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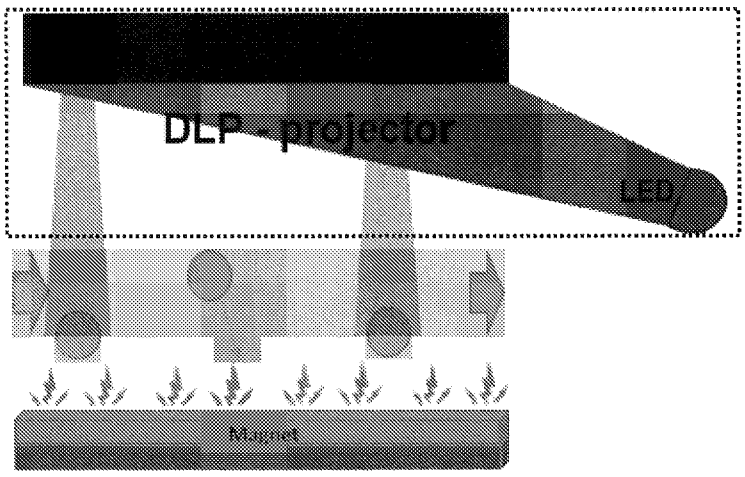

FIG. 1A

(57) Abstract: A system and method for analyzing a phenotype and polynucleotide sequences of a biological particle. The biological particle is optically analyzed by a phenotype analyzer, such as a flow cytometer, to determine a phenotype of the biological particle. A oligonucleotide barcode carrier is generated with the oligonucleotide barcode attached to the carrier. The oligonucleotide barcode has a pre-defined sequence. The biological particle and the oligonucleotide barcode carrier are passed to a microreactor where the biological particle is lysed to release the polynucleotides of the biological particle. The oligonucleotide barcode is ligated to the polynucleotides of the biological particle to form a genetic complex. The resulting genetic complex is sequenced, and the phenotype of the biological particle is matched to the sequence of the polynucleotides of the biological particle.

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SYSTEM AND METHOD OF PHENOTYPE AND POLYNUCLEOTIDE SEQUENCING ANALYSES FOR BIOLOGICAL PARTICLES VIA DETERMINISTIC BARCODING

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/562,115, entitled "On-flow analysis with deterministic barcoding" and filed on September 22, 2017, and U.S. Provisional Application No. 62/577,905, entitled "Combining high-throughput individual particle sequencing with other methods of individual particle analysis via deterministic barcoding" and filed on October 27, 2017. The complete disclosures of said provisional applications are hereby incorporated by reference.

BACKGROUND ART

As used herein, the following terms and abbreviations shall apply:

BP – biological particle with genetic content (e.g., a cell, or an organelle like a mitochondrion).

Phenotype – a set of information regarding functional properties, molecular composition, structure and morphology of an individual BP. For example, a phenotype may be assessable via on-flow optical analysis or imaging.

FC – Flow Cytometry.

FACS – Fluorescently Activated Cell Sorting or, broadly, any FC-based BP sorting.

Genome/transcriptome – all polynucleotide (DNA/RNA) sequences in a given BP.

mSCS – Massive (or high-throughput) Single Cell Sequencing, but for all BPs and not just cells. mSCS reveals genome/transcriptome of each BP in large communities, most commonly via in-drop individual BP barcoding followed by sequencing of combined genetic material. Barcoding of polynucleotide molecules from each BP is performed by using oligonucleotide tags with unique sequences. Most commonly, barcoding systems employ a stream of beads with barcoding tags attached and each bead has random but unique barcoding sequence. Each analyzed BP is paired with a single bead in a microdroplet, where barcoding is performed. Then, microdroplets are combined and sequenced together. After

sequencing of combined polynucleotides from multiple BPs, the barcodes allow identification of sequences from the same particle.

IDBC – Identifiable Deterministically Barcoding Carrier for deterministic barcoding, which consists of an identifiable core and oligonucleotides of a pre-defined sequence attached to this core. “Identifiable” refers to the ability for each particular carrier to be identified (ID), such as by intrinsic unique combination of fluorophores or by RFID, or by its positioning on a chip or in a serial flow.

EOF – ElectroOsmotic Flow.

GIDBC - Generator of Identifiable Deterministically Barcoding Carriers

LIF/LS – Laser Induced Fluorescence/Light Scattering for on-flow detection.

FISH – Fluorescent *In Situ* Hybridization.

Impact of technology to advance biomedical research: With the rapid advancement of bioanalytical and data processing methods, the study of cell and organelle properties in bulk is increasingly becoming obsolete, being replaced by high-throughput analysis of individual BPs. Such a broad-ranging shift in biomedical research is driven by increasing biological evidence emphasizing the significance of BP-to-BP heterogeneity (e.g., cellular heterogeneity within a given population). mSCS now provides unprecedented insight into cellular heterogeneity of gene expression, genetic makeup, extent of DNA damage, regulatory mutations, etc. This genetic heterogeneity extends from the cell in general to cellular organelles like the mitochondrion. In response to environmental, biological, and cellular queues, genetic heterogeneity of BPs is translated to phenotypic heterogeneity. The phenotype of cells can be measured in multiple ways, but FC provides for one of the most high-throughput methods for assessing cellular phenotypes (e.g., functional properties, morphology). Simultaneous knowledge of phenotype and genome/transcriptome of each BP in a large population would be transformative for biomedical research.

The present invention is beneficial for analysis of BPs, including but not limited to: host somatic cells, host transformed cells, pathogenic biota, symbiotic biota, mitochondria (especially in oocytes), spermatozoids, platelets (for mitochondrial content), nuclei, nucleoli, ribosomes, viruses, and exosomes.

What is missing and urgently needed in biomedical research is an instrument or high-throughput technological platform that can unambiguously match mSCS information for a specific cell with phenotypic data for that same cell. The randomness of barcoding oligonucleotide sequences in existing mSCS platforms does not provide for identification of a particular BP of polynucleotide origin, it only establishes if polynucleotides originate from the same BP or not. This prevents matching of FC-measured phenotypic properties to genome/transcriptome of individual BPs. Perhaps, the closest technology that does match some phenotypic properties with transcriptomes of individual BPs is a recently reported approach termed CITE-SEQ where antibody-oligonucleotide tagging enables limited phenotype identification using cell surface markers; however, this platform does not realize comprehensive high-throughput coupling of single cell genomic/transcriptomic and phenotypic data. The present invention overcomes these limitations of current mSCS methodologies available to the biomedical research community.

Emerging opportunities and applicability: Precise knowledge of genome/transcriptome and corresponding phenotype for each BP in a heterogeneous population in essential for deeper insight into many biomedical problems. Some broad ranging opportunities that this invention will advance include: analysis of cancer development such as polyclonal seeding in metastatic cancers, defining links between stem cells and cancer, understanding of therapy resistance development and manifestation of genetic causes of phenotypes, studies of individual virus/bacteria/pathogen-cell interactions, studies of complex microbial communities and their interactions with host, defining roles of mitochondrial heteroplasmy in compromising of apoptotic antiproliferation, etc. In the following paragraphs, we briefly expand on some of these biomedical applications to provide a sampling of the power of this enabling technology:

Defining links between stem cells and cancer: Stem cell therapy is effective for treatment of certain pathologies, limited in part by potential carcinogenicity. The stem cell population is necessarily heterogeneous due to DNA damage, mutation load, and environment-induced transcriptome variability. This heterogeneity

manifests itself in phenotypic variations, sometimes carcinogenic. Hence, in-depth analysis of genome and transcriptome manifestations in phenotype for large populations is essential.

Studies of carcinogenesis: Carcinogenesis is typically a stochastic process where a combination of genetic changes (often mediated via retroviruses, transposons, plasmids, viral factors) and regulatory influence (changing transcriptome) leads to a malignant phenotype. It is critical to reveal genotype-transcriptome-phenotype links at the single-cell level in large cellular communities.

Polyclonal cancer studies: Cancer cells often have increased mutation rate and, as a result, increased adaptability. The mutagenic nature of most cancer therapies increases the adaptability further. Early detection of clones resistant to drugs and immunotherapies is essential, especially for dispersed cancer cells. Detection of cells with an unusual metabolism, apoptotic signature, or senescent response combined with immediate knowledge of corresponding mutations in the genome/transcriptome of the suspected cells would help to identify particularly malignant clones, and subsequently modify therapy.

Studies of cell-virus interactions: Interaction of cells with viral particles, and oncoviruses in particular, is a very heterogeneous process where only a small fraction of cells may be susceptible to the infection depending on cellular status and transcriptome. Detection of cell-virus interactions and of the subsequent phenotypic changes with immediate knowledge of corresponding transcriptome and fate of viral genetic material for each cell would be important for virulence studies.

Bacterial community dynamics: Bacterial communities, including the human microbiome, are highly heterogeneous. The reaction of microbiomes to changing environments such as abundance of proteins, fat, host digestion-resistant and digestible carbohydrates, presence of toxins, therapeutic agents, or introduction of new possibly pathogenic species is extremely complex. Bacteria can adapt their phenotype via individual or collective response, dedicating particular organisms for suicidal release of biocides, or exhibiting regulatory mutations. There is currently no technology available to deeply investigate these complex and cell-specific changes

without directly measuring functional properties, morphology, biomolecule content, transcriptome, and genome of every bacterium in a very large community.

Study of the role of mitochondria heteroplasmy in impairment of mechanisms of apoptosis/senescence/mitogenesis: Mitochondria heteroplasmy studies can define links between mtDNA mutations and damage, transcription, functionality, and biomolecule content. Investigation of how a cell tags individual mitochondria for mitophagy, replication, or initiation of apoptotic processes is essential for understanding anti-cancerous mechanisms and their progressive malfunction over a lifespan, as well as for aging-related mitochondria neural and muscular degeneration, infertility, and inherited pathologies.

DISCLOSURE OF THE INVENTION

The present invention is directed to a system and method for integration of polynucleotide sequencing and phenotype analyses of biological particles via deterministic barcoding. The system and method preserves links between non-destructively assessable (e.g. optically) phenotype of the biological particle and the sequencing data of the polynucleotide content of the biological particle in a high-throughput analysis.

More specifically, the present invention is directed to a system for analyzing the phenotype and the polynucleotide sequences of a biological particle, comprising a phenotype analyzer, wherein the phenotype analyzer is operable to determine a phenotype of the biological particle; a generator of identifiable, deterministically barcoding carriers, wherein a oligonucleotide barcode of pre-defined sequence is attached to the identifiable core of the barcoding carriers; a microreactor configured to receive the biological particle from the phenotype analyzer and to receive the barcoding carrier from the generator, to release the polynucleotides from the biological particle, to perform reverse transcription if needed for RNA analysis, to attach the oligonucleotide barcodes from the carrier to the polynucleotides from the biological particle, and to amplify the resulting barcoded polynucleotides; and a sequencer to sequence the barcoded polynucleotides.

In addition, the present invention is directed to a method for analyzing the phenotype and polynucleotide sequences of a biological particle, comprising the

steps of non-destructively analyzing the biological particle to determine a phenotype of the biological particle; recording the phenotype of the biological particle; generating a barcoding carrier, wherein a oligonucleotide barcode with a pre-defined sequence is attached to the identifiable core of the barcoding carrier; passing the biological particle and the identifiable oligonucleotide barcode carrier to a microreactor and pairing the barcoding carrier with the biological particle; lysing the biological particle to release the polynucleotides of the biological particle; reverse transcribing RNA from the biological particle (if needed), ligating the oligonucleotide barcode to the polynucleotides of the biological particle to form barcoded polynucleotides; sequencing the barcoded polynucleotides to determine sequences of the polynucleotides from the biological particle; and matching the phenotype of the biological particle to the sequences of the polynucleotides of the biological particle.

These and other features, objects and advantages of the present invention will become better understood from a consideration of the following detailed description of the preferred embodiments and appended claims in conjunction with drawings as described following:

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A shows a schematic of a GIDBC. The particular embodiment of the invention in Fig. 1A uses IDBC identification by position, reversible immobilization by magnetic field and photo activation for oligonucleotide growth. Magnetic beads (circles) are shown reversibly trapped in chip surface indentations by external magnetic field (sparks). Trapped beads are individually illuminated for deprotection and nucleotides are subsequently attached.

Fig. 1B is a top view of SU-8 master of a chip prototype with the microfluidic channel meandering over the photoprocessed area and the magnetic traps in the channel marked by arrows.

Figs. 2A-2C show schematics of an element of the cross-flow chip for oligonucleotide synthesis. Fig. 2A shows after the reagent is introduced to the left reservoir and left region of the channels. Fig. 2B shows after the reservoir is filled with insulating media to brake the electrical contact between the channels. Fig. 2C

shows after electric potential is applied to the upper channel's electrodes, which electroosmotically moves the reagent into the working region. The upper horizontal channel in the chip may be used to add a nucleotide to all crossing in the upper row, then de-protect anchors on these crossings to subsequently add a nucleotide to some of them via the vertical channels.

Fig. 2D is a schematic illustrating tracked mixing synthesis. An on-flow detector is used for tracking each IDBC through each round of nucleotide attachment. A four-way valve directs one-quarter of the suspension of IDBCs to chambers for attachment of A, T, G, C nucleotides, correspondingly. After A, T, G and C nucleotide attachment, IDBCs are returned to the mixer from all four chambers and are ready for next step of barcode growth, As a result, a mixture of IDBCs with unique barcodes is complimented by a database where each IDBC's ID is linked to particular barcode sequence.

Fig. 3 is a schematic showing the components of the present invention in five blocks. Block A is a cytometer or fluorescence-activated cell sorting device, Block B is a cell accumulator with a camera for verifying the position of reference beads (the darkest three circles), Block C is a GIDBC with IDBC (e.g. beads) held in individual traps while pre-defined oligonucleotides are grown via selective photo activation using a DLP projector, Block D is a barcoding droplets generator, and Block E is a synchronization detector.

Figs. 4A-4C show examples of integrated chip layouts. Fig. 4A shows a complete instrument-on-chip with hydrodynamic traps. Figs. 4B-4C show complete instrument layouts with magnetic traps. Fig. 4B shows a layout with minimized outlets, while Fig. 4C shows a layout with a dual GIDBC and no loop morphology.

Fig. 5 is an example chip layout with all components integrated except the GIDBC. The chip layout includes a separate on-flow IDBC detection area for synchronization (E1).

Figs. 6A-6E show layouts for two-layer PDMS chips. Fig. 6A shows a mask for a 5 μm thick layer containing traps. Fig. 6B shows a mask for 40 μm thick layer containing channels. This layout is suitable for making both 105 mm (inner circle) and 130 mm (outer circle) 2-layer masters with extra chips at corners. It contains

several kinds of chips, including chips for evaluating optimal geometry of channels and traps. Fig. 6C shows a layout example containing five testing regions (3x3 mm squares filled with a meandering channel with traps to hold 900 and 1800 beads each), oval inlets with filters, rectangular inlets without filters, and on-flow detection region terminated by the outlet (center-left). The top-right insert shows magnified traps. Fig. 6D shows an image of SU-8 master made using masks in Fig. 6A and Fig. 6B. Fig. 6E is an image of a region of a completed PDMS/glass chip that is half-filled with running buffer. The traps are visible only in air-filled half of the channel due to higher difference in refractive index.

Fig. 7A shows an example of a chip mask layout with hydrodynamic traps. Fig. 7B is magnification of the traps from Fig. 7A. Fig. 7C shows an element SU-8 master made with the layout of Fig. 7A and Fig. 7B. The arrow marks a fluidic shortcut and a trap for a bead.

Figs. 8A-8D show magnetic traps of different morphologies. Figs. 8A-8C show closer images of different channel-trap combinations for the chip layouts shown in Figs. 4C, 6C, and 6E. Fig. 8D shows a region of a completed PDMS/glass chip made using the master shown on Fig. 6D and half-filled with running buffer. The traps are visible only in air-filled half due to a higher difference in refractive index.

Fig. 9 shows a schematic of GIDBC embodiment with independent EOF control of media flow in each horizontal channel. The vertically-oriented side channels allow for parallel flushing during oligonucleotide synthesis. Electrodes (double triangles) allow for independent EOF-driving media in individual horizontal channels after the vertical channels are filled with insulator. The large circles represent electrolyte reservoirs. Pulse laser pumping could be used instead of EOF to selectively propel media.

BEST MODE FOR CARRYING OUT THE INVENTION

With reference to Figs. 1A-9, the embodiments of the system and method of the present invention may be described. The present invention is directed to a system and method of phenotype and polynucleotide sequencing analyses for DNA/RNA-containing particles via deterministic barcoding. The system and method

is directed to the goal of preserving links between optically assessable individual BP's phenotypes and the BP's genome/transcriptome in a high-throughput analysis. As opposed to current mSCS methods with beads carrying unique but random oligonucleotide barcodes, the deterministic barcoding approach of the present invention employs an IDBC for each BP and applies a unique and pre-defined barcode to each polynucleotide of the BP. Hence, the IDBC is a key element of this invention.

To design an IDBC, the inventors considered combinations of identifiable cores and deterministic oligonucleotide growing procedures. As identifiable cores, the inventors considered: (1) beads in serial flow where a particular IDBC is identified by a serial number based on its positioning in the consecutive outflow; (2) an array of on-chip micro wells or spots where the identity of a particular spot is defined by coordinates of the spot; (3) and beads with build-in unique identification tags that are identifiable on-flow. The latter includes beads with: (a) multiple optical tags (e.g., fluorescent or surface-enhanced Raman tags); and (b) radio-frequency identifiable (RFID) beads.

Oligonucleotides with known, pre-defined sequences are then grown on the identifiable cores of IDBC discussed above to yield IDBC through one of the following growth procedures: (1) photo activation; (2) cross-flow synthesis; or (3) tracked mixing synthesis.

Photo activation is a well-established technology for generating pre-defined oligonucleotides on surface of a chip. Briefly stated, protective groups are removed in selected spots by light illumination and one nucleotide (A, T, G or C) is attached to growing oligonucleotides in the activated spots. This process is repeated for the other three nucleotides. Next, these four cycles are repeated N-times for N-nucleotide long oligonucleotides, generating up to 4^N unique spots.

Photo-activated synthesis can produce multitude spots with oligonucleotides with spot-specific, pre-defined sequences. The number of the regions and thus different sequences is generally unlimited and depends only on size of the chip and resolution of the process. Currently, the most effective approach for photo activation utilizes Digital Light Processing (DLP), which enables millions of independent spots

for unique sequences in a single processing.

This technology can be applied "as is" to oligonucleotide growth on chip spots (identifiable core option #2), and with changes to oligonucleotide growth on identifiable beads (identifiable core option #1 and #3). For the latter, reversible immobilization of beads in pre-defined spots of a chip is needed, as shown in Figs. 1A-1B, 3, 6C, 6E, and 7A-9.

Beads can be reversibly immobilized (or trapped) in pre-defined spots of a microfluidic channel that are aligned over the photo processed chip area to place maximum number of these spots in the area. Magnetic, hydrodynamic, dielectrophoretic, chemical, vacuum, and inertial-based immobilization approaches are all options. In the magnetic approach, magnetized (e.g. superparamagnetic) beads are held by external magnetic field in surface indentations or traps, as shown in Figs. 1B, 3, 4B, 4C, 6C, and 6E. In the hydrodynamic approach, beads are held in smaller-than-beads microfluidic flow shortcuts, as shown in Figs. 4A and 7A-C. In the dielectrophoretic approach, beads are held in electrode-defined traps by alternating electromagnetic field with frequency defined by bead size. In the chemical approach, beads are chemically immobilized using cleavable bonds, (e.g. covalent, ion-pair, hydrophobic, Van-der-Waals, hydrogen, etc.), but binding should not interfere with oligonucleotide synthesis and should be completely reversible. Gravity force can be used for very low flows; and centrifugal force can be used for chips in rotating holder. In the vacuum approach, beads are held in traps by external suction.

Out of these alternatives, magnetic immobilization as shown in Fig. 1A is the preferred option, closely followed by the hydrodynamic and dielectrophoretic approaches. In summary, the inventors have identified the photo activation method to grow barcoding oligonucleotides on serial-flow IDBC with magnetic, hydrodynamic or dielectrophoretic trapping employed for positioning as the preferred embodiment of the present invention.

Cross-flow synthesis is an alternative for instances where photo activation is not suitable. In this alternative, oligonucleotides are grown on the identifiable cores at intersections of individually-controlled microfluidic flows, as shown in Figs. 2A-C.

A chip with a grid of microfluidic channels with cores at channel intersections and electrodes placed at the end of each channel is suggested. Electrodes are used to generate EOF between each chosen pairs of electrodes and selectively drive media between them. Alternatively, laser pulsing can be used to drive this media. Terminals of channels on each side of the chip are connected to reservoirs. The reservoirs are step-wise filled with reagents for a particular protected nucleotide attachment to the growing tags and then with an insulated media. The combination of alternating filling of intersecting channels and changing nucleotide type results in unique and known sequence on the IDBC at each intersection. For greater flexibility in the resulting sequences, one set of channels may be filled by an anchor deprotecting reagent, while the other with the nucleotide-attaching reagent. Alternatively, media can be driven by pulse laser.

Tracked mixing synthesis is a preferred method of growing oligonucleotides on the IDBC if cores with intrinsic ID (RFID or optical) are used. An example of IDBC with intrinsic ID is a core with ID made by combination of 16 fluorophores, which are simultaneously detectable in many commercial cytometers and allow for $2^{16} = 65,536$ unique IDs. Half of the IDBC sample can be modified by one fluorophore, mixed with an unmodified half, and this cycle repeated with other fluorophores. Other means of identification (e.g. RFID, magnetic, optical non-fluorescence, etc.) are also possible. Combinations of different IDs (e.g. fluorescent and RFID) further increase the number of unique core IDs.

In existing mSCS with random barcoding, a set of four reactors introduces one nucleotide on each step of synthesis. After each step, all beads are mixed and divided into four fractions for the next step. But for IDBCs with intrinsic ID cores, the inventors suggest interconnection of these reactors and a mixer with microfluidic lines with an on-flow detector, as shown in Fig. 2D. As a result, each transition of IDBCs between the mixing chamber and reactors is recorded, and the full history of motion for each IDBC, which defines the barcode sequence, is available. Beads that acquired the same sequence (i.e. passed through exactly the same path at all synthesis cycles) can be sorted out from final mixture using a sorter activated by the same on-flow detector.

IBCs generated through these cores and growth procedures enable several deterministic barcoding approaches which integrate non-disruptive phenotype analyses (e.g. flow cytometer or microscopic imaging) of biological particles with massive or high-throughput single cell sequencing analyses of the genome or transcriptome of the biological particles. IDBC allows for individual labeling of each biological particle after nearly any phenotype analysis, and linking the phenotype of those biological particles to their individual sequencing data. Population size of the analyzed biological particles is limited only by performance of sequencing, which is a fast growing field. This ensures that this invention will not only be useful in current biomedical research applications, but will be even more useful in the future ones. Even with current sequencing technologies, analyses could include millions of particles with smaller genomes/transcriptomes or small targeted genetic elements. The deterministic (or known and pre-defined) barcoding of the present invention is capable of measuring any combination of optically-assessable phenotypic properties including but not limited to: (1) membrane potential, membrane fluidity, and membrane electropermeabilization; (2) intracellular pH, ions, and oxygen; (3) total DNA/RNA content, copy number variation, chromosome analysis, and sorting; (4) protein expression, protein modification, and localization; (5) presence of intracellular, nuclear, and surface antigens; (6) enzymatic activity; (7) cell viability, volume, and morphological complexity; (8) multidrug resistance; (9) adherence (e.g., pathogen-host cell); (10) apoptosis via measurement of DNA degradation, mitochondrial membrane potential, and permeability changes; and (11) cell pigments and transgenic products *in vivo* (e.g., green fluorescent protein).

Preferred Embodiment:

In one preferred embodiment, the IDBCs are generated using beads in serial flow as the identifiable cores with photo activation as the procedure for oligonucleotide growth on the beads. Using these IDBCs in the system of the present invention can establish a one-to-one correspondence between high-throughput individual cell sequence information and flow cytometry data.

As shown in Fig. 3, a FC collects and records single BP specific information and directs sorted BPs into the system via an optional cell accumulator. In parallel, a

microfluidic chip reversibly immobilizes beads in a pre-defined array. Several steps of photo-activated synthesis produce multitude of IDBCs containing oligonucleotides with a pre-defined, unique sequence for each identifiable core (i.e., bead). The outflow of IDBCs from this chip is combined with outflow of BPs from the BP accumulator in a chip in droplets (i.e. microreactors) that are being generated with IDBC-BP pairs for barcoding and sequencing. Deterministic barcoding of BP's polynucleotides allows for matching sequencing data for these polynucleotides with cytometric and imaging data on the BP of polynucleotide origin.

In this preferred embodiment, as shown in Fig. 3 with each component letter-labeled, the system is comprised of the following components:

Component A: Flow cytometer with optional sorter;

Component B: BP accumulator with a BP and reference beads imager to confirm and track the relative positioning of the BPs and reference beads;

Component C: GIDBC with another reference beads imager to confirm and track the relative positioning of the barcoding beads and the reference beads;

Component D: Chip for water-in-oil droplet/microreactor generation, where release and barcoding of BP polynucleotides occurs; and

Component E: On-flow detector of reference beads for error correction if the relative positioning is incorrect.

The system allows for complete integration of all components on a single chip (as shown in Figs. 4A-C), combination of separate chip connected by microfluidic lines, and a combination of on-chip capillary based and macro-scale devices (as shown in Figs. 3 and 5).

The inventors manufactured various PDMS chips (Figs. 6A,B,D), including prototypes of separate GDBBs (Figs. 6C, 6E, and 7A-8D) complete instruments on-chips (Figs. 4A-C), and intermediate variants (e.g. a chip with all components except the GDBB (i.e. including microfluidics for FACS, BP- accumulator, droplet generator, and synchronization detector)) (as shown in Fig. 5).

For chip fabrication, a 2-layer soft lithography process, with 5μm layer of traps and 40μm layer of channels, for core diameter from 5 to 10um was used. The inventors manufactured a lithography mask for each layer (Figs. 6A-6B), SU-8

master (Fig. 6D) and PDMS casts. The inventors cut PDMS casts to individual chip size and glued them to activated glass support or PDMS slabs to fabricate chips (Fig. 6E). Resulting chips have a glass side for optical access and PDMS side for fluid lines connection and magnet influence (Fig. 1B).

A detailed description of each component of the system and its alternatives is provided below:

(A) Cytometer/sorter

A flow cytometer is used for on-flow phenotype analysis and sorting BPs of interest. While most cytometers and other on-flow particle analyzers could be used, either a capillary-based scheme (Fig. 3A) or on-chip scheme (Figs. 4A-5) is preferable.

BPs can be driven through a microfluidic channel by pressure, EOF, laser pulsing or other means well-known to those skilled in the art. The inventors suggest EOF and laser pulsing as primary driving means because they are fast responding and require no moving parts. BPs can be detected in-channel or in a shear-flow cuvette by LIF/LS. In-channel detection is easier to implement and use, while detection in shear-flow cuvette could provide better signal to noise ratio and higher throughput. Sorting can be activated by fluorescent, light scattering or any other signal from the detector and performed using flow redirection via EOF, laser pulsing driving or other means well-known to those skilled in the art. EOF driving is easier to implement, while laser pulse driving is less dependent on channel wall surface chemistry and provide better throughput. As shown in Fig. 3, the portion of the sample that does not meet the sorting criteria is passed out of the system as waste.

(B) Cell accumulator

An accumulator is optionally used to (i) timely decouple sorting and droplet generation to avoid cross-talk between flow drivers of different components, and (ii) imaging of reference beads for error correction.

BPs are loaded in a long microfluidic channel (Fig. 3) or more complex microfluidic systems (similar to shown in Figs. 7A-C and 9) and can be supplied in the same order to component D of the system at any convenient time. While loaded, BPs with reference beads can be imaged on-chip to verify order and load via

number of BPs between reference beads. Deep imaging can also provide additional information about BPs phenotype (e.g. BP shape), complementary to information acquired via on-flow detector.

While this component is recommended to enhance the capabilities of the system, it is not absolutely necessary. If not employed, BPs could be directly supplied to component D after sorting from block A. In contrast, this component with advanced imaging could be used not only in addition to, but also as a substitute to, block A. In this instance, phenotypic data is acquired exclusively via imaging, although this would also decrease capability of the system.

(C) GIDBC

In this preferred embodiment, the core of the IDBCs are beads in serial flow where each particular bead is identified by a serial number (i.e. position in the outflow) to track its position relative to other carrier beads and reference beads (Figs. 3, 4A-C, 6C, 7A-C, and 8A-D). As discussed above, the barcoding oligonucleotides are grown on the beads via photo activation (Fig. 1). In this embodiment, reversible immobilization of beads in “traps” located in pre-defined spots on-chip is essential. These traps are positioned in a microfluidic channel that occupies as much of photo processed chip area as possible. Out of possibilities described above, in this preferred embodiment, magnetic (Figs. 1A-B, 2C, 4A-C, 6C, 6E, and 8A-9) or hydrodynamic (Fig. 7A-C) traps are used.

The geometry of the magnetic traps depends on the size of the beads and the hydrodynamic chip properties. For beads with 8 um diameter, the inventors manufactured chips with channel widths from 20 to 80μm to find an optimal balance between load, seriality control and clogging resistance; 5-20μm size circular, triangular and trapezoidal trap shapes for optimal loading and release control; and 50 to 100μm inter-trap distance to evaluate possible optical cross-talk and to control seriality of elution with the inter-trap distance change (Figs. 6A-E). As an alternative embodiment, the inventors also made chips with a set of hydrodynamic traps (Fig. 4A and Fig. 7A-C). Chips with dielectrophoretic traps were also considered, but were given lower priority.

GIDBC core trapping optimization: For magnetic immobilization, traps can be not only isotropic (i.e. symmetrical relative media flow), but also anisotropic to facilitate immobilization and release, depending on media flow direction, and simplifying IDBC rotation management, if needed. For isotropic traps, circular geometry is preferred, while triangular or trapezoidal traps are suggested for anisotropic traps (Figs. 8A-D). To improve bead coverage by oligonucleotide tags, a bead rotation in traps can be implemented. Rotation can be induced via superposition of sheer force of the media flow and trapping force (magnetic, dielectrophoretic, etc.). Alternatively, optical deprotection of anchors can asymmetrically expose charged groups, creating EOF-based rotation momentum. Furthermore, rotating the electromagnetic field can induce the momentum given appropriate polarizability of beads.

GIDBC bead release optimization: Bead release will be induced by removing the trapping force while shear force of the media flow is present. However, additional means of release control and suppression of unwanted sorption can be implemented as needed. These include (1) inverted or alternating field, (2) reversing media flow for anisotropic magnetic traps as described and hydrodynamic traps, (3) ultrasonic driver and/or pulse laser illumination, (4) reversal of surface charge, (5) use of chaotropic agents, and (6) bead rotation.

GIDBC bead outflow seriality control: For serial flow of the beads, it is important to ensure that the order of beads is retained in the outflow. For the monitoring of this order, the inventors suggest use of optically-labeled beads as a reference to mark positions of the beads on the chip. With these beads it is possible to determine whether the number of untagged beads between optically labeled ones remains the same in the outflow and to compare 2D-imaging of optically-tagged reference beads on traps in the bead processing regions of GIDBC with the data from on-flow LIF/LS detector (Figs. 3-5).

High-throughput bead processing: For thousands of beads, the simplest, single-channel chips (Figs. 3, 4B, 4C, 6C, 6E, 8A-D) with pressure, laser pulse or EOF-driven elution is sufficient. However, for millions of beads and larger chips, flow resistance can be too high for driving via single channel. In this case, channels

can be filled in parallel, and eluted subsequently (see example with hydrodynamic traps in Fig. 4A and Figs. 7A-C). A more universal scheme is presented in Fig. 9. In this design, channels can be filled and processed in parallel, and eluted sequentially. Inter-channel flow junctions can be blocked by solidifying media (e.g., photoresist) followed by pressure-driven elution. EOF-driven chips would also allow for parallel loading and processing. The excessive inter-channel electric junctions can be blocked by insulating media, allowing precisely-controllable, channel-by-channel EOF-driven elution. Pulse-laser pumping could be employed as well. Scaling up would also require higher resolution of optical processing where optical cross-talk could emerge. Undesired light propagation suppression might be required via refractive index control, surface treatment for light extinction, and processing area size increases.

(D) Generator of barcoding microreactors in droplets.

Outlines for a chip for water-in-oil (WIO) droplet/microreactor generation for BP polynucleotides barcoding are well-developed in variety of commercial instruments for mSCS. Briefly, a stream of water-based media, containing barcoding beads, analyzed BPs and all needed reagents meets two streams of oil supplied from the side at a junction, followed by local channel narrowing (Figs. 4A-C and Fig. 5D) where droplets of water in oil are formed.

Each droplet should contain no more than one BP because all polynucleotides in the droplet will have the same droplet-specific barcode and polynucleotides from multiple BPs in the same droplet will not be distinguished after sequencing. A droplet with no BP causes no problems besides wasting efficiency. As result, in commonly employed chips, the ratio of droplets to BPs is maintained high to statistically decrease the number of doublets and multiplets of BPs per droplet. In contrast, in the preferred embodiment, the sorter/accumulator can supply BPs to the droplet generator in a more controlled manner, greatly decreasing the amount of wasted droplets and barcoding beads. This level of the flow control is achievable because driving media by laser pulses or EOF has very low response time to signals from LIF/LS on-flow detector, defined by the rate of pulsing or capacity of electrodes, respectively.

Each droplet should also contain one barcoding bead. In a droplet without a bead, barcoding is impossible and BPs sequencing information will be lost. If a droplet contains multiple beads, different barcodes will be applied to polynucleotides from the same BP, causing problems with sequencing data processing in commonly employed chips. In contrast, in the preferred embodiment, synchronization and error correction (see below) can identify beads participating in such events and sequencing data processing will not suffer.

Each droplet should also contain reagents for BP lysis, polynucleotide-barcode ligation, amplification, and sometimes reverse transcription. These reagents can be supplied with a stream(s) of water-based media, within barcoding beads or migrate into a droplet from oil environment.

Just like in existing mSCS, after co-encapsulation of a BP, IDBC and reagents in a droplet, the BP is lysed to release the polynucleotides, RNA is reverse-transcribed (if necessary), and ligases are used to add the oligonucleotide from the IDBC to the polynucleotide of the BP. In this regard, the polynucleotides of the BP are now barcoded. Amplification, if necessary, can be performed in-droplet or after droplets merging and oil removal in preparation for sequencing.

(E) Auxiliary on-flow synchronization detector for error correction.

A simplified secondary on-flow LIF/LS detector is implemented for droplet outflow analysis (Fig. 3, Fig. 4A-C, Fig. 5) for detecting and counting optically tagged reference beads. While using the same principles with LIF/LS detector of the cytometer/sorter, this detector needs a single scattering channel and two fluorescence (or SERS)-tag detecting channels to detect reference beads, hence sensitivity can be low. The data acquisition rate should correspond to droplet generation rate. Basic imaging could also be beneficial for this detector. As result, low budget solutions such as a 3-color webcamera are sufficient for this component. Use of data acquired in this block is described in the synchronization and error correction section below.

Synchronization and error correction using optical and gene tags: Linking a database of barcode sequences from the GIDBC with cytometric data is prone to errors and provides risk for the analysis. Errors may originate from the bead

processing chip (i.e. GIDBC) because spots of oligonucleotide synthesis may be empty, occupied by multiple beads, or beads may sorb outside the spots. Errors may also originate from the in-drop pairing chip because the ratio of one BP to one bead per drop might be compromised. Errors may originate from the flow cytometer because doublets or multiplets of particles can be detected as a single event. In each of these cases, these errors will manifest themselves in mismatching of the indexes in the barcode database and cytometric data (i.e. phenotype data associated with each BP and tracked by the BP ordering within the system). These mismatches have to be detected and corrected. The inventors suggest two independent correction mechanisms: post-sequencing and pre-sequencing.

For the post-sequencing method, particles (e.g., reference beads, liposomes) containing known polynucleotide tags and optical tags can be added to the BPs to be analyzed. Any mismatch between expected bead sequence (based on FC data and BP-accumulator image) and the sequence of the tagged particle can be detected after sequencing. Then, an appropriate correction can be applied.

For the pre-sequencing method, optically tagged reference beads can be added into the IDBC (one color) and to the BPs (another color), with the secondary on-flow detector (Figs. 3-5) controlling mismatches. For the beads, it will measure the distance (number of un-marked beads) between the marked ones and compare this distance to the initial reference image of the GIDBC. For BPs, it will measure whether the number of detected and sorted barcoding BPs (based on on-flow detector and BP-accumulator image) corresponds to the number of BPs between reference beads. Applied in parallel, these methods provide robust and reliable error correction.

Alternative Embodiments:

In alternative embodiments, other identifiable cores are utilized. For example, when an array of on-chip micro wells or spots is used as the identifiable core where the identity of a particular spot is defined by coordinates of the spot, this invention may be used with chips capable of confining and localizing reaction of biological particles with spots of barcoding media, including, but not limited to, micro wells on the chip surface that confine separate BPs or gel barriers introduced optically,

electromagnetically or mechanically. In this approach, imaging is used to access phenotypes of BPs instead of on-flow methods.

When IDBC with build-in unique identification tags that are identifiable on-flow (e.g. RFID or optical tags, as described above) are used as the identifiable cores, oligonucleotides can be grown using all mentioned methods, but tracked mixing synthesis is the most appropriate (as described above, see Fig. 2D). Solution of these IDBC along with corresponding on-flow ID detector can be employed in the preferred embodiment instrument design instead of chip-based GIDBC, eliminating the need of multi-step oligonucleotide growing for each GIDBC bead load.

The present invention has been described with reference to certain preferred and alternative embodiments that are intended to be exemplary only and not limiting to the full scope of the present invention. While elements of the proposed device are synergistic, some of them could be eliminated without total loss of functionality. For example, flow cytometry-based cell sorting can be eliminated at expense of higher IDBC consumption and sequencer load. Flow cytometry could be eliminated and substituted by image analysis of the biological particle accumulator (as described above). Even flow cytometer, droplet generator and bead-based IDBC could be eliminated if the GIDBC and the biological particle accumulator are integrated and the identifiable barcoding carrier is directly printed on chip surface.

In the preferred or alternative embodiments, it is not necessary to grow oligonucleotide barcodes on the identifiable cores prior to each use of the system. IBCs can instead be prepared in advance or even supplied as a disposable cartridge for analysis when using (i) loaded disposable GIDBC chip; (ii) chip with barcoding spots; or (iii) solution of IDBC with on-flow identifiable cores with appropriate database linking core ID (serial number, position or RFID/optical tags) of each IDBC element with the sequence of its oligonucleotide barcodes, as described above.

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WE CLAIM:

1. A system for analyzing a phenotype and a sequence of polynucleotides of a biological particle, comprising:

an phenotype analyzer, wherein said phenotype analyzer is operable to determine a phenotype of said biological particle;

a generator of a oligonucleotide barcode carrier, wherein a oligonucleotide barcode is attached to said carrier and comprises a pre-defined sequence;

a microreactor configured to receive said biological particle from said phenotype analyzer and to receive said oligonucleotide barcode carrier from said generator; and

a sequencer of said pre-defined sequence of said oligonucleotide barcode and said sequence of polynucleotides of said biological particle after said oligonucleotide barcode is ligated to said polynucleotides of said biological particle.

2. The system of claim 1, wherein said phenotype analyzer is a flow cytometer.

3. The system of claim 1, wherein said phenotype analyzer is a microscope.

4. The system of claim 1, wherein said carrier is a magnetic bead.

5. The system of claim 1, wherein said carrier is an on-chip micro well.

6. The system of claim 1, further comprising a biological particle accumulator between said phenotype analyzer and said microreactor.

7. A method for analyzing a phenotype and a sequence of polynucleotides of a biological particle, comprising the steps of:

optically analyzing said biological particle to determine a phenotype of said biological particle;

recording said phenotype of said biological particle;

generating a oligonucleotide barcode carrier, wherein a oligonucleotide barcode is attached to said carrier and comprises a pre-defined sequence;

passing said biological particle and said oligonucleotide barcode carrier to a microreactor;

lysing said biological particle to release said polynucleotides of said biological particle;

ligating said oligonucleotide barcode to said polynucleotides of said biological particle to form a genetic complex;

sequencing said genetic complex to determine said sequence of said polynucleotides of said biological particle; and

matching said phenotype of said biological particle to said sequence of said polynucleotides of said biological particle.

8. The method of claim 7, wherein said biological particle and said oligonucleotide barcode carrier are encapsulated in a droplet in said microreactor.

9. The method of claim 7, wherein a flow cytometer performs the step of optically analyzing said biological particle to determine a phenotype of said biological particle.

10. The method of claim 7, wherein said biological particle is passed to a biological particle accumulator before said biological particle is passed to said microreactor.

11. The method of claim 7, further comprising the step of analyzing an outflow exiting said microreactor for errors.

12. The method of claim 7, wherein said step of generating an oligonucleotide barcode carrier comprises growing an oligonucleotide on a bead.

13. The method of claim 12, wherein said step of growing an oligonucleotide on a bead comprises photo-activation.

14. The method of claim 12, further comprising the step of immobilizing said bead in a trap on a chip.

15. The method of claim 7, wherein said step of generating an oligonucleotide barcode carrier comprising growing an oligonucleotide on a chip.

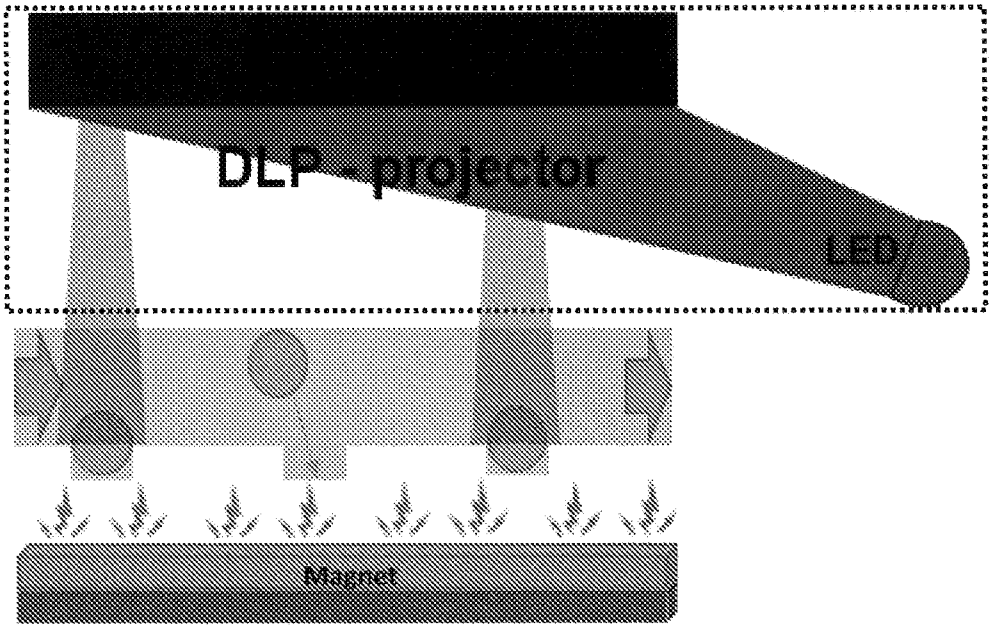

FIG. 1A

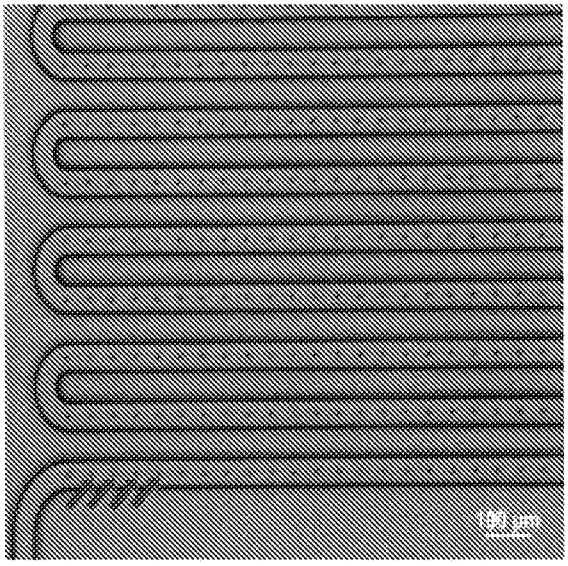

FIG. 1B

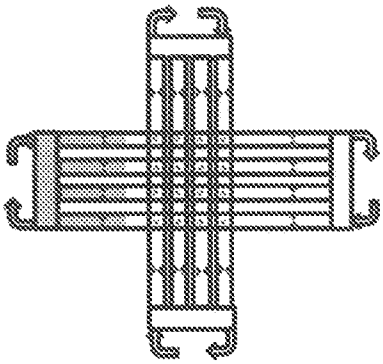

FIG. 2A

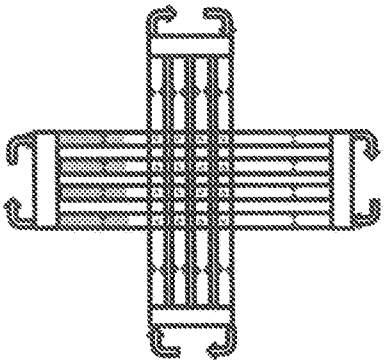

FIG. 2B

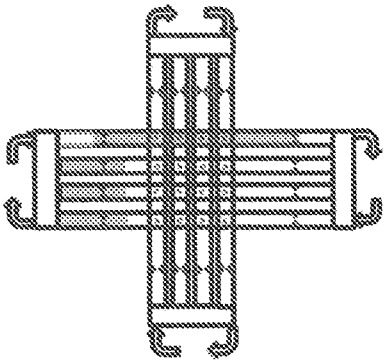

FIG. 2C

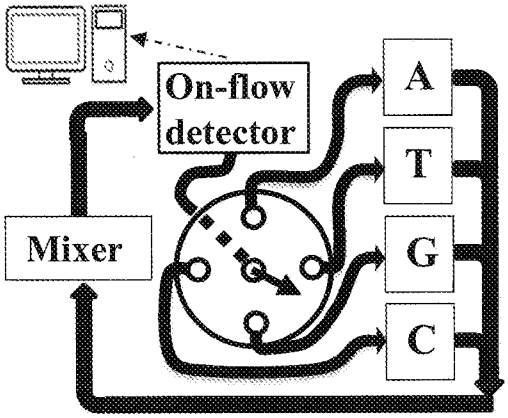

FIG. 2D

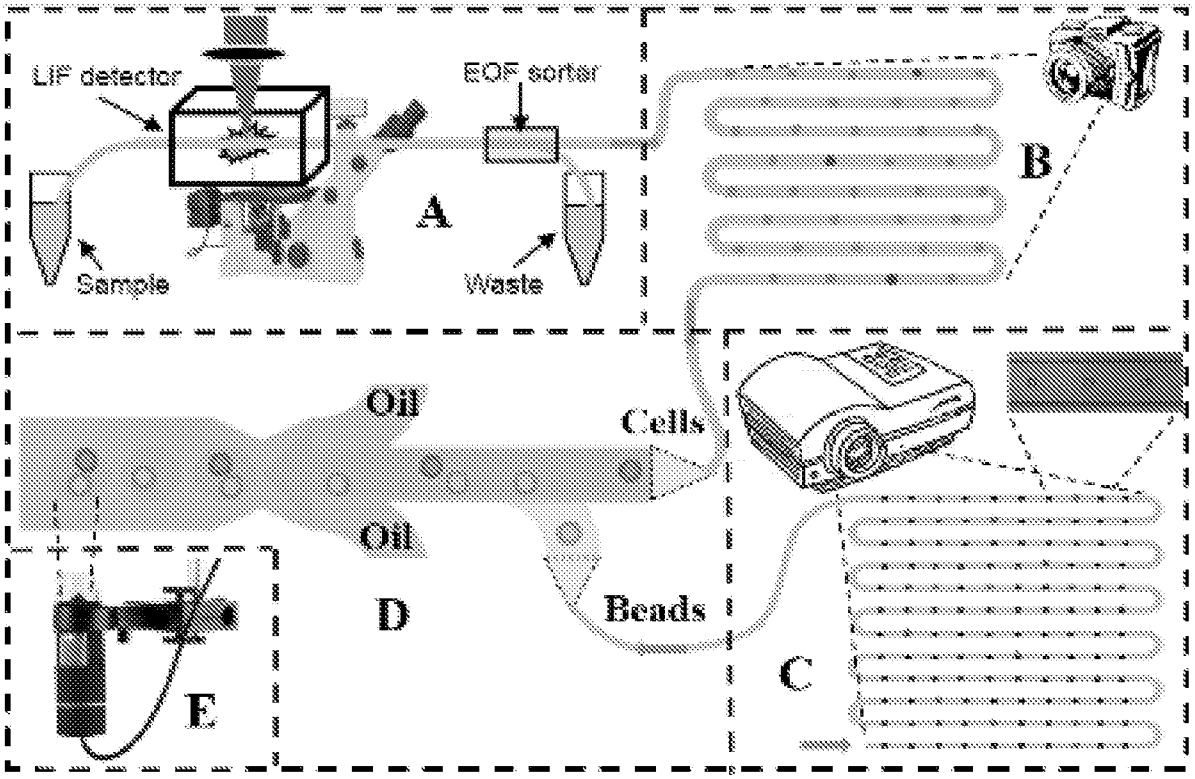
LIF detector
EOF sorter
Sample
Waste
A
B
Oil
Cells
Oil
Beads
D
C
E

FIG. 3

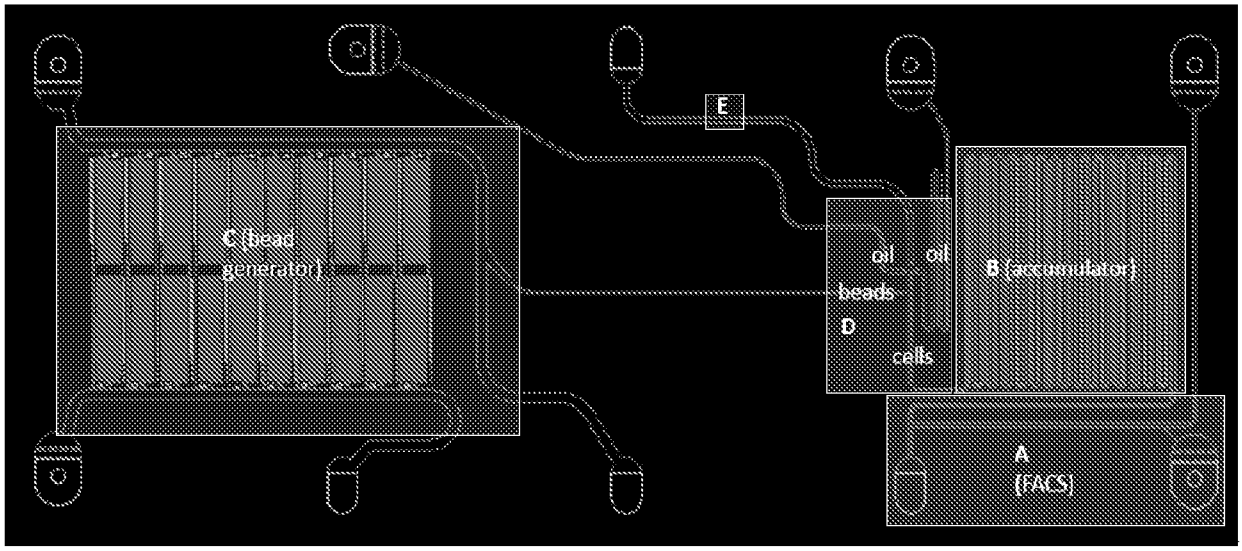

FIG. 4A

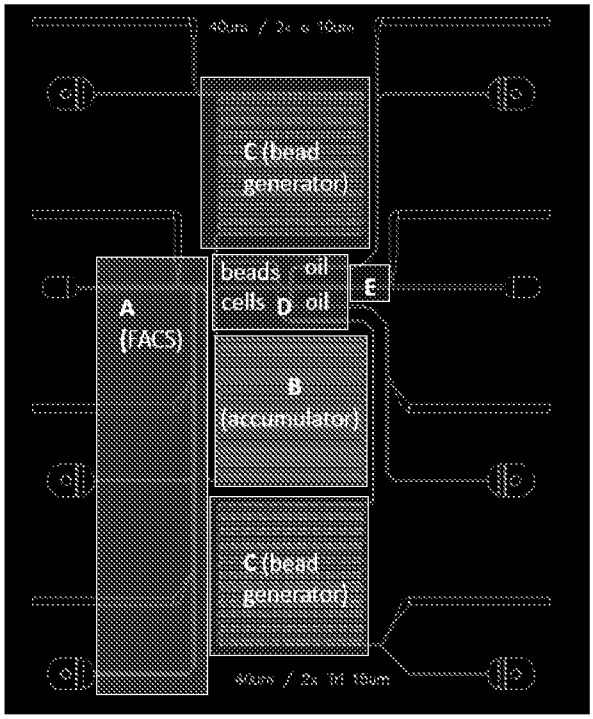

FIG. 4B

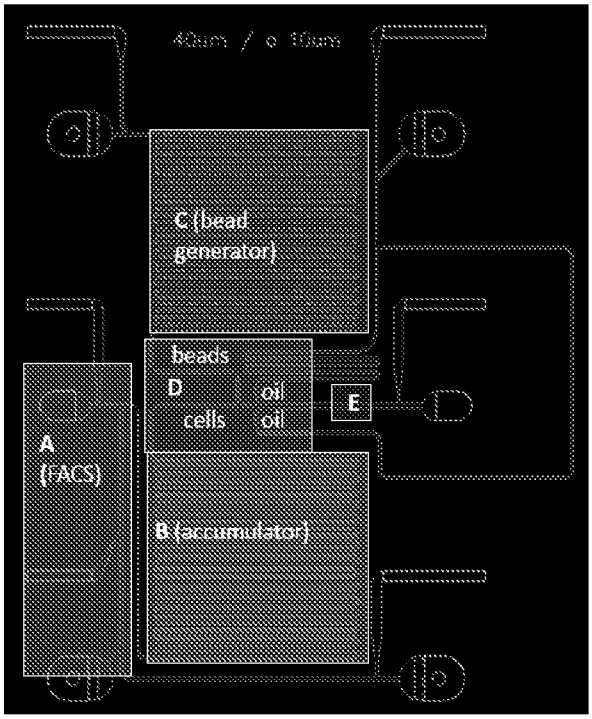

FIG. 4C

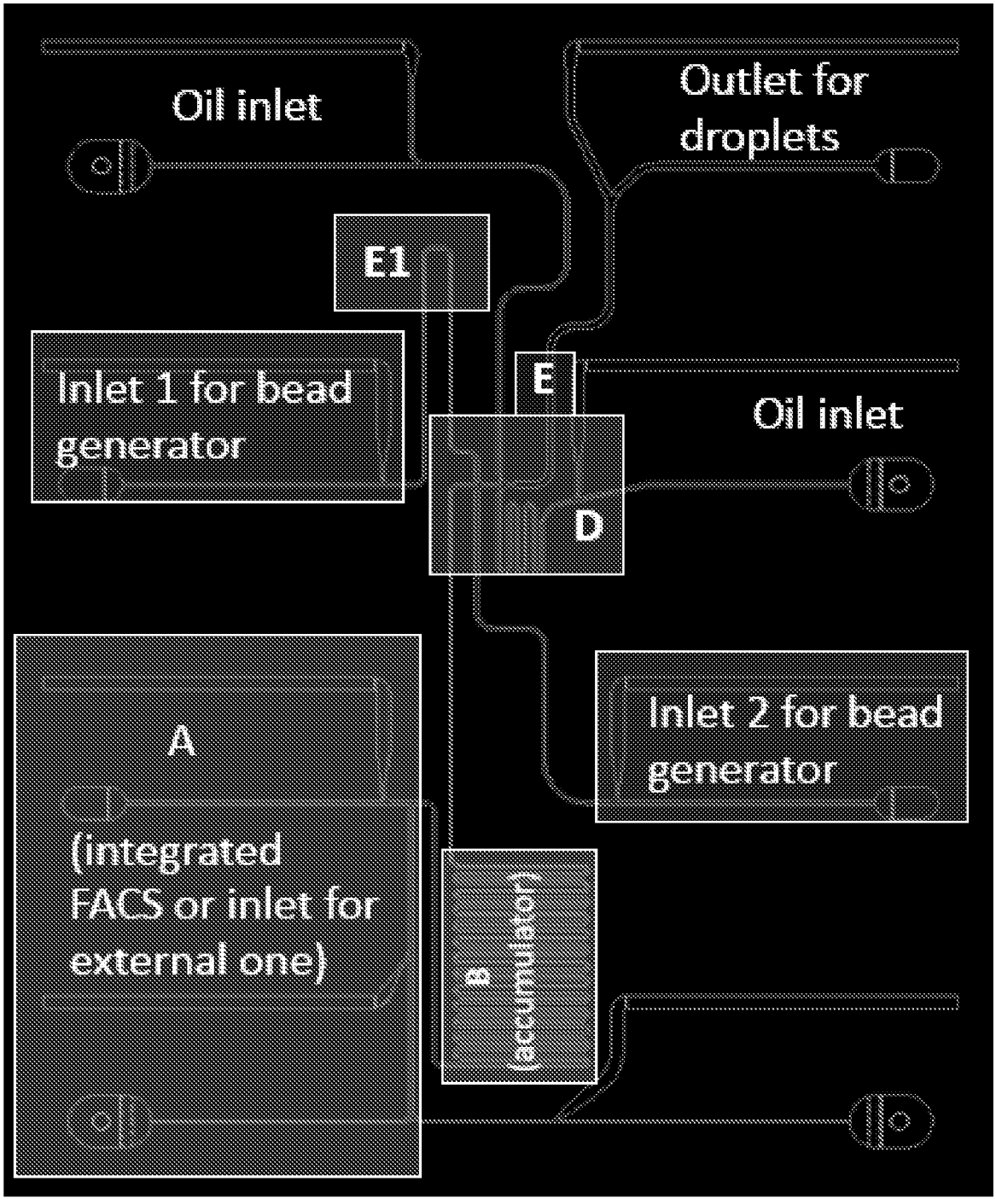
Oil inlet
Outlet for droplets
E1
Inlet 1 for bead generator
E
Oil inlet
D
A
(integrated FACS or inlet for external one)
B (accumulator)
Inlet 2 for bead generator

FIG. 5

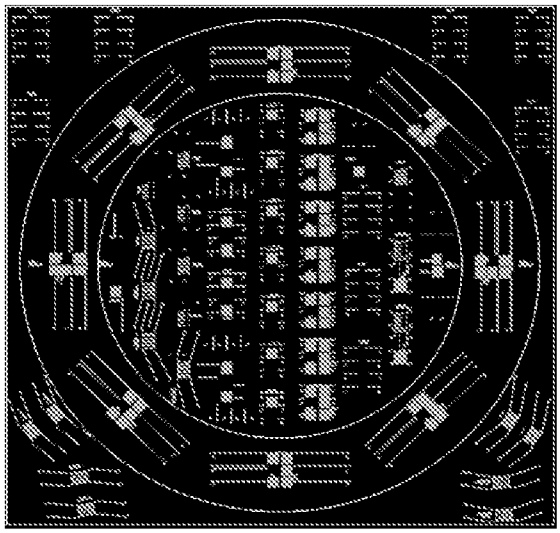

FIG. 6A

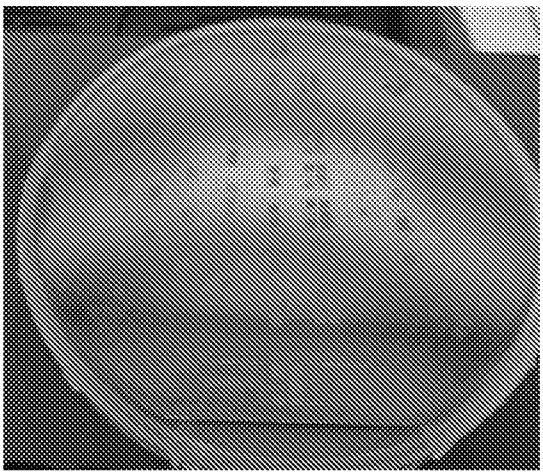

FIG. 6D

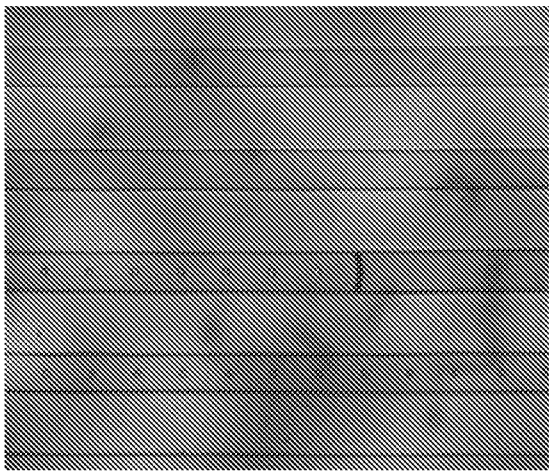

FIG. 6E

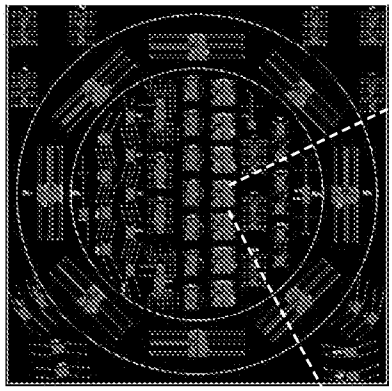

FIG. 6B

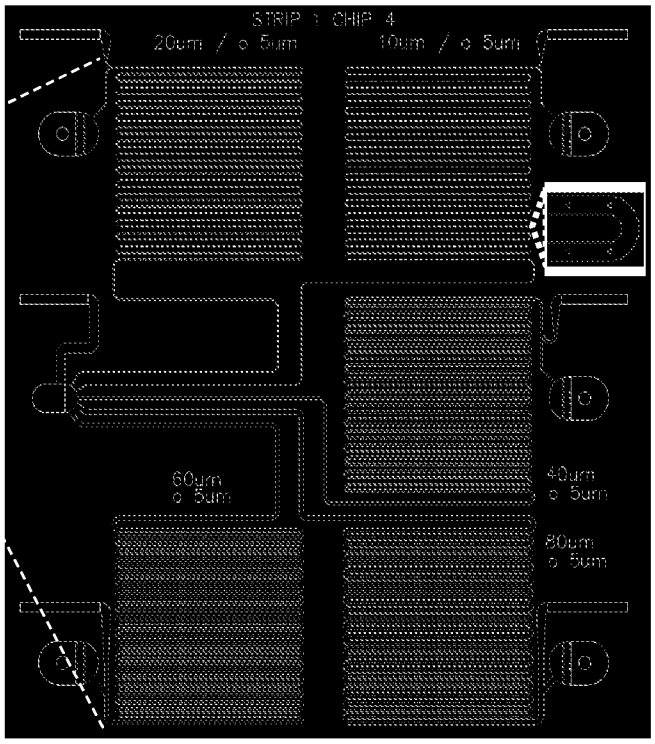
STRIP 1 CHIP 4
20um / o 5um
10um / o 5um
60um
o 5um
40um
o 5um
80um
o 5um

FIG. 6C

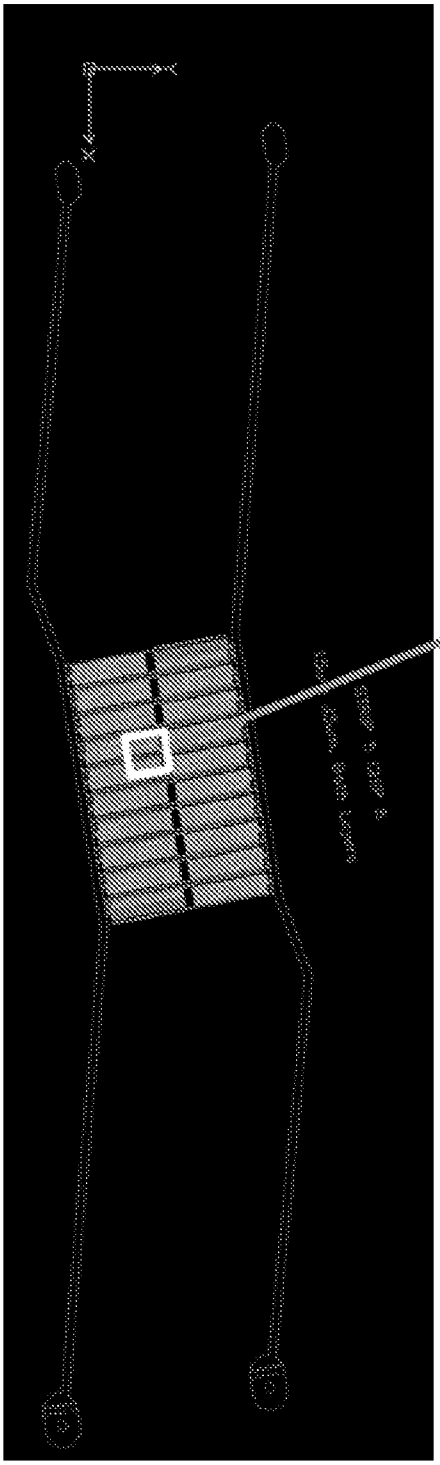

FIG. 7A

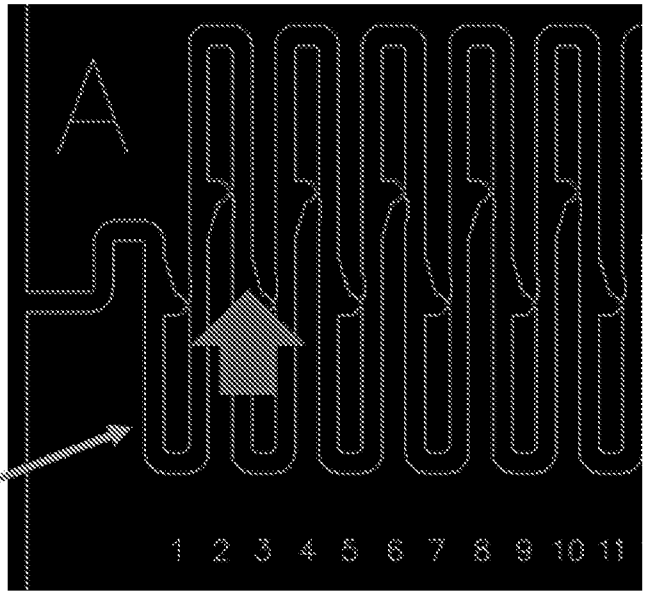

FIG. 7B

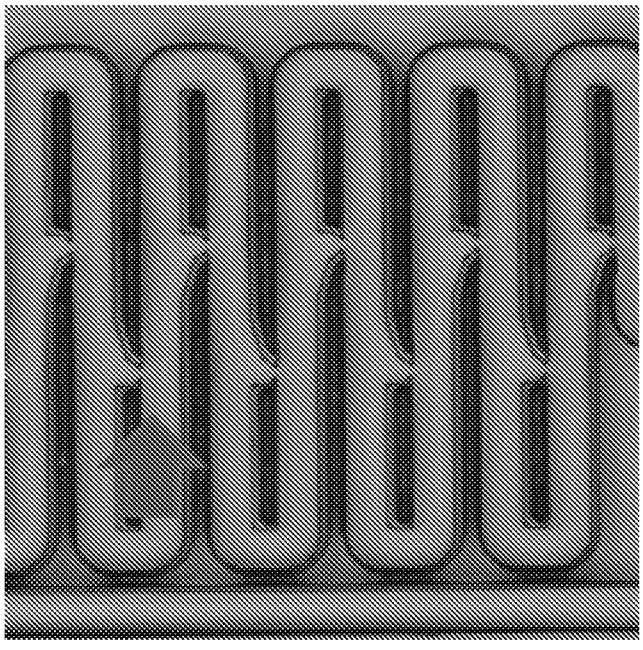

FIG. 7C

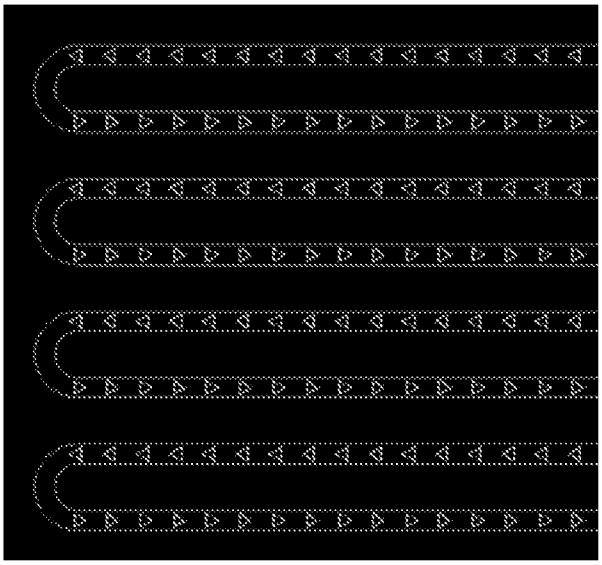

FIG. 8A

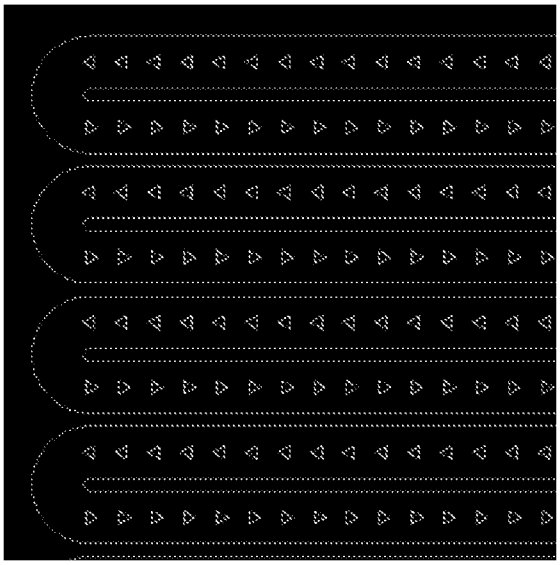

FIG. 8B

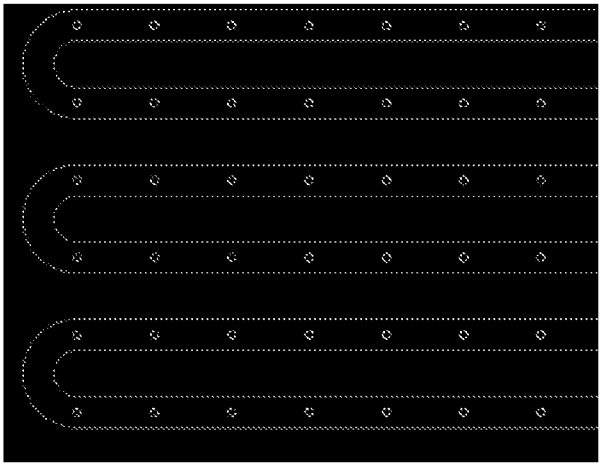

FIG. 8C

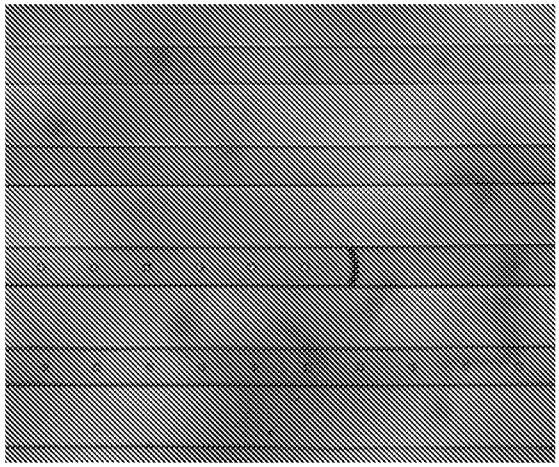

FIG. 8D

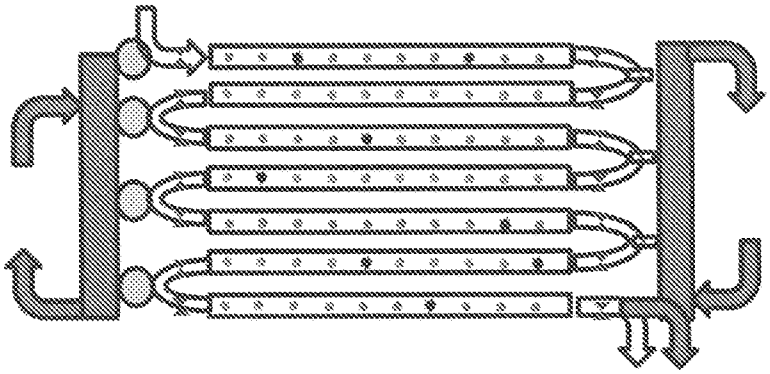

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/052172

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07H 21/00; C12M 1/34; C12M 1/3446; C12P 19/34; C12Q 1/04; C12Q 1/6816 (2018.01)

CPC - C07H 21/00; C12M 1/34; C12M 1/3446; C12Q 1/6806; C12Q 1/6813; C12Q 1/6825; C12Q 1/6827; C12Q 1/6837; G01N 21/00; G01N 33/4833; G01N 33/48735; G01N 33/52; G01N 2570/00 (2018.08)

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

USPC - 422/186; 435/4; 435/182; 435/6.0011; 435/7.5; 436/63; 436/164 (keyword delimited)

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.
X -- Y	US 2013/0323732 A1 (FLUIDIGM CORPORATION) 05 December 2013 (05.12.2013) entire document	1-11 -------- 12-15
Y	US 2010/0112558 A1 (GAO et al) 06 May 2010 (06.05.2010) entire document	12-15
A	US 2016/0060621 A1 (BIO-RAD LABORATORIES, INC.) 03 March 2016 (03.03.2016) entire document	1-15
A	WO 2016/207441 A1 (EUROPEAN MOLECULAR BIOLOGY LABORATORY) 29 December 2016 (29.12.2016) entire document	1-15
A	WO 2015/164212 A9 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE et al) 13 October 2016 (13.10.2016) entire document	1-15
A	KLEIN et al. "Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic Stem Cells," Cell, 21 May 2015 (21.05.2015, Vol. 161, Vol. 5, Pgs. 1187-201. entire document	1-15
A	ZILIONIS et al. "Single-Cell Barcoding and Sequencing Using Droplet Microfluidics," Nature Protocols, 08 December 2016 (08.12.2016), Vol. 12, No. 1, Pgs. 44-73. entire document	1-15

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

* Special categories of cited documents:

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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
28 October 2018	13 DEC 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)