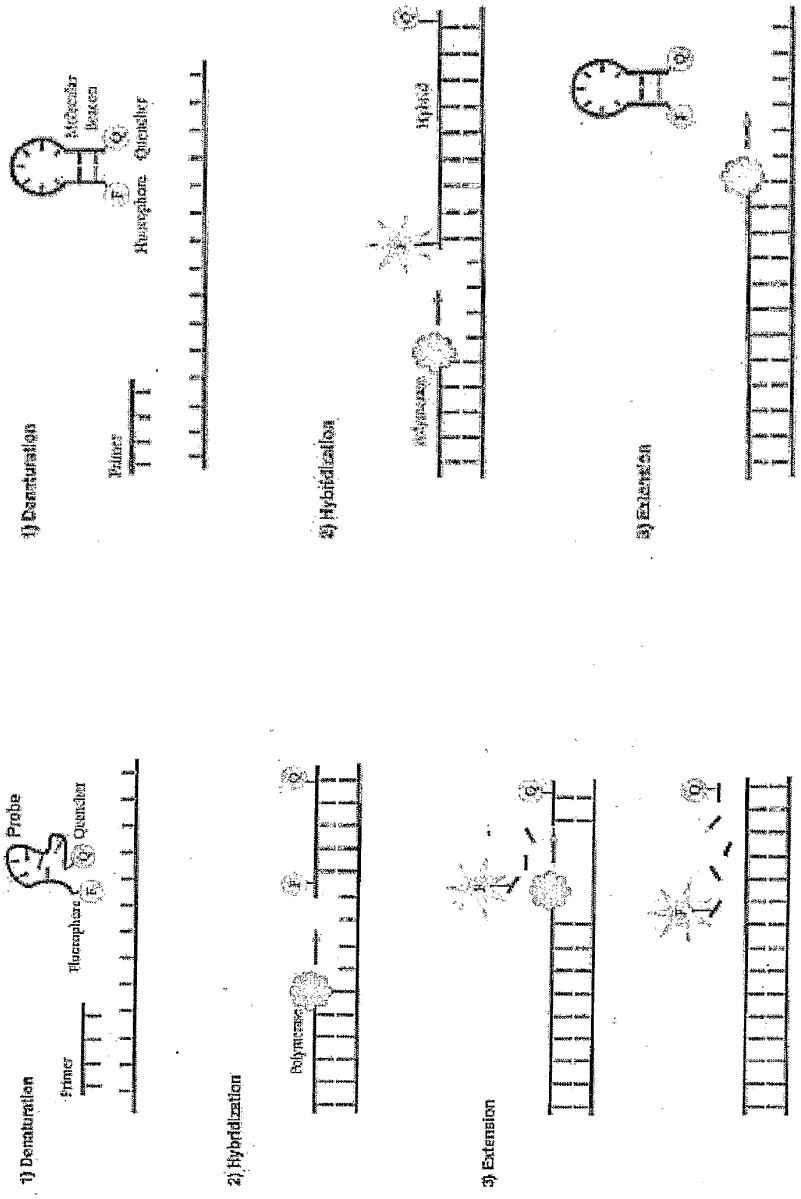

1) Denaturation
Probe
Quencher
Fluorophore
Primer
2) Hybridization
Polymerase
3) Extension
1) Denaturation
Molecular Beacon
Quencher
Fluorophore
Primer
2) Hybridization
Hybrid
Polymerase
3) Extension

Fig. 12

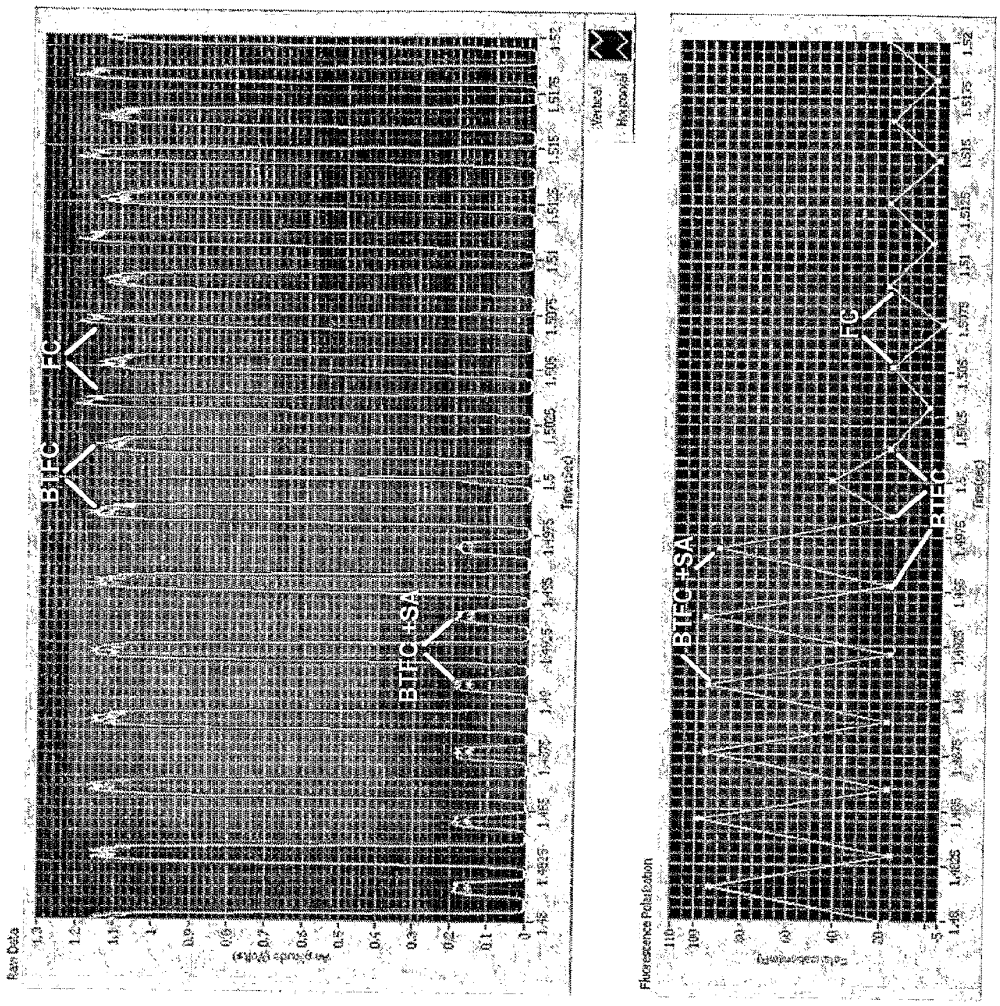

Raw Data
BTFC
FC
BTFC-PSA
Time (sec)
Verbal Approval
Fluorescence Polarization
BTFC-PSA
FC
BTFC
Time (sec)

Fig. 13

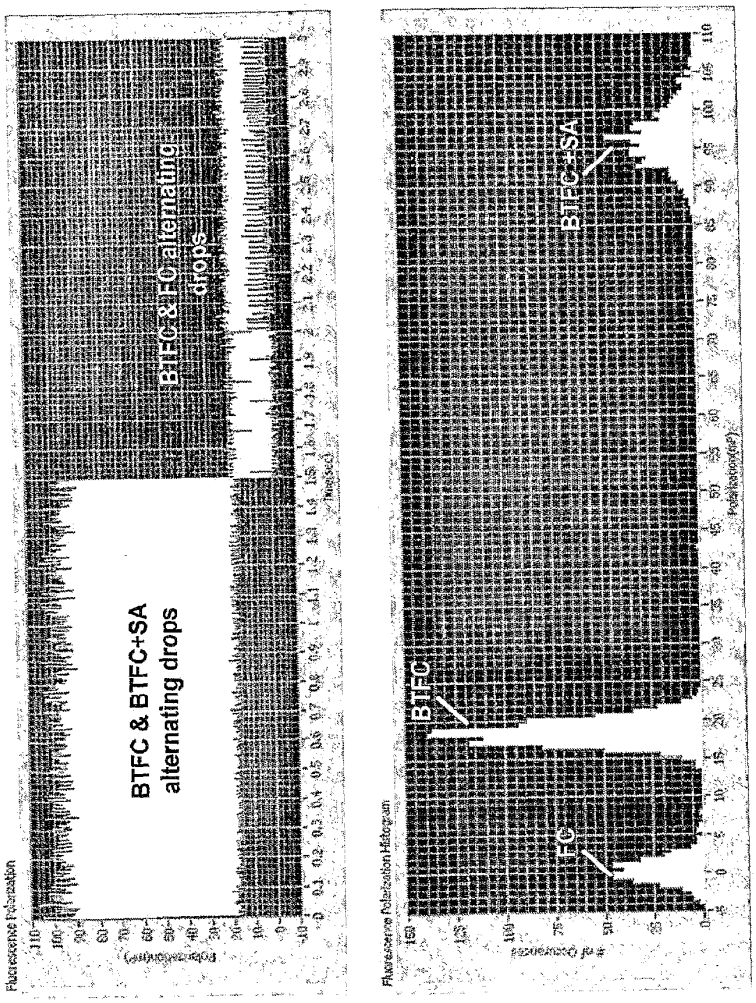

BTFC & BTFC+SA
alternating drops
BTFC & FC alternating
drops
Fluorescence Polarization
Fluorescence Polarization Histogram
BTFC
FC
BTFC+SA

Fig. 14

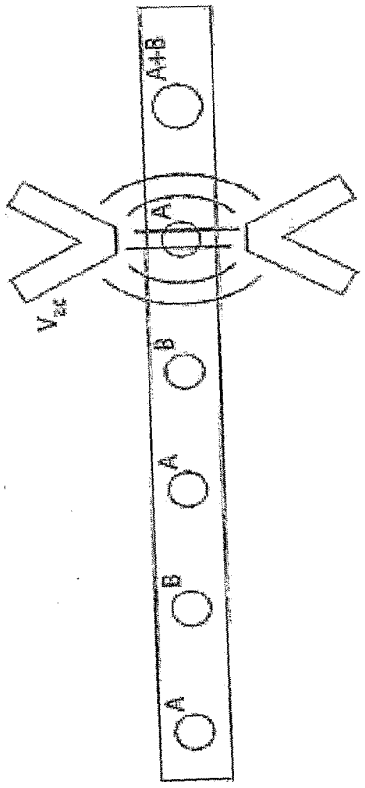


Fig. 15A

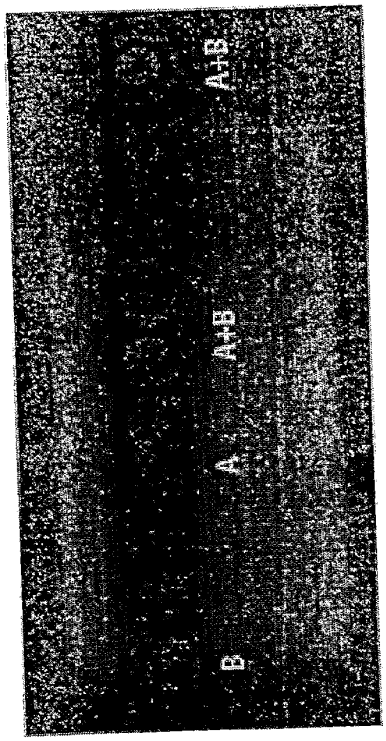


Fig. 15B

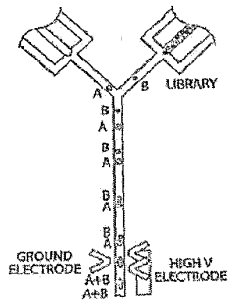


Fig. 16A

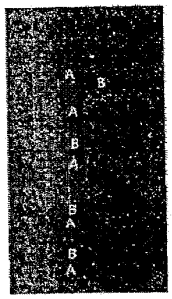


Fig. 16B

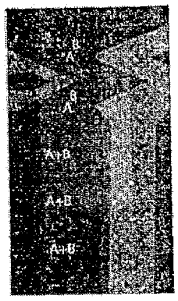


Fig. 16C

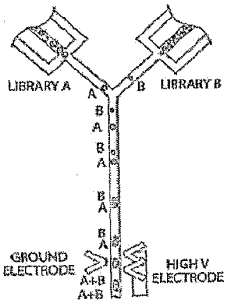


Fig. 16D

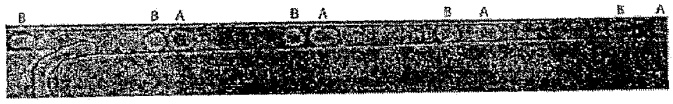


Fig. 16E

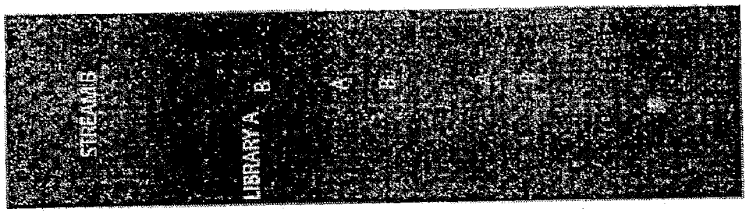


Fig. 17C

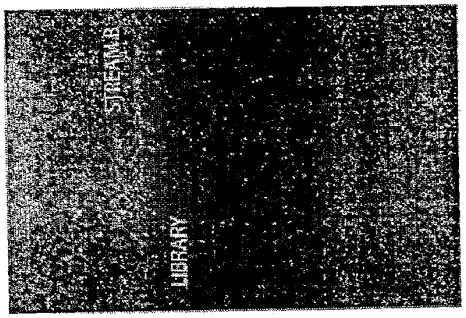


Fig. 17B

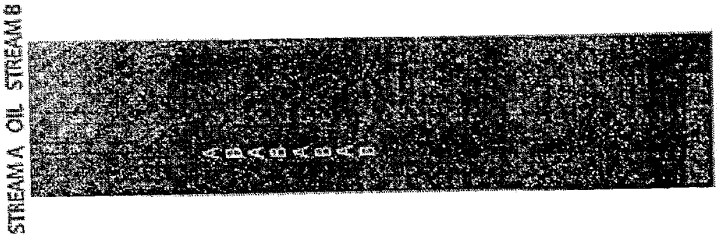
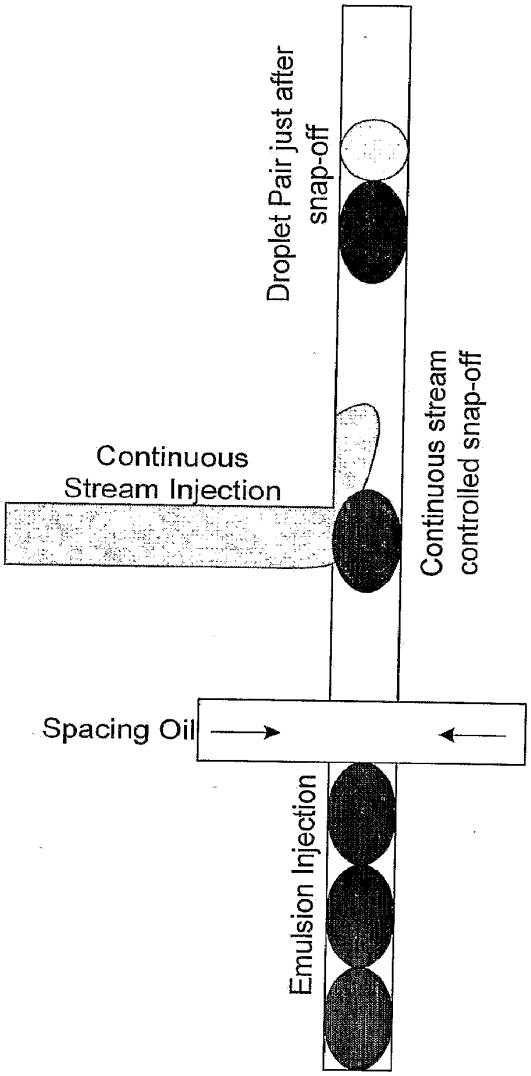


Fig. 17A


Droplet Pair just after snap-off
Continuous stream controlled snap-off
Continuous Stream Injection
Spacing Oil
Emulsion Injection

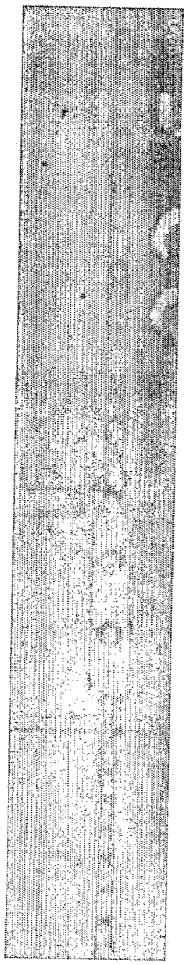


Fig. 17D

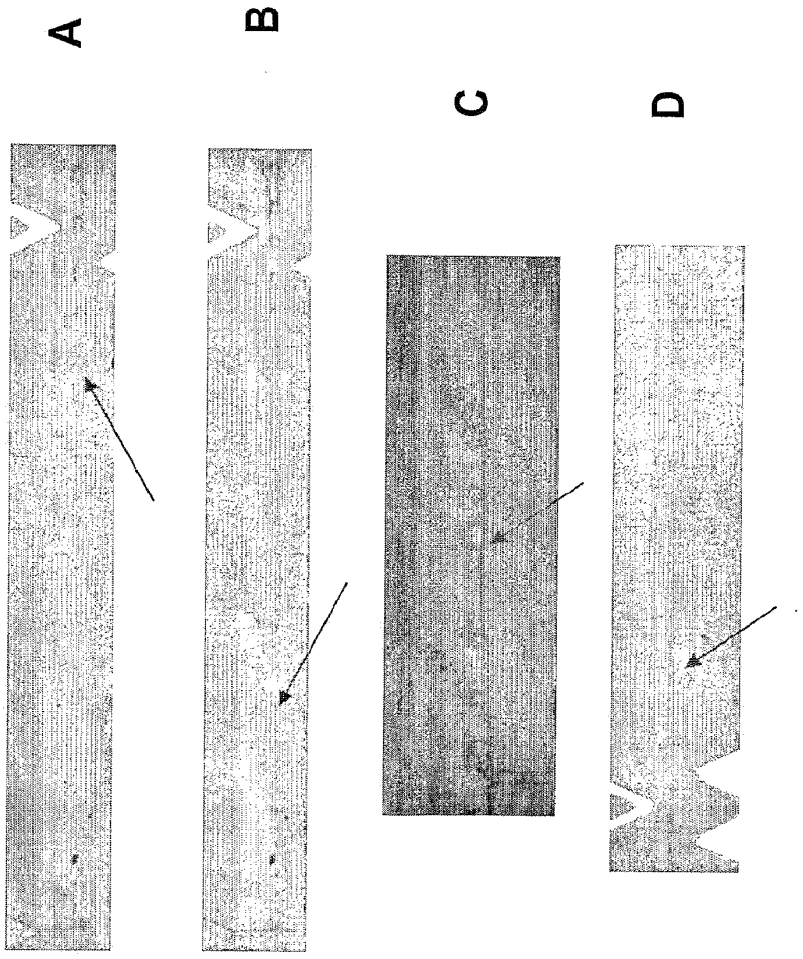


Fig. 18

Fig. 19

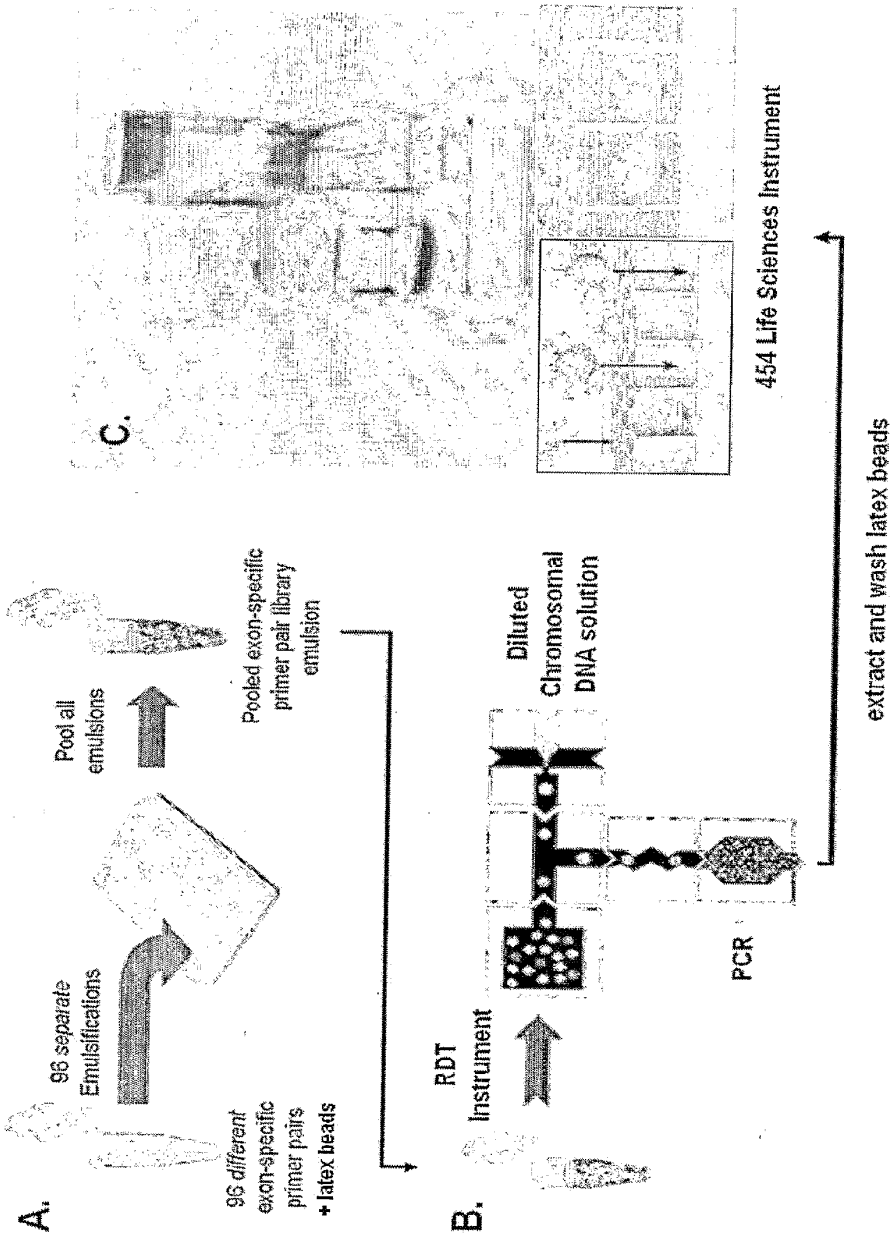

A.
96 different exon-specific primer pairs + latex beads
96 separate Emulsifications
Pool all emulsions
Pooled exon-specific primer pair library emulsion
B.
RDT Instrument
Diluted Chromosomal DNA solution
PCR
extract and wash latex beads
454 Life Sciences Instrument
C.

Fig. 20

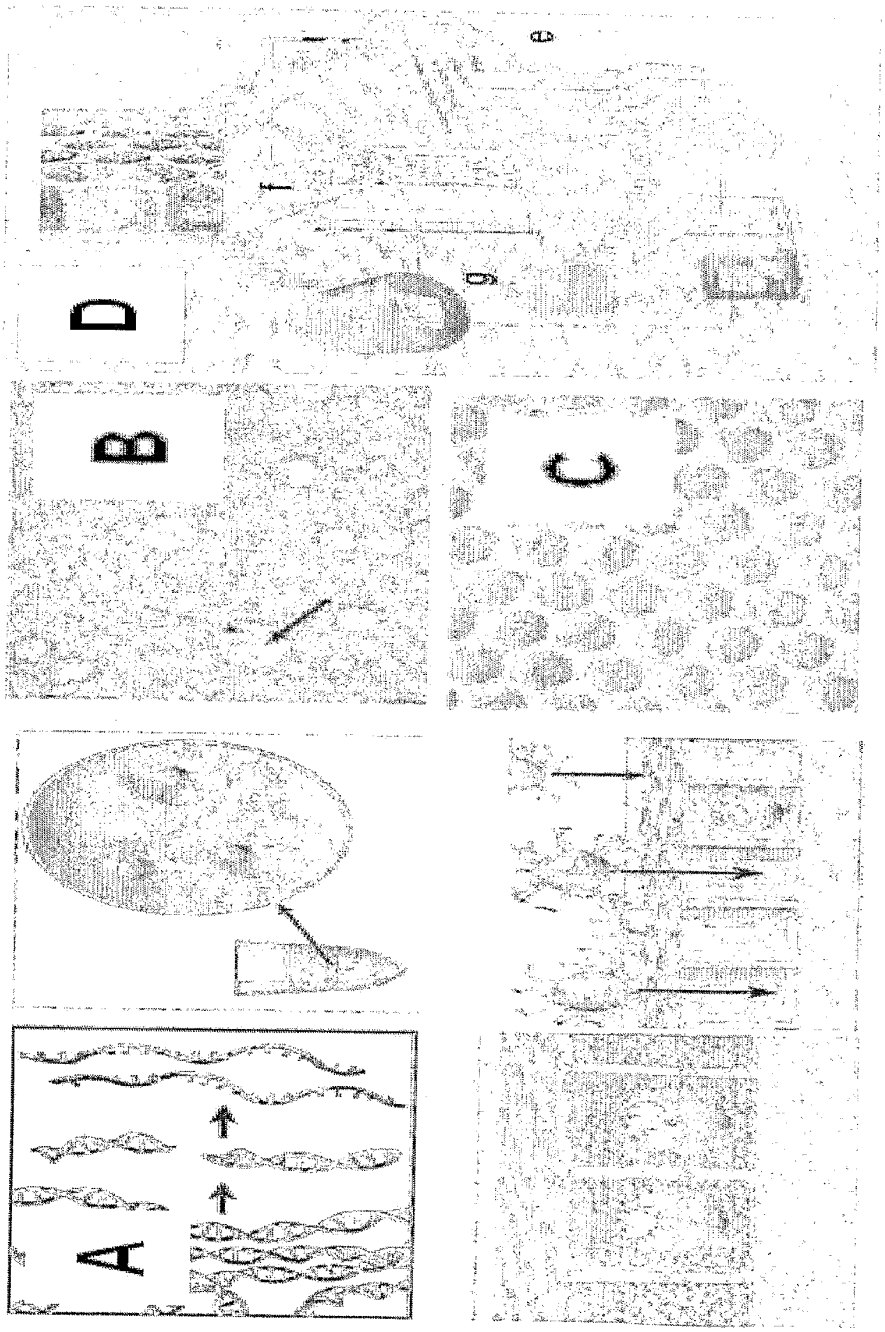
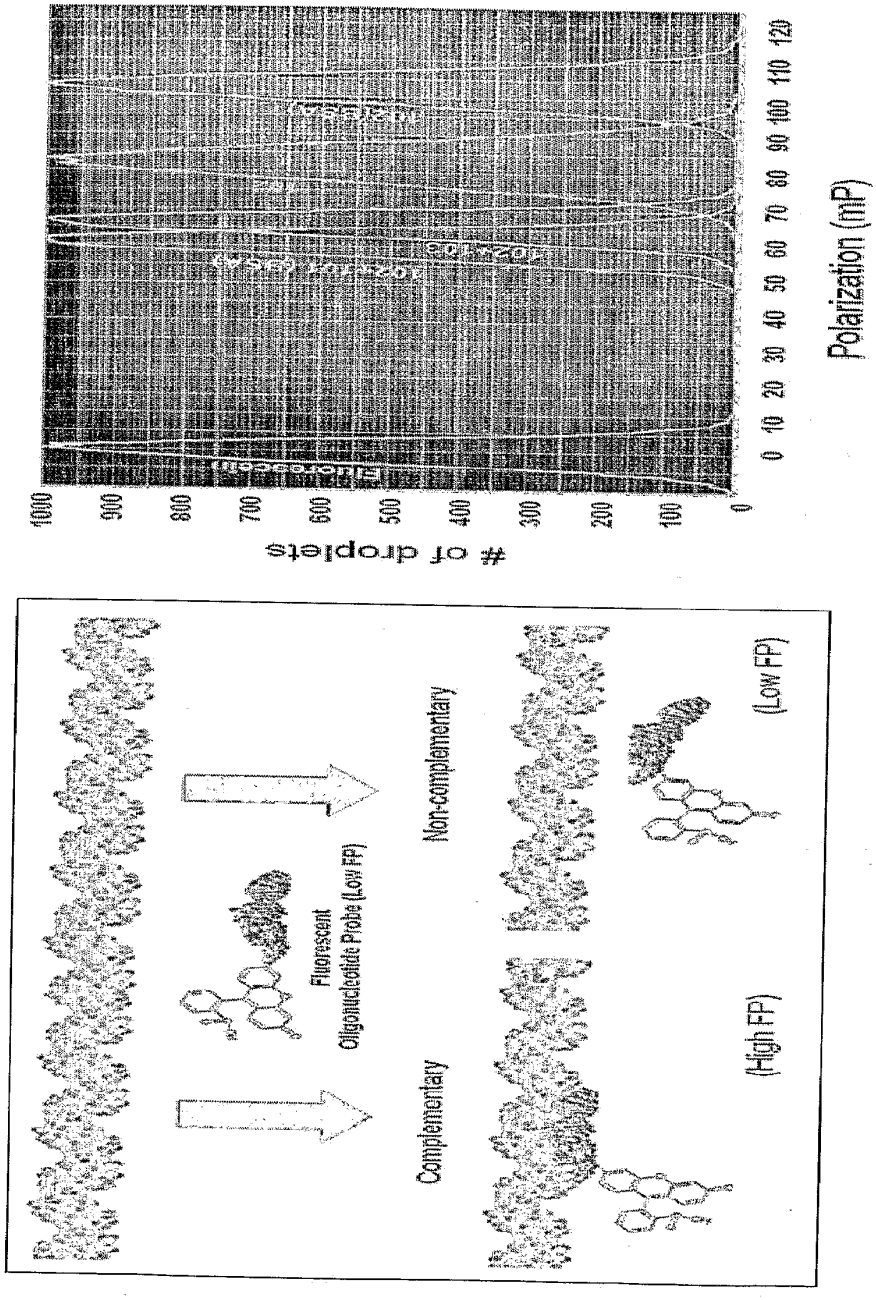


Fig. 21


Fluorescent
Oligonucleotide Probe (Low FP)
Complementary
Non-complementary
(High FP)
(Low FP)
# of droplets
Polarization (mP)

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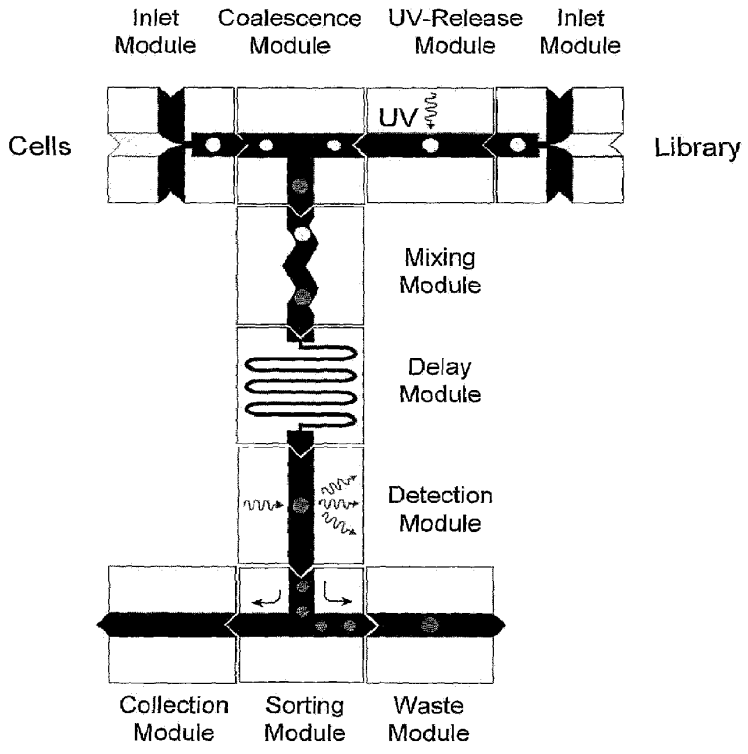
[Continued on next page]

(54) Title: MICROFLUIDIC DEVICES AND METHODS OF USE



(57) Abstract: The present invention provides novel microfluidic devices and methods that are useful for performing high-throughput screening assays and combinatorial chemistry. The device can include 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.

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MICROFLUIDIC DEVICES AND METHODS OF USE

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.

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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 reagents in droplets in an immiscible carrier fluid overcomes these limitations, but demands new fluid-handling technology.

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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 pondermotive function can be carried out following a significantly long delay from droplet formation.

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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 flow of the dispersed phase and continuous phase can be pressure driven, for example.

The substrate can further include at least one detection module downstream from and in fluid communication with the coalescence module. The detection module can include, for example, a detection apparatus for evaluating the contents and/or characteristics of the nanoreactor.

The substrate can further include a sorting module downstream from and in fluid communication with the detection module. The sorting module can include, for example, a sorting apparatus adapted to direct the nanoreactor into or away from a collection module in response to the contents or characterization of the nanoreactor.

The inlet channel can be connected to a means for introducing a sample to the device. The means can include, for example, a well or reservoir, which can be temperature controlled. The well or reservoir can optionally include an acoustic actuator.

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The microfluidic device can further include a means for simultaneously loading multiple samples onto the device. The means can include, for example, a serpentine chip. The samples can include one or more sets of droplets containing a unique set of labels.

The channels of the microfluidic device can be coated with an anti-wetting or blocking agent for the dispersed phase. The anti-wetting or blocking agent can include, for example, a silica primer layer followed by a perfluoroalkylalkylsilane compound, an amorphous soluble perfluoropolymer, BSA, PEG-silane or fluorosilane.

The main channel can be further connected to a means for collecting a sample from the microfluidic device.

The continuous phase used in the channels of the microfluidic device can be a non-polar solvent such as, for example, a fluorocarbon oil. The continuous phase can further include one or more additives such as a surfactant or fluorosurfactant in order to stabilize the droplets. The fluorosurfactant can be a perfluorinated polyether, for example. Accordingly, the invention also includes a composition comprising a fluorinated oil and a fluorosurfactant.

The dispersed phase of the microfluidic device can include a library of droplets of the same or different sizes (*i.e.*, an emulsion stream) or a continuous aqueous stream. The library of droplets can include, for example, a biological or chemical material such as tissues, cells, particles, proteins, antibodies, amino acids, nucleotides, small molecules, and pharmaceuticals. The biological/chemical material can include a label such as a DNA tag, dye, or quantum dot.

The device can further include one or more electrodes, or a patterned electrically conductive layer, which are capable of generating an electric field. The electrodes can be made from metal alloy components such as indium tin oxide, and can be integrally contained in one or more channels isolated from the main and inlet channels of the device.

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. There may be zero, one, or more of each of the modules. The detection module can include a detection apparatus, which can include an optical and/or electrical detector. The optical detector may be an optical waveguide or free space optics. Preferably, free space optics are used. The detection apparatus can also include a two-dimensional sheet of droplets for parallel spectroscopic imaging. The sorting module is downstream from a detection module and can include a sorting apparatus adapted to direct the droplets into or away from a collection module in

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response to the contents or characterization of the droplet or nanoreactor detected. At the sorting module, the fluid stream is split into two or more sorting streams and droplets or nanoreators are directed into one of those sorting streams. The relative split of the volume of the continuous stream into the sorting streams can be controlled by using a controlled length of channel of controlled cross-section, or by controlling the rate at which fluid can exit one channel using a positive displacement pump. Preferably, a positive displacement pump is used to control the rate of flow of the continuous stream into all but one of the sorting channels. Preferably, the mixing module and/or delay module are downstream of the coalescence module and upstream of the detection module. The delay module can be a serpentine channel, a buoyant hourglass, or an off-chip volume. Preferably, a serpentine channel is used to time delays less than 1 hour. Preferably, an off-chip volume is used to time delays longer than 1 hour. The delay module can further include heating and cooling regions. The UV-release module can be downstream of the inlet module and upstream of the coalescence module. The collection module can be connected to a means for storing a sample from the device and/or a waste module connected to a means for collecting a sample discarded from the device. The means can be a well or reservoir, which can be temperature controlled. The acoustic actuator can be in fluid communication with the dispersed fluid in the inlet channel, inlet module and/or mixing module, or combinations thereof.

Where more than one inlet module is used, the dispersed phase can include two aqueous streams, one aqueous stream and an emulsion stream (*i.e.*, a library of droplets of the same or different size), or two emulsion streams. The droplets formed on or entering the device can be paired for coalescence prior to entering the coalescence module. Coalescence of one or more droplets can be achieved via an electric field that generates an electrophoretic force, or it can be achieved passively such as by providing a narrowed portion of the main channel in which the paired droplets flow.

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.

Figures 2A-E depict possible fluidic interconnect designs: (A) A potential fluidic interconnect design when trying to minimize dead volume while connecting to a microfluidic device. Note the fluidic grommet may be placed into either a tapered hole or a hole with perpendicular walls; (B) Alternative design to (A), where the raised contact surface between the two sides is formed on the tubing side instead of the device side; (C) Microfluidic interconnect molded directly into the device; (D) Interconnect created using casting and thermal bonding of PDMS directly to a glass substrate. A thin film of PDMS can be simultaneously formed and bonded to the top of the glass slide and permits the used 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 in this figure could also be built into the tubing side, as shown in (B); and (E) Microfluidic interconnect made to a flat device.

Figure 3A-B depict side (A) and top (B) view of a possible electrical interconnect.

Figure 4 is a photograph and accompanying graphs showing the flow cytometric-cell-based assay for human and bacterial cells.

Figure 5 is a schematic and graph showing simultaneous two color fluorescence detection.

Figure 6 is a schematic illustrating chemical library screening using a nanoreactor of the present invention.

Figure 7 is a schematic illustrating the physical basis of fluorescence polarization assays.

Figure 8 is several graphs showing the absorption and emission spectra of q-dots and organic dye.

Figure 9 is a schematic showing the extension to the fluorescence test station required to perform polarization fluorescence measurements simultaneously with q-dot readout.

Figure 10 is a schematic and accompany graphs showing a fluorescence polarization based kinase assay.

Figure 11 is a schematic illustrating the retrosynthetic analysis of Gleevec.

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Figure 12 is a schematic illustrating the synthesis of a fluorescent and non-fluorescent product from the same precursor.

Figure 13 is schematic showing the use of nucleic acids for chemical encoding and decoding tagging of chemical reactions.

Figure 14 (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 14 (B).

Figure 15 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 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 15 (c). Droplets A and B may both come from libraries (made off-chip) Figure 15 (d), or be made on-chip.

Figure 16 (A-D) shows alternate ways to achieve interdigitation of droplets of different type.

Figure 17A-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 18 depicts multiple drop fluorescence detection using 2D droplet sheet and spectroscopic imaging with unlimited sensitivity.

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

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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 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

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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 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

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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 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

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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.

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.

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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.

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

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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 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. Figure 2A and 2B depict possible designs to provide sealing

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in a device where thru-holes in the device must be relatively large (such as glass). In figure 2A, the fluidic grommet may be placed into either a tapered hole or a hole with perpendicular walls. Figure 2B is an alternative design, wherein 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 (Figure 2C). Figure 2D depicts yet another embodiment, wherein a possible interconnect that could 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 (as shown in Figure 2B). As shown in Figures 2A-D, the sealing surface on the tubing side of the connection is formed directly into the face of the tubing, although a separate piece secured to the tubing assembly/retaining nut may also be used (as shown in Figure 2E).

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 indicated in Figures 2A-E 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

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"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 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

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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.

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.

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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 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

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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. 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

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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 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

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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.

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

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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 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

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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 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

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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 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.

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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 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

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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 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

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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 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

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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 (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.

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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 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

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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.

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

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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 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

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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 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

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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 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

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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 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.

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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 14-16. 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 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).

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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 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.

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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 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

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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 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. Figure 3A-B. 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

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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 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

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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 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 16 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

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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 17A-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 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.

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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 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

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(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 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

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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 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

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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).

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.

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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.

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.

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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. Figure 18. 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 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

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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 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

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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. 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

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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 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.

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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 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

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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, nucleotides, 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 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

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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.

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

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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 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.

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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 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.

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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 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

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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). 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

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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 5, while Figure 4 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 4, 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 5, 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 5 shows excitation and emission spectra for calcein AM and EthD-1 dyes. Normal cytometry protocol excites both at 488 nm. Figure 5 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 6). As shown in Figure 6 (*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 6 (*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 ($\leq$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 7, 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 are 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 8 (Left, Center Panel) shows the absoprtion and emission

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spectra of q-dot 535 nanocrystals and fluorescein, respectively. Figure 8 (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 8, 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 9, 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 10, 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 10, 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 10,

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middle 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 10, 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 11 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 12 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 13 (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 13 (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

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-809M in CT-Solv 180. This solution is injected into the channels of a microfluidic device via

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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.

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CLAIMS

What is claimed is:

1. A microfluidic device comprising a microfabricated substrate, said substrate further comprising:

a) one or more inlet modules having at least one inlet channel adapted to carry at least one dispersed phase fluid;

b) at least one main channel adapted to carry at least one continuous phase fluid, wherein said inlet channel is in fluid communication with said main channel such that said dispersed phase fluid is immiscible with said continuous phase fluid and forms a plurality of droplets in said continuous phase fluid; and

c) a coalescence module downstream from and in fluid communication with said one or more inlet modules via the main channel, wherein two or more droplets passing there through are coalesced to form a nanoreactor.

2. The device of claim 1, wherein said substrate further comprises at least one detection module downstream from and in fluid communication with said coalescence module, said detection module comprising a detection apparatus for evaluating the contents and/or characteristics of the nanoreactor.

3. The device of claim 2, wherein said substrate further comprises a sorting module downstream from and in fluid communication with said detection module, said sorting module comprising a sorting apparatus adapted to direct said nanoreactor into or away from a collection module in response to the contents or characterization of the nanoreactor.

4. The device of claim 1, wherein said inlet channel is further connected to a means for introducing a sample to said device.

5. The device of claim 4, wherein said means is a well or reservoir.

6. The device of claim 4, wherein said means is temperature controlled.

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7. The device of claim 5, wherein said well or reservoir further comprises an acoustic actuator.

8. The device of claim 1 further comprising a means for simultaneously loading multiple samples onto the device.

9. The device of claim 8, wherein said means is a serpentine chip.

10. The device of claim 8, wherein said samples comprise one or more sets of droplets containing a unique set of labels.

11. The device of claim 1, wherein said channels are coated with an anti-wetting or blocking agent for the dispersed phase.

12. The device of claim 1, wherein said channels are coated with a silica primer layer followed by a perfluoroalkylalkylsilane compound, an amorphous soluble perfluoropolymer, BSA, PEG-silane or fluorosilane.

13. The device of claim 12, wherein the channels are coated with a silica primer layer followed by a perfluoroalkylalkylsilane compound.

14. The device of claim 1, wherein said main channel is further connected to a means for collecting a sample from said device.

15. The device of claim 14, wherein said means is a well or reservoir.

16. The device of claim 14, wherein said means is temperature controlled.

17. The device of claim 1, wherein said continuous phase is a non-polar solvent.

18. The device of claim 1, wherein said continuous phase is a fluorocarbon oil.

19. The device of claim 1, wherein said continuous phase further comprises one or more additives.

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20. The device of claim 19, wherein said additive is a fluorosurfactant.

21. The device of claim 20, wherein said fluorosurfactant is a perfluorinated polyether.

22. The device of claim 20, wherein said fluorosurfactant stabilizes said droplets.

23. The device of claim 1, wherein said dispersed phase comprises a library of droplets.

24. The device of claim 23, wherein said library of droplets comprises a biological/chemical material.

25. The device of claim 24, wherein said biological/chemical material is selected from the group consisting of tissues, cells, particles, proteins, antibodies, amino acids, nucleotides, small molecules, and pharmaceuticals.

26. The device of claim 25, wherein said biological/chemical material comprises a label.

27. The device of claim 26, wherein said label is a protein, a DNA tag, a dye or a quantum dot.

28. The device of claim 1, further comprising one or more electrodes that generate an electric field.

29. The device of claim 1, wherein the electric field is generated by a patterned electrically conductive layer.

30. The device of claim 28, wherein the electrodes comprise metal alloy components.

31. The device of claim 30, wherein the metal alloy components are integrally contained in one or more channels and are isolated from the inlet and main channels.

32. The device of claim 1, wherein said substrate further comprises a mixing module in fluid communication with the main channel.

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33. The device of claim 32, wherein the mixing module is downstream of the coalescence module and upstream of the detection module.

34. The device of claim 1, wherein the flow of said dispersed phase fluid and said continuous phase fluid is pressure driven.

35. The device of claim 1, wherein said substrate further comprises a delay module in fluid communication with the main channel.

36. The device of claim 35, wherein the delay module is downstream of the coalescence module and upstream of the detection module.

37. The device of claim 35, wherein said delay module is a serpentine channel.

38. The device of claim 35, wherein said delay module is a chamber designed and situated to cause the plurality of droplets to either cream or sediment with respect to the ambient gravitational field.

39. The device of claim 35, wherein the delay module further comprises heating and cooling regions.

40. The device of claim 1, wherein said substrate further comprises a UV-release module in fluid communication with the main channel.

41. The device of claim 40, wherein the UV-release module is downstream of the inlet module and upstream of the coalescence module.

42. The device of claim 1, further comprising an acoustic actuator in fluid communication with the dispersed phase fluid in the inlet module.

43. The device of claim 1, wherein said substrate further comprises a collection module connected to a means for storing a sample from said device.

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44. The device of claim 43, wherein said means is a well or reservoir.

45. The device of claim 43, wherein said means is temperature controlled.

46. The device of claim 1, wherein said substrate further comprises a waste module connected to a means for collecting a sample discarded from said device.

47. The device of claim 46, wherein said means is a well or reservoir.

48. The device of claim 2, wherein said detection apparatus comprises a two-dimensional sheet of droplets for parallel spectroscopic imaging.

49. The device of claim 2, wherein said detection apparatus comprises an optical detector.

50. The device of claim 49, wherein the optical detector is an optical waveguide.

51. The device of claim 2, wherein said detection apparatus comprises an electrical detector.

52. A microfluidic device comprising a microfabricated substrate, said substrate further comprising:

a) one or more inlet modules having at least one inlet channel adapted to carry at least one dispersed phase fluid;

b) at least one main channel adapted to carry at least one continuous phase fluid, wherein said inlet channel is in fluid communication with said main channel such that said dispersed phase fluid is immiscible with said continuous phase fluid and forms a plurality of droplets in said continuous phase fluid; and

c) a detection module downstream from and in fluid communication with said one or more inlet modules via the main channel, said detection module comprising a detection apparatus for evaluating the contents or characteristics of the droplets.

53. The device of claim 52, wherein said substrate further comprises a coalescence module, wherein two or more droplets passing there through are coalesced to form a nanoreactor.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
1702

54. The device of claim 52, wherein said substrate further comprises a sorting module, said sorting module comprising a sorting apparatus adapted to direct said nanoreactor into or away from a collection module in response to the contents or characterization of the nanoreactor.

55. The device of claim 1, wherein said substrate comprises a first and a second inlet module.

56. The device of claim 55, wherein said dispersed phase is a member selected from the group consisting of: two aqueous streams, one aqueous stream and an emulsion stream, and two emulsion streams.

57. The device of claim 56, wherein said droplets are paired for coalescence.

58. The device of claim 57, wherein said coalescence module comprises a narrowed portion of the main channel, whereby said paired droplets are coalesced in said narrowed portion to form a nanoreactor.

59. A composition comprising a fluorinated oil and a fluorosurfactant.

60. A microfluidic device comprising a microfabricated substrate, said substrate further comprising:

a) one or more inlet modules having at least one inlet channel coated with a silica primer layer followed by a perfluoroalkylalkylsilane compound and adapted to carry at least one dispersed phase fluid;

b) at least one main channel coated with a silica primer layer followed by a perfluoroalkylalkylsilane compound and adapted to carry at least one continuous phase fluid comprising a fluorosurfactant dissolved in a fluorinated oil, wherein said inlet channel is in fluid communication with said main channel such that said dispersed phase fluid is immiscible with said continuous phase fluid and forms a plurality of droplets in said continuous phase fluid; and

c) a coalescence module downstream from said one or more inlet modules, wherein two or more droplets passing there through are coalesced to form a nanoreactor.

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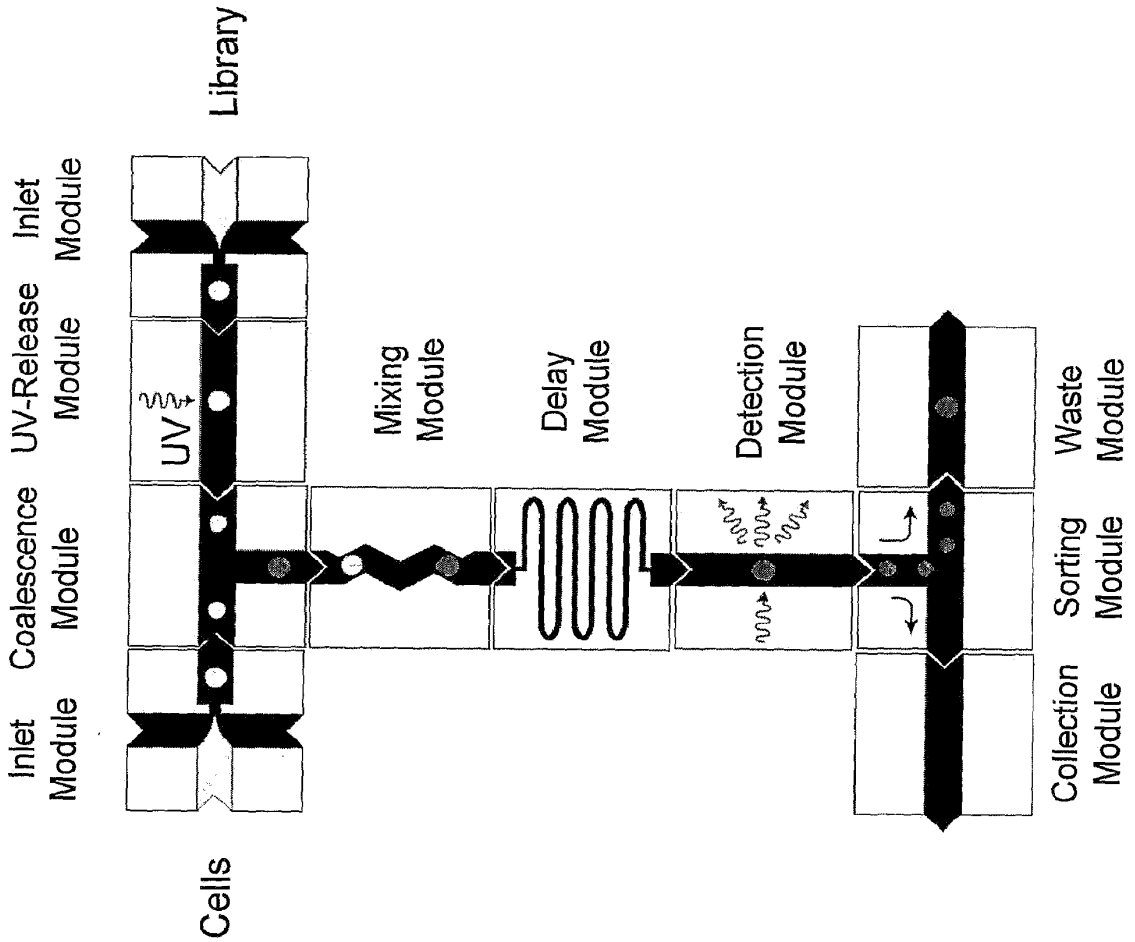

Library
Inlet Module
UV-Release Module
UV
Mixing Module
Delay Module
Detection Module
Waste Module
Inlet Module
Coalescence Module
Sorting Module
Collection Module
Cells

Fig. 1

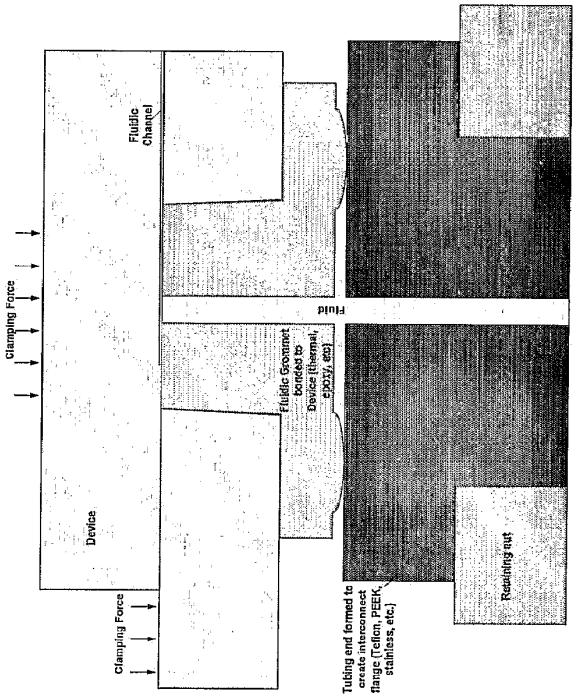

Clamping Force
Fluidic Channel
Fluid
Fluidic Grommet bonded to Device (thermal, epoxy, etc)
Device
Clamping Force
Tubing end formed to create interconnect flange (Teflon, PEEK, stainless, etc.)
Retaining nut

Fig. 2A

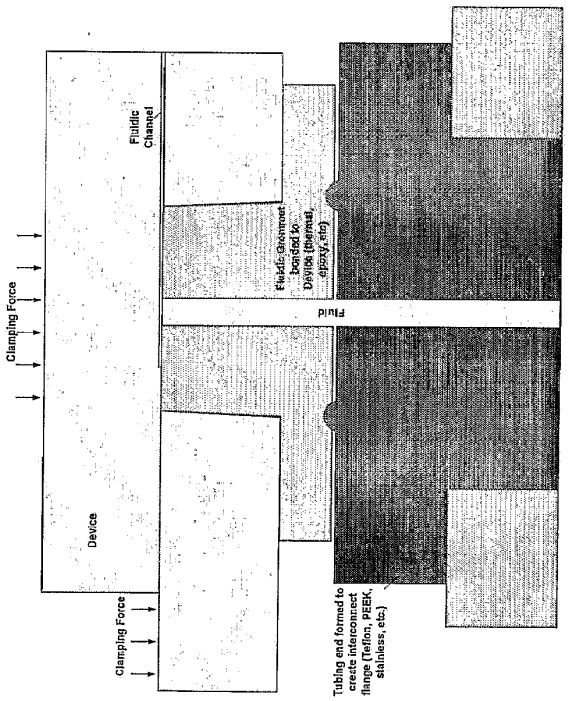

Clamping Force
Device
Fluidic Channel
Fluidic Grommet bonded to Device (thermal, epoxy, etc)
Fluid
Clamping Force
Tubing end formed to create interconnect flange (Teflon, PEEK, stainless, etc.)

Fig. 2B

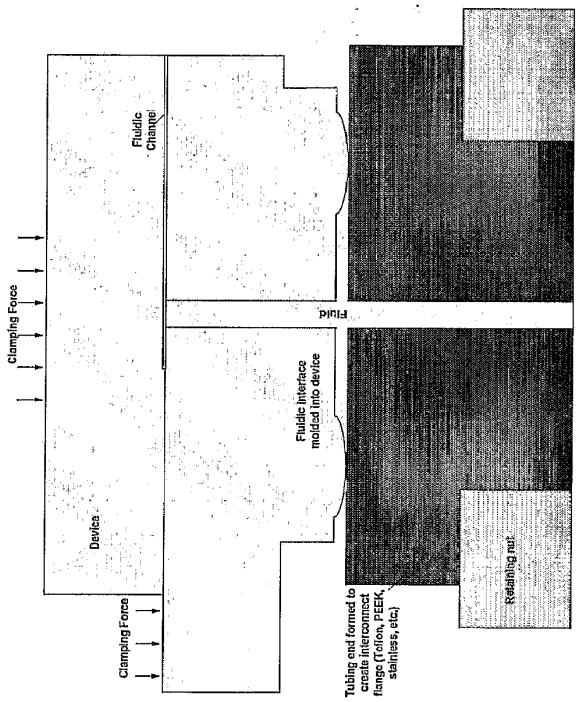

Clamping Force
Fluidic Channel
Fluid
Fluidic interface molded into device
Device
Clamping Force
Tubing end formed to create interconnect flange (Tefzel, PEEK, stainless, etc.)
Retaining nut

Fig. 2C

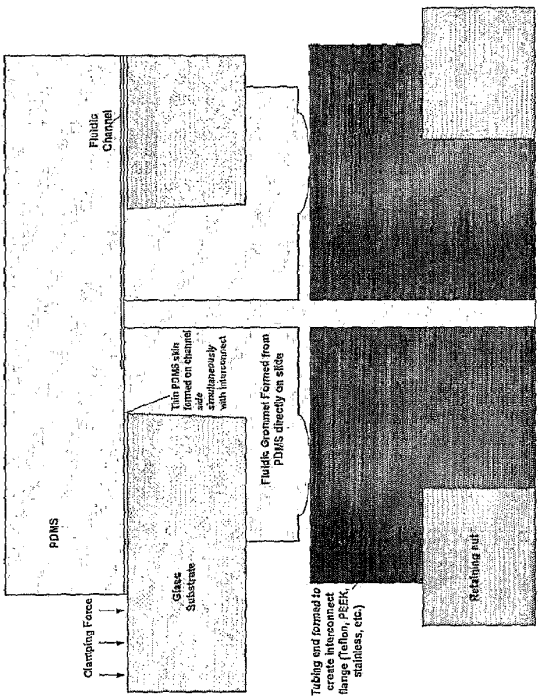

PDMS
Fluidic Channel
Glass Substrate
Clamping Force
Thin PDMS skin formed on channel side simultaneously with interconnect
Fluidic Grommet Formed from PDMS directly on slide
Tubing end formed to create interconnect flange (Teflon, PEEK, stainless, etc.)
Retaining nut

Fig. 2D

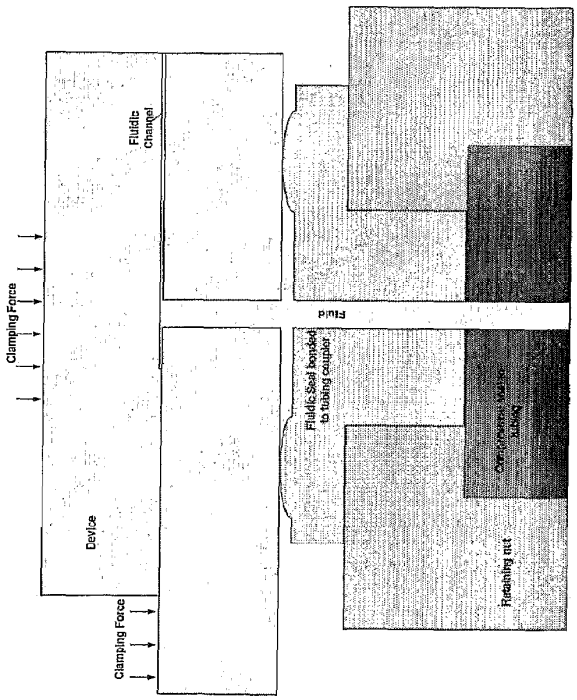

Clamping Force
Fluidic Channel
Device
Clamping Force
Fluid
Fluidic Seal bonded to tubing coupler
Rotating nut

Fig. 2E

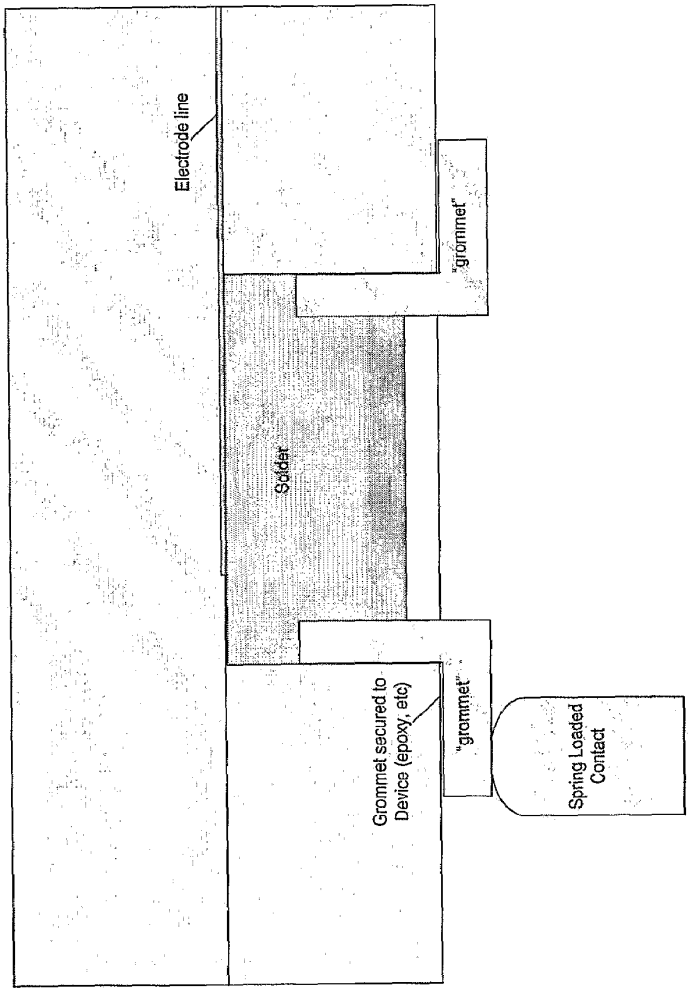

Electrode line
"grommet"
Solder
Grommet secured to Device (epoxy, etc)
"grommet"
Spring Loaded Contact

Fig. 3A

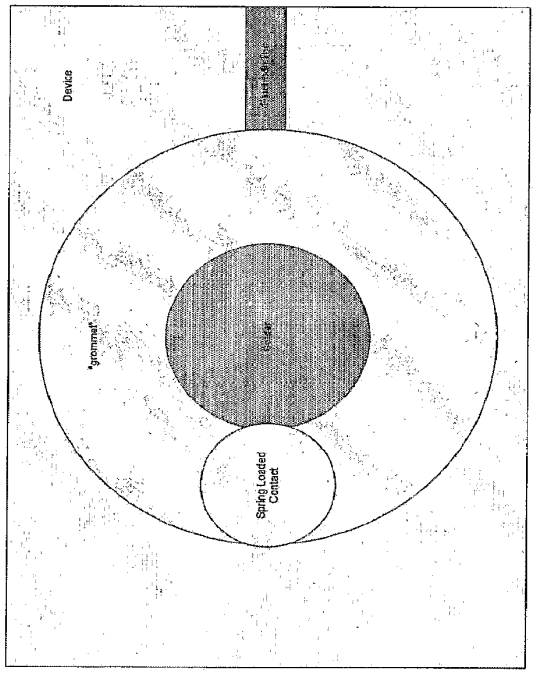


Fig. 3B

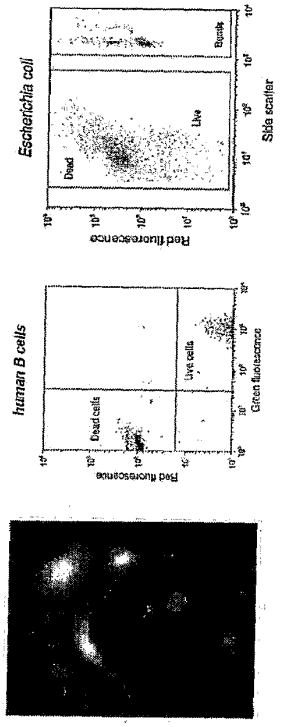

Escherichia coli
Dead
Live
Bursts
Red fluorescence
Side scatter
Human B cells
Dead cells
Live cells
Red fluorescence
Green fluorescence

Fig. 4

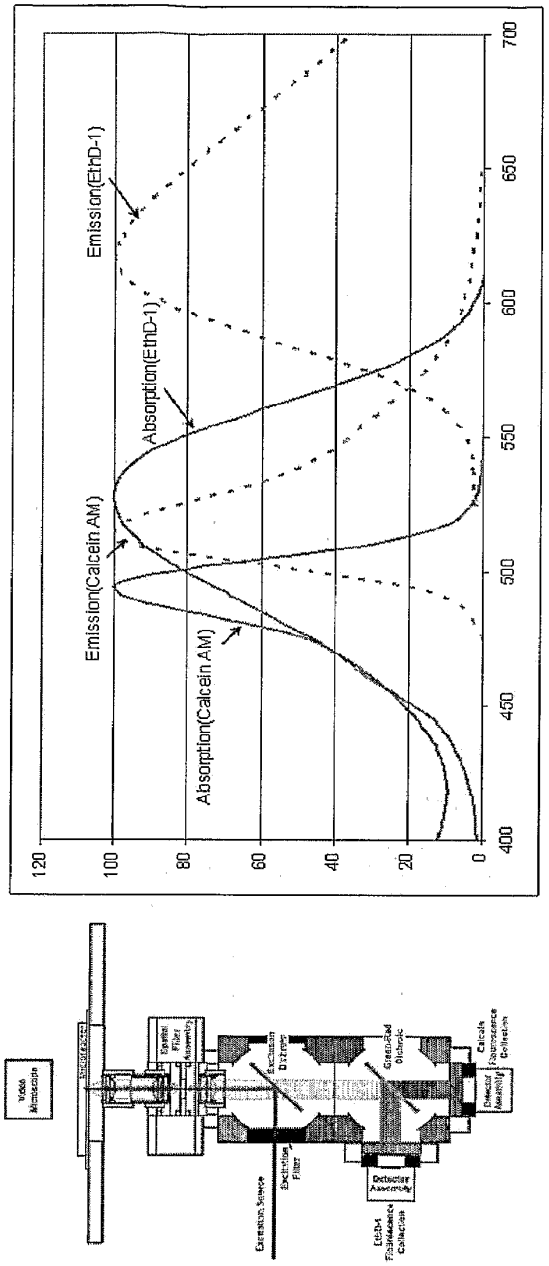

Emission(EthD-1)
Absorption(EthD-1)
Emission(Calcein AM)
Absorption(Calcein AM)
120
100
80
60
40
20
0
400
450
500
550
600
650
700

Fig. 5

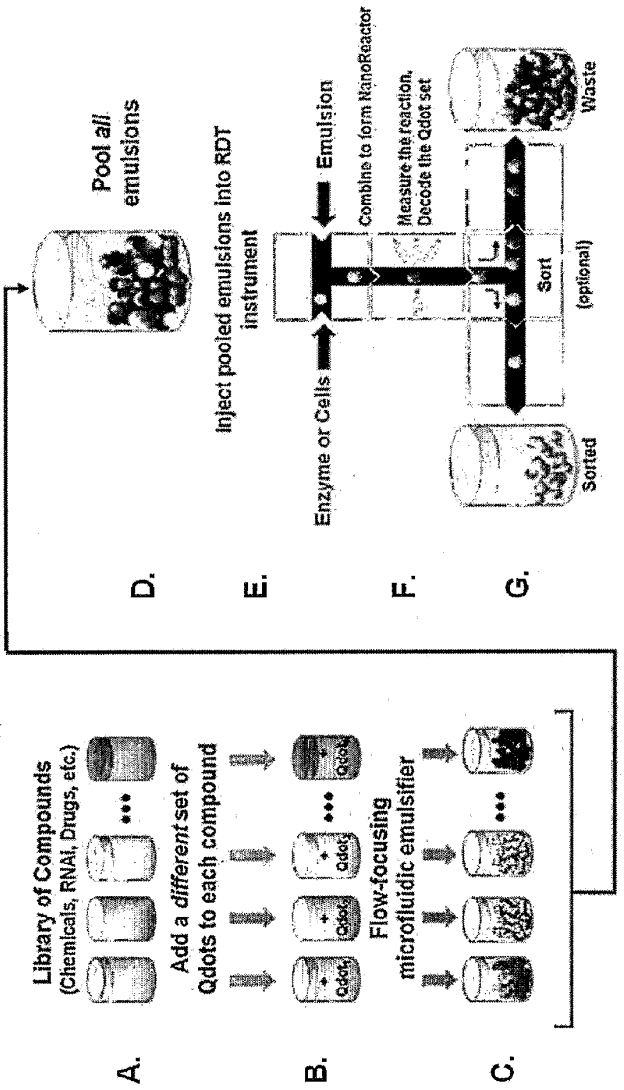

A.
Library of Compounds
(Chemicals, RNAi, Drugs, etc.)
B.
Add a different set of
Qdots to each compound
Qdots
C.
Flow-focusing
microfluidic emulsifier
D.
Pool all
emulsions
E.
Inject pooled emulsions into RDT
instrument
Enzyme or Cells
Emulsion
F.
Combine to form NanoReactor
Measure the reaction,
Decode the Qdot set
G.
Sort
(optional)
Waste
Sorted

Fig. 6

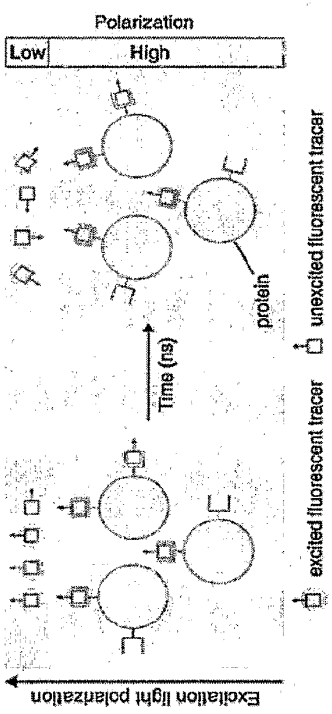

Polarization
Low
High
protein
unexcited fluorescent tracer
Time (ns)
excited fluorescent tracer
Excitation light polarization

Fig. 7

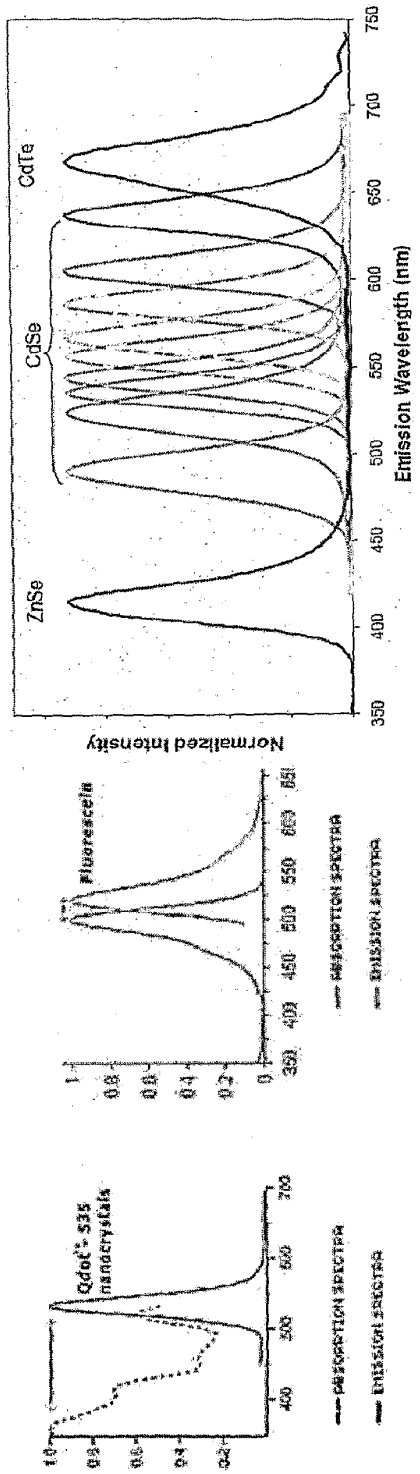

ZnSe
CdSe
CdTe
Normalized Intensity
Emission Wavelength (nm)
Fluorescein
Qdot® 525 nanocrystals
ABSORPTION SPECTRA
EMISSION SPECTRA

Fig. 8

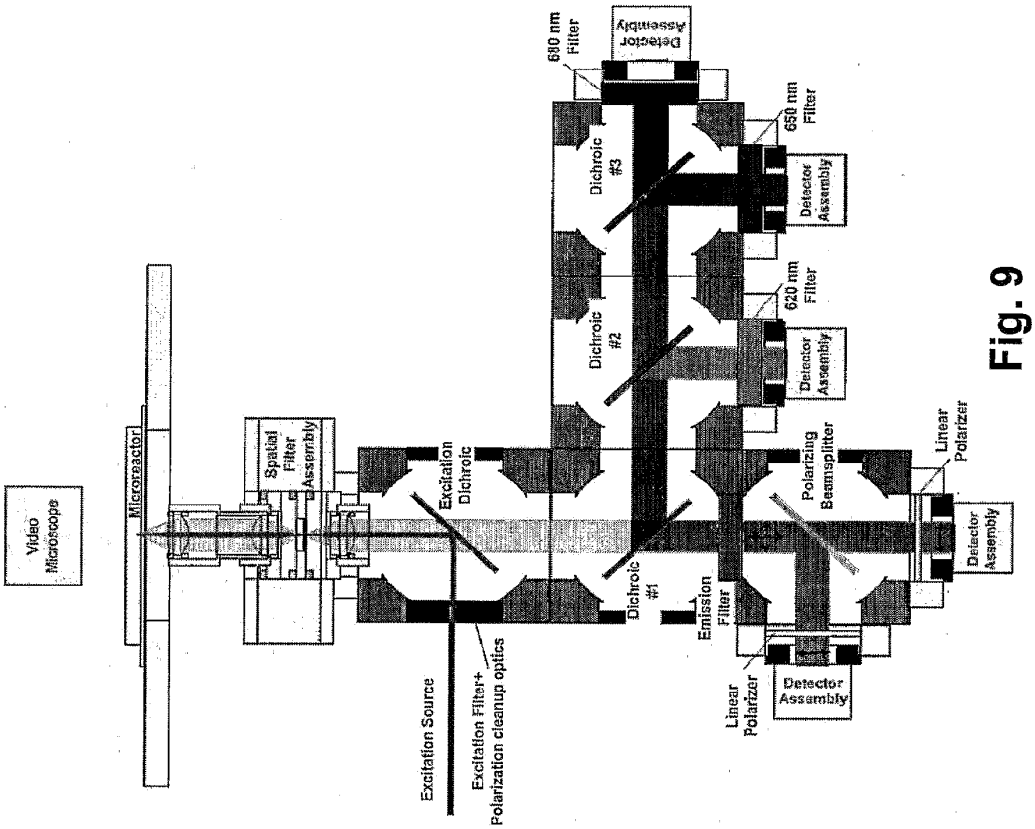

Video Microscope
Microreactor
Spatial Filter Assembly
Excitation Dichroic
Excitation Source
Excitation Filter+ Polarization cleanup optics
Dichroic #1
Emission Filter
Linear Polarizer
Detector Assembly
Polarizing Beamsplitter
Linear Polarizer
Detector Assembly
Dichroic #2
620 nm Filter
Detector Assembly
Dichroic #3
650 nm Filter
Detector Assembly
680 nm Filter

Fig. 9

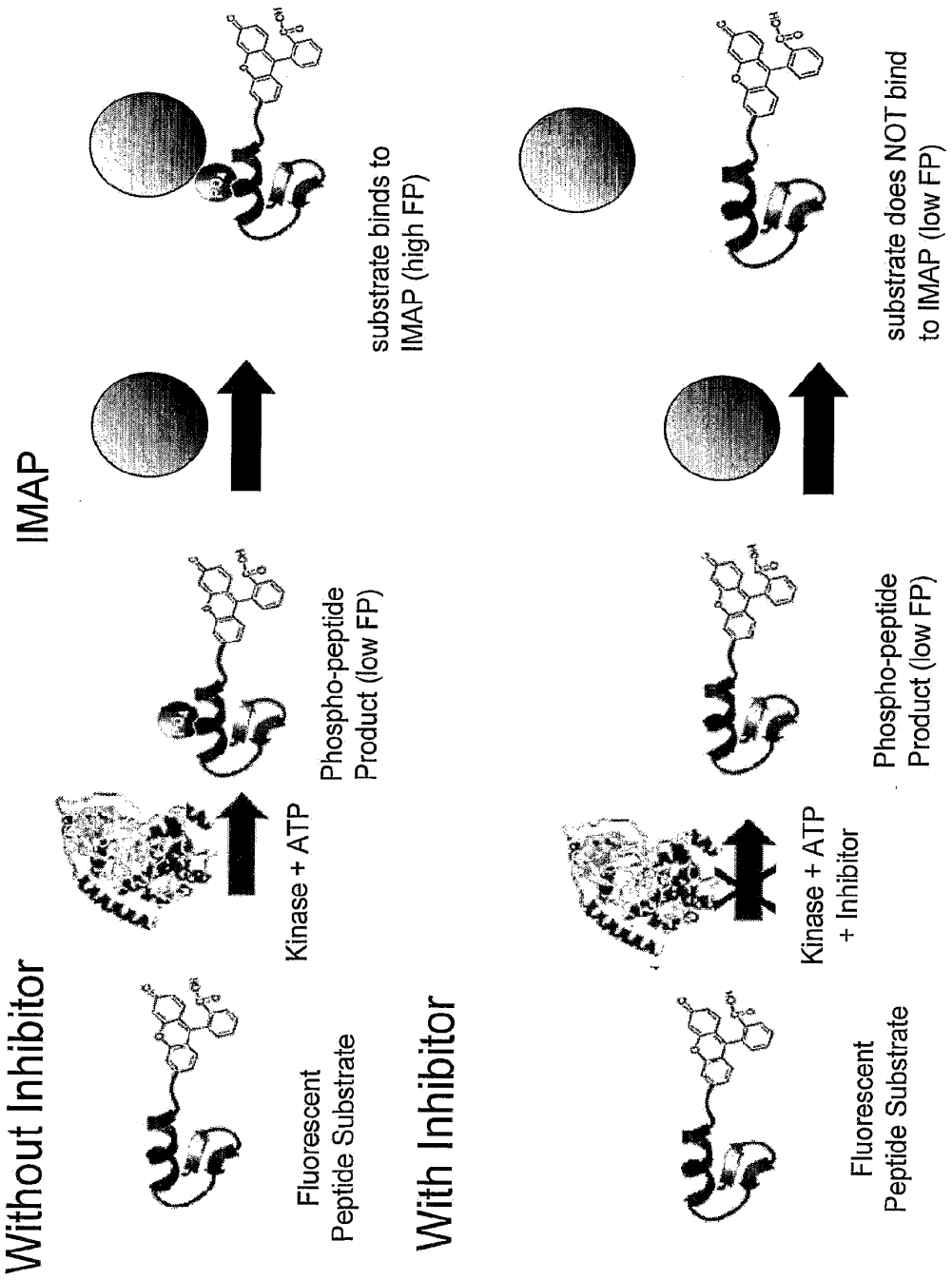

Without Inhibitor
IMAP
Fluorescent
Peptide Substrate
Kinase + ATP
Phospho-peptide
Product (low FP)
substrate binds to
IMAP (high FP)
With Inhibitor
Fluorescent
Peptide Substrate
Kinase + ATP
+ Inhibitor
Phospho-peptide
Product (low FP)
substrate does NOT bind
to IMAP (low FP)

Fig. 10

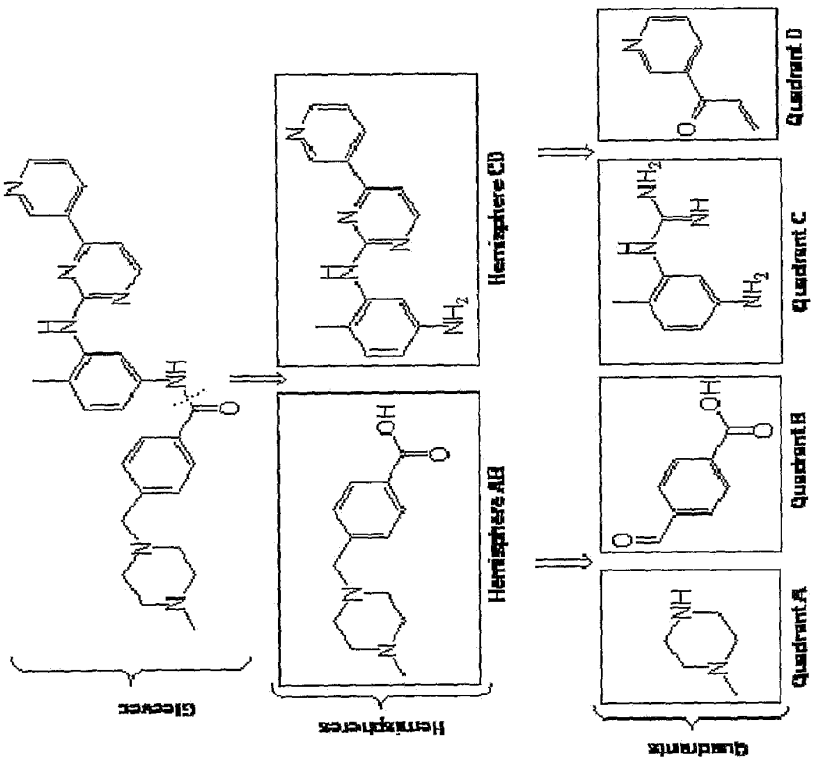
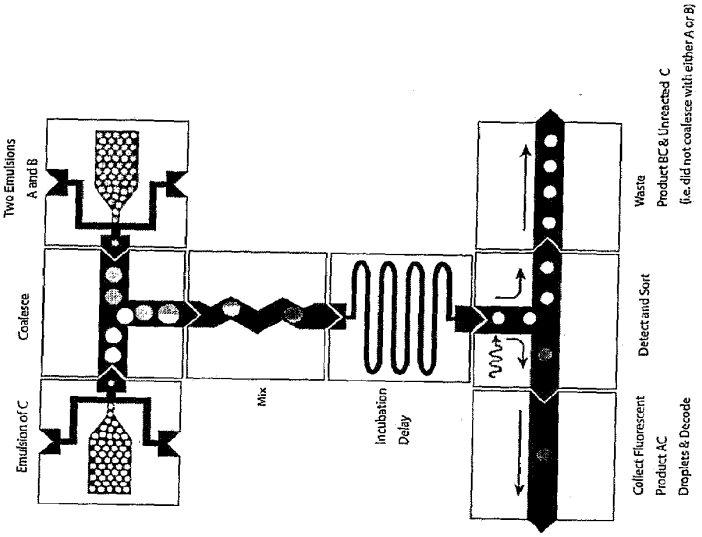

Glycyc
Hemisphere AB
Hemisphere CD
Hemispheres
Quadrant A
Quadrant B
Quadrant C
Quadrant D
Quadrants

Fig. 11


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 (ie. did not coalesce with either A or B)

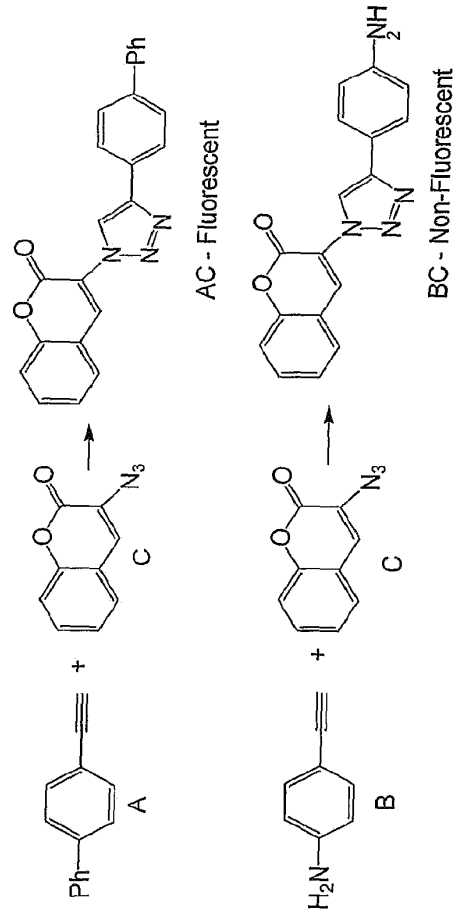

A
B
C
C
AC - Fluorescent
BC - Non-Fluorescent

Fig. 12

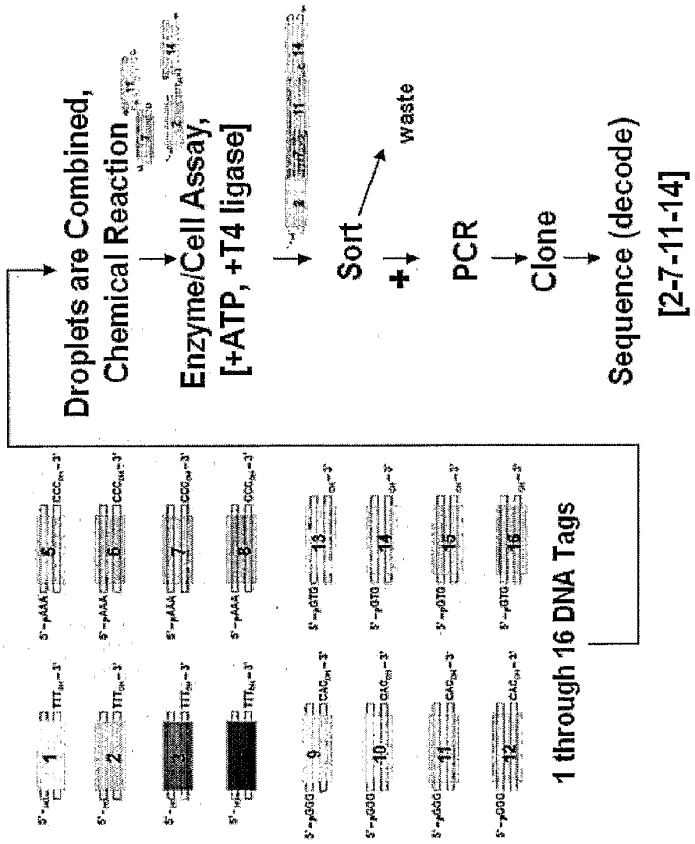

Droplets are Combined,
Chemical Reaction
Enzyme/Cell Assay,
[+ATP, +T4 ligase]
Sort
waste
+
PCR
Clone
Sequence (decode)
[2-7-11-14]
1 through 16 DNA Tags

Fig. 13

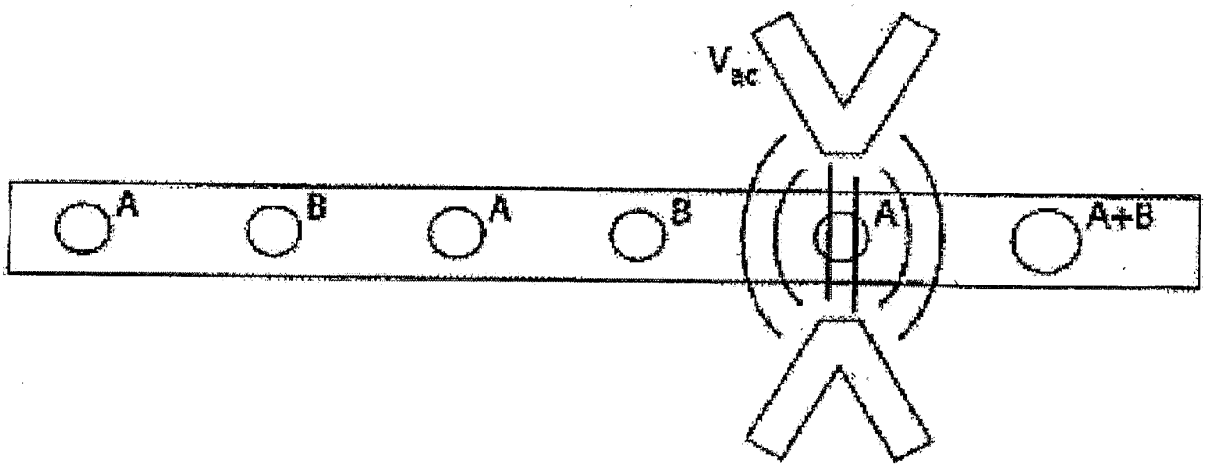


Fig. 14A

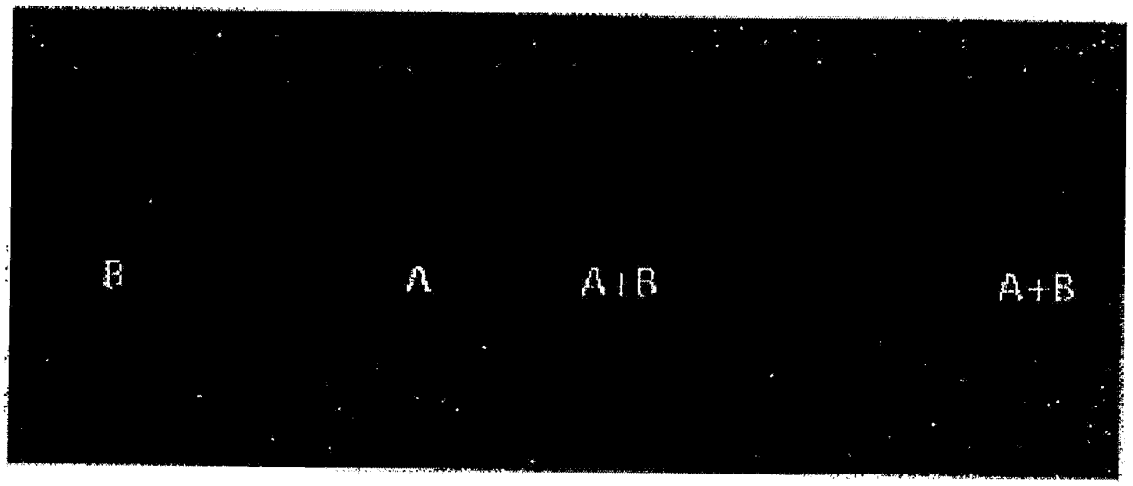


Fig. 14B

Fig. 15A

Fig. 15B

Fig. 15C

Fig. 15D

Fig. 15E

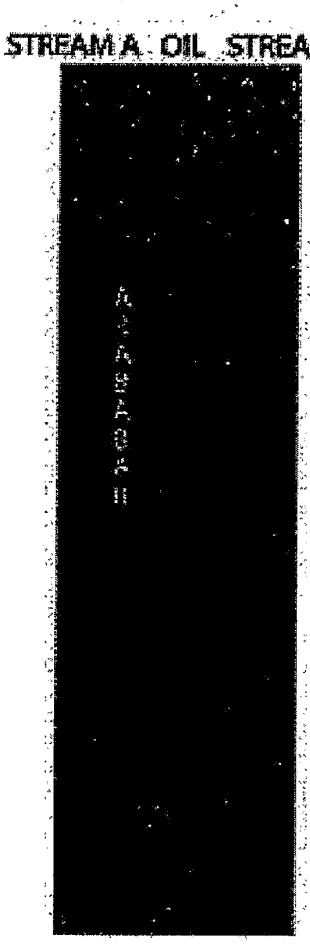


Fig. 16A

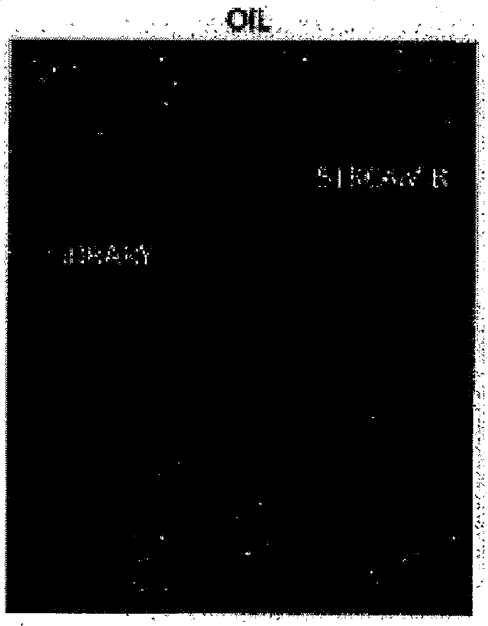


Fig. 16B

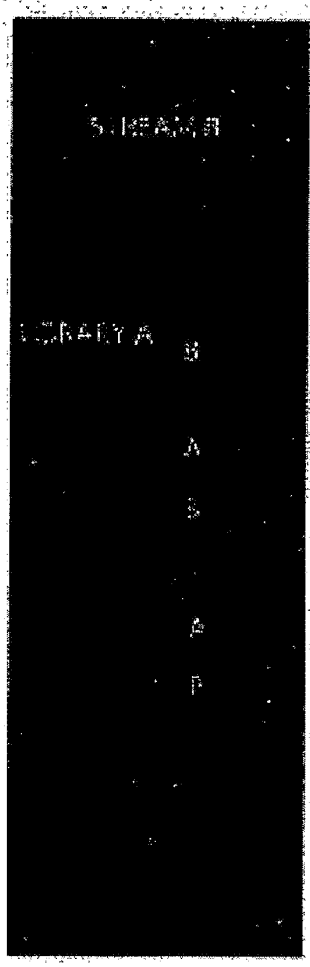
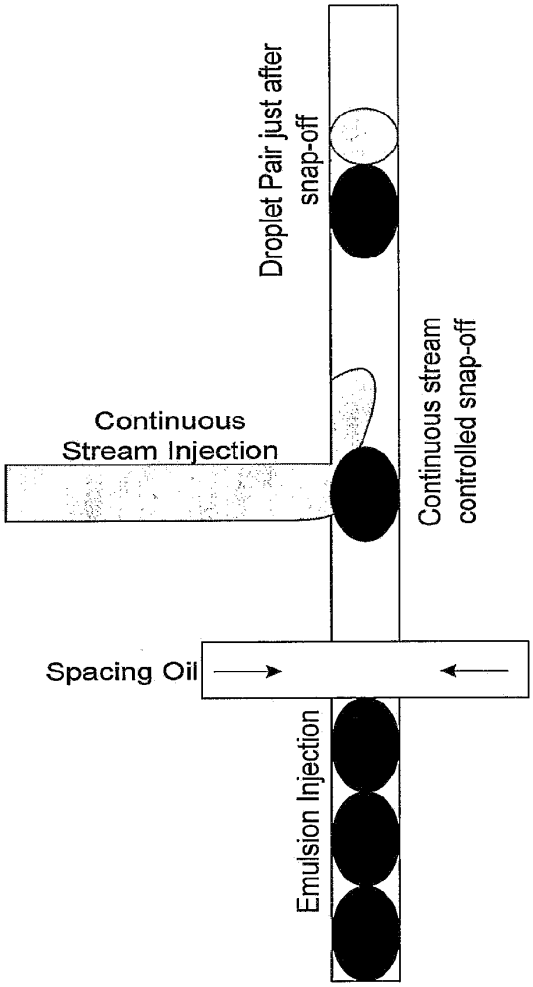


Fig. 16C


Droplet Pair just after snap-off
Continuous Stream Injection
Continuous stream controlled snap-off
Spacing Oil
Emulsion Injection

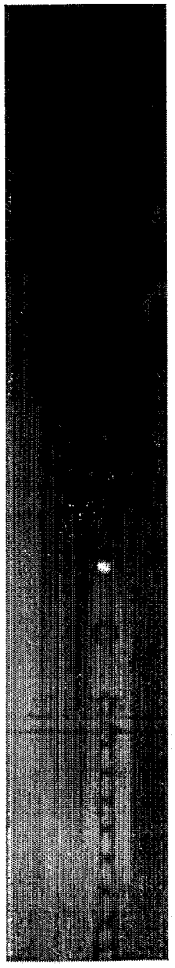


Fig. 16D

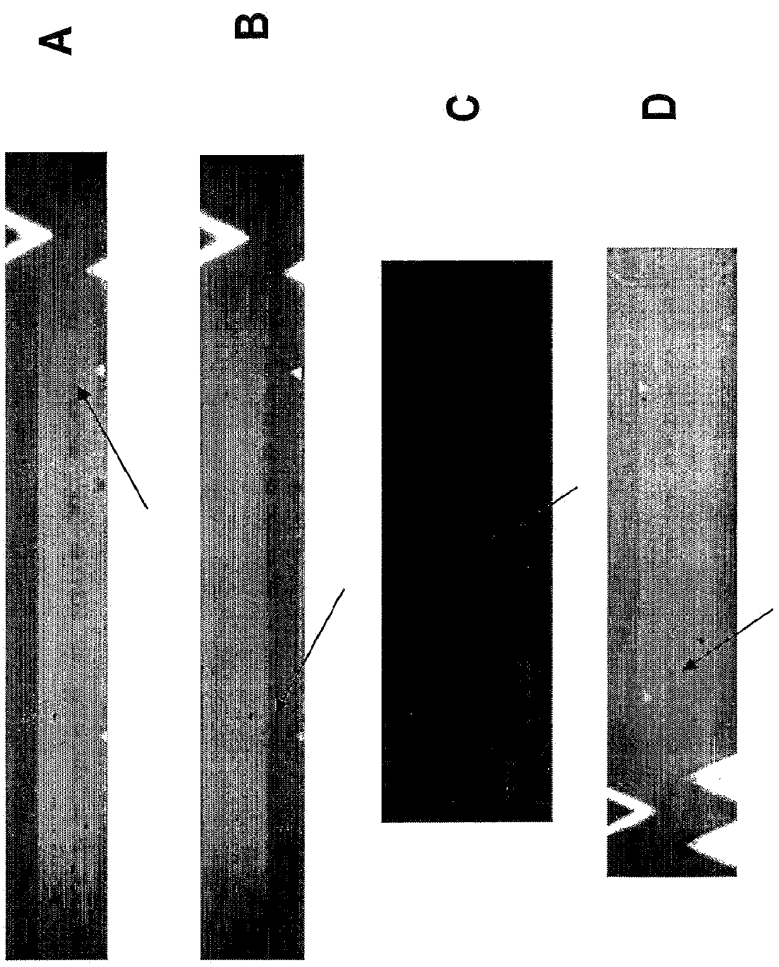


Fig. 17

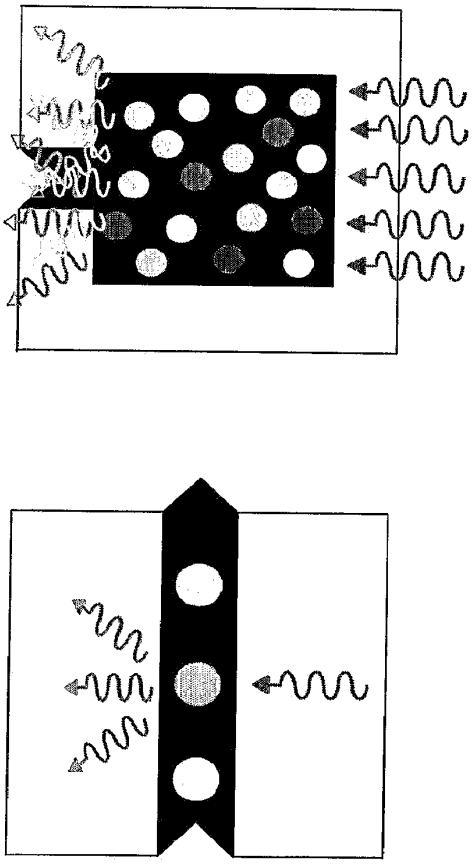

This hvention: Multiple drop fluorescence detection
with unlimited sensitivty
Prior Art: single drop fluorescence detection
with limited sensitivty

Fig. 18

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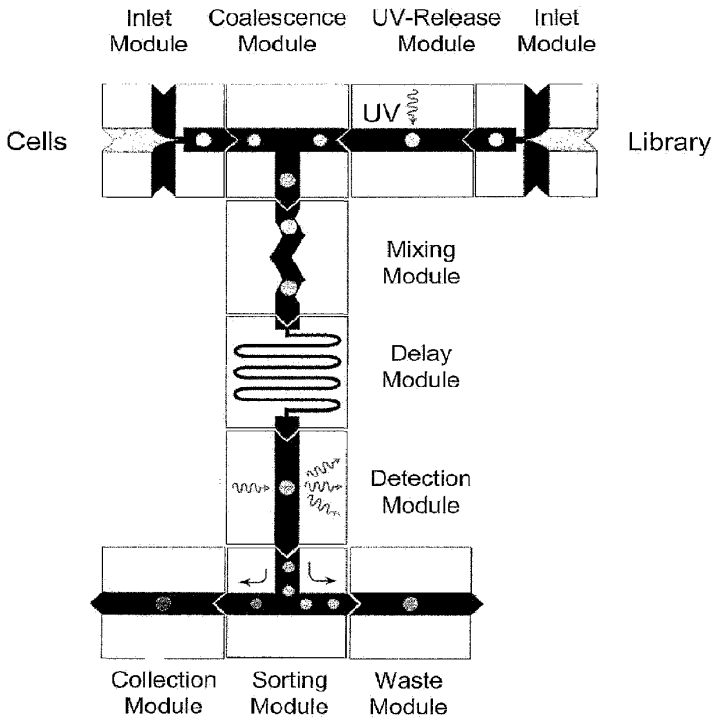
[Continued on next page]

(54) Title: MICROFLUIDIC DEVICES, METHODS OF USE, AND KITS FOR PERFORMING DIAGNOSTICS



(57) Abstract: The present invention provides novel microfluidic devices, kits, and methods that are useful for performing high-throughput screening assays and diagnostics. Such diagnostic methods can include emulsifying an aqueous library of compounds with a set of uniquely dyecoded-labeled droplets in an inert fluorocarbon medium, thereby forming an interactor library, emulsifying an aqueous sample from a subject in an inert fluorocarbon medium, wherein said sample contains a compound that will react with at least one interactor molecule from the interactor library, coalescing the emulsions to form a nanoreactor, and screening the nanoreactors for a desirable reaction between the contents of the nanoreactor, herein one or more steps are performed on a microfluidic device, which can include 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.

WO 2007/081387 A1

Michael [US/US]; 52 Leighton Trail, Guilford, CT 06437 (US). **HINZ, Wolfgang** [DE/US]; 530 Whitfield Street, Guilford, CT 06437 (US). **ROTHBERG, Jonathan, M.** [US/US]; 530 Whitfield Street, Guilford, CT 06437 (US).

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US (patent), UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

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.

MICROFLUIDIC DEVICES, METHODS OF USE, AND KITS FOR PERFORMING DIAGNOSTICS

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.

The invention further provides methods for performing a diagnostic on a microfluidic device. In one embodiment, the method includes emulsifying an aqueous library of compounds with a set of uniquely labeled or dyecoded droplets in an inert fluorocarbon medium (thereby forming an interactor library), emulsifying an aqueous sample from a subject in an inert fluorocarbon medium (such that the sample contains a compound that will react with at least one interactor molecule from the interactor library), combining the emulsions to form a nanoreactor, and screening the nanoreactors for a desirable reaction between the contents of the nanoreactor.

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The library of compounds can include, but is not limited to, antigens to various human allergens, whereas the sample from a subject is serum that contains an antibody to the allergen. The method can further include quantifying the amount of the compound (*e.g.*, antibody) from the subject's sample in the nanoreactor.

The invention still further provides kits for performing the methods described herein. In one embodiment, the kit can include an emulsion library of compounds for performing a diagnostic, wherein each emulsion has been uniquely labeled. The library of compounds can include, for example, antigens to various human allergens. The label can be a liquid label, such as a quantum dot.

In another embodiment, the kit can be provided for synthesizing a compound from two or more reactive substructures. In this embodiment, the kit can include an emulsion library of reactive substructures, wherein each emulsion has been uniquely labeled.

In yet another embodiment, the kit can be provided for sequencing individual exons from individual chromosomes. In this embodiment, the kit can include 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.

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 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.

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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 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-

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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 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

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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 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

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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 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

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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 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

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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.

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.

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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.

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

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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 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

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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.

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

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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 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

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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.

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

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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 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.

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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. 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

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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 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

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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.

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.

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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 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

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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 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

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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 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.

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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 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

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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 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,

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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 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

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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 (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

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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 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

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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.

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

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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 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

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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 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

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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 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

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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 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.

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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 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

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(*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 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

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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 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.

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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 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

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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 reinforce 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 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

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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 electrostatic forces may overcome surface tension to produce a fluid bridge to coalesce and/or neutralize the droplets.

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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 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

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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 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.

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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 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.

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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 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

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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).

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)

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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.

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

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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 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

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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 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.

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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. Afterwards, a low cost circuit can be designed to generate only the optimized waveform in a preferred microfluidic device.

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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 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

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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 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

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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, nucleotides, 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 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

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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 are 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.

The present invention also provides methods of performing proteomics and diagnostics (*e.g.*, to identify markers in serum, or to identify bacteria, viruses, or contaminants either in serum or in solution) utilizing nanoreactors.

A set of dye-labeled (as a specific example through either a direct or indirect attachment of a fluorescein dye) interactors (for example antibodies or almost any other proteins or molecules that bind to another molecule) can be created and separately emulsified with unique droplet dyecodes to create a labeled interactor library wherein the interaction can be monitored by a detection means.

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When the detection means that is done on said interactions is performed in solution it allows one to use fluorescence polarization, which has the advantage of using a generic means for measuring the interaction, as opposed to methods that are performed on a solid phase (for example a glass slide or bead), which generally require a sandwich ELISA-type format. In a proteomics-type analysis, it means that every binding-spot on a glass is performed in a droplet instead of on the chip, and FP is used to determine the level of interaction. As opposed to mass spectrophotometry, this method allows the use of proteins, peptides, and nucleic acids at the same time.

Using protein-protein configured enzyme reactions (as for example lactamase, beta gal, yeast transcription factor, etc) to boost signal in a droplet as a surrogate for interaction prior to detection is also possible.

For example, a serum from a patient is emulsified either on or off the device according to the present invention and said serum droplets are combined on the device with a droplet dyecode-labeled library of interacting proteins, nucleic acids, allergens, small-molecule, etc., and a means of detecting an interaction used to determine if an interactor molecule is interacting with a component of the serum (a specific example of a detection being fluorescence polarization of the dye-labeled interactor combined droplets).

In a specific example, a library of fluorescein-labeled antibodies to human pathogens (for example E.coli strain O, Staphylococcus, AIDS virus, etc) are each separately emulsified in their own uniquely dyecode-labeled droplets. A patient's blood sample is separately emulsified either on or off the machine and combined with the library. FP is used to detect the presence of, (and quantify) the amount of a particular pathogen in a particular droplet.

In a further example, a library of fluorescein-labeled oligonucleotide specific to human pathogens (for example E.coli strain O, Staphylococcus, AIDS virus, etc) are each separately emulsified in their own uniquely dyecode-labeled droplets. A patient's blood sample is separately emulsified either on or off the machine and combined with the library. FP is used to detect the presence of, (and quantify) the amount of a particular pathogen's DNA in a particular droplet.

In yet another example, a library of fluorescein-labeled oligonucleotide specific to human SNPs or other DNA sequence(s) of interest (for example individual cDNAs, CDR sequences from a pre-defined CDR library, etc) are each separately emulsified in their own uniquely dyecode-labeled droplets. A PCR product is separately emulsified either on or off the machine and combined with the library. FP is used to detect the presence of, (and quantify if needed) the amount of a particular DNAsequence in a particular droplet.

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In still another example, a library of fluorescein-labeled mouse monoclonal anti-cytokine antibodies are each separately emulsified in their own uniquely dyecode-labeled droplets. A patient's blood sample is separately emulsified either on or off the machine and combined with the cytokine library. FP is used to detect the presence of, and quantify the amount of a particular cytokine in a particular droplet.

As an example of using the device according to the present invention in a proteomics analysis, a library of fluorescein-labeled monoclonal antibodies to all human proteins (or subset thereof) are each separately emulsified in their own uniquely dyecode-labeled droplets. A tissue or paraffin-embedded sample or a patient's blood sample is separately emulsified either on or off the machine and combined with the library. FP is used to detect the presence of, and quantify the amount of a particular protein in a particular droplet.

In another example, a library of a set of appropriate fluorescein-labeled antibodies to human food-borne pathogens or set of antibodies used for other testing (for example E.coli strain O, Staphylococcus, Salmonella, etc) are each separately emulsified in their own uniquely dyecode-labeled droplets. A food-stuff (for example hamburger, genetically modified organism, etc.) sample is separately emulsified either on or off the machine and combined with the library. FP is used to detect the presence of, (and quantify) the amount of a particular pathogen in a particular droplet.

The device of the present invention can also be used for allergy-testing, wherein a library of a set of appropriate fluorescein-labeled antigens to various common human allergens are each separately emulsified in their own uniquely dyecode-labeled droplets. A patients serum sample is separately emulsified either on or off the machine and combined with the library. FP is used to detect the presence of, (and quantify) the amount of a particular antibody derived from the patient's serum in a particular droplet.

The device of the present invention can also be used to perform an 'inverted assay,' corresponding to a high-throughput automated format for doing protein 'slot-blots' on thousands of normal and/or diseased tissue samples in solution. In such a method, a library of potentially several thousand unlabeled solubilized human tissue samples or serum samples are each separately emulsified in their own uniquely dyecode-labeled droplets. An either directly or indirectly-labeled antibody against an antigen of interest is separately emulsified either on or off the machine and combined with the library. FP is used to detect the presence of, and quantify the amount of a particular protein in a particular droplet.

As another example of an 'inverted assay,' a library of a sub-cellular fractionated solubilized human tissue samples or serum samples are each separately emulsified in their own uniquely

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dyecode-labeled droplets. An either directly or indirectly-labeled antibody against an antigen of interest is separately emulsified either on or off the machine and combined with the library. FP is used to detect the presence of, and quantify the amount of a particular protein in a particular droplet.

Example 10.

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-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 11.

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.

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Example 12.

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 together becomes active) can be attached either covalently or through a reasonably strong interaction.

Example 13.

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

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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 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.

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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 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

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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.

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

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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 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.

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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 for performing a diagnostic, the method comprising:

a) emulsifying an aqueous library of compounds with a set of uniquely dyecoded-labeled droplets in an inert fluorocarbon medium, thereby forming an interactor library;

b) emulsifying an aqueous sample from a subject in an inert fluorocarbon medium, wherein said sample contains a compound that will react with at least one interactor molecule from the interactor library;

c) coalescing the emulsions prepared in steps (a) and (b) to form a nanoreactor; and

d) screening the nanoreactors for a desirable reaction between the contents of the nanoreactor,

wherein one or more steps of (a) - (d) are performed on a microfluidic device.

2. The method of claim 1, wherein the library of compounds comprises antigens to various human allergens.

3. The method of claim 1, wherein the sample from a subject is serum.

4. The method of claim 1, wherein said screening step is performed by fluorescent polarization.

5. The method of claim 1 further comprising quantifying the amount of the compound in the nanoreactor.

6. A kit for performing the method of claim 1, the kit comprising an emulsion library of compounds, wherein each emulsion has been uniquely labeled.

7. The kit of claim 6, wherein the library of compounds comprises antigens to various human allergens.

8. The kit of claim 6, wherein the label is a liquid label.

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9. The kit of claim 8, wherein the liquid label is a quantum dot.

10. A kit for synthesizing a compound from two or more reactive substructures, the kit comprising an emulsion library of reactive substructures, wherein each emulsion has been uniquely labeled.

11. A kit for liquid labeling a library of compounds, the kit comprising an emulsionof unique liquid labels.

12. A kit for sequencing individual exons from individual chromosomes, 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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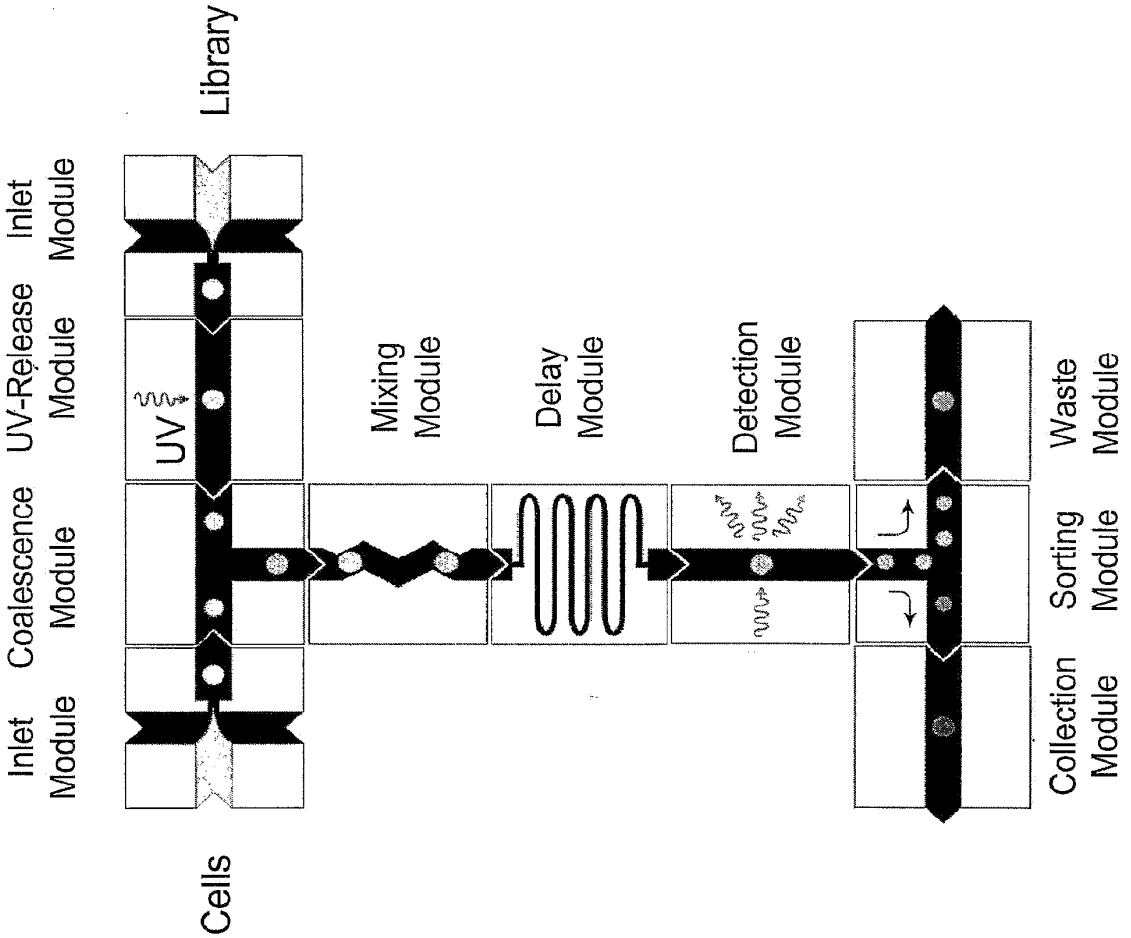

Inlet Module
Coalescence Module
UV-Release Module
Inlet Module
Cells
UV
Library
Mixing Module
Delay Module
Detection Module
Collection Module
Sorting Module
Waste Module

Fig. 1

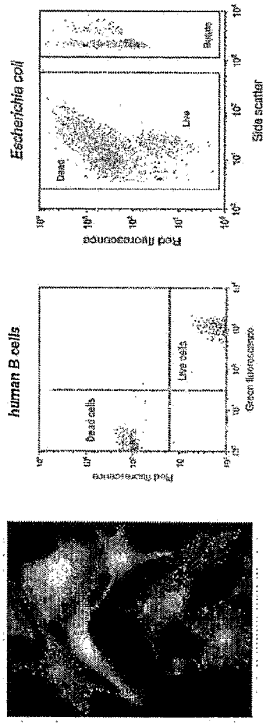

Escherichia coli
Dead
Live
Beads
Side scatter
human B cells
Dead cells
Live cells
Green fluorescence

Fig. 2

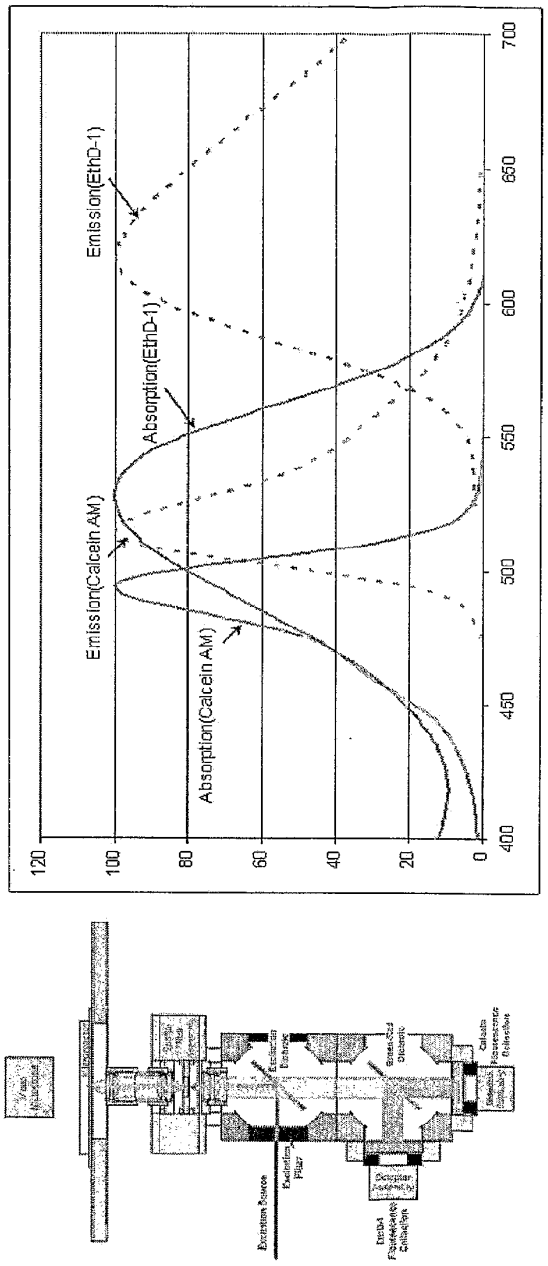

Emission(EthD-1)
Absorption(EthD-1)
Emission(Calcein AM)
Absorption(Calcein AM)
120
100
80
60
40
20
0
400
450
500
550
600
650
700

Fig. 3

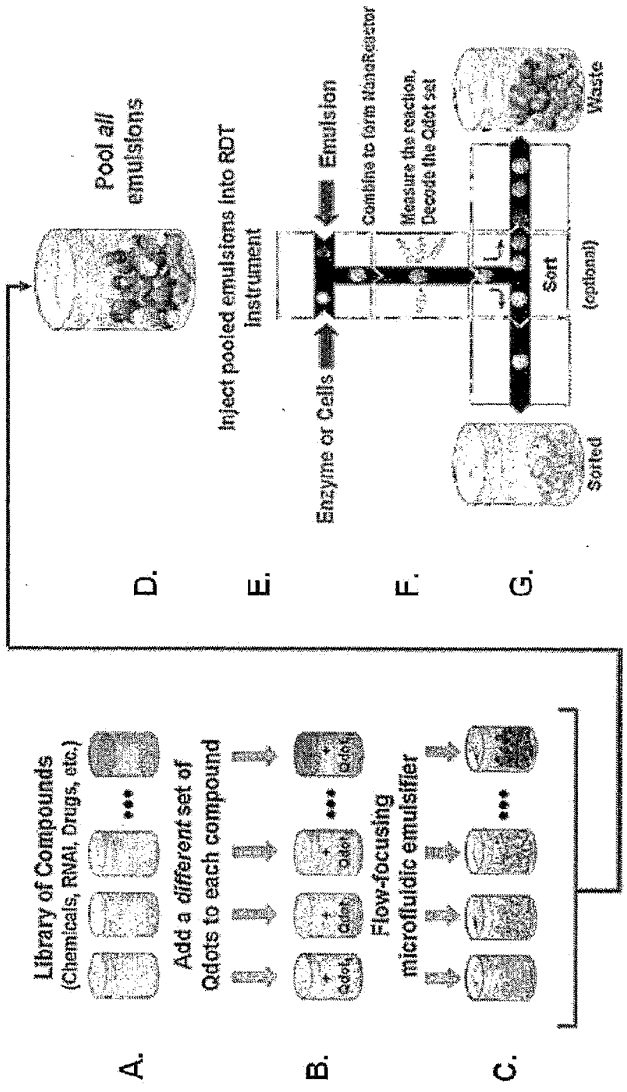

A.
Library of Compounds
(Chemicals, RNAi, Drugs, etc.)
B.
Add a different set of
Qdots to each compound
C.
Flow-focusing
microfluidic emulsifier
D.
Pool all
emulsions
E.
Inject pooled emulsions into RDT
Instrument
Enzyme or Cells
Emulsion
F.
Combine to form NanoReactor
Measure the reaction,
Decode the Qdot set
G.
Sort
(optional)
Waste
Sorted

Fig. 4

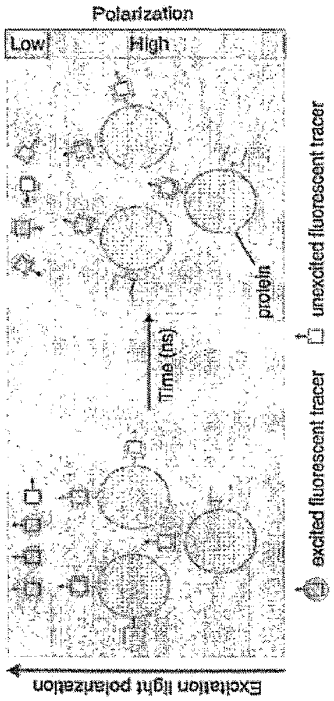
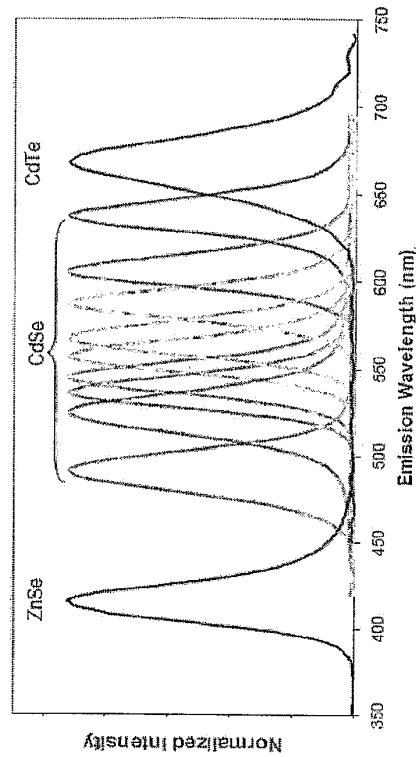
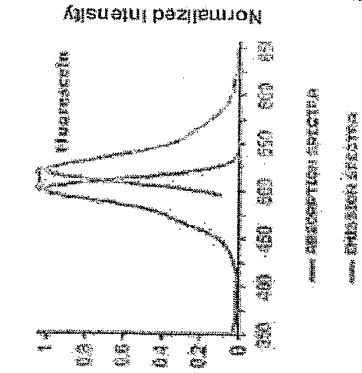

Polarization
Low
High
protein
unexcited fluorescent tracer
Time (ns)
excited fluorescent tracer
Excitation light polarization

Fig. 5


ZnSe
CdSe
CdTe
Normalized Intensity
Emission Wavelength (nm)
350
400
450
500
550
600
650
700
750



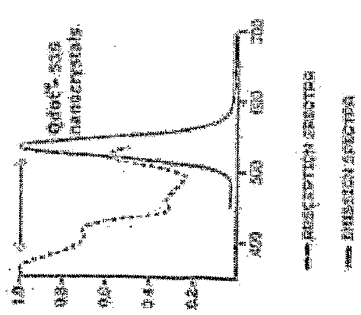


Fig. 6

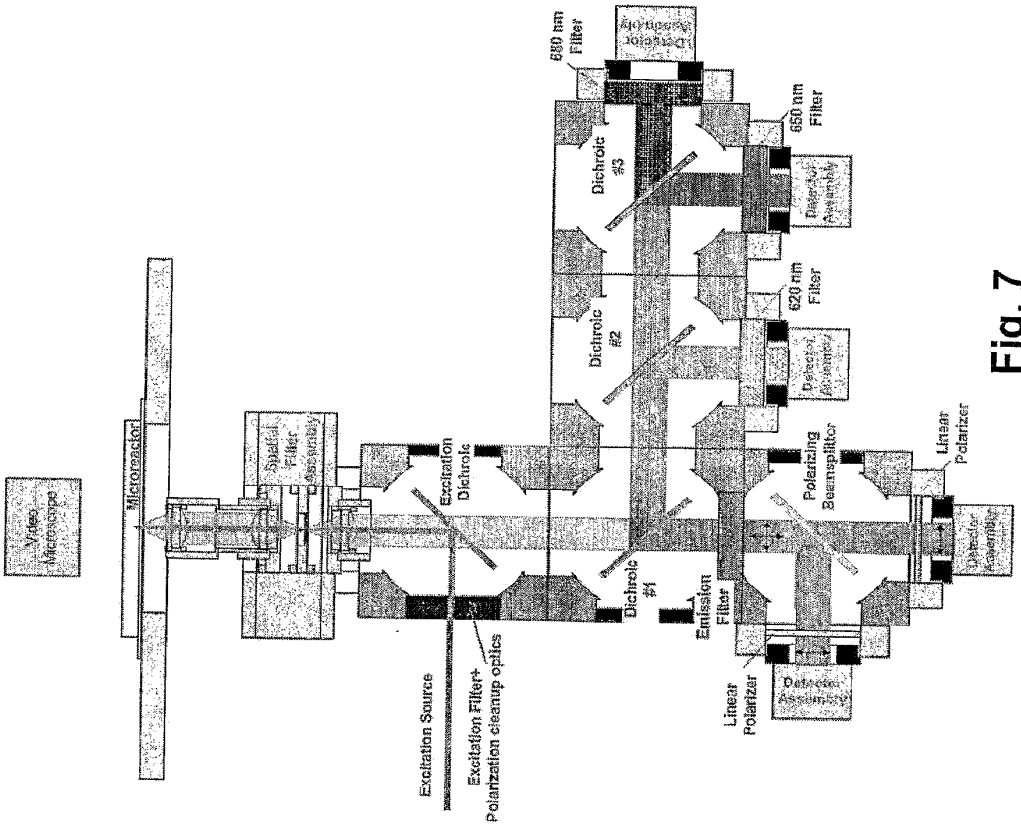

Video Microscope
Microreactor
Spatial Filter Assembly
Excitation Dichroic
Excitation Source
Excitation Filter+ Polarization cleanup optics
Dichroic #1
Dichroic #2
Dichroic #3
680 nm Filter
650 nm Filter
620 nm Filter
Emission Filter
Linear Polarizer
Polarizing Beamsplitter
Linear Polarizer
Detector Assembly
Detector Assembly
Detector Assembly
Detector Assembly

Fig. 7

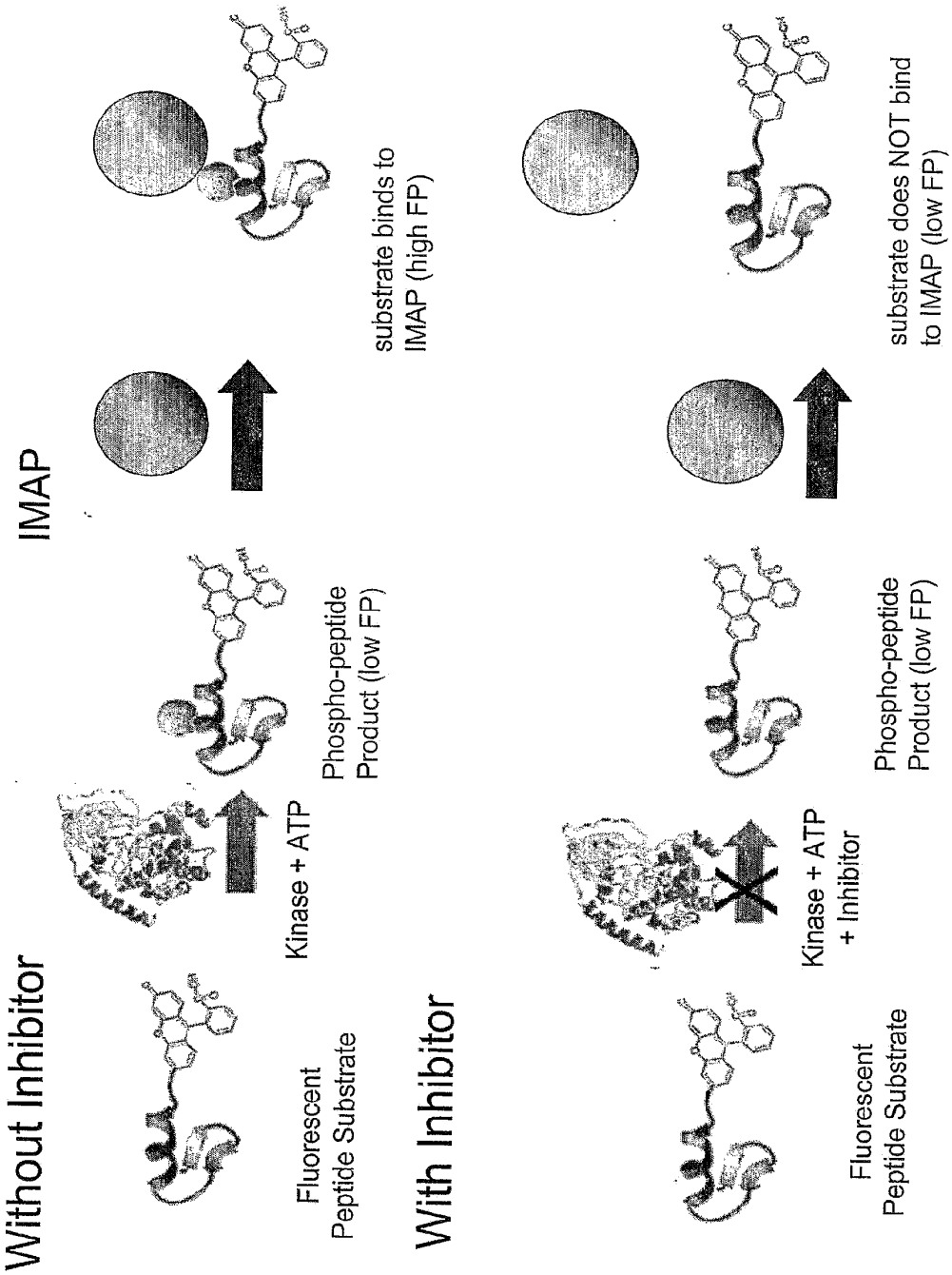

Without Inhibitor
IMAP
Fluorescent
Peptide Substrate
Kinase + ATP
Phospho-peptide
Product (low FP)
substrate binds to
IMAP (high FP)
With Inhibitor
Fluorescent
Peptide Substrate
Kinase + ATP
+ Inhibitor
Phospho-peptide
Product (low FP)
substrate does NOT bind
to IMAP (low FP)

Fig. 8

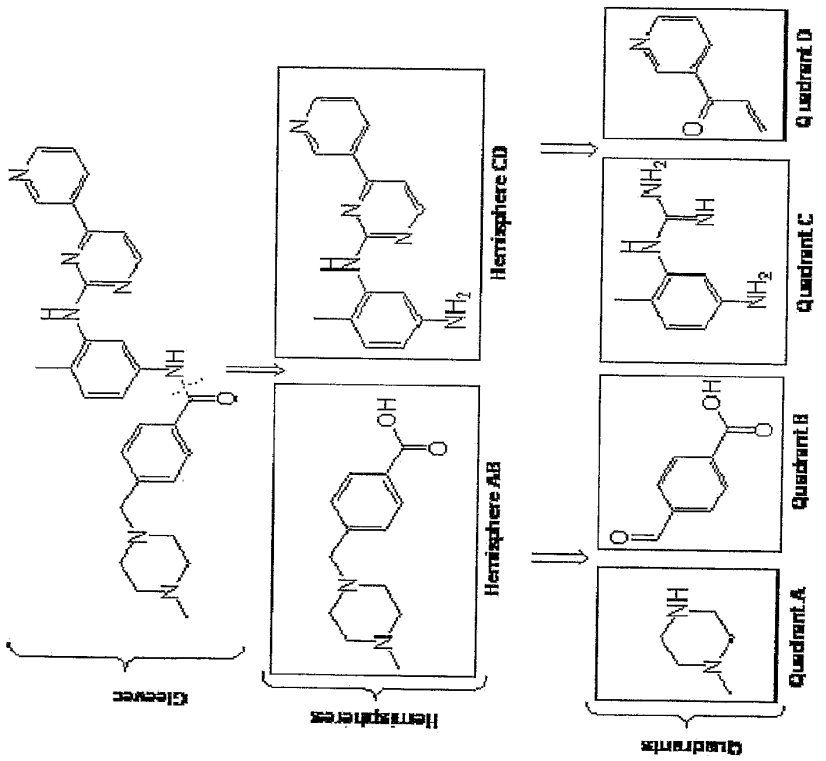

Glecaprevir
Hemispheres
Hemisphere AB
Hemisphere CD
Quadrants
Quadrant A
Quadrant B
Quadrant C
Quadrant D

Fig. 9

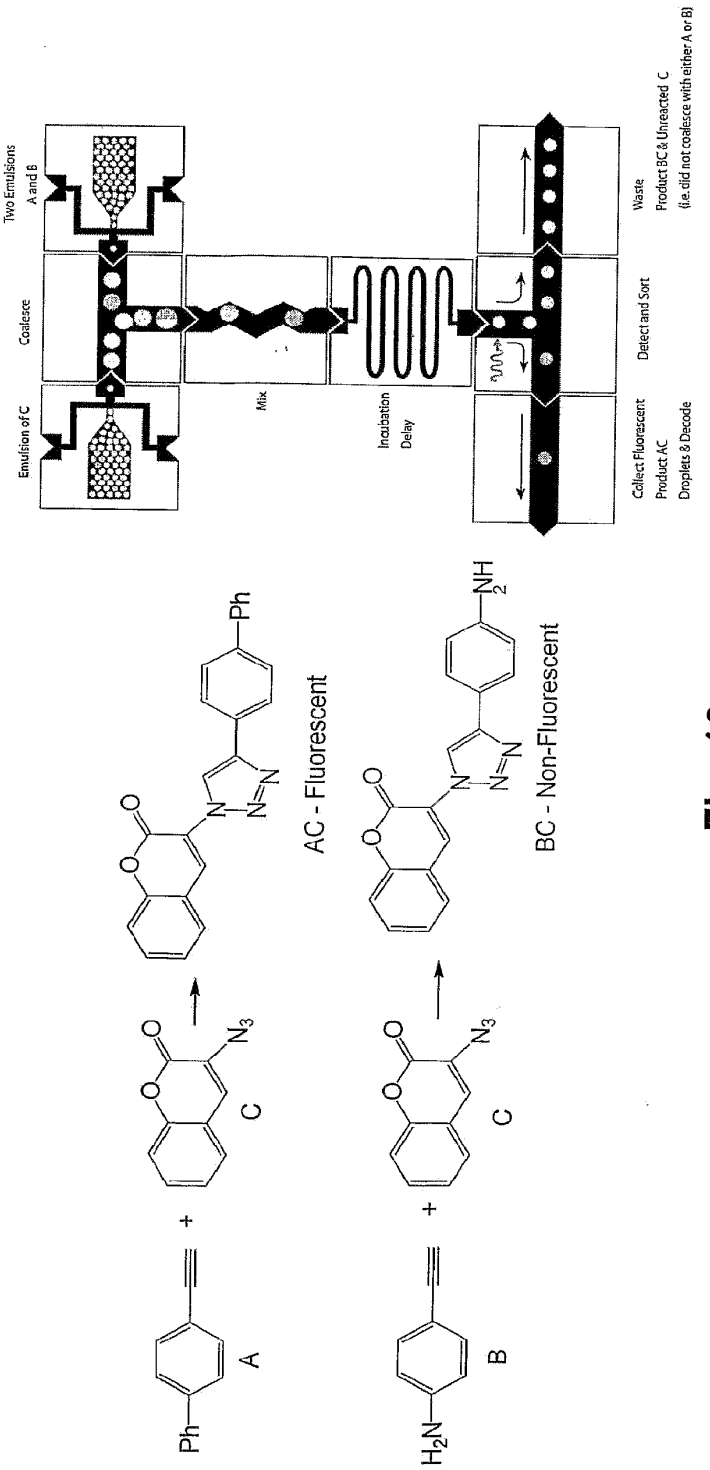

Two Emulsions A and B
Coalesce
Emulsion of C
Mix
Incubation Delay
Detect and Sort
Collect Fluorescent Product AC Droplets & Decode
Waste
Product BC & Unreacted C (i.e. did not coalesce with either A or B)
AC - Fluorescent
BC - Non-Fluorescent
A
B
C

Fig. 10

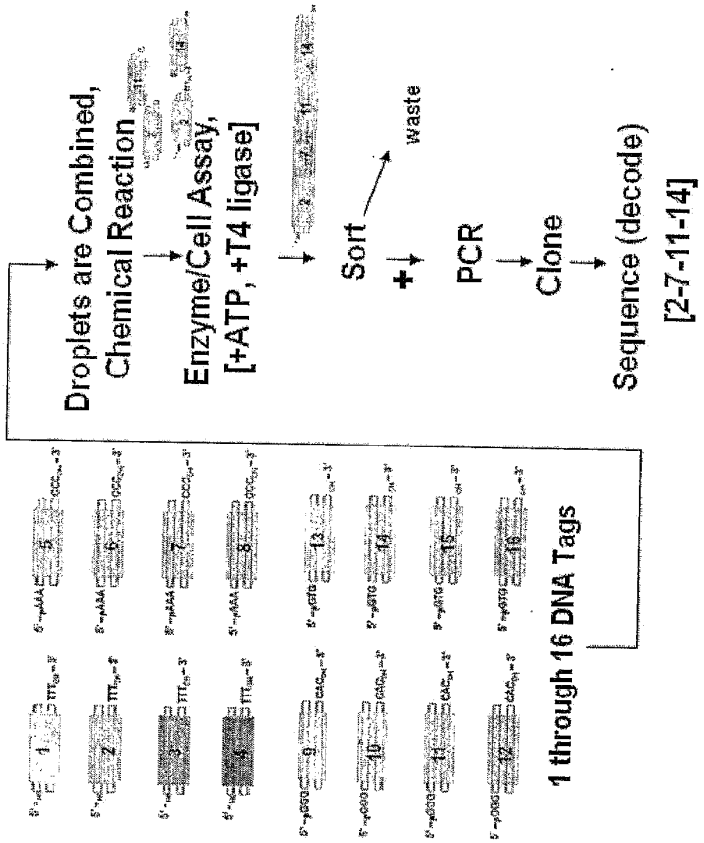

1 through 16 DNA Tags
Droplets are Combined, Chemical Reaction
Enzyme/Cell Assay, [+ATP, +T4 ligase]
Sort
Waste
+
PCR
Clone
Sequence (decode) [2-7-11-14]

Fig. 11

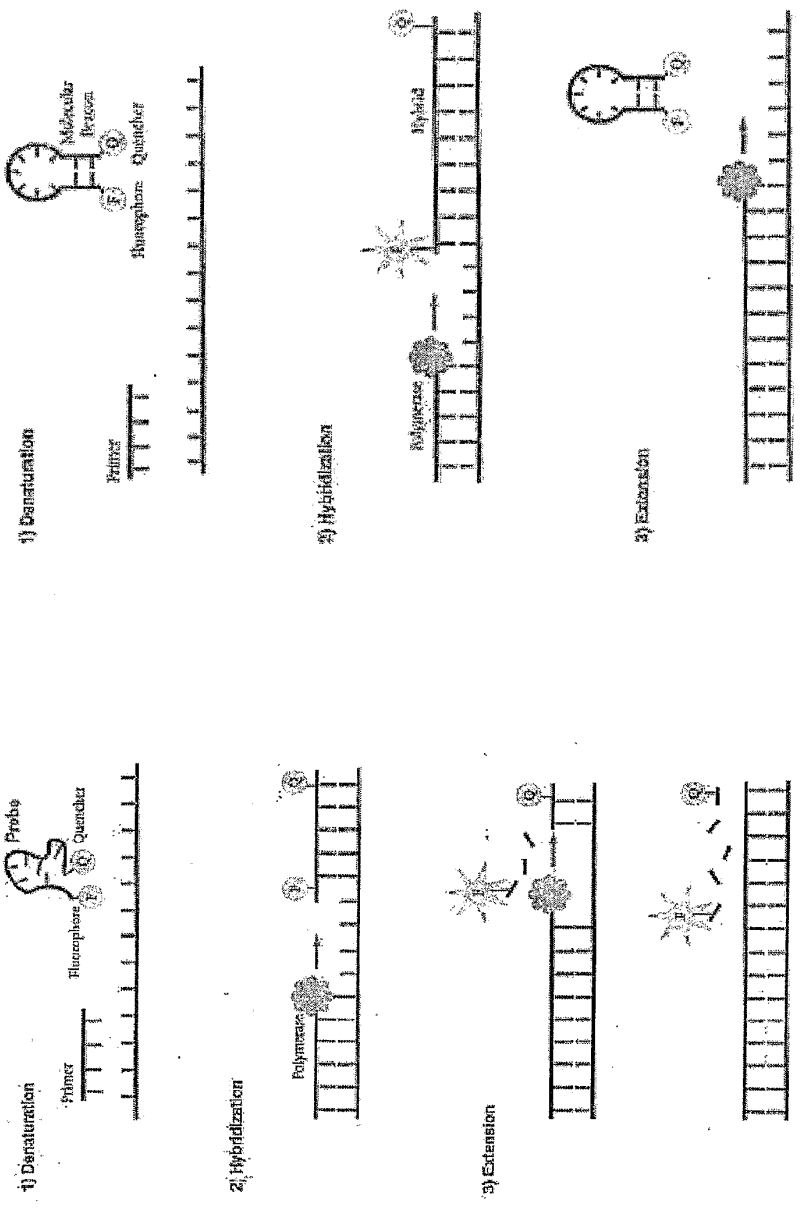

1) Denaturation
Molecular Beacon
Fluorophore
Quencher
Primer
2) Hybridization
Polymerase
Hybrid
3) Extension
1) Denaturation
Probe
Quencher
Fluorophore
Primer
2) Hybridization
Polymerase
3) Extension

Fig. 12

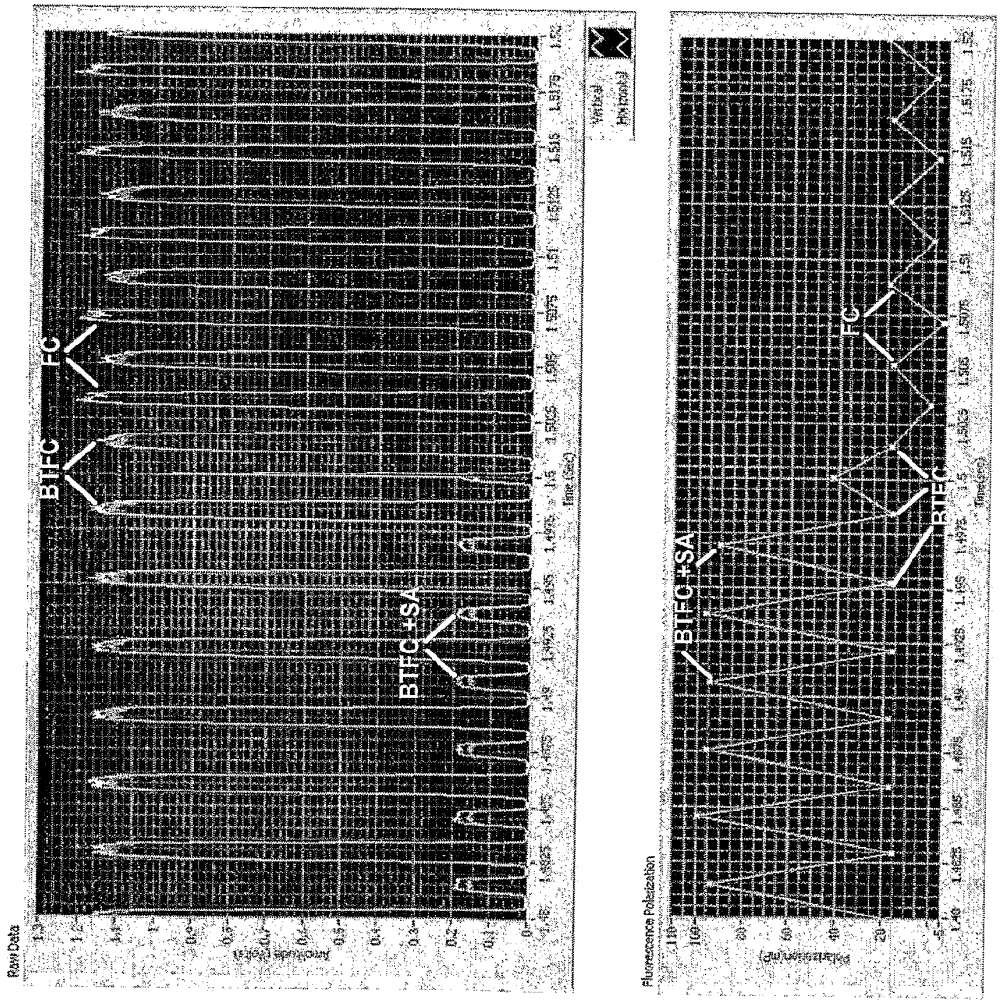

Raw Data
BTFC
FC
BTFC+SA
Vertical
Horizontal
Time (sec)
Fluorescence Polarization
BTFC+SA
FC
BTFC

Fig. 13

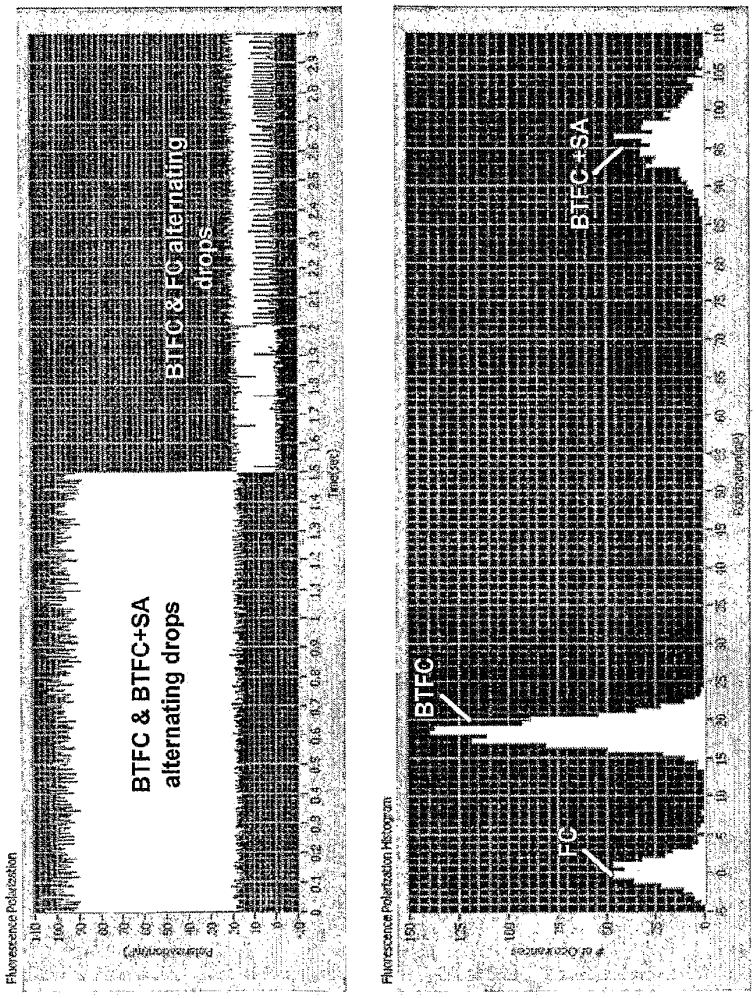

Fluorescence Polarization
BTFC & BTFC+SA
alternating drops
BTFC & FC alternating
drops
Fluorescence Polarization Histogram
BTFC
FC
BTFC+SA

Fig. 14

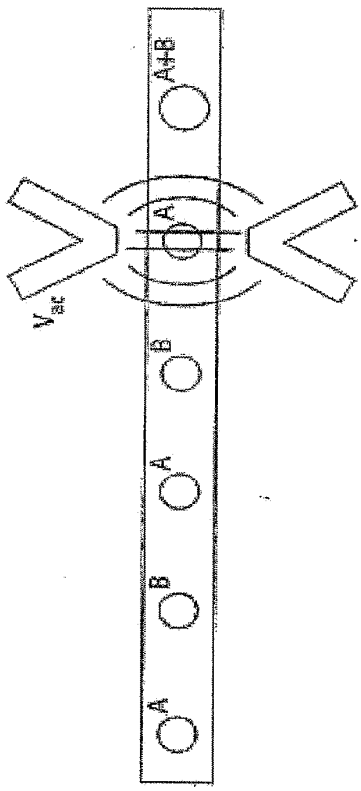


Fig. 15A

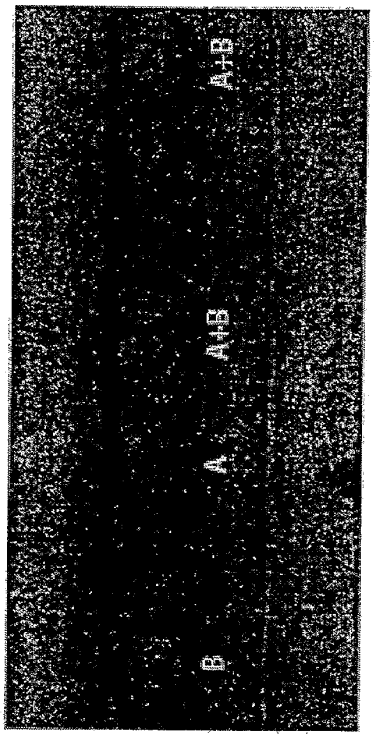


Fig. 15B

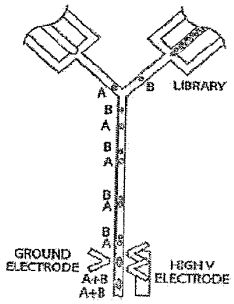


Fig. 16A

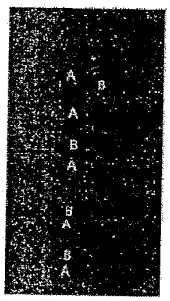


Fig. 16B

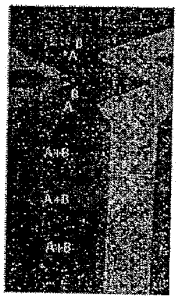


Fig. 16C

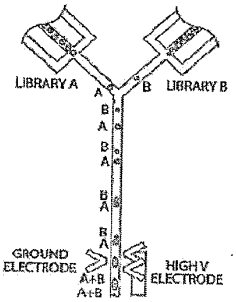


Fig. 16D

Fig. 16E

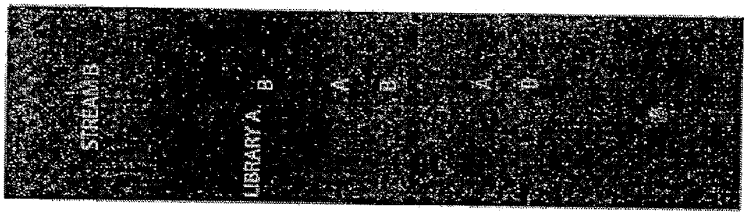


Fig. 17C

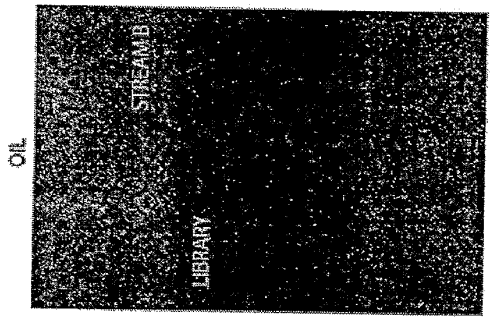


Fig. 17B

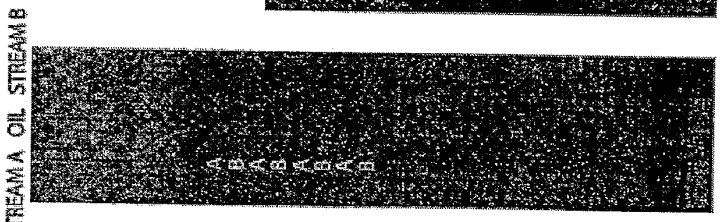
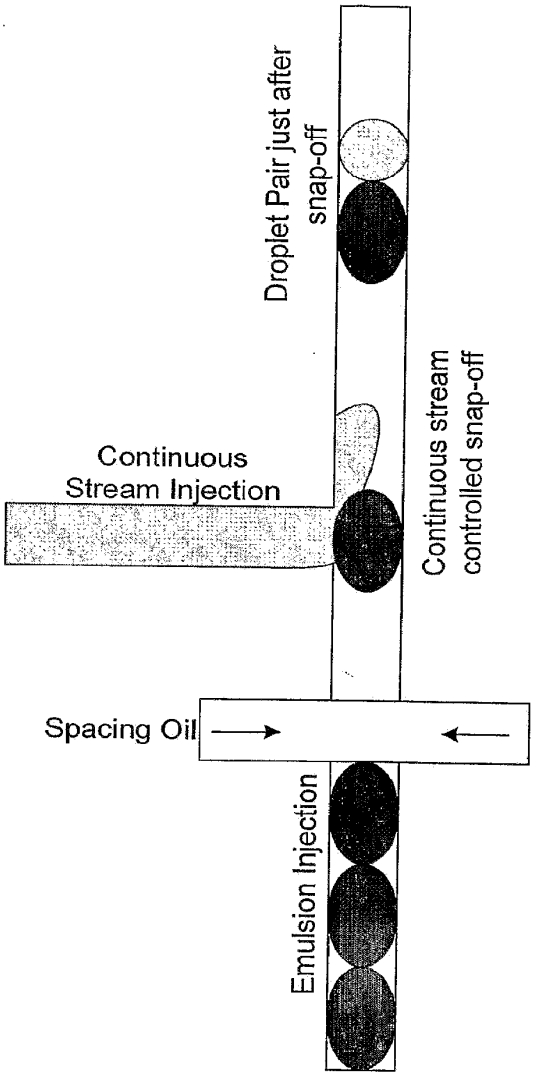


Fig. 17A


Droplet Pair just after snap-off
Continuous Stream Injection
Continuous stream controlled snap-off
Spacing Oil
Emulsion Injection

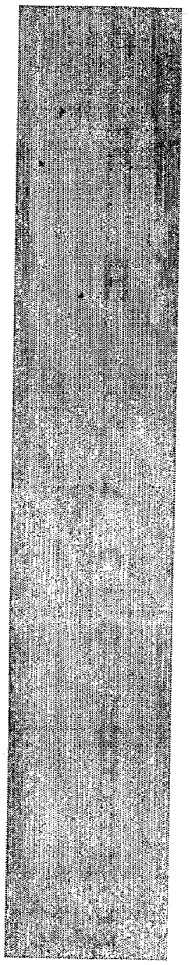


Fig. 17D

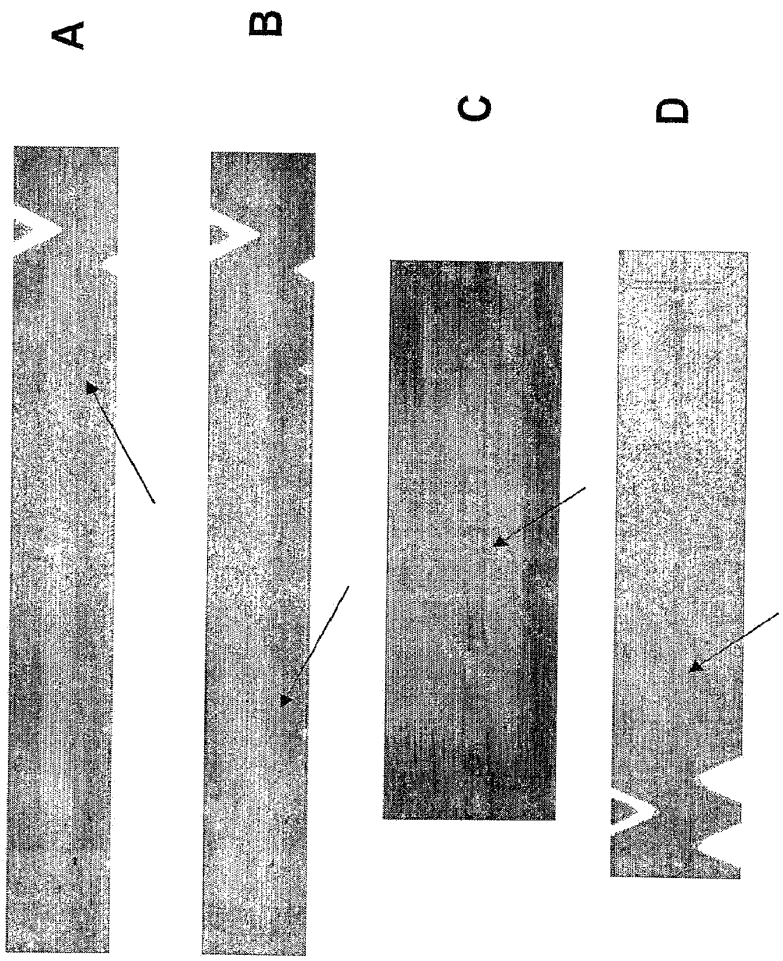


Fig. 18

Fig. 19

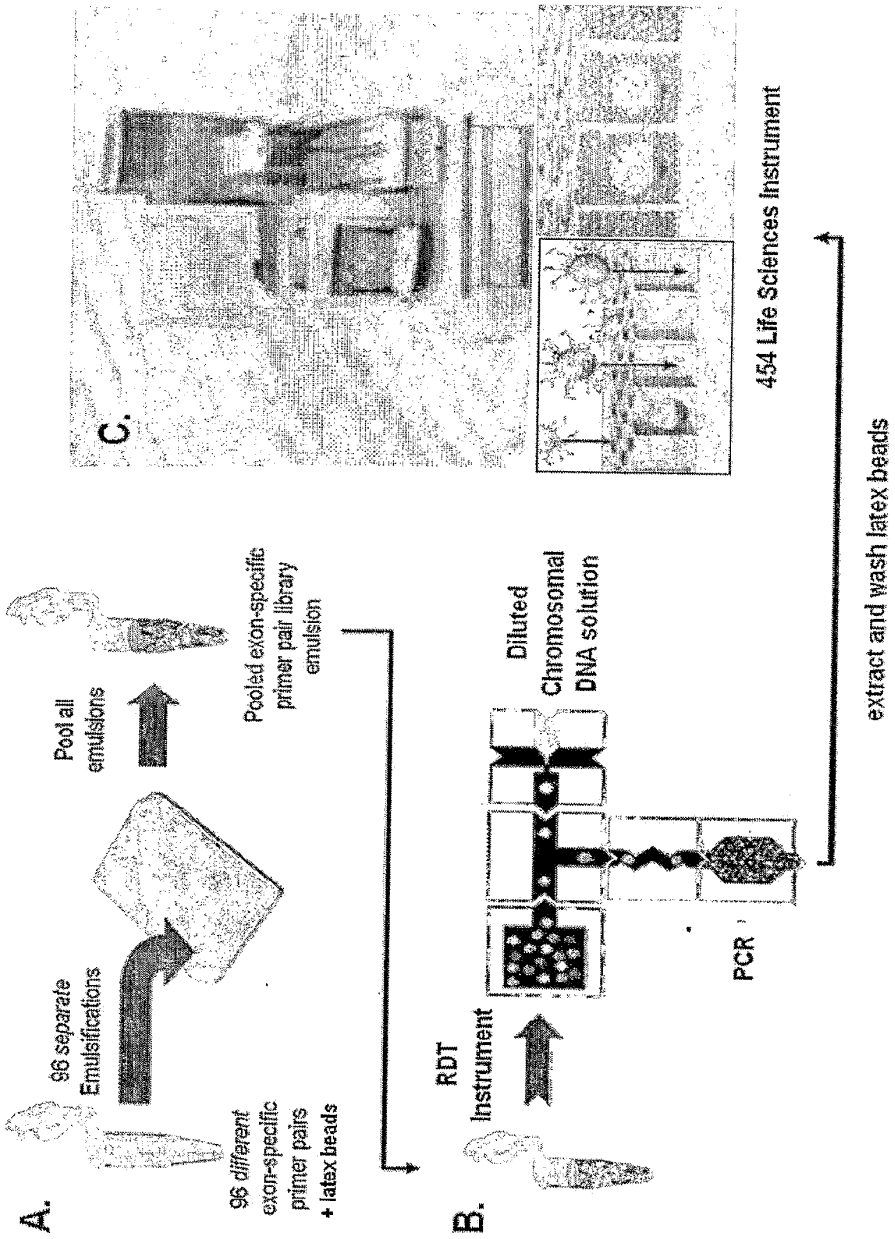

A.
96 different exon-specific primer pairs + latex beads
96 separate Emulsifications
Pool all emulsions
Pooled exon-specific primer pair library emulsion
B.
RDT Instrument
Diluted Chromosomal DNA solution
PCR
extract and wash latex beads
454 Life Sciences Instrument
C.

Fig. 20

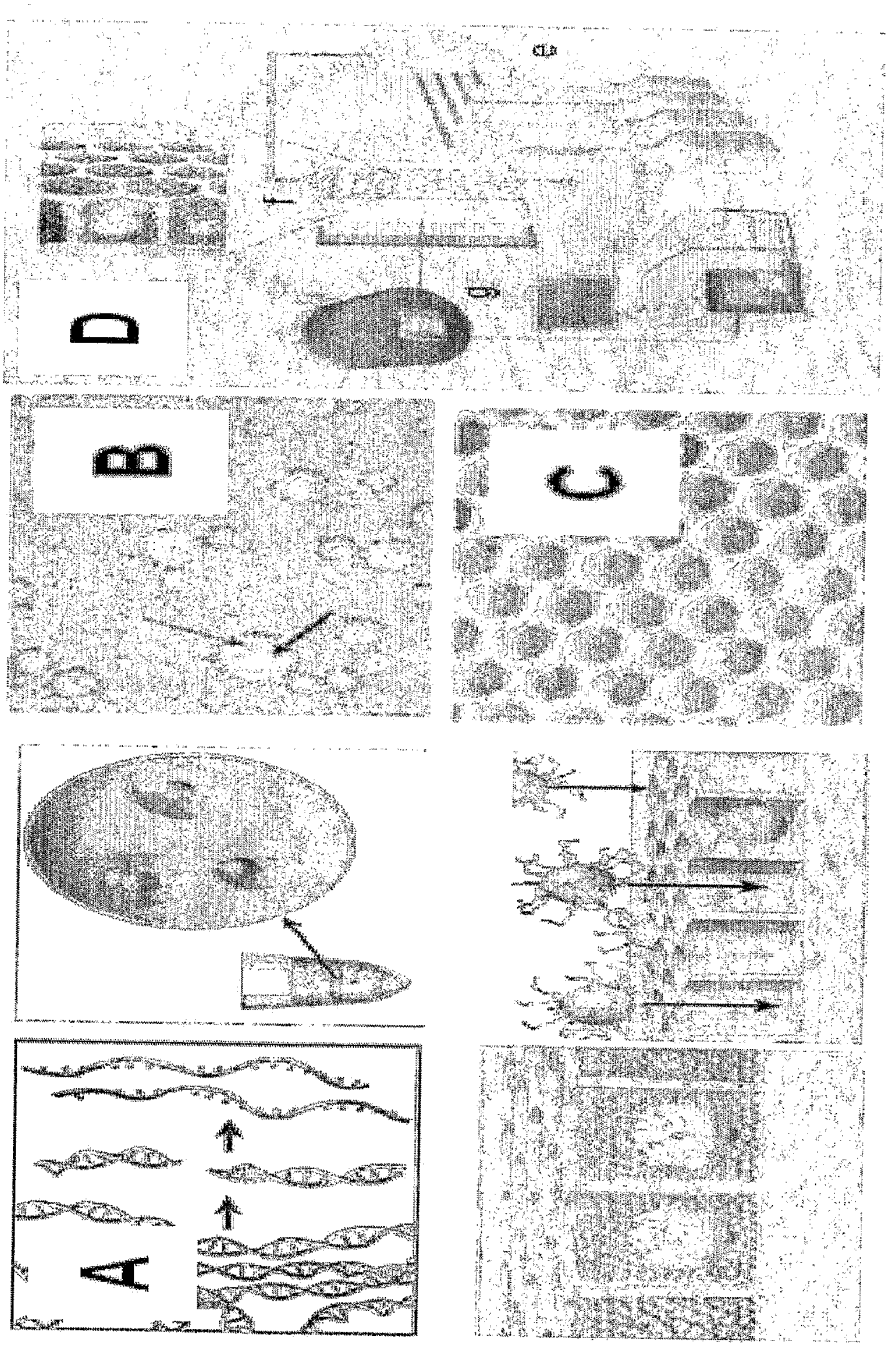
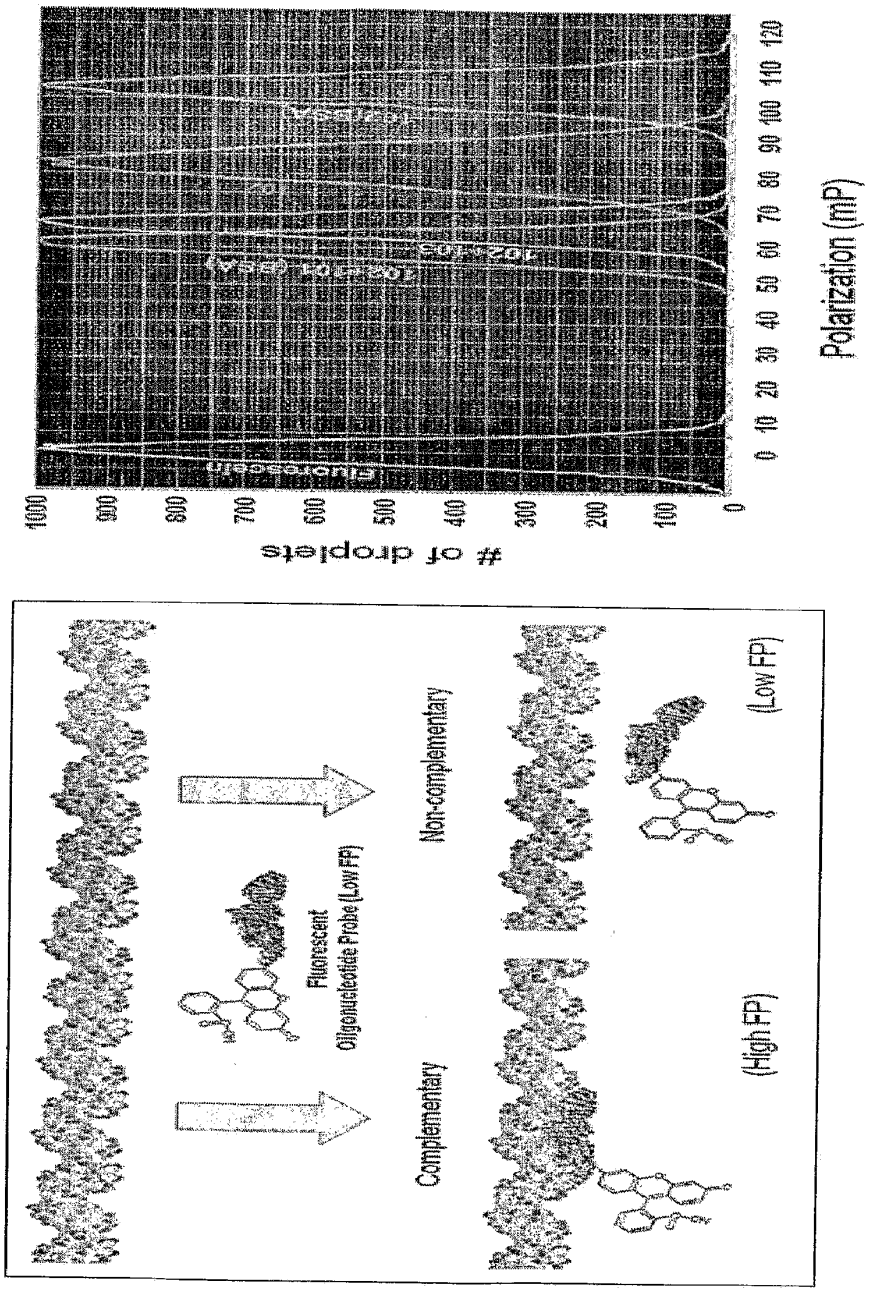


Fig. 21


Fluorescent
Oligonucleotide Probe (Low FP)
Complementary
Non-complementary
(High FP)
(Low FP)
# of droplets
1000
900
800
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Polarization (mP)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/021380

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/536 C12Q1/68 B01J19/00 C07B61/00 C07K1/04
G01N33/68

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)
G01N C12Q B01J C07B C07K B01L

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 practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARGULIES MARCEL ET AL: "Genome sequencing in microfabricated high-density picolitre reactors." NATURE. 15 SEP 2005, vol. 437, no. 7057, 15 September 2005 (2005-09-15), pages 376-380, XP002398505 ISSN: 1476-4687 last page, left column, para.2 ----- -/--	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YONEZAWA MASATO ET AL: "DNA display for in vitro selection of diverse peptide libraries." NUCLEIC ACIDS RESEARCH. 1 OCT 2003, vol. 31, no. 19, 1 October 2003 (2003-10-01), page e118, XP002398594 ISSN: 1362-4962 figure 1	1-12
X	TOKUMITSU H ET AL: "PREPARATION OF GADOPENTETIC ACID-LOADED CHITOSAN MICROPARTICLES FOR GADOLINIUM NEUTRON-CAPTURE THERAPY OF CANCER BY A NOVEL EMULSION-DROPLET COALESCENCE TECHNIQUE" CHEMICAL AND PHARMACEUTICAL BULLETIN, PHARMACEUTICAL SOCIETY OF JAPAN, TOKYO, JP, vol. 47, no. 6, June 1999 (1999-06), pages 838-842, XP000834009 ISSN: 0009-2363 figure 2	1-12

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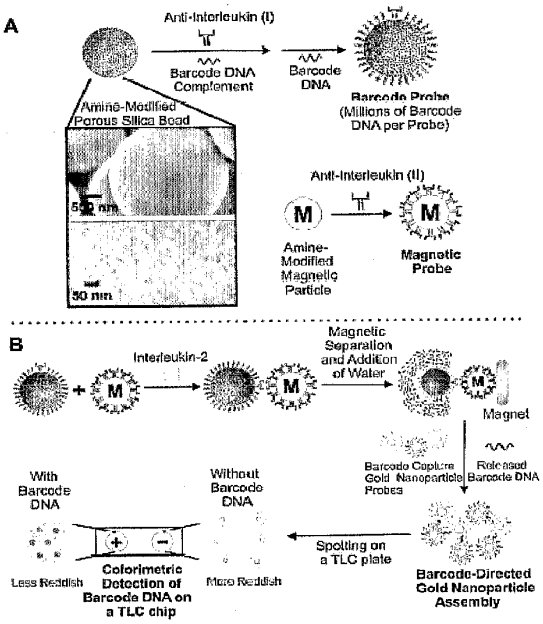
(54) Title: A COLORIMETRIC BIO-BARCODE AMPLIFICATION ASSAY FOR ANALYTE DETECTION



(57) Abstract: The present invention provides a method for detecting an analyte of interest via a bio-barcode assay. The present invention provides a colorimetric bio-barcode method that is capable of detecting minute concentrations of an analyte by relying on porous particles, which enable loading of a large number of barcode DNA per particle, and a metal particle-based colorimetric barcode detection method.

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A Colorimetric Bio-Barcode Amplification Assay for Analyte Detection

Inventors: Jwa-Min Nam and John T. Groves

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims benefit of priority to U.S. Provisional Patent Application No. 60/717,851, filed on September 16, 2005, hereby incorporated by reference in its entirety for all purposes.

STATEMENT OF GOVERNMENTAL SUPPORT

[002] This invention was made during work supported by the U.S. Department of Energy at Lawrence Berkeley National Laboratory under Contract No. DE-AC02-05CH11231. The government has certain rights in this invention.

REFERENCE TO SEQUENCE LISTING

[003] This application incorporates by reference the attached sequence listing found in paper and electronic form.

BACKGROUND OF THE INVENTION

Field of the Invention

[004] The present invention relates to a sensitive screening method for detecting for the presence or absence of one or more target analytes in a sample. In particular, the present invention relates to a method that utilizes reporter oligonucleotides as biochemical barcodes for detecting one or more analytes in a solution.

Related Art

[005] Numerous high sensitivity biomolecule detection methods have been developed, but few have achieved the sensitivity of the polymerase chain reaction (PCR). The bio-barcode amplification assay is the only bio-detection method that has the PCR-like sensitivity for both protein and nucleic acid targets without a need for enzymatic amplification. However, current bio-barcode detection schemes still require microarrayer-based immobilization of oligonucleotide on a glass chip, surface passivation chemistry to minimize nonspecific binding, silver-enhancement of immobilized gold nanoparticles on a chip, light-scattering

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measurement, and a quantification step. Such screening methods and detection schemes have been described by one of the inventors and others in US Pat. Appl. No. 10/ 877,750, published as US20050037397; U.S. Pat. Appl. No. 10/788,414, published as US20050009206; and U.S. Pat. Appl. No. 10/108211, issued as U.S. Pat. No. 6,974,669, all of which are hereby incorporated by reference for all purposes.

[006] Importantly, sophisticated instruments such as microarrayers and chip-imaging tools limit portability, and the assay cost is bound to be expensive. It would be beneficial if one can obviate or minimize the above requirements without sacrificing attomolar sensitivity of the bio-barcode assay.

[007] Others in the art have described colorimetric assays using gold nanoparticle probes capped with oligonucleotides including, Robert Elghanian, et al., Selective Colorimetric Detection of Polynucleotides Based on the Distance-Dependent Optical Properties of Gold Nanoparticles, *Science* 22 August 1997; 277: 1078-1081 (in Reports); James J. Storhoff, et al, One-Pot Colorimetric Differentiation of Polynucleotides with Single Base Imperfections Using Gold Nanoparticle Probes, *J. Am. Chem. Soc.*; (Article); 1998; 120(9); 1959-1964. However, typical detection limit of gold nanoparticle-based colorimetric detection method is ~ nM.

[008] Bio-barcode amplification assays have become a powerful tool in detecting tens to hundreds of biological targets such as proteins and nucleic acids in the entire sample. However, current bio-barcode detection schemes still require many experimental steps including microarrayer-based immobilization of oligonucleotides on a glass chip, silver-enhancement of immobilized gold nanoparticles on a chip, and light-scattering measurement. Thus, there is a need to develop a bio-barcode assay capable of minimizing the above requirements while achieving attomolar sensitivity.

SUMMARY OF THE INVENTION

[009] The present invention provides a method for the detection of analytes in a sample. In one embodiment, the method comprises providing a sample suspected of containing an analyte of interest, contacting a porous particle probe and a magnetic probe particle with the sample, and allowing both the porous particle probe and magnetic probe particle to bind to the analyte of interest. The porous microparticle probe comprises a first ligand that specifically binds the analyte of interest and a barcode oligonucleotide. The magnetic nanoparticle probe comprises a second ligand that also specifically binds the target analyte of interest. If the analyte of interest is present in the sample, a complex is formed between the

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analyte of interest, the porous microparticle probe and the magnetic nanoparticle probe. The complex is separated from the sample, the barcode oligonucleotide is released and collected from the complex, and the barcode oligonucleotide is detected.

[010] In some embodiments, the analyte of interest is a nucleic acid, a protein, a peptide, a metal ion, a hapten, a drug, a metabolite, a pesticide or a pollutant.

[011] In some embodiments, the analyte of interest is a cytokine.

[012] In some embodiments, the analyte of interest is a chemokine.

[013] In some embodiments, the porous microparticle probe is comprised of a material including polystyrene, cellulsose, silica, iron oxide, polyacrylamide, or various polysaccharides, dextran, agarose, cellulose, and derivatives and combinations thereof.

[014] In some embodiments, the porous microparticle probe is modified with an amine.

[015] In some embodiments, the microparticle has a size of about 0.1 micrometers to about 5000 micrometers, preferably a size of about 0.5 micrometers to about 10 micrometers, and even more preferably a size of about 3 micrometers to about 5 micrometers.

[016] In some embodiments, the porous microparticle probe has a pore size of about 50 angstroms to about 150 angstroms, and more preferably about 90 angstroms to about 110 angstroms.

[017] In some embodiments, the porous microparticle probe has a surface area of about $300m^2/g$ to about $500m^2/g$, and more preferably about $400m^2/g$ to about $450m^2/g$.

[018] In some embodiments, the barcode oligonucleotide is a gene, viral RNA or DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA or DNA fragments, natural and synthetic nucleic acids, or aptamers.

[019] In some embodiments, the barcode oligonucleotide is modified with a detectable label. The detectable label may be a biotin, a radiolabel, a fluorescent label, a chromophore, a redox-active group, a group with an electronic signature, a catalytic group, or a Raman label.

[020] In some embodiments, the barcode oligonucleotide and microparticle are members of a universal probe.

[021] In some embodiments, the ligand is a monoclonal or polyclonal antibody.

[022] In some embodiments, detection of the barcode oligonucleotide is performed by a colorimetric assay. In some embodiments, the colorimetric assay comprises detecting the barcode oligonucleotide by providing a solution comprising a first and second particle probe, wherein the first particle probe comprises a capture oligonucleotide complementary to one end of the barcode oligonucleotide, and wherein the second particle probe comprises a capture oligonucleotide complementary to an opposite end of the barcode oligonucleotide;

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contacting the barcode oligonucleotide with the solution and allowing hybridization of the barcode oligonucleotide to the first and second particle probes, whereby the first and second particle probes assemble an aggregate, wherein a color change in the solution indicates formation of said aggregates; and detecting the color change in said solution.

BRIEF DESCRIPTION OF THE DRAWINGS

[023] **Figure 1.** Colorimetric Bio-Barcode Assay. **A.** Probe Preparation and Electron Micrograph Images of Amine-Modified Porous Silica Beads (Inset). **B.** Interleukin-2 Detection Scheme.

[024] **Figure 2.** Quantification Method for Gold Nanoparticle Aggregates Spotted on a TLC Plate. Spot Intensity value is proportional to the number of barcode DNA (the more gold nanoparticles aggregated, the less color appeared) and the number of barcode DNA is proportional to the amount of target proteins present.

[025] **Figure 3.** Gold Nanoparticle-Based Colorimetric Barcode DNA^{IL-2} Detection (Top: Quantification Data; Bottom: Gold Nanoparticle Spots on a TLC Plate). **A.** In Buffer. **B.** In Human Serum Samples.

[026] **Figure 4.** Multiplexed Colorimetric Bio-Barcode Assay. **A.** Scheme showing the assay steps. **B.** Multiple types of nanoparticles that may be used in the assay.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[027] The present invention provides for a simple, ultrasensitive bio-barcode method for detecting an analyte of interest. This bio-barcode approach to analyte detection is important for the following reasons. First, this new method has shown that one can dramatically increase the number of barcode DNA per probe by adjusting surface and size of barcode probe. This allows for various embodiments to detect barcode DNA. In one embodiment, as shown in the examples, a colorimetric assay is used. Second, the detection limit for this assay is orders of magnitude better than other conventional immunoassays. Third, this bio-barcode method does not require complicated instrumentation or experiment steps. Simple mixing and separation of probe solutions would result in attomolar sensitivity without using a microarrayer, complicated signal amplification steps such as enzymatic amplification and silver-enhancement, or sophisticated signal measurement tools. Since the readout is based on color change, minimal expertise is required to perform the assay. Fourth, a quantification

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method using graphic software was developed for quantitative colorimetric barcode DNA detection assay, which was not possible with previous gold nanoparticle-based colorimetric DNA detection schemes. Finally, this assay should be suitable for point-of-care applications with the requirement only for probe solutions and TLC plates.

II. Definitions

[028] As used throughout the invention "barcode", "biochemical barcode", "biobarcode", "barcode oligonucleotide", "barcode DNA", "DNA barcode", "reporter barcode", "reporter barcode DNA", etc. are all interchangeable with each other and have the same meaning. The DNA barcode may be a nucleic acid such as deoxyribonucleic acid or ribonucleic acid. Preferably, the DNA barcode is an oligonucleotide of a predefined sequence. If desired, the DNA barcode may be labeled, for instance, with biotin, a radiolabel, or a fluorescent label.

[029] The term "particle" refers to a small piece of matter that can preferably be composed of metals, silica, silicon-oxide, or polystyrene. A "particle" can be any shape, such as spherical or rod-shaped. The term "particle" as used herein specifically encompasses both nanoparticles and microparticles.

[030] The term "complex" or "probe complex" or "particle complex probe" refers to a conjugate comprised of a porous microparticle comprising a reporter oligonucleotide and a ligand specific for a target analyte conjugated to a magnetic probe particle comprising a ligand specific for the same target analyte, having the target analyte bound thereto to both ligands.

[031] The term "analyte", "analyte of interest", or "target analyte" refers to the compound or composition to be detected, including drugs, metabolites, pesticides, pollutants, and the like. The analyte can be comprised of a member of a specific binding pair (sbp) and may be a ligand, which is monovalent (monoepitopic) or polyvalent (polyepitopic), preferably antigenic or haptenic, and is a single compound or plurality of compounds, which share at least one common epitopic or determinant site. The analyte can be a part of a cell such as bacteria or a cell bearing a blood group antigen such as A, B, D, etc., or an HLA antigen or a microorganism, e.g., bacterium, fungus, protozoan, or virus. If the analyte is monoepitopic, the analyte can be further modified, e.g. chemically, to provide one or more additional binding sites. In practicing this invention, the analyte has at least two binding sites.

[032] The term "ligand" refers to any organic compound for which a receptor naturally exists or can be prepared. The term ligand also includes ligand analogs, which are modified ligands, usually an organic radical or analyte analog, usually of a molecular weight greater

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than 100, which can compete with the analogous ligand for a receptor, the modification providing means to join the ligand analog to another molecule. The ligand analog will usually differ from the ligand by more than replacement of a hydrogen with a bond, which links the ligand analog to a hub or label, but need not. The ligand analog can bind to the receptor in a manner similar to the ligand. The analog could be, for example, an antibody directed against the idiotype of an antibody to the ligand.

[033] The term "receptor" or "antiligand" refers to any compound or composition capable of recognizing a particular spatial and polar organization of a molecule, e.g., epitopic or determinant site. Illustrative receptors include naturally occurring receptors, e.g., thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids, nucleic acid aptamers, avidin, protein A, barsar, complement component C1q, and the like. Avidin is intended to include egg white avidin and biotin binding proteins from other sources, such as streptavidin.

[034] The term "specific binding pair (sbp) member" refers to one of two different molecules, which specifically binds to and can be defined as complementary with a particular spatial and/or polar organization of the other molecule. The members of the specific binding pair can be referred to as ligand and receptor (antiligand). These will usually be members of an immunological pair such as antigen-antibody, although other specific binding pairs such as biotin-avidin, enzyme-substrate, enzyme-antagonist, enzyme-agonist, drug-target molecule, hormones-hormone receptors, nucleic acid duplexes, IgG-protein A/protein G, polynucleotide pairs such as DNA-DNA, DNA-RNA, protein-DNA, lipid-DNA, lipid-protein, polysaccharide-lipid, protein-polysaccharide, nucleic acid aptamers and associated target ligands (e.g., small organic compounds, nucleic acids, proteins, peptides, viruses, cells, etc.), and the like are not immunological pairs but are included in the invention and the definition of sbp member. A member of a specific binding pair can be the entire molecule, or only a portion of the molecule so long as the member specifically binds to the binding site on the target analyte to form a specific binding pair.

[035] The term "specific binding" refers to the specific recognition of one of two different molecules for the other compared to substantially less recognition of other molecules. Generally, the molecules have areas on their surfaces or in cavities giving rise to specific recognition between the two molecules. Exemplary of specific binding are antibody-antigen interactions, enzyme-substrate interactions, polynucleotide interactions, and so forth.

[036] As used herein, a polynucleotide or fragment thereof is "substantially homologous" ("substantially similar") to another if, when optimally aligned (with appropriate nucleotide

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insertions or deletions) with the other polynucleotide (or its complementary strand), using BLASTN (Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." *J. Mol. Biol.* 215:403-410) there is nucleotide sequence identity in at least about 80%, preferably at least about 90%, and more preferably at least about 95-98% of the nucleotide bases. To determine homology between two different polynucleotides, the percent homology is to be determined using an alignment program such as the BLASTN program "BLAST 2 sequences". This program is available for public uses from the National Center for Blotechnolgoy Information (NCBI) over the Internet (Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences – a new tool for comparing protein and nucleotide sequences", *FEMS Microbiol Lett.* 174:247-250). The parameters that can be used are whatever combination of the following yields the highest calculated percent homology (as calculated below) with the default parameters shown in parentheses:

Program – blastn

Reward for a match – 0 or 1 (1)

Penalty for a mismatch – 0, -1, -2 or -3 (-2)

Open gap penalty – 0, 1, 2, 3, 4 or 5 (5)

Extension gap penalty – 0 or 1 (1)

Gap x_dropoff – 0 or 50 (50)

Expect – 10

Word size – 11

Filter – low complexity.

[037] The term "antibody" refers to an immunoglobulin which specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of another molecule. The antibody can be monoclonal or polyclonal and can be prepared by techniques that are well known in the art such as immunization of a host and collection of sera (polyclonal) or by preparing continuous hybrid cell lines and collecting the secreted protein (monoclonal), or by cloning and expressing nucleotide sequences or mutagenized versions thereof coding at least for the amino acid sequences required for specific binding of natural antibodies. Antibodies may include a complete immunoglobulin or fragment thereof, which immunoglobulins include the various classes and isotypes, such as IgA, IgD, IgE, IgG1, IgG2a, IgG2b and IgG3, IgM, etc. Fragments thereof may include Fab, Fv and F(ab[prime])2, Fab[prime], and the like. In addition, aggregates, polymers, and conjugates of immunoglobulins or their fragments can be used where appropriate so long as binding affinity for a particular molecule is maintained.

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III. Method for Detecting Analytes in a Sample

[038] Referring now to Figure 1B, one embodiment of the invention provides methods for detecting analytes of interest from a sample. The method comprises providing a sample suspected of containing an analyte of interest, contacting a porous particle probe and a magnetic probe particle with the sample, and allowing both the porous particle probe and magnetic probe particle to bind to the analyte of interest. The porous particle (i.e. microparticle or nanoparticle) probe comprises a first ligand that specifically binds the analyte of interest and a barcode oligonucleotide. The magnetic probe particle (i.e. nanoparticle) comprises a second ligand that also specifically binds the target analyte of interest. If the analyte of interest is present in the sample, a complex is formed between the analyte of interest, the porous particle probe and the magnetic probe particle. The complex is separated from the sample, the barcode oligonucleotide is released and collected from the complex, and the barcode oligonucleotide is detected.

[039] As shown in Figure 1B, the porous microparticle probe and the magnetic probe particle, both of which are functionalized with a ligand to capture the analyte of interest, are mixed with the sample suspected of containing the analyte of interest. Upon mixing, the analyte of interest, if present, binds to the ligands on both the magnetic probe particle and the porous microparticle probe to form a probe complex comprising the magnetic probe particle and the porous microparticle probe linked together by the ligands bound to the analyte of interest.

[040] In one embodiment, the method utilizes binding events of an analyte of interest to a particle labeled with oligonucleotides, and the subsequent detection of those binding events. The final step of the method described herein relies on the surface chemistry of ordinary DNA. Therefore, it can incorporate many of the high sensitivity aspects of state-of-the-art particle DNA detection methods but allows one to detect a variety of biomolecules, such as proteins, rather than DNA without having the proteins present during the detection event. For surface assays, proteins are typically more difficult to work with than short oligonucleotides because they tend to exhibit greater nonspecific binding to solid supports, which often leads to higher background signals. Finally, for the homogeneous assay, the unusually sharp melting profiles associated with these nanoparticle structures will allow one to design more biobarcodes than what would be possible with probes that exhibit normal and broad DNA melting behavior.

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[041] The present invention contemplates the use of any suitable particle having oligonucleotides attached thereto that are suitable for use in detection assays. As described herein, each microparticle, magnetic probe particle and nanoparticle will have a plurality of oligonucleotides attached to it. As a result, each particle-oligonucleotide conjugate can bind to a plurality of oligonucleotides or nucleic acids having the complementary sequence.

[042] The oligonucleotides are contacted with the particles in aqueous solution for a time sufficient to allow at least some of the oligonucleotides to bind to the nanoparticles by means of the functional groups. Such times can be determined empirically. For instance, it has been found that a time of about 12 to 24 hours gives good results. In some embodiments wherein detection is in the clinic, a preferred time for hybridization may be 10 minutes to 12 hours. Other suitable conditions for binding of the oligonucleotides can also be determined empirically. For instance a concentration of about 10-20nM nanoparticles and incubation at room temperature gives good results.

[043] The probe complex is separated from the sample after formation of the probe complex. In a preferred embodiment, this is carried out by magnetic separation facilitated by exposing the sample to a magnetic field (e.g., via a magnetic separation device) which attracts the magnetic particles in the probe complex and allows isolation or separation from the sample. Thus, in one aspect of the invention, the particle probe complex comprises a microparticle having barcode oligonucleotides and a ligand, wherein the ligand is bound to a specific analyte of interest and the analyte of interest is also bound to another ligand on the magnetic probe particle.

[044] After separation from the sample, the barcode oligonucleotide attached to the porous microparticle in the probe complex is released and captured for further detection or analysis. The barcodes can be released for the particles to which they are attached by a chemical releasing agent that will disrupt binding of the barcode to the surface of the particle. Such agents include, but are not limited to, any molecule that will preferentially bind to a particle through a thiol link such as other thiol- or disulfide-containing molecules, dithiothreitol (DTT), dithioerythritol (DTE), mercaptoethanol and the like, and reducing agents such as sodium borohydride that will cleave a disulfide linkage thereby releasing barcodes from the particles to which they are attached. The barcodes can also be released from the particles by exposing the barcodes to conditions under which the barcodes will dehybridize from oligonucleotides by which the barcodes were attached to the particles.

[045] The barcodes or reporter oligonucleotides may then be detected by any suitable means. Generally, the barcodes are released via dehybridization from the complex prior to

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detection. Any suitable solution or media may be used that dehybridize and release the barcode from the complex. A representative medium is water.

a. Analyte of Interest

[046] The analyte of interest may be nucleic acid molecules, proteins, peptides, haptens, metal ions, drugs, metabolites, pesticide or pollutant. The method can be used to detect the presence of such analytes as toxins, hormones, enzymes, lectins, proteins, signaling molecules, inorganic or organic molecules, antibodies, contaminants, viruses, bacteria, other pathogenic organisms, idiotopes or other cell surface markers. It is intended that the present method can be used to detect the presence or absence of an analyte of interest in a sample suspected of containing the analyte of interest.

[047] In some embodiments, the target analyte is comprised of a nucleic acid and the specific binding complement is an oligonucleotide. Alternatively, the target analyte is a protein or hapten and the specific binding complement is an antibody comprising a monoclonal or polyclonal antibody. Alternatively, the target analyte is a sequence from a genomic DNA sample and the specific binding complement are oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence. The genomic DNA may be eukaryotic, bacterial, fungal or viral DNA.

[048] In one embodiment, detection of a particular cytokine can be used for diagnosis of cancer. Specific analytes of interest include cytokines, such as IL-2 as shown in the examples. Cytokines are important analytes of interest in that cytokines play a central role in the regulation of hematopoiesis; mediating the differentiation, migration, activation and proliferation of phenotypically diverse cells. Improved detection limits of cytokines will allow for earlier and more accurate diagnosis and treatments of cancers and immunodeficiency-related diseases and lead to an increased understanding of cytokine-related diseases and biology, because cytokines are signature biomarkers when humans are infected by foreign antigens.

[049] Chemokines are another important class of analytes of interest. Chemokines are released from a wide variety of cells in response to bacterial infection, viruses and agents that cause physical damage such as silica or the urate crystals. They function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection or damage. They can be released by many different cell types and serve to guide cells involved in innate immunity and also the lymphocytes of the adaptive immune system. Thus, improved detection limits of chemokines will allow for

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earlier and more accurate diagnosis and treatments, i.e. for bacterial infections and viral infections.

[050] In some embodiments, the target analyte may be a variety of pathogenic organisms including, but not limited to, sialic acid to detect HIV, *Chlamydia, Neisseria meningitides, Streptococcus suis, Salmonella,* mumps, newcastle, and various viruses, including reovirus, sendai virus, and myxovirus; and 9-OAC sialic acid to detect coronavirus, encephalomyelitis virus, and rotavirus; non-sialic acid glycoproteins to detect cytomegalovirus and measles virus; CD4, vasoactive intestinal peptide, and peptide T to detect HIV; epidermal growth factor to detect vaccinia; acetylcholine receptor to detect rabies; Cd3 complement receptor to detect Epstein-Barr virus; β-adrenergic receptor to detect reovirus; ICAM-1, N-CAM, and myelin-associated glycoprotein MAb to detect rhinovirus; polio virus receptor to detect polio virus; fibroblast growth factor receptor to detect herpes virus; oligomannose to detect *Escherichia coli*; ganglioside G_{M1} to detect *Neisseria meningitides*; and antibodies to detect a broad variety of pathogens (e.g., *Neisseria gonorrhoeae, V. vulnificus, V. parahaemolyticus, V. cholerae,* and *V. alginolyticus*).

[051] In some embodiments, multiple analytes of interest can be detected by utilizing multiple ligands specific to different analytes of interest and utilizing distinct barcode oligonucleotides corresponding to each analyte of interest.

b. Sample

[052] The analyte of interest may be found directly in a sample such as a body fluid from a host. The host may be a mammal, reptile, bird, amphibian, fish, or insect. In a preferred embodiment, the host is a human. The body fluid can be, for example, urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, pus, phlegm, and the like. The particles can be mixed with live cells or samples containing live cells.

[053] Where the sample is live cells or samples containing live cells, a cell surface protein or other molecule may serve as the analyte of interest. This allows for the detection of cell activation and proliferation events, cellular interactions, multiplexing, and other physiologically relevant events.

c. Porous Microparticle Probe

[054] In a preferred embodiment, the present method utilizes porous microparticles and a metal nanoparticle-based colorimetric DNA detection scheme for straightforward readout (Figure 1). In a preferred embodiment, the porous microparticle probe should feature a ligand

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to capture a target analyte and a barcode oligonucleotide, which is a specific barcode DNA sequence.

[055] In one embodiment, the microparticle is a porous particle having a defined degree of porosity and comprised of pores having a defined size range, wherein the barcode oligonucleotides are impregnated within the pores of the microparticle. The use of a porous microparticle can accommodate millions of barcode DNA per particle, thus allowing the use of a colorimetric barcode DNA detection scheme with attomolar sensitivity. This is an important advance because this scheme has the attomolar (10^{-18} M) sensitivity of the bio-barcode amplification method as well as the simplicity, portability and low cost of gold nanoparticle-based colorimetric detection methods.

[056] In some embodiments, the porous microparticle probe can be comprised of materials including silica and iron oxide. The term "microparticle" as used herein is intended to encompass any particulate bead, sphere, particle or carrier, whether biodegradable or nonbiodegradable, comprised of naturally-occurring or synthetic, organic or inorganic materials that is porous. In particular, the microparticle includes any particulate bead, sphere, particle, or carrier having a diameter of about 0.1 to about 5000 micrometers, more preferably about 1-5μm in diameter, and even more preferably between about 3-4μm in diameter. The term "about" as used herein is meant to include up to ±1 unit of the provided range. In another embodiment, porous silica microparticles (1.57×10^9 ml^{-1} diameter: 3.53 ± 0.49 μm) are used.

[057] The microparticles of the invention are comprised of polystyrene, silica, iron oxide, polyacrylamide, and various polysaccharides including dextran, agarose, cellulose and modified, crosslinked and derivatized embodiments thereof. Specific examples of the microparticles of the invention include polystyrene, cellulose, dextran crosslinked with bisacrylamide (Biogel.TM., Bio-Rad, U.S.A.), agar, glass beads and latex beads. Derivatized microparticles include microparticles derivatized with carboxyalkyl groups such as carboxymethyl, phosphoryl and substituted phosphoryl groups, sulfate, sulfhydryl and sulfonyl groups, and amino and substituted animo groups.

[058] The size, shape and chemical composition of the particles will contribute to the properties of the resulting probe including the barcode DNA. These properties include optical properties, optoelectronic properties, electrochemical properties, electronic properties, stability in various solutions, ability to separate bioactive molecules while acting as a filter, etc. The use of mixtures of particles having different sizes, shapes and/or chemical

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compositions and the use of particles having uniform sizes, shapes and chemical composition, are contemplated.

[059] In some embodiments, the microparticle is amino-functionalized and then reacted with the ligand and the barcode oligonucleotide. In a preferred embodiment, the porous microparticle probes are comprised of silica and iron oxide and functionalized with amine groups for further modification with other biomolecules. For example, such particles can be obtained from PHENOMENEX (Torrance, CA). Analogous glutaraldehyde linker chemistry has been extensively used by others to affect protein linking to amino functionalized particles.

[060] In another embodiment, the methods to functionalize the nanopartices as described infra may be used to functionalize the porous microparticle probe. In some embodiments, the silica coated magnetic particles are functionalized amino-silane molecules to functionalize the silica surface with amines.

[061] Other properties of the porous microparticles that affect the number of barcode oligonucleotides which can be incorporated onto the probe, and therefore sensitivity, include: surface area, pore size, interconnectivity of the pores, hydrophilicity and pore distribution.

i. Surface Area

[062] The number of barcode oligonucleotides per probe is dramatically increased by adjusting the surface and size of the barcode probe which also allows for various embodiments to detect more than one barcode oligonucleotide. In the bio-barcode approach, the number of barcode oligonucleotide per probe is important because the final detection signal is proportional to the amount of captured barcode DNA.

[063] In some embodiments, the surface area of the porous particles is about 300 m^2/g to about 500 m^2/g, more preferably about 400 m^2/g to about 450 m^2/g.

[064] In a preferred embodiment, the large size (a few micrometers) and porosity of probe result in significantly increased barcode oligonucleotide loading relative to past approaches (tens-of-nanometer particle without pores). Using UV-Vis spectroscopy (the UV absorption peak for single stranded DNA is at 260 nm), it was determined the average total number of barcode oligonucleotides per ~3.5 micrometer bead to be ~ 3.6×10^6. Compared with other nanoparticle-based barcode probes which can host only hundreds of barcode DNA per nanoparticle probe, the present microparticles result in several orders of magnitude more amplification in terms of the number of barcode oligonucleotides per barcode probe.

ii. Pore Size

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[065] Pore size is also an important aspect of the porous particles. The pore size must be large enough such that the barcode oligonucleotides can enter the pore during binding of the barcode to the particle and exit the pore when releasing the barcode oligonucleotides for detection.

[066] Therefore, in some embodiments, the pore size is about 50 angstroms to about 150 angstroms, more preferably from about 90 angstroms to about 110 angstroms.

iii. Interconnectivity

[067] Interconnectivity of the pores within the porous particles allows sample or effluent to flow throughout the porous particle. These "channels" provides means for preparing and releasing the barcode DNA from within the pores. Also, by having channels, it prevents air pockets from forming within pores which can interfere with barcode DNA entrance and release.

[068] Thus, in a preferred embodiment, the porous particles have channels to afford greater accommodation of barcode DNA and better binding and release of the barcode DNA from the particle.

iv. Hydrophilicity

[069] In a preferred embodiment, the porous particle is hydrophilic and has little to no hydrophobicity. Hydropholic porous particles allows for effective probe preparation and effective release of barcode DNA for detection.

v. Pore Distribution

[070] In a preferred embodiment, the porous particle will have the greatest number of pores that can be incorporated onto the particle without negatively affecting the structural integrity of each particle.

[071] Pore distribution or the number of pores per particle can also affect the number of barcode DNA that can be accommodated onto the particle. The number of pores has a direct effect on the surface area of each particle. There is, however, a limit to the number of pores that a particle can have. The structural integrity of the particle may be compromised if too many pores are incorporated into each particle.

d. Ligands

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[072] The ligands attached to capture an analyte of interest may be attached, removeably attached, covalently or non-covalently attached to the porous particle probe and magnetic particle probe.

[073] Both the ligand attached to the porous particle probe and the ligand attached to the magnetic particle probe specifically bind to an analyte of interest. Thus, in a preferred embodiment, the analyte of interest has at least two binding sites allowing for each ligand to specifically bind.

[074] A ligand can be any molecule or material having a known analyte as a specific binding pair member. Thus, each member of the specific binding pair may be a nucleic acid, an oligonucleotide, a peptide nucleic acid, a polypeptide, an antigen, a carbohydrate, an amino acid, a hormone, a steroid, a vitamin, a virus, a polysaccharide, a lipid, a lipopolysaccharide, a glycoprotein, a lipoprotein, a nucleoprotein, an albumin, a hemoglobin, a coagulation factor , a peptide hormone, a non-peptide hormone, a biotin, a streptavidin, a cytokine, a chemokine, a peptide compromising a tumor-specific epitope, a cell, a cell surface molecule, a microorganism, a small molecules, an enzyme, a receptor, a channel, a chromophore, a chelating compound, a phosphate and reactive group, a molecular recognition complex, a dinitrophenol, an electron donor or acceptor group, a hydrophobic compound, a hydrophilic compound, an organic molecule, and an inorganic molecule.

[075] In some embodiments, the ligand is a monoclonal antibody or polyclonal antibody where the analyte of interest is a protein, hapten or peptide. Where antibodies are used as the ligands, the epitopes of the antibodies used to functionalize the magnetic probe particle are different from those of the antibodies used to prepare the microparticle probes by using a different coupling chemistry. Therefore in a preferred embodiment, the antibodies chosen as the ligands are already developed antibodies with two different epitopes. For important disease markers, many high quality antibodies with different epitopes are readily available through academic and commercial means. Furthermore, it is recognized in the art that antibodies can be raised to a ligand by one with skill in the art.

[076] In some embodiments, where the analyte of interest is a nucleic acid, the ligand is an oligonucleotide having a sequence that is complementary to at least a portion of the sequence of the nucleic acid.

[077] In some embodiments, where the analyte of interest is from a genomic DNA sample, the ligand is an oligonucleotide having a sequence that is complementary to the genomic sequence.

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[078] Amino-functionalized magnetic particles were linked to ligands for the target analyte. In a preferred embodiment where antibodies are used as the ligand, the epitopes of the antibodies are different from those of the antibodies used to prepare the barcode DNA using glutaraldehyde-amine coupling chemistry.

e. Barcode Oligonucleotide

[079] In a preferred embodiment, the barcode oligonucleotides attached to the porous microparticle probe to capture a target analyte may be attached, removeably attached, covalently or non-covalently attached.

[080] Any suitable method for attaching oligonucleotides onto the nanosphere surface may be used. A particularly preferred method for attaching oligonucleotides onto a surface is based on an aging process described in U.S. application Ser. No. 09/344,667, filed Jun. 25, 1999; Ser. No. 09/603,830, filed Jun. 26, 2000; Ser. No. 09/760,500, filed Jan. 12, 2001; Ser. No. 09/820,279, filed Mar. 28, 2001; Ser. No. 09/927,777, filed Aug. 10, 2001; and in International application nos. PCT/US97/12783, filed Jul. 21, 1997; PCT/US00/17507, filed Jun. 26, 2000; PCT/US01/01190, filed Jan. 12, 2001; PCT/US01/10071, filed Mar. 28, 2001, the disclosures which are incorporated by reference in their entirety. The aging process provides nanoparticle-oligonucleotide conjugates with unexpected enhanced stability and selectivity.

[081] In one embodiment, the method comprises providing barcode oligonucleotides preferably having covalently bound thereto a moiety comprising a functional group which can bind to the nanoparticles. The moieties and functional groups are those that allow for binding (i.e., by chemisorption or covalent bonding) of the oligonucleotides to nanoparticles. For instance, oligonucleotides having an alkanethiol, an alkanedisulfide or a cyclic disulfide covalently bound to their 5' or 3' ends can be used to bind the oligonucleotides to a variety of nanoparticles, including gold nanoparticles. Methods of attaching oligonucleotides to nanoparticles are futher described in U.S. Pat. Appl. Serial No. 10/877,750, published as US20050037397, hereby incorporated by reference.

[082] In some embodiments, the barcode oligonucleotides are attached to the microparticle by means of a linker. There are many amine-reactive linkers (for covalent linking) available commercially. Therefore, it is contemplated that the microparticles are commonly modified with amines. Preferably, the linker further comprises a hydrocarbon moiety attached to the cyclic disulfide. Suitable hydrocarbons are available commercially, and are attached to the cyclic disulfides. Preferably the hydrocarbon moiety is a steroid residue. Oligonucleotide-

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particle conjugates prepared using linker comprising a steroid residue attached to a cyclic disulfide have unexpectedly been found to be remarkably stable to thiols (e.g., dithiothreitol used in polymerase chain reaction (PCR) solutions) as compared to conjugates prepared using alkanethiols or acyclic disulfides as the linker. Indeed, others have found the oligonucleotide-particle conjugates of the invention have been found to be 300 times more stable. See U.S. Pat. Appl. Serial No. 10/877,750. This stability is likely due to the fact that each oligonucleotide is anchored to a microparticle through two sulfur atoms, rather than a single sulfur atom. In particular, it is thought that two adjacent sulfur atoms of a cyclic disulfide would have a chelation effect which would be advantageous in stabilizing the oligonucleotide-microparticle conjugates. The large hydrophobic steroid residues of the linkers also appear to contribute to the stability of the conjugates by screening the microparticles from the approach of water-soluble molecules to the surfaces of the nanoparticles.

[083] In another embodiment, the barcode oligonucleotides are bound to the microparticles using sulfur-based functional groups. U.S. Pat. Appl. Serial No. 09/760,500 and 09/820,279 and international application nos. PCT/US01/01190 and PCT/US01/10071 describe oligonucleotides functionalized with a cyclic disulfide which are useful in practicing this invention. The cyclic disulfides preferably have 5 or 6 atoms in their rings, including the two sulfur atoms. Suitable cyclic disulfides are available commercially or may be synthesized by known procedures. The reduced form of the cyclic disulfides can also be used.

[084] In one embodiment, ethanolamine is used to passivate all unreacted reaction sites on the microparticles. A protein such as bovine serum albumin can also be used in addition or instead to further passivate inactive regions on the microparticle surface.

[085] As described in the definitions, the DNA barcode may be a nucleic acid such as deoxynucleic acid or ribonucleic acid. Preferably, the DNA barcode is an oligonucleotide of a predefined sequence. The DNA barcode oligonucleotide may comprise genes; viral RNA and DNA; bacterial DNA; fungal DNA; mammalian DNA, cDNA, mRNA, RNA and DNA fragments; oligonucleotides; synthetic oligonucleotides; modified nucleotides; single-stranded and double-stranded nucleic acids; natural and synthetic nucleic acids; and aptamers.

[086] Methods of making oligonucleotides of a predetermined sequence are well known. See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed. 1989) and F. Eckstein (ed.) Oligonucleotides and Analogues, 1st Ed. (Oxford University Press, New York, 1991). Solid-phase synthesis methods are preferred for both oligoribonucleotides and oligodeoxyribonuclotides (the well-known methods of synthesizing DNA are also useful for

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synthesizying RNA). Oligonucleotides can also be prepared enzymatically. For oligonucleotides having a specific binding complement to a target analyte bound thereto, any suitable method of attaching the specific binding complement, such as proteins, to the oligonucleotide may be used.

[087] The present invention contemplates using sequences designed by techniques known to those of skill in the art including, optimization for annealing temperatures, the specificity of the sequence to the template, and length of sequence. The design of the sequences can be done using primer prediction software such as Oligo6 (Molecular Biology Insights, Inc., Cascade, CO). Custom scripts and software for primer design can also be used.

[088] Any unique oligonucleotide sequence and its complementary sequence can be used for the barcode oligonucleotide. It is preferred that the oligonucleotide sequences used as barcode oligonucleotides hybridize their complementary sequences under stringent conditions. The term "stringent conditions" as used herein refers to conditions under which a sequence will hybridize to its target subsequence or complement, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 15°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. (As the target sequences are generally present in excess, at Tm, 50% of the probes are occupied at equilibrium.)

[089] In some embodiments, the barcode oligonucleotide is modified with a detectable label. Examples of detectable labels include biotin, radiolabels, fluorescent labels, chromophores, redox-active groups, groups with electronic signatures, catalytic groups and Raman labels.

[090] Examples of such specific barcode DNA sequences can be found e.g. in *Multiplexed Detection of Protein Cancer Markers with Biobarcoded Nanoparticle Probes*, Stoeva et al., 128 J. Am. Chem. Soc. 8378-8379 (2006); *Bio-Bar-Code-Based DNA Detection with PCR-like Sensitivity*, Nam et al. 126 J. Am. Chem. Soc. 5932-5933 (2004); and *Multiplexed DNA Detection with Biobarcoded Nanoparticle Probes*, Soteva et al., 45 Angew. Chem. Int. Ed. 3303-3306 (2006), hereby incorporated by reference.

[091] In a preferred embodiment, barcode DNA are 3' amino-functionalized bar-code DNA complements having a defined sequence (e.g., as an identification tag) to identify the

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microparticle as being used to detect a specific target analyte, thereby permitting the detection of multiple target analytes in a sample.

[092] In one embodiment, the method utilizes oligonucleotides as biochemical barcodes for detecting a single or multiple analytes in a sample. The approach takes advantage of recognition elements (e.g., proteins or nucleic acids) functionalized either directly or indirectly with nanoparticles and the previous observation that hybridization events that result in the aggregation of gold nanoparticles can significantly alter their physical properties (e.g. optical, electrical, mechanical). The general idea is that each recognition element can be associated with a different oligonucleotide sequence (i.e. a DNA barcode) with discrete and tailorable hybridization and melting properties and a physical signature associated with the nanoparticles that change upon melting to decode a series of analytes in a multi-analyte assay. Therefore, one can use the melting temperature of a DNA-linked aggregate and a physical property associated with the nanoparticles that change upon melting to decode a series of analytes in a multiple analyte assay. The barcodes herein are different from the ones based on physical diagnostic markers such as nanorods, fluorophore-labeled beads, and quantum dots, in that the decoding information is in the form of chemical information stored in a predesigned oligonucleotide sequence.

f. Magnetic Probe Particle

[093] The magnetic probe particle can be comprised of magnetic materials including iron oxide and other ferromagnetic materials. The magnetic probe particle can be coated with silica, or polymers such as polyacrylamide, polystyrene, etc. with the surface functionalized as described for the porous microparticles.

[094] In a preferred embodiment, the magnetic probe particles can be nanoparticles or microparticles having a diameter of about 0.1 nanometers to about 5000 micrometers. Suitable magnetic particles are widely used in the art and can be obtained from such vendors as Dynal Biotech (newly acquired by Invitrogen).

[095] In one embodiment, the magnetic particles are prepared as described in the Examples using glutaraldehyde-amine coupling chemistry.

[096] Microparticles and nanoparticles useful in the practice of the invention include metal (e.g., gold, silver, copper and platinum), semiconductor (e.g., CdSe, CdS, and CdS or CdSe coated with ZnS) and magnetic (e.g., ferromagnetite) colloidal materials. Other nanoparticles useful in the practice of the invention include ZnS, ZnO, TiO2, AgI, AgBr, HgI2, PbS, PbSe, ZnTe, CdTe, In2S3, In2Se3, Cd3P2, Cd3As2, InAs, and GaAs. The size of the nanoparticles

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is preferably from about 5 nm to about 150 nm (mean diameter), more preferably from about 5 to about 50 nm, most preferably from about 10 to about 30 nm. The nanoparticles may also be rods, prisms, or tetrahedra.

[097] Methods of making metal, semiconductor and magnetic nanoparticles are well-known in the art. See, e.g., Schmid, G. (ed.) Clusters and Colloids (VCH, Weinheim, 1994); Hayat, M. A. (ed.) Colloidal Gold: Principles, Methods, and Applications (Academic Press, San Diego, 1991); Massart, R., IEEE Taransactions On Magnetics, 17, 1247 (1981); Ahmadi, T. S. et al., Science, 272, 1924 (1996); Henglein, A. et al., J. Phys. Chem., 99, 14129 (1995); Curtis, A. C., et al., Angew. Chem. Int. Ed. Engl., 27, 1530 (1988).

[098] Methods of making ZnS, ZnO, TiO2, AgI, AgBr, HgI2, PbS, PbSe, ZnTe, CdTe, In2S3, In2Se3, Cd3P2, Cd3As2, InAs, and GaAs nanoparticles are also known in the art. See, e.g., Weller, Angew. Chem. Int. Ed. Engl., 32, 41 (1993); Henglein, Top. Curr. Chem., 143, 113 (1988); Henglein, Chem. Rev., 89, 1861 (1989); Brus, Appl. Phys. A., 53, 465 (1991); Bahncmann, in Photochemical Conversion and Storage of Solar Energy (eds. Pelizetti and Schiavello 1991), page 251; Wang and Herron, J. Phys. Chem., 95, 525 (1991); Olshavsky et al., J. Am. Chem. Soc., 112, 9438 (1990); Ushida et al., J. Phys. Chem., 95, 5382 (1992).

[099] Suitable nanoparticles are also commercially available from, e.g., Ted Pella, Inc. (gold), Amersham Corporation (gold) and Nanoprobes, Inc. (gold).

[0100] Presently preferred for use in detecting nucleic acids are gold nanoparticles. Gold colloidal particles have high extinction coefficients for the bands that give rise to their beautiful colors. These intense colors change with particle size, concentration, interparticle distance, and extent of aggregation and shape (geometry) of the aggregates, making these materials particularly attractive for colorimetric assays. For instance, hybridization of oligonucleotides attached to gold nanoparticles with oligonucleotides and nucleic acids results in an immediate color change visible to the naked eye.

[0101] The particles or the oligonucleotides, or both, are functionalized in order to attach the oligonucleotides to the particles. Such methods are known in the art. For instance, oligonucleotides functionalized with alkanethiols at their 3'-termini or 5'-termini readily attach to gold nanoparticles. See Whitesides, Proceedings of the Robert A. Welch Foundation 39th Conference on Chemical Research Nanophase Chemistry, Houston, Tex., pages 109-121 (1995). See also, Mucic et al. Chem. Commun. 555-557 (1996) (describes a method of attaching 3' thiol DNA to flat gold surfaces; this method can be used to attach oligonucleotides to nanoparticles). The alkanethiol method can also be used to attach oligonucleotides to other metal, semiconductor and magnetic colloids and to the other

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nanoparticles listed above. Other functional groups for attaching oligonucleotides to solid surfaces include phosphorothioate groups (see, e.g., U.S. Pat. No. 5,472,881 for the binding of oligonucleotide-phosphorothioates to gold surfaces), substituted alkylsiloxanes (see, e.g. Burwell, Chemical Technology, 4, 370-377 (1974) and Matteucci and Caruthers, J. Am. Chem. Soc., 103, 3185-3191 (1981) for binding of oligonucleotides to silica and glass surfaces, and Grabar et al., Anal. Chem., 67, 735-743 for binding of aminoalkylsiloxanes and for similar binding of mercaptoaklylsiloxanes). Oligonucleotides terminated with a 5' thionucleoside or a 3' thionucleoside may also be used for attaching oligonucleotides to solid surfaces. The following references describe other methods which may be employed to attached oligonucleotides to nanoparticles: Nuzzo et al., J. Am. Chem. Soc., 109, 2358 (1987) (disulfides on gold); Allara and Nuzzo, Langmuir, 1, 45 (1985) (carboxylic acids on aluminum); Allara and Tompkins, J. Colloid Interface Sci., 49, 410-421 (1974) (carboxylic acids on copper); Iler, The Chemistry Of Silica, Chapter 6, (Wiley 1979) (carboxylic acids on silica); Timmons and Zisman, J. Phys. Chem., 69, 984-990 (1965) (carboxylic acids on platinum); Soriaga and Hubbard, J. Am. Chem. Soc., 104, 3937 (1982) (aromatic ring compounds on platinum); Hubbard, Acc. Chem. Res., 13, 177 (1980) (sulfolanes, sulfoxides and other functionalized solvents on platinum); Hickman et al., J. Am. Chem. Soc., 111, 7271 (1989) (isonitriles on platinum); Maoz and Sagiv, Langmuir, 3, 1045 (1987) (silanes on silica); Maoz and Sagiv, Langmuir, 3, 1034 (1987) (silanes on silica); Wasserman et al., Langmuir, 5, 1074 (1989) (silanes on silica); Eltekova and Eltekov, Langmuir, 3, 951 (1987) (aromatic carboxylic acids, aldehydes, alcohols and methoxy groups on titanium dioxide and silica); Lec et al., J. Phys. Chem., 92, 2597 (1988) (rigid phosphates on metals).

g. Universal Probes

[0102] In some embodiments, the barcode oligonucleotide and porous particle are members of a universal probe which may be used in an assay for any target nucleic acid that comprises at least two portions. This "universal probe" comprises oligonucleotides of a single "capture" sequence that is complementary to at least a portion of a reporter oligonucleotide (e.g. barcode DNA), and to a portion of a target recognition oligonucleotide. The target recognition oligonucleotides comprise a sequence having at least two portions; the first portion comprises complementary sequence to the capture sequence attached to the porous particle, and the second portion comprises complementary sequence to the first portion of the particular target nucleic acid sequence. Various types of target recognition oligonucleotides can be used to great advantage with the universal probe, such that a library of target

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recognition oligonucleotides can be switched or interchanged in order to select for particular target nucleic acid sequences in a particular test solution. A capture oligonucleotide, which comprises sequence complementary to the second portion of the target nucleic acid is attached to the magnetic probe particle.

[0103] These universal probes can be manipulated for increased advantage, which depend on the particular assay to be conducted. The probes can be "tuned" to various single target nucleic acid sequences, by simply substituting or interchanging the target recognition oligonucleotides, such that the second portion of the universal probe comprises complementary sequence to different target nucleic acid of interests. Similarly, if multiple target nucleic acid sequences are to be assayed in a single test solution, the reporter oligonucleotides can comprise a sequence that is specific for each target nucleic acid, whereby, detection of the reporter oligonucleotide of known and specific sequence would indicate the presence of the particular target nucleic acid in the test solution. A capture oligonucleotide, which comprises sequence complementary to the second portion of the target nucleic acid is attached to the nanoparticle.

h. Dendrimers

[0104] In one aspect of this embodiment of the invention, particles conjugated with dendrimers labeled with at least two types of oligonucleotides are provided. Dendritic molecules are structures comprised of multiple branching unit monomers, and are used in various applications. See, e.g. Barth et al., *Bioconjugate Chemistry* 5:58-66 (1994); Gitsov & Frechet, *Macromolecules* 26:6536-6546 (1993); Lochmann et al., *J. Amer. Chem. Soc.* 115:7043-7044 (1993); Miller et al., *J. Amer. Chem. Soc.* 114:1018-1025 (1992); Mousy et al., *Macromolecuels* 25:2401-2406 (1992); Naylor et al., *J. Amer. Chem Soc.* 111:2339-2341 (1989); Spindeler & Frechet, *Macromolecules 26:4809-4813* (1993); Turner et al., *Macromolecules* 26:4617-4623 (1993); Wiener et al., *Magnetic Resonance Med.* 31(1):1-8 (1994); Service, 267:458-459 (1995); Tomalia, *Sci. Amer.* 62-66 (1995); and U.S. Pat. Nos. 4,558,120; 4,507,466; 4,568,737; 4,587,329; 4,857,599; 5,527,524; 5,338,532 to Tomalia, and U.S. Pat. No. 6,274,743 to Nilsen, all of which are incorporated by reference in their entirety, for all purposes. Dendritic molecules provide important advantages over other types of supermolecular architectures, such as contacting a maximum volume a minimum of structural units, ability to more easily control size, weight, and growth properties, and the multiple termini can be derivatized to yield highly labeled molecules with defined spacing between the labels, or provide sites of attachment for other molecules, or mixtures thereof.

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See generally U.S. Pat. No. 6,274,723 and the above cited references for methods of synthesis. Nucleic acid dendrimers that are useful in the methods of the invention are any of those known in the art that can be functionalized with nucleic acids or generated from nucleic acids/oligonucleotides. Such dendrimers can be synthesized according to disclosures such as Hudson et al., "Nucleic Acid Dendrimers: Novel Biopolymer Structures," *Am. Chem. Soc.* 115:2119-2124 (1993); U.S. Pat. No. 6,274,723; and U.S. Pat. No. 5,561,043 to Cantor.

IV. Colorimetric Method

[0105] In a preferred embodiment, the present invention provides for a simple, ultrasensitive colorimetric bio-barcode assay. The screening methods and detection schemes of the present invention are based upon those described by one of the inventors and others in US Pat. Appl. No. 10/ 877,750, published as US20050037397; U.S. Pat. Appl. No. 10/788,414, published as US20050009206; and U.S. Pat. Appl. No. 10/108211, published as US20020192687, again all of which are hereby incorporated by reference for all purposes. In a preferred embodiment, the present bio-barcode assay provides an improved bio-barcode approach to analyte detection by providing a colorimetric assay having improved amplification of bio-barcode DNA, and quantification and multiplexing capability.

[0106] In one embodiment, as shown in the examples, a colorimetric assay is used to detect barcode DNA because it does not require complicated instrumentation or experiment steps. Simple mixing and separation of probe solutions would result in attomolar sensitivity without using a microarrayer, complicated signal amplification steps such as enzymatic amplification and silver-enhancement, or sophisticated signal measurement tools. Since the readout is based on color change, minimal expertise is required to perform the assay.

[0107] In some embodiments, the color change can be detected and quantified by use of an image analysis means. In another embodiment, the color change can be visually detected by eye.

[0108] In some embodiments, detection of the barcode oligonucleotide is performed by a colorimetric assay. In some embodiments, the colorimetric assay comprises detecting the barcode oligonucleotide by providing a solution comprising a first and second particle probe, wherein the first particle probe comprises a capture oligonucleotide complementary to one end of the barcode oligonucleotide, and wherein the second particle probe comprises a capture oligonucleotide complementary to an opposite end of the barcode oligonucleotide; contacting the barcode oligonucleotide with the solution and allowing hybridization of the barcode oligonucleotide to the first and second particle probes, whereby the first and second

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particle probes assemble an aggregate, wherein a color change in the solution indicates formation of said aggregates; and detecting the color change in said solution.

[0109] The colorimetric detection of barcode DNA is carried out by visual detection of aggregated nanoparticles. Each type of nanoparticle contains a predetermined capture oligonucleotide complementary to specific barcode oligonucleotide for a particular target analyte. In the presence of target analyte, probe complexes are produced as a result of the binding interactions between the microparticles, magnetic particles and the target analyte. The barcode oligonucleotides are released from the complex and can be isolated and analyzed by any suitable means, e.g., thermal denaturation, to detect the presence of one or more different types of reporter oligonucleotides. However, it is contemplated that further amplification is not necessary for colorimetric detection.

[0110] In a preferred embodiment, the method further comprises contacting a solution containing the particle capture probes with the barcode oligonucleotides under conditions effective to allow specific binding interactions between the oligonucleotides to form an aggregate complex to signal the presence of the target analyte in the sample; detecting for the presence or absence of a color change. In one embodiment, particle probes are used in the step to detect barcode DNA separated from the probe complex.

[0111] Presently preferred for use in detecting nucleic acids are gold or silver nanoparticles. Gold and silver colloidal particles have high extinction coefficients for the bands that give rise to their beautiful colors. These intense colors change with particle size, concentration, interparticle distance, and extent of aggregation and shape (geometry) of the aggregates, making these materials particularly attractive for colorimetric assays. For instance, hybridization of oligonucleotides attached to gold nanoparticles with oligonucleotides and nucleic acids results in an immediate color change visible to the naked eye (see, e.g., the Examples and Figure 4B). Suitable nanoparticles are also commercially available from, e.g., Ted Pella, Inc. (gold), Amersham Corporation (gold) and Nanoprobes, Inc. (gold).

[0112] Methods for using such nanoparticles for colorimetric detection have also been described by Chad A. Mirkin, Robert L. Letsinger, Robert C. Mucic, James J. Storhoff, A DNA-based method for rationally assembling nanoparticles into macroscopic materials, Nature 382, 607-609 (15 Aug 1996) and Selective Colorimetric Detection of Polynucleotides Based on the Distance-Dependent Optical Properties of Gold Nanoparticles, Science 22 August 1997; 277: 1078-1081. In a preferred embodiment where gold nanoparticles probes are used, the color change is observed from red to purple.

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[0113] Referring to Figure 4B, the method can be multiplexed. Multiplexing herein refers to the simultaneous detection of many different targets in one solution. This multiplexing can be done as shown in Figure 4A. One kind of nanostructure (e.g. 13 nm gold nanoparticle) can be used with different spot positions (this is a simpler format). However, multiplexing with multiple labels would be more beneficial (this is true multiplexing since you detect several markers from one test tube by performing one experiment and you can differentiate target by looking color readout). The main idea here is to use different nanostructures (shape, composition, and size are variables) that present different optical properties, and these properties allow for labeling targets molecules with different nanostructures that exhibit many different colors.

[0114] Again, referring to Figure 4B, the method can also be performed using silver nanoparticles and other quantum dots for the readout. In embodiments where silver nanoparticle probes are used, the color change can be from orange, yellow or green and depending on the size, shape, etc of the particles, generally to a darker shade of yellowish or greenish color.

a. *Colorimetric Detection of Barcode Oligonucleotide*

[0115] The DNA barcodes or reporter oligonucleotides once released by dehybridization from the porous microparticles in the probe complex may then be detected by any suitable means. Generally, the DNA barcodes are released via dehybridization from the complex prior to detection. Any suitable solution or media may be used that dehybridize and release the DNA barcode from the complex. A representative medium is water.

[0116] In a preferred embodiment, the barcode DNA oligonucleotide is detected by: (a) providing a solution comprising a first and second nanoparticle probe, wherein the first nanoparticle probe is functionalized with a capture oligonucleotide complementary to one end of said specific DNA sequence of said barcode oligonucleotide, and wherein the second nanoparticle probe is functionalized with a capture oligonucleotide complementary to the opposite end of said specific DNA sequence of said barcode oligonucleotide; (b) mixing said barcode oligonucleotide separated from the probe complex with said solution to allow hybridization of said barcode oligonucleotide to said nanoparticle probes and the assembly of aggregates of said nanoparticle probes, wherein a color change in the solution reflects the formation of said aggregates; (c) spotting said solution on a substrate; (d) detecting a color change in said solution as compared to a control.

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[0117] In another embodiment, the detectable change (the signal) can be amplified and the sensitivity of the assay increased by employing a substrate having the nanoparticle probes bound or attached thereto. A solution containing the barcode oligonucleotides is then deposited on the substrate for subsequent detection.

[0118] In a preferred embodiment, nanoparticle probes functionalized with a capture oligonucleotide complementary to a portion of said specific DNA sequence are provided in a solution. Two sets of nanoparticle probes are provided; each is functionalized with a capture oligonucleotide complementary to one of two ends of a specific DNA sequence of the barcode oligonucleotide released from the probe complexes. Thus, the capture oligonucleotides attached to the one set of nanoparticle probes has a sequence complementary to the 5' end of the sequence of the barcode oligonucleotides to be detected, while the other set of nanoparticle probes has a sequence complementary to the 3' end of the sequence of the barcode oligonucleotides to be detected. The barcode oligonucleotide is then contacted with the two sets of nanoparticle probes under conditions effective to allow hybridization of the capture oligonucleotides on the nanoparticle probes with the barcode oligonucleotides. In this manner the barcode oligonucleotide becomes bound to at least two nanoparticle probes permitting assembly of aggregates of nanoparticle probes. The formation of aggregates of nanoparticle probes is thereby reflected in a colorimetric change of the solution containing the capture nanoparticle probe aggregates. The solution can then be spotted or delivered to a substrate for subsequent detection.

[0119] If sufficient complex is present in the solution, the complex can be observed visually with or without a background substrate. Any substrate can be used which allows observation of the detectable change. Suitable substrates include transparent solid surfaces (e.g., glass, quartz, plastics and other polymers), opaque solid surface (e.g., white solid surfaces, such as TLC silica plates, filter paper, glass fiber filters, cellulose nitrate membranes, nylon membranes), and conducting solid surfaces (e.g., indium-tin-oxide (ITO)). The substrate can be any shape or thickness, but generally will be flat and thin. Preferred are transparent substrates such as glass (e.g., glass slides) or plastics (e.g., wells of microtiter plates). In a preferred embodiment, the substrate is a TLC plate.

[0120] In one embodiment where the detection of the colorimetric change is used for diagnosis of a disease state of a patient, to insure against a false positive rate of occurrence, multiple panels or array should be provided to test. For example, a high-throughput microplate is provided, containing multiple wells each having the same solution of specific barcode and magnetic particle probes to identify target analytes. In another embodiment, the

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detection step of the method is performed multiple times for each single marker or analyte. For example, a clinician would make five spots for barcode analysis, removing the spots of the highest and the lowest spot intensities, and use the other three spots for the final quantification and diagnosis.

[0121] It is also contemplated that the two sets of nanoparticle probes provided for detection may be the same or different types of nanoparticles. This may further permit multiplexing for the purposes of identifying one or more to many different target analytes present in a sample. Referring to Figure 4A, multiplexing with multiple labels would be more beneficial allowing detection of several target analytes in one sample well. Multiplexing with a heterogeneous mixture of nanoparticles may require detection using Rayleigh Light-Scattering or Raman spectroscopy for detection of the specific optical signature or wavelength of each nanoparticle, as is known and practiced in the art.

[0122] The present invention also contemplates providing an array to detect more than one target analyte present in a sample. For example, providing a high-throughput microplate containing multiple wells each having solutions containing specific probes to identify target analytes.

[0123] In another embodiment, microfluidics are employed to automate and make massively parallel arrays. A suitable microfluidics device can be based on that described by one of the inventors and others in Proc. Natl. Acad. Sci. USA, 102, 9745 (2005), which is hereby incorporated by reference in its entirety.

[0124] Referring now to Figure 2, the present invention further provides a quantification method for a quantitative colorimetric barcode DNA detection assay, which was not possible with previous gold nanoparticle-based colorimetric DNA detection schemes. This quantification method can be carried out using graphic software developed using a method comprising the steps: (a) acquire a digital image of the aggregate spots on the substrate; (b) select a spot for analysis; (c) calculate the spot intensity as compared to a control spot. In one embodiment, step (b) further comprises the step of adjust contrast for better visualization and characterization. In a preferred embodiment, where gold nanoparticles are used, the quantification of aggregates and thereby the amount of analyte present in a sample is calculated according to the following:

$$\text{Spot Intensity} = \frac{\text{(Mean Value of Histogram through RED channel for the Control spot)}}{\text{(Mean Value of Histogram through RED channel for a Given spot)}}$$

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[0125] Spot intensity is proportional to the number of barcode DNA oligonucleotides, i.e., the more nanoparticles aggregated, the less red color appeared; and the number of barcode DNA oligonucleotides is proportional to the amount of target proteins present.

[0126] In a preferred embodiment, after adding barcode DNA to gold nanoparticle probes, the solution is spotted and dried on a TLC plate. The plate is scanned to acquire a digital scan of the plate. The scanned image contrast is adjusted using a graphic program such as ADOBE PHOTOSHOP software. Each nanoparticle spot is then selected, and the selected area is quantified using a quantification function such as the Histogram function in PHOTOSHOP with red channel option. The mean value from the Histogram window is used to calculate the spot intensity of each spot.

[0127] Finally, this assay should be suitable for point-of-care applications with the requirement only for probe solutions and TLC plates. Efforts to optimize the detection system for better quantification, and multiplex the system with other cytokines are currently ongoing. It is contemplated that the present embodiments described can be varied or optimized according to concentrations of probe solutions, probe size, reaction time, synthesizing more monodispersed porous microparticles, or by minimizing cross-reactivity for multiplexing (e.g., by further probe passivation or adjusting reaction time).

V. Kit for Detecting Analytes

[0128] In one embodiment, the invention provides for a kit to carry out the present method comprising a high-throughput microplate, containing an array of wells, each well having the same or different solution of specific barcode and magnetic particle probes to identify an analyte of interest. An aliquot of the sample is mixed with each well in the array, thereby allowing the assay to be performed in parallel wells. In another embodiment, the detection step of the method is performed multiple times for each single marker or analyte. For example, a clinician would make five spots for barcode analysis, removing the spots of the highest and the lowest spot intensities, and use the other three spots for the final quantification and diagnosis.

[0129] Optionally, in one embodiment, the invention provides for a device to carry out the image analysis comprised of a means for obtaining digital signal, such as a flatbed scanner or CCD camera, and a means for analysis, such as a computer having graphic software that can analyze pixel intensity. In a preferred embodiment, the device is comprised of a plain flatbed scanner and a computer having software such as ADOBE PHOTOSHOP (Adobe Systems, San Jose, CA) to analyze pixel intensity.

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VI. Examples

[0130] The following examples are meant to exemplify and illustrate, but not to limit the invention.

Example 1: Materials and Methods

[0131] Electron Micrographs. LEO 1550 Scanning Electron Micro-scope (SEM) at UC Berkeley Microlab facility has been used. The images were taken using 3 kV acceleration voltage at a working distance of 3 mm after vapor deposition of ~3 nm Chromium onto the sample.

[0132] Barcode Probe Preparation. To prepare the barcode probes, 1 ml of an aqueous suspension of the amino-functionalized porous silica microparticles (1.57×10^9 ml^{-1} diameter: 3.53 ± 0.49 μm; obtained from Phenomenex, Torrance, CA) was centrifuged for 5 min at 10,000 rpm, and the supernatant was removed. The particles were re-suspended in PBS solution, and the centrifugation step was repeated once more. The resulting polystyrene particle pellet was re-suspended in 1 ml of 8 % glutaraldehyde in PBS solution at pH 7.4. The solution was mixed for 5 hrs on a rocking shaker. Centrifugation followed for 5 min at 10,000 rpm, and the supernatant was discarded (this step was repeated two more times). The resulting pellet was re-suspended in PBS, and 5 μg of monoclonal antibody for IL-2 was added to the solution. The amount of antibody (5 μg) is much less than the amount of antibody recommended by Polysciences, Inc. to fully modify the particle surface (antibodies were purchased from Abcam, Inc, Cambridge, MA). The solution was left on a shaker overnight to link the anti-IL-2 to the activated polystyrene particles. Analogous glutaraldehyde linker chemistry has been extensively used by others to effect protein linking to amino functionalized particles. 3'Amino-functionalized bar-code DNA complements (1 ml at 100 μM; 5' CGTCGCATTCAGGATTCTCAACTCGTAGCT-A_{10}-C6-amine 3' (SEQ ID NO: 1)) were then added to the monoclonal antibody-modified silica particles, and the centrifugation step was repeated twice. The resulting pellet was re-suspended in 1 ml of 0.2 M ethanolamine to passivate all unreacted glutaraldehyde sites on the microparticles for 30 min at room temperature. Centrifugation was performed to remove supernatant. Bovine serum albumin solution (10% BSA) was subsequently added to further passivate the protein-inactive regions of the particle surface. The centrifugation step was repeated twice, and the supernatant was removed. The resulting pellet was re-suspended in 1 ml of 0.15 M PBS solution.

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[0133] Magnetic Probe Preparation. Amino-functionalized magnetic particles (Dynal Biotech, Brown Deer, WI) were linked to monoclonal antibodies for IL-2. The epitopes of these antibodies are different from those of the antibodies used to prepare the barcode probes (Abcam, Cambridge, MA) using glutaraldehyde-amine coupling chemistry. Amino-functionalized magnetic particles in 0.05 mM EDTA solution (5 ml solution at 1 mg/ml) were washed with 10 ml of pyridine wash buffer. The resulting solution was magnetically separated, and the supernatant was removed (repeated two more times). The magnetic particles were then activated with 5 ml of 5 % glutaraldehyde in pyridine wash buffer for 3 hrs at room temperature. The activated magnetic particles were then magnetically separated, and the supernatant was removed. This magnetic separation step was repeated twice, and the magnetic particles were re-suspended in 10 ml of pyridine wash buffer. The monoclonal anti-IL-2 in pyridine wash buffer (1 ml at 750 μg/ml) was then added to magnetic particles, and the solution was mixed for 10 hrs at room temperature. Then, 1 mg of BSA was added to the magnetic particle solution, and the solution was mixed for an additional 10 hrs at room temperature. The magnetic separation step was repeated twice, and the magnetic particles were re-suspended in 5 ml of pyridine wash buffer. Then 3 ml of glycine solution (1 M at pH 8.0) was added to the resulting solution to quench all of the unreacted aldehyde sites, and the resulting solution was stirred for 30 min. After the magnetic separation step, 5 ml of wash buffer was added to the monoclonal antibody-functionalized magnetic particles and mixed vigorously (this step is repeated two more times). The magnetic particles were then magnetically separated and the supernatant was removed. This washing step was repeated three more times. Finally, the magnetic probes were re-suspended in 0.15 M PBS solution.

[0134] Barcode DNA Quantification. After adding barcode DNA to gold nanoparticle probes, the solution was spotted and dried on a TLC plate. The plate was scanned using a flatbed scanner, and the scanned image was adjusted using Adobe Photoshop software (all the spots were adjusted together). Each nanoparticle spot was then selected, and the selected area was quantified using the Histrogram function with red channel option of the Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The mean value from the Histogram window was used to calculate the spot intensity of each spot (Figure 2).

Example 2: Colorimetric Bio-Barcode Amplification Assay for Cytokines

[0135] In this work, our assay target is interleukin-2 (IL-2). IL-2 is a secreted human cytokine protein that mediates local interactions between white blood cells during inflammation and immune responses. Cytokines play a central role in the regulation of

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hematopoiesis; mediating the differentiation, migration, activation and proliferation of phenotypically diverse cells.[21,22] Improved detection limits of cytokines will allow for earlier and more accurate diagnosis and treatments of cancers and immunodeficiency-related diseases and lead to an increased understanding of cytokine-related diseases and biology, because cytokines are signature biomarkers when humans are infected by foreign antigens. Conventional cytokine detection assays have a detection limit of ~50 fM and the detection limit of enzyme-based rolling-circle amplification method is ~500 aM.

[0136] In a typical bio-barcode colorimetric bio-barcode assay, two types of probes were prepared (Figure 1A). The first is the barcode probe, a 3 μm porous silica particle modified with anti-IL-2 and the oligonucleotide which is complementary to a bar-code sequence (5' AGCTACGAGTTGAGAATCCTGAATGCGACG 3' (SEQ ID NO: 2)) that is a unique identification tag for the target molecule. The second probe is a 2.8 μm iron oxide magnetic probe particle, which has a magnetic iron oxide core with an amine-modified silane coating (Dynal Biotech, Brown Deer, WI). These particles were functionalized with anti-IL-2 molecules that can capture IL2 targets.

[0137] The detection limit for this assay is orders of magnitude better than other conventional immunoassays. In one embodiment, the assay is three orders of magnitude better in detecting IL-2 (e.g., 30 aM IL-2 in PBS buffer solution). Significantly, in this embodiment, the detection limit is ~15 times more sensitive than an enzyme-based amplification method in detecting IL-2.

[0138] In the IL-2 detection assay (Figure 1B), 15 μL of magnetic probe solution ($1.5x10^9$ beads/ml) was added to 20 μl of IL-2 solution, followed by the addition of 15 μl of barcode probe solution ($1x10^9$ beads/ml). The resulted solution was incubated at 37 °C for 50 min on an orbital shaker. Next, the solution was placed in a magnetic separator (Dynal Biotech, Brown Deer, WI), and the supernant was removed. Then the probe complex solution was washed with 0.15 M PBS solution three more times. Finally, 50 μl of NANOpure water (18 megohm) was added to the magnetically separated complexes to release the barcode DNA and the complexes were kept on a rocking shaker at 70 °C for 10 min. After magnetic separation, the supernatant including free barcode DNA strands was collected for barcode DNA detection. To detect the barcode DNA, 30 nm gold nanoparticle probes (25 μl at 1 nM for both probe 1 and 2) functionalized for barcode DNA capture (barcode capture probe 1: 5' TCTCAACTCGTAGCTAAAAAAAAAA-triethylene glycol-SH 3' (SEQ ID NO: 3); barcode capture probe 2: 5' SH-triethylene glycol-AAAAAAAAAACGTCGCATTCAGGAT 3' (SEQ ID NO: 4)) were added to the barcode DNA in 0.15 M PBS solution. The resulting solution

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was kept at room temperature for one and half hours. The solution was then centrifuged to increase the concentration of probe complexes and to collect small nanoparticle aggregates (10,000 rpm for 5 min), and the supernatant was discarded. Although a centrifugation step is used here, this step may not be essential for actual implementation of the assay after further optimization. Finally, 5 μl of nanoparticle probe solution from the concentrated nanoparticle solution was spotted on a reverse-phase silica TLC plate (EMD Chemicals, Inc., Gibbstown, NJ) for target verification and quantification (Figure 2A). The spot test was ranged from 30 aM to 300 fM and included a control sample where no IL-2 is present. This assay can detect as low as 30 aM IL-2 targets in the presence of background proteins (1 μl of 5 μM anti-biotin and 1 μl of 5 μM anti-fibronectin per sample). Spotted dots show not only different colors but also different intensities. Each spot intensity was quantified using image analysis software based on the red color intensity that reflects the aggregation of gold nanoparticles (Adobe Photoshop, Adobe Systems Incorporated, San Jose, CA). Because this colorimetric assay is based on the color change from red (without barcode DNA) to purple (with barcode DNA), a lower mean red color channel value is indicative of more barcode DNA present in solution (Figure 2). Spot intensity herein is defined by the mean red channel value of a control spot divided by the mean red channel value of a given sample spot. These spot intensity values are plotted in Figure 3A (experiments were repeated five times, and the highest and the lowest values were not used for the final spot intensity calculation). The spot intensity of a 30 aM target solution is higher than that of the control spot, and the dynamic range of this assay ranges from 30 aM to 300 fM (Figure 3A).

[0139] To validate this colorimetric bio-barcode system for real samples, IL-2 molecules in human serum samples (Cambrex Corp., East Rutherford, NJ) were tested with the same protocol that was used for IL-2 detection in PBS buffer solution. Nanoparticle-based barcode detection spots for 300 aM, 3 fM, 30 fM, and 300 fM IL-2 samples were distinctively different from the control spot (Figure 3B). The spot intensity rapidly saturates after 30 fM.

[0140] Any patents, patent publications, publications, or GenBank Accession numbers cited in this specification are indicative of levels of those skilled in the art to which the patent pertains and are hereby incorporated by reference to the same extent as if each was specifically and individually incorporated by reference.

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CLAIMS

What is claimed is:

1. A method for detecting an analyte of interest comprising the steps of:
   (a) providing a sample suspected of containing said target analyte of interest;
   (b) contacting (I) a porous microparticle probe comprising a first ligand that specifically binds said target analyte of interest, and a barcode oligonucleotide and (II) a magnetic particle probe comprising a second ligand that specifically binds said target analyte of interest with said sample, and allowing said porous microparticle probe and said magnetic particle probe to bind to said analyte of interest, if present in said sample, to form a complex between said porous microparticle probe and said magnetic particle probe;
   (c) separating said complex from said sample;
   (d) releasing and collecting said barcode oligonucleotide from said complex; and
   (e) detecting said barcode oligonucleotide.

2. The method of claim 1 wherein said analyte of interest is selected from the group consisting of nucleic acids, proteins, peptides, metal ions, haptens, drugs, metabolites, pesticides and pollutants.

3. The method of claim 1 wherein said analyte of interest is a cytokine.

4. The method of claim 1 wherein said analyte of interest is a chemokine.

5. The method of claim 1 wherein said porous microparticle probe comprises a material selected from the group consisting of polystyrene, cellulose, silica, iron oxide, polyacrylamide, polysaccharides, dextran, agarose, and cellulose.

6. The method of claim 5 wherein said porous microparticle probe is modified with an amine.

7. The method of claim 1 wherein said microparticle has a size of about 0.1 micrometers to about 5000 micrometers.

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8. The method of claim 1 wherein said microparticle has a size of about 0.5 micrometers to about 10 micrometers.

9. The method of claim 1 wherein said microparticle has a size of about 3 micrometers to about 5 micrometers.

10. The method of claim 1 wherein said porous microparticle probe has a pore size of about 50 angstroms to about 150 angstroms.

11. The method of claim 1 wherein said porous microparticle probe has a pore size of about 90 angstroms to about 110 angstroms.

12. The method of claim 1 wherein said porous microparticle probe has a surface area of about $300m^2/g$ to about $500m^2/g$.

13. The method of claim 1 wherein said porous microparticle probe has a surface area of about $400m^2/g$ to about $450m^2/g$.

14. The method of claim 1 wherein said barcode oligonucleotide is selected from the group consisting of genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, natural and synthetic nucleic acids, and aptamers.

15. The method of claim 14 wherein said barcode oligonucleotide is modified with a detectable label.

16. The method of claim 15 wherein said detectable label is selected from the group consisting of biotin, radiolabel, fluorescent label, chromophore, redox-active group, group with an electrical signature, catalytic group, and Raman label.

17. The method of claim 1 wherein said barcode oligonucleotide and said microparticle are members of a universal probe.

18. The method of claim 1 wherein said ligand is a monoclonal or polyclonal antibody.

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19. The method of claim 1, wherein step (e) is a colorimetric assay.

20. The method of claim 19, wherein said colorimetric assay comprises detecting said barcode oligonucleotide by:

(i) providing a solution comprising a first and second particle probe, wherein said first particle probe comprises a capture oligonucleotide complementary to one end of said barcode oligonucleotide, and wherein said second particle probe comprises a capture oligonucleotide complementary to an opposite end of said barcode oligonucleotide;

(ii) contacting said barcode oligonucleotide with said solution of step (i) and allowing hybridization of said barcode oligonucleotide to said first and second particle probes, whereby said first and second particle probes assemble an aggregate, wherein a color change in the solution indicates formation of said aggregates;

(iii) detecting said color change in said solution.

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Figure 1

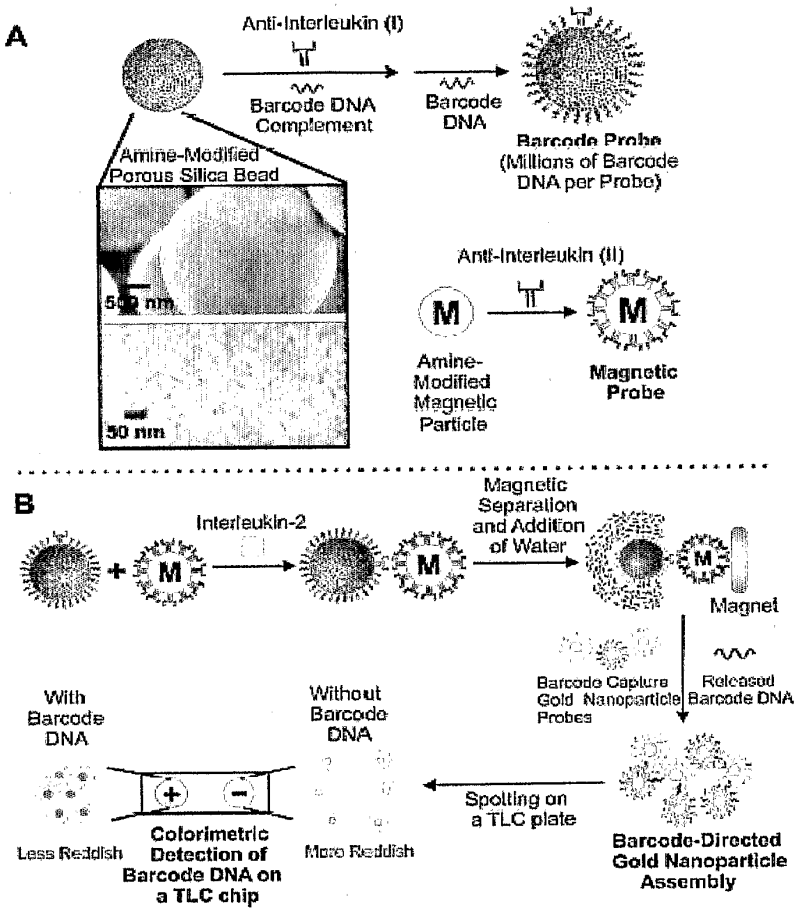
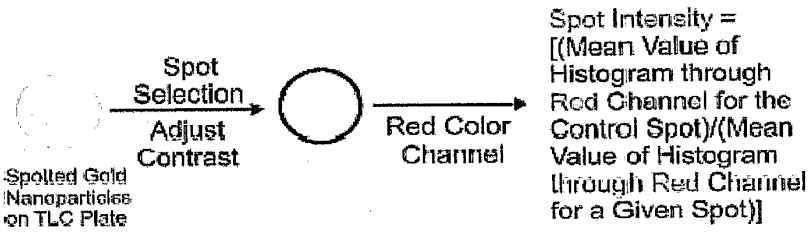

A
Anti-Interleukin (I)
Barcode DNA Complement
Barcode DNA
Barcode Probe
(Millions of Barcode DNA per Probe)
Amine-Modified Porous Silica Bead
50 nm
Anti-Interleukin (II)
M
Amine-Modified Magnetic Particle
Magnetic Probe
B
Interleukin-2
Magnetic Separation and Addition of Water
Magnet
Barcode Capture Gold Nanoparticle Probes
Released Barcode DNA
With Barcode DNA
Without Barcode DNA
Spotting on a TLC plate
Barcode-Directed Gold Nanoparticle Assembly
Less Reddish
Colorimetric Detection of Barcode DNA on a TLC chip
More Reddish

Figure 2


Spot
Selection
Adjust
Contrast
Red Color
Channel
Spot Intensity =
[(Mean Value of
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Control Spot)/(Mean
Value of Histogram
through Red Channel
for a Given Spot)]
Spotted Gold
Nanoparticles
on TLC Plate

Figure 3

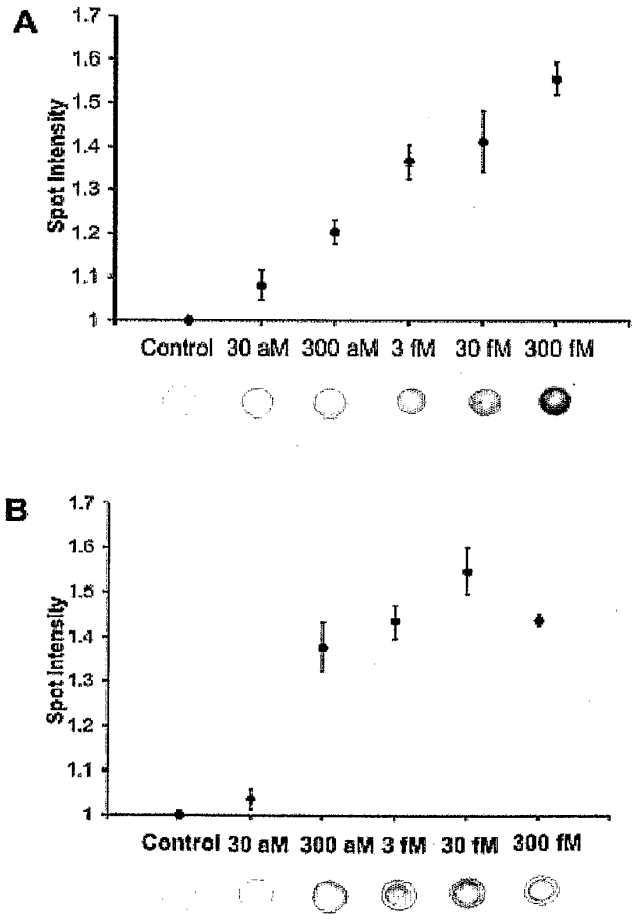

A
Spot Intensity
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
Control 30 aM 300 aM 3 fM 30 fM 300 fM
B
Spot Intensity
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
Control 30 aM 300 aM 3 fM 30 fM 300 fM

Figure 4A

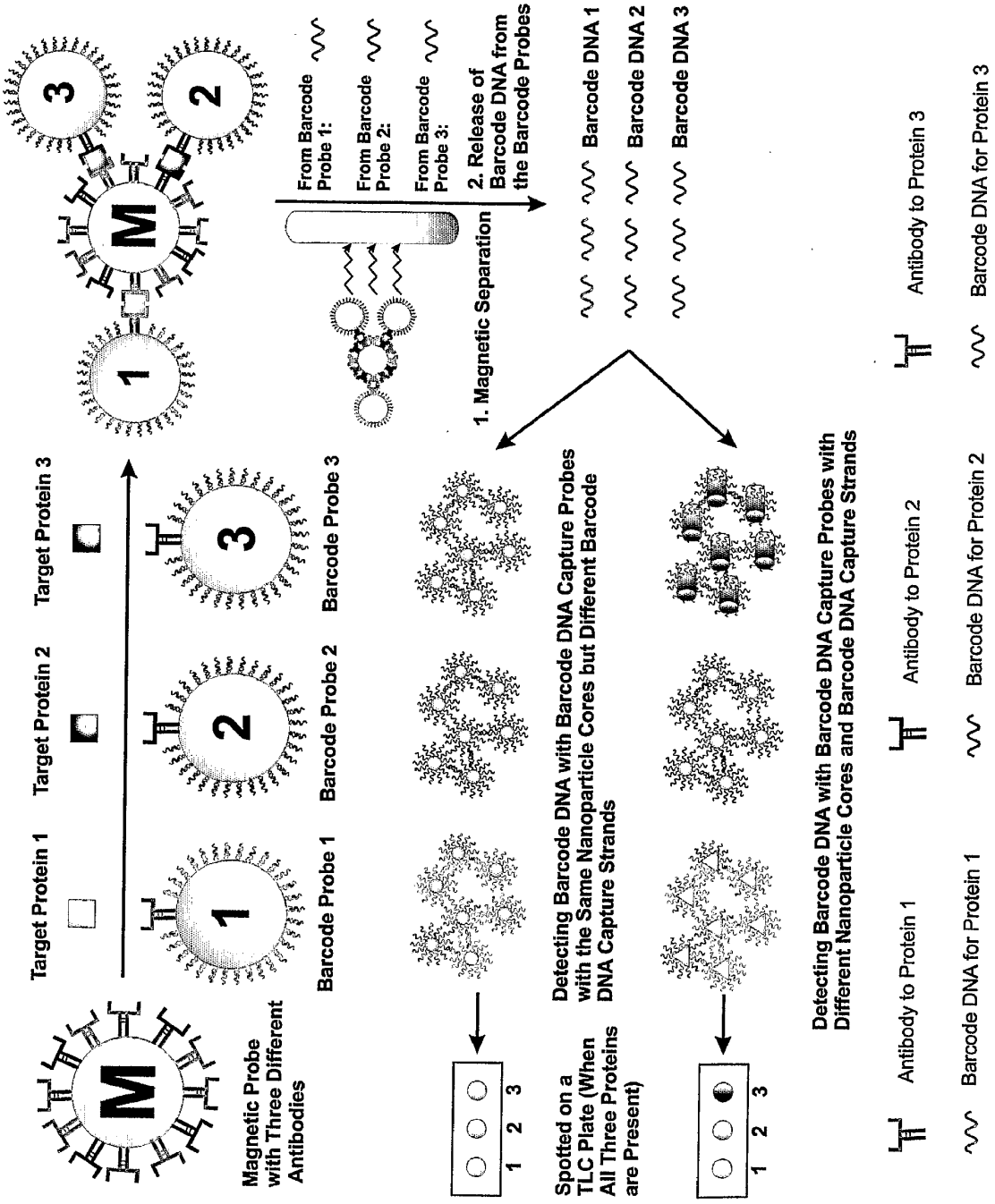
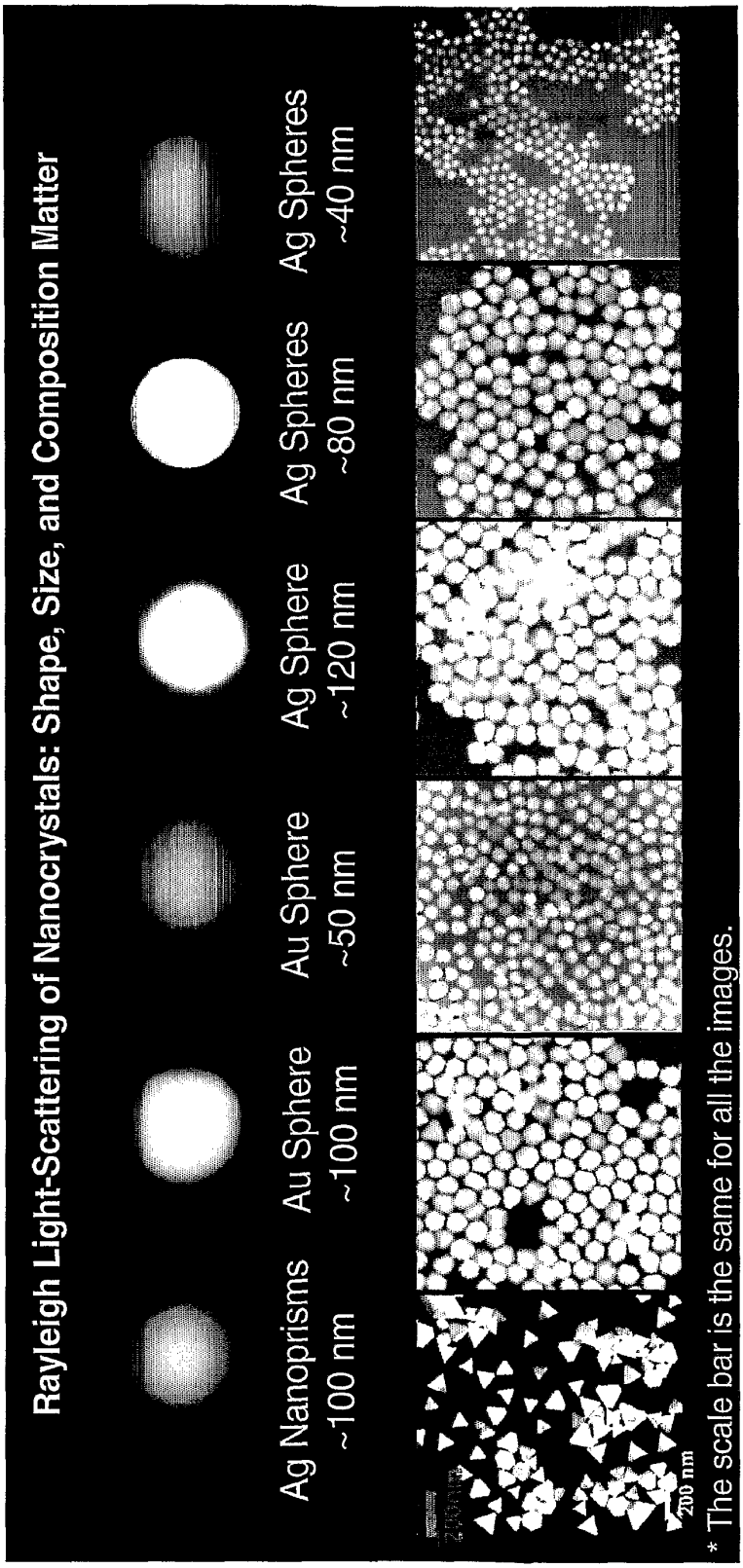

Target Protein 1
Target Protein 2
Target Protein 3
Barcode Probe 1
Barcode Probe 2
Barcode Probe 3
Magnetic Probe with Three Different Antibodies
From Barcode Probe 1:
From Barcode Probe 2:
From Barcode Probe 3:
1. Magnetic Separation
2. Release of Barcode DNA from the Barcode Probes
Barcode DNA 1
Barcode DNA 2
Barcode DNA 3
Spotted on a TLC Plate (When All Three Proteins are Present)
1 2 3
Detecting Barcode DNA with Barcode DNA Capture Probes with the Same Nanoparticle Cores but Different Barcode DNA Capture Strands
Detecting Barcode DNA with Barcode DNA Capture Probes with Different Nanoparticle Cores and Barcode DNA Capture Strands
Antibody to Protein 1
Antibody to Protein 2
Antibody to Protein 3
Barcode DNA for Protein 1
Barcode DNA for Protein 2
Barcode DNA for Protein 3

Figure 4B


Rayleigh Light-Scattering of Nanocrystals: Shape, Size, and Composition Matter
Ag Nanoprisms ~100 nm
Au Sphere ~100 nm
Au Sphere ~50 nm
Ag Sphere ~120 nm
Ag Spheres ~80 nm
Ag Spheres ~40 nm
200 nm
* The scale bar is the same for all the images.

Sequence Listings

<110> The Regents of the University of California
Nam, Jwa-Min
Groves, Jay T.

<120> A Colorimetric Bio-Barcode Amplification Assay for Analyte Detection

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<151> 2005-09-16

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tctcaactcg tagctaaaaa aaaaa 25

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<213> Synthetic

<220>
<221> Functionalized
<222> (1)..(1)
<223> Attached to the 5' end of the sequence is a "SH-triethylene

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<400> 4
aaaaaaaaaa cgtcgcattc aggat 25

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(54) Title: MTHODS AND ARRAYS FOR TARGET ANALYTE DETECTION AND DETERMINATION OF TARGET ANALYTE CONCENTRATION IN SOLUTION

(57) Abstract: Arrays of single molecules and methods of producing an array of single molecules are described. Arrays with defined volumes between 10 attoliters and 50 picoliters enable single molecule detection and quantitation. Biomolecules such as enzymes can be addressed at the single molecule level in order to discover function, detect binding partners or inhibitors, and/or measure rate constants.

Methods and Arrays for Target Analyte Detection and Determination of Target Analyte Concentration in Solution

Government Rights

[001] The United States government may have certain rights in this invention pursuant to Contract No. N00014-01-1 awarded by the Department of Defense, Defense Advanced Research Projects Agency (DARPA) Office of Naval Research.

Background

[002] Methods that implement high-sensitivity and low-level analyte detection in conjunction with rapid and reproducible experimental protocols are the cornerstone of modern analytical measurements. Currently, most known techniques for quantifying low levels of target analyte in a sample matrix use amplification procedures to increase the number of reporter molecules and thereby provide a measurable signal. These known processes include enzyme-linked immunosorbent assays (ELISA) for amplifying the signal in antibody-based assays, as well as the polymerase chain reaction (PCR) for amplifying target DNA strands in DNA-based assays. A more sensitive but indirect protein target amplification technique, called immuno-PCR (*see* Sano, T.; Smith, C. L.; Cantor, C. R. *Science* 1992, *258*, 120-122), makes use of oligonucleotide markers, which can subsequently be amplified using PCR and detected using a DNA assay (*see* Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884-1886; Niemeyer, C. M.; Adler, M.; Pignataro, B.; Lenhert, S.; Gao, S.; Chi, L. F.; Fuchs, H.; Blohm, D. *Nucleic Acids Research* **1999**, *27*, 4553-4561; and Zhou, H.; Fisher, R. J.; Papas, T. S. *Nucleic Acids Research* **1993**, *21*, 6038-6039). While the immuno-PCR method permits ultra low-level protein detection, it is a complex assay procedure, and can be prone to false-positive signal generation (*see* Niemeyer, C. M.; Adler, M.; Wacker, R. *Trends in Biotechnology* **2005**, *23*, 208-216).

[003] One disadvantage of these known methods is their reliance on separate steps to amplify reporter molecules to provide a measurable signal, thereby requiring additional amplification steps and thus additional time, equipment, and materials.

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[004] In addition, known methods for accurately quantifying the concentration of a particular analyte in solution are all based on ensemble responses in which many analyte molecules give rise to the measured signal.

[005] Therefore, there is a need in the art for an improved method and system of target analyte detection.

Brief Summary of the Invention

[006] According to one embodiment, the present invention relates to a method of detecting a target analyte in a sample. The method includes providing an array comprising a plurality of sites, each site comprising a capture component, and contacting the array with the sample such that each site in a subset of the plurality of sites contains a single target analyte. Each target analyte comprises an enzymatic component. The method further includes contacting the array with an enzymatic substrate and detecting a change in an optical property at each of the sites as an indication of the presence of the target analyte.

[007] The present invention, in another embodiment, relates to a method of detecting target analytes in a sample. The method includes providing an array comprising a plurality of sites, and contacting the array with the sample such that each site in a first subset of the plurality of sites contains a single first target analyte and each site in a second subset of the plurality of sites contains a single second target analyte. In this embodiment, each site comprises a capture component and each of the first and second target analytes comprises an enzymatic component. The method further includes contacting the array with a first enzymatic substrate and detecting any change in an optical property as a result of the first enzymatic substrate at each of the sites as an indication of the presence of one of the first or second target analytes. In addition, the method includes washing the array and contacting the array with a second enzymatic substrate. Further, the method includes detecting any change in an optical property as a result of the second enzymatic substrate at each of the sites as an indication of the presence of one of the first or second target analytes.

[008] In accordance with another embodiment, the present invention relates to a method of detecting a target analyte in a sample. The method includes providing an array comprising a plurality of sites and contacting the array with the sample such that each site in a subset of the plurality of sites contains a single target analyte. In this method, each site comprises a capture

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component. The method also includes contacting each of the single target analytes with a binding ligand comprising an enzymatic component and further contacting the array with an enzymatic substrate. In addition, the method includes detecting a change in an optical property at each of the sites as an indication of the presence of the target analyte.

[009] The present invention, according to a further embodiment, is a method of quantifying an amount of a target analyte in a sample. The method includes providing an array comprising a plurality of sites, each site comprising a capture component and contacting the array with the sample such that each site in a subset of the plurality of sites contains a single target analyte. In this embodiment, each target analyte comprises an enzymatic component. The method also includes contacting the array with an enzymatic substrate, detecting a change in an optical property at each of the sites as an indication of the presence of the target analyte, and calculating an amount of the target analyte in the sample.

[010] In one embodiment, the present invention contemplates a method of producing an array of single molecules, comprising: providing a sample and an array, said sample comprising a molecules of target analyte in a concentration, said array comprising at least 1000 sites, each site having a defined volume between 10 attoliters and 50 picoliters; and contacting said array with said sample such that the ratio of molecules of target analyte to each site of said array is less than 1:1, so as to produce an array comprising sites containing single molecules. In one embodiment, the present invention contemplates the array, as a composition of matter, produced according to this method. It is not intended that the present invention be limited by the nature of the target analyte. In one embodiment said target analyte comprises biomolecules (for example, proteins, nucleic acids, lipids, and carbohydrates; alternatively, hormones, cytokines and cellular antigens; preferably, receptors such as neural receptors, hormonal receptors, and nutrient receptors). It is not intended that the present invention be limited by the nature of the biomolecule. In one embodiment, said biomolecule is selected from the group consisting of proteins, nucleic acids, lipids, and carbohydrates. In another embodiment, said biomolecule is selected from the group consisting of hormones, cytokines and cellular antigens. In yet another embodiment, said biomolecule is a receptor (e.g. neural receptors, hormonal receptors, and nutrient receptors). In one embodiment, said target analyte is an environmental pollutant (e.g. pesticides, insecticides and toxins). In one embodiment, said target analyte is a drug. It is not intended that the present invention be limited by the ratio. In one embodiment, the ratio is chosen

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so that one achieves sites with single molecules. In a preferred, embodiment, the ratio is chosen so that the readout will be a digital readout (e.g. each site will have either one molecule or none). In one embodiment, the ratio is less than 1:5. In another embodiment, said ratio is 1:10. In still another embodiment, said ratio is between 1:5 and 1:500. In one embodiment, the percentage of sites containing a single molecule provide a measure of the concentration of molecules in said sample (in other words, by counting and knowing the number of total sites and the defined volume of the sites).

[011] In one embodiment, the method further comprises, after step b), sealing the array such that the contents of each site cannot escape said site. In one embodiment, the present invention contemplates the sealing of molecules in solution, the capture of molecules in solution (e.g. with a capture component) before sealing, and/or the capture of molecules from the solution (and in some embodiments, the removal of molecules from the solution) before sealing. In one embodiment, the present invention contemplates the sealed array, produced according to the method.

[012] It is not intended that the present invention be limited by the nature of the capture component. In one embodiment, wherein each site of said array comprises a capture component. In another embodiment, said target analyte comprises a single-stranded nucleic acid, and said capture component comprises a complementary nucleic acid

[013] It is not intended that the present invention be limited to any particular volume. However, in one embodiment, said defined volume is measured in femtoliters. In one embodiment, the present invention contemplates, as a composition of matter, an array of sites, said sites of defined femtoliter volume.said defined volume is the same at each site and ranges from about 30 femtoliters to about 60 femtoliters. In one embodiment, said defined femtoliter volume is 46 femtoliters.

[014] It is not intended that the present invention be limited to the number of sites. While 1000 sites may be used, preferably more sites are employed (e.g. more than 10,000, between 20,000 and 30,000 sites, and still more preferably between 100,000 and 10,000,000 sites.

[015] In another embodiment, the present invention contemplates a method of detecting a target analyte in a sample, the method comprising: providing a sample and an array, said sample comprising a biomolecule target analyte, said array comprising at least 1000 sites, each

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site having a defined volume between 10 attoliters and 50 picoliters; contacting said array with said sample such that the ratio of biomolecule target analyte to each site of said array is less than 1:1; and determining the number of sites of said array which contain a single biomolecule. Again, it is not intended that the present invention be limited by the ratio. In one embodiment, the ratio is chosen so that one achieves sites with single molecules. In a preferred, embodiment, the ratio is chosen so that the readout will be a digital readout (e.g. each site will have either one molecule or none). In one embodiment, the ratio is less than 1:5. In another embodiment, said ratio is 1:10. In still another embodiment, said ratio is between 1:5 and 1:500. In one embodiment, the percentage of sites containing a single molecule provide a measure of the concentration of molecules in said sample (in other words, by counting and knowing the number of total sites and the defined volume of the sites).

[016] Preferably, the biomolecules are labeled (e.g. with a fluorophore). In one embodiment, said determining comprises detecting a change in an optical property at said sites as an indication of the presence of said target analyte. Again, it is preferred that the percentage of sites containing a single biomolecule provides a measure of the concentration of biomolecules in said sample.

[017] In one embodiment, the method further comprises, after the contacting step but before the determining step, sealing the array such that the contents of each site cannot escape said site. In one embodiment, the present invention contemplates the sealing of molecules in solution, the capture of molecules in solution (e.g. with a capture component) before sealing, and/or the capture of molecules from the solution (and in some embodiments, the removal of molecules from the solution) before sealing. In one embodiment, the present invention contemplates the sealed array, produced according to the method.

[018] In yet another embodiment, the present invention contemplates a method of detecting a target analyte in a sample, the method comprising: providing a sample and an array, said sample comprising a biomolecule target analyte in a first concentration, said array comprising at least 10,000 sites, each site having a defined femtoliter volume; diluting said sample to create a diluted sample, said diluted sample comprising a biomolecule target analyte in a second concentration; contacting said array with said diluted sample such that the ratio of biomolecule target analyte to each site of said array is between 1:5 and 1:500; and determining the number of sites of said array which contain a single biomolecule.

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[019] In still another embodiment, the present invention contemplates a method of producing an array of single cells, the method comprising: providing a sample and an array, said sample comprising cells, said array comprising at least 1000 sites, each site having a defined volume between 10 attoliters and 50 picoliters; and contacting said array with said sample such that the ratio of cells to each site of said array is less than 1:1, so as to produce an array comprising sites containing single molecules. In one embodiment, the present invention contemplates, as a composition of matter, the array produced according to this embodiment of the method. In one embodiment of the method, the present invention further contemplates the step of determining which sites of the array contain single cells. Preferably, the percentage of sites containing a single cell provide a measure of the concentration of cells in said sample. It is not intended that the present invention be limited by the nature of the cells. Prokaryotic cells are contemplates (e.g. bacterial cells) as well as eukaryotic cells (e.g. mammalian cells such as tumor cells and nerve cells. Although it is not necessary to understand the mechanism of an invention, it is believed that an array of single cells allows capture of a single mRNA target per well. In one embodiment, the single cells are trapped and subsequently lysed. In one embodiment, a single well comprises a single cell. In one embodiment, the single cell within the single well is lysed. In one embodiment, the targets from the single cell are trapped within the single well. In another embodiment, a single cell is lysed and the mRNA targets are subsequently captured. For example, one single cell is lysed wherein a plurality of mRNA targets are captured in wells with no more than one copy of each transcript type per well. Although it is not necessary to understand the mechanism of an invention, it is believed that while a cell may comprise thousands of different transcripts with may copies of each per cell, the present invention contemplates capturing no more than one of each different transcript type per well (i.e., for example, each well comprises no more than one copy of each transcript). In one embodiment, the single cell comprises multiple targets (i.e., for example, mRNA types). In one embodiment, the multiple targets (i.e., for example, mRNA types) are sequentially interrogated thereby determining their presence (i.e., for example, by using different colored fluorophores). In one embodiment, the targets (i.e., for example, mRNA types) are quantitatively determined by detecting a digital signal.

[020] In still another embodiment, the present invention contemplates a method of producing an array comprising a plurality of different cells, the method comprising: providing a

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sample and an array, said sample comprising cells, said array comprising at least 1000 sites, each site having a defined volume between 10 attoliters and 50 picoliters; and contacting said array with said sample. In one embodiment, the plurality of different cells are lysed, thereby forming a lysate comprising multiple targets. In one embodiment, each of the plurality of different cells are lysed within different wells. In one embodiment, the plurality of different cells are lysed within a single well. In one embodiment, the lysates from the plurality of different cells are pooled. In one embodiment, the pooled lysates are captured. In one embodiment, the multiple targets (i.e., for example, mRNA types) are sequentially interrogated thereby determining their presence (i.e., for example, by using different colored fluorophores). In one embodiment, the targets (i.e., for example, mRNA types) are quantitatively determined by detecting a digital signal.

[021] In one embodiment, the method further comprises, after contacting the array with the cells, sealing the array such that the contents of each site cannot escape said site. In one embodiment, the present invention contemplates the sealing of cells in solution, the capture of cells in solution (e.g. with a capture component) before sealing, and/or the capture of cells from the solution (and in some embodiments, the removal of cells from the solution) before sealing. In one embodiment, the present invention contemplates the sealed array comprising cells, produced according to the above-described method. In one embodiment, cells are immobilized by said capture component (e.g. a receptor, antibody or protein, such as fibronectin) after contacting said array (e.g. the capture component is present, and preferably attached, at each site of the array).

[022] Again, it is not intended that the present invention be limited by the ratio of cells to sites. In one embodiment, the ratio is chosen so that one achieves sites with single cells. In a preferred, embodiment, the ratio is chosen so that the readout will be a digital readout (e.g. each site will have either one cells or none). In one embodiment, the ratio is less than 1:5. In another embodiment, said ratio is 1:10. In still another embodiment, said ratio is between 1:5 and 1:500. In one embodiment, the percentage of sites containing a single cell provide a measure of the concentration of molecules in said sample (in other words, by counting and knowing the number of total sites and the defined volume of the sites).

[023] In one embodiment, the present invention contemplates a method of detecting cells in a sample, the method comprising: providing a sample and an array, said sample comprising cells in a first concentration, said array comprising at least 1000 sites, each site having a defined volume between 10 attoliters and 50 picoliters; diluting said sample to create a

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diluted sample, said diluted sample comprising cells in a second concentration; contacting said array with said diluted sample such that the ratio of cells to each site of said array is less than 1:1; and determining the number of sites of said array which contain a single cell. This array is preferably sealed prior to said determining step and the present invention contemplates both the array and the sealed array as compositions of matter.

[024] In still another embodiment, the present invention contemplates a method of detecting a target analyte of a cell in a sample, the method comprising: providing a sample and an array, said sample comprising cells in a first concentration, each cell comprising a target analyte, said array comprising at least 1000 sites, each site having a defined femtoliter volume; diluting said sample to create a diluted sample, said diluted sample comprising cells in a second concentration; contacting said array with said diluted sample such that the ratio of cells to each site of said array is less than 1:1; treating said cells in said array under conditions such that said cells are lysed; and determining the number of sites of said array which contain a target analyte of said cells. This array is preferably sealed prior to said determining step and the present invention contemplates, as a composition of matter, the array and the sealed array produced in this fashion.

[025] In alternative embodiment, the present invention contemplates a method of detecting a target analyte of a cell in a sample, the method comprising: providing a sample and an array, said sample comprising cells in a concentration, said array comprising at least 1000 sites, each site having a defined femtoliter volume; treating said cells under conditions such that said cells are lysed so as to create a treated sample; contacting said array with said treated sample such that the ratio of target analyte to each site of said array is less than 1:1; and determining the number of sites of said array which contain a target analyte. Again, the array is preferably sealed prior to said determining step and the present invention contemplates, as a composition of matter, the array and the sealed array produced in this manner.

[026] In one embodiment, the cells are prokaryotic cells. In a particular embodiment, the cells are bacterial cells and the target analyte is a bacterial enzyme released from the cells after lysis. In a preferred embodiment, each site contains a capture reagent directed at capturing the target analyte after lysis.

[027] A method of detecting a reaction component that affects a reaction, the method comprising: providing a reaction component, a sample and an array, said sample comprising a

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target analyte in a first concentration, said array comprising at least 1000 sites, each site having a defined volume between 10 attoliters and 50 picoliters; contacting said array with said sample such that the ratio of target analyte to each site of said array is less than 1:1; introducing said reaction component to each site of said array; and determining whether the reaction component affects a reaction. In a preferred embodiment, said target analyte is an enzyme and substrate for said enzyme is present in each site of said array prior to the determining step. In a particularly preferred embodiment, said reaction component is an enzyme inhibitor and said determining step comprises determining whether said enzyme inhibitor inhibits the reaction of said enzyme with said substrate. In one embodiment, the present invention contemplates sealing the array prior to the determining step, and both the array and the seal array are contemplated as compositions of matter.

[028] Indeed, the present invention specifically contemplate embodiments where the rate constants of enzyme are measured in single enzyme molecule settings. For example, in embodiment, the enzyme and inhibitor are preincubated prior to introduction into the sites of the array (e.g. in an aqueous buffer system) together in bulk, such that the inhibitor concentration is higher than the dissociation constant for inhibitor binding (K_i) and all of the active sites of the enzyme are likely to be blocked or occupied. As K_i differs for different enzyme/inhibitor pairs, the concentration of inhibitor must be adjusted accordingly. With some particular enzyme/inhibitor pairs exemplified herein, a variety of inhibitor starting concentrations can be employed, ranging between 100 mM and 20 μM. In a second step of this embodiment of the method, one or more substrates (e.g. a chromogenic substrate) in an aqueous buffer system are added, preferably in a high volume excess (between 10-fold and 100,000-fold). In a third step of this embodiment of the method, the bulk solution is added to the array of sites, each site having a defined volume (between 10 attoliters and 50 picoliters, more preferably femtoliters). At this point, the inhibitor is not in excess any more; indeed, the concentration of the inhibitor is much less than the K_i (it is preferred that less than 100,000 molecules of inhibitor, and preferably less than 10,000 molecules of inhibitor, and still more preferably less than 3,000 molecules of inhibitor, and even more preferably less than 1,000 molecules of inhibitor, are remaining in the solution). At the point where the solution is added to the sites of the array, it is preferred that the enzyme concentration is such (due to the many fold dilution of step 2) there will be only a single enzyme in every array site (or, more typically, a single enzyme in some sites and no enzyme in

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others). In a preferred embodiment, the array is then monitored for signal over time with a detection means (e.g. so that the utilization of substrate by the enzyme can be detected). When the frequency distribution of substrate turnover onset times is plotted, the K_{off} rate constant can be calculated.

[029] In another embodiment of such a method, the concentration of the inhibitor is chosen such that it is in the same range as K_i. At this inhibitor concentration the inhibitor-bound enzyme is in equilibrium with the inhibitor-free enzyme. The bulk solution is added to the array of sites, each site having a defined volume (between 10 attoliters and 50 picoliters, more preferably femtoliters). It is again preferred that the enzyme concentration be chosen to yield only a single enzyme in every array site (or, more typically, a single enzyme in some sites and no enzyme in other sites). When signal (e.g. fluorescence) is monitored over time, one can see binding, release, and subsequent binding at each site where enzyme is present in the array.

[030] In yet another embodiment, the present invention contempalates a method of detecting a reaction component that affects a reaction, the method comprising: providing a reaction component, a sample and an array, said sample comprising a biomolecule target analyte in a first concentration, said array comprising at least 1000 sites, each site having a defined volume between 10 attoliters and 50 picoliters; diluting said sample to create a diluted sample, said diluted sample comprising a biomolecule target analyte in a second concentration; contacting said array with said diluted sample such that the ratio of biomolecule target analyte to each site of said array is less than 1:1; introducing said reaction component to each site of said array; and determining whether the reaction component affects a reaction.

[031] In still another embodiment, the present invention contemplates a method of detecting enzyme inhibition, the method comprising: providing i) a reaction component, ii) a sample, said sample comprising a enzymatic target analyte in a first concentration, iii) a substrate for said enzymatic target analyte, and iv) an array, said array comprising at least 10,000 sites, each site having a defined femtoliter volume; diluting said sample to create a diluted sample, said diluted sample comprising a enzymatic target analyte in a second concentration; contacting said array, in any order, with said substrate, said reaction component, and said diluted sample such that the ratio of enzymatic target analyte to each site of said array is between 1:5 and 1:500; and determining whether the reaction component inhibits the reaction of said enzyme with said substrate.

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[032] In one embodiment, nanoparticles are utilized for the digital readout of target and concentrations. Nanoparticles have several advantages over an enzyme digital readout. With an enzyme approach, as concentrations increase, the linearity of the digital readout, which follows a Poisson distribution, is lost. Using nanoparticles functionalized with fluorescent species, the present invention contemplates in one embodiment to be able to perform a digital readout as well as the traditional bulk intensity readout, which is used extensively in our laboratory. As the linearity of the digital signal is lost, the analysis can be shifted from a digital readout to a traditional intensity readout using the nanoparticles. The signal can then be read as an average intensity of each site in the array (e.g. average well intensity) correlating to concentration, and will significantly increase the range of concentrations that can be read with this system. In some embodiments employing nanoparticles, the array is not sealed.

[033] A variety of embodiments are contemplated, including but not limited to embodiments where the target is attached to the gold particle and introduced into an array, said array comprising a binding partner (e.g. immobilized ligand). In another embodiment, the target analyte may be attached or otherwise immobilized on the array and the gold particle-binding partner conjugate can be introduced to the array to bind the target analyte. In another embodiment, a first binding ligand may immobilize the target, and the gold-particle-second binding ligand may be introduced into the array to bind the target. In one embodiment, the target is nucleic acid and the binding partners are complementary probes. In another embodiment, a two-step sandwich format is contemplated, where the target can be unlabeled and incubated with a first complementary probe; thereafter, the nanoparticle-second probe conjugate is introduced into the site of the array. The target can be viewed to operate as a linker in this embodiment.

[034] While multiple embodiments are disclosed, still other embodiments of the present invention will become apparent to those skilled in the art from the following detailed description, which shows and describes illustrative embodiments of the invention. As will be realized, the invention is capable of modifications in various obvious aspects, all without departing from the spirit and scope of the present invention. Accordingly, the drawings and detailed description are to be regarded as illustrative in nature and not restrictive.

Brief Description of the Drawings

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[035] FIGS. 1a, 1b, and 1c are side view cross-section schematics representing etched bundle modifications, according to one embodiment of the present invention.

[036] FIGS. 2a, 2b, and 2c are side view cross-section schematics representing a sandwich assay, according to one embodiment of the present invention.

[037] FIGS. 3a and 3b are photographs depicting Streptavidin Alexa Fluor 568® binding to (a) an unpolished biotin modified fiber optic array, and (b) a polished biotin modified fiber optic array, according to one embodiment of the present invention.

[038] FIGS. 4a, 4b, 4c, 4d, 4e, and 4f are photographs depicting experiments according to one embodiment of the present invention in which β-galactosidase hydrolyzes RDG to form resorufin. More specifically, each of these figures depicts a different sample having a different concentration of SβG. The concentrations were: (a) 128 amol, (b) 51 amol, (c) 25 amol, (d) 7.5 amol, and (e) 2.6 amol, and (f) was the control.

[039] FIG. 5 is a chart depicting a log-log plt of the moles of target present in a sample with the resulting percentage of active reaction vessels, according to one embodiment of the present invention.

[040] FIG. 6a is a microscopic photograph of an entire fiber array and an inset close-up of the bundle, according to one embodiment of the present invention.

[041] FIG. 6b is an AFM image of a portion of an etched surface, according to one embodiment of the present invention.

[042] FIGS. 7a, 7b, and 7c depict enclosure of the reaction vessels and evaluation of the seal, according to one embodiment. FIG. 7a is a microscopic photograph of a solution of $Ru(bpy)_3Cl_2$ enclosed in the array of chambers. FIG. 7b is a microscopic photograph of a small octagonal portion of the bundle photobleached with UV light. FIG. 7c is a microscopic photograph of FIG. 7b taken 60 minutes later.

[043] FIGS. 8a, 8b, and 8c are microscopic photographs depicting detection of the activity of single molecules of β-galactosidase, according to various embodiments of the present invention. FIG. 8a is a microscopic photograph of a background image of a portion of an array. FIG. 8b is a microscopic photograph of an image taken of a portion of a 1:5 enzyme to vessel assay. FIG. 8c is a microscopic photograph of a 1:80 enzyme to vessel assay.

[044] FIG. 9 is a schematic of one embodiment for measuring rate constants of enzymes at the single enzyme level.

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[045] FIG. 10 is a representative image of an array wherein signal is detected due to release of inhibitor.

[046] FIGS. 11a, 11b and 11c are time traces showing substrate turnover in the presence of inhibitor or absence (11c) of inhibitor.

[047] FIG. 12 shows the plot of measurements enabling determination of Koff for a particular enzyme/inhibitor pair.

[048] FIGS. 13a and 13b show a plurality of time traces (13a) and a single time trace (13b) plotted to show increased signal upon inhibitor release.

[049] FIG. 14 is a histogram of off-times, permitting a calculation of the half-life values for a particular enzyme/inhibitor pair.

[050] FIG. 15 schematically shows one embodiment wherein nanoparticles are employed to enhance detection.

[051] FIG. 16 schematically shows an alternative embodiment wherein nanoparticles are employed to enhance detection.

[052] FIG. 17 shows the detection of gold nanoparticles at sites in an array.

Detailed Description

[053] The present invention relates to methods, systems, and devices for enzymatic detection and quantification of a target analyte or target analytes in a sample. More specifically, the present invention relates to enzymatic detection and quantification of target analytes using arrays of micron- to nanoscale-sized reaction vessels containing capture components. According to one embodiment, an array of reaction vessels containing capture components is contacted with a sample containing at least one target analyte. A chromogenic substrate is then added and the resulting chromogenic product of the enzymatic reaction allows for detection of the analyte. Further, according to one embodiment, the percentage of reaction vessels with captured target analytes can be used to calculate the amount of target analyte in the sample using a binary readout method.

[054] More specifically, the present invention provides for an array of micron- to nanoscale-sized reaction vessels specifically functionalized and capable of capturing target molecules that are enzymes or enzyme-labelled. The ability to immobilize the target allows the

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use of washing steps and indirect assays, as outlined below. In use, single enzyme (or enzyme-labelled) molecules are captured in individual reaction vessels and catalyze the production of a sufficient number of chromogenic product molecules to generate a detectable signal. In accordance with one embodiment relating to samples having low target analyte concentrations, only a portion of the reaction vessels bind a target molecule, thereby enabling a binary readout of target concentration from the array.

[055] Thus, the direct enzymatic amplification in the method and system of the present invention allows for direct amplification of a detectable signal. Further, unlike the prior art methods, the present invention allows for detection of low concentrations of protein.

[056] The quantification method, according to one embodiment, is a novel method for concentration determination based on statistical analysis. The sample enzyme concentration is determined by distributing the enzyme-containing sample and a suitable substrate, into many nanoscale reaction vessels. In this method, the vessels contain either zero or one enzyme molecule. By observing the presence or absence of a fluorescent product resulting from single enzyme molecule catalysis in each reaction vessel, a binary readout method can be used to count enzyme molecules. Finally, the percentage of reaction vessels occupied by enzyme molecules is correlated to the bulk enzyme concentration.

I. Arrays

[057] The present invention provides array compositions comprising at least a first substrate with a surface comprising a plurality of assay locations. By "array" herein is meant a plurality of capture components in an array format. The size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different capture components to many millions can be made, with very large arrays being possible, including very large fiber optic arrays. Generally, the array will comprise from two to as many as a billion or more capture components, depending on the size of the wells and the substrate, as well as the end use of the array, thus very high density, high density, moderate density, low density and very low density arrays may be made. Preferred ranges for very high density arrays are from about 10,000,000 to about 2,000,000,000, with from about 100,000,000 to about 1,000,000,000 being preferred. High density arrays range about 100,000 to about 10,000,000, with from about 1,000,000 to about 5,000,000 being particularly preferred. Moderate density arrays range from about 10,000 to about 50,000 being particularly preferred, and from about 20,000 to about

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30,000 being especially preferred. Low density arrays are generally less than 10,000, with from about 1,000 to about 5,000 being preferred. Very low density arrays are less than 1,000, with from about 10 to about 1000 being preferred, and from about 100 to about 500 being particularly preferred. In some embodiments, multiple substrates may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller substrates.

[058] The compositions comprise a substrate. By "substrate" , "array substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of target analytes and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates are very large, and include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon™, etc.), polysaccharides, nylon or nitrocellulose, composite materials, ceramics, and plastic resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not appreciably fluoresce.

[059] In one embodiment, the substrate comprises the end of an optical fiber bundle. Alternatively, the substrate does not comprise the ends of an optical fiber bundle. For example, the substrate may be a spotted, printed or photolithographic substrate known in the art; see for example WO 95/25116; WO 95/35505; PCT US98/09163; U.S. Patent Nos. 5,700,637; 5,807,522 and 5,445,934; and U.S.S.N.s 08/851,203 and 09/187,289, and references cited within, all of which are expressly incorporated by reference. One advantage of using the distal end of a optical fiber bundle as a substrate in the present invention is that the individual fibers in contact with each well can be used to carry both excitation and emission light to and from the wells, enabling remote interrogation of the well contents. Further, an array of optical fibers provides the capability for simultaneous excitation of molecules in adjacent vessels, without signal "cross-talk" between fibers. That is, excitation light transmitted in one fiber does not escape to a neighboring fiber.

[060] In one embodiment, the substrate is planar, although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used. Preferred substrates include optical fiber bundles as

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discussed below, and flat planar substrates such as glass, polystyrene and other plastics and acrylics.

[061] In one embodiment, at least one surface of the substrate is modified to contain discrete, individual sites (also referred to herein as "reaction vessels" and "microwells") for later association of target analytes. These sites generally comprise physically altered sites, i.e. physical configurations such as wells or small depressions in the substrate that can retain the beads. The microwells may be formed as is generally known in the art using a variety of techniques, including, but not limited to, photolithography, stamping techniques, molding techniques and microetching techniques. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the substrate.

[062] In one embodiment, physical alterations are made in a surface of the substrate to produce the sites. In a preferred embodiment, the substrate is a fiber optic bundle and the surface of the substrate is a terminal end of the fiber bundle. In this embodiment, wells are made in a terminal or distal end of a fiber optic bundle comprising individual fibers. In this embodiment, the cores of the individual fibers are etched, with respect to the cladding, such that small wells or depressions are formed at one end of the fibers. The required depth of the wells will depend on the size of the beads to be added to the wells. In one aspect of the present invention, the physical alterations can be made as taught in U.S. Patents 6,023,540, 6,327,410, and 6,858,394, which are each incorporated by reference herein in their entirety.

[063] The sites may be a pattern, i.e. a regular design or configuration, or randomly distributed. A preferred embodiment utilizes a regular pattern of sites such that the sites may be addressed in the X-Y coordinate plane. "Pattern" in this sense includes a repeating unit cell, preferably one that allows a high density of beads on the substrate.

[064] In accordance with one embodiment of the present invention, the reaction vessels have a volume ranging from about 10 attoliters to about 50 picoliters. Alternatively, the reaction vessels range in size from about 1 femtoliter to about 1 picoliter. In a further alternative, the reaction vessels range from about 30 femtoliters to about 60 femtoliters.

[065] In one aspect of the present invention, the array is a fiber optic array. The array, according to one embodiment, can be made as follows. First, the reaction vessels are formed on the distal end of a fiber optic bundle. According to one embodiment, the vessels are created using an etching process, such as, for example, an acid etching process, resulting in reaction

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vessels of the desired volume. That is, the etching process creates depressions or holes in the core material at the end of the fiber bundle, while the cladding material is not impacted, thus resulting in reaction vessels. Alternatively, both the core material and cladding material are etched, but the cladding material is etched at a slower rate than the core material, thereby resulting in reaction vessels. One advantage of the fiber optic array format is that it circumvents a complicated microfabrication procedure and provides the ability to observe many reaction vessels simultaneously.

II. Capture Components

[066] The microwells of the present invention comprise at least one capture component. A capture component (also referred to as a "capture binding ligand," "binding ligand," "capture binding species," or "capture probe") is any molecule, compound, or microwell modification that can be used to probe for, attach, bind or otherwise capture a target analyte within a microwell on the substrate, such that the target analyte is immobilized during the assay. Generally, the capture binding ligand or component allows the attachment of a target analyte to the microwell, for the purposes of detection, quantification, or other analysis.

[067] As will be appreciated by those in the art, the composition of the capture component will depend on the composition of the target analyte. Capture components for a wide variety of analytes are known or can be readily found using known techniques. For example, when the analyte is a protein, the capture components or binding ligands include proteins (particularly including antibodies or fragments thereof (FAbs, etc.)) or small molecules. Preferred capture component proteins include peptides. For example, when the analyte is an enzyme, suitable binding ligands include substrates and inhibitors. Antigen-antibody pairs, receptor-ligands, and carbohydrates and their binding partners are also suitable analyte-binding ligand pairs. In addition, when the analyte is a single-stranded nucleic acid, the binding ligand may be a complementary nucleic acid. Similarly, the analyte may be a nucleic acid binding protein and the capture binding ligand is either single-stranded or double stranded nucleic acid; alternatively, the binding ligand may be a nucleic acid-binding protein when the analyte is a single or double-stranded nucleic acid. Alternatively, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867,5,705,337, and related patents, hereby incorporated by reference, nucleic acid "aptomers" can be developed for binding to virtually any target analyte. As will be appreciated by those in the art, any two molecules that

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will associate may be used, either as an analyte or as the capture component. Similarly, there is a wide body of literature relating to the development of capture components based on combinatorial chemistry methods.

[068] Suitable analyte/capture component pairs include, but are not limited to, antibodies/antigens, receptors/ligands, proteins/nucleic acid, enzymes/substrates and/or inhibitors, carbohydrates (including glycoproteins and glycolipids)/lectins, proteins/proteins, proteins/small molecules; and carbohydrates and their binding partners are also suitable analyte-binding ligand pairs. These may be wild-type or derivative sequences. According to one embodiment, the capture components are portions (particularly the extracellular portions) of cell surface receptors that are known to multimerize, such as the growth hormone receptor, glucose transporters (particularly GLUT 4 receptor), transferring receptor, epidermal growth factor receptor, low density lipoprotein receptor, high density lipoprotein receptor, epidermal growth factor receptor, leptin receptor, interleukin receptors including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-13, IL-15, and IL-17 receptors, human growth hormone receptor, VEGF receptor, PDGF receptor, EPO receptor, TPO receptor, ciliary neurotrophic factor receptor, prolactin receptor, and T-cell receptors.

[069] In a preferred embodiment, the capture component is attached to the microwell or reaction vessel as outlined herein, for example via an "attachment component" (also referred to herein as an "attachment linker"). An "attachment component," as used herein, is defined as any component, functionalization, or modification of the microwells that results in the attachment of the capture component, and can include bonds and/or linkers. Alternatively, the capture component may utilize a capture extender component. In this embodiment, the capture component or binding ligand comprises a first portion that will bind the target analyte and a second portion that can be used for attachment to the surface.

[070] The method of attachment of the capture binding ligand to the attachment linker will generally be done as is known in the art, and will depend on the composition of the attachment linker and the capture binding ligand. In general, the capture binding ligands are attached to the attachment linker through the use of functional groups on each that can then be used for attachment. According to one embodiment, the functional group is a chemical functionality. That is, the microwell surface is derivatized such that a chemical functionality is bound to the surface. Preferred functional groups for attachment are amino groups, carboxy

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groups, oxo groups and thiol groups. These functional groups can then be attached, either directly or through the use of a linker, sometimes referred to herein as a "cross-linker." Linkers are known in the art; for example, homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical. Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). Preferred linkers include, but are not limited to, alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), with short alkyl groups, esters, amide, amine, epoxy groups and ethylene glycol and derivatives being preferred. Linkers may also be a sulfone group, forming sulfonamide.

[071] According to one embodiment, the functional group is a light-activated functional group. That is, the functional group can be activated by light to attach to the target analyte or to the crosslinker. One example is the PhotoLink™ technology available from SurModics, Inc. in Eden Prairie, MN.

[072] In one alternative aspect of the invention, the functional group is added without derivatizing the well surface. That is, the functional groups can be added to the surface by adding a molecule having an attached functional group attached, wherein the molecule has a binding affinity for the well surface. The molecule, according to one embodiment is bovine serum albumin. Alternatively, the molecule is any protein capable of binding or sticking to the vessel surface. In a further alternative, the molecule is any molecule capable of binding or sticking to the vessel surface. In one example, the molecule is bovine serum albumin with free amine groups on its surface. The crosslinker can then be added to attach to the amine groups.

[073] According to one exemplary embodiment in which the capture component is a chemical crosslinker, the target analyte is attached using chemical crosslinking in the following manner. First, the reaction vessel surface is derivatized with a functional group such as NH_2. Next, the crosslinker and the target analyte are added to the array such that the crosslinker attaches to the NH_2 and the target analyte attaches to the crosslinker. In an alternative embodiment described in further detail below in which the target analyte is not an enzyme, a label having an enzymatic component can also be attached to the target analyte.

[074] In this way, capture binding ligands comprising proteins, lectins, nucleic acids, small organic molecules, carbohydrates, etc. can be added.

[075] One embodiment utilizes proteinaceous capture components or capture binding ligands. As is known in the art, any number of techniques may be used to attach a proteinaceous

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capture binding ligand. "Protein" in this context includes proteins, polypeptides, peptides, including, for example, enzymes. A wide variety of techniques are known to add moieties to proteins. One preferred method is outlined in U.S. Patent No. 5,620,850, hereby incorporated by reference in its entirety. The attachment of proteins to surfaces is known; see also Heller, Acc. Chem. Res. 23:128 (1990), and related work.

[076] An alternative embodiment utilizes nucleic acids as the capture binding ligand, for example for when the target analyte is a nucleic acid or a nucleic acid binding protein, or when the nucleic acid serves as an aptamer for binding a protein, as is well known in the art.

[077] According to one embodiment, each microwell comprises a plurality of capture components. The plurality of capture components, in one aspect of the invention, are distributed on the surface of the well like a "lawn." Alternatively, the capture components are distributed in any known fashion.

[078] The binding between the capture component and the target analyte, in accordance with one embodiment, is specific and the capture component is part of a binding pair. That is, the capture component is a target specific capture component that specifically binds with or has specificity for the target analyte. More specifically, the capture component binds specifically and directly to the target analyte. By "specifically bind" or "binding specificity" herein is meant that the capture component binds the analyte with specificity sufficient to differentiate between the analyte and other components or contaminants of the test sample. For example, the capture component according to one embodiment is an antibody that binds specifically to some portion of the target analyte. The antibody, according to one embodiment, can be any antibody capable of binding specifically to a target analyte. For example, appropriate antibodies include, but are not limited to, monoclonal antibodies, bispecific antibodies, minibodies, domain antibodies, synthetic antibodies (sometimes referred to as "antibody mimetics"), chimeric antibodies, humanized antibodies, antibody fusions (sometimes referred to as "antibody conjugates"), and fragments of each, respectively.

[079] However, as will be appreciated by those in the art, it is possible to detect analytes using binding which is not highly specific; for example, the systems may use different capture components such as, for example, an array of different ligands, and detection of any particular analyte is via its "signature" of binding to a panel of binding ligands, similar to the manner in which "electronic noses" work. This finds particular utility in the detection of chemical analytes.

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The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding. In some embodiments, for example in the detection of certain biomolecules, the binding constants of the analyte to the binding ligand will be at least about 10^4-10^6 M^{-1}, with at least about 10^5 to 10^9 M^{-1} being preferred and at least about 10^7 -10^9 M^{-1} being particularly preferred.

[080] According to one embodiment in which the target analyte is a cell, including, for example, bacterial cells, the capture component is an adhesin receptor molecule. In use, the adhesin receptor molecule binds with a surface protein called an adhesin on the extracellular surface of the target cell, thereby immobilizing or capturing the cell. Alternatively, in embodiments in which the target analyte is another type of cell (a non-bacterial cell), the capture component is an appropriate cell surface receptor that binds the target analyte cell. In a further embodiment in which the target analyte is a cell, the capture component is fibronectin. For example, fibronectin can be used when the target analyte is a nerve cell.

[081] Alternatively, the capture component is a non-specific capture component. That is, the capture component does not bind specifically to a target analyte, but rather binds to a corresponding binding partner associated with or attached to the target analyte. For example, the non-specific capture component according to one embodiment is a chemical cross-linker as described above. According to one embodiment, every peptide molecule in a target sample can attach to the chemical cross-linker. This type of system can be used to identify enzyme target analytes because the analytes are detected by modifying the substrate.

[082] In one example of a non-specific capture component according to one embodiment, the capture component is streptavidin, which binds with high affinity to biotin, and thus binds to any molecule to which biotin has been attached. Alternatively, the capture component is biotin, and streptavidin is attached to or associated with the target analyte such that the target analyte can be captured by the biotin.

[083] According to one embodiment, the capture component is added to the reaction vessels in the following manner. First, the microwells are prepared for attachment of the capture component(s). That is, the microwells are modified or an attachment component is added to the microwells such that the capture component(s) will attach to the microwells. In one embodiment, the microwells are derivatized with a chemical functionality as described above. Next, the capture component is added.

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[084] One example of capture component attachment is depicted in FIG. 1, in which reaction vessels of the present invention are functionalized with biotin. As shown in FIG. 1a, the array of the present invention in this example is a fiber optic bundle 10. To attach the capture component 18, the microwells are first modified with an attachment component 16, which in this example is an aminopropyl silane 16 that is bound to both the core 12 and cladding 14 surfaces of the distal end of the fiber bundle 10, as shown in FIG. 1b. The modification with aminopropyl silane is effective in this example because NHS-biotin attaches to an amino-silanized surface 16. However, since the capture component 18 should be present only within the reaction vessels, the external surfaces of the substrate, such as the external surfaces of the cladding 14, should not be silanized. That is, the silanization must be removed from the external cladding surface 14 to avoid biotin attachment. In this example as shown in FIG. 1c, the silanization 16 was removed from the external cladding layer 14 by polishing the amino-silanized fibers for 10 seconds with 0.3 μm lapping film, thereby removing the top amino-silanized cladding layer.

[085] After the attachment component 16 has been added to the microwells, the capture component 18 can be attached. In the example in FIG. 1, the capture component 18 is biotin 18. As shown in FIG. 1d, biotin succinimidyl ester 18 is attached to the amino groups 16 on the well surfaces 12.

III. Target Analytes

[086] As discussed herein, the array of the present invention provides for detection, quantification, and further analysis of target analytes. By "target analyte" or "analyte" or grammatical equivalents herein is meant any atom, molecule, ion, molecular ion, compound or particle to be either detected or evaluated for binding partners.

[087] According to one embodiment, the target analyte is an enzyme. For example, the enzyme can be an enzyme from any of the six enzyme classifications: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Thus, appropriate enzymes include, but are not limited to, polymerases, cathepsins, calpains, amino-transferases such as, for example, AST and ALT, proteases such as, for example, caspases, nucleotide cyclases, transferases, lipases, enzymes associated with heart attacks, and the like. When the system of the present invention is used to detect viral or bacterial targets, appropriate enzymes include viral or bacterial polymerases and other such enzymes, including viral or bacterial proteases.

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[088] Alternatively, the target analyte has an enzymatic component. For example, the target analyte can be a cell having an enzyme or enzymatic component present on its extracellular surface. Alternatively, the target analyte is a cell having no enzymatic component. Such a cell is typically identified using an indirect assaying method described below such as a "sandwich" assay.

[089] In accordance with another embodiment, the target analyte is not an enzyme. As will be appreciated by those in the art, a large number of analytes may be used in the present invention; basically, any target analyte can be used which binds a capture component and/or a secondary binding ligand. As will be explained in further detail below, these target analytes are typically identified using an indirect assay such as a "sandwich" assay. As mentioned above, one suitable target analyte is a cell. In addition, suitable analytes include organic and inorganic molecules, including biomolecules. In a preferred embodiment, the target analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous target analytes that may be detected or evaluated for binding partners using the present invention. In addition to enzymes as discussed above, suitable protein target analytes include, but are not limited to, (1) immunoglobulins; (2) hormones and cytokines (many of which serve as ligands for cellular receptors); and (3) other proteins.

[090] According to one embodiment in which the target analyte is not an enzyme and a sandwich assay is performed as described in further detail below, the enzymatic label as described in further detail below can be beta galactosidase. Alternatively, the enzyme label can be, but is not limited to, alkaline phosphatase or horseradish peroxidase.

[091] Further suitable target analytes include, but are not limited to, an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc.

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IV. Enzymatic Substrate

[092] After the target analyte(s) are captured within the microwell(s) (and after a washing step, according to certain embodiments), a reaction component is added to the array. By "reaction component," as used herein, is meant a molecule that affects an enzymatic reaction when contacted with an enzyme or enzymatic molecule. By "affects" a reaction is meant to include, but is not limited to, inducing, activating, or altering (for example, slowing down or speeding up) a reaction, or inhibiting a reaction. According to one embodiment, the reaction component is a chromogenic enzymatic substrate. A "chromogenic enzymatic substrate" as used herein is any molecule that is converted by an enzyme into a chromogenic product as a result of an enzymatic reaction. "Chromogenic" means relating to color or pigment in the optical (visible light) spectrum and includes fluorogenic.

[093] It is understood in the art that chromogenic substrates are known or can be made for enzymes in any of the six enzyme classifications. Thus, any known chromogenic substrate capable of producing a chromogenic product in a reaction with a particular enzyme can be used in the present invention, including any of the chromogenic enzyme substrates disclosed in *The Handbook — A Guide to Fluorescent Probes and Labeling Technologies, Tenth Ed.*, Chapter 10, http://probes.invitrogen.com/handbook/sections/1000.html, which is incorporated herein by reference in its entirety.

[094] According to one embodiment in which the assay of the present invention is a sandwich assay as described further herein in which the enzyme label is beta galactosidase, the substrate added to the array is a beta galactosidase substrate such as resorufin-β-D-galactopyranoside.

V. Assay Methods

[095] The array of the present invention can be used for several different assay methods. More specifically, the present invention provides for both (a) target analyte detection and (b) quantification of target analyte concentration in a sample.

[096] Generally, the system or array of the present invention is exposed to an analyte of interest (or contacted with a sample containing an analyte of interest) and the analyte is immobilized by a capture component in a microwell, under conditions suitable for immobilization of the target analyte to at least one of the capture components, i.e. generally physiological conditions. For purposes of the present application, the term "immobilized" means

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attached, bound, or affixed to a capture component in a microwell. Thus, the interaction between any analyte molecule and the capture component in a microwell results in immobilization of the analyte molecule within that microwell.

[097] According to one aspect of the invention, the sample of interest is placed in contact with the array of the present invention (or the array is incubated in the sample) for a period of from about 45 minutes to about 75 minutes. Alternatively, the array and sample are contacted for a period of from about 50 minutes to about 70 minutes. In a further alternative, the incubation period is about 1 hour.

[098] According to one embodiment, a wash step is performed after contacting the array with the sample. The wash step is intended to wash away any target analytes or non-target molecules that are not bound to a capture component. Alternatively, no wash step is needed.

[099] In one aspect of the invention, a secondary binding ligand is then added to the array. Generally, the secondary binding ligand is added if the assay is an indirect assay such as a "sandwich assay" (when the target analyte is not an enzyme), as described in further detail herein. The secondary binding ligand, as discussed above, will associate with or bind to the bound target analyte and comprises an enzymatic component. The secondary binding ligand is added in an amount sufficient to ensure that a ligand comes into contact with every bound target analyte in the array. Alternatively, no secondary binding ligand is added, such as, for example, when the target analyte is going to be detected directly.

[0100] A chromogenic enzymatic substrate as described above is then introduced or added to the array. The chromogenic enzymatic substrate is provided in an amount sufficient to contact any captured target analyte. The chosen substrate reacts with or is modified by the enzymatic component such that the reaction produces a chromogenic product and thus an optical signal. The presence of the chromogenic product in the array can provide information about the identity and/or concentration of an analyte based on the interaction of the analyte with the capture component and the enzymatic substrate (and the secondary binding ligand, in some cases).

[0101] In one embodiment of the present invention, the microwells are sealed after the enzymatic substrate is added. That is, a sealing component is placed in contact with the face of the substrate, thereby fluidly isolating each microwell and sealing its contents therein. A "sealing component," as used herein, is defined as any material or device large enough to cover

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the entire surface of the array substrate and capable of contacting the array substrate surface such that each reaction vessel is sealed or isolated such that the contents of each vessel cannot escape the vessel. According to one embodiment, the sealing component is a silicone elastomer gasket that is placed against the substrate surface with a uniform pressure across the entire substrate. By sealing the contents in each microwell, the enzymatic reaction can proceed within the microwell, thereby producing a detectable amount of the chromogenic product that is retained in the microwell for detection purposes. That is, the enzyme converts the substrate into a chromogenic product that builds up to a locally high concentration in each sealed vessel, generating a detectable chromogenic signal.

[0102] According to one embodiment, the present invention provides for a microscope system equipped with a mechanical platform that applies the sealing component. The platform is positioned beneath the microscope stage on the microscopy system. After the assay contents have been added to each well, the sealing component is sandwiched between a flat surface (such as, for example, a microscope slide) and the array substrate using uniform pressure applied by the mechanical platform.

[0103] The assays may be run under a variety of experimental conditions, as will be appreciated by those in the art. A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding. Various blocking and washing steps may be utilized as is known in the art.

[0104] The microwells exhibiting activity or changes in their optical signature may be identified by a conventional optical train and optical detection system. Depending on the particular chromogenic enzymatic substrates used and the operative wavelengths of their chromogenic products, optical filters designed for a particular wavelengths may be employed for optical interrogation of the microwells. In a preferred embodiment, the system or array of the present invention is used in conjunction with an optical fiber bundle or fiber optic array as a substrate.

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[0105] According to one embodiment, the array of the present invention can be used in conjunction with an optical detection system such as the system described in U.S. Application No. 09/816,651, which is incorporated herein by reference in its entirety. For example, according to one embodiment, the array of the present invention is the distal end of a fiber optic assembly comprising a fiber optic bundle constructed of clad fibers so that light does not mix between fibers. As depicted in the 09/816,651 Application, the proximal end of the bundle is received by a z-translation stage and x-y micropositioner.

[0106] The optical detection system of U.S. Application No. 09/816,651 operates as follows. Light returning from the distal end of the bundle is passed by the attachment to a magnification changer which enables adjustment of the image size of the fiber's proximal or distal end. Light passing through the magnification changer is then shuttered and filtered by a second wheel. The light is then imaged on a charge coupled device (CCD) camera. A computer executes imaging processing software to process the information from the CCD camera and also possibly control the first and second shutter and filter wheels.

[0107] The array or system of the present invention may be attached to the distal end of the optical fiber bundle using a variety of compatible processes. Wells are formed at the center of each optical fiber of the bundle. Thus, each optical fiber of the bundle conveys light from the single microwell formed at the center of the fiber's distal end. This feature is necessary to enable the interrogation of the optical signature of individual microwells to identify reactions in each microwell. Consequently, by imaging the end of the bundle onto the CCD array, the optical signatures of the microwells are individually interrogatable.

A. Detection

[0108] In one aspect of the present invention, the present array can be used to detect the presence of a target analyte in a sample. More specifically, the invention provides a method for detecting the product of the enzymatic reaction as an indication of the presence of the target analyte.

[0109] The method of detection can proceed either directly or indirectly. If the target analyte is an enzyme, the analyte can be identified by a direct method of detection. Alternatively, if the target analyte is not an enzyme and thus cannot produce a chromogenic product in the presence of a chromogenic enzymatic substrate, the analyte is identified by an indirect method of detection.

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[0110] The direct method of detection, which involves a target analyte that is an enzyme, proceeds as follows. First, the sample of interest and the array are placed in contact as described in further detail above under suitable conditions. Subsequently, the chromogenic enzymatic substrate is added.

[0111] The presence or absence of the target analyte in any given microwell is then detected by optical interrogation. That is, any change in the optical signal caused by production of a chromogenic product is detected. In any microwell containing the target analyte, the analyte modifies or acts upon the substrate in some way, thereby resulting in the release of a chromogenic product, resulting in a change in the optical signal from the microwell. The chromogenic reaction product is then optically detected.

[0112] In one embodiment of the present invention, the microwells are sealed after the enzymatic substrate is added, as described above.

[0113] The indirect methods of detection involve a target analyte that does not have enzymatic properties. Two indirect methods that can be used with the present invention are the "sandwich" assay and the "competitive" assay.

[0114] A sandwich assay can be performed as depicted in FIG. 2. First, the sample of interest and the array 10 are placed in contact as shown in FIG. 2a and as described in further detail above. Under suitable conditions, target analyte 12 present in the sample is captured by the capture components 16 in the microwells 14, as shown in FIG. 2b. According to one embodiment, a wash step is then performed.

[0115] Next, a solution binding ligand 18 (also referred to herein as a "secondary binding ligand") is added to the array 10, as shown in fig. 2c. Solution binding ligands 18 are similar to capture components 16 in that they bind to target analytes 12. The solution binding ligand 18 may be the same or different from the capture binding ligand 16. The binding of the solution binding ligand 18 to a captured target analyte 12 forms a "sandwich" of sorts. In the absence of the target analyte, the solution binding ligand 18 is washed away.

[0116] A solution binding ligand 18 has two components – a binding component 22 and an enzymatic label 24. The binding component 22 is the portion of the solution binding ligand 18 that binds to the target analyte 12. Typically, the solution binding ligand 18 binds to a different portion of the target analyte 12 than the capture component 16, because if both the capture component 16 and solution binding ligand 18 were to bind to the same portion, the

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solution binding ligand 18 would not be capable of binding to a captured target analyte 12. Thus, the chosen secondary binding ligand 18 can bind to the target analyte 12 while the target analyte 12 is bound to a microwell 14 via a capture component 16.

[0117] The enzymatic label 24 is the portion of the solution binding ligand 18 that exhibits enzymatic activity. According to one embodiment, the enzymatic label 24 is an enzyme attached to the solution binding ligand 18.

[0118] Subsequently, the chromogenic enzymatic substrate is added.

[0119] In one embodiment of the present invention, the microwells are sealed after the enzymatic substrate is added, as described above.

[0120] The presence or absence of the target analyte in any given microwell is then detected by optical interrogation. That is, any change in the optical signal caused by production of a chromogenic product is detected. In any microwell containing the target analyte and the secondary binding ligand, the enzyme associated with the secondary binding ligand modifies or acts upon the substrate in some way, thereby producing a chromogenic product, resulting in a change in the optical signal from the microwell. The product is then optically detected.

[0121] The competitive assay operates as follows. First, a labelled molecule is added to the array of the present invention, wherein the label is a enzyme or enzymatic component. In this embodiment, the chosen labelled molecule binds with the capture component such that the addition of the labelled molecule to the array results in labelled molecules being bound to capture components in the microwells.

[0122] Next, the sample of interest and the array are placed in contact as described in further detail above. The presence of the target analyte in the array causes the displacement of the labelled molecule and binding of the analyte to the capture components. The displacement occurs for the following reason: in this embodiment, the chosen capture component is capable of binding to either of the labelled molecule or the target analyte, thus resulting in a competitive binding situation. As a result, if a labelled molecule is bound to a capture component in a microwell and a target analyte is added, the target analyte will displace the labelled molecule under suitable conditions.

[0123] According to one embodiment, a wash step is then performed to remove any non-bound labelled molecules from the array.

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[0124] Subsequently, the chromogenic enzymatic substrate is added. And as discussed above, according to one aspect of the invention, the microwells are sealed after the enzymatic substrate is added. Alternatively, the microwells are not sealed.

[0125] The presence or absence of the target analyte in any given microwell is then detected by optical interrogation. But unlike the optical interrogations that are described above, in this interrogation it is the lack of a chromogenic product that indicates the presence of the target analyte in the microwell. In any microwell containing the target analyte, no enzymatic action occurs and no change occurs in the optical signal from the microwell. In contrast, in any microwell in which the labelled molecule is still present, an optical signal is detected.

[0126] In an alternative version of the competitive assay embodiment, both the labelled molecule and sample of interest are added to the array at the same time in fixed volumes. In this version, the target analyte and labelled molecule compete directly for the binding sites on the capture components.

1. Subpopulations of identical capture components to same target analyte

[0127] In accordance with one detection embodiment, sensor redundancy is used. In this embodiment, a plurality of reaction vessels comprising identical capture components referred to as "subpopulations" are used. That is, each subpopulation comprises a plurality of identical capture components present in microwells of the array. Further, according to one embodiment, each subpopulation comprises a plurality of microwells comprising identical capture components. By using a number of identical capture components for a given array, the optical signal from each microwell can be combined for the subpopulation and any number of statistical analyses run, as outlined below. This can be done for a variety of reasons. For example, in time varying measurements, redundancy can significantly reduce the noise in the system. For non-time based measurements, redundancy can significantly increase the confidence of the data.

[0128] The number of subpopulations, according to one embodiment, can range from 2 to any number of subpopulations possible given the limitations of any known array and the number of different capture components. Alternatively, the number can range from about 2 to about 10. In a further alternative, the number can range from about 2 to about 5.

[0129] In one embodiment, a plurality of identical capture components are used. As will be appreciated by those in the art, the number of identical capture components in a subpopulation will vary with the application and use of the sensor array. In general, anywhere from 2 to

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thousands of identical capture components may be used in a given subpopulation, with from 2 to 100 being preferred, 2 to 50 being particularly preferred and from 5 to 20 being especially preferred. In general, preliminary results indicate that roughly 10 identical capture components in a subpopulation gives a sufficient advantage, although for some applications, more identical capture components can be used.

[0130] Once obtained, the optical response signals from a plurality of microwells within each subpopulation (that is, having the same capture component) can be manipulated and analyzed in a wide variety of ways, including baseline adjustment, averaging, standard deviation analysis, distribution and cluster analysis, confidence interval analysis, mean testing, etc.

2. Multiple different capture components to same target analyte

[0131] In addition to the sensor redundancy, the array of the present invention according to one embodiment utilizes a plurality of capture components that are directed to a single target analyte but are not identical. This embodiment provides for more than one different capture component in each microwell or different capture components in different microwells. In one example, a single target analyte may be provided to which two or more capture components are capable of binding. This adds a level of confidence as non-specific binding interactions can be statistically minimized. In this embodiment, when proteinaceous target analytes are to be evaluated, preferred embodiments utilize capture components that bind to different parts of the target. For example, when two or more antibodies (or antibody fragments) to different portions of the same target protein are used as capture components, preferred embodiments utilize antibodies to different epitopes. Similarly, when nucleic acid target analytes are to be evaluated, the redundant nucleic acid probes may be overlapping, adjacent, or spatially separated. However, it is preferred that two probes do not compete for a single binding site, so adjacent or separated probes are preferred.

[0132] In this embodiment, a plurality of different capture components may be used, with from about 2 to about 20 being preferred, and from about 2 to about 10 being especially preferred, and from 2 to about 5 being particularly preferred, including 2, 3, 4 or 5. However, as above, more may also be used, depending on the application.

3. Multiple different capture components to multiple target analytes

[0133] According to another embodiment, the array of the present invention utilizes a plurality of different capture components that are directed to a plurality of target analytes. This

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embodiment includes more than one different capture component in each microwell or different capture components in different microwells. In one example, two or more target analytes may be provided to which two or more capture components in the same microwells or different microwells are capable of binding.

[0134] In this embodiment, more than one target analyte can be identified. For example, two or more target analytes can be identified so long as each different analyte is a different enzyme or has a different enzymatic component such as a enzymatic surface molecule. In one embodiment, the target analytes are identified using multiple enzymatic substrates wherein each substrate produces a different color upon interaction with the appropriate enzyme. Thus, each target analyte can be distinguished based on the color produced by reaction with the substrate. In an alternative embodiment, the target analytes are identified using multiple substrates that each produce the same color. Thus, each target analyte can be distinguished by added the substrates sequentially.

[0135] In this embodiment, a plurality of different capture components may be used, with from about 2 to about 20 being preferred, and from about 2 to about 10 being especially preferred, and from 2 to about 5 being particularly preferred, including 2, 3, 4 or 5. However, as above, more may also be used, depending on the application.

[0136] Please note that each of the different assay configurations above, including the capture component subpopulations directed to different target analytes and the plurality of capture components directed to the same analyte, can also be utilized for quantification as described below.

B. Quantification

[0137] According to one embodiment of the present invention, the present array cannot only be used for detection of a target analyte in a sample, but also for quantification of the analyte in the sample. That is, there is a correlation between the percentage of reaction vessels containing target analytes and the concentration of the analyte in the sample. Thus, the quantification method of the present invention allows for calculation of the amount of a target analyte in a sample based on the percentage of microwells that captured a target analyte.

[0138] Without being limited by theory, the quantification method is driven in part by the fact that the number and volume of reaction vessels employed govern the dynamic range of concentrations that can be determined by this technique. That is, based on the number and

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volume of the reaction vessels in an array of the present invention, an estimate can be made of the range of concentrations of target analyte in solution that allow for the concentration to be determined using the method of the present invention.

[0139] For example, for an array as disclosed in Example 2 with reaction vessels each having a volume of 46 fL, a solution having a concentration of 3.6×10^{-11} M β-galactosidase will yield, on average, one enzyme molecule per vessel. However, it is important to note that distributing a solution having a target analyte concentration within the appropriate range into an array of reaction vessels will not result in the distribution of exactly one enzyme molecule per vessel; statistically, some vessels will have multiple molecules while others will have zero. In the case where the number of enzyme molecules per vessel is high, the data can be fit to a Gaussian distribution. As the ratio of enzyme molecules to reaction vessels approaches zero, the Poisson distribution applies. This limiting distribution is used to calculate the probability of rare events occurring in a large number of trials. For example, based on Poisson statistics, for a concentration of 3.6×10^{-11} M, a distribution between zero and five enzyme molecules per container is observed, with the most probable values being zero and one.

[0140] Equation 1 can be used to determine the probability of observing v events based on the expected average number of events per trial, μ.

Equation 1: $P_\mu(v) = e^{-\mu}(\mu^v/v!)$

[0141] If the concentrations used are much less than 3.6×10^{-11} M, the expected average becomes exceptionally low, the distribution is narrowed, and the probability of observing anything other than 0 or 1 events per trial is improbable in all experimental cases. At these low concentrations, the relationship between the percentage of active reaction vessels and the bulk enzyme concentration is approximately linear. Thus, based on this knowledge, the array of the present invention can be used to determine the concentration of a target analyte in a sample by a simple digital readout system as described herein.

[0142] According to one embodiment, the quantification method of the present invention can be performed as follows. The method is a digital readout system (also referred to as a "binary readout system") that includes first detecting the target analytes in the array of microwells by any detection method described above. The number of reaction vessels is then counted and a percentage of the total number of reaction vessels is calculated. That is, utilization of a yes or no response, in conjunction with the high-density array of reaction vessels, permits

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the digital readout of bulk concentrations of β-galactosidase. This readout is accomplished by counting the vessels containing an active enzyme molecule across the array, with the resulting "active well" percentage correlating to the enzyme concentration. Given the large number of vessels simultaneously interrogated in the array of the present invention, the ratio of enzyme molecules to reaction vessels could be as low as 1:500, as the large number of wells provides a statistically significant signal even at this low ratio.

[0143] Without being limited by theory, it is believed that the quantification method of the present invention is only limited by the number of individual reaction vessels that can be viewed with an acceptable resolution. Thus, expanding the number of vessels that are interrogated by using higher density CCD chips will decrease the limit of detection as the lower limit is defined by the statistics of the small number of active wells that light up at the lower target concentrations. On the other hand, the upper limit of the dynamic range is controlled by the well-to-well deviation from a binary readout. As target concentrations are increased, the binary readout is lost, as a Gaussian distribution becomes a better approximation of target molecule binding. Higher concentrations of target lead to a broad distribution in the number of enzyme molecules that can occupy each well, and consequently, the transition to a non-linear increase in the percentage of active wells.

[0144] The limitations of this technique are realized above and below the thresholds of the dynamic range. As the concentration goes below the lower limit of the dynamic range, the number of enzyme molecules is too low to observe sufficient occupied wells and, therefore, the number of wells must be increased in order to make sure that a statistically significant number of them are occupied by enzyme molecules. Results for extremely dilute concentrations have large relative errors associated with them, due to the very small number of reaction vessels that are expected to show activity. Slight deviation from the expected Poisson value, in this case, will result in a large error. The ultimate upper limit to this technique occurs when 100% of the reaction vessels contain at least one enzyme molecule. At this limit, discrimination between two solutions of high enzyme concentrations is not feasible. As the percentage of active vessels approaches 100%, the linearity between concentration and active vessel percentage is lost. This situation results in a broadening distribution, as a normal distribution becomes an increasingly better approximation of the results.

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[0145] In one aspect of the present invention, the array can also be used to analyze enzyme kinetics. "Enzyme kinetics" as used herein refers to the study of the rates of enzyme-controlled reactions. It is understood in the art of enzyme kinetics that the rate of an enzymatic reaction at low substrate concentrations is proportional to the substrate concentration (is "substrate dependent"). This is referred to as first order. It is further understood that the rate of the reaction at high substrate concentrations reaches a maximum rate and is independent of substrate concentration because the reaction becomes saturated. Thus, if reaction velocity is plotted as a function of substrate concentration, the line initially increases linearly with an increase in substrate and then begins to level off as substrate concentration approaches saturation.

[0146] Thus, according to one embodiment, the kinetics of any particular enzyme can be studied using the present system and array. Reaction velocity varies across enzymes for various reasons, including, for example, reaction inhibition caused by allosteric inhibition. The array of the present invention allows for study of these varied kinetic characteristics.

[0147] According to one embodiment, kinetics are examined in the following fashion. The target analyte is allowed to bind to the capture component, the substrate is added, and the reaction vessel is sealed. Given that a finite amount of substrate is present in the reaction vessel and that no further substrate can be added due to the sealing of the vessel, the reaction velocity can be determined based on the amount of chromogenic product detected over time.

C. Enhancing Signal And Sensitivity

[0148] The one embodment, the present invention contemplates a metallic or semiconductor nanoparticle attached to a biomolecule, and preferably a plurality of biomolecules (e.g. oligonucleotides) in order to enhance signal and sensitivity for the various embodiments of single molecule detection and quantification describe above. In one embodiment, the biomolecules (e.g. oligonucleotides) are labeled with fluorescent molecules at the ends not attached to the nanoparticle. It is not intended that the present invention be limited to the type of nanoparticle or to the use of merely one type of nanoparticle in an assay. In some embodiments, two, or more different types or sizes of nanoparticle are employed. For example, because different sized (e.g., 50 nm and 100 nm diameter) gold nanoparticles scatter light of different colors (due to their different surface plasmon resonances), they can be used to label different DNA targets hybridized to the same array.

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[0149] Nanoparticles useful in the practice of the invention include metal (e.g., gold, silver, copper and platinum), semiconductor (e.g., CdSe, CdS, and CdS or CdSe coated with ZnS) and magnetic (e.g., ferromagnetite) colloidal materials. Methods of making metal, semiconductor and magnetic nanoparticles are well-known in the art. See, e.g., Schmid, G. (ed.) Clusters and Colloids (VCH, Weinheim, 1994); Hayat, M. A. (ed.) Colloidal Gold: Principles, Methods, and Applications (Academic Press, San Diego, 1991); Massart, R., IEEE Taransactions On Magnetics, 17, 1247 (1981); Ahmadi, T. S. et al., Science, 272, 1924 (1996); Henglein, A. et al., J. Phys. Chem., 99, 14129 (1995); Curtis, A. C., et al., Angew. Chem. Int. Ed. Engl., 27, 1530 (1988). Other nanoparticles useful in the practice of the invention include ZnS, ZnO, TiO_2, AgI, AgBr, HgI_2, PbS, PbSe, ZnTe, CdTe, In_2S_3, In_2Se_3, Cd_3P_2, Cd_3As_2, InAs, and GaAs. See, e.g., Weller, Angew. Chem. Int. Ed. Engl., 32, 41 (1993); Henglein, Top. Curr. Chem., 143, 113 (1988); Henglein, Chem. Rev., 89, 1861 (1989); Brus, Appl. Phys. A., 53, 465 (1991); Bahncmann, in Photochemical Conversion and Storage of Solar Energy (eds. Pelizetti and Schiavello 1991), page 251; Wang and Herron, J. Phys. Chem., 95, 525 (1991); Olshavsky et al., J. Am. Chem. Soc., 112, 9438 (1990); Ushida et al., J. Phys. Chem., 95, 5382 (1992). Suitable gold nanoparticles are also commercially available from, e.g., Ted Pella, Inc., Amersham Corporation and Nanoprobes, Inc.

[0150] There are methods for controlling nanoparticle growth. U.S. Patent 7,033,415 to Mirkin et al. (hereby incorporated by reference) describes new types of plasmon-driven growth mechanisms for silver nanostructures involving the fusion of triangular nanoprisms. This mechanism, which is plasmon excitation-driven and highly cooperative, produces bimodal particle size distributions. In these methods, the growth process can be selectively switched between bimodal and unimodal distributions using dual beam illumination of the nanoparticles. This type of cooperative photo-control over nanostructure growth enables synthesis of monodisperse nanoprisms with a preselected edge length in the 30-120 nm range simply by using one beam to turn off bimodal growth and the other (varied over the 450-700 nm range) for controlling particle size. While a variety of sizes are contemplated, the size of the nanoparticles is preferably from about 5 nm to about 150 nm (mean diameter), more preferably from about 5 to about 50 nm, most preferably from about 10 to about 30 nm. The nanoparticles may also be rods.

[0151] Presently preferred for use in detecting nucleic acids are gold nanoparticles. See U.S. Patent No. 7,169,556 (hereby incorporated by reference). Gold colloidal particles have high

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extinction coefficients for the bands that give rise to their beautiful colors. These intense colors change with particle size, concentration, interparticle distance, and extent of aggregation and shape (geometry) of the aggregates, making these materials particularly attractive for colorimetric assays. For instance, hybridization of oligonucleotides attached to nanoparticles with oligonucleotides and nucleic acids results in an immediate color change visible to the naked eye.

[0152] Gold nanoparticles are also presently preferred for use in nanofabrication for the same reasons given above and because of their stability, ease of imaging by electron microscopy, and well-characterized modification with thiol functionalities. Also preferred for use in nanofabrication are semiconductor nanoparticles because of their unique electronic and luminescent properties.

[0153] Alternatively, in one embodiment, the present invention contemplates the use of nanocrystals as imaging agents, and/or coding agents (i.e. agents which permit the detection and/or identification of compounds, including biomolecules, introduced into in the arrays) as well as substances to be transferred themselves in the sites of the arrays. In some embodiments, detection of single molecules within an array is enhanced by the use of nanocrystals. In one embodiment, the nanocrystals are attached or bind to the target analyte. In another embodiment, nanocrystals are attached to a binding partner, the binding partner in turn binding the target analyte. In a further embodiment, nanocrystals are attached to an inhibitor, a substrate or other ligand. In other embodiments, the nanocrystals are used with fiber-optic sensing techniques. In some embodiments of the present invention, imaging modules comprise surface modifications of quantum dots (See e.g., Chan and Nie, Science 281:2016 (1998)) such as zinc sulfide-capped cadmium selenide coupled to biomolecules (Sooklal, Adv. Mater., 10:1083 (1998)).

[0154] The terms "semiconductor nanocrystal," "quantum dot" and "Qdot.TM. nanocrystal" are used interchangeably herein and refer to an inorganic crystallite between about 1nm and about 1000 nm in diameter or any integer or fraction of an integer therebetween, preferably between about 2 nm and about 50 nm or any integer or fraction of an integer therebetween, more preferably about 2 nm to about 20 nm (such as about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nm). A semiconductornanocrystal is capable of emitting electromagnetic radiation upon excitation (i.e., the semiconductor nanocrystal is luminescent) and includes a "core" of one or more first semiconductor materials, and may be surrounded by a

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"shell" of a second semiconductor material. A semiconductor nanocrystal core surrounded by a semiconductor shell is referred to as a "core/shell" semiconductor nanocrystal. The surrounding "shell" material will preferably have a bandgap energy that is larger than the bandgap energy of the core material and may be chosen to have an atomic spacing close to that of the "core" substrate. The core and/or the shell can be a semiconductor material including, but not limited to, those of the group II-VI (ZnS, ZnSe, ZnTe, CdS, CdSe, CdTe, HgS, HgSe, HgTe, MgS, MgSe, MgTe, CaS, CaSe, CaTe, SrS, SrSe, SrTe, BaS, BaSe, BaTe, and the like) and III-V (GaN, GaP, GaAs, GaSb, InN, InP, InAs, InSb, and the like) and IV (Ge, Si, and the like) materials, and an alloy or a mixture thereof.

[0155] A semiconductor nanocrystal is, optionally, surrounded by a "coat" of an organic capping agent. The organic capping agent may be any number of materials, but has an affinity for the semiconductor nanocrystal surface. In general, the capping agent can be an isolated organic molecule, a polymer (or a monomer for a polymerization reaction), an inorganic complex, and an extended crystalline structure. The coat is used to convey solubility, e.g., the ability to disperse a coated semiconductor nanocrystal homogeneously into a chosen solvent, functionality, binding properties, or the like. For example, binding properties can be introduced such that the nanocrystal(s) bind to the target analyte of choice. In addition, the coat can be used to tailor the optical properties of the semiconductor nanocrystal. Methods for producing capped semiconductor nanocrystals are discussed in U.S. Patent No. 6,274,323, hereby incorporated by reference.

[0156] In yet other embodiments, detection of single molecules within an array is enhanced by the use of dendrimers. In one embodiment, the dendrimers are attached or bind to the target analyte. In another embodiment, dendrimers are attached to a binding partner, the binding partner in turn binding the target analyte. In a further embodiment, dendrimers are attached to an inhibitor, a substrate or other ligand. In other embodiments, dendrimers are used with fiber-optic sensing techniques (See, e.g., Ye et al., U.S. Pat. App. No. 20040131322, PCT App. No. WO/2004057386, and Thomas et al.,, Biophysical Journal, 86(6), 3959 (2004), each of which is herein incorporated by reference in their entireties).

[0157] Dendrimeric polymers have been described extensively (See, Tomalia, Advanced Materials 6:529 (1994); Angew, Chem. Int. Ed. Engl., 29:138 (1990); incorporated herein by reference in their entireties). Dendrimer polymers are synthesized as defined spherical structures

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typically ranging from 1 to 20 nanometers in diameter. Molecular weight and the number of terminal groups increase exponentially as a function of generation (the number of layers) of the polymer Different types of dendrimers can be synthesized based on the core structure that initiates the polymerization process.

[0158] The dendrimer core structures dictate several characteristics of the molecule such as the overall shape, density and surface functionality (Tomalia et al., Chem. Int. Ed. Engl., 29:5305 (1990)). Spherical dendrimers can have ammonia as a trivalent initiator core or ethylenediamine (EDA) as a tetravalent initiator core. Recently described rod-shaped dendrimers (Yin et al., J. Am. Chem. Soc., 120:2678 (1998)) use polyethylene-imine linear cores of varying lengths; the longer the core, the longer the rod. Dendritic macromolecules are available commercially in kilogram quantities and are produced under current good manufacturing processes (GMP) for biotechnology applications.

[0159] Dendrimers may be characterized by a number of techniques including, but not limited to, electrospray-ionization mass spectroscopy, ^{13}C nuclear magnetic resonance spectroscopy, ^{1}H nuclear magnetic resonance spectroscopy, high performance liquid chromatography, size exclusion chromatography with multi-angle laser light scattering, ultraviolet spectrophotometry, capillary electrophoresis and gel electrophoresis. These tests assure the uniformity of the polymer population and are important for monitoring quality control of dendrimer manufacture for GMP applications.

[0160] Numerous U.S. Patents describe methods and compositions for producing dendrimers. Examples of some of these patents are given below in order to provide a description of some dendrimer compositions that may be useful in the present invention, however it should be understood that these are merely illustrative examples and numerous other similar dendrimer compositions could be used in the present invention.

[0161] U.S. Pat. No. 4,507,466, U.S. Pat. No. 4,558,120, U.S. Pat. No. 4,568,737, and U.S. Pat. No. 4,587,329 (each of which is hereby incorporated by reference) each describe methods of making dense star polymers with terminal densities greater than conventional star polymers. These polymers have greater/more uniform reactivity than conventional star polymers, i.e. 3rd generation dense star polymers. These patents further describe the nature of the amidoamine dendrimers and the 3-dimensional molecular diameter of the dendrimers.

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[0162] U.S. Pat. No. 4,631,337 describes hydrolytically stable polymers. U.S. Pat. No. 4,694,064 describes rod-shaped dendrimers. U.S. Pat. No. 4,713,975 describes dense star polymers and their use to characterize surfaces of viruses, bacteria and proteins including enzymes. Bridged dense star polymers are described in U.S. Pat. No. 4,737,550. U.S. Pat. No. 4,857,599 and U.S. Pat. No. 4,871,779 describe dense star polymers on immobilized cores useful as ion-exchange resins, chelation resins and methods of making such polymers. Each of these patents is hereby incorporated by reference.

[0163] U.S. Pat. No. 5,338,532 (hereby incorporated by reference) is directed to starburst conjugates of dendrimer(s) in association with a pharmaceutical or other material. This patent describes the use of dendrimers to provide means of delivery of high concentrations of carried materials per unit polymer, controlled delivery, targeted delivery and/or multiple species such as e.g., drugs antibiotics, general and specific toxins, metal ions, radionuclides, signal generators, antibodies, interleukins, hormones, interferons, viruses, viral fragments, pesticides, and antimicrobials.

[0164] U.S. Pat. No. 6,471,968 (hereby incorporated by reference) describes a dendrimer complex comprising covalently linked first and second dendrimers, with the first dendrimer comprising a first agent and the second dendrimer comprising a second agent, wherein the first dendrimer is different from the second dendrimer, and where the first agent is different than the second agent.

[0165] Other useful dendrimer type compositions are described in U.S. Pat. No. 5,387,617, U.S. Pat. No. 5,393,797, and U.S. Pat. No. 5,393,795 in which dense star polymers are modified by capping with a hydrophobic group capable of providing a hydrophobic outer shell. U.S. Pat. No. 5,527,524 discloses the use of amino terminated dendrimers in antibody conjugates. Each of these patents are hereby incorporated by reference.

[0166] The use of dendrimers as metal ion carriers is described in U.S. Pat. No. 5,560,929. U.S. Pat. No. 5,773,527 discloses non-crosslinked polybranched polymers having a comb-burst configuration and methods of making the same. U.S. Pat. No. 5,631,329 describes a process to produce polybranched polymer of high molecular weight by forming a first set of branched polymers protected from branching; grafting to a core; deprotecting first set branched polymer, then forming a second set of branched polymers protected from branching and grafting

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to the core having the first set of branched polymers, etc. Each of these patents is hereby incorporated by reference.

[0167] U.S. Pat. No. 5,898,005 and U.S. Pat. No. 5,861,319 describe specific immunobinding assays for determining concentration of an analyte. U.S. Pat. No. 5,661,025 provides details of a self-assembling polynucleotide delivery system comprising dendrimer polycation to aid in delivery of nucleotides to target site. This patent provides methods of introducing a polynucleotide into a eukaryotic cell in vitro comprising contacting the cell with a composition comprising a polynucleotide and a dendrimer polyeation non-covalently coupled to the polynucleotide. Each of these patents is hereby incorporated by reference.

[0168] Dendrimer-antibody conjugates for use in in vitro diagnostic applications has previously been demonstrated (Singh et al., Clin. Chem., 40:1845 (1994)), for the production of dendrimer-antibody constructs. Dendrimers have also been conjugated to fluorochromes or molecular beacons and shown to enter cells. They can then be detected within the cell in a manner compatible with sensing apparatus for evaluation of physiologic changes within cells (Baker et al., Anal. Chem. 69:990 (1997)). Finally, dendrimers have been constructed as differentiated block copolymers where the outer portions of the molecule may be digested with either enzyme or light-induced catalysis (Urdea and Hom, Science 261:534 (1993)).

[0169] In one embodiment, the present invention contemplates the use of dendrimers with attached imaging agents, e.g. imaging agents such as fluorophores (e.g. fluorescein isothiocyanate) or quantum dots. Fluorescein is easily attached to the dendrimer surface via the isothiocyanate derivatives, available from Molecular Probes, Inc. Hence, the present invention provides a multifunctional dendrimers to enhance single molecule detection.

VI. Exemplary Uses of the Present Invention

[0170] The system and array of the present invention has many uses. For example, the array has application to fundamental enzymology studies, as well as digital concentration measurements. Further, the array permits studies with multiple different enzymes and extends the limits of ultra-low detection for protein and DNA targets. With the ability to simultaneously monitor a large array of reaction vessels, single molecule enzymology can be used to resolve individual enzyme molecule behavior (e.g. Koff and Kon) from bulk kinetic signal.

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[0171] Another use, for example, is environmental monitoring of bacteria or viruses or both. An environmental sample potentially containing certain bacteria can be placed in contact with an array of the present invention. To detect the bacteria, the bacteria cells are lysed and a bacterial enzyme (or more than one enzyme) is targeted for detection. According to one embodiment, the cells are lysed prior to being added to the array. Alternatively, the cells are captured and a lysing step occurs on the array prior to detection. In a further alternative, no lysis may be necessary if a cell surface marker is targeted. For example, the bacteria or virus of interest can be captured with an antibody that is specific to a surface marker on the target, and then the capture can be detected with a sandwich-type assay by adding an enzyme-labelled antibody that binds to the target in another location.

[0172] Another use, for example, involves measuring gene expression. As noted above, in one embodiment, the target analytes are cells and these are introduced into the sites of the array under conditions such that some sites contain single cells. This is the first step in measuring gene expression, which can be done if desired at the whole cell level by detecting surface proteins and secreted proteins. Alternatively, the cells can be lysed and transcripts (or other components) from the cells addressed. In one embodiment, each site contains one or no transcripts. In one embodiment, normal and cancer cells are compared. In one embodiment, the cell to cell variation in gene expression can be addressed by examining gene expression at the single cell level.

[0173] Please note that all references disclosed herein are incorporated herein by reference in their entirety.

[0174] Although the present invention has been described herein with reference to preferred embodiments, persons skilled in the art will recognize that changes may be made in form and detail without departing from the spirit and scope of the invention.

EXAMPLES

Example 1

[0175] In this example, a proof-of-concept binding assay is performed using enzymatic signal amplification in an array of femtoliter sized reaction vessels. More specifically, various assays are performed to detect varying amounts of streptavidin-β-galactosidase (SβG) in solution using a biotinylated array of the present invention and then the correlation between the number

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of wells with captured SβG molecules and the concentration of the SβG in the sample is examined.

[0176] In this example, an etched fiber optic array is used to create a collection of femtoliter sized reaction vessels, each specifically functionalized and capable of capturing enzyme-labeled target molecules. Single enzyme molecules are confined to individual reaction vessels and catalyze the production of a sufficient number of fluorescent product molecules to generate a positive signal. At low target molecule concentrations, only a percentage of the capture sites bind a target molecule, enabling a binary readout of target concentration from the high-density array.

Materials

[0177] The reactor vessel arrays in this example are generated using an acid etch of the distal face of a polished 1mm fiber optic array, consisting of 24,000 individual 4.5 μm optical fibers. The core fiber material is silica, and the cladding around each fiber is germania-doped silica, which etches at a slower rate. The 4.5 μm fibers are etched to a depth of 2.9 μm, creating an array of reactor vessels, each with a 46 fL volume (*see* Figure 1a).

[0178] The fibers were first modified with an aminopropyl silane bound to both the core and cladding surfaces (*see* Figure 1b). To avoid biotin attachment to the cladding, the amino-silanized fibers were polished for 10 seconds with 0.3 μm lapping film, which removed the top amino-silanized cladding layer from the fiber array (*see* Figure 1c). After polishing, NHS-biotin was attached to the amino groups on the well surfaces (*see* Figure 1d).

Methods

[0179] First, the effectiveness of the capture component was tested. To test the effectiveness of the biotinylation of the substrate, streptavidin Alexa Fluor 568® was attached directly to the biotin groups on the surfaces of both a polished and an unpolished fiber, followed by image acquisition of the modified surface (*see* FIG. 3). FIG. 3 shows Streptavidin Alexa Fluor 568® binding to (a) an unpolished biotin modified fiber optic array, and (b) a polished biotin modified fiber optic array. As seen in image (a), streptavidin binding occurred on all surfaces, in comparison to image (b), where binding occurred only on the surfaces of the microwell reactors. Thus, the unpolished fiber shows dye over the entire array including the cladding surface, while the polished fiber shows dye localized only on the well surfaces.

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[0180] Subsequent to array modification, the biotinylated fiber arrays were incubated for 1 hour at room temperature in 150 μL PBS buffer containing varying amounts of SβG. The concentration of the SβG was chosen so that during the incubation time, statistically either one molecule or no molecules would bind to each well. The arrays were then washed repeatedly in PBS buffer, to ensure that unbound target was removed.

[0181] For a binary readout of SβG binding, the fiber array was loaded and secured on an upright microscope system equipped with a mechanical platform. A solution of β-galactosidase substrate, resorufin-β-D-galactopyranoside (RDG), was introduced to the distal end of the fiber containing the reaction vessels, and subsequently sealed. The substrate was sealed using a 0.01-inch thick silicone elastomer gasket sandwiched between a microscope slide and the fiber array by means of a mechanical platform located beneath the microscope stage. This platform applied a uniform pressure to the gasket material, across the entire bundle, sealing off each reaction chamber and enabling well to well interrogation of enzyme activity. β-galactosidase hydrolyzes RDG to form resorufin, which builds up to a locally high concentration in each sealed reaction vessel, generating a detectable fluorescent signal (Figure 4).

[0182] FIG. 4 depicts a portion of the fiber array for each experiment. Each of the experiments tested a different sample having a different concentration of SβG. The concentrations for each experiment were as follows: (a) 128 amol, (b) 51 amol, (c) 25 amol, (d) 7.5 amol, and (e) 2.6 amol. FIG. 4(f) depicts the control.

[0183] Analysis of over 5000 reaction vessels for each experiment allowed for a correlation between the percentage of reaction vessels that captured an enzyme molecule and the amount of enzyme present in the interrogated sample. The variation seen in the intensity differences from active well to active well is most likely a result of molecule-to-molecule variation in catalytic activity, in combination with surface effects, which may modulate the relative activities of enzyme molecules based on their orientation to the reaction chamber surface.

[0184] Two control experiments were also conducted to ensure that the binding of enzyme to the surface of the reactors was based exclusively on the biotin-streptavidin interaction, and not on non-specific binding to the glass surface. One control experiment consisted of an etched, unmodified fiber incubated with the most concentrated SβG target solution (128 amol in 150 μL). The second control experiment was performed using the modified fiber incubated in a

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solution of β-galactosidase lacking streptavidin (128 amol in 150 μL). Both control experiments generated a negligible active well percentage (less than 0.06%, versus 0.2% for the 2.6 amol experiment discussed below).

Results

[0185] FIG. 5 depicts a log-log plot of the moles of target present in a sample with the resulting percentage of active reaction vessels. The linear relationship between the percentage of active reaction vessels and the moles of target in the log-log plot shown in FIG. 5 suggests that a binary readout detection method can be used for the detection of real targets such as DNA and antigens. This method permits rapid analysis and accurate concentration information via digital readout, while maintaining a straightforward assay procedure.

[0186] It is also interesting to note that the lowest limit of detection (LOD) for binding streptavidin-β-galactosidase (SβG) to a biotinylated femtoliter array in this example was 2.6 amoles (150 μL of 17 fM solution) using a target incubation time of 1 hour.

Example 2

[0187] In this example, single molecules of β-galactosidase were monitored using a 1 mm diameter fiber optic bundle with over 2.0 x 10^5 individually sealed, femtoliter microwell reactors. By observing the buildup of fluorescent products from single enzyme molecule catalysis over the array of reaction vessels and by applying a Poisson statistical analysis, a digital concentration readout was obtained.

Materials

[0188] 1mm bundled 4.5 μm optical fibers were purchased from Illumina (San Diego, CA). β-galactosidase and $Ru(bpy)_3Cl_2$ was obtained from Sigma-Aldrich (St. Louis, MO). Resorufin-D-β-galactopyranoside was purchased from Molecular Probes (Eugene, OR). 0.01-inch non-reinforced gloss silicone sheeting material was purchased from Specialty Manufacturing Inc. (Saginaw, MI). All other chemicals used were of reagent grade and obtained from Sigma-Aldrich (St. Louis, MO).

[0189] A custom-built, upright epifluorescence imaging system acquired all fluorescence images using a mercury light source, excitation and emission filter wheels, microscope objectives, and a CCD camera (QE, Sensicam). Filter wheels and shutters were computer controlled and analysis was performed with IPlab software (Scanalytics, Fairfax, VA). The

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system was equipped with a fastening device to fix the fiber optic array onto the system through the entire experiment. A mechanical platform beneath the stage was used to house the silicone-sealing layer, which was subsequently brought into contact with the distal end of the fiber array, sealing off each reaction vessel. All measurements were performed with femtowell arrays at the distal end of the optical fiber bundle.

[0190] Optical fiber bundles containing approximately 2.4 x 10^5 individual 4.5 μm diameter optical fibers were used as the substrate for fabricating femtoliter reaction vessel arrays. The well volume can be precisely controlled, as etch depth varies with etch time and etchant concentration. The optical fibers used in these experiments were etched to a depth of approximately 2.9 μm, yielding a 46 fL well volume. FIG. 6 depicts images of the etched surface of the fiber optic bundles. More specifically, FIG. 6a depicts the entire fiber array and close-up microscope images of the fiber bundle, emphasizing the regularity of both the array and each individual optical fiber. Further, FIG. 6b is an AFM image of a portion of the etched surface, showing wells created from the etching process.

Methods

[0191] *Assay.* For the β-galactosidase assay, the substrate used was resorufin-β-D-galactopyranoside. After the individual wells in the array were sealed in the presence of enzyme and substrate, the fluorescence intensity was monitored across the array of vessels for the enzymatic product, resorufin (ex 558 nm / em 573 nm). A 100 μM solution of resorufin-D-β-galactopyranoside (RDG) was prepared in 100mM Tris buffer pH 8.0 containing 2.0 mM KCl and 0.1mM $MgCl_2$. All enzyme solutions were prepared from previously aliquoted and frozen stock samples in the same reaction buffer. Just prior to experimentation, the two samples were centrifuged for 2 min at 7000 RPM to remove any particulate material that could interfere with the mechanics of the silicone seal. Approximately 1 cm^2 of silicone and a microscope slide were cleaned with absolute ethanol. The silicone sheeting was placed on the surface of the glass, to which it adhered. Subsequently, 75 μL volumes of enzyme and RDG solutions were mixed on the silicone gasket using a pipette. The gasket was mechanically raised towards the distal end of the fiber bundle until it experienced resistance, suggesting that a seal was formed. An initial fluorescence image was acquired, followed by periodic image acquisition for approximately 2 hr.

[0192] *Sealing component.* To seal the femtoliter array, a 0.01-inch thick silicone elastomer gasket was sandwiched between a microscope slide and the fiber array using a

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mechanical platform. This platform applied uniform pressure to the gasket material, across the entire bundle, sealing off each microwell to create the reaction vessels.

[0193] The silicone/glass seal used to create and isolate the femtoliter containers was inspected for its sealing ability by performing a photobleaching experiment (*see* Fig. 7). FIG. 7 depicts enclosure of a solution into the microchambers and evaluation of the silicone seal for integrity. FIG. 7a depicts a solution of $Ru(bpy)_3Cl_2$ enclosed into the array of chambers as observed by the red fluorescence across the array. FIG. 7b depicts a small octagonal portion of the fiber bundle that was photobleached via UV light. FIG. 7c depicts the array 60 minutes later. As shown in the figure, diffusion of $Ru(bpy)_3Cl_2$ from one well to another as a result of an imperfect silicone seal would display increased fluorescence intensity in photobleached wells and was not observed. This experiment substantiated the integrity of the seal for its ability to successfully isolate the array of vessels. Enzyme molecule denaturation on the glass surface was prevented by blocking with a BSA blocking buffer. Enzyme to vessel ratios used ranged from 1:5, down to 1:500, achieving accurate detection over two orders of magnitude.

[0194] *Photobleaching Experiment.* A solution of 1mM $Ru(bpy)_3Cl_2$ in DI water was used for the photobleaching experiments. A piece of silicone, approximately 1 cm^2, and a microscope slide were cleaned with absolute ethanol using lint-free swabs. The silicone sheeting was placed on the surface of the glass, to which it adhered. 50 μL of the $Ru(bpy)_3Cl_2$ solution was placed on the silicone, and subsequently brought into contact with the fiber bundle, to enclose the solution in the individual vessels. Using a field stop on the imaging system, UV light was used to illuminate a small portion of the array for 10 minutes, photobleaching the $Ru(bpy)_3Cl_2$. The field stop was then opened, and an image was acquired, displaying the difference in fluorescence. The array was then allowed to rest with the seal maintained. A final image was taken after 60 minutes, confirming the integrity of the seal.

[0195] As discussed above, the number and volume of reaction vessels employed govern the dynamic range of concentrations that can be determined by this technique. The reaction vessel volumes employed in this example were 46 fL (vide infra); therefore, it was calculated that a solution of 3.6×10^{-11} M β-galactosidase will yield, on average, one enzyme molecule per vessel. As also discussed above, if the concentrations used are much less than 3.6×10^{-11} M, the expected average becomes exceptionally low, the distribution is narrowed, and the probability of observing anything other than 0 or 1 events per trial is improbable in all experimental cases. At

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these low concentrations, the relationship between the percentage of active reaction vessels and the bulk enzyme concentration is approximately linear. After waiting for sufficient time to allow enzyme catalysis to occur, individual vessels were interrogated for an on/off response, correlating to each vessel either possessing or lacking enzymatic activity.

[0196] The substrate resorufin-D-β-galactopyranoside (RDG) was used as the substrate for experiments, which was sealed into all the vessels, along with the trapped enzyme molecules, using a silicone gasket material and mechanical arm. The expected percentages of active wells were calculated for each concentration used by applying the Poisson distribution statistics.

Results

[0197] As shown in FIG. 8, for the β-galactosidase assay, different bulk solution enzyme concentrations correspond to different ratios of enzyme to vessel volume, resulting in variation in the percentage of vessels that contain an enzyme molecule. Figure 8 depicts the detection of the activity of single molecules of β-galactosidase. FIG. 8a is a background image of a portion of the array, while FIG. 8b depicts an image taken of a portion of a 1:5 enzyme to vessel assay, and FIG. 8c shows a 1:80 enzyme to vessel assay.

[0198] Table 1 is a comparison of each experimental result with the percentage of occupied vessels calculated from the Poisson distribution. As shown by the data in the table, the array measurements successfully correlated with the number of single enzyme β-galactosidase molecules over the entire range of interrogated concentrations. There is minor disparity in the observed signals as a result of molecule-to-molecule variation in catalytic activity. This result is most likely due to the inherent stochastic nature of enzymes, in addition to surface effects, resulting in modulation of enzyme activity.

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[0199]

Digital Readout of Enzyme Concentrations

Enzyme to well ratio	Concentration	Poisson % of active wells	Actual % active
1:5	7.20E-12	18.2	**14.9**
1:10	3.60E-12	9.5	**11.5**
1:20	1.80E-12	4.9	**5.6**
1:40	9.00E-13	2.5	**3.5**
1:80	4.50E-13	1.2	**1.5**
1:100	3.60E-13	1.0	**1.1**
1:200	1.80E-13	0.5	**0.3**
1:500	7.20E-14	0.2	**0.1**

Table 1. Digital readout from the arrays. The actual percentage of chambers exhibiting activity, in comparison to the expected percentage calculated from the Poisson distribution, are listed for the various concentrations analyzed.

[0200] The variation between the calculated and experimental results can be attributed to the intrinsic variability associated with the probability distribution, as well as experimental error in the preparation of enzyme solutions.

Example 3

[0201] In this example, beta-galactosidase was studied in the presence of a competitive inhibitor, D-galactal (a transition state analog of D-galactose) as a model enzyme-inhibitor-pair. The scope of this application shall, however, not be limited to this particular enzyme-inhibitor-pair. Beta-galactosidase is a tetramer and D-galactal is known to exhibit relatively slow binding and release. Figure 9 shows one embodiment of the scheme for measuring single molecule inhibition, as well as detection of inhibitor release. In a first step, the enzyme and inhibitor are preincubated in an aqueous buffer system such as a phosphate buffer (in this particular embodiment PBS/$MgCl_2$-buffer: 2.7 mM KCl, KH_2PO_4, 1.5 mM KH_2PO_4, 136 mM NaCl, 8.1 mM Na_2HPO_4, 1 mM $MgCl_2$, pH 7.3) together in bulk, such that the inhibitor concentration ([inhibitor]) is higher than the dissociation constant for inhibitor binding (K_i) and all of the active

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sites of the enzyme are likely to be blocked or occupied. As K_i differs for different enzyme/inhibitor pairs, the concentration of inhibitor must be adjusted. With this particular enzyme/inhibitor pair used in this example a variety of inhibitor starting concentrations can be employed, ranging between 100 mM and 20 μM. In this embodiment, [inhibitor] was 100 μM (~ 7 x K_i (14 μM)). In a second step, one or more substrates (e.g. rsesorufin-beta-galactopyranoside RGP) in an aqueous buffer system (in this particular embodiment: PBS/$MgCl_2$-buffer) are added, preferably in a high volume excess (between 10-fold and 100,000-fold). In this particular embodiment a 1000-fold volume excess was used, thereby diluting the inhibitor and the enzyme 1000-fold. More components (in this particular embodiment 1% (w/v) bovine serum albumin (BSA) to avoid unspecific binding to the surface of the arrays of sites) may be added to the buffer system. In a third step, the bulk solution is added to the array of sites, each site having a defined volume (between 10 attoliters and 50 picoliters, more preferably femtoliters, in this embodiment: 46 femtoliters). At this point, the inhibitor is not in excess any more; indeed, [inhibitor] << K_i (it is preferred that less than 100,000 molecules of inhibitor, and preferably less than 10,000 molecules of inhibitor, and still more preferably less than 3,000 molecules of inhibitor, are remaining in the solution). The enzyme concentration (1.8 nM) is adjusted so that upon 1,000-fold dilution in step 2 there will be only a single enzyme in every array site in some sites and no enzyme in others. Figure 9 shows schematically the situation where one of the active sites of the tetramer is unblocked and active. This active site would be capable of interacting with a substrate that includes a reporter-moiety. This domain may create a chromogenic or a fluorogenic signal upon enzymatic cleavage but is not limited to these. Preferably a fluorogenic signal is generated, and more preferably RGP is used as the fluorogenic substrate. In this way the site in the array with such activity can be readily detected (Figure 10).

[0202] In one embodiment, the present invention contemplates determining substrate turnover at time points (e.g. intervals of seconds, tens of seconds, 100s of seconds, minutes, tens of minutes, etc.). Figure 11 shows that when assayed according to the scheme of Figures 9 and 10, the presence of an inhibitor delays substrate turnover, as compared to a control (lower left panel) where no inhibitor is present. When the frequency distribution of substrate turnover onset times is plotted (Figure 12), the K_{off} rate constant can be calculated: 4.6×10^{-3} sec. This agrees well with published values.

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[0203] Interestingly, the data suggests that release of inhibitor at each of the four sites of the tetramer is not independent. That is to say, if there are 4 independent catalytic sites of a tetramer and koff is a first order rate constant then koff of an enzyme ensemble is the same as koff of each protomer. But as each single enzyme contains 4 protomers there are 4 possibilities to release a first inhibitor. Therefore: $koff_1 = 4 \times koff$ ($= 1.8 \times 10^{-2}$ sec^{-1}). The data suggests that the inhibitor release from the protomers is non-independent.

[0204] In a second embodiment without preincubation the inhibitor is given to the enzyme in an aqueous buffer system such as a phosphate buffer (in this particular embodiment PBS/$MgCl_2$-buffer. More components (in this particular embodiment 1% (w/v) (BSA) to avoid unspecific binding to the surface of the arrays of sites) may be added to the buffer system. The [inhibitor] is chosen in the same range as Ki (14 μM), preferably between 100 mM and 1 μM, in this embodiment 20 μM. At this inhibitor concentration the inhibitor-bound enzyme is in equilibrium with the inhibitor-free enzyme. The bulk solution is added to the array of sites, each site having a defined volume (between 10 attoliters and 50 picoliters, more preferably femtoliters, in this embodiment: 46 femtoliters). The enzyme concentration (1.8 pM) is chosen to yield only a single enzyme in some sites and no enzyme in other sites. When fluorescence is monitored over time, one can see binding, release, and subsequent binding (Figure 13). Figure 13A (left panel) represents the raw data from various time traces. Figure 13B (the right panel) simply shows one of the time traces of the left panel, where for the sake of clarity, the y-axis is scaled differently. Figure 13B shows that the signal increases after the inhibitor has been released from the enzyme and it decreases after the inhibitor has been bound. One should expect that the fluorescence signal remains constant over time if the inhibitor is bound to the enzyme as there is no enzyme activity. But this is not the case because there is a substantial amount of photobleaching that depletes the fluorophor continuously. This creates a dynamic with the net results (over time) shown in Figure 13.

[0205] Figure 14 shows a histogram of off-times (the time how long the inhibitor stays on the enzyme before it is released. This permits a calculation of the half-life (the half-life of a quantity subject to exponential decay is the time required for the quantity to decay to half of its initial value) which is experimentally determined to be approximately two minutes and thirty seconds. This example demonstrates that a system that measures single enzymes may reveal

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more kinetic data about how the enzyme subunits interact than were previously available from ensemble reactions.

Example 4

[0206] In one embodiment, nanoparticles are utilized for the digital readout of target concentrations. Nanoparticles have several advantages over an enzyme digital readout. With an enzyme approach, as concentrations increase, the linearity of the digital readout, which follows a Poisson distribution, is lost. Using nanoparticles functionalized with fluorescent species, the present invention contemplates in one embodiment to be able to perform a digital readout as well as the traditional bulk intensity readout, which is used extensively in our laboratory. As the linearity of the digital signal is lost, the analysis can be shifted from a digital readout to a traditional intensity readout using the nanoparticles. The signal can then be read as an average intensity of each site in the array (e.g. average well intensity) correlating to concentration, and will significantly increase the range of concentrations that can be read with this system. In some embodiments employing nanoparticles, the array is not sealed.

[0207] Nanoparticles such as gold particles (e.g. between 1 and 500 nanometer, and more preferably between 50 and 250 nanometer, and still more preferably between 80-150 nanometer gold particles) may be attached to the target analyte or a ligand that will bind a target analyte. A variety of embodiments are contemplated, including but not limited to embodiments where the target is attached to the gold particle and introduced into an array, said array comprising a binding partner (e.g. immobilized ligand). In another embodiment, the target analyte may be attached or otherwise immobilized on the array and the gold particle-binding partner conjugate can be introduced to the array to bind the target analyte. In another embodiment, a first binding ligand may immobilize the target, and the gold-particle-second binding ligand may be introduced into the array to bind the target. These embodiments are schematically illustrated in Figures 15 and 16, where the target is nucleic acid and the binding partners are complementary probes. In Figure 15, either probe # 1 or probe # 2 could be replaced with target, if desired. In Figure 16 shows a two-step sandwich format, where the target can be unlabeled and first incubated with probe #1; thereafter, the nanoparticle with probe #2 is introduced. The target can be viewed to operate as a linker in this embodiment.

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[0208] In the present example, the target analyte is nucleic acid, and the gold particles are labeled with a plurality (e.g. approximately 2000-8000 strands) of fluorescent DNA; this DNA-gold particle conjugate will be used to generate signal. Because of the high number of fluorescent DNA strands attached to each nanoparticle, a single particle is sufficient for a signal spike over background in a single site (e.g. microwell) of the array (e.g. the fiber optic array described above). The fluorescence intensity deviation over a population of nanoparticles is very small in comparison to enzymes, whose catalytic rate is highly fluctuating. Because of this high fluctuation rate, only a yes or no response can be deciphered, limiting the range of detection for enzymes to approximately two orders of magnitude. Using nanoparticles that have equivalent signal intensities will allow for a more controlled readout of 0 or 1 as carried out with the enzymes, along with readouts of 2, 3, 4, and so on. The ability to readout discreet intensity levels will enable a superior range of detection, well beyond two orders of magnitude. Analyte-functionalized gold nanoparticles (e.g. DNA-gold particle conjugates or other biomolecule-gold particle conjugates) will improve the limit of detection, as they consistently show extremely low levels of non-specific and cross-reactive binding in analyte (e.g. DNA) detection schemes.

A. Preparation of Gold Particles

[0209] Gold particles can be prepared a number of ways (*Analytical Chemistry*, 2000, 72, 5535-5541; *Science*, 2002, 296, 1836-1838; *Science*, 2002 295, 1503-1506; *J. Am. Chem. Soc.* 2004, 126, 5932-5933). In one case, gold colloids (13 nm diameter) are prepared by reduction of $HAuCl_4$ with citrate as described in Frens, Nature Phys. Sci., 241, 20 (1973) and Grabar, Anal Chem., 67, 735 (1995). Briefly, all glassware is cleaned in aqua regia (3 parts HCl, 1 part HNO_3), rinsed with Nanopure H_2O, then oven dried prior to use. $HAuCl_4$ and sodium citrate are purchased from Aldrich Chemical Company. Aqueous $HAuCl_4$ (1 mM, 500 mL) is brought to reflux while stirring. Then, 38.8 mM sodium citrate (50 mL) is added quickly. The solution color will change from pale yellow to burgundy, and refluxing should continue for 15 min. After cooling to room temperature, the red solution can be filtered through a Micron Separations Inc. 1 micron filter. Au colloids are readily characterized by UV-vis spectroscopy using a Hewlett Packard 8452A diode array spectrophotometer and by Transmission Electron Microscopy (TEM) using a Hitachi 8100 transmission electron microscope. Gold particles with diameters of 13 nm

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will produce a visible color change when aggregated with target and probe oligonucleotide sequences in the 10 to 35 nucleotide range.

B. Fiber Modifications

[0210] In this example, a fiber array is employed. The polished fiber array is etched for 115 seconds in 0.025 M HCl giving 46 fL wells. For cleaning, the etched fibers is incubated in boiling Nanopure water for 10 minutes.

[0211] The surface of the etched fiber can be modified in a number ways. In this example, an amine modification of the fiber is employed. First, a silanization solution is prepared, i.e. a 1% solution of DETA (trimethoxysilylpropyldiethylenetriamine) in a 95:5 solution of ethanol:nanopure water. The etched fiber was incubated in the silanization solution for 20 minutes at room temperature, followed by washing with nanopure water and drying under argon. The fiber was then cured at 120°C for 15 minutes. At this point, the fiber is now modified with amine groups.

[0212] A variety of functional groups can be reacted with the amine groups. In this example, the process involved maleimide modification of the fiber. 3.55 mg of SMPB (Succinimidyl 4-[p-maleimidophenyl]butyrate) was dissolved in 1 mL DMSO to create a stock solution. A cross-linking solution was prepared: 100 μL of this stock solution was added to 900 μL of a 90:10 solution of ethanol:DMSO. The silanized fibers are incubated in the crosslinking solution for 2 hours at room temperature. The array is then washed with an 80:20 solution of ethanol:DMSO and dried under argon. At this point, The fiber is now modified with maleimide groups.

C. DNA Deprotection

[0213] 2 nanomoles of thiol modified (protected) DNA is reconstituted in 110 μL PBS pH 7.4. 10 μL DTT (dithiothreitol) stock (1.0 M) is added to the protected DNA solution and incubated for 2 hours at room temperature. A NAP-5 column (GE Healthcare) was used to purify the deprotected DNA. The column was equilibrated with Nanopure water.

[0214] The 120 μL solution of deprotected thiol DNA was added to the column and allowed to completely enter the gel. 380 μL PBS 7.4 water was added to the column and allowed to completely enter the gel. A collection tube was placed under the column and the thiol DNA

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was eluted by adding 1.0 mL PBS 7.4 water to the column. The first 400 μL was discarded, and DNA was collected from 400 μL to 1 mL, as described in the NAP-5 column protocol (provided by the manufacturer).

D. DNA Attachment

[0215] A ligand (in this case, a probe) specific for the target analyte (in this case, nucleic acid) can be attached directly to the sites of the array or to the nanoparticle (e.g. gold particle) and then the conjugate (DNA-gold nanoparticle) can introduced into the array. In this example, the maleimide-treated fibers were incubated in the thiol deprotected DNA solution (1 μM) for 2 hours at room temperature. The fibers were rinsed with PBS 7.4 to remove unbound DNA and the array was swabbed to remove DNA probes that were bound to the cladding material. Gold nanoparticles (in this case 80 nm) were then incubated with the fiber and preliminary experiments show successful binding (Figure 16).

Example 5

[0216] This example describes the immobilization of synthetic single-stranded DNA on semiconductor nanoparticle quantum dots (QDs). Native CdSe/ZnS core/shell QDs (-4 nm) are soluble only in organic media, making direct reaction with alkylthiol-terminated single-stranded DNA difficult. This problem is circumvented according to Mirkin et al. by first capping the QDs with 3-mercaptopropionic acid. The carboxylic acid group is then deprotonated with 4-(dimethylamino)pyridine, rendering the particles water soluble, and facilitating reaction of the QDs with either 3'-propylthiol- or 5'-hexylthiol-modified oligonucleotide sequences. After DNA modification, the particles are separated from unreacted DNA by dialysis. A "linker" DNA strand can then be hybridized to surface-bound sequences, generating extended assemblies of nanoparticles. The QD assemblies are readily characterized by TEM, UV/Visible spectroscopy, and fluorescence spectroscopy. Reportedly, they can be reversibly assembled by controlling the temperature of the solution. As a result, QD assemblies and composite aggregates can be formed between QDs and nanoparticles (e.g. gold nanoparticles of about.13 nm).

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[0217] Each of the following references is incorporated by reference in their entirety.

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Claims

What is claimed is:

1. A method of producing an array of single molecules, comprising:
   a) providing a sample and an array, said sample comprising a molecules of target analyte in a concentration, said array comprising at least 1000 sites, each site having a defined volume between 10 attoliters and 50 picoliters; and
   b) contacting said array with said sample such that the ratio of molecules of target analyte to each site of said array is less than 1:1, so as to produce an array comprising sites containing single molecules.

2. The method of Claim 1, wherein said target analyte comprises biomolecules.

3. The method of Claim 2, wherein said biomolecule is selected from the group consisting of proteins, nucleic acids, lipids, and carbohydrates.

4. The method of Claim 2, wherein said biomolecule is selected from the group consisting of hormones, cytokines and cellular antigens.

5. The method of Claim 2, wherein said biomolecule is a receptor.

6. The method of Claim 5, wherein said receptor is selected from the group consisting of neural receptors, hormonal receptors, and nutrient receptors.

7. The method of Claim 2, wherein said biomolecules are labeled.

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8. The method of Claim 1, wherein said target analyte is an environmental pollutant.

9. The method of Claim 1, wherein said target analyte is selected from the group consisting of pesticides, insecticides and toxins.

10. The method of Claim 1, wherein said target analyte is a drug.

11. The method of Claim 1, wherein said ratio is less than 1:5.

12. The method of Claim 1, wherein said ratio is 1:10.

13. The method of Claim 11, wherein said ratio is between 1:5 and 1:500.

14. The method of Claim 1, wherein the percentage of sites containing a single molecule provide a measure of the concentration of molecules in said sample.

15. An array produced according to the method of Claim 1.

16. The method of Claim 1, further comprising, after step b), sealing the array such that the contents of each site cannot escape said site.

17. An array produced according to the method of Claim 16.

18. The method of Claim 1, wherein said defined volume is measured in femtoliters.

19. An array produced according to the method of Claim 18.

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20. The method of Claim 18, wherein said defined volume is the same at each site and ranges from about 30 femtoliters to about 60 femtoliters.

21. The method of Claim 20, wherein said defined femtoliter volume is 46 femtoliters.

22. The method of Claim 1, wherein said array comprises between 20,000 and 30,000 sites.

23. The method of Claim 1, wherein said array comprises between 100,000 and 10,000,000 sites.

24. The method of Claim 1, wherein each site of said array comprises a capture component.

25. The method of Claim 24, wherein said target analyte comprises a single-stranded nucleic acid, and said capture component comprises a complementary nucleic acid.

26. A method of detecting a target analyte in a sample, the method comprising:
a) providing a sample and an array, said sample comprising a biomolecule target analyte, said array comprising at least 1000 sites, each site having a defined volume between 10 attoliters and 50 picoliters;
b) contacting said array with said sample such that the ratio of biomolecule target analyte to each site of said array is less than 1:1; and
c) determining the number of sites of said array which contain a single biomolecule.

27. The method of Claim 26, wherein said ratio is less than 1:5.

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28. The method of Claim 26, wherein said ratio is 1:10.

29. The method of Claim 27, wherein said ratio is between 1:5 and 1:500.

30. The method of Claim 26, wherein said biomolecules are labeled.

31. The method of Claim 26, wherein said determining comprises detecting a change in an optical property at said sites as an indication of the presence of said target analyte.

32. The method of Claim 26, wherein the percentage of sites containing a single biomolecule provides a measure of the concentration of biomolecules in said sample.

33. The method of Claim 26, further comprising, after step b), sealing the array such that the contents of each site cannot escape said site.

34. The method of Claim 26, wherein said defined volume is measured in femtoliters.

35. The method of Claim 34, wherein said defined femtoliter volume is the same at each site and ranges from about 30 femtoliters to about 60 femtoliters.

36. The method of Claim 35, wherein said defined femtoliter volume is 46 femtoliters.

37. The method of Claim 26, wherein said array comprises between 20,000 and 30,000 sites.

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38. The method of Claim 26, wherein said array comprises between 100,000 and 10,000,000 sites.

39. The method of Claim 26, wherein each site of said array comprises a capture component.

40. The method of Claim 39, wherein said target analyte comprises a single-stranded nucleic acid, and said capture component comprises a complementary nucleic acid.

41. The method of Claim 26, wherein said biomolecule is selected from the group consisting of proteins, nucleic acids, lipids, and carbohydrates.

42. The method of Claim 26, wherein said biomolecule is selected from the group consisting of hormones, cytokines and cellular antigens.

43. The method of Claim 26, wherein said biomolecule is a receptor.

44. The method of Claim 43, wherein said receptor is selected from the group consisting of neural receptors, hormonal receptors, and nutrient receptors.

45. A method of detecting a target analyte in a sample, the method comprising:

a) providing a sample and an array, said sample comprising a biomolecule target analyte in a first concentration, said array comprising at least 10,000 sites, each site having a defined femtoliter volume;

b) diluting said sample to create a diluted sample, said diluted sample comprising a biomolecule target analyte in a second concentration;

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c) contacting said array with said diluted sample such that the ratio of biomolecule target analyte to each site of said array is between 1:5 and 1:500; and

d) determining the number of sites of said array which contain a single biomolecule.

46. The method of Claim 45, wherein said biomolecules are labeled.

47. The method of Claim 45, wherein said determining comprises detecting a change in an optical property at said sites as an indication of the presence of said target analyte.

48. The method of Claim 45, wherein the percentage of sites containing a single biomolecule provides a measure of the concentration of biomolecules in said diluted sample.

49. The method of Claim 45, further comprising, after step c), sealing the array such that the contents of each site cannot escape said site.

50. The method of Claim 45, wherein said defined volume is measured in femtoliters.

51. The method of Claim 50, wherein said defined femtoliter volume is the same at each site and ranges from about 30 femtoliters to about 60 femtoliters.

52. The method of Claim 51, wherein said defined femtoliter volume is 46 femtoliters.

53. The method of Claim 45, wherein said ratio is 1:10.

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54. The method of Claim 45, wherein said array comprises between 20,000 and 30,000 sites.

55. The method of Claim 45, wherein said array comprises between 100,000 and 10,000,000 sites.

56. The method of Claim 45, wherein each site of said array comprises a capture component.

57. The method of Claim 56, wherein said target analyte comprises a single-stranded nucleic acid, and said capture component comprises a complementary nucleic acid.

58. The method of Claim 45, wherein said biomolecule is selected from the group consisting of proteins, nucleic acids, lipids, and carbohydrates.

59. The method of Claim 45, wherein said biomolecule is selected from the group consisting of hormones, cytokines and cellular antigens.

60. The method of Claim 45, wherein said biomolecule is a receptor.

61. The method of Claim 60, wherein said receptor is selected from the group consisting of neural receptors, hormonal receptors, and nutrient receptors.

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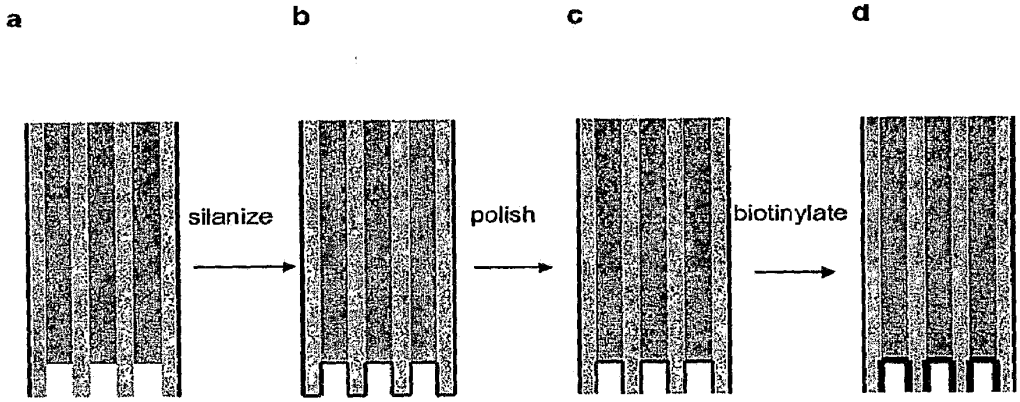
FIG. 1



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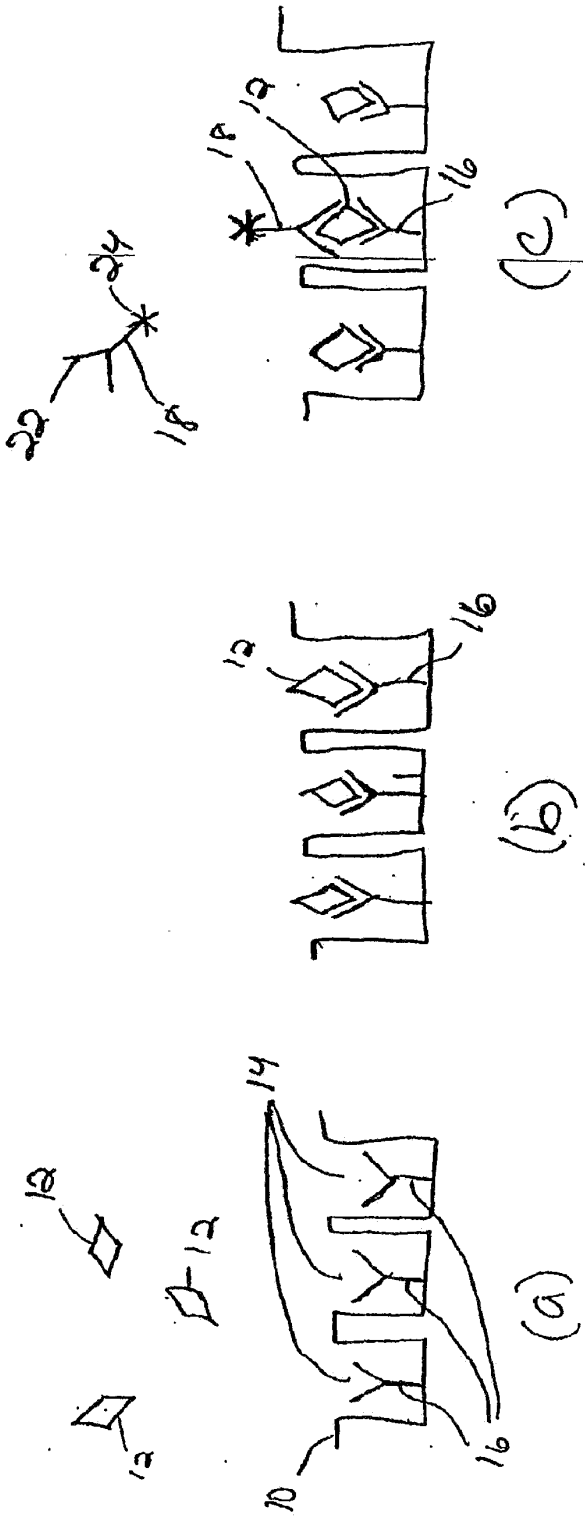

FIG. 2

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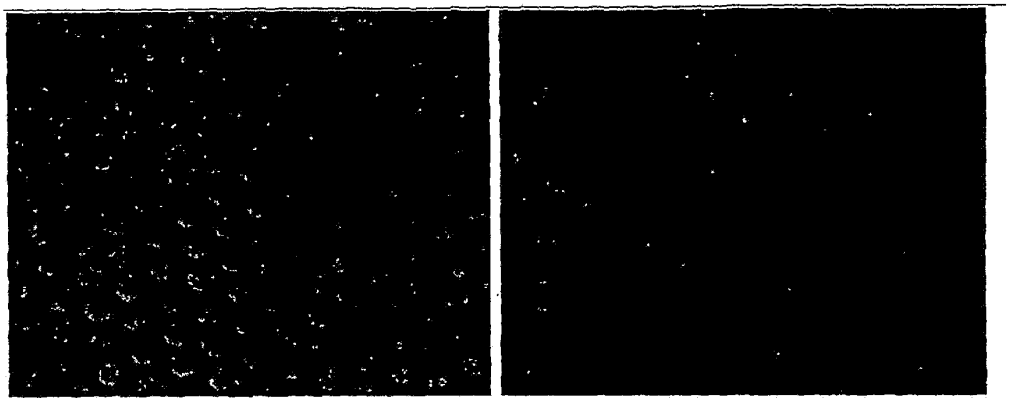
FIG. 3



(a) (b)

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FIG. 4

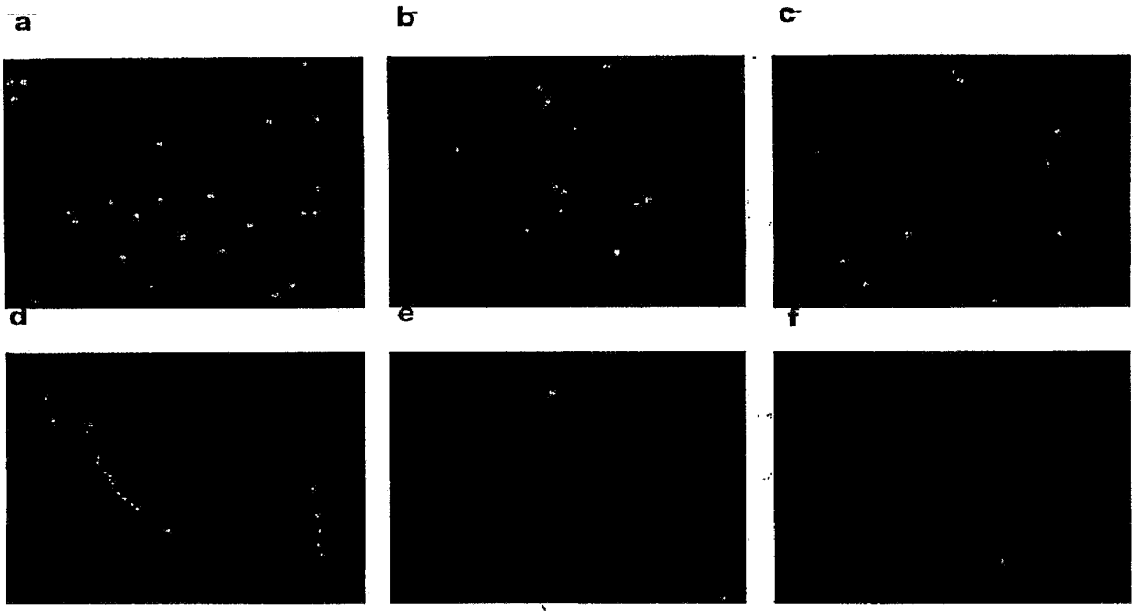

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FIG. 5

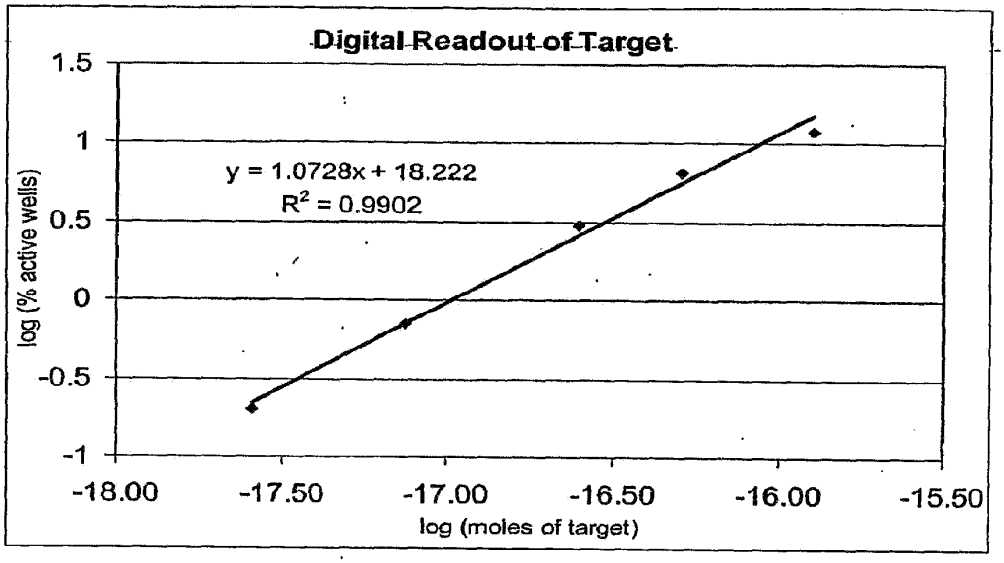

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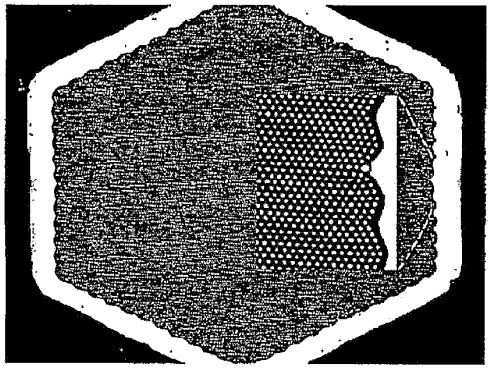
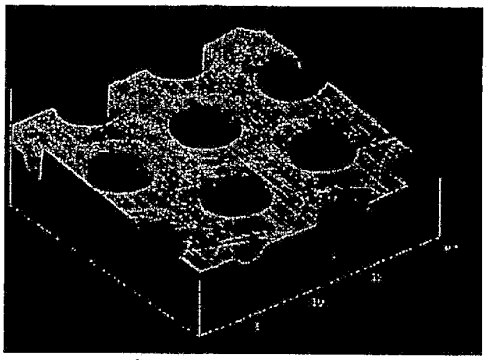
FIG. 6



(a)



(b)

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FIG. 7

(a) (b) (c)

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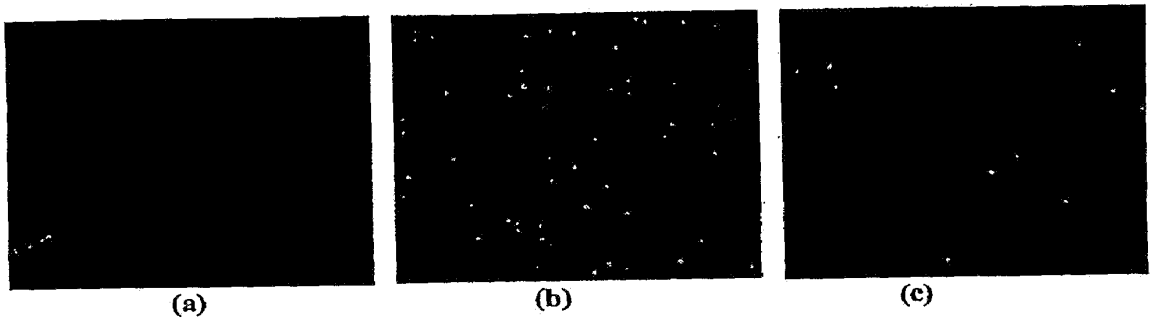
FIG. 8



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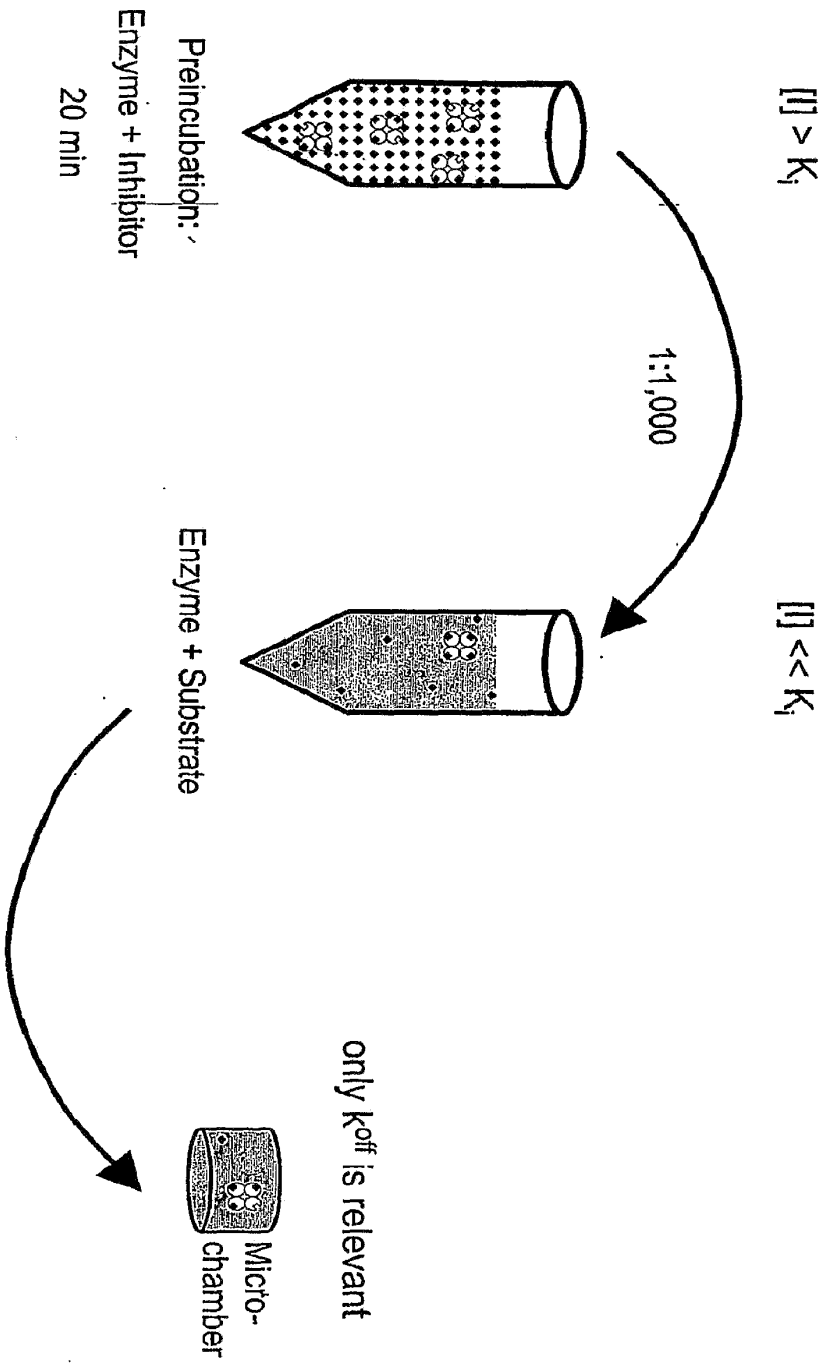

FIG. 9

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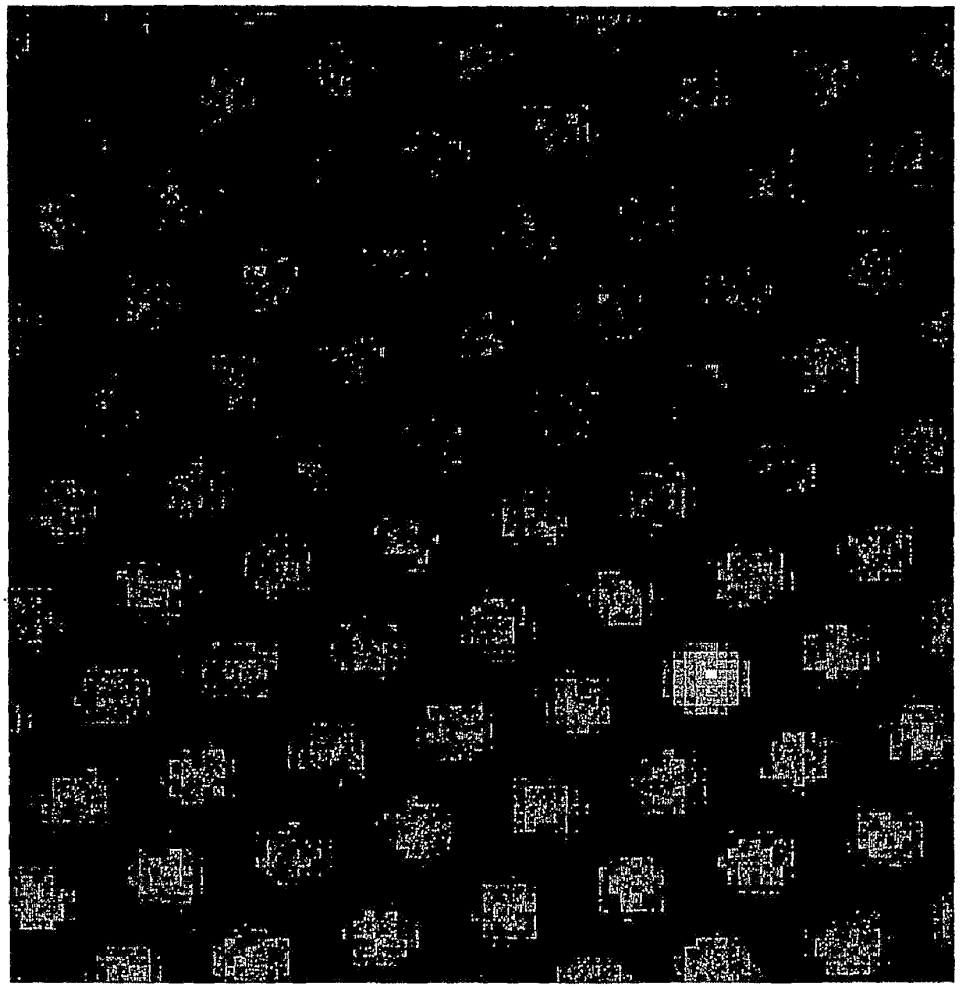

FIG. 10

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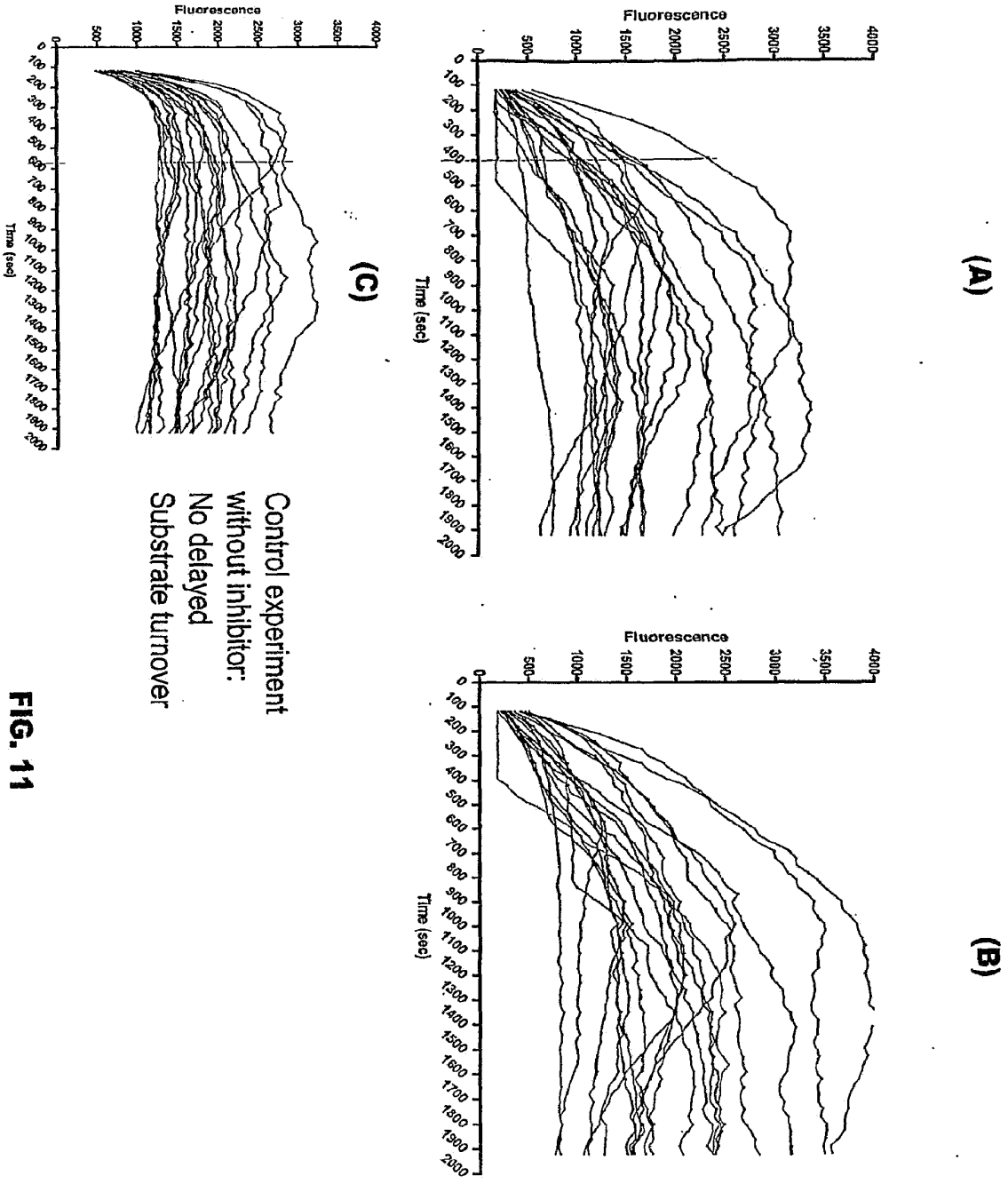

FIG. 11

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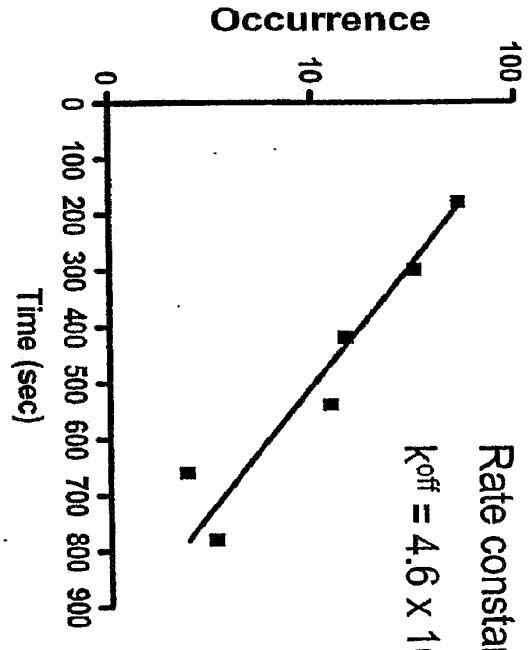

FIG. 12

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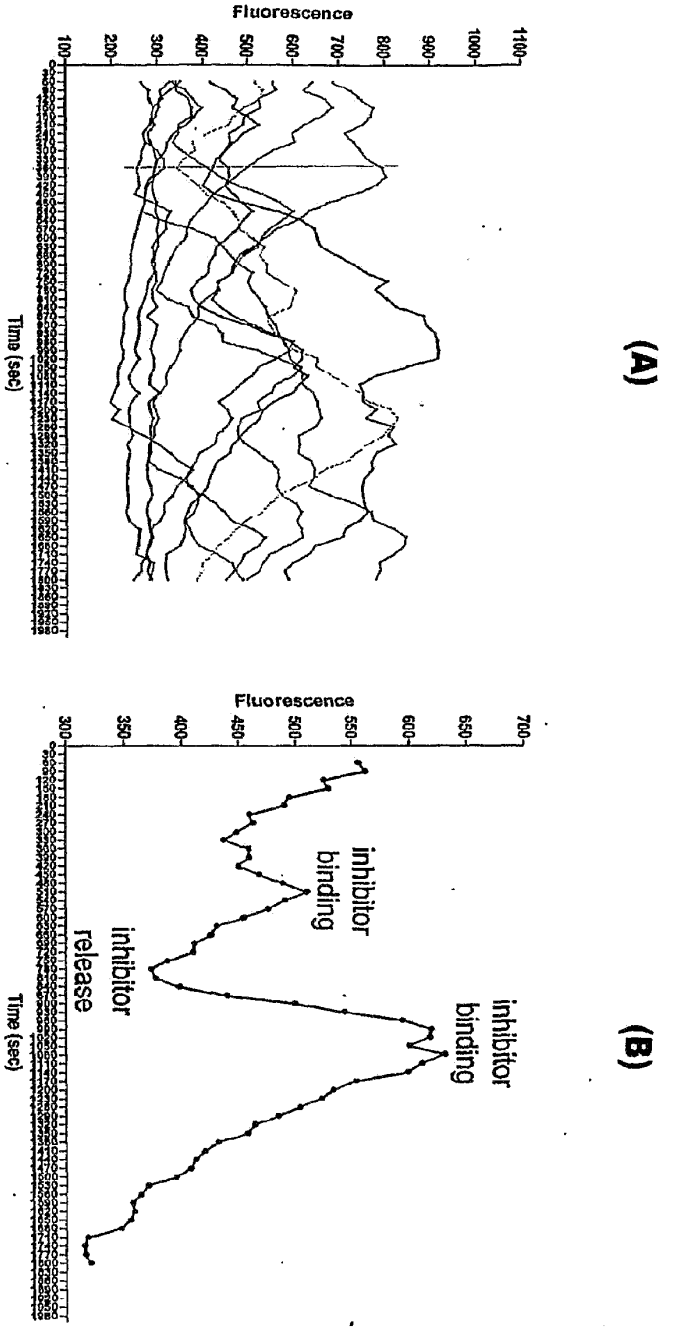

FIG. 13

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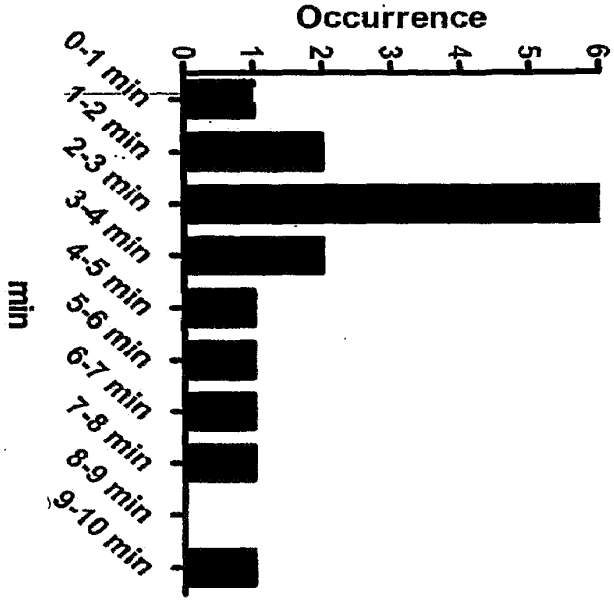

$k^{off} = 4.6 \times 10^{-3}$ sec

=> Half-life: $\ln 2 / k^{off}$

= 151 sec = 2 min, 30 sec

FIG. 14

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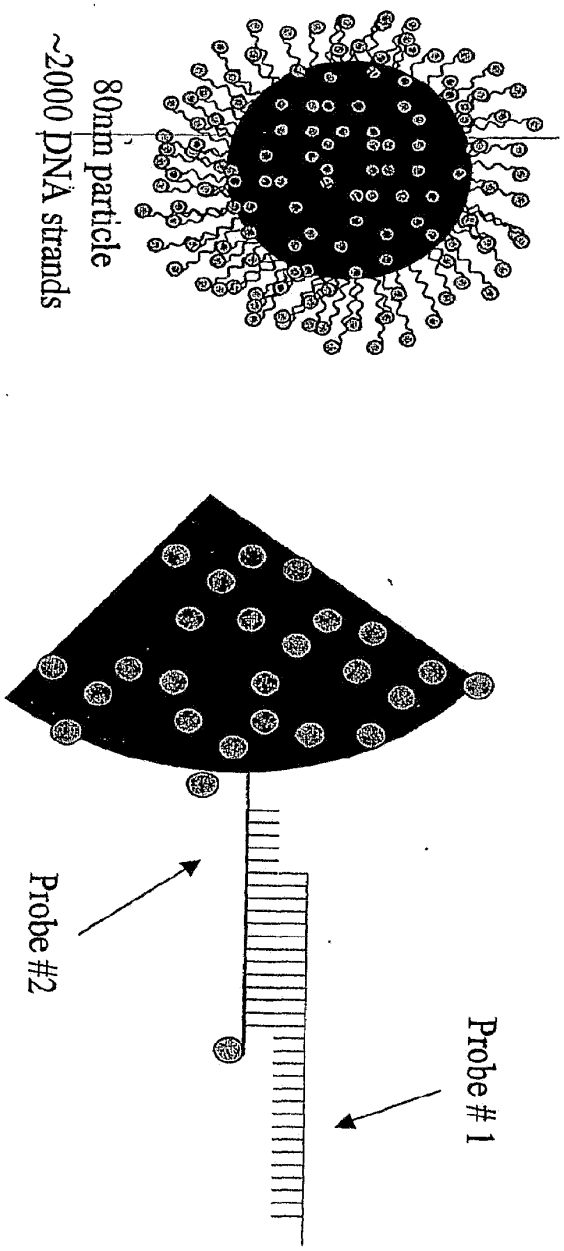

FIG. 15

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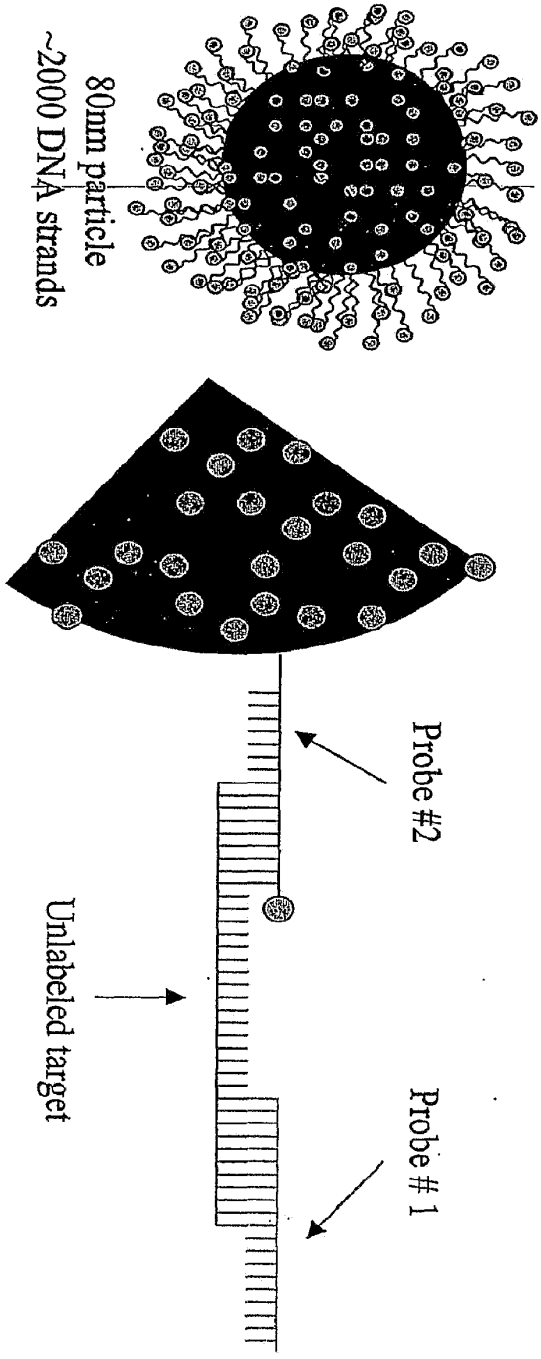

FIG. 16

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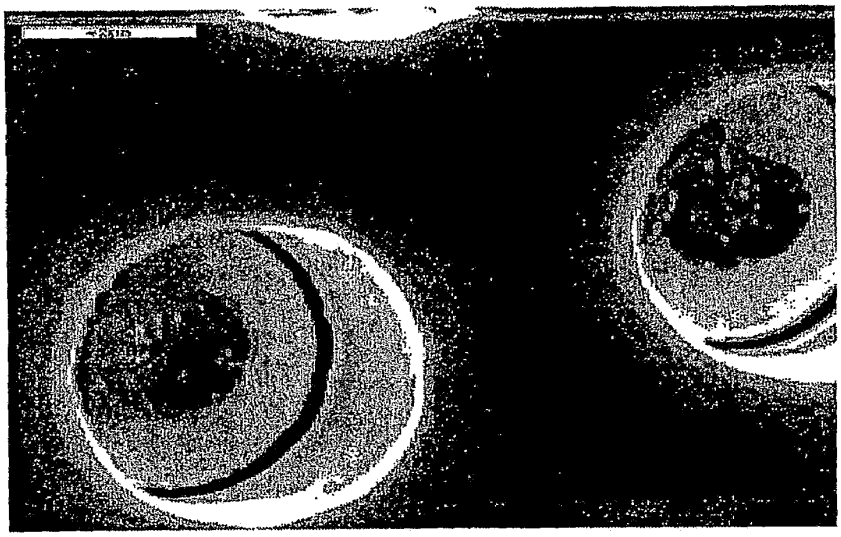

FIG. 17

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60/861,498	28 November 2006 (28.11.2006)	US
60/872,986	4 December 2006 (04.12.2006)	US

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(54) Title: HIGHLY SENSITIVE SYSTEM AND METHODS FOR ANALYSIS OF TROPONIN

(57) Abstract: The invention provides methods, compositions, kits, and systems for the sensitive detection of cardiac troponin, Such methods, compositions, kits, and systems are useful in diagnosis, prognosis, and determination of methods of treatment in conditions that involve release of cardiac troponin.

WO 2007/114947 A2

HIGHLY SENSITIVE SYSTEM AND METHODS FOR ANALYSIS OF TROPONIN

CROSS-REFERENCE

[0001] This application also claims priority under 35 USC § 119 to U.S. Provisional Application No. 60/789,304, filed April 4, 2006, U.S. Provisional Application No. 60/793,664, filed April 19, 2006, U.S. Provisional Application No. 60/808,662, filed May 26, 2006, U.S. Provisional Application No. 60/861,498, filed November 28, 2006, and U.S. Provisional Application No. 60/872,986, filed December 4, 2006, all of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] Each year in the United States, some six million people present to emergency departments with chest pain. Although only 15% to 20% of these patients are ultimately diagnosed with an acute coronary syndrome (ACS), about half are admitted for evaluation. Conversely, 2% of patients with ACS are mistakenly discharged. As patients with ACS have a relatively high risk of major adverse cardiovascular events in the short term, there is a clear need for accurate objective tools by which to identify them

[0003] Currently used markers for cardiac damage suffer disadvantages that limit their clinical usefulness. Cardiac enzyme assays have formed the basis for determining whether or not there is damage to the cardiac muscle. Unfortunately, the standard creatine kinase-MB (CK-MB) assay is not reliable in excluding infarction until 10 to 12 hours after the onset of chest pain. Earlier diagnosis would have very specific advantages with regard to fibrinolytic therapy and triage.

SUMMARY OF THE INVENTION

[0004] In one aspect the invention provides methods.

[0005] In some embodiments, the invention provides a method for determining the presence or absence of a single molecule of troponin or a fragment or complex thereof in a sample, including i) labeling the molecule, fragment, or complex, if present, with a label; and ii) detecting the presence or absence of the label, where the detection of the presence of the label indicates the presence of the single molecule, fragment, or complex of troponin in the sample.In some embodiments of the methods of the invention, the troponin is a cardiac isoform of troponin. In some embodiments of the methods of the invention, the troponin can be cardiac troponin I (cTnI) or cardiac troponin C (cTnC). In some embodiments of the methods of the invention, the troponin is cTnI. In some embodiments of the methods of the invention, a single molecule of troponin can be detected at a limit of detection of less than about 100 pg/ml. In some embodiments of the methods of the invention, a single molecule or troponin can be detected at a level of detection of less than about 20 pg/ml. In some embodiments of the methods of the invention, the label includes a fluorescent moiety. In some embodiments, the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments of the methods of the invention, the fluorescent moiety includes a molecule that contains at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance group. In some embodiments of the methods of the invention, the fluorescent moiety includes a dye. Examples of dyes include, but are not limited to, AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 and AlexaFluor 700. In some embodiments of the methods of the invention, the fluorescent moiety includes

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AlexaFluor 647. In some embodiments, the fluorescent moiety includes a molecule that contains at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance group. In some embodiments of the methods of the invention, the label further includes a binding partner for the troponin molecule, fragment, or complex. In some embodiments of the methods of the invention, the binding partner includes an antibody specific to the troponin molecule, fragment, or complex. In some embodiments of the methods of the invention, the antibody is specific to a specific region of the troponin molecule. In some embodiments of the methods of the invention, the antibody is specific to a region comprising amino acids 27-41 of cardiac troponin I. In some embodiments of the methods of the invention, the antibody can be a polyclonal antibody. In some embodiments of the methods of the invention, the antibody is a monoclonal antibody. In some embodiments of the methods of the invention, the methods further include capturing troponin or troponin complex on a solid support. In some embodiments of the methods of the invention, the solid support can be a microtiter plate or paramagnetic beads. In some embodiments of the methods of the invention, the solid support includes a capture partner specific for the troponin or troponin complex that is attached to the solid support. In some embodiments of the methods of the invention, the attachment of the capture partner to the solid support is noncovalent. In some embodiments of the methods of the invention, the attachment of the capture partner to the solid support is covalent. In some embodiments of the methods of the invention, the covalent attachment of the capture partner is such that the capture partner is attached to the solid support in a specific orientation. In some embodiments of the methods of the invention, the specific orientation serves to maximize specific binding of the troponin or troponin complex to the capture partner. In some embodiments of the methods of the invention, the capture partner comprises an antibody. In some embodiments of the methods of the invention, the antibody is a monoclonal antibody. In some embodiments of the methods of the invention, antibody is specific to amino acids 87-91 of cardiac troponin I. In some embodiments of the methods of the invention, the antibody is specific to amino acids 41-49 of cardiac troponin I. In some embodiments of the methods of the invention, the sample is a blood, serum, or plasma sample. In some embodiments of the methods of the invention, the sample is a serum sample. In some embodiments of the methods of the invention, the label include a fluorescent moiety, and step ii) includes passing the label through a single molecule detector. In some embodiments of the methods of the invention, the single molecule detector include: a) an electromagnetic radiation source for stimulating the fluorescent moiety; b) a capillary flow cell for passing the fluorescent moiety; c) a source of motive force for moving the fluorescent moiety in the capillary flow cell; d) an interrogation space defined within the capillary flow cell for receiving electromagnetic radiation emitted from the electromagnetic source; e) an electromagnetic radiation detector operably connected to the interrogation space for measuring an electromagnetic characteristic of the stimulated fluorescent moiety; and f) a microscope objective lens situated between the interrogation space and the detector, where the lens is a high numerical aperture lens.

[0006] In some embodiments, the invention provides a method for determining a diagnosis, prognosis, or method of treatment in an individual that includes: i) determining a concentration of cardiac troponin in a sample or determining the concentrations of cardiac troponin in a series of samples from the individual, where the concentration is determined by a cardiac troponin assay with a limit of detection for the cardiac troponin in the sample of less than about 50 pg/ml; and ii) determining a diagnosis, prognosis, or method of treatment in the individual, based on the concentration in the sample, or on the concentrations in the series of samples. In some embodiments of the methods of the invention, step ii) includes an analysis such as comparing the concentration or series of concentrations to a normal value for the concentration, comparing the concentration or series of concentrations to a predetermined threshold level, comparing the concentration or series of concentrations to a

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baseline value, and determining a rate of change of concentration for the series of concentrations. In some embodiments of the methods of the invention, step ii) includes comparing the concentration of troponin in the sample with a predetermined threshold concentration, and determining a diagnosis, prognosis, or method of treatment if the sample concentration is greater than the threshold level. In some embodiments of the methods of the invention, the threshold concentration is determined by determining a the 99th percentile concentration of troponin in a group of normal individuals, and setting the threshold concentration at the 99th percentile concentration. In some embodiments of the methods of the invention, at least one sample is taken during or after a cardiac stress test. In some embodiments of the methods of the invention, the cardiac troponin is selected from the group consisting of cardiac troponin I and cardiac troponin T. In some embodiments of the methods of the invention, the cardiac troponin is cardiac troponin I. In some embodiments of the methods of the invention, the concentration of cardiac troponin is a concentration of total cardiac troponin. In some embodiments of the methods of the invention, the concentration of cardiac troponin is a concentration of a cardiac troponin complex, cardiac troponin fragment, phosphorylated cardiac troponin, oxidized cardiac troponin, or a combination thereof. In some embodiments of the methods of the invention, the concentration of cardiac troponin is compared to total cardiac troponin. In some embodiments of the methods of the invention, the diagnosis, prognosis, or method of treatment is a diagnosis, prognosis, or method of treatment of myocardial infarct. In some embodiments of the methods of the invention, the diagnosis, proguosis, or method of treatment comprises risk stratification for level of risk of myocardial infarct. In some embodiments of the methods of the invention, the concentration or series of concentrations is determined at or near the time the individual presents to a health professional with one or more symptoms indicative of myocardial ischemia or infarct or the possibility thereof. In some embodiments, the one or more symptoms can be chest pain, chest pressure, arm pain, abnormal EKG, abnormal enzyme levels, or shortness of breath. In some embodiments, the concentration is determined by a method that includes detecting single molecules of troponin, or complexes or fragments thereof. In some embodiments, the methods of the invention involve labeling troponin or a troponin complex with a label that comprises a fluorescent moiety. In some embodiments of the methods of the invention, the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments of the methods of the invention, the fluorescent moiety includes a molecule that contains at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance group. In some embodiments of the methods of the invention, the fluorescent moiety includes a dye selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments of the methods of the invention, the fluorescent moiety comprises AlexaFluor 647.. In some embodiments of the methods of the invention, the label further comprises a binding partner for the troponin. In some embodiments, the binding partner comprises an antibody specific to the troponin. In some embodiments, the antibody is a polyclonal antibody. In some embodiments of the methods of the invention, the methods further include capturing troponin or troponin complex on a solid support. In some embodiments of the methods of the invention, the solid support can be a microtiter plate or paramagnetic beads. In some embodiments of the methods of the invention, the solid support includes a capture partner specific for the troponin or troponin complex that is attached to the solid support. In some embodiments of the methods of the invention, the attachment of the capture partner to the solid support is noncovalent. In some embodiments of the methods of the invention, the attachment of the capture partner to the solid support is covalent. In some embodiments of the methods of the invention, the covalent attachment of the capture partner is such that the

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capture partner is attached to the solid support in a specific orientation. In some embodiments of the methods of the invention, the specific orientation serves to maximize specific binding of the troponin or troponin complex to the capture partner. In some embodiments of the methods of the invention, step i) further involves assessing another indicator for the individual., and step ii) involves determining a diagnosis, prognosis, or method of treatment in the individual, based on the concentration of troponin and the assessment of the other indicator of the non-troponin marker in the sample, or on the concentrations in the series of samples. In some embodiments, the other indicator is a clinical indicator of myocardial ischemia or infarct. In some embodiments, the other indicator is the concentration of one or more non-troponin markers in the sample or the series of samples. In some embodiments of the methods of the invention, the one or more markers are markers of cardiac ischemia, or markers of inflammation and of plaque instability. In some embodiments, the one or more markers of cardiac ischemia can be creatine kinase (CK) and its myocardial fraction CK myocardial band (MB), aspartate aminotransferase, lactate dehydrogenase (LDH), α-hydroxybutyrate dehaydrogenase, myoglobin, glutamate oxaloacetate transaminase, glycogen phosphorylase BB, unbound free fatty acids, heart fatty acid binding protein (H-FABP), ischemia-modified albumin, myosin light chain 1, or myosin light chain 2. In some embodiments of the methods of the invention, the one or more markers include one or more specific markers of myocardial injury. In some embodiments of the methods of the invention, the diagnosis, prognosis, or method of treatment is a diagnosis, prognosis, or method of treatment of a condition that is not myocardial infarct. In some embodiments, the condition is cardiac toxicity. In some embodiments, the cardiac toxicity is associated with the administration of a drug to the individual. In some embodiments of the methods of the invention, the condition is selected from the group consisting of acute rheumatic fever, amyloidosis, cardiac trauma (including contusion, ablation, pacing, firing, cardioversion, catheterization and cardiac surgery), reperfusion injury, congestive heart failure, end-stage renal failure, glycogen storage disease type II (Pompe's disease), heart transplantation, haemoglobinopathy with transfusion haemosiderosis, hypertension, including gestational hypertension, hypotension, often with arrhythmias, hypothyroidism, myocarditis, pericarditis, post-operative non-cardiac surgery, pulmonary embolism, and sepsis.

[0007] In another aspect the invention includes compositions.

[0008] In some embodiments the invention includes a composition for the detection of a troponin isoform including a binding partner to the troponin isoform attached to a fluorescent moiety, where the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments of the compositions of the invention, the binding partner comprises an antibody to the troponin isoform. In some embodiments, the antibody is a polyclonal antibody. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the troponin isoform is a cardiac isoform. In some embodiments, the cardiac isoform is selected from the group consisting of cTnI and cTnT. In some embodiments, the cardiac isoform is cTnI. In some embodiments, the antibody is specific to a specific region of the troponin molecule. In some embodiments, the antibody is specific to a region comprising amino acids 27-41 of cardiac troponin I. In some embodiments of the compositions of the invention, the fluorescent moiety comprises a molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance group. In some embodiments, the fluorescent moiety includes a dye that can be AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the fluorescent moiety comprises AlexaFluor 647.

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[0009] In some embodiments the invention involves a composition comprising a set of standards for the determination of a concentration of a cardiac troponin, where at least one of the standards is at a concentration of cardiac troponin less than about 10 pg/ml.

[0010] In some embodiments the invention involves a kit containing a composition including an antibody to cardiac troponin attached to a fluorescent dye moiety, where the moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules, where the composition is packaged in suitable packaging. In some embodiments of the kits of the invention, the cardiac troponin is cardiac troponin I or cardiac troponin T. In some embodiments, the cardiac troponin is cardiac troponin I. In some embodiments of the kits of the invention, the kits further include instructions. In some embodiments of the kits of the invention, the kits further include a composition containing a capture antibody for the cardiac troponin I attached to a solid support. In some embodiments, the solid support comprises a microtiter plate or paramagnetic microparticles. In some embodiments of the kits of the invention, the kits further include a component selected from the group consisting of wash buffer, assay buffer, elution buffer, and calibrator diluent. In some embodiments of the kits of the invention, further include a standard for the cardiac troponin.

INCORPORATION BY REFERENCE

[0011] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1A and 1B. Schematic diagram of the arrangement of the components of a single particle analyzer. Figure 3A shows an analyzer that includes one electromagnetic source and one electromagnetic detector; Figure 3B shows an analyzer that includes two electromagnetic sources and one electromagnetic detectors.

[0013] Figures 2A and 2B. Schematic diagrams of a capillary flow cell for a single particle analyzer. Figure 4A shows the flow cell of an analyzer that includes one electromagnetic source; and Figure 4B shows the flow cell of an analyzer that includes two electromagnetic sources.

[0014] Figures 3A and 3B. Schematic diagrams showing the conventional (A) and confocal (B) positioning of laser and detector optics of a single particle analyzer. Figure 3A shows the arrangement for an analyzer that has one electromagnetic source and one electromagnetic detector; Figure 3B shows the arrangement for an analyzer that has two electromagnetic sources and two electromagnetic detectors.

[0015] Figure 4. Linearized standard curve for the range concentrations of cTnI.

[0016] Figure 5. Biological threshold (cutoff concentration) for cTnI is at a cTnI concentration of 7pg/ml, as established at the 99th percentile with a corresponding CV of 10%.

[0017] Figure 6. Correlation of assay results of cTnI determined using the analyzer system of the invention with standard measurements provided by the National Institute of Standards and Technology ($R2 = 0.9999$).

[0018] Figure 7. Detection of cTnI in serial serum samples from patients who presented at the emergency room with chest pain. The measurements made with the analyzer system of the invention were compared to measurements made with a commercially available assay.

[0019] Figure 8. Distribution of normal biological concentrations of cTnI (No Ischemia) and concentrations of cTnI in serum samples from patients presenting with chest pain.

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[0020] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

DETAILED DESCRIPTION OF THE INVENTION

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I. Introduction

[0021] The invention provides compositions and methods for the highly sensitive detection of troponin, e.g., cardiac troponin. The release into the blood of the cardiac isoforms of troponin, which are unique to cardiac muscle (cardiac troponin I and/or T) is indicative of damage to cardiac muscle, and provides the basis for their use as diagnostic or prognostic markers, or to aid in determination of treatment.

[0022] The troponin complex in muscle consists of troponin I, C and T. Troponin C exists as two isoforms, one from cardiac and slow-twitch muscle and one from fast-twitch muscle; because it is found in virtually all striated muscle, its use as a specific marker is limited. In contrast, troponin I and T are expressed as different isoforms in slow-twitch, fast-twitch and cardiac muscle The unique cardiac isoforms of troponin I and T allow them to be distinguished immunologically from the other troponins of skeletal muscle. Therefore, the release into the blood of cardiac troponin I and T is indicative of damage to cardiac muscle, and provides the basis for their use as diagnostic or prognostic markers, or to aid in determination of treatment.

[0023] Currently used markers for cardiac damage suffer disadvantages that limit their clinical usefulness. Cardiac enzyme assays have formed the basis for determining whether or not there is damage to the cardiac muscle. Unfortunately, the standard creatine kinase-MB (CK-MB) assay is not reliable in excluding infarction until 10 to 12 hours after the onset of chest pain. Earlier diagnosis would have very specific advantages with regard to fibrinolytic therapy and triage.

[0024] Because the level of troponin found in the circulation of healthy individuals is very low, and cardiac specific troponins do not arise from extra-cardiac sources, the troponins are very sensitive and specific markers of cardiac injury. In addition to cardiac infarct, a number of other conditions can cause damage to the heart muscle, and early detection of such damage would prove useful to clinicians. However, present methods of detection and quantitation of cardiac troponin do not possess sufficient sensitivity to detect the release of cardiac troponin into the blood until levels have reached abnormally high concentrations, e.g., 0.1 ng/ml or greater.

[0025] The methods and compositions of the invention thus include methods and compositions for the highly sensitive detection and quantitation of cardiac troponin, and compositions and methods for diagnosis, prognosis, and/or determination of treatment based on such highly sensitive detection and quantitation.

II. Cardiac Troponin

[0026] When the two unique forms of cardiac troponin, cardiac troponin I (cTnI) and cardiac troponin (cTnT) are released into the blood from cardiac muscle, several species of each may exist in the blood. These include various complexes of the two forms, with each other and/or with cardiac troponin C (cTnC). In addition, the two forms are subject to virtually immediate proteolytic degradation, resulting in a variety of fragments. Also, various phosphorylated and oxidized forms of the troponins may exist in the blood. See, e.g., U.S. Patent No. 6,991,907, incorporated by reference herein in its entirety. Unless otherwise specified, "cardiac troponin," as used herein, encompasses all forms of cardiac troponin, including

[0027] In some embodiments, the invention provides methods and compositions for the detection and/or determination of concentration of total cardiac troponin, i.e., the sum of all or a substantial portion of the cardiac troponin in a sample, e.g., blood, serum or plasma sample, whether it is free, complexed, a proteotlytic fragment, phosphorylated, oxidized, or otherwise modified. In some embodiments, the cardiac troponin is cTnI, in others, it is cTnT, and in still other embodiments, the cardiac troponin is cTnI and cTnT. It will be appreciated that an absolute total measurement need not be achieved, as long as a consistent proportion of the total is determined, which can be compared to standard values. It will also be appreciated that if a form of troponin is a minor constituent of the total,

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absence or low levels of detection of that form will not appreciably affect measures of total troponin. Thus, as used herein, "total cardiac troponin" refers to a measurement that is intended to measure all or substantially all forms of a particular cardiac troponin, e.g., all cTnI, or all cTnT, in a sample, where the sample-to-sample consistency is such that clinically relevant conclusions may be drawn from comparisons of samples to standards, or comparison of one sample to another.

[0028] In some embodiments, the invention provides methods and compositions for the detection and/or determination of concentration of one or more of the various forms of troponin in the sample as a separate entity, e.g., complexed cTnI, free cTnI, muddied cTnI (e.g., oxidized or phosphorylated), or complexed cTnT, free cTnT, muddied cTnT (e.g., oxidized or phosphorylated), and, typically, can provide a concentration for that form in the sample. In the latter embodiments, ratios or absolute values may be determined for the different entities. Thus, in some embodiments, the invention provides methods of detecting and, typically, determining the concentration of, one or more forms of complexed troponin, or one or more fragments of troponin, or one or more oxidized or phosphorylated forms of troponin. In some embodiments, more than one form is detected, and the concentrations of the various forms may be determined e.g., by performing multiplexed assays on a single sample for the different entities, or by performing separate assays on aliquots from the same or similar samples. Ratios of concentrations of the various forms may be obtained. For example, a ratio of the concentration of a particular form, e.g., a fragment, complex, or modified form, of the cardiac troponin to the concentration of total cardiac troponin, may be determined. These ratios and/or absolute values can provide meaningful clinical information. For example the relative proportion of fragments of cardiac troponin can indicate the length of time since release into the blood and thus, indirectly, length of time since, e.g., myocardial infarct. See, e.g., U.S. Patent No U.S. Patent No. 6,991,907, incorporated by reference herein in its entirety.

III. Labels for Cardiac Troponin

[0029] In some embodiments, the invention provides methods and compositions that include labels for the highly sensitive detection and quantitation of cardiac troponin.

[0030] One skilled in the art will recognize that many strategies can be used for labeling target molecules to enable their detection or discrimination in a mixture of particles. The labels may be attached by any known means, including methods that utilize non-specific or specific interactions of label and target. Labels may provide a detectable signal or affect the mobility of the particle in an electric field. In addition, labeling can be accomplished directly or through binding partners.

[0031] In some embodiments, the label comprises a binding partner to troponin attached to a fluorescent moiety.

A. Binding partners for troponin

[0032] Any suitable binding partner with the requisite specificity for the form of cardiac troponin to be detected may be used. For example, a binding partner specific to all or substantially all forms of cTnI may be used or a binding partner specific to all or substantially all forms of cTnT may be used; typically such binding partners bind to a region of the cardiac troponin that is common to all or most of the different forms likely to be found in a sample. In some embodiments, a binding partner specific to one or more particular forms of cardiac troponin may be used, e.g., a binding partner to complexed cTnI, free cTnI, muddied cTnI (e.g., oxidized or phosphorylated), or complexed cTnT, free cTnT, muddied cTnT (e.g., oxidized or phosphorylated). Binding partners are known in the art and include, e.g., aptamers, lectins, and receptors. A useful and versatile type of binding partner is an antibody.

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1. Antibodies

[0033] In some embodiments, the binding partner is an antibody specific for a cardiac troponin. The term "antibody," as used herein, is a broad term and is used in its ordinary sense, including, without limitation, to refer to naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. In some embodiments, the antibody is specific for cTnI. In some embodiments, the antibody is specific for cTnT. In some embodiments, the label includes antibodies to both cTnI and cTnT. The antibody may be specific to all or substantially all forms the cardiac troponin; e.g., all or substantially all forms of cTnI, or all or substantially all forms of cTnT . In some embodiments, an antibody specific to one or more particular forms of cardiac troponin may be used, e.g., a binding partner to complexed cTnI, free cTnI, muddied cTnI (e.g., oxidized or phosphorylated), or complexed cTnT, free cTnT, muddied cTnT (e.g., oxidized or phosphorylated). Mixtures of antibodies are also encompassed by the invention, e.g., mixtures of antibodies to cTnI and cTnT, or mixtures of antibodies to the various forms of the troponin (free, complexed, etc.), or mixtures of mixtures.

[0034] It will be appreciated that the choice of epitope or region of troponin to which the antibody is raised will determine its specificity, e.g., for total troponin, for certain fragments, for complexed troponin, for modified troponin, and the like. In some embodiments, the antibody is specific to a specific amino acid region of a cardiac troponin. In some embodiments, the antibody is specific to amino acids 27-41 of human cardiac troponin I. Both monoclonal and polyclonal antibodies are useful as binding partners. In some embodiments, the antibody is a polyclonal antibody. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the antibody is a polyclonal antibody specific to amino acids 27-41 of human cardiac troponin I. In some embodiments, this antibody is not affected by heparin, phosphorylation, oxidation and troponin complex formation, and does not cross-react with skeletal muscle troponin I.

[0035] Methods for producing antibodies are well-established. The cardiac specific sequences to troponin I and troponinT are described in FEBS Lett. 270, 57-61 (1990) and Genomics 21, 311-316 (1994). One skilled in the art will recognize that many procedures are available for the production of antibodies, for example, as described in Antibodies, A Laboratory Manual, Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988), Cold Spring Harbor, N.Y. One skilled in the art will also appreciate that binding fragments or Fab fragments which mimic antibodies can also be prepared from genetic information by various procedures (Antibody Engineering: A Practical Approach (Borrebaeck, C., ed.), 1995, Oxford University Press, Oxford; J. Immunol. 149, 3914-3920 (1992)). Methods for raising antibodies to the various complexed, fragment, phosphorylated, and oxidized forms of the troponins are disclosed in US Patent Nos. 5,579,687; 6,991,907; and in US Patent Application No. 20050164317, which are herein incorporated by reference in their entirety. A synthetic peptide comprised of 14 amino acids which mimics a cardiac specific sequence of troponin I and methods used to prepare antibodies to the peptide are described in international Patent Application number PCT/US94/05468. Monoclonal and polyclonal antibodies to free and complexed cardiac troponins are also commercially available (HyTest, HyTest Ltd.,Turku Finland; Abcam Inc., Cambridge, MA, USA, Life Diagnostics, Inc., West Chester, PA, USA; Fitzgerald Industries International, Inc., Concord, MA 01742-3049 USA; BiosPacific, Emeryville, CA).

[0036] In some embodiments, the antibody is a mammalian, e.g., goat polyclonal anti-cTnI antibody. The antibody may be specific to specific regions of the cTnI, e.g., amino acids 27-41 of human cardiac troponin I. Capture binding partners and detection binding partner pairs, e.g., capture and detection antibody pairs, may be used in embodiments of the invention. Thus, in some embodiments, a heterogeneous assay protocol is used in which,

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typically, two binding partners, e.g., two antibodies, are used. One binding partner is a capture partner, usually immobilized on a solid support, and the other binding partner is a detection binding partner, typically with a detectable label attached. In some embodiments, the capture binding partner member of a pair is an antibody that is specific to all or substantially all forms of cardiac troponin. An example is an antibody, e.g., a monoclonal antibody, specific to free cardiac troponin I (cTnI) a.a. 41-49 and cTnI forming complexes with other troponin components. Preferably, this antibody is not affected by heparin, phosphorylation, oxidation and troponin complex formation, and does not cross-react with skeletal muscle troponin I. Thus, it is thought that the antibody binds to total cTnI. Another example is a monoclonal antibody, specific to cardiac troponin I (cTnI) a.a. 87-91 and does not cross-react with skeletal muscle troponin I. Such antibodies are available from BiosPacific, Emeryville, CA. Other antibody pairs are known or can be designed.

[0037] Cross-reacting antibodies In some embodiments it is useful to use an antibody that cross-reacts with a variety of species, either as a capture antibody, a detection antibody, or both. Such embodiments include the measurement of drug toxicity by determining, e.g., release of cardiac troponin into the blood as a marker of cardiac damage. A cross-reacting antibody allows studies of toxicity to be done in one species, e.g. a non-human species, and direct transfer of the results to studies or clinical observations of another species, e.g., humans, using the same antibody or antibody pair in the reagents of the assays, thus decreasing variability between assays. Thus, in some embodiments, one or more of the antibodies for use as a binding partner to the marker, e.g., cardiac troponin, such as cardiac troponin I, may be a cross-reacting antibody. In some embodiments, the antibody cross-reacts with the marker, e.g. cardiac troponin, from at least two species selected from the group consisting of human, monkey, dog, and mouse. In some embodiments the antibody cross-reacts with the marker e.g. cardiac troponin, from all of the group consisting of human, monkey, dog, and mouse.

B. Fluorescent Moieties To Be Used With Binding Partners

[0038] In some embodiments, the binding partner, e.g., antibody, is attached to a fluorescent moiety. The fluorescence of the moiety, will be sufficient to allow detection in a single molecule detector, such as the single molecule detectors described herein. A "fluorescent moiety," as that term is used herein, includes one or more fluorescent entities whose total fluorescence is such that the moiety may be detected in the single molecule detectors described herein. Thus, a fluorescent moiety may comprise a single entity (e.g., a Quantum Dot or fluorescent molecule) or a plurality of entities (e.g., a plurality of fluorescent molecules). It will be appreciated that when "moiety," as that term is used herein, refers to a group of fluorescent entities, e.g., a plurality of fluorescent dye molecules, each individual entity may be attached to the binding partner separately or the entities may be attached together, as long as the entities as a group provide sufficient fluorescence to be detected.

[0039] Typically, the fluorescence of the moiety involves a combination of quantum efficiency and lack of photobleaching sufficient that the moiety is detectable above background levels in a single molecule detector, with the consistency necessary for the desired level of detection, accuracy, and precision of the assay. For example, in some embodiments, the fluorescence of the fluorescent moiety is such that it allows detection and/or quantitation of troponin at a level of detection of less than about 10, 5, 4, 3, 2, or 1 pg/ml and with a coefficient of variation of less than about 20, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1% or less, e.g., about 10% or less, in the instruments described herein. In some embodiments, the fluorescence of the fluorescent moiety is such that it allows detection and/or quantitation of troponin at a limit of detection of less than about 5 pg/ml and with a coefficient of variation of less than about 10%, in the instruments described herein. "Limit of detection," as that term is used herein, includes the lowest concentration at which one can identify a sample as containing a molecule of the substance of interest,

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e.g., the first non-zero value. It can be defined by the variability of zeros and the slope of the standard curve. For example, the limit of detection of an assay may be determined by running a standard curve, determining the standard curve zero value, and adding 2 standard deviations to that value. A concentration of the substance of interest that produces a signal equal to this value is the "lower limit of detection" concentration.

[0040] Furthermore, the moiety has properties that are consistent with its use in the assay of choice. In some embodiments, the assay is an immunoassay, where the fluorescent moiety is attached to an antibody; the moiety must have properties such that it does not aggregate with other antibodies or proteins, or experiences no more aggregation than is consistent with the required accuracy and precision of the assay. In some embodiments, fluorescent moieties that are preferred are fluorescent moieties, e.g., dye molecules that have a combination of 1) high absorption coefficient; 2) high quantum yield; 3) high photostability (low photobleaching); and 4) compatibility with labeling the biomolecule of interest (e.g., protein) so that it may be analyzed using the analyzers and systems of the invention (e.g., does not cause precipitation of the protein of interest, or precipitation of a protein to which the moiety has been attached).

[0041] Fluorescent moieties, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that are useful in some embodiments of the invention may be defined in terms of their photon emission characteristics when stimulated by EM radiation. For example, in some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 350, 400, 500, 600, 700, 800, 900, or 1000, photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. It will be appreciated that the total energy may be achieved by many different combinations of power output of the laser and length of time of exposure of the dye moiety. E.g., a laser of a power output of 1 mW may be used for 3 ms, 3 mW for 1 ms, 6 mW for 0.5 ms, 12 mW for 0.25 ms, and so on.

[0042] In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 50 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 100 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 150 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than

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about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 300 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 500 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

[0043] In some embodiments, the fluorescent moiety comprises an average of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 fluorescent entities, e.g., fluorescent molecules. In some embodiments, the fluorescent moiety comprises an average of no more than about 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 fluorescent entities, e.g., fluorescent molecules. In some embodiments, the fluorescent moiety comprises an average of about 1 to 11, or about 2 to 10, or about 2 to 8, or about 2 to 6, or about 2 to 5, or about 2 to 4, or about 3 to 10, or about 3 to 8, or about 3 to 6, or about 3 to 5, or about 4 to 10, or about 4 to 8, or about 4 to 6, or about 2, 3, 4, 5, 6, or more than about 6 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 2 to 8 fluorescent moieties are attached. In some embodiments, an average of about 2 to 6 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 2 to 4 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 3 to 10 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 3 to 8 fluorescent enties. In some embodiments, the fluorescent moiety comprises an average of about 3 to 6 fluorescent entities. By "average" is meant that, in a given sample that is a representative sample of a group of labels of the invention, where the sample contains a plurality of the binding partner-fluorescent moiety units, the molar ratio of the particular fluorscent entity of which the fluorescent moiety is comprise, to the binding partner, as determined by standard analytical methods, corresponds to the number or range of numbers specified. For example, in embodiments in which the label comprises a binding partner that is an antibody and a fluorescent moiety that comprises a plurality of fluorescent dye molecules of a specifc absorbance, a spectrophometric assay may be used in which a solution of the label is diluted to an appropriate level and the absorbance at 280 nm is taken to determine the molarity of the protein (antibody) and an absorbance at, e.g., 650 nm (for AlexaFluor 647) is taken to determine the molarity of the fluorescent dye molecule. The ratio of the latter molarity to the former represents the average number of fluorescent entities (dye molecules) in the fluorescent moiety attached to each antibody.

1. Dyes

[0044] In some embodiments, the invention utilizes fluorescent moieties that comprise fluorescent dye molecules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of emitting an average of at least about 50 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of emitting an average of at least about 75 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a

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fluorescent dye molecule that is capable of emitting an average of at least about 100 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of emitting an average of at least about 150 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of emitting an average of at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules

A non-inclusive list of useful fluorescent entities for use in the fluorescent moieties of the invention is given in Table 1, below. In some embodiments, the fluorescent entity is selected from the group consisting of Alexa Flour 488, 532, 647, 700, 750, Fluorescein, B-phycoerythrin, allophycocyanin, PBXL-3, and Qdot 605.

TABLE 1

FLUORESCENT ENTITIES

Dye	Ex (nm)	E (M)-1	Em (nm)	Mw
Bimane	380	5,700	458	282.31
Dapoxyl	373	22,000	551	362.83
Dimethylamino coumarin-4-acetic acid	375	22,000	470	344.32
Marina blue	365	19,000	460	367.26
8-Anilino naphthalene-1-sulfonic acid	372		480	
Cascade blue	376	23,000	420	607.42
Alexa Fluor 405	402	35,000	421	1028.26
Cascade blue	400	29,000	420	607.42
Cascade yellow	402	24,000	545	563.54
Pacific blue	410	46,000	455	339.21
PyMPO	415	26,000	570	582.41
Alexa 430	433	15,000	539	701.75
Atto-425	438		486	
NBD	465	22,000	535	391.34
Alexa 488	495	73,000	519	643.41
Fluorescein	494	79,000	518	376.32
Oregon Green 488	496	76,000	524	509.38
Atto 495	495		522	
Cy2	489	150,000	506	713.78
DY-480-XL	500	40,000	630	514.60
DY-485-XL	485	20,000	560	502.59
DY-490-XL	486	27,000	532	536.58
DY-500-XL	505	90,000	555	596.68
DY-520-XL	520	40,000	664	514.60
Alexa Fluor 532	531	81,000	554	723.77
BODIPY 530/550	534	77,000	554	513.31
6-HEX	535	98,000	556	680.07
6-JOE	522	75,000	550	602.34
Rhodamine 6G	525	108,000	555	555.59
Atto-520	520		542	
Cy3B	558	130,000	572	658.00
Alexa Fluor 610	612	138,000	628	
Alexa Fluor 633	632	159,000	647	ca. 1200
Alexa Fluor 647	650	250,000	668	ca. 1250
BODIPY 630/650	625	101,000	640	660.50

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Dye	Ex (nm)	E (M)-1	Em (nm)	Mw
Cy5	649	250,000	670	791.99
Alexa Fluor 660	663	110,000	690	
Alexa Fluor 680	679	184,000	702	
Alexa Fluor 700	702	192,000	723	
Alexa Fluor 750	749	240,000	782	
B-phycoerythrin	546, 565	2,410,000	575	240,000
R-phycoerythrin	480, 546, 565	1,960,000	578	240,000
Allophycocyanin	650	700,000	660	700,000
PBXL-1	545		666	
PBXL-3	614		662	

Atto-tec dyes

Name	Ex (nm)	Em (nm)	QY	□ (ns)
Atto 425	436	486	0.9	3.5
Atto 495	495	522	0.45	2.4
Atto 520	520	542	0.9	3.6
Atto 560	561	585	0.92	3.4
Atto 590	598	634	0.8	3.7
Atto 610	605	630	0.7	3.3
Atto 655	665	690	0.3	1.9
Atto 680	680	702	0.3	1.8

Dyomics Fluors

label	Ex (um)	Molar absorbance* [l·mol-1·cm-1]	Em (nm)	molecular weight# [g·mol-1]
DY-495/5	495	70,000	520	489.47
DY-495/6	495	70,000	520	489.47
DY-495X/5	495	70,000	520	525.95
DY-495X/6	495	70,000	520	525.95
DY-505/5	505	85,000	530	485.49
DY-505/6	505	85,000	530	485.49
DY-505X/5	505	85,000	530	523.97
DY-505X/6	505	85,000	530	523.97
DY-550	553	122,000	578	667.76
DY-555	555	100.000	580	636.18
DY-610	609	81.000	629	667.75
DY-615	621	200.000	641	578.73
DY-630	636	200.000	657	634.84
DY-631	637	185.000	658	736.88
DY-633	637	180.000	657	751.92
DY-635	647	175.000	671	658.86
DY-636	645	190.000	671	760.91
DY-650	653	170.000	674	686.92
DY-651	653	160.000	678	888.96
DYQ-660	660	117,000	-	668.86
DYQ-661	661	116,000	-	770.90
DY-675	674	110.000	699	706.91
DY-676	674	145.000	699	807.95
DY-680	690	125.000	709	634.84
DY-681	691	125.000	708	736.88
DY-700	702	96.000	723	668.86
DY-701	706	115.000	731	770.90

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label	Ex (nm)	Molar absorbance* [l·mol-1·cm-1]	Em (nm)	molecular weight# [g·mol-1]
DY-730	734	185.000	750	660.88
DY-731	736	225.000	759	762.92
DY-750	747	240.000	776	712.96
DY-751	751	220.000	779	814.99
DY-776	771	147.000	801	834.98
DY-780-OH	770	70.000	810	757.34
DY-780-P	770	70.000	810	957.55
DY-781	783	98.000	800	762.92
DY-782	782	102.000	800	660.88
EVOblue-10	651	101.440	664	389.88
EVOblue-30	652	102.000	672	447.51

Quantum Dots :Qdot 525, 565, 585, 605, 655, 705, 800

[0045] Suitable dyes for use in the invention include modified carbocyanine dyes. The modification of carbocyanine dyes includes the modification of an indolium ring of the carbocyanine dye to permit a reactive group or conjugated substance at the number 3 position. The modification of the indolium ring provides dye conjugates that are uniformly and substantially more fluorescent on proteins, nucleic acids and other biopolymers, than conjugates labeled with structurally similar carbocyanine dyes bound through the nitrogen atom at the number one position. In addition to having more intense fluorescence emission than structurally similar dyes at virtually identical wavelengths, and decreased artifacts in their absorption spectra upon conjugation to biopolymers, the modified carbocyanine dyes have greater photostability and higher absorbance (extinction coefficients) at the wavelengths of peak absorbance than the structurally similar dyes. Thus, the modified carbocyanine dyes result in greater sensitivity in assays that use the modified dyes and their conjugates. Preferred modified dyes include compounds that have at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. Other dye compounds include compounds that incorporate an azabenzazolium ring moiety and at least one sulfonate moiety. The modified carbocyanine dyes that can be used to detect individual particles in various embodiments of the invention are described in U.S. Patent 6,977,305, which is herein incorporated by reference in its entirety. Thus, in some embodiments the labels of the invention utilize a fluorescent dye that includes a substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance group.

[0046] In some embodiments, the label comprises a fluorescent moiety that includes one or more Alexa dyes (Molecular Probes, Eugene, OR). The Alexa dyes are disclosed in U.S. Patent 6,977,305; 6,974,874; 6,130,101; and 6,974,305, which are herein incorporated by reference in their entirety. Some embodiments of the invention utilize a dye chosen from the group consisting of AlexaFluor 647, AlexaFluor 488, AlexaFluor 532, AlexaFluor 555, AlexaFluor 610, AlexaFluor 680, AlexaFluor 700, and AlexaFluor 750. Some embodiments of the invention utilize a dye chosen from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 700 and AlexaFluor 750. Some embodiments of the invention utilize the AlexaFluor 647 molecule, which has an absorption maximum between about 650 and 660 nm and an emission maximum between about 660 and 670 nm. The AlexaFluor 647 dye is used alone or in combination with other AlexaFluor dyes.

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[0047] In addition, currently available organic fluors can be improved by rendering them less hydrophobic by adding hydrophilic groups such as polyethylene. Alternatively, currently sulfonated organic fluors such as the AlexaFluor 647 dye can be rendered less acidic by making them zwitterionic. Particles such as antibodies that are labeled with the modified fluors are less likely to bind non-specifically to surfaces and proteins in immunoassays, and thus enable assays that have greater sensitivity and lower backgrounds. Methods for modifying and improving the properties of fluorescent dyes for the purpose of increasing the sensitivity of a system that detects single particles are known in the art. Preferably, the modification improves the Stokes shift while maintaining a high quantum yield.

2 Quantum dots

[0048] In some embodiments, the fluorescent label moiety that is used to detect a molecule in a sample using the analyzer systems of the invention is a quantum dot. Quantum dots (QDs), also known as semiconductor nanocrystals or artificial atoms, are semiconductor crystals that contain anywhere between 100 to 1,000 electrons and range from 2-10 nm.. Some QDs can be between 10-20 nm in diameter. QDs have high quantum yields, which makes them particularly useful for optical applications. QDs are fluorophores that fluoresce by forming excitons, which can be thought of the excited state of traditional fluorophores, but have much longer lifetimes of up to 200 nanoseconds. This property provides QDs with low photobleaching. The energy level of QDs can be controlled by changing the size and shape of the QD, and the depth of the QDs' potential. One of the optical features of small excitonic QDs is coloration, which is determined by the size of the dot. The larger the dot, the redder, or more towards the red end of the spectrum the fluorescence. The smaller the dot, the bluer or more towards the blue end it is. The bandgap energy that determines the energy and hence the color of the fluoresced light is inversely proportional to the square of the size of the QD. Larger QDs have more energy levels which are more closely spaced, thus allowing the QD to absorb photons containing less energy, i.e. those closer to the red end of the spectrum. Because the emission frequency of a dots dependent on the bandgap, it is therefore possible to control the output wavelength of a dot with extreme precision. In some embodiments the protein that is detected with the single particle analyzer system is labeled with a QD. In some embodiments, the single particle analyzer is used to detect a protein labeled with one QD and using a filter to allow for the detection of different proteins at different wavelengths.

QDs have broad excitation and narrow emission properties which when used with color filtering require only a single electromagnetic source for multiplex analysis of multiple targets in a single sample to resolve individual signals. Thus, in some embodiments, the analyzer system comprises one continuous wave laser and particles that are each labeled with one QD. Colloidally prepared QDs are free floating and can be attached to a variety of molecules via metal coordinating functional groups. These groups include but are not limited to thiol, amine, nitrile, phosphine, phosphine oxide, phosphonic acid, carboxylic acids or other ligands. By bonding appropriate molecules to the surface, the quantum dots can be dispersed or dissolved in nearly any solvent or incorporated into a variety of inorganic and organic films. Quantum dots (QDs) can be coupled to streptavidin directly through a maleimide ester coupling reaction or to antibodies through a meleimide-thiol coupling reaction. This yields a material with a biomolecule covalently attached on the surface, which produces conjugates with high specific activity. In some embodiments, the protein that is detected with the single particle analyzer is labeled with one quantum dot. In some embodiments the quantum dot is between 10 and 20 nm in diameter. In other embodiments, the quantum dot is between 2 and 10 nm in diameter. Useful Quantum Dots include QD 605, QD 610, QD 655, and QD 705. A particularly preferred Quantum Dot is QD 605.

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C. Binding Partner-Fluorescent Moiety Compositions (Labels)

[0049] The labels of the invention generally contain a binding partner, e.g., antibody, bound to a fluorescent moiety to provide the requisite fluorescence for detection and quantitation in the instruments described herein. Any suitable combination of binding partner and fluorescent moiety for detection in the single molecule detectors described herein may be used as a label in the invention. In some embodiments, the invention provides a label for a cardiac troponin molecule, or fragment, complex, phosphorylated, or oxidized form thereof, where the label includes an antibody to a cardiac troponin and a fluorescent moiety. The antibody may be any antibody as described above, e.g., an antibody to cTnT or cTnI. In some embodiments, the antibody is an antibody to cTnI. In some embodiments, the antibody is specific to a specific region of the cardiac troponin, e.g., specific to amino acids 27-41 of human cTnI. In some embodiments, the invention provides compositions comprising a fluorescent moiety attached to an anti-cTnI antibody , e.g., a polyclonal antibody such as a goat polyclonal antibody from those designated G129C available from BiosPacific, Emeryville. A fluorescent moiety may be attached such that the label is capable of emitting an average of at least about 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 350, 400, 500, 600, 700, 800, 900, or 1000, photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the label, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the fluorescent moiety may be a fluorescent moiety that is capable of emitting an average of at least about 50, 100, 150, or 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. The fluorescent moiety may be a fluorescent moiety that includes one or more dye molecules with a structure that includes a substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance group.. The label composition may include a fluorescent moiety that includes one or more dye molecules selected from the group consisting of AlexaFluor 488, 532, 647, 700, or 750. The label composition may include a fluorescent moiety that includes one or more dye molecules selected from the group consisting of AlexaFluor 488, 532, 700, or 750. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 488. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 555. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 610. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 647. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 680. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 700. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 750.

[0050] In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an AlexFluor molecule, e.g. an AlexaFluor molecule selected from the described groups, such as an AlexaFluor 647 molecule attached to a to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of 1 to 11, or about 2 to 10, or about 2 to 8, or about 2 to 6, or about 2 to 5, or about 2 to 4, or about 3 to 10, or about 3 to 8, or about 3 to 6, or about 3 to 5, or about 4 to 10, or about 4 to 8, or about 4 to 6, or about 2, 3, 4, 5, 6, or more than about 6 AlexaFluor 647 molecules molecule attached an antibody,

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e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of 1 to 11, or about 2 to 10, or about 2 to 8, or about 2 to 6, or about 2 to 5, or about 2 to 4, or about 3 to 10, or about 3 to 8, or about 3 to 6, or about 3 to 5, or about 4 to 10, or about 4 to 8, or about 4 to 6, or about 2, 3, 4, 5, 6, or more than about 6 AlexaFluor 647 molecules molecule attached to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of about 2 to 10 AlexaFluor 647 molecules molecule attached to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of about 2 to 8 AlexaFluor 647 molecules molecule attached to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of about 2 to 6 AlexaFluor 647 molecules molecule attached to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of about 2 to 4 AlexaFluor 647 molecules molecule attached to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of about 3 to 8 AlexaFluor 647 molecules molecule attached to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of about 3 to 6 AlexaFluor 647 molecules molecule attached to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI. In some embodiments the invention provides a composition for the detection of cardiac troponin I that includes an average of about 4 to 8 AlexaFluor 647 molecules molecule attached to an antibody, e.g., a goat polyclonal anti-cTnI antibody, specific for amino acids 27-41 of human cTnI.

[0051] Attachment of the fluorescent moiety, or fluorescent entities that make up the fluorescent moiety, to the binding partner, e.g., antibody, may be by any suitable means; such methods are well-known in the art and exemplary methods are given in the Examples. In some embodiments, after attachment of the fluorescent moiety to the binding partner to form a label for use in the methods of the invention, and prior to the use of the label for labeling the protein of interest, it is useful to perform a filtration step. E.g., an antibody-dye label may be filtered prior to use, e.g., through a 0.2 micron filter, or any suitable filter for removing aggregates. Other reagents for use in the assays of the invention may also be filtered, e.g., e.g., through a 0.2 micron filter, or any suitable filter. Without being bound by theory, it is thought that such filtration removes a portion of the aggregates of the, e.g., antibody-dye labels. As such aggregates will bind as a unit to the protein of interest, but upon release in elution buffer are likely to disaggregate, false positives may result; i.e., several labels will be detected from an aggregate that has bound to only a single protein molecule of interest. Regardless of theory, filtration has been found to reduce false positives in the subsequent assay and to improve accuracy and precision.

IV. Highly Sensitive Analysis of Cardiac Troponin

[0052] In one aspect, the invention provides a method for determining the presence or absence of a single molecule of cardiac troponin or a fragment or complex thereof in a sample, by i) labeling the molecule, fragment, or complex, if present, with a label; and ii) detecting the presence or absence of the label, where the detection of the presence of the label indicates the presence of the single molecule, fragment, or complex of cardiac troponin in the sample. As used herein, "molecule of cardiac troponin" includes a molecule that contains substantially the entire

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naturally-occurring amino acid sequence of the particular type of cardiac troponin, including post-translationally modified forms, e.g., phosphorylated forms, as well as oxidized or otherwise chemically altered forms. As used herein, a "fragment" of a molecule includes a molecule of cardiac troponin that contains less than the entire naturally-occurring amino acid sequence, including modifications as for the entire molecule. As used herein, a "complex" of a molecule of cardiac troponin includes a molecule of cardiac troponin or a fragment that is associated with one or more other molecules or substances, e.g., that is associated with one or more other molecules of cardiac troponin. In some embodiments, the method is capable of detecting the troponin at a limit of detection of less than about 100, 80, 60, 50, 40, 30, 20, 15, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2. or 0.1 pg/ml. In some embodiments, the method is capable of detecting the troponin at a limit of detection of less than about 100 pg/ml. In some embodiments, the method is capable of detecting the troponin at a limit of detection of less than about 50 pg/ml. In some embodiments, the method is capable of detecting the troponin at a limit of detection of less than about 20 pg/ml. In some embodiments, the method is capable of detecting the troponin at a limit of detection of less than about 10 pg/ml. In some embodiments, the method is capable of detecting the troponin at a limit of detection of less than about 5 pg/ml. In some embodiments, the method is capable of detecting the troponin at a limit of detection of less than about 3 pg/ml. In some embodiments, the method is capable of detecting the troponin at a limit of detection of less than about 1 pg/ml. Detection limits may be determined by use of the appropriate National Institute of Standards and Technology reference standard material, e.g., standard cTnI.

[0053] The methods also provide methods of determining a concentration of cardiac troponin in a sample by detecting single molecules of troponin in the sample. The "detecting" of a single molecule of troponin includes detecting the molecule directly or indirectly. In the case of indirect detection, labels that corresponds to single molecules of cardiac troponin, e.g., a labels that have been attached to the single molecules of cardiac troponin, may be detected.

[0054] Types of cardiac troponin for detection are as described herein, e.g., cTnT, cTnI, total cardiac troponin (e.g., total cTnI or total cTnT) or free, complexed, or fragments of cardiac troponin. In some embodiments, total cardiac troponin is detected and/or quantitated. In some embodiments, total cTnT is detected. In some embodiments, total cTnI is detected and/or quantitated.

A. Sample

[0055] The sample may be any suitable sample. Typically, the sample is a biological sample, e.g., a biological fluid. Such fluids include, without limitation, exhaled breath condensate (EBC), bronchoalveolar lavage fluid (BAL), blood, serum, plasma, urine, cerebrospinal fluid, pleural fluid, synovial fluid, peritoneal fluid, amniotic fluid, gastric fluid, lymph fluid, interstitial fluid, tissue homogenate, cell extracts, saliva, sputum, stool, physiological secretions, tears, mucus, sweat, milk, semen, seminal fluid, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and abscesses, and extracts of tissues including biopsies of normal, malignant, and suspect tissues or any other constituents of the body which may contain the target particle of interest. Other similar specimens such as cell or tissue culture or culture broth are also of interest.

[0056] In some embodiments, the sample is a blood sample. In some embodiments the sample is a plasma sample. In some embodiments the sample is a serum sample.

B. Sample preparation

[0057] In general, any method of sample preparation may be used that produces a label corresponding to a molecule of cardiac troponin that is wished to be measured, where the label is detectable in the instruments

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described herein. As is known in the art, sample preparation in which a label is added to one or more particles may be performed in a homogeneous or heterogeneous format. In some embodiments, the sample preparation is formed in a homogenous format. In analyzer system employing a homogenous format, unbound label is not removed from the sample. See, e.g., U.S. Patent Application No. 11/048,660, incorporated by reference herein in its entirety. In some embodiments, the particle or particles of interest are labeled by addition of labeled antibody or antibodies that bind to the particle or particles of interest.

[0058] In some embodiments, a heterogeneous assay format is used, where, typically, a step is employed for removing unbound label. Such assay formats are well-known in the art. One particularly useful assay format is a sandwich assay, e.g., a sandwich immunoassay. In this format, the molecule of interest, e.g., marker of a biological state, is captured, e.g., on a solid support, using a capture binding partner. Unwanted molecules and other substances may then optionally be washed away, followed by binding of a label comprsing a detection binding partner and a detectable label, e.g., fluorescent moiety. Further washes remove unbound label, then the detectable label is released, usually though not necessarily still attached to the detection binding partner. In alternative embodiments, sample and label are added to the capture binding partner without a wash in between, e.g., at the same time. Other variations will be apparent to one of skill in the art.

[0059] In some embodiments, the method for detecting troponin particles uses a sandwich assay with antibodies, e.g., monoclonal antibodies as capture binding partners. The method comprises binding troponin molecules in a sample to a capture antibody that is immobilized on a binding surface, and binding the detection antibody to the troponin molecule to form a "sandwich" complex. The detection antibody comprises a detectable fluorescent label, as described herein, which is detected, e.g., using the single molecule analyzers of the invention. Both the capture and detection antibodies specifically bind troponin. Many example of sandwich immunoassays are known, and some are described in U.S. Pat. No. 4,168,146 to Grubb et al. and U.S. Pat. No. 4,366,241 to Tom et al., both of which are incorporated herein by reference. Further examples specific to cardiac troponin are described in the Examples.

[0060] The capture binding partner may be attached to a solid support, e.g., a microtiter plate or paramagnetic beads. In some embodiments, the invention provides a binding partner for a cardiac troponin attached to a paramagnetic bead. Any suitable binding partner that is specific for the type of cardiac troponin that it is wished to capture may be used. The binding partner may be an antibody, e.g., a monoclonal antibody. The antibody may be specific for free cardiac troponin (cTnI or cTnT) or for complexed cardiac troponin, modified cardiac troponin, or fragments of cardiac troponin, as described herein, or specific to all or substantially all forms of cardiac troponin, e.g., cTnI or cTnT, likely to be found in the sample of interest. Production and sources of antibodies to cardiac troponin are described elsewhere herein. Preferred antibodies for measuring total troponin are those that not substantially affected by heparin, phosphorylation, oxidation and troponin complex formation, and that do not cross-react with skeletal muscle troponin, e.g., troponin I. In some embodiments, the antibody is specific for a specific region of a cardiac troponin. In some embodiments, the region includes amino acids 41-49 of human cardiac troponin I. In some embodiments, the region includes amino acids 87-91 of human cardiac troponin I. Such antibodies are well-known in the art and are available from, e.g. BiosPacific, Emeryville, CA. An example of a capture antibody useful in embodiments of the invention is an antibody, e.g., a monoclonal antibody, that reacts with free cardiac troponin I (cTnI) a.a. 41-49 and cTnI forming complexes with other troponin components. Preferably, this antibody is not affected by heparin, phosphorylation, oxidation and troponin complex formation, and does not cross-react with skeletal muscle troponin I. An exemplary antibody of this type is Monoclonal Antibody Clone Number A34650228P, available from BiosPacific, Emeryville, CA. Another example of a capture antibody useful

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