



EUROPEAN PATENT APPLICATION

(43) Date of publication:
06.03.2024 Bulletin 2024/10

(51) International Patent Classification (IPC):
B01L 3/00 (2006.01)

(21) Application number: **22306279.5**

(52) Cooperative Patent Classification (CPC):
**B01L 3/502784; B01L 3/502715; B01L 2200/0652;
B01L 2300/0816; B01L 2300/0867**

(22) Date of filing: **29.08.2022**

(84) Designated Contracting States:
**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR**
Designated Extension States:
BA ME
Designated Validation States:
KH MA MD TN

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(54) **METHOD OF EXTRACTING A BEAD FROM A DROPLET**

(57) The invention relates to a method of extracting a bead from a droplet, comprising the steps of:
- providing a droplet of a first fluid within a second fluid, the first fluid being immiscible with the second fluid, the droplet containing a bead;
- passing the droplet through a constriction in a main channel, and supplying a third fluid second fluid immiscible with the first fluid in a downstream channel, downstream of the constriction so as to extract the bead from the droplet.

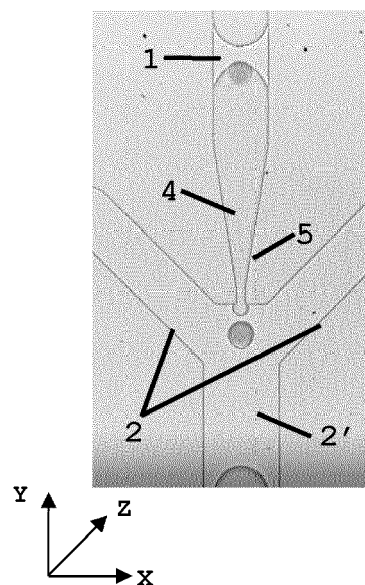


Fig. 3a

Description

TECHNICAL FIELD

[0001] The present invention relates to a method for extracting beads from droplets in a microfluidic channel and a device suitable for carrying out the method. This method is applicable for performing a single-cell multi-omics analysis.

TECHNICAL BACKGROUND

[0002] Recent advances in single-cell sequencing and droplet microfluidics have allowed molecular modalities to be analyzed from a large number of individual cells at a single-cell resolution. The most common method today is based on compartmentalizing single cells within microdroplets forming an emulsion in a microfluidic device, in which the targeted modality, such as transcriptome, genome, proteome or epigenome, is associated with a unique DNA barcode before being subjected to sequencing.

[0003] For example, Klein AM, et al., Cell. 2015;161(5):1187-1201 (2015) proposes a droplet-microfluidic approach for indexing RNA molecules of individual cells with unique DNA barcodes in droplets, followed by next-generation sequencing. DNA barcodes are brought in droplets by polymer beads that are co-encapsulated with cells. The teaching of this document is limited to the analysis of a single cellular modality, e.g., RNA.

[0004] Over the last years, there has been an increasing interest in establishing links between different cellular modalities at a single-cell resolution. Several methods for such single-cell multiomics sequencing protocols have been reviewed in, for example, Macaulay IC, et al., Nat Methods. 2015;12(6):519-22, Matuta, K., Rivello, F., Huck, W. T. S., Adv. Biosys. 4, 1900188 (2020), and Lee, J., Hyeon, D.Y. & Hwang, D. Exp Mol Med 52, 1428-1442 (2020), such as the G&T-seq method for quantifying the genome and transcriptome, the SNARE-seq method for quantifying the transcriptome and epigenome, and the CITE-seq method for quantifying the transcriptome and proteome.

[0005] G&T-seq involves bead-based separation of RNA and DNA, in which the beads on which RNA is captured can be extracted from a supernatant containing DNA. In other words, it requires a physical separation step of the RNA and DNA and it is difficult to be adapted to a droplet microfluidics platform. In SNARE-seq and CITE-seq, which are based on microfluidics-based analysis, barcodes can be released from beads in droplets, and the multiple analytes are treated simultaneously. This approach often lacks versatility as the biochemical reactions and buffers need to be compatible with all the analytes. To improve this aspect, it can be necessary to add a step of physically separating the different cellular modalities or a step of splitting the product including the

different cellular modalities into two fractions to treat them separately.

[0006] None of the above-mentioned methods discloses extracting the beads from the droplets in a microfluidics platform while keeping the emulsion integrity. Thus, there is a need for an upgraded approach to extract a bead from a droplet while keeping the emulsion integrity.

SUMMARY OF THE INVENTION

[0007] It is a first object of the invention to provide a method of extracting a bead from a droplet, comprising the steps of:

- providing a droplet of a first fluid within a second fluid, the first fluid being immiscible with the second fluid, the droplet containing a bead;
- passing the droplet through a constriction in a main channel and supplying a third fluid immiscible with the first fluid in a downstream channel downstream of the constriction so as to extract the bead from the droplet.

[0008] In some embodiments, the third fluid is supplied to the downstream channel *via* two side channels, downstream of the constriction, which are preferably arranged in a symmetrical way with respect to a longitudinal direction of the main channel.

[0009] In some embodiments, the method further comprises a step of positioning the bead at the rear of the droplet before reaching the constriction, preferably by passing the droplet through a narrowed portion of the main channel upstream of the constriction, the narrowed portion having a transverse dimension which is equal to or smaller than the diameter of the bead in a non-constricted state.

[0010] In some embodiments, the constriction has at least one transverse dimension which is equal to or less than the diameter of the bead in a non-constricted state.

[0011] In some embodiments, the constriction has a transverse dimension which is at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50% smaller than the diameter of the bead in a non-constricted state.

[0012] In some embodiments, the constriction has a transverse dimension which is at most 90%, or at most 80%, or at most 70%, or at most 65% smaller than the diameter of the bead in a non-constricted state.

[0013] In some embodiments, the narrowed portion has a transverse dimension which is up to 10 μm smaller than the diameter of the bead in a non-constricted state, preferably up to 5 μm smaller than the diameter of the bead in a non-constricted state.

[0014] In some embodiments, the narrowed portion extends from an inlet of the main channel to the constriction.

[0015] In some embodiments, the main channel comprises a non-narrowed portion in addition to the narrowed portion, wherein the non-narrowed portion extends from

an inlet of the main channel to a transition area, and the narrowed portion extends from the transition area to the constriction.

[0016] In some embodiments, all transverse dimensions of the non-narrowed portion are larger than the diameter of the bead in a non-constricted state.

[0017] In some embodiments, the method further comprises a step of separately collecting the bead from the droplet downstream of the constriction.

[0018] In some embodiments, the bead comprises a magnetic material, and the step of separately collecting is performed using a magnetic field.

[0019] In some embodiments, the method comprises a step of encapsulating the bead in the droplet prior to passing the droplet through the constriction.

[0020] In some embodiments, a first binding assembly and a second binding assembly are tethered to the bead, and the droplet contains at least a first analyte which binds to the first binding assembly and a second analyte which binds to the second binding assembly.

[0021] In some embodiments, the method further comprises, before extracting the bead from the droplet, a step of releasing the second binding assembly from the bead in the droplet, while the first analyte bound to the first binding assembly remains tethered to the bead.

[0022] In some embodiments, the ratio of the flow rate of the third fluid supplied in the downstream channel to the flow rate of the second fluid flowing through the constriction in the main channel ranges from 1 to 30, preferably from 1 to 20, more preferably 2 to 10, still more preferably from 3 to 6 and particularly preferably from 3.5 to 5.

[0023] In some embodiments, the flow rate of the second fluid flowing through the constriction in the main channel is from 50 to 1000 $\mu\text{L}/\text{h}$ and the flow rate of the third fluid supplied in the downstream channel is from 50 to 2000 $\mu\text{L}/\text{h}$, preferably the flow rate of the second fluid flowing through the constriction in the main channel is from 50 to 450 $\mu\text{L}/\text{h}$ and the flow rate of the third fluid is from 200 to 2000 $\mu\text{L}/\text{h}$, more preferably the flow rate of the second fluid flowing through the constriction in the main channel is from 100 to 450 $\mu\text{L}/\text{h}$ and the flow rate of the third fluid is from 500 to 1600 $\mu\text{L}/\text{h}$.

[0024] In some embodiments, the first fluid is aqueous and the second fluid and third fluid are non-aqueous, such as fluorocarbon-based or oil-based.

[0025] In some embodiments, the third fluid supplied in the downstream channel is the same as the second fluid.

[0026] In some embodiments, the third fluid supplied in the downstream channel is different from the second fluid but is miscible with the second fluid.

[0027] In some embodiments, the method comprises a step of introducing a biological sample into the droplet prior to passing the droplet through the constriction, wherein the biological sample is preferably a single cell.

[0028] It is a second object of the invention to provide a microfluidic device for bead extraction, comprising:

- a main channel for passing a droplet of a first fluid within a second fluid, the first fluid being immiscible with the second fluid, the droplet comprising a bead, wherein the main channel includes a constriction; and
- a downstream channel for flowing a third fluid immiscible with the first fluid, fluidically connected to the main channel downstream of the constriction;
- wherein the device is configured for extracting the bead from the droplet at the constriction.

[0029] In some embodiments, the main channel of the microfluidic device comprises a narrowed portion upstream of the constriction, wherein the narrowed portion is configured for positioning the bead at the rear of the droplet before reaching the constriction and has a transverse dimension which is equal to or smaller than the diameter of the bead in a non-constricted state.

[0030] In some embodiments, the constriction of the microfluidic device has at least one transverse dimension which is equal to or less than the diameter of the bead in a non-constricted state.

[0031] In some embodiments, the microfluidic device may comprise two or more main channels in parallel.

[0032] It is a third object of the invention to provide an assembly for bead extraction, comprising:

- the above-mentioned microfluidic device; and
- a collecting reservoir equipped with a magnetic element.

[0033] The present invention makes it possible to overcome the limitations of the prior art such as to perform bead extraction in a single-cell, droplet-based multiomics platform. In particular, the invention provides a simple method for extracting a bead from a droplet while keeping the emulsion integrity, thereby allowing different biological modalities to be separated easily.

[0034] This is achieved by providing a constriction in a main channel and supplying a fluid immiscible with the first fluid in a downstream channel, downstream of the constriction. As a droplet passes through the constriction, it can deform easily while the bead shows more resistance. In other words, the droplet flows faster through the constriction while the flow of the bead slows down. The droplet is then broken by the fluid supplied in downstream channel downstream of the constriction, thus releasing the bead from the droplet. The bead may be then collected separately from the droplet, for example, by using a magnetic field.

[0035] One advantage of the invention is that high-throughput extraction (approximately 100 Hz) is possible while keeping the emulsion integrity. This advantage can be coupled with the concept of the single-cell multiomics analysis in which multimodal barcoded beads index two or more different cellular modalities from each cell. For instance, one cellular modality can be associated with UV-released barcodes and treated conventionally in the

droplet, while the second cellular modality is captured on the bead and extracted out of the droplet to be treated following a different adapted protocol.

[0036] Another advantage of the invention is that this method is simple to implement as it is already fully compatible with conventional methods such as hydrogel beads fabrication, barcoding (split-pool) and encapsulation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037]

Figure 1a shows one example of a step of making beads which may be used in the invention.

Figure 1b shows beads containing a ferrofluid which may be used in the invention.

Figure 2a to **Figure 2c** show an example of a step of encapsulating a bead in a droplet.

Figure 3a to **Figure 3c** show an example of the method of extracting a bead from a droplet according to the present invention.

Figure 4 shows an example of the main channel comprising a narrowed portion and a non-narrowed portion.

Figure 5 is a graph showing how the extraction percentage of beads from droplets varies depending on the flow rate of droplets and the flow rate of second fluid (oil).

Figure 6 shows an example of a collecting reservoir for collecting beads separately from droplets.

Figure 7a shows an example of the step of introducing a biological sample and a bead into a droplet.

Figure 7b shows an example of the step of releasing a second analyte bound to a second binding assembly from a bead into a droplet, while a first analyte bound to a first binding assembly remains tethered to the bead.

Figure 7c shows an example of extracting a bead from a droplet, thereby separating the first analyte on the bead from the second analyte released in the droplet.

Figure 8 shows an example of a microfluidic device having several main channels in parallel.

Figure 9 shows a schematic diagram of a probe which can be used in the present invention.

Figure 10 shows a schematic diagram of an example of the probe which can be used in the present invention.

Figure 11a and **Figure 11b** schematically illustrate one application example of the invention, in which the second analyte is subjected to amplification after the extraction.

Figure 12a to **Figure 12e** schematically illustrate one application example of the invention, in which the first analyte bound on the bead is, after the extraction, subjected to reverse transcription and amplification.

DESCRIPTION OF EMBODIMENTS

[0038] The invention will now be described in more detail without limitation in the following description.

[0039] The term "*microfluidic*" herein means a device or chip in which the minimal channel or chamber dimensions are of the order of 1 to less than 1000 μm . The term "*millifluidic*" herein means a device or chip in which the minimal channel or chamber dimensions are of the order of 1 to 10 mm. The term "*nanofluidic*" herein means a device or chip in which the minimal channel or chamber dimensions are of the order of less than 1 μm .

[0040] The term "*channel*" means an elongated space such as a tube, duct, pipe, or conduit, along which fluids can flow. The channel is delimited by at least one inlet and at least one outlet. Each of the inlet and outlet may correspond to a connection port or may represent a mere junction with another channel for other upstream/downstream microfluidic operations.

[0041] Although the description below makes reference to microfluidic devices or chips, millifluidic or nanofluidic devices or chips may be used equivalently.

Beads and method of making them

Beads

[0042] The term "beads" herein means three-dimensional particles, preferably made from natural or synthetic polymers, having preferably a substantially spherical shape in a non-constricted state. The term "*non-constricted state*" herein refers to the shape of the beads freely suspended in a suspension at zero or a low flow rate. In this state, the beads are not deformed by shear, by contact with a surface, by a magnetic force or the like.

[0043] The beads may be hydrogel beads. Polyacrylamide beads are a preferred example.

[0044] In some embodiments, the beads contain a magnetic material such as magnetic fluid, a magnetic nanoparticle or a magnetic core. Examples of the magnetic fluid is a ferrofluid. The term "*ferrofluid*" refers to a suspension comprising magnetic nanoparticles (i.e. particles having a maximum dimension of less than 1 μm). Examples of magnetic nanoparticles include, but are not limited to, those comprising or consisting of iron, cobalt, zinc, cadmium, nickel, gadolinium, chromium, copper, manganese, terbium, europium, gold, silver, platinum, and alloys thereof. Examples of the magnetic core include an iron core.

[0045] In some embodiments, the beads contain a ferrofluid, as shown in **Fig. 1b**. The proportion of ferrofluid in the beads may for example range from 0.5 to 50%, preferably from 1 to 30 %, more preferably from 2 to 20% (v/v). The proportion of the magnetic nanoparticles in the beads may for example range from 0.01 to 1%, preferably 0.05% to 0.7%, more preferably 0.1% to 0.5% (v/v).

[0046] The beads may be magnetic such as paramagnetic or superparamagnetic, and in particular paramag-

netic.

[0047] A step of adjusting the particle size distribution of the bead population may be provided, which may include for example straining the beads in order to remove all beads having a diameter above a threshold value.

[0048] The diameter of each individual bead may range for example from 10 nm to 1 mm, preferably from 100 nm to 500 μm , more preferably from 1 μm to 250 μm , even more preferably from 10 μm to 150 μm , most preferably from 25 μm to 100 μm . An example of diameter is approximately 50 μm . As a population, the beads may be characterized by a median volume diameter Dv50 ranging for example from 10 nm to 1 mm, preferably from 100 nm to 500 μm , more preferably from 1 μm to 250 μm , even more preferably from 10 μm to 150 μm , most preferably from 25 μm to 100 μm . An example of median volume diameter Dv50 diameter is approximately 50 μm .

[0049] The diameter may be determined by microscope imaging, optionally with fluorescence labeling. For example, two-dimensional images of the beads may be captured and the average diameter or median diameter may be calculated from, for example, 100 beads, based on such microscopy images. The maximum dimension measured on each microscopy image corresponds to the diameter of the bead.

Making beads

[0050] Beads used in the invention may be formed by any conventional method known to the skilled person, as described, for example, in Zilionis R et al., Immunity. 2019;50(5):1317-1334.

[0051] The beads may in particular be formed in a microfluidic chip.

[0052] One example of a method of making beads is illustrated in Fig. 1a.

[0053] In a microfluidic chip, a gel precursor may be passed through a main channel 1 and a fluid immiscible with the gel precursor as a continuous phase may be passed through at least one side channel 2, thereby forming droplets of the gel precursor within the immiscible fluid. The gel precursor may contain the magnetic material as described above.

[0054] Fig. 1a illustrates two side channels for the immiscible fluid, which are perpendicular to the main channel (so-called "flow-focusing" geometry), but the channel geometry is not limited to this flow-focusing geometry, and may be a T-junction in which two incoming flows of fluid are orthogonally joined, or a co-flow geometry in which one fluid flows in an inner channel and the other fluid flows in an outer channel in the same direction, the outlet of the inner channel being disposed in the outer channel.

[0055] As shown in Fig. 1a, a cross-linking initiator may be injected from an additional channel 1' fluidically connected to the main channel 1, but alternatively the cross-linking initiator may be premixed in the gel precursor (in this case, there may be no need for the additional chan-

nel).

[0056] The flow rates of the gel precursor, the immiscible fluid, and possibly the cross-linking initiator may vary depending on the application, and in particular on the dimensions of the channel. The throughput may be approximately from 100 to 6000 droplets/s.

[0057] The formed droplets may then be cross-linked to form beads dispersed in a surrounding fluid, which is preferably an aqueous phase. If the immiscible fluid used for forming the beads is fluorocarbon-based or oil-based, the beads may be transferred to an aqueous phase as a surrounding fluid, or said fluid may be removed and replaced by an aqueous phase as a surrounding fluid. This replacement may take place before, or preferably after cross-linking. The conditions for cross-linking may be selected depending on the composition of the precursor gel and may include for example heating or electromagnetic irradiation such as UV irradiation, or chemical crosslinking such as the addition of calcium in the case of alginate cross-linking.

[0058] The beads may be conditioned in a packed configuration, for example owing to a centrifugation or magnetic sedimentation step.

25 Encapsulating a bead in a droplet

[0059] The beads may be encapsulated in droplets according to any known method in the art, for example, as described in the abovementioned article by Zilionis R et al. and in Abate et al. Lab on a Chip. 2009;9(18):2628-2631, using a commercially available machine, such as 10X Genomics Chromium and a microfluidic chip known from, for example, the abovementioned article by Klein et al.

[0060] Preferably, each droplet contains at most one single bead.

[0061] Specifically, a first fluid comprising beads may be passed through a main channel, and a second fluid immiscible with the first fluid may be passed through at least one side channel in a microfluidic chip. Then, droplets of the first fluid within the second fluid are formed. This may be also referred to as an emulsion of droplets of the first fluid in the second fluid.

[0062] In some embodiments, the volume of the formed droplets may range from 0.05 nL to 3 nL, preferably from 0.1 nL to 1 nL. The volume of the formed droplets may be, for example, 0.8 nL.

[0063] The term "emulsion" as used herein refers to a mixture of two or more fluids that are normally immiscible. In an emulsion, one fluid (dispersed phase) is dispersed in another fluid (continuous phase). The emulsion used in the present invention may be directed to "water-in-oil" emulsions, in which an aqueous fluid (e.g., droplets of the first fluid) is dispersed in an oil-based or fluorocarbon-based fluid (e.g., second fluid). The phrase "keeping the emulsion integrity" means that the aqueous fluid remains dispersed in the oil-based or fluorocarbon-based fluid without destruction of the emulsion, i.e., without any

merging of droplets or any breaking of droplets in several parts.

[0064] In some embodiments, the first fluid is aqueous and the second fluid is non-aqueous, such as fluorocarbon-based or oil-based. The second fluid preferably comprises a fluorinated oil, such as a fluoroether. It may also comprise an emulsion stabilizer and/or a surfactant. When it is simply referred to as "oil", the oil may refer to a fluorocarbon-based fluid or an oil-based fluid.

[0065] Fig. 2a to Fig. 2c show an example of a step of encapsulating a bead in a droplet, at three successive time points.

[0066] As shown in Fig. 2a, the first fluid is passed through a main channel 1. Beads 3 are fed to the main channel 1 via an upstream side channel. The beads 3 within the first side channel may be contained in a fluid which can be the first fluid, or at least is miscible with the first fluid. In particular, both the first fluid and the fluid in the first side channel may be aqueous. A second fluid immiscible with the first fluid (preferably fluorocarbon-based or oil-based) may be passed through at least one downstream side channel 2, e.g., two downstream side channels 2 on either side of the main channel.

[0067] Fig. 2b corresponds to the end of the encapsulation shown in Fig. 2a, where the bead 3 and some first fluid are about to be encapsulated to form a droplet. Fig. 2c corresponds to the beginning of the next encapsulation, where the droplet 4, which is formed in Fig. 2a and Fig. 2b and which contains the bead 3, flows further downstream while a new droplet 4' is being formed.

[0068] Fig. 2a to Fig. 2c illustrate two side channels for the second fluid, but the channel geometry is not limited to this flow-focusing geometry, and may be a T-junction or a co-flow geometry, as described above in connection with the step of making the beads.

[0069] In this example, the beads 3 may be packed in the first fluid or in the fluid at least miscible with the first fluid. This may be achieved by centrifugation, for example. One advantage of packed beads is that the beads can be encapsulated into the droplets in a deterministic way because the bead release can be easily synchronized with the droplet generation by tuning the flow rates. This way, the Poisson distribution resulting from random loading techniques can be avoided, as described more in detail in the above-mentioned article by Abate et al.

[0070] The first fluid may further comprise a buffer for avoiding sedimentation in tubes and syringes, and the like.

[0071] The flow rates of the first fluid, the beads in the first fluid or in the fluid miscible with the first fluid, and the second fluid may vary depending on the application, and in particular on the dimensions of the main channel. The throughput may be generally between 70 and 200 Hz, depending on the flow rates and droplet composition.

Collecting bead-containing droplets

[0072] The droplets of the first fluid within the second

fluid thus obtained may be collected in a reservoir, such as a test tube, equipped with a plug and inlet and outlet conduits connected to the test tube through the plug. The plug may be a polydimethylsiloxane (PDMS) plug.

[0073] The configuration of such a reservoir may be similar to that shown in Fig. 6, which will be explained later in detail.

[0074] The emulsion (droplets of the first fluid within the second fluid) may arrive in the test tube via the inlet conduit, and the droplets may remain packed near the surface as they are generally lighter than the second fluid. The reservoir of this emulsion of droplets of the first fluid in the second fluid may be transported, subjected to different operations such as heat incubation or UV exposure, and reinjected to another microfluidic chip for any additional treatment, such as the subsequent extraction of the beads from the droplets. For this purpose, a syringe filled by a second fluid, e.g., oil, may be plugged to the outlet conduit and mounted on a syringe pump or a pressure controller. The droplets may be flowed backwards in a packed way out of the inlet conduit to a microfluidic chip for bead extraction, for example.

Extracting beads from droplets using a microfluidic device

Extraction of beads from droplets

[0075] The extraction of a bead from a droplet may be performed in a microfluidic device which comprises in particular a main channel.

[0076] The device and in particular the main channel of the device may be prepared for instance by micro-lithography, soft lithography, hot embossing, microcontact printing, direct laser writing, additive or subtractive 3D printing, micromachining, removing sacrificial wires or materials, injection molding or extrusion.

[0077] In other possible embodiments, the main channel and any additional (e.g. side) channel may be tubes which are assembled together.

[0078] Typical but non-exhaustive examples of materials which may be used to make the device and in particular the channels of the device include elastomers, thermoplastics, resins, glass, fused silica, silicone or combinations thereof. Elastomers can be, for instance and in a non-limiting manner, silicones such as polydimethylsiloxane, polyurethanes, acrylic elastomers, fluoroelastomers, polyenes, materials marketed under the brand Tygon® and combinations thereof. Thermoplastic polymers can be, for instance and in a non-limiting manner, polyolefins, such as polyethylene, polypropylene, and more generally polyenes and their copolymers, low or high density, crosslinked or not, cyclic olefin polymers, cyclic olefin copolymers, acrylates such as polymethylmethacrylates, polycarbonates, polyesters, fluorinated polymers, polyamides and combinations thereof. Resins may notably be epoxy, polyester and/or polyurethane resins.

[0079] The method of the invention comprises providing a bead-containing droplet of a first fluid within a second fluid (as defined above), passing the droplet through a constriction in a main channel, and supplying a third fluid immiscible with the first fluid in a downstream channel, downstream of the constriction so as to extract the bead from the droplet.

[0080] The method of the invention may comprise a step of making beads and/or a step of encapsulating a bead in a droplet (as described above), as preliminary steps.

[0081] Alternatively, the method of the invention may not comprise these preliminary steps. In this case, a feed-stock of beads or of bead-containing droplets may be used.

[0082] In some embodiments, the first fluid is aqueous and the second fluid is fluorocarbon-based or oil-based, as described above.

[0083] In some embodiments, the third fluid supplied in a downstream channel downstream of the constriction is the same as the second fluid.

[0084] Alternatively, the third fluid supplied in a downstream channel downstream of the constriction may be different from the second fluid and miscible with the second fluid.

[0085] For the sake of simplicity, it will be considered below that the second fluid is supplied in the downstream channel, but the description applies similarly if a third fluid different from the second fluid is supplied in the downstream channel.

[0086] In some embodiments, the second fluid is supplied in a downstream channel comprising two side channels, downstream of the constriction, which are arranged in a symmetrical way with respect to a longitudinal direction of the main channel.

[0087] The term "*longitudinal direction*" herein refers to the direction of passing the droplet (i.e., direction of the droplet flow) in the constriction of the main channel. The term "*transverse dimension*" refers to a direction perpendicular to the longitudinal direction. When the main channel extends along a length of the microfluidic device, the width direction and the thickness direction are transverse directions.

[0088] The method of extracting a bead from a droplet and the microfluidic device suitable for implementing the method are explained below by reference to **Fig. 3a** to **Fig. 3c**, in which XYZ axes are shown; the X axis corresponds to the width direction, the Y axis to the longitudinal direction, and the Z axis perpendicular to the plane of **Fig. 3a-3c** to the thickness direction.

[0089] As shown in **Fig. 3a**, the microfluidic device comprises a main channel **1** which comprises a constriction **5**; and a downstream channel **2'** fluidically connected to the main channel **1**, downstream of the constriction **5**.

[0090] By "*constriction*" is meant an area of the main channel which has a transverse dimension smaller than the transverse dimension of the main channel in an area immediately upstream of the constriction (in other terms,

a reduced transverse dimension).

[0091] Preferably, the outlet of the main channel is positioned in the constriction, at the junction with the downstream channel.

[0092] The downstream channel may be aligned, i.e. in the same orientation as the main channel, as shown in **Fig. 3a** (i.e., the direction of flow in the downstream channel may be the same as the direction of flow in the main channel). Alternatively, the downstream channel can have a different orientation from the main channel, such as a perpendicular orientation.

[0093] The downstream channel **2'** may further comprise at least one side channel, preferably two side channels. In **Fig. 3a**, for example, the downstream channel **2'** further comprises two side channels **2** which are connected to the main channel at an acute angle (with respect to the portion of the main channel upstream of the junction). In this example, the second fluid may be supplied via the two side channels, and the flow direction of the second fluid may be aligned with the flow direction of the main channel **1** (from top to down in **Fig. 3a**). Alternatively, the two side channels **2** may be arranged in a flow-focusing geometry (arranged perpendicularly to the main channel) or a co-flow geometry.

[0094] In reference to **Fig. 3a**, the droplet **4** flows along the main channel **1** and passes through the constriction **5**, and second fluid is supplied in the downstream channel via two side channels **2** downstream of the constriction.

[0095] As the droplet **4** passes through the constriction, the droplet **4** may deform easily while the bead **3** may show more resistance. As a result, the droplet **4** may flow through the constriction faster than the bead **3** (**Fig. 3b**). The droplet **4** may be then broken by the shearing force exerted by the second fluid supplied in the downstream channel (supplied via the at least one side channel, if present) downstream of the constriction, releasing the bead **3** from the droplet **4** (**Fig. 3c**). Thus, the bead can be extracted from the droplet while keeping the emulsion integrity.

[0096] After the extraction, the bead may be surrounded by a small amount of the first fluid, and therefore may be provided in a reduced droplet of first fluid within the flow of second fluid. The volume of first fluid in this reduced droplet may be less than 10%, preferably less than 5%, or less than 2%, or less than 1%, or less than 0.5%, or less than 0.1%, relative to the volume of first fluid in the (initial) droplet before extraction.

[0097] In some embodiments, the constriction has a transverse dimension which is equal to or less than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state). Preferably, the constriction has a transverse dimension which is at least 10% smaller than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state). The transverse dimension of the constriction (as measured at the longitudinal position wherein the transverse dimension is minimal) may be for example from 10 to 90%, or from 20 to 80%, or from 30 to 75%,

or from 40 to 70%, or from 50 to 65% smaller than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state).

[0098] As shown in **Fig. 3a-3c**, the constriction may have a tapered shape, wherein the transverse dimension gradually decreases from upstream to downstream (towards the junction with the downstream channel). The transverse dimension may be minimal at the junction with the downstream channel. Alternatively, other shapes are possible. For example, the constriction may have a stepped portion, wherein the transverse dimension is reduced in one or more discrete increments from upstream to downstream.

[0099] Only one transverse dimension may be reduced in the constriction. For instance, as shown in **Fig. 3a-3c**, the width of the channel is reduced along the constriction, while the thickness of the channel may remain constant. The opposite is also possible, i.e. the thickness of the channel is reduced along the constriction, while the width of the channel may remain constant. Alternatively, two transverse dimensions may be reduced, e.g. both the thickness and the width of the channel are reduced along the constriction.

[0100] Immediately downstream of the constriction, the transverse dimension of the downstream channel (in which the supplied second fluid, the extracted bead and the remaining droplet continue to flow) is larger than the transverse dimension of the constriction. Preferably, the transverse dimension of the downstream channel immediately downstream of the constriction is equal to or, as illustrated in **Fig. 3a-3c**, larger than the transverse dimension of the main channel upstream of the constriction.

[0101] As shown in **Fig. 3a-3c**, when the downstream channel comprises a side channel, each side channel **2** may be connected to the downstream channel at an acute angle (with respect to the main channel upstream of the junction), which may be between 10 and 85°, preferably between 20 and 75°, more preferably between 30 and 60°, such as between 40 and 50°. Alternatively, each side channel **2** may be connected perpendicularly to the downstream channel. Alternatively, each side channel **2** may be connected to the downstream channel at an obtuse angle (with respect to the portion of the main channel upstream of the junction), which may be between 100 and 175°, preferably between 110 and 165°, more preferably between 120 and 150°, such as between 130 and 140°.

Plurality of main channels

[0102] Although the method of the invention is mostly described above by making reference to one main channel, the microfluidic device of the invention advantageously comprises two or more main channels in parallel.

[0103] Each main channel may be independently connected to a different downstream channel. In this case, each downstream channel may be provided with respec-

tive side channels, as described above. Alternatively, several main channels, for example all main channels, may be connected to the same downstream channel.

[0104] **Fig. 8** shows an example of such a microfluidic device, in which XYZ axes are shown; the X axis corresponds to the width direction, the Y axis to the longitudinal direction, and the Z axis perpendicular to the plane of **Fig. 8** to the thickness direction.

[0105] As shown in **Fig. 8**, the microfluidic device comprises eight main channels **1** each of which comprises a constriction **5**, and a single common downstream channel **2'** downstream of the constrictions. Preferably all the main channels have inlets connected to the same source of fluid.

[0106] The second fluid flows within the downstream channel in one direction from left to right of **Fig. 8**.

[0107] The dimension of each main channel/constriction may be as discussed above. The dimension of each main channel/constriction may be different or preferably the same in the microfluidic device.

[0108] The microfluidic device having two or more main channels makes it possible to parallelize the above-described extraction process, increasing the extraction efficiency. Moreover, even if some of the main channels are blocked or clogged, e.g., at the constrictions, other channels are not affected and keep extracting the beads from the droplets.

[0109] As shown in **Fig. 8**, the common downstream channel **2'** may be oriented perpendicularly to the main channels. It may have, on the side connecting to the main channels, one or more portions which protrude in the longitudinal (Y) direction (hereinafter referred to as "protruded portions"). The protruded portions may be located on either side of each main channel.

[0110] The protruded portions may also protrude in the thickness (Z) direction. Alternatively, the downstream channel may be a straight channel, without containing any protruded portion.

[0111] The downstream channel may have a thickness (Z) equal to or preferably larger than the thickness of the main channel (including upstream of the constriction). This may assist in droplet breakup.

[0112] For the sake of simplicity, the method of the invention will be described below mostly by referring to one main channel, but the description applies similarly if the microfluidic device comprises several main channels.

Positioning of beads

[0113] The method of the invention may further comprise a step of positioning the bead at the rear of the droplet before the droplet reaches the constriction. The rear end of the droplet is the upstream end of the droplet relative to the direction of flow. By "*positioning the bead at the rear of the droplet*" is meant that the bead is displaced within the droplet from any position in the droplet to a position which is at the rear end of the droplet along the longitudinal direction (i.e., upstream end along the

direction of the droplet flow), and which is preferably substantially centered along the transverse directions.

[0114] The positioning step facilitates and improves the quality of the later extraction of the bead from the droplet.

[0115] This positioning step is preferably carried out by passing the droplet through a narrowed portion of the main channel upstream of the constriction, which has a transverse dimension equal to or smaller than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state).

[0116] The narrowed portion may have a transverse dimension which is for example from 0 to 30%, or from 1 to 20%, or from 2 to 15%, or from 5 to 10% smaller than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state). The narrowed portion may have a transverse dimension which is from 0 to 20 μm smaller, or from 1 to 15 μm , or from 2 to 10 μm or from 3 to 8 μm smaller than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state).

[0117] Owing to the narrowed portion, the beads may be slightly compressed in the transverse direction upstream of the constriction, so that a friction force directs the bead to the rear of the droplet and maintains it in this position before the bead is extracted from the droplet.

[0118] The narrowed portion may extend from an inlet of the main channel (by which the droplets are supplied) down to (and possibly including) the constriction.

[0119] Alternatively, the main channel may comprise a non-narrowed portion in addition to the narrowed portion, wherein the non-narrowed portion may extend from an inlet of the main channel (by which the droplets are supplied) to a transition area, and the narrowed portion may extend from the transition area down to (and possibly including) the constriction.

[0120] The non-narrowed portion has at least one transverse dimension which is larger than the narrowed portion. In some embodiments, all transverse dimensions of the non-narrowed portion are larger than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state).

[0121] The transverse dimension of the narrowed portion equal to or smaller than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state) may be in particular the width direction or the thickness direction. In some embodiments, both the width dimension and the thickness dimension are equal to or smaller than the diameter of the bead (or the Dv50 of the beads in a non-constricted state) in a non-constricted state as described above.

[0122] When a non-narrowed portion and a narrowed portion are present, the transition between both portions may be a step or a series of steps (along the width and/or thickness). Alternatively, the main channel may be tapered, the width and/or thickness decreasing gradually in the transition area.

[0123] The narrowed portion may extend down to and

encompass the constriction.

[0124] The transverse dimension in the narrowed portion which is equal to or smaller than the diameter or Dv50 as defined above may be in the same direction as the reduced transverse dimension in the constriction (in which case the transverse dimension in the constriction is even smaller than the transverse dimension in the narrowed portion upstream of the constriction), or it may be in a different direction.

[0125] For example, the transverse dimension in the narrowed portion which is equal to or smaller than the diameter or Dv50 as defined above may be the width, while the reduced transverse dimension in the constriction may be the thickness.

[0126] Alternatively, the transverse dimension in the narrowed portion which is equal to or smaller than the diameter or Dv50 as defined above may be the thickness, while the reduced transverse dimension in the constriction may be the width.

[0127] Alternatively, the transverse dimension in the narrowed portion which is equal to or smaller than the diameter or Dv50 as defined above may be the thickness, while the reduced transverse dimension in the constriction may be the thickness.

[0128] Alternatively, the transverse dimension in the narrowed portion which is equal to or smaller than the diameter or Dv50 as defined above may be the width, while the reduced transverse dimension in the constriction may be the width.

[0129] Alternatively, the transverse dimensions in the narrowed portion which are equal to or smaller than the diameter or Dv50 as defined above may be the width and thickness, while the reduced transverse dimension in the constriction may be the width only, or the thickness only, or both the width and the thickness.

[0130] Alternatively, the transverse dimension(s) in the narrowed portion which is(are) equal to or smaller than the diameter or Dv50 as defined above may be the width only, or the thickness only, or both the width and the thickness, while the reduced transverse dimensions in the constriction may be both the width and the thickness.

[0131] Fig. 4 shows an example of the main channel comprising a narrowed portion 6 and a non-narrowed portion 7 upstream of the narrowed portion 6, and a transition area 8 between the narrowed and non-narrowed portions. In Fig. 4, the narrowed portion 6 has a dimension in the thickness direction which is smaller than the diameter of the bead in a non-constricted state (or the Dv50 of the beads in a non-constricted state), and thus the transition area 8 forms a stepped portion in the thickness direction. This way, the bead upstream of the transition area 8 (bead in the non-narrowed portion 7) is not compressed while the bead downstream of the transition area 8 (in the narrowed portion 6) may be slightly compressed in the thickness direction. Thus, the bead may be positioned at the rear of the droplet and then kept at the rear of the droplet due to the friction force until the droplet reaches the constriction (constriction not shown

in Fig. 4).

[0132] In some embodiments, the main channel may have a width immediately upstream of the constriction from 10 to 500 μm , preferably from 20 to 200 μm , more preferably from 50 to 150 μm , even more preferably from 70 to 120 μm .

[0133] In some embodiments, the main channel may have a thickness immediately upstream of the constriction from 5 to 300 μm , preferably from 10 to 150 μm , more preferably from 20 to 100 μm , even more preferably from 30 to 80 μm .

[0134] In some embodiments, the thickness of the main channel immediately upstream of the constriction is less than the width of the main channel immediately upstream of the constriction.

[0135] In some embodiments, the constriction has a minimum thickness from 5 to 300 μm , preferably from 10 to 150 μm , more preferably from 20 to 100 μm , even more preferably from 30 to 80 μm .

[0136] Alternatively, the constriction may have a minimum thickness from 1 to 100 μm , preferably from 5 to 80 μm , more preferably from 10 to 50 μm , even more preferably from 15 to 40 μm . In some embodiments, the constriction has a minimum width from 1 to 100 μm , preferably from 5 to 80 μm , more preferably from 10 to 50 μm , even more preferably from 15 to 40 μm .

[0137] Alternatively, the constriction may have a minimum width from 5 to 300 μm , preferably from 10 to 150 μm , more preferably from 20 to 100 μm , even more preferably from 30 to 80 μm . In some embodiments, the (minimum) width of the constriction is less than the (minimum) thickness of the constriction.

[0138] Alternatively, the (minimum) thickness of the constriction is less than the (minimum) width of the constriction.

[0139] Alternatively, the positioning of the bead at the rear of the droplet may be achieved differently, such as by applying a magnetic field having a suitable magnitude and orientation to the main channel upstream of the constriction.

Plurality of droplets

[0140] Although the method of the invention is mostly described herein by making reference to one bead-containing droplet, it advantageously comprises providing a plurality of droplets (as described above) and successively passing the droplets through the constriction so as to extract the beads from their respective droplets.

[0141] The method may thus be continuous and involve the processing of a stream of droplets. The entirety of the present description must be interpreted in this context.

Adjustment of flow rates

[0142] The flow rate of the droplets and the flow rate of the second fluid supplied in the downstream channel

may vary depending on the application, and in particular on the dimensions of the channel. They may be selected so that most of the beads (e.g., all the beads) are extracted from the droplets, and so that a low volume of first fluid remains around the extracted beads.

[0143] The term "*flow rate of the droplet(s)*" herein means the flow rate of the second fluid (e.g., oil) carrying the droplets.

[0144] Fig. 5 is a table obtained from experimental data showing how the extraction percentage of beads from droplets varies depending on the flow rate of droplets upstream of the constriction and the flow of the second fluid (oil) supplied in the downstream channel downstream of the constriction (e.g. via one or more side channels), using beads having a volume median diameter (in the non-constricted state) of 47 μm and the arrangement shown in Fig. 3a-3c. Qdrops represents the flow rate of the droplets and Qoil represents the total flow rate of oil (second fluid) supplied in the downstream channel via the side channels. The extraction percentage is measured using a fast camera, which will be explained in detail in the Examples.

[0145] The numbers in the table of Fig. 5 correspond to the proportion of beads which are extracted from the droplets. Ideally, the extraction rate should be 100% or close to 100% (such as from 95 to 100%, or from 98 to 100%, or from 99 to 100%). The color in the table relates to the amount of first fluid remaining around the beads after extraction. When the cells are colored in white, the amount of first fluid remaining around the beads is higher than when the cells are colored in gray. This higher amount of the remaining first fluid is due to the droplets breaking prematurely because of high shearing due to the supply of second fluid via the side channels. This may result in reduced purity of the extraction process.

[0146] The flow rates of the droplets and the flow rate of the second fluid may thus be selected so that a satisfactory extraction rate is achieved with little retention of first fluid around the beads.

[0147] Specifically, the ratio of the flow rate of the second fluid (supplied in the downstream channel) to the flow rate of the droplets flowing through the main channel (upstream of the constriction) may range from 1 to 30, preferably from 1 to 20, more preferably 2 to 10, still more preferably from 3 to 6, particularly preferably from 3.5 to 5.

[0148] The flow rate of the droplets flowing through the main channel (upstream of the constriction) may range from 50 to 1000 $\mu\text{L/h}$ and the flow rate of the second fluid supplied in the downstream channel may be from 50 to 2000 $\mu\text{L/h}$, preferably the flow rate of the droplets flowing through the main channel (upstream of the constriction) is from 50 to 450 $\mu\text{L/h}$ and the flow rate of the second fluid supplied in the downstream channel is from 200 to 2000 $\mu\text{L/h}$, more preferably the flow rate of the droplets flowing through the constriction in the main channel is from 100 to 450 $\mu\text{L/h}$ and the flow rate of the second fluid supplied in the downstream channel is from 500 to 1600 $\mu\text{L/h}$.

[0149] The flow rate of fluid entering the downstream channel from the main channel through the constriction is not taken into account in the flow rate of the second fluid supplied in the downstream channel, in the above.

Collecting beads and droplets

[0150] After the beads are extracted, the method of the invention may further comprise a step of separately collecting the bead from the droplet downstream of the constriction.

[0151] In some embodiments, the bead comprises a magnetic material, and the step of separately collecting the bead is performed using a magnetic field.

[0152] As shown in Fig. 6, for example, the droplets 4 and extracted beads 3 may be collected (from an outlet of the downstream channel) in a collecting reservoir 9.

[0153] The collecting reservoir 9 may be a test tube for example, which may have a volume ranging from 100 μ L to 100 mL, in particular from 500 μ L to 50 mL, such as from 1 to 5 mL. The collecting reservoir 9 may be equipped with a plug 11 and inlet conduit 12 and outlet conduit 13 inserted into the collecting reservoir 9 through the plug 11. The plug may be a PDMS plug. The collecting reservoir 9 may be also equipped or associated with a magnetic element 10, such as a magnet. The magnetic element 10 may optionally be fixed to the collecting reservoir 9.

[0154] Preferably, both the inlet conduit 12 and outlet conduit 13 may have an open end within the collecting reservoir 9. The inlet conduit 12 may extend deeper in the collecting reservoir 9 than the outlet conduit 13, so that the open end of the inlet conduit 12 is below the open end of the outlet conduit 13 as shown in the figure. The stream containing the droplets and extracted beads may be introduced into the collecting reservoir 9 via the inlet conduit 12.

[0155] The extracted beads 3 may be collected using a magnetic element 10 to the collecting reservoir 9 while the droplets 4 may gather at or near the surface of the second fluid, as the first fluid is generally less dense than the second fluid.

[0156] Optionally, the droplets may be withdrawn from the collecting reservoir 9 via the outlet conduit 13 (for example using a syringe or a peristaltic pump or the like), while the beads remain in the collecting reservoir 9 owing to the magnetic element 10.

[0157] The emulsion of droplets may be further transported to another reservoir, such as another test tube. The beads 3 and droplets 4, thus collected separately, may be then subjected to different downstream analyses.

Separating different analytes of a biological sample

[0158] The method of extracting a bead from a droplet may be advantageously applied to perform a biological multiomics analysis, for example a single-cell multiomics analysis, by allowing different analytes of a biological

sample to be separated easily.

Biological sample

[0159] The term "biological sample" means any sample obtained from a biological source. Examples thereof include whole blood, serum, plasma, saliva, urine, sputum, lymph, a cell, an organelle, an organoid, cellular assembly, an aggregate of cells, an island of cells, an embryo, a dendrimer, a tissue slice, a unicellular or multicellular organism, a virus, or any combination of these. Preferably, the biological sample is a single cell or a lysed single cell, or a fraction extracted from a single cell (such as a nucleus from a single cell).

[0160] The cells may include, as an exemplary and non-exhaustive list, eukaryotic cells, including animal cells (such as mammal cells and more specifically human cells), yeast cells, fungal cells, plant cells, protozoa, prokaryotic cells, such as bacteria. Any combination of the above may also be used. The cells may be of any cell type, including circulating tumor cells, hematopoietic cells, red blood cells, circulating endothelial cells, parasites, circulating fetal cells and the like.

[0161] A biological sample may also be obtained from a multicellular organism, which may include animals, notably but not exclusively, laboratory model animals such as nematodes, embryos, notably non-human embryos (such as fish embryos), flies, eggs, plants, fungi, genetically modified organisms (GMOs).

Introducing a biological sample

[0162] In some embodiments, the first fluid may comprise a biological sample.

[0163] In some embodiments, the method of the invention comprises a step of introducing a biological sample into the droplet.

[0164] Alternatively, the method of the invention may not comprise any step of introducing a biological sample. In this case, a feedstock of droplets containing a biological sample and a bead may be used.

[0165] The step of introducing a biological sample into the droplet may be carried out simultaneously with the step of encapsulating the bead in the droplet as described above.

[0166] Fig. 7a shows an example of the step of introducing a biological sample and a bead into a droplet.

[0167] As shown in Fig. 7a, a flow of a first fluid may be passed through a main channel 1. Biological samples (e.g., nuclei, cells and the like) 14 may be fed to the main channel 1 via a first side channel, and beads 3 are fed to the main channel 1 via a second side channel. The beads 3 may be packed in a fluid so that the bead release can be easily synchronized with the droplet generation by tuning the flow rates. The configuration in Fig. 7a is thus similar to the configuration in Fig. 2a-2c, except that an additional side channel is provided in order to supply biological samples.

[0168] The biological samples **14** and beads **3** within the first and second side channels may be contained in a fluid which can be the first fluid, or at least is miscible with the first fluid flowing through the main channel. The fluid containing the beads **3** and the fluid containing the biological sample **14** may be the same or different. In particular, both the first fluid and the fluid in the first and second side channels may be aqueous.

[0169] A second fluid immiscible with the first fluid (preferably fluorocarbon-based or oil-based) may be passed through at least one side channel **2**, e.g., two side channels on either side of the main channel.

[0170] The first fluid passed through the main channel **1** may further comprise a buffer as necessary. The buffer may be for example a lysis buffer, a PCR buffer, a buffer for avoiding sedimentation in tubes and syringes, etc., and may be selected suitably depending on the application. When the first fluid further comprises a lysis buffer, the lysis buffer may be introduced through a channel different from the channel for introducing biological samples **14** to avoid the lysis of the biological samples before encapsulation.

[0171] **Fig. 7a** illustrates two side channels for the second fluid, but the channel geometry is not limited to this geometry, and may be a T-junction or a co-flow geometry.

[0172] The biological samples may be encapsulated in the droplets, following for example a Poisson distribution, by adjusting the flow rate of biological samples relative to the flow rate of the first fluid in the main channel and the concentration (or dilution) of the biological samples within their fluid.

[0173] Preferably, these conditions are selected so that most of the formed droplets (such as at least 80%, or at least 90% or at least 95%) do not contain a biological sample. For example, approximately 10% of the formed droplets contain a biological sample. Preferably, the proportion of formed droplets containing more than one biological sample is less than 5%, preferably less than 1%, more preferably less than 0.5% or less than 0.1%.

[0174] On the other hand, as mentioned above, substantially all droplets may contain a bead.

[0175] **Fig. 7a** illustrates feeding separately the biological samples **14** and packed beads **3**, but alternatively the biological samples and beads may be supplied together (for example via a single side channel).

Binding assemblies for first and second analytes

[0176] In the method of the invention, a first binding assembly and a second binding assembly may be tethered to the bead, and the droplet may contain at least a first analyte which binds to the first binding assembly and a second analyte (different from the first analyte) which binds to the second binding assembly. More than two binding assemblies may be present and bind to respective analytes.

[0177] The first analyte and second analyte may be contained in the biological sample added to the droplet.

[0178] The term "*analyte*" as used herein (also referred to as "*cellular modality*") refers to a variety of biological and chemical molecules including, but not limited to, nucleic acids, polypeptides, amino acids, polysaccharides and lipids. Specific examples thereof include DNA such as genomic and mitochondrial DNA, RNA such as mRNA and microRNA, modified or artificial nucleic acids such as block nucleic acids, peptide nucleic acids, thiolated nucleic acids, epigenetic information such as chromatin and DNA methylation, cell surface, intracellular, or extracellular proteins, lipid messengers involved in cell signaling, steroid hormones, sphingolipids, prostaglandins, phosphatidylserine lipids, oxysterol and cholesterol derivatives.

[0179] The term "*binding assembly*" as used herein refers to a supramolecular assembly, which is attached to the bead and capable of binding to an analyte. The term "*supramolecular assembly*" used herein refers to a structure comprising several bound molecules.

[0180] The first binding assembly may comprise a first bead-binding portion and a first probe portion, and the second binding assembly may comprise a second bead-binding portion, a second probe portion and a cleavable portion between the second bead-binding portion and the second probe portion.

[0181] Below, the term "probe" may be used as an assembly comprising a bead, a first binding assembly and a second binding assembly.

[0182] **Fig. 9** schematically shows an example of the probe **15**. The first binding assembly **17** and the second binding assembly **18** may be attached to the bead **16** via the first bead-binding portion **17a** and the second bead-binding portion **18a**, respectively.

[0183] In some embodiments, the first binding assembly **17** and the second binding assembly **18** may be a single-stranded or double-stranded polynucleotide sequence. Alternatively, the first binding assembly **17** and the second binding assembly **18** may be partially single-stranded and partially double-stranded polynucleotide sequence.

[0184] The term "*bead-binding portion*" refers to a portion which is attached to the bead. The bead-binding portion may be a single-stranded or double-stranded polynucleotide sequence.

[0185] The term "*polynucleotide*" as used herein refers to a nucleic acid sequence. The nucleic acid sequence may be a DNA or a RNA sequence, preferably the nucleic acid sequence is a DNA sequence. This term also encompasses what is sometimes referred to as oligonucleotides. The polynucleotide sequences used in the present invention may be designed and purchased commercially from any DNA synthesis facilities/companies, or synthesized by standard techniques.

[0186] The bead-binding portion may be a single-stranded sequence having a length of 5 to 100 nucleotides (nt), 5 to 90 nt, 5 to 80 nt, 5 to 70 nt, 5 to 60 nt, 5 to 50 nt, 5 to 40 nt, 5 to 30 nt, 5 to 20 nt, 10 to 100 nt, 10 to 90 nt, 10 to 80 nt, 10 to 70 nt, 10 to 60 nt, 10 to 50 nt,

10 to 40 nt, 10 to 30 nt or 10 to 20 nt, for example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 nt. Alternatively, the bead-binding portion may be a double-stranded sequence having a length of 5 to 100 base pairs (bp), 5 to 90 bp, 5 to 80 bp, 5 to 70 bp, 5 to 60 bp, 5 to 50 bp, 5 to 40 bp, 5 to 30 bp, 5 to 20 bp, 10 to 100 bp, 10 to 90 bp, 10 to 80 bp, 10 to 70 bp, 10 to 60 bp, 10 to 50 bp, 10 to 40 bp, 10 to 30 bp or 10 to 20 bp, for example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 bp.

[0187] Preferably, the bead-binding portion is a double-stranded sequence.

[0188] The bead-binding portion may vary depending on the composition of the bead; for example, in the case of polyacrylamide beads, the bead-binding portion may be an Acrydite-modified nucleotide sequence or a nucleotide sequence having an acrylic phosphoramidite moiety.

[0189] In an alternative case, the beads may comprise streptavidin on their surface, and the binding portion may be a biotin-modified nucleotide sequence.

[0190] More generally, the bead-binding portion comprises a chemical moiety which adapted to specifically, covalently or non-covalently, binding to a corresponding chemical moiety on the bead surface.

[0191] The bead-binding portion may be the same or different between the first and second binding assemblies. The bead-binding portion of the first binding assembly and the bead-binding portion of second binding assembly are preferably the same.

[0192] Referring again to **Fig. 9**, the first binding assembly **17** and the second binding assembly **18** may comprise a first probe portion **17b** and a second probe portion **18b**, respectively.

[0193] The term "*probe portion*" refers to a portion which may bind to an analyte in a specific manner. In the present invention, the first probe portion is a portion capable of (specifically) binding to a first analyte and the second probe portion is a portion capable of (specifically) binding to a second analyte.

[0194] For example, the binding between the probe portion and the analyte may occur through the hybridization of the probe portion with the analyte, or through ligation (either by blunt ligation or "sticky end" ligation).

[0195] The term "*hybridization*" refers to the process in which two single-stranded polynucleotide sequences bind via hydrogen bonding between the bases of the nucleotide residues (i.e., base pairing) to form a stable double-stranded complex. The hydrogen bonding may occur by Watson Crick base pairing, Hoogsteen binding, or in any other sequence specific manner. The complex may also comprise three or more strands forming a multi stranded complex.

[0196] The term "*ligation*" as used herein refers to the covalent binding or joining of two polynucleotides to produce a single larger polynucleotide. Ligation can include

chemical as well as enzymatic ligation. In general, the ligation methods discussed herein utilize enzymatic ligation by a ligase. The ligation may be bluntend ligation or sticky-end ligation.

[0197] The probe portions **17b**, **18b** may be located at a distal position with respect to the bead **16**. More specifically, each of the probe portions **17b** and **18b** may be located at the distal end of the binding assembly **17** and **18**, respectively.

[0198] The term "*distal*" as used herein refers to a relative position in a binding assembly, the position being farther from the bead. The term "*proximal*" as used herein refers to a relative position in a binding assembly, the position being closer to the bead.

[0199] When the binding occurs through the hybridization, the probe portion may be a single-stranded or double-stranded polynucleotide sequence having a sufficient length to allow for the hybridization to the analyte.

[0200] The probe portion may be 5'-phosphorylated on the strand(s).

[0201] The probe portion may be a single-stranded sequence having a length of 10 to 100 nt, 10 to 90 nt, 10 to 80 nt, 10 to 70 nt, 10 to 60 nt, 10 to 50 nt, 10 to 40 nt, 10 to 30 nt or 10 to 20 nt, for example, a length of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, or 100 nt. Alternatively, the probe portion may be a double-stranded sequence having a length of 10 to 100 bp, 10 to 90 bp, 10 to 80 bp, 10 to 70 bp, 10 to 60 bp, 10 to 50 bp, 10 to 40 bp, 10 to 30 bp or 10 to 20 bp, for example, a length of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, or 100 bp.

[0202] When the probe portion is double-stranded, the portion may be subjected to a suitable denaturation treatment (i.e., separation of a double-stranded sequence into single, complementary strands by heating or a reagent such as NaOH) prior to the hybridization to the analyte, allowing the resulting single-stranded sequence to bind to the target analyte.

[0203] By way of example, the first analyte may be mRNA and the second analyte may be chromatin - or conversely. In other examples, the first analyte may be mRNA and the second analyte may be membrane proteins - or conversely.

[0204] The probe portions may be designed to have a complementary sequence of a part of the analyte of interest.

[0205] The term "*complementary*" as used herein refers to the hybridization or base pairing between nucleotides or nucleic acids, such as, for instance, between the two strands of a DNA molecule or between an polynucleotide primer and a primer-binding site on a single-stranded nucleic acid to be sequenced or amplified. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single-stranded RNA or DNA molecules are said to be complementary when the nucleotides of one strand pair with at least about 80% of the

nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementarity over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary.

[0206] When the analyte is mRNA, the probe portion may comprise a poly(T) tail (a stretch of thymine nucleotides) which has a sufficient length to allow poly(A)-tailed RNAs to be captured by hybridization.

[0207] The term "*poly(A) tail*" means a chain of adenine nucleotides, and can refer to a poly (A) tail that is to be added to an RNA transcript at the end of transcription, or can refer to the poly (A) tail that already exists at the 3' end of an RNA transcript. A poly (A) tail is typically 5 to 300 nucleotides in length.

[0208] When the analyte is DNA, the probe portion may comprise polynucleotides which are complementary to a specific target sequence, coding or non-coding, contained in the genome. For example, the probe portion may comprise a polynucleotide sequence complementary to repetitive sequences.

[0209] When the analyte is a protein, the protein may be labeled beforehand with a barcoded antibody (an antibody comprising an antibody barcode and a polynucleotide capture sequence, e.g., poly(A) tail), and the probe portion may comprise a sequence complementary to the capture sequence, e.g., poly(T) tail.

[0210] In some embodiments, the analyte may be subjected to a pretreatment (e.g., "*tagmentation*" of chromatin, which will be further described later) to add sequencing adapters. In this case, the probe portion may be designed to contain the same sequencing adapters to hybridize with the analyte.

[0211] The term "*sequencing adapter*" as used herein refers to a molecule (e.g., polynucleotide sequence) which is adapted to allow a sequencing instrument to sequence a target polynucleotide.

[0212] Referring back to **Fig. 9**, the second binding assembly may further comprise a cleavable portion **18c** between the second binding portion and the second probe portion. **Fig. 9** illustrates for convenience a photocleavable portion which is already cleaved, but the photocleavable portion **18c** is not cleaved prior to a suitable cleavage treatment.

[0213] The term "*cleavable portion*" as used herein refers to a portion which can be cleaved under certain conditions, by a specific mechanism. The cleavable portion may be electromagnetically (e.g. by UV light of a specific wavelength), enzymatically, chemically, or thermally cleavable. The conditions applied to cleave the cleavable portions are such that the rest of the binding assembly is not damaged or cleaved.

[0214] Examples of the cleavable portion include a photocleavable spacer (for example, available from In-

tegrated DNA Technologies among other suppliers), a thermally-cleavable linker, a linker containing a disulfide bond which is broken by reduction, a linker containing an azo group which is broken by reduction, and a linker containing a uracil residue which can be excised by Uracil Glycosylase or USER® enzyme (NEB).

[0215] The cleavable portion may be a polynucleotide sequence, single-stranded or double-stranded, which comprises a cleavable molecular moiety.

[0216] As used herein, the term "*cleavable molecular moiety*" refers to any chemical bond that can be cleaved by a cleavage mechanism as explained above. Suitable cleavable chemical bonds are well known in the art and include, but are not limited to, acid labile bonds, protease/peptidase labile bonds, photolabile bonds, disulfide bonds, and esterase labile bonds.

[0217] The cleavable portion may be a single-stranded sequence having a length of 5 to 100 nt, 5 to 90 nt, 5 to 80 nt, 5 to 70 nt, 5 to 60 nt, 5 to 50 nt, 5 to 40 nt, 5 to 30 nt, 5 to 20 nt, 10 to 100 nt, 10 to 90 nt, 10 to 80 nt, 10 to 70 nt, 10 to 60 nt, 10 to 50 nt, 10 to 40 nt, 10 to 30 nt or 10 to 20 nt, for example, a length of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 nt.

Alternatively, the cleavable portion may be a double-stranded sequence having a length of 5 to 100 bp, 5 to 90 bp, 5 to 80 bp, 5 to 70 bp, 5 to 60 bp, 5 to 50 bp, 5 to 40 bp, 5 to 30 bp, 5 to 20 bp, 10 to 100 bp, 10 to 90 bp, 10 to 80 bp, 10 to 70 bp, 10 to 60 bp, 10 to 50 bp, 10 to 40 bp, 10 to 30 bp or 10 to 20 bp, for example, a length of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 bp.

[0218] The cleavable portion is preferably a double-stranded sequence.

[0219] The cleavable portion may be 5'-phosphorylated on the strand(s).

[0220] The cleavable portion may also comprise an additional moiety, such as 3SpC3 (three-carbon group), which prevents the backward extension during amplification (e.g., PCR).

[0221] In some embodiments, the first binding assembly of the probe of the present invention does not comprise a cleavable portion which is cleavable by the same mechanism as the cleavable portion of the second binding assembly.

[0222] In some embodiments, the first binding assembly comprises a cleavable portion different from the cleavable portion of the second binding assembly, the cleavable portion of the first binding assembly is cleavable by a different mechanism from the cleavable portion of the second binding assembly. For example, the first binding assembly may comprise a cleavable portion which is chemically cleavable while the second binding assembly may comprise a cleavable portion which is electromagnetically, e.g. UV-cleavable.

[0223] In other embodiments, the first binding assembly does not comprise any cleavable portion as defined

above. In this case, preferably, the first bead-binding portion **17a** is directly connected to the first probe portion **17b** or at least one barcode portion **17d**, which is described below.

[0224] Referring back to **Fig. 9**, the first and second binding assemblies may further comprise at least one barcode portion **17d**, **18d**. **Fig. 9** illustrates three barcode portions **17d**, **18d** in each binding assembly, but the binding assembly may comprise one barcode portion, two barcode portions, four barcode portions, and so on.

[0225] The term "*barcode portion*" generally refers to a polynucleotide sequence that can be used as an identifier for an associated analyte, or as an identifier of the source of an associated analyte, such as a cell-of-origin (cell barcode).

[0226] The barcode portion(s) of the first binding assembly is/are preferably located between the first bead-binding portion and the first probe portion. The barcode portion(s) of the second binding assembly is/are preferably located between the cleavable portion and the second probe portion.

[0227] The barcode portion(s) are preferably the same between the first binding assembly and the second binding assembly.

[0228] The barcode portion may be a single-stranded or double-stranded polynucleotide sequence. The barcode portion may be a single-stranded having a length of 5 to 100 nt, 5 to 90 nt, 5 to 80 nt, 5 to 70 nt, 5 to 60 nt, 5 to 50 nt, 5 to 40 nt, 5 to 30 nt, 5 to 20 nt, 10 to 100 nt, 10 to 90 nt, 10 to 80 nt, 10 to 70 nt, 10 to 60 nt, 10 to 50 nt, 10 to 40 nt, 10 to 30 nt or 10 to 20 nt, for example, a length of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 nt. Alternatively, the barcode portion may be a double-stranded sequence having a length of 5 to 100 bp, 5 to 90 bp, 5 to 80 bp, 5 to 70 bp, 5 to 60 bp, 5 to 50 bp, 5 to 40 bp, 5 to 30 bp, 5 to 20 bp, 10 to 100 bp, 10 to 90 bp, 10 to 80 bp, 10 to 70 bp, 10 to 60 bp, 10 to 50 bp, 10 to 40 bp, 10 to 30 bp or 10 to 20 bp, for example, a length of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 bp.

[0229] Each barcode portion is preferably a double-stranded polynucleotide sequence.

[0230] Each barcode portion may be 5'-phosphorylated on the strand(s).

[0231] In some embodiments, the first binding assembly and/or the second binding assembly further comprises at least one additional portion selected from a primer-binding site capable of hybridizing a primer, a sequencing adapter, a spacer, a tag, a sample barcode, a unique molecular identifier (UMI), or any combination thereof.

[0232] Examples of the primer include a polymerase chain reaction (PCR) primer, reverse transcription (RT)-PCR primer, library preparation primer, or a sequencing primer.

[0233] Example of the sequencing adapter include a library preparation primer (such as Illumina PE1, PE2 or

PE2-N6), library reading sequences (Read1 (R1), Read2 (R2), Read1N (R1N), Read2N (R2N)) a flow cell binding site (such as Illumina P5 and P7), or an index sequence. The flow cell binding site, as used herein, refers to an polynucleotide sequence which binds to a complementary sequence immobilized at the surface of a flow cell which is a part of a sequencing instrument.

[0234] The term "*tag*" as used herein refers to a moiety or part of a molecule that enables or enhances the ability to detect and/or identify, either directly or indirectly, a molecule or molecular complex (e.g., binding assembly).

[0235] The term "*sample barcode*" (also referred to as "*sample identifier*") is a known polynucleotide sequence that can be used to identify a sample.

[0236] The term "*unique molecular identifier*" or "*UMI*" as used herein refers to a sequencing linker or a subtype of nucleic acid barcode for detecting and quantifying unique amplified products. In theory, no two original fragments should have the same UMI sequence. As such, UMIs can be used to determine if two similar sequence reads are derived from different original fragments or if they are simply duplicates created during amplification (such as by PCR).

[0237] The at least one additional portion may be located at the distal end of the bead-binding portion, or of the cleavable portion if present. Specifically, when the barcode portion(s) is/are present, the additional portion(s) may be located between the bead-binding portion and the barcode portion(s), or between the cleavable portion and the barcode portion(s). Alternatively, the additional portion(s) may be located between the barcode portion(s) and the probe portion.

[0238] In the probe of the present invention, the first binding assembly may further comprise connectors for the first binding assembly, and the second binding assembly may further comprise connectors for the second binding assembly. The connectors for the first binding assembly are preferably different from the connectors for the second binding assembly.

[0239] The term "*connector*" as used herein refers to a molecule or segment of a molecule (e.g., polynucleotide sequence) which is capable of binding to another corresponding (e.g., complementary) connector. In the probe of the invention, at least one connector may be present at one or both ends of each portion. One connector at an end of a portion can bind to a corresponding connector at an end of another portion, thereby allowing the connection of the two portions via the connectors.

[0240] The connectors for the first binding assembly may be the same with each other or different from each other. The connectors for the first binding assembly are preferably different from each other.

[0241] The connectors for the second binding assembly may be the same with each other or different from each other. The connectors for the second binding assembly are preferably different from each other.

[0242] In cases where two or more barcode portion(s) are present, the barcode portions may be attached to

each other via the connectors. In other words, the barcode portions of the first binding assembly may be attached via the connectors for the first binding assembly while the barcode portions of the second binding assembly may be attached via the connectors for the second binding assembly.

[0243] The other portions as explained above may be also attached to each other via connectors. In other words, the portions of the first binding assembly may be attached via the connectors for the first binding assembly while the portions of the second binding assembly may be attached via the connectors for the second binding assembly.

[0244] For example, the barcode portion(s) of the first binding assembly may be attached to each other, to the first bead-binding portion, and/or to the first probe portion via the connectors for the first binding assembly, and the barcode portion(s) of the second binding assembly may be attached to each other, to the cleavable portion, and/or to the second probe portion via the connectors for the second binding assembly.

[0245] The connectors for the first and second binding assemblies may be a single-stranded polynucleotide sequence having a length of 2 to 50 nt, 2 to 40 nt, 2 to 30 nt, 3 to 20 nt, 4 to 10 nt, for example, a length of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50 nt.

[0246] The connectors are preferably single-stranded polynucleotide sequences located at an end of a portion (e.g., 3' overhang or 5' overhang). A single-stranded connector at one end of a portion may bind to a complementary single-stranded connector at one end of another portion to form a double-stranded sequence, thereby allowing the two portions to be connected to each other via the connectors.

[0247] The bead-binding portions may be attached to the bead after the bead formation or during the bead formation (the binding portions may be pre-mixed in the gel precursor for beads, or the gel precursor and the binding portions may be co-injected to the microfluidic chip).

[0248] The first binding assembly may be assembled by successive ligation of the above-described portions for the first binding assembly.

[0249] The second binding assembly may be assembled by successive ligation of the above-described portions for the second binding assembly.

[0250] Alternatively, the first binding assembly and the second binding assembly may be assembled by hybridization and primer extension.

[0251] The term "*primer extension*" as used herein refers to the extension (polymerization) of a nucleic acid sequence from a free 3'-hydroxy group, thereby creating a strand of nucleic acid complementary to an opposing strand.

[0252] As explained above, the first and second binding assemblies may be single-stranded or double-stranded. However, this does not mean that binding assemblies have to be one or another. For example, the binding as-

semblies may be partially single-stranded and partially double-stranded (e.g., the probe portion is single-stranded and the rest is double-stranded). They may be also subjected to denaturation or hybridization treatment depending on the application. For example, if binding assemblies are double-stranded at the time of fabrication, the binding assemblies may be subjected to a suitable denaturation treatment prior to the hybridization to the analytes, allowing the resulting single-stranded sequence to bind to the target analyte.

[0253] Although the above description relates to a probe comprising a first binding assembly and a second binding assembly, it is also possible to have more than two binding assemblies. Each additional (third, fourth, etc.) binding assembly may be similar to the first binding assembly or to the second binding assembly described above. Each binding assembly comprises a probe portion which binds to a different analyte. For example, in one variation, the probe comprises a first binding assembly which does not comprise any cleavable portion, a second binding assembly which comprises a cleavable portion which may be cleaved by a first mechanism, and a third binding assembly which comprises a cleavable portion which may be cleaved by a second mechanism different from the first mechanism. More generally, it is preferred that at most one binding assembly is devoid of a cleavable portion, and that all binding assemblies which comprise a cleavable portion have different cleavable portions, which are cleaved by different mechanisms.

Separating the first analyte and the second analyte

[0254] Referring again to **Fig. 7a**, when a droplet additionally contains a biological sample, at least the first analyte may bind to the first binding assembly and the second analyte may bind to the second binding assembly. The first analyte and the second analytes may be initially dispersed in the droplet for example due to a lysis buffer which may be comprised in or may constitute the first fluid.

[0255] The method of the invention may further comprise, before extracting the bead from the droplet, a step of releasing the second binding assembly from the bead in the droplet, while the first analyte bound to the first binding assembly remains tethered to the bead.

[0256] Preferably, the first analyte binds to the first binding assembly before the release of the second binding assembly from the probe. Preferably, the second analyte binds to the second binding assembly before the release of the second binding assembly from the probe; in this case, during the release step, the second analyte is released from the probe together with the second binding assembly. In other variations, the second analyte may bind to the second binding assembly after it has been released from the probe.

[0257] The second binding assembly may be released from the bead via the cleavage of the cleavable portion in a treatment step. This treatment step may comprise

subjecting the droplets to specific conditions conducive to cleavage, such as exposure to a certain temperature, exposure to electromagnetic radiation (such as UV light), addition of an enzyme and/or of a reactant into the droplets.

[0258] For convenience, the separation of two analytes is explained below, but the separation of two or more analytes is also possible. Specifically, two or more binding assemblies can be tethered to the bead, as described above, allowing for the separation of two or more analytes. Use may be made in particular of different cleavable portions in different binding assemblies, so that that release of binding assemblies for different analytes from the bead may be performed in different treatment steps.

[0259] After the second binding assembly has been released, the beads are extracted from the droplets, as described above. Thus, the first analyte may be recovered with the beads, while the second analyte may be recovered with the droplets.

[0260] Fig. 7b illustrates that the free second binding assembly (not bound with the second analyte) is released and dispersed in the droplet. In this case, the free second binding assembly may then bind to the second analyte. Alternatively, the second binding assembly may capture (bind to) the second analyte before the release in the droplet, and then the second analyte bound to the second binding assembly may be released and dispersed in the droplet.

Biological application for a multiomics analysis

[0261] The method of the present invention can be used for performing multiomics analysis (e.g., transcriptomic, genomic, epigenetic, proteomic; metabolomic, and/or lipidomic analyses). Particularly, the method of the present invention can be used for performing a single-cell multiomics analysis. As an example, the use of the probe for studying chromatin and mRNA will be explained below.

[0262] Fig. 10 schematically illustrates the probe used in this example..

[0263] The probe 15 contains a bead 16, a first binding assembly 17 and a second binding assembly 18. The bead may contain a magnetic material such as magnetic fluid, a magnetic nanoparticle or a magnetic core.

[0264] The first binding assembly 17 may contain, from proximal to distal, a first bead-binding portion BB1, barcode portions BC1 to BC3, additional portions R2 (primer-binding site) and UMI (unique molecular identifier), and a first probe portion polyT. The first binding assembly 17 may be double stranded, except for the single-stranded UMI and polyT.

[0265] The second binding assembly 18 may contain, from proximal to distal, a second bead-binding portion BB2, a cleavable portion PC, an additional portion P5 (illumina flow cell adapter), barcode portions BC1 to BC3, and a second probe portion R1N. The cleavable portion may be photocleavable. The second binding assembly

18 may be double stranded.

[0266] In this example, the first probe portion polyT (poly(T) tail) may capture poly (A)-tailed mRNA, and the second probe portion R1N captures DNA tagged with R1N.

[0267] Fig. 10 illustrates for convenience a photocleavable portion which is already cleaved, but the photocleavable portion is not cleaved prior to a suitable cleavage treatment.

[0268] Connectors are not shown in Fig. 5, but all the portions of the first binding assembly 17 and the second binding assembly 18 may be attached to each other via connectors for the first binding assembly and via connectors for the second binding assembly, respectively.

[0269] For chromatin profiling, nuclei may be isolated from cells by a conventional method, and subjected to tagmentation by CUT&Tag, as described in, for example, Kaya-Okur et al. Nat. Comm. 10, 1930 (2019).

[0270] The tagmentation is a process in which unfragmented DNA is cleaved and tagged for analysis. CUT&Tag is a cleavage and tagmentation method in which a complex of protein A and a Tn5 transposase which is conjugated to sequencing adapters, performs antibody-targeted cleavage of chromatin and simultaneous addition of the adapters.

[0271] In this example, when the isolated nuclei are subjected to CUT&Tag, DNA sequences corresponding to the binding sites of the target protein or histone modification of interest may be modified at 5' and 3' ends with sequencing adapters, such as R1N and R2N. In this case, as shown in Fig. 10, the probe portion of the second binding assembly may comprise the sequencing adapter (for example, R1N).

[0272] Below, the term "bead" may be used as the bead to which the first binding assembly and second binding assembly are attached. In other words, the "bead" may be used interchangeably with the "probe."

[0273] By reference to Fig. 7a, the beads 3 in a fluid, preferably an aqueous fluid, may be fed to the main channel 1 of a microfluidic chip through an upstream side channel. In this example, the beads 3 may be packed in the fluid so that the bead release can be easily synchronized with the droplet generation by tuning the flow rates.

[0274] The tagmented nuclei 14 in a nuclei buffer may be fed to the main channel 1 via another upstream side channel. The nuclei buffer may contain a primer for downstream analysis, such as Reverse i7 primer (5'-P7-i7-R2N-3'; P7 is an illumina flow cell adapter, i7 is a sample barcode, and R2N is an sequencing primer site). A lysis/PCR buffer may be passed through the main channel 1. The fluid containing the beads (probes) 3, the nuclei buffer, and the lysis/PCR buffer may be the same or miscible with each other. A fluid which is immiscible with the fluid(s) passing through/fed to the main channel 1 (preferably fluorocarbon-based or oil-based) may be passed through at least one downstream side channel 2 to form a bead-containing droplet (which may also contain a tagmented nucleus 14).

[0275] In the droplet, mRNA may bind to the first binding assembly at the first probe portion via the hybridization between the poly(A) tail of the mRNA and the poly(T) tail (**polyT**) of the first probe portion. The tagmented nuclei (DNA) of target may bind to the second binding assembly at the second probe portion via the hybridization between the R1N of the tagmented DNA and the **R1N** of the second probe portion. This hybridization between the R1N of the tagmented DNA and the R1N of the second probe portion may occur after the cleavage treatment, such as during the downstream amplification process (e.g., PCR).

[0276] As shown in **Fig. 7b**, the droplets may be then collected in a reservoir. The second binding assembly may be released from the bead via the cleavage of the cleavable portion by a treatment step. This treatment step may comprise subjecting the droplets to specific conditions conducive to cleavage, such as exposure to a certain temperature, exposure to electromagnetic radiation (such as UV light), addition of an enzyme and/or of a reactant into the droplets. The droplet illustrated in **Fig. 7b** shows that the free second binding assembly is released in the droplet (the second binding assembly and the tagmented DNA fragments are dispersed in the droplet) while the first binding assembly remains on the bead, capturing mRNA molecules via the first probe portion. However, the tagmented DNA fragments may be captured by the second binding assembly prior to the treatment step of cleavage, and then after the treatment step, the second analyte bound to the second binding assembly may be released in the droplet.

[0277] Subsequently, the droplets may be reinjected to the microfluidic device of the invention, and beads are extracted from droplets, as explained above. Specifically, as shown in **Fig. 7c**, the droplets in a fluid are reinjected to the microfluidic device of the invention through the main channel, and a fluid which is immiscible with the fluid containing the droplets is supplied to the microfluidic device in a downstream (in this example, via two side channels), and the beads and the droplets are extracted at a constriction **5** of the microfluidic device.

[0278] As shown in **Fig. 6**, for example, the droplets **4** and extracted beads **3** may be collected (from an outlet of the main channel), as explained above, in a collecting reservoir **9**. The collecting reservoir is as described above.

[0279] The extracted beads **3** may be separately collected using a magnetic element **10** to the collecting reservoir **9** while the droplets **4** may gather at or near the surface of the immiscible fluid the as the droplets are generally less dense than the fluid.

[0280] Optionally, the emulsion of the droplets may be withdrawn from the collecting reservoir **9** via the outlet conduit **13** (for example using a syringe or a peristaltic pump or the like), while the beads remain in the collecting reservoir **9** owing to the magnetic element **10**.

[0281] The emulsion of droplets may be further transported to another reservoir, such as another test tube.

The beads **3** and droplets **4**, thus collected separately, may be subjected to different downstream analyses.

[0282] **Fig. 11a** illustrates schematically the content of an extracted droplet. In the droplet, the released second binding assembly **18** (excluding the binding portion and the cleavable portion), DNA fragment **19** tagmented with R1N and R2N at 5' and 3' ends, and a Reverse i7 primer (R2N-i7-P7) contained in the nuclei buffer may be dispersedly present. The droplets are then subjected to droplet PCR of the DNA fragments **19**, using the released partial second binding assembly **18** and Reverse i7 as primers (in other words, the second probe portion binds to the DNA fragment after the release of the binding assembly from the bead).

[0283] The PCR conditions may be optimized to increase the yield of the target sequence of DNA fragments **19**.

[0284] The resulting PCR products **20** (**Fig. 11b**) may be then purified by a conventional cleanup kit, such as Qiagen Minelute or Macherey-Nagel Nucleospin, and/or by size selection with SPRI (Solid-phase reversible immobilization) beads to remove primers. The sample may be also verified for the quality control. The sample may be sequenced by next generation sequencing or high-throughput sequencing.

[0285] The term "*sequencing*" or "*(to) sequence*" as used herein refers to a method by which the identity of at least 10 consecutive nucleotides (e.g., the identity of at least 20, at least 50, at least 100 or at least 200 or more consecutive nucleotides) of a polynucleotide sequence is obtained.

[0286] The terms "*next-generation sequencing*" or "*high-throughput sequencing*", as used herein, refer to the so-called parallelized sequencing-by-synthesis or sequencing-by-ligation platforms currently employed by Illumina, Life Technologies, and Roche, etc. Next-generation sequencing methods may also include nanopore sequencing methods such as that commercialized by Oxford Nanopore Technologies, electronic-detection based methods such as Ion Torrent technology commercialized by Life Technologies, or single-molecule fluorescence-based methods such as that commercialized by Pacific Biosciences.

[0287] The extracted beads may be used for RNA analysis, based on conventional approaches such as drop-seq, smart-seq2 and smart-seq3. Specifically, the beads may be subjected to reverse transcription and template switching.

[0288] The term "*reverse transcription (RT)*" as used herein means a method wherein a complementary DNA (cDNA) copy of an RNA molecule is synthesized. The cDNA product can be used as a template for PCR.

[0289] The term "*template switching*" as used herein refers to an activity of a polymerase that is capable of switching template strands in a homology dependent manner during DNA synthesis. An example of a polymerase with template switching activity is M-MLV reverse transcriptase.

[0290] As shown in Fig. 12a, during the reverse transcription, cDNA 22 may be synthesized by a reverse transcriptase (e.g., M-MLV type reverse transcriptase) using the captured mRNA 21 as a template. A few additional nucleotides may be added by the reverse transcriptase at the 3' end of the newly synthesized cDNA strand (for example, CCC as illustrated in Fig. 12a), which can then anneal to the matching 3'-end riboguanosines (GGG as illustrated in Fig. 12a) of a template switching oligo (TSO), which is complementary to R1. Thus, the reverse transcriptase may switch the template strands from 21 to TSO to continue the polymerization. The TSO may be contained in a reverse transcription buffer. The beads may be treated with exonuclease I (Fig. 12b).

[0291] Subsequently, the cDNA bound on the bead may be amplified, using R1 primer and R2 primer, which results in dispersed double-stranded cDNA in a solution, no longer bound on the bead (Fig. 12c). The cDNA may be then fragmented and tagged with sequencing adapters such as R1N primers on 3' end and R2N primers on 5' end (Fig. 12d). For the tagmentation of cDNA, a conventional kit such as NEBNext Ultra II may be used. As shown in Fig. 12d, at the end of the fragmentation and tagmentation, fragments of various lengths may be obtained: fragments containing R2, barcode portions (BC), UMI, cDNA and R1N, fragments containing R2N, cDNA and R1N, and fragments containing R2N, cDNA and R1.

[0292] Only the fragments containing R2, barcode portions (BC), UMI, cDNA and R1N may be further amplified by PCR using primers containing sequencing adapters (e.g., primers P7-i7-R2 and R1N-P5). Thus, the resulting PCR products may be added with sequencing adapters P7 and P5 at their both ends. The final PCR products may be purified by size selection, using SPRI beads, for example, to remove primers, and subjected to be quality control such as Qubit, TapeStation, Bioanalyzer and a conventional gel electrophoresis. The sample may be then sequenced by next generation sequencing or high-throughput sequencing, for example.

[0293] Separation of mRNA and chromatin has been discussed above, but handling two or more analytes is also possible. For example, DNA/RNA-barcoded antibodies containing a polyA tail may be used in addition. In this case, three binding assemblies, one with C&T and the photocleavable portion (for chromatin) and two with oligoT sequences (for mRNA and protein) may be tethered to each bead. This way, three analytes may be analyzed.

EXAMPLES

[0294] The following examples illustrate the invention without limiting it.

Example 1 - Polyacrylamide beads synthesis

[0295] Polyacrylamide beads were prepared by gen-

erating droplets on a conventional microfluidic chip.

[0296] Specifically, as shown in Fig. 1a, a gel precursor (58% v/v H₂O, 2.3% v/v Ferrofluid (Ferrotec® EMG700SP), 11.3% v/v TBEST buffer (10 mM Tris-HCl [pH 8.0], 137 mM NaCl, 2.7 mM KCl, 10 mM EDTA and 0.1% (v/v) Triton X-100), 28.4% 4X AB solution (36% v/v Acrylamide/Bis solution 40% (w/w) molar ratio 19:1, 25.8% v/v Acrylamide solution 40% (w/w), 38.2% v/v H₂O)) was injected through a main channel 1 at 320 µL/h, 2.5% w/v APS (ammonium persulfate) as a cross-linking initiator was injected through a side channel at 34.5 µL/h, and oil (HFE-7500 with 1.5% Fluosurf (Emulseo) and 0.4% v/v TEMED) as a continuous phase was injected through two side channels 2 at 550 µL/h.

[0297] The median diameter Dv50 of the obtained droplets was approximately 40 µm, and the droplet generation throughput was between 3000 and 4000 droplets/s.

[0298] The resulting droplets were kept at 60°C overnight for cross-linking to form beads. The emulsion of beads in the oil was broken with perfluoro-octan-1-ol and the remaining oil was dissolved in hexane. The beads were suspended in TBEST to let them swell in TBEST, thereby having a final median diameter Dv50 of 50 µm. The beads were passed through a cell strainer to remove any dust and large beads.

Example 2 - Encapsulation of beads and biological samples in droplets

[0299] The beads from Example 1 were packed by centrifugation at 4000 g for 1 min, and the supernatant was removed.

[0300] In a microfluidic chip (similar to the one used in Klein et al. Cell. 2015) equipped with syringe pumps, a buffer containing 0.1% Tween® 20 surfactant was injected at 200 µL/h and co-flowed with a diluted suspension of cell nuclei in phosphate buffer saline and 15% Optiprep™ medium at 200 µL/h and the packed beads at 35 µL/h through a main channel. Oil (HFE-7500 supplied by 3M™ supplemented by 1.5% of stabilizer Fluosurf® (Emulseo) or 0.75% of surfactant supplied by Ran Bio-tech) was injected at 300 µL/h through two side channels to form bead-containing droplets.

[0301] The beads were released at the same frequency as the droplet generation so that the bead loading (proportion of bead-containing droplets) was approximately 90%.

[0302] The generated bead-containing droplets were collected in a reservoir made of an 1.5-mL Eppendorf tube with a PDMS plug and inlet and outlet conduits inserted in the tube through the plug as shown in Fig. 6. The droplets were collected in the reservoir through the inlet conduit and remained packed near the surface as they were lighter in density than the oil.

[0303] The packed bead-containing droplets (the volume of the droplets ranging between 0.5 nL to 1 nL, the bead diameter of 50 µm) were taken out of the reservoir

using an oil-filled syringe connected to the outlet and mounted on a syringe pump, then reinjected to a microfluidic chip for bead extraction by pushing the droplets backward.

Example 3 - Bead extraction using a microfluidic chip

[0304] A microfluidic chip was fabricated by a standard photolithography process. Specifically, a SU8-2050 photoresist mold was spun on a 4-inch (approximately 100 mm) silicon wafer having a thickness of approximately 60 μm , then exposed through a chromium mask and developed according to the manufacturer's instructions. The mold was then silanized with fluorinated silane, and PDMS (curing agent at a ratio 1:10) was poured and baked 2h at 70°C. This process was repeated twice to prepare a chip having two different thicknesses.

[0305] As shown in **Fig. 3a** to **Fig. 3c** and **Fig. 4**, the microfluidic chip had a main channel **1** having an inlet (width: 80 μm , thickness: 60-70) for reinjecting the bead-containing droplet phase and an outlet (width: 110 μm , thickness: 45-50 μm) for recovering beads and droplets, and a downstream channel comprising two side channels **2** (width: 80 μm , thickness: 45-50 μm) for introducing oil to break the droplets. The main channel also comprised a constriction **5** with a gradually reduced width, the minimum width being 20 μm wide and the thickness being 45-50 μm .

[0306] As shown in **Fig. 3a**, the bead-containing droplets in the oil were passed through the main channel **1** from the inlet at a flow rate of 150 $\mu\text{L/h}$. The droplets were then passed through the constriction **5**, and oil (same composition as in Example 2) was supplied through the two side channels **2** downstream of the constriction **5** at a flow rate of 600 $\mu\text{L/h}$.

[0307] The main channel **1** had a non-narrowed portion extending from the inlet of the main channel to a transition area, and a narrowed portion extending from the transition area to the constriction **5**, i.e., the thickness of the main channel was equal to or slightly smaller than the bead diameter (approximately 50 μm) after the transition area, so that the beads were slightly compressed in the thickness direction, and the friction force kept the beads at the rear of the droplets. The beads were then extracted from the droplets upon passing through the constriction **5**.

[0308] The droplets and the extracted beads were collected in a collecting reservoir equipped with a plug and inlet and outlet conduits inserted in the reservoir through the plug, as shown in **Fig. 6**. The extracted beads were separately collected using a magnet while the droplets were packed near the surface as they were lighter than the oil and were withdrawn from the test tube via the outlet conduit.

Example 4 - Characterization of flow rates

[0309] The flow rates of droplets and oil were characterized. As shown in **Fig. 5**, various flow rates of droplets

ranging from 50 to 450 $\mu\text{L/h}$ and flow rates of oil ranging from 50 to 2000 $\mu\text{L/h}$ were tested and the extraction success rate was reported. In **Fig. 5**, Qdrops represents the flow rate of the droplets and Qoil represents the flow rate of the oil (second fluid) supplied via the side channels.

[0310] The beads were prepared as described in Example 1, except that the median diameter Dv50 of the beads was 47 μm and the compositions were changed as follows: droplets composed of 100mM Tris HCl pH8 and 0.1% v/v Tween® 20, with a volume of approximately 0.8 nL; oil composed of HFE-7500 by 3M®+ 1.5% Fluor-surf®.

[0311] Using a fast camera, footage of extracting the beads from the droplets was recorded. In each video (40 to 200 droplets), the ratio of the number of extracted beads to the total number of droplets was determined as a percentage.

[0312] There was a sharp transition in the extraction rate from 0% to 100%, showing that the process is highly stable, and there is almost no region of instability (i.e. where approximately half of the beads were extracted). When the flow rates were properly adjusted, a 100% extraction rate could be kept on extended periods of time.

[0313] It is also noted that Qdrops varied between 50 to 450 $\mu\text{L/h}$, showing a very large operating range of the flow rate. The throughput was high (from approximately 20 droplets/s at 50 $\mu\text{L/h}$ to approximately 150 droplets/s at 450 $\mu\text{L/h}$).

[0314] If the droplet size, bead size, constriction size, surfactant concentration, or stabilizer concentration are changed, slightly different results can be expected as the surface tension and shear force will be modified; however, the trend is expected to remain the same.

Claims

1. A method of extracting a bead from a droplet, comprising the steps of:
 - providing a droplet of a first fluid within a second fluid, the first fluid being immiscible with the second fluid, the droplet containing a bead;
 - passing the droplet through a constriction in a main channel, and supplying a third fluid immiscible with the first fluid in a downstream channel, downstream of the constriction, so as to extract the bead from the droplet.
2. The method of claim 1, comprising a step of positioning the bead at the rear of the droplet before reaching the constriction, preferably by passing the droplet through a narrowed portion of the main channel upstream of the constriction, the narrowed portion having a transverse dimension which is equal to or smaller than the diameter of the bead in a non-constricted state.

3. The method of claim 1 or 2, wherein the constriction has at least one transverse dimension which is equal to or less than the diameter of the bead in a non-constricted state.
4. The method of any one of claims 1 to 3, further comprising a step of separately collecting the bead from the droplet downstream of the constriction.
5. The method of claim 4, wherein the bead comprises a magnetic material, and the step of separately collecting is performed using a magnetic field.
6. The method of any one of claims 1 to 5, comprising a step of encapsulating the bead in the droplet prior to passing the droplet through the constriction.
7. The method of any one of claims 1 to 6, wherein a first binding assembly and a second binding assembly are tethered to the bead, and the droplet contains at least a first analyte which binds to the first binding assembly and a second analyte which binds to the second binding assembly.
8. The method of claim 7, further comprising, before extracting the bead from the droplet, a step of releasing the second binding assembly from the bead in the droplet, while the first analyte bound to the first binding assembly remains tethered to the bead.
9. The method of any one of claims 1 to 8, wherein the ratio of the flow rate of the third fluid to the flow rate of second fluid flowing through the constriction in the main channel ranges from 2 to 10, preferably from 3 to 6, more preferably from 3.5 to 5.
10. The method of any one of claims 1 to 9, wherein the first fluid is aqueous and the second fluid and third fluid are non-aqueous, such as fluorocarbon-based or oil-based; and/or wherein the third fluid is the same as the second fluid.
11. The method of any one of claims 1 to 10, comprising a step of introducing a biological sample into the droplet prior to passing the droplet through the constriction, wherein the biological sample is preferably a single cell.
12. A microfluidic device for bead extraction, comprising:
 - a main channel for passing a droplet of a first fluid within a second fluid, the first fluid being immiscible with the second fluid, the droplet comprising a bead, wherein the main channel includes a constriction; and
 - a downstream channel for flowing a third fluid immiscible with the first fluid fluidically connected to the main channel downstream of the con-
- striction;
 - wherein the device is configured for extracting the bead from the droplet at the constriction.
13. The microfluidic device of claim 12, wherein the main channel comprises a narrowed portion upstream of the constriction, the narrowed portion configured for positioning the bead at the rear of the droplet before reaching the constriction and having a transverse dimension which is equal to or smaller than the diameter of the bead in a non-constricted state.
14. The microfluidic device of claim 12 or 13, wherein the constriction has at least one transverse dimension which is equal to or less than the diameter of the bead in a non-constricted state.
15. An assembly for bead extraction, comprising:
 - the microfluidic device of any one of claims 12 to 14; and
 - a collecting reservoir equipped with a magnetic element.

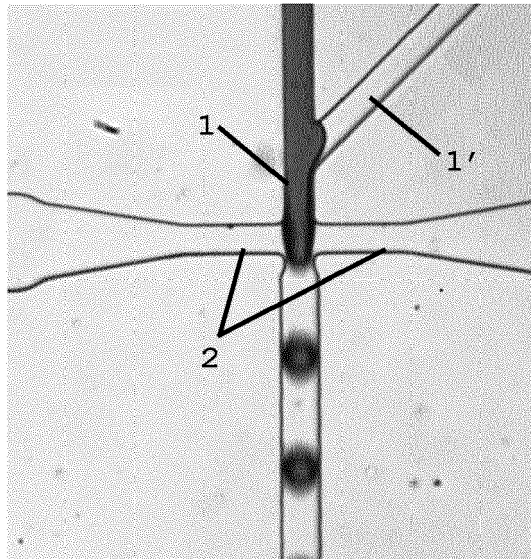


Fig. 1a

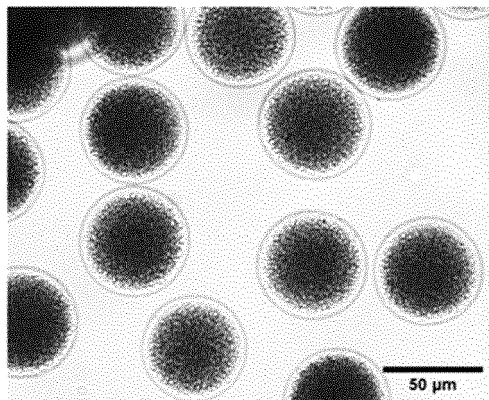


Fig. 1b

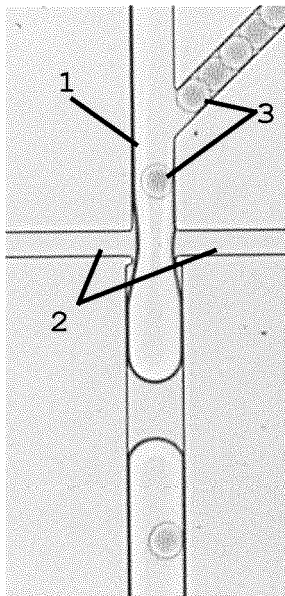


Fig. 2a

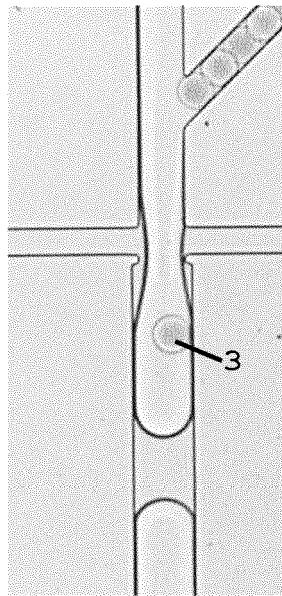


Fig. 2b

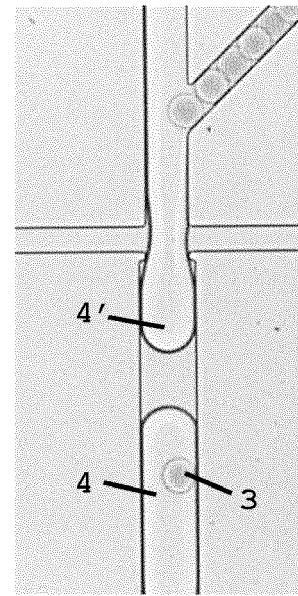


Fig. 2c

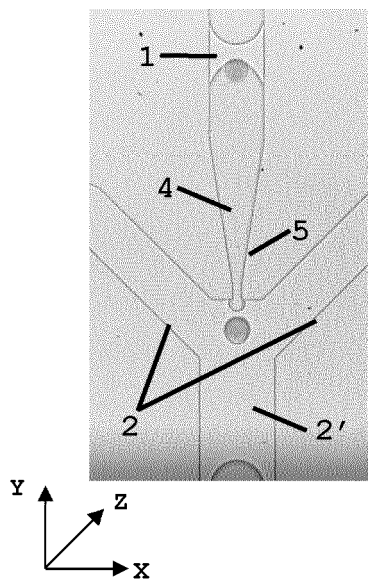


Fig. 3a

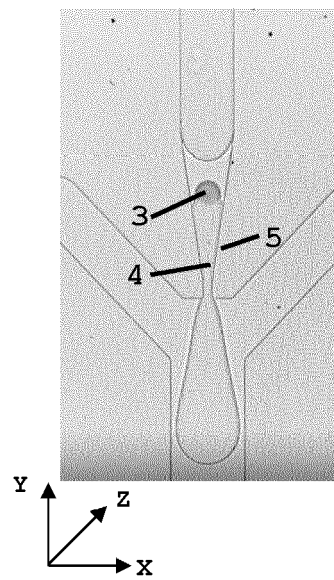


Fig. 3b

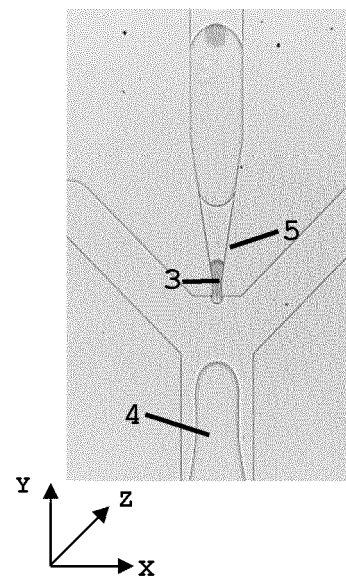


Fig. 3c

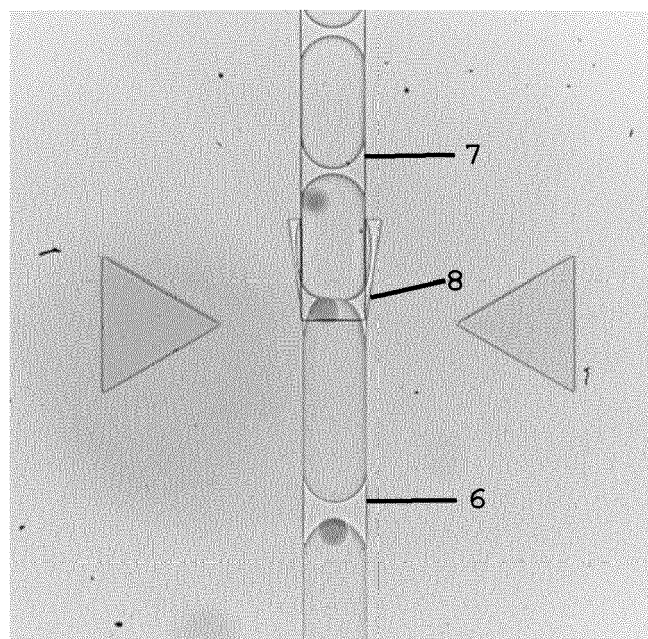


Fig. 4

% of bead extraction

		Qoil (ul/h)												
		50	200	300	400	500	600	800	1000	1200	1400	1600	1800	2000
Qdrops (ul/h)	50	3%	87%	95%	96%	100%	100%	100%						
	100		3%	71%	99%	100%	100%	100%	100%					
	150		1%	1%	44%	99%	100%	100%	100%	100%				
	200				1%	5%	95%	100%	100%	100%				
	250					1%	1%	100%	100%	100%	100%			
	300						0%	32%	93%	100%	100%	100%		
	350							0%	6%	98%	100%	100%		
	400								0%	88%	97%	100%	100%	
	450									17%	60%	100%	100%	100%

Fig. 5

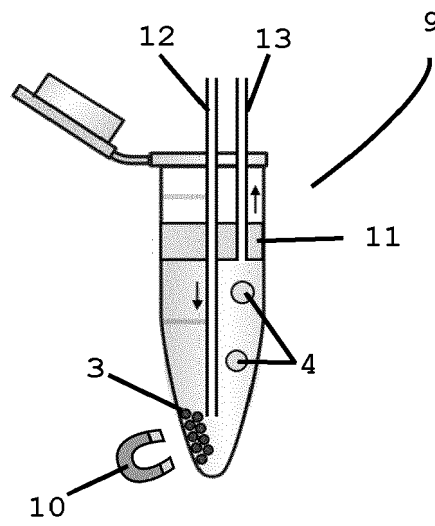


Fig. 6

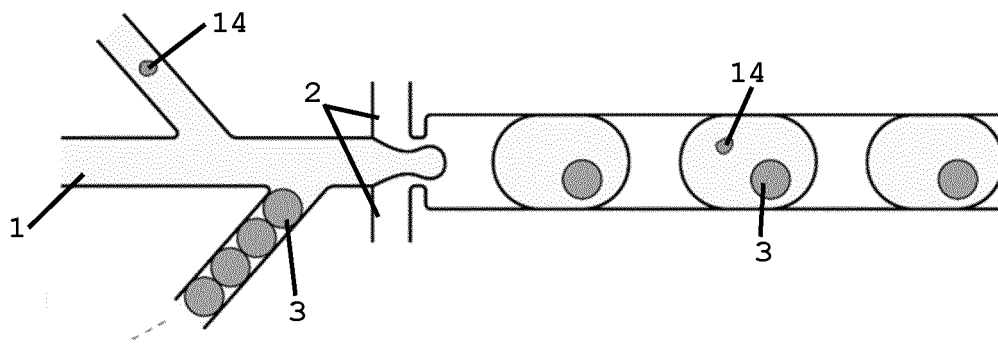


Fig. 7a

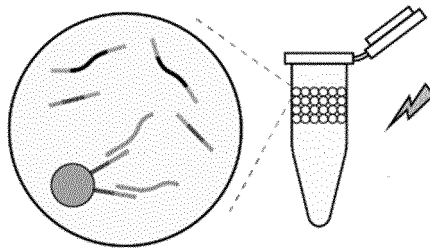


Fig. 7b

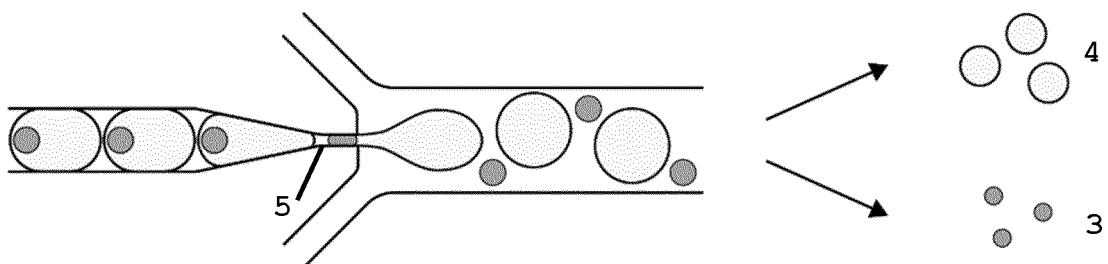


Fig. 7c

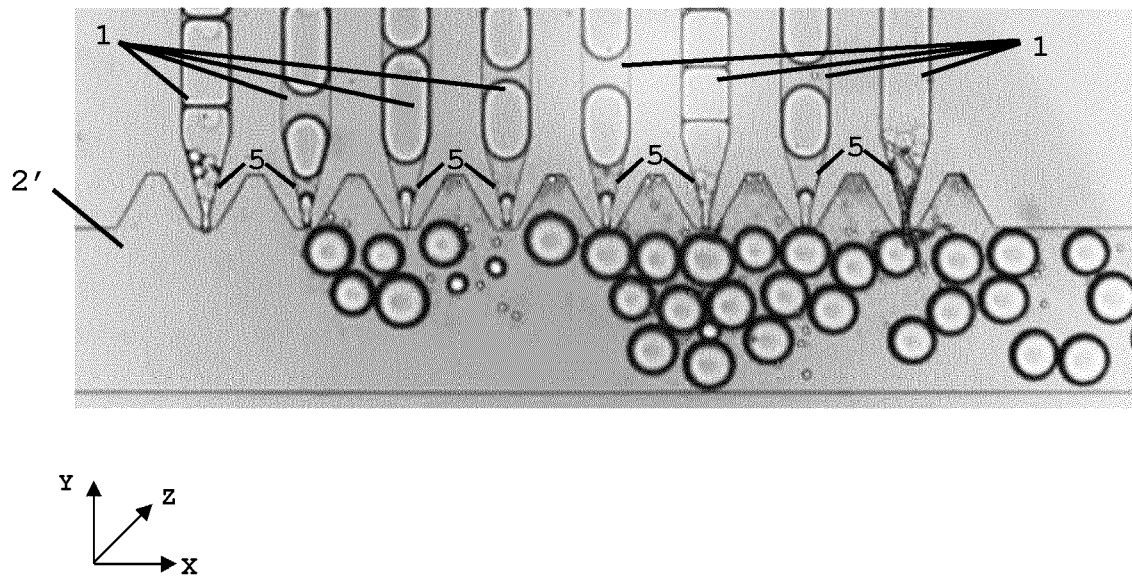


Fig. 8

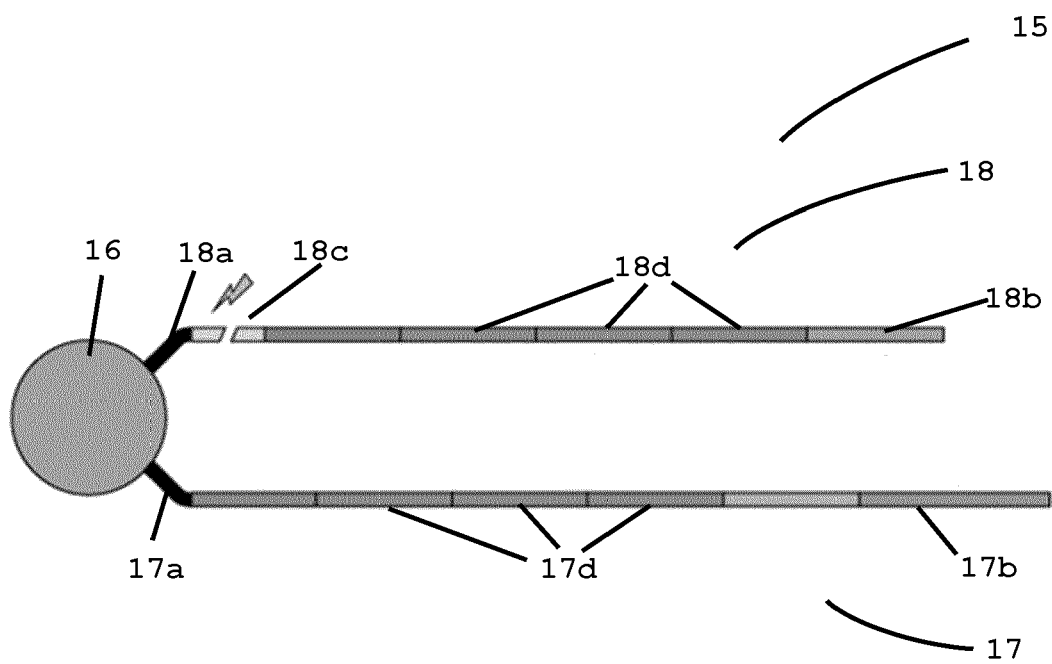


Fig. 9

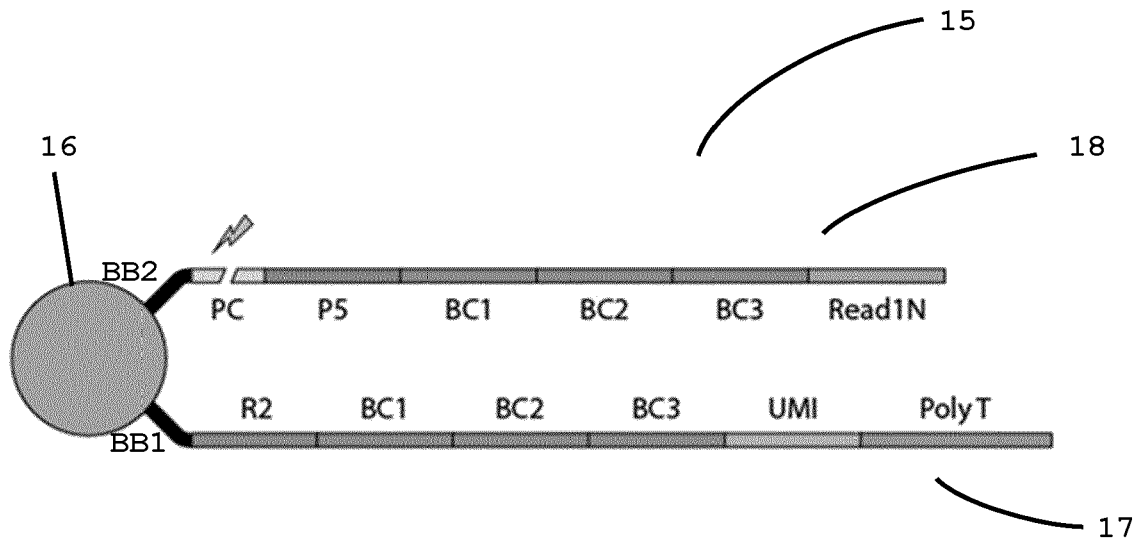


Fig. 10

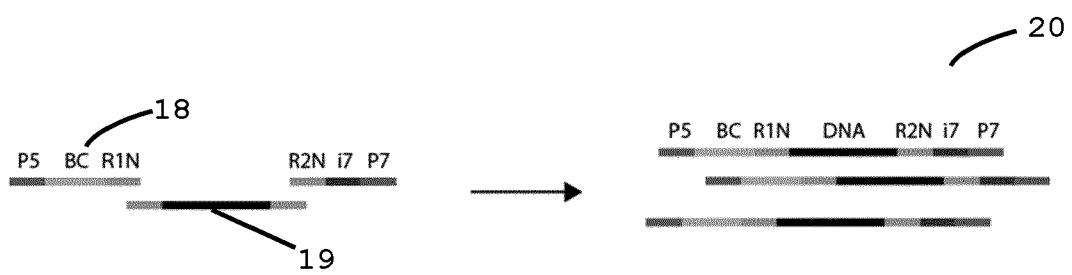


Fig. 11a

Fig. 11b

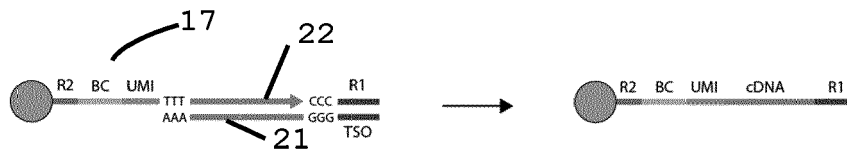


Fig. 12a

Fig. 12b

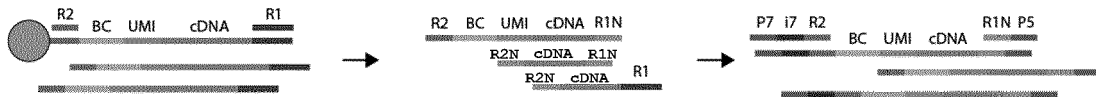


Fig. 12c

Fig. 12d

Fig. 12e



EUROPEAN SEARCH REPORT

Application Number

EP 22 30 6279

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The present search report has been drawn up for all claims			
Place of search The Hague		Date of completion of the search 30 January 2023	Examiner Ruiz-Echarri Rueda
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**ANNEX TO THE EUROPEAN SEARCH REPORT
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