

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

10X GENOMICS, INC.,
Petitioner

v.

PRESIDENT AND FELLOWS OF HARVARD COLLEGE,
Patent Owner

Case No. IPR2023-01299
U.S. Patent No. 11,098,303

DECLARATION OF PAUL T. SPELLMAN, PH.D.

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I, Paul T. Spellman, hereby declare as follows:

I. INTRODUCTION

1. I am over the age of 18 and competent to make this declaration.

2. I have been retained as an expert witness on behalf of 10x Genomics, Inc. (“10x”) for the above-captioned *inter partes* review (IPR) against President and Fellows of Harvard College’s U.S. Patent No. 11,098,303 (“the 303 patent”) (EX1001). I understand that Vizgen, Inc. is the exclusive licensee of the 303 patent. I understand that this Declaration accompanies 10x’s Petition for the IPR. I am being compensated for my time in connection with this IPR.

3. I understand that the 303 patent resulted from U.S. Patent Application No. 15/329,683, which is a National Stage Entry of PCT Appl. No. PCT/US2015/042556, filed on July 29, 2015, which claims benefit to the following provisional applications:

- Provisional Appl. No. 62/142,653 (EX1053), filed on April 3, 2015;
- Provisional Appl. No. 62/050,636 (EX1054), filed September 15, 2014;
- and
- Provisional Appl. No. 62/031,062 (EX1055), filed July 30, 2014.

4. I understand July 30, 2014, is the 303 patent’s earliest alleged priority date. I refer to this date throughout this Declaration.

5. In preparing for this Declaration, I have reviewed the 303 patent (EX1001) and each of the documents cited herein, in light of the general knowledge in the art before July 30, 2014. The documents cited herein are provided as exhibits and each exhibit is a true and correct copy of what it purports to be and contain the types of facts or data an expert in the field would reasonably rely on when forming opinions.

6. In formulating my opinions, I have relied upon my experience, education, and knowledge in the relevant art. In formulating my opinions, I have also considered the viewpoint of a person of ordinary skill in the art (“POSA”), as defined below in Section IV.a before July 30, 2014.

II. SUMMARY OF MY OPINIONS

7. I have been asked to consider whether, before July 30, 2014, U.S. Patent No. 10,829,814 (“Fan”; EX1005) anticipated the challenged claims of the 303 patent. I understand that Fan was filed before July 30, 2014, and thus it qualifies as prior art. For the reasons I explain under Ground 1, in view of the state of the art before July 30, 2014, Fan teaches every element of claims 1-12, arranged as claimed. I understand that Fan is presumed enabled. Thus, for the reasons detailed under Ground 1, Fan anticipated claims 1-12 of the 303 patent under Harvard/Vizgen’s interpretation in a related district court litigation.

8. I have been asked to consider whether, before July 30, 2014, Fan together with Gunderson *et al.*, *Genome Research* 14:870-877 (2004) (“Gunderson”; EX1007) would have rendered the challenged claims of the 303 patent obvious. For the reasons I explain under Ground 2, in view of the state of the art before July 30, 2014, Fan and Gunderson teach or suggest all elements of the methods of claims 1, 3, and 6-12. Further, as I explain under Ground 2, a POSA would have been motivated to perform the claimed methods with a reasonable expectation of success. Thus, for the reasons detailed under Ground 2, Fan and Gunderson would have rendered claims 1, 3, and 6-12 of the 303 patent obvious under Harvard/Vizgen’s interpretation in a related district court litigation.

9. I have been asked to consider whether, before July 30, 2014, Fan together with Gunderson and Larsson *et al.*, *Nat. Methods* 7(5):395-397 (2010) (“Larsson”; EX1008) would have rendered the challenged claims of the 303 patent obvious. I understand that Gunderson and Larsson were both published before July 30, 2014, and thus they qualify as prior art. For the reasons I explain under Ground 3, in view of the state of the art before July 30, 2014, Fan, Gunderson, and Larsson teach or suggest all the elements of the methods of claims 2, 4, and 5. Further, as I explain under Ground 3, a POSA would have been motivated to perform the claimed methods with a reasonable expectation of success. Thus, for the reasons detailed under Ground 3, Fan, Gunderson, and Larsson would have rendered

claims 2, 4, and 5 of the 303 patent obvious under Harvard/Vizgen's interpretation in a related district court litigation.

10. I have been asked to consider whether, before July 30, 2014, Fan together with U.S. Patent No. 8,765,642 ("Curry"; EX1009) would have rendered the challenged claims of the 303 patent obvious. I understand that Curry was both filed and issued before July 30, 2014, and thus it qualifies as prior art. For the reasons I explain under Ground 4, in view of the state of the art before July 30, 2014, Fan and Curry teach or suggest all the elements of the methods of claims 1-12. Further, as I explain under Ground 4, a POSA would have been motivated to perform the claimed methods with a reasonable expectation of success. Thus, for the reasons detailed under Ground 4, Fan and Curry would have rendered claims 1-12 of the 303 patent obvious under Harvard/Vizgen's interpretation in a related district court litigation.

III. MY BACKGROUND AND QUALIFICATIONS

11. I am a Professor of Medicine and Human Genetics in the David Geffen School of Medicine at the University of California, Los Angeles.

12. I have over 20 years of experience in the field of genetics and genomics, including approaches for detecting or monitoring human diseases, such as spatial detection of target molecules. I also have over 15 years of experience in cancer genomics.

13. My current research focuses on computational biology, cancer biology, and cancer genomics. My lab develops technologies and approaches to understand the process by which cancer develops, monitor disease, and identify therapeutic strategies. Within this broad area of research, I have specific experience in using population genetics to help determine who is at risk for cancer, how to computationally analyze genomic data to identify early changes in cancers, and developing new genomic methodologies including those focused on analyzing single cells and the use of DNA barcodes that help identify individual molecules. I have extensive experience in the fields of gene expression profiling by hybridization or sequencing. I also have experience in software engineering principles, codes, and error detection methodologies implemented in molecular biology.

14. I have provided a copy of my *curriculum vitae* as EX1003.

15. I graduated with a Bachelor's degree in Biology from the Massachusetts Institute of Technology in 1995. I then received my Ph.D. in Genetics from Stanford University Medical School in 2000. My Ph.D. thesis topic was "Generating and Analyzing Genome Scale Data."

16. From 2000-2003, I was a postdoctoral fellow in the Department of Molecular and Cellular Biology at the University of California, Berkeley. As a postdoctoral fellow, I was selected for the prestigious National Science Foundation

Biocomputing Fellowship, which covered both my stipend and significant research costs.

17. After my postdoctoral fellowship, I became a Staff Scientist at the Life Science Division of Lawrence Berkeley Lab (2007-2011) and served as a Special Assistant to the Deputy Director of the National Cancer Institute (2011).

Subsequently, I served as an Associate Professor (2011-2013) and as a Full Professor with Tenure (2013-2023) in the Department of Molecular and Medical Genetics at Oregon Health & Science University. In 2023, I was recruited to the University of California, Los Angeles as a Professor of Medicine and Human Genetics.

18. I have authored more than 115 peer-reviewed publications that discuss methods of nucleic acid manipulation and analysis, including nucleic acid sample preparation for hybridization, sequencing, imaging of hybridized nucleic acids by fluorescence detection scanning laser microscopy, DNA amplification (*e.g.*, PCR), methods of DNA sequencing (including NGS and related sequencing methods), and bioinformatics methods for raw data analysis and interpretation, including distinguishing sequences from one another by Hamming Distances.

19. Key among these papers are work on developing the field of gene expression profiling (Spellman *et al.*, *Molecular Biology of The Cell*, 1998; and Eisen *et al.*, *Proceedings of the National Academy of Sciences*, 1998, which

together have been cited in more than 20,000 other works), fabricating DNA microarrays (George, Wooley, and Spellman, *Genome Research*, 2001), ligation-based approaches to detecting and quantifying DNA and DNA methylation (Wang *et al.*, *Genome Biology*, 2008; and Nautiyal *et al.*, *Proceedings of the National Academy of Sciences*, 2011), histopathological classification by gene expression and imaging (*e.g.*, Chang *et al.*, *BMC Bioinformatics*, 2011), and the use of barcodes to identify unique molecules in multiplexed samples (Butler *et al.*, *Cold Spring Harbor Molecular Case Studies*, 2019).

20. In addition, I have presented over 100 invited lectures or conference presentations regarding these topics. I have also supervised five completed Ph.D. theses and have taught graduate-level courses at OHSU in the area of sequencing technologies, computational analysis, and genetics.

21. I have received over \$16 million in federal grants for my research in the area of cancer genomics and cancer precision medicine. I am currently one of 30 principal investigators for the Genome Data Analysis Network, sponsored by the National Cancer Institute. The Genome Data Analysis Network is a consortium of computational researchers from across the United States focused on developing the framework for relating the genomics and outcomes of patients in cancer clinical trials.

22. In 2017, I was named the inaugural holder of the Penny and Phil Knight Endowed Professorship for Cancer Research Innovation. In 2020, I was one of the recipients of the American Association for Cancer Research (AACR) Team Science award for my leadership roles in the Cancer Genome Atlas.

IV. MY UNDERSTANDING OF THE RELEVANT LEGAL PRINCIPLES

23. In formulating my opinions set forth in this Declaration, I have applied the following legal principles.

a. Person of Ordinary Skill in the Art (“POSA”)

24. I understand that patent law analyses are performed from the viewpoint of a POSA. I understand that a POSA is a hypothetical person who is presumed to be aware of all pertinent art, thinks along conventional wisdom in the art, and is a person of ordinary creativity. A POSA, with respect to the 303 patent, would have possessed a strong understanding of genetics and genomics approaches for detecting or monitoring human diseases, such as spatial detection of a target analyte (*e.g.*, mRNA or cDNA) in an individual cell of a biological sample. A POSA would typically have a Ph.D. in a relevant field (*e.g.*, molecular biology, genetics, and bioinformatics) with two years of experience.

b. Claim Construction

25. I understand that in an IPR, patent claim terms are given their ordinary and customary meaning as understood by a POSA at the time of the invention, in

view of the patent's specification and its prosecution history. I also understand that when a patent explicitly defines a claim term in the specification, the patent's definition controls.

26. I understand that claim 1 is the independent claim in the 303 patent, and that claims 2-12 are dependent claims. I understand that claims 2-12 depend directly or indirectly from claim 1. I understand that a dependent claim contains all the elements of the claim from which it depends. Thus, I understand that claims 2-12 contain all the elements of claim 1.

c. Obviousness

27. I understand that an obviousness analysis involves comparing a claim to the prior art to determine whether the claimed subject matter would have been obvious to a POSA in view of the prior art and general knowledge of the prior art. I understand that obviousness must be assessed from the viewpoint of a POSA at the time of the invention. I further understand that to show obviousness a party must perform the following factual inquiries: (a) determining the scope and content of the prior art; (b) ascertaining the differences between the claimed invention and the prior art; (c) resolving the level of skill in the art; and (d) considering any known objective evidence of non-obviousness.

28. I also understand that, for a finding of obviousness, a POSA needs to have (i) a reason or motivation to modify or combine the prior art to arrive at the claimed subject matter and (ii) a reasonable expectation of doing so successfully.

29. I understand that the reason to combine prior-art references can come from a variety of sources, not just the prior art itself or the specific problem the patentee was trying to solve. And I understand that the references themselves need not provide a specific hint or suggestion of the alteration needed to arrive at the claimed invention; the analysis may include recourse to logic, judgment, and common sense available to a person of ordinary skill that does not need to be explicit in any reference.

30. I understand that a “reasonable expectation of success” is assessed in view of the prior art and general knowledge of the art from the viewpoint of a POSA, and it does not require an absolute certainty of success.

31. I also understand that the objective evidence of non-obviousness can include evidence of commercial success attributable to the claimed invention, evidence of industry praise for the claimed invention, evidence of a long-felt need that was solved by the claimed subject matter, evidence that others copied the claimed subject matter, or evidence that the claimed subject matter achieved an unexpected, superior result relative to the closest prior art. I understand that such evidence must have a nexus, or causal relationship, to the claimed subject matter

beyond what was available in the prior art, and must be commensurate in scope with the patent claim(s) at issue.

d. Anticipation

32. I understand that anticipation analysis involves comparing a claim to a single prior art reference to determine whether the claimed subject matter is novel to a POSA in view of the prior art and general knowledge in the prior art. I understand that anticipation must be assessed from the viewpoint of a POSA at the time of the invention.

33. I understand that anticipation analysis requires comparing the claim language to the prior art on an element-by-element basis. I understand that a prior art reference anticipates a claim if all of the claim's elements are expressly or inherently disclosed in a single prior art reference, arranged as in the claim. I understand that the disclosure in a prior art reference does not have to use the same words as the claim, but all of the requirements of the claim must be expressly or inherently disclosed in the prior art reference.

34. I understand that a prior art reference anticipates a patent claim when the claim element not expressly found in that reference are nonetheless inherent in it. I understand that express disclosure means that the subject matter is clearly described in the prior art reference.

35. I understand that an element or the entire invention is inherent and in the public domain if it is the natural result flowing from the explicit disclosure of the prior art. I understand that for a claimed element to be inherently present within a prior art document, it must be necessarily present. That is, the prior art reference must necessarily function in accordance with or include the claimed element. I understand that inherency may not be established by probabilities or possibilities—the mere fact that a certain thing may result from a given set of circumstances is not sufficient.

36. I understand that in some cases, the inherent property corresponds to a claimed new benefit or characteristic of a purported invention otherwise in the prior art. I understand that in such circumstances, the new realization alone does not render the old invention patentable. I further understand that when a prior art method is at issue, the inherent anticipation examines the natural and inherent results in that method without regard to the full recognition of those benefits or characteristics within the art field at the time of the prior art disclosure.

e. Written Description

37. I understand that written description of a subject matter is assessed from the viewpoint of a POSA. I understand that to satisfy written description, the disclosures in the application must convey to a POSA that the inventors had “possession” of the claimed subject matter. I understand that the inventor need not

physically possess the described subject matter to have “possession” in the context of written description. I understand that possession for written description purposes is determined by the sufficiency of the disclosure in the application taken together, and in the context of, the state of the art at the time of the filing. I understand that the state of the art at the time of filing is determined by the general knowledge of a POSA, as evidenced by the published literature at the time.

38. I also understand that a patent application need not disclose a working example of the subject matter to satisfy the written description requirement. I further understand that a subject matter may be fully described in a patent application even if separate parts of it are described in separate parts of the application, as long as there is sufficient direction in the application to guide a reader towards the subject matter.

V. THE 303 PATENT AND ITS CLAIMS

a. The 303 patent

39. The 303 patent relates to spatially detecting target nucleic acids by applying “a plurality of nucleic acid probes” to a sample, using fluorescence to “determine locations of the nucleic acid probes within the sample.” EX1001, Abstract. The patent discloses that it adapts known single-molecule fluorescent in situ hybridization (smFISH) techniques to “enable the simultaneous detection of the number and spatial organization of thousands of distinct mRNAs within single

cells with high efficiency and low error-rate.” EX1001, 34:12-16, Abstract. The patent’s methods use primary probes and secondary probes with fluorescent “readouts” to generate binary “codewords,” compare those codewords to valid codewords in a codebook, and use well-known mathematical techniques such as Hamming codes to detect and/or correct errors when there is no match between the generated codewords and valid codewords in the codebook. EX1001, 8:58-11:28. The 303 patent uses the following terminology.

40. **“Primary probes” or “encoding nucleic acid probes”**: The “primary probes” or “encoding nucleic acid probes” encode “codewords” and bind to nucleic acid targets in a tissue sample. The primary probes comprise the following two regions:

(1) a “RNA targeting sequence” that “targets [the primary] probe to the RNA of interest” (*i.e.*, a target-binding domain), and

(2) one or more “read sequences” which “bind certain of the secondary nucleic acid probes.” EX1001, 35:19-24, 44:67-45:3, 18:50-55.

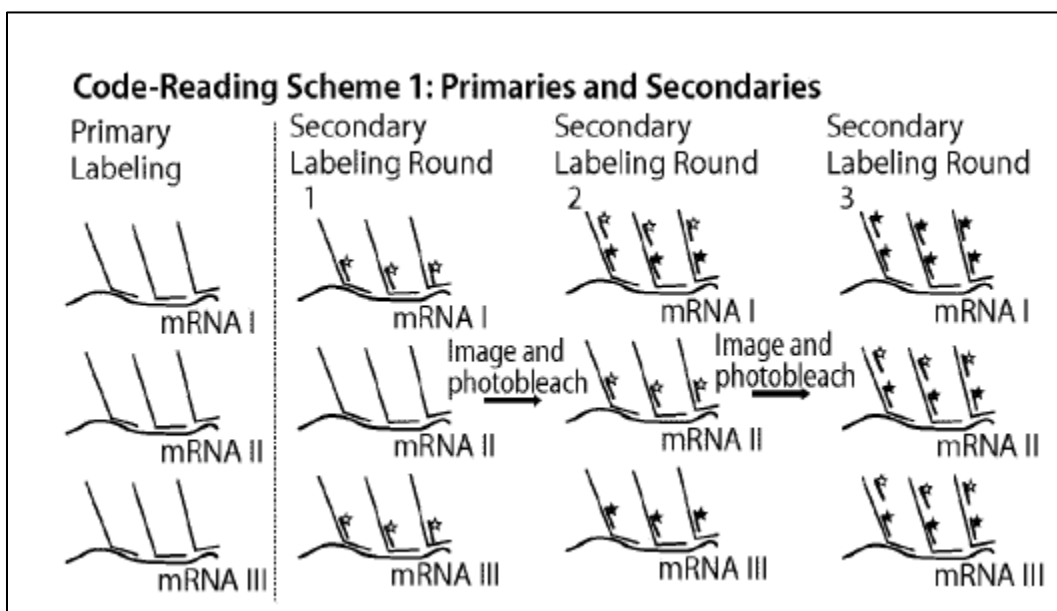
41. **“Secondary probes” or “readout probes”**: The “secondary probes” or “readout probes” are used to “read out the codewords from the primary probes.” EX1001, 8:1-6. The secondary probes comprise the following two regions:

(1) a “recognition sequence” that “recognize[s] one of the read sequences present within the population of primary nucleic acid probes,” and “is able to bind to or hybridize with corresponding nucleic acid probe,”

(2) one or more “signaling entities” such as “a fluorescent entity.” EX1001, 9:30-43.

42. **“Codewords”**: A “codeword” is created based on the fluorescence signal of the secondary nucleic acid probe and is matched to a valid codeword to determine the identity of the target nucleic acid. EX1001, 2:25-36.

43. The 303 patent’s methods follow the workflow exemplified in Figure 1B, which uses the primary probes (*i.e.*, “Primaries” in the figure) and secondary probes (*i.e.*, “Secondaries” in the figure).



EX1001, FIG. 1B.

44. First, the target mRNAs (I-III) are hybridized to primary probes comprising a “primary targeting sequence” (*i.e.*, target-binding domain), which hybridizes to the target RNAs, and a “tail” containing “read sequences” that do not interact with endogenous nucleotide sequences. EX1001 36:18-23.

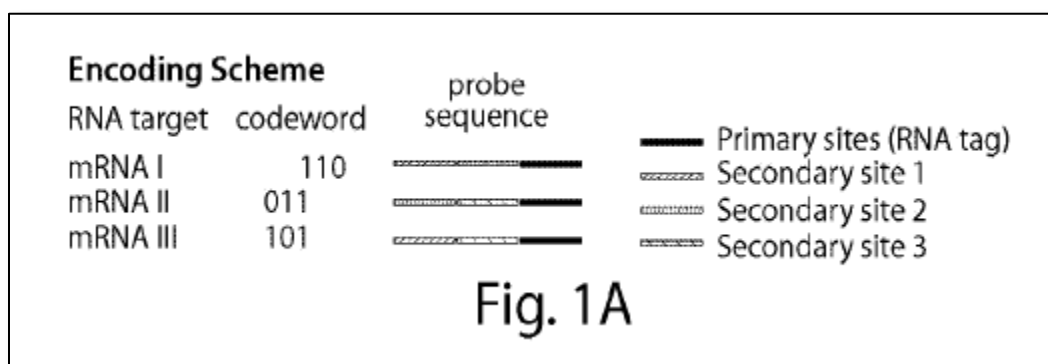
45. Next, the first labeled secondary probe is added that hybridizes to all primary probes whose tails have a corresponding read sequence. EX1001, 36:23-26. The signals from labels on the secondary probes hybridized to the primary probes are imaged and then bleached. EX1001, 36:26-30. A second labeled secondary probe is then added to hybridize to a different read sequence on the primary probes bound to the mRNA. EX1001, 36:26-30. The signals from labels on the secondary probes hybridized to the primary probes are again imaged and bleached. EX1001, 36:26-30. These rounds of sequential hybridizations with secondary probes are repeated until the final step when the microscopy images from each staining and imaging round are aligned and the clusters of localizations (arising from individual target mRNA molecules) at a spot are identified. EX1001, 36:26-37.

46. In a hybridization round, in which, a signal is detected for a spot is given the value “1” and when no signal is detected is given the value “0.” EX1001, 10:43-46. Thus, the sequential hybridizations of the secondary probes generate a

“binary codeword” for each spot (which should correspond to an individual mRNA molecule).

47. These codewords produced from the hybridization rounds are matched with the expected codewords (*i.e.*, valid codewords) collected in a codebook.

EX1001, 36:40-46. A codebook can be prepared by assigning each target mRNA a “binary codeword” before the hybridization rounds. EX1001, 38:4-17. In Figure 1, which describes a scheme with three-letter (*i.e.*, three-position) codewords, the target mRNAs are pre-assigned codewords 110, 101, and 011, which are compiled in a codebook. EX1001, 36:43-46.



EX1005, FIG. 1A.

48. Thus, when overlapping signals are detected in the same spot in only the 1st and 2nd hybridization rounds and not in the 3rd hybridization round (meaning only the secondary probes in 1st and 2nd rounds hybridized to the target mRNAs), codeword 110 is produced. EX1001, 36:37-40, 36:43-54. The produced codeword

can then be matched with the known, pre-assigned, valid codeword for mRNA I to identify the mRNA at that spot as mRNA I. EX1001, 36:43-54.

49. If there are no missed-detection events or false positive signal in the images, a codeword will perfectly match one of the expected codewords. EX1001, 36:40-62. Any missed-detection events or false positive signals are corrected by using an error-detection and error-correction system, such as a Hamming system. EX1001, 11:8-28.

50. The method illustrated in Figure 3A (reproduced below) further details the patent's methods.

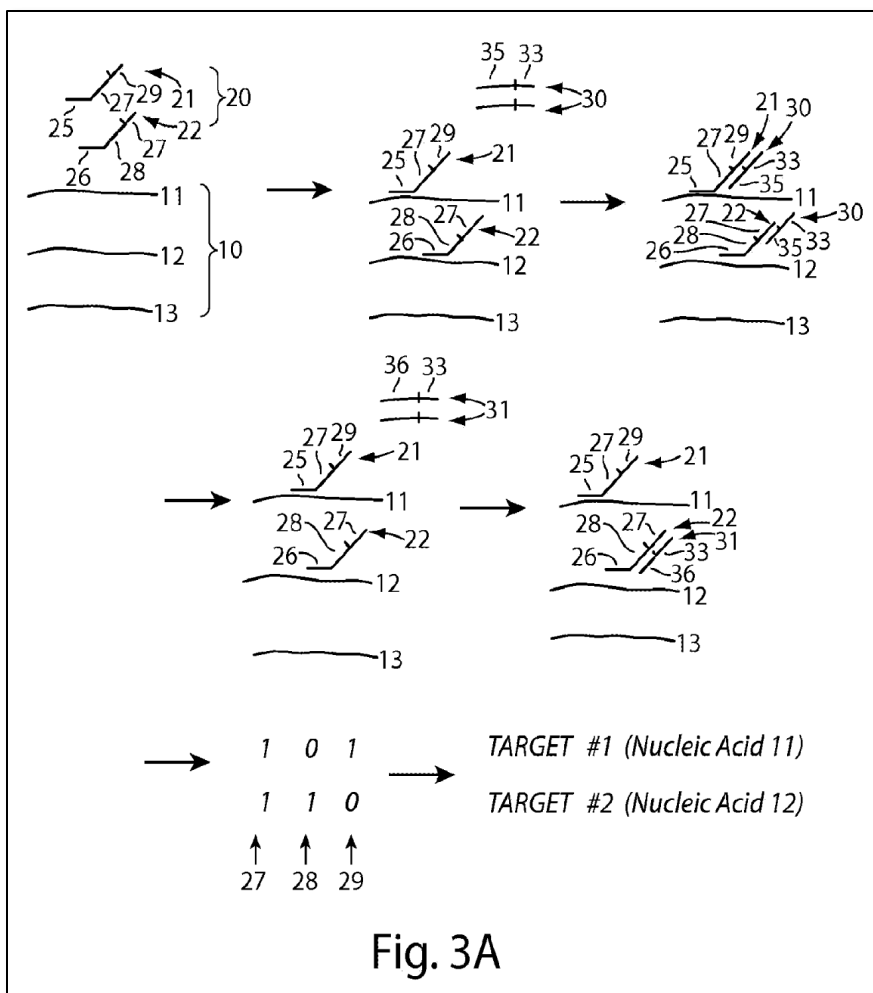


Fig. 3A

EX1001, FIG. 3A.

51. Figure 3A depicts a series of primary and secondary probes, with fluorescent “readouts” tied to the secondary probes that are used to generate “codewords” and identify the location of a target nucleic acid in the sample.

EX1001, 8:48-10:11.

52. First, the primary probes 21 and 22 (also called “encoding nucleic acid probes”) are added to the biological sample, where they bind target nucleic acids 11 and 12 in the sample. EX1001, 8:48-9:8. Primary probe 21 contains (1)

targeting sequence 25 that targets the probe to the nucleic acid 11, and (2) read sequences 27 and 29 that are designed to hybridize to the secondary probes.

EX1001, 8:48-9:25, 18:50-55. Similarly, primary probe 22 contains the following two types of elements (1) targeting sequence 26 that targets the probe to the nucleic acid 12, and (2) read sequences 27 and 28 that are designed to hybridize to the secondary probes. EX1001, 8:48-9:25, 18:50-55.

53. Next, different sets of secondary probe sets 30 (also called “readout” probes) are added to the sample in sequential fashion, where the secondary probes 30 bind to primary probes 21 and 22 (bound to the target nucleic acids 11 and 12) via the read sequence 27. EX1001, 9:26-51. The secondary probes 30 contain a recognition sequence 35 that is able to recognize and bind to read sequence 27. EX1001, 9:26-51. The secondary probes also contain “signaling entities” 33 (*e.g.*, a fluorescent tag) which are used to visualize their location in the sample. EX1001, 9:39-65. For example, a fluorescent tag on the secondary probe can be visualized using fluorescence microscopy. EX1001, 9:39-65.

54. After the initial set of secondary probes is added and detected, they are washed away and the process is repeated with a different set of secondary probes (depicted as secondary probes 31 in the figure above), which bind to one or more primary probes. EX1001, 8:48-10:11. The different set of secondary probes 31 contain a recognition sequence 36 that recognizes and binds to read sequence 28,

which is only located on the primary probe 22. EX1001, 9:43-51. Accordingly, the secondary probe 31 will only bind to primary probe 22. EX1001, 9:43-51. The process is repeated multiple times, which generates a “pattern of binding or hybridization of the secondary nucleic acid probes” that is “converted into a ‘codeword.’” EX1001, 10:41-58. In Figure 3A, the codewords are 101 and 110 for primary probes 21 and 22, respectively, where “1” represents binding and “0” represents no binding. EX1001, 10:41-58.

55. As I discuss above, the generated binary codeword is compared to valid codewords in a codebook to identify the target, wherein the codewords in the codebook are constructed using well-known error detection and correction systems, such as Hamming system, to differentiate the codewords enough to detect and correct potential errors. EX1001, 11:8-28.

b. The 303 patent claims

56. I understand that the 303 patent issued with 12 claims. EX1001, 85:34-88:27. I understand that claim 1 is the sole independent claim, and claims 2-12 depend from claim 1. I understand that the dependent claims incorporate the elements of their respective independent claims.

57. Independent claim 1 recites a “method for detecting a plurality of different nucleic acid targets within a sample by in situ hybridization,” and requires the following steps:

- (a) producing a plurality of first hybridized complexes within a sample containing a plurality of different nucleic acid targets by exposing the sample to a plurality of different *encoding nucleic acid probes*, wherein each of the plurality of encoding nucleic acid probes comprises (i) a target sequence that specifically hybridizes to a different nucleic acid target or a different location of an identical nucleic acid target of the plurality of different nucleic acid targets and (ii) one or more read sequences such that each of the plurality of first hybridized complexes comprises multiple encoding nucleic acid probes of the different encoding nucleic acid probes;
- (b) producing a plurality of readout probe-hybridized complexes by exposing each of the plurality of first hybridized complexes within the sample to a *first readout nucleic acid probe*, wherein the first readout nucleic acid probe comprises a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal;
- (c) detecting a fluorescent signal from each of the plurality of readout probe-hybridized complexes;

- (d) removing the fluorescent signal from each of the plurality of readout probe-hybridized complexes by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the plurality of readout probe-hybridized complexes;
- (e) after step (d), producing a different plurality of readout probe-hybridized complexes by exposing the sample to *a different readout nucleic acid probe*, wherein the different readout nucleic acid probe comprise a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal;
- (f) detecting a fluorescent signal from each of the different plurality of readout probe hybridized complexes;
- (g) removing the fluorescent signal from each of the different plurality of readout probe hybridized complexes by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the different plurality of readout probe hybridized complexes;
- (h) repeating steps (e) to (g) for one or more times using a different readout nucleic acid probe, wherein the

different readout nucleic acid probe comprises a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal;

- (i) producing codewords representing the plurality of different nucleic acid targets at locations within the sample, wherein each of the codeword represents one of the plurality of different nucleic acid targets and comprises multiple binary values 1 and 0, wherein a value of 1 is obtained when the fluorescent signal is detected from one of the plurality of readout probe hybridized complexes or one of the different plurality of readout probe-hybridized complexes at a respective location within the sample while a value of 0 is obtained when the fluorescent signal is not detected from one of the plurality of readout probe-hybridized complexes or one of the different plurality of readout probe-hybridized complexes at the respective location within the sample;
- (j) matching the codewords with valid codewords in a codebook by comparing the codewords to the valid codewords in the codebook, and if one of the codewords is not matched with one of the valid

codewords in the codebook, applying an error detection or correction system, matching the one of the codewords with another of the valid codewords in the codebook, or discarding the one of the codewords, wherein the codebook comprises the valid codewords of the plurality of nucleic acid targets; and

(k) determining the plurality of nucleic acid targets in the sample based on results from the matching step.

EX1001, 85:34-87:18 (emphasis added).

58. Dependent claims 2-12 further specify various elements of the independent claims, *e.g.*, the tissue sample type, methods for determining the fluorescent signal entity, and the comparing codewords steps. EX1001, 87:19-88:27

c. The 303 patent prosecution history

59. I have reviewed the 303 patent's prosecution history (EX1004). The 683 application (that led to the 303 patent) was filed on January 27, 2017. EX1004. I understand from the file history that after initially rejecting the claims, the patent examiner eventually allowed the claims—which issued as the 303 patent claims. I understand that the examiner did not raise any rejections against the claims based on prior art. EX1004.

VI. STATE OF THE ART BEFORE JULY 30, 2014

a. Prior-art references asserted in the Grounds

60. I have relied upon the following asserted prior-art references in Grounds 1-4, which are prior art alone (anticipation under Ground 1) or in combination (obviousness under Grounds 2-4).

61. I understand that in June 2015, Illumina, Inc. filed US Application No. 14/742,027 (“the 027 application”), entitled “Methods and Compositions for Single Cell Genomics,” which issued as US Patent No. 10,829,814 (“Fan”; EX1005) on November 10, 2020. EX1005, face. I understand that the 027 application claims benefit to US Application No. 62/014,538 (“Fan-Provisional”; EX1006), which was filed on June 19, 2014. I understand that Fan is prior art to the 303 patent.

62. Gunderson *et al.*, *Genome Research* 14:870-877 (2004) (“Gunderson”; EX1007), is a printed publication that published in April 2004, which is before the 303 patent’s claimed July 30, 2014 priority date. I understand that Gunderson is prior art to the 303 patent.

63. Larsson *et al.*, *Nat. Methods* 7(5):395-397 (2010) (“Larsson”; EX1008), is a printed publication that published in May 2010, which is before the 303 patent’s claimed July 30, 2014 priority date. I understand that Larsson is prior art to the 303 patent.

64. I understand that in April 2012 Agilent Technologies, Inc. filed US Application No. 13/448,985, entitled “Combinatorial Probe Libraries,” which issued as U.S. Patent No. 8,765,642 (“Curry”; EX1009) on July 1, 2014. I understand that Curry is prior art to the 303 patent.

65. As I discuss above in Section V, the 303 patent’s claims relate to detecting a plurality of nucleic acid targets within individual cells by hybridizing a plurality of primary probes specific to the targets, sequentially hybridizing labeled secondary probes to primary probes, and decoding the pattern of sequential hybridization of secondary probe to identify the nucleic acid targets. EX1001, 85:34-88:27. Thus, the 303 patent claims comprise two basic components: an *assay component* comprising sequential rounds of in situ hybridization and imaging; and an *analysis component* comprising binary coding and error-correction systems. As I explain below, the prior art taught methods comprising both these components.

b. The prior art taught sequential in situ hybridization methods

66. Fan taught in April 2014 “methods and compositions for obtaining sequence information from one or more individual cells.” EX1005, Abstract. Fan discloses that its methods are useful for “obtaining sequence information for a single nucleotide sequence, and for multiplex generation of sequence information from an individual cell.” EX1005, 1:60-62. In Fan’s method, nucleic acids of interest are first amplified within the cell (in situ) to produce a nucleic acid target,

such as a cDNA colony. EX1005, 1:63-2:1, 2:21-36, 4:9-19, 9:1-22, 14:3-48

(Example 1), FIG. 1, Claim 1.

67. The nucleic acid targets are probed in situ with a series of primary nucleic acid probes that hybridize (bind) to the targets. EX1005, 5:17-20, 9:1-22, FIG. 1. POSAs knew that primary probes comprise a portion that hybridizes to its complementary sequence on the target nucleic acids. EX1022, ¶[0062]; EX1021, ¶[00102]; EX1019, 360, FIG. 1; EX1020, FIG. 2; EX1009, 7:24-32. For example, Fan uses primary probes called “barcode probes” that “hybridize specifically” to the target nucleic acids in the cell. EX1005, 5:17-20, FIG. 1.

68. Before July 30, 2014, POSAs knew they could further increase sensitivity and specificity of in situ hybridization methods by using multiple primary probes that bind to different sequences on the same target nucleic acid. EX1015, 836-837, FIG. 1A-1B; EX1025, 66; EX1013, Abstract; EX1026, 3:37-42; EX1020, 4:35-48; EX1009, 6:17-7:13; EX1019, FIG. 1. Fan also suggests this technique in its workflow, stating that “[t]he amplified nucleic acids can then be hybridized with one *or more* unique padlock probes with unique molecular barcodes.” EX1005, 9:8-11. POSAs knew that this approach could help avoid issues with off-target binding for single probes, as well as mitigate different optimal hybridization conditions for different probes. EX1024, 92-93.

69. After hybridizing the barcode probes to the target nucleic acid, Fan teaches adding secondary probes (called “decoder probes”) that hybridize to a specific portion of the barcode probes. EX1005, 8:30-67, FIG. 1, 9:1-22. Fan referred to these portions of its barcode probes that hybridize to decoder probes as “decoder sequences.” EX1005, 8:45-52. This structural design for primary probes was well known and standard in the art and many other prior art workflows detailed similar primary probe structures comprising a portion that specifically hybridizes or binds to a labeled secondary probe. EX1022, ¶[0062]; EX1009, 6:17-31, 7:24-28, 7:32-45; EX1020, 4:35-48; EX1021, [0019], [0016].

70. Fan teaches performing successive rounds of in situ hybridization of fluorescently labeled secondary probes, with each round of hybridization separated by a washing step to remove the previous round’s labeled secondary probes. EX1005, 9:1-22, 14:6-47. In Example 1 (illustrated in Figure 1), Fan hybridizes decoder probes referred to as “fluorescently labeled oligos” in “eight rounds of sequential hybridization.” EX1005, 9:18-22, FIG. 1. Fan’s method uses sequential “cycles” of “hybridization and imaging” of a different sets of decoder probes (e.g., “cycle 1” through “cycle 8” in Figure 1). EX1005, FIG. 1B, 2:21-36, 9:1-22, 14:3-48. This approach of primary and secondary probes was commonly used in sequential hybridization methods in the art. EX1015, 836-837, FIGs. 1A-1B;

EX1025, 66; EX1013, Abstract; EX1020, 4:35-48; EX1009, 6:17-7:13; EX1019, FIG. 1.

71. In between cycles of hybridization, Fan’s method includes a removal step (e.g., washing) to remove the fluorescent signal from that cycle before adding the next set of labeled decoder probes. EX1005, 2:56-58, claim 1. Removing or inactivating the signal in between hybridization rounds was standard procedure in sequential hybridization workflows. EX1005, 2:56-58, claim 1. Indeed, the art taught a variety of routine removal methods, such as “washing, heating, photo-bleaching, displacement..., cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation, or any combinations thereof.” EX1021, ¶[0016]; EX1020, FIG. 2a; EX1005, 2:56-58, FIG. 1; EX1007, 875.

c. Fan and Larsson taught routine detection and imaging methods

72. Fan teaches that its sequential hybridization methods produced a series of fluorescent signal patterns that could be “detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ.” EX1005, 11:11-15. For example, Fan refers to a 2010 publication from Larsson et al., which describes methods for “in situ detection and genotyping of individual mRNA molecules.” EX1008, title. Larsson’s methods involve probing an amplified DNA ball (akin to Fan’s cDNA colony) with fluorescently labeled probes. EX1008, FIG. 1a. To detect the fluorescent signal, Larsson teaches “using

an AxioplanII epifluorescence microscope (Zeiss) equipped with ... a CCD camera (C4742-95, Hamamatsu), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5.”

EX1008, 0005. POSAs understood that imaging technologies such as Larsson’s could provide the desired resolution to identify nucleic acids in cells. EX1008, 0005.

73. The art also provided many other routine techniques for imaging nucleic acids in cells, such as super-resolution microscopy. EX1029, 323; EX1028, 968; EX1030, 16. These art-recognized techniques included stimulated emission depletion (STED), photo-activated localization microscopy (PALM), fluorescence PALM (FPALM), stochastic optical reconstruction microscopy (STORM), direct STORM (dSTORM), and PALM with independent running acquisition (PALMIRA). EX1028, 968; EX1029, 323; EX1030, EX1031, 76; EX1032, 1103; EX1020, 21:16-45; EX1005, 11:11-19; EX1008, 0004; EX1033, 2227.

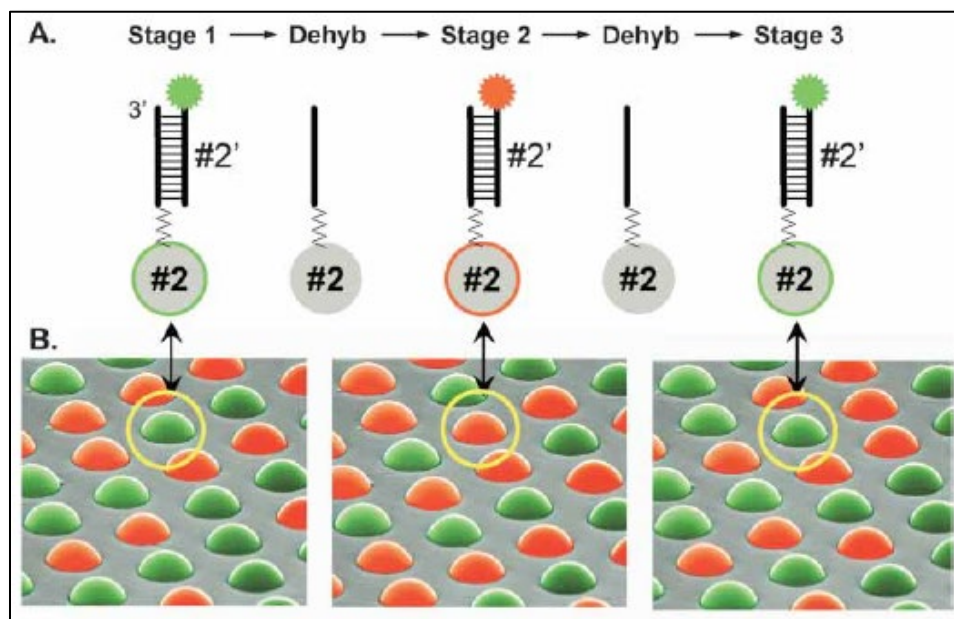
d. Fan and Gunderson taught binary code and error-checking systems

74. The computational aspect of binary codes and error-checking in sequential hybridization methods was well established in the art before July 30, 2014. In Fan’s method, the spectral pattern of fluorescent signals during sequential hybridization produces a “code” that is decoded to identify the different nucleic acid targets. EX1005, 8:30-67, 8:53-55, 14:28-47. Fan discloses that, “[d]uring

decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” EX1005, 8:53-55. Fan also endorses and incorporates by reference a 2004 publication from Gunderson et al. that describes “[s]equential hybridization of decoder probes to decoder sequences.” EX1005, 8:56-59 (citing EX1007), 14:31-34 (same). Fan also applies Gunderson’s “error-checking scheme” in Fan’s sequential hybridization workflow detailed in Example 1. EX1005, 14:2-48; EX1007, 871-872.

75. Gunderson teaches sequential hybridization methods for decoding and error-checking, using codewords of multiple binary values 1 and 0 for a given fluorescent signal detected from labeled secondary probes. EX1007, 871-872. Gunderson demonstrates its decoding method using an array of universal capture oligonucleotides, each individually immobilized on microarray beads. EX1007, 871-872, 875. Gunderson teaches using sequential hybridization of “fluorescently labeled decoder oligonucleotide” that are complementary to the immobilized oligonucleotides on the beads. EX1007, 871-872, 875. As Gunderson explains, in each “stage” of the sequential hybridization, “the bead is ‘colored’ by hybridization to a fluorescently labeled decoder oligonucleotide.” EX1007, 871-872, 875. Gunderson depicts this sequential hybridization and “coloring” in Figure

2A-B, showing an individual bead's signal as green (first stage), red (second stage), and green (third stage):



EX1007, FIG. 2.

76. In between each stage of hybridization, Gunderson removed signal from the arrays by washing to remove the fluorescent signal before the next stage.

EX1007, 875.

77. Gunderson's method uses the "combinatorial assignment of green and red" from the sequential hybridizations to generate a spectral pattern, or code, for each bead sequence. EX1007, 871-872, FIG. 2. Gunderson teaches that a value of "1" is obtained when the fluorescent signal (a red signal) is detected during a stage of sequential hybridization, and a value of "0" is obtained when that same red fluorescent signal is not detected in another stage of sequential hybridization.

EX1007, 871-872, FIG. 2. Gunderson shows this combinatorial assignment of color codes in Figure 2C:

		Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code
Sequences	0				GGG	000	0000
	1				GGR	001	0011
	2				GRG	010	0101
	3				GRR	011	0110
	4				RGG	100	1001
	5				RGR	101	1010
	6				RRG	110	1100
	7				RRR	111	1111

EX1007, FIG. 2C, 871.

78. As Gunderson demonstrated, bead #2 in the example has a color signature of green-red-green, which generated a binary code of 010. EX1007, FIG. 2C, 871. The bead's sequence "can be identified as sequence 2 by referring to the color lookup table in Figure 2C." EX1007, FIG. 2C, 871.

79. Gunderson also teaches methods for error-checking its decoding data, stating that, "assignment of codes to sequences is unambiguous after three stages," but "additional stages can be added for error-checking purposes." EX1007, 871. Gunderson teaches using a collection of "valid codes" (e.g., a codebook) to cross-reference the codewords generated from the sequential hybridization data. EX1007, 871. If a sequence's codeword matched a valid codeword from the codebook, that particular sequence was confirmed; if a sequence's codeword did not match a valid codeword in the codebook, an error was detected. EX1007, 871-

872, FIGs. 2-3, Table 1. Gunderson teaches that its error-checking methods

“achieve a level of quality control that is unprecedented.” EX1007, 871.

80. Similar to Gunderson, Curry teaches a sequential hybridization method for identifying nucleic acids using combinatorial libraries of probes and “determining which of the multiple libraries hybridized to a spatially separate target.” EX1009, 11:46-48. Curry teaches that, after performing the sequential hybridization and detection, “the identities of the targets in the sample can be determined” using a “lookup table,” which contains a binary code of “1” for signal detected and “0” for no signal detected. EX1009, 11:46-60, 6:17-7:23.

81. Curry also discloses an error detection or correction system based on Hamming code that is able to correct certain errors if correctable or discard the code if the error is not correctable. Curry describes “an error correction system so that if any one hybridization fails, useful data can still be obtained.” EX1009, 12:40-44. Curry discloses that its method includes an “an error correcting (7,4) Hamming code, for example” and provides an exemplary error detecting and correcting scheme in Table 2. EX1009, 12:40-13:6.

82. A Hamming code is a well-known algorithm from digital information theory that has been applied in other fields. *See, e.g.*, EX1007, 871. Indeed, Gunderson discloses that its decoding and error-checking methods were based “on algorithms from digital information theory,” referring to publications from 1948 by

Shannon (EX1035, EX1036) and 1986 by Hamming (EX1037). EX1007, 871; *see also*, EX1038, 236; EX1039, 1-2; EX1040, 4:8-18; EX1041, ¶[0073]; EX1042, 192-193; EX1043, ¶¶[0008]-[0011]; EX1044, ¶¶[0097]-[0100], [0108]-[0110], [0135]-[0145], Figs. 1B-1C; EX1009, 6:36-7:23, 11:46-60; EX1034, ¶¶[0082], [0102], [0194]. Thus, the computational aspect of binary codes and error-checking in sequential hybridization methods was well established in the art before July 30, 2014.

VII. CLAIM CONSTRUCTION

83. I understand that Harvard and Vizgen in a related district court litigation alleged that 10x's product infringes the 303 patent claims. Solely for the purposes of this IPR, I have been asked to apply interpretations according to Harvard/Vizgen's infringement positions.

VIII. FAN-PROVISIONAL DISCLOSES THE FAN DISCLOSURES RELIED UPON AS PRIOR ART, AND THE PROVISIONAL PROVIDES WRITTEN DESCRIPTION SUPPORT FOR FAN'S CLAIM 1

84. I understand that in June 2015, Illumina, Inc. filed US Application No. 14/742,027 ("the 027 application"), entitled "Methods and Compositions for Single Cell Genomics," which issued as US Patent No. 10,829,814 ("Fan"; EX1005) on November 10, 2020. EX1005, face. I understand that the 027 application claims benefit to US Application No. 62/014,538 ("Fan-Provisional"; EX1006), which was filed on June 19, 2014. I understand that Fan is prior art to the 303 patent.

85. I have been asked to consider whether the disclosures in Fan that are relied upon as prior art to challenge the 303 patent claims under Grounds 1-4 are also disclosed in Fan-Provisional. In my opinion, the relied-upon disclosures in Fan *are* also disclosed in Fan-Provisional.¹

86. The specification of Fan and Fan-Provisional are substantially the same. *Compare* EX1045 with EX1006; EX1046. I have provided in Appendix B a side-by-side comparison of the relied-upon disclosures in Fan and the corresponding disclosures in Fan-Provisional. As shown in Appendix B and the redline comparison, the relied-upon disclosures in Fan *are* also disclosed in Fan-Provisional. *See* EX1046; Appendix B.

87. I have been asked to consider whether at least one claim of Fan is supported by Fan-Provisional. As shown in Appendix C, Fan-Provisional describes all the elements of claim 1, and thus Fan-Provisional provides written description support for claim 1. *See* Appendix C.

88. Accordingly, I understand that Fan is prior art to the 303 patent as of Fan-Provisional's filing date of June 19, 2014.

¹ Throughout my declaration, I have provided citations to the relied-upon disclosures of Fan (EX1005) and the corresponding disclosures in Fan-Provisional (EX1006).

IX. GROUND 1: CLAIMS 1-12 ARE ANTICIPATED BY FAN

89. In my opinion, claims 1-12 of the 303 patent are anticipated by Fan under Harvard/Vizgen's interpretation. Specifically, Fan discloses a method meeting all the elements of the claimed methods. I understand that Fan is presumed enabled.

a. Claim 1

90. As I discuss below, Fan anticipates claim 1. *See also* Appendix A.

i. Preamble: "A method for detecting a plurality of different nucleic acid targets within a sample by in situ hybridization"

91. Claim 1 recites "[a] method for detecting a plurality of different nucleic acid targets within a sample by in situ hybridization." EX1001, 85:34-36. I understand that this is the preamble of claim 1. I understand that a preamble may be relevant in understanding the scope of the claim. To the extent Claim 1's preamble is relevant in understanding its scope, Fan teaches the claimed methods for detecting a plurality of different nucleic acid targets within a sample by in situ hybridization.

92. Fan discloses a method for "obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell." EX1005, 2:21-23; EX1006, 2:20-21. Fan discloses that its "methods allow for multiplex detection of a plurality of nucleotide species of interest," including "*simultaneous* detection of 2 ... or more than 10,000 unique nucleic acid molecules." EX1005, 9:49-59 (emphasis added); EX1006, 14:15-22. In particular, Fan teaches a method for

“obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell.” EX1005, 2:21-36; EX1006, 2:20-30. Thus, Fan’s method detects a plurality of different nucleic acids within a sample, as claimed.

93. Fan also teaches amplifying a plurality of nucleic acid molecules *in situ* within a cell and “annealing *in situ* a barcode probe” to the amplified nucleic acid molecules. EX1005, 2:21-29; *see also* EX1005, 1:63-2:1, 2:21-36, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1; EX1006, 2:20-26; *see also* EX1006, 2:2-6, 5:17-25, 13:12-27, 21:15-22:16 (Example 1), FIG. 1, Claim 1. Fan further explains that in its method, “cDNA is generated *in situ*,” “amplified,” and the “amplified nucleic acids can then be *hybridized* with one or more unique *padlock probes* with unique molecular barcodes.” EX1005, 9:4-11 (emphasis added), FIG. 1, 14:34-36 (“eight rounds of *hybridization* decoding are performed”); EX1006, 13:14-18, FIG. 1, 22:7-8.

94. Accordingly, Fan’s method detects a plurality of different nucleic acid targets within a sample by *in situ* hybridization.

95. As I explain below, Fan’s method (which is described, *e.g.*, at 1:63-2:1, 2:21-36, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, and Claim 1) includes all the elements of the claim 1. For reference, I have included below a table providing certain terms used in the 303 patent and their counterpart terms in Fan:

Technical Terms	
303 Patent	Fan
Encoding nucleic acid probes or primary probes Primary probes that comprise: (1) a target-binding domain that binds to the target nucleic acids in a tissue sample, and (2) a decoder sequence.	Barcode probes Primary probes that comprise: (1) a target-binding domain that binds to the target nucleic acids in a tissue sample, and (2) a decoder sequence.
Readout probes or readout nucleic acid probes or secondary probes Secondary probes that comprise (1) a sequence that recognizes and binds to the barcode probe, and (2) a signaling entity	Decoder probes Secondary probes that comprise (1) a sequence that recognizes and binds to the barcode probe, and (2) a signaling entity.
Read sequence or readout probe sequence A sequence on the primary probe to which a secondary probe binds	Decoder sequence A sequence on the primary probe to which a secondary probe binds

ii. “(a) producing a plurality of first hybridized complexes within a sample containing a plurality of different nucleic acid targets by exposing the sample to a plurality of different encoding nucleic acid probes”

96. In my opinion, Fan’s method includes this step.

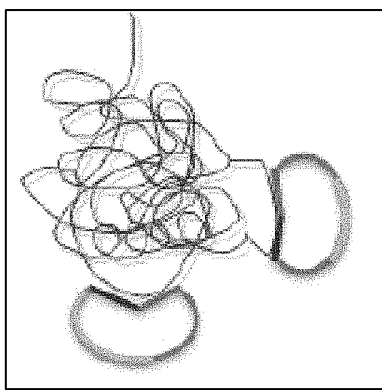
97. As I explain below, Fan’s method uses “barcode probes,” which are the “encoding nucleic acid probes” of the claim. Fan’s method produces a plurality of

first hybridized complexes by exposing a sample containing a plurality of different nucleic acid targets to a plurality of different barcode probes (*i.e.*, encoding nucleic acid probes of the claim).

98. Specifically, Fan’s method amplifies a “*plurality* of nucleic acid molecules in situ within a cell” and “anneal[s] in situ a barcode probe ... to the amplified nucleic acid molecule.” EX1005, 2:21-36 (emphasis added); *see also* EX1005, 1:63-2:1, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1; EX1006, 2:20-30; *see also* EX1006, 2:2-6, 5:17-25, 13:12-27, 21:15-22:16 (Example 1), FIG. 1, Claims 1-3. Thus, Fan’s method produces a plurality of first hybridized complexes within a sample by exposing the sample to a plurality of different barcode probes (*i.e.*, encoding nucleic acid probes of the claim).

99. In Example 1, Fan generates cDNAs from mRNAs *in situ*, and then amplifies the cDNA molecules via rolling circle amplification to produce cDNA “rolonies” again, in situ. EX1005, 9:4-8; EX1006, 13:14-17. Fan tags the cDNA rolonies with “gene specific barcodes using a library of padlock probes” by hybridizing the amplified DNA with “one or more unique padlock probes with unique molecular barcodes [*i.e.*, barcode probes].” EX1005, 14:15-18, 9:8-11; EX1006, 21:23-25, 13:17-18. The padlock probes are barcode probes that are specialized ligation probes. EX1005, 7:36-37 (“Barcode probes ... can include padlock probes.”); EX1006, 21:23-25, 13:17-18, 11:1-2. In other words, Fan’s

method in Figure 1 generates a plurality of nucleic acid targets (*e.g.*, cDNA colonies) within a cell and probes them with a plurality of different barcode probes to produce a plurality of nucleic acid target-barcode probe complexes. EX1005, 7:36-37; EX1006, 11:1-2. Fan's Figure 1 (reproduced below) illustrates one such target nucleic acid-barcode probe complex (*i.e.*, first hybridized complex of the claim) within a sample that is produced by exposing the sample to barcode probes (*i.e.*, encoding nucleic acid probes):



EX1005, FIG. 1; EX1006, FIG. 1.

100. Although Fan's Figure 1 illustrates a single complex with a nucleic acid target (one cDNA colony) hybridized to barcode probes, Fan's method produces a plurality of such complexes. I have depicted this in Figure A below generated based on Fan's Figure 1:

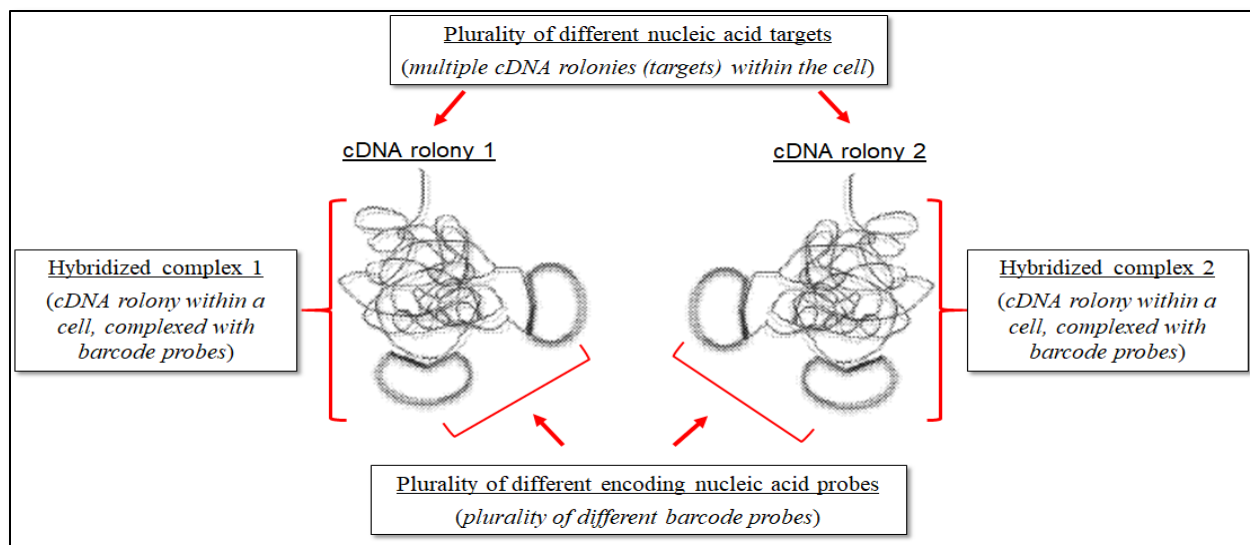


FIG. A.

101. According to the 303 patent, “nucleic acids to be determined may be, for example, DNA, RNA, or *other nucleic acids that are present within a cell* (or other sample).” EX1001, 13:19-21 (emphasis added). Fan’s cDNA rolonies created in situ in Figure 1, are nucleic acids, and are therefore nucleic acids present within a cell or other sample. Thus, Fan’s cDNA rolonies are nucleic acid targets under the 303 patent’s definition.

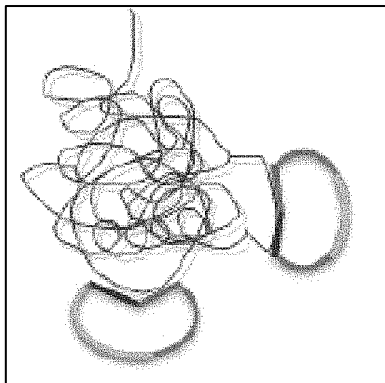
102. Thus, Fan’s method produces a plurality of first hybridized complexes (nucleic acid target-barcode probes complexes) within a sample containing a plurality of different nucleic acid targets (*e.g.*, cDNA rolonies) by exposing the sample to a plurality of different encoding nucleic acid probes (*i.e.*, barcode probes).

“wherein each of the plurality of encoding nucleic acid probes comprises (i) a target sequence that specifically hybridizes to a different nucleic acid target or a different location of an identical nucleic acid target of the plurality of different nucleic acid targets and (ii) one or more read sequences”

103. Fan’s barcode probes (*i.e.*, encoding nucleic acid probes of the claim) comprise a target sequence that specifically hybridizes to a different nucleic acid target or a different location of an identical nucleic acid target of the plurality of different nucleic acid targets and one or more read sequences, as claimed.

104. Fan’s “barcode probe” is a “nucleic acid molecule which can hybridize specifically to a nucleic acid molecule of interest.” EX1005, 5:17-20, 3:32-35; EX1006, 7:13-15, 4:16-18. Fan’s method of Example 1 and Figure 1 uses padlock probes as barcode probes to tag the target nucleic acids (*i.e.*, cDNA colonies) in a “gene specific manner.” EX1005, 3:34-36; EX1006, 4:16-19. In fact, “each [padlock] probe has two gene-specific ... capturing arms” and it “specifically hybridizes to a sequence of interest.” EX1005, 3:40-42, 9:11-12; EX1006, 4:21-22, 13:18-19.

105. Thus, Fan’s barcode probes comprise a target sequence that specifically hybridizes to a different nucleic acid target or a different location of an identical nucleic acid target of the plurality of different nucleic acid targets, as claimed. This is illustrated below in Fan’s Figure 1 and in my Figure B below generated based on Fan’s Figure 1:



EX1005, FIG. 1, EX1006, FIG. 1.

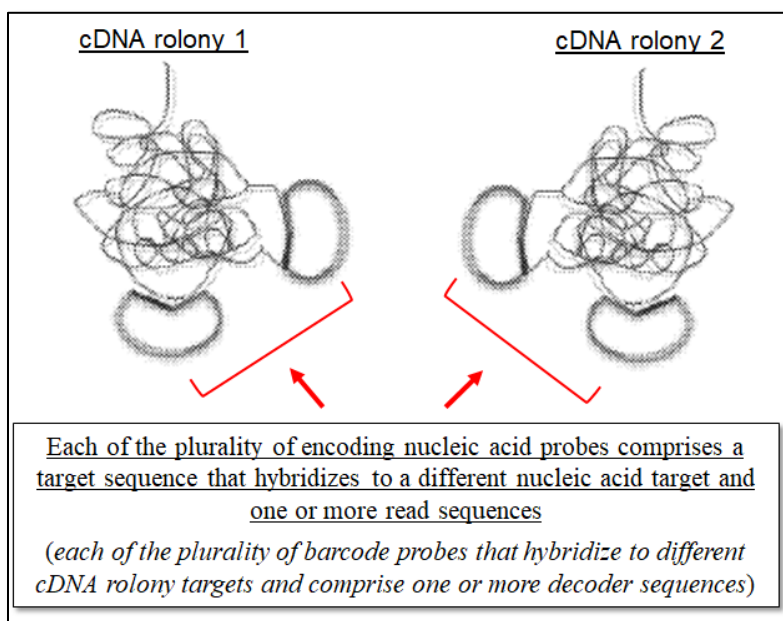


FIG. B.

106. Fan's barcode probes also comprise a "first decoder sequence and a second decoder sequence." EX1005, 5:26-29; *see also* EX1005, 1:63-2:1, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1; EX1006, 7:19-21; *see also* EX1006, 2:2-6, 5:17-25, 13:12-26, 21, 17-22:16 (Example 1), FIG. 1, Claims 1 and 2. A

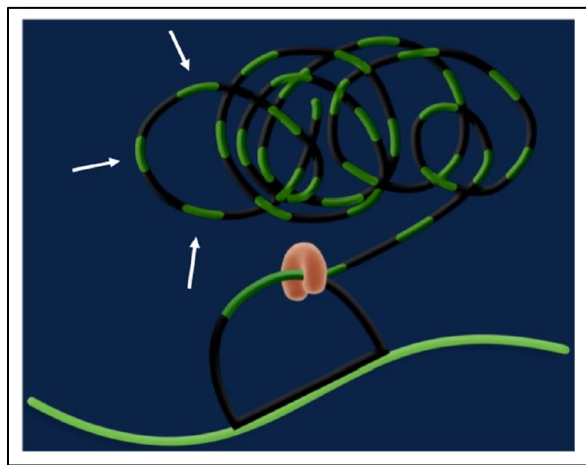
“decoder sequence” is a unique sequence that is “designed to each specifically hybridize to a labeled decoder probe.” EX1005, 8:30-39, 8:45-55, FIG. 1; EX1006, 12:16-21, 12:26-13:3, FIG. 1. Fan’s “decoder probes” are secondary probes that hybridize to its primary probes (barcode probes). EX1005, 8:53-55, 2:14-17; EX1006, 13:1-3, 2:15-17. Thus, a decoder sequence is a sequence in Fan’s primary probes that specifically hybridizes to a sequence on the secondary probes.

107. In the 303 patent’s methods, a “read sequence” on the encoding nucleic acid probe (primary probe) “bind[s] to or hybridize[s] with” a “recognition sequence” present on the readout probe (secondary probe). EX1001, 18:12-14. Thus, the 303 patent’s read sequence is a sequence on primary probes that specifically hybridizes to a sequence on secondary probes. Accordingly, Fan’s decoder sequences—which are present on the primary probes (barcode probes) and hybridize to complementary sequences on the secondary probes (decoder probes)—are the 303 patent’s read sequences. Thus, Fan’s barcode probes comprise one or more read sequences, as claimed.

108. Fan’s barcode probes (*i.e.*, encoding nucleic acid probes) comprise (1) a target sequence that specifically hybridizes to a target nucleic acid, and (2) one or more decoder sequences (*i.e.*, read sequences), as claimed in the 303 patent.

“such that each of the plurality of first hybridized complexes comprises multiple encoding nucleic acid probes of the different encoding nucleic acid probes located in its different locations”

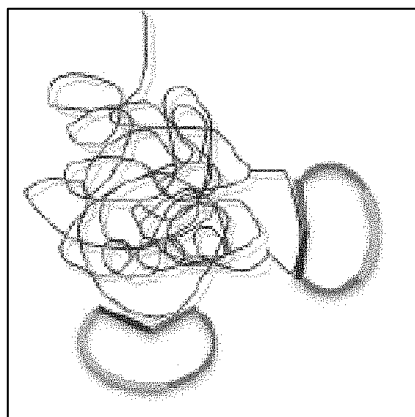
109. I understand that Harvard and Vizgen have alleged in a related litigation that 10x’s product infringes the 303 patent claims. Under Harvard/Vizgen’s interpretation the “such that” clause of claim 1 encompasses a method in which each of the plurality of first hybridized complexes are created by hybridizing multiple copies of the same encoding nucleic acid probe (*i.e.*, barcode probe) at different locations of an identical nucleic acid target



EX1047, 0044.

110. Fan’s method specifically hybridizes barcode probes (*i.e.*, encoding nucleic acid probes of the claim) to a plurality of different nucleic acid targets, such that multiple copies of the same barcode probe are hybridized to multiple locations of an identical nucleic acid target (*e.g.*, cDNA rolony). EX1005, 2:21-36, 7:36-37 9:1-22, FIG. 1, 14:3-48; EX1006, 2:20-30, 21:17-22:16, 13:12-26, 11:1-2,

FIG. 1. For example, the method in Fan's Figure 1 hybridizes multiple padlock probes to each cDNA rolon. This is depicted below in Fan's Figure 1 and my Figure C generated based on Fan's Figure 1:



EX1005, FIG. 1; EX1006, FIG. 1.

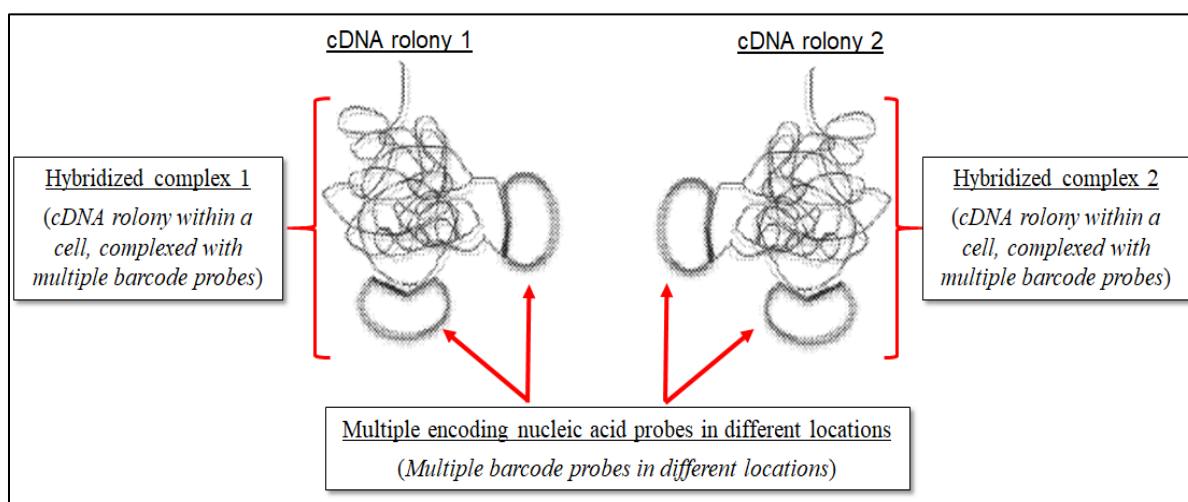


FIG. C.

111. Moreover, Fan discloses hybridizing different barcode probes (*i.e.*, encoding nucleic acid probes of the claim) to multiple locations of a nucleic acid target (e.g, cDNA rolon). For example, Fan discloses that the “amplified nucleic

acids can then be hybridized with *one or more unique padlock probes* with unique molecular barcodes.” EX1005, 9:8-11 (emphasis added); EX1006, 13:17-18.

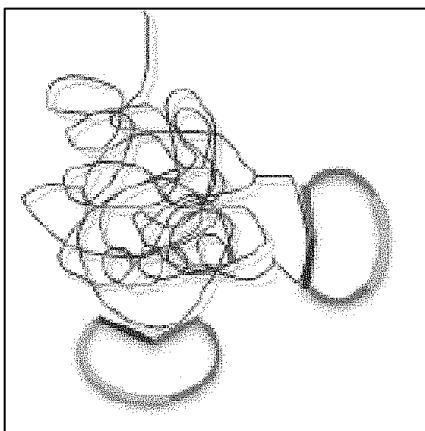
112. Accordingly, Fan’s method produces a plurality of first hybridized complexes (nucleic acid target-barcode probes complex) within a sample, wherein each of the plurality of first hybridized complexes comprises multiple encoding nucleic acid probes (*i.e.*, barcode probes) located in its different locations under Harvard/Vizgen’s interpretation. Thus, in my opinion, Fan’s Method includes this step of claim 1.

iii. “(b) producing a plurality of readout probe-hybridized complexes by exposing each of the plurality of first hybridized complexes within the sample to a first readout nucleic acid probe”

113. In my opinion, Fan’s method includes this step.

114. As I discussed above, the 303 patent’s “readout nucleic acid probe” is a secondary probe that comprises two regions: (1) a “sequence that specifically hybridizes to one of the read sequences” on the encoding nucleic acid probes (*i.e.*, primary probes), and (2) a “fluorescent signaling entity.” EX1001, 8:1-6, 2:28-29 (“secondary nucleic acid probes (*also called readout probes*)” (emphasis added)), 8:3 (“secondary probes (*also called readout probes*)” (emphasis added)), 35:58-60 (“fluorescently labeled secondary nucleic acid probes (*also called readout probes*)” (emphasis added)), 45:7-8 (“*readout probes* (also called secondary probes)” (emphasis added)).

115. After hybridizing the barcode probes to the nucleic acid targets, Fan's method hybridizes a "first labeled decoder probe to the first decoder sequence" of the barcode probes. EX1005, 2:21-49, 8:49-55 ("decoder sequence ... specifically hybridize[s] to a labeled decoder probe"), Claim 1; EX1006, 2:20-30, 12:28-13:3, Claims 1 and 2. Fan also discloses that "[d]uring decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences" in the barcode probes (*i.e.* primary probes). EX1005, 8:49-55; EX1006, 12:28-13:3. Thus, Fan's decoder probes are secondary probes that hybridize to its primary probes (barcode probes), which are bound to the target nucleic acids (cDNA colonies). This is illustrated below in Fan's Figure 1 and in my Figure D generated based on Fan's Figure 1:



EX1005, FIG. 1; EX1006, FIG. 1.

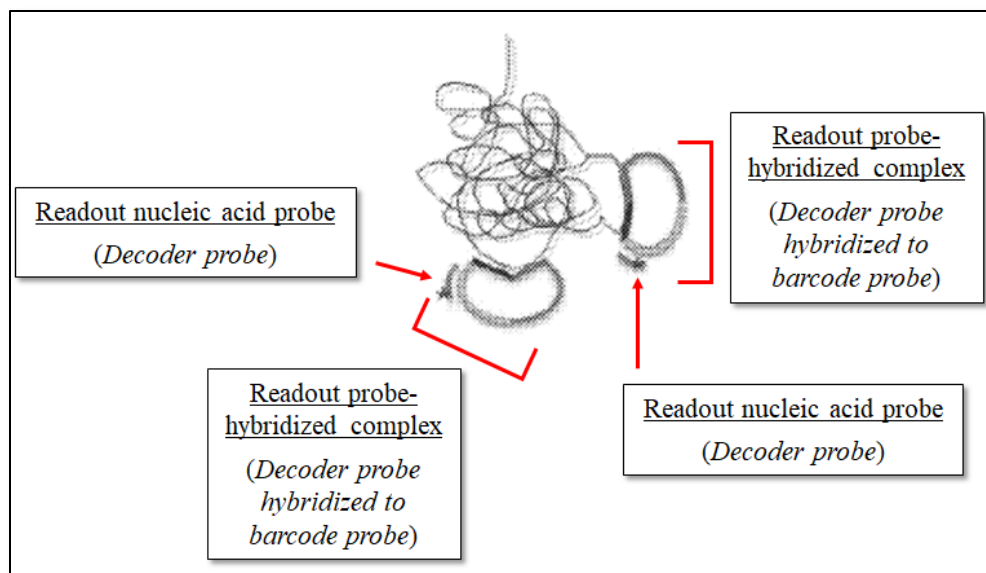


FIG. D.

116. Fan explains that in Figure 1, “a set of barcode probes having decoder sequences [] can be decoded by eight rounds of sequential hybridization with *fluorescently labeled oligos* and imaging of fluorescent images.” EX1005, 9:18-22, FIG. 1, 14:3-48 (Example 1), Claim 1; EX1006, 13:24-26, FIG. 1, 21:15-22:16 (Example 1), Claims 1 and 2.

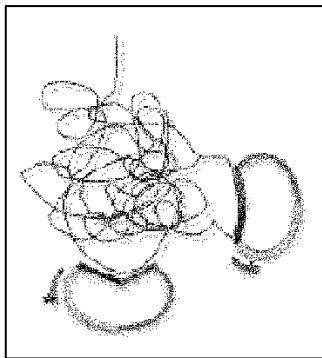
117. Thus, Fan’s method produces a plurality of readout probe-hybridized complexes by exposing each of the plurality of first hybridized complexes within the sample to a first readout nucleic acid probe (*i.e.*, decoder probe).

“wherein the first readout nucleic acid probe comprises a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal”

118. Fan’s decoder probe (*i.e.*, readout nucleic acid probe of the claim) comprises a sequence that specifically hybridizes to decoder sequences (*i.e.*, read sequences of the claim) on the barcode probe (*i.e.*, encoding nucleic acid probe of the claim).

119. Fan explains that, “[d]uring decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences” in the barcode probes. EX1005, 8:49-55; EX1006, 12:28-13:3. Thus, Fan’s decoder probes act as secondary probes and bind the barcode probes, which are bound to the target nucleic acid.

120. Fan’s decoder probes also comprise a second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal because Fan’s “*labeled* decoder probes” are *fluorescently* labeled. EX1005, 9:18-22, FIG. 1, 14:3-48, Claim 1; EX1006, 13:24-26, FIG. 1, 21:17-22:16, Claims 1 and 2. This is depicted below in Fan’s Figure 1 and my Figure E generated based on Fan’s Figure 1:



EX1005, FIG. 1; EX1006, FIG. 1.

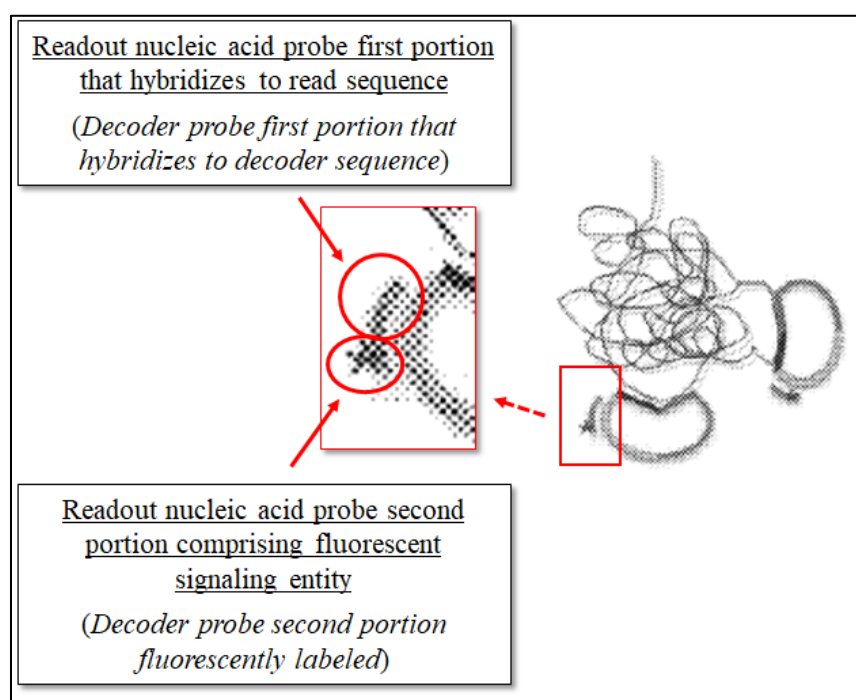


FIG. E

121. Thus, Fan generates a plurality of “readout probe-hybridized complexes” (*i.e.*, complexes of decoder probes hybridized to the barcode probes) by exposing the first hybridized complex (the barcode probes hybridized to the target nucleic acid) to a decoder probe (*i.e.*, a “readout nucleic acid probe”). In addition, Fan’s

decoder probes are fluorescently labeled probes that sequentially hybridize specifically to the decoder sequences (*i.e.*, readout sequence) of the barcode probes to spatially “determine the identity of each barcode probe.” EX1005, 5:14-29, 8:30-39, 8:45-47, 9:18-22, 27:31-28:8; EX1006, 7:12-21, 12:16-21, 12:26-27, 13:23-26.

122. Thus, in my opinion, Fan’s Method includes this step of claim 1.

iv. “(c) detecting a fluorescent signal from each of the plurality of readout probe-hybridized complexes”

123. In my opinion, Fan’s method includes this step.

124. Fan methods include “detecting a plurality of signals indicative of the hybridizing” of the decoder probes to the barcode probes in the previous step. EX1005, 2:21-36; EX1006, 2:20-30. In Figure 1, decoding of the barcode probe takes place by “eight rounds of sequential hybridization [of the decoder probe to the decoder sequence of the barcode probe] with fluorescently labeled oligos and imaging of fluorescent images.” EX1005, 9:18-22, *see also* EX1005, 9:23-43, 11:11-15, 14:3-48; EX1006, 13:24-26, *see also* EX1006, 13:27-14:10, 16:29-17:2, 21:15-22:16.

125. The hybridization of the fluorescently labeled decoder probes to the decoder sequence of the barcode probes emits a fluorescent signal that can be detected and analyzed. If the decoder probe does not hybridize to the first hybridized complex (*i.e.*, the complex formed by hybridizing the barcode probe to

the target nucleic acid), then no fluorescent signal will be generated and detected.

Accordingly, Fan teaches detecting a fluorescent signal from each of the complexes formed by hybridizing the decoder probe to the barcode probe (*i.e.*, the “readout probe-hybridized complexes”). Thus, in my opinion, Fan’s method includes this step of claim 1.

- v. **“(d) removing the fluorescent signal from each of the plurality of readout probe-hybridized complexes by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the plurality of readout probe-hybridized complexes”**

126. In my opinion, Fan’s method includes this step.

127. Under Harvard/Vizgen’s interpretation, this step of claim 1 encompasses a method in which the fluorescently labeled probe is removed. EX1047, 0072-0074 (“the sample then undergoes successive rounds of fluorescent *probe* hybridization, imaging, and *removal*” (emphasis added)), (“sample underwent 15 rounds of fluorescent probe hybridization, imaging, and *probe removal*” (emphasis added)).

128. Fan specifies that “the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” EX1005, 2:56-58; *see also* EX1005, Claim 1 (“de-hybridizing and removing the first fluorescently labeled decoder probe” before hybridizing a second fluorescently labeled decoder probe), 9:25-27 (“decoding process ... involves hybridization and washing of oligo pools at room temperature”); EX1006, 3:14-15, *see also* EX1006, 13:28-30, Claims 1-3.

129. Further, Fan’s methods apply “decoding strategies that entail *sequential hybridizations of labeled decoder probes* to decoder sequences found in immobilized nucleic acids within an individual cell.” EX1005, 8:34-39 (emphasis added), 8:53-59; EX1006, 12:18-21, 13:1-5. Thus, Fan must remove the fluorescently labeled decoder probes from the barcode probes in between rounds of sequential hybridization to accurately detect signal.

130. Indeed, the 303 patent confirms that “removal of the signaling entity” includes removing it “*from the sample, or from the nucleic acid probe, etc.*” and includes using “*washing.*” EX1001, 23:57-24:3 (emphasis added). The patent further states that “the secondary nucleic acid probes are inactivated *or removed.*” EX1001, 8:49-51 (emphasis added), 9:43-51, FIG. 3A.

131. Accordingly, Fan’s method removes the fluorescent signaling entity from each of the plurality of hybridized complexes between the barcode probes and decoder probes (*i.e.*, readout probe-hybridized complexes) by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the plurality of readout probe-hybridized complexes.

132. Thus, Fan teaches claim 1’s Step (d) under Harvard/Vizgen’s interpretation.

- vi. **“(e) after step (d), producing a different plurality of readout probe-hybridized complexes by exposing the sample to a different readout nucleic acid probe,**

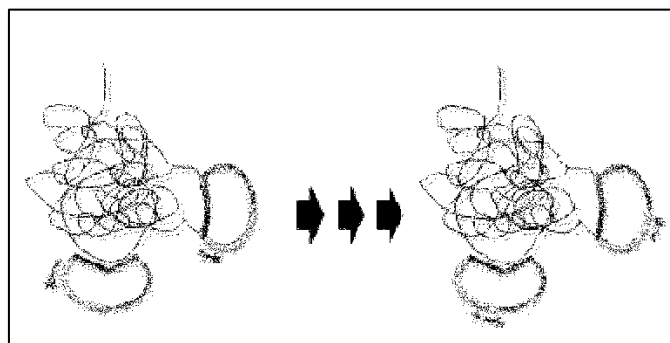
wherein the different readout nucleic acid probe comprise a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal”

133. In my opinion, Fan’s method includes this step.

134. Fan’s barcode probes can comprise “at least a first decoder sequence and a second decoder sequence...” EX1005, 2:8-13, 5:26-29; EX1006, 2:13-14, 7:19-21. Fan’s decoder probes comprise (1) a binding domain that specifically hybridizes to a decoder sequence of the barcode probes, and (2) a fluorescent signaling entity capable of producing a fluorescent signal. EX1005, 27:31-28:8, 8:49-55; EX1006, 12:28-13:3. Accordingly, Fan’s barcode probe can contain two (or more) different decoder sequences, and more than one decoder probe can hybridize to the same barcode probe at different locations (*i.e.*, different decoder sequences) on the barcode probe.

135. After removing the first decoder probe in the previous step, Fan hybridizes a “*second* labeled decoder probe to the *second* decoder sequence” of the barcode probe. EX1005, 2:21-36, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1; EX1006, 2:20-30, 13:12-27, 21:17-22:16 (Example 1), FIG. 1, Claims 1 and 2. Thus, Fan’s method hybridizes in the second hybridization decoder probes that are

different from the decoder probes in the first hybridization. This is shown below in Fan's Figure 1 and my Figure F generated based on Fan's Figure 1:



EX1005, FIG. 1; EX1006, FIG. 1.

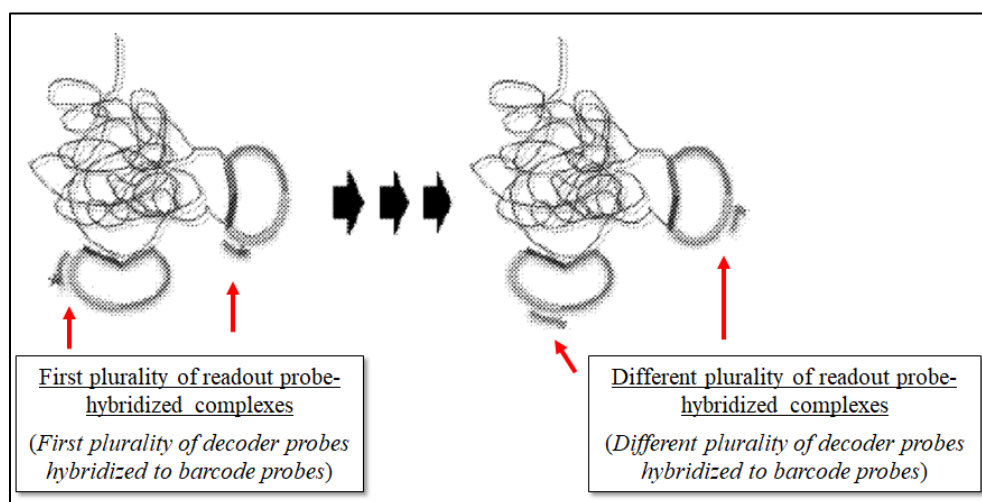


FIG. F.

136. The hybridization of decoder probes to the barcode probes can be repeated several times. For example, Figure 1's method performs "*eight rounds* of sequential hybridization with *fluorescently labeled oligos* and imaging of fluorescent images." EX1005, 9:18-22 (emphasis added); EX1006, 13:23-26. Accordingly, Fan's method produces a different plurality of readout probe-

hybridized complexes by exposing the sample to different readout nucleic acid probes (*i.e.*, decoder probes) comprising a sequence that hybridizes to a read sequence (*i.e.*, decoder sequence) on the encoding nucleic acid probes (*i.e.*, barcode probes) and a fluorescent signaling entity.

137. Thus, Fan teaches claim 1's Step 1(e).

vii. “(f) detecting a fluorescent signal from each of the different plurality of readout probe hybridized complexes”

138. In my opinion, Fan's method includes this step.

139. After the second decoder probe is hybridized to the second decoder sequence of the barcode probe, Fan teaches “detecting a plurality of signals indicative of the hybridizing” of the second decoder probes. EX1005, 2:21-36; EX1006, 2:20-30. As I explained above, this process can occur several times. For example, in Figure 1, “decoding takes place ... by eight rounds of sequential hybridization with *fluorescently labeled oligos* and imaging of *fluorescent images*.” EX1005, 9:18-22, *see also* 9:23-43, 11:11-15, 14:3-48; EX1006, 13:23-27; *see also* EX1006, 13:27-14:10, 16:29-17:2, 21:15-22:16, Claims 1 and 2.

140. Fan also discloses detecting each of the plurality of readout probe hybridized complexes because it specifies separate steps of “detecting a plurality of signals indicative of the hybridizing” after “hybridizing a first labeled decoder probe to the first decoder sequence” and after “hybridizing a second labeled

decoder probe to the second decoder sequence.” EX1005, 2:21-49, Claim 1;

EX1006, 2:20-30, Claims 1 and 2.

141. Accordingly, Fan teaches detecting a fluorescent signal from each of the different plurality of readout probe hybridized complexes formed by hybridizing the second decoder probe to the barcode probe. Thus, Fan teaches claim 1 Step 1(f).

viii. “(g) removing the fluorescent signal from each of the different plurality of readout probe hybridized complexes by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the different plurality of readout probe hybridized complexes”

142. In my opinion, Fan’s method includes this step for the reasons I explain above for claim 1’s Step (d) under Harvard/Vizgen’s interpretation in a related litigation.

143. As I explained above, Fan’s method involves “*sequential hybridizations of labeled decoder probes* to decoder sequences found in immobilized nucleic acids within an individual cell.” EX1005, 8:34-39 (emphasis added), 8:53-59; EX1006, 12:18-21, 13:1-5. For example, Figure 1 utilizes “*eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images*” to decode the barcode probe. EX1005, 9:18-22 (emphasis added), FIG. 1; EX1006, 13:23-26, FIG. 1.

144. Fan teaches removing “the first labeled decoder probe” before “hybridizing the second labeled decoder probe.” EX1005, 2:56-58, 9:25-27 (“decoding process ... involves hybridization and washing of oligo pools at room temperature”), Claim 1 (“de-hybridizing and removing the first fluorescently labeled decoder probe”); EX1006, 3:14-15, 13:28-30, Claims 1-3. Because Figure 1 uses eight rounds of sequential hybridization with fluorescently labeled decoder probes, this process of removing the previous decoder probes before hybridizing the next round of decoder probes must be repeated in between every round of sequential hybridization with fluorescently labeled decoder probes.

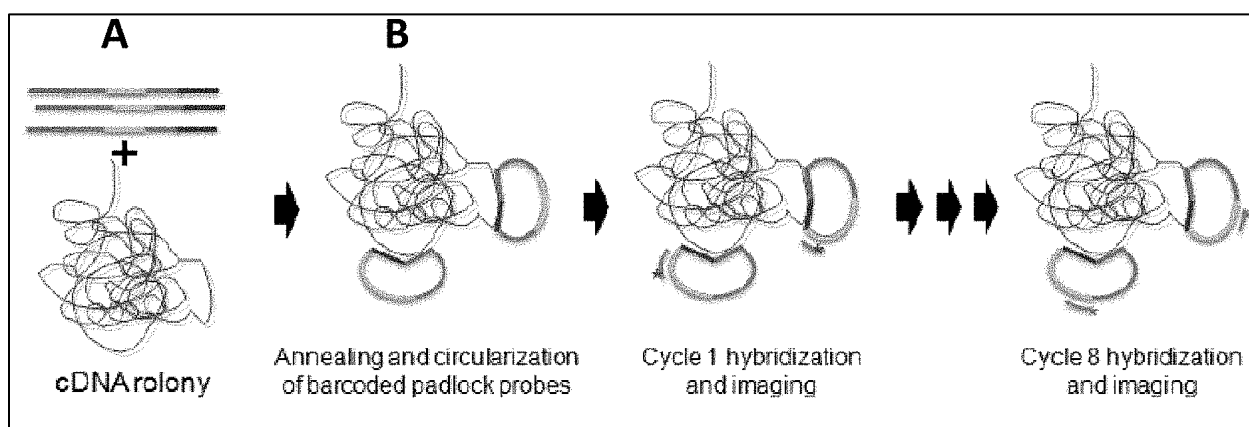
145. Accordingly, Fan’s method includes removing the fluorescent signaling entity the second hybridized complexes between the barcode probes and decoder probes (*i.e.*, second readout probe-hybridized complexes) by removing the fluorescently labeled decoder probes. The 303 patent confirms that “removal of the signaling entity” includes removing it “*from the sample*, or from the nucleic acid probe, *etc.*” and includes using “*washing*,” just like Fan. EX1001, 23:57-66 (emphasis added). The patent further admits that “the secondary nucleic acid probes are inactivated *or removed*.” EX1001, 8:49-51 (emphasis added), 9:43-51, FIG. 3A.

146. Thus, Fan teaches claim 1 Step 1(g) under Harvard/Vizgen’s interpretation.

- ix. **“(h) repeating steps (e) to (g) for one or more times using a different readout nucleic acid probe, wherein the different readout nucleic acid probe comprises a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal”**

147. In my opinion, Fan’s method includes this step.

Fan’s method uses “*sequential hybridizations of labeled decoder probes* to decoder sequences found in immobilized nucleic acids within an individual cell.” EX1005, 8:34-39 (emphasis added), 8:53-59; EX1006, 12:18-21, 13:1-5. For example, Figure 1 utilizes “*eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images*” to decode the barcode probe. EX1005, 9:16-22 (emphasis added); EX1006, 13:22-26. As shown in Figure 1 below, Fan refers to this as “cycle 1” through “cycle 8”:



EX1005, FIG. 1; EX1006, FIG. 1.

148. The decoder probe is shown in Figure 1 as a short bar on top of the barcoded padlock probe (circled in red below). The first decoder probe was

removed from the barcode probe after Cycle 1 because there is no decoder probe in the Cycle 1 position in Cycle 8. I have depicted this in Figure F (copied from above), based on Figure 1.

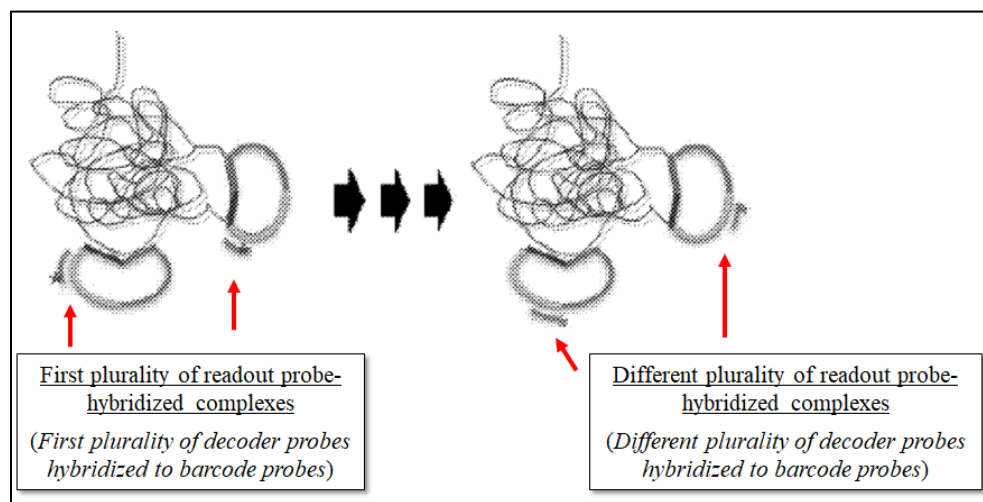


FIG. F.

149. Further, as I discussed above, Fan's methods include removing the fluorescent signal between each round of sequential hybridizations by removing the decoder probes. EX1005, 2:56-58, 9:25-27, Claim 1; EX1006, 3:14-15, 13:28-30, Claims 1-3.

150. Accordingly, Fan's method repeats one or more times hybridizing with fluorescently labeled decoder probes, detecting the fluorescent signal, and removing the fluorescent signal (*i.e.*, Steps (e)-(g)). Thus, Fan discloses claim 1's Step (h).

- x. **“(i) producing codewords representing the plurality of different nucleic acid targets at locations within the sample, wherein each of the codewords represents one of the plurality of different nucleic acid targets and comprises multiple binary values 1 and 0, wherein a value of 1 is obtained when the fluorescent signal is detected from one of the plurality of readout probe hybridized complexes or one of the different plurality of readout probe-hybridized complexes at a respective location within the sample while a value of 0 is obtained when the fluorescent signal is not detected from one of the plurality of readout probe-hybridized complexes or one of the different plurality of readout probe-hybridized complexes at the respective location within the sample”**

151. In my opinion, Fan’s method includes this step.

152. Fan’s method identifies the target nucleic acids by “decoding [] the barcode probes.” EX1005, 1:60-2:1, 4:6-19, 9:1-22, Claim 1; EX1006, 1:30-2:6, 5:16-25, 13:12-27, Claims 1 and 2.

153. When discussing decoding methods, Fan references an article by Gunderson *et al.* (Genome Res. 14:870-877 (2004); “Gunderson”; EX1007) for its decoding and error-checking schemes. EX1005, 3:36-40, 8:56-59, 14:31-34; EX1006, 4:19-21, 13:3-5, 22:3-7. I understand Gunderson’s disclosure about decoding and error-checking schemes is considered part of Fan’s disclosure. I understand that Gunderson is prior art to the 303 patent.

154. Fan identifies with particularity the decoding and error-checking schemes of Gunderson. For example, Fan explains that the “[s]equential hybridization of

decoder probes to decoder sequences [that] is *described in Gunderson et al.*

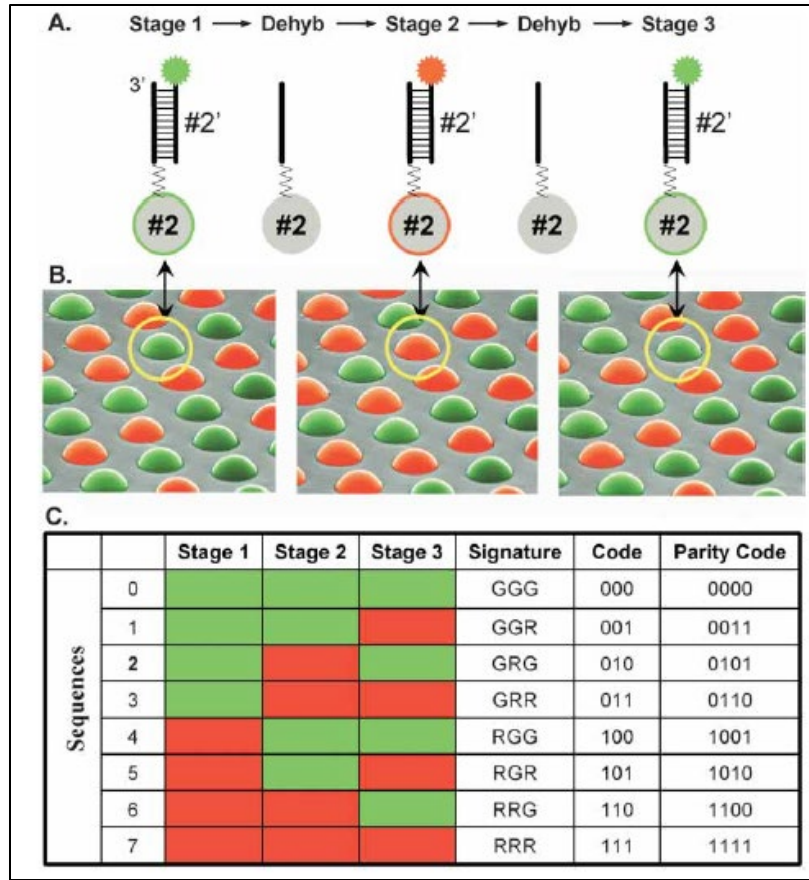
(Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety.” EX1005, 8:56-59 (emphasis added), 3:36-40 (“barcodes can be accurately *decoded by hybridization* similar to the *decoding of ...*

Gunderson et al.”) (emphasis added), 8:34-44, 14:31-36; EX1006, 13:3-5, 4:19-21, 12:18-25, 22:3-17. Further, Fan explains that its “design of the barcoding region *incorporates the error-checking scheme described in the incorporated materials of Gunderson*” and generates a “valid barcode for each gene.” EX1005, 14:31-34 (emphasis added); EX1007; EX1006, 22:3-7. Fan’s Example 1 also specifies using Gunderson’s “three-state, eight-stage decoding scheme” with “two fluorescent (FAM and Cy3) ‘ON’ states and one nonfluorescent OFF state” to decode the nucleic acid targets. EX1005, 14:34-48; EX1006, 22:7-16; EX1007, 871-873.

155. Gunderson discloses an “algorithm to identify each member of a large collection of DNA-linked objects through the use of hybridization.” EX1007, Abstract. Specifically, Gunderson’s “[a]lgorithm uses sequential hybridizations of dye-labeled oligonucleotides, or decoders, complementary to bead sequences to create a combinatorial decoding scheme for arrays.” EX1007, 870. The rounds of sequential hybridization in Gunderson produce a “code” (codeword) for each nucleic acid target on the array.

156. Claim 1 requires “a value of 1 ... when *the fluorescent signal* is detected” and “a value of 0 ... when *the fluorescent signal* is not detected.” EX1001, 86:59-87:6 (emphasis added). Gunderson teaches decoding schemes using codewords comprising binary values of 1 and 0, wherein 1 represents detecting red fluorescence and 0 represents not detecting red fluorescence (and instead detecting green fluorescence). EX1007, 871. This scheme is depicted in Gunderson’s Figure 2. EX1007, 872, FIG. 2, 871.

157. Figure 2 depicts a “[d]ecoding process” where binary codes are generated using sequential hybridizations of fluorophore-labeled decoders to the arrayed beads. EX1007, FIG. 2



EX1007, FIG. 2.

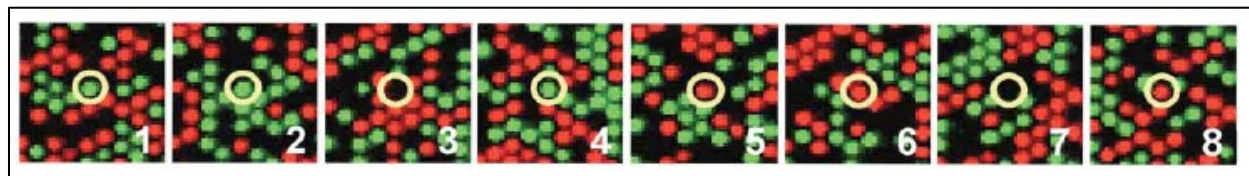
158. Each of Gunderson's beads is a unique DNA sequence that is recognized by its complementary decoder (primary probe). EX1007, 870. The labeled oligonucleotide will generate a response (*e.g.*, emit a fluorescent signal) if it hybridizes to its target sequence. The sequential hybridizations of labeled oligonucleotides (*i.e.*, secondary probe) will produce a pattern that can be converted into a "code" (*i.e.*, codeword) for each target nucleic acid. EX1007, 871. Gunderson teaches reading the fluorescent signal after each hybridization round to reveal a "combinatorial code" (*i.e.*, the codeword from the 303 patent) that

“identifies each bead type,” wherein 1 represents detecting a red signal and 0 represents *not* detecting a red signal (a green signal is detected instead). EX1007, FIG. 2 Legend. Each bead (*i.e.*, location) on the array is assigned a unique code. EX1007, FIG. 2 Legend. For example, the “bead circled in yellow has the color signature GRG or code 010” in binary representation, in which R=1 and G=0. EX1007, 871, FIG. 2 Legend. In other words, this exemplary bead has a color signature of red-signal-*not detected* / red-signal-*detected* / red-signal-*not detected* (code “010”).

159. Under Harvard/Vizgen’s interpretation, Step (i) of claim 1 encompasses a method in which multiple different colors of fluorescent signals are detected and the codeword is made up of different values for each “on” color (A, B, C, and D representing four possible “on” color values in Co-Plaintiffs contentions) and another value is assigned for “off” (no detected fluorescence (E in Co-Plaintiffs’ contentions). EX1047, 0106-0110. Gunderson also discloses a decoding scheme that uses “two fluorescent ‘ON’ states (FAM and CY3 fluorescent labels) and one non-fluorescent ‘OFF’ state.” EX1007, 871, 872; EX1005, 14:34-42; EX1006, 22:7-12. Under this decoding scheme, detection of the green FAM fluorescence or of the Red Cy3 fluorescence are mapped to two different “ON” values (1 and 2 respectively) while detection of no fluorescence “OFF” is mapped to 0. EX1007, 871-873. Gunderson depicts this system in Figure 3, showing 8 cycles of detection

that produce a code of “11012202” (or, On-On-Off-On-On-On-Off-On under

Harvard/Vizgen’s interpretation):



EX1007, FIG. 3A.

160. Gunderson describes a “clear separation between the two modes in the histogram indicat[ing] that most beads can be classified unambiguously as OFF or ON.” EX1007, 872, 873, FIG. 3 Legend, (“[t]he low intensity peak includes beads in the *OFF state* and empty wells. The higher intensity peak includes beads in the *ON state*.”), 876 (“[t]he histogram is *bimodal* because each stage and color channel will have beads that are in the *ON state* and beads that are in the *OFF state*.”) (emphasis added).

161. Accordingly, Fan (incorporating Gunderson) teaches claim 1’s Step (i) under Harvard/Vizgen’s interpretation.

- xi. **“(j) matching the codewords with valid codewords in a codebook by comparing the codewords to the valid codewords in the codebook,**

and if one of the codewords is not matched with one of the valid codewords in the codebook, applying an error detection or correction system, matching the one of the codewords with another of the valid codewords in the codebook, or discarding the one of the codewords,

wherein the codebook comprises the valid codewords of the plurality of nucleic acid targets”

162. In my opinion, Fan, by incorporating Gunderson’s error-detection and error-correction scheme, teaches this step under at least Harvard/Vizgen’s interpretation. As I explain above, Fan incorporates Gunderson’s decoding and error-checking schemes. Gunderson applies “error-checking concepts” “based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986)” “to DNA hybridization.” EX1007, 871, 872; EX1035-EX1037. Gunderson cites specifically to the 1986 book by Hamming that describes Hamming distance and the use of Hamming error detecting and error correcting code systems developed from the earlier Shannon work on information theory.

163. One example of Gunderson’s error-checking system is detailed in Figure 2. Here, the pattern of sequential hybridizations of labeled oligonucleotides (secondary probes) for each bead in the array is converted into a code (codeword) for that bead. EX1007, FIG. 2A-B. Gunderson teaches comparing the generated codewords to the valid codewords in the codebook shown in Figure 2C and

identifying the codewords that match those in the codebook (“valid codes”) and codewords that do not match those in the codebook (“invalid codes”). EX1007, 871-872.

164. Gunderson teaches identifying a sequence (*i.e.*, codeword) for each bead on the array by “referring to the *color-lookup table* in Figure 2C.” EX1007, 871, 872, FIG. 2 (emphasis added). Gunderson’s “color-lookup table” in Figure 2C (shown below) is a codebook because it contains the list of valid codewords for the sequences (*i.e.*, valid codewords of the plurality of nucleic acid targets, as claimed).

C.

		Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code
Sequences	0				GGG	000	0000
	1				GGR	001	0011
	2				GRG	010	0101
	3				GRR	011	0110
	4				RGG	100	1001
	5				RGR	101	1010
	6				RRG	110	1100
	7				RRR	111	1111

EX1007, 871, FIG. 2C.

165. Gunderson’s method uses the color-lookup table (codebook) to identify sequences of beads on the array and determine a “match” by *comparing and determining* whether each bit of a produced codeword is the same as each bit in the

corresponding position of a valid codeword in the codebook. EX1007, 871. For example, Gunderson teaches that the “sequence” of the bead circled in yellow in Figure 2B (with color signature GRG and binary code 010) “can be identified as sequence 2 by referring to the color-lookup table.” EX1007, 871.

166. Harvard/Vizgen’s interpretation of the “and if one of the codewords is not matched...” step encompasses a method that applies “an error detection or correction” system that can detect errors and discard erroneous/unmatched codewords even if that system is not also capable of correcting an error to assign an erroneous/unmatched codeword to a corrected “matched” valid codeword. EX1047, 0114 (“an error detection or correction system is applied (e.g., the decoding algorithm) to either match the decoded transcript to a valid codeword or discard the one of the codewords”).

167. Gunderson also teaches performing an error detection (“error-checking”) step when a codeword from a bead does *not* match the list of valid codewords in the color-lookup table. EX1007, 871. Gunderson explains that “[t]he simplest form of *error checking* we have used has a parity bit and is illustrated in Figure 2C.” EX1007, 871 (emphasis added). In Gunderson’s error-checking scheme, “[t]he assigned four-bit codes all have an even parity bit sum and are termed *valid codes*,” while “[a]n *error* in a single decode stage is a one-bit error that creates an odd parity, or *invalid code*.” EX1007, 871(emphasis added).

168. Similarly, for Figure 3's scheme, Gunderson describes a carefully designed codebook for detecting errors and eliminating "the most common error type." EX1007, 871-72, 875. Rather than using as valid codes all possible codes that could be created using Gunderson's two-color, eight-cycle detection method, Gunderson teaches "designing the decode stages so that every valid code has a fixed number of OFF states" (exactly 6 ON and 2 OFF states at 871-872; and either exactly 6 ON and 2 OFF states or exactly 3 ON and 5 OFF states at 875). EX1007, 871-72, 875. Gunderson teaches that errors are further reduced by removing from the set of valid codes "all codes ending in three or more OFF states" and "any codes that do not have at least one ON state in each of the color channels." EX1007, 875. Gunderson also uses an additional eight detection cycle as an error checking parity bit. EX1007, 871 ("the 1520 bead types can be decoded in only...7 stages. In practice, we use an additional stage to enable error checking.").

169. Thus Gunderson's coding system detects errors at least anytime a detected codeword fails the parity bit check and any time a detected codeword does not contain the predefined required number of ON and OFF states. Gunderson teaches that this technique eliminates the most common error type: "With this scheme, it is theoretically impossible to misclassify a code through any number of occurrences of the most common error type: a transition from an ON state to an OFF state." EX1007, 871. Using Gunderson's code system, Gunderson was able to

detect and calculate a median error rate and determined that Gunderson's system reduced the average misclassification rate by "86-fold" compared to a system without this error detection and prevention rules. EX1007, 872 ("the rate was estimated by using the error checking scheme summarized in Table 1"), Table 1 (disclosing that the "purpose" of the predefined "invalid codes" is to "[d]etect single stage errors."), 873 ("the average misclassification rate would increase 86-fold.").

170. Thus, Fan (incorporating Gunderson) teaches claim 1's Step (j) under at least Harvard/Vizgen's interpretation.

xii. "(k) determining the plurality of nucleic acid targets in the sample based on results from the matching step."

171. In my opinion, Fan's method includes this step.

172. As I explain above, Fan's method teaches determining the "identity of the amplified nucleic acid molecules" "by decoding of the barcode probes." EX1005, 1:60-2:1, 4:6-19; EX1006, 1:30-2:6, 5:16-25. As discussed above, Fan performs decoding by using Gunderson's error-checking schemes that include the matching step. EX1005, 8:53-59 (citing and incorporating by reference EX1007), 3:32-40, 8:34-44, 9:1-22, 14:31-36; EX1006, 13:1-5, 4:16-21, 12:18-25, 13:12-27, 22:3-8; Section IX.a.x and xi. Accordingly, Fan's method determines the plurality of nucleic acid targets in the sample based on the results of the matching step taught in Gunderson's decoding and error-checking schemes.

173. Thus, Fan (incorporating Gunderson) teaches claim 1's Step (k).

174. For the reasons I discuss above, Fan teaches every limitation of claim 1, arranged as claimed, under Harvard/Vizgen's interpretation. I understand that Fan's methods are presumed enabled. Therefore, in my opinion, Fan anticipates claim 1 under Harvard/Vizgen's interpretation.

b. Claim 2

175. Claim 2 depends from claim 1 and further requires that "the fluorescent signal in step (c) and/or step (f) is detected at a resolution better than 300 nm." EX1001, 87:19-21. Fan discloses the elements of claim 2 under Harvard/Vizgen's interpretation.

176. Specifically, Fan teaches detecting the "signals generated by fluorescent imaging of one or more probes during decoding" by "using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ," such as the method disclosed in "Larsson et al., Nat. Methods (2010) 7:395-397 and associated supplemental materials, the entire content of which is incorporated by reference [] in its entirety" in Fan. EX1005, 11:11-19; EX1006, 16:29-7:5. Thus, Fan references Larsson's imaging and detection methods with particularity. I understand Larsson's disclosure is considered to be part of Fan. I understand that Larsson is prior art to the 303 patent.

177. Under Harvard/Vizgen's interpretation, claim 2 encompasses use of a system with a per-pixel resolution of less than 300 nm—an “imager [that] uses a high numerical aperture and a fast area scan camera with a low read noise sensor to achieve ~200 nm per-pixel resolution” is an embodiment that satisfies “wherein the fluorescent signal in step (c) and/or step (f) is detected at a resolution better than 300 nm.” EX1047, 0135.

178. The “per-pixel resolution” is a function of the pixel size of the sensor and the optical magnification of the sample. For example, a camera with a per-pixel size of 4.25 μm (*e.g.*, 4.25 x 4.25 μm square) magnified at 20x has a 0.212 μm (212.5 nm) per-pixel resolution.

179. Under the heading “Image acquisition and analysis,” Larsson also states that, “[i]mages of cultured cells were acquired using an AxioplanII epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp, a *CCD camera (C4742-95, Hamamatsu)*, and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5. A $\times 20$ (*Plan-Apocromat, Zeiss*), $\times 40$ (*Plan-Neofluar, Zeiss*) or $\times 63$ (*Plan-Neofluar, Zeiss*) objective was used for capturing the images.” EX1008, 0005 (emphasis added).

180. The manual for the Hamamatsu C4742-95 CCD camera (“Hamamatsu”) describes the camera as “a high resolution image sensor featuring an effective pixel

number of 4000 (Horizontal) x 2624 (Vertical) pixels.” EX1048, 7. The effective imaging area of the camera is 23.6 mm x 15.5 mm with each pixel having a size of 5.9 μm (5.9 x 5.9 μm square). EX1048, 35. Thus, the resolution of fluorescent signals disclosed in Fan (via Larsson) for the 20x, 40x, and 63x magnifications is 0.295 μm (295 nm), 0.148 μm (148 nm), and 0.094 μm (94 nm) respectively—each of which are at a resolution better than (*i.e.*, less than) 300 nm.

181. Thus, both the maximal optical imaging capabilities and camera collection in Larsson are better than 300 nm. Accordingly, Fan (incorporating Larsson) teaches every element of claim 2 under Harvard/Vizgen’s interpretation.

c. Claim 3

182. Claim 3 depends from claim 1, and further requires that “the sample comprises a cell.” EX1001, 87:22-23. Fan teaches this limitation. Fan discloses the elements of claim 3.

183. Fan discloses detecting the nucleic acid targets within a cell. Indeed, Fan’s method teaches obtaining sequence information for “a plurality of nucleic acid molecules from an individual *cell*.” EX1005, 2:21-36 (emphasis added), 1:58-2:1, 4:6-18, 9:1-22, Example 1; EX1006, 2:20-30, 1:29-2:6, 5:16-17, 13:12-27, Example 1. Fan also explains that its “methods and compositions make use of hybridization, amplification, washing and detection within *individual cells*.” EX1005, 10:62-64 (emphasis added).

184. Thus, Fan teaches every element of claim 3 under Harvard/Vizgen's interpretation.

d. Claim 4

185. Claim 4 depends from claim 3 and further requires that "the cell is fixed on a coverslip." EX1001, 87:24-25. Fan discloses the elements of claim 4.

186. Fan references Larsson's methods for cell permeabilization and fixation. Specifically, Fan teaches using the "[m]ethods for permeabilization and fixation of cells and tissue samples" that were "well-known in the art, as exemplified by ... Larsson et al., Nat. Methods (2010) 7:395-397 and associated supplemental materials, the content of each of which is incorporated herein by reference in its entirety." EX1005, 10:66-11:6; EX1006, 16:19-23. Larsson teaches placing mouse embryo sections on "Superfrost Plus Gold *slides* (Thermo Scientific)" and placing breast tissue sections on "Starfrost microscope slides (Instrumedics)." EX1008, 0004 (emphasis added). Accordingly, Fan (incorporating Larsson) discloses detecting nucleic acid molecules within cells that were fixed on a slide, as claimed. A POSA would have known a cell fixed on a slide includes a cell fixed on a coverslip.

187. Thus, Fan (incorporating Larsson) teaches every element of claim 4 under Harvard/Vizgen's interpretation.

e. Claim 5

188. Claim 5 depends from claim 1. Claim 5 further comprises “determining the fluorescent signal entity by imaging at least a portion of the sample using a light microscope.” EX1001, 87:26-28. Fan discloses the elements of claim 5.

189. Fan teaches detecting the “signals generated by fluorescent imaging of one or more probes during decoding” by “using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ,” such as the method disclosed in “Larsson et al., Nat. Methods (2010) 7:395-397 and associated supplemental materials, the entire content of which is incorporated by reference [] in its entirety” in Fan. EX1005, 11:11-19; EX1006, 16:29-17:5. Thus, Fan identifies with particularity Larsson’s “imaging and detection technologies.”

190. Larsson teaches using “using an AxioplanII epifluorescence microscope (Zeiss) . . . with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5,” to image cultured cells. EX1008, 0005. AxioplanII epifluorescence microscope is a type of fluorescent light microscope. Accordingly, Fan images the sample with a light microscope to detect the fluorescent signal. Thus, Fan (incorporating Larsson) teaches every element of claim 5 under Harvard/Vizgen’s interpretation.

f. Claim 6

191. Claim 6 depends from claim 1. Claim 6 further comprises “determining an abundance and/or spatial distribution of the different nucleic acid targets at locations within the sample.” EX1001, 88:1-3. Fan discloses the elements of claim 6.

192. Fan explains that “the spatial location of nucleic acids can be simultaneously determined for several individual cells in a tissue or other cell-containing sample.” EX1005, 4:32-34; EX1006, 6:5-7. Further, Fan’s methods “comprise evaluating a signal to determine the presence, quantity, frequency, and/or *spatial distribution* of a disease-causing mutation, allele or common variant within a tissue.” EX1005, 3:21-24 (emphasis added); EX1006, 4:8-10. Thus, Fan teaches every element of claim 6 under Harvard/Vizgen’s interpretation.

g. Claim 7

193. Claim 7 depends from claim 1. Claim 7 further comprises “removing the readout nucleic acid probes unhybridized to each of the plurality of first hybridized complex prior to step (c).” EX1001, 88:4-6. Fan discloses the elements of claim 7.

194. As I explain above, Fan’s decoder probes are the 303 patent’s “readout nucleic acid probes.” *See* Section IX.a.iii. Fan teaches removing the decoder probes that are not hybridized to barcode probe-target nucleic acid complexes (*i.e.*, first hybridized complexes of the claim) before detecting the fluorescent signal.

For example, Fan discloses that its methods “make use of hybridization, amplification, washing and detection within individual cells” (i.e., washing takes place *before* detection). EX1005, 10:62-64; EX1006, 16:16-17.

195. In addition, in claim 1, Fan discloses removing the “fluorescently labeled decoder probe that is not hybridized to said first decoder sequence” “by washing.” EX1005, 27:31-34. Fan performs this step before performing step (d), which is “detecting a plurality of fluorescent signals indicative of said hybridizing of the first fluorescently labeled decoder probe to said first decoder sequence.” EX1005, 27:35-37. This process of washing the unhybridized probes from the sample before detecting the fluorescent signals is repeated in the second round of sequential hybridization. EX1005, 28:1-11. Fan further explains that its method “involves hybridization and *washing* of oligo pools at room temperature.” EX1005, 9:25-27 (emphasis added); EX1006, 13:28-30.

196. Moreover, as I discuss above under claim 1, Gunderson’s disclosure about decoding scheme using sequential hybridization is part of Fan’s disclosure. EX1005, 3:36-40, 8:56-59, 14:31-34; EX1006, 4:19-21, 13:3-5, 22:3-7. For example, Fan explains that the “*sequential hybridization* of decoder probes to decoder sequences [that] is *described in Gunderson et al.* (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety.” EX1005, 8:56-59 (emphasis added), 3:36-40, 8:34-44, 14:31-36; EX1006, 13:3-5,

4:19-21, 12:18-25, 22:3-17. Gunderson teaches that “[f]ollowing hybridization, three 1-min washes ... were used to remove unbound oligonucleotides.” EX1007, 875.

197. Accordingly, Fan teaches removing the readout nucleic acid probes (*i.e.*, decoder probes) unhybridized to each of the plurality of first hybridized complex (barcode probe-target nucleic acid complex) prior to detecting the fluorescent signal. Thus, Fan teaches every element of claim 7 under Harvard/Vizgen’s interpretation.

h. Claim 8

198. Claim 8 depends from claim 1. Claim 8 further requires that “the valid codewords form an error-checking or an error-correcting code space.” EX1001, 88:7-8. Fan discloses every element of claim 8.

199. The 303 patent discloses that “codewords may be assigned within a *code space* ... using *an error-detection or an error-correcting system*, such as a Hamming system....” EX1001, 12:46-51 (emphasis added). The patent provides an example in which “the codewords or nucleic acid probes may be assigned within a *code space* such that the assignments are separated by a Hamming distance, which measures the number of incorrect ‘reads’ in a given pattern that cause the nucleic acid probe to be misinterpreted as a different valid nucleic acid probe.” EX1001, 20:51-56 (emphasis added). Thus, claim 8’s requirement that the

“valid codewords form an error-checking or an error-correcting code space”

simply means that valid codewords can be used for error-checking or error-correcting. Fan teaches this limitation through its incorporation of Gunderson, which explicitly references and implements error detection and correction systems of Hamming.

200. As discussed above for claim 1(i) and 1(j), Fan’s method incorporates Gunderson’s (EX1007) error-checking scheme. *See supra*, Section IX.a. Gunderson teaches that its “*error-checking* concepts ... are based on algorithms from digital information theory” and cites Shannon 1948 and Hamming 1986. EX1007, 871. That is, Gunderson teaches using the same error-checking systems disclosed in the 303 patent. Gunderson further discloses that its valid codewords are used for error-checking (*i.e.*, they form an “error-checking codespace”) because Gunderson teaches adding “additional stages ... for error-checking purposes” to its color-lookup table. EX1007, 871. Gunderson further explains that its “assigned four-bit codes all have an even parity bit sum and are termed *valid codes*,” while “[a]n error in a single decode stage is a one-bit *error* that creates an odd parity, or *invalid code*.” EX1007, 871 (emphasis added).

201. Accordingly, Fan (incorporating Gunderson), discloses that the “valid codewords form an error-checking or an error-correcting code space.” Thus, Fan

(incorporating Gunderson) teaches every element of claim 8 under Harvard/Vizgen's interpretation.

i. Claims 9-11

202. Claims 9-11 each depend from claim 1 and further require that “in said comparing the codewords to the valid codewords in the codebook in step (j), one of the codewords is not matched with one of the valid codewords in the codebook when the one of the codewords has one or more errors” (claim 9), or “has one error” (claim 10), or “has two errors” (claim 11). EX1001, 88:9-23. Fan discloses all the elements of claims 9-11.

203. As I explained earlier for claim 1, Fan incorporates Gunderson's decoding and error-checking schemes. Gunderson applies “error-checking concepts” “based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986)” “to DNA hybridization.” EX1007, 871. Gunderson's error-checking system is illustrated in Figures 2 and 3.

204. Gunderson teaches an error-checking scheme that uses a “color-lookup table” (codebook) containing a list of valid codewords, and identifies sequences by comparing and matching a bead's color signature/codeword with valid codewords in the color-lookup table. EX1007, 871, 872. Gunderson discloses that, if a codeword from a bead does *not* match one of the valid codewords in the lookup table, there must be *at least one error*. EX1007, 871 (“[a]n error in a single decode

stage is a one-bit error that creates an odd parity, or invalid code.”), FIG. 2C.

Indeed, Gunderson discloses that one of the “purpose[s]” of the “invalid codes” (codewords that do not match those in the codebook) in Table 1 is to “[d]etect *single* stage errors.” EX1007, 872 (emphasis added). Accordingly, Gunderson teaches that one of the codewords is not matched with one of the valid codewords in the codebook when one of the codewords has one error (claims 9-10) or two errors (claim 11). Gunderson also teaches detecting an error and discarding the erroneous codeword when a detected codeword does not have exactly the prescribed number of ON and OFF cycles; ends in three or more OFF states; or does not have at least one ON state in each of the color channels (any of which can result from either one error or two errors). *See* claim 1(i)-1(j) above.

205. Thus, Fan (incorporating Gunderson) teaches all the elements of claims 9-11 under Harvard/Vizgen’s interpretation.

j. Claim 12

206. Claim 12 depends from claim 1 and further requires that “said comparing the codewords to the valid codewords in the codebook in step (j) comprises comparing the values at each of the positions of the codewords.” EX1001, 88:24-27. Fan discloses all the elements of claim 12.

207. As I explain for claim 1 above, Fan incorporates Gunderson's decoding and error-checking schemes that match the codeword with a valid codeword in a codebook.

208. Gunderson's error-checking scheme in Figure 2 refers to Figure 2C's "color-lookup table" for reference (or valid) codewords for error-checking. EX1007, 871-872, FIG. 2C. Gunderson uses this table as a codebook. Gunderson's Figure 2 identifies a bead on the array by "referring to the color-lookup table in Figure 2C," (*i.e.*, codebook). EX1007, 871, 872, FIG. 2 Legend.

209. To check for errors in Gunderson's error-checking scheme, the values of *each of the positions* of a generated codeword must be compared with that of the valid codewords in the codebook. Accordingly, Gunderson's error-checking scheme inherently compares the values at each position in the codewords for error-checking. Thus, Fan (incorporating Gunderson) teaches every element of claim 12 under Harvard/Vizgen's interpretation.

210. For these reasons, Fan teaches every element of claims 2-12, arranged as claimed, under Harvard/Vizgen's interpretation. I understand that Fan's methods are presumed enabled. Thus, in my opinion, Fan anticipates claims 2-12 under Harvard/Vizgen's interpretation.

X. GROUND 2: CLAIMS 1, 3, and 6-12 WOULD HAVE BEEN OBVIOUS OVER FAN AND GUNDERSON

211. As I explain above under Ground 1, Gunderson's disclosure about decoding and error-checking schemes is considered part of Fan's disclosure. Even if that was not the case and Fan and Gunderson were not considered a single reference, Fan and Gunderson together would have rendered claims 1, 3, and 6-12 obvious under Harvard/Vizgen's interpretation.

212. A POSA would have been motivated to use Gunderson's decoding and error-checking scheme in Fan's methods for several reasons. *Initially*, Fan references Gunderson for its decoding and error-checking system with particularity. EX1005, 3:36-40, 8:53-59, 8:34-44, 9:1-22, 14:31-48; ; EX1006, 4:19-21, 13:1-5, 12:18-25, 13:12-26, 22:5-16. For example, Fan explains that the "sequential hybridization of decoder probes to decoder sequences [that] is described in Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety." EX1005, 8:53-59 (emphasis added); EX1006, 13:1-5. Similarly, Fan explains that its "design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson" and generates a "valid barcode for each gene." EX1005, 14:31-34 (emphasis added); EX1006, 22:5-7.

213. A POSA would have a reasonable expectation of successfully combining the teachings of Fan and Gunderson and using Gunderson's decoding and error-

checking scheme in Fan's method because Fan specifically references those schemes for use in its methods and uses the schemes successfully in its methods. EX1005, 3:36-40, 8:53-59, 14:31-48; EX1006, 4:19-21, 13:1-5, 22:5-16. For example, Fan's method in Example 1 uses Gunderson's ON/OFF decoding scheme for detecting 1700 target genes with eight rounds of hybridization decoding. EX1005, 14:31-48; EX1006, 22:5-16; EX1007, 872-873, FIG. 3.

214. I understand that there is no alleged objective evidence of non-obviousness. Thus, in my opinion, claims 1, 3, and 6-12 would have been obvious over Fan and Gunderson under Harvard/Vizgen's interpretation for the above reasons and the reasons discussed under Ground 1.

XI. GROUND 3: CLAIMS 2, 4, and 5 WOULD HAVE BEEN OBVIOUS OVER FAN, GUNDERSON, AND LARSSON

215. As I explain above under Ground 1, Gunderson's disclosure about decoding and error-checking schemes and Larsson's disclosure about imaging and detection methods are considered part of Fan's disclosure. Even if that was not the case and Fan, Gunderson, and Larsson were not considered a single reference, Fan in combination with Gunderson and Larsson would have rendered claims 2, 4, and 5 obvious under Harvard/Vizgen's interpretation.

216. A POSA would have been motivated to use Gunderson's decoding and error-checking scheme and Larsson's imaging and detection methods in Fan's methods for several reasons. *Initially*, Fan references Gunderson for its decoding

and error-checking system and Larsson's imaging and detection methods with particularity. EX1005, 3:36-40, 8:53-59, 8:34-44, 9:1-22, 11:11-19, 14:31-48; EX1006, 4:19-21, 13:1-5, 12:18-25, 13:12-26, 16:29-17:5, 22:5-16.

217. A POSA would have a reasonable expectation of successfully combining the teachings of Fan, Gunderson, and Larsson and using Gunderson's decoding and error-checking scheme and Larsson's imaging and detection methods in Fan's method. This is because Fan specifically references (i) Gunderson for its decoding and error-checking schemes and successfully uses the schemes in its methods, and (ii) Larsson for its imaging and detection methods. EX1005, 3:36-40, 8:53-59, 11:11-19, 14:31-48; EX1006, 4:19-21, 13:1-5, 16:29-17:5, 22:5-16. For example, Fan's method in Example 1 uses Gunderson's ON/OFF decoding scheme for detecting 1700 target genes with eight rounds of hybridization decoding. EX1005, 14:31-48; EX1006, 22:5-16; EX1007, 872-873, FIG. 3. Similarly, Larsson teaches methods for "Image acquisition and analysis" and specifies acquiring images using an epifluorescence microscope and a computer-controlled filter wheel with excitation and emission filters for visualization of fluorophores. EX1008, 0005.

218. Moreover, the prior art included a host of common microscopy techniques, such as super-resolution microscopy, that used light microscopes and detected molecules within a cell at a resolution better than 300 nm. EX1029, 323; EX1028, 968; EX1030, 16; EX1031, 76; EX1032, 1103; EX1020, 21:16-45;

EX1005, 11:11-19; EX1006, 16:29-17:5; EX1008, 0004; EX1033, 2227. The prior art also included a variety of routine, cell-fixation techniques, including fixing cells on commercially available glass coverslips for imaging under a fluorescent microscope. EX1020, 38:39-52; EX1049, 26:59-61, 27:60-65.

219. I understand that there is no alleged objective evidence of non-obviousness. Thus, claims 2, 4, and 5 would have been obvious over Fan, Gunderson, and Larsson under Harvard/Vizgen's interpretation.

XII. GROUND 4: CLAIMS 1-12 WOULD HAVE BEEN OBVIOUS OVER FAN AND CURRY

220. As I explain above under Ground 1, Fan teaches every element of claims 1-12 under Harvard/Vizgen's interpretation, and thus it anticipates claims 1-12. To the extent it is argued that Fan does not anticipate claims 1-12, the claims would have been obvious over Fan and Gunderson (Ground 2) or Fan, Gunderson, and Larsson (Ground 3) under Harvard/Vizgen's interpretation.

221. Further, as I explain below, Fan and Curry in combination render the claims obvious. Fan's method of imaging sequences and Curry's teachings for generating "combinatorial probe libraries" (similar to those used by Fan) that include multiple different probes to different sequences within the same target analyte (and Curry's accompanying coding/decoding system for Curry's probe libraries) provides additional disclosures relevant to claims 1-12, including but not

limited to step 1(a), the “removing” steps in 1(d) and 1(g), the decoding steps in 1(i) and 1(j), and certain dependent claims.

a. Fan and Curry combination

222. Fan and Curry address the same general concept, problem, and field of endeavor (i.e., multiplexed detection of target molecules in situ) and share many similarities. For example, Fan and Curry are both directed towards detecting a plurality of nucleic acid targets in a biological sample by sequentially hybridizing labeled probes to the sample and determining the identity of an individual target based on the pattern of sequential hybridization. EX1005, 8:31-44, 8:49-59, 9:13-22; EX1006, 12:16-25, 12:28-13:5, 13:17-26,; EX1009, Abstract, 6:17-7:13, 8:19-21, 8:46-9:9, 9:62-10:5, 11:46-60, 12:40-13:6. Both Fan and Curry also apply decoding schemes that generate codewords with on/off values and error detection or error correction schemes that are based on comparing a codeword with valid codewords in a codebook. EX1005, 8:53-59, 3:36-40, 14:31-48; EX1006, 13:1-5, 4:19-21, 22:5-16; EX1009, 12:40-51.

i. Motivation to combine

223. As discussed above, Fan already discloses binding multiple primary probes to a target nucleic acid. *See* Ground 1, claim 1(a). Specifically, Fan discloses that the multiple barcode probes of (encoding nucleic acid probes of the 303 patent’s claim 1) that hybridized to multiple locations of a nucleic acid target

of Fan's method can be different barcode probes. EX1005, 9:8-11 ("The amplified nucleic acids can then be hybridized with *one or more unique padlock probes* with unique molecular barcodes."); *see also* EX1005, 2:1-7 ("the methods comprise first hybridizing one or more different barcode probes to the one or more nucleic acid molecule in situ within a cell..."); EX1006, 13:17-18, 2:6-10.

224. A POSA would have known of multiple advantages from using multiple different probes to different target regions of the same target nucleic acid, including in the context of Fan's teachings. These known benefits include (1) reduced false positive due to off-target binding and reduced false negative due to binding failures and (2) improved signal due to more opportunities for probes to bind to the correct target, both of which improve the quality of the resulting detection and data. In view of Fan's teachings to use multiple probes (including multiple different probes) and/or in view of these advantages known in the art, a POSA would have been motivated to look to Curry. This is because Curry details a detection probe design compatible with Fan that uses multiple different probes to target multiple different locations within the same target nucleic acid analyte.

225. POSAs knew that having multiple distinct sequence binding sites in a target reduces the effect of off-target binding and increases signal specificity. A nucleic acid probe might hybridize off-target to sequences in other molecules that are sufficiently similar to or overlap with the intended target sequence. This would

be particularly problematic if off-target hybridizations occurred in another target, generating false positive signals. A POSA would have known that by binding multiple probes to different sequences of the same target, most probes will bind correctly (even if some probes bind off-target), producing a sufficiently bright signal to identify the target nucleic acid molecule. In contrast, by binding probes to a single sequence on each target creates a bigger risk that the probes will hybridize off-target. This off-target binding problem was well known in the prior art. EX1024, 90-91 (“Typical problems encountered [for FISH probes] include nonspecific binding of probe...”), 82 (recognize the issue of probes binding to “similar nontarget sequences”).

226. Additionally, it was known in the art that different probes require different optimal hybridization conditions (including temperature). Although the optimal conditions can be modeled, that modeling is imprecise and imperfect. Thus, by using multiple different probes to different sequences of the same nucleic acid target increases the chances of successful hybridization of at least some of the probes to a target at the selected hybridization conditions. EX1024, 92 (hybridization conditions are highly dependent on probe characteristics).

227. In a highly multiplexed detection method (such as, in Fan), in which all probes are hybridized simultaneously under the same conditions, some probes will fail to bind. EX1024, 82 (inappropriate hybridization conditions may lower probe

affinity to the target). When probes bind to multiple different sequences of each target, even if some fail to bind, having a greater variety of probes targeting each analyte increases the likelihood that at least some other probe(s) will bind correctly to that target, reducing the chance that probes entirely miss a target.

228. Having looked to Curry for teaching the use of multiple primary probes targeting different target sites (sequences) per nucleic acid target to apply to Fan's method, a POSA would also be motivated to use Curry's teachings for how to decode those same probes using Curry's decoding scheme. Curry's decoding scheme includes Hamming code-based error detection and correction system that uses three parity bits, which also has benefits over Gunderson's disclosed coding schemes—namely, Curry's system allows for the correction of unmatched codewords containing only a single error and the detection of unmatched codewords that contain two errors. EX1009, 12:42-49. As Gunderson, Curry, and the 303 patent all confirm, these Hamming distance code systems (including Hamming-based single error correction, double error detection systems like Curry's) were known in the art, as were the benefits of being able to correct some erroneous detected codewords. EX1007, 871; EX1001, 20:18-24 (Hamming code was known); *see also* EX1044, ¶[0106] (Hamming codes “create a highly robust method that maintains an error-free sample assignment code.”).

ii. Reasonable expectation of success

229. A POSA also would have had a reasonable expectation of success in adapting Fan's primary probes (such as, padlock probes) in view of Curry's teaching to create libraries of primary probes (such as padlock probes) that include multiple different probes to bind to different target sites within the same target analyte. A POSA knew of many examples in the art successfully applying multiple primary probes to different target sequences in the same target nucleic acid. *See, e.g.*, EX1012; EX1015, 836-837, FIGs. 1A-1B; EX1025, 66; EX1013, Abstract; EX1026, 3:37-42; EX1020, 4:35-48; EX1009, 6:17-7:13; EX1019, FIG. 1.

230. Fan's teachings already disclose (at least) the use of multiple barcode probes (such as padlock probes) per target analyte (*see* Ground 1, claim 1(a)). Fan discloses that its methods are compatible with the use of multiple different probes per target (not just multiple instances of the same probe hybridizing with instances of the same sequences within a target). EX1005, 9:8-11; *see also* EX1005, 2:1-7. These art-recognized techniques required no more than ordinary skill and routine methods to implement into Fan's method. *See, e.g.*, EX1012; EX1015, 836-837, FIGs. 1A-1B; EX1025, 66; EX1013, Abstract; EX1026, 3:37-42; EX1020, 4:35-48; EX1009, 6:17-7:13; EX1019, FIG. 1. Researchers already knew how to and had designed multiple padlock probes that hybridize to different target regions of a target nucleic acid. For example, Fan expressly cites and incorporates Deng 2009

when discussing “examples of padlock probes useful for the methods provided herein.” EX1005, 8:1-4. Deng describes the use of padlock probes and teaches that each target “was covered by multiple probes targeting partially overlapping DNA fragments.” EX1027, 355.

231. A POSA would also have a reasonable expectation of success in combining Curry’s error-detection or error-correction system with Fan’s disclosures. Parity-based error detection or correction schemes such as the Hamming code were conventional and widely used, as at least Fan, Gunderson, Curry, and the 303 patent all confirm. EX1005, 14:31-48; EX1006, 22:5-16; EX1007,] 871-872, FIG. 2, 875; EX1009, 12:40-51, Table 2. Gunderson itself already teaches the use of at least one parity bit and describes that Gunderson’s coding system “are based on algorithms from” Hamming. EX1007, 871. Implementing Curry’s Hamming-based coding scheme using three parity bits instead of Gunderson’s one parity bit would have involved only the application of routine and conventional knowledge and would have produced the known and expected benefits of being able to correct single-bit errors while detecting two-bit errors.

b. Claim 1

232. Fan together with Curry’s multiple primary probes to different sequences of the same nucleic acid target and related coding scheme discloses the preamble

and claim elements 1(b)-1(c), 1(e)-1(f), 1(h), and 1(k) under Harvard/Vizgen's interpretation for the same reasons I discuss under Grounds 1-3.

i. Step 1(a)

233. In addition to Fan's teachings discussed under Grounds 1-3, Fan and Curry combined also disclose this step. In my opinion, Fan and Curry together disclose a method that produces a plurality of first hybridized complexes by hybridizing multiple encoding nucleic acid probes at different locations of an identical nucleic acid target (where those locations have different *sequences* that are targeted by the different encoding probes), such that each of complexes comprises multiple encoding nucleic acid probes located in its different location.

234. Fan discloses that in its method, the "amplified nucleic acids can then be hybridized with one or *more unique* padlock probes with unique molecular barcodes." EX1005, 9:8-11 (emphasis added); EX1006, 13:17-18. Thus, Fan discloses hybridizing multiple barcode probes (*i.e.*, encoding nucleic acid probes of the claim) having unique target-binding domains to different sequences of the same nucleic acid target.

235. Curry is directed to identifying nucleic acid targets in biological samples by sequentially hybridizing fluorescently labeled primary probes to the targets and using the hybridization pattern for each target to decode its identity. *See, e.g.*, EX1009, Abstract, 6:17-7:13, 8:19-21, 8:46-9:9, 9:62-10:5, 11:46-60, 12:40-13:6.

Curry's methods "sequentially" hybridize different libraries of probes with a "sample containing a plurality of spatially separated targets," such as RNA and DNA molecules. EX1009, 6:17-35, 10:10-11 ("hybridizing labeled libraries to RNA or DNA molecules contained within the fixed sample"), 11:3-7, Abstract, 1:26-29, 5:8-10, 5:25-31, 9:10-11, 9:21-23. Curry's methods then identify a target "by identifying which combination of the multiple libraries hybridize[s] to that target." EX1009, 6:30-35, 6:22-23 ("The identity of an individual target may be determined by determining which of the libraries hybridize to the target."), Abstract, 5:8-10, 11:46-50.

236. Each of Curry's libraries comprises "probe sets," each of which contains multiple "polynucleotide probes that all specifically hybridize to the same target." EX1009, 5:22-24, 6:23-41, 7:46-47. The libraries also comprise a "different combination of the target-specific probe sets." Thus, the libraries of probes are directed to different targets.

237. Each probe set comprises primary probes that are "*specific for a target*" and "*specifically bind to a particular molecule*," though they "hybridize to different sequences in a target" or "overlap with each other" on a target (so that they are tiled). EX1009, 6:17-31 (emphasis added), 7:24-28, 7:32-45. Each probe set may contain "up to 1,000 or 10,000 or more probes." EX1009, 7:24-28. Thus, each probe set contains probes to the same target. As such, each library comprises

a combination of probe sets, with each probe set directed to a different target and containing multiple probes to different sequences on the same target.

238. Curry's methods sequentially hybridize different primary probe sets directed to different targets, with multiple primary probes hybridizing to the same target at different sequences. A POSA using Curry's teaching of hybridizing multiple probes to different sequences on the same target in Fan's method would have hybridized multiple barcode probes to different sequences on Fan's nucleic acid targets (*i.e.*, amplified nucleic acid targets, such as cDNA colonies).

239. Thus, Fan and Curry combined disclose producing a plurality of first hybridized complexes (*i.e.*, Fan's target-barcode probe complexes modified as in Curry to include probes targeting multiple sequences within a given analyte) within a sample containing a plurality of different nucleic acid targets (*e.g.*, Fan's amplified nucleic acids, such as cDNA colonies) by exposing the sample to a plurality of different encoding nucleic acid probes (*i.e.*, Fan's barcode probes modified as in Curry), such that each of the complexes comprises multiple probes located in its different locations.

ii. Steps 1(d) and 1(g)

240. As I discuss above under Ground 1, steps 1(d) and 1(g) encompass removing the fluorescently labeled probes under Harvard/Vizgen's interpretation. EX1047, 0072-0074. As I explain under Ground 1, Fan's method removes the

fluorescent signaling entity from each of its barcode probe-decoder probe complexes (*i.e.*, readout probe-hybridized complexes of the claim) by removing the decoder probe (*i.e.*, readout probe of the claim) with the signaling entity by washing before the next round of decoder probe hybridization. EX1005, 2:56-58, 9:25-27, Claim 1; EX1006, 3:14-15, 13:28-30, Claims 1- 3. Curry also discloses that its “libraries [of probes] are hybridized successively, measured, then *washed* away to allow the sample to be hybridized to the next library.” EX1009, 9:33-35 (emphasis added). Thus, Fan and Curry combined also disclose steps 1(d) and 1(g) under Harvard/Vizgen’s interpretation.

241. Further, to the extent it is argued that washing and removal in Fan (and, Fan and Curry) do not disclose the “removing...” steps 1(d) and 1(g), these steps were well known to a POSA based on general knowledge (and in view of Fan and/or Curry). A variety of routine methods for removing or inactivating the fluorescent signal (*e.g.*, fluorophore) between rounds of sequential hybridization methods, like those in Fan and Fan and Curry combined, were known in the art. EX1021, ¶[0016]; EX1050, ¶¶[0011], [0097], [00120], [00121], [00164], [00172], [00180]. For example, “removal of the different signal signature produced by the hybridization can be performed by washing, heating, photo-bleaching, displacement (*e.g.*, displacement of decoder probes with another reagent or nucleic acid sequence), cleavage, enzymatic digestion, quenching, chemical degradation,

bleaching, oxidation, or any combinations thereof.” EX1021, ¶[0016]. Photo-bleaching, for example, was known to be fast and effective in inactivating fluorescent signals. EX1050, ¶[0042] (10 seconds of photobleaching reduces signal intensity from ~11% to almost 0).

242. Removing or inactivating fluorescent signals (e.g., by photo-bleaching) is especially suitable for Fan’s probe design because Fan’s barcode probes comprise more than one decoder sequence, and decoder probes from the previous hybridization round need not be physically removed before the next hybridization round is performed. EX1005, 8:47-49.

243. Thus, under Harvard/Vizgen’s interpretation, Fan and Curry together with POSA’s knowledge would render obvious removing the fluorescent signaling entity from each of Fan’s barcode probe-decoder probe complexes (*i.e.*, readout probe-hybridized complexes) by inactivating the signaling entity by photo-bleaching before the next round of decoder probe hybridization.

iii. Steps 1(i) and 1(j)

244. As discussed above under Ground 1, Fan (incorporating Gunderson) discloses decoding the identities of the nucleic acid targets by sequential hybridization of labeled decoder probes (*i.e.*, secondary probes) to the barcode probes (*i.e.*, primary probes) hybridized to the targets according to a decoding scheme with ON/OFF states, such as the decoding and error checking scheme of

Gunderson. EX1005, 8:53-59, 3:36-40, 14:31-48; EX1006, 13:1-5, 4:19-21, 22:5-

16. To the extent it is argued that Fan (incorporating Gunderson) does not disclose these steps under Grounds 1-3 (whether under Harvard/Vizgen's interpretation or some other interpretation), the Fan and Curry combination discloses these steps and renders the challenged claims obvious.

245. Similar to Fan, Curry teaches (1) sequentially hybridizing labeled probes to targets, (2) generating a binary code (codeword) with 1 representing fluorescent signal from probe hybridization and 0 representing absence of fluorescent signal, (3) using a "look-up table" to match detected codewords against valid codewords to determine the identity of the target molecules, and (4) the use of an error detecting and error correcting Hamming code system that is applied to unmatched detected codewords in order to detect errors, correct certain correctable errors, and discard other uncorrectable codewords. EX1009, 11:48-60, Abstract, 6:17-7:13, 5:8-10, 5:17-21, 12:40-51.

246. Curry method uses a "look-up table" that acts as a codebook listing valid binary codes (*i.e.*, codewords) for each target. EX1009, 11:50-60, 4:47-53, 14:29-34, Table 1. The look-up table is prepared before performing the experiment, wherein each target is given a binary code in which 1 represents that the library hybridized in that round included a probe set against that target and 0 represents that the library did not include a probe set against that target. Thus, the codes in

look-up table had binary values with 1 representing detecting a fluorescent signal from a labeled probe to that target and 0 representing *not* detecting a fluorescent signal. Curry provides an exemplary look-up table as Table 1, reproduced below.

TABLE 1				
	L1	L2	L3	L4
P1	0	0	0	1
P2	0	0	1	0
P3	0	0	1	1
P4	0	1	0	0
P5	0	1	0	1

TABLE 1-continued				
	L1	L2	L3	L4
P6	0	1	1	0
P7	0	1	1	1
P8	1	0	0	0
P9	1	0	0	1
P10	1	0	1	0
P11	1	0	1	1
P12	1	1	0	0
P13	1	1	0	1
P14	1	1	1	0
P15	1	1	1	1

EX1009, FIG. 1, 11:50-60.

247. In the above look-up table, a target for probe set P7 has binary code 0111, representing a fluorescent signal in 2nd, 3rd, and 4th hybridization rounds (with probe libraries L2, L3, and L4) and no signal in the 1st hybridization round (with probe library L1). EX1009, 11:50-60.

248. After it is determined which libraries hybridize to a target in the sequential hybridizations, “the identity of the target may be determined matching a ‘code’ which indicates which libraries hybridized to the target (e.g., 1010), with a code in a look-up table.” EX1009, 4:48-53. Thus, Curry’s method performs a step of matching each of the detected codewords against the set of valid codewords in the codebook and determines a “match” by comparing and determining whether each bit of a produced codeword is the same as each bit in the corresponding position of a valid codeword in the codebook (look-up table). When there is a match to a valid codeword (or when an unmatched detected codeword can be corrected), the Fan and Curry system identifies a target analyte based on the analyte identity assigned to that valid codeword in the codebook (look-up table).

249. Fan and Curry also discloses an error detection or correction system based on Hamming code that is able to correct certain errors if correctable or discard the code if the error is not correctable. Curry describes “an error correction system so that if any one hybridization fails, useful data can still be obtained.” EX1009, 12:40-44. Curry discloses that its method includes an “an error correcting (7,4) Hamming code, for example.” EX1009, 12:43-44. A Hamming code is a well-known algorithm from digital information theory that has been applied in other fields. *See, e.g.*, EX1007, 871. Curry provides an exemplary error detecting and correcting scheme in Table 2 (reproduced below).

TABLE 2							
	L1p	L2p	L3	L4p	L5	L6	L7
P1	1	1	0	1	0	0	1
P2	0	1	0	1	0	1	0
P3	1	0	0	0	0	1	1
P4	1	0	0	1	1	0	0
P5	0	1	0	0	1	0	1
P6	1	1	0	0	1	1	0
P7	0	0	0	1	1	1	1
P8	1	1	1	0	0	0	0
P9	0	0	1	1	0	0	1
P10	1	0	1	1	0	1	0
P11	0	1	1	0	0	1	1
P12	0	1	1	1	1	0	0
P13	1	0	1	0	1	0	1
TABLE 2-continued							
	L1p	L2p	L3	L4p	L5	L6	L7
P14	0	0	1	0	1	1	0
P15	1	1	1	1	1	1	1

EX1009, Table 2, 12:40-13:6.

250. In the above table, libraries L3, L5, L6, and L7 encode the data, and libraries L1p, L2p, L4p encode parity bits for error detection and error correction. EX1009, 12:40-13:6. Curry further teaches that using a Hamming system with three parity bits “[a]ny single FP or FN error can be corrected using this scheme, and the occurrence of two errors can be detected.” EX1009, 12:46-49. As Curry teaches, if only one error occurs for a particular detected analyte (“if any one hybridization fails”) then this error detection or correction system means “useful data can still be obtained” because single-error detected codewords can be

corrected to the one (and only one) closest matching valid codeword. EX1009, 12:40-49. Where two errors occur for a particular detected analyte, this system can still detect the error, that is, recognize that the unmatched codeword is erroneous and not valid (as opposed to, *e.g.*, misidentifying the erroneous detected two-error codeword as a valid codeword).

251. A POSA would have recognized that in this Fan and Curry system, detecting two errors in an unmatched codeword means the codeword is not correctable and that the codeword should be discarded. This Fan and Curry system is thus an extended Hamming-based “SECDED” (Single-Error Correction, Double-Error Detection) error detection or correction system of the types disclosed by the 303 patent. EX1001, 11:12-20.

252. Thus, Fan and Curry teach or suggest producing codewords with binary values of 1 and 0, wherein 1 represents a fluorescent signal and 0 represents no signal, matching the codewords with valid codewords in a codebook, and applying an error-detection or correction system to correct errors (if correctable) or discard errors (if not correctable) if one of the codewords is not matched with one of the valid codewords in the codebook.

253. In conclusion, Fan and Curry teach or suggest every limitation of claim 1, and a POSA would have been motivated to perform the methods of claim 1 with a reasonable expectation of success. I understand that there is no alleged objective

evidence of non-obviousness. Thus, in my opinion, claim 1 would have been obvious over Fan and Curry.

c. Claim 2

254. Claim 2 depends from claim 1 and further requires that “the fluorescent signal in step (c) and/or step (f) is detected at a resolution better than 300 nm.” EX1001, 87:19-21. For the reasons I discuss above under Grounds 1 and 3, claim 2 would have been obvious over Fan and Curry under Harvard/Vizgen’s interpretation. If it is argued that Fan and Curry do not render claim 2 obvious for those reasons, the claim would have been obvious over Fan and Curry in view of POSA’s knowledge as discussed below.

255. Fan’s method detects individual nucleic acid molecules in a cell. EX1005, 4:6-9 (“presented herein are methods are[sic] useful for obtaining sequence information at the level of *individual* nucleic acid molecules” (emphasis added)), 9:59-64 (“the methods presented herein have the advantage of virtually limitless numbers of *individually* detected molecules in parallel and in situ” (emphasis added)); EX1006, 5:16-18, 14:22-26. Fan also discloses detecting the fluorescent signals generated in its methods in situ by “using *any suitable imaging and detection technologies* which allow for detection of individual amplicons in situ.” EX1005, 11:11-15 (emphasis added); EX1006, 16:29-17:2.

256. Regular optical microscopes (*e.g.*, fluorescent microscopes) had spatial resolution limited to “approximately half of the wavelength of light used”—*i.e.*, ~ 200 nm. EX1028, 967 (“With the best optics, the resolution of fluorescence microscopy is limited to ~ 200 nm, which cannot resolve many fine cellular structures.”); EX1052, Abstract (“When observed with light, however, structural features smaller than ~ 0.2 μm [*i.e.*, 200 nm)] are blurred and are difficult or impossible to resolve.”); EX1029, 322-323.

257. Further, there were a variety of well-known microscopy and imaging techniques in the prior art, such as super-resolution microscopy, that achieved resolutions better than 200 nm needed for detection of individual molecules. EX1028, 967; EX1031, 76; EX1029, 322-323; EX1052, Abstract, 498; EX1032, 1104. For example, Cai *et al.* in a review article in 2012 noted: “Resolving *molecules* natively in individual cells without separation becomes possible with the advent of *super-resolution microscopy* such as PALM, STORM, FPALM, SSIM and STED, as many cellular components can be resolved down to *nanometer* accuracy.” EX1031, 76 (internal citations omitted) (emphasis added). Further, Leung *et al.* stated in 2011 that STED is “typically limited to 50 to 100 nm for biological samples” and (F)PALM and STORM can easily achieve a “higher resolution (~ 20 nm).” EX1028, 979; *see also* EX1032, 1104 (“For STORM/PALM, the precision to determine the position of a fluorescent molecule

can routinely reach ~10 nm in standard deviation, or ~25 nm in full-width at half-maximum, with some of the best numbers smaller than 5 nm in standard deviation”). Thus, a POSA would have been motivated to use super-resolution microscopy in Fan’s methods for detecting individual nucleic acid molecules at a resolution better than 300 nm.

258. A POSA combining Curry with Fan would have had a reasonable expectation of successfully detecting a fluorescent signal at “a resolution better than 300 nm” because Fan teaches using art-recognized detection and imaging techniques, which include super-resolution microscopy. Super-resolution microscopy techniques were well known in the art and had been successfully used to detect fluorescent signals in a variety of different FISH workflows. EX1049, 24:21-37, 26:9-12, 27:5-9. Accordingly, in my opinion, claim 2 would have been obvious over Fan and Curry under Harvard/Vizgen’s interpretation.

d. Claims 3 and 6

259. As I explain above under Ground 1, Fan discloses all the elements of claims 3 and 6 under Harvard/Vizgen’s interpretation. A POSA would have been motivated to combine Fan and Curry for at least the same reasons I discuss above for claim 1. A POSA would have reasonably expected to successfully arrive at the methods of claims 3 and 6 for at least the same reasons discussed above for claim 1 (Section XII.a) and in Ground 1 (Section IX.c and IXf). Accordingly, claims 3 and

6 would have been obvious over Fan and Curry under Harvard/Vizgen's interpretation.

e. Claim 4

260. Claim 4 depends from claim 3, and further requires that "the cell is fixed on a coverslip." EX1001, 87:24-25. For the reasons I discuss above under Grounds 1 and 3, claim 4 would have been obvious over Fan and Curry under Harvard/Vizgen's interpretation. If it is argued that Fan and Curry do not render claim 4 obvious for those reasons, the claim would have been obvious over Fan and Curry in view of POSA's knowledge as discussed below.

261. Fan discloses using "[m]ethods for permeabilization and fixation of cells and tissue samples" that were "well-known in the art." EX1005, 10:66-11:6; EX1006, 14:19-23. Biological samples (*e.g.*, cells) were routinely fixed on slides or coverslips before they could be visualized under a microscope. EX1008, 0004 (placing tissue sections on "Starfrost microscope *slides*"); EX1050, ¶¶[00167]-[00168], [00208] ("cells and tissues fixed on the bottom coverslip"). Moreover, a POSA would have generally known a variety of routine methods for fixing cells on glass substrates, including glass slides and coverslips. EX1050, ¶¶[00167]-[00168], [00208]; EX1049, 26:59-61. Thus, a POSA would have been motivated to fix cells on a coverslip with a reasonable expectation of success.

262. Accordingly, in my opinion, claim 4 would have been obvious over Fan and Curry.

f. Claim 5

263. Claim 5 depends from claim 1 and further comprises “determining the fluorescent signal entity by imaging at least a portion of the sample using a light microscope.” EX1001, 87:26-28. For the reasons I discuss above under Grounds 1 and 3, claim 5 would have been obvious over Fan and Curry under Harvard/Vizgen’s interpretation. If it is argued that Fan and Curry do not render claim 5 obvious for those reasons, the claim would have been obvious over Fan and Curry in view of POSA’s knowledge as discussed below.

264. As I explain above for claim 2, a POSA would have been motivated to use super-resolution microscopy to detect the fluorescent signal at a resolution better than 300 nm with a reasonable expectation of success. *See* Section XII.c (Ground 4). Super-resolution microscopy techniques, such as STORM and (F)PALM, use a light microscope. Accordingly, in my opinion, claim 5 would have been obvious over Fan and Curry.

g. Claim 7

265. Claim 7 depends from claim 1 and further comprises “removing the readout nucleic acid probes unhybridized to each of the plurality of first hybridized complex prior to step (c).” EX1001, 88:4-6. For the reasons I discuss above under

Grounds 1 and 2, claim 7 would have been obvious over Fan and Curry under Harvard/Vizgen's interpretation. If it is argued that Fan and Curry do not render claim 7 obvious for those reasons, the claim would have been obvious over Fan and Curry in view of POSA's knowledge as discussed below.

266. As I discuss above under Ground 1, Fan discloses that its methods "make use of hybridization, amplification, washing and detection within individual cells." EX1005, 10:62-64, 9:25-27, Claim 1; EX1006, 16:16-17, 13:28-30. Thus, Fan's method performs "washing" before "detection."

267. Moreover, fluorescent in situ hybridization (FISH) techniques require "washing steps to remove unbound probes which would otherwise interfere with the desired signal." EX1024, 81; *see also* EX1007, 875 ("Following hybridization, three 1-min washes ... were used to remove unbound oligonucleotides."). In fact, Curry also discloses that detection assays involve "contacting a labeled target-specific probe ... with a sample" and after "unbound probe is removed, e.g., by washing, the signal emanating from [the] probe's label is detected." EX1009, 1:5-10, 10:8-8-21 ("washing the hybridized sample to remove non-specific binding").

268. Thus, a POSA would have been motivated to remove the readout nucleic acid probes (*i.e.*, Fan's decoder probes) unhybridized to each of the plurality of first hybridized complex (*i.e.*, Fan's target-barcode probe complex) prior to

detecting a fluorescent signal in step (c). Accordingly, in my opinion, claim 7 would have been obvious over Fan and Curry.

h. Claim 8

269. Claim 8 depends from claim 1 and further requires that “the valid codewords form an error-checking or an error-correcting code space.” EX1001, 88:7-8.

270. As I discuss above under Ground 1, claim 8’s requirement that the “valid codewords form an error-checking or an error-correcting code space” simply means that valid codewords can be used for error-checking or error-correcting. For the reasons I discuss above under Grounds 1 and 2, claim 8 would have been obvious over Fan and Curry under Harvard/Vizgen’s interpretation. If it is argued that Fan and Curry do not render claim 8 obvious for those reasons, the claim would have been obvious over Fan and Curry in view of POSA’s knowledge as discussed below.

271. The 303 patent discloses that “codewords may be assigned within a *code space* ... using *an error-detection or an error-correcting system*, such as a Hamming system ... or an extended Hamming system or a SECDED system (single error correction, double error detection.)” EX1001, 12:46-51 (emphasis added).

272. As I explain above for claim 1 under Ground 4, Curry discloses using an “error correction system so that if any one hybridization fails, useful data can still be obtained.” EX1009, 12:40-42; *see* Section XII.b.iii. Further, as I explain above for claim 1, Curry discloses using a Hamming system, including using an extended Hamming-based “SECDDED” (Single-Error Correction, Double-Error Detection) error-detection or error-correction system of the same type disclosed by the 303 patent. EX1009, 12:40-49; *see* Section XII.b.iii. Curry provides an exemplary error-detection or error-correction system in Table 2. EX1009, 12:40-13:6. Table 2 provides valid codes (*i.e.*, codewords) for each target including “parity bits” for error checking and error correction. EX1009, 12:42-44. Curry further states that “[a]ny single FP [*i.e.*, false positive)] or FN [*i.e.*, false negative)] error can be corrected using this scheme, and the occurrence of two errors can be detected.” EX1009, 12:46-48.

273. Thus, Curry discloses that its valid codewords are used for error-checking or error-correcting (*i.e.*, they form an “error-checking codespace”).

274. A POSA would have combined Fan and Curry for at least the reasons I discuss above for claim 1. A POSA would also have had a reasonable expectation of successfully practicing the method of claim 8 because Curry teaches that its valid codewords are used for error-checking (*i.e.*, in a codespace) and provides

examples of successfully using that codespace. Accordingly, in my opinion, claim 8 would have been obvious over Fan and Curry.

i. Claims 9-11

275. Claims 9-11 each depends from claim 1 and further requires that “in said comparing the codewords to the valid codewords in the codebook in step (j), one of the codewords is not matched with one of the valid codewords in the codebook when the one of the codewords has one or more errors” (claim 9), or “has one error” (claim 10), or “has two errors” (claim 11). EX1001, 88:9-23. For the reasons I discuss above under Grounds 1 and 2, claims 9-11 would have been obvious over Fan and Curry under Harvard/Vizgen’s interpretation. If it is argued that Fan and Curry do not render claims 9-11 obvious for those reasons, the claim would have been obvious over Fan and Curry in view of POSA’s knowledge as discussed below.

276. As I discuss above for claims 1 and 8, Curry discloses using a Hamming system, including using an extended Hamming-based “SECDED” (Single-Error Correction, Double-Error Detection) error-detection or error-correction system of the same type disclosed by the 303 patent. EX1009, 12:40-49; *see* Section XII.b.iii. Curry discloses an “error correction system so that if any one hybridization fails, useful data can still be obtained.” EX1009, 12:40-42. Curry provides an exemplary error-correcting system in Table 2 that includes valid codes for each target

including “parity bits” for error checking and error correction. EX1009, 12:40-44.

Curry further states that “[a]ny single FP [(i.e., false positive)] or FN [(i.e., false negative)] error can be corrected using this scheme, and the occurrence of two errors can be detected.” EX1009, 12:46-48.

277. Thus, Curry teaches that one of the codewords is not matched with one of the valid codewords in the codebook when one of the codewords has one error (claims 9-10) or two errors (claim 11). Accordingly, in my opinion, claims 9-11 would have been obvious over Fan and Curry.

j. Claim 12

278. Claim 12 depends from claim 1 and further requires wherein “said comparing the codewords to the valid codewords in the codebook in step (j) comprises comparing the values at each of the positions of the codewords.”

EX1001, 88:9-27. For the reasons I discuss above under Grounds 1 and 2, claim 12 would have been obvious over Fan and Curry under Harvard/Vizgen’s interpretation. If it is argued that Fan and Curry do not render claim 12 obvious for those reasons, the claim would have been obvious over Fan and Curry in view of POSA’s knowledge as discussed below.

279. As I discuss above for claim 1 under Ground 4, Curry discloses decoding and error-checking schemes. Curry’s error-checking scheme is exemplified in a

“look-up table,” Table 1. EX1009, 11:46-60. Curry uses Table 1 as a codebook of valid codes (codewords) for error-checking.

280. Curry further discloses that “the identity of the target may be determined matching a ‘code’ which indicates which libraries hybridized to the target (e.g., 1010), with a code in a look-up table.” EX1009, 4:47-53. As such, to check for errors, Curry compares values of *each of the positions* of a generated code with that of the valid code in the look-up table.

281. Thus, a POSA would have been motivated to compare the values at each of the positions of the codewords (codes) generated by sequential hybridization to each of the positions of the valid codewords (codes) in the look-up table in Fan and Curry’s method with a reasonable expectation of success. Accordingly, in my opinion, claim 12 would have been obvious over Fan and Curry. In conclusion, Fan and Curry teach or suggest every limitation of claims 2-12, and a POSA would have been motivated to perform the methods of these claims with a reasonable expectation of success. I understand that there is no alleged objective evidence of non-obviousness. Thus, in my opinion, claims 2-12 would have been obvious over Fan and Curry.

XIII. CONCLUSION

282. I conclude that in view of the state of the art before July 30, 2014, Fan teaches each and every element of claims 1-12 arranged as claimed. I understand

that Fan is presumed enabled. Thus, for the reasons detailed under Ground 1, Fan anticipated claims 1-12 of the 303 patent under Harvard/Vizgen's interpretation.

283. Fan and Gunderson teach or suggest all the elements of the methods of claims 1, 3, and 6-12 and a POSA would have been motivated to perform the methods of these claims with a reasonable expectation of success. Thus, for the reasons detailed under Ground 2, Fan in view of Gunderson would have rendered claims 1, 3, and 6-12 of the 303 patent obvious under Harvard/Vizgen's interpretation.

284. Fan, Gunderson, and Larsson teach or suggest the all elements of the methods of claims 2, 4, and 5 and a POSA would have been motivated to perform the methods of these claims with a reasonable expectation of success. Thus, for the reasons detailed under Ground 3, Fan in view of Gunderson and Larsson would have rendered claims 2, 4, and 5 of the 303 patent obvious under Harvard/Vizgen's interpretation.

285. Fan and Curry teach or suggest all the elements of the methods of claims 1-12 and a POSA would have been motivated to perform the methods of these claims with a reasonable expectation of success. Thus, for the reasons detailed under Ground 4, Fan in view of Curry would have rendered claims 1-12 of the 303 patent obvious under Harvard/Vizgen's interpretation.

Inter Partes Review of U.S. Patent 11,098,303
Declaration of Paul T. Spellman, Ph.D.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the U.S. Code.

I declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

Respectfully submitted,

A handwritten signature in dark ink, appearing to read 'Paul T. Spellman', followed by a long horizontal flourish line extending to the right.

Paul T. Spellman, Ph.D.

Date: August 30, 2023

APPENDIX A¹

303 Patent Claim	Fan (EX1005)
1. A method for detecting a plurality of different nucleic acid targets within a sample by in situ hybridization, comprising:	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“In some embodiments, the methods provided herein allow for detection of a single target nucleotide species of interest. In other embodiments, the methods allow for multiplex detection of a plurality of nucleotide species of interest. The use of barcode probes with unique identifier sequences as described above allows for simultaneous detection of 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000 or more than 10,000 unique nucleic acid target molecules within a single cell.” (9:49-59)</p>

¹ Emphasis added in bold.

303 Patent Claim	Fan (EX1005)
	<p>“In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes.” (9:4-11)</p> <p>“For up to 1700 target genes, eight rounds of hybridization decoding are performed.” (14:34-36)</p> <p><i>See also</i> 1:63-2:1, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1.</p>
(a) producing a plurality of first hybridized complexes within a sample containing a plurality of different nucleic acid targets by exposing the sample to a plurality of different encoding nucleic acid probes	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p><i>See also</i> 1:63-2:1, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1.</p> <p>“In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific</p>

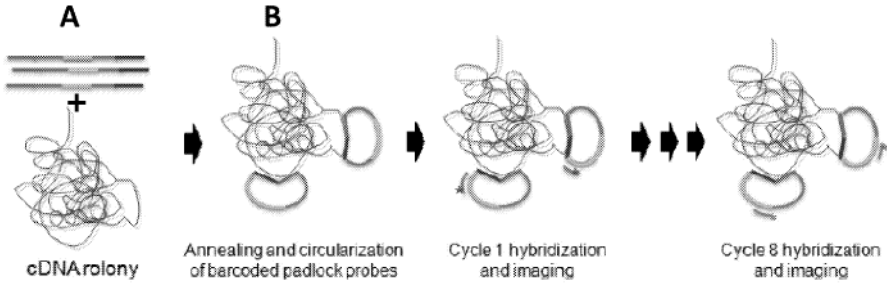
303 Patent Claim	Fan (EX1005)
	<p>barcodes using a library of padlock probes (FIG. 1B).” (14:15-18)</p> <p>“In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes.” (9:4-11)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes. Padlock probes are specialized ligation probes and are known in the art....” (7:36-38)</p> <div data-bbox="516 1018 1396 1302"> <p style="text-align: center;">A B</p> <p style="text-align: center;">cDNA rolony Annealing and circularization of barcoded padlock probes Cycle 1 hybridization and imaging Cycle 8 hybridization and imaging</p> </div> <p>(FIG. 1)</p>
<p>wherein each of the plurality of encoding nucleic acid probes comprises (i) a target sequence that specifically hybridizes to a different nucleic acid target or a</p>	<p>“As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and which comprises a barcode sequence.” (5:17-20)</p> <p>“FIG. 1 describes one embodiment of padlock capture and hybridization decoding of rolones. (A) Molecular barcodes are attached to rolones in a gene specific manner using padlock probes. (B) After padlock capture, each rolony will have a unique barcode in thousands of copies. These barcodes can be accurately decoded by hybridization similar</p>

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<p>different location of an identical nucleic acid target of the plurality of different nucleic acid targets and (ii) one or more read sequences</p>	<p>to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004)). (C) Schematic of padlock probes. In the example shown, each probe has two gene-specific (and/or allele-specific) capturing arms H1/H2.” (3:32-42)</p> <p>“When a padlock probe specifically hybridizes to a sequence of interest....” (9:11-12)</p> <div data-bbox="516 772 1398 1056" data-label="Diagram"> <p>The diagram illustrates the process of using padlock probes for sequencing. It consists of four panels labeled A, B, and two unlabeled panels representing Cycle 1 and Cycle 8. Panel A shows a cDNA library (represented by a tangled line) and a target sequence (represented by three horizontal lines). Panel B shows the padlock probe (a circular structure with two arms) hybridizing to the target sequence. The subsequent panels show the probe circularizing and then hybridizing to the target sequence in subsequent cycles (Cycle 1 and Cycle 8), with imaging at each step.</p> </div> <p>(FIG. 1)</p> <p>“The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (5:26-29)</p> <p><i>See also</i> 1:63-2:1, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1.</p> <p>“The methods and compositions presented herein make use of unique barcode probes having decoder sequences to identify multiple nucleic acid targets in a multiplex fashion. The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to</p>

303 Patent Claim	Fan (EX1005)
	<p>decoder sequences found in immobilized nucleic acids within an individual cell.” (8:30-39)</p> <p>“In some embodiments, a barcode probe can comprise at least a first decoder sequence and a second decoder sequence. In some embodiments, a barcode probe can comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 decoder sequences. As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:45-55)</p> <p>“...hybridizing a first labeled decoder probe to the first decoder sequence; detecting a signal indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence....” (2:14-17).</p>
<p>such that each of the plurality of first hybridized complexes comprises multiple encoding nucleic acid probes of the different encoding nucleic acid probes located in its different locations</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence</p>

303 Patent Claim	Fan (EX1005)
	<p>information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes. Padlock probes are specialized ligation probes and are known in the art....” (7:36-38)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate</p>

303 Patent Claim	Fan (EX1005)
	<p>decoding of randomly assembled bead arrays. In some embodiments, rolonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is</p>

303 Patent Claim	Fan (EX1005)
	<p>sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (14:3-48)</p>  <p>(FIG. 1)</p>
<p>(b) producing a plurality of readout probe-hybridized complexes by exposing each of the plurality of first hybridized complexes within the sample to a first readout nucleic acid probe</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are</p>

303 Patent Claim	Fan (EX1005)
	<p>sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:49-55)</p> <div data-bbox="516 575 1398 856"> <p>The diagram illustrates the decoding process in four stages: A: A cDNA roloni (represented by a tangled line) is shown with a plus sign above it. B: The process moves to 'Annealing and circularization of barcoded padlock probes', showing a padlock probe (a circular structure with a tail) binding to the cDNA roloni. Cycle 1 hybridization and imaging: The first cycle of hybridization and imaging is shown. Cycle 8 hybridization and imaging: The final cycle of hybridization and imaging is shown. Arrows indicate the sequential progression from A to B, then to Cycle 1, and finally to Cycle 8.</p> </div> <p>(FIG. 1)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, rolonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials</p>

303 Patent Claim	Fan (EX1005)
	<p>disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a colony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate (<10⁻⁴).” (14:3-48)</p> <p><i>See also</i> Claim 1.</p>
wherein the first readout nucleic acid probe comprises a first portion and a second portion,	<p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to</p>

303 Patent Claim	Fan (EX1005)
<p>the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal</p>	<p>specifically determine the identity of each barcode sequence.” (8:49-55)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, colonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, colonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated</p>

303 Patent Claim	Fan (EX1005)
	<p>materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a colony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (14:3-48)</p> <p><i>See also</i> Claim 1.</p> <div data-bbox="516 1157 1398 1440" data-label="Diagram"> <p>The diagram illustrates the eight-stage barcoding scheme for transcript decoding. It begins with a cDNA colony (A) and proceeds through four stages: 1. Annealing and circularization of barcoded padlock probes (B), 2. Cycle 1 hybridization and imaging, 3. Cycle 2 hybridization and imaging, and 4. Cycle 8 hybridization and imaging. The diagram shows the progression of the barcoding process, with the final stage showing a complex, multi-colored structure representing the decoded transcript.</p> </div> <p>(FIG. 1)</p> <p>“The methods provided herein further comprise annealing in situ a barcode probe to the amplified nucleic acid molecule. As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and which comprises a barcode sequence. As used herein, the terms barcode, barcode sequence and like terms refer to a unique identifier sequence on a probe</p>

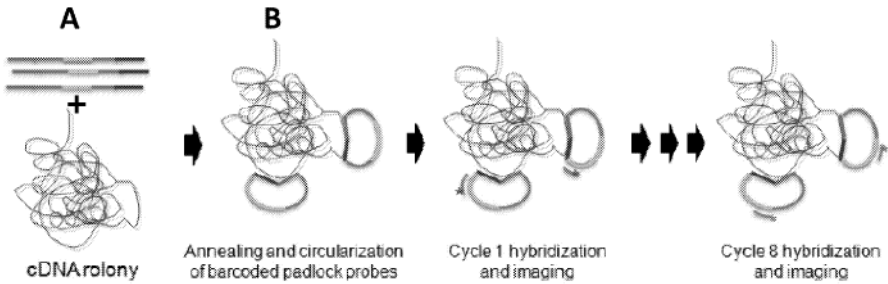
303 Patent Claim	Fan (EX1005)
	<p>molecule. The unique identifier sequence can be, for example, a nucleic acid sequence which associates the barcode probe with the nucleic acid of interest to which it hybridizes. The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence." (5:14-29)</p> <p>"The methods and compositions presented herein make use of unique barcode probes having decoder sequences to identify multiple nucleic acid targets in a multiplex fashion. The instant innovations overcome limitations of existing rolonry identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell." (8:30-39)</p> <p>"In some embodiments, a barcode probe can comprise at least a first decoder sequence and a second decoder sequence." (8:45-47)</p>
(c) detecting a fluorescent signal from each of the plurality of readout probe-hybridized complexes	<p>"Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating</p>

303 Patent Claim	Fan (EX1005)
	<p>sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p><i>See also</i> (9:23-43), (11:11-15), (14:3-48).</p>
(d) removing the fluorescent signal from each of the plurality of readout probe-hybridized complexes by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the plurality of readout probe-hybridized complexes	<p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (2:56-58)</p> <p><i>See also</i> Claim 1, (9:25-27).</p> <p>“The instant innovations overcome limitations of existing colony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (8:34-39)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:53-59)</p>
(e) after step (d), producing a different plurality of readout probe-hybridized	<p>“...annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence....” (2:12-13)</p>

303 Patent Claim	Fan (EX1005)
<p>complexes by exposing the sample to a different readout nucleic acid probe,</p> <p>wherein the different readout nucleic acid probe comprise a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal</p>	<p>“The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (5:26-29)</p> <p><i>See also</i> Claim 1.</p> <p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:49-55)</p> <p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of</p>

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	<p>the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, rolonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009)</p>

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	<p>and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolon can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (14:3-48)</p> <p><i>See also</i> Claim 1.</p>

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	 <p>(FIG. 1)</p>
(f) detecting a fluorescent signal from each of the different plurality of readout probe hybridized complexes	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p><i>See also</i> (9:23-43), (11:11-15), (14:3-48), Claim 1.</p>

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(g) removing the fluorescent signal from each of the different plurality of readout probe hybridized complexes by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the different plurality of readout probe hybridized complexes	<p>“The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (8:34-39)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:53-59)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (2:56-58)</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (9:25-27)</p> <p><i>See also</i> Claim 1.</p>
(h) repeating steps (e) to (g) for one or more times using a different readout nucleic acid	<p>“The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to</p>

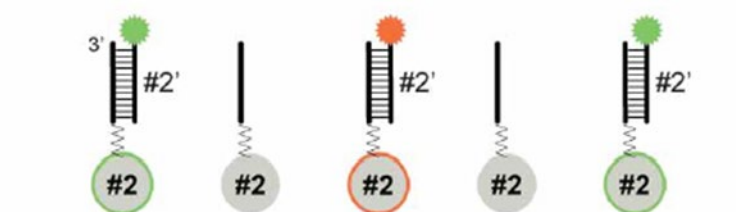
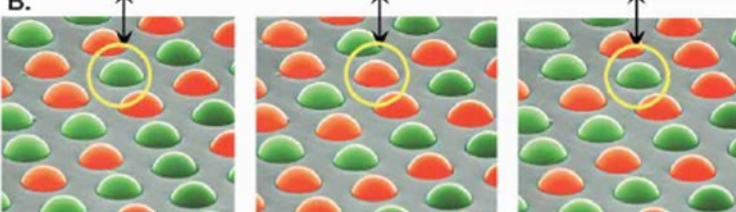
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<p>probe, wherein the different readout nucleic acid probe comprises a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal</p>	<p>decoder sequences found in immobilized nucleic acids within an individual cell.” (8:34-39)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:53-59)</p> <p>“...and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:16-22)</p> <div data-bbox="516 1192 1398 1476" data-label="Diagram"> <p>The diagram illustrates the decoding process in four stages:</p> <ul style="list-style-type: none"> A: A cDNA colony is shown with a plus sign (+) indicating the addition of a probe. B: Annealing and circularization of barcoded padlock probes. The probe is shown hybridizing to the cDNA and forming a circular structure. Cycle 1 hybridization and imaging: The first round of hybridization and imaging is shown. Cycle 8 hybridization and imaging: The eighth round of hybridization and imaging is shown. </div> <p>(FIG. 1)</p> <p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (2:56-58)</p>

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	<p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (9:25-27)</p> <p><i>See also</i> Claim 1.</p>
<p>(i) producing codewords representing the plurality of different nucleic acid targets at locations within the sample,</p> <p>wherein each of the codewords represents one of the plurality of different nucleic acid targets and comprises multiple binary values 1 and 0,</p> <p>wherein a value of 1 is obtained when the fluorescent signal is detected from one of the plurality of readout probe hybridized complexes or one of the different</p>	<p>“The methods are useful for obtaining sequence information for a single nucleotide sequence, and for multiplex generation of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules in situ within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (1:60-2:1)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell. The methods are useful for obtaining sequence information at the level of individual nucleic acid molecules. The methods are especially advantageous for multiplex generation of sequence information from an individual cell. As such, sequence information can be obtained for several individual nucleic acid molecules in an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules in situ within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (4:6-19)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either</p>

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<p>plurality of readout probe-hybridized complexes at a respective location within the sample while a value of 0 is obtained when the fluorescent signal is not detected from one of the plurality of readout probe-hybridized complexes or one of the different plurality of readout probe-hybridized complexes at the respective location within the sample</p>	<p>random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images." (9:1-22)</p> <p><i>See also</i> Claim 1.</p> <p>"These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004))." (3:36-40)</p> <p>"Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety" (8:56-59)</p> <p>"Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of</p>

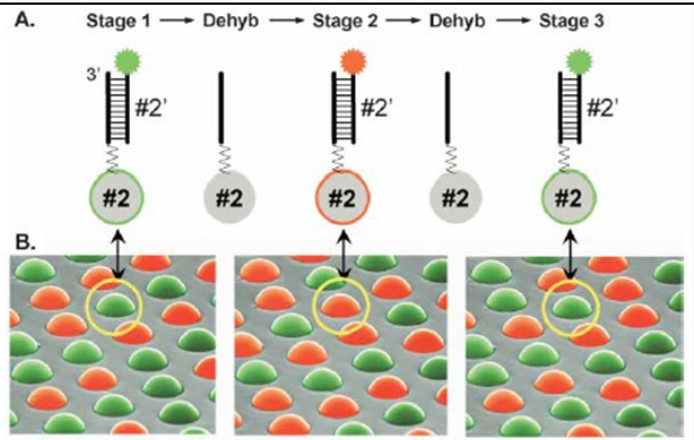
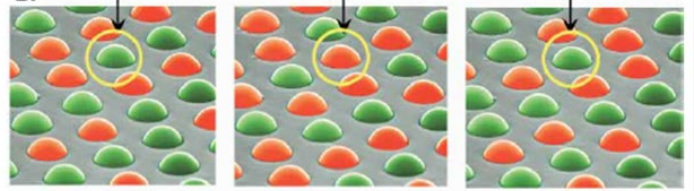
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	<p>Gunderson et al. (Genome Res. (2004) 14:870-877).” (14:31-34)</p> <p>“The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and decode thousands of unique amplified nucleic acid species.” (8:34-44)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential</p>

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	<p>hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p>“For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate (<10-4).” (14:34-48)</p> <p>“...we use three-state eight-stage decoding....” (Gunderson, 871)</p> <p>We use a process that decodes 1520 different bead types by using three states: two fluorescent “ON” states (FAM andCY3 fluorescent labels) and one nonfluorescent “OFF” state” (Gunderson, 871)</p> <p>“We have developed a simple and efficient algorithm to identify each member of a large collection of DNA-linked objects through the use of hybridization....” (Gunderson, Abstract)</p> <p>“Our algorithm uses sequential hybridizations of dye-labeled oligonucleotides, or decoders, complementary to bead sequences to create a combinatorial decoding scheme for arrays.” (Gunderson, 870)</p>

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	<p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“Each bead type is defined by a unique DNA sequence that is recognized by a complementary decoder.” (Gunderson, 870)</p> <div><p>A. Stage 1 → Dehyb → Stage 2 → Dehyb → Stage 3</p><p>B.</p><p>C.</p><table><tr><th></th><th></th><th>Stage 1</th><th>Stage 2</th><th>Stage 3</th><th>Signature</th><th>Code</th><th>Parity Code</th></tr><tr><td rowspan="8">Sequences</td><td>0</td><td></td><td></td><td></td><td>GGG</td><td>000</td><td>0000</td></tr><tr><td>1</td><td></td><td></td><td></td><td>GGR</td><td>001</td><td>0011</td></tr><tr><td>2</td><td></td><td></td><td></td><td>GRG</td><td>010</td><td>0101</td></tr><tr><td>3</td><td></td><td></td><td></td><td>GRR</td><td>011</td><td>0110</td></tr><tr><td>4</td><td></td><td></td><td></td><td>RGG</td><td>100</td><td>1001</td></tr><tr><td>5</td><td></td><td></td><td></td><td>RGR</td><td>101</td><td>1010</td></tr><tr><td>6</td><td></td><td></td><td></td><td>RRG</td><td>110</td><td>1100</td></tr><tr><td>7</td><td></td><td></td><td></td><td>RRR</td><td>111</td><td>1111</td></tr></table></div> <p>(Gunderson, FIG. 2)</p> <p>“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a</p>			Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code	Sequences	0				GGG	000	0000	1				GGR	001	0011	2				GRG	010	0101	3				GRR	011	0110	4				RGG	100	1001	5				RGR	101	1010	6				RRG	110	1100	7				RRR	111	1111
		Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code																																																											
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	3				GRR	011	0110																																																											
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	6				RRG	110	1100																																																											
	7				RRR	111	1111																																																											

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	<p>complementary decoder hybridizes to the oligonucleotide probe that is attached to the bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p> <p>“The clear separation between the two modes in the histogram indicates that most beads can be classified unambiguously as OFF or ON.” (Gunderson, 872).</p> <div data-bbox="516 1396 1360 1837"> <p>A 1 2 3 4 5 6 7 8</p> <p>B Typical Decode Stage</p> <p>C Decode Bead Profile</p> <p>FAM</p> <p>Cy3</p> </div>

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	<p>(Gunderson, FIG. 3)</p> <p>“The low intensity peak includes beads in the OFF state and empty wells. The higher intensity peak includes beads in the ON state.” (Gunderson, FIG. 3, Legend)</p> <p>“The histogram is bimodal because each stage and color channel will have beads that are in the ON state and beads that are in the OFF state.” (Gunderson, 876).</p>
<p>(j) matching the codewords with valid codewords in a codebook by comparing the codewords to the valid codewords in the codebook,</p> <p>and if one of the codewords is not matched with one of the valid codewords in the codebook, applying an error detection or correction system, matching the one of the codewords with another of the valid codewords in the codebook, or discarding the</p>	<p>“Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877).” (14:31-34)</p> <p>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p>

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one of the codewords, wherein the codebook comprises the valid codewords of the plurality of nucleic acid targets	<div><p>A. Stage 1 → Dehyb → Stage 2 → Dehyb → Stage 3</p><p>B.</p><p>C.</p><table><tr><th></th><th>Stage 1</th><th>Stage 2</th><th>Stage 3</th><th>Signature</th><th>Code</th><th>Parity Code</th></tr><tr><td rowspan="8">Sequences</td><td>0</td><td></td><td></td><td>GGG</td><td>000</td><td>0000</td></tr><tr><td>1</td><td></td><td></td><td>GGR</td><td>001</td><td>0011</td></tr><tr><td>2</td><td></td><td></td><td>GRG</td><td>010</td><td>0101</td></tr><tr><td>3</td><td></td><td></td><td>GRR</td><td>011</td><td>0110</td></tr><tr><td>4</td><td></td><td></td><td>RGG</td><td>100</td><td>1001</td></tr><tr><td>5</td><td></td><td></td><td>RGR</td><td>101</td><td>1010</td></tr><tr><td>6</td><td></td><td></td><td>RRG</td><td>110</td><td>1100</td></tr><tr><td>7</td><td></td><td></td><td>RRR</td><td>111</td><td>1111</td></tr></table></div>		Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code	Sequences	0			GGG	000	0000	1			GGR	001	0011	2			GRG	010	0101	3			GRR	011	0110	4			RGG	100	1001	5			RGR	101	1010	6			RRG	110	1100	7			RRR	111	1111
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	6			RRG	110	1100																																																			
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(Gunderson, FIG. 2)

“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to

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	<p>individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p> <p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)</p> <p>“In our implementation of three-state decoding, the most common errors are transitions from an ON state, one or two, to the OFF state, zero. Less common are transitions from OFF to ON. Transitions from one ON state to the other ON state are ex-tremely rare. This can be explained by the fact that such transi-tions require two simultaneous classification errors: a mistaken ON in one color channel and a mistaken OFF call in the other color channel. We used these biases in error rates by designing the decode stages so that every valid code has a fixed number of OFF states. For example, if there are 1520 bead types and we use three-state eight-stage decoding, then each valid code would be designed to be OFF in exactly two stages and ON in exactly six stages (the actual scheme used is a slight variant on this design; see Methods). An example of a valid code would be 21110210. With this</p>

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	<p>scheme, it is theoretically impossible to misclas-sify a code through any number of occurrences of the most com- mon error type: a transition from an ON state to an OFF state. The following events can lead to misclassification: a transition from one ON state to the other, or multiple stage errors with at least one ON-to-OFF transition and at least one OFF-to-ON tran-sition. Both events are extremely rare.” (Gunderson, 871-872)</p> <p>“The strategy for the design of decoder pools to enable error checking, described in the main text, included the condition that all valid codes have exactly two OFF states, which allows for a maximum of 1792 codes. To enable the decoding of more bead types, we pooled the decoders so that valid codes have either exactly two OFF states or exactly five OFF states. The transition events that lead to misclassification are the same as described above, except that errors of the type (ON to OFF) or (OFF to ON) occurring in three different stages could also lead to a misclassi-fication event. Three errors from ON to OFF could occur if a bead falls out of the array in the middle of decoding. To eliminate this source of error, all codes ending in three or more OFF states were removed from the set of valid codes. Finally, we eliminated any codes that do not have at least one ON state in each of the color channels that we use. The fact that each bead must show specific signal in two color channels during the decode process provides an additional quality check. The total number of valid color codes in this scheme is 2012.” (Gunderson, 875)</p> <p>“We use a process that decodes 1520 different bead types by using three states: two fluorescent “ON” states (FAM and CY3 fluorescent labels) and one nonfluorescent “OFF” state. The logarithmic relationship between the number of bead types and the number of decode stages shows that the 1520 bead types can be decoded in only $\log 1520 = 7$ stages. In</p>

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	<p>practice, we use an additional stage to enable error checking.” (Gunderson, 871)</p> <p>“The space of all possible color codes is divided into three categories: used valid, unused valid, and invalid (Table 1).” (Gunderson, 872)</p> <table><tr><th colspan="3">Table 1. Distribution and Use of 6561 Codes in the Three-State, Eight-Stage System</th></tr><tr><th>Type</th><th>Number</th><th>Purpose</th></tr><tr><td>Used valid codes</td><td>1520</td><td>Assigned to actual beads.</td></tr><tr><td>Unused valid codes</td><td>272</td><td>Detect rare two-stage errors.</td></tr><tr><td>Invalid codes</td><td>4769</td><td>Estimate the number of misclassification events. Detect single stage errors. Monitor overall decode process; useful for detecting occasional catastrophic failures.</td></tr></table> <p>(Gunderson, Table 1)</p> <p>“Based on the average number of invalid codes generated by our process, we estimate that the average misclassification rate would increase 86-fold.” (Gunderson, 873)</p>	Table 1. Distribution and Use of 6561 Codes in the Three-State, Eight-Stage System			Type	Number	Purpose	Used valid codes	1520	Assigned to actual beads.	Unused valid codes	272	Detect rare two-stage errors.	Invalid codes	4769	Estimate the number of misclassification events. Detect single stage errors. Monitor overall decode process; useful for detecting occasional catastrophic failures.
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(k) determining the plurality of nucleic acid targets in the sample based on results from the matching step.	<p>“The methods are useful for obtaining sequence information for a single nucleotide sequence, and for multiplex generation of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules in situ within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (1:60-2:1)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell. The methods are useful for obtaining sequence information at the level of individual nucleic acid molecules. The methods are especially advantageous for multiplex generation of sequence information from an individual cell. As such, sequence information can be</p>															

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	<p>obtained for several individual nucleic acid molecules in an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules in situ within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (4:6-19)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res.</p>

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	<p>(2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:53-59)</p> <p>“FIG. 1 describes one embodiment of padlock capture and hybridization decoding of rolones. (A) Molecular barcodes are attached to rolones in a gene specific manner using padlock probes. (B) After padlock capture, each rolony will have a unique barcode in thousands of copies. These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004)).” (3:32-40)</p> <p>“The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and decode thousands of unique amplified nucleic acid species” (8:34-44)</p> <p>“Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed.” (14:31-36)</p>

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<p>2. The method of claim 1, wherein the fluorescent signal in step (c) and/or step (f) is detected at a resolution better than 300 nm.</p>	<p>“The signals generated by fluorescent imaging of one or more probes during decoding, sequencing and other detection methods carried out in situ and can be detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ. Suitable imaging technologies are known in the art, as exemplified by Larsson et al., Nat. Methods (2010) 7:395-397 and associated supplemental materials, the entire content of which is incorporated by reference herein in its entirety.” (11:11-19)</p> <p>“Images of cultured cells were acquired using an AxioplanII epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp, a CCD camera (C4742-95, Hamamatsu), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5. A ×20 (Plan-Apocromat, Zeiss), ×40 (Plan-Neofluar, Zeiss) or ×63 (Plan-Neofluar, Zeiss) objective was used for capturing the images.” (Larsson, 0005)</p> <p>“One of the reagents, Cy3, can be excited with the 488-, 514- and 532-nm laser lines and is optimally excited with the 546-nm mercury arc line.” (Mujumdar, Abstract)</p> <p>“Images of viral particles on cover slips or fixed cells were acquired using an Apotome (Carl Zeiss) structured illumination fluorescence imaging system mounted on an Axioplan 2 imaging microscope equipped with a Plan-Apochromat 63 NA-1.4 oil objective and an MRm charge-coupled device camera piloted by AxioVision software.” (Arhel, 823)</p> <p>“The unit is equipped with a high resolution image sensor featuring an defective pixel number of 4000 (Horizontal) x 2624 (Vertical) pixels.” (Hamamatsu, 7).</p>

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	<div>(1) Electrical specifications</div> <table><tr><td>Imaging device</td><td colspan="2">Interface readout interline CCD solid imaging element</td></tr><tr><td>Effective number of pixels</td><td colspan="2">4000 (H) × 2624 (V)</td></tr><tr><td>Cell size</td><td colspan="2">5.9 μm × 5.9 μm (Square format)</td></tr><tr><td>Effective area</td><td colspan="2">23.6 mm × 15.5 mm (1.8-inch size)</td></tr><tr><td rowspan="4">Frame rate</td><td>Interface readout</td><td>1.7 Hz</td></tr><tr><td>2×2 binning readout</td><td>3.4 Hz</td></tr><tr><td>4×4 binning readout</td><td>6.4 Hz</td></tr><tr><td>Outline readout</td><td>8.9 Hz</td></tr><tr><td>Readout noise (rms.)</td><td colspan="2">20 electrons (typ.) *¹</td></tr><tr><td>A/D converter</td><td colspan="2">12 bit</td></tr><tr><td>Cooling method</td><td colspan="2">Electronic and natural air cooling</td></tr><tr><td>Lens mount</td><td colspan="2">F mount</td></tr><tr><td>Amp gain conversion factor *²</td><td colspan="2">3.2 electrons/ADcounts (13 300 electrons)</td></tr><tr><td>Contrast variable range</td><td colspan="2">1 to approx. 10 times</td></tr></table> <div>(Hamamatsu, 35)</div>	Imaging device	Interface readout interline CCD solid imaging element		Effective number of pixels	4000 (H) × 2624 (V)		Cell size	5.9 μm × 5.9 μm (Square format)		Effective area	23.6 mm × 15.5 mm (1.8-inch size)		Frame rate	Interface readout	1.7 Hz	2×2 binning readout	3.4 Hz	4×4 binning readout	6.4 Hz	Outline readout	8.9 Hz	Readout noise (rms.)	20 electrons (typ.) * ¹		A/D converter	12 bit		Cooling method	Electronic and natural air cooling		Lens mount	F mount		Amp gain conversion factor * ²	3.2 electrons/ADcounts (13 300 electrons)		Contrast variable range	1 to approx. 10 times	
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3. The method of claim 1, wherein the sample comprises a cell.	<div>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</div> <div>“Presented herein are methods and compositions for obtaining sequence information from one or more individual cells. The methods are useful for obtaining sequence</div>																																							

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	<p>information for a single nucleotide sequence, and for multiplex generation of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules in situ within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (1:58-2:1)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell.” (4:6-8)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p>

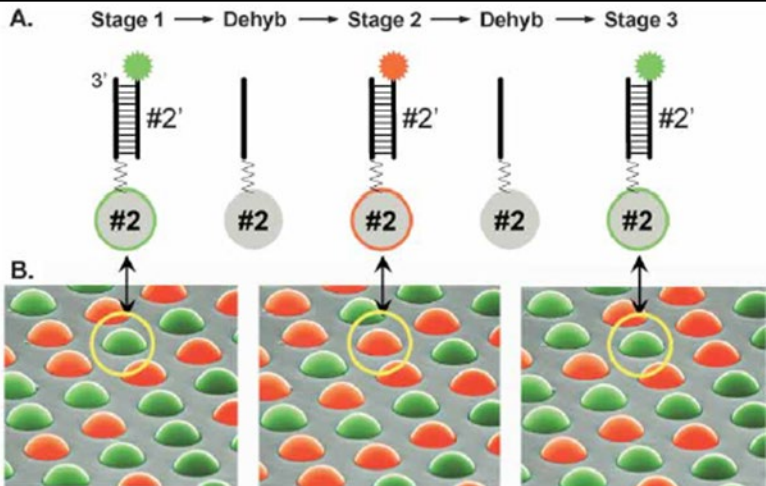
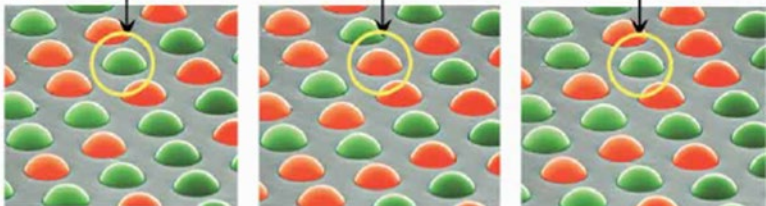
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	<p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, rolonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs</p>

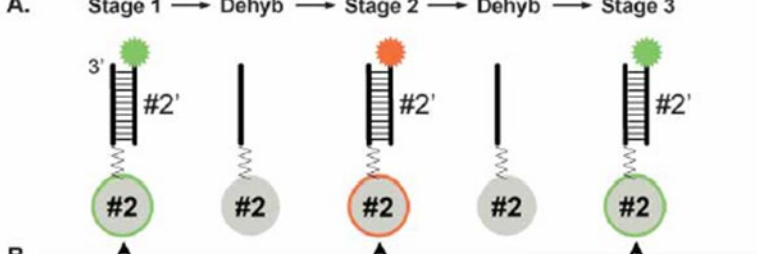
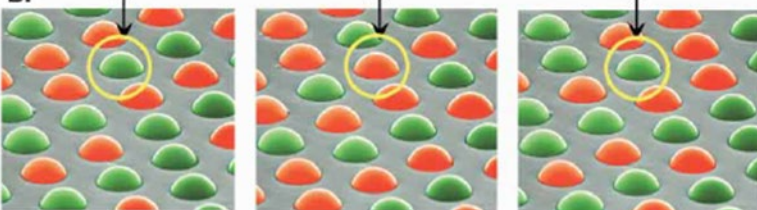
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	<p>six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (Example 1, 14:3-48)</p>
<p>4. The method of claim 3, wherein the cell is fixed on a coverslip.</p>	<p>“Methods for permeabilization and fixation of cells and tissue samples are well-known in the art, as exemplified by Cremer et al., <i>The Nucleus: Volume 1: Nuclei and Subnuclear Components</i>, R. Hancock (ed.) 2008; and Larsson et al., <i>Nat. Methods</i> (2010) 7:395-397 and associated supplemental materials, the content of each of which is incorporated herein by reference in its entirety.” (10:66-11:6)</p> <p>“Fresh frozen 9-μm sections of E14.5 mouse embryos were placed on Superfrost Plus Gold slides (Thermo Scientific). Fully anonymized fresh frozen human tissue sections from a HER2-positive breast cancer were obtained from the Fresh Tissue Biobank at the Department of Pathology, Uppsala University Hospital, in accordance with the Swedish Biobank Legislation. Breast tissue sections of 4 μm thickness were placed on Starfrost microscope slides (Instrumedics).” (Larsson, 0004)</p>
<p>5. The method of claim 1, comprising determining the fluorescent signal entity by imaging at least a portion of the sample</p>	<p>“The signals generated by fluorescent imaging of one or more probes during decoding, sequencing and other detection methods carried out in situ and can be detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ. Suitable imaging technologies are known in the art, as exemplified by Larsson et al., <i>Nat. Methods</i> (2010) 7:395-397 and associated supplemental materials, the entire content of</p>

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using a light microscope.	<p>which is incorporated by reference herein in its entirety.” (11:11-19)</p> <p>“Images of cultured cells were acquired using an AxioplanII epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp, a CCD camera (C4742-95, Hamamatsu), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5. A ×20 (Plan-Apocromat, Zeiss), ×40 (Plan-Neofluar, Zeiss) or ×63 (Plan-Neofluar, Zeiss) objective was used for capturing the images.” (Larsson, 0005)</p>
6. The method of claim 1, further comprising determining an abundance and/or spatial distribution of the different nucleic acid targets at locations within the sample.	<p>“In some embodiments the spatial location of nucleic acids can be simultaneously determined for several individual cells in a tissue or other cell-containing sample.” (4:32-34)</p> <p>“In some aspects, detecting can further comprise evaluating a signal to determine the presence, quantity, frequency, and/or spatial distribution of a disease-causing mutation, allele or common variant within a tissue.” (3:21-24)</p>
7. The method of claim 1, further comprising removing the readout nucleic acid probes unhybridized to each of the plurality of first hybridized	<p>“The methods and compositions make use of hybridization, amplification, washing and detection within individual cells.” (10:62-64)</p> <p><i>See also</i> Claim 1.</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (9:25-27)</p> <p>“These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a</p>

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complex prior to step (c).	<p>combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004)).” (3:36-40)</p> <p>“Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:56-59)</p> <p>“Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877).” (14:31-34)</p> <p>“The instant innovations overcome limitations of existing rolon identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and decode thousands of unique amplified nucleic acid species.” (8:34-44)</p> <p>“Following hybridization, three 1-min washes in wash buffer (167 mM NaCl, 16.7 mM potassium phosphate, 0.017% Tween-20 at pH 7.6) were used to remove unbound oligonucleotides.” (Gunderson, 875)</p>
8. The method of claim 1, wherein the valid	“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information

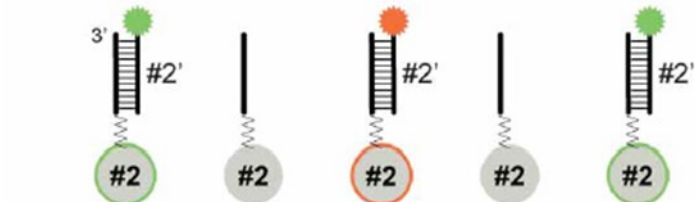
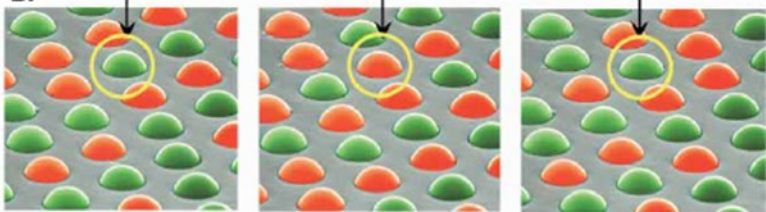
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<p>codewords form an error-checking or an error-correcting code space.</p>	<p>theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p> <p>“Although assignment of codes to sequences is unambiguous after three stages, additional stages can be added for error checking purposes (last column of Fig. 2C) to be described below.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)</p>
<p>9. The method of claim 1, wherein in said comparing the codewords to the valid codewords in the codebook in step (j), one of the codewords is not matched with one of the valid codewords in the codebook when the one of the codewords has one or more errors.</p>	<p>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p> <p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)</p>

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	<div><p>A. Stage 1 → Dehyb → Stage 2 → Dehyb → Stage 3</p><p>B.</p><p>C.</p><table><tr><th></th><th></th><th>Stage 1</th><th>Stage 2</th><th>Stage 3</th><th>Signature</th><th>Code</th><th>Parity Code</th></tr><tr><td rowspan="8">Sequences</td><td>0</td><td></td><td></td><td></td><td>GGG</td><td>000</td><td>0000</td></tr><tr><td>1</td><td></td><td></td><td></td><td>GGR</td><td>001</td><td>0011</td></tr><tr><td>2</td><td></td><td></td><td></td><td>GRG</td><td>010</td><td>0101</td></tr><tr><td>3</td><td></td><td></td><td></td><td>GRR</td><td>011</td><td>0110</td></tr><tr><td>4</td><td></td><td></td><td></td><td>RGG</td><td>100</td><td>1001</td></tr><tr><td>5</td><td></td><td></td><td></td><td>RGR</td><td>101</td><td>1010</td></tr><tr><td>6</td><td></td><td></td><td></td><td>RRG</td><td>110</td><td>1100</td></tr><tr><td>7</td><td></td><td></td><td></td><td>RRR</td><td>111</td><td>1111</td></tr></table></div> <p>(Gunderson, FIG. 2)</p> <div><p>Table 1. Distribution and Use of 6561 Codes in the Three-State, Eight-Stage System</p><table><tr><th>Type</th><th>Number</th><th>Purpose</th></tr><tr><td>Used valid codes</td><td>1520</td><td>Assigned to actual beads.</td></tr><tr><td>Unused valid codes</td><td>272</td><td>Detect rare two-stage errors.</td></tr><tr><td rowspan="3">Invalid codes</td><td rowspan="3">4769</td><td>Estimate the number of misclassification events.</td></tr><tr><td>Detect single stage errors.</td></tr><tr><td>Monitor overall decode process; useful for detecting occasional catastrophic failures.</td></tr></table></div> <p>(Gunderson, Table 1)</p>			Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code	Sequences	0				GGG	000	0000	1				GGR	001	0011	2				GRG	010	0101	3				GRR	011	0110	4				RGG	100	1001	5				RGR	101	1010	6				RRG	110	1100	7				RRR	111	1111	Type	Number	Purpose	Used valid codes	1520	Assigned to actual beads.	Unused valid codes	272	Detect rare two-stage errors.	Invalid codes	4769	Estimate the number of misclassification events.	Detect single stage errors.	Monitor overall decode process; useful for detecting occasional catastrophic failures.
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	3				GRR	011	0110																																																																									
	4				RGG	100	1001																																																																									
	5				RGR	101	1010																																																																									
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	<p>“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p>

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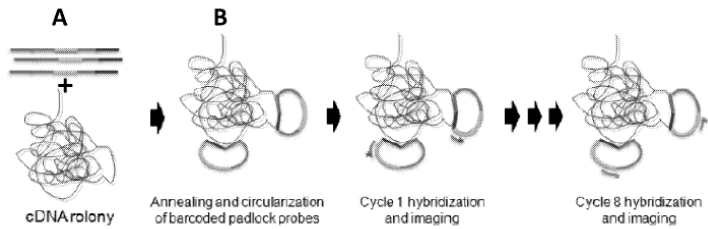
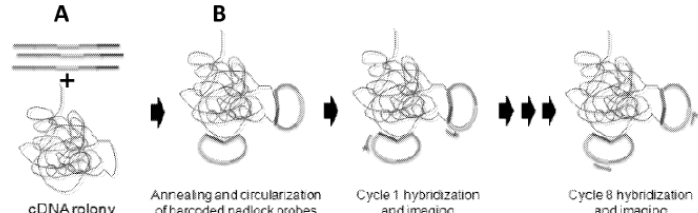
APPENDIX B

Fan (EX1005)	Fan Provisional Application (EX1006)
303 Patent, Claim 1: A method for detecting a plurality of different nucleic acid targets within a sample by in situ hybridization, comprising:	
<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:20-30)</p> <p>“In some embodiments, the methods provided herein allow for detection of a single target nucleotide species of interest. In</p>

Fan (EX1005)	Fan Provisional Application (EX1006)
<p>information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“In some embodiments, the methods provided herein allow for detection of a single target nucleotide species of interest. In other embodiments, the methods allow for multiplex detection of a plurality of nucleotide species of interest. The use of barcode probes with unique identifier sequences as described above allows for simultaneous detection of 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000 or more than 10,000 unique nucleic acid target molecules within a single cell.” (9:49-59)</p> <p>“In the embodiment shown in FIG. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique</p>	<p>other embodiments, the methods allow for multiplex detection of a plurality of nucleotide species of interest. The use of barcode probes with unique identifier sequences as described above allows for simultaneous detection of 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000 or more than 10,000 unique nucleic acid target molecules within a single cell.” (14:15-22)</p> <p>“In the embodiment shown in Fig. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes.” (13:14-18)</p> <p>“For up to ~ 1700 target genes, eight rounds of hybridization decoding are performed.” (22:7-8)</p> <p><i>See also</i> 2:2-6, 5:17-25, 13:12-26, 21:15-22:16 (Example 1), FIG. 1, Claim 1.</p>

Fan (EX1005)	Fan Provisional Application (EX1006)
<p>padlock probes with unique molecular barcodes.” (9:4-11)</p> <p>“For up to 1700 target genes, eight rounds of hybridization decoding are performed.” (14:34-36)</p> <p><i>See also</i> 1:63-2:1, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1.</p>	
<p>303 Patent, Claim 1(a): “producing a plurality of first hybridized complexes within a sample containing a plurality of different nucleic acid targets by exposing the sample to a plurality of different encoding nucleic acid probes”</p>	
<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules <i>in situ</i> within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing <i>in situ</i> a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules <i>in situ</i> within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing <i>in situ</i> a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder</p>

Fan (EX1005)	Fan Provisional Application (EX1006)
<p>decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p><i>See also</i> 1:63-2:1, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1.</p> <p>“In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B).” (14:15-18)</p> <p>“In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique</p>	<p>sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:20-30)</p> <p><i>See also</i> 2:2-6, 5:17-25, 13:12-27, 21:15-22:16 (Example 1), FIG. 1, Claim 1.</p> <p>“In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (Fig. 1B).” (21:23-25)</p> <p>“In the embodiment shown in Fig. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes.” (13:14-18)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes. Padlock probes are specialized ligation probes and are known in the art....” (11:1-2)</p>

Fan (EX1005)	Fan Provisional Application (EX1006)
<p>padlock probes with unique molecular barcodes.” (9:4-11)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes. Padlock probes are specialized ligation probes and are known in the art....” (7:36-38)</p>  <p>(FIG. 1)</p>	 <p>(FIG. 1)</p>
<p>303 Patent, Claim 1(a): “wherein each of the plurality of encoding nucleic acid probes comprises (i) a target sequence that specifically hybridizes to a different nucleic acid target or a different location of an identical nucleic acid target of the plurality of different nucleic acid targets and (ii) one or more read sequences”</p>	
<p>“As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and</p>	<p>“As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic</p>

Fan (EX1005)	Fan Provisional Application (EX1006)
<p>which comprises a barcode sequence.” (5:17-20)</p> <p>“FIG. 1 describes one embodiment of padlock capture and hybridization decoding of rolones. (A) Molecular barcodes are attached to rolones in a gene specific manner using padlock probes. (B) After padlock capture, each rolony will have a unique barcode in thousands of copies. These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004)). (C) Schematic of padlock probes. In the example shown, each probe has two gene-specific (and/or allele-specific) capturing arms H1/H2.” (3:32-42)</p> <p>“When a padlock probe specifically hybridizes to a sequence of interest....” (9:11-12)</p>	<p>acid of interest, and which comprises a barcode sequence.” (7:13-15)</p> <p>“Figure 1 describes one embodiment of padlock capture and hybridization decoding of rolones. (A) Molecular barcodes are attached to rolones in a gene specific manner using padlock probes. (B) After padlock capture, each rolony will have a unique barcode in thousands of copies. These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5):870-877 (2004)). (C) Schematic of padlock probes. In the example shown, each probe has two gene-specific (and/or allele-specific) capturing arms H1/H2” (4:16-23)</p> <p>“When a padlock probe specifically hybridizes to a sequence of interest....” (13:18-19)</p> <div data-bbox="972 1086 1627 1299"> <p>The diagram illustrates the padlock capture and hybridization decoding process in four stages: (A) A cDNA rolony is shown as a cluster of molecules. (B) Padlock probes are added and hybridize to the cDNA, forming a circular structure. (C) The process continues through multiple cycles of hybridization and imaging, with the first cycle labeled 'Cycle 1 hybridization and imaging' and the eighth cycle labeled 'Cycle 8 hybridization and imaging'.</p> </div>

Fan (EX1005)	Fan Provisional Application (EX1006)
<div data-bbox="210 357 913 592"> <p>A cDNA rolony</p> <p>B Annealing and circularization of barcoded padlock probes</p> <p>C Cycle 1 hybridization and imaging</p> <p>D Cycle 8 hybridization and imaging</p> </div> <p>(FIG. 1)</p> <p>“The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (5:26-29)</p> <p><i>See also</i> 1:63-2:1, 4:9-19, 9:1-22, 14:3-48 (Example 1), FIG. 1, Claim 1.</p> <p>“The methods and compositions presented herein make use of unique barcode probes having decoder sequences to identify multiple nucleic acid targets in a multiplex fashion. The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of</p>	<p>(FIG. 1)</p> <p>“The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (7:19-21)</p> <p><i>See also</i> 2:2-6, 5:17-25, 13:12-26, 21, 17-22:16 (Example 1), FIG. 1, Claims 1 and 2.</p> <p>“The methods and compositions presented herein make use of unique barcode probes having decoder sequences to identify multiple nucleic acid targets in a multiplex fashion. The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (12:16-21)</p> <p>“In some embodiments, a barcode probe can comprise at least a first decoder sequence and a second decoder sequence. In some embodiments, a barcode probe can comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 decoder sequences. As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique</p>

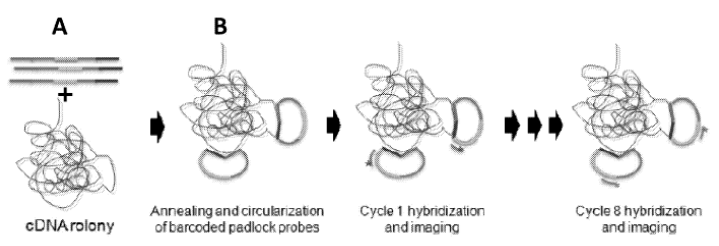
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<p>labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (8:30-39)</p> <p>“In some embodiments, a barcode probe can comprise at least a first decoder sequence and a second decoder sequence. In some embodiments, a barcode probe can comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 decoder sequences. As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:45-55)</p> <p>“...hybridizing a first labeled decoder probe to the first decoder sequence; detecting a signal indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence....” (2:14-17).</p>	<p>sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (12:26-13:3)</p> <p>“...hybridizing a first labeled decoder probe to the first decoder sequence; detecting a signal indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence....” (2:15-17)</p>

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<p style="text-align: center;">303 Patent, Claim 1(a): “such that each of the plurality of first hybridized complexes comprises multiple encoding nucleic acid probes of the different encoding nucleic acid probes located in its different locations”</p>	
<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:20-30)”</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in Fig. 1, cDNA is generated <i>in situ</i>, using either random</p>

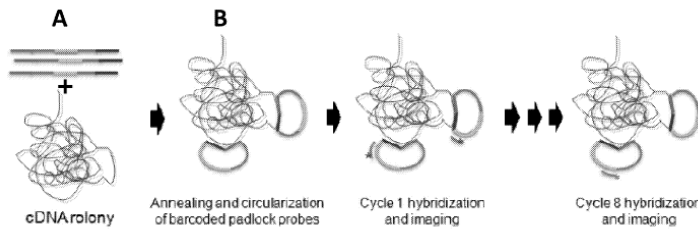
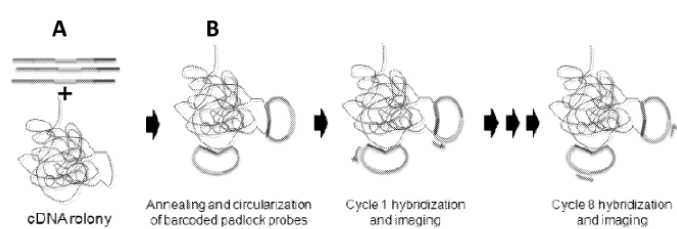
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<p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds</p>	<p>hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:12-27)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes. Padlock probes are specialized ligation probes and are known in the art....” (11:1-2)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (Fig. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses,</p>

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<p>of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes. Padlock probes are specialized ligation probes and are known in the art....” (7:36-38)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, rolonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the</p>	<p>as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, rolonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (Fig. 1A). In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (Fig. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to ~1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolonity can have three states: two fluorescent</p>

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<p>full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolon can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-</p>	<p>(FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking. Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stages x 2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (21:17-22:16)</p> <div data-bbox="970 850 1627 1065"> </div> <p>(FIG. 1)</p>

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<p>state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (14:3-48)</p>  <p>(FIG. 1)</p>	
<p>303 Patent, Claim 1(b): “producing a plurality of readout probe-hybridized complexes by exposing each of the plurality of first hybridized complexes within the sample to a first readout nucleic acid probe”</p>	

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<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:20-30)</p> <p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (12:28-13:3)</p>

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<p>hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:49-55)</p>  <p>(FIG. 1)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This</p>	 <p>(FIG. 1)</p> <p>“In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:23-26)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (Fig. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, r colonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (Fig. 1A). In some embodiments, r colonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library</p>

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<p>method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, colonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, colonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly</p>	<p>of padlock probes (Fig. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to ~1700 target genes, eight rounds of hybridization decoding are performed. In each round, a colony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking. Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization</p>

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<p>optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a colony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to</p>	<p>sequences used in these six slots, a set of 16 (8 stages x 2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$)." (21:17-22:16)</p> <p><i>See also</i> Claims 1 and 2.</p>

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<p>1792 transcripts with a low error rate (<10⁻⁴).” (14:3-48)</p> <p><i>See also</i> Claim 1.</p>	
<p>303 Patent, Claim 1(b): “wherein the first readout nucleic acid probe comprises a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal”</p>	
<p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:49-55)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with</p>	<p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (12:28-13:3)</p> <p>“In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:24-26)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (Fig.</p>

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<p>fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, rolonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature</p>	<p>1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, rolonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (Fig. 1A). In some embodiments, rolonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (Fig. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al.</p>

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<p>478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking. Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages) (21:17-22:16)</p>	<p>(Genome Res. (2004) 14:870-877). For up to ~1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking. Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stages x 2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (21:17-22:16)</p> <p>See also Claims 1 and 2.</p> <div data-bbox="976 1055 1638 1274"> <p>The diagram illustrates the process of transcript identification using padlock probes. It consists of four main stages labeled A, B, C, and D. Stage A shows a 'cDNA rolony' with a plus sign, indicating the starting material. Stage B shows 'Annealing and circularization of barcoded padlock probes', where the probes are binding to the cDNA and forming a circular structure. Stage C shows 'Cycle 1 hybridization and imaging', where the first round of decoding is performed. Stage D shows 'Cycle 8 hybridization and imaging', where the final round of decoding is performed. Arrows indicate the progression from A to B, B to C, and C to D.</p> </div> <p>(FIG. 1)</p>

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<p>six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (14:3-48)</p> <p>See also Claim 1.</p> <div data-bbox="210 808 913 1036"> <p>The diagram illustrates the eight-stage barcoding scheme. It shows four stages: A (cDNA rolony), B (Annealing and circularization of barcoded padlock probes), Cycle 1 hybridization and imaging, and Cycle 8 hybridization and imaging. The diagram illustrates the progression from a linear cDNA molecule to a circularized probe, followed by sequential hybridization and imaging cycles.</p> </div> <p>(FIG. 1)</p> <p>“The methods provided herein further comprise annealing <i>in situ</i> a barcode probe to the amplified nucleic acid molecule. As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and</p>	<p>“The methods provided herein further comprise annealing <i>in situ</i> a barcode probe to the amplified nucleic acid molecule. As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and which comprises a barcode sequence. As used herein, the terms barcode, barcode sequence and like terms refer to a unique identifier sequence on a probe molecule. The unique identifier sequence can be, for example, a nucleic acid sequence which associates the barcode probe with the nucleic acid of interest to which it hybridizes. The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (7:12-22)</p> <p>“The methods and compositions presented herein make use of unique barcode probes having decoder sequences to identify multiple nucleic acid targets in a multiplex fashion. The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (12:16-21)</p>

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<p>which comprises a barcode sequence. As used herein, the terms barcode, barcode sequence and like terms refer to a unique identifier sequence on a probe molecule. The unique identifier sequence can be, for example, a nucleic acid sequence which associates the barcode probe with the nucleic acid of interest to which it hybridizes. The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (5:14-29)</p> <p>“The methods and compositions presented herein make use of unique barcode probes having decoder sequences to identify multiple nucleic acid targets in a multiplex fashion. The instant innovations overcome limitations of existing rolonly identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (8:30-39)</p>	<p>“In some embodiments, a barcode probe can comprise at least a first decoder sequence and a second decoder sequence.” (12:26-27)</p>

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<p>“In some embodiments, a barcode probe can comprise at least a first decoder sequence and a second decoder sequence.” (8:45-47)</p>	
<p style="text-align: center;">303 Patent, Claim 1(c): “detecting a fluorescent signal from each of the plurality of readout probe-hybridized complexes”</p>	
<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules <i>in situ</i> within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing <i>in situ</i> a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules <i>in situ</i> within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing <i>in situ</i> a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:20-30)</p> <p>“In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be</p>

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<p>information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p><i>See also</i> 9:23-43, 11:11-15, 14:3-48.</p>	<p>decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:24-26)</p> <p><i>See also</i> 13:27-14:10, 16:29-17:2, 21:15-22:16.</p>
<p>303 Patent, Claim 1(d): “removing the fluorescent signal from each of the plurality of readout probe-hybridized complexes by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the plurality of readout probe-hybridized complexes”</p>	
<p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (2:56-58)</p> <p><i>See also</i> Claim 1, 9:25-27.</p> <p>“The instant innovations overcome limitations of existing colony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential</p>	<p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (3:14-15)</p> <p><i>See also</i> Claim 1, Claim 3, 13:28-30.</p> <p>“The instant innovations overcome limitations of existing colony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences</p>

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<p>hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (8:34-39)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:53-59)</p>	<p>found in immobilized nucleic acids within an individual cell.” (12:18-21)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety.” (13:1-5)</p>
<p>303 Patent, Claim 1(e): “after step (d), producing a different plurality of readout probe-hybridized complexes by exposing the sample to a different readout nucleic acid probe, wherein the different readout nucleic acid probe comprise a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal”</p>	
<p>“...annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence....” (2:12-13)</p>	<p>“...annealing <i>in situ</i> a barcode probe comprising at least a first decoder sequence and a second decoder sequence....” (2:13-14)</p>

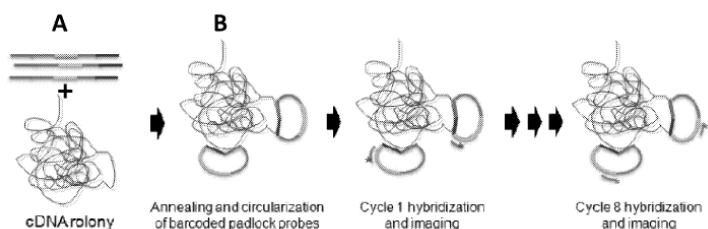
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<p>“The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (5:26-29)</p> <p><i>See also</i> Claim 1.</p> <p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:49-55)</p> <p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules <i>in situ</i> within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified</p>	<p>“The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (7:19-21)</p> <p><i>See also</i> Claims 1 and 2.</p> <p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe. During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (12:28-13:3)</p> <p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules <i>in situ</i> within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing <i>in situ</i> a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing</p>

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<p>nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony".The amplified nucleic acids can then</p>	<p>a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:20-30)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in Fig. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of</p>

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<p>be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well</p>	<p>sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:12-27)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (Fig. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, colonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (Fig. 1A). In some embodiments, colonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (Fig. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300nt in length) acquired from Agilent</p>

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<p>as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, colonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, colonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount</p>	<p>Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to ~1700 target genes, eight rounds of hybridization decoding are performed. In each round, a colony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking. Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stages x 2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (21:17-22:16)</p>

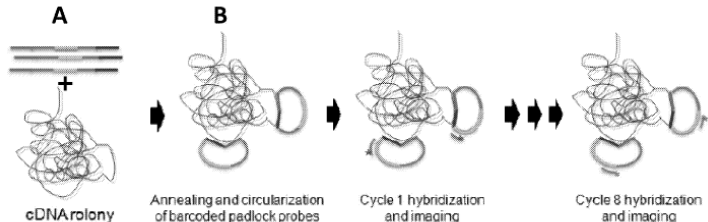
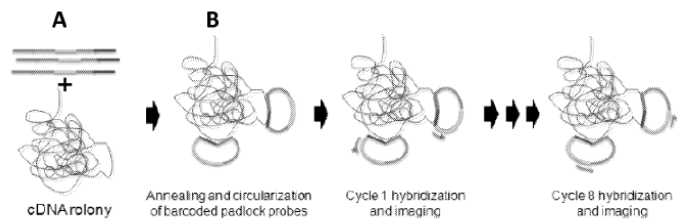
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<p>of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolon can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (14:3-48)</p>	<div data-bbox="976 349 1648 576"> </div> <p>(FIG. 1)</p>

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 <p>(FIG. 1)</p>	
303 Patent, Claim 1(f): “detecting a fluorescent signal from each of the different plurality of readout probe hybridized complexes”	
<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules <i>in situ</i> within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing <i>in situ</i> a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence;</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules <i>in situ</i> within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing <i>in situ</i> a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the</p>

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<p>detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p><i>See also</i> 9:23-43, 11:11-15, 14:3-48, Claim 1.</p>	<p>hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:20-30)</p> <p>“In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:23-27)</p> <p><i>See also</i> 13:27-14:10, 16:29-17:2, 21:15-22:16, Claims 1 and 2.</p>
<p>303 Patent, Claim 1(g): “removing the fluorescent signal from each of the different plurality of readout probe hybridized complexes by inactivating the fluorescent signaling entity or removing the fluorescent signaling entity from each of the different plurality of readout probe hybridized complexes”</p>	
<p>“The instant innovations overcome limitations of existing colony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to</p>	<p>“The instant innovations overcome limitations of existing colony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences</p>

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<p>decoder sequences found in immobilized nucleic acids within an individual cell.” (8:34-39)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:53-59)</p> <p>“In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:18-22)</p> <p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (2:56-58)</p>	<p>found in immobilized nucleic acids within an individual cell.” (12:18-21)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety.” (13:1-5)</p> <p>“In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:23-26)</p> <p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (3:14-15)</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (13:28-30)</p> <p><i>See also</i> Claim 1 and 3.</p>

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<p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (9:25-27)</p> <p><i>See also</i> Claim 1.</p>	
<p>303 Patent, Claim 1(h): “repeating steps (e) to (g) for one or more times using a different readout nucleic acid probe, wherein the different readout nucleic acid probe comprises a first portion and a second portion, the first portion comprising a sequence that specifically hybridizes to one of the read sequences, and the second portion comprising a fluorescent signaling entity capable of producing a fluorescent signal”</p>	
<p>“The instant innovations overcome limitations of existing rolon identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (8:34-39)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to</p>	<p>“The instant innovations overcome limitations of existing rolon identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell.” (12:18-21)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res.</p>

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<p>decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:53-59)</p> <p>“...and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:16-22)</p>  <p>(FIG. 1)</p> <p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior</p>	<p>(2004) 14:870-877), the content of which is incorporated by reference in its entirety.” (13:1-5)</p> <p>“...and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:22-26)</p>  <p>(FIG. 1)</p> <p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (3:14-15)</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (13:28-30)</p>

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<p>to hybridizing the second labeled decoder probe.” (2:56-58)</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (9:25-27)</p> <p><i>See also</i> Claim 1.</p>	<p><i>See also</i> Claims 1 and 3.</p>
<p>303 Patent, Claim 1(i): “producing codewords representing the plurality of different nucleic acid targets at locations within the sample, wherein each of the codewords represents one of the plurality of different nucleic acid targets and comprises multiple binary values 1 and 0, wherein a value of 1 is obtained when the fluorescent signal is detected from one of the plurality of readout probe hybridized complexes or one of the different plurality of readout probe-hybridized complexes at a respective location within the sample while a value of 0 is obtained when the fluorescent signal is not detected from one of the plurality of readout probe-hybridized complexes or one of the different plurality of readout probe-hybridized complexes at the respective location within the sample”</p>	
<p>“The methods are useful for obtaining sequence information for a single nucleotide sequence, and for multiplex generation of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and</p>	<p>“The methods are useful for obtaining sequence information for a single nucleotide sequence, and for multiplex generation of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified</p>

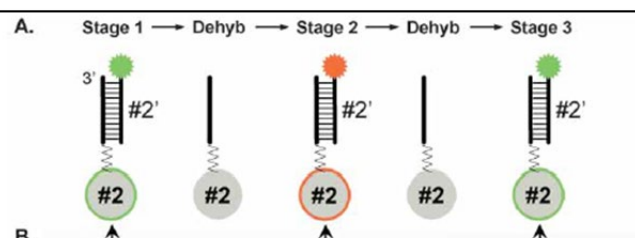
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<p>subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (1:60-2:1)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell. The methods are useful for obtaining sequence information at the level of individual nucleic acid molecules. The methods are especially advantageous for multiplex generation of sequence information from an individual cell. As such, sequence information can be obtained for several individual nucleic acid molecules in an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (4:6-19)</p>	<p>nucleic acid molecules is then determined by decoding of the barcode probes.” (1:30-2:6)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell. The methods are useful for obtaining sequence information at the level of individual nucleic acid molecules. The methods are especially advantageous for multiplex generation of sequence information from an individual cell. As such, sequence information can be obtained for several individual nucleic acid molecules in an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (5:16-25)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in Fig. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified</p>

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<p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds</p>	<p>nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:12-27)</p> <p><i>See also</i> Claims 1 and 2.</p> <p>“These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5):870-877 (2004)).” (4:19-21)</p> <p>“Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety.” (13:3-5)</p>

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<p>of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p><i>See also</i> Claim 1.</p> <p>“These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004)).” (3:36-40)</p> <p>“Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:56-59)</p> <p>“Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the</p>	<p>“Long oligonucleotides (up to 300nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877).” (22:3-7)</p> <p>“The instant innovations overcome limitations of existing rolon identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and decode thousands of unique amplified nucleic acid species.” (12:18-25)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in Fig. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA</p>

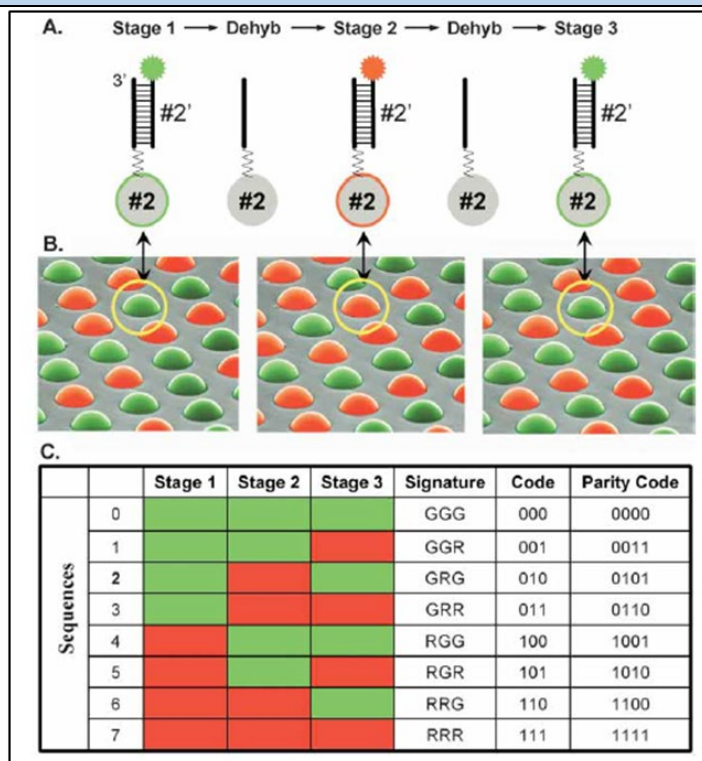
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<p>incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877).” (14:31-34)</p> <p>“The instant innovations overcome limitations of existing rolonity identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and decode thousands of unique amplified nucleic acid species.” (8:34-44)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated in situ, using either random hexamers or oligo dT primers or both. Then the cDNA</p>	<p>molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:12-27)</p> <p>“For up to ~1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolonity can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking. Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8</p>

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<p>molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images." (9:1-22)</p> <p>"For up to 1700 target genes, eight rounds of hybridization decoding are performed. In each round, a rolony can have three states: two fluorescent (FAM and Cy3) "ON" states and</p>	<p>stages x 2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$)." (22:7-16)</p> <p>"...we use three-state eight-stage decoding...." (Gunderson, 871)</p> <p>"We use a process that decodes 1520 different bead types by using three states: two fluorescent "ON" states (FAM and CY3 fluorescent labels) and one nonfluorescent "OFF" state" (Gunderson, 871)</p> <p>"We have developed a simple and efficient algorithm to identify each member of a large collection of DNA-linked objects through the use of hybridization...." (Gunderson, Abstract)</p> <p>"Our algorithm uses sequential hybridizations of dye-labeled oligonucleotides, or decoders, complementary to bead sequences to create a combinatorial decoding scheme for arrays." (Gunderson, 870)</p> <p>"The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R =</p>

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<p>one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate (<10-4).” (14:34-48)</p> <p>“...we use three-state eight-stage decoding....” (Gunderson, 871)</p> <p>We use a process that decodes 1520 different bead types by using three states: two fluorescent “ON” states (FAM andCY3 fluorescent labels) and one nonfluorescent “OFF” state” (Gunderson, 871)</p> <p>“We have developed a simple and efficient algorithm to identify each member of a large</p>	<p>1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“Each bead type is defined by a unique DNA sequence that is recognized by a complementary decoder.” (Gunderson, 870)</p> <div><p>A. Stage 1 → Dehyb → Stage 2 → Dehyb → Stage 3</p><p>B. Three panels showing the bead surface at each stage. Panel 1: Green beads (ON) and red beads (OFF). Panel 2: Red beads (ON) and green beads (OFF). Panel 3: Green beads (ON) and red beads (OFF). A yellow circle highlights a specific bead in each panel.</p><p>C.</p><table><tr><th></th><th></th><th>Stage 1</th><th>Stage 2</th><th>Stage 3</th><th>Signature</th><th>Code</th><th>Parity Code</th></tr><tr><td rowspan="8">Sequences</td><td>0</td><td>Green</td><td>Green</td><td>Green</td><td>GGG</td><td>000</td><td>0000</td></tr><tr><td>1</td><td>Green</td><td>Green</td><td>Red</td><td>GGR</td><td>001</td><td>0011</td></tr><tr><td>2</td><td>Green</td><td>Red</td><td>Green</td><td>GRG</td><td>010</td><td>0101</td></tr><tr><td>3</td><td>Green</td><td>Red</td><td>Red</td><td>GRR</td><td>011</td><td>0110</td></tr><tr><td>4</td><td>Red</td><td>Green</td><td>Green</td><td>RGG</td><td>100</td><td>1001</td></tr><tr><td>5</td><td>Red</td><td>Green</td><td>Red</td><td>RGR</td><td>101</td><td>1010</td></tr><tr><td>6</td><td>Red</td><td>Red</td><td>Green</td><td>RRG</td><td>110</td><td>1100</td></tr><tr><td>7</td><td>Red</td><td>Red</td><td>Red</td><td>RRR</td><td>111</td><td>1111</td></tr></table></div> <p>(Gunderson, FIG. 2)</p>			Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code	Sequences	0	Green	Green	Green	GGG	000	0000	1	Green	Green	Red	GGR	001	0011	2	Green	Red	Green	GRG	010	0101	3	Green	Red	Red	GRR	011	0110	4	Red	Green	Green	RGG	100	1001	5	Red	Green	Red	RGR	101	1010	6	Red	Red	Green	RRG	110	1100	7	Red	Red	Red	RRR	111	1111
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	4	Red	Green	Green	RGG	100	1001																																																											
	5	Red	Green	Red	RGR	101	1010																																																											
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<p>collection of DNA-linked objects through the use of hybridization....” (Gunderson, Abstract)</p> <p>“Our algorithm uses sequential hybridizations of dye-labeled oligonucleotides, or decoders, complementary to bead sequences to create a combinatorial decoding scheme for arrays.” (Gunderson, 870)</p> <p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“Each bead type is defined by a unique DNA sequence that is recognized by a complementary decoder.” (Gunderson, 870)</p>	<p>“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p> <p>“The clear separation between the two modes in the histogram indicates that most beads can be classified unambiguously as OFF or ON.” (Gunderson, 872).</p>

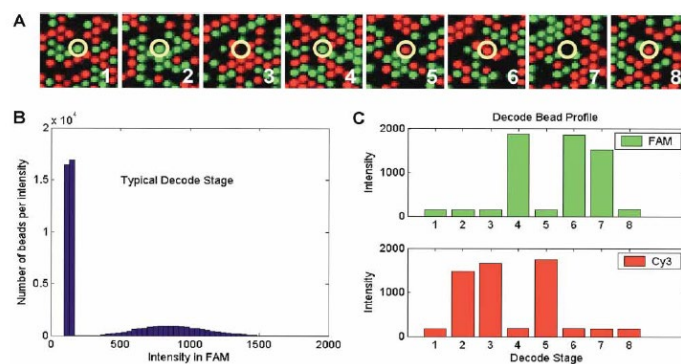
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(Gunderson, FIG. 2)

“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the

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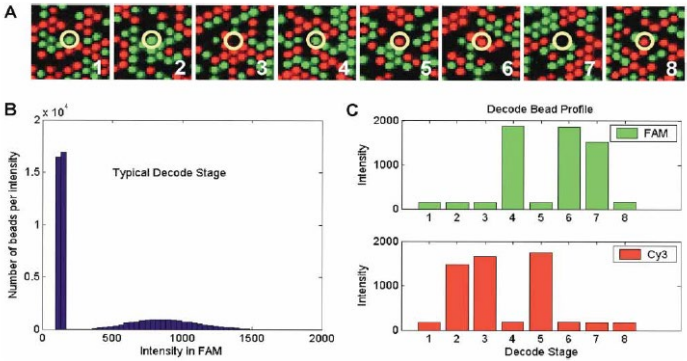


(Gunderson, FIG. 3)

“The low intensity peak includes beads in the OFF state and empty wells. The higher intensity peak includes beads in the ON state.” (Gunderson, FIG. 3, Legend)

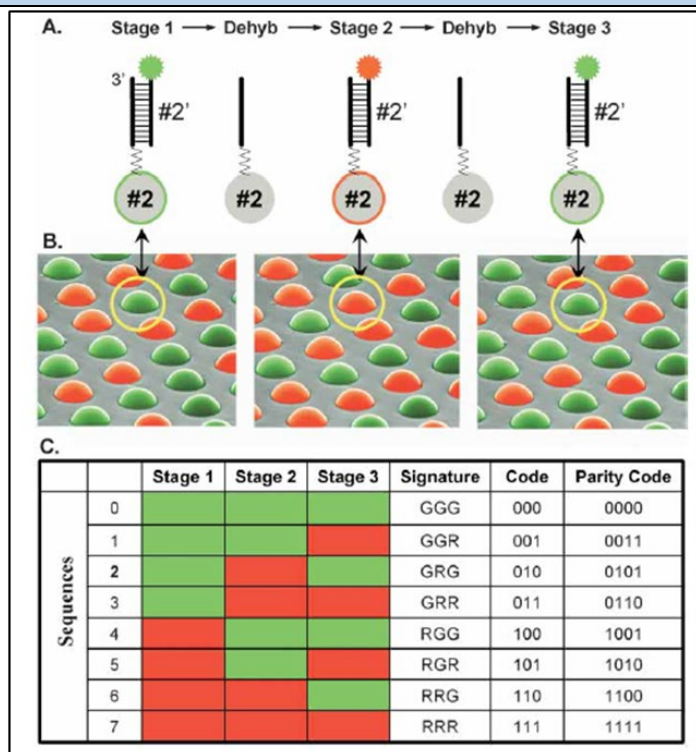
“The histogram is bimodal because each stage and color channel will have beads that are in the ON state and beads that are in the OFF state.” (Gunderson, 876).

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<p>bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p>	

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<p>303 Patent, Claim 1(j): matching the codewords with valid codewords in a codebook by comparing the codewords to the valid codewords in the codebook, and if one of the codewords is not matched with one of the valid codewords in the codebook, applying an error detection or correction system, matching the one of the codewords with another of the valid codewords in the codebook, or discarding the one of the codewords, wherein the codebook comprises the valid codewords of the plurality of nucleic acid targets</p>	
<p>“Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877).” (14:31-34)</p> <p>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p>	<p>“Long oligonucleotides (up to 300nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877).” (22:3-7)</p> <p>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p>

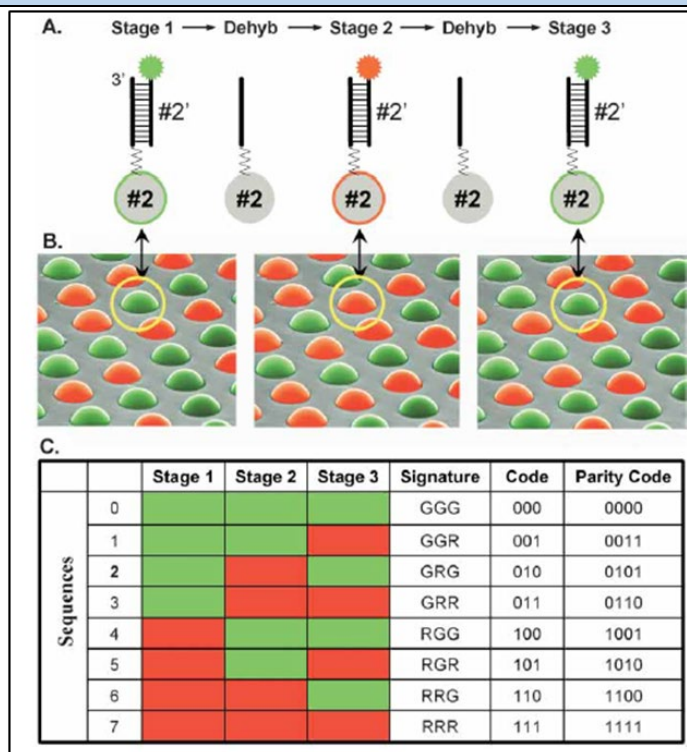
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(Gunderson, FIG. 2)

“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the

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(Gunderson, FIG. 2)

“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore

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<p>bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p>	<p>(green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p> <p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid</p>

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<p>stages so that every valid code has a fixed number of OFF states. For example, if there are 1520 bead types and we use three-state eight-stage decoding, then each valid code would be designed to be OFF in exactly two stages and ON in exactly six stages (the actual scheme used is a slight variant on this design; see Methods). An example of a valid code would be 21110210. With this scheme, it is theoretically impossible to misclassify a code through any number of occurrences of the most common error type: a transition from an ON state to an OFF state. The following events can lead to misclassification: a transition from one ON state to the other, or multiple stage errors with at least one ON-to-OFF transition and at least one OFF-to-ON transition. Both events are extremely rare.” (Gunderson, 871-872)</p> <p>“The strategy for the design of decoder pools to enable error checking, described in the main text, included the condition that all valid codes have exactly two OFF states, which allows for a maximum of 1792 codes. To enable the decoding of more bead types, we pooled the</p>	<p>“The strategy for the design of decoder pools to enable error checking, described in the main text, included the condition that all valid codes have exactly two OFF states, which allows for a maximum of 1792 codes. To enable the decoding of more bead types, we pooled the decoders so that valid codes have either exactly two OFF states or exactly five OFF states. The transition events that lead to misclassification are the same as described above, except that errors of the type (ON to OFF) or (OFF to ON) occurring in three different stages could also lead to a misclassification event. Three errors from ON to OFF could occur if a bead falls out of the array in the middle of decoding. To eliminate this source of error, all codes ending in three or more OFF states were removed from the set of valid codes. Finally, we eliminated any codes that do not have at least one ON state in each of the color channels that we use. The fact that each bead must show specific signal in two color channels during the decode process provides an additional quality check. The total number of valid color codes in this scheme is 2012.” (Gunderson, 875)</p> <p>“We use a process that decodes 1520 different bead types by using three states: two fluorescent “ON” states (FAM and CY3 fluorescent labels) and one nonfluorescent “OFF” state. The logarithmic relationship between the number of bead types and</p>

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303 Patent, Claim 1(k) determining the plurality of nucleic acid targets in the sample based on results from the matching step.																		
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<p>and for multiplex generation of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (1:60-2:1)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell. The methods are useful for obtaining sequence information at the level of individual nucleic acid molecules. The methods are especially advantageous for multiplex generation of sequence information from an individual cell. As such, sequence information can be obtained for several individual nucleic acid molecules in an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and subsequently</p>	<p>of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (1:30-2:6)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell. The methods are useful for obtaining sequence information at the level of individual nucleic acid molecules. The methods are especially advantageous for multiplex generation of sequence information from an individual cell. As such, sequence information can be obtained for several individual nucleic acid molecules in an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (5:16-25)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the</p>

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<p>hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (4:6-19)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the</p>	<p>amplified nucleic acid in tandem. In the embodiment shown in Fig. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:12-27)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res.</p>

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<p>crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence. Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety.” (8:53-59)</p> <p>“FIG. 1 describes one embodiment of padlock capture and hybridization decoding of rolonies. (A) Molecular barcodes are attached to rolonies in a gene specific manner using padlock probes. (B) After padlock capture, each rolony will have a unique barcode in thousands of copies. These</p>	<p>(2004) 14:870-877), the content of which is incorporated by reference in its entirety.” (13:1-5)</p> <p>“Figure 1 describes one embodiment of padlock capture and hybridization decoding of rolonies. (A) Molecular barcodes are attached to rolonies in a gene specific manner using padlock probes. (B) After padlock capture, each rolony will have a unique barcode in thousands of copies. These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5):870-877 (2004)).” (4:16-21)</p> <p>“The instant innovations overcome limitations of existing rolony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and decode thousands of unique amplified nucleic acid species.” (12:18-25)</p>

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<p>barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004)).” (3:32-40)</p> <p>“The instant innovations overcome limitations of existing colony identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and decode thousands of unique amplified nucleic acid species.” (8:34-44)</p> <p>“Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The</p>	<p>“Long oligonucleotides (up to 300nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to ~ 1700 target genes, eight rounds of hybridization decoding are performed.” (22:3-8)</p>

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<p>design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed.” (14:31-36)</p>	
303 Patent, Claim 2	
<p>“The signals generated by fluorescent imaging of one or more probes during decoding, sequencing and other detection methods carried out in situ and can be detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ. Suitable imaging technologies are known in the art, as exemplified by Larsson et al., Nat. Methods (2010) 7:395-397 and associated supplemental materials, the entire content of which is incorporated by reference herein in its entirety.” (11:11-19)</p> <p>“One of the reagents, Cy3, can be excited with the 488-, 514- and 532-nm laser lines and is</p>	<p>“The signals generated by fluorescent imaging of one or more probes during decoding, sequencing and other detection methods carried out in situ and can be detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ. Suitable imaging technologies are known in the art, as exemplified by Larsson et al., Nat. Methods (2010) 7:395-397 and associated supplemental materials, the entire content of which is incorporated by reference herein in its entirety.” (16:29-17:5)</p> <p>“One of the reagents, Cy3, can be excited with the 488-, 514- and 532-nm laser lines and is optimally excited with the 546-nm mercury arc line.” (Mujumdar, Abstract)</p> <p>“Images of viral particles on cover slips or fixed cells were acquired using an Apotome (Carl Zeiss) structured illumination fluorescence imaging system mounted on an</p>

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<p>optimally excited with the 546-nm mercury arc line.” (Mujumdar, Abstract)</p> <p>“Images of viral particles on cover slips or fixed cells were acquired using an Apotome (Carl Zeiss) structured illumination fluorescence imaging system mounted on an Axioplan 2 imaging microscope equipped with a Plan-Apochromat 63 NA-1.4 oil objective and an MRm charge-coupled device camera piloted by AxioVision software.” (Arhel, 823)</p> <p>“Images of cultured cells were acquired using an AxioplanII epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp, a CCD camera (C4742-95, Hamamatsu), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5. A $\times 20$ (Plan-Apocromat, Zeiss), $\times 40$ (Plan-Neofluar, Zeiss) or $\times 63$ (Plan-Neofluar, Zeiss) objective was used for capturing the images.” (Larsson, 5)</p> <p>“The unit is equipped with a high resolution image sensor featuring an defective pixel</p>	<p>Axioplan 2 imaging microscope equipped with a Plan-Apochromat 63 NA-1.4 oil objective and an MRm charge-coupled device camera piloted by AxioVision software.” (Arhel, 823)</p> <p>“Images of cultured cells were acquired using an AxioplanII epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp, a CCD camera (C4742-95, Hamamatsu), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5. A $\times 20$ (Plan-Apocromat, Zeiss), $\times 40$ (Plan-Neofluar, Zeiss) or $\times 63$ (Plan-Neofluar, Zeiss) objective was used for capturing the images.” (Larsson, 5)</p> <p>“The unit is equipped with a high resolution image sensor featuring an defective pixel number of 4000 (Horizontal) x 2624 (Vertical) pixels.” (Hamamatsu, 7).</p>

Fan (EX1005)	Fan Provisional Application (EX1006)																																																		
<p>number of 4000 (Horizontal) x 2624 (Vertical) pixels.” (Hamamatsu, 7).</p> <p>(1) Electrical specifications</p> <table border="1"> <tr><td>Imaging device</td><td>Interface readout interline CCD solid imaging element</td></tr> <tr><td>Effective number of pixels</td><td>4000 (H) × 2624 (V)</td></tr> <tr><td>Cell size</td><td>5.9 μm × 5.9 μm (Square format)</td></tr> <tr><td>Effective area</td><td>23.6 mm × 15.5 mm (1.8-inch size)</td></tr> <tr><td rowspan="4">Frame rate</td><td>Interface readout 1.7 Hz</td></tr> <tr><td>2×2 binning readout 3.4 Hz</td></tr> <tr><td>4×4 binning readout 6.4 Hz</td></tr> <tr><td>Outline readout 8.9 Hz</td></tr> <tr><td>Readout noise (rms.)</td><td>20 electrons (typ.) ^{*1}</td></tr> <tr><td>A/D converter</td><td>12 bit</td></tr> <tr><td>Cooling method</td><td>Electronic and natural air cooling</td></tr> <tr><td>Lens mount</td><td>F mount</td></tr> <tr><td>Amp gain conversion factor ^{*2}</td><td>3.2 electrons/ADcounts (13 300 electrons)</td></tr> <tr><td>Contrast variable range</td><td>1 to approx. 10 times</td></tr> </table> <p>(Hamamatsu, 35)</p>	Imaging device	Interface readout interline CCD solid imaging element	Effective number of pixels	4000 (H) × 2624 (V)	Cell size	5.9 μm × 5.9 μm (Square format)	Effective area	23.6 mm × 15.5 mm (1.8-inch size)	Frame rate	Interface readout 1.7 Hz	2×2 binning readout 3.4 Hz	4×4 binning readout 6.4 Hz	Outline readout 8.9 Hz	Readout noise (rms.)	20 electrons (typ.) ^{*1}	A/D converter	12 bit	Cooling method	Electronic and natural air cooling	Lens mount	F mount	Amp gain conversion factor ^{*2}	3.2 electrons/ADcounts (13 300 electrons)	Contrast variable range	1 to approx. 10 times	<p>(1) Electrical specifications</p> <table border="1"> <tr><td>Imaging device</td><td>Interface readout interline CCD solid imaging element</td></tr> <tr><td>Effective number of pixels</td><td>4000 (H) × 2624 (V)</td></tr> <tr><td>Cell size</td><td>5.9 μm × 5.9 μm (Square format)</td></tr> <tr><td>Effective area</td><td>23.6 mm × 15.5 mm (1.8-inch size)</td></tr> <tr><td rowspan="4">Frame rate</td><td>Interface readout 1.7 Hz</td></tr> <tr><td>2×2 binning readout 3.4 Hz</td></tr> <tr><td>4×4 binning readout 6.4 Hz</td></tr> <tr><td>Outline readout 8.9 Hz</td></tr> <tr><td>Readout noise (rms.)</td><td>20 electrons (typ.) ^{*1}</td></tr> <tr><td>A/D converter</td><td>12 bit</td></tr> <tr><td>Cooling method</td><td>Electronic and natural air cooling</td></tr> <tr><td>Lens mount</td><td>F mount</td></tr> <tr><td>Amp gain conversion factor ^{*2}</td><td>3.2 electrons/ADcounts (13 300 electrons)</td></tr> <tr><td>Contrast variable range</td><td>1 to approx. 10 times</td></tr> </table> <p>(Hamamatsu, 35)</p>	Imaging device	Interface readout interline CCD solid imaging element	Effective number of pixels	4000 (H) × 2624 (V)	Cell size	5.9 μm × 5.9 μm (Square format)	Effective area	23.6 mm × 15.5 mm (1.8-inch size)	Frame rate	Interface readout 1.7 Hz	2×2 binning readout 3.4 Hz	4×4 binning readout 6.4 Hz	Outline readout 8.9 Hz	Readout noise (rms.)	20 electrons (typ.) ^{*1}	A/D converter	12 bit	Cooling method	Electronic and natural air cooling	Lens mount	F mount	Amp gain conversion factor ^{*2}	3.2 electrons/ADcounts (13 300 electrons)	Contrast variable range	1 to approx. 10 times
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303 Patent, Claim 3																																																			
<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first</p>																																																		

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<p>nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“Presented herein are methods and compositions for obtaining sequence information from one or more individual cells. The methods are useful for obtaining sequence information for a single nucleotide sequence, and for multiplex generation of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules in situ within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid</p>	<p>decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:20-30)</p> <p>“Presented herein are methods and compositions for obtaining sequence information from one or more individual cells. The methods are useful for obtaining sequence information for a single nucleotide sequence, and for multiplex generation of sequence information from an individual cell. In some embodiments presented herein, the methods comprise first amplifying one or more nucleic acid molecules <i>in situ</i> within a cell, and subsequently hybridizing a barcode probe to the amplified nucleic acid molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (1:29-2:6)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell.” (5:16-17)</p>

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<p>molecules. The identity of the amplified nucleic acid molecules is then determined by decoding of the barcode probes.” (1:58-2:1)</p> <p>“Accordingly, presented herein are methods and compositions for obtaining sequence information from an individual cell.” (4:6-8)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in FIG. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes</p>	<p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule containing many repeats of the amplified nucleic acid in tandem. In the embodiment shown in Fig. 1, cDNA is generated <i>in situ</i>, using either random hexamers or oligo dT primers or both. Then the cDNA molecules are amplified using rolling circle amplification (RCA) to generate a "DNA ball" or "rolony". The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes. When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe. The amplified nucleic acid molecules and/or the hybridized padlock probes are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in Fig. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (13:12-27)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (Fig. 1). This method is built upon ultra-high degree multiplexed</p>

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<p>are immobilized within the cell using the crosslinking methods described herein, and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:1-22)</p> <p>“This example presents an innovative method based on padlock circularization and hybridization-based decoding (FIG. 1). This method is built upon ultra-high degree multiplexed capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, colonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (FIG. 1A). In some embodiments, colonies for a specific subset of genes of interest can then be</p>	<p>capture (>300,000 per reaction) using padlock probes for useful for genomic, transcriptomic and methylomic analyses, as well as technology for accurate decoding of randomly assembled bead arrays. In some embodiments, colonies for an entire transcriptome can be generated using either random hexamers or oligo-dT primers (Fig. 1A). In some embodiments, colonies for a specific subset of genes of interest can then be tagged with gene-specific barcodes using a library of padlock probes (Fig. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to ~1700 target</p>

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<p>tagged with gene-specific barcodes using a library of padlock probes (FIG. 1B). Padlock probes are designed in a similar manner as the full transcriptome probe sets previously developed for digital RNA allelotyping according to the incorporated materials disclosed in Zhang, K. et al. Nat Methods 6, 613-618 (2009) and Noggle, S. et al. Nature 478, 70-75 (2011). A neural network, trained for predicting probe efficiency using experimental data accumulated on over 400,000 probes, is integrated into a probe design algorithm called ppDesigner. The entire probe design, synthesis and production process has been highly optimized. Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877). For up to 1700 target genes, eight rounds of hybridization decoding are performed. In</p>	<p>genes, eight rounds of hybridization decoding are performed. In each round, a rolony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking. Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stages x 2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (Example 1, 21:17-22:16)</p>

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<p>each round, a colony can have three states: two fluorescent (FAM and Cy3) "ON" states and one nonfluorescent OFF state. In this three-state, eight-stage decoding scheme, the valid barcode for each gene is required to have exactly two OFF stages and six ON stages for error checking Each padlock probe only needs six slots for hybridization (corresponding to the six ON stages). For the hybridization sequences used in these six slots, a set of 16 (8 stagesx2 ON states) 20-mers is selected that are orthogonal to the human genome and have uniform annealing parameters. This eight-stage barcoding scheme is sufficient to decode up to 1792 transcripts with a low error rate ($<10^{-4}$).” (Example 1, 14:3-48)</p>	
303 Patent, Claim 4	
<p>“Methods for permeabilization and fixation of cells and tissue samples are well-known in the art, as exemplified by Cremer et al., The Nucleus: Volume 1: Nuclei and Subnuclear Components, R. Hancock (ed.) 2008; and Larsson et al., Nat. Methods (2010) 7:395-397 and associated supplemental materials, the</p>	<p>“Methods for permeabilization and fixation of cells and tissue samples are well-known in the art, as exemplified by Cremer et al., The Nucleus: Volume 1: Nuclei and Subnuclear Components, R. Hancock (ed.) 2008; and Larsson et al., Nat. Methods (2010) 7:395-397 and associated supplemental</p>

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<p>content of each of which is incorporated herein by reference in its entirety.” (10:66-11:6)</p> <p>“Fresh frozen 9-µm sections of E14.5 mouse embryos were placed on Superfrost Plus Gold slides (Thermo Scientific). Fully anonymized fresh frozen human tissue sections from a HER2-positive breast cancer were obtained from the Fresh Tissue Biobank at the Department of Pathology, Uppsala University Hospital, in accordance with the Swedish Biobank Legislation. Breast tissue sections of 4 µm thickness were placed on Starfrost microscope slides (Instrumedics).” (Larsson, 0004)</p>	<p>materials, the content of each of which is incorporated herein by reference in its entirety.” (16:19-23)</p> <p>“Fresh frozen 9-µm sections of E14.5 mouse embryos were placed on Superfrost Plus Gold slides (Thermo Scientific). Fully anonymized fresh frozen human tissue sections from a HER2-positive breast cancer were obtained from the Fresh Tissue Biobank at the Department of Pathology, Uppsala University Hospital, in accordance with the Swedish Biobank Legislation. Breast tissue sections of 4 µm thickness were placed on Starfrost microscope slides (Instrumedics).” (Larsson, 0004)</p>
303 Patent, Claim 5	
<p>“The signals generated by fluorescent imaging of one or more probes during decoding, sequencing and other detection methods carried out in situ and can be detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ. Suitable imaging technologies are known in the art, as exemplified by Larsson</p>	<p>“The signals generated by fluorescent imaging of one or more probes during decoding, sequencing and other detection methods carried out in situ and can be detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ. Suitable imaging technologies are known in the art, as exemplified by Larsson et al., Nat. Methods (2010) 7:395-397 and associated</p>

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<p>et al., Nat. Methods (2010) 7:395-397 and associated supplemental materials, the entire content of which is incorporated by reference herein in its entirety.” (11:11-19)</p> <p>“Images of cultured cells were acquired using an AxioplanII epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp, a CCD camera (C4742-95, Hamamatsu), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5. A $\times 20$ (Plan-Apocromat, Zeiss), $\times 40$ (Plan-Neofluar, Zeiss) or $\times 63$ (Plan-Neofluar, Zeiss) objective was used for capturing the images.” (Larsson, 0005)</p>	<p>supplemental materials, the entire content of which is incorporated by reference herein in its entirety.” (16:29-17:5)</p> <p>“Images of cultured cells were acquired using an AxioplanII epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp, a CCD camera (C4742-95, Hamamatsu), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5. A $\times 20$ (Plan-Apocromat, Zeiss), $\times 40$ (Plan-Neofluar, Zeiss) or $\times 63$ (Plan-Neofluar, Zeiss) objective was used for capturing the images.” (Larsson, 0005)</p>
303 Patent, Claim 6	
<p>“In some embodiments the spatial location of nucleic acids can be simultaneously determined for several individual cells in a tissue or other cell-containing sample.” (4:32-34)</p> <p>“In some aspects, detecting can further comprise evaluating a signal to determine the presence, quantity, frequency, and/or spatial distribution</p>	<p>“In some embodiments the spatial location of nucleic acids can be simultaneously determined for several individual cells in a tissue or other cell-containing sample.” (6:5-7)</p> <p>“In some aspects, detecting can further comprise evaluating a signal to determine the presence, quantity, frequency, and/or</p>

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of a disease-causing mutation, allele or common variant within a tissue.” (3:21-24)	spatial distribution of a disease-causing mutation, allele or common variant within a tissue.: (4:8-10)
303 Patent, Claim 7	
<p>“The methods and compositions make use of hybridization, amplification, washing and detection within individual cells.” (10:62-64)</p> <p><i>See also</i> Claim 1.</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (9:25-27)</p> <p>“These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004)).” (3:36-40)</p> <p>“Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the</p>	<p>“The methods and compositions make use of hybridization, amplification, washing and detection within individual cells.” (16:16-17)</p> <p><i>See also</i> Claim 1 and Claim 3.</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (13:28-30)</p> <p>“These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5):870-877 (2004)).” (4:19-21)</p> <p>“Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety.” (13:3-5)</p> <p>“Long oligonucleotides (up to 300nt in length) acquired from Agilent Technologies contain amplification adaptors to allow</p>

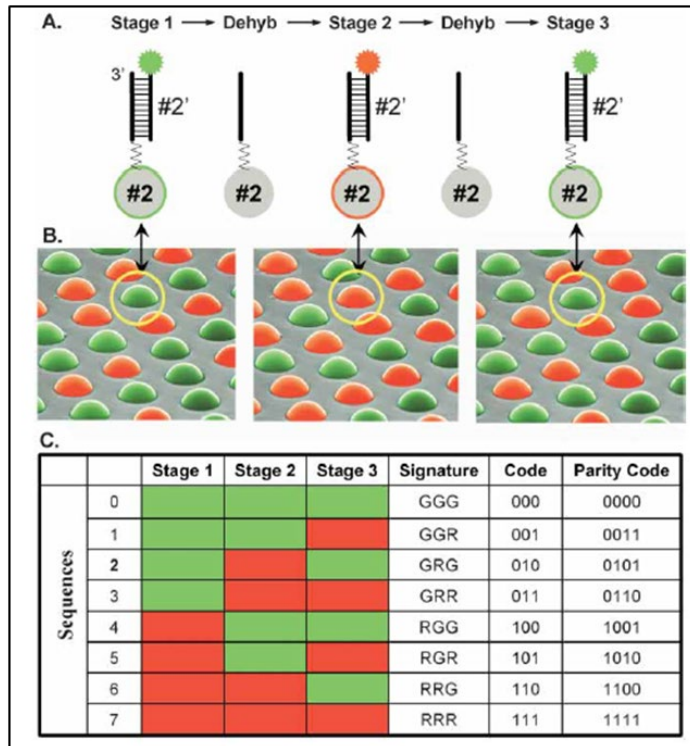
Fan (EX1005)	Fan Provisional Application (EX1006)
<p>content of which is incorporated by reference in its entirety” (8:56-59)</p> <p>“Long oligonucleotides (up to 300 nt in length) acquired from Agilent Technologies contain amplification adaptors to allow production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877).” (14:31-34)</p> <p>“The instant innovations overcome limitations of existing rolon identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and</p>	<p>production of essentially an unlimited amount of functional padlock probes from a set of seed oligos. The design of the barcoding region incorporates the error-checking scheme described in the incorporated materials of Gunderson et al. (Genome Res. (2004) 14:870-877).” (22:3-7)</p> <p>“The instant innovations overcome limitations of existing rolon identification methodologies by surprisingly and successfully applying decoding strategies that entail sequential hybridizations of labeled decoder probes to decoder sequences found in immobilized nucleic acids within an individual cell. Such decoding strategies using decoder sequences have until now been used for decoding of randomly assembled arrays of beads. Thus, the decoding strategies presented herein are flexible and scalable and can be used to detect and decode thousands of unique amplified nucleic acid species.” (12:18-25)</p> <p>“Following hybridization, three 1-min washes in wash buffer (167 mM NaCl, 16.7 mM potassium phosphate, 0.017% Tween-20 at pH 7.6) were used to remove unbound oligonucleotides.” (Gunderson, 875)</p>

Fan (EX1005)	Fan Provisional Application (EX1006)
<p>decode thousands of unique amplified nucleic acid species.” (8:34-44)</p> <p>“Following hybridization, three 1-min washes in wash buffer (167 mM NaCl, 16.7 mM potassium phosphate, 0.017% Tween-20 at pH 7.6) were used to remove unbound oligonucleotides.” (Gunderson, 875)</p>	
303 Patent, Claim 8	
<p>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p> <p>“Although assignment of codes to sequences is unambiguous after three stages, additional stages can be added for error checking purposes (last column of Fig. 2C) to be described below.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even</p>	<p>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p> <p>“Although assignment of codes to sequences is unambiguous after three stages, additional stages can be added for error checking purposes (last column of Fig. 2C) to be described below.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid</p>

Fan (EX1005)	Fan Provisional Application (EX1006)
<p>parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)</p>	<p>codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)</p>
303 Patent, Claim 9	
<p>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p> <p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error</p>	<p>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</p> <p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)</p>

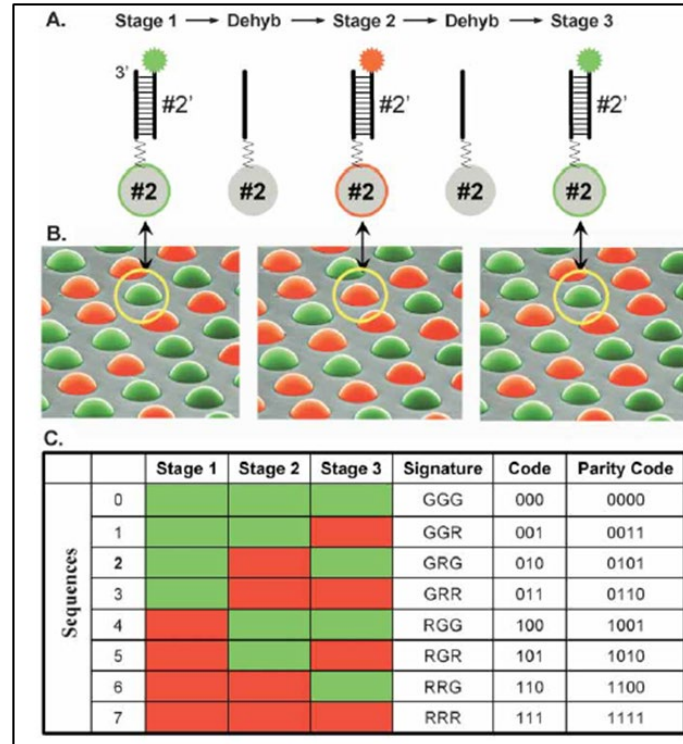
Fan (EX1005)

that creates an odd parity, or invalid code.”
(Gunderson, 871)



(Gunderson, FIG. 2)

Fan Provisional Application (EX1006)



(Gunderson, FIG. 2)

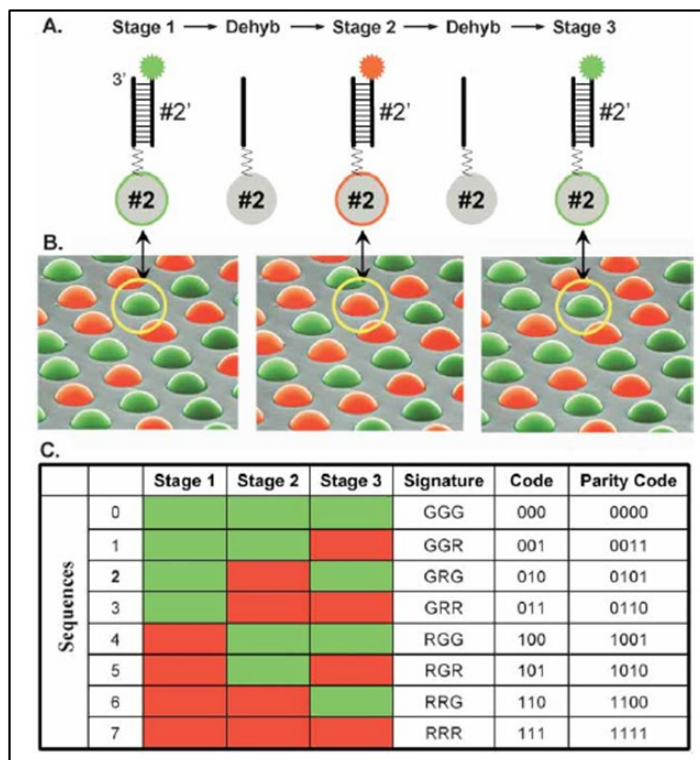
Table 1. Distribution and Use of 6561 Codes In the Three-State, Eight-Stage System

Type	Number	Purpose
Used valid codes	1520	Assigned to actual beads.
Unused valid codes	272	Detect rare two-stage errors.
Invalid codes	4769	Estimate the number of misclassification events.
		Detect single stage errors.
		Monitor overall decode process; useful for detecting occasional catastrophic failures.

Fan (EX1005)	Fan Provisional Application (EX1006)												
<div>Table 1. Distribution and Use of 6561 Codes in the Three-State, Eight-Stage System</div> <table><tr><th>Type</th><th>Number</th><th>Purpose</th></tr><tr><td>Used valid codes</td><td>1520</td><td>Assigned to actual beads.</td></tr><tr><td>Unused valid codes</td><td>272</td><td>Detect rare two-stage errors.</td></tr><tr><td>Invalid codes</td><td>4769</td><td>Estimate the number of misclassification events. Detect single stage errors. Monitor overall decode process; useful for detecting occasional catastrophic failures.</td></tr></table> <div>(Gunderson, Table 1)</div>	Type	Number	Purpose	Used valid codes	1520	Assigned to actual beads.	Unused valid codes	272	Detect rare two-stage errors.	Invalid codes	4769	Estimate the number of misclassification events. Detect single stage errors. Monitor overall decode process; useful for detecting occasional catastrophic failures.	<div>(Gunderson, Table 1)</div>
Type	Number	Purpose											
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Unused valid codes	272	Detect rare two-stage errors.											
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303 Patent, Claim 10													
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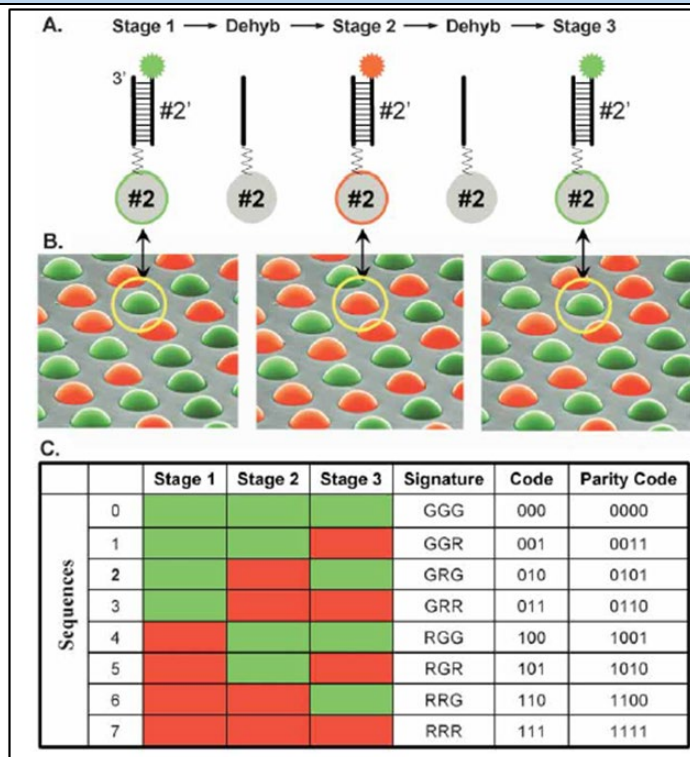
Fan (EX1005)

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(Gunderson, 871)



(Gunderson, FIG. 2)

Fan Provisional Application (EX1006)



(Gunderson, FIG. 2)

Table 1. Distribution and Use of 6561 Codes in the Three-State, Eight-Stage System		
Type	Number	Purpose
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Unused valid codes	272	Detect rare two-stage errors.
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		Detect single stage errors.
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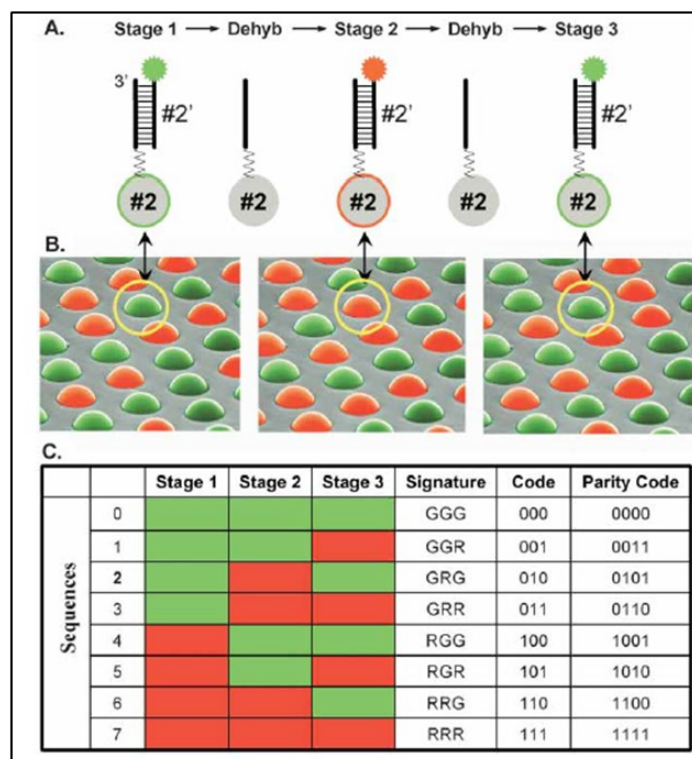
Fan (EX1005)	Fan Provisional Application (EX1006)												
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303 Patent, Claim 11													
<div>“These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial</div> <div>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</div> <div>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</div>	<div>“These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial</div> <div>“The error-checking concepts that we have developed, and that underlie the robustness of our hybridization-based approach, are based on algorithms from digital information theory (Shannon 1948a,b; Hamming 1986).” (Gunderson, 871).</div> <div>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</div> <div>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid</div>												

Fan (EX1005)

“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)

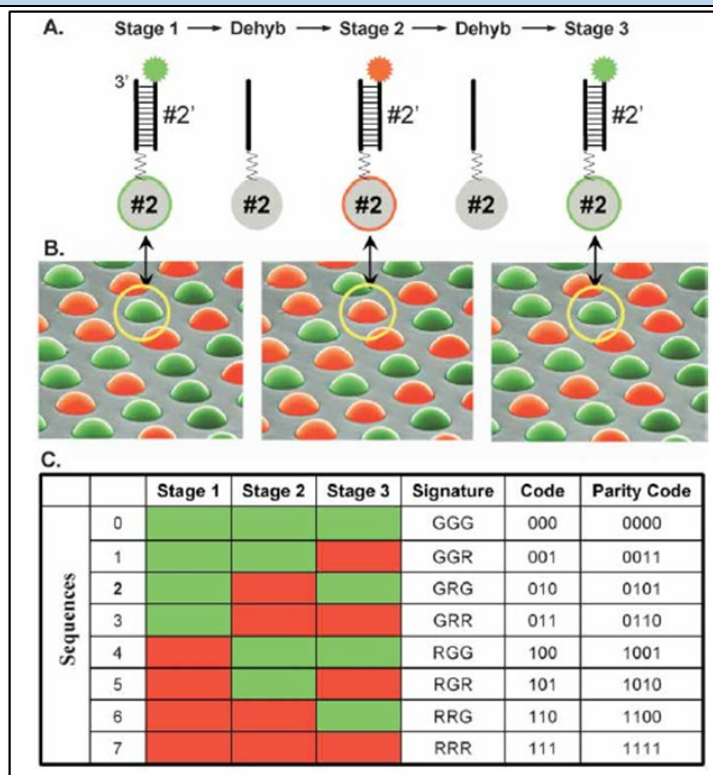
Fan Provisional Application (EX1006)

codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)



(Gunderson, FIG. 2)

Fan (EX1005)



(Gunderson, FIG. 2)

Type	Number	Purpose
Used valid codes	1520	Assigned to actual beads.
Unused valid codes	272	Detect rare two-stage errors. Estimate the number of misclassification events.
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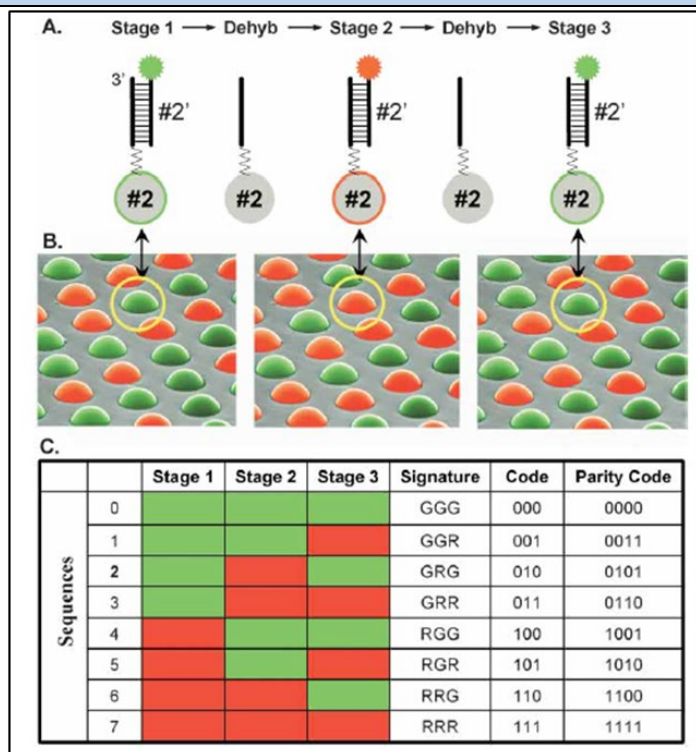
Fan Provisional Application (EX1006)

Type	Number	Purpose
Used valid codes	1520	Assigned to actual beads.
Unused valid codes	272	Detect rare two-stage errors. Estimate the number of misclassification events.
Invalid codes	4769	Detect single stage errors. Monitor overall decode process; useful for detecting occasional catastrophic failures.

(Gunderson, Table 1)

Fan (EX1005)	Fan Provisional Application (EX1006)
(Gunderson, Table 1)	
303 Patent, Claim 12	
<p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)</p>	<p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p> <p>“The simplest form of error checking we have used has a parity bit and is illustrated in Figure 2C. The assigned four-bit codes all have an even parity bit sum and are termed valid codes. An error in a single decode stage is a one-bit error that creates an odd parity, or invalid code.” (Gunderson, 871)</p>

Fan (EX1005)

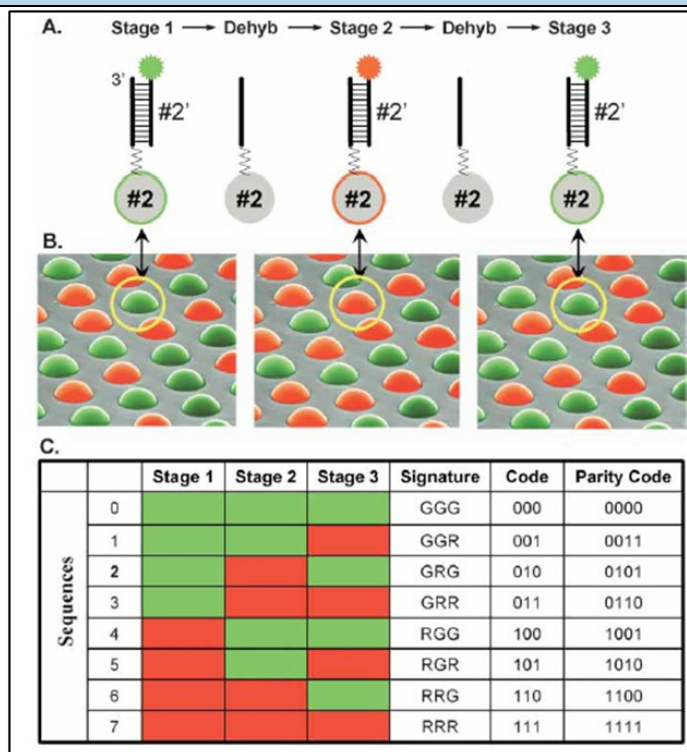


(Gunderson, FIG. 2)

Table 1. Distribution and Use of 6561 Codes In the Three-State, Eight-Stage System

Type	Number	Purpose
Used valid codes	1520	Assigned to actual beads.
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Fan Provisional Application (EX1006)



(Gunderson, FIG. 2)

Table 1. Distribution and Use of 6561 Codes In the Three-State, Eight-Stage System

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Fan (EX1005)	Fan Provisional Application (EX1006)
<p>(Gunderson, Table 1)</p> <p>“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra</p>	<p>(Gunderson, Table 1)</p> <p>“Decoding process. (A) The sequential hybridization process is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. The images, taken collectively, reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p>

Fan (EX1005)	Fan Provisional Application (EX1006)
decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)	

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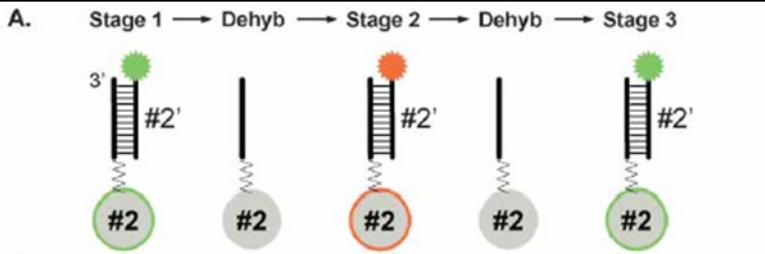
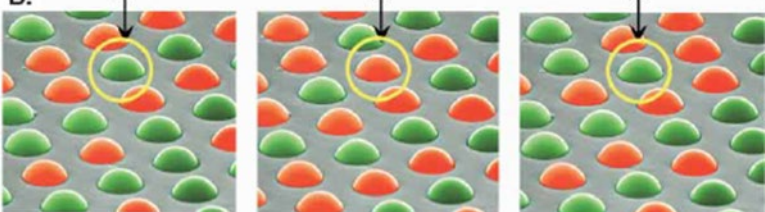
APPENDIX C

Fan Claim 1 (EX1005)	Fan Provisional (EX1006)
1. A method of providing sequence information regarding at least one target nucleic acid sequence in a plurality of nucleic acid molecules from an individual cell comprising:	“Presented herein are methods and compositions for obtaining sequence information from one or more individual cells . The methods are useful for obtaining sequence information for a single nucleotide sequence , and for multiplex generation of sequence information from an individual cell .” (1:58-62)
(a) amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules, wherein the set of amplified nucleic acid molecules comprise the at least one target nucleic acid sequence;	<p>“Accordingly, presented herein is a method of obtaining sequence information from an individual cell comprising: amplifying a nucleic acid molecule in situ within a cell...” (2:8-10)</p> <p>“As an example, one or more target nucleic acid species can be amplified, either specifically or non-specifically, to generate an amplified molecule...” (9:1-3)</p>
(b) for each amplified nucleic acid sequence of said set of amplified nucleic acid molecules,	<p>“The amplified nucleic acids can then be hybridized with one or more unique padlock probes with unique molecular barcodes.” (9:8-11)</p> <p>“The methods provided herein further comprise annealing in situ a barcode probe to the amplified nucleic acid molecule. As used herein, the term barcode probe refers</p>

Fan Claim 1 (EX1005)	Fan Provisional (EX1006)
annealing in situ a padlock probe that is designed to anneal to the at least one target nucleic acid sequence,	<p>to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and which comprises a barcode sequence.” (5:15-20)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes.” (7:36-37)</p>
wherein the padlock probe comprises two sequence-specific capturing arms on the 5' and 3' end of the probe,	<p>“(C) Schematic of padlock probes. In the example shown, each probe has two gene-specific (and/or allele-specific) capturing arms H1/H2.” (3:40-42)</p> <div data-bbox="557 919 1390 1018" data-label="Diagram"> </div> <p>(FIG. 1C)</p>
wherein the capturing arms are ligatable when each are annealed to the target nucleic acid sequence,	<p>“When a padlock probe specifically hybridizes to a sequence of interest, the probe is ligated to form, for example, a circularized barcoded padlock probe.” (9:11-13)</p> <p>“These probes have a first ligation domain that is identical to a first ligation probe, in that it hybridizes to a first target sequence domain, and a second ligation domain, identical to the second ligation probe, that hybridizes to an adjacent target sequence domain. Again, as for OLA, the detection position can be either at the 3' end of the first ligation domain or at the 5' end of the second ligation domain.” (7:40-46)</p> <p>“The configuration of the system is such that upon ligation of the first and second ligation domains of the padlock probe, the probe so forms a circular probe,</p>

Fan Claim 1 (EX1005)	Fan Provisional (EX1006)
	and forms a complex with the target sequence wherein the target sequence is "inserted" into the loop of the circle.” (7:48-53)
wherein the padlock probe further comprises at least a first decoder sequence and a second decoder sequence that do not anneal to said amplified nucleic acid molecules,	<p>“The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (5:26-29)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes.” (7:36-37)</p> <p>“As used herein, the terms decoder sequence, stage, slot and like terms refer to a unique sequence which is part of a plurality of unique sequences are designed to each specifically hybridize to a labeled decoder probe.” (8:49-52)</p>
and wherein the padlock probe can be identified based upon generating a code from decoding the at least first decoder sequence and the second decoder sequence by using sequential hybridization with fluorescently labeled decoder probes, and identifying the padlock probe by matching said code with a reference	<p>“Barcode probes used in the methods presented herein can include padlock probes.” (7:36-37)</p> <p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:53-55)</p> <p>“As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and which comprises a barcode sequence. As used herein, the terms barcode, barcode sequence and like terms refer to a unique identifier sequence on a probe molecule. The unique identifier sequence can be, for example, a nucleic acid sequence which associates the barcode probe with the nucleic acid of interest to which it hybridizes.” (5:17-25)</p>

Fan Claim 1 (EX1005)	Fan Provisional (EX1006)
code that is assigned for each padlock probe,	<p>“...and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:16-22)</p> <p>“These barcodes can be accurately decoded by hybridization similar to the decoding of Illumina bead array, using a combinatorial chemistry approach (Gunderson et al. Genome Res. 14(5): 870-877 (2004)).” (3:36-40)</p> <p>“Sequential hybridization of decoder probes to decoder sequences is described by Gunderson et al. (Genome Res. (2004) 14:870-877), the content of which is incorporated by reference in its entirety” (8:56-59)</p>

Fan Claim 1 (EX1005)	Fan Provisional (EX1006)																																																																	
	<div><p>A. Stage 1 → Dehyb → Stage 2 → Dehyb → Stage 3</p></div> <div><p>B.</p></div> <div><p>C.</p><table><tr><th></th><th></th><th>Stage 1</th><th>Stage 2</th><th>Stage 3</th><th>Signature</th><th>Code</th><th>Parity Code</th></tr><tr><td rowspan="8">Sequences</td><td>0</td><td></td><td></td><td></td><td>GGG</td><td>000</td><td>0000</td></tr><tr><td>1</td><td></td><td></td><td></td><td>GGR</td><td>001</td><td>0011</td></tr><tr><td>2</td><td></td><td></td><td></td><td>GRG</td><td>010</td><td>0101</td></tr><tr><td>3</td><td></td><td></td><td></td><td>GRR</td><td>011</td><td>0110</td></tr><tr><td>4</td><td></td><td></td><td></td><td>RGG</td><td>100</td><td>1001</td></tr><tr><td>5</td><td></td><td></td><td></td><td>RGR</td><td>101</td><td>1010</td></tr><tr><td>6</td><td></td><td></td><td></td><td>RRG</td><td>110</td><td>1100</td></tr><tr><td>7</td><td></td><td></td><td></td><td>RRR</td><td>111</td><td>1111</td></tr></table></div>			Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code	Sequences	0				GGG	000	0000	1				GGR	001	0011	2				GRG	010	0101	3				GRR	011	0110	4				RGG	100	1001	5				RGR	101	1010	6				RRG	110	1100	7				RRR	111	1111
		Stage 1	Stage 2	Stage 3	Signature	Code	Parity Code																																																											
Sequences	0				GGG	000	0000																																																											
	1				GGR	001	0011																																																											
	2				GRG	010	0101																																																											
	3				GRR	011	0110																																																											
	4				RGG	100	1001																																																											
	5				RGR	101	1010																																																											
	6				RRG	110	1100																																																											
	7				RRR	111	1111																																																											

(Gunderson, FIG. 2)

“Decoding process. (A) The **sequential hybridization process** is illustrated for a single bead, of bead type 2. In stage 1, a complementary decoder hybridizes to the oligonucleotide probe that is attached to the bead (for details of the procedure, see Methods). The decoder is labeled with a fluorophore (green in stage 1, red in stage 2, and green in stage 3). The fluorescent signal is read by imaging the entire array. The array is then dehybridized, and the process is repeated for two more stages. (B) A scanning electron micrograph of an array of beads, artificially colored to represent three sequential hybridization stages. **The images, taken collectively,**

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	<p>reveal a combinatorial code for each bead. Note that the bead circled in yellow has the color signature GRG or code 010. (C) Colors, or states, are assigned to individual decoder sequences in each stage to produce a unique combination across stages. This signature, or code, identifies each bead type. As indicated in the parity code column, an extra decoding stage (data not shown) can be performed to provide an error checking parity bit. After three stages of decoding, all the beads are uniquely identified by their color.” (Gunderson, FIG. 2 Legend)</p> <p>“The bead circled in Figure 2B has the color signature (GRG), or 010 code in binary representation, in which G = 0 and R = 1. Its sequence can be identified as sequence 2 by referring to the color-lookup table in Figure 2C.” (Gunderson, 871)</p>
<p>wherein each padlock probe that hybridizes to a different target nucleic acid sequence has a different reference code;</p>	<p>“As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and which comprises a barcode sequence.” (5:17-20)</p> <p>“The barcode probes presented herein can be any probe which specifically hybridizes to a nucleic acid species of interest and which comprises at least a first and a second decoder sequence.” (5:26-29)</p> <p>“In other embodiments presented herein, the methods comprise first hybridizing one or more different barcode probes to one or more nucleic acid molecule in situ within a cell....” (2:1-4)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes.” (7:36-37)</p>
<p>(c) hybridizing a first fluorescently</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid</p>

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<p>labeled decoder probe to said first decoder sequence, wherein the first fluorescently labeled decoder probe that is not hybridized to said first decoder sequence is removed by washing;</p>	<p>molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (9:25-27)</p> <p>“The methods and compositions make use of hybridization, amplification, washing and detection within individual cells.” (10:62-64)</p>
<p>(d) detecting a plurality of fluorescent signals indicative of said hybridizing of the first fluorescently labeled decoder probe to said first decoder sequence;</p>	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of</p>

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	<p>signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p>
(e) de-hybridizing and removing the first fluorescently labeled decoder probe;	<p>“In some aspects of the above embodiments, the first labeled decoder probe is removed prior to hybridizing the second labeled decoder probe.” (2:56-58)</p>
(f) hybridizing a second fluorescently labeled decoder probe to said second decoder sequence, wherein the second fluorescently labeled decoder probe that is not hybridized to said second decoder sequence is removed by washing,	<p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“For example, one advantage is the simplicity and robustness of the decoding process, which involves hybridization and washing of oligo pools at room temperature.” (9:25-27)</p>

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	“The methods and compositions make use of hybridization, amplification, washing and detection within individual cells.” (10:62-64)
and wherein the second fluorescently labeled decoder probe is labeled with a fluor that absorbs light at a different wavelength than the fluor of the first fluorescently labeled decoder probe;	“Further, in many embodiments, only two fluors are needed for decoding, which provides convenient spectral distinction using simple detection devices and leaves enough spectrum for labeling cellular structure using other fluors.” (9:34-37)
(g) detecting a plurality of fluorescent signals indicative of said hybridizing of the second fluorescently labeled decoder probe to said second decoder sequence, and	“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing , thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)

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	<p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:53-55)</p> <p>“...and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:16-22)</p> <p>“The signals generated by fluorescent imaging of one or more probes during decoding, sequencing and other detection methods carried out in situ and can be detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ.” (11:11-15)</p>
<p>(h) determining the identity of the padlock probe based upon decoding the plurality of fluorescent signals generated from hybridization of the first fluorescently labeled decoder probe with the first decoder sequence of the padlock probe, and the plurality of fluorescent signals generated from</p>	<p>“During decoding, labeled decoder probe oligonucleotides are sequentially hybridized to the decoder sequences to specifically determine the identity of each barcode sequence.” (8:53-55)</p> <p>“As used herein, the term barcode probe refers to a nucleic acid molecule which can hybridize specifically to a nucleic acid of interest, and which comprises a barcode sequence.” (5:17-20)</p> <p>“Barcode probes used in the methods presented herein can include padlock probes.” (7:36-37)</p> <p>“Also presented herein is a method of obtaining sequence information regarding a plurality of nucleic acid molecules from an individual cell comprising: amplifying a plurality of nucleic acid molecules in situ</p>

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<p>hybridization of the second fluorescently labeled decoder probe to the second decoder sequence of the padlock probe,</p> <p>wherein identification of the padlock probe provides sequence information regarding the at least one target nucleic acid sequence in the plurality of nucleic acid molecules from an individual cell.</p>	<p>within a cell, thereby generating a set of amplified nucleic acid molecules; for each of a plurality of amplified nucleic acid molecules in the set, annealing in situ a barcode probe comprising at least a first decoder sequence and a second decoder sequence to the amplified nucleic acid molecule; and hybridizing a first labeled decoder probe to the first decoder sequence; detecting a plurality of signals indicative of the hybridizing; hybridizing a second labeled decoder probe to the second decoder sequence; and detecting a plurality of signals indicative of the hybridizing, thereby generating sequence information regarding the plurality of amplified nucleic acid molecules.” (2:21-36)</p> <p>“...and decoding of the barcode probe takes place by multiple rounds of sequential hybridization. In the example shown in FIG. 1, decoding takes place via a set of barcode probes having decoder sequences that can be decoded by eight rounds of sequential hybridization with fluorescently labeled oligos and imaging of fluorescent images.” (9:16-22)</p> <p>“The signals generated by fluorescent imaging of one or more probes during decoding, sequencing and other detection methods carried out in situ and can be detected using any suitable imaging and detection technologies which allow for detection of individual amplicons in situ.” (11:11-15)</p>