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(54) LASER BASED SORTING OF DROPLETS IN MICROFLUIDIC STREAMS

(57) The present invention relates to methods and devices for sorting droplets in microfluidic systems based on manipulation of droplets in motion by pulsed high energy laser irradiation. The methods and devices are useful in various microfluidic settings such as droplet sorting in cell sorting applications and in particular in droplet-based single-cell isolation.

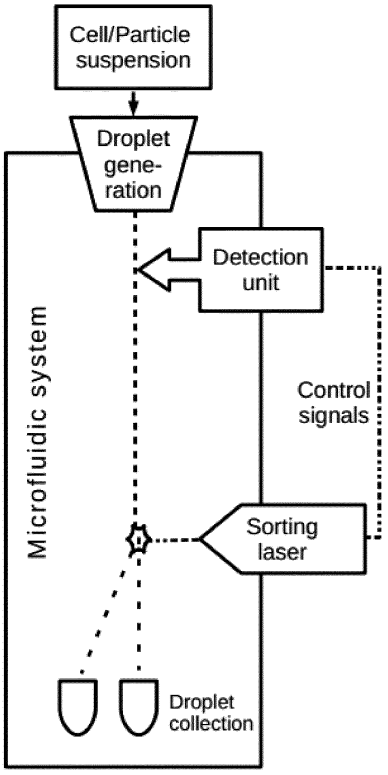

Figure 1

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Description

Field of the Invention

[0001] The present invention relates to methods and devices for sorting droplets in microfluidic systems based on manipulation of droplets in motion by pulsed high energy laser irradiation. The methods and devices are useful in various microfluidic settings such as droplet sorting in cell sorting applications and in particular in droplet-based single-cell isolation.

Introduction

[0002] Conventional cell-based assays mainly measure the average response from a population of cells, assuming the average response is representative of each cell. However, important information about a small but potentially relevant subpopulation gets lost, particularly in cases where that subpopulation determines the behaviour of the whole population. For example, the tumor microenvironment is a complex heterogeneous system that consists of multiple intricate interactions between tumor cells and its neighbouring non-cancerous stromal cells.

[0003] Technologies to isolate individual single cells from a complex sample and study the genomes and proteomes of single cells promise great insights on genome variation and gene expression processes. Single cell analysis gains more and more importance in various fields including life sciences and biomedical research.

[0004] In early times, researchers have applied low-throughput single cell analysis techniques, such as immunofluorescence, fluorescence in situ hybridization (FISH) and single cell PCR, to detect certain molecular markers of single cells. These techniques allow quantification of a limited number of parameters in single cells. On the other hand, high-throughput genomic analysis, such as DNA and RNA sequencing are now widely used. However, classic genomic studies rely on studying collective averages obtained from pooling thousands to millions of cells, precluding genome-wide analysis of cell to cell variability. Therefore, single cell sequencing techniques were developed and were recently awarded "method of the year" by Nature Methods in 2013. By using single cell analysis, researchers have profiled many biological processes and diseases at the single cell level including tumor evolution, circulating tumor cells (CTCs), neuron heterogeneity, early embryo development, and uncultivatable bacteria.

[0005] However, single-cell sequencing relies on the efficient preparation of single cells.

[0006] Before initiating a single cell analysis, one needs to isolate or identify single cells. The performance of cell isolation technology is typically characterized by three parameters: efficiency or throughput (how many cells can be isolated in a certain time), purity (the fraction of the target cells collected after the separation), and recovery (the fraction of the target cells obtained after the separation as compared to initially available target cells in the sample). Hu et al. (Frontiers in Cell and Developmental Biology, Oct. 2017, Vol. 4 Article 116) provides an overview of existing techniques for preparing single cells from a heterogeneous population of cells. One possibility is fluorescence activated cell sorting (FACS) a specialized type of flow cytometry with sorting capacity; in this technique, after labelling the cells with fluorescent labels a cell suspension is prepared and is then entrained in the centre of a flowing stream of liquid. The flow is adjusted so that there is a relatively large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. However, there is a certain fraction of droplets that contain more than one cell and a further fraction with empty droplets. The sorting of the droplets is achieved by an electrical charging ring that is placed just at the point where the stream breaks into droplets. Prior to fluorescence intensity being measured, a charge is placed on the ring and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. However, the physiology of the cells may be deteriorated due to the force acting on the cells by the electric voltage or the electric field which is applied for the separation of droplets from the droplet stream. FACS also requires a huge starting number of cells (more than 10,000) in suspension.

[0007] Magnetic-Activated Cell Sorting (MACS) is another commonly used passive separation technique to isolate different types of cells depending on their cluster of differentiation. It has been reported that MACS is capable of isolating specific cell populations with a purity >90% purification (Miltenyi et al., Cytometry 11, 231-238, 1990). MACS is based on antibodies, enzymes, lectins, or strepavidins conjugated to magnetic beads to bind specific proteins on the target cells. When a mixed population of cells is placed in an external magnetic field, the magnetic beads will activate and the labelled cells will polarize while other cells are washed out. The remaining cells can be acquired by elution after the magnetic field is turned off. However, this technique can only separate cells into positive and negative populations and is also often hampered by the presence of non-specific cell capture.

[0008] Another method for preparing single cells is laser capture microdissection (LCM). The technique utilizes a laser which is fired through a cap placed over the cells of interest to melt the membrane and let the cells adhere to the melted membrane. When the cap is removed, captured cells are removed, leaving the unwanted cells behind. However, this method requires extensive

instrumentation and knowledge to operate and is only suitable to isolate relatively few single cells. Moreover, LCM introduces a number of technical artefacts, including slicing the cells during the preparation of tissue sections and UV damage to DNA or RNA from the laser cutting energy (Allard et al., Clin. Cancer Res. 10, 6897-6904. 2004).

[0009] Microfluidics is recognized as a powerful enabling technology for investigating the inherent complexity of cellular systems as it provides accurate fluid control, low sample consumption, device miniaturization, low analysis cost, and easy handling of nanoliters. Cell Sorting by a microfluidic chip can be divided into four categories: cell-affinity chromatography based microfluidic, physical characteristics of cell based microfluidic separation, immunomagnetic beads based microfluidic separation, and separation methods based on differences between dielectric properties of various cell types. The single-cell trapping in microfluidic systems may be based on on-chip valves and microchambers or by droplet-based microfluidic reaction arrays formed by integrated pneumatic valves. However, the sorting means of existing microfluidic systems are often not able to perform an efficient high throughput sorting of cells.

[0010] Wen et al. (Molecules (2016), Vol. 21, 881, p. 1-13) is an overview on the existing attempts of droplet microfluidics for high-throughput single cell analysis. Accurate control of the number of cells per droplet is a challenging issue due to the nature of Poisson (random) encapsulation where the Poisson probability of a droplet containing one and only one cell is only 36.8%. In practice, in order to make sure that no two cells are confined within one droplet, cell suspensions are further diluted, leading to a large number of empty droplets, which is wasteful. One approach to address this issue was to remove empty droplets after single-cell confinements. One technique is based on a purely hydrodynamic method for encapsulation of single cells into picoliter droplets, followed by spontaneous self-sorting based on the sizes, wherein encapsulation was realized based on a cell-triggered Rayleigh-Plateau instability in a flow-focusing geometry, and self-sorting relied on two extra hydrodynamic mechanisms, which are lateral drift of deformable objects in a shear flow, and sterically-driven dispersion in a compressional flow, respectively. Successful encapsulation and sorting of 70%-80% of the droplets containing one and only one cell was reported, demonstrating a significant improvement in comparison to random cell encapsulation (Chabert, M.; Viovy, J.L. Proc. Natl. Acad. Sci. USA 2008, 105, 3191-3196).

[0011] A passive separation of microfluidic droplets by size can also be achieved by deterministic lateral displacement where a tilted pillar array allows droplets smaller than a certain critical diameter to follow the direction of the incoming fluid flow while larger droplets are constrained to follow the tilted lanes of the pillar array (Joensson, H.N.; et al., Lab Chip 2011, 11, 1305-1310; Jing, T. et al., Biosens. Bioelectron. 2015, 66, 19-23). Based on this microfluidic structure, shrunken yeast-cell containing droplets from 31% larger diameter droplets which were generated at the same time containing only media could be sorted out and large droplets encapsulating tumor cells (diameter, -25 μm) and small empty droplets (diameter, -14 μm), enriching the single-cell encapsulated droplets were sorted to roughly 78% (Jint, T. et al., supra). By forcing a high-density suspension of cells to travel rapidly through a high aspect-ratio straight microchannel to evenly spaced cells, a single-cell encapsulation efficiency of 80% has been reported (Eds, J.F.; di Carlo, et al., Lab Chip 2008, 8, 1262-1264). However, although several studies have been conducted to address the issue of low efficiency of single-cell encapsulation, the optimal encapsulation efficiency was only about 80%.

[0012] As evidenced by the various implementations discussed above and their respective individual drawbacks, the wider application of single-cell analysis is limited by the lack of an efficient strategy for single-cell isolation that can be directly coupled to single-cell sequencing (drop sequencing) and other applications. In view of the considerable costs triggered by drop-sequencing analysis, reducing the number of droplets with no or more than one cell, implies significant cost savings.

[0013] Hence, there remains a need in the art for an efficient sorting mechanism to sort out empty droplets and droplets that contain more than one cell in an efficient and accurate way.

[0014] It is one object of the present invention to provide methods and devices for high throughput isolation of single-cell containing droplets with an improved purity and recovery as compared to the prior art techniques.

[0015] It is a further object of the invention to provide means for sorting single cell containing microdroplets contained in a fluid stream in a high throughput manner with superior accuracy as compared with the prior art techniques and without imposing physiological stress to the desired cells.

Summary of the Invention

[0016] The present invention fulfils this need by providing means and methods for preparing droplets containing a single particle comprising (i) providing a fluid containing a plurality of particles; (ii) generating droplets from the fluid containing the plurality of particles; (iii) analyzing the droplets generated in (ii) for a particular property such as the number of cells within the droplet; and (iv) sorting the droplets using a microfluidic system into at least two groups, depending on the number of particles contained in a droplet and/or depending on an identifiable property of the particles; wherein the sorting of the droplets is effected by laser irradiation of at least one sorting laser. In one specific embodiment the invention relates to a method for preparing single cell droplets, preferably single cell droplets within a fluid stream of a carrier liquid, the method comprising (i) providing a fluid containing a

plurality of cells; (ii) generating droplets from the fluid containing the plurality of cells, the generated droplets being a mixture of droplets with droplets containing no cell, droplets containing one single cell and droplets containing more than one cell, advantageously the fraction of droplets containing one single cell is the predominant fraction; (iii) analyzing the droplets generated in (ii) for a particular property such as the number of cells within the droplet or the presence of intensity of a marker; and (iv) sorting the droplets into at least two groups using a microfluidic system, the method characterized in that the sorting of the droplets comprises laser irradiation by at least one sorting laser. The group of sorted droplets preferably comprises more than 95%, preferably more than 99% of single cell droplets or comprise more than 95%, preferably more than 99% of droplets of a particular property.

[0017] In one aspect, the analyzing of step (iii) comprises the steps of (a) examining each droplet for the presence or absence of particles (such as cells) within the droplet, or for the absence or presence or the intensity of a marker within each droplet, and (b) generating a control signal for the sorting laser depending on the number of particles detected in each droplet or depending on the absence or presence or intensity of the marker detected in each droplet.

[0018] To implement the methods of the invention, the present invention provides a microfluidic device for preparing droplets containing exactly one single particle (e.g. cell) or droplets containing a particular identifiable property comprising (1) at least one inlet for a fluid containing a plurality of particles; (2) at least one inlet for a carrier fluid for transporting droplets of the fluid containing the plurality of particles; (3) microfluidic means for generating a fluid stream of carrier fluid comprising droplets from the fluid containing the plurality of particles, wherein the at least a part of the droplets contain one or more particles; the microfluidic means may for example generate a plurality of droplets which at least partially contain one or more single particle or cell; preferably already at this stage the fraction of droplets containing only one single particle or cell per droplet is the predominant fraction, although the method or device of the invention is operative as long as there is any fraction of droplets containing one single cell per droplet, (4) means for analyzing each droplet in the fluid stream containing the droplets; (5) at least one microfluidic droplet path directing the droplets generated by (3) to a sorting site of the microfluidic device; (6) at least one sorting laser configured to be capable of emitting laser irradiation to the sorting site of the microfluidic device depending on the presence or absence of particles within the droplet, or depending on the absence or presence or the intensity of an identifiable property of the droplets; wherein the laser irradiation is capable of (a) changing the droplets trajectory by generating a vapor bubble close to a droplet or on the surface of a droplet or by acting as an optical tweezer in the sorting site or (b) disintegrating a droplet in the sorting site, (7) (a) at least two or (b) at least one microfluidic droplet path(s) departing from the sorting site of the microfluidic device; and (8) means for collecting droplets specifically containing exactly one single particle or droplets containing exactly one particular identifiable property, for example means for collecting at least one fraction of droplets containing the desired number of cells, preferably droplets containing exactly one single cell. The microfluidic droplet paths departing from the sorting site of the microfluidic device may be configured to allow droplets to take a different route depending on whether they have been deflected through the action of the laser irradiation or the sorting laser or not. The invention is not limited to discriminating between the number of particles or cells per droplet, but may also be used to sort single cell or single particle droplets wherein the single cells or particles are discriminated from each other by way of one or a plurality of different markers.

[0019] The means for analyzing each droplet of the microfluidic device of the invention may comprise, upstream of the sorting site, a detection site and include means for detecting the presence or absence of particles or cells within each droplet, or the presence or absence or intensity of markers within each droplet or associated with the cell or particle. These means for detecting the presence or absence of particles or cells and/or markers within each droplet are configured to generate a control signal depending on the presence of absence of cells and/or the absence, or presence or intensity of markers in a droplet, wherein the control signal triggers that the sorting laser emits a laser pulse to the sorting site when a droplet analyzed passes the sorting site. In the present invention one exemplary way to analyze the droplets prior to sorting is to analyze the droplets by optical means. These optical means exemplarily comprise detecting the presence or absence of cells with a camera with a high frame rate, or by detecting the presence or absence or intensity of a marker associated with the particles. For example the marker may be a cellular label or marker or a dye or a fluorescent group associated with the particle or cell.

[0020] In the method or device of the present invention the sorting laser is a pulsed laser with a pulse rate of 1 Hz to 200 MHz and each laser pulse has an energy of at least 100 nJ, preferably between 1,5 to 15 μJ and up to 100 μJ. The detection and sorting means are configured such as to reach a sorting rate of above 100 droplets/sec, preferably above 5000 droplets/sec, most preferably up to 10.000 droplets/sec.

[0021] In some embodiments of the invention, the laser irradiation (i) generates a vapor bubble in the fluid stream close to the droplet or on the surface of the droplet to be sorted or (ii) acts as an optical tweezer, thereby giving the droplet to be sorted an impulse at least partially lateral to its prior flow direction in the microfluidic system, preferably leading to a deflection of more than 3° (from its previous trajectory to the sorting site) or more than 10 μm. Thus, the laser irradiation generated impulse forces

the droplet to be sorted to take a path different from that of a droplet not having received such an impulse. In some embodiments the laser irradiation directs the droplet to a trajectory that depends on the mass of the droplet.

[0022] Alternatively to changing a droplets path or trajectory the sorting laser can be used to disintegrate droplets containing no particle or cell and/or droplets containing more than one particle or cell (using several, e.g. 1-9 μJ of energy per pulse). In this aspect of the invention, it suffices that the device comprises only a single microfluidic droplet path departing from the sorting site of the microfluidic device.

[0023] The inventive method or device the sorting accuracy is such that one sorted group of droplets comprises more than 95%, preferably more than 99% of droplets containing exactly one single particle or cell or contains to 95%, preferably to more than 99% only particles or cells with exactly one particular identifiable property.

Definitions

[0024] The term "single cell droplets" relates to a droplet containing exactly one single cell. In some aspects, the present invention relates to the sorting of particles. It is to be understood that the term single cell droplet may equivalently be exchanged with the term "single particle droplets", signifying a droplets that contains exactly one particle in those aspects of the invention that relates to the sorting of particles.

[0025] The term "plurality" means for the sake of the present invention at least two. In particular a plurality can be any number higher than two.

[0026] The term "sorting" for the sake of the present invention refers to the act of enriching from a mixture of entities (such as droplets, cells or particles) one fraction of entities having a specific property of interest in common.

[0027] The term "microfluidic system" is used in the broadest sense to include a system for the precise control and manipulation of fluids that are geometrically constrained to a small, typically sub-millimeter, scale in order to enhance efficiency and mobility as well as reducing sample and reagent volumes. In order to consider it microfluidics, at least one dimension of the channel must be in the range of a micrometer or tens of micrometers. Accordingly a "microfluidic droplet path" refer to a channel or chamber of a microfluidic system allowing for a microfluidic stream to pass through it.

[0028] "Optical tweezer" denotes an instrument that use a highly focused laser beam to provide an attractive or repulsive force (typically in the order of piconewtons), depending on the relative refractive index between a particle (such as a microfluidic droplet) and surrounding medium, to physically hold and/or move said particle. An optical tweezer is able to trap and manipulate small particles, typically in the order of micron in size, including dielectric and absorbing particles. "Optical" refers to the interaction with light, be it in the visible or invisible (UV, IR) range.

Description of the Figures

[0029]

Figure 1: Figure 1 is a schematic implementation of one embodiment of the invention comprising a microfluidic system with one detection unit and a sorting laser. It is to be understood that the sorting laser is capable of producing more than one deflection angle depending on the radiation energy emitted.

Figure 2: Figure 2 is an exemplary implementation of the system of Figure 1, wherein the detection unit is based on detecting fluorescent signals from droplets, or cells or particles within the droplets; the detection implies the use of an excitation laser to generate fluorescence signals.

Figure 3: Figure 3 is an exemplary implementation of the system of Figure 1, wherein the detection unit is capable of generating control signals for two different sorting lasers signals from droplets.

Figure 4: Figure 4 is an exemplary implementation of the system of Figure 1, comprising more than one detection unit and more than one sorting laser. It is to be understood that the detection units and sorting lasers do not necessarily have to act in tandems as depicted; each detection unit may be configured to send control signals to both sorting lasers.

Detailed Description of the Invention

[0030] According to the present invention, the laser irradiation is directed onto a fluid stream that contains droplets and has a wavelength and intensity such that liquid (either of the carrier fluid nearby a droplet or of the droplet surface itself) is heated and thereby evaporates such that the irradiated section of the liquid is accelerated and deflected into the opposite direction of the laser irradiation source. The droplets deflected by the laser beam and/or the droplets not deflected from their flow path by the laser irradiation can be collected based on a signal associated with the respective droplets (see **Fig.** 1).

[0031] In a first aspect the invention pertains to a method for preparing droplets with a particular identifiable property, for example droplets containing a single particle (e.g. single cell droplets), preferably single particle droplets within a fluid stream of a carrier liquid, the method comprising (i) providing a fluid containing a plurality of particles or cells; (ii) generating droplets from the fluid containing the plurality of particles or cells, the generated droplets being a mixture of droplets with droplets containing no particle or cell, droplets containing one single particle or cell and droplets containing more than one

particle or cell, advantageously the fraction of droplets containing one single particle or cell is the predominant fraction; in particular, the droplets are produced into a stream of carrier fluid (iii) analyzing the droplets generated in (ii) for a particular property such as the presence (and number), or absence of particles or cells or for the presence (and/or intensity) or absence of a marker and (iv) sorting the droplets into at least two groups, e.g. depending on the number of particles contained in a droplet and/or depending on the particular property of the particles using a microfluidic system; wherein the sorting of the droplets comprises laser irradiation by at least one sorting laser. The group of sorted droplets preferably comprises more than 95%, 96%, 97%, 98% or 99% preferably more than 99% of droplets with the desired property, e.g. droplets containing one single particle or cell, or droplets that contain a particular marker or contain a marker above a predetermined concentration (intensity) threshold.

Droplet generation

[0032] Methods for producing droplets of a uniform volume at a regular frequency are well known in the art. A fluid containing a plurality of particles or cells can be a cell suspension, a slurry containing particles or cells, or a solution of resuspended particles or cells after a centrifugation or filtration or any other particle or cell collection step. The liquid phase of the fluid containing a plurality of particles/cells is essentially water based, but may be of any liquid suitable to host particles/cells in a non-detrimental way. The liquid phase may accordingly be a buffer or a cell medium, and the fluid stream contains water based droplets with particles/cells to be sorted in an hydrophobic carrier fluid. The fluid containing the cells or particles to be sorted may typically comprise an aqueous buffer solution, such as ultrapure water, 10 mM Tris HCl and 1 mM EDTA (TE) buffer, phosphate buffer saline (PBS) or acetate buffer. Any liquid or buffer that is physiologically compatible with the cells to be sorted or the integrity of the particles can be used. The carrier fluid may include one that is immiscible with the fluid containing the cells or particles to be sorted. The carrier fluid can be a non-polar solvent, decane (e.g., tetradecane or hexadecane), fluorocarbon oil, silicone oil, an inert oil such as hydrocarbon, or another oil (for example, mineral oil) as long as it does not negatively affect the cell's physiology or the particle's integrity.

[0033] The carrierfluid may contain one or more additives, such as agents which reduce surface tensions (surfactants). Surfactants can include Tween, Span, fluorosurfactants, and other agents that are soluble in oil relative to water. Surfactants can aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel. This can affect droplet volume and periodicity, or the rate or frequency at which droplets break off into an intersecting channel. Furthermore, the surfactant can serve to stabilize aqueous emulsions in fluorinated oils from coalescing.

[0034] The droplets containing the cells or particles may be surrounded by a surfactant which stabilizes the droplets by reducing the surface tension at the aqueous oil interface. Preferred surfactants that may be added to the carrier fluid include, but are not limited to, surfactants such as sorbitan-based carboxylic acid esters (e.g., the "Span" surfactants, Fluka Chemika), including sorbitan monolaurate (Span 20), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60) and sorbitan monooleate (Span 80), and perfluorinated polyethers (e.g., DuPont Krytox 157 FSL, FSM, and/or FSH). Other non-limiting examples of non-ionic surfactants which may be used include polyoxyethylenated alkylphenols (e.g., nonyl-, p-dodecyl-, and dinonylphenols), polyoxyethylenated straight chain alcohols, polyoxyethylenated polyoxypropylene glycols, polyoxyethylenated mercaptans, long chain carboxylic acid esters (e.g., glyceryl and polyglyceryl esters of natural fatty acids, propylene glycol, sorbitol, polyoxyethylenated sorbitol esters, polyoxyethylene glycol esters, etc.) and alkanolamines (e.g., diethanolamine-fatty acid condensates and isopropanolamine-fatty acid condensates).

[0035] Alternatively, the liquid phase of the fluid containing the plurality of particles/cells is hydrophobic or oil-based and the carrier fluid is hydrophilic (water based). In that case the fluid stream will contain hydrophobic droplets with particles/cells to be sorted in a hydrophilic carrier fluid. Droplet volumes and channel width of the microfluidic system used for sorting can be chosen by design as e.g. disclosed in US 2007/0195127 and WO 2007/089541.

[0036] The concentration of particles or cells in the fluid containing the plurality of particles or cells can be adjusted accordingly so as to favor the generation of droplets containing a single particle or cell. It may also be adjusted to explicitly disfavor the generation of droplets containing more than one single particle or single cell to the expense that more empty droplets are generated. However, since two particles/cells may physically still stick to each other, the need for isolating single particle/cell droplets remains.

[0037] The cells to be sorted are not limited and may be prokaryotic or eukaryotic cells, such as animal cells or plant cells. The cells may also be fungal cells or bacterial cells. The cells or particles may be labeled or unlabeled prior to the sorting, or may be differentially labelled depending on different traits on their cell surface. However, the invention is not necessarily limited to the sorting of cells, in fact, the inventive method may be used to sort any kind of particles that are contained in a droplet. The term "particle" also includes virus particles as well as other unanimate particles.

[0038] In the present invention the droplets are generated from the fluid containing the plurality of particles or cells and subjected to a stream of carrier fluid that surrounds the droplets. The provision of droplets within a fluid stream can be performed by methods known in the

art, e.g. those described in Zhu & Wang (Lab Chip, 2017,17, 34-75), Shelley Lynn Anna (Annual Review of Fluid Mechanics Vol. 48:285-309) or Chen & Jiang (Chinese Journal of Analytical Chemistry Volume 40, Issue 8, August 2012, Pages 1293-1300).

[0039] Droplets can also be generated using hydrodynamic focusing of a dispersed phase fluid and immiscible carrier fluid, such as disclosed in US 2005/0172476 and WO 2004/002627. The droplets may be aqueous droplets that are surrounded by an immiscible carrier fluid. Methods of forming such droplets are shown for example in US 2008/0014589, US 2008/0003142, and US 2010/0137163), and EP2047910.

Droplet analysis. Markers

[0040] In one aspect, the step of analyzing comprises the steps of (a) of analyzing each droplet for the presence or absence of cells or particles within the droplet or analyzing each droplet for the absence or presence or the intensity of a marker within each droplet, and (b) generating a control signal for the sorting laser depending on the number of cells or particles detected in each droplet or depending on the absence or presence or intensity of the marker detected in each droplet. In more general aspect, the droplets are analyzed for the presence or absence of a property of the cell or particle contained in the droplet. This property may be due to the mere presence of the particle/cell or the particles/cells contained in the droplet, or may come from a marker or label that is covalently or non-covalently associated with the particle/cell or particles/cells contained in the droplet.

[0041] A marker is any entity that is suitable to generate a detectable signal either on its own or via interaction with a second or further entity. Exemplary markers are dyes, in particular fluorescent dyes, nanoparticles, magnetic beads, radiolabels, antibodies, proteins such as fluorescent proteins, nucleic acids, positively or negatively charged moieties, lectins, sugar moieties, etc.. In one embodiment the marker is a dye such as a fluorescent dye. In another embodiment the marker can be a conjugate of a specific binding portion (such as e.g. an antibody) and a detectable label. The binding portion can be an antibody or a fragment or binding portion of a natural or synthetic antibody and a dye portion, especially a fluorescence dye. For identification of particles comprising a nucleic acid sequence of interest (e.g. a nucleic acid sequence specific for a single nucleotide mutation (SNP)) the conjugate can comprise a nucleic acid sequence complimentary to the nucleic acid sequence of interest.

[0042] Preferably the marker or label is an entity that does not impact the viability of the cell or the integrity of the particle to which it is attached. Preferably the maker or label is reversibly attached to the cell(s) or particle(s) in order to not interfere with further subsequent analysis steps of the sorted cells or particles. Alternatively, the fluid portion of the droplet may contain a marker, which marker is "diluted" through the presence of one or more cells or particles. In a further alternative embodiment, the marker is a pH sensitive dye which has a different absorption in the presence or absence of the one or more cell or particle in the droplet. The invention is not limited to discriminating between the number of cells or particles per droplet, but may also be used to sort single cell droplets wherein the single cells or particles are discriminated from each other by way of one or a plurality of different detectable properties and/or markers.

Detection means/control signals

[0043] The analysis of the presence or absence of a cell or particle within a droplet or the presence of absence of a property or signal of a cell or particle within a droplet can be carried out by any suitable means including optical means, electrical means, mechanical (acoustic) means or physical means such as size of the droplet or weight of the droplet. Suitable other detection means comprise microelectrodes measuring the conductivity (or resistance, and/or net charge) of each droplet passing the microelectrodes and generating a signal depending on whether the conductivity of the droplet indicates the absence of a cell or particle or the presence of one or more cells or particles or the presence or absence of a suitable marker influencing the conductivity, resistance and/or net charge of a droplet containing said marker. Electrochemical, mechanical and optical detection means for implementation in microfluidic devices and methods are e.g. disclosed in Pires et al. (Sensors, 2014, Vol 14, p. 15458-79).

[0044] In the present invention one preferred way to analyze the droplets prior to sorting is to analyze the droplets by optical means. These optical means exemplarily comprise detecting the presence or absence of cells (or particles) with a camera with a high frame rate, or by detecting at least one (cellular) label or marker or the intensity of a dye or a fluorescent group associated with the cell or particle. Other optical detection means comprise surface Plasmon resonance or surface enhanced Raman spectroscopy.

[0045] Optical detectors that can be utilized to detect the wavelength-separated signals include photodiodes, avalanche photodiodes, (arrays of) photomultiplier tubes, photomultiplier modules, and CCD cameras. These optical detectors are available from suppliers such as Hamamatsu (Bridgewater, NJ). Other detectors suitable for the inventive methods and devices include multielement photodiodes, CCD cameras, back-side thinned CCD cameras, and multi-anode PMTs. Further optical detection methods for microfluidic systems are also known in the prior art such as those discussed in Huo et al. (Chinese Journal of Analytical Chemistry,Volume 38, Issue 9, September 2010, Pages 1357-1365) Kuswandi et al. (Analytica chimica acta Vol. 601(2):141-55, November 2007), Gai &Yeung (Topics in Current Chemistry 2011;304:171-201), or Mogensen & Kutter (Electrophoresis - Microfluidics and Miniaturization, Vol. 30, Is-

sue S1, pages S92-S100).

[0046] The detection may be performed by a single detector or may be performed by more than one detector, including detector arrays. In certain embodiments, particularly embodiments which are directed to the detection of more than one marker or property that may be present on the cells or particles occurring in the plurality of droplets containing the cells or particles distinct wavelength components of one or more markers may be separated by the use of dichroic mirrors and/or bandpass filters and these wavelength components may then be simultaneously detected with Photomultiplier Tube (PMT) detectors (such as a H7732-10 detector from Hamamatsu). One skilled in the art may apply a combination of wavelength dispersion elements and optical detector elements to yield a system that is capable of discriminating wavelengths from the markers labels and/or dyes used in the method or system.

[0047] For embodiments relying on optical detection means the methods and systems of the present invention require a light or excitation source for illuminating or irradiating the droplets to be sorted (see **Fig. 2**). Optical excitation of a droplet or a cell or particle on an associated marker contained in that droplet can preferably be accomplished by at least one light source, preferably a laser, with emission wavelengths in the visible region, between 400 to 650 nm. Exemplary lasers for the detection comprise solid state lasers providing emission wavelengths of approximately 460 nm, 488 nm, and 532 nm. These lasers include, for example, the Compass, Sapphire and Verdi products from Coherent (Santa Clara, CA). Gas lasers include argon-ion and helium neon with emission in the visible wavelengths at approximately 488 nm, 514 nm, 543 nm, 595 nm, and 632 nm. Other exemplary lasers with emission wavelengths in the visible region are available from CrystaLaser (Reno, NV). In another embodiment, a light source with wavelength beyond the visible range can be used for exciting dyes having absorption and/or emission spectra beyond the visible range (*e.g.*, infrared or ultra-violet emitting dyes). Alternatively, optical excitation can be achieved by the use of non-laser light sources with emission wavelengths appropriate for dye excitation, including light emitting diodes, and lamps. As a further alternative, a light source may be configured to provide a sufficient illumination of the droplets passing the detection site to allow the operation of a camera with high frame rates to produce pictures of droplets which allow discriminating empty droplets from droplets containing exactly one single cell or particle or containing more than one cell or particle.

[0048] In one implementation the inventive device may comprise one or more additional detectors (i.e. a second, third, etc. detector) which measure the same or further properties of particles and/or of the fluid stream containing the droplets to be sorted. In one embodiment, the device comprises a second detector, and optionally also a second excitation light source the radiation of which is directed onto the microfluidic stream, wherein the second detector receives e.g. a (further) fluorescence signal for the cells or particles and transmits it to the control unit from which the control unit identifies the relative orientation of the cell(s) or particle(s), especially of non-rotationally symmetrical cells or particles, and the control unit generates the control signal, also on the basis of this second detection signal in combination with the first detection signal of a detector. The two or more detectors may be arranged in the same of different angle relative to the microfluidic stream flow direction. These configurations enhance the accuracy of detection and further increases the yield of the desired droplet fraction.

[0049] The means for detecting the presence or absence of cells and/or markers within each droplet (the detector or detectors) are configured to generate a control signal in dependence to the presence of absence of cells or particles and/or markers in a droplet, wherein the control signal controls whether the sorting laser emits a laser pulse to a sorting site when a droplet analyzed passes the sorting site depending on the presence or absence of cells or particles and/or cell markers in the droplets for each single droplet. In one embodiment, the means for detection, i.e. all necessary components such as light source and associated light detector, or light source and associated high framerate camera and image processing means may be integrated to form a detection unit, which generates control signals for controlling the sorting laser.

[0050] In one embodiment the generation of control signals depends on whether a perceived signal indicates the presence of exactly one single cell in the droplet, as compared to no cell or more than one cell in the droplet.

[0051] The control signal may be provided by the detector on detection unit dependent on the type of detector used. For example, if the detector measures the optical density or absorbance of a droplet at a predetermined wavelength, which optical density or absorbance indicates the presence of a particle or cell to be sorted (e.g. because the cell or particle absorbs this wavelength naturally or has been labeled with a dye absorbing that wavelength), it may directly generate a control signal for the sorting laser either depending on the mere presence of the particular absorbance or only when said absorbance reaches a predetermined threshold. Or, if the particle or the cell to be sorted are capable of emitting a fluorescent signal of a particular wavelength upon irradiation with a suitable excitation wavelength, the detector may, upon detection of the fluorescent signal, generate a control signal for the sorting laser (again, either due to the mere presence of that specific fluorescence or only if it reaches a predetermined threshold value).

[0052] In one embodiment the control signal is generated with an algorithm which in dependence from the intensity of a detection signal perceived by the detector(s) generates and transmits control signals to the sorting laser and/or to optical elements in its beam path which are suitable to direct the radiation of the sorting laser in dependence from the detection signal with temporal delay onto the sorting site of the fluid stream. The algorithm

may e.g. be a simple yes/no logic (presence or absence of a specific wavelength), may be a threshold calculation or may be adapted for more complex sorting logics as needed (e.g. due to a combination of markers used to sort more than one population of droplets).

[0053] The microfluidic devices of the present invention can comprise more than one detector for determining more than one property of the droplets to be sorted. Accordingly the detectors may generate more than one control signal for the one or more sorting laser(s). These control signals may be integrated for controlling one sorting laser or may be processed in parallel by more than one sorting laser.

[0054] In an exemplary embodiment the droplets are, in addition to the presence or absence of a cell or particle, analyzed for their size or volume by an additional detector configured to measure the size or volume of each droplet. The size/volume detector then generates a modulation signal for the sorting laser depending on the size or volume of the droplet. The modulation signal is integrated with the control signal for the sorting laser and can be used to adjust the energy emitted by the sorting laser such that the energy correlates with the volume of the droplet to be sorted. By this measure the device can be configured, e.g. to only deflect droplets containing a single particle or cell and to submit each single particle/cell droplet to an sorting impulse that matches with the volume of each droplet. The deflection of each droplet will then be dependent on the specific weight of the droplet. By this way the droplets can be sorted with only one laser impulse for two properties; (i) the presence of exactly one cell/particle and (ii) the weight of the cell/particle contained in the droplet.

[0055] In a further embodiment the control signal is modulated in dependence of the intensity of a property of a particles such that the control signal causes the sorting laser to emit laser energy that correlates with the intensity of the signal perceived from the droplet by the detector. In this way, for example, droplets with a higher intensity of fluorescence detected become more strongly deflected in the sorting site.

Sorting site

[0056] The at least one sorting site is preferably located in a different position as the detection site(s) (the site(s) that droplets pass the detection means) and is located downstream of the detection site(s) of the microfluidic stream. The distance between the detection site(s) and the sorting site(s) can be chosen by design and depending on the detection means and the speed of the generation of control signals.

[0057] The distance between a detection site and a sorting site together with the speed of the microfluidic stream containing the droplets to be sorted determines the required delay between detection and the provision of the control signal for the sorting laser. These parameters also define the available time frame for generating a control signal from more complex detection means (e.g. image analysis or integration of multiple signals from multiple detectors). A skilled person is able to select an appropriate distance between detection site and sorting site (and microfluidic stream velocity) to allow for the required implementation.

[0058] A sorting site of the microfluidic system comprises at least one channel with a position that receives the irradiation of the sorting laser and at least one outlet channel, preferably at least two outlet channels (bifurcation). The sorting site may comprise more than two (e.g. three, four, five, six, seven, eight, nine, ten, more than 10 between 10 and 20 or more than 20 or between 2 and 100 and any individual integer within that range) outlet channels. For embodiments relying on the disintegration of entire droplets, the sorting site may however comprise just one single outlet channel.

[0059] Depending on the desired implementation and number of sorting lasers, the sorting site may consist of a plurality of sites with positions that receive sorting laser irradiation from one or more sorting lasers (e.g. comprise multiple bifurcations of the microfluidic stream path). The microfluidic device of the present invention may e.g. comprise two, three, four, ... ten or more sorting sites to sort the droplets in accordance with signals from the more than one detectors.

Sorting laser

[0060] The at least one sorting laser that is disposed for the irradiation of the sections of the microfluidic stream containing the droplets to be sorted is directed onto the sorting site(s) of the microfluidic system. The laser is disposed for generation of radiation having a wavelength which is absorbed by the carrier fluid surrounding the droplets or the liquid phase of the droplet to be sorted or of the cell or particle within the droplet, depending on the application.

[0061] In a first embodiment, the emitted laser energy has a sufficient radiation duration and radiation intensity, respectively, to heat a portion the carrier fluid surrounding the droplets nearby a droplet passing the sorting site or the liquid phase of the droplet to be sorted which preferably is only a superficial portion of the droplet. The laser radiation applies a total energy onto the irradiated carrier fluid or droplet which causes a portion of the corresponding liquid to evaporate. By the laser-induced local superficial evaporation the section of the liquid stream irradiated by the laser radiation is accelerated into the direction opposite to the laser radiation.

[0062] The laser may be a pulsed laser having a suitable wavelength and intensity to generate a vapour bubble sufficient to deflect the targeted droplet. The laser may be a continuous laser the wavelength and/or intensity of which only becomes suitable to generate a deflective vapour bubble upon appropriate focussing of the laser to the desired site. In another embodiment the laser may have a wavelength which without focusing on a spe-

cific site in or on the liquid stream could largely pass through the liquid and/or droplets to be sorted. In this embodiment, the laser is equally disposed for superficial heating of portion of liquid of the microfluidic stream (either a carrier liquid or a liquid of the droplet), wherein the focusing onto a specific spot induces a photodisruption by nonlinear multiphoton absorption.

[0063] Preferably, the focusing of the laser onto the surface of the microfluidic stream occurs by means of optical elements having in total a numerical aperture of 0.2 to 1.4, preferably having a numerical aperture of 0.3 to 1.0, especially preferred of 0.4 to 0.8. The optical elements which in their arrangement have such a numerical aperture and which are arranged in the beam path of the sorting laser, can be objectives, single-lenses, a lens combination, mirrors, concave mirrors, parabolic mirrors and/or mirror combinations.

[0064] In the method or device of the present invention the sorting laser is a pulsed laser with a pulse rate of 1 Hz to 200 MHz and each laser pulse has an energy of at least 100 nJ, preferably between 1,5 to 15 μJ and up to 100 μJ.

[0065] Suitable sorting laser emission wavelengths are between 180 and 1800 nm. Preferred emission wavelengths of the sorting laser are between 180 and 400 nm and between 1,2 and 2,7 μm. The invention explicitly includes each and every single value within the respective ranges. Exemplary wavelengths between 180 and 400 nm are 193; 222; 229; 238; 244; 248; 250; 257; 264; 284; 308; 337; 351; and 364 nm. Exemplary wavelengths between 1,2 and 2,7 μm are 1.32; 1.35; 1.3686; 1.3772; 1.444; 1.355; 1.45; 1.53; 1.54; 1.5406; 1.5413; 1.5421; 1.5436; 1.5437; 1.5469; 1.5477; 1.5489; 1.55; 1.553; 1.5553; 1.5586; 1.56; 1.5606; 1.665, 1.9708; 2.088; 2.123; 2.293 μm. The sorting laser may also have emission wavelengths above 2.7 μm, such as between 2.7 and 4.5 μm, especially 2.791; 2.8; 3.5; 3.8 μm, as well as between 4.5, -5.4 μm, especially 4.65; 4.42; 4.48; 4.86; 4.87; 5.25 μm, or between 5.4-6.5 μm, especially 5.45; 6.13; 6.29 μm, or between 6.5-11.5 μm, especially 7.43; 7.62; 7.85; 7.87; 10.09; 10.6 μm.

[0066] If the fluid is water or water based, preferred wavelengths emitted by the sorting laser are in the range of less than 350 nm, especially less than 200 nm or in the range of 1.5 to 6.45 μm, especially of 2 to 3 μm.

[0067] In an alternative embodiment the laser radiation of the sorting laser is focused very narrowly on the surface of the fluid stream, e.g. by optical elements arranged in its beam path and having a numerical aperture in the range from 0.2 to 1.4, especially NA=0.4 to 1.0, are directed onto the microfluidic stream. By this measure the emission wavelength of the sorting laser becomes subsidiary. Due to the focusing of the laser radiation onto a fraction of the microfluidic stream, the laser radiation induces a photodisruption at the site it is focused on by nonlinear multiphoton absorption which generates a local superficial evaporation of the fluid and thereby a deflection of the irradiated section of the fluid even if the fluid is not absorbing at the emission wavelength. For controlling the laser radiation emitted by the sorting laser, its laser medium itself can be controlled by a control unit, especially for generation of laser beam pulses in dependence from control signals or integrated control signals. In one embodiment, the sorting laser is a continuous (CW) laser, especially a solid-state laser or CO_2-laser, in the beam path of which no or optionally at least one optical element connected to the control unit and controlled thereby is arranged, for example an optical switch, the transmission of which is controlled by the control unit. Preferred laser beam pulses have energies of at least 100 nJ and preferably in the range from 1 to 15 μJ, e.g. 1.8 to 5 μJ, especially 3.5 μJ and up to 100 μJ. The irradiation with a pulse energy of 1.8 μJ achieves a deflection of water droplets by at least about half of the droplet size, preferably at least about 1 times the droplet size, and at a pulse energy of 3.5 μJ a deflection of water droplets by at least 1 times the droplet size, preferably more than 1 times the droplet size. Preferably, the laser beam pulses have pulse durations (tp) of from 1×10^{-4} to 1×10^{-15} s, preferably from 1×10^{-6} to 1×10^{-10} s. Preferably, pulsed lasers have repetition rates (pulse repetition rates) of from 1 Hz to 200 MHz, preferably from 100 Hz to 200 kHz, more preferably from 500 Hz to 20 kHz. A pulsed laser can have a connected triggering means or can be formed by a CW laser in the beam path of which an optical switch is arranged which is controlled by a connected triggering means (which in turn is controlled by the control signals of the one or more detectors). Alternatively, it can be formed by a pulsed laser in the beam path of which a pulse picker is arranged which selects individual pulses from a pulse group in a triggered manner and guides them into a beam path having optical elements which direct the selected pulses onto the microfluidic stream. In a preferred embodiment, a focusing means is arranged in the beam path of the sorting laser, for example a microscope objective, which focuses the laser radiation of a sorting laser onto a section of a sorting site.

[0068] In a special embodiment, in the beam path of a sorting laser there is a beam splitter arranged which divides or directs the sorting laser irradiation into a first and a second (partial) path, each of which is directed onto the microfluidic stream. The first and second partial paths can be directed onto one or more sorting sites of the microfluidic system. In this manner, the same section of the microfluidic stream can be irradiated by laser radiation on the first and/or second sorting site at a temporal distance. Preferably, a first optical switch is arranged in the first partial path and a second optical switch is arranged in the second partial path, each of which switches receives control signals from the one or more detectors. For the alignment of at least one of the first and second partial paths onto the fluid stream, mirrors may be arranged within these.

[0069] In a further embodiment the microfluidic system may have apart from the first sorting laser one or more

second sorting lasers the beam path of which is also directed onto the microfluidic stream, wherein second sorting lasers may be disposed as is the first sorting laser and may emit the same or a different wavelength. In this manner, the precision of the sorting method can be increased if the first sorting laser emits radiation having a wavelength which is absorbed by the fluidic portion targeted and results in its sectional deflection, while the beam of a second sorting laser is directed at an angle relative to the beam of the first sorting laser (see **Fig.** 3 and 4). Preferably, the second or further sorting laser is also directed onto the fluid stream and has a wavelength absorbed by the carrier fluid and/or is focused on the surface of the fluid of the droplet to be sorted. The section of the beam path of the second sorting laser may arranged at an angle of from 30° to 180°, more preferred of from 60° to 90° to the section of the beam path of the first sorting laser directed onto the fluid stream.

[0070] A second or further sorting laser may target the same of different sorting site as the first sorting laser.

[0071] Optionally or additionally, a second laser may be disposed for generation of radiation having a wavelength which essentially is not absorbed by the carrier fluid but may be absorbed by the fluid of the droplet or the cell or particle contained in the droplet such as to disintegrate the droplet and/or cell or particle.

Deflection/sorting modes

[0072] In some embodiments of the invention, the sorting laser irradiation generates a vapor bubble in the fluid stream close to the droplet or on the surface of the droplet to be sorted or acts as an optical tweezer, thereby giving the droplet to be sorted an impulse at least partially lateral to its prior flow direction in the microfluidic system, preferably leading to a deflection of more than 3° (from its previous trajectory to the sorting site) or more than 10 μm. Thus, the laser irradiation generated impulse forces the droplet to be sorted to take a path different from that of a droplet not having received such an impulse. In some embodiments the laser irradiation directs the droplet to a trajectory that depends on the mass of the droplet or on the specific weight of the droplet. In further embodiments the sorting laser irradiation directs the droplet to a trajectory that depends on the intensity of a marker contained in that droplet.

[0073] Fluidic droplets may be screened or sorted within a fluidic system of the invention by altering the trajectory of the droplets in the flow of the liquid containing the droplets. For example, by the laser deflection a droplet can be directed toward a channel junction including multiple options for further direction of flow (e.g., directed toward a branch, or fork, in a channel defining optional downstream flow channels) or directing it to a site of aspiration of drops that received a sorting impulse.

[0074] In a further embodiment, the sorting may be effected by producing with the sorting laser a laser induced bubble at the sorting site that effects that droplets passively take a different route depending on the number of cells contained in the droplets. This embodiment is particularly applicable to the sorting of plant cells and is based on the principle that empty drops will be significantly lighter than drops containing one two or more cells. The deflection based on the laser bubble generated at the sorting site is relative to the weight of the droplets to be sorted, so that droplets passing the laser generated bubble take a different trajectory depending on the number of cells contained in them. By use of the sorting laser, the energy may be adjusted in order to allow an efficient passive sorting as described.

[0075] In another embodiment the droplets (or cells or particles contained therein) are disintegrated by way of a pulse of the sorting laser. Especially in embodiments of the invention in which particles are biological cells, especially animal cells, the biological activity of the cell is altered by the heating, in particular the cells are inactivated in a targeted manner by heating. This can be done alternatively to changing a droplets path or trajectory or in addition to this sorting mode. Thus, at least one sorting laser in this disintegration mode is used to disintegrate droplets containing no cell or particle and/or droplets containing more than one cell or particle or droplets containing or not a particular detectable marker. The energy required for disintegrating droplets is several μJ and preferably ranges between 1-9 μJ of energy per pulse (e.g. 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, or 8.5 μJ). In this aspect of the invention, it suffices that the device comprises only a single microfluidic droplet path departing from the sorting site of the microfluidic device, since only desired droplets are allowed to pass the sorting site intact. However, depending on the number of sorting lasers and the number of properties for which the droplets are analyzed, more than one channel departing from the sorting site may be implemented as needed.

[0076] Therefore, for the purposes of the invention, the term of fractionation or sorting also comprises the targeted inactivation of at least a portion of the droplets cells or particles (with undesired or un-elected properties) in the alternative to or in addition to the deflection described above.

[0077] A skilled person will appreciate that the different sorting modes (active deflection, passive deflection, disintegration) may be combined into one microfluidic system using the same or different sorting lasers as required.

Collection

[0078] The collecting of the droplets of interest preferably occurs by receiving sections of the microfluidic system comprising channels, chambers and/or receptacles arranged in the flow path downstream of the sorting site. After the droplets pass the sorting site (having been deflected or not), they may be collected or captured. In one embodiment the droplets that are deflected are collected in a collecting receptacle which is arranged separately from a collecting receptacle arranged in the flow path of

the non-deflected droplets. Collecting receptacles for collecting the non-deflected droplets within the microfluidic stream and the deflected droplets of the microfluidic stream can each be arranged at a distance to one another corresponding to the flow path of the microfluidic stream departing from the sorting site (as indicated in **Figs.** 3 and 4).

[0079] The means for collecting are not limited to receptacles, but can include further channels leading to vessels, containers or repositories or leading to further devices for subsequent analysis (such as single cell sequencing devices).

[0080] The method according to the present invention allows higher sorting rates and at identical sorting rates has a higher yield or purity than the deflection of charged droplets in the electric field (as e.g. used in FACS systems), because a repolarization of the droplet stream which occurs upon application of an electrical charge to the droplet stream tearing off is not necessary. Sorting by electrical means results in that upon deflection in the electric field at usual passage rates of up to 2,500 particles/s 3 to 5 subsequent fluid droplets are subjected to the same charge and therefore usually are sorted into the same fraction independently from the particle. In contrast to this in the methods and devices according to the invention the accurate selection of single droplets is achieved by means of laser irradiation, without influencing neighboring particles by the selection.

[0081] The detection and sorting means are configured such as to reach a sorting rate of above 100 droplets/sec, preferably above 5000 droplets/sec, most preferably up to 10.000 droplets/sec.

[0082] The purity of the sorted droplets preferably exceeds 95%. Preferably the fraction of droplets sorted by the means of the present invention contains more than 95%, 96%, 97%, 98% or 99% preferably more than 99% of droplets of the desired trait for which the sorting system has been adjusted for. A fraction of droplets with a "purity" of e.g. 98% for the sake of the present invention means that the plurality of droplets sorted for a particular property (e.g. droplets containing exactly one single cell) do not contain more than 2% of droplets not having the property for which they were sorted (e.g. droplets with more than one or no cell).

Microfluidic systems

[0083] It is to be understood that each and every aspect taught in relation to the methods of the present invention is equally applicable to the microfluidic devices of the present invention without exception. Thus, each and every of the above aspects can explicitly be combined with the following sections of this description, i.e. applied to the microfluidic system described below. Accordingly, the description of the microfluidic device according to the invention also applies to and complements the steps of the inventive methods described above.

[0084] To implement the methods of the invention, the present invention provides a microfluidic device for preparing single cell droplets comprising (1) at least one inlet for a fluid containing a plurality of cells; (2) at least one inlet for a fluid for generating droplets with the fluid containing the plurality of cells; (3) microfluidic means for generating a plurality of droplets which at least partially contain one or more single cells; preferably already at this stage the fraction of droplets containing only one single cell per droplet is the predominant fraction, although the method or device of the invention is operative as long as there is any fraction of droplets containing one single cell per droplet; (4) at least one microfluidic droplet path directing the droplets generated by (3) to a sorting site of the microfluidic device; (5) at least one sorting laser configured to be capable of emitting laser irradiation to the sorting site of the microfluidic device; wherein the laser is capable of generating a vapor bubble close to a droplet or on the surface of a droplet or acts a s an optical tweezer in the sorting site and/or is capable of disintegrating a droplet in the sorting site; (6) at least one, preferably two microfluidic droplet paths departing from the sorting site of the microfluidic device in order to allow droplet to take a different direction depending on whether they have been deflected through the action of the laser irradiation; and (7) means for collecting at least one fraction of droplets containing the desired number of cells, preferably droplets containing exactly one single cell.

[0085] The microfluidic device of the invention may, upstream of the sorting site, further comprise (4.1) a detection site comprising means for detecting the presence or absence of cells within each droplet, or the presence or absence of cell markers or the intensity of one or more cell markers. As outlined above, the microfluidic device may contain more than one detector and or more than one sorting site.

[0086] Recently, microfluidic chips have been fabricated from silicon or glass, elastomer, thermosets, hydrogel, thermoplastics, and even paper. The properties of the materials used in microfluidic chips have been well summarized previously (Ren et al., Curr. Opin. Biotechnol. 25, 78-85, 2014). Microfluidics are used to manipulate liquids (dimensions from 1 to 1000μm) in networks of microchannels in a single device. The implementation of the above described methods into a microfluidic device is customary practice as described e.g. in "Microdroplet Technology: Principles and Emerging Applications in Biology and Chemistry" (Philip Day, Andreas Manz, Yonghao Zhan, Eds. Springer 2012).

[0087] A skilled person would understand that each of the specific aspects discussed in the various sections of the above description may be readily combined in any way in order to build a microfluidic system for droplet generation and sorting. The present invention therefore includes microfluidic systems integrating any of the above described aspects.

Claims

1. A method for preparing droplets containing a single particle comprising

   (i) providing a fluid containing a plurality of particles;
   (ii) generating droplets from the fluid containing the plurality of particles;
   (iii) analyzing the droplets generated in (ii) for a particular property; and
   (iv) sorting the droplets using a microfluidic system into at least two groups, depending on the number of particles contained in a droplet and/or depending on an identifiable property of the particles;

   wherein the sorting of the droplets is effected by laser irradiation of at least one sorting laser.

2. The method of claim 1, wherein the analyzing comprises the steps of

   (a) analyzing each droplet for the presence or absence of particles within the droplet, or for the absence or presence or the intensity of a marker within each droplet and
   (b) generating a control signal for the sorting laser depending on the number of particles detected in each droplet or depending on the absence or presence or intensity of the marker detected in each droplet.

3. A microfluidic device for preparing droplets containing exactly one single particle or droplets containing exactly one particular identifiable property, comprising

   (1) at least one inlet for a fluid containing a plurality of particles;
   (2) at least one inlet for a carrier fluid for transporting droplets of the fluid containing the plurality of cells;
   (3) microfluidic means for generating a fluid stream of carrier fluid comprising droplets from the fluid containing the plurality of particles, wherein the at least a part of the droplets contain one or more particles;
   (4) means for analyzing each droplet in the fluid stream containing the droplets;
   (5) at least one microfluidic droplet path directing the droplets generated by (3) to a sorting site of the microfluidic device;
   (6) at least one sorting laser configured to be capable of emitting laser irradiation to the sorting site of the microfluidic device depending on the presence or absence of particles within the droplet, or depending on the absence or presence or the intensity of an identifiable property of the droplets; wherein the laser irradiation is capable of (a) changing the droplets trajectory by generating a vapor bubble close to a droplet or on the surface of a droplet or by acting as an optical tweezer in the sorting site or (b) disintegrating a droplet in the sorting site, and
   (7) (a) at least two or (b) at least one microfluidic droplet path(s) departing from the sorting site of the microfluidic device; and
   (8) means for collecting droplets specifically containing exactly one single particle or droplets containing the one particular identifiable property.

4. The microfluidic device of claim 3, wherein the means for analyzing comprise
   a detection site upstream of the sorting site and comprising means for detecting the presence or absence of particles within the droplet, or for the absence or presence or the intensity of a marker within each droplet;
   wherein the means for detecting are configured to generate a control signal depending on the presence of absence of particles in a droplet, or depending on the absence or presence or intensity of a marker within each droplet, wherein the control signal triggers that the sorting laser emits a laser pulse to the sorting site when a droplet analyzed passes the sorting site.

5. The method of claim 1 or 2 or the device of claims 3 or 4, wherein the droplets are analyzed by optical means.

6. The method or device of claim 5, wherein the optical means comprise detecting the presence or absence of particles with a camera with a high frame rate, or by detecting a the presence of absence or intensity of a marker such as a fluorescent group associated with the particle.

7. The method or device of any one of the preceding claims, wherein the laser is a pulsed laser with a pulse rate of 1 Hz to 200 MHz and each laser pulse has an energy of at least 100 nJ, preferably between 1,5 to 15 μJ and up to 100 μJ.

8. The method or device of any one of the preceding claims wherein the sorting rate is above 100 droplets/sec, preferably above 5000 droplets/sec, most preferably up to 10.000 droplets/sec.

9. The method or device of any one of the preceding claims, wherein the laser irradiation generates an impulse that forces the droplet to take a path different from that of a droplet not having received such an impulse.

10. The method or device of claim 9, wherein the laser irradiation (i) generates a vapor bubble in the fluid stream close to the droplet or on the surface of the droplet to be sorted or (ii) acts as an optical tweezer, thereby giving the droplet to be sorted an impulse at least partially lateral to its prior flow direction in the microfluidic system, preferably leading to a deflection of more than 3° or more than 10 μm.

11. The method or device of claim 9 or 10, wherein the laser irradiation directs the droplet to a trajectory that depends on the mass of the droplet.

12. The method or device of any one of claims 1-8, wherein the laser is used to disintegrate droplets containing no particle and/or droplets containing more than one particle.

13. The method or device of claim 12, wherein the device comprises only a single microfluidic droplet path departing from the sorting site of the microfluidic device.

14. The method or device of any of the preceding claims, wherein one sorted group of droplets comprises more than 95%, 96%, 97% or 98%, preferably more than 99% of droplets containing exactly one single particle and/or contain particles with exactly one particular identifiable property.

15. The method or device of any of the preceding claims, wherein the particle is a cell, preferably a eukaryotic cell.

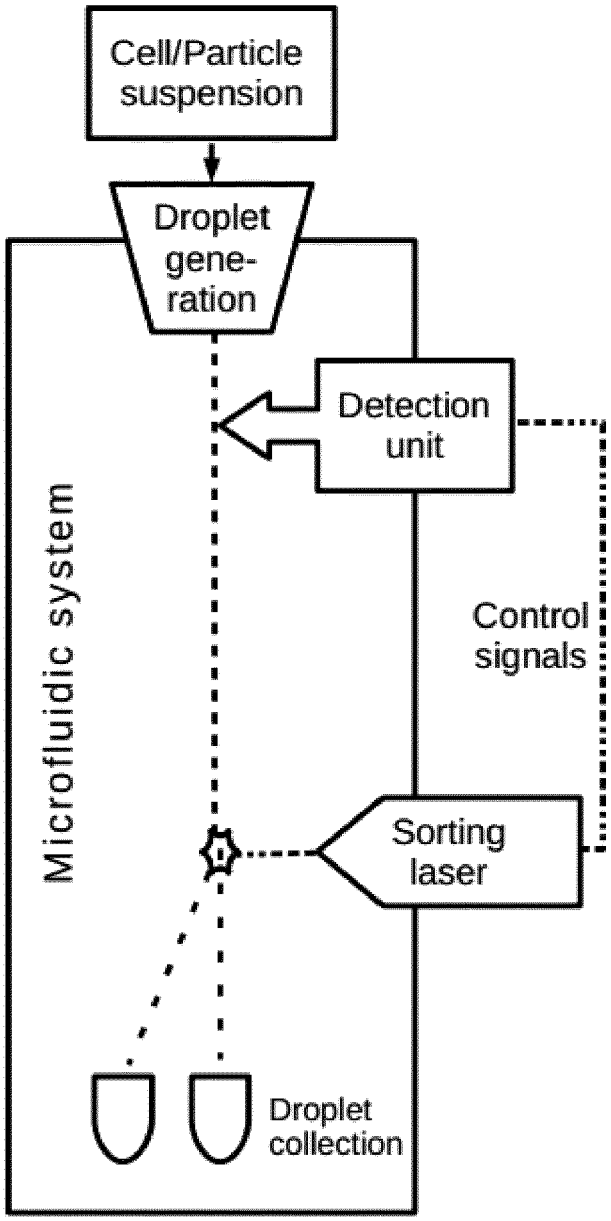
Cell/Particle suspension
Droplet gene-ration
Detection unit
Microfluidic system
Control signals
Sorting laser
Droplet collection

Figure 1

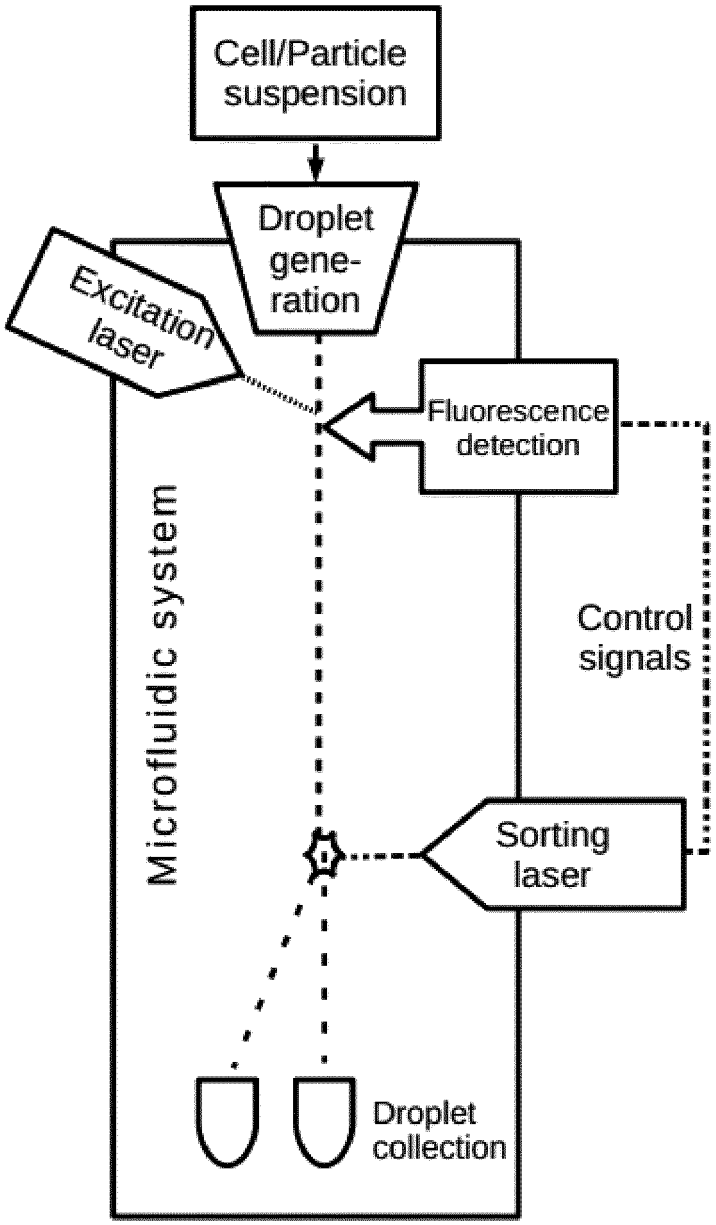
Cell/Particle suspension
Droplet gene-ration
Excitation laser
Fluorescence detection
Microfluidic system
Control signals
Sorting laser
Droplet collection

Figure 2

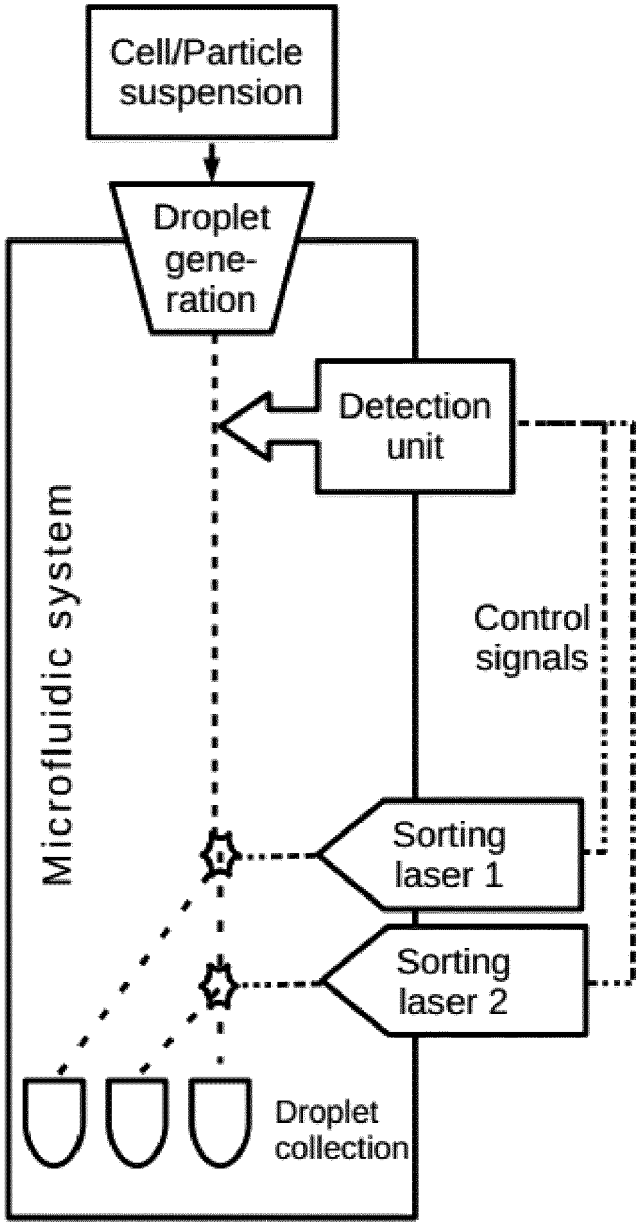
Cell/Particle suspension
Droplet gene-ration
Detection unit
Microfluidic system
Control signals
Sorting laser 1
Sorting laser 2
Droplet collection

Figure 3

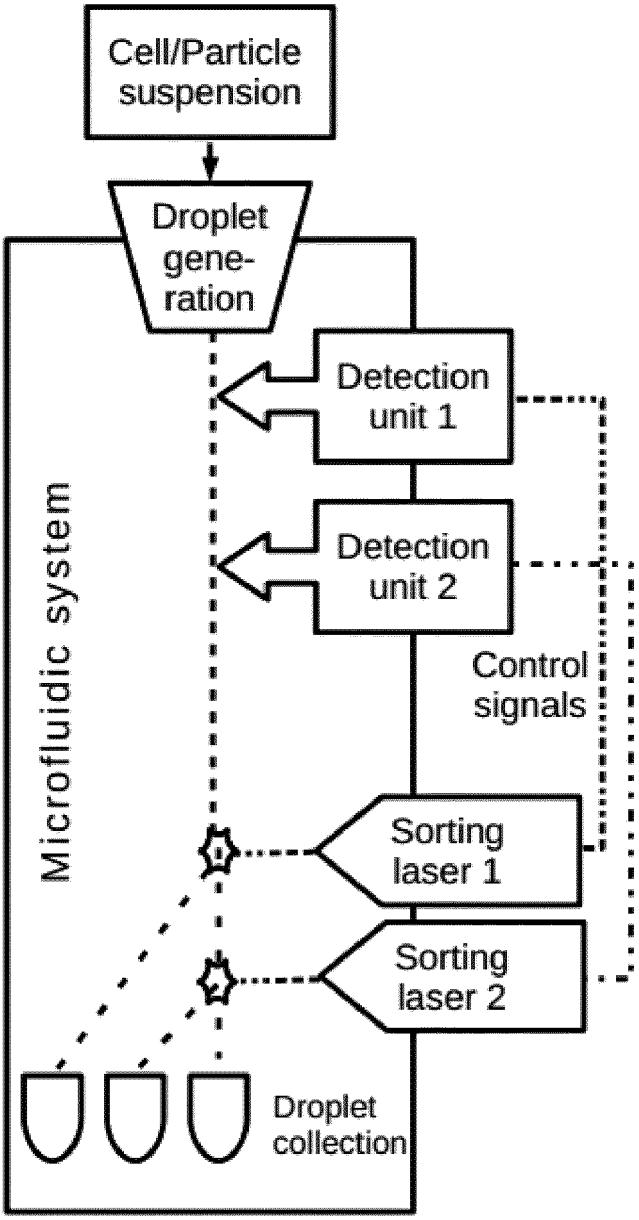
Cell/Particle suspension
Droplet gene-ration
Detection unit 1
Detection unit 2
Microfluidic system
Control signals
Sorting laser 1
Sorting laser 2
Droplet collection

Figure 4

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EUROPEAN SEARCH REPORT

Application Number
EP 19 15 7973

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	2 May 2019	Goodman, Marco

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X : particularly relevant if taken alone
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