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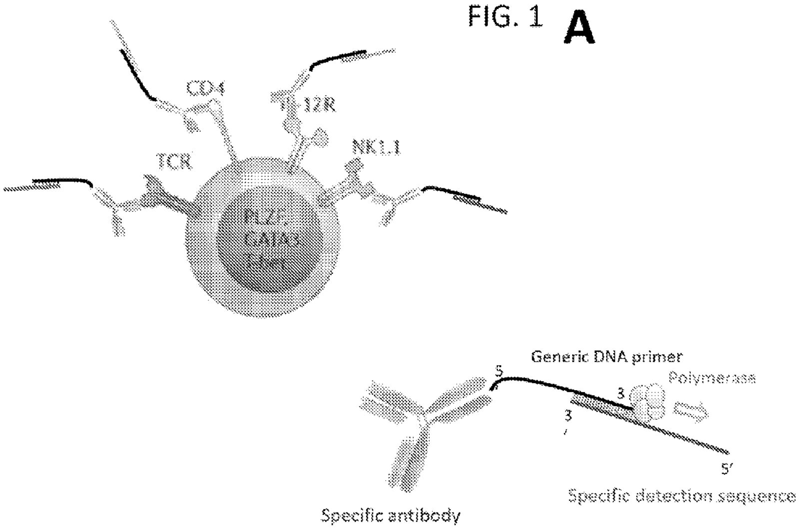
(54) **Title**: ON-SLIDE STAINING BY PRIMER EXTENSION

FIG. 1 A



(57) **Abstract**: A method for analyzing planar sample is provided. In some cases the method comprises: (a) labelling the planar sample with a capture agent that is linked to a nucleic acid, wherein the capture agent specifically binds to complementary sites in the planar sample; (b) reading a fluorescent signal caused by extension of a primer that is hybridized to the nucleic acid, using fluorescence microscopy. Several implementations of the method, and multiplexed versions of the same, are also provided.

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ON-SLIDE STAINING BY PRIMER EXTENSION

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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CROSS-REFERENCING

This patent application claims the benefit of U.S. provisional application serial no. 62/015,799, filed June 23, 2014, and U.S. non-provisional application serial no. 14/560,921, filed on December 4, 2014, which patent applications are incorporated by reference herein in their entireties.

BACKGROUND

Several major approaches have been used so far for single-cell antigen cytometry. Among the most popular are single cell PCR, fluorescence activated flow cytometry, mass cytometry and single cell sequencing. These (fluorescence and mass- based cytometry) approaches are limited from either inability to breach the multiplexing levels of more than 100 parameters per analyte (cell in this case) or from inability to achieve high throughput (single cell sequencing). Also these methods are not appropriate or readily modified to enable cell multiplexed analysis of archived tissues and slide based samples.

Disclosed herein are several related methods for capture agent detection that are based on labeling the capture agent with DNA and subsequent detection of this DNA by primer extension.

SUMMARY

A method for analyzing a planar sample is provided. In certain embodiments, the method may comprise: (a) labeling the planar sample (e.g., a tissue section) with a capture agent (e.g., an antibody or an oligonucleotide probe) in a way that produces a labeled sample in which: (i) the capture agent is linked to a double-stranded nucleic acid that comprises a first strand and a second strand; and (ii) the 3’ end or 5’ end of either the first strand or the second strand is extendible using the other strand as a template; (b) contacting the labeled sample with i. a polymerase and a nucleotide mix and/or ii. a labeled oligonucleotide and a ligase, thereby adding one or more nucleotides and/or a labeled oligonucleotide to an one of

the strands of the double-stranded nucleic acid; and (c) reading a fluorescent signal generated by addition of the one or more nucleotides and/or oligonucleotide to one of the strands of the double-stranded nucleic acid using fluorescence microscopy, thereby producing an image showing the pattern of binding of the capture agent to the planar sample.

The method may be implemented in a variety of different ways. For example, in some embodiments, step (b) may contacting the labeled sample with a polymerase and a nucleotide mix that comprises a fluorescent nucleotide, thereby adding the fluorescent nucleotide to one of the strands (i.e., the top strand or the bottom strand, whichever strand has the extendible 3' end) of the double-stranded nucleic acid; and step (c) may comprise reading a fluorescent signal generated by addition of the fluorescent nucleotide to one of the strands (i.e., the top strand or the bottom strand, whichever strand has the extendible 3' end) of the double-stranded nucleic acid. In this embodiment, the fluorescent signal may: i. emitted directly from the added nucleotide; ii. a FRET signal generated by energy transfer between two fluorescent nucleotides that are added to a 3' end of one of the strands; or iii. a FRET signal generated by energy transfer between a first added fluorescent nucleotide (i.e., a fluorescent nucleotide that has been added to one of the strands) and a second fluorescent nucleotide that is already present in one of the strands.

In alternative embodiments, step (b) comprises contacting the labeled sample with a ligase and a labeled oligonucleotide, thereby adding the labeled oligonucleotide to the 3' or 5' end of one of the strands of the double-stranded nucleic acid; and step (c) comprises reading a fluorescent signal generated by ligation of the labeled oligonucleotide to one of the strands of the double-stranded nucleic acid. In some cases, an extendible 3' end may be extended by a polymerase, and ligated to a labeled oligonucleotide. In these embodiments, the fluorescent signal may be: i. emitted directly from the added nucleotide; ii. a FRET signal generated by energy transfer between two fluorescent nucleotides that are added to one of the strands; or iii. a FRET signal generated by energy transfer between a first fluorescent nucleotide added one of the strands and a second fluorescent nucleotide that is already present in the other strand.

In some embodiments, extension of one of the strands removes a quencher from a quenched fluorescently labeled oligonucleotide that is hybridized to the other strand, downstream from the first strand.

In some embodiments, the first strand is a rolling circle amplification (RCA) product, and the second strand comprises oligonucleotides that are hybridized to multiple sites in the RCA product.

In other embodiments, the first strand is an oligonucleotide, and the second strand is a second oligonucleotide that is hybridized to the first oligonucleotide. In these embodiments, the oligonucleotides may be designed to produce a 5' overhang such that the 3' end of the first strand oligonucleotide is extendible using the other oligonucleotide as a template. In other embodiments, the oligonucleotides may be designed to produce a 3' overhang such that the 5' end of the first strand oligonucleotide is extendible by ligation, using the other oligonucleotide as a template

In any embodiment, the planar sample may be a tissue section, e.g., a formalin-fixed, paraffin-embedded (FFPE) tissue section.

Also provided herein is a capture agent that is linked to a double-stranded nucleic acid, wherein: (i) the double-stranded nucleic acid comprises a first strand and a second strand; (ii) the capture agent is linked to the first strand; and (iii) the 3' end or 5' end of either the first strand or the second strand is extendible using the other strand as a template.

Also provided herein is a capture agent composition comprising a plurality of capture agents that recognize different complementary sites, wherein: each of the capture agents is linked to a double-stranded nucleic acid that comprises a first strand and a second strand; the capture agents are linked to a double-stranded nucleic acid by the first strand; the 3' end or 5' end of the first or second strand is extendible using the other strand as a template; and the templates immediately downstream of the extendible ends are different for each of the capture agents. In these embodiments, the sequence of the first strand is the same for each of the capture agents; and the sequence of the second strand is different for each of the capture agents.

In embodiments that use a reversible terminator ("reversible terminator" approach), the templates immediately adjacent to the template at the extendible 3' end may be of the formula 3'- $N_{4n}N_1/N_2/N_3$ -5'optionally followed by short stretch (e.g., 1-5 residues) of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, where N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 0, 1 or more. In some cases, the population contains single nucleotide overhangs of nucleotides N_1, N_2 and N_3 or the population of overhangs comprises two nucleotide overhangs of sequence 3'- N_4N_1-5', 3'- N_4N_2-5' and 3'- N_4N_3-5' -5' and, optionally overhangs of sequence, 3'- $N_4N_4N_1$-5', 3'-$N_4N_4N_2$-5' and 3'- $N_4N_4N_3$-5' and so on (e.g., four nucleotide overhangs of sequence 3'- $N_4N_4N_4N_1$-5', 3'- $N_4N_4N_4N_2$-5' and 3'- $N_4N_4N_4N_3$-5'). A population of oligonucleotides or RCA products having sequences that are defined by any of these formulas is also provided. In RCA embodiments, the sequence may be found in each repeat of an RCA product.

In these embodiments, the templates immediately adjacent to the extendible 3' end may be of a more general formula 3'- $XN_1/N_2/N_3$-5', where N_1, N_2, N_3 are different nucleotides selected from G, A, T and C and X is a nucleotide stretch of bases Xi (such that Xi are different nucleotides selected from G, A, T and C) of random composition and length. In some cases, the population may comprise comprises two nucleotide overhangs of sequence 3'- X_1N_1-5', 3'- X_1N_2-5' and 3'- X_1N_3-5' and, optionally overhangs of sequence, 3'-$N_1X_1X_2$-5', 3'-$N_2X_1X_2$-5' and 3'-$N_3X_1X_2$-5' and so on (e.g., four nucleotide overhangs of sequence 3'- $N_1X_1X_2X_3$-5', 3'- $N_2X_1X_2X_3$-5' and 3'- $N_3X_1X_2X_3$-5'). In many embodiments, this population additionally contains single nucleotide overhangs of nucleotides N_1, N_2 and N_3. A population of oligonucleotides or RCA products having sequences that are defined by any of these formulas is also provided. In RCA embodiments, the sequence may be found in each repeat of an RCA product.

In embodiments that rely on a "missing base" approach, the template immediately adjacent to the extendible 3' end may be of the formula 3'-YN_1/N_2-5', optionally followed by short stretch (e.g., 1-5 residues) of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is a nucleotide sequence of length n (n is 0, 1 or more) composed of bases N_3 and N_4, wherein nucleotide N_3 is in odd positions and nucleotide N_4 is in even positions, counting from the start of the overhang and N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C. For example, in some cases, the population may comprise 5' overhangs of sequence 3'-N_1-5' and 3'- N_2-5' or optionally 3'-N_3N_1-5' and 3'- N_3N_2-5' or 3'- $N_3N_4N_1$-5' and 3'- $N_3N_4N_2$-5' and, optionally, overhangs of sequence 3'- $N_3N_4N_3N_1$-5' and 3'- $N_3N_4N_3N_2$-5' and so on (e.g., overhangs of sequence 3'- $N_3N_4N_3N_4N_1$-5' and 3'- $N_3N_4N_3N_4N_2$-5' and then 3'-$N_3N_4N_3N_4N_3N_1$-5' and 3'- $N_3N_4N_3N_4N_3N_2$-5'). A population of oligonucleotides or RCA products having sequences that are defined by any of these formulas is also provided. In RCA embodiments, the sequence may be found in each repeat of an RCA product.

In these embodiments the template immediately adjacent to the extendible 3' end may also be of a more general formula 3'-YN_1/N_2-5', wherein Y is a nucleotide sequence of length n (n is 0, 1 or more) composed of alternating random length stretches of bases N_3 and N_4 such that the order number of N_3 - stretches is odd and of N_4 stretches is even and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C. For example, the population may comprise overhangs of sequence 3'-N_1-5' and 3'-N_2-5' or optionally 3'- $N_3N_3N_1$-5' and 3'- $N_3N_3N_2$-5' or 3'- $N_3N_3N_4N_1$-5' and 3'- $N_3N_3N_4N_2$-5' and, optionally, overhangs of sequence 3’- $N_3N_3N_3N_3N_4N_4N_3N_3N_3N_1$-5’ and 3’-$N_3N_3N_3N_3N_4N_4N_3N_3N_3N$-5’ and so on). A population of oligonucleotides or RCA products having sequences that are defined by any of these formulas is also provided. In RCA embodiments, the sequence may be found in each repeat of an RCA product.

A method for analyzing a tissue sample is also provided. In these embodiments, the method may comprise (a) labeling a planar sample with the above-described capture agent composition; (b) contacting the labeled sample with i. a polymerase and either an incomplete nucleotide mix or a nucleotide mix that comprises a reversible terminator nucleotide and/or ii. a labeled oligonucleotide and a ligase; and (c) reading, using fluorescence microscopy, a fluorescent signal generated by addition a nucleotide or a labeled oligonucleotide to some but not all of the capture agents.

In these embodiments, the method may comprises: (c) contacting the planar sample with a polymerase and: (i) a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4 or (ii) a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, and N_2, an unlabeled nucleotide that is complementary to $N_{3,}$ and no nucleotide that is complementary to N_4, thereby adding fluorescent nucleotides onto the double-stranded nucleic acids of some but not all of the capture agents; and (d) reading, using fluorescence microscopy, a fluorescent signal generated by addition of a fluorescent nucleotide to some but not all of the capture agents.

In some embodiments, the templates immediately adjacent to the extendible 3’ end are of the formula 3’-$N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more; and step (c) comprises contacting the planar sample with a polymerase and a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4.

In some embodiments, this method may further comprise: (e) inactivating the fluorescent signal, deprotecting the reversible terminator nucleotide and blocking the sample; and (f) repeating steps (c) and (d). In some cases, step (f) may comprise repeating steps (c), (d) and (e) multiple times.

In some embodiments, the templates immediately adjacent to the extendible 3’ end may be of the formula 3’-YN_1/N_2-5’, optionally followed by short stretch (e.g., 1-5 nucleotides) of random nucleotides on the 5’ end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of bases N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C.

In these embodiments, the method may comprise (e) inactivating the fluorescent signal and contacting the planar sample with a polymerase and a an unlabeled nucleotide that is complementary to N_4; and (f) repeating steps (c) and (d). In certain cases, step (f) may comprise repeating steps (c), (d) and (e) multiple times.

In alternative embodiments, the double-stranded oligonucleotides may each comprise a fluorescently labeled oligonucleotide hybridized to the second strand downstream from first strand, wherein the fluorescently labeled oligonucleotide comprises a quencher and extension of the first strand removes the quencher from some but not all of the quenched fluorescently labeled oligonucleotides, thereby generating a fluorescent signal for some but not all of the capture agents.

In other embodiments, the capture agent is linked to a single stranded oligonucleotide, which can be either unlabeled or labeled with FRET acceptor fluorophore. Such a single stranded nucleotide incorporates a dedicated sequence that hybridizes to a complementary oligonucleotide which is to be extended with unlabeled base or with a base labeled with a FRET excitation fluorophore, thereby generating a fluorescent signal for some but not all of the capture agents.

In some embodiments, a method for analyzing a planar sample. In some embodiments, the method comprises: (a) labeling the planar sample with a capture agent to produce a labeled sample, wherein: (i) the capture agent is linked to a double-stranded nucleic acid that comprises a first strand and a second strand; and (ii) a 3’ end or 5’ end of either the first strand or the second strand is extendible using the other strand as a template; (b) contacting the labeled sample with i. a polymerase and a plurality of nucleotides and/or ii. a labeled oligonucleotide and a ligase, thereby adding one or more nucleotides of the plurality of nucleotides and/or a labeled oligonucleotide to an end of one of the strands of the double-stranded nucleic acid; and (c) reading a signal generated by addition of the one or more nucleotides and/or labeled oligonucleotide to one of the first strand or the second strand of the double-stranded nucleic acid. In some embodiments, the signal may be a fluorescent signal. In some embodiments, the reading may comprises flourescence microscopy. Any embodiment, the method may further comprise producing an image showing the pattern of binding of the capture agent to the planar sample.

In any embodiment, step (b) may comprise contacting the labeled sample with a polymerase and a plurality of nucleotides that comprises a fluorescent nucleotide, thereby adding the fluorescent nucleotide to one of the first strand or the second strand of the double-stranded nucleic acid; and step (c) comprises reading a fluorescent signal generated by addition of the fluorescent nucleotide to one of the first strand or the second strand of the double-stranded nucleic acid. In these embodiment, wherein the fluorescent signal may be: i. emitted directly from the added nucleotide; ii. a FRET signal generated by energy transfer between two fluorescent nucleotides of the plurality of flourescent nucleotides that are added to one of the first strand or second strand of the double-stranded nucleic acid; or iii. a FRET signal generated by energy transfer between the added fluorescent nucleotide and a second fluorescent nucleotide that is present in one of the first strand or second strand double-stranded nucleic acid.

In any embodiment, the method step (b) may comprise contacting the labeled sample with a ligase and a labeled oligonucleotide, thereby adding the labeled oligonucleotide to one of the first strand or second strand of the double-stranded nucleic acid; and step (c) comprises reading a fluorescent signal generated by addition of the labeled oligonucleotide to one of the first strand or second strand of the double-stranded nucleic acid. In this embodiment, the fluorescent signal may be: i. emitted directly from the added labeled nucleotide; ii. a FRET signal generated by energy transfer between two labeled nucleotides that are added to one of the first strand or second strand of the double-stranded nucleic acid; or iii. a FRET signal generated by energy transfer between the labeled nucleotide added to one of the first strand and second strand of the double-stranded nucleic acid and a second labeled nucleotide that is present in the other strand. In these embodiments, the labeled nucleotide may comprise a fluorescent nucleotide.

In any embodiment, extension of one of the first strand or second strand of the double-stranded nucleic acid may remove a quencher from a quenched fluorescently labeled oligonucleotide that is hybridized to the other strand, downstream from the first strand.

In any embodiment, the first strand of the double-stranded nucleic acid may be a rolling circle amplification (RCA) product, and the second strand of the double-stranded nucleic acid comprises oligonucleotides that are hybridized to multiple sites in the RCA product.

In any embodiment, the first strand of the double-stranded nucleic acid may be a first oligonucleotide, and the second strand of the double-stranded nucleic acid is a second oligonucleotide that is hybridized to the first oligonucleotide.

In any embodiment, the planar sample may be a formalin-fixed, paraffin-embedded (FFPE) section.

In any embodiment, the capture agent may be an antibody, an aptamer, or an oligonucleotide probe.

A capture agent that is linked to a double-stranded nucleic acid is also provided. In some embodiments, (i) the double-stranded nucleic acid comprises a first strand and a second strand; (ii) the capture agent is linked to the first strand; and (iii) the 5’ end or the 3’ end of either the first strand or the second strand is extendible using the other strand as a template.

Also provided is a capture agent composition comprising a plurality of capture agents that each recognize different complementary sites. In these embodiments, each of the plurality of capture agents may be linked to a double-stranded nucleic acid that comprises a first strand and a second strand; the 5’ end or 3’ end of the first or second strand may be extendible using the other strand as a template; and the templates immediately downstream of the extendible ends may be different for each of the plurality of capture agents. In these embodiments, the sequence of the first strand may be the same for each of the plurality of capture agents; and the sequence of the second strand may be different for each of the plurality of capture agents.

In some embodiments, the templates immediately adjacent to the extendible 3’ ends may be of the formula 3’- $N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more.

In some embodiments, the templates immediately adjacent to the extendible 3’ ends may be of the formula 3’-YN_1/N_2-5’, optionally followed by a short stretch of random nucleotides on the 5’ end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C.

A method for analyzing a planar sample is provided. This method may comprise (a) labeling the planar sample with a capture agent composition summarized above; (b) contacting the labeled sample with i. a polymerase and either an incomplete nucleotide mix or a nucleotide mix that comprises a reversible terminator nucleotide, thereby adding a nucleotide to the plurality of capture agents; and/or ii. a labeled oligonucleotide and a ligase, thereby adding a labeled oligonucleotide to the plurality of capture agents; and (c) reading a signal generated by addition of the nucleotide or the labeled oligonucleotide to some but not all of the plurality of capture agents. In these embodiments, the signal may be a fluorescent signal. In some embodiments, the reading may be done by fluorescent microscopy.

In some embodiments, the method may be done by (b) contacting the planar sample with a polymerase and: (i) a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4; or (ii) a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, and N_2, an unlabeled nucleotide that is complementary to N_3, and no nucleotide that is complementary to N_4, thereby adding fluorescent nucleotides onto the double-stranded nucleic acids of some but not all of the plurality of capture agents; and (c) reading, using fluorescence microscopy, a fluorescent signal generated by addition of the fluorescent nucleotides to the double-stranded nucleic acids of some but not all of the plurality of capture agents. In these embodiments, the templates immediately adjacent to the extendible 3' end may be of the formula 3'-$N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more; and step (b) comprises contacting the planar sample with a polymerase and a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4. In these embodiments, the method may further comprise: (d) inactivating the fluorescent signal, (e) optionally, deprotecting the reversible terminator nucleotide; (f) blocking the sample; and (g) repeating steps (b) and (c). In some embodiment, step (g) may comprise repeating steps (b)-(f) multiple times.

In some embodiments, the templates immediately adjacent to the extendible 3' end may be of the formula 3'-YN_1/N_2-5', optionally followed by a short stretch of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C. In these embodiments, the method may further comprise: (d) inactivating the fluorescent signal; (e) contacting the planar sample with a polymerase and an unlabeled nucleotide that is complementary to N_4; and (f) repeating steps (b) and (c). In some cases, step (f) may comprise repeating steps (b)-(e) multiple times.

In some embodiments, the double-stranded nucleic acids each comprise a fluorescently labeled oligonucleotide hybridized to the second strand downstream from the first strand, wherein the fluorescently labeled oligonucleotide comprises a quencher and extension of the first strand removes the quencher from some but not all of the quenched fluorescently labeled oligonucleotides, thereby generating a fluorescent signal for some but not all of the plurality of capture agents.

In some embodiments, extension of the double-stranded nucleic acid comprises contacting the planar sample with a mixture of labeled and unlabeled oligonucleotides and a ligase.

In any embodiment, the plurality of capture agents may be selected from the group consisting of: antibodies, aptamers, and oligonucleotide probes.

A kit is also provided. In these embodiments, the kit may comprise: (a) one or more capture agents, wherein the one or more capture agents can specifically bind to complementary sites in a planar sample.(b) one or more double-stranded nucleic acids comprising a first strand a second strand, wherein each of the one or more capture agents is linked to the double-stranded nucleic acid, and wherein a 5' end or 3' end of either the first strand or the second strand is extendible using the other strand as a template. In some embodiments, the kit may further comprise a polymerase or ligase. In some embodiments, the kit may further comprise a nucleotide mix comprising at least one of a fluorescent nucleotide, an unlabeled nucleotide, and a reversible terminator nucleotide. In some embodiments, the one or more capture agents may be selected from the group consisting of: an antibody, an aptamer and an oligonucleotide probe.

In some aspects, a method is provided for analyzing a planar sample. In some cases, the method comprises incubating the planar sample with a capture agent under conditions by which the capture agent specifically binds to complementary sites in the planar sample. In some cases, the capture agent is linked to a double-stranded oligonucleotide that comprises a first strand and a second strand. In some cases, a 3' end of the first strand is recessed relative to a 5' end of the second strand, thereby producing an overhang. In some cases, the method comprises contacting the planar sample with a polymerase and a plurality of nucleotides, thereby adding one or more nucleotides of the plurality of nucleotides to the overhang. In some cases, the method comprises reading a signal generated by addition of the one or more nucleotides to the overhang. In some cases, the plurality of nucleotides comprises a plurality of fluorescent nucleotides. In some cases, a fluorescent nucleotide of the plurality of nucleotides is added to the overhang. In some cases, the signal comprises a fluorescent signal. In some cases, the fluorescent signal is emitted directly from the fluorescent nucleotide added to the overhang. In other cases, two of the plurality of fluorescent nucleotides are added to the overhang. In this example, the fluorescent signal is a FRET signal generated by energy transfer between the two of the plurality of fluorescent nucleotides added to the overhang. In an alternative example, the fluorescent signal is a FRET signal generated by energy transfer between the fluorescent nucleotide from the plurality of fluorescent nucleotides added to the overhang and a fluorescent nucleotide that is present in the second strand. In some cases, extension of the first strand removes a quencher from a quenched fluorescently labeled oligonucleotide that is hybridized to the second strand, downstream from the first strand. In some cases, the planar sample is a formalin-fixed, paraffin-embedded (FFPE) section. In some cases, the capture agent is linked to the double-stranded oligonucleotide by a 5’ end of the first strand. In other cases, the capture agent is linked to the double-stranded oligonucleotide by a 3’ end of the second strand. In some cases, the method further comprises crosslinking the capture agent to the planar sample. In some cases, the reading comprises fluorescence microscopy. In some cases, the method further comprises producing an image showing a pattern of binding of the capture agent to the planar sample. In some cases, the one or more nucleotides of the plurality of nucleotides is added to the overhang by primer extension. In some cases, the capture agent is an antibody, an aptamer or an oligonucleotide probe.

In some aspects, a composition is provided comprising a plurality of capture agents that specifically bind to different complementary sites in a planar sample. In some cases, each of the plurality of capture agents is linked to a double-stranded oligonucleotide that comprises a first strand and a second strand. In some cases, a 3’ end of the first strand in each of the double-stranded oligonucleotides is recessed relative to a 5’ end of the second strand, thereby producing an overhang. In some cases, the overhang is different for each of the plurality of capture agents. In some cases, each of the plurality of capture agents is linked to the double-stranded oligonucleotide by a 5’ end of the first strand. In other cases, each of the plurality of capture agents is linked to the double-stranded oligonucleotide by a 3’ end of the second strand. In some cases, a sequence of the first strand is the same for each of the plurality of capture agents and a sequence of the second strand is different for each of the plurality of capture agents. In some cases, the overhang is of the formula 3’-N4nN1/N2/N3, wherein N1, N2, N3 and N4 are different nucleotides selected from G, A, T and C and n is 1 or more. In other cases, the overhang is of the formula 3’-YN1/N2-5’, optionally followed by a short stretch of random nucleotides on the 5’ end of the first strand to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of N3 and N4, and wherein N1, N2, N3 and N4 are different nucleotides selected from G, A, T and C. In some cases, Y is a nucleotide sequence of length n and wherein n is 0, 1, or more. In some cases, the order number of N3 stretches is odd and wherein the order number of N4 stretches is even. In some cases, the planar sample is a formalin-fixed, paraffin-embedded section (FFPE). In some cases, the plurality of capture agents are antibodies, aptamers, or oligonucleotide probes.

In some aspects, a method is provided for analyzing a planar sample. In some cases, the method comprises incubating the planar sample with the composition described above under conditions by which each of the plurality of capture agents specifically bind to different complementary sites in the planar sample. In some cases, the method comprises contacting the planar sample with a polymerase and a plurality of nucleotides, thereby adding one or more nucleotides of the plurality of nucleotides to the overhang of some, but not all, of the plurality of capture agents. In some cases, the method comprises reading a signal generated by addition of the one or more nucleotides from the plurality of nucleotides to the overhang of some, but not all, of the plurality of capture agents. In some cases, the method further comprises crosslinking the plurality of capture agents to the planar sample. In some cases, the plurality of nucleotides comprises an incomplete nucleotide mix or a nucleotide mix comprising a reversible terminator nucleotide. In some cases, the signal comprises a fluorescent signal. In some cases, the reading comprises fluorescence microscopy. In some cases, the method further comprises producing an image showing a pattern of binding of the plurality of capture agents to the planar sample. In some cases, the plurality of nucleotides comprises: (i) a plurality of fluorescent nucleotides that are complementary to N1, N2 and N3, and a reversible terminator nucleotide that is complementary to N4; or (ii) a plurality of fluorescent nucleotides that are complementary to N1 and N2, an unlabeled nucleotide that is complementary to N3, and no nucleotide that is complementary to N4. In some cases, a fluorescent nucleotide of the plurality of fluorescent nucleotides is added to the overhang of some, but not all, of the plurality of capture agents. In some cases, the signal comprises a fluorescent signal generated by addition of the fluorescent nucleotide of the plurality of fluorescent nucleotides to some, but not all, of the plurality of capture agents. In some cases, the reading comprises fluorescence microscopy. In some cases, the method further comprises producing an image showing the pattern of binding of the plurality of capture agents to the planar sample. In some cases, the overhangs are of the formula 3’-N4nN1/N2/N3, wherein N1, N2, N3 and N4 are different nucleotides selected from G, A, T and C and n is 1 or more, and wherein the plurality of nucleotides comprises a plurality of fluorescent nucleotides that are complementary to N1, N2, N3 and a reversible terminator nucleotide that is complementary to N4. In some cases, the method further comprises inactivating the fluorescent signal, optionally, deprotecting the reversible terminator nucleotide; blocking the planar sample; and repeating the steps of contacting and reading. In some cases, the repeating further comprises repeating the steps of contacting, reading, inactivating, optionally deprotecting, and blocking a plurality of times. In other cases, the overhangs are of the formula 3'-YN1/N2-5', optionally followed by a short stretch of random nucleotides on the 5' end of the first strand to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of N3 and N4, and wherein N1, N2, N3 and N4 are different nucleotides selected from G, A, T and C. In some cases, Y is a nucleotide sequence of length n and wherein n is 0, 1, or more. In some cases, the order number of N3 stretches is odd and wherein the order number of N4 stretches is even. In some cases, the method further comprises inactivating the fluorescent signal, contacting the planar sample with a polymerase and an unlabeled nucleotide that is complementary to N4; and repeating the steps of contacting and reading. In some cases, the repeating comprises repeating the steps of contacting, reading, inactivating, and contacting a plurality of times. In some cases, each of the double-stranded oligonucleotides comprise a fluorescently labeled oligonucleotide hybridized to the second strand downstream from the first strand, wherein the fluorescently labeled oligonucleotide comprises a quencher and extension of the first strand removes the quencher from some, but not all, of the quenched fluorescently-labeled oligonucleotides, thereby generating a fluorescent signal for some, but not all, of the capture agents.

BRIEF DESCRIPTION OF THE FIGURES

The skilled artisan will understand that the drawings, described below, are for illustration purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

FIG. 1A-1B (A) schematically illustrates a detection reagent composed of a combination of a capture agent that is conjugated to a double-stranded oligonucleotide. Upon detection and removal of unbound detection reagent the binding pattern is rendered by polymerase driven primer extension. Panel (B) schematically illustrates three approaches for linking the capture agent (an antibody in this case, but not excluding other possible capture agents) to a double stranded oligonucleotide (i.e., by chemical conjugation of the upper strand oligonucleotide to the capture agent; using streptavidin as an intermediate to connect biotinylated antibody and biotinylated oligonucleotide; and by linking biotinylated oligonucleotide to antibody chemically conjugated to streptavidin).

FIG. 2 schematically illustrates examples of capture agents that are bound to double-stranded oligonucleotides that have different overhangs. Such different overhangs represent a strategy to increase signal harvested from a particular capture agent by multiplication of positions in lower strand oligonucleotide complementary to detector base (dU in this case). The lower panel also shows how a different base labeled with a different fluorophore can be used as a FRET excitation pair for the “Detector” base. SEQ ID NOS: 1-4.

FIG. 3 schematically illustrates several cycles of a multiplexed detection method that relies on reversible dye terminators.

FIG. 4 schematically illustrates several cycles of a multiplexed detection method that relies on leaving out one of the four nucleotides per cycle.

FIG. 5A-5D schematically illustrates an exemplary design of oligonucleotide duplexes for “reversible terminator” and “missing base” multiplexing methods. SEQ ID NOS: 5-12.

FIG. 6 schematically illustrates an exemplary design of oligonucleotide duplexes for a strategy that allows one to reduce the length of the lower strand oligonucleotide, creating an overhang in the case of highly multiplexed capture agent panels. SEQ ID NOS: 13-30.

FIG. 7 schematically illustrates an example of a detection method that relies on removing a quencher from a labeled oligonucleotide by nick translation. SEQ ID NOS: 31-35.

FIG. 8 schematically illustrates a multiplexed detection method that relies on removing quenchers from labeled oligonucleotides. Step 1: SEQ ID NOS 36-44, Step 2: SEQ ID NOS: 45-52, Step 3: SEQ ID NOS: 53-60, Step 4: SEQ ID NOS: 61-67 .

FIGS. 9A and 9B schematically illustrate an embodiment that relies on cyclical re-annealing of polymerase priming nucleotides and a variant of the same approach that utilizes FRET. SEQ ID NOS: 68-80.

FIG. 10 schematically illustrates an embodiment that relies on cyclical re-annealing of polymerase priming nucleotides and a variant of the same approach that utilizes FRET. SEQ ID NOS: 81-86.

FIGS. 11A-11C shows an anti-CD4 antibody linked to oligonucleotide duplex designed for rendering staining by primer extension (panel A) and data obtained from labeled population of spleen cells in suspension in the absence of polymerase (panel B) and in the presence of polymerase (panel C). SEQ ID NOS: 87 and 88.

FIGS. 12A-12D shows data obtained from labeling by primer extension a population of spleen cells preattached on the slide. Cells were co-stained with “regular” TCRb-FITC antibody and CD4 antibody linked to oligonucleotide duplex designed for rendering staining by primer extension.

FIGS. 13A-13D show schematic illustration of two capture agents CD4 and CD8 linked to oligonucleotide duplexes (panel A) and data obtained from a multiplexed method whereby staining by this capture agents was sequentially detected on spleen cells smeared on a slide using a “reversible terminator” method (panels C-D). SEQ ID NOS: 89-92.

Fig.14 shows a schematic diagram of an experiment testing multiplexed staining by “missing base” approach. Mouse spleen samples were barcoded by pan-leukocytic CD45 antibody conjugated to per sample specific oligonucleotide duplexes. Samples were mixed after staining and mixture was resolved by sequential rendering of CD45-oligonucleotide variants.

Fig. 15 is 12 panels of images showing the first 6 cycles of rendering the 30 populations barcoded by CD45 (as per scheme on Fig.14). Two populations were co-detected per cycle of rendering. In each cycle control image was acquired after fluorescence inactivation.

Fig. 16 illustrates enhanced antibody signal with rolling circle amplification. A. Antibody-DNA conjugate that consists of an antibody, a covalently linked linear linker oligonucleotide and a 5’-phosphorylated padlock nucleotide is used to stain the cellular antigens. Padlock probe contains the detection primer sequence (orange) followed by the fluorescent nucleotide incorporation site (T). B. Padlock oligonucleotide is treated with T4 DNA ligase, inducing its circularization. C. Rolling circle amplification with strand-displacing phi29 DNA polymerase created repeats of the reverse-complement of the detection primer sites (green). F-G. Staining of Mouse Spleen cells with antibody-DNA conjugate visualized by primer extension with dUTP-Cy5 without the rolling circle amplification (F) and after rolling circle amplification (G).

Fig. 17 shows fluorescent images of cells, showing the staining of 22 different antigens rendered by the iterative primer extension protocol. At each cycle one antigen-antibody-DNA complex incorporates dUTP-SS-Cy5 fluorophore (red) and one complex incorporates dCTP-SS-Cy3 (green), all other complexes receive an unlabelled ‘walking’ base (dGTP on odd cycles, dATP on even cycles).

Fig. 18 shows A: multipanel design whereby antibody-DNA conjugates are incapable of polymerase extension because of 3’-dideoxy-terminator bases, but each panel can be activated for extension independently of others by an addition of a panel-specific primer. B: 18 aliquotes of mouse spleen cells were independently stained with different CD45 antibody conjugates that were designed such. Aliquots 1-3 (panel 1) can be detected by regular ABseq primer extension (top row), aliquots 4-6 (panel 2) were be extended after addition of Spacer1 oligonucleotide primer and aliquotes 7-9 (panel 3) can be extended after addition of Spacer2 oligonucleotide primer. C: Results of image quantification. Intensities of individual cell intensities displayed as a barcodes, one cell for each row, red color representing higher staining intensity. Columns represent intensities of cells on each extension cycle. The diagonal pattern shows the high specificity of spacer-based extension and the absence of signal cross-talk between panels and extension cycles.

Fig. 19 shows A: A pair of coincidence detection probes is hybridized to the target RNA. Upstream oligonucleotide probe (Splint-primer) serves as a splint for circularization and ligation of the downstream oligonucleotide probe (padlock). Padlock probe contains a detection primer sequence (lilac) followed by the fluorescent nucleotide incorporation site (red) B. Rolling circle amplification is initiated at the 3’ end of the upstream probe and creates multiple copies of the reverse-complement of detection primer sequence (lilac). C. Detection primer is annealed to the multiple sites of the amplification product. D. Polymerase reaction with dUTP-Cy5 results incorporations. E-F: small and bright puncta in NALM cells correspond to single HLADRA RNA molecules, which are absent in the negative control Jurkat cells. Large red blobs present in both panels correspond to apoptotic cells that nonspecifically bind the fluorescent nucleotide.

Fig. 20 shows an alternative method that relies on primer extension and the ligation of a short, labeled oligonucleotide. Left side, from top to bottom: SEQ ID NOS: 93-108; right side, from top to bottom: SEQ ID NOS: 109-124.

Fig. 21 depicts a system to enable a user to detect, analyze, and process images of samples.

DEFINITIONS

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

The headings provided herein are not limitations of the various aspects or embodiments of the invention. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Markham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, N.Y. (1991) provide one of skill with the general meaning of many of the terms used herein. Still, certain terms are defined below for the sake of clarity and ease of reference.

As used herein, the term “biological feature of interest” refers to any part of a cell that can be indicated by binding to a capture agent. Exemplary biological features of interest include cell walls, nuclei, cytoplasm, membrane, keratin, muscle fibers, collagen, bone, proteins, nucleic acid (e.g., mRNA or genomic DNA, etc). fat, etc. A biological feature of interest can also be indicated by immunohistological methods, e.g., a capture agent that is linked to an oligonucleotide. In these embodiments, the capture agent binds to an site, e.g., a protein epitope, in the sample. Exemplary epitopes include, but are not limited to carcinoembryonic antigen (for identification of adenocarcinomas, cytokeratins (for identification of carcinomas but may also be expressed in some sarcomas) CD15 and CD30 (for Hodgkin's disease), alpha fetoprotein (for yolk sac tumors and hepatocellular carcinoma), CD117 (for gastrointestinal stromal tumors), CD10 (for renal cell carcinoma and acute lymphoblastic leukemia), prostate specific antigen (for prostate cancer), estrogens and progesterone (for tumour identification), CD20 (for identification of B-cell lymphomas), CD3 (for identification of T-cell lymphomas). Complementary nucleic acid molecules (e.g., DNA and/or RNA) in the sample provide binding complementary sites for oligonucleotide probes.

As used herein, the term "multiplexing" refers to using more than one label for the simultaneous or sequential detection and measurement of biologically active material.

As used herein, the terms “antibody” and “immunoglobulin” are used interchangeably herein and are well understood by those in the field. Those terms refer to a protein consisting of one or more polypeptides that specifically binds an antigen. One form of antibody constitutes the basic structural unit of an antibody. This form is a tetramer and consists of two identical pairs of antibody chains, each pair having one light and one heavy chain. In each pair, the light and heavy chain variable regions are together responsible for binding to an antigen, and the constant regions are responsible for the antibody effector functions.

The recognized immunoglobulin polypeptides include the kappa and lambda light chains and the alpha, gamma (IgG_1, IgG_2, IgG_3, IgG_4), delta, epsilon and mu heavy chains or equivalents in other species. Full-length immunoglobulin "light chains" (of about 25 kDa or about 214 amino acids) comprise a variable region of about 110 amino acids at the NH_2-terminus and a kappa or lambda constant region at the COOH-terminus. Full-length immunoglobulin "heavy chains" (of about 50 kDa or about 446 amino acids), similarly comprise a variable region (of about 116 amino acids) and one of the aforementioned heavy chain constant regions, e.g., gamma (of about 330 amino acids).

The terms "antibodies" and “immunoglobulin” include antibodies or immunoglobulins of any isotype, fragments of antibodies which retain specific binding to antigen, including, but not limited to, Fab, Fv, scFv, and Fd fragments, chimeric antibodies, humanized antibodies, minibodies, single-chain antibodies, and fusion proteins comprising an antigen-binding portion of an antibody and a non-antibody protein. Also encompassed by the term are Fab’, Fv, $F(ab')_2$, and or other antibody fragments that retain specific binding to antigen, and monoclonal antibodies. Antibodies may exist in a variety of other forms including, for example, Fv, Fab, and $(Fab')_2$, as well as bi-functional (i.e. bi-specific) hybrid antibodies (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)) and in single chains (e. g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et al., Science, 242, 423-426 (1988), which are incorporated herein by reference). (See, generally, Hood et al., "Immunology", Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323, 15-16 (1986),).

The term “specific binding” refers to the ability of a binding reagent to preferentially bind to a particular analyte that is present in a homogeneous mixture of different analytes. In certain embodiments, a specific binding interaction will discriminate between desirable and undesirable analytes in a sample, in some embodiments more than about 10 to 100-fold or more (e.g., more than about 1000- or 10,000-fold).

In certain embodiments, the affinity between a binding reagent and analyte when they are specifically bound in a capture agent/analyte complex is characterized by a K_D (dissociation constant) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-9} M, less than 10^{-11} M, or less than about 10^{-12} M or less.

A “plurality” contains at least 2 members. In certain cases, a plurality may have at least 2, at least 5, at least10, at least 100, at least 1000, at least 10,000, at least 100,000, at least 10^6, at least 10^7, at least 10^8 or at least 10^9 or more members.

As used herein, the term “labeling” refers to attaching a detectable fluorophore to specific sites in a sample (e.g., sites containing an epitope for the antibody being used, for example) such that the presence and/or abundance of the sites can be determined by evaluating the presence and/or abundance of the label.

The term “labelling” refers to a method for producing a labeled sample in which any necessary steps are performed in any convenient order, as long as the required labeled sample is produced. For example, in some embodiments and as will be exemplified below, the capture agent may be already linked to a double-stranded nucleic acid prior to binding of the antibody to the sample, in which case a sample can be labeled using relatively few steps. In other embodiments, the capture agent may be linked to the first strand of the double stranded nucleic acid at the time at which it is incubated with the sample. In these embodiments, the second strand of the double stranded nucleic acid may be hybridized to the first strand of the double stranded nucleic acid after the antibody has bound to the sample. Along similar lines, the capture agent may be linked to a rolling circle amplification (RCA) primer at the time at which it is incubated with the sample. In these embodiments, the double-stranded nucleic acid may be produced by: a) hybridizing the sample with a padlock probe having ends that are complementary to the RCA primer, ligating the ends of the padlock probes together, and copying the padlock probe by rolling circle amplification and b) hybridizing an oligonucleotide to the RCA product, as illustrated in Fig. 16. In this example, the RCA product is the first strand of the double-stranded nucleic acid, and the oligonucleotides that are hybridized to the RCA product are the second strand of the double-stranded nucleic acid. In many embodiments, the labeling step may comprise crosslinking the capture agent to the planar sample so that subsequence manipulations can be done without the capture agent disassociating from its complementary sites in the planar sample. In these embodiments, if the capture agent is linked to the double-stranded nucleic acid prior to binding of the antibody to the sample, then the crosslinking step may be done immediately after binding of the antibody to the sample. In embodiments in which the capture agent is only linked to the first strand (or an RCA primer for making the same) at the time at which it is incubated with the sample, the sample may be cross-linked after binding of the antibody to the sample, and the double-stranded may be produced after crosslinking.

As used herein, the term “planar sample” refers to a substantially planar, i.e., two dimensional, material (e.g. glass, metal, ceramics, organic polymer surface or gel) that contains cells or any combinations of biomolecules derived from cells, such as proteins, nucleic acids, lipids, oligo/polysachharides, biomolecule complexes, cellular organels, cellular debris or excretions (exosomes, microvesicles). A planar cellular sample can be made by, e.g., growing cells on a planar surface, depositing cells on a planar surface, e.g., by centrifugation, by cutting a three dimensional object that contains cells into sections and mounting the sections onto a planar surface, i.e., producing a tissue section, absorbing the cellular components onto the surface that is functionalized with affinity agents (e.g. antibodies, haptens, nucleic acid probes), introducing the biomolecules into a polymer gel or transferring them onto a polymer surface electrophoretically or by other means. The cells or biomolecules may be fixed using any number of reagents including formalin, methanol, paraformaldehyde, methanol:acetic acid, glutaraldehyde, bifunctional crosslinkers such as bis(succinimidyl)suberate, bis(succinimidyl)polyethyleneglycole etc. This definition is intended to cover cellular samples (e.g., tissue sections, etc), electrophoresis gels and blots thereof, Western blots, dot-blots, ELISAs, antibody microarrays, nucleic acid microarrays etc.

As used herein, the term "tissue section" refers to a piece of tissue that has been obtained from a subject, fixed, sectioned, and mounted on a planar surface, e.g., a microscope slide.

As used herein, the term "formalin-fixed paraffin embedded (FFPE) tissue section" refers to a piece of tissue, e.g., a biopsy that has been obtained from a subject, fixed in formaldehyde (e.g., 3%-5% formaldehyde in phosphate buffered saline) or Bouin solution, embedded in wax, cut into thin sections, and then mounted on a microscope slide.

As used herein, the term "spatially-addressable measurements" refers to a set of values that are each associated with a specific position on a surface. Spatially-addressable measurements can be mapped to a position in a sample and can be used to reconstruct an image of the sample.

A "diagnostic marker" is a specific biochemical in the body which has a particular molecular feature that makes it useful for detecting a disease, measuring the progress of disease or the effects of treatment, or for measuring a process of interest.

A "pathoindicative" cell is a cell which, when present in a tissue, indicates that the animal in which the tissue is located (or from which the tissue was obtained) is afflicted with a disease or disorder. By way of example, the presence of one or more breast cells in a lung tissue of an animal is an indication that the animal is afflicted with metastatic breast cancer.

The term "complementary site" is used to refer to an epitope for an antibody or aptamer, or a nucleic acid molecule if the capture agent is an oligonucleotide probe. Specifically, if the capture agent is an antibody, then the complementary site for the capture agent is the epitope in the sample to which the antibody binds. If the capture agent is an oligonucleotide probe, then the complementary site for the capture agent is a complementary sequence in a DNA or RNA molecule in the sample.

The term "epitope" as used herein is defined as small chemical groups on the antigen molecule that is bound to by an antibody. An antigen can have one or more epitopes. In many cases, an epitope is roughly five amino acids or sugars in size. One skilled in the art understands that generally the overall three-dimensional structure or the specific linear sequence of the molecule can be the main criterion of antigenic specificity.

A "subject" of diagnosis or treatment is a plant or animal, including a human. Non-human animals subject to diagnosis or treatment include, for example, livestock and pets.

As used herein, the term "incubating" refers to maintaining a planar sample and capture agent under conditions (which conditions include a period of time, a temperature, an appropriate binding buffer and a wash) that are suitable for specific binding of the capture agent to molecules (e.g., epitopes or complementary nucleic acid) in the planar sample.

As used herein, the term “capture agent” refers to an agent that can specifically bind to complementary sites in a planar sample. Exemplary capture agents include, e.g., an antibody, an aptamer, and a nucleic acid (e.g., oligonucleotide) probe (which may be DNA or RNA) that hybridizes to a binding site. If antibodies are used, in many cases the antibodies may bind to protein epitopes. If nucleic acid probes are used, the nucleic acid probes may bind to, for example, genomic DNA or RNA (such that the location and abundance of intracellular RNAs can be detected).

As used herein, the term “extendible”, in the context of, for example, a 3’ end that is “extendible using the other strand as a template”, means that a polymerase or ligase can add to the 3’ end of a nucleic acid molecule, where the template sequence that is immediately downstream of the 3’ end (i.e., on the other strand) determines which nucleotides (if a polymerase is used) or oligonucleotide (if a ligase is used) is added. A “5’ end that is extendible using the other strand as a template” means that a ligase can add an oligonucleotide to the 5’ end of a nucleic acid molecule, where the template sequence that is immediately downstream of the 5’ end (i.e., on the other strand) determines which oligonucleotide is added.

As used herein, the term “template sequence that is immediately downstream to the 3’ end” refers to the sequence on the other strand that use used as a template for extending the 3’ end, starting with the first nucleotide. In embodiments in which the first strand is an RCA product, the template sequence that is immediately downstream of the 3’ end may be a sequence in the RCA product. In embodiments in which the first strand is an oligonucleotide, the template sequence that is immediately downstream of the 3’ end may be a 5’ overhang.

As used herein, the term “capture agent that is linked to a double stranded nucleic acid” refers to a capture agent, e.g., an antibody or an oligonucleotide probe, that is non-covalently (e.g., via a streptavidin/biotin interaction) or covalently (e.g., via a click reaction or the like) linked to an double-stranded nucleic acid (which may be composed of two single-stranded oligonucleotide strands that are hybridized together, or an RCA product that is hybridized to a plurality of oligonucleotides) in a way that the capture agent can still bind to its binding site and the 3’ end of one of the nucleic acids is accessible to a polymerase and/or ligase. The nucleic acid and the capture agent may be linked via a number of different methods, including those that use maleimide or halogen-containing group, which are cysteine-reactive. The capture agent and the nucleic acid may be linked at, proximal to or at the 5’ end of one of the strands of the double stranded nucleic acid, proximal to or at the 3’ end of one of the strands of the double stranded nucleic acid, or anywhere in-between.

The terms “nucleic acid” and “polynucleotide” are used interchangeably herein to describe a polymer of any length, e.g., greater than about 2 bases, greater than about 10 bases, greater than about 100 bases, greater than about 500 bases, greater than 1000 bases, up to about 10,000 or more bases composed of nucleotides, e.g., deoxyribonucleotides, ribonucleotides or a combination thereof, and may be produced enzymatically or synthetically (e.g., PNA as described in U.S. Patent No. 5,948,902 and the references cited therein) and which can hybridize with naturally occurring nucleic acids in a sequence specific manner analogous to that of two naturally occurring nucleic acids, e.g., can participate in Watson-Crick base pairing interactions. Naturally-occurring nucleotides include guanine, cytosine, adenine, thymine, uracil (G, C, A, T and U respectively). DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, whereas PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. In PNA various purine and pyrimidine bases are linked to the backbone by methylene carbonyl bonds. A locked nucleic acid (LNA), often referred to as an inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformation, which is often found in the A-form duplexes. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. The term “unstructured nucleic acid”, or “UNA”, is a nucleic acid containing non-natural nucleotides that bind to each other with reduced stability. For example, an unstructured nucleic acid may contain a G’ residue and a C’ residue, where these residues correspond to non-naturally occurring forms, i.e., analogs, of G and C that base pair with each other with reduced stability, but retain an ability to base pair with naturally occurring C and G residues, respectively. Unstructured nucleic acid is described in US20050233340, which is incorporated by reference herein for disclosure of UNA.

As used herein, the term “oligonucleotide” refers to a multimer of at least 10, e.g., at least 15 or at least 30 nucleotides. In some embodiments, an oligonucleotide may be in the range of 15-200 nucleotides in length, or more.

As used herein, the term “reading” in the context of reading a fluorescent signal, refers to obtaining an image by scanning or by microscopy, where the image shows the pattern of fluorescence as well as the intensity of fluorescence in a field of view.

As used herein, the term “primer” is an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3’ end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. A primer may be at least 10, e.g., at least 15 or at least 30 nucleotides in length.

As used herein, the term “single nucleotide 5’ overhang” refers to a 5’ overhang, where the overhang is a single nucleotide in length. Likewise, a “two nucleotide 5’ overhang” is a 5’ overhang, where the overhang is two nucleotides in length. The 3’ end is recessed in a 5’ overhang.

In certain cases, the various nucleotides of an overhang may be referred to by their position, e.g., “first position” and “second position”. In these cases, the “position” is relative to the recessed 3’ end. As such, in a multiple base 5’ overhang, the “first” position of the overhang is immediately adjacent to the recessed 3’ end and the “second” position of the overhang is immediately adjacent to the first position.

In certain cases, the complementary strands of a double stranded oligonucleotide or nucleic acid may be referred to herein as being the “first” and “second” or the “top” and “bottom” strands. The assignment of a strand as being a “top” or “bottom” strand is arbitrary and does not imply any particular orientation, function or structure.

As used herein, the term “signal generated by”, in the context of reading a fluorescent signal generated by addition of the fluorescent nucleotide, refers to a signal that is emitted directly from the fluorescent nucleotide, a signal that is emitted indirectly via energy transfer to another fluorescent nucleotide (i.e., by FRET).

As used herein, the term “fluorescently labeled oligonucleotide comprising a quencher” refers to an oligonucleotide that contains a fluorophore and a quencher, wherein the quencher quenches the fluorophore in the same oligonucleotide.

As used herein, the term “different” in the context of different 5’ overhangs that are different, refers to overhangs that have a different sequence. Overhangs of different lengths (e.g., GATC vs GAT) implicitly have a different sequence, even through one sequence may be encompassed by the other.

As used herein, the term “overhang” refers to a structure in which one strand of a double stranded nucleic acid ends such that nucleic acid synthesis can be initiated from that strand by a polymerase (or an oligonucleotide can be ligated to the end by a ligase) using the other strand as a template.

As used herein, the term “adding to the extendible 3’ end”, in the context of adding one or more nucleotides or an oligonucleotide to an extendible 3’ end, refers to adding nucleotides (or an oligonucleotide) to an extendible 3’ end using the other strand as a template (e.g., adding to the recessed 3’ end of a 5’ overhang using the overhang as a template).

As used herein, the term “template of the formula 3’- $N_{4n}N_1/N_2/N_3$ -5’ followed by an optional short stretch (e.g., 1-5 residues) of random nucleotides on the 5’ end to increase the overall polymerase residence on the DNA duplex, where N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 0, 1 or more”, refers to a population of sequences that potentially contains single nucleotide overhangs of nucleotides N_1, N_2 and N_3 or the population of overhangs comprises two nucleotide overhangs of sequence 3’-N_4N_1-5’, 3’- N_4N_2-5’ and 3’- N_4N_3-5’ -5’ and, optionally overhangs of sequence, 3’-$N_4N_4N_1$-5’, 3’-$N_4N_4N_2$-5’ and 3’- $N_4N_4N_3$-5’ and so on (e.g., four nucleotide overhangs of sequence 3’- $N_4N_4N_4N_1$-5’, 3’- $N_4N_4N_4N_2$-5’ and 3’- $N_4N_4N_4N_3$-5’)..

As used herein, the term “template of the formula 3’-YN_1/N_2-5’, optionally followed by short stretch (e.g., 1-5 residues) of random nucleotides on the 5’ end to increase the overall polymerase residence on the DNA duplex, wherein Y is a nucleotide sequence of length n (n is 0, 1 or more) composed of bases N_3 and N_4, wherein nucleotide N_3 is in odd positions and nucleotide N_4 is in even positions, counting from the start of the overhang and N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C” refers to a population of sequences that potentially contain sequences 3’-N_1-5’ and 3’- N_2-5’ or optionally 3’-N_3N_1-5’ and 3’- N_3N_2-5’ or 3’- $N_3N_4N_1$-5’ and 3’- $N_3N_4N_2$-5’ and, optionally, overhangs of sequence 3’- $N_3N_4N_3N_1$-5’ and 3’- $N_3N_4N_3N_2$-5’ and so on (e.g., overhangs of sequence 3’- $N_3N_4N_3N_4N_1$-5’ and 3’- $N_3N_4N_3N_4N_2$-5’ and then 3’-$N_3N_4N_3N_4N_3N_1$-5’ and 3’- $N_3N_4N_3N_4N_3N_2$-5’).

As used herein, the term “alternating stretches” refers to two nucleotides stretches, where one “stretch” is a contiguous sequence of, e.g., up to 10, of the same nucleotide (e.g., a G, A, T or C), and the second stretch is contiguous sequence of, e.g., up to 10, of a different nucleotide, that alternate with one another, i.e., one stretch (e.g., a string of T’s) occupies the odd positions and the other stretch (e.g., a string of A’s) occupies the even positions.

As used herein, the term “incomplete nucleotide mix” comprises a nucleotide mix that contains one, two or three nucleotides (but not all four nucleotides) selected from G, A, T and C. The nucleotides may be labeled or unlabeled.

As used herein, the term “reversible terminator” refers to a chemically modified nucleotide base that when incorporated into growing DNA strand by DNA polymerase blocks further incorporation of bases. Such “reversible terminator” base and DNA strand can be deprotected by chemical treatment and following such deprotection DNA strand can be further extended by DNA polymerase.

As used herein, the term “fluorescently labeled reversible terminator” refers to a “reversible terminator” base which is labeled by fluorophore through linker cleavable by same treatment which is used to deprotect the DNA strand which ends with this base. Deprotecting the “fluorescently labeled reversible terminator” simultaneously activates the DNA strand for further extension and removes the fluorescent label from it.

For ease of description, many of the sequences described herein are written out in the 3’ to 5’ direction. While DNA sequences are routinely set forth in 5’ to 3’ direction, for the ease description, certain DNA sequences in the text below are described in the 3’ to 5’direction. In each such case the directionality is specifically annotated.

Other definitions of terms may appear throughout the specification.

DETAILED DESCRIPTION

In some embodiments the method comprises producing a labeled a planar sample (e.g., an FFPE section mounted on a planar surface such as a microscope slide) using a capture agent that specifically binds to complementary sites in the planar sample. Methods for binding antibodies and/or nucleic acids to sites in the planar sample are well known. In these embodiments, the capture agent in the labeled sample is linked to a double-stranded nucleic acid that comprises a first strand and a second strand (e.g., two oligonucleotide that are hybridized together or an RCA product that is hybridized to oligonucleotides) and the capture agent is linked (covalently or non-covalently via a biotin) to the double-stranded nucleic acid by the first strand of the double-stranded nucleic acid (e.g., by the 5’ end, the 3’ end, or anywhere in-between), and the 3’ end or 5’ end of one of the strands (e.g., the 3’ end of the first strand, any 3’ ends in the second strand, the 5’ end of the first strand or any 5’ ends in the second strand) is extendible using the other strand as a template. In some cases, the 3’ end of the first strand may be recessed relative to the 5’ end of the second strand, thereby defining an overhang. In other cases, the 5’ end of the first strand may be recessed relative to the 3' end of the second strand, thereby defining an overhang. In many embodiments, the capture agent is cross-linked the planar sample, thereby preventing the capture agent from disassociating during subsequent steps. This crosslinking step may be done using any amine-to-amine crosslinker (e.g. formaldehyde, disuccinimiyllutarate or another reagents of similar action) although a variety of other chemistries can be used to cross-link the capture agent to the planar sample if desired. The method comprises reading a fluorescent signal generated by addition of a nucleotide or short oligonucleotide (e.g., of 2-10 bases) to the extendible end (e.g., the 3' end) of one of the strands. This step may be done by contacting the planar sample with a polymerase and a nucleotide mix, a ligase and a labeled oligonucleotide, or a combination of the two, thereby adding one or more nucleotides and/or a labeled oligonucleotide to the extendible end; and reading a fluorescent signal generated by addition of the one or more nucleotides or oligonucleotide to the extendible end.

As will be described in greater detail below, the fluorescent signal may be generated by a variety of different methods. For example, in some embodiments, the fluorescent signal may be fluorescence from a fluorescent nucleotide added to the end of the primer, or a FRET (fluorescence resonance energy transfer) signal resulting from the same. In other embodiments, the signal may generated by removing a quencher from a fluorescently labeled oligonucleotide that is also hybridized to the oligonucleotide.

In any implementation of the method, the reading step may be followed by inactivating the fluorescence after reading so that other binding events can be detected and read. In these embodiments, the fluorescence may be inactivated by peroxide-based bleaching, cleavage of fluorophore linked to nucleotide through cleavable linker (e.g. using TCEP as a cleaving reagent), base-exchange by exo+ polymerase such as Vent, or subsequent incorporation of quencher, for example.

Also, as will be described in greater detailed below, the method may be multiplexed in a way that a single planar sample can be interrogated by a plurality of different capture agents, where each antibody is linked to a different oligonucleotide (i.e., oligonucleotides of different sequence). In multiplex embodiments, the planar sample may be labeled using at least 5, at least 10, at least 20, at least 30, at least 50, or at least 100, up to 150 or more capture agents that are each linked to a different oligonucleotide, and binding of the capture agents can be separately read using a fluorescence microscope equipped with an appropriate filter for each fluorophore, or by using dual or triple band-pass filter sets to observe multiple fluorophores. See, e.g., U.S. Pat. No. 5,776,688. As noted below, the oligonucleotides linked to the capture agent may act as a splint for a padlock probe, and as a primer for initiating rolling circle amplification.

The capture agent used in some embodiments of the method may be linked to a double-stranded oligonucleotide that contains a 5’ overhang (i.e., a recessed 3’ end that can be extended by a polymerase or ligase) or a 3’ overhang (i.e., a recessed 5’ end that can be extended by a ligase). An example of such a capture agent is shown in Figs. 1 and 2. In the example shown in Fig. 1B, the overhang is a single nucleotide overhang (e.g., an A), although a longer overhang (e.g., at least 2, at least 3, at least 4, at least 5, at least 6, at least 8, at least 10, at least 20, or at least at least 30, may be useful for other applications (e.g., multiplexed applications). As shown in Fig. 5 A-D, in certain cases, the overhang may contain a repeated sequence, e.g., 2, 3, 4, 5, or 6 or more repeats of the same sequence of 2, 3, 4, 5 or 6 nucleotides, thereby allowing the capture agent to be used in multiplexed applications as described below. In certain embodiments, the double stranded oligonucleotide may have a recessed 3’ end at the other end of the oligonucleotide (i.e., at the end closest to the capture agent). However, this end may be designed to be not extendible. In certain circumstances, the double-stranded oligonucleotide may contain one or more third oligonucleotides that are hybridized to the overhang. In these embodiments, there will be a gap of 1, 2, 3, 4 or 5 or more nucleotides between the second strand of the double-stranded oligonucleotide and the oligonucleotide that is hybridized to the overhang (see, e.g., Figs. 7 and 8). In multiplex embodiments, the plurality of capture agents may be distinguished by the sequence of the overhang and not by the sequence of the first strand of the double stranded oligonucleotide. In these embodiments, the second strand of the double stranded oligonucleotides is different for each of the capture agents. As shown in other figures, the method may also be implemented using capture agents that are linked to a primer that acts a splint for circularlizing a padlock probe and for priming amplification of circularlized padlock probe by rolling circle amplification. In these embodiments, the capture agents in the labeled sample may be linked to a rolling circle amplification product.

In certain cases, the fluorophore used may be a coumarin, a cyanine, a benzofuran, a quinoline, a quinazolinone, an indole, a benzazole, a borapolyazaindacene and or a xanthene including fluorescein, rhodamine and rhodol. In multiplexing embodiments, fluorophores may be chosen so that they are distinguishable, i.e., independently detectable, from one another, meaning that the labels can be independently detected and measured, even when the labels are mixed. In other words, the amounts of label present (e.g., the amount of fluorescence) for each of the labels are separately determinable, even when the labels are co-located (e.g., in the same tube or in the same area of the section).

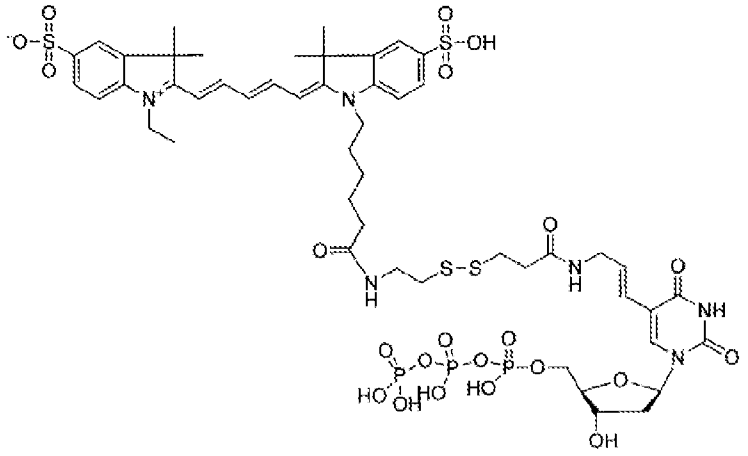
Specific fluorescent dyes of interest include: xanthene dyes, e.g., fluorescein and rhodamine dyes, such as fluorescein isothiocyanate (FITC), 6-carboxyfluorescein (commonly known by the abbreviations FAM and F), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 6-carboxy-4', 5'-dichloro-2', 7'-dimethoxyfluorescein (JOE or J), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA or T), 6-carboxy-X-rhodamine (ROX or R), 5-carboxyrhodamine-6G (R6G^5 or G^5), 6-carboxyrhodamine-6G (R6G^6 or G^6), and rhodamine 110; cyanine dyes, e.g., Cy3, Cy5 and Cy7 dyes; coumarins, e.g., umbelliferone; benzimide dyes, e.g. Hoechst 33258; phenanthridine dyes, e.g., Texas Red; ethidium dyes; acridine dyes; carbazole dyes; phenoxazine dyes; porphyrin dyes; polymethine dyes, e.g., BODIPY dyes and quinoline dyes. Specific fluorophores of interest that are commonly used in subject applications include: Pyrene, Coumarin, Diethylaminocoumarin, FAM, Fluorescein Chlorotriazinyl, Fluorescein, R110, Eosin, JOE, R6G, Tetramethylrhodamine, TAMRA, Lissamine, Napthofluorescein, Texas Red, Cy3, and Cy5, etc.

Suitable distinguishable fluorescent label pairs useful in the subject methods include Cy-3 and Cy-5 (Amersham Inc., Piscataway, NJ), Quasar 570 and Quasar 670 (Biosearch Technology, Novato CA), Alexafluor555 and Alexafluor647 (Molecular Probes, Eugene, OR), BODIPY V-1002 and BODIPY V1005 (Molecular Probes, Eugene, OR), POPO-3 and TOTO-3 (Molecular Probes, Eugene, OR), and POPRO3 and TOPRO3 (Molecular Probes, Eugene, OR). Further suitable distinguishable detectable labels may be found in Kricka et al. (Ann Clin Biochem. 39:114-29, 2002), Ried et al. (Proc. Natl. Acad. Sci. 1992: 89: 1388-1392) and Tanke et al. (Eur. J. Hum. Genet. 1999 7:2-11) and others.

In addition to the labeling methods described above, the sample may be stained using a cytological stain, either before or after performing the method described above. In these embodiments, the stain may be, for example, phalloidin, gadodiamide, acridine orange, bismarck brown, barmine, Coomassie blue, bresyl violet, brystal violet, DAPI, hematoxylin, eosin, ethidium bromide, acid fuchsine, haematoxylin, hoechst stains, iodine, malachite green, methyl green, methylene blue, neutral red, Nile blue, Nile red, osmium tetroxide (formal name: osmium tetraoxide), rhodamine, safranin, phosphotungstic acid, osmium tetroxide, ruthenium tetroxide, ammonium molybdate, cadmium iodide, carbohydrazide, ferric chloride, hexamine, indium trichloride, lanthanum nitrate, lead acetate, lead citrate, lead(II) nitrate, periodic acid, phosphomolybdic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate, sodium chloroaurate, thallium nitrate, thiosemicarbazide, uranyl acetate, uranyl nitrate, vanadyl sulfate, or any derivative thereof. The stain may be specific for any feature of interest, such as a protein or class of proteins, phospholipids, DNA (e.g., dsDNA, ssDNA), RNA, an organelle (e.g., cell membrane, mitochondria, endoplasmic recticulum, golgi body, nulear envelope, and so forth), a compartment of the cell (e.g., cytosol, nuclear fraction, and so forth). The stain may enhance contrast or imaging of intracellular or extracellular structures. In some embodiments, the sample may be stained with haematoxylin and eosin (H&E).

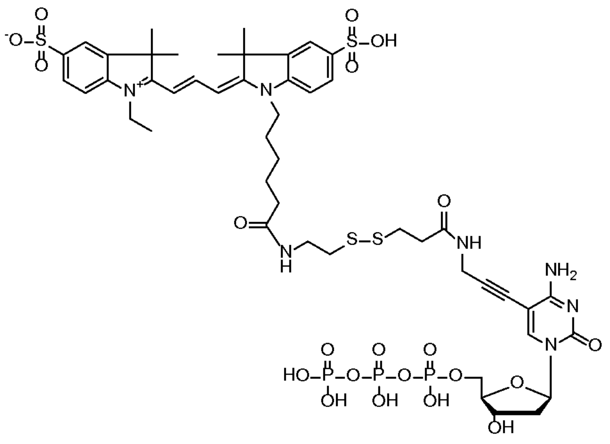
The structures of exemplary sulfhydryl-cleavable deoxynucleotide analogues that can be used in the present method are shown below. As would be recognized, these nucleotides are only exemplary and other nucleotides, including nucleotides that are cleavable by other stimuli (e.g., photocleavable nucleotides) can be used in the present method.

dUTP-SS-Cy5:



Chemical Formula: $C_{50}H_{67}N_6O_{22}P_3S_4$
Molecular Weight: 1325,28

dCTP-SS-Cy3



Chemical Formula: $C_{48}H_{64}N_7O_{21}P_3S_4$
Molecular Weight: 1296,24

In order to further illustrate the present invention, the following specific examples are given with the understanding that they are being offered to illustrate the present invention and should not be construed in any way as limiting its scope.

Implementation I

In this example, the fluorescent signal may be produced by a fluorescent nucleotide that is added to (i.e., added by a polymerase or, if the fluorescent nucleotide is in an oligonucleotide, ligated onto) the 3' end of the primer. This method may comprise reading a signal from the added fluorescent nucleotide, or reading a FRET signal generated by energy transfer between two fluorescent nucleotides that are added to the primer.

The example shown in Figs. 1 and 2 shows how an antibody can be linked to a oligonucleotide chemically, or via biotin/streptavidin interactions (Fig. 1B) and how a fluorescent signal can be generated by adding a fluorescent nucleotide to the end of the primer (Fig. 2). In this example, the antigen is stained by an antibody that is coupled to a DNA dimer with an overhanging 5' end (lower strand) and recessed 3' end (upper strand) either chemically (Fig.1 top panel) or through streptavidin (Fig.1 bottom and middle panels).

After binding the capture agent to the tissue sample, the pattern of binding of the capture agent may be determined using an on-slide end fill-in reaction by using a suitable polymerase (e.g., by exo$^-$ Klenow, Bst, Taq, Klentaq, or an exo$^-$ Klenow–Vent mixture) and fluorescently labeled nucleotide (Fig,1 and Fig.2 top panel).

If necessary, the signal-to-noise ratio can be increased by: a) multimerization of position complementary to labeling nucleotide (Fig. 2, middle panel); or b) by generating a FRET between two nucleotides are incorporated, whereby the emission wavelength of one of the nucleotides (Fig. 2, bottom panel C on the figure) serves as an excitation wavelength for another (Fig. 2, bottom panel U on the figure).

Fluorescence may be inactivated before addition of subsequent staining reagents by any convenient method including, but not limited to photobleaching, peroxide-based bleaching, inactivation by ozone, cleavage of fluorophore linked to nucleotide through cleavable linker (e.g. using TCEP as a cleaving reagent), base-exchange by exo+ polymerase such as Vent, subsequent incorporation of quencher.

In these embodiments, after fluorescence has been inactivated, the method can be repeated, i.e., the planar sample may be re-stained using a different antibody and fluorescence can be read.

Multiplexing

Multiplexing can be implemented using specially designed oligonucleotides using two different approaches, referred to as the "reversible terminator" and "missing base" approaches, which are described in greater detail below. Both of these methods rely on a composition comprising a plurality of (e.g., at least 5, at least 10, at least 20, at least 30, at least 50, or at least 100, up to 150 or more) capture agents that recognize different complementary sites, wherein: each of the capture agents is linked to a double-stranded nucleic acid (e.g., oligonucleotide) that comprises a first strand and a second strand; the capture agents are linked to a double-stranded nucleic acid by the (e.g., the 5' end of) the first strand; the 3' end of one of the strands in each of the double-stranded nucleic acids extendible using the other strand as a template, where the template is different for each of the capture agents. Examples of such compositions are illustrated in Figs. 3 and 4, where the template is an overhang. The general principle shown in Figs. 3 and 4 can be extended to double stranded nucleic acids that comprise RCA products. Fig. 3 shows a population of capture agents that have a template (e.g., overhang) defined by the formula: 3'- $N_{4n}N_1/N_2/N_3$ -5' followed by short stretch of random composition on the 5' end to increase the overall polymerase residence on the DNA duplex, where N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 0, 1 or more. Fig. 4, on the other hand, shows a population of capture agents that have an overhang defined by the formula 3'-YN_1/N_2-5', optionally followed by short stretch (e.g., 1-5 residues) of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is a nucleotide sequence of length n (n is 0, 1 or more) composed of bases N_3 and N_4, wherein nucleotide N_3 is in odd positions and nucleotide N_4 is in even positions, counting from the start of the overhang and N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C. As illustrated in Figs. 3, 4 and 5, the sequence of the first strand is the same for each of the capture agents; and the sequence of the second strand is different for each of the capture agents. In these embodiments, the different second strands make the overhangs different between the different capture agents.

In some embodiments, the multiplex methods may generally comprise: (a) incubating a planar sample with an above-described antibody composition under conditions by which the capture agents bind to complementary sites in the planar sample; (b) cross-linking the capture agents to the planar sample; (c) contacting the planar sample with a polymerase and either an incomplete nucleotide mix of labeled and unlabeled nucleotides or a nucleotide mix where some or all nucleotides are fluorescent and some or all nucleotides are reversible terminator nucleotides or fluorescent reversible terminator nucleotides and optionally, contacting the planar sample with a mixture of labelled and unlabeled oligonucleotides and a DNA ligase enzyme that covalently attaches the short labelled oligonucleotides to the 3'end of the oligonucleotide duplexes that are attached to the specific capture agents. In these embodiments, oligonucleotides are only added to duplexes that an overhang that is complementary to the oligonucleotide. This method further comprises (d) reading, using fluorescence microscopy, a fluorescent signal generated by addition a nucleotide to some but not all of the capture agents. Following signal registration, this method may comprise (e) removing the fluorescent signals by chemical or photocleavage of a labeled nucleotide if the reversible terminator approach is used, followed by deprotecting the 3' ends of the oligonucleotides, enabling the addition of further nucleotides and/or oligonucleotides. Step (c) of this method may comprise (c) contacting the planar sample with a polymerase and: (i) a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4 or (ii) a nucleotide mix that comprises fluorescent reversible terminator nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4 or

(iii) a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, and N_2, an unlabeled nucleotide that is complementary to N_3, and no nucleotide that is complementary to N_4, thereby adding fluorescent nucleotides onto the double-stranded oligonucleotides of some but not all of the capture agents thereby adding fluorescent nucleotides onto the double-stranded oligonucleotides of some but not all of the capture agents; and (d) reading, using fluorescence microscopy, a fluorescent signal generated by addition of a fluorescent nucleotide to some but not all of the capture agents. Step (c) can also be implemented by adding a labeled oligonucleotide to the duplex using a ligase. Examples of such methods are described in greater detail below.

With reference to Fig. 6 it is expected that in the case when larger panels of capture agents are to be employed (e.g. 100 and more) the length of the read over the oligonucleotide overhangs may increase accordingly. This may or may not reduce the efficiency of staining due to accumulation of primer extension errors along the length of the oligonucleotide duplex. To circumvent such potential source of signal loss a slight modification of design can be implemented. The plurality of capture agents can be divided in sets such that number of capture agents in the set does exceed the capacity of the multiplexing protocol to render staining without significant signal loss (e.g.30). Each such set of capture agents will be conjugated to "terminated" (the last 3' base is dideoxy- or propyl- modified) upper strand oligonucleotide of the same sequence as in the original version of the "missing base" approach. The lower strand oligonucleotides will incorporate an additional set-specific region which will serve as a landing spot for an additional primer which is to be on-slide hybridized to the particular subset of the total plurality of the antibodies at the time when they are to be rendered. This approach allows not to extend the reads beyond certain threshold and at the same time have an unlimited potential number of capture agents in the sample.

Reversible terminator method

This implementation of the method relies on reversible terminators, i.e., chain terminator nucleotides that can be de-protected after incorporation, thereby allowing further nucleotides to be added to that nucleotide.

This method can be implemented using a composition comprising a plurality of capture agents that are linked to a double stranded nucleic acid (e.g., oligonucleotides), as illustrated in Fig. 3. In these embodiments, the top strand of the double stranded nucleic acid is linked to the capture agent and may be the same for each antibody, and the sequence of the bottom strand varies between capture agents. As shown on Fig.5A, the 5' end of the lower strand of the double-stranded nucleic acid (which may form an overhang) is of the general 3'- $N_{4n}N_1/N_2/N_3$ -5' followed by short stretch of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, where N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 0, 1 or more. As shown on Fig.5B a more general formula of lower oligonucleotide overhang 3'- $XN_1/N_2/N_3$-5', where N_1, N_2, N_3 are different nucleotides selected from G, A, T and C and X is a nucleotide stretch of bases Xi (such that Xi are different nucleotides selected from G, A, T and C) of random composition and length is also applicable in this method.

In certain embodiments, this method may comprise: (a) incubating a planar sample with a multiplex antibody composition in which the overhangs are of the formula 5'-$N_1/N_2/N_3N_{4n}$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more; under conditions by which the capture agents specifically bind to complementary sites in the planar sample; (b) cross-linking the capture agent to the planar sample; (c) contacting the planar sample with a polymerase and a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4 and/or ligating a oligonucleotide that comprises a labeled nucleotide; and (d) reading, using fluorescence microscopy, a fluorescent signal generated by addition of a nucleotide to some but not all of the capture agents. This cycle may be repeated by (e) inactivating the fluorescent signal, deprotecting the reversible terminator nucleotide and (f) blocking the planar sample; and repeating steps (c) and (d). In certain embodiments, the method may comprise repeating steps (c), (d) (e) and (f) multiple times. The reagent used for blocking may vary depending on the chemistry used. In certain embodiments, the sample may be blocked with a thiol-reactive compounds such as cysteine, glutathione or iodoacetamide.

For example, this method can be implemented using a composition comprising: a first antibody linked to a first double stranded oligonucleotide, wherein the first double stranded oligonucleotide comprises a single nucleotide 5' overhang comprising base N_1; a second antibody linked to a second double stranded oligonucleotide, wherein the second double stranded oligonucleotide comprises a single nucleotide 5' overhang comprising base N_2; a third antibody linked to a third double stranded oligonucleotide, wherein the third double stranded oligonucleotide comprises a single nucleotide 5' overhang comprising base N_3; a fourth antibody linked to a fourth double stranded oligonucleotide comprises a two nucleotide 5' overhang, wherein the first position of the overhang comprises base N_4 and the second position of the overhang is base N_1; a fifth antibody linked to a fifth double stranded oligonucleotide, wherein the fifth double stranded oligonucleotide comprises a two nucleotide 5' overhang, wherein the first position of the overhang comprises base N_4 and the second position of the overhang is base N_2; and a sixth antibody linked to a sixth double stranded oligonucleotide, wherein the sixth double stranded oligonucleotide comprises a two nucleotide 5' overhang, wherein the first position of the overhang comprises base N_4 and the second position of the overhang is base $N_{3,}$ wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C. An example of such a population of capture agents is shown in Fig. 3.

In RCA embodiments, the strand linked to the antibodies may be different for each of the antibodies, where the RCA product contains a sequence conforming to the formula described above in each repeat of the RCA product.

In certain implementations, the composition may also contain a seventh antibody linked to a seventh double stranded oligonucleotide, wherein the seventh double stranded oligonucleotide comprises a multiple nucleotide 5' overhang, wherein the first position of the overhang comprises base $N_{4,}$ the second position of the overhang is base N_4 and third is selected from N_1, N_2, and N_3. The same principle may be applied to overhangs that have more than 7 positions (e.g., 9, 10, 11 up to 20, 30, or 40 ore more) positions.

In this implementation of the method, the planar sample can be co-stained simultaneously using a panel of capture agents, each labeled with one oligonucleotide duplex designed according to the strategy outlined on Fig. 3. The duplexes are designed in such a way that each antibody has the same upper strand sequence linked, covalently or through streptavidin, to an antibody through the 5' end. The lower strand changes from antibody to antibody. In this implementation, the general formula for the lower strand is 3'-dideoxydC -sequence-complimentary-to-upper-strand $G_nA/T/C$-5' . One type of lower strand base (nucleotide G in this example) is reserved for step-wise progression and its complementary pair on the upper strand is never used in labeled form. The other three bases are complementary to labeled nucleotides and can be used to identify three capture agents per cycle. In a more general case the general formula for the lower strand is 3'- dideoxydC-sequence-complimentary-to-upper-strand- X-$N_1/N_2/N_3$-5' where X_i of X is any nucleotide excluding one reserved for "walking base" of this particular cycle and X is any base as shown on Fig. 5B. This design ensures that: a) no two antibody species contain the same duplex and b) only three different capture agents are detected at a time. Each cycle includes: (a) a labeling step in which the three capture agents are labeled and duplexes on the rest are extended one base at a time, (b) an imaging step and (c) a destaining/deprotection step. During cycle to cycle transition the added fluorescent labels from the previous cycle are inactivated by any of the suitable methods, including but not limited to: cleavage of fluorophore off the nucleotide (if the labeled nucleotide is linked to the fluorophore through a cleavable linker); peroxide based bleaching; photobleaching; chemically-assisted photobleaching; labeled base replacement by exo+ polymerase, etc. After or simultaneously with inactivation of the fluorophores added in the previous reaction, the unlabeled "extension" nucleotide that has been added to the remainder of the capture agents is activated by cleavage of the protective group off its 3' end. Cleavage of the protective group, in turn, allows that nucleotide to be extended in the next cycle. Since the A, T and C are reserved for incorporation of a labelled nucleotide, those nucleotides only occur at the end of each lower strand of the duplex. This approach is based on the chemical nature of reversible terminators, which precludes upper strand extension for more than one nucleotide at a time even on polyG stretches of the lower strand. Optionally, a quencher labeled nucleotide can be incorporated following the labeled nucleotide. The performance of "reversible terminator method" as exemplified in sequential detection of CD4 and CD8 positive T-cells in smears of mouse splenocytes is illustrated in fig. 13A-D.

Missing base method

This implementation of the method relies on a "missing" base design in which, in each cycle, two labeled and one unlabeled nucleotides are added to the reaction, and the "missing base" prevents the primers from being extended by more than a single nucleotide.

This method can be implemented using a composition comprising a plurality of capture agents that are linked to double stranded nucleic acids, as illustrated in Fig. 4. In these embodiments, the top strand of the double stranded nucleic acids is linked to the capture agent and may be the same for each antibody, and the sequence of the bottom strand varies between capture agents. As shown in Fig. 4, the 5' end of the lower strand of the double-stranded oligonucleotide (which forms the overhang) is of the general formula 3'-YN_1/N_2-5', optionally followed by short stretch (e.g., 1-5 residues) of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is a nucleotide sequence of length n (n is 0, 1 or more) composed of bases N_3 and N_4, wherein nucleotide N_3 is in odd positions and nucleotide N_4 is in even positions, counting from the start of the overhang and N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C.

Also a more general formula 3'-YN_1/N_2-5', wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and Y is a nucleotide sequence of length n (n is 0, 1 or more) composed of alternating random length stretches of bases N_3 and N_4 such that the order number of N_3 - stretches is odd and of N_4 stretches is even,, may be applicable in this method

In certain embodiments, this method may comprise: (a) incubating a planar sample with a multiplex antibody composition in which the overhangs are of the formula (3'-YN_1/N_2-5') described in the prior paragraph ; under conditions by which the capture agents specifically bind complementary sites in the planar sample; (b) cross-linking the capture agent to the planar sample; (c) contacting the planar sample with a polymerase and a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, and N_2, an unlabeled nucleotide that is complementary to N_3 and no nucleotide that is complementary to N_4 and/or ligating an oligonucleotide that has a labeled nucleotide; and (d) reading, using fluorescence microscopy, a fluorescent signal generated by addition of a nucleotide to some but not all of the capture agents. This cycle may be repeated by (e) inactivating the fluorescent signal, (f) blocking the sample and contacting the planar sample with a polymerase and an unlabeled nucleotide that is complementary to N_4 and/or contacting the sample with a labeled oligonucleotide and a ligase; and repeating steps (c) (d). In certain embodiments, the method may comprise repeating steps (c), (d), (e) and (f) multiple times.

This method can be implemented using a capture agent composition that comprises: a first antibody linked to a first double stranded oligonucleotide, wherein the first double stranded oligonucleotide comprises a single nucleotide 5' overhang comprising base N_1; a second antibody linked to a second double stranded oligonucleotide, wherein the second double stranded oligonucleotide comprises a single nucleotide 5' overhang comprising base N_2; a third antibody linked to a fourth double stranded oligonucleotide, wherein the third double stranded oligonucleotide comprises a two nucleotide 5' overhang, wherein the first from the 3' position of the overhang comprises base N_4 and the second position comprises N_1; and a fourth antibody linked to a fourth double stranded oligonucleotide, wherein the fourth double stranded oligonucleotide comprises a two nucleotide 5' overhang, wherein the first position of the overhang comprises base N_4 and the second position comprises base N_2, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C. An example of such a population of capture agents is shown in Fig. 4.

In certain implementations, the composition may also contain a fifth antibody linked to a fifth double stranded oligonucleotide, wherein the fifth double stranded oligonucleotide comprises a multiple nucleotide 5' overhang, wherein the first position of the overhang comprises base N_4, the second position comprises base N_3, and the third position comprises N_1 or N_2.

Overall there is no theoretical limits to the number of co-detected complementary sites, e.g., antigens, both in the case of "reversible terminator" and of "missing base" approach

The missing base approach does not use reversible terminators. Instead, extension of a single nucleotide is ensured by using two interchanging bases (e.g., T and C as shown in Fig. 4 instead of the corresponding G in the "reversible terminators" approach) and adding only one of the two dNTPs at a time in the primer extension reaction. After the incorporation of the first nucleotide, the absence of the second dNTP causes strand elongation to stall, thereby ensuring that the primers are extended by only a single nucleotide. As in the previous strategy, all complementary sites can be co-stained simultaneously using capture agents, each labeled with a specific oligonucleotide duplex.

In this embodiment, the duplexes can be designed using the strategy shown in Fig. 4, i.e., in such a way that each antibody has the same upper stand oligonucleotide sequence linked to it via covalent bond or through a streptavidin-biotin interaction. In this implementation, the lower strand changes from antibody to antibody. In this method, the general formula for the lower strand is 3' ddC -sequence-complimentary-to-upper-strand -YA/N_2-5' where Y is composed of bases T and C such that T can be found only in even and C only at odd positions. Or in the more general case 3'-YN_1/N_2-5', wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and Y is a nucleotide sequence of length n (n is 0, 1 or more) composed of alternating random length stretches of bases N_3 and N_4 such that the order number of N_3 - stretches is odd and of N_4 stretches is even.. In the first simple implementation two base pairs of the lower strand (T and C as in exemplary design on Fig.4) are reserved for step-wise progression and their complementary pair on the upper strand is never labeled. The other two bases are complementary to labeled nucleotides and can render the staining with two different capture agents per cycle. Such design ensures that a) no two capture agents contain the same duplex and b) only two different antibody are read per cycle. In this implementation, each cycle can have three steps: a labeling step in which the two capture agents are labeled by incorporation of fluorescent dNTPs and all of the other duplexes are extended one base at a time, an imaging step, and a de-staining/reactivation step.

In RCA embodiments, the strand linked to the antibodies may be different for each of the antibodies, where the RCA product contains a sequence conforming to the formula described above in each repeat of the RCA product.

During cycle-to-cycle transition the labeled capture agents from the prior cycle can be bleached/destained in the same way as described above. Optionally, instead of bleaching, a quencher labeled nucleotide can be incorporated after the labeled base. Because, in this embodiment, the position that is labeled is the last position in the overhang, the labeled capture agents from prior cycle cannot be re-labeled in later cycles because all nucleotide positions in the overhang have been filled in. The performance of “reversible terminator method” as exemplified in sequential detection of CD4 and CD8 positive T-cells in smears of mouse splenocytes is illustrated in fig. 13, 15 and figure 16.

Implementation II

In this method, extension of a primer by nick translation removes a quencher from a fluorescently labeled “detector” oligonucleotide that is hybridized to the lower strand oligonucleotide in such a way that is positioned downstream from the upper strand primer. The principles of this method are illustrated in Figs. 7. A multiplexed version of this method is shown in Fig. 8.

In certain embodiments, the multiplexed implementations may comprise: (a) incubating the planar sample with a plurality of capture agents that are linked to a double-stranded oligonucleotide; (b) crosslinking the capture agents to the planar sample; (c) extending a primer that is hybridized to the oligonucleotide of a first set of capture agents of the plurality, thereby generating a first set of fluorescent signals (e.g., by removing the quencher from a labeled oligonucleotide that is hybridized to the oligonucleotide downstream from the primer), e.g., by adding a nucleotide using a polymerase or by adding an oligonucleotide using a ligase; (d) reading the first set of fluorescent signals using fluorescence microscopy; (e) inactivating the fluorescence; (f) extending a primer that is hybridized to the oligonucleotide of a second set of capture agents of the plurality, thereby generating a second set of fluorescent signals (e.g., by removing the quencher from a labeled oligonucleotide that is hybridized to the oligonucleotide downstream from the primer); (g) reading the second set of fluorescent signals using fluorescence microscopy; and (h) comparing the images produce in steps (d) and (g).

In this method, the architecture of the double-stranded oligonucleotides linked to the capture agent has a specific design which is effectively enabling rendering of the capture agent binding pattern by “nick translation”. In particular the duplex of the upper strand and the lower strand oligonucleotide with long 5’ overhang of the lower strand is further hybridized to a small detector oligonucleotide labeled both by fluorescent and the quencher. There is a predesigned gap between the initial upper strand and the upper strand detector oligo. During cyclic staining this gap is “walked” by either “reversible terminator” or “missing base” (similar to described in previous sections) until the gap is reduced to a single base nick. Extension and progression through the nick on the upper strand by “nick translating” polymerase such as DNA pol I removes the quencher from some but not all of the quenched fluorescently labeled oligonucleotides, thereby generating a fluorescent signal for some but not all of the capture agents.

In some embodiments the method generally comprises: (i) labeling a planar sample with: i. a first antibody, wherein the first antibody is linked to a first oligonucleotide duplex comprising, lower strand oligonucleotide with a unique sequence hybridized thereto: (i) an oligonucleotide upper strand “primer” and (ii) a labeled upper strand oligonucleotide comprising a 5’ quencher at a site that is downstream from the primer; and a fluorophore downstream from the quencher and ii. a second antibody, wherein the second antibody is linked to a second oligonucleotide duplex comprising, lower strand oligonucleotide with unique sequence hybridized thereto: (i) an oligonucleotide upper strand “primer” and (ii) an upper strand oligonucleotide labeled both by fluorophore and a quencher; wherein the gap between the 3’ end of the primer and the 5’ end of the labeled oligonucleotide is different for the first and second oligonucleotides; (ii) incubating the tissue sample with a first nucleotide mix and a polymerase, thereby removing the quencher from only the labeled oligonucleotide that is hybridized to the first oligonucleotide and producing a first fluorescent signal; (iii) reading the first fluorescent signal using fluorescence microscopy; (iv) inactivating the fluorescent signal by further progression of nick-translating polymerase; (v) incubating the tissue sample with a second nucleotide mix and a polymerase, thereby removing the quencher from only the labeled oligonucleotide that is hybridized to the second oligonucleotide and producing a first fluorescent signal; and (vi) reading the second fluorescent signal from the planar sample using fluorescence microscopy.

Fig's 7 and 8 show an example of this method. The multiplexing method shown in Fig. 8 has the following steps:

Step 1: The planar sample is stained by capture agents that are coupled to a DNA double-stranded oligonucleotide chemically or through streptavidin (as described in Fig. 1) such that the top strand of the duplex contains a nick or a single base deletion followed by a nucleotide stretch bordered by a fluorophore and its quencher on two ends ("molecular beacon" or Taqman based design).

Step 2: Staining pattern is rendered by a nick-translation reaction carried out by any 5' exo+ polymerase such as DnaPolI Klenow fragment in the presence of a single letter (A as in Fig. 5 for example). Nick translation removes the quencher but stops before removing the part of the duplex with the fluorophore.

Step 3: For rendering of other staining reagents, the fluorescence is removed by continuing nick translation in the presence of the letters of the stretch bearing the fluorophore.

Step 4: When multiplexing is desired, multiplexing can be achieved by special desigm of oligonucleotide duplexes attached to detection reagents. In particular each antibody set (two or three per cycle) has a gap of an increasing length between the top strand priming and the detector oligonucleotide. This sequence gap on the strand bearing the quencher/fluorophore pair is filled up to final nick in such a way that single base is extended per cycle, similar to how it is achieved in method 1 (see Fig.8).

Implementation III

In this implementation, the method comprises rending antibody staining by primer extension with a fluorophore labeled base or otherwise reading a FRET signal generated by energy transfer between a first fluorescent nucleotide added to the primer by primer extension and a second nucleotide that is present in the oligonucleotide Fig.10. The principles of this method are illustrated in Fig. 9A. The multiplexing is achieved by removing the extension priming oligonucleotide by melting the duplex or by exonuclease and reannealing another primer oligonucleotide which is extendable on a different antibody. A multiplexed version of this method is shown in Fig. 9B.In certain embodiments, the multiplexed implementations may comprise: (a) incubating the planar sample with a plurality of capture agents; (b) cross-linking the capture agents to the planar sample; (c) extending a primer that is hybridized to the oligonucleotide of a first set of capture agents of the plurality (e.g., wherein the 3' end of the first primer anneals to only the oligonucleotide of the first population), thereby generating a first set of fluorescent signals (which step can be done by adding a labeled nucleotide using polymerase and/or contacting the sample with a labeled oligonucleotide and a ligase); (d) reading the first set of fluorescent signals using fluorescence microscopy; (e) inactivating the fluorescence; (f) extending a primer that is hybridized to the oligonucleotide of a second set of capture agents of the plurality (e.g., wherein the 3' end of the first primer anneals to only the oligonucleotide of the second population), thereby generating a second set of fluorescent signals (which step can also be done by adding a labeled nucleotide using polymerase and/or contacting the sample with a labeled oligonucleotide and a ligase); (g) reading the second set of fluorescent signals using fluorescence microscopy; and (h) comparing the images produce in steps (d) and (g).

In certain embodiments, this method comprises: (a) incubating the planar sample with (i) a first antibody that is linked to a first labeled oligonucleotide and (ii) a second antibody that is linked to a second labeled oligonucleotide, (b) cross-linking the capture agents to the planar sample; (c) hybridizing the first and second labeled oligonucleotides with a first primer, wherein the 3' end of the first primer anneals to only the first labeled oligonucleotide; (d) extending the primer with a fluorescent nucleotide (which step can be done by adding a labeled nucleotide using polymerase and/or contacting the sample with a labeled oligonucleotide and a ligase); (e) reading, by fluorescence microscopy, a FRET signal generated by energy transfer between the label of the first oligonucleotide and the fluorescent nucleotide added to the first primer; (f) inactivating the fluorescent nucleotide added to the first primer; (g) hybridizing the first and second labeled oligonucleotides with a second primer, wherein the 3' end of the second primer anneals to only the second labeled oligonucleotide; (h) extending the second primer with a fluorescent nucleotide; and (i) reading, by fluorescence microscopy, a FRET signal generated by energy transfer between the label of the second oligonucleotide and the fluorescent nucleotide added to the second primer.

Figs. 9-10 shows an example of this method. The method shown in Figs. 8-11 has the following steps:

Step 1: The planar sample is stained using a capture agent that is coupled to a single stranded oligonucleotide. The oligonucleotide could be either unlabeled or labeled by FRET acceptor (e.g. Cy5) fluorophore on the 3' end.

Step 2: The binding pattern can be determined by an on-slide hybridization of a complementary probe followed a primer extension reaction in which a fluorescently labeled nucleotide fills in the overhang in the extended strand. In this example (see Fig. 10) the extended base is labeled by a FRET donor (e.g. Cy3), which can increase the signal to noise ratio. If the oligonucleotide that is linked to the capture agent is unlabeled, then the fluorescent emission of the nucleotide that has been incorporated by DNA synthesis can be detected directly, without FRET Fig.9.

Step 3: The binding pattern of other capture agents can be determined by removing the fluorescence by cleavage of lower strand by exo+ DNA polymerase such as Vent (Fig. 9). Alternatively, the fluorescence can be removed by raising the temperature beyond the melting point of the DNA strands or by one of the de-staining techniques described previously.

Step 4: Multiplexing can be achieved by staining of the sample with a library of capture agents each labeled with specific oligonucleotides and cycling through Steps 1-3, as described above, each time using a different detection oligonucleotide that is complementary to one of the capture agent-conjugated oligonucleotides. Only duplexes where primers are annealed specifically will be properly extended (Fig. 11). In these embodiments, each primers is designed so that its 3' end hybridizes to only one of the oligonucleotides that are linked to a capture agent.

Further implementations

As schematically illustrated in Fig. 16, the signal may be amplified using rolling circle amplification. In these embodiments, a capture agent that is linked to an oligonucleotide is hybridized to a padlock probe that hybridizes to the oligonucleotide in such a way that the ends of the padlock probe are ligatably adjacent. In this embodiment, after ligation, the padlock probe (which is now circularized) can be copied by a rolling circle amplification reaction that is primed by the oligonucleotide. This reaction results in a concatamer of the padlock probe that contains several (in many cases hundreds or thousands) of copies of the same sequence in tandem that is linked to the capture agent. The rolling circle amplification product (which is linked to the antibody) can be detected using methods described above and, as illustrated, the signal is amplified because the sequence being detected is repeated. In these embodiments, the (i) the capture agent is linked to a double-stranded nucleic acid that comprises a first strand (i.e., the RCA product) and a second strand (comprising the detection oligonucleotides). Single molecules can be detected using such methods.

Fig. 19 shows how RNA molecules can be detected using a padlock probe/RCA amplification approach. In this method, the padlock probe hybridizes to the same mRNA as the capture agent (the “splint-primer”), thereby ensuring that the padlock probe circularizes only in the presence of the target RNA. In this embodiment, the splint-primer hybridizes to the target RNA, acts as a splint for the padlock probe, and also acts as a primer for rolling circle amplification, thereby allowing the signal to be amplified in a similar to Fig. 16.

Fig. 20 shows an alternative method that relies on primer extension and the ligation of a short, labeled oligonucleotide. In this embodiment, ligation of short labeled oligonucleotide to the top strand oligonucleotide only occurs after the overhang has been filed in to a certain point. In embodiments that rely on ligation, a labeled oligonucleotide can be added to either the 3’ end or the 5’ end of the extendible end.

Utility

The methods and compositions described herein find general use in a wide variety of application for analysis of any planar sample (e.g., in the analysis of tissue sections, sheets of cells, spun-down cells, blots of electrophoresis gels, Western blots, dot-blots, ELISAs, antibody microarrays, nucleic acid microarrays etc).

In particular embodiments, the planar sample may be a section of a tissue biopsy obtained from a patient. Biopsies of interest include both tumor and non-neoplastic biopsies of skin (melanomas, carcinomas, etc.), soft tissue, bone, breast, colon, liver, kidney, adrenal, gastrointestinal, pancreatic, gall bladder, salivary gland, cervical, ovary, uterus, testis, prostate, lung, thymus, thyroid, parathyroid, pituitary (adenomas, etc.), brain, spinal cord, ocular, nerve, and skeletal muscle, etc.

In certain embodiments, capture agents specifically bind to biomarkers, including cancer biomarkers, that may be proteinaceous or a nucleic acid. Exemplary cancer biomarkers, include, but are not limited to carcinoembryonic antigen (for identification of adenocarcinomas), cytokeratins (for identification of carcinomas but may also be expressed in some sarcomas), CD15 and CD30 (for Hodgkin's disease), alpha fetoprotein (for yolk sac tumors and hepatocellular carcinoma), CD117 (for gastrointestinal stromal tumors), CD10 (for renal cell carcinoma and acute lymphoblastic leukemia), prostate specific antigen (for prostate cancer), estrogens and progesterone (for tumour identification), CD20 (for identification of B-cell lymphomas) and CD3 (for identification of T-cell lymphomas).

The above-described method can be used to analyze cells from a subject to determine, for example, whether the cell is normal or not or to determine whether the cells are responding to a treatment. In one embodiment, the method may be employed to determine the degree of dysplasia in cancer cells. In these embodiments, the cells may be a sample from a multicellular organism. A biological sample may be isolated from an individual, e.g., from a soft tissue. In particular cases, the method may be used to distinguish different types of cancer cells in FFPE samples.

The method described above finds particular utility in examining planar samples using a plurality of antibodies, each antibodies recognizing a different marker. Examples of cancers, and biomarkers that can be used to identify those cancers, are shown below. In these embodiments, one does not need to examine all of the markers listed below in order to make a diagnosis.

Acute Leukemia IHC Panel	CD3, CD7, CD20, CD34, CD45, CD56, CD117, MPO, PAX-5, and TdT.
Adenocarcinoma vs. Mesothelioma IHC Panel	Pan-CK, CEA, MOC-31, BerEP4, TTF1, calretinin, and WT-1.
Bladder vs. Prostate Carcinoma IHC Panel	CK7, CK20, PSA, CK 903, and p63.
Breast IHC Panel	ER, PR, Ki-67, and HER2. Reflex to HER2 FISH after HER2 IHC is available.
Burkitt vs. DLBC Lymphoma IHC panel	BCL-2, c-MYC, Ki-67.
Carcinoma Unknown Primary Site, Female (CUPS IHC Panel - Female)	CK7, CK20, mammaglobin, ER, TTF1, CEA, CA19-9, S100, synaptophysin, and WT-1.
Carcinoma Unknown Primary Site, Male (CUPS IHC Panel - Male)	CK7, CK20, TTF1, PSA, CEA, CA19-9, S100, and synaptophysin.
GIST IHC Panel	CD117, DOG-1, CD34, and desmin.

Hepatoma/Cholangio vs. Metastatic Carcinoma IHC Panel	HSA (HepPar 1), CDX2, CK7, CK20, CAM 5.2, TTF-1, and CEA (polyclonal).
Hodgkin vs. NHL IHC Panel	BOB-1, BCL-6, CD3, CD10, CD15, CD20, CD30, CD45 LCA, CD79a, MUM1, OCT-2, PAX-5, and EBER ISH.
Lung Cancer IHC Panel	chromogranin A, synaptophysin, CK7, p63, and TTF-1.
Lung vs. Metastatic Breast Carcinoma IHC Panel	TTF1, mammaglobin, GCDFP-15 (BRST-2), and ER.
Lymphoma Phenotype IHC Panel	BCL-2, BCL-6, CD3, CD4, CD5, CD7, CD8, CD10, CD15, CD20, CD30, CD79a, CD138, cyclin D1, Ki67, MUM1, PAX-5, TdT, and EBER ISH.
Lymphoma vs. Carcinoma IHC Panel	CD30, CD45, CD68, CD117, pan-keratin, MPO, S100, and synaptophysin.
Lymphoma vs. Reactive Hyperplasia IHC Panel	BCL-2, BCL-6, CD3, CD5, CD10, CD20, CD23, CD43, cyclin D1, and Ki-67.
Melanoma vs. Squamous Cell Carcinoma IHC Panel	CD68, Factor XIIIa, CEA (polyclonal), S-100, melanoma cocktail (HMB-45, MART-1/Melan-A, tyrosinase) and Pan-CK.
Mismatch Repair Proteins IHC Panel (MMR/Colon Cancer)	MLH1, MSH2, MSH6, and PMS2.
Neuroendocrine Neoplasm IHC Panel	CD56, synaptophysin, chromogranin A, TTF-1, Pan-CK, and CEA (polyclonal).
Plasma Cell Neoplasm IHC Panel	CD19, CD20, CD38, CD43, CD56, CD79a, CD138, cyclin D1, EMA, kappa, lambda, and MUM1.
Prostate vs. Colon Carcinoma IHC Panel	CDX2, CK 20, CEA (monoclonal), CA19-9, PLAP, CK 7, and PSA.
Soft Tissue Tumor IHC Panel	Pan-CK, SMA, desmin, S100, CD34, vimentin, and CD68.
T-Cell Lymphoma IHC panel	ALK1, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD20, CD21, CD30, CD56, TdT, and EBER ISH.
T-LGL Leukemia IHC panel	CD3, CD8, granzyme B, and TIA-1.
Undifferentiated Tumor IHC Panel	Pan-CK, S100, CD45, and vimentin.

In some embodiments, the method may be employed to detect the location and, optionally, the abundance of DNA molecules and/or RNA molecules *in situ*. In one exemplary embodiment, the method may be used to detect intracellular RNAs. In these embodiments, the capture agent may be a nucleic acid, and the intracellular location and, optionally, the abundance of RNA molecules (e.g., mRNAs or lncRNAs) may be detected in situ. Such hybridization methods may be adapted from known RNA or DNA FISH methods (see, e.g., Mahadevaiah et al (Methods Mol Biol. 2009 558:433-44), Shaffer et al (PLoS

One. 2013 8:e75120) and Pollex et al (Methods Mol. Biol. 2013 1042:13-31), which are incorporated by reference herein.

In some embodiments, the method may involve obtaining an image as described above (an electronic form of which may have been forwarded from a remote location) and may be analyzed by a doctor or other medical professional to determine whether a patient has abnormal cells (e.g., cancerous cells) or which type of abnormal cells are present. The image may be used as a diagnostic to determine whether the subject has a disease or condition, e.g., a cancer. In certain embodiments, the method may be used to determine the stage of a cancer, to identify metastasized cells, or to monitor a patient's response to a treatment, for example.

The compositions and methods described herein can be used to diagnose a patient with a disease. In some cases, the presence or absence of a biomarker in the patient's sample can indicate that the patient has a particular disease (e.g., cancer). In some cases, a patient can be diagnosed with a disease by comparing a sample from the patient with a sample from a healthy control. In this example, a level of a biomarker, relative to the control, can be measured. A difference in the level of a biomarker in the patient's sample relative to the control can be indicative of disease. In some cases, one or more biomarkers are analyzed in order to diagnose a patient with a disease. The compositions and methods of the disclosure are particularly suited to identifying the presence or absence of, or determining expression levels, of a plurality of biomarkers in a sample.

In some cases, the compositions and methods herein can be used to determine a treatment plan for a patient. The presence or absence of a biomarker may indicate that a patient is responsive to or refractory to a particular therapy. For example, a presence or absence of one or more biomarkers may indicate that a disease is refractory to a specific therapy and an alternative therapy can be administered. In some cases, a patient is currently receiving the therapy and the presence or absence of one or more biomarkers may indicate that the therapy is no longer effective.

In any embodiment, data can be forwarded to a “remote location”, where “remote location,” means a location other than the location at which the image is examined. For example, a remote location could be another location (e.g., office, lab, etc.) in the same city, another location in a different city, another location in a different state, another location in a different country, etc. As such, when one item is indicated as being "remote" from another, what is meant is that the two items can be in the same room but separated, or at least in different rooms or different buildings, and can be at least one mile, ten miles, or at least one hundred miles apart. "Communicating" information references transmitting the data representing that information as electrical signals over a suitable communication channel (e.g., a private or public network). "Forwarding" an item refers to any means of getting that item from one location to the next, whether by physically transporting that item or otherwise (where that is possible) and includes, at least in the case of data, physically transporting a medium carrying the data or communicating the data. Examples of communicating media include radio or infra-red transmission channels as well as a network connection to another computer or networked device, and the internet or including email transmissions and information recorded on websites and the like. In certain embodiments, the image may be analyzed by an MD or other qualified medical professional, and a report based on the results of the analysis of the image may be forwarded to the patient from which the sample was obtained.

In some cases, the method may be employed in a variety of diagnostic, drug discovery, and research applications that include, but are not limited to, diagnosis or monitoring of a disease or condition (where the image identifies a marker for the disease or condition), discovery of drug targets (where the a marker in the image may be targeted for drug therapy), drug screening (where the effects of a drug are monitored by a marker shown in the image), determining drug susceptibility (where drug susceptibility is associated with a marker) and basic research (where is it desirable to measure the differences between cells in a sample).

In certain embodiments, two different samples may be compared using the above methods. The different samples may be composed of an “experimental” sample, i.e., a sample of interest, and a “control” sample to which the experimental sample may be compared. In many embodiments, the different samples are pairs of cell types or fractions thereof, one cell type being a cell type of interest, e.g., an abnormal cell, and the other a control, e.g., normal, cell. If two fractions of cells are compared, the fractions are usually the same fraction from each of the two cells. In certain embodiments, however, two fractions of the same cell may be compared. Exemplary cell type pairs include, for example, cells isolated from a tissue biopsy (e.g., from a tissue having a disease such as colon, breast, prostate, lung, skin cancer, or infected with a pathogen etc.) and normal cells from the same tissue, usually from the same patient; cells grown in tissue culture that are immortal (e.g., cells with a proliferative mutation or an immortalizing transgene), infected with a pathogen, or treated (e.g., with environmental or chemical agents such as peptides, hormones, altered temperature, growth condition, physical stress, cellular transformation, etc.), and a normal cell (e.g., a cell that is otherwise identical to the experimental cell except that it is not immortal, infected, or treated, etc.); a cell isolated from a mammal with a cancer, a disease, a geriatric mammal, or a mammal exposed to a condition, and a cell from a mammal of the same species, preferably from the same family, that is healthy or young; and differentiated cells and non-differentiated cells from the same mammal (e.g., one cell being the progenitor of the other in a mammal, for example). In one embodiment, cells of different types, e.g., neuronal and non-neuronal cells, or cells of different status (e.g., before and after a stimulus on the cells) may be employed. In another embodiment of the invention, the experimental material is cells susceptible to infection by a pathogen such as a virus, e.g., human immunodeficiency virus (HIV), etc., and the control material is cells resistant to infection by the pathogen. In another embodiment, the sample pair is represented by undifferentiated cells, e.g., stem cells, and differentiated cells.

The images produced by the method may be viewed side-by-side or, in some embodiments, the images may be superimposed or combined. In some cases, the images may be in color, where the colors used in the images may correspond to the labels used.

Cells any organism, e.g., from bacteria, yeast, plants and animals, such as fish, birds, reptiles, amphibians and mammals may be used in the subject methods. In certain embodiments, mammalian cells, i.e., cells from mice, rabbits, primates, or humans, or cultured derivatives thereof, may be used.

Computer Systems

The invention also provides a computer system that is configured to implement the methods of the disclosure. The system can include a computer server ("server") that is programmed to implement the methods described herein. **FIG. 21** depicts a system **1600** adapted to enable a user to detect, analyze, and process images of samples. The system **1600** includes a central computer server **1601** that is programmed to implement exemplary methods described herein. The server **1601** includes a central processing unit (CPU, also "processor") **1605** which can be a single core processor, a multi core processor, or plurality of processors for parallel processing. The server **1601** also includes memory **1610** (e.g. random access memory, read-only memory, flash memory); electronic storage unit **1615** (e.g. hard disk); communications interface **1620** (e.g. network adaptor) for communicating with one or more other systems; and peripheral devices **1625** which may include cache, other memory, data storage, and/or electronic display adaptors. The memory **1610**, storage unit **1615**, interface **1620**, and peripheral devices **1625** are in communication with the processor

1605 through a communications bus (solid lines), such as a motherboard. The storage unit **1615** can be a data storage unit for storing data. The server **1601** is operatively coupled to a computer network ("network") **1630** with the aid of the communications interface **1620**. The network **1630** can be the Internet, an intranet and/or an extranet, an intranet and/or extranet that is in communication with the Internet, a telecommunication or data network. The network **1630** in some cases, with the aid of the server **1601**, can implement a peer-to-peer network, which may enable devices coupled to the server **1601** to behave as a client or a server. A microscope can be a peripheral device **1625** or a remote computer system **1640**.

The storage unit **1615** can store files, such as individual images, time lapse images, data about individual cells, data about individual biomarkers, images showing a pattern of binding of capture agents to a sample, or any aspect of data associated with the invention. The data storage unit **1615** may be coupled with data relating to locations of cells in a virtual grid.

The server can communicate with one or more remote computer systems through the network **1630**. The one or more remote computer systems may be, for example, personal computers, laptops, tablets, telephones, Smart phones, or personal digital assistants.

In some situations the system **1600** includes a single server **1601**. In other situations, the system includes multiple servers in communication with one another through an intranet, extranet and/or the Internet.

Methods as described herein can be implemented by way of machine (e.g., computer processor) computer readable medium (or software) stored on an electronic storage location of the server **1601**, such as, for example, on the memory **1610**, or electronic storage unit **1615**. During use, the code can be executed by the processor **1605**. In some cases, the code can be retrieved from the storage unit **1615** and stored on the memory **1610** for ready access by the processor **1605**. In some situations, the electronic storage unit **1615** can be precluded, and machine-executable instructions are stored on memory **1610**. Alternatively, the code can be executed on a second computer system **1640**.

Aspects of the systems and methods provided herein, such as the server **1601**, can be embodied in programming. Various aspects of the technology may be thought of as "products" or "articles of manufacture" typically in the form of machine (or processor) executable code and/or associated data that is carried on or embodied in a type of machine readable medium (e.g., computer readable medium). Machine-executable code can be stored on an electronic storage unit, such memory (e.g., read-only memory, random-access memory, flash memory) or a hard disk. "Storage" type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical, and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over various air-links. The physical elements that carry such waves, such as wired or wireless likes, optical links, or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" refer to any medium that participates in providing instructions to a processor for execution.

Hence, a machine readable medium, such as computer-executable code, may take many forms, including but not limited to, tangible storage medium, a carrier wave medium, or physical transmission medium. Non-volatile storage media can include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such may be used to implement the system. Tangible transmission media can include: coaxial cables, copper wires, and fiber optics (including the wires that comprise a bus within a computer system). Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include, for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD, DVD-ROM, any other optical medium, punch cards, paper tame, any other physical storage medium with patterns of holes, a RAM, a ROM, a PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables, or links transporting such carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

The results of the sample staining or labeling can be presented to a user with the aid of a user interface, such as a graphical user interface.

Kits

In some aspects, the disclosure herein provides for kits. The kits can comprise any number of compositions to perform the methods of the disclosure, each of which have been described herein. For example, a kit may comprise at least one capture agent. The capture agent can be an antibody, an aptamer, or an oligonucleotide probe. The capture agent can be custom-made to specifically bind to a desired target. For example, a user may custom-order one or more capture agents to be included in the kit. In some cases, the capture agents can be sold separately. The capture agents can specifically bind to a target molecule of interest. Additionally or alternatively, capture agents can be ordered as a panel (i.e., a pre-determined selection of capture agents). The panel can be specific for a particular type of disease (e.g., cancer) or a particular sub-type of a disease (e.g., colon cancer). A kit of the disclosure can also include one or more oligonucleotides. The oligonucleotides can comprise a first strand and a double strand, as described herein. The oligonucleotides can be provided as single-stranded oligonucleotides or as double-stranded olgionucleotides. In the latter case, the kit can include reagents and/or instructions for annealing the first strand and the second strand of oligonucleotides to produce double-stranded oligonucleotides. The single- or double-stranded oligonucleotides can be conjugated to the capture agents or can be provided unconjugated. In the latter case, reagents can be included in the kit for conjugating the double-stranded oligonucleotides to the capture agents (e.g., reagents to perform Click chemistry). In some cases, the kit may provide a plurality of oligonucleotides wherein each of the first strands is the same and each of the second strands is different. The kit can further comprise any nucleotide mixture disclosed herein. Nucleotide mixtures can comprise any combination of fluorescent nucleotides, unlabeled nucleotides, reversible terminator nucleotides, and the like. Generally, the nucleotide mixture provided in the kit will be compatible with the provided oligonucleotides. The kit can further comprise, without limitation, a polymerase for performing primer extension, a reagent for inactivating a signal (e.g., TCEP), a blocking solution (e.g., iodoacetamide solution), and any buffer or solution suitable to perform the methods herein. The kit can comprise any reagent for preparing a sample for labeling such as a fixative (e.g., formaldehyde) or reagents for embedding a sample (i.e., paraffin wax). The kit can further comprise a control sample for comparison with a test sample. The control sample can be a healthy sample or a diseased sample. The control sample may be matched to the tissue or cell type under investigation or to the disease being studied. In some cases, the control sample may be a positive control or a negative control.

EMBODIMENTS

A method for analyzing a planar sample is provided. In certain embodiments, the method comprises: (a) incubating the planar sample with a capture agent under conditions by which the capture agent specifically binds to complementary sites in the planar sample, wherein: (i) the capture agent is linked to a double-stranded oligonucleotide that comprises a first strand and a second strand; (ii) the capture agent is linked to a double-stranded oligonucleotide by the 5' end of the first strand; and (iii) the 3' end of the first strand is recessed relative to the 5' end of the second strand, thereby producing an overhang; (b) crosslinking the capture agent to planar sample; (c) contacting the planar sample with a polymerase and a nucleotide mix, thereby adding one or more nucleotides to the overhang; and/orcontacting the planar sample with a mixture of short oligonucleotides, some of which may be labelled or not, and a DNA ligase and (d) reading a fluorescent signal generated by addition of the one or more nucleotides to the overhang using fluorescence microscopy, thereby producing an image showing the pattern of binding of the capture agent to the planar sample. In some embodiments, after the sample has been read, this method may involve removing the fluorescent moiety and deprotecting an added fluorescent nucleotide, thereby allowing the method to be repeated.

In any embodiment, step (c) may comprise contacting the planar sample with a polymerase and a nucleotide mix that comprises a fluorescent nucleotide, thereby adding the fluorescent nucleotide to the overhang, or contacting te planar sample with one or more fluorescently labeled oligonucleotides, thereby adding a fluorescently labeled oligonucleotide to the overhang; and step (d) comprises reading a fluorescent signal generated by addition of the fluorescent nucleotide or oligonucleotide to the overhang. In this embodiment, the fluorescent signal may be: emitted directly from the added nucleotide or oligonucleotide, a FRET signal generated by energy transfer between two fluorescent nucleotides that are added to the overhang or a FRET signal generated by energy transfer between a first fluorescent nucleotide added to overhang and a second fluorescent nucleotide that is present in the second strand.

In some embodiments, extension of the first strand removes a quencher from a quenched fluorescently labeled oligonucleotide that is hybridized to the second strand, downstream from the first strand.

In any embodiment, the sample may be a formalin-fixed, paraffin-embedded (FFPE) section.

Also provided herein is a capture agent that is linked to a double-stranded oligonucleotide, wherein: (i) the double-stranded oligonucleotide comprises a first strand and a second strand; (ii) the capture agent is linked to the 5’ end of the first strand; and (iii) the 3’ end of the first strand is recessed relative to the 5’ end of the second strand, thereby producing an overhang or (iiii) the 5’ end is recessed relative to the 3’end of the second strand, there producing and overhang

Also provided herein is a capture agent composition comprising a plurality of capture agents that recognize different complementary sites, wherein: each of the capture agents is linked to a double-stranded oligonucleotide that comprises a first strand and a second strand; the capture agents are linked to a double-stranded oligonucleotide by the 5’ end of first strand; the 3’ end of the first strand in each of the double-stranded oligonucleotides is recessed relative to the 5’ end of the second strand, thereby producing an overhang; and the overhang is different for each of the capture agents. Alternatively, the 5’ end is recessed relative to the 3’ end of the second strand, there producing and overhang that is specific to each capture agent. In this embodiment, the sequence of the first strand may be the same for each of the capture agents; and the sequence of the second strand may be different for each of the capture agents.

In these embodiments, the overhangs may be of the formula 3’- $N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more, or the formula 3’-YN_1/N_2-5’, optionally followed by short stretch of random nucleotides on the 5’ end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of bases N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C.

A method for analyzing a tissue sample is also provided. In these embodiments, the method may comprise (a) incubating a planar sample with a capture agent composition of a prior embodiment under conditions by which the capture agents specifically bind to sites in the planar sample; (b) crosslinking capture agents to planar sample; (c) contacting the planar sample with a polymerase and either an incomplete nucleotide mix or a nucleotide mix that comprises a reversible terminator nucleotide and/or a ligase and a labeled oligonucleotide; and (d) reading, using fluorescence microscopy, a fluorescent signal generated by addition a nucleotide to some but not all of the capture agents.

In this embodiment, the method may comprise: (c) contacting the planar sample with a polymerase and: (i) a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4 or (ii) a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, and N_2, an unlabeled nucleotide that is complementary to N_3, and no nucleotide that is complementary to N_4, thereby adding fluorescent nucleotides onto the double-stranded oligonucleotides of some but not all of the capture agents. This step can also be done by ligation, i.e., by contacting the planar sample with a labeled oligonucleotide (or a mixture of the same), where addition of the labeled oligonucleotide depends on the underlying sequence of the overhang. This method also comprises: (d) reading, using fluorescence microscopy, a fluorescent signal generated by addition of a fluorescent nucleotide to some but not all of the capture agents. In these embodiments, the overhangs may be of the formula 3'-$N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more; and step (c) comprises contacting the planar sample with a polymerase and a nucleotide mix that comprises fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4. This step can also be implemented by addition of a labeled oligonucleotide using a ligase. In these embodiments, the method may comprise (e) inactivating the fluorescent signal, deprotecting the reversible terminator nucleotide and blocking the sample; and (f) repeating steps (c) and (d). In some cases, step (f) may comprise repeating steps (c), (d) and (e) multiple times. Alternatively, the overhangs may be of the formula 3'-YN_1/N_2-5', optionally followed by short stretch of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of bases N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C. In these embodiments, the method may further comprise (e) inactivating the fluorescent signal and contacting the planar sample with a polymerase and an unlabeled nucleotide that is complementary to N_4; and (f) repeating steps (c) and (d). In some cases, step (f) may comprise repeating steps (c), (d) and (e) multiple times.

In some embodiments, the double-stranded oligonucleotides each comprise a fluorescently labeled oligonucleotide hybridized to the second strand downstream from first strand, wherein the fluorescently labeled oligonucleotide comprises a quencher and extension of the first strand removes the quencher from some but not all of the quenched fluorescently labeled oligonucleotides, thereby generating a fluorescent signal for some but not all of the capture agents.

EXAMPLE I

Materials and Methods

Spleen cells fixed in 2% formaldehyde, permeablized and stored in methanol at -80 were spun from methanol, resuspended and washed with buffer 4 (10mM Tris &.5, 10mM MgCl2, 150mM NaCl, 0.1% Triton x100) for 5min on a rotator. To block against non-specific binding of ab-oligonucleotide complexes cells were further spun, resuspended in 1ml PBS, 0.5% BSA (SM) and supplemented up to additional 0.5M NaCl (0.9ml SM + 100ul 5M NaCl). 20ul of sheared ssDNA (10mg/ml), 50ul of mouse IgG (10mg/ml) and 20ul of 0.5M EDTA were further added to 1ml of cells and the mix was incubated for 30min on a rotator. For staining cells were redistributed into 30 250ul tubes (PCR strip tubes is a convenient choice for that matter) with premade antibody/oligonucleotide complexes (0.2ug of CD45-146 complex was annealed with 1ul of specific oligonucleotide (147 etc) per tube 30min at 40C) and incubated for 1h with rotation. Cells were washed in (PBS, 0.1%Triton 0.5M salt 5mM EDTA) twice, placed on poly-lysine treated glass coverslips, allowed to stand/attach for 10min and further fixed with 5mM BS3 (7.4mg per 4ml) in PBS, 0.1%Triton, 0.5M NaCl, 5mM EDTA for 1hour.

Staining was rendered in cycles. For odd cycles (1,3,5,7,9,11,13,15) coverslips were incubated for 2min in dG/dU mix (150nM dG, 150nM dUssCy5, 150nM dCssCy3, 25ul NEB exo- Klenow per ml in buffer#4 (10mM Tris 7.5, 0.5M NaCl, 0.1% Triton x100, 10mM MgCl2)), washed twice with 405 (buffet#4 supplemented up to 0.65M NaCl); and imaged by confocal microscopy. Following imaging the fluorophores were cleaved off cells by incubation in 50mM TCEP for 2min in buffer 405E (10mM Tris 7.5, 0.5M NaCl, 0.1% Triton x100, 5mM EDTA). After cleavage cells were washed in 405E and blocked for for 1min in iodoacetamide solution (FRESHLY made 100mM iodoacetamide in buffer 405E). The blocking solution was removed by two washes with buffer#4. Before proceeding to next cycle cells were again imaged by confocal microscopy. Even cycles (2,4,6,8,10,12, 14) were performed same as odd cycles except for substitution of dG with dA in labeling step and extension of cleavage to 4min at room temperature.

Preliminary data.

To explore the possibility of *in situ* staining by primer extension expression of CD4 was visualized in mouse spleen cells in suspension (Figure 11) or immobilized on a slide. (Fig. 12). To visualize the T lymphocytes spleen cells were co-stained with conventional TcrB-Ax488 antibody. Both samples were stained with CD4 antibody conjugated to oligonucleotide duplex as in (Figure 11 A). No Klenow polymerase was added in control samples which results in no separation of TcrB positive T-cells into subsets (Figure 11 B).When Klenow polymerase was supplied. CD4 positive T-cells could be observed as a Cy5 positive subset of TcrB positive T-cells (Fig. 11 C and Fig. 12). Clear membrane staining pattern was observed by confocal imaging of cells stained on-slide (Figure 12 A). Taken together this data shows that on-slide primer extension reaction can be used for rendering the capture agent binding pattern

Fig. 11. **Flow cytometric analysis of mouse spleen cells stained by primer extension.** Mouse spleen cells were fixed and permeabilized with methanol as done for intracellular protein staining. Cells were co-stained with conventional TcrB-Ax488 antibody and CD4 antibody conjugated to oligonucleotide duplex as in (**A**). After staining cells were either incubated in extension buffer with dUTP-Cy5 without (**B**) or with (**C**) Klenow exo$^-$ polymerase. Note that TcrB positive T-cells in (**B**) are indicated by Ax-488 staining. Dependent upon the addition of Klenow, TcrB positive CD4 positive T-cells are seen as a Cy5 positive subset of TcrB positive T-cells in (**C**).

Fig. 12. **On-slide analysis of mouse spleen cells stained by primer extension.** Mouse spleen cells were fixed and permeabilized with methanol as done for intracellular protein staining. Cells were attached to poly-Lysine coated slide and co-stained with conventional TcrB-Ax488 antibody and CD4 antibody conjugated to oligonucleotide duplex as in Fig.12 A. After staining, cells were incubated in extension buffer with dUTP-Cy5 Klenow exo$^-$ polymerase and visualized by confocal microscopy. Shown are DIC image in **C**, Cy5 channel in **A**, Ax488 channel in **B** and merged Ax488 and Cy5 channels in **D**. Note that only a subset of TcrB-Ax488 positive T-cells in (**B**) are rendered Cy5 positive CD4 positive T-cells by primer extension as seen in (**A**). The membrane pattern of CD4 points to specificity of staining by primer extension as it takes place at a particular expected subcellular localization.

To prove the possibility of multiplexed detection of several antigens by primer extension, the expression of CD4 and CD8 was co-analyzed in mouse spleen cells immobilized on a slide by Method 1 and, specifically, the multiplexing approach based on “reversible terminators”. The cells were simultaneously stained by CD4 and CD8 antibodies conjugated to oligonucleotide duplexes as in (Fig. 14, A) simultaneously. Two cycles of rendering were performed such that CD8 was visualized in the first cycle (Fig. 14, C) and CD4 in the second (Fig. 14, D). Cells were counterstained with TcrB-Ax488 to delineate T-lynphocytes in the spleen cells. As expected CD4 positive cells were rendered as a subset of TcrB positive T-cells mutually exclusive with CD8-positive subset of T-lymphocytes (Fig.

14, A-D). The data suggests that rendering antibody staining by polymer (DNA-duplex) extension is an approach enabling sensitive antigen detection and multiplexing.

Fig. 13. **Two cycle analysis of CD4 and CD8 staining in mouse spleen using Method 1 with reversible terminators.** Mouse spleen cells were fixed and permeabilized with methanol as done for intracellular protein staining. Cells were attached to poly-Lysine coated slide and co-stained with conventional TcrB-Ax488 antibody and a mixtue of CD4 and CD8 antibodies conjugated to oligos as indicated on (**A**). For the first cycle of staining the cells were incubated in extension buffer with Illumina reversible terminators and Klenow exo$^-$ polymerase and visualized by confocal microscopy (**C**). Following the imaging after the first cycle, cells were destained by Illumina cleavage buffer containing TCEP. Following destaining-terminator reactivation, cells were again incubated in extension buffer with Illumina reversible terminators and Klenow exo$^-$ polymerase and visualized by confocal microscopy (**D**) Note that four T-cells identified by high levels of TcrB and marked by four white arrows on (**B**). It becomes evident after the first cycle of staining that two of these cells are CD8a positive (marked by purple arrows on (**C**). Second cycle of staining reveals that the other two cells are CD4 positive (marked by green arrows on (**D**). The expected mutual exclusivity of CD4 and CD8a as well as membrane pattern of incorporated labeled nucleotide further supports the specificity of staining by cycles of primer extension.

The “missing base” multiplexing approach was tested on a model of heterogeneous tissue containing multiplicity of distinct cellular subsets (Fig.14). To this end leukocytes from homogenized mouse spleen were divided into 30 samples. 30 different versions of CD45 were made by conjugating purified CD45 to common upper strand oligonucleotide and then separately annealing 30 different lower strand oligonucleotides designed to create overhangs that can be sequentially rendered (two per cycle) in the multiplexed version of “missing base approach”. The samples were individually stained (barcoded) by 30 CD45 antibody conjugates, the unbound CD45 was washed off the barcoded samples were mixed and attached to a slide. The staining of this mixture of pseudotyped cells was rendered by “missing base” approach. Six first cycles (12 populations, 2 red and green per cycle) as well as inactivation of fluorescence by cleaving the fluorophore off the modified base by TCEP between the cycles is shown on Fig. 15. As can be seen no same two cells are stained in each cycle and between the cycles proving that on-cell primer extension reliably renders the specific antibody staining.

In order to test the performance and multiplexing capacity of “missing base” method the following model approach was employed, as shown in Figs. 14 and 15. Mouse CD45 antibody was chemically conjugated to an "upper strand" oligonucleotide (oligonucleotide id-146). The conjugated antibody was further divided and separately annealed (by 30min co-incubation at 40C) to 30 different "lower strand" oligonucleotides – thus effectively creating 30 different species of CD45 antibody. The 30 "lower strand" oligonucleotides were designed in accordance with "missing base" strategy and in addition in such a way that 2 antibodies could be rendered per cycle using two bases (dUTP and dCTP) reversibly (through s-s linker) coupled with distinct fluorophores (Cy5 and Cy3). 30 samples of homogenized mouse spleen have been "barcoded" with these CD45-oligonucleotide duplex complexes such way that majority of cells in each sample became labeled with a particular CD45-upper/lower oligonucleotide combination. Following staining and washing the samples were combined to mimic a tissue with 30 different cellular subsets. The mixture was smeared on a slide and rendered by cycling staining with a "missing base" approach such that two subsets per staining cycle were co-visualized on different imaging channels.

EXAMPLE 2

Antibody signal amplification in situ with Rolling Circle Amplification

Materials and methods

Rat anti-mouse B220 antibody conjugate to oligonucleotide 146v2 was prepared as described. The conjugate were hybridized to a padlock oligonucleotide (PatgctaccgttAATTATTACTGAAACATACACTAAAGATAACATTA ttctgcaag; SEQ ID NO:125) that is designed to form a circular hybrid on 146v2. Mouse spleen cells were stained with either of the conjugates and then those cells that were stained with the padlock construct were incubated with T4 RNA ligase (NEB) in manufacturer's ligation buffer at 37C for 1 hour and then with phi29 polymerase and dNTP mix for 15 minutes. Cells were washed 3 times with PBS and then incubated with 10 nM RCA product detection oligonucleotide (TGAAACATACACTAAAGA; SEQ ID NO:126) for 10 minutes. After that, cells were incubated with fluorescent dUTP-Cy5 (Jena Biosciences) was incorporated into the cells by incubating with 200nM dUTP-Cy5 in buffer#4 and 1ul of exo- Klenow polymerase (Thermo Scientific). An aliquot of cells was left out and not subject to the rolling circle amplification (RCA) step and then used as a reference to assess the effect of RCA on staining.

Results

The efficiency of rolling circle amplification of multiplexing DNA barcode attached to the antibody for enhancing antibody staining was tested. A special antibody-DNA conjugate based on anti-B220 antibody that contained a circularized 'padlock' oligonucleotide annealed to the linker (146v2) oligonucleotide hybrid was assembled and mouse cells were stained with it. After staining, the padlock oligonucleotide was ligated with T4 ligase and amplified using the rolling circle protocol with phi29 polymerase, resulting in a long repetitive DNA stretch attached to each antibody that contained the repetitive sequence complementary to the detection primer. After annealing of the primer, multiple molecules of dUTP-Cy5 could be incorporated into the amplified DNA molecule, due to its repetitive nature. Fig. 16 panels A-E schematically illustrate this method. Fig. 16 panels F-G shows that the cells staining with the rolling circle amplification is much stronger than without it.

EXAMPLE 3

Co-detecting 22 antigens on dispersed spleen cells

Materials and Methods

Antibody conjugates were prepared using the following protocol. Antibodies were subject to partial reduction of disulfide by 30min incubation at room temperature with TCEP (final concentration 1mM) in PBS pH 7.4. The antibodies were purified from TCEP by buffer exchange on BioGel P-30 spin-columns saturated with conjugation buffer (PBS pH 7.0). Oligonucleotide 146v2 (5'Maleimide- ATAGCAGTCCAGCCGAACGGTA GCATCTTGCAGAA; SEQ ID NO:127) bearing a protected maleimide group were ordered from Trilink Inc. To prepare for covalent crosslinking to antibodies per instruction from manufacturer the maleimide group residing on an oligonucleotide was de-protected/activated by Adler reaction (4h at 90C in toluene). Toluene was removed from the oligonucleotide by several washes in absolute ethanol. Activated oligonucleotides were dissolved in conjugation buffer and mixed with reduced antibodies at a molar ratio of 50:1. Sodium Chloride was added to conjugation reaction to final concentration of 1M. Conjugation reaction was allowed to proceed for 1h. To remove the unbound oligonucleotide the conjugated antibodies were filtered 4 times on molecular weight cutoff filters (Amicon 50KDa). Final wash and storage were performed in phosphate buffer with 0.5M sodium chloride and 0.1% Tween-20.

To assemble DNA duplex tag 0.2ug of conjugated antibodies was mixed with 100pmoles of bottom strand oligonucleotide in phosphate buffer with 0.6M Sodium Chloride and incubated for 30min at 40°C.

v2_C_cycle1 SEQ ID NO: 128: TTTTGTTCTGCAAGATGCTACCGTTCGGz
v2_C_cycle2 SEQ ID NO: 129: TTTTGtTTCTGCAAGATGCTACCGTTCGGz
v2_C_cycle3 SEQ ID NO: 130: TTTTGCtTTCTGCAAGATGCTACCGTTCGGz

v2_C_cycle4	SEQ ID NO: 131: TTTTGTCtTTCTGCAAGATGCTACCGTTCGGz
v2_C_cycle5	SEQ ID NO: 132: TTTTGCcTCtTTCTGCAAGATGCTACCGTTCGGz
v2_C_cycle6	SEQ ID NO: 133: TTTTGtttCcTCtTTCTGCAAGATGCTACCGTTCGGz
v2_C_cycle7	SEQ ID NO: 134: TTTTGcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz
v2_C_cycle8	TTTTGttcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz (SEQ ID NO: 135)
v2_C_cycle9	TTTTGcttcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz (SEQ ID NO: 136)
v2_C_cycle10	TTTTGtcttcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz (SEQ ID NO: 137)
v2_C_cycle11	TTTTGcctcttcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz (SEQ ID NO: 138)
v2_U_cycle1	SEQ ID NO: 139: TTTTATTCTGCAAGATGCTACCGTTCGGz
v2_U_cycle2	SEQ ID NO: 140: TTTTAtTTCTGCAAGATGCTACCGTTCGGz
v2_U_cycle3	SEQ ID NO: 141: TTTTACtTTCTGCAAGATGCTACCGTTCGGz
v2_U_cycle4	SEQ ID NO: 142: TTTTATCtTTCTGCAAGATGCTACCGTTCGGz
v2_U_cycle5	SEQ ID NO: 143: TTTTACcTCtTTCTGCAAGATGCTACCGTTCGGz
v2_U_cycle6	SEQ ID NO: 144: TTTTAtttCcTCtTTCTGCAAGATGCTACCGTTCGGz
v2_U_cycle7	SEQ ID NO: 145: TTTTAcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz
v2_U_cycle8	TTTTAttcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz (SEQ ID NO: 146)
v2_U_cycle9	TTTTActtcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz (SEQ ID NO: 147)
v2_U_cycle10	TTTTAtcttcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz (SEQ ID NO: 148)
v2_U_cycle11	TTTTAcctcttcctttCcTCtTTCTGCAAGATGCTACCGTTCGGz (SEQ ID NO: 149)

Mouse spleen and bone marrow cells were prepared according to standard procedure and fixed in 2% formaldehyde for 10min at room temperature. Following fixation, cells were spun and either stored frozen at -80 in PBS with 5% DMSO or permeabilized by incubation in ice-cold methanol for 10 min. and further stored at -80 °C in methanol.

Before staining stored cells were washed with SM (0.5%BSA in PBS, 5mMEDTA) once and blocked for 30min at room temperature in staining buffer (0.6M NaCl, 0.5%BSA, 50ug/ml rat IgG, 200ug/ml ssDNA, 5mMEDTA, 3nmoles per ml of blocking oligonucleotide TTTTccctctcctcttcctttCcTCt-ddC in phosphate buffer pH 7.4). In case frozen sections were used –tissue sections were picked by warm coverslips and immediately placed into dry ice without allowing the section to dry. Coverslips with sections were dipped for 30 sec into ethanol pre-chilled to dry-ice temperature, and transferred to SME with 4% formaldehyde for 20min. After that the fixed sections were washed twice in SM and further blocked in staining buffer for 30min. Mouse spleen cells were stained in staining buffer for 2-3h at room temperature with a mixture of conjugated antibodies taken at 0.2ug of each antibody per 100ul of solution. After staining cells were washed twice with SM05 (SM supplemented with NaCl up to 0.65M final concentration), then were allowed to adhere to poly-L-lysin coated coverslips and further fixed to coverslip surface by 20min incubation with 5mM BS3 crosslinker in PBS. Following methanol fixation/permeabilization cells were washed once with SM05, allowed to adhere to coverslip surface and further fixed to coverslip surface by 20min incubation with 5mM BS3 crosslinker in PBS. If frozen sections were used – following staining sections were washed twice by SM05 and fixed by 20min incubation with 5mM BS3 crosslinker in PBS. Following staining procedure and converting into planar form (in case of suspension cells) all kinds of samples were subjected to similar ABseq rendering protocol.

Coverslips with cells were washed twice with buffer 4 (10mM Tris pH 6.5, 10mM MgCl2, 150mM NaCl, 0.1% Triton x100).

Staining was rendered by iterative incubation with polymerase reaction mixes. In odd cycles (1,3,5…) - cells were incubated for 2min in G-mix (150nM dG, 150nM dUssCy5, 150nM dCssCy3, 25ul NEB exo- Klenow per ml in buffer 4); wash 3 times with 405 (buffet 4 without MgCl and supplemented with NaCl up to final 0.65M); photographed; incubated 2min in 50mM TCEP in buffer 405; washed twice with 405; photographed; incubated for 1min in freshly made 100mM iodoacetamide in buffer 405; washed three times with buffer 4. In even cycles (2,4,6…) - cells were incubated for 2min in A- mix (150nM dATP, 150nM dUTPssCy5, 150nM dCTPssCy3, 25ul NEB exo- Klenow per ml in buffer 4); wash 3times with 405 (buffet 4 without MgCl and supplemented with NaCl up to final 0.65M); photographed; incubated 4min in 50mM TCEP in buffer 405; washed twice with 405; photographed; incubated for 1min in freshly made 100mM iodoacetamide in buffer 405; washed three times with buffer #4. Reversibly labelled fluorescent nucleotide triphosphates were custom synthesized by Jena Bioscience.

Results

ABseq was used to explore the variety of cellular subsets in mouse spleen and bone marrow using 22-antibody panel. Isolated spleen and bone marrow cells were barcoded by whole cell staining with NHS-PacBlu and NHS-Ax-488 dyes, mixed, stained with a panel of 22 antibodies tagged with DNA duplexes, attached to slide and rendered by ABseq in 11 primer extension and imaging iterations (Fig. 17). Conspicuously 22-color marker expression data on pseudocolored image bearing all marker expressing data proved to be impossible to parse visually due to proximity of colors in multi-color palette (Fig. 17, bottom right panel).

EXAMPLE 4

Multipanel design with spacers

Materials and Methods

Antibody conjugation, cell staining and rendering was performed following the same experimental procedures as in section 4 (Co-detecting 22 antigens on dispersed spleen cells). Nine aliquots of spleen cells were stained separately with a different CD45 antibody-DNA conjugate. Conjugates for each panel were formed in the following way.

Panel1: CD45 conjugated to 146v2 (5'Maleimide-ATAGCAGTCCAGCCGAACGGTAGCATCTTGCAGAA (SEQ ID NO:174) and forming a DNA duplex with:

1. TTTTATTCTGCAAGATGCTACCGTTCGG-dideoxyC (SEQ ID NO:150)
2. TTTTAtTTCTGCAAGATGCTACCGTTCGGz-dideoxyC (SEQ ID NO:151)
3. TTTTACtTTCTGCAAGATGCTACCGTTCGGz-dideoxyC (SEQ ID NO:152)

Panel2 CD45 conjugated to 146v2-ddC(5'Maleimide-ATAGCAGTC CAGCCGAACGGTAGCATCTTGCAGAA-dideoxyC) (SEQ ID NO:153) and forming a DNA duplex with:

4. TTTTAGCGATTAAGCGTGAACTTCTGCAAGATGCTACCGTTCGG-dideoxyC (SEQ ID NO:154)
5. TTTTAtGCGATTAAGCGTGAACTTCTGCAAGATGCTACCGTTCGGz-dideoxyC (SEQ ID NO:155)
6. TTTTACtGCGATTAAGCGTGAACTTCTGCAAGATGCTACCGTTCGGz-dideoxyC (SEQ ID NO:156)

Panel3 CD45 conjugated to 146v2-ddC(5'Maleimide-ATAGCAGTCCAGCCGA ACGGTAGCATCTTGCAGAA-dideoxyC) (SEQ ID NO:157) and forming a DNA duplex with:

7. TTTTACGCTAATTCGCACTTGTTCTGCAAGATGCTACCGTTCGG-dideoxyC (SEQ ID NO:158)
8. TTTTAtCGCTAATTCGCACTTGTTCTGCAAGATGCTACCGTTCGGz-dideoxyC (SEQ ID NO:159)
9. TTTTACtCGCTAATTCGCACTTGTTCTGCAAGATGCTACCGTTCGGz-dideoxyC (SEQ ID NO:160)

After staining the cells were washed with washed twice with buffer 4 (10mM Tris pH 6.5, 10mM MgCl2, 150mM NaCl, 0.1% Triton x100) to remove unbound antibody-DNA conjugates and then the aliquots of cells were mixed together and attached to a lysine-coated coverslip. Antigen staining was rendered in the following sequence of incubations: dGTP+dUTP-Cy5 -> dATP+dUTP-Cy5 -> dGTP+dUTP-Cy5 -> Incubation with 1uM spacer1 (GTTCACGCTTAATCGC; SEQ ID NO:161) in buffer#4 for 20 minutes -> dGTP+dUTP-Cy5 -> dATP+dUTP-Cy5 -> dGTP+dUTP-Cy5 -> Incubation with 1uM spacer2 (CGCTAATTCGCACTTG; SEQ ID NO:162) in buffer#4 for 20 minutes -> dGTP+dUTP-Cy5 -> dATP+dUTP-Cy5 -> dGTP+dUTP-Cy5. Imaging, fluorophore inactivation with 50mM TCEP pH 7.0 and background blocking with iodoacetamide were performed after each step of rendering.

Results

Due to polymerase misincorporation errors the signal intensity of rendering by ABseq is expected to fall with increasing cycle numbers as observed in other studies on development of deep sequencing protocols utilizing sequential addition of individual nucleotides. To circumvent that and to avoid the use of extensively long DNA fragments linked to antibody the following amendment to the design was tested (Fig. 18, panel A). Large antibody panels can be split into subpanels such that the extension reaction on these subpanels is precluded by termination of the upper strand oligonucleotide with ddC, propyl or any other 3’ terminating group. After finishing the extension of each subpanel, the next subpanel is activated by *in situ* hybridization of a short “activation” spacer, which does not bear any terminating moiety on its 3’ end and thus initiates the consecutive cycles of primer extensions. This design was tested experimentally on 3 sequential 3-cycle panels (9 extension cycles in total) (Fig. 18, panel B). Image quantification showed no significant reduction of ABseq rendering efficiency associated with on-slide hybridization of panel activating spacer oligonucleotide was observed and no signal carryover between the individual panels (Fig. 18, panel C).

EXAMPLE 5

Multiplexed single molecule RNA detection

Materials and Methods

NALM and Jurkat cell lines were grown to a density of 1 million/ml, fixed with 1.6% formaldehyde for 10 minutes and then transferred to ice-cold methanol. An aliquot of 200K cells was washed with PBSTR (PBS, 0.1% Tween-20 and 1:1000 Rnasin) and transferred to a hybridization buffer (1x SSC, 10% formamide, 10% vanadyl-ribonucleotide complex, 10% polyvinylsulfonic acid). DNA probe mixture was added to the final concentration of 100 nM and incubated at 40C for 1 hour. Cells were washed 2 times with PBSTR at room temperature for 5 minutes and 2 times with a high salt buffer (4x SSC in PBSTR) at 40 degrees for 20 minutes, once again washed with PBSTR and transferred to a ligation solution (0.1ul T4 DNA ligase (New England Biosciences), 5 ul 10x T4 ligase buffer (New England Biosciences), 45ul H2O). Ligation proceeded for 1h at 37C. Then cells were transferred to amplification solution (1 ul of phi29 polymerase (Thermo Scientific), 5 ul of 10x polymerase buffer (Thermo Scientific), 1ul of 10mM dNTP mix, 43 ul of H2O) and incubated at 30C for 1h. Cells were washed with PBSTR and incubated with 1mM “RCA detection” oligonucleotide for 10 minutes at 37C and transferred to Sequencing buffer (10mM Tris pH 7.5, 10mM MgCl2, 150mM NaCl, 0.1% Triton x100, 1:50 Klenow polymerase (Thermo Scientific), 200mM dUTP-Cy5 (Jena Biosciences)). Cells were washed twice with high salt wash buffer (10mM Tris pH 7.5, 10mM MgCl2, 650mM NaCl, 0.1% Triton x100) and imaged using a florescent microscope.

HLA-DR padlock1	PACATTAaaatcctagcacagggactcAATTATTACTGAAACATACACTAAAGATApa (SEQ ID NO:163)
HLA-DR splint-primer1	ctcatcagcacagctatgatgaTAATGTTATCTT (SEQ ID NO:164)
HLA-DR padlock2	PACATTAtagaactcggcctggatgatAATTATTACTGAAACATACACTAAAGATA (SEQ ID NO:165)
HLA-DR splint-primer2	ctgattggtcaggattcagaTAATGTTATCTT (SEQ ID NO:166)
HLA-DR padlock3	PACATTAtcaaagctggcaaatcgtccAATTATTACTGAAACATACACTAAAGATA (SEQ ID NO:167)
HLA-DR splint-primer2	tggccaatgcaccttgagccTAATGTTATCTT (SEQ ID NO:168)
HLA-DR padlock4	PACATTAtgatttccaggttggctttgAATTATTACTGAAACATACACTAAAGATA (SEQ ID NO:169)
HLA-DR splint-primer2	atagttggagcgctttgtcaTAATGTTATCTT (SEQ ID NO:170)
HLA-DR padlock5	PACATTAtttcgaagccacgtgacattAATTATTACTGAAACATACACTAAAGATA (SEQ ID NO:171)
HLA-DR splint-primer2	ctgtggtgacaggttttccaTAATGTTATCTT (SEQ ID NO:172)
RCA detect	CATACACTAAAGATAACAT (SEQ ID NO:173)

Results

An on-slide primer extension protocol was applied to detect single molecules of human HLADRA mRNA in NALM pro-B-cell line. Jurkat T-cell lymphoma line was used as a negative control to assess the background. In order to enable single molecule mRNA detection, a signal amplification system was designed based on proximity ligation and rolling circle amplification (RCA). Five pairs of probes were designed in a way that the two oligos of each pair were complementary to directly adjacent 20-nt stretches of HLADRA mRNA and that the 3' region of the upstream oligonucleotide served as a splint for circularization of the downstream padlock oligonucleotide (Fig. 19, A) and also as a primer for the rolling circle amplification. After the complex assembly the cells were washed and treated with T4 DNA ligase to circularize the padlock oligonucleotide and the incubated with phi29 polymerase and dNTP mix to carry out the rolling circle amplification (Fig. 19, B). Amplification products were incubated with "RCA detect" oligonucleotide (Fig. 19, C) and then fluorescent dUTP-Cy5 was incorporated by a single base extension with Klenow polymerase (Fig. 19, D). Cells were washed and imaged with a fluorescent microscope. Images of NALM cells that express HLADR show abundant punctate staining in the cytoplasm that corresponds to the RCA products (Fig. 19, E) and the Jurkat cells that are negative for HLADR show very few puncta (Fig. 19, F), demonstrating the high specificity of the proximity ligation-based detection of HLADRA mRNA.

What is claimed is:

1. A method for analyzing a planar sample, the method comprising:

(a) labeling the planar sample with a capture agent to produce a labeled sample, wherein:

(i) the capture agent is linked to a double-stranded nucleic acid that comprises a first strand and a second strand; and

(ii) a 3’ end or 5’ end of either the first strand or the second strand is extendible using the other strand as a template;

(b) contacting the labeled sample with i. a polymerase and a plurality of nucleotides and/or ii. a labeled oligonucleotide and a ligase, thereby adding one or more nucleotides of the plurality of nucleotides and/or a labeled oligonucleotide to an end of one of the strands of the double-stranded nucleic acid; and

(c) reading a signal generated by addition of the one or more nucleotides and/or labeled oligonucleotide to one of the first strand or the second strand of the double-stranded nucleic acid.

2. The method of claim 1, wherein the signal is a fluorescent signal.

3. The method of claim 2, wherein reading comprises flourescence microscopy.

4. The method of any prior claim, further comprising producing an image showing the pattern of binding of the capture agent to the planar sample.

5. The method of any prior claim, wherein:

step (b) comprises contacting the labeled sample with a polymerase and a plurality of nucleotides that comprises a fluorescent nucleotide, thereby adding the fluorescent nucleotide to one of the first strand or the second strand of the double-stranded nucleic acid; and

step (c) comprises reading a fluorescent signal generated by addition of the fluorescent nucleotide to one of the first strand or the second strand of the double-stranded nucleic acid.

6. The method of claim 5, wherein the fluorescent signal is: i. emitted directly from the

added nucleotide; ii. a FRET signal generated by energy transfer between two fluorescent nucleotides of the plurality of flourescent nucleotides that are added to one of the first strand or second strand of the double-stranded nucleic acid; or iii. a FRET signal generated by energy transfer between the added fluorescent nucleotide and a second fluorescent nucleotide that is present in one of the first strand or second strand double-stranded nucleic acid.

7. The method of any of claims 1-4, wherein:

step (b) comprises contacting the labeled sample with a ligase and a labeled oligonucleotide, thereby adding the labeled oligonucleotide to one of the first strand or second strand of the double-stranded nucleic acid; and

step (c) comprises reading a fluorescent signal generated by addition of the labeled oligonucleotide to one of the first strand or second strand of the double-stranded nucleic acid.

8. The method of claim 7, wherein the fluorescent signal is: i. emitted directly from the added labeled nucleotide; ii. a FRET signal generated by energy transfer between two labeled nucleotides that are added to one of the first strand or second strand of the double-stranded nucleic acid; or iii. a FRET signal generated by energy transfer between the labeled nucleotide added to one of the first strand and second strand of the double-stranded nucleic acid and a second labeled nucleotide that is present in the other strand.

9. The method of claim 8, wherein the labeled nucleotide comprises a fluorescent nucleotide.

10. The method of claim 1, wherein extension of one of the first strand or second strand of the double-stranded nucleic acid removes a quencher from a quenched fluorescently labeled oligonucleotide that is hybridized to the other strand, downstream from the first strand.

11. The method of any of claims 1-10, wherein the first strand of the double-stranded nucleic acid is a rolling circle amplification (RCA) product, and the second strand of the double-stranded nucleic acid comprises oligonucleotides that are hybridized to multiple sites in the RCA product.

12. The method of any of claims 1-10, wherein the first strand of the double-stranded nucleic acid is a first oligonucleotide, and the second strand of the double-stranded nucleic acid is a second oligonucleotide that is hybridized to the first oligonucleotide.

13. The method of any of claims 1-12, wherein the planar sample is a formalin-fixed, paraffin-embedded (FFPE) section.

14. The method of claim 1, wherein the capture agent is an antibody, an aptamer, or an oligonucleotide probe.

15. A capture agent that is linked to a double-stranded nucleic acid, wherein:

(i) the double-stranded nucleic acid comprises a first strand and a second strand;

(ii) the capture agent is linked to the first strand; and

(iii) the 5' end or the 3' end of either the first strand or the second strand is extendible using the other strand as a template.

16. A capture agent composition comprising a plurality of capture agents that each recognize different complementary sites, wherein:

each of the plurality of capture agents is linked to a double-stranded nucleic acid that comprises a first strand and a second strand;

the 5' end or 3' end of the first or second strand is extendible using the other strand as a template; and

the templates immediately downstream of the extendible ends are different for each of the plurality of capture agents.

17. The capture agent composition of claim 16, wherein:

the sequence of the first strand is the same for each of the plurality of capture agents; and

the sequence of the second strand is different for each of the plurality of capture agents.

18. The composition of claim 16, wherein the templates immediately adjacent to the extendible 3' ends are of the formula 3'- $N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more.

19. The composition of claim 16, wherein the templates immediately adjacent to the extendible 3' ends are of the formula 3'-YN_1/N_2-5', optionally followed by a short stretch of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C.

20. A method for analyzing a planar sample comprising:

(a) labeling the planar sample with a capture agent composition of any of claims 16-19;

(b) contacting the labeled sample with i. a polymerase and either an incomplete nucleotide mix or a nucleotide mix that comprises a reversible terminator nucleotide, thereby adding a nucleotide to the plurality of capture agents; and/or ii. a labeled oligonucleotide and a ligase, thereby adding a labeled oligonucleotide to the plurality of capture agents; and

(c) reading a signal generated by addition of the nucleotide or the labeled oligonucleotide to some but not all of the plurality of capture agents.

21. The method of claim 20, wherein the signal comprises a fluorescent signal.

22. The method of claim 21, wherein the reading comprises fluorescent microscopy.

23. The method of claim 20, comprising:

(b) contacting the planar sample with a polymerase and: (i) a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4; or (ii) a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, and N_2, an unlabeled nucleotide that is complementary to N_3, and no nucleotide that is complementary to N_4, thereby adding fluorescent nucleotides onto the double-stranded nucleic acids of some but not all of the plurality of capture agents; and

(c) reading, using fluorescence microscopy, a fluorescent signal generated by addition of the fluorescent nucleotides to the double-stranded nucleic acids of some but not all of the plurality of capture agents.

24. The method of claim 23, wherein the templates immediately adjacent to the extendible 3' end are of the formula 3'-$N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more; and step (b) comprises contacting the planar sample with a polymerase and a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4.

25. The method of claim 23, further comprising:

(d) inactivating the fluorescent signal,

(e) optionally, deprotecting the reversible terminator nucleotide;

(f) blocking the sample; and

(g) repeating steps (b) and (c).

26. The method of claim 25, wherein step (g) comprises repeating steps (b)-(f) multiple times.

27. The method of claim 23, wherein the templates immediately adjacent to the extendible 3' end are of the formula 3'-YN_1/N_2-5', optionally followed by a short stretch of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C.

28. The method of claim 27, further comprising:

(d) inactivating the fluorescent signal;

(e) contacting the planar sample with a polymerase and an unlabeled nucleotide that is complementary to N_4; and

(f) repeating steps (b) and (c).

29. The method of claim 28, wherein step (f) comprises repeating steps (b)-(e) multiple times.

30. The method of claim 20, wherein the double-stranded nucleic acids each comprise a fluorescently labeled oligonucleotide hybridized to the second strand downstream from the first strand, wherein the fluorescently labeled oligonucleotide comprises a quencher and

extension of the first strand removes the quencher from some but not all of the quenched fluorescently labeled oligonucleotides, thereby generating a fluorescent signal for some but not all of the plurality of capture agents.

31. The method of claim 20, wherein extension of the double-stranded nucleic acid comprises contacting the planar sample with a mixture of labeled and unlabeled oligonucleotides and a ligase.

32. The method of claim 20, wherein the plurality of capture agents are selected from the group consisting of: antibodies, aptamers, and oligonucleotide probes.

33. A kit comprising:

(a) one or more capture agents, wherein the one or more capture agents can specifically bind to complementary sites in a planar sample.

(b) one or more double-stranded nucleic acids comprising a first strand a second strand,

wherein each of the one or more capture agents is linked to the double-stranded nucleic acid, and wherein a 5’ end or 3’ end of either the first strand or the second strand is extendible using the other strand as a template.

34. The kit of claim 33, further comprising a polymerase.

35. The kit of claim 34, further comprising a nucleotide mix comprising at least one of a fluorescent nucleotide, an unlabeled nucleotide, and a reversible terminator nucleotide.

36. The kit of claim 33, wherein the one or more capture agents is selected from the group consisting of: an antibody, an aptamer and an oligonucleotide probe.

AMENDED CLAIMS

received by the International Bureau on 09 December 2015 (09.12.2015).

1. A method for analyzing a planar sample, the method comprising:

(a) labeling the planar sample with a capture agent to produce a labeled sample, wherein:

(i) the capture agent is linked to a double-stranded nucleic acid that comprises a first strand and a second strand; and

(ii) a 3' end or 5' end of either the first strand or the second strand is extendible using the other strand as a template;

(b) contacting the labeled sample with i. a polymerase and a plurality of nucleotides and/or ii. a labeled oligonucleotide and a ligase, thereby adding one or more nucleotides of the plurality of nucleotides and/or a labeled oligonucleotide to an end of one of the strands of the double-stranded nucleic acid; and

(c) reading a signal generated by addition of the one or more nucleotides and/or labeled oligonucleotide to one of the first strand or the second strand of the double-stranded nucleic acid.

2. The method of claim 1, wherein the signal is a fluorescent signal.

3. The method of claim 2, wherein reading comprises flourescence microscopy.

4. The method of any prior claim, further comprising producing an image showing the pattern of binding of the capture agent to the planar sample.

5. The method of any prior claim, wherein:

step (b) comprises contacting the labeled sample with a polymerase and a plurality of nucleotides that comprises a fluorescent nucleotide, thereby adding the fluorescent nucleotide to one of the first strand or the second strand of the double-stranded nucleic acid; and

step (c) comprises reading a fluorescent signal generated by addition of the fluorescent nucleotide to one of the first strand or the second strand of the double-stranded nucleic acid.

6. The method of claim 5, wherein the fluorescent signal is: i. emitted directly from the added nucleotide; ii. a FRET signal generated by energy transfer between two fluorescent nucleotides of the plurality of flourescent nucleotides that are added to one of the first strand or second strand of the double-stranded nucleic acid; or iii. a FRET signal generated by energy

transfer between the added fluorescent nucleotide and a second fluorescent nucleotide that is present in one of the first strand or second strand double-stranded nucleic acid.

7. The method of any of claims 1-4, wherein:

step (b) comprises contacting the labeled sample with a ligase and a labeled oligonucleotide, thereby adding the labeled oligonucleotide to one of the first strand or second strand of the double-stranded nucleic acid; and

step (c) comprises reading a fluorescent signal generated by addition of the labeled oligonucleotide to one of the first strand or second strand of the double-stranded nucleic acid.

8. The method of claim 7, wherein the fluorescent signal is: i. emitted directly from the added labeled nucleotide; ii. a FRET signal generated by energy transfer between two labeled nucleotides that are added to one of the first strand or second strand of the double-stranded nucleic acid; or iii. a FRET signal generated by energy transfer between the labeled nucleotide added to one of the first strand and second strand of the double-stranded nucleic acid and a second labeled nucleotide that is present in the other strand.

9. The method of claim 8, wherein the labeled nucleotide comprises a fluorescent nucleotide.

10. The method of claim 1, wherein extension of one of the first strand or second strand of the double-stranded nucleic acid removes a quencher from a quenched fluorescently labeled oligonucleotide that is hybridized to the other strand, downstream from the first strand.

11. The method of any of claims 1-10, wherein the first strand of the double-stranded nucleic acid is a rolling circle amplification (RCA) product, and the second strand of the double-stranded nucleic acid comprises oligonucleotides that are hybridized to multiple sites in the RCA product.

12. The method of any of claims 1-10, wherein the first strand of the double-stranded nucleic acid is a first oligonucleotide, and the second strand of the double-stranded nucleic acid is a second oligonucleotide that is hybridized to the first oligonucleotide.

13. The method of any of claims 1-12, wherein the planar sample is a formalin-fixed, paraffin-embedded (FFPE) section.

14. The method of claim 1, wherein the capture agent is an antibody, an aptamer, or an oligonucleotide probe.

15. A capture agent that is linked to a double-stranded nucleic acid, wherein:

(i) the double-stranded nucleic acid comprises a first strand and a second strand;

(ii) the capture agent is linked to the first strand; and

(iii) the 5' end or the 3' end of either the first strand or the second strand is extendible using the other strand as a template.

16. A capture agent composition comprising a plurality of capture agents that each recognize different complementary sites, wherein:

each of the plurality of capture agents is linked to a double-stranded nucleic acid that comprises a first strand and a second strand;

the 5' end or 3' end of the first or second strand is extendible using the other strand as a template; and

the templates immediately downstream of the extendible ends are different for each of the plurality of capture agents.

17. The capture agent composition of claim 16, wherein:

the sequence of the first strand is the same for each of the plurality of capture agents; and

the sequence of the second strand is different for each of the plurality of capture agents.

18. The composition of claim 16, wherein the templates immediately adjacent to the extendible 3' ends are of the formula 3'- $N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more.

19. The composition of claim 16, wherein the templates immediately adjacent to the extendible 3' ends are of the formula 3'-YN_1/N_2-5', optionally followed by a short stretch of

random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C.

20. A method for analyzing a planar sample comprising:
    (a) labeling the planar sample with a capture agent composition of any of claims 16-19;
    (b) contacting the labeled sample with i. a polymerase and either an incomplete nucleotide mix or a nucleotide mix that comprises a reversible terminator nucleotide, thereby adding a nucleotide to the plurality of capture agents; and/or ii. a labeled oligonucleotide and a ligase, thereby adding a labeled oligonucleotide to the plurality of capture agents; and
    (c) reading a signal generated by addition of the nucleotide or the labeled oligonucleotide to some but not all of the plurality of capture agents.

21. The method of claim 20, wherein the signal comprises a fluorescent signal.

22. The method of claim 21, wherein the reading comprises fluorescent microscopy.

23. The method of claim 20, comprising:
    (b) contacting the planar sample with a polymerase and: (i) a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4; or (ii) a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, and N_2, an unlabeled nucleotide that is complementary to N_3, and no nucleotide that is complementary to N_4, thereby adding fluorescent nucleotides onto the double-stranded nucleic acids of some but not all of the plurality of capture agents; and
    (c) reading, using fluorescence microscopy, a fluorescent signal generated by addition of the fluorescent nucleotides to the double-stranded nucleic acids of some but not all of the plurality of capture agents.

24. The method of claim 23, wherein the templates immediately adjacent to the extendible 3' end are of the formula 3'-$N_{4n}N_1/N_2/N_3$, wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C and n is 1 or more; and step (b) comprises contacting the planar sample with a polymerase and a nucleotide mix that comprises a plurality of fluorescent nucleotides that are complementary to N_1, N_2 and N_3 and a reversible terminator nucleotide that is complementary to N_4.

25. The method of claim 23, further comprising:

(d) inactivating the fluorescent signal,

(e) optionally, deprotecting the reversible terminator nucleotide;

(f) blocking the sample; and

(g) repeating steps (b) and (c).

26. The method of claim 25, wherein step (g) comprises repeating steps (b)-(f) multiple times.

27. The method of claim 23, wherein the templates immediately adjacent to the extendible 3' end are of the formula 3'-YN_1/N_2-5', optionally followed by a short stretch of random nucleotides on the 5' end to increase the overall polymerase residence on the DNA duplex, wherein Y is composed of alternating stretches of N_3 and N_4, and wherein N_1, N_2, N_3 and N_4 are different nucleotides selected from G, A, T and C.

28. The method of claim 27, further comprising:

(d) inactivating the fluorescent signal;

(e) contacting the planar sample with a polymerase and an unlabeled nucleotide that is complementary to N_4; and

(f) repeating steps (b) and (c).

29. The method of claim 28, wherein step (f) comprises repeating steps (b)-(e) multiple times.

30. The method of claim 20, wherein the double-stranded nucleic acids each comprise a fluorescently labeled oligonucleotide hybridized to the second strand downstream from the first strand, wherein the fluorescently labeled oligonucleotide comprises a quencher and extension of the first strand removes the quencher from some but not all of the quenched fluorescently labeled oligonucleotides, thereby generating a fluorescent signal for some but not all of the plurality of capture agents.

31. The method of claim 20, wherein extension of the double-stranded nucleic acid comprises contacting the planar sample with a mixture of labeled and unlabeled oligonucleotides and a ligase.

32. The method of claim 20, wherein the plurality of capture agents are selected from the group consisting of: antibodies, aptamers, and oligonucleotide probes.

33. A kit comprising:

(a) one or more capture agents, wherein the one or more capture agents can specifically bind to complementary sites in a planar sample.

(b) one or more double-stranded nucleic acids comprising a first strand a second strand,

wherein each of the one or more capture agents is linked to the double-stranded nucleic acid, and wherein a 5' end or 3' end of either the first strand or the second strand is extendible using the other strand as a template.

34. The kit of claim 33, further comprising a polymerase.

35. The kit of claim 34, further comprising a nucleotide mix comprising at least one of a fluorescent nucleotide, an unlabeled nucleotide, and a reversible terminator nucleotide.

36. The kit of claim 33, wherein the one or more capture agents is selected from the group consisting of: an antibody, an aptamer and an oligonucleotide probe.

37. The method of any of claims 1-12, wherein the planar sample is a blot of a gel.

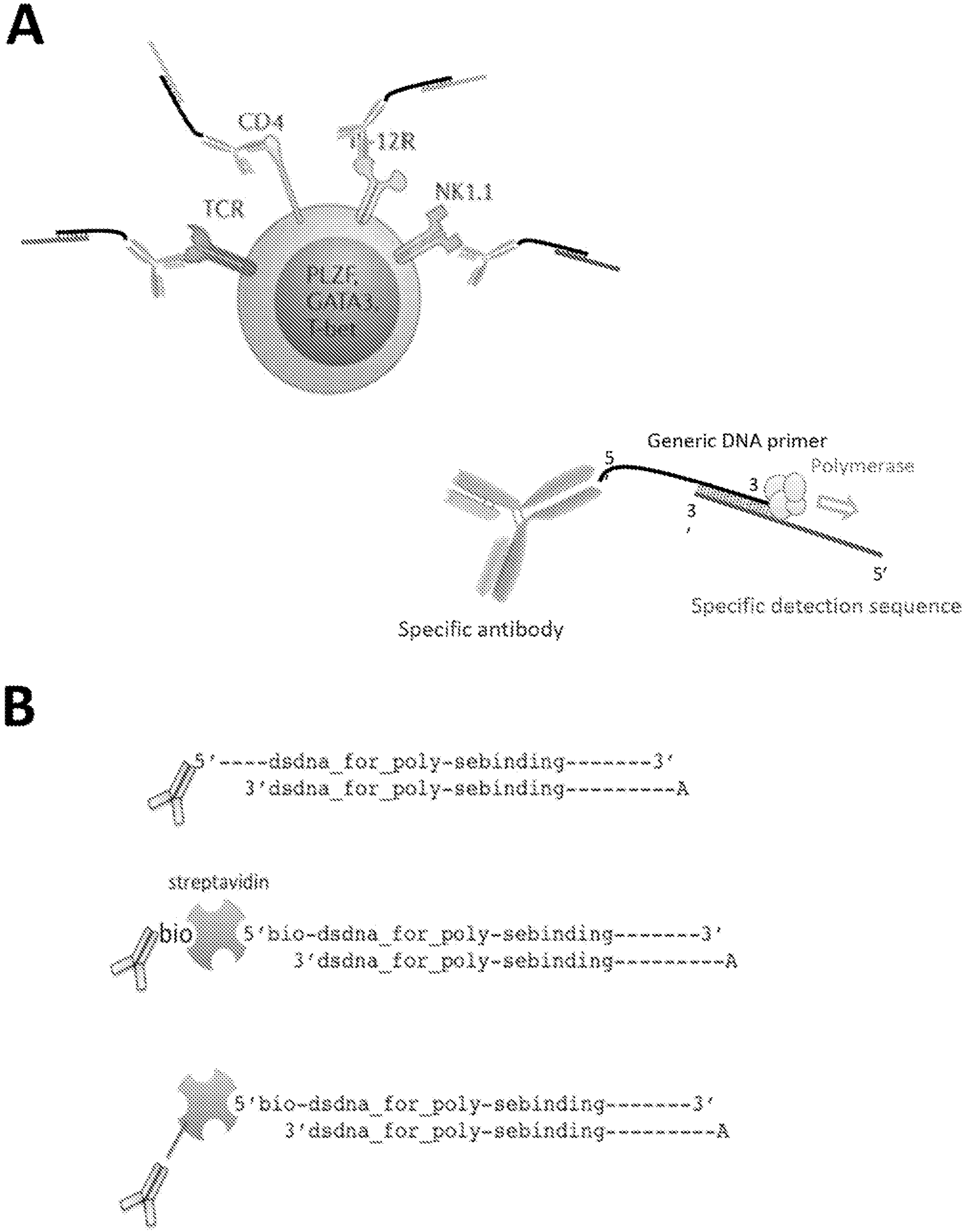

A
CD4
IL-12R
TCR
NK1.1
PLZF,
GATA3,
T-bet
Generic DNA primer
5
3
Polymerase
3
'
5'
Specific detection sequence
Specific antibody
B
5'----dsdna_for_poly-sebinding--------3'
3'dsdna_for_poly-sebinding----------A
streptavidin
bio
5'bio-dsdna_for_poly-sebinding--------3'
3'dsdna_for_poly-sebinding----------A
5'bio-dsdna_for_poly-sebinding--------3'
3'dsdna_for_poly-sebinding----------A

FIG. 1

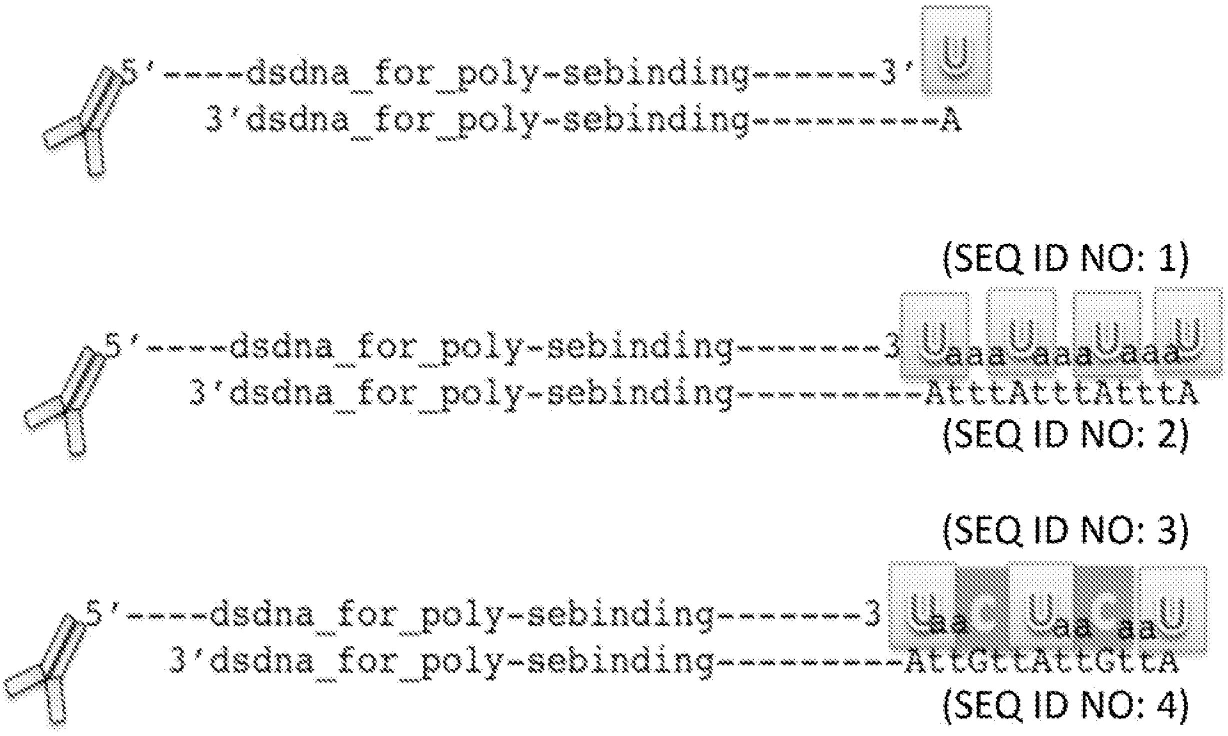

5'----dsdna_for_poly-sebinding------3'
U
3'dsdna_for_poly-sebinding---------A
(SEQ ID NO: 1)
5'----dsdna_for_poly-sebinding-------3
UaaaUaaaUaaaU
3'dsdna_for_poly-sebinding---------AtttAtttAtttA
(SEQ ID NO: 2)
(SEQ ID NO: 3)
5'----dsdna_for_poly-sebinding-------3
Uaa Uaa aaU
3'dsdna_for_poly-sebinding---------AttGttAttGttA
(SEQ ID NO: 4)

FIG. 2

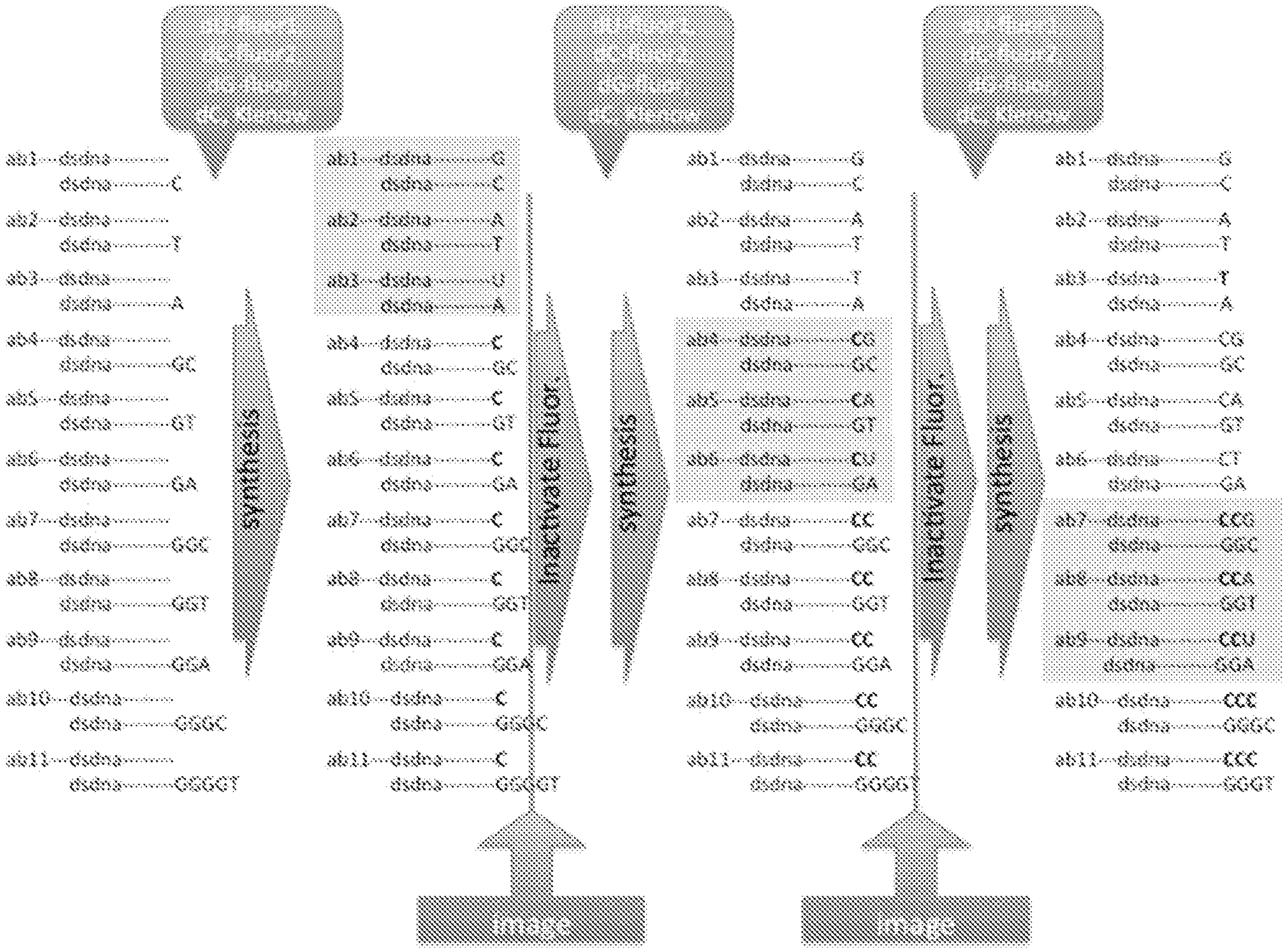

ab1---dsdna---------
dsdna---------C
ab2---dsdna---------
dsdna---------T
ab3---dsdna---------
dsdna---------A
ab4---dsdna---------
dsdna---------GC
ab5---dsdna---------
dsdna---------GT
ab6---dsdna---------
dsdna---------GA
ab7---dsdna---------
dsdna---------GGC
ab8---dsdna---------
dsdna---------GGT
ab9---dsdna---------
dsdna---------GGA
ab10---dsdna---------
dsdna---------GGGC
ab11---dsdna---------
dsdna---------GGGGT
synthesis
ab1---dsdna---------G
dsdna---------C
ab2---dsdna---------A
dsdna---------T
ab3---dsdna---------U
dsdna---------A
ab4---dsdna---------C
dsdna---------GC
ab5---dsdna---------C
dsdna---------GT
ab6---dsdna---------C
dsdna---------GA
ab7---dsdna---------C
dsdna---------GGC
ab8---dsdna---------C
dsdna---------GGT
ab9---dsdna---------C
dsdna---------GGA
ab10---dsdna---------C
dsdna---------GGGC
ab11---dsdna---------C
dsdna---------GGGGT
Inactivate Fluor.
Image
synthesis
ab1---dsdna---------G
dsdna---------C
ab2---dsdna---------A
dsdna---------T
ab3---dsdna---------T
dsdna---------A
ab4---dsdna---------CG
dsdna---------GC
ab5---dsdna---------CA
dsdna---------GT
ab6---dsdna---------CU
dsdna---------GA
ab7---dsdna---------CC
dsdna---------GGC
ab8---dsdna---------CC
dsdna---------GGT
ab9---dsdna---------CC
dsdna---------GGA
ab10---dsdna---------CC
dsdna---------GGGC
ab11---dsdna---------CC
dsdna---------GGGGT
Inactivate Fluor
Image
synthesis
ab1---dsdna---------G
dsdna---------C
ab2---dsdna---------A
dsdna---------T
ab3---dsdna---------T
dsdna---------A
ab4---dsdna---------CG
dsdna---------GC
ab5---dsdna---------CA
dsdna---------GT
ab6---dsdna---------CT
dsdna---------GA
ab7---dsdna---------CCG
dsdna---------GGC
ab8---dsdna---------CCA
dsdna---------GGT
ab9---dsdna---------CCU
dsdna---------GGA
ab10---dsdna---------CCC
dsdna---------GGGC
ab11---dsdna---------CCC
dsdna---------GGGT

FIG. 3

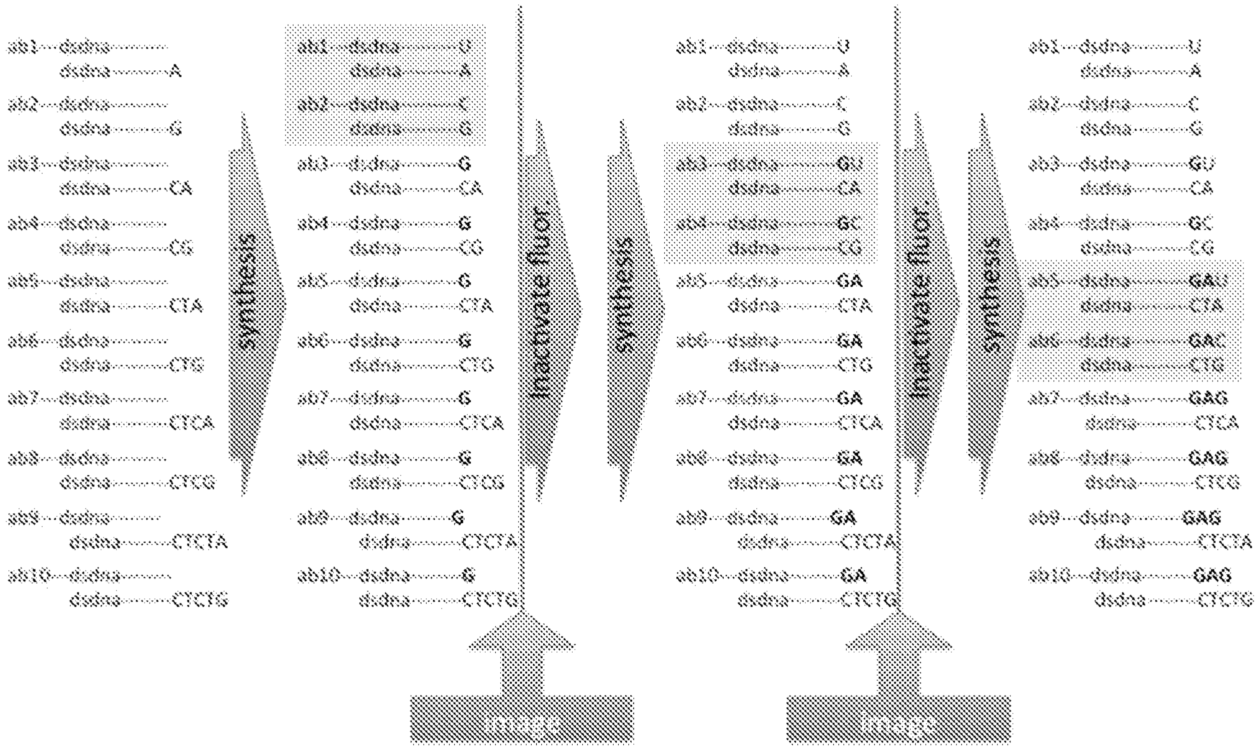
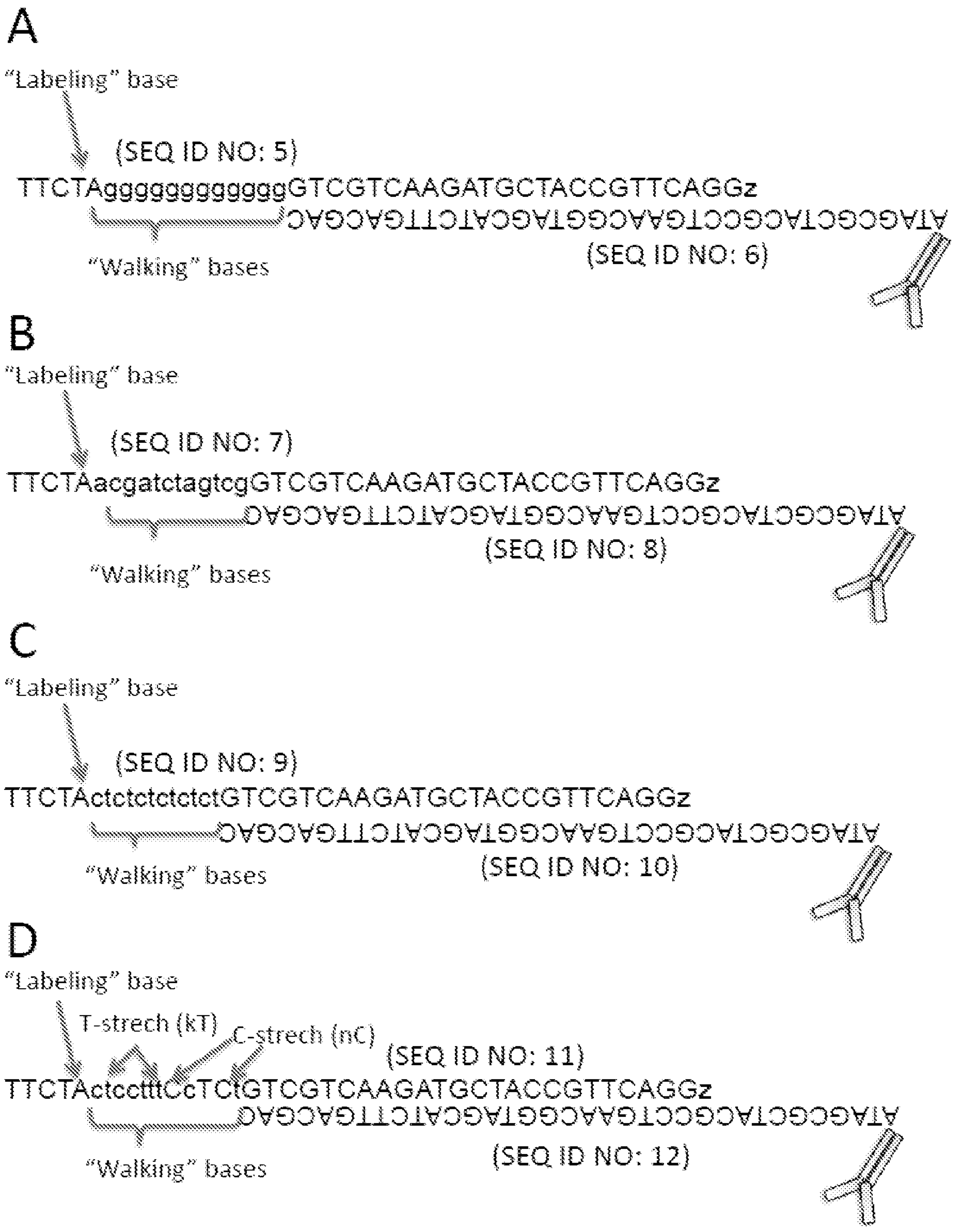

ab1—dsdna———
dsdna———A
ab2—dsdna———
dsdna———G
ab3—dsdna———
dsdna———CA
ab4—dsdna———
dsdna———CG
ab5—dsdna———
dsdna———CTA
ab6—dsdna———
dsdna———CTG
ab7—dsdna———
dsdna———CTCA
ab8—dsdna———
dsdna———CTCG
ab9—dsdna———
dsdna———CTCTA
ab10—dsdna———
dsdna———CTCTG
synthesis
ab1—dsdna———U
dsdna———A
ab2—dsdna———C
dsdna———G
ab3—dsdna———G
dsdna———CA
ab4—dsdna———G
dsdna———CG
ab5—dsdna———G
dsdna———CTA
ab6—dsdna———G
dsdna———CTG
ab7—dsdna———G
dsdna———CTCA
ab8—dsdna———G
dsdna———CTCG
ab9—dsdna———G
dsdna———CTCTA
ab10—dsdna———G
dsdna———CTCTG
Inactivate fluor.
image
synthesis
ab1—dsdna———U
dsdna———A
ab2—dsdna———C
dsdna———G
ab3—dsdna———GU
dsdna———CA
ab4—dsdna———GC
dsdna———CG
ab5—dsdna———GA
dsdna———CTA
ab6—dsdna———GA
dsdna———CTG
ab7—dsdna———GA
dsdna———CTCA
ab8—dsdna———GA
dsdna———CTCG
ab9—dsdna———GA
dsdna———CTCTA
ab10—dsdna———GA
dsdna———CTCTG
Inactivate fluor.
image
synthesis
ab1—dsdna———U
dsdna———A
ab2—dsdna———C
dsdna———G
ab3—dsdna———GU
dsdna———CA
ab4—dsdna———GC
dsdna———CG
ab5—dsdna———GAU
dsdna———CTA
ab6—dsdna———GAC
dsdna———CTG
ab7—dsdna———GAG
dsdna———CTCA
ab8—dsdna———GAG
dsdna———CTCG
ab9—dsdna———GAG
dsdna———CTCTA
ab10—dsdna———GAG
dsdna———CTCTG

FIG. 4


A
"Labeling" base
(SEQ ID NO: 5)
TTCTAgggggggggggggGTCGTCAAGATGCTACCGTTCAGGz
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAC
"Walking" bases
(SEQ ID NO: 6)
B
"Labeling" base
(SEQ ID NO: 7)
TTCTAacgatctagtcgGTCGTCAAGATGCTACCGTTCAGGz
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAC
(SEQ ID NO: 8)
"Walking" bases
C
"Labeling" base
(SEQ ID NO: 9)
TTCTActctctctctctGTCGTCAAGATGCTACCGTTCAGGz
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAC
"Walking" bases
(SEQ ID NO: 10)
D
"Labeling" base
T-strech (kT)
C-strech (nC)
(SEQ ID NO: 11)
TTCTActcctttCcTCtGTCGTCAAGATGCTACCGTTCAGGz
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAC
"Walking" bases
(SEQ ID NO: 12)

FIGS. 5A-5D


Ab1
(SEQ ID NO: 13)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAddC
TTTTTA------GTCGTCAAGATGCTACCGTTCAGG
(SEQ ID NO: 14)
Spacer1 landing
Ab2
(SEQ ID NO: 15)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAddC
TTTTTAC------GTCGTCAAGATGCTACCGTTCAGGz
(SEQ ID NO: 16)
Spacer1 landing
Ab3
(SEQ ID NO: 17)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAddC
TTTTTACT------GTCGTCAAGATGCTACCGTTCAGG
(SEQ ID NO: 18)
Spacer1 landing
Ab4
(SEQ ID NO: 19)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAddC
TTTTTA------GTCGTCAAGATGCTACCGTTCAGGz
(SEQ ID NO: 20)
Spacer2 landing
Ab5
(SEQ ID NO: 21)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAdd
C
TTTTTAC------GTCGTCAAGATGCTACCGTTCAGG
(SEQ ID NO: 22)
Spacer2 landing
Ab6
(SEQ ID NO: 23)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAddC
TTTTTACT------GTCGTCAAGATGCTACCGTTCAGG
(SEQ ID NO: 24)
Spacer2 landing
Ab7
(SEQ ID NO: 25)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAddC
TTTTTA------GTCGTCAAGATGCTACCGTTCAGG
(SEQ ID NO: 26)
Spacer3 landing
Ab8
(SEQ ID NO: 27)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAddC
TTTTTAC------GTCGTCAAGATGCTACCGTTCAGG
(SEQ ID NO: 28)
Spacer3 landing
Ab9
(SEQ ID NO: 29)
ATAGCGCTACGCCTGAACGGTAGCATCTTGACGAddC
TTTTTACT------GTCGTCAAGATGCTACCGTTCAGG
(SEQ ID NO: 30)
Spacer3 landing

FIG. 6

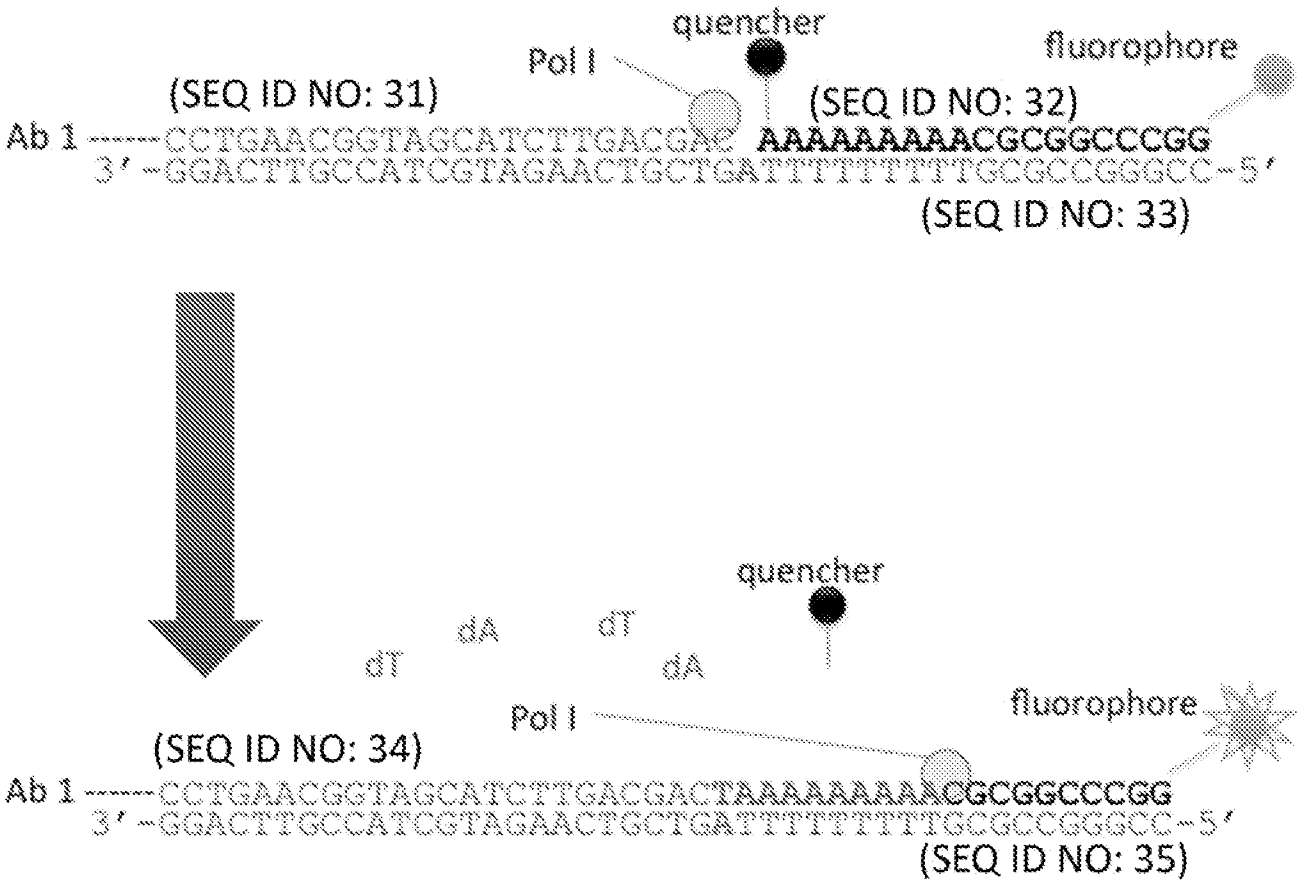

quencher
Pol I
fluorophore
(SEQ ID NO: 31)
(SEQ ID NO: 32)
Ab 1 -----CCTGAACGGTAGCATCTTGACGAC AAAAAAAAACGCGGCCCGG
3'-GGACTTGCCATCGTAGAACTGCTGATTTTTTTTTGCGCCGGGCC-5'
(SEQ ID NO: 33)
quencher
dA
dT
dT
dA
Pol I
fluorophore
(SEQ ID NO: 34)
Ab 1 -----CCTGAACGGTAGCATCTTGACGACTAAAAAAAAAACGCGGCCCGG
3'-GGACTTGCCATCGTAGAACTGCTGATTTTTTTTTTGCGCCGGGCC-5'
(SEQ ID NO: 35)

FIG. 7

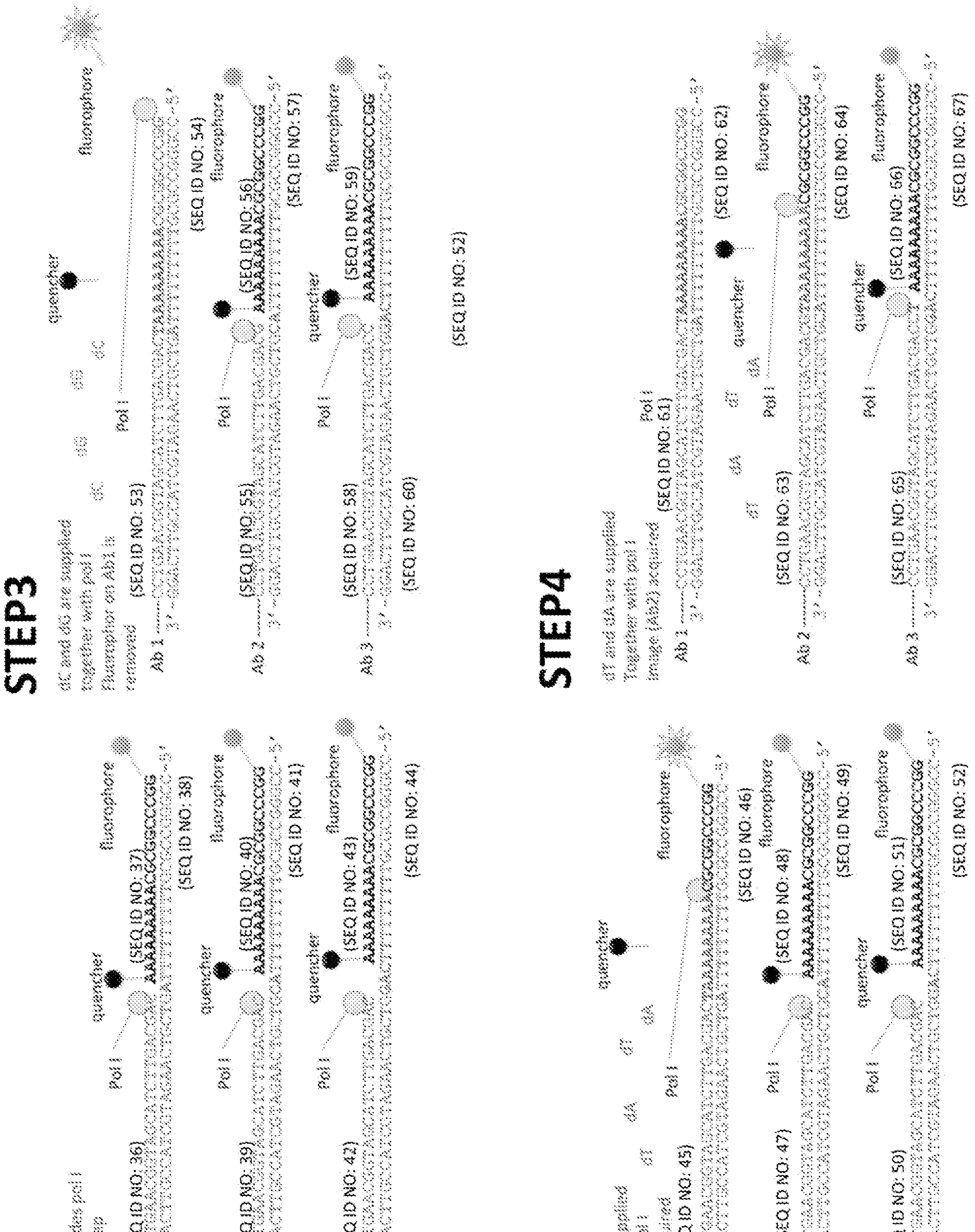
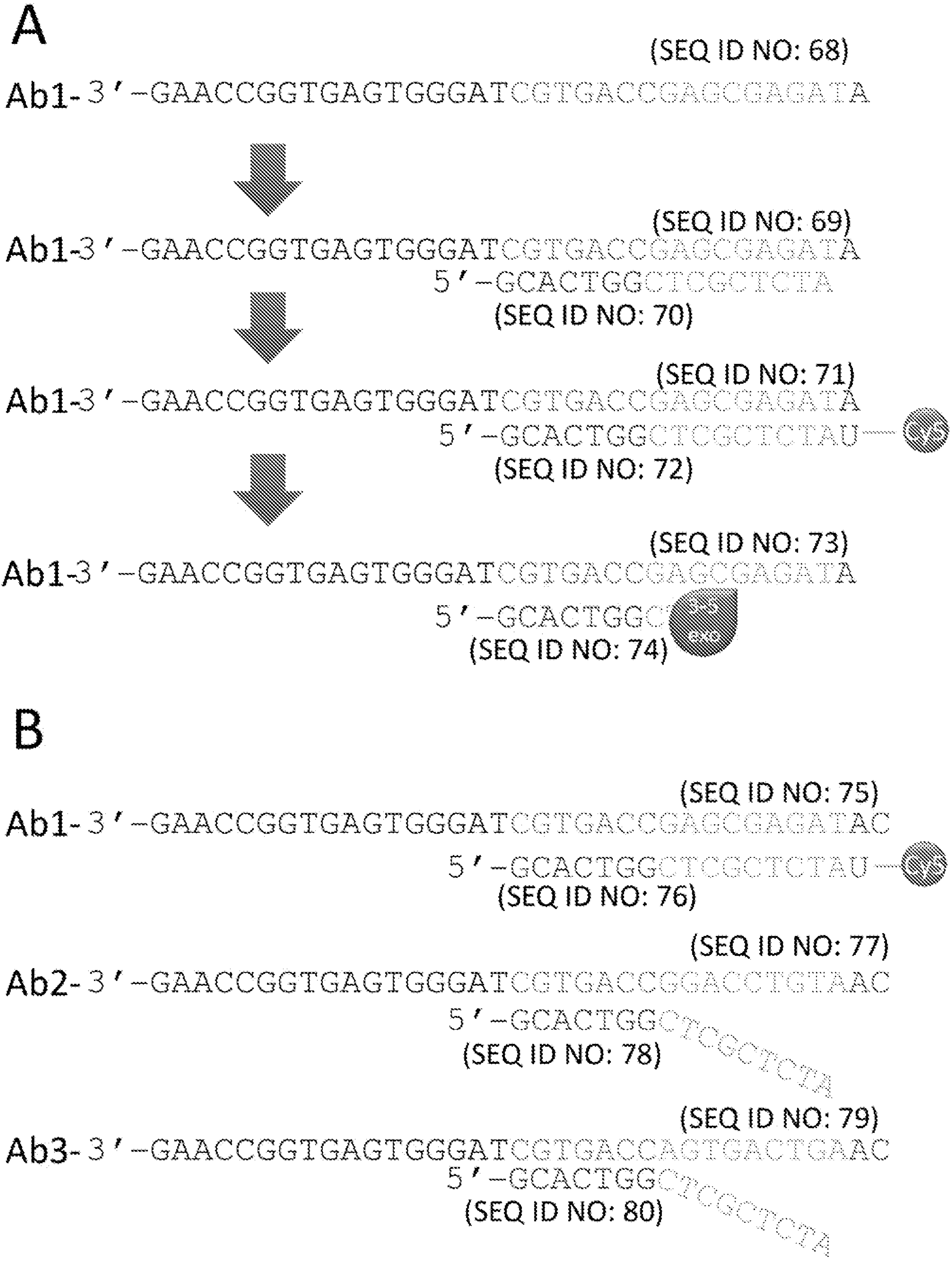

STEP1
Without nucleotides pol I is stalled at the gap
Ab 1
(SEQ ID NO: 36)
(SEQ ID NO: 37)
AAAAAAAAACGCGGCCCGG
(SEQ ID NO: 38)
Ab 2
(SEQ ID NO: 39)
(SEQ ID NO: 40)
AAAAAAAAACGCGGCCCGG
(SEQ ID NO: 41)
Ab 3
(SEQ ID NO: 42)
(SEQ ID NO: 43)
AAAAAAAAACGCGGCCCGG
(SEQ ID NO: 44)
Pol I
quencher
fluorophore
STEP2
dT and dA are supplied
Together with pol I
image (Ab1) acquired
dT
dA
Ab 1
(SEQ ID NO: 45)
(SEQ ID NO: 46)
Ab 2
(SEQ ID NO: 47)
(SEQ ID NO: 48)
AAAAAAAAACGCGGCCCGG
(SEQ ID NO: 49)
Ab 3
(SEQ ID NO: 50)
(SEQ ID NO: 51)
AAAAAAAAACGCGGCCCGG
(SEQ ID NO: 52)
STEP3
dC and dG are supplied
together with pol I
Fluorophor on Ab1 is
removed
dC
dG
Ab 1
(SEQ ID NO: 53)
(SEQ ID NO: 54)
Ab 2
(SEQ ID NO: 55)
(SEQ ID NO: 56)
AAAAAAAAACGCGGCCCGG
(SEQ ID NO: 57)
Ab 3
(SEQ ID NO: 58)
(SEQ ID NO: 59)
AAAAAAAAACGCGGCCCGG
(SEQ ID NO: 60)
(SEQ ID NO: 52)
STEP4
dT and dA are supplied
Together with pol I
image (Ab2) acquired
Ab 1
(SEQ ID NO: 61)
(SEQ ID NO: 62)
Ab 2
(SEQ ID NO: 63)
(SEQ ID NO: 64)
Ab 3
(SEQ ID NO: 65)
(SEQ ID NO: 66)
AAAAAAAAACGCGGCCCGG
(SEQ ID NO: 67)
3'
5'

FIG. 8


A
(SEQ ID NO: 68)
Ab1- 3′-GAACCGGTGAGTGGGATCGTGACCGAGCGAGATA
(SEQ ID NO: 69)
Ab1-3′-GAACCGGTGAGTGGGATCGTGACCGAGCGAGATA
5′-GCACTGGCTCGCTCTA
(SEQ ID NO: 70)
(SEQ ID NO: 71)
Ab1-3′-GAACCGGTGAGTGGGATCGTGACCGAGCGAGATA
5′-GCACTGGCTCGCTCTAU
Cys
(SEQ ID NO: 72)
(SEQ ID NO: 73)
Ab1-3′-GAACCGGTGAGTGGGATCGTGACCGAGCGAGATA
5′-GCACTGGC
(SEQ ID NO: 74)
B
(SEQ ID NO: 75)
Ab1- 3′-GAACCGGTGAGTGGGATCGTGACCGAGCGAGATAC
5′-GCACTGGCTCGCTCTAU
Cys
(SEQ ID NO: 76)
(SEQ ID NO: 77)
Ab2- 3′-GAACCGGTGAGTGGGATCGTGACCGGACCTGTAAC
5′-GCACTGGCTCGCTCTA
(SEQ ID NO: 78)
(SEQ ID NO: 79)
Ab3- 3′-GAACCGGTGAGTGGGATCGTGACCAGTGACTGAAC
5′-GCACTGGCTCGCTCTA
(SEQ ID NO: 80)

FIGS. 9A-9B

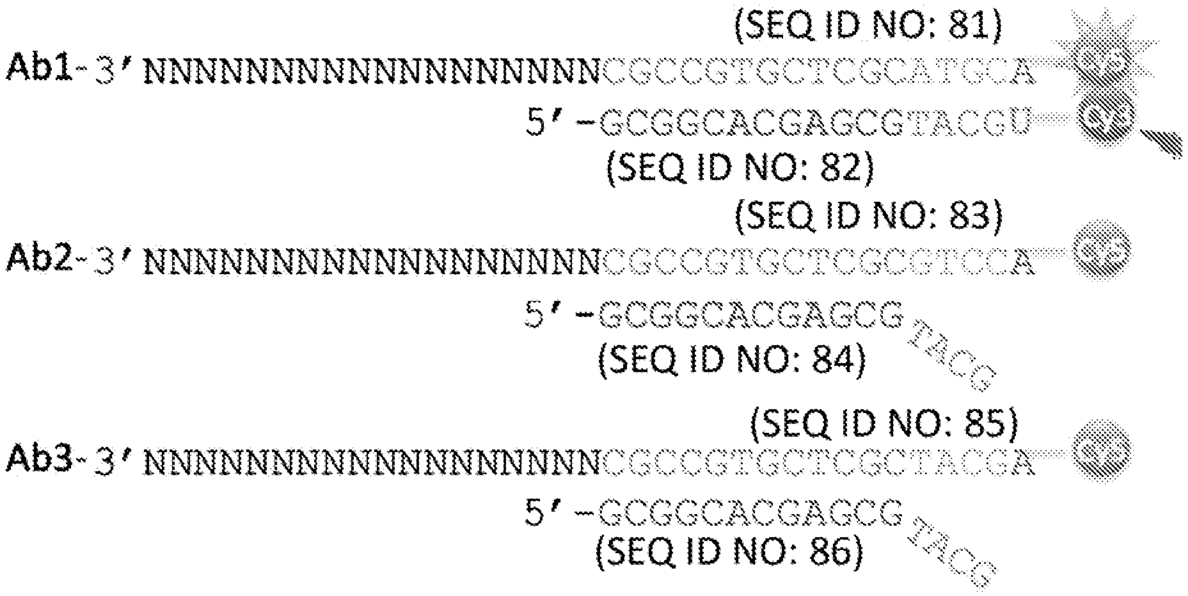

(SEQ ID NO: 81)
Ab1- 3′ NNNNNNNNNNNNNNNNNNCGCCGTGCTCGCATGCA
5′ -GCGGCACGAGCGTACGU
(SEQ ID NO: 82)
(SEQ ID NO: 83)
Ab2- 3′ NNNNNNNNNNNNNNNNNNCGCCGTGCTCGCGTCCA
5′ -GCGGCACGAGCG TACG
(SEQ ID NO: 84)
(SEQ ID NO: 85)
Ab3- 3′ NNNNNNNNNNNNNNNNNNCGCCGTGCTCGCTACGA
5′ -GCGGCACGAGCG TACG
(SEQ ID NO: 86)

FIG. 10

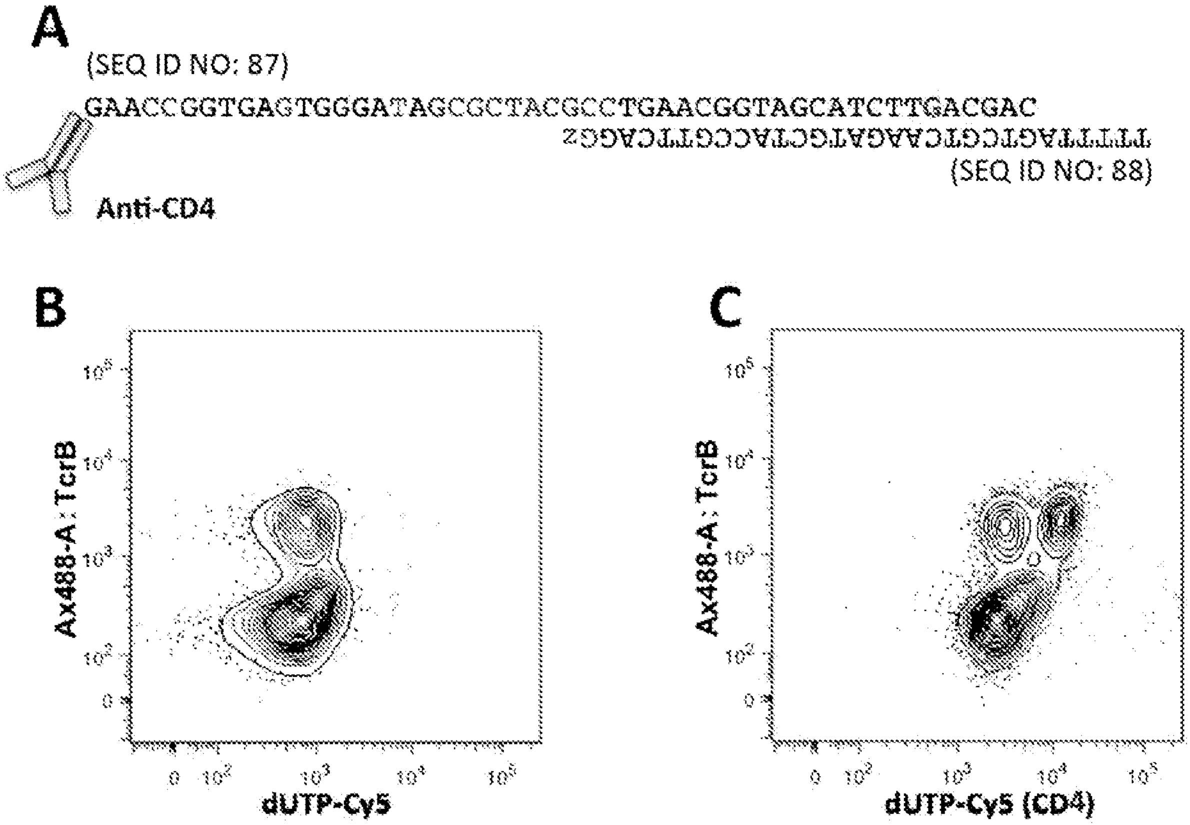
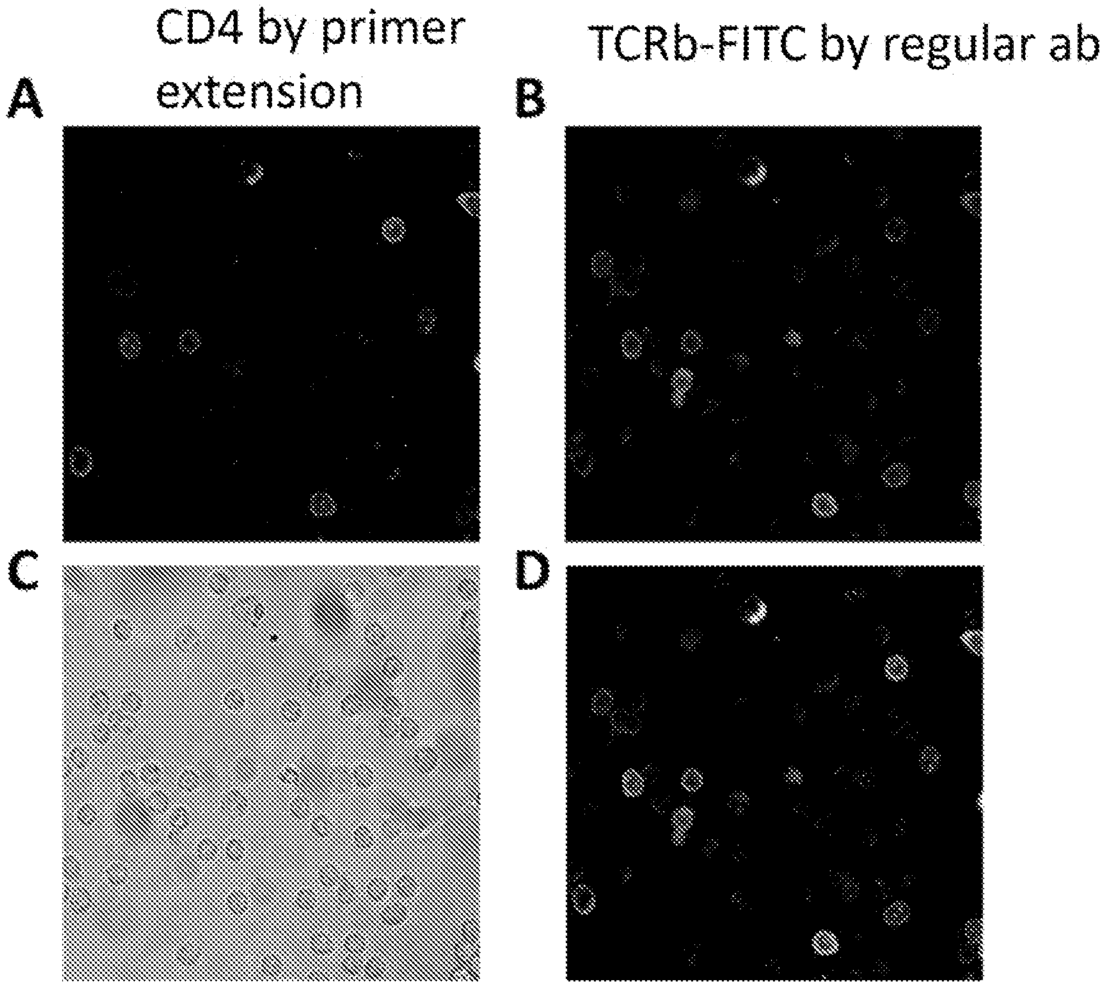
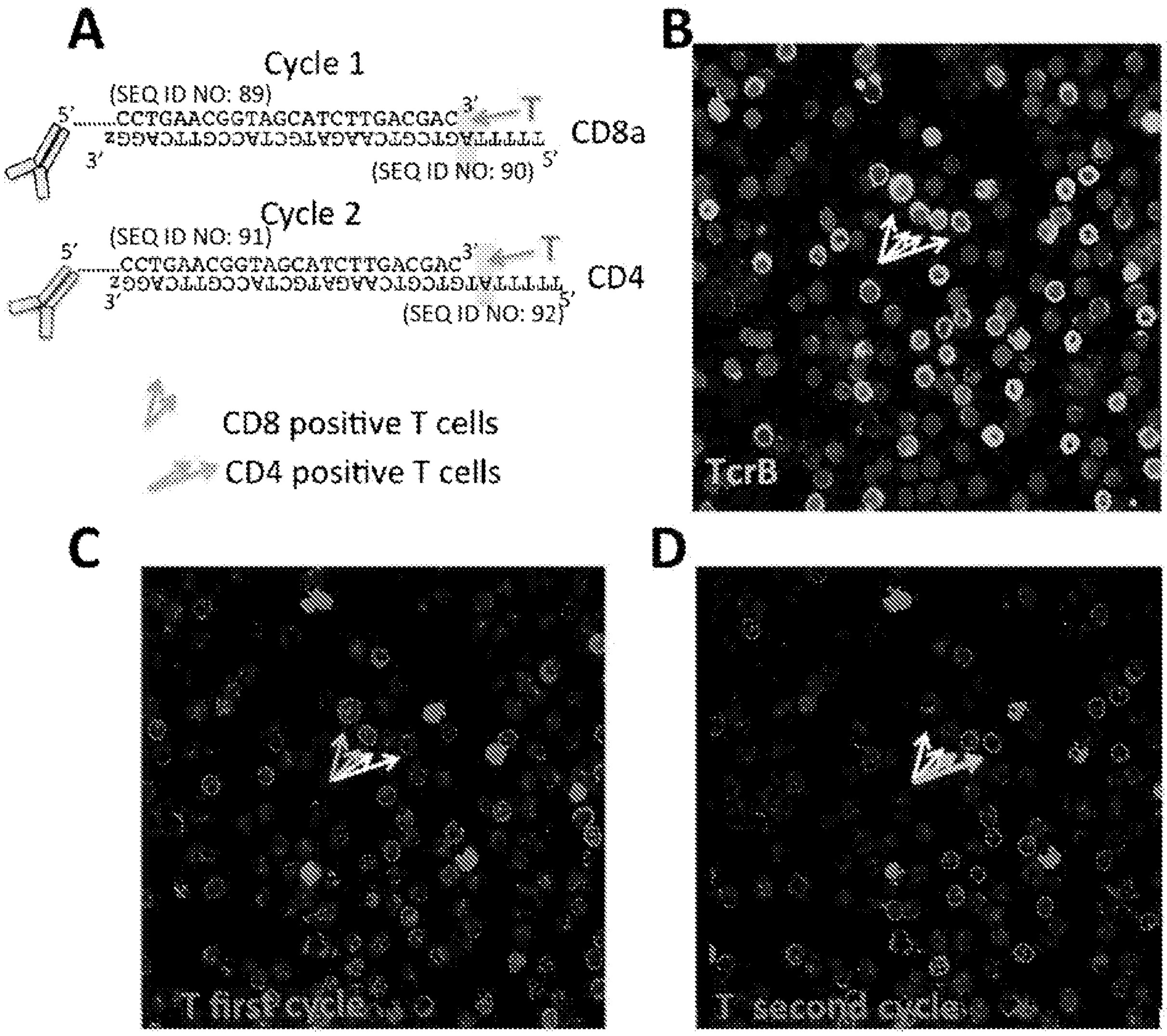

A
(SEQ ID NO: 87)
GAACCGGTGAGTGGGATAGCGCTACGCCTGAACGGTAGCATCTTGACGAC
TTTTTAGTCGTCAAGATGCTACCGTTCAGGz
(SEQ ID NO: 88)
Anti-CD4
B
10^5
10^4
10^3
10^2
0
Ax488-A : TcrB
0
10^2
10^3
10^4
10^5
dUTP-Cy5
C
10^5
10^4
10^3
10^2
0
Ax488-A : TcrB
0
10^2
10^3
10^4
10^5
dUTP-Cy5 (CD4)

FIG. 11A-11C



FIGS. 12A-12D


A
Cycle 1
(SEQ ID NO: 89)
5'
CCTGAACGGTAGCATCTTGACGAC
3'
T
CD8a
TTTTTAGTCGTCAAGATGCTACCGTTCAGG
3'
(SEQ ID NO: 90)
5'
Cycle 2
(SEQ ID NO: 91)
5'
CCTGAACGGTAGCATCTTGACGAC
3'
T
CD4
TTTTTATGTCGTCAAGATGCTACCGTTCAGG
3'
(SEQ ID NO: 92)
5'
CD8 positive T cells
CD4 positive T cells
B
TcrB
C
T first cycle
D
T second cycle

FIGS. 13A-13D

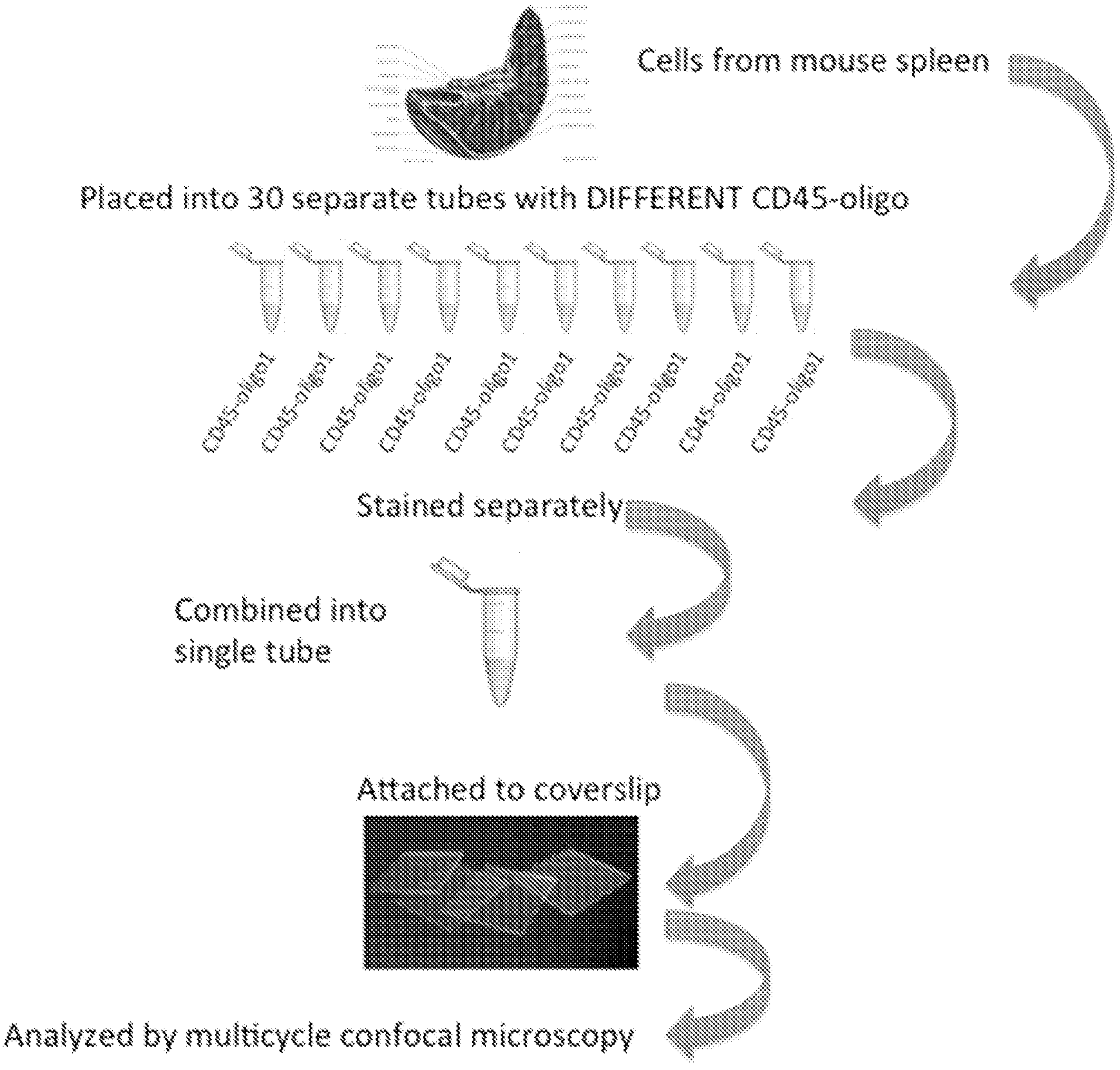

Cells from mouse spleen
Placed into 30 separate tubes with DIFFERENT CD45-oligo
CD45-oligo1
CD45-oligo1
CD45-oligo1
CD45-oligo1
CD45-oligo1
CD45-oligo1
CD45-oligo1
CD45-oligo1
CD45-oligo1
CD45-oligo1
Stained separately
Combined into single tube
Attached to coverslip
Analyzed by multicycle confocal microscopy

FIG. 14

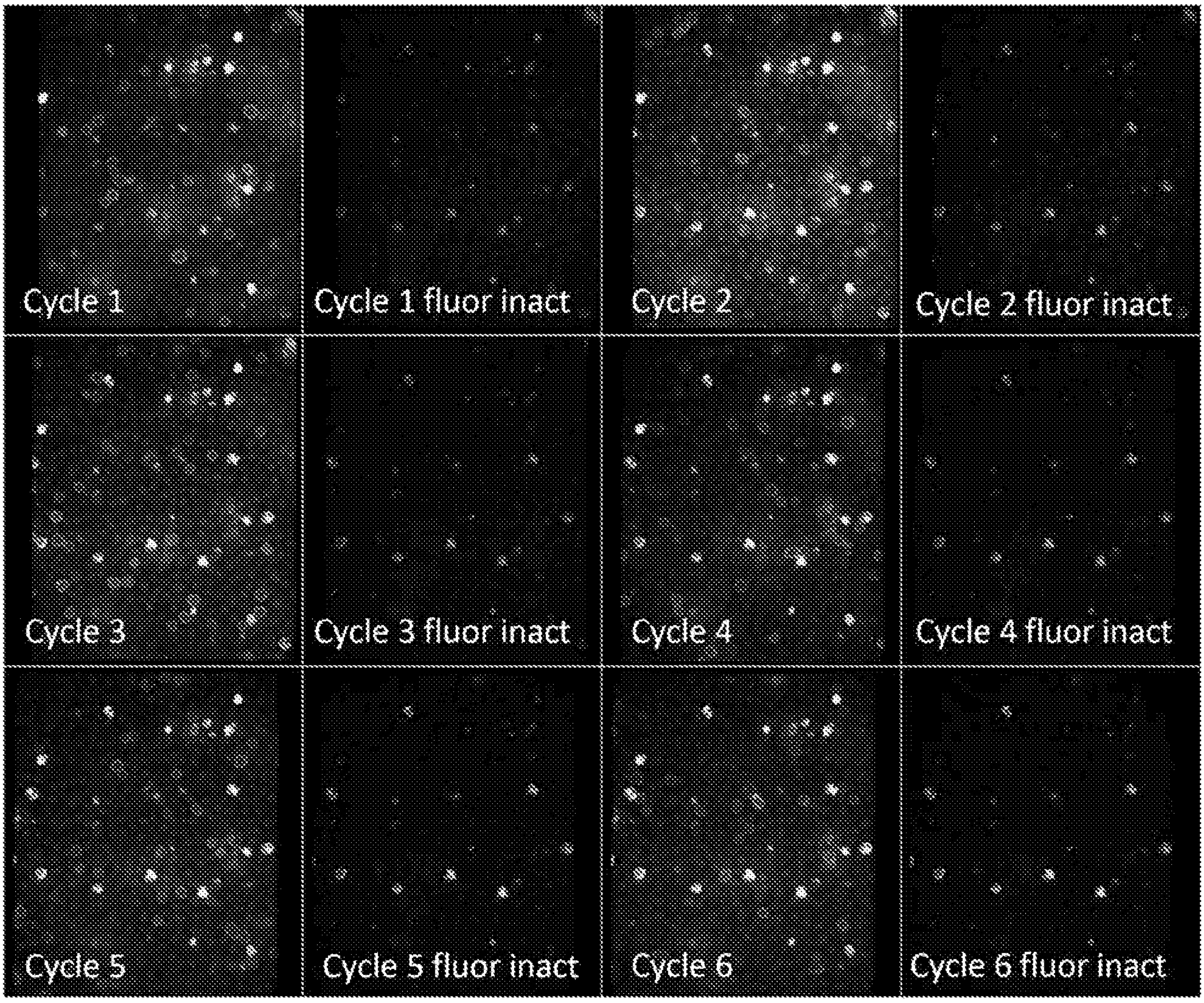
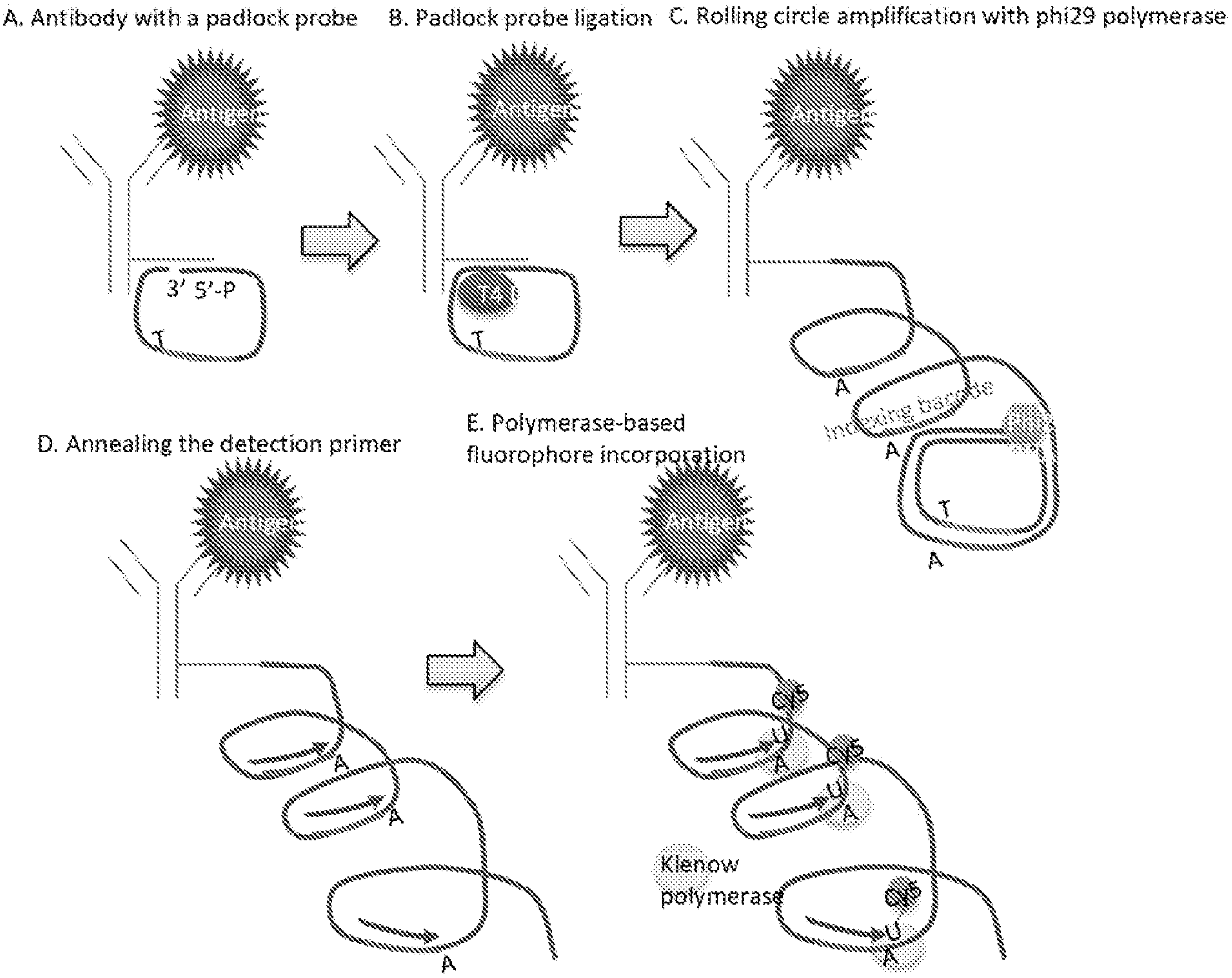

Cycle 1
Cycle 1 fluor inact
Cycle 2
Cycle 2 fluor inact
Cycle 3
Cycle 3 fluor inact
Cycle 4
Cycle 4 fluor inact
Cycle 5
Cycle 5 fluor inact
Cycle 6
Cycle 6 fluor inact

FIG. 15


A. Antibody with a padlock probe
B. Padlock probe ligation
C. Rolling circle amplification with phi29 polymerase
Antigen
3' 5'-P
T
T4
A
Indexing barcode
D. Annealing the detection primer
E. Polymerase-based fluorophore incorporation
Cy5
U
Klenow polymerase

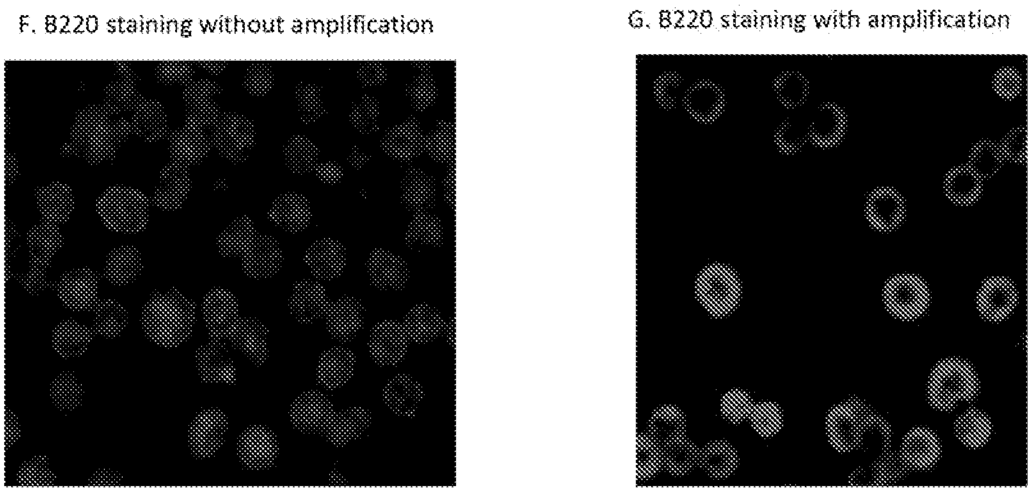

F. B220 staining without amplification
G. B220 staining with amplification

FIG. 16

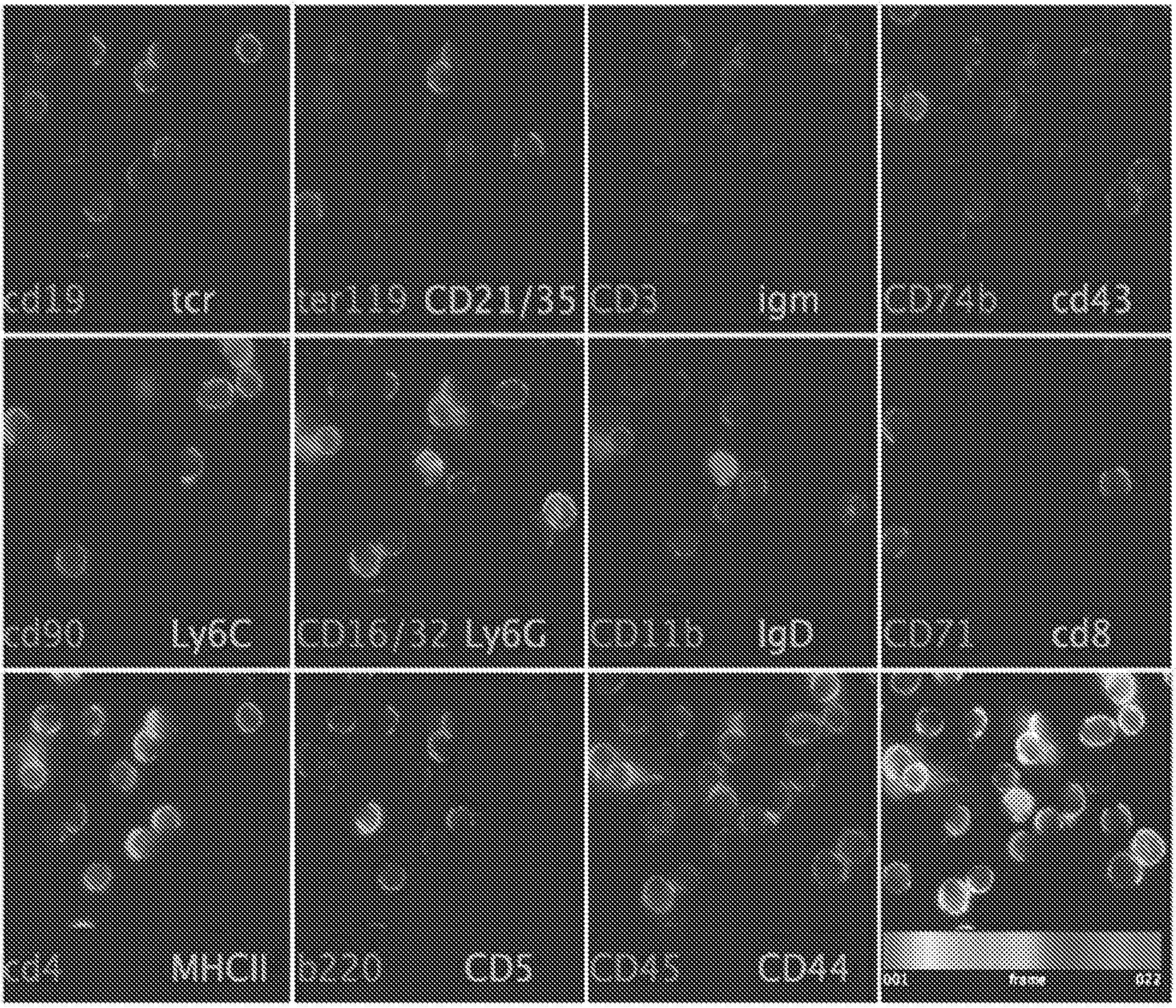

cd19
tcr
ter119
CD21/35
CD3
igm
CD74b
cd43
cd90
Ly6C
CD16/32
Ly6G
CD11b
IgD
CD71
cd8
cd4
MHCII
b220
CD5
CD45
CD44
001
frame
033

FIG. 17

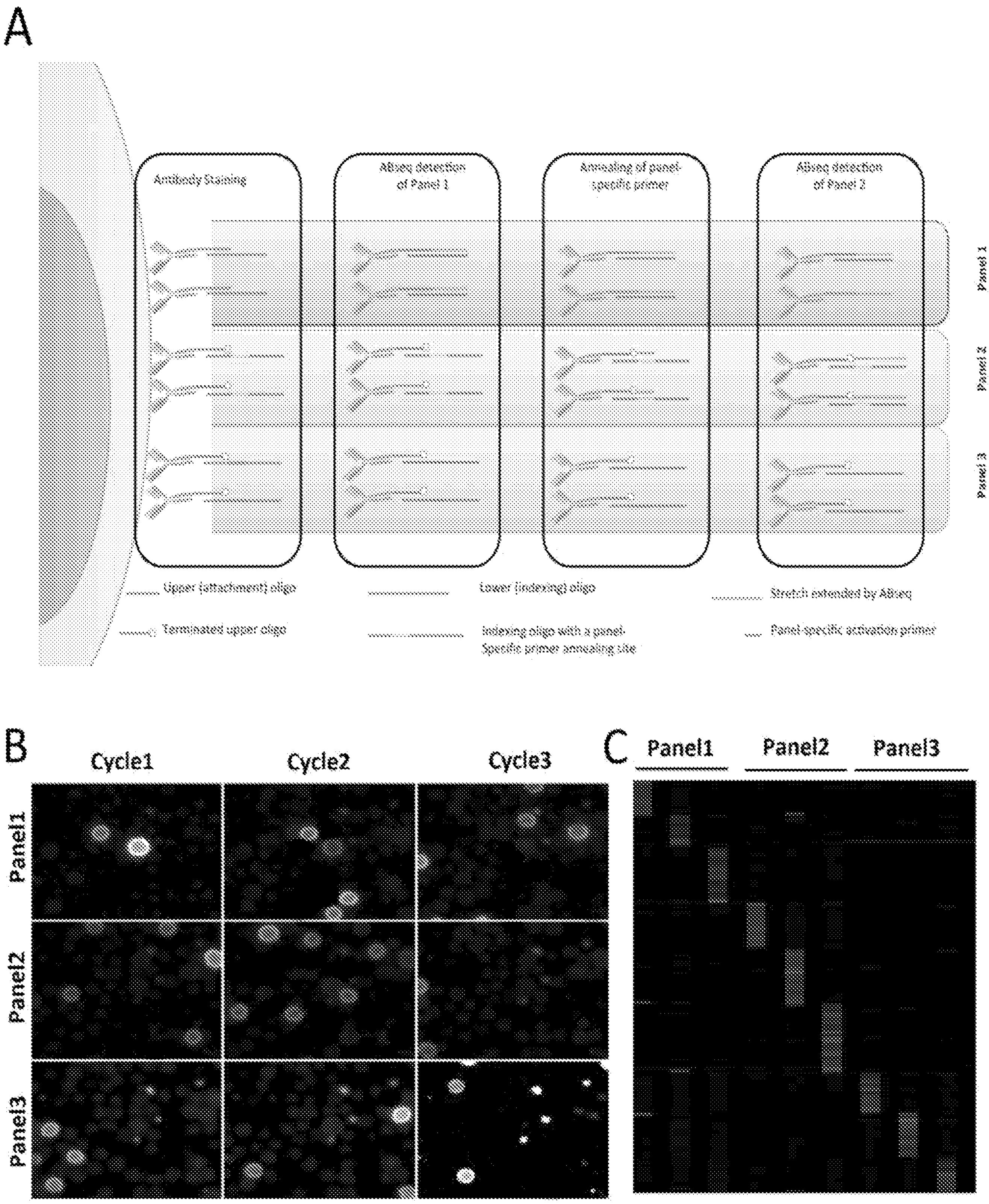

A
Antibody Staining
ABseq detection of Panel 1
Annealing of panel-specific primer
ABseq detection of Panel 2
Panel 1
Panel 2
Panel 3
Upper (attachment) oligo
Lower (indexing) oligo
Stretch extended by ABseq
Terminated upper oligo
Indexing oligo with a panel-Specific primer annealing site
Panel-specific activation primer
B
Cycle1
Cycle2
Cycle3
Panel1
Panel2
Panel3
C
Panel1
Panel2
Panel3

FIG. 18

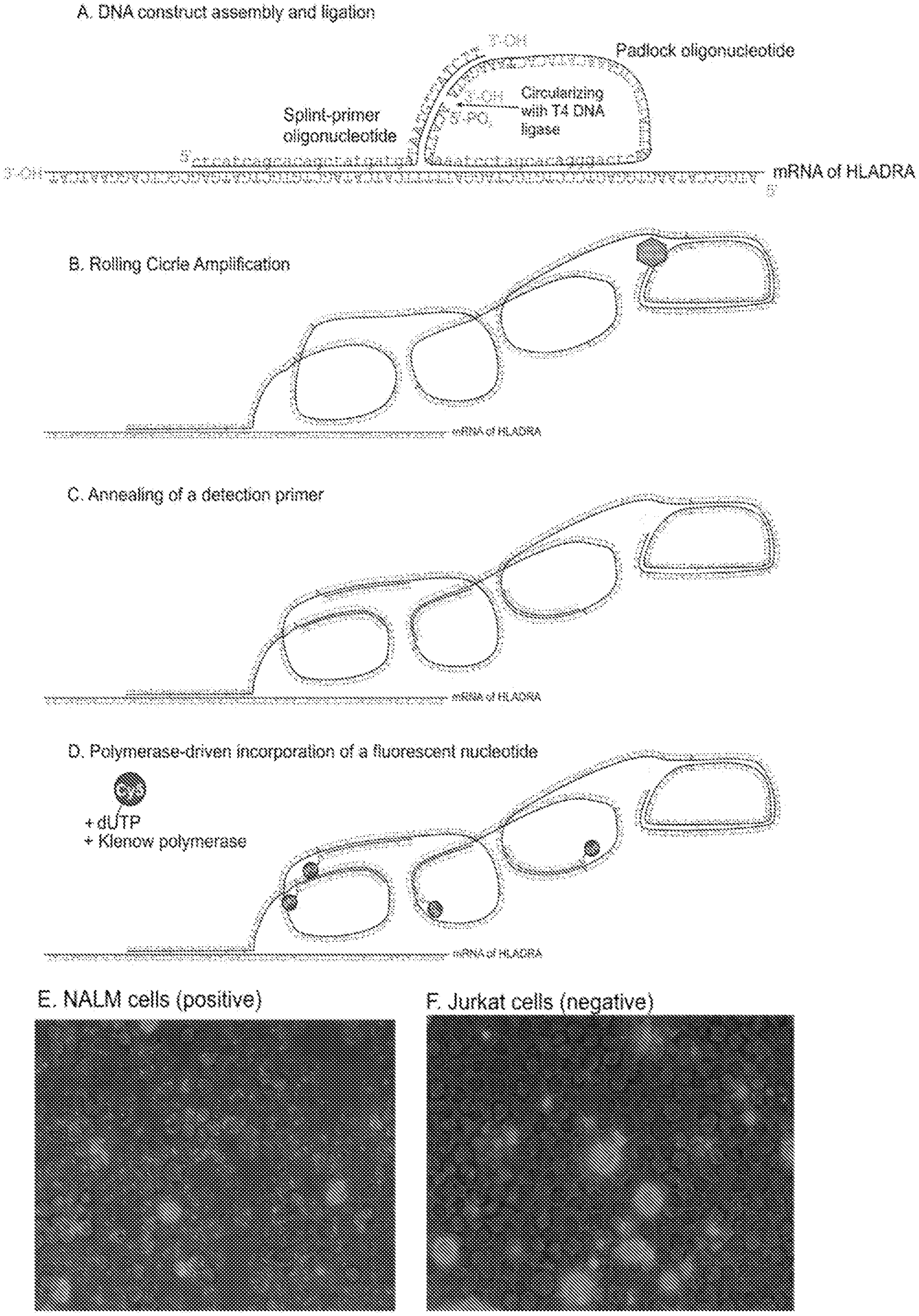

A. DNA construct assembly and ligation
3'-OH
Padlock oligonucleotide
3'-OH
Circularizing with T4 DNA ligase
5'-PO4
Splint-primer oligonucleotide
5'
3'-OH
mRNA of HLADRA
5'
B. Rolling Cicrle Amplification
mRNA of HLADRA
C. Annealing of a detection primer
mRNA of HLADRA
D. Polymerase-driven incorporation of a fluorescent nucleotide
Cy5
+ dUTP
+ Klenow polymerase
mRNA of HLADRA
E. NALM cells (positive)
F. Jurkat cells (negative)

FIG. 19

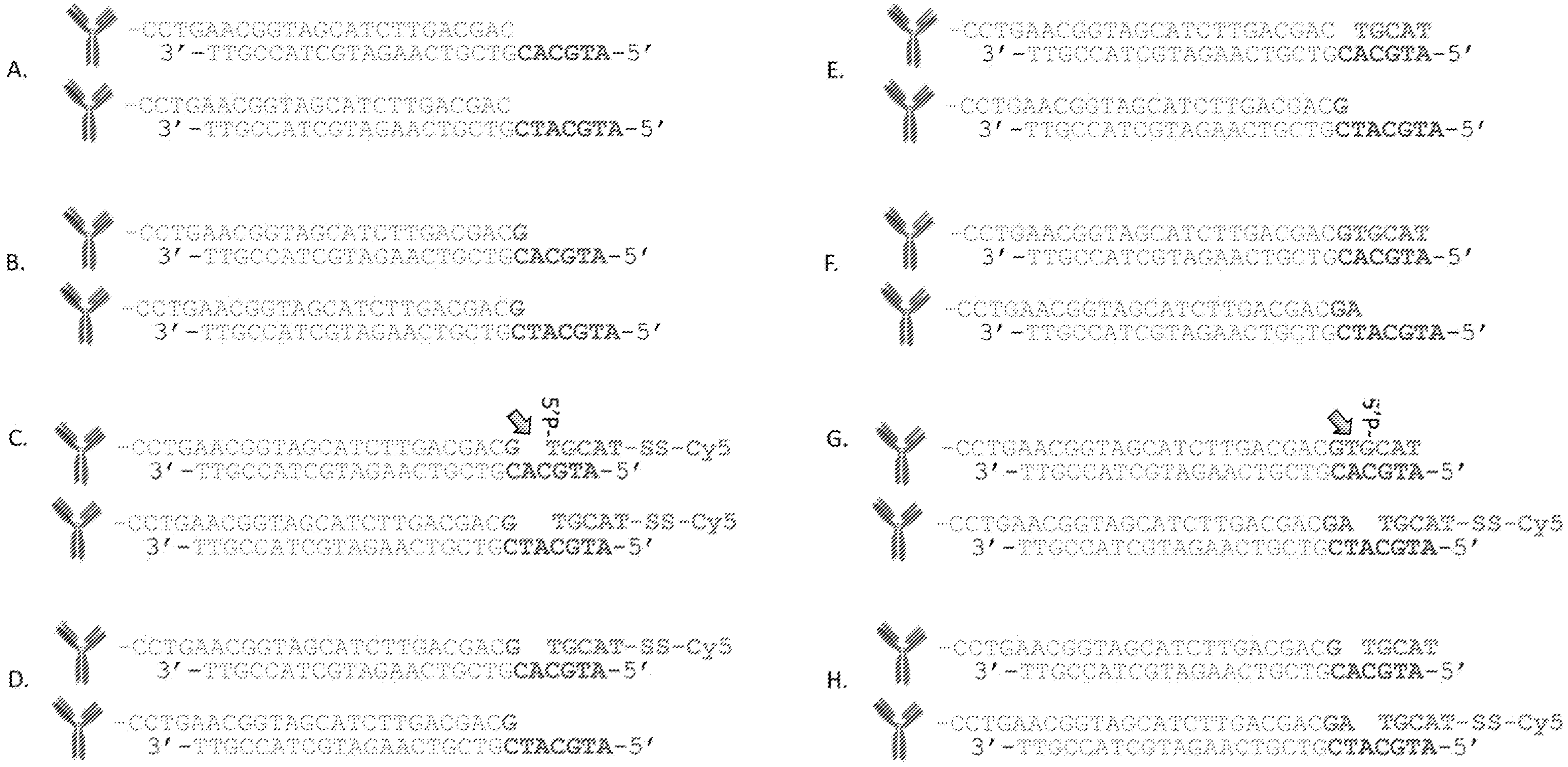

A.
-CCTGAACGGTAGCATCTTGACGAC
3'-TTGCCATCGTAGAACTGCTGCACGTA-5'
-CCTGAACGGTAGCATCTTGACGAC
3'-TTGCCATCGTAGAACTGCTGCTACGTA-5'
B.
-CCTGAACGGTAGCATCTTGACGACG
3'-TTGCCATCGTAGAACTGCTGCACGTA-5'
-CCTGAACGGTAGCATCTTGACGACG
3'-TTGCCATCGTAGAACTGCTGCTACGTA-5'
C.
5'P-
-CCTGAACGGTAGCATCTTGACGACG TGCAT-SS-Cy5
3'-TTGCCATCGTAGAACTGCTGCACGTA-5'
-CCTGAACGGTAGCATCTTGACGACG TGCAT-SS-Cy5
3'-TTGCCATCGTAGAACTGCTGCTACGTA-5'
D.
-CCTGAACGGTAGCATCTTGACGACG TGCAT-SS-Cy5
3'-TTGCCATCGTAGAACTGCTGCACGTA-5'
-CCTGAACGGTAGCATCTTGACGACG
3'-TTGCCATCGTAGAACTGCTGCTACGTA-5'
E.
-CCTGAACGGTAGCATCTTGACGAC TGCAT
3'-TTGCCATCGTAGAACTGCTGCACGTA-5'
-CCTGAACGGTAGCATCTTGACGACG
3'-TTGCCATCGTAGAACTGCTGCTACGTA-5'
F.
-CCTGAACGGTAGCATCTTGACGACGTGCAT
3'-TTGCCATCGTAGAACTGCTGCACGTA-5'
-CCTGAACGGTAGCATCTTGACGACGA
3'-TTGCCATCGTAGAACTGCTGCTACGTA-5'
G.
5'P-
-CCTGAACGGTAGCATCTTGACGACGTGCAT
3'-TTGCCATCGTAGAACTGCTGCACGTA-5'
-CCTGAACGGTAGCATCTTGACGACGA TGCAT-SS-Cy5
3'-TTGCCATCGTAGAACTGCTGCTACGTA-5'
H.
-CCTGAACGGTAGCATCTTGACGACG TGCAT
3'-TTGCCATCGTAGAACTGCTGCACGTA-5'
-CCTGAACGGTAGCATCTTGACGACGA TGCAT-SS-Cy5
3'-TTGCCATCGTAGAACTGCTGCTACGTA-5'

FIG. 20

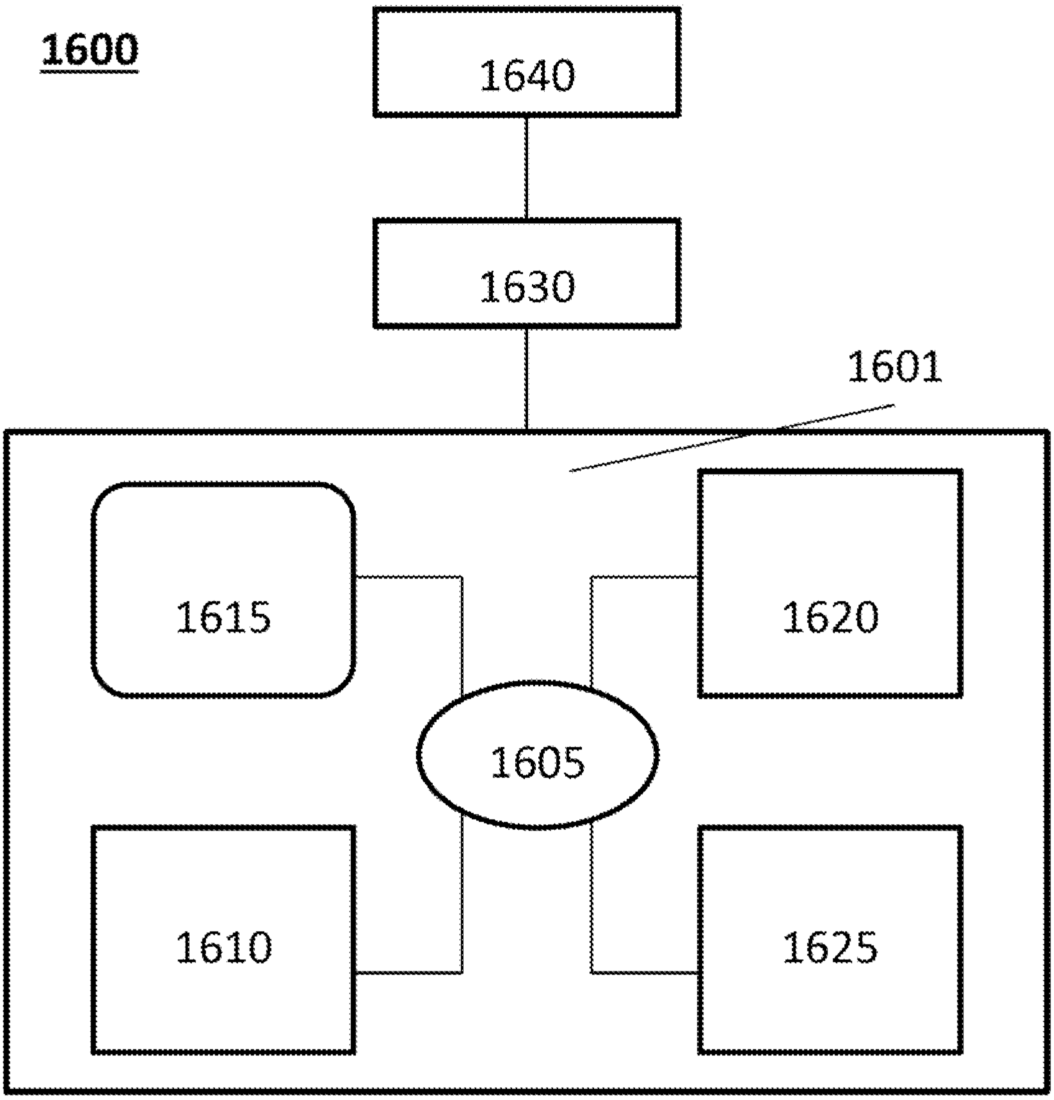


FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/36763

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68, G01N 33/52, A61K 31/7088 (2015.01)
CPC - C12Q 1/6855, C12Q 1/6853, C12N 15/115
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12Q 1/68, G01N 33/52, A61K 31/7088 (2015.01)
CPC: C12Q1/6855, C12Q1/6853, C12N15/115

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 436/501, 435/6.11, 435/325

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, Google Web, search terms: label, fluorescent, planar, microscopy, nucleic acid, DS DNA, DSDNA, double strand, fill in, polymerase, ligase, signal, nucleotide mix, complementary terminator, quencher, labeled oiligonucleotide, plurality, in situ, fixed cells, cultured cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0261781 A1 (GMEINER) 14 October 2010 (14.10.2010) para [0006], [0010], Figs. 1, 3	1-4, 10, 14, 20-32
Y	US 2005/0186572 A1 (EGHOLM et al.) 25 August 2005 (25.08.2005) para [0010], [0015], [0016], [0024], [0027], [0029], [0065], [0094], [0110], Fig. 2	1-4, 10, 14, 20-32
Y	US 2011/0046359 A1 (LEE et al.) 24 February 2011(24.02.2011) para [0058]	20-32
Y	US 2011/0033846 A1 (DATTAGUPTA) 10 February 2011 (10.02.2011) para [0007], [0004]	10, 30
Y	US 2013/0172213 A1 (OLIPHANT et al.) 04 July 2013 (04.07.2013) para [0011], [00121]	31

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search 17 October 2015 (17.10.2015)	Date of mailing of the international search report 23 NOV 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/36763

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:
      - ☒ in the form of an Annex C/ST.25 text file.
      - ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
      - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
      - ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/36763

Box No. II **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-9, 11-13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

************************** See Supplemental Sheet to continue *************************

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 10, 14, 20-32

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box No. III, Observations where unity of invention is lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-4, 10, 14, 20-32, drawn to a method on analyzing a planar surface.

Group II: Claims 15-19, drawn to a capture agent composition

Group III: Claims 33-36, drawn to a kit.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I has the special technical feature of analyzing a planar surface, not required by Groups II or III.

Group I has the special technical feature of labeling a planar surface with a capture agent, not required by Groups II or III.

Group I has the special technical feature of reading a signal generated by addition of the one or more nucleotides and/or labeled oligonucleotide to one of the first strand or the second strand of the double-stranded nucleic acid, not required by Groups II or III.

Group II has the special technical feature of a capture agent composition that is linked to a double stranded nucleic acid through a first strand, not required by Groups I or III.

Group III has the specific technical feature of being a kit, not required by Groups I or II.

Common Technical Features:

1. Groups I-III share the common technical feature of a double stranded nucleic acid comprises a first strand and a second strand.

2. Groups I-III share the common technical feature of a capture agent that is linked to the first strand of the nucleic acid.

3. Groups I-III share the common technical feature of the 5' or the 3' end of either the first or the second strand is extendable using the other strand as a template.

4. Some inventions in Group I (claims 20-32) and Group II share the common technical feature of a capture agent as described in claim 16.
However, said common technical features do not represent a contribution over the prior art, and is obvious over US 2004/0038194 A1 (HOFMANN).

Concerning common technical features #1, #2, #3, Hofmann teaches (claim 1; "A method for simultaneously detecting and capturing a double-stranded DNA sequence, which comprises: providing a sample; adding a forward primer for the double-stranded DNA sequence and a reverse primer for the double-stranded DNA sequence; one of the forward primer and the reverse primer having a capture agent, the other of the forward primer and the reverse primer having a detection agent; replicating the double-stranded DNA sequence; binding the capture agent to a capture medium [see para [0007] for description of capture medium]; rinsing the sample; and detecting the detection agent").

As to common technical features #4, Hofmann teaches (claim 16) a capture agent composition comprising a plurality of capture agents that each recognize different complementary sites, wherein:
each of the plurality of capture agents is linked to a double-stranded nucleic acid that comprises a first strand and a second strand (para [0012-0013]; "In accordance with a further object of the invention, the method includes detecting a plurality of double stranded DNA sequence by adding a forward primer and a reverse primer for each additional double-stranded DNA sequence. One of each pair of a forward primers and a reverse primer has a capture agent, and the other of the pair has a second detection agent"); the 5' end or 3' end of the first or second strand is extendible using the other strand as a template (para [0005]; " The next step is replicating the double-stranded DNA sequence"); and the templates immediately downstream of the extendible ends are different for each of the plurality of capture agents (para [0012-0013]; "In accordance with a further object of the invention, the method includes detecting a plurality of double stranded DNA sequence by adding a forward primer and a reverse primer for each additional double-stranded DNA sequence. One of each pair of a forward primers and a reverse primer has a capture agent, and the other of the pair has a second detection agent. [0013] In addition, each detection agent is different").

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I-III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.