

CLAIMS

1. A method for analyzing a biological sample, comprising:
 - (a) contacting a biological sample with a circular probe or circularizable probe or probe set that hybridizes to a target nucleic acid in the biological sample,
wherein the circular probe or circularizable probe or probe set comprises a first hybridizing region and a second hybridizing region capable of hybridizing to each other;
 - (b) generating a rolling circle amplification product (RCP) at a location in the biological sample using the circular probe or a circularized probe generated from the circularizable probe or probe set as a template,
wherein the RCP comprises multiple copies of complementary sequences of the first and second hybridizing regions, and
wherein the complementary sequences of the first and second hybridizing regions from different copies hybridize to one another, thereby compacting and/or stabilizing the RCP at the location; and
 - (c) detecting the RCP at the location in the biological sample.
2. The method of claim 1, wherein the first and second hybridizing regions are complementary to each other.
3. The method of claim 1 or 2, wherein the first and second hybridizing regions are adjacent and form a palindromic sequence.
4. The method of any of claims 1-3, wherein the circular probe or circularizable probe or probe set further comprises a third hybridizing region and a fourth hybridizing region capable of hybridizing to each other.
5. The method of claim 4, wherein the first and second hybridizing regions are different in sequence from the third and fourth hybridizing regions, respectively.
6. The method of claim 4 or 5, wherein the first and second hybridizing regions do not hybridize to the third or fourth hybridizing region.
7. The method of claim 4, wherein the first and second hybridizing regions are the same in sequence as the third and fourth hybridizing regions, respectively.
8. The method of any of claims 1-7, wherein the circular probe or circularizable probe or probe set further comprises one or more barcode sequences.

9. The method of any of claims 1-8, wherein the first and second hybridizing regions are linked by a loop region.
10. The method of claim 9, wherein the loop region is no more than 5, no more than 10, no more than 15, no more than 20, no more than 25, or no more than 30 nucleotides in length.
11. The method of any of claims 1-10, wherein the melting temperature of a duplex of the first and second hybridizing regions is in thermodynamic equilibrium with the temperature at which the RCP is generated.
12. The method of claim 11, wherein the melting temperature is between about 15°C and about 45°C and/or wherein the temperature at which the RCP is generated is between about 15°C and about 45°C.
13. The method of claim 11 or 12, wherein the melting temperature is about 30°C, and the RCP is generated at about 30°C.
14. The method of any of claims 1-13, wherein the RCP is:
- i) a product of an endogenous nucleic acid molecule in the biological sample; or
 - ii) a product of a labelling agent that directly or indirectly binds to a target nucleic acid in the biological sample.
15. The method of any of claims 1-14, wherein the target nucleic acid is cDNA or mRNA.
16. The method of any of claims 1-15, wherein the RCP is immobilized in the biological sample.
17. The method of any of claims 1-16, wherein the detecting comprises imaging the biological sample using fluorescent microscopy.
18. The method of any one of claims 1-17, wherein a sequence of the RCP is analyzed at the location in the biological sample by sequential hybridization, sequencing by hybridization, sequencing by ligation, sequencing by synthesis, sequencing by binding, or a combination thereof.
19. A method for analyzing a biological sample, comprising:
- (a) contacting the biological sample with a padlock probe comprising a sequence that hybridizes to a target nucleic acid in the biological sample,

wherein the padlock probe comprises:

(i) a first hybridizing region and a second hybridizing region capable of hybridizing to each other, and

(ii) one or more barcode sequences,

wherein the first and second hybridizing regions are linked by a loop region, optionally wherein the loop region comprises the one or more barcode sequences, and optionally wherein the target nucleic acid is a cDNA or an mRNA;

(b) circularizing the padlock probe to generate a circularized padlock probe using the target nucleic acid/or a splint as template, and generating a rolling circle amplification product (RCP) at a location in the biological sample using the circularized padlock probe as a template,

wherein the RCP comprises multiple copies of complementary sequences of the first and second hybridizing regions, and

wherein the complementary sequences of the first and second hybridizing regions from different copies hybridize to one another, thereby compacting and/or stabilizing the RCP at the location in the biological sample; and

(c) detecting the RCP at the location in the biological sample.

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

(a) contacting the biological sample with a padlock probe comprising a sequence that hybridizes to a target nucleic acid in the biological sample,

wherein the padlock probe comprises:

(i) a first hybridizing region and a second hybridizing region capable of hybridizing to each other, wherein the first and second hybridizing regions are linked by a first loop region;

(ii) a third hybridizing region and a fourth hybridizing region capable of hybridizing to each other, wherein the third and fourth hybridizing regions are linked by a second loop region; and

(iii) one or more barcode sequences in the first and/or second loop regions;

(b) circularizing the padlock probe to generate a circularized padlock probe using the target nucleic acid/or a splint as template, and generating a rolling circle amplification

product (RCP) at a location in the biological sample using the circularized padlock probe as a template,

wherein the RCP comprises multiple copies of complementary sequences of the first, second, third, and fourth hybridizing regions, and

wherein the complementary sequences of the first and second hybridizing regions from two or more different copies hybridize to one another and the complementary sequences of the third and fourth hybridizing regions from two or more different copies hybridize to one another, thereby compacting and/or stabilizing the RCP at the location in the biological sample; and

(c) detecting the RCP at the location in the biological sample.

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

(a) contacting the biological sample with a padlock probe comprising a sequence that hybridizes to a target nucleic acid in the biological sample,

wherein the padlock probe comprises:

(i) a first hybridizing region and a second hybridizing region capable of hybridizing to each other, wherein the first and second hybridizing regions are linked by a loop region; and

(ii) one or more barcode sequences;

(b) circularizing the padlock probe to generate a circularized padlock probe using the target nucleic acid/or a splint as template, and generating a rolling circle amplification product (RCP) at a location in the biological sample using the circularized padlock probe as a template,

wherein the RCP comprises multiple copies of a unit sequence comprising complementary sequences of the first and second hybridizing regions, and

wherein the complementary sequence of the first hybridizing region from a first copy hybridizes to the complementary sequence of the second hybridizing region from a second copy, and the complementary sequence of the second hybridizing region from the first copy hybridizes to the complementary sequence of the first hybridizing region from the second copy, thereby compacting and/or stabilizing the RCP at the location in the biological sample; and

- (c) detecting the RCP at the location in the biological sample.
22. A method for analyzing a biological sample, comprising:
- (a) contacting a biological sample with a circular probe or circularizable probe or probe set that hybridizes to a target nucleic acid in the biological sample,
- wherein the circular probe or circularizable probe or probe set comprises a palindromic sequence;
- (b) generating a rolling circle amplification product (RCP) at a location in the biological sample using the circular probe or a circularized probe generated from the circularizable probe or probe set as a template,
- wherein the RCP comprises multiple copies of a unit sequence comprising a complementary sequence of the palindromic sequence, and
- wherein the complementary sequences of the palindromic sequence from different copies of the unit sequence hybridize to one another, thereby compacting and/or stabilizing the RCP at the location; and
- (c) detecting the RCP at the location in the biological sample.
23. The method of claim 21 and 22, wherein hybridization between sequences in different copies of the unit sequence is favored over hybridization between sequences in the same copy of the unit sequence.
24. The method of any one of claims 21-23, wherein the melting temperature of a duplex formed between sequences in different copies of the unit sequence is higher than the melting temperature of a duplex formed between sequences in the same copy of the unit sequence.
25. The method of any one of claims 1-24, wherein the biological sample is a cell or tissue sample.
26. The method of any one of claims 1-25, wherein the biological sample is a tissue section.
27. The method of any one of claims 1-26, wherein the biological sample is embedded in a hydrogel and cleared.
28. The method of any of claims 19-27, wherein in the circularized padlock probe, the first and second hybridizing regions linked by the loop region form a hairpin structure,

wherein the first and second hybridizing regions form a duplex stem region of the hairpin structure.

29. The method of claim 28, wherein the loop region is about four nucleotides in length, and the duplex stem region is between about 10 and about 40 base pairs.

30. The method of claim 29, wherein in addition to the hairpin structure, the circularized padlock probe further comprises a sequence between about 50 and about 120 nucleotides in length.

31. The method of any of claims 19-27, wherein the circularized padlock probe forms a dumbbell structure, wherein the first and second hybridizing regions form a duplex stem region of the dumbbell structure flanked by the first loop region and a second loop region.

32. The method of claim 31, wherein the first loop region is between about 15 and about 60 nucleotides in length, the second loop region is between about 30 and about 60 nucleotides in length, and the duplex stem region is between about 5 and about 30 base pairs.