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(54) Title: DROPLET ACTUATORS AND TECHNIQUES FOR DROPLET-BASED ASSAYS

(57) Abstract: A method of conducting an assay, the method comprising incubating a droplet in oil, the droplet comprising an umbelliferone substrate, a sample potentially comprising an enzyme which cleaves the umbelliferone substrate, and zwitterionic surfactant and detecting a signal emitted from the droplet.

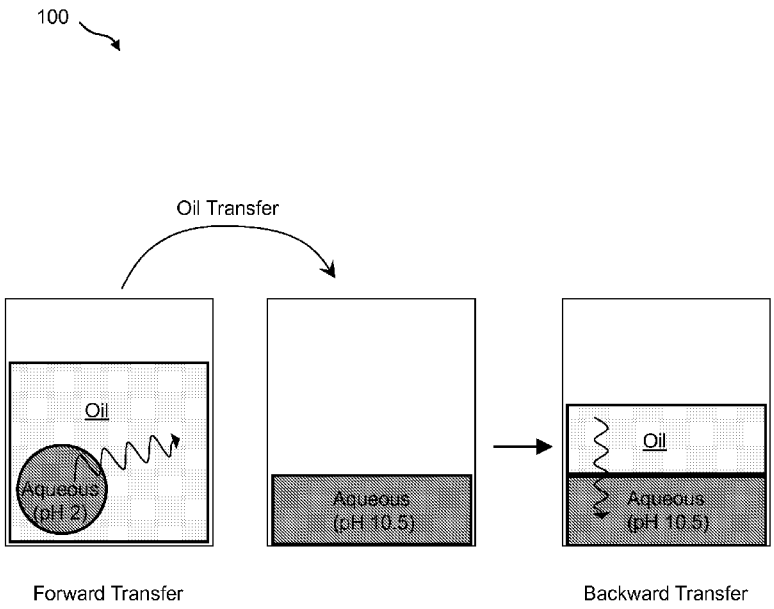

Figure 1

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Droplet Actuators and Techniques for Droplet-Based Assays

1 Related Applications

This application claims the benefit of provisional U.S. Patent Application Nos. 61/506,369 and 61/506,359, both filed on July 11, 2011.

2 Grant Information

This invention was made with government support under HG004354 awarded by the National Institutes of Health of the United States.

3 Field of the Invention

The invention relates to a droplet actuator with a modified droplet operations surface.

4 Background

A droplet actuator typically includes one or more substrates configured to form a surface or gap for conducting droplet operations. The one or more substrates establish a droplet operations surface or gap for conducting droplet operations and may also include electrodes arrange to conduct the droplet operations. The droplet operations substrate or the gap between the substrates may be coated or filled with a filler fluid that is immiscible with the liquid that forms the droplets.

Droplet actuators are used in a variety of applications, including molecular diagnostic assays, such as enzymatic assays. In one example, lysosomal enzyme tests used in newborn testing assays (NBS) may be performed on a droplet actuator. The NBS assays are fluorescent based tests which measure the release of 4-methylumbelliferone (4-MU) or other umbelliferyl derivatives (e.g., 6-hexadecanoylamido-4-methylumbelliferone; HMU) after enzymatic hydrolysis of the substrates. In the droplet operations environment of a droplet actuator, partitioning of 4-MU (or derivatives) between the aqueous phase (i.e., droplet) and the organic phase (filler fluid) may result in a reduction in the assay signal and potential contamination of neighboring samples. Therefore, there is a need for improved methods for reducing partitioning of 4-MU (or derivatives) in droplet-based bioassays on a droplet actuator.

Summary of the Invention

The invention provides a method of conducting an assay. The method may, for example, include incubating a droplet in oil, the droplet including an umbelliferone substrate, a sample potentially including an enzyme which cleaves the umbelliferone substrate, and a zwitterionic surfactant, and detecting a signal emitted from the droplet.

In certain embodiments, the droplet further includes a cyclodextrin compound. In certain embodiments, the cyclodextrin compound is selected from the group consisting of α-cyclodextrins, β-cyclodextrins, and γ-cyclodextrins, and analogs and derivatives of the foregoing. In some cases, the umbilliferone substrate is selected from the group consisting of alkylumbelliferyl-α-L-iduronides, 4-methylumbelliferyl-α-L-iduronide, 4-methylumbelliferyl-α-L-iduronide-2-sulfate, 4-methylumbelliferyl-α-L-idopyranosiduronic acid, 4-methylumbelliferyl-α-L-fucoside, 4-methylumbelliferyl-α-L-mannoside, 4-methylumbelliferyl-β-D-mannoside, 4-methylumbelliferyl β-D-N-acetylglucosaminide, 4-methylumbelliferyl β-D-N-acetylglucosaminide sulfate, alkylumbelliferyl-β-D-glycosides, methylumbelliferyl-β-D-glycosides, 4-methylumbelliferyl-α-D-galactoside, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-β-D-glucouronic acid, phenolphthalein-β-D-glucuronic acid, ethylumbelliferyl-β-D-glycosides, multifluoroethylumbelliferyl-β-D-glycosides, pentafluoroethylumbelliferyl-β-D-glycosides, pentafluoroethylumbelliferyl-β-D-glucoside, umbelliferylchiotriosides, 4-alkyumbelliferylchiotrioside, 4-methylumbelliferylchiotrioside, 4-methylumbelliferyl-β-galactose, 4-alkyumbeliferrone phosphates, 4-methylumbeliferrone phosphate, 6-alkanoylamido-4-methylumbelliferones, substrates including a 4-methyllumbelliferyl group, 6-hexadecanoylamido-4-methylumbelliferone, 4-methyllumbelliferyl-β-D-glucosaminide, 4-methylumbelliferyl-α-neuraminic acid, 4-methylumbelliferyl-α-D-N-acetylgalactosaminide, and their functional analogs and derivatives. In certain embodiments, the steps are performed within droplets controlled by a droplet actuator. In certain embodiments, the droplet actuator controls the steps using electrode mediated droplet operations. In certain embodiments, the droplet actuator controls the steps using electrowetting mediated droplet operations. In certain embodiments, the droplet actuator controls the steps using dielectrophoresis mediated droplet operations.

The invention provides a method of conducting a droplet-based enzyme assay. For example, the method may include providing an immiscible fluid including a sample droplet including an enzyme of interest, and one or more reagent droplets including a substrate which is potentially

modified in the presence of the enzyme yielding one or more signal-producing products, a zwitterionic surfactant, and optionally, other reagents sufficient to produce the activity of the target enzyme under ordinary conditions. The method may include combining the sample droplet and the one or more reagent droplets in the immiscible fluid to yield a reaction droplet effecting an enzyme reaction in the immiscible fluid, and measuring any signal produced by the one or more signal producing products. In certain embodiments, the zwitterionic surfactant is selected form the group consisting of n-Octylphosphocholine, n-Nonylphosphocholine, n-decylphosphocholine, n-dodecylphosphocholine, 3-cyclohexyl-1-propylphosphocholine, decylphospho-N-methylethanolamine, n-decyl-N,N-dimethylglycine, n-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, n-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, n-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, n-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, dimethylbenzylammonium propane sulfonate, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate, and 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate.

The invention provides a method of providing diagnostic information, the method including conducting an assay according to a method of the invention, wherein the sample droplet includes a clinical sample from a subject, the sample including the enzyme of interest, and providing to the subject diagnostic information based on the activity of the enzyme of interest from the human clinical sample. In certain embodiments, the diagnostic information includes information diagnostically relevant to a glycogen storage disease. In certain embodiments, the assay is conducted and the diagnostic information is provided at a point of sample collection. In certain embodiments, the point of sample collection is in the presence of the subject. In certain embodiments, the clinical sample includes a sample substance selected from the group consisting of: blood, plasma, serum, tears, saliva, and urine. In certain embodiments, the clinical sample includes a dried blood sample. In certain embodiments, the clinical sample includes a fresh blood sample. In certain embodiments, the fresh blood sample is collected from the subject and immediately loaded onto a droplet actuator for conducting the assay. In certain embodiments, time from collection of the blood sample to providing diagnostic information is less than about 12 hours. In certain embodiments, time from collection of the blood sample to providing diagnostic information is less than about 6 hours. In certain embodiments, the clinical sample includes a human clinical sample. In certain embodiments, the clinical sample includes a non-human animal clinical sample. In certain embodiments, the substrate includes a glycoside substrate. In certain embodiments, the substrate releases a fluorophore upon contact with the enzyme of

interest. In certain embodiments, two or more assays are conducted simultaneously using different fluorophores for each enzyme tested. In certain embodiments, the fluorophore includes 4-methylumbelliferyl. In certain embodiments, the substrate includes a glycoside substrate which releases a fluorophore upon contact with the enzyme of interest. In certain embodiments, the substrate includes a glycoside substrate including glucose, galactose, fucose, mannose, sialic acid, hexose, hexosamine and/or N-acetylated hexosamine. In certain embodiments, the substrate includes a 4-methylumbelliferyl glycoside. In certain embodiments, the method further includes reducing or eliminating reaction contaminants associated with the substrate prior to yielding the assay droplet. In certain embodiments, the reducing or eliminating reaction contaminants includes photobleaching the substrate prior to yielding the assay droplet. In certain embodiments, the photobleaching is effected prior to providing the droplet including the substrate on the droplet actuator. In certain embodiments, the photobleaching is effected after to providing the droplet including the substrate on the droplet actuator. In certain embodiments, the substrate includes a 4-methylumbelliferyl glycoside substrate. In certain embodiments, the method includes photobleaching the substrate prior to yielding the assay droplet. In certain embodiments, the immiscible liquid includes a filler fluid. In certain embodiments, the immiscible liquid includes a silicone oil. In certain embodiments, the filler fluid includes a surfactant. In certain embodiments, the surfactant includes nonionic low hydrophile-lipophile balanced (HLB) surfactant. In certain embodiments, the HLB is less than about 10. In certain embodiments, the HLB is less than about 5. In certain embodiments, the surfactant is selected from the group consisting of Triton X-15, Span 85, Span 65, Span 83, Span 80, Span 60, and fluorinated surfactants. In certain embodiments, the sample droplet includes a reconstituted blood sample, the blood sample is reconstituted using a single universal reconstitution solution, the blood sample is divided to yield two or more reaction droplets, and two or more of the reaction droplets are each combined with one or more sets of one or more reagent droplets, each such set including reagents selected for establishing reaction conditions for a different enzyme assay. In certain embodiments, the universal reconstitution solution includes a saline solution. In certain embodiments, the universal reconstitution solution includes water. In certain embodiments, the enzyme assay is selected to provide diagnostic information about an enzyme deficiency. In certain embodiments, the enzyme deficiency is selected from lysosomal storage diseases. In certain embodiments, the enzyme deficiency is selected from the group consisting of Pompe, Niemann-Pick, Fabry, Krabbe, and Gaucher. In certain embodiments, the method also includes providing therapeutic treatment to a subject based on the diagnostic information. In certain embodiments, the sample droplet including an enzyme of interest includes cultured cells and/or

supernatant from a cell culture. In certain embodiments, the substrate is selected from the group consisting of 4-methylumbelliferyl-α-L-iduronide, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-β-D-glucuronic acid, 4-methylumbelliferyl-α-L-fucoside, 4-methylumbelliferyl-α-mannoside, 4-methylumbelliferyl-β-D-mannoside, 4-nitrocathecol sulfate, 4-methylumbelliferyl-β-D-N-acetylglucosaminide, 4-methylumbelliferyl-β-D-N-acetylglucosaminide sulfate, 4-methylumbelliferyl-β-D-glucosaminide, 4-methylumbelliferyl-α-D-galactoside, 4-methylumbelliferyl-α-D-neuraminic acid, 4-methylumbelliferyl-α-D-N-acetylgalactosaminide, phenolphthalein β-D-glucuronic acid, and mixtures and derivatives thereof. In certain embodiments, the substrate includes a fluorophoric moiety. In certain embodiments, the fluorophoric moiety includes 4-methyllumbelliferyl. In certain embodiments, the substrate includes a chromophoric moiety. In certain embodiments, the chromophoric moiety includes 4-nitrocathecol or phenolphthalein. In certain embodiments, the substrate includes a radioactive moiety. In certain embodiments, the radioactive moiety includes 14C sphingomyeline or 3H galactosylceramide. In certain embodiments, incubating the reaction droplet for a period of less than about 12 hours.

The invention provides a method of conducting an assay in a droplet in oil, the droplet including a lipophilic moiety, the method including including in the droplet a nonionic surfactant selected from the group consisting of n-hexyl-ß-D-glucopyranoside, 2-cyclohexyl-1-ethyl-ß-D-maltoside, 3-cyclohexyl-1-propyl-ß-D-maltoside, octanoyl-N-methylglucamide, nonanoyl-N-methylglucamide, octanoyl-N-hydroxyethylglucamide, nonanoyl-N-hydroxyethylglucamide, n-hexyl-ß-D-glucopyranoside, and α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxy-poly(oxy-1,2-ethanediyl). In certain embodiments, the lipophilic moiety includes an umbilliferone substrate. In certain embodiments, umbilliferone substrate is selected from the group consisting of alkylumbelliferyl-α-L-iduronides, 4-methylumbelliferyl-α-L-iduronide, 4-methylumbelliferyl-α-L-iduronide-2-sulfate, 4-methylumbelliferyl-α-L-idopyranosiduronic acid, 4-methylumbelliferyl-α-L-fucoside, 4-methylumbelliferyl-α-L-mannoside, 4-methylumbelliferyl-β-D-mannoside, 4-methylumbelliferyl β-D-N-acetylglucosaminide, 4-methylumbelliferyl β-D-N-acetylglucosaminide sulfate, alkylumbelliferyl-β-D-glycosides, methylumbelliferyl-β-D-glycosides, 4-methylumbelliferyl-α-D-galactoside, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-β-D-glucouronic acid, phenolphthalein-β-D-glucuronic acid, ethylumbelliferyl-β-D-glycosides, multifluoroethylumbelliferyl-β-D-glycosides, pentafluoroethylumbelliferyl-β-D-glycosides, pentafluoroethylumbelliferyl-β-D-glucoside, umbelliferylchiotriosides, 4-alkyumbelliferylchiotrioside, 4-methylumbelliferylchiotrioside, 4-

methylumbelliferyl-β-galactose, 4-alkyumbeliferrone phosphates, 4-methylumbeliferrone phosphate, 6-alkanoylamido-4-methylumbelliferones, substrates including a 4-methyllumbelliferyl group, 6-hexadecanoylamido-4-methylumbelliferone, 4-methyllumbelliferyl-β-D-glucosaminide, 4-methylumbelliferyl-α-neuraminic acid, 4-methylumbelliferyl-α-D-N-acetylgalactosaminide, and their functional analogs and derivatives.

The invention provides a droplet actuator including one or more substrates arranged to form a droplet operations gap, a fluoropolymer surface on one or more droplet operations gap-facing surfaces of the one or more substrates, where the fluoropolymer surface is deposited using a plasma-enhanced chemical vapor deposition process, a perfluorinated oil filler fluid in the droplet operations gap. In certain embodiments, the fluoropolymer surface includes an amorphous fluoropolymer. In certain embodiments, the fluoropolymer surface includes an amorphous fluoropolymer including carboxyl end-groups. In certain embodiments, the fluoropolymer surface includes an amorphous fluoropolymer including amino-silane coupling agents. In certain embodiments, the fluoropolymer surface includes an amorphous fluoropolymer including perfluoro groups. In certain embodiments, the fluoropolymer surface includes CYTOP® Type A. In certain embodiments, the fluoropolymer surface includes CYTOP® Type M. In certain embodiments, the fluoropolymer surface includes CYTOP® or Type S. In certain embodiments, the one or more substrates comprise one or more electrodes arranged for conducting droplet operations. In certain embodiments, the one or more substrates comprise an arrangement of electrodes arranged for conducting electrowetting-mediated droplet operations. In certain embodiments, the one or more substrates includes a printed circuit board substrate. In certain embodiments, the one or more substrates includes a silicone substrate. In certain embodiments, the one or more substrates includes a glass substrate. In certain embodiments, a droplet in the droplet operations gap, the droplet including a lipophilic substance. In certain embodiments, the water contact angle (θ) of the surface is greater than about 100.

6 Definitions

As used herein, the following terms have the meanings indicated.

"Activate," with reference to one or more electrodes, means affecting a change in the electrical state of the one or more electrodes which, in the presence of a droplet, results in a droplet operation. Activation of an electrode can be accomplished using alternating or direct current.

Any suitable voltage may be used. For example, an electrode may be activated using a voltage which is greater than about 150 V, or greater than about 200 V, or greater than about 250 V, or from about 275 V to about 1000 V, or about 300 V. Where alternating current is used, any suitable frequency may be employed. For example, an electrode may be activated using alternating current having a frequency from about 1 Hz to about 10 MHz, or from about 10 Hz to about 60 Hz, or from about 20 Hz to about 40 Hz, or about 30 Hz.

"Bead," with respect to beads on a droplet actuator, means any bead or particle that is capable of interacting with a droplet on or in proximity with a droplet actuator. Beads may be any of a wide variety of shapes, such as spherical, generally spherical, egg shaped, disc shaped, cubical, amorphous and other three dimensional shapes. The bead may, for example, be capable of being subjected to a droplet operation in a droplet on a droplet actuator or otherwise configured with respect to a droplet actuator in a manner which permits a droplet on the droplet actuator to be brought into contact with the bead on the droplet actuator and/or off the droplet actuator. Beads may be provided in a droplet, in a droplet operations gap, or on a droplet operations surface. Beads may be provided in a reservoir that is external to a droplet operations gap or situated apart from a droplet operations surface, and the reservoir may be associated with a flow path that permits a droplet including the beads to be brought into a droplet operations gap or into contact with a droplet operations surface. Beads may be manufactured using a wide variety of materials, including for example, resins, and polymers. The beads may be any suitable size, including for example, microbeads, microparticles, nanobeads and nanoparticles. In some cases, beads are magnetically responsive; in other cases beads are not significantly magnetically responsive. For magnetically responsive beads, the magnetically responsive material may constitute substantially all of a bead, a portion of a bead, or only one component of a bead. The remainder of the bead may include, among other things, polymeric material, coatings, and moieties which permit attachment of an assay reagent. Examples of suitable beads include flow cytometry microbeads, polystyrene microparticles and nanoparticles, functionalized polystyrene microparticles and nanoparticles, coated polystyrene microparticles and nanoparticles, silica microbeads, fluorescent microspheres and nanospheres, functionalized fluorescent microspheres and nanospheres, coated fluorescent microspheres and nanospheres, color dyed microparticles and nanoparticles, magnetic microparticles and nanoparticles, superparamagnetic microparticles and nanoparticles (e.g., DYNABEADS® particles, available from Invitrogen Group, Carlsbad, CA), fluorescent microparticles and nanoparticles, coated magnetic microparticles and nanoparticles, ferromagnetic microparticles and nanoparticles, coated ferromagnetic microparticles and

nanoparticles, and those described in U.S. Patent Publication Nos. 20050260686, entitled "Multiplex flow assays preferably with magnetic particles as solid phase," published on November 24, 2005; 20030132538, entitled "Encapsulation of discrete quanta of fluorescent particles," published on July 17, 2003; 20050118574, entitled "Multiplexed Analysis of Clinical Specimens Apparatus and Method," published on June 2, 2005; 20050277197. Entitled "Microparticles with Multiple Fluorescent Signals and Methods of Using Same," published on December 15, 2005; 20060159962, entitled "Magnetic Microspheres for use in Fluorescence-based Applications," published on July 20, 2006; the entire disclosures of which are incorporated herein by reference for their teaching concerning beads and magnetically responsive materials and beads. Beads may be pre-coupled with a biomolecule or other substance that is able to bind to and form a complex with a biomolecule. Beads may be pre-coupled with an antibody, protein or antigen, DNA/RNA probe or any other molecule with an affinity for a desired target. Examples of droplet actuator techniques for immobilizing magnetically responsive beads and/or non-magnetically responsive beads and/or conducting droplet operations protocols using beads are described in U.S. Patent Application No. 11/639,566, entitled "Droplet-Based Particle Sorting," filed on December 15, 2006; U.S. Patent Application No. 61/039,183, entitled "Multiplexing Bead Detection in a Single Droplet," filed on March 25, 2008; U.S. Patent Application No. 61/047,789, entitled "Droplet Actuator Devices and Droplet Operations Using Beads," filed on April 25, 2008; U.S. Patent Application No. 61/086,183, entitled "Droplet Actuator Devices and Methods for Manipulating Beads," filed on August 5, 2008; International Patent Application No. PCT/US2008/053545, entitled "Droplet Actuator Devices and Methods Employing Magnetic Beads," filed on February 11, 2008; International Patent Application No. PCT/US2008/058018, entitled "Bead-based Multiplexed Analytical Methods and Instrumentation," filed on March 24, 2008; International Patent Application No. PCT/US2008/058047, "Bead Sorting on a Droplet Actuator," filed on March 23, 2008; and International Patent Application No. PCT/US2006/047486, entitled "Droplet-based Biochemistry," filed on December 11, 2006; the entire disclosures of which are incorporated herein by reference. Bead characteristics may be employed in the multiplexing aspects of the invention. Examples of beads having characteristics suitable for multiplexing, as well as methods of detecting and analyzing signals emitted from such beads, may be found in U.S. Patent Publication No. 20080305481, entitled "Systems and Methods for Multiplex Analysis of PCR in Real Time," published on December 11, 2008; U.S. Patent Publication No. 20080151240, "Methods and Systems for Dynamic Range Expansion," published on June 26, 2008; U.S. Patent Publication No. 20070207513, entitled "Methods, Products, and Kits for Identifying an Analyte

in a Sample," published on September 6, 2007; U.S. Patent Publication No. 20070064990, entitled "Methods and Systems for Image Data Processing," published on March 22, 2007; U.S. Patent Publication No. 20060159962, entitled "Magnetic Microspheres for use in Fluorescence-based Applications," published on July 20, 2006; U.S. Patent Publication No. 20050277197, entitled "Microparticles with Multiple Fluorescent Signals and Methods of Using Same," published on December 15, 2005; and U.S. Patent Publication No. 20050118574, entitled "Multiplexed Analysis of Clinical Specimens Apparatus and Method," published on June 2, 2005.

"Droplet" means a volume of liquid on a droplet actuator. Typically, a droplet is at least partially bounded by a filler fluid. For example, a droplet may be completely surrounded by a filler fluid or may be bounded by filler fluid and one or more surfaces of the droplet actuator. As another example, a droplet may be bounded by filler fluid, one or more surfaces of the droplet actuator, and/or the atmosphere. As yet another example, a droplet may be bounded by filler fluid and the atmosphere. Droplets may, for example, be aqueous or non-aqueous or may be mixtures or emulsions including aqueous and non-aqueous components. Droplets may take a wide variety of shapes; nonlimiting examples include generally disc shaped, slug shaped, truncated sphere, ellipsoid, spherical, partially compressed sphere, hemispherical, ovoid, cylindrical, combinations of such shapes, and various shapes formed during droplet operations, such as merging or splitting or formed as a result of contact of such shapes with one or more surfaces of a droplet actuator. For examples of droplet fluids that may be subjected to droplet operations using the approach of the invention, see International Patent Application No. PCT/US 06/47486, entitled, "Droplet-Based Biochemistry," filed on December 11, 2006. In various embodiments, a droplet may include a biological sample, such as whole blood, lymphatic fluid, serum, plasma, sweat, tear, saliva, sputum, cerebrospinal fluid, amniotic fluid, seminal fluid, vaginal excretion, serous fluid, synovial fluid, pericardial fluid, peritoneal fluid, pleural fluid, transudates, exudates, cystic fluid, bile, urine, gastric fluid, intestinal fluid, fecal samples, liquids containing single or multiple cells, liquids containing organelles, fluidized tissues, fluidized organisms, liquids containing multi-celled organisms, biological swabs and biological washes. Moreover, a droplet may include a reagent, such as water, deionized water, saline solutions, acidic solutions, basic solutions, detergent solutions and/or buffers. Other examples of droplet contents include reagents, such as a reagent for a biochemical protocol, such as a nucleic acid amplification protocol, an affinity-based assay protocol, an enzymatic assay protocol, a sequencing protocol, and/or a protocol for analyses of biological fluids. A droplet may include one or more beads.

"Droplet Actuator" means a device for manipulating droplets. For examples of droplet actuators, see Pamula et al., U.S. Patent 6,911,132, entitled "Apparatus for Manipulating Droplets by Electrowetting-Based Techniques," issued on June 28, 2005; Pamula et al., U.S. Patent Application No. 11/343,284, entitled "Apparatuses and Methods for Manipulating Droplets on a Printed Circuit Board," filed on filed on January 30, 2006; Pollack et al., International Patent Application No. PCT/US2006/047486, entitled "Droplet-Based Biochemistry," filed on December 11, 2006; Shenderov, U.S. Patents 6,773,566, entitled "Electrostatic Actuators for Microfluidics and Methods for Using Same," issued on August 10, 2004 and 6,565,727, entitled "Actuators for Microfluidics Without Moving Parts," issued on January 24, 2000; Kim and/or Shah et al., U.S. Patent Application Nos. 10/343,261, entitled "Electrowetting-driven Micropumping," filed on January 27, 2003, 11/275,668, entitled "Method and Apparatus for Promoting the Complete Transfer of Liquid Drops from a Nozzle," filed on January 23, 2006, 11/460,188, entitled "Small Object Moving on Printed Circuit Board," filed on January 23, 2006, 12/465,935, entitled "Method for Using Magnetic Particles in Droplet Microfluidics," filed on May 14, 2009, and 12/513,157, entitled "Method and Apparatus for Real-time Feedback Control of Electrical Manipulation of Droplets on Chip," filed on April 30, 2009; Velev, U.S. Patent 7,547,380, entitled "Droplet Transportation Devices and Methods Having a Fluid Surface," issued on June 16, 2009; Sterling et al., U.S. Patent 7,163,612, entitled "Method, Apparatus and Article for Microfluidic Control via Electrowetting, for Chemical, Biochemical and Biological Assays and the Like," issued on January 16, 2007; Becker and Gascoyne et al., U.S. Patent Nos. 7,641,779, entitled "Method and Apparatus for Programmable fluidic Processing," issued on January 5, 2010, and 6,977,033, entitled "Method and Apparatus for Programmable fluidic Processing," issued on December 20, 2005; Decre et al., U.S. Patent 7,328,979, entitled "System for Manipulation of a Body of Fluid," issued on February 12, 2008; Yamakawa et al., U.S. Patent Pub. No. 20060039823, entitled "Chemical Analysis Apparatus," published on February 23, 2006; Wu, International Patent Pub. No. WO/2009/003184, entitled "Digital Microfluidics Based Apparatus for Heat-exchanging Chemical Processes," published on December 31, 2008; Fouillet et al., U.S. Patent Pub. No. 20090192044, entitled "Electrode Addressing Method," published on July 30, 2009; Fouillet et al., U.S. Patent 7,052,244, entitled "Device for Displacement of Small Liquid Volumes Along a Micro-catenary Line by Electrostatic Forces," issued on May 30, 2006; Marchand et al., U.S. Patent Pub. No. 20080124252, entitled "Droplet Microreactor," published on May 29, 2008; Adachi et al., U.S. Patent Pub. No. 20090321262, entitled "Liquid Transfer Device," published on December 31, 2009; Roux et al., U.S. Patent Pub. No. 20050179746, entitled "Device for Controlling the Displacement of a Drop Between two or Several Solid

Substrates," published on August 18, 2005; Dhindsa et al., "Virtual Electrowetting Channels: Electronic Liquid Transport with Continuous Channel Functionality," Lab Chip, 10:832–836 (2010); the entire disclosures of which are incorporated herein by reference, along with their priority documents. Certain droplet actuators will include one or more substrates arranged with a droplet operations gap therebetween and electrodes associated with (e.g., layered on, attached to, and/or embedded in) the one or more substrates and arranged to conduct one or more droplet operations. For example, certain droplet actuators will include a base (or bottom) substrate, droplet operations electrodes associated with the substrate, one or more dielectric layers atop the substrate and/or electrodes, and optionally one or more hydrophobic layers atop the substrate, dielectric layers and/or the electrodes forming a droplet operations surface. A top substrate may also be provided, which is separated from the droplet operations surface by a gap, commonly referred to as a droplet operations gap. Various electrode arrangements on the top and/or bottom substrates are discussed in the above-referenced patents and applications and certain novel electrode arrangements are discussed in the description of the invention. During droplet operations it is preferred that droplets remain in continuous contact or frequent contact with a ground or reference electrode. A ground or reference electrode may be associated with the top substrate facing the gap, the bottom substrate facing the gap, in the gap. Where electrodes are provided on both substrates, electrical contacts for coupling the electrodes to a droplet actuator instrument for controlling or monitoring the electrodes may be associated with one or both plates. In some cases, electrodes on one substrate are electrically coupled to the other substrate so that only one substrate is in contact with the droplet actuator. In one embodiment, a conductive material (e.g., an epoxy, such as MASTER BOND™ Polymer System EP79, available from Master Bond, Inc., Hackensack, NJ) provides the electrical connection between electrodes on one substrate and electrical paths on the other substrates, e.g., a ground electrode on a top substrate may be coupled to an electrical path on a bottom substrate by such a conductive material. Where multiple substrates are used, a spacer may be provided between the substrates to determine the height of the gap therebetween and define dispensing reservoirs. The spacer height may, for example, be from about 5 μm to about 600 μm, or about 100 μm to about 400 μm, or about 200 μm to about 350 μm, or about 250 μm to about 300 μm, or about 275 μm. The spacer may, for example, be formed of a layer of projections form the top or bottom substrates, and/or a material inserted between the top and bottom substrates. One or more openings may be provided in the one or more substrates for forming a fluid path through which liquid may be delivered into the droplet operations gap. The one or more openings may in some cases be aligned for interaction with one or more electrodes, e.g., aligned such that liquid flowed through the opening will come

into sufficient proximity with one or more droplet operations electrodes to permit a droplet operation to be effected by the droplet operations electrodes using the liquid. The base (or bottom) and top substrates may in some cases be formed as one integral component. One or more reference electrodes may be provided on the base (or bottom) and/or top substrates and/or in the gap. Examples of reference electrode arrangements are provided in the above referenced patents and patent applications. In various embodiments, the manipulation of droplets by a droplet actuator may be electrode mediated, e.g., electrowetting mediated or dielectrophoresis mediated or Coulombic force mediated. Examples of other techniques for controlling droplet operations that may be used in the droplet actuators of the invention include using devices that induce hydrodynamic fluidic pressure, such as those that operate on the basis of mechanical principles (e.g. external syringe pumps, pneumatic membrane pumps, vibrating membrane pumps, vacuum devices, centrifugal forces, piezoelectric/ultrasonic pumps and acoustic forces); electrical or magnetic principles (e.g. electroosmotic flow, electrokinetic pumps, ferrofluidic plugs, electrohydrodynamic pumps, attraction or repulsion using magnetic forces and magnetohydrodynamic pumps); thermodynamic principles (e.g. gas bubble generation/phase-change-induced volume expansion); other kinds of surface-wetting principles (e.g. electrowetting, and optoelectrowetting, as well as chemically, thermally, structurally and radioactively induced surface-tension gradients); gravity; surface tension (e.g., capillary action); electrostatic forces (e.g., electroosmotic flow); centrifugal flow (substrate disposed on a compact disc and rotated); magnetic forces (e.g., oscillating ions causes flow); magnetohydrodynamic forces; and vacuum or pressure differential. In certain embodiments, combinations of two or more of the foregoing techniques may be employed to conduct a droplet operation in a droplet actuator of the invention. Similarly, one or more of the foregoing may be used to deliver liquid into a droplet operations gap, e.g., from a reservoir in another device or from an external reservoir of the droplet actuator (e.g., a reservoir associated with a droplet actuator substrate and a flow path from the reservoir into the droplet operations gap). Droplet operations surfaces of certain droplet actuators of the invention may be made from hydrophobic materials or may be coated or treated to make them hydrophobic. For example, in some cases some portion or all of the droplet operations surfaces may be derivatized with low surface-energy materials or chemistries, e.g., by deposition or using in situ synthesis using compounds such as poly- or per-fluorinated compounds in solution or polymerizable monomers. Examples include TEFLON® AF (available from DuPont, Wilmington, DE), members of the cytop family of materials, coatings in the FLUOROPEL® family of hydrophobic and superhydrophobic coatings (available from Cytonix Corporation, Beltsville, MD), silane coatings, fluorosilane coatings, hydrophobic phosphonate derivatives

(e.g.., those sold by Aculon, Inc), and NOVEC™ electronic coatings (available from 3M Company, St. Paul, MN), other fluorinated monomers for plasma-enhanced chemical vapor deposition (PECVD), and organosiloxane (e.g., SiOC) for PECVD. In some cases, the droplet operations surface may include a hydrophobic coating having a thickness ranging from about 10 nm to about 1,000 nm. Moreover, in some embodiments, the top substrate of the droplet actuator includes an electrically conducting organic polymer, which is then coated with a hydrophobic coating or otherwise treated to make the droplet operations surface hydrophobic. For example, the electrically conducting organic polymer that is deposited onto a plastic substrate may be poly(3,4-ethylenedioxythiophene) poly(styrenesulfonate) (PEDOT:PSS). Other examples of electrically conducting organic polymers and alternative conductive layers are described in Pollack et al., International Patent Application No. PCT/US2010/040705, entitled "Droplet Actuator Devices and Methods," the entire disclosure of which is incorporated herein by reference. One or both substrates may be fabricated using a printed circuit board (PCB), glass, indium tin oxide (ITO)-coated glass, and/or semiconductor materials as the substrate. When the substrate is ITO-coated glass, the ITO coating is preferably a thickness in the range of about 20 to about 200 nm, preferably about 50 to about 150 nm, or about 75 to about 125 nm, or about 100 nm. In some cases, the top and/or bottom substrate includes a PCB substrate that is coated with a dielectric, such as a polyimide dielectric, which may in some cases also be coated or otherwise treated to make the droplet operations surface hydrophobic. When the substrate includes a PCB, the following materials are examples of suitable materials: MITSUI™ BN-300 (available from MITSUI Chemicals America, Inc., San Jose CA); ARLON™ 11N (available from Arlon, Inc, Santa Ana, CA).; NELCO® N4000-6 and N5000-30/32 (available from Park Electrochemical Corp., Melville, NY); ISOLA™ FR406 (available from Isola Group, Chandler, AZ), especially IS620; fluoropolymer family (suitable for fluorescence detection since it has low background fluorescence); polyimide family; polyester; polyethylene naphthalate; polycarbonate; polyetheretherketone; liquid crystal polymer; cyclo-olefin copolymer (COC); cyclo-olefin polymer (COP); aramid; THERMOUNT® nonwoven aramid reinforcement (available from DuPont, Wilmington, DE); NOMEX® brand fiber (available from DuPont, Wilmington, DE); and paper. Various materials are also suitable for use as the dielectric component of the substrate. Examples include: vapor deposited dielectric, such as PARYLENE™ C (especially on glass), PARYLENE™ N, and PARYLENE™ HT (for high temperature, ~300°C) (available from Parylene Coating Services, Inc., Katy, TX); TEFLON® AF coatings; cytop; soldermasks, such as liquid photoimageable soldermasks (e.g., on PCB) like TAIYO™ PSR4000 series, TAIYO™ PSR and AUS series (available from Taiyo America, Inc. Carson City, NV) (good thermal

characteristics for applications involving thermal control), and PROBIMER™ 8165 (good thermal characteristics for applications involving thermal control (available from Huntsman Advanced Materials Americas Inc., Los Angeles, CA); dry film soldermask, such as those in the VACREL® dry film soldermask line (available from DuPont, Wilmington, DE); film dielectrics, such as polyimide film (e.g., KAPTON® polyimide film, available from DuPont, Wilmington, DE), polyethylene, and fluoropolymers (e.g., FEP), polytetrafluoroethylene; polyester; polyethylene naphthalate; cyclo-olefin copolymer (COC); cyclo-olefin polymer (COP); any other PCB substrate material listed above; black matrix resin; and polypropylene. Droplet transport voltage and frequency may be selected for performance with reagents used in specific assay protocols. Design parameters may be varied, e.g., number and placement of on-actuator reservoirs, number of independent electrode connections, size (volume) of different reservoirs, placement of magnets/bead washing zones, electrode size, inter-electrode pitch, and gap height (between top and bottom substrates) may be varied for use with specific reagents, protocols, droplet volumes, etc. In some cases, a substrate of the invention may derivatized with low surface-energy materials or chemistries, e.g., using deposition or in situ synthesis using poly- or per-fluorinated compounds in solution or polymerizable monomers. Examples include TEFLON® AF coatings and FLUOROPEL® coatings for dip or spray coating, other fluorinated monomers for plasma-enhanced chemical vapor deposition (PECVD), and organosiloxane (e.g., SiOC) for PECVD. Additionally, in some cases, some portion or all of the droplet operations surface may be coated with a substance for reducing background noise, such as background fluorescence from a PCB substrate. For example, the noise-reducing coating may include a black matrix resin, such as the black matrix resins available from Toray industries, Inc., Japan. Electrodes of a droplet actuator are typically controlled by a controller or a processor, which is itself provided as part of a system, which may include processing functions as well as data and software storage and input and output capabilities. Reagents may be provided on the droplet actuator in the droplet operations gap or in a reservoir fluidly coupled to the droplet operations gap. The reagents may be in liquid form, e.g., droplets, or they may be provided in a reconstitutable form in the droplet operations gap or in a reservoir fluidly coupled to the droplet operations gap. Reconstitutable reagents may typically be combined with liquids for reconstitution. An example of reconstitutable reagents suitable for use with the invention includes those described in Meathrel, et al., U.S. Patent 7,727,466, entitled "Disintegratable films for diagnostic devices," granted on June 1, 2010.

"Droplet operation" means any manipulation of a droplet on a droplet actuator. A droplet operation may, for example, include: loading a droplet into the droplet actuator; dispensing one or more droplets from a source droplet; splitting, separating or dividing a droplet into two or more droplets; transporting a droplet from one location to another in any direction; merging or combining two or more droplets into a single droplet; diluting a droplet; mixing a droplet; agitating a droplet; deforming a droplet; retaining a droplet in position; incubating a droplet; heating a droplet; vaporizing a droplet; cooling a droplet; disposing of a droplet; transporting a droplet out of a droplet actuator; other droplet operations described herein; and/or any combination of the foregoing. The terms "merge," "merging," "combine," "combining" and the like are used to describe the creation of one droplet from two or more droplets. It should be understood that when such a term is used in reference to two or more droplets, any combination of droplet operations that are sufficient to result in the combination of the two or more droplets into one droplet may be used. For example, "merging droplet A with droplet B," can be achieved by transporting droplet A into contact with a stationary droplet B, transporting droplet B into contact with a stationary droplet A, or transporting droplets A and B into contact with each other. The terms "splitting," "separating" and "dividing" are not intended to imply any particular outcome with respect to volume of the resulting droplets (i.e., the volume of the resulting droplets can be the same or different) or number of resulting droplets (the number of resulting droplets may be 2, 3, 4, 5 or more). The term "mixing" refers to droplet operations which result in more homogenous distribution of one or more components within a droplet. Examples of "loading" droplet operations include microdialysis loading, pressure assisted loading, robotic loading, passive loading, and pipette loading. Droplet operations may be electrode-mediated. In some cases, droplet operations are further facilitated by the use of hydrophilic and/or hydrophobic regions on surfaces and/or by physical obstacles. For examples of droplet operations, see the patents and patent applications cited above under the definition of "droplet actuator." Impedance or capacitance sensing or imaging techniques may sometimes be used to determine or confirm the outcome of a droplet operation. Examples of such techniques are described in Sturmer et al., International Patent Pub. No. WO/2008/101194, entitled "Capacitance Detection in a Droplet Actuator," published on August 21, 2008, the entire disclosure of which is incorporated herein by reference. Generally speaking, the sensing or imaging techniques may be used to confirm the presence or absence of a droplet at a specific electrode. For example, the presence of a dispensed droplet at the destination electrode following a droplet dispensing operation confirms that the droplet dispensing operation was effective. Similarly, the presence of a droplet at a detection spot at an appropriate step in an assay protocol may confirm that a previous set of droplet operations

has successfully produced a droplet for detection. Droplet transport time can be quite fast. For example, in various embodiments, transport of a droplet from one electrode to the next may exceed about 1 sec, or about 0.1 sec, or about 0.01 sec, or about 0.001 sec. In one embodiment, the electrode is operated in AC mode but is switched to DC mode for imaging. It is helpful for conducting droplet operations for the footprint area of droplet to be similar to electrowetting area; in other words, 1x-, 2x- 3x-droplets are usefully controlled operated using 1, 2, and 3 electrodes, respectively. If the droplet footprint is greater than the number of electrodes available for conducting a droplet operation at a given time, the difference between the droplet size and the number of electrodes should typically not be greater than 1; in other words, a 2x droplet is usefully controlled using 1 electrode and a 3x droplet is usefully controlled using 2 electrodes. When droplets include beads, it is useful for droplet size to be equal to the number of electrodes controlling the droplet, e.g., transporting the droplet.

"Filler fluid" means a fluid associated with a droplet operations substrate of a droplet actuator, which fluid is sufficiently immiscible with a droplet phase to render the droplet phase subject to electrode-mediated droplet operations. For example, the droplet operations gap of a droplet actuator is typically filled with a filler fluid. The filler fluid may, for example, be a low-viscosity oil, such as silicone oil or hexadecane filler fluid. The filler fluid may fill the entire gap of the droplet actuator or may coat one or more surfaces of the droplet actuator. Filler fluids may be conductive or non-conductive. Filler fluids may, for example, be doped with surfactants or other additives. For example, additives may be selected to improve droplet operations and/or reduce loss of reagent or target substances from droplets, formation of microdroplets, cross contamination between droplets, contamination of droplet actuator surfaces, degradation of droplet actuator materials, etc. Composition of the filler fluid, including surfactant doping, may be selected for performance with reagents used in the specific assay protocols and effective interaction or non-interaction with droplet actuator materials. Examples of filler fluids and filler fluid formulations suitable for use with the invention are provided in Srinivasan et al, International Patent Pub. Nos. WO/2010/027894, entitled "Droplet Actuators, Modified Fluids and Methods," published on March 11, 2010, and WO/2009/021173, entitled "Use of Additives for Enhancing Droplet Operations," published on February 12, 2009; Sista et al., International Patent Pub. No. WO/2008/098236, entitled "Droplet Actuator Devices and Methods Employing Magnetic Beads," published on August 14, 2008; and Monroe et al., U.S. Patent Publication No. 20080283414, entitled "Electrowetting Devices," filed on May 17, 2007; the entire disclosures of

which are incorporated herein by reference, as well as the other patents and patent applications cited herein.

"Immobilize" with respect to magnetically responsive beads, means that the beads are substantially restrained in position in a droplet or in filler fluid on a droplet actuator. For example, in one embodiment, immobilized beads are sufficiently restrained in position in a droplet to permit execution of a droplet splitting operation, yielding one droplet with substantially all of the beads and one droplet substantially lacking in the beads.

"Magnetically responsive" means responsive to a magnetic field. "Magnetically responsive beads" include or are composed of magnetically responsive materials. Examples of magnetically responsive materials include paramagnetic materials, ferromagnetic materials, ferrimagnetic materials, and metamagnetic materials. Examples of suitable paramagnetic materials include iron, nickel, and cobalt, as well as metal oxides, such as Fe3O4, BaFe12O19, CoO, NiO, Mn2O3, Cr2O3, and CoMnP.

"Reservoir" means an enclosure or partial enclosure configured for holding, storing, or supplying liquid. A droplet actuator system of the invention may include on-cartridge reservoirs and/or off-cartridge reservoirs. On-cartridge reservoirs may be (1) on-actuator reservoirs, which are reservoirs in the droplet operations gap or on the droplet operations surface; (2) off-actuator reservoirs, which are reservoirs on the droplet actuator cartridge, but outside the droplet operations gap, and not in contact with the droplet operations surface; or (3) hybrid reservoirs which have on-actuator regions and off-actuator regions. An example of an off-actuator reservoir is a reservoir in the top substrate. An off-actuator reservoir is typically in fluid communication with an opening or flow path arranged for flowing liquid from the off-actuator reservoir into the droplet operations gap, such as into an on-actuator reservoir. An off-cartridge reservoir may be a reservoir that is not part of the droplet actuator cartridge at all, but which flows liquid to some portion of the droplet actuator cartridge. For example, an off-cartridge reservoir may be part of a system or docking station to which the droplet actuator cartridge is coupled during operation. Similarly, an off-cartridge reservoir may be a reagent storage container or syringe which is used to force fluid into an on-cartridge reservoir or into a droplet operations gap. A system using an off-cartridge reservoir will typically include a fluid passage means whereby liquid may be transferred from the off-cartridge reservoir into an on-cartridge reservoir or into a droplet operations gap.

"Transporting into the magnetic field of a magnet," "transporting towards a magnet," and the like, as used herein to refer to droplets and/or magnetically responsive beads within droplets, is intended to refer to transporting into a region of a magnetic field capable of substantially attracting magnetically responsive beads in the droplet. Similarly, "transporting away from a magnet or magnetic field," "transporting out of the magnetic field of a magnet," and the like, as used herein to refer to droplets and/or magnetically responsive beads within droplets, is intended to refer to transporting away from a region of a magnetic field capable of substantially attracting magnetically responsive beads in the droplet, whether or not the droplet or magnetically responsive beads is completely removed from the magnetic field. It will be appreciated that in any of such cases described herein, the droplet may be transported towards or away from the desired region of the magnetic field, and/or the desired region of the magnetic field may be moved towards or away from the droplet. Reference to an electrode, a droplet, or magnetically responsive beads being "within" or "in" a magnetic field, or the like, is intended to describe a situation in which the electrode is situated in a manner which permits the electrode to transport a droplet into and/or away from a desired region of a magnetic field, or the droplet or magnetically responsive beads is/are situated in a desired region of the magnetic field, in each case where the magnetic field in the desired region is capable of substantially attracting any magnetically responsive beads in the droplet. Similarly, reference to an electrode, a droplet, or magnetically responsive beads being "outside of" or "away from" a magnetic field, and the like, is intended to describe a situation in which the electrode is situated in a manner which permits the electrode to transport a droplet away from a certain region of a magnetic field, or the droplet or magnetically responsive beads is/are situated away from a certain region of the magnetic field, in each case where the magnetic field in such region is not capable of substantially attracting any magnetically responsive beads in the droplet or in which any remaining attraction does not eliminate the effectiveness of droplet operations conducted in the region. In various aspects of the invention, a system, a droplet actuator, or another component of a system may include a magnet, such as one or more permanent magnets (e.g., a single cylindrical or bar magnet or an array of such magnets, such as a Halbach array) or an electromagnet or array of electromagnets, to form a magnetic field for interacting with magnetically responsive beads or other components on chip. Such interactions may, for example, include substantially immobilizing or restraining movement or flow of magnetically responsive beads during storage or in a droplet during a droplet operation or pulling magnetically responsive beads out of a droplet.

"Washing" with respect to washing a bead means reducing the amount and/or concentration of one or more substances in contact with the bead or exposed to the bead from a droplet in contact with the bead. The reduction in the amount and/or concentration of the substance may be partial, substantially complete, or even complete. The substance may be any of a wide variety of substances; examples include target substances for further analysis, and unwanted substances, such as components of a sample, contaminants, and/or excess reagent. In some embodiments, a washing operation begins with a starting droplet in contact with a magnetically responsive bead, where the droplet includes an initial amount and initial concentration of a substance. The washing operation may proceed using a variety of droplet operations. The washing operation may yield a droplet including the magnetically responsive bead, where the droplet has a total amount and/or concentration of the substance which is less than the initial amount and/or concentration of the substance. Examples of suitable washing techniques are described in Pamula et al., U.S. Patent 7,439,014, entitled "Droplet-Based Surface Modification and Washing," granted on October 21, 2008, the entire disclosure of which is incorporated herein by reference.

The terms "top," "bottom," "over," "under," and "on" are used throughout the description with reference to the relative positions of components of the droplet actuator, such as relative positions of top and bottom substrates of the droplet actuator. It will be appreciated that the droplet actuator is functional regardless of its orientation in space.

When a liquid in any form (e.g., a droplet or a continuous body, whether moving or stationary) is described as being "on", "at", or "over" an electrode, array, matrix or surface, such liquid could be either in direct contact with the electrode/array/matrix/surface, or could be in contact with one or more layers or films that are interposed between the liquid and the electrode/array/matrix/surface. In one example, filler fluid can be considered as a film between such liquid and the electrode/array/matrix/surface.

When a droplet is described as being "on" or "loaded on" a droplet actuator, it should be understood that the droplet is arranged on the droplet actuator in a manner which facilitates using the droplet actuator to conduct one or more droplet operations on the droplet, the droplet is arranged on the droplet actuator in a manner which facilitates sensing of a property of or a signal from the droplet, and/or the droplet has been subjected to a droplet operation on the droplet actuator.

7 Brief Description of the Drawings

Figure 1 shows a diagram of an example of an on-bench assay protocol for evaluating the effect of aqueous surfactants on 4-MU partitioning;

Figure 2 shows an example of a plot of relative fluorescence readings for backward transfer partitioning (backward extraction; BE) of a 4-MU partitioning assay used to evaluate the effect of aqueous phase surfactants on 4-MU containment;

Figure 3 illustrates a side view of a portion of an example of a droplet actuator that has hydrophobic coatings on the droplet operations surfaces thereof and the gap may be filled with perfluorinated oil;

Figures 4A and 4B illustrate water contact angle (θ) with respect to hydrophilicity and hydrophobicity, respectively;

Figures 5A and 5B show top down views of a portion of a droplet actuator that includes M-type CYTOP® coatings applied using the liquid deposition process;

Figure 6 shows a top down view of a portion of a droplet actuator that includes hydrophobic coatings applied using the Plasmatreat PECVD process;

Figure 7 shows a top down view of a portion of a droplet actuator that includes hydrophobic coatings applied using the Triton PECVD process;

Figure 8 shows a top down view of a portion of a droplet actuator that includes hydrophobic coatings applied using the GVD PECVD process at room temperature;

Figure 9 shows a top down view of a portion of a droplet actuator that includes hydrophobic coatings applied using the GVD PECVD process at high temperature; and

Figure 10 illustrates a functional block diagram of an example of a microfluidics system that includes a droplet actuator.

8 Description

The present invention provides methods to reduce, preferably substantially or entirely eliminate, partitioning of 4-MU (or derivatives) in droplet-based bioassays on a droplet actuator. In one embodiment, surfactants (detergents) may be used to retain 4-MU (or derivatives) within an aqueous phase droplet. The methods of the invention provide significantly improved signal retention and substantially reduced cross-contamination between droplets. The methods of the invention also provide significantly improved discrimination between a positive signal and a negative signal in 4-MU-based bioassays.

Further, the invention provides a droplet actuator with a droplet operations surface having a hydrophobic coating that is chemically compatible with the perfluorinated oil (or solvents) and that also ensure suitable droplets operations performance. The invention provides methods of depositing hydrophobic coatings on the droplet operations surfaces of a droplet actuator to render them suitable for use with perfluorinated solvents. The invention also provides hydrophobic coatings for improving operations performance. For example, the invention provides droplet actuators with coatings applied by liquid deposition processes as well as coatings applied by plasma-enhanced chemical vapor deposition (PECVD)-based processes.

8.1 Reducing Partitioning of 4-MU

4-MU-containing substrates (a.k.a 7-hydroxy 4-methylcoumarin) are used in a number of fluorometric enzyme assays (e.g., enzymatic assays for the detection of lysosomal storage disorders in newborns). The fluorometric enzyme assays are based on the hydrolysis of a 4-MU-containing substrate by a specific enzyme to yield the fluorescent molecule 4-MU. In the droplet operations environment of a droplet actuator, partitioning of 4-MU between the aqueous phase (i.e., droplet) and the organic phase (filler fluid) may result in a reduction in the assay signal and potential contamination of neighboring samples. The enzymatic turnover of the 4-MU substrate requires a low-pH environment (acidic environment). At low pH (pK of 4-MU = 7.9), 4-MU is non-ionic and hydrophobic and partitions preferentially from the aqueous droplet phase into the oil filler phase (100:1). Droplets subsequently prepared for the detection step of the bioassay are at a high pH. Fluorescence of 4-MU is optimal at elevated pH (pH > 10). A high pH (pH >10) facilitates reverse partitioning of 4-MU from the oil phase back into an aqueous phase droplet. The potential for droplet cross-contamination occurs when an acidic droplet with elevated

enzyme concentration (producing significant amounts of 4-MU product) is in proximity of a basic droplet with substantially lower 4-MU concentrations.

The efficacy of different surfactants (detergents) in containing 4-MU (or derivatives) within an aqueous phase may, for example, be evaluated using a partitioning assay. Parameters that may be varied in the assay for evaluation of surfactants in aqueous containment of 4-MU (or derivatives) include, but are not limited to, the pH of the aqueous phase solution, and the critical micelle concentration of the surfactant.

Figure 1 shows a diagram of an example of an on-bench assay protocol 100 for evaluating the effect of aqueous surfactants on 4-MU partitioning. The assay format includes forward transfer partitioning (forward extraction; FE) of 4-MU from an aqueous phase to an oil phase and backward transfer partitioning (backward extraction; BE) of 4-MU from an oil phase to an aqueous phase. The assay is performed in 96-well microtiter substrates; clear 96-well substrates (e.g., Costar 3631) for evaluation of forward transfer partitioning with bottom probe fluorescence detection and solid black 96-well substrates (e.g., Costar 3915) for evaluation of backward transfer partitioning with top probe fluorescence detection. A BioTek Synergy HT instrument with 3 mm top probe and 5 mm bottom probe, may, for example, be used for fluorescence measurements.

An example of an assay format used for testing the effect of surfactants on contamination through 4-MU partitioning includes, but is not limited to, the following steps: Pipette an aliquot (20 µL) of an aqueous phase solution (e.g., at pH 2 to pH 10.5) containing 0.01% Tween® 20 in a well of a 96-well clear microtiter plate. The aqueous phase solution may also include 4-MU (e.g., 100 µM), NaCl (e.g., 50 mM), and BSA (e.g., 1 mg/mL). Add 130 µL of oil (e.g., silicone oil 5 cSt, 0.1% Triton X-15) to each well that contains an aqueous phase droplet. Seal the plate with aluminum foil and shake using a bench top shaker (e.g., Thermofisher shaker at speed setting 5) for 30 min at room temperature. Carefully remove the aluminum foil and observe each well to note and record any defects in droplet quality (minimize light exposure during this step). Measure the fluorescence of each well using a bottom probe at gains 40, 45, and 50. Transfer, without disturbing the aqueous droplet, 75 µL of the oil phase (FE oil) from each well into the respective well of a solid black microtiter plate that contains 75 µL of 200 mM $NaHCO_3$ in each well. Seal the plate with aluminum foil and shake using, for example, a Thermofisher bench top

shaker (e.g., speed setting 5) for 60 min at 40 °C. Remove the aluminum foil and measure the fluorescence of each well using a top probe at gains 50, 60, 70, and 80.

Figure 2 shows a bar graph 200 of relative fluorescence readings for backward transfer partitioning (backward extraction; BE) of a 4-MU partitioning assay used to evaluate the effect of aqueous phase surfactants on 4-MU containment. In this example, surfactants were selected from an array of different surfactants available in the detergent screening kit HR2-408 from Hampton Research, Inc. The experiment was performed using 5 cSt silicone oil with 0.1% w/v Triton X-15 as the organic phase (oil phase). Each surfactant was used at 1.5 times the surfactant's critical micellar concentration (CMC). The identity of each surfactant is listed in Tables 1 and 2. AS17 (ANAPOE®-20 or Tween® 20) was used as a reference signal (43,000-44,000 RFU) and reflects an example of the level of droplet cross-contamination that may be observed in a 4-MU-based bioassay (e.g., newborn screening assay) performed on a droplet actuator. Surfactants that retained 4-MU more efficiently than AS17 (ANAPOE®-20 or Tween® 20), i.e., to the left of the arrow in Figure 2, are listed in Table 1. Surfactants that were less efficient than AS17 in retaining 4-MU, i.e., to the right of the arrow in Figure 2, are listed in Table 2.

Table 1. Aqueous surfactants (AS) in 4-MU retention assay of Figure 2 (left of arrow)			
AS #	**Surfactant**	**AS #**	**Surfactant**
AS 93	FOS-Choline®-8	AS 64	HEGA®-9
AS 91	ZWITTERGENT® 3-10	AS 63	C-HEGA®-10
AS 1	BAM	AS 84	DDMAB
AS 90	FOS-Choline®-9	AS 81	FOS-Choline®-8, fluorinated
AS 70	n-Hexyl-ß-D-glucopyranoside	AS 95	LysoFos™ Choline 12
AS 69	CYMAL®-2	AS 2	n-Dodecyl-ß-iminodipropionic acid,
AS 66	MEGA-8	AS 79	n-Dodecyl-N,N-dimethylglycine
AS 68	HEGA®-8	AS 82	n-Undecyl-N,N-Dimethlamine-oxide
AS 89	n-Decyl-N,N-dimethylglycine	AS 60	n-Heptyl-ß-D-thioglucopyranoside
AS 67	HEGA®-9	AS 25	ANAPOE®-$C_{12}E_{10}$
AS 88	FOS-Choline®-10	AS 54	Pluronic® F-68
AS 83	ZWITTERGENT® 3-12	AS 53	C-HEGA®-11
AS 7	Sodium cholate	AS 55	HECAMEG®
AS 9	ANAPOE®-X-305	AS 29	ANAPOE®-X-405
AS 92	CYCLOFOS™-3	AS 17	ANAPOE®-20 (Tween® 20)
AS 62	CYMAL®-3	AS 56	n-Octyl-ß-D-glucoside
AS 86	CHAPS	AS 96	LysoFos™ Choline 10
AS 94	ZWITTERGENT® 3-08	AS 59	2,6-Dimethyl-4-heptyl-ß-D-malto-
AS 77	NDSB-256	AS 57	n-Octanoylsucrose
AS 80	FOS-Choline®-12	AS 31	ANAPOE®-$C_{10}E_6$
AS 58	MEGA-9	AS 74	NDSB-201
AS 87	CHAPSO	AS 12	ANAPOE®-58
AS 85	FOS-MEA®-10		
AS 78	ZWITTERGENT® 3-14		

Table 2. Aqueous surfactants (AS) in 4-MU retention assay of Figure 2 (right of arrow)			
AS #	**Surfactant**	**AS #**	**Surfactant**
AS 17	ANAPOE®-20 (Tween® 20)	AS 21	ANAPOE®-$C_{12}E_8$
AS 34	ANAPOE®-$C_{10}E_9$	AS 22	n-Dodecyl-ß-D-maltoside
AS 35	ANAPOE®-35	AS 75	NDSB-211
AS 24	ANAPOE®-X-114	AS 36	n-Decyl-ß-D-maltoside
AS 20	ANAPOE®-$C_{13}E_8$	AS 11	n-Hexadecyl-ß-D-maltoside
AS 76	NDSB-221	AS 42	n-Nonyl-ß-D-maltoside
AS 49	n-Octyl-ß-D thiomaltoside	AS 50	n-Octyl-ß-D-thioglucoside
AS 10	IPTG	AS 61	n-Octyl-ß-D-galactopyranoside
AS 73	NDSB-195	AS 37	LDAO
AS 71	C-HEGA®-8	AS 41	CYMAL®-5
AS 14	ANAPOE®-80	AS 38	n-Decanoylsucrose
AS 4	CTAB	AS 35	Big CHAP, deoxy
AS 52	DDAO	AS 6	Sodium dodecyl sulfate
AS 72	CYMAL®-2	AS 32	n-Decyl-ß-D-thiomaltoside
AS 47	CYMAL®-4	AS 18	Thesit®
AS 16	ANAPOE®-$C_{12}E_9$	AS 44	HEGA®-10
AS 5	Deoxycholic acid, sodium salt	AS 28	n-Undecyl-ß-D-maltoside
AS 45	MEGA-10	AS 33	Octyl maltoside, fluorinated
AS 40	n-Nonyl-ß-D-thiomaltoside	AS 23	CYMAL®-7
AS 15	n-Tridecyl-ß-D-maltoside	AS 26	Sucrose monolaurate
AS 8	Sodium dodecanoyl sarcosine	AS 27	CYMAL®-6
AS 43	n-Nonyl-ß-D-glucoside	AS 3	Dodecyltrimethylammonium chloride
AS 30	TRITON® X-100	AS 46	C_8E_5
AS 13	n-Tetradecyl-ß-D-maltoside	AS 51	Hexaethylene glycol monooctyl ether
AS 39	n-Nonyl-ß-D-thioglucoside	AS 48	C_8E_4

Classification of the most efficient surfactants (detergents) in retaining 4-MU in an aqueous droplet is shown in Table 3. The zwitterionic surfactant group (14) includes sulfobetaines, betaine and lipid-like phosphocholine and phosphoethanolamine. The non-ionic surfactant group (9) includes sugar-based surfactants (glycosides, glucamides).

Table 3. Classification of top 25 aqueous surfactants in 4-MU retention assay (< 32,000 RFU)			
AS #	**Surfactant**	**Type**	**Chemical Class**
AS 93	FOS-Choline®-8	Zwitterionic	Alkyl phosphocholine
AS 91	ZWITTERGENT® 3-10	Zwitterionic	Alkyl sulfobetaine
AS 1	BAM	Cationic	Quaternary ammonium
AS 90	FOS-Choline®-9	Zwitterionic	Alkyl phosphocholine
AS 70	n-Hexyl-ß-D-glucopyranoside	Non-Ionic	Alkyl glycoside†
AS 69	CYMAL®-2	Non-Ionic	Cycloalkyl glycoside†
AS 66	MEGA-8	Non-Ionic	Alkanoyl-N-methylglucamide*†
AS 68	HEGA®-8	Non-Ionic	Alkanoyl-N-hydroxyethylglucamide*†
AS 89	n-Decyl-N,N-dimethylglycine	Zwitterionic	Alkyl betaine
AS 67	C-HEGA®-9	Non-Ionic	Cycloalkanoyl hydroxyethylglucamide†
AS 88	FOS-Choline®-10	Zwitterionic	Alkyl phosphocholine
AS 83	ZWITTERGENT® 3-12	Zwitterionic	Alkyl sulfobetaine
AS 7	Sodium cholate	Anionic	Bile salts
AS 9	ANAPOE®-X-305	Non-Ionic	Polyoxyethylene
AS 92	CYCLOFOS™-3	Zwitterionic	Cycloalkyl phosphocholine
AS 62	CYMAL®-3	Non-Ionic	Cycloalkyl glycoside†
AS 86	CHAPS	Zwitterionic	Sulfobetaine
AS 94	ZWITTERGENT® 3-08	Zwitterionic	Alkyl sulfobetaine
AS 77	NDSB-256	Zwitterionic	Non-detergent sulfobetaine
AS 80	FOS-Choline®-12	Zwitterionic	Alkyl phosphocholine
AS 58	MEGA-9	Non-Ionic	Alkanoyl-N-methylglucamide*†
AS 87	CHAPSO	Zwitterionic	Sulfobetaine
AS 85	FOS-MEA®-10	Zwitterionic	Lipid-like (phosphoethanolamine)
AS 78	ZWITTERGENT® 3-14	Zwitterionic	Alkyl sulfobetaine
AS 64	HEGA®-9	Non-Ionic	Alkanoyl-N-hydroxyethylglucamide*†

*Fatty acid glucamide or alkanoyl glucamide; †Sugar-based surfactants

8.2 Assay Methods

Enzymatic indicators of lysosomal storage diseases (LSDs) can be identified using droplet based assays on a droplet actuator. In one embodiment, assays of the appropriate glycosidase activity may be used to detect altered activity of a particular glycosidase, which may be an indicator of a particular lysosomal storage disease. Examples of enzyme deficiencies and LSDs include, but are not limited to, the following: a deficiency in iduronate-2-sulfae sulphatase is a diagnostic indicator of Hunter disease; a deficiency in acid ß-D-glucosidase or chitotriosidase is a diagnostic indicator of Gaucher disease; a deficiency in acid sphingomyelinase or chitotriosidase is a diagnostic indicator of Niemann-Pick disease; a deficiency in α-glucosidase activity is a diagnostic indicator of Pompe disease; a deficiency in α-galactosidase activity is a diagnostic

indicator of Fabry disease; a deficiency in α-L-iduronidase is a diagnostic indicator of Hurler disease; a deficiency in heparan sulfate sulfamidase is a diagnostic indicator of Sanfilippo A (MPS IIIA); a deficiency in alpha-N-acetylglucosaminidase is a diagnostic indicator of Sanfilippo B (MPS IIIB); and a deficiency in arylsulfatase A is a diagnositic indicator of metachromatic leukodystrophy. Multiple diseases and/or multiple samples can be tested simultaneously on a single droplet actuator.

The lysosomal enzyme tests are performed in aqueous droplets within an oil filled gap of the droplet actuator. Samples and assay reagents are manipulated as discrete droplets upon an electrode array (digital electrowetting). Sample droplets are blood or blood-derived samples, such as plasma, serum, tissue, cell fractions, and treated, fractionated, concentrated and/or diluted forms of the foregoing. For example, diagnosis for Pompe disease is performed on fibroblasts. Other biological fluids may be used as samples; nonlimiting examples include tears, semen, urine, saliva, amniotic liquid and cerebrospinal fluid. For example, in the testing to diagnose Fabry disease, tears may be used as the input sample droplet. Biological fluids may be treated as necessary to prepare them for being subjected to the protocols of the invention. For example, samples may be diluted or buffered, heated or cooled; pH may be adjusted; and/or blood samples may be treated with one or more anticoagulants. In some embodiments, the sample includes a reconstituted dried blood spot and/or dried plasma spot. Samples may be loaded into a reservoir associated with a droplet actuator, and may be dispensed into one or more subsamples. In some cases, the subsamples are unit-sized subsamples. The subsamples may be in contact with or surrounded with one or more filler fluids.

Assay reagents for testing for lysosomal storage disorders (e.g., LSDs) on a droplet actuator may include any one or more of the following: reaction buffer, 4-MU enzyme substrate, supplemented secondary enzyme, assay-specific inhibitor, and stop buffer (e.g., 0.2M Sodium bicarbonate pH 10.0 with 0.01% Tween® 20). Examples of 4-MU substrates include, but are not limited to, 4-Methylumbelliferyl-α-L-Iduronide-2-Sulfate (4-MU-αIdoA-2S), Hunter substrate; 4-Methylumbelliferyl α-D-Galactopyranoside (4-MU-α Gal), Fabry substrate; 4-MU-α-D-glucopyranoside (4-MU-α-Gluc), Pompe substrate; 4-Methyumbelliferyl-β-D-Glucopyranoside (4-MU-β-Gluc), Gaucher substrate; 4-Methylumbelliferyl-α-L-Iduronide Sodium Salt (4-MU-α-Idu), Hurler substrate; 4-Trifluoromethylumbelliferylchitroside, Gaucher and Niemann-Pick substrate; 4-Methylumbelliferyl-ß-Galactose (4-MU-ß-Galactose), Morquio B substrate; 4-Methylumbelliferyl-α-N-Sulpho-D-Glucosaminide (MU-αGlcNS), Sanfilippo A (MPS IIIA)

substrate; 4-Methylumbelliferyl-α-D-N-Acetylglucosamine, Sanfilippo B (MPS IIIB); and 3-O-Sulfate-β-D-Galactosyl-4-Methylumbelliferyl), metachromatic leukodystrophy (MLD) substrate.

In one embodiment, the invention provides a 4-MU assay in which a droplet comprising assay reagents and a zwitterionic surfactant is dispensed and merged using droplet operations with a sample droplet in a droplet operations gap or on a droplet operations surface. The combined reaction droplet is split using droplet operations into 2 reaction droplets. One reaction droplet is combined using droplet operations with a stop buffer droplet. Fluorescence of the combined droplet is measured (t = 0 h). The second reaction droplet is incubated for a predetermined time and then the reaction droplet is combined with a stop buffer droplet. End point fluorescence is measured (t = END h). In this example, a single sample droplet is dispensed and analyzed. However, any number of sample droplets may be dispensed and analyzed. The concentration of zwitterionic surfactant is preferably about in the range of 1.5 times the surfactant's critical micellar concentration (CMC). Examples of suitable zwitterionic surfactants (detergents) include n-octylphosphocholine (FOS-Choline®-8), n-nonylphosphocholine (FOS-Choline®-9), n-decylphosphocholine (FOS-Choline®-10), n-dodecylphosphocholine (FOS-Choline®-12), 3-Cyclohexyl-1-propylphosphocholine (CYCLOFOS™-3), decylphospho-N-methylethanolamine (FOS-MEA®-10), n-Decyl-N,N-dimethylglycine, n-Octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (ZWITTERGENT® 3-8), n-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (ZWITTERGENT® 3-10), n-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (ZWITTERGENT® 3-12), n-Tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (ZWITTERGENT® 3-14), dimethylbenzylammonium propane sulfonate (NDSB-256), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), and 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO). The droplet operations gap or surface may be coated, filled or partially filled with a filler fluid. For example, the filler fluid may be 5 cSt Silicone oil with 0.1% Triton X15.

In another embodiment, the invention provides a 4-MU assay in which a droplet comprising assay reagents and a non-ionic surfactant is dispensed and merged using droplet operations with a sample droplet in a droplet operations gap or on a droplet operations surface. The combined reaction droplet is split using droplet operations into 2 reaction droplets. One reaction droplet is combined using droplet operations with a stop buffer droplet. Fluorescence of the combined droplet is measured (t = 0 h). The second reaction droplet is incubated for a predetermined time and then the reaction droplet is combined with a stop buffer droplet. End point fluorescence is

measured (t = END h). In this example, a single sample droplet is dispensed and analyzed. However, any number of sample droplets may be dispensed and analyzed. The concentration of non-ionic surfactant is preferably about in the range of 1.5 times the surfactant's critical micellar concentration (CMC). Examples of suitable non-ionic surfactants (detergents) include n-hexyl-ß-D-glucopyranoside, 2-cyclohexyl-1-ethyl-ß-D-maltoside (CYMAL®-2), 3-cyclohexyl-1-propyl-ß-D-maltoside (CYMAL®-3), octanoyl-N-methylglucamide (MEGA-8), nonanoyl-N-methylglucamide (MEGA-9), octanoyl-N-hydroxyethylglucamide (HEGA®-8), nonanoyl-N-hydroxyethylglucamide (HEGA®-9), n-hexyl-ß-D-glucopyranoside, and α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxy-poly(oxy-1,2-ethanediyl) (ANAPOE®X-305). The droplet operations gap or surface may be coated, filled or partially filled with a filler fluid. For example, the filler fluid may be 5 cSt Silicone oil with 0.1% Triton X15.

In yet another embodiment, the invention provides a 4-MU assay in which a droplet comprising assay reagents and an ionic surfactant is dispensed and merged using droplet operations with a sample droplet in a droplet operations gap or on a droplet operations surface. The combined reaction droplet is split using droplet operations into 2 reaction droplets. One reaction droplet is combined using droplet operations with a stop buffer droplet. Fluorescence of the combined droplet is measured (t = 0 h). The second reaction droplet is incubated for a predetermined time and then the reaction droplet is combined with a stop buffer droplet. End point fluorescence is measured (t = END h). In this example, a single sample droplet is dispensed and analyzed. However, any number of sample droplets may be dispensed and analyzed. The concentration of ionic surfactant is preferably about in the range of 1.5 times the surfactant's critical micellar concentration (CMC). Examples of suitable ionic surfactants (detergents) include cationic Benzyldimethyldodecylammonium bromide (BAM) and anionic 3α, 7α, 12α-Trihydroxy-5ß-cholan-24-oic acid, monosodium salt (Sodium cholate). The droplet operations gap or surface may be coated, filled or partially filled with a filler fluid. For example, the filler fluid may be 5 cSt Silicone oil with 0.1% Triton X15.

8.3 Droplet Actuators and Hydrophobic Coatings

Figure 3 illustrates cross section of a portion of an example of a droplet actuator 300 that has hydrophobic coatings on the droplet operations surfaces. The gap in droplet actuator 300 may be filled with perfluorinated oil. Droplet actuator 300 may include a bottom substrate 310 and a top substrate 312 that are separated by a gap 314. Bottom substrate 310 may, for example, be a

printed circuit board (PCB), plastic, silicon, glass, or other suitable material. Top substrate 312 may, for example, be formed of glass, injection-molded plastic, silicon, or other suitable material. Bottom substrate 310 may include an arrangement of droplet operations electrodes 316 (e.g., electrowetting electrodes). Droplet operations electrodes 316 are arranged in a manner that permits them to be used to mediate droplet operations using droplets in the droplet operations gap. Top substrate 312 may include a conductive layer 318. Conductive layer 318 is on the side of top substrate 312 that is facing gap 314. In one example, conductive layer 318 is formed of indium tin oxide (ITO), which is a material that is electrically conductive and substantially transparent to light. Droplet operations are conducted between droplet operations electrodes 316 and conductive layer 318. Droplet operations electrodes 316 of bottom substrate 310 are coated with a dielectric material 328. A hydrophobic layer 320 is provided atop dielectric material 328 of bottom substrate 310. A hydrophobic layer 322 is provided atop conductive layer 318 of top substrate 312.

In operation, gap 314 of droplet actuator 300 is filled or partially filled with a filler fluid 324. Filler fluid 324 may be a perfluorinated oil or solvent, such as perfluorinated silicone oil. One or more droplets 326 are provided in gap 314. Droplet 326 may be subjected to droplet operations the filler fluid 324. These droplet operations are mediated by droplet operations electrodes 316.

8.3.1 Water Contact Angle

Figures 4A and 4B illustrate water contact angle (θ) with respect to hydrophilicity and hydrophilicity, respectively. Referring to Figure 4A, a water droplet tends to spread on a hydrophilic surface. The evaluation of hydrophilicity is made through water contact angle (θ) measurements. Referring to Figure 4B, hydrophobicity refers to the physical property of a material that repels a mass of water. Because a water droplet is repelled by hydrophobic material, a water droplet tends to contact only a small area of the surface and the shape of the droplet is spherical.

8.3.2 Evaluation of CYTOP®liquid deposition and other PECVD processes

Details of examples of evaluating various chemistries and various methods of depositing hydrophobic coatings are described with reference to Tables 4, 5, and 6 and Figures 5A through 9 below. Water contact angle (θ) and hysteresis evaluations as well as droplet operations tests were

performed on M-type CYTOP® coating (an amorphous fluoropolymer) that is deposited on a substrate via a liquid deposition process. Additionally, water contact angle (θ) and hysteresis evaluations as well as droplet operations tests were performed on hydrophobic coatings deposited on a substrate via various plasma-enhanced chemical vapor deposition (PECVD) processes. PECVD is a process used to deposit thin films from a gas state (vapor) to a solid state on a substrate. For example, tests were performed using the PECVD system from Plasmatreat US LP (Elgin, IL). Other tests were performed using the PECVD system from Triton Systems, Inc. (Chelmsford, MA), which is a spray-based process. Yet other tests were performed using the PECVD system from GVD Corporation (Cambridge, MA), which is a chamber-based process. The M-type CYTOP® coating evaluation may be considered the standard against which the PECVD processes are evaluated. In all cases, the droplet operations performance is evaluated with respect to perfluorinated oil or solvent, such as silicone oil, in the gap of a droplet actuator.

Water contact angle (θ) and respective hysteresis values were determined for various treatments of a glass surface. For example, the surface of a glass slide (e.g., 25 mm x 25 mm x 1 mm thick) was treated and evaluated with respect to water contact angle (θ) and hysteresis. For each sample, a water droplet of known volume is deposited on the glass and then the water contact angle (θ) is measured using a goniometer. When liquid is added to the droplet, the volume of the droplet increases and, therefore, the water contact angle (θ) also increases. This is referred to as theta advancing (θa). Theta advancing (θa) is greater than the static water contact angle (θs), or $\theta a > \theta s$. By contrast, when liquid is removed from the droplet, the volume of the droplet decreases and, therefore, the water contact angle (θ) also decreases. This is referred to as theta receding (θr). Theta receding (θr) is less than the static water contact angle (θs), or $\theta r < \theta s$. A hysteresis value is determined by θa minus θr, or hysteresis = $\theta a - \theta r$.

Table 4 shows an example of evaluation results for five initial screening tests. The five tests are summarized as follows.

- Test #1 is M-type CYTOP® coating liquid deposition process on glass at high temperature (i.e., 120 °C). Figures 3A and 3B show droplet operations tests related to Test #1.

- Test #2 is hydrophobic coatings deposited on glass using the Plasmatreat PECVD process. Figure 4 shows a droplet operations test related Test #2.

- Test #3 is hydrophobic coatings deposited on glass using the Triton PECVD process. Figure 5 shows a droplet operations test related Test #3.

- Test #4 is hydrophobic coatings deposited on glass using the GVD PECVD process at room temperature. Figure 6 shows a droplet operations test related Test #4.

- Test #5 is hydrophobic coatings deposited on glass using the GVD PECVD process at high temperature (i.e., 125 °C). Figure 7 shows a droplet operations test related Test #5.

Table 4. Evaluation results: Water contact angle (θ) and Hysteresis values

Test	Process	Water Contact Angle (θ)	Hysteresis
#1	M-type CYTOP® coating (120 °C)	114	15
#2	Plasmatreat PECVD	85	12
#3	Triton PECVD	104	20
#4	GVD PECVD (room temp)	122	65
#5	GVD PECVD (125 °C)	150	30

Following the aforementioned tests, droplet operations performance was tested on five droplet actuators. The five droplet actuators had bottom substrates coated with M-type CYTOP® coating per the liquid deposition process. However, the respective top substrates are unique in each test, as described with reference to Figures 5A through 9. This evaluation is performed to determine whether the hydrophobic coatings perform well under standard droplet operations conditions.

Figures 5A and 5B show top down views of a portion of a droplet actuator 500 that includes M-type CYTOP® coatings applied using the liquid deposition process, which substantially corresponds to the process used in Test #1 of Table 4. Droplet actuator 500 includes bottom substrate 510. An electrode arrangement 520 is patterned on bottom substrate 510. Electrode

arrangement 520 may include, for example, various reservoir electrodes (of on-actuator reservoirs) and droplet operations electrodes. In the top down view of droplet actuator 500, bottom substrate 510 is visible through a substantially transparent top substrate, which is present but not visible. Bottom substrate 510 and the top substrate (not visible) are separated by a gap.

In this example, bottom substrate 510 is a PCB that is coated with M-type CYTOP® coating per the liquid deposition process. The top substrate (not visible) is formed of ITO-coated glass that is also coated with M-type CYTOP® coating per the liquid deposition process. The gap between bottom substrate 510 and the top substrate is filled with silicone oil.

In a test performed on droplet actuator 500, droplet operations are conducted atop the various electrodes to determine the droplet operations performance of M-type CYTOP® coating with respect to silicone oil. Figures 5A and 5B show a volume of fluid 522 at a reservoir electrode, which is the electrode of an on-actuator reservoir. A droplet 524 is dispensed from the fluid 522 at the on-actuator reservoir and transported along a line of droplet operations electrodes. Figures 5A and 5B show that a droplet of fluid is successfully dispensed from the on-actuator reservoir. Further, the size of the droplet is suitable for use in assay protocols and the droplet transported well.

Figure 6 shows a top down view of a portion of a droplet actuator 600 that includes hydrophobic coatings applied using the Plasmatreat PECVD process, which substantially corresponds to the process used in Test #2 of Table 4. Droplet actuator 600 includes bottom substrate 610. An electrode arrangement 620 is patterned on bottom substrate 610. Electrode arrangement 620 may include, for example, various reservoir electrodes (of on-actuator reservoirs) and droplet operations electrodes. In the top down view of droplet actuator 600, bottom substrate 610 is visible through a substantially transparent top substrate, which is present but not visible. Bottom substrate 610 and the top substrate (not visible) are separated by a gap.

In this example, bottom substrate 610 is a PCB that is coated with M-type CYTOP® coating per the liquid deposition process. However, the top substrate (not visible) is formed of ITO-coated glass that is coated with hydrophobic material per the Plasmatreat PECVD process. The gap between bottom substrate 610 and the top substrate is filled with silicone oil.

In a test performed on droplet actuator 600, droplet operations are conducted atop the various electrodes to determine the droplet operations performance of the hydrophobic material deposited via the Plasmatreat PECVD process with respect to silicone oil. Figure 6 shows a volume of fluid 622 at a reservoir electrode, which is the electrode of an on-actuator reservoir. A droplet 624 is dispensed from the fluid 622 at the on-actuator reservoir and transported along a line of droplet operations electrodes. Figure 6 shows that a droplet of fluid is successfully dispensed from the on-actuator reservoir. However, the size of the droplet is not suitable for use in assay protocols and the droplet did not transport well.

Figure 7 shows a top down view of a portion of a droplet actuator 700 that includes hydrophobic coatings applied using the Triton PECVD process, which substantially corresponds to the process used in Test #3 of Table 4. Droplet actuator 700 includes bottom substrate 710. An electrode arrangement 720 is patterned on bottom substrate 710. Electrode arrangement 720 may include, for example, various reservoir electrodes (of on-actuator reservoirs) and droplet operations electrodes. In the top down view of droplet actuator 700, bottom substrate 710 is visible through a substantially transparent top substrate, which is present but not visible. Bottom substrate 710 and the top substrate (not visible) are separated by a gap.

In this example, bottom substrate 710 is a PCB that is coated with M-type CYTOP® coating per the liquid deposition process. However, the top substrate (not visible) is formed of ITO-coated glass that is also coated with hydrophobic material per the Triton PECVD process. The gap between bottom substrate 710 and the top substrate is filled with silicone oil.

In a test performed on droplet actuator 700, droplet operations are conducted atop the various electrodes to determine the droplet operations performance of the hydrophobic material deposited via the Triton PECVD process with respect to silicone oil. Figure 7 shows a volume of fluid 722 at a reservoir electrode, which is the electrode of an on-actuator reservoir. A droplet 724 is dispensed from the fluid 722 at the on-actuator reservoir and transported along a line of droplet operations electrodes. Figure 7 shows that a droplet of fluid is successfully dispensed from the on-actuator reservoir. Further, the size of the droplet is suitable for use in assay protocols and the droplet transported well.

Figure 8 shows a top down view of a portion of a droplet actuator 800 that includes hydrophobic coatings applied using the GVD PECVD process at room temperature, which substantially

corresponds to the process used in Test #4 of Table 4. Droplet actuator 800 includes bottom substrate 810. An electrode arrangement 820 is patterned on bottom substrate 810. Electrode arrangement 820 may include, for example, various reservoir electrodes (of on-actuator reservoirs) and droplet operations electrodes. In the top down view of droplet actuator 800, bottom substrate 810 is visible through a substantially transparent top substrate, which is present but not visible. Bottom substrate 810 and the top substrate (not visible) are separated by a gap.

In this example, bottom substrate 810 is a PCB that is coated with M-type CYTOP® coating per the liquid deposition process. However, the top substrate (not visible) is formed of ITO-coated glass that is also coated with hydrophobic material per the GVD PECVD process at room temperature. The gap between bottom substrate 810 and the top substrate is filled with silicone oil.

In a test performed on droplet actuator 800, droplet operations are conducted atop the various electrodes to determine the droplet operations performance of the hydrophobic material deposited via the GVD PECVD process at room temperature with respect to silicone oil. Figure 8 shows a volume of fluid 822 at a reservoir electrode, which is the electrode of an on-actuator reservoir. A droplet 824 is dispensed from the fluid 822 at the on-actuator reservoir and transported along a line of droplet operations electrodes. Figure 8 shows that a droplet of fluid is successfully dispensed from the on-actuator reservoir. Further, the size of the droplet is suitable for use in assay protocols and the droplet transported well.

Figure 9 shows a top down view of a portion of a droplet actuator 900 that includes hydrophobic coatings applied using the GVD PECVD process at high temperature (e.g., about 125 °C), which substantially corresponds to the process used in Test #5 of Table 4. Droplet actuator 900 includes bottom substrate 910. An electrode arrangement 920 is patterned on bottom substrate 910. Electrode arrangement 920 may include, for example, various reservoir electrodes (of on-actuator reservoirs) and droplet operations electrodes. In the top down view of droplet actuator 900, bottom substrate 910 is visible through a substantially transparent top substrate, which is present but not visible. Bottom substrate 910 and the top substrate (not visible) are separated by a gap.

In this example, bottom substrate 910 is a PCB that is coated with M-type CYTOP® coating per the liquid deposition process. However, the top substrate (not visible) is formed of ITO-coated glass that is also coated with hydrophobic material per the GVD PECVD process at high

temperature. The gap between bottom substrate 910 and the top substrate is filled with silicone oil.

In a test performed on droplet actuator 900, droplet operations are conducted atop the various electrodes to determine the droplet operations performance of the hydrophobic material deposited via the GVD PECVD process at high temperature with respect to silicone oil. Figure 9 shows a volume of fluid 922 at a reservoir electrode, which is the electrode of an on-actuator reservoir. A droplet 924 is dispensed from the fluid 922 at the on-actuator reservoir and transported along a line of droplet operations electrodes. Figure 9 shows that a droplet of fluid is successfully dispensed from the on-actuator reservoir. Further, the size of the droplet is suitable for use in assay protocols and the droplet transported well.

Referring again to Table 4 and Figures 5A through 9, the samples having a water contact angle (θ) greater than about 100 correlate to the samples having the most suitable droplet operations performance. For example, the samples of Tests #1, #3, #4, and #5 have water contact angles (θ) greater than about 100, while the corresponding droplet actuators 300, 500, 600, and 700, respectively, demonstrated suitable droplet operations performance. By contrast, the sample of Test #2 has a water contact angle (θ) of about 85 and the corresponding droplet actuator 400 did not demonstrate suitable droplet operations performance. Therefore, in certain circumstances, the Plasmatreat PECVD process may be a less desirable process for depositing hydrophobic material on a droplet actuator.

Additionally, while the hydrophobic material deposited via the GVD PECVD process at room temperature and at high temperature provides a good hydrophobic surface, the hydrophobic surface is brittle. Therefore, in certain circumstances, the GVD PECVD process may not be a preferred process for depositing hydrophobic material on a droplet actuator. As a result, the M-type CYTOP® coating liquid deposition process and the Triton PECVD process may be a less desirable processes for depositing hydrophobic material on a droplet actuator.

8.3.3 Evaluation of Solubility

With respect to the M-type CYTOP® coating liquid deposition process, currently the M-type CYTOP® coating is cured at about 93 °C for 1 hour. Tests were performed to evaluate the water contact angle (θ) and hysteresis when M-type CYTOP® coating samples are cured at up to about

200 °C, before and after exposure to Galden® HT170 fluid (a perfluoropolyether) for about 2 hours. The Galden® HT170 fluid is an example of a specific brand of perfluorinated solvent that is available from Solvay Solexis (West Deptford, NJ). M-type CYTOP® coating samples were prepared and cured at various temperatures in order to evaluate the chemical stability to Galden® HT170 fluid. The preference is that the high temperature cure results in little or no change in water contact angle (θ) and hysteresis. Table 5 shows chemical stability results of samples treated with M-type CYTOP® coating process vs. GVD PECVD process vs. Triton PECVD process before and after exposure to Galden® HT170 fluid.

Table 5. Chemical stability results of samples treated with M-type CYTOP® coating process vs. GVD process vs. Triton process before and after exposure to Galden® HT170 fluid

	M-type CYTOP®(LT)	**M-type CYTOP®(MT)**	**M-type CYTOP®(HT)**	**M-type CYTOP®(XHT)**	**GVD (HT)**	**Triton**
Cure Temp (°C)/Time (minutes)	93/60	120/60	80/30 + 160/30	80/30 + 200/60	125 °C	N/A
Thickness (nanometers)	200	200	400	200	250	200
*Water contact angle (Θ) before exposure to HT170 @ 60 °C for 2 hrs	113/1	113/1	112/1	113/2	146/3	104/1
*Hysteresis before exposure to HT170 @ 60 °C for 2 hrs	12/1	12/1	12/1	13/1	35/2	22/2

*Water contact angle (Θ) after exposure to HT170 @ 60 °C for 2 hrs	107/4	113/1	112/1	110/5	138/3	104/1
*Hysteresis after exposure to HT170 @ 60 °C for 2 hrs	12/1	18/2	15/3	15/2	37/6	23/0
* xx/x means value/standard deviation						

In each test, a droplet was deposited on the glass slide and the water contact angle (θ) and hysteresis were measured. In the process of testing the samples before and after exposure to Galden® HT170 fluid, the samples were washed with isopropyl alcohol between runs.

Referring to Table 5, the test results show a slight decrease in water contact angle (θ) after exposure to Galden® HT170 fluid. The HT170 did not affect the droplet operations performance for M-type CYTOP® coating cured at low temperature (93 °C), mid-temperature (120 °C), and high temperature (160 °C).

Referring now to Table 6, tests were performed before and after exposure to Novec™ 7500 engineered fluid (available from 3M of St. Paul, MN) for about 2 hours. M-type CYTOP® coating samples were prepared and cured at various temperatures in order to evaluate the chemical stability to the Novec™ 7500 fluid.

Table 6. Chemical stability results of samples treated with M-type CYTOP® coating process before and after exposure to Novec™ 7500 fluid

	M-type CYTOP®(LT)	**M-type CYTOP®(MT)**	**M-type CYTOP®(HT)**	**M-type CYTOP®(XHT)**
Cure Temp (ºC)/Time (minutes)	93/60	120/60	80/30 + 160/30	80/30 + 200/60
Thickness (nanometers)	200	200	400	200
*Water contact angle (Θ) before exposure to Novec™ 7500 @ 60 ºC for 2 hrs	113/1	113/1	112/1	113/2
*Hysteresis before exposure to Novec™ 7500 @ 60 ºC for 2 hrs	12/1	12/1	12/1	13/1
*Water contact angle (Θ) after exposure to Novec™ 7500 @ 60 ºC for 2 hrs	102/1	102/3	109/3	111/2
*Hysteresis after exposure to Novec™ 7500 @ 60 ºC for 2 hrs	41/6	37/7	15/4	13/4

* xx/x means value/standard deviation

Referring again to Tables 4 and 5, with respect to the M-type CYTOP® coating liquid deposition process, the extra-high temperature process (i.e., 160 °C to 200 °C) for about 1 hour may be the preferred process. Optionally, the high temperature cure step and/or the extra-high temperature cure step may be preceded by a prebake step at 80 °C for about 30 minutes.

8.4 Systems

Figure 10 illustrates a functional block diagram of an example of a microfluidics system 1000 that includes a droplet actuator 1005. Digital microfluidic technology conducts droplet operations on discrete droplets in a droplet actuator, such as droplet actuator 1005, by electrical control of their surface tension (electrowetting). The droplets may be sandwiched between two substrates of droplet actuator 1005, a bottom substrate and a top substrate separated by a droplet operations gap. The bottom substrate may include an arrangement of electrically addressable electrodes. The top substrate may include a reference electrode plane made, for example, from conductive ink or indium tin oxide (ITO). The bottom substrate and the top substrate may be coated with a hydrophobic material. Droplet operations are conducted in the droplet operations gap. The space around the droplets (i.e., the gap between bottom and top substrates) may be filled with an immiscible inert fluid, such as silicone oil, to prevent evaporation of the droplets and to facilitate their transport within the device. Other droplet operations may be effected by varying the patterns of voltage activation; examples include merging, splitting, mixing, and dispensing of droplets.

Droplet actuator 1005 may be designed to fit onto an instrument deck (not shown) of microfluidics system 1000. The instrument deck may hold droplet actuator 1005 and house other droplet actuator features, such as, but not limited to, one or more magnets and one or more heating devices. For example, the instrument deck may house one or more magnets 1010, which may be permanent magnets. Optionally, the instrument deck may house one or more electromagnets 1015. Magnets 1010 and/or electromagnets 1015 are positioned in relation to droplet actuator 1005 for immobilization of magnetically responsive beads. Optionally, the positions of magnets 1010 and/or electromagnets 1015 may be controlled by a motor 1020. Additionally, the instrument deck may house one or more heating devices 1025 for controlling

the temperature within, for example, certain reaction and/or washing zones of droplet actuator 1005. In one example, heating devices 1025 may be heater bars that are positioned in relation to droplet actuator 1005 for providing thermal control thereof.

A controller 1030 of microfluidics system 1000 is electrically coupled to various hardware components of the invention, such as droplet actuator 1005, electromagnets 1015, motor 1020, and heating devices 1025, as well as to a detector 1035, an impedance sensing system 1040, and any other input and/or output devices (not shown). Controller 1030 controls the overall operation of microfluidics system 1000. Controller 1030 may, for example, be a general purpose computer, special purpose computer, personal computer, or other programmable data processing apparatus. Controller 1030 serves to provide processing capabilities, such as storing, interpreting, and/or executing software instructions, as well as controlling the overall operation of the system. Controller 1030 may be configured and programmed to control data and/or power aspects of these devices. For example, in one aspect, with respect to droplet actuator 1005, controller 1030 controls droplet manipulation by activating/deactivating electrodes.

In one example, detector 1035 may be an imaging system that is positioned in relation to droplet actuator 1005. In one example, the imaging system may include one or more light-emitting diodes (LEDs) (i.e., an illumination source) and a digital image capture device, such as a charge-coupled device (CCD) camera.

Impedance sensing system 1040 may be any circuitry for detecting impedance at a specific electrode of droplet actuator 1005. In one example, impedance sensing system 1040 may be an impedance spectrometer. Impedance sensing system 1040 may be used to monitor the capacitive loading of any electrode, such as any droplet operations electrode, with or without a droplet thereon. For examples of suitable capacitance detection techniques, see Sturmer et al., International Patent Publication No. WO/2008/101194, entitled "Capacitance Detection in a Droplet Actuator," published on Aug. 21, 2008; and Kale et al., International Patent Publication No. WO/2002/080822, entitled "System and Method for Dispensing Liquids," published on Oct. 17, 2002; the entire disclosures of which are incorporated herein by reference.

Droplet actuator 1005 may include disruption device 1045. Disruption device 1045 may include any device that promotes disruption (lysis) of materials, such as tissues, cells and spores in a droplet actuator. Disruption device 1045 may, for example, be a sonication mechanism, a heating

mechanism, a mechanical shearing mechanism, a bead beating mechanism, physical features incorporated into the droplet actuator 1005, an electric field generating mechanism, a thermal cycling mechanism, and any combinations thereof. Disruption device 1045 may be controlled by controller 1030.

It will be appreciated that various aspects of the invention may be embodied as a method, system, computer readable medium, and/or computer program product. Aspects of the invention may take the form of hardware embodiments, software embodiments (including firmware, resident software, micro-code, etc.), or embodiments combining software and hardware aspects that may all generally be referred to herein as a "circuit," "module" or "system." Furthermore, the methods of the invention may take the form of a computer program product on a computer-usable storage medium having computer-usable program code embodied in the medium.

Any suitable computer useable medium may be utilized for software aspects of the invention. The computer-usable or computer-readable medium may be, for example but not limited to, an electronic, magnetic, optical, electromagnetic, infrared, or semiconductor system, apparatus, device, or propagation medium. The computer readable medium may include transitory and/or non-transitory embodiments. More specific examples (a non-exhaustive list) of the computer-readable medium would include some or all of the following: an electrical connection having one or more wires, a portable computer diskette, a hard disk, a random access memory (RAM), a read-only memory (ROM), an erasable programmable read-only memory (EPROM or Flash memory), an optical fiber, a portable compact disc read-only memory (CD-ROM), an optical storage device, a transmission medium such as those supporting the Internet or an intranet, or a magnetic storage device. Note that the computer-usable or computer-readable medium could even be paper or another suitable medium upon which the program is printed, as the program can be electronically captured, via, for instance, optical scanning of the paper or other medium, then compiled, interpreted, or otherwise processed in a suitable manner, if necessary, and then stored in a computer memory. In the context of this document, a computer-usable or computer-readable medium may be any medium that can contain, store, communicate, propagate, or transport the program for use by or in connection with the instruction execution system, apparatus, or device.

Program code for carrying out operations of the invention may be written in an object oriented programming language such as Java, Smalltalk, C++ or the like. However, the program code for carrying out operations of the invention may also be written in conventional procedural

programming languages, such as the "C" programming language or similar programming languages. The program code may be executed by a processor, application specific integrated circuit (ASIC), or other component that executes the program code. The program code may be simply referred to as a software application that is stored in memory (such as the computer readable medium discussed above). The program code may cause the processor (or any processor-controlled device) to produce a graphical user interface ("GUI"). The graphical user interface may be visually produced on a display device, yet the graphical user interface may also have audible features. The program code, however, may operate in any processor-controlled device, such as a computer, server, personal digital assistant, phone, television, or any processor-controlled device utilizing the processor and/or a digital signal processor.

The program code may locally and/or remotely execute. The program code, for example, may be entirely or partially stored in local memory of the processor-controlled device. The program code, however, may also be at least partially remotely stored, accessed, and downloaded to the processor-controlled device. A user's computer, for example, may entirely execute the program code or only partly execute the program code. The program code may be a stand-alone software package that is at least partly on the user's computer and/or partly executed on a remote computer or entirely on a remote computer or server. In the latter scenario, the remote computer may be connected to the user's computer through a communications network.

The invention may be applied regardless of networking environment. The communications network may be a cable network operating in the radio-frequency domain and/or the Internet Protocol (IP) domain. The communications network, however, may also include a distributed computing network, such as the Internet (sometimes alternatively known as the "World Wide Web"), an intranet, a local-area network (LAN), and/or a wide-area network (WAN). The communications network may include coaxial cables, copper wires, fiber optic lines, and/or hybrid-coaxial lines. The communications network may even include wireless portions utilizing any portion of the electromagnetic spectrum and any signaling standard (such as the IEEE 802 family of standards, GSM/CDMA/TDMA or any cellular standard, and/or the ISM band). The communications network may even include powerline portions, in which signals are communicated via electrical wiring. The invention may be applied to any wireless/wireline communications network, regardless of physical componentry, physical configuration, or communications standard(s).

Certain aspects of invention are described with reference to various methods and method steps. It will be understood that each method step can be implemented by the program code and/or by machine instructions. The program code and/or the machine instructions may create means for implementing the functions/acts specified in the methods.

The program code may also be stored in a computer-readable memory that can direct the processor, computer, or other programmable data processing apparatus to function in a particular manner, such that the program code stored in the computer-readable memory produce or transform an article of manufacture including instruction means which implement various aspects of the method steps.

The program code may also be loaded onto a computer or other programmable data processing apparatus to cause a series of operational steps to be performed to produce a processor/computer implemented process such that the program code provides steps for implementing various functions/acts specified in the methods of the invention.

9 Concluding Remarks

The foregoing detailed description of embodiments refers to the accompanying drawings, which illustrate specific embodiments of the invention. Other embodiments having different structures and operations do not depart from the scope of the present invention. The term "the invention" or the like is used with reference to certain specific examples of the many alternative aspects or embodiments of the applicants' invention set forth in this specification, and neither its use nor its absence is intended to limit the scope of the applicants' invention or the scope of the claims. This specification is divided into sections for the convenience of the reader only. Headings should not be construed as limiting of the scope of the invention. The definitions are intended as a part of the description of the invention. It will be understood that various details of the present invention may be changed without departing from the scope of the present invention. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

The Claims

We claim:

1. A method of conducting an assay, the method comprising:

   (a) incubating a droplet in oil, the droplet comprising an umbelliferone substrate, a sample potentially comprising an enzyme which cleaves the umbelliferone substrate, and a zwitterionic surfactant; and

   (b) detecting a signal emitted from the droplet.

2. The method of claim 1 wherein the droplet further comprises a cyclodextrin compound.

3. The method of any of claims 2 and following wherein the cyclodextrin compound is selected from the group consisting of α-cyclodextrins, β-cyclodextrins, and γ-cyclodextrins, and analogs and derivatives of the foregoing.

4. The method of any of claims 2 and following wherein the umbilliferone substrate is selected from the group consisting of alkylumbelliferyl-α-L-iduronides, 4-methylumbelliferyl-α-L-iduronide, 4-methylumbelliferyl-α-L-iduronide-2-sulfate, 4-methylumbelliferyl-α-L-idopyranosiduronic acid, 4-methylumbelliferyl-α-L-fucoside, 4-methylumbelliferyl-α-L-mannoside, 4-methylumbelliferyl-β-D-mannoside, 4-methylumbelliferyl β-D-N-acetylglucosaminide, 4-methylumbelliferyl β-D-N-acetylglucosaminide sulfate, alkylumbelliferyl-β-D-glycosides, methylumbelliferyl-β-D-glycosides, 4-methylumbelliferyl-α-D-galactoside, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-β-D-glucouronic acid, phenolphthalein-β-D-glucuronic acid, ethylumbelliferyl-β-D-glycosides, multifluoroethylumbelliferyl-β-D-glycosides, pentafluoroethylumbelliferyl-β-D-glycosides, pentafluoroethylumbelliferyl-β-D-glucoside, umbelliferylchiotriosides, 4-alkyumbelliferylchiotrioside, 4-methylumbelliferylchiotrioside, 4-methylumbelliferyl-β-galactose, 4-alkyumbeliferrone phosphates, 4-methylumbeliferrone phosphate, 6-alkanoylamido-4-methylumbelliferones, substrates comprising a 4-methyllumbelliferyl group, 6-hexadecanoylamido-4-methylumbelliferone, 4-methyllumbelliferyl-β-D-glucosaminide, 4-methylumbelliferyl-

α-neuraminic acid, 4-methylumbelliferyl-α-D-N-acetylgalactosaminide, and their functional analogs and derivatives.

5. The method of any of claims 1 and following, wherein the steps are performed within droplets controlled by a droplet actuator.

6. The method of any of claims 5 and following, wherein the droplet actuator controls the steps using electrode mediated droplet operations.

7. The method of any of claims 5 and following, wherein the droplet actuator controls the steps using electrowetting mediated droplet operations.

8. The method of any of claims 5 and following, wherein the droplet actuator controls the steps using dielectrophoresis mediated droplet operations.

9. A method of conducting a droplet-based enzyme assay, the method comprising:

(a) providing an immiscible fluid comprising:

(i) a sample droplet comprising an enzyme of interest; and

(ii) one or more reagent droplets comprising:

(1) a substrate which is potentially modified in the presence of the enzyme yielding one or more signal-producing products;

(2) a zwitterionic surfactant; and

(3) optionally, other reagents sufficient to produce activity of the target enzyme;

(b) combining the sample droplet and the one or more reagent droplets in the immiscible fluid to yield a reaction droplet effecting an enzyme reaction in the immiscible fluid; and

(c) measuring any signal produced by the one or more signal producing products.

10. The method of any of claims 1 and following or 9 and following, wherein the zwitterionic surfactant is selected form the group consisting of n-Octylphosphocholine, n-Nonylphosphocholine, n-decylphosphocholine, n-dodecylphosphocholine, 3-cyclohexyl-1-propylphosphocholine, decylphospho-N-methylethanolamine, n-decyl-N,N-dimethylglycine, n-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, n-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, n-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, n-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, dimethylbenzylammonium propane sulfonate, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate, and 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate.

11. A method of providing diagnostic information, the method comprising:

(a) conducting an assay according to the method of any of claims 9 and following, wherein the sample droplet comprises a clinical sample from a subject, the sample comprising the enzyme of interest; and

(b) providing to the subject diagnostic information based on the activity of the enzyme of interest from the human clinical sample.

12. The method of claim 11 wherein the diagnostic information comprises information diagnostically relevant to a glycogen storage disease.

13. The method of claim 11 wherein the assay is conducted and the diagnostic information is provided at a point of sample collection.

14. The method of claim 13 wherein the point of sample collection is in the presence of the subject.

15. The method of claim 11 wherein the clinical sample comprises a sample substance selected from the group consisting of: blood, plasma, serum, tears, saliva, and urine.

16. The method of claim 11 wherein the clinical sample comprises a dried blood sample.

17. The method of claim 11 wherein the clinical sample comprises a fresh blood sample.

18. The method of claim 17 wherein the fresh blood sample is collected from the subject and immediately loaded onto a droplet actuator for conducting the assay.

19. The method of claim 11 wherein time from collection of the blood sample to providing diagnostic information is less than about 12 hours.

20. The method of claim 11 wherein time from collection of the blood sample to providing diagnostic information is less than about 6 hours.

21. The method of claim 11 wherein the clinical sample comprises a human clinical sample.

22. The method of claim 11 wherein the clinical sample comprises a non-human animal clinical sample.

23. The method of any of claims 1 and following or 9 and following, wherein the substrate comprises a glycoside substrate.

24. The method of any of claims 1 and following or 9 and following, wherein the substrate releases a fluorophore upon contact with the enzyme of interest.

25. The method of claim 24 wherein two or more assays are conducted simultaneously using different fluorophores for each enzyme tested.

26. The method of claim 24 wherein the fluorophore comprises 4-methylumbelliferyl.

27. The method of any of claims 1 and following or 9 and following, wherein the substrate comprises a glycoside substrate which releases a fluorophore upon contact with the enzyme of interest.

28. The method of any of claims 1 and following or 9 and following, wherein the substrate comprises a glycoside substrate comprising glucose, galactose, fucose, mannose, sialic acid, hexose, hexosamine and/or N-acetylated hexosamine.

29. The method of claim 28 wherein the substrate comprises a 4-methylumbelliferyl glycoside.

30. The method of any of claims 1 and following or 9 and following, further comprising reducing or eliminating reaction contaminants associated with the substrate prior to yielding the assay droplet.

31. The method of claim 30 wherein the reducing or eliminating reaction contaminants comprises photobleaching the substrate prior to yielding the assay droplet.

32. The method of claim 39 wherein the photobleaching is effected prior to providing the droplet comprising the substrate on the droplet actuator.

33. The method of claim 39 wherein the photobleaching is effected after to providing the droplet comprising the substrate on the droplet actuator.

34. The method of any of claims 1 and following or 9 and following, wherein the substrate comprises a 4-methylumbelliferyl glycoside substrate.

35. The method of claim 34 further comprising photobleaching the substrate prior to yielding the assay droplet.

36. The method of any of claims 9 and following, wherein the immiscible liquid comprises a filler fluid.

37. The method of any of claims 9 and following, wherein the immiscible liquid comprises a silicone oil.

38. The method of claim 37 wherein the filler fluid comprises a surfactant.

39. The method of claim 38 wherein the surfactant comprises nonionic low hydrophile-lipophile balanced (HLB) surfactant.

40. The method of claim 39 wherein the HLB is less than about 10.

41. The method of claim 39 wherein the HLB is less than about 5.

42. The method of claim 39 wherein the surfactant is selected from the group consisting of Triton X-15; Span 85; Span 65; Span 83; Span 80; Span 60; and fluorinated surfactants.

43. The method of any of claims 9 and following, wherein:

(a) the sample droplet comprises a reconstituted blood sample;

(b) the blood sample is reconstituted using a single universal reconstitution solution;

(c) the blood sample is divided to yield two or more reaction droplets; and

(d) two or more of the reaction droplets are each combined with one or more sets of one or more reagent droplets, each such set comprising reagents selected for establishing reaction conditions for a different enzyme assay.

44. The method of claim 43 wherein the universal reconstitution solution comprises a saline solution.

45. The method of claim 43 wherein the universal reconstitution solution comprises water.

46. The method of any of claims 9 and following, wherein the enzyme assay is selected to provide diagnostic information about an enzyme deficiency.

47. The method of claim 46 wherein the enzyme deficiency is selected from lysosomal storage diseases.

48. The method of claim 46 wherein the enzyme deficiency is selected from the group consisting of Pompe, Niemann-Pick, Fabry, Krabbe, and Gaucher.

49. The method of claim 46 further comprising providing therapeutic treatment to a subject based on the diagnostic information.

50. The method of any of claims 9 and following, wherein the sample droplet comprising an enzyme of interest comprises cultured cells and/or supernatant from a cell culture.

51. The method of any of claims 9 and following, wherein the substrate is selected from the group consisting of 4-methylumbelliferyl-α-L-iduronide, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-β-D-glucuronic acid, 4-methylumbelliferyl-α-L-fucoside, 4-methylumbelliferyl-α-mannoside, 4-methylumbelliferyl-β-D-mannoside, 4-nitrocathecol sulfate, 4-methylumbelliferyl-β-D-N-acetylglucosaminide, 4-methylumbelliferyl-β-D-N-acetylglucosaminide sulfate, 4-methylumbelliferyl-β-D-glucosaminide, 4-methylumbelliferyl-α-D-galactoside, 4-methylumbelliferyl-α-D-neuraminic acid, 4-methylumbelliferyl-α-D-N-acetylgalactosaminide, phenolphthalein β-D-glucuronic acid, and mixtures and derivatives thereof.

52. The method of any of claims 9 and following, wherein the substrate comprises a fluorophoric moiety.

53. The method of claim 52 wherein the fluorophoric moiety comprises 4-methyllumbelliferyl.

54. The method of any of claims 9 and following, wherein the substrate comprises a chromophoric moiety.

55. The method of claim 54 wherein the chromophoric moiety comprises 4-nitrocathecol or phenolphthalein.

56. The method of any of claims 9 and following, wherein the substrate comprises a radioactive moiety.

57. The method of claim 56 wherein the radioactive moiety comprises ^{14}C sphingomyeline or ^{3}H galactosylceramide.

58. The method of any of claims 9 and following, comprising incubating the reaction droplet for a period of less than about 12 hours.

59. A method of conducting an assay in a droplet in oil, the assay comprising a lipophilic moiety, the method comprising including in the droplet a nonionic surfactant selected from the group consisting of n-hexyl-ß-D-glucopyranoside, 2-cyclohexyl-1-ethyl-ß-D-maltoside, 3-cyclohexyl-1-propyl-ß-D-maltoside, octanoyl-N-methylglucamide, nonanoyl-N-methylglucamide, octanoyl-N-hydroxyethylglucamide, nonanoyl-N-hydroxyethylglucamide, n-hexyl-ß-D-glucopyranoside, and α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxy-poly(oxy-1,2-ethanediyl).

60. The method of claim 59 wherein the lipophilic moiety comprises an umbilliferone substrate.

61. The method of claim 60 wherein umbilliferone substrate is selected from the group consisting of alkylumbelliferyl-α-L-iduronides, 4-methylumbelliferyl-α-L-iduronide, 4-methylumbelliferyl-α-L-iduronide-2-sulfate, 4-methylumbelliferyl-α-L-idopyranosiduronic acid, 4-methylumbelliferyl-α-L-fucoside, 4-methylumbelliferyl-α-L-mannoside, 4-methylumbelliferyl-β-D-mannoside, 4-methylumbelliferyl β-D-N-acetylglucosaminide, 4-methylumbelliferyl β-D-N-acetylglucosaminide sulfate, alkylumbelliferyl-β-D-glycosides, methylumbelliferyl-β-D-glycosides, 4-methylumbelliferyl-α-D-galactoside, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-β-D-glucouronic acid, phenolphthalein-β-D-glucuronic acid, ethylumbelliferyl-β-D-glycosides, multifluoroethylumbelliferyl-β-D-glycosides, pentafluoroethylumbelliferyl-β-D-glycosides, pentafluoroethylumbelliferyl-β-D-glucoside, umbelliferylchiotriosides, 4-alkyumbelliferylchiotrioside, 4-methylumbelliferylchiotrioside, 4-methylumbelliferyl-β-galactose, 4-alkyumbeliferrone phosphates, 4-methylumbeliferrone phosphate, 6-alkanoylamido-4-methylumbelliferones, substrates comprising a 4-methyllumbelliferyl group, 6-hexadecanoylamido-4-methylumbelliferone, 4-methyllumbelliferyl-β-D-glucosaminide, 4-methylumbelliferyl-α-neuraminic acid, 4-methylumbelliferyl-α-D-N-acetylgalactosaminide, and their functional analogs and derivatives.

62. A droplet actuator comprising:

(a) one or more substrates arranged to form a droplet operations gap;

(b) a fluoropolymer surface on one or more droplet operations gap-facing surfaces of the one or more substrates, where the fluoropolymer surface is deposited using a plasma-enhanced chemical vapor deposition process;

(c) a perfluorinated oil filler fluid in the droplet operations gap.

63. The method of claim 62 wherein the fluoropolymer surface comprises an amorphous fluoropolymer.

64. The method of claim 62 wherein the fluoropolymer surface comprises an amorphous fluoropolymer comprising carboxyl end-groups.

65. The method of claim 62 wherein the fluoropolymer surface comprises an amorphous fluoropolymer comprising amino-silane coupling agents.

66. The method of claim 62 wherein the fluoropolymer surface comprises an amorphous fluoropolymer comprising perfluoro groups.

67. The method of claim 62 wherein the fluoropolymer surface comprises CYTOP® Type A.

68. The method of claim 62 wherein the fluoropolymer surface comprises CYTOP® Type M.

69. The method of claim 62 wherein the fluoropolymer surface comprises CYTOP® or Type S.

70. The method of any of claims 62 and following, wherein the one or more substrates comprise one or more electrodes arranged for conducting droplet operations.

71. The method of any of claims 62 and following, wherein the one or more substrates comprise an arrangement of electrodes arranged for conducting electrowetting-mediated droplet operations.

72. The method of any of claims 62 and following, wherein the one or more substrates comprises a printed circuit board substrate.

73. The method of any of claims 62 and following, wherein the one or more substrates comprises a silicone substrate.

74. The method of any of claims 62 and following, wherein the one or more substrates comprises a glass substrate.

75. The method of any of claims 62 and following, comprising a droplet in the droplet operations gap, the droplet comprising a lipophilic substance.

76. The method of any of claims 62 and following, wherein the water contact angle (θ) of the surface is greater than about 100.

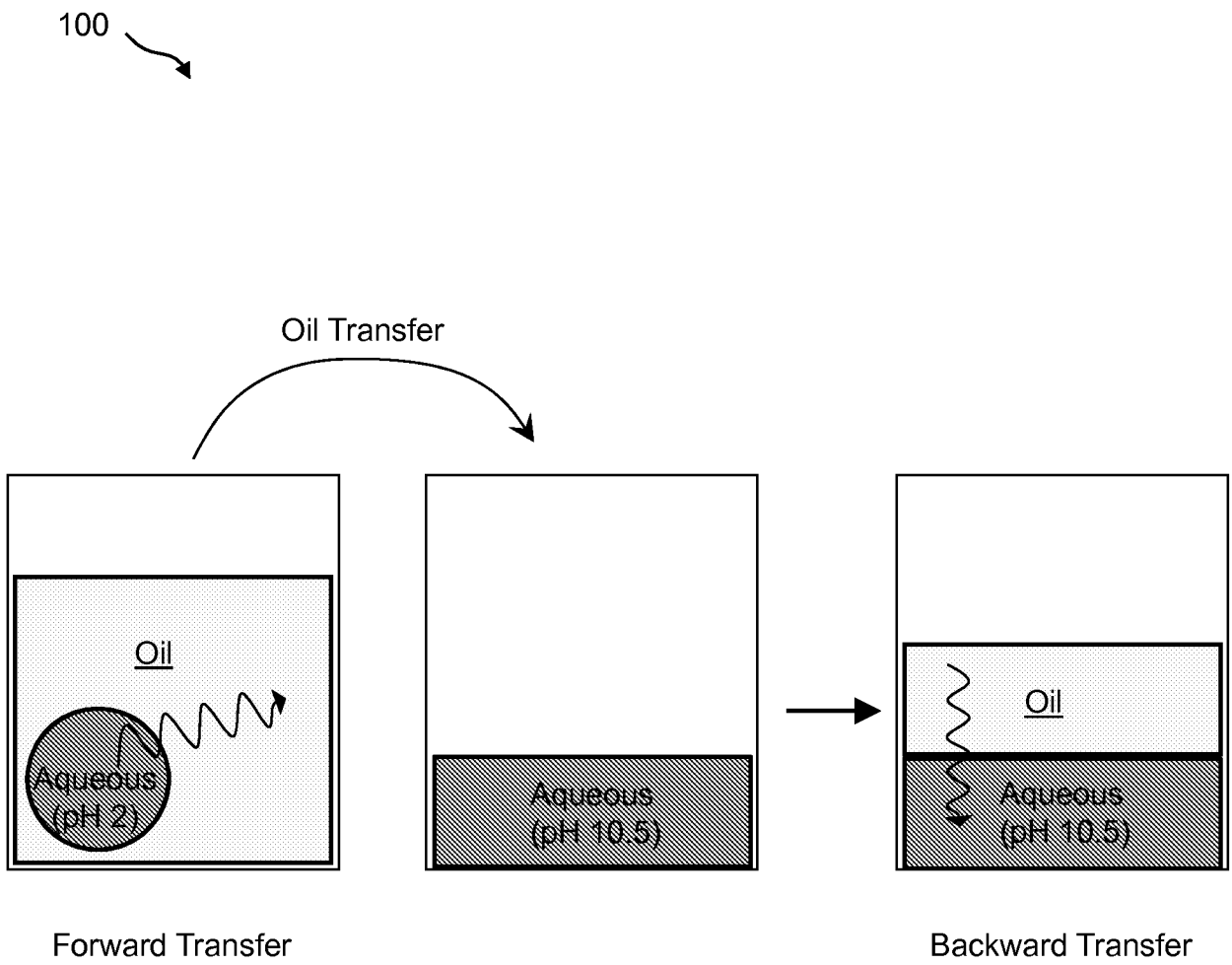

100
Oil Transfer
Oil
Aqueous
(pH 2)
Aqueous
(pH 10.5)
Oil
Aqueous
(pH 10.5)
Forward Transfer
Backward Transfer

Figure 1

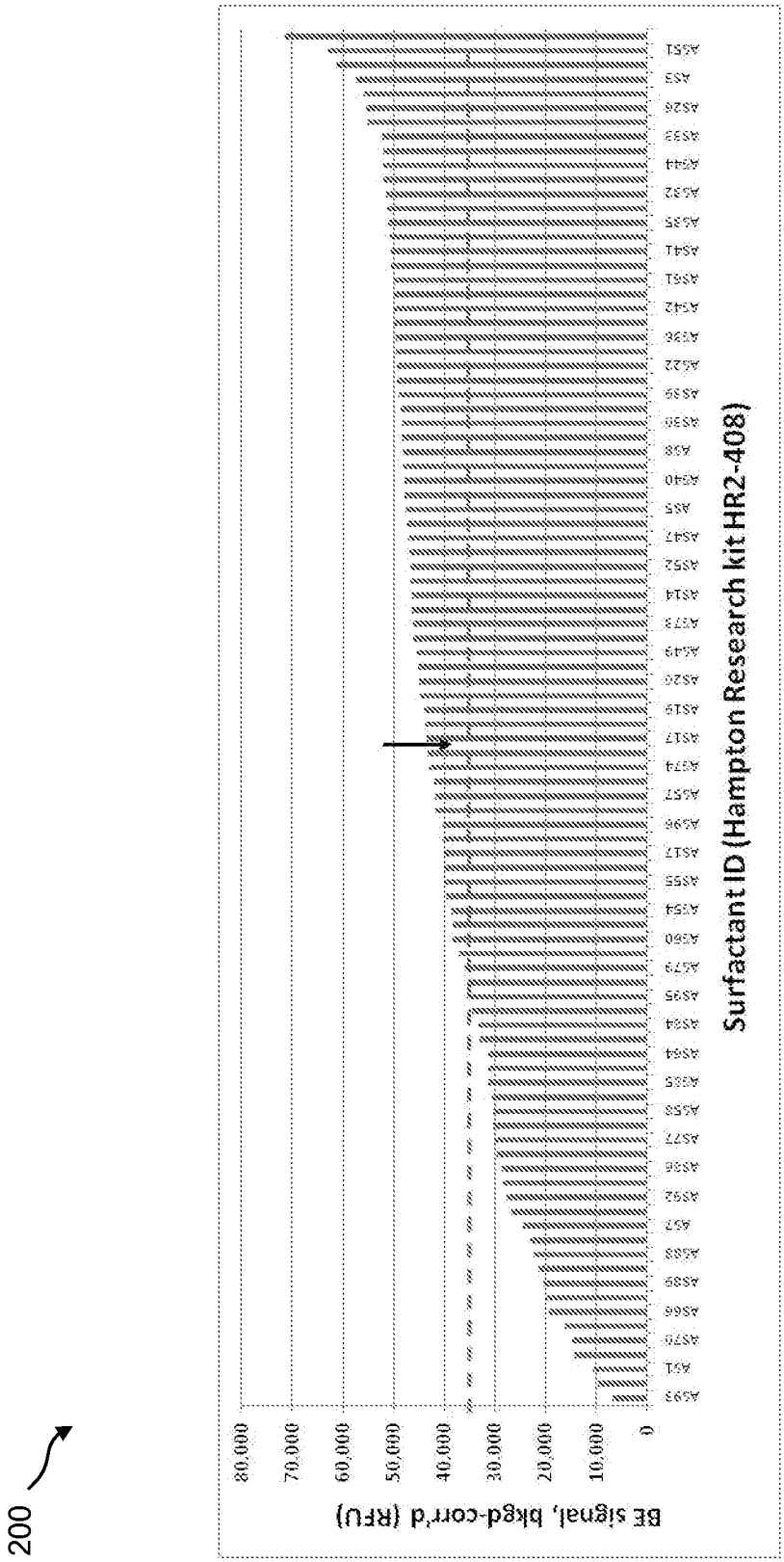

200
BE signal, bkgd-corr'd (RFU)
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
Surfactant ID (Hampton Research kit HR2-408)

Figure 2

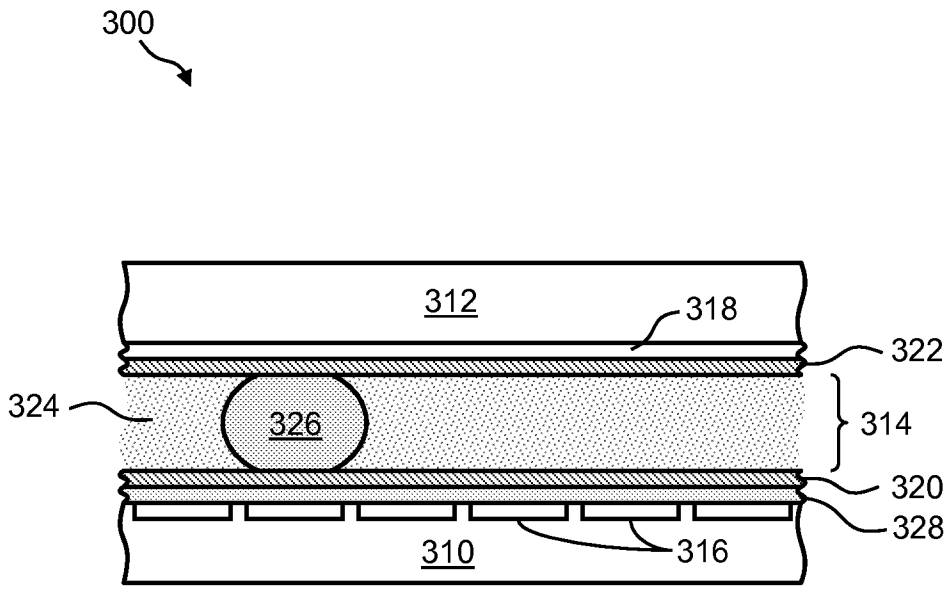
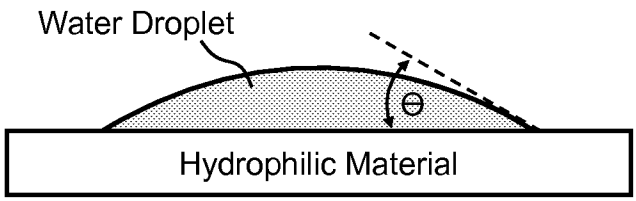
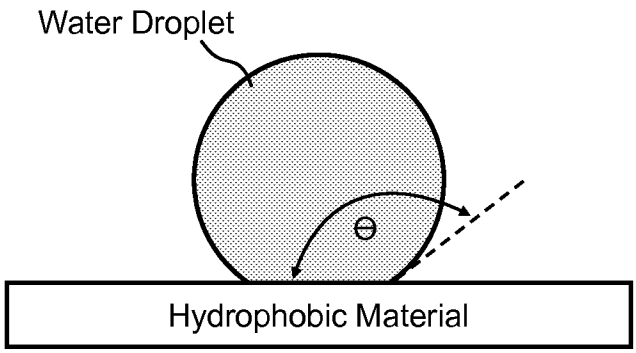


Figure 3



Water Contact Angle (Θ) → 0º

Figure 4A



Water Contact Angle (Θ) > 90º

Figure 4B

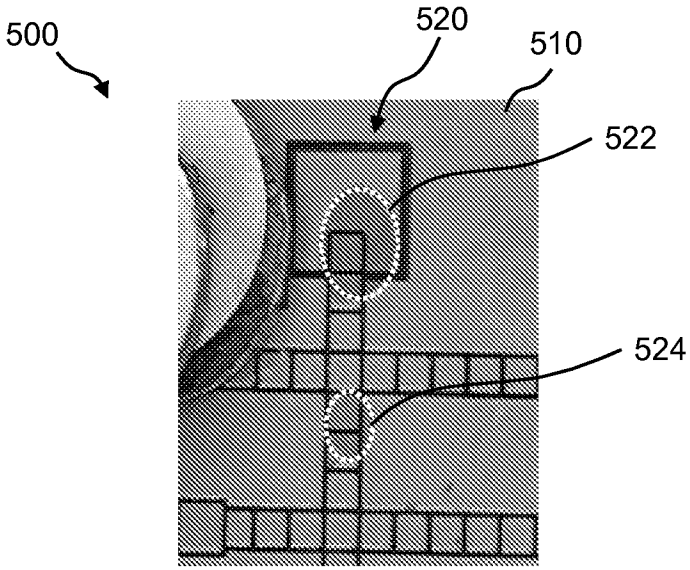


Figure 5A

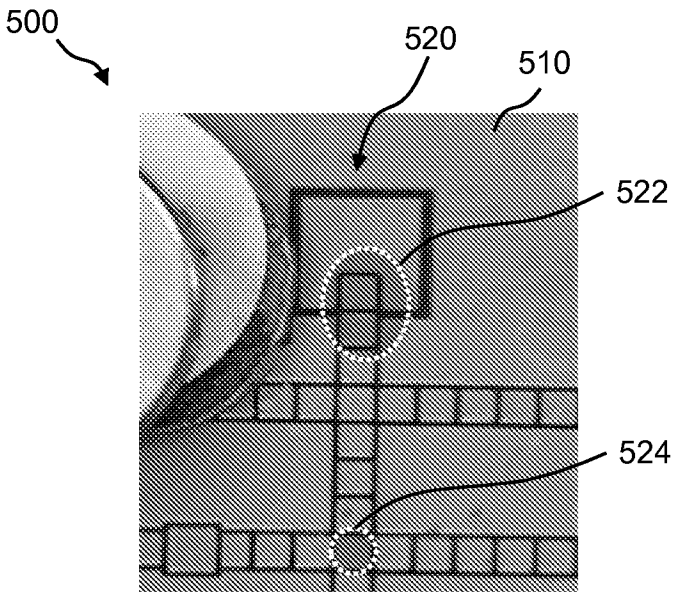


Figure 5B

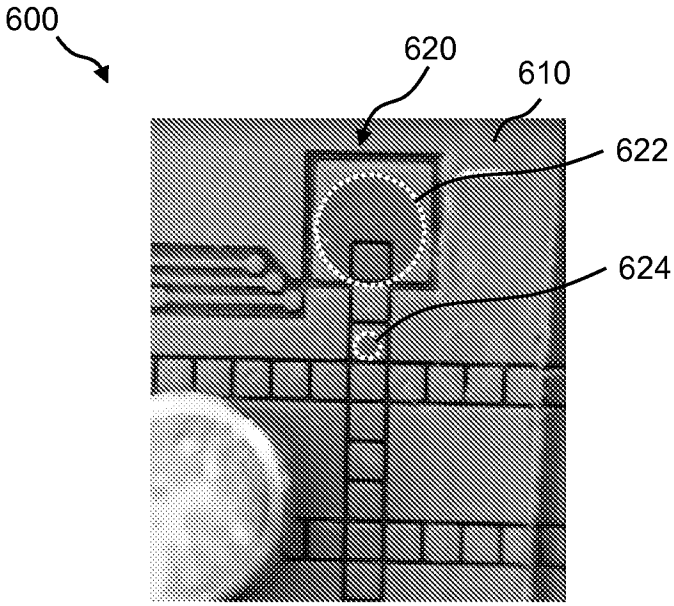


Figure 6

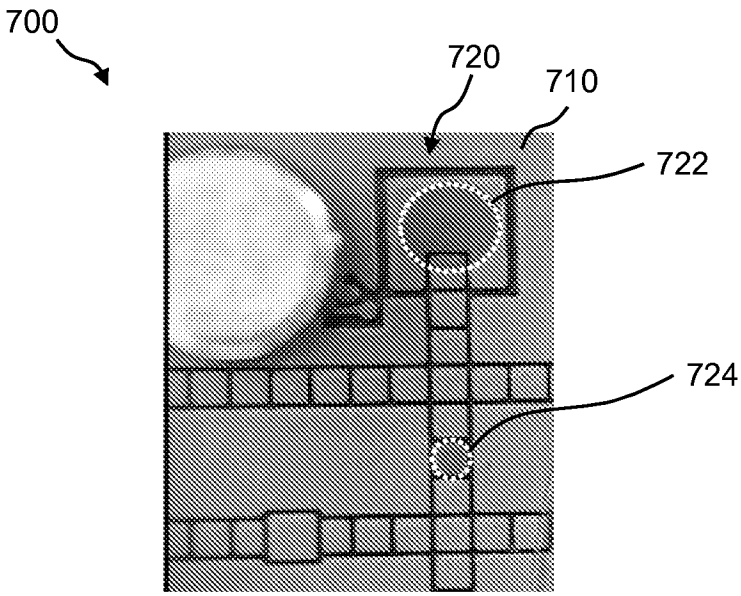


Figure 7

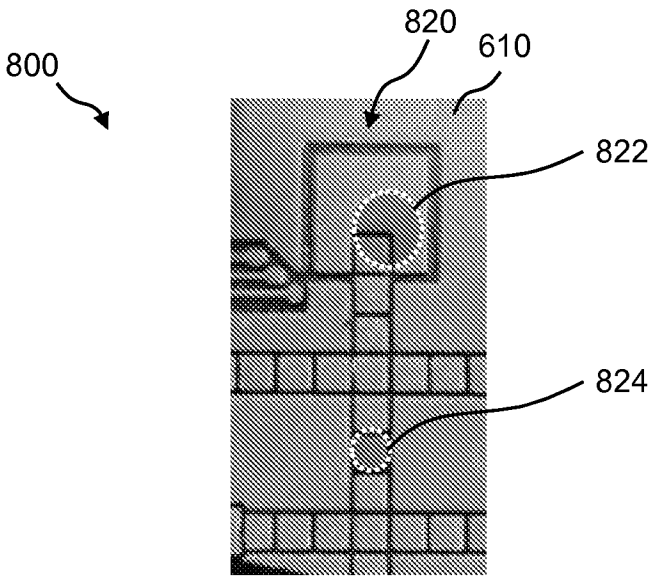


Figure 8

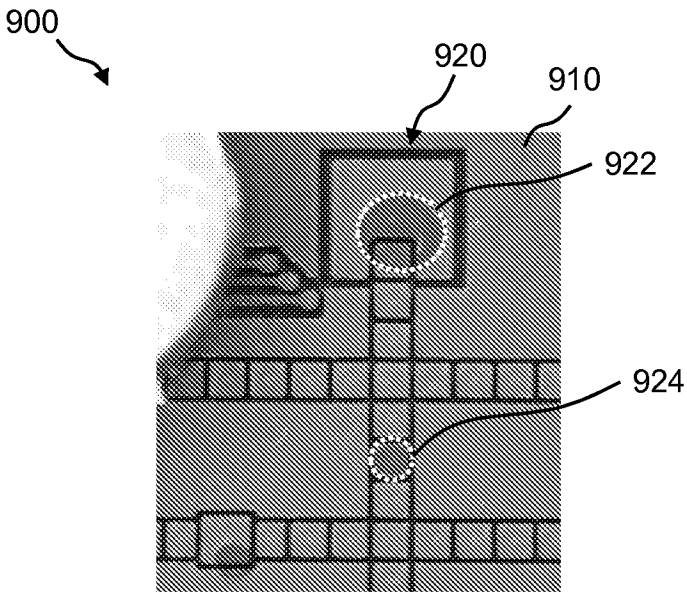


Figure 9

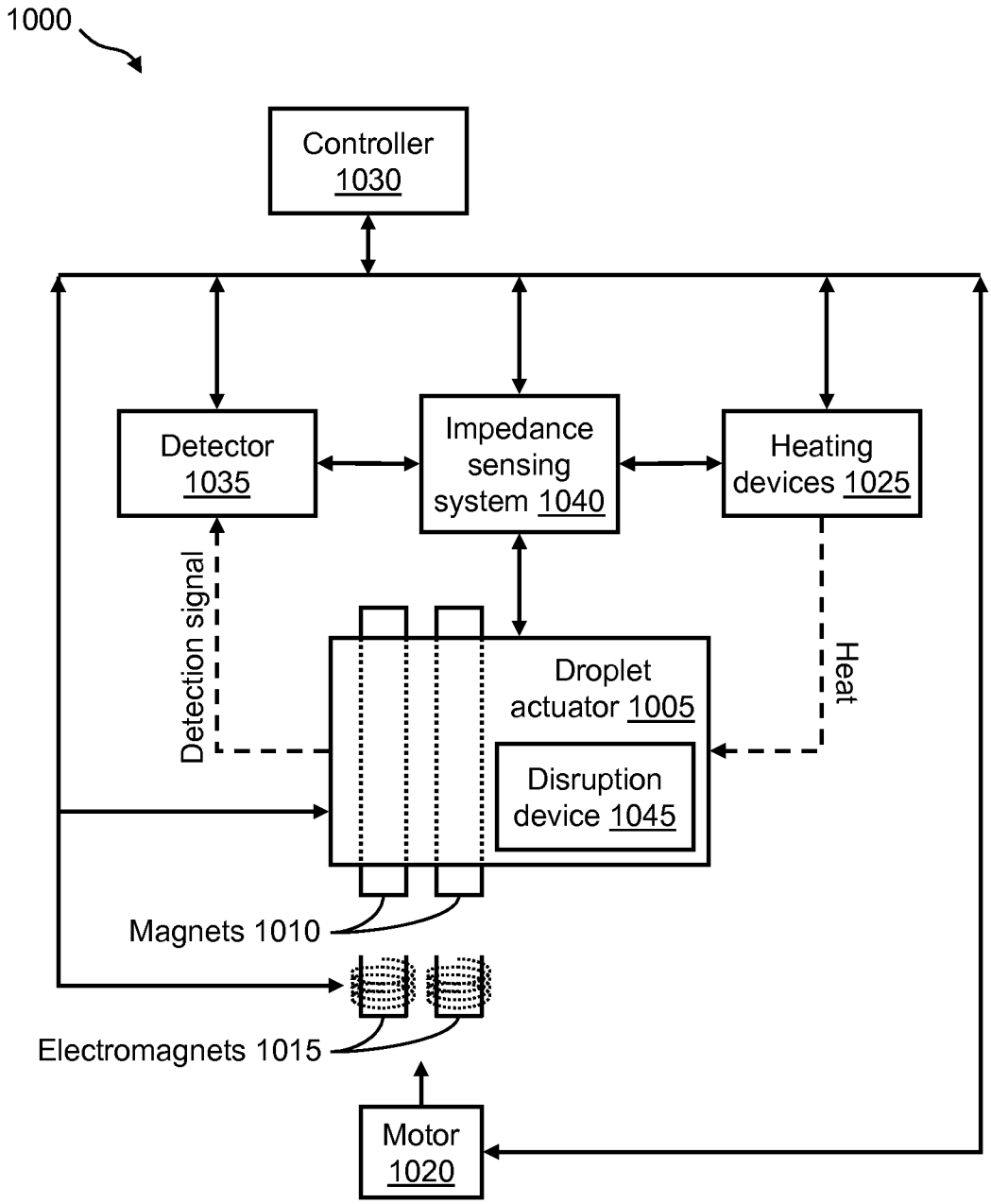

1000
Controller 1030
Detector 1035
Impedance sensing system 1040
Heating devices 1025
Detection signal
Heat
Droplet actuator 1005
Disruption device 1045
Magnets 1010
Electromagnets 1015
Motor 1020

Figure 10