

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

[0005a] In one aspect, the present disclosure refers to a method for assaying ribonucleic acid (RNA) molecules, comprising:

- (a) providing a cell, nucleus, or cell bead that comprises ribonucleic acid (RNA) molecules;
- (b) depleting ribosomal RNA (rRNA) molecules from said RNA molecules, in said cell, nucleus, or cell bead, to yield a plurality of remaining RNA molecules;
- (c) fragmenting, in said cell, nucleus, or cell bead, said plurality of remaining RNA molecules to yield a plurality of RNA fragments;

(d) providing a plurality of nucleic acid barcode molecules, wherein nucleic acid barcode molecules of said plurality of nucleic acid barcode molecules comprise a common barcode sequence; and

(e) using a RNA fragment of said plurality of RNA fragments and a nucleic acid barcode molecule of said plurality of nucleic acid barcode molecules to generate a barcoded nucleic acid molecule.

[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 example, 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 examples, (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 examples, the nucleic acid reaction is a nucleic acid extension reaction. In some examples, the barcoded nucleic acid molecule is a barcoded complementary DNA (cDNA) molecule. In some examples, the nucleic acid reaction is performed using an enzyme. In some examples, the enzyme is a thermostable group II intron reverse transcriptase (TGIRT). In some examples, the method further comprises, subsequent to the nucleic acid reaction, adding an adaptor molecule to the barcoded nucleic acid molecule. In some examples, the adaptor molecule comprises a sequence comprising a 5'-App and 3'-blocker. In some examples, the adaptor molecule is ligated to the barcoded nucleic acid molecule using a 5'-App DNA/RNA ligase. In some examples, the adaptor molecule comprises a splint oligonucleotide. In some examples, the splint oligonucleotide comprises a random N-mer. In some examples, the splint oligonucleotide comprises a 3' blocker. In some examples, the adaptor molecule is ligated to the barcoded nucleic acid molecule using a T4 DNA ligase. In

some examples, (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 examples, the nucleic acid barcode molecules are barcode DNA molecules. In some examples, the nucleic acid reaction is a ligation reaction. In some examples, the ligation reaction is performed using an enzyme. In some examples, the enzyme is a T4KQ ligase. In some examples, 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 examples, the adaptor sequence comprises a sequencing primer sequence. In some examples, the sequencing primer sequence is used for template switching. In some examples, the adaptor sequence comprises a sequencing primer sequence, a reverse transcription primer sequence, and a uridine residue. In some examples, the method further comprises, subsequent to the reverse transcription reaction, subjecting the barcoded nucleic acid molecule to circularization, clean up, and linearization. In some examples, the circularization is performed using a T4 DNA ligase, a splint oligonucleotide, or a CircLigase. In some examples, 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 examples, the first enzyme is a T4 polynucleotide kinase and the second enzyme is a T4 RNA ligase. In some examples, the method further comprises subjecting the barcoded nucleic acid molecule to conditions sufficient for amplification and library preparation. In some examples, the method further comprises sequencing the barcoded nucleic acid molecule or a derivative thereof.

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

fragmentation process. In some examples, the enzymatic fragmentation process comprises RNase III. In some examples, the plurality of nucleic acid barcode molecules comprises a barcode sequence, a unique molecular identifier sequence, and a sequencing primer sequence. In some examples, 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 examples, the plurality of RNA fragments further comprises a poly-adenylated RNA fragment. In some examples, the method further comprises performing (d)-(e) with the poly-adenylated RNA fragment. In some examples, the cell is provided as part of a cell bead. In some examples, the partition is a droplet. In some examples, the partition is a well. In some examples, the plurality of nucleic acid barcode molecules is releasably attached to a bead. In some examples, the bead is a gel bead. In some examples, the method further comprises releasing the plurality of nucleic acid barcode molecules from the bead using a stimulus. In some examples, the stimulus is a biological, chemical, photo, or thermal stimulus. In some examples, 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 examples, the stimulus is a chemical stimulus which comprises a reducing reagent. In some examples, 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 examples, the fragmenting occurs in the cell bead. In some examples, the cell bead comprises the cell. In some examples, the cell bead comprises constituents of the cell. In some examples, the cell bead is configured to release the plurality of RNA fragments. In some examples, the conditions sufficient to deplete the rRNA comprise using an enzyme configured for selective digestion. In some examples, the enzyme is an exonuclease. In some examples, the method further comprises subjecting the cell to conditions sufficient for permeabilization.

[0011] In some examples, 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 examples, the plurality of nucleic acid barcode molecules comprises a capture sequence. In some examples, the capture sequence comprises a poly-T sequence configured to couple to the polyadenylated RNA fragment. In some examples, the method further comprises performing a reverse transcription reaction to generate the barcoded nucleic acid molecule. In some examples, the reverse transcription

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

[0012] In another example, 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 example, 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 examples, (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 examples, the nucleic acid reaction is a nucleic acid extension reaction. In some examples, (d) comprises covalently attaching the RNA fragment to the nucleic acid barcode molecule to generate the barcoded nucleic acid molecule. In some examples, the barcoded nucleic acid molecule is a barcoded complementary DNA (cDNA) molecule. In some examples, the nucleic acid reaction is performed using an enzyme. In some examples, the enzyme is a thermostable group II intron reverse transcriptase (TGIRT). In some examples, the method further comprises, subsequent to the nucleic acid reaction, adding an adaptor molecule to the barcoded nucleic acid molecule. In some examples, the adaptor

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

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

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

[0017] In some examples, the method further comprises subjecting the plurality of analytes to conditions sufficient to deplete a nucleic acid species. In some examples, the nucleic acid species is ribosomal RNA (rRNA). In some examples, 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 examples, the plurality of cellular analytes is provided as part of a cell. In some examples, the method further comprises, prior to (a), subjecting the cell to conditions sufficient for fixation.

[0019] In some examples, the fragmenting comprises a chemical fragmentation process. In some examples, 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 examples, the method further comprises, subsequent to the chemical fragmentation process, cleaving a 3' phosphate group from the RNA fragment. In some examples, the chemical fragmentation process comprises using a reactive oxygen species. In some examples, the reactive oxygen species comprises hydrogen peroxide.

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

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

[0022] In some examples, 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 examples, the plurality of RNA fragments comprises an RNA fragment that is poly-adenylated. In some examples, the method further comprises performing (c)-(d) with the RNA fragment that is poly-adenylated.

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

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

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

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

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

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

[0029] In some examples, 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 examples, the plurality of nucleic acid barcode molecules comprises a capture sequence. In some examples, the capture sequence comprises a poly-T sequence configured to couple to the polyadenylated RNA fragment. In some examples, the method further comprises performing a reverse transcription reaction to generate the barcoded nucleic acid molecule. In some examples, the reverse transcription reaction is performed using an adaptor molecule. In some examples, the adaptor molecule comprises a template switching sequence.

[0030] In another example, 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 examples, the fragmenting occurs in the nucleus or cell bead.

[0032] In another example, 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 example, 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 example, 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 examples, the plurality of cellular analytes is from a single cell.

[0036] Another example 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 example 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 examples and advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description, wherein only illustrative examples of the present disclosure are shown and described. As will be realized, the present disclosure is capable of other and different examples, 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.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for assaying ribonucleic acid (RNA) molecules, comprising:
 - (a) providing a cell, nucleus, or cell bead that comprises ribonucleic acid (RNA) molecules;
 - (b) depleting ribosomal RNA (rRNA) molecules from said RNA molecules, in said cell, nucleus, or cell bead, to yield a plurality of remaining RNA molecules;
 - (c) fragmenting, in said cell, nucleus, or cell bead, said plurality of remaining RNA molecules to yield a plurality of RNA fragments;
 - (d) providing a plurality of nucleic acid barcode molecules, wherein nucleic acid barcode molecules of said plurality of nucleic acid barcode molecules comprise a common barcode sequence; and
 - (e) using a RNA fragment of said plurality of RNA fragments 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 RNA fragment with 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 barcode molecule has, hybridized thereto, an RNA molecule, and wherein said nucleic acid reaction is performed using a thermostable group II intron reverse transcriptase (TGIRT).
4. The method of claim 2, further comprising, subsequent to said nucleic acid reaction, adding an adaptor molecule to said barcoded nucleic acid molecule.
5. The method of claim 4, wherein said adaptor molecule comprises a sequence comprising a 5'-App and 3'-blocker.
6. The method of claim 4, wherein said adding comprises ligating said adaptor molecule to said barcoded nucleic acid molecule using a 5'-App DNA/RNA ligase.
7. The method of claim 4, wherein said adaptor molecule comprises a splint oligonucleotide.
8. The method of claim 7, wherein said splint oligonucleotide comprises a random N-mer or a 3' blocker.

9. The method of claim 7, wherein said adding comprises ligating said adaptor molecule to said barcoded nucleic acid molecule using a T4 DNA ligase.
10. The method of claim 2, wherein said nucleic acid reaction is performed using a T4KQ ligase.
11. The method of claim 10, further comprising, subsequent to said nucleic acid reaction, adding an adaptor sequence to said barcoded nucleic acid molecule using a reverse transcription reaction.
12. The method of claim 11, wherein said adaptor sequence comprises a template switching oligonucleotide.
13. The method of claim 11, wherein said adaptor sequence comprises a sequencing primer sequence, a reverse transcription primer sequence, and a uridine residue.
14. The method of claim 13, further comprising, subsequent to said reverse transcription reaction, subjecting said barcoded nucleic acid molecule to circularization, clean up, and linearization.
15. The method of claim 14, wherein said circularization is performed using a T4 DNA ligase, a splint oligonucleotide, or a single-stranded DNA ligase.
16. The method of claim 15, wherein said circularization is performed using said single-stranded DNA ligase, wherein said single-stranded DNA ligase is Circligase.
17. The method of claim 13, 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.
18. The method of claim 17, wherein said first enzyme is a T4 polynucleotide kinase and said second enzyme is a T4 RNA ligase.
19. The method of claim 1, wherein said cell, nucleus, or cell bead is a fixed and permeabilized cell, nucleus or cell bead.
20. The method of claim 1, wherein said fragmenting comprises a chemical fragmentation process or an enzymatic fragmentation process.
21. The method of claim 20, wherein said chemical fragmentation process comprises using divalent metal cations and heating or using a reactive oxygen species.
22. The method of claim 20, further comprising, subsequent to said fragmentation process, cleaving a 3' phosphate group from said RNA fragment.
23. The method of claim 20, wherein said enzymatic fragmentation process comprises using RNase III.

24. The method of claim 1, wherein said plurality of nucleic acid barcode molecules comprises a capture sequence.
25. The method of claim 1, wherein said plurality of remaining RNA molecules comprises non-poly-adenylated RNA molecules, wherein said RNA fragment is a non-poly-adenylated RNA molecule of said non-poly-adenylated RNA molecules.
26. The method of claim 25, 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.
27. The method of claim 1, wherein said plurality of RNA fragments further comprises a poly-adenylated RNA fragment.
28. The method of claim 1, wherein (d) is performed within a partition.
29. The method of claim 28, wherein said partition is a droplet or a well.