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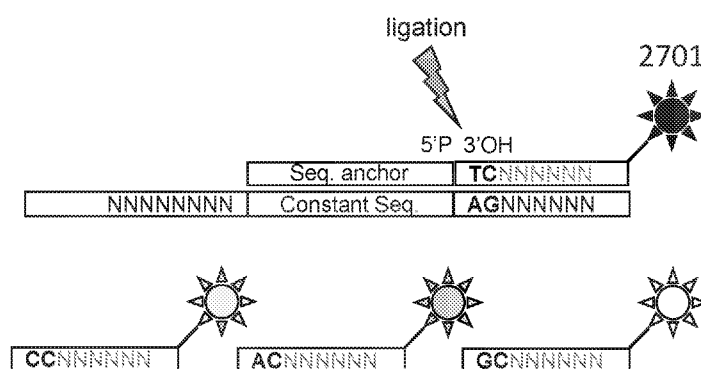


FIG. 23

(57) **Abstract:** Methods for determining a location of a feature in a spatial array of features include: providing a spatial array including a plurality of features on a substrate, where a feature of the plurality of features has a capture probe, and where the capture probe has a spatial barcode and a constant sequence; hybridizing a first sequencing probe to the spatial barcode, where the first sequencing probe has a first label and a first nucleotide sequence; obtaining a first image of the first label with the first sequencing probe hybridized to the spatial barcode; determining, based on the first image, a first portion of a sequence of the spatial barcode; and associating the feature with a location in the spatial array based on a location of the first label in the first image.



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1. A method for determining a location of a feature in a spatial array of features, the method comprising:

(a) providing a spatial array comprising a plurality of features on a substrate, wherein a feature of the plurality of features comprises a capture probe, and wherein the capture probe comprises a spatial barcode;

(b) hybridizing a first sequencing probe to the spatial barcode, wherein the first sequencing probe comprises a first label and a first nucleotide sequence;

(c) obtaining a first image of the first label with the first sequencing probe hybridized to the spatial barcode;

(d) determining, based on the first image, a first portion of a sequence of the spatial barcode;

(e) hybridizing a second sequencing probe to the spatial barcode, wherein the second sequencing probe comprises a second label and a second nucleotide sequence different from the first nucleotide sequence;

(f) obtaining a second image of the second label with the second sequencing probe hybridized to the spatial barcode;

(g) determining, based on the second image, a second portion of the sequence of the spatial barcode; and

(h) associating the feature with a location in the spatial array based on a location of the first label in the first image.

2. The method of claim 1, further comprising repeating steps (e)-(g) with one or more additional sequencing probes, each additional sequencing probe comprising a label and a nucleotide sequence different from the first and second nucleotide sequences and nucleotide sequences of other additional sequencing probes, until the sequence of the spatial barcode is completely determined.

3. The method of any one of claims 1-2, wherein one or more features of the plurality of features comprises a plurality of capture probes.
4. The method of claim 1, further comprising repeating steps (a)-(h) for multiple features in the spatial array, each of the multiple features comprising a capture probe comprising a spatial barcode, to associate each of the multiple features with a location in the spatial array.
5. The method of claim 2, further comprising, for multiple features in the spatial array each comprising a capture probe comprising a spatial barcode, determining the complete sequence of the capture probe of each multiple feature, and associating each of the multiple features with a location in the spatial array.
6. The method of claim 3, wherein for each capture probe associated with one of the features and comprising a spatial barcode, the spatial barcode is unique relative to spatial barcodes of capture probes associated with other features in the spatial array.
7. The method of any one of claims 1-6, wherein the capture probe further comprises a constant sequence comprising a sequence for initiating a sequencing reaction.
8. The method of claim 7, further comprising hybridizing a sequencing anchor to the constant sequence prior to hybridizing the first sequencing probe to the spatial barcode.
9. The method of any one of claims 1-8, further comprising amplifying at least a portion of the capture probe before determining the first and second portions of the sequence of the spatial barcode.
10. The method of claim 9, further comprising amplifying the at least a portion of the capture probe using isothermal or non-isothermal amplification.

11. The method of claim 2, wherein determining the complete sequence of the spatial barcode comprises sequentially hybridizing each of three or more sequencing probes to the spatial barcode.
12. The method of claim 2, wherein determining the complete sequence of the spatial barcode comprises sequentially hybridizing each of ten or more sequencing probes to portions of the spatial barcode.
13. The method of claim 2, wherein the spatial barcode comprises N nucleic acids, and determining the complete sequence of the spatial barcode comprises sequentially hybridizing N different sequencing probes to the spatial barcode.
14. The method of claim 2, wherein the spatial barcode comprises N nucleic acids, and determining the complete sequence of the spatial barcode comprises sequentially hybridizing fewer than N different sequencing probes to the spatial barcode.
15. The method of any one of claims 1-2, wherein the first and second sequencing probes hybridize to a common portion of the spatial barcode.
16. The method of claim 15, wherein the common portion of the spatial barcode comprises the entire spatial barcode.
17. The method of any one of claims 11-14, wherein each of the sequencing probes hybridizes to a common portion of the spatial barcode.
18. The method of claim 17, wherein the common portion of the spatial barcode comprises the entire spatial barcode.
19. The method of claim 7 or 8, further comprising, prior to hybridizing the first sequencing probe to the spatial barcode, hybridizing a sequencing anchor to at least a portion of the constant

sequence, wherein the sequencing anchor comprises a sequence that is complementary to at least a portion of the constant sequence.

20. The method of claim 19, wherein hybridizing the first sequencing probe to the spatial barcode comprises:

contacting the spatial barcode with a composition comprising the first sequencing probe and a first set of three auxiliary sequencing probes, wherein the first sequencing probe and each member of the first set of three auxiliary sequencing probes each comprises a known, different nucleic acid at a first common sequence position and a known, different label; and

coupling the first sequencing probe to a free 5' end of the sequencing anchor.

21. The method of claim 20, wherein determining the first portion of the sequence of the spatial barcode comprises identifying the nucleic acid at the first common sequence position of the first sequencing probe based on the first image of the first label, and identifying the first portion of the sequence of the spatial barcode as a nucleic acid complementary to the nucleic acid at the first common sequence position of the first sequencing probe.

22. The method of claim 21, wherein hybridizing the second sequencing probe to the spatial barcode comprises:

contacting the spatial barcode with a composition comprising the second sequencing probe and a second set of three auxiliary sequencing probes, wherein the second sequencing probe and each member of the second set of three auxiliary sequencing probes each comprises a known, different nucleic acid at a second common sequence position and a known, different label; and

coupling the second sequencing probe to a free 5' end of the sequencing anchor or to a free 5' end of at least a portion of the first sequencing probe.

23. The method of claim 22, wherein determining the second portion of the sequence of the spatial barcode comprises identifying the nucleic acid at the second common sequence position of the second sequencing probe based on the second image of the second label, and identifying

the second portion of the sequence of the spatial barcode as a nucleic acid complementary to the nucleic acid at the second common sequence position of the second sequencing probe.

24. The method of claim 23, wherein the second common sequence position is different from the first common sequence position.

25. The method of claim 23, further comprising removing all of the first sequencing probe from the spatial barcode, and coupling the second sequencing probe to the free 5' end of the sequencing anchor.

26. The method of claim 23, further comprising removing a portion of the first sequencing probe from the spatial barcode, and coupling the second sequencing probe to a free 5' end of a portion of the first sequencing probe that remains hybridized to the spatial barcode.

27. The method of any one of claims 20-26, further comprising coupling the first sequencing probe to the free 5' end of the sequencing anchor using a ligation reaction.

28. The method of any one of claims 22-27, further comprising coupling the second sequencing probe to the free 5' end of the sequencing anchor or to the free 5' end of at least a portion of the first sequencing probe using a ligation reaction.

29. The method of any one of claims 1-28, wherein the first sequencing probe comprises a first nucleic acid sequence having a 3' end and a 5' end, and wherein a known nucleic acid is located at a first sequence position relative to the 3' end of the first nucleic acid sequence.

30. The method of claim 29, wherein each of the nucleic acids in the first nucleic acid sequence other than the known nucleic acid is a universal base.

31. The method of any one of claims 29-30, wherein a length of the first nucleic acid sequence is the same as a length of the spatial barcode.

32. The method of any one of claims 29-31, wherein the second sequencing probe comprises a second nucleic acid sequence having a 3' end and a 5' end, and wherein a known nucleic acid is located at a second sequence position relative to the 3' end of the second nucleic acid sequence.

33. The method of claim 32, wherein each of the nucleic acids in the second nucleic acid sequence other than the known nucleic acid is a universal base.

34. The method of claim 33, wherein a length of the second nucleic acid sequence is the same as the length of the spatial barcode.

35. The method of claim 32, wherein each of the first and second sequencing probes comprises a label coupled to the 5' end of the first and second nucleic acid sequences, respectively.

36. The method of any one of claims 1-35, wherein the first sequencing probe comprises a first nucleic acid sequence having a 3' end and a 5' end, and wherein known nucleic acids are located at two sequence positions of the first nucleic acid sequence.

37. The method of claim 36, wherein the two sequence positions are first and second sequence positions relative to the 3' end of the first nucleic acid sequence.

38. The method of any one of claims 36-37, wherein each of the nucleic acids in the first nucleic acid sequence other than the known nucleic acids is a universal base.

39. The method of any one of claims 36-38, wherein a length of the first nucleic acid sequence is the same as a length of the spatial barcode.

40. The method of any one of claims 36-39, wherein the second sequencing probe comprises a second nucleic acid sequence having a 3' end and a 5' end, and wherein known nucleic acids are located at two sequence positions of the second nucleic acid sequence.
41. The method of claim 40, wherein the two sequence positions are third and fourth positions relative to the 3' end of the second nucleic acid sequence.
42. The method of any one of claims 40-41, wherein each of the nucleic acids in the second nucleic acid sequence other than the known nucleic acids is a universal base.
43. The method of any one of claims 40-42, wherein a length of the second nucleic acid sequence is the same as the length of the spatial barcode.
44. The method of any one of claims 1-43, wherein the label is selected from the group consisting of an optical label, a radioactive label, a fluorescent label, an enzymatic label, a chemiluminescent label, a bioluminescent label, or a dye.
45. The method of any one of claims 1-44, further comprising, prior to hybridizing the second sequencing probe to the spatial barcode, removing at least a portion of the first sequencing probe from the spatial barcode.
46. The method of claim 45, wherein removing the at least a portion of the first sequencing probe from the spatial barcode comprises cleaving the at least a portion of the first sequencing probe to generate a free 5' end capable of serving as substrate in a ligation reaction.
47. The method of any one of claims 45-46, wherein the removed at least a portion of the first sequencing probe comprises the first label.
48. The method of any one of claims 45-47, further comprising cleaving the at least a portion of the first sequencing probe with a nuclease.

49. The method of any one of claims 1-48, wherein the first sequencing probe comprises a nuclease recognition site.

50. The method of any one of claims 6-8, further comprising removing the sequencing anchor after determining the second portion of the sequence of the spatial barcode, or after the sequence of the spatial barcode is completely determined.

51. The method of any one of claims 1-50, wherein the capture probe further comprises a capture domain.

52. The method of claim 51, wherein the capture probe comprises, from a 5' end to a 3' end, the spatial barcode and the capture domain.

53. The method of any one of claims 1-52, wherein the spatial barcode is a first spatial barcode, and wherein the capture probe further comprises a second spatial barcode different from the first spatial barcode.

54. The method of claim 53, wherein the capture probe comprises, from a 5' end to a 3' end, the second spatial barcode, and the first spatial barcode.

55. The method of any one of claims 53-54, wherein the capture probe further comprises a capture domain.

56. The method of claim 55, wherein the capture probe comprises, from a 5' end to a 3' end, the second spatial barcode, the first spatial barcode, and the capture domain.

57. The method of any one of claims 53-56, further comprising determining at least a portion of a sequence of the second spatial barcode.

58. The method of claim 55, wherein determining the at least a portion of the sequence of the second spatial barcode comprises:

(i) hybridizing a third sequencing probe to the second spatial barcode, wherein the third sequencing probe comprises a third label and a third nucleotide sequence;

(j) obtaining a third image of the third label with the third sequencing probe hybridized to the second spatial barcode; and

(k) determining, based on the third image, a first portion of a sequence of the second spatial barcode.

59. The method of claim 58, further comprising repeating steps (i)-(k) with one or more additional sequencing probes, each additional sequencing probe comprising a label and a nucleotide sequence different from the third nucleotide sequence and nucleotide sequences of other additional sequencing probes, until the sequence of the second spatial barcode is completely determined.

60. The method of claim 58, wherein hybridizing the third sequencing probe to the second spatial barcode comprises:

contacting the spatial barcode with a composition comprising the third sequencing probe and a third set of three auxiliary sequencing probes, wherein the third sequencing probe and each member of the third set of three auxiliary sequencing probes each comprises a known, different nucleic acid at a first common sequence position and a known, different label; and

coupling the third sequencing probe to a free 3' end of the sequencing anchor.

61. The method of claim 60, wherein determining the first portion of the sequence of the second spatial barcode comprises identifying the nucleic acid at the first common sequence position of the third sequencing probe based on the third image of the third label, and identifying the first portion of the sequence of the second spatial barcode as a nucleic acid complementary to the nucleic acid at the first common sequence position of the third sequencing probe.

62. The method of any one of claims 58-61, wherein the third sequencing probe comprises a nucleic acid sequence having a 5' end and a 3' end, wherein a known nucleic acid is located at first sequence position relative to the 5' end.

63. The method of claim 62, wherein the third sequencing probe comprises universal bases at positions other than the first sequence position relative to the 5' end.

64. The method of any one of claims 62-63, wherein the third sequencing probe comprises a label coupled to the 3' end of the nucleic acid sequence.

65. A method for associating an analyte of a biological sample with a spatial location in the biological sample, the method comprising:

providing a spatial array comprising a plurality of features on a substrate, wherein a feature of the plurality of features comprises a capture probe, and wherein the capture probe comprises a spatial barcode, a constant sequence, and a capture domain;

determining a sequence of the spatial barcode and associating the feature with a location in the spatial array;

capturing an analyte of a biological sample with the capture domain; and

determining a location of the captured analyte in the biological sample based on the location of the feature in the spatial array,

wherein determining the sequence of the spatial barcode and associating the feature with the location in the spatial array comprises:

(a) hybridizing a sequencing probe to the spatial barcode, wherein the sequencing probe comprises a label and a nucleotide sequence;

(b) obtaining an image of the label with the sequencing probe hybridized to the spatial barcode;

(c) determining, based on the image, a portion of a sequence of the spatial barcode;

(d) repeating steps (a)-(c) until the sequence of the spatial barcode is completely determined; and

(e) associating the feature with a location in the spatial array based on a location of the label in the image.

66. The method of claim 65, wherein the capture domain can hybridize to a nucleic acid sequence present on or associated with the analyte.

67. The method of any one of claims 65-66, wherein capturing the analyte of the biological sample with the capture domain comprises contacting the spatial array with the biological sample and allowing the analyte to interact with the capture domain.

68. The method of any one of claims 65-67, wherein capturing the analyte of the biological sample with the capture domain comprises releasing the capture probe from the spatial array and contacting the biological sample with the released capture probe.

69. The method of any one of claims 65-68, wherein the analyte comprises DNA or RNA.

70. The method of any one of claims 65 and 67-68, wherein the analyte comprises a protein.

71. A method for preparing a spatial array, the method comprising:

(a) providing a substrate comprising a plurality of oligonucleotides attached to a surface of the substrate, wherein an oligonucleotide of the plurality of oligonucleotides comprises, from a 5' end to a 3' end, a spatial barcode, a constant sequence, and a capture domain;

(b) determining a location of the spatial barcode in the array and a sequence of the spatial barcode;

(c) attaching a feature comprising a plurality of capture probes to the substrate by coupling the feature to the oligonucleotide; and

(d) associating the feature with a location in the spatial array based on the location of the spatial barcode in the array.

72. The method of claim 71, wherein determining the location of the spatial barcode in the array and the sequence of the spatial barcode comprises:

(a) hybridizing a sequencing probe to the spatial barcode, wherein the sequencing probe comprises a label and a nucleotide sequence;

(b) obtaining an image of the label with the sequencing probe hybridized to the spatial barcode;

(c) determining, based on the image, a portion of a sequence of the spatial barcode;

(d) repeating steps (a)-(c) until the sequence of the spatial barcode is determined; and

(e) determining a location of the spatial barcode based on a location of the label in the image.

73. The method of claim 71, wherein the spatial barcode of the oligonucleotide is a first spatial barcode, and wherein one or more of the capture probes comprises a second spatial barcode.

74. The method of claim 73, wherein the first and second spatial barcodes are the same.

75. The method of claim 73, wherein the first and second spatial barcodes are different.

76. The method of any one of claims 71-75, wherein the constant sequence comprises a sequence for initiating a sequencing reaction.

77. The method of claim 72, further comprising hybridizing a sequencing anchor to at least a portion of the constant sequence prior to hybridizing the sequencing probe to the spatial barcode.

78. The method of any one of claims 71-77, further comprising amplifying a portion of one of the plurality of capture probes.

79. The method of claim 78, further comprising amplifying the portion of one of the plurality of capture probes using isothermal or non-isothermal amplification.

80. The method of any one of claims 71-79, wherein a capture probe of the plurality of capture probes comprises a universal molecular identifier (UMI).
81. The method of any one of claims 71-80, wherein a capture probe of the plurality of capture probes comprises a cleavage domain.
82. The method of any one of claims 71-81, wherein:
the capture probe comprises a primer sequence; and
the oligonucleotide comprises a template sequence.
83. The method of claim 82, wherein the primer sequence comprises a reverse transcriptase primer.
84. The method of any one of claims 82-83, wherein the template sequence comprises a sequence for a reverse transcription assay.
85. The method of claim 72, wherein the spatial barcode comprises N nucleic acids, and determining the sequence of the spatial barcode comprises sequentially hybridizing N different sequencing probes to the spatial barcode.
86. The method of claim 72, wherein the spatial barcode comprises N nucleic acids, and determining the sequence of the spatial barcode comprises sequentially hybridizing fewer than N different sequencing probes to the spatial barcode.
87. The method of claim 72, wherein each of the sequencing probes hybridizes to a common portion of the spatial barcode.
88. The method of claim 87, wherein the common portion of the spatial barcode comprises the entire spatial barcode.

89. The method of claim 72, wherein hybridizing the sequencing probe to the spatial barcode comprises:

contacting the spatial barcode with a composition comprising the sequencing probe and a set of three auxiliary sequencing probes, wherein the sequencing probe and each member of the set of three auxiliary sequencing probes each comprises a known, different nucleic acid at a common sequence position and a known, different label; and

coupling the sequencing probe to a free 5' end of the sequencing anchor or to a free 5' end of a portion of another sequencing probe.

90. The method of claim 72, wherein determining the portion of the sequence of the spatial barcode comprises identifying the nucleic acid at the common sequence position based on the image of the label, and identifying the portion of the sequence of the spatial barcode as a nucleic acid complementary to the nucleic acid at the common sequence position of the sequencing probe.

91. The method of claim 89, further comprising removing all of the sequencing probe from the spatial barcode prior to hybridizing another sequencing probe to the spatial barcode.

92. The method of claim 89, further comprising removing a portion of one sequencing probe from the spatial barcode, and coupling a successive sequencing probe to a free 5' end of a portion of the one sequencing probe that remains hybridized to the spatial barcode.

93. The method of claim 89, further comprising coupling the sequencing probe to the free 5' end of the sequencing anchor or to the free 5' end of the portion of another sequencing probe using a ligation reaction.

94. The method of claim 72, wherein the sequencing probe comprises a nucleic acid sequence having a 3' end and a 5' end, and wherein a known nucleic acid is located at a first sequence position relative to the 3' end of the first nucleic acid sequence.

95. The method of claim 94, wherein each of the nucleic acids in the nucleic acid sequence other than the known nucleic acid is a universal base.

96. The method of claim 94, wherein a length of the nucleic acid sequence is the same as a length of the spatial barcode.

97. The method of claim 72, wherein the label is selected from the group consisting of an optical label, a radioactive label, a fluorescent label, an enzymatic label, a chemiluminescent label, a bioluminescent label, or a dye.

98. The method of claim 92, further comprising further comprising removing the portion of the one sequencing probe from the spatial barcode by cleaving the one sequencing probe with a nuclease.

99. The method of claim 98, wherein the one sequencing probe comprises a nuclease recognition site.

100. The method of any one of claims 71-99, further comprising:
capturing an analyte of a biological sample with a capture probe of the plurality of capture probes; and
determining a location of the captured analyte in the biological sample based on the location of the feature in the spatial array.

101. The method of claim 100, wherein the capture probe comprises a capture domain that can hybridize to a nucleic acid sequence present on or associated with the analyte.

102. The method of any one of claims 100-101, wherein capturing the analyte of the biological sample with the capture probe comprises contacting the spatial array with the biological sample and allowing the analyte to interact with the capture probe.

103. The method of any one of claims 100-101, wherein capturing the analyte of the biological sample with the capture probe comprises releasing the capture probe from the spatial array and contacting the biological sample with the released capture probe.

104. The method of any one of claims 100-103, wherein the analyte comprises DNA or RNA.

105. The method of claim 100, wherein the analyte comprises a protein.

106. A method of determining a location of a feature in a spatial array of features, the method comprising:

- (a) providing a spatial array comprising:
 - a plurality of a first set of features on a substrate, wherein a feature of the plurality of the first set of features comprises a first probe, and wherein the first probe comprises a first spatial barcode and a first capture domain; and
 - a plurality of a second set of features on the substrate, wherein a feature of the plurality of the second set of features comprises a second probe, and wherein the second probe comprises a second spatial barcode and a second capture domain;
- (b) determining the sequence of the first spatial barcode based on the method of any one of claims 1-64, thereby determining the location of a feature of the plurality of the first set of features on the substrate;
- (c) coupling the first capture domain of the first probe to the second capture domain of the second probe, thereby producing an oligonucleotide comprising the first spatial barcode of the first probe and the second spatial barcode of the second probe;
- (d) determining all or a portion of the sequence of the oligonucleotide or a complement thereof; and
- (e) associating the second spatial barcode of the second probe with the first spatial barcode of the first probe having a known location on the substrate, thereby determining the location of a feature of the plurality of the second set features on the substrate.

107. The method of claim 106, wherein the coupling comprises proximity ligation.
108. The method of claim 107, wherein the proximity ligation comprises ligating together the first capture probe and the second capture probe.
109. The method of any one of claims 106-108, wherein the coupling comprises a splint oligonucleotide.
110. The method of claim 109, wherein the splint oligonucleotide comprises a sequence that is at least partially complementary to the first capture domain and a sequence that is at least partially complementary to the second capture domain.
111. The method of any one of claims 106-110, wherein the first probe comprises a constant sequence.
112. The method of any one of claims 106-111, wherein the first and second spatial barcodes are different.