METHODS AND SYSTEMS FOR SAMPLE PROCESSING OR ANALYSIS

CROSS-REFERENCE

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/711,994, filed July 30, 2018, which is entirely incorporated herein by reference.

BACKGROUND

[0002] A padlock probe may be a linear circularizable oligonucleotide which has free 5' and 3' ends which are available for ligation, to result in the adoption of a circular conformation. For circularization (e.g., by ligation) to occur, the padlock probe may have a free 5' phosphate group or 5' adenylated end. To allow the juxtaposition of the ends of the padlock probe for ligation, the padlock probe may be configured to have its 5' and 3' terminal regions complementary to its target sequence (e.g., a ribonucleic acid or synthesized complementary deoxyribonucleic acid (cDNA) molecule in the cell sample to be analyzed). These regions of complementarity may allow specific binding of the padlock probe to its target sequence by virtue of hybridization to specific sequences in the target.

SUMMARY

[0003] The present disclosure provides methods and systems for nucleic acid sequence detection in a biological sample having a three-dimensional matrix. The present disclosure also provides methods and systems for sample processing for use in target analysis or detection in a downstream application, such as in situ sequencing.

[0003a] In an aspect of the present disclosure, there is provided a method for identification of a nucleic acid sequence in a biological sample, comprising:

a) providing said biological sample comprising a ribonucleic acid (RNA) molecule hybridized to a deoxyribonucleic acid (DNA) molecule in a three-dimensional (3D) matrix, wherein said RNA molecule comprises said nucleic acid sequence and wherein said DNA molecule comprises an additional nucleic acid sequence that is a reverse complement of said nucleic acid sequence;

b) degrading or digesting at least a portion of said RNA molecule hybridized to said DNA molecule, wherein said at least said portion of said RNA molecule is degraded or digested non-enzymatically at conditions suitable for conducting a click reaction; and

c) detecting said additional nucleic acid sequence, thereby identifying said nucleic acid sequence.

[0003b] In another aspect of the present disclosure, there is provided a method for processing a biological sample, comprising:

(a) providing said biological sample comprising a ribonucleic acid (RNA) molecule in a three-dimensional (3D) matrix, wherein said RNA molecule comprises a nucleic acid sequence;

(b) hybridizing a primer to said RNA molecule, which primer does not include a functional moiety for immobilization to said 3D matrix;

(c) using a reverse transcriptase to reverse transcribe said RNA molecule by extending said primer to generate a complementary deoxyribonucleic acid (cDNA) molecule hybridized to said RNA molecule, which cDNA molecule comprises a functional moiety that immobilizes said cDNA molecule to said 3D matrix;

(d) contacting said cDNA molecule with a probe, which probe comprises an additional functional moiety; and

(e) covalently or ionically coupling said additional functional moiety to said 3D matrix.

[0004] In an example, the disclosure provides a method for identification of a nucleic acid sequence in a biological sample. In one example, the method comprises: (a) providing the biological sample comprising a ribonucleic acid (RNA) molecule hybridized to a deoxyribonucleic acid molecule (DNA) in a three-dimensional (3D) matrix, wherein the RNA molecule comprises the nucleic acid sequence; (b) using a reverse transcriptase to degrade or digest at least a portion of the RNA molecule hybridized to the DNA molecule, which DNA molecule comprises an additional nucleic acid sequence that is a reverse complement of the nucleic acid sequence; and (c) detecting the additional nucleic acid sequence in the biological sample, thereby identifying the nucleic acid sequence.

[0005] In some examples, the DNA molecule is a complementary deoxyribonucleic acid (cDNA) molecule. In some examples, the method further comprises, prior to (a),

using an additional reverse transcriptase to reverse transcribe the RNA molecule to generate the DNA molecule hybridized to the RNA molecule in the biological sample. In some examples, the method further comprises, prior to (a), using the reverse transcriptase to reverse transcribe the RNA molecule to generate the DNA molecule hybridized to the RNA molecule in the biological sample.

[0006] In some examples, the DNA molecule is immobilized to the 3D matrix. In some examples, the DNA molecule comprises a functional moiety, and wherein the DNA molecule is immobilized to the 3D matrix via the functional moiety. In some examples, the RNA molecule is immobilized to the 3D matrix. In some examples, the RNA molecule comprises a functional moiety, and wherein the RNA molecule is immobilized to the 3D matrix via the functional moiety. In some examples, the method further comprises using a matrix-forming material to form the 3D matrix.

[0007] In some examples, (c) comprises contacting the cDNA molecule with a probe. In some examples, the probe comprises a functional moiety, wherein the probe is immobilized to the 3D matrix via the functional moiety. In some examples, the probe is a padlock probe, wherein the padlock probe comprises 5' and 3' terminal regions complementary to the cDNA molecule. In some examples, the method further comprises hybridizing the 5' and 3' terminal regions of the padlock probe to the cDNA molecule. In some examples, the method further comprises circularizing the padlock probe by ligating two ends of the padlock probe together, to yield a circularized padlock probe. In some examples, the two ends of the padlock probe are contiguous. In some examples, the two ends of the padlock probe are separated by a gap region comprising at least one nucleotide. In some examples, the gap region comprises from 2 to 500 nucleotides. In some examples, the method further comprises filling the gap region by incorporating at least one nucleotide in an extension reaction. In some examples, the method further comprises filling the gap region by at least one additional nucleotide or an additional oligonucleotide sequence. In some examples, the additional oligonucleotide sequence is from 2 to 500 nucleotides in length.

[0008] In some examples, the method further comprises subjecting the circularized padlock probe to rolling circle amplification (RCA) to generate an amplification product of a sequence of the circularized padlock probe, which amplification product comprises a nucleic acid sequence corresponding to the nucleic acid sequence of the RNA molecule. In some

examples, the method further comprises detecting the nucleic acid sequence of the amplification product, thereby identifying the nucleic acid sequence of the RNA molecule.

[0009] In some examples, the reverse transcriptase or the additional reverse transcriptase has RNA catalytic cleavage activity. In some examples, the reverse transcriptase or the additional reverse transcriptase has RNA catalytic cleavage activity of an RNA/DNA duplex. In some examples, the reverse transcriptase or the additional reverse transcriptase is an Avian myeloblastosis virus (AMV) reverse transcriptase, a wild type human immunodeficiency virus-1 (HIV-1) reverse transcriptase, or a Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase.

[0010] In some examples, the method further comprises, prior to (a), hybridizing a reverse transcription primer to the RNA molecule. In some examples, the reverse transcription primer is hybridizable to the 5' terminal region of the padlock probe. In some examples, the reverse transcription primer comprises a functional moiety, wherein the cDNA molecule is immobilized to the 3D matrix via the function moiety. In some examples, the biological sample comprises a plurality of RNA molecules, which plurality of RNA molecules has a relative 3D spatial relationship.

[0011] In some examples, (b) is performed under a first set of conditions and using an additional reverse transcriptase or the reverse transcriptase to reverse transcribe the RNA molecule is performed under a second set of conditions, wherein the first set of conditions is different than the second set of conditions. In some examples, the first set of conditions or the second set of conditions is selected from the group consisting of pH, temperature, cofactor concentration, and cation concentration. In some examples, the cofactor or cation comprises Mg^{2+}, Mn^{2+}, Na^{+}, ATP, NADPH. In some examples, the second set of conditions inhibits RNase activity of the reverse transcriptase. In some examples, the second set of conditions comprises an RNase inhibitor. In some examples, the RNase inhibitor is a small molecule inhibitor or a polypeptide.

[0012] In another example, the disclosure provides a method for identification of a nucleic acid sequence in a biological sample. In one example, the method comprises: (a) providing the biological sample comprising a ribonucleic acid (RNA) molecule hybridized to a deoxyribonucleic acid molecule (DNA) in a three-dimensional (3D) matrix, wherein the RNA molecule comprises the nucleic acid sequence; (b) using a deoxyribonucleic acid (DNA) binding protein that is not a reverse transcriptase or a ribonuclease to degrade or digest at least a portion of the RNA molecule hybridized to the DNA molecule, which DNA

molecule comprises an additional nucleic acid sequence that is a reverse complement of the nucleic acid sequence; and (c) detecting the additional nucleic acid sequence in the biological sample, thereby identifying the nucleic acid sequence.

[0013] In some examples, the DNA molecule is a complementary deoxyribonucleic acid (cDNA) molecule. In some examples, the method further comprises, prior to (a), using a reverse transcriptase to reverse transcribe the RNA molecule to generate the DNA molecule hybridized to the RNA molecule in the biological sample.

[0014] In some examples, the DNA molecule is immobilized to the 3D matrix. In some examples, the DNA molecule comprises a functional moiety, and wherein the DNA molecule is immobilized to the 3D matrix via the functional moiety. In some examples, the RNA molecule is immobilized to the 3D matrix. In some examples, the RNA molecule comprises a functional moiety, and wherein the RNA molecule is immobilized to the 3D matrix via the functional moiety. In some examples, the method further comprises using a matrix-forming material to form the 3D matrix.

[0015] In some examples, (c) comprises contacting the cDNA molecule with a probe. In some examples, the probe comprises a functional moiety, wherein the probe is immobilized to the 3D matrix via the functional moiety. In some examples, the probe is a padlock probe, wherein the padlock probe comprises 5’ and 3’ terminal regions complementary to the cDNA molecule, and hybridizing the 5’ and 3’ terminal regions of the padlock probe to the cDNA molecule. In some examples, the method further comprises circularizing the padlock probe by ligating two ends of the padlock probe together, to yield a circularized padlock probe. In some examples, the two ends of the padlock probe are contiguous. In some examples, the two ends of the padlock probe are separated by a gap region comprising at least one nucleotide. In some examples, the gap region comprises from 2 to 500 nucleotides. In some examples, the method further comprises filling the gap region by incorporating at least one nucleotide in an extension reaction. In some examples, the method further comprises filling the gap region by at least one additional nucleotide or an additional oligonucleotide sequence. In some examples, the additional oligonucleotide sequence is from 2 to 500 nucleotides in length.

[0016] In some examples, the method further comprises subjecting the circularized padlock probe to rolling circle amplification (RCA) to generate an amplification product of a sequence of the circularized padlock probe, which amplification product comprises a nucleic acid sequence corresponding to the nucleic acid sequence of the RNA molecule. In some

examples, the method further comprises detecting the nucleic acid sequence of the amplification product, thereby identifying the nucleic acid sequence of the RNA molecule.

[0017] In some examples, the DNA binding protein has RNA catalytic cleavage activity. In some examples, the DNA binding protein stabilizes the DNA molecule. In some examples, the DNA binding protein increases a melting temperature of the DNA molecule. In some examples, the DNA binding protein is Sso7d.

[0018] In some examples, the method further comprises, prior to (a), hybridizing a reverse transcription primer to the RNA molecule. In some examples, the reverse transcription primer is hybridizable to the 5' terminal region of the padlock probe. In some examples, the reverse transcription primer comprises a functional moiety, wherein the reverse transcription primer or the DNA molecule is immobilized to the 3D matrix via the function moiety. In some examples, the biological sample comprises a plurality of RNA molecules, which plurality of RNA molecules has a relative 3D spatial relationship.

[0019] In another example, the disclosure provides a method for identification of a nucleic acid sequence in a biological sample. In one example, the method comprises: (a) providing the biological sample comprising a ribonucleic acid (RNA) molecule hybridized to a deoxyribonucleic acid molecule (DNA) in a three-dimensional (3D) matrix, wherein the RNA molecule comprises the nucleic acid sequence; (b) non-enzymatically degrading at least a portion of the RNA molecule hybridized to the DNA molecule, which DNA molecule comprises an additional nucleic acid sequence that is a reverse complement of the nucleic acid sequence; (c) contacting the DNA molecule with a probe; and (d) detecting a sequence of the probe or a derivative thereof, thereby identifying the nucleic acid sequence of the RNA molecule.

[0020] In some examples, the DNA molecule is a complementary deoxyribonucleic acid (cDNA) molecule. In some examples, the method further comprises, prior to (a), using a reverse transcriptase to reverse transcribe the RNA molecule to generate the DNA molecule hybridized to the RNA molecule in the biological sample. In some examples, the DNA molecule is immobilized to the 3D matrix. In some examples, the DNA molecule comprises a functional moiety, wherein the DNA molecule is immobilized to the 3D matrix via the functional moiety. In some examples, the RNA molecule is immobilized to the 3D matrix. In some examples, the RNA molecule comprises a functional moiety, wherein the RNA molecule is immobilized to the 3D matrix via the functional moiety.

[0021] In some examples, the probe is a padlock probe. In some examples, the probe comprises a functional moiety, wherein the probe is immobilized to the 3D matrix via the functional moiety. In some examples, the method further comprises using a matrix-forming material to form the 3D matrix. In some examples, the padlock probe comprises 5' and 3' terminal regions complementary to the DNA molecule. In some examples, the method further comprises hybridizing the 5' and 3' terminal regions of the padlock probe to the DNA molecule.

[0022] In some examples, the method further comprises circularizing the padlock probe by coupling two ends of the padlock probe together, to yield a circularized padlock probe, and detecting a nucleic acid sequence of the circularized padlock probe or a derivative thereof, thereby identifying the nucleic acid sequence of the RNA molecule. In some examples, the two ends of the padlock probe are contiguous. In some examples, the two ends of the padlock probe are separated by a gap region comprising at least one nucleotide. In some examples, the gap region comprises from 2 to 500 nucleotides. In some examples, the method further comprises filling the gap region by incorporating at least one nucleotide in an extension reaction. In some examples, the method further comprises filling the gap region by at least one additional nucleotide or an additional oligonucleotide sequence. In some examples, the additional oligonucleotide sequence is from 2 to 500 nucleotides in length.

[0023] In some examples, (c) comprises subjecting the circularized padlock probe to rolling circle amplification (RCA) to generate an amplification product of a sequence of the circularized padlock probe, which amplification product comprises a nucleic acid sequence corresponding to the nucleic acid sequence of the RNA molecule. In some examples, (d) comprises detecting the nucleic acid sequence of the amplification product, thereby identifying the nucleic acid sequence of the RNA molecule.

[0024] In some examples, the method further comprises, prior to (a), hybridizing a reverse transcription primer to the RNA molecule. In some examples, the reverse transcription primer is hybridizable to the 5' terminal region of the padlock probe. In some examples, the reverse transcription primer comprises a functional moiety, wherein the DNA molecule is immobilized to the 3D matrix via the function moiety. In some examples, (b) comprises subjecting the RNA molecule to chemical degradation under a condition selected from the group consisting of a pH having value from 6 to 14, a

temperature from 10 °C to 100 °C, in the presence of a heavy metal ion, in the presence of a divalent cation, and any combination thereof.

[0025] In another example, the disclosure provides a method for processing a biological sample. In one example, the method comprises: (a) providing the biological sample comprising a ribonucleic acid (RNA) molecule in a three-dimensional (3D) matrix, wherein the RNA molecule comprises a nucleic acid sequence; (b) hybridizing a primer to the RNA molecule, which primer does not include a functional moiety for immobilization to the matrix; (c) using a reverse transcriptase to reverse transcribe the RNA molecule by extending the primer to generate a complementary deoxyribonucleic acid (cDNA) molecule hybridized to the RNA molecule in the biological sample, which cDNA molecule comprises a functional moiety that immobilizes the cDNA molecule to the 3D matrix.

[0026] In some examples, the method further comprises degrading the RNA molecule hybridized to the cDNA molecule, to provide the cDNA molecule immobilized to the 3D matrix through the functional moiety, which cDNA molecule comprises an additional nucleic acid sequence that is a reverse complement of the nucleic acid sequence. In some examples, the RNA molecule comprises a functional moiety, wherein the RNA molecule is immobilized to the 3D matrix via the functional moiety.

[0027] In some examples, degrading comprises degrading the RNA molecule by a non-ribonuclease enzyme. In some examples, the non-ribonuclease enzyme is a reverse transcriptase or a DNA binding protein. In some examples, degrading comprises degrading the RNA molecule by a non-enzymatic reaction. In some examples, the non-enzymatic reaction is under a condition selected from the group consisting of a pH having value from 6 to 14, a temperature from 10 °C to 100 °C, in the presence of a heavy metal ion, in the presence of a divalent cation, and any combination thereof.

[0028] In some examples, the method further comprises contacting the cDNA molecule with a probe. In some examples, the probe comprises a region that is not hybridizable with the cDNA molecule. In some examples, the probe is a padlock probe, wherein the padlock probe comprises 5’ and 3’ terminal regions complementary to the cDNA molecule, and hybridizing the 5’ and 3’ terminal regions of the padlock probe to the cDNA molecule. In some examples, the method further comprises circularizing the padlock probe by coupling two ends of the padlock probe together, to yield a circularized padlock probe, and detecting a nucleic acid sequence of the circularized padlock probe or a derivative thereof, thereby identifying the nucleic acid sequence of the RNA molecule. In some

examples, the two ends of the padlock probe are contiguous. In some examples, the two ends of the padlock probe are separated by a gap region comprising at least one nucleotide. In some examples, the gap region comprises from 2 to 500 nucleotides. In some examples, the method further comprises filling the gap region by incorporating at least one nucleotide in an extension reaction. In some examples, the method further comprises filling the gap region by at least one additional nucleotide or an additional oligonucleotide sequence. In some examples, the additional oligonucleotide sequence is from 2 to 500 nucleotides in length. In some examples, the method further comprises subjecting the circularized padlock probe to rolling circle amplification (RCA) to generate an amplification product of a sequence of the circularized padlock probe, which amplification product comprises a nucleic acid sequence corresponding to the nucleic acid sequence of the RNA molecule. In some examples, the method further comprises detecting the nucleic acid sequence of the amplification product, thereby identifying the nucleic acid sequence of the RNA molecule. In some examples, the probe comprises a functional moiety, wherein the probe is immobilized to the 3D matrix via the functional moiety. In some examples, the functional moiety is directly conjugated on the probe. In some examples, the probe hybridizes to a tethering oligonucleotide comprising the functional moiety. In some examples, the tethering oligonucleotide hybridizes to the region of the probe that is not hybridizable to the cDNA molecule. In some examples, the method further comprises detecting a sequence of the probe or a derivative thereof, thereby identifying the nucleic acid sequence of the RNA molecule.

[0029] In some examples, (c) comprises using the reverse transcriptase to incorporate a nucleotide analog comprising the functional moiety into a growing strand, to yield the cDNA molecule comprising the nucleotide. In some examples, the nucleotide analog comprises amino-allyl dUTP, 5-TCO-PEG4-dUTP, C8-Alkyne-dUTP, 5-Azidomethyl-dUTP, 5-Vinyl-dUTP, 5-Ethynyl dUTP, or a combination thereof. In some examples, the method further comprises, subsequent to (b), modifying the primer or the cDNA molecule to include the functional moiety. In some examples, the primer is modified to include the functional moiety prior to generating the cDNA molecule. In some examples, the primer comprises a region that is not hybridizable to the RNA molecule, wherein the region hybridizes to an additional tethering oligonucleotide that comprises the functional moiety.

[0030] In some examples, (c) comprises attaching the functional moiety to the cDNA molecule through an enzymatic reaction or a non-enzymatic reaction. In some examples,

the enzymatic reaction comprises using an enzyme to attach a nucleotide or an oligonucleotide having the functional moiety to the cDNA molecule. In some examples, the enzyme is a ligase, a polymerase, or a combination thereof. In some examples, the non-enzymatic reaction comprises attaching a chemical reagent having the functional moiety to the cDNA molecule by alkylation or oxymercuration. In some examples, the cDNA molecule further hybridizes to a tethering oligonucleotide having the functional moiety. In some examples, the tethering oligonucleotide hybridizes to the primer. In some examples, the 3D matrix further comprises an additional functional moiety, which additional functional moiety reacts with the function moiety of the cDNA molecule, thereby immobilizing the cDNA molecule.

[0031] 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.

[0032] 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.

[0033] 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

[0034] 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.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for identification of a nucleic acid sequence in a biological sample, comprising:
   (a) providing said biological sample comprising a ribonucleic acid (RNA) molecule hybridized to a deoxyribonucleic acid (DNA) molecule in a three-dimensional (3D) matrix, wherein said RNA molecule comprises said nucleic acid sequence and wherein said DNA molecule comprises an additional nucleic acid sequence that is a reverse complement of said nucleic acid sequence;
   (b) degrading or digesting at least a portion of said RNA molecule hybridized to said DNA molecule, wherein said at least said portion of said RNA molecule is degraded or digested non-enzymatically at conditions suitable for conducting a click reaction; and
   (c) detecting said additional nucleic acid sequence, thereby identifying said nucleic acid sequence.
2. The method of claim 1, wherein said DNA molecule is immobilized to said 3D matrix.
3. The method of claim 2, wherein said DNA molecule comprises a functional moiety, and wherein said DNA molecule is immobilized to said 3D matrix via said functional moiety.
4. The method of claim 1, wherein said RNA molecule is immobilized to said 3D matrix.
5. The method of claim 4, wherein said RNA molecule comprises a functional moiety, and wherein said RNA molecule is immobilized to said 3D matrix via said functional moiety.
6. The method of claim 1, wherein (c) comprises contacting said DNA molecule with a probe.
7. The method of claim 6, wherein said probe comprises a functional moiety, and wherein said probe is immobilized to said 3D matrix via said functional moiety.
8. The method of claim 6, wherein said probe is a padlock probe, wherein said padlock probe comprises 5’ and 3’ terminal regions complementary to said DNA

molecule, and wherein said 5’ and 3’ terminal regions of said padlock probe are hybridized to said DNA molecule.

9. The method of claim 8, further comprising circularizing said padlock probe by ligating two ends of said padlock probe together, to yield a circularized padlock probe.
10. The method of claim 9, wherein said two ends of said padlock probe are contiguous.
11. The method of claim 9, wherein said two ends of said padlock probe are separated by a gap region comprising at least one nucleotide.
12. The method of claim 11, wherein said gap region comprises from 2 to 500 nucleotides.
13. The method of claim 11, further comprising filling said gap region by incorporating at least one nucleotide in an extension reaction.
14. The method of claim 11, further comprising filling said gap region by at least one additional nucleotide or an oligonucleotide sequence.
15. The method of claim 9, further comprising subjecting said circularized padlock probe to rolling circle amplification (RCA) to generate a nucleic acid molecule using said circularized padlock probe as a template, which nucleic acid molecule comprises a sequence corresponding to said nucleic acid sequence of said RNA molecule.
16. The method of claim 15, further comprising detecting said sequence of said nucleic acid molecule, thereby identifying said nucleic acid sequence of said RNA molecule.
17. The method of claim 1, further comprising, prior to (a), reverse transcribing said RNA molecule to generate said DNA molecule hybridized to said RNA molecule in said biological sample.
18. The method of claim 17, wherein said RNA molecule is reverse transcribed using a reverse transcriptase.
19. The method of claim 17, further comprising, prior to (a), hybridizing a reverse transcription primer to said RNA molecule, wherein said reverse transcription primer comprises a functional moiety, and wherein said DNA molecule is immobilized to said 3D matrix via said functional moiety.

20. The method of claim 17, wherein (b) is performed under a first set of conditions and reverse transcribing said RNA molecule is performed under a second set of conditions, wherein said first set of conditions is different than said second set of conditions.

21. The method of claim 20, wherein said first set of conditions or said second set of conditions is selected from the group consisting of pH, temperature, cofactor concentration, and cation concentration.

22. The method of claim 1, wherein in (b) said at least said portion of said RNA molecule is degraded or digested non-enzymatically in a presence of a metal ion suitable for conducting said click reaction.

23. The method of claim 22, wherein said metal ion is a copper ion.

24. The method of claim 1, wherein said biological sample comprises a plurality of RNA molecules, wherein in (a) said plurality of RNA molecules has a fixed relative 3D spatial relationship.

25. The method of claim 1, wherein (b) comprises degrading said at least said portion of said RNA molecule non-enzymatically by subjecting said at least said portion of said RNA molecule to (1) a pH in a range from 6 to 14, (2) a temperature from 10. °C to 100 °C., (3) a heavy metal ion, or (4) a divalent cation.

26. A method for processing a biological sample, comprising:

(a) providing said biological sample comprising a ribonucleic acid (RNA) molecule in a three-dimensional (3D) matrix, wherein said RNA molecule comprises a nucleic acid sequence;

(b) hybridizing a primer to said RNA molecule, which primer does not include a functional moiety for immobilization to said 3D matrix;

(c) using a reverse transcriptase to reverse transcribe said RNA molecule by extending said primer to generate a complementary deoxyribonucleic acid (cDNA) molecule hybridized to said RNA molecule, which cDNA molecule comprises a functional moiety that immobilizes said cDNA molecule to said 3D matrix;

(d) contacting said cDNA molecule with a probe, which probe comprises an additional functional moiety; and

(e) covalently or ionically coupling said additional functional moiety to said 3D matrix.

27. The method of claim 26, further comprising degrading said RNA molecule hybridized to said cDNA molecule, to provide said cDNA molecule immobilized to said 3D matrix through said functional moiety.

28. The method of claim 26, wherein said probe comprises a sequence region that is not hybridizable with said cDNA molecule.

29. The method of claim 28, wherein said probe hybridizes to a tethering oligonucleotide comprising said additional functional moiety.