CLAIMS

1. A method for analyzing a biological sample, comprising:

(a) contacting the biological sample with:

(i) a first probe comprising a first hybridization region and a first portion of a barcode region, and

(ii) a second probe comprising a second hybridization region and a second portion of the barcode region,

wherein the first and second hybridization regions are complementary to target sequences in a target nucleic acid molecule in the biological sample, and

wherein the barcode region comprises one or more barcode sequences corresponding to the target nucleic acid molecule;

(b) connecting the first and second probes hybridized to the target nucleic acid molecule to form a composite probe by connecting the first and second portions of the barcode region; and

(c) contacting the biological sample with one or more detectable probes that hybridize to the barcode region at sequences corresponding to both the first portion of the barcode region and the second portion of the barcode region, or a complement thereof,

wherein a signal associated with the one or more detectable probes is detected in the biological sample, thereby detecting the target nucleic acid molecule in the biological sample.

2. The method of claim 1, further comprising connecting the first hybridization region and the second hybridization region, wherein the ends of the first hybridization region and the second hybridization region are ligated using enzymatic ligation or chemical ligation without gap filling prior to ligation.

3. The method of claim 1 or claim 2, wherein the ends of the first and second portions of the barcode region are ligated using a splint that hybridizes to (i) the first portion or a subportion thereof and (ii) the second portion or a subportion thereof.

4. The method of claim 3, wherein the method comprises removing the splint comprising a mismatch with (i) the first portion or a subportion thereof and/or (ii) the second portion or a subportion thereof, whereas under the same conditions, the splint complementary to both (i) the first portion or a subportion thereof and (ii) the second portion or a subportion thereof remains hybridized to the barcode region for subsequent ligation.

5. The method of any of claims 1-4, wherein the first portion of the barcode region comprises one, two, or more barcode sequences, and/or wherein the second portion of the barcode region comprises one, two, or more barcode sequences.

6. The method of any of claims 1-5, wherein the barcode region comprises two or more adjacent barcode sequences.

7. The method of any of claims 1-6, wherein the barcode region comprises two or more non-overlapping barcode sequences and/or two or more overlapping barcode sequences.

8. The method of any of claims 1-7, wherein the method further comprises performing an extension reaction using the composite probe as template to generate the complement of the barcode region.

9. The method of claim 8, wherein the method further comprises circularizing the composite probe and generating a rolling circle amplification (RCA) product of the circularized composite probe, wherein the RCA product comprises multiple copies of the complement of the barcode region.

10. The method of any of claims 1-9, wherein the one or more detectable probes comprise: (i) a detectable label; or (ii) a sequence complementary to a sequence of a detectably labelled probe.

11. The method of any of claims 1-10, wherein the target nucleic acid molecule comprises an RNA.

12. The method of any of claims 1-11, wherein the composite probe or a product thereof is generated *in situ* in the biological sample and/or in a matrix embedding the biological sample or molecules thereof.

13. The method of any of claims 1-12, wherein the composite probe or a product thereof is immobilized in the biological sample and/or in a matrix embedding the biological sample or molecules thereof.

14. The method of any of claims 1-13, wherein the method comprises imaging the biological sample to detect the signal associated with the one or more detectable probes.

15. A method for analyzing a biological sample, comprising:

(a) contacting the biological sample with:

(i) a first probe comprising a first hybridization region and a first barcode sequence, and

(ii) a second probe comprising a second hybridization region and a second barcode sequence,

wherein the first and second hybridization regions are complementary to adjacent target sequences in a target RNA molecule in the biological sample, and

wherein the first and second barcode sequences each corresponds to the target RNA molecule;

(b) ligating the first and second probes hybridized to the target RNA molecule to form a circular probe, wherein the ligating comprises:

(i) ligating the first and second hybridization regions hybridized to the adjacent target sequences using the target RNA molecule as a template, and

(ii) ligating the first and second probes to form a composite probe comprising the first and second barcode sequences corresponding to the target RNA molecule; and

(c) generating a rolling circle amplification product of the composite probe;

(d) contacting the biological sample with a detectable probe that hybridizes to the composite probe or a complement thereof in the rolling circle amplification product at sequences corresponding to both the first and second barcode sequences,

wherein a signal associated with the detectable probe is detected in the biological sample, thereby detecting the target RNA molecule in the biological sample.

16. The method of claim 15, wherein the first probe and/or the second probe further comprise one or more additional barcode sequences which alone or in combination correspond to the target RNA molecule, optionally wherein the method further comprises contacting the biological sample with an additional detectable probe that hybridizes to any one or more of the additional barcode sequences.

17. The method of claim 15 or claim 16, comprising:

contacting the biological sample with the detectable probe which is an intermediate probe, wherein the intermediate probe is detectable using a detectably-labeled probe, and

dehybridizing the intermediate probe and/or the detectably-labeled probe,

wherein the contacting and dehybridizing steps are repeated with the intermediate probe, the detectably-labeled probe, one or more other intermediate probes, and/or one or more other detectably-labeled probes.

18. The method of any of claims 1-17, wherein the biological sample is a formalin-fixed, paraffin-embedded (FFPE) sample, a frozen tissue sample, or a fresh tissue sample.

19. The method of any of claims 1-18, wherein the biological sample includes one or more diseased cells.

20. A kit for analyzing a biological sample, comprising:

(a) a first probe comprising a first hybridization region and a first portion of a barcode sequence, and

(b) a second probe comprising a second hybridization region and a second portion of the barcode sequence,

wherein the first and second hybridization regions are complementary to adjacent target sequences in a target RNA molecule, and the barcode sequence corresponds to the target RNA molecule, and

wherein the kit comprises:

(c) a splint that hybridizes to the first and second probes for ligation of the first and second portions to form a composite probe comprising the barcode sequence,

optionally wherein: (i) the first and second portions of the barcode sequence are no more than 10 nucleotides in length, and the barcode sequence is at least 15 nucleotides in length, and/or (ii) the splint is single-stranded.