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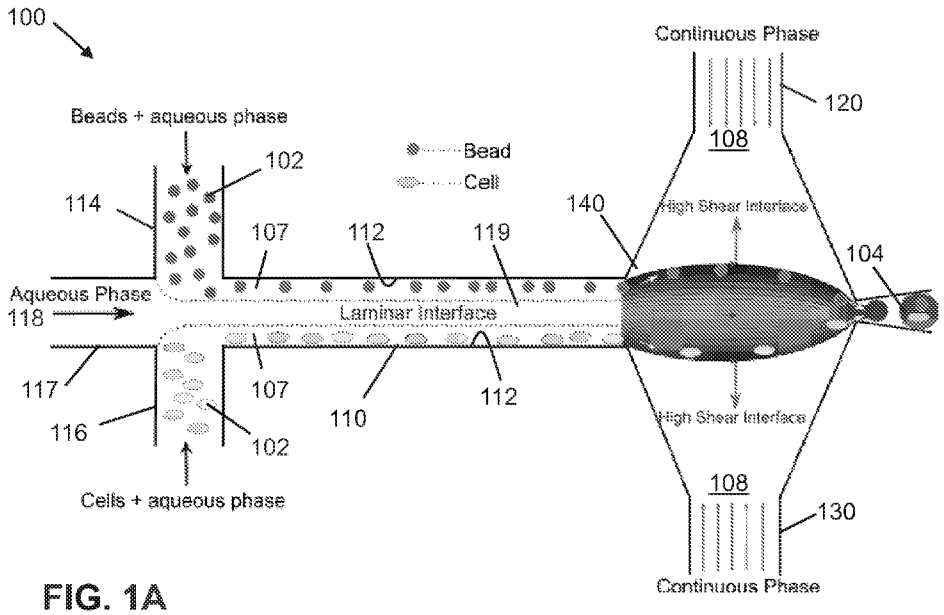

FIG. 1A

(57) Abstract: A passive, hydrodynamic technique to perform co-encapsulation of one cell-one bead in droplets is described herein. The hydrodynamic technique utilizes laminar flows and high shear liquid-liquid interfaces at a microfluidic junction to achieve a one-one-one encapsulation efficiency of about 30%. This technique may he implemented using a microfluidic device to significantly improve the efficiency of droplet sequencing and other bead based single cell assays.

[Continued on next page]

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CONTROLLED ENCAPSULATION IN DROPLETS BY LIQUID-LIQUID INTERFACIAL SHEARING

CROSS REFERENCE

[0001] This application claims priority to U.S. Provisional Application No. 62/517,775 filed June 09, 2017, the specification(s) of which is/are incorporated herein in their entirety by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to microfluidic devices, namely, to encapsulation of samples using droplet-based microfluidic devices.

GOVERNMENT SUPPORT

[0003] The inventions were made with government support under Grant No. 1362165 awarded by the National Science Foundation. The government may have certain rights in the inventions.

BACKGROUND OF THE INVENTION

[0004] Single-cell analysis is a field that studies the genomics, transcriptomics, proteomics and metabolomics at the single cell level. Conventional techniques to perform single cell analysis are flow cytometry and automated microscopy. To address the wide range of applications for single cell analysis, these conventional methods are often coupled with microfluidic devices. Microfluidic devices and systems are configured to process (e.g., move, mix, separate) small volumes of fluid, typically in the range of picoliters to microliters. In addition, the microfluidic devices can spatially collect single cells in micro wells, patterned surfaces, and various traps based on mechanical, magnetic, hydrodynamic, optical, dielectrophoretic, and acoustic principles. These microfluidic devices can be used for various applications including printing, bio-chemical assays, drug discovery, etc. A class of microfluidic devices and systems includes microfluidic droplet generating and manipulating devices configured to manipulate discrete droplets. Droplet-based microfluidic devices can be configured to perform a variety of operations, such as, for example, transportation of droplets, storage of droplets, mixing of droplets, analysis of droplets, etc. For example, these devices can be used as microreactors to achieve controlled and rapid mixing of fluids and/or to synthesize droplets and encapsulate various biological entities for biomedicine and biotechnology applications.

[0005] Droplet-based single cell assays are based on the ability to encapsulate and confine single cells in individual droplets and enable genome wide expression profiling. One-one-one encapsulation in droplets is a critical unit operation in single cell high-throughput screening and droplet sequencing. Most single cell encapsulations in droplets are performed randomly and are dictated by Poisson statistics. One of the current challenges in performing droplet sequencing (drop-seq) operation in droplets is achieving high efficiency one cell-one-bead encapsulation. It has been recently reported that for genome wide expression profiling, the encapsulation efficiency is as low as 0.1%, or 1 in 1000 droplets will have a cell therein. Hence, there is a need for improved microfluidic device and method for encapsulation in single droplets.

[0006] The present invention features a passive, hydrodynamic technique to perform encapsulation in droplets utilizing laminar flows and high shear liquid-liquid interface at a microfluidic junction, with an

encapsulation efficiency of 30%, which could significantly improve the efficiency of drop-seq and other bead based single cell assays.

SUMMARY OF THE INVENTION

[0007] It is an objective of the present invention to provide for microfluidic devices and methods for encapsulating biomolecules in droplets. Embodiments of the invention are given in the dependent claims. Embodiments of the present invention can be freely combined with each other if they are not mutually exclusive.

[0008] In some aspects, the present invention provides an interfacial hydrodynamic technique that combines the effects of laminar flow and liquid-liquid interfacial shearing, resulting in one-one-one encapsulation in droplets. Beads, cells and aqueous phase introduced through upper, lower and middle inlets respectively, create distinct laminar flow streams at a junction. The flow rates at three inlets are kept equal to prevent the bead/cell migration across the streamlines due to Magnus forces. The beads and cells self-assemble in a single file along the channel wall while moving toward the droplet generation junction. Upon reaching the droplet generation junction, the beads and cells get pulled from either side of the channel wall toward the symmetrical high shear interfaces into the droplet. The droplet diameter has to be large enough to accommodate one cell and one bead in it. This can be achieved by precisely tuning the dispersed to continuous phase pressure ratio (φ).

[0009] Various embodiments discussed herein comprise microfluidic devices that are configured to encapsulate single particle or cells with high through-put. Various embodiments of the microfluidic devices can be configured to provide encapsulation efficiencies of 30% or higher. For example, various microfluidic devices discussed herein can be configured to encapsulate particles or cells with an encapsulation efficiency of about 30% or greater. In some embodiments, the encapsulation efficiency is maximized in the squeezing regime to near dripping regime, where $Ca < 10^{-1}$, and φ is about 0.5-1.0.

[0010] One of the unique inventive features of the present invention is the formation of high shear interfaces between the continuous phase and dispersed phase fluid streams can be formed, which increases the encapsulation efficiency. Without wishing to limit the present invention to a particular mechanism or theory, laminar flow guides the particles/cells along the channel wall toward the high shear interface, and the high shear interface draws the cells or particles toward it at a higher velocity resulting in self-spacing of the cells, thereby reducing and even eliminating the possibility of doublets. An important advantage of this technique is that it can be modified based on the desired application including single cell or bead encapsulation (1-1), and 1 cell–1 bead–1droplet encapsulation or 1 cell–1 cell–1droplet encapsulation (1-1-1) for different cell types and cell sizes.

[0011] In some aspects, the method of encapsulating one or more solid samples (e.g., biological material comprising cellular material, one or more cells, one or more particles, one or more beads, etc.) in a droplet of a fluid (e.g., water) may comprise flowing a dispersed phase fluid stream comprising a solid sample (e.g., a biological sample comprising cellular material or one or more cells) dispersed in a fluid

(e.g., water) through a combining channel; controlling the flow rates of the flow stream to establish laminar flow through the combining channel; flowing a continuous phase fluid through a continuous phase channel that intersects the combining channel, the continuous phase fluid being immiscible with the dispersed phase fluid; controlling the flow rate of the continuous phase fluid to shear the laminar flow of the dispersed phase fluid stream and generating droplets encapsulating the solid sample in an output microfluidic channel.

[0012] Another innovative aspect of the subject matter of this application is embodied in a microfluidic device, comprising: a combining channel configured to transport a dispersed phase fluid stream comprising a solid sample (e.g., a biological sample comprising cellular material or one or more cells) dispersed in a fluid (e.g., water); and continuous phase channels intersecting the combining channel to form an intersection region. Each continuous phase channel is configured to transport a continuous phase fluid stream. The intersection region is configured to open into an output microfluidic channel through an orifice. The microfluidic device further comprises a fluid controller to control the flow rates of the fluid streams to generate droplets encapsulating the solid sample. The flow rate of the dispersed phase flow stream can be controlled to establish laminar flow, and the flow rate of the continuous phase fluid stream can be controlled to shear the laminar flow of the dispersed phase fluid stream in the intersection region, thus generating droplets that enter the output microfluidic channel through the orifice.

[0013] Any feature or combination of features described herein are included within the scope of the present invention provided that the features included in any such combination are not mutually inconsistent as will be apparent from the context, this specification, and the knowledge of one of ordinary skill in the art. Additional advantages and aspects of the present invention are apparent in the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The features and advantages of the present invention will become apparent from a consideration of the following detailed description presented in connection with the accompanying drawings in which:

[0015] FIG. 1A shows an exemplary mechanism of one-cell-one-bead encapsulation in droplets using interfacial shearing technique, according to an embodiment of the present invention. Beads and cells introduced from upper and lower inlets self-assemble along the channel wall while moving toward high shear interfaces. At the droplet generation junction, both the beads and cells get pulled toward the high shear interface symmetrically from both the channel boundaries resulting in one-one-one encapsulation.

[0016] FIG. 1B is a schematic of the encapsulation process using interfacial shearing.

[0017] FIG. 2 shows a non-limiting computational fluid dynamic model (CFD) of interfacial shearing.

[0018] FIG. 3 shows a non-limiting embodiment of one-one-one (1-1-1) encapsulation of 10 μm beads and k-562 cells. The 10 μm beads were introduced from an upper inlet while the k-562 cells are flowed in through a lower inlet. Both the beads and cells align close to the channel wall on the way toward a flow focusing junction. At the junction, the beads and the cells are pulled toward a symmetrical shear interface and encapsulated into droplets.

[0019] FIGs. 4A-4D show steps of 1-1-1 encapsulation of 10 μm beads from the top and Hela cells from the bottom. The encircled droplet indicates the 1-1-1 droplet.

[0020] FIG. 5A shows the 10 μm beads self-assemble along the top channel wall while Hela cells align along the bottom wall.

[0021] FIG. 5B shows the one cell-one bead encapsulation in droplets. The encircled droplets indicate the 1-1-1 droplets.

[0022] FIGs. 6A-6E show steps of one-one-one encapsulation of 10 μm beads. In FIG. 6A, beads self-align along the channel wall. In FIG. 6B, beads gets pulled by the high shear flow at the boundary. In FIGs. 6C-6D, beads enter the droplets from two sides and are encapsulated in FIG. 6E. The encircled droplet shows the 1-1-1 droplets.

[0023] FIG. 7A is a graph of encapsulation efficiency vs. droplet diameter. The encapsulation efficiency increases with the droplet diameter, reaches a maximum, and decreases thereafter due to multiple encapsulations in one droplet.

[0024] FIG. 7B is a graph of 1-1-1 encapsulation efficiency vs. pressure ratio of dispersed phase to continuous phase (φ). The encapsulation efficiency increases with the dispersed to continuous pressure ratio (φ), reaches a maximum, and decreases thereafter due to multiple encapsulations in one droplet.

[0025] FIG. 7C is a graph of concentration optimization for encapsulation efficiency vs. cell concentration. The encapsulation efficiency increases with cell concentration, reaches a maximum, and decreases thereafter due to multiple encapsulations in one droplet.

[0026] FIG. 8A is a schematic illustration of single cell encapsulation in droplets (1-1) using interfacial shearing. The cells were introduced from a single inlet.

[0027] FIG. 8B shows one cell encapsulation in droplets of cells introduced from a single inlet. The circles indicate the single cells.

[0028] FIG. 9A is an alternative schematic of single cell encapsulation in droplets (1-1) using interfacial shearing. The cells were introduced from two inlets.

[0029] FIG. 9B shows one cell encapsulation in droplets of cells introduced from two inlets. The circles indicate the single cells.

[0030] DESCRIPTION OF PREFERRED EMBODIMENTS

[0031] Following lists elements corresponding to a particular element referred to herein:

[0032] 100 microfluidic device

[0033] 102 solid sample

[0034] 104 droplet

[0035] 106 dispersed phase fluid

[0036] 107 flow stream

[0037] 108 continuous phase fluid

[0038] 109 high shear interface

[0039] 110 combining channel

[0040] 112 channel sidewall

[0041] 114 first dispersed phase channel

[0042] 116 second dispersed phase channel
[0043] 117 aqueous phase channel
[0044] 118 aqueous phase fluid
[0045] 119 laminar interface stream
[0046] 120 first continuous phase channel
[0047] 130 second continuous phase channel
[0048] 140 intersection region
[0049] 145 droplet shearing junction
[0050] 147 orifice
[0051] 150 output channel
[0052] 160 fluid flow controller

[0053] As used herein, the microfluidic devices employ fluid volumes on the scale of microliters (10^{-6}) to picoliters (10^{-12}) that are contained within sub-millimeter scale channels. The structural or functional features may be dimensioned on the order of mm-scale or less, preferably in the micron scale or less. For example, a diameter or width of a channel or a dimension of an intersection or junction may range from <0.1 μm to greater than 1000 μm. Alternatively or in addition, a length of a channel may range from 0.1 μm to greater than cm-scale. The microfluidic device may employ active or passive techniques for fluid transport and droplet production. Compared to the active approach, which fluid manipulation involves the use of micropumps and microvalves, the passive approach takes advantage of the characteristic flow field in microfluidics to control the interface and capillary instability, and consequently to produce droplets.

[0054] As used herein, the term "high shear interface" refers to a high velocity liquid-liquid interface formed between two immiscible liquids. Generally, the continuous phase flow rate is greater than the flow rate of the dispersed phase. For instance, the continuous phase flow rate may be about 2-5 times greater. At the aqueous-oil interface, the high continuous phase flow rate imparts the same velocity to the dispersed phase at the interface. Hence, the dispersed phase at the interface is at a higher velocity (shear) than the bulk. As used herein, the term "laminar flow" refers to flow of a fluid in layers that do not mix. One of ordinary skill in that art would understand that at lower Reynold's numbers (<10), a laminar flow is always established in the microfluidic channel. The fluid flows in parallel layers with no lateral mixing but with some minor diffusion.

[0055] As known to one of ordinary skill in the art, in a geometry-mediated regime, or squeezing regime, the droplet generation depends only on the size of the orifice and the flow rate ratio of the dispersed phase to the continuous phase flow rate, whereas interfacial tension and viscosity has no significant influence. The transition between the geometry-mediated regime to a dripping regime is dictated by the Capillary number (Ca), $Ca = \frac{\mu v}{\sigma}$, where μ is the viscosity, v is the velocity of the continuous phase, and σ is the interfacial tension between the two fluid phases. Generally, in the geometry-mediated regime, Ca is $<10^{-1}$. In the dripping regime, Ca may be $\geq 10^{-1}$ and interfacial tension and viscosity can predict the formation of droplets.

[0056] *SAMPLES FOR ENCAPSULATION*

[0057] In various embodiments of the present invention, the samples for encapsulation may be microparticles. The microparticles may be beads. Examples of beads include, but are not limited to, polymer beads, bar-coded beads, functionalized beads, and magnetic beads. In some embodiments, the beads may have a size or dimension, such as a diameter or width, ranging from about 0.01 μm to about 20 μm.

[0058] In other various embodiments of the present invention, the samples for encapsulation may be cells. Any particular cell type from any organism may be used in the methods and systems of the present invention. The cells may have a size or dimension, such as a diameter or width, ranging from about 0.1 μm to about 20 μm. In some embodiments, the cells may be wild type cells or genetically modified cells. In other embodiments, the cells may be cells harboring one or more mutations, healthy cells, diseased cells or unhealthy cells, etc. For example, in some embodiments, the cells may be prokaryotic cells (e.g., bacteria, archaebacteria, etc.). In other embodiments, the cells may be eukaryotic cells such as single-celled eukaryotes, fungal cells (e.g. yeast, mold, etc.), animal cells, mammalian cells (e.g. cells from a human, non-human primate, rodent, rabbit, sheep, dog, cat, etc), and non-mammalian cells (e.g. cells from insects, reptiles, amphibians, birds, etc.).

[0059] In some embodiments, the cells used in the present invention may be other eukaryotic cells such as plant cells or algal cells. Non-limiting and non-exhaustive examples of plant cells include cells from corn, soybean, wheat, cotton, grass, flowering plants, fruit-bearing plants, trees, tuberous plants, potatoes, root plants, carrots, peanut, nuts, beans, legumes, and squashes. It is to be understood that the term "plant cell" encompasses all types and stages of plant cells and is not limited to the aforementioned examples. Non-limiting and non-exhaustive examples of algal cells include cells from Chlorella sp., Nannochloropsis sp. and Botryococcus sp. It is to be understood that the term "algal cell" encompasses all types of algal cells and is not limited to the aforementioned examples. One of the distinguishing characteristics that plant and algal cells have over animal cells is a cell wall that surrounds a cell membrane to provide rigidity, strength, and structure to the cell. The cell wall may be comprised of polysaccharides including cellulose, hemicellulose, and pectin. Similar to plant and algal cells, the fungal cells also have a cell wall, which may be comprised of polysaccharides including glucans, mannans, and chitin.

[0060] In other embodiments, the cells used in the present invention may be protoplasts, which are intact plant, bacterial or fungal cells that had its cell wall completely or partially removed using either mechanical or enzymatic means.

[0061] In yet other embodiments, the cells used in the present invention may be a tetrad. The term "tetrad" is used to herein to refer to a single structure comprised of four individual physically attached components. A "microspore" is an individual haploid structure produced from diploid sporogenous cells (e.g., microsporoyte, pollen mother cell, or meiocyte) following meiosis. A microspore tetrad refers to four individual physically attached microspores. A "pollen grain" is a mature gametophyte containing vegetative

(non-reproductive) cells and a generative (reproductive) cell. A pollen tetrad refers to four individual physically attached pollen grains.

[0062] *ENCAPSULATION*

[0063] Microfluidic devices including droplet generation portions can be used to create droplets of a fluid (e.g., oil or water). Microfluidic devices that include droplet generation portions can be used: to study chemical reactions, in drug delivery, in drug discovery, etc. One method of generating droplets in microfluidic devices comprises flowing a first liquid (e.g., water) through a first channel and a second liquid (e.g., oil) that is immiscible with the first liquid through channels intersecting the first channel. The first liquid flowing through the first channel (e.g., water) is broken up to form discrete droplets as a result of shear forces from the second liquid. The size of the generated first liquid droplets generated can depend on a variety of factors including velocity of the second liquid. For example, as the velocity of the second liquid is increased, the size of the first liquid droplets is reduced.

[0064] Referring now to FIG. 1A-1B, in one embodiment, the present invention features a method for encapsulating a solid sample (102) in a droplet (104). The method may comprise flowing a first fluid (106) through a first microfluidic channel (110) at a first flow rate (v_d) such that flow of the first fluid is laminar, and co-flowing a second fluid (108) through each of a second microfluidic channel (120) and a third microfluidic channel (130) at a second flow rate (v_c). In one embodiment, the first fluid (106) may comprise at least two flow streams (107). One or both of said flow streams (107) may comprise dispersed solid samples (102) that self-assemble near a sidewall (112) of the first microfluidic channel while flowing towards an intersection region (140). The second and third microfluidic channels (120, 130) can intersect the first microfluidic channel (110) at the intersection region (140) such that the second fluid streams (108) intersect the first fluid (106) and merge to form a droplet shearing junction (145) within the intersection region (140). In some embodiments, the method further comprises adjusting v_d, v_c, or both such that each of the second fluid streams (108) forms a high shear interface (109) with the first fluid (106), and the solid samples (102) are drawn to the high shear interface (109), and generating droplets (104) at the droplet shearing junction (145) such that each droplet (104) is substantially sized to encapsulate one solid sample or co-encapsulate two different solid samples.

[0065] According to some embodiments, the method for encapsulating a solid sample (102) in a droplet (104) may include providing a microfluidic device (100). In some embodiments, the microfluidic device (100) may comprise a combining channel (110), a first continuous phase channel (120) having a portion thereof disposed on one side of the combining channel, a second continuous phase channel (130) having a portion thereof disposed on an opposite side of the combining channel, and an output channel (150). The portions of the first and second continuous phase channels can intersect at a terminal end of the combining channel to form an intersection region (140) to which the output channel (150) is fluidly coupled thereto. In one embodiment, the portions of the first and second continuous phase channels can intersect the combining channel (110) orthogonally such that the continuous phase channels and combining channel form a T-junction. Alternatively, the continuous phase channels can intersect the combining channel (110) at an acute angle such that the continuous phase channels and output channel form a Y-

junction.

[0066] In some embodiments, the microfluidic device (100) may further comprise a first dispersed phase channel (114) comprising one of the flow streams (107) forming the dispersed phase fluid (106), and a second dispersed phase channel (116) comprising the other flow stream (107). The first and second dispersed phase channels (114, 116) can merge to form the combining channel (110). In other embodiments, the microfluidic device (100) may further comprise an aqueous phase channel (117) intersecting with the first and second dispersed phase channels (114, 116). The aqueous phase channel (117) may comprise aqueous phase fluid (118), which flows in the combining channel (110) such that the aqueous phase fluid (118) forms a laminar interface stream (119) between the two flow streams (107).

[0067] In other embodiments, the device (100) may further comprise a fluid flow controller (160) configured to perform operations. These operation can include adjusting v_d of the dispersed phase fluid to establish laminar flow in the combining channel (110) such that the solid samples (102) assemble near a sidewall (112) of the combining channel while flowing towards the intersection region (140), adjusting v_{c1}, v_{c2}, or both such that each continuous phase fluid stream (108) forms a high shear interface (109) with the dispersed phase fluid (106) at the intersection region (140) and the solid samples (102) are drawn to the high shear interface (109) while flowing through the intersection region (140), and adjusting v_d, v_{c1}, or both to generate droplets (104) at the droplet shearing junction (145) such that each droplet (104) is substantially sized to encapsulate a solid sample (102). In one embodiment, the flow in the microfluidic device (100) and adjustment of the flow rates may be pressure-driven. Preferably, the microfluidic device (100) utilizes passive techniques to control fluid flow.

[0068] In various embodiments, the width of the various microfluidic channels (e.g., the first and second dispersed phase and aqueous phase channels (114, 116, 117); the combining channel (110); and the continuous phase channels (120, 130)) can range from about 25 μm to about 75 μm. For examples, the width of the various microfluidic channels can be in a range between about 30 μm to about 60 μm.

[0069] In other embodiments, a width and/or length of the intersection region can be about 3-6 times the width of the various microfluidic channels (e.g., the combining channel, the first continuous phase channel, or the second continuous phase channel). For example, the width of the intersection region may be about 150 μm, which is about three times the width of a 50 μm incoming microfluidic channel. In another embodiment, the length of the intersection region may be about 200 μm, which is about four times the width of a 50 μm incoming microfluidic channels.

[0070] In some embodiments, the width of the orifice may be about 5-40 μm. For example, in one embodiments, the width of the orifice may be about 5-15 μm, about 10-20 μm, about 20-30 μm, or about 30-40 μm. In other embodiments, the width of the output channel may widen from the width of the orifice to a maximum width. The maximum width of the output channel can be about 2-10 times the width of the orifice. For examples, for a 30 μm orifice, the output channel widens from a minimum width of 30 μm to a maximum width of about 120 μm. In further embodiments, the width of the output channel may be reduced

after reaching its maximum.

[0071] Consistent with the embodiments described above, an exemplary implementation of the method may comprise flowing a dispersed phase fluid (106) through the combining channel (110) at a first flow rate (v_d), and adjusting v_d of the dispersed phase fluid (106) to establish laminar flow in the combining channel (110) such that the solid samples (102) assemble near a sidewall (112) of the combining channel while flowing towards the intersection region (140). In one embodiment, the dispersed phase fluid (106) may comprise at least two flow streams (107), with one or both of the flow streams (107) having dispersed solid samples (102). Continuous phase fluid streams (108) co-flow through each of the first and second continuous phase channels (120, 130) at a second flow rate (v_c). The continuous phase fluid streams (108) can intersect the dispersed phase fluid (106) at the intersection region (140) such that a droplet shearing junction (145) is formed within the intersection region (140) as the continuous phase fluid streams (108) merge with the dispersed phase fluid (106). The droplet shearing junction (145) can comprise an orifice (147) that fluidly couples the output channel (150) to the intersection region (140). The method continues with adjusting v_d, v_c, or both such that each continuous phase fluid stream (108) forms a high shear interface (109) with the dispersed phase fluid (106) at the intersection region (140). The solid samples (102) are drawn to the high shear interface (109) while flowing through the intersection region (140). v_d, v_c, or both are further adjusted to generate droplets (104) encapsulating one solid sample (102) at the droplet shearing junction (145). Preferably, each droplet (104) can be substantially sized to encapsulate said solid sample (102).

[0072] In one embodiment, as shown in FIG. 3-6E, the method and microfluidic device can be adapted to co-encapsulate two different samples in one droplet. For example, the dispersed solid samples (102) may comprise a plurality of cells flowing in one of the flow streams (107), and a plurality of particles flowing in the other flow stream (107). When flowing through the combining channel (110), laminar flow of the dispersed phase fluid causes the cells to assemble near the sidewall (112a) and the particles to assemble near an opposing sidewall (112b). At the intersection region (140), the cells are drawn to one high shear interface (109a) and the particles are drawn to the other high shear interface (109b), thereby enabling one cell and one particle to be co-encapsulated in one droplet (104) as said droplet (104) is formed at the droplet shearing junction (145). The droplet (104) co-encapsulating the one cell and one particle can then be released from the orifice (147) into the output channel (150).

[0073] In another embodiment, as shown in FIGs. 8A-9B, the method and microfluidic device can be adapted to encapsulate a single sample in one droplet. The dispersed solid samples (102) may comprise either cells or particles. The cells or particles enter the combining channel (110) from one or both of the first and second dispersed phase channels, and one solid cell or particle (102) is encapsulated as the droplet (104) is formed at the droplet shearing junction (145). The droplet (104) encapsulating the one solid sample (102) is released from the orifice (147) into the output channel (150).

[0074] Microfluidic droplet generators utilizing the droplet generation method described above can be used to compartmentalize or encapsulate a single cell or a bead comprising single cell, cellular material or

some other biological material in a single water droplet. Droplets encapsulating a single cell or bead can be useful for single cell assays of cells (e.g., cancer cells or immune cells) that exhibit biological heterogeneity for which assays that provide a population average may be insufficient. Encapsulation of a single cell (one cell) and/or a single bead (one-bead) in a single droplet can be useful for high-throughput screening of single cell. As previously described of prior technoologies, the efficiency of encapsulating a single cell (one cell) and/or a single bead (one-bead) in a single droplet can be as low as 0.1%, i.e. 1 in 1000 droplets may have a single cell (one cell) and/or a single bead (one-bead) while the remaining droplets may have no cells and/or beads or have more than one cell and/or one bead. Without wishing to limit the present invention, this application provides a passive, hydrodynamic technique which can achieve a 'one-one-one' (one cell and/or one bead in one droplet) encapsulation efficiency of 30% or higher, which could significantly improve the biomolecular capture efficiency of various bead-based single cell assays.

[0075] The device can be configured to encapsulate one cell and/or one bead in a single droplet of a fluid (e.g., water) by the combined effect of laminar flow and the high shear liquid-liquid interfacial boundary. In the illustrated device of FIG. 1A, a first fluid stream comprising a first solid sample (e.g., cells or cellular material) dispersed in a first fluid (e.g., water) is introduced through a first incoming microfluidic channel and a second fluid stream comprising a second solid sample (e.g., beads or particles) dispersed in the first fluid (e.g., water) is introduced through the second incoming microfluidic channel. A third fluid stream comprising the first fluid (e.g., water) is introduced through the third incoming microfluidic channel. The first, second and third flow streams, collectively referred to as a dispersed phase fluid stream, flow into the combining channel.

[0076] In some embodiments, the velocities of the first, second and third flow streams can be adjusted such that laminar flow is established in the combining channel. For example, the flow rates of the first, second and third flow streams can be equal to each other such that laminar flow is established in the combining channel. By maintaining equal flow rates in the three incoming microfluidic channels, bead/cell migration across the streamlines due to Magnus force can be prevented. The first and the second fluid streams in the combining channel can be separated by a laminar interface as a result of the laminar flow. The constituents of the first solid sample (e.g., cells or cellular material) self-assemble on one side of the laminar interface and the constituents of the second solid sample (e.g., particles or beads) self-assemble on another side of the laminar interface. For example, beads or particles self-assemble in a single row along a channel wall of the combining channel adjacent to the incoming microfluidic channel of the bead or particles, and cells self-assemble in a single row along the opposite channel wall of the combining channel adjacent to the incoming microfluidic channel of the cells.

[0077] The laminar flow of the dispersed phase fluid stream enters the intersection region. In the intersection region, the flow rate of the continuous phase fluid streams can be adjusted to create a high shear interface between the laminar flow of the dispersed phase fluid stream. Cells in the first flow stream and the beads or particles in the second flow stream are pulled towards the high shear interface as shown in FIG. 1B. The flow rates of the dispersed phase fluid stream and the continuous phase fluid streams can be adjusted to generate droplets having a droplet size large enough to encapsulate a single cell from the

first flow stream and a single bead/particle from the second fluid stream. The size of the droplet can depend on the capillary number Ca = μV/σ, where μ is the viscosity of the continuous phase comprising the second fluid, V is the superficial velocity (flow rate) of the continuous phase comprising the second fluid, and σ is the equilibrium surface tension between the continuous phase and the dispersed phase fluid streams. To generate droplets having an appropriate size to encapsulate a single cell and/or single bead, the capillary number can be in the range of about 0.01 and about 1 (e.g., about 0.1). In various embodiments, the velocity of the continuous phase fluid streams can be about 2-10 times greater that the velocity of the dispersed phase fluid stream.

[0078] The droplet size can also be controlled by controlling the pressure ratio between the dispersed phase fluid stream and the continuous phase fluid stream. In various embodiments, a droplet encapsulating a single cell and a single bead/particle can be achieved by controlling the pressure ratio (φ) and/or the flow rate ratio between the dispersed phase and the continuous phase. In some embodiments, the pressure ratio and/or the flow rate ratio between the dispersed phase and the continuous phase may be about 0.1 to about 0.5 (e.g., about 0.3) in order to maximize encapsulation efficiency. Depending on the pressure ratio and/or the flow rate ratio, the generated droplets can be configured to have a diameter of about 20 μm to about 100 μm to match the size and/or concentration of the incoming cells and/or beads.

[0079] In various embodiments, the height of the various microfluidic channels is less than twice the diameter of the solid samples (e.g., cells, beads, particles, etc.) that are configured to be dispersed in dispersed phase fluid. Restricting the height of the various microfluidic channels to be less than twice the diameter of the solid samples can advantageously reduce the chance that the solid samples roll over each other and/or stack over each other.

[0080] FIGs. 4A-4D and 5A-5B illustrate experimental results showing encapsulation of single beads and cells in a single droplet using a microfluidic device of the present invention. In the experiment, the first flow stream comprises Hela cells having a size of about 10 microns dispersed in water and the second flow stream comprises particles/beads having a size of about 10 microns dispersed in water. The concentration of the cells or beads in water can be in the range between about 10^6 – 10^9 cell or beads in 1 ml of water. FIG. 5A illustrates the self-assembly of 10 μm beads along the channel wall and the self-assembly of the Hela cells along the channel wall. FIG. 5B illustrates the droplets encapsulating a single 10 micron particle/bead and a single Hela cell.

[0081] FIG. 8A illustrates another embodiment of a microfluidic device that is configured to encapsulate a single cell in a single droplet. In contrast to the device depicted in FIG. 1A, the device of FIG. 8A comprises only two incoming microfluidic channels instead of three. Cells dispersed in a first fluid (e.g., water) is introduced through a first microfluidic channel and the first fluid is introduced through the through a second microfluidic channel. Laminar flow is established in the combining channel such that the cells self-assemble along the channel wall. By controlling the pressure ratio between the dispersed phase and the continuous phase, the concentration of cells in the dispersed phase and the droplet size,

encapsulation of a single cell in a single droplet can be achieved as shown in the droplets of FIG. 8B.

[0082] It is noted that cells can be introduced through both the microfluidic channels. For example, as shown in FIG. 9A, cells can be introduced through both the first and the second incoming channels. In such embodiments, the pressure ratio between the dispersed phase and the continuous phase can be adjusted to change the size of the generated droplets to facilitate encapsulation of a single cell in a single droplet, as shown in the droplets of FIG. 9B. In various embodiments, the size of the generated droplets can be tuned by adjusting the droplet generation regimes. The encapsulation of a single cell in a single droplet and/or a single bead and a single cell in a single droplet can be achieved in both geometry-mediated and dripping regimes. For example, when the droplets are generated in the geometry-mediated regime, the size of the droplet can be greater than or equal to the size of orifice diameter. In the dripping regime, where the droplet break-off occurs due to interfacial instability, the droplet size can be less than the size of the orifice. In both regimes, the beads and cells that assemble in single row along the channel wall are pulled into the droplets by the symmetrical high shear zone resulting in encapsulation. The droplet size can be tailored to the size of the incoming cells and/or concentrations by controlling the pressure and/or flow rate ratio between the dispersed phase and the continuous phase and the capillary number. Without wishing to limit the present invention, the encapsulation efficiency achieved using the methods described herein can be 10% or higher. More preferably, the encapsulation efficiency achieved using the methods described herein can be 30% or higher.

[0083] **EXAMPLES**

[0084] The following are non-limiting examples of encapsulation using the interfacial shearing technique of the present invention. It is to be understood that the examples are for illustrative purposes only and are not intended to limit the present invention in any way. Equivalents or substitutes are within the scope of the invention.

[0085] **Materials and Methods**

[0086] The microfluidic devices were fabricated in polydimethylsiloxane (PDMS) using soft lithography. The PDMS molded imprints and another plain PDMS layer were plasma treated and brought together to form a permanent seal. The device was left in an oven at 120 °C overnight to regain its natural hydrophobicity. Ethyl oleate and 2% ABIL EM 90 formed the continuous phase, and mixture of water, lipids (DSPC and DSPE-PEG 2000), glycerol and surfactant (Pluronic F-68) form the dispersed phase. Hela cells and 10 μm beads were suspended in freshly prepared dispersed phase.

[0087] Both the continuous phase and the dispersed phase were introduced into the microfluidic chip using a constant pressure source via high speed solenoid valves controlled by a custom-built lab view program. One-one-one encapsulation was monitored using a Nikon 100-S inverted microscope and recorded using a Phantom camera V-310. Image J software was used to analyze the videos frame by frame, and yield the encapsulation data.

[0088] **Results**

[0089] A one cell-one bead encapsulation process is shown in FIGs. 4A-4D and FIGs. 5A-5B. To demonstrate the one-cell-one-bead encapsulation in droplets, 10 μm beads were introduced through an upper inlet while Hela cells entered through a lower inlet. Referring to FIG. 5A, the beads and cells then assemble single file along the channel wall while moving towards a symmetrical high shear zone. Referring to FIGs. 7A-7B, the droplet diameter can be tuned by adjusting (Dp/Cp) to achieve maximum encapsulation efficiency. The encapsulation efficiency increases with the droplet diameter or (Dp/Cp); however, it starts decreasing beyond a threshold because of the multiple encapsulations. By arranging the cells/ beads single file along the channel wall using laminar flow, along with the high shear interface, the randomness involved in the encapsulation process is overcome to a considerable extent.

[0090] Referring now to FIGs. 6A-6E, 10 μm beads were introduced from both the upper and lower inlets and were encapsulated into the droplets from either side of the channel. Referring As shown in FIGs. 8A-8B, the cells were introduced from only one inlet and were encapsulated into single-cell droplets. As shown in FIGs. 9A-9B, the cells were introduced from two inlets and were encapsulated into single-cell droplets.

[0091] Based on the examples described herein, the present invention has been demonstrated to perform one-one or one-one-one encapsulation in droplets utilizing the combined effect of laminar flow and high shear liquid-liquid interface at the microfluidic junction. These results suggest that this technique can be applied to droplet-based high-throughput genomic workflows.

[0092] As used herein, the term "about" refers to plus or minus 10% of the referenced number.

[0093] Various modifications of the invention, in addition to those described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Each reference cited in the present application is incorporated herein by reference in its entirety.

[0094] Although there has been shown and described the preferred embodiment of the present invention, it will be readily apparent to those skilled in the art that modifications may be made thereto which do not exceed the scope of the appended claims. Therefore, the scope of the invention is only to be limited by the following claims. Reference numbers recited in the below claims are solely for ease of examination of this patent application, and are exemplary, and are not intended in any way to limit the scope of the claims to the particular features having the corresponding reference numbers in the drawings. In some embodiments, the figures presented in this patent application are drawn to scale, including the angles, ratios of dimensions, etc. The figures are understood to be representative only and the claims are not limited by the dimensions of the figures. In some embodiments, descriptions of the inventions described herein using the phrase "comprising" includes embodiments that could be described as "consisting of", and as such the written description requirement for claiming one or more embodiments of the present invention using the phrase "consisting of" is met.

WHAT IS CLAIMED IS:

1. A method for encapsulating a solid sample (102) in a droplet (104), comprising:
    a. providing a microfluidic device (100) comprising:
        i. a combining channel (110);
        ii. a first continuous phase channel (120) having a portion thereof disposed on one side of the combining channel;
        iii. a second continuous phase channel (130) having a portion thereof disposed on an opposite side of the combining channel, wherein said portions of the first and second continuous phase channels intersect at a terminal end of the combining channel to form an intersection region (140); and
        iv. an output channel (150) fluidly coupled to the intersection region(140);
    b. flowing a dispersed phase fluid (106) through the combining channel (110) at a first flow rate (v_d), wherein the dispersed phase fluid (106) comprises at least two flow streams (107), wherein one or both of said flow streams (107) comprises dispersed solid samples (102);
    c. adjusting v_d of the dispersed phase fluid (106) to establish laminar flow in the combining channel (110) such that the solid samples (102) assemble near a sidewall (112) of the combining channel while flowing towards the intersection region (140);
    d. co-flowing a continuous phase fluid stream (108) through each of the first and second continuous phase channels (120, 130) at a second flow rate (v_c), wherein the continuous phase fluid streams (108) intersect the dispersed phase fluid (106) at the intersection region (140), wherein a droplet shearing junction (145) is formed within the intersection region (140) as the continuous phase fluid streams (108) merge with the dispersed phase fluid (106), wherein the droplet shearing junction (145) comprises an orifice (147) fluidly coupling the output channel (150) to the intersection region (140);
    e. adjusting v_d, v_c, or both such that each continuous phase fluid stream (108) forms a high shear interface (109) with the dispersed phase fluid (106) at the intersection region (140), wherein the solid samples (102) are drawn to the high shear interface (109) while flowing through the intersection region (140); and
    f. adjusting v_d, v_c, or both to generate droplets (104) encapsulating one solid sample (102) at the droplet shearing junction (145), wherein each droplet (104) is substantially sized to encapsulate said solid sample (102).

2. The method of claim 1, wherein the microfluidic device (100) comprises a first dispersed phase channel (114) comprising one of the flow streams (107) forming the dispersed phase fluid (106), and a second dispersed phase channel (116) comprising the other flow stream (107), wherein the first and second dispersed phase channels (114, 116) merge to form the combining channel (110).

3. The method of claim 2, wherein the microfluidic device (100) further comprises an aqueous phase channel (117) intersecting with the first and second dispersed phase channels (114, 116),

wherein the aqueous phase channel (117) comprises aqueous phase fluid (118), wherein the aqueous phase fluid (118) flows in the combining channel (110) such that the aqueous phase fluid (118) forms a laminar interface stream (119) between the two flow streams (107).

4. The method of claim 2, wherein the dispersed solid samples (102) comprises a plurality of cells flowing in one of the flow streams (107), and a plurality of particles flowing in the other flow stream (107), wherein when flowing through the combining channel (110), laminar flow of the dispersed phase fluid causes the cells to assemble near the sidewall (112a) and the particles to assemble near an opposing sidewall (112b), wherein at the intersection region (140), the cells are drawn to one high shear interface (109a) and the particles are drawn to the other high shear interface (109b), wherein one cell and one particle are co-encapsulated in one droplet (104) as said droplet (104) is formed at the droplet shearing junction (145), wherein the droplet (104) co-encapsulating the one cell and one particle is released from the orifice (147) into the output channel (150).

5. The method of claim 2, wherein the dispersed solid samples (102) are either cells or particles, wherein the dispersed solid samples (102) enter the combining channel (110) from one or both of the first and second dispersed phase channels, wherein one solid sample (102) is encapsulated as the droplet (104) is formed at the droplet shearing junction (145), wherein the droplet (104) encapsulating the one solid sample (102) is released from the orifice (147) into the output channel (150).

6. The method of claim 4, wherein the cells are eukaryotic cells, prokaryotic cells, or a combination thereof.

7. The method of claim 6, wherein the eukaryotic cells are animal cells, plant cells, algae cells, fungal cells, or a combination thereof.

8. The method of claim 6, wherein the prokaryotic cells are bacterial cells.

9. The method of claim 6, wherein the cells are protoplasts, pollen grains, microspores, or tetrads.

10. The method of claim 4, wherein the particles are beads.

11. The method of claim 1, wherein the portions of the first and second continuous phase channels intersect the combining channel (110) orthogonally.

12. The method of claim 1, wherein flow in the microfluidic device (100) is pressure-driven.

13. The method of claim 1, wherein a width of the combining channel, the first continuous phase channel, or the second continuous phase channel ranges from about 30 μm to about 60 μm.

14. The method of claim 13, wherein a length and a width of the intersection region are each about 3

to 5 times the width of the combining channel, the first continuous phase channel, or the second continuous phase channel.

15. A method for encapsulating a solid sample (102) in a droplet (104), comprising:
    a. flowing a first fluid (106) through a first microfluidic channel (110) at a first flow rate (v_d) such that flow of the first fluid is laminar, wherein the first fluid (106) comprises at least two flow streams (107), wherein one or both of said flow streams (107) comprises dispersed solid samples (102), wherein the solid samples (102) assemble near a sidewall (112) of the first microfluidic channel while flowing towards an intersection region (140);
    b. co-flowing a second fluid (108) through each of a second microfluidic channel (120) and a third microfluidic channel (130) at a second flow rate (v_c), wherein the second and third microfluidic channels (120, 130) intersect the first microfluidic channel (110) at the intersection region (140) such that the second fluid streams (108) intersect the first fluid (106) and merge to form a droplet shearing junction (145) within the intersection region (140);
    c. adjusting v_d, v_c, or both such that each of the second fluid streams (108) forms a high shear interface (109) with the first fluid (106), wherein the solid samples (102) are drawn to the high shear interface (109); and
    d. generating droplets (104) at the droplet shearing junction (145) such that each droplet (104) is substantially sized to encapsulate one solid sample or co-encapsulate two different solid samples.

16. A microfluidic device (100) for encapsulating a solid sample (102) in a droplet (104), said microfluidic device (100) comprising:
    a. a combining channel (110) having a dispersed phase fluid (106) flowing therein at a first flow rate (v_d), wherein the dispersed phase fluid (106) comprises at least two flow streams (107), wherein one or both of said flow streams (107) comprises dispersed solid samples (102);
    b. a first continuous phase channel (120) having a continuous phase fluid stream (108) flowing therein at a second flow rate (v_c), wherein a portion thereof is disposed on one side of the combining channel;
    c. a second continuous phase channel (130) having a continuous phase fluid stream (108) flowing therein at the second flow rate (v_c), wherein a portion thereof is disposed on an opposite side of the combining channel;
    d. an intersection region (140) formed by said portions of the first and second continuous phase channels intersecting at a terminal end of the combining channel, wherein the continuous phase fluid streams (108) intersect the dispersed phase fluid (106) at the intersection region (140), wherein a droplet shearing junction (145) is formed within the intersection region (140) as the continuous phase fluid streams (108) merge with the dispersed phase fluid (106), wherein the droplet shearing junction (145) comprises an orifice (147);

e. an output channel (150) fluidly coupled to the intersection region (140) via the orifice (147); and

f. a fluid flow controller (160) configured to perform operations comprising:

i. adjusting v_d of the dispersed phase fluid to establish laminar flow in the combining channel (110) such that the solid samples (102) assemble near a sidewall (112) of the combining channel while flowing towards the intersection region (140);

ii. adjusting v_d, v_c, or both such that each continuous phase fluid stream (108) forms a high shear interface (109) with the dispersed phase fluid (106) at the intersection region (140), wherein the solid samples (102) are drawn to the high shear interface (109) while flowing through the intersection region (140); and

iii. adjusting v_d, v_c, or both to generate droplets (104) at the droplet shearing junction (145) such that each droplet (104) is substantially sized to encapsulate a solid sample (102).

17. The device of claim 16 further comprising a first dispersed phase channel (114) comprising one of the flow streams (107) forming the dispersed phase fluid (106), and a second dispersed phase channel (116) comprising the other flow stream (107), wherein the first and second dispersed phase channels (114, 116) merge to form the combining channel (110).

18. The device of claim 17 further comprising an aqueous phase channel (117) intersecting with the first and second dispersed phase channels (114, 116), wherein the aqueous phase channel (117) comprises aqueous phase fluid (118), wherein the aqueous phase fluid (118) flows in the combining channel (110) such that the aqueous phase fluid (118) forms a laminar interface stream (119) between the two flow streams (107)

19. The device of claim 17, wherein the dispersed solid samples (102) comprises a plurality of cells flowing in one of the flow streams (107), and a plurality of particles flowing in the other flow stream (107), wherein when flowing through the combining channel (110), laminar flow of the dispersed phase fluid causes the cells to assemble near the sidewall (112a) and the particles to assemble near an opposing sidewall (112b), wherein at the intersection region (140), the cells are drawn to one high shear interface (109a) and the particles are drawn to the other high shear interface (109b), wherein one cell and one particle are co-encapsulated in one droplet (104) as said droplet (104) is formed at the droplet shearing junction (145), wherein the droplet (104) co-encapsulating the one cell and one particle is released from the orifice (147) into the output channel (150).

20. The device of claim 17, wherein the dispersed solid samples (102) are either cells or particles, wherein the dispersed solid samples enter the combining channel (110) from one or both of the first and second dispersed phase channels, wherein one solid sample is encapsulated as the droplet (104) is formed at the droplet shearing junction (145), wherein the droplet encapsulating the one solid sample is released from the orifice (147) into the output channel (150).

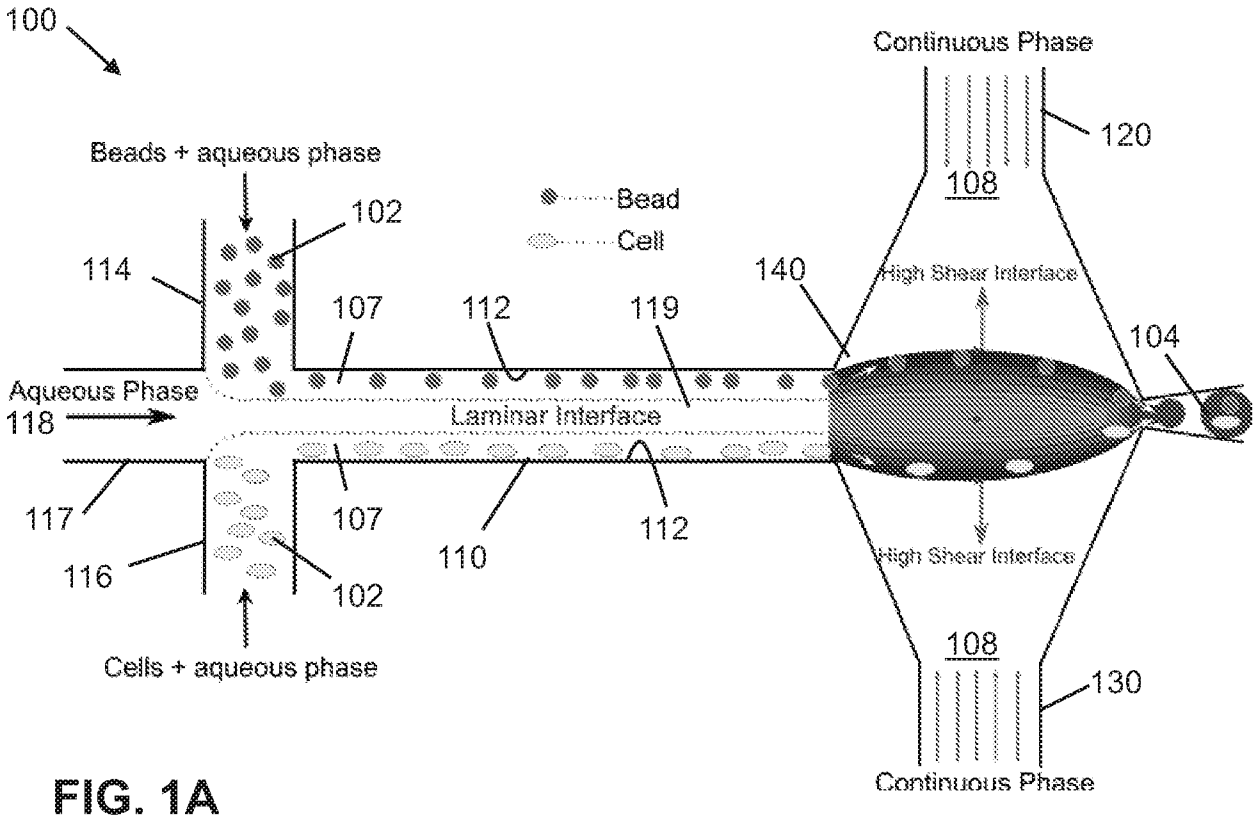
100
Continuous Phase
120
Beads + aqueous phase
102
Bead
Cell
108
114
140
High Shear Interface
107
112
119
104
Aqueous Phase
118
Laminar Interface
117
107
110
112
High Shear Interface
116
102
108
130
Cells + aqueous phase
Continuous Phase

FIG. 1A

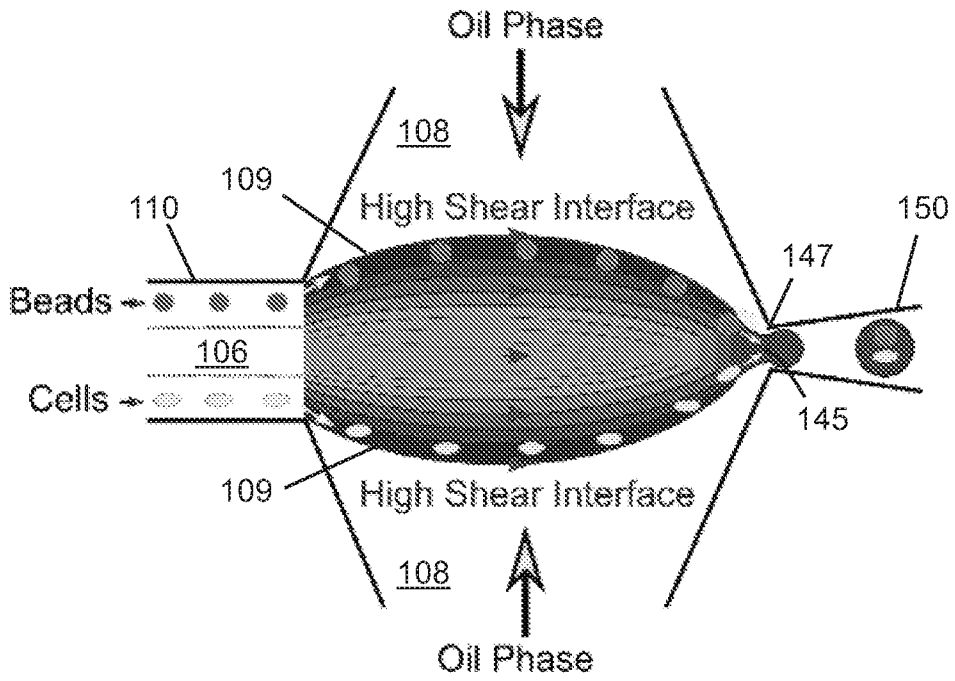
Oil Phase
108
109
110
High Shear Interface
150
147
Beads
106
Cells
145
109
High Shear Interface
108
Oil Phase

FIG. 1B

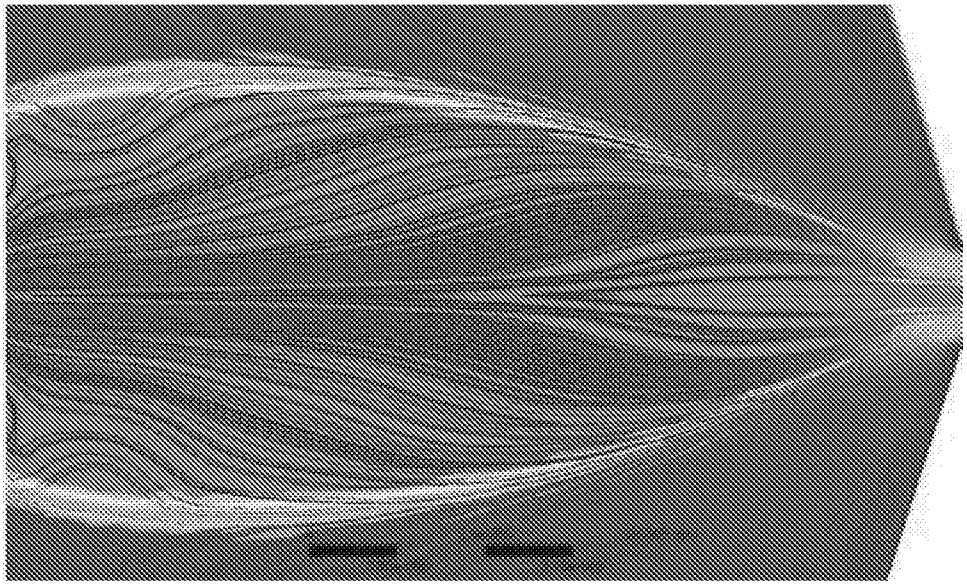

FIG. 2

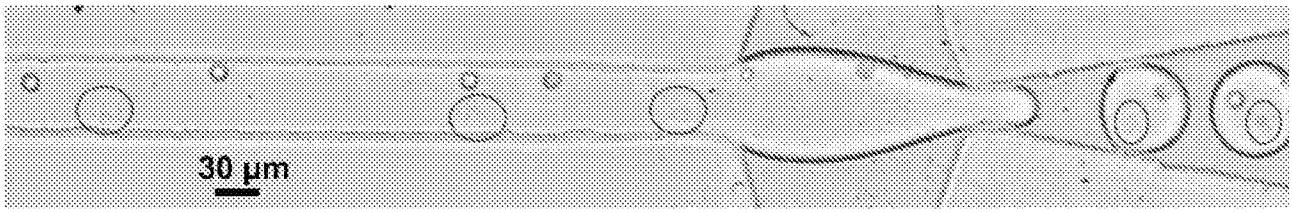

FIG. 3

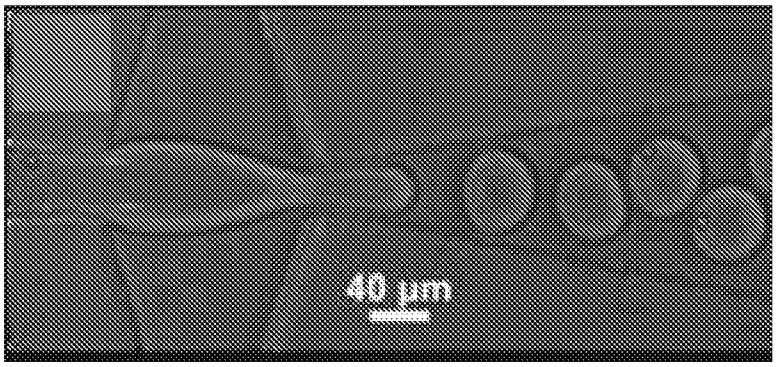

FIG. 4A

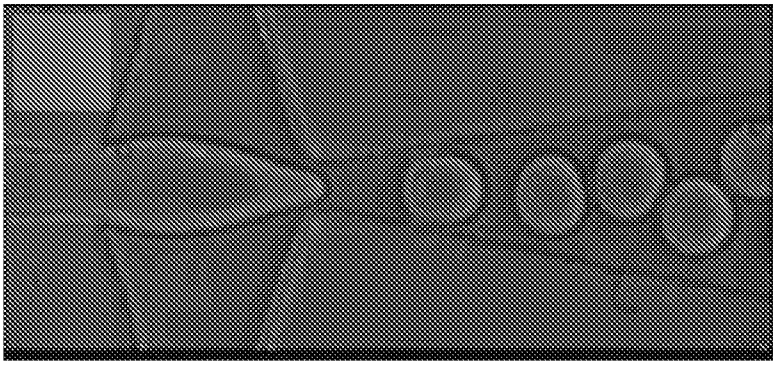

FIG. 4B

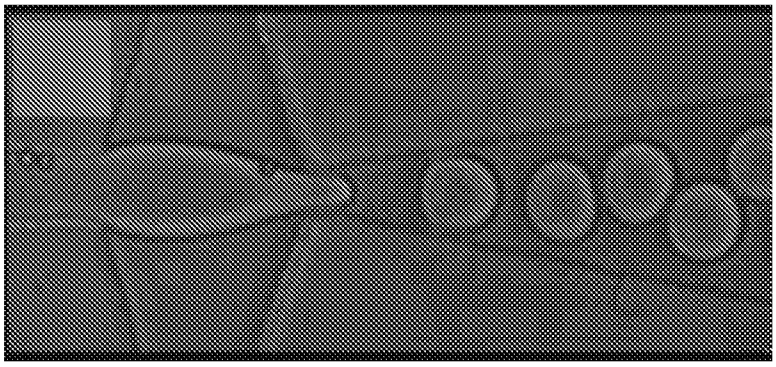

FIG. 4C

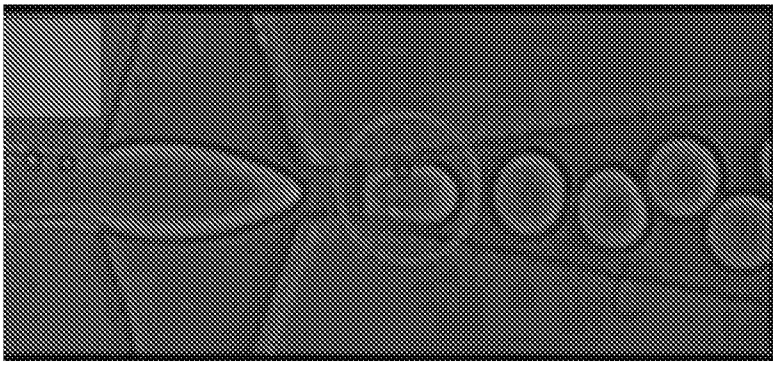

FIG. 4D

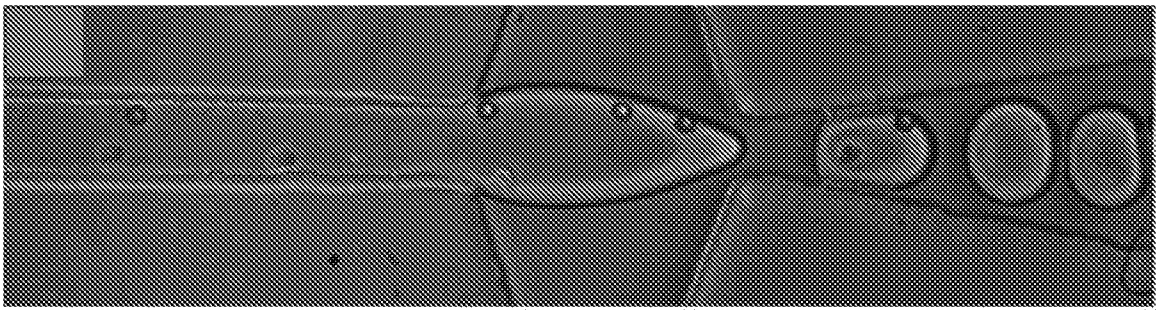

FIG. 5A

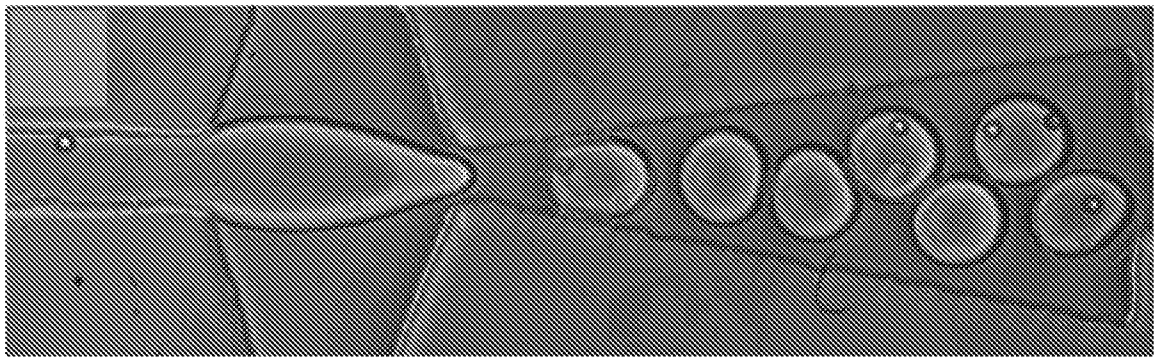

FIG. 5B

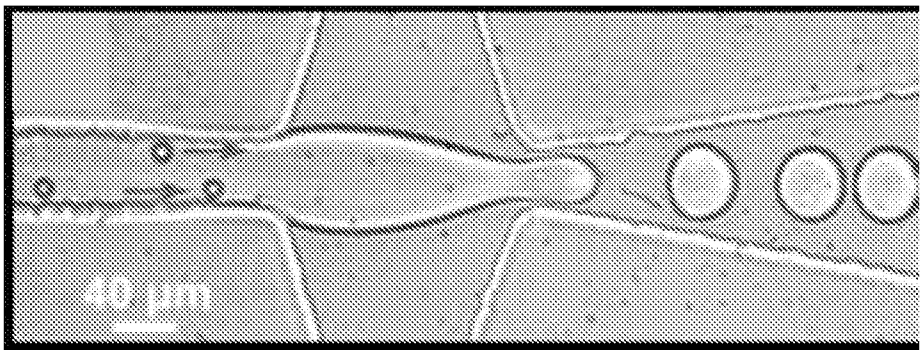

FIG. 6A

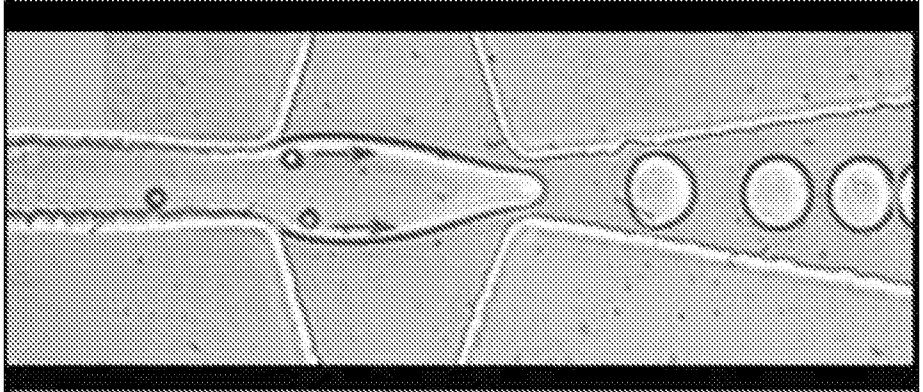

FIG. 6B

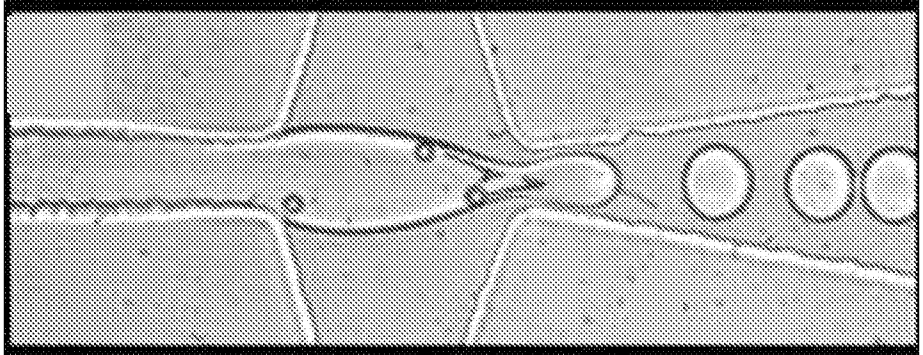

FIG. 6C

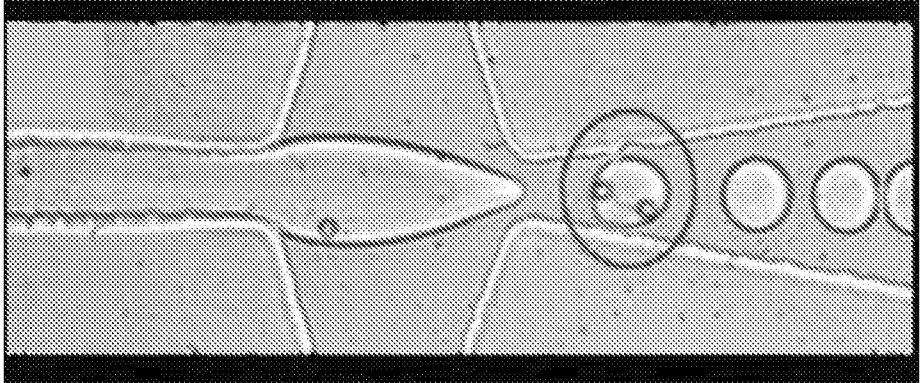

FIG. 6D

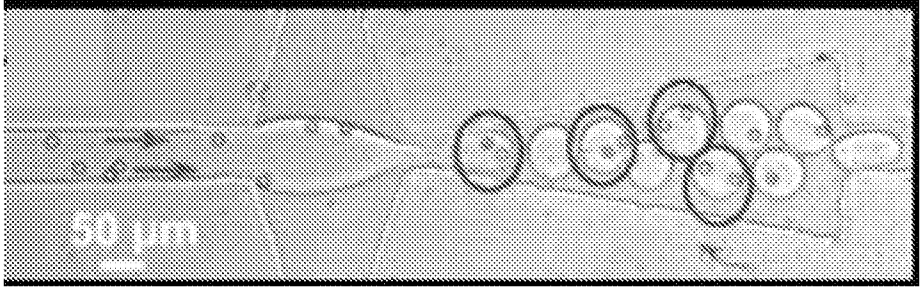

FIG. 6E

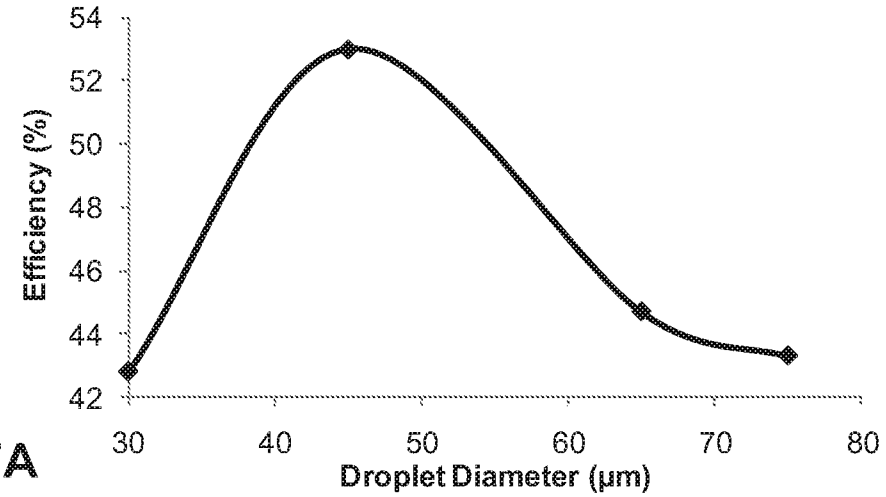

FIG. 7A

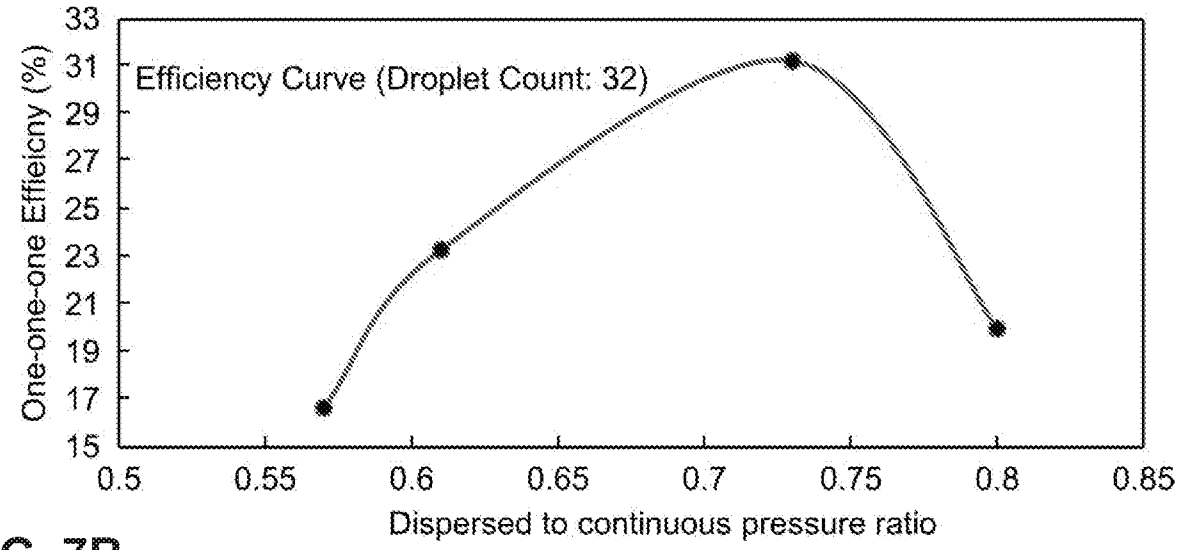

FIG. 7B

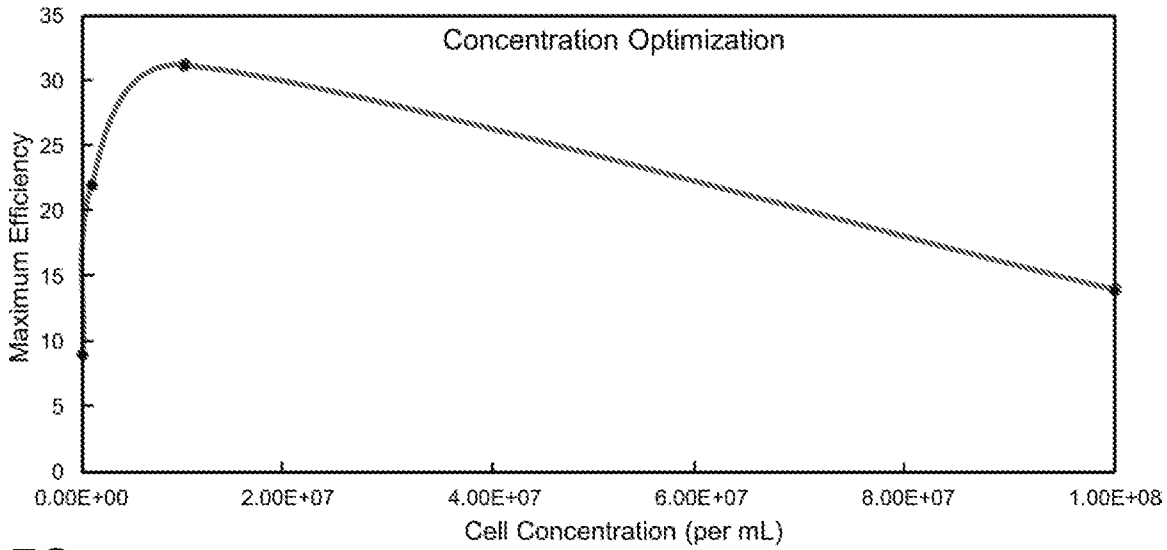

FIG. 7C

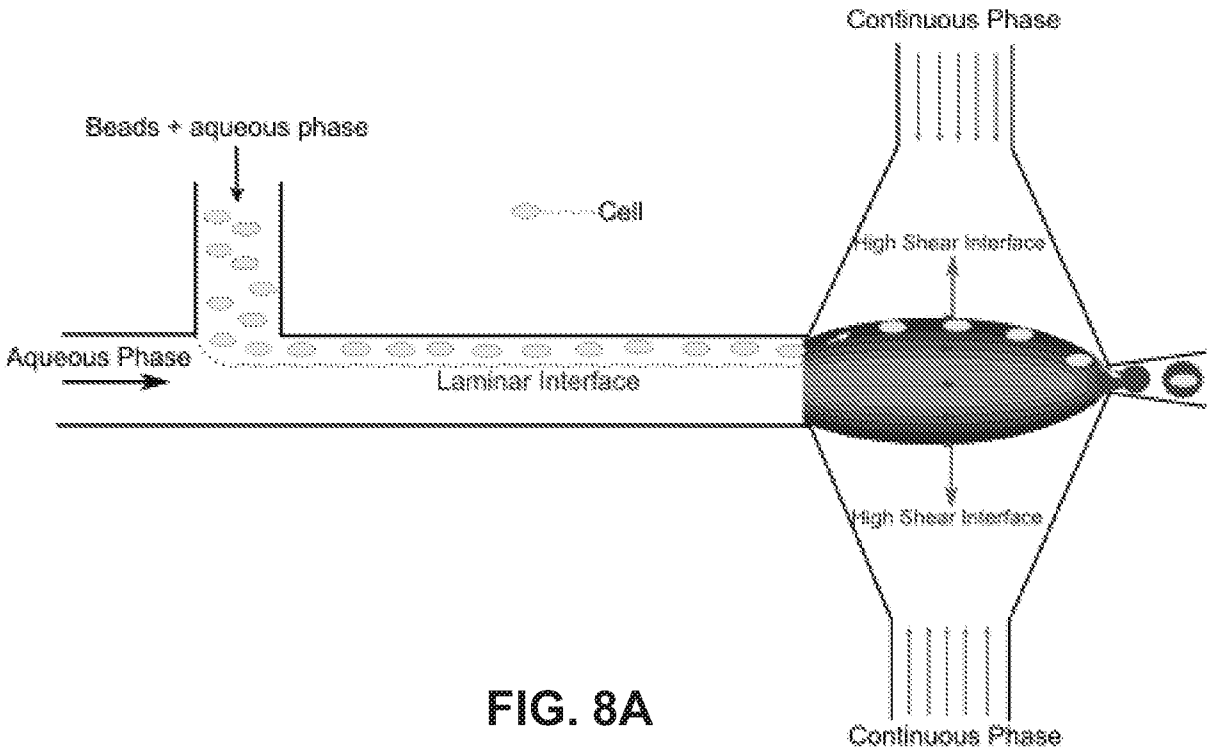
Continuous Phase
Beads + aqueous phase
Cell
High Shear Interface
Aqueous Phase
Laminar Interface
High Shear Interface
Continuous Phase

FIG. 8A

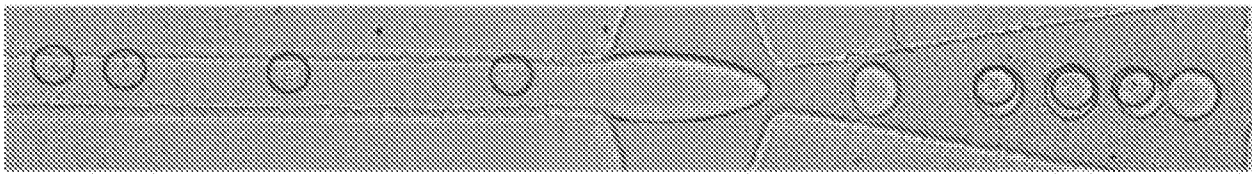

FIG. 8B

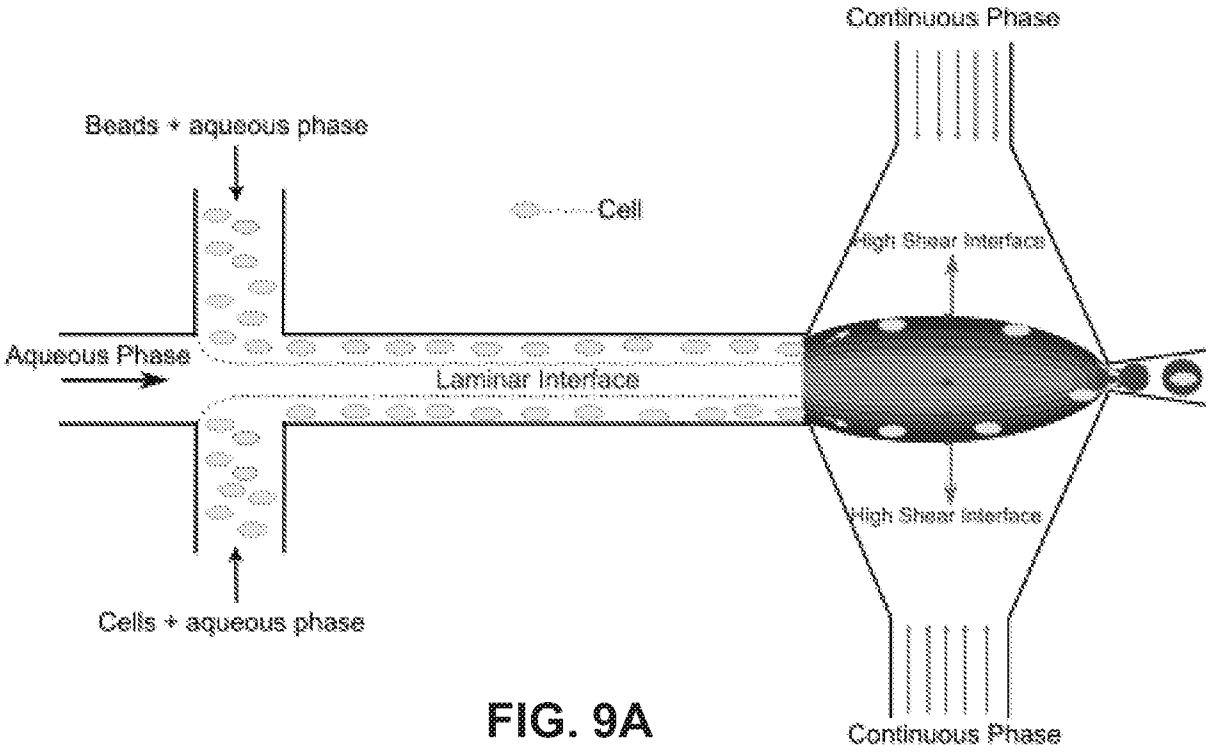
Continuous Phase
Beads + aqueous phase
Cell
High Shear Interface
Aqueous Phase
Laminar Interface
High Shear Interface
Cells + aqueous phase
Continuous Phase

FIG. 9A

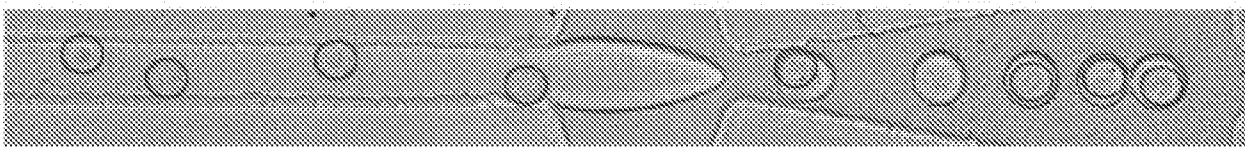

FIG. 9B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/36952

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - B01F 13/00, C12N 5/00 (2018.01)
CPC - B01L 3/5027, B01L 3/502761, B01F 13/0062, B01L 3/502776, B01J 2219/00468, B01L 2200/0636

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2017/0128940 A1 (ILLUMINA, INC.) 11 May 2017 (11.05.2017) the entire document, and more specifically: para [0003], [0053], [0072], [0080], [0132]-[0134], [0182]-[0183]; figure 6; abstract	1-20
A	US 2006/0051329 A1 (LEE et al.) 09 March 2006 (09.03.2006) the entire document, and more specifically: para [0009], [0041], [0044]-[0045], [0047], [0050]; figures 2, 5, 6A and 9; abstract	1-20
A	US 2016/0123858 A1 (THE GENERAL HOSPITAL CORPORATION) 05 May 2016 (05.05.2016); the entire document	1-20
A	US 9,176,504 B2 (CHIOU et al.) 03 November 2015 (03.11.2015); the entire document	1-20
A	US 2011/0285042 A1 (VIOVY et al.) 24 November 2011 (24.11.2011); the entire document	1-20
A	US 2014/0076430 A1 (RAINDANCE TECHNOLOGIES, INC.) 20 March 2014 (20.03.2014); the entire document	1-20

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

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 August 2018	18 SEP 2018

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774