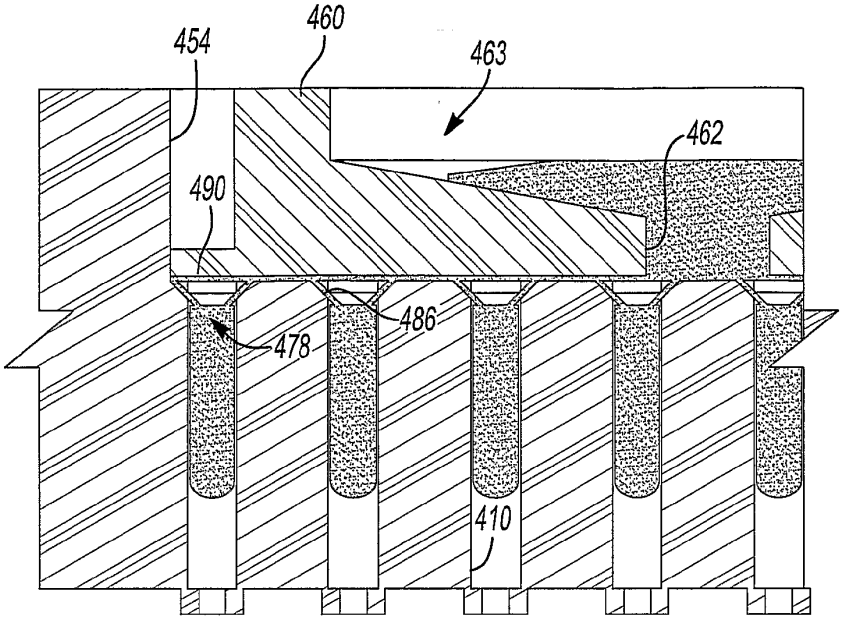


Fig-151

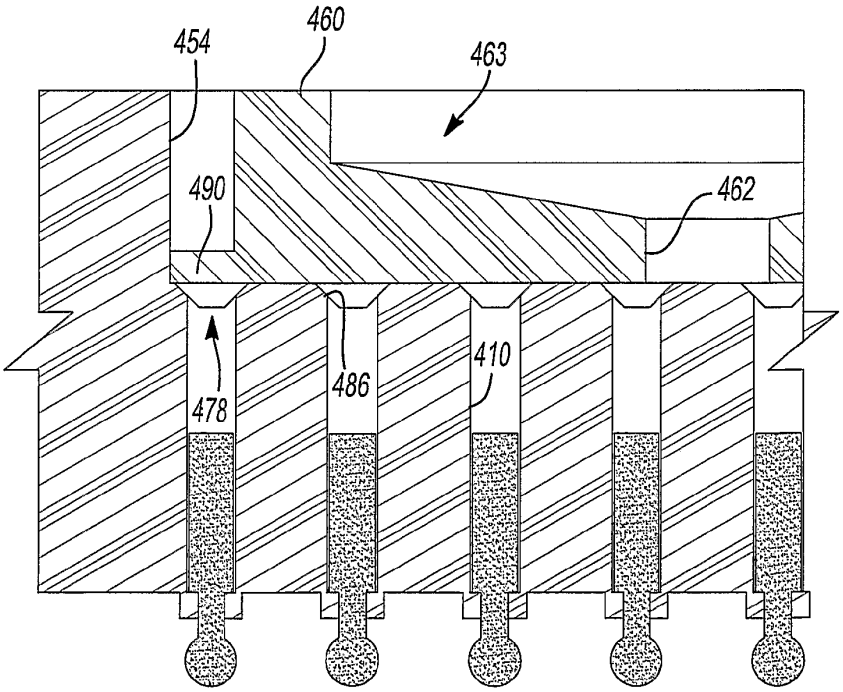


Fig-152

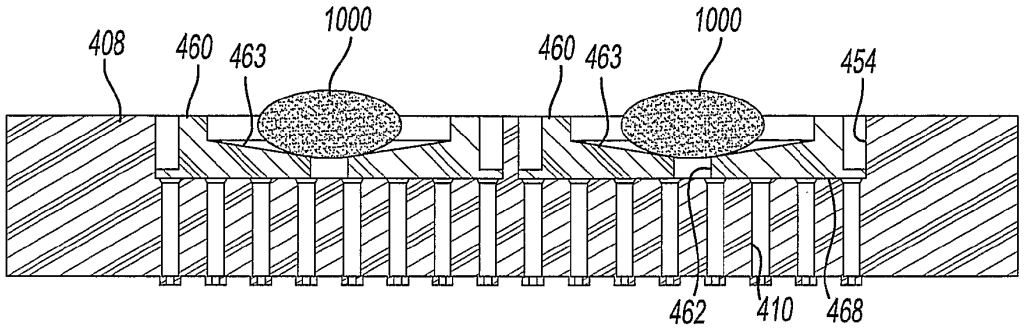


Fig-153

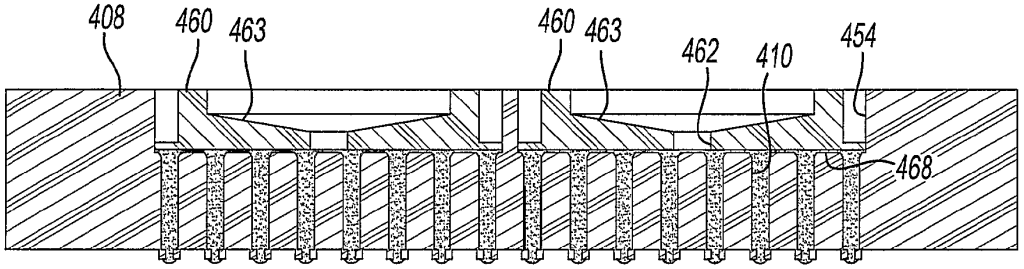


Fig-154

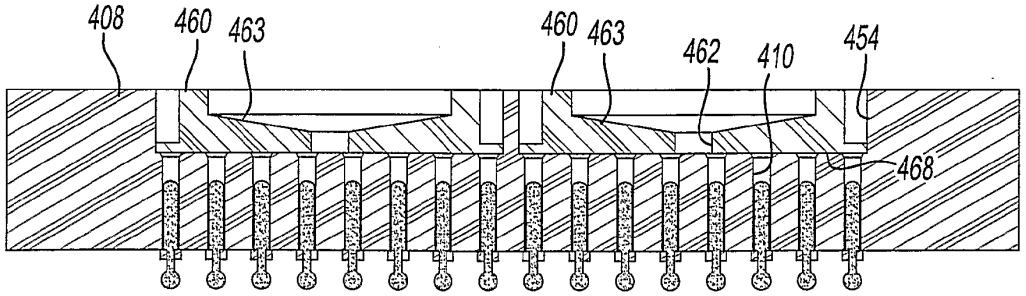


Fig-155

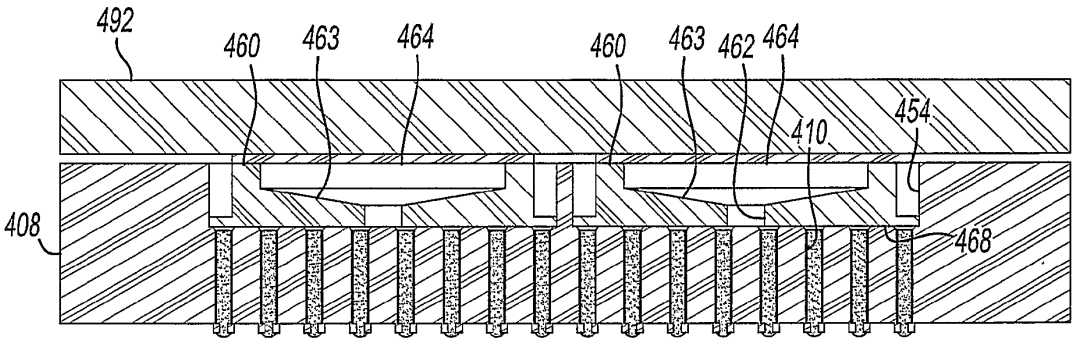


Fig-156

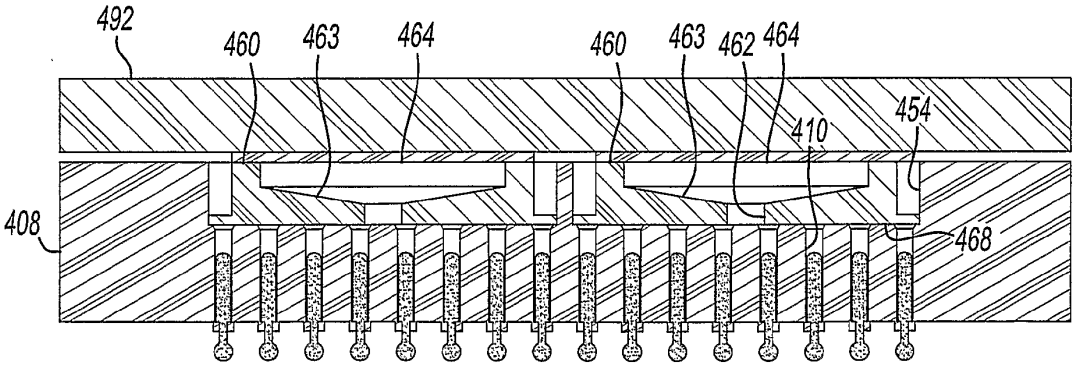


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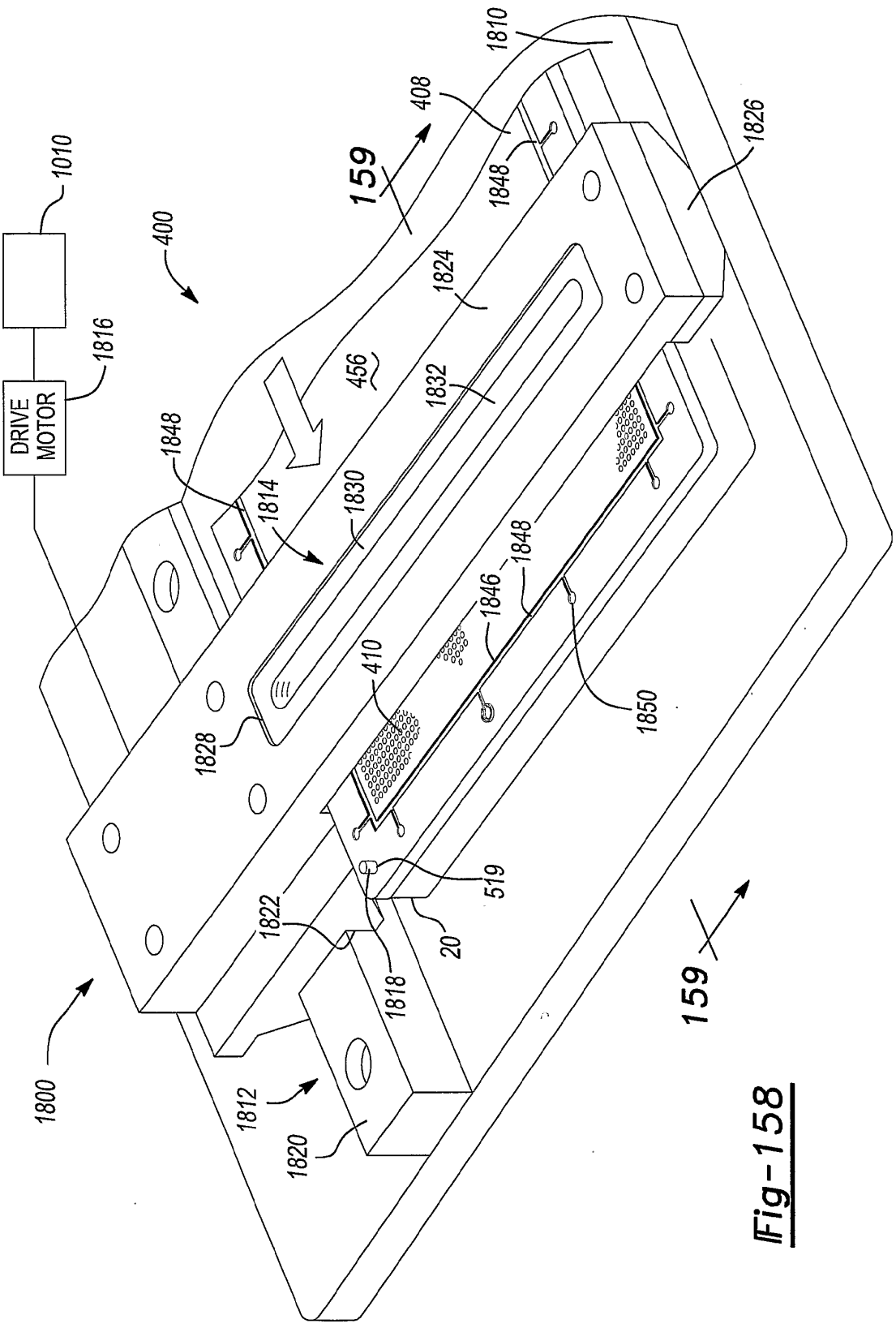

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Fig-158

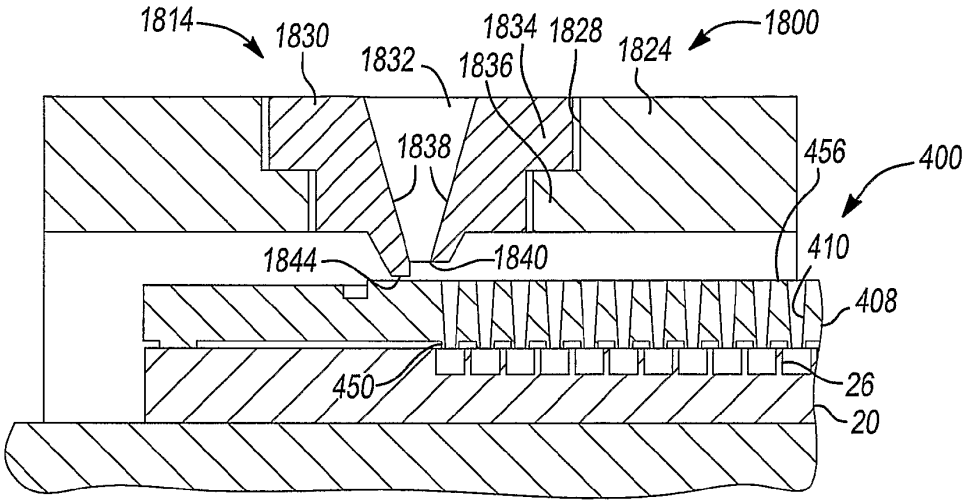


Fig-159

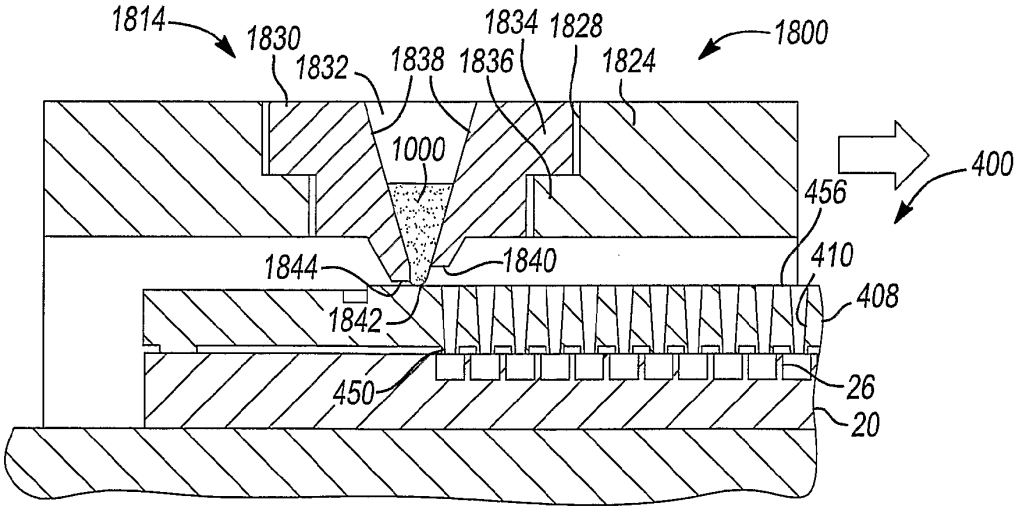


Fig-160

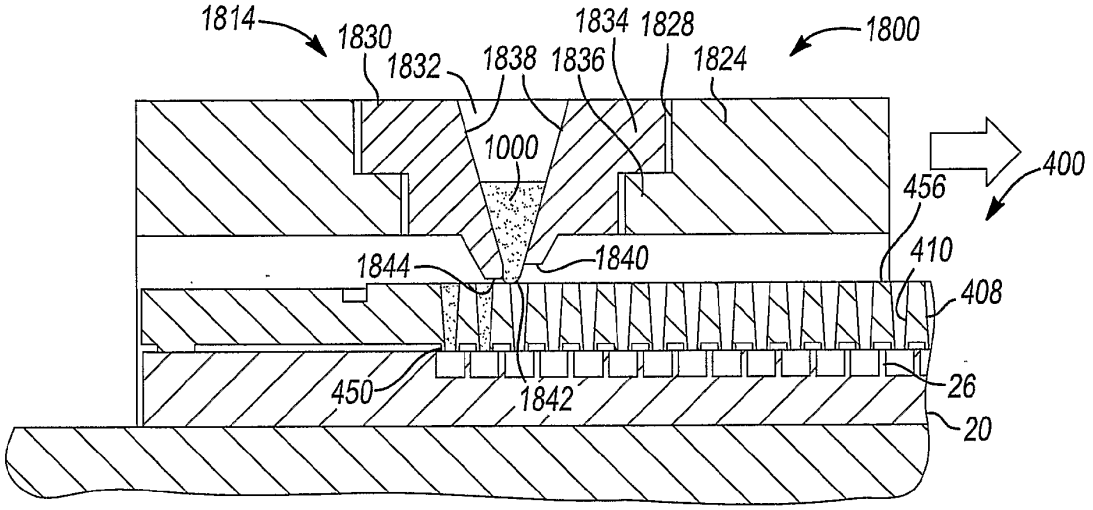


Fig-161

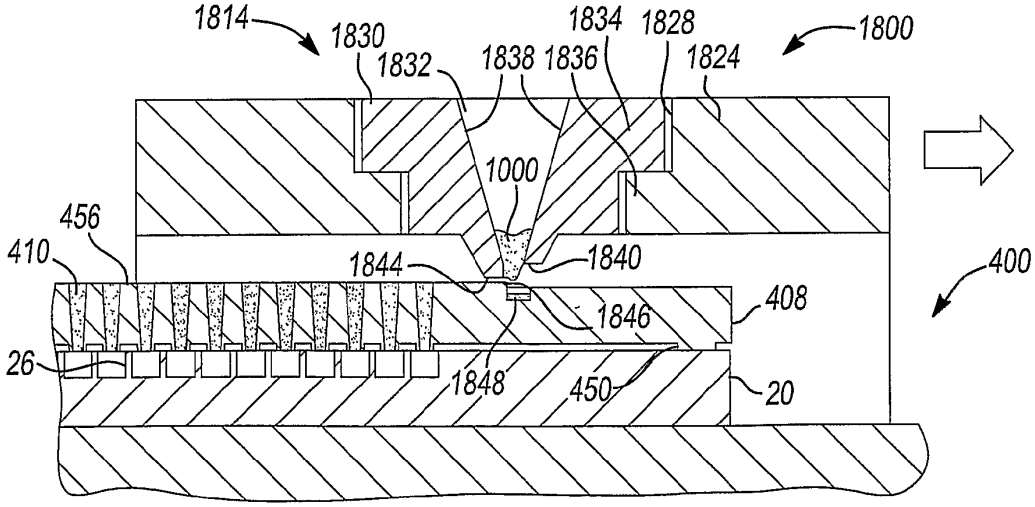


Fig-162

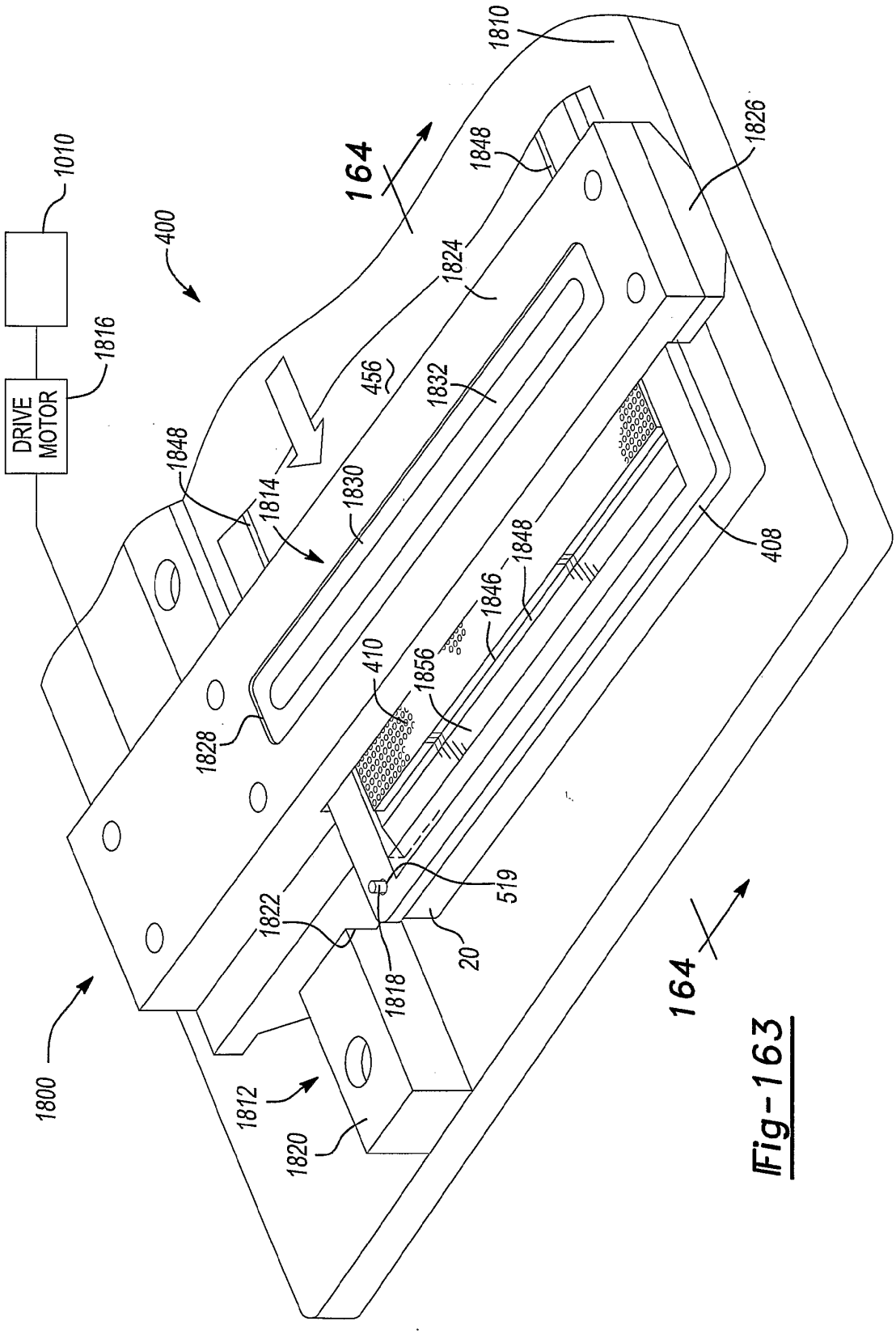

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Fig-163

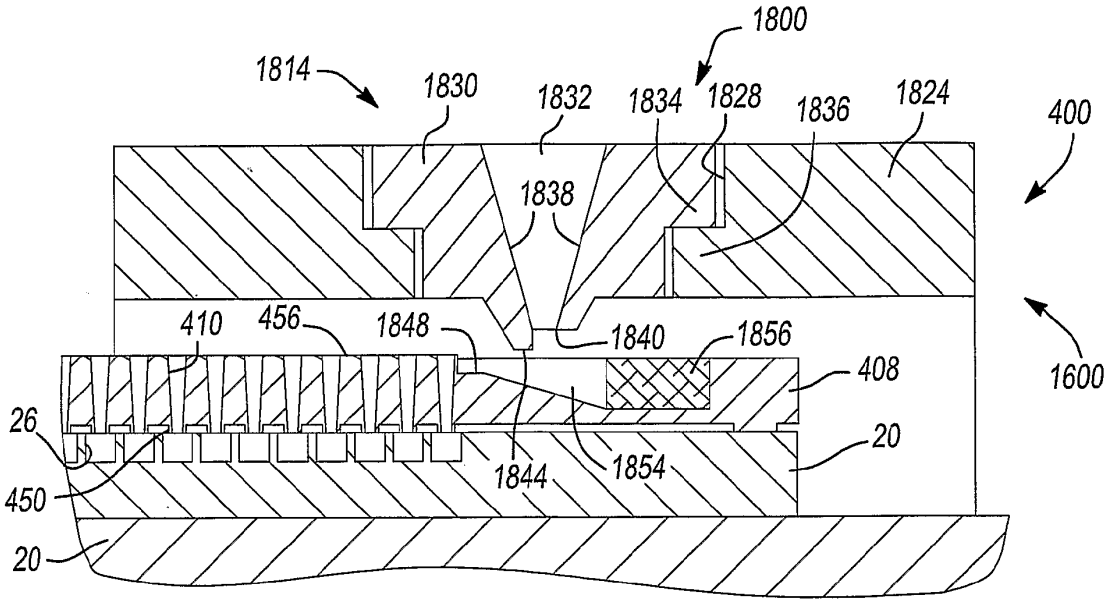


Fig-164

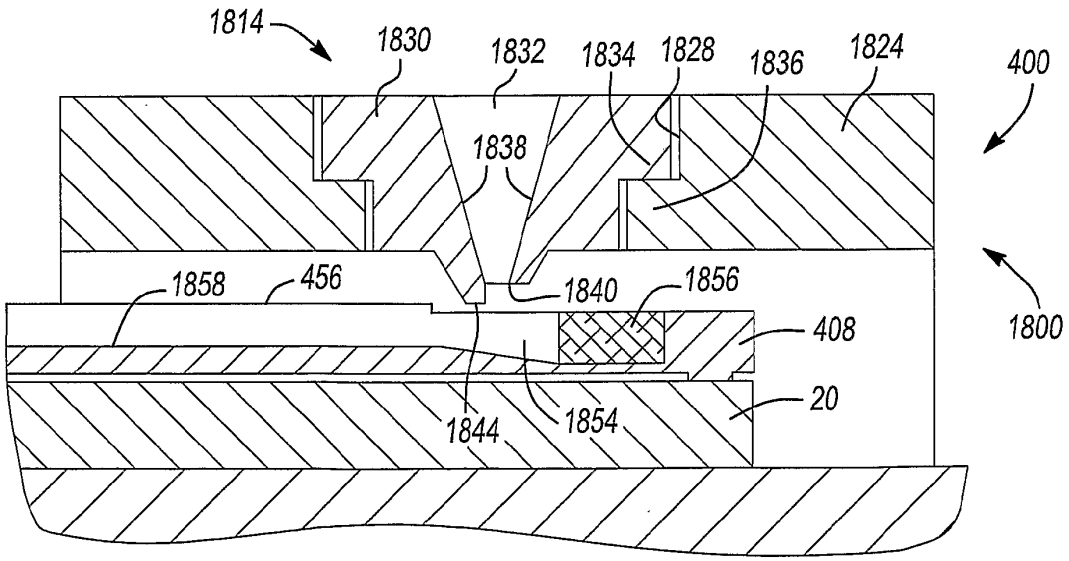


Fig-166

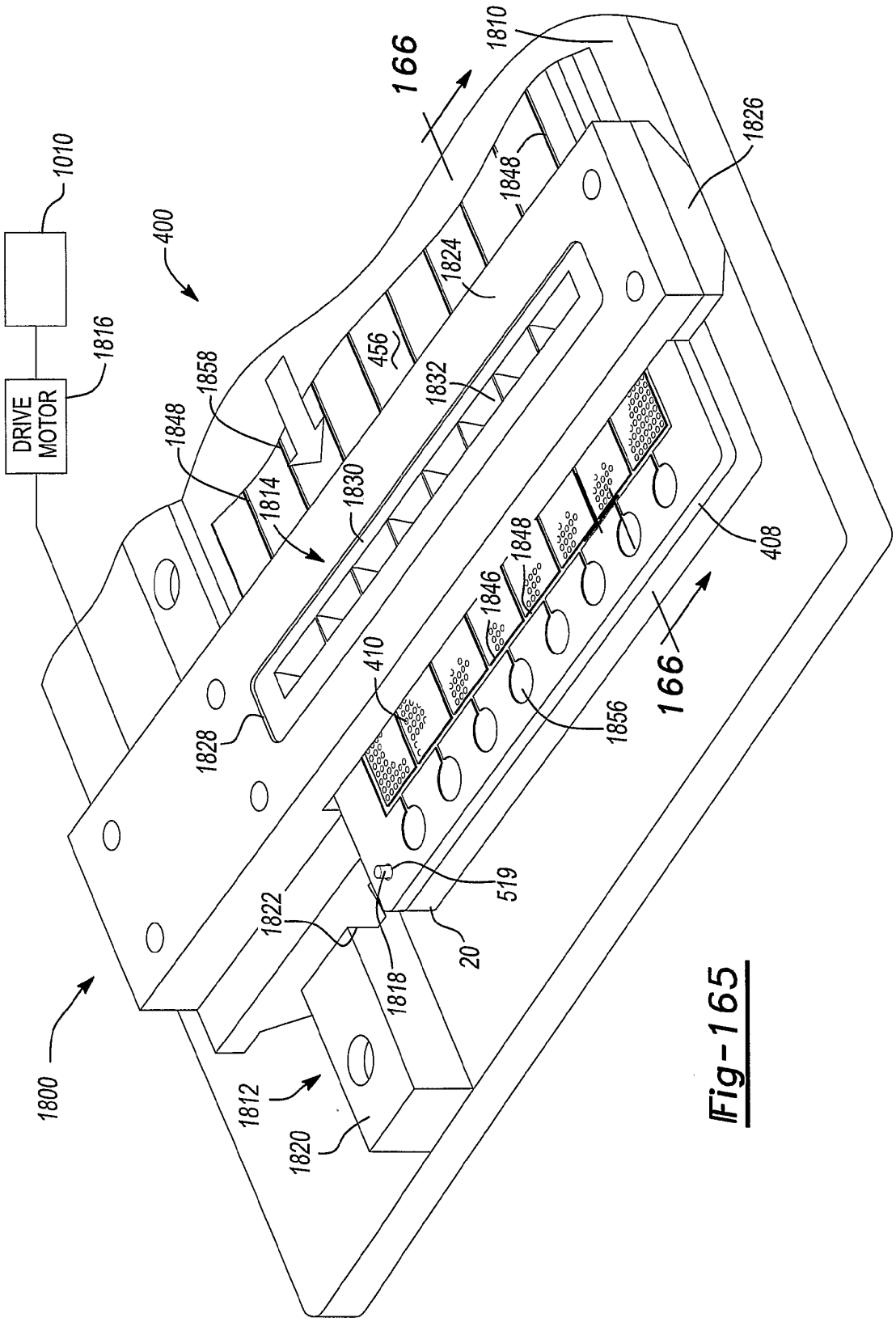

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Fig-165

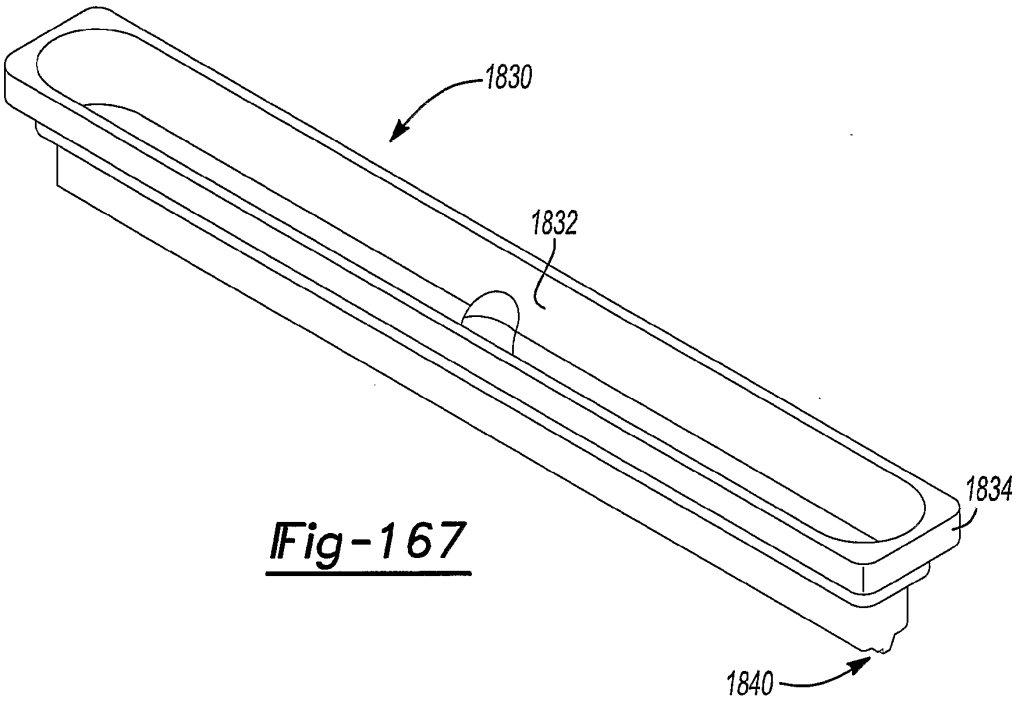


Fig-167

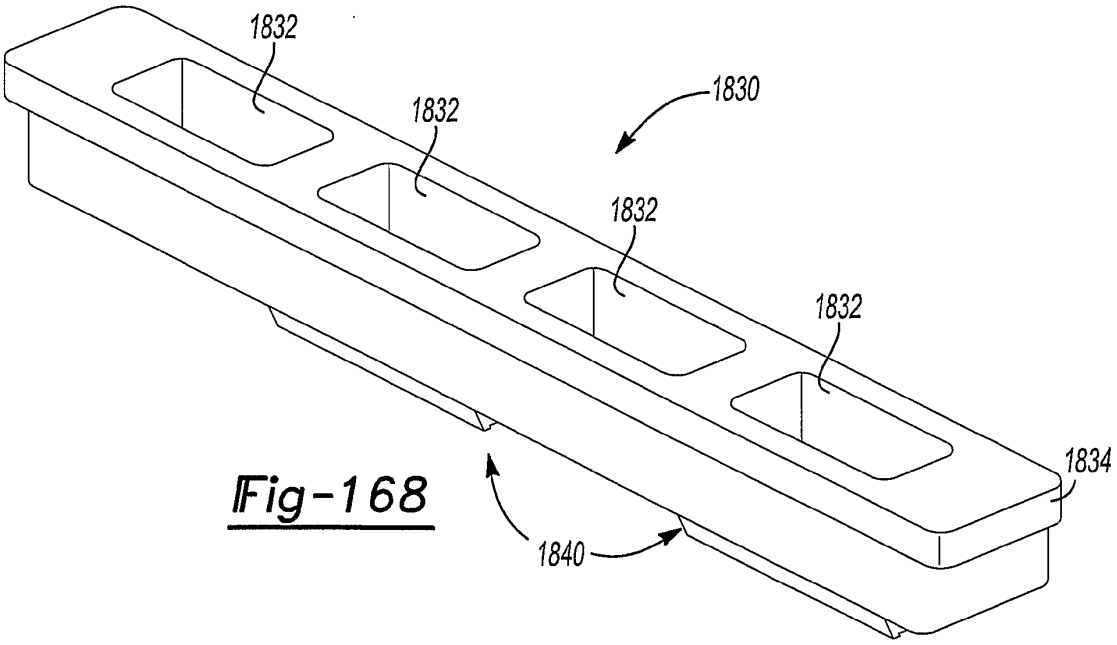


Fig-168

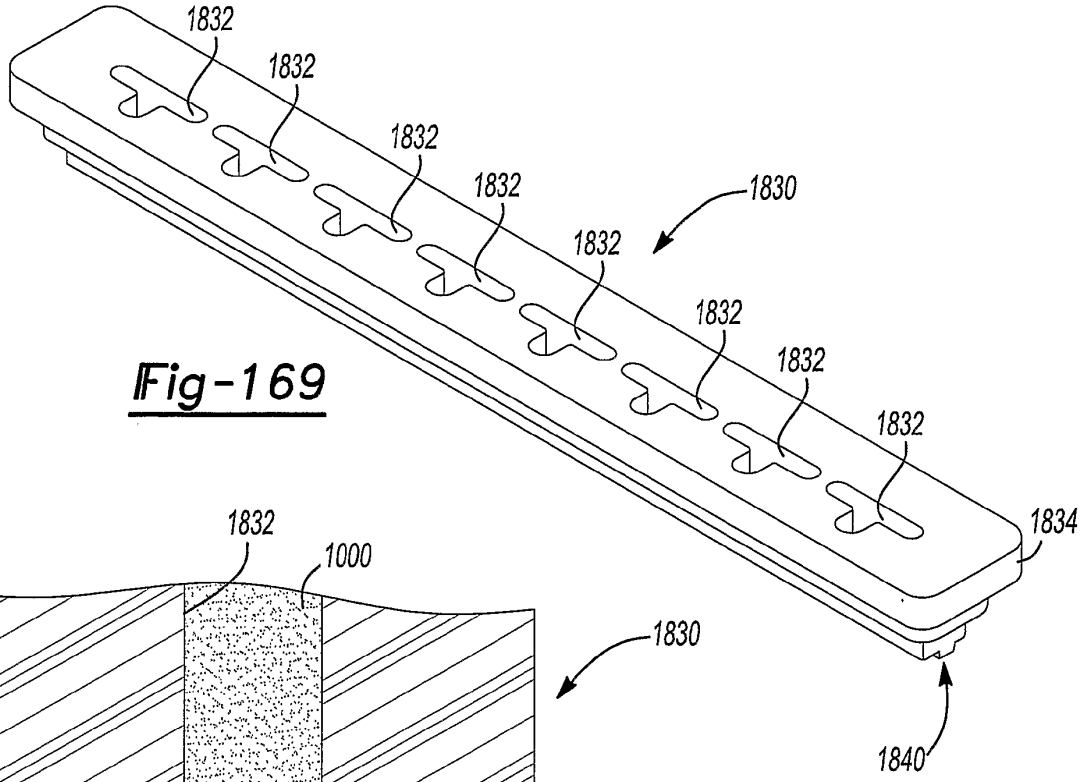


Fig-169

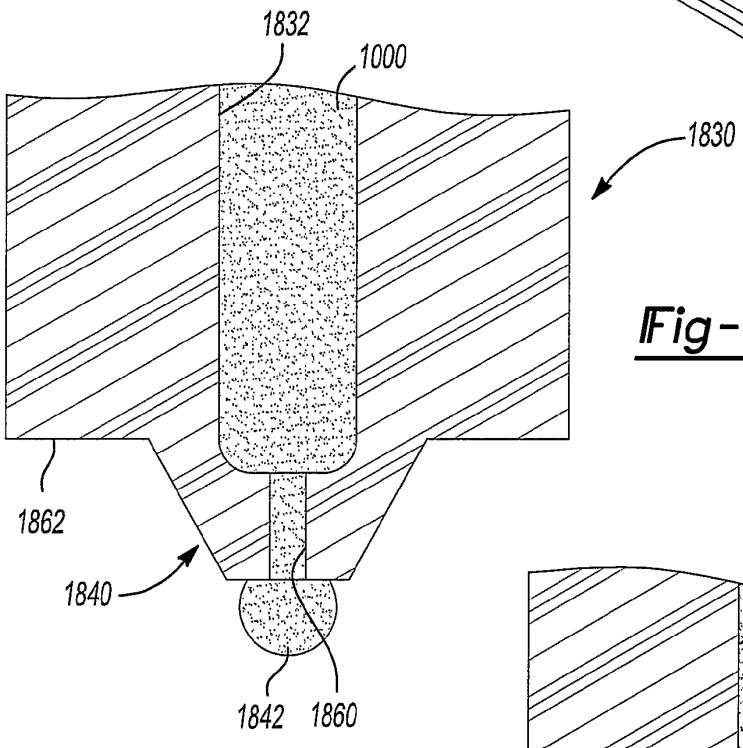


Fig-170

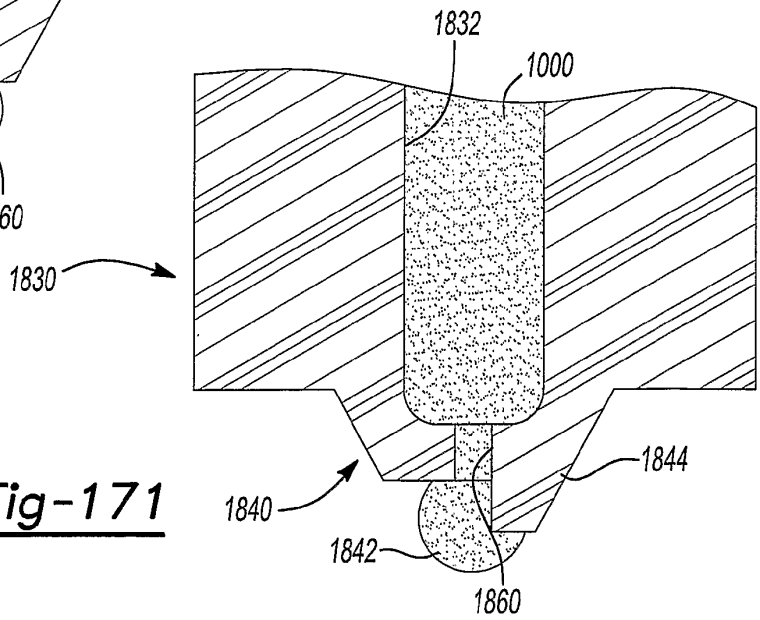


Fig-171

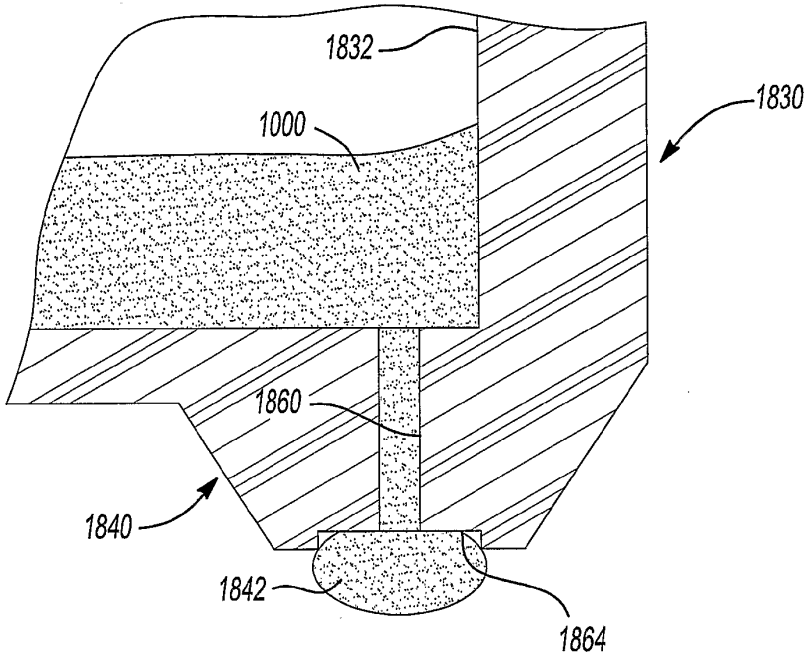


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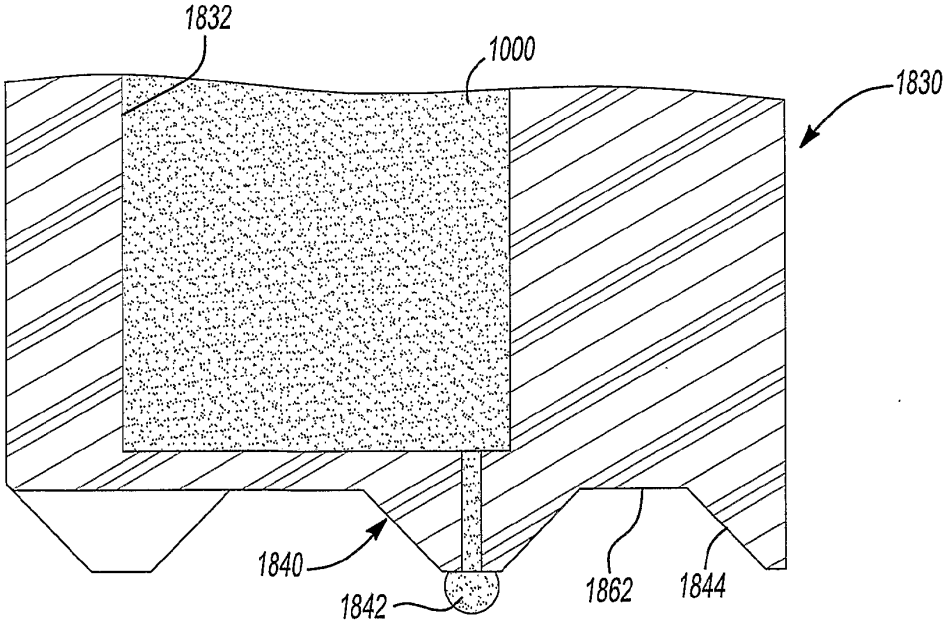


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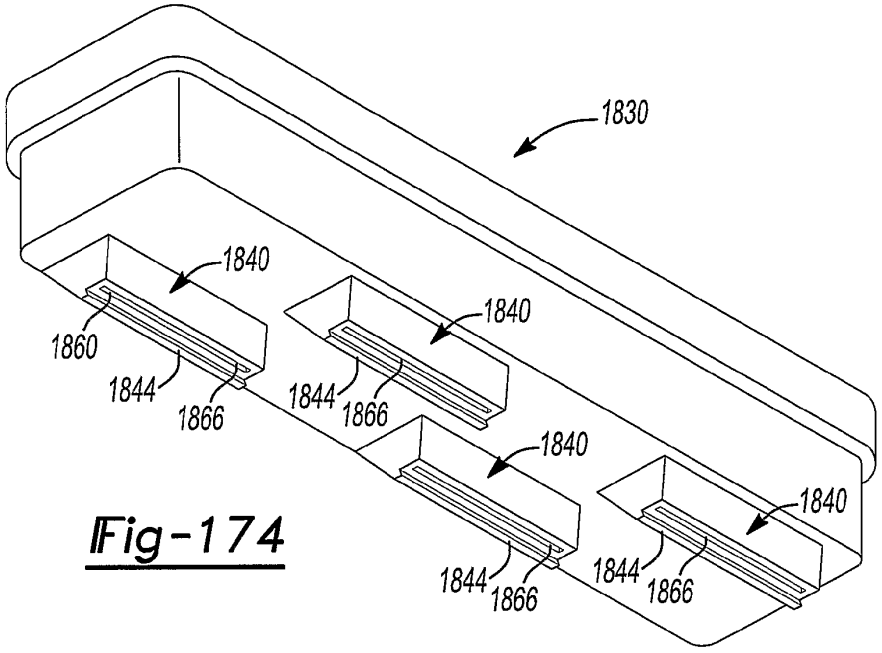


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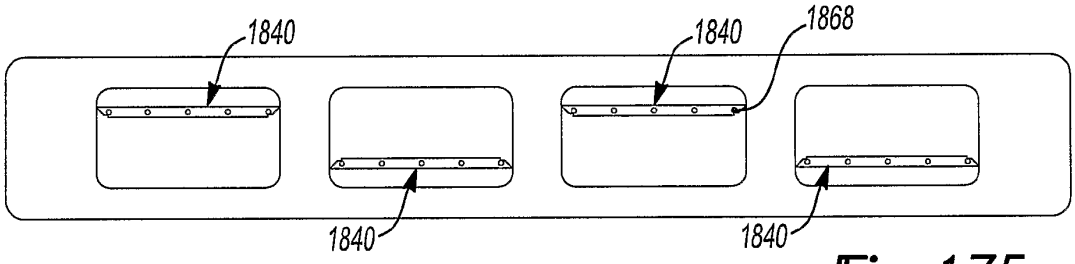


Fig-175

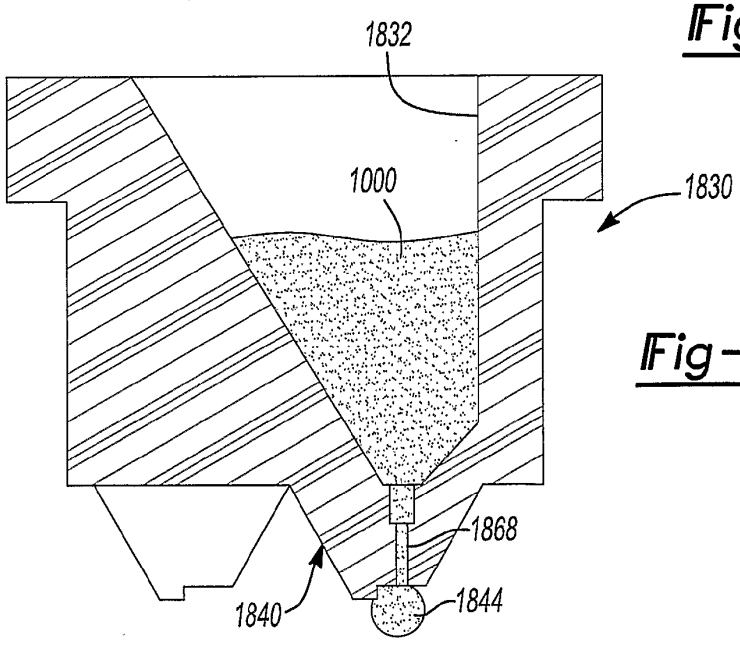


Fig-176

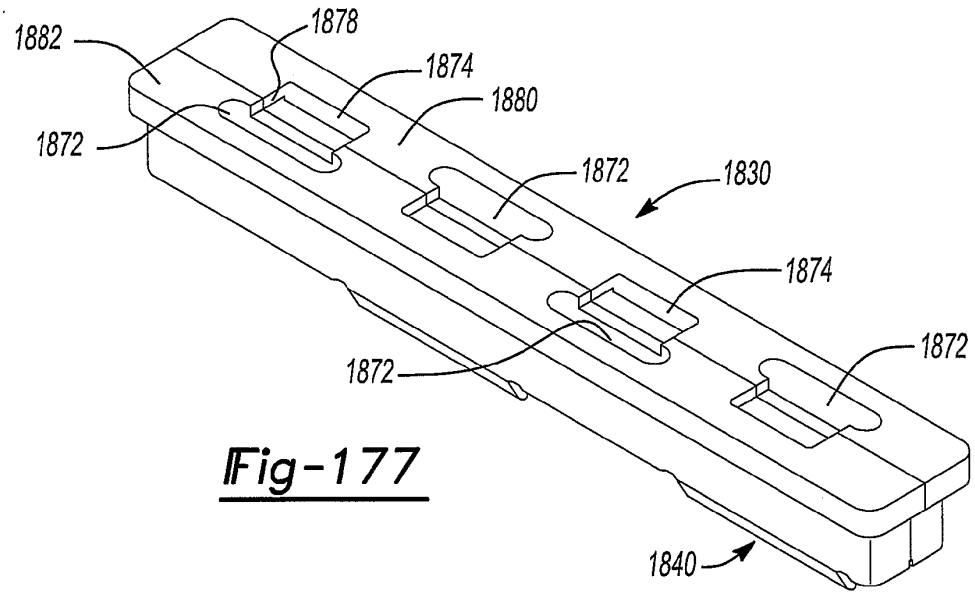


Fig-177

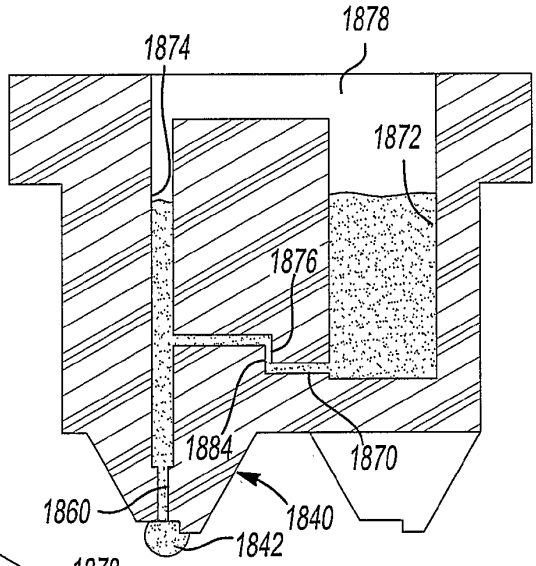


Fig-178

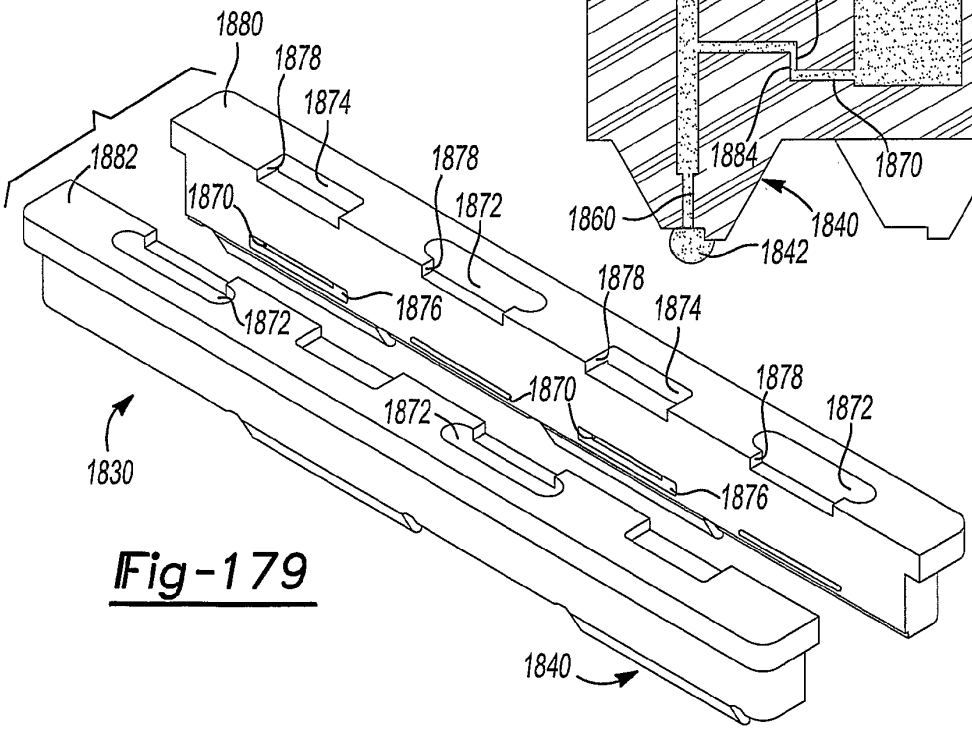


Fig-179

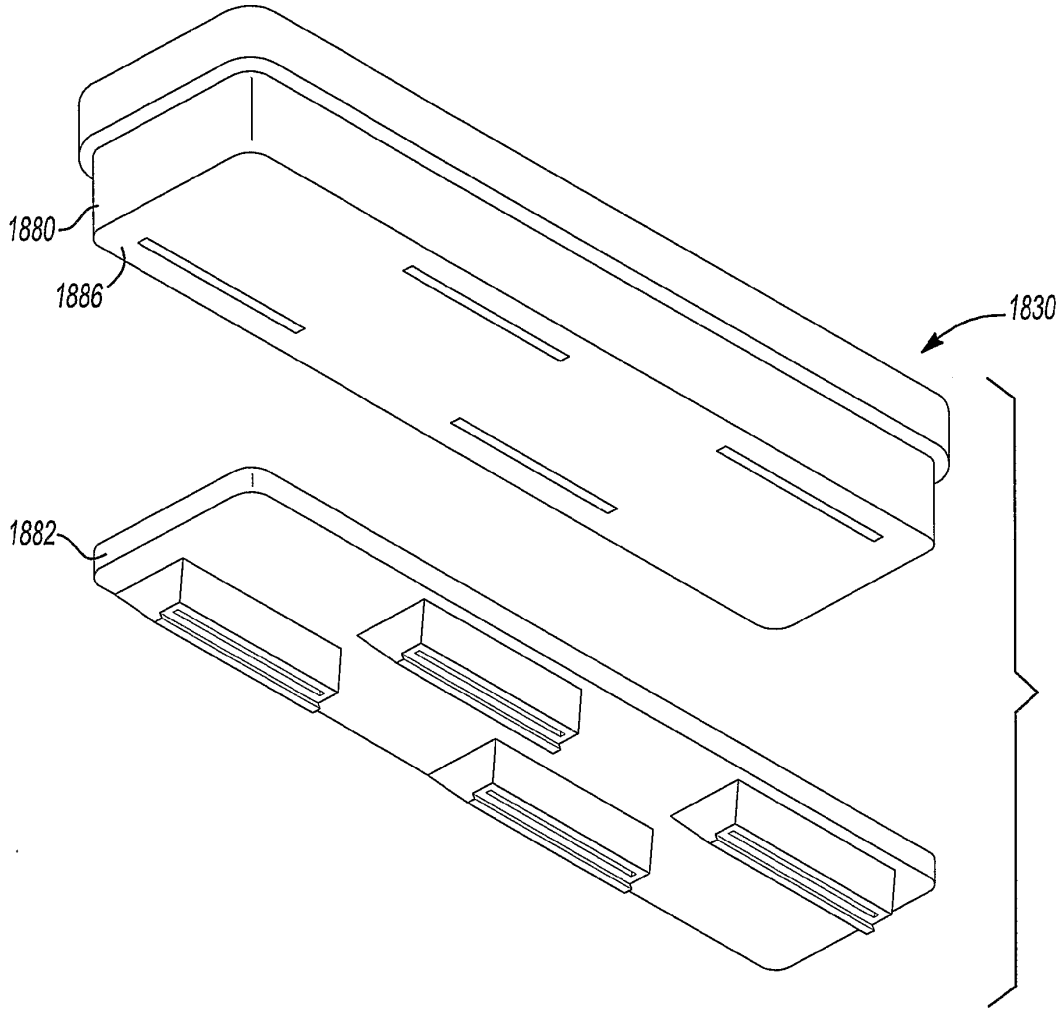


Fig-180

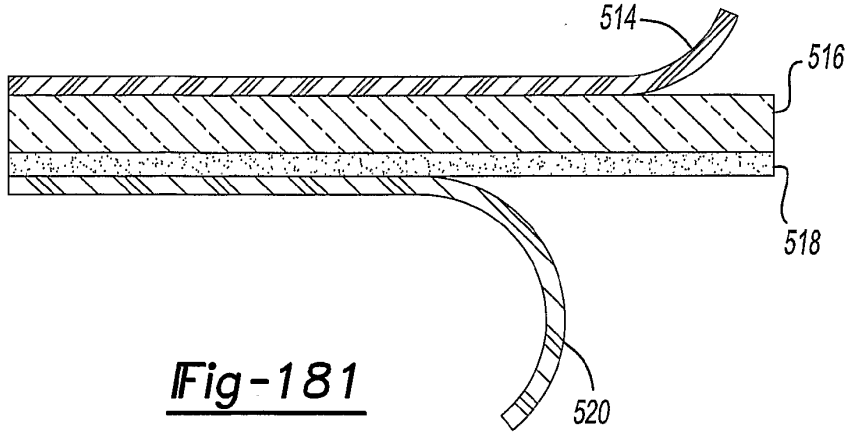


Fig-181

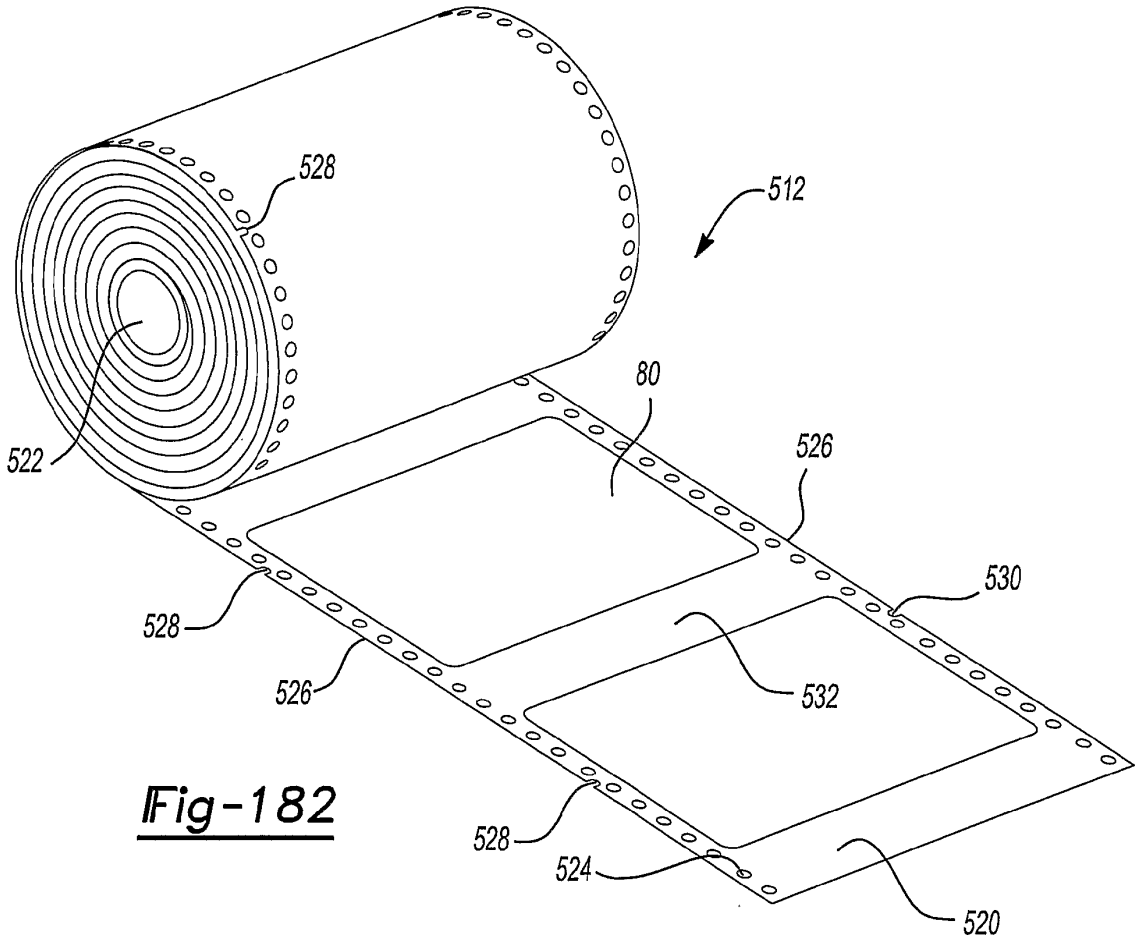


Fig-182

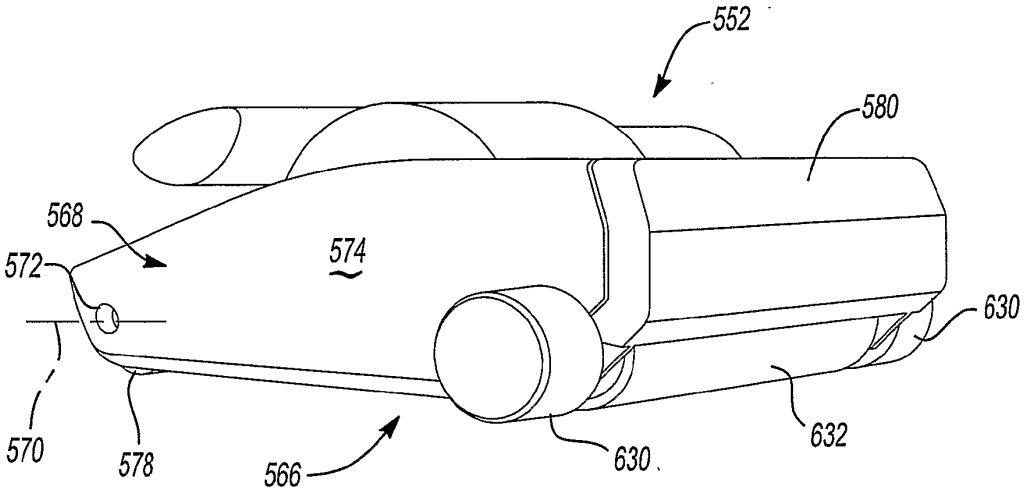


Fig-183

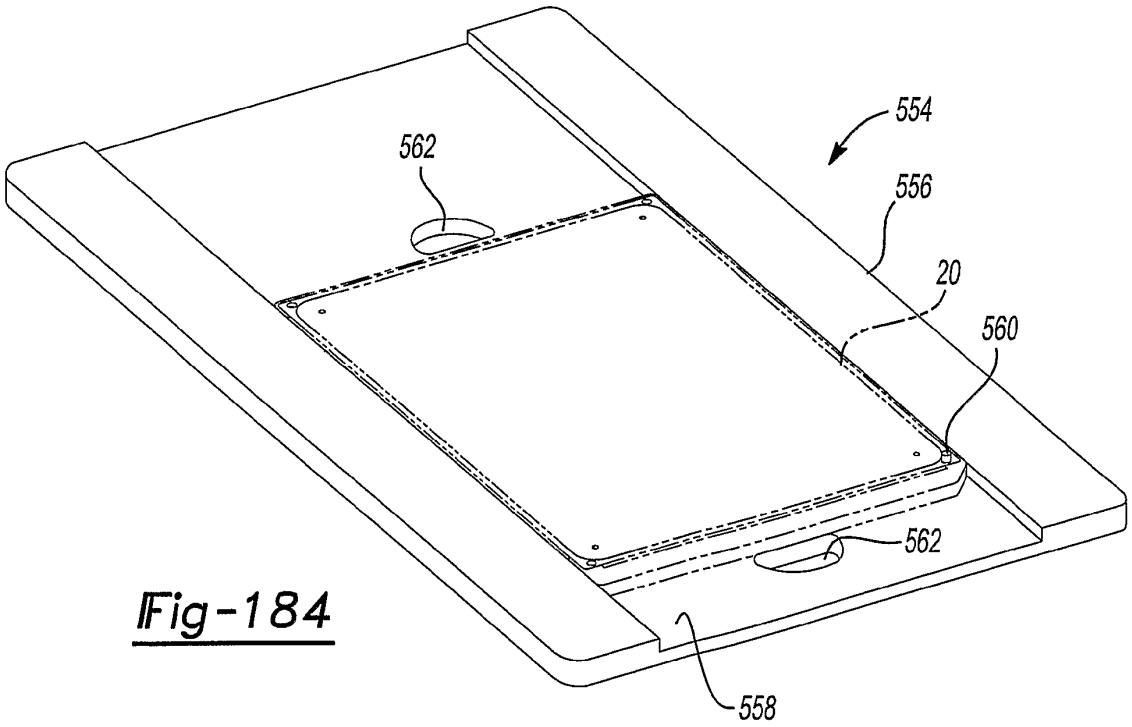


Fig-184

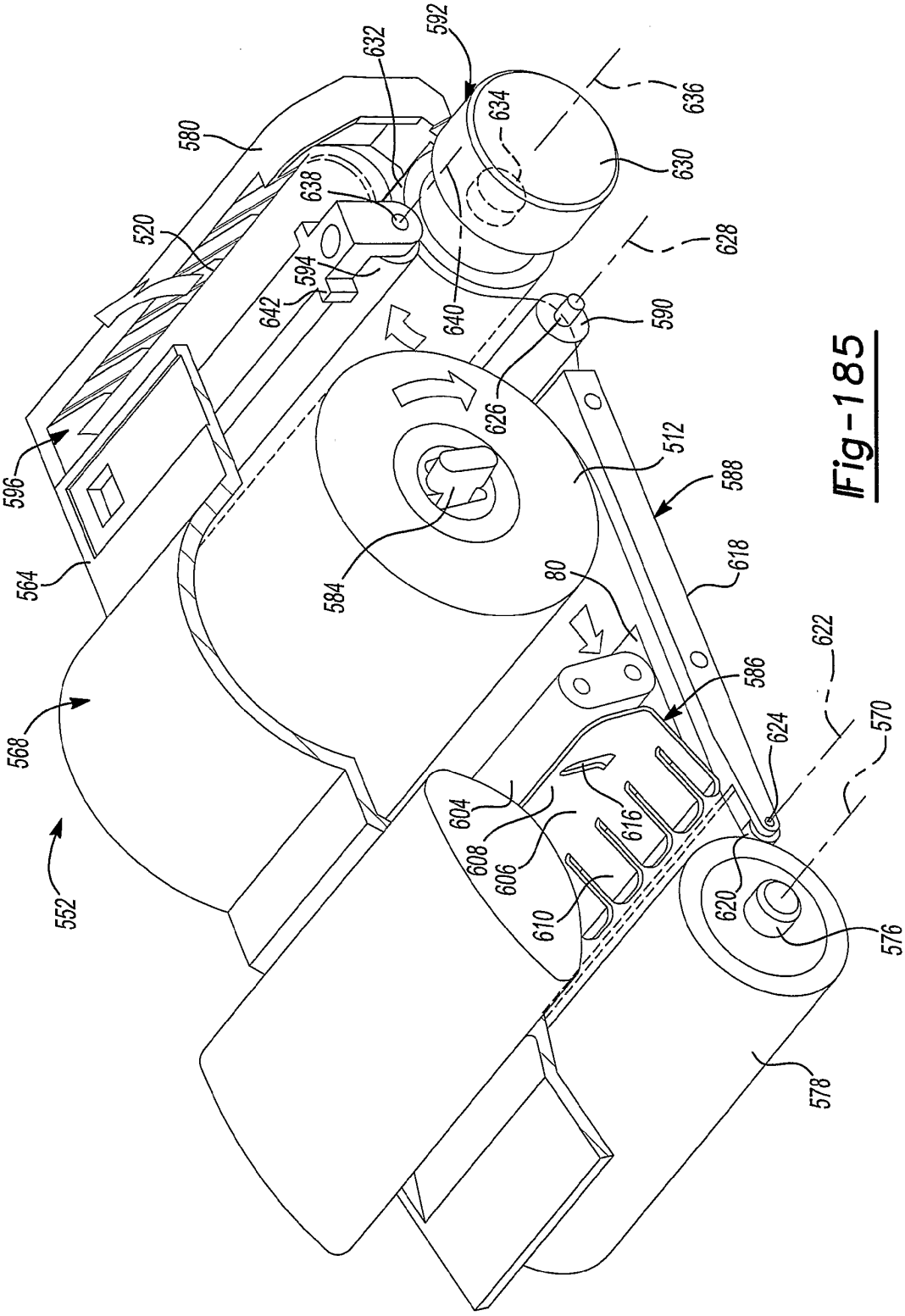

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Fig-185

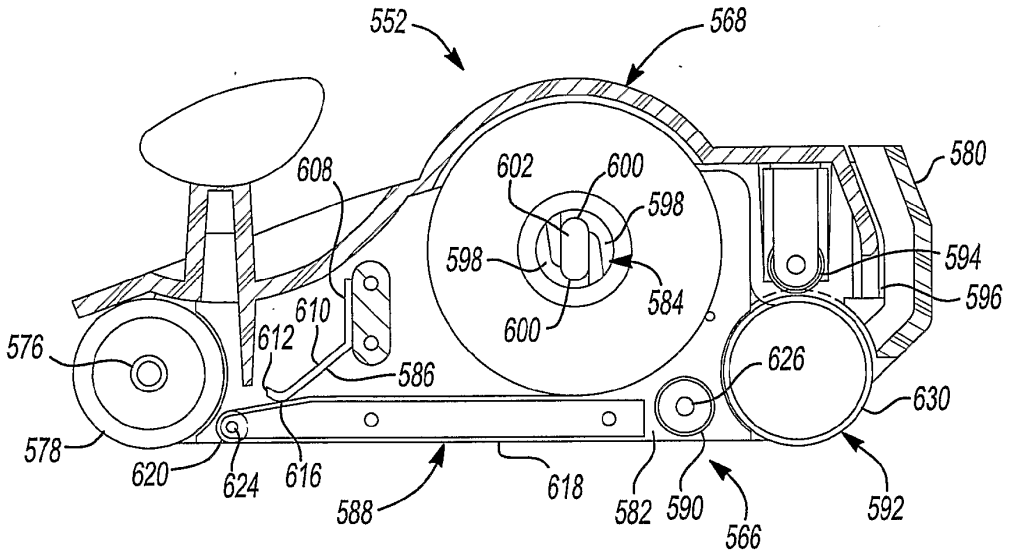


Fig-186

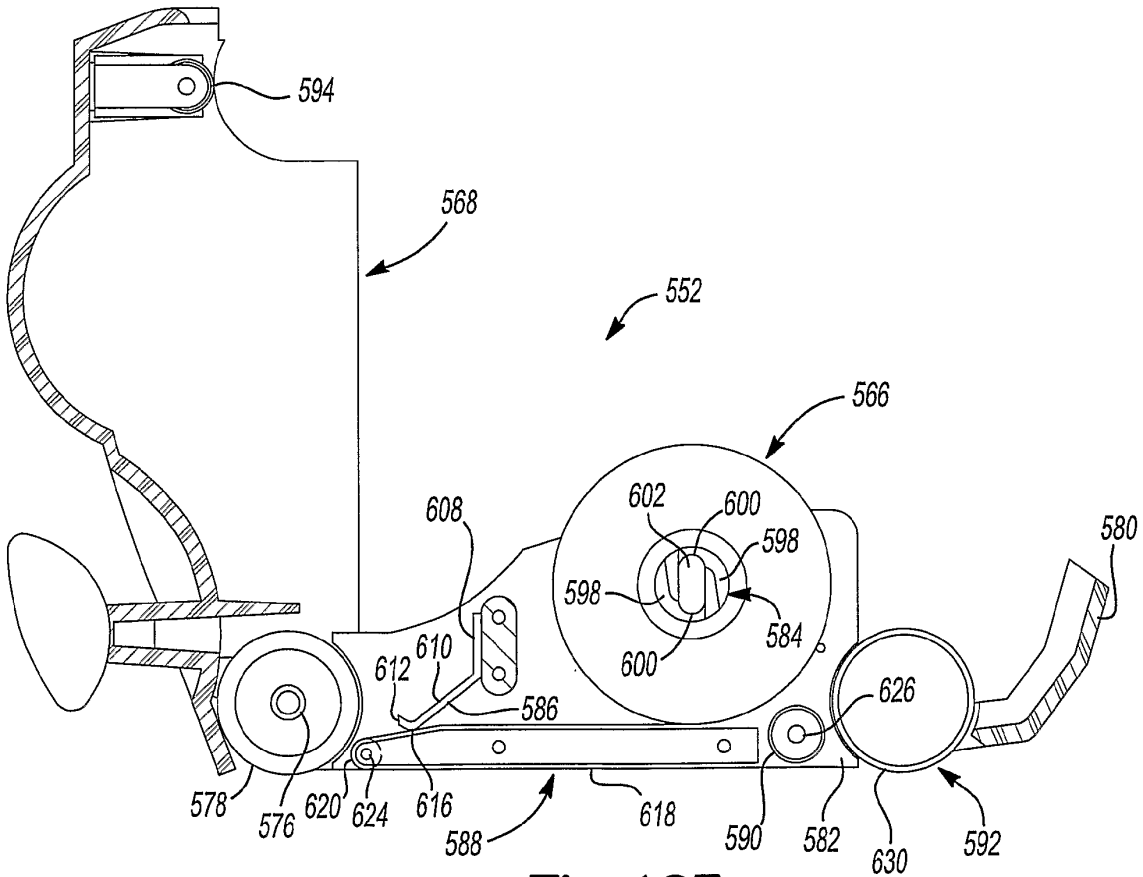


Fig-187

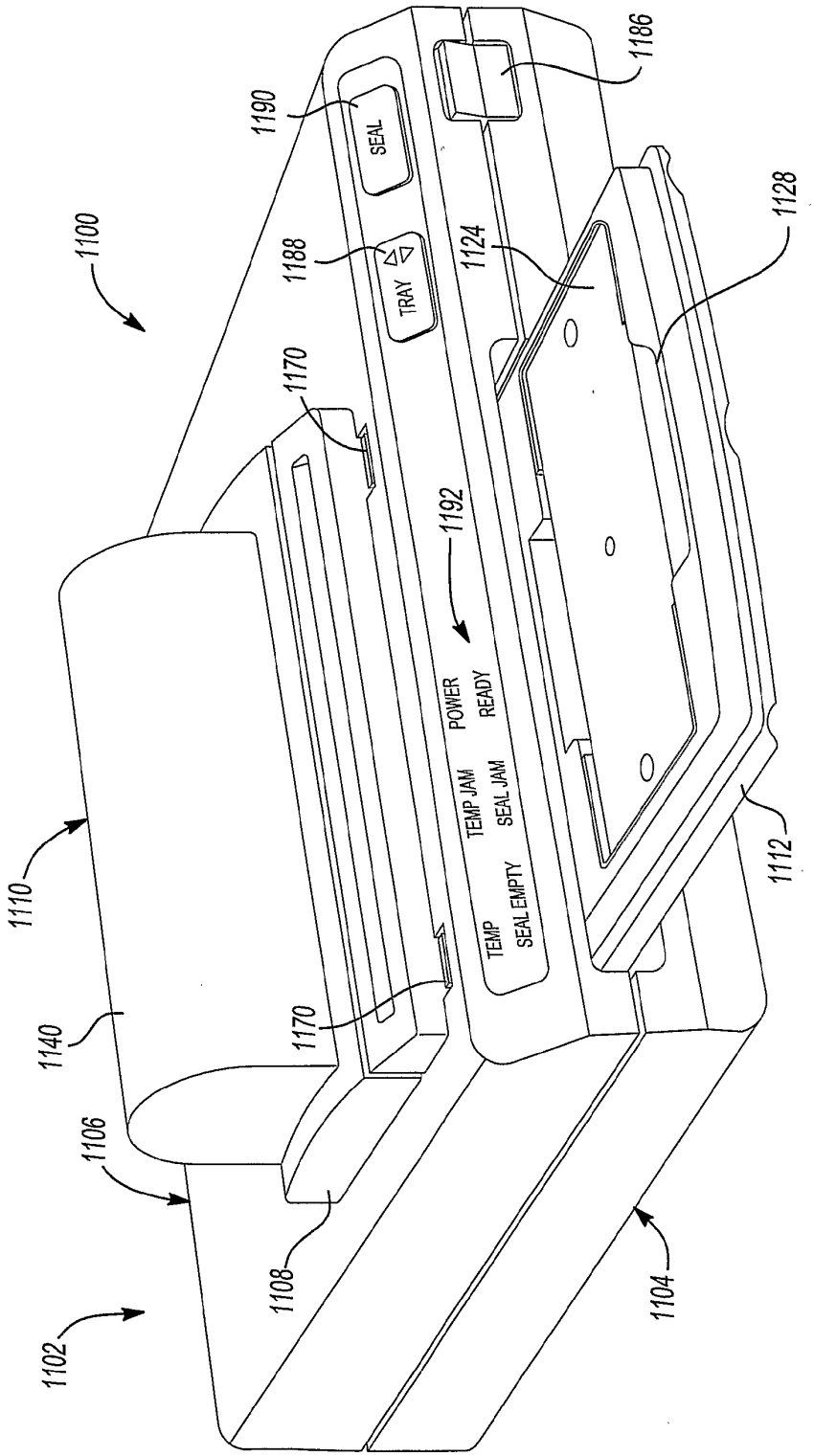

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Fig-188

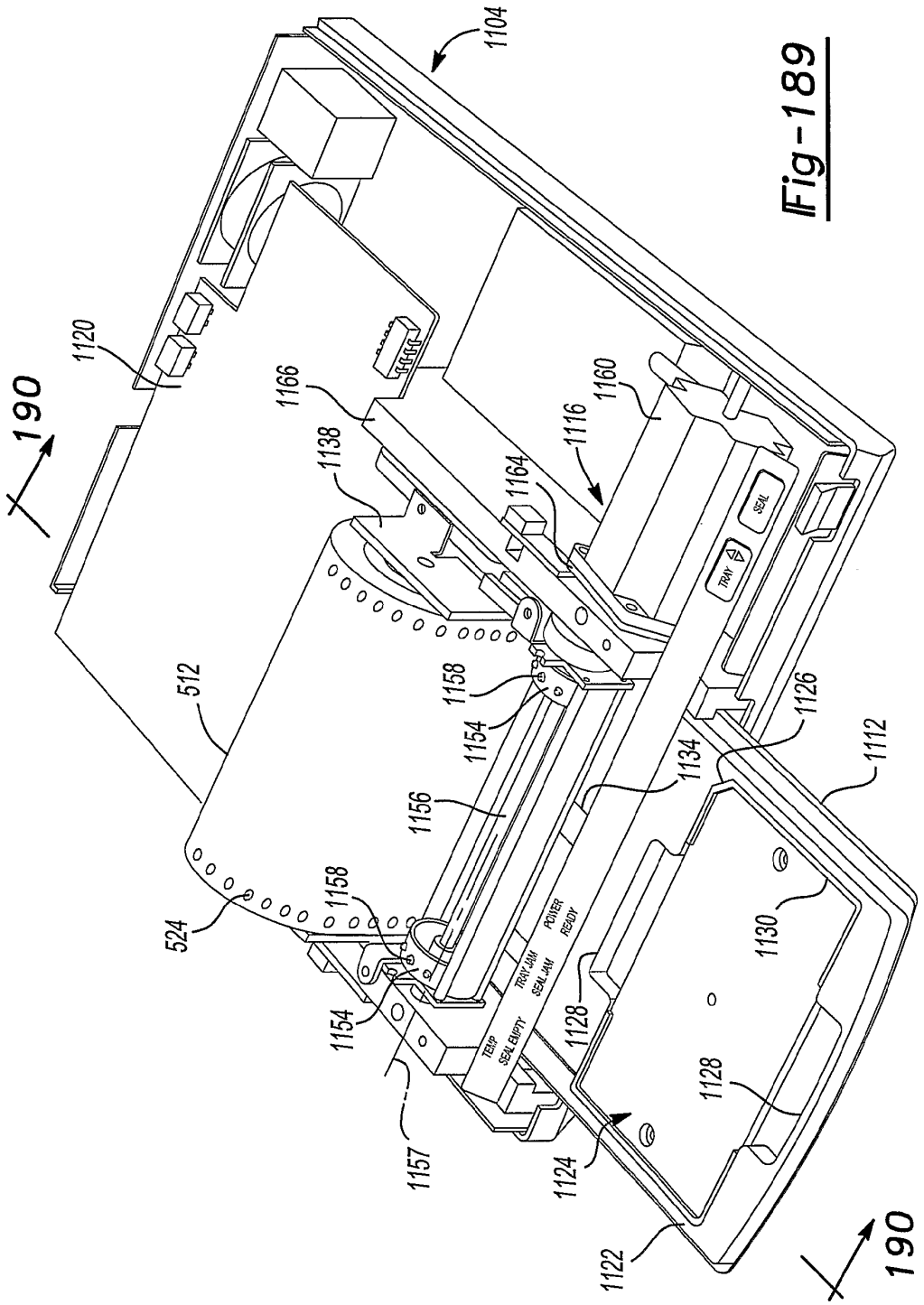

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Fig-189

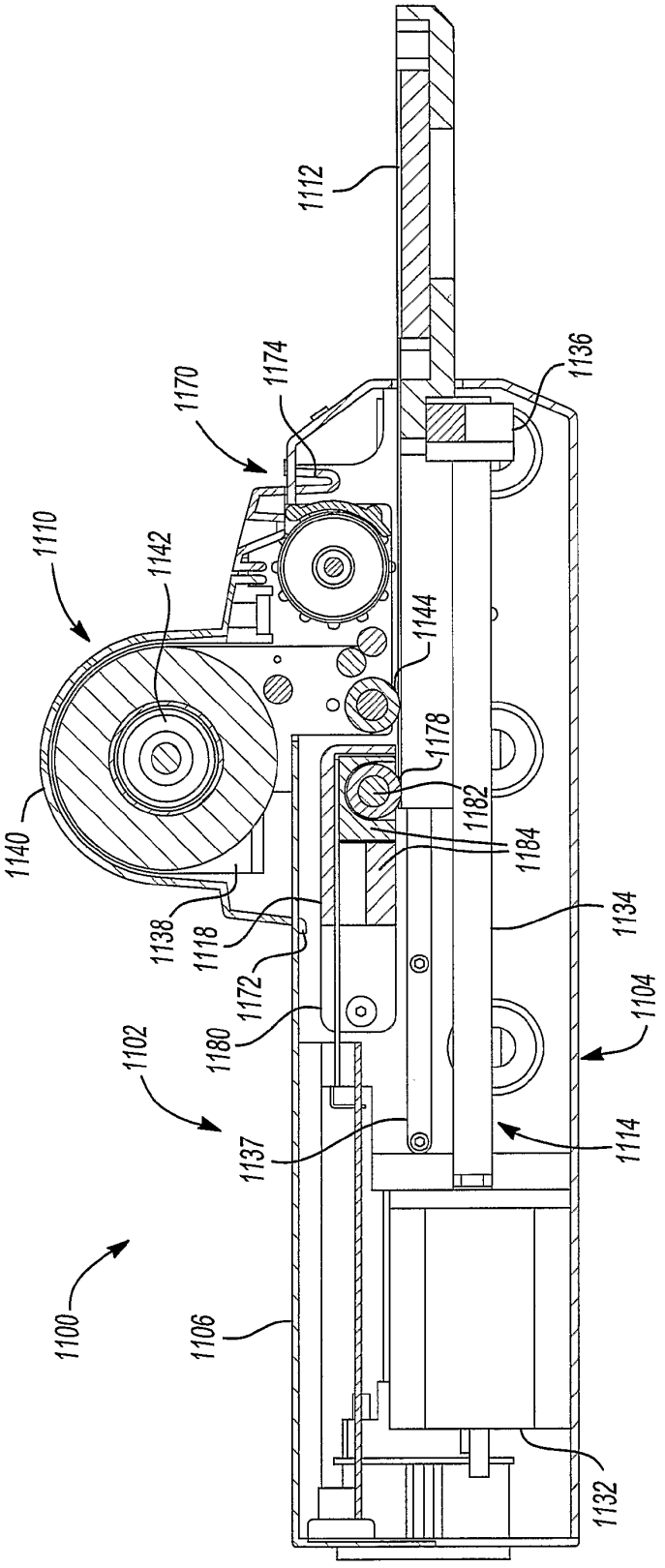

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Fig-190

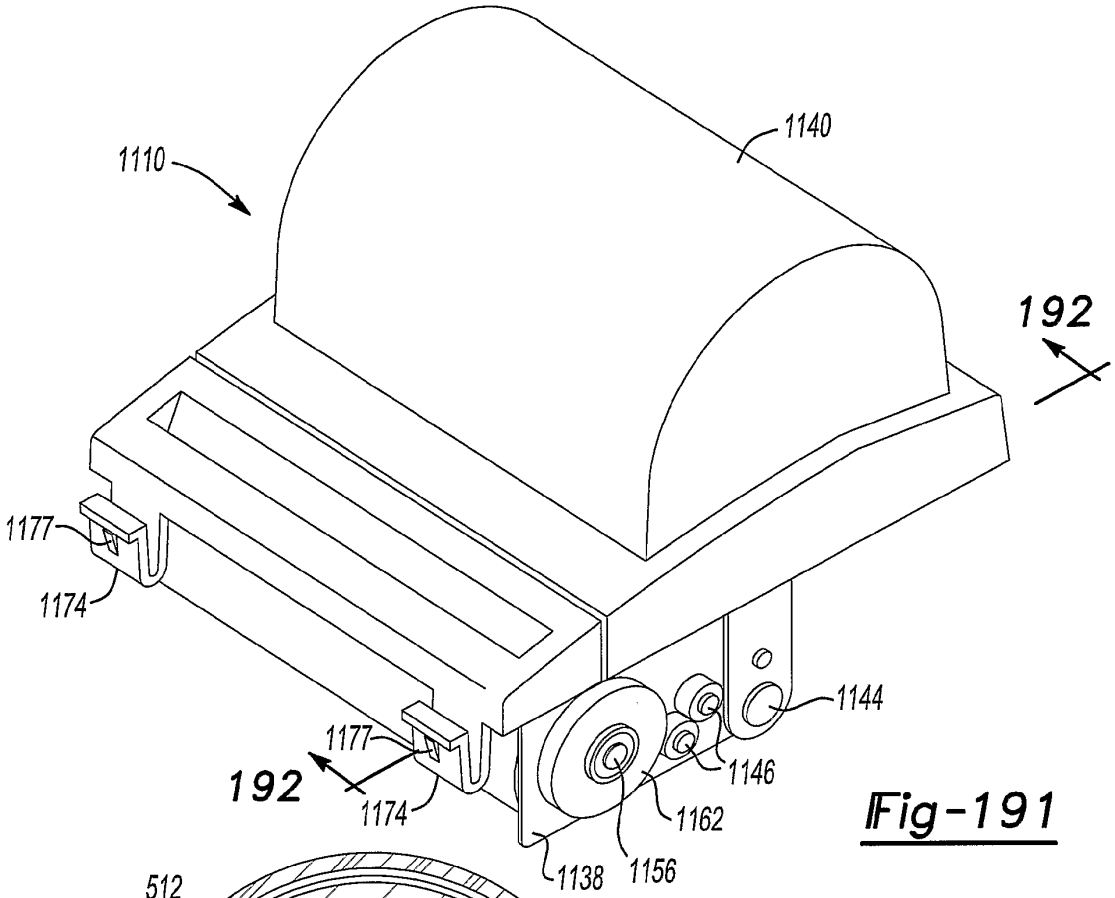


Fig-191

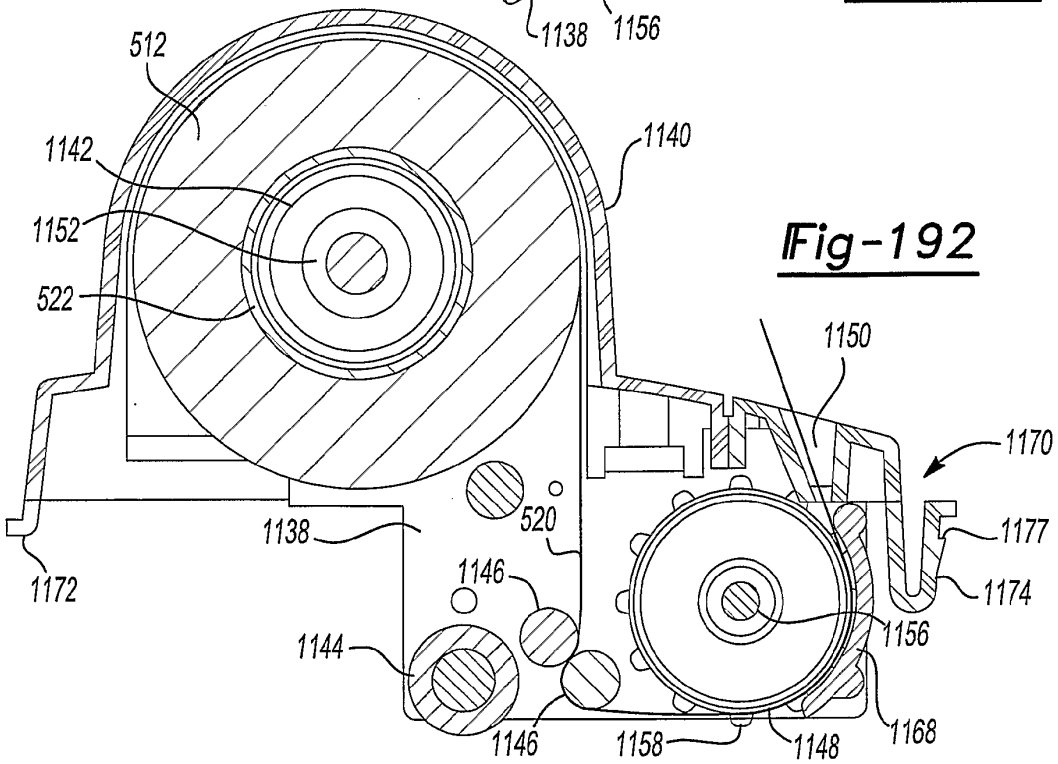


Fig-192

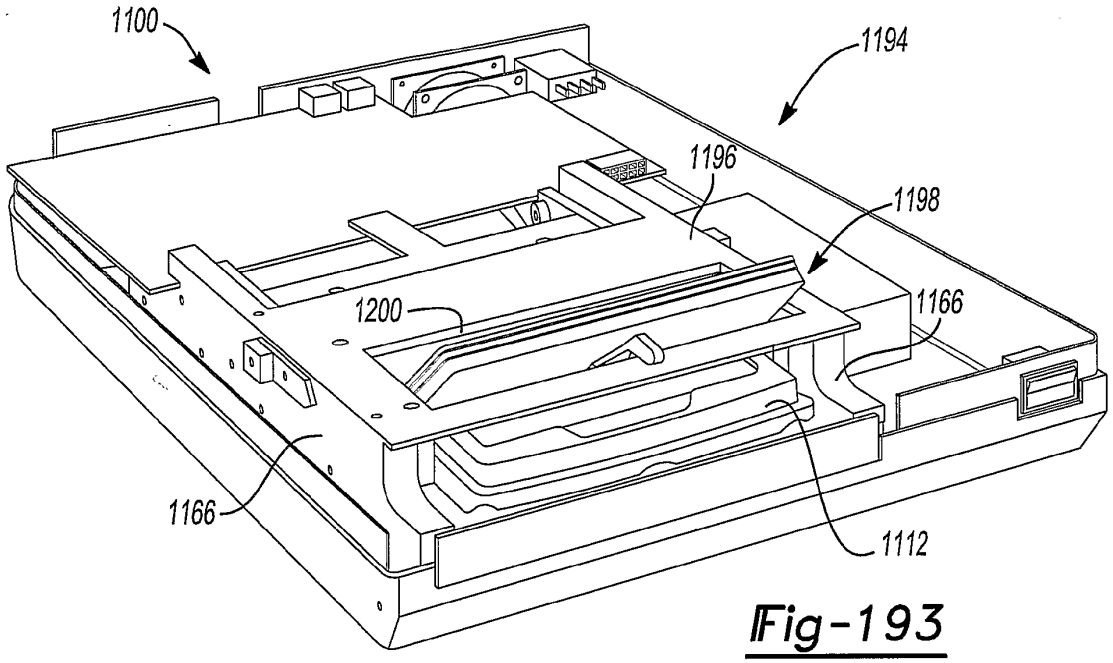


Fig-193

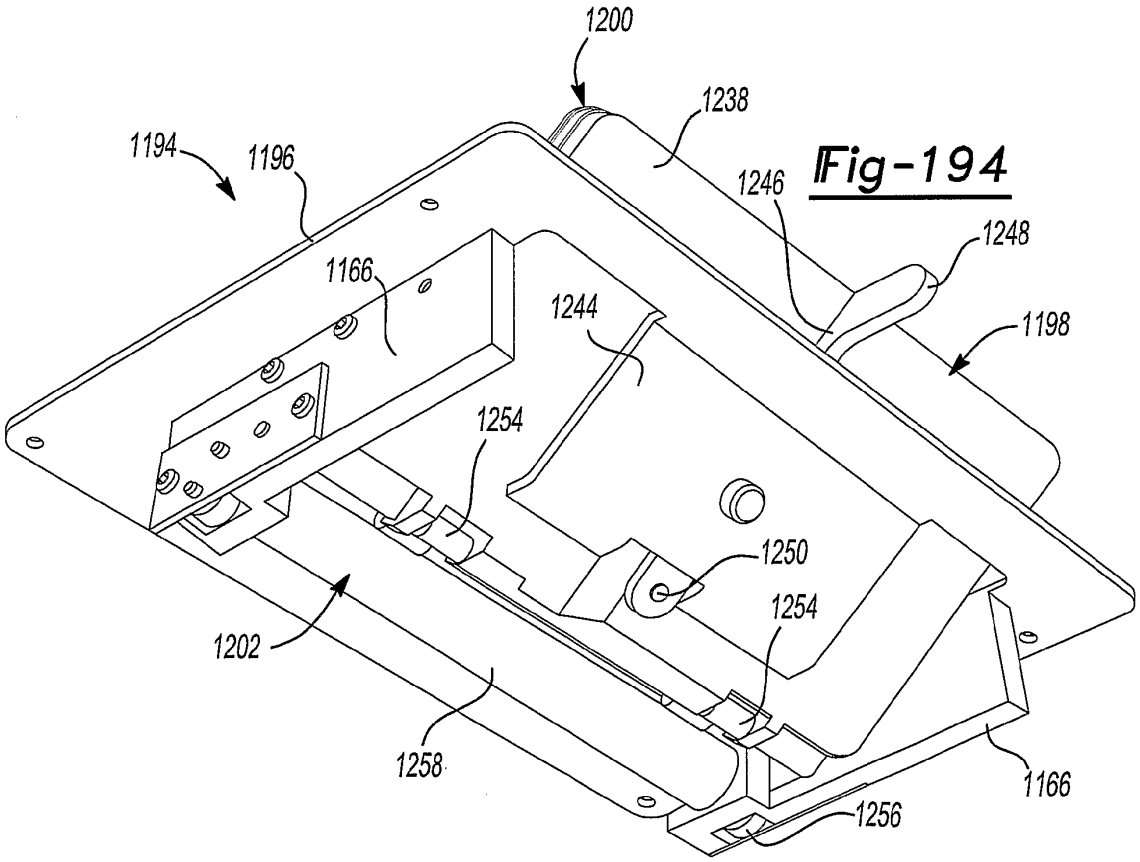


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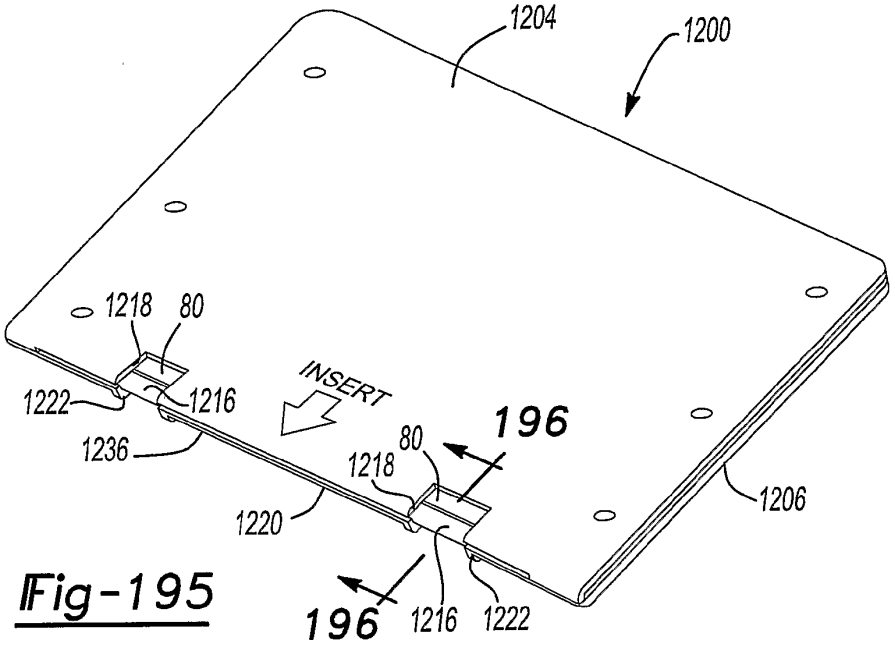


Fig-195

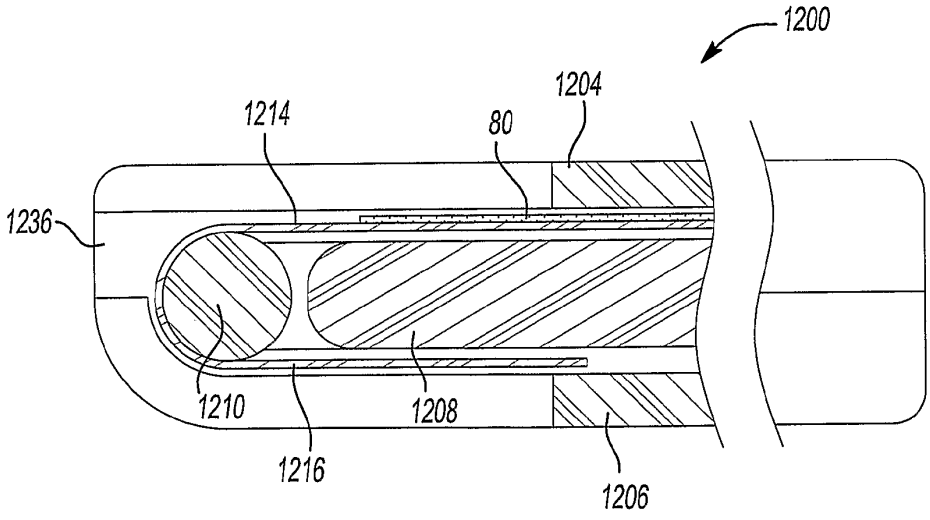


Fig-196

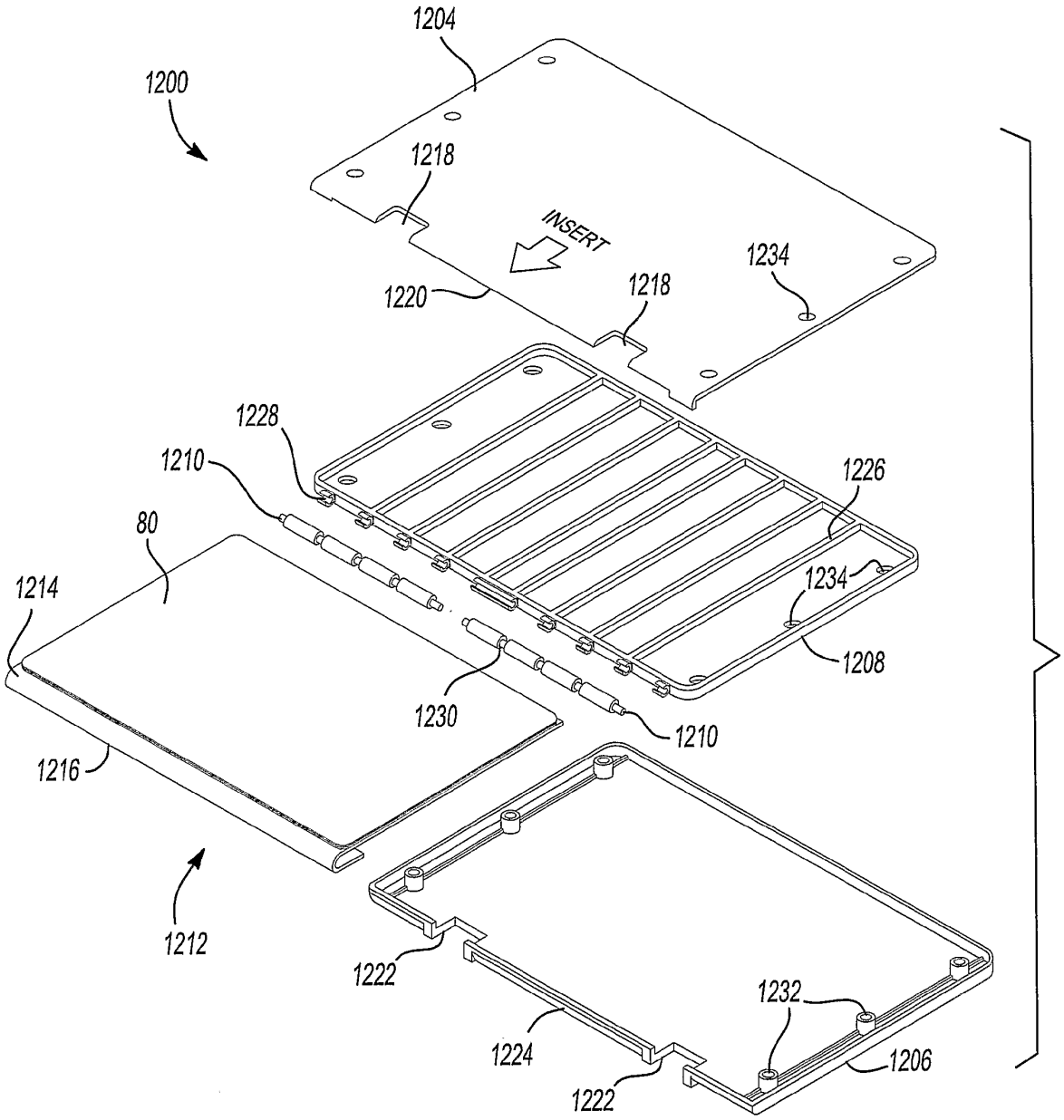

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Fig-197

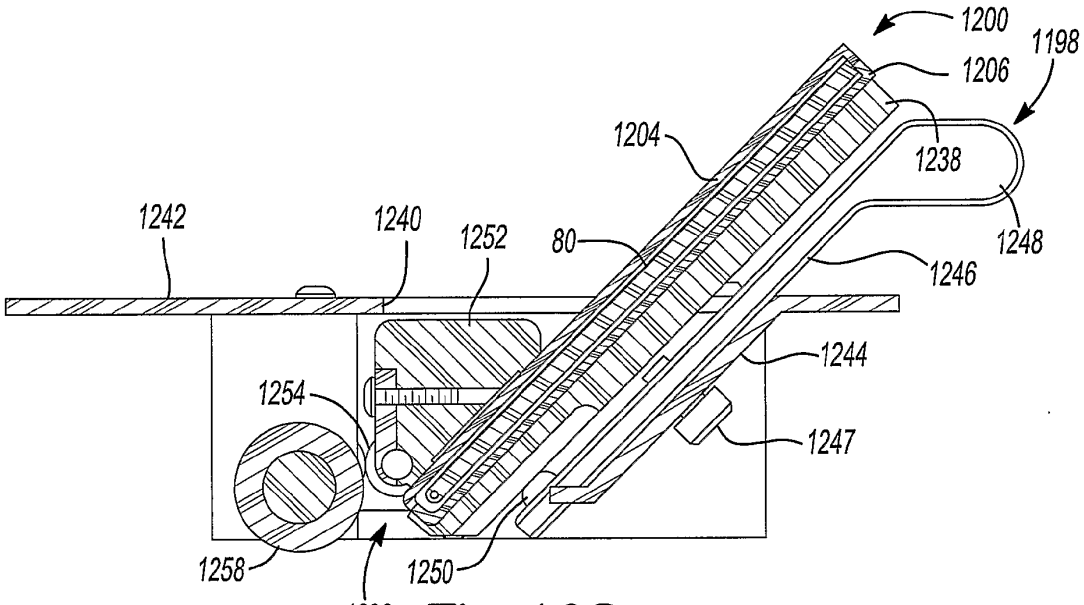


Fig-198

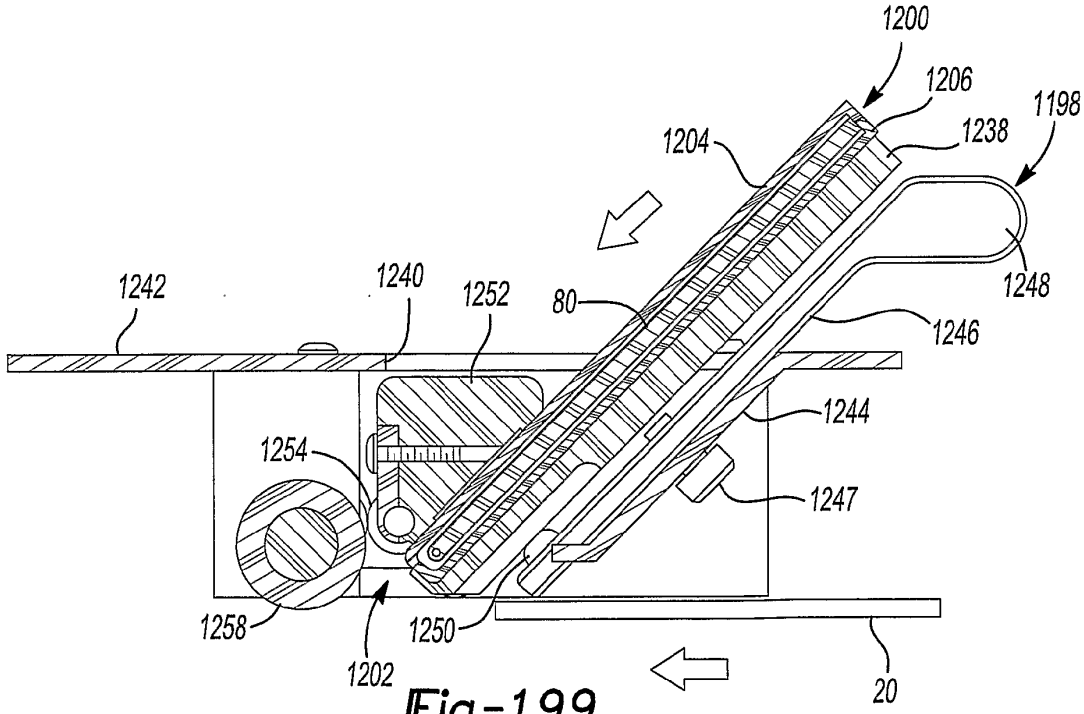


Fig-199

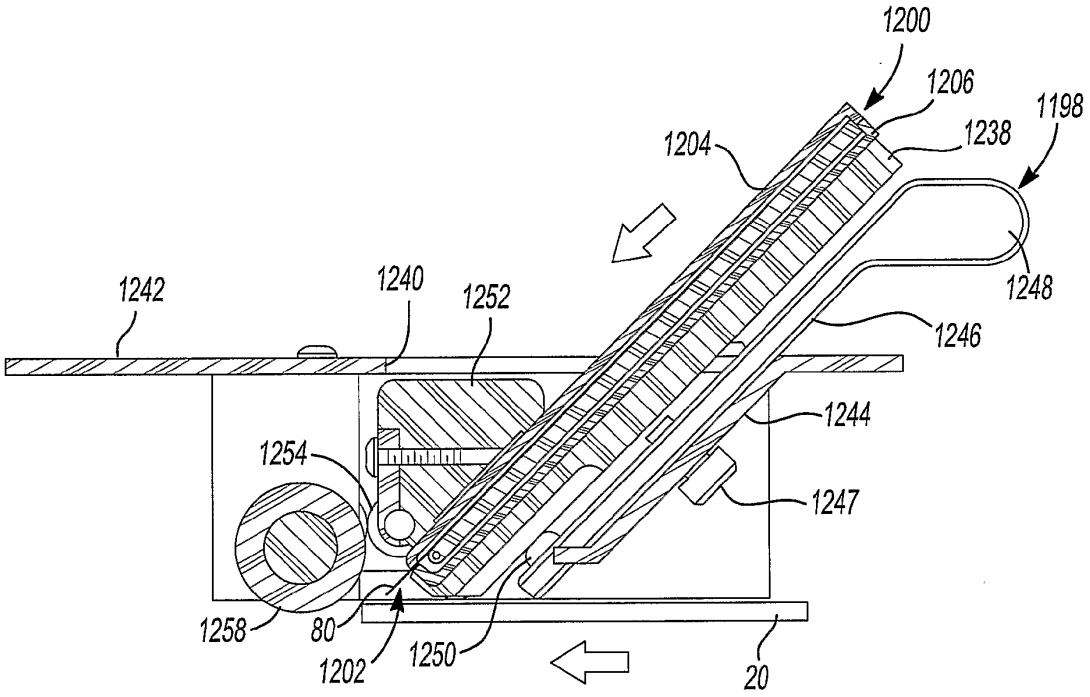


Fig-200

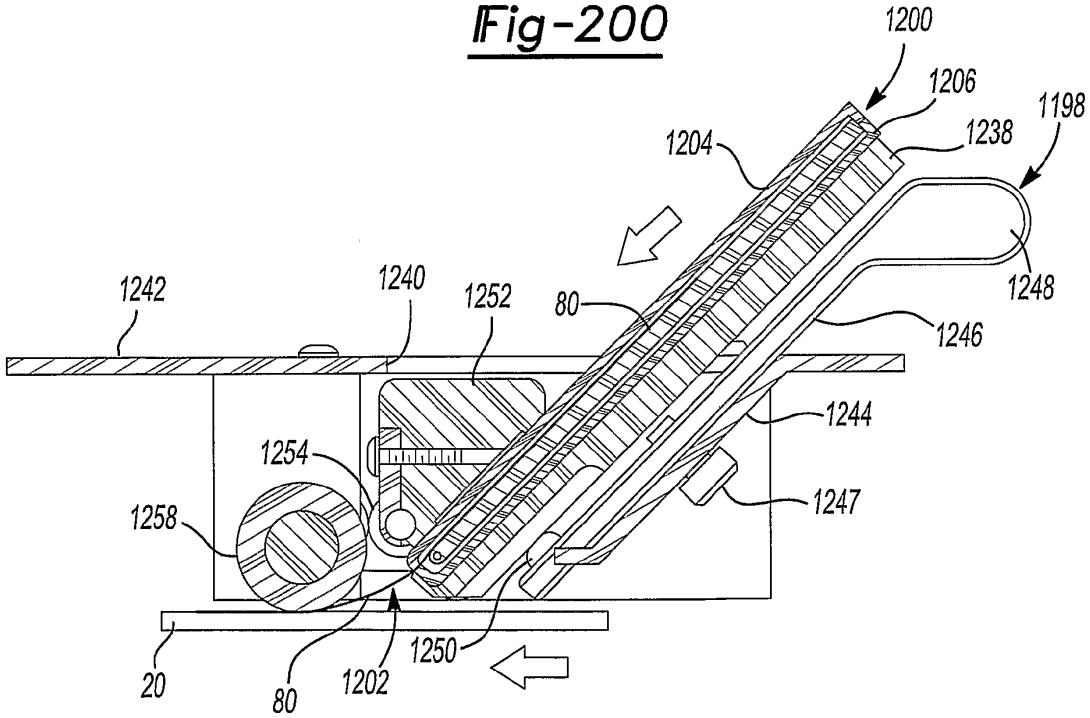


Fig-201

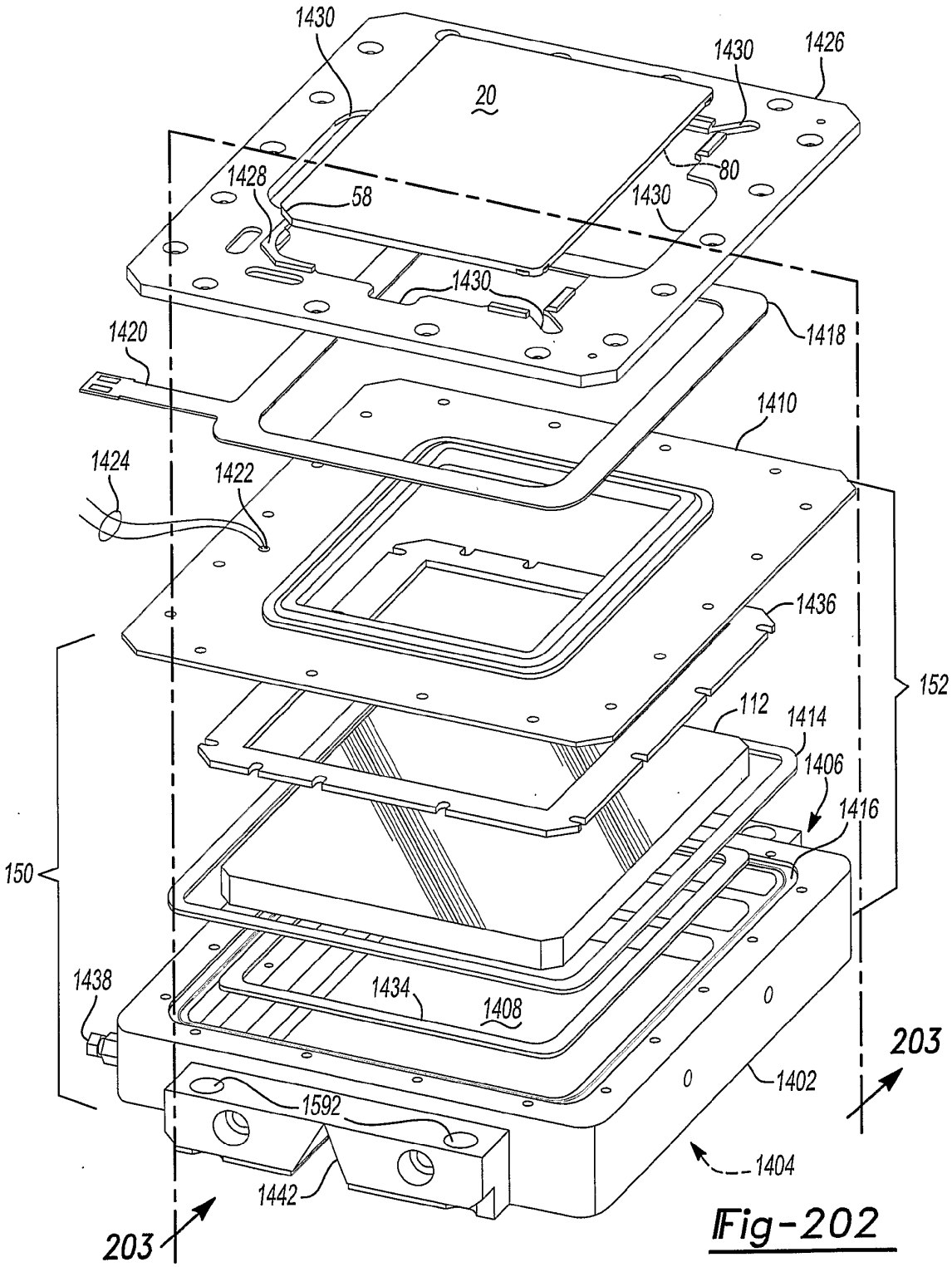

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Fig-202

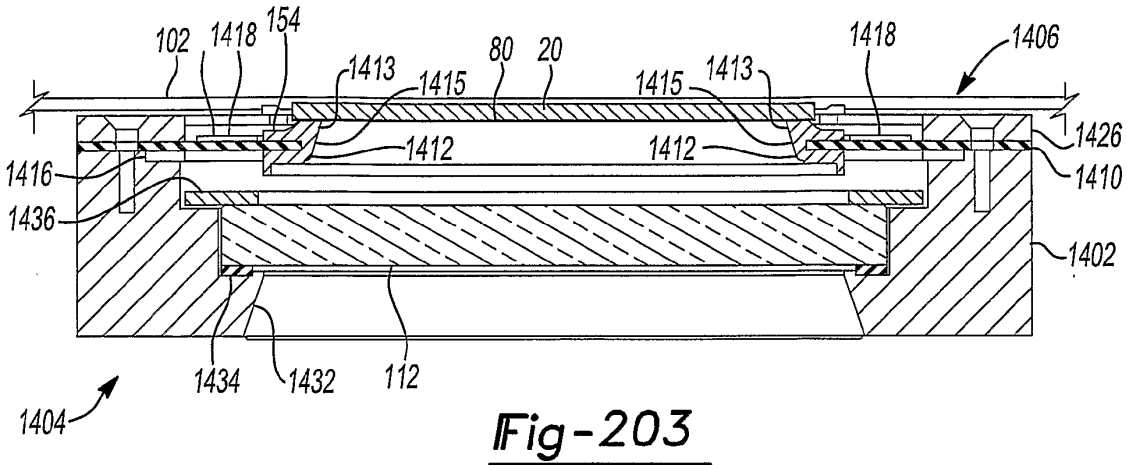


Fig-203

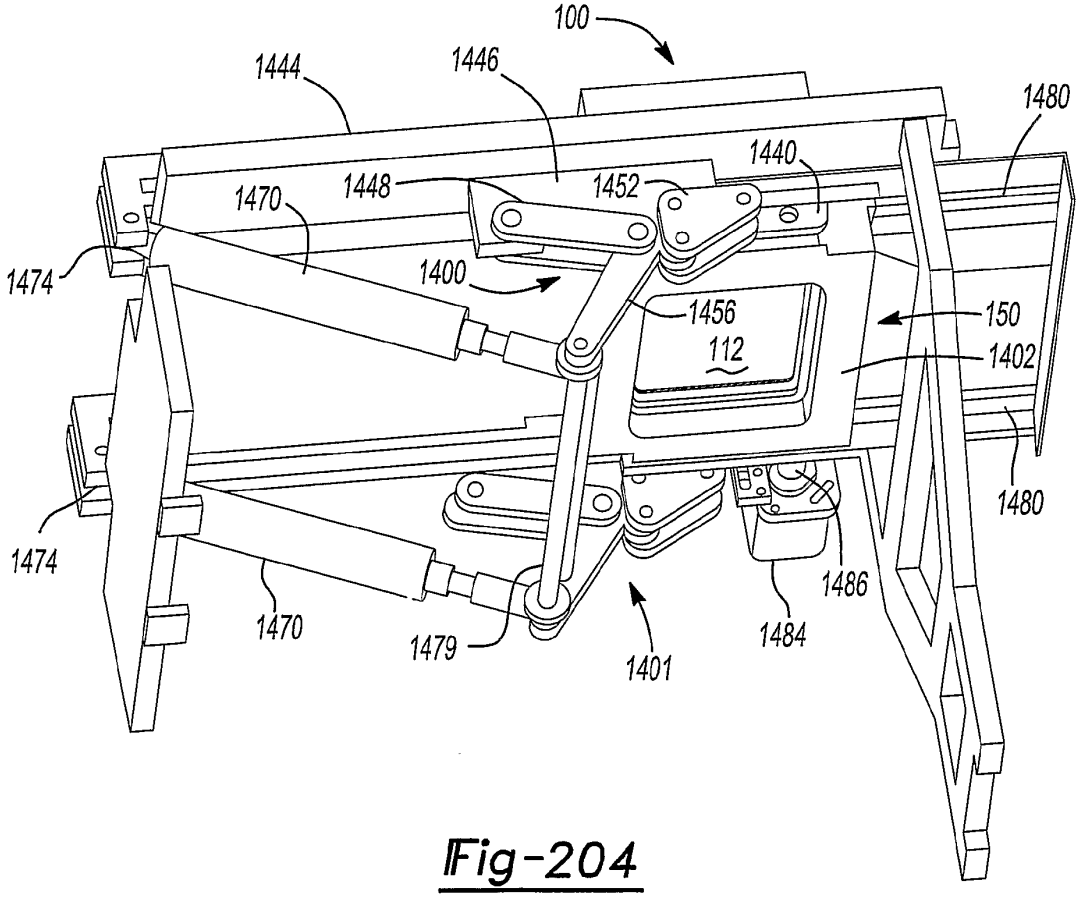


Fig-204

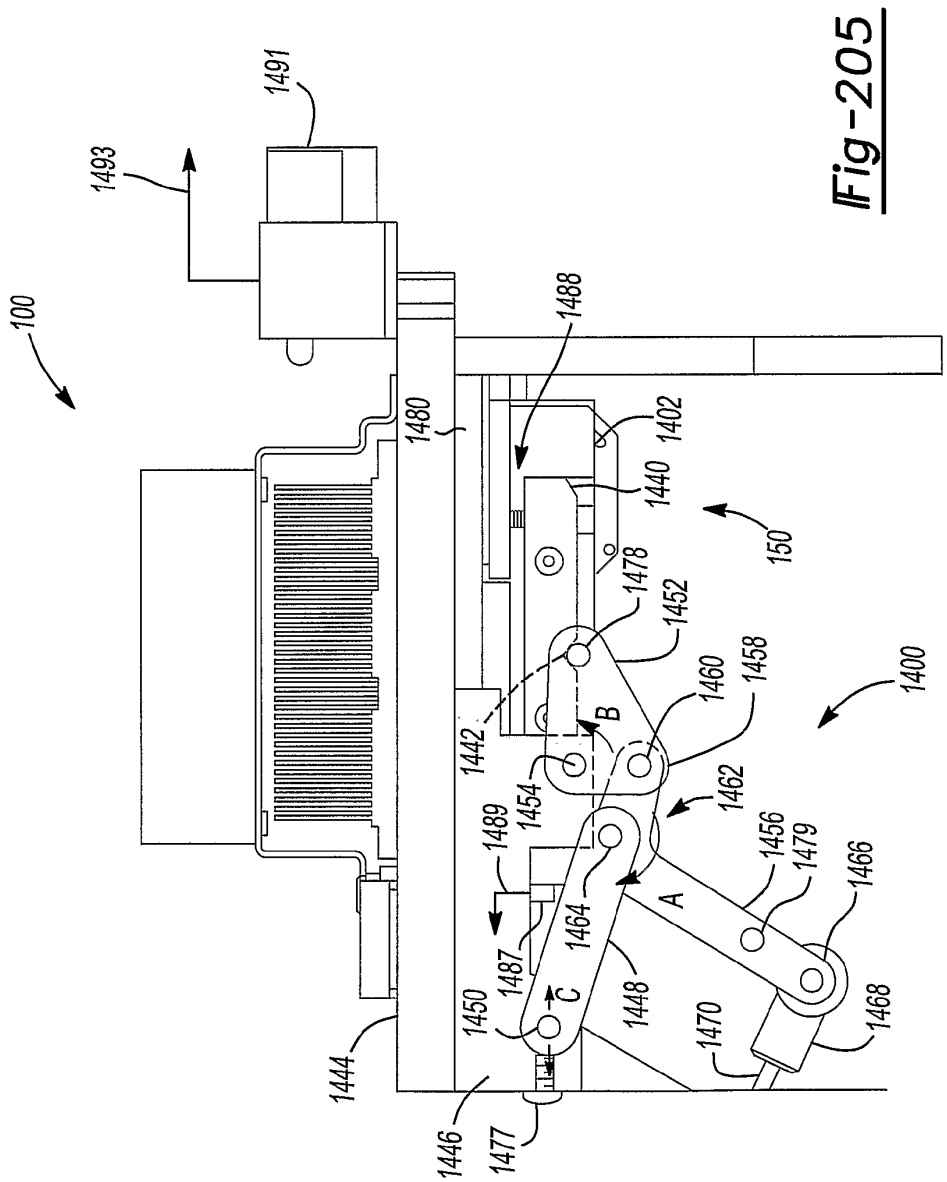

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Fig-205

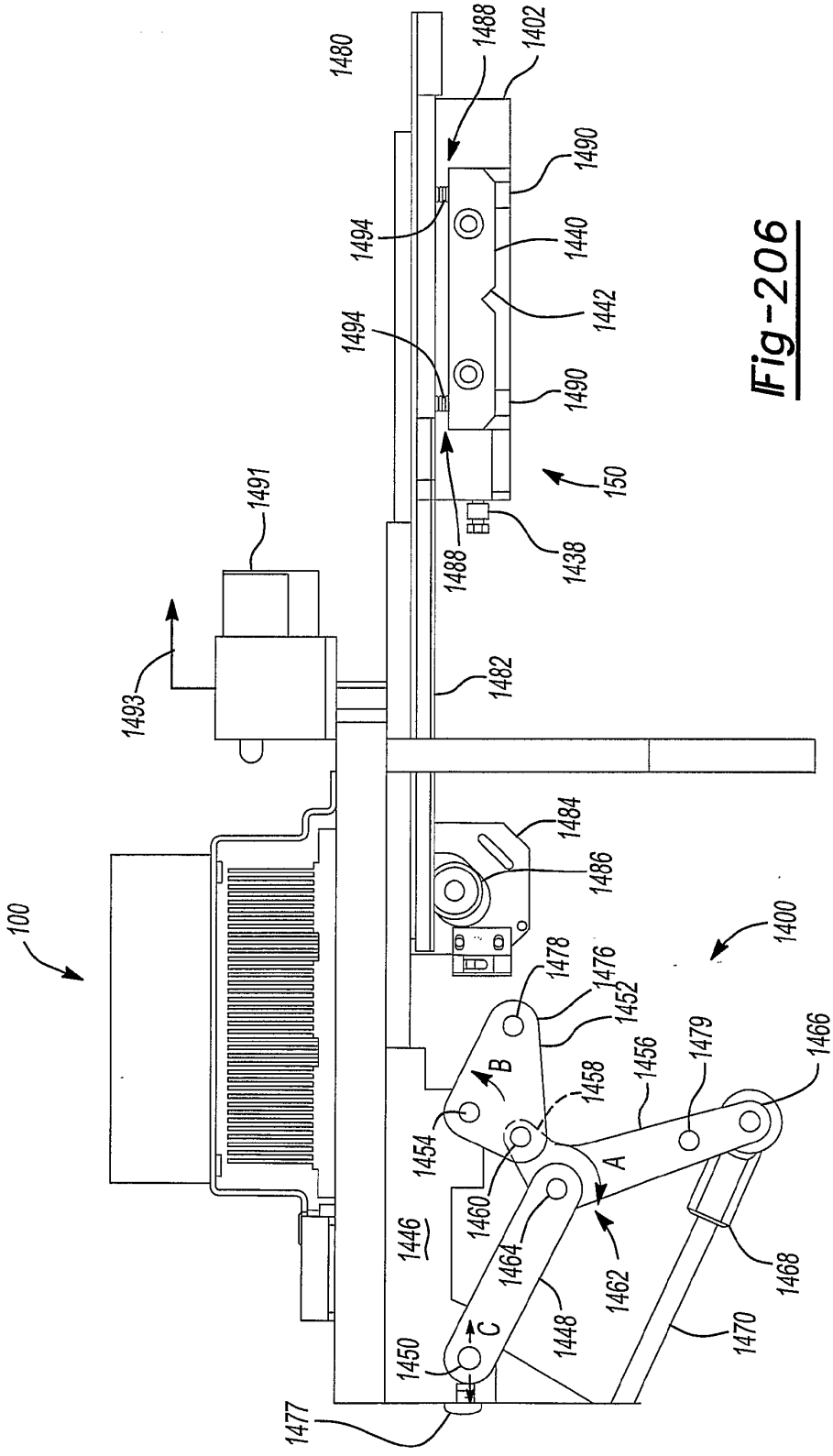

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Fig-206

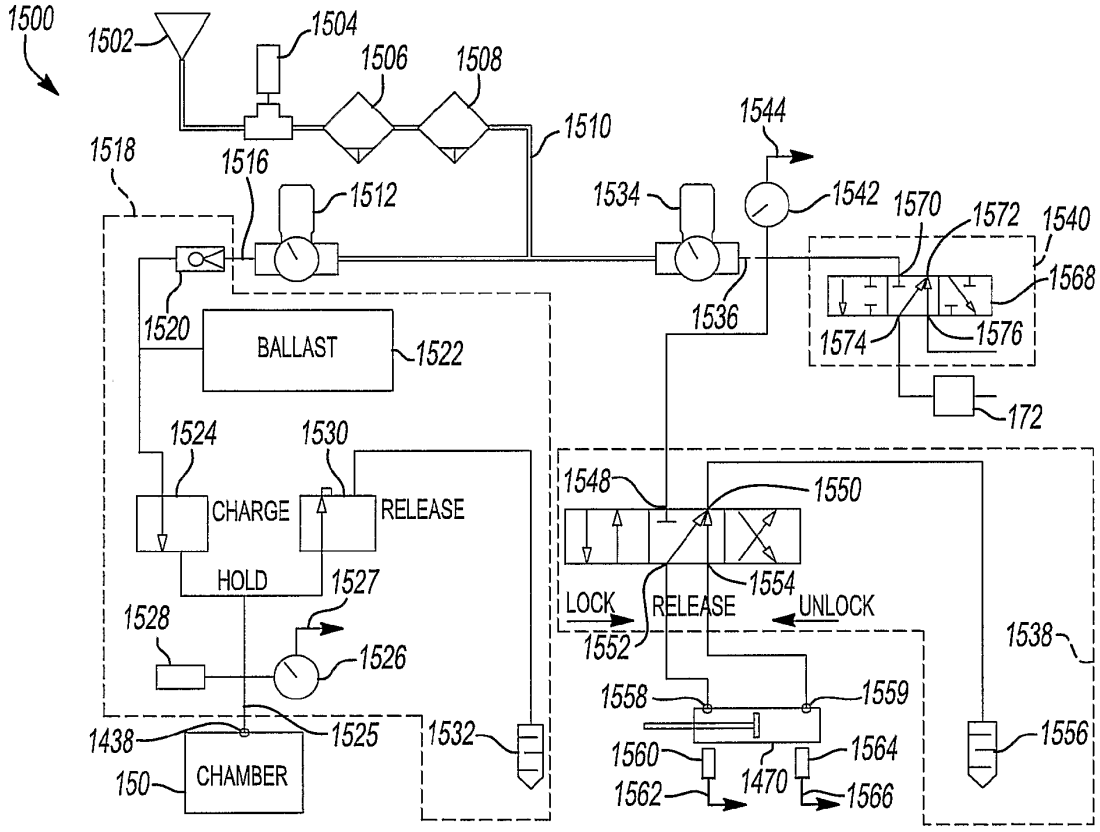

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RELEASE
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HOLD
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LOCK
RELEASE
UNLOCK
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Fig-207

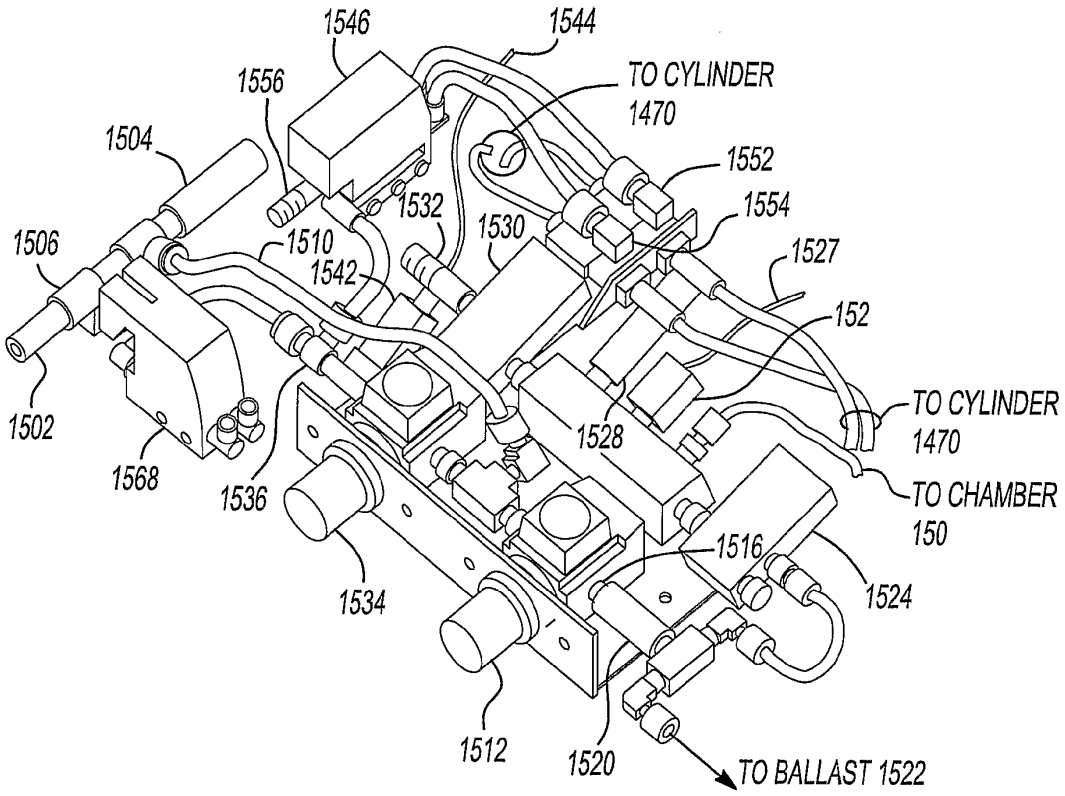

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Fig-208

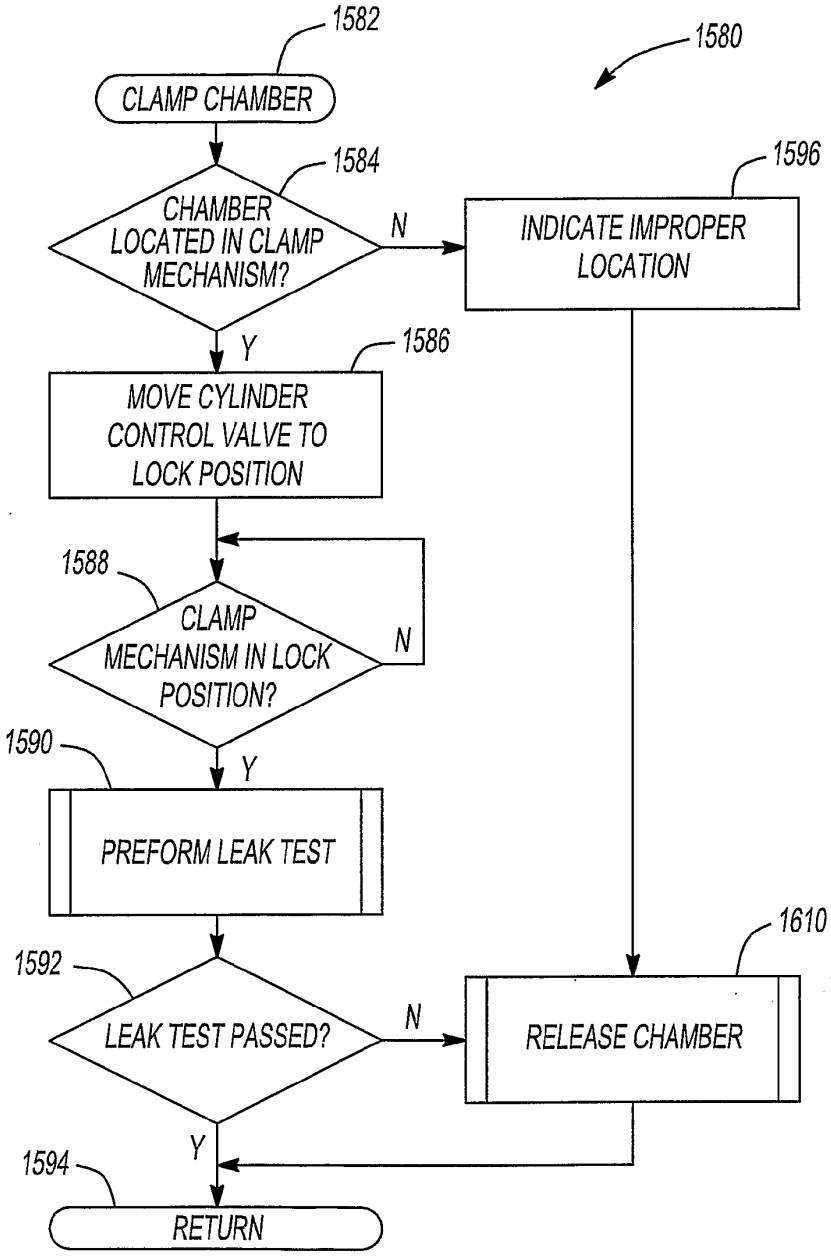

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CLAMP CHAMBER
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CHAMBER LOCATED IN CLAMP MECHANISM?
N
INDICATE IMPROPER LOCATION
Y
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MOVE CYLINDER CONTROL VALVE TO LOCK POSITION
1588
CLAMP MECHANISM IN LOCK POSITION?
N
1590
Y
PREFORM LEAK TEST
1610
1592
LEAK TEST PASSED?
N
RELEASE CHAMBER
Y
1594
RETURN

Fig-209

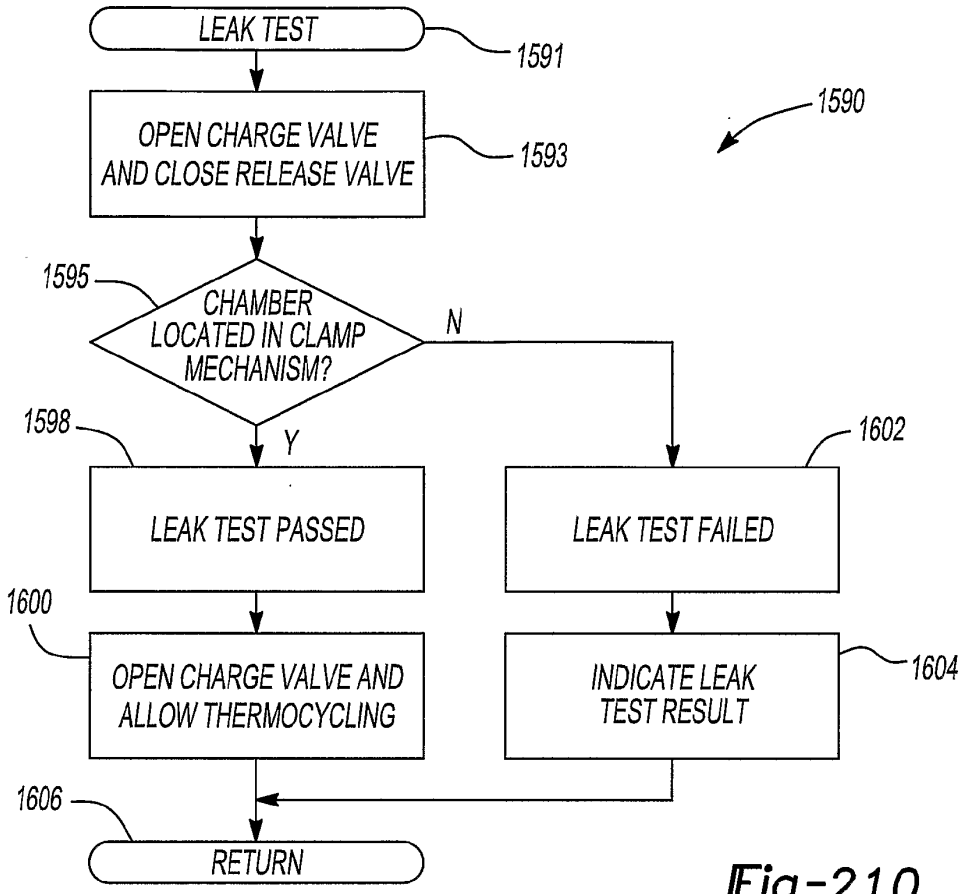


Fig-210

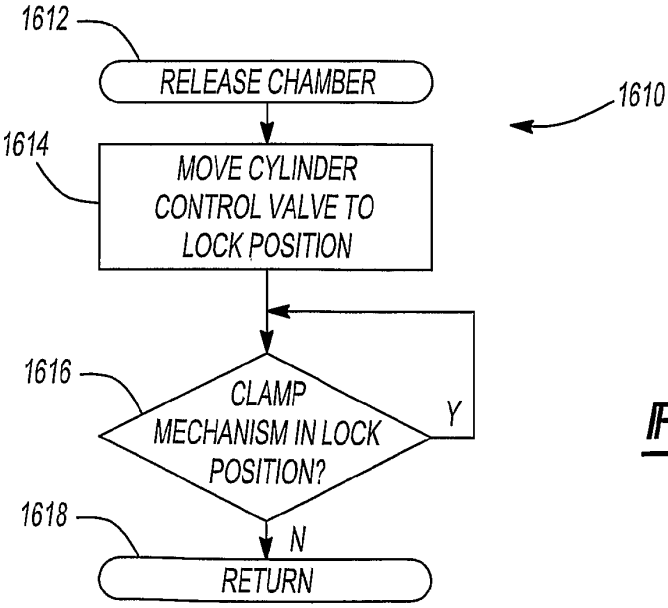


Fig-211

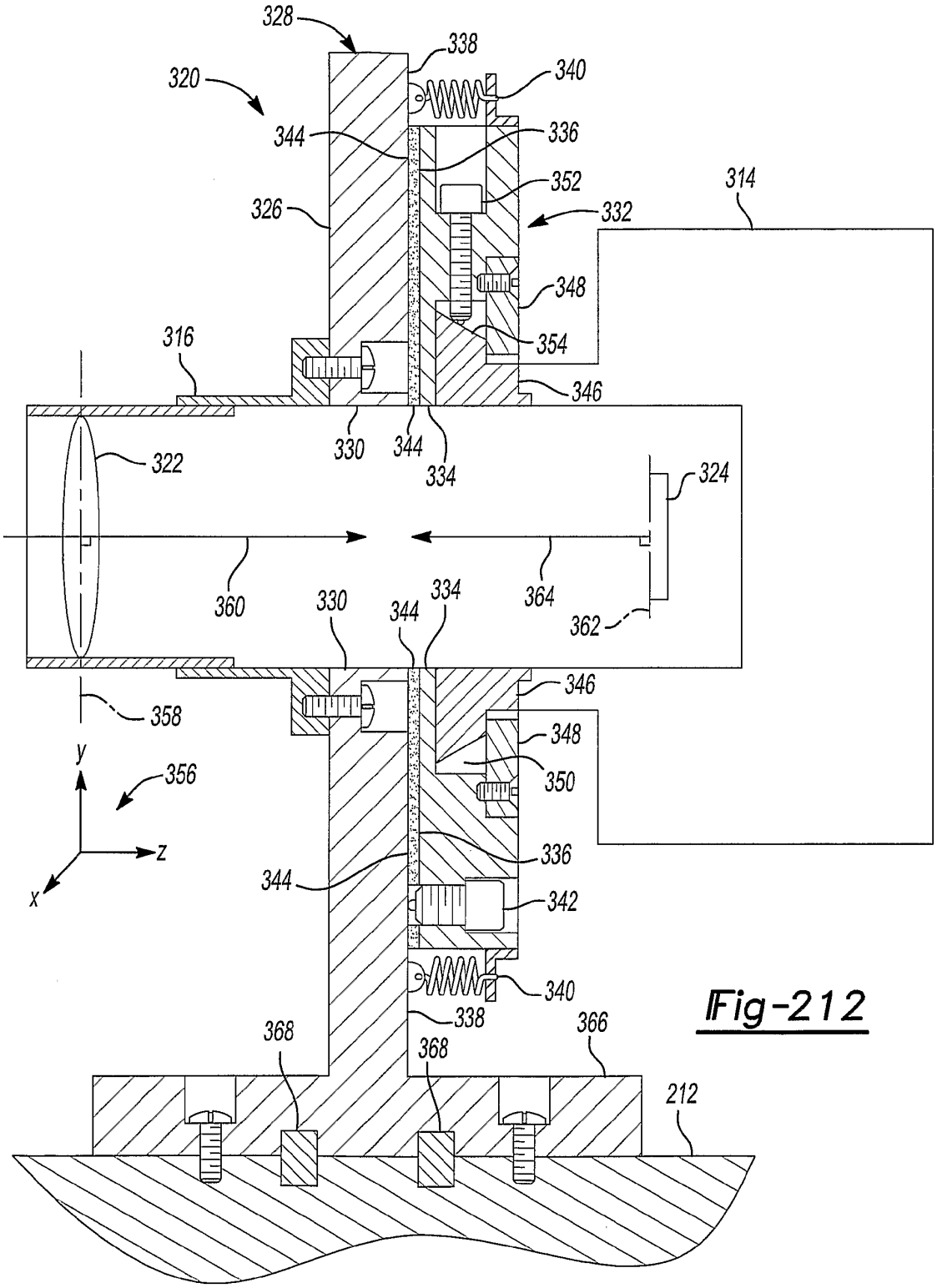

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Fig-212

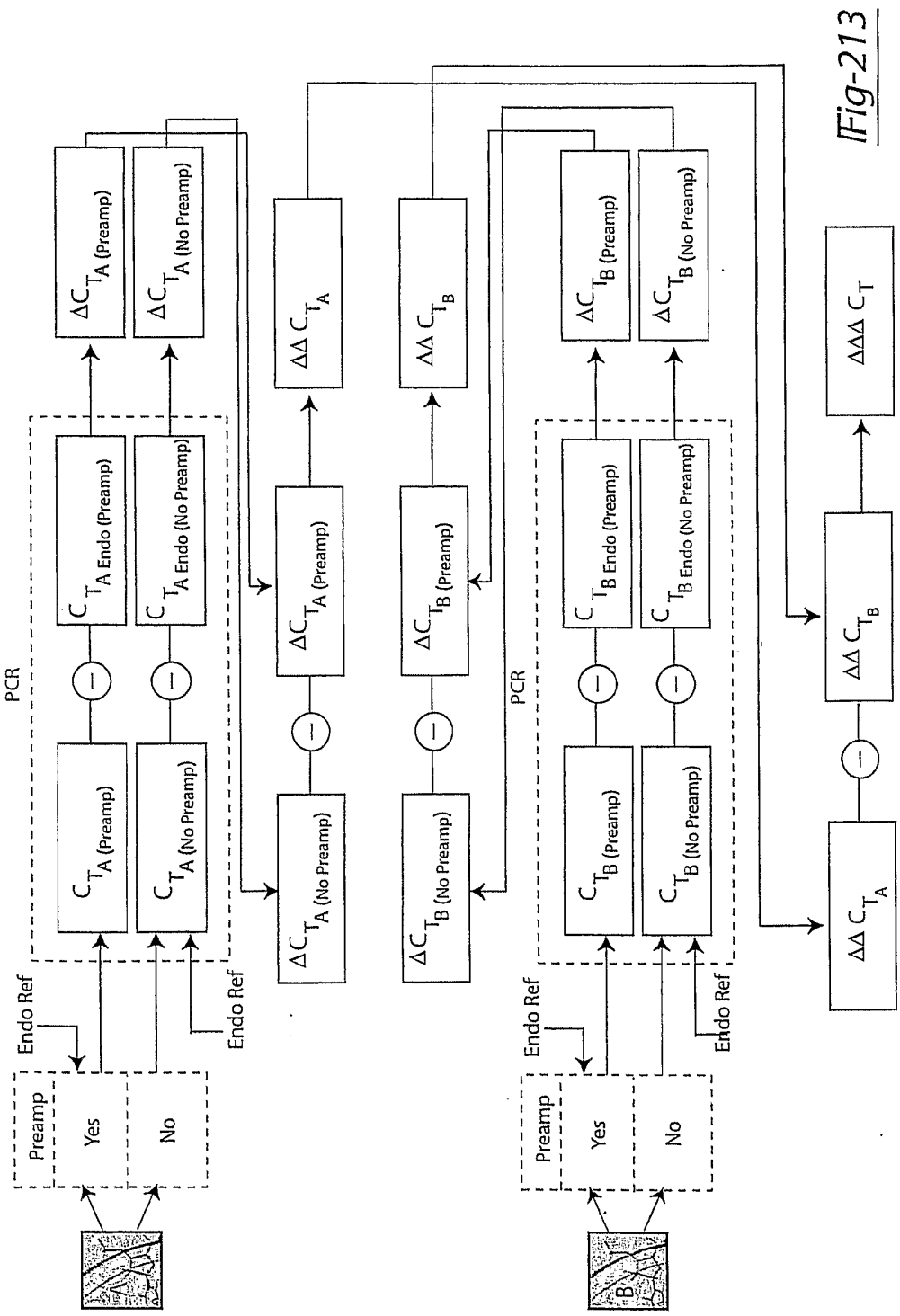

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Endo Ref
Endo Ref
PCR
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$C_{T_{A\ Endo\ (Preamp)}}$
$C_{T_{A\ Endo\ (No\ Preamp)}}$
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$\Delta C_{T_{A\ (No\ Preamp)}}$
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$\Delta C_{T_{B\ (No\ Preamp)}}$
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$\Delta\Delta\Delta\ C_T$

Fig-213

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/10172

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **C12M 1/32**(2006.01);**B29C 65/02**(2006.01)
C12M 1/14(2006.01);B32B 1/08(2006.01)

USPC: 156/381,389,443,499;422/99
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 156/381,389,443,499;422/99

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 03/099988 A (HENRIKSEN) 04 December 2003 (04.12.2003), page 4, lines 21-31 and figure 1.	1-9,11-29,31-40,43-45
Y	US 3,944,455 A (FRENCH) 16 March 1976 (16.03.1976), abstract, col 4, lines 25 - 40, col 5, lines 47 - 49, and figure 3.	1-9,11-29,31-40,43-45
Y	US 6,827,906 B1 (BJORNSON et al) 07 December 2004 (07.12.2004), column 4, lines 50-67, column 12, lines 29-33, column 17, lines 20-28, and figures 5, 7a, & 7b.	2,3,11-23,28
Y	US 5,645,800 A (MASTERSON et al) 08 July 1997 (08.07.1997), column 4, lines 59-67 and column 8, lines 46-59.	6,7,32,33,45
Y	US 5,080,582 A (SALZBERG) 14 January 1992 (14.01.1992), column 2, lines 3-12.	8,34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
15 July 2006 (15.07.2006)	16 AUG 2006
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Christopher Fiorilla Telephone No. (571) 272-6019

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KIM, Jong-Bum** [KR/US]; 53 Brushy Plain Road #5A, Branford, Connecticut 06405 (US). **LEFKOWITZ, Steven, Martin** [US/US]; 41 Victoria Drive, Branford, Connecticut 06405 (US). **NOBILE, John** [US/US]; 65 Forest Avenue, Fairfield, Connecticut 06824 (US). **ROTH, George, Thomas** [US/US]; 88 Buena Vista Road, Fairfield, Connecticut 06825 (US). **YU, Pengguang** [CA/US]; 20 Commercial Street, Branford, Connecticut 06405 (US).

(74) Agent: **ELRIFI, Ivor, R.**; Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, PC, One Financial Center, Boston, Massachusetts 02111 (US).

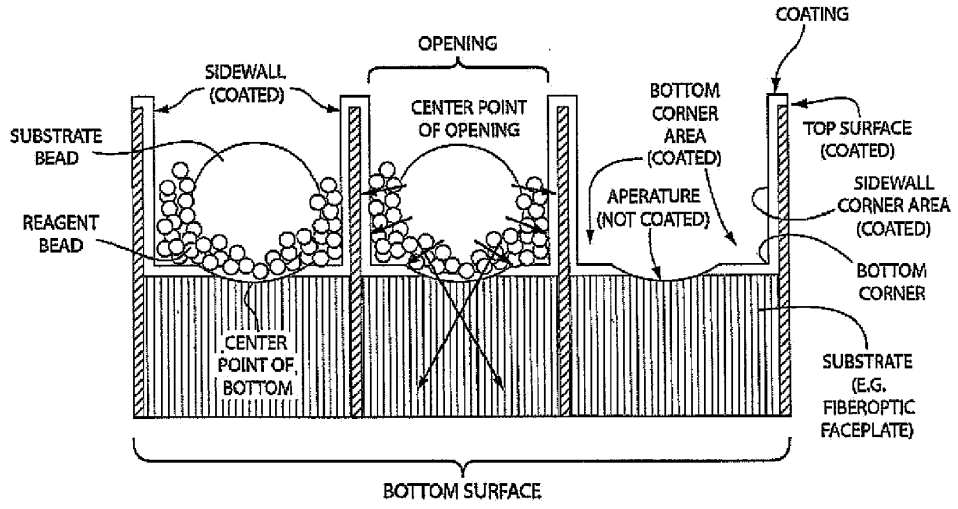
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[Continued on next page]

(54) Title: THIN FILM COATED MICROWELL ARRAYS AND METHODS OF MAKING SAME



(57) Abstract: This invention relates to microwell array compositions which are coated with one or more thin film coatings. The invention includes the process of fabricating and using thin film coated microwell arrays including methods of etching, depositing one or more thin film coatings, preparing and using the array.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

THIN FILM COATED MICROWELL ARRAYS AND METHODS OF MAKING SAME

FIELD OF THE INVENTION

This invention relates to microwell array compositions comprised of a substrate coated with one or more thin film coatings. The invention includes the process of fabricating and using the thin film coated microwell array.

BACKGROUND OF THE INVENTION

The ability to perform parallel microanalysis on minute quantities of sample is important to the advancement of chemistry, biology, drug discovery and medicine. Today, the traditional 1536-well microtitre plate has been surpassed by microwell arrays which have an even greater number of reaction chambers and use lesser amounts of reagents due to efforts focused on maximizing time and cost efficiencies. Although there are several types of microwell arrays available, many microwell materials prove to be incompatible with the components of bioassays and chemical reactions and result in problems such as low sensitivity, high background signal, and lack of reproducibility. Thus, there continues to be a need for the development of improved microwell arrays.

One solution to the problem of incompatible materials is to apply a thin film coating of a compatible material to the microwell array to enhance its surface properties and function. Patil, *et al.* U.S. Pat. No. 6,395,483 has disclosed a method to coat polymeric substrates with mask layers comprised of metallic and metal-oxide for use in high-density microarray applications. Yon-Hin, *et al.* U.S. Pat. No. 6,440,645 has described a process to use a photoimageable thin film on a polymer substrate to form microwells or channels. Heller, *et al.* U.S. Pat. No. 5,632,957 describes the deposition of metal, insulator and passivation coatings of substrates to form microelectrode arrays, and to form microwells over the individual microelectrodes. Walt, *et al.* U.S. Patent 6,377,721 has disclosed coating the interior surfaces of the microwells on fiber optic arrays with a thin film or a layer of biologically compatible material.

Certain fiber optic bundles have been used to create arrays. Several methods are known in the art for attaching functional groups (and detecting the attached functional groups) to reaction chambers etched in the ends of fiber optic bundles. See, *e.g.*, Michael, *et al.*, *Anal. Chem.* 70: 1242-1248 (1998); Ferguson, *et al.*, *Nature Biotechnology* 14: 1681-

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1684 (1996); Healey and Walt, *Anal. Chem.* 69: 2213-2216 (1997). A pattern of reactive functional groups can also be created in the reaction chamber, using photolithographic techniques similar to those used in the generation of a pattern of reaction pads on a planar support. See, Healey, *et al., Science* 269: 1078-1080 (1995); Munkholm and Walt, *Anal. Chem.* 58: 1427-1430 (1986), and Bronk, *et al., Anal. Chem.* 67: 2750-2757 (1995).

An array of functional groups on a substrate can be constructed using lithographic techniques commonly used in the construction of electronic integrated circuits as described in, *e.g.*, techniques for attachment described in U.S. Patent Nos. 5,143,854, 5,445,934, 5,744,305, and 5,800,992; Chee *et al., Science* 274: 610-614 (1996); Fodor *et al., Nature* 364: 555-556 (1993); Fodor *et al., Science* 251: 767-773 (1991); Gushin, *et al., Anal. Biochem.* 250: 203-211 (1997); Kinosita *et al., Cell* 93: 21-24 (1998); Kato-Yamada *et al., J. Biol. Chem.* 273: 19375-19377 (1998); and Yasuda *et al., Cell* 93: 1117-1124 (1998). Photolithography and electron beam lithography sensitize the substrate with a functional group that allows attachment of a reactant (*e.g.*, proteins or nucleic acids). See *e.g.*, Service, *Science* 283: 27-28 (1999); Rai-Choudhury, HANDBOOK OF MICROLITHOGRAPHY, MICROMACHINING, AND MICROFABRICATION, VOLUME I: MICROLITHOGRAPHY, Volume PM39, SPIE Press (1997). Alternatively, an array of functional groups can be generated using thin film technology as described in Zasadzinski *et al., Science* 263: 1726-1733 (1994).

One major disadvantage of this type of fiber optic array is the constraint of the materials comprising the fiber optic bundle. To act as an efficient waveguide, each fiber element should include a high refractive index core surrounded by a low refractive index cladding. These fiber optic materials are often incompatible with many reaction conditions, particularly bioassays which are often conducted in aqueous solutions and contain sensitive enzymatic reagents. Two major sources of incompatibility are the dissolution of the fiber optic substrate into the solution contained in the reaction chamber and the actual chemical reaction of the fiber optic substrate with components contained in the chamber. For example, core components, such as barium and lanthanum oxides, can form hydroxides which are water soluble, particularly at elevated temperatures. Multivalent heavy metals, such as barium and lanthanum, can interact unfavorably with enzymes, especially those enzymes with metal ion co-factors. Heavy metal oxide surfaces tend to be positively charged at the solution interface and tend to non-specifically bind negatively charged species such as nucleic acids. All of these effects will tend to degrade the performance of assays and

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reactions conducted in the fiber optic reaction chambers. Increasing miniaturization also tends to exacerbate these unfavorable effects.

The fact that the fiber optic substrate is comprised of two materials (core and cladding) also can limit the effectiveness of any surface modification of the reaction chambers with a monolayer (*e.g.* functional groups). For example, a singly charged surface is modified by binding to the charged surface of functionalized polyelectrolytes which contain an opposite charge. The core and cladding materials of the fiber optic substrate each have different types of charges. Thus, any modification of the fiber optic substrate with a single polyelectrolyte is impossible since the substrate does not contain a single uniform charge.

The optical properties of a fiber optic substrate are also limited. During a photochemical reaction carried out in the reaction chamber of a fiber optic faceplate, photons are generated which run through the fiber core and eventually reach through the other end of the fiber. At the same time, photons can also penetrate through the cladding material and travel until they are trapped by another fiber of an adjoining reaction chamber. These photons which travel through the transparent cladding are often referred to as optical scattering and result in problems such as optical bleeding and physical interferences (*e.g.*, cross-talk) between neighboring reaction chambers.

There is a clear need for microwell arrays which are compatible for any bioassay or reaction condition and which have superior optical properties.

SUMMARY OF THE INVENTION

One way to alleviate the problems associated with using fiber optic faceplates for chemical reactions and bioassays is to coat the array substrate with one or more thin film coatings using methods described herein. In general, the discussion herein is focused on fiber optic substrates, although other substrates as described below may be used in any embodiment described herein.

This invention relates to array compositions which include a substrate coated with one or more thin film coatings wherein at least one of the coatings is non-transparent. One aspect of the invention is an array which includes a substrate that is a fiber optic faceplate having a top surface with a plurality of reaction chambers and a bottom surface. Each reaction chamber of the array has a bottom and a sidewall. At least one of the bottom or sidewall of substantially all of the reaction chambers or the top surface of the array is coated with a non-transparent thin film coating, wherein the non-transparent coating is opaque, semi-opaque,

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shiny opaque, or translucent and at least one of the bottom or sidewall of substantially all of the reaction chambers or the top surface of the array is coated with a transparent thin film coating. The transparent coating differs from the non-transparent coating, and the non-transparent coating is optically transparent, 0.1-5.0 microns thick, and is impermeable to water.

In one embodiment, the transparent coating is silicon dioxide. In a further embodiment, when present, the thickness of the transparent coating is about 200-400 nm on the top surface of the array, 50-100 nm on the sidewall, and 100-300 nm on the bottom.

In another embodiment, the non-transparent coating is opaque. In another embodiment, the non-transparent coating is semi-opaque. In another embodiment, the non-transparent coating is shiny opaque. In another embodiment, the non-transparent coating is translucent. In one embodiment, when present, the thickness of the non-transparent coating is about 200-300 nm on the top surface of the array, 60-120 nm on the sidewall, and no less than 50 nm on the corner area, wherein the corner area is the comprised of the junction formed between the bottom and sidewall of the reaction chamber.

The non-transparent coating is selected from an organic compound, an inorganic compound, and a non-metal oxide. In one embodiment, the inorganic compound is a metal. In another embodiment, the non-transparent coating is selected from chromium, gold, silver, titanium, platinum and aluminum. In one embodiment, the non-transparent coating is conductive. In another embodiment, the non-transparent coating is dielectric.

In another embodiment, the sidewall of substantially all of the reaction chambers is coated with the transparent coating. In another embodiment, the top surface of the array is coated with the transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers is coated with the transparent coating. In one embodiment, each one of the sidewall and bottom of substantially all of the reaction chambers and the top surface of the array is coated with the transparent coating.

In one embodiment, the bottom and sidewall of substantially all of the reaction chambers are partially coated with a transparent coating, such that the transparent coating is applied to the corner area formed at the junction between the bottom and sidewall of the reaction chamber, and the coating is absent from the center of the bottom of the reaction chamber and forms an aperture near the center of the bottom. In one embodiment, the diameter of the aperture is about 28 microns. In another embodiment, a ring of coating is formed around the aperture on the bottom of the reaction chamber when the coating is

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applied to the corner area and the ring of coating has a width of 8.5 microns.

In another embodiment, the sidewall of substantially all of the reaction chambers is coated with the non-transparent coating. In another embodiment, the top surface of the array is coated with the non-transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers is coated with the non-transparent coating. In one embodiment, the bottom and sidewall of substantially all of the reaction chambers are partially coated with a non-transparent coating, such that the non-transparent coating is applied to the corner area formed at the junction between the bottom and sidewall of the reaction chamber, and the non-transparent is absent from the center of the bottom of the reaction chamber and forms an aperture near the center of the bottom. In a further embodiment, the transparent coating is applied to the array that is already partially coated with a non-transparent coating and the transparent coating is applied to at least one of the bottom or sidewall of substantially all of the reaction chambers or top surface of the array.

In one embodiment, the sidewall of substantially all of the reaction chambers and the top surface of the array are coated with the transparent coating, and the bottom is coated with the non-transparent coating. In one embodiment, the sidewall and bottom of substantially all of the reaction chambers are coated with the transparent coating, and the top surface of the array is coated with the non-transparent coating. In another embodiment, the top surface of the array and bottom of substantially all of the reaction chambers are coated with the transparent coating, and the sidewall of substantially all of the reaction chambers is coated with the non-transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers is coated with the transparent coating, and the sidewall of substantially all of the reaction chambers and the top surface of the array are coated with the non-transparent coating. In another embodiment, the top surface of the array is coated with the transparent coating, and the sidewall and bottom of substantially all of the reaction chambers are coated with the non-transparent coating. In another embodiment, the sidewall of substantially all of the reaction chambers is coated with the transparent coating, and the bottom of substantially all of the reaction chambers and the top surface of the array are coated with the non-transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers is coated with the transparent coating, the sidewall of substantially all of the reaction chambers is coated with the non-transparent coating, and the top surface of the array is not coated. In another embodiment, the transparent coating includes at least a first transparent coating. In another embodiment, the non-transparent coating includes at least a

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first non-transparent coating.

In another embodiment, the non-transparent coating is applied to the array before any transparent coating is applied. In one embodiment, at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array is coated with the non-transparent coating before any transparent coating is applied. In another embodiment, the transparent coating is applied to the array before any non-transparent coating is applied. In another embodiment, at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array is coated with the transparent coating before any non-transparent coating is applied.

In one embodiment, an enzyme is immobilized on the non-transparent coating. In another embodiment, an enzyme is immobilized on the transparent coating. In one embodiment, the reaction chambers of the array are arranged in a regular pattern. In another embodiment, the reaction chambers of the array are arranged in an irregular pattern. In another embodiment, the spacing between the center points of two adjoining reaction chambers on the array is between 5 μm to 200 μm, and each reaction chamber has a width in at least one dimension of between 4μm and 190 μm. In one embodiment, the number of reaction chambers is 10,000 or less. In another embodiment, the number of reaction chambers is greater than 10,000. In one embodiment, the depth of substantially all of the reaction chambers is between 10-100 μm. In another embodiment, the depth of substantially all of the reaction chambers is between 50-55 μm.

In another embodiment, the array is comprised of a top surface comprising a plurality of reaction chambers and an opposed, planar, polished bottom surface without reaction chambers, wherein the polished bottom surface is optically transmissive such that optical signals from the reaction chambers are detected through the polished surface, wherein the distance between the top surface comprising the reaction chambers and the polished bottom surface is no greater than 5 mm in thickness.

In one embodiment, the array comprises an index feature. In another embodiment, the array substrate comprises one or more side edges beveled at an angle. In another embodiment, the angle is 45 degrees. In another embodiment, the substrate is marked with one or more identifier codes.

Another aspect of the invention is an array which includes a substrate that is a fiber optic faceplate having a top surface which includes a plurality of reaction chambers and a bottom surface. Each reaction chamber of the array has a bottom and sidewall. At least one

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of the bottom or sidewall of substantially all of the reaction chambers or top surface of the array is coated with a non-transparent, thin film coating. The non-transparent coating is opaque, semi-opaque, shiny opaque, or translucent.

In one embodiment, at least one of the bottom or sidewall of substantially all of the reaction chambers or top surface of the array is not coated. In one embodiment, the non-transparent coating is opaque. In another embodiment, the non-transparent coating is semi-opaque. In another embodiment, the non-transparent coating is shiny opaque. In another embodiment, the non-transparent coating is translucent.

In one embodiment, when present, the thickness of the non-transparent coating is about 200-300 nm on the top surface of the array, 60-120 nm on the sidewall, and no less than 50 nm on the corner area, wherein the corner area is the comprised of the junction between the bottom and sidewall of the reaction chamber. In another embodiment, the non-transparent coating is selected from an organic compound, an inorganic compound, and a non-metal oxide. In one embodiment, the inorganic compound is a metal. In another embodiment, the non-transparent coating is selected from chromium, gold, silver, titanium, platinum, and aluminum. In one embodiment, the non-transparent coating is conductive. In another embodiment, the non-transparent coating is dielectric.

In another embodiment, the sidewall of substantially all of the reaction chambers is coated. In another embodiment, the top surface of the array is coated. In another embodiment, the bottom of substantially all of the reaction chambers is coated. In another embodiment, the bottom and sidewall of substantially all of the reaction chambers are partially coated with a non-transparent coating, such that the non-transparent coating is applied to the corner area formed at the junction between the bottom and sidewall of the reaction chamber, and the non-transparent coating is absent from the center of the bottom of the reaction chamber and forms an aperture near the center of the bottom. In another embodiment, the diameter of the aperture is 28 microns. In another embodiment, a ring of coating is formed around the aperture when the coating is applied to the corner area and the ring of coating has a width of 8.5 microns.

In one embodiment, the sidewall of substantially all of the reaction chambers and the top surface of the array are coated. In another embodiment, the sidewall and bottom of substantially all of the reaction chambers are coated. In another embodiment, the bottom of substantially all of the reaction chambers and the top surface of the array are coated.

In one embodiment, the non-transparent coating comprises at least a first non-

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transparent coating. In another embodiment, the non-transparent coating comprises at least a first non-transparent coating and a second non-transparent coating, further wherein the first and second non-transparent coatings are not the same. In another embodiment, an enzyme is immobilized on the non-transparent coating. In one embodiment, the reaction chambers of the array are arranged in a regular pattern. In another embodiment, the reaction chambers of the array are arranged in an irregular pattern. In another embodiment, the spacing between the center points of two adjoining reaction chambers of the array is between 5μm to 200 μm, and each reaction chamber has a width in at least one dimension of between 4μm and 190 μm.

In one embodiment, the number of reaction chambers of the array is 10,000 or less. In another embodiment, the number of reaction chambers of the array is greater than 10,000. In one embodiment, the depth of substantially all of the reaction chambers of the array is between 10-100 μm. In another embodiment, the depth of substantially all of the reaction chambers of the array is between 50-55 μm. In another embodiment, the array includes a top surface which contains a plurality of reaction chambers and an opposed, planar, polished bottom surface without reaction chambers, wherein the polished bottom surface is optically transmissive such that optical signals from the reaction chambers are detected through the polished bottom surface, wherein the distance between the top surface comprising the reaction chambers and the polished bottom surface is no greater than 5 mm in thickness.

In one embodiment, the array includes an index feature. In another embodiment, the array substrate includes one or more side edges beveled at an angle. In another embodiment, the angle of the beveled side edge is 45 degrees.

Another aspect of the invention is a process for depositing a non-transparent thin film coating on a top surface of a substrate containing a plurality of reaction chambers, where each reaction chamber has a bottom and sidewall. The non-transparent coating is opaque, semi-opaque, shiny opaque, or translucent and is deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array. The steps of the process include: (a) mounting a substrate onto a substrate carrier at an angle, (b) spinning the mounted substrate in a vacuum chamber, (c) depositing the non-transparent coating onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array, (d) removing the substrate from the vacuum chamber, and (e) dismounting the substrate coated with the non-transparent coating from the substrate carrier. In one embodiment, the substrate is a fiber optic faceplate. In another embodiment, at least one

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bottom or sidewall of substantially all of the reaction chambers or top surface of the array is coated with a transparent coating before the non-transparent coating is applied. In another embodiment, at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the substrate is coated with a non-transparent coating prior to the application of a transparent coating to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the substrate. In another embodiment, the transparent coating is optically transparent, 0.1-5.0 microns thick, and impermeable to water.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a series of SEM images at 5k and 10k magnification of etched fiber optic faceplates coated with SiO_2 using the sputtering (Figures 1a and 1b), PECVD (Figures 1c and 1d), and ion-plating (Figures 1e and 1f) vapor deposition methods;

Figure 2 is a series of cross-sectional SEM images of fiber optic faceplates having reaction chambers which have been coated with SiO_2 showing coating thickness and quality for three vapor deposition coating methods. Figure 2a is a schematic of a deposition area in a reaction chamber. Figure 2b is an image of coating applied by sputtering. Figure 2c is an image of a coating applied by PECVD. Figure 2d is an image of a coating applied by ion-platting;

Figure 3 is a schematic diagram of the ion-plating process;

Figure 4 is a bar graph showing PCR-induced sequencing backgrounds of SiO_2 coated and uncoated fiber optic faceplates comprising reaction chambers;

Figure 5 is a series of graphs showing sequencing performance metrics of SiO_2 coated and uncoated fiber optic faceplates comprising reaction chambers for accuracy (Figure 5a), "normalized" keypass fraction (Figure 5b), and pyrophosphate (PPi) signal (Figure 5c);

Figure 6 is a series of Scanning Electron Micrographs of a portion of a fiber optic faceplate surface (uncoated) comprising reaction chambers;

Figure 7a is a drawing of a PEEK clamp (top view);

Figure 7b is a drawing of a PEEK clamp (side view);

Figure 7c is a drawing of a PEEK clamp (front end view);

Figure 7d is a drawing of a PEEK clamp (back end view);

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Figure 8a is a drawing of a 25 x 75 mm fiber optic faceplate (top view);

Figure 8b is a drawing of a 25 x 75 mm fiber optic faceplate (front edge view);

Figure 8c is a drawing of a 25 x 75 mm fiber optic faceplate (back edge view);

Figure 9 is a process flow diagram illustrating the RER cleaning procedure;

Figure 10 is a process flow diagram illustrating the single-sided etching process;

Figure 11a is a drawing of an etch gasket (top view);

Figure 11b is a drawing of an etch gasket (back end view);

Figure 11c is a drawing of an etch gasket (front end view);

Figure 11d is a drawing of an etch gasket (first side view);

Figure 11e is a drawing of an etch gasket (second side view);

Figure 12 is a drawing of an assembled fiber optic faceplate "clamped sandwich" held together with two clamps and ready for the etch process (top view);

Figure 13 is an exploded view drawing of a fiber optic faceplate "clamped sandwich".

Figure 14 is a side view of a fiber optic faceplate containing reaction chambers in which the sidewall of the reaction chambers and top surface of the array are coated with a non-transparent thin film.

Figure 15a illustrates the set-up used for the process of angled metal deposition using an angled jig. Shown is a side view of a fiber optic faceplate containing reaction chambers mounted onto the substrate carrier of a jig.

Figure 15b illustrates the rotary and angled jig assembly.

Figure 16a is a side view of a fiber optic faceplate containing three reaction chambers where one chamber is empty and the other two chambers are charged with reagent substrate beads (larger) and reagent beads (smaller). The reaction chambers have a coating on the sidewall of the reaction chamber and top surface of the array, and the bottom of the reaction chamber is partially coated. The coating shown is applied to the corner area of the reaction chamber, such that the coating is applied to the "sidewall corner area" and the "bottom corner area" where there is an absence of coating on the bottom of the reaction chamber.

Figure 16b is a top view of three reaction chambers having a coating applied to the bottom corner area of the reaction chamber, such that there is an absence of coating on the bottom of the reaction chamber which forms an aperture near the center of the bottom. The diameter of the aperture and width of the ring of the corner

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area are shown.

Figure 17 is a side view of a fiber optic faceplate containing three reaction chambers, charged with substrate beads (larger) and reagent beads (smaller), and the sidewall of each chamber is coated with a coating. The coating has been modified to contain an enzyme (e.g., apyrase) on its surface.

Figure 18 illustrates "angle effects" that can occur when the ion source is considerably smaller than the substrate during vapor deposition of a coating. Angle effects produce an array with reaction chambers that are not uniformly coated. For example, A shows the coating being deposited in the corner area of three reaction chambers located on one end of the substrate, and B shows the coating being deposited on the bottom of three reaction chambers located on the other end of the substrate

Figure 19a illustrates the use of a shadow mask during the deposition process to direct the coating to a selected area (*e.g.*, the "metal coated zone") of the fiber optic faceplate and to eliminate angle effects.

Figure 19b illustrates the use of a shutter during the deposition process to direct the coating to a selected area (the "metal coated zone") of the fiber optic faceplate and to eliminate angle.

Figure 20a shows a fiber optic faceplate having fiber strands, which are oriented at an acute angle ("acute angle (#1)") which is less than 90 degrees to the bottom surface of the fiber optic faceplate (i.e. the fiber strands are not perpendicular).

Figure 20b shows a titled reaction chamber where a bead is located in one corner of the reaction chamber having a sidewall which is slanted at an acute angle (#2) to the bottom of the reaction chamber. The bead can be fixed in this corner position using centrifugal force.

Figure 20c is a sketch of a fiber optic bundle which is marked with two the lines showing an example of where to cut the fiber optic bundle to make a non-perpendicular cut.

Figure 21a is a sketch of an array where the reaction chambers of the array are each formed by 4 boundary posts built on top of the substrate, such boundary posts "trap" each bead or particle in the space defined by the set of 4 boundary posts. The bottom and sidewall of the reaction chamber holding a bead is shown.

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Figure 21b illustrates the metal deposition process for an array where the reaction chambers are formed by a series of boundary posts on top of a substrate. The figure 21b shows overlapping shadows during multiple depositions of thin film coatings at different angles. Shadows created by the boundary posts produce unique patterns and shapes on overlapping areas.

Figure 22 shows optical images taken of two areas of a fiber optic faceplate that has been etched and coated on one half. Area A is metal (silver) coated and Area B is uncoated. The silver was applied to the array using a thermal evaporation process.

Figure 23 shows two microscopic photos of the same etched fiber optic faceplate which has been coated with a non-transparent, silver coating. The top surface area of the array, the sidewall and corner area of each reaction chamber is coated with silver, such that the coating is absent in the center of the bottom which creates an aperture near the center of the bottom. Photo A is focuses on the bottom of the chamber and photo B is focused on the cladding.

Figure 24 A and B are graphs illustrating how a non-transparent, metal coating that is applied to the sidewall of the reaction chambers of an array can reduce optical bleeding between two adjacent chambers on an array. The two graphs represent two different nucleotide flow orders.

Figure 25a shows three views of a silver coating on the corner of a reaction chamber of a fiber optic faceplate, the silver coating covers the bottom corner of the chamber. The thickness of the coating is such that it blocks light and shields photons from leaking into adjacent chambers. View 1 shows the entire reaction chamber. View 2 is a close-up of the corner of the chamber, and View 3 is a magnified view of this corner.

Figure 25b shows three views of a silver coating on the upper corner of a reaction chamber and the top surface of a fiber optic faceplate, where the thickness of the coating on the sidewall is different from the thickness on the top surface of the cladding. This difference in thickness is due to the spinning of the substrate during the metal deposition process. View 1 shows the entire reaction chamber. View 2 is a close-up of the upper corner and the top of the reaction chamber, and the View 3 is a magnified view of this upper corner.

Figure 26a shows an array where the reaction chambers have a sidewall that is not

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continuous i.e., the sidewall is interrupted with a slit in the sidewall between two adjacent reaction chambers.

Figure 26b illustrates the pattern formed on the bottom of a reaction chamber (with a non-continuous sidewall) when a coating is deposited through a slit opening in the sidewall of a reaction chamber. The coating is deposited in a gradient such that the coating is thicker near the center line of the bottom of the reaction chamber and the coating becomes thinner away from the center line to the edge.

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, and exemplified suitable methods and materials are described below. For example, methods may be described which comprise more than two steps. In such methods, not all steps may be required to achieve a defined goal and the invention envisions the use of isolated steps to achieve these discrete goals. The disclosures of all publications, patent applications, patents and other references are incorporated *in toto* herein by reference. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

"Analyte" means a molecule, compound, composition or complex, either naturally occurring or synthesized, to be detected or measured in or separated from a sample of interest. Analytes include, without limitation, proteins, peptides, amino acids, fatty acids, nucleic acids, carbohydrates, hormones, steroids, compounds, lipids, vitamins, bacteria, viruses, pharmaceuticals, and metabolites.

"Clamped Sandwich" refers to a combination of two FOFs with a gasket positioned in between the two FOFs, wherein the corner notch of each FOF is properly aligned with the appropriate gasket corner barrier, such that the polished surface of each FOF is facing towards the gasket, wherein a seal is formed between the polished surface of the FOF and the gasket which is impermeable to liquid.

"Core material" refers to the inner component of a fiber optic fiber. The material is transparent and has a high refractive index.

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"Cladding material" refers to the outer component of a fiber optic fiber. The material is transparent and has a low refractive index.

"Corner Area" refers to the inner surface of the reaction chamber where a junction is formed between the sidewall and bottom of the reaction chamber. (see Figure 16a).

"Corner barrier" refers to a feature on a gasket, which includes a band that is placed at an angle in the corner of a gasket and provides a physical basis for orienting an FOF when the FOF is mounted in the gasket.

"Corner notch" refers to one corner of a fiber optic faceplate which is cut at angle and removed to provide a physical basis for orienting the fiber optic faceplate, for example when a fiber optic faceplate is mounted on a system for analysis or in a gasket for the etch process, the corner notch of the fiber optic faceplate is matched with a complementary feature located on the analysis system or gasket.

"Etch process" refers to a chemical process using acid to create reaction chambers in an array substrate.

"Fiber optic faceplate" or "FOF" refers to a bundle of fiber optic cables which are fused together to form a monolithic structure which is then "sliced" to form a "wafer" of required thickness.

"Functional groups" means any chemical or biological species capable of affixing a reactant, reaction substrate, or analyte to the inside surface of the reaction chamber.

"Gasket" refers to one component of the apparatus for the etch process. The gasket is aligned between two FOFs and protects one side of each FOF (*e.g.*, the polished side) from exposure to acid by forming a fluid tight seal.

"Gasket Index Feature" refers to a band positioned in one corner of a gasket which creates a notch that is complementary to the corner notch of a FOF and that provides a physical basis for orienting a FOF.

"Index Feature" refers to a structure that provides a physical basis for orienting a FOF.

"FOF Index Feature" refers to a corner notch on the FOF which provides a physical basis for properly orienting a FOF.

"Ion-Plating" means a method of vapor deposition used for depositing a thin film coating on an array substrate where first, electrically charged and neutral atoms are used to remove contaminants from the substrate and second, the coating material is evaporated, enhanced by the interaction with energetic inert gas or reactive gas atoms and ions, and

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deposited on the substrate.

"Impermeable to Water" refers to the ability of a thin film coating to provide a barrier to an aqueous solution contained in the reaction chamber and to prevent leaching of the chamber solution into the sidewall components of the reaction chamber.

"Non-transparent Thin Film Coating" or "Non-transparent Coating" refers to a coating of material that modulates *e.g.*, blocks, substantially blocks, or diffuses the passage of light through there through. For example, the non-transparent thin film coating or non-transparent coating is opaque, semi-opaque, shiny opaque, or translucent.

"Opaque" means that the passage of light is blocked such that less than 2 out of 1000 photons pass through a thin film material.

"Optically Transparent" refers to the ability of light to transmit through a thin film coating. "PEEK clamp" refers to a clamp made of poly ether ether ketone which is used to firmly hold two FOFs and a gasket together during the etch process.

"Partial Coating" means less than the entire surface is coated e.g., less than the entire surface of a bottom or sidewall of a reaction chamber is coated. "PicoTiter Plate™" or "PTP" means an array substrate which includes an etched FOF.

"Plasma-Enhanced Chemical Vapor Deposition" or "PECVD" means a method of vapor deposition used for depositing a thin film coating on an array substrate, where the thin film coating is produced from the chemical reaction of gases.

"Reactant" means any chemical or biological molecule, compound, composition or complex, either naturally occurring or synthesized, that is capable of binding, forming, or reacting with an analyte in a sample of interest either alone or in conjunction with another reactant. The reactants of the present invention are useful for chemical reaction or biochemical measurement, detection or separation. Examples of reactants include, without limitation, amino acids, nucleic acids, including oligonucleotides and cDNA, carbohydrates, and proteins such as enzymes and antibodies.

"Reaction Chamber" means a localized well or chamber comprised of a bottom and a sidewall (i.e. a hollowed-out space, having width and depth) that is found on a substrate.

"RCA cleaner" means a solution of ammonium hydroxide and hydrogen peroxide.

"RER cleaning process" refers to a process for cleaning an etched FOF using RCA cleaner, EDTA, and RCA cleaner.

"Sandwich" refers to a combination of two FOFs with a gasket positioned in between the two FOFs, wherein the corner notch of each FOF is properly aligned with the appropriate

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gasket index feature, such that the polished surface of each FOF is facing towards the gasket and the unpolished surface is exposed.

"Scanning Electron Microscopy" or "SEM" refers to a method for high resolution imaging.

"Semi-opaque" means that the passage of light is nearly blocked such that less than 10% of the light is passed through the thin film coating.

"Shiny Opaque" means the passage of light is blocked such that less than 2 out of 1000 photons pass through a thin film coating and the light is reflected off of the shiny opaque surface.

"Sputtering" means a method of vapor deposition used for depositing a thin film coating on an array substrate.

"Substrate" refers to a solid support or any material that can be modified to contain discrete individual reaction chambers and is amenable to at least one detection method.

"Thin Film" or "Thin Film Coating" refers to a coating of material deposited on the surface of the substrate.

"Transparent" means that light is permitted to pass through a thin film coating without appreciable scattering such that objects can be seen clearly.

"Transparent Thin Film Coating" or "Transparent Coating" refers to a coating of material deposited on the surface of the substrate wherein the coating is less than 5.0 microns thick, optically transparent, and impermeable to water.

"Translucent" means that light is permitted to pass through a thin film coating, but the light is diffuse such that objects can not be seen clearly.

"Vapor deposition" refers to a method for depositing a thin film coating on the array.

The present invention provides array compositions including a substrate which has individual reaction chambers and is coated with one or more thin film coatings. The invention includes the process of fabricating and using the coated array. By "array" herein is meant a plurality of reaction chambers, which are localized wells or chambers in an array format on the surface of a substrate. Each reaction chamber includes a bottom and sidewall. The size of the array and its reaction chambers will depend on the composition and end use of the array. In one embodiment, the array, including at least one bottom or sidewall of substantially all of the reaction chambers of the array or top surface of the array, is coated with one or more thin film coatings to enhance the properties and functions of the array and

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its reaction chambers. The thin film coating or coatings protect the contents of the solution in the reaction chamber from the deleterious effects of the array substrate, without compromising the utility or ease of fabrication of the array. The thin film coatings also provide a uniform surface composition allowing for uniform modification of the reaction chamber surface (*e.g.*, with a monolayer). In one embodiment, the thin film coating produces an optical obstacle in order to eliminate optical scattering of light through the cladding materials into adjacent reaction chambers.

The invention described herein can be a component of a larger system and methods. Such system and methods can be used to process nucleic acids in a multitude of ways. For example, methods are performed to determine the identity of a sequence of nucleic acids, or for single nucleotide polymorphism (SNP) detection in nucleic acid fragments, for nucleic acid expression profiling (comparing the nucleic acid expression profile between two or more states *e.g.*, comparing between diseased and normal tissue or comparing between untreated tissue and tissue treated with drug, enzymes, radiation or chemical treatment), for haplotying (comparing genes or variations in genes on each of the two alleles present in a human subject), for karyotyping (diagnostically comparing one or more genes in a test tissue, typically from an embryo or fetus prior to conception to detect birth defects, with the same genes from "normal" karyotyped subjects), and for genotyping (comparing one or more genes in a first individual of a species with the same genes in other individuals of the same species).

A typical analytical system has a number of components. These include, for example, (1) a nucleic acid template that is to be processed, (2) a delivery chamber, wherein the chamber comprises a coated array of the invention, an apparatus for containing the nucleic acid template, (3) a flow chamber and reagent delivery means that permits flow of nucleic acid processing reagents over the nucleic acid template where the assay reagents generate a detectable signal, *e.g.*, light, as the nucleic acid is processed, (4) an imaging system in communication with the reagent delivery chamber that detects the signal emitted as the nucleic acid is processed and that converts the captured light into data, and (5) a data collection system in communication with the reagent delivery chamber that processes the data to yield meaningful information about the nucleic acid that has been processed.

1. Thin Film Coated Arrays

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The present invention includes array compositions which include a substrate with a plurality of reaction chambers on its top surface. Each of the reaction chambers on the array is made up of a bottom and a sidewall, and at least one part, a bottom, sidewall, or top surface of the array is coated with one or more thin film coatings. In one embodiment, the array substrate has a non-transparent thin film coating on at least one bottom or sidewall of substantially all of the reaction chambers or the top surface, and the non-transparent coating is opaque, semi-opaque, shiny opaque, or translucent. In another embodiment, the substrate has a non-transparent coating on at least one bottom or sidewall of substantially all of the reaction chambers or the top surface of the array, and the substrate has a transparent coating on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array, where the transparent coating is from 0.1-5.0 microns thick and impermeable to water.

A. The Array Substrate

The array substrate is the solid support that can be modified to contain individual reaction chambers. Any material is used as the substrate. Substrate materials include, but are not limited to, organic polymers and plastics, such as vinyl polymers including polystyrene, polyethylene, polypropylene, polybutylene, polyvinyl chloride, and Teflon®, including copolymers and blends, as well as condensation polymers including polyethylene terephthalate, polyurethanes, polycarbonates, acrylics, polyamides, polyimides, polyesters, and epoxies, and silicones including copolymers and blends. Substrate materials may also include inorganic materials including ceramics, glasses, modified or functionalized glasses, silica or silica-based materials, silicon and modified silicon. Substrate materials may also include fiber optic bundles. In general, all of these types of materials are easily formed into arrays with reaction chambers. However, arrays made from such materials are often incompatible with many organic solvents and thus, applying a thin film coating to such an array substrate enhances the solvent compatibility of the array. Arrays also encounter problems such as physical interference and optical bleeding between neighboring reaction chambers. These types of problems may be solved by applying one or more thin film coatings which are opaque, semi-opaque, shiny opaque, or translucent to prevent photons from traveling through the transparent cladding material into the adjoining reaction chamber.

Generally, the substrate is planar (flat), although other configurations of substrates are used; for example, substrates are concave, convex, three-dimensional, *e.g.* spherical, textured,

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or cavitated, *e.g.*, in a cavitated terminus of a fiber optic fiber or in a microwell etched, molded, or otherwise micromachined into the planar surface, *e.g.* using techniques commonly used in the construction of microelectromechanical systems. See *e.g.*, Rai-Choudhury, HANDBOOK OF MICROLITHOGRAPHY, MICROMACHINING, AND MICROFABRICATION, VOLUME I: MICROLITHOGRAPHY, Volume PM39, SPIE Press (1997); Madou, CRC Press (1997), Aoki, *Biotech. Histochem.* 67: 98-9 (1992); Kane *et al.*, Biomaterials. 20: 2363-76 (1999); Deng *et al.*, *Anal. Chem.* 72:3176-80 (2000); Zhu *et al.*, *Nat. Genet.* 26:283-9 (2000). In one embodiment, the substrate is planar. A substrate may also contain features *e.g.*, spherical cavities, cylinder wells, columns, posts, and tilted cylinders. Such features on the surface of the substrate are formed by etching, plating, imprinting, stamping, molding, machining, etc.

In general, the substrate allows optical detection and does not itself appreciably fluoresce. The substrate is made of a material that facilitates detection of the chemical reaction event or assay result. For example, in a typical nucleic acid sequencing reaction, binding of a dNTP to a sample nucleic acid to be sequenced can be monitored by detection of photons generated by enzyme action on phosphate liberated in the sequencing reaction. Thus, having the substrate made of a transparent or light conductive material facilitates detection of the photons. In some embodiments, the substrate is optically transparent. In another embodiment, the surface of the substrate is optically interrogated.

1. Fiber Optic Faceplate ("FOF")

In one embodiment, the substrate of the array is fashioned from a "sliced" fiber optic bundle or a fiber optic faceplate ("FOF"). A FOF is fabricated by fusing many optical fibers together into a monolithic structure (i.e. a bundle) which retains the light transmission properties of the individual fibers. A fiber bundle is "sliced" to form a "wafer," a FOF. The resulting FOF possesses similar handling properties to that of a plane of glass or microscope slide. A FOF is a substrate onto which wells or chambers are etched to create an array of reaction chambers.

The individual fiber optic fiber strands which make up a FOF are composed of two materials, an inner "core" material and an outer layer "cladding" material (Figure 2). The fiber optic core includes a material which is transparent and has a high refractive index. Examples of core materials include heavy metal oxides such as lead, barium, lanthanum, and niobium oxides. The fiber optic cladding includes a material which is transparent and has a low refractive index. One typical cladding material is doped silicon dioxide. Doping agents

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include metal oxides, such as boron, and aluminum.

The individual fiber strands which make up a FOF each have a diameter. The individual fiber strands can be any size in diameter (*e.g.*, between 3 μm to 100 μm). In one embodiment, the individual fibers are between 6 μm to 12 μm in diameter. Once a fiber optic bundle has been fused to form a monolithic structure, the fiber strands are not individually manipulated; that is, one fiber strand generally cannot be physically separated at any point along its length from another fiber strand. Fused fiber optic bundles and face plates are obtained commercially from manufacturers.

A fiber optic bundle is "sliced" to form a "wafer," a FOF. In one embodiment, the fiber optic bundle is sliced perpendicular to the fused optical fibers. In another embodiment, the fiber optic bundle is sliced at an angle which is not perpendicular to the fused optical fibers (see, *e.g.*, Figures 20a-c). When the fiber optic bundle is sliced at an angle (the "slice angle") which is not 90 degrees to the fused optical fibers, the FOF produced contains fiber strands that are not perpendicular to the bottom of the FOF. When the FOF is etched, the reaction chambers that are formed are also not 90 degrees to the bottom of the FOF. The reaction chambers that are formed have a sidewall that is slanted ("tilted reaction chambers").

One surface of a FOF (i.e., the non-reaction chamber side) is typically highly polished so as to allow optical-coupling (*e.g.*, by immersion optic or other optical coupling fluids) to a detection device (Figure 2). In one embodiment, optical-coupling may be facilitated by a second fused fiber bundle. This second fused fiber bundle typically has a significantly smaller fiber size than the first FOF containing the reaction chambers, and serves to act as a conduit for the transmission of light product to the attached detection device, such as a CCD imaging system or camera.

In one embodiment, the array substrate is a FOF. The overall shape of a FOF is rectangular, although it is understood by those skilled in the art that a FOF is not limited to a specific shape and suitable FOFs include a variety of other shapes and overall dimensions. A FOF has at least a top and bottom surface which are opposed, wherein there is a distance between the top surface and the bottom surface. In one embodiment, the fiber strands within the FOF are oriented substantially perpendicular to the top and bottom surfaces of the FOF. In another embodiment, the fiber strands within the FOF are not oriented perpendicular to the top and bottom surfaces of the FOF. In one embodiment, a FOF further includes at least one index feature to provide a physical basis for ensuring proper orientation of a FOF in both automated equipment and for manual processes. For example, an index feature is used to

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properly position a FOF in the instrument for analysis.

Figures 8a-8c illustrate a FOF **1** for use with this invention. In one embodiment, a FOF **1** has a planar first surface **2** and a planar second surface **3**, which are opposed. A FOF **1** is optically conductive such that optical signals from the reaction chambers are detected through the first planar surface **3**, wherein there is a distance D_1 between the first surface and the second surface. The distance D_1 is any distance. In one embodiment, typically the distance D_1 between the first surface **2** and the second surface **3** is no greater than 10 cm. In another embodiment, the distance D_1 is no greater than 5 mm. In another embodiment, D_1 is between 0.5 mm to 5 mm. In a further embodiment, D_1 is 2 mm. The first surface **2** and second surface **3** of a FOF **1** are polished or alternatively non-polished. In one embodiment, one surface is polished and the opposing surface is non-polished. In one aspect of this invention, the first surface **2** is polished and the second surface **3** is non-polished. In a further embodiment, the first surface **2** is polished and the second surface **3** has reaction chambers. In another embodiment, the first surface **2** has reaction chambers and the second surface **3** is polished.

A FOF **1** has at least a first and second side and at least a first and second end. In one embodiment, a FOF **1** has a first end **4** and a second end **5** separated by a distance; a first side **6** and a second side **7** separated by a distance; and one corner side **8** extending between an end and a side. In one embodiment, shown in Figure 8a, a corner edge **8** extends between a second side **7** and a second end **5**. The ends and sides are connected to form the outer perimeter of a FOF **1**. For example, a first side **6** and the second side **7** both extend perpendicularly between the first and second ends **4, 5**.

The sides and/or ends of an FOF may be beveled at an angle in order to make it possible for the FOF to be mounted in an instrument. Any combination of sides and ends are beveled or alternatively, none of the sides or ends are beveled. In one embodiment, all of the sides and ends are beveled at an angle. A beveled side edge is beveled at an angle to form an inclined portion and a flat portion or alternatively, the beveled side has no flat portion. In one embodiment, the first side **6** is beveled at an angle A_1 to form an angled first side edge **17** (Figure 8b). The angled first side edge **17** is formed by an incline portion **15** and a flat portion **12**. The flat portion **12** of the angled first side edge **17** has a height of L_8. Similarly, the second side **7** is beveled at an angle to form an angled second side edge **16**. The first and second side edges **6** and **7** are beveled to any angle. In one embodiment, the side edges are beveled to an angle between 10 and 80 degrees. In another embodiment, the angle is

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substantially 45 degrees, wherein substantially means that the angle is a little more or a little less than 45 degrees. In one aspect of the invention, the opposing side edges are beveled to allow the FOF to slide into a suitable retaining structure (*e.g.*, a cartridge) located inside the analytical instrument that will capture, properly locate and mount the FOF relative to the fluidic reaction chamber and the camera.

In one embodiment, the first and second sides 6 and 7 are each beveled to form a 45 degree angle and the length L_8 of the resulting flat portion is 0.20 mm - 0.45 mm. The beveled sides 6 and 7 are located along the planar optically conductive surface 2.

Those of ordinary skill in the art will appreciate that the first surface and second surface of a FOF is any length and any width. In one embodiment, the length and width are the same. In embodiments where at least one of the sides is beveled, the width of the first surface is slightly smaller than the width of the second surface due to the angled side edge. For example, in one embodiment the width W_2 of the first surface 2 is about 38 mm and the width W_3 of the second surface 3 is about 40 mm (Figure 8b). In similar embodiments where at least one of the ends is beveled, the length of the first surface is slightly smaller than the length of the second surface.

In a first embodiment, second surface 3 has a length L_3 of about 75 mm, the width W_3 of the second surface 3 is about 40 mm. The first surface 2 has the same length L_3 as the second surface 3 and the width W_2 of the first surface 2 is about 38 mm.

In a second embodiment, second surface 3 has a length L_3 of about 75 mm, the width W_3 first surface 2 is about 25 mm. The first surface 2 has the same length L_3 as second surface 3 and the width W_2 of the first surface 2 is about 22 mm.

Suitable FOFs may include, for example, one or more index features. An index feature is located anywhere on a FOF and is not limited to any specific shape or size. In one embodiment, an index feature is located on the perimeter of the FOF. In a further embodiment, the index feature is located in a corner. The purpose of an index feature is to provide a physical basis to allow engagement of a FOF to ensure its proper orientation. As shown in Figure 8a, a suitable FOF 1 includes an index feature that is a corner notch 13 formed by removing a portion of the corner of the FOF where the side and end of the FOF connect perpendicularly. A FOF 1 has one or more corner notches. In one embodiment, a FOF 1 has one corner notch 13. In another embodiment, one corner notch 13 is formed by removing the corner, where the second side 7 connects the second end 5. The corner portion is cut off at an angle A_2 to create a corner notch 13. An angle A_2 is not limited to a particular

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degree and includes other angles and shapes. In one embodiment, an angle A_2 is removed from the corner portion of FOF 1 where the second side 7 connects to the second end 5 and the angle A_2 is about 45 degrees. The resulting corner side 8 has a length L_{11} which is any length. In one embodiment, L_{11} is about 6 mm. The corner notch 13 is matched with a complementary feature designed into the component in which a FOF 1 fits *e.g.*, a feature designed into the FOF mounting hardware of the analysis system or a feature designed into a gasket of an etching apparatus.

A FOF 1 may be labeled with one or more identifier codes. A FOF is marked with an identifier code for a variety of purposes. For example, an identifier code enables tracking and/or authenticating of a FOF. An identifier code also allows visual orientation of a FOF 1 when it is mounted in the analysis system (i.e., the system does not operate to analyze a FOF 1, unless the analysis system can properly read the identifier code). The identifier code may be any type of code *e.g.*, a bar-code, a two dimensional bar-code such as a Data Matrix code, etc. In one embodiment, a FOF 1 is coded with a bar-code. In another embodiment, a FOF 1 is coded with a Data Matrix code. In one embodiment, a FOF 1 has both a bar-code and Data Matrix code. An alpha-numeric code that can be read by a human may also be incorporated. The identifier code is read by an instrument, for example, a CCD camera, or a bar-code reader.

In one embodiment, an identifier code can be directly etched into the FOF surface with a laser. In an alternative embodiment, the identifier code is printed onto the surface of the FOF.

B. The Array Surface

Prior to coating with one or more thin film coatings, at least one surface of the substrate is modified to contain one or more individual reaction chambers, arranged so as to allow for the discrete localization of each reaction mixture or assay solution in a defined space, as well as for detection of the analytical result. The top or the bottom surface, or both surfaces can be modified to contain individual reaction chambers. Thus, as used herein, the term "reaction chamber" refers to a localized "well" or "chamber" on the substrate that facilitates interaction of reactants, *e.g.*, in a nucleic acid sequencing reaction. Typically, the reactants are distributed into the reaction chambers of the array in a medium which facilitates the chemical reaction or bioassay and which flows through the reaction chamber. For example, for DNA sequencing, a nucleic acid template is distributed into each reaction

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chamber on one or more solid supports, beads, or particles in a solution which flows through the reaction chamber.

The reaction chambers can be formed on either the top or bottom surface or both surfaces of the array substrate. A reaction chamber is a hollowed-out space having a width, a depth, and an opening. The opening of a reaction chamber can be any shape *e.g.*, substantially round, square, oval, rectangular, hexagonal, crescent, or star-shaped. The reaction chamber can be any shape *e.g.*, substantially round, square, oval, rectangular, hexagonal, crescent, or star-shaped. In one embodiment, the reaction chamber is circular or cylindrical. In another embodiment, the reaction chamber is multi-sided so as to approximate the shape of a square or rectangular box. Referring to Figure 6, the shape of the reaction chamber in one embodiment is substantially hexagonal. In one embodiment, the reaction chambers are uniform in shape.

Each reaction chamber on the array includes a bottom and a sidewall which define the boundary of the reaction chamber. The bottom of the reaction chamber can be any shape *e.g.*, substantially round, square, oval, rectangular, hexagonal, crescent, or star-shaped. The bottom of the reaction chamber can be either planar (i.e. flat), concave or convex. The bottom of the reaction chamber is opposed to the opening of the reaction chamber. The sidewall of the reaction chamber can be any shape. For example, the sidewall can be cylindrical in shape and connected to a round bottom. The sidewall of the reaction chamber can also have multiple sides *e.g.*, the sidewall of a reaction chamber with a hexagonal-shaped bottom has six sides which make up the sidewall of the reaction chamber. The sides of the reaction chamber which make up the sidewall are continuous or alternatively, the sides are discontinuous. For example, in a hexagonal shaped reaction chamber, reaction chamber can be closed, such that the sides are continuous and connect to each other or the reaction chamber can be open, such that the sides are not continuous and do not connect to each other. The sidewall can have a smooth surface or an irregular surface.

The bottom and the sidewall connect to form the bottom corner of the reaction chamber at the junction where the bottom and sidewall are joined. The angle at which the sidewall and bottom are connected can be substantially 90 degrees around the perimeter of the bottom or alternatively, the angle can vary from less than 90 degrees to greater than 90 degrees around the perimeter of the bottom. The connection between the bottom and sidewall can be continuous around the perimeter of the bottom. Alternatively, in some embodiments, the connection between the bottom and the sidewall is not continuous around

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the perimeter of the bottom.

In one embodiment, the inner surface of the reaction chamber takes the form of a well or chamber in the substrate, having a width and depth, into which reaction mixtures or assay solutions are deposited (Figure 2). In one embodiment, the reaction chambers are of sufficient dimension and order to allow for (i) the introduction of the necessary reactants into the chambers, (ii) chemical reactions or assays to take place within the chamber and (iii) inhibition of mixing of reactants and/or analytes between chambers.

In another embodiment, the sidewall of the reaction chamber is not continuous i.e., the sidewall is interrupted with an opening between adjacent reaction chambers such that reactants can flow from one reaction chamber to another.

The opening between the chambers can be any size and shape. For example, the opening can be one or more holes, channels, tubes, or slits. Such an opening in the sidewall facilitates the fast exchange of reagents. For example, reagents immobilized on the surface of beads ("reagent beads") which are small enough to fit the opening in the sidewall are free to flow between adjacent reaction chambers of the array, while reaction substrates immobilized on larger beads, which can not fit through the opening in the sidewall, are retained within the individual reaction chambers *e.g.* reaction substrates bound to beads ("substrate beads") or alternatively, reaction substrate beads can be bound to the bottom of the reaction chamber. The efficiency of reagent exchange between reaction chambers with an opening in the sidewall is generally improved and the time for chemical reactions or bioassays is reduced. In addition, the opening in the sidewall of the reaction chamber permits faster and more complete removal of reaction products and by-products from the chamber. In one embodiment, the opening is a slit in the cladding material between two adjacent reaction chambers on a FOF (Figure 26a-b).

In another embodiment, the sidewall is not continuous and contains one or more boundary posts that define the geometry of the reaction chamber. The boundary posts have any shape (*e.g.*, cylindrical, hemispherical, crescent-shaped, etc.). In one embodiment, the reaction chambers on the array are formed by a series of cylindrical boundary posts which extend upward from the substrate (*e.g.*, glass, non-fiber optic faceplate, etc.) to form a "post-array." In one embodiment, each chamber is defined by four boundary posts and the posts form the non-continuous sidewall of each reaction chamber on a post-array. In one embodiment, the post-array is used to "trap" a reaction substrate bead (Figure 21a-b). The number and size of the boundary posts, as well as the pattern of the boundary posts on the

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substrate can vary. This "open" type of reaction chamber facilitates a fast exchange between reagents that are able to flow through the reaction chambers of the array and the reaction substrates which are retained in the individual reaction chambers e.g., reaction substrates attached to beads or particles which are trapped between boundary posts. For more examples see, e.g., US Patent 10/260,704, Pengguang Yu, Kevin Kornelsen. US patent application number 20030091475, May 5, 2003.

In one embodiment, substantially all of the reaction chambers of the array have a sidewall that is connected at a perpendicular angle to the bottom of the substrate, and the opening of the reaction chamber and the bottom are opposed such that the center points of the bottom and the opening are aligned. Alternatively, in another embodiment, substantially all of the reaction chambers of the array have a sidewall that is not connected at a perpendicular angle to the bottom of the substrate, and the opening of the reaction chamber and the bottom are opposed such that the center points of the bottom and the opening are offset i.e., not aligned (see, *e.g.*, Figure 20b). The reaction chamber that is formed when the angle is not perpendicular and the center points are not aligned is referred to as a "tilted reaction chamber." The sidewall of a tilted reaction chamber is slanted and the angle at which the bottom and sidewall connect varies from between less than 90 degrees to greater than 90 degrees around the perimeter of the bottom corner of the reaction chamber. In one embodiment, the angle formed at the bottom corner area of the reaction chamber varies from between about 60 to 120 degrees. In another embodiment, the angle formed at the bottom corner area of the reaction chamber varies from between 80 to 100 degrees. In another embodiment, the angle formed at the bottom corner area of the reaction chamber varies from between 85 to 95 degrees. In another embodiment, the angle formed at the bottom corner area of the reaction chamber varies from between 88 to 92 degrees. In another embodiment, the angle formed at the bottom corner area of the reaction chamber varies from at 89 to 91 degrees.

In one embodiment, an array with tilted reaction chambers is formed when a FOF is sliced at a non-perpendicular angle, and the resulting FOF has optical fiber strands that are not perpendicular to the bottom of the faceplate (Figure 20a-b). When this FOF with non-perpendicular fibers is chemically etched, the reaction chambers that are formed each have a sidewall which is slanted i.e., a tilted reaction chamber. Tilted reaction chambers can be used to "fix" or "hold" a bead in a specific position within a tilted reaction chamber (Figure 20b). In one embodiment, centrifugal force is used to fix the position of a bead in a tilted reaction

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chamber. In another embodiment, the bead is fixed or held in one corner of the tilted reaction chamber. In another embodiment, centrifugal force is used to deposit a first bead (*e.g.*, a substrate bead) into each reaction chamber of the array). In another embodiment, centrifugal force is used to deposit a second bead, smaller than the first bead (*e.g.*, a reagent bead), into each chamber. In a further embodiment, centrifugal force is used deposit more than one second bead, which is smaller than the first bead, into each chamber.

The "Corner Area" of a reaction chamber is the inner surface of the bottom and the sidewall of the reaction chamber, where the bottom and sidewall of the reaction chamber connect to form a junction ("the junction") at the bottom corner (Figures 16a-b). The corner area includes both the "bottom corner area" and "sidewall corner area." The corner area extends from the junction along the length of the sidewall towards the top surface of the array and along the length of the bottom towards the center of the reaction chamber. The corner area does not extend the entire length of the sidewall or the entire length of the bottom. In one embodiment, the sidewall corner area extends from the junction to a length at least 90% of the total length of the sidewall. In another embodiment, the sidewall corner area extends from the junction to a length at least 60% of the total length of the sidewall. In another embodiment, the sidewall corner area extends from the junction to a length at least 40% of the total length of the sidewall. In another embodiment, the sidewall corner area extends from the junction to a length at least 20% of the total length of the sidewall. In another embodiment, the sidewall corner area extends from the junction to at least 10% of the total length of the sidewall. In another embodiment, the sidewall corner area extends from the junction to at least 5% of the total length of the sidewall. In another embodiment, the sidewall corner area extends less than 5% of the total length of the sidewall (see, *e.g.*, Figures 16a and 16b).

In another embodiment, the bottom corner area extends from the junction to a length at least 40% of the total length of the bottom. In another embodiment, the bottom corner area extends from the junction to a length at least 20% of the total length of the bottom. In another embodiment, the bottom corner area extends from the junction to a length of at least 10% of the total length of the bottom. In another embodiment, the bottom corner area extends from the junction to a length of at least 5% of the total length of the bottom. In another embodiment, the bottom corner area extends from the junction to a length of less than 5% of the total length of the bottom. In another aspect of the invention, the corner area forms the shape of a ring on the bottom of the reaction chamber, where the corner area does not

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include the area near the center of the bottom of the reaction chamber. In a further embodiment, an opening is formed near the center of the bottom of the reaction chamber by the coating that is partially applied to the bottom of the reaction chamber. In one embodiment, the reaction chambers of the array are in a pattern, i.e. a regular design or configuration, or the chambers can be randomly distributed on the array surface. In one embodiment, there is a regular pattern of reaction chambers on the array such that the chambers may be addressed in the X-Y coordinate plane. "Pattern" in this sense includes a repeating unit. In another embodiment, the array contains a high density of reaction chambers arranged in a pattern on the substrate. In one embodiment, an irregular hexagonal packed array of reaction chambers is on the surface of the array substrate as illustrated by Figure 6.

The reaction chambers may be spaced any suitable distance apart. Spacing is determined by measuring the distance between the center points of two adjoining reaction chambers (Figure 2). The reaction chambers are generally spaced between 5 μm and 200 μm apart. In one embodiment, the reaction chambers are spaced between 10 μm and 150 μm apart. In one embodiment, the reaction chambers are between 20 μm and 100 μm apart. In another embodiment, the reaction chambers are between 40 and 60 μm apart. In another embodiment, the reaction chambers have a spacing between the center points of two adjoining chambers of about 43 μm to 50 μm. The size of the reaction chamber is made to accommodate any volume. In one embodiment, the reaction chamber volume is between 10 to 150 pL. In another embodiment, the reaction chamber volume is between 20 to 90 pL. In a further embodiment, the reaction chamber volume is between 40 to 85 pL. In another embodiment, the reaction chamber volume is about 75 pL.

The reaction chambers can have any suitable width. In one embodiment, substantially all of the reaction chambers of the array have a diameter (width) in one dimension of between 3 μm and 100 μm. In another embodiment, substantially all of the reaction chambers have a diameter between 20 μm and 70 μm. In another embodiment, substantially all of the reaction chambers of the array have a diameter between about 30 μm and 50 μm. In a further embodiment, substantially all of the reaction chambers of the array have a diameter of between 38 μm to 44 μm.

The reaction chambers may have any suitable depth. The depth of substantially all of the reaction chambers is generally between 10 μm and 100 μm. In one embodiment, the depth of substantially all of the reaction chambers is between 20 μm and 60 μm. In another

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embodiment, the depth of substantially all of the reaction chambers is between 50-55 μm. Alternatively, substantially all of the reaction chambers have a depth that is between 0.25 and 5 times the width in one dimension of the reaction chamber or, in another embodiment, between 0.3 and 1 times the width in one dimension of the reaction chamber. For the purposes of this invention, substantially all of the reaction chambers means at least 90% of the reaction chambers. In another embodiment, substantially all of the reaction chambers means at least 95%. In another embodiment, substantially all of the reaction chambers means at least 97%. In a further embodiment, substantially all of the reaction chambers means at least 99%. In another embodiment, substantially all of the reaction chambers means 100% of the reaction chambers. In one embodiment, the reaction chamber ranges in depth on a FOF from approximately one-half the diameter of an individual optical fiber up to two to three times the diameter of the fiber. The depth of a reaction chamber is measured, for example using a MicroXam 3-D interferometric surface profiler (ADE Phase shift, San Jose, CA). Routine reaction chamber depth measurements are made using the area difference plot feature of the instrument. The instrument compares the depth of eight chambers to a reference point on the FOF cladding to provide an average chamber depth.

The array comprises a sufficient number of reaction chambers to carry out such numerous individual assays. The array contains any number of reaction chambers. Depending on the end use of the array, substrates are made to contain a very high density (*e.g.*, greater than 200,000), high density (*e.g.*, at least 100,000), moderate density (*e.g.*, at least 50,000), low density (*e.g.*, at least 10,000), and very low density (*e.g.*, less than 10,000) of reaction chambers. Low density arrays have a small number of reaction chambers. In one embodiment there are less than 10,000 reaction chambers. For example, the array contains between 1-96 reaction chambers. In one embodiment, the array contains between 96-384 reaction chambers. In another embodiment, the array contains between 384-1536 reaction chambers. In a further embodiment, the array contains greater than 1536 reaction chambers.

In one aspect of the invention, a FOF is made to contain a very large number of reaction chambers. In one embodiment, there are at least 10,000 reaction chambers. In another embodiment, there are at least 50,000 reaction chambers. In a further embodiment, there are greater than 100,000 reaction chambers. In another embodiment, there are greater than 200,000 reaction chambers on the surface of the substrate. Since the number of simultaneous analytical measurements is limited by the number of reaction chambers, the throughput of analytical measurement performed using an array may be increased by

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fabricating array substrates containing increasing densities of reaction chambers. Table 1 shows this progression for a 14 x 43 mm and 30 x 60 mm active areas, derived from 25 x 75 mm and 40 x 75 mm FOFs, respectively. See, for example, co-pending U.S. Patent Application Serial No.10/767,779, herein incorporated by reference. Pitch is the distance between fibers, measured 'center to center' (Table 1). Pitch and fiber size are generally equivalent.

Table 1: Development of arrays with a higher number of reaction chambers.

Pitch (um)	Reaction Chamber Diameter (um)	# of Reaction Chambers (14 x 43 mm)	# of Reaction Chambers (30 x 60 mm)
50	44	275K	800K
43	38	375K	1.2M
35	31	575K	1.6M
25	22	1.1M	3.2M

As illustrated above, one particular advantage of the present invention is that, particularly through the use of fiber optic technology, improved extremely high density arrays can be made. Thus, for example, it is possible to have as many as 50,000 different fibers and cells in a 1 mm^2 fiber optic bundle, with densities of greater than 250,000 individual fibers per 0.5 cm^2 obtainable.

For example, a wide channel reaction chamber can have dimensions of approximately 14 mm x 43 mm. Thus, with this approximate dimension and at approximately 4.82 x 10^{-4} chambers/um^2 density, the array can have approximately 290,000 reaction chambers.

Reaction chambers are formed in the surface of the substrate as is generally known in the art using a variety of techniques, including but not limited to, chemical etching, photolithography, stamping techniques, pressing, casting, molding, microetching, electrolytic deposition, chemical or physical vapor deposition employing masks or templates, electrochemical machining, laser machining or ablation, electron beam machining or ablation, and conventional machining. The technique will depend on the composition and shape of the substrate. In one embodiment, reaction chambers are formed using chemical etching. Reaction chambers are typically formed in the substrate prior to coating the array with any thin film coating.

C. The Thin Film Coatings

The present invention provides for the application of one or more thin film coatings to

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the array substrate. A thin film coating is a transparent or a non-transparent coating. Such transparent and non-transparent thin film coatings are designed to improve the properties and functions of the array, including to improve the compatibility of the reaction mixture or assay solution with the array substrate and to reduce problems such as optical bleed and physical interference between neighboring reaction chambers. The transparent thin film coating provides a barrier between the solution contained in the reaction chamber and the substrate and prevents both leaching of the substrate material into the solution and contact between the contents in the reaction chamber and the substrate. The non-transparent thin film coating provides a photon barrier which prevents the leaking of photons from one reaction chamber into another adjoining reaction chamber (i.e. cross-talk). The non-transparent coating creates a barrier which does not permit light to pass between neighboring reaction chambers and thus, restrains the photons and keeps the light in the reaction chamber to eliminate optical scattering. A non-transparent coating can be used to improve the retention of beads or particles in the reaction chamber (*e.g.*, solid-support beads for carrying out a chemical reaction).

The thin film coatings of the present invention are typically applied to coat the bottom or sidewall of the reaction chambers or the top surface of the array. For the purposes of this invention, when a bottom or sidewall of the reaction chamber or the top surface of the array is coated with a thin film coating, the coating partially, substantially, or completely covers the surface of the bottom, sidewall or top surface. In one embodiment, the bottom, sidewall, or top surface is completely covered when the surface of the bottom, sidewall, or top surface is covered 100% with the thin film coating. In another embodiment, the bottom, sidewall, or top surface is substantially coated when the surface of the bottom, sidewall, or top surface is covered 97-100% with thin film coating. In another embodiment, the bottom, sidewall, or top surface is partially coated when the surface of the bottom, sidewall, or top is covered less than 97% with thin film coating. In another embodiment, the bottom, sidewall, or top surface is partially coated when at least 80% of the bottom, sidewall, or top is covered with thin film coating. In another embodiment, the bottom, sidewall, or top surface is partially coated when at least 60% of the bottom, sidewall, or top is covered with thin film coating. In another embodiment, the bottom, sidewall, or top surface is partially coated when at least 40% of the bottom, sidewall, or top is covered with thin film coating. In another embodiment, the bottom, sidewall, or top surface is partially coated when at least 20% of the bottom, sidewall, or top is covered with thin film coating. In another embodiment, the bottom, sidewall, or top

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surface is partially coated when at least 10% of the bottom, sidewall, or top is covered with thin film coating.

In one aspect of the invention, the non-transparent coating is deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array. In another embodiment, the non-transparent coating is deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array, and the non-transparent coating is deposited on the array prior to a transparent coating being deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array. In another embodiment, a transparent coating is deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array, and the transparent coating is deposited on the array prior to a non-transparent coating being deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array.

The Transparent Thin Film Coating

In one embodiment, the substrate of the array is coated with a non-transparent thin film coating in combination with a transparent thin film coating, which is comprised of a material typically known to be compatible with components found in assay solutions and chemical reaction mixtures. The transparent coating can be deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array prior to the non-transparent coating being deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array. Alternatively, the transparent coating can be applied after the non-transparent coating is deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array.

In one embodiment, the transparent coating is impermeable to water. In another embodiment, the transparent coating provides for a uniform surface composition. In one embodiment, the transparent coating is optically transparent. Other desirable properties of the transparent coating include durability, compatibility with the substrate materials, well-understood deposition parameters, and resistance to high temperatures. In one embodiment, the transparent coating is adhesive to glassy materials. Typically, the transparent coating minimizes non-specific absorption of macromolecules. The array substrate can be coated

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completely with the transparent thin film coating. In one embodiment, the entire array substrate, including of each bottom and sidewall of substantially all of the reaction chambers and top surface of the array has a transparent coating. Alternatively, the array substrate may not be coated completely with the transparent coating. For example, at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array is coated with a transparent coating (Figure 2). Alternatively, the transparent coating is absent. In another embodiment, the bottom of substantially all of the reaction chambers of the array has a transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers of the array has a transparent coating, and the sidewall of substantially all of the reaction chambers and top surface of the array do not have a transparent coating. In another embodiment, the sidewall of substantially all of the reaction chambers of the array has a transparent coating. In another embodiment, the sidewall of substantially all of the reaction chambers of the array has a transparent coating, and the bottom of substantially all of the reaction chambers of the array and top surface of the array do not have a transparent coating. In another embodiment, the sidewall of substantially all of the reaction chambers of the array and top surface of the array have a transparent coating. In a further embodiment, the sidewall of substantially all of the reaction chambers and top surface of the array have a transparent coating, and the bottom of substantially all of the reaction chambers does not have a transparent coating.

In another embodiment, at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array is partially coated. In one embodiment, the bottom and sidewall of substantially all of the reaction chambers of the array are partially coated with a transparent coating, such that the transparent coating is applied to the corner area formed at the junction between the bottom and the sidewall and is absent from the center of the bottom such that the absence to the transparent coating forms an aperture (i.e., an opening) near the center of the bottom, (*e.g.*, see Figures 16a and16b). The size of the aperture can be adjusted during the coating process such that the amount of light allowed through the bottom of the reaction chamber can be modulated and concentrated.

In one aspect of the invention, the transparent coating is applied to a feature located on a substrate. Examples of such features which may be coated on a substrate include: spherical cavities, cylinder wells, columns, posts, tilted cylinders, etc. In another embodiment, an array made of a substrate and boundary posts built on top of the substrate to form reaction chambers i.e., a "post-array" is coated with a transparent coating (Figures 21a-

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b). The size, number, and pattern of the boundary posts on the substrate can vary. The boundary posts can take any number of shapes (e.g., circular, rectangular, hexagonal, etc.). In one embodiment, a first transparent coating is applied to the post-array. In another embodiment, a second transparent coating is applied to the post-array after the first transparent coating has been applied, such that the first and second transparent coatings are different (*e.g.*, see Figure 21b). In another embodiment, deposition of the first and second transparent coatings onto the post-array array creates a "shadow area" wherein no coating is applied *e.g.*, see Figure 21b.

In another aspect of the invention, the transparent coating is applied to an array where substantially all of the reaction chambers have a sidewall that is not continuous i.e., the sidewall is interrupted with an opening between adjacent reaction chambers. The opening can be any size and shape. In one embodiment, the opening is a slit as shown in Figure 26a. The opening in the sidewall of the reaction chamber provides a means for depositing the coating in a particular pattern on the bottom of the reaction chamber (e.g., Figure 26b). In one embodiment, the pattern on the bottom of the reaction chamber is cone shaped. In another embodiment, the transparent coating is deposited in a gradient, such that the thickness of the coating varies. In one embodiment, the transparent coating is thicker near the center line of the bottom of the reaction chamber, and the coating becomes thinner away from the center line towards the edge.

In another aspect of the invention, the transparent coating is applied to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array where the reaction chambers have sidewalls that are not perpendicular to the bottom of the substrate i.e., tilted reaction chambers. In one embodiment, the transparent coating is applied to a fiber optic faceplate with optical fiber strands that are not perpendicular to the bottom of the faceplate, where at least one bottom or sidewall of the reaction chambers or top surface of the fiber optic faceplate is coated with the transparent coating.

The term "transparent thin film" refers to a transparent coating with a thickness that is significantly smaller than other characteristic dimensions of the array. In one embodiment, the thickness of the transparent coating is from 0.1-5.0 microns. The thickness of the transparent coating may be non-uniform over the surface of the array, including on the inner surface of the reaction chamber. For example, in one embodiment, when present, the thickness of the transparent coating of substantially all of the reaction chambers of the array is approximately 200-400 nm on the top surface of the array. In another embodiment, when

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present, the thickness of the transparent coating of substantially all of the reaction chambers of the array is approximately 50-100 nm on the sidewall of the reaction chamber. In another embodiment, when present, the thickness of the transparent coating of substantially all of the reaction chambers of the array is approximately 100-300 nm on the bottom of the reaction chamber.

Many different types of materials can be used as a transparent coating. The composition of a transparent thin film coating will depend on the array substrate, the application, and the method of thin film deposition. In one embodiment, the transparent coating is a polymer. In one embodiment, the polymer is an inorganic polymer. In another embodiment, the transparent coating is a non-metal oxide (*e.g.* silicon dioxide (SiO_2)). Other transparent coatings are, for example, a metal alloy, a metal or semi-conductor oxide, nitride, carbide, or boride. Many transparent coatings are commercially available.

Transparent coating materials also include those materials used to attach an anchor primer to a substrate. Organosilane reagents, which allow for direct covalent coupling of proteins via amino, sulfhydryl or carboxyl groups, can also be used as a transparent coating to coat the array substrate. Additional transparent coatings include photoreactive linkers, *e.g.* photobiotin, (Amos et al., "Biomaterial Surface Modification Using Photochemical Coupling Technology," in *Encyclopedic Handbook of Biomaterials and Bioengineering, Part A: Materials*, Wise et al. (eds.), New York, Marcel Dekker, pp. 895926, 1995).

Other transparent coating materials include polymeric materials such as hydrophilic polymer gels such as polyacrylamide and polysaccharides, which are polymerized directly on the surface of the substrate or polymer chains that are covalently attached to the substrate directly (Hjerten, *J. Chromatogr.* 347,191 (1985); Novotny, *Anal. Chem.* 62,2478 (1990), as well as pluronic polymers (triblock copolymers, *e.g.* PPO-PEO-PPO, also known as F-108), specifically adsorbed to either polystyrene or silanized glass surfaces (Ho et al., *Langmuir* 14:3889-94, 1998), as well as passively adsorbed layers of biotin-binding proteins. The surface can also be coated with a transparent coating comprising an epoxide which allows the coupling of reagents via an amine linkage. In one embodiment, the transparent thin film coating is SiO_2.

Prior to applying any thin film coating, the FOF is cleaned by sonication in an aqueous, basic solution *e.g.*, 5% Contrad® solution. Contrad® solution is a cleaning solution made up of surfactants in an alkaline basic aqueous solution. The 5% Contrad® solution contains substantially 5 percent Contrad®. Substantially 5% means that the solution may be

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a little more or a little less than 5% Contrad®.

In one embodiment, after cleaning, an ion-plating process is typically used to coat the etched FOFs with a transparent coating of SiO_2, wherein the thickness of the transparent coating is from 0.1-5.0 microns. In one embodiment, when present, the thickness of the transparent coating is 200-400 nm on the top surface of the array. In another embodiment, when present, the thickness of the transparent coating is 50-100 nm on the sidewall and when present, is 100-300 nm on the bottom of substantially all of the reaction chambers of the array. SiO_2 is transparent, has a very efficient water barrier thicknesses down to 10 nm, adheres to glassy materials, and with stands harsh cleaning procedures and high temperatures. Further, the surface properties of SiO_2 are well known, as are methods for modifying these properties. Further, SiO_2 has also been shown to be compatible with microscale polymerase chain reaction ("PCR") conditions.

The Non-Transparent Thin Film Coating

In another embodiment, the substrate of the array is coated with a non-transparent thin film coating made up of a material that modulates the passage of light there through e.g., blocks, substantially blocks or diffuses light to prevent, inhibit, or reduce optical bleeding of photons into adjacent reaction chambers and physical interference between neighboring reaction chambers.

The thickness of the non-transparent coating can be varied and controlled such that an opaque, semi-opaque, shiny opaque, or translucent non-transparent coating is obtained. In one embodiment, the non-transparent coating is opaque. In another embodiment, the non-transparent coating is semi-opaque. In another embodiment, the non-transparent coating is shiny opaque. In a further embodiment, the non-transparent coating is translucent. Other desirable properties of the non-transparent coating include durability, compatibility with substrate materials, well-understood deposition parameters, and resistance to high temperatures. In one embodiment, the non-transparent coating is adhesive to glassy materials. The non-transparent coating minimizes non-specific absorption of macromolecules. The array substrate can be coated in its entirety with the non-transparent thin film coating. Alternatively, the array substrate may not be coated in its entirely with the non-transparent coating. In one embodiment, at least one of the bottom or sidewall of substantially all of the reaction chambers or top surface of the array has a non-transparent coating (Figure 2). In one embodiment, the entire array substrate, including each bottom and

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sidewall of substantially all of the reaction chambers and top surface of the array has a non-transparent coating. In another embodiment, the top surface of the array and the sidewall and bottom of substantially all of the reaction chambers is coated with a non-transparent coating.

In another embodiment, the bottom of substantially all of the reaction chambers of the array has a non-transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers of the array has a non-transparent coating, and the sidewall of substantially all of the reaction chambers and the top surface of the array do not have a non-transparent coating. In another embodiment, the sidewall of substantially all of the reaction chambers has a non-transparent coating. In another embodiment, the sidewall of substantially all of the reaction chambers has a non-transparent coating, and the bottom of substantially all of the reaction chambers and the top surface do not have a non-transparent coating. In another embodiment, the sidewall of substantially all of the reaction chambers and the top surface of the array has a non-transparent coating. In a further embodiment, the sidewall of substantially all of the reaction chambers and the top surface of the array have a non-transparent coating, and the bottom of substantially all of the reaction chambers does not have a non-transparent coating.

In another embodiment, at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array is partially coated with a non-transparent coating. In one embodiment, the bottom and sidewall of substantially all of the reaction chambers of the array is partially coated with a non-transparent coating, such that the non-transparent coating is applied to the corner area formed at the junction between the bottom and the sidewall, and the non-transparent coating is absent from the center of the bottom such that the absence of the non-transparent coating forms an aperture (i.e., an opening) near the center of the bottom (e.g., see Figure 16a and Figure 16b). The size of the aperture can be adjusted such that the amount of light allowed through the bottom of the reaction chamber can be modulated and concentrated.

The non-transparent coating can be used to coat, i.e. cover, a feature located on a substrate. Examples of such features which can be coated on a substrate include: spherical cavities, cylinder wells, columns, posts, tilted cylinders, etc. In another embodiment, an array comprising a substrate with boundary posts built on top of the substrate to form reaction chambers (a "post-array") is coated with a non-transparent coating. The size, number, and pattern of the boundary posts on the substrate can vary. In one embodiment, a first non-transparent coating is applied to the post-array. In another embodiment, a second non-

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transparent coating is applied to the post-array after the first non-transparent coating is applied, and the first and second non-transparent coatings are different (Figure 21b). In another embodiment, deposition of the first and second non-transparent coatings onto the post-array array creates a "shadow area" where no coating is applied *e.g.*, see Figure 21b.

In another aspect of the invention, the non-transparent coating is applied to an array where substantially all of the reaction chambers have a sidewall that is not continuous i.e., the sidewall is interrupted with an opening between adjacent reaction chambers. The opening can be any size and shape. In one embodiment, the opening is a slit as shown in Figure 26a. The opening in the sidewall of the reaction chamber provides a means for depositing the non-transparent coating in a particular pattern on the bottom of the reaction chamber (*e.g.*, Figure 26b). In one embodiment, the pattern is cone shaped. In another embodiment, the non-transparent coating is deposited in a gradient such that the thickness of the coating varies where the non-transparent coating is thicker near the center line of the bottom of the reaction chamber, and the non-transparent coating becomes thinner away from the center line towards the edge.

In another aspect of the invention, the non-transparent coating is applied to at least one bottom or sidewall of the substantially all of the reaction chambers or the top surface of the array which has reaction chambers with sidewalls that are not perpendicular to the bottom of the substrate i.e., tilted reaction chambers. In one embodiment, the non-transparent coating is applied to a fiber optic faceplate with optical fiber strands that are not perpendicular to the bottom of the faceplate where at least one bottom or sidewall of substantially all of the reaction chamber or the top surface of the fiber optic faceplate is coated with the non-transparent coating.

The term "non-transparent thin film" refers to a non-transparent coating with a thickness that is significantly smaller than other characteristic dimensions of the array (Figure 14). In one embodiment, the thickness of the non-transparent coating determines whether the coating is opaque, semi-opaque, translucent, or shiny opaque.

Many different types of materials can be used as non-transparent thin film coatings. The composition of the non-transparent thin film coating will depend on the array substrate, the application, and the method of thin film deposition. A non-transparent thin film coating can be selected from an organic compound, an inorganic compound, and a non-metal oxide. In one embodiment, the non-transparent coating is a polymer. In another embodiment, the non-transparent coating is an organic or inorganic compound. In one embodiment, the

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inorganic compound is a metal. Metals that are used as non-transparent thin film coatings are selected from chromium, gold, silver, aluminum, titanium, copper, iron, nickel, zinc, cadmium, tin, lead, antimony, cobalt, platinum, and any alloys thereof, e.g., titanium/lead. In one embodiment, the non-transparent coating is selected from chromium, gold, silver, aluminum, titanium, and platinum. In one embodiment, the non-transparent coating is chromium. In another embodiment, the non-transparent coating is silver.

In one embodiment, a first non-transparent coating is applied to the array, and a second non-transparent coating is applied to the array after the first non-transparent coating is applied. In one embodiment, the first and second non-transparent coatings are the same. Alternatively, the first and second non-transparent coatings are different. In one embodiment, the first non-transparent coating is chromium, and the second non-transparent coating is gold. In another embodiment, the first non-transparent coating is gold and the second non-transparent coating is chromium. In another embodiment, the first non-transparent coating is titanium and the second non-transparent coating is platinum. In another embodiment, the first non-transparent coating is platinum and the second non-transparent coating is titanium. The first and second non-transparent coatings can be applied in separate processes. Alternatively, the first and second metal coatings can be applied in the same process by switching to different metal targets.

In another embodiment, the coating is dielectric. The dielectric coating can be non-transparent or transparent. In one embodiment, the dielectric coating is non-transparent. In another embodiment, the dielectric coating is transparent. A coating which is dielectric has a high electrical resistance, for example, SiO_2. In one embodiment, a transparent, dielectric coating is applied on top of a non-transparent coating and is used to protect the non-transparent coating from erosion. For example, a SiO_2 coating is applied after a metal coating is applied to an array, in order to protect the metal coating from erosion while the SiO_2 coating remains transparent.

In other embodiment, the coating is conductive. A coating which is conductive is electrically conductive, for example, a gold thin film. A conductive coating can be used as a first coating for electroplating or used as an electrode surface in a solution. In one embodiment, a non-transparent coating which is conductive is applied to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array. In another embodiment, a non-transparent coating, which is conductive, is applied to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array and a

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transparent coating is applied to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array after the non-transparent coating is applied. In one embodiment, the array is coated with a conductive coating which allows for the chemical reactions or bioassays carried out in the reaction chambers to be electrochemically enhanced. For example, a metal coated, etched FOF can be used as an anode or a cathode for analysis using electrochemical methods in conjunction with illuminochemistry. In another embodiment, a SiO_2 coating is applied on top of a conductive metal coating and the SiO_2 coating of the array is polarized which enhances the movement of ions near the surface in solution. Many non-transparent coatings are commercially available.

In another aspect of the invention, the array substrate is coated with both a transparent and non-transparent coating. In one embodiment, the sidewall of substantially all of the reaction chambers and top surface of the array are coated with the transparent coating, and the bottom is coated with the non-transparent coating. In another embodiment, the sidewall and bottom of substantially all of the reaction chambers are coated with the transparent coating, and the top surface of the array is coated with the non-transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers and the top surface of the array are coated with the transparent coating, and the sidewall of substantially all of the reaction chambers is coated with the non-transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers is coated with the transparent coating, and the sidewall of substantially all of the reaction chambers and top surface of the array are coated with the non-transparent coating. In another embodiment, the top surface of the array is coated with the transparent coating, and the sidewall and bottom of substantially all of the reaction chambers are coated with the non-transparent coating. In another embodiment, the sidewall of substantially all of the reaction chambers is coated with the transparent coating, and the bottom of substantially all of the reaction chambers and the top surface of the array are coated with the non-transparent coating. In another embodiment, the bottom of substantially all of the reaction chambers is coated with the transparent coating, the sidewall of substantially all of the reaction chambers is coated with the non-transparent coating, and the top surface of the array is not coated.

Multiple layers of transparent and non-transparent coatings can be deposited on the array substrate. In one embodiment, the transparent coating includes at least a first transparent coating. In another embodiment, the non-transparent coating includes at least a first non-transparent coating. Two or more coatings of transparent and/or non-transparent

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coatings can be deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array. In one embodiment, the first non-transparent coating, *e.g.*, chromium, is deposited on one side of the sidewall of substantially all of the reaction chambers of the array, and the second non-transparent coating, *e.g.*, gold, is deposited on the opposite side of the sidewall of substantially all of the reaction chambers of the array. In another embodiment, at least one of the bottom or sidewall of substantially all of the reaction chambers or top surface of the array is coated with the first and second non-transparent coating. In one embodiment, the bottom of substantially all of the reaction chambers of the array is coated with a first transparent coating, and the bottom and sidewall of substantially all of the reaction chambers of the array are coated with a partial transparent second coating, further wherein the second transparent coating is applied to the corner area formed at the junction between the bottom and sidewall of the reaction chamber, and the second transparent coating is absent from the center of the bottom such that the absence of the second transparent coating on the bottom of the reaction chamber forms an aperture near the center of the bottom. In one embodiment, the bottom and sidewall of substantially all of the reaction chambers of the array are coated with a partial non-transparent coating, further wherein the transparent coating is applied to the corner area formed at the junction between the bottom and sidewall of the reaction chamber, and the non-transparent coating is absent from the center of the bottom such that the absence of the non-transparent coating on the bottom of the reaction chamber forms an aperture near the center of the bottom.

Functionalization of the Transparent and Non-transparent Thin Film Coatings

Both the transparent and non-transparent thin film coatings allow for easy attachment of reactants (*e.g.* proteins, enzymes, and nucleic acids) and do not negatively affect the activity of immobilized reactants. In some instances, the thin film coating can increase the stability of reactants.

Transparent and non-transparent coatings allow for surface modification which can provide additional functions to enhance chemical reactions or to eliminate unwanted effects such as cross-talk between adjacent reaction chambers.

Generally, the reactants and analytes are non-covalently associated in the reaction chambers. However, thin film coated chambers can be biologically or chemically functionalized. For example, any of the transparent or non-transparent thin film coatings discussed can be derivatized with one or more functional groups, commonly known in the art

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for the immobilization of enzymes and nucleotides, *e.g.* metal chelating groups (*e.g.* nitrilo, triacetic acid, iminodiacetic acid, pentadentate chelator, etc.). In one embodiment, substantially all of the reaction chambers of the thin film coated array, coated with either a transparent or non-transparent coating, are modified to contain functional groups that are used to attach or capture, either covalently or non-covalently, reactants or analytes to the sidewall or bottom. "Chemically modified reaction chambers" in this context include, but are not limited to, the addition of functional groups including amino groups, carboxy groups, oxo groups and thiol groups, that are attached to the reaction chamber thin film coated surface and used to attach or capture reactants or analytes on the same surface. Alternatively, "biologically modified reaction chambers" include the attachment of binding ligands or binding partner pairs, including but not limited to, antigen/antibody pairs, enzyme/substrate or inhibitor pairs, receptor-ligand pairs, carbohydrates and their binding partners (lectins, etc.).

In one embodiment, the transparent coating generates a surface for chemical or biological modification. In another embodiment, the non-transparent coating generates a surface for chemical or biological modification. In a further embodiment, the transparent coating provides a surface for enzyme immobilization. In one embodiment, the enzyme for immobilization is selected from sulfurylase, luciferase, polymerase, hypoxanthine, phosphoribosyltransferase, xanthine oxidase, uricase, apyrase, and peroxidase. In another embodiment, the non-transparent coating provides a surface for enzyme immobilization. In another embodiment, the first coating provides a means for trapping reactants or analytes in the reaction chamber. In another embodiment, the non-transparent coating provides a means for trapping reactants or analytes in the reaction chamber.

2. Process for Single Sided Chemical Etching of a Fiber Optic Faceplate

The present invention is further directed to a process for producing reaction chambers on an array substrate and an apparatus for performing such a process. In one embodiment, reaction chambers are formed on a FOF substrate. In one embodiment, reaction chambers are formed using a selective chemical etching process which takes advantage of the difference in etch rates between core and cladding materials. See, *e.g.*, Pantano, et al., *Chem. Mater.* 8:2832 (1996), and Walt, et al., U.S. Patent Publication No. 20020015146. Reaction chambers may be formed on one or both sides of a FOF. Methods for forming reaction chambers on both sides of the substrate require no special hardware or an apparatus beyond

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an etching bath. However, in one embodiment, the reaction chambers etched on one of the side of the FOF subsequently need to be removed so that the surface will be smooth enough for optical coupling to a camera system. This removal process involves an expensive and time-consuming polishing step. If reaction chambers are formed only on a single side, the FOF is almost immediately ready for cleaning and surface coating. The present invention provides a both an apparatus and a chemical etching process which are designed to produce reaction chambers on only one side of a FOF.

A. Etch Apparatus: Clamps and Gasket

Generally, an apparatus used to etch a FOF on a single side includes the following pieces of hardware: at least one clamp and a gasket (Figures 12 and 13). The term "sandwich" refers to two FOFs with a gasket positioned in between the two FOFs. A clamp **39** is a device which is used to press or hold firmly the two FOFs **1** together with a gasket **75** to form a "clamped sandwich" which is used during the etch process. A gasket **75** is designed to create a fluid tight seal in a clamped sandwich, *e.g.*, a gasket is placed in between two FOFs in order to protect one side of each FOF from exposure to acid. Both the FOFs and gasket each contain an index feature which provides a physical basis for mounting the FOFs so that only one side of each FOF is subjected to acid treatment.

1. Clamp

The present invention comprises a clamp which includes two joined components, a base and prongs. Both components are constructed from a single suitable material, or alternatively, each component is constructed separately from suitable materials and mated together using any variety of mating techniques known in the art (*e.g.*, adhesively attached, mechanically mated etc.). A base is generally the shape of a rectangular box having at least a top surface and a bottom surface which are opposed, wherein there is a distance between the top and bottom surface. A bottom surface is permanently joined to a top surface by sides and ends which are permanently joined to form the outer perimeter of the base. A base has at least a first and second side and at least a first and second end. A surface of a base is generally planar.

At least two prongs are aligned on a surface of a base. In this version of the invention, at least two prongs are aligned on a top surface of a base and extend radially outward a distance. Any number of prongs are aligned on a top surface. A suitable prong is

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any shape or size. For example, a prong is a tapered rectangle having at least four edges, including an external and internal edge and a first and second edge. External and internal edges are opposed and first and second edges are opposed. The edges of a prong are connected to form a tapered rectangular shaped prong. An internal edge of a prong is flat and attaches perpendicularly to a top surface of a base. A prong has a top and a height. A pair of prongs is positioned along a top surface, with the internal edge of one prong opposed to the internal edge of a second prong to form a slot down the middle of the top surface of a base. A slot with a width W_{11} is where FOFs and a gasket are securely held by the prongs of a clamp. It is understood by those in the art that a clamp is not limited to a specific shape and includes other shapes and overall dimensions.

Referring to Figures 7a-d, one embodiment of the invention includes a unitary clamp **39** comprised of a rectangular shaped base **40** and at least two prongs **43, 44**. A base has a top surface **41** and a bottom surface **42**, which are opposed. A bottom surface **42** is permanently joined to a top surface **41** by any number of sides and ends, which are connected to form the outer boundary of a base **40**. The distance D_2 extending between the top surface **41** and the bottom surface **42** is any distance. In one embodiment, the distance between the top and bottom surfaces **41, 42** is no greater than 10 cm. In another embodiment, D_2 is 0.5 mm to 5 mm. In a further embodiment, the distance D2 is about 4 mm.

In one embodiment, a rectangular base **40** has a first side **35** and a second side **36** separated by a distance W_{12} and a first end **37** and a second end **38** separated by a distance L_{10}. In a more preferred embodiment, a base **40** is rectangular shaped with flattened corners. For example, a base **40** has a first side **35** and a second side **36** separated by a distance W_{12}; a first end **37** and a second end **38** separated by a distance L_{10}; and four corner ends, including a first corner end **45**, a second corner end **46**, a third corner end **47**, and a fourth corner end **48**.

Those of ordinary skill in the art will appreciate that the sides, ends, and corner ends of a base **40** are any length. In one embodiment, the first and second sides **35, 36** are the same length; the first and second ends **37, 38** are the same length; and the four corner ends **45, 46, 47, 48** are the same length. In one embodiment, the length and width are the same. In another embodiment, the sides are longer in length than the ends. For example, the first side **35** and second side **36** each have a length L_9 of about 58 mm. The first end **37** and second end **38** each have a length L_{11} that is about 12 mm. The four corner ends, including **45, 46, 47**, and **48** each have a width L_{13} that is about 2 mm.

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A prong is any shape and has any dimensions, including height. In this version of the invention, the general shape of the prong is a tapered rectangle. A prong comprises multiple edges, a prong top and a height. In one embodiment, a prong **43** has four edges, including an internal edge **49** and an external edge **52** which are opposed, and a first edge **50** and a second edge **51** which are opposed. In one embodiment, the surface of an inner edge **49** is flat and perpendicular to the top surface **41**. The surface of the external edge **52** is flat and forms an angle A_4 with the top surface **41**. In one embodiment, the angle A_4 is 45 degrees. A first edge **50** has a surface that is concave and a second edge **51** has a surface that is concave. A prong top may be any shape and have any dimensions. In one embodiment, a prong top **55** is pointed. Alternatively, a prong top is rounded or flat. In one aspect of the invention, the prong top **55** is flat and rectangular shaped. In a further embodiment, the rectangular area of the prong top **55** is about 1 mm X 2 mm. A prong (*e.g.*, 44) may be any height. In one embodiment, the height H_6 of a prong is about 5-6 mm.

In one invention, a clamp **39** uses a set of at least two prongs to compress FOFs and a gasket together for the etching process. The surface of a base **40** has any number of prongs. Generally, prongs are aligned along the top surface **41**. In one embodiment, a prong **43** is aligned on a top surface **41** with its external side **52** along a first side **35** and another prong **44** is opposed and aligned on a top surface **41** with its external side along a second side **36**. A clamp **39** may have one or more pairs of prongs. In one invention, a clamp **39** has multiple pairs of prongs. In one aspect of the invention, a clamp **39** has one to six pairs of prongs aligned on its top surface **41**. It is understood by one skilled in the art that the number of pairs of prongs can vary depending on the size of the FOF.

A pair of at least two prongs **43, 44** and base **40** form a unitary clamp **39** comprised from a single suitable material or from separate suitable materials which are integrally formed or mated together. In this version, the clamp **39** has a slot **56** located down the center of the top surface **41**. The slot **56** is where the FOFs and gasket are held by the clamp **39**. The slot **56** is created by the alignment of the prongs **43** and **44** on the top surface **41** along the first and second sides **35, 36**. The length L_{10} of slot **56** extends from the first end **37** to the second end **38**. The width W_{11} of slot **56** is the distance between the internal edges of a prong **44** and the internal edge of a prong **43**. The slot can have any width and any length. The width W_{11} of a slot **56** is generally equivalent to the width of two FOFs and a gasket sandwiched together. In one embodiment, the width W_{11} of the slot is about 6 mm. In one embodiment, the length L_{10} of the slot **56** is about 62 mm.

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A clamp is comprised of any suitable material. In one embodiment, a clamp is fabricated from any acid resistant material. In a further embodiment, the clamp is fabricated from a plastic material. In a most preferred embodiment, the clamp is fabricated poly ether ether ketone ("PEEK clamp").

2. Gasket

The present invention includes a gasket as one of the pieces of hardware comprising an etch apparatus. The general purpose of a gasket is to form a seal between the gasket and one FOF surface in order to protect the other surface of the FOF from exposure to liquid (*e.g.*, acid). A gasket is fabricated from a suitable material with properties compatible with the conditions of the chemical etching process. A gasket is generally comprised of an acid resistant material. In one embodiment, a material comprising a gasket is flexible. Those of ordinary skill in the art will appreciate that a gasket is any shape or size which is suitably shaped to form a seal with the FOF, such that the sealed area is correctly located on the FOF and protects the desired area from etching. A gasket has at least two surfaces *e.g.*, a top and bottom surface. A gasket surface has a ridge, which is a flat, raised surface that makes contact with the FOF along the perimeter of the FOF around the area to be protected from the etching process. In one embodiment, a gasket has at least two opposed ridges which are flat raised surfaces that make contact with the FOF along the perimeter of the FOF around the area to be protected. In one embodiment, a gasket is rectangular in shape having two flat opposed surfaces with a distance in between the surfaces and each surface contains a ridge. The gasket has a protruding outer frame feature on both its top and bottom surface that serves to position the FOF in the proper location when it is mounted on the gasket. The outer frame of the gasket is comprised of walls which are permanently joined to the top and bottom surface. A gasket has any number of walls. In one embodiment, a gasket has four walls, including a first wall, a second wall, a third wall and a fourth wall. A gasket wall has a height. The gasket walls are connected at perpendicular angles to form a rectangular outer frame. The gasket walls, which comprise the outer frame of the FOF, extend above the top surface and below the bottom surface to form a tray-like structure into which each FOF is placed during the etch process. In one aspect of the invention, a gasket is the same general shape as a FOF.

At least the top surface or the bottom surface or alternatively, both surfaces of a gasket include a raised portion which forms a ridge elevated above the surface, wherein the

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reason for such a ridge is to facilitate the formation of a tight seal between a FOF and the gasket surface and prevent one side of each FOF from exposure to liquid. The ridge is smooth and uniform in cross section and forms a continuous barrier around the perimeter of the FOFs. In one embodiment, a ridge is a rectangular shaped border which has a height above the gasket surface and a width. A ridge is located a distance inside the gasket walls.

A gasket further includes at least one index feature to provide a physical basis for properly orienting a FOF during the etch process, so that a FOF is only allowed to be mounted in a gasket in only one orientation. Proper positioning of a FOF ensures consistency that the same side of the FOF is etched. An index feature is any shape or form. In one embodiment, a band is positioned at an angle in one corner of a gasket to form a gasket index feature, wherein the band forms a corner barrier which restricts how a FOF is mounted into a gasket (i.e. the corner notch of a FOF must be matched to the gasket index feature).

Referring to Figures 11a-e, this version of the invention includes a gasket **75**, including a top surface **76** and a bottom surface **77**. The top and bottom surfaces **76, 77** are directly opposed, wherein there is a distance between the top surface **76** and the bottom surface **77**. The distance between the two surfaces is any distance. In one embodiment, the distance H_{10} between the top surface **76** and the bottom surface **77** is about 1 mm. A surface of a gasket is any shape. In one embodiment, the top and bottom surfaces of a gasket are the same. In preferred embodiment, the top and bottom surfaces of a gasket are rectangular in shape.

It is understood by one skilled in the art that a gasket has any number of walls. In one embodiment, a gasket **75** has four walls, including a first wall **78**, a second wall **79**, a third wall **80**, and a fourth wall **81**. In one embodiment, all of the walls of a gasket **75** are connected permanently. The walls are connected to form an outer frame permanently joined to opposed top and bottom surfaces **76, 77**. In one embodiment, the walls **78, 79, 80, 81** of a gasket form a rectangular shaped frame around opposed top and bottom surfaces **76, 77**. For example, in Figure 11a, a first wall **78** and third wall **80** extend perpendicular between a second wall **79** and fourth wall **81**.

The walls of a gasket **75** have a length which is any length. In one embodiment, the walls **78, 79, 80, 81** are the same length or alternatively different lengths. Suitable gaskets have walls that are different lengths. In one embodiment, the length L_4 of first wall **78** is about 78 mm and the length of a third wall **80** is the same as length L_4 of the first wall **78**. The width L_6 of a second wall **79** is about 42 mm and the length of a fourth wall **81** is the

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same as the length L_6 of a second wall **79**.

In one embodiment, one or more walls of a gasket **75** extend to a height above the top surface **76**. In one embodiment, all of the walls **78, 79, 80, 81** extend to a height above and below the surfaces **76, 77**. In a more preferred embodiment, a fourth wall **81** extends above the top surface **76** to a height H_8; a second wall **79** extends above the top surface **76** to a height H_{12}; a third wall **80** extends above the top surface **76** to a height H_{14}; and a first wall **78** extends above the top surface **76** to a height H_{17}. In a more preferred embodiment, the heights of each of the sides above the top surface H_8, H_{12}, H_{14}, and H_{17} are the same. In a most preferred embodiment, the height H_8, H_{12}, H_{14}, and H_{17} of each of the walls **81, 79, 80, 78** which extends above the top surface **76** is the same and about 2 mm.

In another embodiment, one or more walls of a gasket **75** have a height that extends below the bottom surface **77**. In one embodiment, a fourth wall **81** extends below the bottom surface **77** to a height H_9; a second wall **79** extends below the bottom surface **77** to a height H_{13}; a third wall **80** extends below the bottom surface **76** to a height H_{15}; and a first wall **78** extends below the bottom surface **77** to a height H_{16}. In a more preferred embodiment, the height H_9, H_{13}, H_{15}, and H_{16} of each of the walls **81, 79, 80, 78** which extends below the bottom surface **77** is the same and about 2 mm.

Both surfaces **76** and **77** of a gasket contain a raised portion which is a ridge elevated above the surface. In one embodiment, both the top surface **76** and bottom surface **77** include a raised ridge. For example, a suitable ridge **85** is raised a height H_7 on a top surface **76** and a same ridge is found on a bottom surface **77**. In one embodiment, the height H_7 of a ridge **85** is about 0.5 mm. A ridge **85** is continuous and the shape of a rectangle. A ridge **85** forms a border inside the walls of a gasket and is a distance D_3 from the walls of the gasket. In one embodiment, the distance D_3 of a ridge **85** from the wall of a gasket is about 1 mm. A ridge **85** has any width. In one embodiment, the width W_{15} of a ridge is about 2 mm. In some suitable gaskets, there is at least one additional feature of the ridge which ensures that an effective seal is formed near the index feature between a FOF and the gasket. For example, a ridge **85** has an additional crossbar **84**. Crossbar **84** has the same height and width as the rest of the ridge **85**. A gasket has any number of cross bars.

In this invention, a gasket has at least one index features. An index feature of a gasket provides a physical basis for orienting a FOF so that the FOF mounts into a gasket in only one orientation. Consistent orientation of a FOF in a gasket ensures that the same single side of a FOF is protected from exposure to liquid. An index feature is located on the top surface

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76 or the bottom surface 77 or alternatively, on both surfaces 76, 77 of a gasket. In one embodiment, a gasket includes at least one index feature which is a corner barrier 82, comprising a band 83 placed at an angle A_5 that provides a physical basis for orienting a FOF when the FOF is mounted into a gasket. In one embodiment, the angle A_5 is 45 degrees. As a result of having a suitable index feature on a FOF (*e.g.*, a corner notch) and a complementary index feature on a gasket (*e.g.*, a corner barrier), proper mounting of a FOF 1 into a gasket occurs when the notched corner 13 of a FOF and a corner barrier 82 of a gasket are appropriately aligned. When a FOF and gasket are properly assembled, the polished surface 2 of a FOF 1 is consistently placed against a gasket 75 and therefore, the polished surface 2 is not exposed to acid and does not get etched.

A gasket is fabricated from a suitable material wherein the material is compatible with the conditions of the etch process. In one embodiment, a gasket is fabricated from an acid resistant material. Suitable materials for fabrication of a gasket are flexible. In one embodiment, the gasket is constructed of a flexible material. In one embodiment, the gasket is comprised of silicone.

B. Chemical Etch Process

The present invention provides a process that produces reaction chambers onto a single surface or side of a FOF. One suitable process is chemical etching, wherein the reaction chambers are etched onto a FOF using chemicals. In one embodiment, acid is the chemical used to etch the reaction chambers onto the FOF. In a further embodiment, a process for etching a single surface of a FOF requires that one surface of the FOF is protected such that the protected surface is not exposed to chemicals and therefore, does not get etched. A suitable process for etching a single surface of a FOF uses an apparatus (*e.g.*, the apparatus described above, comprised of a set of clamps and a gasket) to protect one surface of a FOF from chemical exposure. In one embodiment, a FOF with reaction chambers etched onto one surface is produced using the process outlined in Figure 10. Prior to chemical exposure, unetched FOFs are assembled using an etch apparatus comprised of a set of clamps and a gasket.

Figure 13 shows an exploded view which illustrates the relationship of the various components utilized in the etching process, including a gasket 75 which is placed in between two unetched FOFs 1. A first surface 2 of a FOF 1 is mounted opposed to a top surface 76 of

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a gasket **75**, wherein the corner notch **13** of the FOF **1** is matched with the complementary corner barrier **82** on the gasket **75**. Two PEEK clamps **39** are affixed along the two longer, opposite sides of the sandwich **100** to firmly hold the FOFs **1** and gasket **75** together and to prevent the first surface **2** of the FOF from being exposed to acid. The entire assembly shown in Figure 12 is referred to as a "clamped sandwich". The clamped sandwich **101** is transferred to an acid bath. In one embodiment, the acid bath is comprised of 20% nitric acid (w/v aqueous solution). The time and conditions of the chemical etching reaction are adjusted to achieve control of the size and volume of the resultant reaction chambers. The clamped sandwich remains in the acid bath for a time sufficient to allow reaction chambers of the desired depth to form. In one embodiment, the etch time is about 3 hours and 30 minutes, to produce reaction chambers with a reaction chamber depth of 55 μm. The process of fabricating reaction chambers is adapted to any fiber size so as to provide a wide range of appropriately sized chambers. Generally, chambers are introduced into the termini of the fibers by placing the FOF into an acid bath for a variable amount of time. The amount of time is varied, depending upon the overall depth of the reaction chamber desired (see *e.g.*, Walt, *et al.*, 1996. *Anal. Chem.* 70: 1888).

3. Process for RER Cleaning

Prior to applying one or more thin film coatings, all substrates (*e.g.*, etched FOFs) are thoroughly washed to make the array surface free of gross particulate contaminants, and relatively free of oily contamination, such as fingerprints, prior to applying the thin film coating. In one embodiment, the FOF is thoroughly cleaned after the process etching of forming the reaction chambers is complete as described in Example 1 (Figure 9). In one embodiment, after cleaning at least one of the bottom, top, or sidewall of substantially all of the reaction chambers, is coated with a transparent thin film coating where the transparent thin film coating comprises SiO_2 which is 0.1 – 5.0 microns thick, optically transparent, and impermeable to water and the transparent coating is applied prior to apply the non-transparent coating. In another embodiment, at least one of the bottom, top, or sidewall of substantially all of the reaction chambers is coated with a non-transparent thin film coating where the non-transparent coating is chromium or silver.

4. Process for Coating Arrays

Several methods are used for depositing one or more thin film coatings onto the

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surface of an array substrate. These methods include vapor and liquid deposition processes such as thermal evaporation, e-beam evaporation, sputtering, spray, and electrostatic plating and can be used to deposit both transparent and non-transparent thin film coatings. Typically, the transparent and non-transparent coatings are applied in separate steps. These methods are described in further detail below.

A. Vapor Deposition

Vapor deposition is a method widely used in the semiconductor and optical components industry for which controlled processes are commercially available. Vapor deposition can be used to deposit the transparent and non-transparent coating. In one embodiment, when present, the transparent and/or non-transparent thin film coatings are deposited from the vapor phase onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array. Vapor phase deposition processes are typically described as either physical or chemical in nature, depending on the extent to which the deposited film material is chemically transformed from its precursors. In one embodiment, when present, the transparent and/or non-transparent thin film coatings are deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using a physical vapor deposition process known as sputtering or evaporation, where the chemical reagent, which is the precursor to the thin film, is thermally evaporated in a vacuum chamber. The "pre-film" vapor coats at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array and forms the thin film coating. See *e.g.*, Plummer et al., Silicon VLSI Technology, Chapter 9, Prentice Hall, 2000. In one embodiment, a non-metal oxide is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the sputtering or evaporation method of vapor deposition. In a further embodiment, the non-metal oxide SiO_2 is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the sputtering or evaporation method (Figures 1a, 1b, and 2b). In another embodiment, a metal is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the sputtering or evaporation method of vapor deposition. In a further embodiment, the metal chromium is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the sputtering or evaporation method of vapor deposition. In another embodiment, the metal silver is deposited onto at least one bottom or sidewall of

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substantially all of the reaction chambers or top surface of the array using the sputtering or evaporation method of vapor deposition.

Chemical vapor deposition can be used to deposit the transparent and/or non-transparent coating. In one embodiment, when present, the transparent and/or non-transparent thin film coatings are deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using a chemical vapor deposition process known as plasma-enhanced chemical vapor deposition (PECVD), where two chemicals are reacted unto a heated substrate to produce a thin film coating. PECVD is performed in a reaction chamber and gases are injected into the chamber. A chemical reaction occurs at typically 400 degrees C which results in a thin film coating being deposited onto the array. A plasma is generated in the chamber to increase the energy available for the chemical reaction at a given temperature. The PECVD process is typically performed on one side of the substrate at a time. See *e.g.*, Plummer et al., Silicon VLSI Technology, Chapter 9, Prentice Hall, 2000. In another embodiment, a non-metal oxide is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the PECVD method of vapor deposition. In one embodiment, the non-metal oxide SiO_2 is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the PECVD method (Figures 1c, 1d, and 2c). In another embodiment, a metal is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the PECVD method of vapor deposition. In a further embodiment, the metal chromium is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the PECVD method of vapor deposition. In another embodiment, the metal silver is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the PECVD method.

Ion-plating vapor deposition can be used to deposit the transparent and/or non-transparent coating. The ion-plating process typically incorporates characteristics of two other technologies, sputter etching and ion-beam mixing. In the ion-plating process, the substrate to be coated and the source of the coating material are held in a vacuum chamber within a low-pressure gaseous environment. Prior to being coated with the thin film coating, the substrate is "sputter cleaned." Energetic ions (electrically charged atoms) and neutral atoms of an activated inert gas strike the of the substrate to remove contaminants. Sputter cleaning is important to the ion-plating process because it produces a very reactive and

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atomically clean surface. The thin film coating material is evaporated, enhanced by the interaction with energetic inert gas (or reactive gas) atoms and ions, and deposited on the surface of the item. In one embodiment, when present the transparent and/or non-transparent thin film coating is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using an ion-plating vapor deposition method which is a hybrid chemical-physical process. Figure 3 shows a schematic of the ion-plating vapor deposition method. In one embodiment, a non-metal oxide is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the ion-plating method of vapor deposition. In one embodiment, the non-metal oxide, SiO_2 is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or a top surface of an FOF using the ion-plating method of vapor deposition (Figures 1e, 1f, and 2d). In another embodiment, a metal is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the ion-plating method of vapor deposition. In a further embodiment, the metal chromium is deposited onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array using the ion-plating method of vapor deposition. In another embodiment, the metal silver is deposited onto at least one bottom, or sidewall of substantially all of the reaction chambers or top surface of the array using the ion-plating method of vapor deposition (Figure 23).

B. Liquid Deposition

Many liquid phase processes are used to apply the transparent or non-transparent thin film coating. In one embodiment, when present, the transparent and non-transparent thin film coatings or their precursor materials are applied in liquid form to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of an array, and the material subsequently solidifies. Liquid phase processes are either physical or chemical or some combination. In one embodiment, when present, the transparent and non-transparent thin film material is dissolved in a volatile solvent, the resulting solution is applied to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array and the solvent is allowed to evaporate, yielding a thin film coating via a physical liquid phase process. In another embodiment, when present, the transparent and non-transparent thin film coatings can be formed by a sol gel process, where inorganic silicates or organic siloxanes are dissolved in an appropriate solvent and applied to at least one bottom or

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sidewall of substantially all of the reaction chambers or top surface of the array. Upon drying and heating, the low molecular weight silicates/siloxanes undergo chemical condensation reactions to polymerize into a glass-like film via a hybrid physical-chemical liquid phase process.

Transparent and non-transparent thin film coatings deposited by liquid phase deposition are applied to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array in a variety of ways. In one embodiment, array substrates are dipped into the coating solution and withdrawn in a controlled fashion, leaving a liquid coating with thickness controlled by the rate of withdraw of the substrate and the angle. In another embodiment, liquids are applied by spin coating, where the thin film solution is applied to at least one top or sidewall of substantially all of the reaction chambers or top surface of the array, which is then spun to spread the liquid evenly and to remove any excess. In another embodiment, the thin film solution is sprayed onto at least one top or sidewall of substantially all of the reaction chambers or top surface of the array, where the coalescence of droplets produces a transparent or non-transparent thin film coating. In another embodiment, a technique known as capillary coating is used to coat at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array with a transparent or non-transparent thin film. Capillary coating encompasses the use of a rolling cylinder partially immersed in the thin film solution. The substrate is moved close to the cylinder so that a meniscus is formed to the cylinder, and the cylinder is rotated at the same rate as the substrate is translated.

The invention provides processes for depositing a transparent or non-transparent thin film coating on the surface of an array which has a top surface and a bottom surface. In one embodiment, the invention provides processes is for depositing a transparent or non-transparent thin film coating on the bottom and sidewall of substantially all of the reaction chambers and top surface of the array. The invention also provides processes for depositing a transparent or non-transparent thin film coating on at least one sidewall or bottom of substantially all of the reaction chambers or top surface of the array of the array. For example, the invention provides a method for depositing a transparent or non-transparent thin film coating on the sidewall of the reaction chamber without depositing a the same thin film coating on the bottom of the reaction chamber. In one embodiment, the bottom of the reaction chamber is protected during the coating process and is not coated, because the bottom of the reaction chamber is in the shadow area (i.e., the coating is blocked by the

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sidewall of the reaction chamber and is therefore, not deposited onto the bottom of the reaction chamber). For example, Figure 15a illustrates a reaction chamber where the sidewall creates a shadow, thereby protecting the shadow area on the bottom of the reaction chamber, such that no metal is deposited in the shadow area on the bottom of the reaction chamber. In one embodiment, the method of the invention utilizes shadows created by the sidewall of the reaction chamber and the rotation of the array at a tilt angle to control where the transparent or non-transparent thin film coating is deposited. In another embodiment, the array substrate is spinning during the process of depositing the transparent or non-transparent thin film coating. Spinning the array substrate allows for the thin film coating to be uniformly deposited.

The set-up for the process of depositing a metal on the sidewall and corner area of substantially all of the reaction chambers and top surface of the array is illustrated in Figure 15a. The process involves tilting or angling the array substrate mounted on a carrier and rotating the substrate with a motor. As the array substrate rotates, the thin film coating is deposited on the sidewall and corner area of substantially all of the reaction chambers and top surface of the array, and the thin film coating is absent from the center of the bottom such that the absence of the coating creates an aperture near the center of the bottom of the reaction chamber. Changing the tilt angle of the rotating substrate results in the thin film coating being deposited in different patterns on the array substrate *e.g.*, different shapes, gradients of thickness, or overlap of two coatings (Figure 15a).

In one embodiment, the process of the invention deposits the transparent or non-transparent thin film coating over the entire surface of at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array *e.g.*, the bottom surface of substantially all of the reaction chambers is completely coated with a thin film.

In another embodiment, the transparent or non-transparent thin film coating is deposited in the corner area of the reaction chamber such that the corner area is formed at the junction between the bottom and sidewall. The thin film coating is partially deposited on the bottom and partially deposited on the sidewall of the reaction chamber, such that the coating is absent from the center of the bottom of substantially all of the reaction chambers and forms an aperture (Figure 16). In one embodiment, the angle at which the substrate is held (i.e., the tilt angle) can be adjusted to control the size of the aperture (Figure 15a). Partial deposition of a non-transparent thin film coating on the corner area such that there is an absence of coating on the bottom of the reaction chamber further eliminates optical bleeding between

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adjacent reaction chambers and restrains any light beams which are directed down into the fiber strands for example when the array is being used for analysis (*e.g.*,. DNA sequencing).

In one embodiment, the process of the invention is used to partially deposit (i.e., to coat) an opaque, non-transparent thin film coating over a portion of the surface of at least one bottom or sidewall of substantially all of the reaction chambers or top surface of an array *e.g.*, the bottom of substantially all of the reaction chambers is partially coated with a thin film. In one embodiment, at least 80% of the bottom of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film. In another embodiment, at least 60% of the bottom of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film. In another embodiment, at least 40% of the bottom of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film. In another embodiment, at least 20% of the bottom of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film. In another embodiment, at least 10% of the bottom of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film.

In another embodiment, the opaque non-transparent thin film coating is applied to the sidewall of substantially all of the reaction chambers on a fiber optic faceplate. The non-transparent coating completely covers the entire surface of the sidewall or alternatively, the non-transparent coating partially covers the surface of the sidewall. In one embodiment, at least 80% of the sidewall of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film. In another embodiment, at least 60% of the sidewall of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film. In another embodiment, at least 40% of the sidewall of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film. In another embodiment, at least 20% of the sidewall of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film. In another embodiment, at least 10% of the sidewall of substantially all of the reaction chambers of the array is coated with a transparent or non-transparent thin film.

In another embodiment, the opaque non-transparent partial coating on the bottom corner area and the sidewall corner area produces an optical obstacle and eliminates optical scattering through the cladding material and allows light to come through the bottom which is not covered with opaque second coating.

The method for depositing a transparent or non-transparent thin film can also be used

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to apply a thin film coatings to features found on a substrate *e.g.*, boundary posts or column structures. The method is also used for coating the spaces on the bottom of the reaction chamber between the boundary post or column structures. The patterns of the posts produce different shadow patterns on the bottom.

As shown in Figure 18, angle effects can occur during the deposition process when the source (*e.g.*, a metal ion source) is considerably smaller in size in comparison to the surface of the substrate for coating. Angle effects mean that the transparent or non-transparent thin film coating profile is varied (i.e. not uniform) at different locations on a array substrate. Angle effects produce an array with reaction chambers that are not uniformly coated. Figure 18A shows the coating being deposited in the corner area of three reaction chambers located at one end of the substrate, and Figure 18b shows the coating being deposited on the bottom of three reaction chambers located on the other end of the same array substrate. Strategies to eliminate angle effects include increasing the distance between the source and the substrate to be coated, putting a shadow mask in front of a substrate to shift and scan an entire substrate during deposition, introducing a shutter between the source and the substrate, or reducing the size of the shutter (aperture). Figure 19a shows the use of a shadow mask during the deposition process to help direct the coating to a selected area (*e.g.*, the "metal coated zone") of the substrate and to eliminate angle effects. The shadow mask is placed between the ion source and the substrate and can be positioned any distance from the substrate. Figure 19b shows the use of a shutter during the deposition process to help direct the metal coating to a selected area, the metal coated zone, of the substrate.

One embodiment of the invention includes a process for depositing a non-transparent thin film coating on a substrate with a top surface containing a plurality of reaction chambers, where each reaction chamber is comprised of a bottom or sidewall; and the non-transparent coating is opaque, semi-opaque, shiny opaque, or translucent and is deposited on at least one bottom or sidewall, of substantially all of the reaction chambers or top surface of the array. The process involves (a) mounting a substrate onto a substrate carrier at an angle, (b) spinning the mounted substrate in a vacuum chamber, (c) depositing the non-transparent thin film onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the substrate, (d) removing the substrate from the vacuum chamber, and (e) dismounting the substrate coated with the non-transparent substrate from the substrate carrier. In one embodiment, the process is used to deposit a non-transparent coating on the substrate which is a fiber optic faceplate. In another embodiment, the process is used to deposit a non-

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transparent coating onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of an array that is already coated on one bottom or sidewall of substantially all of the reaction chambers or top surface of the array with a transparent coating prior to the non-transparent coating being applied. In another embodiment, the process is used to deposit a non-transparent coating on to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of an array that is coated on at least one bottom or sidewall of substantially all of its reaction chambers or top surface of the array with a transparent thin film that is optically transparent, 0.1-5.0 microns thick, and impermeable to water. In another embodiment, at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array is coated with a non-transparent coating prior to a transparent coating being applied to at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the array.

In one embodiment, the process of angled metal deposition uses an angled jig and jig carrier assembly. Figure 15a shows a side view of a fiber optic faceplate containing reaction chambers which is mounted onto the substrate carrier of a jig. The rotary and angled jig assembly is shown in Figure 15b. The fiber optic faceplate is held at an angle (the "Tilt Angle") and rotated while a non-transparent coating *e.g.*, a metal is evaporated from a source and deposited onto the sidewall and corner area of each reaction chamber and the top surface of the array. The entire process can be carried out in a metal deposition chamber and the rotary disc can be driven by a vacuum compatible motor, which can rotate at less than 10 rpm.

C. Quality Control

After the coating process is complete, the quality of the resulting thin film coatings is evaluated using a number of different techniques. Both direct and functional methods are used to detect the presence of intact thin film coatings and to evaluate performance of the coated array. In one aspect of the invention, the coating on the array is uniform. Initially, a visual quality control inspection is performed which consists of visually inspecting each film coated array with a microscope in order to detect any gross defects. Small holes ("pin holes") in the coating indicate that the coating is of poor quality. In some embodiments, where the bottom is partially coated, the center of the reaction chamber bottom should be clear from any non-transparent coating to ensure that light can pass through the center of the chamber without any reduction in intensity. The diameter of the aperture formed on a partially coated

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bottom can be measured to determine whether the tilt angle used in the deposition process is correct. In one embodiment, diameter of the aperture formed by the partial coating on the bottom of the reaction chamber is about 28 microns. In another embodiment, the width of the ring formed when the coating is applied to the corner area is about 8.5 microns (Figure 16b). In some embodiments, it is critical that the corner area of the reaction chamber is uniformly coated with the non-transparent coating so that light does not leak out to adjacent chambers. In one embodiment, the thickness of the non-transparent coating in the corner area of substantially all of the reaction chambers of the array is not less than 500 Angstroms (Figure 16a)

Second, Scanning Electron Microscopy or "SEM" analysis is performed on the thin film coated array. SEM analysis is a key analysis method used to determine the quality of both transparent and non-transparent coatings. Typically, SEM images of both the thin film coated surface and a prepared cross-section are collected and analyzed. Surface images are analyzed for defects and coating damage as well as for overall morphology of the thin film coated surface, while cross-sections are measured for thickness. Select arrays are also evaluated for any potential effects by components used in a chemical reaction or bioassay and contained in the reaction chamber (*e.g.*, the thin film coating is examined both before and after "mock" PCR conditions, See, Example 3). The thickness of the thin film coating can be measured. In one embodiment, SEM is used to determine thickness of the thin film coating. In another embodiment, the thickness of the thin film coating is measured by adding a sapphire coupon (Corion Division, Franklin, MA) to the batch, and determining the thin film (*e.g.* SiO_2) thickness after the coating process is complete by measuring the wavelength dependence on its light transmittance. The thickness of coatings is also followed by electroplating for thicker thin film deposition in order to produce additional features on the array.

The thin film coated arrays are also evaluated "functionally" regarding their performance for specific applications, *e.g.* DNA sequencing, to determine the effects of the thin film coating on PCR-induced sequencing background and overall quality of sequencing results. Such functional test can provide the advantage of single chamber resolution across the entire array surface. See, Example 4 and Figure 4. A comparison of the light intensity generated during DNA sequencing reactions at two adjacent reaction chambers from coated and uncoated reaction chambers on the same array showed that a non-transparent coating can help to reduce optical bleeding between two adjacent chambers (see, Example 6 and Figures

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24a-b).

5. Methods of Using Arrays

Thin film coated arrays can contain a number of different reactants and analytes in their reaction chambers. In one embodiment, each reaction chamber of the thin film coated array contains reagents for analyzing a nucleic acid or protein. Typically those reaction chambers that contain a nucleic acid (not all reaction chambers in the array are required to) contain only a single species of nucleic acid (i.e., a single sequence that is of interest). There may be a single copy of this species of nucleic acid in any particular reaction chamber, or there may be multiple copies. In one embodiment, a reaction chamber contains at least 100,000 copies of the nucleic acid template sequence. In another embodiment, the reaction chamber contains at least 1,000,000 copies. In further embodiment, the reaction chamber contains between 2,000,000 to 20,000,000 copies. In another embodiment, the reaction chamber contains between 5,000,000 to 15,000,000 copies of the nucleic acid. For example, if the apparatus of the invention is to be used for a pyrosequencing reaction, the ordinarily skilled artisan will appreciate that changes in the number of copies of a nucleic acid species in any one reaction chamber will affect the number of photons generated in a pyrosequencing reaction, and is routinely adjusted to provide more or less photon signal as is required. In one embodiment the nucleic acid species is amplified to provide the desired number of copies using PCR, RCA, ligase chain reaction, other isothermal amplification, or other conventional means of nucleic acid amplification. In one embodiment, the nucleic acid is single stranded.

The thin film coating provides an array with enhanced compatibility and functionality. One particular assay that has encountered problems due to array incompatibility is the analysis of nucleic acid molecules, specifically pyrophosphate sequencing (PPS) of PCR amplified nucleic acids. In one embodiment the thin film coated array is used to overcome a difficulty encountered in the PPS of PCR amplified nucleic acids. The PPS method used is according to the methods of U.S. Patent Application Ser. No. 10/767,779. See also, U.S. Patent No. 4,863,849 and U.S. Patent No. 4,971,903. When amplification is performed, followed by sequencing in the same reaction chamber, a "background" signal is observed. The source of this background is the released pyrophosphate (PPi) which remains strongly bound to the reaction chamber of the FOF such that it persists even after extensive washing. Therefore, a "background" assay was developed which involves performing solution phase PCR, washing the FOF, loading the FOF with Dynal sulforylase (S) and luciferase (L) beads

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(Fisher Scientific, Pittsburgh, PA), and measuring the signal (normalized to an earlier pyrophosphate flow). A reduction in the background noise in PPS would result in better sequencing results at a given signal level or allow equivalent sequencing result to be obtained at lower signal levels (Example 5).

Thin film coatings on the reaction chamber of a microwell array can significantly reduce PCR-induced sequencing background as shown in Figure 4. In one embodiment, a thin film coating of SiO_2 with a thickness of 0.1- 5.0 microns on at least one or sidewall of substantially all of the reaction chambers or top surface of a FOF significantly reduces the PCR-induced sequencing background and provides for the same sequencing results as obtained in an uncoated array as shown in Figure 5.

This invention includes a method for sequencing a nucleic acid using the arrays, the method comprises: providing a plurality of single-stranded nucleic acid templates deposited within a plurality of reaction chambers on an array of any one of the embodiments described herein; performing a pyrophosphate sequencing reaction simultaneously on substantially all reaction chambers by annealing an effective amount of a sequencing primer to the nucleic acid templates and extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product such that, if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer, a sequencing reaction byproduct is produced; and identifying the sequencing reaction byproduct, thereby determining the sequence of the nucleic acid in each reaction chamber.

Another aspect of this invention is an apparatus for analyzing a nucleic acid sequence. The apparatus comprises: a delivery chamber, wherein the chamber comprises an array of any one of embodiments described herein; a reagent delivery device in communication with the reagent delivery chamber; an imaging system in communication with reagent delivery chamber; and a data collection system in communication with the imaging system.

Examples

The following examples have been selected merely to further illustrate features, advantages, and other details of the invention. It is to be expressly understood, however, that while the examples serve this purpose, the particular conditions and details are not to be construed in a manner that would unduly limit the scope of this invention.

Example 1: General Etching Process and RER Cleaning Procedure

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This example describes the general single-sided etching process for FOFs (Incom Corporation, Charlton, MA) sizes 25 x 75 mm or 40 x 75 using silicon etch gaskets and PEEK (Poly Ether Ether Ketone) plastic clamps (Figs. 11a and 11b). Distilled water for the Branson Ultrasonic bath (Fischer Scientific, Hampton, NH), from a PicoPure™ water purification unit ("PicoPure water"), was degassed for 10 minutes. The NesLab recirculator (Portsmouth, NH) was set to 57.2 °C (at this setting, water inside the ultrasonic bath was maintained at a constant temperature of 55 °C). Four stainless steel containers were covered and placed in the ultrasonic bath. Each container was charged with 1000 mL (for a 25 x 75 mm FOF) and 1200 mL (for a 40 x 75 mm FOF) of 20% HNO_3 and warmed to 55 °C. Ninety-six unetched FOFs were loaded into each of the 96 positions of four etch trays. A designated FOF was selected and weighed. The difference in FOF weight before and after etching was plotted in order to correlate weight loss due to reaction chamber depth.

A pair of unetched FOFs was combined by positioning a silicon gasket (Figure 13) in between the two FOFs and properly aligning each, such that the polished side of each FOF is facing the gasket and within the edge of the gasket. The corner notch of the FOF is aligned with the corner barrier on the gasket. The first PEEK clamp was affixed to hold the two FOFs and gasket together vertically and squeeze the plates together. The clamp was snapped on down the center of the long end of the FOFs starting with the bottom-most clip. The second PEEK clamp was applied. The finished "clamped sandwich" is shown in Figure 12. The remaining unetched FOFs were combined and clamped as described above. Next, 12 clamped sandwiches were positioned into each of the four stainless steel racks. Once the temperature of the HNO_3 reached 55 °C, the ultrasonic bath was turned on and the lids of the other 3 stainless steel containers removed. Each of the four stainless steel racks holding FOFs was transferred into the acid at 55 °C. The FOFs were kept in the 55 °C acid bath. The etch rate of the FOFs was approximately 0.245 μm/min.The length of time that the FOFs remained in the bath depends on the desired reaction chamber depth. For example, an etch time of 3 hours and 44 minutes resulted in a reaction chamber depth of 55 μm.

The etched FOFs (i.e. FOFs) were removed from the acid bath and placed directly into stainless steel containers filled with 1.0 L of PicoPure water, and sonicated for 5 minutes. The water was discarded and the containers were filled with 1.0 L of PicoPure water, and sonicated for an additional 5 minutes. The FOFs were removed from the rinse water and PEEK clamps and silicon gaskets were detached from each pair of FOFs.

To complete the post etch testing, the designated FOF was re-weighed and change in

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weight due to the etching was recorded. A count of the number of individual fibers with impaired optical transmission (a "dark fiber count") was preformed using a microscope.

A five step RER cleaning procedure using RCA cleaner, and ethylenediamine tetra-acetic acid (EDTA) ("RER cleaning procedure") for etched FOFs was conducted. "RCA" is the abbreviation for a solution of ammonium hydroxide (NH_4OH) and hydrogen peroxide (H_2O_2). To prepare RCA cleaner 1.0L of NH_4OH and 1.0L of H_2O_2 were combined under a hood and the solution was mixed using a magnetic stir bar. Ninety-six (96) FOFs from the same lot were placed into the glass slide racks.

Step 1 – First RCA Wash

RCA cleaner was prepared by combining a 1:1 solution of NH_4OH and H_2O_2. For the 25 x 75 mm FOF, 200 mL of RCA cleaner was added to each of the glass staining dishes (Fisher Scientific, Pittsburgh, PA). For the 40 x 75 mm FOF, 200 mL of RCA cleaner was added to the polypropylene staining dishes. Six staining dishes were placed into the container of one rotator of an Ocelot Rotator (Boekel Scientific, Feasterville, PA) and the other dishes were placed on the other rotator. The shake speed was set at C. After 30 minutes, the RCA cleaner was disposed. Each set of 10 FOFs was rinsed 5 times with 200 mL of PicoPure water.

Step 2 – First EDTA Wash

EDTA (200 mL) was added to each staining dish. The six dishes were placed into the container on one of the rotators and the other 4 dishes were placed into the container on the other rotator. The shake speed was set at C. After 30 minutes, the EDTA was disposed. Each set of 10 FOFs was rinsed with 200 mL of PicoPure water 5 times.

Step 3– Second RCA Wash

A fresh solution of RCA cleaner (1:1 NH_4OH and H_2O_2) was prepared and 200 mL of RCA cleaner was added to each of the staining dishes. The six dishes were placed into the container on one of the rotators and the other 4 dishes were placed into the container on the other rotator. The shake speed was set at C. After 30 minutes, the RCA cleaner was disposed. Each set of 10 FOFs was rinsed with 200 mL of PicoPure water 5 times.

Step 4 – Second EDTA Wash

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EDTA (200 mL) was added to each staining dish. The six dishes were placed into the container on one of the rotators and the other 4 dishes were placed into the container on the other rotator. The shake speed was set at C. After 30 minutes, the EDTA was disposed. Each set of 10 FOFs was rinsed with 200 mL of PicoPure water 5 times.

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Step 5 - Sonicating the FOFs

The NesLab recirculator was charged with the appropriate amount of water and the temperature point was set to 40 °C with the low temperature alarm at 5 °C and the high temperature alarm at 100 °C. The FOFs were placed into a stainless steel rack (Fisher 10 Scientific, Pittsburgh, PA) loading the FOFs into every other slot to allow for good flow of the cleaning solution. The filled racks were placed into a polypropylene pan filled with a 5% Contrad® solution with the solution completely covering the FOFs. The 5% Contrad® solution was prepared by combining 1000 mL of deionized water with 50 mL of Contrad® (Fisher Scientific). The pans were then covered with a HDPE (high density polyethylene) 15 cover. When the temperature of the recirculator reached 40°C, two polypropylene pans containing the FOFs were submerged in the water. The FOFs were sonicated for 90 minutes and then removed from the sonicator. The racks were removed from the pans and the Contrad® solution drained. The polypropylene pans were filled with PicoPure water. The stainless steel racks were placed back in the pan and the FOFs were rinsed 2 times. The pan 20 was refilled a third time with PicoPure water and taken to the Ocelot Rotator and rotated on speed C. After 5 minutes, the FOFs were rinsed with water 2 times. The FOFs were covered with tin foil and allowed to dry in the stainless steel racks.

Example 2: FOF Coating Using Ion-Plating Deposition Method

25 FOFs were immediately cleaned prior to thin film coating. The detergent used was Contrad®. The Branson sonicator was set to 40 °C. Two FOFs were placed back to back in a Falcon tube, and 40-45 mL of 5% Contrad® solution was added in the tube and the cap closed. The tube was loaded in the sonicator and sonicated for 90 min. FOFs were removed from the tube, rinsed thoroughly with fresh deionized water, and transferred to a new Falcon 30 tube and filled with deionized water. The detergent solution was disposed in the sink and FOFs kept in de-ionized water at 4 °C.

After cleaning, the ion-plating process for coating a chemically etched FOF with a thin film SiO_2 coating was performed. The FOF to be coated was placed on an electrically

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isolated holder in a vacuum chamber, along with an electrically isolated heated silicon target, an argon plasma source, and a source of oxygen. The argon plasma source was ignited, which generated an overall negative charge on the FOF. Silicon vapor was produced by evaporation of the heated target, and reacted with the argon plasma, as well as the oxygen to form positively charged SiO_2 species. These SiO_2 species attract to the negatively charged FOF, where they energetically condense, forming a glassy, unstructured SiO_2 film. The film morphology and homogeneity is a complex function of many process variables, but the film thickness was precisely controlled by the exposure time in the chamber. See Figures 1 and 2.

Example 3: Exposure of the Transparent Thin Film Coated FOF to "Mock" PCR Conditions

Transparent thin film coated, etched FOFs were tested for environmental robustness by exposing the etched FOF coated with SiO_2 to deionized water at temperatures (approximating PCR thermal exposure), and examining SEM images of both the surfaces and cross-sections for signs of coating damage according to the following procedure. One milliliter of 1xHiFi PCR buffer (Invitrogen, Carlsbad. CA) was prepared. The thin film, etched FOF was placed on top of tissues and using a cell scraper, the excess water was removed. Quickly, 400 µL of the buffer was added on top of the FOF surface and the solution was evenly spread out with a cell scraper. After 1 minute, the excess solution was removed. The procedure of adding 400 µL of the buffer followed by removing the solution was repeated. The FOF was immediately placed in an in-house amplification device (the "AMP jig") (454 Life Sciences, Branford, CT), covered with a silicon rubber plate and foam, and the AMP jig screws were tightened as described in Leamon *et al.*, Electrophoresis 24: 3769-3777 (2003). The AMP jig was loaded into a Thermocycler MJ PTC 225 Tetrad (MJ Research, Waltham, MA) and the thermocycling program was run. The details of the temperature profile were as follows: total run time is 4.5 hours, 1) 40 short cycles: 94 °C: 15 sec; 58 °C: 15 sec; 68 °C: 15 sec; 2) 10 additional long cycles: 95 °C: 30 sec; 58 °C: 10 min. The procedure was repeated for a total of 80 cycles. After thermocycling, the FOF surface was rinsed with fresh water. The surface was dried with nitrogen flow and the SiO_2 coating was measured by optical microscopy and SEM analysis. Since SEM analysis is a destructive technique, "before" and "after" analysis of the same thin film etched FOF could not be performed. However, all thin film etched FOFs examined by SEM after the above "mock-PCR" procedure showed no apparent damage to the SiO_2 coating on the FOFs.

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Example 4: Evaluation of PCR-Induced Sequencing Background

A 1 mL LuerLock® syringe (20G1) (Becton Dickinson, Franklin Lakes, NJ) was charged with 525μL of PCR solution (1 x Platinum HiFi Buffer (Invitrogen, Carlsbad, CA), 2.5 mM MgSO4, 0.5% BSA, 1 mM dNTPs (MBI Fermentas, Hanover, MD) and the syringe needle was connected to an in-house loading device (the "loading jig") (454 Life Sciences, Branford, CT). dNTP refers to the 4 deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP). An etched FOF coated with SiO_2 was removed from a deionized water bath, placed on the lab bench and using a cell scraper, excess water was removed from the FOF surface. The FOF was quickly assembled on the loading jig with 4 plastic clips. The fluid chamber was filled to the top with PCR solution by pushing the syringe forward. After 3 minutes, the solution had diffused into the reaction chambers. The plunger was pulled back and the loading jig disassembled. The FOF was immediately placed in an AMP jig and covered with a silicon rubber plate and foam. Jig screws were put in place and tightened. The jig assembly was loaded in the Thermocycler and the thermocycling program was run. The details of the temperature profile were as follows: total run time is 4.5 hours, 1) 40 short cycles: 94 °C: 15 sec; 58 °C: 15 sec; 68 °C: 15 sec; 2) 10 additional long cycles: 95 °C: 30 sec; 58 °C: 10 min.

After thermocycling, the AMP jig was opened, the FOF was removed and placed in a 50 mL Falcon tube (Becton Dickinson, Franklin Lakes, NJ) containing 50 mL of deionized water. The tube was then positioned on a Clay Adams® Nutator Mixer and nutated for 20 min to dissolve the PCR product. Nutation is a gentle orbital motion that can assure uniform mixing without foaming. The FOF was transferred into 50 mL of Assay Buffer ("AB") with BSA (bovine serum albumin). AB is a buffer solution containing tricine and magnesium acetate. The resulting solution was collected for the PCR sequencing analysis described in the following example (Example 5).

The bead mixture was prepared (Bangs beads 175 μL + Dynal beads 175 μL) and diluted with 700 μL of deionized water. Bangs beads are microspheres carrying immobilized sulfurylase and luciferase enzymes, and dynal beads are magnetic beads carrying the bound enzymes of luciferase and sulfurylase. Nineteen adhesive pads (3M VHS, St. Paul, MN) containing 13.2 μL of bead mixture each were used to seal the inlet holes. The FOF was then spun using an Allegra 6R Centrifuge (Beckman Coulter, Fullerton, CA) for 8 minutes at 2000 rpm. The background run was performed and total time was 24 min: 1) wash 5 min; 2) PPi 2

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min; 3) wash 10 min; 4) PPi 2 min; and 5) wash 5 min). After the run, a trace analysis was performed using Kangaroo software (454 Life Sciences, Branford, CT). Adjusted counts were obtained by subtracting 500 from raw counts. The whole procedure was repeated for both uncoated and SiO_2 coated FOFs for background comparison.

Example 5: PCR Sequencing Results

For validation of PCR results, uncoated and SiO_2 coated FOFs were paired and tested simultaneously using the solution collected for analysis in Example 5. Each solution (for both SiO_2 coated and uncoated arrays) was doubled by dilution with deionized water. Then, the PCR product in each was quantitated using an iCycler® RealTime PCR unit (Bio-Rad, Hercules, CA). Using fluorescence measurements, the amount of amplified product was determined. Finally, the number of molecules per reaction chamber after PCR was calculated. Normal range of the number is 10^3-10^9 order. The results are shown in Table 2 below and the results are in the range of 10^6-10^8 and are acceptable. All of the SiO_2 coated FOFs produced equal level of the PCR product compared to the uncoated FOFs.

Table 2: Results of in-reaction chamber PCR

Uncoated (Control)		SiO_2-coated	
FOF Number	Molecules/Reaction Chamber	FOF Number	Molecules/Reaction Chamber
FOF #1	8.21×10^7	FOF #3	1.70×10^8
FOF #2	1.10×10^8	FOF #4	3.98×10^7
		FOF #5	1.70×10^8
		FOF #6	4.40×10^7
		FOF #7	8.16×10^7
		FOF #8	6.04×10^6

Example 6: Comparison of Profile of Light Intensities in Adjacent Reaction Chamber

A FOF array (40x75mm) was prepared, where the first half of the array was coated with two coatings, a non-transparent coating and a transparent coating, and the second half of the array was coated with only one transparent coating. DNA sequencing reactions were carried out in reaction chambers on both halves of the array and the light intensities generated were compared.

A non-transparent, aluminum metal coating was applied to the first half of the array and then a transparent SiO_2 coating was applied on top of the aluminum coating to the first half of the array. The aluminum coating was applied to the top surface of the array and the

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sidewall of the reaction chambers and partially applied to the corner area of the reaction chambers, such that the diameter formed by the metal coating near the center of the bottom was 28.77 microns. The thickness of the aluminum coating measured on the top surface of the array was 200 nm. While the metal coating was being applied the second half of the array was protected by a shadow mask. After the metal coating was applied, the transparent, SiO_2 coating was applied on the top surface of the array and the bottom and sidewall of the reaction chambers of both the first and second halves of the array.

Pyrophosphate sequencing analysis of PCR-amplified nucleic acids as described in U.S. Patent Application Ser. No. 10/767,779 was conducted in reaction chambers located on both halves of the array. Figures 24a and 24b show the results of these experiments.

In Figure 24a, the two arrows indicate the center points of two adjacent reaction chambers located on the second half of the array. The center points of these two adjacent chambers are at pixel No. 5 and pixel No. 2, respectively. One reaction chamber (pixel No. 5) contained Test Fragment 10 DNA ("TF10") and the other chamber (pixel No. 2) contained Test Fragment 12 DNA ("TF12"). The sequencing process was conducted at a nucleotide flow order of 43. The nucleotide flow order is defined as a specific nucleotide injection into a stream of a solution running through a DNA sequencing machine (*e.g.*, a 454 Squencer 1.0). During the DNA sequencing process, a series of specific nucleotide solutions are injected into the solution stream and each injection is given a flow number. At a nucleotide flow order of 43, the TF10 DNA underwent 5 multiple DNA incorporations and produced a high intensity light in the chamber. At the same nucleotide flow order, the TF12 DNA underwent no incorporation of DNA and no high intensity light was produced.

Curve 1 shows a profile of six pixels (Nos. 1-6) which cross the center points of the two adjacent SiO_2 coated chambers. For example, Pixel No 1 is the first pixel which is offset one pixel from the center of the TF12 chamber in an upstream direction, Pixel No. 2 is one pixel to the right after Pixel No. 1 in the downstream direction. From Pixel No. 1 to Pixel No. 6, a scanning path can be drawn which passes the center points of the two chambers (TF10 and TF2) in a downstream direction. Pixel No. 3 is located inside the TF12 chamber and in theory the TF12 chamber in theory should not give any signal because there is no DNA incorporation in that chamber. However, an increased intensity of light was observed at pixel No. 3 in the TF12 chamber due to light from the adjacent TF10 chamber which bled photons over into the TF12 chamber.

For comparison, on the metal-coated first half of the array, sequencing reactions were

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conducted in two adjacent metal-coated chambers which contained the same TF10 and TF12 DNA fragments, one in each chamber, and the sequencing process was conducted at the same nucleotide flow order of 43. The signal observed at pixel No. 3 in the metal coated chambers as shown by curve 3 is non-linearly smaller than the signal measured in the SiO_2 coated chambers on the first half of the array, thus indicating that the metal coated, opaque sidewalls reduce optical bleeding of photons between two adjacent metal coated chambers.

Figure 24b shows the results of the same experiment, but the sequencing reactions were conducted at a nucleotide flow order of 35. At this nucleotide flow order, TF10 underwent 4 DNA incorporations and again at pixel No. 3, there is less signal due to reduced optical bleeding on the metal coated first half of the array compared to the second half of the array.

Example 7: SiO_2 Thin Film Coated Microwell Array

In one embodiment, the array is formed from a commercial FOF (Incom, Charlton, MA) which has been chemically etched on a single side with acid to yield individual reaction chambers. Each reaction chamber formed by etching has a volume of about 75 pL. The FOF is cleaned prior to coating with a thin film. The preferred thin film coating is the non-metal oxide SiO_2 with a thickness measuring 0.1-5.0 microns thick. The SiO_2 coating is optically transparent and impermeable to water. The thin film coating is applied using an ion-plating vapor deposition method. The use of thin film coated reaction chambers on a FOF surface serves several purposes; i) delayed diffusion of the luminescence from emitting light in a different region of the array, ii) isolation of reaction chambers that contain components of the assay solution or reaction mixture protected from any deleterious effects of the array substrate material, and iii) prevention of any leaching of substrate materials into the chamber solution, and iv) very efficient, high numerical aperture optical coupling to the CCD. Finally, the larger the amount of reactant (*e.g.* immobilized sequencing template) or analyte within a reaction mixture or assay solution, the more optical signal one is able to achieve.

Example 8: Metal Thin Film Coated Microwell Array

The array is formed from a commercial FOF (Incom, Charlton, MA) which has been chemically etched on a single side with acid to yield individual reaction chambers. Substantially all of the reaction chambers formed by the etch process have a volume of

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about 75 pL and a depth of between 50-55 μm. The FOF is cleaned prior to coating with a non-transparent thin film coating. The non-transparent thin film coating is chromium with a thickness measuring about 200-300 nm on the top surface of the array and 60-120 nm on the sidewall and no less than 50 nm on the corner area of substantially all of the reaction chambers. The coating is opaque. The thin film coating is applied using a ion-deposition method. The use of an opaque thin film coated reaction chambers on a FOF surface provides an array with several advantages. Optical problems such as optical bleeding, light scattering, and interference between neighboring reaction chambers are eliminated and light is concentrated. Bead loading efficiency is maximized to close to 100%.

Example 9: Metal and SiO_2 Thin Film Coated Microwell Array

The array apparatus for nucleic acid sequencing is formed from a commercial FOF (Incom, Charlton, MA) which has been chemically etched on a single side with acid to yield individual reaction chambers. The apparatus used to etch an FOF includes two PEEK clamps and a silicone etch gasket. Substantially all of the reaction chambers formed by the etch process have a volume of about 75 pL and a depth of between 50-55 μm. The FOF is cleaned prior to coating with a non-transparent thin film coating. The non-transparent thin film coating is chromium with a thickness measuring about 200-300 nm on the top surface of the array, 60-120 nm on the sidewall and no less than 50 nm on the corner area of substantially all of the reaction chambers of the array. The coating is opaque. The thin film coating is applied using an ion-deposition method. After the non-transparent thin film coating is applied, a transparent thin film coating is applied. The transparent thin film coating is SiO_2 with a thickness measuring about 200-400 nm on the top surface of the array, 50-100 nm on the sidewall and no less than 50 nm on the corner area of substantially all of the reaction chambers. An array with reaction chambers coated with first, a non-transparent film coating and second, a transparent thin film coating provides an array with several advantages. Optical problems such as optical bleeding, light scattering, and interference between neighboring reaction chambers are eliminated, and the transparent SiO_2 coating applied on top of the non-transparent coating also protects the chromium coating from erosion.

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CLAIMS

What is claimed:

1. An array comprising a substrate, wherein said substrate is a fiber optic faceplate, having a surface comprising a plurality of reaction chambers and a thin film coating on said surface, wherein the thin film is from 0.1 – 5.0 microns thick, and impermeable to water.

2. The array of claim 1, wherein the thin film coating is optically transparent.

3. The array of claim 1, wherein the thin film coating is silicon dioxide.

4. The array of claim 1, wherein the spacing between the center points of two adjoining reaction chambers is between 5 μm to 200 μm, and each reaction chamber has a width in at least one dimension of between 4 μm and 190 μm.

5. The array of claim 1, wherein the number of reaction chambers is less than 10,000.

6. The array of claim 1, wherein the number of reaction chambers is greater than 10,000.

7. The array of claim 1, wherein the depth of substantially all of the reaction chambers is between 10 - 100 μm.

8. The array of claim 1, wherein the depth of substantially all of the reaction chamber is 50-55 μm.

9. The array of claim 1, wherein the reaction chambers are formed on one side of the fiber optic faceplate.

10. The array of claim 1, wherein the array is comprised of a surface comprising a plurality of reaction chambers and an opposed, planar, polished surface without reaction chambers, wherein the polished surface is optically transmissive such that optical signals from the reaction chambers are detected through the polished surface, wherein the distance between the surface comprising the reaction chambers and the polished surface is no greater than 5 mm in thickness.

11. An array comprising a substrate having a surface comprising a plurality of reaction chambers and a thin film coating on said surface, wherein the thin film coating is from 0.1 – 5.0 microns thick, and impermeable to water.

12. The array of claim 11, wherein the thin film coating is optically transparent.

13. The array of claim 11, wherein the thin film coating is 200-400 nm thick on the surface of the substrate.

14. The array of claim 11, wherein the thin film coating is 50-100 nm thick on the side walls

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of the reaction chambers and 100-300 nm thick on the bottom of the reaction chambers.

15. The array of claim 11, wherein the thin film coating is an inorganic polymer.

16. The array of claim 11, wherein the thin film coating is silicon dioxide.

17. An array comprising:

(a) a substrate having a surface with a plurality of reaction chambers, wherein said substrate is a fiber optic faceplate; and

(b) an index feature.

18. The array of claim 17, wherein the array substrate comprises one or more side edges beveled at an angle.

19. The array of claim 17, wherein the angle is substantially 45 degrees.

20. The array of claim 17, wherein the index feature comprises a corner notch.

21. The array of claim 17, wherein the substrate is marked with one or more identifier codes.

22. The array of claim 17, wherein the spacing between the center points of two adjoining reaction chambers is between 5 μm to 200 μm, and each reaction chamber has a width in at least one dimension of between 4 μm and 190 μm.

23. The array of claim 17, wherein the number of reaction chambers is less than 10,000.

24. The array of claim 17, wherein the number of reaction chambers is greater than 10,000.

25. The array of claim 17, wherein the depth of substantially all of the reaction chambers is 10 - 100 μm.

26. The array of claim 17, wherein the depth of substantially all of the reaction chambers is 50-55 μm.

27. The array of claim 17, wherein the reaction chambers are formed on one surface of the fiber optic faceplate.

28. The array of claim 17, wherein the array is comprised of a surface comprising a plurality of reaction chambers and an opposed, planar, polished surface without reaction chambers, wherein the polished surface is optically transmissive such that optical signals from the reaction chambers are detected through the polished surface, wherein the distance between the surface comprising the reaction chambers and the polished surface is no greater than 5 mm in thickness.

29. An array comprising a substrate, wherein the substrate is a fiber optic faceplate having a top surface comprising a plurality of reaction chambers and a bottom surface; wherein, each reaction chamber is comprised of a bottom and a sidewall;

further wherein at least one of the bottom or the sidewall of substantially all of the reaction

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chambers or the top surface is coated with a non-transparent thin film coating, wherein the non-transparent coating is opaque, semi-opaque, shiny opaque, or translucent; and at least one of the bottom or sidewall of substantially all of the reaction chambers or top surface is coated with a transparent thin film coating, which differs from the non-transparent coating, and wherein the transparent coating is optically transparent, 0.1- 5.0 microns thick, and is impermeable to water.

30. The array of claim 29, wherein the transparent coating is silicon dioxide.

31. The array of claim 29, wherein, when present, the thickness of the transparent coating is about 200-400 nm on the top surface, 50-100 nm on the sidewall, and 100-300 nm on the bottom.

32. The array of claim 29, wherein the non-transparent coating is opaque.

33. The array of claim 29, wherein the non-transparent coating is semi-opaque.

34. The array of claim 29, wherein the non-transparent coating is shiny opaque

35. The array of claim 29, wherein the non-transparent coating is translucent.

36. The array of claim 29, wherein, when present, the thickness of the non-transparent coating is about 200-300 nm on the top surface, 60-120 nm on the sidewall, and no less than 50 nm on the corner area, wherein the corner area is the comprised of the junction of the bottom and sidewall of the reaction chamber.

37. The array of claim 29, wherein the non-transparent coating is selected from an organic compound, an inorganic compound, and a non-metal oxide.

38. The array of claim 37, wherein the inorganic compound is a metal.

39. The array of claim 38, wherein the metal is selected from chromium, gold, silver, titanium, platinum and aluminum.

40. The array of claim 29, wherein the sidewall of substantially all of the reaction chambers is coated with the transparent coating.

41. The array of claim 29, wherein the top surface is coated with the transparent coating.

42. The array of claim 29, wherein the bottom of substantially all of the reaction chambers is coated with the transparent coating.

43. The array of claim 29, wherein each one of the sidewall and bottom of substantially all of the reaction chambers and top surface is coated with the transparent coating.

44. The array of claim 29, wherein the bottom and sidewall of substantially all of the reaction chambers are partially coated with a transparent coating, such that the transparent coating is applied to the corner area formed at the junction between the bottom and sidewall

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of the reaction chamber and is absent from the center of the bottom of the reaction chamber which forms an aperture near the center of the bottom.

45. The array of claim 44, wherein the diameter of the aperture is 28 microns and a ring formed around the aperture when the coating is applied to the corner area has a width of 8.5 microns.

46. The array of claim 29, wherein the sidewall of substantially all of the reaction chambers is coated with the non-transparent coating.

47. The array of claim 29, wherein the top surface is coated with the non-transparent coating.

48. The array of claim 29, wherein the bottom of substantially all of the reaction chambers is coated with the non-transparent coating.

49. The array of claim 29, wherein the bottom and sidewall of substantially all of the reaction chambers are partially coated with a non-transparent coating, such that the non-transparent coating is applied to the corner area formed at the junction between the bottom and sidewall of the reaction chamber and is absent from the center of the bottom of the reaction chamber which forms an aperture near the center of the bottom.

50. The array of claim 49, wherein the transparent coating is applied after the non-transparent coating is applied, and the transparent coating is applied to at least one of the bottom or sidewall of substantially all of the reaction chambers or top surface of the array

51. The array of claim 29, wherein the top surface and bottom of substantially all of the reaction chambers are coated with the transparent coating, and the sidewall of substantially all of the reaction chambers is coated with the non-transparent coating.

52. The array of claim 29, wherein the bottom of substantially all of the reaction chambers is coated with the transparent coating, and the sidewall of substantially all of the reaction chambers and the top surface are coated with the non-transparent coating.

53. The array of claim 29, wherein the bottom of substantially all of the reaction chambers is coated with the transparent coating, the sidewall of substantially all of the reaction chambers is coated with the non-transparent coating, and the top surface is not coated.

54. The array of claim 29, wherein the transparent coating comprises at least a first transparent coating.

55. The array of claim 29, wherein the non-transparent coating comprises at least a first non-transparent coating.

56. The array of claim 29, wherein the non-transparent coating is applied before any transparent coating is applied.

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57. The array of claim 29, wherein the bottom or sidewall of substantially all of the reaction chambers or top surface is coated with the non-transparent coating before any transparent coating is applied.

58. The array of claim 29, wherein the bottom or sidewall of substantially all of the reaction chambers or top surface is coated with the transparent coating before any non-transparent coating is applied.

59. The array of claim 29, wherein an enzyme is immobilized on the non-transparent coating.

60. The array of claim 29, wherein an enzyme is immobilized on the transparent coating.

61. The array of claim 29, wherein the number of reaction chambers is greater than 10,000.

62. An array comprising a substrate, wherein the substrate is a fiber optic faceplate having a top surface comprising a plurality of reaction chambers and a bottom surface; wherein each reaction chamber is comprised of a bottom and a sidewall;
further wherein at least one of the bottom or sidewall of substantially all of the reaction chambers or top surface is coated with a non-transparent, thin film coating, wherein the non-transparent coating is opaque, semi-opaque, shiny opaque, or translucent.

63. The array of claim 62, wherein at least one of the bottom or sidewall of substantially all of the reaction chambers or the top surface is not coated.

64. The array of claim 62, wherein the non-transparent coating is opaque.

65. The array of claim 62, wherein the non-transparent coating is semi-opaque.

66. The array of claim 62, wherein the non-transparent coating is shiny opaque.

67. The array of claim 62, wherein the non-transparent coating is translucent.

68. The array of claim 62, wherein, when present, the thickness of the non-transparent coating is about 200-300 nm on the top surface, 60-120 nm on the sidewall, and no less than 50 nm on the corner area, wherein the corner area is the comprised of the junction of the bottom and sidewall of the reaction chamber.

69. The array of claim 62, wherein the non-transparent coating is selected from organic compound, inorganic compound, and non-metal oxide.

70. The array of claim 69, wherein the inorganic compound is a metal.

71. The array of claim 70, wherein the metal is selected from chromium, gold, silver, titanium, platinum, and aluminum.

72. The array of claim 62, wherein the sidewall of substantially all of the reaction chambers is coated.

73. The array of claim 62, wherein top surface is coated.

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74. The array of claim 62, wherein the bottom of substantially all of the reaction chambers is coated.

75. The array of claim 62, wherein the bottom and sidewall of substantially all of the reaction chambers are partially coated with a non-transparent coating, such that the non-transparent coating is applied to the corner area formed at the junction between the bottom and sidewall of the reaction chamber and is absent from the center of the bottom of the reaction chamber which forms an aperture near the center of the bottom.

76. The array of claim 75, wherein the diameter of the aperture is 28 microns and a ring formed by the aperture when the coating is applied to the corner area has a width of 8.5 microns.

77. The array of claim 62, wherein the sidewall and bottom of substantially all of the reaction chambers are coated.

78. The array of claim 62, wherein the non-transparent coating comprises at least a first non-transparent coating.

79. The array of claim 62, wherein the non-transparent coating comprises at least a first non-transparent coating and a second non-transparent coating, further wherein the first and second coatings are not the same.

80. The array of claim 62, wherein an enzyme is immobilized on the non-transparent coating.

81. The array of claim 62, wherein the number of reaction chambers is greater than 10,000.

82. A process for coating a substrate containing a plurality of reaction chambers, wherein said coating is deposited onto the surface of the substrate using a method of deposition selected from vapor and liquid, wherein said coating is a thin film that has a thickness of 0.1 – 5.0 microns, is optically transparent, and is impermeable to water.

83. The process of claim 82, wherein the substrate is a fiber optic faceplate.

84. The process of claim 82, wherein there are at least 10,000 reaction chambers.

85. The process of claim 82, wherein the depth of substantially all of the reaction chambers is 10 - 100 μm.

86. The process of claim 82, wherein the reaction chambers are formed on one side of the fiber optic faceplate.

87. The process of claim 82, wherein the thin film coating is silicon dioxide.

88. The process of claim 82, wherein the thin film coating is deposited using a vapor deposition method.

89. A process for depositing a non-transparent thin film coating on a substrate having a top

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surface comprising a plurality of reaction chambers and a bottom surface, wherein each reaction chamber is comprised of a bottom or sidewall; and the non-transparent coating is opaque, semi-opaque, shiny opaque, or translucent and is deposited on at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the substrate comprising:

(a) mounting the substrate onto a substrate carrier at an angle;

(b) spinning the mounted substrate in a vacuum chamber;

(c) depositing the non-transparent coating onto at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the substrate;

(d) removing the substrate from the vacuum chamber; and

(e) dismounting the substrate coated with the non-transparent coating from the substrate carrier.

90. The process of claim 89, wherein the substrate is a fiber optic faceplate.

91. The process of claim 89, wherein at least one bottom or sidewall of substantially all of the reaction chambers or top surface of the substrate is coated with a non-transparent coating prior to the application of a transparent coating.

92. The process of claim 89, wherein the transparent coating is optically transparent, 0.1-5.0 microns thick, and impermeable to water.

93. An apparatus for analyzing a nucleic acid sequence, the apparatus comprising

(a) a delivery chamber, wherein the chamber comprises the array of any one of claims 1-81;

(b) a reagent delivery device in communication with the reagent delivery chamber;

(c) an imaging system in communication with reagent delivery chamber; and

(d) a data collection system in communication with the imaging system.

94. A method for sequencing a nucleic acid, the method comprising:

(a) providing a plurality of single-stranded nucleic acid templates deposited within a plurality of reaction chambers on the array of any one of claims 1-81;

(b) performing a pyrophosphate sequencing reaction simultaneously on substantially all reaction chambers by annealing an effective amount of a sequencing primer to the nucleic acid templates and extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product such that, if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer, a

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sequencing reaction byproduct is produced; and

(c) identifying the sequencing reaction byproduct, thereby determining the sequence of the nucleic acid in each reaction chamber.

95. An apparatus comprising:

(a) at least one clamp; fabricated from a first acid resistant material, comprising a base having an opposed top surface, bottom surface, and sides and ends, and prongs, wherein said prongs are integral with said top surface; and

(b) a gasket, fabricated from a second acid resistant material, comprising an index feature, a top surface and a bottom surface, wherein said surfaces are opposed.

96. The apparatus of claim 95, wherein said first acid resistant material is poly ether ether ketone.

97. The apparatus of claim 95, wherein said second acid resistant material is silicone.

98. The apparatus of claim 95, wherein the index feature of the gasket comprises a corner barrier.

99. A method of etching one side of a fiber optic face plate comprising the steps of:

(a) combining two fiber optic face plates with a gasket fabricated from a second acid resistant material, wherein said gasket is positioned in between the fiber optic plates to form a sandwich;

(b) affixing clamps, fabricated from a first acid resistant material, to hold the fiber optic face plates and gasket together, thereby forming a clamped sandwich having a seal that is impermeable to liquid;

(c) transferring the clamped sandwich to an acid bath for a time sufficient to form reaction chambers of the desired depth;

(d) removing the clamped sandwich from the acid bath;

(e) transferring the clamped sandwich to a first water bath;

(f) sonicating the clamped sandwich for about 5 minutes;

(g) replacing said first water bath with a second water bath;

(h) sonicating the clamped sandwich for about 5 minutes;

(i) removing the clamped sandwich from the second water bath; and

(j) detaching the clamps and gasket.

100. The method of claim 99, wherein the acid bath is nitric acid.

101. The method of claim 99, wherein said first acid resistant material is poly ether ether ketone.

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102. The apparatus of claim 99, wherein said second acid resistant material is silicone.

103. A method of cleaning a fiber optic faceplate comprising a plurality of reaction chambers:

(a) washing the fiber optic faceplate in a solution comprising ammonium hydroxide and hydrogen peroxide;

(b) washing the fiber optic faceplate in ethylenediaminetetraacetic acid;

(c) rinsing the fiber optic faceplate in water;

(d) washing the fiber optic faceplate in a solution comprising ammonium hydroxide and hydrogen peroxide;

(e) rinsing the fiber optic faceplate in water;

(f) washing the fiber optic faceplate in ethylenediaminetetraacetic acid;

(g) rinsing the fiber optic faceplate in water;

(h) sonicating the fiber optic faceplate in a basic solution;

(i) rinsing the fiber optic faceplate in water; and

(j) repeating steps g and h.

104. The method of claim 103, wherein said basic solution is substantially 5% Contrad®.

105. The method of claim 103, wherein step i is repeated once.

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Figure 1
SEMs of Coated FOFs

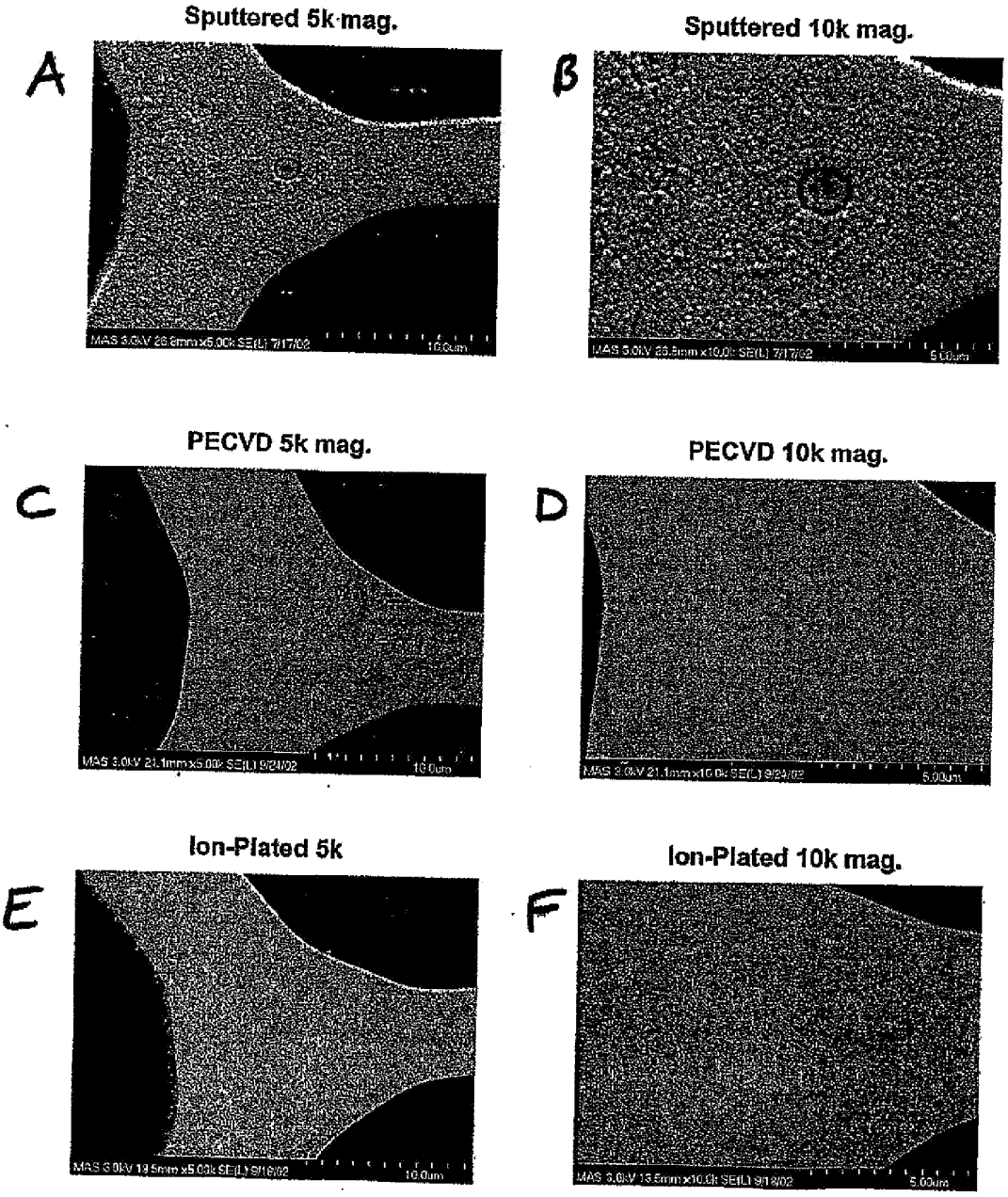
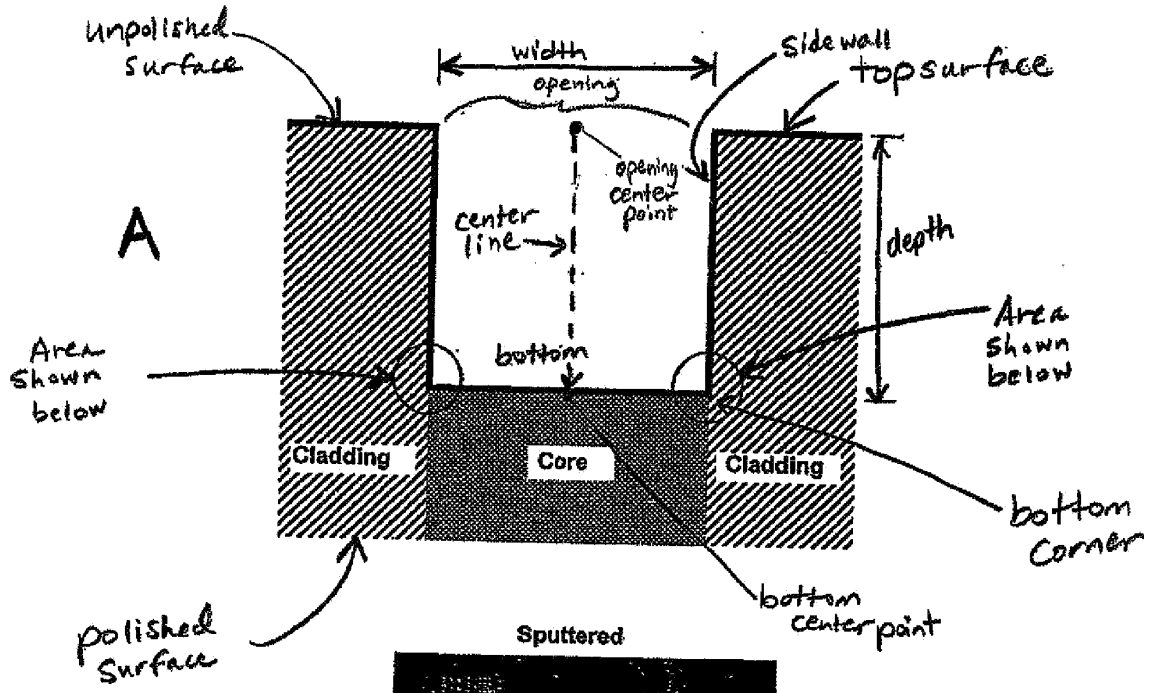
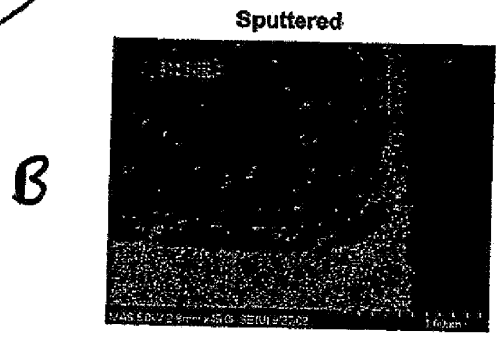
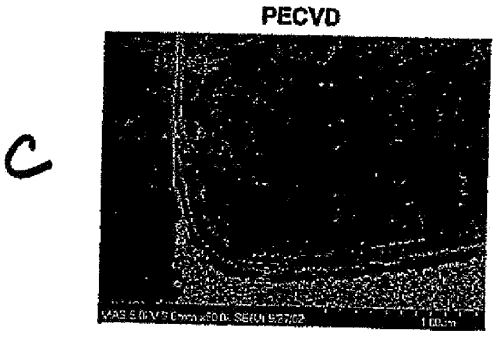


Figure 2
Cross-Section SEMs of Coated FOFs







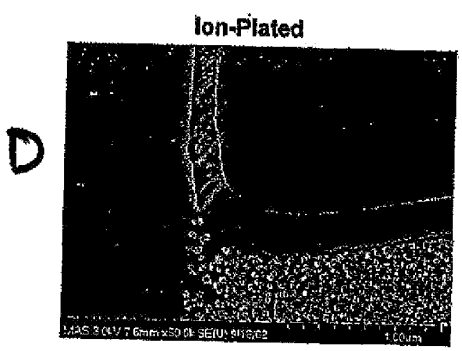


Figure 3

Schematic Diagram Of Ion-Plating Process

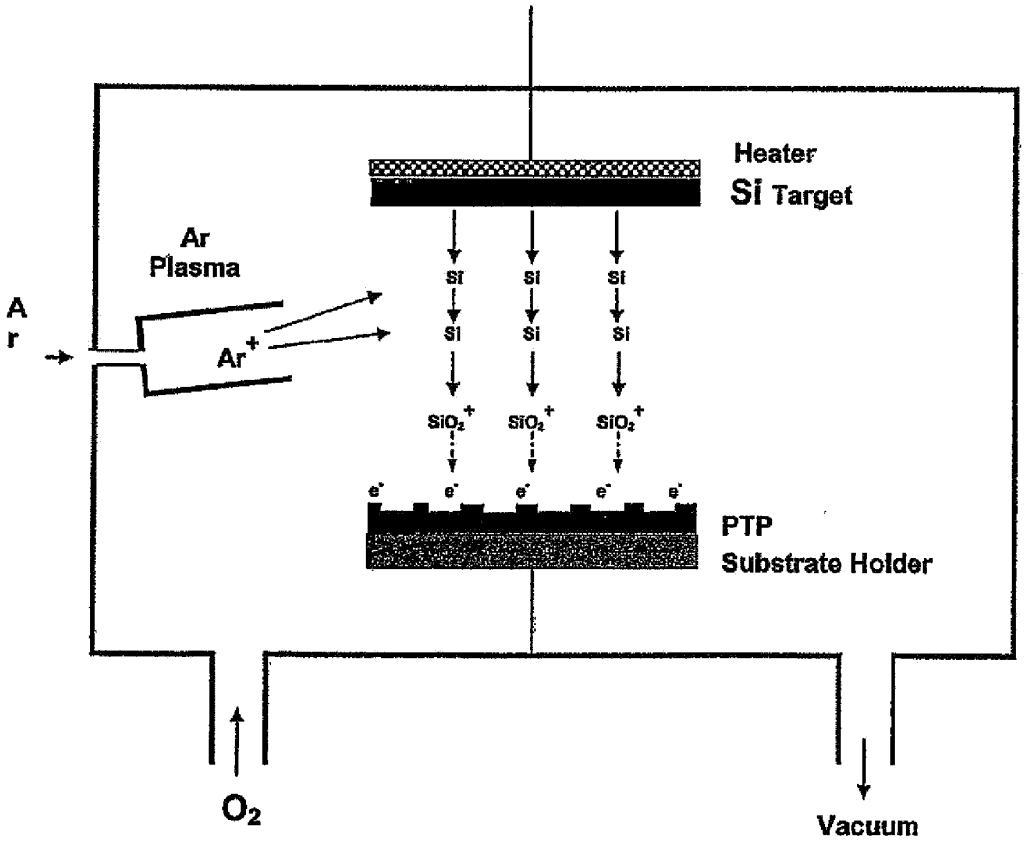


Figure 4
PCR-Induced Sequencing Background of SiO_2-Coated and Uncoated FOFs

(error bars = 95% CL for 3 samples)

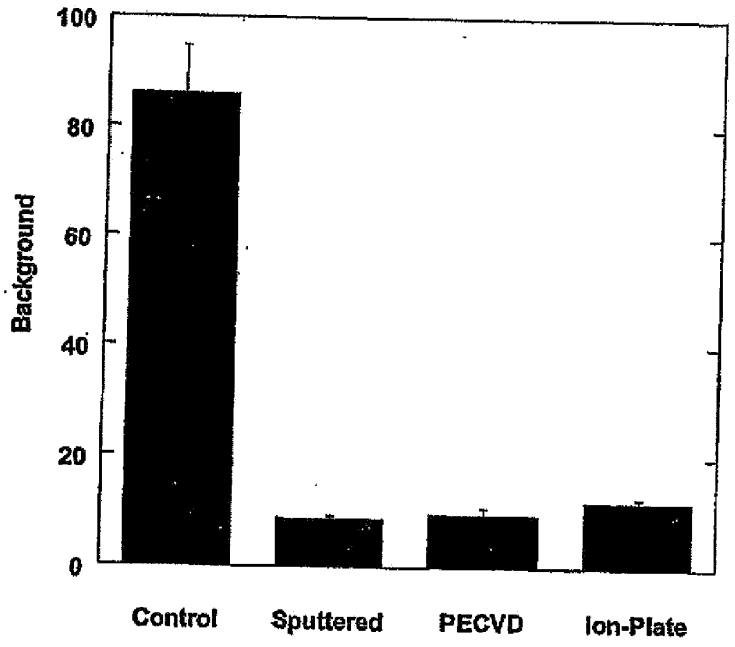


Figure 5
Sequencing Performance Metrics of SiO_2-Coated and Uncoated FOFs

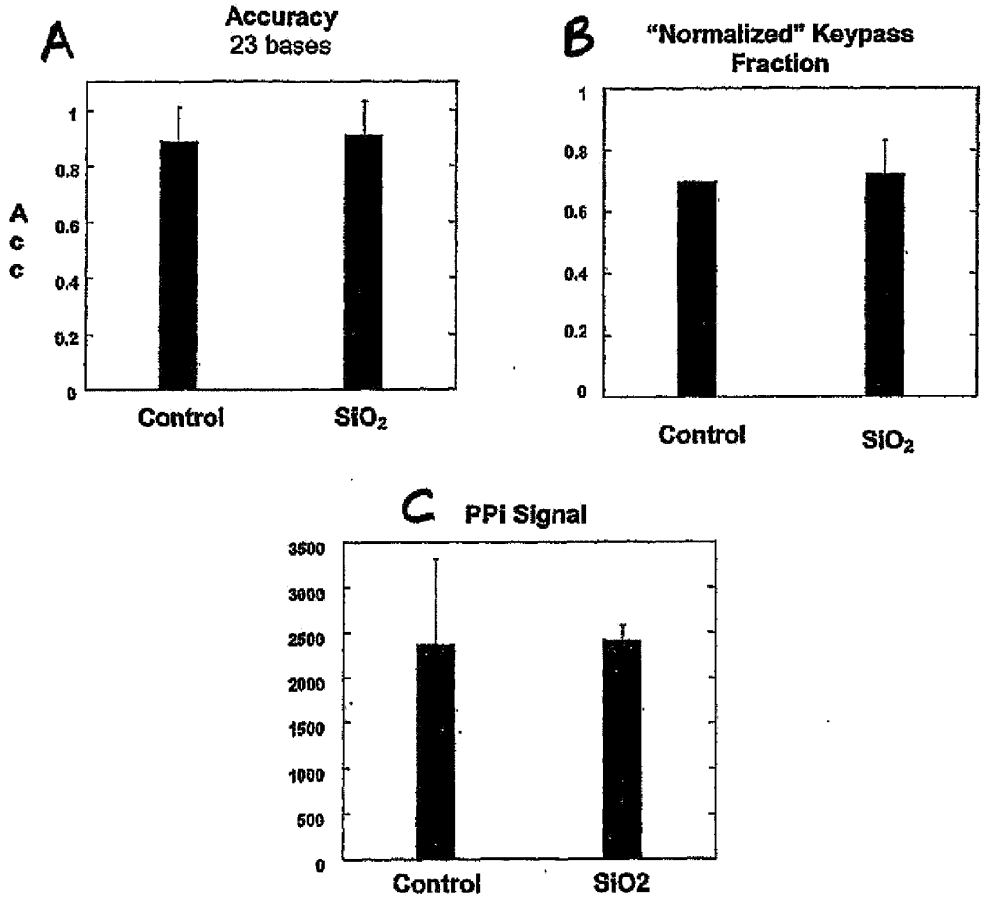
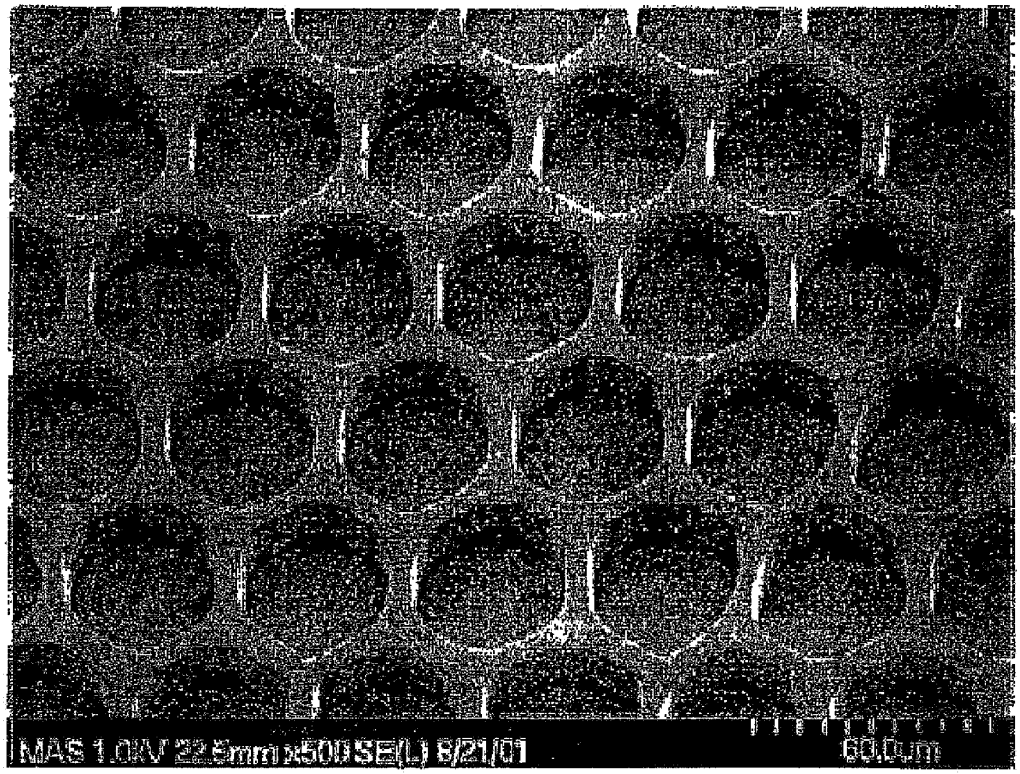
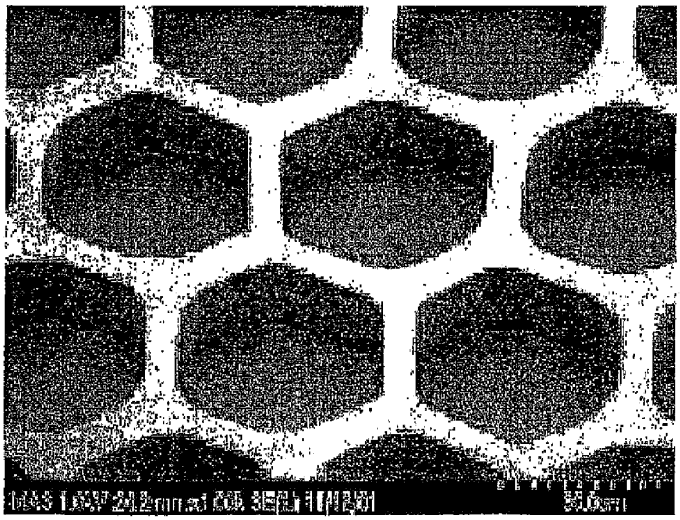


Figure 6

A



B



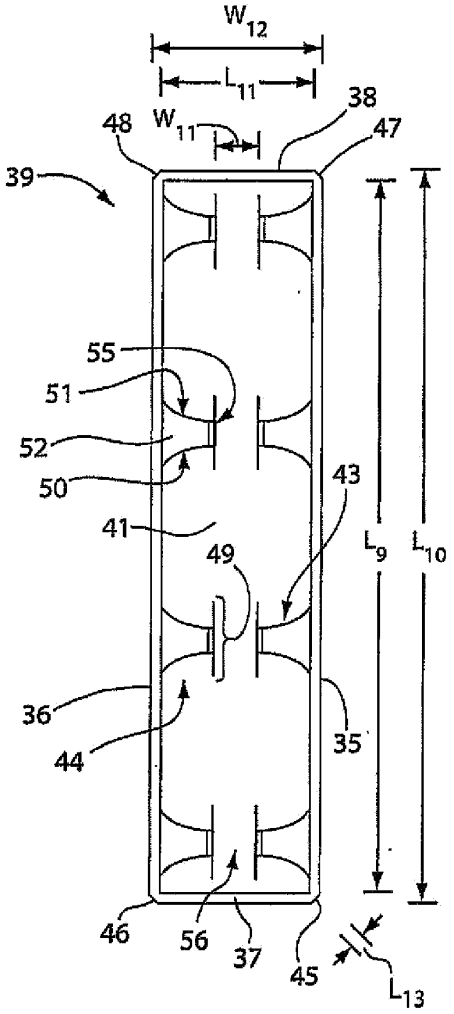

W12
L11
38
48
W11
47
39
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52
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43
41
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L9
L10
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44
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56
37
45
L13

Fig. 7a

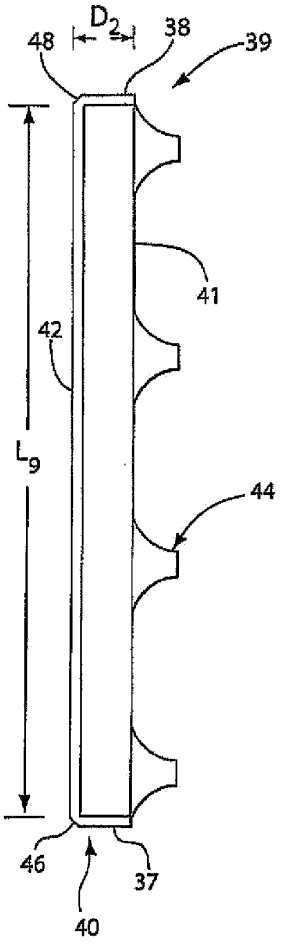


Fig. 7b

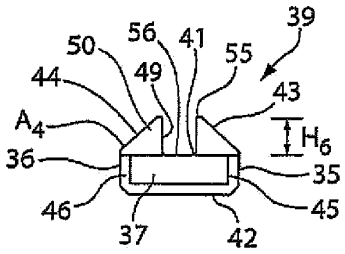

39
50
56
41
55
44
49
43
A_4
H_6
36
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46
37
42
45

Fig. 7c

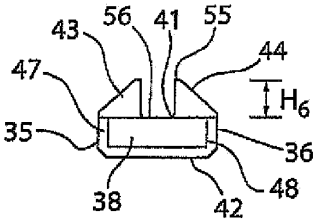

43
56
41
55
44
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H_6
35
36
38
42
48

Fig. 7d

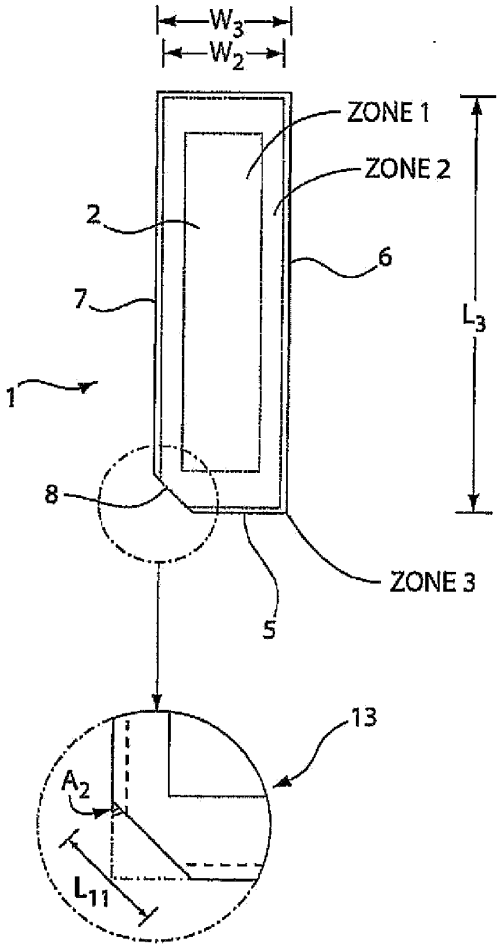


Fig. 8a

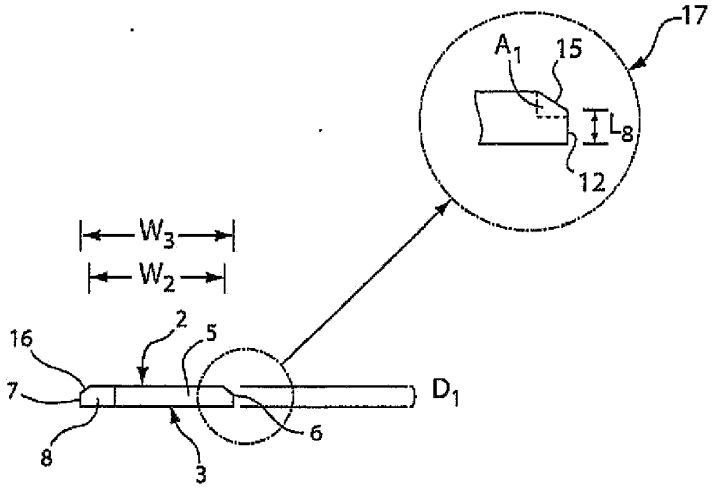


Fig. 8b

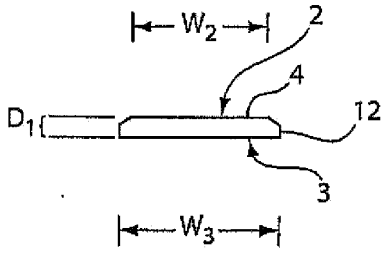


Fig. 8c

Figure 9

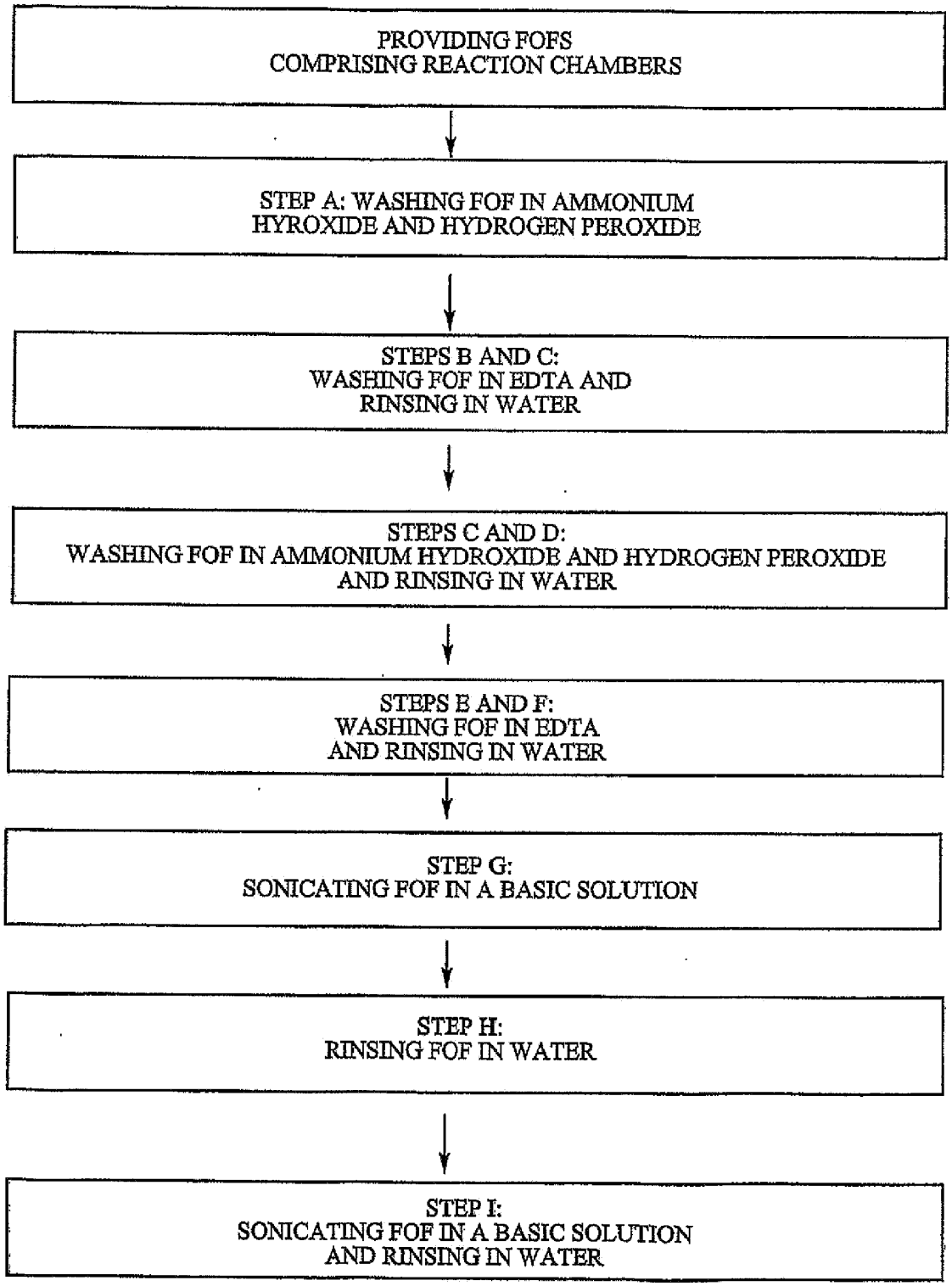
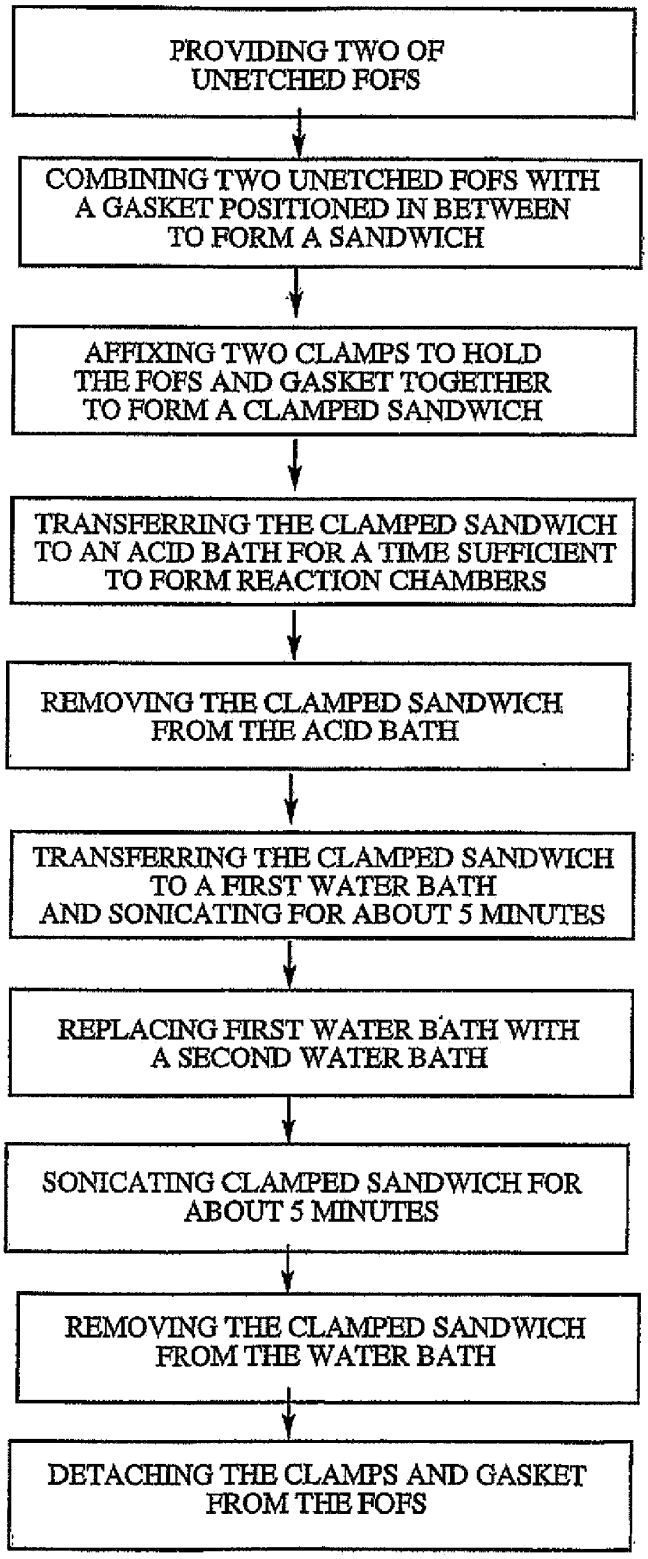

PROVIDING FOFS
COMPRISING REACTION CHAMBERS
STEP A: WASHING FOF IN AMMONIUM
HYROXIDE AND HYDROGEN PEROXIDE
STEPS B AND C:
WASHING FOF IN EDTA AND
RINSING IN WATER
STEPS C AND D:
WASHING FOF IN AMMONIUM HYDROXIDE AND HYDROGEN PEROXIDE
AND RINSING IN WATER
STEPS E AND F:
WASHING FOF IN EDTA
AND RINSING IN WATER
STEP G:
SONICATING FOF IN A BASIC SOLUTION
STEP H:
RINSING FOF IN WATER
STEP I:
SONICATING FOF IN A BASIC SOLUTION
AND RINSING IN WATER

Figure 10


PROVIDING TWO OF UNETCHED FOFS
COMBINING TWO UNETCHED FOFS WITH A GASKET POSITIONED IN BETWEEN TO FORM A SANDWICH
AFFIXING TWO CLAMPS TO HOLD THE FOFS AND GASKET TOGETHER TO FORM A CLAMPED SANDWICH
TRANSFERRING THE CLAMPED SANDWICH TO AN ACID BATH FOR A TIME SUFFICIENT TO FORM REACTION CHAMBERS
REMOVING THE CLAMPED SANDWICH FROM THE ACID BATH
TRANSFERRING THE CLAMPED SANDWICH TO A FIRST WATER BATH AND SONICATING FOR ABOUT 5 MINUTES
REPLACING FIRST WATER BATH WITH A SECOND WATER BATH
SONICATING CLAMPED SANDWICH FOR ABOUT 5 MINUTES
REMOVING THE CLAMPED SANDWICH FROM THE WATER BATH
DETACHING THE CLAMPS AND GASKET FROM THE FOFS

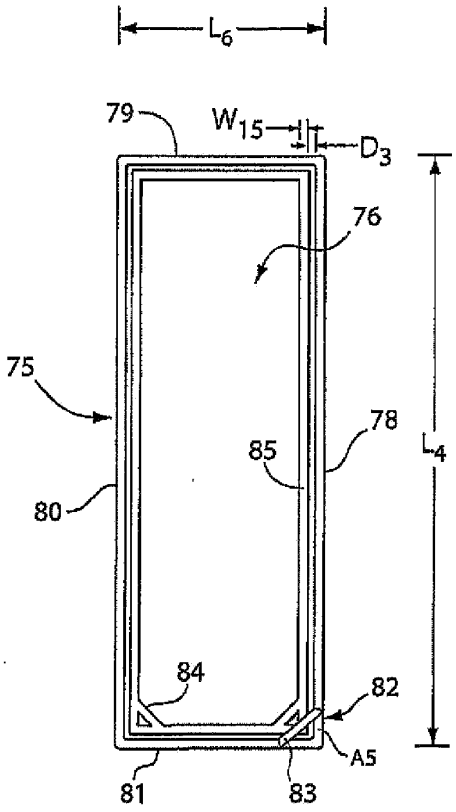


Fig. 11a

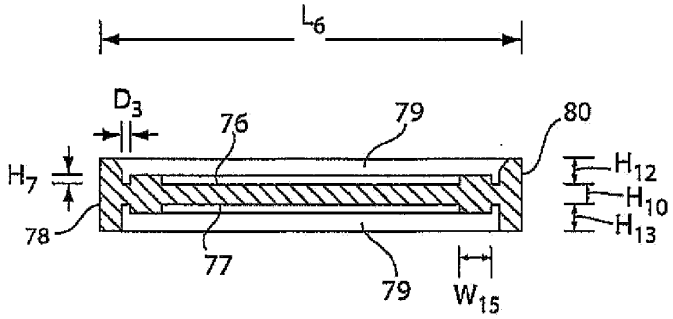


Fig. 11b

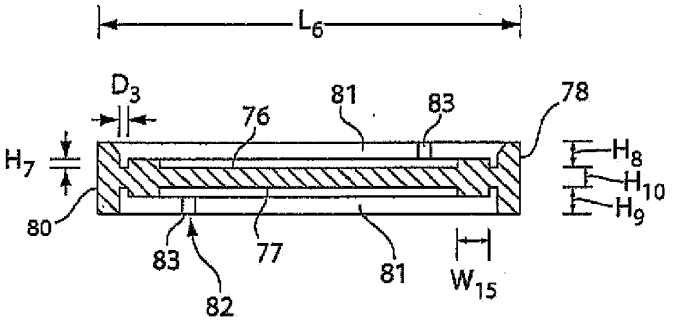


Fig. 11c

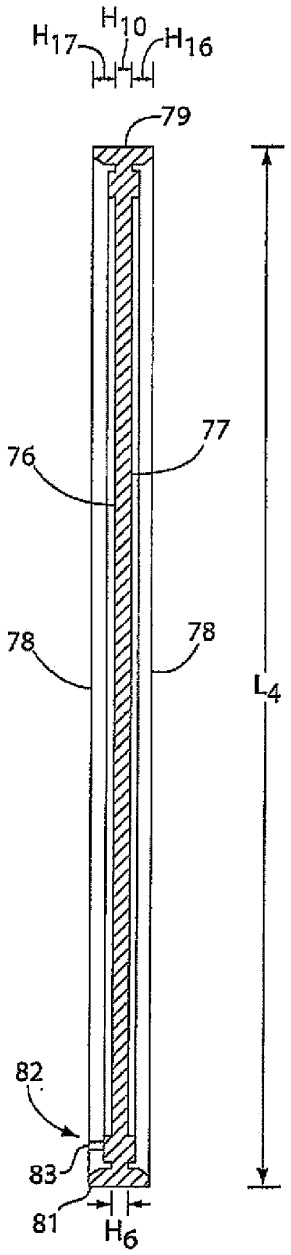


Fig. 11d

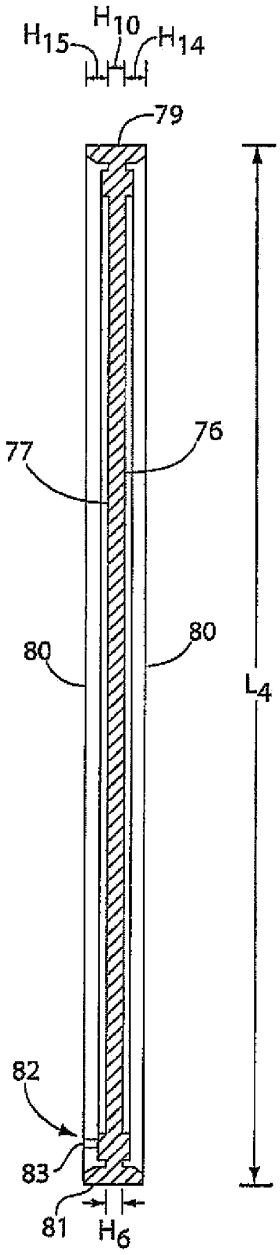


Fig. 11e

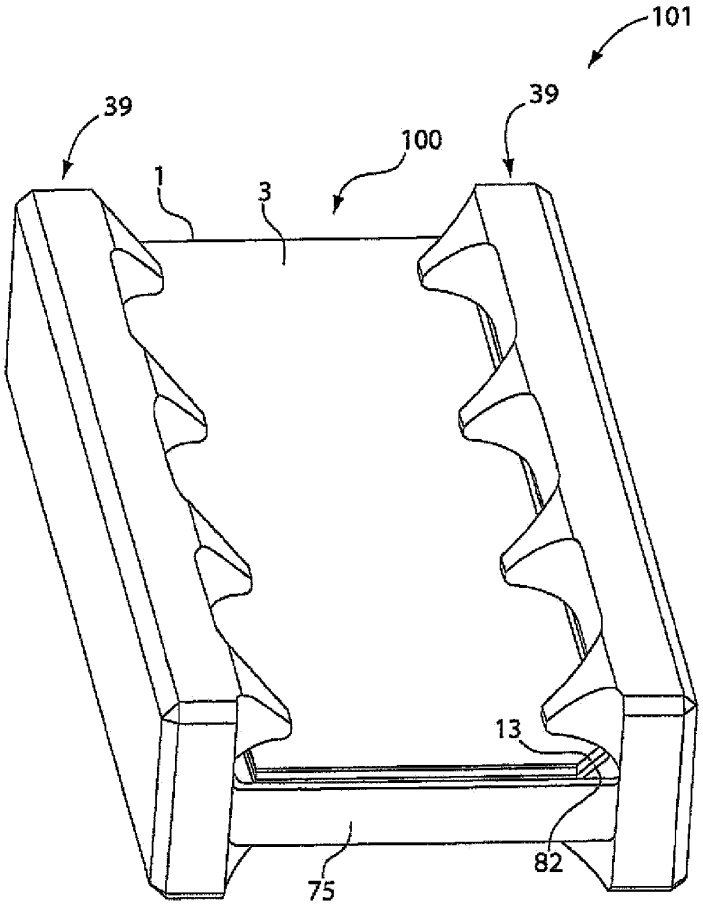


Fig. 12

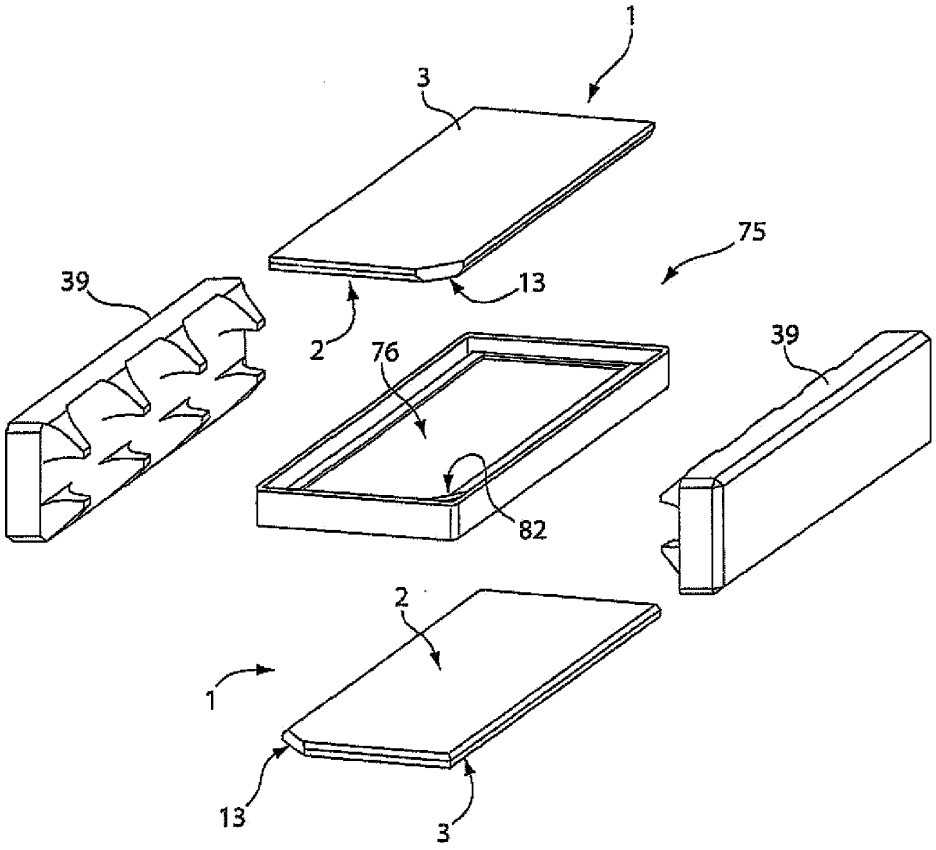


Fig. 13

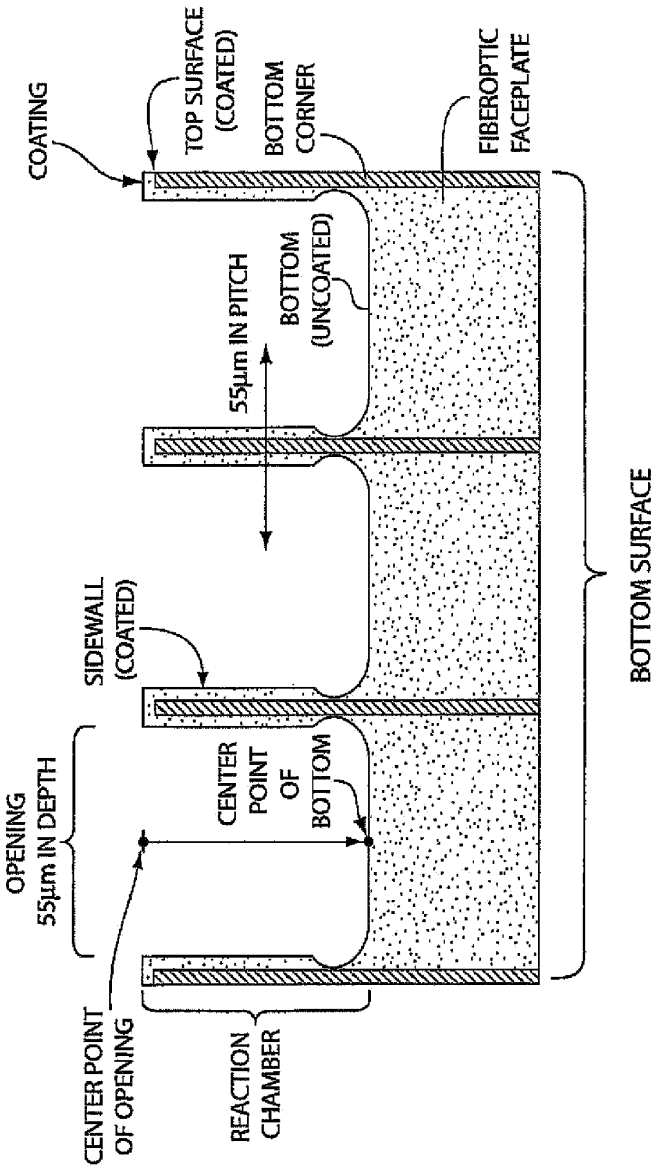

COATING
TOP SURFACE (COATED)
BOTTOM CORNER
FIBEROPTIC FACEPLATE
55µm IN PITCH
BOTTOM (UNCOATED)
SIDEWALL (COATED)
OPENING 55µm IN DEPTH
CENTER POINT OF BOTTOM
BOTTOM SURFACE
CENTER POINT OF OPENING
REACTION CHAMBER

Fig. 14

Figure 15a

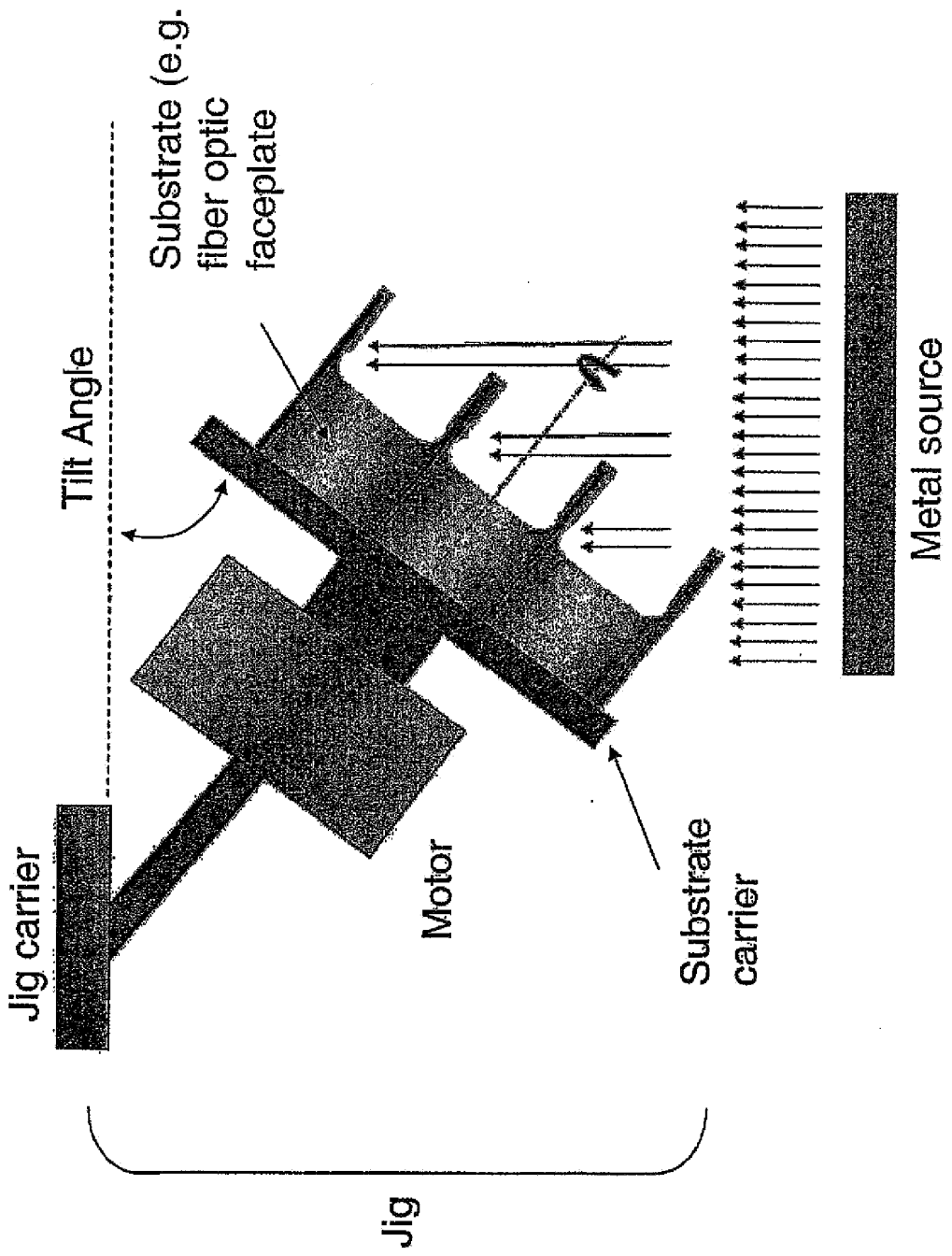
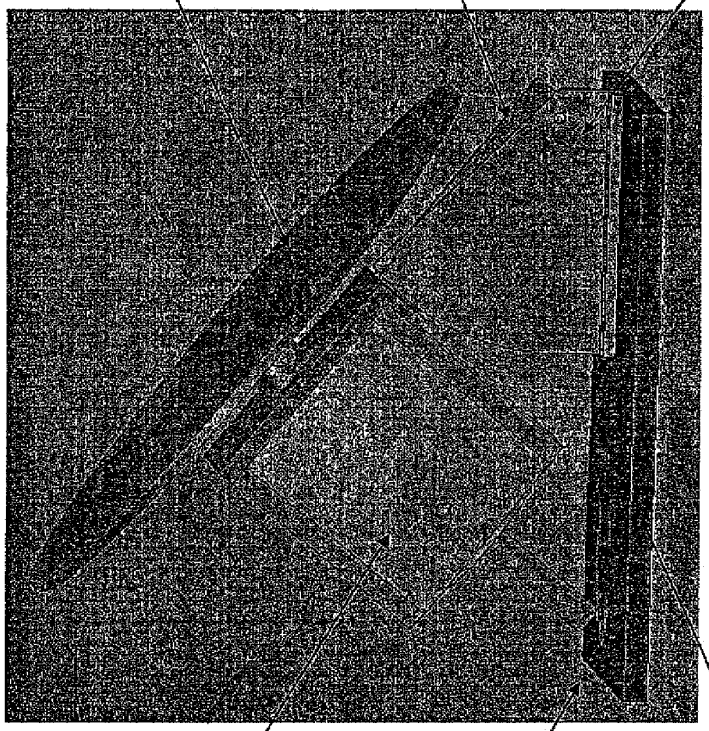

Substrate (e.g. fiber optic faceplate
Tilt Angle
Metal source
Jig carrier
Motor
Substrate carrier
Jig

Figure 15b

Rotary and Angled Jig Assembly



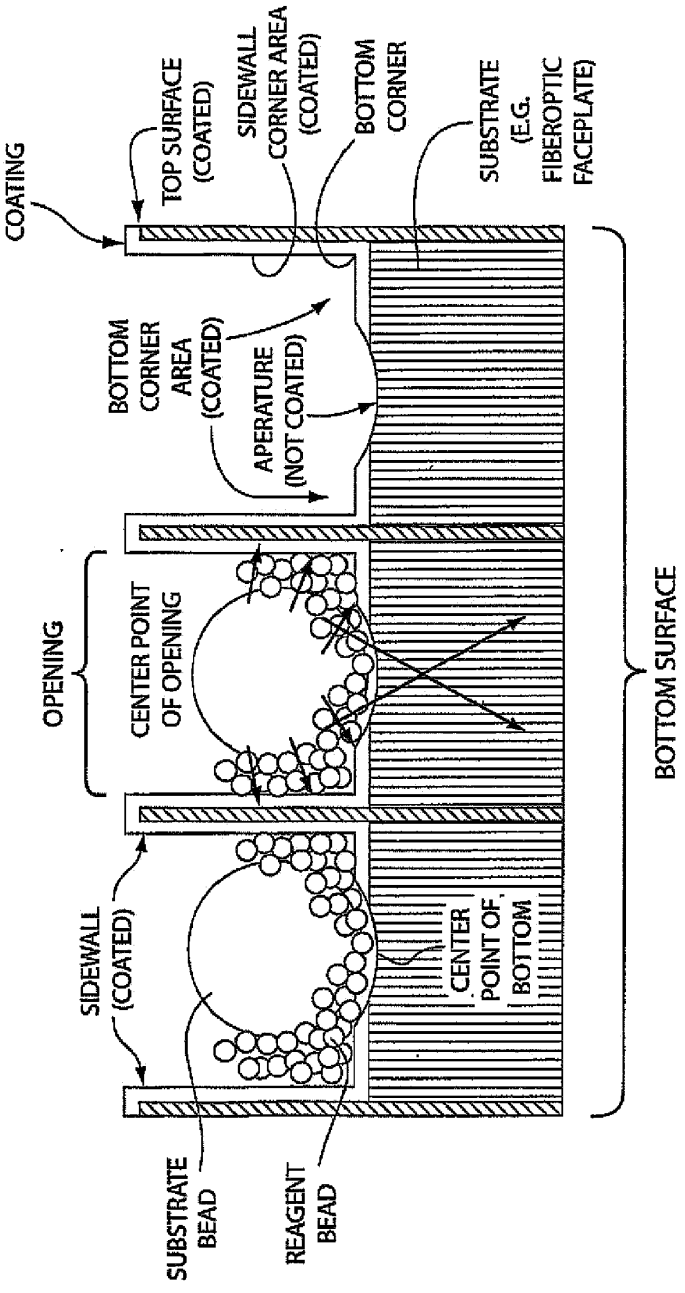

COATING
TOP SURFACE (COATED)
SIDEWALL CORNER AREA (COATED)
BOTTOM CORNER
SUBSTRATE (E.G. FIBEROPTIC FACEPLATE)
BOTTOM CORNER AREA (COATED)
APERATURE (NOT COATED)
OPENING
CENTER POINT OF OPENING
SIDEWALL (COATED)
CENTER POINT OF BOTTOM
BOTTOM SURFACE
SUBSTRATE BEAD
REAGENT BEAD

Fig. 16a

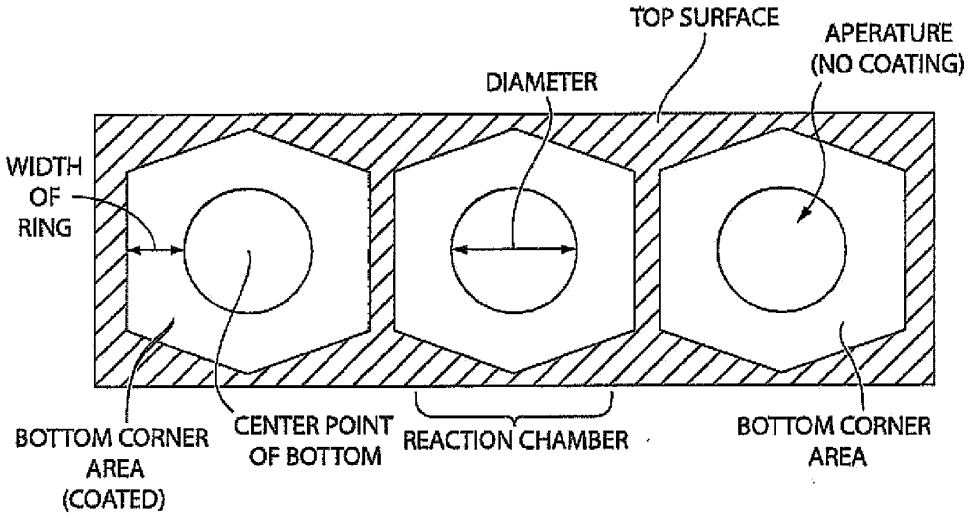

TOP SURFACE
APERATURE
(NO COATING)
DIAMETER
WIDTH
OF
RING
BOTTOM CORNER
AREA
(COATED)
CENTER POINT
OF BOTTOM
REACTION CHAMBER
BOTTOM CORNER
AREA

Fig. 16b

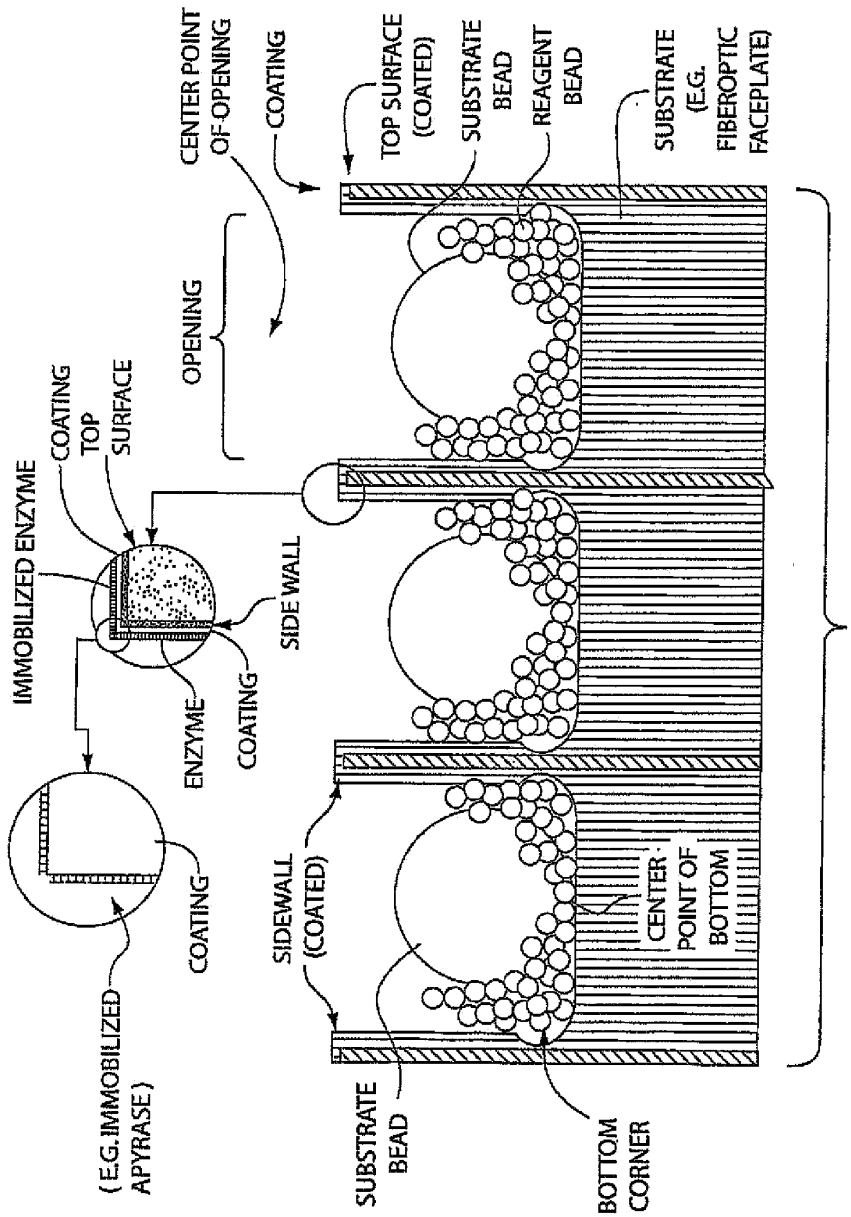

CENTER POINT OF OPENING
COATING
TOP SURFACE (COATED)
SUBSTRATE BEAD
REAGENT BEAD
SUBSTRATE (E.G. FIBEROPTIC FACEPLATE)
OPENING
COATING
TOP SURFACE
IMMOBILIZED ENZYME
SIDE WALL
ENZYME
COATING
SIDEWALL (COATED)
CENTER POINT OF BOTTOM
BOTTOM SURFACE
COATING
(E.G. IMMOBILIZED APYRASE)
SUBSTRATE BEAD
BOTTOM CORNER

Fig. 17

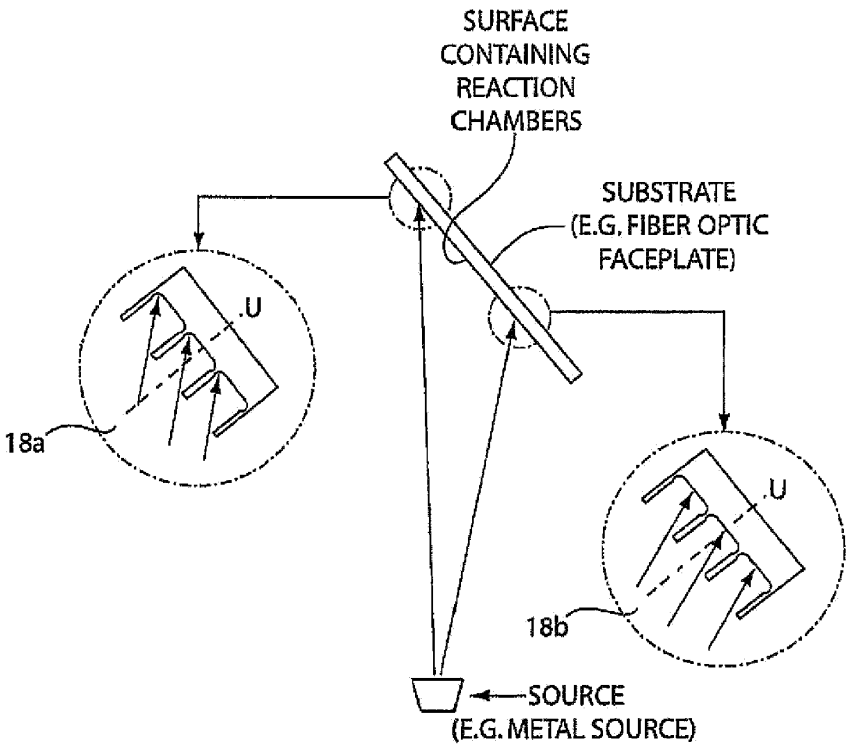

SURFACE
CONTAINING
REACTION
CHAMBERS
SUBSTRATE
(E.G. FIBER OPTIC
FACEPLATE)
U
18a
U
18b
SOURCE
(E.G. METAL SOURCE)

Fig. 18

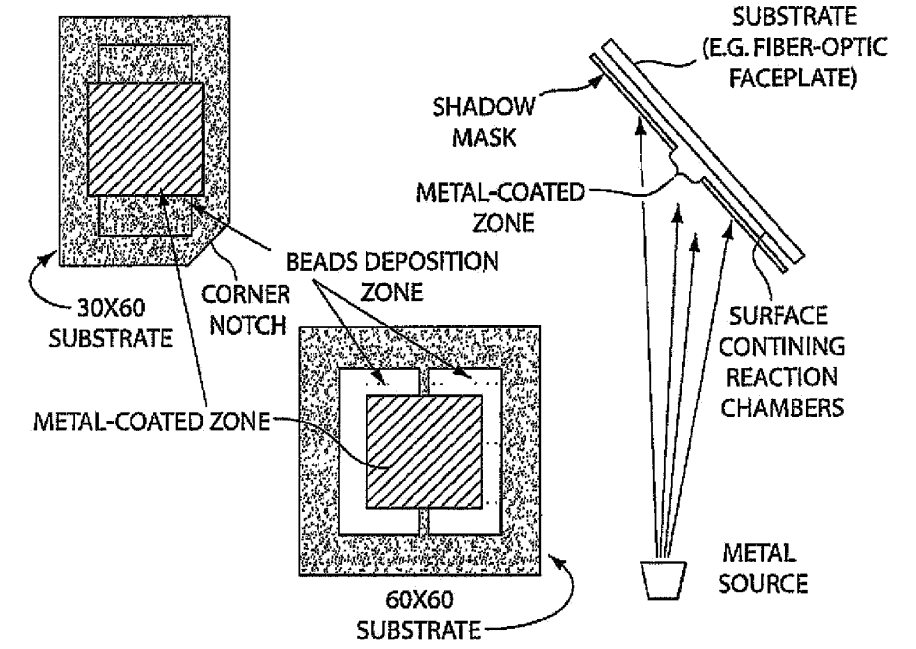

SUBSTRATE
(E.G. FIBER-OPTIC
FACEPLATE)
SHADOW
MASK
METAL-COATED
ZONE
BEADS DEPOSITION
ZONE
CORNER
NOTCH
30X60
SUBSTRATE
METAL-COATED ZONE
SURFACE
CONTINING
REACTION
CHAMBERS
METAL
SOURCE
60X60
SUBSTRATE

Fig. 19a

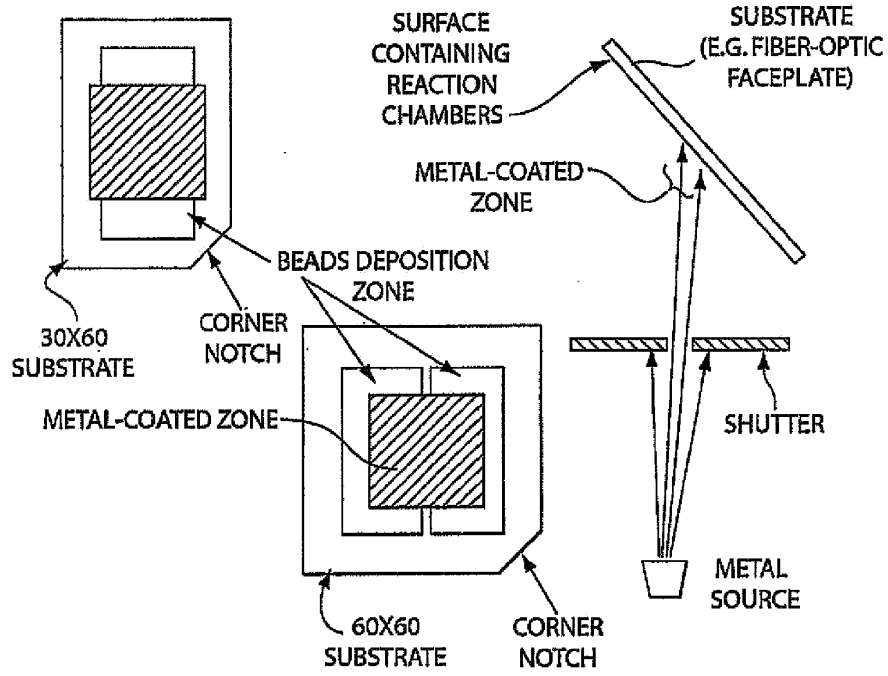

SURFACE CONTAINING REACTION CHAMBERS
SUBSTRATE (E.G. FIBER-OPTIC FACEPLATE)
METAL-COATED ZONE
BEADS DEPOSITION ZONE
30X60 SUBSTRATE
CORNER NOTCH
METAL-COATED ZONE
SHUTTER
METAL SOURCE
60X60 SUBSTRATE
CORNER NOTCH

Fig. 19b

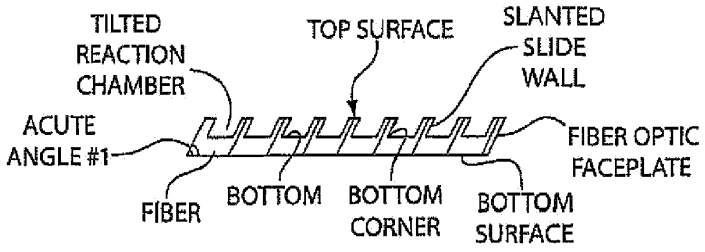


Fig. 20a

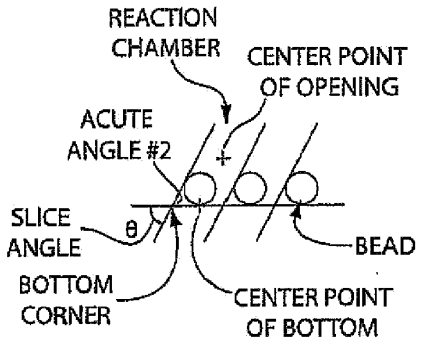


Fig. 20b

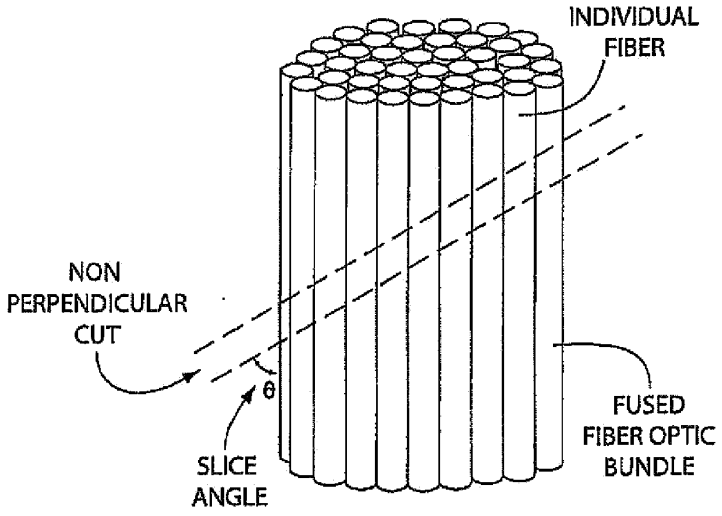


Fig. 20c

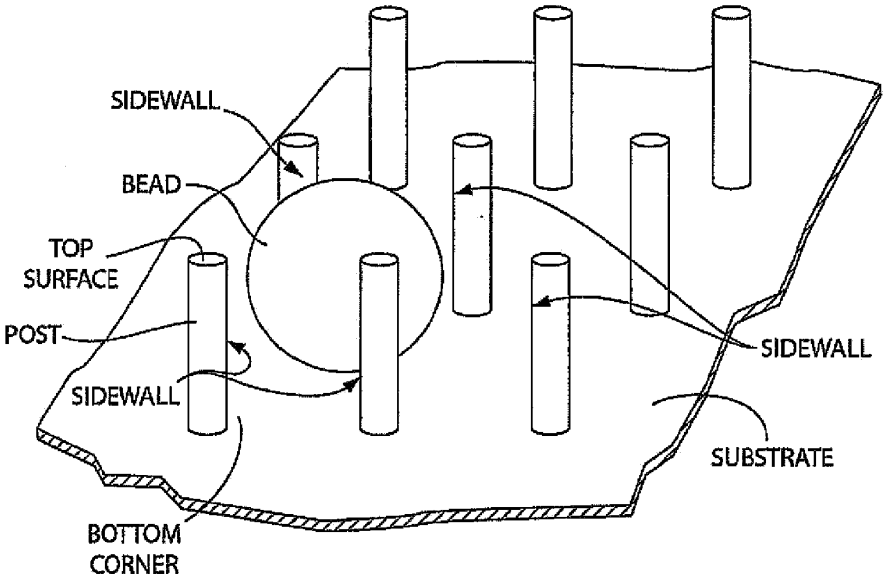

SIDEWALL
BEAD
TOP SURFACE
POST
SIDEWALL
SIDEWALL
SUBSTRATE
BOTTOM CORNER

Fig. 21a

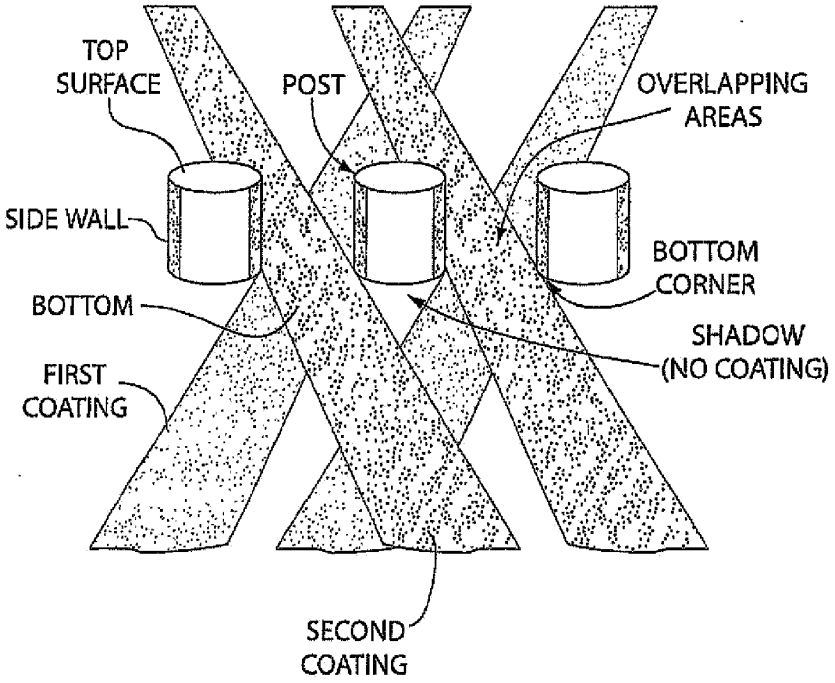

TOP SURFACE
POST
OVERLAPPING AREAS
SIDE WALL
BOTTOM CORNER
BOTTOM
SHADOW (NO COATING)
FIRST COATING
SECOND COATING

Fig. 21b

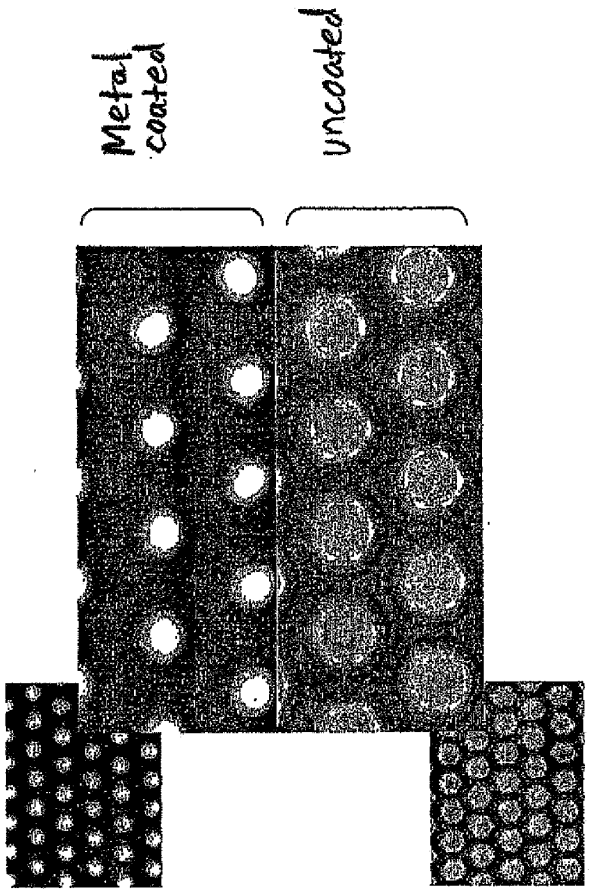
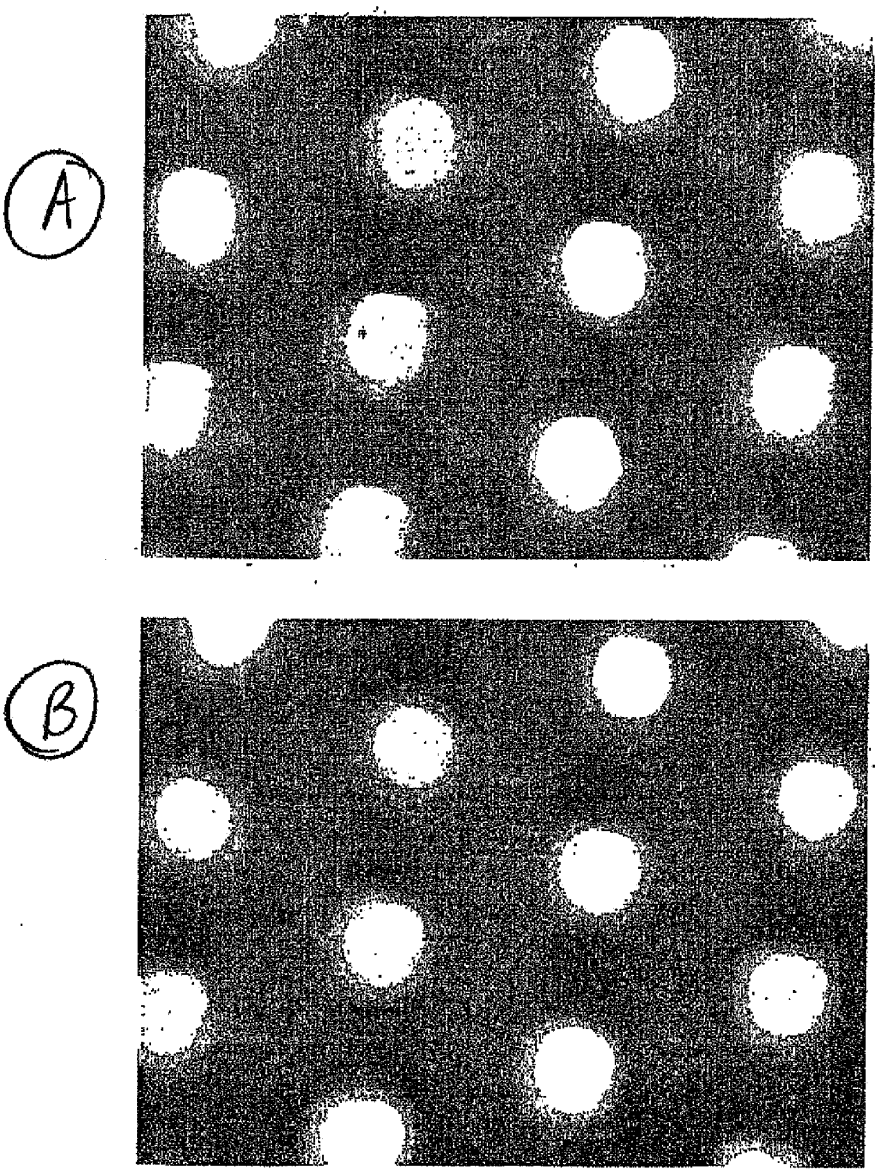


Figure 22

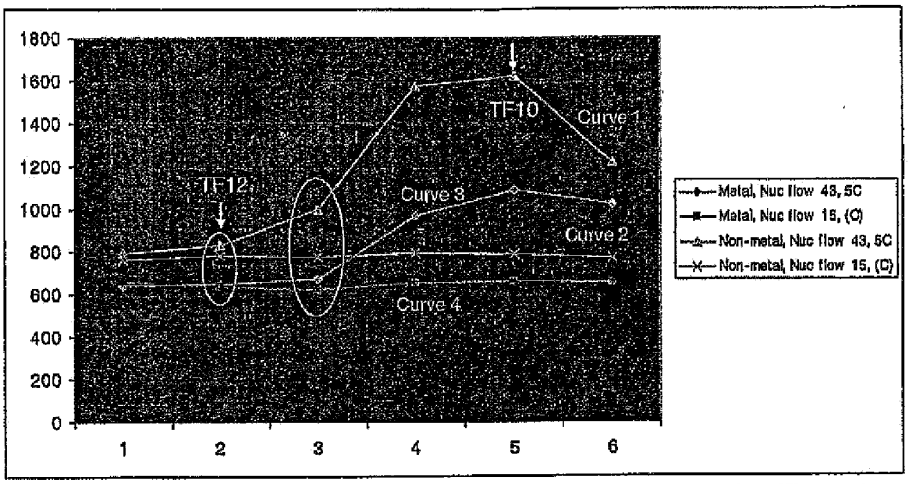
Figure 23



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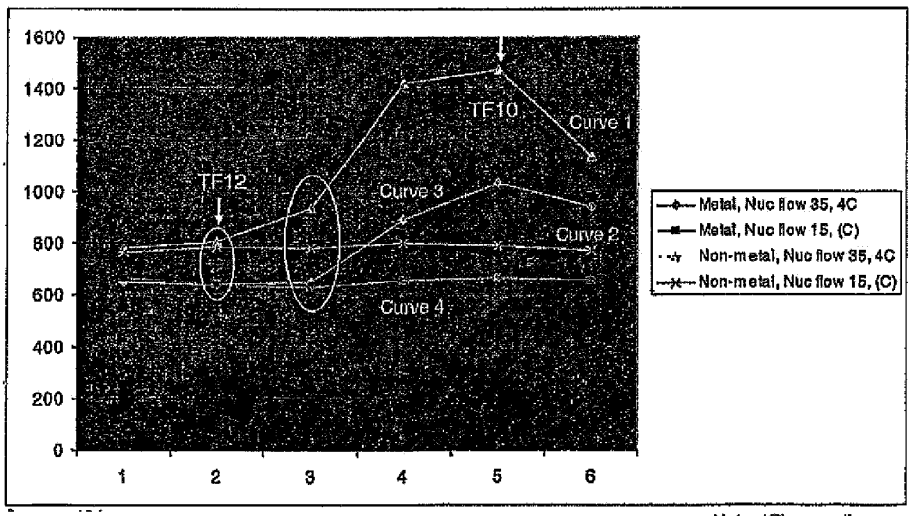
Figure 24

A



Note: (C) - negative

B



Note: (C) - negative

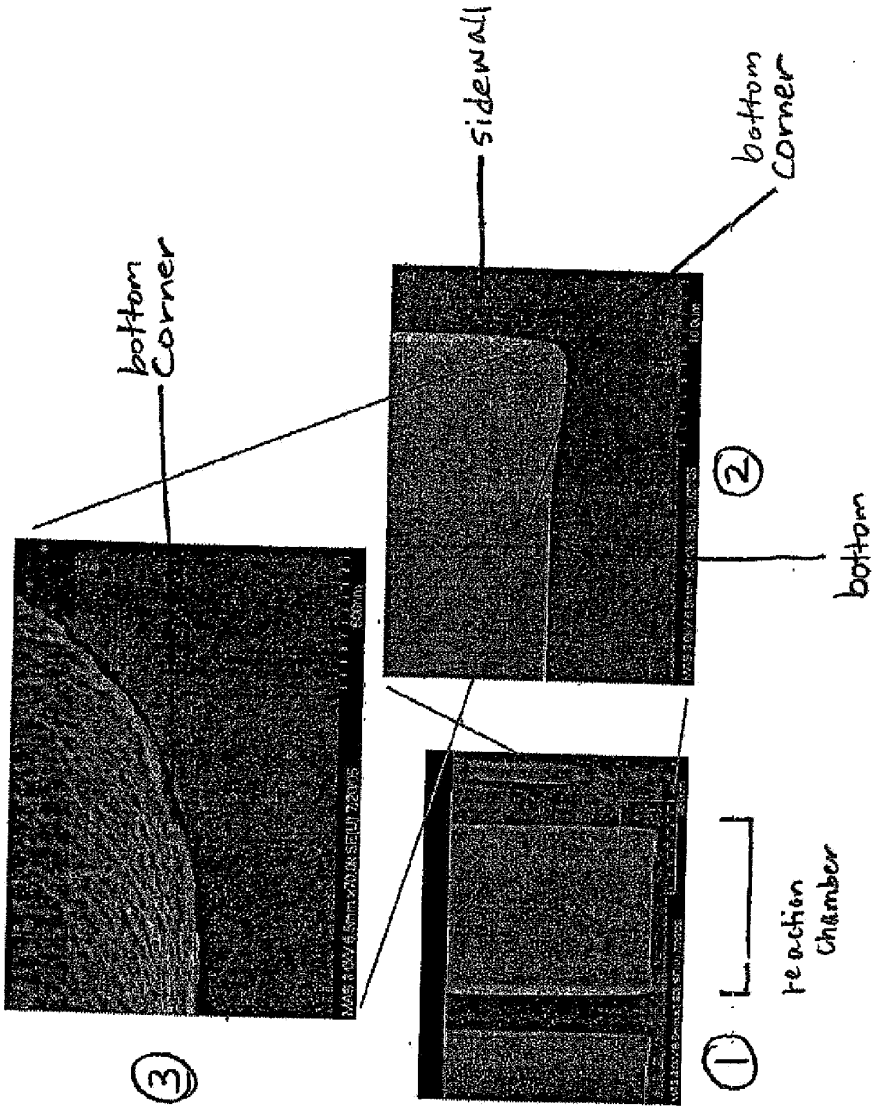

sidewall
bottom corner
bottom corner
bottom
reaction chamber
1
2
3

Figure 25a

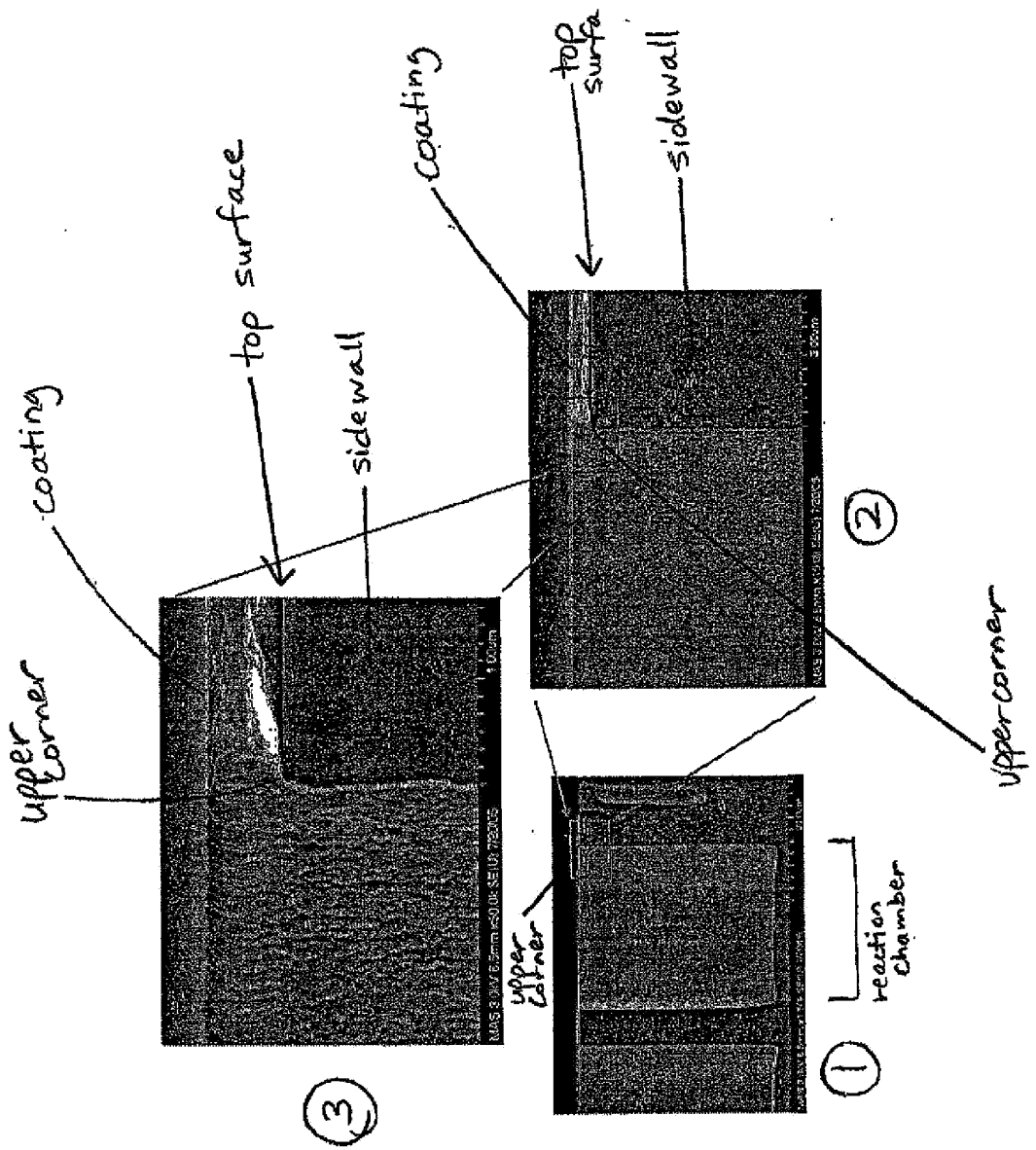

coating
top surface
sidewall
upper corner
coating
top surfa
sidewall
upper corner
upper corner
reaction chamber
1
2
3

Figure 25b

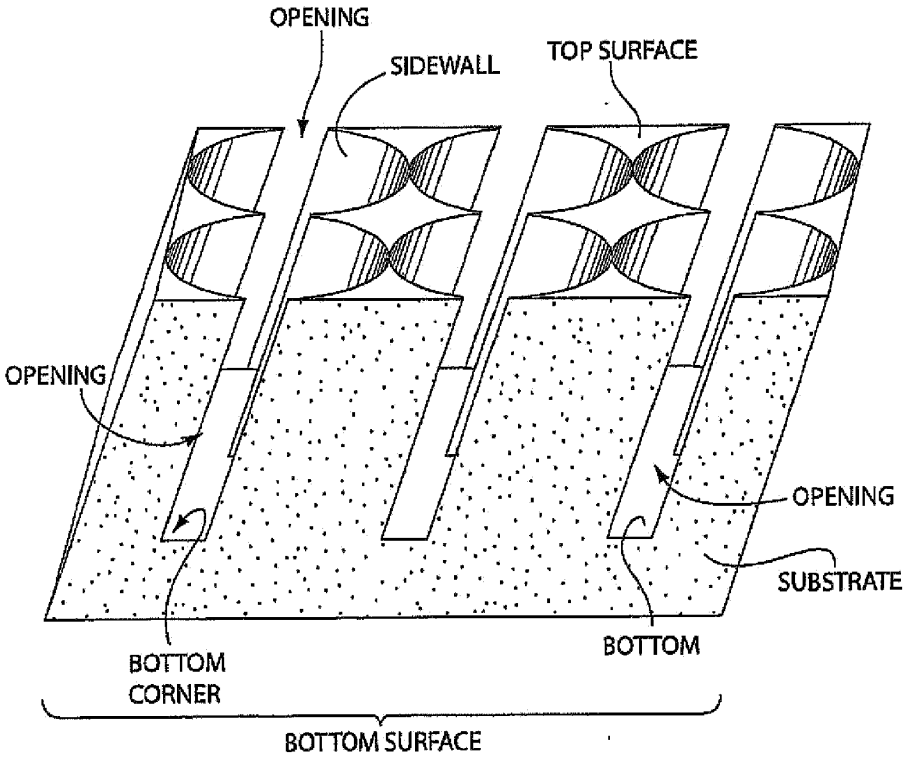

OPENING
SIDEWALL
TOP SURFACE
OPENING
OPENING
SUBSTRATE
BOTTOM
BOTTOM
CORNER
BOTTOM SURFACE

Fig. 26a

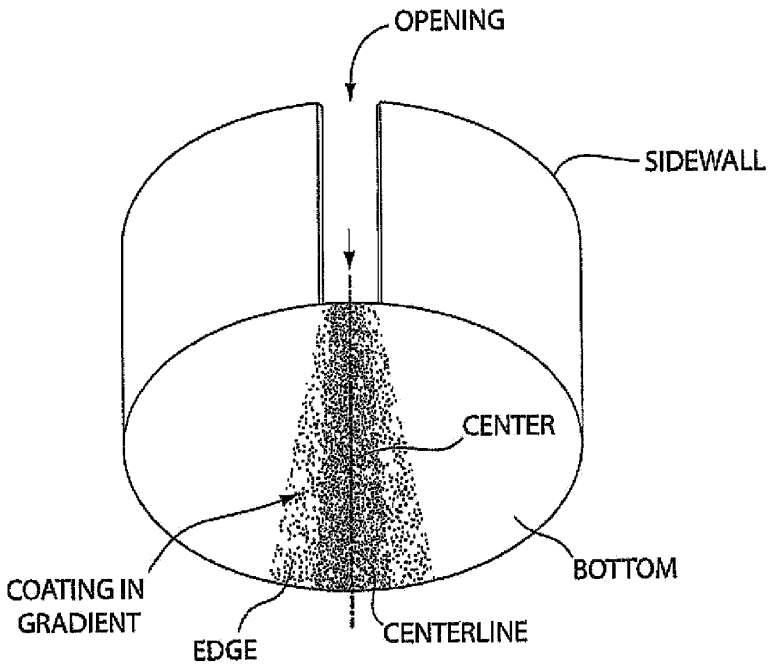

OPENING
SIDEWALL
CENTER
BOTTOM
COATING IN GRADIENT
EDGE
CENTERLINE

Fig. 26b

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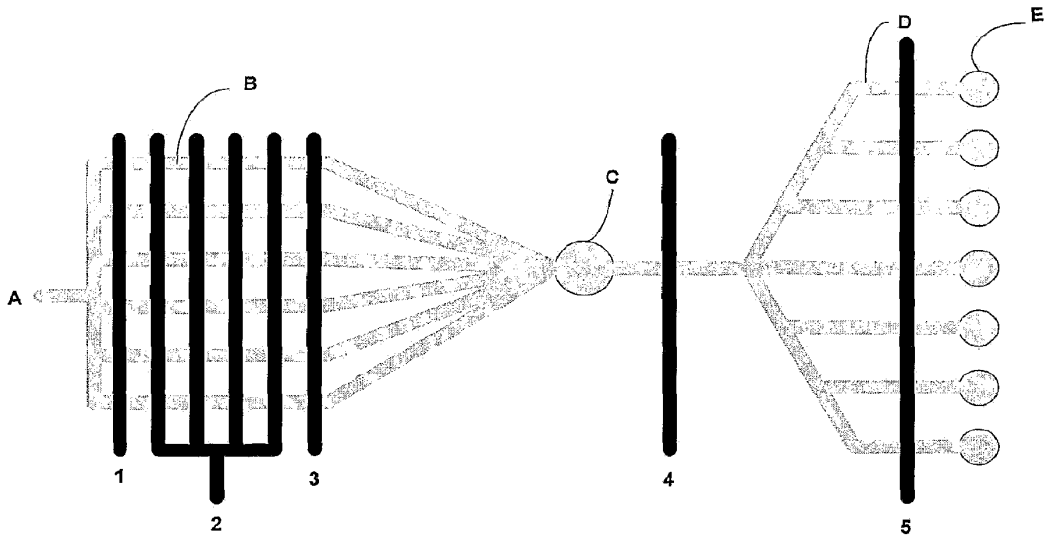
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(54) Title: ANALYSIS USING MICROFLUIDIC PARTITIONING DEVICES



(57) Abstract: The invention relates to methods, reagents and devices for detection and characterization of nucleic acids, cells, and other biological samples. Assay method are provided in which a sample is partitioned into sub-samples, and analysis of the contents of the sub-samples carried out. The invention also provides microfluidic devices for conducting the assay. The invention also provides an analysis method using a universal primers and probes for amplification and detection.

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PATENT APPLICATION

ANALYSIS USING MICROFLUIDIC PARTITIONING DEVICES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of United States provisional application No. 60/687,010, filed June 2, 2005, the entire contents of which are herein incorporated by reference.

FIELD OF THE INVENTION

[0002] The invention relates to methods, reagents and devices for detecting and characterizing nucleic acids, cells, and other biological samples.

BACKGROUND

[0003] A variety of nucleic acid amplification assays and immunological assays are used for analysis of cells and nucleic acids. These assays can be used to detect or characterize nucleic acid sequences associated with particular diseases or genetic disorders, for genotyping, for gene expression analyses, to detect and identify pathogens such as viruses, bacteria and fungi), for paternity and forensic identification, and for many other purposes. However, in some applications the efficiency and sensitivity of these assays is reduced, which may render the assays useless or at minimum require that additional manipulations and/or significant amounts of expensive reagents be used. For example, when a cell or molecule to be analyzed is from a sample with a large excess of non-target cells or molecules (*e.g.*, as in genetic or phenotypic analysis of a rare cell in a background of other cells) conventional assay methods are inadequate. Similarly, when a number of different targets must be detected in a single sample, conventional approaches (*e.g.*, multiplex PCR) are expensive, inefficient or not sufficiently sensitive. Thus, new methods, reagents and devices for detection and characterization of nucleic acids, cells, and other biological molecules will find broad application in biomedicine and other fields.

BRIEF SUMMARY

[0004] The invention relates to methods, reagents and devices for detection and characterization of nucleic acids, cells, and other biological samples. In one aspect, the invention provides an assay method including the following steps (a) partitioning a sample into a plurality of sub-samples, where said sample comprises a plurality of nucleic acid molecules, and where at least two sub-samples comprise at least one nucleic acid molecule; (b) providing sufficient reagents in each sub-sample to amplify a target sequence or sequences; (c) amplifying the target sequence(s) in the sub-sample(s) containing target sequence(s) thereby producing amplicons in the sub-sample; (d) distributing the amplicons into a plurality of aliquots; and, (e) for each aliquot, determining a property of amplicons in the aliquot.

[0005] In a related aspect, the invention provides an assay method including the following steps (a) partitioning a sample into a plurality of sub-samples, where said sample comprises a plurality of nucleic acid molecules, and where at least two sub-samples comprise at least one nucleic acid molecule; (b) providing sufficient reagents in each sub-sample to amplify at least two different target sequences; (c) amplifying target sequence(s) in at least two sub-sample(s) thereby producing amplicons in the sub-sample(s); (d) combining the amplicons from said at least two sub-samples to create an amplicon pool; (d) dividing the amplicon pool into a plurality of aliquots; and, (e) for each aliquot, determining a property of amplicons in the aliquot. In one embodiment, the sample is partitioned into at least 10^4 sub-samples. In one embodiment, each subsample has a volume of less than one nanoliter. In one embodiment, the nucleic acid molecules comprise DNA and/or mRNA. In one embodiment, the amplification is by PCR or RT-PCR. In one embodiment, sufficient reagents are provided to amplify at least 10, 20, or 50 different target sequences, if present. In one embodiment, the amplicon pool is divided into at least 10, 20, 50 or 100 aliquiots. In one embodiment, the sample contains a plurality of cells having nucleic acid molecules, and partitioning the sample involves partitioning intact cells into a plurality of sub-samples. In one embodiment, the sample contains only one cell.

[0006] In another aspect, the invention provides an assay method including the following steps (a) partitioning a sample comprising a plurality of separable cells into at least 1000 separate reaction chambers in a massively partitioning device (MPD), where after partitioning at least two reaction chambers each comprise exactly one cell; (b) providing in each reaction chamber one or more reagents for determining a property or properties of a cell, where the same reagents are provided in each chamber; and (c) determining at least two

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different properties of a single cell in a chamber and/or determining at least one property for at least two different cells in different chambers. In one embodiment, at least 99% of the reaction chambers contain zero or one cell. In one embodiment, the cells are bacterial cells. In one embodiment, the reagents include reagents for nucleic acid amplification. In one embodiment, at least one property is the presence or absence in the cell of a nucleic acid having a specified sequence. In one embodiment, at least one property is other than the presence or absence in the cell of a nucleic acid having a specified sequence.

[0007] In another aspect, the invention provides a method for amplification and detection of multiple target DNA sequences in a sample, including the following steps: (a) providing a sample containing (i) multiple target DNA sequences, (ii) a primer pair corresponding to each of said multiple target DNA sequences, each pair consisting of a first primer comprising U_1, B_1 and F domains in the order 5'-U_1-B_1-F-3' and a second primer comprising U_2 and R domains in the order 5'-U_2-R-3', where each pair of F and R primers is capable of annealing specifically to a different target DNA sequence under stringent annealing conditions; (iii) a universal primer pair capable of amplifying a double stranded DNA molecule with the structure

$$5'\text{-}\ U_1\ \text{--}\ U_2'\ \text{-}\ 3'$$
$$3'\text{-}\ U_1'\ \text{---}\ U_2\ \text{-}\ 3'$$

where U_1' is the sequence complementary to U_1 and U_2' is the sequence complementary to U_2; (b) subjecting the sample to multiple cycles of melting, reannealing, and DNA synthesis thereby producing amplicons for each of said multiple target DNA sequences, and (c) detecting the amplicons using a probe that anneals to sequence of the amplicon having the sequence of the B_1 domain or its complement. In one embodiment, the sample also contains a second set of multiple target sequences, a primer pair corresponding to to each of the target sequences in the second set, each pair consisting of a first primer comprising U_1, B_2 and F domains in the order 5'-U_1-B_2-F-3' and a second primer comprising U_2 and R domains in the order 5'-U_2-R-3', where each pair of F and R oligonucleotides is capable of annealing specifically to a different target DNA sequence in the second set of multiple target sequences under stringent annealing conditions; and where amplicons for each of the multiple target DNA sequences of the second set are produced; and detecting the amplicons for each of the multiple target DNA sequences using a probe that anneals to sequence of the amplicon having the sequence of the B_2 domain or its complement. In one embodiment, U_1, B_1, F_1, U_2

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and R_1 domains are between 6 and 30 nucleotides in length. In one embodiment the probe is a molecular beacon. In one embodiment, the probe is a a Taqman™-type probe.

[0008] In another aspect, the invention provides a microfluidic device, having (a) a first region comprising (i) a flow channel formed within an elastomeric material and having a first end and a second end in fluid communication with each other through said channel, where said channel may be branched or unbranched; (ii) an inlet for introducing a sample fluid in communication with said channel, said inlet; (iii) an outlet in communication with said flow channel; (iv) a plurality of control channels overlaying the flow channel(s), where an elastomeric membrane separates the control channels from the flow channels at each intersection, the elastomeric membrane disposed to be deflected into or withdrawn from the flow channel in response to an actuation force, and where, when the control channels are actuated the flow channel is partitioned into at least 1000 reaction chambers not in fluidic communication with each other; (b) a second region compromising a channel or chamber interposed between and in communication with said outlet in (a) and a flow channel in the third region; (c) a third region comprising a plurality of flow channels (e.g., blind flow channels), in fluidic communication with the channel or chamber of the second region, with a region of each flow channel defining a reaction site; (d) a control channel or channels that when actuated separates the first and second regions; (e) a control channel or channels that when actuated separates the second and third regions; and (f) a control channel or channels that when actuated separates the reaction sites of said flow channels from the other portions of control channels.

BRIEF DESCRIPTION OF THE FIGURES

[0009] Figures 1A and 1B show an exemplary design of a massively partitioning device (MPD) in valve off (Fig. 1A) and valve actuated (Fig. 1B) states.

[0010] Figure 2 shows an exemplary design of a MPD with two banks: a first bank in which nucleic acids are partitioned and amplified in individual chambers, and a second bank in which subsequent analysis of the amplicon pool occurs.

[0011] Figures 3A-C are flow charts illustrating partition and analysis of nucleic acids using methods of the invention. Fig. 3A illustrates partition and analysis of nucleic acids in which multiple target sequences are amplified. Fig. 3B illustrates partition and analysis acids in which target sequences in only a single chamber are amplified. Fig. 3C illustrates analysis of nucleic acids of a single cell.

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[0012] Figure 4 is a flow chart illustrating partition of cells and analysis of their properties.

[0013] Figure 5 is an illustration of primers used in the universal amplification method.

DETAILED DESCRIPTION

Definitions

[0014] The term "elastomer" has the general meaning used in the art. Thus, for example, Allcock et al. (Contemporary Polymer Chemistry, 2nd Ed.) describes elastomers in general as polymers existing at a temperature between their glass transition temperature and liquefaction temperature. Elastomeric materials exhibit elastic properties because the polymer chains readily undergo torsional motion to permit uncoiling of the backbone chains in response to a force, with the backbone chains recoiling to assume the prior shape in the absence of the force. In general, elastomers deform when force is applied, but then return to their original shape when the force is removed. The elasticity exhibited by elastomeric materials can be characterized by a Young's modulus. The elastomeric materials utilized in the microfluidic devices disclosed herein typically have a Young's modulus of between about 1 Pa – 1 TPa, in other instances between about 10 Pa – 100 GPa, in still other instances between about 20 Pa – 1 GPa, in yet other instances between about 50 Pa – 10 MPa, and in certain instances between about 100 Pa – 1 MPa. Elastomeric materials having a Young's modulus outside of these ranges can also be utilized depending upon the needs of a particular application. Microfluidic devices can be fabricated from an elastomeric polymer such as GE RTV 615 (formulation), a vinyl-silane crosslinked (type) silicone elastomer (family). However, elastomeric microfluidic systems are not limited to this one formulation, type or even this family of polymer; rather, nearly any elastomeric polymer is suitable. Given the tremendous diversity of polymer chemistries, precursors, synthetic methods, reaction conditions, and potential additives, there are a large number of possible elastomer systems that can be used to make monolithic elastomeric microvalves and pumps (including, for example, perfluoropolyethers, polyisoprene, polybutadiene, polychloroprene, polyisobutylene, poly(styrene-butadiene-styrene), polyurethanes, and silicones, for example, or poly(bis(fluoroalkoxy)phosphazene) (PNF, Eypel-F), poly(carborane-siloxanes) (Dexsil), poly(acrylonitrile-butadiene) (nitrile rubber), poly(1-butene), poly(chlorotrifluoroethylene-vinylidene fluoride) copolymers (Kel-F), poly(ethyl vinyl ether), poly(vinylidene fluoride), poly(vinylidene fluoride-hexafluoropropylene) copolymer (Viton), elastomeric compositions

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of polyvinylchloride (PVC), polysulfone, polycarbonate, polymethylmethacrylate (PMMA), polytertrafluoroethylene (Teflon), polydimethylsiloxane, polydimethylsiloxane copolymer, and aliphatic urethane diacrylate). The choice of materials typically depends upon the particular material properties (e.g., solvent resistance, stiffness, gas permeability, and/or temperature stability) required for the application being conducted. Additional details regarding the type of elastomeric materials that can be used in the manufacture of the components of the microfluidic devices disclosed herein are set forth in Unger et al. (2000) *Science* 288:113-116, PCT Publications WO 02/43615, WO 2005030822, WO 2005084191and WO 01/01025; and U.S. patent publication No. 20050072946.

[0015] A "reagent" refers broadly to any agent used in a reaction, other than the analyte (*e.g.*, cell or nucleic acid being analyzed). Exemplary reagents for a nucleic acid amplification reaction include, but are not limited to, buffer, metal ions, polymerase, reverse transcriptase, primers, template nucleic acid, nucleotides, labels, dyes, nucleases and the like. Reagents for enzyme reactions include, for example, substrates, cofactors, buffer, metal ions, inhibitors and activators. Reagents for cell-based reactions include, but are not limited to, cells, cell specific dyes and ligands (e.g., agonists and antagonists) that bind to cellular receptors.

[0016] The terms "nucleic acid," "polynucleotide," and "oligonucleotide" include a polymeric form of nucleotides of any length, including, but not limited to, ribonucleotides or deoxyribonucleotides. There is no intended distinction in length between these terms. Further, these terms refer only to the primary structure of the molecule. Thus, in certain embodiments these terms can include triple-, double- and single-stranded DNA, as well as triple-, double- and single-stranded RNA. They also include modifications, such as by methylation and/or by capping, and unmodified forms of the polynucleotide. More particularly, the terms "nucleic acid," "polynucleotide," and "oligonucleotide," include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), any other type of polynucleotide which is an N– or C-glycoside of a purine or pyrimidine base, and other polymers containing nonnucleotidic backbones, for example, polyamide (e.g., peptide nucleic acids (PNAs)) and polymorpholino (commercially available from the Anti-Virals, Inc., Corvallis, Oregon, as Neugene) polymers, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA.

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[0017] A "primer" is a single-stranded polynucleotide capable of acting as a point of initiation of template-directed DNA or RNA synthesis under appropriate conditions (*i.e.*, in the presence of four different nucleoside triphosphates and an agent for polymerization, such as, DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer but typically is at least 7 nucleotides long and, more typically range from 10 to 30 nucleotides in length. Other primers can be somewhat longer such as 30 to 50 nucleotides long. In this context, primer "length" refers to the portion of an oligo- or polynucleotide that hyrbidizes to a complementary "target" sequence and primes synthesis. For example, in the primer 5'-U_1-B_1-F1-3' the length of F_1 might be 20 nucleotides and the combined length of U, B and F1 could be 60 nucleotides or more (typically between 30 and 100 nucleotides). Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. The term "primer site" or "primer binding site" refers to the segment of the target DNA to which a primer hybridizes. The term "primer pair" means a set of primers including a 5' "upstream primer" that hybridizes with the complement of the 5' end of the DNA sequence to be amplified and a 3' "downstream primer" that hybridizes with the 3' end of the sequence to be amplified.

[0018] A primer or probe anneals or hybrdizes to a complementary target sequence. The primer or probe may be exactly complementary to the target sequence or can be less than perfectly complementary. Typically the primer has at least 65% identity to the complement of the target sequence over a region of at least 7 nucleotides, more typically over a region in the range of 10-30 nucleotides, and often over a region of at least 14-25 nucleotides, and more often has at least 75% identity, at least 85% identity or 90% identity. It will be understood that certain bases (e.g., the 3' base of a primer) generally should be exactly complementary to corresponding base of the target sequence. Primer and probes generally anneal to the target sequence under stringent conditions. Stringent annealing conditions refers to conditions in a range from about 5°C to about 20°C or 25°C below the melting temperature (T_m) of the target sequence and a probe with exact or nearly exact complementarity to the target. As used herein, the melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half-dissociated into single strands. Methods for calculating the T_m of nucleic acids are well known in the art (see, e.g., Berger and Kimmel (1987) METHODS IN ENZYMOLOGY, VOL.152: GUIDE TO

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MOLECULAR CLONING TECHNIQUES, San Diego: Academic Press, Inc. and Sambrook et al. (1989) MOLECULAR CLONING: A LABORATORY MANUAL, 2ND ED., VOLS. 1-3, Cold Spring Harbor Laboratory), both incorporated herein by reference). As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: T_m =81.5+0.41(% G+C), when a nucleic acid is in aqueous solution at 1 M NaCl (see e.g., Anderson and Young, Quantitative Filter Hybridization in NUCLEIC ACID HYBRIDIZATION (1985)). The melting temperature of a hybrid (and thus the conditions for stringent hybridization) is affected by various factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, and the like), and the concentration of salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol). The effects of these factors are well known and are discussed in standard references in the art

[0019] A "probe" is an nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation, thus forming a duplex structure. The probe binds or hybridizes to a "probe binding site." The probe can be labeled with a detectable label to permit facile detection of the probe, particularly once the probe has hybridized to its complementary target. The label attached to the probe can include any of a variety of different labels known in the art that can be detected by chemical or physical means, for example. Suitable labels that can be attached to probes include, but are not limited to, radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, and enzyme substrates. Probes can vary significantly in size. Some probes are relatively short. Generally, probes are at least 7 to 15 nucleotides in length. Other probes are at least 20, 30 or 40 nucleotides long. Still other probes are somewhat longer, being at least 50, 60, 70, 80, 90 nucleotides long. Yet other probes are longer still, and are at least 100, 150, 200 or more nucleotides long. Probes can be of any specific length that falls within the foregoing ranges as well.

[0020] The term "label" refers to a molecule or an aspect of a molecule that can be detected by physical, chemical, electromagnetic and other related analytical techniques. Examples of detectable labels that can be utilized include, but are not limited to, radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic

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particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, enzymes linked to nucleic acid probes and enzyme substrates. The term "detectably labeled" means that an agent has been conjugated with a label or that an agent has some inherent characteristic (e.g., size, shape or color) that allows it to be detected without having to be conjugated to a separate label.

[0021] A "polymorphic marker" or "polymorphic site" is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%, and more preferably greater than 10% or 20% of a selected population. A polymorphic locus may be as small as one base pair. Polymorphic markers include restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. The first identified allelic form is arbitrarily designated as the reference form and other allelic forms are designated as alternative or variant alleles. The allelic form occurring most frequently in a selected population is sometimes referred to as the wildtype form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic polymorphism has two forms. A triallelic polymorphism has three forms.

[0022] A "single nucleotide polymorphism" (SNP) occurs at a polymorphic site occupied by a single nucleotide, which is the site of variation between allelic sequences. The site is usually preceded by and followed by highly conserved sequences of the allele (e.g., sequences that vary in less than 1/100 or 1/1000 members of the populations). A single nucleotide polymorphism usually arises due to substitution of one nucleotide for another at the polymorphic site. A transition is the replacement of one purine by another purine or one pyrimidine by another pyrimidine. A transversion is the replacement of a purine by a pyrimidine or vice versa. Single nucleotide polymorphisms can also arise from a deletion of a nucleotide or an insertion of a nucleotide relative to a reference allele.

[0023] The term "haplotype" refers to the designation of a set of polymorphisms or alleles of polymorphic sites within a gene of an individual.

[0024] A used herein, "plurality" means at least three. In general a plurality of cells, nucleic acid molecules, etc., will contain at least 10, at least about 10^2, at least about 10^3, or at least about 10^4 different cells, molecules, etc.

[0025] A used herein, "entities" refers to a plurality of structurally similar biological molecules or structures (e.g., macromolecules such as nucleic acids, protein, carbohydrates and lipids; cells or subcellular structures or components; viruses) or nonbiological particles

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that are separate and distinct from each other in the sense that they can be separated into separate reaction chambers using a MPD. "Entity" refers to a single such molecule or structure.

[0026] The term "biological sample", refers to a sample obtained from an organism or from components of an organism, such as cells, biological tissues and fluids. In some methods, the sample is from a human patient. Such samples include sputum, blood, blood cells (*e.g.*, white cells), tissue or fine needle biopsy samples, urine, peritoneal fluid, and fleural fluid, or cells therefrom.

A. INTRODUCTION

[0027] The invention relates generally to analysis of macromolecules and small particles, and particularly to analysis of nucleic acids, proteins, and individual cells. In certain aspects, the invention relates to analysis methods involving massive partitioning. Massive partitioning of liquid samples, *i.e.*, dividing the sample into thousands of isolated reaction volumes, has been made possible by the development of specially designed elastomeric microfluidic devices. These devices can be referred to as "massively partitioning devices" or MPDs and are sometimes referred to as "chips" or Digital Isolation and Detection Integrated Fluidic Circuits (DID IFCs). Exemplary devices are described in McBride *et al.* (PCT publication WO 2004/089810, published on October 21, 2004; copending, commonly assigned U.S. Pat. App. No. 10/ 819088 published as patent publication No. 20050019792 on January 27, 2005; and copending, commonly assigned U.S. Pat. App. No. 10/ 819088 published as patent publication No. 20050252773 on November 17, 2005, each of which is incorporated by reference in its entirety for all purposes and the specific purposes describe therein and herein; hereinafter referred to together as "McBride *et al.*"). Using MPDs, a sample can be partitioned into a multitude of isolated reaction chambers, and reactions carried out simultaneously in each chamber. For example, McBride *et al.*, *supra*, describes the performance of 21,000 simultaneous PCR reactions in a single microfluidic chip, in a volume of 90 picoliters per reaction and with single template molecule sensitivity.

[0028] In a first broad aspect, the invention provides new methods and devices for analysis of a sample containing nucleic acids, proteins, other biomolecules, cells, microorganisms, viruses, and other biological or nonbiological entities, in which the sample undergoes massive partitioning as part of the analysis process.

[0029] In a second broad aspect, the invention provides methods and reagents for amplification and/or detection of a nucleic acid. These methods and reagents find particular

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application in the analysis of nucleic acids partitioned using a MPD, but may be used in amplification-based analysis of any nucleic acid.

[0030] These and other inventions are described in the following sections.

B. MASSIVELY PARTITIONING DEVICES

[0031] Methods described in this disclosure can be, and in some cases are necessarily, carried out using an elastomeric microfluidic device. Methods for fabricating elastomeric microfluidic devices capable of separating molecules or cells and for carrying out reactions are known in the art (see, *e.g.*, Unger et al., 2000, *Science* 288:113-116, PCT Publications WO 01/01025 and WO/02/43615; and US patent application No. 10/306798 published as Pat App. No. 20030138829 on July 24, 2003). In particular, exemplary elastomeric massively partitioning devices (MPDs) are described in McBride *et al.*, *supra* and references cited therein. Based on these and other publications, one of ordinary skill in the art guided by this disclosure will be able to practice all aspects of the inventions described herein. Accordingly, elastomeric microfluidic devices are described only briefly below.

General Structure of Microfluidic Devices

[0032] Elastomeric microfluidic devices are characterized in part by utilizing various components such as flow channels, control channels, valves, pumps, vias, and/or guard channels from elastomeric materials. Figures 1A and 1B show an exemplary design of a massively partitioning device.

[0033] A "flow channel" refers generally to a flow path through which a solution can flow. A "blind channel" refers to a flow channel which has an entrance but not a separate exit. A "control channel" is a channel separated from a flow channel by an elastomeric membrane that can be deflected into or retracted from the flow channel in response to an actuation force. The term "valve" refers to a configuration in which a flow channel and a control channel intersect and are separated by an elastomeric membrane that can be deflected into or retracted from the flow channel in response to an actuation force. An "isolated reaction site" or "reaction chamber" refers to a reaction site that is not in fluid communication with other reactions sites present on the device, and which is created by the actuation of control channels in the device. A "via" refers to a channel formed in an elastomeric device to provide fluid access between an external port of the device and one or more flow channels. Thus, a via can serve as a sample input or output, for example. "Guard channels" may be included in elastomeric microfluidic devices for use in heating applications to minimize

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evaporation of sample from the reaction sites. Guard channels are channels formed within the elastomeric device through which water can be flowed to increase the water vapor pressure within the elastomeric material from which the device is formed, thereby reducing evaporation of sample from the reaction sites. The guard channels are similar to the control channels in that typically they are formed in a layer of elastomer that overlays the flow channels and/or reaction site. Typically, the guard channels are placed adjacent and over flow channels and reaction sites as these are the primary locations at which evaporation is the primary concern. Guard channels are typically formed in the elastomer utilizing the MSL techniques and/or sacrificial-layer encapsulation methods cited above. The solution flowed through the guard channel includes any substance that can reduce evaporation of water.

[0034] The devices incorporate flow channels, control channels and valves to isolate selectively a reaction site at which reagents are allowed to react. Figures 1A and 1B depict an exemplary design of a partitioning microfluidic device 01 in a valve off and valve actuated state. Referring to the figure, a sample is injected into inlet 02 which is in communication with branched partitioning channel system 03 of the device. Solution flow through flow channels of the device is controlled, at least in part, with one or more control channels that are separated from the flow channel by an elastomeric membrane or segment. This membrane or segment can be deflected into or retracted from the flow channel with which a control channel is associated by applying an actuation force to the control channels so that solution flow can be entirely blocked by valves. Actuating the control valves creates isolated reaction chambers 05 in which individual reactions can be conducted. The reaction chambers can number from 10^3 to 10^5 or more be at a density of at least 100 sites/cm^2 and can range up to at least 2000, 3000, 4000 or more than 4000 sites/cm^2. Very small wells or cavities can be formed within an elastomeric material to increase the volume of the reaction chamber. Valves can be actuated by injecting gases (*e.g.*, air, nitrogen, and argon), liquids (*e.g.*, water, silicon oils and other oils), solutions containing salts and/or polymers (including but not limited to polyethylene glycol, glycerol and carbohydrates), and the like into the port.

[0035] Although Figure 1 illustrates a MPD with branched flow channels, any channel configuration (flow channel path) that can be partitioned by control channels can be used in accordance with the invention, including, for example, square, spiral or serpentine configurations.

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[0036] The dimensions of flow channels in a MPD can vary widely. Typically channels are from about 0.1 μm to about 1000 μm in any dimension, sometimes from about 0.1 to about 100 μm, and sometimes from about 0.1 to about 10 μm. In one embodiment the channels have a high aspect ratio (*e.g.*, a height to width ratio of from about 2:1 to about 10:1) to increase channel density and/or to increase signal collection from channels containing a detectably labeled moiety. For example, in some embodiments the channel has a columnar shape in which the dimensions of floor and ceiling are smaller that the dimensions of the walls, and a signal (*e.g.*, fluorescence, infra red or visible radiation) is detected through the celing or floor. Appropriate channel dimensions will depend in part on the nature of the entities being partitioned. For partition of eukaryotic cells, for example, a dimension should be at least sufficient for passage of the cell (*e.g.*, 2-5 times the dimension of the cell). However, for the purpose of restricting movement the dimensions can be on the order of 0.75 times the smallest dimension of the particle. Microfluidic manipulation and analysis of particles is also described in U.S. Patent Pub. 20040229349 entitled "Microfluidic particle-analysis systems" and incorporated herein by reference.

[0037] Reactions (*e.g.*, nucleic acid amplification, protein binding, etc.) are allowed to occur in each chamber. For example, PCR reactions can be initiated by heating the chambers (*e.g.*, placing the device on a suitably programmed flat plate thermocycler.

[0038] The results or products of the reaction can be detected using any of a number of different detection strategies. Because the MPDs are usually made of elastomeric materials that are relatively optically transparent, reactions can be readily monitored using a variety of different detection systems at essentially any location on the microfluidic device. Most typically, however, detection occurs at the reaction site itself.

[0039] The nature of the signal to be detected will, of course, determine, to a large extent, the type of detector to be used. The detectors can be designed to detect a number of different signal types including, but not limited to, signals from radioisotopes, fluorophores, chromophores, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, enzymes linked to nucleic acid probes and enzyme substrates. Illustrative detection methodologies suitable for use with the present microfluidic devices include, but are not limited to, light scattering, multichannel fluorescence detection, infra-red, UV and visible wavelength absorption, luminescence, differential reflectivity, and confocal laser scanning. Additional detection methods that can be used in certain application include scintillation proximity assay

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techniques, radiochemical detection, fluorescence polarization, fluorescence correlation spectroscopy (FCS), time-resolved energy transfer (TRET), fluorescence resonance energy transfer (FRET) and variations such as bioluminescence resonance energy transfer (BRET). Additional detection options include electrical resistance, resistivity, impedance, and voltage sensing.

[0040] A detector can include a light source for stimulating a reporter that generates a detectable signal. The type of light source utilized depends in part on the nature of the reporter being activated. Suitable light sources include, but are not limited to, lasers, laser diodes and high intensity lamps. If a laser is utilized, the laser can be utilized to scan across a set of detection sections or a single detection section. Laser diodes can be microfabricated into the microfluidic device itself. Alternatively, laser diodes can be fabricated into another device that is placed adjacent to the microfluidic device being utilized to conduct a thermal cycling reaction such that the laser light from the diode is directed into the detection section.

[0041] Detectors can be microfabricated within the microfluidic device, or can be a separate element. A number of commercially-available external detectors can be utilized. Many of these are fluorescent detectors because of the ease in preparing fluorescently labeled reagents. Specific examples of detectors that are available include, but are not limited to, Applied Precision ArrayWoRx (Applied Precision, Issaquah, WA) and the ABI 7700 (Applied Biosystems, Inc., Foster City, CA).

Fabrication

[0042] Microfluidic devices are generally constructed utilizing single and multilayer soft lithography (MSL) techniques and/or sacrificial-layer encapsulation methods. The basic MSL approach involves casting a series of elastomeric layers on a micro-machined mold, removing the layers from the mold and then fusing the layers together. In the sacrificial-layer encapsulation approach, patterns of photoresist are deposited wherever a channel is desired. These techniques and their use in producing microfluidic devices is discussed in detail, for example, by Unger et al., 2000, *Science* 288:113-116; by Chou, et al., 2000, "Integrated Elastomer Fluidic Lab-on-a-chip-Surface Patterning and DNA Diagnostics, in Proceedings of the Solid State Actuator and Sensor Workshop, Hilton Head, S.C.; in PCT Publication WO 01/01025; and in published U.S. patent application No. 20050072946 (each incorporated herein by reference).

[0043] In one approach, the foregoing fabrication methods initially involve fabricating mother molds for top layers (e.g., the elastomeric layer with the control channels)

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and bottom layers (e.g., the elastomeric layer with the flow channels) on silicon wafers by photolithography with photoresist (Shipley SJR 5740). Channel heights can be controlled precisely by the spin coating rate. Photoresist channels are formed by exposing the photoresist to UV light followed by development. Heat reflow process and protection treatment is typically achieved as described by Unger et al. *supra*. A mixed two-part-silicone elastomer (GE RTV 615) is then spun into the bottom mold and poured onto the top mold, respectively. Spin coating can be utilized to control the thickness of bottom polymeric fluid layer. The partially cured top layer is peeled off from its mold after baking in the oven at 80 °C for 25 minutes, aligned and assembled with the bottom layer. A 1.5-hour final bake at 80 °C is used to bind these two layers irreversibly. Once peeled off from the bottom silicon mother mold, this RTV device is typically treated with HCL (0.1N, 30 min at 80 °C). This treatment acts to cleave some of the Si-O-Si bonds, thereby exposing hydroxy groups that make the channels more hydrophilic.

[0044] The device can then optionally be hermetically sealed to a support. The support can be manufactured of essentially any material, although the surface should be flat to ensure a good seal, as the seal formed is primarily due to adhesive forces. Examples of suitable supports include glass, plastics and the like.

[0045] In certain devices, the devices formed according to the foregoing method result in the substrate (e.g., glass slide) forming one wall of the flow channel. Alternatively, the device once removed from the mother mold is sealed to a thin elastomeric membrane such that the flow channel is totally enclosed in elastomeric material. For certain uses, e.g., PCR amplification, flow channels and chambers enclosed in elastomeric material (i.e., without a glass wall) are preferred. The resulting elastomeric device can then optionally be joined to a substrate support. In some cases, the device is made as described in U.S. patent publication No. 20050072946. In some cases, the device uses "push-up valves" described in U.S. patent publication No. 20050072946 (e.g., Fig. 37B). "Push-up" refers to low actuation pressure geometry in which the membrane deflects upwards to seal off the upper fluid channel. In this geometry, the deflectable membrane is featureless and exhibits a substantially constant thickness.

[0046] Reagents can be deposited in reaction chambers before addition of a sample to the MPD. A number of commercially available reagent spotters and established spotting techniques can be used to deposit the reagent(s). Microfluidic devices in which reagents are deposited at the reaction sites during manufacture are typically formed of three layers. The bottom layer is the layer upon which reagents are deposited. The bottom layer can be formed

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from various elastomeric materials as described in the references cited above on MLS methods. Typically, the material is polydimethylsiloxane (PDMS) elastomer. Based upon the arrangement and location of the reaction sites that is desired for the particular device, one can determine the locations on the bottom layer at which the appropriate reagents should be spotted. Because PDMS is hydrophobic, the deposited aqueous spot shrinks to form a very small spot. The deposited reagents are deposited such that a covalent bond is not formed between the reagent and the surface of the elastomer because, as described earlier, the reagents are intended to dissolve in the sample solution once it is introduced into the reaction site. In some versions, the reagent is designed to be inactive or unavailable to a reaction until a specified condition occurs (e.g., a polymerase not activated until heated or until the addition of a necessary cofactor).

[0047] The other two layers of the device are the layer in which the flow channels are formed and the layer in which the control and optionally guard channels are formed. These two layers are prepared according to the general methods set forth earlier in this section. The resulting two layer structure is then placed on top of the first layer onto which the reagents have been deposited. A specific example of the composition of the three layers is as follows (ration of component A to component B): first layer (sample layer) 30:1 (by weight); second layer (flow channel layer) 30:1; and third layer (control layer) 4:1. It is anticipated, however, that other compositions and ratios of the elastomeric components can be utilized as well. During this process, the reaction sites are aligned with the deposited reagents such that the reagents are positioned within the appropriate reaction site.

C. PARTITIONING, DETECTION AND ANALYSIS OF NUCLEIC ACIDS

[0048] In this section, methods for analysis of nucleic acids in a sample are described. The methods involve massive partitioning of the sample and any nucleic acid molecules it contains, and amplification (as defined herein) of target sequences in the partitioned nucleic acid molecules. Various versions of the methods may also involve application of particular amplication strategies, pooling of amplification products, analysis of pooled amplification products and/or other features that will be apparent upon reading this disclosure. This section also describes devices (*i.e.*, massively partitioning devices, MPDs) useful in carrying out analyses according to the method.

[0049] Analytical methods described in this section can be used for deleting the presence or absence of a target sequence, detection of polymorphisms; single polynucleotide polymorphism (SNP) analysis; haplotype analysis; amplification of a segment for sequence

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determination, gene expression analysis, quantification of nucleic acids, analysis of cells (see Section D, below), as well as other applications that will be apparent to one of skill guided by this disclosure. Although this section focuses on analysis of nucleic acids it will be appreciated by the reader that many aspects of the description in this section will be applicable, with appropriate modification, to analysis of other molecules and of cells. Figures 3A and 3B are flow charts illustrating partition and analysis of nucleic acids in which multiple targets are amplified in which amplicons may be pooled. Fig. 3B illustrates partition and analysis of nucleic acids in which, in one embodiment, target molecules in only a single chamber are amplified.

i) Samples Containing Nucleic Acids

[0050] In one step of the assay method, a sample containing a plurality of nucleic acid molecules is partitioned into a plurality of sub-samples, at least two of which each comprise at least one nucleic acid molecule.

[0051] Samples that may be analyzed according to the invention are any fluid sample that contains nucleic acids. A variety of types of samples can be used, so long as at least some nucleic acids can be partitioned from each other by the MPD. The nucleic acids can be free in solution or can be contained in particles or within cells suspended in a fluid. Samples may be processed so that any nucleic acids in the sample can be amplified. For example, in samples containing cells or viruses, the cells or viruses can be lysed or disrupted, using such routine methods as exposure to enzymes (such as lysozyme), detergents, denaturants (such as guanidine salts) and/or physical disruption) before the sample. Any method of liberating nucleic acids that results in nucleic acid molecules sufficiently intact and purified to amplify fragments is suitable. Examples of samples containing nucleic acids are cell lysates or cell fractions, water samples containing microorganisms, purified DNA resuspended in an aqueous buffer, sputum, blood, nucleated blood cells, tissue or fine needle biopsy samples, urine, peritoneal fluid, fecal samples, and fleural fluid, or cells therefrom. Exemplary samples include cells and cell lysates (e.g., eukaryotic cells, human cells, animal cells, plant cells, fetal cells, embryonic cells, stem cells, blood cells, lymphocytes, bacterial cells, recombinant cells and cells infected with a pathogen. tissue samples), viruses, purified or partially purified DNA or RNA, environmental samples (*e.g.*, water samples), food samples, forensic samples, plant samples and the like. It will apparent that the sample can contain other compounds and macromolecules in addition to nucleic acids. If necessary for the

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functioning of a microfluidic device non-nucleic acid components and/or particulates can be removed by filtration, sedimentation or other methods.

[0052] In one embodiment of the invention, the nucleic acids are contained in cells, organelles, or viruses and the nucleic acids are not released (*e.g.*, the cells are not lysed) until at least after a partitioning step. Particular aspects of this embodiment are discussed in Section D, below.

[0053] Analysis of nucleic acids in a sample generally involves determining whether the sample contains a nucleic acid having a particular target sequence. A target sequence may be predefined (*i.e.*, known prior to analysis) or may be a sequence in a segment of a nucleic acid defined by other parameters (*e.g.*, defined as the segment of a gene that can be amplified by a particular primer pair).

[0054] A target sequence can be any nucleic acid sequence of interest, such as a sequence associated with a gene, a sequence that identifies a particular allele or polymorphism, a sequence that, alone in combination with other genotypic or phenotypic markers, identifies the presence in the sample of a particular organism or strain, and the like. A target sequence can also include sequences flanking a sequence of interest, such as the sequences flanking a SNP. In addition, in some cases as will be recognized from context, a "target sequence" is a sequence added during an amplification step. For example, the B and U sequences discussed below in the context of a Universal Amplification method can be referred to as "target sequences" recognized by a probe or amplification primer.

[0055] A target sequence can be found in DNA (including genomic, mitochondrial DNA, viral DNA, recombinant DNA and complementary cDNA made from RNA) or in RNA (including rRNA, mRNA and iRNA). If a target sequence is detected in a sample it is possible to deduce that the sample contains a nucleic acid molecule containing the detected sequence or its complement. For example, a sample from a human patient can be analyzed to determine whether a viral nucleotide sequence (the target sequence) is detectable in the sample, in order to diagnose (or rule out) viral infection. As another example, genomic DNA from a human patient can be analyzed to determine whether a particular polymorphism is or is not present in a subject's genome.

[0056] In some cases, it will be advantageous to fragment the nucleic acid molecules prior to the partitioning step. For example, to characterize two genes on different regions of a eukaryotic chromosome it may be useful to fragment the DNA to produce smaller nucleic acid molecules so that the genes can be separately partitioned (*i.e.*, partitioned into different sub-samples). Fragmentation can be accomplished enzymatically (*e.g.*, using restriction

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enzymes), mechanically or chemically. In one embodiment, shearing is accomplished by passing the DNA through a channel of a MPD with a diameter (bore size) that is sufficiently small, or which varies in diameter along the length of the channel, so as to sheer large nucleic acids as they pass through. A sample containing a single DNA molecule (*e.g.*, a single chromosome) contains a plurality of nucleic acids upon fragmentation of the single molecule.

ii) Partitioning of a Sample Containing Nucleic Acid Molecules

[0057] Methods for partitioning a sample using a MPD are provided in Section B, above. The terms "to partition," "partitioning," "partitioned," and grammatical equivalents refer to the process of separating a sample into a plurality of sub-samples using a MPD. A sample is partitioned by introducing the sample into the flow channels and reaction sites with valves open and then closing the valves to isolate each sub-sample. It will be recognized that each sub-sample is contained (for at least a period of time) in a separate reaction chamber such that the sample is isolated from (not in fluidic communication with) other sub-samples. It is sometimes convenient to refer to the "sample" even after it has been partitioned into sub-samples. Thus, in some contexts "sample" can refer to the aggregate contents of the sub-samples or chambers after partition, as well as before partition.

[0058] When a sample containing a complex mixture of nucleic acid molecules it is partitioned into very small-volume sub-samples, the effective concentration of the target sequence in the sub-sample(s) in which it is located is significantly increased. Effective concentration of the target occurs because, while the number of molecules of target in the sample does not change as a result of the partitioning, the number of other molecules (including molecules that can produce side reactions, *e.g.*, primer-dimers and non-complementary DNA sequences in the sample) is linearly proportional to volume. For example, if a 30 microliter sample containing one molecule of interest is partitioned into ten thousand subsamples (each with a volume of 3 nanoliters) the effective concerntration of molecule of interest is enriched by a factor of 10^4 in the chamber in which it is located. Since the ratio of target to side reactions is inversely proportional to volume, partitioning into a small volume increases this ratio (*i.e.*, effectively concentrates). As noted by McBride *et al.*, such an increase in effective concentration results in remarkable sensitivity and fidelity of PCR-based detection.

[0059] Typically the sample is partitioned into at least about 10^3 different sub-samples or reaction chambers, sometimes at least about 5×10^3 different sub-samples or chambers, sometimes 10^4 different sub-samples or chambers, often at least about 2×10^4

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different sub-samples or chambers, sometimes at least about 3 x 10^4 different sub-samples or chambers, and sometimes at least about 10^5 different sub-samples or chambers. In certain embodiments the sample is partitioned into between 100 and 100,000 sub-samples, more often between 1000 and 50,000 sub-samples, and sometimes between 1000 and 20,000 sub-samples.

[0060] Typically the volume of each sub-sample is less than about 1000 picoliters (pL), often less than about 500 pL, sometimes less than about 100 pL, and sometimes less than about 50 pL.

[0061] The relationship between the number of nucleic acid molecules (or non-nucleic acid macromolecules, particles or cells) in a sample, the number of chambers into which the sample is partitioned, and the distribution of number of nucleic acid molecules or other entities in each chamber can be estimated using well know methods. For example, to determine the number of chambers (C) into which the number (N) particles (e.g. cells, nucleic acid molecules, etc.) would be partitioned so that most or essentially all of the chambers contained either 0 or 1 particle can be determined using a Poisson Distribution: [1]

$P(x,\lambda)=\frac{e^{-\lambda}\lambda^{x}}{x!}$. P(x,λ) is the probability of finding x particles if the average number of particles in a box is λ. We can get this by setting the average number (λ) such that

$$P(0,\lambda)+P(1,\lambda)\geq x$$

$$\frac{e^{-\lambda}\lambda^{0}}{0!}+\frac{e^{-\lambda}\lambda^{1}}{1!}\geq x$$

$$e^{-\lambda}+e^{-\lambda}\lambda\geq x$$

$$e^{-\lambda}(1+\lambda)\geq x$$

The average number of particles in a box is λ= N/C, so if you know λ and N, you can find C:

$$C=\frac{N}{\lambda}$$

λ is easily determined using eqn. [1] above.

For instance, if x is .99 (i.e. 99% chance of a chamber containing a 0 or 1 particle),

$$e^{-\lambda}(1-\lambda)\geq .99$$

$$\lambda\leq\sim 0.1487$$

If N is 10,000, then

$$C\cong\frac{10000}{0.1487}$$

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$C \geq 67250$ chambers are required to have 99% likelihood of 0 or 1 particles per chamber. Although this calculation is provided for illustration it will be understood that any method (emperical or analytical) may be used. In some applications it will be useful to use such a calculation and adjust (e.g., dilute) the sample and/or select a MPD with an appropriate number of chambers for an increased likelihood there will be few, if any, chambers with more than a predetermined number of target molecules (e.g., 1) per chamber.

iii) Amplification of Partitioned Nucleic Acids

[0062] Following the partitioning step, any target sequences of interest that are in the sample are amplified. As used herein, nucleic acid "amplification" is a process that produces multiple nucleic acid molecules (called "amplicons") based on the presence of a particular target sequence. Most often the amplicons include a base sequence that is the same as, or complementary to, the target sequence so that amplification means that the number of copies of the target sequence increases. These identical or complementary amplicons are the products, for example and without limitation, of the Polymerase Chain Reaction (PCR) [see, Dieffenbach and Dvksler, 1995, PCR Primer: A Laboratory Manual. CSHL press, Cold Spring Harbor, USA]; Nucleic Acid Sequence Based Amplification (NASBA) [see Sooknanan and Malek, 1995, *BioTechnology* 13:563-65] SPIA™ Isothermal Linear Amplification, Ribo-SPIA, X-SPIA™ [Nugen Technologies, San Carlos CA, see Pat. No. 6,251,639, WO 02/72772; US2003/0017591 A1]; the Ligase Chain Reaction (LCR) [Wu and Wallace, 1989, Genomics 4:560; Landegren et al., 1988, *Science* 241:1077]; Transcription amplification [Kwoh et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:1173]; Self-sustained sequence replication [Guatelli et al., 1990, *Proc. Nat. Acad. Sci. USA* 87:1874]. In some embodiments, however, an amplicon is a nucleic acid with a sequence different from the target sequence and the process of amplification consists of increasing the number of amplicons if the target is present in a chamber, but not in the absence of the target sequence.

[0063] As noted above, typically the invention is used to simultaneously assay for multiple different target sequences in the same sample (*e.g.*, sequences of multiple different genes or gene segments, or alternative sequences of a single gene). For example, provided with a patient blood sample, it may be useful to assay the sample for the presence of several (*e.g.*, 10, 20 or 100) different sequences each characteristic of a different pathogen. In one embodiment, the different sequences are amplified in different reaction chambers. For example, an assay to detect the presence of multiple mutations in different genes from an individual sample will result in analysis of products in different reaction chambers if the

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genes are on fragments distributed to different sub-samples. In other embodiments, multiple different target sequences are assayed for and/or detected in a single reaction chamber, such as, for example, when the reaction chamber contains a single rare cell and the assay is designed to analyse several genetic loci in the cell genome. For example, an assay to determine which of several possible polymorphisms (e.g. defining a haplotype) are represented at a specific genomic site, multiple primers or probes may be used to determine which of several possible target sequences are present in a single reaction chamber. Very often both types of amplification are used.

[0064] A variety of methods are known for "multiplex" analysis (amplification of multiple sequences from one reaction) and can be adapted for use in a single reaction chamber MPDs. In one embodiment amplification is conducted using a universal primer strategy in which each target sequence is initially amplified by a pair of target sequence specific primers that include a 3-prime domain with a gene-specific sequence and a 5-prime domain with a universal (not target specific) sequence. For example, N different target sequences can be detected using primers 5'-U_1-F_N-3' and 5'-U_2-R_N-3' where F_N and R_N are forward and reverse PCR primers for each gene N, and U_1 and U_2 are sequences common to all of the primer pairs and U_1 and U_2 may be the same or different. Subsequent rounds of amplification can be conducted using primers 5'-U_1-3' and 5'-U_2-3'. See, e.g.,. Zhenwu Lin et al., 1996, "Multiplex genotype determination at a large number of gene loci" *Proc. Nat'l Acad. Sci USA* 93: 2582-87. When used in an MPD this strategy allows use of a low concentration of target specific primers (thereby reducing expense and the chances of primer dimer and other unintended side reactions) and a higher concentration of universal primers.

[0065] In one embodiment, the multiplex amplification is carried out in a MPD using the primers and strategy described below in Section C(v).

[0066] Amplification reagents, including primers, can be provided by prepositioning reagents in reaction chambers, by combining reagents with the sample before partitioning, by a combination of prepositioning and combining, or by any other suitable method. Specific amplification reagents will depend on the amplification method and sample, but can include primers, polymerase, reverse transcriptase, nucleotides, cofactors, metal ions, buffers, and the like. Methods for prepositioning reagents in a microfluidic device have been described (see McBride *et al., supra*). In addition, detection reagents such as labeled probes can be provided by prepositioning and/or combining.

[0067] To produce amplicons the environment of the MPD reaction chambers is manipulated as required to accomplish amplification by the amplification method selected.

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For example, thermocycling necessary for a PCR-type amplification reaction can be accomplished by placing the device on a thermocycling plate and cycling the device between the various required temperatures for melting of the DNA duplex (either target or amplicon), annealing of primers, and DNA synthesis. For example a protocol with an initial ramp to 95°C and maintain for 1 m; three step thermocycling for 40 cycles [92°C for 30 s, 54°C for 30 s, and 72°C for 1 m] or two step thermocycling for 40 cycles [92°C for 30 s and 60°C for 60 s] can be used. A variety of thermocycling plates are available from commercial sources, including for example the ThermoHybaid Px2 (Franklin, MA), MJ Research PTC-200 (South San Francisco, CA), Eppendorf Part# E5331 (Westbury, NY), Techne Part# 205330 (Princeton, NJ).

[0068] Although in some cases a single target sequence in the sample is amplified, more often at least 2, at least 3, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, or at least 100 different target sequences are amplified. Thus, in some embodiments, sufficient reagents are provided to amplify more than one target sequence.

[0069] Thus, the amplification procedure can produce from zero (if no target sequence is present) to 100 or more different amplicons. Any particular chamber may have zero, one or more than one amplicons species (where amplicons corresponding to the same target sequence are of the same "species") depending on the nature of the assay and sample.

[0070] Usually, at least about 10^3, at least about 10^4, at least about 10^5, at least about 10^6, at least about 10^7, or at least about 10^8 amplicon molecules are produced corresponding to some or all of the target sequences present in the sample. Most typically, from about 10^7 to about 10^9 amplicon molecules are produced for each target sequence, although the number may be lower when multiple reactions are conducted in a single reaction chamber.

iv) Detection of Amplicons

[0071] The amplicon products can be detected in individual chambers and/or they can be pooled for subsequent detection and analysis (as described below in Section C(vi)). That is, amplicons can be detected and then pooled, pooled without previously being detected, or detected and not subsequently pooled.

[0072] Amplicons can be detected and distinguished (whether isolated in a reaction chamber or at any subsequent time) using routine methods for detecting nucleic acids. Amplicons comprising double-stranded DNA can be detected using intercalation dyes such as SYBRTM, Pico Green (Molecular Probes, Inc., Eugene, OR), ethidium bromide and the like

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(see Zhu et al., 1994, *Anal. Chem.* 66:1941-48) and/or gel electrophoresis. More often, sequence-specific detection methods are used (*i.e.*, amplicons are detected based on their nucleotide sequence). Examples of detection methods include hybridization to arrays of immobilized oligo or polynucleotides, and use of differentially labeled molecular beacons or other "fluorescence resonance energy transfer" (FRET) -based detection systems. FRET - based detection is a preferred method for detection. In FRET- based assays a change in fluorescence from a donor (reporter) and/or acceptor (quencher) fluorophore in a donor/acceptor fluorophore pair is detected. The donor and acceptor fluorophore pair are selected such that the emission spectrum of the donor overlaps the excitation spectrum of the acceptor. Thus, when the pair of fluorophores are brought within sufficiently close proximity to one another, energy transfer from the donor to the acceptor can occur and can be detected. A variety of assays are known including, for example and not limitation, template extension reactions, quantitative RT-PCR, Molecular Beacons, and Invader assays, these are described briefly below.

[0073] *FRET and template extension reactions* utilize a primer labeled with one member of a donor/acceptor pair and a nucleotide labeled with the other member of the donor/acceptor pair. Prior to incorporation of the labeled nucleotide into the primer during an template-dependent extension reaction, the donor and acceptor are spaced far enough apart that energy transfer cannot occur. However, if the labeled nucleotide is incorporated into the primer and the spacing is sufficiently close, then energy transfer occurs and can be detected. These methods are particularly useful in conducting single base pair extension reactions in the detection of single nucleotide polymorphisms and are described in U.S. Patent No. 5,945,283 and PCT Publication WO 97/22719.

[0074] *Quantitative Real Time PCR*. A variety of so-called "real time amplification" methods or "real time quantitative PCR" methods can also be used to determine the quantity of a target nucleic acid present in a sample by measuring the amount of amplification product formed during or after the amplification process itself. Fluorogenic nuclease assays are one specific example of a real time quantitation method which can be used successfully with the devices described herein. This method of monitoring the formation of amplification product involves the continuous measurement of PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe -- an approach frequently referred to in the literature as the "TaqMan" method. See U.S. Pat. No. 5,723,591

[0075] *Molecular Beacons*: With molecular beacons, a change in conformation of the probe as it hybridizes to a complementary region of the amplified

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product results in the formation of a detectable signal. The probe itself includes two sections: one section at the 5' end and the other section at the 3' end. These sections flank the section of the probe that anneals to the probe binding site and are complementary to one another. One end section is typically attached to a reporter dye and the other end section is usually attached to a quencher dye. In solution, the two end sections can hybridize with each other to form a hairpin loop. In this conformation, the reporter and quencher dye are in sufficiently close proximity that fluorescence from the reporter dye is effectively quenched by the quencher dye. Hybridized probe, in contrast, results in a linearized conformation in which the extent of quenching is decreased. Thus, by monitoring emission changes for the two dyes, it is possible to indirectly monitor the formation of amplification product. Probes of this type and methods of their use are described further, for example, by Piatek et al., 1998, Nat. Biotechnol. 16:359-63; Tyagi, and Kramer, 1996, Nat. Biotechnology 14:303-308; and Tyagi, et al., 1998, Nat. Biotechnol. 16:49-53 (1998).

[0076] *Scorpion:* The Scorpion detection method is described, for example, by Thelwell et al. 2000, Nucleic Acids Research, 28:3752-3761 and Solinas et al., 2001, "Duplex Scorpion primers in SNP analysis and FRET applications" Nucleic Acids Research 29:20. Scorpion primers are fluorogenic PCR primers with a probe element attached at the 5'-end via a PCR stopper. They are used in real-time amplicon-specific detection of PCR products in homogeneous solution. Two different formats are possible, the 'stem–loop' format and the 'duplex' format. In both cases the probing mechanism is intramolecular. The basic elements of Scorpions in all formats are: (i) a PCR primer; (ii) a PCR stopper to prevent PCR read-through of the probe element; (iii) a specific probe sequence; and (iv) a fluorescence detection system containing at least one fluorophore and quencher. After PCR extension of the Scorpion primer, the resultant amplicon contains a sequence that is complementary to the probe, which is rendered single-stranded during the denaturation stage of each PCR cycle. On cooling, the probe is free to bind to this complementary sequence, producing an increase in fluorescence, as the quencher is no longer in the vicinity of the fluorophore. The PCR stopper prevents undesirable read-through of the probe by Taq DNA polymerase.

[0077] *Invader:* Invader assays (Third Wave Technologies, Madison, WI) are used particularly for SNP genotyping and utilize an oligonucleotide, designated the signal probe, that is complementary to the target nucleic acid (DNA or RNA) or polymorphism site. A second oligonucleotide, designated the Invader Oligo, contains the same 5' nucleotide sequence, but the 3' nucleotide sequence contains a nucleotide polymorphism. The Invader

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Oligo interferes with the binding of the signal probe to the target nucleic acid such that the 5' end of the signal probe forms a "flap" at the nucleotide containing the polymorphism. This complex is recognized by a structure specific endonuclease, called the Cleavase enzyme. Cleavase cleaves the 5' flap of the nucleotides. The released flap binds with a third probe bearing FRET labels, thereby forming another duplex structure recognized by the Cleavase enzyme. This time the Cleavase enzyme cleaves a fluorophore away from a quencher and produces a fluorescent signal. For SNP genotyping, the signal probe will be designed to hybridize with either the reference (wild type) allele or the variant (mutant) allele. Unlike PCR, there is a linear amplification of signal with no amplification of the nucleic acid. Further details sufficient to guide one of ordinary skill in the art are provided by, for example, Neri, B.P., et al., *Advances in Nucleic Acid and Protein Analysis* 3826:117-125, 2000) and U.S. Patent No. 6,706,471.

Padlock probes: Padlock probes (PLPs) are long (e.g., about 100 bases) linear oligonucleotides. The sequences at the 3' and 5' ends of the probe are complementary to adjacent sequences in the target nucleic acid. In the central, noncomplementary region of the PLP there is a "tag" sequence that can be used to identify the specific PLP. The tag sequence is flanked by universal priming sites, which allow PCR amplification of the tag. Upon hybridization to the target, the two ends of the PLP oligonucleotide are brought into close proximity and can be joined by enzymatic ligation. The resulting product is a circular probe molecule catenated to the target DNA strand. Any unligated probes (i.e., probes that did not hybridize to a target) are removed by the action of an exonuclease (which may be introduced before or after a pooling step). Hybridization and ligation of a PLP requires that both end segments recognize the target sequence. In this manner, PLPs provide extremely specific target recognition.

Using universal primers, the tag regions of circularized PLPs can be amplified and resulting amplicons detected. For example, TaqMan real time PCR can be carried out to detect and quantitate the amplicon. The presence and amount of amplicon can be correlated with the presence and quantity of target sequence in the sample. For descriptions of PLPs see, e.g., Landegren et al., 2003, Padlock and proximity probes for in situ and array-based analyses: tools for the post-genomic era, *Comparative and Functional Genomics* 4:525-30; Nilsson et al., 2006, Analyzing genes using closing and replicating circles *Trends Biotechnol.* 24:83-8; Nilsson et al., 1994, Padlock probes: circularizing oligonucleotides for localized DNA detection, *Science* 265:2085-8.

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v) Detection of Multiple Different Target Nucleic Acid Sequences Using The "Universal Amplification" Method

[0078] As described above, a variety of multiplex amplification systems can be used in conjunction with the present invention. In one type, several different targets can be detected simultaneously by using multiple differently labeled probes each of which is designed to hybridize only to a particular target. Since each probe has a different label, binding to each target to be detected based on the fluorescence signals. By judicious choice of the different labels that are utilized, analyses can be conducted in which the different labels are excited and/or detected at different wavelengths in a single reaction. See, e.g., Fluorescence Spectroscopy (Pesce et al., Eds.) Marcel Dekker, New York, (1971); White et al., Fluorescence Analysis: A Practical Approach, Marcel Dekker, New York, (1970); Berlman, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd ed., Academic Press, New York, (1971); Griffiths, Colour and Constitution of Organic Molecules, Academic Press, New York, (1976); Indicators (Bishop, Ed.). Pergamon Press, Oxford, 19723; and Haugland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Eugene (1992).

[0079] Conventional multiplex fluorescence detection using many different probes is limited, however, by fluorescence background because probe concentration must be high enough to allow detection of many different probes (*i.e.*, one for each sequence to be detected). Combining many probes results in fluorescence that is the sum of all the probes. This also results in a fluorescence background that is the sum of the background from all of the probes. This background may be so high as to interfere with detection of the reaction product(s). An alternative approach, referred to as the Universal Amplification ("UA") method uses multiple sets of primer pairs, referred to here as "UA primer" amplification in a PCR-type reaction. Universal amplification allows many different sequences in a sample to be amplified using a single reaction mixture, with lower background and cost than conventional systems, and is particularly well suited for use with an MPD.

[0080] UA primers can be used to determine whether or not any one or more of a number of different target nucleic acid sequences are present in a sample (without necessarily identifying which of the several target sequences is present). If the assay indicates that at least one of the different nucleic acid sequences is present in the sample, subsequent analysis can be conducted to determine which of the different sequences is present. Such two-step analysis is advantageous in many applications. For example, using the present invention, in a first step a sample can be assayed to determine whether any of 100 (for example) different

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pathogenic agents is present in the sample. If it is determined in the first step that at least one pathogenic agent is present, the sample can be subjected to further analysis to identify and characterize the particular pathogen.

[0081] In one aspect the invention provides a method for detecting multiple amplification products (*i.e.*, amplicons) using the Universal Amplification method. The total number of target sequences detected is usually at least two, and is sometimes at least 5, more often at least 10, at least 20 or at least 30. In some embodiments the total number of target sequences is between 2 and 100, between 5 and 100, between 10 and 100, between 20 and 100, or between 30 and 100. In some embodiments the total number of target sequences is between 2 and 50, between 5 and 50, between 10 and 50, between 20 and 50, or between 30 and 50. In some embodiments the total number of target sequences is more than 100. The UA method makers use of three or more types of primers:

[0082] • The Type 1 primer has the structure 5'-U_F-B_x-F_N-3' where U_F is a universal forward primer sequence, B_x is a sequence recognized by a detectable (e.g., detectably labeled) labeled probe, Probe X, and F is a forward primer sequence specific to a target sequence N so that F_1 is primer for a first target sequence, F_2 is a primer sequence for a second target sequence, and so on. Probe X can be a molecular beacon, Taqman-type probe, or other probe (such as, but not limited to, those described above) that specifically binds or hybridizes to sequence B_x.

[0083] • The Type 2 primer has the structure 5'-U_R-R_N-3' where U_R is a universal reverse primer sequence and R_N is a primer specific to a target sequence N so that R_1 is primer for a first target sequence, R_2 is a primer for a second target sequence and so on. U_R may or may not be the same sequence as U_F. In one embodiment, 5'-U_R-3' has the same sequence as 5'-U_F-3'.

[0084] • The Type 3 primer comprises the sequence 5'-U_F-3' (or 5'-U_1-3').

[0085] • The Type 4 primer comprises the sequence 5'-U_R-3' (or 5'-U_2-3').

[0086] Each pair of Type 1 and Type 2 primers is specific to a particular target. The cognate pair of primers that amplify the same target is a "UA primer pair." Thus, if there are 20 target sequences to be detected (for example, sequences corresponding to 20 different pathogens) twenty different primer pairs can be prepared, i.e., 5'-U_1-B_x-$F_{[1\rightarrow 20]}$-3' and 5'-U_2'-$R_{[1\rightarrow 20]}$-3'. The various pairs of Type 1 and Type 2 primers are combined at low concentrations with the sample, and Type 3 and Type 4 primers are added at a higher concentration. During the initial rounds of amplification, the Type 1 and Type 2 primers will

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amplify any target sequences present in a sample or sub-sample. It will be appreciated that U_1 and U_2 sequences can be designed with sequences not present (or unlikely to be present) in the initial sample nucleic acid, to avoid amplification of non-target sequences in the sample. For example, for analysis of human DNA, U_1 and U_2 can be selected to have sequences not found on the human genome. During subsequent rounds of amplification, the amplification products generated in the first rounds of amplification are themselves amplified by the Type 3 and Type 4 primers. The resulting double stranded amplification products will have the structure (showing one strand):

$^{5'}$- U_1-B_x- F_1- target sequence1-R_1'-U_2'-$^{3'}$

$^{5'}$- U_1-B_x- F_2-target sequence2- R_2'- U_2'-$^{3'}$

$^{5'}$- U_1-B_x- F_3-target sequence3- R_3'-U_2'-$^{3'}$

$^{5'}$- U_1-B_x- F_4-target sequence4- R_4'-U_2'-$^{3'}$

$^{5'}$- U_1-B_x- F_5-target sequence5- R_5'-U_2'-$^{3'}$

$^{5'}$- U_1-B_x- F_6-target sequence6- R_6'-U_2'-$^{3'}$

etc.

where U_1' is the complement of U_1 and U_2' is the complement of U_2. Each of the amplification products shown above can be detected by a probe (*e.g.*, molecular beacon, Invader probe, Scorpion probe) that hybridizes to B_x. Thus, using the UA universal probes described herein, a multiplicity of target sequences can be detected using a single probe. Most of the amplification steps involve amplification using a single primer (if U_1 and U_2 are the same) or primer pair. Methods for designing probes that recognize a specified sequence (e.g., B_x) are well known. For example, Molecular Beacons can be designed as described in Marras et al., 2003, Genotyping single nucleotide polymorphisms with molecular beacons. In Kwok, P. Y. (ed.), Single nucleotide polymorphisms: methods and protocols. The Humana Press Inc., Totowa, NJ, Vol. 212, pp. 111-128. Molecular Beacons can also be designed with the help of a dedicated software package called 'Beacon Designer,' which is available from Premier Biosoft International (www.premierbiosoft.com). However, it will be appreciated that the sequence of B_x can be, and generally is, an artificial sequence (i.e., not found in the in the initial sample nucleic acid) that can be recognized by the probe.

[0087] Primer concentration(s) will vary with the length, composition, and nature of the sample and targets. Those primer pairs with sequences specific to each of the targets (e.g., Type 1 and 2 primers) are required only in the first few rounds of amplification and can be provided in very small quantities (for example and not limitation, e.g., typically less than about 50 nM, more often less than about 30 nM and sometimes less than about 20 nM). Type

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3 and 4 primers can be provided at somewhat higher concentration (for example and not limitation, e.g., typically from about 100 nM to 1 uM, such as from about 200 nM to about 900 nM). The practicioner guided by this disclosure will be able to select appropriate concentration using routine methods.

[0088] The method can be modified in a variety of ways to achieve particular results. In one version of the method, a relatively small number of probe sequences can be used with a larger number of unique target sequences, with different *classes* of target specific sequences associated with differently labeled probes. For example, if target sequences 1-20 are characteristic of a viral pathogen, target sequences 21-40 are characteristic of a bacterial pathogen, and target sequences 41-45 are positive control sequences (human genes), amplification of a human patient sample could give zero, one or more than one of the following 45 amplification products:

Set 1: 20 products (*from* $^{5'}$- U_F-B_V- target sequence1 -$U_R{}'$-$^{3'}$ *to* $^{5'}$- U_F-B_V- target sequence20-$U_R{}'$-$^{3'}$)

Set 2: 20 products (*from* $^{5'}$- U_F-B_B- target sequence21-$U_R{}'$-$^{3'}$ *to* $^{5'}$- U_F-B_B- target sequence40-$U_R{}'$-$^{3'}$)

Set 1: 5 products (*from* $^{5'}$- U_F-B_C- target sequence41-$U_R{}'$-$^{3'}$ *to* $^{5'}$- U_F-B_C- target sequence45-$U_R{}'$-$^{3'}$)

[0089] By using differently labeled probes B_V (hybridizes to Set 1 products), B_B (hybridizes to Set 2 products), and B_C (hybridizes to Set 3 products) the classes of amplification products can be detected and distinguished. Thus, if the human sample produced any amplicon to which Probe B_V hybridized and emitted signal it would indicate that the patient was infected with one of 20 viruses. If desired, the precise identity of the viral pathogen could be determined in a second assay step. Similarly, if the human sample produced any amplicon to which Probe B_B hybridized and emitted signal (different from the signal emitted by Probe B_V) it would indicate that the patient was infected with one of 20 bacteria. Other types of samples, such as food or agricultural sample can be screened for many different pathogens simultaneously and if *any* hits are detected the sample can be selected for further analysis to determine which of the many pathogen(s) was responsible for the signal. If there is no signal, the sample can be concluded to be pathogen free.

[0090] Numerous primer pairs are know for detection and analysis of pathogens, and other primer combinations can be prepared using well established methods. For illustration and not limitation see, e.g., U.S. Pat. No. 6,503,722 "Detection of toxigenic strains of Clostridium difficile using a PCR-based assay" [e.g., 5'

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CCCCAATAGAAGATTCAATATTAAG with 5'ATGTAGAAGTAAACTTACT TGGATG to detect strains expressing toxin A; 5' GGTGGAGCTTCAATTGGAGAG with 5' GTGTAACCTACTTTCATAACACCA to detect strains expressing toxin B; and 5' AAGTGTTCTGTAACAGGTATACC with 5' GGTCCATTAGCAGCCTCACA to detect glutamate dehydrogenase (positive control for presence of bacteria)]; U.S. Pat. No. 6,723,505 "Method for identification of the indicators of contamination in liquid samples"; U.S. Pat. No. 6,632,642 "Genes for detecting bacteria and detection method by using the same"; U.S. Pat. No. 6,387,652 "Method of identifying and quantifying specific fungi and bacteria"; U.S. Pat. No. 6,013,435 "Drug resistance screening method using multiplex amplification"; U.S. Pat. No. 5,932,415 "Processes and agents for detecting listerias"; U.S. Pat. No. 6,225,094 "Method for the genus-specific or/and species-specific detection of bacteria in a sample liquid"; Di Pinto, 2005, A collagenase-targeted multiplex PCR assay for identification of Vibrio alginolyticus, Vibrio cholerae, and Vibrio parahaemolyticus", *J Food Prot.* 68(1):150-3; Maher et al., 2003, " Use of PCR to detect Campylobacter species in samples" *J. Clin. Micro.* 41(7):2980 [5' AGTCGTAACA AGGTAGCCG with 5' CYRYTGCCAAGGCATCCACC]; Lim et al., "Use of PCR to detect heliobacter pylori in gastric mucosa of patients" *J. Clin. Micro.* 41(7):3387 [5' ACTTTAAACGCATGAAGATAT with 5' ATATTTTGACCTTCTGGGGT]; and Wilson et al., 2003, "Use of PCR to detect Legionella pneumophila" *J. Clin. Micro.* 41(7):3327 [5' GCAATGTCAACAGCAA with 5' CATAGCGTCTTGCATG].

Although the "Universal Amplification" method described above is suited for use in MPD-based analyses, it will be appreciated that this method can be used in a variety of formats (both microfluidic and nonmicrofluidic).

vi) Pooling of Amplicons

[0091] In some embodiments of the invention, following amplification and optional detection of amplicons, the contents of the sub-samples, including any amplicons in them, are pooled (*i.e.*, allowed to combine or mix at least partially). Pooling combines the amplicons (if present) from multiple sub-samples. Pooling can be accomplished by, for example, opening the valves of an elastomeric microfluidic device in which partitioning and amplification occurred such that the contents of multiple sub-samples (e.g., at least about 10^3, at least about 5×10^3, at least about 10^4, at least about 2×10^4, at least about 3×10^4, or at least about 10^5 sub-samples) are in fluidic communication with each other, constituting a "post-amplification sample" that consists of the contents of all of the chambers. Amplicons

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can mix by diffusion, which can be accelerated using thermal, mechanical, acoustic, or chemical energy. In one embodiment, pooling occurs primarily as a result of active mixing (*e.g.*, by pumping the fluid through flow channels in the device using a rotary peristaltic pump or other mechanism). Alternatively or in combination with the methods above, a portion, all or substantially all of the post-amplification sample can be pumped out of or otherwise withdrawn from the device, thereby pooling and mixing any amplicons present in the sample. Any method that results in a distribution of amplicons sufficient to carry out subsequent detection steps may be used.

[0092] It is not necessary that the various amplicons diffuse (or are mixed) to equilibrium in the post-amplification sample, but it is desirable that sufficient mixing occur so that an aliquot of post-amplification sample contains a number of molecules of each amplicon (e.g., at least 10, at least 100, at least 1000, or at least 5000 molecules) from each sub-sample in which amplicons were produced. For illustration, consider carrying out an amplification reaction that produces 100,000 copies of each of three distinct amplicons, *i.e.*, amplicon A in chamber 1, amplicon B in chamber 2, and amplicon C in chamber 3. The sub-samples are pooled (by releasing valves) and one-fifth of the volume of the post-amplification sample removed from the device or from the partition region of the device (see Section C(viii), below). If the amplicons had diffused to equilibrium in the post-amplification sample, and assuming no loss of material, the one-fifth volume would contain about 20,000 molecules of each of the three amplicons. If diffusion was less than complete, the one-fifth volume could contain unequal amounts of each amplicon, for example 50,000 molecules of amplicon A, 20,000 molecules of amplicon B and 15,000 molecules of amplicon C.

[0093] Alternatively, all or substantially all, of the post-amplification sample (or substantially all of it) can be withdrawn from the device, thereby mixing any amplicons present in the sample.

vii) Subsequent Analysis of Amplicons

[0094] Following pooling (*e.g.*, diffusion and/or mixing) all or a portion of the amplicon pool can be used for subsequent analyses. Typically the amplicon pool is divided into a plurality of aliquots and each aliquot separately analyzed to determine a property (*e.g.*, a nucleotide sequence or the presence or absence of a predetermined nucleotide sequence) of an amplicon or amplicons in that aliquot. If desired, the volume of the amplicon pool can be increased by addition of a suitable solution such as aqueous buffer or a reaction mixture

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containing amplification and/or detection reagents. The amplicon pool can be divided into aliquots manually or can be divided using an appropriately designed MPD (see Section C(viii), below).

[0095] It will be apparent from the discussion above that each of the aliquots from the amplicon pool will contain essentially the same set of amplicons (*i.e.*, the same amplicon species will be represented in each aliquot). Each of the aliquots can be used for a different analysis. For example, a first aliquot can be assayed for the presence of a first target sequence (*e.g.*, a first SNP in an amplified gene sequence), a second aliquot can be assayed for the presence of a second target sequence (*e.g.*, a second SNP in the same amplified gene sequence), a third aliquot can be assayed for the presence of a third target sequence (*e.g.*, a first SNP of a different amplified gene) and so on. It will be appreciated that target sequences of interest are not limited to SNPs.

[0096] The subsequent analysis of amplicons can be carried out using any desired technique including, without limitation, hybridization to a target nucleic acid or array of targets, PCR amplification, FRET-assays, hybridization to probes, and the like.

viii) Devices

[0097] As noted above, massive partitioning is accomplished using an elastomeric MPD. Subsequent analyses can also be accomplished using any suitable assay. In certain embodiments, an elastomeric microfluidic device is used for subsequent analyses. In some embodiments the initial partitioning and amplification, the subsequent mixing of applications, and the subsequent analysis of amplicons are carried out using different sections (*i.e.*, different banks) of the same the same device. For example, an elastomeric device can be fabricated with three regions: a first region that is a MPD in which target sequences, cells or molecules are amplified in individual chambers to produce amplicons and then allowed to mix to produce an amplicon pool, a second region (which can be as small as a single flow channel) by which the amplicon pool is transferred to the third region, and a third region having a plurality of flow channels with a region of each flow channel defining a reaction site in which subsequent analysis of the amplicon pool occurs. In one embodiment, the flow channels in the third region are blind flow channels with reaction sites near the channel terminus. A schematic of an exemplary device is shown in Figure 2. In this schematic five control channels (1, 2, 3, 4 and 5) are shown in black. A branched flow channel system is shown in gray. It will be appreciated, as discussed above, that the flow channel configuration need not be branched. In the device shown, a sample containing an amplification mixture

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and nucleic acids is injected into inlet A with the values formed by control channels 1 and 2 open, and control channel 3 actuated (closed). Control channel 2 is then actuated to isolate the sample in multiple chambers (B). The samples are then subject to thermocycling and optionally detection of the amplification products (e.g., using a commercially available fluorescence reader). Control channel 1 is then closed and control channel 2 opened to allow mixing of amplicons in the various chambers to produce an amplicon pool, a portion of which is then pumped into blind channels (D) with control channels 3, 4 (if present) and 5 open. Alternatively, control channels 2 and 3 are both opened and a portion of the amplicon pool is pumped to a mixing chamber (C) with control channel 4 closed. Control channel 3 is then closed and channels 4 and 5 are opened and portion of the amplicon pool is pumped into blind channels (D). Control channel 5 is then closed isolating blind reaction chambers (E) and the subsequent round of analysis takes place.

[0098] In one embodiment the device is configured so that reagents can be added to the pooled amplicon sample; for example, the mixing chamber (C) shown in Fig. 2 may be fluidically linked to a reservoir containing reagents (e.g., nuclease, probes or primers) that can be added prior to distributing the amplicon pool into a reaction chambers.

ix) Systems

[0099] In one aspect, the invention provides a system for analysis of nucleic acids, proteins or cells comprising a massively partioning device and an external collection reservoir for collection of an amplicon pool. The external reservoir can be any type of container or tube that is fluidically connected to the MPD, so that the contents of the MPD chambers can be transferred from the MPD to the reservioir. Transfer can be by displacement of the MPD contents using a displacement fluid, by active pumping, or by other means.

[0100] In one aspect, the invention provides a system for analysis of nucleic acids, proteins or cells comprising a massively partitioning device, optionally including an integral or external reservoir for collection of an amplicon pool, and an (additional) external component. In one embodiment the MPD includes a region with a plurality of flow channels defining a reaction sites for subsequent analysis of the amplicon pool aliquots (e.g., the "third" region of the device described above in Section (viii).

[0101] In one aspect, the invention provides a system for analysis of nucleic acids, proteins or cells comprising a massively partitioning device as described in Section (viii) above, where the MPD has three regions: a first region that is a MPD in which target

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sequences, cells or molecules are amplified in individual chambers to produce amplicons and then allowed to mix to produce an amplicon pool, a second region (which can be as small as a single flow channel) by which the amplicon pool is transferred to the third region, and a third region having a plurality of flow channels with a region of each flow channel defining a reaction site in which subsequent analysis of the amplicon pool occurs.

[0102] Additional external components of the system may include sensors, actuators (e.g., pumps; see US 6,408,878), control systems for actuating valves, data storage systems, reagent storage units (reservoirs), monitoring devices and signal detectors Signal detectors may detect visible, fluorescent, and UV light (intensity, scattering, absorption) luminescence, differential reflectivity, electrical resistance, resistivity, impedance, or voltage, in chambers or reaction sites. In one embodiment the external component is a temperature control component such as a thermocycler (e.g., Peltier device, resitive heaters, and heat exchangers; see e.g., US 6,960,437 B2).

[0103] In one aspect, the invention provides a system for analysis of nucleic acids, proteins or cells comprising a massively partitioning device, optionally including an integral or external reservoir for collection of an amplicon pool, optionally including a plurality of flow channels defining a reaction sites for subsequent analysis of the amplicon pool aliquots, and optionally including and an additional reagent positioned in the chambers of the MPD and/or the reaction sites for subsequent analysis of the amplicon pool aliquots. Exemplary additional reagents include enzymes (e.g., nuclease, polymerase, or ligase); primers and probes (PCR primers. molecular beacons, padlock probes, proximity ligation probes, Universal Amplification primers), amplification reagents and the like.

[0104] For illustration and not limitation, particular systems may comprise an MPD and a heat source (e.g., thermocycler) positioned to regulate the temperature of the contents of reaction chambers. In one embodiment, heat is transmitted from the heat source to the MPD by conduction (e.g., the heat source being adjacent and in contact with the MPD). In one embodiment the MPD is fixed (e.g., clamped) to the heat source.

[0105] For illustration and not limitation, particular systems may comprise an MPD and a signal detector positioned to detect signal emanating from reaction chambers in the MPD system. In one embodiment, a fluorescent signal is detected. In one embodiment the system includes an appropriately programmed computer coupled to the signal detector capable of storing information such as the position, intensity and/or duration of a signal emanating from reaction chambers in the MPD system.

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[0106] For example, when the MPD comprises reaction chambers for analysis of amplicon pool aliquots, the signal detector, heat source, or other component of the system may be associated with those reaction chambers, reaction chambers produced in the partitioning step, or both. Other particular systems may comprise a MPD comprising prepositioned reagents in one or more reaction chambers or mixing chambers.

x) Illustrative Examples

[0107] The following prophetic examples are intended to illustrate aspects of the invention. However, they are for illustration only and are not intended to limit the invention in any fashion.

1. SNP Analysis

[0108] In this illustration, 200 different genes of an individual are screened for the presence of mutations.

[0109] A sample containing genomic DNA from the subject is obtained. A small number of genome equivalents is sufficient for analysis. Thus the sample may be from a small number of cells (for example, fewer than 10 cells, and as few as one cell) which may be treated to release and fragment genomic DNA. Alternatively isolated or purified DNA may be used. Usually the DNA is fragmented by shearing, enzymatic or chemical cleavage, or other methods known in the art. In one embodiment the DNA is sheared by transport through a channel with varying cross-dimensions.

[0110] The reagents for amplification of target sequences are added to the sample. The amplification reagents include the following:

[0111] a) Primer pairs for each of the 200 gene segments to be analyzed. The primer pairs can be selected, for example, to (i) amplify a target polymorphic site sequence only if site has a particular sequence (*i.e.*, a specified SNP allele is present) or (ii) amplify the target polymorphic site sequence using primers that flank the SNP site, so that the segment is amplified without regard to what SNP is present. Each primer pair includes both a target specific sequence and one of two 5' universal sequences shared by all of the forward or all of the reverse primers, allowing all of the amplicons produced using the target specific primers to be amplified with the same universal primers.

[0112] b) A pair of universal primers capable of amplifying all of the amplicons produced using the target specific primers.

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[0113] Primers are selected so that all of the first round amplifications (using target specific primers) can occur under the same reaction conditions.

[0114] c) Amplification reagents (polymerase, cofactors, nucleotides, metal ions, buffer, etc.). The reagents may be added to the sample before partition, may be pre-positioned in the reaction chambers, or some reagents may be added and others pre-positioned.

[0115] The sample is injected into the partitioning channel system of a MPD having 40,000 chambers. After injection of the sample, valves are closed creating 40,000 isolated reaction chambers. Each reaction chamber contains all of the probes described above, and some of the chambers contain a nucleic acid molecule with a target sequence of interest as a consequence of the partitioning. The MPD is placed on a thermocycler and cycled using an appropriate protocol (e.g., 2 min at 51°C, 1 sec at 96°C and 59 sec at 95°C, followed by 40 cycles of 1 min at 58°C, 1 sec at 96°C and 59 sec at 95°C). Following amplification, the values are opened and sub-samples allowed to mix (e.g., by diffusion or active mixing) producing the amplicon pool.

[0116] A portion of the resulting amplicon pool is withdrawn from the MPD and distributed into two hundred (200) aliquots, and each aliquot is subjected to a different SNP assay. Alternatively, different sets of SNP assays can be conducted using multiplex methods. The individual SNP assays can be carried out using, for example, a Taqman™-type probe, Molecular Beacon, Scorpion, or other detection methods and detected using a fluorescence detector.

2. Analysis of Many Sequences Using Nucleic Acids from a Single Cell or Very Few Cells

[0117] In one embodiment, a nucleic acid analysis is conducted on a single cell. Such an analysis is useful for diagnostic or prognostic methods when tissue is limited such as, for example, genetic testing of a single blastocyst of a pre-implantation embryo produced using *in vitro* fertilization techniques. Such testing is also useful in the study or cloning of non-human animals. For example, blastocyst cells obtained from a non-human animal can be assayed for the presence, expression or characteristics of a transgene or endogenous gene. It can be verified that the genotype or expression profile of the embryo is consistent with the goals of the researcher prior to implantation into a surrogate mother, resulting in savings of time and resources. Such testing is also useful in forensic analysis in which very few cells

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may be available or in which cells must be analyzed individually because a sample is contaminated with cells from multiple sources.

[0118] Analysis of nucleic acids of a single cell is illustrated by the flow chart in Figure 3C. It will be appreciated that the figure is provided to assist the reader in understanding the invention, and is not intended to limit the invention in any fashion.

[0119] In this method, the single cell (or small number of cells) is provided in a solution or combined with a solution. The cell is treated to release DNA. Any number of methods for cell lysis (e.g., using sonication, denaturants, etc.) are suitable. If genomic DNA is being analyzed it is fragmented. The desired fragment size will be based on the method of detection of the target sequence and the number of reaction chambers on the chip. The goal is to end up with large enough fragments so that target sequences can be amplified (typically > 300 bp) and enough fragments so that different amplicons (i.e., amplicons corresponding to different sequences) will be generated is separate reaction chambers. Thus, the intended average size will depend on the number of target sequences to be detected and the number of partitions available. Fragments can be created by restriction digestion or other DNA fragmentation methods. In one embodiment, DNA is sheared by driving it thorough a narrow opening. Thus, a MPD can be designed with a via or flow channel of sufficiently small diameter narrowness to shear DNA to the desired fragment size. In an embodiment the diameter of the via or flow channel varies across its length (e.g., narrow-wide-narrow-wide) to drive the fragmentation. The sample is introduced into a MPD and the MPD valves are actuated to partition the DNA fragments, or RNA molecules, into separate reaction chambers.

[0120] Reagents sufficient to amplify each of the target sequences of interest are provided in each reaction chamber. For purposes of this example, assume 50 different loci containing SNPs are of interest, and at each of the 50 polymorphic loci there are two different possible sequences at the SNP site, with the 100 total different target sequences designated SNP 1A, 1B, 2A, 2B, ... 50A, and 50B. As discussed above, the reagents can be added to the solution containing the intact cell, can be prelocated in the reaction chambers, or some combination of the two. In this example, the amplification reagents include primers sufficient to amplify gene segments spanning each of the 50 loci to produce the SNP site and 40 basepairs of flanking sequence on each side. UA amplification primers may be used. Amplification is then carried out (*e.g.*, by thermocycling if the amplification method is PCR or reverse transcription-PCR). Amplicons are produced in those reaction chambers that contain a nucleic acid molecule (i.e., DNA fragment or RNA molecule) comprising one of the target sequences.

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[0121] As described above, following amplification the valves are opened and amplicons allowed to mix to produce an amplicon pool. The pool is than divided into 100 different aliquots (optionally using a duel bank MPD as described in Section C(viii), above). In each aliquot a single assay is carried out for an individual SNP using, for example, a TaqmanTM-type probe, Molecular Beacon, Scorpion, or other detection methods known in the art.

3. Detection of Pathogens Using the UA System

[0122] In this illustration, a sample is assayed for the presence of 150 different pathogens. Exemplary samples for the method include (i) a sample is obtained from a patient, (ii) an environmental sample (*e.g.*, from a pond or reservoir) and (iii) a sample from a poultry processing facility.

[0123] The reagents for amplification of target sequences are added to the sample. The amplification reagents include the following:

[0124] a) Fifty UA primer pairs for gene segments found in fifty different bacterial pathogens. The forward primer of these UA primer pairs includes a recognition site for a molecular beacon labeled with the blue fluorescing dye Cy 5.5.

[0125] b) Fifty UA primer pairs for gene segments found in fifty different fungal pathogens. The forward primer of these UA primer pairs includes a recognition site for a molecular beacon labeled with the green fluorescing dye 6-FAM (Fluorescein).

[0126] c) Fifty UA primer pairs for gene segments found in fifty different viral pathogens. The forward primer of these UA primer pairs includes a recognition site for a molecular beacon labeled with the red fluorescing dye Cy 3.

[0127] d) Type 3 and Type 4 primers corresponding to the UA primer pairs Primers 5'-U_A -3' and 5'-U_B -3'.

[0128] Primer sequences are selected so that all of the UA primer pairs produce amplicons that can be amplified using the same Type 3 and Type 4 primers; and all of the first round amplifications can occur under the same reaction conditions.

[0129] e) Molecular beacons that recognize the recognition sites of the three UA forward primers and are labeled as indicated above.

[0130] f) Amplification reagents (polymerase, cofactors, nucleotides, metal ions, buffer, etc.). The reagents may be added to the sample before partition, may be prepositioned in the reaction chambers, or some reagents may be added and others prepositioned.

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[0131] The sample is injected into the partitioning channel system of a MPD having 40,000 chambers. After injection of the sample each valves are closed creating 40,000 isolated reaction chambers. Each reaction chamber contains all of the probes described above, and some of the chambers contain a nucleic acid molecule with a target sequence of interest. The MPD is placed on a thermocycler and cycled using an appropriate protocol (e.g., 2 min at 51°C, 1 sec at 96°C and 59 sec at 95°C, followed by 40 cycles of 1 min at 58°C, 1 sec at 96°C and 59 sec at 95°C). Following amplification, the device is imaged using a commercially available, modified, or custom made fluorescence reader.

[0132] The appearance of chambers fluorescening blue indicates that there is at least one bacterial pathogen present in the sample. The appearance of chambers fluorescening green indicates that there is at least one fungal pathogen present in the sample. The appearance of chambers fluorescesing red indicates that there is at least one viral pathogen present in the sample.

[0133] If none of the chambers is emits blue, green or red fluorescence when illuminated at the proper wavelengths this is an indication that none of the pathogens tested for are present (typically assays would also include a positive control). If, however, red fluorescence was detected, this would be an indication that a virus is present. Further testing could then be carried out to identify the viral pathogen. For further testing, the values of the MPD are opened and sub-samples allowed to pool by diffusion or active mixing, producing the amplicon pool. A portion of the resulting amplicon pool is withdrawn from the MPD and divided into fifty (50) aliquots each containing reagents for identification of one of the 50 viruses assayed for in the initial part of the screen. An exemplary detection method uses 50 molecular beacons (one in each aliquot) that each recognize a different virus specific sequence in the amplicon. By determining which of the molecular beacons bind a target sequence present in the amplicon pool (or the portion contained in an aliquot) the identity of the pathogen is determined.

4. Additional Applications

[0134] It will be appreciated to the reader that the methods of the invention can be used in many applications not specifically described, including, for example and not limitation, detection of gene mutations (substitutions, deletions, translocations, amplifications, etc.) in samples from cancer patients and others. Many of these assays can be carried out without using the optional pooling step and subsequent analysis steps.

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D. PARTITIONING, DETECTION AND ANALYSIS OF PROTEINS AND OTHER BIOMOLECULES

[0135] Proteins and other biomolecules can be partitioned in a manner analogous to that described above for nucleic acid molecules. Any suitable method can be used to produce an amplification product indicative of the presence of a protein. In one method, a proximity ligation procedure is used. The proximity ligation procedure is analogous in certain respects to the use of padlock probes, described above in Section C, but is used for detecting proteins and other analytes. The proximity ligation procedure uses specific protein binding agents linked to oligonucleotides. Examples of specific protein binding agents include, but are not limited to, antibodies (defined as any specific binding agent comprising a CDR, including phage display antibodies, single chain antibodies, monoclonal antibodies, and the like) and nucleic acid aptamers. The proximity ligation procedure is described in Landegren et al., 2003, *supra*; Landegren et al., 2004, Molecular tools for a molecular medicine: analyzing genes, transcripts and proteins using padlock and proximity probes, *J Mol Recognit.* 7:194-7; Gullberg et al., 2004, Cytokine detection by antibody-based proximity ligation, *Proc Natl Acad Sci USA* 101(22):8420-4; Fredriksson et al., 2002, Protein detection using proximity-dependent DNA ligation assays, *Nat Biotechnol.* 20:448-9; and Landegren, 2002, Methods and kits for proximity probing *United States Patent Application 20020064779*. Briefly, a pair of protein binding agents that recognize different epitopes of a target protein are used. Each of the binding agents is attached (e.g., via streptavidin-biotin linkage) to a synthetic DNA strand that includes a PCR primer binding site. The synthetic DNA strands are brought into proximity when both binding agents bind the same target molecule. A connector oligonucleotide that hybridizes to sequences at the ends of both of the synthetic DNAs is added in excess, bringing termini of the DNA strands together so that they can be joined by ligase. In the presence of PCR reagents and primers that recognize the primer binding sites on the two DNA strands, a region of the ligated sequence may be amplified and detected by real time PCR. In contrast, unligated strands are not amplified and therefore not detected in the assay.

[0136] It will be appreciated that the assay also may be used to assay for non-protein molecules that are specifically bound by a nucleic acid aptamer, antibody or other binding agent. Proximity ligation methods can also be used to detect nucleic acid targets. In this approach, nucleotide sequences complementary to the target are used rather than protein binding agents.

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E. PARTITIONING OF CELLS

[0137] In another aspect of the invention, individual cells are isolated by partitioning using a MPD, and one or more properties of one or more of the individual cells are determined. Using this method, analysis of individual cells can be carried out without background from other cells in a sample.

[0138] Virtually any property of an individual cell can be assayed. For illustration, cell properties include

[0139] • the presence or absence of a target nucleic acid sequence in the cell (where the nucleic acid is RNA or DNA; recombinant or naturally occurring; cellular or viral; nuclear, cytoplasmic or from an organelle);

[0140] • the presence or absence of a protein or epitope in the cell or on the cell surface;

[0141] • secretion by the cell of a protein or non-protein molecule, for example in response to a stimulus;

[0142] • metabolic reactions or changes in cell metabolism, for example in response to a stimulus;

[0143] • other properties of cells (which will be recognized by those of skill in the art);

[0144] • combinations of two, three, or more than three different properties (*e.g.*, the presence in a cell of two different target nucleic acid sequences; the presence in a cell of a target nucleic acid sequence and a protein epitope; a change in a metabolic property of a cell and expression of a nucleic acid sequence in the cell; a cell surface epitope and secretion of a cytokine by the cell in response to a stimulus, etc.).

[0145] For this analysis, a liquid sample containing a plurality of separable cells is introduced into the MPD and the cells partitioned. A "separable cell" is a cell that is physically separated from other cells and can be partitioned into a chamber without other cells. In some cases (e.g., blood cells, lymphocytes, spermatocytes, oocytes, yeast, certain bacteria or other microorganisms) seperable cells can be obtained from a patient or other source with little processing. In other cases (e.g., liver biopsy, cultured cells, blastocyte) it will be necessary to disrupt a tissue or aggregate mechanically, enzymatically, or using other methods well known in the art. See, e.g., Ausubel *et al.*, 2004, Current Protocols In Molecular Biology, Greene Publishing and Wiley-Interscience, New York; Chapter 25. Examples of cells that can be assayed in this method include eukaryotic cells, human cells,

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animal cells, plant cells, fetal cells, embryonic cells, stem cells, blood cells, lymphocytes, bacterial cells, recombinant cells and cells infected with a pathogen. Further, although this section describes analysis of cells, the reader will appreciate that the same methods can be used for analysis of other biological entities, such as viruses and organelles.

[0146] In one aspect, the method includes partitioning a sample comprising a plurality of seperable cells into at least 10^3 separate reaction chambers in an MPD, where after partitioning at least two chambers comprise exactly one cell each. Often the sample is partitioned into at least 10^4 separate reaction chambers, at least 2×10^4 separate reaction chambers or at least 3×10^4 separate reaction chambers. The number of cells introduced into the MPD and/or the number of chambers in the MPD are selected so that most or virtually all of the chambers contain either no cells or a single cell. This can be determined from the Poisson distribution (based on the number of chambers in the device and number of cells injected into the device) or empirically (e.g., by detecting the number of chambers that contain cells). Usually at least 90% of the chambers contain zero or one cell, often at least 99% of the chambers contain zero or one cell, and in some cases virtually all of the chambers contain zero or one cell.

[0147] Each of the plurality of chambers contains the same reagents for conducting the analysis. All or some of these reagents can added to the sample or cells prior to injection into the device and/or can be prepositioned in the chambers and/or provided in inactive form, as described above. Because the reagents are constant, any chamber-to-chamber differences in analytical results are due to the presence of different cells (or no cell) and reflect differences in the properties of the cells. By detecting different signals from different chambers, a property or properties of cells in chambers can be determined and compared. This method finds a variety of applications in which it is informative to determine that a sample contains a cell having two or more properties detectable in separate assays. This method also finds a variety of applications in which the cell of interest is a rare cell in a background of many other cells.

[0148] The nature or type of reagents used will depend on the type of assay contemplated and specific properties to be detected. Generally the properties that can be assayed can be divided into two groups: properties determined based on the presence or absence of a nucleic acid target sequence and properties determined based on something other than the presence or absence of a nucleic acid target sequence. In many applications both a nucleic acid analysis and detection of a different type of property are carried out.

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[0149] In embodiments in which the analysis of cell properties includes a detecting a nucleic acid sequence (i.e., one, two or more target sequences are detected for an isolated cell) reagents suitable for nucleic acid analysis include those used for nucleic acid amplification (including but not limited to the PCR, SPIA, Invader, and other amplification methods described in this disclosure or known in the art) and those used for detection (including but not limited to, FRET based methods and other detection methods described in this disclosure or known in the art). In one embodiment the UA amplification/detection methods described in Section C(v) are used.

[0150] Methods are known in the art for assay of a multitude of cell properties other than or in addition to the characteristics of nucleic acids. For example, proximity ligation and FRET-based assays can be used to detect the presence of proteins or epitopes in a cell; presence, activation or change in enzymatic activities; intracellular organelle function; pathogen (e.g., viral) infection; intracellular signaling; protein-protein interactions; protein-DNA interactions; colocalization of proteins cell cycle; metabolic reactions such as generation of reactive oxygen species; mitochondrial membrane potential; apotosis; intracellular organelle function; changes in representations of cell types in cell populations; and subcellular localization of macromolecules.

[0151] In some embodiments of the invention the cell(s) is destroyed in the course of the process of detecting the cell property. Alternatively, numerous fluorescence based assays that can be carried out on living cells can readily be adapted for use in the present invention and/or reagents used in such assays may be used in the methods of the present invention. See, e.g., Dirks et al., 2003, *Visualizing RNA molecules inside the nucleus of living cells,* Methods, 29:51-7; Santangelo *et al.*, 2004, *Dual FRET molecular beacons for mRNA detection in living cells*, Nucleic Acids Res., 32:e57; Awais *et al.*, 2004, *A genetically encoded fluorescent indicator capable of discriminating estrogen agonists from antagonists in living cells,* Anal Chem., 76:2181-6; Nohe *et al.*, 2004, *Analyzing for co-localization of proteins at a cell membrane*, Curr Pharm Biotechnol., 5:213-20; Thoren *et al.*, 2004, *Membrane binding and translocation of cell-penetrating peptides,* Biochemistry, 43:3471-89; Balaji *et al.*, 2004, *Live cell ultraviolet microscopy: a comparison between two- and three-photon excitation*, Microsc Res Tech, 63:67-71; Gardiner, 2002, *Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis*, Curr Biol., 12:2029-34; Moshinsky *et al.*, 2003, *A Widely Applicable, High-Throughput TR-FRET Assay for the Measurement of Kinase Autophosphorylation: VEGFR-2 as a Prototype Journal of Biomolecular Screening*, 8:447-452; U.S. patents 4,822,733, 5,622,821, 5,639,615, and

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[0152] In addition, many cell-based assays developed for use in Laser Scanning Cytometry technology (e.g., CompuCyte Corp., Cambridge, MA) can be readily adapted for use in the methods of the invention and/or regents used in such assays may be used in the methods of the present invention. See, e.g., Adams *et al.*, 2004, *Increase of Circulating Endothelial Progenitor Cells in Patients With Coronary Artery Disease After Exercise-Induced Ischemia*, Arterioscler. Thromb. Vasc. Biol.; Akimitsu *et al.*, 2003, *Enforced cytokinesis without complete nuclear division in embryonic cells depleting the activity of DNA topoisomerase IIalpha*, Genes Cells, 8:393-402; Amirlak and Couldwell, 2003, *Apoptosis in glioma cells: review and analysis of techniques used for study with focus on the laser scanning cytometer*, J Neurooncol., 63:129-45; Balmer *et al.*, 2003, *Elevated methyl-CpG-binding protein 2 expression is acquired during postnatal human brain development and is correlated with alternative polyadenylation*, J Mol Med., 81:61-8; Baskin *et al.*, 2003, *Thimerosal Induces DNA Breaks, Caspase-3 Activation, Membrane Damage, and Cell Death in Cultured Human Neurons and Fibroblasts*, Toxicol. Sci., 74:361-368; Beliakoff *et al.*, 2003, *Hormone-Refractory Breast Cancer Remains Sensitive to the Antitumor Activity of*

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2004, *Calphostin-C Induction of Vascular Smooth Muscle Cell Apoptosis Proceeds through Phospholipase D and Microtubule Inhibition*, J. Biol. Chem., 279:7112-18.

[0153] Many other assay methods are known or can be developed. Reagents appropriate for each reaction type will be provided in the sample and/or prepositioned in the reaction chamber. Exemplary reagents include antibodies, ligands, enzyme substrates, effectors and the like.

Exemplary Applications

[0154] The following prophetic examples are intended to illustrate aspects of the invention. However, these examples are for illustration only and are not intended to limit the invention in any fashion.

1. Detection and characterization of pathogens

[0155] The methods of the invention may be used for detection, identification and characterization of pathogens. There are many situations in which a sample contains a heterogeneous mixture of microorganisms (e.g., various bacterial species or strains, viruses, fungi) for which rapid detection and identification would be advantageous. For example, clinical (patient) samples often contain small numbers of microorganisms (e.g., bacteria, fungi) or viruses. Rapid characterization would permit earlier administration of appropriate drugs, if necessary. Similarly, the ability to rapidly detect and identify cellular and viral pathogens would be of value in the medical, veterinary, and agricultural fields, as well as in response to actual or suspected bioterrorism and for rapid detection water or food contaminants. In many cases a relatively small number of cells are available to work with, and, as noted, the cells are often available as a heterogeneous mixture with other cells.

[0156] In one illustrative embodiment, the method is used to determine whether a patient is infected with methicillin-resistance *S. aureus*. *S. aureus* can be identified using a bacteria-specific probe (*e.g.* to a rRNA gene). An methicillin resistant strain is distinguished from non-resistant strains based on a characteristic genetic sequence such as an open reading frame (gene or gene seqment) or single polynucleotide polymorphism. A sample containing bacteria cells is obtained from a patient (*e.g.*, a nose swab containing about 100 bacterial cells) and diluted into a reaction mixture containing nucleic acid primers and other reagents for amplification and detection of target sequences (e.g., PCR reagents, molecular beacons, polymerase, nucleotides, agents that lyse cells for nucleic acid release, etc.). Exemplary PCR primers are described in Huletsky et al., 2004, "New real-time PCR assay for rapid detection

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of methicillin-resistant Staphylococcus aureus directly from specimens containing a mixture of staphylococci." *J Clin Microbiol.*42:1875-84. One primer/probe set in the reaction mixture emits a red fluorescence signal in the presence of a *S. aureus* target sequence found in both resistant and non-resistant stains while the second primer/probe set emits a green fluorescence signal only in the presence of a *S. aureus* target sequence found in the resistant strain. The cells are injected into a MPD and control channels actuated to create separate reaction chambers (*e.g.*, a sample containing about 100 bacterial cells is partitioned into 2000 chambers) all or most of which contain zero or one cell. The device is placed on the thermocycler or amplification is otherwise initiated. Detection in a chamber of only a red signal indicates the presence in the sample of non-resistant *S. aureus*; detection in a chamber of both a red and green signal indicates the presence in the sample of drug resistant *S. aureus*; detection or no signal indicates no *S. aureus* bacteria are present in the sample.

2. Quantitation of cells in a population having specific properties

[0157] In one aspect, the method is used for quantization of cells in a heterogeneous population having specific properties. For illustration, a cell population (e.g., peripheral blood mononuclear cells (PBMC)) containing cytotoxic T lymphocytes (CTL) (effector cells) can be partitioned and the ability of the cells to be stimulated by an antigen tested. The antigen reagent can be prepositioned in chambers or combined with cells immediately before partition. The proportion or type of cells activated in the presence can be assayed using any of a variety of assays for effector cell activation. For example, by performing *in vitro* stimulation after limiting dilution of circulating CTLs with the gag antigens of human immunodeficiency virus (HIV), the precursor population of gag-specific CTL can be quantitated and/or characterized. See, e.g., Koup "Limiting dilution analysis of cytotoxic T lymphocytes to human immunodeficiency virus gag antigens in infected persons: in vitro quantitation of effector cell populations with p17 and p24 specificities" *J Exp Med.* 1991 Dec 1;174(6):1593-600.

3. Characterization of a rare cell in a background of other cells

[0158] It is often advantageous to quantitate and/or characterize rare cells in a background of other cells. For example, in cancer, individual disseminated cancer cells may be found in blood. Further, biopsies may recover only a few malignant cells in a background of normal cells. The methods of the present invention allow the malignant cells to be

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isolated, identified based on a property (e.g., antigen, mutation or expression pattern) unique to the cancer cell, and then a different property of the cell determined.

[0159] In another example, nucleated fetal red blood cells are found at low levels in the blood of pregnant women and are a potential source of information about the fetal genome including any sequences associated with disease or propensity to disease. However, even enriched 10,000-fold fetal cells may be less than 0.1% of a sample making analysis by conventional methods difficult. Cells from a sample enriched for fetal NRBCs can be partitioned using the methods disclosed herein. Chambers containing fetal cells can be identified using a fetal-specific probe (e.g., a probe specific for the Y chromosome; abundance of RNA encoding fetal forms of hemoglobin) and assayed for several genetic characteristics using a multiplex assay. Other examples of rare cells in a background of different cells include, for example, a virally infected cell in a background of uninfected cells, a cell expressing a gene in a background of cells not expressing the gene; and the like.

[0160] In one embodiment, the MPD is used to partition a mixed population of cells to detect a property characteristic of a rare cell type in the population, i.e., cells comprising less than about 1%, more often less than about 0.1%, and very often less than about 0.01% of the cells in the population. There are many cases in which it advantageous to determine the properties of a rare cell in a population of other cells. The methods of the present invention enable analysis of a rare cell without background or interference for other cells. In general, the method involves partitioning cells and assaying individual cells for at least two properties at least one of which identifies the rare cell.

4. Expression Analysis of Individual Cells

[0161] In one embodiment the nucleic acid being analyzed is or includes RNA, and the expression level of specified genes in an individual cell is determined. Again using the example of a virus-infected cell, the expression profile for several host genes in a single cell can be correlated with the presence or absence of virus or with viral load. Gene expression profiles can also be correlated with cell identity (*e.g.*, different expression profiles for different cells in a sample containing a heterogeneous mixture of cells) or cell response to a stimulus (*e.g.*, the presence of a ligand that binds a cell receptor).

[0162] Because expression analysis typically involves a quantitative analysis, detection is typically achieved using one of the quantitative real time reverse transcriptase PCR methods described above. Thus, if a TaqMan approach is utilized, the reagents that are introduced (or previously spotted) in the reaction sites can include one or all of the following:

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primer, labeled probe, nucleotides and polymerase. Another approach is Ribo-SPIA (see above).

***

[0163] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes can be made and equivalents can be substituted without departing from the scope of the invention. In addition, many modifications can be made to adapt a particular situation, material, composition of matter, process, process step or steps, to achieve the benefits provided by the present invention without departing from the scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

[0164] All publications and patent documents cited herein are incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an indication that any such document is pertinent prior art, nor does it constitute any admission as to the contents or date of the same.

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CLAIMS

1. An assay method comprising
   (a) partitioning a sample into a plurality of sub-samples,
      wherein said sample comprises a plurality of nucleic acid molecules, and wherein at least two sub-samples comprise at least one nucleic acid molecule;
   (b) providing sufficient reagents in each sub-sample to amplify a target sequence or sequences;
   (c) amplifying the target sequence(s) in the sub-sample(s) containing target sequence(s)
      thereby producing amplicons in the sub-sample;
   (d) distributing the amplicons into a plurality of aliquots; and,
   (e) for each aliquot, determining a property of amplicons in the aliquot.

2. An assay method comprising
   (a) partitioning a sample into a plurality of sub-samples,
      wherein said sample comprises a plurality of nucleic acid molecules, and wherein at least two sub-samples comprise at least one nucleic acid molecule;
   (b) providing sufficient reagents in each sub-sample to amplify at least two different target sequences;
   (c) amplifying target sequence(s) in at least two sub-sample(s)
      thereby producing amplicons in the sub-sample(s);
   (d) combining the amplicons from said at least two sub-samples to create an amplicon pool;
   (d) dividing the amplicon pool into a plurality of aliquots; and,
   (e) for each aliquot, determining a property of amplicons in the aliquot.

3. The method of claim 2 wherein the sample is partitioned into at least 10^4 sub-samples.

4. The method of claim 3 wherein each subsample has a volume of less than 1 nanoliter.

5. The method of claim 2 wherein said nucleic acid molecules comprise RNA.

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6. The method of claim 2 wherein sufficient reagents are provided to amplify at least 10, 20, or 50 different target sequences, if present.

7. The method of claim 2 wherein said amplification is by PCR or RT-PCR.

8. The method of claim 2 wherein the amplicon pool is divided into at least 10, 20, 50 or 100 aliquiots.

9. The method of claim 2 wherein the sample comprises a plurality of cells comprising nucleic acid molecules, and wherein partitioning the sample comprises partitioning intact cells into a plurality of sub-samples.

10. The method of claim 2 wherein the sample comprises only one cell.

11. A method for conducting an analysis, comprising:

(a) partitioning a sample comprising a plurality of separable cells into at least 1000 separate reaction chambers in a MPD, wherein after partitioning at least two reaction chambers each comprise exactly one cell;

(b) providing in each reaction chamber one or more reagents for determining a property or properties of a cell, wherein the same reagents are provided in each chamber; and

(c) determining at least two different properties of a single cell in a chamber and/or determining at least one property for at least two different cells in different chambers.

12. The method of claim 11 wherein at least 99% of the reaction chambers contain zero or one cell.

13. The method of claim 11 wherein the cells are bacterial cells.

14. The method of claim 11 wherein said reagents comprise reagents for nucleic acid amplification.

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15. The method of claim 11 wherein at least one property is the presence or absence in the cell of a nucleic acid having a specified sequence.

16. The method of claim 11 wherein at least one property is other than the presence or absence in the cell of a nucleic acid having a specified sequence.

17. A method for amplification and detection of multiple target DNA sequences in a sample, said method comprising

a) providing a sample containing

i) multiple target DNA sequences,

ii) a primer pair corresponding to each of said multiple target DNA sequences, each pair consisting of a first primer comprising U_1, B_1 and F domains in the order 5'-U_1-B_1-F-3' and a second primer comprising U_2 and R domains in the order 5'-U_2-R-3', wherein each pair of F and R primers is capable of annealing specifically to a different target DNA sequence under stringent annealing conditions;

iii) a universal primer pair capable of amplifying a double stranded DNA molecule with the structure

5'- U_1 ----- U_2' - 3'
3'- U_1' ----- U_2 - 3'

where U_1' is the sequence complementary to U_1 and U_2' is the sequence complementary to U_2;

b) subjecting the sample to multiple cycles of melting, reannealing, and DNA synthesis thereby producing amplicons for each of said multiple target DNA sequences, and

c) detecting the amplicons using a probe that anneals to sequence of the amplicon having the sequence of the B_1 domain or its complement.

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18. The method of claim 17

wherein the sample also contains a second set of multiple target sequences, a primer pair corresponding to to each of the target sequences in the second set, each pair consisting of a first primer comprising U_1, B_2 and F domains in the order 5'-U_1-B_2-F-3' and a second primer comprising U_2 and R domains in the order 5'-U_2-R-3', wherein each pair of F and R oligonucleotides is capable of annealing specifically to a different target DNA sequence in the second set of multiple target sequences under stringent annealing conditions; and

wherein amplicons for each of the multiple target DNA sequences of the second set are produced; and

detecting the amplicons for each of the multiple target DNA sequences using a probe that anneals to sequence of the amplicon having the sequence of the B_2 domain or its complement.

19. The method of claim 17 wherein U_1, B_1, F_1, U_2 and R_1 domains are between 6 and 25 nucleotides in length and the probe is a molecular beacon.

20. A microfluidic device, comprising

(a) a first region comprising

(i) a flow channel formed within an elastomeric material and having a first end and a second end in fluid communication with each other through said channel, wherein said channel may be branched or unbranched;

(ii) an inlet for introducing a sample fluid in communication with said channel, said inlet;

(iii) an outlet in communication with said flow channel;

(iv) a plurality of control channels overlaying the flow channel(s),

wherein an elastomeric membrane separates the control channels from the flow channels at each intersection, the elastomeric membrane disposed to be deflected into or withdrawn from the flow channel in response to an actuation force, and

wherein, when the control channels are actuated the flow channel is partitioned into at least 1000 reaction chambers not in fluidic communication with each other;

(b) a second region compromising

a channel or chamber interposed between and in communication with said outlet in (a) and a flow channel in the third region;

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(c) a third region comprising a plurality of flow channels in fluidic communication with the channel or chamber of the second region, with a region of each flow channel defining a reaction site;

(d) a control channel or channels that when actuated separates the first and second regions;

(e) a control channel or channels that when actuated separates the second and third regions; and

(f) a control channel or channels that when actuated separates the reaction sites of said flow channels from the other portions of control channels.

21. The device of claim 20 wherein the flow channels in the third region are blind flow channels.

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Figure 1A

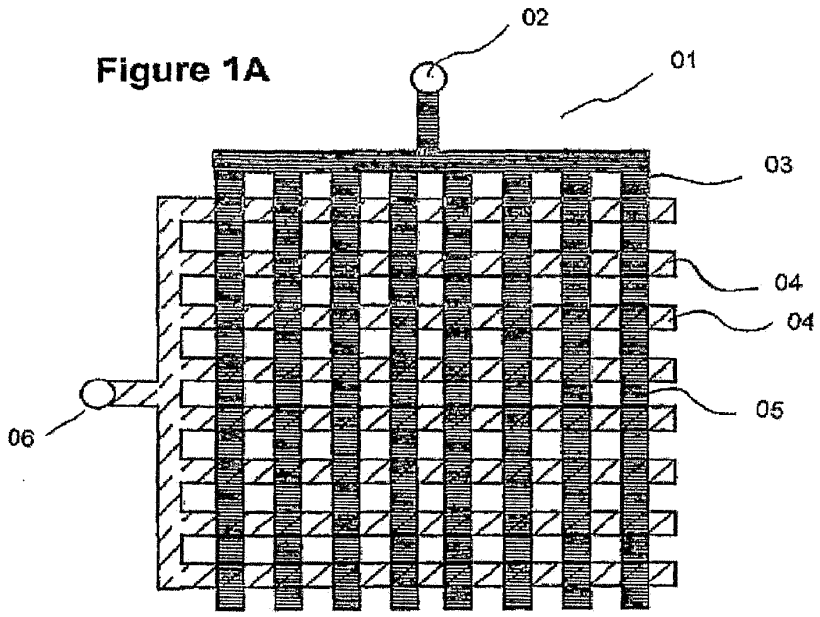
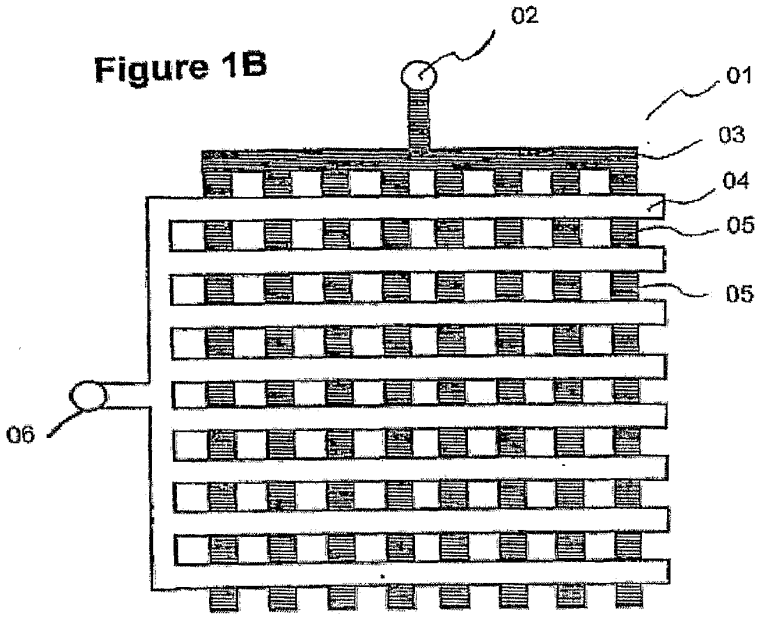


Figure 1B



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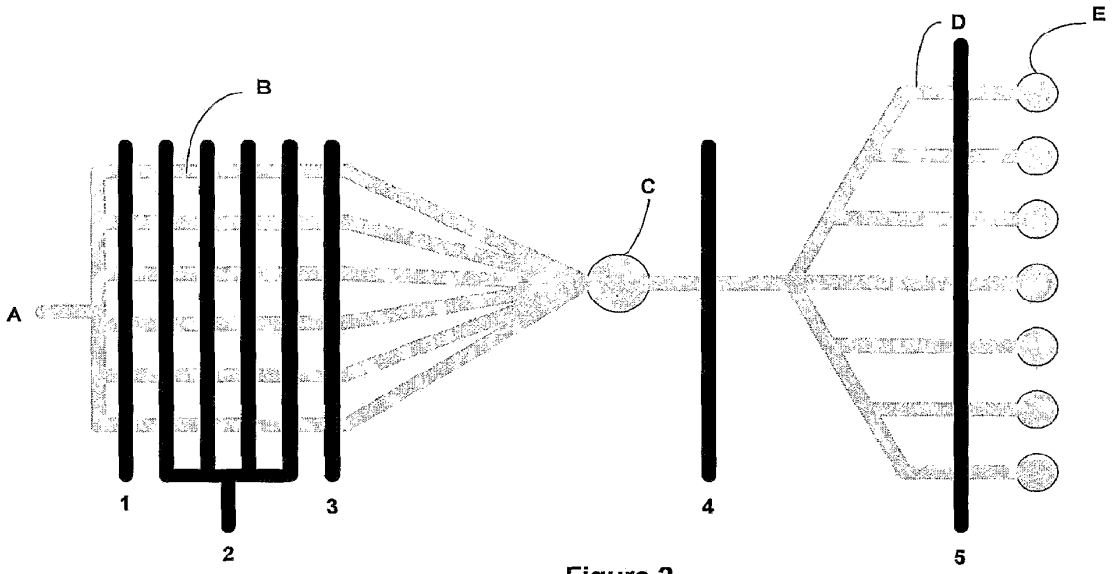
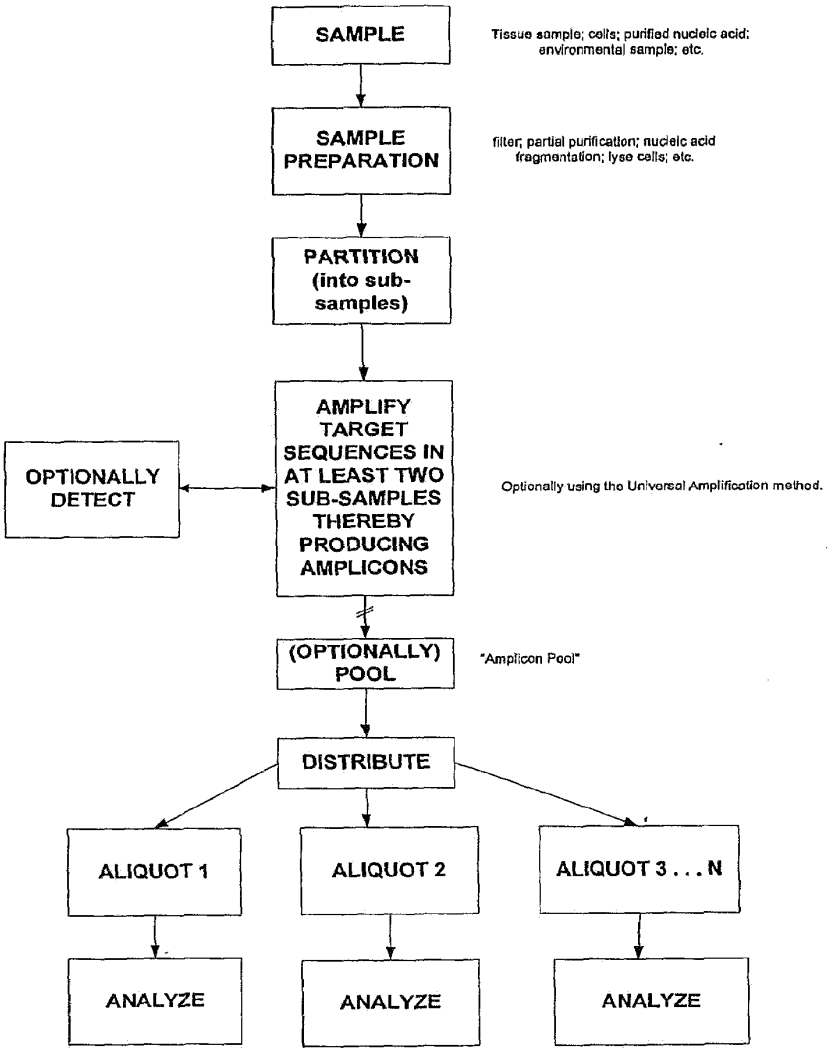


Figure 2

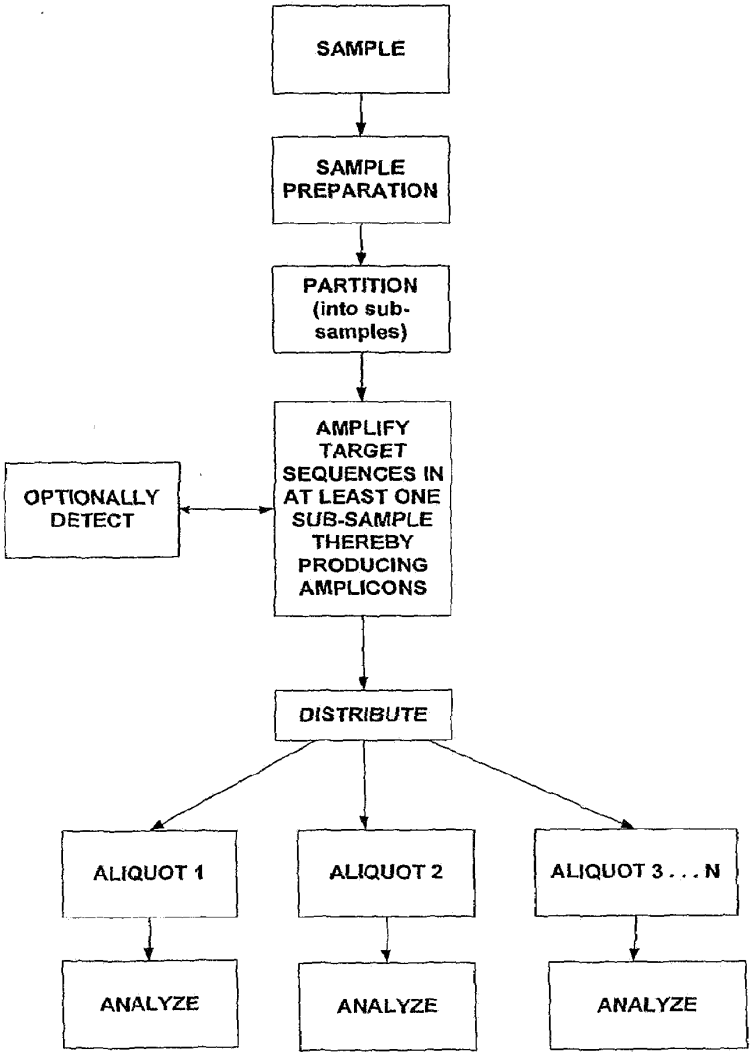
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Figure 3A



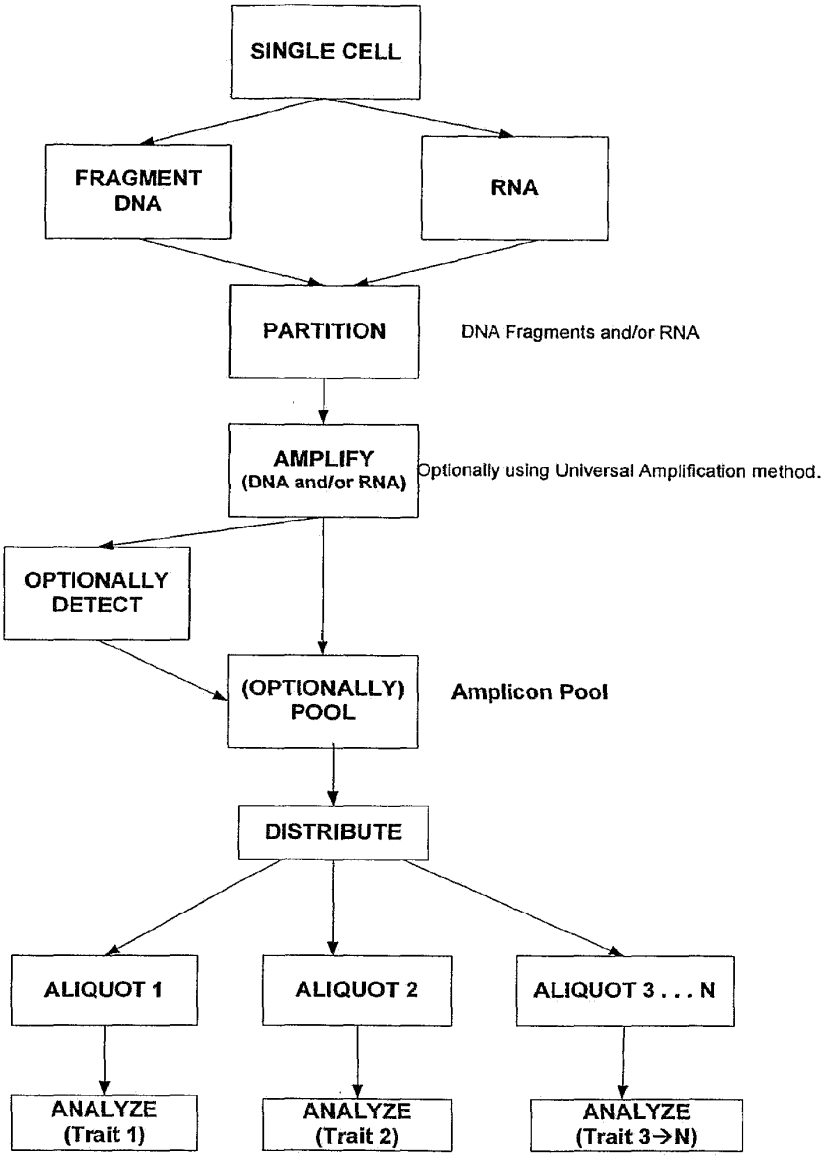
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Figure 3B



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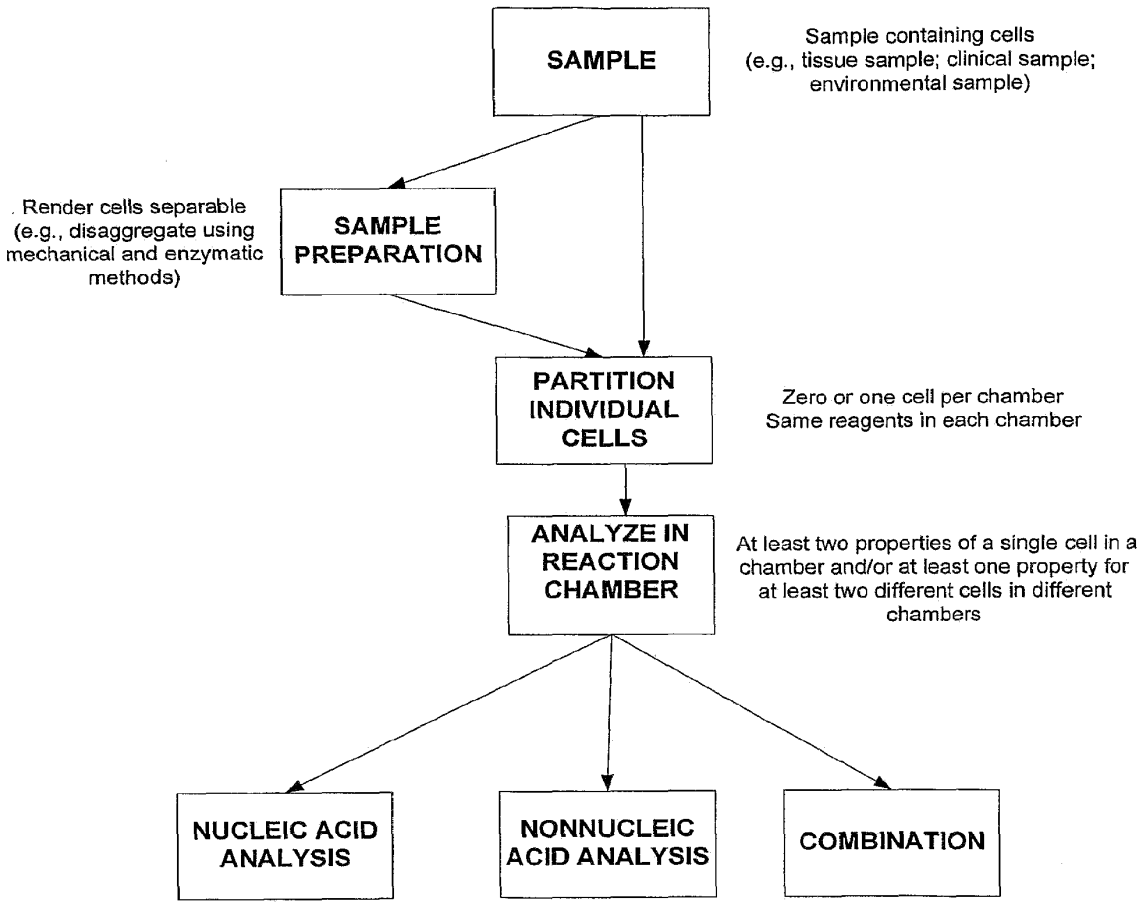
Figure 3C



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Figure 4

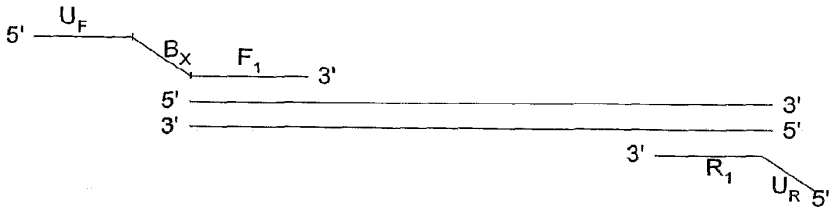
Partition of Cells



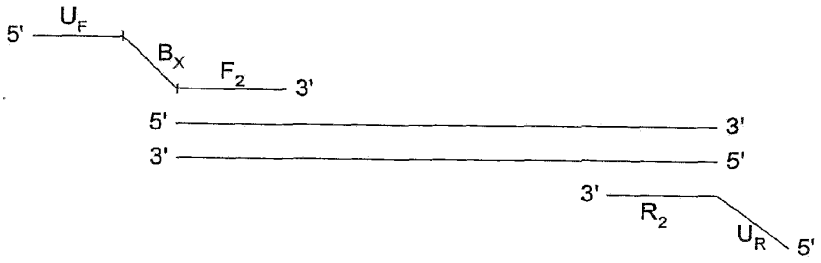
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Figure 5

Target 1



Target 2



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(54) Title: ABSOLUTE PCR QUANTIFICATION

(57) Abstract: The present application provides methods and devices for absolute quantification of polymerase chain reaction target nucleic acids. In particular, the methods and devices of the present application provide for splitting a nucleic acid sample to be analyzed into small, isolated volumes, conducting the method of polymerase chain reaction (PCR) on said volumes, detecting PCR amplification products, analyzing said detected PCR amplification products, performing absolute quantification of the PCR target and presenting said quantification results.

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ABSOLUTE PCR QUANTIFICATION

The present application claims priority to Unites States Provisional Application Number 60/725,899, filed October 12, 2005, which is incorporated herein by reference.

FIELD OF THE INVENTION

The present application provides methods and devices for absolute quantification of polymerase chain reaction target nucleic acids. In particular, the methods and devices of the present application provide for splitting a nucleic acid sample to be analyzed into small, isolated volumes, conducting the method of polymerase chain reaction (PCR) on said volumes, detecting PCR amplification products, analyzing said detected PCR amplification products, performing absolute quantification of the PCR target and presenting said quantification results.

BACKGROUND OF THE INVENTION

Real-time PCR has become the method of choice in various gene-related applications because it is conceptually straightforward, flexible and sensitive while generating quantitative data (Heid et al., 1996, Genome Res. 6:986). Its applicability as an important diagnostic tool has been demonstrated in many clinical applications (*e.g.*, Bustin, 2002, J. Mol. Endo. 29:23). However, its inherent wide variability of results (Reinhold et al., 2001, J. Clin.Onc. 19:1723) still makes it unreliable in clinical diagnostics. The major drawback is that all known real-time PCR methods (and instruments) have not yet achieved the levels of sensitivity, fidelity, accuracy, reproducibility and resolution necessary for true absolute quantification of low abundance targets. Moreover, new reagents, chemistries and instruments continuously introduced into the real-time PCR field make it increasingly difficult to compare results obtained in different laboratories and at different times (Bustin, 2002).

Quantification of an amplified target in "real-time" PCR is based on measuring the reaction product by sampling its fluorescence in the reaction mix during the course of the amplification reaction, generally at each cycle (Heid et al., 1996). The fluorescence gives a measure of the reaction's kinetics, which allows building a

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kinetic curve for the reaction and assessing a linearity range for the reaction (*e.g.*, in logarithmic scale). In order to estimate the amount of the target nucleic acid prior to amplification (*e.g.*, the number of original copies of the target, prior to amplification), one only needs a calibration curve, which is built from a series of kinetic curves recorded for the target nucleic acid at known target dilutions. The calibration curve is then used to estimate the concentration of target in unknown samples, on the assumption that the reaction kinetics (the efficiencies of amplifying the target materials) for each calibration dilution are the same, and that they are equal to those in the experimental sample. This in turn requires the calibration curve to be linear on a logarithmic scale.

Quantification of the real-time PCR is centered on the assumption that the fluorescence recorded at each cycle represents the amount of the amplified target DNA. Fluorescence of a sample is normalized to that of an internal reference fluorophore to correct for fluctuations in volume and/or concentration and recorded as a point on a kinetic curve. The point at which the fluorescent signal is first recorded as statistically significant above the background is defined as threshold cycle (Ct). It is assumed that Ct occurs during the exponential phase of the PCR, so that quantification is not affected by biases of the plateau phase. The Ct value is reported for each sample and then made into a quantitative result, *e.g.*, the initial target concentration or number of target copies is estimated by comparison to an external calibration (or "standard") curve.

An inherent problem when using a calibration or standard curve is that the reaction saturates very quickly, and background fluorescence in the absence of amplification is typically very high. Early detection is precluded by high background fluorescence that occursin the absence of amplification. This is because the level of noise associated with the background is higher than the useful signal generated by the PCR product on early cycles, which makes the PCR product effectively undetectable at the beginning of the reaction. Also, for the product quantification using a calibration or standard curve, it is assumed that during the early amplification cycles the reaction efficiency (*i.e.*, fold increase per cycle) is the same as in later cycles.

There are currently two major approaches to PCR quantification: relative and absolute. Relative quantification is used to determine the changes in nucleic acid levels (*e.g.*, differences between samples of different kinds, or in cells that have been

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differently treated). For a relative calibration curve, a series of dilutions of a calibrator sample are used. The calibrator can be any nucleic acid with known concentration and amplicon length. During PCR amplification, Ct numbers for the calibrator dilutions are detected and plotted against arbitrary units. The target Ct numbers are applied directly to the calibration curve, and the result is expressed as fold increase or decrease relative to the reference measurement.

In contrast, absolute quantification seeks to determine the number of target copies initially present in a sample. It requires the building of an absolute calibration curve for each individual amplicon as a measure of the efficiencies of amplification during all amplification steps, including the reverse transcription step (*e.g.*, when performing reverse transcription PCR, RT-PCR) and during PCR amplification. Since the standard template must be pure (*i.e.*, a standard free from non-target RNA/DNA contamination), it is usually prepared from recombinant DNA or RNA. Serial dilutions of the DNA/RNA standard are prepared, and each dilution is re-assayed in the same PCR run along with experimental samples and positive and negative controls. Ct values for each standard dilution are recorded and a standard curve is generated by plotting the Ct values against the logarithm of the initial copy numbers, which are inversely proportional to each other. The initial target copy number (TCN) of the experimental sample is calculated using a linear regression equation of that calibration curve and Ct values for the experimental sample. Because of the sample-to-sample variations in the amount of starting material, especially in clinical samples, the results are normalized by tissue mass, cell number, or nucleic acid amount (*e.g.*, total DNA or RNA, ribosomal RNA, or cDNA/mRNA of, for example, housekeeping genes) (Bustin, 2002; Bustin, 2002, J. Mol. Endo. 25:169).

Although the objective is to identify a precise initial TCN, absolute quantification of the real-time PCR method has a principle flaw in that it is based on an external calibration. The external calibration requires ideal validation of identical reaction kinetics (*i.e.* amplification efficiencies) for the calibrator template and the target template (Pfaffl and Hageleit, 2001, Biotech.Lett. 23:275; Klein, 2002, Trends Mol. Med. 8:257). Such validation is extremely difficult, even for ideal samples, let alone experimental and clinical samples from different laboratories and testing sites, different tissues, animals and patients. In addition, clinical samples can vary widely

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in purity, making it very difficult to provide truly identical reaction conditions for both the purified control and the clinical sample.

The accuracy of the absolute quantification method depends heavily on a few critical assumptions. The first assumption is that targets and calibrators are amplified with the same efficiency. Another assumption is that the efficiencies for calibrator and target detected at Ct are identical to those in the earlier cycles. However, clinical samples often contain contaminants and/or inhibitors that reduce the efficiency of the amplification reaction compared to the pristine samples used as calibrator templates. In addition, inhibitors appear to have a stronger effect in the earlier cycles (they may degrade, *e.g.*, by exposure to the extreme temperature of PCR, by the later cycles), which then results in underestimation of the copy number for a reaction. The exponential nature of the PCR, combined with a small number of the target molecules leads to a situation where small variations in efficiencies during the early cycles causes great variations of the final yield of the amplified product (Bustin, 2002). As a result, PCR-based quantification is often characterized by significant variations and non-reproducibility. Therefore, a large variety of enzymes, primers, and test samples, and the absence of acceptable validation and normalization procedures leads to poor reproducibility of data in different laboratories and raises serious doubts about how quantitative, reproducible or statistically informative real-time PCR is (*e.g.*, Bustin 2002). Therefore, the usefulness and reliability of using quantitative real-time PCR as a routine clinical diagnostic is questionable (Bustin 2000, 2002; Klein 2002; Pfaffl and Hageleit, 2001).

PCR is, to date, the best method for detecting low abundance nucleic acid molecules. However, the statistics of particle distribution predicts that quantification of small numbers of molecules makes Ct data less reproducible due to stochastic effects (Rasmussen, R (2001) Quantification on the LightCycler. In: Meuer, S, Wittwer, C, Nakagawara, K, eds. Rapid Cycle Real-time PCR, Methods and Applications. Springer Press, Heidelberg; p. 21-34) which, in combination with all the other drawbacks as previously listed, makes quantification of the low abundance target even less reliable.

There exists a PCR-based method to assess directly the small numbers of DNA/RNA molecules. The approach is based on competitive end-point PCR of multiple sub-microliter samples containing two terminally diluted targets labeled with

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different fluorescent markers excitable at different wavelengths (*e.g.*, a control target of known TCN and an unknown target of unknown TCN). Following PCR amplification, the samples undergo relative quantification of the two targets (*e.g.*, either by capillary electrophoresis, Lagally et al., 2001, Anal. Chem. 73:565; Lagally et al., 2001, Lab on a Chip, 1:102, or fluorescence detection by confocal microscope, Kalinina et al., 1997, Nucl. Acids Res. 25:1999). The distribution function of the amplified product is analyzed using Poisson statistics best-fit method. Although the described approach enables a statistically significant absolute quantification of the TCN in the original sample and does not require any external calibration, it relies on the relative quantification of the control and the target components after the end-point PCR. This by itself lowers the quantification accuracy and makes the method both time and labor consuming. This approach has been utilized to demonstrate single copy PCR amplification (Lagally et al., 2001; Kalinina et al., 1997). It has not become a routine quantitative method because, in order to collect statistics sufficient for accurate target quantification, it requires rather complicated sample handling and the detection of hundreds of separate PCR amplification reactions.

Efforts in improving quantitative PCR also include the desire to minimize the time-to-result and the cost per result. Approaches generally include decreasing the amplification reaction volume and at the same time increasing the number of reactions that are performed simultaneously (*e.g.*, by use of high throughput systems). The microchamber array for PCR amplification described by Nagai et al. (2001, Biosens. Bioelec. 16:1015) demonstrated that high-throughput, rapid, synchronous amplification of multiple amplification targets was possible. The PicoTiterPlate™ as described by Leamon et al., 2003, Electophoresis 24:3769, further demonstrated that amplification using low reaction volumes (as low as 39.5 µl) and low starting template copy number (calculated as 5 copies of template per reaction) in a high throughput format (up to 300,000 discrete reactions on one plate) was possible. Leamon goes one step further and describes his method as being a method for quantitative PCR. However, the quantitative PCR of Leamon is a relative and not absolute quantification, in that a standard curve was generated and fluorescence readings were applied to the curve to define amplicon fold increase in an end-point PCR assay.

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The BioMark™ system developed by Fluidigm Corporation (San Francisco, CA) advertises a nanofluidic chip reported to absolutely quantify target nucleic acids (Application Marketing Note MRKT00047.VD). The literature describes constant amplification monitoring via fluorescence detection, such that the system is a real-time PCR system. However, when calculations are performed on the data as described, mathematical analysis does not bear out the conclusions, *i.e.*, absolute quantification of the sample template was not, in fact, realized. The results as listed do not satisfy a Poisson distribution curve (*i.e.*, bell-shaped curve). For example, when taking the white counts found in Figure 2, 5pg DNA slide (count=310) and applying the Poisson probability equation, the λ parameter (*i.e.*, a shape parameter that indicates the average number of events in a given time period) is calculated to be λ=0.25, indicating that the white compartment counts found in Figure 2 should be around 273, which differs from the reported results by two standard deviations-outside the range of random probability. When calculating the λ parameter of the 1pg DNA slide (count=62), λ=0.50. Therefore, the disparity in the average number of events in a given time period and data reported that is actually outside the range of random probability demonstrates that, under the experimental conditions reported, absolute quantification of target nucleic acids has not occurred.

As such, the lack of automated, reliable, absolute quantification in nucleic acid amplification methods is a huge obstacle for introducing these methods in clinical diagnostics where the problems are multiplied by, for example, variations in samples from different patients, sample collection conditions, and technical loading errors. This variation makes it practically impossible to relate the results from, for example, different laboratories, methods, patients and tissues in terms of target copy number. Consequently, it is impossible to establish databases originating from PCR results obtained in various studies and proves to be a critical impediment for utilizing real time PCR as a clinical tool.

Therefore, what are needed are new methods and devices that provide approaches for high-throughput, highly accurate nucleic acid quantification such that direct, absolute quantification of target amplicons is realized. Such methods and devices will not only aide in bringing continuity and consistency to clinical diagnostics, but also serve as improved research tools for the scientific community as they perform the needed research to decipher the genes and related sequences that impact daily lives.

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SUMMARY OF THE INVENTION

The present application provides methods and devices for absolute quantification of target nucleic acids. In particular, the methods and devices of the present application provide for splitting a nucleic acid sample to be analyzed into small, isolated volumes, conducting the method of nucleic acid detection on said volumes, analyzing the detection results, performing absolute quantification of initial amount of target nucleic acid in the sample, and presenting said quantification results.

In one embodiment, the present invention provides for a method of counting target nucleic acid molecules comprising providing a sample containing a plurality of target nucleic acid molecules, applying said sample to a multiple vessel array wherein the multiple vessel array comprises a plurality of reaction vessels, wherein each of said reaction vessels receives an essentially equal sub-volume of the aforementioned sample, wherein the combined volumes of said sub-volumes defines a tested volume and wherein the number of said plurality of reaction vessels is comparable with the number of target nucleic acid molecules in said tested volume, treating said multi vessel array to conditions to produce a detectable signal in the presence of said target nucleic acid, and determining how many of said reaction vessels contain reactions producing said detectable signal. In some embodiments, the ratio of target molecules to reaction vessels is 10:1, 3:1, 2:1, 1:1, 0.5:1, 0.1:1 or 0.01:1. In some embodiments, said multiple vessel array comprise at least 100 reaction vessels, at least 500 reaction vessels, or at least 1000 reaction vessels. In some preferred embodiments, said multiple vessel array is a multiple capillary array, wherein said reaction vessels are capillary reaction vessels. In some preferred embodiments, the individual reaction vessels have volumes of less than 1 µl. In particularly preferred embodiments, the individual reaction vessels have volumes of less than 1 nanoliter.

In some embodiments, said determining comprises determining how many of said reaction vessels contain reactions that do not produce said detectable signal.

In some embodiments, said conditions comprise exposure of said target nucleic acid to reagents for producing a detectable signal in the presence of said target nucleic acid. In some embodiments, the sample further comprises the nucleic acid detection reagents. In some embodiments, said multiple vessel array further comprises said reagents. In some preferred embodiments, the multiple vessel array comprises the reagents and the reagents are dried prior to exposure to a liquid sample.

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In some preferred embodiments, the reagents are reagents for performing a nucleic acid amplification reaction. In particularly preferred embodiments, the nucleic acid amplification reaction is a polymerase chain reaction. In some embodiments, said determining is conducted after the completion of a polymerase chain reaction.

In some embodiments, the detectable signal comprises fluorescence. In some embodiments, the reagents comprise an oligonucleotide probe comprising a fluorescent moiety. In some preferred embodiments, the oligonucleotide probe further comprises a quencher moiety. In some embodiments, the reagents comprise a nucleic acid intercalating dye.

In some embodiments, the polymerase chain reaction is conducted for a predetermined number of cycles, wherein said determining is conducted after completion of the predetermined number of cycles.

In some embodiments, the number of target molecules is calculated using the equation:

$$M_0 = N_{\text{sub-volumes}} \times \left[-\ln(P)\right]$$

wherein M_0 is the number of target molecules in said tested volume,

wherein N is the number of sub-volumes, and

wherein P is the percent of sub-volumes lacking the detectable signal.

In some embodiments, a standard deviation for the number of target molecules in a tested volume is calculated using the equation:

$$\sigma_N = \sqrt{N_{sub-volumes} P(1-P)}$$

wherein σ_N is the standard deviation.

In some embodiments, the sample is a test sample, and the method further comprises providing a negative control sample lacking said target nucleic acid, wherein said negative control sample is tested in parallel and is treated identically to said test sample, wherein a Poisson parameter for said test sample is estimated using the equation:

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$$\lambda_{EST} = \ln\left[P_{CONTROL}(0) / P_{SAMPLE}(0)\right]$$

wherein

λ_{EST} is the estimated Poisson parameter for said test sample

$P_{CONTROL}(0)$ is the percent of reaction vessels lacking signal in the control negative assay

$P_{SAMPLE}(0)$ is the percent of reaction vessels lacking signal in said test DNA sample.

In some embodiments, the method further comprises communication of the number of capillary reaction vessels in which it is determined that the reagents produced no detectable signal to a computer, wherein the calculations described above are performed by the computer.

In one embodiment, the present invention provides a device for testing a nucleic acid sample wherein said device comprises a multiple capillary array comprising a first plurality of capillary reaction vessels, wherein each of said first plurality of capillary reaction vessels is configured to receive an essentially equal sub-volume of an applied liquid sample. In some embodiments, said capillary reaction vessels are essentially parallel to each other. In some embodiments, said device further comprises a filling groove and at least one vent groove, wherein said filling groove is fluidically connected to each of said capillary reaction vessels in said first plurality of capillary reaction vessels. In some embodiments, said multiple capillary array comprises one or more additional pluralities of capillary reaction vessels wherein each of the additional pluralities of capillary reaction vessels is fluidically connected to a filling groove to which no other pluralities of capillary reaction vessels are connected. In some embodiments, said pluralities of capillary reaction vessels are separated from one another by zone separators.

In some embodiments, said device further comprises a component for temperature cycling said capillary reaction vessels. In some embodiments, said device further comprises a fluorescence detector. In some embodiments, said device further comprises a computer operably linked to said fluorescence detector. In further embodiments, the fluorescence detector is configured to detect fluorescence during temperature cycling, while in other embodiments, the fluorescence detector is

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configured to detect fluorescence only when the component is not temperature cycling.

In one embodiment, the present invention provides a method of sampling a target nucleic acid, comprising providing a sample tray comprising a plurality of isolated reaction zones, wherein each isolated reaction zone contains a nucleic acid binding agent having a specific nucleic acid binding rate, and wherein the binding rates of the binding agents in different isolated reaction zones are variable binding rates, exposing the sample tray to a sample comprising target nucleic acid under conditions wherein each isolated reaction zone is exposed to said sample for essentially the same amount of time, removing unbound target nucleic acid from said sample tray, and detecting the target nucleic acid in said isolated reaction zones. In some embodiments, said variable binding rates are serial binding rates. In further embodiments, the serial binding rates differ between isolated reaction zones by factors of 2, by factors of 5, by factors of 10, by factors of 100, or by factors of 1000.

In some embodiments, said nucleic acid binding agent comprises a gel matrix. In some embodiments, the specific nucleic acid binding rate of said gel matrix is determined by the rate at which nucleic acid diffuses through said gel matrix. In some embodiments, the gels matrices in said different isolated reaction zones have different gel porosities. In some embodiments, the gels matrices in said different isolated reaction zones have different gel volumes.

In one embodiment, the present invention provides a method of sampling a target nucleic acid comprising providing a sample tray comprising a plurality of isolated reaction zones wherein each isolated reaction zone contains a nucleic acid binding agent having a specific nucleic acid binding capacity and wherein the binding capacities of the binding agents in different isolated reaction zones are variable binding capacities, exposing the sample tray to a sample comprising target nucleic acid under conditions wherein said binding agent binds to said target nucleic acid, removing unbound target nucleic acid from said sample tray, and detecting the target nucleic acid in said isolated reaction zones. In some embodiments, said variable binding capacities are serial binding capacities. In some embodiments, the serial binding capacities differ between isolated reaction zones by factors of 2, by factors of 5, by factors of 10, by factors of 100, or by factors of 1000.

In one embodiment, the present invention provides a method of sampling a target nucleic acid comprising providing a sample tray comprising a plurality of

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isolated reaction zones wherein each isolated reaction zone defines a specific reaction sub-volume and wherein the reaction sub-volumes of said different isolated reaction zones are variable reaction sub-volumes, exposing the sample tray to a sample comprising one or more target nucleic acid molecules under conditions wherein sample fills said plurality of isolated reaction zones, and detecting the target nucleic acid in said isolated reaction zones. In some embodiments, said variable reaction sub-volumes are serial reaction sub-volumes. In some embodiments, the serial reaction sub-volumes differ between isolated reaction zones by factors of 2, by factors of 5, by factors of 10, by factors of 100, or by factors of 1000.

In some embodiments, said sample comprises reagents for a nucleic acid detection reaction. In further embodiments, said detecting comprises performing a nucleic acid detection reaction. In some embodiments, said nucleic acid detection reaction comprises a nucleic acid amplification reaction. In some embodiments, said nucleic acid amplification reaction is a polymerase chain reaction. In some embodiments, said detecting is conducted after the completion of said polymerase chain reaction. In further embodiments, said detecting for said sampling a target nucleic acid comprises detection of fluorescence. In some embodiments, said reagents comprise an oligonucleotide probe comprising a fluorescent moiety. In some embodiments, said oligonucleotide probe further comprises a quencher moiety. In some embodiments, said reagents comprise a nucleic acid intercalating dye. In some embodiments, said polymerase chain reaction is conducted for a predetermined number of cycles and said detecting is conducted after completion of said predetermined number of cycles.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows a characterization of PCR products (gray circles) obtained in small isolated sub-volumes from different number of target copies (black circles).

Figures 2A and B show real-time PCR curves (fluorescence on the y-axis vs. PCR cycle number on the x-axis) recorded from positive or "YES" (A) and negative, or "NO" (B) cells.

Figured 3A, B and C demonstrate typical PCR curves; (A) of different starting template copy number (fluorescence on the y-axis vs. PCR cycles number on the x-axis), (B) a histogram of fluorescence signal with fluorescence minus background B

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(x-axis) detected in individual sub-volumes (y-axis), and (C) a Poisson distribution of initial copy numbers (ONTMs) in individual sub-volumes for average λ=1.

Figures 4A, B and C show Monte-Carlo simulations of detecting no target in k of N sub-volumes for mean copy number λ= 0.5, 1, 1.5, and 2. Distribution of 'NO' results, N=96 (A), distribution of estimated λ_{EST}, N=96 (B), and 95% range depending on number of trials N (C).

Figure 5 graph shows the difference between the PCR detection threshold cycle C_{T0} for the original PCR sample and the detection threshold cycle $C_{Tsub\text{-}vol}$ for the sample subdivided into small sub-volumes. Note that when M_0 is smaller than the number of sub-volumes, the difference in detection threshold can be very significant. The graph shows that the lower the number of original DNA molecules M_0 the greater is the increase in the sensitivity of the PCR detection.

Figure 6 illustrates an embodiment of the present invention. The template sample is diluted in PCR mix containing fluorescent labels and distributed on the substrate between small-volume cells. It is done so that the average number of target copies per one cell λ is low. The substrate undergoes thermal cycling. Cells on the substrate are illuminated by an illumination system (*e.g.*, laser) and emitted fluorescence image of the device is projected through the optical system onto multi-pixel photodetector (*e.g.*, CCD, CMOS detector, PMT, or APD array) and recorded by the computer system. Statistical analysis of "+" and "-" events yields λ, from which ONTM is calculated and reported.

Figure 7 illustrates one embodiment of the present invention. For example, a monolith multi-capillary array with nanoliter compartments is shown. PCR zones consist of capillary tubes for target samples that are bordered on either side by filling and vent grooves and are separated from adjacent capillary tubes and grooves by zone separators for separation of target samples.

Figure 8 illustrates one embodiment of the present invention. For example, a disposable sample tray for PCR amplification in polonies is shown. Arrays of gel micro-pads (*e.g.*, gel-substrates) are deposited in the individual compartments of a plastic sample tray. Said arrays may differ both in volumes of individual pads and in composition (porosity) of gels of which the pads are formed. DNA samples are loaded in the compartments of the tray, the tray is covered by a lid, and thermal cycling using an end-point cycler is performed. The sample tray is made of scannable

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optically transparent plastic such that scanning can be performed from above or below with a laser beam or LED arrays.

Figure 9 demonstrates that the calculation of the number of polonies based on the count of empty cells as described herein yields a more accurate polony count than the manually counted (observed count) polonies as performed in Metra and Church (1999, Nucl. Acids Res. 27:e34). For example, comparing the left panel plot and m_1 values obtained through calculated Poisson statistics to observed polonies for the number of template molecules (graph) shows a discrepancy between the the polonies counted and those calculated. The discrepancy between the number of template DNA molecules and the observed (counted) number of polonies increases with an increase in the concentration of template DNA, as shown when comparing the 180 and 360 template molecule panel calculations with those observed as plotted in the graph.

Figure 10 diagrams steps for PCR on beads immersed in a liquid polymer. Beads with attached DNA are put into a vial with PCR mix diluted in liquid polymer having a certain DNA diffusion coefficient. Conditions are selected to assure that concentration of beads is such that the average distance between beads significantly exceeds the possible diffusion distance of amplified DNA segments in the PCR mix diluted in liquid polymer. After PCR is completed, the beads with DNA samples from the vial are loaded into a capillary and PCR product is detected. Determined percent P of beads that yielded no PCR amplification allows calculation of pre-PCR number of DNA molecules M_0 using statistical analysis.

Figure 11 demonstrates an embodiment of the present invention. For example, manufacture and characteristics of monolith multi-capillary arrays is illustrated; a) shows a general flow scheme of how a multiple micro-capillary array (MMCA) substrate is created, b) shows the general view of the capillary tubes, (c) shows the capillary tube array in cross section; d) and f) shows top view fluorescence of 16- and 32-capillary arrays; and (e) shows a 32-capillary array with opened filling V-groove.

DEFINITIONS

As used herein, the term "nucleic acid molecule" refers to any nucleic acid containing molecule, including but not limited to, DNA or RNA. The term encompasses sequences that include any of the known base analogs of DNA and RNA

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including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudo-uracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methyl-cytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-amino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

As used herein, the term "Poisson statistics", or "Poisson distribution" describes, through mathematics, a discrete probability distribution. It expresses the probability of a number of events occurring in a fixed period of time if these events occur with a known average rate and are independent of the time since the last event. For example, the present invention presents devices and methods for performing and calculating the original number of template molecules in a PCR reaction that satisfy Poisson statistics, thereby yielding absolute quantification of the original number of template molecules present at the beginning of an amplification reaction.

The term "target," when used in reference to a nucleic acid detection or analysis method, refers to a nucleic acid having a particular sequence of nucleotides to detected or analyzed, *e.g.*, in a sample suspected of containing the target nucleic acid. When used in reference to the polymerase chain reaction, "target" generally refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences that may be present in a sample. A "segment" is defined as a region of nucleic acid within the target sequence. The term "sample template" refers to nucleic acid originating from a sample that is analyzed for the presence of a target.

As used herein, the term "genotype" refers to the actual genetic make-up of an organism (*e.g.*, in terms of the particular alleles carried at a genetic locus). Expression of the genotype gives rise to an organism's physical appearance and characteristics–the "phenotype."

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As used herein, the term "locus" refers to the position of a gene or any other characterized sequence on a chromosome.

As used herein the term "disease" or "disease state" refers to a deviation from the condition regarded as normal or average for members of a species, and which is detrimental to an affected individual under conditions that are not inimical to the majority of individuals of that species (*e.g.*, diarrhea, nausea, fever, pain, and inflammation etc).

As used herein, the term "treatment" in reference to a medical course of action refer to steps or actions taken with respect to an affected individual as a consequence of a suspected, anticipated, or existing disease state, or wherein there is a risk or suspected risk of a disease state. Treatment may be provided in anticipation of or in response to a disease state or suspicion of a disease state, and may include, but is not limited to preventative, ameliorative, palliative or curative steps. The term "therapy" refers to a particular course of treatment.

The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide, RNA (*e.g.*, rRNA, tRNA, etc.), or precursor. The polypeptide, RNA, or precursor can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (*e.g.*, ligand binding, signal transduction, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene and the including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb on either end such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' untranslated sequences. The sequences that are located 3' or downstream of the coding region and that are present on the mRNA are referred to as 3' untranslated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments included when a gene is transcribed into heterogeneous nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are generally absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of

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amino acids in a nascent polypeptide. Variations (*e.g.*, mutations, SNPS, insertions, deletions) in transcribed portions of genes are reflected in, and can generally be detected in corresponding portions of the produced RNAs (*e.g.*, hnRNAs, mRNAs, rRNAs, tRNAs).

Where the phrase "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, amino acid sequence and like terms, such as polypeptide or protein are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences that are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers that control or influence the transcription of the gene. The 3' flanking region may contain sequences that direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or gene product that has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the terms "modified," "mutant," and "variant" refer to a gene or gene product that displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. In this case, the DNA sequence thus codes for the amino acid sequence.

The term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, that describe a method

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for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing, and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" ("PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified" and are "PCR products" or "amplicons."

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ^{32}P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment, fluorescently labeled primers, incorporation of fluorophore into PCR products during the amplification process or after cycling is completed). In addition to genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

The terms "PCR product," "PCR fragment," and "amplification product" or "amplicon" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms

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encompass the case where there has been amplification of one or more segments of one or more target sequences.

As used herein, the term "real-time PCR" is used to describe the detection of amplification products throughout the amplification process. For example, it may be desirable to measure the accumulation of PCR products after each cycle, or after selected numbers of cycles during the ongoing reaction (*e.g.*, after 4 cycles, after 6 cycles, after 8 cycles, *etc.*). Using real time PCR, the progress and efficiency of the amplification reaction can be monitored while it is occurring.

As used herein, the term "end point PCR" is used to describe the detection of the amplification products at the end of a PCR assay. For example, if a PCR assay was designed to contain 30 PCR cycles, an end point PCR assay would detect the accumulation of amplification products after the 30 cycles has been completed. Using end point PCR, accumulation of amplicons is determined only when PCR is completed.

As used herein, the term "amplification reagents" refers to those reagents (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel, for example a device of the present invention.

The term "reverse-transcriptase" or "RT-PCR" refers to a type of PCR where the starting material is mRNA. The starting mRNA is enzymatically converted to complementary DNA or "cDNA" using a reverse transcriptase enzyme. The cDNA is then used as a "template" for a "PCR" reaction.

The term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer is sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

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As used herein, the term "gel substrate" refers to a substrate that is capable of allowing the diffusion of nucleic acid molecules, while allowing for PCR to occur within the substrate. For example, a gel substrate of the present invention could be gelatin, agarose, Matrigel®, collagen, and other types of polymers capable of allowing for nucleic acid diffusion and PCR amplification.

As used herein, the term "probe" or "hybridization probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, that is capable of hybridizing, at least in part, to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular sequences. In some preferred embodiments, probes used in the present invention will be labeled with a "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

The term "sample" as used herein is used in its broadest sense. For example, a sample suspected of containing a human gene or chromosome or sequences associated with a human chromosome may comprise a cell, chromosomes isolated from a cell (*e.g.*, a spread of metaphase chromosomes), genomic DNA (in solution or bound to a solid support such as for Southern blot analysis), RNA (in solution or bound to a solid support such as for Northern blot analysis), cDNA (in solution or bound to a solid support) and the like.

The term "signal" as used herein refers to any detectable effect, such as would be caused or provided by a label or an assay reaction.

As used herein, the term "distinct" in reference to signals refers to signals that can be differentiated one from another, *e.g.*, by spectral properties such as fluorescence emission wavelength, color, absorbance, mass, size, fluorescence polarization properties, charge, etc., or by capability of interaction with another moiety, such as with a chemical reagent, an enzyme, an antibody, etc.

As used herein, the term "detector" refers to a system or component of a system, *e.g.*, an instrument (*e.g.* a camera, fluorimeter, charge-coupled device, scintillation counter, *etc.*) or a reactive medium (X-ray or camera film, pH indicator, *etc.*), that can convey to a user or to another component of a system (*e.g.*, a computer

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or controller) the presence of a signal or effect. A detector can be a photometric or spectrophotometric system, which can detect ultraviolet, visible or infrared light, including fluorescence or chemiluminescence; a radiation detection system; a spectroscopic system such as nuclear magnetic resonance spectroscopy, mass spectrometry or surface enhanced Raman spectrometry; a system such as gel or capillary electrophoresis or gel exclusion chromatography; or other detection system known in the art, or combinations thereof.

.The term "detection" as used herein refers to quantitatively or qualitatively identifying an analyte (*e.g.*, DNA, RNA or a protein) within a sample. The term "detection assay" as used herein refers to a kit, test, or procedure performed for the purpose of detecting an analyte nucleic acid within a sample. Detection assays produce a detectable signal or effect when performed in the presence of the target analyte, and include but are not limited to assays incorporating the processes of hybridization, nucleic acid cleavage (*e.g.*, exo- or endonuclease), nucleic acid amplification, nucleotide sequencing, primer extension, or nucleic acid ligation.

As used herein, the term "detection assay component" refers to a component of a system capable of performing a detection assay. Detection assay components include, but are not limited to, hybridization probes, buffers, and the like.

As used herein, the term "a detection assay configured for target detection" refers to a collection of assay components that are capable of producing a detectable signal when carried out using the target nucleic acid. For example, a detection assay that has empirically been demonstrated to detect a particular single nucleotide polymorphism is considered a detection assay configured for target detection.

As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (*e.g.*, oligonucleotides, enzymes, etc. in the appropriate containers) and/or supporting materials (*e.g.*, buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (*e.g.*, boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the term "fragmented kit" refers to a delivery systems comprising two or more separate containers that each contain a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second

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container contains oligonucleotides. The term "fragmented kit" is intended to encompass kits containing Analyte specific reagents (ASR's) regulated under section 520(e) of the Federal Food, Drug, and Cosmetic Act, but are not limited thereto. Indeed, any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included in the term "fragmented kit." In contrast, a "combined kit" refers to a delivery system containing all of the components of a reaction assay in a single container (*e.g.*, in a single box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

As used herein, the term "information" refers to any collection of facts or data. In reference to information stored or processed using a computer system(s), including but not limited to internets, the term refers to any data stored in any format (*e.g.*, analog, digital, optical, etc.). As used herein, the term "information related to a subject" refers to facts or data pertaining to a subject (*e.g.*, a human, plant, or animal). The term "genomic information" refers to information pertaining to a genome including, but not limited to, nucleic acid sequences, genes, allele frequencies, RNA expression levels, protein expression, phenotypes correlating to genotypes, etc. "Allele frequency information" refers to facts or data pertaining allele frequencies, including, but not limited to, allele identities, statistical correlations between the presence of an allele and a characteristic of a subject (*e.g.*, a human subject), the presence or absence of an allele in a individual or population, the percentage likelihood of an allele being present in an individual having one or more particular characteristics, etc.

As used herein, the term "reaction vessel" refers to a system in which a reaction may be conducted, including but not limited to test tubes, wells, microwells (*e.g.*, wells in microtitre assay plates such as, 96-well, 384-well and 1536-well assay plates), capillary tubes, ends of fibers such as optical fibers, microfluidic devices such as fluidic chips, cartridges and cards (including but not limited to those described, *e.g.*, in US Patent No. 6,126,899, to Woudenberg, *et al.*, U.S. Patent Nos. 6,627,159, 6,720,187, and 6,734,401 to Bedingham, *et al.*, U.S. Patent Nos. 6,319,469 and 6,709,869 to Mian, *et al.*, U.S. Patent Nos. 5,587,128 and 6,660,517 to Wilding, *et al.*), or a test site on any surface (including but not limited to a glass, plastic or silicon surface, a bead, a microchip, or an non-solid surface, such as a gel or a dendrimer).

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As used herein, the terms "computer memory" and "computer memory device" refer to any storage media readable by a computer processor. Examples of computer memory include, but are not limited to, RAM, ROM, computer chips, digital video disc (DVDs), compact discs (CDs), hard disk drives (HDD), and magnetic tape.

As used herein, the term "computer readable medium" refers to any device or system for storing and providing information (*e.g.*, data and instructions) to a computer processor. Examples of computer readable media include, but are not limited to, DVDs, CDs, hard disk drives, magnetic tape and servers for streaming media over networks.

As used herein, the terms "processor" and "central processing unit" or "CPU" are used interchangeably and refers to a device that is able to read a program from a computer memory (*e.g.*, ROM or other computer memory) and perform a set of steps according to the program.

As used herein, the term "fluidic connection" refers to a continuous fluid path between components, *e.g.*, between a filling groove and a reaction vessel such as a capillary reaction vessel.

As used herein, the term "parallel" in reference to processes, reactions, *etc.*, refers to processes or actions functioning in an essentially simultaneous, side-by-side, manner (*e.g.*, parallel PCR reactions).

As used herein, the term "parallel" as used in reference to a plurality of physical components or objects refers to the geometrical relationship between the objects. For example, a capillary tube or vessel having an essentially linear form is said to be parallel to a second capillary of the same form when the pair of capillaries are oriented in the same direction, such that they are geometrically parallel to each other.

As used herein, the term "multiple vessel array" refers to a collection of reaction vessels configured for simultaneous processing of multiple sub-volumes of a sample, *e.g.*, for conducting multiple detection reactions on sub-volumes of the same sample, in parallel (at the same time, under the same conditions). A multiple vessel array is further configured to distribute said sample into each of said reaction vessels, *e.g.*, through fluidic connection to a sample loading system such as a filling groove, such that a single application of sample to the array can fill each vessel in said multiple vessel array. The multiple vessel array is not limited to any particular number or configuration of vessels, *e.g.*, it may comprise, 2, 10, 100, 1000, or any

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other number greater than 2. A single device may comprise more than one multiple vessel array. For example, a fluidic chip or slide may comprise, *e.g.*, 2 or more multiple vessel arrays, each of which is served by its own filling system.

As used herein a "multiple capillary array" refers to multiple vessel array, wherein the reaction vessels of the array are capillary vessels.

As used herein, the term "sub-volume" as used in reference to a sample or a reaction, refers to a partitioned portion of a sample or vessel. For example, in a multiple vessel array, each of the reaction vessels in the array defines a sub-volume of the total volume in the array. As used in reference to a sample or a reaction, *e.g.*, a nucleic acid detection reaction on a test sample, the term sub-volume refers to a partitioned portion of the reaction, wherein the combined volumes of all the sub-volumes is the total volume of the test reaction on that sample. The total reaction volume is referred to as the "test volume" for that sample. Different sub-volumes of a test volume may be of different volume, or they may be of equal volume.

As used herein, the term "sampling" as used in reference to samples or reactions refers to taking from or partitioning a portion of a sample. For example, sampling a solution containing target nucleic acid molecules may comprise taking or partitioning a liquid portion of the sample, or it may comprise taking or partitioning a portion of the nucleic acid in said sample (*e.g.*, by capturing a sample of the nucleic acid from said solution).

As used herein, the term "sample tray" is used in its broadest sense to refer to any system or substrate wherein a plurality of partitioned reactions may be conducted, including but not limited to microtitre assay plates, microwell slides, fluidic chips, fluidic cartridges and cards, *etc.* A sample tray is not limited by the nature of the partition, which may comprise closed or opened physical containment (*e.g.*, walls, wells, tubes, *etc.*) or chemical containment (*e.g.*, in emulsions or suspensions, by use of hydrophobic or hydrophilic zones) or containment by semi-solid containment, *e.g.*, by use of media or components that slows movement, *e.g.*, by restricting diffusion, such as gels or polymers.

As used herein, the term "isolated reaction zone" refers to a portion of a sample tray wherein a reaction is effectively partitioned from other reactions.

As used herein, the term "binding agent," as used in reference to binding molecules, *e.g.*, nucleic acid, encompasses any agent capable of capturing or holding on to a molecule. For example, binding may be by formation of a covalent bond with

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a molecule, or may be by formation of a non-covalent linkage (*e.g.*, by antibody binding, hybridization, intercalation, binding to a binding partner) or any of the other affinity interactions well known in the art). Binding also encompasses, *e.g.*, capture in a substrate such as a gel matrix, *e.g.*, by diffusion, in which a slow rate of diffusion out of the matrix restricts the bound molecule.

As used herein, the term "variable," as used in reference to binding capacities, binding rates, volumes or sub-volumes, refers to a collection of two or more binding capacities, binding rates, volumes or sub-volumes that differ one from another.

As used herein, the term "serial," as used in reference to binding capacities, binding rates, volumes or sub-volumes refers to variable binding capacities, binding rates, volumes and sub-volumes, wherein, within a collection of three or more binding capacities, binding rates, volumes or sub-volumes, the binding capacities, binding rates, volumes or sub-volumes differ one from another by a multiple of the same factor. For example, a collection of sub-volumes having volumes V, 10V, 100V and 1000V differ by multiples of the factor of 10.

As used herein, the term "binding rate" refers to the rate at which a binding agent will bind a given binding partner, such that the binding agent will bind a particular amount of partner in a particular amount of time, until, *e.g.*, the binding capacity is reached.

As used herein, the term "binding capacity" refers to the total amount of a binding partner that an amount (*e.g.*, a unit or a portion) of binding agent can bind.

As used herein, the term "comparable with," as used in reference to numbers of entities, indicates that the numbers of entities are similar, *e.g.*, that there is less than about 2 orders of magnitude difference between the numbers of each entity. For example, if a number of reaction vessels is comparable to a number of target molecules, then the numbers of reaction vessels and target molecules are within about 2 orders of magnitude of each other.

DETAILED DESCRIPTION OF THE INVENTION

Particular embodiments of the invention are described in the Summary, and in this Detailed Description of the Invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. For example,

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the devices and methods of the present invention are described in connection with particular nucleic acid detection methods such as polymerase chain reaction. It should be understood that the present invention is not limited to methods and applications related to the polymerase chain reaction.

For example, in addition to PCR, numerous technologies are known for generating a detectable signal in the presence of a target nucleic acid are known in the art. Some methods comprise amplification using RNA-DNA composite primers (*e.g.*, as disclosed in U.S. Patent No. 6,251,639, herein incorporated by reference in its entirety). Some methods comprises the use of rolling circle amplification, (*e.g.*,as in U.S. Pat. Nos. 6,210,884, 6,183,960 and 6,235,502, herein incorporated by reference in their entireties), while other methods comprise the creation of DNA product using nucleic acids comprising loop-forming sequences, *e.g.*, as described in U.S. Patent No. 6,410,278, herein incorporated by reference in its entirety. Other methods that find application with the devices and methods of the present invention include but are not limited to the INVADER assay (Third Wave Technologies, Madison WI; see *e.g.*, U.S. Pat. Nos. 5,994,069, and 7,011,944, and de Arruda et al., Expert. Rev. Mol. Diagn. 2(5), 487-496 (2002), all of which are incorporated herein by reference in their entireties)

The present application provides methods and devices for absolute quantification of polymerase chain reaction target nucleic acids. In particular, the methods and devices of the present application provide for splitting a nucleic acid sample to be analyzed into small, isolated volumes, conducting the method of polymerase chain reaction (PCR) on said volumes, detecting PCR amplification products, analyzing said detected PCR amplification products, performing absolute quantification of the PCR target and presenting said quantification results.

Highly accurate, absolute quantification of a PCR target is very important for studies of gene function and pathology in such fields as for example, basic research and biotechnology, and it is mandatory for clinical application of a PCR method. A current approach to absolute PCR quantification is not based on direct measurement of the target copy number, but relies on certain assumptions about efficiency of the reaction at early cycles and on building reliable calibration curves. Methodologically, therefore, real-time PCR analysis is only relatively quantitative.

The method of the present invention provides ultra-sensitive detection and highly accurate absolute quantification of real-time or end point PCR at the single

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molecule level in nanoliter or sub-nanoliter volumes. One embodiment of the method of the present invention comprises the steps of: 1) serial dilution of a template sample with an unknown original number of template molecules (ONTM) in a PCR reaction mixture, 2) sub-dividing each of said dilutions into a large number of discrete, spatially isolated small sub-volumes, 3) perform PCR amplification in each of said sub-volumes simultaneously such that amplification products generated in each sub-volume are confined in said sub-volumes (Figure 1), detect the generated amplification products in each discrete, spatially isolated sub-volume, and 4) statistically analyze each of said sub-volumes for: a) the original starting number of template molecules (or concentration) in the PCR mixture and b) the probability of PCR contamination.

It is contemplated that the serial dilution of the template sample ensures that a statistically significant number of the sub-volumes contain single template molecules. As well, sub-division of the template sample into small, isolated sub-volumes further serves to distribute the template sample so that a statistically significant number of said sub-volumes contained single template molecules. It is contemplated that the sub-division of the template sample can be achieved by various methods, including, but not limited to, those methods as described herein. It is contemplated that conducting PCR in said isolated sub-volumes such that the PCR products generated in one sub-volume are confined in that sub-volume enables detection of said single template molecules. It is further contemplated that detection of the PCR products in said isolated sub-volumes is performed either during the PCR cycles (*e.g.*, "real time" detection) or after the PCR amplification is completed (*e.g.*, "end-point" detection). It is additionally contemplated that subsequent statistical analysis of the data generated by detection methods as described herein is targeted to determine the average number of template molecules per one sub-volume λ, and, based on Poisson statistics, calculate the ONTM and probability of PCR contamination.

The devices for practicing the methods of the present invention are provided to allow for high throughput amplification of multiple target molecules simultaneously. There are several ways to capture data generated in the amplification process whereby detection of template copies can be measured. One such data capture, real-time data capture, occurs throughout the amplification process. The other alternative is to capture data at the end of the amplification, end point data capture.

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An example of a device that performs real-time absolute quantitative PCR using the methods of the present invention is depicted in Figure 6. The template sample is diluted in a PCR mix containing a nucleic acid affinity fluorescent dye (*e.g.*, for example, SYBR® Green) and distributed on a substrate located in proximity, for example, between the aforementioned sub-volume cells. It is contemplated that by performing the aforementioned sample distribution, the average number of target copies per one cell λ is low, such that there will be many sub-volume cells with negative responses (*i.e.*, no amplification). Once distributed, the substrate undergoes thermal cycling (*i.e.* polymerase chain reaction). The sub-volumes on the substrate are illuminated by an illumination system (*e.g.*, for example, laser) and emitted fluorescence of the device is projected through an optical system onto a multi-pixel photodetector (*e.g.*, for example, a charge coupled device (CCD), complementary metal oxide semiconductor (CMOS) detector, photomultiplier tube (PMT), or avalanche photodiode (APD) array) and recorded by a computer system. Statistical analysis of sub-volumes that contain "YES" (*e.g.*, amplification products) and "NO" (*e.g.*, no amplification products) events, yields λ from which ONTM is calculated. Figure 2 depicts the two sorts of results, either YES or NO, that are recorded from sub-volumes in a real-time PCR device, depending whether a template molecule was present (YES, Figure 2A) or not (NO, Figure 2B).

An example of a device of that serves to perform end point absolute quantitative PCR using the methods of the present invention is similar to the above mentioned device for performing real time PCR. However in end point PCR, illumination of the sub-volumes is only performed after completion of the amplification reaction, and not during the reaction.

For real-time PCR amplification, if it is assumed that the sensitivity of the real time detection system is sufficient for detection of the PCR amplification products obtained from a single copy, then the expected real time PCR curves detected from individual sub-volumes are seen in Figure 2. It is contemplated that as very low ONTMs per sub-volume ($0.5<\lambda<2$) are used, large variation of the fluorescent signal between individual sub-volumes are realized. Indeed, in most compartments there will not be a target molecule at all, thereby yielding no signal. In other compartments there will be a single copy of the template molecule, in still others there will be two, three, or four copies or more. As the amount of the PCR product is contemplated to be proportional to the original number of copies in the exponential, or semi-log linear,

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region of PCR curve, the observed PCR curves will fall into several distinct groups, corresponding to different original copy numbers (Figure 3A). When the separation between the groups is well pronounced, several peaks on a histogram of fluorescence signal F detected in individual sub-volumes (Figure 3B) are seen. It is equally important that for different λ values, there are distinct (characteristic) proportions of sub-volumes with no signal (Figure 3C).

The distribution function *P* of highly diluted targets are described by Poisson statistics, such that:

$$P(k) = \frac{\lambda^k}{k!} e^{-\lambda}$$

wherein *P(k)* is a probability to find a sub-volume containing *k* copies,
wherein λ is an average ONTM per one sub-volume, and
wherein e is the natural log

Figure 3C illustrates the distribution function for λ =1. Thus, for any given number of detected sub-volumes, by integrating the histogram zones corresponding to different ONTMs per small volume (*e.g.*, 0, 1, 2, 3, 4, etc.), a distribution of the original target molecules in individual sub-volumes is obtained.

Using a log-linear least squares regression, the obtained event frequency can be fit to a Poisson distribution and the mean value for λ and test goodness of the fit using X^2 can be determined. From λ, ONTM or initial concentration based on total number of sub-volumes and their total volume is calculated. Comparison of the obtained distribution of the target over sub-volumes with Poisson distribution allows the determination of PCR contamination. It is contemplated that for λ <2, statistical analysis requires detecting at least 100-200 sub-volumes. For example, if a sub-volume is 10nl, the total reaction volume necessary for absolute target quantification will be 1-2μl. Therefore, when λ is determined, the original target concentration or ONTM can be easily calculated since the entire reaction volume used for detection or total number of sub-volumes and dilution degree for the native sample are known.

Real-time PCR quantification procedures can be complicated if real time detection data does not yield a histogram with clearly separated maxima. However, it is contemplated that for highly diluted samples, a significant number of sub-volumes will not exhibit any amplification, as no target molecules will be contained therein. In such a scenario, quantification of the target molecules is based on statistical analysis

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of 'YES'/'NO' results at the end of PCR amplification. For end-point PCR quantification, fluorescence measurement at the end of the PCR cycles indicates either a product of PCR amplification is present in a compartment, meaning this compartment confined one or more target molecules initially ('YES' result), or there is no PCR amplification in a compartment, and thus it did not contain target DNA before PCR ('NO result').

It is contemplated that when a series of N measurements, such that a sample is distributed, or sub-divided, into N sub-volumes, PCR amplification is performed and the presence or absence of amplification product (*e.g.*, via fluorescence measurement) is detected in each sub-volume, the result of each measurement in the series will be 'NO', if there are no PCR products detected in the sub-volume and 'YES', otherwise. Thus, the series can be considered as a sequence of N Bernoulli trials. The result of each series of experiments represents the number of 'empty' sub-volumes k, such that the number of 'NO' results, k, is distributed according to binomial law:

$P(k) = \binom{N}{k} p^k (1-p)^{N-k}$, with mean value $E(k) = Np$, variance $\mathrm{var}(n) = Np(1-p)$, and standard deviation $\sigma_n = \sqrt{Np(1-p)}$ where p is the probability of 'NO' result. The estimate for concentration is evaluated as $\lambda_{EST} = -\log\left(\frac{k}{N}\right)$.

A Monte-Carlo simulation of a series of independent experiments, N experiments, in each series are seen in Figure 4. Figure 4 demonstrates that even in cases when real-time PCR does not give a clear distinction of signals obtained from each sub-volume during exponential phase of the amplification of single target copies, statistics of 'YES'/'NO' data at the end of PCR allows accurate target quantification for a sufficient number of trials (*i.e.*, sub-volumes) N. For example, Figure 4C demonstrates that at N=300, 95% confidence intervals are distinct enough to give statistically significant λ values, from which ONTM per sample can be easily derived. It is contemplated that for an individual sub-volume of 10nl where N=1000, total required reaction volume will be only 10μl, and an entire amplification array (*e.g.*, NMCA, chip) with all 1000 sub-volumes (*e.g.*, in capillaries, chambers, etc.) can be cycled within about 1hour. The present invention described herein differs from currently available quantification techniques in real-time or end-point PCR

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instruments, such that in order to apply the same quantification techniques in commercial real-time or end-point PCR instruments, at least 10ml of a PCR mixture with template would be required and the experiment would take around 10-20 hours.

Calculations to determine the pre-PCR number of target DNA molecules (M_0) in a pre-amplification target sample is preformed using the equation:

$$M_0 = N_{\text{sub-volumes}} \times \left[-\ln(P)\right]$$

wherein N is the number of sub-volumes, and

wherein P is the percent of sub-volumes that contained to amplification products

Standard deviations are further estimated using the equation:

$$\sigma_N = \sqrt{N_{sub-volumes} P(1-P)}$$

The PCR quantification methods of the present invention collect statistics for single molecule PCR amplification. To obtain accurate statistics, it is contemplated that the method requires at least 100, at least 200, at least 300 hundred DNA molecules amplified in isolated small sub-volumes. Using the methods and devices of the present invention, PCR contamination by contaminant DNA molecules is insignificant if the number of contaminant molecules is smaller than standard deviation σ_N of the calculated number of molecules in the sample. Therefore, the present invention differs from current PCR protocols whereby the entire sample is amplified in a single reaction tube, and the contaminants are amplified together with the sample, such that even one contaminant DNA molecule may cause false results because the PCR on that mixture could favor the contaminant, causing the amplified contaminant to mask the presence of the DNA species of interest.

For the present invention, to identify sample contaminants that could impact the outcome of M_0, a negative control is assayed along with the unknown template

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samples, such that all conditions are the same, except the negative control contains no target nucleic acid.

The estimated Poisson parameter λ_{EST} is then determined using the following equation:

$$\lambda_{EST} = \ln\left[P_{\text{CONTROL}}(0) \Big/ P_{\text{SAMPLE}}(0) \right]$$

where:

λ_{EST} is estimated Poisson parameter λ taking into account sample contamination probability

$P_{\text{CONTROL}}(0)$ is percent of "0" results in control negative

$P_{\text{SAMPLE}}(0)$ is percent of "0" results in tested DNA sample

As used herein, a "0" result is wherein there is no amplicon in a sub-volume. The high level of sensitivity provided by the methods and devices of the present invention for determining the absolute number of pre-PCR target nucleic acid molecules is seen in Figure 5. In Figure 5, the calculated difference between the threshold PCR cycle C_{T0} of the original diluted sample and the threshold cycle in sub-volumes $C_{T\,subvol}$ is provided by application of the data to the equation:

$$C_{T\,0} - C_{T\,subvol} = \frac{\log\left[V_0 \Big/ (V_{subvol} \times M_0) \right]}{\log(E)}$$

where:

E - PCR efficiency, C_T - threshold cycle, V - sample volume

M_0 - pre-PCR number of target copies

Therefore, in low volume PCR, amplification products become detectable in earlier cycles and/or at lower reaction efficiencies thereby yielding significantly enhanced sensitivity when utilizing the methods of the present invention.

In one embodiment, the present invention provides for methods and devices performing absolute quantitative end-point PCR (qe-PCR). In some embodiments, a serial dilution of a nucleic acid template, or target, sample which contains a known or unknown original number of target molecules (ONTM) is created in a mixture of PCR reaction components. In some embodiments, the serial dilution is created external to a high-throughput amplification substrate (*e.g.*, slide, gel, tube), whereas in other

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embodiments the dilutions are created by the partitioning of the sample into sub-volumes, as described herein. In some embodiments, real-time measurements of PCR amplification products (*e.g.*, via fluorescence detection) are taken. In some embodiments, amplification products are measured (*e.g.*, via fluorescence detection) at the end of the PCR cycling. In some embodiments, the amplification of all sub-volumes occurs simultaneously. In some embodiments, the percent of sub-volumes *P* that yield no amplification products (*i.e.*, 'NO' determination) and the percent of sub-volumes (*1-P*) that yield amplification products (*i.e.*, 'YES' determination) are determined.

In one embodiment, the present invention provides for a device for performing absolute real-time and end-point PCR. In some embodiments, the device provides for serial dilution of the template sample containing an unknown original number of the template (target) molecules in a PCR mix. In some embodiments, the device provides for a dispenser (*e.g.*, automated dispensing device) for subdividing each serial dilution of the template sample into spatially isolated sub-volumes on a substrate. In some embodiments, the substrate is, for example a micro-well plate, a slide, a chip, or a capillary tube. In some embodiments, the substrate is pre-coated with a matrix (*e.g.*, agarose, MATRIGEL®, collagen) prior to dispensing the samples. In some embodiments, PCR amplification is conducted in said sub-volumes on said substrate, such that the PCR products generated in said sub-volumes are confined to said sub-volumes. In some embodiments, the generated PCR products are detected in each of said sub-volumes after each amplification cycle is completed (*e.g.*, real-time PCR). In some embodiments, the generated PCR products are detected in each of said sub-volumes after completion of the PCR assay (*e.g.*, end point PCR). In some embodiments, the method of detection is fluorescence detection. In some embodiments, the device of the present invention comprises devices that include an excitation light source, a photo receiving system including an optical system for controlling and synchronizing the thermal cycling and the PCR detection processes, processes for transfer, recording and processing of the acquired data.

Method of detecting accumulated PCR product using fluorescence are well known in the art. When the devices provided herein are utilized to conduct nucleic acid amplification reactions, a number of different approaches can be utilized to detect amplified product. Examples of suitable approaches include, for example, the

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use of intercalating dye, the use of labeled probes in conjunction with 5' nuclease cleavage, and the use of structured probes.

The use of intercalating dyes utilizes fluorogenic compounds that only bind to double stranded DNA. In this type of approach, amplification product (which is generally double stranded) binds dye molecules in solution to form a complex. With the appropriate dyes, it is possible to distinguish between dye molecules remaining free in solution and dye molecules bound to amplification product. For example, certain dyes fluoresce efficiently only when bound to double stranded DNA, such as amplification product. Examples of such dyes include, but are not limited to, SYBR Green and Pico Green (from Molecular Probes, Inc., Eugene, OR), ethidium bromide, propidium iodide, chromomycin, acridine orange, Hoechst 33258, TOTO-1, YOYO-1, and DAPI (4',6-diamidino-2-phenylindole hydrochloride). Additional discussion regarding the use of intercalation dyes is provided, *e.g.*, by Zhu et al., Anal. Chem. 66:1941-1948 (1994), which is incorporated by reference in its entirety.

Fluorogenic nuclease assays are another example of a product quantitation method that can be used successfully with the devices and methods described herein. The basis for this method of monitoring the formation of amplification product is to measure PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe, an approach frequently referred to in the literature as the "TaqMan" method. The probe used in such assays is typically a short (ca. 20-25 bases) polynucleotide that is labeled with two different fluorescent dyes. The 5' terminus of the probe is typically attached to a reporter dye and the 3' terminus is attached to a quenching moiety, although the dyes can be attached at other locations on the probe as well. The probe is designed to have at least substantial sequence complementarity with the probe-binding site on the target nucleic acid. Upstream and downstream PCR primers that bind to regions that flank the probe binding site are also included in the reaction mixture.

When the fluorogenic probe is intact, energy transfer between the fluorophore and quencher moiety occurs and quenches emission from the fluorophore. During the extension phase of PCR, the probe is cleaved, *e.g.*, by the 5' nuclease activity of a nucleic acid polymerase such as Taq polymerase, or by a separately provided nuclease activity that cleaves bound probe, thereby separating the fluorophore and quencher moieties. This results in an increase of reporter emission intensity that can be measured by an appropriate detector. Additional details regarding fluorogenic

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methods for detecting PCR products are described, for example, in U.S. Pat. No. 5,210,015 to Gelfand, U.S. Pat. No. 5,538,848 to Livak, et al., and U.S. Pat. No. 5,863,736 to Haaland, as well as Heid, C. A., et al., Genome Research, 6:986-994 (1996); Gibson, U. E. M, et al., Genome Research 6:995-1001 (1996); Holland, P. M., et al, Proc. Natl. Acad. Sci. USA 4 88:7276-7280, (1991); and Livak, K. J., et al., PCR Methods and Applications 357-362 (1995), each of which is incorporated by reference in its entirety.

Structured probes (*e.g.*, "molecular beacons") provide another method of detecting accumulated PCR product. With molecular beacons, a change in conformation of the probe as it hybridizes to a complementary region of the amplified product results in the formation of a detectable signal. In addition to the target-specific portion, the probe includes addition sections, generally one section at the 5' end and another section at the 3' end, that are complementary to each other. One end section is typically attached to a reporter dye and the other end section is usually attached to a quencher dye.

In solution, the two end sections can hybridize with each other to form a stem loop structure. In this conformation, the reporter dye and quencher are in sufficiently close proximity that fluorescence from the reporter dye is effectively quenched by the quencher. Hybridized probe, in contrast, results in a linearized conformation in which the extent of quenching is decreased. Thus, by monitoring emission changes for the reporter dye, it is possible to indirectly monitor the formation of amplification product. Probes of this type and methods of their use is described further, for example, by Piatek, A. S., et al., Nat. Biotechnol. 16:359-63 (1998); Tyagi, S. and Kramer, F. R., Nature Biotechnology 14:303-308 (1996); and Tyagi, S. et al., Nat. Biotechnol. 16:49-53 (1998), each of which is incorporated by reference herein in their entirety for all purposes.

It is contemplated that the detection device of the present invention is not limited to fluorescence detection. Indeed, those skilled in the art will understand that other means of detection for monitoring the accumulation of PCR amplifications are equally applicable to the present invention. In some embodiments, a computer and software for statistical analysis capable of performing the calculations as described herein to determine the original number (or concentration) of target molecules initially contained in the template sample further comprise the device of the present invention.

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As noted above, methods of quantitating the number of initial target nucleic acid molecules in a sample often comprises performing variable dilutions of the sample, in which an aliquot of the sample is diluted by a factor to make a first diluted sample, the an aliquot of the first diluted sample is then diluted by a factor to make a second diluted sample, and so forth. These are referred to herein as "variable dilutions."

Any desired dilution factor can be used. For example, in some instances, the successive dilutions are performed using dilution factors that vary between the successive dilutions. For example, an initial aliquot may be subject to dilution by a factor of 10 to make a first diluted sample, an aliquot of the first diluted sample may then be diluted by a factor of 5 to make a second diluted sample, an aliquot of which may then be diluted by a factor of 7.5 to make a third diluted sample, and so forth.

In some instances, the same dilution factor is used for each successive dilution. For example, if the factor is 2, the first dilution contains ½ the concentration of analyte as the starting material, the second dilution contains ¼, and so on. Sequential dilutions in which the same dilution factor is used for each dilution are referred to herein as "serial" dilutions. Serial dilutions using factors of 5, 10, or 100 are common in assays of biological molecules.

In any such sequential dilutions, the total dilution factor at any point is the product of the individual dilution factors in each step up to that point.

One source of variation in quantitation of arises from such sequential dilutions. Generally, samples are diluted using a purified diluent, such a saline solution or a buffer. In many instances, samples such as clinical samples comprise a number of components in addition to the nucleic acid in the sample. For example, a sample may contain contaminating (non-target) nucleic acids, proteins, salts, heme, heparin, *etc.* When such samples are sequentially diluted, the contaminants are also sequentially diluted such that the reaction conditions under which the different dilutions of target nucleic acids are being tested are not truly identical. In addition, introduction of exogenous materials into the samples (*e.g.,* diluents) increases the likelihood of introducing contaminants into the reactions.

One embodiment of the present invention is to provide methods and devices for sampling a test sample without conducting serial dilutions. The invention comprises several different ways of partitioning different volumes of a sample, *e.g.*, serially smaller sub-volumes, so that the benefits of sequential dilution (*e.g.*, finding

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an amount of a test sample that provides a Poisson parameter λ that is in the desired range for a particular method) can be had without the need for actual dilution of the sample.

In one embodiment, the present invention provides for a sample tray, *e.g.*, for performing PCR. In some embodiments, the sample tray is disposable. In some embodiments, the sample tray is divided into many distinct, isolated compartments or zones. In some embodiments, the number of compartments, or wells, in the sample tray is at least 48, at least 96, at least 384. In some embodiments, the wells in the sample tray are coated with a gel substrate such that the volume of the gel substrate is variable from well to well. In some embodiments, the gel substrate volume increases in a manner such that a serial dilution of a nucleic acid sample, when applied, is realized over a series of wells. In some embodiments, the gel substrate is gelatin, agarose, collagen, or Matrigel®. The present invention is not limited to the type of gel substrate used, it is only required that the nucleic acid sample, when applied, is able to diffuse through the gel and that the gel allows for amplification to occur (*e.g.*, does not inhibit the PCR reaction). In some embodiments, the tray is treated with a gel substrate and a nucleic acid sample is applied such that a "dilution" series of the DNA sample is realized, amplification in the tray is allowed to proceed (*e.g.*, by application of the tray to a PCR instrument). In some embodiments, end point PCR product detection is performed (*e.g.*, via fluorescence detection), the data is applied to the Poisson equations as described herein and an absolute quantification and reporting of sample ONTM is performed. In some embodiments, the data acquisition, calculations, quantification and reporting of ONTM results is performed automatically (*e.g.*, on a computer).

In some embodiments, a sample tray is provided that comprises isolated reaction zones that will contain variable quantities of target nucleic acid. In some embodiments, the reaction zones differ in volume, such that, although the absolute concentrations of all components in the reactions in the different zones are identical, the reactions contain different numbers of target molecules. Use of very low volume trays, *e.g.* fluidic chips, or multiple capillary arrays, means that the variations in the heating dynamics of the different volumes will be negligible, and will have negligible effects on the efficiency, *e.g.*, of PCR.

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Similarly, a sample tray can be configured such that the isolated reaction zones comprise a binding agent configured to bind variable amounts of target nucleic acid from a sample. In some embodiments, the binding agents can bind nucleic acid at different, particular rates, such that exposure of the sample tray to a sample for a fixed amount of time permits the isolated reaction zones to bind variable amounts of nucleic acid. In other embodiments, the isolated reaction zones comprise binding agents having different binding capacities, such that exposure of the sample tray to a sample for a period of time to bind available nucleic acids produces isolated reaction zones having variable amounts of bound target nucleic acid. In each of these embodiments, the variability in binding can be by any desired factor, including but not limited to serial factors in which the binding capability of multiple isolated reaction zones each differ by multiples of the same factor.

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLES

Example 1

Multi-capillary arrays (MCAs) or chips as PCR vessels

The detection and absolute quantification of DNA samples will be performed during a real-time PCR amplification of the samples loaded into nano- or picoliter volume capillaries of disposable multi-capillary arrays (Figure 7), or chips. Capillary electrophoresis will be used in the system that will help to increase the system sensitivity and specificity and to avoid false negative and false positive results.

The general view and the enlarged fragment of the MCA are shown in Figure 7. The array can be divided into many PCR zones. The array in Figure 7 shows 24 zones (approximately 3mm long and 10mm wide), although it is contemplated that MCA may comprise a single zone, or may comprise fewer or more than 24 zones. Each zone is a row of parallel nanoliter to picoliter capillaries, known as a sub-array. By way of example but not by way of limitation, a sub-arrays as described here contains 32 capillaries of 10nl volume each (100 μm wide, square cross-section, and 1mm long). More or fewer capillaries may be used a sub-array, depending on the

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specific configuration of the thermal cycler and/or photo detector. In this embodiment, the capillaries are arrayed in parallel and are bordered by two grooves; a filling groove and a vent groove. The PCR zones are separated by zone separators which block penetration of the DNA samples between individual zones. Similar structures can be organized on glass or silicon chips with scalable microfabrication technology.

A DNA sample to be amplified is loaded the filling grooves of an individual PCR zones. For a serial dilution panel, a DNA sample to be amplified is serially diluted and different dilutions are loaded into different filling grooves of individual PCR zones, one per zone. When the DNA samples are applied to the zone(s) the sample enters (*e.g.*, is taken up by) the capillaries of the sub-arrays due to capillary force. Thus, for example, 32 capillaries of a sub- array are filled with the same DNA sample (total sample volume loaded in one sub-array is approximately 320nl). Depending on the PCR purpose, one or more zones can be loaded with the same DNA sample. When sample loading is completed, both filling and vent groves may be sealed, *e.g.*, with PCR oil. The loaded MCA is covered with a glass slide and transferred to a PCR device where it undergoes thermal cycling, and either real-time or end point fluorescence is detected for the PCR products generated in all PCR zones.

Example 2

PCR on matrix coated substrates

PCR can be performed in a disposable sample tray with arrays of gel micro-pads as depicted in Figure 8. For example, serial dilutions of DNA samples in a PCR mix are made, aliquoted to a multi-well plate and covered with mineral oil in order to avoid the sample evaporation. The samples are then loaded from the plate into individual cells of the disposable sample tray with the gel pad arrays. The DNA samples are allowed to infuse into the gel pads. For example, the samples are allowed in infuse into the gel pads at 60°C for 5-30 minutes. After infusion of the sample DNA into the gel pad, the pad is washed to remove any remaining sample. Mineral oil or another sealing compound is applied to the top of the gel pad to seal the PCR template and generated PCR products in the gel pad (Tillib et al., 2001, Anal. Biochem. 292:155). If a polymer is used to seal the gel pad, the polymer may be

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allowed to solidify before proceeding with PCR. PCR is carried out to an end point read. PCR generated products are detected in the gel pads, and *P* percent is determined and ONTM calculated for each individual gel pad array.

PCR amplification can also occur on glass or plastic substrates with deposited non-denaturing gel/matrix as depicted in Figure 9. The present invention takes advantage of the idea that the progeny of a single template molecule (called a 'polony') occupies the smallest possible volume due to poor diffusion of DNA molecules through the gel/matrix. As a result, the polony's spot has very a high concentration of PCR generated products (Mitra and Church, 1999, Nucl. Acids Res. 27:e34).

Example 3

PCR in water/oil emulsions

Serial dilutions of a DNA sample in PCR mix are prepared and placed into a vial and covered with oil. Emulsification of the PCR mix and oil is preformed such that an emulsion is created and the bubbles are capable of withstanding PCR thermal cycling. The emulsified samples are loaded into a capillary tube and end point PCR is carried out in the tube. PCR generated products are detected in the bubbles, *P* percent of bubbles with no PCR amplification products associated with them is determined and ONTM is calculated as described herein.

Example 4

PCR on beads in water/oil emulsions

Serial dilutions of a DNA sample in PCR mix are prepared, placed into a vial and covered with oil. Beads are added to the dilutions and the DNA is allowed to attach to the beads. Emulsification is carried out in that the beads are surrounded by water, and the water surrounded by oil so that an emulsion is created and the bubbles are capable of withstanding PCR thermal cycling. The emulsified samples are loaded into a capillary tube, and end point PCR is performed. The PCR generated products are detected in the water bubbles, *P* percent of beads with no amplification products associated with them is determined and ONTM is calculated.

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Example 5

PCR on liquid polymer beads

In a vial (see, *e.g.*, Figure 10), a PCR mix is prepared which includes a liquid polymer with a known DNA diffusion coefficient. The target DNA is attached to beads, and the DNA beads are added to the PCR mix with liquid polymer. The concentration of beads should be such that the average distance between beads significantly exceeds the diffusion length of target DNA fragments in said PCR mix diluted in liquid polymer. Aliquots of the samples are loaded into a capillary tube and end point PCR is performed. PCR products generated are detected and *P* percent of beads which yielded no PCR amplification products is determined and ONTM calculated.

Example 6

qPCR and reduction of manual serial dilutions

On the same tray, gel substrates in different cells of the sample tray (Figure 8) can differ in thickness, geometry (*e.g.*, solid substrates or arrays of gel micro-pads), and/or composition (*e.g.*, gels can differ in content of polymer, cross-linker, etc.). Specific configuration of the gel substrates and specific sets of gel substrates on the sample tray provides different ways for performing PCR quantification.

For example, arrays of gel micro-pads that differ in pad volume (Figure 8) can be used to avoid manual serial dilution of a DNA sample. Indeed, an original DNA concentration per one pad in pads of volume V1 will differ from the original DNA concentration per one pad in pads of volume V2 by the factor V1/V2. Effectively, this is the same as placing different dilutions of DNA sample in arrays with identical pad volume V. As well, using solid gel substrates that differ in gel composition (*e.g.*, for example, different content of polymer and cross-linker) is equally amenable. The infusion of DNA samples into gel proceeds at different speed depending on the gel concentration, and by using an appropriate calibration the same density of polonies for different DNA content can be obtained thereby avoiding the necessity of preparing and distributing serial dilutions.

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Example 7

Design, fabrication and use of multi-capillary arrays

A process that produces monolith capillary arrays with or without split ends is illustrated in Figure 11. For example, a set of glass ferrules, their number equal to the desired number of capillaries required, is utilized to create the multi-capillary array. The size of the ferrules and the thickness of their walls is chosen depending on the desired inner size of the capillaries and the spacing between them. The ferrules are pressed together into a planar array and are drawn at an elevated temperature. Due to adhesion, the resulting array has a truly monolithic structure. The production process allows formation of extremely regular arrays of square or rectangular capillaries with an ideal translational symmetry. Being monolithic, the array acts as a low loss medium for the propagation of light. Its optical properties ensure a uniform illumination of all capillaries, thereby facilitating focusing a laser beam on the center of the capillary. Significant advantages of the multi capillary arrays include low cost of manufacture and the absence of any specially adjusted parts in the detection zone.

Thirty-two capillary arrays are fabricated with 100, 150 and 200μm square capillary cross section, 300 μm array pitch, 0.5-1mm thickness, 10mm width and 90mm length. Using laser engraving instrumentation, sub-arrays can be formed of nano-volume capillaries by making V-grooves across the top surface of the MCA for loading of nucleic acid samples into nano-volume wells and for venting air (see Figure 7). The size of the V-grooves can be chosen to accommodate nucleic acid samples volumes that allow for filling of the 32 capillaries. For example, for a 100 μm cross-section and a 10nl capillary volume, the V-grooves will be 500 μm wide, 200 μm deep and separated by 1.2mm. In order to create the multiple PCR zones (Figure 7), separation zones can be formed across the MCA. These zones will define size/volume of the sub-arrays and sub-volumes, respectively. For example, the zones can be formed either by melting glass with a laser across the arrays thereby creating focal plugs inside the capillaries, or by laser engraving grooves across the array and filling the grooves with UV curable optical glue. Both laser engraving and filling separators with UV curable glue can be performed automatically. The automatic procedures utilized yield a low cost, disposable device of the present invention.

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Example 8

An application for calculating results

The reported results from Mitra and Church, 1999, Nucl. Acids Res. 27:e34 were applied to the Poisson equations of the present invention to demonstrate the increased accuracy for determining ONTM using the methods of the present invention for processing experimental results. Images of gel slides with polonies (Figure 9) were used. The slide surface was divided into cells of the size of polonies (approximately 300mkm) and the fraction of empty cells was found in order to determine λ_{EST}. The calculated number of polonies grown on gel slides were determined by using the methods of the present invention and the numbers were compared with the number of polonies as manually counted by Mitra and Church (see graph, Figure 9). The calculated polony number was more accurate that just counting colonies, and as the concentration of template DNA increased (*e.g.*, increase in ONTM), the discrepancy between counted colonies and the calculated colonies increased.

All publications and patents mentioned in the present application are herein incorporated by reference. Various modification and variation of the described methods and compositions of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.

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CLAIMS

We claim:

1. A method of counting target nucleic acid molecules, comprising:

a) providing a sample containing a plurality of target nucleic acid molecules;

b) applying said sample to a multiple vessel array comprising a plurality of reaction vessels, wherein each of said reaction vessels receives an essentially equal sub-volume of said sample, wherein the combined volumes of said sub-volumes defines a tested volume, and wherein the number of said plurality of reaction vessels is comparable with the number of target nucleic acid molecules in said tested volume;

c) treating said multiple vessel array to conditions to produce a detectable signal in the presence of said target nucleic acid; and

d) determining how many of said reaction vessels contain reactions that produce said detectable signal.

2. The method of Claim 1, wherein said multiple vessel array is a multiple capillary array, and wherein said reaction vessels are capillary reaction vessels.

3. The method of Claim 1 or Claim 2, wherein said determining comprises determining how many of said reaction vessels contain reactions that do not produce said detectable signal.

4. The method of Claim 1 or Claim 2, wherein said conditions comprise exposure of said target nucleic acid to reagents for producing a detectable signal in the presence of said target nucleic acid.

5. The method of Claim 4, wherein said sample further comprises said reagents.

6. The method of Claim 4, wherein said multiple vessel array comprises said reagents.

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7. The method of Claim 6, wherein said reagents are dried prior to exposure to said sample.

8. The method of Claim 4, wherein said reagents are reagents for performing a nucleic acid amplification reaction.

9. The method of Claim 8, wherein said nucleic acid amplification reaction is a polymerase chain reaction.

10. The method of Claim 9, wherein said determining is conducted after the completion of said polymerase chain reaction.

11. The method of Claim 1 or Claim 2, wherein said detectable signal comprises fluorescence.

12. The method of Claim 1 or Claim 2, wherein said reagents comprise an oligonucleotide probe comprising a fluorescent moiety.

13. The method of Claim 12, wherein said oligonucleotide probe further comprises a quencher moiety.

14. The method of Claim 1 or Claim 2, wherein said reagents comprise a nucleic acid intercalating dye.

15. The method of Claim 9, wherein said polymerase chain reaction is conducted for a predetermined number of cycles, and wherein said determining is conducted after completion of said predetermined number of cycles.

16. The method of Claim 1 or 2, wherein the ratio of target molecules to reaction vessels is 10:1.

17. The method of Claim 1 or 2, wherein the ratio of target molecules to reaction vessels is 3:1.

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18. The method of Claim 1 or 2, wherein the ratio of target molecules to reaction vessels is 2:1.

19. The method of Claim 1 or 2, wherein the ratio of target molecules to reaction vessels is 1:1.

20. The method of Claim 1 or 2, wherein the ratio of target molecules to reaction vessels is 0.5:1.

21. The method of Claim 1 or 2, wherein the ratio of target molecules to reaction vessels is 0.1:1.

22. The method of Claim 1 or 2, wherein the ratio of target molecules to reaction vessels is 0.01:1.

23. The method of Claim 1 or 2, wherein the number of target molecules is calculated using the equation:

$$M_0 = N_{\text{sub-volumes}} \times \left[-\ln(P)\right]$$

wherein M_0 is the number of target molecules in said tested volume,

wherein N is the number of sub-volumes, and

wherein P is the percent of sub-volumes lacking said detectable signal.

24. The method of Claim 1 or 2, wherein a standard deviation in the number of target molecules in a sample is calculated using the equation:

$$\sigma_N = \sqrt{N_{sub-volumes} P(1-P)}$$

wherein σ_N is the standard deviation.

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25. The method of Claim 1 or 2, wherein said sample is a test sample, further comprising providing a negative control sample lacking said target nucleic acid, wherein said negative control sample is treated identically to said test sample, wherein the Poisson parameter for said test sample is estimated using the equation:

$$\lambda_{EST} = \ln\left[P_{CONTROL}(0) \Big/ P_{SAMPLE}(0) \right]$$

wherein:

λ_{EST} is the estimated Poisson parameter for said test sample;

$P_{CONTROL}(0)$ is the percent of reaction vessels lacking signal in the control negative assay; and

$P_{SAMPLE}(0)$ is the percent of reaction vessels lacking signal in said test DNA sample.

26. The method of Claims 12 and 13, further comprising communication of the determined number of capillary reaction vessels containing reactions producing said detectable signal to a computer, wherein the calculation is performed by said computer.

27. The method of Claim 1 or 2, wherein said multiple vessel array comprises at least 100 reaction vessels.

28. The method of Clam 1 or 2, wherein said multiple vessel array comprises at least 500 reaction vessels.

29. The method of Claim 1 or Claim 2, wherein said multiple vessel array comprises at least 1000 reaction vessels.

30. A device for testing a nucleic acid sample, said device comprising a multiple capillary array comprising a first plurality of capillary reaction vessels, wherein each of said first plurality of capillary reaction vessels is configured to receive an essentially equal sub-volume of an applied liquid sample.

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31. The device of Claim 30, wherein said capillary reaction vessels are essentially parallel to each other.

32. The device of Claim 30, further comprising a filling groove and at least one vent groove, wherein said filling groove is fluidically connected to each of said capillary reaction vessels in said first plurality of capillary reaction vessels.

33. The device of Claim 30, wherein said multiple capillary array comprises one or more additional pluralities of capillary reaction vessels, wherein each of the additional pluralities of capillary reaction vessels is fluidically connected to a filling groove to which no other pluralities of capillary reaction vessels are connected.

34. The device of Claim 33, wherein the said pluralities of capillary reaction vessels are separated from one another by zone separators.

35. The device of Claim 30, further comprising a component for temperature cycling said capillary reaction vessels.

36. The device of Claim 30, further comprising a fluorescence detector.

37. The device of Claim 30, further comprising a computer operably linked to said fluorescence detector.

38. The device of Claim 36, wherein said fluorescence detector is configured to detect fluorescence during temperature cycling.

39. The device of Claim 36, wherein said fluorescence detector is configured to detect fluorescence only when said device is not temperature cycling.

40. A method of sampling a target nucleic acid, comprising:

a) providing a sample tray comprising a plurality of isolated reaction zones, wherein each isolated reaction zone contains a nucleic acid binding agent having a specific nucleic acid binding rate, wherein

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the binding rates of the binding agents in different isolated reaction zones are variable binding rates;

b) exposing the sample tray to a sample comprising target nucleic acid under conditions wherein each isolated reaction is exposed to said sample for essentially the same amount of time;

c) removing unbound target nucleic acid from said sample tray;

d) detecting the target nucleic acid in said isolated reaction zones.

41. The method of Claim 40, wherein said variable binding rates are serial binding rates.

42. The method of Claim 40, wherein said nucleic acid binding agent comprises a gel matrix.

43. The method of Claim 42, wherein the specific nucleic acid binding rate of said gel matrix is determined by the rate at which nucleic acid diffuses through said gel matrix.

44. The method of Claim 42, wherein the gels matrices in said different isolated reaction zones have differing gel porosities.

45. The method of Claim 42, wherein the gels matrices in said different isolated reaction zones have differing gel volumes.

46. The method of Claim 41, wherein the serial binding rates differ between isolated reaction zones by factors of 2.

47. The method of Claim 41, wherein the serial binding rates differ between isolated reaction zones by factors of 5.

48. The method of Claim 41, wherein the serial binding rates differ between isolated reaction zones by factors of 10.

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49. The method of Claim 41, wherein the serial binding rates differ between isolated reaction zones by factors of 100.

50. The method of Claim 41, wherein the serial binding rates differ between isolated reaction zones by factors of 1000.

51. A method of sampling a target nucleic acid, comprising:

a) providing a sample tray comprising a plurality of isolated reaction zones, wherein each isolated reaction zone contains a nucleic acid binding agent having a specific nucleic acid binding capacity, wherein the binding capacities of the binding agents in different isolated reaction zones are variable binding capacities;

b) exposing the sample tray to a sample comprising target nucleic acid under conditions wherein said binding agent binds to said target nucleic acid;

c) removing unbound target nucleic acid from said sample tray;

d) detecting the target nucleic acid in said isolated reaction zones.

52. The method of Claim 51, wherein said variable binding capacities are serial binding capacities.

53. The method of Claim 52, wherein the serial binding capacities differ between isolated reaction zones by factors of 2.

54. The method of Claim 52, wherein the serial binding capacities differ between isolated reaction zones by factors of 5.

55. The method of Claim 52, wherein the serial binding capacities differ between isolated reaction zones by factors of 10.

56. The method of Claim 52, wherein the serial binding capacities differ between isolated reaction zones by factors of 100.

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57. The method of Claim 52, wherein the serial binding capacities differ between isolated reaction zones by factors of 1000.

58. A method of sampling a target nucleic acid, comprising:

a) providing a sample tray comprising a plurality of isolated reaction zones, wherein each isolated reaction zone defines a specific reaction sub-volume, wherein the reaction sub-volumes of said different isolated reaction zones are variable reaction sub-volumes;

b) exposing the sample tray to a sample comprising one or more target nucleic acid molecules under conditions wherein said sample fills said plurality of isolated reaction zones;

c) detecting the target nucleic acid in said isolated reaction zones.

59. The method of Claim 58, wherein said variable reaction sub-volumes are serial reaction sub-volumes.

60. The method of Claim 59, wherein the serial reaction sub-volumes differ between isolated reaction zones by factors of 2.

61. The method of Claim 59, wherein the serial reaction sub-volumes differ between isolated reaction zones by factors of 5.

62. The method of Claim 59, wherein the serial reaction sub-volumes differ between isolated reaction zones by factors of 10.

63. The method of Claim 59, wherein the serial reaction sub-volumes differ between isolated reaction zones by factors of 100.

64. The method of Claim 59, wherein the serial reaction sub-volumes differ between isolated reaction zones by factors of 1000.

65. The method of Claim 58, wherein said sample comprises reagents for a nucleic acid detection reaction.

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66. The method of Claim 40, 51, or 58, wherein said detecting comprises performing a nucleic acid detection reaction.

67. The method of Claim 66, wherein said nucleic acid detection reaction comprises a nucleic acid amplification reaction.

68. The method of Claim 67, wherein said nucleic acid amplification reaction is a polymerase chain reaction.

69. The method of Claim 68, wherein said detecting is conducted after the completion of said polymerase chain reaction.

70. The method of Claim 40, 51, or 58, wherein said detecting comprises detection of fluorescence.

71. The method of Claim 65, wherein said reagents comprises an oligonucleotide probe comprising a fluorescent moiety.

72. The method of Claim 71, wherein said oligonucleotide probe further comprises a quencher moiety.

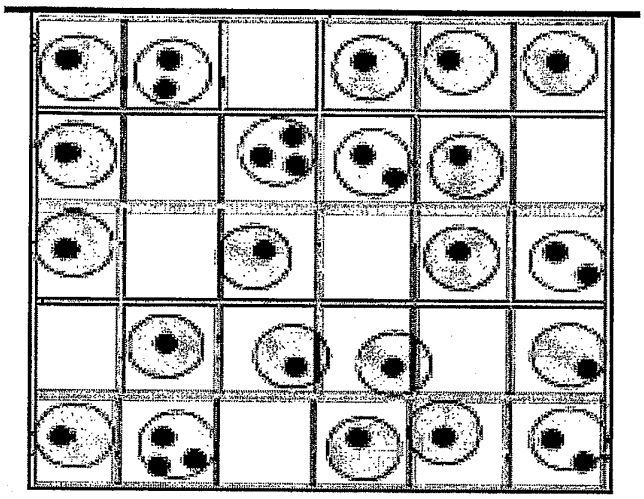
73. The method of Claim 65, wherein said reagents comprise a nucleic acid intercalating dye.

74. The method of Claim 68, wherein said polymerase chain reaction is conducted for a predetermined number of cycles, and wherein said detecting is conducted after completion of said predetermined number of cycles.

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FIGURE 1

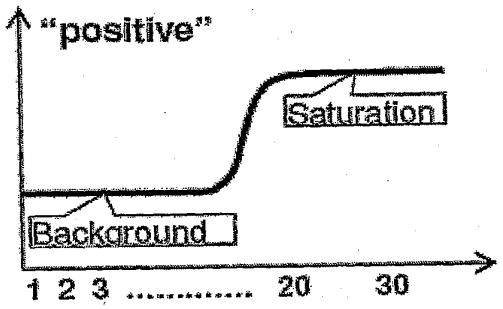


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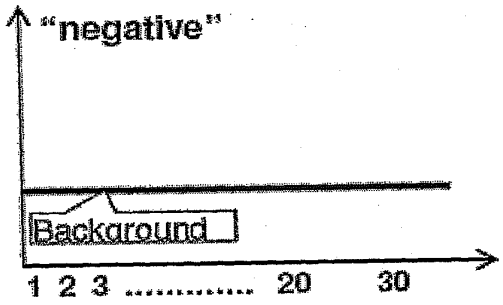
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FIGURE 2

A



B

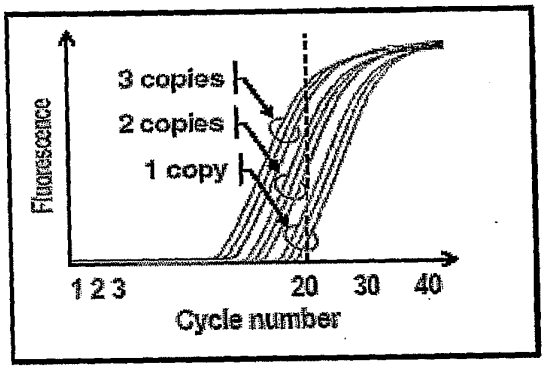


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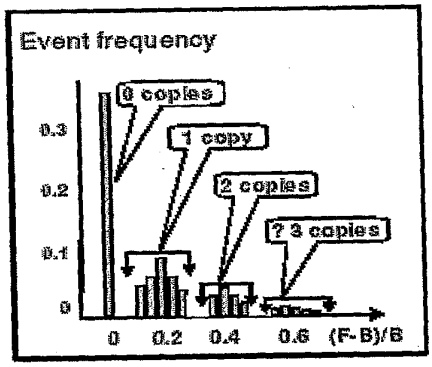
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FIGURE 3

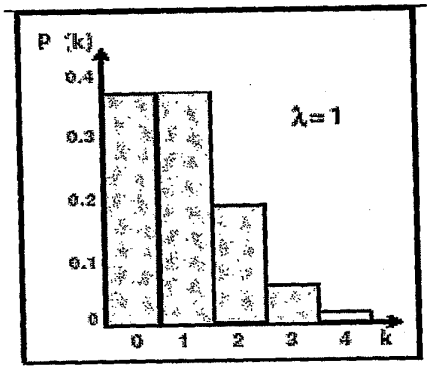
A



B



C

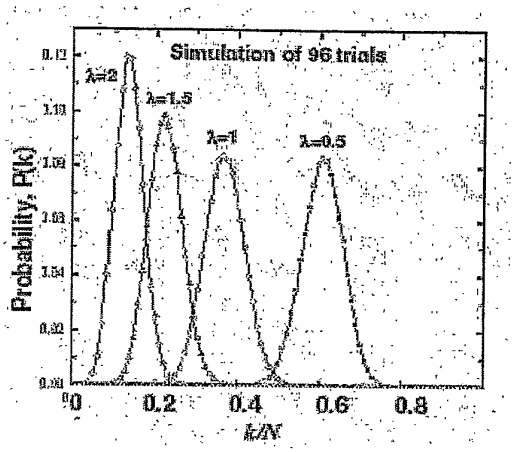


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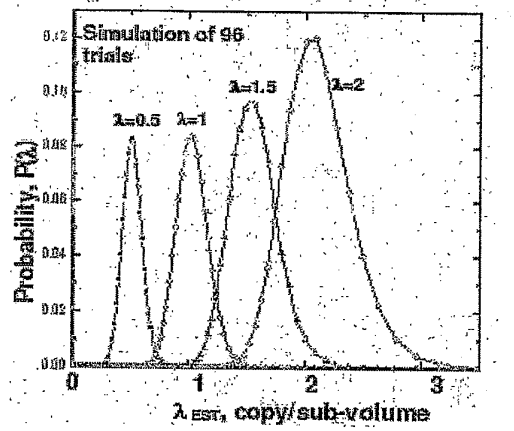
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FIGURE 4

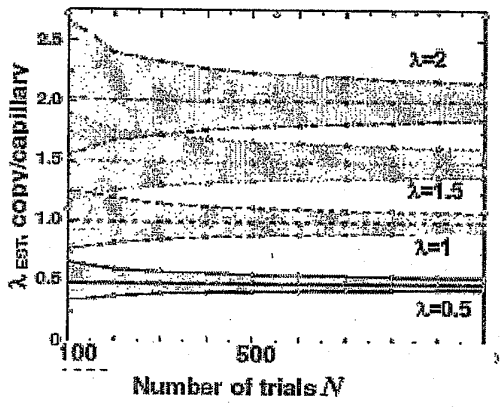
A



B



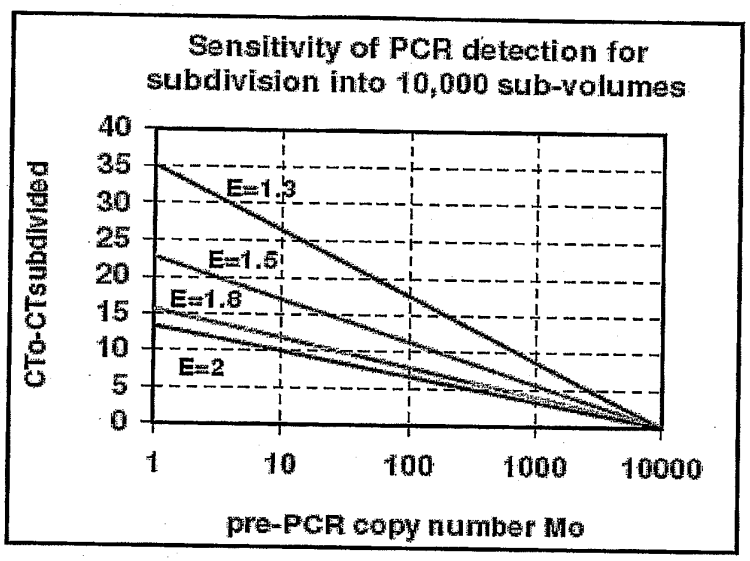
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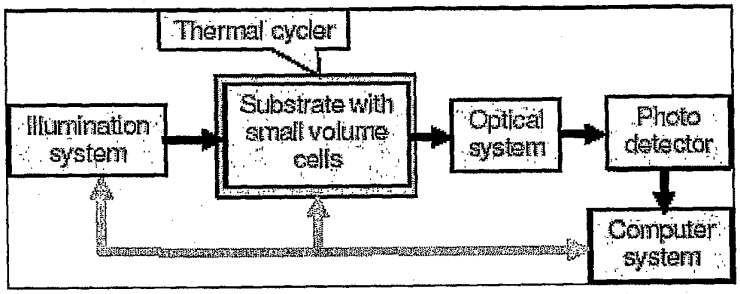
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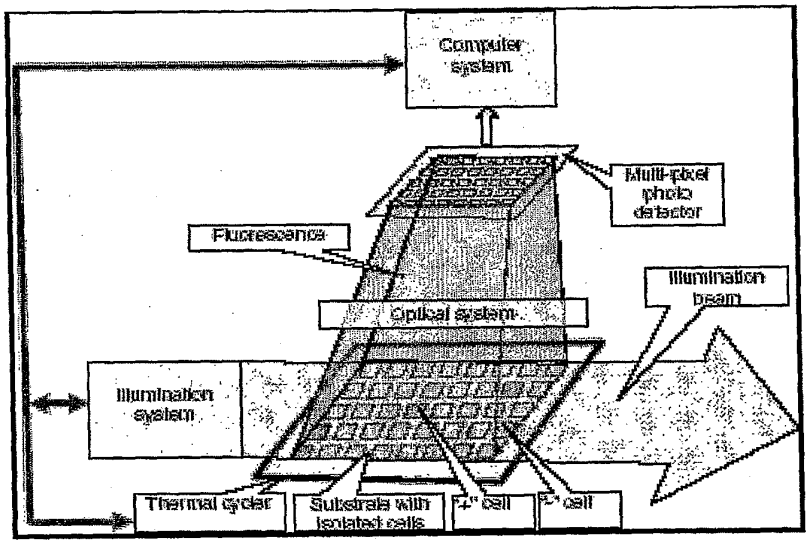


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FIGURE 6

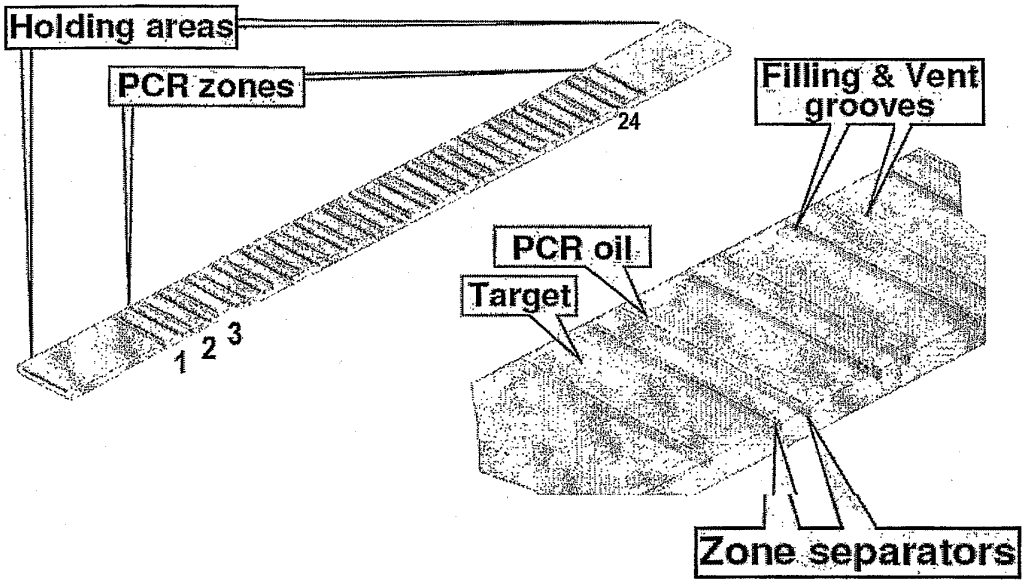




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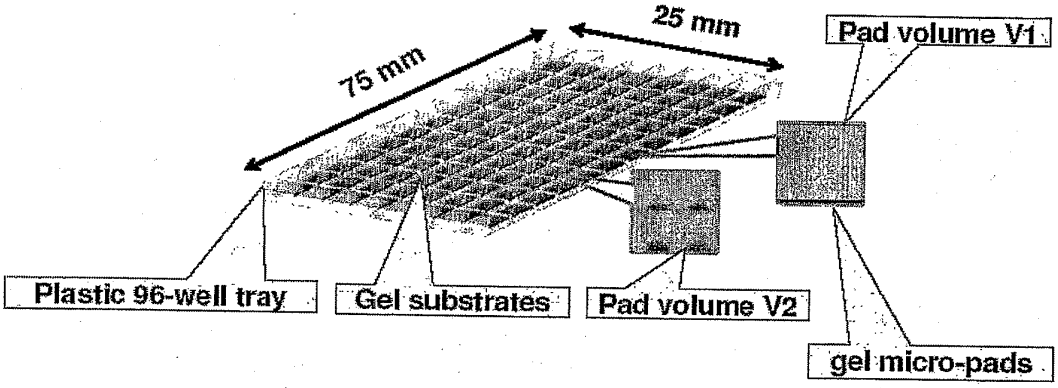
FIGURE 7



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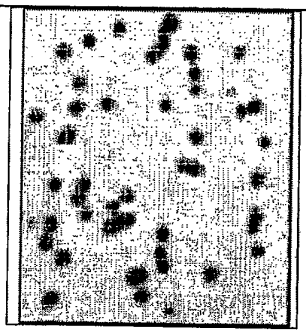
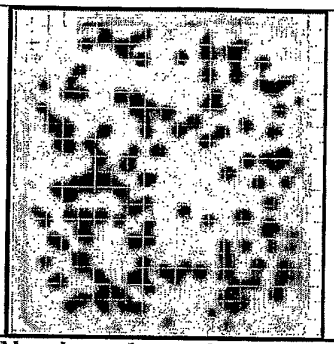
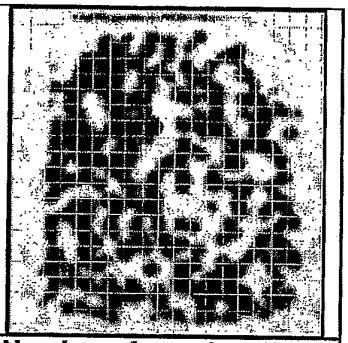
FIGURE 8

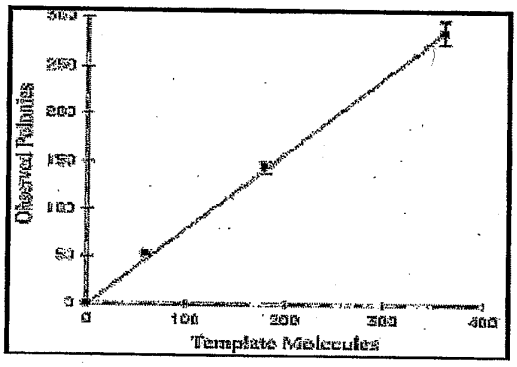


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FIGURE 9

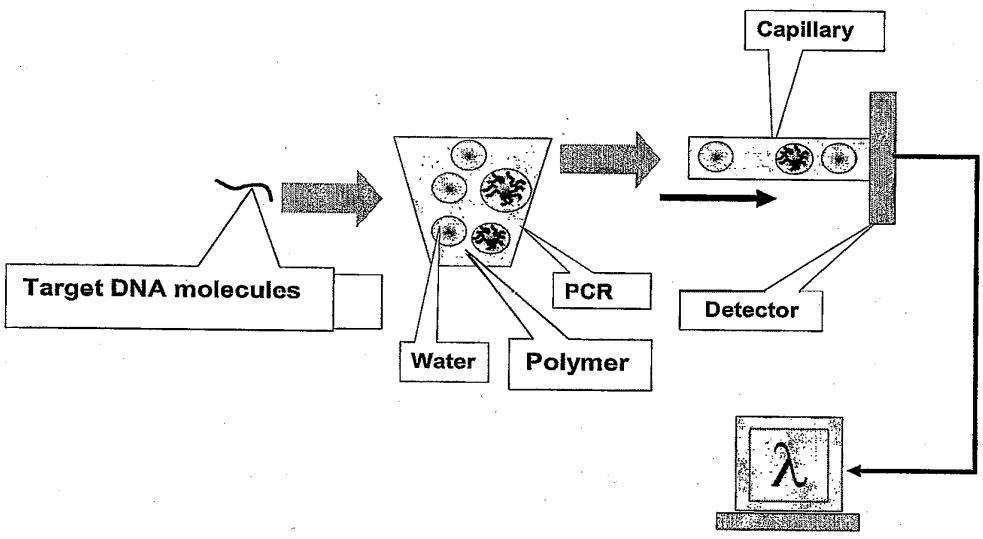
		
Number of template DNA molecules =60	**Number of template DNA molecules =180**	**Number of template DNA molecules =360**
Calculation Total grid cells N=342 Empty cells m=290 λ_{EST}=-ln(290/342)=0.165 m_1=342×0.165≈56	**Calculation** Total grid cells N=360 Empty cells m=220 λ_{EST} =-ln(220/360)=0.492 m_1=360×0.492≈177	**Calculation** Total grid cells N=360 Empty cells m=132 λ_{EST} =-ln(132/360)=1.03 m_1=360×1.03≈361



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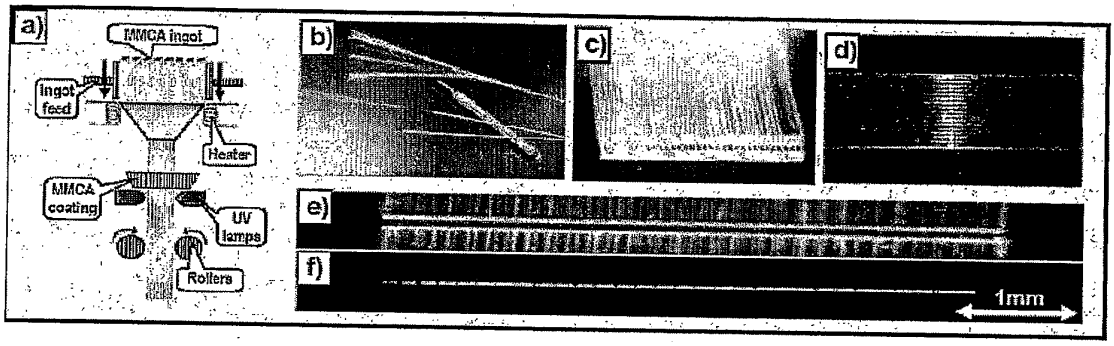
FIGURE 10



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FIGURE 11



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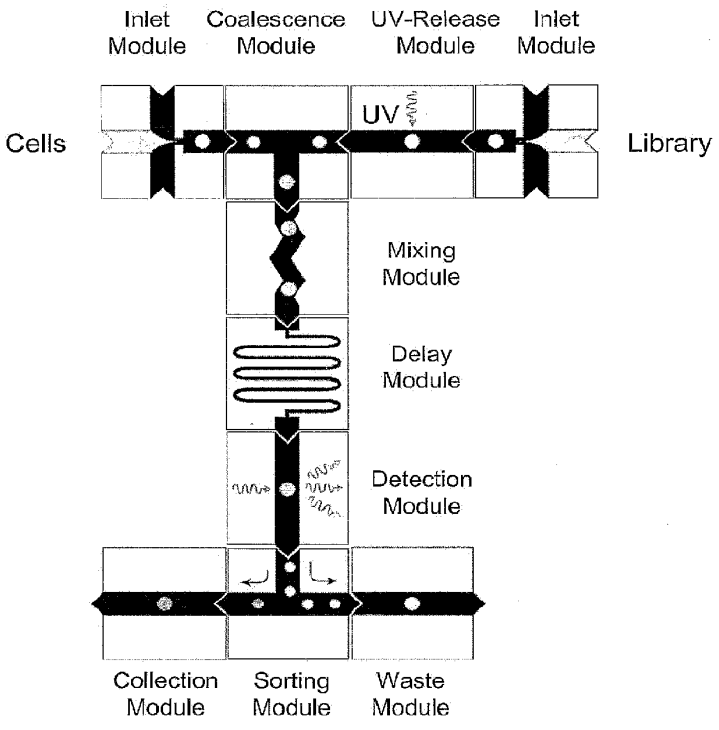
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[Continued on next page]

(54) Title: MICROFLUIDIC DEVICES AND METHODS OF USE IN THE FORMATION AND CONTROL OF NANOREACTORS



(57) Abstract: The present invention provides novel microfluidic devices and methods that are useful for performing high-throughput screening assays and combinatorial chemistry. Such methods can include labeling a library of compounds by emulsifying aqueous solutions of the compounds and aqueous solutions of unique liquid labels on a microfluidic device, which includes a plurality of electrically addressable, channel bearing fluidic modules integrally arranged on a microfabricated substrate such that a continuous channel is provided for flow of immiscible fluids, whereby each compound is labeled with a unique liquid label, pooling the labeled emulsions, coalescing the labeled emulsions with emulsions containing a specific cell or enzyme, thereby forming a nanoreactor, screening the nanoreactors for a desirable reaction between the contents of the nanoreactor, and decoding the liquid label, thereby identifying a single compound from a library of compounds.

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(74) **Agent: ELRIFI, Ivor, R.**; MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO PC, One Financial Center, Boston, MA 02111 (US).

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MICROFLUIDIC DEVICES AND METHODS OF USE IN THE FORMATION AND CONTROL OF NANOREACTORS

FIELD OF INVENTION

The present invention generally relates to systems and methods for the formation and/or control of fluidic species, and articles produced by such systems and methods. More particularly, the present invention relates to the development of high throughput microfluidic devices for precision fluid handling and use of such systems in various biological, chemical, or diagnostic assays.

BACKGROUND

High throughput molecular screening (HTS) is the automated, rapid testing of thousands of distinct small molecules or probes in cellular models of biological mechanisms or disease, or in biochemical or pharmacological assays. Active compounds identified through HTS can provide powerful research tools to elucidate biological processes through chemical genetic approaches, or can form the basis of therapeutics or imaging agent development programs. HTS has experienced revolutionary changes in technology since the advent of molecular biology and combinatorial chemistry, and the incorporation of modern information management systems. Current HTS instrumentation allows screening of hundreds of thousands of compounds in a single day at a rate orders of magnitude greater than was possible a decade ago. However, there are still bottlenecks which currently limit HTS capacity, such as (a) compound collection maintenance, tracking, and disbursement, and (b) rapidity, accuracy, and content of assay instrumentation.

The manipulation of fluids to form fluid streams of desired configuration, discontinuous fluid streams, droplets, particles, dispersions, etc., for purposes of fluid delivery, product manufacture, analysis, and the like, is a relatively well-studied art. For example, highly monodisperse gas bubbles, less than 100 microns in diameter, have been produced using a technique referred to as capillary flow focusing. In this technique, gas is forced out of a capillary tube into a bath of liquid, where the tube is positioned above a small orifice, and the contraction flow of the external liquid through this orifice focuses the gas into

a thin jet which subsequently breaks into equal-sized bubbles via a capillary instability. A similar arrangement can be used to produce liquid droplets in air.

Microfluidic systems have been described in a variety of contexts, typically in the context of miniaturized laboratory (e.g., clinical) analysis. Other uses have been described as well. For example, International Patent Application Publication No. WO 01/89788 describes multi-level microfluidic systems that can be used to provide patterns of materials, such as biological materials and cells, on surfaces. Other publications describe microfluidic systems including valves, switches, and other components.

Precision manipulation of streams of fluids with microfluidic devices is revolutionizing many fluid-based technologies. Networks of small channels are a flexible platform for the precision manipulation of small amounts of fluids. The utility of such microfluidic devices depends critically on enabling technologies such as the microfluidic peristaltic pump, electrokinetic pumping, dielectrophoretic pump or electrowetting driven flow. The assembly of such modules into complete systems provides a convenient and robust way to construct microfluidic devices. However, virtually all microfluidic devices are based on flows of streams of fluids; this sets a limit on the smallest volume of reagent that can effectively be used because of the contaminating effects of diffusion and surface adsorption. As the dimensions of small volumes shrink, diffusion becomes the dominant mechanism for mixing leading to dispersion of reactants; moreover, surface adsorption of reactants, while small, can be highly detrimental when the concentrations are low and volumes are small. As a result, current microfluidic technologies cannot be reliably used for applications involving minute quantities of reagent; for example, bioassays on single cells or library searches involving single beads are not easily performed. An alternate approach that overcomes these limitations is the use of aqueous droplets in an immiscible carrier fluid; these provide a well defined, encapsulated microenvironment that eliminates cross contamination or changes in concentration due to diffusion or surface interactions. Droplets provide the ideal microcapsule that can isolate reactive materials, cells, or small particles for further manipulation and study. However, essentially all enabling technology for microfluidic systems developed thus far has focused on single phase fluid flow and there are few equivalent active means to manipulate droplets requiring the development of droplet handling technology. While significant advances have been made in dynamics at the macro-or microfluidic scale, improved techniques and the results of these techniques are still needed. For example, as the scale of these reactors shrinks, contamination effects due to surface adsorption and diffusion limit the smallest quantities that can be used. Confinement of

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reagents in droplets in an immiscible carrier fluid overcomes these limitations, but demands new fluid-handling technology.

Furthermore, the underlying physics of the influence of electric fields on fluids is well known. The attractive and repulsive forces produced by an electric field on positive or negative charges give rise to the forces on charged fluid elements, the polarization of non-polar molecules, and the torque on polar molecules which aligns them with the field. In a non-uniform field, because the force on the positively charged portion of the distribution is different than the force on the negatively charged portion, polar molecules will also experience a net force toward the region of higher field intensity. In the continuum limit, the result is a pondermotive force in the fluid. In the limit of high droplet surface tension, it is useful to describe the net pondermotive force on a droplet as if it were a rigid sphere:

$$F = qE + 2\pi\Re(\varepsilon_m)r^3\Re(K)\nabla E^2,$$

where the first term is the electrophoretic force on the droplet (q is the net droplet charge and E is the electric field), and the second term is the dielectrophoretic force (r is the radius of the sphere, $\Re(K)$ is the real part of the Clausius-Mossotti factor

$$K = (\varepsilon^*_p - \varepsilon^*_m)/(\varepsilon^*_p + 2\varepsilon^*_m),$$

and ε^*_p and ε^*_m are the complex permittivities of the droplet and carrier fluid).

Although utility of electrophoretic control of droplets is great, it does have significant limitations. First, the charging of droplets is only effectively accomplished at the nozzle. Second, the discharge path required to eliminate screening effects also discharges the droplets. Third, finite conductivity of the carrier fluid, however small, will eventually discharge the droplets. Therefore, once the droplet is formed, there is essentially only one opportunity to perform any pondermotive function which relies on the droplet's charge density (such as coalescing oppositely charged droplets through their mutual Coulombic attraction, or electrophoretically sorting a droplet), and that function can only be performed as long as sufficient charge has not leaked off of the droplet.

Thus, it would be desirable to develop an electrically addressable emulsification system that combines compartmentalization and electrical manipulation, which allows for multi-step chemical processing, including analysis and sorting, to be initiated in confinement with exquisite timing and metering precision, for use in a variety of chemical, biological, and screening assays, in which the cost and time to perform such assays would be drastically reduced. It would also be desirable to develop a device using dielectrophoretic force (which does not rely on charge density) to manipulate droplets so that more than one electrical

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pondermotive function can be carried out following a significantly long delay from droplet formation.

SUMMARY OF THE INVENTION

The present invention provides devices having individual fluid handling modules that can be combined into fluid processing systems so as to perform multi-step processing of isolated components, which is essential for searching through molecular libraries for rare interactions with cells, nucleic acids, enzymes, coded microbeads, and other biomaterials. Using principles based on the electrostatic and dieletrophoretic manipulation of charged and neutral droplets 20 to 100 microns in diameter, the microfluidic devices as described herein can inexpensively encapsulate reagents, combine same, analyze, and sort in the range of 1 x 10^9 droplets per day. The present invention provides a microfluidic device that includes a microfabricated substrate. The substrate can include a plurality of electrically addressable channel bearing microfluidic modules integrally arranged with each other so as to be in fluid communication. The microfabricated substrate can have, for example, (i) one or more inlet modules that have at least one inlet channel adapted to carry a dispersed phase fluid, (ii) at least one main channel adapted to carry a continuous phase fluid, wherein the inlet channel is in fluid communication with the main channel such that the dispersed phase fluid is immiscible with the continuous phase fluid and forms a plurality of droplets in the continuous phase fluid, and (iii) a coalescence module downstream from and in fluid communication with the inlet modules via the main channel, wherein two or more droplets passing there through are coalesced to form a nanoreactor. The microfluidic device of the present invention can further include a sorting module, mixing module, delay module, UV-release module, detection module, collection module, waste module and/or acoustic actuator, and or combinations thereof, in any order. These modules are in fluid communication with the main channel. The flow of the dispersed phase and continuous phase can be pressure driven, for example.

The present invention also provides methods of creating a nanoreactor. The method includes, for example, a) providing a microfabricated substrate having a plurality of electrically addressable channel bearing microfluidic modules integrally arranged on the substrate so as to be in fluid communication with each other, thereby forming at least one main channel adapted to carry at least one continuous phase fluid; b) flowing a first dispersed phase fluid through a first inlet channel into the main channel such that one or more droplets

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is formed in the continuous phase fluid flowing therein; c) flowing a second dispersed phase fluid through a second inlet channel into the main channel such that one or more droplets is formed in the continuous phase fluid flowing therein; and d) coalescing at least one droplet formed in step (b) with at least one droplet formed in step (c) as the droplets pass through a coalescence module of the microfabricated substrate, thereby producing a nanoreactor. The coalescing step can be achieved by an electric field or passively. The first and second dispersed phase fluids can include a biological or chemical material, which can include, for example, tissues, cells, particles, proteins, antibodies, amino acids, nucleotides, small molecules, and pharmaceuticals. The nanoreactor can further be incubated within a delay module, and then interrogated for a predetermined characteristic within a detection module.

The present invention also includes methods of synthesizing a compound from two or more reactive substructures. In a particular aspect the method includes a) labeling the reactive substructures with a label unique to the substructure; b) emulsifying aqueous solutions of the labeled reactive substructures on a microfluidic device to form droplets; and c) randomly combining the droplets on the microfluidic device to form a compound. The one embodiment, the method further includes d) screening the compound formed in step (c) based on a desirable chemical or biological property exhibited by the compound; and e) identifying the structure of the compound by decoding the label. In another embodiment, steps (a) and (b) are alternatively performed by introducing a preformed labeled emulsion.

In another aspect, the present invention provides methods for identifying a single compound from a library on a microfluidic device. The method can include a) labeling a library of compounds by emulsifying aqueous solutions of the compounds and aqueous solutions of unique liquid labels, whereby each compound is labeled with a unique liquid label; b) pooling the labeled emulsions resulting from step (a); c) coalescing the labeled emulsions with emulsions containing a specific cell or enzyme, thereby forming a nanoreactor; d) screening the nanoreactors for a desirable reaction between the contents of the nanoreactor; and e) decoding the liquid label, thereby identifying a single compound from a library of compounds. In various embodiments of the method, the contents of the nanoreactor can be incubated prior to screening. The screening step can be performed by fluorescent polarization, for example. The liquid label can be a quantum dot (q-dot) or a dye.

In yet another aspect, the present invention includes methods for controlling the quality of a library of emulsified compounds. The method can include, for example, a) providing a library of emulsified compounds; b) emulsifying a q-dot encoded aqueous buffer in an inert fluorocarbon medium, thereby forming droplets; c) incubating the q-dot encoded

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droplet with the library of emulsified compounds; d) sorting the q-dot encoded droplet away from the library; e) analyzing the q-dot encoded droplet for the presence of any of the compounds emulsified in the library; and f) eliminating the compounds identified in step (e) from the library of emulsified compounds, wherein one or more of steps (a) - (f) are performed on a microfluidic device. In one embodiment, the analyzing step is performed by mass spectroscopy.

In still a further aspect, the present invention provides methods for sorting cells. The method can include a) fusing an affinity-reagent to an enzyme; b) mixing the fusion product of step (a) with a cell population; c) isolating cells attached to the fusion product; d) emulsifying the cells of step (c) in an inert fluorocarbon medium; e) coalescing the cell emulsion of step (d) with an emulsion comprising a substrate corresponding to the enzyme of step (a), thereby forming a nanoreactor; and f) screening the nanoreactor for a desirable reaction between the contents of the nanoreactor, wherein one or more steps of (a) - (f) are performed on a microfluidic device. In one embodiment, the affinity-reagent can be an antibody that is specific for a cell-surface cancer marker. The enzyme can include alk/phos, β-galactosidase, or horseradish peroxidase. The affinity-reagent can be fused to multiple enzymes, and multiple substrates can be emulsified and coalesced with the cell emulsions.

Another aspect of the present invention provides methods for sequencing individual exons from individual chromosomes. The method can include, for example, a) emulsifying specific primer-pairs to an exon with beads that can bind to said primer-pairs; b) pooling the emulsions of step (a) to create a library emulsion; c) providing a separate chromosomal DNA emulsion; d) coalescing the library emulsion of step (b) with the chromosomal emulsion of step (c), thereby forming a nanoreactor; e) amplfying the DNA in the nanoreactor; f) isolating the beads; g) screening for beads containing DNA; and h) sequencing the beads containing DNA, wherein one or more steps of (a) - (h) are performed on a microfluidic device.

BRIEF DESCRIPTION OF DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying drawings, which are schematic and are not intended to be drawn to scale. In the drawings, each identical or nearly identical component illustrated is typically represented by a single numeral. For the purposes of clarity, not every component is labeled in every drawing, nor is every component of each embodiment of the

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invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. In the drawings:

Figure 1 is an schematic illustrating the interacting modules of a microfluidic device of the present invention.

Figure 2 is a photograph and accompanying graphs showing the flow cytometric-cell-based assay for human and bacterial cells.

Figure 3 is a schematic and graph showing simultaneous two color fluorescence detection.

Figure 4 is a schematic illustrating chemical library screening using a nanoreactor of the present invention.

Figure 5 is a schematic illustrating the physical basis of fluorescence polarization assays.

Figure 6 is several graphs showing the absorption and emission spectra of q-dots and organic dye.

Figure 7 is a schematic showing the extension to the fluorescence test station required to perform polarization fluorescence measurements simultaneously with q-dot readout.

Figure 8 is a schematic and accompany graphs showing a fluorescence polarization based kinase assay.

Figure 9 is a schematic illustrating the retrosynthetic analysis of Gleevec.

Figure 10 is a schematic illustrating the synthesis of a fluorescent and non-fluorescent product from the same precursor.

Figure 11 is schematic showing the use of nucleic acids for chemical encoding and decoding tagging of chemical reactions.

Figure 12 is a schematic illustrating a taqman assay and molecular beacon probes.

Figure 13 is a graph showing polarized fluorescence signals.

Figure 14 is a graph showing a time trace and histogram of fluorescence polarization calculated from analyzed droplets.

Figure 15 (A) is a schematic illustrating dielectrophoretic stopping of droplet A allowing droplet B to contact A and coalesce. The dielectrophoretic force is not strong enough to stop the combined A+B and they move off in the stream. This is shown in the photomicrograph, Figure 15 (B).

Figure 16 is a schematic (a) and photographs (b, e) showing droplets made on-chip (A) being interdigitated with library droplets (B). The droplets are of different size and the

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smaller droplets B move at a higher velocity than droplets A until they catch up after which they move together. An electric field causes the droplets to coalesce, Figure 16 (c). Droplets A and B may both come from libraries (made off-chip) Figure 16 (d), or be made on-chip.

Figure 17 (A-D) shows alternate ways to achieve interdigitation of droplets of different type.

Figure 18A-D highlight observed passive coalescence of coupled droplet pairs - (A) Tee 0.25mM FC-1%E5-5%PVP 70 - FF 1%E5-5%PVP-0.1mM FC-PBS 70-11172005-nozzles-3.cin; (B) Tee 0.25mM FC-1%E5-5%PVP 90 - FF 1%E5-5%PVP-0.1mM FC-PBS 70-11172005-nozzles-5.cin; (C) Tee 0.25mM FC-1%E5-5%PVP 90 - FF 1%E5-5%PVP-0.1mM FC-PBS 70-11172005-nozzles-prior to coalesce-8.cin; (D) 1%E5-5%PVP-0.25mM FC- 50 - 1%E5-5%PVP-0.1mM FC-PBS 50-11172005-overview-1.cin.

Figure 19 shows a schematic diagram of the assembly of modules used for sequencing exons of individual chromosomes. (A) Individual specific primer-pairs to different exons along with a primer-bound bead are each *separately* emulsified and then pooled to create a library emulsion (a set of 96 exon primer pairs are shown for illustrative purposes); (B) Individual modules are strung together in a sequence of droplet operations. A chromosomal DNA solution is diluted such that a 30 micron drop contains, on average, slightly less than a half-genome's concentration of DNA. Droplets from the pooled emulsion library set of exon-specific primers are combined with droplets containing the diluted solution of chromosomal DNA and used in a bead-based DNA amplification reaction (i.e., PCR); (C) The DNA-containing beads will be randomly placed into a picotiter plate and sequenced using a 454 Corp.'s Life Sciences DNA sequencing instrument.

Figure 20 describes sample preparation and DNA sequencing on the 454 Instrument. A) Genomic DNA is isolated, fragmented, ligated to adapters and separated into single strands (top left). Fragments are bound to beads under conditions that favor one fragment per bead, the beads are captured in the droplets of a PCR-reaction-mixture-in-oil emulsion and PCR amplification occurs within each droplet, resulting in beads each carrying ten million copies of a unique DNA template (top, second from the left). The emulsion is broken, the DNA strands are denatured, and beads carrying single-stranded DNA clones are deposited into wells of a fiber-optic slide (bottom left). Smaller beads carrying immobilized enzymes required for pyrophosphate sequencing are deposited into each well (bottom, second from the left); B) Microscope photograph of emulsion showing droplets containing a bead and empty droplets. The thin arrow points to a 28-mm bead; the thick arrow points to an approximately 100-mm droplet; C) Scanning electron micrograph of a portion of a fiber-optic slide, showing

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fiber-optic cladding and wells before bead deposition; D) The sequencing instrument consists of the following major subsystems: a fluidic assembly; E) a flow chamber that includes the well-containing fiber-optic slide; F) a CCD camera-based imaging assembly; G) and a computer that provides the necessary user interface and instrument control.

Figure 21 shows measurement of DNA hybridization using fluorescence polarization (FP) on the microfluidic device described herein. *(A)* FP can be used to measure the binding of 2 DNA molecules on the microfluidic device. (*B*) Oligonucleotide 102 is complement to both 101 and 103. The addition of either oligonucleotide to the labeled 102 shifts the mP value, indicative of binding. Addition of 102 to non-complementing oligos does not change its mP (data not shown). Oligonucleotides; #101:5' Biotin- ATCCGCCCCAGCA GCTGCCAGGCACAGCCCCTAAACTCCTGATTTATGCTGCATCCATTTTGC 3'; #102: 5' Fluorescein- GCAAAATGGATGCAGCATAAATCAGGAGTTTAG 3'; #103: 5' Fluorescein- CTAAACTCCTGATTTA TGCTGCATCCATTTTGC-3'.

DETAILED DESCRIPTION

The microfluidic devices and methods of use described herein are based on the creation and electrical manipulation of aqueous phase droplets completely encapsulated by an inert fluorocarbon oil stream. This combination enables electrically addressable droplet generation, highly efficient droplet coalescence, precision droplet breaking and recharging, and controllable single droplet sorting. Additional passive modules include multi-stream droplet formulations, mixing modules, and precision break-up modules. The integration of these modules is an essential enabling technology for a droplet based, high-throughput microfluidic reactor system. The microfluidic devices of the present invention can use a flow-focusing geometry to form the droplets. For example, a water stream can be infused from one channel through a narrow constriction; counter propagating oil streams (preferably fluorinated oil) hydrodynamically focus the water stream and stabilize its breakup into micron size droplets as it passes through the constriction. In order to form droplets, the viscous forces applied by the oil to the water stream must overcome the water surface tension. The generation rate, spacing and size of the water droplets is controlled by the relative flow rates of the oil and the water streams and nozzle geometry. While this emulsification technology is extremely robust, droplet size and rate are tightly coupled to the fluid flow rates and channel dimensions. Moreover, the timing and phase of the droplet

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production cannot be controlled. To overcome these limitations, the microfluidic devices of the present invention can incorporate integrated electric fields, thereby creating an electrically addressable emulsification system. In one embodiment, this can be achieved by applying high voltage to the aqueous stream and charge the oil water interface. The water stream behaves as a conductor while the oil is an insulator; electrochemical reactions charge the fluid interface like a capacitor. At snap-off, charge on the interface remains on the droplet. The droplet size decreases with increasing field strength. At low applied voltages the electric field has a negligible effect, and droplet formation is driven exclusively by the competition between surface tension and viscous flow, as described above. The microfluidic, droplet-based reaction-confinement system of the present invention can further include a mixer which combines two or more reagents to initiate a chemical reaction. Multi-component droplets can easily be generated by bringing together streams of materials at the point where droplets are made. However, all but the simplest reactions require multiple steps where new reagents are added during each step. In droplet-based microfluidic devices, this can be best accomplished by combining (*i.e.* coalescing) different droplets, each containing individual reactants. However, this is particularly difficult to achieve in a microfluidic device because surface tension, surfactant stabilization, and drainage forces all hinder droplet coalescence; moreover, the droplets must cross the stream lines that define their respective flows and must be perfectly synchronized to arrive at a precise location for coalescence. The microfluidic devices of the present invention overcome these difficulties by making use of electrostatic charge, placing charges of opposite sign on each droplet, and applying an electric field to force them to coalesce. By way of non-limiting example, a device according to the present invention can include two separate nozzles that generate droplets with different compositions and opposite charges. The droplets are brought together at the confluence of the two streams. The electrodes used to charge the droplets upon formation also provide the electric field to force the droplets across the stream lines, leading to coalescence. In the absence of an electric field, droplets in the two streams do not in general arrive at the point of confluence at exactly the same time. When they do arrive synchronously the oil layer separating the droplets cannot drain quickly enough to facilitate coalescence and as a result the droplets do not coalesce. In contrast, upon application of an electric field, droplet formation becomes exactly synchronized, ensuring that droplets each reach the point of confluence simultaneously (*i.e.*, paired droplets).

Moreover, since the droplets are oppositely charged they are attracted to one another, which forces them to traverse the fluid stream lines and contact each other, thereby causing

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them to coalesce. The remarkable synchronization of the droplet formation results from coupling of the break-off of each of the pair of droplets as mediated by the electric field. The use of oppositely charged droplets and an electric field to combine and mix reagents is extremely robust, and 100% of the droplets coalesce with their partner from the opposite stream.

Other embodiments of the microfluidic devices of the present invention can include a droplet sorter. The contents of individual droplets must be probed, and selected droplets sorted into discreet streams. In one embodiment, such sorting in microfluidic devices can be accomplished through the use of mechanical valves. In another embodiment of the present invention, the use of electrostatic charging of droplets provides an alternate means that can be precisely controlled, can be switched at high frequencies, and requires no moving parts. Electrostatic charge on the droplets enables drop-by-drop sorting based on the linear coupling of charge to an external electric field. As an example, a T-junction bifurcation that splits the flow of carrier fluid equally will also randomly split the droplet population equally into the two streams. However, a small electric field applied at the bifurcation precisely dictates which channel the drops enter. Varying the direction of the field varies the direction of the sorted droplets. The large forces that can be imparted on the droplets and the short time required to switch the field make this a fast and robust sorting engine with no moving parts; thus the processing rate is limited only by the rate of droplet generation and electric field switching time, and can easily exceed 20,000 per second.

Accordingly, in one embodiment the present invention provides a microfluidic device comprising a microfabricated substrate comprising at least one inlet channel adapted to carry at least one dispersed phase fluid and at least one main channel adapted to carry a continuous phase fluid, where inlet channel is in fluid communication with the main channel at one or more inlet modules such that the dispersed phase fluid is immiscible with the continuous phase fluid and forms a plurality of droplets in the continuous phase fluid; a coalescence module, where an electric field is applied to cause two or more droplets to coalesce; and c) a detection module including a detection apparatus for evaluating the contents and/or characteristics of the coalesced droplets produced in the coalescence module. The microfabricated substrate can further comprise one or more sorting modules, collection modules, waste modules, branch channels, delay modules, mixing modules and/or UV release modules, or any combinations thereof in any order. Figure 1.

The present invention also provides methods of creating a nanoreactor. The method includes a) providing a microfabricated substrate comprising at least one inlet channel

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adapted to carry at least one dispersed phase fluid and at least one main channel adapted to carry a continuous phase fluid, where the inlet channel is in fluid communication with the main channel at one or more inlet modules, and where the dispersed phase fluid is immiscible with the continuous phase fluid; b) flowing a first dispersed phase fluid through a first inlet channel such that the first dispersed phase fluid forms one or more droplets in the continuous phase fluid; c) flowing at least a second dispersed phase fluid through an at least second inlet channel such that the second dispersed phase fluid forms one or more droplets in the continuous phase fluid; and d) coalescing at least one droplet formed in step (b) with at least one droplet formed in step (c) under the influence of an electric field, thereby producing a nanoreactor.

The present invention also provides a method for manipulating a nanoreactor. The method includes providing a nanoreactor as described herein; providing a plurality of electrically addressable channel bearing microfluidic modules integrally arranged with each other on a microfabricated substrate so as to be in fluid communication and providing a control system for manipulating the nanoreactor.

The present invention also provides methods of manipulating biological/chemical material. The method includes a) providing a microfabricated substrate comprising at least one inlet channel adapted to carry at least one dispersed phase fluid and at least one main channel adapted to carry a continuous phase fluid, where the inlet channel is in fluid communication with the main channel at one or more inlet modules, and where the dispersed phase fluid is immiscible with the continuous phase fluid; b) flowing a first dispersed phase fluid comprising a first biological/chemical material through a first inlet channel such that the first dispersed phase fluid resides as one or more droplets in the continuous phase fluid; c) flowing at least a second dispersed phase fluid comprising a second biological/chemical material through a second inlet channel such that the second dispersed phase fluid resides as one or more droplets in the continuous phase fluid; d) slowing or stopping at least one droplet formed in step (b) by exerting a dielectrophoretic force onto the droplet; e) coalescing at least one droplet formed in step (c) with the droplet slowed or stopped in step (d) under the influence of an electric field within a coalescence module, thereby producing a nanoreactor; f) incubating the nanoreactor within a delay module; and g) interrogating the nanoreactor for a predetermined characteristic within a detection module. Slowing or stopping the droplets from step (b) allows pairing of the droplets from step (c) before they move to the location (e) where they are driven to coalesce by an electric field, or passively by passing through a narrowing of the channel.

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In step (d), the pairing of droplets from (b) and (c) may be achieved in one of three ways: (i) using the dielectrophoretic force produced by the electric field gradient; (ii) using droplets of two different sizes, which works best when one droplet is comparable to the channel width and one droplet is smaller than the channel width, so that the smaller droplet catches up to the larger droplet; and (iii) the droplet in steps (b) and (c) have different viscosities, and thus, move at different velocities. Preferably, the droplets are of different sizes, and more preferably, the larger droplet has enough volume so that it would have a diameter greater than the channel width if it were spherical.

Methods of sorting biological/chemical material, although frequently desired, is not necessary in order to use the devices or practice the methods of the present invention. In particular, the devices and methods of the invention also include embodiments wherein the biological/chemical material is analyzed and/or identified, but is not sorted. The generation of nanoreactors through the coalescence of two droplets, although frequently desired, is not necessary in order to use the devices or practice the methods of the present invention. In particular, the devices and methods of the invention also include embodiments wherein the biological/chemical material is sorted without a coalescence event.

Substrates

The present invention also provides methods of producing a microfluidic device. The method of producing a microfluidic device comprises one or more of the following steps in any combination: 1) hard lithography, 2) soft lithography, 3) extraction and/or punch though, 4) bonding, 5) channel coating, 6) interconnect assembly, 7) electrode injection and 8) waveguide injection and fiber installation. The foregoing steps are described in more detail herein.

An "analysis unit" is a microfabricated substrate, e.g., a microfabricated chip, having at least one inlet channel, at least one main channel, at least one coalescence module, and at least one detection module. The analysis unit can further contain one or more sorting module. The sorting module can be in fluid communication with branch channels in communication with one or more outlet modules (collection module or waste module). For sorting, at least one detection module cooperates with at least one sorting module to divert flow via a detector-originated signal. It shall be appreciated that the "modules" and "channels" are in fluid communication with each other and therefore may overlap; i.e., there may be no clear boundary where a module or channel begins or ends. A device according to the invention may comprise a plurality of analysis units.

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A variety of channels for sample flow and mixing can be microfabricated on a single chip and can be positioned at any location on the chip as the detection or sorting modules, e.g., for kinetic studies. A plurality of analysis units of the invention may be combined in one device. Microfabrication applied according to the invention eliminates the dead time occurring in conventional gel electrophoresis or flow cytometric kinetic studies, and achieves a better time-resolution. Furthermore, linear arrays of channels on a single chip, i.e., a multiplex system, can simultaneously detect and sort a sample by using an array of photo multiplier tubes (PMT) for parallel analysis of different channels. This arrangement can be used to improve throughput or for successive sample enrichment, and can be adapted to provide a very high throughput to the microfluidic devices that exceeds the capacity permitted by conventional flow sorters. Circulation systems can be used in cooperation with these and other features of the invention. Positive displacement pressure driven flow is a preferred way of controlling fluid flow and electric fields and electric field gradients are a preferred way of manipulating droplets within that flow.

Microfabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR, moving cells using optical tweezer/cell trapping, transformation of cells by electroporation, μTAS, and DNA hybridization. Detectors and/or light filters that are used to detect cellular characteristics of the reporters can also be fabricated directly on the chip. Preferably, detectors are off-chip free space optics or off-chip electronics with on-chip leads.

A device of the invention can be microfabricated with a sample solution reservoir or well or other apparatus for introducing a sample to the device, at the inlet module, which is typically in fluid communication with an inlet channel. A reservoir may facilitate introduction of molecules or cells into the device and into the sample inlet channel of each analysis unit. An inlet module may have an opening such as in the floor of the microfabricated chip, to permit entry of the sample into the device. The inlet module may also contain a connector adapted to receive a suitable piece of tubing, such as liquid chromatography or HPLC tubing, through which a sample may be supplied. Such an arrangement facilitates introducing the sample solution under positive pressure in order to achieve a desired infusion rate at the inlet module.

A microfabricated device of the invention is preferably fabricated from a silicon microchip or silicon elastomer. The dimensions of the chip are those of typical microchips, ranging between about 0.5 cm to about 7.5 cm per side and about 1 micron to about 1 cm in thickness. A microfabricated device can be transparent and can be covered with a material

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having transparent properties, such as a glass coverslip, to permit detection of a reporter, for example, by an optical device such as an optical microscope.

The device of the present invention can comprise inlet and outlet interconnects. The interconnections, including tubes, must be extremely clean and make excellent bonding with the PDMS surface in order to allow proper operation of the device. The difficulty in making a fluidic connection to a microfluidic device is primarily due to the difficulty in transitioning from a macroscopic fluid line into the device while minimizing dead volume. Development of a commercial microfluidic platform requires a simple, reliable fluidic interconnect in order to reduce the chance of operator and error leaks. The curing and manufacturing of the PDMS slab with the tubes already placed on the silicon wafer accomplish these goals.

The template process can include, but is not limited to, the following features. In order to minimize contamination and leakage, process operations that allow for greater reproducibility and reliability are improved. Tubes and interconnects for the PDMS slab can be cured in place. The tubes and interconnects can be placed in position by applying a UV-cured adhesive to allow for holding the tubes in place on the silicone wafer. Once the tubes are placed in position, PDMS can be poured over the wafer and cured. The cured PDMS, along with the tubes in place, can be peeled off of the silicone wafer easily. This process can be applied to fluidics channels as well as other connection channels. Once the adhesive is applied onto the wafer, the process will allow for quick templating of PDMS slabs with exact reproducibility of channel locations and cleanliness. Tubes of any size can be implemented for this process. This process allows for less stress on the interconnection joints and smaller interconnection footprints in the device.

In one embodiment, small interconnects based on creating a face seal between the tubing and the device are used. A grommet may be placed into either a tapered hole or a hole with perpendicular walls. In one embodiment, the raised contact surface between the two sides is formed on the tubing side instead of the device side. In another embodiment, the sealing feature can be molded into the device. In yet another embodiment, a possible interconnect can be molded and bonded on a glass substrate directly from PDMS. In this embodiment, a thin film of PDMS can be simultaneously formed and bonded to the top of the glass slide and permits the use of isolated patterned electrodes and heating elements beneath the fluid channels. If not required, the seals could be made without the top skin. The raised contact surface could also be built into the tubing side. The sealing surface on the tubing side of the connection can be formed directly into the face of the tubing, although a separate piece secured to the tubing assembly/retaining nut may also be used.

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The tubing side of the interconnect can be mounted into a retaining block that provides precise registration of the tubing, while the microfluidic device can be positioned accurately in a carrier that the retaining block would align and clamp to. The total dead volume associated with these designs would be critically dependent on how accurately the two mating surfaces could be positioned relative to each other. the maximum force required to maintain the seal would be limited by the exact shape and composition of the sealing materials as well as the rigidity and strength of the device itself. The shapes of the mating surfaces can be tailored to the minimal leakage potential, sealing force required, and potential for mis-alignment. By way of non-limiting example, the single ring used in the fluidic interconnects can be replaced with a series of rings of appropriate cross-sectional shape.

The device of the present invention can comprise a layer, such as a glass slide, which is perforated for functional interconnects, such as fluidic, electrical, and/or optical interconnects, and sealed to the back interface of the device so that the junction of the interconnects to the device is leak-proof. Such a device can allow for application of high pressure to fluid channels without leaking.

A silicon substrate containing the microfabricated flow channels and other components is preferably covered and sealed, most preferably with a transparent cover, e.g., thin glass or quartz, although other clear or opaque cover materials may be used. When external radiation sources or detectors are employed, the detection module is covered with a clear cover material to allow optical access to the cells. For example, anodic bonding 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.

The present invention provides improved methods of bonding PDMS to incompatible media. Normal methods of bonding various materials (plastic, metals, etc) directly to materials such as PDMS, silicone, Teflon, and PEEK using traditional bonding practices (adhesives, epoxies, etc) do not work well due to the poor adhesion of the bonding agent to materials such as PDMS. Normal surface preparation by commercially available surface activators has not worked well in microfluidic device manufacturing. This problem is eliminated by treating the PDMS surface to be bonded with high intensity oxygen or air plasma. The process converts the top layer of PDMS to glass which bonds extremely well with normal adhesives. Tests using this method to bond external fluid lines to PDMS using a UV-cure adhesive (Loctite 352, 363, and others) resulted in a bond that is stronger than the PDMS substrate, resulting in fracture of the PDMS prior to failure of the bond. The present

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method combines high radiant flux, wavelength selection, and cure exposure time to significantly enhance the bond strength of the adhesive.

Channels

The invention provides microfluidic devices having channels that form the boundary for a fluid. The channels of the device carry a mixture of incompatible or immiscible fluids, such as an oil-water mixture. Droplets of aqueous solution containing a biological/chemical material are dispersed within the oil or other incompatible solvent. Each droplet of this multi-phase mixture can encapsulate one or more molecules, particles, or cells. The droplets are trapped and their boundaries are defined by channel walls, and therefore they do not diffuse and/or mix. Individual particles or molecules can be separately compartmentalized inside individual droplets. These droplets can be analyzed, combined with other droplets (e.g. to react droplet contents) and analyzed, and then sorted. Thus, the invention also provides methods for analyzing, combining, detecting and/or sorting of biological/chemical materials.

The channels present in the device can be made with micron dimensions and the volume of the detection module is precisely controlled. The planar geometry of the device allows the use of high numerical aperture optics, thereby increasing the sensitivity of the system. Because the system is entirely self-contained, there is no aerosol formation, allowing for much safer sorting of biohazardous materials. Materials sorted in the device are compartmentalized within individual droplets of an aqueous solution traveling in a flow of a second, incompatible or immiscible solution. Thus, there is no problem with the material diffusing or exchanging positions, even when sorting or analyzing extremely small particles, molecules, or reagents. In a preferred embodiment, water droplets are extruded into a flow of oil, but any fluid phase may be used as a droplet phase and any other incompatible or immiscible fluid or phase may be used as a barrier phase.

A "channel," as used herein, means a feature on or in a device (e.g. , a substrate) that at least partially directs the flow of a fluid. In some cases, the channel may be formed, at least in part, by a single component, e.g., an etched substrate or molded unit. The channel can have any cross-sectional shape, for example, circular, oval, triangular, irregular, square or rectangular (having any aspect ratio), or the like, and can be covered or uncovered (i.e., open to the external environment surrounding the channel). In embodiments where the channel is completely covered, at least one portion of the channel can have a cross-section that is completely enclosed, and/or the entire channel may be completely enclosed along its entire length with the exception of its inlet and outlet.

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A channel may have an aspect ratio (length to average cross-sectional dimension) of at least 2: 1, more typically at least 3: 1,5 : 1, or 10: 1. As used herein, a "cross-sectional dimension," in reference to a fluidic or microfluidic channel, is measured in a direction generally perpendicular to fluid flow within the channel. An open channel generally will include characteristics that facilitate control over fluid transport, e.g., structural characteristics (an elongated indentation) and/or physical or chemical characteristics (hydrophobicity vs. hydrophilicity) and/or other characteristics that can exert a force (e.g., a containing force) on a fluid. The fluid within the channel may partially or completely fill the channel. In some cases the fluid may be held or confined within the channel or a portion of the channel in some fashion, for example, using surface tension (e.g., such that the fluid is held within the channel within a meniscus, such as a concave or convex meniscus). In an article or substrate, some (or all) of the channels may be of a particular size or less, for example, having a largest dimension perpendicular to fluid flow of less than about 5 mm, less than about 2 mm, less than about 1 mm, less than about 500 microns, less than about 200 microns, less than about 100 microns, less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about 30 microns, less than about 25 microns, less than about 10 microns, less than about 3 microns, less than about 1 micron, less than about 300 nm, less than about 100nm, less than about 30 nm, or less than about 10 nm or less in some cases. In one embodiment, the channel is a capillary. Of course, in some cases, larger channels, tubes, etc. can be used to store fluids in bulk and/or deliver a fluid to the channel.

In some embodiments, the dimensions of the channel may be chosen such that fluid is able to freely flow through the channel, for example, if the fluid contains cells. The dimensions of the channel may also be chosen, for example, to allow a certain volumetric or linear flow rate of fluid in the channel. Of course, the number of channels and the shape of the channels can be varied by any method known to those of ordinary skill in the art. In some cases, more than one channel or capillary may be used. For example, two or more channels may be used, where they are positioned inside each other, positioned adjacent to each other, etc.

A "main channel" is a channel of the device of the invention which permits the flow of molecules, cells, small molecules or particles past a coalescence module for coalescing one or more droplets, a detection module for detection (identification) or measurement or a droplet and a sorting module, if present, for sorting a droplet based on the detection in the detection module. The coalescence, detection and/or sorting modules can be placed or fabricated into the main channel. The main channel is typically in fluid communication with

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an inlet channel or inlet module. An "inlet channel" permits the flow of molecules, cells, small molecules or particles into the main channel. One or more inlet channels communicate with one or more means for introducing a sample into the device of the present invention. The inlet channel communicates with the main channel at an inlet module. The main channel is also typically in fluid communication with an outlet module and optionally with branch channels, each of which may have a collection module or waste module. These channels permit the flow of cells out of the main channel.

Channels of the device of the present invention can be formed from silicon elastomer (e.g. RTV), urethane compositions, of from silicon-urethane composites such as those available from Polymer Technology Group (Berkeley, Calif.), e.g. PurSil™ and CarboSil™. The channels may also be coated with additives or agents, such as surfactants, TEFLON, or fluorinated oils such as octadecafluoroctane (98%, Aldrich), Fluorinert (FC-3283; 3M), or fluorononane, any of which can be modified to contain a fluorosurfactant. Fluorinated oils have favorable properties including chemical inertness, high gas permeability, and biocompatibility, which are desirable in micrfluidic applications. TEFLON is particularly suitable for silicon elastomer (RTV) channels, which are hydrophobic and advantageously do not absorb water, but they may tend to swell when exposed to an oil phase. Swelling may alter channel dimensions and shape, and may even close off channels, or may affect the integrity of the chip, for example by stressing the seal between the elastomer and a coverslip. Urethane substrates do not tend to swell in oil but are hydrophillic, they may undesirably absorb water, and tend to use higher operating pressures. Hydrophobic coatings may be used to reduce or eliminate water absorption. Absorption or swelling issues may also be addressed by altering or optimizing pressure or droplet frequency (e.g. increasing periodicity to reduce absorption). RTV-urethane hybrids may be used to combine the hydrophobic properties of silicon with the hydrophilic properties of urethane.

The channels of the invention are microfabricated, for example by etching a silicon chip using conventional photolithography techniques, or using a micromachining technology called "soft lithography" as described by Whitesides and Xia, Angewandte Chemie International Edition 37, 550 (1998). These and other microfabrication 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. When optical detection is employed, the invention also provides minimal light scatter from molecule, cell, small molecule or particle suspension and chamber material. Devices according to the invention are relatively inexpensive and easy to set up.

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They can also be disposable, which greatly relieves many of the concerns of gel electrophoresis (for molecules), and of sterilization and permanent adsorption of particles into the flow chambers and channels of conventional FACS machines.

The channels of the device of the present invention can be of any geometry as described. However, the channels of the device can comprise a specific geometry such that the contents of the channel are manipulated, e.g., sorted, mixed, prevent clogging, etc.

For particles (e.g., cells) or molecules that are in droplets (i.e., deposited by the inlet module) within the flow of the main channel, the channels of the device are preferably rounded, with a diameter between about 2 and 100 microns, preferably about 60 microns, and more preferably about 30 microns at the cross-flow area or droplet extrusion region. This geometry facilitates an orderly flow of droplets in the channels. Similarly, the volume of the detection module in an analysis device is typically in the range of between about 10 femtoliters (fl) and 5000 fl, preferably about 40 or 50 fl to about 1000 or 2000 fl, most preferably on the order of about 200 fl. In preferred embodiments, the channels of the device, and particularly the channels of the inlet connecting to a droplet extrusion region, are between about 2 and 50 microns, most preferably about 30 microns.

A microfluidic device can include a bifurcation geometry designed in such a manner as to minimize fluidic shear forces on droplets during sorting. Known devices describe bifurcation geometries in which significant shear forces affect droplets during sorting. Specifically droplets may experience shear forces when moving under the influence of the sorting force across the width of the input channel prior to encountering the bifurcation, and droplets may experience shear forces at the bifurcation point which are applied in such a manner as to elongate or even tear the droplet apart.

A microfluidic device comprising channels having a bifurcation geometry can minimize these shear forces by (i) including a necked-down segment of the input channel upstream of the bifurcation where the droplet is diagnosed to make the sorting decision, and/or by (ii) including a flaired-out segment of the input channel immediately prior to the bifurcation, and/or by (iii) including a fork on the far wall of the bifurcation. The shear forces are minimized by component (i) because the sorting field is applied while the droplet is in the necked-down segment. Therefore, when the droplet exits the necked-down segment, the droplet is placed on fluid streamlines, which will carry it out the desired branch of the bifurcation. Furthermore, the droplet does not significantly encounter fluid streamlines, which follow the undesired branch of the bifurcation. The shear forces are minimized by component (ii) because the droplet does not significantly impact the far wall of the

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bifurcation at a point where it would experience fluid streamlines, which follow the undesired branch of the bifurcation. The shear forces are minimized by component (iii) because the fork serves to focus the two sets of fluid streamlines (i.e., the one set which follows one branch of the bifurcation, and the other set which follows the other branch of the bifurcation) away from each other.

A microfluidic device can include a specific geometry designed in such a manner as to prevent the aggregation of biological/chemical material and keep the biological/chemical material separated from each other prior to encapsulation in droplets. The geometry of channel dimension can be changed to disturb the aggregates and break them apart by various methods, that can include, but is not limited to, geometric pinching (to force cells through a (or a series of) narrow region(s), whose dimension is smaller or comparable to the dimension of a single cell) or a barricade (place a series of barricades on the way of the moving cells to disturb the movement and break up the aggregates of cells).

Channel design can force biological/chemical material moving along the center streamline through flow focus, e.g., using two dilution channels at the entrance of the channel to prevent attachment to the channel surface. This can also be used to prevent the surface attachment by cells.

Droplets at these dimensions tend to conform to the size and shape of the channels, while maintaining their respective volumes. Thus, as droplets move from a wider channel to a narrower channel they become longer and thinner, and vice versa. Droplets can be at least about four times as long as they are wide. This droplet configuration, which can be envisioned as a lozenge shape, flows smoothly and well through the channels. Longer droplets, produced in narrower channels, provides a higher shear, meaning that droplets can more easily be sheared or broken off from a flow, i.e. using less force. Droplets can also tend to adhere to channel surfaces, which can slow or block the flow, or produce turbulence. Droplet adherence is overcome when the droplet is massive enough in relation to the channel size to break free. Thus, droplets of varying size, if present, can combine to form uniform droplets having a so-called critical mass or volume that results in smooth or laminar droplet flow. Droplets that are longer than they are wide, preferably about four times longer than they are wide, generally have the ability to overcome channel adherence and move freely through the microfluidic device. Thus, in an exemplary embodiment with 60 micron channels, a typical free-flowing droplet is about 60 microns wide and 240 microns long. Droplet dimensions and flow characteristics can be influenced as desired, in part by changing the channel dimensions, e.g. the channel width.

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The microfabricated devices of this invention most preferably generate round, monodisperse droplets. The droplets can have a diameter that is smaller than the diameter of the microchannel; i.e., preferably 40 to 100 μm when cells are used or 5 to 40 μm when reagents are used. Monodisperse droplets may be particularly preferably, e.g., in high throughput devices and other embodiments where it is desirable to generate droplets at high frequency and of high uniformity.

To prevent material (e.g., cells and other particles or molecules) from adhering to the sides of the channels, the channels (and coverslip, if used) may have a coating which minimizes adhesion. Such a coating may be intrinsic to the material from which the device is manufactured, or it may be applied after the structural aspects of the channels have been microfabricated. "TEFLON" is an example of a coating that has suitable surface properties.

The surface of the channels of the microfluidic device can be coated with any anti-wetting or blocking agent for the dispersed phase. The channel can be coated with any protein to prevent adhesion of the biological/chemical sample. For example, in one embodiment the channels are coated with BSA, PEG-silane and/or fluorosilane. For example, 5mg/ml BSA is sufficient to prevent attachment and prevent clogging. In another embodiment, the channels can be coated with a cyclized transparent optical polymer obtained by copolymerization of perfluoro (alkenyl vinyl ethers), such as the type sold by Asahi Glass Co. under the trademark ***Cytop***. In such an embodiment, the coating is applied from a 0.1-0.5 wt% solution of Cytop CTL-809M in CT-Solv 180. This solution can be injected into the channels of a microfluidic device via a plastic syringe. The device can then be heated to about 90 °C for 2 hours, followed by heating at 200 °C for an additional 2 hours. In another embodiment, the channels can be coated with a hydrophobic coating of the type sold by PPG Industries, Inc. under the trademark ***Aquapel*** (*e.g.*, perfluoroalkylalkylsilane surface treatment of plastic and coated plastic substrate surfaces in conjunction with the use of a silica primer layer) and disclosed in U.S. Pat. No. 5,523,162, which patent is hereby incorporated by reference. By fluorinating the surfaces of the channels, the continuous phase preferentially wets the channels and allows for the stable generation and movement of droplets through the device. The low surface tension of the channel walls thereby minimizes the accumulation of channel clogging particulates.

The surface of the channels in the microfluidic device can be also fluorinated to prevent undesired wetting behaviors. For example, a microfluidic device can be placed in a polycarbonate dessicator with an open bottle of (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane. The dessicator is evacuated for 5 minutes, and then sealed for

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20-40 minutes. The dessicator is then backfilled with air and removed. This approach uses a simple diffusion mechanism to enable facile infiltration of channels of the microfluidic device with the fluorosilane and can be readily scaled up for simultaneous device fluorination.

The microfluidic device can include a syringe (or other glass container) that is treated with a vapor or solution of an appropriate PEG-silane to effect the surface PEG functionalization. The purpose for treating the walls of glass containers (e.g., syringes) with a PEG functionality is to prevent biological adhesion to the inner walls of the container, which frustrates the proper transfer of biological/chemical materials into the microfluidic device of the present invention.

The device of the present invention can comprise one or more fluid channels to inject or remove fluid in between droplets in a droplet stream for the purpose of changing the spacing between droplets.

The invention provides methods of cell manipulation by channel geometry. Most cells, especially mammalian cells intend to attach each other in suspension. The purpose of changing channel geometry is to detach the cell from aggregates and keep them separated from each other before they are encapsulated in the drops. The geometry of channel dimension can be changed to disturb the aggregates and break them apart by various methods, that can include, for example, geometric pinching and/or barricades. With geometric pinching, cells are forced through one or more narrow regions, whose dimension is smaller or comparable to the dimension of a single cell. With a barricade, a series of obstacles/impediments (barricades) are placed in the way of the moving cells to disturb the movement and break up the aggregates of cells.

The present invention provides methods to prevent channel clogging including methods of fluid pinching and surface coating. Some cells and polystyrene beads tend to attach to the PDMS/Glass surface. This is an undesired result as the accumulated beads can clog the channel, especially the narrow region (i.e. nozzle). Channel design and blocking reagent can be used in some embodiments to prevent the beads' attachment to the channel surface and to each other. Non-limiting examples include coating reagents and channel design. A coating reagent, such as BSA (or any other protein), is added to the bead buffer to coat the channel surface as well as the beads' surface. 5mg/ml BSA has shown to be sufficient to prevent the beads' attachment. No clogging is observed in an experiment with 10um diameter beads in a 30um wide and 25um deep nozzle device. With channel design, cells/beads are forced to move along the center streamline through flow focus – Using two

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dilution channels at the entrance of the beads' channel to prevent beads' attachment to the channel surface. This can also used to prevent the surface attachment by cells.

A typical analysis unit of the invention comprises a main inlet channel that is part of and feeds or communicates directly with a main channel, along with one or more sample inlet channels in communication with the main channel at a inlet module situated downstream from the main inlet. In one embodiment, each different sample inlet channel preferably communicates with the main channel at a different inlet module. In another embodiment, different sample inlet channels can communication with the main channel at the same inlet module. The inlet channel is further connected to a means for introducing a sample to said device. The means can be a well or reservoir. The well or reservoir further include an acoustic actuator. The means can be temperature controlled. The main channel is further connected to a means for collecting a sample from said device. The means can be a well or reservoir. The means can be temperature controlled.

The inlet module generally comprises a junction between the sample inlet channel and the main channel such that a solution of a sample (i.e., a fluid containing a sample such as molecules, cells, small molecules (organic or inorganic) or particles) is introduced to the main channel and forms a plurality of droplets. The sample solution can be pressurized. The sample inlet channel can intersect the main channel such that the sample solution is introduced into the main channel at an angle perpendicular to a stream of fluid passing through the main channel. For example, the sample inlet channel and main channel intercept at a T-shaped junction; i.e., such that the sample inlet channel is perpendicular (90 degrees) to the main channel. However, the sample inlet channel can intercept the main channel at any angle, and need not introduce the sample fluid to the main channel at an angle that is perpendicular to that flow. The angle between intersecting channels is in the range of from about 60 to about 120 degrees. Particular exemplary angles are 45, 60, 90, and 120 degrees.

The main channel in turn can communicate with two or more branch channels at the sorting module or "branch point", if present, forming, for example, a T-shape or a Y-shape. Other shapes and channel geometries may be used as desired.

The device of the present invention can comprise one more means for chromatographically sorting the sample prior to droplet formation. The means can be in fluid communication with the inlet channel and/or the inlet module. Preferably, the means is a channel. The sample can be sorted by size, charge, hydrophobicity, atomic mass, etc. The separating can be done isocratic or by generating a gradient chemically, (for example using salt or hydrophobicity), electrically, by pressure, or etc. For size exclusion, the channel can

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be preloaded with Sepharose. The sample is then loaded at one end, and the droplets are formed at an opposing end. The sample separates by size prior to becoming incorporated within a droplet.

Fluids

The term "flow" means any movement of liquid or solid through a device or in a method of the invention, and encompasses without limitation any fluid stream, and any material moving with, within or against the stream, whether or not the material is carried by the stream. For example, the movement of molecules, beads, cells or virions through a device or in a method of the invention, e.g. through channels of a microfluidic chip of the invention, comprises a flow. This is so, according to the invention, whether or not the molecules, beads, cells or virions are carried by a stream of fluid also comprising a flow, or whether the molecules, cells or virions are caused to move by some other direct or indirect force or motivation, and whether or not the nature of any motivating force is known or understood. 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, so long as molecules, cells or virions are directed for detection, measurement or sorting according to the invention.

The flow stream in the main channel is typically, but not necessarily, continuous and may be stopped and started, reversed or changed in speed. Prior to sorting, a liquid that does not contain sample molecules, cells or particles can be introduced into a sample inlet well or channel and directed through the inlet module, 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 region that communicates directly with the main channel to purge the device (e.g., or "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 module.

The pressure at the inlet module can also be regulated by adjusting the pressure on the main and sample inlet channels, for example, with pressurized syringes feeding into those inlet channels. By controlling the pressure difference between the oil and water sources at the inlet module, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the inlet module or the sample inlet channel connected thereto to control the flow of solution into the inlet module, thereby controlling the size and periodicity of the droplets. Periodicity and droplet volume may also

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depend on channel diameter, the viscosity of the fluids, and shear pressure.

As used herein, the term "fluid stream" or "fluidic stream" refers to the flow of a fluid, typically generally in a specific direction. The fluidic stream may be continuous and/or discontinuous. A "continuous" fluidic stream is a fluidic stream that is produced as a single entity, e. g. , if a continuous fluidic stream is produced from a channel, the fluidic stream, after production, appears to be contiguous with the channel outlet. The continuous fluidic stream is also referred to as a continuous phase fluid or carrier fluid. The continuous fluidic stream may be laminar, or turbulent in some cases. The continuous fluidic stream may be, e.g., solid or hollow (i.e., containing a second fluid internally, for example, as in a hollow tube). It is to be understood that wherever "tube" is used herein, the structure can be a hollow, a solid or filled (i.e., not hollow) stream, a stream that includes a central core and a surrounding layer or layers, any of which can be selectively reacted with any others, or solidified, or the like. In some cases, the central core is hollow, and/or fluid may be removed from a hardened surrounding fluid to produce a hollow tube. The continuous phase fluid can be a non-polar solvent. The continuous phase fluid can be a fluorocarbon oil.

Similarly, a "discontinuous" fluidic stream is a fluidic stream that is not produced as a single entity. The discontinuous fluidic stream is also referred to as the dispersed phase fluid or sample fluid. A discontinuous fluidic stream may have the appearance of individual droplets, optionally surrounded by a second fluid. A "droplet," as used herein, is an isolated portion of a first fluid that completely surrounded by a second fluid. In some cases, the droplets may be spherical or substantially spherical ; however, in other cases, the droplets may be non-spherical, for example, the droplets may have the appearance of "blobs" or other irregular shapes, for instance, depending on the external environment. As used herein, a first entity is "surrounded" by a second entity if a closed loop can be drawn or idealized around the first entity through only the second entity. The dispersed phase fluid can include a biological/chemical material. The biological/chemical material can be tissues, cells, particles, proteins, antibodies, amino acids, nucleotides, small molecules, and pharmaceuticals. The biological/chemical material can include one or more labels. The label can be a DNA tag, dyes or quantum dot, or combinations thereof.

The term “emulsion” refers to a preparation of one liquid distributed in small globules (also referred to herein as drops, droplets or NanoReactors) in the body of a second liquid. The first and second fluids are immiscible with each other. For example, the discontinuous phase can be an aqueous solution and the continuous phase can a hydrophobic fluid such as an oil. This is termed a water in oil emulsion. Alternatively, the emulsion may

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be a oil in water emulsion. In that example, the first liquid, which is dispersed in globules, is referred to as the discontinuous phase, whereas the second liquid is referred to as the continuous phase or the dispersion medium. The continuous phase can be an aqueous solution and the discontinuous phase is a hydrophobic fluid, such as an oil (e.g., decane, tetradecane, or hexadecane). The droplets or globules of oil in an oil in water emulsion are also referred to herein as "micelles", whereas globules of water in a water in oil emulsion may be referred to as "reverse micelles".

As used herein, the term "NanoReactor" and its plural encompass the terms "droplet", "microdrop" or "microdroplet" as defined herein, as well as an integrated system for the manipulation and probing of droplets, as described in detail herein. Nanoreactors as described herein can be 0-100 μm (*e.g.*, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100)

The droplet forming liquid is typically an aqueous buffer solution, such as ultrapure water (e.g., 18 mega-ohm resistivity, obtained, for example by column chromatography), 10 mM Tris HCl and 1 mM EDTA (TE) buffer, phosphate buffer saline (PBS) or acetate buffer. Any liquid or buffer that is physiologically compatible with the population of molecules, cells or particles to be analyzed and/or sorted can be used. The fluid passing through the main channel and in which the droplets are formed is one that is immiscible with the droplet forming fluid. The fluid passing through the main channel can be a non-polar solvent, most preferably decane (e g., tetradecane or hexadecane), fluorocarbon oil or another oil (for example, mineral oil).

The dispersed phase fluid may also contain biological/chemical material (e.g., molecules, cells, or other particles) for combination, analysis and/or sorting in the device. The droplets of the dispersed phase fluid can contain more than one particle or can contain no more than one particle. For example, where the biological material comprises cells, each droplet preferably contains, on average, no more than one cell. The droplets can be detected and/or sorted according to their contents.

The fluids used in the invention may contain one or more additives, such as agents which reduce surface tensions (surfactants). Surfactants can include Tween, Span, fluorosurfactants, and other agents that are soluble in oil relative to water. Surfactants can aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel. This can affect droplet volume and periodicity, or the rate or frequency at which droplets break off into an intersecting channel. Furthermore, the surfactant can serve to stabilize aqueous emulsions in

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fluorinated oils from coalescing.

The droplets may be coated with a surfactant. Preferred surfactants that may be added to the continuous phase fluid include, but are not limited to, surfactants such as sorbitan-based carboxylic acid esters (e.g., the "Span" surfactants, Fluka Chemika), including sorbitan monolaurate (Span 20), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60) and sorbitan monooleate (Span 80), and perfluorinated polyethers (*e.g.*, DuPont Krytox 157 FSL, FSM, and/or FSH). Other non-limiting examples of non-ionic surfactants which may be used include polyoxyethylenated alkylphenols (for example, nonyl-, p-dodecyl-, and dinonylphenols), polyoxyethylenated straight chain alcohols, polyoxyethylenated polyoxypropylene glycols, polyoxyethylenated mercaptans, long chain carboxylic acid esters (for example, glyceryl and polyglycerl esters of natural fatty acids, propylene glycol, sorbitol, polyoxyethylenated sorbitol esters, polyoxyethylene glycol esters, etc.) and alkanolamines (e.g., diethanolamine-fatty acid condensates and isopropanolamine-fatty acid condensates). In addition, ionic surfactants such as sodium dodecyl sulfate (SDS) may also be used. However, such surfactants are generally less preferably for many embodiments of the invention. For instance, in those embodiments where aqueous droplets are used as nanoreactors 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 droplet.

The carrier fluid can be an oil (e.g., decane, tetradecane or hexadecane) or fluorocarbon oil that contains a surfactant (e.g., a non-ionic surfactant such as a Span surfactant) as an additive (preferably between about 0.2 and 5% by volume, more preferably about 2%). A user can preferably cause the carrier fluid to flow through channels of the microfluidic device so that the surfactant in the carrier fluid coats the channel walls.

In one embodiment, the fluorosurfactant can be prepared by reacting the perflourinated polyether DuPont Krytox 157 FSL, FSM, or FSH with aqueous ammonium hydroxide in a volatile fluorinated solvent. The solvent and residual water and ammonia can be removed with a rotary evaporator. The surfactant can then be dissolved (*e.g.*, 2.5 wt%) in a fluorinated oil (*e.g.*, Flourinert (3M)), which then serves as the continuous phase of the emulsion.

The invention can use pressure drive flow control, e.g., utilizing valves and pumps, to manipulate the flow of cells, particles, molecules, enzymes or reagents in one or more directions and/or into one or more channels of a microfluidic device. However, other methods may also be used, alone or in combination with pumps and valves, such as electro-osmotic flow control, electrophoresis and dielectrophoresis (Fulwyer, *Science* 156, 910

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(1974); Li and Harrison, *Analytical Chemistry* 69, 1564 (1997); Fiedler, et al. *Analytical Chemistry* 70, 1909-1915 (1998); U.S. Patent No. 5,656,155). Application of these techniques according to the invention provides more rapid and accurate devices and methods for analysis or sorting, for example, because the sorting occurs at or in a sorting module that can be placed at or immediately after a detection module. This provides a shorter distance for molecules or cells to travel, they can move more rapidly and with less turbulence, and can more readily be moved, examined, and sorted in single file, i.e., one at a time.

Without being bound by any theory, electro-osmosis is believed to produce motion in a stream containing ions e.g. a liquid such as a buffer, by application of a voltage differential or charge gradient between two or more electrodes. Neutral (uncharged) molecules or cells can be carried by the stream. Electro-osmosis is particularly suitable for rapidly changing the course, direction or speed of flow. Electrophoresis is believed to produce movement of charged objects in a fluid toward one or more electrodes of opposite charge, and away from one on or more electrodes of like charge. Where an aqueous phase is combined with an oil phase, aqueous droplets are encapsulated or separated from each other by oil. Typically, the oil phase is not an electrical conductor and may insulate the droplets from the electro-osmotic field. In this example, electro-osmosis may be used to drive the flow of droplets if the oil is modified to carry or react to an electrical field, or if the oil is substituted for another phase that is immiscible in water but which does not insulate the water phase from electrical fields.

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, non-homogeneous electric fields in the presence of droplets and/or particles, such as cells or molecules, cause the droplets 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. For example, the polarizability of living cells depends on their composition, morphology, and phenotype and is highly dependent on the frequency of the applied electrical field. Thus, cells of different types and in different physiological states generally possess distinctly different dielectric properties, which may provide a basis for cell separation, e.g., by differential dielectrophoretic forces. Likewise, the polarizability of droplets also depends upon their size, shape and composition. For example, droplets that contain salts can be polarized. According to formulas provided in Fiedler, et al. *Analytical Chemistry* 70, 1909-1915 (1998), individual manipulation of single droplets requires field differences (inhomogeneities) with dimensions

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close to the droplets.

Manipulation is also dependent on permittivity (a dielectric property) of the droplets and/or particles with the suspending medium. Thus, polymer particles, living cells show negative dielectrophoresis at high-field frequencies in water. For example, dielectrophoretic forces experienced by a latex sphere in a 0.5 MV/m field (10 V for a 20 micron electrode gap) in water are predicted to be about 0.2 piconewtons (pN) for a 3.4 micron latex sphere to 15 pN for a 15 micron latex sphere (Fiedler, et al. *Analytical Chemistry* 70, 1909-1915 (1998)). These values are mostly greater than the hydrodynamic forces experienced by the sphere in a stream (about 0.3 pN for a 3.4 micron sphere and 1.5 pN for a 15 micron sphere). Therefore, manipulation of individual cells or particles can be accomplished in a streaming fluid, such as in a cell sorter device, using dielectrophoresis. Using conventional semiconductor technologies, electrodes can be microfabricated onto a substrate to control the force fields in a microfabricated sorting device of the invention. 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. See U.S. Patent No. 5,454,472.

Radiation pressure can also be used in the invention to deflect and move objects, e.g. droplets and particles (molecules, cells, particles, etc.) contained therein, with focused beams of light such as lasers. Flow can also be obtained and controlled by providing a pressure differential or gradient between one or more channels of a device or in a method of the invention.

Molecules, cells or particles (or droplets containing molecules, cells or particles) can be moved by direct mechanical switching, e.g., with on-off valves or by squeezing the channels. Pressure control may also be used, for example, by raising or lowering an output well to change the pressure inside the channels on the chip. See, e.g., the devices and methods described U.S. Patent No. 6,540,895. These methods and devices can further be used in combination with the methods and devices described in pending U.S. Patent Application Publication No. 20010029983 and 20050226742. The "pump and valve" drive systems are particularly preferred. They are rapid, efficient, economical, and relatively easy to fabricate and control. Additionally, they do not rely on electrical fields or electrical charges, which may be harder to control and in some cases may potentially affect the droplet contents. Different switching and flow control mechanisms can be combined on one chip or in one device and can work independently or together as desired.

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The device can exchange constituents within a droplet through the use of fluid flow in such a way that the droplet, while in a first immiscible fluid, is exposed to a second immiscible fluid such that constituents within the droplet that are immiscible in the first immiscible fluid are soluble in the second immiscible fluid. In one example, an aqueous droplet containing a chemical reaction produces by-products that are soluble in a lipid solvent. The chemical reaction is performed in a water-environment in a silicon-based solvent. After the chemical reaction occurs, the droplet is exposed to an organic-oil based solvent where the chemical byproducts are allowed to diffuse out of the droplet. The resulting droplet is then assayed for cell-killing activity by combining the droplet with live cells. Alternatively, the change in the non-aqueous fluid flow is used to add a particular constituent from the second immerscible fluid to diffuse into the aqueous drop before the droplet is returned to the 100% first immiscible fluid flow.

The concentration (i.e., number) of molecules, cells or particles in a droplet can influence sorting efficiently and therefore is preferably optimized. In particular, the sample concentration should be dilute enough that most of the droplets contain no more than a single molecule, cell or particle, with only a small statistical chance that a droplet will contain two or more molecules, cells or particles. This is to ensure that for the large majority of measurements, the level of reporter measured in each droplet as it passes through the detection module corresponds to a single molecule, cell or particle and not to two or more molecules, cells or particles.

The parameters which govern this relationship are the volume of the droplets and the concentration of molecules, cells or particles in the sample solution. The probability that a droplet will contain two or more molecules, cells or particles ($P_{\leq 2}$) can be expressed as

$$P_{\leq 2}=1-\{1+[\text{cell}]\times V\}\times e^{-[\text{cell}]\times V}$$

where "[cell]" is the concentration of molecules, cells or particles in units of number of molecules, cells or particles per cubic micron (μm^3), and V is the volume of the droplet in units of μm^3.

It will be appreciated that $P_{\leq 2}$ can be minimized by decreasing the concentration of molecules, cells or particles in the sample solution. However, decreasing the concentration of molecules, cells or particles in the sample solution also results in an increased volume of solution processed through the device and can result in longer run times. Accordingly, it is desirable to minimize to presence of multiple molecules, cells or particles in the droplets (thereby increasing the accuracy of the sorting) and to reduce the volume of sample, thereby permitting a sorted sample in a reasonable time in a reasonable volume containing an

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acceptable concentration of molecules, cells or particles.

The maximum tolerable $P_{\leq 2}$ depends on the desired "purity" of the sorted sample. The "purity" in this case refers to the fraction of sorted molecules, cells or particles that posses a desired characteristic (e.g., display a particular antigen, are in a specified size range or are a particular type of molecule, cell or particle). The purity of the sorted sample is inversely proportional to $P_{\leq 2}$. For example, in applications where high purity is not needed or desired a relatively high $P_{\leq 2}$ (e.g., $P_{\leq 2}$=0.2) may be acceptable. For most applications, maintaining $P_{\leq 2}$ at or below about 0.1, preferably at or below about 0.01, provides satisfactory results.

A sample solution containing a mixture or population of molecule, cells or particles in a suitable fluid (such as a liquid or buffer described above) is supplied to the sample inlet channel, and droplets of the sample solution are introduced, at the inlet module, into the flow passing through the main channel. The force and direction of flow can be controlled by any desired method for controlling flow, for example, by a pressure differential, by valve action or by electro-osmotic flow (e.g., produced by one or more electrodes or patterned electrically conductive layers at inlet and/or outlet modules). This permits the movement of the cells into one or more desired branch channels or outlet modules.

Both the fluid comprising the droplets and the fluid carrying the droplets (i.e., the aqueous and non-polar fluids) have, preferably, 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 length. More viscous, less dense, slower moving fluids over a shorter distance 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, microfabricated fluid systems are often in a low Reynolds number regime ($Re<<1$). In this regime, inertial effects, which cause turbulence and secondary flows, are negligible; viscous effects dominate the dynamics. These conditions are advantageous for sorting, and are provided by microfabricated devices of the invention. Accordingly the microfabricated devices of the invention are preferably if not exclusively operated at a low or very low Reynold's number.

The device of the present invention can be used to generate droplets whose composition may vary from one droplet to the next droplet due to any number of reasons (chemical reaction, sample preparation, etc). Within the same device, the droplets can be passed through a measurement volume in which the contents can be interrogated using various means (optical or electrical). The result of the measurement can be used to decide which flow path the droplets should take. The means of changing the flow path can be

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accomplished through mechanical, electrical, optical, or other technique as described herein or well known in the art.

The present invention provides methods for the determination of droplet size and rate information without the need for optical measurements on a microfluidic device. The need to control the timing between multiple events requires the determination of the exact time when a droplet passes a given point. It is also essential to know which channel a droplet enters. This method can significantly reduce the cost and complexity of such measurements.

The fluids used to generate droplets in microfluidic devices are typically immiscible liquids such as oil and water. These two materials generally have very different dielectric constants associated with them. These differences can be exploited to determine droplet rate and size for every drop passing through a small section of a microfluidic device. One method to directly monitor this variation in the dielectric constant measures the change in capacitance over time between a pair of closely spaced electrodes. This change in capacitance can be detected by the change in current measured in these electrodes:

$$i = V\frac{dC}{dt}$$

Where i is the current, V is the voltage applied across the electrodes, and dC/dt is the change in capacitance with time. Alternatively, the capacitance can be measured directly if a time varying voltage is applied to these same electrodes: i=CdV/dt Where C is the measured capacitance, and dV/dt is the change in voltage with time.

As a first approximation, the electrode pair can be determiend as a parallel plate capacitor:

$$C = \varepsilon_0 k\frac{A}{d}$$

Where ε_0 is the permittivity of free space, k is the effective dielectric constant (this changes every time a droplet passes through), A is the area of the capacitor and d is the electrode separation. The current measured in the device is then plotted as a function of time.

Inlet Module

An "inlet module" is an area of a microfabricated device that receives molecules, cells, small molecules or particles for coalescence, detection and/or sorting. The inlet module can contain one or more inlet channels, wells or reservoirs, openings, and other features which facilitate the entry of molecules, cells, small molecules or particles into the device. A chip may contain more than one inlet module if desired. The inlet module is in fluid communication with the main channel. The inlet module can include a junction between an

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inlet channel and the main channel of a device of the invention. The junction can permit the introduction of a pressurized fluid to the main channel. The inlet channel can be at an angle perpendicular to the flow of fluid in the main channel. The fluid introduced to the main channel through the inlet module is "incompatible" (i.e., immiscible) with the fluid in the main channel so that droplets of the fluid introduced through the inlet module are formed in the stream of continuous fluid in the main channel.

Embodiments of the invention are also provided in which there are two or more inlet modules introducing droplets of samples into the main channel. For example, a first inlet module may introduce droplets of a first sample into a flow of fluid (e.g., oil) in the main channel and a second inlet module may introduce droplets of a second sample into the flow of fluid in main channel, and so forth. The second inlet module is preferably downstream from the first inlet module (e.g., about 30 μm). The fluids introduced into the two or more different inlet modules can comprise the same fluid or the same type of fluid (e.g., different aqueous solutions). For example, droplets of an aqueous solution containing an enzyme are introduced into the main channel at the first inlet module and droplets of aqueous solution containing a substrate for the enzyme are introduced into the main channel at the second inlet module. Alternatively, the droplets introduced at the different inlet modules may be droplets of different fluids which may be compatible or incompatible. For example, the different droplets may be different aqueous solutions, or droplets introduced at a first inlet module may be droplets of one fluid (e.g., an aqueous solution) whereas droplets introduced at a second inlet module may be another fluid (e.g., alcohol or oil).

To obtain one droplet comprising a single element of a specific biological/chemical material (e.g., a cell), separation of biological/chemical material, and uniformity of the number density of biological/chemical materials in a microfluidic channel is desirable. Accordingly, the microfluidic device can include an acoustic actuator. The loaded sample (biological/chemical material) can be well mixed and separated in a small chamber by acoustic wave before sending out to the nozzle region for encapsulation. The frequency of the acoustic wave should be fine tuned so as not to cause any damage to the cells. The biological effects of acoustic mixing have been well studied (e.g., in the ink-jet industry) and many published literatures also showed that piezoelectric microfluidic device can deliver intact biological payloads such as live microorganisms and DNA.

The design of the acoustic resonant can use a Piezoelectric bimorph flat plate located on the side of the carved resonant in the PDMS slab. The resonant inlet can connect to the cell flow input channel and the outlet can connect to the cell flow pinching channel. The

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piezoelectric driving waveform can be carefully optimized to select the critical frequencies that can separate cells in fluids. There are five parameters to optimize beyond the frequency parameter and Lab electronics can be used to optimize the piezoelectric driving waveform. Afterwards, a low cost circuit can be designed to generate only the optimized waveform in a preferred microfluidic device.

Coalescence Module

The device of the invention also comprises one or more coalescence modules. A "coalescence module" is within or coincident with at least a portion of the main channel at or downstream of the inlet module where molecules, cells, small molecules or particles comprised within droplets are brought within proximity of other droplets comprising molecules, cells, small molecules or particles and where the droplets in proximity coalesce or combine their contents. The coalescence module can also include an apparatus, preferably one or more electrodes or patterned electrically conductive layers for generating a dielectrophoretic force. The dielectrophoretic force generated by one or more electrodes or patterned electrically conductive layers can slow or stop the droplets within the main channel thereby facilitating their proximity and resulting coalescence or combination.

Two or more precursor droplets in one or more droplet streams can be coalesced into a larger droplet by applying a voltage to produce an electric field. The voltage can be alternating. The electric field can be an AC electric field, or a DC electric field.

The coalescing influence can create a dielectrophoretic force that slows or stops a first precursor droplet relative to the velocity of the stream that carries the droplet. The first precursor droplet will remain slow or stopped until a second (or more) precursor droplet arrives and coalesces with the first precursor droplet due to interactions between the field induced dipoles in the droplets. The new droplet of increased volume is then too large to be held by the dielectric field and moves off under the influence of the flow of the continuous phase fluid. See Figures 15-17. No change in the applied voltage is required and the electric field remains constant. Both the trapping (slowing or stopping) of the precursor droplets and the release of the coalesced droplet can be passive. Once the new droplet of increased volume moves off, the next precursor droplet is then trapped in the field and the process repeated. The voltage can be tuned such that more than one droplet is coalesced with a trapped droplet. An advantage of coalescing more than one droplet with another is that it allows for pairwise combinations. Alternatively, a variation on this geometry will allow

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precise control of the droplet phase by temporarily shifting droplets to low velocity streamlines in the flow.

The precursor droplets can come at different times in the same fluid stream and subsequently coalesce. Alternatively, the precursor droplets can arrive together in different (*e.g.*, two or more) fluid streams so that the droplets are in a substantially adjacent position with respect to each other when they come under the influence of the dielectrophoretic force and then subsequently coalesce due to the interactions between the field induced dipoles in the droplets. The different fluid streams can be substantially parallel.

The electric field gradient can be stronger for precursor droplets in a first parallel fluid stream than for precursor droplets in a second (or more) parallel fluid stream. Accordingly, it is only the precursor droplets from the first stream that are trapped, thereby preventing coalescence of precursor droplets in the other fluid stream(s) among each other in the case where the frequency of precursor droplets in the other stream(s) is greater than the frequency of precursor droplets in the first stream. In this manner the electric field can be changed to cause coalescence of only correct pairs of precursor droplets. Thus, in some embodiments the trapping and release of the droplets can be non-passive (*i.e.*, based on whether the electric field is on or off).

The device can include channels for use in fluid control and other channels filled with a metal alloy for casting integrated metal alloy components (*i.e.*, electrodes). Alternatively, the electrodes can be manufactured using other technologies (e.g., lithographically patterned electrodes made from indium tin oxide or a metal such as platinum). The microfluidic device can include metal alloy components useful for performing electrical functions on fluids, including but not limited to, coalescing droplets, charging droplets, sorting droplets, detecting droplets and shaking droplets to mix the contents of coalesced droplets. The device can contain more than one of the above mentioned components for more than one of the above mentioned functions.

The present invention also provides methods of manipulating biological/chemical material. In one embodiment, the first and second droplets can be brought into proximity prior to coalescence by slowing or stopping at least one droplet comprising a first biological/chemical material by exerting a dielectrophoretic force onto the droplet produced by an electric field gradient. In another embodiment, the first and second droplets can be brought into proximity prior to coalescence where the first and second droplet are of different size. In some embodiments, one of the first and second droplets can be the size of the channel width and the other droplet can be smaller than the channel width. In other

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embodiments, the larger droplet has enough volume so that it would have a diameter greater than the channel width if it were spherical. In a further embodiment, the first and second droplets can be brought into proximity prior to coalescence where the first and second droplet are of different viscosities and thus move at different velocities. Viscosity of a droplet can be changed by changing the content of the droplet. For example, glycerol can be added to a droplet to give it an increased viscosity.

In one embodiment, the method of manipulating biological and chemical material, further comprises coalescing at least one droplet with a droplet slowed or stopped under the influence of a dielectrophoretic force from an electric field gradient created within a coalescence module, thereby producing a nanoreactor.

The droplet size can be controlled such that the droplet formed from flowing a first dispersed phase fluid in a continuous phase fluid moves at a different velocity with respect to a droplet formed from flowing a second dispersed phase fluid in a continuous phase fluid, such that droplets arrive in pairs at a region where an electric field induces them to coalesce, thereby producing a nanoreactor. In some embodiments, greater than 50% of the droplets are paired. In other embodiments, greater than 75% of the droplets are paired.

The droplet viscosity can be controlled such that the droplet formed from flowing a first dispersed phase fluid in a continuous phase fluid moves at a different velocity with respect the droplet formed from flowing a second dispersed phase fluid in a continuous phase fluid, such that droplets arrive in pairs at a region where an electric field induces them to coalesce, thereby producing a nanoreactor. In some embodiments, greater than 50% of the droplets are paired. In other embodiments, greater than 75% of the droplets are paired.

The electrodes comprising metal alloy components may either terminate at fluid channels or be isolated from fluid channels. The electrodes can be constructed by filling the appropriate channels with metal alloy. One way this can be accomplished to use positive pressure injection of the metal alloy in a melted state, such as with a syringe, into the channels, and then cool the metal alloy to a solid form. Another example is to use negative pressure to suck the metal alloy in a melted state into the channels, and then cool the metal alloy to a solid form. This can be accomplished for example by use of capillary forces. Another method of construction can use any of the above mentioned embodiments, and then flush out the metal alloy in a melted state with another liquid to define the geometry of the metal alloy components. Another example is to use any of the above mentioned embodiments, and then use a localized cold probe to define a solid termination point for the metal alloy, and then cool the remaining metal alloy to a solid form. A further example is to

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use another material, such as microscopic solder spheres or UV curable conductive ink, to form a barrier between fluid and metal alloy channels, to define the geometry of the metal alloy components.

The device can include a combination of both integrated metal alloy components and a patterned electrically conductive layer. The patterned electrically conductive layer can have features patterned such that their boundaries are within a leak-proof seal. The device can have a patterned electrically conductive feature as one of two charging electrodes and one integrated metal alloy component as the other of two charging electrodes. Alternatively, the device can have metal alloy components as the two halves of a bowtie antenna and patterned electrically conductive features as the two halves of a pickup antenna for dielectric droplet detection.

The device can include a plurality of electrodes that are insulated from the fluid present in the device, and the method of operation including appropriate application of dielectrical signals and appropriate fluids. In known devices, the electrodes are typically in contact with the fluids in order to allow discharge of species that would otherwise screen the applied dielectric field. Whereas, in devices where the electrodes have been insulated from the fluid, this screening effect typically arises so quickly that the device is not useful for any significantly extended period of time. The drawbacks of electrodes in contact with the fluids vs. insulated electrodes are (a) degraded reliability against leaking (since the interface between the electrodes and the other components of the device may be more difficult to effect a leak-proof seal), and (b) degraded reliability against electrode corrosion (whose failure mode effects include failure of application of dielectric fields, and fluid channel contamination).

The device of the present invention comprising a plurality of electrodes that are insulated from the fluid present in the device counteracts this screening effect by extending the screening rise time and including a polarity switch for all of the different dielectric fields applied in the device. The screening rise time is extended by using fluids with dielectrical properties. A polarity switch for all of the different dielectric fields applied in the device is achieved by using an algorithm for dielectrical control, which switches the polarity of the dielectrical fields at a frequency sufficiently high to maintain proper dielectrical function of the device. This dielectrical control algorithm may also switch the polarity for the dielectric fields in a cascading, time controlled manner starting at the fluid origin point and progressing downstream, so that given fluid components experience one polarity at every point along

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their course. The device of the present invention can be used with metal alloy electrodes or using a combination of metal alloy electrodes and patterned conductive film electrodes.

In one embodiment, the invention provides a microfluidic device using injected electrodes. The interface between the microscopic electrode (typicaly 25μm thick) and the macroscopic interconnect can easily fail if the joint between the two is flexed. The flexing of the joint can be eliminated by securing a firm material that serves to fasten, support, and re-inforce the joint (*i.e.*, a grommet) into the interface. In order to prevent flexing, the mating surface of the device can be manufactured from a hard material such as glass or plastic. The electrical connection with the external system can be made by securing the device such that it connects to a spring loaded contact, which is either offset from the grommet (thereby minimizing the force applied to the solder region), or centered on the grommet (as long as the contact does not touch the solder).

The metal alloy components are also useful for performing optical functions on fluids, including but not limited to, optical detection of droplets in a geometry which may include a mirror.

To prevent leakage of fluid out of electrodes placed within microfluidic channels, the microfluidic device can include a layer patterned with channels for fluid control, and another layer with patterned electrically conductive features, where the features are patterned such that their boundaries are within a leak-proof seal. The leak-proof seal can be achieved at the interface between the unpatterned areas of the fluid control layer and the unpatterned areas of the electrically conductive layer. The leak-proof seal can also be achieved by a third interfacial layer between the fluid control layer and the unpatterned areas of the electrically conductive layer. The third interfacial layer can or can not be perforated at specific locations to allow contact between the fluid and the electrically conductive layer. Electrical access ports can also be patterned in the fluid control layer.

The electrodes and patterned electrically conductive layers as described can be associated with any module of the device (inlet module, coalescence module, mixing module, delay module, detection module and sorting module) to generate dielectric or electric fields to manipulate and control the droplets and their contents.

The microfluidic device can combine dielectric or electric fields with droplet fission to separate ionic species during droplet breakup.

The present invention provides methods of controlling droplets using fringing fields. Effective control of uncharged droplets within microfluidic devices can require the generation of extremely strong dielectric field gradients. The fringe fields from the edges of

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a parallel plate capacitor can provide an excellent topology to form these gradients. The microfluidic device according to the present invention can include placing a fluidic channel between two parallel electrodes, which can result in a steep electric field gradient at the entrance to the electrodes due to edge effects at the ends of the electrode pair. Placing these pairs of electrodes at a symmetric channel split can allow precise bi-directional control of droplet within a device. Using the same principle, only with asymmetric splits, can allow single ended control of the droplet direction in the same manner. Alternatively, a variation on this geometry will allow precise control of the droplet phase by shifting.

A device of the invention can be used for the application of an electric field at a junction between two immiscible fluids. The electric field created charged droplets and large forces necessary for emulsification, while the junction stabilized droplet production even at high fields, when a Taylor cone was present. Applications of this technology include, but are not limited to, the generation of charged droplets with a narrow distribution in radius down to submicron sizes and controlled droplet coalescence by oppositely charged droplets.

The device of this embodiment can be created by patterning PDMS on a glass substrate having electrodes formed from indium tin oxide ("ITO"). A voltage difference can be applied to the electrodes to create an applied dielectric field. The device can include a two- fluid injection system where a conductive fluid can be injected into a non-conductive fluid in the presence of the electric field to generate droplets of the conductive fluid dispersed in the non- conductive fluid. Droplets can be created having diameters of less than about 1 micron to about 100 microns. These droplets can remain charged with the sign of the charge dependent on the sign of the dielectric field with respect to the direction of flow.

In the absence of an electric field, large droplets can be generated, while in the presence an electric field (E = 2 V/micron), a Taylor cone can be stabilized with uniform submicron droplets being emitted from the tip. The droplets may also be discharged on a ground electrode located further downstream. Such a device can have many applications, for example, in generating well controlled nanoemulsions.

Oppositely oriented devices can also be used to generate droplets having opposite sign of charge. Using this charge, the droplets can coalesce at a precise or generally predetermined location. If there is no electric field applied, the droplets cannot coalesce. The electrostatic attraction can cause the drops to coalesce. The electric field, in some cases, can be used to control the phase between when the droplets are generated to ensure simultaneous arrival at a central location and subsequent coalescence, for example, through an auto feedback mechanism or a using an AC dither. The surface of the droplets can be deformed and

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electrostatic forces may overcome surface tension to produce a fluid bridge to coalesce and/or neutralize the droplets.

Interdigitation and Coalescence of Droplets

Particular design embodiments of the microfluidic device described herein allow for a more reproducible and controllable interdigitation of droplets of specific liquids followed by pair-wise coalescence of these droplets. The droplet pairs can contain liquids of different compositions and/or volumes, which would then combine to allow for a specific reaction to be investigated. The pair of droplets can come from any of the following: (i) two continuous aqueous streams and an oil stream; (ii) a continuous aqueous stream, an emulsion stream, and an oil stream, or (iii) two emulsion streams and an oil stream. Figure 17 A-D.

The nozzle design enhances the interdigitation of droplets and further improves coalescence of droplets due to the better control of the interdigitation and smaller distance between pairs of droplets. The greater control over interdigitation allows for a perfect control over the frequency of either of the droplets. Coalescence can be accomplished by localized electric field application, as described above. Coalescence may also be accomplished by passive coalescence of droplets (*i.e.*, without application of any external effects for the appropriate mix). Passive coalescence significantly simplifies the device operation and control, which is critical as the same procedure is repeated multiple times in a given process. To obtain the optimum operation, the spacing between droplets and coupling of the droplets can be adjusted by adjusting flow of any of the streams, viscosity of the streams, nozzle design (including orifice diameter, the channel angle, and post-orifice neck of the nozzle).

In one embodiment, passive coalescence of paired droplets can be achieved by passing the droplets through a narrowing of a channel (or a neck-down or a pinch). Figure 18A-D. In such an embodiment, droplets passing through the pinch are touching while being elongated as they are passing through the channel. Due to the elongation and redistribution of surface activities at the elongated ends, the droplet pair coalesces spontaneously and passively.

Detection Module

A "detection module" is a location within the device, typically within the main channel where molecules, cells, small molecules or particles are to be detected, identified, measured or interrogated on the basis of at least one predetermined characteristic. The molecules, cells, small molecules or particles can be examined one at a time, and the

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characteristic is detected or measured optically, for example, by testing for the presence or amount of a reporter. For example, the detection module is in communication with one or more detection apparatuses. The detection apparatuses can be optical or electrical detectors or combinations thereof. Examples of suitable detection apparatuses include optical waveguides, microscopes, diodes, light stimulating devices, (e.g., lasers), photo multiplier tubes, and processors (e.g., computers and software), and combinations thereof, which cooperate to detect a signal representative of a characteristic, marker, or reporter, and to determine and direct the measurement or the sorting action at the sorting module.

A detection module is within, communicating or coincident with a portion of the main channel at or downstream of the inlet module and, in sorting embodiments, at, proximate to, or upstream of, the sorting module or branch point. Precise boundaries for the detection module are not required, but are preferred. The sorting module may be located immediately downstream of the detection module or it may be separated by a suitable distance consistent with the size of the molecules, the channel dimensions and the detection system. It will be appreciated that the channels may have any suitable shape or cross-section (for example, tubular or grooved), and can be arranged in any suitable manner so long as flow can be directed from inlet to outlet and from one channel into another.

The detection module can have features to detect the droplets, including but not limited to, integrated metal alloy components and/or features patterned in an electrically conductive layer, to broadcast a signal around a droplet and pick up an electrical signal in proximity to the droplet.

As each droplet passes into the detection module, it is examined for a predetermined characteristic (i.e., using the detector) and a corresponding signal is produced, for example indicating that "yes" the characteristic is present, or "no" it is not. The signal may correspond to a characteristic qualitatively or quantitatively. That is, the amount of the signal can be measured and can correspond to the degree to which a characteristic is present. For example, the strength of the signal may indicate the size of a molecule, or the potency or amount of an enzyme expressed by a cell, or 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 control system in the sorting module, if present, can be activated to divert a droplet into one branch channel or another for delivery to the collection module or waste module. Thus, in sorting embodiments, molecules or cells within a droplet at a sorting module can be sorted into an appropriate branch channel according to a signal produced by the corresponding examination

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at a detection module. The detection can be optical detection of molecular, cellular or other characteristics, for example directly or by use of a reporter associated with a characteristic chosen for sorting. However, other detection techniques can also be employed.

The device can be used to generate droplets whose composition may vary from one to the next due to any number of reasons (chemical reaction, sample preparation, etc). Within the same device, the droplets are passed through a measurement volume in which the contents are interrogated using various means (optical or electrical). The result of the measurement is used to decide which flow path the droplets should take. The means of changing the flow path can be accomplished through mechanical, electrical, optical, or some other technique as described herein.

The device can provide an accurate means of precisely aligning optical waveguides and their associated optical elements (lenses, prisms, mirrors, interconnects, etc.) to the fluidic channels contained within the microfluidic device. Such waveguides can be used to provide well defined optical access to the fluidic channels to permit optical scattering, absorption, fluorescence, or any other optical measurement technique.

Channels within the device are typically made using semiconductor lithographic processes. In order to create the waveguides, a separate series of channels and useful shapes (lenses, mirrors, etc) can be created either simultaneously (i.e. in the same processing step) or in successive steps. The reusable master created in this way can then used to fabricate the waveguide components and fluid channels without the need for special fixturing or careful alignment in subsequent steps. The extra channels or shapes can then filled with a high index of refraction liquid (for waveguides) or reflective material (for mirrors) through injection into the channel or void. The liquid can either remain as a fluid or be allowed to solidify. UV cure epoxies used by the telecommunications industry are excellent choices for the waveguide materials. Possible waveguide geometry can include a focusing lens and a back-reflecting mirror.

The device of the present invention also comprises the use of beads and methods for analyzing and sorting beads (i.e, bead reader device). The device can read and either sort or not sort droplets containing one or more of a set of two or more beads. Each bead can be differentiated from each other bead within a set. Beads can be separated by several tags including, but not limited to, quantum dyes, fluorescent dyes, ratios of fluorescent dyes, radioactivity, radio-tags, etc. For example, a set of beads containing a ratio of two dyes in discrete amounts with an apparatus for detecting and differentiating beads containing one discrete ratio from the other beads in this set having a different ratio of the two dyes. The

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microfluidic device can include a paramagnetic beads. The paramagnetic beads can introduce and remove chemical components from droplets using droplet coalescence and breakup events. The paramagnetic beads can also be used for sorting droplets.

The present invention provides methods of screening molecular libraries on beads through limited-dilusion-loading and then chemical or optical release inside of droplets. Provided are methods for chemical synthesis on a bead and releasing said chemical attached to the bead using a releasing means (chemical, UV light, heat, etc) within a droplet, and then combining a second droplet to the first droplet for further manipulation. For example, tea-bag synthesis of chemicals on a bead simultaneously with a means for identifying said bead (using, for example, a mass spec tag). Using the resulting mixed-chemistry beads in a droplet within a fluid flow, and exposing the beads to UV light to release the chemical synthesized from the bead into the droplet environment. Combining the droplet containing the released chemical with a droplet containing a cell, and performing a cell-based assay. Sorting droplets having the desired characteristics (for example, turn on of a reporter gene), and then analyzing the sorted beads using mass spectroscopy.

The device of the present invention can comprise column separation prior to bead sorting. A device containing a channel loaded with a separating means for chromatographically sorting the sample prior to droplet formation. Such separating means could include size, charge, hydrophobicity, atomic mass, etc. The separating can be done isocratic or by use of a means for generating a gradient chemically, (for example using salt or hydrophobicity), electrically, by pressure, or etc. For example, a channel is preloaded with Sepharose size exclusion media. A sample is loaded at one end, and the droplets are formed at an opposing end. The sample separates by size prior to becoming incorporated within a droplet.

The detector can be any device or method for interrogating a molecule, a cell or particle as it passes through the detection module. Typically, molecules, cells or particles (or droplets containing molecules, cells or particles) are to be analyzed or sorted according to a predetermined characteristic that is directly or indirectly detectable, and the detector is selected or adapted to detect that characteristic. 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. Enzymes can be analyzed and/or sorted by the extent to which they catalyze chemical reaction of a substrate (conversely, substrate can be analyzed and/or sorted

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by the level of chemical reactivity catalyzed by an enzyme). Cells can be sorted according to whether they contain or produce a particular protein, by using an optical detector to examine each cell for an optical indication of the presence or amount of that protein. The protein may itself be detectable, for example by a characteristic fluorescence, or it may be labeled or associated with a reporter that produces a detectable signal when the desired protein is present, or is present in at least a threshold amount. There is no limit to the kind or number of characteristics that can be identified or measured using the techniques of the invention, which include without limitation surface characteristics of the cell and intracellular characteristics, provided only that the characteristic or characteristics of interest for sorting can be sufficiently identified and detected or measured to distinguish cells having the desired characteristic(s) from those which do not. For example, any label or reporter as described herein can be used as the basis for analyzing and/or sorting molecules or cells, i.e. detecting molecules or cells to be collected.

The molecules or cells or particles (or droplets containing them) are analyzed and/or separated based on the intensity of a signal from an optically-detectable reporter bound to or associated with them as they pass through a detection module in the device. Molecules or cells or particles having an amount or level of the reporter at a selected threshold or within a selected range are diverted into a predetermined outlet or branch channel of the device. The reporter signal may be collected by a microscope and measured by a photo multiplier tube (PMT). A computer digitizes the PMT signal and controls the flow via valve action or electro-osmotic potentials. Alternatively, the signal can be recorded or quantified as a measure of the reporter and/or its corresponding characteristic or marker, e.g., for the purpose of evaluation and without necessarily proceeding to sort the molecules or cells.

The chip can be mounted on an inverted optical microscope. Fluorescence produced by a reporter is excited using a laser beam focused on molecules (e.g., DNA, protein, enzyme or substrate) or cells passing through a detection region. Fluorescent reporters can include, but are not limited to, rhodamine, fluorescein, Texas red, Cy 3, Cy 5, phycobiliprotein (*e.g.*, phycoerythrin), green fluorescent protein (GFP), YOYO-1 and PicoGreen. In molecular fingerprinting applications, the reporter labels can be fluorescently labeled single nucleotides, such as fluorescein-dNTP, rhodamine-dNTP, Cy3-dNTP, etc.; where dNTP represents dATP, dTTP, dUTP or dCTP. The reporter can also be chemically-modified single nucleotides, such as biotin-dNTP. The reporter can be fluorescently or chemically labeled amino acids or antibodies (which bind to a particular antigen, or fragment thereof, when expressed or displayed by a cell or virus).

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The device can analyze and/or sort cells based on the level of expression of selected cell markers, such as cell surface markers, which have a detectable reporter bound thereto, in a manner similar to that currently employed using fluorescence-activated cell sorting (SACS) machines. Proteins or other characteristics within a cell, and which do not necessarily appear on the cell surface, can also be identified and used as a basis for sorting. The device can also determine the size or molecular weight of molecules such as polynucleotides or polypeptides (including enzymes and other proteins) or fragments thereof passing through the detection module. Alternatively, the device can determine the presence or degree of some other characteristic indicated by a reporter. If desired, the cells, particles or molecules can be sorted based on this analysis. The sorted cells, particles or molecules can be collected from the outlet channels in collection modules (or discarded in wasted modules) and used as needed. The collected cells, particles or molecules can be removed from the device or reintroduced to the device for additional coalescence, analysis and sorting.

To detect a reporter or determine whether a molecule, cell or particle has a desired characteristic, the detection module may include an apparatus for stimulating a reporter for that characteristic to emit measurable light energy, e.g., a light source such as a laser, laser diode, light emitting diode (LED), high-intensity lamp, (e.g., mercury lamp), and the like. Where a lamp is used, the channels are preferably shielded from light in all regions except the detection module. Where a laser is used, the laser can be set to scan across a set of detection modules from different analysis units. In addition, laser diodes or LED's may be microfabricated into the same chip that contains the analysis units. Alternatively, laser diodes or LED's may be incorporated into a second chip (i.e., a laser diode chip) that is placed adjacent to the microfabricated analysis or sorter chip such that the laser light from the diodes shines on the detection module(s).

An integrated semiconductor laser and/or an integrated photodiode detector can be included on the silicon wafer in the vicinity of the detection module. This design provides the advantages of compactness and a shorter optical path for exciting and/or emitted radiation, thus minimizing distortion and losses.

The present invention provides methods of droplet detection using electrical signal broadcasting. The device of the present invention can comprise features, such as integrated metal alloy components and/or features patterned in an electrically conductive layer, for detecting droplets by broadcasting a signal around a droplet and picking up an electrical signal in proximity to the droplet.

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The present invention provides self-aligning optical waveguides and optical elements for detection and control of droplets. The device of the present invention can comprise an accurate means of precisely aligning optical waveguides and their associated optical elements (lenses, prisms, mirrors, interconnects, etc.) to the fluidic channels contained within the device. Such waveguides can be used to provide well defined optical access to the fluidic channels to permit optical scattering, absorption, fluorescence, or any other optical measurement technique.

Fluidic channels within a microfluidic device are typically made using semiconductor lithographic processes. In order to create the waveguides, a separate series of channels and useful shapes (lenses, mirrors, etc) can be created either simultaneously (i.e. in the same processing step) or in successive steps. The reusable master created in this way can then used to fabricate the waveguide components and fluid channels without the need for special fixturing or careful alignment in subsequent steps. The extra channels or shapes can then filled with a high index of refraction liquid (for waveguides) or reflective material (for mirrors) through injection into the channel or void. The liquid can either remain as a fluid or be allowed to solidify. UV cure epoxies used by the telecommunications industry are excellent choices for the waveguide materials. Possible waveguide geometries can include a focusing lens and a back-reflecting mirror.

The dimensions of the detection module are influenced by the nature of the sample under study and, in particular, by the size of the droplets, beads, particles, molecules or cells (including virions) under study. For example, mammalian cells can have a diameter of about 1 to 50 microns, more typically 10 to 30 microns, although some mammalian cells (e.g., fat cells) can be larger than 120 microns. Plant cells are generally 10 to 100 microns. However, other molecules or particles can be smaller with a diameter from about 20 nm to about 500 nm.

Detection modules used for detecting molecules and cells have a cross-sectional area large enough to allow a desired molecule, cells, bead, or particles to pass through without being substantially slowed down relative to the flow carrying it.

In another embodiment, the droplet content detection can be achieved by simultaneous detection of contents of multiple droplets in parallel using spectroscopic fluorescence imaging with sensitivity as high as single-molecule limit. In this embodiment, one can spatially distribute droplets containing fluorescent entities such as Fluorophore biological markers and/or quantum dots in a two-dimesional sheet in a microscopic field-of-view. The filed-of-view of those droplets can then be illuminated by a fluorescence

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excitation source and the resulting fluorescence can be spectroscopically imaged. Therefore, for a given fluorescence detection sensitivity, the throughput of fluorescence detection compared to a single-drop fluorescence detection method can be increased by a factor of a/b for a given sensitivity, where a is the number of droplets that can be imaged within a given field-of-view, and b is the ratio of the fluorescence sensitivity of a single-drop fluorescence detector compared to that of the multiple drop fluorescence detector. Furthermore, unlike the prior art single-drop fluorescent detection method where the drops are flowed through a detection volume so that their residence time in the detection volume, and hence the signal integration time and sensitivity, is limited, the residence time of the droplet in the field-of-view can be unlimited, thereby allowing sensitivity as high as the single-molecule limit.

Sorting Module

The device of the present invention can further include one or more sorting modules. A "sorting module " is a junction of a channel where the flow of molecules, cells, small molecules or particles can change direction to enter one or more other channels, e.g., a branch channel for delivery to an outlet module (i.e., collection or waste module), depending on a signal received in connection with an examination in the detection module. Typically, a sorting module is monitored and/or under the control of a detection module, and therefore a sorting module may "correspond" to such detection module. The sorting region is in communication with and is influenced by one or more sorting apparatuses. A sorting apparatus comprises techniques or control systems, e.g., dielectric, electric, electro-osmotic, (micro-) valve, etc. A control system can employ a variety of sorting techniques to change or direct the flow of molecules, cells, small molecules or particles into a predetermined branch channel. A "branch channel" is a channel which is in communication with a sorting region and a main channel. Typically, a branch channel receives molecules, cells, small molecules or particles depending on the molecule, cells, small molecules or particles characteristic of interest as detected by the detection module and sorted at the sorting module. A branch channel can have an outlet module and/or terminate with a well or reservoir to allow collection or disposal (collection module or waste module, respectively) of the molecules, cells, small molecules or particles. Alternatively, a branch channel may be in communication with other channels to permit additional sorting.

The device of the present invention can further include one or more outlet modules. An "outlet module" is an area of a microfabricated device that collects or dispenses molecules, cells, small molecules or particles after coalescence, detection and/or sorting. The

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outlet module can include a collection module and/or a waste module. The collection module can be connected to a means for storing a sample. The collection module can be a well or reservoir for collecting and containing droplets detected to have a specific predetermined characteristic in the detection module. The collection module can be temperature controlled. The waste module can be connected to a means for discarding a sample. The waste module can be a well or reservoir for collecting and containing droplets detected to not have a specific predetermined characteristic in the detection module. The outlet module is downstream from a sorting module, if present, or downstream from the detection module if a sorting module is not present. The outlet module may contain branch channels or outlet channels for connection to a collection module or waste module. A device can contain more than one outlet module.

Mixing Module

Although coalescence of one or more droplets in one or more coalescence modules can be sufficient to mix the contents of the coalesced droplets (e.g., through rotating vortexes existing within the droplet), the device of the present invention can further include one or more mixing modules. A "mixing module" can comprise features for shaking or otherwise manipulate droplets so as to mix their contents. The mixing module is preferably downstream from the coalescing module and upstream from the detection module. The mixing module can include, but is not limited to, the use of metal alloy component electrodes or electrically conductive patterned electrodes to mix the contents of droplets and to reduce mixing times for fluids combined into a single droplet in the microfluidic device.

The device of the present invention can comprise features, such as, acoustic actuators, metal alloy component electrodes or electrically conductive patterned electrodes, for shaking droplets to reduce mixing times for fluids combined into a single droplet.

For acoustic manipulation, the frequency of the acoustic wave should be fine tuned so as not to cause any damage to the cells. The biological effects of acoustic mixing have been well studied (e.g., in the ink-jet industry) and many published literatures also showed that piezoelectric microfluidic device can deliver intact biological payloads such as live microorganisms and DNA. In an exemplary embodiment, the design of the acoustic resonant uses a Piezoelectric bimorph flat plate located on the side of the carved resonant in the PDMS slab. The piezoelectric driving waveform is carefully optimized to select the critical frequencies that can separate cells in fluids. There are five parameters to optimize beyond the frequency parameter. Lab electronics is used to optimize the piezoelectric driving waveform.

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Afterwards, a low cost circuit can be designed to generate only the optimized waveform in a preferred microfluidic device.

Delay Module

The device of the present invention can further include one or more delay modules. The "delay module" can be a delay line. The operation of a microfluidics device where a reaction within a droplet is allowed to occur for a non-trivial length of time requires a delay line to increase the residence time within the device. For reactions demanding extensive residence time, longer or larger delay lines are required. Accordingly, the invention provides methods to increase residence times within microfluidic devices.

The delay module is in fluid communication with the main channel. The delay module can be located downstream of the coalescence module and upstream of the detection module. The delay module can be a serpentine channel or a buoyant hourglass. The delay module can further comprise heating and cooling regions. The heating and cooling regions can be used for performing on-chip, flow-through PCR with the devices described herein.

The channel dimensions and configurations can be designed to accommodate the required residence time with minimum pressure drops across the device. For example, to accommodate very long delay lines within the microfluidic device, the device can comprise a multilayered PDMS slab which is composed of several patterned PDMS slabs.

The channel dimensions can also be designed so as to allow for required flow, residence time and pressure drop. Some channels may be required to be very large in width and height. In order to avoid collapse of the channels, the device includes support posts within the channel design. In order to reduce dead volume behind posts and further improve droplet stability, the support posts are designed to optimize a streamlined flow within the channel. These designs can include curved features as opposed to sharp edges.

To allow for longer period of device operation, delay lines can also be extended to the outside of the chip. The off-chip delay lines can be tubes within micron-sized internal diameter.

In order to allow more efficient use of available space and faster operation, in methods where droplets are charged, after charging, asymmetric splitting of oil and drops can be accommodated by siphoning off oil from channels after droplets are charged.
The delay lines can be in the form of a tower (i.e., a structure which is vertical with respect to the ambient gravitational field) as to allow buoyant forces to assist controlled droplet transport. Known delay lines involve transporting droplets by emulsifying them in a carrier

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fluid flowing in a channel and/or tube. Because the velocity profile of the carrier fluid through the cross-section of the channel and/or tube is not uniform, the velocity distribution of the droplets will not be narrow, which causes the delay time distribution of the droplets to not be narrow (i.e., some droplets will be delayed more or less than others).

The devices of the present invention can also include buoyancy-assisted microfluidic delay lines. In buoyancy-assisted microfluidic delay lines, buoyant forces act on droplets emulsified in a fluid in one or more towers. This can include allowing the tower to fill for the desired delay time, and then releasing the droplets. The tower can or can not continue to fill and release droplets as needed. In this example, one may desire to have a cylindrical tower section that is capped by a pyramidal funnel section. The tower can effectively functions as an hourglass. Droplets that have a density less than their carrier fluid are fed into the base of the tower, buoyantly rise to the top of the tower with a substantially uniform velocity distribution, and are funneled into a functional component of the microfluidic device (such as a y-branch). Carrier fluid is exhausted at the base of the tower at the same rate as it is introduced at the apex so that the net flow of carrier fluid through the delay line is zero. The tower and funnel sections can have any cross-sectional shape, such as circular, elliptical, or polygonal. The microfluidic device can include a tower with adjustable length.

The device can also include a switching network of twenty towers to guarantee a delay time dispersion of 5% (because 1/20 = 0.05). The capacity of each tower is 0.05*T, where T is the delay time. The concept includes, for example: (a) upon device start-up, filling the first tower for 0.05*T, but stop-cock its exhaust, and also have the other nineteen towers closed; (b) after 0.05*T, closing the first tower and filling the second between 0.05*T and 0.10*T; (c) repeating step (b) for the remaining eighteen towers; (d) at time T, allowing the first tower to exhaust; (e) at time 1.05*T, stop-cocking the exhaust of the first tower, allowing the second tower to exhaust, and allowing the first tower to fill; (f) at time 1.10*T, stop-cocking the exhaust of the second tower, allowing the third tower to exhaust, closing the first tower, and allowing the second tower to fill, and (g) repeating step (f) ad infinitum. More than twenty towers may provide an even tighter control over the width of the delay time dispersion. This scheme may require a valve network. This network of towers can be outside the microfluidic device.

UV-Release Module

The device of the present invention can further include one or more UV-release modules. The "UV-release module" is in fluid communication with the main channel. The

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UV-release module is located downstream of the inlet module and upstream of the coalescence module. The UV-module can be a used in bead assays. Compounds from encapsulated beads can be cleaved in a UV-releasing module using UV light. Photolabile linkers can be broken down on demand after a single bead has been encapsulated thus releasing multiple copies of a single compound into solution. In the cell based assay disclosed herein the chemical compound assayed is desired to be in solution in order to penetrate the cell membrane. Furthermore, to ensure compartmentalization of a single compound with a cell the cleavage of the compound from the solid support can only be done after the bead has been encapsulated. Photocleavable linkers can be utilized to cleave the compounds of the bead after drop formation by passing the drop through a UV-release module (i.e., laser of the appropriate wavelength).

The present invention also provides methods for chemical synthesis on a bead and releasing said chemical attached to the bead using a releasing means (chemical, UV light, heat, etc) within a droplet, and then combining a second droplet to the first droplet for further manipulation. Preferably, the releasing means is a UV-module. For example, tea-bag synthesis of chemicals on a bead simultaneously with a means for identifying said bead (using, for example, a mass spec tag). Using the resulting mixed-chemistry beads in a droplet within a fluid flow, and exposing the beads to UV light to release the chemical synthesized from the bead into the droplet environment. Combining the droplet containing the released chemical with a droplet containing a cell, and performing a cell-based assay. Sorting droplets having the desired characteristics (for example, turn on of a reporter gene), and then analyzing the sorted beads using mass spectroscopy.

Kits

As a matter of convenience, predetermined amounts of the reagents, compound libraries, and/or emulsions described herein and employed in the present invention can be optionally provided in a kit in packaged combination to facilitate the application of the various assays and methods described herein. Such kits also typically include instructions for carrying out the subject assay, and may optionally include the fluid receptacle, e.g., the cuvette, multiwell plate, microfluidic device, etc. in which the reaction is to be carried out.

Typically, reagents included within the kit are uniquely labeled emulsions containing tissues, cells, particles, proteins, antibodies, amino acids, nucloetides, small molecules, substrates, and/or pharmaceuticals. These reagents may be provided in pre-measured container (*e.g.*, vials or ampoules) which are co-packaged in a single box, pouch or the like

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that is ready for use. The container holding the reagents can be configured so as to readily attach to the fluid receptacle of the device in which the reaction is to be carried out (*e.g.*, the inlet module of the microfluidic device as described herein). In one embodiment, the kit can include an RNAi kit. In another embodiment, the kit can include a chemical synthesis kit. It will be appreciated by persons of ordinary skill in the art that these embodiments are merely illustrative and that other kits are also within the scope of the present invention.

Definitions

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and in the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the devices and methods of the invention and how to make and use them. It will be appreciated that the same thing can typically be described in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein. Synonyms for certain terms are provided. However, a recital of one or more synonyms does not exclude the use of other synonyms, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

The invention is also described by means of particular examples. However, the use of such examples anywhere in the specification, including examples of any terms discussed herein, is illustrative only and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to any particular preferred embodiments described herein. Indeed, many modifications and variations of the invention will be apparent to those skilled in the art upon reading this specification and can be made without departing from its spirit and scope. The invention is therefore to be limited only by the terms of the appended claims along with the full scope of equivalents to which the claims are entitled.

As used herein, "about" or "approximately" shall generally mean within 20 percent, preferably within 10 percent, and more preferably within 5 percent of a given value or range.

The term "molecule" means any distinct or distinguishable structural unit of matter comprising one or more atoms, and includes for example polypeptides and polynucleotides.

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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.

The term "polynucleotide" as used herein refers to a polymeric molecule having a backbone that supports bases capable of hydrogen bonding to typical polynucleotides, where the polymer backbone presents the bases in a manner to permit such hydrogen bonding in a sequence specific fashion between the polymeric molecule and a typical polynucleotide (e.g., single-stranded DNA). Such bases are typically inosine, adenosine, guanosine, cytosine, uracil and thymidine. Polymeric molecules include double and single stranded RNA and DNA, and backbone modifications thereof, for example, methylphosphonate linkages.

Thus, a "polynucleotide" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") generally in DNA and RNA, and means any chain of two or more nucleotides. A nucleotide sequence typically carries genetic information, including the information used by cellular machinery to make proteins and enzymes. These terms include double or single stranded genomic and cDNA, RNA, any synthetic and genetically manipulated polynucleotide, and both sense and anti-sense polynucleotide (although only sense stands are being represented herein). This includes single- and double-stranded molecules, i.e., DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases, for example thio-uracil, thio-guanine and fluoro-uracil.

The polynucleotides herein may be flanked by natural regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5′- and 3′- non-coding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Polynucleotides may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The polynucleotides may be derivatized by formation of a methyl or ethyl

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phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the polynucleotides herein may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The term "interdigitation" as used herein means pairing of droplets from separate aqueous streams, or from two separate inlet nozzles, for eventual coalescence.

The term "dielectrophoretic force gradient" means a dielectrophoretic force is exerted on an object in an electric field provided that the object has a different dielectric constant than the surrounding media. This force can either pull the object into the region of larger field or push it out of the region of larger field. The force is attractive or repulsive depending respectively on whether the object or the surrounding media has the larger dielectric constant.

"DNA" (deoxyribonucleic acid) means any chain or sequence of the chemical building blocks adenine (A), guanine (G), cytosine (C) and thymine (T), called nucleotide bases, that are linked together on a deoxyribose sugar backbone. DNA can have one strand of nucleotide bases, or two complimentary strands which may form a double helix structure. "RNA" (ribonucleic acid) means any chain or sequence of the chemical building blocks adenine (A), guanine (G), cytosine (C) and uracil (U), called nucleotide bases, that are linked together on a ribose sugar backbone. RNA typically has one strand of nucleotide bases.

A "polypeptide" (one or more peptides) is a chain of chemical building blocks called amino acids that are linked together by chemical bonds called peptide bonds. A "protein" is a polypeptide produced by a living organism. A protein or polypeptide may be "native" or "wild-type", meaning that it occurs in nature; or it may be a "mutant", "variant" or "modified", meaning that it has been made, altered, derived, or is in some way different or changed from a native protein, or from another mutant.

An "enzyme" is a polypeptide molecule, usually a protein produced by a living organism, that catalyzes chemical reactions of other substances. The enzyme is not itself altered or destroyed upon completion of the reaction, and can therefore be used repeatedly to catalyze reactions. A "substrate" refers to any substance upon which an enzyme acts.

As used herein, "particles" means any substance that may be encapsulated within a droplet for analysis, reaction, sorting, or any operation according to the invention. Particles are not only objects such as microscopic beads (e.g., chromatographic and fluorescent beads), latex, glass, silica or paramagnetic beads, but also includes other encapsulating porous and/or biomaterials such as liposomes, vesicles and other emulsions. Beads ranging in size from 0.1 micron to 1 mm can be used in the devices and methods of the invention and are therefore encompassed with the term "particle" as used herein. The term particle also encompasses

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biological cells, as well as beads and other microscopic objects of similar size (e.g., from about 0.1 to 120 microns, and typically from about 1 to 50 microns) or smaller (e.g., from about 0.1 to 150 nm). The devices and methods of the invention are also directed to sorting and/or analyzing molecules of any kind, including polynucleotides, polypeptides and proteins (including enzymes) and their substrates and small molecules (organic or inorganic). Thus, the term particle further encompasses these materials.

The particles (including, e.g., cells and molecules) are sorted and/or analyzed by encapsulating the particles into individual droplets (e.g., droplets of aqueous solution in oil), and these droplets are then sorted, combined and/or analyzed in a microfabricated device. Accordingly, the term "droplet" generally includes anything that is or can be contained within a droplet.

A "small molecule" as used herein, is meant to refer to a composition that has a molecular weight of less than about 5 kD and most preferably less than about 4 kD. Small molecules can be, *e.g.*, nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic or inorganic molecules. Libraries of chemical and/or biological mixtures, such as fungal, bacterial, or algal extracts, are known in the art.

As used herein, "cell" means any cell or cells, as well as viruses or any other particles having a microscopic size, e.g. a size that is similar to or smaller than that of a biological cell, and includes any prokaryotic or eukaryotic cell, e.g., bacteria, fungi, plant and animal cells. Cells are typically spherical, but can also be elongated, flattened, deformable and asymmetrical, i.e., non-spherical. The size or diameter of a cell typically ranges from about 0.1 to 120 microns, and typically is from about 1 to 50 microns. A cell may be living or dead. Since the microfabricated device of the invention is directed to sorting materials having a size similar to a biological cell (e.g. about 0.1 to 120 microns) or smaller (e.g., about 0.1 to 150 nm) any material having a size similar to or smaller than a biological cell can be characterized and sorted using the microfabricated device of the invention. Thus, the term cell shall further include microscopic beads (such as chromatographic and fluorescent beads), liposomes, emulsions, or any other encapsulating biomaterials and porous materials. Non-limiting examples include latex, glass, orparamagnetic beads; and vesicles such as emulsions and liposomes, and other porous materials such as silica beads. Beads ranging in size from 0.1 micron to 1 mm can also be used, for example in sorting a library of compounds produced by combinatorial chemistry. As used herein, a cell may be charged or uncharged. For example, charged beads may be used to facilitate flow or detection, or as a reporter. Biological cells, living or dead, may be charged for example by using a surfactant, such as

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SDS (sodium dodecyl sulfate). The term cell further encompasses "virions", whether or not virions are expressly mentioned.

A "virion", "virus particle" is the complete particle of a virus. Viruses typically comprise a nucleic acid core (comprising DNA or RNA) and, in certain viruses, a protein coat or "capsid". Certain viruses may have an outer protein covering called an "envelope". A virion may be either living (i.e., "viable") or dead (i.e., "non-viable"). A living or "viable" virus is one capable of infecting a living cell. Viruses are generally smaller than biological cells and typically range in size from about 20-25 nm diameter or less (parvoviridae, picornoviridae) to approximately 200-450 nm (poxviridae). However, some filamentous viruses may reach lengths of 2000 nm (closterviruses) and are therefore larger than some bacterial cells. Since the microfabricated device of the invention is particularly suited for sorting materials having a size similar to a virus (i.e., about 0.1 to 150 nm), any material having a size similar to a virion can be characterized and sorted using the microfabricated device of the invention. Non-limiting examples include latex, glass or paramagnetic beads; vesicles such as emulsions and liposomes; and other porous materials such as silica beads. Beads ranging in size from 0.1 to 150 nm can also be used, for example, in sorting a library of compounds produced by combinatorial chemistry. As used herein, a virion may be charged or uncharged. For example, charged beads may be used to facilitate flow or detection, or as a reporter. Biological viruses, whether viable or non-viable, may be charged, for example, by using a surfactant, such as SDS.

A "reporter" is any molecule, or a portion thereof, that is detectable, or measurable, for example, by optical detection. In addition, the reporter associates with a molecule, cell or virion or with a particular marker or characteristic of the molecule, cell or virion, or is itself detectable to permit identification of the molecule, cell or virion's, or the presence or absence of a characteristic of the molecule, cell or virion. In the case of molecules such as polynucleotides such characteristics include size, molecular weight, the presence or absence of particular constituents or moieties (such as particular nucleotide sequences or restrictions sites). In the case of cells, characteristics which may be marked by a reporter includes antibodies, proteins and sugar moieties, receptors, polynucleotides, and fragments thereof. The term "label" can be used interchangeably with "reporter". The reporter is typically a dye, fluorescent, ultraviolet, or chemiluminescent agent, chromophore, or radio-label, any of which may be detected with or without some kind of stimulatory event, e.g., fluoresce with or without a reagent. In one embodiment, the reporter is a protein that is optically detectable without a device, e.g. a laser, to stimulate the reporter, such as horseradish peroxidase (HRP).

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A protein reporter can be expressed in the cell that is to be detected, and such expression may be indicative of the presence of the protein or it can indicate the presence of another protein that may or may not be coexpressed with the reporter. A reporter may also include any substance on or in a cell that causes a detectable reaction, for example by acting as a starting material, reactant or a catalyst for a reaction which produces a detectable product. Cells may be sorted, for example, based on the presence of the substance, or on the ability of the cell to produce the detectable product when the reporter substance is provided.

A "marker" is a characteristic of a molecule, cell or virion that is detectable or is made detectable by a reporter, or which may be coexpressed with a reporter. For molecules, a marker can be particular constituents or moieties, such as restrictions sites or particular nucleic acid sequences in the case of polynucleotides. For cells and virions, characteristics may include a protein, including enzyme, receptor and ligand proteins, saccharrides, polynucleotides, and combinations thereof, or any biological material associated with a cell or virion. The product of an enzymatic reaction may also be used as a marker. The marker may be directly or indirectly associated with the reporter or can itself be a reporter. Thus, a marker is generally a distinguishing feature of a molecule, cell or virion, and a reporter is generally an agent which directly or indirectly identifies or permits measurement of a marker. These terms may, however, be used interchangeably.

The invention is further described below, by way of the following examples. The examples include descriptions of particular, exemplary embodiments of the devices and methods of the present invention, including particular embodiments of channel architectures, valves, switching and flow control devices and methods which may be implemented as part of the devices and methods of the invention. The examples are provided for illustrative purposes only and are not limiting of the above-described invention in any way. For example, many of these specific embodiments are described and discussed primarily in terms of detecting and sorting cells suspended directly in the fluid that flows through a main channel of the device. Nevertheless, it will be appreciated by persons of ordinary skill in the art that these preferred embodiments are merely illustrative and that the invention may be practiced in a variety of embodiments that share the same inventive concept. In particular, the devices and methods described in this example (including the channel architectures, valves, switching and flow control devices and methods) may be readily adapted to a multi-phased device so that droplets which contain, e.g., molecules, cells or virions may be analyzed and/or sorted as desired by a user.

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EXAMPLES

Example 1

The device of the present invention can be used for Live/Dead Cell Based Assays. In one example, the assay uses two fluorophores; one is permeable across cell membranes, and a second dye binds DNA and can enter the cell only if the membrane is compromised. Similar Live/Dead assays exist for bacteria and yeast. Tagged chemical libraries and photocleavable linkers can be used in such assays. Combinatorial one-bead-one-compound libraries obtained through split-bead synthesis require a tag which describes their synthetic history in order to identify the compound reliably. Several encoding technologies for microcarriers such as beads, rods and crowns have been developed over the last decade to address this need. A simple and effective method relies on spectrometric chemical tags which are generated in parallel to the chemical entity of interested utilizing orthogonal chemistry. Alternatives include the use of nucleic acids such as DNA, followed by the use of the polymerase chain reaction (PCR) to decode the encoded beads.

In the cell based assay disclosed herein the chemical compound assayed is desired to be in solution in order to penetrate the cell membrane. Furthermore, to ensure compartmentalization of a single compound with a cell the cleavage of the compound from the solid support can only be done after the bead has been encapsulated. Photocleavable linkers can be utilized to cleave the compounds of the bead after drop formation by passing the drop through a UV-release module (i.e., laser of the appropriate wavelength).

To evaluate the effect of individual components contained in the molecular library, a two color fluorescence detection for standard cytotoxicity assays can be used [available from Invitrogen (Carlsbad, CA) or Cell Technology]. While any cells can be used, for illustrative purposes, the Invitrogen LIVE/DEAD Viability/Cytotoxicity Kit #L3224 for animal cells are used here. This kit contains two probes that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. Live cells are identified by the presence of intracellular esterase activity, detected by the enzymatic conversion of the almost nonfluorescent cell-permeant calcein AM to the extremely fluorescent calcein. The calcein is retained within live cells, producing an intense uniform green fluorescence. EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells. EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of

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cells. Background fluorescence levels are inherently low with this assay technique because the dyes are essentially non-fluorescent before interacting with cells.

The spectral absorption and emission characteristics for both the calcein and EthD-1 are presented in Figure 3, while Figure 2 plots results presented by Molecular probes when a 50/50 mix of live and dead cells are run through a flow cytometer. The absorption characteristics of both dyes makes it possible to excite fluorescence using the existing 488 nm excitation source. Figure 2, left panel, shows a mixture of live and ethanol-killed bovine pulmonary artery epithelial cells stained with the reagents in Molecular Probes Live/Dead Cell Viability/Cytotoxicity Assay Kit (L3224). Live cells fluoresce bright green, whereas dead cells with compromised membranes fluoresce red-orange. (Molecular Probes). The middle panel shows a viability assay using Molecular Probes' LIVE/DEAD Viability/Cytotoxicity Kit on a flow cytometer. A 1:1 mixture of live and ethanol-fixed human B cells was stained with calcein AM and ethidium homodimer-1, flow cytometric analysis was carried out with excitation at 488 nm. The right panel shows analysis of bacterial cultures using the Live/Dead BacLight Bacterial Viability and Counting Kit available from www.molecular probes.com. As shown in Figure 3, left panel, the present invention further provides a fluorescence detection system comprising a fluorescence detection stand capable of measuring green fluorophores within microfluidic channels while simultaneously permitting visual monitoring via a high speed video microscope. The optical components of this system are commercially available. The modular layout of this system permits straightforward modification of the excitation and detection wavelengths. This modularity also makes it possible to upgrade the system to multi-wavelength excitation, multiwavelength detection, and detection of orthogonal polarization states. Currently, the 488 nm transition of a multiline Argon-Ion Laser is used as the excitation source for Fluorescein (FITC). The laser provides between 3 and 20 milliwatts of power and is focused to a spot approximately 17 microns in diameter (full width half maximum, FWHM). When the stand is configured to use a photomultiplier tube, it is able to detect less than 10,000 FITC molecules at a 10 kHz droplet rate. The sensitivity of this system is limited by fluorescence interference generated by the microfluidic device itself. The right panel of Figure 3 shows excitation and emission spectra for calcein AM and EthD-1 dyes. Normal cytometry protocol excites both at 488 nm. Figure 3 indicated the changes required to convert the single flurophore station to a two color fluorescence station. The calcein fluorescence can be collected using filters designed for fluorescein detection, while the EthD-1 can be monitored using filters designed for propidium iodide or Texas Red.

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Using water droplets doped with fluorescien and propidium iodide over a range of concentrations from $1x10^{-3}$M to $1x10^{-8}$M, both dyes have similar absorption and fluorescence properties to calcein and Ethd-1. In seveteen micron droplets, this corresponds to a range of $1.5x10^{9}$ to $1.5x10^{4}$ molecules within the measurement volume. Once the baseline performance has been verified, tests can begin on droplets containing live cells, dead cells, and mixtures of the two. This establishes selectivity and detection limits on the two types of cells.

The dyes selected have been used extensively in flow cytometry and are commonly used in most cell-based assays. They are designed not to overlap significantly with each other and can be evaluated both independently and together to assess the cross-talk. The status of (potentially) many cells within one drop can thus be determined. The use of inexpensive optics on our instrument will be more than compensated for by the theoretical increase of dye molecules in the nanoreactor. Optics with higher efficiencies can be used.

Compounds from encapsulated beads can be cleaved in a UV-releasing module using UV light. Photolabile linkers can be broken down on demand after a single bead has been encapsulated thus releasing multiple copies of a single compound into solution.

Synthetic chemistry relies on the differential activity of chemical groups in order to control bond breaking and forming processes. Photolabile protecting groups form a fourth orthogonal type of functionality which survive reaction conditions capable of cleaving protective groups of the other types. Several of these photolabile protecting groups have been used to link organic molecules to solid support and their use as linkers has been reviewed. This allows the synthesis of solid supported molecules with the option of releasing the final product by irradiation with the appropriate wavelength. The repertoire of chemical groups for which photocleavalbe protecting groups have been devised is extensive, which allows the synthesis of diverse combinatorial libraries.

In lieu of the high sample rate a triazene-based photolabile linker, which is cleaved by irradiation with a 355 nm 3w Nd-YAG laser, can be used. This linker is stable under a wide range of reaction conditions with the exception of strong acids lending itself to solid supported split-bead synthesis.

If the residence time of the bead inside the UV laser is insufficient to cleave all of the compound off the substrate bead, the residence time can be increased by slowing down the flow of the bead containing drops by widening the channel. Alternatively the intensity of laser beam can be increased to ensure complete cleavage.

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As previously discussed, long incubation times are desirable for cytotoxicity studies, but cannot be easily achieved in known micofulidic channel layouts. Accordingly, one embodiment of the device used in the live/dead cell-based assay disclosed herein uses a passive means to achieve uniform droplet residency times exceeding one hour in a delay line module located directly before the sorting module. It is possible to achieve a delay time of one hour between droplet generation and detection without stopping droplet generation.

In one example, a "buoyancy hourglass" delay line can be used, wherein, similar to sands in an hourglass which depend on gravity, the droplets will rise from a large reservoir to an exhaust port due to their density mismatch with the carrier oil. Microfluidic modules (e.g., inlet module, UV-releasing module, coalescence module, and mixing module) which are utilized before the delay module can be patterned at the bottom of the stack, and microfluidic modules which are utilized after the delay module (e.g., detection module and sorting module) can be patterned at the top of the stack. Upon start-up, the hourglass will be stop-cocked to allow droplets to fill until the desired delay time is reached and then droplet will be removed from the device at the same rate that they enter, thereby ensuring essentially the same residency time for all droplets. Spontaneous droplet coalescence in the hourglass can be prevented by using one or more surfactants to stabilize the droplets.

The shape and timing of electric field gradients through the use of computer modeling can be optimized by tailoring the geometry of the electrodes and the fluid channels and the synchronization of the applied voltages to the droplets.

The FEMLAB (COMSOL, Inc.) partial differential equation solver software can be used to model the combination of fluid dynamics and electrostatics. The model can include "still-frame captures" of the trajectory of droplets through bifurcations, and can optimize the electrode geometry, the fluid channel geometry, and the distribution of applied voltages as a function of the incremental droplet trajectory. Further, a high-speed digital camera and driving electronics can be used to acquire "still-frame captures" of the actual droplet trajectories and comparing those captures to those produced by the model. The model and the electrode and fluid channel geometries can be iteratively optimized using inexpensive rapid prototyping capability (24 hours from design to test-results). Finally, the electric field gradients can be satisfactorily optimized when bidirectional sorting at rates of 1000 droplets/second or greater, without breaking the droplets and with an acceptably low error rate for the given application, is achieved. If the electromechanical relay network is not fast enough to operate with the optimized timing parameters, a solid-state relay network (e.g., using Behlke electronic relays) can be used to increase the speed of the driving electronics.

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Droplets containing beads can be sorted using dielectrophoretic and electrostrictive forces based on a fluorescence probe at rates of 1000 droplets/s or greater. A fluorescence detection system and Electrical Control System can be used to trigger the optimal "pulse" (i.e., distribution of applied voltages as a function of time) to sort neutral droplets based on the fluorescence probe. Dielectrophoretic/electrostrictive sorting of droplets containing fluorescent dye can be performed, wherein the sorting is triggered by the droplet number (e.g., every nth droplet is sorted in one direction, or every nth or mth droplet is sorted in one direction, etc.). Fluorescent dye can be used to perform dielectrophoretic sorting of droplets because it is convenient and inexpensive; the trigger signal for the dielectrophoretic/electrostrictive sorting can be exactly the same as was used for electrophoretic sorting. This process is the direct logical consequence of optimizing the electric field gradients.

Additionally, dielectrophoretic/electrostrictive sorting of droplets containing fluorescent beads can be performed. This step is intermediate between droplets containing fluorescent dye and droplets containing cells and beads laden with chemical libraries. Finally, dielectrophoretic/electrostrictive sorting of droplets containing fluorescent cells can be performed. This step is intermediate between droplets containing fluorescent dye and droplets containing cells and beads laden with chemical libraries.

In the event that the solutions containing the beads or cells have dielectric properties so different than the solution containing fluorescent dye that the dielectric field gradients are not optimal, the dielectric field gradients can be optimized separately for each solution. The fluorescent dye solution can be modified to better resemble the bead or cell solutions in order to continue to take advantage of the convenience of the fluorescent dye for the development of the sorting parameters.

Droplets sorted based on a particular phenotype (for example, dead cells) will be decoded (by using a decoding scheme) to identify the compound added in that droplet.

In some embodiments, the assay can be based on a nucleic-acid based encoded bead system. Two types of beads can be used for example - one contains a cytotoxic compound and oligonucleotide tag, and a second bead contains only a different oligonucleotide tag. The two types of beads may (optionally) also be encoded by a different fluorescent tag (i.e., other than the ones being used for the cell-based assays, as an example, two different Q-dots) so that the beads can be examined under a fluorescent microscope after sorting to determine the sorting efficiency.

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The sorted beads from dead-cell containing nanoreactors can then be taken, and using the polymerase chain reaction (PCR), the tags on the beads can be amplified using PCR primers. These tags can be 'hard-copied' by cloning them into a plasmid vector, transforming them into E. coli, and the tag sequence of 100 different E. coli transformants determined by DNA sequencing.

Additionally, more complex libraries using T-bag synthesis on beads, can be constructed, wherein oligonucleotide tags are specific for each round of synthesis for a monomer. The same monomer used in two different rounds can have two separate tags. As a non-limiting example, if 30 monomers in a bead-based T-bag synthesis were used for 5 rounds, 5x30, or 150 different tags will be required. The complexity of a library of 30 monomers after 5 rounds is 30^5, or nearly 25 million compounds. The beads in a specific T-bag after each round of monomer synthesis can have a specific oligonucleotide tag ligated, using T4 DNA ligase, onto the beads. These tags, from sorted beads, can be amplified, cloned and sequenced. By knowing what tags were used in which round of synthesis, an internal check of validation of the bead that was positive in that droplet is achieved. The sequencing reaction can be eliminated by using a hybridization chip containing the 150 tags.

Example 2.

The present invention provides methods for performing polymerase chain reaction in nanoreactors of the present invention as described. PCR can be performed on a drop-by-drop basis in a microfluidic device according to the present invention. A monolithic chip can be provided wherein the heating and cooling lines are built into the chip and a sorting means is provided. Advantages of performing PCR in droplets on such a chip are that the chip is disposable and the reaction can be repeated without cleaning the device between reactions. Furthermore, the chip provides a convenient way of getting all the components to perform PCR in the droplets in the right concentration. Additionally, the PCR is more efficient because the heat transfer is more efficient due to the small volume. This provides for shorter incubation/residence times. Droplets containing the nucleic acids, all PCR primers, and, if present, beads are generated one at a time at rates between 100 and 20,000 droplets per second. The droplets can then be sent through a serpentine path between heating and cooling lines to amplify the genetic material inside the droplets. Upon exiting the device the droplets may be sent for further on-chip or off-chip processing, directed into another chip, or the emulsion may be broken to release the PCR product. If present, beads may be harvested by passing the emulsion through a filtration device, sedimentation, or centrifugation.

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The width and depth of the channel can be adjusted to set the residence time at each temperature, which can be controlled to anywhere between less than a second and minutes. At a typical rate of 1000 drops per second, 1 million strands of DNA can be amplified in approximately 20 minutes on one device. A typical flow rate of 250 μL/hour would correspond to 1000 drops of 50 microns in diameter being generated every second. Flow rates and droplet sizes can be adjusted as needed by controlling the nozzle geometry.

In an example bead based application, the purpose is to amplify at most one DNA fragment in a droplet containing a single micro-bead (1 to 100 microns in diameter) and then separate and collect only the beads coated with DNA. This is achieved by starting with a dilute mixture of DNA fragments and beads in a solution containing the appropriate PCR primers. Droplets are then made in the limited dilution regime where most of the droplets are empty, but some droplets have a DNA strand in them and some droplets have beads in them. The target droplets have both a single DNA fragment and a single bead. After PCR amplification of the DNA on the surface of the beads a fluorescence activated sorting module (NanoFACS) can be added to the end of the device to separate the droplets into two populations, one containing amplified DNA and one without amplified DNA. The beads are then removed from the emulsion where the droplets all contain DNA to achieve a collection of beads where essentially all beads are coated with only one type of DNA fragment.

The quality of the collection of beads where each fragment is amplified in the presence of only one bead can be enhanced by ensuring that each droplet contains at most one bead. Droplets containing more than one bead can be removed using a fluorescence-based sorting step.

Along with PCR, nucleic acid based signal methods such as tyramide assays using an appropriate enzyme reaction, oligonucleotides decorated with two or more detecting groups, or other amplification means, for example, rolling circle amplification, ligase chain reaction, and NASBA can be used to increase the signal within a droplet.

Example 3.

The device of the present invention can be used to screen chemical libraries composed of at least 10^6 molecules against an established cell line. In this manner, positive and negative nanoreactors can be tracked and sorted using either a nucleic-acid based, or multi-colored bead-based encoding scheme For example, a control library with known hits can be screened against a human cancer cell line.

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In one embodiment, a chemical library can be screened using a nanoreactor as described in detail herein. The power of the present invention comes from a combination of compartmentalization and electrical manipulation that enables multi-step chemical processing, including analysis and sorting, to be initiated in confinement with exquisite timing and metering precision. This multi-step processing of isolated components is essential for searching through molecular libraries for rare interactions with cells, nucleic acids, enzymes, coded microbeads, and other biomaterials. For example, a set of encoding nucleic acids, (*i.e.*, DNA tags) can be combined into solutions of unique chemical compounds such that the DNA tags and chemicals are emulsified together. In one embodiment, the DNA tag acts as a surrogate identifier to track the associated chemical compound in droplets sorted by a nanoreator described herein. After sorting, the emulsion can be broken and the nucleic acids can be decoded (Figure 4). As shown in Figure 4 (*Left panel*), (**A**) An individual compound from a library of compounds will each be combined (**B**) with a unique, differentiatable set of q-dots. The combined mixture will each (**C**) be separately emulsified off-line using a flow-focusing microfluidics emulsifier to synthesize individual droplets containing both a specific compound and a unique set of q-dots. As shown in Figure 4 (*Right panel*), the set of individually-emulsified encoded compounds will be (**D**) pooled together and injected, along with either cells or enzymes, into the RDT instrument and (**E**) the two droplets combined to form individual NanoReactors. Depending on the reaction being monitored, a separate combining (not shown in the figure) of these nanoreactors with droplets containing assay components may be needed. In addition, a delay loop may be placed between these combinings and the detector (**F**) to allow sufficient time to occur in the droplet as to allow any potential chemical/cellular/enzymatic reaction to occur. The nanoreactors are next sent past a detector to both monitor the reaction and decode the q-dots contained within it. The nanoreactors can be further sorted (**G**) if necessary. In designing the device to be placed on the Instrument, individual modules are strung together in a sequence of droplet operations. Operations can be used to encapsulate cells or enzyme, inject the labeled pre-formed compound library emulsion, coalesce pairs of droplets, mix the contents of droplets, incubate reactions over time, detect fluorescence, decode the liquid label, sort (if needed) based on the detected signal, and transport droplets to collection and waste streams. The individual modules operate independently, much like resisters and capacitors in an electrical circuit, to collectively perform complex fluid processing operations. Several methods encompassing various chemical library screening embodiments of the invention as described. In one example, a kinase enzyme assay is used as an enzyme model, three different quantum

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dots (q-dots) for the liquid label, and a set of 96 different chemical compounds (in which 1-2 will be preferred kinase substrates) as the library. Fluorescence polarization is preferred since the argument can be made that it can be adapted to many different types of assays. In one example, water-soluble q-dots that emit at 620 nm, 650 nm and 680 nm are used. These emission bands are well outside of spectral region where the target enzyme assays emit (below 580 nm), so these q-dots are an excellent choice for In another example, near-IR q-dots are used to enhance their water solubility for the purpose of expanding the non-overlapping spectral region of the target assays. Moving the q-dot readout to this "unused" wavelength band can permit virtually any fluorescence assay of interest to be adapted to the nanoreactors of the present invention without modification, tremendously expanding the application space immediately available.

The nucleic acid can be a linear molecule wherein the ends can be used as priming sites for PCR, and the middle sequence is unique to each chemical compound; it is this middle sequence that is used as the encode. The nucleic acid and chemical compound are together combined into one droplet by pre-emulsifying the nucleic acid and chemical together and then adding them to a microfluidic device as described herein, as a pre-made, compound droplet. The compound droplet can be combined with a another droplet on the instrument. This other droplet can contain an item under investigation (including for example, but not limited to, a cell or enzyme), which, when combined with the compound droplet forms an 'assay' droplet. The assay droplets having a desired detected property (for example, inhibition of enzyme activity through the use of a fluorescent substrate added to the compound microdrop) can then be sorted. The sorted assay droplets can be collected, the emulsion broken, and the nucleic acid sequence can be decoded. The decoding can be performed by emulsion PCR (as described in U.S. Application Publication No. 2005-0227264) and sequencing on a sequencing instrument. Alternatively, the decoding can be performed by cloning the PCR product into an appropriate host (for example, *E. coli*), and the resultant clones subjected to DNA sequencing.

The nucleic acid can be a linear molecule having a region of uniqueness, and the decoding can be performed by cloning and subsequently transforming the DNA obtained from sorted assay droplets into an appropriate host (*e.g.*, *E. coli*). The resultant clones can then be subjected to decoding by hybridizing a PCR product containing the unique identifier to a complementary strand of nucleic acid fixed to a solid support (for example a chip, wafer, or bead).

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The nucleic acid can be a plasmid having a region of uniqueness, and the decoding can be performed by transforming the DNA obtained from sorted assay droplets into an appropriate host (*e.g.*, *E. coli*). The resultant clones can then be subjected to DNA sequencing to identify the encoded sequence.

The nucleic acid can be a plasmid having a region of uniqueness, and the decoding can be performed by transforming the DNA obtained from sorted assay droplets into an appropriate host (*e.g.*, *E. coli*). The resultant clones can then be subjected to decoding by hybridizing a labeled-PCR product containing the unique identifier to a complementary strand of nucleic acid fixed to a solid support (for example a chip, wafer, or bead).

The nucleic acid can be either a plasmid or linear fragment having a region of uniqueness, and the decoding can be performed by transforming the DNA obtained from sorted assay droplets into an appropriate host (*e.g.*, *E. coli*). The resultant clones can then be subjected to decoding by hybridizing a labeled-PCR product containing the unique identifier to a complementary strand of nucleic acid fixed to a solid support (for example a chip, wafer, or bead). Preferably, the bead can be encoded with dyes or Qdots, and the decoding can be performed on a microfluidic device according to the present invention, or on a Qdot or Luminex instrument.

A set of unique nucleic acids can be added to a set of unique chemical entities, wherein each combined set is separately emulsified. The separately emulsified combined set can be further combined to generate an emulsified mixed solution of droplets, wherein each droplet can contain both a nucleic acid and a unique chemical entity. This combined mixed solution can be injected into a microfluidic device according to the present invention for use in various assays contemplated by one of ordinary skill in the art.

The nucleic acid containing unique identifiers can be generated by PCR of an antibiotic resistance or other selectable gene with a set of the forward and reverse PCR primers each containing a 5' nucleotide sequence common to each other, forward and downstream primers, respectively, a unique sequence 3' to the common sequence, and a region of the antibiotic or other selectable gene. Said primers can be used in a PCR reaction to generate an antibiotic resistance or other selectable gene bracketed by unique identifiers which in turn can be bracketed by either a forward or reverse common sequence. The PCR product can then be cloned into a vector having a second antibiotic resistance or other selectable gene, and the vector can be cloned into an appropriate host (*e.g.*, *E. coli*), thereby selecting for antibiotic resistance and another selectable gene simultaneously.

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The label can also be a solution containing a dye such as an organic dye (for example cy3, cy5, flourescein) or inorganic label such as a quantum dot. The dot can be further coated or encapsulated by hydrophobic residues. More than one dye can be added to a solution prior to emulsification and the ratio of one or more dyes can be used to decode the droplet.

Additionally, many bead-encoded assays have already been developed for microspheres that should be directly ported to the devices and systems disclosed herein. Such assays include, for example: allergy testing, disease markers (including, autoimmune, cancer and cardiac), cytokine, genotyping, gene expression, infectious disease, kinase/phosphorylated proteins, metabolic markers, tissue typing, transcription factors/nuclear receptors and others.

The present invention also provides methods of using a drop-washer for combinatorial chemistry/biology. A device of the present invention capable of exchanging constituents within a droplet through the use of fluid flow in such a way that the micropdrop, while in a first immiscible fluid, is exposed to a second immiscible fluid such that constituents within the droplet that are immiscible in the first immiscible fluid are soluble in the second immiscible fluid.

For example, an aqueous droplet containing a chemical reaction produces by-products that are soluble in a lipid solvent. The chemical reaction is performed in a water-environment in a silicon-based solvent. After the chemical reaction occurs, the droplet is exposed to an organic-oil based solvent where the chemical byproducts are allowed to diffuse out of the droplet. The resulting droplet is then assayed for cell-killing activity by combining the droplet with live cells.

Similar to the preceding example, but the change in the non-aqueous fluid flow is used to add a particular constituent from the second immiscible fluid to diffuse into the aqueous drop before the droplet is returned to the 100% first immiscible fluid flow.

Example 4.

The present invention also provides methods of performing biological assays in nanoreactors using fluorescence polarization (FP). Fluorescence polarization technology has been used in basic research and commercial diagnostic assays for many decades, but has begun to be widely used in drug discovery only in the past six years. Originally, FP assays for drug discovery were developed for single-tube analytical instruments, but the technology was rapidly converted to high-throughput screening assays when commercial plate readers with equivalent sensitivity became available. These assays include such well-known

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pharmaceutical targets such as kinases, phosphatases, proteases, G-protein coupled receptors, and nuclear receptors.

Nuclear Receptors; FP has been used to develop high throughput screening (HTS) assays for nuclear receptor-ligand displacement (Parker GJ, et al., Development of high throughput screening assays using fluorescence polarization: nuclear receptor-ligand-binding and kinase/phosphatase assays.). The FP-based estrogen receptor (ER) assay is based on the competition of fluorescein-labeled estradiol and estrogen-like compounds for binding to ER. In a screen of 50 lead compounds from a transcriptional activation screen, 21 compounds had IC50 values below 10 microM, with one exhibiting roughly a 100-fold higher affinity for ERbeta over ERalpha. An FP-based competitive binding assay can be used to screen diverse compounds with a broad range of binding affinities for ERs.

Phosphatases and Kinases; A nonradioactive, simple, sensitive fluorescence polarization assay has been developed to assay protein tyrosine kinase activity (Seethala R.; Menzel R. A Homogeneous, Fluorescence Polarization Assay for Src-Family Tyrosine Kinases. Analytical Biochemistry, November 1997, vol. 253, no. 2, pp. 210-218(9)). This assay involves incubation of a fluorescenylated peptide substrate with the kinase, ATP, and anti-phosphotyrosine antibody. The phosphorylated peptide product is immunocomplexed with the anti-phosphotyrosine antibody resulting in an increase in the polarization signal as measured in a fluorescence polarization analyzer. These results show that the fluorescence polarization assay can detect inhibitors and is comparable to the $^{32}PO_4$ transfer assay. The fluorescence polarization method is advantageous compared to the $^{32}PO_4$ transfer assay or ELISA or DELFIA because it is a one-step assay that does not involve several washings, liquid transfer, and sample preparation steps. It has the added advantage of using nonisotopic substrates. The fluorescence polarization assay thus is environmentally safe and minimizes handling problems.

G-protein coupled receptors; High-throughput fluorescence polarization (FP) assays offer a nonradioactive, homogeneous, and low-cost alternative to radioligand binding assays for cell surface receptors (G protein-coupled receptors and ligand-gated ion channels) (Allen M, Reeves J, Mellor G.. High throughput fluorescence polarization: a homogeneous alternative to radioligand binding for cell surface receptors. J Biomol Screen. 2000 Apr;5(2):63-9.). FP assays were shown to work across a range of both peptide (vasopressin V1a and delta-opioid) and nonpeptide (beta1-adrenoceptor, 5-hydroxytryptamine3) receptors. Assays could be run in 384-well plates with little loss of signal window or sensitivity

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compared to 96-well plate assays. New advances in FP measurement have therefore enabled FP to offer a high throughput alternative to radioligand binding for cell surface receptors.

GTPases; A 30,000-member compound library was screened using filter binding [FB (^{33}P)] and FP detection systems, and compounds that were active in either assay were retested in 5-point curve confirmation assays (C.L. Hubert et al. Data Concordance from a Comparison between Filter Binding and Fluorescence Polarization Assay Formats for Identification of ROCK-II Inhibitors). Analysis of these data showed an approximate 95% agreement of compounds identified as active in both assay formats. Also, compound potency determinations from FB and FP had a high degree of correlation and were considered equivalent. These data suggest that the assay methodology has little impact on the quality and productivity of the screen, provided that the assays are developed to standardize kinetic conditions.

Diagnostics using Antibodies; The control of equine infectious anemia virus (EIAV) infections of horses has been over the past 20 years based primarily on the identification and elimination of seropositive horses, predominantly by a standardized agar gel immunodiffusion (AGID) assay in centralized reference laboratories. Peptides derived from antigenic regions of EIAV core and envelope proteins were initially screened for their utility as probes in an FP assay to select the best peptide antigen candidates (S.B. Tencza, et al. Development of a Fluorescence Polarization-Based Diagnostic Assay for Equine Infectious Anemia Virus. Journal of Clinical Microbiology, May 2000, p. 1854-1859, Vol. 38, No. 5). The FP assay was optimized to detect the presence of EIAV-specific antibodies by a change in the FP of a fluorescein-labeled immunoreactive peptide diagnostic antigen. The most sensitive and specific peptide probe was a peptide corresponding to the immunodominant region of the EIAV transmembrane protein, gp45. This probe was tested for its reactivity in the optimized FP assay with 151 AGID-positive horse sera and 106 AGID-negative serum samples. The results of these studies demonstrated that the FP assay reactivity correlated with reported AGID results in 106 of 106 negative serum samples (100% specificity) and in 135 of 151 positive serum samples (89.4% sensitivity). The FP assay was also found to have a very low background reactivity and to readily detect antibodies produced early in infection (≤3 weeks postinfection).

FP is a homogeneous technology with very rapid reactions; seconds to minutes suffice to reach equilibrium. As the reagents are stable, large highly reproducible batches may be prepared. Because of these properties, FP has proven to be highly automatable, often performed with a single incubation with a single, premixed, tracer-receptor reagent. The fact

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that there are no washing steps increases the precision and speed over heterogeneous technologies and dramatically reduces waste.

Other homogeneous technologies based on fluorescence intensity have been developed. These include energy transfer, quenching, and enhancement assays. FP offers several advantages over these. The assays are usually easier to construct, since the tracers do not have to respond to binding by intensity changes. In addition, only one tracer is required and crude receptor preparations may be utilized. Furthermore, since FP is independent of intensity, it is relatively immune to colored solutions and cloudy suspensions. FP offers several advantages in the area of instrumentation. Because FP is a fundamental property of the molecule, and the reagents are stable, little or no standardization is required. FP is relatively insensitive to drift in detector gain settings and laser power.

The concept of molecular movement and rotation is the basis of fluorescence polarization. By using a fluorescent dye to label a small molecule, its binding to another molecule of equal or greater size can be monitored through its speed of rotation. As shown in Figure 5, dye molecules with their absorption transition vectors (arrows) aligned parallel to the electric vector of linearly polarized light (along the vertical page axis) are selectively excited. For dyes attached to small, rapidly rotating molecules, the initially photoselected orientational distribution becomes randomized prior to emission, resulting in low fluorescence polarization. Conversely, binding of the low molecular weight tracer to a large, slowly rotating molecule results in high fluorescence polarization. Fluorescence polarization therefore provides a direct readout of the extent of tracer binding to proteins, nucleic acids and other biopolymers.

Fluorescence polarization, first described in 1926 by Perrin, has a long history. FP theory and the first instrument for measuring was developed by Weber. This work was expanded to biological systems, such as antigen-antibody reactions and hormone-receptor interactions by Dandliker. The first commercial systems, aimed at monitoring drugs in body fluids come from Jolley and co-workers.

Fluorescence polarization is defined by the following equation: $P = (V - H) / (V + H)$ where P is the polarization unit, V is the intensity of the vertical component of the emitted light, and H is the intensity of horizontal component of the emitted light of a fluorophore excited by vertical plane polarized light. The "polarization unit" P is a dimensionless entity and is not dependent on the intensity of the emitted light or on the concentration of the fluorophore. This is the fundamental power of FP. The term "mP" is now in general use, where 1 mP equals one thousandth of a P.

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The excitation dipole is the direction in which the molecule prefers to absorb light. The emission dipole is the direction in which a molecule prefers to emit light. This assumed (for the sake of simplicity) that these directions are parallel. In one experiment, if the fluorescent molecules are fixed so that all excitation dipoles are aligned in the vertical plane and assume there is only fluoresces with a polarization along the emission dipole then a maximum polarization unit of 1000 mP is observed. If, however, the excitation dipoles were randomly oriented this maximum polarization unit is reduced to 500mP. In another experiment, if the requirement that the dipoles arc fixed was removed and they are allowed to reorient between the time when they are excited and the time when they fluoresce the polarization unit falls below 500 mP.

In another experiment, a collection of randomly oriented transition moments are free to rotate. In this case, the polarization unit is between 0 and 500 mP and is dependent on how far the molecule has rotated during the fluorescence lifetime of the excited state. The smaller the molecule, the faster it rotates, and so the lower the FP will be.

The rate of rotation of a molecule is described by the Stokes equation: $\rho = (3\eta V) / (RT)$ where ρ is the rotational relaxation time (the time required to rotate through an angle whose cosine is 1/e, or approximately 68.5°), η is the viscosity of the medium, V is the molecular volume of the molecule, R is the gas constant, and T is the temperature in degrees Kelvin. From the previous equations we can see that the higher the molecular weight of a molecule, the higher the rotational relaxation time will be; V=vM where M (Perrin equation) is the molecular weight of the molecule in Daltons and v is its partial specific volume ($cm^3\ g^{-1}$). The Perrin equation was first described in 1926, and describes the relationship between the observed FP, the limiting polarization, the fluorescence lifetime of the fluorophore (τ), and its rotational relaxation time. $((1/P) - (1/3)) = ((1/P_0) - (1/3)) \times ((1 + (3\tau / P))$.

The shorter the fluorescence lifetime, the higher the FP will be. Conversely, the shorter the rotational relaxation time, the smaller the FP will be. Combining the Stokes equation and the Perrin equation, and substituting M for V and rearranging, we get the relationship between the molecular weight of a molecule and its FP (1 / P is proportional to 1 / M); $(1/P) = (1/P_0) + ((1/P_0) - (1/3)) \times (RT/vM) \times (\tau/\eta)$. From this equation we can see that P equals P_0 in the limiting cases of high molecular weight, high viscosity, and short lifetime. In fact, P_0 can be determined by measuring FP at various viscosities, plotting P against $1/\eta$, and determining the intercept on the ordinate.

Organic fluorophores have characteristics, such as narrow excitation bands and broad red-tailing emission band. Figure 6 (Left, Center Panel) shows the absoprtion and emission

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spectra of q-dot 535 nanocrystals and fluorescein, respectively. Figure 6 (Right panel) shows the emission spectra of a several sizes of CdSe-ZnS quantum dots, with excitation of ZnSe at 290 nm, all others at 365 nm. nm in all cases. These bands often limit their effectiveness. This makes concurrent resolution of multiple light-emitting probes problematic due to spectral overlap. Also, many organic dyes exhibit low resistance to photodegradation.

Luminescent colloidal semiconductor nanocrystals called quantum dots or q-dots (QD) are inorganic fluorophores that have the potential to circumvent some of the functional limitations encountered by organic dyes. In particular, CdSe-ZnS core-shell QDs exhibit size-dependent tunable photoluminescence (PL) with narrow emission bandwidths (FWHM ~ 30 to 45 nm) that span the visible spectrum and broad absorption bands. These allow simultaneous excitation of several particle sizes (colors) at a common wavelength. This, in turn, allows simultaneous resolution of several colors using standard instrumentation (Figure 6, right panel) . CdSe-ZnS QDs also have high quantum yields, are resistant to photodegradation, and can be detected optically at concentrations comparable to organic dyes.

Quantum dots are nano-scale semiconductors typically consisting of materials such as crystalline cadmium selenide. The term 'q-dot' emphasizes the quantum confinement effect of these materials, and typically refers to fluorescent nanocrystals in the quantum confined size range. Quantum confinement refers to the light emission from bulk (macroscopic) semiconductors such as LEDs which results from exciting the semiconductor either electrically or by shining light on it, creating electron-hole pairs which, when they recombine, emit light. The energy, and therefore the wavelength, of the emitted light is governed by the composition of the semiconductor material. If, however, the physical size of the semiconductor is considerably reduced to be much smaller than the natural radius of the electron-hole pair (Bohr radius), additional energy is required to "confine" this excitation within the nanoscopic semiconductor structure leading to a shift in the emission to shorter wavelengths.

Fluorescence polarization assays can be used in a microfluidics device to monitor the activity of kinase enzymes, phosphatases, proteases, ligand-ligand binding, and others. Extension of the existing fluorescence detection system to perform fluorescence polarization measurements requires the incorporation of a linearly polarized laser and polarizing optics into the design. As shown in Figure 7, linearly polarized laser and polarizing optics is incorporated into the design. A linearly polarized frequency doubled diode laser operating at 488 nm passes through a ½ waveplate and linear polarizer (Meadowlark Optics, >2000:1

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contrast ratio). This makes it possible to orient and lock the exciting laser polarization as required for FP. The laser is reflected and focused into the sample using a dichroic beamsplitter and anti-reflection coated lenses. Fluorescence from the sample is transmitted back through the lenses and dichroic beamsplitter and isolated using the emission filter. This fluorescence signal is then split into orthogonal polarizations using a polarizing beamsplitter (Meadowlark Optics polarizing cube beamsplitter, contrast ratio >500:1 transmitted, >20:1 reflected). Contrast is further enhanced with linear polarizers (Meadowlark Optics, >2000:1 contrast ratio). Finally, each polarization signal is measured using a pair of photomultiplier tubes (Hamamastsu H5789), digitized and analyzed by computer. A linearly polarized (>200:1) frequency doubled diode laser operating at 488 nm from Picarro is used for this purpose. As seen in the figure, the laser passes through a ½ waveplate and linear polarizer (Meadowlark Optics, >2000:1 contrast ratio). This makes it possible to orient and lock the exciting laser polarization as required for FP. As with the standard station, the laser is reflected and focused into the sample using a dichroic beamsplitter and anti-reflection coated lenses. Fluorescence from the sample is transmitted back through the lenses and dichroic beamsplitter and isolated using the emission filter. This fluorescence signal is then split into orthogonal polarizations using a polarizing beamsplitter (Meadowlark Optics polarizing cube beamsplitter, contrast ratio >500:1 transmitted, >20:1 reflected) and contrast is further enhanced with linear polarizers (Meadowlark Optics, >2000:1 contrast ratio). Finally, each polarization signal is measured using a pair of photomultiplier tubes (Hamamastsu H5789), digitized and analyzed on the computer. It is expected that these optics will permit better than mP sensitivity.

These fluorescence polarization systems were tested using a model enzyme system; Src-family tyrosine kinase. A nonradioactive, simple, sensitive fluorescence polarization assay has been developed to assay protein tyrosine kinase activity (Seethala R.; Menzel R. A Homogeneous, Fluorescence Polarization Assay for Src-Family Tyrosine Kinases. Analytical Biochemistry, November 1997, vol. 253, no. 2, pp. 210-218(9)). This assay involves incubation of a fluorescenylated peptide substrate with the kinase, ATP, and anti-phosphotyrosine antibody. As shown in Figure 8, the phosphorylated peptide product is immunocomplexed with the anti-phosphotyrosine antibody resulting in an increase in the polarization signal as measured in a fluorescence polarization analyzer. Figure 8, left panel, shows the IMAP principle of operation. When a fluorescent substrate is phosphorylated by a kinase, it can bind to the IMAP binding reagent, whose molecular size is large relative to the substrate. This gives a large increase in the polarization of the fluorescence. Figure 8, middle

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panel, shows the IMAP assay of MAPKAP-K2, a serine/threonine kinase. MAPKAP-K2, from Upstate, was assayed in a volume of 20 μL using the amounts of enzyme indicated. Concentrations of ATP and substrate were 5.0 and 0.5 μM, respectively. Incubation was 60 minutes at room temperature, followed by the addition of 60 μL IMAP binding reagent. FP was read on an Analyst system 30 minutes later. Figure 8, right panel, shows the IMAP quantification of kinase inhibition. MAPKAP-K2 (0.25 units/mL) was incubated using the amounts of enzyme shown for 15 minutes. The activity of the enzyme was then assessed as described in above. These results show that the fluorescence polarization assay can detect inhibitors and is comparable to the $^{32}PO_4$ transfer assay. The fluorescence polarization method is advantageous compared to the $^{32}PO_4$ transfer assay or ELISA or DELFIA because it is a one-step assay that does not involve several washings, liquid transfer, and sample preparation steps. It has the added advantage of using nonisotopic substrates. The fluorescence polarization assay thus is environmentally safe and minimizes handling problems.

The dyes that are chosen are used extensively in flow cytometry and in our instrument will be determining the status of (potentially) many dyes within one drop. The use of inexpensive optics on our instrument will be more than compensated for by the theoretical increase of dye molecules in the nanoreactor.

FP assays have been shown to tolerate up to 5% DMSO with no loss in sensitivity or signal window. From a random set of 1,280 compounds, Allen et al found that 1.9% significantly interfere with FP measurement (J Biomol Screen. 2000 Apr;5(2):63-9. High throughput fluorescence polarization: a homogeneous alternative to radioligand binding for cell surface receptors. Allen M, Reeves J, Mellor G. Receptor & Enzyme Screening Technologies, Glaxo Wellcome Medicines Research Centre, Stevenage, Herts, UK.). If fluorescent or quenching compounds were eliminated (3% of all compounds), less than 0.4% of compounds were found to interfere with FP measurement. Compounds are assayed *a priori* and those that have these undesirable characteristics are eliminated.

In some enzymatic assays, a delay module (i.e., delay line) will be utilized. This is less true for enzyme reaction mechanisms in a small volume. And even many cell-based assays can be measured within 5 minutes. Longer assay times can be accomplished by collecting the droplets, incubating them for an appropriate amount of time, and then re-injecting them into the device.

Three different q-dots in several concentrations each can be placed in a microdroplet, and can then be used with the device of the present invention to decode what is in the drop. In

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one experiment, the initial labeling scheme used three colors of q-dots having emission wavelengths of 620 nm (CdSe/ZnS), 650 nm (InGaP/ZnS), and 680 nm (InGaP/ZnS) (excitation at 488 nm is appropriate for all). In one specific example, one q-dot was maintained at a constant concentration and varying the second and third q-dots at least 10 different concentrations giving 100 different encodes (1 x 10 x 10). Decoding will be computed by referencing the intensity of the second and third q-dots relative to the first q-dot. Other labeling schemes can be used during the course of these experiments.

The Q-dot readout extension to the fluorescence station is described herein and is easily incorporated into the design due to the modular layout developed. As seen, a series of dichroic beamsplitters, emission filters, and detectors are stacked onto the system, allowing measurement of the required five emission channels (two fluorescence polarization signals and three q-dot bands). Dichroic beamsplitters and emission filters capable of separating the q-dot wavelength bands from each other are readily available, so it is a straightforward process to configure the station appropriately.

The residence time can be increased by slowing down the flow of drops by widening the channel. Alternatively the intensity of the laser beam can be increased to compensate or increase the concentration of the q-dots within the droplet.

As described herein, the dyes chosen for FP are commonly used in most cell- and enzyme-based assays and are designed not to overlap significantly with the q-dots. The dyes are evaluated both independently and together with the q-dots (at first off-instrument) to assess the cross-talk. Preferably, the liquid q-dot labels are read outside a spectral wavelength band currently used in FACS analysis and sorting (i.e., the dyes flourescein, Cy3, Cy5, etc). This permits the use of currently-available assays (dependent on these dyes). Using specific q-dots, crosstalk is minimized. Several commercial entities sell q-dots that can be read by the optics being designed. The three colors of q-dots used currently are the non-functionalized T2 EviTags having emission wavelengths of 620 nm (CdSe/ZnS), 650 nm (InGaP/ZnS), and 680 nm (InGaP/ZnS) (excitation at 488 nm is appropriate for all).

It is possible to generate 96 types of droplets, each droplet containing both a unique set of q-dot labels and a chemical compound, and as the droplet flows through the device of the present invention kinase enzyme activity can be analyzed using FP and the q-dot label can be decoded. This method allows for scaling to more complex and interesting libraries.

FP assays have been shown to tolerate up to 5% DMSO with no loss in sensitivity or signal window. From a random set of 1,280 compounds, Allen et al. found that 1.9% significantly interfere with FP measurement (J Biomol Screen. 2000 Apr;5(2):63-9. High-

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throughput fluorescence polarization: a homogeneous alternative to radioligand binding for cell surface receptors. Allen M, et al. Receptor & Enzyme Screening Technologies, Glaxo Wellcome Medicines Research Centre, Stevenage, Herts, UK.). If fluorescent or quenching compounds are eliminated (3% of all compounds) then less than 0.4% of compounds are found to interfere with FP measurements. Compounds are assayed a priori and those that quench FP are eliminated.

The three colors of q-dots we will use are the non-functionalized T2 EviTags having emission wavelengths of 620 nm, 650 nm, and 680 nm; excitation at 488 nm is appropriate for all. The >620 nm liquid labeling emission band was chosen not to interfere with the FP assay band found between 488 and 620 nm. These q-dots are commercially-available, stable in some buffers and remain suspended in aqueous solution.

A mixture of two types of droplets, buffer-only and fluorescein-containing, are stable for at least 1 month without any detectable diffusion of the organic dye into the buffer-only droplets. Other surfactants may be substituted for different kinds of compounds. For other compound testing, i) similar mixtures of compound-containing and buffer-only droplets can be created, ii) they can be sorted based on their q-dot labels, and iii) Mass Spectrometry can be used on the buffer-only droplets to quantitatively detect the presence of other chemicals compounds.

In some embodiments, a delay module (i.e., delay line) can be utilized. This will be true for enzyme reaction mechanisms in a small volume. But even many cell-based assays can be measured within 5 minutes. Droplets can also be taken off-line and stored for at least a month a month before re-injection into the device of the present invention with no apparent change in the droplets. Longer delay times can be achieved by taking mixed droplets off-line, and then re-injected them.

Example 5.

The present invention provides methods for performing condensation chemistry in nanoreactors of the present invention as described to synthesize libraries of drug-like molecules in a highly convergent manner.

All life processes can be reduced to chemical reactions that take place in aqueous media. Hence water is considered to be the universal ultimate solvent and inevitably biological experiments are performed in aqueous media. Furthermore, organic solvents of all types are detrimental to biochemical reactions limiting their use to small percentages of water miscible solvents such as dimethyl sulfoxide and ethylene glycol. On chip synthesis in our

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system therefore requires that all chemical reactions are performed in biologically compatible aqueous media. In contrast, conventional synthetic organic chemistry relies on highly activated substrates and highly reactive reagents to conduct bond forming processes. Typically these substrates and/or reagents are unstable in the presence of and react with water rendering them useless. An ever increasing effort to reduce cost, enhance safety and to address environmental concerns w.r.t. solvent choice has driven the development of synthetic methods that utilize water as the primary and in many cases the only solvent (Li). In the case in which water is not the only solvent, water miscible organic solvents are used to aid the dissolution of substrates.

While material can be removed from NanoReactors by breaking them in a controlled way, it is preferable to avoid having to do so. To eliminate the requirements of removing material from doplests, we have identified 5 reaction types, which can be performed in aqueous media, that can be used to "stitch" drug-like molecules together from a highly diverse library of sub-structural components. These reactions generate commonly occurring functional groups in drug-like molecules and include: i) N-Acylation ii) N-Sulfonylation iii) Cycloadditions iv) Reductive alkylation of amines and v) SNAr reactions (Morgan). Random combination of sub-structures will yield a library of all possible combinations. The reactions are sufficiently orthogonal to perform multi-step reactions. Furthermore, simple protection and deprotection schemes can be used to increase the number of condensations. A sufficient number excess of component nanoreactors will yield a library of all possible combinations with multiple copies of each combination for testing in a biomolecular assay. This redundancy is required to reduce the impact of false positive sorting events.

This technology is based on the assembly of drug-like chemical entities by two or more step convergent syntheses from diverse sub-structural components. A two step process would assemble the final chemical species from four substructures. An example of such a synthesis is the construction of the kinase inhibitor Gleevec from relatively simple building blocks. Gleevec is the first of a class of kinase inhibitors which targets the chimeric tyrosine kinase bcr-abl. Bcr-abl is constitutively active causing a rare life-threatening form of cancer called chronic myeloid leukemia (CML). Gleevec was given FDA approval in a record breaking three months in May of 2001. Analogues to a sphere, the three dimensional structure can be broken down into hemispheres and quadrant by the appropriate disconnections. Figure 9 shows that Quadrants A and B are combined utilizing a reductive alkylation. Quadrant C and D are combined utilizing a 1,3 dipolar cycloaddtion. The Hemisphere AB and CD are combined utilizing an N-acylation

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Chemical Diversity is achieved by varying the sub-structural units (Quadrants) and randomly combining to achieve all possible combinations. Table 1 shows the theoretical diversity for number of each quadrant (e.g. 4 different Quadrants A, with 4 different Quadrants B, with 4 different Quadrants C and 4 different Quadrants D yield 256 unique products after two steps as described above.

Table 1

Diversity of quadrants (equal number of diverse members for each quadrant)	Number of Unique solutions
1	1
2	16
3	81
4	256
5	625
6	1,295
7	2,401
8	4,096
9	6,561
10	10,000

Sufficiently diverse condensation chemistry in aqueous media exists to synthesize diverse libraries of drug-like molecules in a highly convergent manner.

The majority of synthetic reactions routinely used in the synthesis of complex drug-like molecules rely on reagents and substrates which are sensitive to hydrolysis in the presence of water and hence require stringent exclusion of water from the reaction vessel. Furthermore, condensation reaction often require the equivalent of a dehydration to proceed, with one or both of the condensation substrates being sensitive to degradation by water. The pursuit of "greener" chemistry, using benign and environmentally friendly solvents such as water, has resulted in the development of several condensation reactions which take place in the presence of water. Aqueous organic chemistry has been extensively reviewed in the primary literature and books.

The encapsulation technology of the present invention which relies on the addition of reagents and/or substrates to nanoreactors without the option for reaction work-up (i.e. purification of product). This precludes the use of reactions which have side products which could potentially interfere with subsequent steps or the biological assay intended to be performed on the final product. Furthermore, in multistep reactions, the two steps will have to be orthogonal with respect to their coupling chemistry, i.e. the functional groups for

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consecutive reactions may not interfere with each other. Five reaction types have been identified which can be performed in aqueous media and which do not require purification of the product prior to the next synthetic step or testing in biological assays. These reactions include: i) N-Acylation ii) N-Sulfonylation iii) Cycloadditions iv) reductive alkylation of amines and v) SNAr reactions.

The present invention provides methods of performing these condensation reactions in a highly convergent, "one pot" synthesis to stitch together complex drug-like molecules from at least 2 – 16 substructures.

The solubility of organic compounds in aqueous media is strongly dependent on their structure. To enhance solubility of the library compounds in aqueous media, it is common in the biomolecular screening community to dissolve library compounds in DMSO and subsequently dilute the DMSO solution with water. DMSO is compatible with the nanoreactors described herein.

A fluorescent product resulting from the condensation of two suitable fragments can be distinguished from droplets that have components which did not react to form the fluorescent product thus enabling the optical readout to distinguish between the two cases and sort the droplets accordingly. The components able to form the fluorescent product would contain a different tag from the components which are not able to form a fluorescent product. Hence this system can be used to test the tagging strategy chosen to identify the composition of the final product.

Traditional combinatorial chemistry relies on complex deconvolution methods to determine the structural identity of the final product once it has been determined to be active in any particular biomolecular assay. Massively parallel synthetic approaches use encoding technologies to infer the structure of any particular product from the tag associated with it which typically identifies the reaction history of that particular compound. In one example, the reagent droplets are encoded with nucleic acids tag which will provide a unique PCR signature for the final product from which the reactant composition and hence structure can be inferred.

The synthesis of a fluorescent molecule which will be the product of one particular component with a common reaction partner will be used to test the tagging technology. Figure 10 shows three tags denoted A, B and C label one of each of the following unique components: A is a fragment which if combined with C will yield a fluorescent molecule. B is a fragment which if combined with C will yield a non-fluorescent molecule. The fluorescence detector will be able to distinguish between drops that contain A,B, C or the

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mixture BC and between the drops which contain the mixture AC (if the reaction has taken place and the fluorescent product is formed). The fluorescence based sorting will yield a population of AC tags which are completely devoid of B. The second (waste) population of drops may contain B, C and A tag if not every drop containing the tag A has been fused to a drop containing C.

Multistep convergent syntheses of drug-like compounds can be performed in nano-reactors by the selective fusion of droplets. These compounds can then be tested in a biomolecular screen immediately after being synthesized on chip.

The synthesis of the bcr-abl kinase inhibitor Gleevec has been described herein. The synthesis of this inhibitor from four sub-structural units followed by an assay determining its ability to inhibit the bcr-abl kinase in a fluorescence polarization based assay would serve as proof of principle for this technology.

Two different sub-structures will be used for each quadrant such that at least 16 possible products can be formed of which one is Gleevec. Although some of the other products will have sub-structural elements of Gleevec, the "alternative" quadrants will be considerably different to ensure that a completely non-active product will be amongst the possible combinations. Each unique quadrant will be tagged with a suitable nuclei acid oligomer.

The products will be tested in a fluorescence polarization based kinase assay with the expectation that Gleevec will strongly inhibit the activity of bcr-abl. Based on the assay readout, the drops containing Gleevec will be sorted and collected separately. Analysis of the nucleic acid tags of those drops can reveal the composition of the hit compound.

The present invention also pvides methods of using nucleic acids for chemical encoding and decoding tagging of chemical reactions Current technology exists for the tagging of beads with chemical tags which "record" the synthetic history of any particular bead thereby allowing the deconvolution of the active small molecule's structure. The encapsulation of the reagents used to assemble the library members enables the use of homogeneous nucleic acid based tags to determine the structure of any particular quaternary reaction combination. Positive hits from the biomolecular screen would be cloned into E. coli and decoded using polymerase chain reaction (PCR) to determine the composition of quadrants used to assemble the bio-active molecule. Figure 11 (left panel) shows four groups of DNA tags. One of sixteen double-stranded oligodeoxynucleotide 'surrogate' tags will be added to each of four 'groups' of the sixteen different chemicals being used for chemical synthesis (see text for details). Each group of tags will have unique overlapping 5' and 3'

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ends that are the same for each member of the group, but complementary between adjacent groups. The tags within the groups are designed with asymmetric 5' overhangs such that they can ligate once with a member of an adjacent group. The first and fourth groups will additionally contain 5' and 3' sequences (respectively) that can be used as priming sites to PCR up final products containing all four groups. The top-strand in groups 2, 3 and 4 will contain a 5' phosphate needed for DNA ligation. Figure 11 (right panel) shows the tags in each reaction are sorted based on the enzyme assay. In the example shown, chemical synthesis is allowed to occur (see text for details) and droplets (in this example) containing tags 2, 7, 11 and 14 have within them a synthesized compound that reacts positively in an enzyme/cell-based assay. The positive droplets are then subjected to a polymerase chain reaction (PCR) using primers complementary to the ends of groups 1 and 4. The resulting PCR product will next be cloned into an appropriate DNA vector. Finally, colonies of transformed E.coli containing the catenated tags will be DNA-sequenced to decode the synthesis history of the compound associated with positively-sorted droplets.

Alternatively soluble quantum dot dyes can be used to encode the input emulsions which can identify the chemical composition of a positive hit by measuring relative fluorescence signals of multicolored quantum dots eliminating the need for sorting. An assay point with appropriate signal from a fluorescent marker (in the case of the kinase assay proposed here we would measure changes in fluorescence polarization) the synthetic history of the molecule responsible for this signal would be read out by determining relative levels of dyes. This tagging technology is limited by the number of unique combinations that can be discerned with appropriate confidence and hence would be applied to smaller, more focused libraries typically used to explore a sub-set of chemical space surrounding an early lead.

Example 6.

The present invention provides methods of isolating self-antigens. A first sample droplet set consisting of a tumor obtained from a multicellular organism treated in such a way as to create single cells that are then each separately or multiply contained within said first droplet set are combined with a second set of droplets consisting of one or more t-cells isolated from the organism, and the resulting combined droplets are analyzed for t-cell killing of the tumor cells contained within the combined droplets using a detecting means. The detecting means can include analysis for cytoplasmic enzymes that would be released to the droplet environment upon cell lysis. The droplets can be either sorted or not sorted and then further analyzed for identification of tumor cell epitopes recognized by the t-cell.

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Example 7.

The present invention provides methods of matrix screening using a phased-drop approach or derivatives thereof. A device composed of a multitude of samples each separately contained within sample wells connected to one or more inlet channels such that that can be operated in such a way that each sample can be encapsulated within a droplet within a fluid-flow and be both sequentially and separately combined with each of the other samples by varying the phase of the combining of the separate, sequential droplets.

For example, by changing the phase of the combining of the drops it is possible to have, for example, with five separate samples each combine with the other samples, in this example in pairs, to yield drops containing a mixture of all possible pairs of compounds 1+2, 2+3, 3+4, 4+5, 5+1, ... 1+4. The phasing can be by one of several means, including channel length, valves, pressure, etc.

In another example, a matrix of 100 chemical compounds are loaded into 100 separate wells and are each combined in separate pairs to yield 100^2 different pairwise combinations. These 10^3 combinations are each separately used in a cell-based assay to determine their combined effects on cell survival.

The devices and systems disclosed herein have several distinct advantages over current devices and methods for analyzing samples. These advantages include, for example: reliability and reproducibility, flexibility (the ability to 'swap out'), the greatly reduced cost of an assay, speed and handling, reduced skill-level required needed to perform the an analysis, scalability of assays from one to many nanoreactors, automatable with current liquid-handling robotics, multiple sort capability and previously unachievable assay architecture enabled by NanoRector confinement and manipulation

The enhanced functionality that electrostatic charge brings to droplets in microfluidic devices has the potential to enable an expansive list of microfluidics applications. This toolkit of techniques for manipulating droplets described herein can enable modular integration of systems for transporting and reacting small numbers of molecules. High throughput screening, combinatorial chemistry, and the search for rare biological function in libraries of biomolecules all benefit from electrostatic manipulation of droplets in microchannels. Droplet-based microfluidic technology can also be used to develop a chip-scale fluorescence activated cell sorter (FACS) with enhanced activation functionality that goes beyond fluorescence to include multiple reagent-based assays between the droplet formation and sorting steps. Moreover by using femtoliter droplets, which are a few microns in diameter,

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even a single biomolecule represents concentrations of »1 nM, sufficient for efficient chemical reactivity and single-molecule assays.

Many of the potential uses of droplet-based microfluidic devices are driven by a need to encapsulate a varied population or library of molecules, cells or particles into microreactors, perform an assay on the contents, perhaps through the addition of reagents, and then, finally, to selectively remove specific microreactors from the collection in a search for rare events. This requires a processing rates of 10^3 per second to sort through the smallest libraries in a reasonable time while rates on the order 10^5 per second are desirable for larger libraries. These rates are feasible using the charged droplet paradigm. Moreover, because the microfluidic devices are stamped, parallel flow streams can be fabricated, further enhancing the total throughput. Combined, the advantages of droplets and high throughput manipulation provide significant opportunity for widespread application. The inventions presented and described in detail herein will facilitate the application of droplet-based microfluidic technology.

Example 8.

The present invention also provides adaptations of known assays for use on the microfluidic device according to the present invention. For example, fluorescence polarization, molecular beacons, and taqman assays can be adapted for use in SNP, DGE, and nucleic acid identification. In a high-throughput mode the individual droplets can be labeled with either organic or inorganic dyes, or colored beads. A distinct advantage is that beads are not required and the entire assay can be performed in solution. Some exemplary assays are described.

The present invention can be used to identify CDRs in a pre-defined CDR library. In one example, there can be 100 pre-defined CDRs for each of the 6 CDRs in an scFv (i.e., 3 in VH, and 3 in VL). 600 molecular beacons can be created, each beacon separately emulsified with a different (for example, q-dot) LiquidLabel. The 600 separate emulsions can be pooled to create one emulsion library mixture (composed of 600 different types of droplets, and as stated each droplet containing both a molecular beacon specific to a specific CDR, and a LiquidLabel specific to droplets containing that molecular beacon). scFv Ab genes from antigen-interacting antibodies isolated by either phage display or yeast two-hybrid can be amplified by PCR using 5' and 3' flanking primers. The PCR product of the Ab gene can be either emulsified on the RDT Instrument prior to combining with the library mixture, or, in a separate example, combined with it's own unique LiquidLabel off-line, and mixed with

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several (other) amplified Ab fragments, thereby allowing *several* PCR fragments to be analyzed simultaneously. The amplified fragment will then be combined with the library mixture, and run past the detector. The detector will identify the molecular beacon within the droplet using the LiquidLabel and further detect whether hybridization has occurred by examining the status of the fluorophore relative to the probe-containing quencher.

An oligonucleotide assay can be used to generate a product against which an fluorescence polarization (FP) organic-dye type tag, molecular beacon or taqman oligonucleotide can be used in an assay as described above. Other assays are also possible.

The present invention can also be utilized in differential gene expression. Taqman or molecular beacons can be used in a modification of the methods as described herein.

The TaqMan system requires the use of a polymerase with 5 ' to 3 ' nuclease activity, such as Taq DNA polymerase, and a short oligonucleotide probe labeled with a reporter dye and a quencher dye that anneals to the target downstream from one of the primers (See, Figure 12, left panel). If the probe is hybridized to the target, the polymerase cleaves the hybridized probe, separating the reporter from the quencher, which results in a higher fluorescent signal. The fluorescent signal increases proportionally to the number of amplicons generated during the log-linear phase of amplification. It is important that the probe hybridizes before the primers so the polymerase can cleave the probe and release the reporter dye as primers are extended. Otherwise, amplification occurs but is not monitored because the probe is not cleaved.

Molecular beacon probes are hairpin-shaped oligonucleotide molecules that have a fluorophore and a non-fluorescent quencher dye attached to the 5 ' and 3 ' ends (See, Figure 12, right panel). Generally, DABCYL is the non-fluorescent universal quencher and the other dye is a reporter fluorophore such as, FAM, TET, TAMRA or ROX. The molecular beacon is in a hairpin configuration when it is not hybridized to the target site. It is designed to have two "arms" with complementary sequences that form a very stable hybrid or stem. The close proximity of the quencher and reporter suppresses reporter fluorescence when the beacon is in a hairpin configuration. When the beacon hybridizes to the target during the annealing step the reporter dye is separated from the quencher, which allows the reporter to fluoresce. In order for the beacon to anneal to the target sequence, it must form a hybrid that is even more stable than the hairpin to remain in the hybridized conformation. Therefore, the probe is less likely to form a hybrid with the target if there are mismatched base pairs.

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In addition to molecular beacons and Taqman, the devices of the present invention can be used to carry out fluorescence polarization as described herein. Most SNP assays can be adapted for both mini-sequencing and gene expression analysis.

A series of fluorescence polarization measurements have been made inside a microfluidic device according to the present invention while looking at droplets containing Fluorescein, Fluorescein bound to biotin, and Fluorescein bound to biotin + Steptaviden. The fluorescence signal was split into two orthogonal polarizations: one parallel to the laser excitation polarization, and one perpendicular to the polarization. These signal were collected and analyzed to determine the change in polarization of the fluorescence for each of these binding conditions.

The Polarization is calculated from:

$$P = \frac{(V - H)}{(V + H)}$$

Where V=fluorescence signal polarized parallel to laser excitation polarization, and H=fluorescence signal polarized perpendicular. A *mP* ("milli-P") is 1000*P. The Polarization is equal to zero when the fluorescence is completely depolarized, and has a maximum of 500 mP when the fluorescent molecule is "frozen" (i.e. bound to a large molecule that does not rotate between excitation and emission).

The fluorescence station was modified to include cleanup polarizer for the laser and a polarizing beamsplitter with cleanup polarizers for collection. The two resulting fluorescence channels collect light with orthogonal polarizations ("Vertical" is parallel to the laser polarization, "Horizontal" is perpendicular to the laser polarization). The device used to generate alternating droplets is built from RDT Master #257 (50 um deep channels). Table 2 lists the fluids used for these tests.

Table 2. Fluids ran through the double nozzle device.

Fluid Name	Composition
Oil	FC3283+10% "Avocado"
BTFC	$1x10^{-6}$ Molar Biotinylated Fluorescein in 10mM Borate pH 9
BTFC+SA	$1x10^{-6}$ Molar Biotinylated Fluorescein in 10mM Borate pH 9 + $0.5x10^{-6}$ Molar Steptaviden (4 binding sites per molecule)

During these experiments, the fluid flow rates were 200 ul/hr for FC, BTFC, and BTFC+SA, and 600 ul/hr for the oil (FC3283+10% "Avacado"). Droplets ranged in size from 65 to 75 um in diameter, depending on which water solution was used. Time between

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droplets varied from 1300 us to 2200 us, again depending on which fluid was used. Fluorescence polarization measurements were made by injecting BTFC in one nozzle, and BTFC+SA in the other. Once these measurements were completed, the BTFC+SA was replaced with FC and a second series of data was collected. Figure 13 plots typical fluorescence data collected from both these runs, as well as the calculated Polarization for each of the droplets shown. Figure 13 (top panel) shows raw polarized fluorescence signals collected on the test station when measuring BTFC and BTFC+SA, then BTFC and FC (the data is normalized so P(Fluorescein) = 0.0). Figure 13 (bottom panel) shows polarization calculated from each droplet in the top plot. The transition at t=1.5 sec is a mathematical artifact where the data collected for the two conditions were concatenated in software (the data after t=1.5 was collected approximately 30 minutes after the data collected before t=1.5). In this data, Vertical is fluorescence parallel to laser polarization, Horizontal is polarization perpendicular. In this data, the "horizontal" polarization has been normalized such that the Polarization is equal to zero for Fluorescein. The Polarization was calculated by integrating the fluorescence signal across the droplet for each polarization, then plugging the results into Equation 1. Figure 14 plots the Polarization measured for a longer number of droplets, along with the histogram created from this data. Figure 14 (top panel) shows polarization calculated from each droplet for a longer time period than in figure 3. Figure 14 (bottom panel) shows histogram generated from the data in the top figure. The data after t=1.5 was collected approximately 30 minutes after the data collected before t=1.5.

As seen in the histograms of the Polarization, the polarization clusters tightly around three different mean values. The zero-centered mean (σ=1.7) corresponds to Fluorescein, while the 18.4 mP (σ=1.6) grouping corresponds to Biotinalated Fluorescein and the 96 mP (σ=3.46) grouping corresponds to Biotinalated Fluorescein bound to Steptaviden.

Example 9.

Fluorosurfactants are synthesized by reacting Krytox 157 FSL, FSM, or FSH with aqueous ammonium hydroxide in a volatile fluorinated solvent. The solvent and residual water and ammonia are removed with a rotary evaporator. The surfactant can then be dissolved in a fluorinated oil (*e.g.*, FC-3283 from 3M), which can then be used as the continuous phase of the emulsion. A typical concentration is 2.5 wt% of surfactant dissolved in the oil.

The channels of the microfluidic device are also coated with a fluorinated surface product. For example, the coating is applied from a 0.1-0.5 wt% solution of Cytop CTL-

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809M in CT-Solv 180. This solution is injected into the channels of a microfluidic device via a plastic syringe. The device is then heated to 90 °C for two hours, followed by 200 °C for an additional two hours. These surfactants in the fluorinated oil stabilize the aqueous droplets from spontaneously coalescing. By fluorinating the channel surfaces, the oil phase preferentially wets the channels and allows for the stable generation and movement of droplets through the device. the low surface tension of the channel walls minimizes the accumulation of channel clogging particulates.

Example 10.

The quality of libraries of emulsified compounds can also be controlled so as to eliminate from the library those compounds that cross between droplets. By emulsifying a q-dot encoded, buffer-only droplet and allowing it to incubate with 1-1000 separately emulsified compounds quality control of libraries can be achieved. After incubation, the uniquely encoded q-dot droplet is sorted away from the compound-containing droplets and analyzed (*e.g.*, by mass spec) for the presence of any of the compounds emulsified in the other 1-1000 types of droplets. Compounds that cross between droplets are identified and eliminated from the library.

Example 11.

The present invention also enables the user to sort cells based on binding of an affinity-reagent attached to a means for signal amplification. As a non-limiting example, an antibody fused to an enzyme (*e.g.*, alk/phos, β-gal, horseradish peroxidase, etc.) is added to a mix of cells and incubated. The antibody can be against a particular cell-surface marker, for example, such as a cancer marker. The cell suspension can be washed or unwashed (if the antibody is in low concentration, *i.e.*, less than 1 antibody per droplet, and the antibody has good binding properties).

The cells that have antibodies attached to them are then emulsified into droplets and an appropriate enzyme-substrate is added. The presence of a fluorigenic substrate product is amplified from one to many copies by the enzyme turning-over the substrate. Multiple enzymes and multiple substrates can be used to allow analysis of multiple samples with multiple fluorophores at the same time or sequentially. The affinity-reagent can be a protein, nucleic acid, or other molecule to which an enzyme (or portion thereof that when brought

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together becomes active) can be attached either covalently or through a reasonably strong interaction.

Example 12.

The device of the present invention can also be used to sequence individual exons from individual chromosomes or tumor cells. A schematic diagram for performing this method is provided in Figure 19. Individual specific primer-pairs to different exons (*e.g.*, epidermal growth factor receptor (EGFR) exon-specific primer pairs) along with a primer-bound bead (*e.g.*, a Dynal strepavidin bead) are each emulsified and then pooled to create a library emulsion (in Fig 19 a set of 96 exon primer pairs are shown for illustrative purposes). *Separately*, a chromosomal DNA solution is diluted in an aqueous buffer such that upon emulsification on a microfluidic device described herein, a 30-50 micron droplet contains, on average, slightly less than a half-genome's concentration of DNA. Droplets from the pooled emulsion library set of exon-specific primers are coalesced with droplets containing the diluted solution of chromosomal DNA on a microfluidic device as described herein, and used in a bead-based DNA amplification reaction (*i.e.*, PCR). The microfluidic device as described herein collects 1×10^9 of these droplets in 24 hours, which results in an emulsion of droplets, some of which contain beads with amplified exon-DNA attached. After PCR, the emulsions are broken by centrifugation, the beads are isolated, washed, and then enriched for DNA-containing beads on a microfluidic device as described herein. The exon- and chromosome-specific DNA-containing beads are randomly placed into a picotiter plate (454 Corp.) and sequenced using a Life Sciences DNA sequencing instrument (as provided by 454 Corp. and described in any of U.S. Application No. 09/814,338, filed March 21, 2001; U.S. Application No. 10/104,280, filed March 21, 2002; U.S. Application No. 10/767,899, filed January 28, 2004; U.S. Application No. 11/045,678, filed January 28, 2005; or U.S. Application No. 11/195,254, filed August 1, 2005, each of which are incorporated herein by reference in their entirety). A summary of this process is provided by Figure 20. The emulsion PCR amplification reaction can be performed off-chip using control chromosomal DNA as template and a single set of exon-specific primers, or on-chip (*i.e.*, on the microfluidic devices of the present invention as described herein).

In a more specific example, the microfluidic devices and methods of the present invention have been used to develop individual exon-, and chromosome-specific sequencing

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methods with off-line emulsion PCR using chromosomal DNA as template and a single set of exon-specific primers.

The ability to combine (i.e., coalesce) two droplets together can be used to amplify an exon from an individual chromosome.

a. PCR amplification of DNA within a droplet. 454 Life Sciences has previously demonstrated emulsion solid-phase PCR in droplets of a size range anticipated for the microfluidic devices according to the present invention. Successful DNA amplification using an emulsion of a PCR with the perfluorocarbon oils and surfactants used to generate and manipulate droplets on microfluidic devices of the present invention have also been demonstrated. Several polymerases (notably those from archea, *e.g.*, Thermal Ace DNA Pol; Pfu Turbo Pol; Advantage 2-CG Taq Pol; and Advantage Taq Pol) work well in the buffers and oils used in the devices described herein.

b. Setting up the exon-specific PCR reactions. In the first set of experiments, robust bulk conditions for the droplet-based exon amplification are developed. Primers within an exon in the EGFR gene can be used. Approximately 10% of patients with non-small-cell lung cancer (NSCLC) show responsiveness to targeted tyrosine kinase inhibitor (TKI) chemotherapy regimens. Response in patients has been strongly associated with somatic heterozygous mutations in the ATP cleft of the EGFR gene.

Wild-type and mutant chromosomal DNAs containing an 18 base pair (bp) deletion in an EGFR exon encoding this ATP cleft are used. Initial experiments can be performed in a bulk (i.e., off-instrument) solution using the perfluorocarbon oils and surfactants as described herein. Once conditions have been established with limiting chromosomal DNA these amplification experiments in mono-dispersed droplets formulated on-instrument are repeated. These droplets are collected and the DNA contained within amplified. The emulsion containing the amplified droplets are broken, and the aqueous phase analyzed by gel electrophoresis.

c. Measurement of solid phase amplification. Once the exon PCR reactions are working in solution from droplets formed on the instrument, these experiments will be repeated with droplets containing both primers and beads. The beads are emulsified *with* the exon primer pairs. One of the primers is attached to the bead using standard oligonucleotide coupling chemistry. Both primers will also be in solution (those skilled in the art will

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appreciate that the goal of solid-phase amplification is to generate enough amplified product in *solution* such that *some* of it is driven to the oligonucleotide primer attached to the bead).

A serial dilution of chromosomal DNA in several trials is then added to the primer-bead solution. Droplets are formulated at a concentration of less than one bead per droplet. The DNA/primer/bead solution is gently shaken to keep the beads in suspension as the droplets are being formed on the microfluidic devices of the present invention. The droplets are collected from the instrument and the DNA within them is amplified by PCR off-line.

d. Measurement of single-chromosome PCR. On-bead hybridization of two separately-labeled cy3- and cy5-containing oligonucleotide probes is used to measure amplified-DNA attachment efficiency. A cy3-labeled probe is synthesized complementary to the sequence within the 18 bp deletion region, and a second cy5-labeled oligonucleotide probe is synthesized that will span this deletion (with complementarity to both 5' and 3' sides). The probes are designed such that at 30 °C they do not cross-hybridize. The quantitation and ratio of cy3:cy5 dyes on the bead is a measure of the amount of each specific allele of the DNA present on the bead.

As a control, a fluorescein-labeled oligonucleotide complementary to the oligonucleotide attached to the bead is used. This control oligonucleotide is used to estimate the *maximum* amount of cy3 or cy5-labeled probe that can attach to the bead. The hybridized beads are washed to remove un-hybridized probe and the amount of fluorescein still attached to individual beads is compared to fluorescein-standard concentrations. Other methods and controls for estimating attached DNA (such as fluorescence polarization) can be used in conjunction with the microfluidic devices according to the present invention. Figure 21. The chromosomal DNAs are diluted and added to exon-primer containing droplets on the microfluidic devices of the present invention using conditions established in the bulk emulsions. After PCR amplification, the beads are isolated, washed, and hybridized in solution to the cy3-, cy5-labeled probes. The hybridized beads are washed to remove un-hybridized and non-specifically-bound labeled nucleotide and the amount of dye still attached to individual beads is determined using a fluorescent microscope. The % synthesized is estimated from the maximum estimated to be able to be synthesized. The 454 instrument requires $1x10^7$ copies of DNA per bead for accurate reads. Using the methods described herein, more than $1x10^7$ DNA molecules per bead can be attached.

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PCR is a typical temperature-controlled and enzyme catalyzed biochemical reaction that consists of the periodical repetition of three different temperatures (melting, annealing and extension temperature). Alternatively, two temperatures can be applied by combining the annealing and extension temperatures, thus further reducing the complexity of the thermal cycling profile and increasing the speed and efficiency of the PCR reaction. Because of the temperature-sensitivity of the PCR system a minor temperature difference may significantly affect the efficiency of DNA amplification, especially in emulsion PCR microfluidic systems.

Accordingly, the effects of temperature on enzyme kinetics, heating and temperature-measuring methods in emulsion PCR microfluidics are critical in order to gain a better understanding of PCR kinetics in microfluidics.

Several thermostable polymerases that work well in oils used in the microfluidic devices have been idientified herein. A syringe pump attached to a microfluidic device of the present invention with appropriate sensors and heating elements (described below) can be used to model the ability of the polymerases to generate PCR product off-instrument.

The choice of a heating method for PCR microfluidics is of importance for achieving faster temperature ramping rates. In one embodiment, a contact-heating method (e.g., the use of hot air) canbe used. Contact-heating methods utilize electrothermal conversion to heat the PCR solution, in which the thermal components embedding the heating element are in direct contact with the components of the PCR amplification. To date, along with the thin film heaters, metallic heating blocks and Peltier-effect-based thermo-electric ceramic heating blocks have been widely applied in temperature control of PCR.

In one embodiment, 2 Kaptan Thermofoil heaters from Minco and a two-step PCR cycling method can be used. Thermofoil heaters are thin, flexible heating elements consisting of an etched foil resistive element laminated between layers of flexible insulation. Thermofoil heaters are applied to the surface of the part to be heated. Their thin profile gives close thermal coupling between the heater and heat sink. The flat foil element of thermofoil heaters transfers heat more efficiently and over a larger surface area than round wire. Thermofoil heaters, therefore, develop less thermal gradient between the resistive element and heat sink.

Methods of temperature measurement for PCR microfluidics. In emulsion PCR microfluidics, it is critical to select methods for temperature measurement to accurately control temperature during PCR cycling. Temperature measurement methods are usually

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divided into two categories: contact and non-contact temperature measurement. The former includes thin-film type temperature sensing and non-thin-film-type temperature sensing.

In one embodiment, temperature measurement can be performed by using the Minco Non-Invasive Sensors Design Kit. This kit comes with thermal-ribbon, thermal-tab, and bolt-on resistance temperature detectors that will allow us to accurately sense temperature without having to drill or tap into the chip. The detectors are accurate to +/- 0.25 °C.
In another embodiment, temperature measurements using a temperature dependent fluorescent dye (*e.g.*, a dilute fluorophore such as rhodamine B or rhodamine 3B) can constitute a second technique for measuring temperature in microfluidic structures.

The methods discussed above can be repeated with a second primer set, which consists of 96 different exons. Primers can be designed and tested on an MJ Research PCR instrument *a priori* to establish suitability to the two-step PCR conditions to be used on-chip.

All exons to be amplified are first sequenced by traditional Sanger methods to establish a base-line read. Where possible, exons with known polymorphisms within the to-be-sequenced DNA (i.e., the individual will be polymorphic at a site within the exon) are selected. We expect 50:50 for each polymorphism, this analysis will enable us to gather information about the bias in sequencing during the on-chip amplification reactions. The controls for attachment are the same as that described above.

A pool of primer sets are tested on a diluted genomic DNA solution whereby the genomic DNA is at several concentrations.

Once the ideal amount and quality of DNA attached to the beads is achieved, the 454 instrument will be used to sequence the beads. DNA-containing beads are enriched for on microfluidic devices according to the present invention either using a DNA staining agent (ex, Syber green) or by hybridization to a fluorescent oligonucleotide probe. Appropriate controls are used to estimate the number of exon copies per bead.

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CLAIMS

What is claimed is:

1. A method of creating a nanoreactor, the method comprising:

a) providing a microfabricated substrate comprising a plurality of electrically addressable channel bearing microfluidic modules integrally arranged on said substrate so as to be in fluid communication with each other, thereby forming at least one main channel adapted to carry at least one continuous phase fluid;

b) flowing a first dispersed phase fluid through a first inlet channel into the main channel such that one or more droplets is formed in said continuous phase fluid flowing therein;

c) flowing a second dispersed phase fluid through a second inlet channel into the main channel such that one or more droplets is formed in said continuous phase fluid flowing therein; and

d) coalescing at least one droplet formed in step (b) with at least one droplet formed in step (c) as the droplets pass through a coalescence module of the microfabricated substrate, thereby producing a nanoreactor.

2. The method of claim 1, wherein the coalescing step is achieved by an electric field.

3. The method of claim 1, wherein the coalescing step is passive.

4. The method of claim 1, wherein said first and second dispersed phase fluids comprise a biological or chemical material.

5. The method of claim 4, wherein the biological or chemical material is a member selected from the group consisting of: tissues, cells, particles, proteins, antibodies, amino acids, nucleotides, small molecules, and pharmaceuticals.

6. The method of claim 4, further comprising: e) incubating the nanoreactor within a delay module, and f) interrogating the nanoreactor for a predetermined characteristic within a detection module.

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7. The method of claim 4, wherein the biological or chemical material is labeled

8. The method of claim 7, wherein said label is a protein, DNA tag, dye, or quantum dot.

9. The method of claim 1, wherein said first and second dispersed phase fluid is a member selected from the group consisting of: two aqueous streams, one aqueous stream and an emulsion stream, and two emulsion streams.

10. A method of synthesizing a compound from two or more reactive substructures, the method comprising:

a) labeling the reactive substructures with a label unique to the substructure;

b) emulsifying aqueous solutions of the labeled reactive substructures on a microfluidic device to form droplets; and

c) randomly combining the droplets on said microfluidic device to form a compound.

11. The method of claim 10, wherein steps (a) and (b) are alternatively performed by introducing a preformed labeled emulsion.

12. The method of claim 10, further comprising:

d) screening the compound formed in step (c) based on a desirable chemical or biological property exhibited by the compound; and

e) identifying the structure of the compound by decoding the label.

13. A method for identifying a single compound from a library on a microfluidic device, the method comprising:

a) labeling a library of compounds by emulsifying aqueous solutions of the compounds and aqueous solutions of unique liquid labels, whereby each compound is labeled with a unique liquid label;

b) pooling the labeled emulsions resulting from step (a);

c) coalescing the labeled emulsions with emulsions containing a specific cell or enzyme, thereby forming a nanoreactor;

d) screening the nanoreactors for a desirable reaction between the contents of the nanoreactor; and

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e) decoding the liquid label, thereby identifying a single compound from a library of compounds.

14. The method of claim 13 further comprising incubating the contents of the nanoreactor prior to performing the screening step.

15. The method of claim 13, wherein the liquid label is a quantum dot or dye.

16. The method of claim 13, wherein the liquid label is organic or inorganic.

17. The method of claim 13, wherein the library comprises a biological or chemical material selected from a member of the group consisting of: tissues, cells, particles, proteins, antibodies, amino acids, nucleotides, small molecules, and pharmaceuticals.

18. The method of claim 13, wherein step (d) is performed by fluorescent polarization.

19. A method for controlling the quality of a library of emulsified compounds, the method comprising:

a) providing a library of emulsified compounds;

b) emulsifying a q-dot encoded aqueous buffer in an inert fluorocarbon medium, thereby forming droplets;

c) incubating the q-dot encoded droplet with the library of emulsified compounds;

d) sorting the q-dot encoded droplet away from the library;

e) analyzing the q-dot encoded droplet for the presence of any of the compounds emulsified in the library; and

f) eliminating the compounds identified in step (e) from the library of emulsified compounds,

wherein one or more of steps (a) - (f) are performed on a microfluidic device.

20. The method of claim 19, wherein the analyzing step is performed by mass spectroscopy.

21. A method for sorting cells, the method comprising:

a) fusing an affinity-reagent to an enzyme;

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b) mixing the fusion product of step (a) with a cell population;

c) isolating cells attached to the fusion product;

d) emulsifying the cells of step (c) in an inert fluorocarbon medium;

e) coalescing the cell emulsion of step (d) with an emulsion comprising a substrate corresponding to the enzyme of step (a), thereby forming a nanoreactor; and

f) screening the nanoreactor for a desirable reaction between the contents of the nanoreactor,

wherein one or more steps of (a) - (f) are performed on a microfluidic device.

22. The method of claim 21, wherein the affinity-reagent is a protein, nucleic acid, antibody, or other molecule to which an enzyme can be attached.

23. The method of claim 22, wherein the antibody is specific for binding a cell-surface marker.

24. The method of claim 23, wherein the cell-surface marker is a cancer marker.

25. The method of claim 21, wherein said enzyme is alk/phos, β-galactosidase, or horseradish peroxidase.

26. The method of claim 21, wherein the antibody is fused to multiple enzymes.

27. The method of claim 21, wherein multiple substrates are emulsified and coalesced with the cell emulsions formed in step (d).

28. A method for sequencing individual exons from individual chromosomes, the method comprising:

a) emulsifying specific primer-pairs to an exon with beads that can bind to said primer-pairs;

b) pooling the emulsions of step (a) to create a library emulsion;

c) providing a separate chromosomal DNA emulsion;

d) coalescing the library emulsion of step (b) with the chromosomal emulsion of step (c), thereby forming a nanoreactor;

e) amplfying the DNA in the nanoreactor;

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f) isolating the beads;

g) screening for beads containing DNA; and

h) sequencing the beads containing DNA,

wherein one or more steps of (a) - (h) are performed on a microfluidic device.

29. A kit for performing the method of claim 28, the kit comprising:

a) an emulsion library of specific primer-pairs to an exon and beads that can bind to said primer-pairs; and

b) a chromosomal DNA emulsion.

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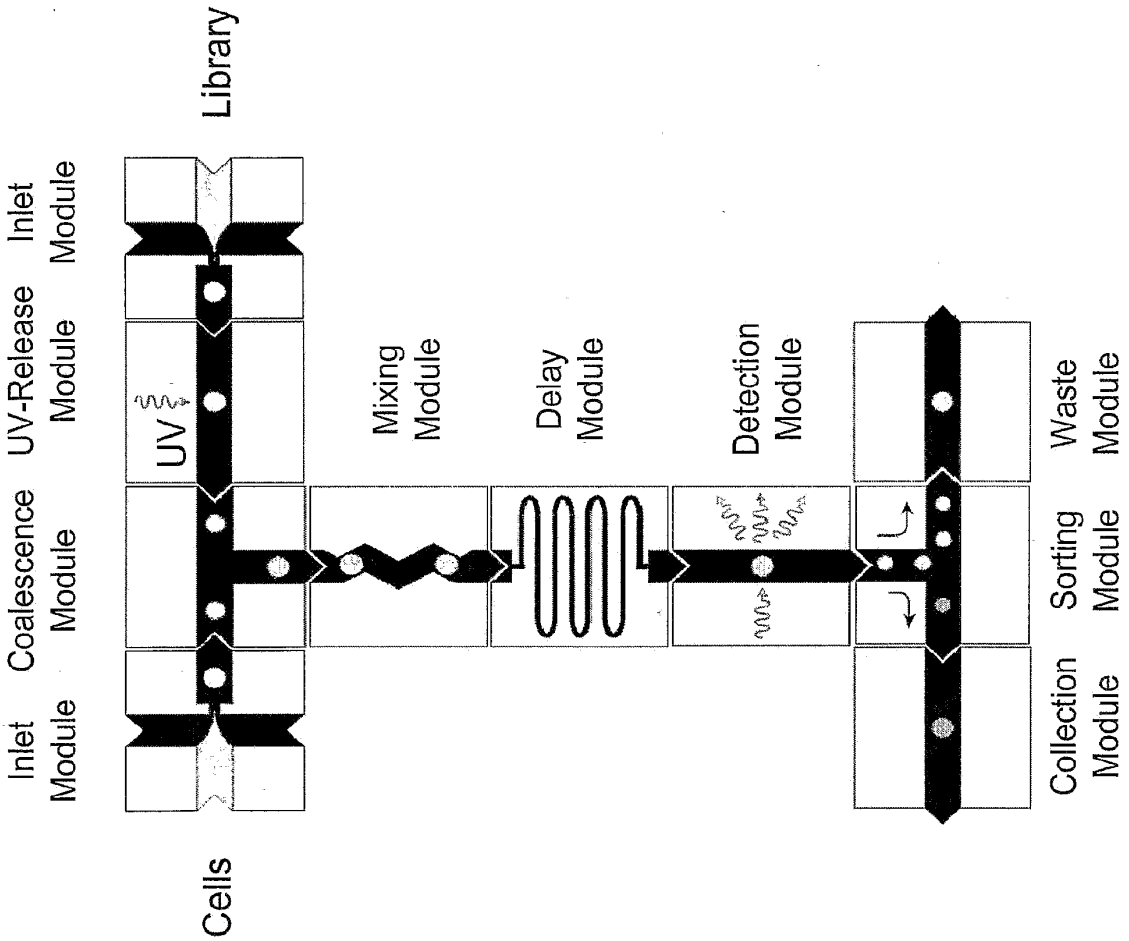

Library
Inlet Module
UV-Release Module
Coalescence Module
Inlet Module
UV
Mixing Module
Delay Module
Detection Module
Waste Module
Sorting Module
Collection Module
Cells

Fig. 1

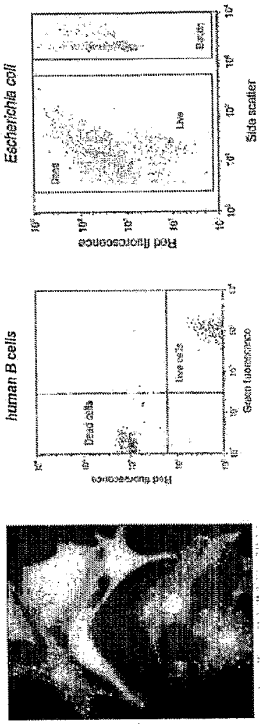

Escherichia coli
Dead
Live
Bacteria
Red fluorescence
Side scatter
human B cells
Dead cells
Live cells
Green fluorescence

Fig. 2

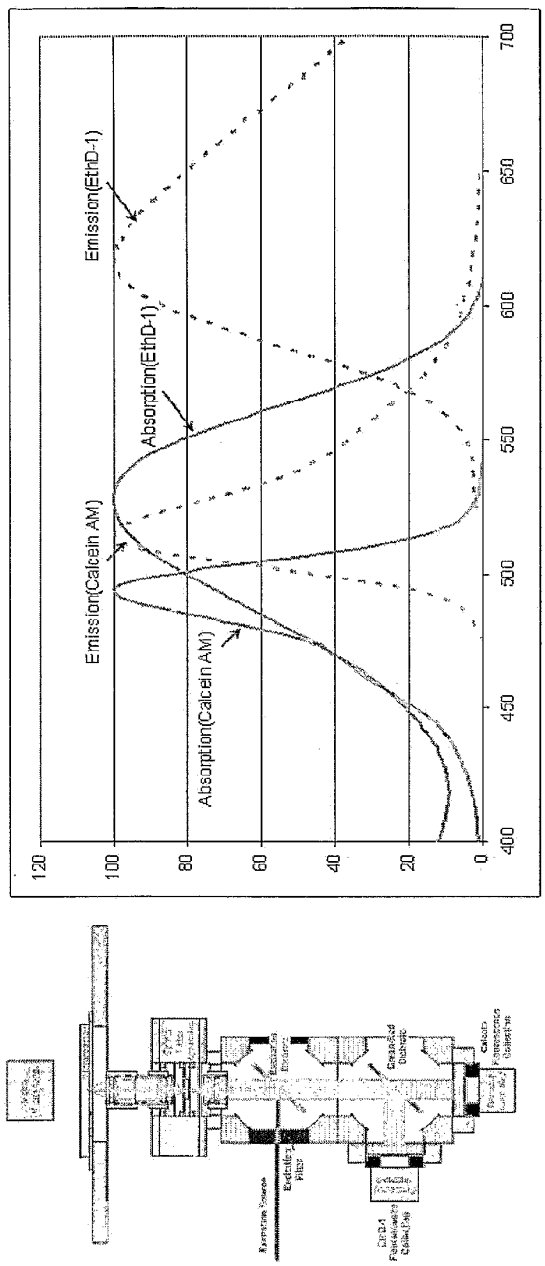

Emission(EthD-1)
Absorption(EthD-1)
Emission(Calcein AM)
Absorption(Calcein AM)
120
100
80
60
40
20
0
400
450
500
550
600
650
700

Fig. 3

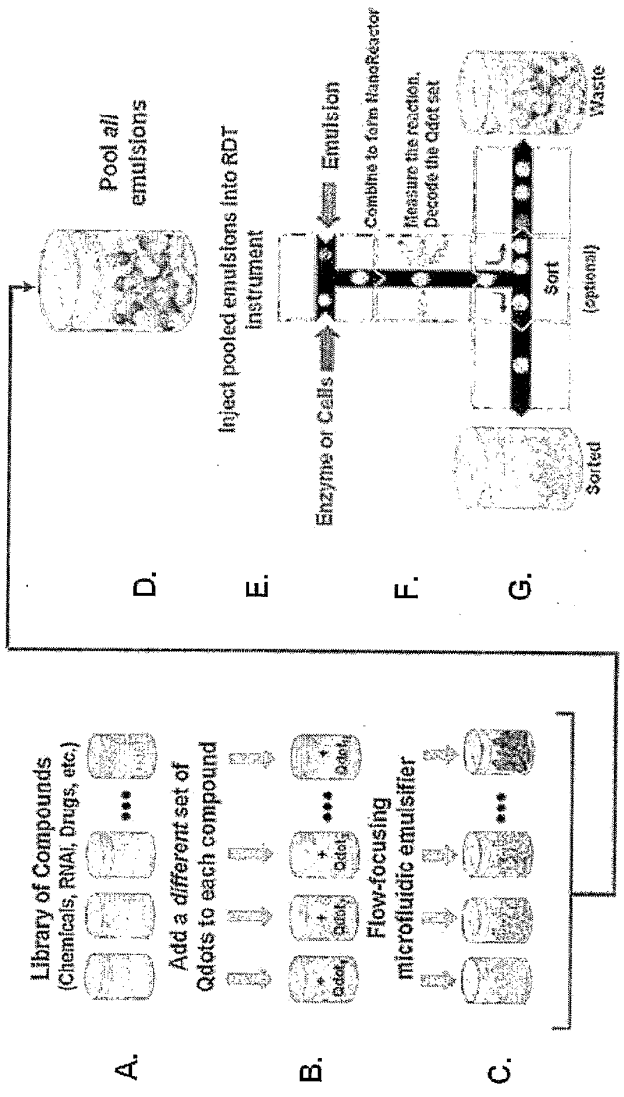

A.
Library of Compounds
(Chemicals, RNAi, Drugs, etc.)
B.
Add a different set of
Qdots to each compound
Qdots
C.
Flow-focusing
microfluidic emulsifier
D.
Pool all
emulsions
E.
Inject pooled emulsions into RDT
instrument
F.
Enzyme or Cells
Emulsion
Combine to form NanoReactor
Measure the reaction,
Decode the Qdot set
G.
Sort
(optional)
Sorted
Waste

Fig. 4

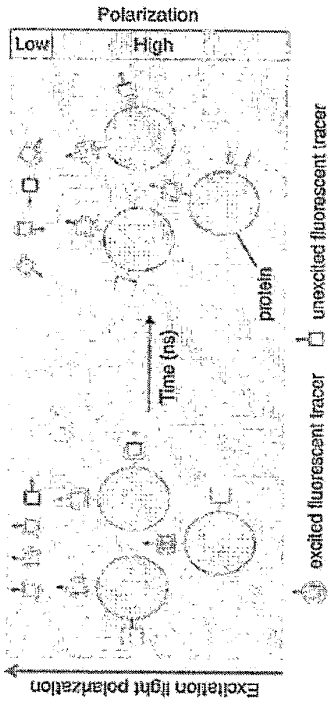

Polarization
Low
High
Time (ns)
protein
unexcited fluorescent tracer
excited fluorescent tracer
Excitation light polarization

Fig. 5

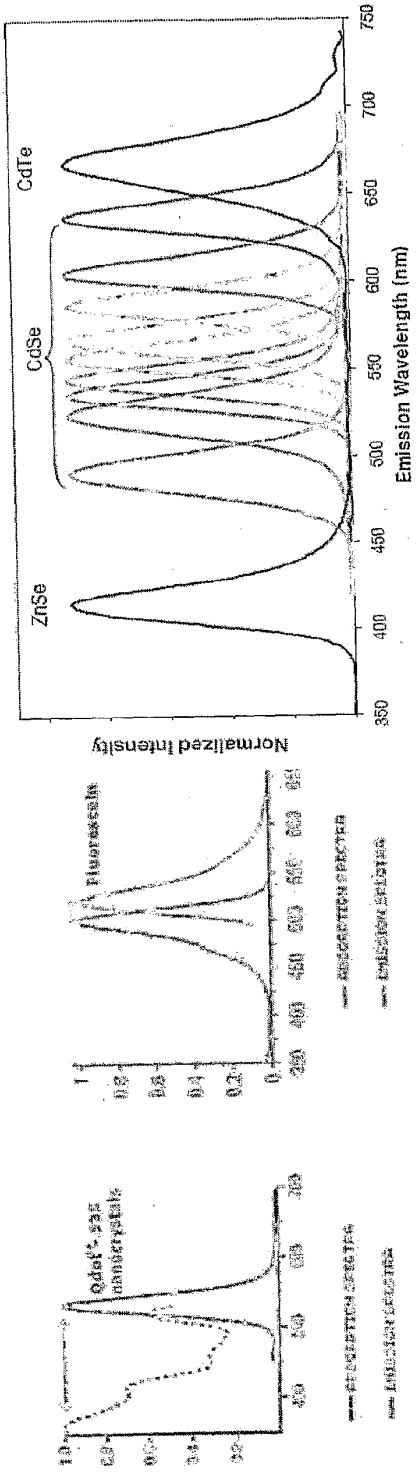

ZnSe
CdSe
CdTe
Normalized Intensity
Emission Wavelength (nm)
360
400
450
500
550
600
650
700
750
Fluorescence
Qdot® 525 nanocrystals

Fig. 6

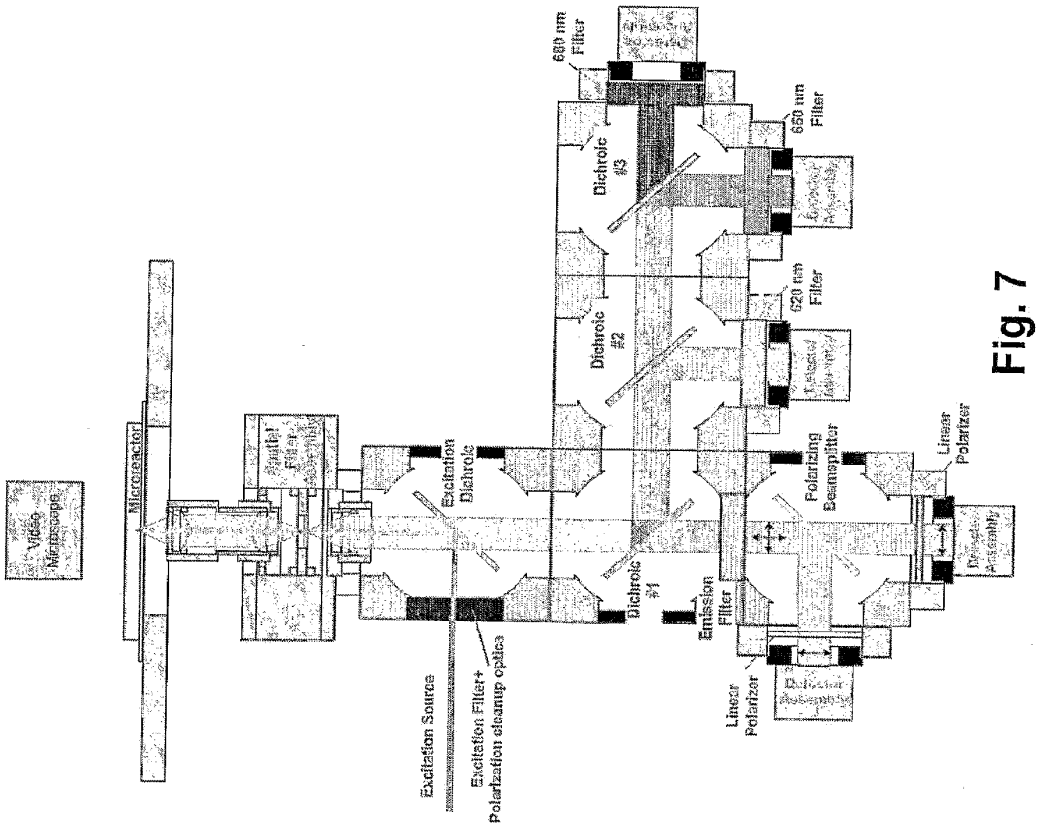

Video Microscope
Microreactor
Excitation Dichroic
Excitation Source
Excitation Filter+ Polarization cleanup optics
Dichroic #1
Dichroic #2
Dichroic #3
680 nm Filter
650 nm Filter
620 nm Filter
Emission Filter
Linear Polarizer
Polarizing Beamsplitter
Linear Polarizer

Fig. 7

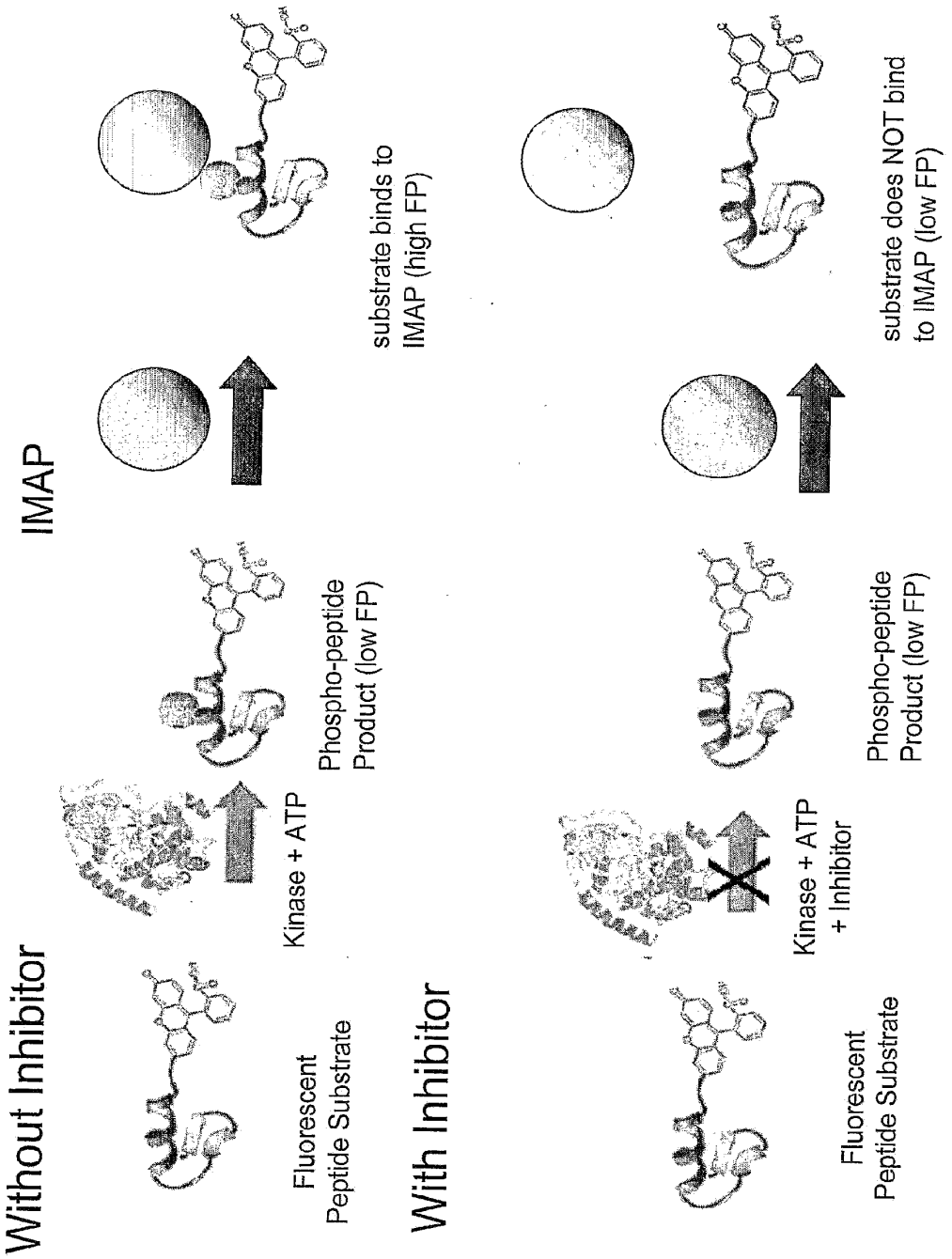

Without Inhibitor
IMAP
Fluorescent
Peptide Substrate
Kinase + ATP
Phospho-peptide
Product (low FP)
substrate binds to
IMAP (high FP)
With Inhibitor
Fluorescent
Peptide Substrate
Kinase + ATP
+ Inhibitor
Phospho-peptide
Product (low FP)
substrate does NOT bind
to IMAP (low FP)

Fig. 8

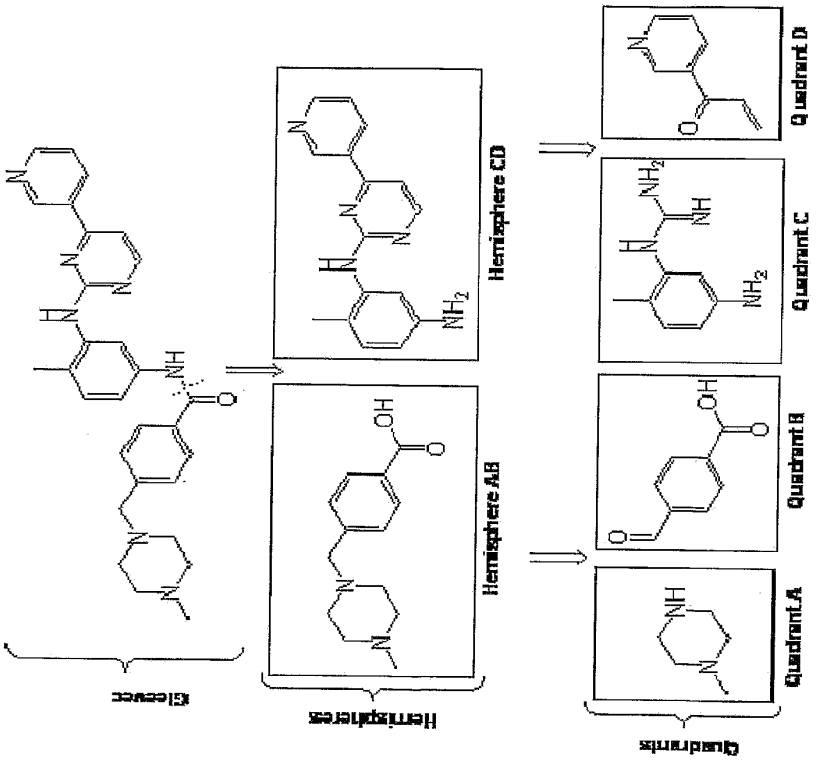
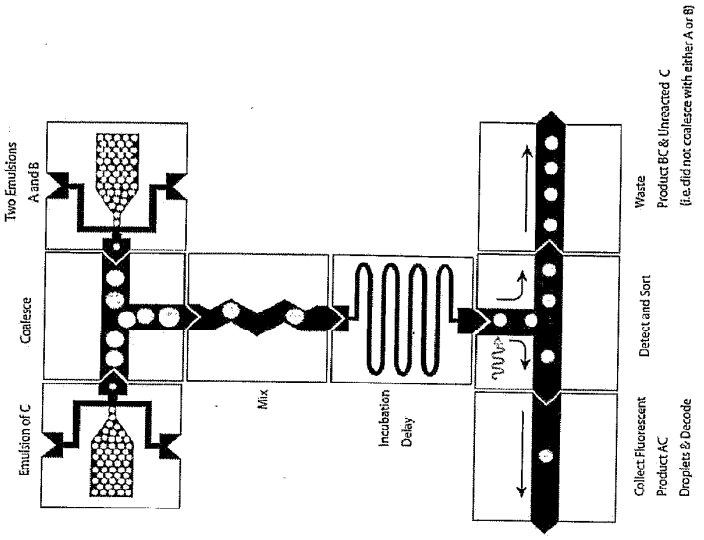

Gleevec
Hemispheres
Hemisphere AB
Hemisphere CD
Quadrants
Quadrant A
Quadrant B
Quadrant C
Quadrant D

Fig. 9


Two Emulsions A and B
Coalesce
Emulsion of C
Mix
Incubation Delay
Detect and Sort
Collect Fluorescent Product AC Droplets & Decode
Waste
Product BC & Unreacted C
(i.e. did not coalesce with either A or B)

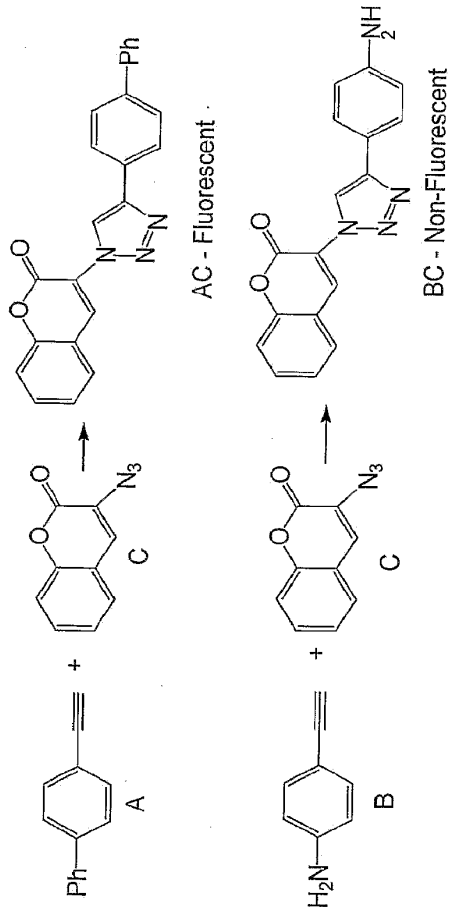

Ph
A
+
C
AC - Fluorescent
B
BC - Non-Fluorescent

Fig. 10

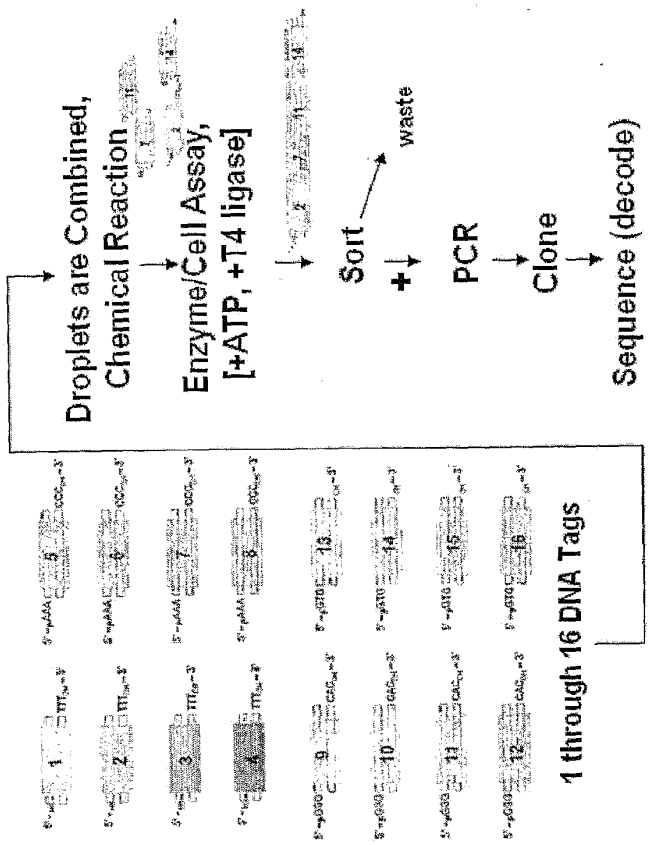

Droplets are Combined,
Chemical Reaction
Enzyme/Cell Assay,
[+ATP, +T4 ligase]
Sort
waste
+
PCR
Clone
Sequence (decode)
[2-7-11-14]
1 through 16 DNA Tags

Fig. 11