

SYSTEMS AND METHODS FOR PROCESSING RNA FROM CELLS**CROSS-REFERENCE**

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/824,851, filed March 27, 2019, which is entirely incorporated herein by reference for all purposes.

BACKGROUND

[0002] A sample may be processed for various purposes, such as identification 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.

[0003] 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.

[0004] 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.

[0005] 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

[0006] A variety of diseases can be diagnosed using genetic tests, and personalized medicine benefits from analysis of individual genomes and transcriptomes. In analyzing transcriptomes, messenger RNA (mRNA) is commonly studied. However, other species of RNA are important in disease progression, diagnosis, and treatment. Current methods for obtaining full-length transcript information are limited. Moreover, ribosomal RNA (rRNA) is abundant and may exhaust resources when assessing total RNA from samples or introduce bias. Provided herein are methods for streamlined sample processing to obtain full-length transcript information while avoiding rRNA. These methods may provide information on underexplored RNA species, e.g., microRNA, long non-coding RNA, transfer RNA, small nucleolar RNA, circular RNA, RNA isoforms, etc.

[0007] 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 operations 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.

[0008] In an aspect, disclosed herein is a method for assaying ribonucleic acid (RNA) molecules, comprising: (a) providing a cell that comprises a plurality of cellular analytes, wherein the plurality of cellular analytes comprises ribonucleic acid (RNA) molecules; (b) depleting ribosomal RNA (rRNA) molecules from the RNA molecules, in the cell, to yield a plurality of remaining RNA molecules, wherein the plurality of remaining RNA molecules comprises non-poly-adenylated RNA molecules; (c) fragmenting, in the cell, the plurality of remaining RNA molecules to yield a plurality of RNA fragments, wherein the plurality of RNA fragments comprises a non-poly-adenylated RNA fragment from the non-poly-adenylated RNA molecules; (d) partitioning the cell, after (c), with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of the plurality of nucleic acid barcode molecules comprise a common barcode sequence; and (e) using the non-poly-adenylated RNA fragment and a nucleic acid barcode molecule of the plurality of nucleic acid barcode molecules to generate a barcoded nucleic acid molecule.

[0009] In some embodiments, (e) comprises (i) associating the non-poly-adenylated RNA fragment with an RNA molecule hybridized to the nucleic acid barcode molecule, and (ii) performing a nucleic acid reaction, to generate the barcoded nucleic acid molecule. In some embodiments, the nucleic acid reaction is a nucleic acid extension reaction. In some embodiments, the barcoded nucleic acid molecule is a barcoded complementary DNA (cDNA) molecule. In some embodiments, the nucleic acid reaction is performed using an enzyme. In some embodiments, the enzyme is a thermostable group II intron reverse transcriptase (TGIRT). In some embodiments, the method further comprises, subsequent to the nucleic acid reaction, adding an adaptor molecule to the barcoded nucleic acid molecule. In some embodiments, the adaptor molecule comprises a sequence comprising a 5'-App and 3'-blocker. In some embodiments, the adaptor molecule is ligated to the barcoded nucleic acid molecule using a 5'-App DNA/RNA ligase. In some embodiments, the adaptor molecule comprises a splint oligonucleotide. In some embodiments, the splint oligonucleotide comprises a random N-mer. In some embodiments, the splint oligonucleotide comprises a 3' blocker. In some embodiments, the adaptor molecule is ligated to the barcoded nucleic acid molecule using a T4 DNA ligase. In

some embodiments, (e) comprises (i) associating the non-poly-adenylated RNA fragment with the nucleic acid barcode molecule, and (ii) performing a nucleic acid reaction to generate the barcoded nucleic acid molecule. In some embodiments, the nucleic acid barcode molecules are barcode DNA molecules. In some embodiments, the nucleic acid reaction is a ligation reaction. In some embodiments, the ligation reaction is performed using an enzyme. In some embodiments, the enzyme is a T4KQ ligase. In some embodiments, the method further comprises subsequent to the nucleic acid reaction, adding an adaptor sequence to the barcoded nucleic acid molecule using a reverse transcription reaction. In some embodiments, the adaptor sequence comprises a sequencing primer sequence. In some embodiments, the sequencing primer sequence is used for template switching. In some embodiments, the adaptor sequence comprises a sequencing primer sequence, a reverse transcription primer sequence, and a uridine residue. In some embodiments, the method further comprises, subsequent to the reverse transcription reaction, subjecting the barcoded nucleic acid molecule to circularization, clean up, and linearization. In some embodiments, the circularization is performed using a T4 DNA ligase, a splint oligonucleotide, or a CircLigase. In some embodiments, the method further comprises, prior to the reverse transcription, subjecting the barcoded nucleic acid molecule to (i) phosphorylation using a first enzyme, and (ii) ligation of the sequencing primer sequence using a second enzyme. In some embodiments, the first enzyme is a T4 polynucleotide kinase and the second enzyme is a T4 RNA ligase. In some embodiments, the method further comprises subjecting the barcoded nucleic acid molecule to conditions sufficient for amplification and library preparation. In some embodiments, the method further comprises sequencing the barcoded nucleic acid molecule or a derivative thereof.

[0010] In some embodiments, the cell is a fixed and permeabilized cell. In some embodiments, the cell is fixed and permeabilized prior to step (a). In some embodiments, the depleting in (b) comprises subjecting the cell to conditions sufficient to deplete the rRNA molecules. In some embodiments, the conditions sufficient to deplete the rRNA comprise selective precipitation with lithium chloride, treatment with a RNase, or a pull-down assay. In some embodiments, the method further comprises, prior to (a), subjecting the cell to conditions sufficient for fixation. In some embodiments, the fragmenting comprises a chemical fragmentation process. In some embodiments, the chemical fragmentation process comprises using divalent metal cations and heating. In some embodiments, the method further comprises, subsequent to the chemical fragmentation process, cleaving a 3' phosphate group from the non-poly-adenylated RNA fragment. In some embodiments, the chemical fragmentation process comprises using a reactive oxygen species. In some embodiments, the reactive oxygen species comprises hydrogen peroxide. In some embodiments, the fragmenting comprises an enzymatic

fragmentation process. In some embodiments, the enzymatic fragmentation process comprises RNase III. In some embodiments, the plurality of nucleic acid barcode molecules comprises a barcode sequence, a unique molecular identifier sequence, and a sequencing primer sequence. In some embodiments, the non-poly-adenylated RNA molecules comprises miRNA, long non-coding RNA, short non-coding RNA, tRNA, RNA isoforms, snoRNA, small RNA, piRNA, or circular RNA. In some embodiments, the plurality of RNA fragments further comprises a poly-adenylated RNA fragment. In some embodiments, the method further comprises performing (d)-(e) with the poly-adenylated RNA fragment. In some embodiments, the cell is provided as part of a cell bead. In some embodiments, the partition is a droplet. In some embodiments, the partition is a well. In some embodiments, the plurality of nucleic acid barcode molecules is releasably attached to a bead. In some embodiments, the bead is a gel bead. In some embodiments, the method further comprises releasing the plurality of nucleic acid barcode molecules from the bead using a stimulus. In some embodiments, the stimulus is a biological, chemical, photo, or thermal stimulus. In some embodiments, the stimulus is a photo-stimulus which comprises photo-activated cleavage to release the plurality of nucleic acid barcode molecules from the bead. In some embodiments, the stimulus is a chemical stimulus which comprises a reducing reagent. In some embodiments, the plurality of nucleic acid barcode molecules is releasably attached to the bead via disulfide bonds, and the reducing agent reduces the disulfide bonds to release the plurality of nucleic acid barcodes from the bead. In some embodiments, the fragmenting occurs in the cell bead. In some embodiments, the cell bead comprises the cell. In some embodiments, the cell bead comprises constituents of the cell. In some embodiments, the cell bead is configured to release the plurality of RNA fragments. In some embodiments, the conditions sufficient to deplete the rRNA comprise using an enzyme configured for selective digestion. In some embodiments, the enzyme is an exonuclease. In some embodiments, the method further comprises subjecting the cell to conditions sufficient for permeabilization.

[0011] In some embodiments, the method further comprises subjecting the non-poly-adenylated RNA fragment to conditions sufficient for polyadenylation to generate a polyadenylated RNA fragment from the non-poly-adenylated RNA fragment, wherein (e) comprises using the polyadenylated RNA fragment and the nucleic acid barcode molecule to generate the barcoded nucleic acid molecule. In some embodiments, the plurality of nucleic acid barcode molecules comprises a capture sequence. In some embodiments, the capture sequence comprises a poly-T sequence configured to couple to the polyadenylated RNA fragment. In some embodiments, the method further comprises performing a reverse transcription reaction to generate the barcoded nucleic acid molecule. In some embodiments, the reverse transcription

reaction is performed using an adaptor molecule. In some embodiments, the adaptor molecule comprises a template switching sequence.

[0012] In another aspect, disclosed herein is a method for assaying RNA molecules, comprising: (a) providing a cell that comprises a plurality of cellular analytes, wherein said plurality of cellular analytes comprises ribonucleic acid (RNA) molecules, wherein the RNA molecules comprise ribosomal RNA (rRNA) and non-poly-adenylated RNA molecules; (b) fragmenting said RNA molecules to yield a plurality of RNA fragments; (c) partitioning said cell after (b) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of said plurality of nucleic acid barcode molecules comprise a common barcode sequence, wherein a nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules is a double-stranded molecule comprising an RNA strand and a deoxyribonucleic acid (DNA) strand; and wherein said DNA strand comprises said common barcode sequence and an overhang sequence; and (d) allowing an RNA fragment of said plurality of RNA fragments to hybridize to said overhang sequence and extending said RNA fragment, thereby generating a barcoded nucleic acid molecule.

[0013] In another aspect, disclosed herein is a method for assaying RNA molecules, comprising: (a) providing a cell that comprises a plurality of cellular analytes, wherein said plurality of cellular analytes comprises ribonucleic acid (RNA) molecules; (b) fragmenting said RNA molecules to yield a plurality of RNA fragments; (c) partitioning said cell after (b) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of said plurality of nucleic acid barcode molecules comprise a common barcode sequence and an adaptor sequence; and (d) covalently attaching said RNA fragment to a nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules, thereby generating a barcoded nucleic acid molecule.

[0014] In some embodiments, (d) comprises (i) associating the RNA fragment of the non-poly-adenylated RNA molecule with an RNA molecule hybridized to the nucleic acid barcode molecule, and (ii) performing a nucleic acid reaction, to generate the barcoded nucleic acid molecule. In some embodiments, the nucleic acid reaction is a nucleic acid extension reaction. In some embodiments, (d) comprises covalently attaching the RNA fragment to the nucleic acid barcode molecule to generate the barcoded nucleic acid molecule. In some embodiments, the barcoded nucleic acid molecule is a barcoded complementary DNA (cDNA) molecule. In some embodiments, the nucleic acid reaction is performed using an enzyme. In some embodiments, the enzyme is a thermostable group II intron reverse transcriptase (TGIRT). In some embodiments, the method further comprises, subsequent to the nucleic acid reaction, adding an adaptor molecule to the barcoded nucleic acid molecule. In some embodiments, the adaptor

molecule comprises a sequence comprising a 5'-App and 3'-blocker. In some embodiments, the adaptor molecule is ligated to the barcoded nucleic acid molecule using a 5'-App DNA/RNA ligase. In some embodiments, the adaptor molecule comprises a splint oligonucleotide. In some embodiments, the splint oligonucleotide comprises a random N-mer. In some embodiments, the splint oligonucleotide comprises a 3' blocker. In some embodiments, the adaptor molecule is ligated to the barcoded nucleic acid molecule using a T4 DNA ligase.

[0015] In some embodiments, (d) comprises (i) associating the RNA fragment of the non-poly-adenylated RNA molecule with the barcode molecule, and (ii) performing a nucleic acid reaction, to generate the barcoded nucleic acid molecule. In some embodiments, the barcode molecules are barcode DNA molecules. In some embodiments, the nucleic acid reaction is a ligation reaction. In some embodiments, the ligation reaction is performed using an enzyme. In some embodiments, the enzyme is a T4KQ ligase. In some embodiments, the method further comprises, subsequent to the nucleic acid reaction, adding an adaptor sequence to the barcoded nucleic acid molecule using reverse transcription. In some embodiments, adaptor sequence comprises a sequencing primer sequence. In some embodiments, the sequencing primer sequence is used for template switching. In some embodiments, the adaptor sequence comprises a sequencing primer sequence, a reverse transcription primer sequence, and a uridine residue. In some embodiments, the method further comprises, subsequent to the reverse transcription reaction, subjecting the barcoded nucleic acid molecule to circularization, clean up, and linearization. In some embodiments, the circularization is performed using a T4 DNA ligase, a splint oligonucleotide, or a CircLigase. In some embodiments, the method further comprises, prior to the reverse transcription, subjecting the barcoded nucleic acid molecule to (i) phosphorylation using a first enzyme, and (ii) ligation of the sequencing primer sequence using a second enzyme. In some embodiments, the first enzyme is a T4 polynucleotide kinase and the second enzyme is a T4 RNA ligase.

[0016] In some embodiments, the method further comprises subjecting the barcoded nucleic acid molecule to conditions sufficient for amplification and library preparation. In some embodiments, the method further comprises sequencing the barcoded nucleic acid molecule or a derivative thereof.

[0017] In some embodiments, the method further comprises subjecting the plurality of analytes to conditions sufficient to deplete a nucleic acid species. In some embodiments, the nucleic acid species is ribosomal RNA (rRNA). In some embodiments, the conditions sufficient to deplete the rRNA comprise selective precipitation with lithium chloride, treatment with a RNase, or a pull-down assay.

[0018] In some embodiments, the plurality of cellular analytes is provided as part of a cell. In some embodiments, the method further comprises, prior to (a), subjecting the cell to conditions sufficient for fixation.

[0019] In some embodiments, the fragmenting comprises a chemical fragmentation process. In some embodiments, the chemical fragmentation process comprises using divalent metal cations (e.g., magnesium or zinc) and heat. For instance, the chemical fragmentation process may involve the use of magnesium chloride and heat. In some embodiments, the method further comprises, subsequent to the chemical fragmentation process, cleaving a 3' phosphate group from the RNA fragment. In some embodiments, the chemical fragmentation process comprises using a reactive oxygen species. In some embodiments, the reactive oxygen species comprises hydrogen peroxide.

[0020] In some embodiments, the fragmenting comprises an enzymatic fragmentation process. In some embodiments, the enzymatic fragmentation process comprises RNase III.

[0021] In some embodiments, the plurality of barcode molecules comprises a barcode sequence, a unique molecular identifier sequence, and a sequencing primer sequence.

[0022] In some embodiments, the non-poly-adenylated RNA molecule comprises miRNA, long non-coding RNA, short non-coding RNA, tRNA, RNA isoforms, snoRNA, small RNA, piRNA, or circular RNA.

[0023] In some embodiments, the plurality of RNA fragments comprises an RNA fragment that is poly-adenylated. In some embodiments, the method further comprises performing (c)-(d) with the RNA fragment that is poly-adenylated.

[0024] In some embodiments, the plurality of cellular analytes is provided as part of a cell bead.

[0025] In some embodiments, the partition is a droplet. In some embodiments, the partition is a well.

[0026] In some embodiments, the plurality of nucleic acid barcode molecules is releasably attached to a bead. In some embodiments, the bead is a gel bead. In some embodiments, the method further comprises releasing the plurality of nucleic acid barcode molecules from the bead using a stimulus. In some embodiments, the stimulus is a biological, chemical, photo, or thermal stimulus.

[0027] In some embodiments, the plurality of cellular analytes is provided as part of a nucleus.

[0028] In some embodiments, the fragmenting occurs in the cell. In some embodiments, the fragmenting occurs in the cell bead. In some embodiments, the fragmenting occurs in the nucleus. In some embodiments, the cell bead comprises the cell. In some embodiments, the cell

is absent from the cell bead. In some embodiments, the cell bead is configured to release the plurality of RNA fragments. In some embodiments, the conditions sufficient to deplete the rRNA comprise using an enzyme configured for selective digestion. In some embodiments, the enzyme is an exonuclease. In some embodiments, the method further comprises, subjecting the cell to conditions sufficient for permeabilization.

[0029] In some embodiments, the method further comprises subjecting the RNA fragment to conditions sufficient for polyadenylation to generate a polyadenylated RNA fragment, wherein (d) comprises using the polyadenylated RNA fragment and the nucleic acid barcode molecule to generate the barcoded nucleic acid molecule. In some embodiments, the plurality of nucleic acid barcode molecules comprises a capture sequence. In some embodiments, the capture sequence comprises a poly-T sequence configured to couple to the polyadenylated RNA fragment. In some embodiments, the method further comprises performing a reverse transcription reaction to generate the barcoded nucleic acid molecule. In some embodiments, the reverse transcription reaction is performed using an adaptor molecule. In some embodiments, the adaptor molecule comprises a template switching sequence.

[0030] In another aspect, provided herein is a method for assaying ribonucleic acid (RNA) molecules, comprising: (a) providing a nucleus or cell bead that comprises a plurality of cellular analytes, wherein the plurality of cellular analytes comprises ribonucleic acid (RNA) molecules; (b) depleting rRNA from the nucleus or the cell bead to yield a plurality of remaining RNA molecules, wherein the plurality of remaining RNA molecules comprises non-poly-adenylated RNA molecules; (c) fragmenting the plurality of remaining RNA molecules to yield a plurality of RNA fragments, wherein the plurality of RNA fragments comprises a non-poly-adenylated RNA fragment from the non-poly-adenylated RNA molecules; (d) partitioning the nucleus or cell bead after (c) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of the plurality of nucleic acid barcode molecules comprise a common barcode sequence; and (e) using the non-poly-adenylated RNA fragment and a nucleic acid barcode molecule of the plurality of nucleic acid barcode molecules to generate a barcoded nucleic acid molecule.

[0031] In some embodiments, the fragmenting occurs in the nucleus or cell bead.

[0032] In another aspect, provided herein is a method for assaying ribonucleic acid molecules, comprising: (a) providing a cell that comprises a plurality of cellular analytes, wherein the plurality of cellular analytes comprises ribonucleic acid (RNA) molecules; (b) fragmenting the RNA molecules to yield a plurality of RNA fragments, wherein the RNA molecules comprise a non-poly-adenylated RNA molecule, and wherein the plurality of RNA fragments comprises a RNA fragment of the non-poly-adenylated RNA molecule; (c)

partitioning the plurality of cellular analytes after (b) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of the plurality of nucleic acid barcode molecules comprise a common barcode sequence; and (d) using the RNA fragment of the non-poly-adenylated RNA molecule and a nucleic acid barcode molecule of the plurality of nucleic acid barcode molecules to generate a barcoded nucleic acid molecule.

[0033] In another aspect, provided herein is a method for assaying ribonucleic acid molecules, comprising: (a) providing a plurality of cellular analytes from a cell, wherein the plurality of cellular analytes comprises ribonucleic acid (RNA) molecules; (b) fragmenting the RNA molecules to yield a plurality of RNA fragments; (c) partitioning the plurality of cellular analytes after (b) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of the plurality of nucleic acid barcode molecules comprise a common barcode sequence, wherein a nucleic acid barcode molecule of the plurality of nucleic acid barcode molecules is a double-stranded molecule comprising an RNA strand and a deoxyribonucleic acid (DNA) strand; and wherein the DNA strand comprises the common barcode sequence and an overhang sequence; and (d) allowing an RNA fragment of the plurality of RNA fragments to hybridize to the overhang sequence and extending the RNA fragment, thereby generating a barcoded nucleic acid molecule.

[0034] In yet another aspect, provided herein is a method for assaying ribonucleic acid molecules, comprising: (a) providing a plurality of cellular analytes from a cell, wherein the plurality of cellular analytes comprises ribonucleic acid (RNA) molecules; (b) fragmenting the RNA molecules to yield a plurality of RNA fragments; (c) partitioning the plurality of cellular analytes after step (b) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of the plurality of nucleic acid barcode molecules comprise a common barcode sequence and an adaptor sequence; and (d) covalently attaching the RNA fragment to a nucleic acid barcode molecule of the plurality of nucleic acid barcode molecules, thereby generating a barcoded nucleic acid molecule.

[0035] In some embodiments, the plurality of cellular analytes is from a single cell.

[0036] 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.

[0037] 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.

[0038] 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

[0039] 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

[0040] 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:

[0041] **FIG. 1** shows an example of a microfluidic channel structure for partitioning individual biological particles.

[0042] **FIG. 2** shows an example of a microfluidic channel structure for delivering barcode carrying beads to droplets.

[0043] **FIG. 3** shows an example of a microfluidic channel structure for co-partitioning biological particles and reagents.

[0044] **FIG. 4** shows an example of a microfluidic channel structure for the controlled partitioning of beads into discrete droplets.

[0045] **FIG. 5** shows an example of a microfluidic channel structure for increased droplet generation throughput.

[0046] **FIG. 6** shows another example of a microfluidic channel structure for increased droplet generation throughput.

[0047] 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.

[0048] FIG. 8 illustrates an example of a barcode-carrying bead.

[0049] FIG. 9 shows an example workflow of a method for barcoding nucleic acids in cells.

[0050] FIG. 10 shows an example of a nucleic acid reaction comprising a thermostable reverse transcriptase.

[0051] FIG. 11 shows an example of a method for barcoding nucleic acid molecules.

[0052] FIG. 12 shows another example of a method for barcoding nucleic acid molecules.

[0053] FIG. 13 shows another example of a method for barcoding nucleic acid molecules.

[0054] FIG. 14 shows another example of a method for barcoding nucleic acid molecules.

[0055] FIG. 15 shows another example of a method for barcoding nucleic acid molecules.

[0056] FIG. 16 shows a computer system that is programmed or otherwise configured to implement methods provided herein.

DETAILED DESCRIPTION

[0057] 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.

[0058] 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.

[0059] The terms “a,” “an,” and “the,” as used herein, generally refers to singular and plural references unless the context clearly dictates otherwise.

[0060] 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.

[0061] 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.

[0062] 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 or partial sequencing primer sequences, etc.

[0063] 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.

[0064] 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).

[0065] 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.

[0066] The terms “adaptor(s)”, “adapter(s)”, “adaptor molecule(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.

[0067] 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.

[0068] 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®), MGI, Complete Genomics, 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. Sequencing can comprise short-read sequencing or long-read sequencing, or both. In some situations, systems and methods provided herein may be used with proteomic information.

[0069] 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.

[0070] As used herein, the term “barcoded nucleic acid molecule” generally refers to a nucleic acid molecule that results from, for example, the processing of a nucleic acid barcode molecule with a nucleic acid sequence (e.g., nucleic acid sequence complementary to a nucleic acid primer sequence encompassed by the nucleic acid barcode molecule). The nucleic acid sequence may be a targeted sequence (e.g., targeted by a primer sequence) or a non-targeted sequence. For example, in the methods and systems described herein, hybridization and reverse transcription of the nucleic acid molecule (e.g., a messenger RNA (mRNA) molecule) of a cell with a nucleic acid barcode molecule (e.g., a nucleic acid barcode molecule containing a barcode sequence and a nucleic acid primer sequence complementary to a nucleic acid sequence of the mRNA molecule) results in a barcoded nucleic acid molecule that has a sequence corresponding to the nucleic acid sequence of the mRNA and the barcode sequence (or a reverse complement thereof). A barcoded nucleic acid molecule may serve as a template, such as a template polynucleotide, that can be further processed (e.g., amplified) and sequenced to obtain the target nucleic acid sequence. For example, in the methods and systems described herein, a barcoded nucleic acid molecule may be further processed (e.g., amplified) and sequenced to obtain the nucleic acid sequence of the mRNA.

[0071] 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.

[0072] 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 combination 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.

[0073] 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.

[0074] 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 primer. The molecular tag may be, or comprise, a protein. The molecular tag may comprise a polypeptide. The molecular tag may be a barcode.

[0075] 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 (e.g., a microwell). 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.

Methods for full length, total RNA sequencing

[0076] Provided herein are methods for sample processing and/or analysis, such as analysis of nucleic acid molecules from cells. A method of the present disclosure may allow for depletion of unwanted nucleic acid species (e.g., ribosomal ribonucleic acid (rRNA)) and barcoding a nucleic acid molecule (e.g., a non-rRNA molecule). A method of the present disclosure may be useful in preparation of libraries (e.g., for sequencing) with a workflow that may obviate certain processes involved in RNA processing (e.g., cDNA amplification, fragmentation, end-repair, A-tailing, etc.). One or more of the operations in these methods may occur within a partition.

[0077] The method may comprise barcoding of a nucleic acid molecule to generate a barcoded nucleic acid molecule. The barcoded nucleic acid molecule may comprise a target nucleic acid molecule. Such a method may involve attaching a nucleic acid barcode molecule to

the target nucleic acid molecules. In some cases, barcoding may occur through ligation of the nucleic acid barcode molecule to the target nucleic acid molecule. For example, a barcode DNA molecule may be ligated to a target RNA molecule. In other cases, the target nucleic acid molecule may hybridize to the nucleic acid barcode molecule, and a nucleic acid reaction may extend the target nucleic acid molecules. For example, the nucleic acid barcode molecule may be double-stranded and may attach to an overhanging sequence of the target nucleic acid molecule; an extension reaction may then generate a complementary barcoded nucleic acid molecule comprising both a sequence complementary to the barcode sequence and a sequence complementary to a target region of the nucleic acid molecule. 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.

[0078] One or more of these operations may be mediated by enzymes, such as ligases, polymerases, reverse transcriptases, exonucleases, and engineered variants thereof. One or more of the operations in these methods may occur outside a partition (e.g., before partitioning or after partitioning). In certain instances, the depletion of an unwanted nucleic acid species (e.g., rRNA) is an optional step according to the methods of the present invention.

[0079] In an aspect, the present disclosure provides a method for assaying ribonucleic acid molecules, comprising (a) providing a plurality of cellular analytes from a cell or cell bead, wherein the plurality of cellular analytes comprises RNA molecules (b) fragmenting RNA molecules in the cell or cell bead to yield a plurality of RNA fragments contained within the cell or the cell bead, wherein the RNA molecules comprise a non-poly-adenylated RNA molecule, and wherein the plurality of RNA fragments comprises a RNA fragment derived from the non-polyadenylated RNA molecule, (c) partitioning the cell or cell bead with a plurality of nucleic acid barcode molecules in a partition, wherein a subset of the plurality of nucleic acid barcode molecules comprise a common barcode sequence, and (d) using the RNA fragment derived from the non-poly-adenylated RNA molecule and a subset of the plurality of nucleic acid barcode molecules to generate a barcoded nucleic acid molecule.

[0080] In some instances, the method comprises subjecting a cell or a cell bead under conditions sufficient to deplete ribosomal ribonucleic acid (rRNA). In some instances, the cell is pre-processed, which may include fixation, permeabilization, or both. In some instances, the method does not comprise a fragmenting process, and entire RNA molecules may be processed (e.g., barcoded, sequenced). In some instances, entire RNA molecules or fragmented RNA molecules may be subjected to reverse transcription, which may be barcoded. In some instances, the fragmented RNA molecules may be treated to generate a poly-A tail (e.g., polyadenylation

using an enzyme, e.g., poly-ADP ribose polymerase), which may be used for downstream operations, such as barcoding.

[0081] In some instances, generating the barcoded nucleic acid molecule comprises associating the RNA fragment with a synthetic RNA molecule hybridized to the nucleic acid barcode molecule and performing a nucleic acid reaction, to generate the barcoded nucleic acid molecule. In some instances, the nucleic acid reaction is a nucleic acid extension reaction. In some instances, the barcoded nucleic acid molecule is a barcoded complementary DNA (cDNA) molecule. In some instances, the nucleic acid reaction is performed using an enzyme. In some cases, the enzyme is a thermostable group II intron reverse transcriptase (TGIRT). In some instances, subsequent to the nucleic acid reaction, the method further comprises adding an adaptor sequence to the barcoded nucleic acid molecule. In some cases, the adaptor sequence comprises a 5'-App and 3'-blocker. In some cases, the adaptor sequence is ligated to the barcoded nucleic acid molecule using a 5'-App DNA/RNA ligase.

[0082] In some instances, generating the barcoded nucleic acid molecule comprises associating the RNA fragment with the barcode molecule and performing a nucleic acid reaction, to generate the barcoded nucleic acid molecule. In some cases, the barcode molecules are barcode DNA molecules. In some instances, the nucleic acid reaction is a ligation reaction. In some cases, the ligation reaction is performed using an enzyme. In some cases, the enzyme is a T4KQ ligase. In some instances, subsequent to the nucleic acid reaction, the method comprises adding an adaptor sequence to the barcoded nucleic acid molecule using reverse transcription. In some cases, the adaptor sequence comprises a sequencing primer sequence or a partial sequencing primer sequence. In some cases, the sequencing primer sequence or partial sequencing primer sequence is used for template switching. In some cases, the adaptor sequence comprises a sequencing primer sequence or partial sequencing primer sequence, a reverse transcription primer sequence, and a uridine residue. In some cases, subsequent to the reverse transcription reaction, the barcoded nucleic acid molecule is subjected to circularization, cleanup, and linearization. In some cases, prior to the reverse transcription, the barcoded nucleic acid molecule is subjected to phosphorylation using a first enzyme and ligation of the sequencing primer sequence or partial sequencing primer sequence using a second enzyme. In some cases, the first enzyme is a T4 polynucleotide kinase and the second enzyme is a T4 RNA ligase.

[0083] In some instances, the method further comprises subjecting the barcoded nucleic acid molecule to conditions sufficient for amplification and library preparation. In some cases, the method further comprises sequencing the barcoded nucleic acid molecule or a derivative thereof.

[0084] In some instances, the conditions sufficient to deplete the rRNA comprise using an enzyme configured for selective digestion. In some cases, the enzyme is an exonuclease. In some

cases, the enzyme is a RNase. In some cases, the RNase is RNase H. In such cases, the use of RNase may be pre-pended by a process to anneal rRNA molecules to DNA molecules. For instance, DNA molecules comprising sequences complementary to one or more sequences of the rRNA molecules may be added to a sample (e.g., cell, cell bead, or population of cells or cell beads). The DNA molecules can hybridize with the rRNA. Treatment of the sample with RNase (e.g., RNase H) may then be used to selectively digest the RNA-DNA hybrids or the RNA in the RNA-DNA hybrids but not the unhybridized RNA molecules, thereby depleting rRNA molecules.

[0085] In some instances, the conditions sufficient to deplete the rRNA comprise selective precipitation with lithium chloride. In some instances, the conditions sufficient to deplete the rRNA comprise a pull-down assay. In some examples, the pull-down assay may comprise non-covalent pull-down assays. For example, the pull-down assay can comprise the use of a first binding moiety comprising or coupled to a nucleic acid molecule that can hybridize to rRNA. For instance, the nucleic acid molecule (e.g., DNA, RNA) may comprise sequences complementary to one or more sequences of the rRNA molecules, and the nucleic acid molecules may be added to a sample (e.g., cell, cell bead, or population of cell or cell beads). The binding moiety comprising or coupled to the nucleic acid molecule may comprise, for example, a biotin moiety, a streptavidin moiety, antibody, or a sugar or polysaccharide (e.g., maltose). The nucleic acid molecules may hybridize to one or more rRNA molecules and the hybridized molecules may then be removed using another moiety, e.g., a streptavidin moiety, a biotin moiety, an antibody, a protein, a molecule, a sugar-binding protein (e.g., maltose-binding protein), etc., which may selectively bind to the binding moiety. In some cases, the pull-down assay may be performed using a bead, e.g., magnetic bead. In some instances, the bead may comprise the moiety that is used to capture or bind to the binding moiety that comprises or is coupled to the nucleic acid molecule, which nucleic acid molecule is configured to hybridize to one or more rRNA molecules. In an example, the binding moiety comprises a biotin moiety, which is captured by a streptavidin moiety coupled to a magnetic bead. The assembly is isolated using a magnet or otherwise applying a magnetic field.

[0086] In some instances, prior to subjecting the cell or the cell bead under conditions sufficient to deplete rRNA, the method further comprises subjecting the cell or the cell bead to conditions sufficient for fixation. In some cases, the method further comprises subjecting the cell or the cell bead to conditions sufficient for permeabilization.

[0087] In some instances, the fragmenting comprises a chemical fragmentation process. In some cases, the chemical fragmentation process comprises using divalent cations, e.g., magnesium chloride and heat. In some instances, the fragmentation comprises the use of a

reactive oxidative species. In some cases, subsequent to the chemical fragmentation process, the fragmenting further comprises cleaving a 3' phosphate group from the RNA fragment.

[0088] In some instances, the fragmenting comprises an enzymatic fragmentation process.

In some cases, the enzymatic fragmentation process comprises RNase (e.g., RNase III).

[0089] In some instances, the plurality of barcode molecules comprises a barcode sequence, a unique molecular identifier sequence, and a sequencing primer sequence.

[0090] In some instances, the non-poly-adenylated RNA molecule comprises miRNA, long non-coding RNA, short non-coding RNA, tRNA, RNA isoforms, snoRNA, small RNA, piRNA, or circular RNA.

[0091] In some instances, the plurality of RNA fragments comprises an RNA fragment that is poly-adenylated. In some cases, the method further comprises repeating (iii) and (iv) with the RNA fragment that is poly-adenylated.

[0092] In some instances, the cell bead is configured to release the plurality of RNA fragments.

[0093] In some instances, the partition is a droplet. In some instances, the partition is a well.

[0094] In some instances, the plurality of nucleic acid barcode molecules is releasably attached to a bead. In some cases, the bead is a gel bead. In some cases, the method further comprises releasing the plurality of nucleic acid barcode molecules from the bead using a stimulus. In some cases, the stimulus is a biological, chemical, photo, or thermal stimulus.

[0095] In some instances, a cell selected for generation of a cell bead is a eukaryotic cell (e.g., fungal, yeast, insect, and mammalian cells). Non-limiting examples of mammalian cells include HEK293 cells, HeLa cells, CHO cells, COS cells, Jurkat cells, U20 cells, SKBR3 cells, NS0 hybridoma cells, baby hamster kidney (BHK) cells, MDCK cells, NIH-3T3 fibroblast cells, and any other immortalized cell line derived from a mammalian cell or mammalian cells derived from primary cell culture. In some instances, a cell selected for generation of a cell bead is a non-eukaryotic cell (e.g., archaeal cells, bacterial cells). A cell may be alive or dead when the cell bead is generated.

[0096] A cell bead can be any cell bead, such as a bead that contains biological particles (e.g., a cell) or macromolecular constituents (e.g., RNA, DNA, proteins, etc.) of biological particles. A cell bead may include a single cell or multiple cells, or a derivative of the single cell or multiple cells. For example after lysing and washing the cells, inhibitory components from cell lysates can be washed away and the macromolecular constituents can be bound as cell beads. Systems and methods disclosed herein can be applicable to both cell beads (and/or droplets or other partitions) containing biological particles and cell beads (and/or droplets or other partitions) containing macromolecular constituents of biological particles.

[0097] In some cases, cells or cell beads may be treated for subsequent analysis. In some cases, the cells may be fixed with, e.g., paraformaldehyde, or reducing agent-labile groups such as dithiobis(succinimidyl propionate) (DSP). The cells may then be washed to remove the fixative. The cells may then be permeabilized using a low concentration of detergents, e.g., Triton-X 100. The cells may then be washed again and subjected to conditions sufficient for depletion of a subset or type of nucleic acid molecules, ribosomal RNA (rRNA).

[0098] Conditions sufficient for depletion of a subset or type of nucleic acid molecules, such as rRNA, may comprise using an enzyme capable of selectively digesting one or more molecules of the cell. For example, the enzyme may be an exonuclease, e.g., a 5'-to-3' exonuclease (e.g., a 5'-phosphate dependent exonuclease). The activity of such an enzyme may be modulated by an agent such as an inorganic ion such as a magnesium ion. The enzyme may digest molecules having particular features. For example, the enzyme may digest RNA molecules having a 5'-monophosphate moiety. Such molecules include prokaryotic 16S and 23S rRNA and eukaryotic 18S and 28S rRNA. Similarly, the enzyme may not digest molecules having other particular features. For example, the enzyme may not digest RNA molecules having a 5' cap structure (e.g., eukaryotic RNA with a 5' cap structure), a 5'-triphosphate moiety (e.g., prokaryotic mRNA with a 5-triphosphate moiety), or a 5'-hydroxyl moiety (e.g., degraded RNA with a 5'-hydroxyl moiety). The enzyme may not be capable of digesting, for example, 5S rRNA molecules, which have a 5'-triphosphate moiety, and tRNA, which has an inaccessible 5'-monophosphate moiety. Such species may be removable from a sample using, for example, selective precipitation with lithium chloride. The enzyme may not be inhibited by proteinaceous RNase inhibitors. Such an enzyme may allow for selective digestion of RNA molecules without the use of columns, beads, or immobilized oligo(dT) matrices. For example, the enzyme may be capable of selectively digesting RNA molecules that are not mRNA molecules, thereby enriching mRNA molecules within a container or partition. An example of such an enzyme is the Terminator Exonuclease (Epicentre Biotechnologies). Subjecting RNA molecules to conditions suitable for enzymatic digestion may comprise incubating the sample and/or a partition at a particular temperature for a period of time.

[0099] An incubation temperature for any of the reactions disclosed herein may be, for example, about 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 100°C or higher. An incubation temperature may be at least 25°C, at least 30°C, at least 35°C, at least 40°C, at least 45°C, at least 50°C, at least 55°C, at least 60°C, at least 70°C, at least 75°C, at least 80°C, at least 85°C, at least 90°C, at least 95°C, at least 100°C or higher. An incubation temperature may be at most 100°C, at most 95°C, at most 95°C, at most 85°C, at most 80°C, at most 75°C, at most 70°C, at most 60°C, at most 55°C, at most 50°C, at

most 45°C, at most 40°C, at most 35°C, at most 30°C, at most 25°C. An incubation temperature may fall in a range of useful temperatures, e.g., between 25°C and 30 °C, between 60°C and 75°C, etc. Incubation durations can be for about 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, or more minutes. Incubation durations can be for at least 5 minutes, at least 10 minutes, at least 15 minutes, at least 20 minutes, at least 25 minutes, at least 30 minutes, at least 35 minutes, at least 40 minutes, at least 45 minutes, at least 50 minutes, at least 55 minutes, at least 60 minutes, or more minutes. Incubation durations may fall in a range of durations, e.g., about 30 minutes to 60 minutes. For example, an incubation condition may comprise treatment of the sample for about 30°C for about 30-60 minutes. In some cases, the sample and/or partition may optionally be incubated again (or in multiple cycles). For example, the sample and/or partition may be incubated again at about 53°C for about 30-60 minutes. The second incubation period may allow for further digestion of RNA molecules and/or may facilitate other processes such as, for example, other nucleic acid reactions. Subjecting RNA molecules to conditions suitable for enzymatic digestion may further comprise subjecting RNA molecules to a useful pH. For example, a buffer may be used to adjust the pH of the partition to a useful value such as about 5.5, 6, 6.5, 7, 7.5, or 8. The concentration of the enzyme may also be altered. For example, the concentration of the enzyme may be determined based on the size or other characteristics of the partition or biological particle.

[00100] Depletion of the rRNA may be capable of increasing the concentration of a first type of RNA molecules relative to a second type of RNA molecules (rRNA, or types thereof), and/or decreasing the concentration of the second type of RNA molecules (rRNA, or types thereof) relative to the first type of RNA molecules. Digestion of RNA molecules by an enzyme may take place within a partition (e.g., by co-partitioning the enzyme with the cell and/or the RNA molecules) or outside the partition, such as prior to partitioning. Digestion of all or a portion of a second set of molecules (e.g., within a partition) may increase the relative concentration or amount of a first set of molecules. For example, the relative concentration of the first set of RNA molecules may increase by at least 5%, 10%, 15%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more. In some cases, the relative concentration of the first set of RNA molecules may increase by at least twofold, such as at least threefold, fourfold, fivefold, tenfold, or more. Correspondingly, the relative concentration of the second set of RNA molecules may decrease by at least 5%, 10%, 15%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%. In some cases, all of the molecules of the second set of molecules may be digested. Alternatively, at least 1%, 2%,

5%, 10%, 15%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the molecules of the second set of molecules may be digested.

[00101] In some cases, treated cells (e.g., cells that are fixed, permeabilized, and rRNA-depleted) may be further subjected to conditions sufficient to fragment nucleic acid molecules within the cells. In some cases, the nucleic acid molecules (i.e., target nucleic acid molecules) are RNA molecules. The nucleic acid molecules may be fragmented chemically or enzymatically. In some instances, the nucleic molecules may be fragmented chemically through the introduction of ions, e.g., from magnesium chloride (MgCl_2), and heat for a defined duration to produce fragmented nucleic acid molecules. In one non-limiting example, the conditions sufficient to fragment the nucleic acid molecules may comprise the use of 10 millimolar (mM) of MgCl_2 at 70°C for about 15 minutes. It will be appreciated that the conditions sufficient to fragment the nucleic acid molecules may comprise a higher or lower concentration of reagent (e.g., MgCl_2), a higher or lower temperature, or a longer or shorter duration. Alternatively or in addition to, other reagents may be used for fragmentation of the nucleic acid molecules. For example, reactive oxygen species (e.g., hydrogen peroxide, superoxide ion) or other oxidative species may be used. Subsequently, the fragmented nucleic acid molecules may be subjected to a reaction sufficient to dephosphorylate the 3' ends.

[00102] In other instances, the nucleic acid molecules may be fragmented enzymatically through the introduction of an enzyme, such as an RNase (e.g., RNase III). Following RNA fragmentation, and in some cases, 3' dephosphorylation, the cells may then be washed with an appropriate buffer (e.g., phosphate buffered saline with a low concentration of bovine serum albumin, HEPES, etc.). In some cases, the treatment of the cells may occur in a partition. In other cases, the treatment of the cells may occur outside a partition (e.g., before or after partitioning).

[00103] **FIG. 9** shows an example workflow for sample processing. In process **902**, cells or cell beads may be fixed. The cells may be permeabilized in process **904** and subjected to conditions sufficient for rRNA depletion in process **906**. In some instances, RNA is fragmented in process **908** after rRNA depletion (e.g., process **906**). In some cases, RNA is fragmented in process **908** before rRNA depletion in process **906** and may occur inside or outside of a partition. The RNA may be further processed inside or outside a partition. In some instances, fragmented RNA is barcoded in process **910**. The barcoded RNA may then be amplified and sequenced or may be used for library preparation.

[00104] In some cases, the target nucleic acid molecules or nucleic acid molecules that are barcoded include RNA molecules. RNA molecules may include various types of RNA. For example, RNA molecules may comprise mRNA, transfer RNA (tRNA), and/or small RNAs. 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). Other types of RNA molecules include, but are not limited to: long non-coding RNA (lncRNA), RNA isoforms, non-poly(a) RNA, etc. The RNA may be double-stranded RNA or single-stranded RNA. The RNA may be circular RNA. Multiple types of RNA molecules may be targeted. Target RNA molecules may range in size. For example, a target RNA molecule may comprise 10 base pairs, 20 base pairs, 30 base pairs, 40 base pairs, 50 base pairs, 60 base pairs, 70 base pairs, 80 base pairs, 90 base pairs, 100 base pairs, 120 base pairs, 130 base pairs, 140 base pairs, 150 base pairs, 160 base pairs, 170 base pairs, 180 base pairs, 190 base pairs, 200 base pairs, or more base pairs. In some examples, target nucleic acid molecules may be heterogeneous in size and comprise a range of approximately 20 to 400 base pairs. In some cases, larger nucleic acid molecules may be target nucleic acid molecules.

[00105] In some instances, the treated, RNA-fragmented cells or cell beads, or the RNA fragments from the cells or cell beads, may be co-partitioned (e.g., a first partition) with a gel bead comprising a plurality of nucleic acid barcode molecules. Gel beads, as described elsewhere herein, may comprise hydrogels, crosslinkers, and/or any combinations or derivatives thereof. In some instances, the plurality of nucleic acid barcode molecules may be bound to the gel bead through a cleavable linker (e.g., disulfide bonds). Upon, during, or following partitioning, a subset of the plurality of nucleic acid molecules barcode may be released from the bead. In such an embodiment, release of the nucleic acid barcode molecules may occur through a dissolution reaction, such as a reducing reaction (e.g., using DTT, TCEP, etc.) for release of the nucleic acid barcode molecules (e.g., reduction of a thio-acrydite moiety). In some instances, the dissolution reaction may de-crosslink the cells or cell beads. In some cases, the dissolution reaction may dissolve both the gel beads (e.g., for release of the nucleic acid barcode molecules) and the cell beads. In some cases, the dissolution reaction may only dissolve the gel beads or only the cell beads. In other instances, barcodes may be released through other mechanisms, such as through an oxidation reaction (e.g., sodium periodate) of, e.g., a dihydroxybutane linkage. Other mechanisms of cleavage of the cleavable linker, such as through photo-labile linkers, may also be used, as described elsewhere herein.

[00106] In some cases, target nucleic acid molecules, e.g., fragmented, 3'-desphosphorylated RNA molecules, may be barcoded and subjected to a nucleic acid reaction sufficient for sequencing and/or library preparation. In some instances, target RNA molecules may be co-partitioned with nucleic acid barcode molecules, which may be coupled to a bead. The nucleic acid barcode molecules may be single-stranded, double-stranded, or partially double-stranded.

For instance, the nucleic acid barcode molecule may comprise a double-stranded nucleic acid molecule comprising an RNA strand hybridized with a DNA strand. The double-stranded or partially double-stranded nucleic acid barcode molecules may also comprise a spacer and/or other adapter sequences. The double-stranded nucleic acid barcode molecules may comprise a nucleotide overhang sequence that may associate (e.g., hybridize) with the target RNA molecules. In some cases, the overhang sequence comprises one nucleotide. In other cases, the overhang sequence may comprise more than one nucleotide. A plurality of nucleic acid barcode molecules with varying overhanging sequences may be used. For example, a plurality of nucleic acid barcode molecules with an overhanging adenine nucleotide may be used along with or separately from a plurality of double-stranded nucleic acid barcode molecules with an overhanging adenine, guanine, cytosine, thymine, uracil, etc.

[00107] In some instances, the nucleic acid barcode molecule and associated target RNA molecule may be subjected to conditions sufficient to generate a barcoded nucleic acid molecule (e.g., barcoded RNA). In some instances, the conditions comprise using an enzyme (e.g., polymerases, reverse transcriptases, which can include thermostable group II intron reverse transcriptases (TGIRTs), and/or any variations (e.g., engineered variants) or derivative thereof). For example, in cases where the nucleic acid barcode molecule comprises an RNA-DNA hybrid with an optional overhang sequence, the nucleic acid barcode molecule may couple to the target nucleic acid molecule (e.g., target RNA). In some instances, the nucleic acid barcode molecule may couple to the target RNA via hybridization of the overhang sequence. An enzyme that can associate with nucleic acid hybrids (e.g., RNA- DNA hybrids) and that can template switch to the target nucleic acid molecule and extend the complementary strand (e.g., DNA) may be used, thereby generating complementary nucleic acid molecules (e.g., barcoded cDNA or barcoded hybrid nucleic acid molecules comprising RNA and DNA). The barcoded nucleic acid molecules may then be further subjected to conditions sufficient for amplification and library preparation. For example, the barcoded nucleic acid molecules may be subjected to treatment in an alkaline solution (e.g., sodium hydroxide) followed by neutralization in an acidic solution (e.g., hydrochloric acid). Subsequently, adaptor molecules that comprise spacers and/or sequence priming sites may be ligated (e.g., enzymatically with a DNA or RNA ligase) onto the barcoded nucleic acid molecules. The adaptor-ligated, barcoded nucleic acid molecules may then be amplified and prepared for sequencing.

[00108] Partitions may be broken, ruptured, or disrupted at any convenient step in the process. For example, in some instances, the partitions may be disrupted following barcoding of the target nucleic acid molecules. In these instances, the contents within the partitions may be collected prior to further treatment (e.g., adaptor ligation, amplification, and/or library preparation).

[00109] Enzymes may be used for template switching and/or nucleic acid extension. In some cases, reverse transcriptases may be used. Derivatives of reverse transcriptases may also be used for optimal reaction conditions. In some cases, thermostable group II intron reverse transcriptases (TGIRT), or a derivative or variant thereof (e.g., engineered variant) may be used. Other examples of reverse transcription enzymes include, but are not limited to: SuperScript III, Affinityscript, Maxima, Rocketscript, Thermoscript, Monsterscript, and/or engineered modifications of reverse transcriptases. Enzymes may be selected for a particular application. For example, TGIRT enzymes may be advantageous over traditional reverse transcriptases as they have higher processivity, fidelity, thermostability, and flexibility. Moreover, the modified enzymes may not require the use of template switch nucleic acid molecules or may have template switching capabilities (e.g., from RNA to DNA, or DNA to RNA). In some instances, the modified enzymes may not require the use of RNA ligase or can be expressed in bacteria or offer other additional advantages.

[00110] A variety of enzymes may be used in the methods described herein. Examples of enzymes which may be used include, but are not limited to: polymerases, reverse transcriptases, restriction enzymes (e.g., endonuclease), transposase, ligases (e.g., T4KQ ligase, EvoT4 ligase, CirculigaseTM, ThermoPhageTM, T4 RNA ligase 1, T4 RNA ligase 2, mutants of T4 RNA ligases 1 and 2, Mth RNA ligase, a T7 DNA ligase, a T3 DNA ligase, *E.coli* DNA ligase, *Taq* ligase, a 9°N DNA ligase, thermostable ligases, etc.), proteinase K, DNase, RNase, uracil glycosylase, USER, etc. The ligase may be a ligase described in U.S. Patent Publication No. 201880320162A1, which is entirely incorporated herein by reference for all purposes.

[00111] **FIG. 10** schematically illustrates a nucleic acid reaction comprising a template-switching mechanism using a reverse transcriptase (e.g., TGIRT). In a process **1000**, a first nucleic acid molecule (e.g. RNA) **1002** hybridizes with a second nucleic acid molecule **1004** (e.g., DNA) to form a hybrid nucleic acid molecule **1006**. The hybrid nucleic acid molecule **1006** may comprise a barcode sequence and/or an adaptor or functional sequence. The second nucleic acid molecule **1004**, which may form a strand of the hybrid nucleic acid molecule **1006**, may comprise an overhang sequence comprising one or more nucleotides that does not hybridize with the first nucleic acid molecule **1002**, which first nucleic acid molecule **1002** may form another strand of the hybrid nucleic acid molecule **1006**. The overhang sequence may be any nucleotide base pair (A,T,C,G, etc.). In some cases, the first nucleic acid molecule **1002** may also comprise a blocker sequence (e.g., at the 3' end) that may prevent extension of the nucleic acid in that direction. An enzyme **1008** binds to or associates with the hybrid nucleic acid molecule **1006** in process **1010**. The enzyme may be a reverse transcriptase or a variant reverse transcriptase, e.g., TGIRT or variant thereof. The enzyme **1008**, upon or after associating with the hybrid nucleic

acid molecule **1006** may move to the 3' overhang sequence of the hybrid nucleic acid molecule **1006**. In process **1012**, a third nucleic acid molecule **1014** (e.g., target RNA) may hybridize with the overhang sequence of hybrid nucleic acid molecule **1006**. The enzyme **1008** may then switch template strands to the third nucleic acid molecule **1014** and extend the first nucleic acid molecule **1002**. For example, a complementary DNA (cDNA) strand may be generated to provide a linked nucleic acid molecule **1018**.

[00112] **FIG. 11** schematically shows an example method of barcoding nucleic acid molecules. In some cases, target RNA molecules **1102** comprising a hydroxyl 3' end (e.g., RNA molecules cleaved of the 3' phosphate groups) **1108** are co-partitioned in partition **1100** with hybrid nucleic acid barcode molecules that are double-stranded and that comprise a RNA molecule **1106** hybridized to a DNA molecule **1104**. As described herein, the target RNA molecules **1102** may be derived from a cell and may be generated through one or more pre-processing operations, e.g., fixation and permeabilization of the cell, rRNA depletion of the cell, RNA fragmentation, cleavage of 3' phosphate groups, or a combination thereof. The target RNA molecules **1102** may be a part of or included in a cell or cell bead.

[00113] In some cases, the hybrid nucleic acid barcode molecules are bound to a bead and are releasable upon application of a stimulus (e.g., thermal stimulus, photo-stimulus, chemical stimulus, biological stimulus). The double-stranded nucleic acid barcode molecules comprise a barcode sequence and may also comprise a spacer and/or other adapter sequences, such as a constant sequence, a unique molecular identifier, and/or primer sequencing sites. The double-stranded nucleic acid barcode molecules may also comprise a nucleotide overhang sequence that may associate (e.g., hybridize) with the target RNA molecules **1102**. Although one hybrid nucleic acid molecule is depicted in **FIG. 11**, it may be appreciated that more than one type of hybrid nucleic acid molecule, may be used, e.g., hybrid nucleic acid molecules comprising an overhang sequence of an adenine, hybrid nucleic acid molecules comprising an overhang sequence of a cytosine, and so on, may be used within the partition **1100**. In some cases, the overhang sequence comprises one nucleotide. In other cases, the overhang sequence may comprise more than one nucleotide. e.g., 2 nucleotides, 3 nucleotides, 4 nucleotides, 5 nucleotides, 10 nucleotides, etc. For example, a plurality of double-stranded nucleic acid barcode molecule with an overhanging adenine nucleotide may be used along with or separately from a plurality of double-stranded nucleic acid barcode molecules with an overhanging guanine, cytosine, thymine, uracil, etc.

[00114] In process **1110**, an enzyme (e.g., TGIRT) can associate with the hybrid nucleic acid molecules and can template switch to the target nucleic acid molecule (e.g., RNA) and extend the complementary strand, generating barcoded nucleic acid molecules (e.g., barcoded cDNA)

1112. The barcoded nucleic acid molecules **1112** may then be removed from the partition and undergo process **1114**, for example, to denature the enzymes and/or nucleic acid molecules. In one example, the barcoded nucleic acid molecules **1112** may be subjected to treatment **1114** comprising an alkaline solution (e.g., sodium hydroxide) followed by neutralization in an acidic solution (e.g., hydrochloric acid). In process **1116**, adaptor molecules **1118** that comprise a blocked 3' end, a 5'-App sequence, and a primer sequence may be introduced with enzymes (e.g., 5'-App DNA/RNA ligase) to generate a 3' primed barcoded nucleic acid molecule. In some cases, the adaptor **1118** may comprise a sequencing primer sequence. In some cases, the adaptor **1118** may be double-stranded or partially double-stranded and may comprise a splint oligonucleotide, which may serve as a primer. For instance, the splint oligonucleotide may comprise a first strand comprising a sequencing primer sequence, a random or targeted N-mer, and optionally a 3' blocking moiety. The splint oligonucleotide may comprise a second strand comprising a complement to the sequencing primer sequence, and optionally, a 5'-phosphorylated end and/or a 3' blocking moiety. In such cases, the adaptor **1118** may be coupled to the barcoded nucleic acid molecule **1112** (e.g., via ligation using an enzyme, e.g., T4 DNA ligase). In process **1120**, additional adaptor molecules **1122** may be added. The adaptor molecules may comprise, for example, primers and sequences for amplification and/or sequencing of the barcoded nucleic acid molecule. In cases where adaptor **1118** in process **1116** comprises a splint oligonucleotide, the 3' primed barcoded nucleic acid molecule may be primed (e.g., via the N-mer) for a nucleic acid reaction (e.g., nucleic acid extension reaction), which may not require the use of additional adaptor molecules **1122** or a subset of adaptor molecules **1122** (e.g. comprising a sequencing primer sequence). In another example, prior to process **1116**, a nucleic acid reaction (e.g., extension or amplification) may be performed to generate a double-stranded barcoded nucleic acid molecule (e.g., double-stranded cDNA); in such cases, in process **1116**, the adaptor molecules **1118** may be double-stranded and may be ligated to the double-stranded barcoded nucleic acid molecule. In some instances, the adaptor molecules **1118** may comprise a 5'-App sequence or 5' phosphate group, which may facilitate ligation (e.g., via a ligase) of the adaptor molecules **1118** to the double-stranded barcoded nucleic acid molecule.

[00115] The 5'-App sequence, as described herein, may be an adenylated nucleic acid molecule. The adenylation may occur on the 5' end of the nucleic acid molecule. In some cases, the adenylated nucleic acid molecule is a DNA molecule. In some cases, the adenylated nucleic acid molecule is an RNA molecule. In some cases, the 5'-App sequence comprises a nucleoside. For example, the 5'-App sequence may comprise a riboadenosine that is linked to the 5' end of a nucleic acid molecule, e.g., via a phosphodiester bond. In other non-limiting examples, the 5'-App sequence may comprise a ribo-cytidine, ribo-uridine, ribo-guanosine, ribo-thymidine, ribo-

inosine, a deoxy-cytidine, a deoxy-uridine, a deoxy-guanosine, a deoxy-thymidine, a deoxy-inosine, a deoxy-adenosine, etc.

[00116] In some cases, target nucleic acid molecules, e.g., fragmented, 3'-desphosphorylated RNA molecules, may be barcoded and subjected to a nucleic acid reaction sufficient for sequencing and/or library preparation. In some instances, a nucleic acid molecule (e.g., target RNA molecules) may be co-partitioned with nucleic acid barcode molecules (e.g., barcode DNA molecules). In some cases, the barcode nucleic acids are single-stranded. The nucleic acid barcode molecules may be bound to a bead and may be releasable upon application of a stimulus (e.g., thermal stimulus, photo-stimulus, chemical stimulus). In some cases, following release of the barcode from the bead, the nucleic acid barcode molecules (e.g., barcode DNA molecules) are ligated to the target nucleic acid molecules (e.g. RNA molecules), generating barcoded target nucleic acid molecules. Ligation of the nucleic acid barcode molecule to the target nucleic acid molecule may occur using an enzyme, as described elsewhere herein. Following barcoding of the target nucleic acid molecules, the products may be cleaned up. The barcoded target nucleic acid molecules may then be subjected to conditions sufficient to generate barcoded target complementary nucleic acid molecules (e.g., barcoded cDNA molecules). In some cases, these conditions comprise reverse transcription with template switching. Template switching may comprise reverse transcription primers, template switching nucleic acid molecules, and/or other adaptor sequences. Barcoded target complementary nucleic acid molecules may then be amplified and prepared for sequencing.

[00117] **FIG. 12** schematically illustrates another example method for barcoding nucleic acid molecules. In this embodiment, target RNA molecules **1202** with a 3' hydroxyl (-OH) group **1208** are co-partitioned with nucleic acid barcode molecules (e.g., barcode DNA molecule) **1204**. As described herein, the target RNA molecules **1202** may be derived from a cell and may be generated through one or more pre-processing operations, e.g., fixation and permeabilization of the cell, rRNA depletion of the cell, RNA fragmentation, cleavage of 3' phosphate groups, or a combination thereof. The target RNA molecules **1202** may be a part of or included in a cell or cell bead. In some cases, the nucleic acid barcode molecules **1204** are bound to a bead and are releasable upon application of a stimulus (e.g., thermal stimulus, photo-stimulus, chemical stimulus, biological stimulus). The nucleic acid barcode molecules comprise a barcode sequence and may also comprise a spacer and/or other adapter sequences, such as a constant sequence, a unique molecular identifier, and/or primer sequencing sites. The nucleic acid barcode molecules may also comprise a 5'-App sequence that may be used to ligate to target RNA molecules **1202**. In some instances, an enzyme (e.g., ligase, e.g., T4KQ ligase, T4RNA ligase or engineered variants thereof, etc.) can ligate, in process **1210**, the target RNA molecules **1202** and the nucleic

acid barcode molecules (e.g., barcode DNA) **1204**, generating a barcoded nucleic acid molecule **1212**. The barcoded nucleic acid molecules **1212** may then be removed from the partition and undergo process **1214**, comprising cleanup of reaction products, for example, to remove unwanted reaction byproducts. In process **1216**, adaptor molecules **1218** and **1219** may be added to the barcoded nucleic acid molecules. Adaptor **1218** may comprise a primer sequence for reverse transcription, and adaptor **1219** may comprise a sequencing primer sequence or partial sequencing primer sequence and may, in some instances, be used for template switching. Process **1216** may further comprise enzymes (e.g., reverse transcriptases with terminal transferase (TdT) activity) to provide a barcoded complementary DNA (barcoded cDNA) molecule **1212**, which may be single-stranded. In process **1220**, additional adaptor molecules **1222** may be added. The adaptor molecules may be, for example, primers and sequences for amplification and/or sequencing of the barcoded nucleic acid molecules. In some cases, adaptor molecule **1219** comprises a splint oligonucleotide, which may prime the barcoded nucleic acid molecule for a nucleic acid reaction (e.g., nucleic acid extension reaction), which may not require or may only require a subset of adaptor molecules **1222** in process **1220**.

[00118] In some cases, following barcoding of the target nucleic acid molecules, the barcoded target nucleic acid molecules may be further processed. In some cases, the barcoded target nucleic acid molecules may be phosphorylated on the 5' end using an enzyme (e.g., T4 polynucleotide kinase) and adenosine triphosphate (ATP). The phosphorylation may facilitate ligation of an adapter sequence to the phosphorylated end. In some cases, the ligated adapter sequence is a PCR priming sequence, which may be ligated using an enzyme (e.g., T4 RNA ligase). Following ligation of the adapter sequence, the barcoded target nucleic acid molecules may be subjected to a reverse transcription reaction using an enzyme (e.g., reverse transcriptase) to generate complementary barcoded molecules (e.g., barcoded cDNA molecules). The products of the reverse transcription reaction may then be amplified and prepared for sequencing.

[00119] **FIG. 13** schematically illustrates an example method for barcoding nucleic acid molecules by ligation and reverse transcription. In such an example, target RNA molecules **1302** with 3'hydroxyl (-OH) groups **1308** are co-partitioned with nucleic acid barcode molecules (e.g., barcode DNA molecules) **1304**. As described herein, the target RNA molecules **1302** may be derived from a cell and may be generated through one or more pre-processing operations, e.g., fixation and permeabilization of the cell, rRNA depletion of the cell, RNA fragmentation, cleavage of 3' phosphate groups, or a combination thereof. The target RNA molecules **1302** may be a part of or included in a cell or cell bead. In some cases, the nucleic acid barcode molecules **1304** are bound to a bead and are releasable upon application of a stimulus (e.g., thermal stimulus, photo-stimulus, chemical stimulus, biological stimulus). The nucleic acid barcode

molecules comprise a barcode sequence and may also comprise a spacer and/or other adapter sequences, such as a constant sequence, a unique molecular identifier, and/or primer sequencing sites. The nucleic acid barcode molecules may also comprise a 5'-App sequence that may be used to ligate to target RNA molecules **1302**. In some instances, an enzyme (e.g., T4KQ ligase) can ligate, in process **1310**, the target RNA molecules **1302** and the nucleic acid barcode molecules (e.g., barcode DNA) **1304**, generating a barcoded nucleic acid molecule **1312**. The barcoded nucleic acid molecules **1312** may be removed from the partition and undergo process **1314**, comprising an enzyme (e.g., T4 polynucleotide kinase) and reagents (e.g., ATP) to provide a 5'-phosphorylated barcoded nucleic acid molecule **1315**. The 5'-phosphorylated barcoded nucleic acid molecule **1315** may undergo process **1316**, wherein an adaptor nucleic acid molecule **1318** comprising a primer sequencing site may be attached to the 5'-end of the 5'-phosphorylated barcoded nucleic acid molecule **1315**. Ligation of the adaptor nucleic acid molecule may occur through use of an enzyme, e.g., a T4 RNA ligase. In process **1320**, a nucleic acid reaction, e.g., reverse transcription, occurs through addition of a primer sequence **1322** and may be mediated by an enzyme (e.g., reverse transcriptase). The nucleic acid reaction may result in a barcoded complementary nucleic acid molecule, e.g. barcoded cDNA. In process **1324**, additional adaptor molecules **1326** may be added. The adaptor molecules may be, for example, primers and sequence amplification and/or sequencing of the barcoded nucleic acid molecules.

[00120] In some cases, following barcoding of the target nucleic acid molecules and reaction cleanup, the barcoded target nucleic acid molecules may be further processed. In some cases, the barcoded target nucleic acid molecules may be subjected to a reverse transcription reaction. In some cases, the reverse transcription reaction comprises reverse transcription nucleic acid primer sequences that are complementary to a sequence of the barcoded nucleic acid molecule. The reverse transcription nucleic acid primer sequences may additionally comprise a 5' phosphate group, a sequencing primer sequence or partial sequencing primer sequence, and a residue (e.g., uridine) that may, downstream, promote circularization of the nucleic acid molecule. The reverse transcription nucleic acid primer sequences may hybridize to a sequence of the barcoded nucleic acid molecule. The reverse transcription reaction may comprise using one or more enzymes (e.g., reverse transcriptase, RNase H, etc.) to generate complementary barcoded molecules (e.g., barcoded cDNA molecules) that comprise the 5' phosphate group and the residue. The barcoded cDNA molecules may then be subjected to conditions sufficient to circularize the nucleic acid molecules, generating circularized, barcoded cDNA molecules. The conditions may comprise using an enzyme (e.g., CircLigase or T4 ligase (e.g., in the presence of a cation, e.g., magnesium)), or the use of a splint oligonucleotide. Following circularization, the circularized, barcoded cDNA molecules may be subjected to cleanup and linearization. Linearization may

occur at any convenient step and may comprise using an enzyme (e.g., USER enzyme for uracil excision). The linearized nucleic acid molecules (e.g., barcoded cDNA molecules) may then be amplified and prepared for sequencing.

[00121] FIG. 14 illustrates schematically a method for barcoding nucleic acid molecules by ligation and circularization. In this example, target RNA molecules **1402** with a free 3'hydroxyl (-OH) group **1408** are co-partitioned with nucleic acid barcode molecules (e.g., barcode DNA molecules) **1404**. As described herein, the target RNA molecules **1402** may be derived from a cell and may be generated through one or more pre-processing operations, e.g., fixation and permeabilization of the cell, rRNA depletion of the cell, RNA fragmentation, cleavage of 3' phosphate groups, or a combination thereof. The target RNA molecules **1402** may be a part of or included in a cell or cell bead. In some cases, the nucleic acid barcode molecules **1404** are bound to a bead and are releasable upon application of a stimulus (e.g., thermal stimulus, photo-stimulus, chemical stimulus, biological stimulus). The nucleic acid barcode molecules **1404** comprise a barcode sequence and may also comprise a spacer and/or other adapter sequences, such as a constant sequence, a unique molecular identifier, and/or primer sequencing sites. In some cases, the nucleic acid barcode molecules **1404** may also comprise an adenine nucleotide at a known or random location. In some instances, the nucleic acid barcode molecules **1404** comprise two sequencing primer sequences, wherein the moiety (e.g., adenine) is disposed between the two sequencing primer sequences. The nucleic acid barcode molecules **1404** may also comprise a 5'-App sequence that may be used to ligate to target RNA molecules **1402**. In some instances, an enzyme (e.g., T4KQ ligase) can ligate, in process **1410**, the target RNA molecules **1402** and the nucleic acid barcode molecules (e.g., barcode DNA) **1404**, generating a barcoded nucleic acid molecule **1412**. The barcoded nucleic acid molecules **1412** may then be removed from the partition and undergo process **1414**, comprising cleanup of reaction products, for example, to remove unwanted reaction byproducts. In process **1416**, a nucleic acid reaction, e.g., reverse transcription, occurs through addition of an adaptor sequence **1418** and may be mediated by one or more enzymes (e.g., reverse transcriptase and RNase H). In some cases, process **1416** also comprises use of another enzyme (e.g. RNase H), which can remove RNA byproducts. Adaptor sequence **1418** may comprise a cleavable moiety (e.g., uridine residue, cleavable linker) and sequences complementary to the barcoded nucleic acid molecule **1412**. The adaptor sequence **1418** may additionally comprise sequencing primers sequences or partial sequencing primer sequences, and, in some instances, a phosphorylated 5' end. The nucleic acid reaction may result in a barcoded complementary nucleic acid molecule **1420**, e.g. barcoded cDNA, comprising the cleavable moiety (e.g., a uridine residue). In process **1422**, the barcoded cDNA comprising the uridine residue may be circularized to form a circular barcoded nucleic

acid molecule **1424**. Circularization may occur through the use of an enzyme (e.g., CircLigase, T4 ligase in the presence of a cation, e.g., magnesium) and/or a splint oligonucleotide. Examples of using T4 ligase for circularization can be found, for example, in R. An, et al. "Highly efficient preparation of single-stranded DNA rings by T4 ligase at abnormally low Mg(II) concentration," Nucleic Acids Res. 2017 Sep 6, which is incorporated by reference herein. In process **1426**, a second cleanup step may occur and the circular barcoded nucleic acid molecule **1424** may be linearized, e.g., via cleavage of the cleavable moiety, e.g., uridine residue. In some cases, the cleavable moiety is cleaved using an enzyme (e.g., UDG or USER enzyme). In other instances, the cleavable moiety may comprise a labile linker (e.g. photo-labile, thermo-labile, etc. linkage) that may be cleaved in the presence of a stimulus (e.g., light, heat, etc.). In process **1430**, additional adaptor molecules **1432** may be added. The adaptor molecules may be, for example, primers and sequence amplification and/or sequencing of the barcoded nucleic acid molecules.

[00122] In some cases, following fragmentation of the nucleic acid molecules (e.g., RNA molecules), which may optionally be performed following rRNA depletion or other pre-processing of the cells or cell beads, the fragmented nucleic acid molecules (e.g., fragmented RNA molecules) may be treated to generate a poly-A tail. The poly-A tail may be generated using poly-adenylation of the RNA fragments. For instance, the fragmented RNA molecules may be contacted with a poly-ADP ribose polymerase or a poly-A polymerase, which may be used to generate a poly-A tail on a fragmented RNA molecule. In some cases, the poly-A polymerase may preferentially polyadenylate mRNA. Following generation of the poly-A tail, the fragmented RNA molecules may be used for downstream processing, e.g., barcoding. For example of barcoding schemes, see, e.g., U.S. Patent No. 9,951,386, which is incorporated by reference herein in its entirety.

[00123] **FIG. 15** schematically illustrates an example method for barcoding nucleic acid molecules comprising polyadenylation and reverse transcription. In such an example, target RNA molecules **1502** with a 3'hydroxyl (-OH) groups **1508** are co-partitioned with nucleic acid barcode molecules (e.g., barcode DNA molecules) **1504**. As described herein, the target RNA molecules **1502** may be derived from a cell and may be generated through one or more pre-processing operations, e.g., fixation and permeabilization of the cell, rRNA depletion of the cell, RNA fragmentation, cleavage of 3' phosphate groups, or a combination thereof. For example, the cell or cell bead comprising RNA molecules **1501**, or RNA molecules **1501** derived from a cell or cell bead, may be partitioned and subjected to RNA fragmentation in process **1503**, thereby generating fragmented target RNA molecules **1502**. The fragmented target RNA molecules **1502** may be subjected to conditions sufficient to generate a poly-A tail, in process **1510**, on the fragmented target RNA molecules **1502** to generate polyadenylated nucleic acid

molecules **1506**. In some cases, the nucleic acid barcode molecules **1504** are bound to a bead and are releasable upon application of a stimulus (e.g., thermal stimulus, photo-stimulus, chemical stimulus, biological stimulus). The nucleic acid barcode molecules **1504** comprise a barcode sequence and may also comprise a spacer and/or other adapter sequences, such as a constant sequence, a unique molecular identifier, and/or primer sequencing sites. In some instances, the nucleic acid barcode molecules **1504** comprise a capture sequence, e.g., a poly-T sequence that can anneal to the polyadenylated nucleic acid molecule **1506**. In some instances, the polyadenylated nucleic acid molecule **1506** may undergo process **1510**, which can include annealing of the polyadenylated nucleic acid molecule **1506** to a nucleic acid barcode molecule (e.g., barcode DNA) **1504**, and, in some cases, reverse transcription (e.g., using a template switching oligonucleotide (TSO)), thereby generating a barcoded nucleic acid molecule **1512**. The barcoded nucleic acid molecules **1512** may be removed from the partition and undergo process **1514**, which can be mediated by an enzyme (e.g., reverse transcriptase) to generate a double-stranded barcoded nucleic acid molecule **1515**. The double-stranded barcoded nucleic acid molecule **1515** may undergo process **1520**, where additional adaptor molecules **1526** may be added. The adaptor molecules may be, for example, primers and sequence amplification and/or sequencing of the barcoded nucleic acid molecules.

[00124] Partitions (e.g., droplets) may be broken, ruptured, or disrupted at any convenient step in the process (e.g., the processes in **FIGs. 11-15**). For example, in some instances, the partitions may be disrupted following barcoding of the target nucleic acid molecules. In these instances, the contents within the partitions may be collected prior to further treatment (e.g., enzymatic treatments, cleanup, adaptor ligation, reverse transcription, amplification, and/or library preparation). In another example, the partitions may be disrupted prior to further analysis, e.g., sequencing. In yet another example, the partitions may be used for pre-processing (e.g., fragmentation, polyadenylation, etc.) of the nucleic acid molecules in addition to or alternatively to barcoding in the partitions.

Systems and methods for sample compartmentalization

[00125] 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.

[00126] 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.

[00127] 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.

[00128] 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 or reservoir of a non-aqueous fluid, such that droplets are generated (see generally, e.g., **FIGS. 1-7B**). 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.

[00129] 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.

[00130] 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.

[00131] 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.

[00132] 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.

[00133] In some cases, it may be useful 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.

[00134] In some cases, the flow of one or more of the biological particles (e.g., in channel segment **102**), or other fluids 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 described above. For example, in many cases, the use of the 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%, 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.

[00135] As will be appreciated, the above-described occupancy 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 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.

[00136] In another aspect, in addition to or as an alternative to 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 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 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 crosslinking, polymerization initiation of the precursor (e.g., through added initiators)), mechanical stimuli, or a combination thereof.

[00137] 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 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 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.

[00138] 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.

[00139] 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^{2+} 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.

[00140] 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.

[00141] 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.

[00142] 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.

[00143] 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.

[00144] 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 useful 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

[00145] In some cases, nucleic acid barcode molecules are delivered to a partition (e.g., a droplet or well) via a solid support or carrier (e.g., a bead). In some cases, nucleic acid barcode molecules are initially associated with the solid support and then released from the solid support upon application of a stimulus, which allows the nucleic acid barcode molecules to dissociate or to be released from the solid support. In certain examples, nucleic acid barcode molecules are initially associated with the solid support (e.g., bead) and then released from the solid support upon application of a biological stimulus, a chemical stimulus, a thermal stimulus, an electrical stimulus, a magnetic stimulus, and/or a photo stimulus.

[00146] In some cases, a nucleic acid barcode molecule contains a barcode sequence and a functional sequence, such as a nucleic acid primer sequence or a template switch oligonucleotide (TSO) sequence.

[00147] In some instances, the solid support is a bead. A solid support, e.g., a bead, may be porous, non-porous, hollow (e.g., a microcapsule), solid, semi-solid, and/or a combination thereof. In addition, beads may be solid, semi-solid, semi-fluidic, fluidic, and/or a combination thereof. In some instances, a solid support, e.g., a bead, may be dissolvable, disruptable, and/or degradable. In some cases, a solid support, e.g., a bead, may not be degradable. In some cases, the solid support, e.g., a 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 support, e.g., a bead, may be a liposomal bead. Solid supports, e.g., beads, may comprise metals including iron oxide, gold, and silver. In some cases, the solid support, e.g., the bead, may be a silica bead. In some cases, the solid support, e.g., a bead, can be rigid. In other cases, the solid support, e.g., a bead, may be flexible and/or compressible.

[00148] 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.

[00149] 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.

[00150] 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.

[00151] 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.

[00152] 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.

[00153] 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, perfluorooctanol (PFO), perfluoropolyethers, etc. may also be employed to prevent coalescence of droplets.

[00154] 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).

[00155] 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.

[00156] 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 **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.

[00157] 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.

[00158] 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.

[00159] 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 (μm), 5 μm , 10 μm , 20 μm , 30 μm , 40 μm , 50 μm , 60 μm , 70 μm , 80 μm , 90 μm , 100 μm , 250 μm , 500 μm , 1mm, or greater. In some cases, a bead may have a diameter of less than about 10 nm, 100 nm, 500 nm, 1 μm , 5 μm , 10 μm , 20 μm , 30 μm , 40 μm , 50 μm , 60 μm , 70 μm , 80 μm , 90 μm , 100 μm , 250 μm , 500 μm , 1mm, or less. In some cases, a bead may have a diameter in the range of about 40-75 μm , 30-75 μm , 20-75 μm , 40-85 μm , 40-95 μm , 20-100 μm , 10-100 μm , 1-100 μm , 20-250 μm , or 20-500 μm .

[00160] In certain aspects, beads can be provided as a population or plurality of beads having a relatively monodisperse size distribution. Where it may be useful 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.

[00161] 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, sterculia 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, 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.

[00162] 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.

[00163] 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.

[00164] 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.

[00165] 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.

[00166] 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.

[00167] 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.

[00168] 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) that comprises one or more functional sequences, such as a TSO sequence or a primer sequence (e.g., a poly T sequence, or a nucleic acid primer sequence complementary to a target nucleic acid sequence and/or for amplifying a target nucleic acid sequence, a random primer, or a primer sequence for messenger RNA) that is useful for incorporation into the bead 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.

[00169] 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 (or a portion thereof) 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 (or a portion thereof) 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 nucleic acid molecule can further comprise a unique molecular identifier (UMI). In some cases, the nucleic acid molecule can comprise an R1 primer sequence for Illumina sequencing. In some cases, the nucleic acid molecule 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.

[00170] 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) or partial sequencing primer sequence. 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.

[00171] 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 compress 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.

[00172] 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.

[00173] 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.

[00174] 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.

[00175] 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.

[00176] 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.

[00177] 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.

[00178] 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.

[00179] 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.

[00180] 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.

[00181] 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.

[00182] In other instances, oligonucleotides and other reagents for barcoding of cellular analytes (single or multiple) can be provided in a partition (e.g., a microwell) in the absence of a solid support (e.g., a bead). The partition may comprise such oligonucleotides and other reagents in solution (e.g., in a well or a droplet). Alternatively, the oligonucleotides may be attached (e.g., releasably, cleavably, reversibly, or irreversibly) to a surface of the partition (e.g., a well) according to any of the strategies described herein.

[00183] 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.

[00184] 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 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 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 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 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.

[00185] 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 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 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.

[00186] The addition of multiple types of labile bonds to a gel 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 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.

[00187] 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.

[00188] 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., tagmentation) 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.

[00189] 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.

[00190] 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.

[00191] 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 useful 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 useful 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 1/10th, less than about 1/50th, or even less than about 1/100th 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.

[00192] 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.

[00193] 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 β -mercaptoethanol, (2S)-2-amino-1,4-dimercaptobutane (dithiobutylamine 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 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.

[00194] 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.

[00195] 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), β -mercaptoethanol, (2S)-2-amino-1,4-dimercaptobutane (dithiobutylamine or DTBA), tris(2-carboxyethyl) phosphine (TCEP), or combinations thereof. The reducing agent may be present at a concentration of about 0.1mM, 0.5mM, 1mM, 5mM, 10mM. The reducing agent may be present at a concentration of at least about 0.1mM, 0.5mM, 1mM, 5mM, 10mM, or greater than 10 mM. The reducing agent may be present at concentration of at most about 10mM, 5mM, 1mM, 0.5mM, 0.1mM, or less.

[00196] Although **FIG. 1** and **FIG. 2** have been described in terms of providing substantially singly occupied partitions, above, in certain cases, it may be useful to provide multiply occupied partitions, e.g., containing two, three, four or more cells and/or microcapsules (e.g., beads) comprising barcoded 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.

[00197] 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).

[00198] The partitions described herein may comprise small volumes, for example, less than about 10 microliters (μL), 5 μL , 1 μL , 500 nanoliters (nL), 100 nL, 50 nL, 900 picoliters (pL), 800 pL, 700 pL, 600 pL, 500 pL, 400pL, 300 pL, 200 pL, 100pL, 50 pL, 20 pL, 10 pL, 1 pL, or less.

[00199] 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, 400pL, 300 pL, 200 pL, 100pL, 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.

[00200] 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

[00201] 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.

[00202] 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.

[00203] 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.

[00204] 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.

[00205] 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.

[00206] 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).

[00207] 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.

[00208] 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.

[00209] 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 useful 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.

[00210] Alternatively or in addition to the lysis agents co-partitioned with the analyte carriers described above, other reagents can also be co-partitioned with the analyte carriers, including,

for example, DNase and RNase inactivating agents or inhibitors, such as proteinase K, 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 analyte carriers (e.g., a cell or a nucleus in a polymer matrix), the analyte carriers may be exposed to an appropriate stimulus to release the analyte carriers or their contents from a co-partitioned microcapsule. For example, in some cases, a chemical stimulus may be co-partitioned along with an encapsulated analyte carrier 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 examples, this may be a different and non-overlapping stimulus, in order to allow an encapsulated analyte carrier to be released into a partition at a different time from the release of nucleic acid molecules into the same partition. For a description of methods, compositions, and systems for encapsulating cells (also referred to as a “cell bead”), see, e.g., U.S. Pat. 10,428,326 and U.S. Pat. Pub. 20190100632, which are each incorporated by reference in their entirety.

[00211] 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-hydroxybutynl-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.

[00212] 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.

[00213] 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.

[00214] 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.

[00215] 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.

[00216] 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.

[00217] 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 nucleic acids (e.g., mRNA, 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. 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 (e.g., attached to a bead) into partitions, e.g., droplets within microfluidic systems.

[00218] In an example, microcapsules, such as beads, are provided that each include large numbers of the above described nucleic acid barcode 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.

[00219] 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.

[00220] In some cases, it may be useful 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.

[00221] 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.

[00222] 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.

[00223] 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_0 , 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**.

[00224] 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.

[00225] 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.

[00226] 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.

[00227] 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.

[00228] 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.

[00229] 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, α . The expansion angle, α , 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 α :

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

[00230] 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 $124 \mu\text{m}$.

[00231] In some instances, the expansion angle, α , 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° , 80° , 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° , 0.01° , or less. In some instances, the width, w , can be between a range of from about 100 micrometers (μm) to about 500 μm . In some instances, the width, w , can be between a range of from about 10 μm to about 200 μm . Alternatively, the width can be less than about 10 μm . Alternatively, the width can be greater than about 500 μm . In some instances, the flow rate of the aqueous fluid **408** entering the junction **406** can be between about 0.04 microliters (μL)/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 (μL)/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**.

[00232] 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.

[00233] 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**.

[00234] **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.

[00235] 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.

[00236] 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_0 , 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 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.

[00237] The geometric parameters, w , h_0 , 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 useful to have a different distribution of droplet sizes, the geometric parameters for the plurality of channel segments **502** may be varied accordingly.

[00238] 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.

[00239] **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.

[00240] 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.

[00241] 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.

[00242] The reservoir **604** may have an expansion angle, α (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 α , 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**.

[00243] 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 useful to have a different distribution of droplet sizes, the geometric parameters for the plurality of channel segments **602** may be varied accordingly.

[00244] 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.

[00245] **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**.

[00246] 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., Δh , 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.

[00247] 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.

[00248] 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.

[00249] 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.

[00250] 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 Δh . 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, β , at or near the junction **706**. The height difference, Δh , and/or expansion angle, β , 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.

[00251] The height difference, Δh , can be at least about 1 μm . 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 μm 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 μm or less. In some instances, the expansion angle, β , 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° , 80° , 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° , 0.01° , or less.

[00252] In some instances, the flow rate of the aqueous fluid **712** entering the junction **706** can be between about 0.04 microliters (μL)/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 (μL)/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**.

[00253] 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.

[00254] While **FIGs. 7A** and **7B** illustrate the height difference, Δh , being abrupt at the junction **706** (e.g., a step increase), the height difference may increase gradually (e.g., from about 0 μm 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 at an angle greater than 90°. 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, β), 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.

[00255] 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 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.

[00256] The methods and systems described herein may be used to greatly increase the efficiency of single-cell applications 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 (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 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 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 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.

[00257] 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.

Computer systems

[00258] The present disclosure provides computer systems that are programmed to implement methods of the disclosure. **FIG. 16** shows a computer system **1601** that is programmed or otherwise configured to control a microfluidics system (e.g., fluid flow) and perform sequencing applications. The computer system **1601** can regulate various aspects of the present disclosure. The computer system **1601** 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.

[00259] The computer system **1601** includes a central processing unit (CPU, also “processor” and “computer processor” herein) **1605**, which can be a single core or multi core processor, or a plurality of processors for parallel processing. The computer system **1601** also includes memory or memory location **1610** (e.g., random-access memory, read-only memory, flash memory), electronic storage unit **1615** (e.g., hard disk), communication interface **1620** (e.g., network adapter) for communicating with one or more other systems, and peripheral devices **1625**, such as cache, other memory, data storage and/or electronic display adapters. The memory **1610**, storage unit **1615**, interface **1620** and peripheral devices **1625** are in communication with the CPU **1605** through a communication bus (solid lines), such as a motherboard. The storage unit **1615** can be a data storage unit (or data repository) for storing data. The computer system **1601**

can be operatively coupled to a computer network (“network”) **1630** with the aid of the communication interface **1620**. The network **1630** can be the Internet, an internet and/or extranet, or an intranet and/or extranet that is in communication with the Internet. The network **1630** in some cases is a telecommunication and/or data network. The network **1630** can include one or more computer servers, which can enable distributed computing, such as cloud computing. The network **1630**, in some cases with the aid of the computer system **1601**, can implement a peer-to-peer network, which may enable devices coupled to the computer system **1601** to behave as a client or a server.

[00260] The CPU **1605** 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 **1610**. The instructions can be directed to the CPU **1605**, which can subsequently program or otherwise configure the CPU **1605** to implement methods of the present disclosure. Examples of operations performed by the CPU **1605** can include fetch, decode, execute, and writeback.

[00261] The CPU **1605** can be part of a circuit, such as an integrated circuit. One or more other components of the system **1601** can be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC).

[00262] The storage unit **1615** can store files, such as drivers, libraries and saved programs. The storage unit **1615** can store user data, e.g., user preferences and user programs. The computer system **1601** in some cases can include one or more additional data storage units that are external to the computer system **1601**, such as located on a remote server that is in communication with the computer system **1601** through an intranet or the Internet.

[00263] The computer system **1601** can communicate with one or more remote computer systems through the network **1630**. For instance, the computer system **1601** 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 **1601** via the network **1630**.

[00264] 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 **1601**, such as, for example, on the memory **1610** or electronic storage unit **1615**. 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 **1605**. In some cases, the code can be retrieved from the storage unit **1615** and stored on the memory **1610** for ready access by the processor **1605**. In

some situations, the electronic storage unit **1615** can be precluded, and machine-executable instructions are stored on memory **1610**.

[00265] 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.

[00266] Aspects of the systems and methods provided herein, such as the computer system **1201**, 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.

[00267] 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.

[00268] The computer system **1601** can include or be in communication with an electronic display **1635** that comprises a user interface (UI) **1640** for providing, for example, results of sequencing analysis. Examples of UIs include, without limitation, a graphical user interface (GUI) and web-based user interface.

[00269] 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 **1605**. The algorithm can, for example, perform sequencing.

[00270] 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.

[00271] 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.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for assaying ribonucleic acid (RNA) molecules, comprising:
 - (a) providing a cell that comprises a plurality of cellular analytes, wherein said plurality of cellular analytes comprises ribonucleic acid (RNA) molecules;
 - (b) depleting ribosomal RNA (rRNA) molecules from said RNA molecules, in said cell, to yield a plurality of remaining RNA molecules, wherein said plurality of remaining RNA molecules comprises non-poly-adenylated RNA molecules;
 - (c) fragmenting, in said cell, said plurality of remaining RNA molecules to yield a plurality of RNA fragments, wherein said plurality of RNA fragments comprises a non-poly-adenylated RNA fragment from said non-poly-adenylated RNA molecules;
 - (d) partitioning said cell, after (c), with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of said plurality of nucleic acid barcode molecules comprise a common barcode sequence; and
 - (e) using said non-poly-adenylated RNA fragment and a nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules to generate a barcoded nucleic acid molecule.
2. The method of claim 1, wherein (e) comprises (i) associating said non-poly-adenylated RNA fragment with an RNA molecule hybridized to said nucleic acid barcode molecule, and (ii) performing a nucleic acid reaction, to generate said barcoded nucleic acid molecule.
3. The method of claim 2, wherein said nucleic acid reaction is a nucleic acid extension reaction.
4. The method of claim 2, wherein said barcoded nucleic acid molecule is a barcoded complementary DNA (cDNA) molecule.
5. The method of claim 2, wherein said nucleic acid reaction is performed using an enzyme.
6. The method of claim 5, wherein said enzyme is a thermostable group II intron reverse transcriptase (TGIRT).
7. The method of claim 2, further comprising, subsequent to said nucleic acid reaction, adding an adaptor molecule to said barcoded nucleic acid molecule.
8. The method of claim 7, wherein said adaptor molecule comprises a sequence comprising a 5'-App and 3'-blocker.
9. The method of claim 7, wherein said adaptor molecule is ligated to said barcoded nucleic acid molecule using a 5'-App DNA/RNA ligase.

10. The method of claim 7, wherein said adaptor molecule comprises a splint oligonucleotide.
11. The method of claim 10, wherein said splint oligonucleotide comprises a random N-mer.
12. The method of claim 10, wherein said splint oligonucleotide comprises a 3' blocker.
13. The method of claim 7, wherein said adaptor molecule is ligated to said barcoded nucleic acid molecule using a T4 DNA ligase.
14. The method of claim 1, wherein (e) comprises (i) associating said non-poly-adenylated RNA fragment with said nucleic acid barcode molecule, and (ii) performing a nucleic acid reaction to generate said barcoded nucleic acid molecule.
15. The method of claim 14, wherein said nucleic acid barcode molecules are barcode DNA molecules.
16. The method of claim 14, wherein said nucleic acid reaction is a ligation reaction.
17. The method of claim 16, wherein said ligation reaction is performed using an enzyme.
18. The method of claim 17, wherein said enzyme is a T4KQ ligase.
19. The method of claim 14, further comprising, subsequent to said nucleic acid reaction, adding an adaptor sequence to said barcoded nucleic acid molecule using a reverse transcription reaction.
20. The method of claim 19, wherein said adaptor sequence comprises a sequencing primer sequence.
21. The method of claim 20, wherein said sequencing primer sequence is used for template switching.
22. The method of claim 19, wherein said adaptor sequence comprises a sequencing primer sequence, a reverse transcription primer sequence, and a uridine residue.
23. The method of claim 22, further comprising, subsequent to said reverse transcription reaction, subjecting said barcoded nucleic acid molecule to circularization, clean up, and linearization.
24. The method of claim 23, wherein said circularization is performed using a T4 DNA ligase, a splint oligonucleotide, or a CircLigase.
25. The method of claim 20, further comprising, prior to said reverse transcription, subjecting said barcoded nucleic acid molecule to (i) phosphorylation using a first enzyme, and (ii) ligation of said sequencing primer sequence using a second enzyme.
26. The method of claim 25, wherein said first enzyme is a T4 polynucleotide kinase and said second enzyme is a T4 RNA ligase.
27. The method of claim 1, further comprising subjecting said barcoded nucleic acid molecule to conditions sufficient for amplification and library preparation.

28. The method of claim 27, further comprising sequencing said barcoded nucleic acid molecule or a derivative thereof.
29. The method of claim 1, wherein said cell is a fixed and permeabilized cell.
30. The method of claim 29, wherein said cell is fixed and permeabilized prior to step (a).
31. The method of claim 1, wherein said depleting in (b) comprises subjecting said cell to conditions sufficient to deplete said rRNA molecules.
32. The method of claim 31, wherein said conditions sufficient to deplete said rRNA comprise selective precipitation with lithium chloride, treatment with a RNase, or a pull-down assay.
33. The method of claim 1, further comprising, prior to (a), subjecting said cell to conditions sufficient for fixation.
34. The method of claim 1, wherein said fragmenting comprises a chemical fragmentation process.
35. The method of claim 34, wherein said chemical fragmentation process comprises using divalent metal cations and heating.
36. The method of claim 34, further comprising, subsequent to said chemical fragmentation process, cleaving a 3' phosphate group from said non-poly-adenylated RNA fragment.
37. The method of claim 34, wherein said chemical fragmentation process comprises using a reactive oxygen species.
38. The method of claim 37, wherein said reactive oxygen species comprises hydrogen peroxide.
39. The method of claim 1, wherein said fragmenting comprises an enzymatic fragmentation process.
40. The method of claim 39, wherein said enzymatic fragmentation process comprises RNase III.
41. The method of claim 1, wherein said plurality of nucleic acid barcode molecules comprises a barcode sequence, a unique molecular identifier sequence, and a sequencing primer sequence.
42. The method of claim 1, wherein said non-poly-adenylated RNA molecules comprises miRNA, long non-coding RNA, short non-coding RNA, tRNA, RNA isoforms, snoRNA, small RNA, piRNA, or circular RNA.
43. The method of claim 1, wherein said plurality of RNA fragments further comprises a poly-adenylated RNA fragment.
44. The method of claim 43, further comprising performing (d)-(e) with said poly-adenylated RNA fragment.

45. The method of claim 1, wherein said cell is provided as part of a cell bead.
46. The method of claim 1, wherein said partition is a droplet.
47. The method of claim 1, wherein said partition is a well.
48. The method of claim 1, wherein said plurality of nucleic acid barcode molecules is releasably attached to a bead.
49. The method of claim 48, wherein said bead is a gel bead.
50. The method of claim 48, further comprising releasing said plurality of nucleic acid barcode molecules from said bead using a stimulus.
51. The method of claim 50, wherein said stimulus is a biological, chemical, photo, or thermal stimulus.
52. The method of claim 51, wherein said stimulus is a photo-stimulus which comprises photo-activated cleavage to release said plurality of nucleic acid barcode molecules from said bead.
53. The method of claim 51, wherein said stimulus is a chemical stimulus which comprises a reducing reagent.
54. The method of claim 53, wherein plurality of nucleic acid barcode molecules is releasably attached to said bead via disulfide bonds, and said reducing agent reduces said disulfide bonds to release said plurality of nucleic acid barcodes from said bead.
55. The method of claim 45, wherein said fragmenting occurs in said cell bead.
56. The method of claim 45, wherein said cell bead comprises said cell.
57. The method of claim 45, wherein said cell bead comprises constituents of said cell.
58. The method of claim 45, wherein said cell bead is configured to release said plurality of RNA fragments.
59. The method of claim 31, wherein said conditions sufficient to deplete said rRNA comprise using an enzyme configured for selective digestion.
60. The method of claim 59, wherein said enzyme is an exonuclease.
61. The method of claim 33, further comprising, subjecting said cell to conditions sufficient for permeabilization.
62. The method of claim 1, further comprising subjecting said non-poly-adenylated RNA fragment to conditions sufficient for polyadenylation to generate a polyadenylated RNA fragment from said non-poly-adenylated RNA fragment, wherein (e) comprises using said polyadenylated RNA fragment and said nucleic acid barcode molecule to generate said barcoded nucleic acid molecule.
63. The method of claim 62, wherein said plurality of nucleic acid barcode molecules comprises a capture sequence.

64. The method of claim 63, wherein said capture sequence comprises a poly-T sequence configured to couple to said polyadenylated RNA fragment.
65. The method of claim 62, further comprising, performing a reverse transcription reaction to generate said barcoded nucleic acid molecule.
66. The method of claim 65, wherein said reverse transcription reaction is performed using an adaptor molecule.
67. The method of claim 66, wherein said adaptor molecule comprises a template switching sequence.
68. A method for assaying RNA molecules, comprising:
- (a) providing a cell that comprises a plurality of cellular analytes, wherein said plurality of cellular analytes comprises ribonucleic acid (RNA) molecules, wherein said RNA molecules comprise ribosomal RNA (rRNA) and non-polyadenylated RNA molecules;
 - (b) fragmenting said RNA molecules to yield a plurality of RNA fragments;
 - (c) partitioning said cell after (b) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of said plurality of nucleic acid barcode molecules comprise a common barcode sequence, wherein a nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules is a double-stranded molecule comprising an RNA strand and a deoxyribonucleic acid (DNA) strand; and wherein said DNA strand comprises said common barcode sequence and an overhang sequence; and
 - (d) allowing an RNA fragment of said plurality of RNA fragments to hybridize to said overhang sequence and extending said RNA fragment, thereby generating a barcoded nucleic acid molecule.
69. A method for assaying RNA molecules, comprising:
- (a) providing a cell that comprises a plurality of cellular analytes, wherein said plurality of cellular analytes comprises ribonucleic acid (RNA) molecules;
 - (b) fragmenting said RNA molecules to yield a plurality of RNA fragments;
 - (c) partitioning said cell after (b) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of said plurality of nucleic acid barcode molecules comprise a common barcode sequence and an adaptor sequence; and
 - (d) covalently attaching said RNA fragment to a nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules, thereby generating a barcoded nucleic acid molecule.

70. The method of any one of claims 1, 68, and 69, wherein said plurality of cellular analytes is from a single cell.
71. A method for assaying ribonucleic acid (RNA) molecules, comprising:
- (a) providing a nucleus or cell bead that comprises a plurality of cellular analytes, wherein said plurality of cellular analytes comprises ribonucleic acid (RNA) molecules;
 - (b) depleting rRNA from said nucleus or said cell bead to yield a plurality of remaining RNA molecules, wherein said plurality of remaining RNA molecules comprises non-poly-adenylated RNA molecules;
 - (c) fragmenting said plurality of remaining RNA molecules to yield a plurality of RNA fragments, wherein said plurality of RNA fragments comprises a non-poly-adenylated RNA fragment from said non-poly-adenylated RNA molecules;
 - (d) partitioning said nucleus or cell bead after (c) with a plurality of nucleic acid barcode molecules in a partition, wherein nucleic acid barcode molecules of said plurality of nucleic acid barcode molecules comprise a common barcode sequence; and
 - (e) using said non-poly-adenylated RNA fragment and a nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules to generate a barcoded nucleic acid molecule.
72. The method of claim 71, wherein said fragmenting occurs in said nucleus or cell bead.