid stream through channel **349** resulting from a 1:1 oil-to-water volumetric ratio was 4.0 mm/s, while that through channel **350** corresponding to the 4:1 oil-to-water volumetric ratio was 6.9 mm/s. Each branch channel of the

Y-shaped portion of the network (magnified view shown) had a dimension of 50×50 μm^2 while the common channel **351** (the channel to which the branch channels merge) was 125×50 dm^2.

FIG. **33***d* illustrates defects (i.e., plugs that fail to undergo merging when they would normally merge under typical or ideal conditions) produced by fluctuations in the relative velocity of the two incoming streams of plugs. The oil streams were introduced into inlets **342**, **344**, while the aqueous streams were introduced into inlets **343**, **345**. In this experiment, the flow rate corresponding to the fluid stream through channel **352** resulting from a 1:1 oil-to-water volumetric ratio was 4.0 mm/s, while that through channel **353** corresponding to the 4:1 oil-to-water volumetric ratio was 6.9 mm/s. Each branch channel that formed one of the two branches of the Y-shaped intersection (magnified view shown) was 50×50 μm^2 while the common channel **354** (the channel to which the two branch channels merge) is 125×50 μm^2.

Example 5

Splitting Plugs Using a Constricted Junction

The splitting of plugs was investigated using a channel network with a constricted junction. In this case, the plugs split and flowed past the junction into two separate branch channels (in this case, branch channels are the channels to which a junction branches out) that are at a 180°-angle to each other (see FIGS. **34***a-c* each of which show a channel network viewed from the top). In these experiments, the outlet pressures, P_1 and P_2, past the constricted junction were varied such that either $P_1 \approx P_2$ (FIG. **34***b*) or $P_1 < P_2$ (FIG. **34***c*). Here, the relative pressures were varied by adjusting the relative heights of the channels that were under pressures P_1 and P_2. Since longer plugs tend to split more reliably, this branching point (or junction) was made narrower than the channel to elongate the plugs. FIG. **34***a* shows a schematic diagram of the channel network used in the experiment. The oil and water were introduced into inlets **3400** and **3401**, respectively. The oil-to-water ratio was 4:1 while the flow rate past the junction where the oil and water meet was 4.3 mm/s.

FIG. **34***b* is a microphotograph showing the splitting of plugs into plugs of approximately one-half the size of the initial plugs. The channels **3404**, which were rectangular, had a cross-section that measured 50×50 μm^2. The constricted section of the channel **3402** right next to the branching point measured 25×50 μm^2. The outlet pressures, P_1 and P_2, were about the same in both branch channels. Here, the plugs split into plugs of approximately the same sizes.

FIG. **34***c* is a microphotograph showing the asymmetric splitting of plugs (i.e., the splitting of plugs into plugs of different sizes or lengths) which occurred when $P_1 < P_2$. The microphotograph shows that larger plugs (somewhat rectangular in shape) flowed along the channel with the lower pressure P_1; while smaller plugs (spherical in shape) flowed along the channel with the higher pressure P_2. As in FIG. **34***b*, each of the channel **3405** cross-section measured 50×50 μm^2. The constricted section of the channel **3403** at the junction measured 25×50 μm^2.

Example 6

Splitting Plugs without Using a Constricted Junction

The splitting of plugs was investigated using a channel network without a constriction such as the one shown in FIGS. **35***b-c*. The channel network used was similar to that shown in FIG. **34**(*a*) except that here the plugs split and flowed past the junction in two separate channels at a 90°-angle to each other (the plug flow being represented by arrows). The oil and aqueous streams (4:1 oil:aqueous stream ratio) were introduced into inlets **3500** and **3501**, respectively. An oil-only stream flowed through channel **3502**. All channels had a cross-section of 50×50 μm^2. The flow rate used was 4.3 mm/s. FIGS. **35***a-c*, which represent top views of a channel network, show that plugs behave differently compared to the plugs in Example 3 when they flow past a junction in the absence of a channel constriction, such as a constriction shown in FIGS. **35***b-c*. As FIG. **35***c* shows, when $P_1 < P_2$, the plugs remained intact after passing through the junction. Further, the plugs traveled along the channel that had the lower pressure (P_1 in FIG. **35***c*) while the intervening oil stream split at the junction. The splitting of the oil stream at the junction gives rise to a shorter separation between plugs flowing along the channel with pressure P_1 compared to the separation between plugs in the channel upstream of the branching point or junction.

Example 7

Monitoring Autocatalytic Reactions Using a Microfluidic System

FIG. **37** illustrates the design of an experiment involving chemical amplification in microfluidic devices according to the invention that involves an investigation of a stochastic autocatalytic reaction. This example illustrates how the devices of the present invention can be used to study the acid-sensitive autocatalytic reaction between $NaClO_2$ and NaS_2O_3. On the left side of the microfluidic network, a three-channel inlet introduces an aqueous stream through channel **3702**, an ester through channel **3701**, and an esterase through channel **3703**. Oil flowed through channels **3713**, **3714**. The reaction between ester and esterase yield plugs **3704** that contain a small amount of acid. On the right side of the microfluidic network, the five-channel inlet introduces $NaClO_2$ through inlet **3705**, an aqueous stream through inlet **3706**, a pH indicator through inlet **3707**, a second aqueous stream through inlet **3708**, and NaS_2O_3 through channel **3709**. A carrier fluid flows through channels **3713**, **3714**. Unstirred mixtures of $NaClO_2$ and NaS_2O_3 are highly unstable and even a slight concentration fluctuation within that mixture leads to rapid decomposition. Thus, the plugs **3710** containing $NaClO_2/NaS_2O_3$ mixture must not only be quickly mixed but also promptly used after formation. In this proposed experiment, the curvy channels promote chaotic mixing. When a slightly acidic plug of the ester-esterase reaction is merged with a plug of an unstable $NaClO_2/NaS_2O_3$ mixture at the contact region **3712**, an autocatalytic reaction will generally be triggered. Upon rapid mixing of these two plugs, the resulting plugs **3711** become strongly acidic. The pH indicator introduced in the five-channel inlet is used to visualize this entire amplification process.

Example 8

Using Chemical Reactions as Highly Sensitive Autoamplifying Detection Elements in Microfluidic Devices

In one aspect according to the invention, a sequential amplification using controlled autocatalytic systems is used to amplify samples that contain single molecules of autocatalysts into samples containing a sufficiently high concentra-

tion of an autocatalyst such that the amplified autocatalyst can be detected with the naked eye can be detected with the naked eye. Although systems displaying stochastic behavior are expected to display high sensitivity and amplification, various autocatalytic systems can be used in accordance with the invention. A sequential amplification using the microfluidic devices according to the invention can be illustrated using a reaction that has been characterized analytically: the autocatalytic decomposition of violet bis[2-(5-bromo-pyridylazo)-5-(N-propyl-N-sulfopropyl-amino-phenolato]cobaltate, (Co(III)-5-Br-PAPS), upon oxidation with potassium peroxomonosulfate to produce colorless Co^{2+} ions. Here, the Co^{2+} ions serve as the autocatalyst (the order of autocatalysis, m, has not been established for this reaction).

$$\text{Co(III)-[5-Br—PAPS]reduced} + HSO_5^- \longrightarrow \text{Co}^{2+} + \text{[5-Br—PAPS]oxidized} + HSO_4^- \quad (3)$$

Addition of small amounts of Co^{2+} to the violet mixture of (Co(III)-5-Br-PAPS and peroxomonosulfate produces an abrupt loss of color to give a colorless solution. The time delay before this decomposition depends on the amount of the Co^{2+} added to the solution. This reaction has been used to detect concentrations of Co^{2+} as low as about 1×10^{-10} mole/L. The reaction shows good selectivity in the presence of other ions (V(V), Cr(III), Cr(VI), Mn(II), Fe(II), Ni(II), Cu(II) and Zn(II)).

To use this reaction for amplification, a microfluidic network as shown in FIG. **38** is preferably used. An unstable solution of Co(III)-[5-Br-PAPS]reduced and peroxomonosulfate at pH=7 buffer in large plugs are preferably formed in a channel. These large plugs are preferably split in accordance with the invention into three different sizes of plugs. Preferably, the plug sizes are $(1\ \mu m)^3=10^{-15}$ L in the first channel; $(10\ \mu m)^3=10^{-12}$ L in the second channel; and $(100\ \mu m)^3=10^{-9}$ L in the third channel. A three-step photolithography is preferably used in the fabrication of masters for these microfluidic channels.

Example 9

Multi-Stage Chemical Amplification in Microfluidic Devices for Single Molecule Detection

FIG. **38** illustrates a method for a multi-stage chemical amplification for single molecule detection using microfluidic devices according to the invention. This example illustrates the use of an autocatalytic reaction between Co(III)-5-Br-PAPS (introduced through inlet **3803**) and $KHSO_5$ (introduced through inlet **3801**) in a pH=7 buffer (introduced through inlet **3802**) that is autocatalyzed by Co^{2+} ions. Oil streams are allowed to flow through channels **3804**, **3805**. This reaction mixture (contained in plugs **3811**) is unstable and decomposes rapidly (shown in red) when small amounts of Co^{2+} **3810** are added. Thus, this reaction mixture is preferably mixed quickly and used immediately. The reaction mixture is preferably transported through the network in $(1\ \mu m)^3$, $(10\ \mu m)^3$, $(100\ \mu m)^3$ size plugs. On the left side of the microfluidic network, the approximately 1 μm^3 plugs of the sample to be analyzed form at a junction of two channels (shown in green). The merging of plugs containing Co^{2+} ions and plugs containing the reaction mixture results in a rapid autocatalytic reaction. By using an amplification cascade in which larger and larger plugs of the reaction mixture are used for amplification, each Co^{2+} ion in a plug can be amplified to about 10^{10} Co^{2+} ions per plug. The result of amplification is visually detectable.

The $(10\ \mu m)^3$ plugs are preferably merged with larger $(100\ \mu m)^3$ plugs in the third channel to give approximately 4×10^{-8} mole/L solution of Co^{2+} ions. Autocatalytic decomposition in the approximately 10^{-9} L plugs will produce plugs **3809** with about 2.4×10^{10} Co^{2+} ions (4×10^{-5} mole/L). The flow rates in this system are preferably controlled carefully to control the time that plugs spend in each branch. The time provided for amplification is preferably long enough to allow amplification to substantially reach completion, but short enough to prevent or minimize slow decomposition.

Using different plug sizes is advantageous when merging plugs. Plugs with a size of about $(1\ \mu m)^3$ are preferably formed by flowing a sample containing about 3×10^{-9} mole/L Co^{2+} through channel **3806**. This reaction can be used to detect Co^{2+} at this, or lower, concentration (Endo et al., "Kinetic determination of trace cobalt(II) by visual autocatalytic indication," *Talanta,* 1998, vol. 47, pp. 349-353; Endo et al., "Autocatalytic decomposition of cobalt complexes as an indicator system for the determination of trace amounts of cobalt and effectors," *Analyst,* 1996, vol. 121, pp. 391-394.). These plugs have a corresponding volume of about 10^{-15} L and carry just a few cobalt ions, on average about 1.8 ions per plug (corresponding to a Poisson distribution). These plugs **3810** are preferably merged with the $(1\ \mu m)^3$ plugs **3811** containing the Co(III)-5-Br-PAPS/peroxomonosulfate mixture (about 4×10^{-5} mole/L).

Upon autocatalytic decomposition of the complex, the number of Co^{2+} ions in the merged plug **3807** will increase by a factor of between about 10^4 to 1.2×10^4 Co^{2+} ions (2×10^{-5} mole/L in 2 μm^3). These plugs **3807** are preferably merged with the $(10\ \mu m)^3$ plugs **3811** containing the unstable mixture (about 4×10^{-5} mole/L). The concentration of Co^{2+} ions in these approximately 10^{-12} L plugs is preferably about 2×10^{-8} mole/L, which is sufficient to induce autocatalytic decomposition. The number of Co^{2+} ions will increase by a factor of between about 10^3 to about 2.4×10^7 ions/plug in plugs **3808**. The starting solution is dark violet ($\epsilon=9.8\times10^4$ L $mol^{-1}cm^{-1}$ for Co(III)-5-Br-PAPS). Channels are preferably designed to create an optical path through at least ten consecutive 100 μm plugs. These plugs will provide an approximately 1-mm long optical path, with absorbance of the starting 4×10^{-5} mole/L solution of about 0.4. This absorbance can be detected by an on-chip photodetector or with the naked eye. If Co^{2+} is present in the sample solution, an autocatalytic cascade will result in the disappearance of the color of the reaction mixture.

At low concentrations of Co^{2+} in the sample, the system may show stochastic behavior, that is, not every Co^{2+} ion would give rise to a decomposition cascade. However, the attractive feature of this system is that thousands of tests can be carried out in a matter of seconds, and statistics and averaging can be performed. Preferably, a sequence of controlled autocatalytic amplification reactions leads to a visual detection of single ions.

Example 10

Enzyme Kinetics

A microfluidic chip according to the invention was used to measure millisecond single-turnover kinetics of ribonuclease A (RNase A; EC 3.1.27.5), a well-studied enzyme. Submicroliter sample consumption makes the microfluidic chip especially attractive for performing such measurements

because they require high concentrations of both the enzyme and the substrate, with the enzyme used in large excess.

The kinetic measurements were performed by monitoring the steady-state fluorescence arising from the cleavage of a fluorogenic substrate by RNase A as the reaction mixture flowed down the channel (see FIG. **40**(*a*)). In FIG. **40**, a substrate, buffer, and RNase A were introduced into inlets **401**, **401**, and **403**, respectively. A carrier fluid flowed through channel **404**. The amount of the product at a given reaction time t [s] was calculated from the intensity of fluorescence at the corresponding distance point d [m] (t=d/U where U=0.43 m/s is the velocity of the flow). The channels were designed to wind so that rapid chaotic mixing was induced, and were designed to fit within the field of view of the microscope so that the entire reaction profile could be measured in one spatially resolved image. Selwyn's test (Duggleby, R. G., *Enzyme Kinetics and Mechanisms, Pt D*; Academic Press: San Diego, 1995, vol. 249, pp. 61-90; Selwyn, M. J. *Biochim. Biophys. Acta,* 1965, vol. 105, pp. 193-195) was successfully performed in this system to establish that there were no factors leading to product inhibition or RNase A denaturation.

The flow rate of the stock solution of 150 μM of RNase A was kept constant to maintain 50 μM of RNase A within the plugs. By varying the flow rates of the buffer and substrate (see FIG. **45**), progress curves were obtained for eight different substrate concentrations. For $[E]_o >> [S]_o$, the simple reaction equation is $[P]_t = [S]_o(1-\mathrm{Exp}(-kt))$, where $[E]_o$ is the initial enzyme concentration, $[S]_o$ is the initial substrate concentration, $[P]_t$ is the time-dependent product concentration and k [s^{-1}] is the single-turnover rate constant. To obtain a more accurate fit to the data, the time delay Δt_n required to mix a fraction of the reaction mixture f_n was accounted for.

An attractive feature of the microfluidic system used is that the reaction mixture can be observed at time t=0 (there is no dead-time). This feature was used to determine Δt_n and f_n in this device by obtaining a mixing curve using fluo-4/Ca^{2+} system as previously described (Song et al., *Angew. Chem. Int. Ed.* 2002, vol. 42, pp. 768-772), and correcting for differences in diffusion constants (Stroock et al., *Science,* 2002, vol. 295, pp. 647-651). All eight progress curves gave a good fit with the same rate constant of 1100±250 s^{-1}. The simpler theoretical fits gave indistinguishable rate constants. These results are in agreement with previous studies, where cleavage rates of oligonucleotides by ribonucleases were shown to be ~10^3 s^{-1}.

Thus, this example demonstrates that millisecond kinetics with millisecond resolution can be performed rapidly and economically using a microchannel chip according to the invention. Each fluorescence image was acquired for 2 s, and required less than 70 nL of the reagent solutions. These experiments with stopped-flow would require at least several hundreds of microliters of solutions. Volumes of about 2 μL are sufficient for ~25 kinetic experiments over a range of concentrations. Fabrication of these devices in PDMS is straightforward (McDonald, et al., *Accounts*

$$[P]_t = \sum_n f_n [S]_0 (1 - \mathrm{Exp}(-k(t - \Delta t_n)))$$

Chem. Res. 2002, vol. 35, pp. 491-499) and no specialized equipment except for a standard microscope with a CCD camera is needed to run the experiments. This system could serve as an inexpensive and economical complement to stopped-flow methods for a broad range of kinetic experiments in chemistry and biochemistry.

Example 11

Kinetics of RNA Folding

The systems and methods of the present invention are preferably used to conduct kinetic measurements of, for example, folding in the time range from tens of microseconds to hundreds of seconds. The systems and methods according to the invention allow kinetic measurements using only small amounts of sample so that the folding of hundreds of different RNA mutants can be measured and the effect of mutation on folding established. In one aspect according to the invention, the kinetics of RNA folding is preferably measured by adding Mg^{2+} to solutions of previously synthesized unfolded RNA labeled with FRET pairs in different positions. In accordance with the invention, the concentrations of Mg^{2+} are preferably varied in the 0.04 to 0.4 μM range by varying the flow rates (see, for example, FIGS. **25***a*)-*c*)) to rapidly determine the folding kinetics over a range of conditions. The ability to integrate the signal over many seconds using the steady-flow microfluidic devices according to the invention can further improve sensitivity.

As shown in FIGS. **25***a*)-*c*), the concentrations of aqueous solutions inside the plugs can be controlled by changing the flow rates of the aqueous streams. In FIGS. **25***a*)-*c*), aqueous streams were introduced into inlets **251**-**258** wherein flow rates of about 0.6 μL/min for the two aqueous streams and 2.7 μL/min was used for the third stream. The stream with the 2.7 μL/min volumetric flow rate was introduced in the left, middle, and right inlet in FIGS. **25***a*)-*c*), respectively. A carrier fluid in the form of perfluorodecaline was introduced into channel **259**, **260**, **261**. The corresponding photographs on each of the right side of FIGS. **25***a*)-*c*) illustrate the formation of plugs with different concentrations of the aqueous streams. The various shadings inside the streams and plugs arise from the use of aqueous solutions of food dyes (red/dark and green/light), which allowed visualization, and water were used as the three streams, the darker shading arising mainly from the red dye color while the lighter shading arising mainly from the green dye color. The dark stream is more viscous than the light stream, therefore it moves slower (in mm/s) and occupies a larger fraction of the channel at a given volumetric flow rate (in μL/min).

Example 12

Nanoparticle Experiments with and Without Plugs

FIG. **15** illustrates a technique for the synthesis of CdS nanoparticles **155**. In one experiment, nanoparticles were formed in a microfluidic network. The channels of the microfluidic device had 50 μm×50 μm cross-sections. A fluorinated carrier-fluid (10:1 v/v mixture of perfluorohexane and 1H,1H,2H,2H-perfluorooctanol) was flowed through the main channel at 15 μm min^{-1}. An aqueous solution, pH=11.4, of 0.80 mM $CdCl_2$ and 0.80 mM 3-mercaptopropionic acid was flowed through the left-most inlet channel **151** at 8 μL min^{-1}. An aqueous solution of 0.80 mM polyphosphates $Na(PO_3)_n$ was flowed through the central inlet channel **152** at 8 μL min^{-1}, and an aqueous solution of 0.96 mM Na_2S was flowed through the right-most inlet channel **153** at 8 μL min^{-1}. To terminate the growth of nanoparticles, an aqueous solution of 26.2 mM 3-mercaptopropionic acid, pH=12.1, was flowed through the bottom inlet of the device **157** at 24 μM

min^{-1}. FIG. **15** shows various regions or points along the channel corresponding to regions or points where nucleation **154**, growth **158**, and termination **156** occurs. Based on the UV-VIS spectrum, substantially monodisperse nanoparticles formed in this experiment.

Nanoparticles were also formed without microfluidics. Solutions of $CdCl_2$, polyphosphates, Na_2S, and 3-mercaptopropionic acid, identical to those used in the microfluidics experiment, were used. 0.5 mL of the solution of $CdCl_2$ and 3-mercaptopropionic acid, 0.5 mL of polyphosphates solution, and 0.5 mL of Na_2S solution were combined in a cuvette, and the cuvette was shaken by hand. Immediately after mixing, 1.5 mL of 26.2 mM 3-mercaptopropionic acid was added to the reaction mixture to terminate the reaction, and the cuvette was again shaken by hand. Based on the UV-VIS spectrum, substantially polydisperse nanoparticles formed in this experiment.

Example 13

Networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The PDMS was purchased from Dow Corning Sylgard Brand 184 Silicone Elastomer. The PDMS devices were sealed after plasma oxidation treatment in Plasma Prep II (SPI Supplies). The devices were rendered hydrophobic by baking the devices at 120° C. for 2-4 hours. Microphotographs were taken with a Leica MZ12.5 stereomicroscope and a SPOT Insight color digital camera (Model#3.2.0, Diagnostic Instruments, Inc.). Lighting was provided from a Machine Vision Strobe X-strobe X1200 (20 Hz, 12 μF, 600V, Perkin Elmer Optoelectronics). To obtain an image, the shutter of the camera was opened for 1 second and the strobe light was flashed once with the duration of approximately 10 μs.

Aqueous solutions were pumped using 10 μl or 50 μl Hamilton Gastight syringes (1700 series). Carrier-fluid was pumped using 50 μl Hamilton Gastight syringes (1700 series). The syringes were attached to microfluidic devices by means of Teflon tubing (Weico Wire & Cable Inc., 30 gauge). Syringe pumps from Harvard Apparatus (PHD 2000) were used to inject the liquids into microchannels.

Microbatch crystallization conditions can be achieved. This experiment shows that size of plugs can be maintained and evaporation of water prevented. In this case, the PDMS device has been soaked in water overnight before the experiment in order to saturate PDMS with water. The device was kept under water during the experiment. During the experiment, the flow rates of carrier-fluid and NaCl solution were 2.7 μL/min and 1.0 μL/min, respectively. The flow was stopped by cutting off the Teflon tubing of both carrier-fluid and NaCl solution.

FIG. **16** shows a schematic illustration of a microfluidic device according to the invention and a microphotograph of plugs of 1M aqueous NaCl sustained in oil. The carrier-fluid is perfluorodecaline with 2% 1H,1H,2H,2H-perfluorooctanol. Inside a microchannel, plugs showed no appreciable change in size.

B. Vapor Diffusion Crystallization in Microchannels: Controlling Evaporation of Water from Plugs

This experiment shows that evaporation of water from plugs can be controlled by soaking devices in water for shorter amounts of time or not soaking at all. The rate of evaporation can be also controlled by the thickness of PDMS used in the fabrication of the device. Evaporation rate can be increased by keeping the device in a solution of salt or other substances instead of keeping the device in pure water.

The plug traps are separated by narrow regions that help force the plugs into the traps.

In this experiment, a composite glass/PDMS device was used. PDMS layer had microchannel and a microscopy slide (Fisher, 35x50-1) was used as the substrate. Both the glass slide and the PDMS were treated in plasma cleaner (Harrick) then sealed. The device was made hydrophobic by first baking the device at 120° C. for 2-4 hours then silanizing it by (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.).

During the experiment, a flow of carrier-fluid at 1.0 μL/min was established, then flow of aqueous solution was established at a total rate of 0.9 μL/min. Plug formation was observed inside the microchannel. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **41** shows a microphotograph (middle and right side) of the water plugs region of the microfluidic network. FIG. **41**(*b*)-(*c*) show the plugs at time t=0 and t=2 hours, respectively. Red aqueous solution is 50% waterman red ink in 0.5 M NaCl solution. Ink streams were then introduced into inlets **411**, **412**, **413**. An oil stream flowed through channel **414**. The carrier-fluid is FC-3283 (3M Fluorinert Liquid) with 2% 1H,1H,2H,2H-perfluorodecanol. This photograph demonstrates that the evaporation of water through PDMS can be controlled, and thus the concentration of the contents inside the drops can be increased (this is equivalent to microbatch crystallization). FIG. **41**(*a*) shows a diagram of the microfluidic network.

C. Controlling Shape and Attachment of Water Plugs

During the experiment, a flow of carrier fluid at 1.0 μL/min was established, then flow of aqueous solution was established at a total rate of 2.1 μL/min. Plug formation was observed inside the microchannel. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **39** shows a diagram (left side) of a microfluidic network according to the invention. Aqueous streams were introduced into inlets **3901**, **3902**, **3903** while an oil stream flowed through channel **3904**. FIG. **39** also shows a microphotograph (right side) of the water plug region of the microfluidic network. This image shows water plugs attached to the PDMS wall. This attachment occurs when low concentrations of surfactant, or less-effective surfactants are used. In this case 1H,1H,2H,2H-perfluorooctanol is less effective than 1H,1H,2H,2H-perfluorodecanol. In this experiment the oil is FC-3283 (3M Fluorinert Liquid) with 2% 1H,1H,2H,2H-perfluorooctanol as the surfactant.

D. Examples of Protein Crystallization

During the experiment, a flow of oil at 1.0 μL/min was established. Then the flow of water was established at 0.1 μL/min. Finally flows of lysozyme and precipitant were established at 0.2 μL/min. Plug formation was observed inside the microchannel. The flow of water was reduced to zero after the flow inside the channel became stable. The flow was stopped approximately 5-10 minutes afterwards by applying a pressure from the outlet and stopping the syringe pumps at the same time.

FIG. **36** depicts lysozyme crystals grown in water plugs in the wells of the microfluidic channel. Lysozyme crystals started to appear inside aqueous plugs both inside and outside plug traps in approximately 10 minutes. The image of the three crystals in FIG. **36** was taken 1 hour after the flow was

stopped. Lysozyme crystals appear colored because they were observed under polarized light. This is common for protein crystals.

The left side of FIG. **36** is a diagram of a microfluidic network according to the invention while the right side is microphotograph of the crystals formed in plugs in the microfluidic network. A precipitant, lysozyme, and water were introduced into inlets **3601**, **3602**, and **3603**, respectively. Oil was flowed through channel **3604**. The lysozyme solution contains 100 mg/ml lysozyme in 0.05 M sodium acetate (pH 4.7); the precipitant solution contains 30% w/v PEG (M.W. 5000), 1.0 M NaCl and 0.05 M sodium acetate (pH 4.7); The carrier-fluid is FC-3283 (3M Fluorinert Liquid) with 10% 1H,1H,2H,2H-perfluoro-octanol. The microchannel device was soaked in FC-3283/H2O for one hour before experiment.

FIG. **32** shows that plug traps are not required for formation of crystals in a microfluidic network. FIG. **32** shows a diagram (left side) of the microfluidic network. A precipitant was introduced into inlet **321**, lysozyme was introduced into inlet **322**, and an aqueous stream was introduced into inlet **323**. Oil was flowed through channel **324**. FIG. **32** also shows microphotographs (middle and right side) of lysozyme crystals grown inside the microfluidic channel. The experimental condition is same as in FIG. **36**.

Example 14

Oil-soluble Surfactants for Charged Surfaces

In accordance with the invention, neutral surfactants that are soluble in perfluorinated phases are preferably used to create positively and negatively-charged interfaces. To create charged surfaces, neutral surfactants that can be charged by interactions with water, e.g., by protonation of an amine or a guanidinium group (FIG. **24**B), or deprotonation of a carboxylic acid group (FIG. **24**C), are preferably used. Preferably, charged surfaces are used to repel, immobilize, or stabilize charged biomolecules. Negatively charged surfaces are useful for handling DNA and RNA without surface adsorption. Preferably, both negatively and positively-charged surfaces are used to control the nucleation of protein crystals. Many neutral fluorinated surfactants with acidic and basic groups ($RfC(O)OH$, $Rf(CH_2)_2NH_2$, $Rf(CH_2)_2C(NH)NH_2$) are available commercially (Lancaster, Fluorochem, Aldrich).

To synthesize oligoethylene-glycol terminated surfactants, a modification and improvement of a procedure based on the synthesis of perfluoro non-ionic surfactants is preferably used. In one aspect, the synthesis relies on the higher acidity of the fluorinated alcohol to prevent the polycondensation of the oligoethylene glycol. The modified synthesis uses a selective benzylation of one of the alcohol groups of oligoethylene glycol, followed by activation of the other alcohol group as a tosylate. A Williamson condensation is then performed under phase transfer conditions followed by a final deprotection step via catalytic hydrogenation using palladium on charcoal.

Example 15

Formation of Plugs in the Presence of Fluorinated Surfactants and Surface Tension

The surface tension of the oil/water interface has to be sufficiently high in order to maintain a low value of capillary number, C.n. The fluorosurfactant/water interfaces for water-insoluble fluorosurfactants have not been characterized, but these surfactants are predicted to reduce surface tension similar to that observed in a system involving Span on hexane/water interface (about 20 mN/m). The surface tensions of the aqueous/fluorous interfaces are preferably measured in the presence of fluorosurfactants using the hanging drop method. A video microscopy apparatus specifically constructed for performing these measurements has been used to successfully characterize interfaces. FIG. **24** illustrates the synthesis of fluorinated surfactants containing perfluoroalkyl chains and an oligoethylene glycol head group.

Example 16

Forming Gradients by Varying Flow Rates

FIG. **42** shows an experiment involving the formation of gradients by varying the flow rates. In this experiment, networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The width and height of the channel were both 50 μm. 10% 1H,1H,2H,2H-perfluorodecanol in perfluoroperhydrophenanthrene was used as oil. Red aqueous solution prepared from 50% Waterman red ink in 0.5 M NaCl solution was introduced into inlet **421**. The oil flowed through channel **424** at 0.5 μl/min. Aqueous streams were introduced into inlets **422**, **423**. To generate the gradient of ink in the channel, the total water flow rate was gradually increased from 0.03 μl/min to 0.23 μl/min in 20 seconds at a ramp rate of 0.01 μl/min per second. At the same time, ink flow rate was gradually decreased from 0.25 μl/min to 0.05 μl/min in 20 seconds at a ramp rate of −0.01 μl/min per second. The total flow rate was constant at 0.28 μl/min. The established gradient of ink concentration inside the plugs can be clearly seen from FIG. **42**: the plugs further from the inlet are darker since they were formed at a higher ink flow rate.

Example 17

Lysozome Crystallization Using Gradients

FIG. **43** illustrates an experiment involving the formation of lysozome crystals using gradients. The channel regions **435**, **437** correspond to channel regions with very low precipitant concentration while channel region **436** corresponds to optimal range of precipitant concentration. In this experiment, networks of microchannels were fabricated using rapid prototyping in polydimethylsiloxane (PDMS). The width of the channel was 150 μm and the height was 100 μm. 10% 1H,1H,2H,2H-perfluorodecanol in perfluoroperhydrophenanthrene was used as oil.

During the experiment, a flow of oil through channel **434** at 1.0 μl/min was established. Then the flow of water introduced through inlet **432** was established at 0.2 μl/min. The flows of lysozyme introduced through inlet **431** and precipitant introduced through inlet **433** were established at 0.2 μl/min. Plugs formed inside the channel. To create the gradient, water flow rate was first gradually decreased from 0.35 μl/min to 0.05 μl/min over 45 seconds at a ramp rate of (−0.01 μl/min per 1.5 seconds), then increased back to 0.35 μl/min in 45 seconds at a ramp rate of (0.01 μl/min per 1.5 seconds). At the same time, precipitant flow rate was gradually increased from 0.05 μl/min to 0.35 μl/min in 45 seconds at a ramp rate of (0.01 μl/min per 1.5 seconds), then decreased to 0.05 μl/min in 45 seconds at a ramp rate of (−0.01 μl/min per 1.5 seconds). The flow was stopped by pulling out the inlet tubing immediately after water and precipitant flow rates returned to the starting values. The plugs created in this way contained constant concentration of the protein. but variable concentration of the

precipitant: the concentration of the precipitant was lowest in the beginning and the end of the channel, and it peaked in the middle of the channel (the center row). Only the plugs in the middle of the channel have the optimal concentration of precipitant for lysozyme crystallization, as confirmed by observing lysozyme crystals inside plugs in the center row. Visualization was performed under polarized light. Preferably, all flow rates would be varied, not just the precipitant and water.

We claim:

1. A microfluidic system comprising:

a non-fluorinated microchannel;

a carrier fluid comprising a fluorinated oil and a fluorinated surfactant comprising a hydrophilic head group in the microchannel;

at least one plug comprising an aqueous plug-fluid in the microchannel and substantially encased by the carrier-fluid, wherein the fluorinated surfactant is present at a concentration such that surface tension at the plug-fluid/microchannel wall interface is higher than surface tension at the plug-fluid/carrier fluid interface.

2. The microfluidic system of claim **1**, wherein the at least one plug contains at least one of a cell, a virion, an enzyme, DNA, and RNA.

3. The microfluidic system of claim **2** wherein the cell is a blood cell.

4. The microfluidic system of claim **2** wherein the cell is a bacterium.

5. The microfluidic system of claim **1**, further comprising a detection region of the microchannel, in which whether the contents of the at least one plug have a selected characteristic is determined.

6. The microfluidic system of claim **5**, further comprising a discrimination region of the system, in which the microchannel divides into at least a first and second branch, the discrimination region being downstream of the detection region.

7. The microfluidic system of claim **6**, wherein the at least one plug is directed into one of the at least first and second branches of the microchannel in the discrimination region based on whether the contents of the at least one plug have the selected characteristic.

8. The microfluidic system of claim **6**, wherein the first branch differs from the second branch in at least one of the following features:

(a) diameter,

(b) hydrophilicity,

(c) net charge,

(d) temperature, or

(e) pressure.

9. The microfluidic system of claim **1**, wherein the fluorinated surfactant comprises an oligoethylene glycol.

10. The microfluidic system of claim **1**, wherein the at least one plug contains at least one reagent for an autocatalytic reaction.

11. The microfluidic system of claim **10**, wherein the autocatalytic reaction is a polymerase-chain reaction.

12. The microfluidic system of claim **1**, wherein the volume of the at least one plug is between about two femtoliters and about one hundred nanoliters.

13. The microfluidic system of claim **1**, wherein the microchannel is made from a polymer, a glass or a metal.

14. The microfluidic system of claim **13**, wherein the polymer is a polysiloxane.

15. The microfluidic system of claim **1**, further comprising at least one detector.

16. The microfluidic system of claim **15**, wherein the detector is selected from the group consisting of an optical detector, a fluorescence detector, an electrochemical detector, a nuclear magnetic resonance detector, and a mass spectrometer detector.

17. The microfluidic system of claim **1**, wherein the fluorinated oil is perfluorinated.

18. The microfluidic system of claim **17**, wherein the fluorinated oil is selected from the group consisting of perfluorohexane, perfluoro(methylcyclohexane), perfluoro(1,3-dimethylcyclohexane), perfluorodecaline, perfluoroperhydrofluorene, perfluoroperhydrophenanthrene, perfluorotoluene, hexafluorobenzene, and combinations thereof.

19. The microfluidic system of claim **1**, wherein the fluorinated oil comprises a plurality of fluorinated substances of differing viscosity.

20. A method of conducting a reaction within at least one plug, comprising the steps of:

introducing a carrier-fluid comprising a fluorinated oil and a fluorinated surfactant comprising a hydrophilic head group into a first non-fluorinated microchannel of a device;

introducing at least one stream of plug-fluid into a first inlet in fluid communication with the first microchannel so that at least one plug forms in the carrier-fluid after the at least one stream contacts the carrier-fluid; wherein:

the at least one plug-fluid comprises an aqueous fluid and at least one reagent for an autocatalytic reaction; the at least one plug-fluid is immiscible with the carrier-fluid; each plug is substantially surrounded on all sides by carrier-fluid; and the fluorinated surfactant is present at a concentration such that surface tension at the plug-fluid/microchannel wall interface is higher than surface tension at the plug-fluid/carrier fluid interface.

21. The method of claim **20**, wherein the autocatalytic reaction is a polymerase-chain reaction.

22. The method of claim **20**, wherein the carrier-fluid comprises a fluorinated compound.

23. The method of claim **22**, wherein the fluorinated compound is perfluorinated.

24. The method of claim **22**, wherein the fluorinated compound is selected from the group consisting of perfluorohexane, perfluoro(methylcyclohexane), perfluoro(1,3-dimethylcyclohexane), perfluorodecaline, perfluoroperhydrofluorene, perfluoroperhydrophenanthrene, perfluorotoluene, hexafluorobenzene, and combinations thereof.

25. The method of claim **22**, wherein the fluorinated compound comprises a plurality of fluorinated substances of differing viscosity.

26. The method of claim **20**, wherein the at least one plug contains at least one of a cell, a virion, an enzyme, DNA and RNA.

27. The method of claim **26**, wherein the cell is a blood cell.

28. The method of claim **26**, wherein the cell is a bacterium.

29. The method of claim **20**, further comprising determining whether the contents of the at least one plug have a selected characteristic.

30. The method of claim **29**, further comprising directing the at least one plug into one of at least two branches of the microchannel based on whether the contents of the at least one plug have the selected characteristic.

31. A microfluidic system comprising:

a non-fluorinated microchannel;

a fluorinated carrier fluid;

a fluorinated surfactant comprising a hydrophilic head group in the carrier fluid; and

at least one plug comprising an aqueous plug-fluid in the microchannel and substantially encased by the carrier-

fluid, wherein the fluorinated surfactant is present at a concentration such that surface tension at the plug-fluid/microchannel wall interface is higher than surface tension at the plug-fluid/carrier fluid interface.

* * * * *

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

BIO-RAD LABORATORIES INC. and THE UNIVERSITY OF CHICAGO, Plaintiffs, v. 10X GENOMICS, INC., Defendant.	No. 15-cv-152-RGA

MEMORANDUM OPINION

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July 3, 2019

ANDREWS, U.S. DISTRICT JUDGE:

Presently before the Court is Defendant's motion for judgment as a matter of law under Federal Rule of Civil Procedure 50(b), new trial under Federal Rule of Civil Procedure 59, and remittitur. (D.I. 509). I have reviewed the parties' briefing. (D.I. 510, 530, 535). I have also reviewed Plaintiffs' notice of subsequent development. (D.I. 551). For the following reasons, Defendant's motion is **DENIED**.

I. BACKGROUND

On February 12, 2015, RainDance Technologies, Inc. and the University of Chicago filed suit against 10X Genomics, Inc. alleging infringement of several patents. On May 30, 2017, Bio-Rad Laboratories Inc. substituted for RainDance. (D.I. 180). I held a jury trial from November 5 to 13, 2018.[1] Only three patents remained at issue—U.S. Patent Nos. 8,889,083 ("the '083 patent"), 8,304,193 ("the '193 patent"), and 8,329,407 ("the '407 patent"). (*See* D.I. 499). The jury found all three patents valid and infringed, that the infringement was willful, and that Plaintiffs were entitled to $23,930,718 in damages. (D.I. 476).

10X now moves for judgment as a matter of law that the accused products do not infringe, that infringement was not willful, that the asserted claims are invalid, and that Plaintiffs failed to present a legally sufficient damages case. Where appropriate, 10X requests remittitur of damages. (D.I. 510 at 30). In the alternative, 10X moves for a new trial. (*Id.*).

II. LEGAL STANDARDS

A. Judgment as a Matter of Law

Judgment as a matter of law is appropriate if "the court finds that a reasonable jury would not have a legally sufficient evidentiary basis to find for [a] party" on an issue. Fed. R. Civ. P.

[1] I cite to the trial transcript as "Tr."

50(a)(1). "Entry of judgment as a matter of law is a 'sparingly' invoked remedy, 'granted only if, viewing the evidence in the light most favorable to the nonmovant and giving it the advantage of every fair and reasonable inference, there is insufficient evidence from which a jury reasonably could find liability.'" *Marra v. Phila. Hous. Auth.*, 497 F.3d 286, 300 (3d Cir. 2007) (citation omitted).

"To prevail on a renewed motion for JMOL following a jury trial, a party must show that the jury's findings, presumed or express, are not supported by substantial evidence or, if they were, that the legal conclusion(s) implied [by] the jury's verdict cannot in law be supported by those findings." *Pannu v. Iolab Corp.*, 155 F.3d 1344, 1348 (Fed. Cir. 1998). "'Substantial' evidence is such relevant evidence from the record taken as a whole as might be accepted by a reasonable mind as adequate to support the finding under review." *Perkin-Elmer Corp. v. Computervision Corp.*, 732 F.2d 888, 893 (Fed. Cir. 1984).

In assessing the sufficiency of the evidence, the Court must give the non-moving party, "as [the] verdict winner, the benefit of all logical inferences that could be drawn from the evidence presented, resolve all conflicts in the evidence in his favor and, in general, view the record in the light most favorable to him." *Williamson v. Consol. Rail Corp.*, 926 F.2d 1344, 1348 (3d Cir. 1991). The Court may "not determine the credibility of the witnesses [nor] substitute its choice for that of the jury between conflicting elements in the evidence." *Perkin-Elmer*, 732 F.2d at 893. Rather, the Court must determine whether the evidence supports the jury's verdict. *See Dawn Equip. Co. v. Ky. Farms Inc.*, 140 F.3d 1009, 1014 (Fed. Cir. 1998); *Gomez v. Allegheny Health Servs. Inc.*, 71 F.3d 1079, 1083 (3d Cir. 1995) (describing standard as "whether there is evidence upon which a reasonable jury could properly have found its verdict"); 9B *Charles Alan Wright & Arthur R. Miller, Federal Practice and Procedure* § 2524

(3d ed. 2008) ("The question is not whether there is literally no evidence supporting the party against whom the motion is directed but whether there is evidence upon which the jury might reasonably find a verdict for that party.").

Where the moving party bears the burden of proof, the Third Circuit applies a different standard. This standard "requires the judge to test the body of evidence not for its insufficiency to support a finding, but rather for its overwhelming effect." *Fireman's Fund Ins. Co. v. Videfreeze Corp.*, 540 F.2d 1171, 1177 (3d Cir. 1976) (quoting *Mihalchak v. Am. Dredging Co.*, 266 F.2d 875, 877 (3d Cir. 1959)). The Court "'must be able to say not only that there is sufficient evidence to support the finding, even though other evidence could support as well a contrary finding, but additionally that there is insufficient evidence for permitting any different finding.'" *Id.* at 1171 (quoting *Mihalchak*, 266 F.2d at 877).

B. New Trial

Federal Rule of Civil Procedure 59(a)(1)(A) provides, in pertinent part: "The court may, on motion, grant a new trial on all or some of the issues—and to any party— . . . after a jury trial, for any reason for which a new trial has heretofore been granted in an action at law in federal court . . . ." The decision to grant or deny a new trial is committed to the sound discretion of the district court. *See Allied Chem. Corp. v. Daiflon, Inc.*, 449 U.S. 33, 36 (1980); *Olefins Trading, Inc. v. Han Yang Chem. Corp.*, 9 F.3d 282, 289 (3d Cir. 1993) (reviewing district court's grant or denial of new trial motion under the "abuse of discretion" standard). Although the standard for granting a new trial is less rigorous than the standard for granting judgment as a matter of law—in that the Court need not view the evidence in the light most favorable to the verdict winner—a new trial should only be granted where "a miscarriage of justice would result if the verdict were

to stand," the verdict "cries out to be overturned," or where the verdict "shocks [the] conscience." *Williamson*, 926 F.2d at 1352-53.

III. ASSERTED CLAIMS

A. The '083 Patent

Plaintiffs asserted claims 1 and 9 of the '083 patent. The claims provide:

1. A microfluidic system comprising:

a non-fluorinated microchannel;

a carrier fluid comprising a fluorinated oil and a fluorinated surfactant comprising a hydrophilic head group in the microchannel;

at least one plug[2] comprising an aqueous plug-fluid in the microchannel and substantially encased by the carrier-fluid, wherein the fluorinated surfactant is present at a concentration such that surface tension at the plug-fluid/microchannel wall interface is higher than surface tension at the plug-fluid/carrier fluid interface.

9. The microfluidic system of claim 1, wherein the fluorinated surfactant comprises an oligoethylene glycol.

B. The '193 Patent

Plaintiffs asserted claims 1, 6, and 8 of the '193 patent. The relevant claims provide:

1. A method for conducting an autocatalytic reaction in plugs in a microfluidic system, comprising the steps of:

providing the microfluidic system comprising at least two channels having at least one junction;

flowing an aqueous fluid containing at least one substrate molecule and reagents for conducting an autocatalytic reaction through a first channel of the at least two channels;

flowing an oil through the second channel of the at least two channels;

forming at least one plug of the aqueous fluid containing the at least one substrate molecule and reagents by partitioning the aqueous fluid with the flowing oil at the junction of the at least two channels, the plug being substantially surrounded by an oil flowing through the channel, wherein

[2] The parties also refer to plugs as "droplets."

the at least one plug comprises at least one substrate molecule and reagents for conducting an autocatalytic reaction with the at least one substrate molecule; and

providing conditions suitable for the autocatalytic reaction in the at least one plug such that the at least one substrate molecule is amplified.

6. The method of claim 1, wherein the oil is fluorinated oil.

7. The method of claim 1, wherein the carrier fluid further comprises a surfactant.

8. The method of claim 7, wherein the surfactant is fluorinated surfactant.

C. The '407 Patent

Plaintiffs asserted claims 1, 10, and 11 of the '407 patent. The relevant claims provide:

1. A method for conducting a reaction in plugs in a microfluidic system, comprising the steps of:

providing the microfluidic system comprising at least two channels having at least one junction;

continuously flowing an aqueous fluid containing at least one biological molecule and at least one reagent for conducting the reaction between the biological molecule and the at least one reagent through a first channel of the at least two channels;

continuously flowing a carrier fluid immiscible with the aqueous fluid through the second channel of the at least two channels;

forming at least one plug of the aqueous fluid containing the at least one biological molecule and the at least one reagent by partitioning the aqueous fluid with the flowing immiscible carrier fluid at the junction of the at least two channels, the plug being substantially surrounded by the immiscible carrier fluid flowing through the channel, wherein the at least one plug comprises at least one biological molecule and the at least one reagent for conducting the reaction with the at least one biological molecule; and

providing conditions suitable for the reaction in the at least one plug involving the at least one biological molecule and the at least one reagent to form a reaction product.

8. The method according to claim 1, wherein the immiscible carrier fluid is an oil.

9. The method according to claim 8, wherein the oil comprises a surfactant.

10. The method according to claim 9, wherein the surfactant is a fluorosurfactant.

11. The method according to claim 8, wherein the oil is a fluorinated oil.

IV. NON-INFRINGEMENT

The jury found direct, induced, and contributory infringement of each asserted claim relating to each of 10X's accused products. (D.I. 476). In addition, for liability under 35 U.S.C. § 271(f)(2), the jury found that 10X supplies from the United States a component of the invention claimed in the '083 patent. (*Id.* at 3). Lastly, the jury found that infringement was willful. (*Id.* at 8).

A. '083 Patent

10X's motion addresses two limitations in claim 1 of the '083 patent—(1) the "non-fluorinated microchannel" and (2) the claimed surface tension relationship between the "plug-fluid/microchannel wall interface" and the "plug-fluid/carrier fluid interface." (D.I. 510 at 1-5, 10-11). For the following reasons, 10X's motion is **DENIED** with respect to both limitations.

1. "non-fluorinated microchannel"

Three out of the six accused products are modified to include 0.02% Kynar, a substance containing fluorine. Tr. at 368:15-369:19 (Dr. Sia). The jury found the products with Kynar did not literally satisfy the "non-fluorinated microchannel" limitation, but did meet the limitation under the doctrine of equivalents. (D.I. 476 at 3). 10X argues that the jury verdict is wrong as matter of law, or in the alternative, that it is based on insufficient evidence. (D.I. 510 at 1-4).

"[T]he doctrine of equivalents cannot be employed in a manner that wholly vitiates a claim limitation." *SciMed Life Sys., Inc. v. Advanced Cardiovascular Sys., Inc.*, 242 F.3d 1337, 1346 (Fed. Cir. 2001). For example:

> [I]f a patent states that the claimed device must be "non-metallic," the patentee cannot assert the patent against a metallic device on the ground that a metallic device is equivalent to a non-metallic device. The unavailability of the doctrine of equivalents could be explained either as the product of an impermissible vitiation of the "non-metallic" claim limitation, or as the product of a clear and binding statement to the public that metallic structures are excluded from the protection of the patent.

Id. at 1347. "'Vitiation' is not an exception to the doctrine of equivalents." *Deere & Co. v. Bush Hog, LLC*, 703 F.3d 1349, 1356 (Fed. Cir. 2012). "The proper inquiry for the court is to apply the doctrine of equivalents, asking whether an asserted equivalent represents an 'insubstantial difference' from the claimed element, or 'whether the substitute element matches the function, way, and result of the claimed element.'" *Id.* (quoting *Warner-Jenkinson Co. v. Hilton Davis Chem. Co.*, 520 U.S. 17, 40 (1997)). The argument against equivalence is especially strong where the prosecution history indicates "particular advantages arising from the absence of" the allegedly equivalent feature. *Moore U.S.A., Inc. v. Standard Register Co.*, 229 F.3d 1091, 1115 n.5 (Fed. Cir. 2000); *see also SciMed*, 242 F.3d at 1347 (finding strong support for not applying the doctrine of equivalents where the asserted patents "specifically recognized and disclaimed" the allegedly equivalent structure, making clear that the patentee regarded it as "significantly inferior" to the structure used in the invention).

Here, it is undisputed that the accused products with Kynar have microchannels with some amount of fluorine. Tr. at 369:14-19 (Dr. Sia). 10X argues that the jury's finding of equivalence thus vitiates the "non-fluorinated microchannel" limitation. (D.I. 510 at 2). 10X further asserts that the patentee disclaimed fluorinated microchannels during prosecution by adding "non-fluorinated" to avoid prior art. (*Id.* at 3).

I considered the prosecution history for "non-fluorinated" during summary judgment. I found the patentee "sought to distinguish the 'microchannel' in its system from the channels described in [the prior art], which may be 'coated with . . . fluorinated oils.'" (D.I. 351 at 9).

Thus, the patentee only disclaimed "'microchannel[s]' 'coated' with fluorine for a purpose—not those containing *de minimis* amounts of fluorine that have no effect on how the microchannel functions in the system." (*Id.*). Similarly, in denying 10X's Rule 50(a) motion, I noted that Plaintiffs could meet the doctrine of equivalents for "non-fluorinated microchannel" with "a product that has the absence of fluorine atoms other than minute quantities that have no function in the accused product." (D.I. 504, Ex. B at 61).

10X argues that my prior ruling is inconsistent with the Federal Circuit's standard that hinges on whether the asserted equivalent and claimed element are "insubstantially different." (D.I. 510 at 3). 10X relies on *Moore*, which addressed a limitation requiring adhesive strips to extend the "majority of the lengths" of a sheet. 229 F.3d at 1105. The accused product had strips extending about 48% of the length. *Id.* at 1106. The Federal Circuit affirmed the district court's summary judgment finding of no infringement by equivalents, because "to allow what is undisputedly a minority (*i.e.*, 47.8%) to be equivalent to a majority would vitiate the [claim] requirement," and "it would defy logic to conclude that a minority—the very antithesis of a majority—could be insubstantially different from a claim limitation requiring a majority, and no reasonable juror could find otherwise." *Id.* 10X argues that, like in *Moore*, no reasonable juror could find a microchannel "containing quintillions of fluorine atoms" to be "insubstantially different" from a "non-fluorinated microchannel." (D.I. 510 at 4).

I agree with 10X that the proper inquiry is whether the asserted equivalent—a microchannel with 0.02% Kynar—is "insubstantially different" from the claimed element—a "non-fluorinated microchannel." *See Bush Hog*, 703 F.3d at 1356. However, unlike in *Moore*, I do not think having 0.02% Kynar is "the very antithesis" of "non-fluorinated." *See* 229 F.3d at 1106. In *Bush Hog*, the Federal Circuit made clear that equivalence may be determined by

asking "whether the substitute element matches the function, way, and result of the claimed element." 703 F.3d at 1356. Although 10X emphasizes that the accused products contain "quintillions" of fluorine atoms, Plaintiffs' expert, Dr. Sia, noted that "[a]toms are really, really small" and "millions of atoms is not a lot." Tr. at 457:1-13. In addition, Dr. Sia testified that the addition of Kynar did not change how the microchannels worked, as evidenced by 10X's documents and testimony from Dr. Lowe, a 10X scientist. Tr. at 370:19-373:8. Therefore, a reasonable juror could find that a 0.02% Kynar microchannel is "insubstantially different" from a "non-fluorinated microchannel," because the Kynar microchannel contains negligible amounts of fluorine and "matches the function, way, and result" of a non-fluorinated microchannel.

In the alternative, 10X argues that there is insufficient evidence to support the jury's verdict, because Dr. Sia merely "reiterated his opinions on literal infringement under the label of the doctrine of equivalents." (D.I. 510 at 4-5).[3] I disagree. By testifying on how the addition of Kynar had no effect on the microchannels in 10X's products, Dr. Sia gave sufficiently particularized testimony to support the jury's verdict. *See* Tr. at 370:19-373:8.

2. Surface Tension Relationship

The '083 patent requires that "the fluorinated surfactant is present at a concentration such that surface tension at the plug-fluid/microchannel wall interface is higher than surface tension at the plug-fluid/carrier fluid interface." 10X argues that no reasonable juror could find 10X's products meet such surface tension relationship. (D.I. 510 at 10-11).

Dr. Sia presented substantial evidence that the accused products meet the surface tension limitation. First, he opined that the surface tension relationship means that "the droplet will then

[3] 10X also argues that Dr. Sia's testimony is insufficient because he is neither a person of ordinary skill in the art nor testified from the perspective of one. (D.I. 510 at 4). This argument is unavailing. *See supra* Section V.A.

not stick to the channel wall, and instead, it's going to be encased in the carrier fluid." Tr. at 381:12-19. It is undisputed that the droplet does not touch the channel wall in 10X products. *Id.* at 382:25-383:7, 383:24-384:9. Second, Dr. Sia presented results from surface tension testing done by Bio-Rad personnel. *Id.* at 385:13-15. He explained that they first measured the surface tension between the plug fluid and microchannel wall by placing the plug fluid on the 10X chips. *Id.* at 385:19-23, 1298:1-2. They then measured the surface tension between the plug fluid and carrier fluid by placing the carrier fluid on top of the plug fluid. *Id.* at 385:24-386:2. The measurements were made using standard lab instruments. *Id.* at 386:2-4. Dr. Sia explained that, because the chips are made of the same material throughout, measurements taken on the surface of the chip are an accurate reflection of what happens inside the microchannel. *Id.* at 1297:23-1298:3.

10X argues that the Bio-Rad tests are insufficient to show the requisite surface tension relationship. (D.I. 510 at 10). 10X's expert, Dr. Huck, testified that due to differences such as surface contaminations, surface roughness, surfactants in the plug fluids, or presence of gel beads, one "really would have to do experiments inside the microchannel." Tr. at 1089:18-21. However, Dr. Huck agreed that "if a sufficient concentration of surfactant is present such that the plug flowed smoothly without adhering to the channel walls[,] then the surface tension at the plug[/]wall interface will be higher than at the plug[/]carrier interface." *Id.* at 1097:2-11.

The jury's verdict is supported by substantial evidence. Given Dr. Sia's testimony, the jury was not required to accept Dr. Huck's opinion that the claimed surface tension could only be measured by testing inside the microchannel. *See id.* at 1089:18-21, 1297:23-1298:3. Further, it is undisputed that droplets in 10X products do not adhere to the channel wall. *Id.* at 382:25-

383:7, 383:24-384:9. Therefore, it would be consistent with Dr. Huck's testimony to find that the accused products meet the claimed surface tension relationship. *See id.* at 1097:2-11.

B. '193 Patent—"autocatalytic reaction"

For the following reasons, 10X's motion is **DENIED** with respect to the '193 patent's "autocatalytic reaction" limitation.

I construed "autocatalytic reaction" to mean "a reaction in which a product of the reaction is also a reagent for the same reaction." (D.I. 469 ¶ 8). Dr. Sia testified that the PHASE and Landlord reactions in 10X products are autocatalytic. Tr. at 417:13-418:15, 422:1-21. Although 10X moves for JMOL with respect to both reactions, its substantive arguments only address the Landlord reaction. (D.I. 510 at 9-10).

Dr. Sia explained that the Landlord reaction starts with a single DNA strand, which combines with enzymes to create new DNA strands. The new DNA strands are both products and reagents of the Landlord reaction—they are a product of the reaction between the first DNA strand and the enzymes, but will also undergo the same reaction to produce more DNA strands. Tr. at 422:12-423:21. In support, Dr. Sia relies on 10X internal documents and Rule 30(b)(6) testimony. *Id.* at 422:1-3 (PTX-1204-011), 423:25-424:7 (deposition video).

10X argues that Dr. Sia failed to present sufficient evidence to support his opinions. (D.I. 510 at 9-10). 10X's expert, Dr. Quackenbush, opined that the Landlord reaction is not autocatalytic because each reaction produces a different fragment of DNA. Tr. at 1129:18-23. The reaction copies sections of a DNA strand, wherein each section is randomly selected by enzymes. The fragments that are copied from the original DNA strand feed back into the reaction by acting as templates from which new fragments are copied. *Id.* at 1130:4-22.

The jury's verdict is supported by substantial evidence. Aside from their ultimate conclusions, I think Dr. Sia and Dr. Quackenbush gave comparable testimony. They agreed that the products of the Landlord reaction, DNA fragments, are used to create more DNA fragments. *See id.* at 422:12-423:21, 1130:4-22. Thus, the DNA fragments are both products and reagents of the reaction. That is sufficient to meet my construction of an autocatalytic reaction. Dr. Quackenbush assumed that the Landlord reaction could not be autocatalytic because each copied DNA fragment is different. I do not think my construction is that limiting—it requires that a product of the reaction be a reagent for the same reaction, but does not specify that each product and reagent be identical. (*See* D.I. 469 ¶ 8).

C. '407 and '193 Patent Preambles

For the following reasons, 10X's motion is **DENIED** with respect to the '407 and '193 patent preambles.

The preambles in claim 1 of the '407 and '193 patents describe a method for conducting a reaction "in plugs in a microfluidic system." The preambles are identical except that the '193 patent specifies "an autocatalytic reaction." '407 patent at 78:54-55; '193 patent at 78:8-9. I will refer to the '407 patent for simplicity, but the same analysis applies to both patents. (D.I. 510 at 5 n.2).

10X argues that under the correct claim construction, the preambles limit the claims to methods of conducting reactions in a microfluidic system. (D.I. 510 at 6). During claim construction, I found each preamble "limiting only to the extent that it provides antecedent basis for the terms 'microfluidic system' and 'reaction.'" (D.I. 116 at 13). Specifically, I noted:

> While portions of a preamble may be limiting where those portions provide an antecedent basis for terms appearing in the body of the claim, it is inappropriate to construe an entire preamble as limiting if the rest of the preamble language is not limiting. Here, the preamble language states an

> intended use for the invention, "followed by the body of the claim, in which the claim limitations describing the invention are recited." Furthermore, the invention as claimed is "structurally complete" without the remaining preamble language. The claim elements are duplicative of the preamble in that it is clear that the reaction in question takes place "in the at least one plug." Nothing in the body of the claims further limits the location of the reaction.

(*Id.* at 12 (quoting *TomTom, Inc. v. Adolph*, 790 F.3d 1315, 1323 (Fed. Cir. 2015)). I later prohibited 10X from arguing at trial that reactions must occur in the microfluidic system as inconsistent with my claim construction order. Tr. at 21:18-22:13, 275:3-6.

If a preamble "recites essential structure or steps, or if it is 'necessary to give life, meaning, and vitality' to the claim," then the preamble can limit the scope of a claim. *Catalina Mktg. Int'l, Inc. v. Coolsavings.com, Inc.,* 289 F.3d 801, 808 (Fed. Cir. 2002). "When limitations in the body of the claim rely upon and derive antecedent basis from the preamble, then the preamble may act as a necessary component of the claimed invention." *Eaton Corp. v. Rockwell Int'l Corp.*, 323 F.3d 1332, 1339 (Fed. Cir. 2003). However, that a phrase in the preamble "provides a necessary structure for [the claim] does not necessarily convert the entire preamble into a limitation, particularly one that only states the intended use of the invention." *TomTom*, 790 F.3d at 1323.

The Federal Circuit's analysis in *TomTom* is informative. In *TomTom*, the asserted claim provided, "A method for generating and updating data for use in a destination tracking system of at least one mobile unit," comprising steps of "generating and storing traveled distance data," "generating and storing section data," and "generating a section data file." 790 F.3d at 1318. The Federal Circuit agreed with the district court that the phrase "destination tracking system of at least one mobile unit" in the preamble was limiting, because it provides an antecedent basis for the later use of "mobile unit" in the body of the claim. *Id.* at 1323. However, the Federal Circuit went on to find the phrase "[a] method for generating and updating data for use in" ("the

generating language") was not limiting and did not provide an antecedent basis for any of the claims. *See id.* at 1323-24. "Rather, it [was] language stating a purpose or intended use and employs the standard pattern of such language: the words 'a method for a purpose or intended use comprising,' followed by the body of the claim, in which the claim limitations describing the invention are recited." *Id.* at 1324. Therefore, the Federal Circuit found the claim "directed to a method for generating and updating travel-related data and [did] not require the data to be used later." *Id.* at 1324. The claim only required "that the data be generated, selected, stored, and continuously updated," all of which were performed within the body of the claim. *Id.* "Though the collected data could at some point be used in the context of a navigation system, this [was] not required of [the claim], and [did] not convert it into a claim limitation." *Id.*

As discussed in my claim construction opinion, I do not think the '407 patent preamble requires the reaction to occur in the microfluidic system. (*See* D.I. 116 at 12-13). That "reaction" and "microfluidic system" provide antecedent basis for the use of those terms in the body of the claim does not necessarily convert the entire preamble into a limitation. *See TomTom*, 790 F.3d at 1323. Specifically, the portion of the preamble that states "*conducting* a reaction in plugs *in a* microfluidic system" is not limiting. Like the generating language in *TomTom*, the conducting language does not provide an antecedent basis for the rest of the claim and follows the standard pattern of "a method for a purpose or intended use comprising," followed by the body of the claim. *See id.* at 1324. The body of the claim requires "providing the microfluidic system comprising at least two channels having at least one junction," forming a plug at the junction, and "providing conditions suitable for the reaction" in the plug. '407 patent at 78:53-79:12. It says nothing about where the reaction would take place. Therefore, like in *TomTom*, though the plug having "conditions suitable for the reaction" could at some point be

used to conduct reactions in the microfluidic system, "this is not required of [claim 1], and does not convert it into a claim limitation." *See* 790 F.3d at 1324.

D. Direct Infringement

For the following reasons, 10X's motion is **DENIED** with respect to direct infringement.

All of the asserted claims are method claims except claim 1 of the '083 patent, which covers a microfluidic system. However, the '083 patent system must include "at least one plug." '083 patent at 73:10-17. It is undisputed that 10X does not sell its products with plugs—the plugs are formed by running the products. Tr. at 721:19-23, 1326:15-21; (*see also* D.I. 510 at 11; D.I. 530 at 8-9). Therefore, to be a direct infringer, 10X had to have used its accused products. 10X argues that there was insufficient evidence to support the jury's verdict that 10X directly infringed through use of the accused products. (D.I. 510 at 11-12).

The jury heard relevant testimony from Dr. Ness, co-founder and former Chief Technology Officer of 10X,[4] and Dr. Huck, 10X's infringement expert. Dr. Ness stated that 10X ran its products "as of 2016" for testing purposes in California. Tr. at 956:1-15. He further stated that they ran the "actual product according to how it's supposed to be used in the product literature." *Id.* at 956:25-957:6. Plaintiffs assert that the 10X products "as of 2016" are the accused products. (D.I. 530 at 9). 10X argues that that cannot be true because the Chromium Single Cell V(D)J product was not launched until 2017. (D.I. 535 at 5 (citing PTX 1255)). However, Dr. Huck also testified that 10X set up its systems in a demonstration lab in California where he used the products. Tr. at 1108:2-1109:5. Plaintiffs argue that this occurred after Dr. Ness left. (D.I. 530 at 9).[5]

[4] Dr. Ness left 10X in September 2016. Tr. at 907:19-908:1.

[5] I believe Plaintiffs' theory is that 10X did not specifically set up the demonstration lab for Dr. Huck as an expert in this case, but maintained it for general use. Therefore, 10X employees would have directly infringed by using the products in the lab.

The testimony from Drs. Ness and Huck is substantial evidence sufficient to support the jury's verdict. "Direct infringement can be proven by circumstantial evidence." *Toshiba Corp. v. Imation Corp.*, 681 F.3d 1358, 1364 (Fed. Cir. 2012). From the fact that 10X ran its products "as of 2016" for testing purposes and maintained a "demonstration lab" in California where Dr. Huck used 10X products, a reasonable juror could infer that 10X used each of its accused products.[6]

E. Indirect Infringement

For the following reasons, 10X's motion is **DENIED** with respect to indirect infringement.

The jury found both induced and contributory infringement of each asserted patent. (D.I. 476). 10X argues that, at most, Plaintiffs' evidence shows that 10X knew of the asserted patents, which is insufficient as a matter of law for either induced or contributory infringement. (D.I. 510 at 12-14).

The Supreme Court clarified the knowledge requirement for indirect infringement in *Commil USA, LLC v. Cisco Systems, Inc.*, 135 S. Ct. 1920 (2015). While direct infringement is a "strict-liability offense," "liability for inducing infringement attaches only if the defendant knew of the patent and that the induced acts constitute patent infringement." *Id.* at 1926 (internal quotation marks omitted). "Like induced infringement, contributory infringement requires knowledge of the patent in suit and knowledge of patent infringement." *Id.* Therefore, knowledge of the patents alone cannot support a finding of indirect infringement. The requisite

[6] Given that I find sufficient evidence to support indirect infringement, *see infra* Section IV.E, even if I were wrong about direct infringement relating to the Chromium Single Cell V(D)J product, I do not believe any of the other issues the jury decided, such as willfulness and damages, would be affected.

knowledge, however, can be met with circumstantial evidence. *See Enplas Display Device Corp. v. Seoul Semiconductor Co., Ltd.*, 909 F.3d 398, 408 (Fed. Cir. 2018).

Plaintiffs argue that substantial evidence supports the jury's finding that 10X knew of both the asserted patents and its infringement. (D.I. 530 at 9-13). 10X was on notice of its alleged infringement for the entire damages period. (D.I. 5); Tr. at 651:12-16 (10X did not begin selling its accused products until after Plaintiffs served their initial complaint). Dr. Hindson, co-founder and Chief Scientific Officer of 10X,[7] was the "point person for IP in the early stages of 10X." *Id.* at 694:14-15, 781:9-10. He admitted that 10X monitored the asserted patents as patent applications were filed and patents issued. *Id.* at 787:14-22. He also stated that he looked at the asserted patents for "intellectual property reasons," "as [10X] was adopting droplets." *Id.* at 786:24-787:3.

Dr. Ness explained that 10X first tried other approaches such as capsules and wells but moved to droplets after the other approaches were unsuccessful. *Id.* at 953:1-954:13. Both Drs. Hindson and Ness had prior experience with droplets. They previously co-founded QuantaLife, which developed a product to perform polymerase chain reaction ("PCR") in droplets. *Id.* at 700:3-23, 907:19. Dr. Hindson was also Chief Scientific Officer at QuantaLife. *Id.* at 700:10-12. He explained that he and Dr. Ness built the QuantaLife product and "were really the only ones who really knew the nuts and bolts from start to finish of that product." *Id.* at 703:4-7. Bio-Rad acquired QuantaLife in 2011 to develop its droplet business. *Id.* at 47:23-48:2, 82:11-19, 120:8-121:5, 701:6-10. Drs. Hindson and Ness stayed at Bio-Rad for about a year before leaving to found 10X. *Id.* at 705:5-6, 707:2-7, 907:17-24.

[7] Dr. Hindson described his role as "the head science guy at the company." Tr. at 694:16-18.

There is no real dispute that 10X knew of the asserted patents. As to knowledge of infringement, neither party presented direct evidence of 10X's state of mind, at least with respect to the '407 and '193 patents.[8] The testimony from Drs. Hindson and Ness, however, provides enough circumstantial evidence to support the jury's verdict. Both were experts in droplets and had succeeded in using droplets at QuantaLife. In fact, they were so successful that Bio-Rad acquired QuantaLife to develop its droplet business. Yet, when they left Bio-Rad to start 10X, they avoided using droplets in their new system. Only after failing with other approaches did they return to droplets. Dr. Hindson admitted that, as the "point person for IP," he looked at the asserted patents as 10X made the move to droplets. A reasonable juror could thus conclude that 10X knew its droplet products were infringing the asserted patents.

The '083 patent is a somewhat different situation because it requires a microfluidic system with a "non-fluorinated microchannel." As discussed, 10X deliberately added 0.02% Kynar, a substance containing fluorine, to its microfluidic chips. *See supra* Section IV.A.1. 10X presented evidence that it did not believe its products infringed after the addition of Kynar. Dr. Stuelpnagel, the Chairman of the Board, said that he came up with and advocated for the addition of Kynar as a means to "intentionally add some fluorine and take [the] issue off the table." *Id.* at 604:11-21. He also noted that 10X "wanted to make sure that whatever [it] did intentionally to the chip would cause no problems with [its] current product." *Id.* at 604:22-24. Likewise, Dr. Saxonov, the CEO, testified that the Kynar was added because "while [10X] felt like [its] position as far as patent infringement was very strong, this was going to make it even stronger."

[8] 10X argues that Dr. Hindson stated that 10X didn't think it infringed. (D.I. 510 at 13). The testimony 10X refers to is said in passing in response to a question about the addition of Kynar to avoid infringement of the '083 patent. He responds, "We didn't think we infringed any way. To make a lot of fluorine into the chip, then it's a fluorinated microchannel." Tr. at 792:8-15. Dr. Hindson appears to be referring to the '083 patent, not making a general statement about infringement of all the asserted patents.

Id. at 602:1-15. Neither Drs. Stuelpnagel nor Saxonov could identify any technical benefits from the addition of Kynar. *Id.* at 605:2-12, 602:16-20.

Plaintiffs argue that the jury could have found "the inclusion of a meaningless chemical to try to create a non-infringement argument was evidence of culpability." (D.I. 530 at 12). I agree, although I think this is a close case.[9] 10X has not shown that no reasonable juror could find, given the small amount of fluorine added and lack of identified benefits, that 10X knew its post-Kynar products infringed the '083 patent.

Therefore, the jury's indirect infringement verdicts are supported by substantial evidence.

F. Section 271(f)(2) Infringement

For the following reasons, 10X's motion is **DENIED** with respect to § 271(f)(2) infringement.

35 U.S.C. § 271(f)(2) provides:

> Whoever without authority supplies or causes to be supplied in or from the United States any component of a patented invention that is especially made or especially adapted for use in the invention and not a staple article or commodity of commerce suitable for substantial noninfringing use, where such component is uncombined in whole or in part, knowing that such component is so made or adapted and intending that such component will be combined outside of the United States in a manner that would infringe the patent if such combination occurred within the United States, shall be liable as an infringer.

The jury's verdict under § 271(f)(2) is based solely on the '083 patent. (D.I. 476 at 3; D.I. 510 at 14; D.I. 530 at 13). 10X argues that there is insufficient evidence that 10X supplied components from the United States or knew that such components would be combined in an infringing manner. (D.I. 510 at 14-16).

[9] Credibility is an issue for the jury. *Perkin-Elmer*, 732 F.2d at 893. It did not have to accept any protestations of innocence by 10X executives.

There was substantial evidence to support finding that 10X supplies components of the accused products from the United States to customers abroad. 10X's damages expert, Dr. Sullivan, acknowledged that "10X manufactures and/or assembles its products in the United States." Tr. at 1242:1-4. Dr. Sullivan relied on testimony from Ms. Osborn, 10X's Vice President of Finance. *Id.* at 1242:5-9, 1243:12-14. The jury heard deposition testimony from Ms. Osborn that "final assembly" of 10X reagents occurs at 10X's California office. *Id.* at 1244:11-16. Likewise, Dr. Hindson testified that the reagents used with the accused products are provided by 10X. *Id.* at 716:4-7. Dr. Hindson also stated that 10X formulates a fluorinated oil with its specialized surfactants and "ship[s] it out to [10X's] customers to use in [10X's system]." *Id.* at 733:1-9, 734:16-735:3. It is undisputed that 10X sells its products to customers worldwide. *Id.* at 1244:19-1245:6, 1322:12-16. Therefore, a reasonable juror could conclude that 10X ships its fluorinated oil, a "component of [the] patented invention that is especially made or especially adapted for use in the invention," from California to customers abroad for use in the accused products.

I addressed 10X's knowledge of infringement with respect to induced and contributory infringement. For the reasons discussed, a reasonable juror could find that 10X knew its customers would use the accused products in an infringing manner. *See supra* Section IV.E.

Therefore, there is substantial evidence to support to the jury's finding that 10X supplies from the United States a component of the '083 patented invention. 10X has thus failed to show that it does not infringe under § 271(f)(2) as a matter of law.

G. Willful Infringement

For the following reasons, 10X's motion is **DENIED** with respect to willful infringement.

Under *Halo Electronics, Inc. v. Pulse Electronics*, "[t]he subjective willfulness of a patent infringer, intentional or knowing, may warrant enhanced damages, without regard to whether his infringement was objectively reckless." 136 S. Ct. 1923, 1933 (2016). Subjective willfulness is met with proof, by a preponderance of the evidence, that "the defendant acted despite a risk of infringement that was either known or so obvious that it should have been known to the accused infringer." *WesternGeco L.L.C. v. ION Geophysical Corp.*, 837 F.3d 1358, 1362, 1364 (Fed. Cir. 2016) (internal citation and quotation marks omitted).

Again, based on testimony from Drs. Hindson and Ness, a reasonable juror could conclude that 10X knew its customers would use the accused products in a manner that infringed the asserted patents. *See supra* Section IV.E. Therefore, a reasonable juror could also conclude that, by selling those products, 10X acted despite a known risk of infringement. Thus, the jury's finding of willful infringement is supported by substantial evidence.

V. INVALIDITY

10X bears the burden of proof by clear and convincing evidence on invalidity. Therefore, to prevail on JMOL, 10X must show "not only that there is sufficient evidence to support the finding, even though other evidence could support as well a contrary finding, but additionally that there is insufficient evidence for permitting any different finding." *Fireman's Fund*, 540 F.2d at 1171.

A. Dr. Sia and the Person of Ordinary Skill in the Art

For the following reasons, 10X's motion with respect to Dr. Sia's testimony is **DENIED**.

10X argues that Dr. Sia, Plaintiffs' invalidity expert, was neither a person of ordinary skill in the art, nor testified from the perspective of a person of ordinary skill in the art. (D.I. 510 at 17-18). 10X did not raise this issue under *Daubert* or at trial. Plaintiffs offered Dr. Sia as an

expert in the subject matter of the patents-in-suit at trial without objection. Tr. at 357:16-19. Therefore, 10X waived any argument relating to Dr. Sia's alleged failings as a person of ordinary skill in the art. *See MobileMedia Ideas, LLC v. Apple Inc.*, 966 F. Supp. 2d 439, 476 (D. Del. 2013), *aff'd in part, rev'd in part*, 780 F.3d 1159 (Fed. Cir. 2015) ("A party's failure to object at trial to the issue it wishes to raise post-trial is fatal to its argument.").

B. Anticipation—'407 Patent, Claim 1

For the following reasons, 10X's motion is **DENIED** with respect to anticipation.

10X argues that no reasonable juror could have rejected 10X's evidence that the Quake reference (DTX 13) anticipates claim 1 of the '407 patent. (D.I. 510 at 18-19). 10X's expert, Dr. Chang, testified that paragraph 170 of Quake discloses the claim element of "continuously flowing an aqueous fluid containing at least one biological molecule and at least one reagent for conducting the reaction between the biological molecule and the at least one reagent through a first channel of the at least two channels." Tr. at 971:6-980:1; (D.I. 510 at 18). However, Dr. Sia testified that paragraph 170 does not disclose a channel with both the biological molecule and reagent as required by the claim. *Id.* at 1269:21-1270:25. He further noted that paragraph 170 "talks about what's going on before the operation of the microfluidic chip." *Id.* at 1271:4-9. A reasonable juror could have relied on Dr. Sia's testimony to find Quake did not disclose the "continuously flowing" claim element. Therefore, 10X has not met its burden of showing that there is insufficient evidence to support the jury's verdict that 10X had not proved by clear and convincing evidence that Quake anticipates claim 1 of the '407 patent.

C. Obviousness

For the following reasons, 10X's motion is **DENIED** with respect to obviousness.

1. '407 Patent, Claims 10 and 11

Claims 10 and 11 of the'407 patent depend from claim 1 and require the use of a "fluorosurfactant" and "fluorinated oil," respectively. '407 patent at 80:9-12. 10X argues that claims 10 and 11 must be invalid as obvious in view of the Quake and Schubert (DTX 16) references. (D.I. 510 at 19-20).

Dr. Chang testified that a person of ordinary skill in the art would have had both motivation to combine and a reasonable expectation of success in using the fluorinated oil and fluorinated surfactant disclosed in Schubert in the microfluidic device disclosed in Quake. Tr. at 980:20-984:6. However, Dr. Sia testified that a person of ordinary skill in the art would not have combined the two references, because "the Schubert reference talks about painting, coatings, polymer technology, metal working in uranium recovered" and did not mention microfluidic devices. *Id.* at 1275:8-16. He also explained that Schubert was a "totally different system," and thus there would be no reasonable expectation of success in using the fluorinated oils for droplets. *Id.* at 1276:1-12. Dr. Sia further noted that Schubert was published before Quake, and thus opined, "[I]f it was so obvious to use the fluorinated compounds disclosed in [Schubert] that was really about paint, and coatings, and so forth in [Quake's] system, [Quake] would have, I'm sure done it." *Id.* at 1275:17-1276:4. Based on Dr. Sia's testimony, a reasonable juror could conclude that 10X did not prove that a person of ordinary skill in the art had motivation to, and a reasonable expectation of success in, combining Quake and Schubert.

2. '193 Patent, Claims 6 and 8

Claims 6 and 8 depend from claim 1 of the '193 patent. Analogous to claims 10 and 11 of the '407 patent, claims 6 and 8 require a "fluorinated oil" and "fluorinated surfactant," respectively. '193 patent at 78:41-45. However, unlike claim 1 of the '407 patent, claim 1 of the '193 patent requires conditions suitable for an "autocatalytic reaction." '193 patent at 78:27-29.

10X argues that claims 6 and 8 of the '193 patent must be invalid as obvious in view of the Quake, Corbett (DTX 18), and Schubert references. (D.I. 510 at 20-21).

Dr. Chang testified that Corbett disclosed conducting PCR, an autocatalytic reaction, in "slugs." Tr. at 996:4-8, 997:3-20. In contrast to droplets, slugs are not encapsulated by oil and thus touch the microchannel wall. *Id.* at 997:11-20. Dr. Chang opined that a person of ordinary skill in the art would have been motivated to conduct the PCR reactions from Corbett in the Quake droplet system to avoid the contamination that occurred from the slugs touching the channel walls. *Id.* at 997:22-998:13.

Dr. Sia gave substantial testimony to the contrary. Dr. Sia opined that the slug and droplet systems are "totally different" and "fundamentally different" from a "flow perspective." *Id.* at 1277:25-1278:2. Further, like Schubert, Corbett predates Quake. Thus, Dr. Sia testified that Quake would have combined the references had there been motivation to do so. *Id.* at 1278:10-14. I do not think Dr. Sia's testimony is rendered insufficient by his later statement on cross-examination that a person of ordinary skill in the art "wouldn't need much motivation" to use PCR. *Id.* at 1318:3-6. Dr. Sia appears to have been commenting on the use of PCR generally, as opposed specifically in the context of combining the Corbett and Quake systems. *See id.* at 1317:17-1318:6. Therefore, based on Dr. Sia's testimony, a reasonable juror could conclude that 10X did not prove that a person of ordinary skill in the art had motivation to combine Corbett and Quake.

Therefore, 10X has failed to show that Dr. Sia's testimony is so insufficient that the jury could have only concluded that 10X proved obviousness.

D. Lack of Enablement—'407 and '193 Patents

For the following reasons, 10X's motion is **DENIED** with respect to lack of enablement.

10X argues that claims 1, 10, and 11 of the '407 patent and claims 6 and 8 of the '193 patent are invalid for lack of enablement. (D.I. 510 at 21-23). 10X asserts that the claims cover methods for conducting reactions once the droplets are removed from the microfluidic chips and transported outside the microfluidic system. (*Id.* at 21). It is undisputed that surfactants are needed to stabilize the droplets once removed from the microfluidic system. (D.I. 510 at 22; D.I. 530 at 22-23); Tr. at 1008:3-23 (Chang), 410:9-411:19 (Sia). 10X argues that the patents thus fail to enable the claims, because the necessary surfactants were not available until 2008, well after the date of invention. (D.I. 510 at 22).

10X raises two distinct issues for enablement—off-chip reactions and reactions outside the microfluidic system. Plaintiffs address each separately. (D.I. 530 at 22; D.I. 535 at 10).

Regarding off-chip reactions, Dr. Chang admitted that the asserted patents teach reactions in droplets wherein the droplets are contained in a capillary that is removed from the microfluidic chip. Tr. at 1054:2-19. Based on that testimony, a reasonable juror could conclude that the asserted patents teach off-chip reactions.

Regarding reactions outside the microfluidic system, Plaintiffs argue that the necessary surfactants were available at the time of the invention. 10X asserts that the surfactants first became available in 2008, citing to the Holtze paper (DTX 93). (D.I. 510 at 22). However, Dr. Ismagilov, a named inventor of the asserted patents, testified that the patents teach "exactly" the same surfactants disclosed in the Holtze paper—"fluoro-surfactants with non-ionic category groups for doing reaction." Tr. at 221:11-222:16. In particular, he stated, "The patents teach that longer [fluorocarbon] tails stabilize droplets." *Id.* at 222:15-16. Dr. Sia agreed, opining that the asserted patents teach "the defining feature of what the Holtze paper says about its own surfactants, having long fluorophillic tails." *Id.* at 1287:22-1288:16. A reasonable juror could

thus conclude that the asserted patents taught the same surfactants taught in the Holtze paper, meaning that the necessary surfactants were available at the time of the invention. Therefore, 10X has failed to show that the '407 and '193 patent claims are not enabled as a matter of law.

E. Indefiniteness—'083 Patent

For the following reasons, 10X's motion is **DENIED** with respect to indefiniteness.

As discussed, the '083 patent claims require the surface tension at the plug-fluid/microchannel wall interface to be higher than that at the plug-fluid/carrier fluid interface. *See supra* Section IV.A.2. 10X argues that claims 1 and 9 of the '083 patent are indefinite because a person of ordinary skill in the art cannot determine whether the claimed surface tension relationship is met in a system where there is no plug-fluid/microchannel wall interface. (D.I. 510 at 23-24).

It is undisputed that the plug does not touch the microchannel wall during flow. Tr. at 381:12-19, 382:25-383:7, 383:13-384:9. Dr. Chang testified that a person of ordinary skill in the art would thus not know where or how to measure the claimed surface tension. *Id.* at 1003:10-19. Dr. Sia, however, offered substantial opposing testimony. Dr. Sia presented the Bio-Rad data measuring the relevant surface tensions on the top and bottom of 10X's microfluidic chips. *Id.* at 384:14-393:13. He explained that surface tension is an intrinsic property—"as long as you have the same two phases" to measure surface tension between, "then that surface tension is going to be the same . . . no matter where it appears." *Id.* at 386:5-19. Dr. Sia also identified portions of the '083 patent specification that state specific surface tension values relevant to the invention. *Id.* at 1295:1-19. A reasonable juror could rely on Dr. Sia's testimony to find that a person of ordinary skill in the art would know how to measure the surface tension at the plug-

fluid/microchannel wall interface. Therefore, 10X has failed to show that the '083 patent claims are indefinite as a matter of law.

VI. DAMAGES

10X argues that the damages award is based on legally insufficient testimony and is not supported by substantial evidence. The jury awarded $23,930,718 in damages, which corresponds to a 15% royalty on 10X's worldwide sales, through the second quarter of 2018. (D.I. 476); Tr. at 611:20-613:2. 10X argues that Plaintiffs' damages expert, Mr. Malackowski, gave insufficient testimony to support the jury's verdict, because (1) he relied on noncomparable prior licenses, (2) he failed to properly apportion damages based on the value of the patented technology, and (3) there was no basis to include 10X's foreign sales in the royalty base. (D.I. 510 at 24-30). For the following reasons, 10X's motion is **DENIED** with respect to damages.

A. Prior Licenses

10X previously moved to exclude Mr. Malackowski's comparable license opinions as inadmissible under *Daubert*. Mr. Malackowski relies on three prior licenses—Caliper/RainDance, Applera/Bio-Rad, and Applied BioSystems/QuantaLife. I denied 10X's motion on the basis that Mr. Malackowski provided "reasonable and specific explanations for selecting the agreements he did." (D.I. 361 at 14; *see also* D.I. 425 at 5 ("I rejected Defendant's arguments in my prior *Daubert* order, which found that Mr. Malackowski met a showing of baseline comparability between the licenses, and that the degree of comparability is a factual issue best addressed through cross examination.")). Now, 10X argues that Plaintiffs failed to present sufficient evidence at trial to establish comparability. (D.I. 510 at 24-26). Although I think 10X is correct with respect to the Applera/Bio-Rad license, that alone does not warrant

granting JMOL. The Caliper/RainDance and Applied BioSystems/QuantaLife licenses provide sufficient support for Mr. Malackowski's reasonable royalty opinions.

1. Caliper/RainDance (PTX 413)

The Caliper/RainDance license covered a large portfolio of patents relating to microfluidics. (D.I. 510 at 25; D.I. 530 at 25); Tr. at 137:15-22, 626:25-627:3. There were separate competitor and noncompetitor royalty rates of 15% and 2%, respectively. Tr. at 140:11-17 (Tumolo).[10] The same rate applied regardless of the number of patents actually used. *Id.* at 140:4-7; 1239:15-1240:1. 10X argues that Plaintiffs failed to present sufficient evidence of technological comparability, or that the 15% competitor rate applies. (D.I. 510 at 25).

First, 10X argues that Plaintiffs did not go beyond "surface similarity" to support technological comparability. (D.I. 510 at 25 (quoting *Finjan, Inc. v. Blue Coat Sys., Inc.*, 879 F.3d 1299 (Fed. Cir. 2018))). In *Finjan*, the court found insufficient evidence to support a damages award based in part on a prior jury verdict. 879 F.3d at 1312. The court found no evidence showing the patents in the prior case were economically or technologically comparable to the asserted patent in *Finjan*. *Id.* Rather, the fact that the infringing products in the prior case "were also in the computer security field" and that the parties were competitors, was mere "surface similarity" and "far too general to be the basis for a reasonable royalty calculation." *Id.*

Regarding technological comparability, Dr. Sia testified that "the Caliper patents dealt with microfluidics and all sorts of ways to control fluids really accurately and so forth," and the asserted patents dealt with the "same subject matter, but with droplets." Tr. at 441:13-442:2. Dr. Sia also described a few Caliper patents, noting that, although they were not doing droplets, they "dealt with manipulating tiny amounts of fluids, mixing, performing nucleic acid reactions, a

[10] Ms. Tumolo, president of life sciences at Bio-Rad, became familiar with the Caliper/RainDance agreement through diligence related to Bio-Rad's acquisition of RainDance. *Id.* at 113:8-9, 138:7-139:1.

whole tool box of reactions and things you can do on a chip," which is "what the [asserted] patents deal with." *Id.* at 442:11-24. Dr. Sia's testimony goes beyond "surface similarity." In *Finjan*, the only technological similarity was the field of "computer security." 879 F.3d at 1312. Here, Dr. Sia testified that both sets of patents related not only to the field of microfluidics, but specific ways to control fluids and conduct reactions in small amounts of fluids. Based on Dr. Sia's testimony, a reasonable juror could conclude that the Caliper patents and the asserted patents are technologically comparable.

Second, 10X argues that the competitor rate cannot apply because RainDance paid Caliper the noncompetitor rate. (D.I. 510 at 25). Although RainDance may have paid the lower rate, it is undisputed that RainDance and Caliper agreed to the competitor rate as the result of an arms-length negotiation. (D.I. 510 at 25; D.I. 530 at 26; D.I. 535 at 11). Further, Ms. Tumolo testified that once RainDance became a competitor, the higher rate would apply. Tr. at 140:11-18. Therefore, Mr. Malackowski had sufficient support to rely on the competitor rate as a prior license relevant to the hypothetical negotiation.

2. Applera/Bio-Rad (PTX 128)

10X asserts that the Applera/Bio-Rad license covered "Nobel Prize-winning technology, licensed for over $2 billion, related to real-time PCR and thermal cycler instruments, including the 'foundational' Higuchi patent." (D.I. 510 at 25). In contrast, 10X argues that its products do not use PCR reactions, it does not make thermal cyclers, and the asserted patents brought in less than $2 million in royalties. (*Id.* at 26).

10X conflates early PCR patents and the Higuchi patent licensed in the Applera/Bio-Rad agreement. Kary Mullis won the Nobel Prize for his PCR inventions made in the 1980s. Tr. at 1161:24-1162:3. There was no evidence that any of the Mullis patents were licensed as part of

the Applera agreement. *Id.* at 1236:17-1237:3. Ms. Tumolo explained that, in contrast, the Higuchi patent "enabled monitoring of a PCR reaction," and was licensed for "a relatively new technology at the time called real[-]time PCR." *Id.* at 154:16-23. She further testified that even with the Higuchi technology, Bio-Rad "had to do a lot of heavy lifting" and "ended up with a lot of patents [itself] around the product that [it] developed using this license." *Id.* at 155:15-24.

On reply, 10X argues that the Higuchi patent must be considered with Applera's entire PCR patent portfolio, because the Applera agreement was part of a global settlement. (D.I. 535 at 12). Mr. Malackowski agreed that the Applera agreement was part of a "three-part resolution" comprising three separate agreements. Tr. at 660:7-24. However, there was no evidence that those other agreements addressed the Higuchi patent or included patents licensed for $2 billion. *See id.* at 196:19-197:3, 1161:1-20. Dr. Sullivan merely made the conclusory statement that the agreements "all are surrounding certain technology that was foundational to the industry." *Id.* at 1161:23-24. Therefore, there was sufficient evidence for the jury to find the Applera/Bio-Rad agreement economically comparable to the hypothetical license.[11]

Plaintiffs presented very little, however, regarding technological comparability. Dr. Sia testified that the Higuchi patent was for "an instrument for doing real-time PCR," and the asserted patents "allow you to do a lot of different types of reactions, and PCR is one of those important reactions." *Id.* at 443:4-13. That is not sufficient to support a finding of technological comparability. The fact that the asserted patents cover droplet technology, which may be used to conduct many types of reactions, does not mean that any patent relating to those reactions is

[11] 10X also raises the new argument on reply that Plaintiffs failed to account for the "offset provision" in the agreement, which resulted in an effective rate of 7-8%, rather than 15%. (D.I. 535 at 12). The issue is waived since it was not raised in the opening brief. In any event, there was conflicting expert testimony at trial. Tr. at 665:13-666:15, 1163:10-22, 1237:22-1238:22. 10X has not shown that the jury had insufficient support to find the 15% rate applied.

technologically comparable. (*See* D.I. 535 at 13). Therefore, Plaintiffs failed to provide substantial evidence that the Applera/Bio-Rad license is technologically comparable such that it can support a reasonable royalty calculation.

3. Applied BioSystems/QuantaLife (PTX 412)

10X argues that the Applied BioSystems license related to the use of the taq polymerase enzyme in PCR and is not technologically comparable to the hypothetical license. 10X relies on the testimony of its expert, Dr. Quackenbush. Dr. Quackenbush opined that, of the patents in the Applied BioSystems agreement, "the only one that's really worth considering is the taq polymerase license." Tr. at 1126:7-19. He went on to find that the asserted patents "just aren't comparable," as they "discuss changes to microfluidic technology, but they haven't transformed the field in a way that taq polymerase has." Tr. at 1126:19-1127:2.

It appears undisputed that the Applied BioSystems license covered patents necessary for PCR. Ms. Tumolo described the patents as "sort of basic rights if you want to do PCR."[12] "They are surrounding polymerases, especially enzymes, things that makes the reaction go. . . . [S]o if you wanted to do PCR and you needed that enzyme, which you do, you needed these patents." *Id.* at 146:4-10. Likewise, Dr. Sia stated that the license was "for some reagents for doing . . . PCR," and specifically "that would help you to do PCR in an improved manner." *Id.* at 443:14-20.

Regarding technological comparability, Dr. Sia stated that the asserted patents "also deal with the subject [of] trying to do PCR and trying to do it better using the droplet technologies." *Id.* at 443:20-22. He also noted that QuantaLife was "licensing the reagents for doing . . . PCR in droplets." *Id.* at 444:4-8. Based on Dr. Sia's testimony, a reasonable juror could conclude that

[12] Ms. Tumolo became familiar with the Applied BioSystems/QuantaLife agreement through diligence related to Bio-Rad's acquisition of QuantaLife. Tr. at 145:19-22.

the Applied BioSystems license, like the hypothetical license, covered technology necessary to conduct reactions in droplets, and thus is technologically comparable.

B. Apportionment

For the following reasons, 10X's motion is **DENIED** with respect to apportionment.

10X argues that Mr. Malackowski's testimony should never have been admitted because he applied an "untested and unreliable theory of 'comparable apportionment.'" (D.I. 510 at 27). 10X raised the same argument under *Daubert*. I held, "The Federal Circuit does not limit apportionment to specific methodologies, because flexibility is required to determine fact-dependent damages. As a methodology, I see no problem with using comparable licenses to establish a reasonable royalty rate, without performing a separate apportionment analysis, where there is a logical basis for doing so." (D.I. 361 at 16 (citations omitted)). I excluded Mr. Malackowski's initial opinion because he failed to explain how the royalty rates in the prior licenses were "apportioned in a comparable fashion to the contribution of the patented technology to the accused products." (D.I. 361 at 17 n.3). I allowed Mr. Malackowski to submit a supplemental report, however, which I found "fill[ed] the gaps in his initial report." (D.I. 425 at 4-5). "Mr. Malackowski compared the unpatented features of the accused product with what he considered to be the unlicensed features of the products in the [prior] licenses." (*Id.* at 5). Specifically, he matched an analogous unlicensed feature to each unpatented feature identified by 10X's damages expert, Dr. Sullivan. (*Id.* at 5 & n.2). Mr. Malackowski's trial testimony was consistent with the methodology in his expert reports. Tr. at 623:21-624:20, 625:24-626:627:3, 630:3-12. Therefore, I find his testimony was properly admitted.

C. Foreign Sales

For the following reasons, 10X's motion is **DENIED** with respect to foreign sales damages.

10X argues that no reasonable juror could have found infringement under § 271(f)(2), the only basis for damages on 10X's foreign sales, or infringement of the '083 patent, the only patent to which § 271(f)(2) applies. (D.I. 510 at 30). I found infringement under § 271(f)(2), including infringement of the '083 patent, supported by substantial evidence. *See supra* Sections IV.A, IV.F. Therefore, 10X's argument is moot.

VII. NEW TRIAL

The decision to grant or deny a new trial is within the discretion of the district court. *Allied Chem.*, 449 U.S. at 36. 10X has not shown that "a miscarriage of justice would result if the verdict were to stand," the verdict "cries out to be overturned," or the verdict "shocks [the] conscience." *Williamson*, 926 F.2d at 1352-53. Therefore, 10X's motion for a new trial is **DENIED**.

VIII. CONCLUSION

A separate order will be entered.

CERTIFICATE OF SERVICE

I hereby certify that I electronically filed the foregoing with the Clerk of the Court for the United States Court of Appeals for the Federal Circuit by using the appellate CM/ECF system on October 18, 2019.

I certify that all participants in the case are registered CM/ECF users and that service will be accomplished by the appellate CM/ECF system.

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CERTIFICATE OF COMPLIANCE

The brief complies with the type-volume limitation of Fed. Cir. R. 32(a) because this brief contains 13,934 words, excluding the parts of the brief exempted by Fed. R. App. P. 32(f) and Fed. Cir. R. 32(b).

This brief complies with the typeface requirements of Fed. R. App. P. 32(a)(5) and the type style requirements of Fed. R. App. P. 32(a)(6) because this brief has been prepared in a proportionally spaced typeface using Microsoft Word 2016 in Century Schoolbook 14-point font.

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