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Belhocine et al.(10) **Patent No.:** US 12,391,975 B2  
(45) **Date of Patent:** Aug. 19, 2025(54) **SYSTEMS AND METHODS FOR TRANSPOSON LOADING**(71) Applicant: **10X Genomics, Inc.**, Pleasanton, CA (US)(72) Inventors: **Zahra Kamila Belhocine**, Pleasanton, CA (US); **Jason Bell**, Lafayette, LA (US)(73) Assignee: **10X GENOMICS, INC.**, Pleasanton, CA (US)

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CPC ..... C12Q 1/6806; C12Q 2521/531; C12Q 2525/191; C12Q 2525/301; C12Q 2525/313; C12Q 2535/122; C12Q 2543/10

See application file for complete search history.

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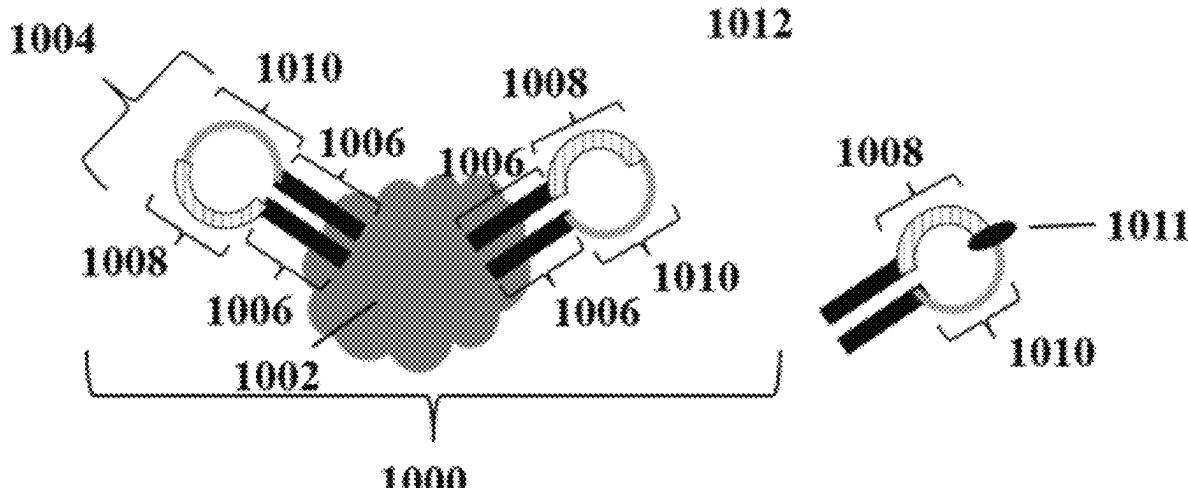
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*Primary Examiner* — Aaron A Priest*Assistant Examiner* — Matthew Harold Raymonda(74) **Attorney, Agent, or Firm** — WILSON SONSINI GOODRICH & ROSATI(57) **ABSTRACT**

Provided herein are methods, compositions, and systems for transposon loading. Transposons are loaded with nucleic acid molecules, allowing for transposition reactions of cellular nucleic acids. The present invention may improve transposon loading, yield of productive fragments, while minimizing potential nucleic acid fragment loss or cross-contamination.

**20 Claims, 13 Drawing Sheets**

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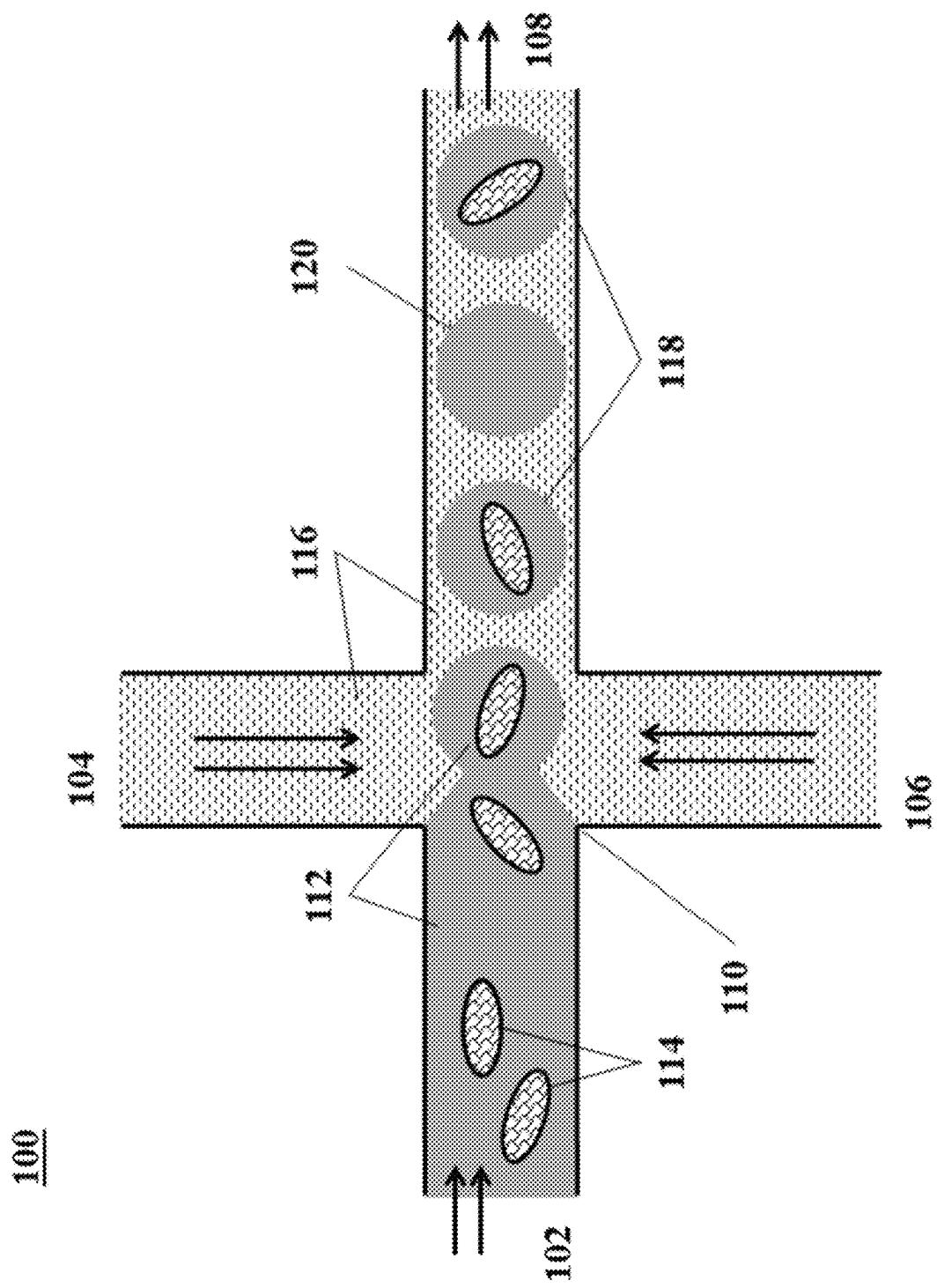
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*FIG. 1*

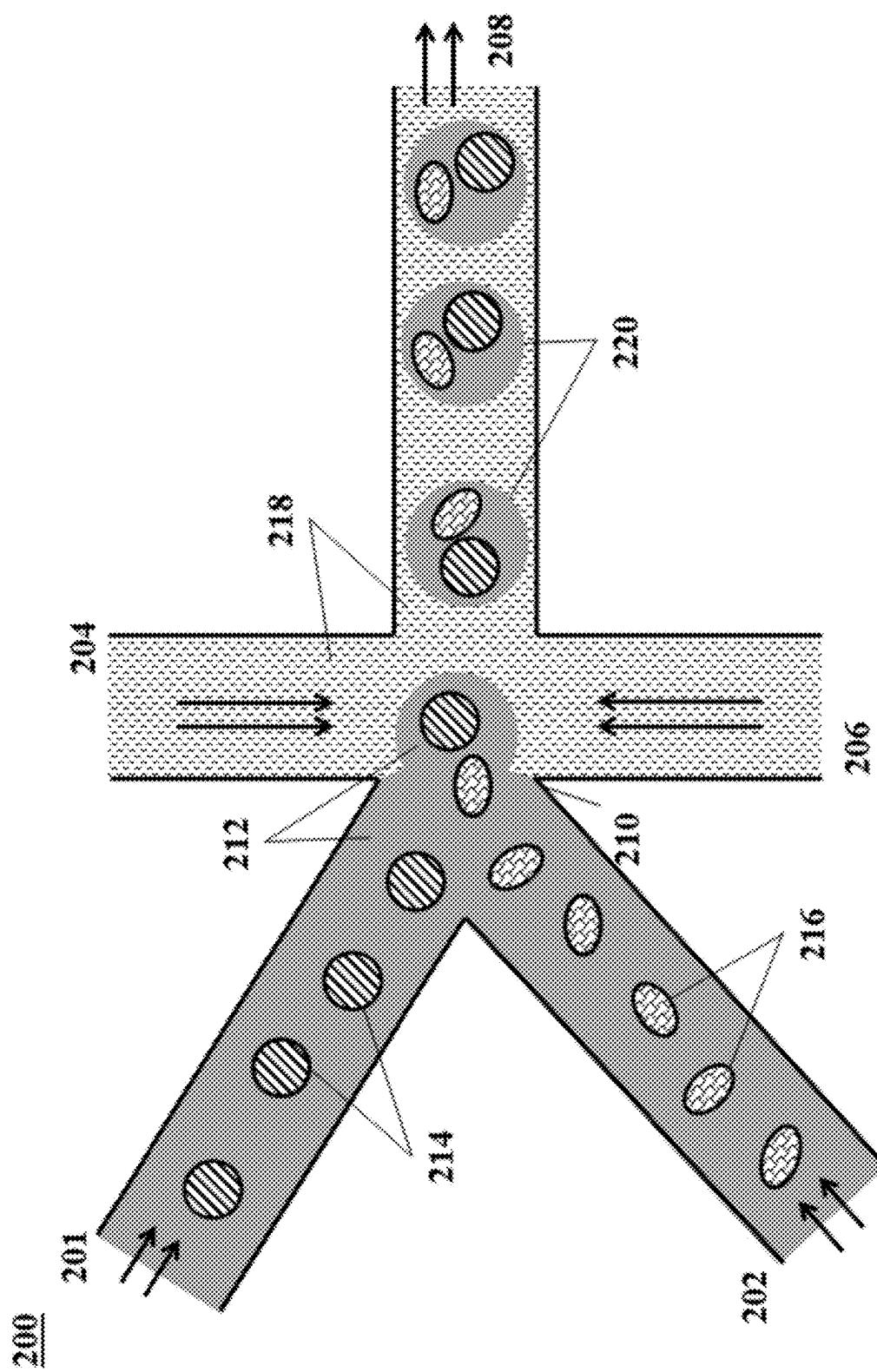


FIG. 2

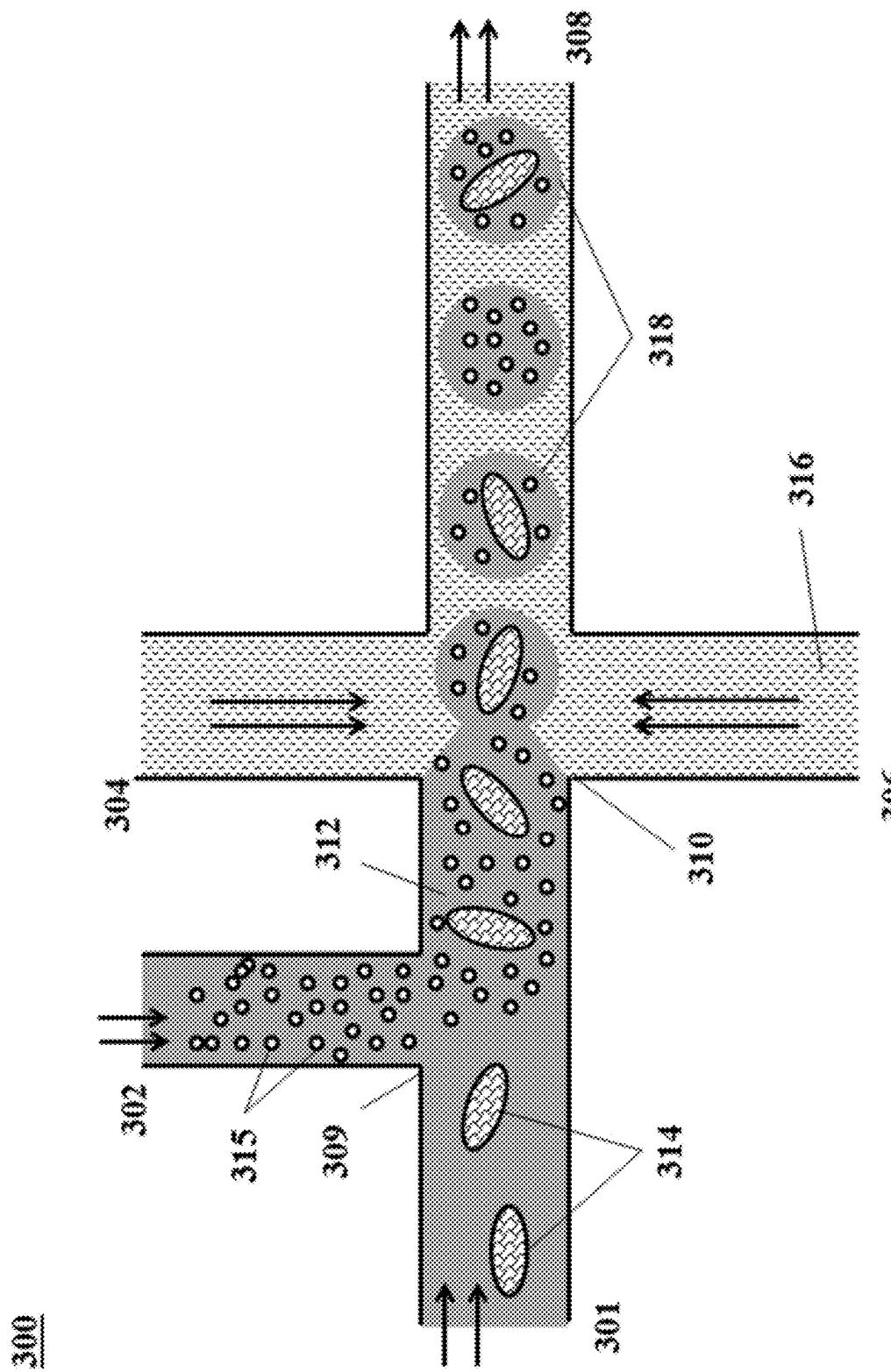
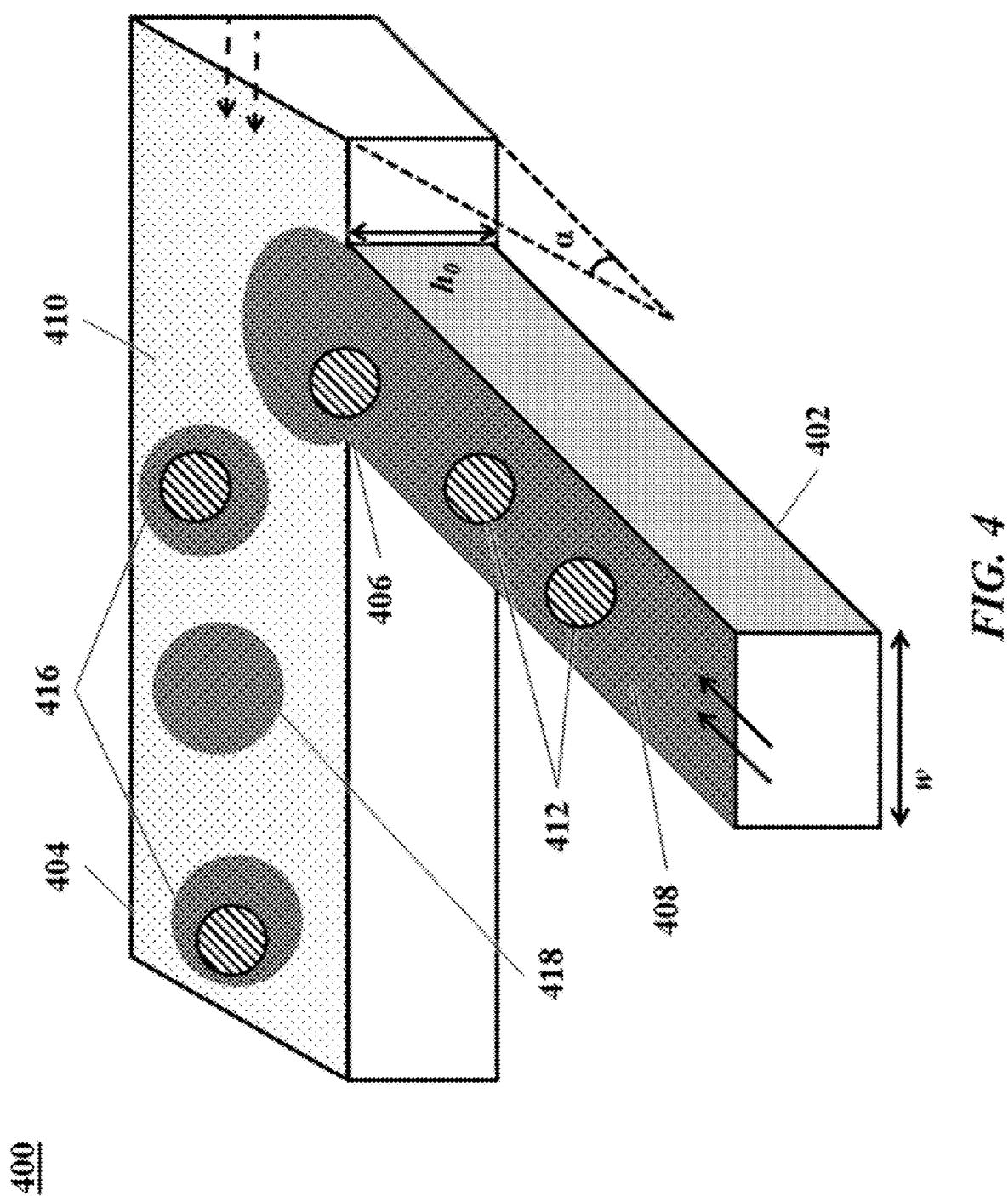


FIG. 3



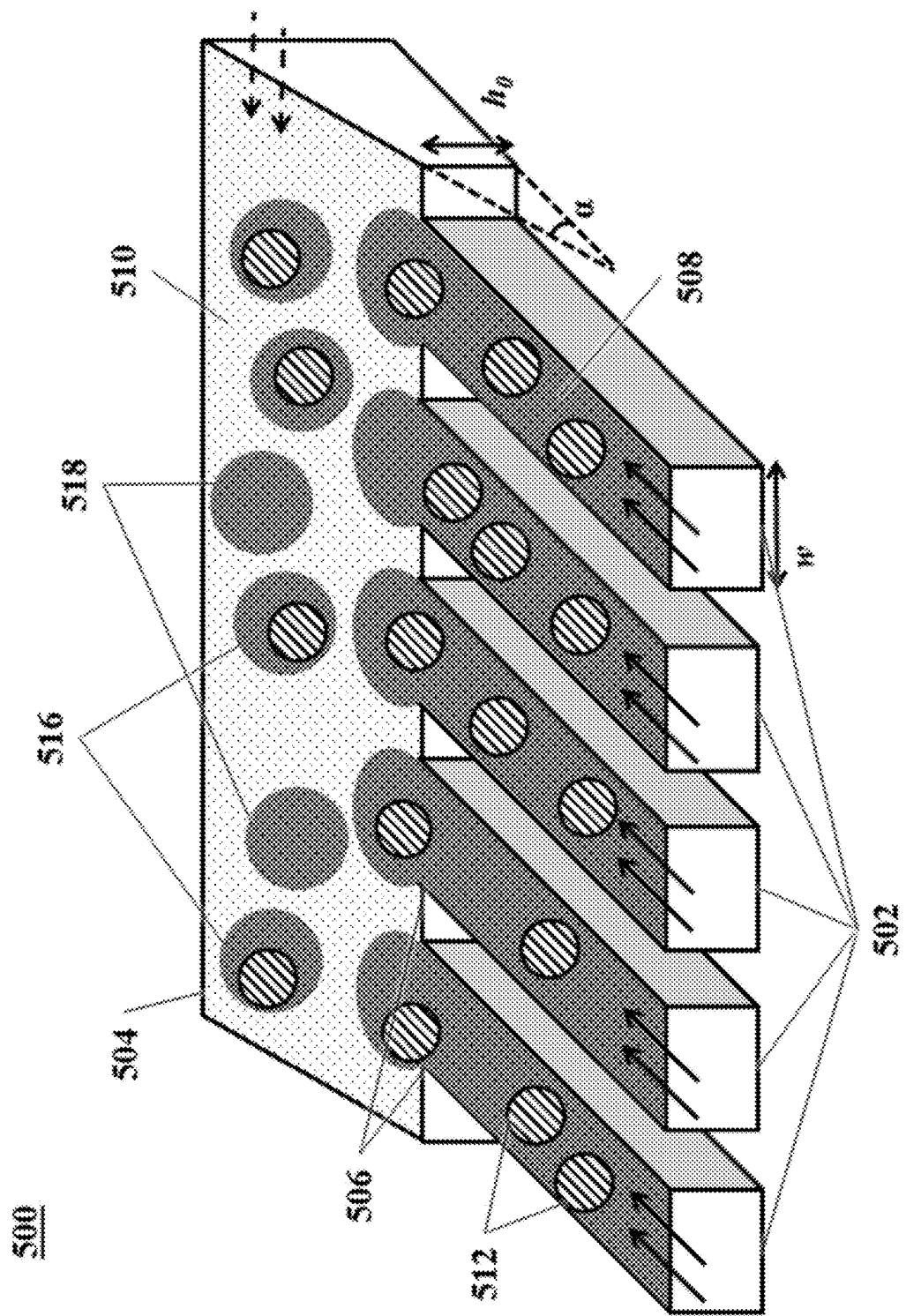


FIG. 5

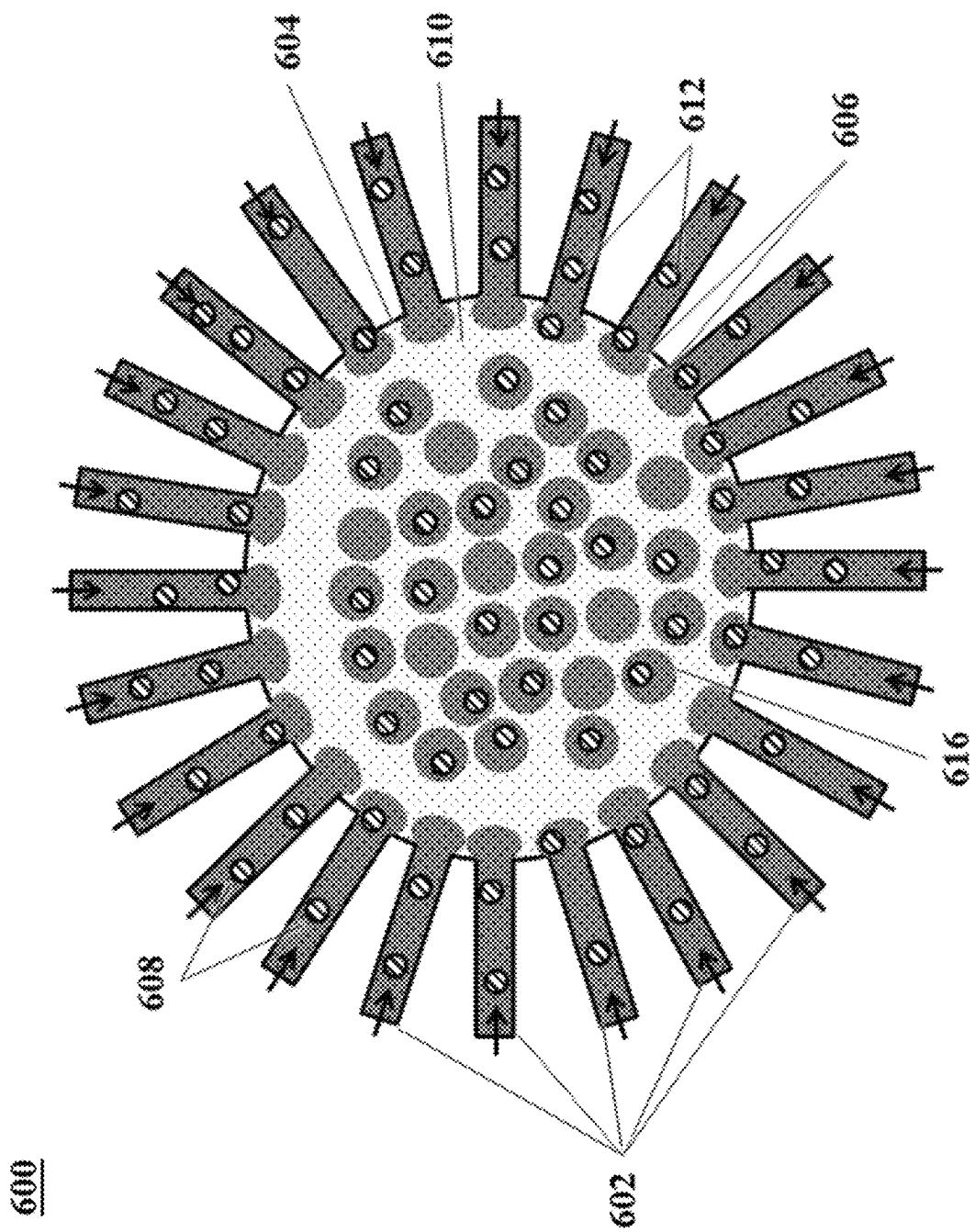


FIG. 6

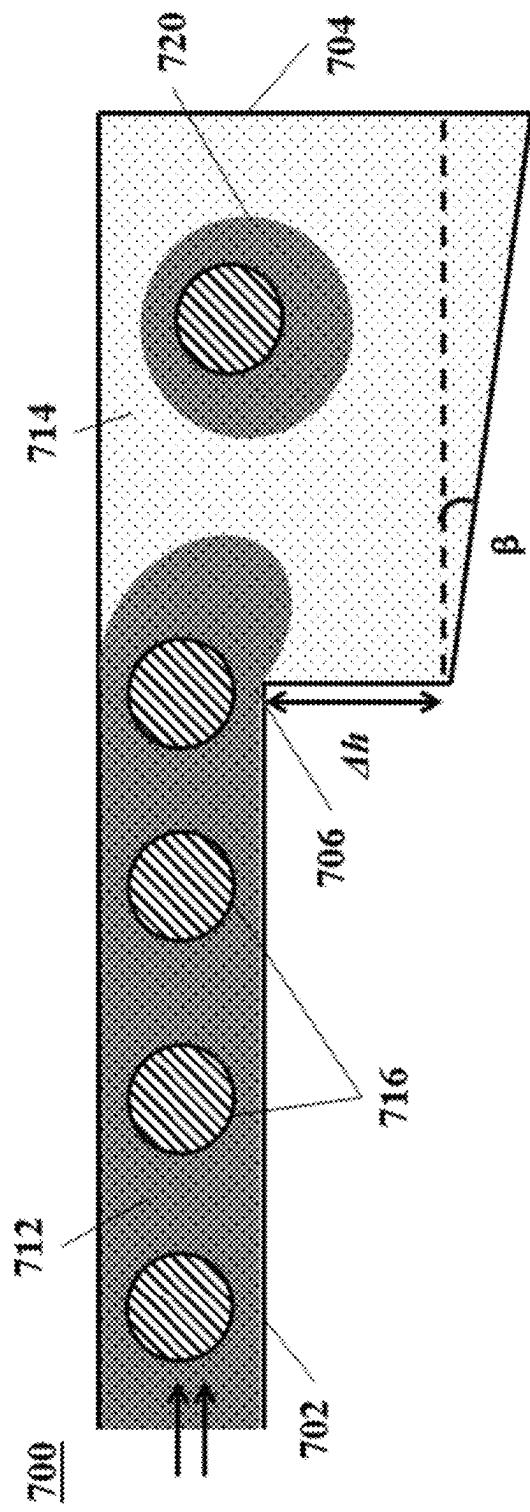


FIG. 7A

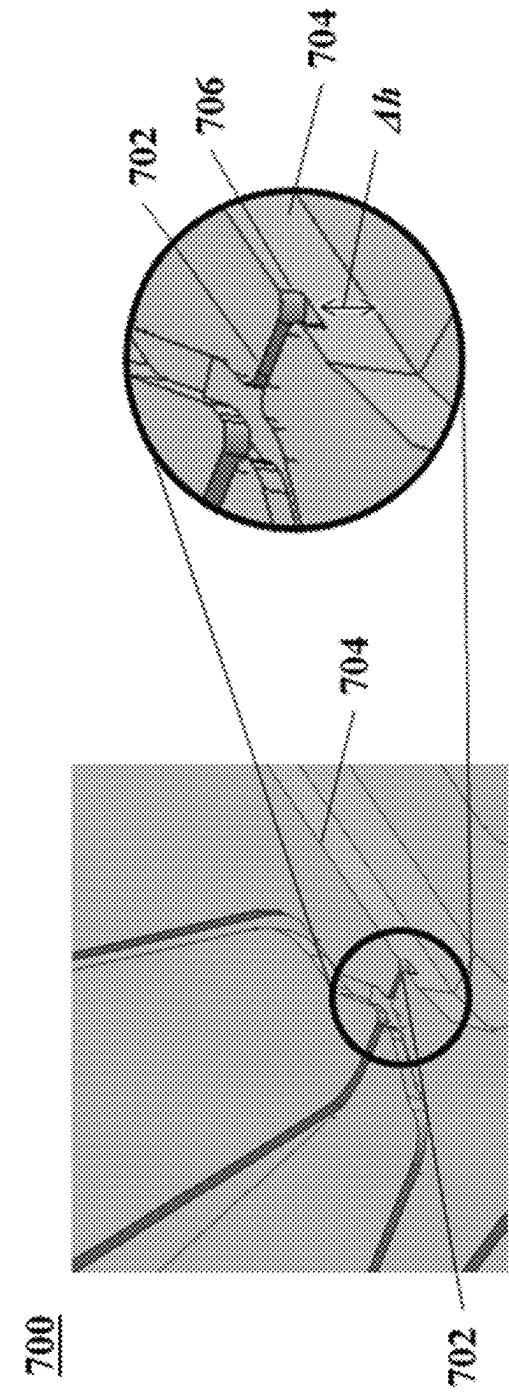
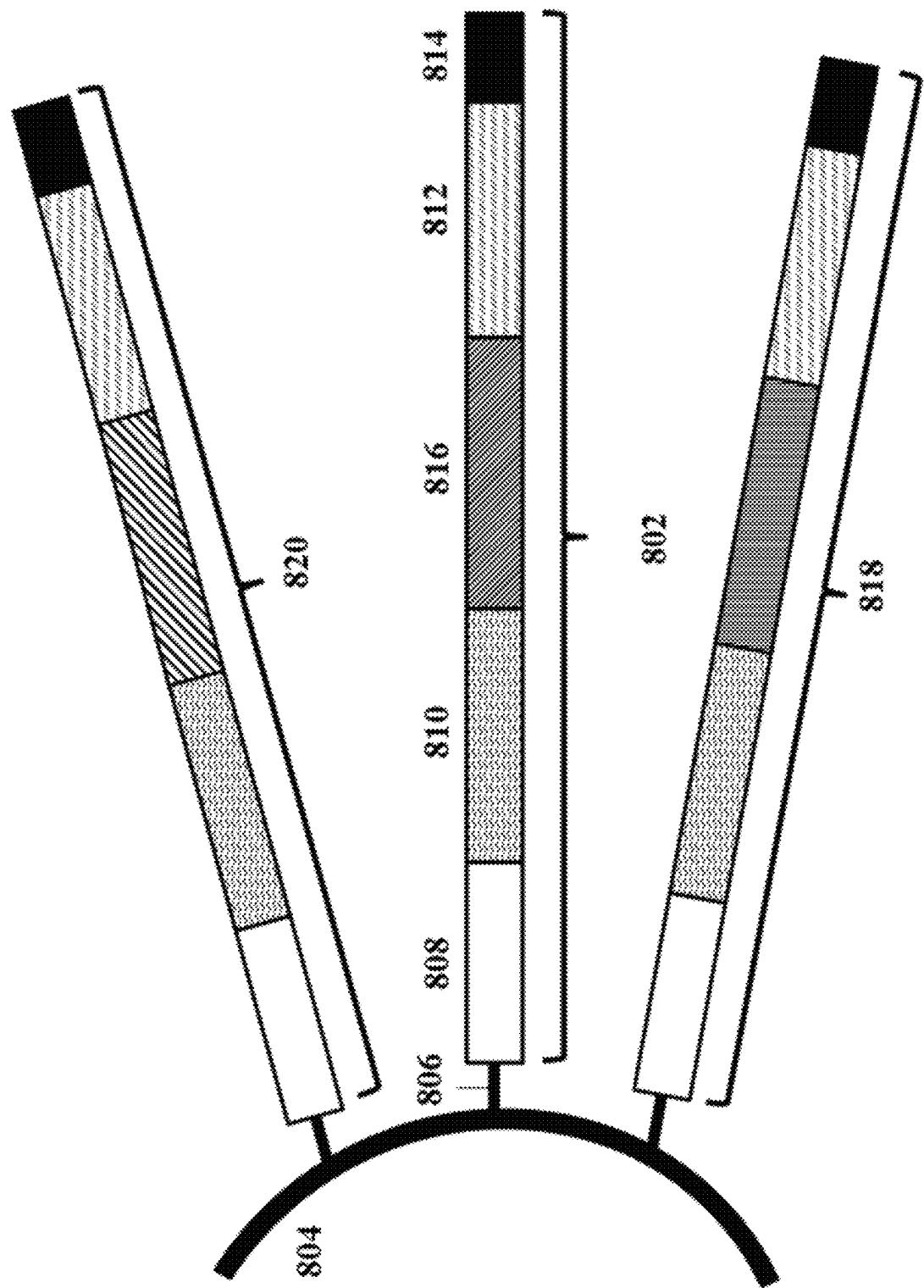


FIG. 7B



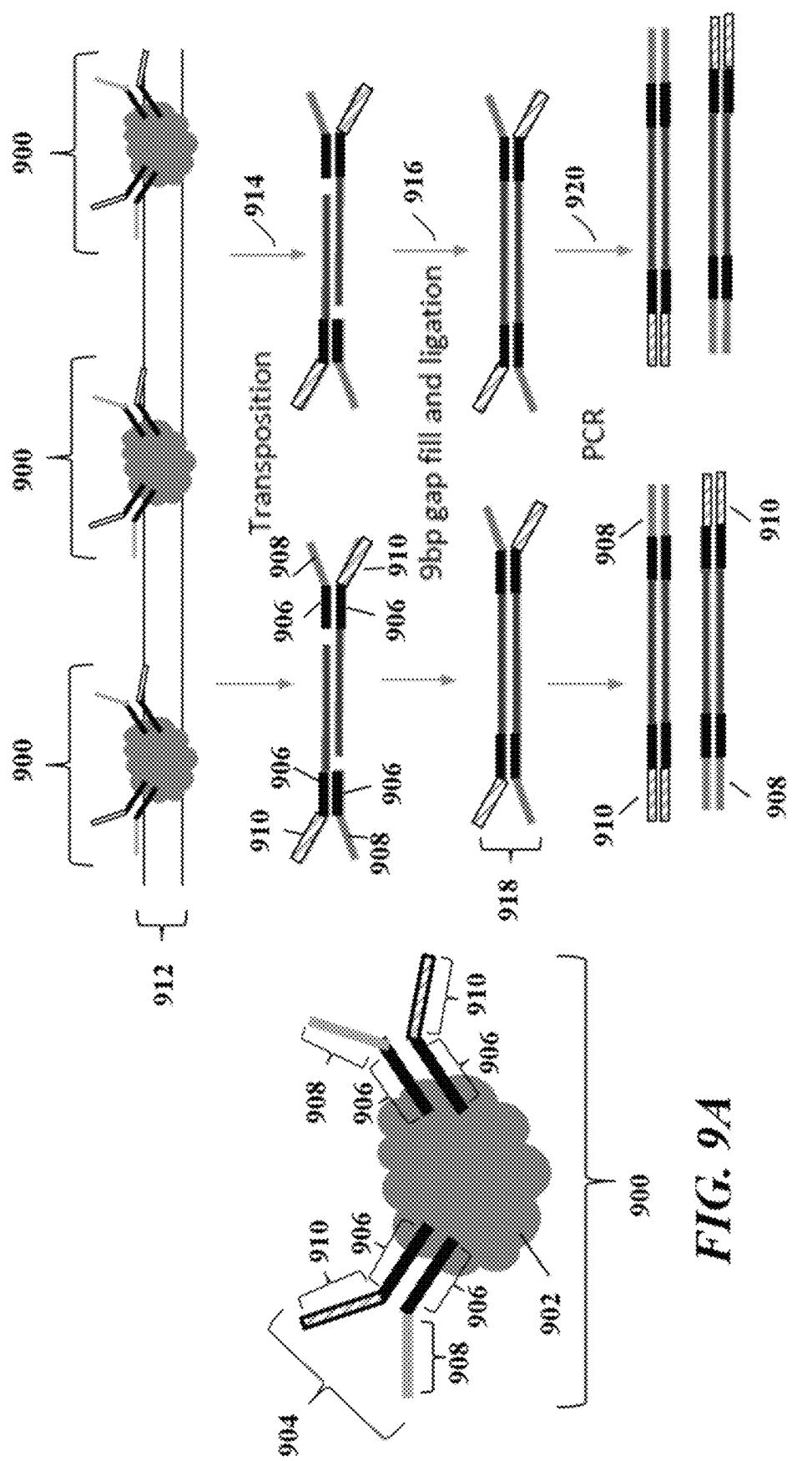
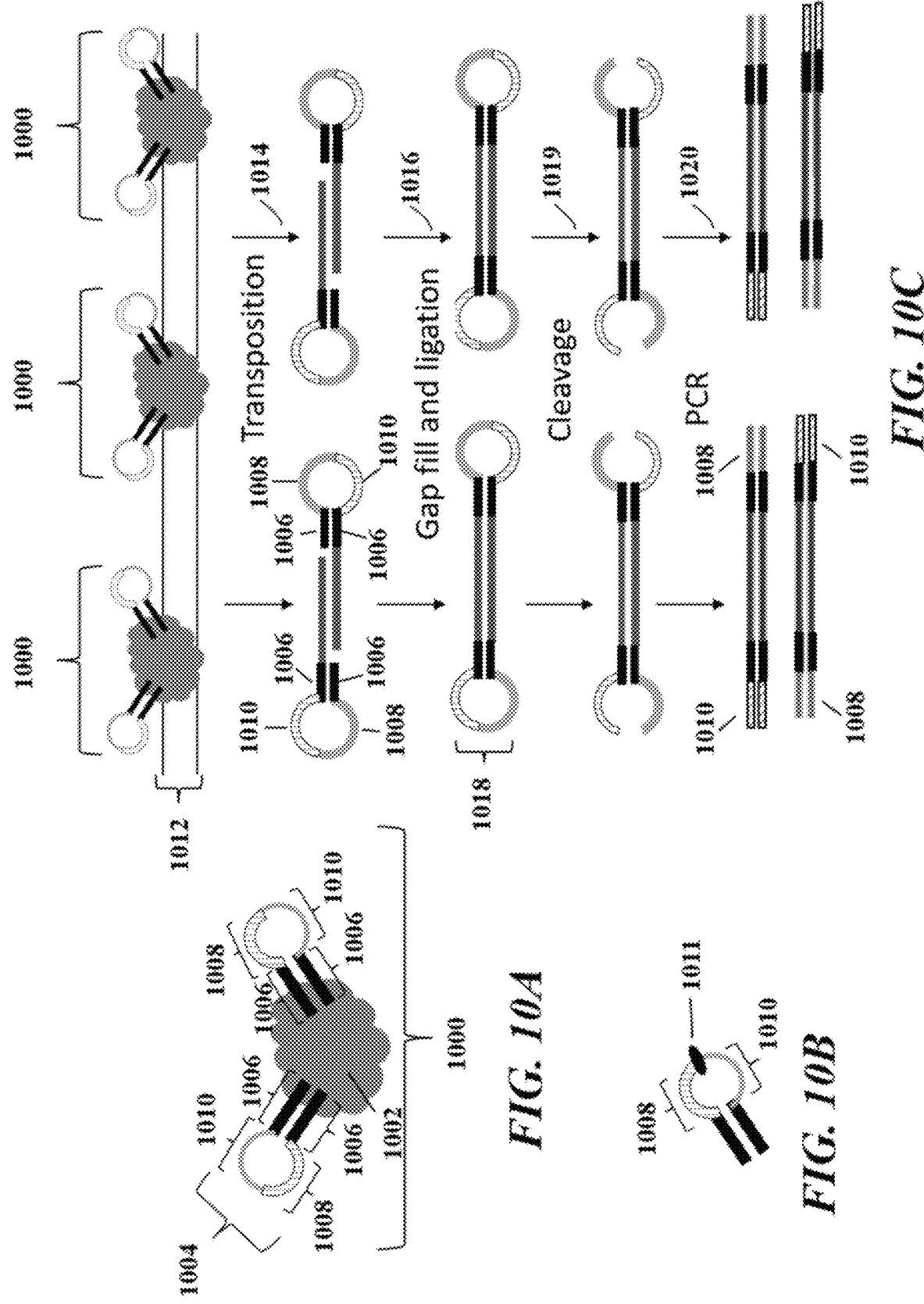
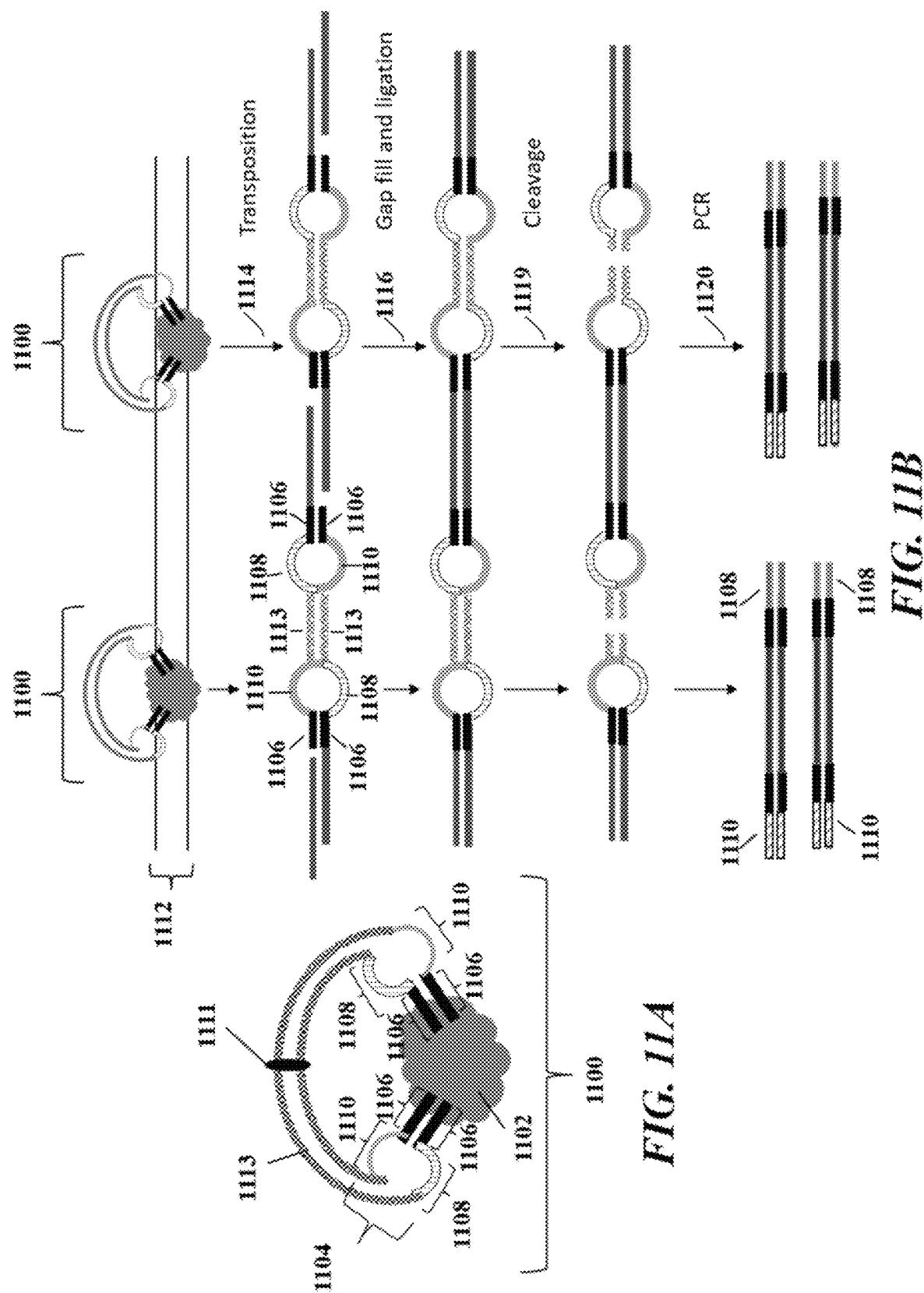
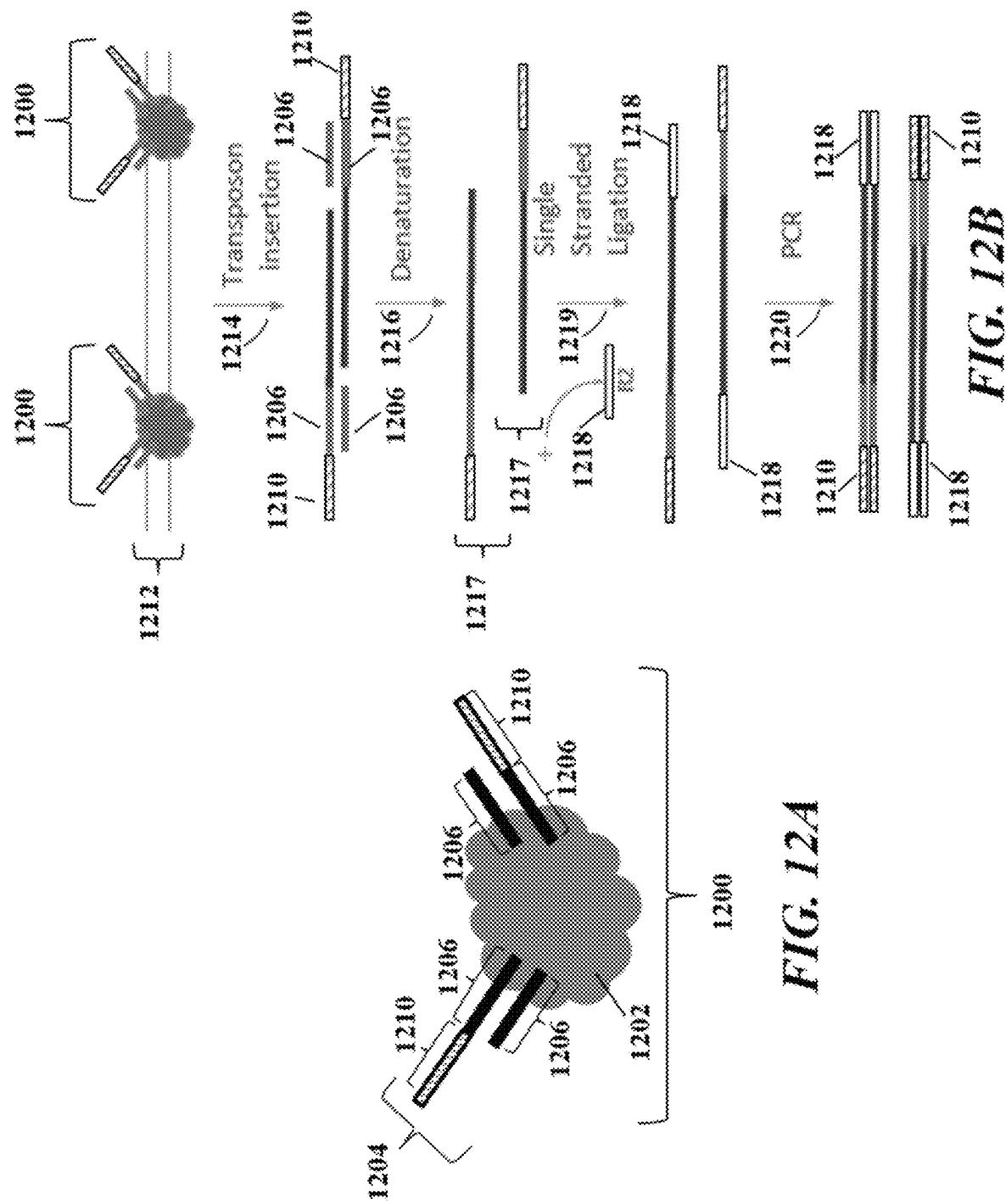


FIG. 9A

FIG. 9B







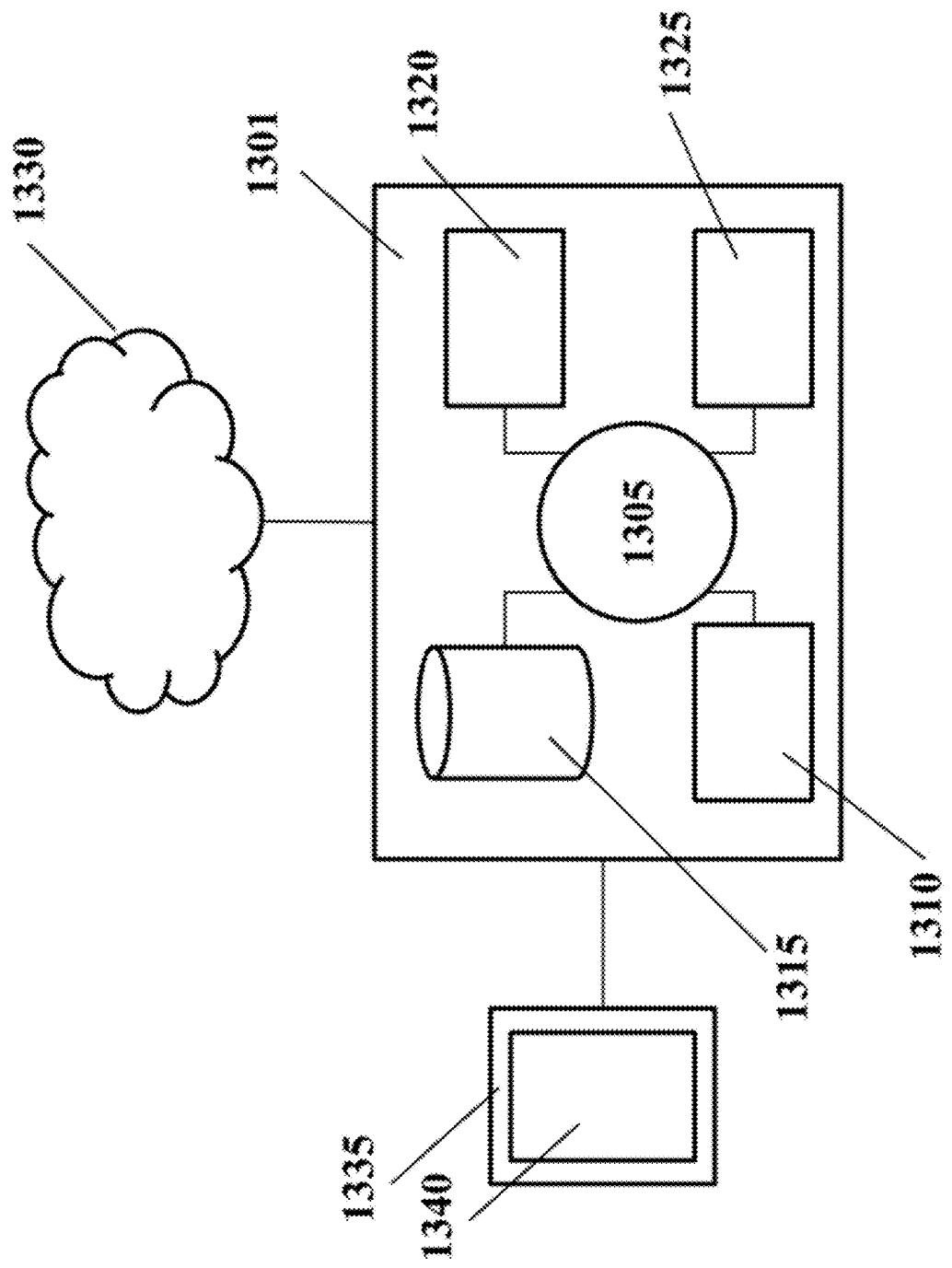


FIG. 13

## 1

SYSTEMS AND METHODS FOR  
TRANSPOSON LOADING

## CROSS-REFERENCE

This application is a continuation of International Application No. PCT/US2020/017789, filed Feb. 11, 2020, which claims the benefit of U.S. Provisional Patent Application No. 62/804,499, filed Feb. 12, 2019, which are incorporated by reference herein in their entirety for all purposes.

## BACKGROUND

A sample may be processed for various purposes, such as detection, identification, quantitation and/or characterization of nucleic acid molecules within the sample. The sample may be a biological sample. Biological samples may be processed, such as for detection of a disease (e.g., cancer) or identification of a particular species.

Biological samples may comprise nucleic acid molecules. Nucleic acid molecules such as DNA, RNA, etc. may be analyzed. There are various approaches for processing samples, such as polymerase chain reaction (PCR) and sequencing.

Biological samples may be processed within various reaction environments, such as partitions. Partitions may be wells or droplets. Droplets or wells may be employed to process biological samples in a manner that enables the biological samples to be partitioned and processed separately. For example, such droplets may be fluidically isolated from other droplets, enabling accurate control of respective environments in the droplets.

Biological samples in partitions may be subjected to various processes, such as chemical processes or physical processes. Samples in partitions may be subjected to heating or cooling, or chemical reactions, such as to yield species that may be qualitatively or quantitatively processed.

## SUMMARY

The manipulation and processing of biological samples may comprise the use of enzymes. In certain cases, transposases may be used for processing of nucleic acid molecules. Loading of transposases with nucleic acid sequences may have limitations; for example, sub-optimal transposon loading, low yield of productive fragments, potential fragment loss from cells (e.g., cell nuclei) and/or cross-contamination may occur (e.g., between cells, cell nuclei, or partitions) during sample processing. Provided herein are methods for transposon loading that can address these and other issues.

The present disclosure also provides methods for use in various sample processing and analysis applications. The methods provided herein may involve assaying nucleic acid molecules of interest. A method may comprise depletion of unwanted nucleic acid species and barcoding of target nucleic acid molecules to provide barcoded target nucleic acid molecules. The barcoded target nucleic acid molecules may be prepared for further analysis, e.g., sequencing, via steps such as amplification and library preparation. One or more processes of the methods provided herein may be performed within a partition such as a droplet or a well.

In an aspect, provided herein is a method for processing a nucleic acid molecule, comprising: (a) loading a transposase molecule with a pair of nucleic acid adapters, wherein each of the pair of nucleic acid adapters comprises a first single-stranded portion comprising a first nucleic acid

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sequence and a second single-stranded portion comprising a second nucleic acid sequence, wherein the first nucleic acid sequence is different from the second nucleic acid sequence; (b) bringing the transposase molecule in contact with the nucleic acid molecule under conditions sufficient to generate a nucleic acid fragment, wherein the nucleic acid fragment (i) comprises at each of a first end and a second end, both the first nucleic acid sequence and the second nucleic acid sequence and (ii) is at least partially double stranded; and (c) subjecting the nucleic acid fragment under conditions sufficient to generate a processed nucleic acid fragment, wherein the processed nucleic acid fragment comprises (i) the first nucleic acid sequence at the first end and the second nucleic acid sequence at the second end, or (ii) the second nucleic acid sequence at the first end and the first nucleic acid sequence at the second end.

In some embodiments, each of the pair of nucleic acid adapter comprises a double-stranded portion. In some embodiments, the first single-stranded portion and the second single-stranded portion are adjacent to a same end of the double-stranded portion. In some embodiments, the first single-stranded portion is not attached to the second single-stranded portion. In some embodiments, the first single-stranded portion and the second single-stranded portion are cleavably attached as a loop sequence. In some embodiments, the first single-stranded portion and the second single-stranded portion are cleavably attached by a linker comprising a uracil. In some embodiments, the loop sequence is cleavable by application of a photo-stimulus, biological stimulus, thermal stimulus or chemical stimulus. In some embodiments, (c) comprises cleaving the loop sequence.

In some embodiments, a first nucleic acid adapter and a second nucleic acid adapter of the pair of nucleic acid adapters are adjacent to a first end and a second end of another double-stranded portion, respectively. In some embodiments, the another double stranded portion comprises a cleavable sequence, and (b) comprises cleaving the another double stranded portion. In some embodiments, the cleavable sequence comprises a uracil.

In some embodiments, (c) comprises filling a gap in the nucleic acid fragment. In some embodiments, the gap is a 9 base pair (bp) gap.

In some embodiments, both (b) and (c) are performed in a partition. In some embodiments, the partition comprises a droplet. In some embodiments, the partition comprises a well.

In some embodiments, (b) comprises bringing the transposase molecule in contact with a nucleus of a cell comprising the nucleic acid molecule. In some embodiments, the cell is permeabilized. In some embodiments, the nucleus is permeabilized.

In some embodiments, the method further comprises (d) reacting a barcode molecule with the processed nucleic acid fragment to generate a barcoded nucleic acid fragment. In some embodiments, (d) is performed in a partition. In some embodiments, (d) further comprises filling a gap in the barcoded nucleic acid fragment. In some embodiments, the barcode molecule is attached to a bead. In some embodiments, the barcode molecule is releasably attached to the bead. In some embodiments, the barcode molecule comprises a common barcode sequence that is common to a plurality of barcode molecules attached to the bead. In some embodiments, the barcode molecule comprises a functional sequence. In some embodiments, the barcode molecule comprises a unique molecular identifier that is unique within a plurality of barcode molecules attached to the bead. In

some embodiments, the method further comprises sequencing the barcoded nucleic acid fragment or a derivative thereof.

In another aspect, provided herein is a method for processing a nucleic acid molecule, comprising: (a) loading a transposase molecule with a pair of first nucleic acid adapters, wherein the pair of first nucleic acid adapters comprises a single stranded portion comprising a first nucleic acid sequence; (b) bringing the transposase molecule in contact with the nucleic acid molecule under conditions sufficient to generate a nucleic acid fragment, wherein the nucleic acid fragment (i) comprises, at each of a first end and a second end, the first nucleic acid sequence and (ii) is at least partially double stranded; (c) denaturing the nucleic acid fragment to generate a first single stranded fragment and a second single stranded fragment, wherein the first single stranded fragment and the second single stranded fragment comprise the first nucleic acid sequence; and (d) bringing the first single stranded fragment and the second single stranded fragment in contact with a plurality of second nucleic acid adapters to generate processed nucleic acid fragments, wherein the plurality of second nucleic acid adapters comprises a second nucleic acid sequence, and wherein the processed nucleic acid fragments comprises (i) the first nucleic acid sequence at the first end and the second nucleic acid sequence at the second end, or (ii) the second nucleic acid sequence at the first end and the first nucleic acid sequence at the second end.

In some embodiments, (d) comprises ligating the first single stranded fragment to a second nucleic acid adapter of the plurality of second nucleic acid adapters and ligating the second single-stranded fragment to another second nucleic acid adapter of the plurality of second nucleic acid adapters.

In some embodiments, (d) is performed at a temperature that prevents reannealing of the first single stranded fragment and the second single stranded fragment.

Another aspect of the present disclosure provides a non-transitory computer readable medium comprising machine executable code that, upon execution by one or more computer processors, implements any of the methods above or elsewhere herein.

Another aspect of the present disclosure provides a system comprising one or more computer processors and computer memory coupled thereto. The computer memory comprises machine executable code that, upon execution by the one or more computer processors, implements any of the methods above or elsewhere herein.

Additional aspects and advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description, wherein only illustrative embodiments of the present disclosure are shown and described. As will be realized, the present disclosure is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, all without departing from the disclosure. Accordingly, the drawings and description are to be regarded as illustrative in nature, and not as restrictive.

#### INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the

specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings (also "Figure" and "FIG." herein), of which:

FIG. 1 shows an example of a microfluidic channel structure for partitioning individual biological particles.

FIG. 2 shows an example of a microfluidic channel structure for delivering barcode carrying beads to droplets.

FIG. 3 shows an example of a microfluidic channel structure for co-partitioning biological particles and reagents.

FIG. 4 shows an example of a microfluidic channel structure for the controlled partitioning of beads into discrete droplets.

FIG. 5 shows an example of a microfluidic channel structure for increased droplet generation throughput.

FIG. 6 shows another example of a microfluidic channel structure for increased droplet generation throughput.

FIG. 7A shows a cross-section view of another example of a microfluidic channel structure with a geometric feature for controlled partitioning. FIG. 7B shows a perspective view of the channel structure of FIG. 7A.

FIG. 8 illustrates an example of a barcode-carrying bead.

FIG. 9A schematically shows an example of loading nucleic acid molecules on a transposase. FIG. 9B schematically shows an example method of processing nucleic acid molecules.

FIG. 10A schematically shows another example of loading nucleic acid molecules on a transposase. FIG. 10B schematically shows a linker molecule on a nucleic acid adapter. FIG. 10C schematically shows another example method of processing nucleic acid molecules.

FIG. 11A schematically shows another example of loading nucleic acid molecules on a transposase. FIG. 11B schematically shows another example method of processing nucleic acid molecules.

FIG. 12A schematically shows another example of loading nucleic acid molecules on a transposase. FIG. 12B schematically shows another example method of processing nucleic acid molecules.

FIG. 13 shows a computer system that is programmed or otherwise configured to implement methods provided herein.

#### DETAILED DESCRIPTION

While various embodiments of the invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

Where values are described as ranges, it will be understood that such disclosure includes the disclosure of all possible sub-ranges within such ranges, as well as specific

numerical values that fall within such ranges irrespective of whether a specific numerical value or specific sub-range is expressly stated.

The term “barcode,” as used herein, generally refers to a label, or identifier, that conveys or is capable of conveying information about an analyte. A barcode can be part of an analyte. A barcode can be independent of an analyte. A barcode can be a tag attached to an analyte (e.g., nucleic acid molecule) or a combination of the tag in addition to an endogenous characteristic of the analyte (e.g., size of the analyte or end sequence(s)). A barcode may be unique. Barcodes can have a variety of different formats. For example, barcodes can include: polynucleotide barcodes; random nucleic acid and/or amino acid sequences; and synthetic nucleic acid and/or amino acid sequences. A barcode can be attached to an analyte in a reversible or irreversible manner. A barcode may be single- or double-stranded. A barcode can be added to, for example, a fragment of a deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sample before, during, and/or after sequencing of the sample. Barcodes can allow for identification and/or quantification of individual sequencing-reads. Barcodes may comprise other sequences, such as a unique molecular identifier (UMI), spacer sequences, sequencing primer sequences, etc.

The term “real time,” as used herein, can refer to a response time of less than about 1 second, a tenth of a second, a hundredth of a second, a millisecond, or less. The response time may be greater than 1 second. In some instances, real time can refer to simultaneous or substantially simultaneous processing, detection or identification.

The term “subject,” as used herein, generally refers to an animal, such as a mammal (e.g., human) or avian (e.g., bird), or other organism, such as a plant. For example, the subject can be a vertebrate, a mammal, a rodent (e.g., a mouse), a primate, a simian or a human. Animals may include, but are not limited to, farm animals, sport animals, and pets. A subject can be a healthy or asymptomatic individual, an individual that has or is suspected of having a disease (e.g., cancer) or a pre-disposition to the disease, and/or an individual that is in need of therapy or suspected of needing therapy. A subject can be a patient. A subject can be a microorganism or microbe (e.g., bacteria, fungi, archaea, viruses).

The term “genome,” as used herein, generally refers to genomic information from a subject, which may be, for example, at least a portion or an entirety of a subject’s hereditary information. A genome can be encoded either in DNA or in RNA. A genome can comprise coding regions (e.g., that code for proteins) as well as non-coding regions. A genome can include the sequence of all chromosomes together in an organism. For example, the human genome ordinarily has a total of 46 chromosomes. The sequence of all of these together may constitute a human genome.

The terms “adaptor(s),” “adapter(s)” and “tag(s)” may be used synonymously. An adaptor or tag can be coupled to a polynucleotide sequence to be “tagged” by any approach, including ligation, hybridization, or other approaches. Adaptors may also be used to refer to a nucleic acid sequence or segment, such as a functional sequence. These adaptors may comprise nucleic acid sequences that may add a function, e.g., spacer sequence, primer sequencing site, barcode sequence, unique molecular identifier sequence, etc.

The terms “transposase” and “transposase molecule” may be used interchangeably herein. A transposase generally refers to an enzyme that is configured to bind a nucleic acid molecule, cleave the nucleic acid molecule and insert a

nucleic acid sequence into the nucleic acid molecule (e.g., via a fragmentation reaction). In some cases, a transposase can be configured to bind to a specific site on the nucleic acid molecule. In some cases, a transposase can be configured to bind to a random site on the nucleic acid molecule. Moreover, in some cases, a transposase can be configured to bind chromatin (e.g., euchromatin). Non-limiting examples of transposases include: a Tn transposase (e.g., Tn3, Tn5, Tn7, Tn10, Tn552, Tn903), a MuA transposase, a Vibhar transposase (e.g., from *Vibrio harveyi*), a prokaryotic transposase, any member of the hAT superfamily of transposases (e.g., Hermes), Ac-Ds, Ascot-1, Bs1, Cin4, Copia, En/Spm, F element, hobo, Hsmar1, Hsmar2, IN (HIV), IS1, IS2, IS3, IS4, IS5, IS6, IS10, IS21, IS30, IS50, IS51, IS150, IS256, IS407, IS427, IS630, IS903, IS911, IS982, IS1031, ISL2, L1, Mariner, P element, Tam3, Tc1, Tc3, Tel, THE-1, Tn/O, TnA, Tol1, Tol2, Tn1O, and Ty1. In some cases, the transposase may be derived from any of the above, such as a transposase including one or more mutations or modifications. In certain instances, a transposase related to and/or derived from a parent transposase can comprise a peptide fragment with at least about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% amino acid sequence homology to a corresponding peptide fragment of the parent transposase. The peptide fragment can be at least about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 60, about 70, about 80, about 90, about 100, about 150, about 200, about 250, about 300, about 400, or about 500 amino acids in length. For example, a transposase derived from Tn5 can comprise a peptide fragment that is 50 amino acids in length and about 80% homologous to a corresponding fragment in a parent Tn5 transposase. Action of a transposase (e.g., insertion) may be facilitated and/or triggered by addition of one or more cations, such as one or more divalent cations (e.g., Ca<sup>2+</sup>, Mg<sup>2+</sup>, or Mn<sup>2+</sup>). In a particular aspect, the transposase can include a hyperactive transposase, such as Tn5.

The terms “primer sequencing site” and “sequencing primer sequence” may be used interchangeably herein. Primer sequencing sites generally refer to nucleic acid sequences that can be used for sequencing.

The term “sequencing,” as used herein, generally refers to methods and technologies for determining the sequence of nucleotide bases in one or more polynucleotides. Polynucleotides may comprise nucleic acid molecules, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), including variants or derivatives thereof (e.g., single-stranded DNA). Sequencing can be performed by various systems currently available, such as, without limitation, a sequencing system by Illumina®, Pacific Biosciences (PacBio®), Oxford Nanopore®, or Life Technologies (Ion Torrent®). Alternatively or in addition, sequencing may be performed using nucleic acid amplification, polymerase chain reaction (PCR) (e.g., digital PCR, quantitative PCR, or real time PCR), or isothermal amplification. Such systems may provide a plurality of raw genetic data corresponding to the genetic information of a subject (e.g., human), as generated by the systems from a sample provided by the subject. In some examples, such systems provide sequencing reads (also “reads” herein). A read may include a string of nucleic acid bases corresponding to a sequence of a nucleic acid molecule that has been sequenced. In some situations, systems and methods provided herein may be used with proteomic information.

The term "bead," as used herein, generally refers to a particle. The bead may be a solid or semi-solid particle. A bead may be hollow. The bead may be a gel bead. The gel bead may include a polymer matrix (e.g., matrix formed by polymerization or cross-linking). The polymer matrix may include one or more polymers (e.g., polymers having different functional groups or repeat units). Polymers in the polymer matrix may be homogeneous or heterogeneous. Polymers within a polymer matrix may be randomly arranged, such as in random copolymers, and/or have ordered structures, such as in block copolymers. Cross-linking can be via covalent, ionic, or inductive, interactions, or physical entanglement. The cross-linking may be reversible. The bead may be a macromolecule. The bead may be a sol-gel. The bead may be formed from biomolecules such as peptides, carbohydrates, lipids, etc. The bead may be formed via covalent or non-covalent assembly of molecules (e.g., macromolecules), such as monomers or polymers. Such polymers or monomers may be natural or synthetic. Such polymers or monomers may be or include, for example, nucleic acid molecules (e.g., DNA or RNA). The bead may be formed of a polymeric material. The bead may be magnetic or non-magnetic. The bead may be rigid. The bead may be flexible and/or compressible. The bead may be disruptable or dissolvable. The bead may be a solid particle (e.g., a metal-based particle including but not limited to iron oxide, gold or silver) covered with a coating comprising one or more polymers. Such coating may be disruptable or dissolvable.

The term "sample," as used herein, generally refers to a biological sample of a subject. The biological sample may comprise any number of macromolecules, for example, cellular macromolecules. The sample may be a cell sample. The sample may be a cell line or cell culture sample. The sample can include one or more cells. The sample can include one or more microbes. The biological sample may be a nucleic acid sample or protein sample. The biological sample may also be a carbohydrate sample, a protein sample, or a lipid sample. The biological sample may be derived from another sample. The sample may be a tissue sample, such as a biopsy, core biopsy, needle aspirate, or fine needle aspirate. The sample may be a fluid sample, such as a blood sample, urine sample, or saliva sample. The sample may be a skin sample. The sample may be a cheek swab. The sample may be a plasma or serum sample. The sample may be a cell-free sample. A cell-free sample may include extracellular polynucleotides. Extracellular polynucleotides may be isolated from a bodily sample that may be selected from the group consisting of blood, plasma, serum, urine, saliva, mucosal excretions, sputum, stool and tears. Samples may be enriched prior to processing.

The term "biological particle," as used herein, generally refers to a discrete biological system derived from a biological sample. The biological particle may be a macromolecule. The biological particle may be a small molecule. The biological particle may be a virus. The biological particle may be a cell or derivative of a cell. The biological particle may be a subcellular component, such as an organelle. The biological particle may be a rare cell from a population of cells. The biological particle may be any type of cell, including without limitation prokaryotic cells, eukaryotic cells, bacterial, fungal, plant, mammalian, or other animal cell type, mycoplasmas, normal tissue cells, tumor cells, or any other cell type, whether derived from single-cell or multicellular organisms. The biological particle may be a constituent of a cell. The biological particle may be or may include DNA, RNA, organelles, proteins, or any combina-

tion thereof. The biological particle may be or may include a matrix (e.g., a gel or polymer matrix) comprising a cell or one or more constituents from a cell (e.g., cell bead), such as DNA, RNA, organelles, proteins, or any combination thereof, from the cell. The biological particle may be obtained from a tissue of a subject. The biological particle may be a hardened cell. Such hardened cell may or may not include a cell wall or cell membrane. The biological particle may include one or more constituents of a cell, but may not include other constituents of the cell. An example of such constituents is a nucleus or an organelle. A cell may be a live cell. The live cell may be capable of being cultured, for example, being cultured when enclosed in a gel or polymer matrix, or cultured when comprising a gel or polymer matrix.

The term "macromolecular constituent," as used herein, generally refers to a macromolecule contained within or from a biological particle. The macromolecular constituent may comprise a nucleic acid. In some cases, the biological particle may be a macromolecule. The macromolecular constituent may comprise DNA. The macromolecular constituent may comprise RNA. The RNA may be coding or non-coding. The RNA may be messenger RNA (mRNA), ribosomal RNA (rRNA) or transfer RNA (tRNA), for example. The RNA may be a transcript. The RNA may be small RNA that are less than 200 nucleic acid bases in length, or large RNA that are greater than 200 nucleic acid bases in length. Small RNAs may include 5.8S ribosomal RNA (rRNA), 5S rRNA, transfer RNA (tRNA), microRNA (miRNA), small interfering RNA (siRNA), short hairpin RNA (shRNA), small nucleolar RNA (snoRNAs), Piwi-interacting RNA (piRNA), tRNA-derived small RNA (tsRNA) and small rDNA-derived RNA (srRNA). The RNA may be double-stranded RNA or single-stranded RNA. The RNA may be circular RNA. The macromolecular constituent may comprise a protein. The macromolecular constituent may comprise a peptide. The macromolecular constituent may comprise a polypeptide.

The term "molecular tag," as used herein, generally refers to a molecule capable of binding to a macromolecular constituent. The molecular tag may bind to the macromolecular constituent with high affinity. The molecular tag may bind to the macromolecular constituent with high specificity or selectivity. The molecular tag may comprise a nucleotide sequence. The molecular tag may comprise a nucleic acid sequence. The nucleic acid sequence may be at least a portion or an entirety of the molecular tag. The molecular tag may be a nucleic acid molecule or may be part of a nucleic acid molecule. The molecular tag may be an oligonucleotide or a polypeptide. The molecular tag may comprise a DNA aptamer. The molecular tag may be, or comprise, a protein. The molecular tag may comprise a polypeptide. The molecular tag may be a barcode.

The term "partition," as used herein, generally, refers to a space or volume that may be suitable to contain one or more species or conduct one or more reactions. A partition may be a physical compartment, such as a droplet or well. The partition may isolate space or volume from another space or volume. The droplet may be a first phase (e.g., aqueous phase) in a second phase (e.g., oil) immiscible with the first phase. The droplet may be a first phase in a second phase that does not phase separate from the first phase, such as, for example, a capsule or liposome in an aqueous phase. A partition may comprise one or more other (inner) partitions. In some cases, a partition may be a virtual compartment that can be defined and identified by an index (e.g., indexed

libraries) across multiple and/or remote physical compartments. For example, a physical compartment may comprise a plurality of virtual compartments.

The terms “a,” “an,” and “the,” as used herein, generally refers to singular and plural references unless the context clearly dictates otherwise.

Whenever the term “at least,” “greater than,” or “greater than or equal to” precedes the first numerical value in a series of two or more numerical values, the term “at least,” “greater than” or “greater than or equal to” applies to each of the numerical values in that series of numerical values. For example, greater than or equal to 1, 2, or 3 is equivalent to greater than or equal to 1, greater than or equal to 2, or greater than or equal to 3.

Whenever the term “no more than,” “less than,” or “less than or equal to” precedes the first numerical value in a series of two or more numerical values, the term “no more than,” “less than,” or “less than or equal to” applies to each of the numerical values in that series of numerical values. For example, less than or equal to 3, 2, or 1 is equivalent to less than or equal to 3, less than or equal to 2, or less than or equal to 1.

#### Methods for Transposon Loading

Provided herein are methods for processing samples, which may comprise nucleic acid molecules, for analysis. A method of the present disclosure may allow for loading of adapter nucleic acid sequences onto transposases. One or more methods provided herein may allow for loading of a first adapter nucleic acid sequence and a second adapter nucleic acid sequence onto a transposase. In some embodiments, the first adapter nucleic acid sequence may comprise a sequencing primer sequence. In some embodiments, the second adapter nucleic acid sequence may comprise a different sequencing primer sequence. In some embodiments, the first adapter sequence may comprise a target-specific or capture sequence. In some embodiments, the second adapter sequence may comprise a target-specific or capture sequence. In such an embodiment, the first or second adapter sequence may comprise a sequence that may hybridize with a target nucleic acid molecule (e.g., DNA, RNA). Additional adapter sequences (e.g., an adapter comprising a sequencing primer sequence) may be hybridized to the first and/or second adapter sequences. One or more methods provided herein may produce a nucleic acid fragment comprising only the first adapter nucleic acid sequence at one end of the nucleic acid fragment and only the second adapter nucleic acid sequence at the other end of the nucleic acid fragment. One or more methods provided herein may produce a nucleic acid fragment comprising the first adapter nucleic acid sequence at one end of the nucleic acid fragment and the second adapter nucleic acid sequence at the other end of the nucleic acid fragment. One or more methods provided herein may prevent potential nucleic acid fragment loss or exchange during sample processing. Any or all of these methods may be performed within a partition. A product or a plurality of products from a reaction of the methods described herein may be further processed. For example, the product or the plurality of products may be barcoded within or outside a partition. The product or the plurality of products may then be prepared for sequencing.

In an aspect, the present disclosure provides a method for processing a nucleic acid molecule, comprising (a) loading a transposase molecule with a pair of nucleic acid adapters, where each of the pair of nucleic acid adapters comprises a first single-stranded portion comprising a first nucleic acid sequence and a second single-stranded portion comprising a second nucleic acid sequence, where the first nucleic acid

sequence is different from the second nucleic acid sequence; (b) bringing the transposase molecules in contact with the nucleic acid molecule under conditions sufficient to generate a nucleic acid fragment, where the nucleic acid fragment (i) comprises at each of the first end and the second end, both the first nucleic acid sequence and the second nucleic acid sequence and (ii) is at least partially double-stranded; and (c) subjecting the nucleic acid fragment under conditions sufficient to generate a processed nucleic acid fragment, where the processed nucleic acid fragment comprises (i) the first nucleic acid sequence at the first end and the second nucleic acid sequence at the second end, or (ii) the second nucleic acid sequence at the first end and the first nucleic acid sequence at the second end.

In some embodiments, each of the pair of nucleic acid adapter comprises a double-stranded portion. In some embodiments, the double-stranded portion comprises between about 4 and about 50 basepairs in length. For example, the double-stranded portion may comprise about 2, 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides (or basepairs) in length. In some embodiments, the nucleic acid insert may comprise at least about 1, 2, 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides (or basepairs) in length. In some embodiments, the double-stranded portion is 19 basepairs in length. In some embodiments, the double-stranded portion comprises a mosaic end (ME) sequence. In some cases, the first single-stranded portion and the second double-stranded portion are adjacent to a same end of the double-stranded portion. In some cases, the first single-stranded portion and the second single-stranded portion are not attached. In some cases, the first single-stranded portion and the second single-stranded portion are cleavably attached as a loop sequence, or a portion thereof. In such embodiments, the first single-stranded portion and the second single-stranded portion may be cleavably attached by a linker. In one embodiment, the first single-stranded portion and the second single-stranded portion may be cleavably attached by a linker comprising a uracil. In another embodiment, the first single-stranded portion and the second single-stranded portion may be cleavably attached by a linker comprising a restriction enzyme recognition site. In another embodiment, the first single-stranded portion and the second single-stranded portion may be cleavably attached by a chemical linker (e.g., polyethylene glycol (PEG)). In another embodiment, the first single-stranded portion and the second single-stranded portion may be cleavable by application of a stimulus, where the stimulus comprises a photo-stimulus, thermal stimulus, biological stimulus, or chemical stimulus. In some embodiments, (c) comprises cleaving the loop sequence. In some embodiments, (c) comprises cleaving the linker. In some embodiments, the linker comprises a uracil. In some embodiments, the linker comprises PEG. In some embodiments, the linker comprises a restriction enzyme recognition site. In such cases, one or more suitable restriction enzymes may be used. Non-limiting examples of restriction enzymes include: MspI, Nari, BfaI, NdeI, HinP1I, ClaI, MseI, CviQI, TaqI, AcII, RsaI, PmeI, AluI, EcoRV, BstUI, PmeI, DpnI, StuI, HaeIII, HpyCH4V, SfoI, rare-cutter enzymes, e.g., NotI, XmaIII, SstII, SalI, NruI, NheI, Nb.BbvCI, BbvCI, Ascl, AsiSI, Fsel, PacI, PmeI, SbfI, SgrAI, SwaI, BspQI, SapI, SfiI, CspCI, AbsI, CciNI, FspAI, MauBI, MreI, MssI, PalAI, RgaI, RigI, SdaI, SfaAI, SgfI, SgrDI, SgsI, SmI,

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SrfI, Sse2321, Sse83871, LgI, PciSI, AarI, AjI, AloI, BarI, PpiI, Psrl, and any variants thereof.

In some cases, a suitable stimulus may be used to cleave a nucleic acid molecule (e.g., the first single-stranded portion or the second single-stranded portion of the nucleic acid adapter), as described elsewhere herein. In some cases, the nucleic acid molecule (e.g., the first single-stranded portion or the second single-stranded portion of the nucleic acid adapter) may comprise a site that is cleavable upon application of a biological stimulus (e.g., restriction enzyme). In such cases, the first single-stranded portion or the second single-stranded portion may comprise a restriction recognition site and may be cleaved upon addition of one or more restriction enzymes. In some embodiments, the nucleic acid molecule may comprise a linker that is cleavable upon application of a thermal or chemical stimulus. In one non-limiting example, an amino group on a nucleotide (e.g., a dC or dT nucleotide or base pair) may be cleaved. In another non-limiting example, the nucleic acid molecule may comprise a thiol linkage that may be cleaved upon addition of a reducing agent.

The loop sequence can comprise a nucleic acid sequence of any suitable length. In some embodiments, the loop sequence may comprise a continuous nucleic acid sequence. In some embodiments, a loop sequence may comprise a nucleic acid sequence having a double-stranded (e.g., paired or hybridized) and single-stranded (e.g., hairpin) configuration (see, e.g., FIGS. 10A and 10B). In some embodiments, the loop sequence may comprise a continuous nucleic acid sequence of about 10 nucleotides to about 3,500 nucleotides (or basepairs) in length. In another embodiment, the loop sequence can comprise a nucleic acid sequence of about 50 nucleotides to about 500 nucleotides (or basepairs) in length. In another embodiment, the loop sequence can comprise a nucleic acid sequence of about 10 nucleotides to about 100 nucleotides (or basepairs) in length. In some embodiments, the loop sequence can comprise single-stranded DNA. In some embodiments, the loop sequence can comprise double-stranded DNA. In some embodiments, the loop sequence can comprise single-stranded RNA. In some embodiments, the loop sequence can comprise a RNA/DNA hybrid. In some embodiments, the loop sequence may comprise a linker such as, but not limited to, a chemical linker (e.g., polyethylene glycol). In some embodiments, the linker can include a cleavage moiety (e.g., uracil, restriction enzyme recognition site or PEG) to facilitate cleavage of the loop sequence, or a portion thereof, from the nucleic acid adapter. In some embodiments, the loop sequence may comprise a nucleic acid sequence having a greater double-stranded configuration (e.g., 60% base paired) as compared to single-stranded configuration (e.g., 5%) (See, e.g., FIGS. 11A-C).

In some embodiments, (c) comprises filling a gap in the nucleic acid fragment. In some cases, the gap is a 9-base pair (bp) gap. In some embodiments, filling the gap in the nucleic acid fragment comprises contacting the gap with one or more nucleotides (e.g., dNTPs) and a polymerase. In some embodiments, the gap filling includes ligation, e.g., using a ligase.

In some embodiments, one or both (b) and (c) are performed in a partition. In some cases, the partition comprises a droplet. In some cases, the partition comprises a well.

In some embodiments, (b) comprises bringing the transposase molecule in contact with a nucleus of a cell comprising the nucleic acid molecule. In some cases, the cell is permeabilized. In some cases, the nucleus is permeabilized.

In some embodiments, the method further comprises (d) reacting a barcode molecule with the processed nucleic acid

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fragment, where the barcode molecule comprises a sequence complementary to the first nucleic acid sequence or the second nucleic acid sequence, to generate a barcoded nucleic acid fragment. In some cases, the barcode molecule may be ligated to the processed nucleic acid fragment, thereby generating the barcoded nucleic acid fragment. In some cases, the barcode molecule may hybridize to a portion of the processed nucleic acid fragment and be subjected to a nucleic acid extension reaction, thereby generating the barcoded nucleic acid fragment.

In some embodiments, (d) is performed in a partition. In some cases, (d) further comprises filling a gap in the barcoded nucleic acid fragment. In some embodiments, filling the gap in the barcoded nucleic acid fragment comprises contacting the gap with one or more nucleotides (e.g., dNTPs) and a polymerase. In some embodiments, the contacting further includes a ligase.

In some embodiments, the barcode molecule is attached to a bead. In some cases, the barcode molecule is releasably attached to the bead. In some cases, the barcode molecule comprises a common barcode sequence that is common to a plurality of barcode molecules attached to the bead. In some cases, the barcode molecule comprises a functional sequence, as described elsewhere herein. In some cases, the barcode molecule comprises a unique molecular identifier that is unique within a plurality of barcode molecules attached to the bead.

In some embodiments, the method further comprises sequencing the barcoded nucleic acid fragment or a derivative thereof.

In another aspect, the present disclosure provides a method for processing a nucleic acid molecule, comprising (a) loading a transposase molecule with a pair of first nucleic acid adapters, where each of the pair of first nucleic acid adapters comprises a single-stranded portion comprising a first nucleic acid sequence (b) bringing the transposase molecules in contact with the nucleic acid molecule under conditions sufficient to generate a nucleic acid fragment, where the nucleic acid fragment (i) comprises, at each of the first end and the second end, the first nucleic acid sequence and (ii) is at least partially double-stranded; and (c) denaturing the nucleic acid fragment to generate a first single-stranded fragment and a second single-stranded fragment, where each of the first single-stranded fragment and the second single-stranded fragment comprises the first nucleic acid sequence; and (d) bringing the first single-stranded fragment and the second single-stranded fragment in contact with a plurality of second nucleic acid adaptors to generate processed nucleic acid fragments, where the plurality of second nucleic acid adaptors each comprises a second nucleic acid sequence, and where each of the processed nucleic acid fragments comprises the first nucleic acid sequence at the first end and the second nucleic acid sequence at the second end, or the first nucleic acid sequence at the second end and the second nucleic acid sequence at the first end.

In some embodiments, (d) comprises ligating the first single-stranded fragment to a second nucleic acid adapter of the plurality of second nucleic acid adaptors and ligating the second single-stranded fragment to another second nucleic acid adapter of the plurality of second nucleic acid adaptors.

In some embodiments, (d) is performed at a temperature that prevents reannealing of the first single-stranded fragment and the second single-stranded fragment.

The nucleic acid molecule may be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), including variants or derivatives thereof (e.g., single-stranded DNA). In some

cases, the nucleic acid molecule may comprise genomic DNA. In some cases, the nucleic acid molecule may be RNA. The RNA may be coding or non-coding. The RNA may be messenger RNA (mRNA), ribosomal RNA (rRNA) or transfer RNA (tRNA), for example. The RNA may be a transcript. The RNA may be small RNA that are less than 200 nucleic acid bases in length, or large RNA that are greater than 200 nucleic acid bases in length. Small RNAs may include 5.8S ribosomal RNA (rRNA), 5S rRNA, transfer RNA (tRNA), microRNA (miRNA), small interfering RNA (siRNA), short hairpin RNA (shRNA), small nucleolar RNA (snoRNAs), Piwi-interacting RNA (piRNA), tRNA-derived small RNA (tsRNA) and small rDNA-derived RNA (srRNA). The RNA may be double-stranded RNA or single-stranded RNA. The RNA may be circular RNA. Other variants and derivatives of nucleic acid molecules may also be processed and analyzed.

In some cases, the transposase may be loaded with a pair of nucleic acid adapters where each of the pair of nucleic acid adapters comprises a first single-stranded portion comprising a first nucleic acid sequence and a second single-stranded portion comprising a second nucleic acid sequence. The first and second nucleic acid sequences may be a first type of sequencing primer sequence and a second type of sequencing primer sequence, respectively. The pair of nucleic acid adapters may further comprise a loading sequence that allow for loading of the nucleic acid adapters onto the transposase. In some cases, the loading sequence may comprise an inverted repeat sequence. In some cases, in one or more nucleic acid adapters, both the first single-stranded portion comprising the first nucleic acid sequence and the second single-stranded portion comprising the second nucleic acid sequence may each comprise an inverted repeat sequence (also referred to herein as a "mosaic end sequence"). In some cases, the mosaic end sequence of the first single-stranded portion may hybridize with the mosaic end sequence of the second single-stranded portion, forming a "Y-like" nucleic acid adapter. In some embodiments, the transposase may be loaded with two identical species of Y-like nucleic acid adapters, generating a Y-adapter transposase (see, e.g., FIGS. 9A and 9B).

The Y-adapter transposase may then be brought in contact with the nucleic acid molecule under conditions sufficient to generate a nucleic acid fragment, where the nucleic acid fragment (i) comprises at each of the first end and the second end, both the first nucleic acid sequence and the second nucleic acid sequence and (ii) is at least partially double-stranded. In some cases, the conditions sufficient to generate a nucleic acid fragment may comprise a transposition or tagmentation reaction. The nucleic acid fragment from the transposition reaction may be partially double-stranded, and each strand may comprise the first nucleic acid sequence on one end and the second nucleic acid sequence at the other end. The nucleic acid fragment may also comprise a gap region (e.g., approximately 9 base-pairs in length). The nucleic acid fragment may then be subjected to a nucleic acid reaction, e.g., a gap-fill and ligation reaction to generate a gap-filled nucleic acid fragment. The gap-filled nucleic acid fragment may then be subjected under conditions sufficient to generate a processed nucleic acid fragment, where the processed nucleic acid fragment comprises (i) the first nucleic acid sequence at the first end and the second nucleic acid sequence at the second end, or (ii) the second nucleic acid sequence at the first end and the first nucleic acid sequence at the second end.

Gap filling may occur through a variety of mechanisms. In one example, the gap region may be extended using an

enzyme (e.g., a polymerase) to add one or more nucleotides to the gap region. Ligation may then occur, for example, using another enzyme (e.g., ligase). Examples of suitable ligases include, but are not limited to, T4 RNA ligase, T4 DNA ligase, T7 DNA ligase, *E. coli* DNA ligase, Thermostable 5' App DNA/RNA ligase (New England Biolabs, Catalog No. M0319S), or CircLigase™ ssDNA Ligase (Epicentre, Catalog No. CL4111K). In some embodiments, the ligase can comprise a single-stranded DNA ligase.

FIG. 9 schematically illustrates an example of a method for nucleic acid processing. In FIG. 9A, a transposase 902 is loaded with a pair of Y-like nucleic acid adapters 904 to generate a Y-adapter transposase 900. The Y-like nucleic acid adapters may comprise a first and a second nucleic acid strand. The first nucleic acid strand may comprise a first nucleic acid sequence, such as a mosaic end sequence 906, which allows for loading of the nucleic acid on the transposase, and a second nucleic acid sequence 908, which may comprise a first sequencing primer sequence. The second nucleic acid strand may comprise (i) a third nucleic acid sequence, which may be also a mosaic end sequence 906 that is complementary to the mosaic end sequence 906 of the first nucleic acid strand and (ii) a fourth nucleic acid sequence 910, which may comprise a second sequencing primer sequence. In FIG. 9B, the Y-adapter transposase 900 may be brought in contact with a nucleic acid molecule 912. In process 914, the Y-adapter transposase 900 may interact with the nucleic acid molecule 912, e.g., in a transposition reaction, to generate a nucleic acid fragment comprising the mosaic end sequences 906 of the first and the second nucleic acid strands, and, at each end, the first sequencing primer sequence 908 and the second sequencing primer sequence 910. In process 916, the nucleic acid fragment may be subjected to a nucleic acid reaction that allows for gap-fill and ligation to generate a gap-filled nucleic acid fragment 918. The gap-filled nucleic acid fragment can comprise a double-stranded gap-filled nucleic acid fragment. In process 920, the gap-filled nucleic acid fragment 918 may then be further processed. In some cases, further processing comprises an amplification reaction (e.g., PCR). The further processing may generate a processed nucleic acid fragment comprising the second nucleic acid sequence 908 (e.g., comprising the first sequencing primer sequence) on one end and the fourth nucleic acid sequence 910, e.g., comprising the second sequencing primer sequence at the opposite end.

The nucleic acid fragment may be further processed. For instance, the gap-filled nucleic acid fragment 918 or the further processed nucleic acid fragment may be subjected to barcoding, as described elsewhere herein. In such cases, the nucleic acid fragment (e.g., pre- or post-processing) or a derivative thereof (e.g., a complement, an amplified molecule, etc.) may be contacted or reacted with a barcode molecule. The barcode molecule may comprise a sequence that is configured to anneal or hybridize to a portion of the processed nucleic acid fragment or derivative thereof. For example, the barcode molecule may comprise a sequence that is complementary to the first sequencing primer sequence or second sequencing primer sequence. The barcode molecule may be ligated to the nucleic acid fragment or derivative thereof to generate a barcoded nucleic acid fragment. Alternatively or in addition to, the barcode molecule and the nucleic acid fragment or derivative thereof may be subjected to a nucleic acid extension reaction to generate a barcoded nucleic acid fragment. For a description of example barcoding schemes using, e.g., linear amplification or ligation, see, e.g., U.S. Pat. Pub. 20180340171, which is hereby incorporated by reference in its entirety.

One or more processes described herein, e.g., as shown in FIG. 9B, may be performed in a partition (e.g., droplet or well). For example, processes 914, 916, and 920 and the further processing (e.g., barcoding) may be performed in a partition. In some instances, only certain operations are performed in a partition. For example, process 914 may be performed outside a partition (e.g., in bulk), whereas processes 916 and 920 may be performed in a partition. The further processing (e.g., amplification or barcoding) may also be performed in the same or a different partition.

In some cases, the transposase may be loaded with a pair of nucleic acid adapters, where each of the pair of nucleic acid adapters comprises a first single-stranded portion comprising a first nucleic acid sequence and a second single-stranded portion comprising a second nucleic acid sequence, and where the first nucleic acid sequence is connected to the second nucleic acid sequence. The first nucleic acid and the second nucleic acid sequences may be a first type of sequencing primer sequence and a second type of sequencing primer sequence, respectively. The pair of nucleic acid adapters may further comprise a loading sequence that allow for loading of the nucleic acid adapters onto the transposase. In some cases, the loading sequence may comprise an inverted repeat sequence (e.g., "mosaic end sequence"). In some cases, in one or more nucleic acid adapters, both the first single-stranded portion comprising the first nucleic acid sequence and the second single-stranded portion comprising the second nucleic acid sequence may each comprise a mosaic end sequence. In some cases, the mosaic end sequence of the first single-stranded portion may hybridize with the mosaic end sequence of the second single-stranded portion, and the first nucleic acid sequence is connected to the second nucleic acid sequence, forming a hairpin nucleic acid adapter. In some cases, the first single-stranded portion and the second single-stranded portion of the hairpin nucleic acid adapter may be cleavably attached as a loop sequence. In some embodiments, the loop sequence may comprise a uracil that may be excised using an enzyme, as described elsewhere herein. In some embodiments, the loop sequence may be cleavable by application of a stimulus, as described elsewhere herein. In some embodiments, the transposase may be loaded with two identical species of hairpin nucleic acid adapters, generating a hairpin-adapter transposase (See, FIGS. 10A-10C).

The hairpin-adapter transposase may then be brought in contact with the nucleic acid molecule under conditions sufficient to generate a nucleic acid fragment, where the nucleic acid fragment (i) comprises at each of the first end and the second end, both the first nucleic acid sequence and the second nucleic acid sequence and (ii) is at least partially double-stranded. In some cases, the conditions sufficient to generate a nucleic acid fragment may comprise a transposition reaction. The nucleic acid fragment from the transposition reaction may be partially double-stranded, and each strand may comprise the first nucleic acid sequence on one end and the second nucleic acid sequence at the other end. The nucleic acid fragment may also comprise a gap region (e.g., approximately 9 base-pairs in length). The nucleic acid fragment may then be subjected to a nucleic acid reaction, e.g., gap-fill and ligation reaction to generate a gap-filled nucleic acid fragment. At any convenient point in the process (e.g., after gap-fill and ligation), the first nucleic acid sequence and the second nucleic acid sequence may be disconnected, e.g., via cleavage. Subsequent to the disconnection process, the nucleic acid fragment may comprise a nucleic acid molecule that is partially hybridized (i.e., double-stranded). The gap-filled, cleaved nucleic acid frag-

ment may then be subjected to conditions sufficient to generate a processed nucleic acid fragment, where the processed nucleic acid fragment comprises (i) the first nucleic acid sequence at the first end and the second nucleic acid sequence at the second end, or (ii) the second nucleic acid sequence at the first end and the first nucleic acid sequence at the second end.

Gap filling may occur through a variety of mechanisms. In one example, the gap region may be extended using an enzyme (e.g., a polymerase) to add one or more nucleotides to the gap region. Ligation may then occur, for example, using another enzyme (e.g., ligase).

Cleavage of the nucleic acid fragment may occur through a variety of strategies. In one non-limiting example, a uracil base may be included in the loop sequence of the hairpin adapter, e.g., between the first nucleic acid sequence and the second nucleic acid sequence. Cleavage may then be initiated, for example, using a polyamine (e.g., DMED) or an enzyme, e.g., a uracil glycosylase. In another non-limiting example, a restriction sequence may be included in the hairpin adapter, e.g., between the first nucleic acid sequence and the second nucleic acid sequence. Cleavage may then occur using a restriction enzyme. In other non-limiting examples, the first nucleic acid sequence and the second nucleic acid sequence may be linked by a labile linkage, such that exposure to a stimulus (e.g., photo, thermal, chemical, or biological) may result in cleavage of the hairpin nucleic acid adapter.

FIG. 10 schematically illustrates an example of a method 30 for nucleic acid processing. In FIG. 10A, a transposase 1002 is loaded with a pair of hairpin nucleic acid adapters 1004 to generate a hairpin transposase 1000. The hairpin nucleic acid adapters may comprise a first and a second nucleic acid strand. The first nucleic acid strand may comprise a first 35 nucleic acid sequence, such as a mosaic end sequence 1006, which allows for loading of the nucleic acid on the transposase, and a second nucleic acid sequence 1008, such as a first sequencing primer sequence. The second nucleic acid strand may comprise (i) a third nucleic acid sequence, which 40 may be also a mosaic end sequence 1006 that is complementary to the mosaic end sequence 1006 of the first nucleic acid strand and (ii) a fourth nucleic acid sequence 1010, which may comprise a second sequencing primer sequence. The second nucleic acid sequence and the fourth nucleic 45 acid sequence, which may comprise the first and second sequencing primer sequences, respectively, may be connected via a linker 1011, as shown in FIG. 10B. In FIG. 10C, the hairpin transposase 1000 may be brought in contact with a nucleic acid molecule 1012. In process 1014, the hairpin transposase 1000 may interact with the nucleic acid molecule 1012, e.g., in a transposition reaction, to generate a 50 nucleic acid fragment comprising the mosaic end sequences 1006 of the first and the second nucleic acid strands, and, at each end, the second nucleic acid sequence 1008 (e.g., comprising the first sequencing primer sequence) and the fourth nucleic acid sequence 1010, (e.g., comprising the second sequencing primer sequence). In process 1016, the nucleic acid fragment may be subjected to a nucleic acid 55 reaction that allows for gap-fill and ligation to generate a gap-filled nucleic acid fragment 1018. In some cases, process 1019 may occur following gap-fill and may comprise cleavage of the linker 1011 between the second 1008 and fourth 1010 nucleic acid sequences. In other embodiments, process 1019 may occur at any convenient operation in the 60 process. In process 1020, further processing may occur. In some cases, further processing comprises an amplification reaction (e.g., PCR). The further processing may generate a 65

processed nucleic acid fragment comprising the second nucleic acid sequence **1008** (e.g., comprising the first sequencing primer sequence) on one end and the fourth nucleic acid sequence, **1010** (e.g., comprising the second sequencing primer sequence) at the opposite end.

In some cases, the nucleic acid fragment may be further processed. For instance, the gap-filled nucleic acid fragment **1018** or the further processed nucleic acid fragment may be subjected to barcoding, as described elsewhere herein. In such cases, the nucleic acid fragment (e.g., pre- or post-processing) or a derivative thereof (e.g., a complement, an amplified molecule, etc.) may be contacted or reacted with a barcode molecule. The barcode molecule may comprise a sequence that is configured to anneal or hybridize to a portion of the processed nucleic acid fragment or derivative thereof. For example, the barcode molecule may comprise a sequence that is complementary to the first sequencing primer sequence or second sequencing primer sequence. The barcode molecule may be ligated to the nucleic acid fragment or derivative thereof to generate a barcoded nucleic acid fragment. Alternatively or in addition to, the barcode molecule and the nucleic acid fragment or derivative thereof may be subjected to a nucleic acid extension reaction to generate a barcoded nucleic acid fragment. For a description of example barcoding schemes using, e.g., linear amplification or ligation, see, e.g., U.S. Pat. Pub. 20180340171, which is hereby incorporated by reference in its entirety.

One or more processes described herein, e.g., as shown in FIG. 10C, may be performed in a partition (e.g., droplet or well). For example, processes **1014**, **1016**, **1019**, and **1020** and the further processing (e.g., barcoding) may be performed in a partition. In some instances, only certain operations are performed in a partition. For example, process **1014** may be performed outside a partition (e.g., in bulk), whereas processes **1016** and **1020** may be performed in a partition. The further processing (e.g., amplification or barcoding) may also be performed in the same or a different partition.

In some cases, the transposase may be loaded with a pair of nucleic acid adapters, where each of the pair of nucleic acid adapters comprises a first single-stranded portion comprising a first nucleic acid sequence and a second single-stranded portion comprising a second nucleic acid sequence, and where the pair of nucleic acid adapters are connected. The first and second nucleic acid sequences may be a first type of sequencing primer sequence and a second type of sequencing primer sequence, respectively. The pair of nucleic acid adapters may further comprise a loading sequence that allow for loading of the nucleic acid adapters onto the transposase. In some cases, the loading sequence may comprise an inverted repeat sequence (e.g., “mosaic end sequence”). In some cases, in one or more nucleic acid adapters, both the first single-stranded portion comprising the first nucleic acid sequence and the second single-stranded portion comprising the second nucleic acid sequence may each comprise a mosaic end sequence. In some cases, the mosaic end sequence of the first single-stranded portion may hybridize with the mosaic end sequence of the second single-stranded portion, and (i) the first nucleic acid sequence of one of the nucleic acid adapters is connected to the second nucleic acid sequence of the other nucleic acid adapter, forming a hairpin nucleic acid adapter and (ii) the second nucleic acid sequence of one of the nucleic acid adapters is connected to the first nucleic acid sequence of the other nucleic acid adapter. In some embodiments, the transposase may be loaded with the connected

pair of nucleic acid adapters, generating a continuous-adapter transposase (See, FIGS. 11A-11B).

The continuous-adapter transposase may then be brought in contact with the nucleic acid molecule under conditions sufficient to generate a nucleic acid fragment, where the nucleic acid fragment (i) comprises at each of the first end and the second end, both the first nucleic acid sequence and the second nucleic acid sequence and (ii) is at least partially double-stranded. In some cases, the conditions sufficient to generate a nucleic acid fragment may comprise a transposition reaction. The nucleic acid fragment may also comprise a gap region (e.g., approximately 9 base-pairs in length). The nucleic acid fragment may then be subjected to a nucleic acid reaction, e.g., gap-fill and ligation reaction to generate a gap-filled nucleic acid fragment. At any convenient point in the process, the pair of nucleic acid adapters may be disconnected, e.g., via cleavage. The nucleic acid fragment may be subjected under conditions sufficient to generate a processed nucleic acid fragment, where the processed nucleic acid fragment comprises the first nucleic acid sequence at the first end and the second nucleic acid sequence at the second end, or (ii) the second nucleic acid sequence at the first end and the first nucleic acid sequence at the second end. In some embodiments, the continuous-adapter transposase can comprise a nucleic acid sequence (e.g., a nucleic acid insert) flanked by a mosaic end sequence. The nucleic acid insert can comprise any suitable length. In some embodiments, the nucleic acid insert can be prepared such that it is suitable for incorporation into the genome of an animal, such as a mammal, e.g., via a transposition reaction. In some embodiments, the nucleic acid insert can comprise about 10 nucleotides to about 3,500 nucleotides (or basepairs) in length. For example, the nucleic acid insert may comprise about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, or 5000 nucleotides (or basepairs) in length. In some embodiments, the nucleic acid insert may comprise at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, or 5000 nucleotides (or basepairs) in length. In some embodiments, the nucleic acid insert may comprise at most 5000, 4500, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 nucleotides (or basepairs) in length. In another embodiment, the nucleic acid insert can comprise about 50 nucleotides to about 500 nucleotides (or basepairs) in length. In another embodiment, the nucleic acid insert can comprise about 10 nucleotides to about 1 kilobase in length. In some embodiments, the nucleic acid insert can comprise single-stranded DNA. In some embodiments, the nucleic acid insert can comprise double-stranded DNA. In some embodiments, the nucleic acid insert can comprise single-stranded RNA. In some embodiments, the nucleic acid insert can comprise a RNA/DNA hybrid. In some embodiments, the nucleic acid insert may comprise a single-stranded nucleic acid sequence and a double-stranded nucleic acid sequence. In some embodiments, the nucleic acid insert can further comprise a linker, such as but not limited to a chemical linker. In some cases, the linker may comprise a linear polymer material, such as a linear polyacrylamide, poly-ethylene glycol (PEG), (PEG)-diacrylate, PEG-acrylate, PEG-thiol, PEG-azide, PEG-alkyne, other acrylates or other linear polymeric material, as described elsewhere herein. In some cases, the linker may be cleavable upon application of a stimulus.

Gap filling may occur through a variety of mechanisms. In one example, the gap region may be extended using an enzyme (e.g., a polymerase) to add one or more nucleotides to the gap region. Ligation may then occur, for example, using another enzyme (e.g., ligase). In some embodiments, the gap filling may comprise a polymerizing enzyme (e.g., a reverse transcriptase or polymerase) and a ligase. In some aspects, the gap filling does not include a polymerizing enzyme having strand displacement activity. In some aspects, the gap filling does not include a polymerizing enzyme having exonuclease activity. In some aspects, the gap filling does not include a polymerizing enzyme having strand displacement and exonuclease activity.

Cleavage of the nucleic acid fragment may occur through a variety of strategies. In one non-limiting example, a uracil base may be included in the hairpin adapter, e.g., between the first nucleic acid sequence and the second nucleic acid sequence. Cleavage may then be initiated, for example, using a polyamine (e.g., DMED) or an enzyme, e.g., uracil-n-glycosylase. In another non-limiting example, a restriction sequence may be included in the hairpin adapter, e.g., between the first nucleic acid sequence and the second nucleic acid sequence. Cleavage may then occur using a restriction enzyme. In other non-limiting examples, the first nucleic acid sequence and the second nucleic acid sequence may be linked by a labile linkage, such that exposure to a stimulus (e.g., photo, thermal, chemical, or biological) may result in cleavage of the hairpin nucleic acid adapter.

FIG. 11 schematically illustrates an example of a method for nucleic acid processing. In FIG. 11A, a transposase **1102** is loaded with a connected pair of nucleic acid adapters **1104** to generate a continuous-adapter transposase **1100**. The continuous-adapter transposase **1100** may comprise a first nucleic acid strand comprising a first nucleic acid sequence, such as a mosaic end sequence **1106**, which allows for loading of the nucleic acid on the transposase, and a second nucleic acid sequence **1108**, such as a first sequencing primer sequence. The second nucleic acid strand may comprise (i) a third nucleic acid sequence, which may be also a mosaic end sequence **1006** that is complementary to the mosaic end sequence **1006** of the first nucleic acid strand and (ii) a fourth nucleic acid sequence **1010**, which may comprise a second sequencing primer sequence. The second nucleic acid sequence and the fourth nucleic acid sequence of the first nucleic acid adapter, which may comprise the first and second sequencing primer sequences, respectively, may be connected to the fourth nucleic acid sequence and second nucleic acid sequence of the second nucleic acid adapter via a nucleic acid sequence **1113** and a cleavable linker **1111**. In FIG. 11B, the continuous-adapter transposase **1100** may be brought in contact with a nucleic acid molecule **1112**. In process **1114**, the continuous-adapter transposase **1100** may interact with the nucleic acid molecule **1112**, e.g., in a transposition reaction, to generate a nucleic acid fragment comprising the mosaic end sequences **1106** of the first and the second nucleic acid strands and the second nucleic acid sequence **1108** (e.g., comprising the first sequencing primer sequence) and the fourth nucleic acid sequence **1110**, (e.g., comprising the second sequencing primer sequence). The second nucleic acid sequence **1108** and the fourth nucleic acid sequence **1110** may be adjacent to the fourth nucleic acid sequence **1110** and the second nucleic acid **1108**, respectively. In process **1116**, the nucleic acid fragment may be subjected to a nucleic acid reaction that allows for gap-fill and ligation to generate a gap-filled nucleic acid fragment. In some cases, process **1119** may occur following gap-fill and may comprise cleavage of the linker **1111** between the

pair of nucleic acid adapters **1104**. For example, the linker **1111** may comprise one or more uracil moieties, and the linker **1111** may be cleaved using an enzyme (e.g., uracil glycosylase). In other embodiments, process **1119** may occur at any convenient operation in the process. In process **1120**, further processing may occur. In some cases, further processing comprises an amplification reaction (e.g., PCR). The further processing may generate a processed nucleic acid fragment comprising the second nucleic acid sequence **1108** (e.g., comprising the first sequencing primer sequence) on one end and the fourth nucleic acid sequence, **1110** (e.g., comprising the second sequencing primer sequence) at the opposite end.

In some cases, the nucleic acid fragment may be further processed. For instance, the gap-filled nucleic acid fragment **1118** or the further processed nucleic acid fragment may be subjected to barcoding, as described elsewhere herein. In such cases, the nucleic acid fragment (e.g., pre- or post-processing) or a derivative thereof (e.g., a complement, an amplified molecule, etc.) may be contacted or reacted with a barcode molecule. The barcode molecule may comprise a sequence that is configured to anneal or hybridize to a portion of the processed nucleic acid fragment or derivative thereof. For example, the barcode molecule may comprise a sequence that is complementary to the first sequencing primer sequence or second sequencing primer sequence. The barcode molecule may be ligated to the nucleic acid fragment or derivative thereof to generate a barcoded nucleic acid fragment. Alternatively or in addition to, the barcode molecule and the nucleic acid fragment or derivative thereof may be subjected to a nucleic acid extension reaction to generate a barcoded nucleic acid fragment. For a description of example barcoding schemes using, e.g., linear amplification or ligation, see, e.g., U.S. Pat. Pub. 20180340171, which is hereby incorporated by reference in its entirety.

One or more processes described herein, e.g., as shown in FIG. 11C, may be performed in a partition (e.g., droplet or well). For example, processes **1114**, **1116**, **1119**, and **1120** and the further processing (e.g., barcoding) may be performed in a partition. In some instances, only certain operations are performed in a partition. For example, process **1114** may be performed outside a partition (e.g., in bulk), whereas processes **1116** and **1120** may be performed in a partition. The further processing (e.g., amplification or barcoding) may also be performed in the same or a different partition.

In some cases, the transposase may be loaded with a pair of first nucleic acid adapters, where each of the pair of first nucleic acid adapters comprises a single-stranded portion comprising a first nucleic acid sequence. The first nucleic acid sequences may comprise a first type of sequencing primer sequence. The pair of first nucleic acid adapters may further comprise a loading sequence that allow for loading of the nucleic acid adapters onto the transposase. In some cases, the loading sequence may comprise an inverted repeat sequence (e.g., “mosaic end sequence”). In some cases, the mosaic end sequence is double-stranded. In some cases, the pair of first nucleic acid adapters loaded onto the transposase may be identical, forming a single-adapter transposase. The single-adapter transposase may then be brought in contact with the nucleic acid molecule under conditions sufficient to generate a nucleic acid fragment, where the nucleic acid fragment (i) comprises at each of the first end and the second end, the first nucleic acid and (ii) is at least partially double-stranded. In some cases, the conditions sufficient to generate a nucleic acid fragment may comprise a transposition reaction. The nucleic acid fragment may also comprise a gap region (e.g., approximately 9 base-pairs in length). The

nucleic acid fragment may then be subjected to a nucleic acid reaction, e.g., gap-fill and ligation reaction to generate a gap-filled nucleic acid fragment. The nucleic acid fragment may be subjected under conditions sufficient to denature the nucleic acid fragment to generate a first single-stranded fragment and a second single-stranded fragment, where each of the first single-stranded fragment and the second single-stranded fragment comprises the first nucleic acid sequence. The first single-stranded fragment and the second single-stranded fragment may then be brought in contact with a plurality of second nucleic acid adapters to generate processed nucleic acid fragments, where the plurality of second nucleic acid adapters each comprises a second nucleic acid sequence (e.g., a second sequencing primer sequence), and where the processed nucleic acid fragments comprises the first nucleic acid sequence at the first end (e.g., the first sequencing primer sequence) and the second nucleic acid sequence (e.g., the second sequencing primer sequence) at the second end, or (ii) the second nucleic acid sequence at the first end and the first nucleic acid sequence at the second end. In some embodiments, processing of the nucleic acid fragments comprises ligation of the second nucleic acid adapters to the single-stranded nucleic acid fragments.

Ligation of the nucleic acid adapters may occur through a variety of mechanisms. In some cases, the nucleic acid adapter may comprise a reactive moiety. Similarly, the first single-stranded fragment and the second single-stranded fragment may each comprise a second reactive moiety. A reactive moiety may be selected from the non-limiting group consisting of azides, alkynes, nitrones (e.g., 1,3-nitrones), strained alkenes (e.g., trans-cycloalkenes such as cyclooctenes or oxanorbornadiene), tetrazines, tetrazoles, iodides, thioates (e.g., phosphorothioate), acids, amines, and phosphates. For example, the first reactive moiety may comprise an azide moiety, and the second reactive moiety may comprise an alkyne moiety. The first and second reactive moieties may react to form a linking moiety. A reaction between the first and second reactive moieties may be, for example, a cycloaddition reaction such as a strain-promoted azide-alkyne cycloaddition, a copper-catalyzed azide-alkyne cycloaddition, a strain-promoted alkyne-nitrona cycloaddition, a Diels-Alder reaction, a [3+2] cycloaddition, a [4+2] cycloaddition, or a [4+1] cycloaddition; a thiol-ene reaction; a nucleophilic substitution reaction; or another reaction. In some cases, reaction between the first and second reactive moieties may yield a triazole moiety or an isoxazoline moiety. A reaction between the first and second reactive moieties may involve subjecting the reactive moieties to suitable conditions such as a suitable temperature, pH, or pressure and providing one or more reagents or catalysts for the reaction. For example, a reaction between the first and second reactive moieties may be catalyzed by a copper catalyst, a ruthenium catalyst, or a strained species such as a difluorooctyne, dibenzylcyclooctyne, or biarylazacyclooctynone. In some embodiments, reaction between a first reactive moiety of the nucleic acid adapter and a second reactive moiety of the first single-stranded fragment may link the nucleic acid adapter and the first single-stranded fragment to form an adapter-linked nucleic acid molecule. In some embodiments, reaction between a first reactive moiety of the nucleic acid adapter and a second reactive moiety of the second single-stranded fragment may link the nucleic acid adapter and the second single-stranded fragment to form an adapter-linked nucleic acid molecule. Upon linking, the nucleic acid adapter and the first single-stranded fragment or the second single-stranded nucleic acid fragment may be considered ligated. In some embodiments, the

nucleic acid adapter may be linked to an at least partially double-stranded nucleic acid fragment. Accordingly, reaction of the first and second reactive moieties may comprise a chemical ligation reaction such as a copper-catalyzed 5' azide to 3' alkyne "click" chemistry reaction to form a triazole linkage between the nucleic acid adapter and the first single-stranded fragment or the second single-stranded fragment. In other non-limiting examples, an iodide moiety may be chemically ligated to a phosphorothioate bond, an acid may be ligated to an amine to form an amide bond, and/or a phosphate and amine may be ligated to form a phosphoroamidate bond.

In some embodiments, the nucleic acid adapter may be ligated to the first single-stranded fragment or the second single-stranded fragment. The first or the second single-stranded fragment and the nucleic acid adapter may be subjected to an enzymatic ligation reaction, using a ligase, e.g., SplintR ligases, T4 ligases, Mu polymerase, PBCV1 enzymes, and/or any combinations, derivatives, and variants thereof. In some embodiments, ribonucleotides are ligated between the adapter and the first or the second single-stranded fragments. In some embodiments, deoxyribonucleotides are ligated between the adapter and the first or the second single-stranded fragments. In some embodiments, the nucleic acid adapter may be ligated to a double-stranded fragment. In some embodiments, the nucleic acid adapter may be double-stranded.

FIG. 12 schematically illustrates an example of a method for nucleic acid processing. In FIG. 12A, a transposase 1202 is loaded with a pair of nucleic acid adapters 1204 to generate a single-adapter transposase 1200. The nucleic acid adapters 1204 may comprise a first and a second nucleic acid strand. The first nucleic acid strand may comprise a first nucleic acid sequence, such as a mosaic end sequence 1206, which allows for loading of the nucleic acid on the transposase, and a second nucleic acid sequence 1210, which may comprise a first sequencing primer sequence. The second nucleic acid strand may comprise a mosaic end sequence 1206 that is complementary to the mosaic end sequence 1206 of the first nucleic acid strand. In FIG. 12B, the single-adapter transposase 1200 may be brought in contact with a nucleic acid molecule 1212. In process 1214, the single-adapter transposase 1200 may interact with the nucleic acid molecule 1212, e.g., in a transposition reaction, to generate a nucleic acid fragment comprising the mosaic end sequences 1206 of the first and the second nucleic acid strands, and, at each end, the first sequencing primer sequence 1210. In process 1216, the nucleic acid fragment may be subjected to a nucleic acid reaction that allows for denaturation of the nucleic acid fragment into single-stranded nucleic acid fragments 1217. In process 1219, a second nucleic acid sequence 1218 (e.g., comprising a second sequencing primer site) may be added (e.g., ligated) to the single-stranded nucleic acid fragments 1217. In some cases, process 1219 comprises the use of enzyme (e.g., a DNA ligase). In other embodiments, click-chemistry or other ligation strategies may be employed. In process 1220, further processing (e.g., PCR) may occur to generate nucleic acid fragments that comprise the first nucleic acid sequence 1210 (e.g., the first sequencing primer sequence) at the first end and the sequence of the second nucleic acid sequence 1218 (e.g., the second sequencing primer sequence) at the second end 1220, or the first nucleic acid sequence 1210 at the second end and the second nucleic acid sequence 1218 at the first end 1220.

In some cases, the nucleic acid fragment may be further processed. For instance, the gap-filled nucleic acid fragment

**1218** or the further processed nucleic acid fragment may be subjected to barcoding, as described elsewhere herein. In such cases, the nucleic acid fragment (e.g., pre- or post-processing) or a derivative thereof (e.g., a complement, an amplified molecule, etc.) may be contacted or reacted with a barcode molecule. The barcode molecule may comprise a sequence that is configured to anneal or hybridize to a portion of the processed nucleic acid fragment or derivative thereof. For example, the barcode molecule may comprise a sequence that is complementary to the first sequencing primer sequence or second sequencing primer sequence. The barcode molecule may be ligated to the nucleic acid fragment or derivative thereof to generate a barcoded nucleic acid fragment. Alternatively or in addition to, the barcode molecule and the nucleic acid fragment or derivative thereof may be subjected to a nucleic acid extension reaction to generate a barcoded nucleic acid fragment. For a description of example barcoding schemes using, e.g., linear amplification or ligation, see, e.g., U.S. Pat. Pub. 20180340171, which is hereby incorporated by reference in its entirety.

One or more processes described herein, e.g., as shown in FIG. 12C, may be performed in a partition (e.g., droplet or well). For example, processes **1214**, **1216**, **1219**, and **1220** and the further processing (e.g., barcoding) may be performed in a partition. In some instances, only certain operations are performed in a partition. For example, process **1214** may be performed outside a partition (e.g., in bulk), whereas processes **1216** and **1220** may be performed in a partition. The further processing (e.g., amplification or barcoding) may also be performed in the same or a different partition.

In some cases, and in reference to FIGS. 9-12, the barcode molecule may be single-stranded. In other cases, the barcode molecules may be double-stranded or partially double-stranded. In some cases, the barcoding may be performed in a partition. In some embodiments, one or more operations in the methods provided herein may be performed in a partition. In other embodiments, one or more operations in the methods provided herein may be performed outside a partition (e.g., in bulk).

#### Systems and Methods for Sample Compartmentalization

In an aspect, the systems and methods described herein provide for the compartmentalization, depositing, or partitioning of one or more particles (e.g., biological particles, macromolecular constituents of biological particles, beads, reagents, etc.) into discrete compartments or partitions (referred to interchangeably herein as partitions), where each partition maintains separation of its own contents from the contents of other partitions. The partition can be a droplet in an emulsion. A partition may comprise one or more other partitions.

A partition may include one or more particles. A partition may include one or more types of particles. For example, a partition of the present disclosure may comprise one or more biological particles and/or macromolecular constituents thereof. A partition may comprise one or more gel beads. A partition may comprise one or more cell beads. A partition may include a single gel bead, a single cell bead, or both a single cell bead and single gel bead. A partition may include one or more reagents. Alternatively, a partition may be unoccupied. For example, a partition may not comprise a bead. A cell bead can be a biological particle and/or one or more of its macromolecular constituents encased inside of a gel or polymer matrix, such as via polymerization of a droplet containing the biological particle and precursors capable of being polymerized or gelled. Unique identifiers, such as barcodes, may be injected into the droplets previous

to, subsequent to, or concurrently with droplet generation, such as via a microcapsule (e.g., bead), as described elsewhere herein. Microfluidic channel networks (e.g., on a chip) can be utilized to generate partitions as described herein. Alternative mechanisms may also be employed in the partitioning of individual biological particles, including porous membranes through which aqueous mixtures of cells are extruded into non-aqueous fluids.

The partitions can be flowed within fluid streams. The partitions may comprise, for example, micro-vesicles that have an outer barrier surrounding an inner fluid center or core. In some cases, the partitions may comprise a porous matrix that is capable of entraining and/or retaining materials within its matrix. The partitions can be droplets of a first phase within a second phase, wherein the first and second phases are immiscible. For example, the partitions can be droplets of aqueous fluid within a non-aqueous continuous phase (e.g., oil phase). In another example, the partitions can be droplets of a non-aqueous fluid within an aqueous phase. In some examples, the partitions may be provided in a water-in-oil emulsion or oil-in-water emulsion. A variety of different vessels are described in, for example, U.S. Patent Application Publication No. 2014/0155295, which is entirely incorporated herein by reference for all purposes. Emulsion systems for creating stable droplets in non-aqueous or oil continuous phases are described in, for example, U.S. Patent Application Publication No. 2010/0105112, which is entirely incorporated herein by reference for all purposes.

In the case of droplets in an emulsion, allocating individual particles to discrete partitions may in one non-limiting example be accomplished by introducing a flowing stream of particles in an aqueous fluid into a flowing stream of a non-aqueous fluid, such that droplets are generated at the junction of the two streams. Fluid properties (e.g., fluid flow rates, fluid viscosities, etc.), particle properties (e.g., volume fraction, particle size, particle concentration, etc.), microfluidic architectures (e.g., channel geometry, etc.), and other parameters may be adjusted to control the occupancy of the resulting partitions (e.g., number of biological particles per partition, number of beads per partition, etc.). For example, partition occupancy can be controlled by providing the aqueous stream at a certain concentration and/or flow rate of particles. To generate single biological particle partitions, the relative flow rates of the immiscible fluids can be selected such that, on average, the partitions may contain less than one biological particle per partition in order to ensure that those partitions that are occupied are primarily singly occupied. In some cases, partitions among a plurality of partitions may contain at most one biological particle (e.g., bead, DNA, cell or cellular material). In some embodiments, the various parameters (e.g., fluid properties, particle properties, microfluidic architectures, etc.) may be selected or adjusted such that a majority of partitions are occupied, for example, allowing for only a small percentage of unoccupied partitions. The flows and channel architectures can be controlled as to ensure a given number of singly occupied partitions, less than a certain level of unoccupied partitions and/or less than a certain level of multiply occupied partitions.

FIG. 1 shows an example of a microfluidic channel structure **100** for partitioning individual biological particles. The channel structure **100** can include channel segments **102**, **104**, **106** and **108** communicating at a channel junction **110**. In operation, a first aqueous fluid **112** that includes suspended biological particles (or cells) **114** may be transported along channel segment **102** into junction **110**, while

a second fluid 116 that is immiscible with the aqueous fluid 112 is delivered to the junction 110 from each of channel segments 104 and 106 to create discrete droplets 118, 120 of the first aqueous fluid 112 flowing into channel segment 108, and flowing away from junction 110. The channel segment 108 may be fluidically coupled to an outlet reservoir where the discrete droplets can be stored and/or harvested. A discrete droplet generated may include an individual biological particle 114 (such as droplets 118). A discrete droplet generated may include more than one individual biological particle 114 (not shown in FIG. 1). A discrete droplet may contain no biological particle 114 (such as droplet 120). Each discrete partition may maintain separation of its own contents (e.g., individual biological particle 114) from the contents of other partitions.

The second fluid 116 can comprise an oil, such as a fluorinated oil, that includes a fluorosurfactant for stabilizing the resulting droplets, for example, inhibiting subsequent coalescence of the resulting droplets 118, 120. Examples of particularly useful partitioning fluids and fluorosurfactants are described, for example, in U.S. Patent Application Publication No. 2010/0105112, which is entirely incorporated herein by reference for all purposes.

As will be appreciated, the channel segments described herein may be coupled to any of a variety of different fluid sources or receiving components, including reservoirs, tubing, manifolds, or fluidic components of other systems. As will be appreciated, the microfluidic channel structure 100 may have other geometries. For example, a microfluidic channel structure can have more than one channel junction. For example, a microfluidic channel structure can have 2, 3, 4, or 5 channel segments each carrying particles (e.g., biological particles, cell beads, and/or gel beads) that meet at a channel junction. Fluid may be directed to flow along one or more channels or reservoirs via one or more fluid flow units. A fluid flow unit can comprise compressors (e.g., providing positive pressure), pumps (e.g., providing negative pressure), actuators, and the like to control flow of the fluid. Fluid may also or otherwise be controlled via applied pressure differentials, centrifugal force, electrokinetic pumping, vacuum, capillary or gravity flow, or the like.

The generated droplets may comprise two subsets of droplets: (1) occupied droplets 118, containing one or more biological particles 114, and (2) unoccupied droplets 120, not containing any biological particles 114. Occupied droplets 118 may comprise singly occupied droplets (having one biological particle) and multiply occupied droplets (having more than one biological particle). As described elsewhere herein, in some cases, the majority of occupied partitions can include no more than one biological particle per occupied partition and some of the generated partitions can be unoccupied (of any biological particle). In some cases, though, some of the occupied partitions may include more than one biological particle. In some cases, the partitioning process may be controlled such that fewer than about 25% of the occupied partitions contain more than one biological particle, and in many cases, fewer than about 20% of the occupied partitions have more than one biological particle, while in some cases, fewer than about 10% or even fewer than about 5% of the occupied partitions include more than one biological particle per partition.

In some cases, it may be desirable to minimize the creation of excessive numbers of empty partitions, such as to reduce costs and/or increase efficiency. While this minimization may be achieved by providing a sufficient number of biological particles (e.g., biological particles 114) at the partitioning junction 110, such as to ensure that at least one

biological particle is encapsulated in a partition, the Poissonian distribution may expectedly increase the number of partitions that include multiple biological particles. As such, where singly occupied partitions are to be obtained, at most about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or less of the generated partitions can be unoccupied.

In some cases, the flow of one or more of the biological particles (e.g., in channel segment 102), or other fluids 10 directed into the partitioning junction (e.g., in channel segments 104, 106) can be controlled such that, in many cases, no more than about 50% of the generated partitions, no more than about 25% of the generated partitions, or no more than about 10% of the generated partitions are unoccupied. These flows can be controlled so as to present a non-Poissonian distribution of single-occupied partitions while providing lower levels of unoccupied partitions. The above noted ranges of unoccupied partitions can be achieved while still providing any of the single occupancy rates 15 described above. For example, in many cases, the use of the 20 systems and methods described herein can create resulting partitions that have multiple occupancy rates of less than about 25%, less than about 20%, less than about 15%, less than about 10%, and in many cases, less than about 5%, 25 while having unoccupied partitions of less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 10%, less than about 5%, or less.

As will be appreciated, the above-described occupancy 30 rates are also applicable to partitions that include both biological particles and additional reagents, including, but not limited to, microcapsules or beads (e.g., gel beads) carrying barcoded nucleic acid molecules (e.g., oligonucleotides) (described in relation to FIG. 2). The occupied 35 partitions (e.g., at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% of the occupied partitions) can include both a microcapsule (e.g., bead) comprising barcoded nucleic acid molecules and a biological particle.

In another aspect, in addition to or as an alternative to 40 droplet based partitioning, biological particles may be encapsulated within a microcapsule that comprises an outer shell, layer or porous matrix in which is entrained one or more individual biological particles or small groups of biological particles. The microcapsule may include other 45 reagents. Encapsulation of biological particles may be performed by a variety of processes. Such processes may combine an aqueous fluid containing the biological particles with a polymeric precursor material that may be capable of being formed into a gel or other solid or semi-solid matrix 50 upon application of a particular stimulus to the polymer precursor. Such stimuli can include, for example, thermal stimuli (e.g., either heating or cooling), photo-stimuli (e.g., through photo-curing), chemical stimuli (e.g., through cross-linking, polymerization initiation of the precursor (e.g., 55 through added initiators)), mechanical stimuli, or a combination thereof.

Preparation of microcapsules comprising biological particles may be performed by a variety of methods. For example, air knife droplet or aerosol generators may be used 60 to dispense droplets of precursor fluids into gelling solutions in order to form microcapsules that include individual biological particles or small groups of biological particles. Likewise, membrane based encapsulation systems may be used to generate microcapsules comprising encapsulated 65 biological particles as described herein. Microfluidic systems of the present disclosure, such as that shown in FIG. 1, may be readily used in encapsulating cells as described

herein. In particular, and with reference to FIG. 1, the aqueous fluid 112 comprising (i) the biological particles 114 and (ii) the polymer precursor material (not shown) is flowed into channel junction 110, where it is partitioned into droplets 118, 120 through the flow of non-aqueous fluid 116. In the case of encapsulation methods, non-aqueous fluid 116 may also include an initiator (not shown) to cause polymerization and/or crosslinking of the polymer precursor to form the microcapsule that includes the entrained biological particles. Examples of polymer precursor/initiator pairs include those described in U.S. Patent Application Publication No. 2014/0378345, which is entirely incorporated herein by reference for all purposes.

For example, in the case where the polymer precursor material comprises a linear polymer material, such as a linear polyacrylamide, PEG, or other linear polymeric material, the activation agent may comprise a cross-linking agent, or a chemical that activates a cross-linking agent within the formed droplets. Likewise, for polymer precursors that comprise polymerizable monomers, the activation agent may comprise a polymerization initiator. For example, in certain cases, where the polymer precursor comprises a mixture of acrylamide monomer with a N,N'-bis-(acryloyl) cystamine (BAC) comonomer, agents such as ammonium persulfate (APS) and tetraethylmethylenediamine (TEMED) and may be provided within the second fluid streams 116 in channel segments 104 and 106, which can initiate and catalyze the copolymerization of the acrylamide and BAC into a cross-linked polymer network, or hydrogel. Other non-limiting examples of initiators include azide-based reagents (e.g., VA-086) and lithium phenyl-trimethylbenzoylphosphinate.

Upon contact of the second fluid stream 116 with the first fluid stream 112 at junction 110, during formation of droplets, the TEMED may diffuse from the second fluid 116 into the aqueous fluid 112 comprising the linear polyacrylamide, which will activate the crosslinking of the polyacrylamide within the droplets 118, 120, resulting in the formation of gel (e.g., hydrogel) microcapsules, as solid or semi-solid beads or particles entraining the cells 114. Although described in terms of polyacrylamide encapsulation, other ‘activatable’ encapsulation compositions may also be employed in the context of the methods and compositions described herein. For example, formation of alginate droplets followed by exposure to divalent metal ions (e.g., Ca<sup>2+</sup> ions), can be used as an encapsulation process using the described processes. Likewise, agarose droplets may also be transformed into capsules through temperature based gelling (e.g., upon cooling, etc.). In another example, addition of a complementary nucleic acid (e.g., DNA) may be used to crosslink or un-crosslink nucleic acid molecules that are conjugated to a polymer network.

In some cases, encapsulated biological particles can be selectively releasable from the microcapsule, such as through passage of time or upon application of a particular stimulus, that degrades the microcapsule sufficiently to allow the biological particles (e.g., cell), or its other contents to be released from the microcapsule, such as into a partition (e.g., droplet). For example, in the case of the polyacrylamide polymer described above, degradation of the microcapsule may be accomplished through the introduction of an appropriate reducing agent, such as DTT or the like, to cleave disulfide bonds that cross-link the polymer matrix. See, for example, U.S. Patent Application Publication No. 2014/0378345, which is entirely incorporated herein by reference for all purposes.

The biological particle can be subjected to other conditions sufficient to polymerize or gel the precursors. The conditions sufficient to polymerize or gel the precursors may comprise exposure to heating, cooling, electromagnetic radiation, and/or light. The conditions sufficient to polymerize or gel the precursors may comprise any conditions sufficient to polymerize or gel the precursors. Following polymerization or gelling, a polymer or gel may be formed around the biological particle. The polymer or gel may be diffusively permeable to chemical or biochemical reagents. The polymer or gel may be diffusively impermeable to macromolecular constituents of the biological particle. In this manner, the polymer or gel may act to allow the biological particle to be subjected to chemical or biochemical operations while spatially confining the macromolecular constituents to a region of the droplet defined by the polymer or gel. The polymer or gel may include one or more of disulfide cross-linked polyacrylamide, agarose, alginate, polyvinyl alcohol, polyethylene glycol (PEG)-diacrylate, PEG-acrylate, PEG-thiol, PEG-azide, PEG-alkyne, other acrylates, chitosan, hyaluronic acid, collagen, fibrin, gelatin, or elastin. The polymer or gel may comprise any other polymer or gel.

The polymer or gel may be functionalized to bind to targeted analytes, such as nucleic acids, proteins, carbohydrates, lipids or other analytes. The polymer or gel may be polymerized or gelled via a passive mechanism. The polymer or gel may be stable in alkaline or acidic conditions or at elevated temperature. The polymer or gel may have mechanical properties similar to the mechanical properties of the bead. For instance, the polymer or gel may be of a similar size to the bead. The polymer or gel may have a mechanical strength (e.g., tensile strength, compressive strength, stiffness, toughness, etc.) similar to that of the bead. The polymer or gel may be of a lower density than an oil. The polymer or gel may be of a density that is roughly similar to that of a buffer. The polymer or gel may have a tunable pore size. The pore size may be chosen to, for instance, retain denatured nucleic acids. The pore size may be chosen to maintain diffusive permeability to exogenous chemicals such as sodium hydroxide (NaOH) and/or endogenous chemicals such as inhibitors. The polymer or gel may be biocompatible. The polymer or gel may maintain or enhance cell viability. The polymer or gel may be biochemically compatible. The polymer or gel may be polymerized and/or depolymerized thermally, chemically, enzymatically, and/or optically.

The polymer may comprise poly(acrylamide-co-acrylic acid) crosslinked with disulfide linkages. The preparation of the polymer may comprise a two-step reaction. In the first activation step, poly(acrylamide-co-acrylic acid) may be exposed to an acylating agent to convert carboxylic acids to esters. For instance, the poly(acrylamide-co-acrylic acid) may be exposed to 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM). The polyacrylamide-co-acrylic acid may be exposed to other salts of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium. In the second cross-linking step, the ester formed in the first step may be exposed to a disulfide crosslinking agent. For instance, the ester may be exposed to cystamine (2,2'-dithiobis(ethylamine)). Following the two steps, the biological particle may be surrounded by polyacrylamide strands linked together by disulfide bridges. In this manner, the biological particle may be encased inside of or comprise a gel or matrix (e.g., polymer matrix) to form a “cell bead.” A cell bead can contain biological particles (e.g., a cell) or

macromolecular constituents (e.g., RNA, DNA, proteins, etc.) of biological particles, as described elsewhere herein.

Encapsulated biological particles can provide certain potential advantages of being more storable and more portable than droplet-based partitioned biological particles. Furthermore, in some cases, it may be desirable to allow biological particles to incubate for a select period of time before analysis, such as in order to characterize changes in such biological particles over time, either in the presence or absence of different stimuli. In such cases, encapsulation may allow for longer incubation than partitioning in emulsion droplets, although in some cases, droplet partitioned biological particles may also be incubated for different periods of time, e.g., at least 10 seconds, at least 30 seconds, at least 1 minute, at least 5 minutes, at least 10 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 5 hours, or at least 10 hours or more. The encapsulation of biological particles may constitute the partitioning of the biological particles into which other reagents are co-partitioned. Alternatively or in addition, encapsulated biological particles may be readily deposited into other partitions (e.g., droplets) as described above.

#### Beads

A partition may comprise one or more unique identifiers, such as barcodes. Barcodes may be previously, subsequently or concurrently delivered to the partitions that hold the compartmentalized or partitioned biological particle. For example, barcodes may be injected into droplets previous to, subsequent to, or concurrently with droplet generation. The delivery of the barcodes to a particular partition allows for the later attribution of the characteristics of the individual biological particle to the particular partition. Barcodes may be delivered, for example on a nucleic acid molecule (e.g., an oligonucleotide), to a partition via any suitable mechanism. Barcoded nucleic acid molecules can be delivered to a partition via a microcapsule. A microcapsule, in some instances, can comprise a bead. Beads are described in further detail below.

In some cases, barcoded nucleic acid molecules can be initially associated with the microcapsule and then released from the microcapsule. Release of the barcoded nucleic acid molecules can be passive (e.g., by diffusion out of the microcapsule). In addition or alternatively, release from the microcapsule can be upon application of a stimulus which allows the barcoded nucleic acid nucleic acid molecules to dissociate or to be released from the microcapsule. Such stimulus may disrupt the microcapsule, an interaction that couples the barcoded nucleic acid molecules to or within the microcapsule, or both. Such stimulus can include, for example, a thermal stimulus, photo-stimulus, chemical stimulus (e.g., change in pH or use of a reducing agent(s)), a mechanical stimulus, a radiation stimulus; a biological stimulus (e.g., enzyme), or any combination thereof.

FIG. 2 shows an example of a microfluidic channel structure 200 for delivering barcode carrying beads to droplets. The channel structure 200 can include channel segments 201, 202, 204, 206 and 208 communicating at a channel junction 210. In operation, the channel segment 201 may transport an aqueous fluid 212 that includes a plurality of beads 214 (e.g., with nucleic acid molecules, oligonucleotides, molecular tags) along the channel segment 201 into junction 210. The plurality of beads 214 may be sourced from a suspension of beads. For example, the channel segment 201 may be connected to a reservoir comprising an aqueous suspension of beads 214. The channel segment 202 may transport the aqueous fluid 212 that includes a plurality of biological particles 216 along the channel segment 202

into junction 210. The plurality of biological particles 216 may be sourced from a suspension of biological particles. For example, the channel segment 202 may be connected to a reservoir comprising an aqueous suspension of biological particles 216. In some instances, the aqueous fluid 212 in either the first channel segment 201 or the second channel segment 202, or in both segments, can include one or more reagents, as further described below. A second fluid 218 that is immiscible with the aqueous fluid 212 (e.g., oil) can be delivered to the junction 210 from each of channel segments 204 and 206. Upon meeting of the aqueous fluid 212 from each of channel segments 201 and 202 and the second fluid 218 from each of channel segments 204 and 206 at the channel junction 210, the aqueous fluid 212 can be partitioned as discrete droplets 220 in the second fluid 218 and flow away from the junction 210 along channel segment 208. The channel segment 208 may deliver the discrete droplets to an outlet reservoir fluidly coupled to the channel segment 208, where they may be harvested.

As an alternative, the channel segments 201 and 202 may meet at another junction upstream of the junction 210. At such junction, beads and biological particles may form a mixture that is directed along another channel to the junction 210 to yield droplets 220. The mixture may provide the beads and biological particles in an alternating fashion, such that, for example, a droplet comprises a single bead and a single biological particle.

Beads, biological particles and droplets may flow along channels at substantially regular flow profiles (e.g., at regular flow rates). Such regular flow profiles may permit a droplet to include a single bead and a single biological particle. Such regular flow profiles may permit the droplets to have an occupancy (e.g., droplets having beads and biological particles) greater than 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95%. Such regular flow profiles and devices that may be used to provide such regular flow profiles are provided in, for example, U.S. Patent Publication No. 2015/0292988, which is entirely incorporated herein by reference.

The second fluid 218 can comprise an oil, such as a fluorinated oil, that includes a fluorosurfactant for stabilizing the resulting droplets, for example, inhibiting subsequent coalescence of the resulting droplets 220. Other surfactants such as Span80, Triton X-100, SDS, perfluoroctanol (PFO), perfluoropolyethers, etc. may also be employed to prevent coalescence of droplets.

A discrete droplet that is generated may include an individual biological particle 216. A discrete droplet that is generated may include a barcode or other reagent carrying bead 214. A discrete droplet generated may include both an individual biological particle and a barcode carrying bead, such as droplets 220. In some instances, a discrete droplet may include more than one individual biological particle or no biological particle. In some instances, a discrete droplet may include more than one bead or no bead. A discrete droplet may be unoccupied (e.g., no beads, no biological particles).

Beneficially, a discrete droplet partitioning a biological particle and a barcode carrying bead may effectively allow the attribution of the barcode to macromolecular constituents of the biological particle within the partition. The contents of a partition may remain discrete from the contents of other partitions.

As will be appreciated, the channel segments described herein may be coupled to any of a variety of different fluid sources or receiving components, including reservoirs, tubing, manifolds, or fluidic components of other systems. As

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will be appreciated, the microfluidic channel structure 200 may have other geometries. For example, a microfluidic channel structure can have more than one channel junctions. For example, a microfluidic channel structure can have 2, 3, 4, or 5 channel segments each carrying beads that meet at a channel junction. Fluid may be directed flow along one or more channels or reservoirs via one or more fluid flow units. A fluid flow unit can comprise compressors (e.g., providing positive pressure), pumps (e.g., providing negative pressure), actuators, and the like to control flow of the fluid. Fluid may also or otherwise be controlled via applied pressure differentials, centrifugal force, electrokinetic pumping, vacuum, capillary or gravity flow, or the like.

A bead may be porous, non-porous, solid, semi-solid, semi-fluidic, fluidic, and/or a combination thereof. In some instances, a bead may be dissolvable, disruptable, and/or degradable. In some cases, a bead may not be degradable. In some cases, the bead may be a gel bead. A gel bead may be a hydrogel bead. A gel bead may be formed from molecular precursors, such as a polymeric or monomeric species. A semi-solid bead may be a liposomal bead. Solid beads may comprise metals including iron oxide, gold, and silver. In some cases, the bead may be a silica bead. In some cases, the bead can be rigid. In other cases, the bead may be flexible and/or compressible.

A bead may be of any suitable shape. Examples of bead shapes include, but are not limited to, spherical, non-spherical, oval, oblong, amorphous, circular, cylindrical, and variations thereof.

Beads may be of uniform size or heterogeneous size. In some cases, the diameter of a bead may be at least about 10 nanometers (nm), 100 nm, 500 nm, 1 micrometer ( $\mu\text{m}$ ), 5  $\mu\text{m}$ , 10  $\mu\text{m}$ , 20  $\mu\text{m}$ , 30  $\mu\text{m}$ , 40  $\mu\text{m}$ , 50  $\mu\text{m}$ , 60  $\mu\text{m}$ , 70  $\mu\text{m}$ , 80  $\mu\text{m}$ , 90  $\mu\text{m}$ , 100  $\mu\text{m}$ , 250  $\mu\text{m}$ , 500  $\mu\text{m}$ , 1 mm, or greater. In some cases, a bead may have a diameter of less than about 10 nm, 100 nm, 500 nm, 1  $\mu\text{m}$ , 5  $\mu\text{m}$ , 10  $\mu\text{m}$ , 20  $\mu\text{m}$ , 30  $\mu\text{m}$ , 40  $\mu\text{m}$ , 50  $\mu\text{m}$ , 60  $\mu\text{m}$ , 70  $\mu\text{m}$ , 80  $\mu\text{m}$ , 90  $\mu\text{m}$ , 100  $\mu\text{m}$ , 250  $\mu\text{m}$ , 500  $\mu\text{m}$ , 1 mm, or less. In some cases, a bead may have a diameter in the range of about 40-75  $\mu\text{m}$ , 30-75  $\mu\text{m}$ , 20-75  $\mu\text{m}$ , 40-85  $\mu\text{m}$ , 40-95  $\mu\text{m}$ , 20-100  $\mu\text{m}$ , 10-100  $\mu\text{m}$ , 1-100  $\mu\text{m}$ , 20-250  $\mu\text{m}$ , or 20-500  $\mu\text{m}$ .

In certain aspects, beads can be provided as a population or plurality of beads having a relatively monodisperse size distribution. Where it may be desirable to provide relatively consistent amounts of reagents within partitions, maintaining relatively consistent bead characteristics, such as size, can contribute to the overall consistency. In particular, the beads described herein may have size distributions that have a coefficient of variation in their cross-sectional dimensions of less than 50%, less than 40%, less than 30%, less than 20%, and in some cases less than 15%, less than 10%, less than 5%, or less.

A bead may comprise natural and/or synthetic materials. For example, a bead can comprise a natural polymer, a synthetic polymer or both natural and synthetic polymers. Examples of natural polymers include proteins and sugars such as deoxyribonucleic acid, rubber, cellulose, starch (e.g., amylose, amylopectin), proteins, enzymes, polysaccharides, silks, polyhydroxyalkanoates, chitosan, dextran, collagen, carrageenan, ispaghula, acacia, agar, gelatin, shellac, sternaria gum, xanthan gum, Corn sugar gum, guar gum, gum karaya, agarose, alginic acid, alginate, or natural polymers thereof. Examples of synthetic polymers include acrylics, nylons, silicones, spandex, viscose rayon, polycarboxylic acids, polyvinyl acetate, polyacrylamide, polyacrylate, polyethylene glycol, polyurethanes, polylactic acid, silica, polystyrene, polyacrylonitrile, polybutadiene, polycarbonate,

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polyethylene, polyethylene terephthalate, poly(chlorotrifluoroethylene), poly(ethylene oxide), poly(ethylene terephthalate), polyethylene, polyisobutylene, poly(methyl methacrylate), poly(oxymethylene), polyformaldehyde, polypropylene, polystyrene, poly(tetrafluoroethylene), poly(vinyl acetate), poly(vinyl alcohol), poly(vinyl chloride), poly(vinylidene dichloride), poly(vinylidene difluoride), poly(vinyl fluoride) and/or combinations (e.g., co-polymers) thereof. Beads may also be formed from materials other than polymers, including lipids, micelles, liposomes, ceramics, glass-ceramics, material composites, metals, other inorganic materials, and others.

In some instances, the bead may contain molecular precursors (e.g., monomers or polymers), which may form a polymer network via polymerization of the molecular precursors. In some cases, a precursor may be an already polymerized species capable of undergoing further polymerization via, for example, a chemical cross-linkage. In some cases, a precursor can comprise one or more of an acrylamide or a methacrylamide monomer, oligomer, or polymer. In some cases, the bead may comprise prepolymers, which are oligomers capable of further polymerization. For example, polyurethane beads may be prepared using prepolymers. In some cases, the bead may contain individual polymers that may be further polymerized together. In some cases, beads may be generated via polymerization of different precursors, such that they comprise mixed polymers, co-polymers, and/or block co-polymers. In some cases, the bead may comprise covalent or ionic bonds between polymeric precursors (e.g., monomers, oligomers, linear polymers), nucleic acid molecules (e.g., oligonucleotides), primers, and other entities. In some cases, the covalent bonds can be carbon-carbon bonds, thioether bonds, or carbon-heteroatom bonds.

Cross-linking may be permanent or reversible, depending upon the particular cross-linker used. Reversible cross-linking may allow for the polymer to linearize or dissociate under appropriate conditions. In some cases, reversible cross-linking may also allow for reversible attachment of a material bound to the surface of a bead. In some cases, a cross-linker may form disulfide linkages. In some cases, the chemical cross-linker forming disulfide linkages may be cystamine or a modified cystamine.

In some cases, disulfide linkages can be formed between molecular precursor units (e.g., monomers, oligomers, or linear polymers) or precursors incorporated into a bead and nucleic acid molecules (e.g., oligonucleotides). Cystamine (including modified cystamines), for example, is an organic agent comprising a disulfide bond that may be used as a crosslinker agent between individual monomeric or polymeric precursors of a bead. Polyacrylamide may be polymerized in the presence of cystamine or a species comprising cystamine (e.g., a modified cystamine) to generate polyacrylamide gel beads comprising disulfide linkages (e.g., chemically degradable beads comprising chemically-reducible cross-linkers). The disulfide linkages may permit the bead to be degraded (or dissolved) upon exposure of the bead to a reducing agent.

In some cases, chitosan, a linear polysaccharide polymer, may be crosslinked with glutaraldehyde via hydrophilic chains to form a bead. Crosslinking of chitosan polymers may be achieved by chemical reactions that are initiated by heat, pressure, change in pH, and/or radiation.

In some cases, a bead may comprise an acrydite moiety, which in certain aspects may be used to attach one or more nucleic acid molecules (e.g., barcode sequence, barcoded nucleic acid molecule, barcoded oligonucleotide, primer, or

other oligonucleotide) to the bead. In some cases, an acrydite moiety can refer to an acrydite analogue generated from the reaction of acrydite with one or more species, such as, the reaction of acrydite with other monomers and cross-linkers during a polymerization reaction. Acrydite moieties may be modified to form chemical bonds with a species to be attached, such as a nucleic acid molecule (e.g., barcode sequence, barcoded nucleic acid molecule, barcoded oligonucleotide, primer, or other oligonucleotide). Acrydite moieties may be modified with thiol groups capable of forming a disulfide bond or may be modified with groups already comprising a disulfide bond. The thiol or disulfide (via disulfide exchange) may be used as an anchor point for a species to be attached or another part of the acrydite moiety may be used for attachment. In some cases, attachment can be reversible, such that when the disulfide bond is broken (e.g., in the presence of a reducing agent), the attached species is released from the bead. In other cases, an acrydite moiety can comprise a reactive hydroxyl group that may be used for attachment.

Functionalization of beads for attachment of nucleic acid molecules (e.g., oligonucleotides) may be achieved through a wide range of different approaches, including activation of chemical groups within a polymer, incorporation of active or activatable functional groups in the polymer structure, or attachment at the pre-polymer or monomer stage in bead production.

For example, precursors (e.g., monomers, cross-linkers) that are polymerized to form a bead may comprise acrydite moieties, such that when a bead is generated, the bead also comprises acrydite moieties. The acrydite moieties can be attached to a nucleic acid molecule (e.g., oligonucleotide), which may include a priming sequence (e.g., a primer for amplifying target nucleic acids, random primer, primer sequence for mRNA) and/or one or more barcode sequences. The one or more barcode sequences may include sequences that are the same for all nucleic acid molecules coupled to a given bead and/or sequences that are different across all nucleic acid molecules coupled to the given bead. The nucleic acid molecule may be incorporated into the bead.

In some cases, the nucleic acid molecule can comprise a functional sequence, for example, for attachment to a sequencing flow cell, such as, for example, a P5 sequence for Illumina® sequencing. In some cases, the nucleic acid molecule or derivative thereof (e.g., oligonucleotide or polynucleotide generated from the nucleic acid molecule) can comprise another functional sequence, such as, for example, a P7 sequence for attachment to a sequencing flow cell for Illumina sequencing. In some cases, the nucleic acid molecule can comprise a barcode sequence. In some cases, the primer can further comprise a unique molecular identifier (UMI). In some cases, the primer can comprise an R1 primer sequence for Illumina sequencing. In some cases, the primer can comprise an R2 primer sequence for Illumina sequencing. Examples of such nucleic acid molecules (e.g., oligonucleotides, polynucleotides, etc.) and uses thereof, as may be used with compositions, devices, methods and systems of the present disclosure, are provided in U.S. Patent Pub. Nos. 2014/0378345 and 2015/0376609, each of which is entirely incorporated herein by reference.

FIG. 8 illustrates an example of a barcode carrying bead. A nucleic acid molecule **802**, such as an oligonucleotide, can be coupled to a bead **804** by a releasable linkage **806**, such as, for example, a disulfide linker. The same bead **804** may be coupled (e.g., via releasable linkage) to one or more other nucleic acid molecules **818**, **820**. The nucleic acid molecule **802** may be or comprise a barcode. As noted elsewhere

herein, the structure of the barcode may comprise a number of sequence elements. The nucleic acid molecule **802** may comprise a functional sequence **808** that may be used in subsequent processing. For example, the functional sequence **808** may include one or more of a sequencer specific flow cell attachment sequence (e.g., a P5 sequence for Illumina® sequencing systems) and a sequencing primer sequence (e.g., a R1 primer for Illumina® sequencing systems). The nucleic acid molecule **802** may comprise a barcode sequence **810** for use in barcoding the sample (e.g., DNA, RNA, protein, etc.). In some cases, the barcode sequence **810** can be bead-specific such that the barcode sequence **810** is common to all nucleic acid molecules (e.g., including nucleic acid molecule **802**) coupled to the same bead **804**. Alternatively or in addition, the barcode sequence **810** can be partition-specific such that the barcode sequence **810** is common to all nucleic acid molecules coupled to one or more beads that are partitioned into the same partition. The nucleic acid molecule **802** may comprise a specific priming sequence **812**, such as an mRNA specific priming sequence (e.g., poly-T sequence), a targeted priming sequence, and/or a random priming sequence. The nucleic acid molecule **802** may comprise an anchoring sequence **814** to ensure that the specific priming sequence **812** hybridizes at the sequence end (e.g., of the mRNA). For example, the anchoring sequence **814** can include a random short sequence of nucleotides, such as a 1-mer, 2-mer, 3-mer or longer sequence, which can ensure that a poly-T segment is more likely to hybridize at the sequence end of the poly-A tail of the mRNA.

The nucleic acid molecule **802** may comprise a unique molecular identifying sequence **816** (e.g., unique molecular identifier (UMI)). In some cases, the unique molecular identifying sequence **816** may comprise from about 5 to about 8 nucleotides. Alternatively, the unique molecular identifying sequence **816** may comprise less than about 5 or more than about 8 nucleotides. The unique molecular identifying sequence **816** may be a unique sequence that varies across individual nucleic acid molecules (e.g., **802**, **818**, **820**, etc.) coupled to a single bead (e.g., bead **804**). In some cases, the unique molecular identifying sequence **816** may be a random sequence (e.g., such as a random N-mer sequence). For example, the UMI may provide a unique identifier of the starting mRNA molecule that was captured, in order to allow quantitation of the number of original expressed RNA. As will be appreciated, although FIG. 8 shows three nucleic acid molecules **802**, **818**, **820** coupled to the surface of the bead **804**, an individual bead may be coupled to any number of individual nucleic acid molecules, for example, from one to tens to hundreds of thousands or even millions of individual nucleic acid molecules. The respective barcodes for the individual nucleic acid molecules can comprise both common sequence segments or relatively common sequence segments (e.g., **808**, **810**, **812**, etc.) and variable or unique sequence segments (e.g., **816**) between different individual nucleic acid molecules coupled to the same bead.

In operation, a biological particle (e.g., cell, DNA, RNA, etc.) can be co-partitioned along with a barcode bearing bead **804**. The barcoded nucleic acid molecules **802**, **818**, **820** can be released from the bead **804** in the partition. By way of example, in the context of analyzing sample RNA, the poly-T segment (e.g., **812**) of one of the released nucleic acid molecules (e.g., **802**) can hybridize to the poly-A tail of a mRNA molecule. Reverse transcription may result in a cDNA transcript of the mRNA, but which transcript includes each of the sequence segments **808**, **810**, **816** of the nucleic

acid molecule 802. Because the nucleic acid molecule 802 comprises an anchoring sequence 814, it will more likely hybridize to and prime reverse transcription at the sequence end of the poly-A tail of the mRNA. Within any given partition, all of the cDNA transcripts of the individual mRNA molecules may include a common barcode sequence segment 810. However, the transcripts made from the different mRNA molecules within a given partition may vary at the unique molecular identifying sequence 812 segment (e.g., UMI segment). Beneficially, even following any subsequent amplification of the contents of a given partition, the number of different UMIs can be indicative of the quantity of mRNA originating from a given partition, and thus from the biological particle (e.g., cell). As noted above, the transcripts can be amplified, cleaned up and sequenced to identify the sequence of the cDNA transcript of the mRNA, as well as to sequence the barcode segment and the UMI segment. While a poly-T primer sequence is described, other targeted or random priming sequences may also be used in priming the reverse transcription reaction. Likewise, although described as releasing the barcoded oligonucleotides into the partition, in some cases, the nucleic acid molecules bound to the bead (e.g., gel bead) may be used to hybridize and capture the mRNA on the solid phase of the bead, for example, in order to facilitate the separation of the RNA from other cell contents.

In some cases, precursors comprising a functional group that is reactive or capable of being activated such that it becomes reactive can be polymerized with other precursors to generate gel beads comprising the activated or activatable functional group. The functional group may then be used to attach additional species (e.g., disulfide linkers, primers, other oligonucleotides, etc.) to the gel beads. For example, some precursors comprising a carboxylic acid (COOH) group can co-polymerize with other precursors to form a gel bead that also comprises a COOH functional group. In some cases, acrylic acid (a species comprising free COOH groups), acrylamide, and bis(acryloyl)cystamine can be co-polymerized together to generate a gel bead comprising free COOH groups. The COOH groups of the gel bead can be activated (e.g., via 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) or 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM)) such that they are reactive (e.g., reactive to amine functional groups where EDC/NHS or DMTMM are used for activation). The activated COOH groups can then react with an appropriate species (e.g., a species comprising an amine functional group where the carboxylic acid groups are activated to be reactive with an amine functional group) comprising a moiety to be linked to the bead.

Beads comprising disulfide linkages in their polymeric network may be functionalized with additional species via reduction of some of the disulfide linkages to free thiols. The disulfide linkages may be reduced via, for example, the action of a reducing agent (e.g., DTT, TCEP, etc.) to generate free thiol groups, without dissolution of the bead. Free thiols of the beads can then react with free thiols of a species or a species comprising another disulfide bond (e.g., via thiol-disulfide exchange) such that the species can be linked to the beads (e.g., via a generated disulfide bond). In some cases, free thiols of the beads may react with any other suitable group. For example, free thiols of the beads may react with species comprising an acrydite moiety. The free thiol groups of the beads can react with the acrydite via Michael addition chemistry, such that the species comprising the acrydite is linked to the bead. In some cases,

uncontrolled reactions can be prevented by inclusion of a thiol capping agent such as N-ethylmaleimide or iodoacetate.

Activation of disulfide linkages within a bead can be controlled such that only a small number of disulfide linkages are activated. Control may be exerted, for example, by controlling the concentration of a reducing agent used to generate free thiol groups and/or concentration of reagents used to form disulfide bonds in bead polymerization. In some cases, a low concentration (e.g., molecules of reducing agent:gel bead ratios of less than or equal to about 1:100,000,000,000, less than or equal to about 1:10,000,000,000, less than or equal to about 1:1,000,000,000, less than or equal to about 1:100,000,000, less than or equal to about 1:10,000,000, less than or equal to about 1:1,000,000, less than or equal to about 1:100,000, less than or equal to about 1:10,000) of reducing agent may be used for reduction. Controlling the number of disulfide linkages that are reduced to free thiols may be useful in ensuring bead structural integrity during functionalization. In some cases, optically-active agents, such as fluorescent dyes, may be coupled to beads via free thiol groups of the beads and used to quantify the number of free thiols present in a bead and/or track a bead.

In some cases, addition of moieties to a gel bead after gel bead formation may be advantageous. For example, addition of an oligonucleotide (e.g., barcoded oligonucleotide) after gel bead formation may avoid loss of the species during chain transfer termination that can occur during polymerization. Moreover, smaller precursors (e.g., monomers or cross linkers that do not comprise side chain groups and linked moieties) may be used for polymerization and can be minimally hindered from growing chain ends due to viscous effects. In some cases, functionalization after gel bead synthesis can minimize exposure of species (e.g., oligonucleotides) to be loaded with potentially damaging agents (e.g., free radicals) and/or chemical environments. In some cases, the generated gel may possess an upper critical solution temperature (UCST) that can permit temperature driven swelling and collapse of a bead. Such functionality may aid in oligonucleotide (e.g., a primer) infiltration into the bead during subsequent functionalization of the bead with the oligonucleotide. Post-production functionalization may also be useful in controlling loading ratios of species in beads, such that, for example, the variability in loading ratio is minimized. Species loading may also be performed in a batch process such that a plurality of beads can be functionalized with the species in a single batch.

A bead injected or otherwise introduced into a partition may comprise releasably, cleavably, or reversibly attached barcodes. A bead injected or otherwise introduced into a partition may comprise activatable barcodes. A bead injected or otherwise introduced into a partition may be degradable, disruptable, or dissolvable beads.

Barcodes can be releasably, cleavably or reversibly attached to the beads such that barcodes can be released or be releasable through cleavage of a linkage between the barcode molecule and the bead, or released through degradation of the underlying bead itself, allowing the barcodes to be accessed or be accessible by other reagents, or both. In non-limiting examples, cleavage may be achieved through reduction of di-sulfide bonds, use of restriction enzymes, photo-activated cleavage, or cleavage via other types of stimuli (e.g., chemical, thermal, pH, enzymatic, etc.) and/or reactions, such as described elsewhere herein. Releasable barcodes may sometimes be referred to as being activatable, in that they are available for reaction once released. Thus,

for example, an activatable barcode may be activated by releasing the barcode from a bead (or other suitable type of partition described herein). Other activatable configurations are also envisioned in the context of the described methods and systems.

In addition to, or as an alternative to the cleavable linkages between the beads and the associated molecules, such as barcode containing nucleic acid molecules (e.g., barcoded oligonucleotides), the beads may be degradable, disruptable, or dissolvable spontaneously or upon exposure to one or more stimuli (e.g., temperature changes, pH changes, exposure to particular chemical species or phase, exposure to light, reducing agent, etc.). In some cases, a bead may be dissolvable, such that material components of the beads are solubilized when exposed to a particular chemical species or an environmental change, such as a change temperature or a change in pH. In some cases, a gel bead can be degraded or dissolved at elevated temperature and/or in alkaline conditions. In some cases, a bead may be thermally degradable such that when the bead is exposed to an appropriate change in temperature (e.g., heat), the bead degrades. Degradation or dissolution of a bead bound to a species (e.g., a nucleic acid molecule, e.g., barcoded oligonucleotide) may result in release of the species from the bead.

As will be appreciated from the above disclosure, the degradation of a bead may refer to the dissociation of a bound or entrained species from a bead, both with and without structurally degrading the physical bead itself. For example, the degradation of the bead may involve cleavage of a cleavable linkage via one or more species and/or methods described elsewhere herein. In another example, entrained species may be released from beads through osmotic pressure differences due to, for example, changing chemical environments. By way of example, alteration of bead pore sizes due to osmotic pressure differences can generally occur without structural degradation of the bead itself. In some cases, an increase in pore size due to osmotic swelling of a bead can permit the release of entrained species within the bead. In other cases, osmotic shrinking of a bead may cause a bead to better retain an entrained species due to pore size contraction.

A degradable bead may be introduced into a partition, such as a droplet of an emulsion or a well, such that the bead degrades within the partition and any associated species (e.g., oligonucleotides) are released within the droplet when the appropriate stimulus is applied. The free species (e.g., oligonucleotides, nucleic acid molecules) may interact with other reagents contained in the partition. For example, a polyacrylamide bead comprising cystamine and linked, via a disulfide bond, to a barcode sequence, may be combined with a reducing agent within a droplet of a water-in-oil emulsion. Within the droplet, the reducing agent can break the various disulfide bonds, resulting in bead degradation and release of the barcode sequence into the aqueous, inner environment of the droplet. In another example, heating of a droplet comprising a bead-bound barcode sequence in basic solution may also result in bead degradation and release of the attached barcode sequence into the aqueous, inner environment of the droplet.

Any suitable number of molecular tag molecules (e.g., primer, barcoded oligonucleotide) can be associated with a bead such that, upon release from the bead, the molecular tag molecules (e.g., primer, e.g., barcoded oligonucleotide) are present in the partition at a pre-defined concentration. Such pre-defined concentration may be selected to facilitate certain reactions for generating a sequencing library, e.g.,

amplification, within the partition. In some cases, the pre-defined concentration of the primer can be limited by the process of producing nucleic acid molecule (e.g., oligonucleotide) bearing beads.

5 In some cases, beads can be non-covalently loaded with one or more reagents. The beads can be non-covalently loaded by, for instance, subjecting the beads to conditions sufficient to swell the beads, allowing sufficient time for the reagents to diffuse into the interiors of the beads, and 10 subjecting the beads to conditions sufficient to de-swell the beads. The swelling of the beads may be accomplished, for instance, by placing the beads in a thermodynamically favorable solvent, subjecting the beads to a higher or lower temperature, subjecting the beads to a higher or lower ion concentration, and/or subjecting the beads to an electric field. The swelling of the beads may be accomplished by various swelling methods. The de-swelling of the beads may be accomplished, for instance, by transferring the beads in a thermodynamically unfavorable solvent, subjecting the 15 beads to lower or high temperatures, subjecting the beads to a lower or higher ion concentration, and/or removing an electric field. The de-swelling of the beads may be accomplished by various de-swelling methods. Transferring the beads may cause pores in the bead to shrink. The shrinking 20 may then hinder reagents within the beads from diffusing out of the interiors of the beads. The hindrance may be due to steric interactions between the reagents and the interiors of the beads. The transfer may be accomplished microfluidically. For instance, the transfer may be achieved by moving 25 the beads from one co-flowing solvent stream to a different co-flowing solvent stream. The swellability and/or pore size of the beads may be adjusted by changing the polymer composition of the bead.

30 In some cases, an acrydite moiety linked to a precursor, another species linked to a precursor, or a precursor itself can comprise a labile bond, such as chemically, thermally, or photo-sensitive bond e.g., disulfide bond, UV-sensitive bond, or the like. Once acrydite moieties or other moieties comprising a labile bond are incorporated into a bead, the 35 bead may also comprise the labile bond. The labile bond may be, for example, useful in reversibly linking (e.g., covalently linking) species (e.g., barcodes, primers, etc.) to a bead. In some cases, a thermally labile bond may include a nucleic acid hybridization based attachment, e.g., where an oligonucleotide is hybridized to a complementary sequence 40 that is attached to the bead, such that thermal melting of the hybrid releases the oligonucleotide, e.g., a barcode containing sequence, from the bead or microcapsule.

The addition of multiple types of labile bonds to a gel 45 bead may result in the generation of a bead capable of responding to varied stimuli. Each type of labile bond may be sensitive to an associated stimulus (e.g., chemical stimulus, light, temperature, enzymatic, etc.) such that release of species attached to a bead via each labile bond may be controlled by the application of the appropriate stimulus. Such functionality may be useful in controlled release of species from a gel bead. In some cases, another species comprising a labile bond may be linked to a gel bead after 50 gel bead formation via, for example, an activated functional group of the gel bead as described above. As will be appreciated, barcodes that are releasably, cleavably or reversibly attached to the beads described herein include barcodes that are released or releasable through cleavage of a linkage between the barcode molecule and the bead, or that are released through degradation of the underlying bead itself, allowing the barcodes to be accessed or accessible by other reagents, or both.

In addition to thermally cleavable bonds, disulfide bonds and UV sensitive bonds, other non-limiting examples of labile bonds that may be coupled to a precursor or bead include an ester linkage (e.g., cleavable with an acid, a base, or hydroxylamine), a vicinal diol linkage (e.g., cleavable via sodium periodate), a Diels-Alder linkage (e.g., cleavable via heat), a sulfone linkage (e.g., cleavable via a base), a silyl ether linkage (e.g., cleavable via an acid), a glycosidic linkage (e.g., cleavable via an amylase), a peptide linkage (e.g., cleavable via a protease), or a phosphodiester linkage (e.g., cleavable via a nuclease (e.g., DNAase)). A bond may be cleavable via other nucleic acid molecule targeting enzymes, such as restriction enzymes (e.g., restriction endonucleases), as described further below.

Species may be encapsulated in beads during bead generation (e.g., during polymerization of precursors). Such species may or may not participate in polymerization. Such species may be entered into polymerization reaction mixtures such that generated beads comprise the species upon bead formation. In some cases, such species may be added to the gel beads after formation. Such species may include, for example, nucleic acid molecules (e.g., oligonucleotides), reagents for a nucleic acid amplification reaction (e.g., primers, polymerases, dNTPs, co-factors (e.g., ionic co-factors), buffers) including those described herein, reagents for enzymatic reactions (e.g., enzymes, co-factors, substrates, buffers), reagents for nucleic acid modification reactions such as polymerization, ligation, or digestion, and/or reagents for template preparation (e.g., fragmentation) for one or more sequencing platforms (e.g., Nextera® for Illumina®). Such species may include one or more enzymes described herein, including without limitation, polymerase, reverse transcriptase, restriction enzymes (e.g., endonuclease), transposase, ligase, proteinase K, DNase, etc. Such species may include one or more reagents described elsewhere herein (e.g., lysis agents, inhibitors, inactivating agents, chelating agents, stimulus). Trapping of such species may be controlled by the polymer network density generated during polymerization of precursors, control of ionic charge within the gel bead (e.g., via ionic species linked to polymerized species), or by the release of other species. Encapsulated species may be released from a bead upon bead degradation and/or by application of a stimulus capable of releasing the species from the bead. Alternatively or in addition, species may be partitioned in a partition (e.g., droplet) during or subsequent to partition formation. Such species may include, without limitation, the abovementioned species that may also be encapsulated in a bead.

A degradable bead may comprise one or more species with a labile bond such that, when the bead/species is exposed to the appropriate stimuli, the bond is broken and the bead degrades. The labile bond may be a chemical bond (e.g., covalent bond, ionic bond) or may be another type of physical interaction (e.g., van der Waals interactions, dipole-dipole interactions, etc.). In some cases, a crosslinker used to generate a bead may comprise a labile bond. Upon exposure to the appropriate conditions, the labile bond can be broken and the bead degraded. For example, upon exposure of a polyacrylamide gel bead comprising cystamine crosslinkers to a reducing agent, the disulfide bonds of the cystamine can be broken and the bead degraded.

A degradable bead may be useful in more quickly releasing an attached species (e.g., a nucleic acid molecule, a barcode sequence, a primer, etc) from the bead when the appropriate stimulus is applied to the bead as compared to a bead that does not degrade. For example, for a species bound to an inner surface of a porous bead or in the case of an

encapsulated species, the species may have greater mobility and accessibility to other species in solution upon degradation of the bead. In some cases, a species may also be attached to a degradable bead via a degradable linker (e.g., disulfide linker). The degradable linker may respond to the same stimuli as the degradable bead or the two degradable species may respond to different stimuli. For example, a barcode sequence may be attached, via a disulfide bond, to a polyacrylamide bead comprising cystamine. Upon exposure of the barcoded-bead to a reducing agent, the bead degrades and the barcode sequence is released upon breakage of both the disulfide linkage between the barcode sequence and the bead and the disulfide linkages of the cystamine in the bead.

Where degradable beads are provided, it may be beneficial to avoid exposing such beads to the stimulus or stimuli that cause such degradation prior to a given time, in order to, for example, avoid premature bead degradation and issues that arise from such degradation, including for example poor flow characteristics and aggregation. By way of example, where beads comprise reducible cross-linking groups, such as disulfide groups, it will be desirable to avoid contacting such beads with reducing agents, e.g., DTT or other disulfide cleaving reagents. In such cases, treatment to the beads described herein will, in some cases be provided free of reducing agents, such as DTT. Because reducing agents are often provided in commercial enzyme preparations, it may be desirable to provide reducing agent free (or DTT free) enzyme preparations in treating the beads described herein. Examples of such enzymes include, e.g., polymerase enzyme preparations, reverse transcriptase enzyme preparations, ligase enzyme preparations, as well as many other enzyme preparations that may be used to treat the beads described herein. The terms "reducing agent free" or "DTT free" preparations can refer to a preparation having less than about  $\frac{1}{10}$ th, less than about  $\frac{1}{50}$ th, or even less than about  $\frac{1}{100}$ th of the lower ranges for such materials used in degrading the beads. For example, for DTT, the reducing agent free preparation can have less than about 0.01 millimolar (mM), 0.005 mM, 0.001 mM DTT, 0.0005 mM DTT, or even less than about 0.0001 mM DTT. In many cases, the amount of DTT can be undetectable.

Numerous chemical triggers may be used to trigger the degradation of beads. Examples of these chemical changes may include, but are not limited to pH-mediated changes to the integrity of a component within the bead, degradation of a component of a bead via cleavage of cross-linked bonds, and depolymerization of a component of a bead.

In some embodiments, a bead may be formed from materials that comprise degradable chemical crosslinkers, such as BAC or cystamine. Degradation of such degradable crosslinkers may be accomplished through a number of mechanisms. In some examples, a bead may be contacted with a chemical degrading agent that may induce oxidation, reduction or other chemical changes. For example, a chemical degrading agent may be a reducing agent, such as dithiothreitol (DTT). Additional examples of reducing agents may include  $\beta$ -mercaptoethanol, (2S)-2-amino-1,4-dimercaptobutane (dithiobutyamine or DTBA), tris(2-carboxyethyl) phosphine (TCEP), or combinations thereof. A reducing agent may degrade the disulfide bonds formed between gel precursors forming the bead, and thus, degrade the bead. In other cases, a change in pH of a solution, such as an increase in pH, may trigger degradation of a bead. In other cases, exposure to an aqueous solution, such as water, may trigger hydrolytic degradation, and thus degradation of the bead. In some cases, any combination of stimuli may

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trigger degradation of a bead. For example, a change in pH may enable a chemical agent (e.g., DTT) to become an effective reducing agent.

Beads may also be induced to release their contents upon the application of a thermal stimulus. A change in temperature can cause a variety of changes to a bead. For example, heat can cause a solid bead to liquefy. A change in heat may cause melting of a bead such that a portion of the bead degrades. In other cases, heat may increase the internal pressure of the bead components such that the bead ruptures or explodes. Heat may also act upon heat-sensitive polymers used as materials to construct beads.

Any suitable agent may degrade beads. In some embodiments, changes in temperature or pH may be used to degrade thermo-sensitive or pH-sensitive bonds within beads. In some embodiments, chemical degrading agents may be used to degrade chemical bonds within beads by oxidation, reduction or other chemical changes. For example, a chemical degrading agent may be a reducing agent, such as DTT, wherein DTT may degrade the disulfide bonds formed between a crosslinker and gel precursors, thus degrading the bead. In some embodiments, a reducing agent may be added to degrade the bead, which may or may not cause the bead to release its contents. Examples of reducing agents may include dithiothreitol (DTT),  $\beta$ -mercaptoethanol, (2S)-2-amino-1,4-dimercaptobutane (dithiobutyramine or DTBA), tris(2-carboxyethyl) phosphine (TCEP), or combinations thereof. The reducing agent may be present at a concentration of about 0.1 mM, 0.5 mM, 1 mM, 5 mM, 10 mM. The reducing agent may be present at a concentration of at least about 0.1 mM, 0.5 mM, 1 mM, 5 mM, 10 mM, or greater than 10 mM. The reducing agent may be present at concentration of at most about 10 mM, 5 mM, 1 mM, 0.5 mM, 0.1 mM, or less.

Although FIG. 1 and FIG. 2 have been described in terms of providing substantially singly occupied partitions, above, in certain cases, it may be desirable to provide multiply occupied partitions, e.g., containing two, three, four or more cells and/or microcapsules (e.g., beads) comprising bar-coded nucleic acid molecules (e.g., oligonucleotides) within a single partition. Accordingly, as noted above, the flow characteristics of the biological particle and/or bead containing fluids and partitioning fluids may be controlled to provide for such multiply occupied partitions. In particular, the flow parameters may be controlled to provide a given occupancy rate at greater than about 50% of the partitions, greater than about 75%, and in some cases greater than about 80%, 90%, 95%, or higher.

In some cases, additional microcapsules can be used to deliver additional reagents to a partition. In such cases, it may be advantageous to introduce different beads into a common channel or droplet generation junction, from different bead sources (e.g., containing different associated reagents) through different channel inlets into such common channel or droplet generation junction (e.g., junction 210). In such cases, the flow and frequency of the different beads into the channel or junction may be controlled to provide for a certain ratio of microcapsules from each source, while ensuring a given pairing or combination of such beads into a partition with a given number of biological particles (e.g., one biological particle and one bead per partition).

The partitions described herein may comprise small volumes, for example, less than about 10 microliters ( $\mu$ L), 5  $\mu$ L, 1  $\mu$ L, 500 nanoliters (nL), 100 nL, 50 nL, 900 picoliters (pL), 800 pL, 700 pL, 600 pL, 500 pL, 400 pL, 300 pL, 200 pL, 100 pL, 50 pL, 20 pL, 10 pL, 1 pL, or less.

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For example, in the case of droplet based partitions, the droplets may have overall volumes that are less than about 1000 pL, 900 pL, 800 pL, 700 pL, 600 pL, 500 pL, 400 pL, 300 pL, 200 pL, 100 pL, 50 pL, 20 pL, 10 pL, 1 pL, or less. Where co-partitioned with microcapsules, it will be appreciated that the sample fluid volume, e.g., including co-partitioned biological particles and/or beads, within the partitions may be less than about 90% of the above described volumes, less than about 80%, less than about 70%, less than about 60%, less than about 50%, less than about 40%, less than about 30%, less than about 20%, or less than about 10% of the above described volumes.

As is described elsewhere herein, partitioning species may generate a population or plurality of partitions. In such cases, any suitable number of partitions can be generated or otherwise provided. For example, at least about 1,000 partitions, at least about 5,000 partitions, at least about 10,000 partitions, at least about 50,000 partitions, at least about 100,000 partitions, at least about 500,000 partitions, at least about 1,000,000 partitions, at least about 5,000,000 partitions, at least about 10,000,000 partitions, at least about 50,000,000 partitions, at least about 100,000,000 partitions, at least about 500,000,000 partitions, at least about 1,000,000,000 partitions, or more partitions can be generated or otherwise provided. Moreover, the plurality of partitions may comprise both unoccupied partitions (e.g., empty partitions) and occupied partitions.

#### Reagents

In accordance with certain aspects, biological particles may be partitioned along with lysis reagents in order to release the contents of the biological particles within the partition. In such cases, the lysis agents can be contacted with the biological particle suspension concurrently with, or immediately prior to, the introduction of the biological particles into the partitioning junction/droplet generation zone (e.g., junction 210), such as through an additional channel or channels upstream of the channel junction. In accordance with other aspects, additionally or alternatively, biological particles may be partitioned along with other reagents, as will be described further below.

FIG. 3 shows an example of a microfluidic channel structure 300 for co-partitioning biological particles and reagents. The channel structure 300 can include channel segments 301, 302, 304, 306 and 308. Channel segments 301 and 302 communicate at a first channel junction 309. Channel segments 302, 304, 306, and 308 communicate at a second channel junction 310.

In an example operation, the channel segment 301 may transport an aqueous fluid 312 that includes a plurality of biological particles 314 along the channel segment 301 into the second junction 310. As an alternative or in addition to, channel segment 301 may transport beads (e.g., gel beads). The beads may comprise barcode molecules.

For example, the channel segment 301 may be connected to a reservoir comprising an aqueous suspension of biological particles 314. Upstream of, and immediately prior to reaching, the second junction 310, the channel segment 301 may meet the channel segment 302 at the first junction 309. The channel segment 302 may transport a plurality of reagents 315 (e.g., lysis agents) suspended in the aqueous fluid 312 along the channel segment 302 into the first junction 309. For example, the channel segment 302 may be connected to a reservoir comprising the reagents 315. After the first junction 309, the aqueous fluid 312 in the channel segment 301 can carry both the biological particles 314 and the reagents 315 towards the second junction 310. In some instances, the aqueous fluid 312 in the channel segment 301

can include one or more reagents, which can be the same or different reagents as the reagents 315. A second fluid 316 that is immiscible with the aqueous fluid 312 (e.g., oil) can be delivered to the second junction 310 from each of channel segments 304 and 306. Upon meeting of the aqueous fluid 312 from the channel segment 301 and the second fluid 316 from each of channel segments 304 and 306 at the second channel junction 310, the aqueous fluid 312 can be partitioned as discrete droplets 318 in the second fluid 316 and flow away from the second junction 310 along channel segment 308. The channel segment 308 may deliver the discrete droplets 318 to an outlet reservoir fluidly coupled to the channel segment 308, where they may be harvested.

The second fluid 316 can comprise an oil, such as a fluorinated oil, that includes a fluorosurfactant for stabilizing the resulting droplets, for example, inhibiting subsequent coalescence of the resulting droplets 318.

A discrete droplet generated may include an individual biological particle 314 and/or one or more reagents 315. In some instances, a discrete droplet generated may include a barcode carrying bead (not shown), such as via other microfluidics structures described elsewhere herein. In some instances, a discrete droplet may be unoccupied (e.g., no reagents, no biological particles).

Beneficially, when lysis reagents and biological particles are co-partitioned, the lysis reagents can facilitate the release of the contents of the biological particles within the partition. The contents released in a partition may remain discrete from the contents of other partitions.

As will be appreciated, the channel segments described herein may be coupled to any of a variety of different fluid sources or receiving components, including reservoirs, tubing, manifolds, or fluidic components of other systems. As will be appreciated, the microfluidic channel structure 300 may have other geometries. For example, a microfluidic channel structure can have more than two channel junctions. For example, a microfluidic channel structure can have 2, 3, 4, 5 channel segments or more each carrying the same or different types of beads, reagents, and/or biological particles that meet at a channel junction. Fluid flow in each channel segment may be controlled to control the partitioning of the different elements into droplets. Fluid may be directed flow along one or more channels or reservoirs via one or more fluid flow units. A fluid flow unit can comprise compressors (e.g., providing positive pressure), pumps (e.g., providing negative pressure), actuators, and the like to control flow of the fluid. Fluid may also or otherwise be controlled via applied pressure differentials, centrifugal force, magnetic force, electrokinetic pumping, vacuum, capillary or gravity flow, or the like.

Examples of lysis agents include bioactive reagents, such as lysis enzymes that are used for lysis of different cell types, e.g., gram-positive or gram-negative bacteria, plants, yeast, mammalian, etc., such as lysozymes, achromopeptidase, lysostaphin, labiase, kitalase, lyticase, and a variety of other lysis enzymes available from, e.g., Sigma-Aldrich, Inc. (St Louis, MO), as well as other commercially available lysis enzymes. Other lysis agents may additionally or alternatively be co-partitioned with the biological particles to cause the release of the biological particle's contents into the partitions. For example, in some cases, surfactant-based lysis solutions may be used to lyse cells, although these may be less desirable for emulsion-based systems where the surfactants can interfere with stable emulsions. In some cases, lysis solutions may include non-ionic surfactants such as, for example, TritonX-100, CHAPS, and Tween 20. In some cases, lysis solutions may include ionic surfactants

such as, for example, sarcosyl and sodium dodecyl sulfate (SDS). In some cases, lysis may be achieved through osmotic pressure, e.g., using a hypotonic lysis buffer. Electroporation, thermal, acoustic or mechanical cellular disruption may also be used in certain cases, e.g., non-emulsion based partitioning such as encapsulation of biological particles that may be in addition to or in place of droplet partitioning, where any pore size of the encapsulate is sufficiently small to retain nucleic acid fragments of a given size, following cellular disruption.

Alternatively or in addition to the lysis agents co-partitioned with the biological particles described above, other reagents can also be co-partitioned with the biological particles, including, for example, DNase and RNase, inactivating agents or inhibitors, such as proteinase K and/or other protease inhibitors, phosphatase inhibitors, chelating agents, such as EDTA, and other reagents employed in removing or otherwise reducing negative activity or impact of different cell lysate components on subsequent processing of nucleic acids. In addition, in the case of encapsulated biological particles, the biological particles may be exposed to an appropriate stimulus to release the biological particles or their contents from a co-partitioned microcapsule. For example, in some cases, a chemical stimulus may be co-partitioned along with an encapsulated biological particle to allow for the degradation of the microcapsule and release of the cell or its contents into the larger partition. In some cases, this stimulus may be the same as the stimulus described elsewhere herein for release of nucleic acid molecules (e.g., oligonucleotides) from their respective microcapsule (e.g., bead). In alternative aspects, this may be a different and non-overlapping stimulus, in order to allow an encapsulated biological particle to be released into a partition at a different time from the release of nucleic acid molecules into the same partition.

Additional reagents may also be co-partitioned with the biological particles, such as endonucleases to fragment a biological particle's DNA, DNA polymerase enzymes and dNTPs used to amplify the biological particle's nucleic acid fragments and to attach the barcode molecular tags to the amplified fragments. Other enzymes may be co-partitioned, including without limitation, polymerase, transposase, ligase, proteinase K, DNase, etc. Additional reagents may also include reverse transcriptase enzymes, including enzymes with terminal transferase activity, primers and oligonucleotides, and switch oligonucleotides (also referred to herein as "switch oligos" or "template switching oligonucleotides") which can be used for template switching. In some cases, template switching can be used to increase the length of a cDNA. In some cases, template switching can be used to append a predefined nucleic acid sequence to the cDNA. In an example of template switching, cDNA can be generated from reverse transcription of a template, e.g., cellular mRNA, where a reverse transcriptase with terminal transferase activity can add additional nucleotides, e.g., polyC, to the cDNA in a template independent manner. Switch oligos can include sequences complementary to the additional nucleotides, e.g., polyG. The additional nucleotides (e.g., polyC) on the cDNA can hybridize to the additional nucleotides (e.g., polyG) on the switch oligo, whereby the switch oligo can be used by the reverse transcriptase as template to further extend the cDNA. Template switching oligonucleotides may comprise a hybridization region and a template region. The hybridization region can comprise any sequence capable of hybridizing to the target. In some cases, as previously described, the hybridization region comprises a series of G bases to complement the

overhanging C bases at the 3' end of a cDNA molecule. The series of G bases may comprise 1 G base, 2 G bases, 3 G bases, 4 G bases, 5 G bases or more than 5 G bases. The template sequence can comprise any sequence to be incorporated into the cDNA. In some cases, the template region comprises at least 1 (e.g., at least 2, 3, 4, 5 or more) tag sequences and/or functional sequences. Switch oligos may comprise deoxyribonucleic acids; ribonucleic acids; modified nucleic acids including 2-Aminopurine, 2,6-Diaminopurine (2-Amino-dA), inverted dT, 5-Methyl dC, 2'-deoxyinosine, Super T (5-hydroxybutyln-2'-deoxyuridine), Super G (8-aza-7-deazaguanosine), locked nucleic acids (LNAs), unlocked nucleic acids (UNAs, e.g., UNA-A, UNA-U, UNA-C, UNA-G), Iso-dG, Iso-dC, 2' Fluoro bases (e.g., Fluoro C, Fluoro U, Fluoro A, and Fluoro G), or any combination.

In some cases, the length of a switch oligo may be at least about 2, 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249 or 250 nucleotides or longer.

In some cases, the length of a switch oligo may be at most about 2, 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249 or 250 nucleotides.

Once the contents of the cells are released into their respective partitions, the macromolecular components (e.g., macromolecular constituents of biological particles, such as RNA, DNA, or proteins) contained therein may be further processed within the partitions. In accordance with the methods and systems described herein, the macromolecular component contents of individual biological particles can be provided with unique identifiers such that, upon characterization of those macromolecular components they may be attributed as having been derived from the same biological

particle or particles. The ability to attribute characteristics to individual biological particles or groups of biological particles is provided by the assignment of unique identifiers specifically to an individual biological particle or groups of biological particles. Unique identifiers, e.g., in the form of nucleic acid barcodes can be assigned or associated with individual biological particles or populations of biological particles, in order to tag or label the biological particle's macromolecular components (and as a result, its characteristics) with the unique identifiers. These unique identifiers can then be used to attribute the biological particle's components and characteristics to an individual biological particle or group of biological particles.

In some aspects, this is performed by co-partitioning the individual biological particle or groups of biological particles with the unique identifiers, such as described above (with reference to FIG. 2). In some aspects, the unique identifiers are provided in the form of nucleic acid molecules (e.g., oligonucleotides) that comprise nucleic acid barcode sequences that may be attached to or otherwise associated with the nucleic acid contents of individual biological particle, or to other components of the biological particle, and particularly to fragments of those nucleic acids. The nucleic acid molecules are partitioned such that as between nucleic acid molecules in a given partition, the nucleic acid barcode sequences contained therein are the same, but as between different partitions, the nucleic acid molecule can, and do have differing barcode sequences, or at least represent a large number of different barcode sequences across all of the partitions in a given analysis. In some aspects, only one nucleic acid barcode sequence can be associated with a given partition, although in some cases, two or more different barcode sequences may be present.

The nucleic acid barcode sequences can include from about 6 to about 20 or more nucleotides within the sequence of the nucleic acid molecules (e.g., oligonucleotides). The nucleic acid barcode sequences can include from about 6 to about 20, 30, 40, 50, 60, 70, 80, 90, 100 or more nucleotides. In some cases, the length of a barcode sequence may be about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or longer. In some cases, the length of a barcode sequence may be at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or longer. In some cases, the length of a barcode sequence may be at most about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 nucleotides or shorter. These nucleotides may be completely contiguous, i.e., in a single stretch of adjacent nucleotides, or they may be separated into two or more separate subsequences that are separated by 1 or more nucleotides. In some cases, separated barcode subsequences can be from about 4 to about 16 nucleotides in length. In some cases, the barcode subsequence may be about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or longer. In some cases, the barcode subsequence may be at least about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or longer. In some cases, the barcode subsequence may be at most about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 nucleotides or shorter.

The co-partitioned nucleic acid molecules can also comprise other functional sequences useful in the processing of the nucleic acids from the co-partitioned biological particles. These sequences include, e.g., targeted or random/universal amplification primer sequences for amplifying the genomic DNA from the individual biological particles within the partitions while attaching the associated barcode sequences, sequencing primers or primer recognition sites, hybridization or probing sequences, e.g., for identification of presence of the sequences or for pulling down barcoded nucleic acids,

or any of a number of other potential functional sequences. In one example, functional sequences such as targeted primer sequences can be prepared for use in assays for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq). Primer sequences can be designed for targeted loci (e.g., for biomarker or functional studies). For example, Yost describes a primer tool for targeted analysis of accessible chromatin suitable for ATAC-Seq (see, Yost et al., *Nature Methods*, (2018), 15(5):304-305). Other mechanisms of co-partitioning oligonucleotides may also be employed, including, e.g., coalescence of two or more droplets, where one droplet contains oligonucleotides, or microdispensing of oligonucleotides into partitions, e.g., droplets within microfluidic systems.

In an example, microcapsules, such as beads, are provided that each include large numbers of the above described barcode nucleic acid molecules (e.g., barcoded oligonucleotides) releasably attached to the beads, where all of the nucleic acid molecules attached to a particular bead will include the same nucleic acid barcode sequence, but where a large number of diverse barcode sequences are represented across the population of beads used. In some embodiments, hydrogel beads, e.g., comprising polyacrylamide polymer matrices, are used as a solid support and delivery vehicle for the nucleic acid molecules into the partitions, as they are capable of carrying large numbers of nucleic acid molecules, and may be configured to release those nucleic acid molecules upon exposure to a particular stimulus, as described elsewhere herein. In some cases, the population of beads provides a diverse barcode sequence library that includes at least about 1,000 different barcode sequences, at least about 5,000 different barcode sequences, at least about 10,000 different barcode sequences, at least about 50,000 different barcode sequences, at least about 100,000 different barcode sequences, at least about 1,000,000 different barcode sequences, at least about 5,000,000 different barcode sequences, or at least about 10,000,000 different barcode sequences, or more. Additionally, each bead can be provided with large numbers of nucleic acid (e.g., oligonucleotide) molecules attached. In particular, the number of molecules of nucleic acid molecules including the barcode sequence on an individual bead can be at least about 1,000 nucleic acid molecules, at least about 5,000 nucleic acid molecules, at least about 10,000 nucleic acid molecules, at least about 50,000 nucleic acid molecules, at least about 100,000 nucleic acid molecules, at least about 500,000 nucleic acids, at least about 1,000,000 nucleic acid molecules, at least about 5,000,000 nucleic acid molecules, at least about 10,000,000 nucleic acid molecules, at least about 50,000,000 nucleic acid molecules, at least about 100,000,000 nucleic acid molecules, at least about 250,000,000 nucleic acid molecules and in some cases at least about 1 billion nucleic acid molecules, or more. Nucleic acid molecules of a given bead can include identical (or common) barcode sequences, different barcode sequences, or a combination of both. Nucleic acid molecules of a given bead can include multiple sets of nucleic acid molecules. Nucleic acid molecules of a given set can include identical barcode sequences. The identical barcode sequences can be different from barcode sequences of nucleic acid molecules of another set.

Moreover, when the population of beads is partitioned, the resulting population of partitions can also include a diverse barcode library that includes at least about 1,000 different barcode sequences, at least about 5,000 different barcode sequences, at least about 10,000 different barcode sequences, at least at least about 50,000 different barcode

sequences, at least about 100,000 different barcode sequences, at least about 1,000,000 different barcode sequences, at least about 5,000,000 different barcode sequences, or at least about 10,000,000 different barcode sequences. Additionally, each partition of the population can include at least about 1,000 nucleic acid molecules, at least about 5,000 nucleic acid molecules, at least about 10,000 nucleic acid molecules, at least about 50,000 nucleic acid molecules, at least about 100,000 nucleic acid molecules, at least about 500,000 nucleic acids, at least about 1,000,000 nucleic acid molecules, at least about 5,000,000 nucleic acid molecules, at least about 10,000,000 nucleic acid molecules, at least about 50,000,000 nucleic acid molecules, at least about 100,000,000 nucleic acid molecules, at least about 250,000,000 nucleic acid molecules and in some cases at least about 1 billion nucleic acid molecules.

In some cases, it may be desirable to incorporate multiple different barcodes within a given partition, either attached to a single or multiple beads within the partition. For example, in some cases, a mixed, but known set of barcode sequences may provide greater assurance of identification in the subsequent processing, e.g., by providing a stronger address or attribution of the barcodes to a given partition, as a duplicate or independent confirmation of the output from a given partition.

The nucleic acid molecules (e.g., oligonucleotides) are releasable from the beads upon the application of a particular stimulus to the beads. In some cases, the stimulus may be a photo-stimulus, e.g., through cleavage of a photo-labile linkage that releases the nucleic acid molecules. In other cases, a thermal stimulus may be used, where elevation of the temperature of the beads environment will result in cleavage of a linkage or other release of the nucleic acid molecules from the beads. In still other cases, a chemical stimulus can be used that cleaves a linkage of the nucleic acid molecules to the beads, or otherwise results in release of the nucleic acid molecules from the beads. In one case, such compositions include the polyacrylamide matrices described above for encapsulation of biological particles, and may be degraded for release of the attached nucleic acid molecules through exposure to a reducing agent, such as DTT.

In some aspects, provided are systems and methods for controlled partitioning. Droplet size may be controlled by adjusting certain geometric features in channel architecture (e.g., microfluidics channel architecture). For example, an expansion angle, width, and/or length of a channel may be adjusted to control droplet size.

FIG. 4 shows an example of a microfluidic channel structure for the controlled partitioning of beads into discrete droplets. A channel structure 400 can include a channel segment 402 communicating at a channel junction 406 (or intersection) with a reservoir 404. The reservoir 404 can be a chamber. Any reference to "reservoir," as used herein, can also refer to a "chamber." In operation, an aqueous fluid 408 that includes suspended beads 412 may be transported along the channel segment 402 into the junction 406 to meet a second fluid 410 that is immiscible with the aqueous fluid 408 in the reservoir 404 to create droplets 416, 418 of the aqueous fluid 408 flowing into the reservoir 404. At the junction 406 where the aqueous fluid 408 and the second fluid 410 meet, droplets can form based on factors such as the hydrodynamic forces at the junction 406, flow rates of the two fluids 408, 410, fluid properties, and certain geometric parameters (e.g., w, h<sub>o</sub>, α, etc.) of the channel structure 400. A plurality of droplets can be collected in the

reservoir 404 by continuously injecting the aqueous fluid 408 from the channel segment 402 through the junction 406.

A discrete droplet generated may include a bead (e.g., as in occupied droplets 416). Alternatively, a discrete droplet generated may include more than one bead. Alternatively, a discrete droplet generated may not include any beads (e.g., as in unoccupied droplet 418). In some instances, a discrete droplet generated may contain one or more biological particles, as described elsewhere herein. In some instances, a discrete droplet generated may comprise one or more reagents, as described elsewhere herein.

In some instances, the aqueous fluid 408 can have a substantially uniform concentration or frequency of beads 412. The beads 412 can be introduced into the channel segment 402 from a separate channel (not shown in FIG. 4). The frequency of beads 412 in the channel segment 402 may be controlled by controlling the frequency in which the beads 412 are introduced into the channel segment 402 and/or the relative flow rates of the fluids in the channel segment 402 and the separate channel. In some instances, the beads can be introduced into the channel segment 402 from a plurality of different channels, and the frequency controlled accordingly.

In some instances, the aqueous fluid 408 in the channel segment 402 can comprise biological particles (e.g., described with reference to FIGS. 1 and 2). In some instances, the aqueous fluid 408 can have a substantially uniform concentration or frequency of biological particles. As with the beads, the biological particles can be introduced into the channel segment 402 from a separate channel. The frequency or concentration of the biological particles in the aqueous fluid 408 in the channel segment 402 may be controlled by controlling the frequency in which the biological particles are introduced into the channel segment 402 and/or the relative flow rates of the fluids in the channel segment 402 and the separate channel. In some instances, the biological particles can be introduced into the channel segment 402 from a plurality of different channels, and the frequency controlled accordingly. In some instances, a first separate channel can introduce beads and a second separate channel can introduce biological particles into the channel segment 402. The first separate channel introducing the beads may be upstream or downstream of the second separate channel introducing the biological particles.

The second fluid 410 can comprise an oil, such as a fluorinated oil, that includes a fluorosurfactant for stabilizing the resulting droplets, for example, inhibiting subsequent coalescence of the resulting droplets.

In some instances, the second fluid 410 may not be subjected to and/or directed to any flow in or out of the reservoir 404. For example, the second fluid 410 may be substantially stationary in the reservoir 404. In some instances, the second fluid 410 may be subjected to flow within the reservoir 404, but not in or out of the reservoir 404, such as via application of pressure to the reservoir 404 and/or as affected by the incoming flow of the aqueous fluid 408 at the junction 406. Alternatively, the second fluid 410 may be subjected and/or directed to flow in or out of the reservoir 404. For example, the reservoir 404 can be a channel directing the second fluid 410 from upstream to downstream, transporting the generated droplets.

The channel structure 400 at or near the junction 406 may have certain geometric features that at least partly determine the sizes of the droplets formed by the channel structure 400. The channel segment 402 can have a height,  $h_0$  and width,  $w$ , at or near the junction 406. By way of example, the channel segment 402 can comprise a rectangular cross-

section that leads to a reservoir 404 having a wider cross-section (such as in width or diameter). Alternatively, the cross-section of the channel segment 402 can be other shapes, such as a circular shape, trapezoidal shape, polygonal shape, or any other shapes. The top and bottom walls of the reservoir 404 at or near the junction 406 can be inclined at an expansion angle,  $\alpha$ . The expansion angle,  $\alpha$ , allows the tongue (portion of the aqueous fluid 408 leaving channel segment 402 at junction 406 and entering the reservoir 404 before droplet formation) to increase in depth and facilitate decrease in curvature of the intermediately formed droplet. Droplet size may decrease with increasing expansion angle. The resulting droplet radius,  $R_d$ , may be predicted by the following equation for the aforementioned geometric parameters of  $h_0$ ,  $w$ , and  $\alpha$ :

$$R_d \approx 0.44 \left(1 + 2.2\sqrt{\tan \alpha}\right) \frac{w}{h_0} \frac{h_0}{\sqrt{\tan \alpha}}$$

By way of example, for a channel structure with  $w=21$   $\mu\text{m}$ ,  $h=21$   $\mu\text{m}$ , and  $\alpha=3^\circ$ , the predicted droplet diameter is 121  $\mu\text{m}$ . In another example, for a channel structure with  $w=25$   $\mu\text{m}$ ,  $h=25$   $\mu\text{m}$ , and  $\alpha=5^\circ$ , the predicted droplet diameter is 123  $\mu\text{m}$ . In another example, for a channel structure with  $w=28$   $\mu\text{m}$ ,  $h=28$   $\mu\text{m}$ , and  $\alpha=7^\circ$ , the predicted droplet diameter is 124  $\mu\text{m}$ .

In some instances, the expansion angle,  $\alpha$ , may be between a range of from about  $0.5^\circ$  to about  $4^\circ$ , from about  $0.1^\circ$  to about  $10^\circ$ , or from about  $0^\circ$  to about  $90^\circ$ . For example, the expansion angle can be at least about  $0.01^\circ$ ,  $0.1^\circ$ ,  $0.2^\circ$ ,  $0.3^\circ$ ,  $0.4^\circ$ ,  $0.5^\circ$ ,  $0.6^\circ$ ,  $0.7^\circ$ ,  $0.8^\circ$ ,  $0.9^\circ$ ,  $1^\circ$ ,  $2^\circ$ ,  $3^\circ$ ,  $4^\circ$ ,  $5^\circ$ ,  $6^\circ$ ,  $7^\circ$ ,  $8^\circ$ ,  $9^\circ$ ,  $10^\circ$ ,  $15^\circ$ ,  $20^\circ$ ,  $25^\circ$ ,  $30^\circ$ ,  $35^\circ$ ,  $40^\circ$ ,  $45^\circ$ ,  $50^\circ$ ,  $55^\circ$ ,  $60^\circ$ ,  $65^\circ$ ,  $70^\circ$ ,  $75^\circ$ ,  $80^\circ$ ,  $85^\circ$ , or higher. In some instances, the expansion angle can be at most about  $89^\circ$ ,  $88^\circ$ ,  $87^\circ$ ,  $86^\circ$ ,  $85^\circ$ ,  $84^\circ$ ,  $83^\circ$ ,  $82^\circ$ ,  $81^\circ$ ,  $80^\circ$ ,  $75^\circ$ ,  $70^\circ$ ,  $65^\circ$ ,  $60^\circ$ ,  $55^\circ$ ,  $50^\circ$ ,  $45^\circ$ ,  $40^\circ$ ,  $35^\circ$ ,  $30^\circ$ ,  $25^\circ$ ,  $20^\circ$ ,  $15^\circ$ ,  $10^\circ$ ,  $9^\circ$ ,  $8^\circ$ ,  $7^\circ$ ,  $6^\circ$ ,  $5^\circ$ ,  $4^\circ$ ,  $3^\circ$ ,  $2^\circ$ ,  $1^\circ$ ,  $0.1^\circ$ ,  $0.01^\circ$ , or less. In some instances, the width,  $w$ , can be between a range of from about 100 micrometers ( $\mu\text{m}$ ) to about 500  $\mu\text{m}$ . In some instances, the width,  $w$ , can be between a range of from about 10  $\mu\text{m}$  to about 200  $\mu\text{m}$ . Alternatively, the width can be less than about 10  $\mu\text{m}$ . Alternatively, the width can be greater than about 500  $\mu\text{m}$ . In some instances, the flow rate of the aqueous fluid 408 entering the junction 406 can be between about 0.04 microliters ( $\mu\text{L}$ )/minute (min) and about 40  $\mu\text{L}/\text{min}$ . In some instances, the flow rate of the aqueous fluid 408 entering the junction 406 can be between about 0.01 microliters ( $\mu\text{L}$ )/minute (min) and about 100  $\mu\text{L}/\text{min}$ . Alternatively, the flow rate of the aqueous fluid 408 entering the junction 406 can be less than about 0.01  $\mu\text{L}/\text{min}$ . Alternatively, the flow rate of the aqueous fluid 408 entering the junction 406 can be greater than about 40  $\mu\text{L}/\text{min}$ , such as 45  $\mu\text{L}/\text{min}$ , 50  $\mu\text{L}/\text{min}$ , 55  $\mu\text{L}/\text{min}$ , 60  $\mu\text{L}/\text{min}$ , 65  $\mu\text{L}/\text{min}$ , 70  $\mu\text{L}/\text{min}$ , 75  $\mu\text{L}/\text{min}$ , 80  $\mu\text{L}/\text{min}$ , 85  $\mu\text{L}/\text{min}$ , 90  $\mu\text{L}/\text{min}$ , 95  $\mu\text{L}/\text{min}$ , 100  $\mu\text{L}/\text{min}$ , 110  $\mu\text{L}/\text{min}$ , 120  $\mu\text{L}/\text{min}$ , 130  $\mu\text{L}/\text{min}$ , 140  $\mu\text{L}/\text{min}$ , 150  $\mu\text{L}/\text{min}$ , or greater. At lower flow rates, such as flow rates of about less than or equal to 10 microliters/minute, the droplet radius may not be dependent on the flow rate of the aqueous fluid 408 entering the junction 406.

In some instances, at least about 50% of the droplets generated can have uniform size. In some instances, at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or greater of the droplets generated can have uniform size. Alternatively, less than about 50% of the droplets generated can have uniform size.

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The throughput of droplet generation can be increased by increasing the points of generation, such as increasing the number of junctions (e.g., junction 406) between aqueous fluid 408 channel segments (e.g., channel segment 402) and the reservoir 404. Alternatively or in addition, the throughput of droplet generation can be increased by increasing the flow rate of the aqueous fluid 408 in the channel segment 402.

FIG. 5 shows an example of a microfluidic channel structure for increased droplet generation throughput. A microfluidic channel structure 500 can comprise a plurality of channel segments 502 and a reservoir 504. Each of the plurality of channel segments 502 may be in fluid communication with the reservoir 504. The channel structure 500 can comprise a plurality of channel junctions 506 between the plurality of channel segments 502 and the reservoir 504. Each channel junction can be a point of droplet generation. The channel segment 402 from the channel structure 400 in FIG. 4 and any description to the components thereof may correspond to a given channel segment of the plurality of channel segments 502 in channel structure 500 and any description to the corresponding components thereof. The reservoir 404 from the channel structure 400 and any description to the components thereof may correspond to the reservoir 504 from the channel structure 500 and any description to the corresponding components thereof.

Each channel segment of the plurality of channel segments 502 may comprise an aqueous fluid 508 that includes suspended beads 512. The reservoir 504 may comprise a second fluid 510 that is immiscible with the aqueous fluid 508. In some instances, the second fluid 510 may not be subjected to and/or directed to any flow in or out of the reservoir 504. For example, the second fluid 510 may be substantially stationary in the reservoir 504. In some instances, the second fluid 510 may be subjected to flow within the reservoir 504, but not in or out of the reservoir 504, such as via application of pressure to the reservoir 504 and/or as affected by the incoming flow of the aqueous fluid 508 at the junctions. Alternatively, the second fluid 510 may be subjected and/or directed to flow in or out of the reservoir 504. For example, the reservoir 504 can be a channel directing the second fluid 510 from upstream to downstream, transporting the generated droplets.

In operation, the aqueous fluid 508 that includes suspended beads 512 may be transported along the plurality of channel segments 502 into the plurality of junctions 506 to meet the second fluid 510 in the reservoir 504 to create droplets 516, 518. A droplet may form from each channel segment at each corresponding junction with the reservoir 504. At the junction where the aqueous fluid 508 and the second fluid 510 meet, droplets can form based on factors such as the hydrodynamic forces at the junction, flow rates of the two fluids 508, 510, fluid properties, and certain geometric parameters (e.g., w, h<sub>0</sub>, α, etc.) of the channel structure 500, as described elsewhere herein. A plurality of droplets can be collected in the reservoir 504 by continuously injecting the aqueous fluid 508 from the plurality of channel segments 502 through the plurality of junctions 506. Throughput may significantly increase with the parallel channel configuration of channel structure 500. For example, a channel structure having five inlet channel segments comprising the aqueous fluid 508 may generate droplets five times as frequently than a channel structure having one inlet channel segment, provided that the fluid flow rate in the channel segments are substantially the same. The fluid flow rate in the different inlet channel segments may or may not be substantially the same. A channel

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structure may have as many parallel channel segments as is practical and allowed for the size of the reservoir. For example, the channel structure may have at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 500, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1500, 5000 or more parallel or substantially parallel channel segments.

The geometric parameters, w, h<sub>0</sub>, and α, may or may not be uniform for each of the channel segments in the plurality of channel segments 502. For example, each channel segment may have the same or different widths at or near its respective channel junction with the reservoir 504. For example, each channel segment may have the same or different height at or near its respective channel junction with the reservoir 504. In another example, the reservoir 504 may have the same or different expansion angle at the different channel junctions with the plurality of channel segments 502. When the geometric parameters are uniform, beneficially, droplet size may also be controlled to be uniform even with the increased throughput. In some instances, when it is desirable to have a different distribution of droplet sizes, the geometric parameters for the plurality of channel segments 502 may be varied accordingly.

In some instances, at least about 50% of the droplets generated can have uniform size. In some instances, at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or greater of the droplets generated can have uniform size. Alternatively, less than about 50% of the droplets generated can have uniform size.

FIG. 6 shows another example of a microfluidic channel structure for increased droplet generation throughput. A microfluidic channel structure 600 can comprise a plurality of channel segments 602 arranged generally circularly around the perimeter of a reservoir 604. Each of the plurality of channel segments 602 may be in fluid communication with the reservoir 604. The channel structure 600 can comprise a plurality of channel junctions 606 between the plurality of channel segments 602 and the reservoir 604. Each channel junction can be a point of droplet generation. The channel segment 402 from the channel structure 400 in FIG. 4 and any description to the components thereof may correspond to a given channel segment of the plurality of channel segments 602 in channel structure 600 and any description to the corresponding components thereof. The reservoir 404 from the channel structure 400 and any description to the components thereof may correspond to the reservoir 604 from the channel structure 600 and any description to the corresponding components thereof.

Each channel segment of the plurality of channel segments 602 may comprise an aqueous fluid 608 that includes suspended beads 612. The reservoir 604 may comprise a second fluid 610 that is immiscible with the aqueous fluid 608. In some instances, the second fluid 610 may not be subjected to and/or directed to any flow in or out of the reservoir 604. For example, the second fluid 610 may be substantially stationary in the reservoir 604. In some instances, the second fluid 610 may be subjected to flow within the reservoir 604, but not in or out of the reservoir 604, such as via application of pressure to the reservoir 604 and/or as affected by the incoming flow of the aqueous fluid 608 at the junctions. Alternatively, the second fluid 610 may be subjected and/or directed to flow in or out of the reservoir 604. For example, the reservoir 604 can be a channel directing the second fluid 610 from upstream to downstream, transporting the generated droplets.

In operation, the aqueous fluid 608 that includes suspended beads 612 may be transported along the plurality of

channel segments 602 into the plurality of junctions 606 to meet the second fluid 610 in the reservoir 604 to create a plurality of droplets 616. A droplet may form from each channel segment at each corresponding junction with the reservoir 604. At the junction where the aqueous fluid 608 and the second fluid 610 meet, droplets can form based on factors such as the hydrodynamic forces at the junction, flow rates of the two fluids 608, 610, fluid properties, and certain geometric parameters (e.g., widths and heights of the channel segments 602, expansion angle of the reservoir 604, etc.) of the channel structure 600, as described elsewhere herein. A plurality of droplets can be collected in the reservoir 604 by continuously injecting the aqueous fluid 608 from the plurality of channel segments 602 through the plurality of junctions 606. Throughput may significantly increase with the substantially parallel channel configuration of the channel structure 600. A channel structure may have as many substantially parallel channel segments as is practical and allowed for by the size of the reservoir. For example, the channel structure may have at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1500, 5000 or more parallel or substantially parallel channel segments. The plurality of channel segments may be substantially evenly spaced apart, for example, around an edge or perimeter of the reservoir. Alternatively, the spacing of the plurality of channel segments may be uneven.

The reservoir 604 may have an expansion angle,  $\alpha$  (not shown in FIG. 6) at or near each channel junction. Each channel segment of the plurality of channel segments 602 may have a width,  $w$ , and a height,  $h_0$ , at or near the channel junction. The geometric parameters,  $w$ ,  $h_0$ , and  $\alpha$ , may or may not be uniform for each of the channel segments in the plurality of channel segments 602. For example, each channel segment may have the same or different widths at or near its respective channel junction with the reservoir 604. For example, each channel segment may have the same or different height at or near its respective channel junction with the reservoir 604.

The reservoir 604 may have the same or different expansion angle at the different channel junctions with the plurality of channel segments 602. For example, a circular reservoir (as shown in FIG. 6) may have a conical, dome-like, or hemispherical ceiling (e.g., top wall) to provide the same or substantially same expansion angle for each channel segments 602 at or near the plurality of channel junctions 606. When the geometric parameters are uniform, beneficially, resulting droplet size may be controlled to be uniform even with the increased throughput. In some instances, when it is desirable to have a different distribution of droplet sizes, the geometric parameters for the plurality of channel segments 602 may be varied accordingly.

In some instances, at least about 50% of the droplets generated can have uniform size. In some instances, at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or greater of the droplets generated can have uniform size. Alternatively, less than about 50% of the droplets generated can have uniform size. The beads and/or biological particle injected into the droplets may or may not have uniform size.

FIG. 7A shows a cross-section view of another example of a microfluidic channel structure with a geometric feature for controlled partitioning. A channel structure 700 can include a channel segment 702 communicating at a channel junction 706 (or intersection) with a reservoir 704. In some instances, the channel structure 700 and one or more of its components can correspond to the channel structure 100 and

one or more of its components. FIG. 7B shows a perspective view of the channel structure 700 of FIG. 7A.

An aqueous fluid 712 comprising a plurality of particles 716 may be transported along the channel segment 702 into the junction 706 to meet a second fluid 714 (e.g., oil, etc.) that is immiscible with the aqueous fluid 712 in the reservoir 704 to create droplets 720 of the aqueous fluid 712 flowing into the reservoir 704. At the junction 706 where the aqueous fluid 712 and the second fluid 714 meet, droplets can form based on factors such as the hydrodynamic forces at the junction 706, relative flow rates of the two fluids 712, 714, fluid properties, and certain geometric parameters (e.g.,  $\Delta h$ , etc.) of the channel structure 700. A plurality of droplets can be collected in the reservoir 704 by continuously injecting the aqueous fluid 712 from the channel segment 702 at the junction 706.

A discrete droplet generated may comprise one or more particles of the plurality of particles 716. As described elsewhere herein, a particle may be any particle, such as a bead, cell bead, gel bead, biological particle, macromolecular constituents of biological particle, or other particles. Alternatively, a discrete droplet generated may not include any particles.

In some instances, the aqueous fluid 712 can have a substantially uniform concentration or frequency of particles 716. As described elsewhere herein (e.g., with reference to FIG. 4), the particles 716 (e.g., beads) can be introduced into the channel segment 702 from a separate channel (not shown in FIG. 7). The frequency of particles 716 in the channel segment 702 may be controlled by controlling the frequency in which the particles 716 are introduced into the channel segment 702 and/or the relative flow rates of the fluids in the channel segment 702 and the separate channel. In some instances, the particles 716 can be introduced into the channel segment 702 from a plurality of different channels, and the frequency controlled accordingly. In some instances, different particles may be introduced via separate channels. For example, a first separate channel can introduce beads and a second separate channel can introduce biological particles into the channel segment 702. The first separate channel introducing the beads may be upstream or downstream of the second separate channel introducing the biological particles.

In some instances, the second fluid 714 may not be subjected to and/or directed to any flow in or out of the reservoir 704. For example, the second fluid 714 may be substantially stationary in the reservoir 704. In some instances, the second fluid 714 may be subjected to flow within the reservoir 704, but not in or out of the reservoir 704, such as via application of pressure to the reservoir 704 and/or as affected by the incoming flow of the aqueous fluid 712 at the junction 706. Alternatively, the second fluid 714 may be subjected and/or directed to flow in or out of the reservoir 704. For example, the reservoir 704 can be a channel directing the second fluid 714 from upstream to downstream, transporting the generated droplets.

The channel structure 700 at or near the junction 706 may have certain geometric features that at least partly determine the sizes and/or shapes of the droplets formed by the channel structure 700. The channel segment 702 can have a first cross-section height,  $h_1$ , and the reservoir 704 can have a second cross-section height,  $h_2$ . The first cross-section height,  $h_1$ , and the second cross-section height,  $h_2$ , may be different, such that at the junction 706, there is a height difference of  $\Delta h$ . The second cross-section height,  $h_2$ , may be greater than the first cross-section height,  $h_1$ . In some instances, the reservoir may thereafter gradually increase in

cross-section height, for example, the more distant it is from the junction 706. In some instances, the cross-section height of the reservoir may increase in accordance with expansion angle,  $\beta$ , at or near the junction 706. The height difference,  $\Delta h$ , and/or expansion angle,  $\beta$ , can allow the tongue (portion of the aqueous fluid 712 leaving channel segment 702 at junction 706 and entering the reservoir 704 before droplet formation) to increase in depth and facilitate decrease in curvature of the intermediately formed droplet. For example, droplet size may decrease with increasing height difference and/or increasing expansion angle.

The height difference,  $\Delta h$ , can be at least about 1  $\mu\text{m}$ . Alternatively, the height difference can be at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500  $\mu\text{m}$  or more. Alternatively, the height difference can be at most about 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1  $\mu\text{m}$  or less. In some instances, the expansion angle,  $\beta$ , may be between a range of from about 0.5° to about 4°, from about 0.1° to about 10°, or from about 0° to about 90°. For example, the expansion angle can be at least about 0.01°, 0.1°, 0.2°, 0.3°, 0.4°, 0.5°, 0.6°, 0.7°, 0.8°, 0.9°, 1°, 2°, 3°, 4°, 5°, 6°, 7°, 8°, 9°, 10°, 15°, 20°, 25°, 30°, 35°, 40°, 45°, 50°, 55°, 60°, 65°, 70°, 75°, 85°, or higher. In some instances, the expansion angle can be at most about 89°, 88°, 87°, 86°, 85°, 84°, 83°, 82°, 81°, 80°, 75°, 70°, 65°, 60°, 55°, 50°, 45°, 40°, 35°, 30°, 25°, 20°, 15°, 10°, 9°, 8°, 7°, 6°, 5°, 4°, 3°, 2°, 1°, 0.1°, or less.

In some instances, the flow rate of the aqueous fluid 712 entering the junction 706 can be between about 0.04 microliters ( $\mu\text{L}$ )/minute (min) and about 40  $\mu\text{L}/\text{min}$ . In some instances, the flow rate of the aqueous fluid 712 entering the junction 706 can be between about 0.01 microliters ( $\mu\text{L}$ )/minute (min) and about 100  $\mu\text{L}/\text{min}$ . Alternatively, the flow rate of the aqueous fluid 712 entering the junction 706 can be less than about 0.01  $\mu\text{L}/\text{min}$ . Alternatively, the flow rate of the aqueous fluid 712 entering the junction 706 can be greater than about 40  $\mu\text{L}/\text{min}$ , such as 45  $\mu\text{L}/\text{min}$ , 50  $\mu\text{L}/\text{min}$ , 55  $\mu\text{L}/\text{min}$ , 60  $\mu\text{L}/\text{min}$ , 65  $\mu\text{L}/\text{min}$ , 70  $\mu\text{L}/\text{min}$ , 75  $\mu\text{L}/\text{min}$ , 80  $\mu\text{L}/\text{min}$ , 85  $\mu\text{L}/\text{min}$ , 90  $\mu\text{L}/\text{min}$ , 95  $\mu\text{L}/\text{min}$ , 100  $\mu\text{L}/\text{min}$ , 110  $\mu\text{L}/\text{min}$ , 120  $\mu\text{L}/\text{min}$ , 130  $\mu\text{L}/\text{min}$ , 140  $\mu\text{L}/\text{min}$ , 150  $\mu\text{L}/\text{min}$ , or greater. At lower flow rates, such as flow rates of about less than or equal to 10 microliters/minute, the droplet radius may not be dependent on the flow rate of the aqueous fluid 712 entering the junction 706. The second fluid 714 may be stationary, or substantially stationary, in the reservoir 704. Alternatively, the second fluid 714 may be flowing, such as at the above flow rates described for the aqueous fluid 712.

In some instances, at least about 50% of the droplets generated can have uniform size. In some instances, at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or greater of the droplets generated can have uniform size. Alternatively, less than about 50% of the droplets generated can have uniform size.

While FIGS. 7A and 7B illustrate the height difference,  $\Delta h$ , being abrupt at the junction 706 (e.g., a step increase), the height difference may increase gradually (e.g., from about 0  $\mu\text{m}$  to a maximum height difference). Alternatively, the height difference may decrease gradually (e.g., taper) from a maximum height difference. A gradual increase or decrease in height difference, as used herein, may refer to a continuous incremental increase or decrease in height difference, wherein an angle between any one differential segment of a height profile and an immediately adjacent differential segment of the height profile is greater than 90°. For example, at the junction 706, a bottom wall of the

channel and a bottom wall of the reservoir can meet at an angle greater than 90°. Alternatively or in addition, a top wall (e.g., ceiling) of the channel and a top wall (e.g., ceiling) of the reservoir can meet an angle greater than 90°.

5 A gradual increase or decrease may be linear or non-linear (e.g., exponential, sinusoidal, etc.). Alternatively or in addition, the height difference may variably increase and/or decrease linearly or non-linearly. While FIGS. 7A and 7B illustrate the expanding reservoir cross-section height as linear (e.g., constant expansion angle,  $\beta$ ), the cross-section height may expand non-linearly. For example, the reservoir may be defined at least partially by a dome-like (e.g., hemispherical) shape having variable expansion angles. The cross-section height may expand in any shape.

The channel networks, e.g., as described above or elsewhere herein, can be fluidically coupled to appropriate fluidic components. For example, the inlet channel segments are fluidly coupled to appropriate sources of the materials they are to deliver to a channel junction. These sources may include any of a variety of different fluidic components, from simple reservoirs defined in or connected to a body structure of a microfluidic device, to fluid conduits that deliver fluids from off-device sources, manifolds, fluid flow units (e.g., actuators, pumps, compressors) or the like. Likewise, the outlet channel segment (e.g., channel segment 208, reservoir 604, etc.) may be fluidically coupled to a receiving vessel or conduit for the partitioned cells for subsequent processing. Again, this may be a reservoir 20 defined in the body of a microfluidic device, or it may be a fluidic conduit for delivering the partitioned cells to a subsequent process operation, instrument or component.

The methods and systems described herein may be used to greatly increase the efficiency of single-cell applications 35 and/or other applications receiving droplet-based input. For example, following the sorting of occupied cells and/or appropriately-sized cells, subsequent operations that can be performed can include generation of amplification products, purification (e.g., via solid phase reversible immobilization 40 (SPRI)), further processing (e.g., shearing, ligation of functional sequences, and subsequent amplification (e.g., via PCR)). These operations may occur in bulk (e.g., outside the partition). In the case where a partition is a droplet in an emulsion, the emulsion can be broken and the contents of the 45 droplet pooled for additional operations. Additional reagents that may be co-partitioned along with the barcode bearing bead may include oligonucleotides to block ribosomal RNA (rRNA) and nucleases to digest genomic DNA from cells. Alternatively, rRNA removal agents may be applied during 50 additional processing operations. The configuration of the constructs generated by such a method can help minimize (or avoid) sequencing of the poly-T sequence during sequencing and/or sequence the 5' end of a polynucleotide sequence. The amplification products, for example, first 55 amplification products and/or second amplification products, may be subject to sequencing for sequence analysis. In some cases, amplification may be performed using the Partial Hairpin Amplification for Sequencing (PHASE) method.

A variety of applications require the evaluation of the presence and quantification of different biological particle or organism types within a population of biological particles, including, for example, microbiome analysis and characterization, environmental testing, food safety testing, epidemiological analysis, e.g., in tracing contamination or the like.

The present disclosure provides computer systems that are programmed to implement methods of the disclosure. FIG. 13 shows a computer system 1301 that is programmed or otherwise configured to control a microfluidics system (e.g., fluid flow) and perform sequencing applications. The computer system 1301 can regulate various aspects of the present disclosure. The computer system 1301 can be an electronic device of a user or a computer system that is remotely located with respect to the electronic device. The electronic device can be a mobile electronic device.

The computer system 1301 includes a central processing unit (CPU, also “processor” and “computer processor” herein) 1305, which can be a single core or multi core processor, or a plurality of processors for parallel processing. The computer system 1301 also includes memory or memory location 1310 (e.g., random-access memory, read-only memory, flash memory), electronic storage unit 1315 (e.g., hard disk), communication interface 1320 (e.g., network adapter) for communicating with one or more other systems, and peripheral devices 1325, such as cache, other memory, data storage and/or electronic display adapters. The memory 1310, storage unit 1315, interface 1320 and peripheral devices 1325 are in communication with the CPU 1305 through a communication bus (solid lines), such as a motherboard. The storage unit 1315 can be a data storage unit (or data repository) for storing data. The computer system 1301 can be operatively coupled to a computer network (“network”) 1330 with the aid of the communication interface 1320. The network 1330 can be the Internet, an internet and/or extranet, or an intranet and/or extranet that is in communication with the Internet. The network 1330 in some cases is a telecommunication and/or data network. The network 1330 can include one or more computer servers, which can enable distributed computing, such as cloud computing. The network 1330, in some cases with the aid of the computer system 1301, can implement a peer-to-peer network, which may enable devices coupled to the computer system 1301 to behave as a client or a server.

The CPU 1305 can execute a sequence of machine-readable instructions, which can be embodied in a program or software. The instructions may be stored in a memory location, such as the memory 1310. The instructions can be directed to the CPU 1305, which can subsequently program or otherwise configure the CPU 1305 to implement methods of the present disclosure. Examples of operations performed by the CPU 1305 can include fetch, decode, execute, and writeback.

The CPU 1305 can be part of a circuit, such as an integrated circuit. One or more other components of the system 1301 can be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC).

The storage unit 1315 can store files, such as drivers, libraries and saved programs. The storage unit 1315 can store user data, e.g., user preferences and user programs. The computer system 1301 in some cases can include one or more additional data storage units that are external to the computer system 1301, such as located on a remote server that is in communication with the computer system 1301 through an intranet or the Internet.

The computer system 1301 can communicate with one or more remote computer systems through the network 1330. For instance, the computer system 1301 can communicate with a remote computer system of a user (e.g., operator). Examples of remote computer systems include personal computers (e.g., portable PC), slate or tablet PC's (e.g., Apple® iPad, Samsung® Galaxy Tab), telephones, Smart

phones (e.g., Apple® iPhone, Android-enabled device, Blackberry®), or personal digital assistants. The user can access the computer system 1301 via the network 1330.

Methods as described herein can be implemented by way of machine (e.g., computer processor) executable code stored on an electronic storage location of the computer system 1301, such as, for example, on the memory 1310 or electronic storage unit 1315. The machine executable or machine readable code can be provided in the form of software. During use, the code can be executed by the processor 1305. In some cases, the code can be retrieved from the storage unit 1315 and stored on the memory 1310 for ready access by the processor 1305. In some situations, the electronic storage unit 1315 can be precluded, and machine-executable instructions are stored on memory 1310.

The code can be pre-compiled and configured for use with a machine having a processor adapted to execute the code, or can be compiled during runtime. The code can be supplied in a programming language that can be selected to enable the code to execute in a pre-compiled or as-compiled fashion.

Aspects of the systems and methods provided herein, such as the computer system 1301, can be embodied in programming. Various aspects of the technology may be thought of as “products” or “articles of manufacture” typically in the form of machine (or processor) executable code and/or associated data that is carried on or embodied in a type of machine readable medium. Machine-executable code can be stored on an electronic storage unit, such as memory (e.g., read-only memory, random-access memory, flash memory) or a hard disk. “Storage” type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over various air-links. The physical elements that carry such waves, such as wired or wireless links, optical links or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible “storage” media, terms such as computer or machine “readable medium” refer to any medium that participates in providing instructions to a processor for execution.

Hence, a machine readable medium, such as computer-executable code, may take many forms, including but not limited to, a tangible storage medium, a carrier wave medium or physical transmission medium. Non-volatile storage media include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such as may be used to implement the databases, etc. shown in the drawings. Volatile storage media include dynamic memory, such as main memory of such a computer platform. Tangible transmission media include coaxial cables; copper wire and fiber optics, including the wires that comprise a bus within a computer system. Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those

generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD or DVD-ROM, any other optical medium, punch cards paper tape, any other physical storage medium with patterns of holes, a RAM, a ROM, a PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables or links transporting such a carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

The computer system 1301 can include or be in communication with an electronic display 1335 that comprises a user interface (UI) 1340 for providing, for example, results of sequencing analysis. Examples of UIs include, without limitation, a graphical user interface (GUI) and web-based user interface.

Methods and systems of the present disclosure can be implemented by way of one or more algorithms. An algorithm can be implemented by way of software upon execution by the central processing unit 1305. The algorithm can, for example, perform sequencing.

Devices, systems, compositions and methods of the present disclosure may be used for various applications, such as, for example, processing a single analyte (e.g., RNA, DNA, or protein) or multiple analytes (e.g., DNA and RNA, DNA and protein, RNA and protein, or RNA, DNA and protein) from a single cell. For example, a biological particle (e.g., a cell or cell bead) is partitioned in a partition (e.g., droplet), and multiple analytes from the biological particle are processed for subsequent processing. The multiple analytes may be from the single cell. This may enable, for example, simultaneous proteomic, transcriptomic and genomic analysis of the cell.

While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. It is not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the embodiments herein are not meant to be construed in a limiting sense. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is therefore contemplated that the invention shall also cover any such alternatives, modifications, variations or equivalents. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A method for processing a nucleic acid molecule, comprising:
  - (a) providing (I) a plurality of cells, nuclei, or cell beads, wherein a cell, nucleus, or cell bead of said plurality of cells, nuclei, or cell beads comprises said nucleic acid molecule and (II) a transposase molecule comprising a

nucleic acid adapter, wherein said nucleic acid adapter comprises (i) a first single-stranded portion comprising a first nucleic acid sequence and (ii) a second single-stranded portion comprising a second nucleic acid sequence, wherein said first nucleic acid sequence is different from said second nucleic acid sequence, wherein said first single-stranded portion and said second single-stranded portion are in a nucleic acid loop;

- (b) using said transposase molecule and said nucleic acid molecule to generate a nucleic acid fragment in said cell, nucleus, or cell bead, wherein said nucleic acid fragment (i) comprises a first end comprising said first nucleic acid sequence and said second nucleic acid sequence, (ii) comprises a second end comprising said first nucleic acid sequence and said second nucleic acid sequence, and (iii) is at least partially double stranded;
- (c) after (b), partitioning, into a plurality of partitions, said plurality of cells, nuclei, or cell beads, wherein a partition of said plurality of partitions comprises (I) said cell, nucleus, or cell bead comprising said nucleic acid fragment and (II) a nucleic acid barcode molecule; and
- (d) in said partition, cleaving said nucleic acid loop in said nucleic acid fragment to generate a processed nucleic acid fragment and reacting said nucleic acid barcode molecule with said processed nucleic acid fragment to generate a barcoded nucleic acid fragment.

2. The method of claim 1, wherein said nucleic acid adapter comprises a first nucleic acid adapter and a second nucleic acid adapter, wherein said first nucleic acid adapter and said second nucleic acid adapter comprise a double-stranded portion.

3. The method of claim 1, wherein said first single-stranded portion and said second single-stranded portion are cleavably attached by a linker comprising a uracil.

4. The method of claim 1, wherein said nucleic acid loop is cleavable by application of a photo-stimulus, biological stimulus, thermal stimulus, or chemical stimulus.

5. The method of claim 2, wherein said first nucleic acid adapter and said second nucleic acid adapter are connected via a double-stranded nucleic acid sequence.

6. The method of claim 5, wherein said double-stranded nucleic acid sequence comprises a cleavable sequence, and wherein (b) comprises cleaving said double-stranded nucleic acid sequence.

7. The method of claim 6, wherein said cleavable sequence comprises a uracil.

8. The method of claim 1, further comprising filling a gap in said nucleic acid fragment.

9. The method of claim 8, wherein said gap is a 9 base-pair (bp) gap.

10. The method of claim 1, wherein said plurality of partitions comprises a plurality of droplets.

11. The method of claim 1, wherein said plurality of partitions comprises a plurality of wells.

12. The method of claim 1, wherein said cell, nucleus, or cell bead is permeabilized.

13. The method of claim 1, wherein said barcoded nucleic acid fragment comprises a gap, and wherein said method further comprises filling said gap in said barcoded nucleic acid fragment.

14. The method of claim 1, wherein said nucleic acid barcode molecule is attached to a bead.

15. The method of claim 14, wherein said nucleic acid barcode molecule is releasably attached to said bead.

**16.** The method of claim **15**, wherein said bead comprises a plurality of nucleic acid barcode molecules, wherein said plurality of nucleic acid barcode molecules comprise a common barcode sequence.

**17.** The method of claim **1**, wherein said nucleic acid barcode molecule comprises a functional sequence.

**18.** The method of claim **1**, wherein said partition comprises a plurality of nucleic acid barcode molecules comprising said nucleic acid barcode molecule, wherein said nucleic acid barcode molecule comprises a unique molecular identifier that is unique within said plurality of barcode molecules.

**19.** The method of claim **1**, further comprising, sequencing said barcoded nucleic acid fragment or a derivative thereof.

**20.** The method of claim **1**, wherein said nucleic acid adapter does not comprise a barcode sequence.

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