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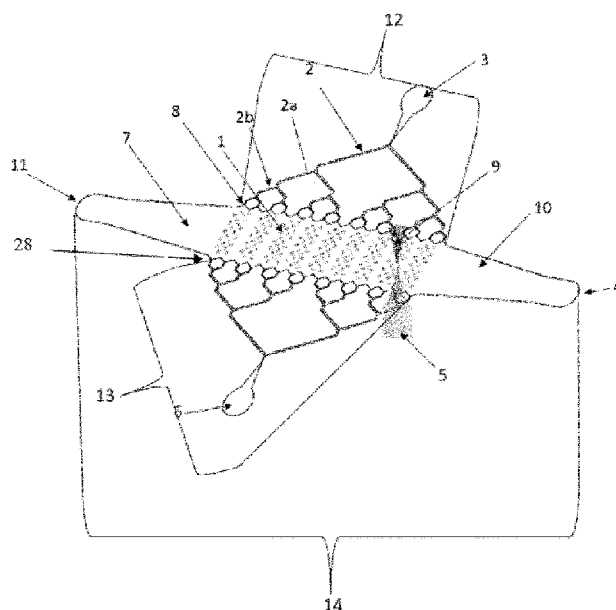


Figure 18

(57) Abstract: In general the present invention concerns 1) single cell trapping of a viable cell in separate well from a plurality of wells in an array of wells, 2) single cell analysis for the selected cell and 3) single cell lifting of the yet viable cell from the well by an optical tweezer. Furthermore present invention concerns a cell trap and lift device for B lymphocytes, the device comprising an array of wells in in polymer matrix comprising an off-stoichiometry thiol-ene polymer of the group consisting of off- stoichiometry thiol-ene (OSTE) and off- stoichiometry thiol-ene-epoxy (OSTE+) or a combination thereof that have been grafted with methacrylated polyethylene glycol (methoxy polyethylene glycol methacrylate or (M-PEG-M)) of a number average molecular weight of Mn 2000. It furthermore concerns using the B lymphocyte trap and lift device for trapping single B lymphocyte cells in wells of the device of present invention and lifting said cell from the cell trapping well by optical tweezers, preferably single beam tweezers.



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Screening and sorting of single cells

Background and Summary

5 BACKGROUND OF THE INVENTION

A. Field of the Invention

In general the present invention concerns 1) single cell trapping of a viable cell in separate well from a plurality of wells in an array of wells, 2) single cell analysis for the selected
10 cell and 3) single cell lifting of the yet viable cell from the well by an optical tweezer.

Furthermore present invention concerns a cell trap and lift device for B lymphocytes, the device comprising an array of wells in polymer matrix composed of alternating copolymers with thiol-ene groups for instance a polymer matrix comprising an off-
15 stoichiometry thiol-ene polymer of the group consisting of off-stoichiometry thiol-ene (OSTE) and off-stoichiometry thiol-ene-epoxy (OSTE+) or a combination thereof that have been grafted with methacrylated polyethylene glycol (methoxy polyethylene glycol methacrylate or (M-PEG-M)) of a number average molecular weight of Mn 2 000. It furthermore concerns using the B lymphocyte trap and lift device for trapping single B
20 lymphocyte cells in wells of the device of present invention and lifting said cell from the cell trapping well by optical tweezers, preferably single beam tweezers.

B. Description of the Related Art

25 FACS sorting and single cell sequencing, have allowed to progress in the discovery of new antibodies and therefore to improve immunoassays for disease diagnostics. However, faster screening tools and higher efficiency rates are required and efficient sorting systems for n single B cells.

30 Present invention concerns a system for identification and sorting of viable individual cells in a high-throughput fashion.

SUMMARY OF THE INVENTION

- 5 The present invention solves the problems of the related art by of manipulating (human) B cells for single B cell selection without losing its viability.

In accordance with the purpose of the invention, as embodied and broadly described herein, the invention is broadly drawn to the integration of optical tweezers with a PEG-
10 grafted OSTE+ microwell array, this array being grafted with methacrylated polyethylene glycol (methoxy polyethylene glycol methacrylate or (M-PEG-M)) of a number average molecular weight of around Mn 2000, for instance 1800 – 2200 and preferably 1950 – 2050. This system is retrieving single cells out of microwells for high-throughput screening of single cell responses to delivered reagents and allows collecting cells with
15 an positive signal for further analysis.

Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the
20 invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

- 25 The present invention relates to a cell sorting device that comprises 1) a microarray [1] of micro-wells [e.g. 1a], 2) an elongate or oblong conduit [14], the micro-well array [1] being comprised in the elongate or oblong conduit [14], and 3) channels, characterised in that the micro-well array [1] is positioned between a) at one side the upstream part of the conduit [7] with at least one inlet port [11] of the conduit [14] and b) at another side
30 the downstream part [10] of the conduit [14] with at least one outlet port [4] of the conduit [14] and further characterised in that the micro-well microarray [1] is being positioned between the ports or apertures (e.g. [8] or [28]) of i) a first set of branching channels

[12] and ii) a second set of branching channels [13], and whereby these two sets of branching channels are outside the conduit [14] but connect with or are engaged with the conduit [14] via ports or apertures (e.g. [8] or [28]) in the conduit [14]. In an embodiment the present invention also provides that each fluid channel set ([12] & [13]) comprises a fluid inlet ([3] & fluid outlet [6]). In an embodiment the present invention also provides that the conduit [14] is a conduit for an aqueous liquid and the branched channels [12] and [13] are channels for an aqueous liquid. In a further embodiment of the invention, the cell sorting device according to any one of the above embodiments also provides that micro-wells are organized in parallel arrays (e.g. [1b]) of micro-wells or rows of micro-wells which are arrays or rows separated with parallel partitions or spaces (e.g. [1c]) and which are positioned longitudinal between a ports or apertures (e.g. [8]) of the first set of branching channels and a ports or apertures (e.g. [28]) of the second set of branching channels or that the micro-wells which are organized in parallel arrays (e.g. [1b]) of micro-wells or rows of micro-wells which arrays or rows are separated with parallel partitions or spaces (e.g. [1c]) and which are positioned crosswise the elongate or oblong conduit [14] in an intermediate zone between the upstream part [7] and the downstream part [10] of the conduit [14]. In yet a further embodiment of the invention, the cell sorting device according to any one of the above embodiments also provides that in the first set of branching channels each channel [2] that extends from the fluid inlet port [3] branch out in additional channels [2a] which again branch out in additional channels [2b] and which at the distal end connect with or are engaged with the conduit [14] via ports or apertures (e.g. [8]) and whereby in the second set of branching channels each channel that extends from the fluid outlet port [6] branch out in additional channels which branch out in additional channels and which at the distal end connect with or are engaged with the conduit [14] via ports or apertures [e.g. 28]).

This invention accordingly provides the advantage of a uniform flow velocity distribution over the array of wells. The velocity increase in branched side channels as compared to the array ensures that cells do not dislodge from the micro wells but can be transported to the outlet. This helps us to work without contamination of unwanted cells and to minimize interaction of the optical tweezers with the cell. Moreover it limits interaction times with optical tweezers and it allows to find the port easily so minimizes interaction time with optical tweezers. The cells are prevented from entering in the branched fluid channels so

ensures that there is no contamination of unwanted cells and there will be no loss of cells in dead volumes of the device or in tubing, collection of single cell in cell by cell manner is possible, in low volumes.

In a further embodiment of the invention, this cell sorting device according to here above
5 described is a microfluidic cell sorting device.

In an embodiment of the cell sorting device there is a space [24 & 23] between the microarray of micro-wells [1] and each zone of the wall of the conduit [14] where the branching channels are connect with or are engaged with the conduit [14] via ports or apertures (e.g. [8] or [28]), the cell sorting device being furthermore characterised in that
10 in the upstream part [7] of the conduit [14] it comprises a first fluid inlet port [15] more distal from said the microarray of micro-wells [1] and a second fluid inlet port [11] more proximate to the microarray of micro-wells [1], whereby the first fluid inlet port [15] connects with or is engaged with two fluid channels [16] which each extend lateral and longitudinal with a space [24 or 23] and open approximate to the space [24 & 23] so to
15 create when operational a lateral flow of a sheath fluid that sandwiches a core fluid and whereby the second fluid inlet port [11] opens more in the core of the upstream part [7] of the conduit [14] so that when operational it creates a core fluid stream towards the microarray of micro-wells [1].

In yet an embodiment of the cell sorting device, it comprises a solid object [26] in the
20 downstream part [10] of the conduit [14] with a space between its rim and part of the wall of the conduit [14] so to form the channels [16] extending from the first fluid inlet port [15] and whereby the second fluid inlet port [11] opens in a cavity [27] formed by recess in the edge of solid object [26] which is faced to the microarray of micro-wells [1] so that when operational a core fluid stream with cells is released in said cavity [27]
25 towards the microarray of micro-wells [1] and by lateral flow of a sheath fluid that sandwiches a core fluid is directed onto the microarray of micro-wells [1]. This solid object [26] can be a Y shaped solid plate. Moreover the second set of branching channels [13] at its end distal from the conduit [14] can be connected with or engaged with the a water-in-oil droplets generator. In one embodiment, the a second set of branching
30 channels [13] at its end distal from the conduit [14] connects with or is engaged with a fluid channel [21] that opens approximately to the outlet of an oil channel [18] so that when operational a flow with aqueous fluid comprising cells is delivered into a flow of

oil from an oil fluid channel [18] to form water-in-oil droplets in an reservoir or chamber [20] with hydrophobic internal walls. This droplet based cell retrieval from sample outlet channel is advantageous in that retrieved cells can be isolated in very small volumes, much smaller than by using the other devices.

5 In a further embodiment of the invention, the micro-well array [1] in the conduit [14] is positioned in a plane with two opposing fluid channels sets ([12] & [13]), or the micro-well array [1] is a micro-well array plate that at its edge site is aligned between two opposing fluid channels sets ([12] & [13]) and between the two opposing fluid conduits ports ([11] & [4]). In a further embodiment of the invention, an optical tweezer [5] is
10 positioned under the plane or under the bottom of the microarray of micro-wells [1], the optical tweezer [5] mouth can be directed towards the bottom of the microarray of micro-wells [1] and the optical tweezer [5] when operational sends a light beam, preferably perpendicular, through the plane of the microarray of micro-wells [1].

In a further embodiment of the invention, each fluid channel set comprises a fluid inlet
15 ([3] & fluid outlet [6]) and from there on the fluid channel branches out into at least 2 channels which can branches out in other at least 2 channels whereby the end channels each are engaged with a port (e.g. [8] in the conduit [14]).

In a further embodiment of the invention, at least one fluid inlet [11] is at one distal end of the elongated conduit [14] opposing the at least one fluid outlet [4] at the other distal
20 end of the elongated conduit [14].

In a particular embodiment of the invention the elongate or oblong conduit is an enclosure or the elongate or oblong conduit is a sleeve or the elongate or oblong conduit is a groove or the elongate or oblong conduit is a liquid passage.

In yet another particular embodiment of the invention the channel port is an aperture
25 and/or the inlet is an aperture.

In another aspect, the present invention provides that the microwell array [1] is for single cell per well trapping and lifting of viable cell and is characterised in that the wells are in a matrix having a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains having a number average molecular weight of a Mn value in a
30 range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.

In yet another aspect, the present invention provides that the microwell array [1] for single cell per well trapping and lifting of viable cell, characterised in that the wells are in a matrix consisting essentially of a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains having a number average molecular weight of a Mn value in a range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.

In yet another aspect, the present invention provides that the microwell array [1] is for single cell per well trapping and lifting of viable (human) B cell and is characterised in that the wells are in a matrix having a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains having a number average molecular weight of a Mn value in a range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.

In yet another aspect, the present invention provides that the microwell array [1] is for single cell per well trapping and lifting of viable (human) B cell, characterised in that the wells are in a matrix consisting essentially of a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains having a number average molecular weight of a Mn value in a range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.

These microwells preferably have a diameter of a value in the range between 9 – 14 μm (12 μm), preferably between 10 – 13 μm and a depth of a value in the range between 9 – 14 μm (12 μm), preferably between 10 – 13 μm .

In yet another aspect, the present invention provides that the methoxy polyethylene glycol methacrylate chains at the surface of said thiol polymer matrix are being bound with the thiol polymer matrix with at least one end of the methoxy polyethylene glycol methacrylate chain with a sulphur atom-containing group there between. The thiol polymer can be a thiol/ene polymer.

In yet another aspect, the present invention provides that the polymer matrix comprises a polymer derived from a thiol terminated polymer whereby thiol groups reacted with a methoxy polyethylene glycol methacrylate. The polymer matrix can comprise oxiranyl group on its surface. Moreover the polymer matrix can comprise a thiol-ene polymer of the group consisting of off-stoichiometry thiol-enes polymer and off-stoichiometry thiol-ene-epoxies polymer.

In yet another aspect, the present invention provides that the microwell array is comprised in an apparatus for trapping of viable single human B cell each in a well and selective lifting viable single human B cell for from it well without affecting viability, the apparatus comprising 1) a single beam optical tweezer in the 900 – 1200 range wave length of and with a laser power of a value between 400 mW and 600 mW or that the microwell array is comprised in an apparatus for trapping of viable single human B cell each in a well and selective lifting viable single human B cell for from it well without affecting viability, the apparatus comprising 1) a single beam optical tweezer in the 1000 – 1210 range wave length of and with a laser power of a value between 450 mW and 550 mW.

A further disadvantageous aspect is also, the use of the cell sorting device of present invention for manipulation viable single cells will save guarding the viability, the manipulation comprising single cell in single well trapping, single cell analysis for the selected cell, identification B cells expressing a selected protein, optical trapping and lifting said selected cell by the optical tweezer for further manipulating of said viable cell, the use thereof for bidirectional flow or the use thereof for cell seeding, washing of non-seeded cells and delivery of reagents for the identification of the cell.

The size of the microwells (withd-depth) of a certain aspect of the invention is advantageous providing that we can have B-cells seeded as single cells in the wells in a way that they can be analysed and still retrieved from the wells. The size of the mixture of PEG500/2000 of a certain aspect of the invention is advantageous providing that the availability of the different functional groups on both PEGs leads to both good PDMS binding and the right hydrophilicity in order to keep the cells in motion and not adhering to the surface so that they can be lifted. The branched sample outlet of a certain aspect of the invention is advantageous providing that cell retrieval does not lead to additional cell contamination and that the process will be faster as sample outlets are closer to the microwell holes. The droplet based cell retrieval from sample outlet channel of a certain aspect of the invention is advantageous providing that retrieved cells can be isolated in very small volumes, much smaller than by using the other devices. The branched channels of a certain aspect of the invention is advantageous providing a uniform flow velocity distribution over array. It is providing a velocity increase in branched side channels as compared to the array ensures that cells do not dislodge from

the microwells but can be transported to the outlet. This helps us to work without contamination of unwanted cells and to minimize interaction times of the optical tweezers with the cell

5 The grouping of array of a certain aspect of the invention is advantageous providing that it limited interaction times with optical tweezers

The port of branched channels in 'funnel shape' of a certain aspect of the invention is advantageous that it allows to find the port easily so minimizes interaction time with optical tweezers.

10 The extra buffer inlet for sheath flow [15,16] of a certain aspect of the invention is advantageous providing that cells are prevented from entering in the branched fluid channels so ensures that there is no contamination of unwanted cells.

The cell retrieval by droplets of a certain aspect of the invention is advantageous providing that there is no loss of cells in dead volumes of the device or in tubing, collection of single cell in cell by cell manner is possible, in low volumes

15

Some embodiments of the invention are set forth in claim format directly below:

1) A microwell array (or microtray) for single cell per well trapping and lifting of viable (human) B cell, characterized in that the wells are in a matrix having a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains
20 having have a number average molecular weight of a Mn value in a range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.

2) A microwell array (or microtray) for single cell per well trapping and lifting of viable (human) B cell, characterized in that the wells are in a matrix consisting essentially of a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface,
25 this chains having have a number average molecular weight of a Mn value in a range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.

3) The microwell array according to any one of the embodiments 1 to 2, whereby the microwells have a diameter of a value in the range between 9 – 14 μm (12 μm), preferably
30 between 10 – 13 μm and a depth of a value in the range between 9 – 14 μm (12 μm), preferably between 10 – 13 μm .

- 4) The microwell array according to any one of the embodiments 1 to 3, whereby methoxy polyethylene glycol methacrylate chains at the surface of said thiol polymer matrix are being bound with the thiol polymer matrix with at least one end of the methoxy polyethylene glycol methacrylate chain with a sulfur atom-containing group there
5 between.
- 5) The microwell array according to any one of the embodiments 1 to 4, whereby the thiol polymer is a thiol/ene polymer
- 6) The microwell array according to any one of the embodiments 1 to 5, whereby polymer matrix comprises a polymer derived from a thiol terminated polymer whereby
10 thiol groups reacts with a methoxy polyethylene glycol methacrylate.
- 7) The microwell array according to any one of the embodiments 1 to 6, whereby the polymer matrix also comprises oxiranyl group on its surface.
- 8) The microwell array according to any one of the embodiments 1 to 6, whereby the polymer matrix comprises a thiol-ene polymer of the group consisting of off-
15 stoichiometry thiol-enes polymer and off-stoichiometry thiol-ene-epoxies polymer
- 9) The microwell array according to any one of the embodiments 1 to 8, whereby the microwell array is comprised in an apparatus for trapping of viable single human B cell each in a well and selective lifting viable single human B cell for from its well without affecting viability, the apparatus comprising 1) a single beam optical tweezer in the 900
20 – 1200 range wave length of and with a laser power of a value between 400 mW and 600 mW. 700 nanometers (nm) to 1 millimeter (mm)
- 10) The microwell array according to any one of the embodiments 1 to 8, whereby the microwell array is comprised in an apparatus for trapping of viable single human B cell each in a well and selective lifting of a viable single human B cell from a well without
25 affecting viability, the apparatus comprising 1) a single beam optical tweezer in the 1000 – 1210 range wave length of and with a laser power of a value between 450 mW and 550 mW.
- 11) The use of the apparatus according to any one of the embodiments 9 to 10, for manipulation viable single human B cells will save guarding the viability, the
30 manipulation comprising single cell in single well trapping, single cell analysis for the selected cell, identification B cells with a selected membrane bound immunoglobulin,

optical trapping and lifting said selected cell by the optical tweezer for further manipulating of said viable cell.

12) The specific design of the microfluidic apparatus that can be used. The design consists of a bidirectional flow. The horizontal channels will be used for cell seeding, washing of non-seeded cells and delivery of reagents for the identification of the cell. The vertical channels will remain completely clear of cells. This vertical direction is used for transport of the tweezed cell.

Particular and preferred aspects of the invention are set out in the accompanying independent and dependent claims. Features from the dependent claims may be combined with features of the independent claims and with features of other dependent claims as appropriate and not merely as explicitly set out in the claims.

Detailed Description

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The following detailed description of the invention refers to the accompanying drawings. The same reference numbers in different drawings identify the same or similar elements. Also, the following detailed description does not limit the invention. Instead, the scope of the invention is defined by the appended claims and equivalents thereof.

The following detailed description of the invention refers to the accompanying drawings. The same reference numbers in different drawings identify the same or similar elements. Also, the following detailed description does not limit the invention. Instead, the scope of the invention is defined by the appended claims and equivalents thereof.

Several documents are cited throughout the text of this specification. Each of the documents herein (including any manufacturer's specifications, instructions etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention.

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn to scale for illustrative

purposes. The dimensions and the relative dimensions do not correspond to actual reductions to practice of the invention.

Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

Moreover, the terms top, bottom, over, under and the like in the description and the claims are used for descriptive purposes and not necessarily for describing relative positions. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other orientations than described or illustrated herein.

It is to be noticed that the term “comprising”, used in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps. It is thus to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more other features, integers, steps or components, or groups thereof. Thus, the scope of the expression “a device comprising means A and B” should not be limited to the devices consisting only of components A and B. It means that with respect to the present invention, the only relevant components of the device are A and B.

Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to one of ordinary skill in the art from this disclosure, in one or more embodiments.

Similarly it should be appreciated that in the description of exemplary embodiments of the invention, various features of the invention are sometimes grouped together in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure

and aiding the understanding of one or more of the various inventive aspects. This method of disclosure, however, is not to be interpreted as reflecting an intention that the claimed invention requires more features than are expressly recited in each claim. Rather, as the following claims reflect, inventive aspects lie in less than all features of a single
5 foregoing disclosed embodiment. Thus, the claims following the detailed description are hereby expressly incorporated into this detailed description, with each claim standing on its own as a separate embodiment of this invention.

Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different
10 embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

In the description provided herein, numerous specific details are set forth. However, it is understood that embodiments of the invention may be practiced without these specific
15 details. In other instances, well-known methods, structures and techniques have not been shown in detail in order not to obscure an understanding of this description.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein.

It is intended that the specification and examples be considered as exemplary only.

20 Each and every claim is incorporated into the specification as an embodiment of the present invention. Thus, the claims are part of the description and are a further description and are in addition to the preferred embodiments of the present invention.

Each of the claims set out a particular embodiment of the invention.

The following terms are provided solely to aid in the understanding of the invention.

25

Definitions

OSTE+ is an off-stoichiometry thiol-ene-epoxy (TEE). Off-stoichiometry thiol-ene polymer polymer comprise off-stoichiometry thiol-enes (OSTE) and off-stoichiometry
30 thiol-ene-epoxies (OSTE+). OSTE resins are cured via a rapid thiol-ene “Click” reaction between thiols and allyls. The thiols and allyls react in a perfectly alternating fashion and has a very high conversion rate (up to 99%), the initial off-stoichiometry of the monomers

will exactly define the number off unreacted groups left after the polymerization. With the right choice of monomers very high off-stoichiometry ratios can be attained while maintaining good mechanical properties. The off-stoichiometry thiol-ene-epoxies, or OSTE+ polymers, are created in a two-step curing process where a first rapid thiol-ene
5 reaction defines the geometric shape of the polymer while leaving an excess of thiols and all the epoxy unreacted. In a second step all the remaining thiol groups and the epoxy groups are reacted to form an inert polymer [Saharil, Journal of Micromechanics and Microengineering 23, 025021 (2013)]

To achieve excellent processing properties of TEE thermosets the reactions of Thiol-ene coupling (TEC) and thiol-epoxy coupling (TEpC) should be temporally separated and
10 individually controlled. This can be done by inducing sequential crosslinking of the TEE network by first curing thiol-ene groups followed by thiol-epoxy groups, or vice versa [J. A. Carioscia, et al Polymer, vol. 48, no. 6, pp. 1526 – 1532, 2007]. Since thiol groups are involved in both of the two curing steps proper control mechanisms for the separation of
15 each curing stage have to be chosen. This effectively include external control of the initiation mechanisms for each ideally orthogonal reaction type (e.g. radical TEC and base catalyzed TEpC) by careful selection of monomers and initiators. More specifically a two-component thiol-ene-epoxy is used prepared by mixing the OSTEmerX Crystal Clear (322–40) compounds A (harder) and B (base) with a mass ratio of 1.1:1. In the
20 first step epoxy monomers added form the ternary thiol-ene-epoxy monomer systems, where the epoxy in a second step reacts with the excess of thiols creating a final polymer article that is completely inert. Throughout the document, the first reaction step in which the thiol groups and the ene groups form bonds, is named ‘UV cure’ or ‘First UV cure’. The second reaction step in which the excess thiol groups form bonds with the epoxy
25 groups, is called the ‘thermal cure’.

‘Grafting of polyethylene glycol (PEG) on the OSTE+ surface’ means that the OSTE+ surface is incubated with a solution containing certain percentage of PEG dissolved in ethanol, containing also a percentage of UV initiator (1-hydroxycyclohexyl phenyl ketone
30 99%, CAS Number 947-19-3, Linear Formula HOC6H10COC6H5, Molecular Weight 204.26). The OSTE+ in contact with the PEG solution only had a first UV cure, meaning that allyl and thiol groups have reacted, and there are still free thiol and epoxy groups on

the surface. The different types of PEG described here, have a methacrylate group that will react with the free thiol on the surface when exposed to UV.

When a 'PDMS channel', or 'microfluidic channel', or 'microfluidic PDMS channel', or
5 'microfluidic channel in PDMS' or 'channel' is described, this refers to a microfluidic channel in the Polydimethylsiloxane polymer which is a very common technique for prototyping microfluidics in research. For this microfluidic device fabrication, the PDMS base is mixed with a curing agent and poured into a microstructured mold. Then, the PDMS is heated to have an elastomeric replica of the mold.

10

Present invention successfully demonstrated collection of single B lymphocyte cells, from microwells by designing and stamp-molding a polyethyleneglycol (PEG)-grafted microwell array and combining it with an optical tweezers set-up. In a particular set up the microwell array was composed of an off-stoichiometry thiol-ene polymer, more
15 particularly an off-stoichiometry thiol-ene-epoxy. The PEG molecules coated on the microwell surface led to enhanced Brownian motion of the cells by avoiding its adhesion to surface, resulting in higher performance of the optical tweezers for single cell trapping and sorting. By integrating the microwell array with a channel in Polydimethylsiloxane (PDMS), the PEG molecules also led to efficient washing of non-seeded cells. Thus, the
20 PEG molecules were required for the development of a high-throughput screening of single cell responses to delivered reagents and collecting cells.

Phenotypic and genetic diversity at the single cell level is often overlooked in bulk assays. Recently, platforms for single cell studies have arisen to provide relevant information of
25 rare cell subpopulations. In particular, studies of single B cells, e.g. FACS sorting and single cell sequencing, have allowed to progress in the discovery of new antibodies and therefore to improve immunoassays for disease diagnostics. However, faster screening tools and higher efficiency rates are still required.

Single cell screening platforms such as microwell arrays could solve this technical
30 problem, however, collecting viable cells with a positive signal for further analysis is still challenging, especially in combination with a sealed microfluidic device. Present invention used an optical tweezer system for lifting a single cell that was first captured in

microwells with suitable dimensions to trap one single cell. We performed crosslinking of poly(ethylene glycol) (PEG) polymer chains onto a surface of the off-stoichiometry thiol-ene epoxy (OSTE+) microwells (surface grafting of PEG on the off-stoichiometry thiol-ene epoxy (OSTE+) microwells) and combined with optical forces of which we
5 found that can overcome the interaction forces between the cell and the microwell surface single cell trapped in a cell could be lifted and translocated successfully without viability loss by lysis.

Present invented technology enables the identification, sorting and analysis of individual
10 cells in a high-throughput fashion and B cells, isolated from fresh blood or from cryopreserved human peripheral blood mononuclear cells (PBMCs), can be used for the identification of specific antibodies. In order to achieve single cell resolution, the cells are seeded individually in microwell arrays. Subsequently the ones that present specific membrane immunoglobulins are identified by means of a sandwich ELISA based assay,
15 which results in a fluorescent signal. Next, an optical tweezers set-up is used to retrieve the positive B cells out of the microwells and transport them across the array. Using a unique microfluidic design, the selected B cells are collected in a tube or 96 well plate for further single cell sequencing.

20 Different combinations of microwell diameter, microwell depth and interwell distance have been tested to optimize the cell seeding. A seeding efficiency of up to 85% was achieved with a single cell seeding of up to 64%, enabling the simultaneous analysis of up to 35.000 individual cells. After cell incubation of at least 1 hour, the optical tweezers were used to elevate the B cells of interest out of the microwells. So far up to 95% of
25 them could be retrieved from the microwells and transported across the array. Furthermore, it was experimentally confirmed that tweezing cells for 10 minutes did not induce cell lysis.

Next to the ability of fast screening and isolation of specific B cells, this versatile platform
30 can be used in many other single cell studies that require high-throughput cell identification and collection.

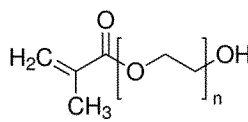
EXAMPLES

Example 1 – Successful integration microwells for B cells with optical tweezers

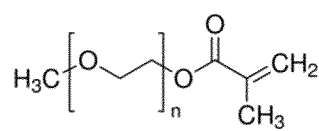
5 OSTE+ microwell arrays with different well diameter (ϕ) and depth ($\downarrow$) were designed considering the dimensions of human B cells (Figure 1). The process of microwell array fabrication in OSTE+ using a stamp-molding technique is shown in Figure 2. The human B cells were prepared at concentration of 10^7 cells/ml and stained against DNA (DAPI). Ten μ l was then pipetted into the arrays and incubated for 30mins. After incubation time,
 10 the arrays are rinsed with PBS to remove unseeded cells. Human B cell seeding efficiency was evaluated using fluorescence microscopy. Arrays with microwells of 12 ± 1 μ m diameter demonstrated a seeding of up to 80% in which 60% represent single cells. Smaller diameters lead to decreased seeding (Figure 3). It was observed that arrays with 11 ± 1 μ m diameter and 11 ± 1 μ m depth leads to an optimal ratio of single cell seeding and
 15 low number of wells with two or more cells. Plus, preliminary experiments demonstrated that a depth of 11 ± 1 μ m is more suitable for single cell lifting when compared with 14 ± 1 μ m.

Seeding efficiency of human B cells in different microwell arrays is displayed in Figure 4. A) DNA stained B cells seeded in microwells; B) Bright field image of microwell array
 20 with seeded B cells.

The microwell array and integration with optical tweezers is shown in figure 5. The optical tweezers set-up is described in [1. Decrop, D. et al. Anal. Chem. 88, 8596–8603 (2016).]. It is a 500 mW single beam optical tweezers of 1064 nm. It was seen that the
 25 optical tweezers were not efficient for lifting single B cells out of the OSTE+ microwells, because of interaction forces between the B cells and the microwell surface. To tune these interaction forces, surface grafting of two types of Poly(ethylene glycol) (PEG) methacrylate was performed on the OSTE+ surface using UV initiated thiol-ene click chemistry (Figure 6). The first type of PEG was **Poly(ethylene glycol) methacrylate**
 30 **with average Mn 360** (Synonym: PEGMA, CAS Number 25736-86-1, Linear Formula $H_2C=C(CH_3)CO(OCH_2CH_2)_nOH$, MDL number MFCD00081879 and with end

standing hydroxyl group as in the formula ). This PEGMA Mn 360 was diluted in ethanol, having 10 w/w% PEGMA, 2 w/w% UV initiator and 88 w/w% ethanol. After the first UV cure of the OSTE+ microwell array, the OSTE+ array with free thiol and epoxy groups is completely submerged in the described solution with PEGMA Mn 360. Then, this is exposed to UV of 12 mW/cm² for 5 minutes. After this, the OSTE+ array is rinsed thoroughly first with ethanol and then with water. Next, the OSTE+ array is blow dried using a N₂ gun. The thermal cure of the OSTE+ array is performed in an oven at 60°C overnight.

The second type of PEG was **Poly(ethylene glycol) methyl ether methacrylate solution average Mn 2,000**, 50 wt. % in H₂O (Synonym: M-PEG-M, CAS Number 26915-72-0, Linear Formula H₂C=C(CH₃)CO₂(CH₂CH₂O)_nCH₃, MDL number MFCD00241432, PubChem Substance ID 24869402) and with end standing methyl group as in the

formulation, ). This M-PEG-M solution Mn 2,000 50 wt% in H₂O was diluted in ethanol, having 50 w/w% of the M-PEG-M in H₂O solution, 1 w/w% UV initiator and 49 w/w% ethanol. After the first UV cure of the OSTE+ microwell array, the OSTE+ array with free thiol and epoxy groups is completely submerged in the described ethanol solution with M-PEG-M Mn 2,000. Then, this is exposed to UV of 12 mW/cm² for 5 minutes. After this, the OSTE+ array is rinsed thoroughly first with ethanol and then with water. Next, the OSTE+ array is blow dried using an N₂ gun. The thermal cure of the OSTE+ array is performed in an oven at 60°C overnight.

The successful grafting of the two types of PEG on the OSTE+ surface was validated using static contact angle measurements (Figure 7). The contact angle is significantly lower for PEGMA Mn 360 grafted microwell arrays compared to OSTE+ arrays. Furthermore, the contact angle is significantly lower for M-PEG-M Mn 2,000 grafted microwell arrays compared to OSTE+ arrays and compared to PEGMA Mn 360 grafted microwell arrays (Figure 7A). To validate that the decrease in contact angle is due to the chemical reaction between the PEG methacrylate and the free thiol and not due to physical adsorption, a control was included for each PEG. In this control, OSTE+ surfaces were simply submerged in the same PEG solutions, but without exposure to UV light. As seen

in the figure 7B, no decrease in contact angle was observed for the controls, which confirms the specificity of the grafting process.

To study the effect of the PEG-grafting on the surface interactions between the cells and the microwells, the Brownian motion (BM) of the cells and the efficiency of the optical tweezers to lift cells out of the microwells were determined (Figure 8 and Figure 9, respectively). For BM study, B cells were seeded in $11 \pm 1 \mu\text{m}$ depth microwells and, after 2 hours incubation, imaged under the microscope at 100x objective. Microwell arrays without treatment (Control, OSTE+ arrays), with PEGMA-360 grafted and with M-PEG-M-2,000 grafted were tested and videos of at least 15 seconds were recorded for the different arrays. A random motion of cells in wells for more than 10 seconds was classified as “BM movement” (classified as 1), absence of movement was taken as “without BM movement” (classified as 0). This study gives an indication if the cells were in suspension or were adhered to surface of microwells and can be used as a prediction if the cells would be elevated by optical tweezers or not. These results show that M-PEG-M Mn 2,000 on the surface significantly increase Brownian motion (Figure 8). Then, cells with Brownian motion were selected and it was attempted to optically tweeze the cell out of the microwell. The efficiency of B cells lifted from the microwell array was increased for arrays grafted with M-PEG-M Mn 2,000 as compared to OSTE+ microwells without PEG or with PEGMA Mn 360 (Figure 9). PEGMA Mn 360 did not have the same desired effect as M-PEG-M Mn 2,000.

Upon the presence of 7-AAD, the viability of cells was evaluated before and after trapping with optical tweezer. It was observed that trapping a single viable cell for up to 10 mins did not compromised the cell membrane integrity (Figure 10).

Example 2 – Microfluidic channel for controlled B cell seeding and B cell identification for specific antibodies

To have more control over the B cell seeding and B cell identification, we bonded a PDMS (Polydimethylsiloxane) microfluidic channel on top of the OSTE+ microwell array (Figure 11 and Figure 12). The PDMS (SYLGARD 184, 1.1KG) was made in 10:1 ratio base:curing agent and cured for 5 hours at 60 °C in an oven. Two access holes were punched in the PDMS using a biopsy puncher of 1mm diameter. Then, the PDMS was activated using oxygen plasma for 30 seconds at 200mTorr and 30W. The PDMS channel

was placed over the OSTE+ microwell array that only had the first UV cure, not yet the thermal cure. The PDMS on OSTE+ was placed overnight in the oven at 60 °C.

Similar grafting of PEG as described in example 1 of PEGMA Mn 360 and M-PEG-M Mn 2,000 was also performed in combination with the PDMS channel. Similar as
5 example 1, the PEGMA Mn 360 was grafted onto the OSTE+ surface by submerging the first cured OSTE+ microwell array in the solution with PEGMA Mn 360, ethanol and UV initiator. After the 5min UV exposure at 12 mW/cm², the microwell array was thoroughly rinsed with ethanol and water. Then, the PDMS channel was activated with oxygen plasma as described above, and the PDMS channel was placed on top of the microwell
10 array with PEGMA Mn 360. Since PEGMA Mn 360 has endstanding hydroxyl groups, the activated PDMS binds efficiently to these PEGMA Mn 360 on the surface in an oven overnight at 60 °C. Since M-PEG-M Mn 2,000 does not have an endstanding hydroxyl group, the PDMS channel has to be attached to the OSTE+ prior to the M-PEG-M Mn 2,000 grafting. Therefore, first the PDMS channel was activated and placed on the
15 OSTE+ surface after its first UV cure. The PDMS channel was tightened to the OSTE+ surface using 4 foldback clamps, one on each side. The M-PEG-M Mn 2,000 solution with ethanol and UV initiator as described above was then pipetted through the access holes of the PDMS channel until the entire channel was filled with the solution. Then, this set-up was exposed to UV for 5 min at 12 mW/cm². Then, the channel was thoroughly
20 rinsed by pipetting ethanol and water through the channel. Next, the construct with OSTE+, PDMS and the clamps was placed in an oven overnight at 60 °C.

Microwells of 11±1 µm deep and 11±1 µm diameter were fabricated in OSTE+ and combined with the PDMS channel (Figure 11). Syringe pumps (Nemesys) were used for flow-based seeding of single B cells in the microwells. The B cells at concentration of
25 10⁷ cells/ml were fluxed at flow rate -5 µl/min. After 5 mins, the flow was stopped, and cells were incubated in the channel for 30 mins. As a washing step, Medium (RPMI+10% fetal calf serum) or PBS can be used to wash the array by flushing the solutions for 30 mins at flow rate of -5 µl/min.

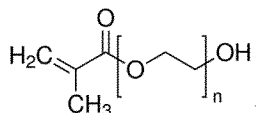
Due to interaction forces between the B cells and the OSTE+ surface, it was difficult to
30 wash away non-seeded B cells as they were sticking to the OSTE+ surface. Therefore, the washing efficiency was compared between OSTE+ surface and OSTE+ surface grafted with PEG. The efficiency of washing non-seeded B cells was evaluated using

fluorescence microscopy (Figure 13). In this figure, you can see the border of a microwell arrays without PEG (control), with PEGMA Mn 360 and M-PEG-M Mn 2,000. Images were captured under the microscope with bright field and fluorescence light source. For the non-treated OSTE+, cells were found next to the microwell array, after attempted washing for 30 mins at -5 μ l/min. An improvement was observed with microwell array treated with PEG-360 however still not optimal. For the OSTE+ with M-PEG-M Mn 2,000, the area next to the array is very clean, meaning that all non-seeded cells are washed away, which is desirable. Therefore, the surface that was grafted with M-PEG-M Mn 2,000 clearly improved the washing of the non-seeded B cells compared to OSTE+ and OSTE+ grafted with PEGMA Mn 360.

Using this microfluidic channel, the identification of specific B cells can performed using an ELISA based assay as illustrated in Figure 14. By labelling a specific receptor on B cells surface (e.g. membrane bounded immunoglobulin) with biotinylated antibody, streptavidin with beta-galactosidase can be bounded. Upon the presence of a substrate (Fluorescein di(B-D-galactopyranoside), a fluorescent product is generated. B cells labelled against surface IgG were seeded in microfluidic channel and after adding 10 μ g/ml of substrate for 1min, the array was sealed using FC-40. In this scenario, individual well are sealed and fluorescence can be detected beneath the presence of a positive cell. Preliminary results are shown in Figure 15 but further optimization is required. Later, an ELISA assay can be implemented to identify B cells expressing antibodies against a target protein.

Example 3 – Adapted PEG grafting enabling both efficient optical tweezing and efficient bonding of the PDMS microfluidic channel

Since the end standing methyl group of M-PEG-M Mn 2,000 complicates the bonding of a microfluidic PDMS channel with the grafted OSTE+ microwell array, as described in example 2, further investigation on another PEGMA was performed. A third type of PEG was used: **Poly(ethylene glycol) methacrylate with average Mn 500** (Synonym: PEGMA, CAS Number 25736-86-1, Linear Formula $\text{H}_2\text{C}=\text{C}(\text{CH}_3)\text{CO}(\text{OCH}_2\text{CH}_2)_n\text{OH}$, MDL number MFCD00081879 and with end

standing hydroxyl group as in the formula ). This PEGMA Mn 500

was diluted in ethanol, having 10 w/w% PEGMA, 2 w/w% UV initiator and 88 w/w% ethanol. After the first UV cure of the OSTE+ microwell array, the OSTE+ array with free thiol and epoxy groups is completely submerged in the described solution with PEGMA Mn 500. Then, this is exposed to UV of 12 mW/cm² for 5 minutes. After this, the OSTE+ array is rinsed thoroughly first with ethanol and then with water. Then, the same PDMS channel as in example 2 was activated with oxygen plasma as described above, and the PDMS channel was placed on top of the microwell array with PEGMA Mn 500. Since PEGMA Mn 500 has an end standing hydroxyl group, the activated PDMS binds efficiently to these PEGMA Mn 500 on the surface in an oven overnight at 60 °C. A similar BM and optical tweezing study as in example 2 was carried out (Figure 16 and Figure 17). Grafting with PEGMA with Mn 500 was not sufficient to increase BM of cells compared to the non-grafted OSTE+ microwell array or the microwell array grafted with PEGMA Mn 360 (Figure 16). However, optical tweezing efficiencies of cells with BM increased for PEGMA Mn 500 as compared to PEGMA Mn 360, which indicates that PEGMA Mn 500 reduced aspecific cell adhesion more than PEGMA Mn 360 (Figure 17).

To combine the low biofouling properties of M-PEG-M Mn 2,000 and the possibility of PDMS bonding with a surface grafted with PEGMA Mn 500, mixtures of these two PEG types were made. Several mixtures of different molar PEG ratios were tested as mentioned in Table 1. The corresponding weight percentages of the two PEG types, UV initiator and ethanol are mentioned in Table 1 as well. These mixtures were grafted to an OSTE+ microwell array by submerging the array in the solution and applying 5min UV exposure at 12 mW/cm², after which the microwell array was thoroughly rinsed with ethanol and water. Then, a PDMS channel was activated with oxygen plasma as described above, and the PDMS channel was placed on top of the microwell array. It was found that for a PEGMA 500/M-PEG-M 2,000 ratio of 1 on 2 and 1 on 4, the PDMS could not bond to the grafted microwell array. For a PEGMA 500/M-PEG-M 2,000 ratio of 4 on 1, 2 on 1 and 1 on 1, the PDMS microfluidic channel could bond to the grafted microwell array. Since the equimolar ratio of 1 on 1 relatively contains most M-PEG-M 2,000 molecules, this ratio was selected for further experiments. This equimolar mixture will now be referred to as PEG 500/2,000.

As shown in the BM study, the same low biofouling performance was reached with microwell arrays grafted with PEG 500/2,000 as compared to arrays grafted with M-PEG-M 2,000 (Figure 16). The optical tweezing study showed that 97 ± 3 % of the BM cells could be lifted out of the microwells using this surface chemistry (Figure 17).

5

Example 4 – Single B cell transfer from the array to the outlet of the microfluidic chip

After the identification of the B cell with specific antibody and the optical tweezing of this cell out of the microwell, the cell still needs to be transferred to a reservoir such as a tube or a 96 well plate. In order to transfer a cell from the microwell array to a reservoir, the M-PEG-M Mn 2,000 or PEG 500/2,000 grafted array was integrated with a channel in PDMS as described in example 2 (Figure 11).

This unidirectional flow for B cell seeding, washing and identification works well. However, a unidirectional channel is not sufficient to optically tweeze and collect only a single cell. The problem is that, for the tweezed cell to exit the channel, a continuous flow needs to be administered to the channel. The flow velocity needs to be high enough to transport the cell until the end of the channel. However, at this flow velocity, other cells also unwantedly pop out of the microwells and will thus also be transported until the end of the channel. This is contamination of unwanted cells which needs to be avoided.

Therefore, a new design was used for the cell transfer with a bidirectional flow. As can be seen in Figure 18, the microfluidic design consists of an oblong conduit [14] in which the microwell array [1] is positioned. As shown in Figure 19, the microwell array [1] is designed in such a way that a series of microwell array groups are equally spaced from each other with a spacing that is at least double the size of a B cell (e.g. 30 μm). Each microwell columns are composed by microwells with size of 11 ± 1 μm diameter and depth for single B cell seeding. The oblong conduit [14] consists of a fluid inlet port [11] for the delivery of cells and a fluid outlet port [4] for the waste collection (Figure 18).

At both sides of the oblong conduit [14], a set of branched fluid channels [12,13] are positioned. The first set of branched fluid channels [12] is composed by one buffer inlet port [3] that splits multiple times into two fluid channels of the same width [2, 2a, 2b].

These fluid channels [2b] are connected to the oblong conduit [14] via a port [8] through a connection that is shaped like a funnel [25] (Figure 20). The second set of branched fluid channels [13] is composed in the same manner. The two sets of branched fluid channels [12,13] are symmetrically positioned around the oblong conduit [14] and the microwell array [1] is positioned in between the two sets of fluid channels [12,13]. The sum of the cross-sections of every port [8] at the set of branched fluid channels [12] or [13] is 10-30 times smaller than the cross-section of the conduit above the microwell array [1] (Figure 18).

Every inlet and outlet port of the conduit [14] is connected via microfluidic tubing to a syringe pump, pressure pump or peristaltic pump. The conduit [14] is produced in PDMS and is bonded to a microwell array in OSTE+ with M-PEG-M 2,000 or PEG 500/2,000 surface chemistry as described in example 1-3. The microfluidic chip is placed on a Nikon epifluorescence microscope equipped with an optical tweezers set-up as described in [1. Decrop, D. et al. Anal. Chem. 88, 8596–8603 (2016).].

As a first step, illustrated in Figure 21, single B cells are seeded in the microwell array [1] by operating buffer inlet port [11] and outlet port [4]. A reservoir, e.g. syringe or pipet tip, with B cells is connected to the inlet port [11] via microfluidic tubing and the cells are pushed into the conduit [14] using the pump. A buffer reservoir, e.g. syringe, is connected to the buffer inlet port [3] and buffer outlet port [6] that pushes buffer into the conduit [14], i.e., buffer outlet port [6] is now used as an inlet port. Because of the design of the fluid channels, the buffer will flow at the outer sides of the a port [8] and act as a sheath flow or curtain flow that focuses the cells over the microwell array [1] and prevents the cells from flowing into the two sets of branched fluid channels [12,13]. This ensures that unwanted cells do not flow into the branched fluid channels [12,13] and uniquely the desired cells are captured at the outlet port [6]. The buffer and non-seeded cells flow through the outlet port [4] via tubing to a waste reservoir (e.g. syringe). The design and connection to syringe pumps balances all flows, meaning that if e.g. $2\text{ }\mu\text{L/min}$ is pushed through cell inlet port [11] and $0.5\text{ }\mu\text{L/min}$ is pushed through the buffer inlet and outlet port [3,6], then liquid is removed through the outlet port [4] at a rate of $3\text{ }\mu\text{L/min}$ ($= 2 + 0.5 + 0.5\text{ }\mu\text{L/min}$). After a certain short periods of flow, e.g. 2 minutes, all flows are stopped by stopping the syringe. Extra stability can be gained by including valves at the

tubing that are closed manually or electronically when the flow needs to be stopped. When the fluid flows are stopped, cells can sediment into the microwells for a certain period of incubation, e.g. 5 minutes. Several cycles, e.g. 5, of cell delivery by the flows and cell sedimentation by stopping the flows can be performed to increase single cell seeding efficiency in the microwell [1].

After the cycles for cell delivery and seeding, B cells that are not seeded in the microwell array [1] need to be washed away, illustrated in Figure 21. Washing buffer is pushed into the conduit [14] through buffer inlet port [11]. This can be performed by switching the syringe at inlet port [11] or by having a valve connected to the syringe that switches the cell suspension to buffer. Consequently, the non-seeded cells are pulled out of the chip through buffer outlet port [4]. A sheath flow from buffer inlet port [3] and buffer outlet port [6] is kept to avoid capture of undesired cell at the collection outlet. Again, all inlet and outlet flow rates are balanced.

Once cells are seeded in the microwell array [1] and non-seeded cells are washed away, fluorescently-labeled B cells (e.g. antigen-specific B cells) can be identified using fluorescence microscopy as explained in example 2. Then, a desired single B cell needs to be retrieved from the microfluidic chip. For this, the optical tweezers [5],[9] are used and the flow is controlled by operating buffer inlet port [3] and buffer outlet port [6], as illustrated in Figure 22. Buffer is pushed through buffer inlet port [3] and distributed through the first set of branched fluid channels [12], through the conduit over the microwell array [1] through the second set of branched fluid channels [13] towards the outlet port [6]. Because of the branched structure of the fluid channel sets [12] and [13], the flow speed is uniformly distributed over the microwell array [1]. Because the sum of the cross-sections of all the ports [8] is 10-30 times smaller than the cross-section of the conduit [14] above the microwell array [1], there is a 10-30 times drop in flow speed from the first set of fluid channels [12] to the conduit above the microwell array [1] and there is again a 10-30 times increase in flow speed from the conduit above the microwell array [1] to the second set of fluid channels [13]. Thanks to this feature, the flow speed above the microwell array is low enough so seeded cells are not disturbed and do not dislodge randomly from their microwell [1]. This low flow speed also allows single cell

manipulation since the force exerted by the optical tweezers [5], [9] on the cell is larger than the forces exerted by the fluid flow. The microwell array [1] is specifically designed to minimize the exposure times of the focused beam laser of optical tweezers to the cell. Since the microwells are placed in microwell groups with spacing in between, the cell
5 can be lifted out of the microwell by the optical tweezers [5],[9], and transported to the nearest spacing. Then, the cell can be transported by the optical tweezers [5],[9] towards the nearest port [8] of the second set of fluid channels [13]. Because the spacing in between the microwell groups is at least double the cell size ($> 20 \mu\text{m}$), the lifting and transport of the cell from microwells arrays by optical tweezing to the nearest port [8] can
10 be performed in several seconds (5-30s). By tweezing the cell towards a port [8], the wall of the conduit [14] will be encountered. The presence of multiple ports and their specifically designed funnel shape [25] enables fast localization of the nearest port (1-5s) and thus minimize interaction time of the optical tweezers with the cell. The cell is released at this port [8] by turning off the optical tweezers [5],[9]. Because of the increase
15 in flow speed in the second set of channels [13], the cell is transported by the flow towards the outlet for cell collection [6]. Because of the fluid transport, interference with the optical tweezers is not needed anymore, so interaction times with the optical tweezers are minimized, cell viability is maintained and cells can be retrieved within several seconds. The process of lifting and transporting the cell is illustrated in Figure 23.

20

Because of the combined features of 1) The grouped microwell array [1b] with spacing [1c] and 2) The ports [8] that are designed as funnels for fast localization of the ports [8] and 3) The flow increase in the second set of branched channels [13], interaction time with the optical tweezers and the target cell are minimized and thus cell viability is
25 maintained.

Because of the combined features of 1) The sheath flow preventing cells to enter in the branched fluid channels [12] and [13], 2) The uniform flow speed distribution above the microwell array [1], and 3) The 10-30 folds increase in flow speed in the second set of branched channels [13] allow to transport only the target cells to the outlet [6] without
30 having any contamination from other unwanted cells.

The combination of all these features enables the selection of rare cells from a large population and collect the desired cells in a cell-by-cell manner with following

advantages: 1) without contamination of other cells, 2) without losing cells in microfluidic tubing or in dead volumes in the microfluidic chip, 3) in collection volumes that are much lower than those achieved by other technologies.

5 Example 5 – Retrieval of single B cells from the microfluidic chip

Additional features were added to the design explained in example 4 to on the one hand apply the sheath flow in a different way and on the other hand to retrieve single cells from the microfluidic chip to an off-chip reservoir for further analysis. This design can be found
10 in Figure 24.

The adapted microfluidic conduit [14] consists of an oblong conduit [14] in which the microwell array [1] is positioned (Figure 24). The microwell array [1] (Figure 19) is designed in such a way that a series of microwell array groups [1b] are equally spaced
15 from each other with a spacing [1c] that is at least double the size (e.g. 30 μm) as the B cell. Each microwell group [1b] is composed by microwells [1a] with size of $11 \pm 1 \mu\text{m}$ diameter and depth for optimal single B cell seeding. The oblong conduit [14] consists of a cell inlet port [11] for the delivery of cells and a fluid outlet port [4] for the waste collection. The cell inlet port [11] can also be used as a fluid outlet port when needed. An
20 extra buffer inlet port [15] is connected to two fluid channels [16] in which the buffer will be flowing at the outer sides of the oblong conduit [23], [24] towards the outlet port [4]. At both sides of the oblong conduit [14], a set of branched fluid channels [12,13] are positioned. The first set of branched fluid channels [12] starts from one buffer inlet port [3] and splits multiple times into two fluid channels with the same width [2, 2a, 2b]. These
25 fluid channels [2b] are connected to the oblong conduit [14] via a port [8] through a connection that is shaped like a funnel [25] (Figure 20). The second set of branched fluid channels [13] is composed in the same manner. The two sets of branched fluid channels [12,13] are symmetrically positioned around the oblong conduit [14] and the microwell array [1] is positioned in between the two sets of fluid channels [12],[13]. The sum of the
30 cross-sections of every port [8] at the set of branched fluid channels [12] or [13] is 10-30 times smaller than the cross-section of the conduit above the microwell array [1]. A space of $> 100 \mu\text{m}$ separates the microwell array [1] from the two sets of branched fluid channels [12],[13] as indicated by region [23] and [24]. The second set of fluid channels

[13] is connected to a droplet generation geometry [22]. In the latter, an oil inlet port [17] is connected to two oil fluid channels [18] that intersect with the fluid channel before droplet generation geometry [21] which is an elongation of the set of fluid channels [13]. Buffer flowing from the buffer inlet [3] through the first set of fluid channels [12] over
5 the microwell array [1] through the second set of fluid channels [13] to the channel before droplet generation geometry [21] will contact the oil coming from oil fluid channels [18] at the position of droplet generation [20] to form buffer-in-oil droplets. These formed buffer-in-oil droplets will flow to the outlet port [19] in which tubing is connected to an off-chip reservoir. The width of the fluid channel at the outlet port [19] is the same (± 25
10 μm) as the inner diameter of the microfluidic tubing.

Every inlet and outlet port of the conduit [14] is connected via microfluidic tubing to a syringe pump, pressure pump or peristaltic pump. The conduit [14] is produced in PDMS and is bonded to a microwell array in OSTe+ with M-PEG-M 2,000 or PEG 500/2,000 surface chemistry as described in example 1-3. The microfluidic chip is placed on a Nikon
15 epifluorescence microscope equipped with an optical tweezers set-up as described in [1. Decrop, D. et al. Anal. Chem. 88, 8596–8603 (2016).].

As a first step, single B cells are seeded in the microwell array [1] by operating buffer inlet port [15], cell inlet port [11] and outlet port [4], as illustrated in Figure 25. A
20 reservoir, e.g. syringe or pipet tip, with cells is connected to the cell inlet port [11] via microfluidic tubing and the cells are pushed into the conduit [14] using the pump. A buffer reservoir, e.g. syringe, is connected to the buffer inlet port [15] and buffer is pushed into the conduit [14]. Because of the design of the fluid channels [16], the buffer will flow at the outer sides of the conduit [23], [24] and act as a sheath flow or curtain flow that
25 focuses the cells over the microwell array [1] and prevents the cells from flowing into the two sets of branched fluid channels [12,13]. This prevents unwanted, random B cells from flowing in the branched fluid channels and causing contamination in the second set of branched fluid channels and the droplet generation geometry. The buffer and non-seeded cells flow through the outlet port [4] via tubing to a waste reservoir (e.g. syringe). The
30 design and connection to syringe pumps enables all flows to be balanced meaning that if for example $2 \mu\text{L}/\text{min}$ is pushed through cell inlet port [11] and $1 \mu\text{L}/\text{min}$ is pushed through the buffer inlet port [15], then liquid is removed through the outlet port [4] at a

rate of 3 $\mu\text{L}/\text{min}$. After a certain period of flow, e.g. 2 minutes, all flows are stopped by stopping the syringes. Extra flow stability can be gained by including valves at the tubing that are closed manually or electronically when the flow needs to be stopped. When the fluid flows are stopped, cells can sediment into the microwells [1a] for a certain period of time, for example 5 minutes. Several cycles, e.g. 5, of cell delivery by the flows and cell sedimentation by stopping the flows can be performed to increase single cell seeding efficiency in the microwell [1].

After the cycles for cell delivery and seeding, B cells that are not seeded in the microwell array [1] need to be washed away, as illustrated in Figure 26. This can be performed by switching on the flows at the inlet port [15] and [11] and outlet port [4]. Washing buffer is pushed into the conduit [14] through buffer inlet port [15]. The buffer and non-seeded cells are pulled out of the chip through buffer outlet port [4] and by pulling through the cell inlet port [11]. The cell inlet port [11] is now thus used as an outlet. This is done because it was observed that cells sediment below the tubing of inlet port [11] during cell seeding, so if the inlet port [11] was used as an inlet for pushing in washing buffer, the sedimented cells were gradually taken along with the flow and cells were constantly delivered to the region of the microwell array [1]. Since this inhibited washing of all non-seeded cells, the washing buffer was pulled into port [11] during washing. Again all inlet and outlet flow rates are balanced.

Once cells are seeded in the microwell array [1] and non-seeded cells are washed away, fluorescently labeled B cells (e.g. antigen-specific B cells) can be identified using fluorescence microscopy as explained in example 2. Then, a B cell of interest needs to be retrieved from the microfluidic chip. For this, the optical tweezers [5],[9] are used and the flow is controlled by operating buffer inlet port [3], oil inlet port [17] and the outlet port for buffer-in-oil droplets [19], as illustrated in Figure 27. Buffer is pushed through buffer inlet port [3] and distributed through the first set of branched fluid channels [12], through the conduit over the microwell array [1] through the second set of branched fluid channels [13] towards the outlet for buffer-in-oil droplets [19]. Because of the branched structure of the fluid channel sets [12] and [13], the flow speed is uniformly distributed over the microwell array [1]. Because the sum of the cross-sections of all the ports [8] is

10-30 times smaller than the cross-section of the conduit [14] above the microwell array [1], there is a 10-30 times drop in flow speed from the first set of fluid channels [12] to the conduit above the microwell array [1] and there is again a 10-30 times increase in flow speed from the conduit above the microwell array [1] to the second set of fluid channels [13]. Thanks to this feature, the flow speed above the microwell array is selected low enough so seeded cells are not disturbed and do not dislodge randomly from their microwell [1], preventing contamination with unwanted cells. This low flow speed also allows the single cells manipulation since the force exerted by the optical tweezers [5], [9] on the cell is larger than the forces exerted by the fluid flow. The microwell array [1] is specifically designed to minimize interaction times of the optical tweezers with the cell. Since the microwells [1a] are placed in microwell groups [1b] with spacing [1c] in between, the cell can be lifted out of the microwell [1a] by the optical tweezers [5],[9], and transported to the nearest spacing [1c]. Then, the cell can be transported by the optical tweezers [5],[9] towards the nearest port [8] of the second set of fluid channels [13]. Because the spacing [1c] in between the microwell groups [1b] is at least double the cell size ($> 20 \mu\text{m}$), the transport of the cell by optical tweezing to the port [8] can be performed in several seconds (5-30s) since there is no hindrance of movement by other cells or microwells or cells in microwells. By tweezing the cell towards a port [8], the wall of the conduit [14] will be encountered. The presence of multiple ports and their specifically designed funnel shape [25] enables fast localization of the nearest port (1-5s) and thus minimize interaction time of the optical tweezers with the cell. The cell is released at the nearest port [8] by turning off the optical tweezers [5],[9]. Because of the increase in flow speed in the second set of channels [13], the cell is transported by the flow towards the droplet generation geometry [22]. Because of the fluid transport, interference with the optical tweezers is not needed anymore, so interaction times with the optical tweezers are minimized, cell viability is maintained and cells can be retrieved within several seconds. Microscopy images of the tweezing process can be found in Figure 28.

Once the desired B cell is optically tweezed out of the microwell and transported by the flow in the into the droplet generation geometry [22], the cell needs to be transferred from the chip to an off-chip reservoir such as a tube, 96 well plate or other, for further

downstream analysis. Conventionally, holes are punched in the PDMS for connecting the chip via tubing to a pump or reservoir [2. Wang, X. et al. Lab Chip 11, 3656 (2011)]. This tubing is inserted via the top of the PDMS microfluidic channel. Using this standard set-up, it was observed that the isolated B cells sediment below the lumen of the tubing and consequently do not enter the tubing (Figure 29), even though there is a continuous microfluidic flow. Consequently, using this traditional set-up, cells are residing in dead volumes in the chip and cannot be transported to an off-chip reservoir. Moreover, if a cell would enter in the tubing, the cell can still be lost in the tubing because of sedimentation in the tubing or adhesion to the tubing wall. As an alternative, in literature, cells were retrieved from microfluidic sorting chips by manually pipetting the cells out of an opening in the chip using a micropipette [3. Kovac, J. R. & Voldman, J. Anal. Chem. 79, 9321–9330 (2007).]. However, such a pipetting step complicates automation and requires manual intervention with the microfluidic chip, which caused random dislodging of B cells from microwells in the case of this invention. Moreover, it cannot be ensured that all cells are effectively collected from the chip by pipetting, since cells might adhere to the bottom of the chip or not all liquid is effectively pipetted.

To enable single cell retrieval in automated and efficient manner through a tubing, a droplet generation geometry [22] was implemented in the microfluidic design (Figure 24). Herein, the flow in the second set of branched fluid channels [13] that carries the tweezed cell towards the outlet, is pinched off into droplets by the oil at [20] (Flow scheme in Figure 27). Similar to droplet microfluidics, the oil acts as the continuous phase or the carrier phase, in which the buffer is dispersed [4. Joensson, H. N. & Andersson Svahn, H. Angew. Chemie - Int. Ed. 51, 12176–12192 (2012).]. The buffer droplets are mostly empty, but will contain a single cell when a desired B cell is tweezed to a port [8]. The oil carrier phase then pushes the droplets into the tubing connected to the outlet port [19]. When the droplet contains a cell, this cell is also pushed into the tubing connected to the outlet port [19] and can be transported to an off-chip reservoir. To illustrate this, Figure 30 shows how a droplet containing multiple B cells and platelets is pushed into the tubing by the oil carrier phase. The outlet of this tubing is then positioned in or above a reservoir, such as a tube or 96-well plate, to capture the generated droplets. To push the droplets into the microfluidic tubing, it is important that the width of the fluid channel at

the outlet port [19] is the same ($\pm 25 \mu\text{m}$) as the inner diameter of the microfluidic tubing. Therefore, the generation of droplets allow the retrieval of desired single cells in a cell-by-cell manner without losing cells in dead volumes in the fluid channels or at the outlet port [19]. Moreover, since the droplet volume is in between $0.01 \mu\text{L}$ and $0.05 \mu\text{L}$, this feature allows the collection of very low volumes which is not possible by pipetting or by buffer flows in tubing. This feature makes the current invention suitable for single cell retrieval for further single cell analysis approaches (e.g. single cell sequencing) at which reduced volumes are required.

- 10 Because of the combined features of 1) The grouped microwell array [1b] with spacing [1c] and 2) The multiple ports [8] that are designed as funnels for fast localization of the ports [8] and 3) The flow increase in the second set of branched channels [13], interaction time with the optical tweezers and the target cell are minimized and thus cell viability is maintained. In addition, because of the combined features of 1) The sheath flow preventing cells to enter in the branched fluid channels [12] and [13], 2) The uniform flow speed distribution above the microwell array [1], and 3) The 10-30 folds increase in flow speed in the second set of branched channels [13], only transport of the target cells to the outlet [19] is allowed, without having any contamination from other unwanted cells. Because of the droplet generation geometry, the target cell can be transported from the conduit [14] via a tubing to an off-chip reservoir for further analysis. These collected volumes are small (0.01 to $0.5 \mu\text{L}$) so cellular RNA is hardly diluted, which is necessary for RNA sequencing.

- 25 The combination of all these features enables the selection of very rare cells from a large population of cells and the collection of the target cells in a cell-by-cell manner without contamination of other cells and without losing cells in microfluidic tubing or in dead volumes in the microfluidic chip, and in collection volumes that are much lower than those achieved by other technologies.

- 30 Based on the flow rates used in the conduit at inlet port [3] and oil inlet port [17], and the diameter of the tubing, the time for the droplet to arrive at the end of the collection tubing can be determined. Then, during the correct time interval, the droplets can be captured in

the off-chip reservoir. This reservoir can be a tube, 96 well plate, or other. This reservoir will then contain a number of empty droplets and one droplet containing the target cell. For further analysis, such as single cell RNA sequencing, the cell needs to be brought in contact with reagents, such as lysis buffer and PCR reagents, for which the droplets can be merged using for example a chemical such as perfluorooctanol and chloroform, or an antistatic gun [5. Karbaschi, M., Shahi, P. & Abate, A. R., Biomicrofluidics 11, (2017)].

The oil used for droplet generation in these experiments was QX200™ Droplet Generation Oil for EvaGreen from Bio Rad, but other oil with surfactants can be applied for generation of stable water-in-oil droplets.

The geometry for the droplet generation [22] used here is drawn in Figure 24, but can be any other design with which droplets can be generated, such as a T-junction or a flow focusing junction [4. Joensson, H. N. & Andersson Svahn, H. Angew. Chemie - Int. Ed. 51, 12176–12192 (2012).].

To produce stable buffer-in-oil droplets, the surface of the droplet generation module had to be hydrophobic. Since the PEG grafted surface is hydrophilic, a hydrophobic treatment had to be applied on this part of the design, as indicated in Figure 31. This was done by flushing an Aquapel solution or a solution of 1% Trichloro(1H,1H,2H,2H-perfluorooctyl)silane in HFE 7500 through the chip and incubating the chip for 30 min in an oven at 65°C.

Thus, the claims following the detailed description are hereby expressly incorporated into this detailed description, with each claim standing on its own as a separate embodiment of this invention.

Drawing Description and tables

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given herein below and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein:

Figure 1 is a size distribution of human B cells isolated from PBMCs. Three samples from different healthy donors were tested and human B cells isolated. Cells were imaged under the microscope and size calculated using cell profile software. Size in μm scale (n=3)

5 **Figure 2** is a schematic showing the fabrication of OSTE+ microwell arrays, similar as described in [6. Decrop, D. et al.. *ACS Appl. Mater. Interfaces* 9, 10418–10426 (2017).]. First, photoresist S1818 or AZ6632 are spin coated on a silicon wafer and photopatterned with circles corresponding the desired microwell diameter. Then, Deep Reactive Ion Etch (DRIE) is used to obtain microwell with the desired microwell depth. In a next step,
10 PDMS in 5:1 ratio for base:curing agent is poured on the silicon wafer and cured in an oven overnight at 60 °C. Then, the PDMS is peeled off of the silicon wafer. This is the ‘PDMS stamp’, which has pillars that correspond do the desired microwell depth and diameter. Then, a glass slide is silanized by submerging for 10 minutes in a 5% solution of 3-(Trimethoxysilyl)propyl methacrylate in methanol followed by 10 min baking at 110
15 °C. Then, OSTE+ is spin coated on the glass slide for 30seconds at 2500 rpm. Next, the PDMS stamp is pushed on the OSTE+ film on the glass slide. Air bubbles between the PDMS and the OSTE+ are pushed out by pressing the PDMS with a miniature rolling pin. After 5 min, the OSTE+ with PDMS stamp on top is exposed to UV at 12 mW/cm² for 2 minutes. Then, the PDMS stamp is peeled off from the OSTE+. The thermal cure
20 can be done overnight at room temperature or for at least 2 hours in an oven at 60 °C.

Figure 3 is a graphic representation of seeding efficiency of human B cells in microwell arrays with different well diameter (ϕ) and depth ($\downarrow$). B cells isolated from human PBMCs were stained with DAPI and seeded in microwell arrays. After 30 minutes incubation, the arrays were washed, and images captured with Nikon fluorescent microscope in bright
25 field and 400 nm laser. Number of well and respective presence of single or more cells per well was registered. Legend: gray bars () indicate the percentage of single cells per well; white bars () indicate the presence of two or more cells per array. Size in μm scale (n=2).

Figure 4 is a set of two complementary pictures with B cells seeded in microwell arrays of $12 \pm 1 \mu\text{m}$ diameter and $14 \pm 1 \mu\text{m}$ depth. A) DNA stained B cells seeded in microwell array. Each blue spot corresponds to a single cell; B) Bright field image of microwell
30 array with seeded B cells.

Figure 5 is a graphic showing the integration of a microwell array with an optical tweezers to lift single B cells from a microwell.

Figure 6 displays grafting of the OSTE+ microwell surfaces with methacrylated PEG (Poly(ethylene glycol) methacrylate with average Mn 360 (PEGMA) using UV thiol-ene click chemistry.

Figure 7 A) demonstrates static contact angle measurements for OSTE+ surface without treatment, OSTE+ grafted with PEGMA Mn 360 and OSTE+ grafted with M-PEG-M Mn 2,000. Legend: white bar correspond to measurements for OSTE+ surface without treatment (); white bar with black stripes correspond to OSTE+ grafted with PEGMA Mn 360 (); black bar correspond to OSTE+ grafted with M-PEG-M Mn 2,000 (). **B)** demonstrates static contact angle measurements of OSTE+ surface, and OSTE+ after submersion in the PEGMA Mn 360 solution and OSTE+ after submersion in the M-PEG-M Mn 2,000 solution without UV exposure. For each condition, 3 samples were fabricated. For each sample, 3 static contact angle measurements were performed. For each sample, the average of these 3 measurements represents the measurement of that sample, thus n=3. Statistical significance was obtained using t tests. Legend: white bar correspond to measurements for OSTE+ surface (); white bar with black stripes correspond to OSTE+ after submersion in the PEGMA Mn 360 solution (); black bar correspond to OSTE+ after submersion in the M-PEG-M Mn 2,000 solution without UV exposure ().

Figure 8 corresponds to study of Brownian motion (BM) of B cells seeded in $11 \pm 1 \mu\text{m}$ well after 2 hours incubation. B cells isolated from human PBMCs were seeded in microwell arrays with different treatments: without PEG treatment (control), with PEGMAM-360 and with M-PEG-M 2,000. After 2 hours incubation, videos were recorded with fluorescent microscope. Random movement of a single cell for more than 10 seconds was taken as a BM. By applying t-test, statistical significance was obtained when comparing the BM of cells seeded in control and M-PEG-M 2,000, with p value<0.05; and when comparing the BM of cells seeded in PEGMA-360 and M-PEG-M 2,000, with p value<0.001. Experiments performed in triplicates (n=3). Legend: white bar correspond to BM of cells seeded in microwell arrays without treatment (); white bar with black stripes correspond to BM of cells seeded in microwell arrays with

PEGMA-360 (); black bar correspond to BM of cells seeded in microwell arrays with M-PEG-M 2,000 ().

Figure 9 is a graphic showing the percentage of B cells lifted from 11 ± 1 μm depth well using optical tweezers. B cells isolated from human PBMCs were seeded in microwell arrays with different treatments: without PEG treatment (control), with PEGMA-360 and with M-PEG-M 2,000. Optical tweezers were applied in B cells, seeded in microwell, that demonstrated BM. An attempt to trap and lifting a single cell using the laser was made. In case of success, the attempt was registered as (1); in case the cell could not be lifted out of the well, the attempt was registered as (0). By applying t-test, statistical significance was obtained when comparing the lifting success of cells seeded in control and M-PEG-M 2,000, with p value < 0.001 ; when comparing the lifting success of cells seeded in PEGMA-360 and M-PEG-M 2,000, with p value < 0.05 . T test was applied on described data. Experiments performed in triplicates ($n=3$). Legend: white bar correspond to BM of cells seeded in microwell arrays without treatment (); white bar with black stripes correspond to BM of cells seeded in microwell arrays with PEGMA-360 (); black bar correspond to BM of cells seeded in microwell arrays with M-PEG-M 2,000 ().

Figure 10 displays B cell viability after 10 mins exposition to an optical tweezer laser. A) B cells in cover slide before applying optical tweezer laser. Cells were resuspended in medium with 7-AAD dye. This dye only penetrates compromised cell membranes and labels the cells of red when excited at 550 nm. The B cell at which the laser was applied is indicated with arrow (). B) B cells in cover slide after applying optical tweezer laser. The absence of red staining in the cell in analysis indicates that membrane integrity was maintained after 10 mins exposition to optical tweezer laser. The B cell at which the laser was applied is indicated with arrow ().

Figure 11 is a graphic showing a PDMS channel on top of the OSTE+ microwell array that is used for seeding of single cells and can be used for controlled reagent delivery. The platform is integrated with an optical tweezers set-up to elevate single cells with the response of interest out of a microwell

Figure 12 shows the top view of the PDMS channel from the schematic in Figure 11 with its dimensions. The total volume of the channel is proximately 20 ± 1 μL .

Figure 13 corresponds to a set of microscope images showing a comparison of washing efficiency in a channel for non-treated OSTE+, OSTE+ grafted with PEGMA-360 and

OSTE+ grafted with M-PEG-M 2,000. B cells were isolated from human PBMCs and stained with commercial kit celltracker. After B cells incubation and washing step in microfluidic channel, images of the array were captured in both bright field and fluorescence.

5 **Figure 14** is a schematic representation of the ELISA based assay for the identification of specific B cells. B cells isolated from human PBMCs are blocked with 1% BSA and incubated with biotinylated anti-human IgG followed by streptavidin- β -galactosidase. Labeled B cells are seeded in microwell arrays and substrate (Fluorescein di(B-D-galactopyranoside)) is added. The microwell arrays are sealed with oil (FC-40). In case a
10 cell expressing IgG on surface is seeded in the well, it becomes green due to substrate cleavage by β -galactosidase.

Figure 15 demonstrates preliminary data obtained in ELISA based assay for the identification of IgG B cells. A) Microwell arrays with streptavidin- β -galactosidase labeled B cells. The green fluorescence was obtained with arrays were sealed with FC-40
15 oil. The fluorescence corresponds to the presence of a B cell with IgG on membrane. B) Measurement of labeled B cells and respective controls in spectrophotometer. 1×10^6 cells were added to 96 well and 1 ug/ml of substrate added before the measurement. Legend: The filled line (**■**) corresponds to B cells labeled with Biotinylated anti-IgG antibody and streptavidin- β -galactosidase. The striped line (**▨**) corresponds to B cells
20 without biotinylated anti-IgG antibody but with streptavidin- β -galactosidase. The dashed line (**▤**) corresponds to B cells medium.

Figure 16 corresponds to study of Brownian motion (BM) of B cells seeded in $11 \pm 1 \mu\text{m}$ well after 2 hours of incubation. B cells isolated from human PBMCs were seeded in microwell arrays with different treatments: without PEG treatment (OSTE+), with
25 PEGMA 360, PEGMA 500, PEG 500/2,000 and M-PEG-M 2,000. After 2 hours incubation, videos were recorded with fluorescent microscope. Random movement of a single cell for more than 10 seconds was taken as a BM. (For all graphs, the line represents the mean and the error bars show standard deviations for 3 measurements. $n=3$, * represents $p < 0.05$).

30 **Figure 17** is a graphic showing the percentage of B cells lifted from $11 \pm 1 \mu\text{m}$ depth well using optical tweezers. B cells isolated from human PBMCs were seeded in microwell arrays with different treatments: without PEG treatment (OSTE+), with PEGMA 360,

PEGMA 500, PEG 500/2,000 and M-PEG-M 2,000. Optical tweezers were applied to B cells, seeded in microwell, that demonstrated BM. An attempt to trap and lifting a single cell using the laser was made. In case of success, the attempt was registered as (1); in case the cell could not be lifted out of the well, the attempt was registered as (0). (For all
 5 graphs, the line represents the mean and the error bars show standard deviations for 3 measurements. $n=3$, * represents $p < 0.05$).

Figure 18 shows a schematic representation of the microfluidic platform for single cell seeding and retrieval with integrated optical tweezer set-up. The microwell array and optical tweezers are not drawn to scale. Each number represent an element and a detailed
 10 description can be found in the text.

Figure 19 shows a schematic representation of the microwell array design in the microfluidic platform for single cell seeding and retrieval.

Figure 20 shows a close-up of a the microfluidic platform to show the detailed design of the connection between the set of branched fluid channels [12],[13] and the main conduit
 15 [14] through the port [8].

Figure 21 displays a schematic representation of fluid flow during cell seeding and washing step. Syringe pumps are connected to each inlet/outlet and the arrows represent the flow direction ($\longrightarrow$). The numbers represent an element of the microfluidic platform and full description can be found in the text.

Figure 22 shows a schematic representation of fluid flow during tweezing of a single cell. Syringe pumps are connected to each inlet/outlet and the arrows ($\longleftrightarrow$) represent the flow direction. The numbers represent an element of the microfluidic platform and full
 20 description can be found in the text.

Figure 23 represents the process of transport a cell from the microwell array to the port using optical tweezers [8] (path indicated by dotted line) and the transport of the cell from the port [8] to the outlet by the microfluidic flow (path indicated by dashed line
 25 --- --- ---).

Figure 24 is a representation of different elements of microfluidic platform with integrated droplet generation for single cell seeding and retrieval. The microwell array and optical tweezers are not drawn to scale. The numbers represent an element of the
 30 microfluidic platform and full description can be found in the text.

Figure 25 shows a schematic representation of fluid flow in the microfluidic platform with integrated droplet generation during the cell seeding step. Syringe pumps are connected to each inlet/outlet and the arrows (→) represent the flow direction. The numbers represent an element of the microfluidic platform and full description can be found in the text.

Figure 26 shows a schematic representation of fluid flow in the microfluidic platform with integrated droplet generation during the washing step. Syringe pumps are connected to each inlet/outlet and the arrows (→) represent the flow direction. The numbers represent an element of the microfluidic platform and full description can be found in the text.

Figure 27 shows a schematic representation of fluid flow in the microfluidic platform with integrated droplet generation during the tweezing step. Syringe pumps are connected to each inlet/outlet and the arrows (→) represent the flow direction. The numbers represent an element of the microfluidic platform and full description can be found in the text.

Figure 28 shows microscopy images of the concept of identification and isolation of a single B cell. i.) The cell is identified using fluorescence microscopy; ii.) The cell is tweezed out of the microwell by the optical tweezers; iii.) The cell is tweezed to the funnel; iv.) The cell is released by the optical tweezers and taken along by the microfluidic flow. This can be seen in the image since the optical tweezers is located in the middle of the field of view but the cell already moved further in the channel.

Figure 29 shows a microscopy image of the buffer-in-oil droplet outlet [19] to which a tubing is connected. Cells can be seen as bright white dots. Cells do not enter the tubing but sediment below the lumen of the tubing because of gravity.

Figure 30 illustrates with microscopy images how cells are taken out of the microfluidic chip into the tubing by using droplets. In this case, multiple B cells and platelets were encapsulated into the medium droplet. At the droplet outlet, no cells can be observed below the lumen of the tubing, which is in large contrast with figure 29. The cells are indicated by white arrows.

Figure 31 indicates which part of the microfluidic design should be treated with a hydrophobic coating to enable droplet formation. This is indicated by grey filling.

Tables in this application

Table 1: Different ratios of PEGMA 500 and M-PEG-M 2,000

Ratio PEGMA 500 /M-PEG-M 2,000	4 on 1	2 on 1	1 on 1	1 on 2	1 on 4
w/w% PEGMA 500	10	10	6,25	3,125	1,5625
w/w% M-PEG-M 2,000 solution in H₂O	20	40	50	50	50
w/w% UV initiator	1	1	1	1	1
w/w% ethanol	69	49	42,75	45,875	47,4375

Screening and sorting of single cells

Claims

What is claimed is:

- 5 1. A cell sorting device that comprises
 - 1) a microarray [1] of micro-wells [e.g. 1a],
 - 2) an elongate or oblong conduit [14], the micro-well array [1] being comprised in the elongate or oblong conduit [14], and
 - 3) channels,
- 10 characterised in that the micro-well array [1] is positioned between
 - a) at one side the upstream part of the conduit [7] with at least one inlet port [11] of the conduit [14] and
 - b) at another side the downstream part [10] of the conduit [14] with at least one outlet port [4] of the conduit [14]
- 15 and further characterised in that the micro-well microarray [1] is being positioned between the ports or apertures (e.g. [8] or [28]) of
 - i) a first set of branching channels [12] and
 - ii) a second set of branching channels [13],
- 20 and whereby these two sets of branching channels are outside the conduit [14] but connect with or are engaged with the conduit [14] via ports or apertures (e.g. [8] or [28]) in the conduit [14]
2. The cell sorting device according to claim 1, whereby each fluid channel set ([12] & [13]) comprises a fluid inlet ([3] & fluid outlet [6]).
3. The cell sorting device according to any one of the claims 1 to 2, whereby the
- 25 cell sorting device is a microfluidic cell sorting device.
4. The cell sorting device according to any one of the claims 1 to 3, whereby the conduit [14] is a conduit for an aqueous liquid and the branched channels [12] and [13] are channels for an aqueous liquid.
5. The cell sorting device according to any one of the claims 1 to 4, whereby the
- 30 micro-wells which are organized in parallel arrays (e.g. [1b]) of micro-wells or rows of micro-wells which are arrays or rows separated with parallel partitions or spaces (e.g. [1c]) and which are positioned longitudinal between a ports or

apertures (e.g. [8]) of the first set of branching channels and a ports or apertures (e.g. [28]) of the second set of branching channels.

6. The cell sorting device according to any one of the claims 1 to 5, whereby the micro-wells which are organized in parallel arrays (e.g. [1b]) of micro-wells or rows of micro-wells which arrays or rows are separated with parallel partitions or spaces (e.g. [1c]) and which are positioned crosswise the elongate or oblong conduit [14] in an intermediate zone between the upstream part [7] and the downstream part [10] of the conduit [14].
7. The cell sorting device according to any one of the claims 1 to 6, whereby in the first set of branching channels each channel [2] that extends from the fluid inlet port [3] branch out in additional channels [2a] which again branch out in additional channels [2b] and which at the distal end connect with or are engaged with the conduit [14] via ports or apertures (e.g. [8]) and whereby in the second set of branching channels each channel that extends from the fluid outlet port [6] branch out in additional channels which branch out in additional channels and which at the distal end connect with or are engaged with the conduit [14] via ports or apertures [e.g. 28]).
8. The cell sorting device according to any one of the claims 1 to 7, whereby there is a space [24 & 23] between the microarray of micro-wells [1] and each zone of the wall of the conduit [14] where the branching channels are connect with or are engaged with the conduit [14] via ports or apertures (e.g. [8] or [28]), the cell sorting device being furthermore characterised in that in the upstream part [7] of the conduit [14] it comprises a first fluid inlet port [15] more distal from said the microarray of micro-wells [1] and a second fluid inlet port [11] more proximate to the microarray of micro-wells [1], whereby the first fluid inlet port [15] connects with or is engaged with two fluid channels [16] which each extend lateral and longitudinal with a space [24 or 23] and open approximate to the space [24 & 23] so to create when operational a lateral flow of a sheath fluid that sandwiches a core fluid and whereby the second fluid inlet port [11] opens more in the core of the upstream part [7] of the conduit [14] so that when operational it creates a core fluid stream towards the microarray of micro-wells [1]

9. The cell sorting device according to any one of the claims 1 to 8, which comprises a solid object [26] in the downstream part [10] of the conduit [14] with a space between its rim and part of the wall of the conduit [14] so to form the channels [16] extending from the first fluid inlet port [15] and whereby the second fluid inlet port [11] opens in a cavity [27] formed by recess in the edge of solid object [26] which is faced to the microarray of micro-wells [1] so that when operational a core fluid stream with cells is released in said cavity [27] towards the microarray of micro-wells [1] and by lateral flow of a sheath fluid that sandwiches a core fluid is directed onto the microarray of micro-wells [1].
10. The cell sorting device according to claim 9, whereby the solid object [26] is a Y shaped solid plate.
11. The cell sorting device according to any one of the claims 1 to 10, whereby a second set of branching channels [13] at its end distal from the conduit [14] connects with or are engaged with the a water-in-oil droplets generator.
12. The cell sorting device according to any one of the claims 1 to 10, whereby a second set of branching channels [13] at its end distal from the conduit [14] connects with or is engaged with a fluid channel [21] that opens approximately to the outlet of an oil channel [18] so that when operational a flow with aqueous fluid comprising cells is delivered into a flow of oil from an oil fluid channel [18] to form water-in-oil droplets in an reservoir or chamber [20] with hydrophobic internal walls.
13. The cell sorting device according to any one of the claims 1 to 12, whereby the micro-well array [1] in the conduit [14] is positioned in a plane with two opposing fluid channels sets ([12] & [13]).
14. The cell sorting device according to any one of the claims 1 to 13, whereby the micro-well array [1] is a micro-well array plate that at its edge site is aligned between two opposing fluid channels sets ([12] & [13]) and between the two opposing fluid conduits ports ([11] & [4]).
15. The cell sorting device according to any one of the claims 1 to 14, whereby an optical tweezer [5] is positioned under the plane or under the bottom of the microarray of micro-wells [1].

16. The cell sorting device according to any to claim 15, whereby the optical tweezer [5] mouth directs towards the bottom of the microarray of micro-wells [1].
17. The cell sorting device according to any one of the claim 15 to 16, whereby the optical tweezer [5] when operational sends a light beam, preferably perpendicular, through the plane of the microarray of micro-wells [1].
18. The cell sorting device according to any one of the claims 1 to 17, whereby each fluid channel set comprises a fluid inlet ([3] & fluid outlet [6]) and from there on the fluid channel branches out into at least 2 channels which can branches out in other at least 2 channels whereby the end channels each are engaged with a port (e.g. [8] in the conduit [14].
19. The cell sorting device according to any one of the claims 1 to 18, whereby at least one fluid inlet [11] is at one distal end of the elongated conduit [14] opposing the at least one fluid outlet [4] at the other distal end of the elongated conduit [14].
20. The cell sorting device according to any one of the claims 1 to 19, whereby the elongate or oblong conduit is an enclosure.
21. The cell sorting device according to any one of the claims 1 to 19, whereby the elongate or oblong conduit is a sleeve.
22. The cell sorting device according to any one of the claims 1 to 8, whereby the elongate or oblong conduit is a groove.
23. The cell sorting device according to any one of the claims 1 to 19, whereby the elongate or oblong conduit is a liquid passage.
24. The cell sorting device according to any one of the claims 1 to 8, whereby the channel port is an aperture.
25. The cell sorting device according to any one of the claims 1 to 19, whereby the inlet is an aperture.
26. The cell sorting device according to any one of the claims 1 to 25, whereby the microwell array [1] is for single cell per well trapping and lifting of viable cell and is characterised in that the wells are in a matrix having a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains having a number average molecular weight of a Mn value in a range

between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.

27. The cell sorting device according to any one of the claims 1 to 25, whereby the microwell array [1] for single cell per well trapping and lifting of viable cell, characterised in that the wells are in a matrix consisting essentially of a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains having a number average molecular weight of a Mn value in a range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.
28. The cell sorting device according to any one of the claims 1 to 25, whereby the microwell array [1] is for single cell per well trapping and lifting of viable (human) B cell and is characterised in that the wells are in a matrix having a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains having a number average molecular weight of a Mn value in a range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.
29. The cell sorting device according to any one of the claims 1 to 25, whereby the microwell array [1] is for single cell per well trapping and lifting of viable (human) B cell, characterised in that the wells are in a matrix consisting essentially of a thiol polymer with methoxy polyethylene glycol methacrylate chains at the surface, this chains having a number average molecular weight of a Mn value in a range between 1 500 and 2 500, preferably between 1 900 and 2 100 and most preferably 2 000.
30. The cell sorting device according to any one of the claims 1 to 29, whereby the microwells have a diameter of a value in the range between 9 – 14 μm (12 μm), preferably between 10 – 13 μm and a depth of a value in the range between 9 – 14 μm (12 μm), preferably between 10 – 13 μm .
31. The cell sorting device according to any one of the claims 29 to 30, whereby the methoxy polyethylene glycol methacrylate chains at the surface of said thiol polymer matrix are being bound with the thiol polymer matrix with at least one end of the methoxy polyethylene glycol methacrylate chain with a sulfur atom-containing group there between.

32. The cell sorting device according to any one of the claims 29 to 31, whereby the thiol polymer is a thiol/ene polymer.
33. The cell sorting device according to any one of the claims 29 to 32, whereby the polymer matrix comprises a polymer derived from a thiol terminated polymer whereby thiol groups reacted with a methoxy polyethylene glycol methacrylate.
34. The cell sorting device according to any one of the claims 29 to 33, whereby the polymer matrix also comprises oxiranyl group on its surface.
35. The cell sorting device according to any one of the claims 29 to 32, whereby the polymer matrix comprises a thiol-ene polymer of the group consisting of off-stoichiometry thiol-enes polymer and off-stoichiometry thiol-ene-epoxies polymer.
36. The cell sorting device according to any one of the claims 1 to 35, whereby the microwell array is comprised in an apparatus for trapping of viable single human B cell each in a well and selective lifting viable single human B cell for from it well without affecting viability, the apparatus comprising 1) a single beam optical tweezer in the 900 – 1200 range wave length of and with a laser power of a value between 400 mW and 600 mW.
37. The cell sorting device according to any one of the claims 1 to 35, whereby the microwell array is comprised in an apparatus for trapping of viable single human B cell each in a well and selective lifting viable single human B cell for from it well without affecting viability, the apparatus comprising 1) a single beam optical tweezer in the 1000 – 1210 range wave length of and with a laser power of a value between 450 mW and 550 mW.
38. The use of the cell sorting device according to any one of the claims 1 to 37, for manipulation viable single cells will save guarding the viability, the manipulation comprising single cell in single well trapping, single cell analysis for the selected cell, identification B cells expressing a selected protein, optical trapping and lifting said selected cell by the optical tweezer for further manipulating of said viable cell.
39. The use of the cell sorting device according to any one of the claims 1 to 38, for bidirectional flow.

40. The use of the cell sorting device according to any one of the claims 1 to 39, for cell seeding, washing of non-seeded cells and delivery of reagents for the identification of the cell.

screening and sorting of single cells

FIGURES

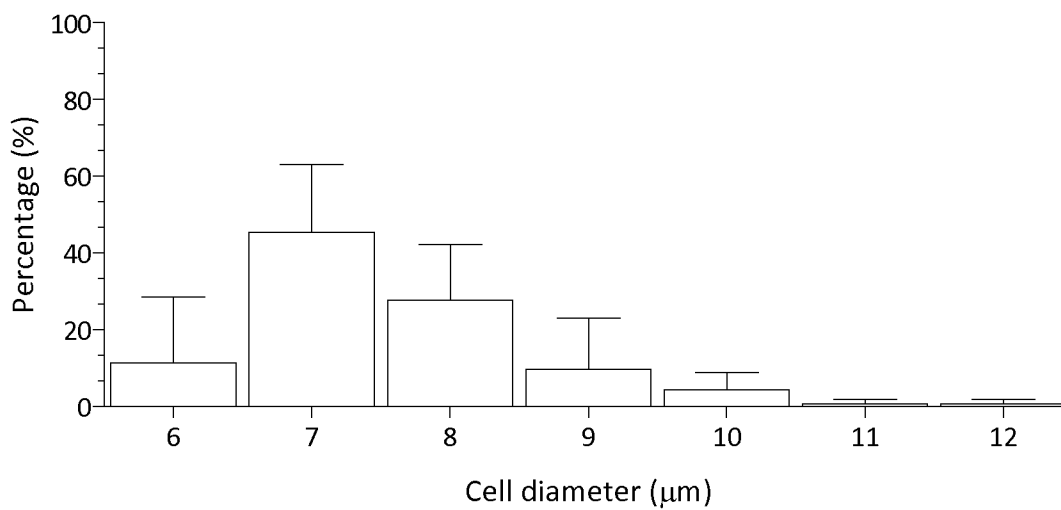


Figure 1

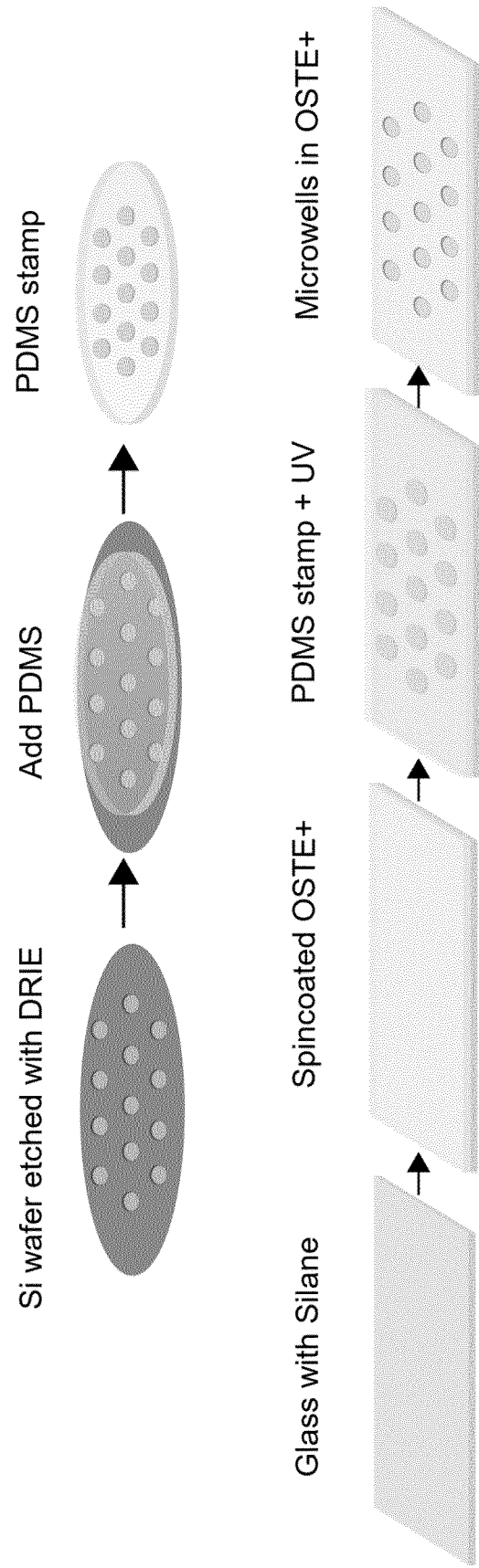


Figure 2

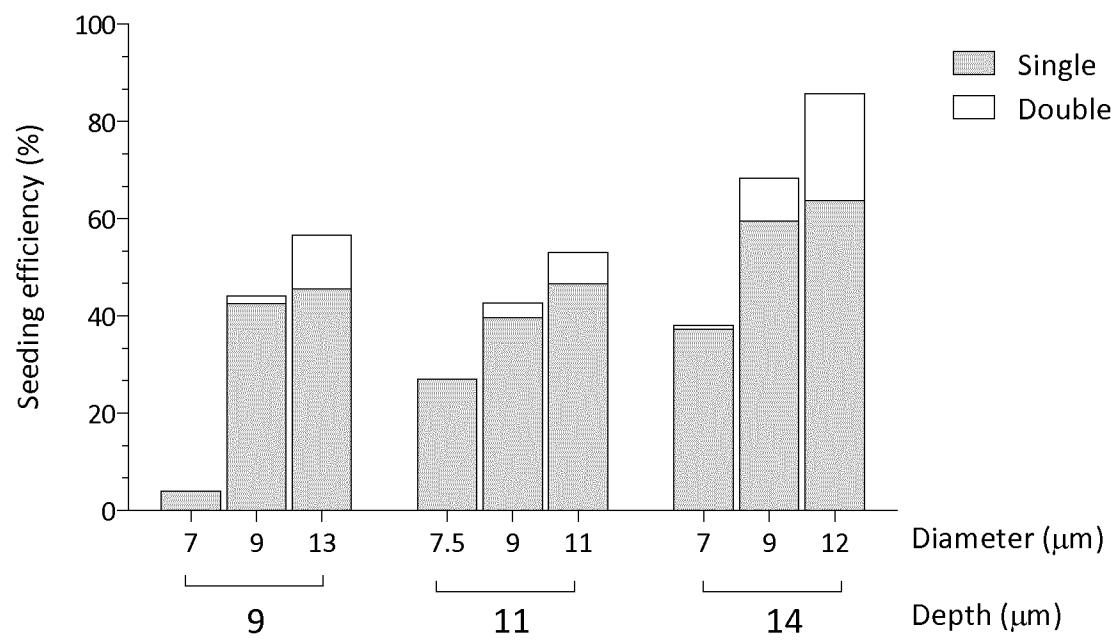


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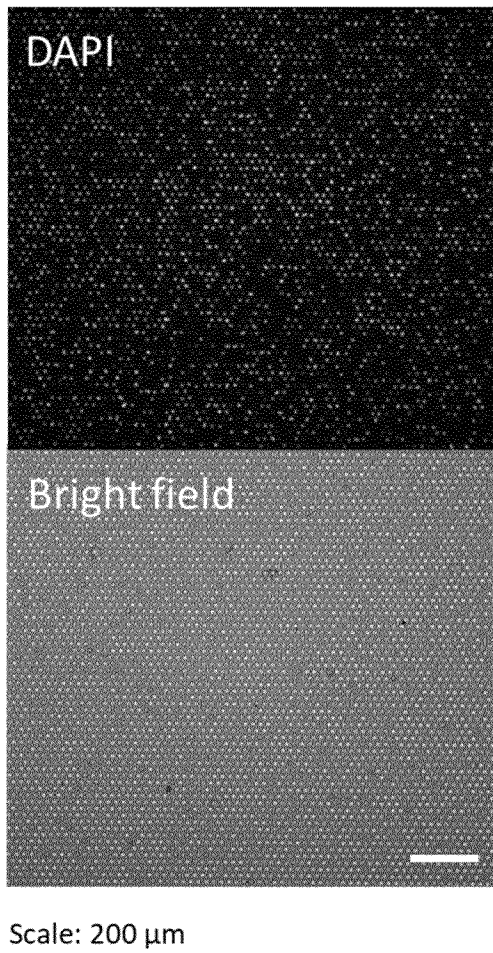


Figure 4

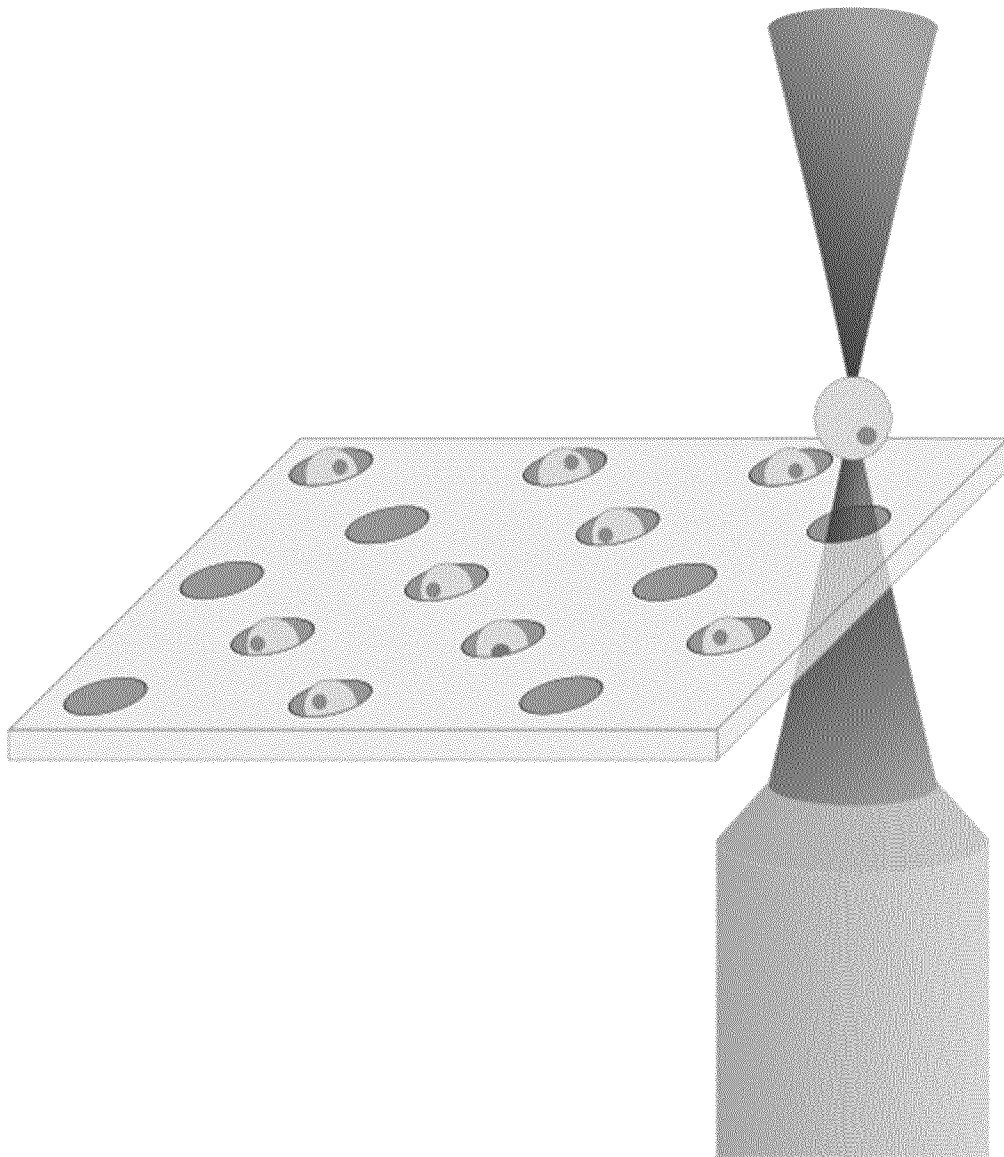


Figure 5

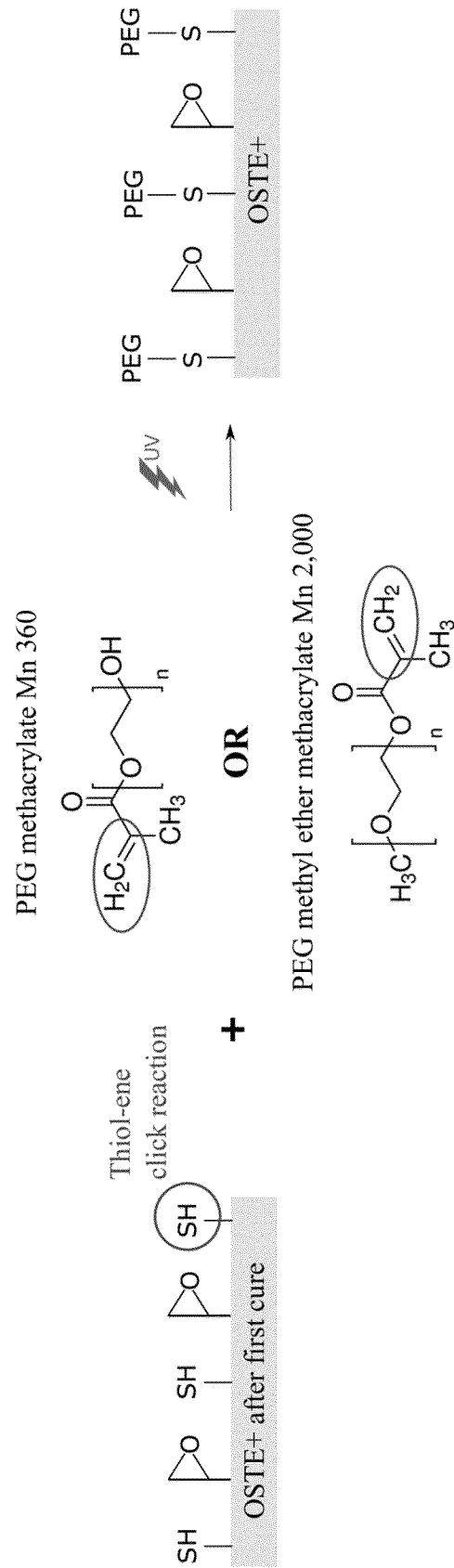
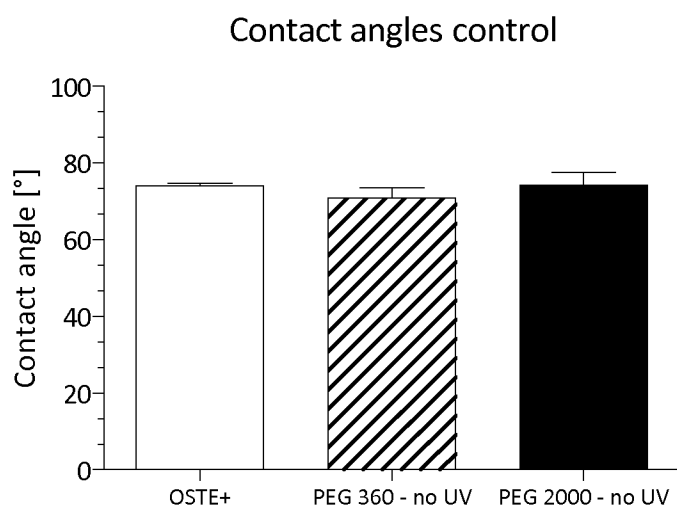
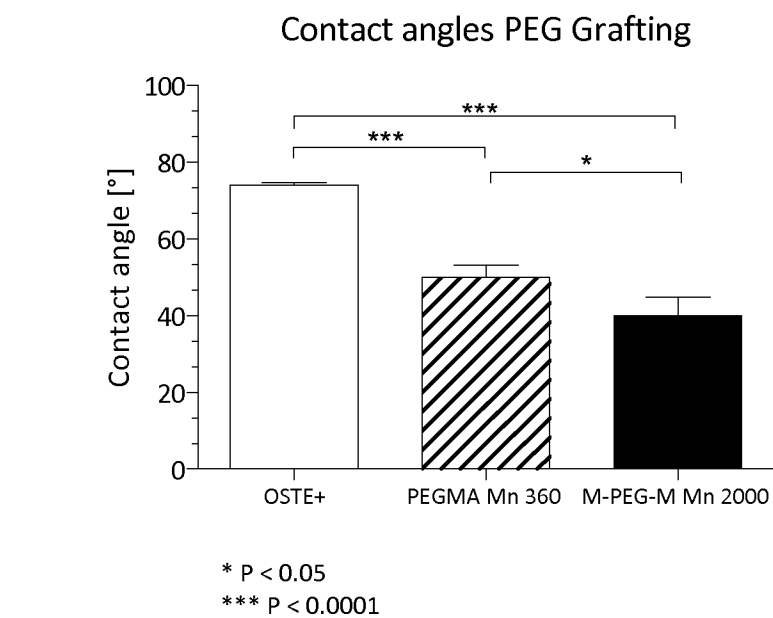


Figure 6

**Figure 7**

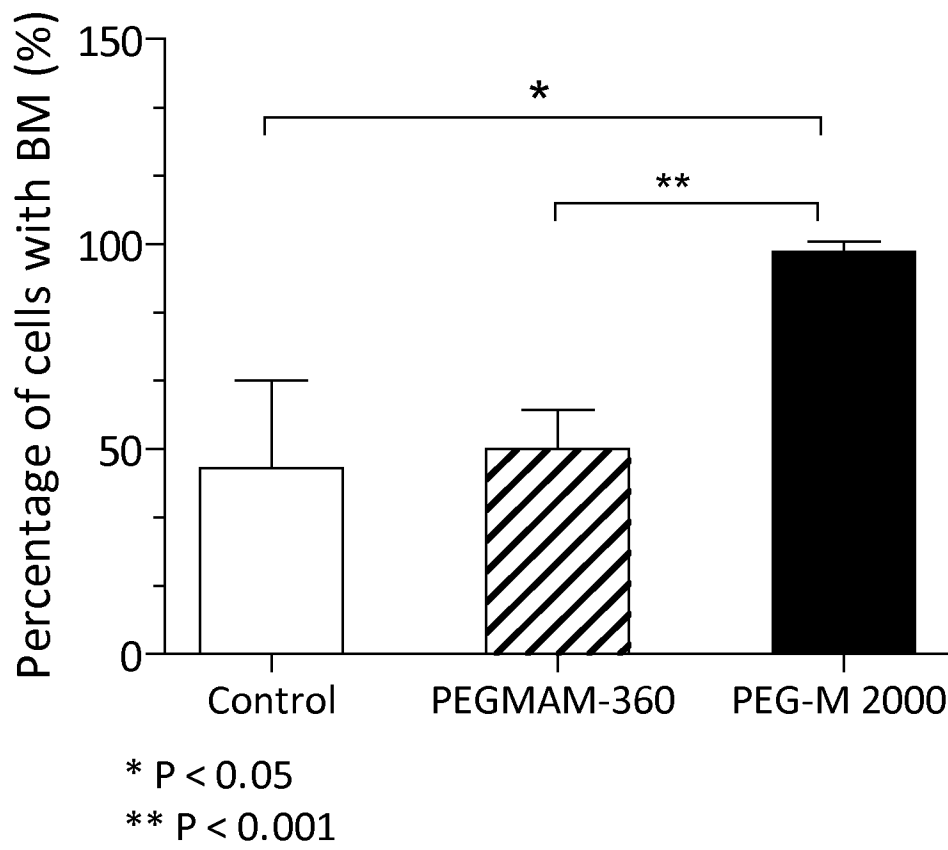


Figure 8

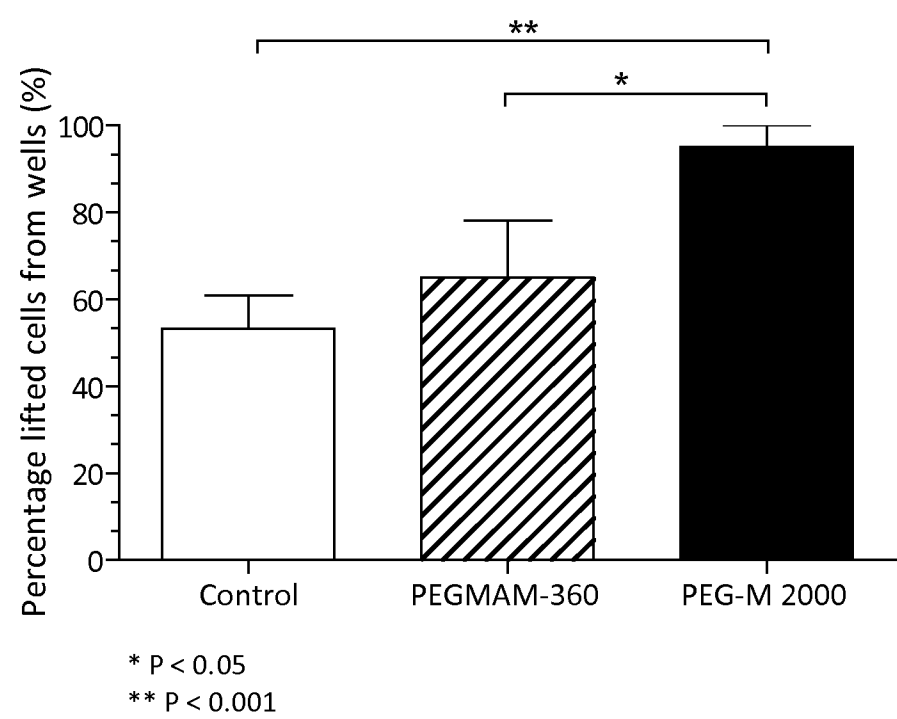


Figure 9

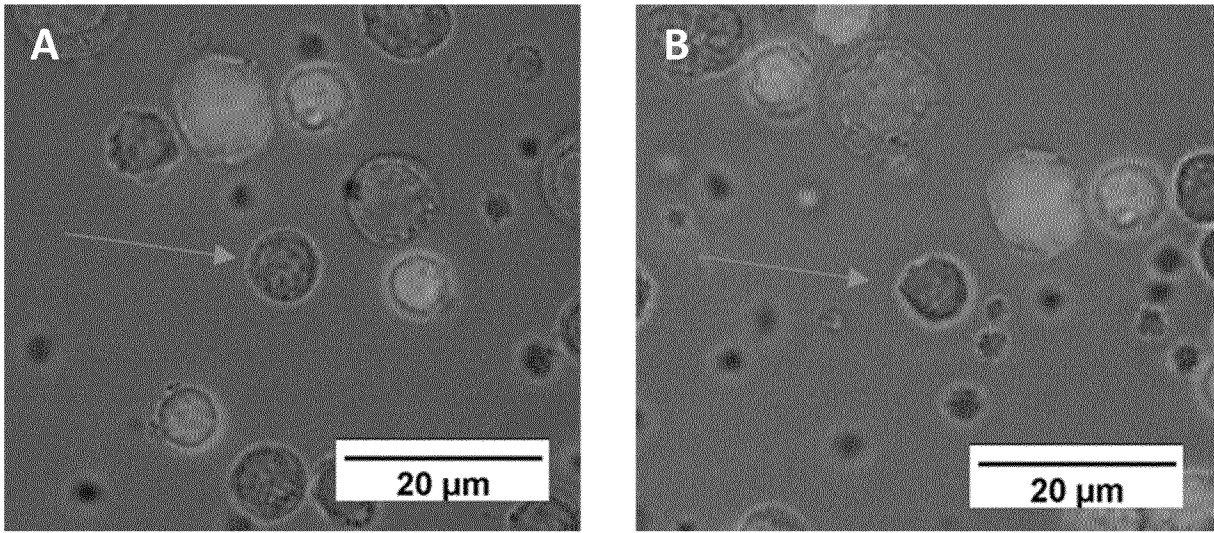


Figure 10

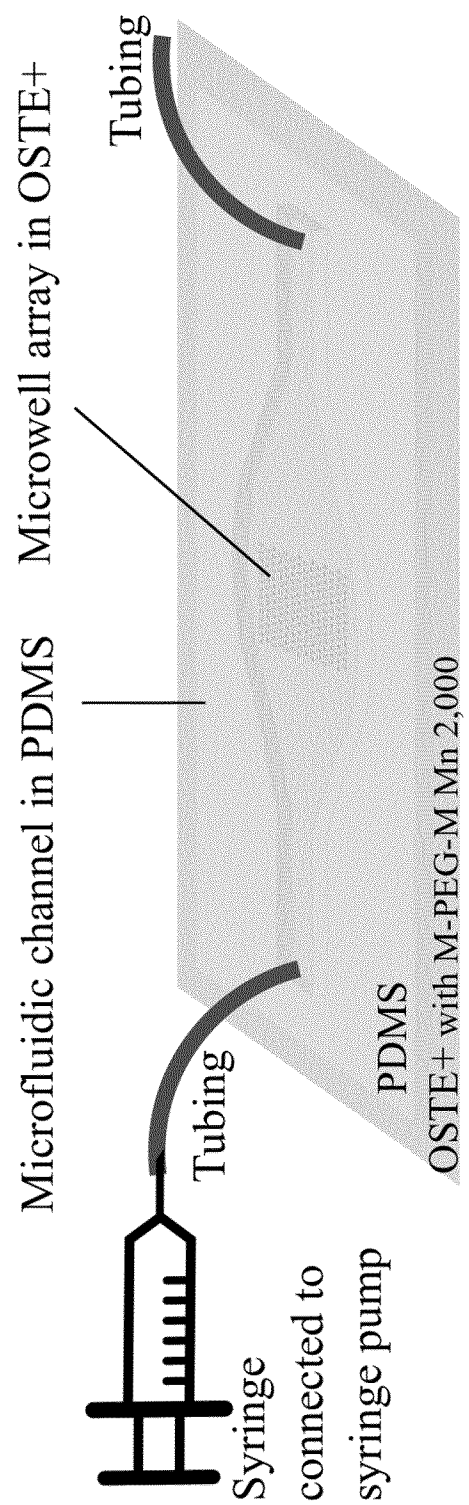


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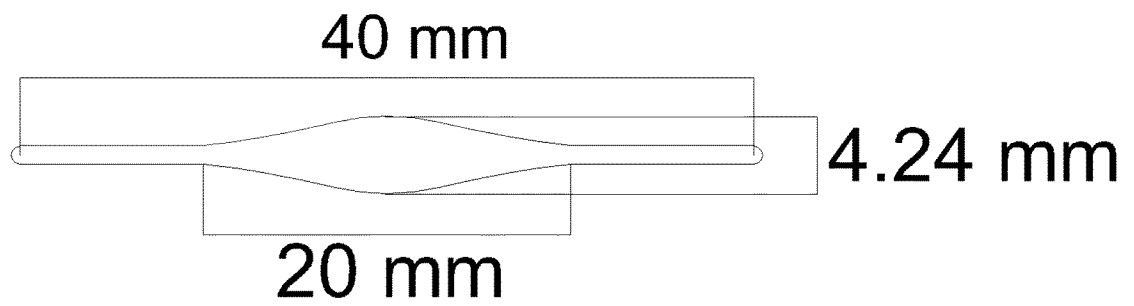


Figure 12

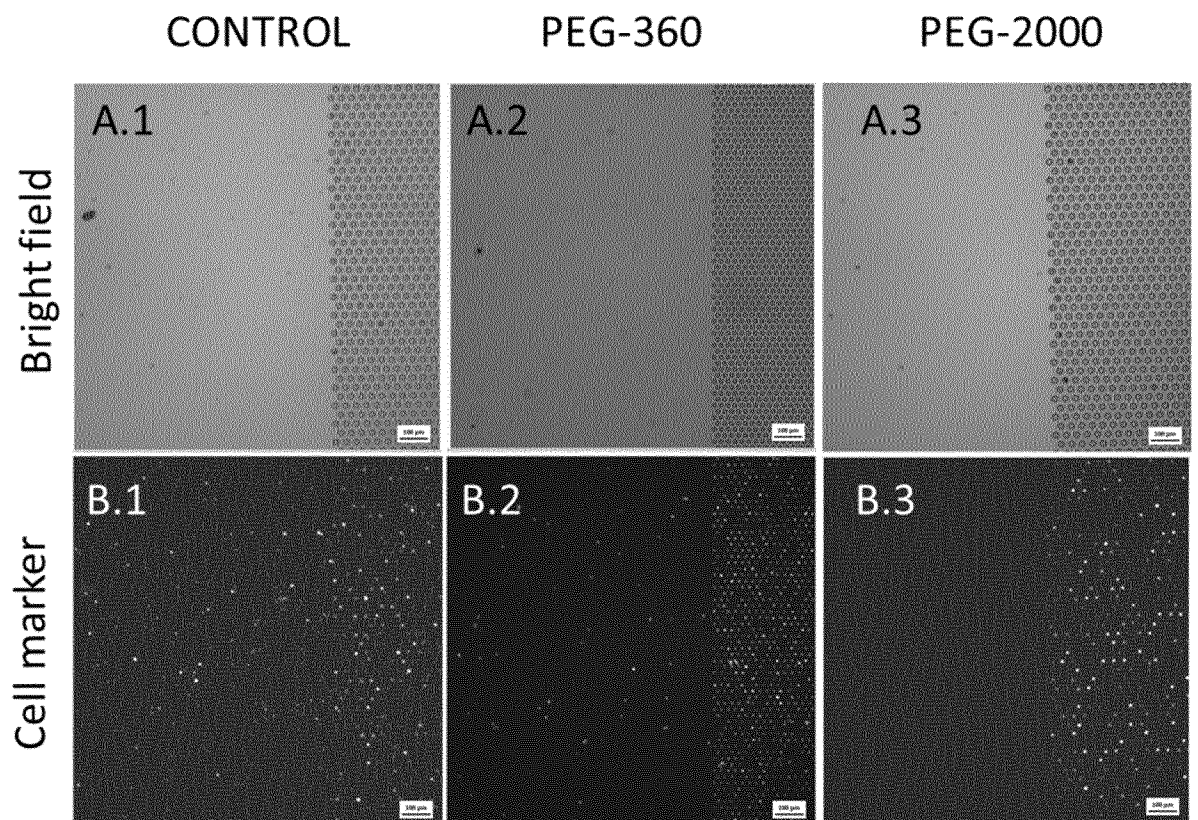


Figure 13

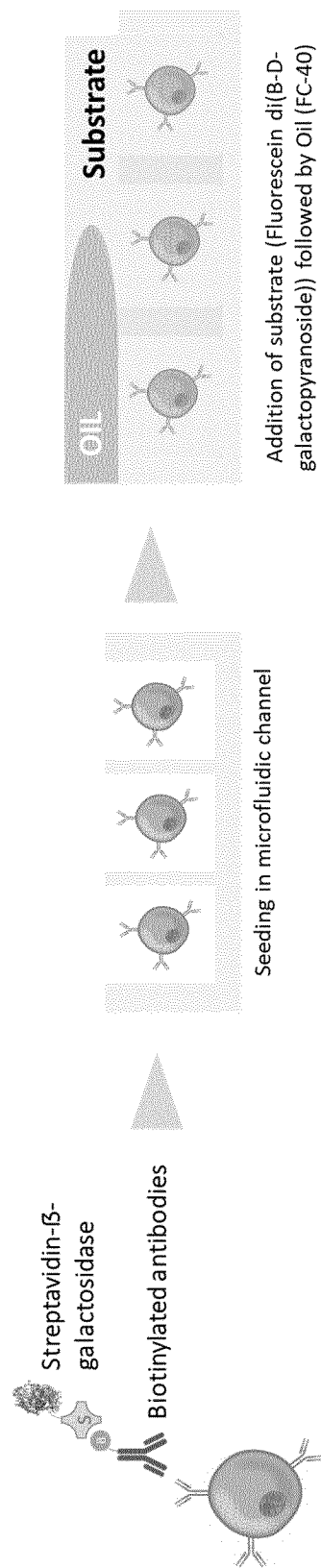


Figure 14

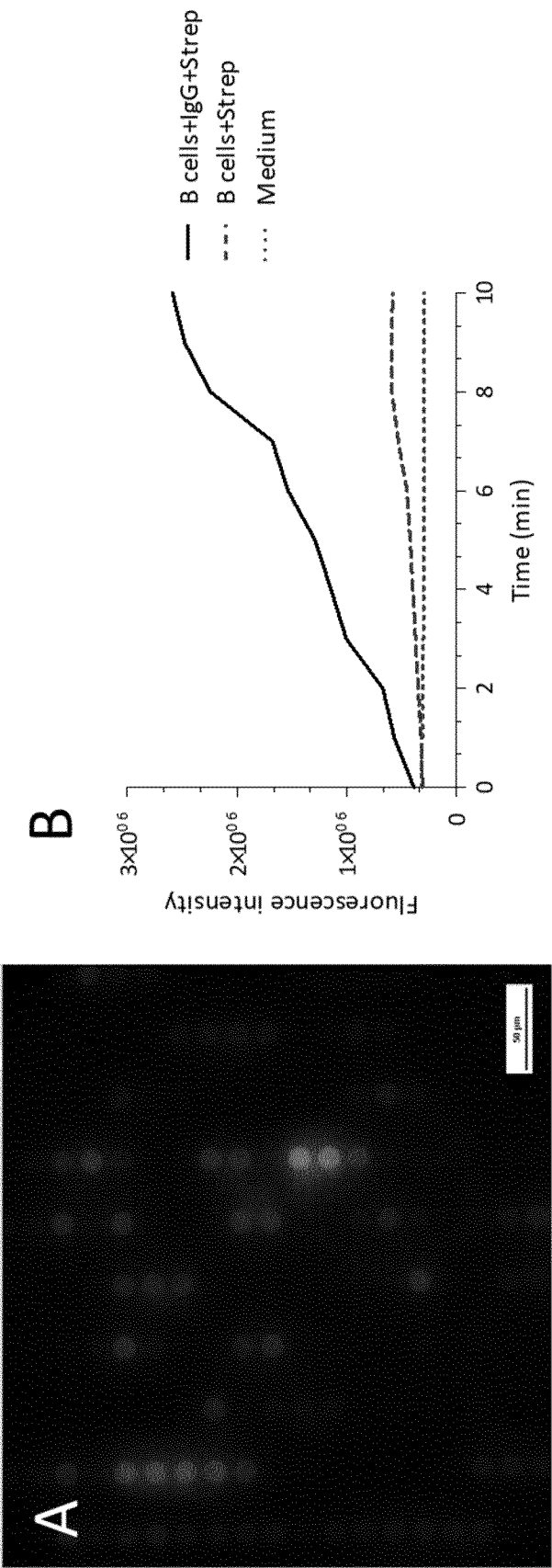


Figure 15

Brownian motion cells

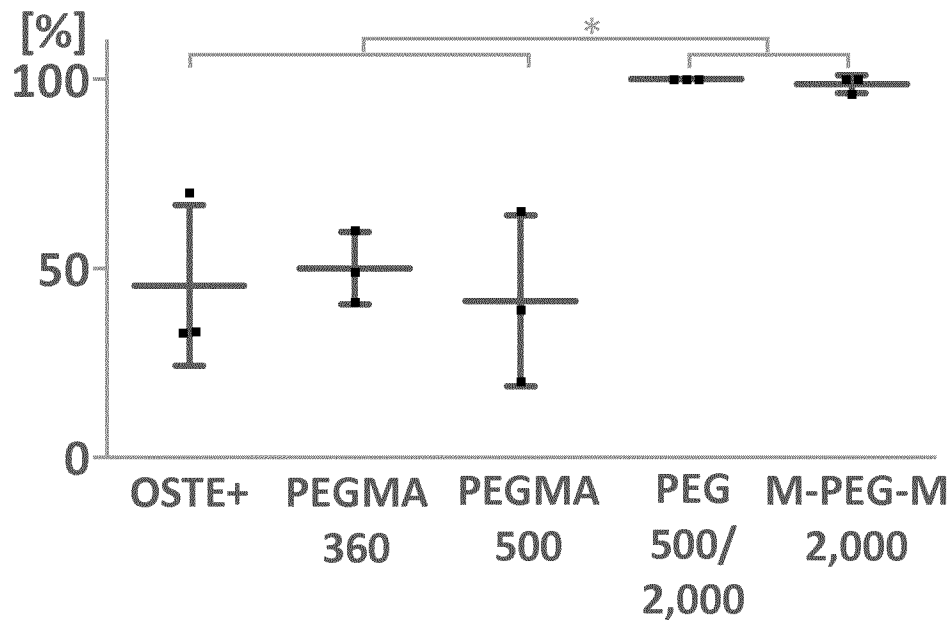


Figure 16

Optically lifted cells

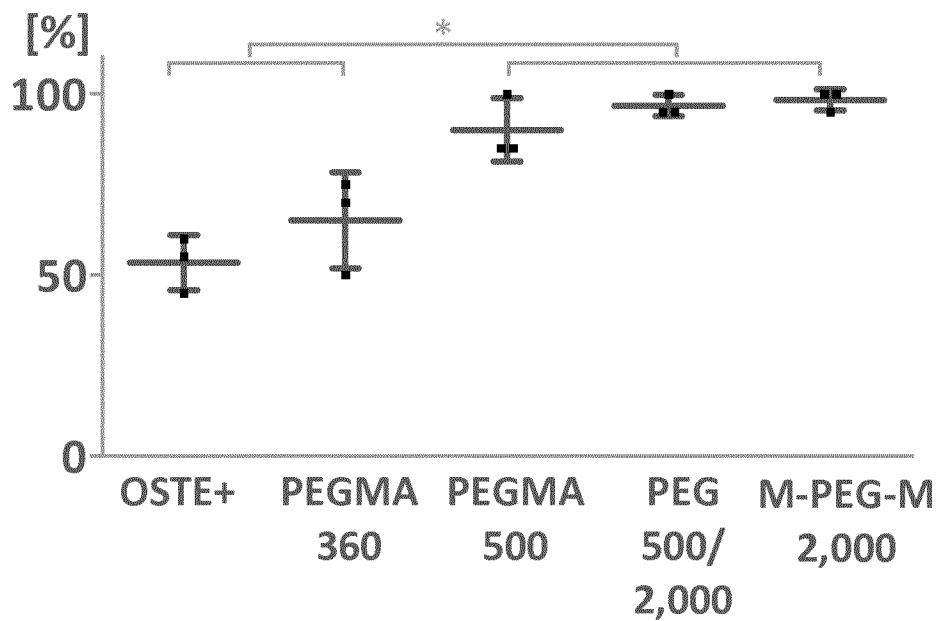


Figure 17

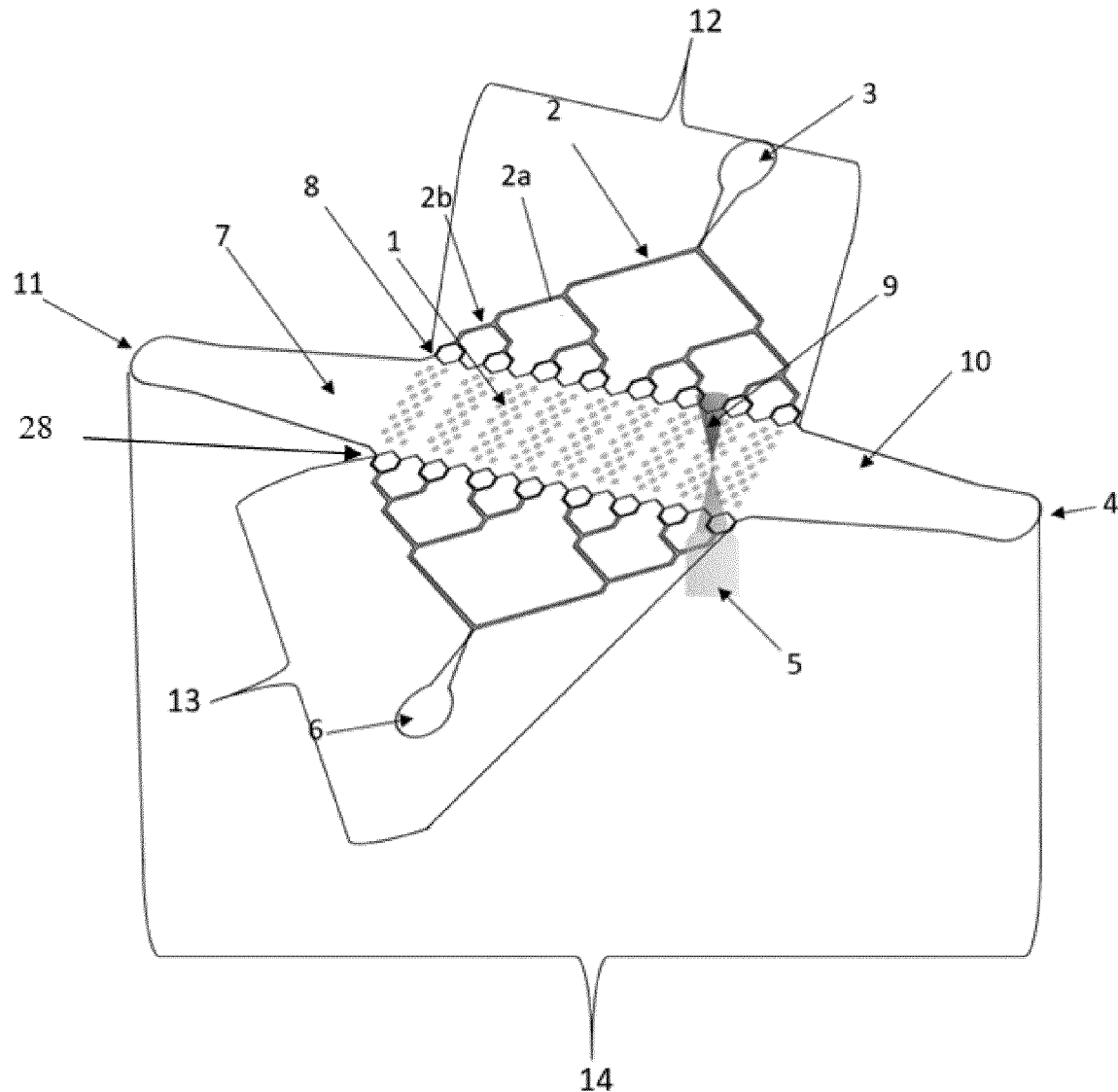


Figure 18

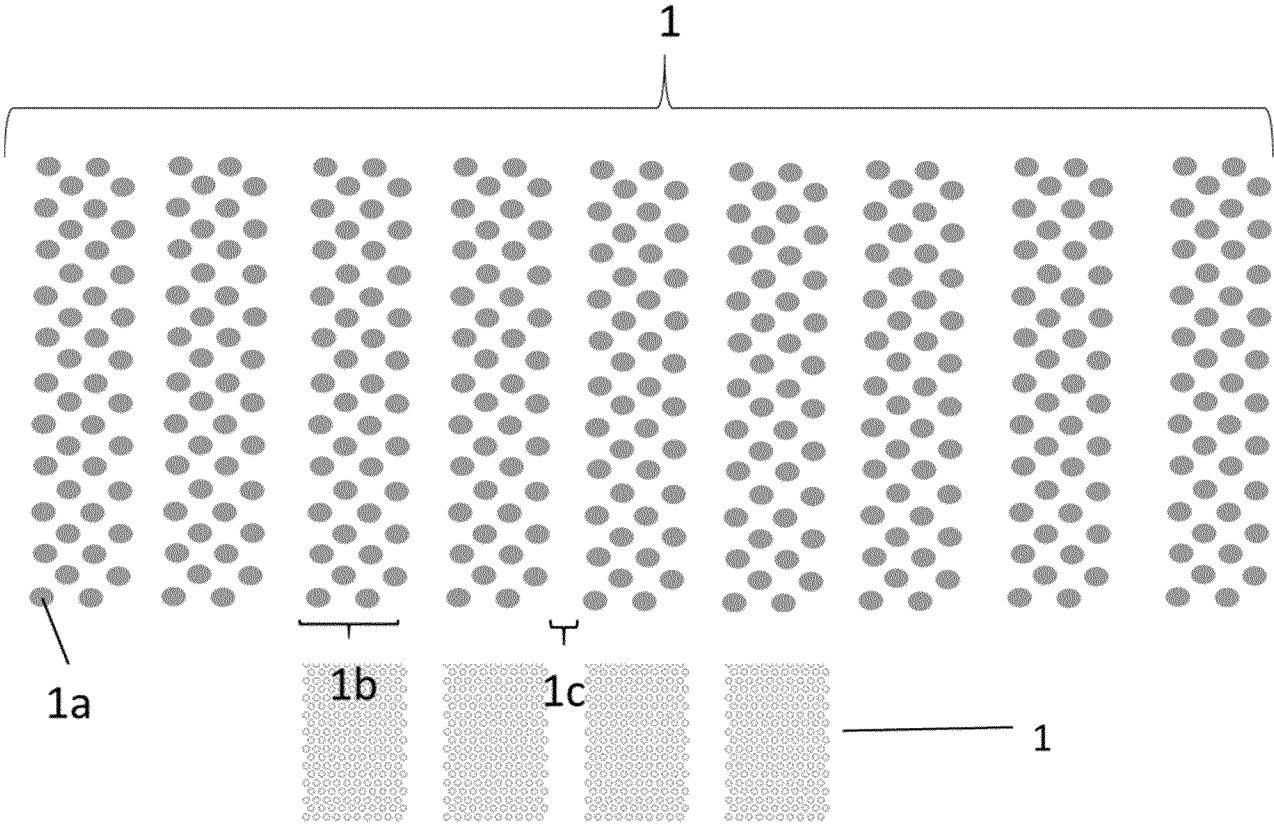


Figure 19

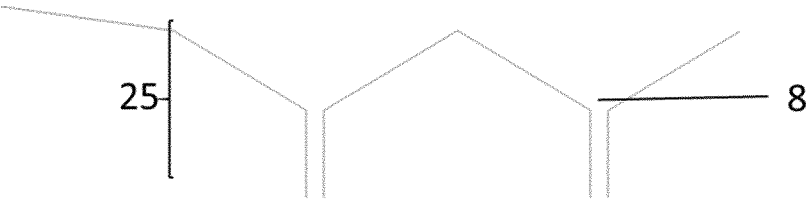


Figure 20

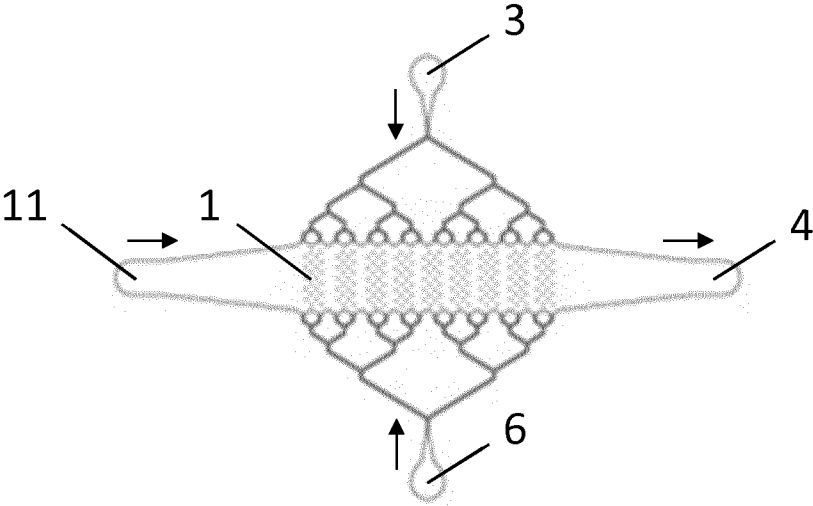


Figure 21

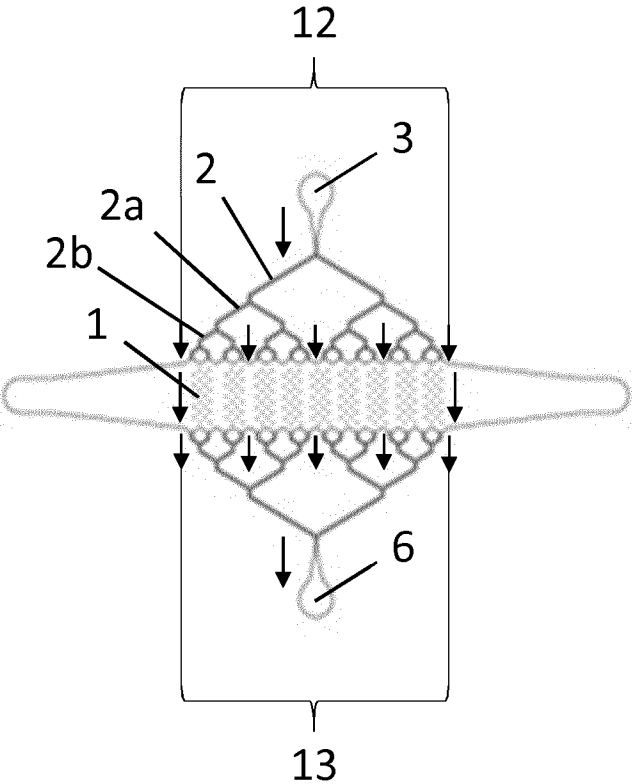


Figure 22

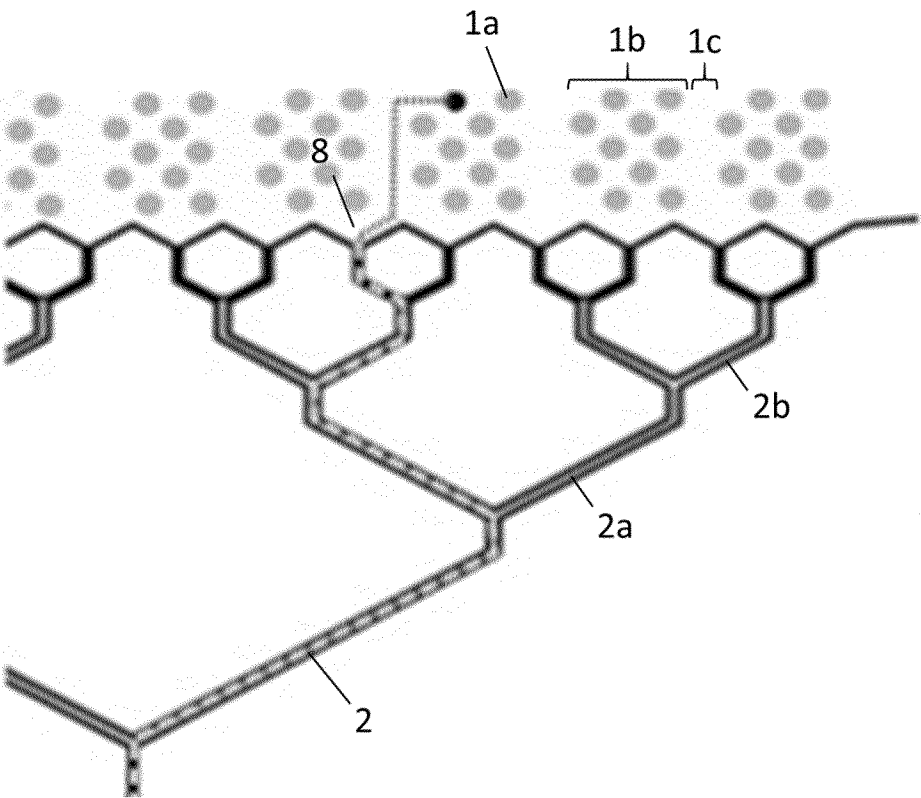


Figure 23

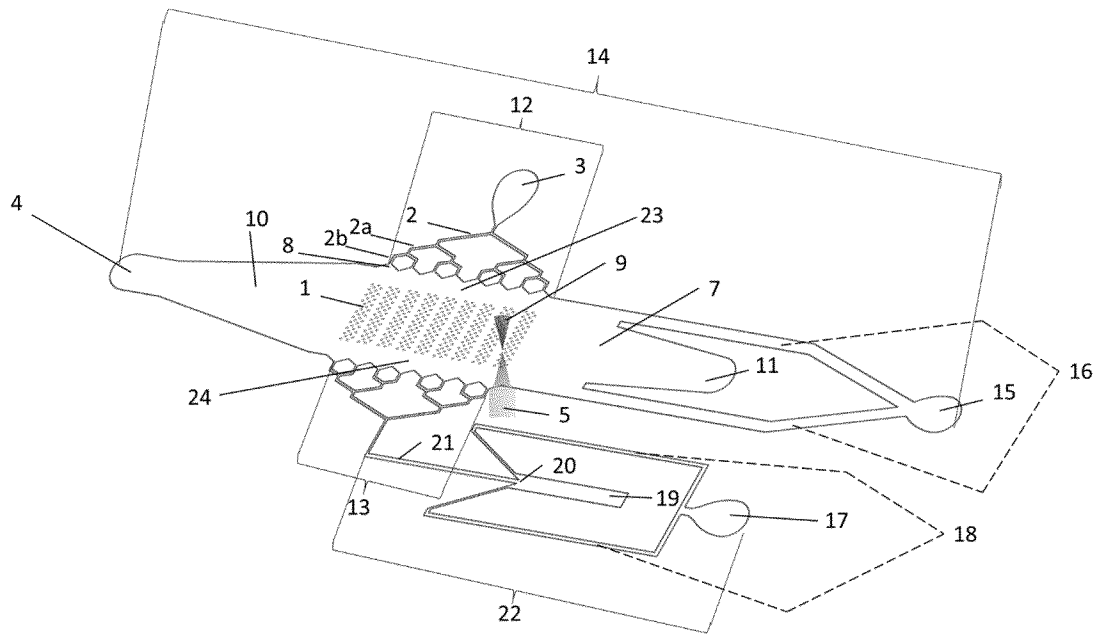


Figure 24

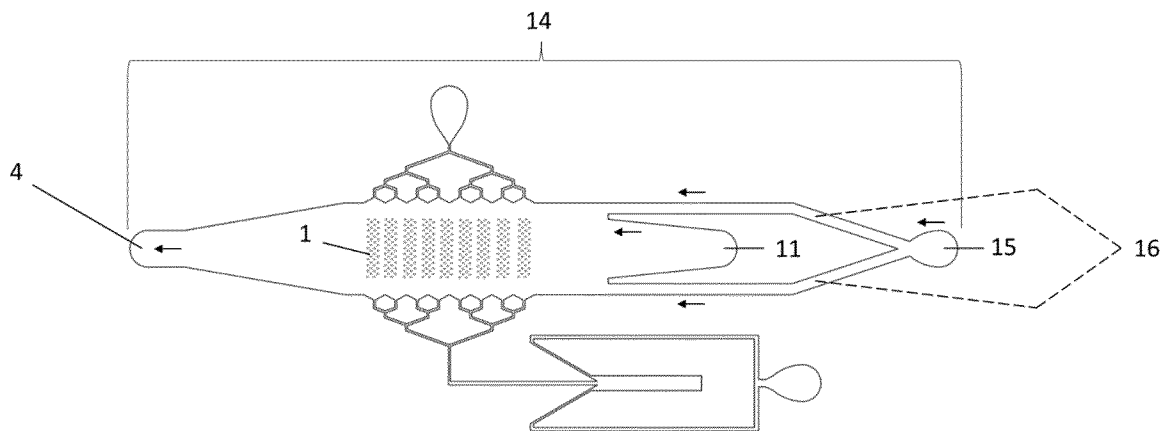


Figure 25

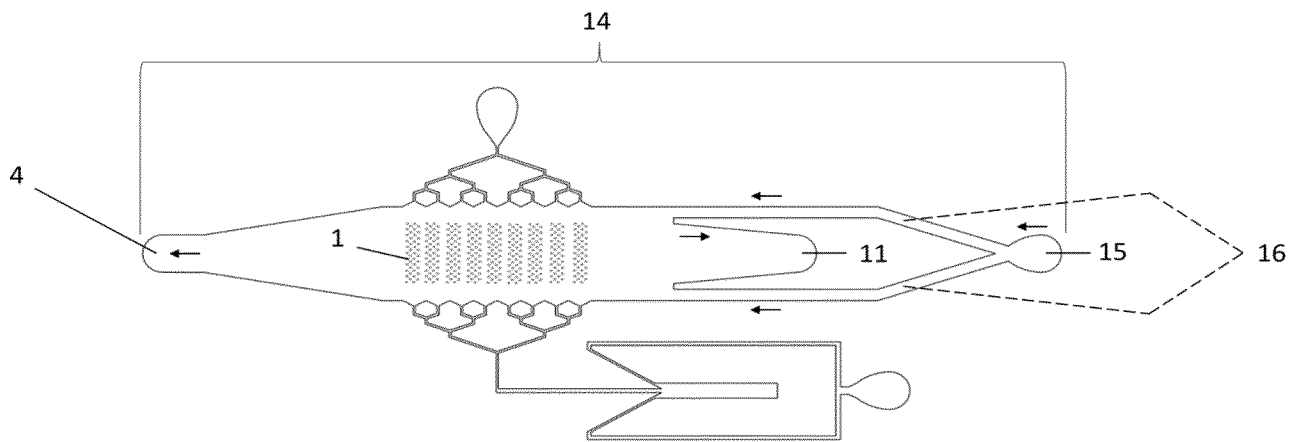


Figure 26

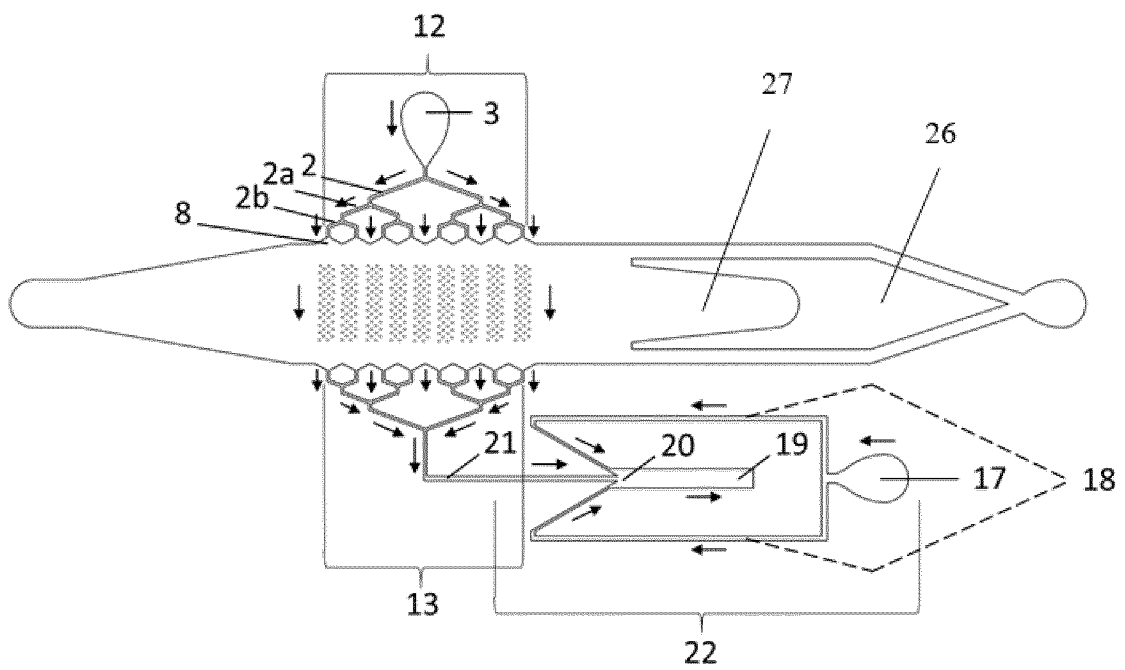
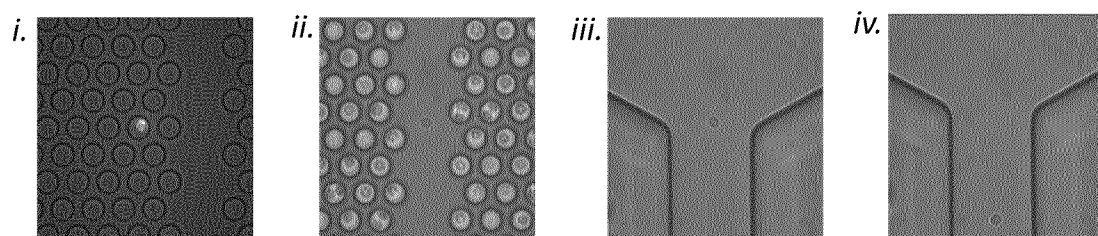
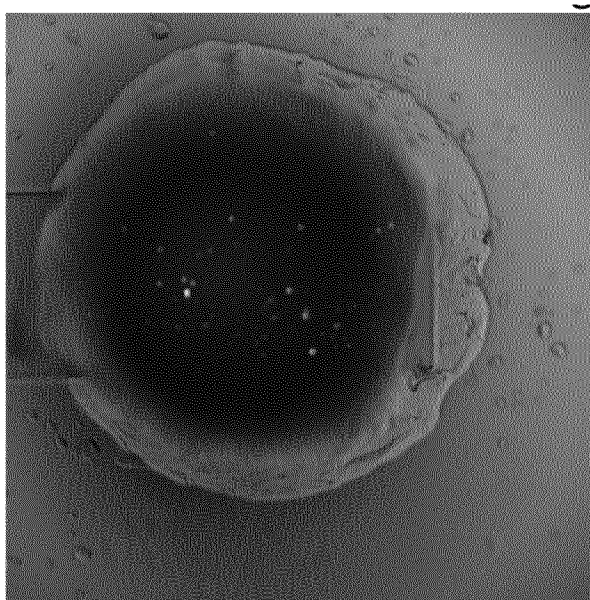


Figure 27

**Figure 28****Figure 29**

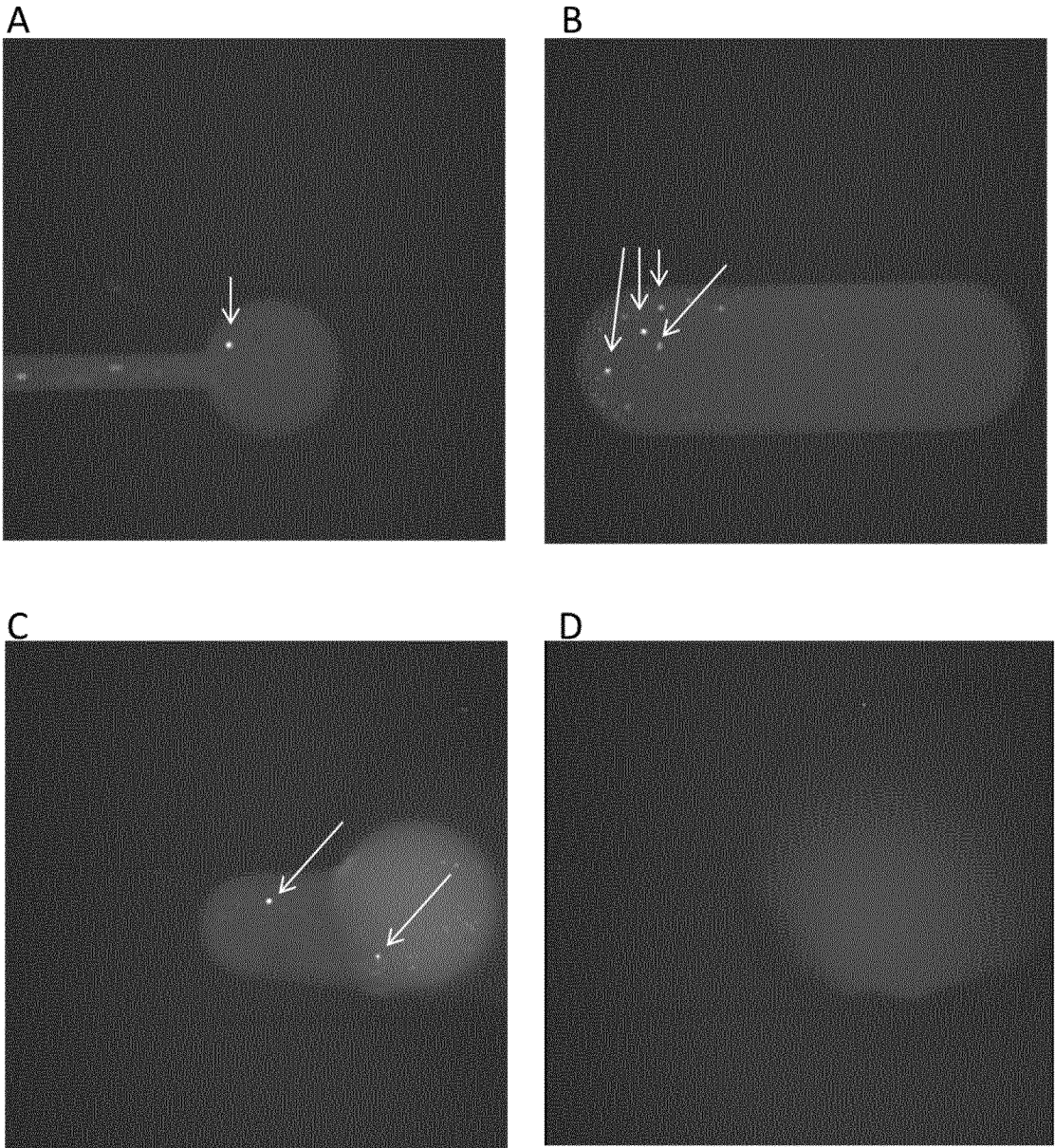


Figure 30

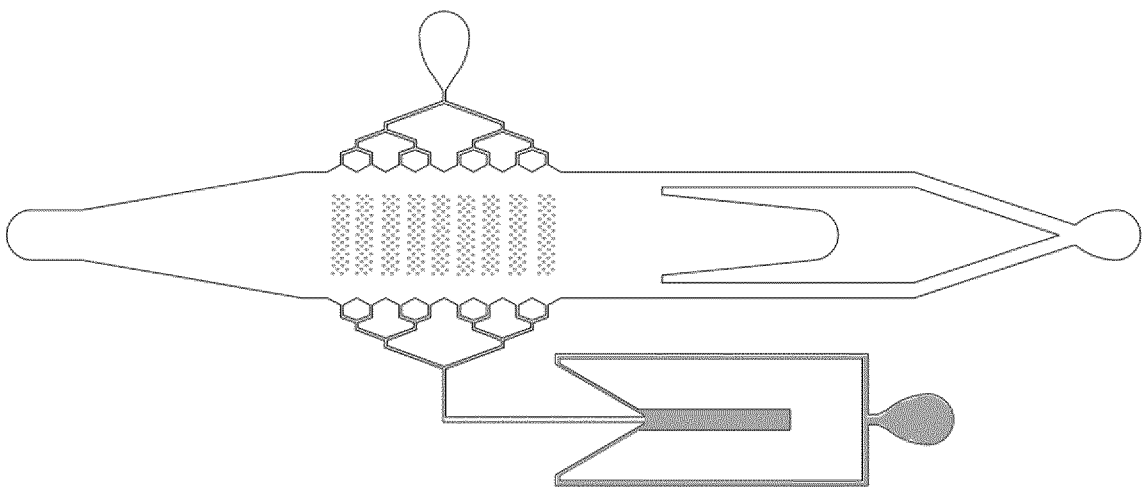


Figure 31

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/080702

A. CLASSIFICATION OF SUBJECT MATTER

INV. B01L3/00

ADD. C12M1/00 G01N15/10 C12M3/06

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)

B01L G01N C12M B01J

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 104 232 468 A (UNIV NORTH CHINA) 24 December 2014 (2014-12-24)	1-4, 11-17, 19-40
A	claim 2; figure 2 -----	8-10
X	US 2015/298121 A1 (COOPER-WHITE JUSTIN JOHN [AU] ET AL) 22 October 2015 (2015-10-22)	1-4,7, 11-40
A	figures 3,8 -----	8-10
X	WO 2018/189532 A1 (UNIV STRATHCLYDE [GB]) 18 October 2018 (2018-10-18)	1-6,11, 13-17, 19-40
A	pages 6,7; figure 1 ----- -/-	8-10



Further documents are listed in the continuation of Box C.



See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"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

12 February 2020

Date of mailing of the international search report

19/02/2020

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Veefkind, Victor

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/080702

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OKUYAMA T ET AL: "Preparation of arrays of cell spheroids and spheroid-monolayer cocultures within a microfluidic device", JOURNAL OF BIOSCIENCE AND BIOENGINEERING, ELSEVIER, AMSTERDAM, NL, vol. 110, no. 5, 1 November 2010 (2010-11-01), pages 572-576, XP027447203, ISSN: 1389-1723, DOI: 10.1016/J.JBIOISC.2010.05.013 [retrieved on 2010-07-01] page 537; figure 1 -----	1-40
A	XIAOLIN WANG ET AL: "Enhanced cell sorting and manipulation with combined optical tweezer and microfluidic chip technologies", LAB ON A CHIP, vol. 11, no. 21, 1 January 2011 (2011-01-01), page 3656, XP055251284, ISSN: 1473-0197, DOI: 10.1039/c1lc20653b the whole document -----	1-40

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/080702

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2018189532	A1	18-10-2018	EP 3609618 A1 19-02-2020
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