CLAIMS

WHAT IS CLAIMED IS:

1. A method for multiplexed nucleic acid assays, comprising:

a. contacting a cell with a first probe, a second probe, and a third probe under conditions sufficient to generate a first probe-associated molecule and a second probe-associated molecule, wherein said cell comprises (i) a nucleic acid molecule comprising a first target region and a second target region and (ii) a feature coupled to a feature binding group,

wherein said feature binding group comprises (i) a reporter oligonucleotide associated with said feature and (ii) a feature probe binding sequence,

wherein said first probe comprises (i) a first probe sequence complementary to said first target region and (ii) a probe capture sequence,

wherein said second probe comprises a second probe sequence complementary to said second target region,

wherein said third probe comprises a (i) third probe sequence complementary to said feature probe binding sequence and (ii) said probe capture sequence

b. in a first partition of a first set of partitions, contacting said first probe-associated molecule and said second probe-associated molecule to probe binding molecules and barcode molecules under conditions sufficient to generate a first barcoded nucleic acid molecule and a second barcoded nucleic acid molecule,

wherein said barcode molecules comprise (i) a common sequence common to a plurality of barcode molecules comprising said barcode molecules and (ii) a first barcode sequence common to said first partition of said first set of partitions,

wherein said probe binding molecule comprises (i) a probe binding sequence complementary to said probe capture sequence and (ii) a barcode binding sequence complementary to said common sequence; and

c. in a second partition of a second set of partitions, (i) contacting said first barcoded nucleic acid molecule, or derivative thereof, to a first capture molecule of a plurality of capture molecules under conditions sufficient to generate a third barcoded nucleic acid molecule, and (ii) contacting said second barcoded nucleic

acid molecule, or derivative thereof, to a second capture molecule of said plurality of capture molecules under conditions sufficient to generate a fourth barcoded nucleic acid molecule, wherein said plurality of capture molecules comprise a second barcode sequence,

wherein each of said third barcoded nucleic acid molecule and said fourth barcoded molecule comprises a sequence corresponding to said first barcode sequence and a sequence corresponding to said second barcode sequence.

2. The method of claim 1, wherein said first target region and said second target region are on a same strand of said nucleic acid molecule.

3. The method of claim 1 or 2, wherein said probe capture sequence is common to a plurality of first probes including said first probe, wherein one or more additional partitions of said first set of partitions comprise one or more additional probe-associated nucleic acid molecules, wherein each of said one or more additional probe-associated nucleic acid molecules comprises said probe capture sequence.

4. The method of any one of claims 1-3, wherein said second probe comprises a second probe capture sequence complementary to a capture sequence of said plurality of capture molecules, and wherein (c) comprises hybridizing said second probe capture sequence to said capture sequence.

5. The method of any one of claims 1-4, wherein said barcode molecules comprise a capture binding sequence complementary to a capture sequence of said plurality of capture molecules, and wherein (c) comprises hybridizing said capture binding sequence to said capture sequence.

6. The method of any one of claims 1-5, wherein said first set of partitions are a plurality of wells.

7. The method of any one of claims 1-6, wherein said second set of partitions are a plurality of droplets.

8. The method of any one of claims 1-6, wherein said second set of partitions are a plurality of wells.

9. The method of any one of claims 1-8, wherein said plurality of capture molecules is coupled to a particle.

10. The method of claim 9, wherein said particle is a bead.

11. The method of claim 10, wherein said bead is a gel bead.

12. The method of claim 11, wherein each capture molecule of said plurality of capture molecules coupled to said gel bead comprises said second barcode sequence.

13. The method of claim 12, wherein one or more additional partitions of said second set of partitions comprise one or more additional gel beads of a plurality of gel beads, and wherein said second barcode sequence is unique to said gel bead among said plurality of gel beads.

14. The method of claim 12, wherein a capture molecule of said plurality of capture molecules comprises a third barcode sequence unique to said capture molecule among said plurality of capture molecules.

15. The method of any one of claims 1-14, wherein one or more additional partitions of said second set of partitions comprise one or more additional capture molecules, and wherein said second barcode sequence is unique to said second partition among said second set of partitions.

16. The method of any one of claims 1-15, wherein (a) comprises hybridizing said first probe and said second probe to said first target region and said second target region, respectively.

17. The method of any one of claims 1-16, further comprising subjecting said first probe-associated molecule to conditions sufficient to yield a probe-linked nucleic acid molecule comprising said first probe linked to said second probe.

18. The method of claim 17, wherein said probe-linked nucleic acid molecule is generated via chemical or enzymatic ligation of said first probe and said second probe.

19. The method of claim 17, wherein said chemical or enzymatic ligation occurs subsequent to (b).

20. The method of any one of claims 1-19, wherein said first target region and said second target region are adjacent.

21. The method of any one of claims 1-19, wherein said first target region and said second target region are non-adjacent, and the method further comprises (i) extending said first probe or said second probe annealed to said first target region or said second target region, respectively, towards the second target region or said first target region, respectively, to generate an extended probe, and (ii) ligating said extended probe to said second probe or said first probe, respectively.

22. The method of any one of claims 1-21, wherein (a) comprises contacting said first probe and said second probe to said nucleic acid molecule inside said cell.

23. The method of claim 22, wherein said first partition comprises a plurality of cells.

24. The method of any one of claims 1-23, wherein said cell is permeabilized.

25. The method of any one of claims 1-24, wherein said cell is fixed.

26. The method of claim any one of claims 1-25, further comprising releasing said first probe-associated molecule, or derivative thereof, from said cell.

27. The method of claim 26, wherein said releasing comprises lysing said cell.

28. The method of any one of claims 1-27, wherein said reporter oligonucleotide comprises said feature probe binding sequence.

29. The method of any one of claims 1-28, further comprising, subsequent to (b) and prior to (c), pooling said first barcoded nucleic acid molecule, said second barcoded nucleic acid molecule, additional first barcoded nucleic acid molecules from said first set of partitions, and additional second barcoded nucleic acid molecules form said first set of partitions.

30. The method of any one of claims 1-28, further comprising, subsequent to (c) and prior to sequencing, pooling said third barcoded nucleic acid molecule, said fourth barcoded nucleic acid molecule, additional third barcoded nucleic acid molecules from said second set of partitions, and additional fourth barcoded nucleic acid molecules from said second set of partitions.

31. The method of claim any one of claims 1-30, wherein said probe capture sequence is from 8 to 50 bp.

32. A method, comprising:

a. contacting a nucleic acid molecule with a first probe to generate a probe-associated nucleic acid molecule, wherein said nucleic acid molecule comprises a first target region and a second target region not adjacent to said first target region, wherein said first probe comprises a first probe sequence complementary to said first target region

b. extending said first probe under conditions sufficient to generate an extended probe molecule comprising a sequence complementary to said second target region;

c. in a partition of a plurality of partitions, providing said extended probe molecule, a second probe, and a barcode molecule and a probe binding molecule, under conditions sufficient to generate a barcoded molecule,

wherein said second probe comprises a second probe sequence corresponding to said second target region,

wherein said first probe or said second probe comprises a probe capture sequence,

wherein said barcode molecule comprises (i) a barcode capture sequence and (ii) a barcode sequence

wherein said probe binding molecule comprises (i) a probe binding sequence complementary to said probe capture sequence and (ii) a barcode binding sequence complementary to said barcode capture sequence,

wherein said barcoded molecule comprises a sequence corresponding to said first target region, a sequence corresponding to said second target region, a sequence corresponding to said probe capture sequence, and a sequence corresponding to said barcode sequence

33. The method of claim 32, wherein (c) comprises ligating said second probe and said barcode molecule

34. The method of claim 33, wherein said ligating comprises chemical or enzymatic ligation

35. The method of any one of claims 32-34 , wherein said particle is a bead.

36. The method of claim 35, wherein said bead is a gel bead.

37. The method of any one of claims 32-36, wherein said cell is permeabilized.

38. The method of any one of claims 32-37, wherein said cell is fixed.

39. The method of any one of claims 32-38, wherein said first probe comprises said probe capture sequence.

40. The method of any one of claims 32-39, further comprising, subsequent to (b), releasing said extended probe molecule from said nucleic acid molecule.

41. The method of claim 40, wherein said releasing comprises freeing a ribonucleic acid (RNA) strand using RNase

42. The method of claim 40, wherein said releasing comprises heat cycling

43. The method of any one of claims 32-42, wherein (c) comprises (i) hybridizing said second probe sequence of said second probe to said sequence complementary to said second target region and (ii) extending said second probe

44. The method of any one of claims 32-43, wherein in (c) said barcode molecule and said probe binding molecule are provided as a pre-annealed complex, wherein said barcode capture sequence is annealed to said barcode binding sequence in said pre-annealed complex

45. The method of any one of claims 32-44, wherein said first probe comprises said probe capture sequence, wherein (c) comprises (i) annealing said probe binding sequence and said barcode binding sequence to said probe capture sequence and said barcode capture sequence, respectively (ii) ligating said barcode molecule and said extended probe molecule, to generate a first barcoded molecule and (iii) annealing said second probe to said first barcoded molecule and initiating an extension reaction to generate said barcoded molecule.

46. The method of claim 45, wherein (ii) and (iii) are preformed outside said partition

47. The method of any one of claims 32-44, wherein said second probe comprises said probe capture sequence, wherein (c) comprises (i) annealing said second probe to said extended probe molecule and initiating an extension reaction to generate an extension molecule, (ii) annealing

said probe binding sequence and said barcode binding sequence to said probe capture sequence and said barcode capture sequence, and (iii) ligating said barcode molecule and said extension molecule.

481. A method of analyzing a samplecell, comprising:

(a). providing a sample comprising a cell, wherein the cell comprises:

(i) a feature binding group, bound to at least a portion of said sample, wherein said feature binding group comprises a reporter oligonucleotide, wherein said reporter oligonucleotide comprises a reporter barcode sequence, a first target region and a second target region, wherein said first target region and said second target region are disposed on a same strand of said reporter oligonucleotide wherein the feature comprises a protein, a peptide, a lipid, or a carbohydrate; and

(ii) a first probe comprising a first probe sequence, wherein said first probe sequence of said first probe is complementary to said first target region of said reporter oligonucleotide; andnucleic acid molecule; and

(iii) a second probe comprising a second probe sequence, wherein said second probe sequence of said second probe is complementary to said second target region of said reporter oligonucleotide;

(b). subjecting said sample to conditions sufficient to (i) hybridize said first probe sequence of said first probe to said first target region of said reporter oligonucleotide, and (ii) hybridize said second probe sequence of said second probe to said second target region of said reporter oligonucleotide to yield a probe-associated reporter oligonucleotide complexcontacting the cell with a feature binding group and coupling the feature binding group to the feature, wherein the feature binding group comprises an oligonucleotide comprising a feature nucleic acid sequence that identifies the feature binding group; and

(c). subjecting said probe-associated reporter oligonucleotide complex to conditions sufficient to yield a probe-linked nucleic acid molecule comprising said first probe linked to said second probehybridizing a first nucleic acid probe to the nucleic acid molecule; wherein the first nucleic acid probe comprises (i) a hybridizing sequence that hybridizes to a first target sequence of the nucleic acid molecule and (ii) a non-hybridizing sequence.;

(d) using the feature nucleic acid sequence and a first barcode molecule comprising a first barcode sequence to generate a first barcoded nucleic acid molecule comprising (i) the feature nucleic acid sequence or reverse complement thereof, and (ii) the first barcode sequence or reverse complement thereof; and

(e) using the non-hybridizing sequence and a second barcode molecule comprising a second barcode sequence to generate a second barcoded nucleic acid molecule comprising (i) the first target sequence or reverse complement thereof and (ii) the second barcode sequence or reverse complement thereof.

~~49. The method of claim 48, further comprising, (d) attaching a barcode molecule to said probe-linked nucleic acid molecule.~~

~~50. The method of claim 48 or 49, wherein (d) occurs in a partition.~~

~~51. The method of claim 50, wherein said partition is a droplet or a well.~~

~~52. The method of claim 49, wherein said sample comprises a nucleic acid molecule, and wherein (d) further comprises: attaching an additional barcode molecule to said nucleic acid molecule or derivative thereof.~~

~~53~~2. The method of ~~any one of claims 48-52~~claim 1, wherein ~~said~~ the feature-binding group ~~is~~ comprises an antibody.

~~54. The method of any one of claims 48-53, wherein said first probe or said second probe comprises an additional probe sequence.~~

~~55. The method of claim 54, further comprising, attaching a barcode sequence to said additional probe sequence.~~

~~56. The method of claim 54, further comprising, (d) providing a barcode molecule and a probe binding molecule comprising (i) a first sequence complementary to said additional probe sequence and (ii) a second sequence complementary to a capture sequence of said barcode molecule.~~

~~57. The method of claim 56, further comprising, providing conditions sufficient to hybridize said first sequence to said additional probe sequence and said second sequence to said capture sequence of said barcode molecule, thereby generating a barcoded probe-associated complex.~~

~~58. The method of claim 57, wherein (d) occurs in a partition among a plurality of partitions.~~

~~59. The method of claim 58, further comprising, pooling said barcoded probe-associated complex from said partition with other barcoded probe-associated complexes from other partitions of said plurality of partitions to generate a pooled set of barcoded probe-associated complexes.~~

~~60. The method of claim 59, further comprising, (i) partitioning said pooled set of barcoded probe-associated complexes into a plurality of additional partitions, wherein an additional partition of said plurality of additional partitions comprises said barcoded probe-associated complex and an additional barcode molecule comprising an additional barcode sequence and (ii) attaching said additional barcode molecule to said barcoded probe-associated complex.~~

~~61. The method of claim 60, wherein said additional barcode molecule is coupled to a bead.~~

~~62. The method of claim 61, wherein said bead is a gel bead.~~

~~63. The method of claim 61, wherein said additional barcode molecule is releasably coupled to said bead.~~

~~64. The method of claim 56, wherein (d) occurs prior to (c).~~

~~65. The method of claim 56, wherein (d) occurs subsequent to (c).~~

~~66. The method of any one of claims 48-65, wherein said at least said portion of said sample comprises a feature.~~

~~67. The method of claim 66, wherein said feature is a protein.~~

~~683~~. The method of claim ~~671~~, wherein ~~said protein is~~ the feature comprises a cell surface receptor or an intracellular protein.

~~69. The method of any one of claims 48-68, wherein said sample comprises a cell or cell bead.~~

~~70. The method of claim 69, wherein said cell is a formalin-fixed, paraffin-embedded cell.~~

4. The method of claim 1, further comprising, prior to or during (c), permeabilizing the cell.

5. The method of claim 1, further comprising, prior to (d), providing a splint molecule, wherein the splint molecule comprises a first splint sequence and a second splint sequence, wherein a first portion of the first barcode molecule hybridizes to the first splint sequence in the splint molecule, and a second portion of the oligonucleotide hybridizes to the second splint sequence on the splint molecule.

6. The method of claim 5, wherein the first splint sequence and the second splint sequence are adjacent on the splint molecule.

7. The method of claim 5, wherein the first splint sequence and the second splint sequence are separated by a gap region of at least 1 nucleotide on the splint molecule.

8. The method of claim 5, wherein (d) comprises linking the first barcode molecule and the oligonucleotide together to generate the first barcoded nucleic acid molecule.

9. The method of claim 1, wherein the first barcode sequence in the first barcode molecule identifies the sample.

10. The method of claim 1, further comprising (f) using the first barcoded nucleic acid molecule and a third barcode molecule comprising a third barcode sequence to generate a third barcoded nucleic acid molecule comprising (i) the feature nucleic acid sequence or reverse complement thereof, (ii) the first barcode sequence or reverse complement thereof; and (iii) the third barcode sequence or reverse complement thereof.

11. The method of claim 10, wherein (f) occurs in a partition among a plurality of partitions.

12. The method of claim 11, wherein the third barcode sequence identifies the partition from other partitions of the plurality of partitions.

13. The method of claim 10, wherein (f) comprises hybridizing a first capture sequence in the third barcode molecule to a first capture binding sequence in the first barcoded nucleic acid molecule; and extending the third barcode molecule to generate the third barcoded nucleic acid molecule.

14. The method of claim 13, wherein the third barcode molecule is coupled to a bead.

15. The method of claim 10, further comprising detecting the third barcoded nucleic acid molecule, thereby identifying (i) the feature nucleic acid sequence, (ii) the first barcode sequence; and (iii) the third barcode sequence.

16. The method of claim 1, wherein the nucleic acid molecule is an RNA transcript.

17. The method of claim 1, wherein (c) further comprises hybridizing a second nucleic acid probe to a second target sequence of the nucleic acid molecule.

18. The method of claim 17, wherein, in (c), wherein the first target sequence and the second target sequence are adjacent on the nucleic acid molecule.

19. The method of claim 17, wherein, in (c), the first target sequence and the second target sequence are separated by a gap region of at least 1 nucleotide on the nucleic acid molecule.

20. The method of claim 17, further comprising, linking the first nucleic acid probe and the second nucleic acid probe together, thereby generating a linked nucleic acid molecule comprising (i) the first nucleic acid probe and (ii) the second nucleic acid probe.

21. The method of claim 20, wherein the linked nucleic acid molecule comprises a fourth barcode sequence that identifies the sample.

22. The method of claim 21, wherein the fourth barcode sequence is the same as the first barcode sequence.

23. The method of claim 21, wherein (e) comprises using the non-hybridizing sequence in the linked nucleic acid molecule and the second barcode molecule to generate the second barcoded nucleic acid molecule, wherein the second barcoded nucleic acid molecule comprises (i) the first target sequence or reverse complement thereof, (ii) the second barcode sequence or reverse complement thereof, and (iii) the fourth barcode sequence or reverse complement thereof.

~~71~~24. The method of ~~any one of claims 48-69~~claim 1, ~~wherein (c)~~ wherein (e) occurs in a partition among a plurality of partitions.

25. The method of claim 24, wherein the second barcode sequence identifies the partition among the plurality of partitions.

~~72. The method of claim 71, wherein said partition is among a plurality of partitions.~~

~~73. The method of any one of claims 48-72, wherein (c) comprises enzymatic or chemical ligation.~~

~~74. The method of claim 73, wherein said ligation is performed in the absence of adenosine triphosphate.~~

~~75. The method of any one of claims 48-74, wherein said first probe or said second probe comprises an adenylated end, a phosphorylated end, a ribonucleotide, a dideoxynucleotide, or a flap sequence.~~

~~76. The method of any one of claims 48-75, wherein said first target region and said second target region are separated by a gap region disposed between said first target region and said second target region.~~

~~77. The method of claim 76, wherein (c) comprises performing an extension reaction to fill said gap region to yield said probe-linked nucleic acid molecule.~~

~~78. The method of claim 76, wherein (c) comprises providing a third probe comprising a third probe sequence complementary to said gap region, hybridizing said third probe sequence to said gap region, and providing conditions sufficient to yield said probe-linked nucleic acid molecule comprising said first probe linked to said second probe via said third probe.~~

26. The method of claim 23, wherein (e) comprises hybridizing a second capture sequence in the second barcode molecule to a second capture binding sequence in the non-hybridizing sequence of the first nucleic acid probe; and extending the second barcode molecule to generate the second barcoded nucleic acid molecule.

27. The method of claim 26, wherein the second barcode molecule is coupled to a bead.

28. The method of claim 23, further comprising detecting the second barcoded nucleic acid molecule, thereby identifying (i) the first target sequence, (ii) the second barcode sequence, and (iii) the fourth barcode sequence.

29. The method of claim 1, wherein (d) is performed in a partition among a plurality of partitions.

30. The method of claim 29, wherein the first barcode sequence identifies the partition from other partitions of the plurality of partitions.