



(51) International Patent Classification:

C07K 16/00 (2006.01) C12N 15/09 (2006.01)
C07K 16/40 (2006.01) C12N 15/11 (2006.01)
C12N 9/22 (2006.01)

(21) International Application Number:

PCT/US2021/044994

(22) International Filing Date:

06 August 2021 (06.08.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/062,779 07 August 2020 (07.08.2020) US

(71) Applicant: YALE UNIVERSITY [US/US]; Two Whitney Avenue, New Haven, Connecticut 06510 (US).

(72) Inventors: WANG, Siyuan; 277 Greene St, New Haven, Connecticut 06511 (US). CHEN, Yanbo; 18 Linden Street, Apt. 3, New Haven, Connecticut 06511 (US).

(74) Agent: HONGFAN, PH.D, Chen et al.; Troutman Pepper Hamilton Sanders LLP, 875 Third Avenue, New York, New York 10022 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,

HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: SINGLE-CELL LOCUS-SPECIFIC PROFILING OF EPIGENETIC MARKS

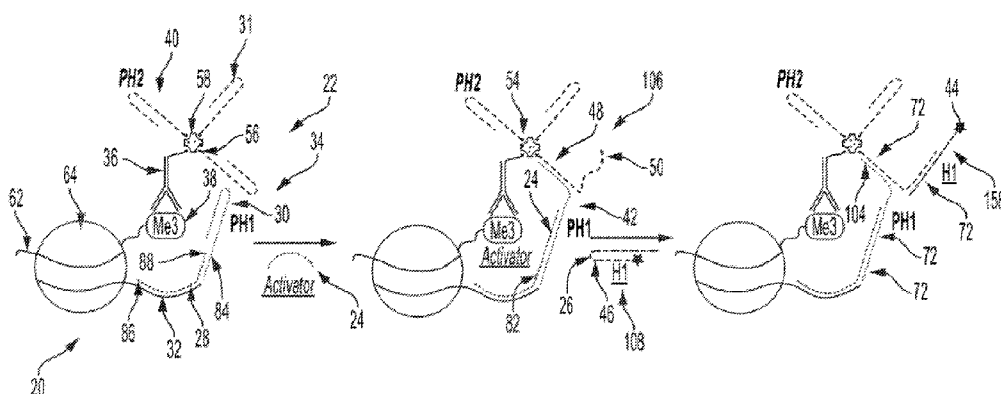


FIG. 1A

(57) **Abstract:** Provided herein are methods for in situ visualization of chromatin modifications of a cell. The methods comprise providing at least one first probe targeting a genomic locus of interest, a second probe comprising an antibody targeting a chromatin modification of interest, and a labeled hybridization oligonucleotide. When the first probe and the second probe can undergo a proximity hybridization reaction making available a sequence to which the labeled hybridization oligonucleotide may then bind to allow for visualization in three dimensions of the co-localization of the first and second probes. The methods allow for detection of single or multiple chromatin modifications of interest at single or multiple genomic locations of interest in a single experiment.

SINGLE-CELL LOCUS-SPECIFIC PROFILING OF EPIGENETIC MARKS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 63/062,779, filed August 7, 2020, the disclosure of which is herein incorporated by reference in its entirety.

GOVERNMENT FUNDING

[0002] This invention was made with government support under HG011245 and GM137414 awarded by National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on August 5, 2021, is named 251609_000055_SL.txt and is 1,499,751 in size.

FIELD OF THE INVENTION

[0004] Provided herein are methods for detecting a locus-specific epigenetic mark(s) in single cells at a given genomic location(s) using a proximity hybridization reaction (PHR), optionally in combination with a multiplexed sequential DNA fluorescence *in situ* hybridization (FISH). The methods enable profiling of an epigenetic mark(s) at a genomic location(s) and imaging of chromatin organization.

BACKGROUND

[0005] Gene expression and phenotype may be influenced by epigenetic factors that do not involve changes to the underlying DNA and may be heritable. Additionally, gene expression and cell division are supported by a complex of DNA and protein called chromatin. Thus, the functional outputs of a particular genome depend on both the local epigenetic states and the three-dimensional (3D) chromatin organization [1, 2]. There is a close relationship between the epigenome and 3D chromosome organization [3]. Aberrant epigenome and chromatin organization changes are associated with many diseases including cancer [4-7]. Changes in chromatin organization are also associated with aging. Currently, distinct sequencing-based methodologies are required to separately profile epigenetic modifications or 3D chromatin organization, and these

methods usually do not have sufficient single-cell resolution [8-10]. These technical limitations hinder research and medical applications that aim to 1) profile epigenetic marks along the genome in single cells, and to 2) profile epigenetic marks together with chromatin organization, in heterogeneous cell culture or tissue samples.

[0006] Therefore, there exists a need for imaging-based methods to profile epigenetic marks and determine 3D chromatin organization at the single-cell level *in situ*.

SUMMARY OF THE INVENTION

[0007] Herein is provided an imaging-based method to profile epigenetic marks at the single-cell level *in situ*. Specifically, herein is provided an epigenetic proximity hybridization reaction (Epi-PHR) method to detect a locus-specific epigenetic mark in single cells at a given genomic locus. Further provided is Epi-PHR combined with multiplexed sequential DNA fluorescence *in situ* hybridization (FISH), called Epi-mFISH, a method that enables combined profiling of epigenetic marks at multiple genomic loci and imaging of chromatin organization. Simplified or “EZ” embodiments, so named for being comparatively “easy” to carry out, of the methods employ less components and require less steps.

[0008] In one aspect, provided is a method for *in situ* visualization of a chromatin modification at a genomic locus of a cell comprising the steps of:

- a) providing at least one first probe comprising a first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein said first oligonucleotide binds to a genomic locus of interest,
- b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest,
- c) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is labeled directly or indirectly,
- d) contacting the cell with the first probe under conditions that allow binding of said first oligonucleotide of said first probe to said genomic locus of the cell,
- e) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,

- f) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to (i) a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, or to (ii) a sequence made available when said PH1 and PH2 oligonucleotides have hybridized, and
- g) detecting the label of the third probe.

[0009] In some embodiments of the method described above, wherein

step a) comprises providing a plurality of first probes, each of which targets a genomic locus of interest;

step d) comprises contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell, and

wherein the method further comprises the steps of:

- h) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,
- i) contacting the cell with each labeled readout probe, and
- j) detecting each label of each readout probe.

[0010] In some embodiments of the methods described above, the plurality of readout probes are labeled with a plurality of dyes. In some embodiments, the plurality of readout probes are labeled with the same dye. In some embodiments, the dye(s) are fluorescent dye(s).

[0011] In some embodiments of the methods described above, each of said plurality of first oligonucleotides comprises one or more readout probe binding sites each selectively bound by one of the plurality of labeled readout probes. In some embodiments, the one or more readout probe binding sites comprises a nucleic acid sequence of any one of SEQ ID NOs: 9 to 11 and 13 to 147, the reverse complement of any one of SEQ ID NOs: 9 to 11 and 13 to 147, or a nucleic acid sequence sharing at least about 50%, 55%, 60%, 65%, 70%, 75%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity with any one of SEQ ID NOs: 9-11 and 13 to 147 or with the reverse complement of any one of SEQ ID NOs: 9 to 11 and 13 to 147. In some embodiments, the one or more readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 9, the reverse complement of SEQ ID NO: 9, a nucleotide sequence with at

least 75% sequence identity to SEQ ID NO: 9, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9. In some embodiments, the one or more readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 10, the reverse complement of SEQ ID NO: 10, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10. In some embodiments, the one or more readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 11, the reverse complement of SEQ ID NO: 11, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11.

[0012] In some embodiments of the methods described above, the chromatin modification is a DNA modification, a DNA-binding protein (including proteins that bind directly or indirectly to DNA), or a modification to a DNA-binding protein. In some embodiments, the chromatin modification is a histone modification or a histone variant.

[0013] In some embodiments of the methods described above, the cell is fixed. In some embodiments, the genomic locus of interest is disposed within the nucleus of the cell.

[0014] In some embodiments of the methods described above, the method further comprises determining in three dimensions a location of the third probe. In some embodiments, the method further comprises using the location of the third probe to analyze chromatin structure. In some embodiments, the method further comprises determining in three dimensions a location of the readout probe. In some embodiments, the method further comprises using the location of the readout probe to analyze chromatin structure.

[0015] In some embodiments of the methods described above, the antibody is coupled to the PH2 oligonucleotide by a biotin-streptavidin bridge. In some embodiments, the antibody is coupled to the PH2 oligonucleotide by a covalent bond. In some embodiments, coupling of the antibody to the PH2 oligonucleotide comprises nucleotide hybridization.

[0016] In some embodiments of the methods described above, the H1 oligonucleotide is labeled with a first dye. In some embodiments, the first dye is a fluorescent dye. In

some embodiments, the first dye is Alexa Fluor 647.

[0017] In some embodiments of the methods described above, the coupling of the first probe to the PH1 oligonucleotide comprises nucleotide hybridization. In some embodiments, the coupling of the first probe to the PH1 oligonucleotide comprises a covalent bond.

[0018] In some embodiments of the methods described above, the H1 oligonucleotide forms a hairpin loop structure.

[0019] In some embodiments of the methods described above, a signal generated by the third probe is amplified through sequential hybridization comprising binding a fourth probe comprising a hybridization 2 (H2) oligonucleotide to the H1 oligonucleotide, wherein said H2 oligonucleotide is labeled.

[0020] In some embodiments of the methods described above, the H1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 6, the reverse complement of SEQ ID NO: 6, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 6, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 6.

[0021] In some embodiments of the methods described above, the H1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3857, the reverse complement of SEQ ID NO: 3857, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3857, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3857.

[0022] In various embodiments of the methods described above, the H2 oligonucleotide forms a hairpin loop structure. In some embodiments the H2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 8, the reverse complement of SEQ ID NO: 8, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 8, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 8.

[0023] In various embodiments of the methods described above, the H2 oligonucleotide forms a hairpin loop structure. In some embodiments the H2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3858, the reverse complement of SEQ ID NO: 3858, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3858, or a nucleotide sequence complementary to a nucleotide

sequence with at least 75% sequence identity to SEQ ID NO: 3858.

[0024] In various embodiments of the methods described above, the H2 oligonucleotide is labeled with a second dye. In some embodiments, the second dye is a fluorescent dye. In some embodiments, the second dye is Alexa Fluor 647.

[0025] In some embodiments of the methods described above, the PH1 oligonucleotide and the PH2 oligonucleotide each form hairpin loop structures. In some embodiments, the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 4, the reverse complement of SEQ ID NO: 4, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4. In some embodiments, the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3854, the reverse complement of SEQ ID NO: 3854, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854. In some embodiments, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 2, the reverse complement of SEQ ID NO: 2, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 2, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 2. In some embodiments, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3855, the reverse complement of SEQ ID NO: 3855, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3855, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3855.

[0026] In some embodiments of the methods described above, a plurality of PH1 oligonucleotides are coupled to the first oligonucleotide through a linker oligonucleotide. In some embodiments, four PH1 oligonucleotides are coupled to the first oligonucleotide. In some embodiments, the linker oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 12, the reverse complement of SEQ ID NO: 12, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 12, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 12. In some embodiments, the first oligonucleotide is coupled to the linker oligonucleotide through nucleotide hybridization. In some

embodiments, the first oligonucleotide is coupled to the linker oligonucleotide through a covalent bond.

[0027] In some embodiments of the methods described above, the method further comprises contacting the cell with an activator oligonucleotide under conditions allowing the activator oligonucleotide to bind the PH1 or PH2 oligonucleotide. In some embodiments, the method further comprises the activator oligonucleotide binding to the PH1 oligonucleotide causing a first hairpin formed by the PH1 oligonucleotide to open, and a portion of the PH1 oligonucleotide made available by the opening of the first hairpin binding to the PH2 oligonucleotide causing a second hairpin formed by the PH2 oligonucleotide to open and make available said sequence made available when said PH1 and PH2 oligonucleotides have hybridized. In some embodiments, the method further comprises the activator oligonucleotide binding to the PH2 oligonucleotide causing a first hairpin formed by the PH2 oligonucleotide to open, and a portion of the PH2 oligonucleotide made available by the opening of the first hairpin binding to the PH1 oligonucleotide causing a second hairpin formed by the PH1 oligonucleotide to open and make available said sequence made available when said PH1 and PH2 oligonucleotides have hybridized. In some embodiments, the activator oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 1, the reverse complement of SEQ ID NO: 1, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 1, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 1 when the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 4, the reverse complement of SEQ ID NO: 4, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4. In some embodiments, the activator oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3856, the reverse complement of SEQ ID NO: 3856, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3856, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3856 when the PH1 oligonucleotide comprises a nucleotide sequences of SEQ ID NO: 3854, the reverse complement of SEQ ID NO: 3854, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854, or a nucleotide sequence complementary to a nucleotide sequence with

at least 75% sequence identity to SEQ ID NO: 3854.

[0028] In some embodiments of the methods described above, wherein, step b) comprises providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a unique chromatin modification or a set of chromatin modifications of interest, step c) comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes, step f) comprises contacting the cell with each third probe of the plurality of third probes, and step g) comprises detecting each label of each third probe. In some embodiments, the plurality of third probes are labeled with a plurality of dyes. In some embodiments, the plurality of third probes are labeled with the same dye. In some embodiments, the dye(s) are fluorescent dye(s). In some embodiments, a signal generated by the plurality of third probes is amplified through sequential hybridization comprising binding a plurality of fourth probes, each of which comprises a hybridization 2 (H2) oligonucleotide to the H1 oligonucleotide, wherein said H2 oligonucleotide is labeled. In some embodiments, the H2 oligonucleotide is labeled with a dye different from the dye(s) used to label the plurality of third probes. In some embodiments, the dye is a fluorescent dye.

[0029] In various embodiments of the methods described above, the H1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 7, the reverse complement of SEQ ID NO: 7, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 7, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 7.

[0030] In some embodiments of the methods described above, the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 5, the reverse complement of SEQ ID NO: 5, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 5, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 5. In some embodiments, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3, the reverse complement of SEQ ID NO: 3, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3.

[0031] In some embodiments of the methods described above, when the H1 oligonucleotide binds to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, the 3' end of the PH1 oligonucleotide is disposed proximal to the 5' end of the PH2 oligonucleotide or the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide. In some embodiments, the method further comprises covalently coupling the PH1 oligonucleotide to the PH2 oligonucleotide through a click reaction. In some embodiments, the click reaction is copper-catalyzed. In some embodiments, the method comprises covalently coupling the PH1 oligonucleotide to the PH2 oligonucleotide through an enzymatic ligation reaction. In some embodiments, when the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide, the PH1 oligonucleotide comprises a phosphate modification at its 5' end. In some embodiments, the 5' phosphate-modified PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3860, the reverse complement of SEQ ID NO: 3860, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3860, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3860. In one embodiment, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3861, the reverse complement of SEQ ID NO: 3861, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3861, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3861. In alternative embodiments, when the 5' end of the PH2 oligonucleotide is disposed proximal to the 3' end of the PH1 oligonucleotide, the PH2 oligonucleotide comprises a phosphate modification at its 5' end. In some embodiments, the 5' phosphate-modified PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3, the reverse complement of SEQ ID NO: 3, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3. In various embodiments, the enzymatic ligation reaction is catalyzed by a T4 DNA ligase, T7 DNA ligase, T3 DNA ligase, Taq DNA ligase, Ampligase, *E. coli* DNA ligase, or SplintR ligase.

[0032] In some embodiments of the methods described above, the PH1 oligonucleotide, the PH2 oligonucleotide, and the H1 oligonucleotide each comprise

DNA.

[0033] In some embodiments of the methods described above, a signal generated by the third probe is amplified through branched amplification.

[0034] In some embodiments of the methods described above, the antibody is biotinylated. In some embodiments, the PH2 oligonucleotide is biotinylated.

[0035] In some embodiments of the methods described above, the first probe does not comprise biotin.

[0036] In some embodiments of the methods described above, the cell is a mammalian cell.

[0037] In some embodiments of the methods described above, the method further comprises quantitating an epigenetic modification level of the genomic locus of interest.

[0038] In some embodiments of the methods described above, the method further comprises identifying each of the plurality of first probes using a barcoding scheme.

[0039] In some embodiments of the methods described above, step d) precedes step e).

[0040] In some embodiments of the methods described above, steps d) and e) take place simultaneously.

[0041] In some embodiments of the methods described above, step e) precedes step d).

[0042] In some embodiments of the methods described above, steps f) and g) precede steps i) and j).

[0043] In some embodiments of the methods described above, steps f) and g) take place simultaneously with steps i) and j).

[0044] In some embodiments of the methods described above, steps i) and j) precede steps f) and g).

[0045] In some embodiments of the methods described above, the method is carried out at a genomic locus within the cell. In some embodiments, the method is carried out at a genomic locus on a chromosome spread, extracted chromatin or extracted DNA outside of the cell or extra-cellular DNA, for example circulating free DNA, cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA).

[0046] In another aspect, provided herein is a method for diagnosing, prognosing, and/or predicting treatment response of a disease in a subject, the method comprising

in situ visualization of a chromatin modification of a cell of the subject according to any one of the methods described above. In some embodiments, the disease is a cancer.

[0047] In another aspect, provided herein is a method of *in situ* visualization of a chromatin modification at a genomic locus in a cell comprising the steps of:

- a) providing at least one first probe comprising a first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein said first oligonucleotide binds to a genomic locus of interest, and wherein said PH1 oligonucleotide forms a first hairpin loop structure,
- b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest, and wherein said PH2 oligonucleotide forms a second hairpin loop structure,
- c) providing an activator oligonucleotide, wherein the activator oligonucleotide is capable of binding to either the PH1 oligonucleotide or the PH2 oligonucleotide, wherein binding of the activator oligonucleotide with the PH1 oligonucleotide causes the first hairpin loop structure to open, and wherein binding of the activator oligonucleotide with the PH2 oligonucleotide causes the second hairpin loop structure to open,
- d) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is labeled,
- e) contacting the cell with the first probe under conditions that allow binding of said first oligonucleotide of said first probe to said genomic locus of the cell,
- f) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
- g) contacting the cell with the activator oligonucleotide under conditions that allow binding of said activator oligonucleotide to said PH1 oligonucleotide or said PH2 oligonucleotide, wherein said binding of said activator causes PH1 and PH2 to hybridize,
- h) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to a sequence made

available when said PH1 and PH2 oligonucleotides have hybridized, and

i) detecting the label of the third probe.

[0048] In some embodiments of the method described above, step a) comprises providing a plurality of first probes, each of which targets a genomic locus of interest; wherein step e) comprises contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell, and wherein the method further comprises the steps of:

j) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,

k) contacting the cell with each labeled readout probe, and

l) detecting each label of each readout probe.

[0049] In some embodiments of the methods described above, the plurality of readout probes are labeled with a plurality of dyes. In some embodiments, the plurality of readout probes are labeled with the same dye. In some embodiments, the dye(s) are fluorescent dye(s).

[0050] In some embodiments of the methods described above, each of said plurality of first oligonucleotides comprises one or more readout probe binding sites each selectively bound by one of the plurality of labeled readout probes. In some embodiments, the one or more readout probe binding sites comprises a nucleic acid sequence of any one of SEQ ID NOs: 9 to 11 and 13 to 147, the reverse complement of any one of SEQ ID NOs: 9 to 11 and 13 to 147, or a nucleic acid sequence sharing at least about 50%, 55%, 60%, 65%, 70%, 75%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity with any one of SEQ ID NOs: 9 to 11 and 13 to 147 or with the reverse complement of any one of SEQ ID NOs: 9 to 11 and 13 to 147. In some embodiments, the one or more readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 9, the reverse complement of SEQ ID NO: 9, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9. In some embodiments, the one or more readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 10, the reverse complement of SEQ ID NO: 10, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO:

10, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10. In some embodiments, the one or more readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 11, the reverse complement of SEQ ID NO: 11, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11.

[0051] In some embodiments of the methods described above, the chromatin modification is a DNA modification, a DNA-binding protein (including proteins that bind directly or indirectly to DNA), or a modification to a DNA-binding protein. In some embodiments, the chromatin modification is a histone modification.

[0052] In some embodiments of the methods described above, the cell is fixed. In some embodiments the genomic locus of interest is disposed within the nucleus of the cell.

[0053] In some embodiments of the methods described above, the method further comprises determining in three dimensions a location of the third probe. In some embodiments, the method further comprises using the location of the third probe to analyze chromatin structure. In some embodiments the method further comprises determining in three dimensions a location of the readout probe. In some embodiments, the method further comprises using the location of the readout probe to analyze chromatin structure.

[0054] In some embodiments of the methods described above, the antibody is coupled to the PH2 oligonucleotide by a biotin-streptavidin bridge. In some embodiments, the antibody is coupled to the PH2 oligonucleotide by a covalent bond. In some embodiments, coupling of the antibody to the PH2 oligonucleotide comprises nucleotide hybridization.

[0055] In some embodiments of the methods described above, the H1 oligonucleotide is labeled with a first dye. In some embodiments, the first dye is a fluorescent dye. In some embodiments, the first dye is Alexa Fluor 647.

[0056] In some embodiments of the methods described above, coupling of the first probe to the PH1 oligonucleotide comprises nucleotide hybridization. In some embodiments, coupling of the first probe to the PH1 oligonucleotide comprises a covalent bond.

[0057] In some embodiments of the methods described above, the H1 oligonucleotide forms a hairpin loop structure.

[0058] In some embodiments of the methods described above, a signal generated by the third probe is amplified through sequential hybridization comprising binding a fourth probe comprising a hybridization 2 (H2) oligonucleotide to the H1 oligonucleotide, wherein said H2 oligonucleotide is labeled.

[0059] In some embodiments of the methods described above, the H1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 6, the reverse complement of SEQ ID NO: 6, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 6, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 6.

[0060] In some embodiments of the methods described above, the H1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3857, the reverse complement of SEQ ID NO: 3857, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3857, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3857.

[0061] In various embodiments of the methods described above, the H2 oligonucleotide forms a hairpin loop structure. In some embodiments, the H2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 8, the reverse complement of SEQ ID NO: 8, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 8, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 8.

[0062] In various embodiments of the methods described above, the H2 oligonucleotide forms a hairpin loop structure. In some embodiments, the H2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3858, the reverse complement of SEQ ID NO: 3858, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3858, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3858.

[0063] In various embodiments of the methods described above, the H2 oligonucleotide is labeled with a second dye. In some embodiments, the second dye is a fluorescent dye. In some embodiments, the second dye is Alexa Fluor 647.

[0064] In some embodiments of the methods described above, the PH1

oligonucleotide and the PH2 oligonucleotide each form hairpin loop structures. In some embodiments, the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 4, the reverse complement of SEQ ID NO: 4, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4.

[0065] In some embodiments of the methods described above, the PH1 oligonucleotide and the PH2 oligonucleotide each form hairpin loop structures. In some embodiments, the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3854, the reverse complement of SEQ ID NO: 3854, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854.

[0066] In some embodiments of the methods described above, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 2, the reverse complement of SEQ ID NO: 2, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 2, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 2.

[0067] In some embodiments of the methods described above, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3855, the reverse complement of SEQ ID NO: 3855, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3855, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3855.

[0068] In some embodiments of the methods described above, a plurality of PH1 oligonucleotides are coupled to the first oligonucleotide through a linker oligonucleotide. In some embodiments, four PH1 oligonucleotides are coupled to the first oligonucleotide. In some embodiments, the linker oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 12, the reverse complement of SEQ ID NO: 12, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 12, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 12. In some embodiments, the first oligonucleotide is coupled to the linker oligonucleotide through nucleotide hybridization. In some embodiments, the first oligonucleotide is coupled to the linker oligonucleotide through a

covalent bond.

[0069] In some embodiments of the methods described above, step g) further comprises, the activator oligonucleotide hybridizing with the PH1 oligonucleotide and causing the first hairpin loop structure to open, and a portion of the PH1 oligonucleotide being made available by the opening of the first hairpin loop structure subsequently hybridizing to the PH2 oligonucleotide and causing the second hairpin loop structure to open and make available said sequence made available when said PH1 and PH2 oligonucleotides have hybridized.

[0070] In some embodiments of the methods described above, the activator oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 1, the reverse complement of SEQ ID NO: 1, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 1, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 1 when the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 4, the reverse complement of SEQ ID NO: 4, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4.

[0071] In some embodiments of the methods described above, the activator oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3856, the reverse complement of SEQ ID NO: 3856, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3856, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3856 when the PH1 oligonucleotide comprises a nucleotide sequences of SEQ ID NO: 3854, the reverse complement of SEQ ID NO: 3854, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854.

[0072] In some embodiments of the methods described above,

- step a) comprises providing a plurality of first probes each comprising a first oligonucleotide coupled to a unique proximity hybridization (PH1) oligonucleotide,
- step d) comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the

- unique PH1 oligonucleotides of said plurality of first probes,
- step h) comprises contacting the cell with each third probe of the plurality of third probes, and
- step i) comprises detecting each label of each third probe.

[0073] In some embodiments of the methods described above, the antibody is coupled to a plurality of unique PH2 oligonucleotides, wherein each unique PH2 oligonucleotide comprises a nucleotide sequence capable of binding to one of the unique PH1 oligonucleotides, thereby causing a first hairpin loop structure formed by the unique PH1 oligonucleotide to open. In some embodiments, the antibody is coupled to the plurality of unique PH2 oligonucleotides through nucleotide hybridization of unique PH2 oligonucleotides to an antibody linker oligonucleotide that is covalently coupled to the antibody.

[0074] In some embodiments of the methods described above, the method further comprises providing a plurality of unique activator oligonucleotides, wherein each unique activator oligonucleotide is capable of binding to one of the unique PH2 oligonucleotide and thereby causing a second hairpin loop structure formed by the unique PH2 oligonucleotide to open and make available said nucleotide sequence capable of binding to one of the unique PH1 oligonucleotides.

[0075] In some embodiments of the methods described above,

step b) comprises providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a unique chromatin modification or set of chromatin modifications of interest,

step d) comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes,

step h) comprises contacting the cell with each third probe of the plurality of third probes, and

step i) comprises detecting each label of each third probe.

[0076] In some embodiments of the methods described above, the first oligonucleotide is coupled to a plurality of unique PH1 oligonucleotides, wherein each unique PH1 oligonucleotide comprises a nucleotide sequence capable of binding to one

of the unique PH2 oligonucleotides, thereby causing a second hairpin loop structure formed by the unique PH2 oligonucleotide to open.

[0077] In some embodiments of the methods described above, the first oligonucleotide is coupled to the plurality of unique PH1 oligonucleotides through nucleotide hybridization to a linker oligonucleotide.

[0078] In some embodiments of the methods described above, the method further comprises providing a plurality of unique activator oligonucleotides, wherein each unique activator oligonucleotide is capable of binding to one of the unique PH1 oligonucleotide and thereby causing a first hairpin loop structure formed by the unique PH1 oligonucleotide to open and make available said nucleotide sequence capable of binding to one of the unique PH2 oligonucleotides.

[0079] In some embodiments of the methods described above, each of said plurality of second probes comprises a unique antibody covalently coupled to an antibody linker oligonucleotide, and wherein a unique PH2 oligonucleotide is coupled to the antibody through nucleotide hybridization to the antibody linker oligonucleotide.

[0080] In some embodiments of the methods described above, the plurality of third probes is labeled with a plurality of dyes. In some embodiments, the plurality of third probes is labeled with the same dye. In some embodiments, the dye(s) are fluorescent dye(s).

[0081] In some embodiments of the methods described above, the first oligonucleotide is coupled to a plurality of PH1 oligonucleotides by branched amplification.

[0082] In some embodiments of the methods described above, the antibody is coupled to a plurality of PH2 oligonucleotides by branched amplification.

[0083] In some embodiments of the methods described above, the PH1 oligonucleotide, the PH2 oligonucleotide, and the H1 oligonucleotide each comprise DNA.

[0084] In some embodiments of the methods described above, a signal generated by the third probe is amplified through branched amplification.

[0085] In some embodiments of the methods described above, the antibody is biotinylated. In some embodiments, the PH2 oligonucleotide is biotinylated.

[0086] In some embodiments of the methods described above, the first probe does

not comprise biotin.

[0087] In some embodiments of the methods described above, the cell is a mammalian cell.

[0088] In some embodiments of the methods described above, the method further comprises quantitating an epigenetic modification level of the genomic locus of interest.

[0089] In some embodiments of the methods described above, the method further comprises identifying each of the plurality of first probes using a barcoding scheme.

[0090] In some embodiments of the methods described above, step e) precedes step f).

[0091] In some embodiments of the methods described above, steps e) and f) take place simultaneously.

[0092] In some embodiments of the methods described above, step f) precedes step e).

[0093] In some embodiments of the methods described above, steps g), h) and i) precede steps k) and l).

[0094] In some embodiments of the methods described above, steps g), h) and i) take place simultaneously with steps k) and l).

[0095] In some embodiments of the methods described above, steps k) and l) proceed steps g), h) and i).

[0096] In some embodiments of the methods described above, the method is carried out at a genomic locus within the cell. In some embodiments, the method is carried out at a genomic locus on a chromosome spread, extracted chromatin or extracted DNA outside of the cell or extra-cellular DNA, for example circulating free DNA, cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA).

[0097] In a related aspect, provided herein is a kit for *in situ* visualization of a chromatin modification of a cell according to any one of the methods described above, the kit comprising the first probe, the second probe, the activator oligonucleotide, and the third probe. In some embodiments, the kit comprises one or more said labeled readout probes.

[0098] In another aspect, provided herein is a method for *in situ* visualization of a chromatin modification at a genomic locus in a cell comprising the steps of:

a) providing at least one first probe comprising a first oligonucleotide coupled

- to a proximity hybridization 1 (PH1) oligonucleotide, wherein said first oligonucleotide binds to a genomic locus of interest,
- b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest,
 - c) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is coupled to a label,
 - d) contacting the cell with the first probe under conditions that allow binding of said first oligonucleotide of said first probe to said genomic locus of the cell,
 - e) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
 - f) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, wherein when the H1 oligonucleotide binds to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, the 3' end of the PH1 oligonucleotide is disposed proximal to the 5' end of the PH2 oligonucleotide or the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide, and
 - g) detecting the label coupled to the third probe.

[0099] In some embodiments of the method described above,

- step a) comprises providing a plurality of first probes, each of which targets a genomic locus of interest; wherein step d) comprises contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell, and wherein the method further comprises the steps of:
- h) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,
 - i) contacting the cell with each labeled readout probe, and

j) detecting each label of each readout probe.

[00100] In some embodiments of the methods described above, the plurality of readout probes are labeled with a plurality of dyes. In some embodiments, the plurality of readout probes are labeled with the same dye. In some embodiments, the dye(s) are fluorescent dye(s).

[00101] In some embodiments of the methods described above, each of said plurality of first oligonucleotides comprises one or more readout probe binding sites each selectively bound by one of the plurality of labeled readout probes. In some embodiments, the one or more readout probe binding sites comprises a nucleic acid sequence of any one of SEQ ID NOs: 9 to 11 and 13 to 147, the reverse complement of any one of SEQ ID NOs: 9 to 11 and 13 to 147, or a nucleic acid sequence sharing at least about 50%, 55%, 60%, 65%, 70%, 75%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity with any one of SEQ ID NOs: 9 to 11 and 13 to 147 or with the reverse complement of any one of SEQ ID NOs: 9 to 11 and 13 to 147. In some embodiments, the one or more readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 9, the reverse complement of SEQ ID NO: 9, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9. In some embodiments, the one or more readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 10, the reverse complement of SEQ ID NO: 10, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10. In some embodiments, the one or more unique readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 11, the reverse complement of SEQ ID NO: 11, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11.

[00102] In some embodiments of the methods described above, the chromatin modification is a DNA modification, a DNA-binding protein (including proteins that bind directly or indirectly to DNA), or a modification to a DNA-binding protein. In some embodiments, the chromatin modification is a histone modification.

[00103] In some embodiments of the methods described above, the cell is fixed.

[00104] In some embodiments of the methods described above, the genomic locus of interest is disposed within the nucleus of the cell.

[00105] In some embodiments of the methods described above, the method further comprises determining in three dimensions a location of the third probe. In some embodiments, the method further comprises using the location of the third probe to analyze chromatin structure. In some embodiments, the method further comprises determining in three dimensions a location of the readout probe. In some embodiments, the method further comprises using the location of the readout probe to analyze chromatin structure.

[00106] In some embodiments of the methods described above, the antibody is coupled to the PH2 oligonucleotide by a biotin-streptavidin bridge. In some embodiments, the antibody is coupled to the PH2 oligonucleotide by a covalent bond. In some embodiments, coupling of the antibody to the PH2 oligonucleotide comprises nucleotide hybridization. In some embodiments the antibody is coupled to the PH2 oligonucleotide through a DBCO-mediated copper-free click reaction.

[00107] In some embodiments of the methods described above, the H1 oligonucleotide is labeled with or coupled to a first dye. In some embodiments, the first dye is a fluorescent dye. In some embodiments, the first dye is Alexa Fluor 647. In some embodiments, the H1 oligonucleotide is labeled with or coupled to the first dye by being bound by a readout probe comprising the first dye. In some embodiments, the H1 oligonucleotide is labeled with or coupled to the first dye by a covalent bond.

[00108] In some embodiments of the methods described above, coupling of the first probe to the PH1 oligonucleotide comprises nucleotide hybridization. In some embodiments, coupling of the first probe to the PH1 oligonucleotide comprises a covalent bond.

[00109] In some embodiments of the methods described above, the H1 oligonucleotide forms a hairpin loop structure.

[00110] In some embodiments of the methods described above, a signal generated by the third probe is amplified through sequential hybridization comprising binding a fourth probe comprising a hybridization 2 (H2) oligonucleotide to the H1 oligonucleotide, wherein said H2 oligonucleotide is labeled. In some embodiments, the H2 oligonucleotide is labeled with a second dye. In some embodiments, the second dye is

a fluorescent dye. In some embodiments, the second dye is Alexa Fluor 647. In some embodiments, the H2 oligonucleotide is labeled with the second dye by being bound by a readout probe comprising the second dye. In some embodiments, the H2 oligonucleotide is labeled with the second dye by a covalent bond. In some embodiments, the H2 oligonucleotide forms a hairpin loop structure.

[00111] In some embodiments of the methods described above, the H1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 7, the reverse complement of SEQ ID NO: 7, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 7, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 7.

[00112] In some embodiments of the methods described above, the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 5, the reverse complement of SEQ ID NO: 5, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 5, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 5.

[00113] In some embodiments of the methods described above, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3, the reverse complement of SEQ ID NO: 3, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3.

[00114] In some embodiments of the methods described above, the method further comprises covalently coupling the PH1 oligonucleotide to the PH2 oligonucleotide through a click reaction. In some embodiments, the click reaction is copper-catalyzed. In some embodiments, the PH1 oligonucleotide is azide-modified and the PH2 oligonucleotide is hexynyl-modified, or the PH1 oligonucleotide is hexynyl-modified and the PH2 oligonucleotide is azide-modified. In some embodiments, the method comprises covalently coupling the PH1 oligonucleotide to the PH2 oligonucleotide through an enzymatic ligation reaction. In some embodiments, when the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide, the PH1 oligonucleotide comprises a phosphate modification at its 5' end. In some embodiments, the 5' phosphate-modified PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3860, the reverse complement of SEQ ID NO: 3860, a

nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3860, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3860. In one embodiment, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3861, the reverse complement of SEQ ID NO: 3861, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3861, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3861. In alternative embodiments, when the 5' end of the PH2 oligonucleotide is disposed proximal to the 3' end of the PH1 oligonucleotide, the phosphate-modified PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3, the reverse complement of SEQ ID NO: 3, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3. In various embodiments, the enzymatic ligation reaction is catalyzed by a T4 DNA ligase, T7 DNA ligase, T3 DNA ligase, Taq DNA ligase, Ampligase, E. coli DNA ligase, or SplintR ligase.

[00115] In some embodiments of the methods described above,

step b) comprises providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a unique chromatin modification or set of chromatin modifications of interest,

step c) comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes,

step f) comprises contacting the cell with each third probe of the plurality of third probes, and

step i) comprises detecting each label coupled to each third probe.

[00116] In some embodiments of the methods described above, each third probe is coupled to a label by being bound by a labeled readout probe.

[00117] In some embodiments of the methods described above, the first oligonucleotide is coupled to a plurality of unique PH1 oligonucleotides, wherein each unique H1 oligonucleotide selectively binds to a sequence of each of one of the unique PH2 oligonucleotides and one of the unique PH1 oligonucleotides, wherein when each

unique H1 oligonucleotide binds to a nucleotide sequence of each of said one of the unique PH1 and PH2 oligonucleotides, the 3' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 5' end of the one of the unique PH2 oligonucleotides or the 5' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 3' end of the one of the unique PH2 oligonucleotides.

[00118] In some embodiments of the methods described above, the first oligonucleotide is coupled to the plurality of unique PH1 oligonucleotides through nucleotide hybridization to a linker oligonucleotide.

[00119] In some embodiments of the methods described above, the plurality of third probes is coupled to a plurality of dyes. In some embodiments, the plurality of third probes is coupled to the same dye. In some embodiments, the dye(s) are fluorescent dye(s).

[00120] In some embodiments of the methods described above, each unique PH1 oligonucleotide is covalently coupled to a corresponding unique PH2 oligonucleotide by a click reaction. In some embodiments of the methods described above, each unique PH1 oligonucleotide is covalently coupled to a corresponding unique PH2 oligonucleotide by an enzymatic ligation reaction.

[00121] In some embodiments of the methods described above, the PH1 oligonucleotide, the PH2 oligonucleotide, and the H1 oligonucleotide each comprise DNA.

[00122] In some embodiments of the methods described above, a signal generated by the third probe is amplified through branched amplification.

[00123] In some embodiments of the methods described above, the antibody is biotinylated. In some embodiments, the PH2 oligonucleotide is biotinylated.

[00124] In some embodiments of the methods described above, the first probe does not comprise biotin.

[00125] In some embodiments of the methods described above, the cell is a mammalian cell.

[00126] In some embodiments of the methods described above, the method further comprises quantitating an epigenetic modification level of the genomic locus of interest.

[00127] In some embodiments of the methods described above, the method further comprises identifying each of the plurality of first probes using a barcoding scheme.

[00128] In some embodiments of the methods described above, step d) precedes step e).

[00129] In some embodiments of the methods described above, steps d) and e) take place simultaneously.

[00130] In some embodiments of the methods described above, step e) precedes step d).

[00131] In some embodiments of the methods described above, steps f) and g) precede steps i) and j).

[00132] In some embodiments of the methods described above, steps f) and g) take place simultaneously with steps i) and j).

[00133] In some embodiments of the methods described above, steps i) and j) precede step f) and g).

[00134] In some embodiments of the methods described above, the method is carried out at a genomic locus within the cell. In some embodiments, the method is carried out at a genomic locus on a chromosome spread, extracted chromatin or extracted DNA outside of the cell or extra-cellular DNA, for example circulating free DNA, cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA).

[00135] In a related aspect, provided herein is a kit for *in situ* visualization of a chromatin modification of a cell according to any of the methods described herein, the kit comprising the first probe, the second probe, and the third probe. In some embodiments, the kit comprises one or more said labeled readout probes.

[00136] In another aspect, provided herein is a method of *in situ* visualization of a chromatin modification of a cell at a plurality of genomic loci comprising the steps of:

- a) providing a plurality of first probes each comprising a unique first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein each said unique first oligonucleotide binds to a genomic locus of interest, and wherein said PH1 oligonucleotide forms a first hairpin loop structure,
- b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest, and wherein said PH2 oligonucleotide forms a second hairpin loop structure,

- c) providing an activator oligonucleotide, wherein the activator oligonucleotide is capable of binding to either the PH1 oligonucleotide or the PH2 oligonucleotide, wherein binding of the activator oligonucleotide with the PH1 oligonucleotide causes the first hairpin loop structure to open, and wherein binding of the activator oligonucleotide with the PH2 oligonucleotide causes the second hairpin loop structure to open,
- d) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is labeled,
- e) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said unique first oligonucleotides of the said plurality of first probes,
- f) contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell,
- g) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
- h) contacting the cell with the activator oligonucleotide under conditions that allow binding of said activator oligonucleotide to said PH1 oligonucleotide or said PH2 oligonucleotide, wherein said binding of said activator causes PH1 and PH2 to hybridize,
- i) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to a sequence made available when said PH1 and PH2 oligonucleotides have hybridized,
- j) detecting the label of the third probe,
- k) contacting the cell with each labeled readout probe, and
- l) detecting each label of each readout probe.

[00137] In another aspect, provided herein is a method of *in situ* visualization of a plurality of chromatin modifications at a genomic locus of a cell comprising the steps of:

- a) providing at least one first probe comprising a first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides, wherein said first oligonucleotide binds to a genomic locus of interest, and

wherein each of said plurality of unique PH1 oligonucleotides forms a hairpin loop structure,

- b) providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each of said unique antibodies recognizes a unique chromatin modification or set of chromatin modifications of interest, and wherein each of said PH2 oligonucleotides forms a hairpin loop structure,
- c) providing a plurality of unique activator oligonucleotides, wherein each unique activator oligonucleotide is capable of binding to a corresponding one of the plurality of unique PH1 or PH2 oligonucleotides, and wherein binding of one of the plurality of unique activator oligonucleotides with the corresponding one of the plurality of PH1 or PH2 oligonucleotides causes the hairpin loop structure formed by the one of the plurality of PH1 or PH2 oligonucleotides to open,
- d) providing a plurality of third probes each comprising a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes or to one of the unique PH1 oligonucleotides, wherein each said unique H1 oligonucleotides is labeled,
- e) contacting the cell with the first probe under conditions that allow binding of said first oligonucleotide of said first probe to said genomic locus of the cell,
- f) contacting the cell with the plurality of second probes under conditions that allow each of said unique antibodies of said plurality of second probes to bind to said unique chromatin modification or set of chromatin modifications,
- g) contacting the cell with each unique activator oligonucleotide under conditions that allow binding of each said unique activator oligonucleotide to said corresponding one of the plurality of PH1 or PH2 oligonucleotides, wherein said binding of said activator causes one of the unique PH1 oligonucleotides and one of the unique PH2 oligonucleotides to hybridize,
- h) contacting the cell with each of the plurality of third probes under

conditions that allow binding of the unique H1 oligonucleotide of each said third probe to a sequence made available when one of the plurality of unique PH1 oligonucleotides and one of the plurality of unique PH2 oligonucleotides have hybridized, wherein the one of the plurality of unique PH2 or PH1 oligonucleotides comprises said sequence made available, and

- i) detecting the label of each of the third probes.

[00138] In another aspect, provided herein is a method for *in situ* visualization of a plurality of chromatin modifications at a plurality of genomic loci in a cell comprising the steps of:

- a) providing a plurality of first probes each comprising a unique first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides, wherein each said unique first oligonucleotide binds to a genomic locus of interest, and wherein each of said plurality of unique PH1 oligonucleotides forms a hairpin loop structure,
- b) providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each of said unique antibodies recognizes a unique chromatin modification or set of chromatin modifications of interest, and wherein each of said PH2 oligonucleotides forms a hairpin loop structure,
- c) providing a plurality of unique activator oligonucleotides, wherein each unique activator oligonucleotide is capable of binding to a nucleotide sequence of one of the plurality of unique PH1 or PH2 oligonucleotides, and wherein binding of one of the plurality of unique activator oligonucleotides with a corresponding one of the plurality of unique PH1 or PH2 oligonucleotides causes the hairpin loop structure formed by the unique PH1 or PH2 oligonucleotide to open,
- d) providing a plurality of third probes each comprising a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes or to one of the unique PH1 oligonucleotides, wherein each said unique H1 oligonucleotides is labeled,

- e) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said unique first oligonucleotides of the said plurality of first probes,
- f) contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell,
- g) contacting the cell with the plurality of second probes under conditions that allow each of said unique antibodies of said plurality of second probes to bind to said unique chromatin modification or set of chromatin modifications,
- h) contacting the cell with each unique activator oligonucleotide under conditions that allow binding of each said unique activator oligonucleotide to the corresponding one of the plurality of PH1 or PH2 oligonucleotides, wherein said binding of said activator causes one of the unique PH1 oligonucleotides and one of the unique PH2 oligonucleotides to hybridize,
- i) contacting the cell with each of the plurality of third probes under conditions that allow binding of the unique H1 oligonucleotide of each said third probe to a sequence made available when one of the plurality of unique PH1 and one of the plurality of unique PH2 oligonucleotides have hybridized, wherein the one of the plurality of unique PH2 or PH1 oligonucleotides comprises said sequence made available,
- j) detecting the label of each of the third probes,
- k) contacting the cell with each labeled readout probe, and
- l) detecting each label of each readout probe.

[00139] In another aspect, provided herein is a method for *in situ* visualization of a chromatin modification at a plurality of genomic loci in a cell comprising the steps of:

- a) providing a plurality of first probes each comprising a unique first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein each said unique first oligonucleotide binds to a genomic locus of interest,
- b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a

- chromatin modification or set of chromatin modifications of interest,
- c) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is coupled to a label,
 - d) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,
 - e) contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell,
 - f) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
 - g) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, wherein when the H1 oligonucleotide binds to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, the 3' end of the PH1 oligonucleotide is disposed proximal to the 5' end of the PH2 oligonucleotide or the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide,
 - h) detecting the label coupled to each third probe,
 - i) contacting the cell with each labeled readout probe, and
 - j) detecting each label of each readout probe.

[00140] In another aspect, provided herein is a method for *in situ* visualization of a plurality of chromatin modifications at a genomic locus in a cell comprising the steps of:

- a) providing at least one first probe comprising a first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides, wherein said first oligonucleotide binds to a genomic locus of interest,
- b) providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a chromatin modification or set of chromatin modifications of interest,

- c) providing a plurality of third probes, each of which comprises a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes and to one of the unique PH1 oligonucleotides, wherein said H1 oligonucleotide is coupled to a label,
- d) contacting the cell with the first probe under conditions that allow binding of said first oligonucleotide of said first probe to said genomic locus of the cell,
- e) contacting the cell with each of the plurality of second probes under conditions that allow binding of said unique antibodies of said second probes to said chromatin modification or set of chromatin modifications,
- f) contacting the cell with each third probe of the plurality of third probes under conditions that allow binding of each said unique H1 oligonucleotide of each of said plurality of third probes to a nucleotide sequence of each of one of the unique PH2 oligonucleotides and one of the unique PH1 oligonucleotides, wherein when the unique H1 oligonucleotide binds to a nucleotide sequence of each of said one of the unique PH1 and PH2 oligonucleotides, the 3' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 5' end of the one of the unique PH2 oligonucleotides or the 5' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 3' end of the one of the unique PH2 oligonucleotides, and
- g) detecting each label coupled to each third probe.

[00141] In another aspect, provided herein is a method for *in situ* visualization of a plurality of chromatin modifications at a plurality of genomic loci in a cell comprising the steps of:

- a) providing a plurality of first probes each comprising a unique first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides, wherein each said unique first oligonucleotide binds to a genomic locus of interest,
- b) providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide,

wherein each unique antibody recognizes a chromatin modification or set of chromatin modifications of interest,

- c) providing a plurality of third probes, each of which comprises a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes and to one of the unique PH1 oligonucleotides, wherein said H1 oligonucleotide is coupled to a label,
- d) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,
- e) contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell,
- f) contacting the cell with each of the plurality of second probes under conditions that allow binding of said unique antibodies of said second probe to said chromatin modification or set of chromatin modifications,
- g) contacting the cell with each third probe of the plurality of third probes under conditions that allow binding of each said unique H1 oligonucleotide of each of said plurality of third probes to a nucleotide sequence of each of one of the unique PH2 oligonucleotides and one of the unique PH1 oligonucleotides, wherein when the unique H1 oligonucleotide binds to a nucleotide sequence of each of said one of the unique PH1 and PH2 oligonucleotides, the 3' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 5' end of the one of the unique PH2 oligonucleotides or the 5' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 3' end of the one of the unique PH2 oligonucleotides,
- h) detecting each label coupled to each third probe,
- i) contacting the cell with each labeled readout probe, and
- j) detecting each label of each readout probe.

[00142] In various embodiments of the methods described above, the method is carried out at a genomic locus within the cell.

[00143] In various embodiments of the methods described above, the method is carried out at a genomic locus on a chromosome spread, extracted chromatin or extracted DNA outside of the cell or extra-cellular DNA, for example circulating free DNA, cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA).

[00144] In various embodiments of the methods described above, the step of contacting the cell with the first probe(s) precedes the step of contacting the cell with the second probe(s).

[00145] In various embodiments of the methods described above, the step of contacting the cell with the first probe(s) and the step of contacting the cell with the second probe(s) take place simultaneously.

[00146] In various embodiments of the methods described above, the step of contacting the cell with the second probe(s) precedes the step of contacting the cell with the first probe(s).

[00147] In various embodiments of the methods described above, the steps of contacting the cell with the third probe(s) and detecting the label of the third probe(s) precede the steps of contacting the cell with the readout probe(s) and detecting the label of the readout probe(s).

[00148] In various embodiments of the methods described above, the steps of contacting the cell with the third probe(s) and detecting the label of the third probe(s) take place simultaneously with the steps of contacting the cell with the readout probe(s) and detecting the label of the readout probe(s).

[00149] In various embodiments of the methods described above, the steps of contacting the cell with the readout probe(s) and detecting the label of the readout probe(s) precede the steps of contacting the cell with the third probe(s) and detecting the label of the third probe(s).

BRIEF DESCRIPTION OF THE DRAWINGS

[00150] **Figs. 1A to 1C. Schematic representation of core designs. (Fig. 1A)**

Principle of Epi-PHR system. The PH1 probes can be hybridized to FISH probes' overhang region. The antibodies can be labeled with biotin molecules. Bridged by streptavidin, the biotin-labeled PH2 probes can be indirectly linked to the antibodies. Upon activator hybridization to PH1, the activator opens PH1 hairpin, and the released PH1 structure invades PH2 hairpin in a proximity-dependent manner. Then dye-labeled

H1 can be introduced to generate a readout fluorescent signal. (**Fig. 1B**) Multiplexed sequential DNA FISH probes can consist of a primary targeting sequence, a PH1 docking site, and a readout probe binding sequence. Each group of FISH probes can target to a specific genomic locus and have the same readout probe binding region. By sequentially hybridizing the readout probes to the sample, imaging each readout probe, and bleaching the sample before the next round of hybridization, the spatial organization of chromatin can be traced from the FISH signals. (**Fig. 1C**) In the EZ-Epi-PHR system, the FISH probes can contain primary targeting and EZ-PH1 regions, and the FISH probe can be 3' Azide labeled. The antibodies can be directly labeled with EZ-PH2 probes via a dibenzocyclooctyne (DBCO) mediated copper-free click reaction, and the 5' end of the EZ-PH2 probes can have a hexynyl modification or phosphate modification. Only when EZ-PH1 and EZ-PH2 are collinear, can the EZ-PH1 and EZ-PH2 form a platform to bind and open a dye-labeled EZ-H1. After a hybridization reaction, a copper-catalyzed click reaction or T4 ligase-catalyzed ligation reaction can be triggered, and the EZ-PH1 probes covalently linked with EZ-PH2 to stabilize the structure, then high stringent washing steps can be applied to eliminate non-specific EZ-Epi-PHR signals.

[00151] Figs. 2A to 2C. Demonstration of Epi-PHR by detecting H3K9Me3, MacroH2A, H3K27Me3, and H3K27Ac at human chromosome 9 alpha satellite locus in RPE1 cells. (Fig. 2A) Epi-PHR robustly detected H3K9Me3 marks at alpha satellite locus and showed no detectable Epi-PHR signals in this region when the H3K27Ac antibody was applied instead, indicating the Epi-PHR system can detect epigenetic marks at a specific genomic locus. (**Fig. 2B**) Schematic representation of a signal amplification design. Instead of directly docking PH1, a 150-base linker probe is hybridized to the FISH probe. The linker probe contains four PH1 docking sites. By this design, the detection efficiency of Epi-PHR can be increased. (**Fig. 2C**) After applying the signal amplification design, Epi-PHR robustly detected MacroH2A and H3K27Me3 at human chromosome 9 alpha satellite locus. Scale bar = 2 μ m.

[00152] Fig. 3. Systematic control experiments omitting indicated component in a mock H3K9Me3 Epi-PHR experiment at human chromosome 9 alpha satellite locus. Results showed that the H3K9Me3 Epi-PHR signals in **Fig. 2** were not false positive signals, and that a whole Epi-mFISH assembly is required for generating an

Epi-PHR signal, which validated the Epi-PHR design. Scale bar = 2 μ m.

[00153] Figs. 4A to 4C. Detection of H3K27Me3 and H3K9Me3 at the central 300-kilobase region of Xq22.3 by Epi-PHR. (Fig. 4A) Schematic representation of controlled signal amplification design, the H1 probes, and H2 probes can be introduced in an alternating sequence. By using this design, an uncontrolled chain reaction can be avoided and the signal-to-noise ratio improved. **(Fig. 4B)** The Epi-PHR detected H3K27Me3 at the central 300-kilobase region of Xq22.3 region. As a negative control, the Epi-PHR could not detect H3K9Me3 marks. These results demonstrated that the Epi-PHR doesn't cross-react with epigenetic marks outside the assigned region. **(Fig. 4C)** The H3K27Me3 mark was only detected at one copy of the X chromosome, which is consistent with previous studies that only the inactive X is enriched with the repressive mark. All experiments used the 4-PH1 docking scheme shown in **Fig. 2B** and 2 rounds of controlled signal amplification. Scale bar = 2 μ m.

[00154] Figs. 5A and 5B. Demonstration of Epi-mFISH by detecting H3K9Me3 at different human satellite loci in RPE1 cells. (Fig. 5A) Schematic representation of multiplexed sequential FISH probe design and readout probe hybridization scheme. Each FISH probe can consist of a targeting sequence for a specific satellite region in the genome, and a unique readout probe binding region. All probes can contain a PH1 docking site. After three rounds of readout probe hybridization, imaging and bleaching, the multiplexed sequential FISH signals can pinpoint corresponding satellite regions. **(Fig. 5B)** Epi-mFISH robustly detected H3K9Me3 marks at different satellite loci. According to the mFISH signals, the Epi-PHR signals could be assigned to corresponding genomic loci. Since Hsat2A2 and Hsat2B generated colocalized mFISH signals, the composite images were generated by merging Hsat2A2, Hsat3B5, and Epi-PHR signals. As depicted in the left panels of **Fig. 5B**, Z-stacks of images were acquired using an epifluorescence microscope. Two optical sections from the same cell are shown in the images shown in **Fig. 5B**. Scale bar = 2 μ m.

[00155] Figs. 6A and 6B. Demonstration of EZ-Epi-PHR strategy by detecting H3K9Me3 at the human chromosome 9 alpha satellite locus in RPE1 cells. (Fig. 6A) Schematic representation of experimental design, the EZ- PH1 probes were hybridized to FISH probes' overhang region. The antibodies were labeled with biotin molecules. Bridged by streptavidin, the biotin-labeled EZ-PH2 probes were indirectly

linked to the antibodies. Then dye-labeled EZ-H1 was introduced to generate a readout fluorescent signal. (**Fig. 6B**) The EZ-Epi-PHR strategy detected H3K9Me3 marks at the alpha satellite locus and showed no or weak EZ-Epi-PHR signal when omitting H3K9Me3 antibody. Scale bar = 2 μ m.

[00156] Figs. 7A to 7C. Schematic representation of branched signal

amplification schemes. (**Fig. 7A**) Instead of directly docking PH1, the DNA FISH probe can comprise 4 binding sites for PH1-docking linker oligonucleotides, each of which can dock 4 PH1 oligonucleotides. By this design, each FISH probe can bind 16 copies of PH1. (**Fig. 7B**) Branched amplification strategy to increase the copy number of PH2 on antibodies. This design can label 48 PH2 to each antibody. (**Fig. 7C**) The same amplification scheme can also be applied to Epi-PHR signal amplification.

[00157] Fig. 8. Schematic representation of multiple epigenetic marks (mEpi)

detection scheme. For simplicity, the depicted example employs two sets of proximity hairpin probes (PH1_1/PH2_1 and PH1_2/PH2_2) to detect two epigenetic marks at the same time. Two antibodies can be labeled with PH2_1 and PH2_2 respectively, and each FISH probe can comprise both PH1_1 and PH1_2. After PHR, the dye-labeled readout probes (H1_1 and H1_2) can be sequentially introduced to the system, and the two epigenetic marks can be sequentially detected. The two epigenetic marks can be detected simultaneously in two fluorescent channels if H1_1 and H1_2 are labeled with different fluorescent colors.

[00158] Fig. 9. Schematic representation of combinatorial barcoding scheme. A unique combination of readout regions can be added to each set of FISH probes. After multiple rounds of hybridization, imaging, and bleaching, the specific round numbers in which a genomic locus is detected can form a unique barcode for the genomic locus. In this example, locus 1 has the barcode '100', locus 2 has the barcode '110', and locus 3 has the barcode '011'. By applying this scheme, the capacity to profile epigenetic marks can be increased by hundreds to thousands of genomic loci with tens of rounds of hybridization and imaging.

[00159] Fig. 10. Schematic representation of "EZ" multiple epigenetic marks (EZ-mEpi) detection scheme. For simplicity, the depicted example shows a scheme for detecting two chromatin modifications at the same time. As shown in **Fig. 10**, two second probes can each comprise an antibody coupled to EZ-PH2_1 and EZ-PH2_2,

respectively, and a first probe can comprise both EZ-PH1_1 and EZ-PH1_2. According to the depicted scheme, a cell can be simultaneously or sequentially contacted with EZ-H1_1 and EZ-H1_2 after being contacted with the first and second probes. After a click reaction and washes, the cell can be contacted with dye-labeled readout probes (readout probe 1 and readout probe 2) sequentially or simultaneously, and the two chromatin modifications can be detected. In some embodiments, the two chromatin modifications can be detected simultaneously in two fluorescent channels if the two readout probes are labeled with different fluorescent dyes. In some embodiments, the EZ-H1_1 and EZ-H1_2 can be labeled, in which case readout probes are not necessary.

[00160] Figs. 11A to 11D. Demonstration of Epi-mFISH by profiling H3K9me3 and H3K27ac marks at 22 non-repetitive regions in IMR90 cells. (Fig. 11A) Schematic representation of multiplexed sequential FISH probe design and imaging pipeline. For simplicity, the Epi-PHR assemblies are not shown in the secondary FISH probe hybridization steps (Hyb1-22). Each set of primary FISH probes contains targeting sequences for a selected genomic region, and a unique secondary probe binding site. All primary FISH probes contain a PH1 linker docking site. The imaging pipeline starts with imaging Epi-PHR signals from all targeted genomic regions, then the signals were bleached. After 22 rounds of secondary FISH probe hybridization, imaging and bleaching, the multiplexed sequential FISH signals can pinpoint the corresponding 22 targeted genomic regions. **(Fig. 11B)** Example raw data from H3K9me3 profiling experiment. Based on the mFISH signals, the Epi-PHR signals could be assigned to the corresponding genomic loci. In this example, locus 1 and locus 2 showed colocalizing Epi-PHR signals, which indicated that H3K9me3 marks were enriched in these regions. However, locus 22 did not show colocalizing Epi-PHR signal, which indicated that H3K9me3 marks were depleted in this region. **(Fig. 11C)** Scatter plots of the Epi-mFISH signal rates of each target regions versus the normalized ChIP-seq peak heights of same targeted regions. The Pearson correlation coefficients are 0.934 for H3K9me3 mark (left), and 0.932 for H3K27ac mark (right). **(Fig. 11D)** Box plots of the normalized Epi-PHR fluorescence intensities of H3K9me3 mark (left) and H3K27ac mark (right). In the left panel, loci 1-10 are H3K9me3 enriched regions, and loci 11-22 are H3K9me3 depleted regions based on ChIP-seq data. In the right panel, loci 1-12

are H3K27ac enriched regions, and loci 13-22 are H3K27ac depleted regions based on ChIP-seq data. In the box plots, the notch and middle line represents the median, boxes show the interquartile range, whiskers show values within 1.5 times the interquartile range and black points represent outliers.

[00161] Figs. 12A and 12B. Demonstration of EZ-Epi-PHR strategy by detecting H3K9me3 at the human chromosome 9 alpha satellite locus in IMR90 cells. (Fig. 12A) The EZ-Epi-PHR strategy without enzymatic ligation detected H3K9me3 marks at the alpha satellite locus, but showed weak, false-positive EZ-Epi-PHR signals when omitting H3K9me3 antibody in a negative control. **(Fig. 12B)** After T4 ligation and highly stringent washes, the weak background signals were completely suppressed when omitting H3K9me3 antibody, while strong, true-positive EZ-Epi-PHR signals could still be observed.

[00162] Figs. 13A and 13B. A-B compartment identity depends on the epigenetic state of topologically associating domains (TADs). (Fig. 13A) Left: Example raw images from multiplexed sequential FISH for chromatin folding measurement, and H3K27ac Epi-PHR signal from one targeted region. 14 selected TADs on human chromosome 20 are imaged by multiplexed sequential FISH in 14 rounds of sequential hybridization and imaging (Hyb1-14). TAD # 8 was targeted by Epi-PHR. Right: Analyzed 3D chromatin folding conformation. 3D positions of the 14 TADs in the left panels are plotted as pseudo-colored spheres connected with a smooth curve showing the folding conformation of this copy of chromatin. **(Fig. 13B)** Compartment scores of the 14 selected TADs on human chromosome 20 from two groups of traces with opposite H3K27ac epigenetic states of TAD # 8 (Epi-PHR target). The positive and negative compartment scores indicate the A and B compartment identities, respectively. The black arrow indicates the Epi-PHR targeted TAD # 8. The compartment scores of TAD # 8 are opposite between the two trace groups, showing the A-B compartment identity of the TAD depends on its H3K27ac state.

[00163] Fig. 14. Demonstration of 2-mark profiling strategy by simultaneously profiling H3K9me3 and H3K27ac at 8 non-repetitive regions in IMR90 cells.

Schematic representation of PH1 docking linker design. Instead of docking one version of PH1, a 150-base linker probe is hybridized to the FISH probe. The linker probe contains two PH1_1 docking sites and two PH1_2 docking sites. By this design, two

sets of PH1 can hybridize to one FISH probe.

DETAILED DESCRIPTION

[00164] The present invention may address major barriers in the field of epigenetic/epigenomic profiling including: **1)** Current single-cell sequencing methods in the field are limited to specific aspects of the epigenome such as DNA accessibility, DNA methylation, and chromatin organization and do not extend to profiling of histone modifications, histone variants, and non-histone DNA binding proteins [12-14]. While profiling of histone modifications was recently attempted, the attempt was limited by an extremely low number of sequencing reads from each cell, making it unreliable in detecting epigenetic marks at given genomic loci of interest [15]. **2)** Sample preparation for prior methods requires cells to be dissociated from each other and lysed, which leads to the loss of important *in situ* spatial information. **3)** It is difficult to multiplex these methods to obtain multiple types of epigenetic information from the same cell. Overcoming this limitation is critical, particularly as there is evidence to suggest that epigenetic marks may generate combinatorial effects, as in the case of “bivalent” histone modifications [16, 17]. And epigenetic marks and 3D chromatin organization may jointly affect genome functions. In various embodiments, “genome functions” include, as non-limiting examples, gene expression, DNA replication, DNA repair, and recombination. The genome provides all information an organism requires to function. Thus, genome functions are outputs of information extraction and information flow. The outputs of information are crucial signals for various important biological processes (e.g., transcription control, DNA replication, DNA damage repair, etc.).

[00165] Herein are provided imaging-based methods to profile epigenetic marks *in situ* at the single-cell level at one or multiple specific genomic loci. Provided is a single-locus epigenetic mark detection method termed ‘Epi-PHR’. Further provided is the Epi-PHR method combined with multiplexed sequential DNA fluorescence *in situ* hybridization to detect epigenetic marks at multiple genomic loci, and to allow simultaneous profiling of epigenetic marks and chromatin organization. The combined technique is termed ‘Epi-mFISH’. Data provided in the Examples provided herein demonstrate that Epi-PHR can robustly detect single epigenetic marks across a broad range of genomic resolutions. The methods provided herein have potential to transform the field of spatial epigenomics, and may lead to numerous new opportunities in both

scientific and medical research (e.g., on the cell-cell variation of epigenetic profiles in normal tissues and clonal epigenetic diversity in cancers) and development of novel strategies for disease prediction, diagnosis, and prognosis.

[00166] In one aspect, provided herein is a technology to allow combinatorial profiling of epigenetic marks and 3D chromatin organization in single cells *in situ*. This may overcome several technical limitations in the field and establish – at single-cell resolution and in the native tissue context – a method for analyzing the combinatorial code of epigenetic marks that regulate cell state. In various embodiments, “native tissue context” is used to refer to fixed histological samples (e.g., tissue sections). In various embodiments, the methods of the present disclosure do not require cells to be dissociated from tissue samples or molecules to be extracted from cells; therefore, special information of individual cells and molecules can be preserved. Native tissue context can be preserved by histological sample preparation.

[00167] The Epi-PHR method may allow for detection of an epigenetic mark in proximity to specific genomic loci, and Epi-PHR has the advantage of using a controlled signal amplification method. An advantage of the proximity-dependent Epi-PHR and Epi-mFISH methods is that they can limit detection of epigenetic marks to those that are adjacent to the genomic loci of interest, thus the methods can be better than simply colocalizing DNA FISH signals with immunofluorescence signals of densely represented epigenetic marks. The close proximity between two hairpin oligonucleotides that enables Epi-PHR is established when specific antibodies bind epigenetic marks adjacent to *in-situ* hybridization probes that bind a DNA locus. Further, the non-enzymatic nature of the methods of signal amplification provided herein can offer better detection efficiency than methods using enzymatic methods for signal amplification [19]. Uncontrolled signal amplification schemes cannot be used to quantitatively profile epigenetic marks, whereas a controlled signal amplification scheme (e.g., in embodiments of the invention) may be used to quantitatively measure epigenetic modification levels of genomic loci (rather than giving an “all-or-none” output).

[00168] Also provided herein are timesaving versions of Epi-PHR and Epi-mFISH, termed ‘EZ-Epi-PHR’ and ‘EZ-Epi-mFISH’ respectively. The modified methods allow for rapid profiling of epigenetic marks at specific genomic loci.

Definitions

[00169] Unless defined otherwise, 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.

[00170] As used herein, the term “proximity-dependent hybridization reaction (PHR)” refers to an interaction made possible only through sufficient spatial proximity of two different oligonucleotides (e.g., PH1 and PH2) whereby a nucleotide platform, optionally comprising a gap(s), is made available for hybridization (e.g., by Watson-Crick base pairing) to an oligonucleotide probe (e.g., H1) through the interaction. The nucleotide platform is obstructed from or unavailable for base pairing with the oligonucleotide probe prior to the PHR. As described further below, in various embodiments, one or both of the two different oligonucleotides forms a hairpin loop structure. As described further below, in various non-limiting embodiments, one or both of the different oligonucleotides does not form a hairpin loop structure.

[00171] As used herein, the term “activator” or “activator oligonucleotide” refers to a molecule or compound that initiates a PHR through binding to a PH1 or PH2 oligonucleotide. In various non-limiting embodiments, the PHR is obstructed from taking place until binding of the activator to the PH1 or PH2 oligonucleotide. In various embodiments, the activator binds to only one PH1 oligonucleotide or one PH2 oligonucleotide participating in a PHR but not to both oligonucleotides.

[00172] As used herein, the term “coupled” or “coupling” refers to any method for pairing a first oligonucleotide to either a second oligonucleotide or to an amino acid sequence (e.g., an antibody). As non-limiting examples, the term “coupling” can include pairing through non-covalent bonding (e.g., hybridization or docking by Watson-Crick base pairing of one oligonucleotide to another, a biotin-streptavidin bridge, binding between an antigen and antibody, binding of one oligonucleotide to another, and hydrogen bonding) and/or covalent bonding (e.g., a covalent bond formed through a click reaction). The coupling can be direct or indirect. As a non-limiting example of indirect coupling, a first oligonucleotide may be coupled to a second oligonucleotide by binding both the first oligonucleotide and the second oligonucleotide to a third oligonucleotide. As a non-limiting example of direct coupling, the first oligonucleotide can be coupled to the second oligonucleotide through a covalent bond formed in a click

reaction. Further non-limiting examples of coupling are provided throughout the present disclosure.

[00173] In cases where the present specification and a document incorporated by reference include conflicting and/or inconsistent disclosure, the present specification shall control. If two or more documents incorporated by reference include conflicting and/or inconsistent disclosure with respect to each other, then the document having the later effective date shall control.

[00174] All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

[00175] The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

[00176] When the word “about” is used herein in reference to a number, it should be understood that still another embodiment of the invention includes that number not modified by the presence of the word “about.”

[00177] If aspects or embodiments of the disclosure are described as "comprising", or versions thereof (e.g., comprises), a feature, embodiments also are contemplated "consisting of" or "consisting essentially of" the feature.

[00178] It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

[00179] In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

[00180] The terms and expressions which have been employed herein are used as terms of description and not of limitation, and use of such terms and expressions do not

exclude any equivalents of the features shown and described or portions thereof, and various modifications are possible within the scope of the technology claimed.

[00181] The practice of the present disclosure employs, unless otherwise indicated, conventional techniques of statistical analysis, molecular biology (including recombinant techniques), microbiology, cell biology, and biochemistry, which are within the skill of the art. Such tools and techniques are described in detail in e.g., Sambrook et al. (2001) *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York; Ausubel et al. eds. (2005) *Current Protocols in Molecular Biology*. John Wiley and Sons, Inc.: Hoboken, NJ; Bonifacino et al. eds. (2005) *Current Protocols in Cell Biology*. John Wiley and Sons, Inc.: Hoboken, NJ; Coligan et al. eds. (2005) *Current Protocols in Immunology*, John Wiley and Sons, Inc.: Hoboken, NJ; Coico et al. eds. (2005) *Current Protocols in Microbiology*, John Wiley and Sons, Inc.: Hoboken, NJ; Coligan et al. eds. (2005) *Current Protocols in Protein Science*, John Wiley and Sons, Inc.: Hoboken, NJ; and Enna et al. eds. (2005) *Current Protocols in Pharmacology*, John Wiley and Sons, Inc.: Hoboken, NJ. Additional techniques are explained, e.g., in U.S. Patent No. 7,912,698 and U.S. Patent Appl. Pub. Nos. 2011/0202322 and 2011/0307437.

Methods of the Disclosure

[00182] Throughout the present disclosure, various embodiments and aspects of the invention of the disclosure will be described with reference to the Figures, where like reference numbers may indicate functionally similar elements. The embodiments and aspects as generally described and illustrated in the Figures provided herein can be modified in a wide variety of manners, a selection of such modifications being described further below. Thus, the Figures are not intended to limit scope, as claimed, but are merely representative of various embodiments of the invention of the disclosure and are provided merely to facilitate a description of various aspects and embodiments of the invention.

[00183] In one aspect, the present disclosure provides a method for *in situ* visualization of a chromatin modification of a cell. Various components of the method are illustrated in **Figs. 1A to 1C, 2B, 4A, 5A, 6A, 7A to 7C, and 8-10**. Description of reference numbers employed in the Figures are provided in **Table 1**.

Table 1. Description of reference numbers employed in the Figures

Element Name	Reference No.
first probe	20
second probe	22
activator oligonucleotide	24
third probe	26
first oligonucleotide	28
proximity hybridization 1 (PH1) oligonucleotide	30
proximity hybridization 2 (PH2) oligonucleotide	31
genomic locus	32
first hairpin loop structure	34
antibody	36
chromatin modification of interest	38
second hairpin loop	40
opening of first hairpin loop structure	42
label of the third probe	44
hybridization 1 (H1) oligonucleotide	46
hybridization of PH1 and PH2 oligonucleotides	48
sequence made available when PH1 oligonucleotide and PH2 oligonucleotide have hybridized	50
nucleotide sequence of each of the PH1 (or PH1_1) and PH2 (or PH2_1) oligonucleotides	52
nucleotide sequence of each of the PH1_2 and PH2_2 oligonucleotides	53
biotin-streptavidin bridge	54
Biotin	56
streptavidin	58
covalent bond	60
genome	62
Histone	64
fourth probe	66
hybridization 2 (H2) oligonucleotide	68
second dye	70
nucleotide hybridization	72
readout probe	76
label	78
readout probe binding site	80
overhang region	82
docking sequence	84
5' terminus	86
3' terminus	88
first contiguous nucleotide of the first oligonucleotide not complementary to a nucleotide sequence corresponding to a genomic locus of interest	90
inactivated label	92

first readout probe	94
second readout probe	96
third readout probe	98
linker oligonucleotide	100
docking regions	102
portion of the PH1 oligonucleotide that binds to the PH2 oligonucleotide	104
opening of second hairpin loop structure	106
hairpin loop structure formed by the H1 oligonucleotide	108
first unique antibody	110
first unique PH2 oligonucleotide (PH2_1 or EZ-PH2_1)	112
second unique antibody	114
second unique PH2 oligonucleotide (PH2_2 or EZ-PH2_2)	116
antibody linker oligonucleotide	118
PH1_1 or EZ-PH1_1	120
PH1_2 or EZ-PH1_2	122
first activator oligonucleotide	124
second activator oligonucleotide	126
H1_1 or EZ-H1_1	128
H1_2 or EZ-H1_2	130
sequence made available upon the opening of a second hairpin loop structure of PH2_1	132
sequence made available upon the opening of a second hairpin loop structure of PH2_2	134
primary amplification oligonucleotide	136
secondary amplification oligonucleotide	138
first terminus of the PH1 oligonucleotide	140
second terminus of the PH2 oligonucleotide	142
EZ-PH1 oligonucleotide	144
EZ-PH2 oligonucleotide	146
EZ-H1 oligonucleotide	148
covalent bond formed by a click reaction or enzymatic ligation reaction.	150
first sequential hybridization step	152
second sequential hybridization step	154
hairpin loop structure formed by the H2 oligonucleotide	156
opening of H1 oligonucleotide hairpin loop structure	158
readout region	160
opening of H2 oligonucleotide hairpin loop structure	162

[00184] The method comprises providing a first probe **20**, a second probe **22**, and a third probe **26**. The first probe **20** comprises a first oligonucleotide **28** coupled to a proximity hybridization 1 (PH1) oligonucleotide **30**. The first oligonucleotide **28** binds to a genomic locus **32** of interest. The second probe **22** comprises an antibody **36** coupled to a proximity hybridization 2 (PH2) oligonucleotide **31**. The antibody **36** recognizes a

chromatin modification or set of chromatin modifications of interest **38**. The third probe comprises a hybridization 1 (H1) oligonucleotide **46**. The H1 oligonucleotide **46** is labeled **44** directly or indirectly (i.e., the H1 oligonucleotide **46** is coupled to a label **44**). The method further includes contacting the cell with the first probe **20** under conditions that allow binding of the first oligonucleotide **28** of the first probe **20** to the genomic locus **32** of the cell. The method further includes contacting the cell with the second probe **22** under conditions that allow binding of the antibody **36** of the second probe **22** to the chromatin modification or set of chromatin modifications **38**. The method also includes contacting the cell with the third probe **26** under conditions that allow binding of the H1 oligonucleotide **46** of the third probe **26** to (i) a nucleotide sequence of each of the PH1 and PH2 oligonucleotides **52** or to (ii) a sequence made available **50** when the PH1 and PH2 oligonucleotides have hybridized **48**. The method includes detecting the label **44** of the third probe **26**.

[00185] In various embodiments, the H1 oligonucleotide **46** is labeled indirectly by being bound by a labeled readout probe. In some embodiments the H1 oligonucleotide **46** is labeled **44** directly by being covalently coupled to a label **44**.

[00186] In various embodiments, the antibody **36** binds to a set of chromatin modifications of interest **38**.

[00187] It is to be understood that, throughout the present disclosure, PH1 is used to designate a proximity hybridization oligonucleotide coupled to the first oligonucleotide and PH2 is used to designate a proximity hybridization oligonucleotide coupled to an antibody. It is also to be understood that the functions and features described herein in reference to the PH1 oligonucleotide and to the PH2 oligonucleotide, respectively, are interchangeable so that a function or feature described for the PH2 oligonucleotide may apply in some embodiments to the PH1 oligonucleotide and a function or feature described for the PH1 oligonucleotide may apply in some embodiments to the PH2 oligonucleotide insofar as the PH1 oligonucleotide and the PH2 oligonucleotide used in a given embodiment may participate in a proximity hybridization reaction.

[00188] In various embodiments, the method comprises providing a plurality of first probes, wherein each first probe comprises a unique first oligonucleotide that binds to a genomic locus of interest, optionally wherein the genomic locus comprises a non-redundant nucleotide sequence. In some embodiments, the method further comprises

contacting the cell with each of the plurality of first probes under conditions that allow binding of each or some of the plurality of first probes to the genomic locus of interest.

[00189] In various embodiments, the genomic locus of interest comprises a non-redundant nucleotide sequence. In some embodiments, the genomic locus of interest comprises a redundant nucleotide sequence.

[00190] In various embodiments, the method further comprises providing an activator oligonucleotide **24**, which is described further below, and contacting the cell with the activator oligonucleotide **24** under conditions allowing the activator oligonucleotide **24** to bind to either the PH1 oligonucleotide **30** or the PH2 oligonucleotide **31**. In various embodiments, the activator oligonucleotide **24** does not form a hairpin loop structure.

[00191] The “nucleotide sequence of each of the PH1 and PH2 oligonucleotides” and the “sequence made available” each correspond to the “nucleotide platform” referenced in the definition of PHR provided above.

[00192] In various embodiments, the first oligonucleotide comprises about or at least about 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, or 500 nucleotides. In various embodiments, the first oligonucleotide comprises no more than about 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, or 500 nucleotides. In various embodiments, the first oligonucleotide is able to bind at least a portion of the genomic locus of interest, in some embodiments, the first oligonucleotide can be a FISH probe.

[00193] In various embodiments, coupling of the first probe **20** to the PH1 oligonucleotide **30** comprises nucleotide hybridization **72**, see **Fig. 1A**, optionally comprising Watson-Crick base pairing. In various embodiments, the first oligonucleotide **28** comprises an overhang region **82** to which the PH1 oligonucleotide **30** can bind (alternatively “dock”) through nucleotide hybridization. In some embodiments, the PH1 oligonucleotide **30** can comprise a docking sequence **84** that can be the reverse complement of the overhang region **82**. In some embodiments, the overhang region **82** comprises the 5' end **86** of the first oligonucleotide **28**. In some embodiments, the

overhang region comprises the 3' end **88** of the first oligonucleotide **28**. In some embodiments, coupling of the first oligonucleotide **28** to the PH1 oligonucleotide **30** comprises a covalent bond **60**, see **Fig. 1C**. In some embodiments, the PH1 oligonucleotide **30** is coupled to the 3' end **88** of the first oligonucleotide **28**. In some embodiments, the PH1 oligonucleotide **30** is coupled to the 5' end **86** of the first oligonucleotide. In various embodiments, the first probe does not comprise biotin.

[00194] It is to be understood in view of the present disclosure that various oligonucleotides of the methods described herein are intended to bind or otherwise interact with one another; therefore, it is to be understood further that alterations in one sequence may necessarily require corresponding modifications to another sequence. Thus, as a non-limiting example, when a reverse complement of a particular oligonucleotide is used in an embodiment, a corresponding oligonucleotide with which the reverse complement is intended to bind or otherwise interact with must necessarily include corresponding modifications to a sequence comprised by the oligonucleotide involved in the binding or other interaction.

[00195] In various embodiments, the antibody **36** is coupled to the PH2 oligonucleotide **31** by a biotin-streptavidin bridge **54**, see **Fig. 1A**, comprising biotin **56** bound by streptavidin **58**. In some embodiments, the antibody **36** is coupled to the PH2 oligonucleotide **31** by a covalent bond **60**. In some embodiments, the covalent bond is formed through a DBCO-mediated copper-free click reaction. The antibody may be coupled to the PH2 oligonucleotide by nucleotide hybridization, as shown in **Fig. 8**, optionally comprising Watson-Crick base pairing. In some embodiments, the PH2 oligonucleotide **31** can hybridize to an antibody linker oligonucleotide **118** covalently coupled to the antibody **36**. The antibody linker oligonucleotide **118** can be NHS ester modified. In various embodiments, the PH2 oligonucleotide **31** is biotinylated **56**. In some embodiments, the antibody **36** is biotinylated **56**. In some embodiments, the antibody **36** is coupled directly or indirectly to biotin **56**.

[00196] In various embodiments where the PH1 oligonucleotide and/or the PH2 oligonucleotide forms hairpin loop structures, indirect coupling of the PH1 oligonucleotide with the first oligonucleotide and/or of the PH2 oligonucleotide with the antibody allows for the method to include a step of refolding any hairpins loop structures formed by the PH1 oligonucleotide and/or the PH2 oligonucleotide to thereby

prevent incorrect hairpin conformations.

[00197] In some embodiments, the genomic locus of interest **32** is disposed within the nucleus of the cell. In various embodiments, the genomic locus of interest **32** is encoded by a genome **62** of the cell. In various embodiments, the genome **62** is disposed in the nucleus of the cell. In some embodiments, the chromatin modification **38** is disposed within the nucleus of the cell. In some embodiments, the genomic locus **32** and/or the chromatin modification **38** is disposed in an organelle of the cell or in the cytosol of the cell. In some embodiments, the genomic locus **32** and/or the chromatin modification **38** is disposed in a mitochondrion of the cell.

[00198] In some embodiments, the chromatin modification **38** is disposed on human chromosome X, Y, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, and/or 22. In some embodiments, the genomic locus and/or the chromatin modification **38** of interest is encoded by chromosome X, Y, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, and/or 22.

[00199] In various embodiments, the chromatin modification **38** is a histone **64** modification **38**. In some embodiments, the chromatin modification is a DNA modification. Non-limiting examples of DNA modifications include 5-methylcytosine and N6-methyladenine. In various embodiments, the chromatin modification **38** is an epigenetic mark **38**. In various embodiments, the epigenetic mark is a histone modification **38** or a histone variant. Non-limiting examples of histone modifications **38** or histone variants include histone H3 lysine 27 trimethylation (H3K27Me3), histone H3 lysine 9 trimethylation (H3K9Me3), H3K4me1, H3K4me3, H3K79me3, H3K36me3, histone variant MacroH2A, histone variant H3.3K27M, histone variant H3.3K36M, and histone H3 lysine 27 acetylation (H3K27Ac). In various embodiments, the chromatin modification comprises a non-histone DNA binding protein, non-limiting examples of which include DNA polymerases, RNA polymerases, transcription factors, DNA repair proteins, DNA recombination proteins, and chromatin remodelers. In some embodiments, the chromatin modification is a DNA modification, a DNA-binding protein (including proteins that bind directly or indirectly to DNA), or a modification to a DNA-binding protein.

[00200] In various embodiments, the antibody is a polyclonal antibody, monoclonal antibody, or an antibody binding fragment or derivative or mimetic thereof, where these

fragments, derivatives and mimetics bind a chromatin modification of interest. For example, antibody fragments, such as Fv, F(ab)₂, and Fab may be prepared by cleavage of an intact antibody, e.g. by protease or chemical cleavage. In some embodiments, the antibody is a synthetically produced antibody fragment or derivative, such as single-chain antibodies or scFvs, or other antibody derivatives such as chimeric antibodies or CDR-grafted antibodies. Such antibody fragments or derivatives can include VH and VL domains. Antibody fragments, derivatives or mimetics of the present disclosure may be readily prepared using any appropriate methodology, such as the methodology disclosed in U.S. Pat. Nos. 5,851,829 and 5,965,371; the disclosures of which are herein incorporated by reference in their entireties for all purposes. In various embodiments, the antibody recognizes (i.e. binds) a chromatin modification of interest. Non-limiting examples of antibodies suitable for use in the methods of the present disclosure include ab4729 (anti-histone H3 (acetyl K27) antibody), ab8898 (anti-histone H3 (tri methyl K9) antibody), ab222481 (anti-histone H3 (tri methyl K27) antibody), and ab183041 (anti-mH2A1 antibody).

[00201] In some embodiments, the PH1 oligonucleotide **30**, the PH2 oligonucleotide **31**, the activator oligonucleotide **24**, the H1 oligonucleotide **46**, an H2 oligonucleotide **68** (discussed further below), and a readout probe **76** (discussed further below) each comprise DNA. In some embodiments, the PH1 oligonucleotide **30**, the PH2 oligonucleotide **31**, the activator oligonucleotide **24**, the H1 oligonucleotide **46**, an H2 oligonucleotide **68** (discussed further below), and a readout probe **76** (discussed further below) each individually comprise DNA and/or any of various other entities that can hybridize to a nucleic acid, such as, to provide non-limiting examples, DNA, RNA, LNA (locked nucleic acids), PNA (peptide nucleic acids), or combinations thereof. In some embodiments, additional components may also be present within the PH1 oligonucleotide **30**, the PH2 oligonucleotide **31**, the activator oligonucleotide **24**, the H1 oligonucleotide **46**, the H2 oligonucleotide **68**, and/or the readout probe **76**.

[00202] In various embodiments, the H1 oligonucleotide **46** is labeled with a first dye **44**. The dye **44** can be a fluorescent dye. In some embodiments, the first dye **44** is 3' Alexa Fluor 647 (NHS Ester). In some embodiments, the first dye **44** is Alexa Fluor 647.

[00203] In certain embodiments, a label is any entity able to emit light. For instance, in one embodiment, the label is fluorescent. In other embodiments, the label may be

phosphorescent, radioactive, absorptive, etc. In some cases, the label is any entity that can be determined within a sample at relatively high resolutions, e.g., at resolutions better than the wavelength of visible light or the diffraction limit. The label may be, for example, a dye, a small molecule, a peptide or protein, or the like. The label may be a single molecule in some cases.

[00204] Non-limiting examples of labels include fluorescent entities (fluorophores) or phosphorescent entities, for example, cyanine dyes (e.g., Cy2, Cy3, Cy3B, Cy5, Cy5.5, Cy7, etc.), Alexa Fluor dyes, Atto dyes, photoswitchable dyes, photoactivatable dyes, fluorescent dyes, metal nanoparticles, semiconductor nanoparticles or “quantum dots”, fluorescent proteins such as GFP (Green Fluorescent Protein), or photoactivatable fluorescent proteins, such as PAGFP, PSCFP, PSCFP2, Dendra, Dendra2, EosFP, tdEos, mEos2, mEos3, PAmCherry, PAtagRFP, mMaple, mMaple2, and mMaple3. Other suitable labels are known to those of ordinary skill in the art. See, e.g., U.S. Pat. No. 7,838,302 or U.S. Pat. Apl. Ser. No. 61/979,436, each incorporated herein by reference in its entirety for all purposes.

[00205] In some embodiments, a label may be attached to an oligonucleotide via a bond that can be cleaved to release the label. In one set of embodiments, a fluorophore may be conjugated to an oligonucleotide via a cleavable bond, such as a photocleavable bond. Non-limiting examples of photocleavable bonds include, but are not limited to, 1-(2-nitrophenyl)ethyl, 2-nitrobenzyl, biotin phosphoramidite, acrylic phosphoramidite, diethylaminocoumarin, 1-(4,5-dimethoxy-2-nitrophenyl)ethyl, cyclo-dodecyl (dimethoxy-2-nitrophenyl)ethyl, 4-aminomethyl-3-nitrobenzyl, (4-nitro-3-(1-chlorocarbonyloxyethyl)phenyl)methyl-S-acetylthioic acid ester, (4-nitro-3-(1-thlorocarbonyloxyethyl)phenyl)methyl-3-(2-pyridyldithiopropionic acid) ester, 3-(4,4'-dimethoxytrityl)-1-(2-nitrophenyl)-propane-1,3-diol-[2-cyanoethyl-(N,N-diisopropyl)]-phosphoramidite, 1-[2-nitro-5-(6-trifluoroacetylcaproamidomethyl)phenyl]-ethyl-[2-cyano-ethyl-(N,N-diisopropyl)]-phosphoramidite, 1-[2-nitro-5-(6-(4,4'-dimethoxytrityloxy)butyramidomethyl)phenyl]-ethyl-[2-cyanoethyl-(N,N-diisopropyl)]-phosphoramidite, 1-[2-nitro-5-(6-(N-(4,4'-dimethoxytrityl))-biotinamidocaproamidomethyl)phenyl]-ethyl-[2-cyanoethyl-(N,N-diisopropyl)]-phosphoramidite, or similar linkers. In another set of embodiments, the fluorophore may be conjugated to an oligonucleotide via a disulfide bond. The disulfide bond may be cleaved by a variety of

reducing agents such as, but not limited to, dithiothreitol, dithioerythritol, beta-mercaptoethanol, sodium borohydride, thioredoxin, glutaredoxin, trypsinogen, hydrazine, diisobutylaluminum hydride, oxalic acid, formic acid, ascorbic acid, phosphorous acid, tin chloride, glutathione, thioglycolate, 2,3-dimercaptopropanol, 2-mercaptoethylamine, 2-aminoethanol, tris(2-carboxyethyl)phosphine, bis(2-mercaptoethyl) sulfone, N,N'-dimethyl-N,N'-bis(mercaptoacetyl)hydrazine, 3-mercaptopropanoate, dimethylformamide, thiopropyl-agarose, tri-n-butylphosphine, cysteine, iron sulfate, sodium sulfite, phosphite, hypophosphite, phosphorothioate, or the like, and/or combinations of any of these. In another embodiment, the fluorophore may be conjugated to an oligonucleotide via one or more phosphorothioate modified nucleotides in which the sulfur modification replaces the bridging and/or non-bridging oxygen. The fluorophore may be cleaved from the oligonucleotide, in certain embodiments, via addition of compounds such as but not limited to iodoethanol, iodine mixed in ethanol, silver nitrate, or mercury chloride. In some embodiments, the label may be photobleached through for example high-intensity or prolonged laser illumination. In some embodiments, the label may be chemically inactivated through reduction or oxidation. For example, in one embodiment, a chromophore such as Cy5 or Cy7 may be reduced using sodium borohydride to a stable, non-fluorescence state. In some embodiments, a fluorophore may be conjugated to an oligonucleotide via an azo bond, and the azo bond may be cleaved with 2-[(2-N-arylamino)phenylazo]pyridine. In some embodiments, a fluorophore may be conjugated to an oligonucleotide via a suitable nucleic acid segment that can be cleaved upon suitable exposure to DNase, e.g., an exodeoxyribonuclease or an endodeoxyribonuclease. Examples include, but are not limited to, deoxyribonuclease I or deoxyribonuclease II. In some embodiments, the cleavage may occur via a restriction endonuclease. Non-limiting examples of potentially suitable restriction endonucleases include BamHI, BsrI, NotI, XmaI, PspAI, DpnI, MboI, MnlI, Eco57I, Ksp632I, DraIII, AhaII, SmaI, MluI, HpaI, ApaI, BclI, BstEII, TaqI, EcoRI, SacI, HindIII, HaeII, DraII, Tsp509I, Sau3AI, PaeI, etc. Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially. In some embodiments, a fluorophore may be conjugated to biotin, and the oligonucleotide conjugated to avidin or streptavidin. An interaction between biotin and avidin or streptavidin allows the fluorophore to be conjugated to the

oligonucleotide, while sufficient exposure to an excess of addition, free biotin could “outcompete” the linkage and thereby cause cleavage to occur. In some embodiments, the probes may be removed by conditions that disrupt base pairing, such as high temperature and/or high concentration of formamide. In addition, in some embodiments, the probes may be removed using corresponding “toe-hold-probes,” which comprise the same sequence as the probe, as well as an extra number of bases of homology to the encoding probes (e.g., 1-20 extra bases, for example, 5 extra bases). These probes may remove the labeled readout probe through a strand-displacement interaction.

[00206] As used herein, the term “light” generally refers to electromagnetic radiation, having any suitable wavelength (or equivalently, frequency). For instance, in some embodiments, the light may include wavelengths in the optical or visual range (for example, having a wavelength of between about 400 nm and about 700 nm, i.e., “visible light”), infrared wavelengths (for example, having a wavelength of between about 300 micrometers and 700 nm), ultraviolet wavelengths (for example, having a wavelength of between about 400 nm and about 10 nm), or the like. In certain cases, more than one label may be used, i.e., labels that are chemically different or distinct, for example, structurally. However, in other cases, the labels may be chemically identical or at least substantially chemically identical.

[00207] In some embodiments, the label is “switchable,” i.e., the label can be switched between two or more states, at least one of which emits light having a desired wavelength. In the other state(s), the label may emit no light, or emit light at a different wavelength. For instance, a label may be “activated” to a first state able to produce light having a desired wavelength, and “deactivated” to a second state not able to emit light of the same wavelength. A label is “photoactivatable” if it can be activated by incident light of a suitable wavelength. As a non-limiting example, Cy5 can be switched between a fluorescent and a dark state in a controlled and reversible manner by light of different wavelengths, i.e., 633 nm (or 642 nm, 647 nm, 656 nm) red light can switch or deactivate Cy5 to a stable dark state, while 405 nm purple light can switch or activate the Cy5 back to the fluorescent state. In some cases, the label can be reversibly switched between the two or more states, e.g., upon exposure to the proper stimuli. For example, a first stimulus (e.g., a first wavelength of light) may be used to activate the

switchable label, while a second stimulus (e.g., a second wavelength of light) may be used to deactivate the switchable label, for instance, to a non-emitting state. Any suitable method may be used to activate the label. For example, in one embodiment, incident light of a suitable wavelength may be used to activate the label to emit light, i.e., the label is “photoswitchable.” Thus, the photoswitchable label can be switched between different light-emitting or non-emitting states by incident light, e.g., of different wavelengths. The light may be monochromatic (e.g., produced using a laser) or polychromatic. In another embodiment, the label may be activated upon stimulation by electric field and/or magnetic field. In other embodiments, the label may be activated upon exposure to a suitable chemical environment, e.g., by adjusting the pH, or inducing a reversible chemical reaction involving the label, etc. Similarly, any suitable method may be used to deactivate the label, and the methods of activating and deactivating the label need not be the same. For instance, the label may be deactivated upon exposure to incident light of a suitable wavelength, or the label may be deactivated by waiting a sufficient time.

[00208] A “switchable” label can be identified by one of ordinary skill in the art by determining conditions under which a label in a first state can emit light when exposed to an excitation wavelength, switching the label from the first state to the second state, e.g., upon exposure to light of a switching wavelength, then showing that the label, while in the second state can no longer emit light (or emits light at a much-reduced intensity) when exposed to the excitation wavelength.

[00209] In one set of embodiments, as discussed, a switchable label may be switched upon exposure to light. In some cases, the light used to activate the switchable label may come from an external source, e.g., a light source such as a laser light source, a second light-emitting label proximate to the switchable label, etc. The second, light-emitting label, in some cases, may be a fluorescent label, and in certain embodiments, the second, light-emitting label may itself also be a switchable label.

[00210] In some embodiments, the switchable label includes a first, light-emitting portion (e.g., a fluorophore), and a second portion that activates or “switches” the first portion. For example, upon exposure to light, the second portion of the switchable label may activate the first portion, causing the first portion to emit light. Examples of activator portions include, but are not limited to, Alexa Fluor 405 (Invitrogen), Alexa

Fluor 488 (Invitrogen), Cy2 (GE Healthcare), Cy3 (GE Healthcare), Cy3B (GE Healthcare), Cy3.5 (GE Healthcare), or other suitable dyes. Examples of light-emitting portions include, but are not limited to, Cy5, Cy5.5 (GE Healthcare), Cy7 (GE Healthcare), Alexa Fluor 647 (Invitrogen), Alexa Fluor 680 (Invitrogen), Alexa Fluor 700 (Invitrogen), Alexa Fluor 750 (Invitrogen), Alexa Fluor 790 (Invitrogen), DiD, DiR, YOYO-3 (Invitrogen), YO-PRO-3 (Invitrogen), TOT-3 (Invitrogen), TO-PRO-3 (Invitrogen) or other suitable dyes. These may be linked together, e.g., covalently, for example, directly, or through a linker, e.g., forming compounds such as, but not limited to, Cy5-Alexa Fluor 405, Cy5-Alexa Fluor 488, Cy5-Cy2, Cy5-Cy3, Cy5-Cy3.5, Cy5.5-Alexa Fluor 405, Cy5.5-Alexa Fluor 488, Cy5.5-Cy2, Cy5.5-Cy3, Cy5.5-Cy3.5, Cy7-Alexa Fluor 405, Cy7-Alexa Fluor 488, Cy7-Cy2, Cy7-Cy3, Cy7-Cy3.5, Alexa Fluor 647-Alexa Fluor 405, Alexa Fluor 647-Alexa Fluor 488, Alexa Fluor 647-Cy2, Alexa Fluor 647-Cy3, Alexa Fluor 647-Cy3.5, Alexa Fluor 750-Alexa Fluor 405, Alexa Fluor 750-Alexa Fluor 488, Alexa Fluor 750-Cy2, Alexa Fluor 750-Cy3, or Alexa Fluor 750-Cy3.5. Those of ordinary skill in the art will be aware of the structures of these and other compounds, many of which are available commercially. The portions may be linked via a covalent bond, or by a linker, such as those described in detail below. Other light-emitting or activator portions may include portions having two quaternized nitrogen atoms joined by a polymethine chain, where each nitrogen is independently part of a heteroaromatic moiety, such as pyrrole, imidazole, thiazole, pyridine, quinoine, indole, benzothiazole, etc., or part of a non-aromatic amine. In some cases, there may be 5, 6, 7, 8, 9, or more carbon atoms between the two nitrogen atoms.

[00211] In certain cases, the light-emitting portion and the activator portions, when isolated from each other, may each be fluorophores, i.e., entities that can emit light of a certain, emission wavelength when exposed to a stimulus, for example, an excitation wavelength. However, when a switchable label is formed that comprises the first fluorophore and the second fluorophore, the first fluorophore forms a first, light-emitting portion and the second fluorophore forms an activator portion that activates or “switches” the first portion in response to a stimulus. For example, the switchable label may comprise a first fluorophore directly bonded to the second fluorophore, or the first and second label may be connected via a linker or a common label. Whether a pair of light-emitting portion and activator portion produces a suitable switchable label can be

tested by methods known to those of ordinary skills in the art. For example, light of various wavelengths can be used to stimulate the pair and emission light from the light-emitting portion can be measured to determined wither the pair makes a suitable switch.

[00212] As a non-limiting example, Cy3 and Cy5 may be linked together to form such a label. In this example, Cy3 is an activator portion that is able to activate Cy5, the light-emission portion. Thus, light at or near the absorption maximum (e.g., near 532 nm light for Cy3) of the activation or second portion of the label may cause that portion to activate the first, light-emitting portion, thereby causing the first portion to emit light (e.g., near 647 nm for Cy5). See, e.g., U.S. Pat. No. 7,838,302, incorporated herein by reference in its entirety. In some cases, the first, light-emitting portion can subsequently be deactivated by any suitable technique (e.g., by directing 647 nm red light to the Cy5 portion of the molecule).

[00213] Other non-limiting examples of potentially suitable activator portions include 1,5 IAEDANS, 1,8-ANS, 4-Methylumbelliferone, 5-carboxy-2,7-dichlorofluorescein, 5-Carboxyfluorescein (5-FAM), 5-Carboxynaphthofluorescein, 5-Carboxytetramethylrhodamine (5-TAMRA), 5-FAM (5-Carboxyfluorescein), 5-HAT (Hydroxy Tryptamine), 5-Hydroxy Tryptamine (HAT), 5-ROX (carboxy-X-rhodamine), 5-TAMRA (5-Carboxytetramethylrhodamine), 6-Carboxyrhodamine 6G, 6-CR 6G, 6-JOE, 7-Amino-4-methylcoumarin, 7-Aminoactinomycin D (7-AAD), 7-Hydroxy-4-methylcoumarin, 9-Amino-6-chloro-2-methoxyacridine, ABQ, Acid Fuchsin, ACMA (9-Amino-6-chloro-2-methoxyacridine), Acridine Orange, Acridine Red, Acridine Yellow, Acriflavin, Acriflavin Feulgen SITSA, Alexa Fluor 350, Alexa Fluor 405, Alexa Fluor 430, Alexa Fluor 488, Alexa Fluor 500, Alexa Fluor 514, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 610, Alexa Fluor 633, Alexa Fluor 635, Alizarin Complexon, Alizarin Red, AMC, AMCA-S, AMCA (Aminomethylcoumarin), AMCA-X, Aminoactinomycin D, Aminocoumarin, Aminomethylcoumarin (AMCA), Anilin Blue, Anthrocyol stearate, APTRA-BTC, APTS, Astrazon Brilliant Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, ATTO 390, ATTO 425, ATTO 465, ATTO 488, ATTO 495, ATTO 520, ATTO 532, ATTO 550, ATTO 565, ATTO 590, ATTO 594, ATTO 610, ATTO 611X, ATTO 620, ATTO 633, ATTO 635, ATTO 647, ATTO 647N, ATTO 655, ATTO 680,

ATTO 700, ATTO 725, ATTO 740, ATTO-TAG CBQCA, ATTO-TAG FQ, Auramine, Aurophosphine G, Aurophosphine, BAO 9 (Bisaminophenyloxadiazole), BCECF (high pH), BCECF (low pH), Berberine Sulphate, Bimane, Bisbenzamide, Bisbenzimidazole (Hoechst), bis-BTC, Blancophor FFG, Blancophor SV, BOBO -1, BOBO -3, Bodipy 492/515, Bodipy 493/503, Bodipy 500/510, Bodipy 505/515, Bodipy 530/550, Bodipy 542/563, Bodipy 558/568, Bodipy 564/570, Bodipy 576/589, Bodipy 581/591, Bodipy 630/650-X, Bodipy 650/665-X, Bodipy 665/676, Bodipy FI, Bodipy FL ATP, Bodipy FI-Ceramide, Bodipy R6G, Bodipy TMR, Bodipy TMR-X conjugate, Bodipy TMR-X, SE, Bodipy TR, Bodipy TR ATP, Bodipy TR-X SE, BO-PRO -1, BO-PRO -3, Brilliant Sulphoflavin FF, BTC, BTC-5N, Calcein, Calcein Blue, Calcium Crimson, Calcium Green, Calcium Green-1 Ca^{2+} Dye, Calcium Green-2 Ca^{2+} , Calcium Green-5N Ca^{2+} , Calcium Green-C18 Ca^{2+} , Calcium Orange, Calcofluor White, Carboxy-X-rhodamine (5-ROX), Cascade Blue, Cascade Yellow, Catecholamine, CCF2 (GeneBlazer), CFDA, Chromomycin A, Chromomycin A, CL-NERF, CMFDA, Coumarin Phalloidin, CPM Methylcoumarin, CTC, CTC Formazan, Cy2, Cy3.1 8, Cy3.5, Cy3, Cy5.1 8, cyclic AMP Fluorosensor (FiCRhR), Dabcyl, Dansyl, Dansyl Amine, Dansyl Cadaverine, Dansyl Chloride, Dansyl DHPE, Dansyl fluoride, DAPI, Dapoxyl, Dapoxyl 2, Dapoxyl 3' DCFDA, DCFH (Dichlorodihydrofluorescein Diacetate), DDAO, DHR (Dihydorhodamine 123), Di-4-ANEPPS, Di-8-ANEPPS (non-ratio), DiA (4-Di-16-ASP), Dichlorodihydrofluorescein Diacetate (DCFH), DiD-Lipophilic Tracer, DiD (DiIC18(5)), DIDS, Dihydorhodamine 123 (DHR), DiI (DiIC18(3)), Dinitrophenol, DiO (DiOC18(3)), DiR, DiR (DiIC18(7)), DM-NERF (high pH), DNP, Dopamine, DTAF, DY-630-NHS, DY-635-NHS, DyLight 405, DyLight 488, DyLight 549, DyLight 633, DyLight 649, DyLight 680, DyLight 800, ELF 97, Eosin, Erythrosin, Erythrosin ITC, Ethidium Bromide, Ethidium homodimer-1 (EthD-1), Euchrysin, EukoLight, Europium (III) chloride, Fast Blue, FDA, Feulgen (Pararosaniline), FIF (Formaldehyd Induced Fluorescence), FITC, Flazo Orange, Fluo-3, Fluo-4, Fluorescein (FITC), Fluorescein Diacetate, Fluoro-Emerald, Fluoro-Gold (Hydroxystilbamidine), Fluor-Ruby, FluorX, FM 1-43, FM 4-46, Fura Red (high pH), Fura Red/Fluo-3, Fura-2, Fura-2/BCECF, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow SGF, GeneBlazer (CCF2), Gloxalic Acid, Granular blue, Haematoporphyrin, Hoechst 33258, Hoechst 33342, Hoechst 34580, HPTS, Hydroxycoumarin, Hydroxystilbamidine (FluoroGold),

Hydroxytryptamine, Indo-1, high calcium, Indo-1, low calcium, Indodicarbocyanine (DiD), Indotricarbocyanine (DiR), Intrawhite Cf, JC-1, JO-JO-1, JO-PRO-1, LaserPro, Laurodan, LDS 751 (DNA), LDS 751 (RNA), Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine Rhodamine, Lissamine Rhodamine B, Calcein/Ethidium homodimer, LOLO-1, LO-PRO-1, Lucifer Yellow, Lyso Tracker Blue, Lyso Tracker Blue-White, Lyso Tracker Green, Lyso Tracker Red, Lyso Tracker Yellow, LysoSensor Blue, LysoSensor Green, LysoSensor Yellow/Blue, Mag Green, Magdala Red (Phloxin B), Mag-Fura Red, Mag-Fura-2, Mag-Fura-5, Mag-Indo-1, Magnesium Green, Magnesium Orange, Malachite Green, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, Merocyanin, Methoxycoumarin, Mitotracker Green FM, Mitotracker Orange, Mitotracker Red, Mitramycin, Monobromobimane, Monobromobimane (mBBBr-GSH), Monochlorobimane, MPS (Methyl Green Pyronine Stilbene), NBD, NBD Amine, Nile Red, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant lavin E8G, Oregon Green, Oregon Green 488-X, Oregon Green, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, Pararosaniline (Feulgen), PBFI, Phloxin B (Magdala Red), Phorwite AR, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, PKH26 (Sigma), PKH67, PMIA, Pontochrome Blue Black, POPO-1, POPO-3, PO-PRO-1, PO-PRO-3, Primuline, Procion Yellow, Propidium Iodid (PI), PyMPO, Pyrene, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, QSY 7, Quinacrine Mustard, Resorufin, RH 414, Rhod-2, Rhodamine, Rhodamine 110, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B extra, Rhodamine BB, Rhodamine BG, Rhodamine Green, Rhodamine Phallicidine, Rhodamine Phalloidine, Rhodamine Red, Rhodamine WT, Rose Bengal, S65A, S65C, S65L, S65T, SBFI, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS, SITS (Primuline), SITS (Stilbene Isothiosulphonic Acid), SNAFL calcein, SNAFL-1, SNAFL-2, SNARF calcein, SNARF1, Sodium Green, SpectrumAqua, SpectrumGreen, SpectrumOrange, Spectrum Red, SPQ (6-methoxy-N-(3-sulfopropyl)quinolinium), Stilbene, Sulphorhodamine B can C, Sulphorhodamine Extra, SYTO 11, SYTO 12, SYTO 13, SYTO 14, SYTO 15, SYTO 16, SYTO 17, SYTO 18, SYTO 20, SYTO 21, SYTO 22, SYTO 23, SYTO 24, SYTO 25, SYTO 40, SYTO 41, SYTO 42, SYTO 43, SYTO 44, SYTO 45, SYTO 59, SYTO 60, SYTO 61, SYTO 62,

SYTO 63, SYTO 64, SYTO 80, SYTO 81, SYTO 82, SYTO 83, SYTO 84, SYTO 85, SYTOX Blue, SYTOX Green, SYTOX Orange, Tetracycline, Tetramethylrhodamine (TAMRA), Texas Red, Texas Red-X conjugate, Thiadicarbocyanine (DiSC3), Thiazine Red R, Thiazole Orange, Thioflavin 5, Thioflavin S, Thioflavin TCN, Thiolyte, Thiozole Orange, Tinopol CBS (Calcofluor White), TMR, TO-PRO-1, TO-PRO-3, TO-PRO-5, TOTO-1, TOTO-3, TRITC (tetramethylrhodamine isothiocyanate), True Blue, TruRed, Ultralite, Uranine B, Uvitex SFC, WW 781, X-Rhodamine, XRITC, Xylene Orange, Y66F, Y66H, Y66W, YO-PRO-1, YO-PRO-3, YOYO-1, YOYO-3, SYBR Green, Thiazole orange (interchelating dyes), or combinations thereof.

[00214] In various embodiments, the method includes inactivating a label. Inactivation may be caused by removal of the signaling entity (e.g., from the cell, or from a probe, etc.), and/or by chemically altering the label entity in some fashion, e.g., by photobleaching the label, bleaching or chemically altering the structure of the label entity, e.g., by reduction, etc.). For instance, in one set of embodiments, a fluorescent label may be inactivated by chemical or optical techniques such as oxidation, photobleaching, chemically bleaching, stringent washing or enzymatic digestion or reaction by exposure to an enzyme, dissociating the label from other components (e.g., a probe), chemical reaction of the label (e.g., to a reactant able to alter the structure of the signaling entity) or the like. For instance, bleaching may occur by exposure to oxygen, reducing agents, or the signaling entity could be chemically cleaved from the nucleic acid probe and washed away via fluid flow.

[00215] In various embodiments, the cell is fixed. As non-limiting examples, in some embodiments, the cell is fixed prior to being contacted with a probe, e.g., to preserve the positions of the nucleic acids within the cell. Techniques for fixing cells are known to those of ordinary skill in the art. As non-limiting examples, a cell may be fixed using chemicals such as formaldehyde, paraformaldehyde, glutaraldehyde, ethanol, methanol, acetone, acetic acid, or the like. In some embodiments, a cell may be fixed using HEPES-glutamic acid buffer-mediated organic solvent (HOPE). In some embodiments, the cell may be immobilized or fixed to a substrate.

[00216] In some embodiments, the cell is a mammalian cell. The cell may be any suitable cell, for example, a bacterial cell (e.g., *E. coli*), a mammalian cell (e.g., human or non-human cells), a eukaryotic cell, a prokaryotic cell, a yeast cell, an

archaeobacterial cell, or other types of cells. The cell may arise from any suitable source, for example, a cell culture. In some cases, the cell may be taken from a tissue sample, e.g., from a biopsy, artificially grown or cultured, etc. In some cases, the cells are genetically engineered. In some cases, the cell is from a tissue sample. The method can be used for visualization of a chromatin modification(s) in a plurality of cells in parallel, optionally in a single reaction chamber. The reaction chamber can be defined by a microfluidic device. In various embodiments, the plurality of cells comprises about or at least about 10, 100, 1,000, 10,000, 100,000 cells. In some embodiments, the cell comprises part of an intact tissue sample.

[00217] In various embodiments, as shown in **Fig. 4A**, a signal generated by the third probe **26** can be amplified through sequential hybridization (alternatively referred to as “sequential hybridization chain reaction (seqHCR)”) comprising binding a fourth probe **66** comprising a hybridization 2 (H2) oligonucleotide **68** to the H1 oligonucleotide **46**, wherein the H2 oligonucleotide **68** is labeled **70**. In sequential hybridization, the cell can be contacted with the third probe **26** and the fourth probe **66** in sequence, wherein the method includes a washing step prior to each sequential probe addition. As a non-limiting example, the cell can be first contacted with the third probe **26** followed by a washing step and then by contacting the cell with the fourth probe **66**, and this contacting may be referred to as a first sequential hybridization step **152**, this may then be followed by another washing step followed by contacting the cell again with the third probe **26**, and this contacting may be referred to as a second sequential hybridization step **154**, this may then be followed by another washing step followed by contacting the cell again with the fourth probe **66**, and this contacting may be referred to as a third sequential hybridization step, etc. In various embodiments, the signal generated is a fluorescent signal. In various embodiments, the signal generated comprises electromagnetic radiation (e.g., photons). In various embodiments, the methods of the present disclosure do not comprise an uncontrolled hybridization chain reaction.

[00218] In various embodiments, binding of the H1 oligonucleotide **46** to the PH2 oligonucleotide **31** can cause a hairpin loop structure **108** formed by the H1 oligonucleotide **46** to open **158**. When the hairpin loop structure **108** of the H1 oligonucleotide **46** opens **158**, a portion of the H1 oligonucleotide **46** becomes available for binding to the H2 oligonucleotide **68**, wherein the portion of the H1 oligonucleotide

46 was previously obstructed from binding through formation of the hairpin loop structure **108**. When the H2 oligonucleotide **68** binds to the H1 oligonucleotide **46**, the binding can cause a hairpin loop structure **156** formed by the H2 oligonucleotide **68** to open **162** thereby making available a portion of the H2 oligonucleotide **68** for binding to the H1 oligonucleotide **46**, wherein the portion of the H2 oligonucleotide **68** was previously obstructed from binding through formation of the hairpin loop structure **156**.

[00219] In some embodiments, conditions that allow binding of the H2 oligonucleotide of the fourth probe to an H1 oligonucleotide sequence of the third probe comprise an incubation temperature of from about 10°C to about 60°C, from about 15°C to about 45°C, or from about 20°C to about 27°C. In some embodiments, the conditions comprise an incubation time ranging from about 10 seconds to about 4 hours, from about 15 minutes to about 4 hours, or from about 30 minutes to about 2 hours. In various embodiments, the conditions comprise using a buffer. In various embodiments, the buffer comprises from about 100 mM to about 2 M, from about 140 mM to about 2M, or from about 300 mM to about 1M NaCl. In some embodiments, the buffer comprises from about 0% (vol/vol) to about 40% (vol/vol) formamide, from about 5% (vol/vol) to about 35% (vol/vol) formamide, from about 10% (vol/vol) to about 30% (vol/vol) formamide, from about 15% (vol/vol) to about 25% (vol/vol) formamide, or from about 15% (vol/vol) to about 20% (vol/vol) formamide. In some embodiments, the buffer comprises from about 0% (vol/vol) to about 35% (vol/vol) formamide. In some embodiments, the buffer comprises 0% formamide, or formamide is absent from the buffer. In some embodiments the buffer comprises about or at least about 1% (vol/vol), 2% (vol/vol), 3% (vol/vol), 4% (vol/vol), 5% (vol/vol), 6% (vol/vol), 7% (vol/vol), 8% (vol/vol), 9% (vol/vol), 10% (vol/vol), 11% (vol/vol), 12% (vol/vol), 13% (vol/vol), 14% (vol/vol), 15% (vol/vol), 16% (vol/vol), 17% (vol/vol), 18% (vol/vol), 19% (vol/vol), 20% (vol/vol), 21% (vol/vol), 22% (vol/vol), 23% (vol/vol), 24% (vol/vol), 25% (vol/vol), 26% (vol/vol), 27% (vol/vol), 28% (vol/vol), 29% (vol/vol), 30% (vol/vol), 31% (vol/vol), 32% (vol/vol), 33% (vol/vol), 34% (vol/vol), 35% (vol/vol), 36% (vol/vol), 37% (vol/vol), 38% (vol/vol), 39% (vol/vol), or 40% (vol/vol) formamide. In some embodiments the buffer comprises less than about 1% (vol/vol), 2% (vol/vol), 3% (vol/vol), 4% (vol/vol), 5% (vol/vol), 6% (vol/vol), 7% (vol/vol), 8% (vol/vol), 9% (vol/vol), 10% (vol/vol), 11% (vol/vol), 12% (vol/vol), 13% (vol/vol), 14% (vol/vol), 15% (vol/vol), 16% (vol/vol), 17%

(vol/vol), 18% (vol/vol), 19% (vol/vol), 20% (vol/vol), 21% (vol/vol), 22% (vol/vol), 23% (vol/vol), 24% (vol/vol), 25% (vol/vol), 26% (vol/vol), 27% (vol/vol), 28% (vol/vol), 29% (vol/vol), 30% (vol/vol), 31% (vol/vol), 32% (vol/vol), 33% (vol/vol), 34% (vol/vol), 35% (vol/vol), 36% (vol/vol), 37% (vol/vol), 38% (vol/vol), 39% (vol/vol), or 40% (vol/vol) formamide. In some embodiments, the buffer comprises from about 10 nM to about 50 mM Na_2HPO_4 . In some embodiments, the buffer comprises from about 5% to about 40% or from about 10% (wt/vol) to about 20% (wt/vol) dextran sulfate. In some embodiments the buffer comprises about or at least about 1% (wt/vol), 2% (wt/vol), 3% (wt/vol), 4% (wt/vol), 5% (wt/vol), 6% (wt/vol), 7% (wt/vol), 8% (wt/vol), 9% (wt/vol), 10% (wt/vol), 11% (wt/vol), 12% (wt/vol), 13% (wt/vol), 14% (wt/vol), 15% (wt/vol), 16% (wt/vol), 17% (wt/vol), 18% (wt/vol), 19% (wt/vol), 20% (wt/vol), 21% (wt/vol), 22% (wt/vol), 23% (wt/vol), 24% (wt/vol), 25% (wt/vol), 26% (wt/vol), 27% (wt/vol), 28% (wt/vol), 29% (wt/vol), or 30% (wt/vol) dextran sulfate. In some embodiments the buffer comprises less than about 1% (wt/vol), 2% (wt/vol), 3% (wt/vol), 4% (wt/vol), 5% (wt/vol), 6% (wt/vol), 7% (wt/vol), 8% (wt/vol), 9% (wt/vol), 10% (wt/vol), 11% (wt/vol), 12% (wt/vol), 13% (wt/vol), 14% (wt/vol), 15% (wt/vol), 16% (wt/vol), 17% (wt/vol), 18% (wt/vol), 19% (wt/vol), 20% (wt/vol), 21% (wt/vol), 22% (wt/vol), 23% (wt/vol), 24% (wt/vol), 25% (wt/vol), 26% (wt/vol), 27% (wt/vol), 28% (wt/vol), 29% (wt/vol), or 30% (wt/vol) dextran sulfate. Not wishing to be bound by theory, dextran sulfate can increase the kinetics of hairpin loop structure opening.

[00220] In various embodiments, the signal generated by the third probe **26** is amplified by about or at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 50, 75, 100, 150, 200, 300, 350, 400, 450, 500, or 1000 sequential hybridization steps. In various embodiments, the signal generated by the third probe **26** is amplified by less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 50, 75, 100, 150, 200, 300, 350, 400, 450, 500, or 1000 sequential hybridization steps.

[00221] Sequential hybridization can allow for controlled signal amplification. In sequential hybridization, an uncontrolled chain reaction is avoided and the signal-to-noise ratio is improved. Not wishing to be bound by theory, sequential hybridization helps to avoid signal amplification biases that can occur in uncontrolled signal amplification reactions. Signal amplification biases can occur in uncontrolled signal

amplification reactions when molecular crowding or various other factors leads to one genomic locus of interest being more accessible to a probe than another. Sequential hybridization controls sequence amplification so that any potential biases in signal amplification resulting from differences in accessibility of various genomic loci of interest in the cell can be avoided or reduced. Also, sequential hybridization allows for precise quantitative control of how many labels are coupled to a genomic locus and/or chromatin modification of interest. This can allow for quantification of an epigenetic modification level of a genetic locus of interest, as described further below.

[00222] The H2 oligonucleotide **68** can be labeled with a second dye **70**. The second dye can be a fluorescent dye. In some embodiments, the second dye is 3' Alexa Fluor 647 (NHS Ester). In some embodiments, the second dye is Alexa Fluor 647. In some embodiments, the second dye is not the same as the first dye. In some embodiments, the second dye is the same as the first dye.

[00223] In some embodiments, as shown in **Fig. 7C**, a signal generated by the third probe **26** can be amplified through branched amplification, which is described further below.

[00224] In some embodiments, as shown in **Fig. 1**, the H1 oligonucleotide **46** forms a hairpin loop structure **108**. In various embodiments, as shown in **Fig. 4A**, the H2 oligonucleotide **68** forms a hairpin loop structure **156**. In various embodiments, the PH1 oligonucleotide **30** and the PH2 oligonucleotide **31** each form hairpin loop structures **34**, **40**. In various embodiments, as shown in **Fig. 1C**, neither the PH1 oligonucleotide **30** nor the PH2 oligonucleotide **31** form a hairpin loop structure.

[00225] A hairpin loop structure can be a secondary structure formed by a nucleic acid molecule. The hairpin loop structure can comprise at least one loop region, which may be a single-stranded loop region, and at least one double-stranded stem region. The double-stranded stem region may be formed by hybridization of two regions complementary to one another within the same oligonucleotide. The hairpin loop structure may alternatively be referred to as a stem-loop structure or a hairpin structure. In some embodiments, an oligonucleotide forming a hairpin loop structure does not comprise a sticky end (i.e., a single-stranded end region) when the hairpin loop structure is closed (i.e., when the oligonucleotide is forming the hairpin loop structure). In some embodiments, an oligomer does form a hairpin loop structure comprising a

sticky end, e.g. an H1 oligomer or an H2 oligomer. In some embodiments, an oligomer forms a bulge-loop structure. For example, a “bulge” may be formed within a region of non-complementarity within a stem of a hairpin loop structure. In some embodiments, a hairpin structure may comprise regions of mismatch between two strands forming a stem of the structure. In some embodiments, two hairpin structures may be connected by a single-stranded region. In various embodiments, a portion of an oligonucleotide complementary to and capable of binding another oligonucleotide is disposed within a stem of a hairpin loop structure. In some embodiments, a portion of an oligonucleotide complementary to and capable of binding another oligonucleotide is disposed within a loop of a hairpin loop structure. In some embodiments, an activator oligonucleotide binds to a portion of a PH1 oligonucleotide disposed at least partially within a hairpin loop structure formed by the PH1 oligonucleotide.

[00226] In some embodiments, a hairpin loop structure is cross-linked by a reversible covalent bond, e.g., by a disulfide bond. In various embodiments, a PHR reaction between a PH1 oligonucleotide and a PH2 oligonucleotide may be initiated through breaking of the reversible covalent bond, optionally by reducing the bond. In some embodiments, a hairpin loop structure may be designed such that a change in physical or chemical conditions may induce or promote opening of the hairpin loop structure; for example, a change in pH, temperature, magnetic field, conductivity or redox conditions, or the addition of a chemical reagent. For example, the hairpin loop structure may be associated with a molecule or moiety which is responsive to the change in physical or chemical conditions, and undergoes or causes a conformational change to occur in response to the change in condition.

[00227] In various embodiments, a hairpin loop structure formed by the PH1 oligonucleotide and/or PH2 oligonucleotide has a stem length of about or at least about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, or 50 nt. In various embodiments, a hairpin loop structure formed by the PH1 oligonucleotide and/or PH2 oligonucleotide has a stem length of less than 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, or 50 nt. In various embodiments, a hairpin loop structure formed by the PH1 oligonucleotide and/or the PH2 oligonucleotide has a stem length of from about 10 nt to about 70 nt, from about 15 nt

to about 50 nt, or from about 20 nt to about 35 nt.

[00228] In various embodiments, a hairpin loop structure formed by the PH1 oligonucleotide and/or PH2 oligonucleotide has a melting temperature of about or at least about 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C, or 80°C. In various embodiments, a hairpin loop structure formed by the PH1 oligonucleotide and/or PH2 oligonucleotide has a melting temperature of less than about 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C, or 80°C. In various embodiments, a hairpin loop structure formed by the PH1 oligonucleotide and/or PH2 oligonucleotide has a melting temperature of from about 25°C to about 75°C, from about 35°C to about 75°C, or from about 45°C to about 75°C, or from about 50°C to about 70°C.

[00229] In various embodiments, the H1 oligomer and/or the H2 oligomer are shorter than the PH1 oligonucleotide and/or the PH2 oligonucleotide. In various embodiments, the melting temperatures of the hairpin loop structure formed by the PH1 oligonucleotide and the hairpin loop structure formed by the PH2 oligonucleotide are higher than the melting temperatures of the hairpin loop structure formed by the H1 oligomer and/or the hairpin loop structure formed by the H2 oligomer. In various embodiments, stably formed, closed hairpin loop structures used in the methods of the present disclosure do not invade one another. In various embodiments, a hairpin loop structure formed by the H1 oligonucleotide and/or H2 oligonucleotide has a stem length of about or at least about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, or 50 nt. In various embodiments, a hairpin loop structure formed by the H1 oligonucleotide and/or H2 oligonucleotide has a stem length of less than 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, or 50 nt. In various embodiments, a hairpin loop structure formed by the H1 oligonucleotide and/or the H2 oligonucleotide has a stem length of from about 1 nt to about 50 nt, from

about 5 nt to about 45 nt, or from about 10 nt to about 25 nt.

[00230] In various embodiments, a hairpin loop structure formed by the H1 oligonucleotide and/or H2 oligonucleotide has a melting temperature of about or at least about 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C, or 80°C. In various embodiments, a hairpin loop structure formed by the H1 oligonucleotide and/or H2 oligonucleotide has a melting temperature of less than about 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C, or 80°C. In various embodiments, a hairpin loop structure formed by the H1 oligonucleotide and/or H2 oligonucleotide has a melting temperature of from about 15°C to about 70°C, from about 20°C to about 60°C, or from about 30°C to about 55°C, or from about 37°C to about 50°C.

[00231] In various embodiments, a hairpin loop structure formed by an oligonucleotide of the present disclosure may include a loop comprising from any one of about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17, or 18 to about or at least about any one of 30, 28, 25, 24, 23, 22, 21, 20, or 19 nucleotides. In some embodiments, a stem length of a hairpin loop structure formed by an oligonucleotide of the present disclosure is from any one of about 12, 15, 18, 19, 20, 21, 22, 23, or 24 nucleotides to any one of about 45, 40, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 18, or 18 nucleotides.

[00232] In some embodiments, a stem-loop structure comprises stem:loop ratio (i.e., the ratio of nucleotides in the stem to nucleotides in the loop) of about or at least about 1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, or 5. In some embodiments, a stem-loop structure comprises a ratio of nucleotides in the stem to nucleotides in the loop of less than about 1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, or 5. In some embodiments, the PH1 oligonucleotide forms a hairpin loop structure comprising a first stem:loop ratio and the PH2 oligonucleotide forms a hairpin loop structure comprising a second stem:loop ratio. In some embodiments, the first stem:loop ratio is not equal to

the second stem:loop ratio. In some embodiments the second stem:loop ratio is greater than the first stem:loop ratio. In some embodiments, the first stem:loop ratio is greater than the second stem:loop ratio. In some embodiments, the first stem:loop ratio is equal to the second stem:loop ratio.

[00233] In some embodiments, a reversible chemical cross-link may be used to maintain a hairpin loop structure in a closed configuration. As a non-limiting example, a disulfide bridge can be introduced into a stem of a hairpin loop structure, e.g. by incorporation of disulfide bond-forming groups or moieties at the 5' and 3' ends of an oligonucleotide. Such a covalent bridge may be disrupted or broken to cause unfolding of the hairpin loop structure. This can be achieved by introducing an appropriate chemical reagent or appropriate conditions, for example by adding a reducing agent (e.g. DTT) in the case of a disulfide bond. A range of different groups forming reversible covalent bonds are known in the art which can be used to cross-link a nucleic acid domain and generate, or fix or hold in place, a hairpin loop structure. As well as disulfide bonds, such bonds may be created using boronate-based linking technology, or other chemical reactions or methods known or used in the art to create chemical cross-links. Boronate conjugation chemistry works for example by reacting boronic acid groups with alcohol (e.g. diol) groups (Weith et al. 1970. Biochemistry 9, 4396-4401, U.S. Pat. No. 5,777,148).

[00234] In some embodiments, a probe or oligonucleotide of the present disclosure can form one, two, or more hairpin loops. As a non-limiting example, a hairpin may comprise a bulge-loop and/or two linked hairpins.

[00235] In various embodiments, a hairpin loop structure of an oligonucleotide of the present disclosure is so designed to be sufficiently stable that the hairpin loop structure will not unfold, or open up, until bound by a complementary oligonucleotide sequence or somehow alternatively "activated" and caused to unfold (e.g., reduction of the disulfide bonds discussed above). That is, in some embodiments, a hairpin loop structure of an oligonucleotide of the present disclosure does not open by itself. This can be achieved through optimization of various parameters, such as G/C ratio or length of the stem (double-stranded regions) of a hairpin loop structure, or size of the loop.

[00236] A probe or oligonucleotide may be introduced into the cell using any suitable

method. In various embodiments, contacting the cell with a probe or oligonucleotide comprises introducing the probe or oligonucleotide into the cell. In some embodiments, the cell may be sufficiently permeabilized such that the probe or oligonucleotide may be introduced into the cell by flowing a fluid containing the probe or oligonucleotide around the cell. In some cases, the cell may be sufficiently permeabilized as part of a fixation process; in other embodiments, the cell may be permeabilized by exposure to certain chemicals such as ethanol, methanol, Triton, or the like. In addition, in some embodiments, techniques such as electroporation or microinjection may be used to introduce a probe or oligonucleotide into the cell or other sample.

[00237] In some embodiments, the conditions that allow binding of the first oligonucleotide of the first probe to the genomic locus of interest comprise a denaturation step carried out at a temperature of about or of at least about 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, or 95°C, in a hybridization buffer. In some embodiments, the denaturation step is carried out at a temperature of less than about 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, or 95°C, in the first hybridization buffer. In some embodiments, the denaturation step is carried out at a temperature of from about 50°C to about 95°C, from about 65°C to about 95°C, or from about 70°C to about 90°C, in the hybridization buffer.

[00238] In some embodiments, the cell is incubated during denaturation while in contact with the hybridization buffer for a period of time of about or of at least about 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, or 15 min prior to a wash step. In some embodiments, the cell is incubated during denaturation while in contact with the hybridization buffer for a period of time of less than about 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, or 15 min prior to a wash step. In some embodiments, the cell is incubated during denaturation while in contact with the hybridization buffer for a period of time from about 30 seconds to about 30 minutes, from about 1 minute to about 15 minutes, or from about 3 minutes to about 10 minutes.

[00239] In various embodiments, the hybridization buffer comprises from about 300 mM to about 600 mM NaCl, from about 10 mM to about 50 mM sodium citrate, from about 40% (vol/vol) to about 50% (vol/vol) formamide, and/or from about 10% (wt/vol) to about 20% (wt/vol) dextran sulfate.

[00240] In various embodiments, the conditions that allow binding of the first oligonucleotide of the first probe to the genomic locus of interest comprise a hybridization step. In various embodiments, the hybridization step comprises an incubation at a temperature ranging from about 15°C to about 50°C, from about 25°C to about 37°C, or from about 30°C to about 37°C for about or at least about 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 7 hr, 8 hr, 9 hr, 10 hr, 11 hr, 12 hr, 13 hr, 14 hr, 15 hr, 16 hr, 17 hr, 18 hr, 19 hr, 20 hr, 21 hr, 22 hr, 23 hr, 24 hr, 25 hr, 26 hr, 27 hr, 28 hr, 29 hr, 30 hr, 31 hr, 32 hr, 33 hr, 34 hr, 35 hr, 36 hr, 37 hr, 38 hr, 39 hr, 40 hr, 41 hr, 42 hr, 43 hr, 44 hr, 45 hr, 46 hr, 47 hr, or 48 hr.

[00241] In some embodiments, the conditions that allow binding of the antibody to the chromatin modification comprise a temperature ranging from about 4°C to about 37°C, in a first incubation buffer. In some embodiments, the cell is incubated with in contact with the first incubation buffer for a period of time ranging from 1 hr to 12 hr prior to a wash step. In some embodiments, the first incubation buffer comprises from about 0.05% (vol/vol) to about 0.1% (vol/vol) TWEEN, from about 1% (wt/vol) to about 5% (wt/vol) BSA, from about 20 mg/mL to about 25 mg/mL glycine, and/or 1x Dulbecco's phosphate buffered saline.

[00242] In some embodiments, the conditions that allow binding of the H1 oligonucleotide of the third probe to a nucleotide sequence of each of the PH1 and PH2 oligonucleotides comprises a temperature ranging from 20°C to about 37°C, in a second incubation buffer. In some embodiments, the cell is incubated in contact with the second incubation buffer for a period of time ranging from 30 min to 2 hr prior to a wash step.

[00243] In some embodiments, the conditions that allow binding of the H1 oligonucleotide of the third probe to the sequence made available when the PH1 and PH2 oligonucleotides have hybridized comprises a temperature ranging from about 20°C to about 37°C, in a third incubation buffer. In some embodiments, the cell is incubated in contact with the third incubation buffer for a period of time ranging from 30 min to 2 hr prior to a wash step.

[00244] In some embodiments, the conditions that allow binding of the activator oligonucleotide to the PH1 oligonucleotide comprises a temperature ranging from about 20°C to about 37°C, in a fourth incubation buffer. In some embodiments, the cell is

incubated in contact with the fourth incubation buffer for a period of time ranging from 30 min to 2 hr prior to a wash step.

[00245] In various embodiments, the second incubation buffer, the third incubation buffer, and/or the fourth incubation buffer comprises from about 300 mM to about 1 M NaCl, from about 0% (vol/vol) to about 50% (vol/vol) formamide, from about 10% (wt/vol) to about 20% (wt/vol) dextran sulfate, and/or from about 10 nM to about 50 mM Na₂HPO₄.

[00246] In various embodiments, the method includes one or more wash steps. The wash step(s) can be used to remove unbound probes or oligonucleotides after contacting the cell with the probe or oligonucleotide. Stringency of wash conditions (e.g., temperature, time, amount of buffer used) can be optimized to remove unbound or non-specifically bound probes or oligonucleotides from the cell.

[00247] In some embodiments, a wash step is a low stringent wash step carried out at a temperature ranging from about 22°C to about 30°C and for a time duration of from about 15 minutes to about 45 minutes. In some embodiments, a low stringent wash buffer used in the low stringent wash step comprises from about 35% (vol/vol) to about 55% (vol/vol) formamide, from about 0.01% (vol/vol) to about 1% (vol/vol) TWEEN 20, from about 300 mM to about 600 mM NaCl, and/or from about 10 nM to about 50 mM sodium citrate. In some embodiments, the low stringent wash buffer comprises about 0.1% (vol/vol) TWEEN.

[00248] In some embodiments, a wash step is a high stringent wash step carried out at a temperature ranging from about 50°C to about 85°C and for a time duration of from about 15 minutes to about 45 minutes. In some embodiments, a high stringent wash buffer used in the high stringent wash step comprises from about 0.01% (vol/vol) to about 1% (vol/vol) TWEEN 20, from about 300 mM to about 600 mM NaCl, and/or from about 10 nM to about 50 mM sodium citrate. In some embodiments, the high stringent wash buffer comprises about 0.1% (vol/vol) TWEEN. In various embodiments, the high stringent wash step is carried out at a temperature greater than 59°C.

[00249] In some embodiments, see **Figs. 1C** and **6A**, when the H1 oligonucleotide **46** binds to a nucleotide sequence **52** of each of said PH1 **30** and PH2 **31** oligonucleotides, a terminus of the PH1 oligonucleotide **140** is proximal to a terminus of the PH2 oligonucleotide **142**, such that the 3' end of the PH1 oligonucleotide **30** is

disposed proximal to the 5' end of the PH2 oligonucleotide **31** or the 5' end of the PH1 oligonucleotide **30** is disposed proximal to the 3' end of the PH2 oligonucleotide **31**.

[00250] In various embodiments, the cell is contacted with the first probe before the cell is contacted with the second probe. In some embodiments, the cell is contacted with the second probe before the cell is contacted with the first probe. In some embodiments, the cell is contacted with the first probe and the second probe simultaneously. In various embodiments, the cell is contacted with an activator oligonucleotide only after the cell has been contacted with a first probe and a second probe. In some embodiments, the cell is contacted with a third probe only after the cell has been contacted with a first probe, a second probe, and an activator probe. In some embodiments, the cell is contacted with a third probe only after the cell has been contacted with a first probe and a second probe.

[00251] In some embodiments, the cell is positioned on a microscope. In some cases, the microscope may contain one or more channels, such as fluidic or microfluidic channels, to direct or control fluid to or from the cell. For instance, in some embodiments, probes, such as those discussed herein, may be introduced and/or removed from contact with the cell by flowing fluid through one or more channels to or from the cell. In some cases, there may also be one or more chambers or reservoirs for holding fluid, e.g., in fluidic communication with the channel, and/or with the cell. Those of ordinary skill in the art will be familiar with channels, including fluidic or microfluidic channels, for moving fluid to or from a cell. In various embodiments, a fluidic system used in the methods of the present disclosure is the fluidic system depicted in Fig. 4 of Chapter One - RNA Imaging with Multiplexed Error-Robust Fluorescence in situ Hybridization (MERFISH) J.R. Moffitt, X. Zhuang *Methods in Enzymology* 572 1 (2016), the disclosure of which is incorporated herein by reference for all purposes.

[00252] As used herein, "microfluidic," "microscopic," "microscale," the "micro-" prefix (for example, as in "microchannel"), and the like generally refer to elements or articles having widths or diameters of less than about 1 mm, or less than about 100 microns (micrometers) in some embodiments. In some embodiments, larger channels may be used instead of, or in conjunction with, microfluidic channels for any of the embodiments of the methods provided herein. For examples, channels having widths or diameters of less than about 10 mm, less than about 9 mm, less than about 8 mm, less

than about 7 mm, less than about 6 mm, less than about 5 mm, less than about 4 mm, less than about 3 mm, or less than about 2 mm may be used in certain embodiments. In some embodiments, the element or article includes a channel through which a fluid can flow. In all embodiments, specified widths can be a smallest width (i.e. a width as specified where, at that location, the article can have a larger width in a different dimension), or a largest width (i.e. where, at that location, the article has a width that is no wider than as specified, but can have a length that is greater). Thus, for instance, the microfluidic channel may have an average cross-sectional dimension (e.g., perpendicular to the direction of flow of fluid in the microfluidic channel) of less than about 1 mm, less than about 500 microns, less than about 300 microns, or less than about 100 microns. In some cases, the microfluidic channel may have an average diameter of less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about 30 microns, less than about 25 microns, less than about 10 microns, less than about 5 microns, less than about 3 microns, or less than about 1 micron.

[00253] A “channel,” as used herein, is used to refer to a feature on or in an article (e.g., a substrate) that at least partially directs the flow of a fluid. In some cases, the channel may be formed, at least in part, by a single component, e.g. an etched substrate or molded unit. The channel can have any cross-sectional shape, for example, circular, oval, triangular, irregular, square, or rectangular (having any aspect ratio), or the like, and can be covered or uncovered (i.e., open to the external environment surrounding the channel). In embodiments where the channel is completely covered, at least one portion of the channel can have a cross-section that is completely enclosed, and/or the entire channel may be completely enclosed along its entire length with the exception of its inlet and outlet.

[00254] A channel may have any aspect ratio, e.g., an aspect ratio (length to average cross-sectional dimension) of at least about 2:1, more typically at least about 3:1, at least about 5:1, at least about 10:1, etc. In various embodiments, the channel is a flow chamber. As used herein, a “cross-sectional dimension,” in reference to a fluidic or microfluidic channel, is measured in a direction generally perpendicular to fluid flow within the channel. A channel can include characteristics that facilitate control over fluid transport, e.g., structural characteristics and/or physical or chemical characteristics

(hydrophobicity vs. hydrophilicity) and/or other characteristics that can exert a force (e.g., a containing force) on a fluid. The fluid within the channel may partially or completely fill the channel. In some cases the fluid may be held or confined within the channel or a portion of the channel in some fashion, for example, using surface tension (e.g., such that the fluid is held within the channel within a meniscus, such as a concave or convex meniscus). In an article or substrate, some (or all) of the channels may be of a particular size or less, for example, having a largest dimension perpendicular to fluid flow of less than about 10 mm, less than about 9 mm, less than about 8 mm, less than about 7 mm, less than about 6 mm, less than about 5 mm, less than about 4 mm, less than about 3 mm, less than about 2 mm, less than about 1 mm, less than about 500 microns, less than about 200 microns, less than about 100 microns, less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about 30 microns, less than about 25 microns, less than about 10 microns, less than about 3 microns, less than about 1 micron, less than about 300 nm, less than about 100 nm, less than about 30 nm, or less than about 10 nm or less in some cases. In one embodiment, the channel is a capillary.

[00255] A variety of materials and methods, according to certain aspects of the invention, can be used to form devices or components containing microfluidic channels, chambers, etc. For example, various devices or components can be formed from solid materials, in which the channels can be formed via micromachining, film deposition processes such as spin coating and chemical vapor deposition, physical vapor deposition, laser fabrication, photolithographic techniques, etching methods including wet chemical or plasma processes, electrodeposition, and the like. See, for example, *Scientific American*, 248:44-55, 1983 (Angell, et al).

[00256] In some embodiments, various structures or components can be formed of a polymer, for example, an elastomeric polymer such as polydimethylsiloxane ("PDMS"), polytetrafluoroethylene ("PTFE" or Teflon®), or the like. For instance, in some embodiments, a channel such as a microfluidic channel may be implemented by fabricating the fluidic system separately using PDMS or other soft lithography techniques (details of soft lithography techniques suitable for this embodiment are discussed in the references entitled "Soft Lithography," by Younan Xia and George M. Whitesides, published in the *Annual Review of Material Science*, 1998, Vol. 28, pages

153-184, and “Soft Lithography in Biology and Biochemistry,” by George M. Whitesides, Emanuele Ostuni, Shuichi Takayama, Xingyu Jiang, and Donald E. Ingber, published in the *Annual Review of Biomedical Engineering*, 2001, Vol. 3, pages 335-373; each of these references is incorporated herein by reference in its entirety for all purposes).

[00257] Further non-limiting examples of potentially suitable polymers include, but are not limited to, polyethylene terephthalate (PET), polyacrylate, polymethacrylate, polycarbonate, polystyrene, polyethylene, polypropylene, polyvinylchloride, cyclic olefin copolymer (COC), polytetrafluoroethylene, a fluorinated polymer, a silicone such as polydimethylsiloxane, polyvinylidene chloride, bis-benzocyclobutene (“BCB”), a polyimide, a fluorinated derivative of a polyimide, or the like. Combinations, copolymers, or blends involving polymers including those described above are also envisioned. The device may also be formed from composite materials, for example, a composite of a polymer and a semiconductor material.

[00258] In some embodiments, various microfluidic structures or components of the device are fabricated from polymeric and/or flexible and/or elastomeric materials, and can be conveniently formed of a hardenable fluid, facilitating fabrication via molding (e.g. replica molding, injection molding, cast molding, etc.). The hardenable fluid can be essentially any fluid that can be induced to solidify, or that spontaneously solidifies, into a solid capable of containing and/or transporting fluids contemplated for use in and with the fluidic network. In some embodiments, the hardenable fluid comprises a polymeric liquid or a liquid polymeric precursor (i.e. a “prepolymer”). Suitable polymeric liquids can include, for example, thermoplastic polymers, thermoset polymers, waxes, metals, or mixtures or composites thereof heated above their melting point. As another example, a suitable polymeric liquid may include a solution of one or more polymers in a suitable solvent, which solution forms a solid polymeric material upon removal of the solvent, for example, by evaporation. Such polymeric materials, which can be solidified from, for example, a melt state or by solvent evaporation, are well known to those of ordinary skill in the art. A variety of polymeric materials, many of which are elastomeric, are suitable, and are also suitable for forming molds or mold masters, for embodiments where one or both of the mold masters is composed of an elastomeric material. A non-limiting list of examples of such polymers includes polymers of the general classes of silicone polymers, epoxy polymers, and acrylate polymers. Epoxy polymers are

characterized by the presence of a three-membered cyclic ether group commonly referred to as an epoxy group, 1,2-epoxide, or oxirane. For example, diglycidyl ethers of bisphenol A can be used, in addition to compounds based on aromatic amine, triazine, and cycloaliphatic backbones. Another example includes the well-known Novolac polymers. Non-limiting examples of silicone elastomers suitable for use according to the invention include those formed from precursors including the chlorosilanes such as methylchlorosilanes, ethylchlorosilanes, phenylchlorosilanes, etc.

[00259] Silicone polymers can be used in certain embodiments, for example, the silicone elastomer polydimethylsiloxane. Non-limiting examples of PDMS polymers include those sold under the trademark Sylgard by Dow Chemical Co., Midland, Mich., and particularly Sylgard 182, Sylgard 184, and Sylgard 186. Silicone polymers including PDMS have several beneficial properties simplifying fabrication of various structures of the invention. For instance, such materials are inexpensive, readily available, and can be solidified from a prepolymeric liquid via curing with heat. For example, PDMSs are typically curable by exposure of the prepolymeric liquid to temperatures of about, as non-limiting examples, about 65°C to about 75°C for exposure times of, for example, at least about an hour. Also, silicone polymers, such as PDMS, can be elastomeric and thus may be useful for forming very small features with relatively high aspect ratios, necessary in certain embodiments of the invention. Flexible (e.g., elastomeric) molds or masters can be advantageous in this regard.

[00260] A potential advantage of forming structures such as microfluidic structures or channels from silicone polymers, such as PDMS, is the ability of such polymers to be oxidized, for example by exposure to an oxygen-containing plasma such as an air plasma, so that the oxidized structures contain, at their surface, chemical groups capable of cross-linking to other oxidized silicone polymer surfaces or to the oxidized surfaces of a variety of other polymeric and non-polymeric materials. Thus, structures can be fabricated and then oxidized and essentially irreversibly sealed to other silicone polymer surfaces, or to the surfaces of other substrates reactive with the oxidized silicone polymer surfaces, without the need for separate adhesives or other sealing means. In most cases, sealing can be completed simply by contacting an oxidized silicone surface to another surface without the need to apply auxiliary pressure to form the seal. That is, the pre-oxidized silicone surface acts as a contact adhesive against

suitable mating surfaces. Specifically, in addition to being irreversibly sealable to itself, oxidized silicone such as oxidized PDMS can also be sealed irreversibly to a range of oxidized materials other than itself including, for example, glass, silicon, silicon oxide, quartz, silicon nitride, polyethylene, polystyrene, glassy carbon, and epoxy polymers, which have been oxidized in a similar fashion to the PDMS surface (for example, via exposure to an oxygen-containing plasma). Oxidation and sealing methods useful in the context of the present invention, as well as overall molding techniques, are described in the art, for example, in an article entitled "Rapid Prototyping of Microfluidic Systems and Polydimethylsiloxane," *Anal. Chem.*, 70:474-480, 1998 (Duffy et al.), incorporated herein by reference.

[00261] Various technologies may be used to detect a signal generated by the label of a probe. In various embodiments, the signal is detected using an imaging technology. Non-limiting examples of imaging technologies suitable for use in the methods of the present disclosure include Non-limiting examples include STORM (stochastic optical reconstruction microscopy), STED (stimulated emission depletion microscopy), NSOM (Near-field Scanning Optical Microscopy), 4Pi microscopy, SIM (Structured Illumination Microscopy), SMI (Spatially Modulated Illumination) microscopy, RESOLFT (Reversible Saturable Optically Linear Fluorescence Transition Microscopy), GSD (Ground State Depletion Microscopy), SSIM (Saturated Structured- Illumination Microscopy), SPDM (Spectral Precision Distance Microscopy), Photo-Activated Localization Microscopy (PALM), Fluorescence Photoactivation Localization Microscopy (FPALM), LIMON (3D Light Microscopical Nanosizing Microscopy), Super-resolution optical fluctuation imaging (SOFI), or the like. See, e.g., U.S. Pat. No. 7,838,302, issued Nov. 23, 2010, entitled "Sub-Diffraction Limit Image Resolution and Other Imaging Techniques," by Zhuang, et al.; U.S. Pat. No. 8,564,792, issued Oct. 22, 2013, entitled "Sub-diffraction Limit Image Resolution in Three Dimensions," by Zhuang, et al.; or Int. Pat. Apl. Pub. No. WO 2013/090360, published Jun. 20, 2013, entitled "High-Resolution Dual-Objective Microscopy," by Zhuang, et al., each incorporated herein by reference in their entireties for all purposes. In various embodiments, detecting the signal comprises acquiring an image, optionally a digital image. In various embodiments, multiple images can be taken of the cell, optionally at different depths. Images may be acquired manually or in an automated manner.

[00262] In some embodiments, the method includes taking at least 1, 2, 3, 4, 5, 10, 15, 20, 30, 50, 75, 100, 150, 200, 300, 400, 500, 1,000, 1,500, or 2,000 images of the cell from different fields of view to produce an overall image, optionally a three-dimensional image. In some embodiments, the method includes taking at least 1, 2, 3, 4, 5, 10, 15, 20, 30, 50, 75, 100, 150, 200, 300, 400, 500, 1,000, 1,500, or 2,000 images of the cell from the same field of view. In some embodiments, multiple images are taken at each field of view imaged. In some embodiments, different wavelengths may be used. For example, in some cases, images may be collected with different illumination sources, and captured using different optical filters so as to produce different colors of images that probe the presence of different fluorescent compounds. Thus, in some embodiments, multiple images may be taken at different wavelengths, e.g., to view the images in different colors (for example, red-green-blue, red-yellow-blue, cyan-magenta-yellow, or the like). In some embodiments, images are taken using a plurality of fluorescent channels of an imaging device.

[00263] In addition, in some embodiments, multiple images may be collected with different imaging modalities, e.g. super-resolution optical microscopy, conventional epifluorescence microscopy, confocal microscopy, etc., including those described herein. Such images may be combined, in some cases, to create high content optical measurements.

[00264] In various embodiments, the method includes a multiplex fluorescence *in situ* hybridization (mFISH), an embodiment of which is shown in **Fig. 1B**. In various embodiments, the method further comprises providing a plurality of first probes **20** each of which comprises a first oligonucleotide **28**. Each of the first probes **20** targets a genomic locus **32** of interest. The method also includes providing a plurality of labeled **78** readout probes **76**. The labeled readout probes **76** each comprise a label **78**. Each labeled readout probe **76** selectively binds to at least one of the plurality of first oligonucleotides **28** of the plurality of first probes **20**. The method further comprises contacting the cell with each of the plurality of first probes **20** under conditions that allow binding of the unique first oligonucleotide **28** of the first probes **20** to the genomic locus **32** of the cell. The method further comprises contacting the cell with each labeled **78** readout probe **76**. The method includes detecting each label **78** of each readout probe **76**.

[00265] In some embodiments, the readout probes **76** are labeled with a plurality of dyes **78**. The dyes **78** can be fluorescent dyes.

[00266] In some embodiments, the readout probes **76** are labeled the same type of dye. Such readout probes can be applied sequentially and imaged in the same fluorescence channel.

[00267] In various embodiments, the first oligonucleotide(s) comprises one or more unique readout probe binding sites **80**, see **Fig. 1B**, selectively bound by one of the plurality of labeled readout probes **76**. In some embodiments, each of the plurality of first oligonucleotides comprises one or more readout probe binding sites each selectively bound by one of the plurality of labeled readout probes.

[00268] In various embodiments, see **Fig. 1B**, the readout probe binding site **80** comprises the 3' end **88** of the first oligonucleotide **28**. In various embodiments, the readout probe binding site comprises the 5' end **86** of the first oligonucleotide **28**. In some embodiments, the readout probe binding site comprises the 3' terminus **88** of the first oligonucleotide **28** and extends to a first contiguous nucleotide **90** of the first oligonucleotide **28** that is not complementary to a nucleotide sequence corresponding to the genomic locus of interest **32** in the cell. In some embodiments, the readout probe binding site **80** comprises the 5' terminus **86** of the first oligonucleotide and extends to a first contiguous nucleotide **90** of the first oligonucleotide that is not complementary to a nucleotide sequence corresponding to the genomic locus of interest **32** in the cell.

[00269] In various embodiments, a readout probe binding sites comprises a nucleotide sequence of SEQ ID NO: 9, the reverse complement of SEQ ID NO: 9, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 77%, 80%, 83%, 87%, 90%, 93%, or 97% sequence identity to SEQ ID NO: 9. In various embodiments, the readout probe binding site comprises a sequence complementary to SEQ ID NO: 9 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 77%, 80%, 83%, 87%, 90%, 93%, or 97% sequence identity to a sequence complementary to SEQ ID NO: 9. In various embodiments, the sequence complementary to SEQ ID NO: 9 is the reverse complement of SEQ ID NO: 9.

[00270] In various embodiments, a readout probe binding site comprises a nucleotide sequence of SEQ ID NO: 10, the reverse complement of SEQ ID NO: 10, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 77%, 80%,

83%, 87%, 90%, 93%, or 97% sequence identity to SEQ ID NO: 10. In various embodiments, the readout probe binding site comprises a sequence complementary to SEQ ID NO: 10 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 77%, 80%, 83%, 87%, 90%, 93%, or 97% sequence identity to a sequence complementary to SEQ ID NO: 10. In various embodiments, the sequence complementary to SEQ ID NO: 10 is the reverse complement of SEQ ID NO: 10.

[00271] In various embodiments, a readout probe binding site comprises a nucleotide sequence of SEQ ID NO: 11, the reverse complement of SEQ ID NO: 11, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 77%, 80%, 83%, 87%, 90%, 93%, or 97% sequence identity to SEQ ID NO: 11. In various embodiments, the readout probe binding site comprises a sequence complementary to SEQ ID NO: 11 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 77%, 80%, 83%, 87%, 90%, 93%, or 97% sequence identity to a sequence complementary to SEQ ID NO: 11. In various embodiments, the sequence complementary to SEQ ID NO: 11 is the reverse complement of SEQ ID NO: 11.

[00272] In various embodiments, the readout probe binding site comprises about or at least about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 80, 85, 90, 95, or 100 nucleotides. In various embodiments, the readout probe binding site comprises no more than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 80, 85, 90, 95, or 100 nucleotides. In various embodiments, the readout probe binding site comprises from about 3 to about 300 nucleotides, from about 15 to about 60 nucleotides, or from about 20 to about 40 nucleotides. The readout probe binding site can comprise about 30 nucleotides.

[00273] In various embodiments, contacting the cell with the third probe precedes contacting the cell with each labeled readout probe. In various embodiments, contacting the cell with one or more labeled readout probes precede contacting the cell with the third probe. In various embodiments, the cell is individually contacted with each labeled

readout probe in sequence. In various embodiments, a washing step is completed after subjecting the cell to a first readout probe and before contacting the cell with a second readout probe. In some embodiments, the cell is contacted with a plurality of labeled readout probes and the third probe simultaneously, optionally wherein each of the plurality of readout probes and the third probe is labeled with a unique dye.

[00274] In some embodiments, the cell is contacted with sub-set of the plurality of labeled readout probes in sequence. As a non-limiting example, the cell may be contacted with about or at least about 2, 3, 4, 5, 6, 7, 8, 9, or 10 unique readout probes at a time in sequence until the cell has been contacted with all of the plurality of labeled readout probes.

[00275] In some embodiments, the method further comprises inactivating, see **Fig. 1B**, the label of a first readout probe **94**, optionally by photobleaching, to yield an inactivated label **92**, prior to contacting the cell with a second readout probe **96**. In some embodiments, the method includes inactivating the label **78** of the second readout probe **96** prior to contacting the cell with a third readout probe **98**, inactivating the label **78** of the third readout probe **98** prior to contacting the cell with a fourth readout probe, etc. In various embodiments, the method includes contacting the cell with about or at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1,100, 1,200, 1,300, 1,400, 1,500, 2,000, 3,000, 4,000, 5,000, or 10,000 labeled readout probes. In various embodiments, the method includes contacting the cell with not more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1,100, 1,200, 1,300, 1,400, 1,500, 2,000, 3,000, 4,000, 5,000, or 10,000 labeled readout probes.

[00276] In various embodiments, the plurality of first probes comprises about or at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 100, 150, 200, 300, 400, 500, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500, 5,000, 10,000, , 20,000, 30,000, 40,000, 50,000, 100,000, 150,000, 160,000, 170,000, 180,000, 190,000, 200,000, 250,000, 300,000, or 500,000 unique first probes. In various embodiments, the plurality of first probes comprises no more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 100, 150, 200, 300, 400, 500, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500, 5,000, 10,000, 20,000, 30,000, 40,000, 50,000, 100,000, 150,000, 160,000, 170,000,

180,000, 190,000, 200,000, 250,000, 300,000, or 500,000 unique first probes.

[00277] In some embodiments, the cell is contacted with a plurality of unique first probes simultaneously. In some embodiments, the cell is contacted individually with a plurality of first probes in sequence. In some embodiments, the cell is contacted with a plurality of readout probes simultaneously. In some embodiments, the cell is individually contacted with a plurality of readout probes in sequence. In some embodiments, the cell is simultaneously contacted with a plurality of readout probes, wherein each of the plurality of readout probes is labeled with a unique dye.

[00278] In various embodiments, the readout probes may be of any length. The readout probes may independently be of the same or different lengths. In some embodiments, the readout probe may be at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, or at least 50 nucleotides in length. In some cases, the readout probe may be no more than 75, no more than 60, no more than 65, no more than 60, no more than 55, no more than 50, no more than 45, no more than 40, no more than 35, no more than 30, no more than 20, or no more than 10 nucleotides in length. In some embodiments, the readout probe may be at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% complementary to the readout probe binding site on a first oligonucleotide.

[00279] In some embodiments, conditions that allow binding of a readout probe to a corresponding readout probe binding site comprises a temperature ranging from about 20°C to about 37°C., in a buffer comprising from about 300 mM to about 600 mM NaCl, from about 10nM to about 50 mM sodium citrate, and from about 5% (vol/vol) to about 30% (vol/vol) ethylene carbonate. In some embodiments, the cell is incubated with the buffer for a period of time ranging from 10 min to 1 hr prior to a wash step.

[00280] In some embodiments, the cell is contacted with the second probe prior to being contacted with the first probe. In various embodiments, the cell is contacted with the second probe and the second probe is post fixed prior to the cell being contacted with the first probe. In various embodiments, the second probe is post fixed using paraformaldehyde and BS(PEG)5 (PEGylated bis(sulfosuccinimidyl)suberate), optionally 4% (wt/vol) paraformaldehyde and 1.5mM BS(PEG)5. In some embodiments,

the second probe is post fixed by contacting the cell with paraformaldehyde for a period of time of about or of at least about 5 min, 10 min, 15 min, 20 min, 23 min, 25 min, or 30 min. In some embodiments, contacting the cell with the second probe prior to contacting the cell with the first probe can prevent damage to sensitive chromatin modifications that may be caused by the conditions that allow binding of the first oligonucleotide to the genomic locus of interest. In some embodiments, a chromatin modification of interest is not sensitive to conditions that allow binding of the first oligonucleotide to the genomic locus of interest and, therefore, the order in which the cell is contacted with the first probe and the second probe can be varied.

[00281] In various embodiments, the method further comprises identifying each of the plurality of first probes using a barcoding scheme see **Fig. 9**. In various embodiments, the method comprises identifying a probe using a barcoding scheme. In various embodiments, the barcoding scheme is a combinatorial barcoding scheme. Any of the various embodiments of combinatorial barcoding schemes and various components thereof disclosed in U.S. Patent Application Publication Nos. US 2020/0095630 and US 2017/0220733, each of which are incorporated herein by reference in their entirety for all purposes, may be used in combination with the methods of the present disclosure.

[00282] A non-limiting example of a barcoding scheme (e.g. see Example 9 below) that can be used to identify a first probe **20** is shown in **Fig. 9**. Each unique first oligomer **28** can comprise a unique combination of several unique readout regions **160**. In some embodiments, one or a plurality of first oligomers **28** comprises a unique set of unique readout regions **160**. Use of the barcoding scheme to identify the first probe **20** can allow for the genomic locus **32** of interest to which the first probe **20** is bound to be detected through a combination of binding and imaging rounds. Binding may comprise nucleotide hybridization and, thus, be referred to as “hybridization.” Each readout region **160** can be bound by a different unique readout probe **76**. In some embodiments, the cell is individually contacted with each unique readout probe **76**, one at a time, in a sequence of rounds of hybridization, each round having a designated number (e.g., Hyb 1, Hyb 2, and Hyb 3 as shown in **Fig. 9**), and imaging. Following each round of hybridization, a label **78** of the readout probe **76** used in the round of hybridization is inactivated, optionally through photobleaching, to yield an inactivated label **92**. The specific round number(s) in which a first probe **20** is bound or not bound

by a readout probe **76** can be used to determine a barcode corresponding to the first probe **20**. As a non-limiting example, a first probe **20** bound by a readout probe **76** in only rounds 1, 2, 5, and 10 in 14 total rounds of hybridization has the barcode “11001000010000,” where for each round the first probe **20** can be assigned a “0” (no binding) or “1” (binding). In various embodiments, the barcode may be referred to as a codeword unique to a probe or a plurality of probes to which the barcode is coupled. Each “0” or “1” assigned to a first probe **20** can be referred to as a “bit.” Using a combinatorial barcode as shown in **Fig. 9** can allow for a large number of first probes **20** to be identified using only a few rounds of hybridization. Also, such identification can be achieved even when the label **78** of each readout probe **76** comprises the same dye **78**; for example, four readout regions **160** can be used to identify 1001 (14-choose-4) unique codewords assigned to first probes **20** with 14 Alexa Fluor 647 labeled readout probes **76** in 14 rounds of hybridization. By incorporating multicolor imaging, more bits (0’s and 1’s, corresponding to “no binding” and “binding”, respectively) can be assigned to a first probe **20** in less hybridization rounds; for example, four readout regions **160** can be used to identify 1001 unique codewords assigned to first probes **20** using 2-color imaging in 7 rounds of hybridization. This can be done by using two unique readout probes in each round of hybridization, wherein each of the two unique readout probes used in each round of hybridization is labeled with a spectrally distinct fluorescent dye.

[00283] In some embodiments, the codeword may define an error-correcting code, as disclosed in U.S. Patent Application Nos. 2017/0220733 A1 and 2017/0095630 A1, which are incorporated herein in their entirety for all purposes, to reduce or prevent misidentification of a sequence coupled to the codeword. In some embodiments, the codewords may be subjected to error detection and/or correction.

[00284] In various embodiments, the readout regions may be concatenated together to produce a barcode. In some embodiments, one or more of the readout regions may be separated with constant portions of oligonucleotides. Any method may be used for the concatenation; for example, readout regions can be concatenated together using ligation, overlap PCR, oligonucleotide pool synthesis, or other techniques known to those of ordinary skill in the art for joining or concatenating nucleic acids together. In some embodiments, a readout region comprises a nucleic acid sequence of any one of

SEQ ID NOs: 9 to 11 and 13 to 147, the reverse complement of any one of SEQ ID NOs: 9 to 11 and 13 to 147, or a nucleic acid sequence sharing at least about 50%, 55%, 60%, 65%, 70%, 75%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity with any one of SEQ ID NOs: 9 to 11 and 13 to 147 or with the reverse complement of any one of SEQ ID NOs: 9 to 11 and 13 to 147.

[00285] The readout regions may individually be of any length. If more than one readout region is used, the readout regions may independently have the same or different lengths. For instance, a readout region may be about or at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 65, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, or 450 nucleotides in length. In some cases, a readout region may be no more than about 500, 450, 400, 350, 300, 250, 200, 175, 150, 125, 100, be 75, 60, 65, 60, 55, 50, 45, 40, 35, 30, 20, or 10 nucleotides in length. In some embodiments, the readout regions are about 20 nucleotides in length. In some embodiments, the readout regions are 30 nucleotides in length.

[00286] The readout regions and various sequences of the present disclosure may be arbitrary or random in some embodiments. In certain cases, the readout regions and/or various other sequences are chosen so as to reduce or minimize homology with other components of the cell or of the methods of the present disclosure e.g., such that the readout regions and readout probes do not themselves bind to or hybridize with nucleic acids suspected of being within the cell. In some cases, the homology may be less than 10%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1%. In some cases, there may be a homology of less than 20 base pairs, less than 18 base pairs, less than 15 base pairs, less than 14 base pairs, less than 13 base pairs, less than 12 base pairs, less than 11 base pairs, or less than 10 base pairs. In some cases, the base pairs are sequential.

[00287] In various embodiments, the methods of the present disclosure may be applied for epigenetic/epigenomic profiling within the cell. In various embodiments, the methods of the present disclosure may be applied for epigenetic/epigenomic profiling on a chromosome spread extracted chromatin, extracted DNA outside of the cell or extra-cellular DNA, for example circulating free DNA, cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA).

Epi-PHR

[00288] In another aspect, the present disclosure provides an Epi-PHR (epigenetic mark – proximity-dependent hybridization reaction) method for *in situ* visualization of a chromatin modification at a genomic locus of interest of a cell. Various components of the Epi-PHR method are illustrated in **Fig. 1A**.

[00289] The Epi-PHR method, see **Fig. 1A**, includes providing a first probe **20**, a second probe **22**, an activator oligonucleotide **24**, and a third probe **26**. The first probe **20** comprises a first oligonucleotide **28** coupled to a proximity hybridization 1 (PH1) oligonucleotide **30**. The first oligonucleotide **28** binds to a genomic locus of interest **32**. The PH1 oligonucleotide **30** forms a first hairpin loop structure **34**. The second probe **22** comprises an antibody **36** coupled to a proximity hybridization 2 (PH2) oligonucleotide **31**. The PH2 oligonucleotide **31** forms a second hairpin loop structure **40**. The antibody **36** recognizes a chromatin modification or set of chromatin modifications of interest **38**. The PH2 oligonucleotide **31** forms a second hairpin loop structure **40**. The activator oligonucleotide **24** is capable of binding either the PH1 oligonucleotide **30** or the PH2 oligonucleotide **31**. Binding of the activator oligonucleotide **24** with the PH1 oligonucleotide **30** causes the first hairpin loop structure to open **42**. Binding of the activator oligonucleotide **24** with the PH2 oligonucleotide **31** causes the second hairpin loop structure **40** to open **106**. The third probe **26** comprises a labeled **44** hybridization 1 (H1) oligonucleotide **46**. The method further comprises contacting the cell with the first probe **20** under conditions that allow binding of the first oligonucleotide **28** of the first probe **20** to the genomic locus **32** of the cell. The method also includes contacting the cell with the second probe **22** under conditions that allow binding of the antibody **36** of the second probe **22** to the chromatin modification or set of chromatin modifications **38**. The method also includes contacting the cell with the activator oligonucleotide **24** under conditions that allow binding of the activator oligonucleotide **24** to the PH1 oligonucleotide **30** or to the PH2 oligonucleotide **31**. Binding of the activator oligonucleotide **24** to the PH1 oligonucleotide **30** or to the PH2 oligonucleotide **31** causes PH1 **30** and PH2 **31** to hybridize **72**. The method includes contacting the cell with the third probe **26** under conditions that allow binding of the H1 oligonucleotide **46** of the third probe **26** to a sequence made available **50** when the PH1 oligonucleotide **30** and PH2 oligonucleotide **31** have hybridized. The method also includes detecting the label **44** of the third probe **26**.

[00290] In various embodiments, the PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 4, the reverse complement of SEQ ID NO: 4, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to SEQ ID NO: 4. In various embodiments, the PH1 oligonucleotide comprises a sequence complementary to SEQ ID NO: 4 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 4. In various embodiments, the sequence complementary to SEQ ID NO: 4, is the reverse complement of SEQ ID NO: 4. In various embodiments, the PH1 oligonucleotide comprises about or at least about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the PH1 oligonucleotide comprises no more than about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the PH1 oligonucleotide comprises from about 10 to about 1000 nucleotides, from about 40 to about 160 nucleotides, from about 60 to about 100 nucleotides, or from about 70 to about 90 nucleotides. The PH1 oligonucleotide can comprise about 83 nucleotides.

[00291] In various embodiments, the PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 2, the reverse complement of SEQ ID NO: 2, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to SEQ ID NO: 2. In various embodiments, the PH2 oligonucleotide comprises a sequence complementary to SEQ ID NO: 2 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 2. In various embodiments, the sequence complementary to SEQ ID NO: 2, is the reverse complement of SEQ ID NO: 2. In various embodiments, the PH2 oligonucleotide comprises about or at least about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100,

105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the PH2 oligonucleotide comprises no more than about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the PH2 oligonucleotide comprises from about 10 to about 700 nucleotides, from about 40 to about 150 nucleotides, from about 50 to about 100 nucleotides, or from about 60 to about 80 nucleotides. The PH2 oligonucleotide can comprise about 72 nucleotides. In one embodiment, the PH2 oligonucleotide comprises a modified nucleotide sequence of SEQ ID NO: 3866.

[00292] In some embodiments, the activator binds a sequence disposed in the loop and stem of the hairpin loop structure formed by the PH1 oligonucleotide. In various embodiments, a portion of the PH1 oligonucleotide binds a sequence disposed in the loop and stem of the hairpin loop structure formed by the PH2 oligonucleotide. In various embodiments, a portion of the PH2 oligonucleotide binds a sequence disposed in the loop and stem of the hairpin loop structure formed by the H1 oligomer. In various embodiments, the loop and stem of the hairpin loop structure formed by the H1 oligomer binds a sequence disposed in the loop and stem of the hairpin loop structure formed by the H2 oligomer. In various embodiments, the loop and stem of the hairpin loop structure formed by the H2 oligomer binds a sequence disposed in the loop and stem of the hairpin loop structure formed by the H1 oligomer.

[00293] In various embodiments, the activator oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 1, the reverse complement of SEQ ID NO: 1, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to SEQ ID NO: 1. In various embodiments, the activator oligonucleotide comprises a sequence complementary to SEQ ID NO: 1 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 1. In various embodiments, the sequence complementary to SEQ ID NO: 1 is the reverse complement of SEQ ID NO: 1. In various embodiments, the activator oligonucleotide comprises about or at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides. In various embodiments, the H1 oligonucleotide comprises no more than

about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides. In various embodiments, the activator oligonucleotide comprises from about 4 to about 400 nucleotides, from about 20 to about 80 nucleotides, or from about 35 to about 55 nucleotides. The activator oligonucleotide can comprise about 44 nucleotides.

[00294] In various embodiments, the H1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 6, the reverse complement of SEQ ID NO: 6, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to SEQ ID NO: 6. In various embodiments, the H1 oligonucleotide comprises a sequence complementary to SEQ ID NO: 6 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 6. In various embodiments, the sequence complementary to SEQ ID NO: 6 is the reverse complement of SEQ ID NO: 6. In various embodiments, the H1 oligonucleotide comprises about or at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides. In various embodiments, the H1 oligonucleotide comprises no more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides. In various embodiments, the H1 oligonucleotide comprises from about 5 to about 500 nucleotides, from about 25 to about 100 nucleotides, or from about 40 to about 60 nucleotides. The H1 oligonucleotide can comprise about 50 nucleotides. In one embodiment, the H1 oligonucleotide comprises a modified nucleotide sequence of SEQ ID NO: 3862.

[00295] In various embodiments, the H2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 8, the reverse complement of SEQ ID NO: 8, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to SEQ ID NO: 8. In various embodiments, the H2 oligonucleotide comprises a sequence complementary to SEQ ID NO: 8 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 8. In various embodiments, the sequence complementary to SEQ ID NO: 8 is the reverse complement of SEQ ID NO: 8. In

various embodiments, the H2 oligonucleotide comprises about or at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides. In various embodiments, the H2 oligonucleotide comprises no more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides. In various embodiments, the H2 oligonucleotide comprises from about 5 to about 500 nucleotides, from about 25 to about 100 nucleotides, or from about 40 to about 60 nucleotides. The H2 oligonucleotide can comprise about 50 nucleotides. In one embodiment, the H2 oligonucleotide comprises a modified nucleotide sequence of SEQ ID NO: 3864.

[00296] In some embodiments, as shown in **Fig. 2B**, a plurality of PH1 oligonucleotides **30** are coupled to the first oligonucleotide **28**. In some embodiments, about or at least about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, or 200 PH1 oligonucleotides **30** are coupled to the first oligonucleotide **28**. In some embodiments, not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, or 200 PH1 oligonucleotides **30** are coupled to the first oligonucleotide **28**. In some embodiments about 4 PH1 oligonucleotides **30** are coupled to the first oligonucleotide **28**. In some embodiments, a plurality of PH1 oligonucleotides **30** are coupled to the first oligonucleotide **28** through a linker oligonucleotide **100**. The linker oligonucleotide **100**, can comprise a plurality of docking regions **102** to which a PH1 oligonucleotide **30** may bind. In some embodiments, a plurality of PH1 oligonucleotides **30** are coupled to the first oligonucleotide **28** through branched amplification, see **Fig. 7A**. Branched amplification is described further below. As will be become clear in view of the disclosure of branched amplification provided below, use of the linker oligonucleotide **100** is a special instance of branched amplification wherein only a primary amplification oligonucleotide (described further below) is used; thus, the linker oligonucleotide **100** may alternatively be referred to as a primary amplification oligonucleotide.

[00297] In some embodiments, see **Fig. 2B**, the linker oligonucleotide **100** comprises four docking regions **102** to which a PH1 oligonucleotide **30** may bind. In some embodiments, the linker oligonucleotide **100** comprises a nucleotide sequence of SEQ

ID NO: 12, the reverse complement of SEQ ID NO: 12, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 12. In some embodiments, the linker oligonucleotide **100** comprises a nucleotide sequence complementary to SEQ ID NO: 12 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to a sequence complementary to SEQ ID NO: 12. In some embodiments, the sequence complementary to SEQ ID NO: 12 is the reverse complement of SEQ ID NO: 12. In various embodiments, the linker oligonucleotide **100** comprises about or at least about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the linker oligonucleotide **100** comprises no more than about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the linker oligonucleotide **100** comprises from about 15 to about 1,500 nucleotides, from about 75 to about 300 nucleotides, from about 125 to about 175 nucleotides, or from about 140 to about 160 nucleotides. The linker oligonucleotide **100** can comprise about 150 nucleotides.

[00298] In various embodiments, the first oligonucleotide **28** can be coupled to the linker oligonucleotide **100** through nucleotide hybridization **72**. In some embodiments, the first oligonucleotide **28** can be coupled to the linker oligonucleotide **100** through a covalent bond. In various embodiments, the first oligonucleotide **28** can be coupled to the linker nucleotide **100** through binding of the linker oligonucleotide **100** to the overhang region **82** of the first oligonucleotide **28**.

[00299] In various embodiments, branched amplification, embodiments of which are shown in **Figs. 7A to 7C**, can be used to increase the number of oligonucleotide sequences associated with a particular oligonucleotide sequence. As non-limiting examples, in some embodiments branched amplification can be used to 1) couple a plurality of PH1 oligonucleotides to the first probe (**Fig. 7A**), 2) couple a plurality of PH2 oligonucleotides to the second probe (**Fig. 7B**), and/or 3) couple a plurality of H1 oligomers to the third probe and thereby amplify a signal generated by the third probe

(Fig. 7C).

[00300] In various embodiments, branched amplification comprises providing a primary amplification oligonucleotide **136** and a secondary amplification oligonucleotide **138**.

The primary amplification oligonucleotide **136** can be coupled to an antibody or oligonucleotide directly or indirectly by any of the various methods described herein. In various embodiments, the peptide is an antibody **36**. In various embodiments, see Fig. **7A**, the primary amplification oligonucleotide **136** can be the first oligonucleotide **28**. In some embodiments, see Fig. **7C**, the primary amplification oligonucleotide **136** can comprise a sequence complementary to the portion **50** of the PH2 oligonucleotide **31** made available for hybridization **72** when the second hairpin loop structure **40** opens **106**. In some embodiments, see Fig. **7B**, a coupling of the primary amplification oligonucleotide **136** to the antibody **36** can comprise a biotin-streptavidin bridge **54**.

[00301] The primary amplification oligonucleotide **136** can comprise multiple docking regions **102** that may each be bound by a secondary amplification oligonucleotide **138**. The secondary amplification oligonucleotide may comprise a plurality of docking regions **102** to which PH1 oligonucleotides **30**, PH2 oligonucleotides **31**, H1 oligonucleotides **46**, H2 oligonucleotides **68**, any of various other oligonucleotides, or various combinations thereof may bind. In various embodiments, the docking regions of the secondary amplification oligonucleotide bind only PH1 oligonucleotides **30**, only PH2 oligonucleotides **31**, only H1 oligonucleotides **46**, or only H2 oligonucleotides **68**. In various embodiments, branched amplification comprises providing a tertiary amplification oligonucleotide, a quaternary amplification oligonucleotide, etc. wherein tertiary amplification oligonucleotides bind to docking sites on a secondary amplification oligonucleotide, quaternary oligonucleotides bind to tertiary oligonucleotides, etc. The highest order (e.g., secondary, tertiary, quaternary, etc.) amplification oligonucleotide used in the method can comprise docking sites for only PH1 oligonucleotides **30**, only PH2 oligonucleotides **31**, only H1 oligonucleotides **46**, only H2 oligonucleotides **68**, any of various other oligonucleotides, or various combinations thereof.

[00302] In various embodiments, the primary, secondary, tertiary, etc. amplification oligonucleotide can comprise about or at least about or at least about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, or 200 PH1 docking regions. In

various embodiments, the primary, secondary, tertiary, etc. amplification oligonucleotide can comprise less than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, or 200 PH1 docking regions. In some embodiments the primary, secondary, tertiary, etc. amplification oligonucleotide can comprise from about 2 to about 20 docking regions, from about 2 to about 10 docking regions, or from about 2 to about 6 docking regions. In some embodiments, the amplification oligonucleotide(s) comprise 4 docking regions.

[00303] In various embodiments, the primary amplification oligonucleotide comprises a nucleotide sequence of any one of SEQ ID NOs: 3820-3836, the reverse complement to any one of SEQ ID NOs: 3820-3836, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any one of SEQ ID NOs: 3820-3836. In various embodiments, the primary amplification oligonucleotide comprises a sequence complementary to any one of SEQ ID NOs: 3820-3836 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to a sequence complementary to any one of SEQ ID NOs: 3820-3836. In various embodiments, the sequence complementary to any one of SEQ ID NOs: 3820-3836 is the reverse complement to the any one of SEQ ID NOs: 3820-3836.

[00304] In various embodiments, the secondary amplification oligonucleotide comprises a nucleotide sequence of any one of SEQ ID NOs: 3837-3853, the reverse complement to any one of SEQ ID NOs: 3837-3853, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any one of SEQ ID NOs: 3837-3853. In various embodiments, the secondary amplification oligonucleotide comprises a sequence complementary to any one of SEQ ID NOs: 3837-3853 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,

97%, 98%, or 99% sequence identity to a sequence complementary to any one of SEQ ID NOs: 3837-3853. In various embodiments, the sequence complementary to any one of SEQ ID NOs: 3837-3853 is the reverse complement to the any one of SEQ ID NOs: 3837-3853.

[00305] Overall sensitivity of the methods of the disclosure can depend on the number of first probes and second probes disposed at the genomic locus of interest. For a given genomic locus of interest, the quantity of probes may be limited by region size, and nucleosome counts. Combining branched amplification with the methods of the disclosure this limitation can be circumvented.

[00306] Also, a precise physical distance between a first probe and a second probe can be difficult to predict, due to some uncertainty of nucleosome positioning on the genome. Thus, branched amplification can be used to increase a distance over which PHR may take effectively take place between a first probe and a second probe, hence improving sensitivity.

[00307] In some embodiments, see **Fig. 1A**, the activator oligonucleotide **24** binding to the PH1 oligonucleotide **30** can cause a first hairpin loop structure **34** formed by the PH1 oligonucleotide **30** to open **42**. This binding of the activator oligonucleotide **24** may be referred to as “triggering” (or “causing”) a proximity hybridization reaction (PHR) between the PH1 oligonucleotide **30** and the PH2 oligonucleotide **31**. A portion **104** of the PH1 oligonucleotide **30** can be made available for nucleotide hybridization **72** by the opening **42** of the first hairpin **34**. Binding **72** of the portion **104** of the PH1 oligonucleotide **30** to the PH2 oligonucleotide **31** can cause the second hairpin loop structure **40** formed by the PH2 oligonucleotide **31** to open **106**, thereby causing a sequence **50** (alternatively, “a portion”) of the PH2 oligonucleotide **31** to be made available for binding to the H1 oligonucleotide **46**. The H1 oligonucleotide **46** can bind to the sequence **50** made available. In some embodiments, binding of the sequence **50** made available to the H1 oligonucleotide **46** can cause a hairpin loop structure **108** formed by the H1 oligonucleotide **46** to open.

[00308] In various embodiments, the method comprises a multiple epigenetic marks (mEpi) detection method, see **Figs. 8** and **10**, where **Fig. 8** depicts an embodiment of the method comprising hairpin loop structures and **Fig. 10** depicts an embodiment, referred to herein as an “EZ” embodiment of the method, that does not comprise hairpin

loop structures. In some embodiments, the method comprises providing a plurality of second probes **22** and providing a plurality of third probes **26**. Each second probe **22** can comprise a unique antibody **36** coupled to a unique PH2 oligonucleotide **31**. In various embodiments, each unique antibody **36** recognizes a unique chromatin modification or set of chromatin modifications of interest **38**. In some embodiments, each third probe **26** can comprise a unique H1 oligonucleotide **46** that can selectively bind to one of the unique PH2 oligonucleotides **31** of the plurality of second probes **22**. In some embodiments, see **Fig. 10**, each third probe **26** can comprise a unique H1 oligonucleotide **46** that can selectively bind to a sequence **52**, **53**, of each of the PH1 oligonucleotide **30** and the PH2 oligonucleotide **31**. In various embodiments, the third probe **26** is not labeled. As described further below, the method can further comprise covalently coupling each unique PH1 oligonucleotide **30** to a corresponding PH2 oligonucleotide **31** using a click reaction or enzymatic ligation reaction. The method can comprise contacting the cell with each third probe **26** of the plurality of third probes **26**. In some embodiments, each third probe **26** comprises a label. The method can include detecting each label **44** of each third probe **26**. In some embodiments, as shown in **Fig. 10**, the method can include providing a plurality of labeled **78** unique readout probes **76**, wherein each unique readout probe **76** binds to at least one of the plurality of third probes **26**. The method can further comprise contacting the cell with each readout probe **76** of the plurality of readout probes **76**. The method can include detecting each label **78** of each readout probe **76**. In some embodiments, as shown in **Figs. 8** and **10**, a first unique antibody **110** is coupled to a first unique PH2 oligonucleotide **112** (PH2_1 or EZ-PH2_1) and a second unique antibody **114** is coupled to a second unique PH2 oligonucleotide **116** (PH2_2 or EZ-PH2_2).

[00309] In some embodiments, see **Figs. 8** and **10**, the first oligonucleotide **28** is coupled to a plurality of unique PH1 oligonucleotides **30**, optionally by nucleotide hybridization **72** to a linker oligonucleotide **100**. In some embodiments, the plurality of unique PH1 oligonucleotides **30** comprises a first PH1 oligonucleotide **120** (PH1_1 or EZ-PH1_1) and a second PH1 oligonucleotide **122** (PH1_2 or EZ-PH1_2).

[00310] In some embodiments, each unique PH1 oligonucleotide **30** comprises a nucleotide sequence capable of binding to one of the unique PH2 oligonucleotides **31**. For example, in the embodiment shown in **Fig. 8**, PH1_1 **120** comprises a nucleotide

sequence capable of binding to PH2_1 **112** and PH1_2 **122** comprises a nucleotide sequence capable of binding to PH2_2 **116**. Binding of a unique PH1 oligonucleotide (**120**, **122**) to a corresponding unique PH2 oligonucleotide (**112**, **116**) causes a second hairpin loop structure **40** formed by the unique PH2 oligonucleotide **31** to open **106**. Opening **106** of the second hairpin loop structure **40** can make a sequence **50** available to which one of the plurality of third probes **26** may bind. For example, in the embodiment shown in **Fig. 8**, a third probe H1_1 **128** can bind to a sequence **132** made available upon the opening **106** of a second hairpin loop structure **40** of PH2_1, and a third probe H1_2 **130** can bind to a sequence **134** made available upon the opening **106** of a second hairpin loop structure **40** of PH2_2. In various embodiments, each of the plurality of third probes **26** is unique.

[00311] In some embodiments, see **Fig. 10**, a third probe EZ-H1_1 **128** selectively binds to a first sequence **52** of PH1_1 **120** and PH2_1 **112** and a third probe EZ-H1_2 **130** selectively binds to a second sequence **53** of PH1_2 **122** and PH2_2 **116**.

[00312] In some embodiments, see **Fig. 8**, the method further comprises providing a plurality of unique activator oligonucleotides **24**. Each unique activator oligonucleotide **24** is capable of binding to one of the unique PH1 oligonucleotides **30**, thereby causing a first hairpin loop structure **34** formed by the unique PH1 oligonucleotide **30** to open and make available the nucleotide sequence **104** capable of binding **72** to one of the unique PH2 oligonucleotides **31**. In the embodiment depicted in **Fig. 8**, the plurality of unique activator oligonucleotides **24** comprises a first activator oligonucleotide **124** (Activator_1) capable of binding to PH1_1 **120** and a second activator oligonucleotide **126** (Activator_2) capable of binding to PH1_2 **122**.

[00313] In some embodiments, the mEpi detection method comprises providing a plurality of first probes each comprising a first oligonucleotide coupled to a unique PH1 oligonucleotide. In some embodiments, the method comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the unique PH1 oligonucleotides of the plurality of first probes. The method can further comprise contacting the cell with each third probe of the plurality of third probes and detecting each label of each third probe. In some embodiments, the antibody is coupled to a plurality of unique PH2 oligonucleotides and each unique PH2 oligonucleotide comprises a nucleotide sequence capable of binding to one of the

unique PH1 oligonucleotides, thereby causing a first hairpin loop structure formed by the unique PH1 oligonucleotide to open. In some embodiments, the antibody is coupled to the plurality of unique PH2 oligonucleotides through nucleotide hybridization of unique PH2 oligonucleotides to an antibody linker oligonucleotide that is covalently coupled to the antibody. In some embodiments, the method further comprises providing a plurality of unique activator oligonucleotides where each unique activator oligonucleotide is capable of binding to one of the unique PH2 oligonucleotides and thereby causing a second hairpin loop structure formed by the unique PH2 oligonucleotide to open and make available the nucleotide sequence capable of binding to one of the unique PH1 oligonucleotides.

[00314] In various embodiments, non-limiting examples of sequences of PH1_1, PH2_1, activator_1, H1_1, and H2_1 include those sequences described herein for PH1, PH2, activator, H1, and H2, respectively.

[00315] In various embodiments, PH1_2 comprises a nucleotide sequence of SEQ ID NO: 3854, the reverse complement to SEQ ID NO: 3854, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 3854. In various embodiments, PH1_2 comprises a sequence complementary to SEQ ID NO: 3854 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to a sequence complementary to SEQ ID NO: 3854. In various embodiments, the sequence complementary to SEQ ID NO: 3854 is the reverse complement to SEQ ID NO: 3854.

[00316] In various embodiments, PH2_2 comprises a nucleotide sequence of SEQ ID NO: 3855, the reverse complement to SEQ ID NO: 3855, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 3855. In various embodiments, PH2_2 comprises a sequence complementary to SEQ ID NO: 3855 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to a sequence complementary to SEQ ID NO: 3855. In various embodiments, the sequence complementary to SEQ ID NO: 3855 is the reverse complement to SEQ ID NO: 3855. In one embodiment, PH2_2 comprises a modified nucleotide sequence of SEQ ID NO: 3867.

[00317] In various embodiments, activator_2 comprises a nucleotide sequence of SEQ ID NO: 3856, the reverse complement to SEQ ID NO: 3856, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 3856. In various embodiments, activator_2 comprises a sequence complementary to SEQ ID NO: 3856 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to a sequence complementary to SEQ ID NO: 3856. In various embodiments, the sequence complementary to SEQ ID NO: 3856 is the reverse complement to SEQ ID NO: 3856.

[00318] In various embodiments, the third probe is H1_2 and comprises a nucleotide sequence of SEQ ID NO: 3857, the reverse complement to SEQ ID NO: 3857, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 3857. In various embodiments, the third probe H1_2 comprises a sequence complementary to SEQ ID NO: 3857 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to a sequence complementary to SEQ ID NO: 3857. In various embodiments, the sequence complementary to SEQ ID NO: 3857 is the reverse complement to SEQ ID NO: 3857. In one embodiment, the third probe H1_2 comprises a modified nucleotide sequence of SEQ ID NO: 3863.

[00319] In various embodiments, the third probe is H2_2 and comprises a nucleotide sequence of SEQ ID NO: 3858, the reverse complement to SEQ ID NO: 3858, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%,

77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 3858. In various embodiments, the third probe H2_2 comprises a sequence complementary to SEQ ID NO: 3858 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to a sequence complementary to SEQ ID NO: 3858. In various embodiments, the sequence complementary to SEQ ID NO: 3858 is the reverse complement to SEQ ID NO: 3858. In one embodiment, the third probe H2_2 comprises a modified nucleotide sequence of SEQ ID NO: 3865.

[00320] In various embodiments, each of the plurality of second probes **22** comprises a unique antibody **36** covalently coupled to an antibody linker oligonucleotide **118**. In some embodiments, a unique PH2 oligonucleotide **31** is coupled to each unique antibody **36** through nucleotide hybridization **72** to the antibody linker oligonucleotide **118**. In various embodiments, each unique PH2 oligonucleotide **31** binds to a corresponding unique antibody linker oligonucleotide **118**.

[00321] In some embodiments, the plurality of third probes is labeled with a plurality of unique dyes. In some embodiments, the dyes are fluorescent dyes.

[00322] In some embodiments, the plurality of third probes comprises about or at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 250, 500, 750, 1,000, 2,000, 3,000, 4,000, 5,000, or 10,000 unique third probes. In some embodiments, some of the third probes bind the same genomic locus or different genomic loci.

[00323] The mEpi detection method allows for the identification of multiple epigenetic marks at the same genomic locus/loci in the same cell. This method can be combined with Epi-PHR, Epi-mFISH, EZ-Epi-PHR, and EZ-Epi-mFISH, all of which methods are described herein.

Epi-mFISH, mEpi-PHR, mEpi-mFISH

[00324] In one aspect, the present disclosure provides an Epi-mFISH (epigenetic mark – multiplex fluorescence *in situ* hybridization) method of *in situ* visualization of a chromatin modification at a plurality of genomic loci of interest of a cell. The method

comprises providing a plurality of first probes, a second probe, an activator oligonucleotide, a third probe, and a plurality of labeled readout probes. Each of the first probes comprises a first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide. Each unique first oligonucleotide binds to a genomic locus of interest. The PH1 oligonucleotide forms a first hairpin loop structure. The second probe comprises an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide. The antibody recognizes a chromatin modification or set of chromatin modifications of interest. The PH2 oligonucleotide forms a second hairpin loop structure. The activator oligonucleotide is capable of binding to either the PH1 oligonucleotide or to the PH2 oligonucleotide. Binding of the activator oligonucleotide with the PH1 oligonucleotide or with the PH2 oligonucleotide causes the first hairpin loop structure or the second hairpin loop structure to open, respectively. The third probe comprises a labeled hybridization 1 (H1) oligonucleotide. Each readout probe selectively binds to at least one of the plurality of unique first oligonucleotides of the plurality of first probes. The method includes contacting the cell with each of the plurality of first probes under conditions that allow binding of the unique first oligonucleotides of the first probes to the genomic locus of the cell. The method also includes contacting the cell with the second probe under conditions that allow binding of the antibody of the second probe to the chromatin modification or set of chromatin modifications. The method further includes contacting the cell with the activator oligonucleotide under conditions that allow binding of the activator oligonucleotide to the PH1 oligonucleotide or to the PH2 oligonucleotide. Binding of the activator causes PH1 and PH2 to hybridize. The method includes contacting the cell with the third probe under conditions that allow binding of the H1 oligonucleotide of the third probe to a sequence made available when the PH1 and PH2 oligonucleotides have hybridized. The method includes detecting a label of the third probe. The method includes contacting the cell with each labeled readout probe. The method also includes detecting each label of each readout probe.

[00325] In another aspect, the present disclosure provides a mEpi-PHR (multiple epigenetic marks – proximity-dependent hybridization reaction) method of *in situ* visualization of multiple chromatin modifications at a genomic locus of interest of a cell. The method comprises providing a first probe, a plurality of second probes, a plurality of unique activator oligonucleotides, and a plurality of third probes. The first probe

comprises a first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides. The first oligonucleotide binds to a genomic locus of interest. Each unique PH1 oligonucleotide forms a hairpin loop structure. Each of the second probes comprises a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide. Each of the unique antibodies recognizes a unique chromatin modification or set of chromatin modifications of interest. Each of the PH2 oligonucleotides forms a hairpin loop structure. Each unique activator oligonucleotide is capable of binding to a corresponding one of the plurality of unique PH1 or PH2 oligonucleotides. Binding of one of the plurality of unique activator oligonucleotides with the corresponding one of the plurality of PH1 or PH2 oligonucleotides causes the hairpin loop structure formed by one of the plurality of PH1 or PH2 oligonucleotides to open. Each third probe comprises a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of the plurality of second probes or to one of the unique PH1 oligonucleotides. Each H1 oligonucleotide is labeled. The method also includes contacting the cell with the first probe under conditions that allow binding of the first oligonucleotide of the first probe to the genomic locus of the cell. The method further comprises contacting the cell with the plurality of second probes under conditions that allow binding of each of the unique antibodies of the plurality of second probes to bind to the unique chromatin modification. The method further comprises contacting the cell with each unique activator oligonucleotide under conditions that allow binding of each unique activator oligonucleotide to the corresponding one of the plurality of PH1 or PH2 oligonucleotides. Binding of the activator causes one of the unique PH1 oligonucleotides and one of the unique PH2 oligonucleotides to hybridize with one another. The method also includes contacting the cell with each of the plurality of third probes under conditions that allow binding of the unique H1 oligonucleotide of each third probe to a sequence made available when one of the plurality of unique PH1 oligonucleotides and one of the plurality of unique PH2 oligonucleotides have hybridized. One of the plurality of unique PH2 or PH1 oligonucleotides comprises the sequence made available. The method further comprises detecting the label of each of the third probes.

[00326] In another aspect, the present disclosure provides a mEpi-mFISH (multiple epigenetic marks – multiplex fluorescence *in situ* hybridization) method of *in situ*

visualization of multiple chromatin modifications at a plurality of genomic loci of interest of a cell. The method comprises providing a plurality of first probes, a plurality of second probes, a plurality of activator oligonucleotides, a plurality of third probes, and a plurality of labeled readout probes. Each of the first probes comprises a unique first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides. Each unique first oligonucleotide binds to a genomic locus of interest. Each of the plurality of unique PH1 oligonucleotides forms a hairpin loop structure. Each second probe comprises a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide. Each of the unique antibodies recognizes a unique chromatin modification or set of chromatin modifications of interest. Each PH2 oligonucleotide forms a hairpin loop structure. Each activator oligonucleotide is capable of binding to a nucleotide sequence of one of the plurality of unique PH1 or PH2 oligonucleotides. Binding of one of the plurality of unique activator oligonucleotides with a corresponding one of the plurality of unique PH1 or PH2 oligonucleotides causes the hairpin loop structure formed by the unique PH1 or PH2 oligonucleotide to open. Each third probe comprises a labeled unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 or PH1 oligonucleotides of the plurality of second probes. Each labeled readout probe selectively binds to at least one of the plurality of unique first oligonucleotides of the plurality of first probes. The method comprises contacting the cell with each of the plurality of first probes under conditions that allow binding of the unique first oligonucleotides of the first probes to the genomic locus of the cell. The method includes contacting the cell with the plurality of second probes under conditions that allow each of the unique antibodies of the plurality of second probes to bind to the unique chromatin modification or set of chromatin modifications. The method also includes contacting the cell with each unique activator oligonucleotide under conditions that allow binding of each unique activator oligonucleotide to the corresponding one of the plurality of PH1 or PH2 oligonucleotides. Binding of the activator causes one of the unique PH1 oligonucleotides and one of the unique PH2 oligonucleotides to hybridize with one another. The method also includes contacting the cell with each of the plurality of third probes under conditions that allow binding of the unique H1 oligonucleotide of each third probe to a sequence made available when one of the plurality of unique PH1 and

one of the plurality of unique PH2 oligonucleotides have hybridized. One of the plurality of unique PH2 or PH1 oligonucleotides comprises the sequence made available. The method includes detecting the label of each of the third probes. The method also comprises contacting the cell with each labeled readout probe. The method further comprises detecting each label of each readout probe.

EZ-Epi-PHR

[00327] In another aspect, the present disclosure provides an EZ-Epi-PHR (easy (EZ) – epigenetic mark – proximity-dependent hybridization reaction) method for *in situ* visualization of a chromatin modification at a genomic locus of interest of a cell. Various components of the EZ-Epi-PHR method are illustrated in **Figs. 1C, 6A, and 10**.

[00328] The EZ-Epi-PHR method comprises providing a first probe **20**, a second probe **22**, and a third probe **26**. The first probe **20** comprises a first oligonucleotide **28** coupled to a proximity hybridization 1 (PH1) oligonucleotide **30**. The first oligonucleotide **28** binds to a genomic locus **32** of interest. The second probe **22** comprises an antibody **36** coupled to a proximity hybridization 2 (PH2) oligonucleotide **31**. The antibody **36** recognizes a chromatin modification or set of chromatin modifications **38** of interest. The third probe **26** comprises a hybridization 1 (H1) oligonucleotide **46** coupled to a label **78**. The method includes contacting the cell with the first probe **20** under conditions that allow binding of the first oligonucleotide **28** of the first probe to the genomic locus **32** of the cell. The method further includes contacting the cell with the second probe **22** under conditions that allow binding of the antibody **36** of the second probe **22** to the chromatin modification or set of chromatin modifications **38**. The method also includes contacting the cell with the third probe **26** under conditions that allow binding of the H1 oligonucleotide **46** of the third probe **26** to a nucleotide sequence **52** of each of the PH1 **30** and PH2 **31** oligonucleotides. When the H1 oligonucleotide **46** binds to a nucleotide sequence **52** of each of the PH1 **30** and the PH2 **31** oligonucleotides, a first terminus **140** of the PH1 oligonucleotide **30** is proximal to a second terminus **142** of the PH2 oligonucleotide **31**, such that the 3' end of the PH1 oligonucleotide **30** is disposed proximal to the 5' end of the PH2 oligonucleotide **31** or the 5' end of the PH1 oligonucleotide **30** is disposed proximal to the 3' end of the PH2 oligonucleotide **31**. The method includes detecting the label **44** of the third probe **26**.

[00329] In various embodiments, see **Figs. 1C** and **6A**, when the PH1 oligonucleotide **30** and the PH2 oligonucleotide **31** are bound to the H1 oligonucleotide **46** the first terminus **140** and the second terminus **142** are adjacent to one another. In some embodiments, the first terminus **140** and the second terminus **142** are separated by about or by at least about 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides when the PH1 oligonucleotide **30** and the PH2 oligonucleotide **31** are bound to the H1 oligonucleotide **46**.

[00330] In various embodiments, the PH1 oligonucleotide **30** is an EZ-PH1 oligonucleotide **144**. In various embodiments, the PH2 oligonucleotide **31** is an EZ-PH2 oligonucleotide **146**. In some embodiments, neither the EZ-PH1 oligonucleotide **144** nor the EZ-PH2 oligonucleotide **146** forms a hairpin loop structure. In various embodiments, the H1 oligonucleotide **46** is an EZ-H1 oligonucleotide **148**. Each of the EZ-PH1 and EZ-PH2 oligonucleotides may be referred to as a “proximity oligonucleotide.”

[00331] In some embodiments, the EZ-PH1 oligonucleotide **144** comprises a nucleotide sequence of SEQ ID NO: 5, the reverse complement of SEQ ID NO: 5, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to SEQ ID NO: 5. In some embodiments, the EZ-PH1 oligonucleotide **144** comprises a nucleotide sequence complementary to SEQ ID NO: 5 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 5. In some embodiments, the sequence complementary to SEQ ID NO: 5 is the reverse complement of SEQ ID NO: 5. In various embodiments, the EZ-PH1 oligonucleotide **144** comprises about or at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the EZ-PH1 oligonucleotide **144** comprises no more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the EZ-PH1 oligonucleotide **144** comprises from about 5 to about 500 nucleotides, from about 25 to about 100 nucleotides, or from about 40 to about 60 nucleotides. The EZ-PH1 oligonucleotide can comprise about 50

nucleotides.

[00332] In some embodiments, the EZ-PH2 oligonucleotide **146** comprises a nucleotide sequence of SEQ ID NO: 3, the reverse complement of SEQ ID NO: 3, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to SEQ ID NO: 3. In some embodiments, the EZ-PH2 oligonucleotide **146** comprises a nucleotide sequence complementary to SEQ ID NO: 3 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 3. In some embodiments, the sequence complementary to SEQ ID NO: 3 is the reverse complement of SEQ ID NO: 3. In various embodiments, the EZ-PH2 oligonucleotide **146** comprises about or at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the EZ-PH2 oligonucleotide **146** comprises no more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the EZ-PH2 oligonucleotide **146** comprises from about 5 to about 500 nucleotides, from about 25 to about 100 nucleotides, or from about 40 to about 60 nucleotides. The EZ-PH2 oligonucleotide can comprise about 50 nucleotides. In one embodiment, the EZ-PH2 oligonucleotide comprises a modified nucleotide sequence of SEQ ID NO: 3869.

[00333] In some embodiments, the EZ-H1 oligonucleotide **148** comprises a nucleotide sequence of SEQ ID NO: 7, the reverse complement of SEQ ID NO: 7, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to SEQ ID NO: 7. In some embodiments, the EZ-H1 oligonucleotide **148** comprises a nucleotide sequence complementary to SEQ ID NO: 7 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 7. In some embodiments, the sequence complementary to SEQ ID NO: 7 is the reverse

complement of SEQ ID NO: 7. In various embodiments, the EZ-H1 oligonucleotide **148** comprises about or at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the EZ-H1 oligonucleotide **148** comprises no more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the EZ-H1 oligonucleotide **148** comprises from about 5 to about 800 nucleotides, from about 35 to about 140 nucleotides, or from about 60 to about 80 nucleotides. The EZ-H1 oligonucleotide can comprise about 72 nucleotides. In one embodiment, the EZ-H1 oligonucleotide comprises a modified nucleotide sequence of SEQ ID NO: 3868.

[00334] In various embodiments, see **Figs. 1C** and **10**, the method can include using a click reaction to covalently couple the EZ-PH1 oligonucleotide **144** to the EZ-PH2 oligonucleotide **146** after binding the EZ-H1 oligonucleotide **148** to both EZ-PH1 **144** and EZ-PH2 **146** oligonucleotides. The click reaction results in the formation of a covalent bond **150** between the EZ-PH1 oligonucleotide **144** and the EZ-PH2 **146** oligonucleotide. In various embodiments, the click reaction is a Cu(I) catalyzed click reaction. In various embodiments, the click reaction is an Alkyne-Azide click chemistry reaction. In some embodiments, BTAA is used as a ligand for the Cu(I) catalyzed click chemistry reaction. In various embodiments, the PH1 oligonucleotide is azide-modified and the PH2 oligonucleotide is hexynyl-modified, or the PH1 oligonucleotide is hexynyl-modified and the PH2 oligonucleotide is azide-modified.

[00335] Covalent coupling of the EZ-PH1 oligonucleotide to the EZ-PH2 oligonucleotide can be used to suppress background signals, which can be generated through non-specific binding of the third probe, by allowing for use of a high stringent wash to wash off background signals.

[00336] In various embodiments, the method may include addition of a phosphate modification to the 5' of the EZ-PH2 oligonucleotide **146** to covalently couple the EZ-PH2 oligonucleotide **146** to the EZ-PH1 oligonucleotide **144** using an enzymatic ligation reaction. When a phosphate modification is added to the 5' of the EZ-PH2 oligonucleotide **146**, the resultant 5' phosphate-modified EZ-PH2 oligonucleotide **146**

(or 5' phosphate-modified PH2 oligonucleotide) may be termed a PHOS-EZ-PH2 oligonucleotide. In various embodiments, the PHOS-EZ-PH2 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3, the reverse complement of SEQ ID NO: 3, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to SEQ ID NO: 3. In some embodiments, the PHOS-EZ-PH2 oligonucleotide comprises a nucleotide sequence complementary to SEQ ID NO: 3 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 3. In some embodiments, the sequence complementary to SEQ ID NO: 3 is the reverse complement of SEQ ID NO: 3. In various embodiments, the PHOS-EZ-PH2 oligonucleotide comprises about or at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the PHOS-EZ-PH2 oligonucleotide comprises no more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the PHOS-EZ-PH2 oligonucleotide comprises from about 5 to about 500 nucleotides, from about 25 to about 100 nucleotides, or from about 40 to about 60 nucleotides. The PHOS-EZ-PH2 oligonucleotide can comprise about 50 nucleotides. In one embodiment, the PHOS-EZ-PH2 comprises a modified nucleotide sequence of SEQ ID NO: 3870. In various embodiments, the PHOS-EZ PH2 oligonucleotide and the EZ-PH1 oligonucleotide **144** may be covalently coupled using an enzymatic ligation reaction comprising a ligase such as, but not limited to, a T4 DNA ligase (i.e., using a T4 ligation), T7 DNA ligase, T3 DNA ligase, Taq DNA ligase, Ampligase, E. coli DNA ligase, or SplintR ligase. In various embodiments, T4 ligation may be used to covalently couple the EZ-PH1 oligonucleotide **144** to the PHOS-EZ-PH2 oligonucleotide following incubation with EZ-H1 oligonucleotide **148**. In various embodiments, T4 ligation may be used to covalently couple the EZ-PH1 oligonucleotide **144** to the PHOS-EZ-PH2 oligonucleotide after binding the EZ-H1 oligonucleotide **148** to both EZ-PH1 **144** and PHOS-EZ-PH2 oligonucleotides.

[00337] In various embodiments, the method may include addition of a phosphate modification to the 5' of the EZ-PH1 oligonucleotide **144** to covalently couple the EZ-PH1 oligonucleotide **144** to the EZ-PH2 oligonucleotide **146** using an enzymatic ligation reaction. When a phosphate modification is added to the 5' of the EZ-PH1 oligonucleotide **144**, the resultant 5' phosphate-modified EZ-PH1 oligonucleotide **144** (or 5' phosphate-modified PH1 oligonucleotide) may be termed a PHOS-EZ-PH1 oligonucleotide. In various embodiments, the PHOS-EZ-PH1 oligonucleotide comprises a nucleotide sequence of SEQ ID NO: 3860, the reverse complement of SEQ ID NO: 3860, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to SEQ ID NO: 3860. In some embodiments, the PHOS-EZ-PH1 oligonucleotide comprises a nucleotide sequence complementary to SEQ ID NO: 3860 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 3860. In some embodiments, the sequence complementary to SEQ ID NO: 3860 is the reverse complement of SEQ ID NO: 3860. In various embodiments, the PHOS-EZ-PH1 oligonucleotide comprises about or at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the PHOS-EZ-PH1 oligonucleotide comprises no more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the PHOS-EZ-PH1 oligonucleotide comprises from about 5 to about 500 nucleotides, from about 25 to about 100 nucleotides, or from about 40 to about 60 nucleotides. The PHOS-EZ-PH1 oligonucleotide can comprise about 50 nucleotides. In one embodiment, the PHOS-EZ-PH1 comprises a modified nucleotide sequence of SEQ ID NO: 3871. In some embodiments, the EZ-PH2 oligonucleotide **146** comprises a nucleotide sequence of SEQ ID NO: 3861, the reverse complement of SEQ ID NO: 3861, or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, or 98% sequence identity to SEQ ID NO: 3861. In some embodiments, the EZ-PH2 oligonucleotide **146** comprises a nucleotide sequence

complementary to SEQ ID NO: 3861 or a nucleotide sequence with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to a sequence complementary to SEQ ID NO: 3861. In some embodiments, the sequence complementary to SEQ ID NO: 3861 is the reverse complement of SEQ ID NO: 3861. In various embodiments, the EZ-PH2 oligonucleotide **146** comprises about or at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the EZ-PH2 oligonucleotide **146** comprises no more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, or 200 nucleotides. In various embodiments, the EZ-PH2 oligonucleotide **146** comprises from about 5 to about 500 nucleotides, from about 25 to about 100 nucleotides, or from about 40 to about 60 nucleotides. The EZ-PH2 oligonucleotide can comprise about 50 nucleotides. In one embodiment, the EZ-PH2 oligonucleotide comprises a modified nucleotide sequence of SEQ ID NO: 3872. In various embodiments, the PHOS-EZ-PH1 oligonucleotide and the EZ-PH2 oligonucleotide **146** may be covalently coupled using an enzymatic ligation reaction comprising a ligase such as, but not limited to, a T4 DNA ligase (i.e., using a T4 ligation), T7 DNA ligase, T3 DNA ligase, Taq DNA ligase, Ampligase, E. coli DNA ligase, or SplintR ligase. In various embodiments, T4 ligation may be used to covalently couple the PHOS-EZ-PH1 oligonucleotide to the EZ-PH2 oligonucleotide **146** following incubation with EZ-H1 oligonucleotide **148**. In various embodiments, T4 ligation may be used to covalently couple the PHOS-EZ-PH1 oligonucleotide to the EZ-PH2 oligonucleotide **146** after binding the EZ-H1 oligonucleotide **148** to both PHOS-EZ-PH1 and EZ-PH2 oligonucleotides **146**.

EZ-Epi-mFISH, EZ-mEpi-PHR, EZ-mEpi-mFISH

[00338] In another aspect, the present invention provides an EZ-Epi-mFISH (easy [EZ] – epigenetic mark – multiplex fluorescence *in situ* hybridization) method for *in situ* visualization of a chromatin modification at a plurality of genomic loci of interest of a cell. The method comprises providing a plurality of first probes, a second probe, a third probe, and a plurality of labeled readout probes. Each of the plurality of first probes

comprises a unique first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide. Each of the first oligonucleotides binds to a genomic locus of interest. The second probe comprises an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide. The antibody recognizes a chromatin modification or set of chromatin modifications of interest. The third probe comprises a labeled hybridization 1 (H1) oligonucleotide. The plurality of labeled readout probes each selectively binds to at least one of the plurality of the first oligonucleotides of the plurality of first probes. The method further comprises contacting the cell with each of the plurality of first probes under conditions that allow binding of the first oligonucleotides of the first probes to the genomic locus of the cell. The method also includes contacting the cell with the second probe under conditions that allow binding of the antibody of the second probe to the chromatin modification or set of chromatin modifications. The method includes contacting the cell with the third probe under conditions that allow simultaneous binding of the H1 oligonucleotide of the third probe to a nucleotide sequence of each of the PH1 and PH2 oligonucleotides. When the H1 oligonucleotide binds to a nucleotide sequence of each of the PH1 and PH2 oligonucleotides, the 3' end of the PH1 oligonucleotide is disposed proximal to the 5' end of the PH2 oligonucleotide or the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide. The method includes detecting the label of the third probe. The method also includes contacting the cell with each labeled readout probe. The method further includes detecting each label of each readout probe.

[00339] In another aspect, the present disclosure provides an EZ-mEpi-PHR (easy (EZ) – multiple epigenetic mark – proximity hybridization reaction) method, see **Fig. 10**, for in situ visualization of multiple chromatin modifications **38** (epigenetic marks) at a genomic locus **32** of interest in a cell. The method comprises providing a first probe **20**, providing a plurality of second probes **22**, and providing a plurality of third probes **26**. The first probe **20** comprises a first oligonucleotide **28** coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides **120**, **122**. The first oligonucleotide **28** binds to a genomic locus of interest **32**. The plurality of second probes **22** each comprise a unique antibody **110**, **114** coupled to a unique proximity hybridization 2 (PH2) oligonucleotide **112**, **116**. Each unique antibody **110**, **114** recognizes a chromatin modification **38** or set or chromatin modifications **38** of interest. Each of the plurality of

third probes **26** comprises a unique hybridization 1 (H1) oligonucleotide **128, 130** that selectively binds to one of the unique PH2 oligonucleotides **112, 116** of the plurality of second probes **22** and one of the unique PH1 oligonucleotides **120, 122**. The H1 oligonucleotide **128, 130** is coupled to a label **78**. The method further comprises contacting the cell with the first probe **20** under conditions that allow binding of the first oligonucleotide **28** of the first probe **20** to the genomic locus **32** of the cell. The method also comprises contacting the cell with each of the plurality of second probes **22** under conditions that allow binding of the unique antibodies **110, 114** of the second probes **22** to the chromatin modification **38** or set of chromatin modifications **38**. The method also includes contacting the cell with each third probe **26** of the plurality of third probes **26** under conditions that allow binding of each unique H1 oligonucleotide **128, 130** of each of the plurality of third probes **26** to a nucleotide sequence **52, 53** of one of the unique PH2 oligonucleotides **112, 116** and of one of the unique PH1 oligonucleotides **120, 122**, wherein when the unique H1 oligonucleotide **128, 130** binds to a nucleotide sequence of the unique PH1 oligonucleotide **120, 122** and unique PH2 oligonucleotide **112, 116**, a first terminus **140** of the unique PH1 oligonucleotide **30** is proximal to a second terminus **142** of the unique PH2 oligonucleotide **31**, such that the 3' end of the one of the unique PH1 oligonucleotides **120, 122** is disposed proximal to the 5' end of the one of the unique PH2 oligonucleotides **112, 116** or the 5' end of the one of the unique PH1 oligonucleotides **120, 122** is disposed proximal to the 3' end of the one of the unique PH2 oligonucleotides **112, 116**. The method also comprises detecting each label **78** coupled to each third probe **26**. In some embodiments, a third probe **26** is coupled to a label **78** indirectly by being bound by a labeled **78** readout probe **76**.

[00340] In another aspect, the present disclosure provides an EZ-mEpi-mFISH (easy (EZ) – multiple epigenetic marks – multiplex fluorescence *in situ* hybridization) method for *in situ* visualization of multiple chromatin modifications at a plurality of genomic loci of interest in a cell. The method comprises providing a plurality of first probes, a plurality of second probes, a plurality of third probes, and a plurality of readout probes. Each of the first probes comprises a unique first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides. Each unique first oligonucleotide binds to a genomic locus of interest. Each second probe comprises a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide.

Each unique antibody recognizes a chromatin modification or set of chromatin modifications of interest. Each third probe comprises a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of the plurality of second probes and one of the unique PH1 oligonucleotides. Each readout probe selectively binds to at least one of the plurality of first oligonucleotides of the plurality of first probes. The method includes contacting the cell with each of the plurality of first probes under conditions that allow binding of each first oligonucleotide of the first probes to the genomic locus of the cell. The method further comprises contacting the cell with each of the plurality of second probes under conditions that allow binding of the unique antibodies of the second probe to the chromatin modification or set of chromatin modifications. The method also comprises contacting the cell with each third probe of the plurality of third probes under conditions that allow binding of each unique H1 oligonucleotide of each of the plurality of third probes to a nucleotide sequence of each of one of the unique PH2 oligonucleotides and one of the unique PH1 oligonucleotides. When the unique H1 oligonucleotide binds to a nucleotide sequence of each of the one of the unique PH1 and PH2 oligonucleotides, the 3' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 5' end of the one of the unique PH2 oligonucleotides or the 5' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 3' end of the one of the unique PH2 oligonucleotides. The method includes detecting each label coupled to each third probe. The method further comprises contacting the cell with each labeled readout probe. The method also includes detecting each label of each readout probe.

[00341] In various embodiments, the present invention provides for various combinations of MERFISH with Epi-PHR, Epi-mFISH, mEpi-PHR, mEpi-mFISH, EZ-Epi-PHR, EZ-Epi-mFISH, EZ-mEpi-PHR, and EZ-mEpi-mFISH methods.

[00342] In one aspect, the present invention provides a branched amplification method in combination with Epi-PHR, Epi-mFISH, mEpi-PHR, mEpi-mFISH, EZ-Epi-PHR, EZ-Epi-mFISH, EZ-mEpi-PHR, and EZ-mEpi-mFISH.

Diagnostic Methods

[00343] In a further embodiment, the present disclosure provides a method for diagnosing, prognosing, and/or predicting treatment response of a disease in a subject. The method comprises *in situ* visualization of a chromatin modification of a cell of the

subject according to a method of the present disclosure.

[00344] In various embodiments, the disease is a cancer or a tumor. Examples of a cancer or tumor that can be diagnosed using methods of the present disclosure include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include breast cancer (including ductal carcinoma in situ (DCIS), invasive breast cancer (ILC or IDC), triple-negative breast cancer, inflammatory breast cancer, Paget disease of the breast, angiosarcoma, and phyllodes tumor), squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, gastric cancer, melanoma, and various types of head and neck cancer. Additional examples of cancer can be found in The Merck Manual of Diagnosis and Therapy, 19th Edition, § on Hematology and Oncology, published by Merck Sharp & Dohme Corp., 2011 (ISBN 978-0-911910-19-3); The Merck Manual of Diagnosis and Therapy, 20th Edition, § on Hematology and Oncology, published by Merck Sharp & Dohme Corp., 2018 (ISBN 978-0-911-91042-1) (2018 digital online edition at internet website of Merck Manuals); and SEER Program Coding and Staging Manual 2016, each of which is incorporated by reference in their entirety for all purposes. A cancer may be diagnosed using criteria generally accepted in the art, including the presence of a malignant tumor.

Automation and Data Analysis

[00345] Colocalization of a readout probe and a third probe, or lack of colocalization, can allow for identification of a chromatin modification of interest (epigenetic mark), or lack of the chromatin modification of interest, at a genomic location of interest. This can allow for the determination of a locus-specific epigenetic state of the cell. Thus, the methods of the present disclosure can allow for the profiling of locus-specific epigenetic states in the cell. In some embodiments, the method allows for detection of a genomic locus comprising a chromatin modification that activates transcription of a gene and a chromatin modification that represses transcription of a gene.

[00346] In various embodiments, the method includes quantitating an epigenetic modification level of the genomic locus of interest. The epigenetic modification level of the genomic locus of interest can comprise the number of individual locations within a cell each meeting conditions necessary for PHR at the genomic locus of interest. The epigenetic modification level can be a quantitative indication of the number of genetic loci recognized by the first probe that are sufficiently proximal to a chromatin modification of interest for PHR to take place between the first probe and the second probe. The epigenetic modification level can be a quantitative indication of a number of chromatin modifications at a genomic locus of interest. The epigenetic modification level can be a quantification of the number of instances of conditions necessary for PHR having been fulfilled at the genomic locus of interest. In some embodiments, quantitating the epigenetic modification level comprises quantifying the intensity of a fluorescent signal.

[00347] In various embodiments, detecting a probe may comprise a spatial and/or quantitative determination. The spatial determination may be in two or three dimensions. The quantitative determination may comprise calculating an amount or concentration of a probe, chromatin modification of interest, genomic locus, and/or chromatin modification of interest proximal to or within a genomic locus of interest.

[00348] In various embodiments, the spatial positions of a probe may be determined at relatively high resolutions. For instance, the positions may be determined at spatial resolutions of better than about 100 micrometers, better than about 30 micrometers, better than about 10 micrometers, better than about 3 micrometers, better than about 1 micrometer, better than about 800 nm, better than about 600 nm, better than about 500 nm, better than about 400 nm, better than about 300 nm, better than about 200 nm, better than about 100 nm, better than about 90 nm, better than about 80 nm, better than about 70 nm, better than about 60 nm, better than about 50 nm, better than about 40 nm, better than about 30 nm, better than about 20 nm, or better than about 10 nm, etc.

[00349] In some embodiments, the centroids of the spatial positions of a signal generated by a label may be determined. For example, a centroid of a signal may be determined within an image or series of images using image analysis algorithms known to those of ordinary skill in the art. In some cases, the algorithms may be selected to

determine non-overlapping single emitters and/or partially overlapping single emitters in a sample. Non-limiting examples of suitable techniques include a maximum likelihood algorithm, a least-squares algorithm, a Bayesian algorithm, a compressed sensing algorithm, or the like. Combinations of these techniques may also be used in some embodiments.

[00350] In some embodiments, the method further comprises determining in three dimensions a location of the third probe and/or the first probe. The location of the first probe can be used to analyze chromatin structure. The location of the third probe can be used to analyze chromatin structure. In some embodiments, the analysis of chromatin structure comprises determining a three-dimensional positioning of a chromatin modification(s) and a genomic locus (or loci) of interest.

[00351] In a further aspect, the present disclosure provides a computer-implemented method. For instance, as a non-limiting example, a computer and/or an automated system may be provided that is able to automatically and/or repetitively perform any of the methods described herein. As used herein, “automated” devices refer to devices that are able to operate without human direction, i.e., an automated device can perform a function during a period of time after any human has finished taking any action to promote the function, e.g. by entering instructions into a computer to start the process. Typically, automated equipment can perform repetitive functions after this point in time. Processing steps may also be recorded onto a machine-readable medium in some cases.

[00352] In some embodiments, a computer may be used to control imaging of the sample, e.g., using fluorescence microscopy, STORM, or other super-resolution techniques such as those described herein. In some embodiments, the computer may also control operations such as drift correction, physical registration, hybridization and cluster alignment in image analysis, cluster decoding (e.g., fluorescent cluster decoding), error detection or correction (e.g., as discussed herein), noise reduction, identification of foreground features from background features (such as noise or debris in images), or the like. As an example, the computer may be used to control activation and/or excitation of a label within the sample, and/or the acquisition of images of a label. In some embodiments, a sample may be excited using light having various wavelengths and/or intensities, and the sequence of the wavelengths of light used to

excite the sample may be correlated, using a computer, to the images acquired of the sample containing the label. For instance, the computer may apply light having various wavelengths and/or intensities to a sample to yield different average numbers of labels in each region of interest (e.g., one activated entity per location, two activated entities per location, etc.). In some cases, this information may be used to construct an image and/or determine the locations of the labels, in some cases at high resolutions, as noted above. In some embodiments, the computer may execute any of the calculations discussed herein.

Kits and Systems of the Disclosure

[00353] In another aspect, the present disclosure provides a kit for *in situ* visualization of a chromatin modification of a cell according to a method provided herein. The kit comprises the first probe(s), the second probe(s), the activator oligonucleotide(s), and the third probe(s). In some embodiments, the kit further comprises the plurality of labeled readout probes. In some embodiments, the kit further comprises one or more labeled readout probes.

[00354] In another aspect, the present disclosure provides a kit for *in situ* visualization of a chromatin modification of a cell according to a method provided herein. The kit comprises the first probe, the second probe, and the third probe. In various embodiments, the kit further comprises the plurality of labeled readout probes.

[00355] In various embodiments, a kit of the present disclosure comprises an RNase, or a nickase. In some embodiments, the kit comprises a solid support for immobilization of the cell, means for immobilization, detection means e.g. probes or reagents required to detect a label, buffers, cations, etc. and the like. In some embodiments, the kit may include a volume excluder. In some embodiments, components of the kit may be present in the same container or in different containers, where the containers may be storage containers and/or containers that are employed during use of reagents of the kit.

[00356] In some embodiments, a kit may further include instructions for practicing a method of the present disclosure. These instructions may be present in the kits in a variety of forms, one or more of which may be present in the kit. One form in which these instructions may be present is as printed information on a suitable medium or substrate, e.g., a piece or pieces of paper on which the information is printed, in the

packaging of the kit, in a package insert, etc. Yet another means would be a computer-readable medium, e.g., diskette, CD, etc., on which the information has been recorded. Yet another means that may be present is a website address which may be used via the internet to access the information at a removed site. Any convenient means may be present in the kits.

[00357] In one aspect, the present invention provides an Epi-PHR system comprising: a DNA FISH probe for targeting repetitive genomic sequence or DNA FISH probes for targeting non-repetitive genomic sequence, a biotinylated antibody, streptavidin, proximity hairpin oligonucleotides, an activator oligonucleotide, and dye-labeled hybridization oligonucleotide.

[00358] In one aspect, the present invention provides an Epi-mFISH system comprising: multiplexed sequential DNA FISH (mFISH) probes, a biotinylated antibody, streptavidin, proximity hairpin oligonucleotides, an activator oligonucleotide, and dye-labeled hybridization oligonucleotide and readout probes.

[00359] In one aspect, the present invention provides an mEpi-mFISH system comprising: mFISH probes, a plurality of antibodies, a plurality of proximity hairpin oligonucleotides, a plurality of activator oligonucleotides, and a plurality of dye-labeled hybridization oligonucleotides and readout probes.

[00360] In one aspect, the present invention provides an EZ-Epi-PHR system comprising: a DNA FISH probe for targeting repetitive genomic sequence or DNA FISH probes for targeting non-repetitive genomic sequence, an antibody, proximity (non-hairpin) oligonucleotides, and dye-labeled hybridization oligonucleotide. An azide-modified (optional) proximity oligonucleotide is directly conjugated to a DNA FISH probe during probe synthesis, or may be hybridized to an overhang region on the DNA FISH probe. A hexynyl-modified (optional) or phosphate-modified (optional) proximity oligonucleotide is directly linked to the antibody via DBCO mediated copper-free click reaction (or other chemistry), or indirectly linked to the antibody via a streptavidin bridge (or other bridge).

[00361] In one aspect, the present invention provides an EZ-Epi-mFISH system comprising: mFISH probes, an antibody, proximity (non-hairpin) oligonucleotides, and dye-labeled hybridization oligonucleotide and readout probes. An azide-modified (optional) proximity oligonucleotide is directly conjugated to mFISH probe during probe

synthesis, or may be hybridized to an overhang region on mFISH probe. A hexynyl-modified (optional) or phosphate-modified (optional) proximity oligonucleotide is directly linked to the antibody via DBCO mediated copper-free click reaction (or other chemistry), or indirectly linked to the antibody via a streptavidin bridge (or other bridge).

EXAMPLES

[00362] The present invention is also described and demonstrated by way of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to any particular preferred embodiments described here. Many modifications and variations of the invention may be apparent to those skilled in the art upon reading this specification, and such variations can be made without departing from the invention in spirit or in scope. The invention is to be limited only by the appended claims, including the full scope of equivalents to which those claims are entitled.

Example 1. Design of Epi-PHR system

[00363] The Epi-PHR system was designed such that a core mechanism of Epi-PHR was a proximity-dependent *in situ* hybridization reaction (**Fig. 1A**), which was triggered by the spatial proximity of two different DNA hairpin oligonucleotides (PH1 and PH2). PH1 (SEQ ID NO: 4) and PH2 (SEQ ID NO: 2) were 'linked' to FISH probes and antibodies respectively. The proximity between the two hairpin oligonucleotides that enabled Epi-PHR was established when the antibodies bound epigenetic marks adjacent to FISH probes binding the DNA locus. Next, an activator oligonucleotide (SEQ ID NO: 1) was introduced to the system. The activator hybridized to PH1 and opened the PH1 hairpin. The opened end of PH1 then invaded the PH2 in proximity and open the PH2 hairpin. A fluorescent-dye-labeled oligonucleotide probe (H1; modified nucleotide sequence (SEQ ID NO: 3862); unmodified nucleotide sequence (SEQ ID NO: 6)) was then hybridized to the opened end of PH2 and generated a fluorescent readout of the locus-specific epigenetic mark signal.

[00364] In a proxHCR system [18], an additional fluorescent-dye-labeled hairpin oligonucleotide (H2) was invaded by and bound to H1, and the opened end of H2 opened and bound by another H1 in a continuing chain reaction. This uncontrolled chain reaction would continue until there was no longer sufficient H1 and H2 in solution.

Epi-PHR did not inherit this uncontrolled signal amplification scheme.

[00365] Since the hairpin oligonucleotides required *in vitro* refolding through controlled heating and cooling to ensure correct folding conformation, to avoid experimental complications, PH1 and PH2 were not directly linked to FISH probes and antibodies. Instead, to the FISH probes was added another short nongenomic overhang region carrying the complementary sequence to PH1, so PH1 was introduced to the system and bind to this overhang region after a harsh FISH procedure and the hairpin conformation would thus not be affected by the FISH procedure. To fulfill a PH2 refolding requirement without denaturing the antibodies, antibodies were labeled with N-hydroxysuccinimide (NHS) ester biotin molecules, then Alexa Fluor 488 labeled streptavidin was used as a bridge. The streptavidin was labeled as a control. Since streptavidin molecules form tetramers, biotin-labeled PH2 was used to occupy free binding sites of the streptavidin tetramers that were bound to the biotin-antibodies. With these strategies, both PH1 and the biotin-labeled PH2 were refolded *in vitro* before being introduced to the Epi-PHR reaction, thus the refolding procedure (which involves heating) did not affect antibody stability. In addition, all hairpin probes were introduced after the FISH procedure, hence circumventing the concern that the stringent FISH procedure could open PH1 and PH2 hairpins.

Example 2. Design of Epi-mFISH system

[00366] The Epi-mFISH system comprised a multiplexed sequential DNA FISH technique to target more than one specific genomic loci. The probes consisted of a primary targeting sequence, and a short nongenomic overhang region (SEQ ID NOs: 9-11) that allowed visualization via dye-labeled secondary oligos (Fig. 1B). High multiplexity was achieved by assigning different nongenomic overhang regions for each group of probes that targeted one specific genomic region of interest, and then sequentially hybridizing the readout probes to visualize each group of probes [11]. The 3D centroid positions of individual regions were extracted and recorded from the images, then the chromatin folding organization was directly traced by linking the 3D positions of the imaged genomic loci.

Example 3. Design of EZ-Epi-PHR and EZ-Epi-mFISH

[00367] The EZ-Epi-PHR and the EZ-Epi-mFISH also employed a proximity-dependent *in situ* hybridization strategy, but the reaction in these examples did not employ DNA

hairpin structures to initiate the reaction (Fig. 1C). Instead of using a DNA hairpin, a single-stranded DNA sequence (the reverse complement of EZ-H1 introduced below) was split into two parts, termed EZ-PH1 (SEQ ID NO: 5) and EZ-PH2 (SEQ ID NO: 3). FISH probes were extended with the EZ-PH1, and the antibodies were directly labeled with NHS ester modified EZ-PH2. When the FISH probes and antibodies were in spatial proximity, the EZ-PH1 and EZ-PH2 could stably co-bind a dye-labeled EZ-H1 oligonucleotide probe (modified nucleotide sequence (SEQ ID NO: 3868); unmodified nucleotide sequence (SEQ ID NO: 7)), thus generating a fluorescent readout of the locus-specific epigenetic state. To improve the detection specificity of the EZ-Epi-PHR and EZ-Epi-mFISH, click chemistry or enzymatic ligation was applied to covalently link the EZ-PH1 and the EZ-PH2 together after the *in situ* hybridization reaction, then high stringent washing steps were applied to suppress the background signals. Compared to the Epi-PHR and Epi-mFISH, the EZ-Epi-PHR and EZ-Epi-mFISH had fewer incubation steps, thus less time cost.

Example 4. The Epi-PHR can robustly detect epigenetic marks across different genomic resolution

[00368] As a proof-of-principle demonstration of the experimental design, the Epi-PHR method was applied to detect epigenetic marks at the human chromosome 9 alpha satellite region. The alpha satellite locates inside the heterochromatic pericentromeric region, which contains high-levels of repressive marks such as histone H3 lysine 27 trimethylation (H3K27Me3), histone H3 lysine 9 trimethylation (H3K9Me3) and histone variant MacroH2A [28-30]. Moreover, the region is devoid of active epigenetic marks such as histone H3 lysine 27 acetylation (H3K27Ac) [28]. Since the alpha satellite is constituted by highly repetitive sequence arrays, it was possible to use just one FISH probe (SEQ ID NO: 148) to target the pericentromeric region and have multiple copies of the probe bound to the region to ensure detection efficiency.

[00369] H3K9Me3 and H3K27Ac were detected at the pericentromeric region of chromosome 9 in the human female RPE1 cell line, using antibodies targeting the two epigenetic marks, respectively. The Epi-PHR results were consistent with previous studies: The repressive mark H3K9Me3 generated strong Epi-PHR signals in the cell nucleus at the alpha satellite region, and the active mark H3K27Ac showed no detectable signal at the alpha satellite region (**Fig. 2A**). To further demonstrate that Epi-

PHR can be applied to detect other epigenetic marks, the Epi-PHR was used to detect H3K27Me3 and MacroH2A at the alpha satellite region. However, the version of Epi-PHR above did not show any strong signal, possibly because the levels of H3K27me3 and MacroH2A were lower than H3K9me3 at the alpha satellite region. To address this low-signal issue, the FISH probe's overhang region was redesigned: Instead of directly docking PH1 to the overhang region, a 150-base linker oligonucleotide (SEQ ID NO: 12) was included to dock four PH1 hairpins on one FISH probe (**Fig. 2B**). After applying this detection amplification scheme, Epi-PHR signals of H3K27me3 and MacroH2A were robustly detected at the alpha satellite region (**Fig. 2C**). Systematic control experiments showed that omitting any Epi-PHR component would demolish H3K9Me3 Epi-PHR signals (**Fig. 3**). A signal was considered as being robustly detected when there was a high detection efficiency (more than 0.8), where detection efficiency was defined as *(number of FISH foci that correspond to an Epi-PHR signal) / (total number of FISH foci)*. The term "FISH foci" refers to fluorescent signals produced by FISH probes. These control results suggested that FISH probes and antibodies in proximity specifically generated the Epi-PHR signals observed in the full experiments and validated the core design of the Epi-PHR. Together, these results above demonstrated that Epi-PHR can robustly detect different epigenetic marks at a large repetitive genomic region.

[00370] Epi-PHR could detect the epigenetic state of non-repetitive genomic regions. Previous studies showed that the inactive X chromosome (Xi) of the RPE1 cell line has alternating multi-megabase intervals marked by either H3K9Me3 or H3K27Me3, and the Xq 22.3 region of the chromosome is H3K27me3 enriched, with low population heterogeneity [31]. Thus, 3668 FISH probes (SEQ ID NOs: 152-3819) were designed to target the central 300-kilobase of the Xq22.3 region. The four-PH1 docking scheme was applied as in Figure 2B to improve the detection efficiency. Moreover, a controlled signal amplification scheme was added to the Epi-PHR system. By sequentially hybridizing dye-labeled imaging probes H1 (modified nucleotide sequence (SEQ ID NO: 3862); unmodified nucleotide sequence (SEQ ID NO: 6)) and H2 (modified nucleotide sequence (SEQ ID NO: 3864); unmodified nucleotide sequence (SEQ ID NO: 8)) to the sample (**Fig. 4A**), uncontrolled chain-reaction signal amplification [18] was avoided, and a good signal-to-noise ratio was achieved (**Fig. 4**). Upon hybridizing the FISH probes to

the target region, 2-4 bright DNA FISH foci were consistently observed in each cell nucleus, thus both Xq22.3 regions on the active and inactive copies of X chromosomes were labeled by FISH probes. Using the H3K27me3 antibody, a bright Epi-PHR signal was detected that co-localized with only half of the DNA FISH foci, which was consistent with previous studies that only Xi is enriched with the repressive mark (**Figs. 4B and 4C**). (The optical section of the imaging system does not cover the whole depth of a cell nucleus, thus sometimes not all DNA FISH foci are displayed in the Figures.) Moreover, the H3K9me3 Epi-PHR signal was not observed in this region, which is consistent with the alternating distribution of H3K27me3 and H3K9me3 marks on the inactive X chromosome (**Fig. 4B**). These results demonstrated that Epi-PHR can robustly detect an epi-genetic modification at a non-repetitive genomic region.

Example 5. The Epi-mFISH can detect epigenetic marks at more than one genomic loci in the same cell

[00371] To demonstrate the multiplexing capacity of the Epi-mFISH, the method was applied to detect H3K9me3 marks at different human satellite sequences on different chromosomes. Human satellite regions are constituted by large repeated arrays of sequences, known as satellite sequences. Each human satellite region is normally constituted by various satellite sequences and one satellite sequence may appear on different satellite regions, and the total sizes of the repeated arrays also differ among different satellite regions [32]. Human satellite subfamilies 2A2, 2B, and 3B5 (Hsat2A2, Hsat2B, Hsat3B5) have similar repeated array size, and they are predominantly localized inside single genomic regions in different human chromosomes [33], so these sequences were selected to ensure that detected regions had unique locations and similar sizes. Moreover, the Hsat2A2, Hsat2B, and Hsat3B5 locate inside constitutive heterochromatin adjacent to centromeric regions [33], and the constitutive heterochromatin is enriched with repressive mark H3K9me3 [28]. Therefore, the Epi-mFISH was applied to simultaneously detect the H3K9me3 mark at the three different human satellite regions.

[00372] In order to target different satellite sequences, three FISH probes (SEQ ID NOs: 149-151) were designed. Each probe contained a sequence for targeting a specific satellite subfamily, and a readout probe binding region for multiplexed sequential FISH imaging. Different probes used different readout probe binding regions

to distinguish the satellite subfamilies. In addition, all the probes had the same overhang region for the four PH1 docking strategy (**Fig. 5A**). To simultaneously detect the H3K9me3 mark at different satellite regions, all three FISH probes were hybridized to the genome all together, and Epi-PHR signals of all three targeting regions were generated. After imaging of Epi-PHR signals, the dye-labeled readout probes were sequentially flowed into the system to generate FISH signals of each satellite sequence. During the imaging analysis, the Epi-PHR images were aligned with FISH images, then the Epi-PHR signals were assigned to the corresponding satellite regions by colocalization.

[00373] During an initial test, strong H3K9me3 Epi-PHR signals were observed, and clear FISH signals were observed from all three rounds of readout probe hybridization, and the Epi-PHR signals could be assigned to different loci by colocalization analysis (**Fig. 5B**). However, upon further examining the raw data, it was found that the FISH signals of the Hsat2A2 and the Hsat2B overlapped. The result indicated that the Hsat2A2 and the Hsat2B FISH probes could cross hybridize to both the Hsat2A2 and the Hsat2B regions. This non-specific hybridization was largely due to the high degree of sequence similarity between the Hsat2A2 and the Hsat2B [33], and that the FISH hybridization condition was not stringent enough to specifically hybridize the FISH probes to targeted genomic loci. To address this problem, hybridization stringency was increased during the FISH procedure to improve the FISH probes' specificity. Despite overlapping Hsat2A2 and Hsat2B FISH signals, the Hsat3B5 showed strong FISH signals that did not colocalize with the Hsat2A2 and Hsat2B FISH signals, and the Epi-PHR signals could be confidentially assigned to the Hsat3B5 region. The initial test demonstrated that the Epi-mFISH could simultaneously detect epigenetic marks at multiple genomic loci.

Example 6. An EZ version of Epi-PHR

[00374] The core design of EZ-Epi-PHR and EZ-Epi-mFISH is splitting a single-stranded DNA sequence into two parts, termed EZ-PH1 and EZ-PH2. When EZ-PH1 and EZ-PH2 are in spatial proximity, the two oligonucleotides form a platform to co-bind a dye-labeled oligonucleotide probe and generate a PHR signal. To test this design, a split initiator was applied to detect an H3K9me3 mark at the human chromosome 9 alpha satellite locus in RPE1 cells.

[00375] To demonstrate the split-sequence scheme, the Epi-PHR labeling strategy was used (**Fig. 6A**). The EZ-PH1 were hybridized to FISH probes via an overhang region, and biotin-labeled EZ-PH2 probes (SEQ ID NO: 3869) were linked to biotinylated antibodies via streptavidin (**Fig. 6A**). The initial results were consistent with the Epi-PHR results: The repressive H3K9me3 mark was enriched inside the human alpha satellite locus. However, the negative control also showed very weak signals in a small portion of the cells (**Fig. 6B**). This background problem may be resolved by linking EZ-PH1 and EZ-PH2 together via click chemistry and then applying high stringent wash steps afterward. Overall, these results validated the core mechanism of the EZ-Epi-PHR and EZ-Epi-mFISH.

Example 7. Improving genomic resolution by branched amplification

[00376] As demonstrated in the above examples, the above-described system can detect epigenetic marks at a 300-kilobase nonrepetitive genomic region, but the sizes of many biologically significant genomic loci are smaller than 300-kilobase. In order to resolve the epigenetic states of these small genomic loci, genomic resolution of the methods is further improved. A challenge to achieving higher genomic resolutions is that fewer FISH probes can be designed to target shorter genomic loci, and the shorter loci contain fewer copies of histone modifications. Hence, shorter genomic loci may not be capable of binding enough PH1 and PH2 to allow for generation of detectable Epi-PHR signals.

[00377] In order to increase the number of PH1 and PH2 in short genomic loci, a branched amplification strategy is incorporated, which is similar to the four-PH1-docking scheme. A primary amplification oligonucleotide is designed that is directly linked to a FISH probe or contains a FISH probe docking sequence, and contains 4 additional binding sites. The binding sites are recognized by another set of linker oligonucleotides, each of which contains 4 PH1 docking regions. By applying this scheme, each FISH probe carries 16 PH1 molecules (**Fig. 7A**). Moreover, the detection amplification is further improved by designing a secondary amplification oligonucleotide, which contains one primary-amplification-oligonucleotide docking site (each primary amplification oligonucleotide will bind 4 secondary amplification oligonucleotides), and 4 binding sites for PH1-docking linker oligonucleotides. This design allows each FISH probe to carry 64 PH1 molecules. Moreover, this amplification scheme is used to increase the number

of PH2 molecules on each antibody. The above-described design uses a biotinylated PH2 to label antibodies. Branched amplification is added to this design through the following modification: Instead of using biotinylated PH2, biotin-labeled primary amplification oligonucleotides are designed and a branched amplification scheme applied to dock 48 or 192 PH2 molecules to each antibody without or with a secondary amplification oligonucleotide (**Fig. 7B** for the case without secondary amplification oligonucleotide). The branched amplification scheme is used to amplify Epi-PHR signals. By replacing the H1 with a primary amplification oligonucleotide that binds to an opened end of the PH2, branched amplification is triggered and generates amplified Epi-PHR signals (**Fig. 7C**). Moreover, these amplification schemes are applied individually or in combinations to Epi-PHR, Epi-mFISH, EZ-Epi-PHR, and EZ-Epi-mFISH.

Example 8. Simultaneously detect multiple epigenetic marks from the same cell

[00378] Some genomic regions have multiple epigenetic modifications, and the combinations of these modifications contain crucial biological information. Thus, the above-described methods are modified in the present example to simultaneously detect multiple epigenetic marks from the same cell. The above-described methods employ one pair of proximity hairpin oligonucleotides (PH1 and PH2) to detect a single epigenetic mark. In order to detect multiple epigenetic marks, more pairs of proximity hairpin oligonucleotides are designed, among which one hairpin only invades a corresponding hairpin, and each pair of proximity hairpin oligonucleotides is responsible for detecting one specified epigenetic mark. For example, two pairs of proximity hairpin oligonucleotides are designed, PH1_1/PH2_1 and PH1_2/PH2_2. By design, the PH1_1 only invades the PH2_1 but cannot invade the PH2_2, so the PH1_1 and PH1_2 can be docked to the same FISH probes, and one antibody labeled with PH2_1 and another antibody labeled with PH2_2. After triggering PHR, the Epi-PHR signals from PH1_1/PH2_1 and PH1_2/PH2_2 are sequentially detected (**Fig. 8**). Since each pair of proximity hairpin oligonucleotides corresponds to one specific epigenetic mark, the Epi-PHR signals are assigned to corresponding epigenetic marks.

[00379] In order to hybridize different PH1 oligonucleotides to one FISH probe, a unique overhang region is added to each version of PH1, and a linker oligonucleotide is designed that contains a FISH probe docking site and multiple binding sites, each of

which specifically binds to one version of PH1. By this design, all versions of PH1 can be introduced to the system at the same time, and each FISH probe has all versions of PH1 bound to it. To label each specific antibody with one version of PH2, a unique overhang region is added to each version of PH2, and different linker oligonucleotides designed for linking to an antibody. Each version of the linker oligonucleotide contains a binding site(s) that bind to one specific version of PH2. Moreover, these linker oligonucleotides are NHS ester modified, and, thus each version of linker oligonucleotide is directly conjugated to a specific antibody. Since each version of PH2 has a unique sequence, multiple dye-labeled oligonucleotides (H1_1, H1_2) are designed to bind different versions of PH2. These dye-labeled oligonucleotides will be simultaneously hybridized to the targets and imaged in different fluorescent channels, or sequentially hybridized to the targets, imaged and photobleached. In the latter case, each round of hybridization and imaging generates the Epi-PHR signals for one specific epigenetic mark. Moreover, all these strategies are applied to Epi-mFISH, EZ-Epi-PHR, and EZ-Epi-mFISH.

Example 9. Simultaneously detect epigenetic marks at hundreds to thousands of genomic loci

[00380] The above Examples 1 through 6 demonstrated that the Epi-mFISH can detect an epigenetic mark at multiple loci. The number of genomic loci to be detected in the same cell is further increased in the present example. One challenge of imaging hundreds to thousands of genomic loci is that the number of hybridization rounds increases substantially. To address this problem, a combinatorial barcoding strategy is used [35 and U.S. Pat. Appl. Pub. No. US20170220733, both of which are incorporated herein by reference in their entirety for all purposes]. In this example, each genomic locus is labeled with a unique combination of several readout regions, each of which hybridizes to a different readout probe in a different round of readout hybridization. In other words, this genomic locus is repeatedly detected in a number of rounds. The specific round numbers in which the genomic locus is detected form a unique barcode for this genomic locus. For example, a genomic locus probed in Rounds 1, 2, 5, and 10 in 14 total rounds of readout hybridization has the barcode “11001000010000”. Using a combinatorial barcode as shown in **Fig. 9**, a large number of genomic loci can be easily identified with only a few rounds of sequential FISH and

merely single-color imaging (e.g. a “14-choose-4” coding scheme can encode 1001 RNA species with 14 Alexa Fluor 647 labeled readout probes in 14 rounds of readout hybridization). This strategy is applicable to the mFISH portion of the Epi-mFISH, mEpi-mFISH, EZ-Epi-mFISH, and EZ-mEpi-mFISH: As shown in **Fig. 9**, additional readout regions are added to the primary FISH probes, so that each genomic locus is detected in a unique combination of readout hybridization rounds. By incorporating multicolor imaging, more bits (binary digits) are imaged in a barcode with less hybridization rounds (e.g. a “14-choose-4” coding scheme is decoded with 2-color imaging in 7 rounds of readout hybridization of two readout probes in each round, labeled with two spectrally distinct fluorescent dyes).

Example 10. Profile transcriptome, epigenome, and chromosome structure in single cells

[00381] MERFISH is combined with the Epi-mFISH to simultaneously profile transcriptome, epigenome, and chromosome structure at the single-cell level in the same cells. Multiplexed Imaging of Nucleome Architectures (MINA) combines the multiplexed sequential DNA FISH with MERFISH to measure multiscale chromatin folding and RNA copy numbers from over one hundred genes at the single-cell level [26]. The MINA technique demonstrates a capacity to integrate multiplexed sequential DNA FISH with MERFISH, and the initial Epi-mFISH results also demonstrate the feasibility of combining the Epi-PHR with the multiplexed sequential DNA FISH. All of these techniques are integrated in the present example. Similarly, EZ-Epi-mFISH is combined with MERFISH.

Example 11. Epi-mFISH can profile epigenetics marks at more than one non-repetitive genomic loci in the same cell

[00382] The Examples described above demonstrate that Epi-mFISH can simultaneously detect epigenetic marks at several repetitive satellite genomic regions in the same single cell, but many biologically important genomic loci are non-repetitive regions. To further demonstrate the multiplexing and profiling capacities of the Epi-mFISH for non-repetitive sequences, the method was applied to profile H3K9me3 and H3K27ac marks at 22 non-repetitive regions in the human lung fibroblast IMR-90 cells.

[00383] To select non-repetitive regions of interest, IMR-90 ChIP-seq data were analyzed [36], 12 H3K27ac enriched regions and 10 H3K9me3 enriched regions were

selected for the Epi-mFISH probe design. To target the 22 different genomic regions, 22 sets of FISH probes were designed. Each set of FISH probes contained 5800 oligonucleotide probes targeting a 500-kb selected genomic region, and a common readout probe binding region was added to these oligonucleotide probes for multiplexed sequential FISH imaging. Different sets of probes consisted of different readout probe binding regions to distinguish among different genomic loci. Moreover, all FISH probes contained the same overhang regions for the 4 PH1 docking strategy (**Fig. 11A**). The experimental procedures were similar to the previously described repetitive region Epi-mFISH example (Example 5). In this demonstration, all 22 sets of FISH probes were hybridized to the genome, then Epi-PHR signals of all the targeted regions were generated, and then multiplexed sequential FISH were performed to identify the genomic loci. By colocalization analysis of the Epi-PHR signals and the multiplexed sequential FISH signals, the epigenetic states of all 22 targeted genomic regions in single cells were observed (**Fig. 11B**). The quality of the Epi-mFISH signals were evaluated in two ways: First, the signal rate for each targeted region was calculated. The signal rate was defined as (the number of FISH foci from one genomic region that colocalize with Epi-PHR signals) / (the total number of FISH foci from the same genomic region). Second, the normalized fluorescent intensities of Epi-mFISH signals was calculated by (the fluorescent intensity of an Epi-PHR signal) / (the fluorescent intensity of the colocalizing multiplexed sequential FISH signal). In some cases, FISH foci do not have colocalizing Epi-PHR foci, likely due to a lack of the epigenetic mark at the genomic region, in which cases zero values were assigned as the normalized fluorescent intensities.

[00384] H3K9me3 and H3K27ac epigenetic marks were separately profiled using Epi-mFISH design and analyses above. The Epi-mFISH signal rates were then compared with IMR-90 ChIP-seq data [36] by correlating the Epi-mFISH signal rates of the 22 genomic loci with the normalized ChIP-seq peak height of same targeted regions, where normalized ChIP-seq peak height is defined as (the summed ChIP-seq peaks within the selected 500-kb region) / (the highest summed ChIP-seq peak value among all the 500-kb regions). Notably, the Epi-mFISH signal rates of H3K9me3 and H3K27ac marks showed high correlations with the corresponding normalized ChIP-seq peak heights, with Pearson correlation coefficients of 0.934 and 0.932, respectively (**Fig.**

11C). The strong correlations between the results from two different methods validated the Epi-mFISH results. Similarly, the normalized fluorescent intensities showed clear differences between the expected targeted mark-enriched regions and targeted mark-depleted regions (**Fig. 11D**). These results demonstrated that the Epi-mFISH can profile epigenetic modifications at multiple non-repetitive genomic regions in the same single cells.

Example 12. The EZ-Epi-PHR can robustly detect epigenetic marks after enzymatic ligation

[00385] As demonstrated in Example 6, the split-sequence design of EZ-Epi-PHR can detect the H3K9me3 mark at the human alpha satellite locus, but the negative control showed weak yet detectable signals. In order to suppress the false positive signals, the click chemistry-based ligation reaction was proposed to link EZ-PH1 and EZ-PH2, then false positive signals can be removed by highly stringent wash. Besides the click ligation, an enzymatic ligation reaction can also be applied to link EZ-PH1 and EZ-PH2.

[00386] In order to enzymatically ligate EZ-PH1 and EZ-PH2, a phosphorylation modification was added to the 5' of the EZ-PH2. After incubation of EZ-H1, T4 ligase was introduced to the system to link EZ-PH1 and EZ-PH2, then highly stringent washes were applied to remove unwanted background signals. To demonstrate this design, the EZ-Epi-PHR was applied to detect the H3K9me3 mark at the human chromosome 9 alpha satellite locus in IMR90 cells. The FISH probe design and antibody labeling were the same as previously described in Example 6. After incubation of EZ-H1, the bright EZ-Epi-PHR signals can be observed in the experimental group, and the negative control samples showed some weak false-positive signals (**Fig. 12A**). T4 ligation was then applied to another set of experimental and negative control samples. After highly stringent washes, strong EZ-Epi-PHR signals can still be observed in the experimental group, while no visible background signals can be detected in the negative control samples (**Fig. 12B**). These data validated the enzymatic ligation design, and demonstrated that EZ-Epi-PHR could robustly detect epigenetic marks at a given genomic locus.

Example 13. Simultaneously detect epigenetic mark and measure chromatin folding in single cells

[00387] The multiplexed sequential FISH is a powerful tool for simultaneously imaging

and unambiguously identifying many targeted genomic regions. As demonstrated in the previous sections, multiplexed sequential FISH was successfully integrated into the Epi-mFISH system to profile epigenetic marks at multiple genomic loci. In addition to this application, the multiplexed sequential FISH were repurposed for measuring chromatin folding in this demonstration, and combined it with Epi-PHR to simultaneously detect epigenetic marks and measure chromatin folding in the same single cells.

[00388] To measure chromatin folding, the 3D folding path of 14 selected topologically associating domains (TADs) along human chromosome 20 was traced by labeling the central 100-kb region of each TAD with FISH probes. In order to target the 14 different TADs, 14 sets of FISH probes were designed. Each set of FISH probes contained 1000 oligonucleotide probes to target a 100-kb selected genomic region, and a common readout probe binding region was added to these oligonucleotide probes for multiplexed sequential FISH imaging. Different sets of probes contained different readout probe binding regions to distinguish the different genomic loci. Then 5800 Epi-PHR probes were designed to target a 500-kb region (Chr20: 48133407-48633407/hg18) within one of the selected TADs, the ChIP-seq data [36] indicates this region is H3K27ac-enriched at the population-averaged level. The overall construct of the Epi-PHR probes was the same as described in the Epi-mFISH demonstration.

[00389] To simultaneously detect epigenetic marks and measure chromatin folding, all FISH probes were hybridized to the genome at the same time, H3K27ac Epi-PHR and multiplexed sequential FISH signals were sequentially imaged. The 3D coordinates of each TAD center were extracted from the multiplexed sequential FISH images, then the chromatin folding traces of the TADs were linked as described in [26, 36] (**Fig. 13A**). At the same time, the colocalization of Epi-PHR signals and the FISH signals from the Epi-PHR targeted TAD was analyzed, and the observed chromatin traces grouped into two groups by asking whether the targeted TAD in each trace showed the Epi-PHR signal (or in other words, had the epigenetic mark). Finally, for each group of traces, the A-B compartmentalization scheme was analyzed using an analysis pipeline introduced previously [26, 36]. A-B compartment identity of the Epi-PHR targeted TAD was opposite between the two group of traces, indicating the A-B compartment identity depends on the epigenetic state of the targeted TAD (**Fig. 13B**), consistent with prior knowledge. These results validated and demonstrated the system's capacity to

simultaneously detect epigenetic marks and measure chromatin folding.

Example 14. The mEpi-mFISH can simultaneously profile more than one epigenetic mark from the same cell

[00390] As previously illustrated in Example 8, the present example further demonstrates detection of 2 histone modifications by 2 pairs of oligonucleotide hairpins. For this demonstration, 8 genomic regions are selected from the previous 22-region Epi-mFISH demonstration (4 of the regions are H3K9me3-enriched; 4 are H3K27ac-enriched), and are targeted with the FISH probes from the previous Epi-mFISH experiment. Two versions of PH1 probes (PH1_1 and PH1_2) are used for the two epigenetic marks. In order to hybridize the two different PH1 probes to the same set of FISH probes, a unique overhang region is added to each version of PH1, and a linker oligonucleotide is designed to contain a FISH probe hybridization site and 4 PH1 docking sites. 2 of the docking sites can bind to the PH1_1 overhang region; the other 2 docking sites can bind to the PH1_2 overhang region (**Fig. 14**). The antibody labeling and PH2 docking scheme are the same as described in the previous example (Example 8). In this demonstration, the H3K9me3 antibody is conjugated with a linker that contains the PH2_2 docking site, and the H3K27ac antibody is conjugated with a linker that contains the PH2_1 docking site. Next activator_1 and activator_2 are simultaneously introduced to initiate the hairpin invasions. After the activators, Alexa 647 labeled H1_1 and Atto 565 labeled H1_2 are introduced to the system, they are then followed by Alexa 647 labeled H2_1 and Atto 565 labeled H2_2. The signals from two pairs of hairpins are imaged in two different fluorescent channels, and individually mapped back to the corresponding multiplexed sequential FISH signals based on colocalization. Eventually, for each targeted mark, the signal rates and normalized fluorescent intensities are calculated as described in the previous example.

[00391] To validate this method, the signal rates of the two marks are individually compared with normalized ChIP-seq peak heights of the two marks. The correlation of the signal rates with population-averaged normalized ChIP-seq peak heights and the difference in the normalized fluorescent intensities between mark-enriched regions and mark-depleted regions would validate the design, and demonstrate the capacity of mEpi-mFISH to simultaneously detect and profile multiple epigenetic marks at multiple genomic regions in the same single cells.

Methods

[00392] The following materials and methods were used, unless described otherwise in a specific Example.

Imaging

[00393] For imaging, a microscope with a Nikon Ti2-U body, a Nikon CFI Plan Apo Lambda 60x Oil (NA1.40) objective lens, and an active auto-focusing system via an infrared 980nm laser (LP980-SF15, Thorlabs) was used. A 647-nm laser (2RU-VFL-P-1000-647-B1R, MPB Communications) was used to excite and image Alexa Fluor 647. A 560-nm laser (2RU-VFL-P-1000-560-B1R, MPB Communications) was used to excite and image ATTO 565. A 488-nm laser (2RU-VFL-P-500-488-B1R, MPB Communications) was used to excite and image the Alexa Fluor 488. The three laser lines were directed to a sample using a multi-band dichroic mirror (ZT405/488/561/647/752rpc-UF2, Chroma) on the excitation path. On the emission path, a multi-band emission filter (ZET405/488/561/647-656/752m, Chroma) was used. A Hamamatsu Orca Flash 4.0 V3 camera was used to record images.

Design of hairpin loop structures

[00394] Thermodynamic parameters for hairpin loop structures and hairpin invasion processes were calculated or simulated using NUPAK.

References

1. Simon, J. A. & Kingston, R. E. Mechanisms of Polycomb gene silencing: knowns and unknowns. *Nat. Rev. Mol. Cell Biol.* **10**, 697 (2009).
2. Soshnev, A. A., Josefowicz, S. Z. & Allis, C. D. Greater Than the Sum of Parts: Complexity of the Dynamic Epigenome. *Mol. Cell* **62**, 681–694 (2016).
3. Boettiger, A. N. *et al.* Super-resolution imaging reveals distinct chromatin folding for different epigenetic states. *Nature* **529**, 418–422 (2016).
4. Sharma, S., Kelly, T. K. & Jones, P. A. Epigenetics in cancer. *Carcinogenesis* **31**, 27–36 (2010).
5. Misteli, T. Higher-order genome organization in human disease. *Cold Spring Harb. Perspect. Biol.* **2**, a000794–a000794 (2010).
6. Moosavi, A. & Motevalizadeh Ardekani, A. Role of Epigenetics in Biology and Human Diseases. *Iran. Biomed. J.* **20**, 246–258 (2016).
7. Babu, D. & Fullwood, M. J. 3D genome organization in health and disease:

emerging opportunities in cancer translational medicine. *Nucleus* **6**, 382–393 (2015).

8. Schmitt, A. D., Hu, M. & Ren, B. Genome-wide mapping and analysis of chromosome architecture. *Nat. Rev. Mol. Cell Biol.* **17**, 743 (2016).

9. Lieberman-Aiden, E. *et al.* Comprehensive Mapping of Long-Range Interactions Reveals Folding Principles of the Human Genome. *Science* (80-.). **326**, 289 LP – 293 (2009).

10. Zhu, Y. *et al.* Constructing 3D interaction maps from 1D epigenomes. *Nat. Commun.* **7**, 10812 (2016).

11. Wang, S. *et al.* Spatial organization of chromatin domains and compartments in single chromosomes. *Science* **353**, 598–602 (2016).

12. Smallwood, S. *et al.* Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. *Nat. Methods* **11**, 817–20 (2014).

13. Buenrostro, J. D. *et al.* Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature* **523**, 486–490 (2015).

14. Jin, W. *et al.* Genome-wide detection of DNase I hypersensitive sites in single cells and FFPE tissue samples. *Nature* **528**, 142–146 (2015).

15. Rotem, A. *et al.* Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. *Nat. Biotechnol.* **33**, 1165 (2015).

16. Bernstein, B. E. *et al.* A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. *Cell* **125**, 315–326 (2006).

17. Voigt, P., Tee, W.-W. & Reinberg, D. A double take on bivalent promoters. *Genes Dev.* **27**, 1318–1338 (2013).

18. Koos, B. *et al.* Proximity-dependent initiation of hybridization chain reaction. *Nat. Commun.* **6**, 7294 (2015).

19. Gomez, D., Shankman, L. S., Nguyen, A. T. & Owens, G. K. Detection of histone modifications at specific gene loci in single cells in histological sections. *Nat. Methods* **10**, 171–177 (2013).

20. Choi, H. M. T. *et al.* Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* **145**, dev165753 (2018).

21. Besanceney-Webler, C. *et al.* Increasing the Efficacy of Bioorthogonal Click Reactions for Bioconjugation: A Comparative Study. *Angew. Chemie Int. Ed.* **50**, 8051–

8056 (2011).

22. Rouhanifard, S. H. *et al.* ClampFISH detects individual nucleic acid molecules using click chemistry-based amplification. *Nat. Biotechnol.* **37**, 84–89 (2019).
23. Beliveau, B. J. *et al.* Versatile design and synthesis platform for visualizing genomes with Oligopaint FISH probes. *Proc. Natl. Acad. Sci.* **109**, 21301 LP – 21306 (2012).
24. Boyle, S., Rodesch, M. J., Halvensleben, H. A., Jeddloh, J. A. & Bickmore, W. A. Fluorescence in situ hybridization with high-complexity repeat-free oligonucleotide probes generated by massively parallel synthesis. *Chromosome Res.* **19**, 901–909 (2011).
25. Yamada, N. A. *et al.* Visualization of Fine-Scale Genomic Structure by Oligonucleotide-Based High-Resolution FISH. *Cytogenet. Genome Res.* **132**, 248–254 (2011).
26. Liu, M., Lu, Y., Yang, B. *et al.* Multiplexed imaging of nucleome architectures in single cells of mammalian tissue. *Nat Commun* **11**, 2907 (2020).
<https://doi.org/10.1038/s41467-020-16732-5>.
27. Mateo, L. J. *et al.* Visualizing DNA folding and RNA in embryos at single-cell resolution. *Nature* **568**, 49–54 (2019).
28. Saksouk, N., Simboeck, E. & Déjardin, J. Constitutive heterochromatin formation and transcription in mammals. *Epigenetics Chromatin* **8**, 3 (2015).
29. Martins, N. M. C. *et al.* Epigenetic engineering shows that a human centromere resists silencing mediated by H3K27me3/K9me3. *Mol. Biol. Cell* **27**, 177–196 (2016).
30. Douet, J. *et al.* MacroH2A histone variants maintain nuclear organization and heterochromatin architecture. *J. Cell Sci.* **130**, 1570 LP – 1582 (2017).
31. Darrow, E. M. *et al.* Deletion of DXZ4 on the human inactive X chromosome alters higher-order genome architecture. *Proc. Natl. Acad. Sci.* **113**, E4504 LP-E4512 (2016).
32. Garrido-Ramos, M. A. Satellite DNA: An Evolving Topic. *Genes (Basel)*. **8**, 230 (2017).
33. Altemose, N., Miga, K. H., Maggioni, M. & Willard, H. F. Genomic Characterization of Large Heterochromatic Gaps in the Human Genome Assembly. *PLOS Comput. Biol.* **10**, e1003628 (2014).

34. Xia, C., Babcock, H. P., Moffitt, J. R. & Zhuang, X. Multiplexed detection of RNA using MERFISH and branched DNA amplification. *Sci. Rep.* **9**, 7721 (2019).
35. Chen, K. H., Boettiger, A. N., Moffitt, J. R., Wang, S. & Zhuang, X. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* **348**, aaa6090–aaa6090 (2015).
36. Kundaje, A., et al., Integrative analysis of 111 reference human epigenomes. *Nature*, 2015. 518(7539): p. 317-330

* * *

[00395] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims. It is further to be understood that all values are approximate, and are provided for description.

[00396] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range and each endpoint, unless otherwise indicated herein, and each separate value and endpoint is incorporated into the specification as if it were individually recited herein.

[00397] Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

Claims

1. A method for *in situ* visualization of a chromatin modification at a genomic locus of a cell comprising the steps of:
 - a) providing at least one first probe comprising a first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein said first oligonucleotide binds to a genomic locus of interest,
 - b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest,
 - c) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is labeled directly or indirectly,
 - d) contacting the cell with the first probe under conditions that allow binding of said first oligonucleotide of said first probe to said genomic locus of the cell,
 - e) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
 - f) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to (i) a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, or to (ii) a sequence made available when said PH1 and PH2 oligonucleotides have hybridized, and
 - g) detecting the label of the third probe.
2. The method of claim 1, wherein
 - step a) comprises providing a plurality of first probes, each of which targets a genomic locus of interest;
 - step d) comprises contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell, andwherein the method further comprises the steps of:
 - h) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,

- i) contacting the cell with each labeled readout probe, and
 - j) detecting each label of each readout probe.
3. The method of claim 2, wherein the plurality of readout probes are labeled with a plurality of dyes.
 4. The method of claim 2, wherein the plurality of readout probes are labeled with the same dye.
 5. The method of claim 3 or 4, wherein the dye(s) are fluorescent dye(s).
 6. The method of any one of claims 2 to 5, wherein each of said plurality of first oligonucleotides comprises one or more readout probe binding sites each selectively bound by one of the plurality of labeled readout probes.
 7. The method of claim 6 wherein the one or more readout probe binding sites comprises a nucleotide sequence of
AATCGATCCACTACCGTCGATCCCGTGTCC (SEQ ID NO: 9), the reverse complement of SEQ ID NO: 9, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9.
 8. The method of claim 6 or 7, wherein the one or more readout probe binding sites comprises a nucleotide sequence of
AACCGGTACATGACGCGGAACCTAAGGTCG (SEQ ID NO: 10), the reverse complement of SEQ ID NO: 10, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10.
 9. The method of any one of claims 6 to 8, wherein the one or more readout probe binding sites comprises a nucleotide sequence of
TAGCAAAGCCGGTAGCGACAACCGTTTCCC (SEQ ID NO: 11), the reverse complement of SEQ ID NO: 11, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11.
 10. The method of any one of claims 1 to 9, wherein the chromatin modification is a DNA modification, a DNA-binding protein, or a modification to a DNA-binding protein.
 11. The method of claim 10, wherein the chromatin modification is a histone

modification or a histone variant.

12. The method of any one of claims 1 to 11, wherein the cell is fixed.
13. The method of any one of claims 1 to 12, wherein the genomic locus of interest is disposed within the nucleus of the cell.
14. The method of any one of claims 1 to 13, wherein the method further comprises determining in three dimensions a location of the third probe.
15. The method of claim 14, wherein the method further comprises using the location of the third probe to analyze chromatin structure.
16. The method of any one of claims 2 to 15, wherein the method further comprises determining in three dimensions a location of the readout probe.
17. The method of claim 16, wherein the method further comprises using the location of the readout probe to analyze chromatin structure.
18. The method of any one of claims 1 to 17, wherein the antibody is coupled to the PH2 oligonucleotide by a biotin-streptavidin bridge.
19. The method of any one of claims 1 to 17, wherein the antibody is coupled to the PH2 oligonucleotide by a covalent bond.
20. The method of any one of claims 1 to 19, wherein coupling of the antibody to the PH2 oligonucleotide comprises nucleotide hybridization.
21. The method of any one of claims 1 to 19, wherein the H1 oligonucleotide is labeled with a first dye.
22. The method of claim 21 wherein the first dye is a fluorescent dye.
23. The method of claim 21 or claim 22, wherein the first dye is Alexa Fluor 647.
24. The method of any one of claims 1 to 23, wherein the coupling of the first probe to the PH1 oligonucleotide comprises nucleotide hybridization.
25. The method of any one of claims 1 to 23, wherein the coupling of the first probe to the PH1 oligonucleotide comprises a covalent bond.
26. The method of any one of claims 1 to 25, wherein the H1 oligonucleotide forms a hairpin loop structure.
27. The method of any one of claims 1 to 26, wherein a signal generated by the third probe is amplified through sequential hybridization comprising binding a fourth probe comprising a hybridization 2 (H2) oligonucleotide to the H1 oligonucleotide, wherein said H2 oligonucleotide is labeled.

28. The method of any one of claims 1 to 27, wherein the H1 oligonucleotide comprises a nucleotide sequence of
ACAGACGACTCCCACATTCTCCAGGTGGGAGTCGTCTGTAACATGAAGTA
(SEQ ID NO: 6), the reverse complement of SEQ ID NO: 6, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 6, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 6.
29. The method of any one of claims 1 to 27, wherein the H1 oligonucleotide comprises a nucleotide sequence of
GCGAATCAGTCAGACGTACCTCATGTCTGACTGATTCGCAACTCCCTCTA
(SEQ ID NO: 3857), the reverse complement of SEQ ID NO: 3857, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3857, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3857.
30. The method of any one of claims 27 to 29, wherein the H2 oligonucleotide forms a hairpin loop structure.
31. The method of any one of claims 27, 28, and 30, wherein the H2 oligonucleotide comprises a nucleotide sequence of
CTGGAGAATGTGGGAGTCGTCTGTTACTTCATGTTACAGACGACTCCCAC
(SEQ ID NO: 8), the reverse complement of SEQ ID NO: 8, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 8, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 8.
32. The method of any one of claims 27, 29 and 30, wherein the H2 oligonucleotide comprises a nucleotide sequence of
ATGAGGTACGTCTGACTGATTCGCTAGAGGGAGTTGCGAATCAGTCAGAC
(SEQ ID NO: 3858), the reverse complement of SEQ ID NO: 3858, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3858, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3858.
33. The method of any one of claims 27 to 32, wherein the H2 oligonucleotide is labeled with a second dye.

34. The method of claim 33 wherein the second dye is a fluorescent dye.
35. The method of claim 33 or claim 34, wherein the second dye is Alexa Fluor 647.
36. The method of any one of claims 1 to 35, wherein the PH1 oligonucleotide and the PH2 oligonucleotide each form hairpin loop structures.
37. The method of any one of claims 1 to 28, 30-31, and 33-36, wherein the PH1 oligonucleotide comprises a nucleotide sequence of
AAAAATCGTCTGTGGCATGAAGGCCCGCTGTATTCAGTGAATGCGAGTCAG
ACGAATACAGCGGGCCTTCATGCCACAGACGA (SEQ ID NO: 4), the reverse complement of SEQ ID NO: 4, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4.
38. The method of any one of claims 1 to 27, 29-30, and 32-36, wherein the PH1 oligonucleotide comprises a nucleotide sequences of
AAAAATGATTCGCTTCTCCCTCAGAGTGGTGTGCAGAACTGTGCGAGAGTC
GATGCACACCACTCTGAGGGAGAAGCGAATCA (SEQ ID NO: 3854), the reverse complement of SEQ ID NO: 3854, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854.
39. The method of any one of claims 1 to 28, 30-31, and 33-37, wherein the PH2 oligonucleotide comprises a nucleotide sequence of
AAAAAGTGGGAGTCGTCTGTAACATGAAGGCCCGCTGTATTCGTCTTACTT
CATGTTACAGACGACTCCAC (SEQ ID NO: 2), the reverse complement of SEQ ID NO: 2, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 2, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 2.
40. The method of any one of claims 1 to 27, 29-30, 32-36 and 38, wherein the PH2 oligonucleotide comprises a nucleotide sequence of
AAAAAGTCTGACTGATTCGCAACTCCCTCAGAGTGGTGTGCATCGATAGAG
GGAGTTGCGAATCAGTCAGAC (SEQ ID NO: 3855), the reverse complement of SEQ ID NO: 3855, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3855, or a nucleotide sequence complementary to a nucleotide

- sequence with at least 75% sequence identity to SEQ ID NO: 3855.
41. The method of any one of claims 1 to 40, wherein a plurality of PH1 oligonucleotides are coupled to the first oligonucleotide through a linker oligonucleotide.
 42. The method of claim 41, wherein four PH1 oligonucleotides are coupled to the first oligonucleotide.
 43. The method of claim 41 or claim 42, wherein the linker oligonucleotide comprises a nucleotide sequence of
GCTATCGTTCGTTTCGAGGCCAGAGCATTCTGGCTATCGTTCGTTTCGAGGCCA
GAGCATTCTGGCTATCGTTCGTTTCGAGGCCAGAGCATTCTGGCTATCGTTCGT
TCGAGGCCAGAGCATTCTGCGCAACGCTTGGGACGGTTCCAATCGGATC
(SEQ ID NO: 12), the reverse complement of SEQ ID NO: 12, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 12, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 12.
 44. The method of any one of claims 41 to 43, wherein the first oligonucleotide is coupled to the linker oligonucleotide through nucleotide hybridization.
 45. The method of any one of claims 41 to 43, wherein the first oligonucleotide is coupled to the linker oligonucleotide through a covalent bond.
 46. The method of any one of claims 1 to 45, wherein the method further comprises contacting the cell with an activator oligonucleotide under conditions allowing the activator oligonucleotide to bind the PH1 or PH2 oligonucleotide.
 47. The method of claim 46, wherein the method further comprises:
the activator oligonucleotide binding to the PH1 oligonucleotide causing a first hairpin formed by the PH1 oligonucleotide to open,
a portion of the PH1 oligonucleotide made available by the opening of the first hairpin binding to the PH2 oligonucleotide causing a second hairpin formed by the PH2 oligonucleotide to open and make available said sequence made available when said PH1 and PH2 oligonucleotides have hybridized.
 48. The method of claim 46, wherein the method further comprises:
the activator oligonucleotide binding to the PH2 oligonucleotide causing a first hairpin formed by the PH2 oligonucleotide to open,

- a portion of the PH2 oligonucleotide made available by the opening of the first hairpin binding to the PH1 oligonucleotide causing a second hairpin formed by the PH1 oligonucleotide to open and make available said sequence made available when said PH1 and PH2 oligonucleotides have hybridized.
49. The method of any one of claims 46 to 48, wherein the activator oligonucleotide comprises a nucleotide sequence of
GACTCGCATTCACTGAATACAGCGGGCCTTCATGCCACAGACGA (SEQ ID NO: 1), the reverse complement of SEQ ID NO: 1, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 1, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 1 when the PH1 oligonucleotide comprises a nucleotide sequence of
AAAAATCGTCTGTGGCATGAAGGCCCGCTGTATTCAGTGAATGCGAGTCAG
ACGAATACAGCGGGCCTTCATGCCACAGACGA (SEQ ID NO: 4), the reverse complement of SEQ ID NO: 4, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4.
50. The method of any one of claims 46 to 48, wherein the activator oligonucleotide comprises a nucleotide sequence of
CTCTCGCACAGTTCTGCACACCACTCTGAGGGAGAAGCGAATCA (SEQ ID NO: 3856), the reverse complement of SEQ ID NO: 3856, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3856, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3856 when the PH1 oligonucleotide comprises a nucleotide sequences of
AAAAATGATTCGCTTCTCCCTCAGAGTGGTGTGCAGAACTGTGCGAGAGTC
GATGCACACCACTCTGAGGGAGAAGCGAATCA (SEQ ID NO: 3854), the reverse complement of SEQ ID NO: 3854, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854.
51. The method of any one of claims 1 to 50, wherein, step b) comprises providing a

- plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a unique chromatin modification or a set of chromatin modifications of interest, step c) comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes, step f) comprises contacting the cell with each third probe of the plurality of third probes, and step g) comprises detecting each label of each third probe.
52. The method of claim 51, wherein the plurality of third probes are labeled with a plurality of dyes.
53. The method of claim 51, wherein the plurality of third probes are labeled with the same dye.
54. The method of claim 52 or 53, wherein the dye(s) are fluorescent dye(s).
55. The method of any one of claims 51 to 54, wherein a signal generated by the plurality of third probes is amplified through sequential hybridization comprising binding a plurality of fourth probes, each of which comprises a hybridization 2 (H2) oligonucleotide to the H1 oligonucleotide, wherein said H2 oligonucleotide is labeled.
56. The method of any one of claims 1 to 27, wherein the H1 oligonucleotide comprises a nucleotide sequence of
AGATATAGAGGCAGGGACCGGGTTAAAGTTGAGTGGCCAGTCTAATAACCA
CTCAACTTTAACCCGGTCCCT (SEQ ID NO: 7), the reverse complement of
SEQ ID NO: 7, a nucleotide sequence with at least 75% sequence identity to
SEQ ID NO: 7, or a nucleotide sequence complementary to a nucleotide
sequence with at least 75% sequence identity to SEQ ID NO: 7.
57. The method of any one of claims 1 to 27, and 56, wherein the PH1
oligonucleotide comprises a nucleotide sequence of
CGCAACGCTTGGGACGGTTCCAATCGGATCTTCCACTCAACTTTAACCCG
(SEQ ID NO: 5), the reverse complement of SEQ ID NO: 5, a nucleotide
sequence with at least 75% sequence identity to SEQ ID NO: 5, or a nucleotide
sequence complementary to a nucleotide sequence with at least 75% sequence
identity to SEQ ID NO: 5.

58. The method of any one of claims 1 to 27, and 56 to 57, wherein the PH2 oligonucleotide comprises a nucleotide sequence of
GTCCCTGCCTCTATATCTTTAATCCGGCGTACGTAAGGCAGCTTGCGTTA
(SEQ ID NO: 3), the reverse complement of SEQ ID NO: 3, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3.
59. The method of any one of claims 1 to 27, and 56 to 58, wherein when the H1 oligonucleotide binds to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, the 3' end of the PH1 oligonucleotide is disposed proximal to the 5' end of the PH2 oligonucleotide or the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide.
60. The method of claim 59, wherein the method further comprises covalently coupling the PH1 oligonucleotide to the PH2 oligonucleotide through a click reaction.
61. The method of claim 60, wherein the click reaction is copper-catalyzed.
62. The method of claim 59, wherein the method further comprises covalently coupling the PH1 oligonucleotide to the PH2 oligonucleotide through an enzymatic ligation reaction.
63. The method of claim 62, wherein the PH2 oligonucleotide comprises a phosphate modification at its 5' end.
64. The method of claim 63, wherein the 5' phosphate-modified PH2 oligonucleotide comprises a nucleotide sequence of
GTCCCTGCCTCTATATCTTTAATCCGGCGTACGTAAGGCAGCTTGCGTTA
(SEQ ID NO: 3), the reverse complement of SEQ ID NO: 3, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3.
65. The method of claim 62, wherein the PH1 oligonucleotide comprises a phosphate modification at its 5' end.
66. The method of claim 65, wherein the 5' phosphate-modified PH1 oligonucleotide comprises a nucleotide sequence of

GTCCCTGCCTCTATATCTTTTCGCAACGCTTGGGACGGTTCCAATCGGATC

(SEQ ID NO: 3860), the reverse complement of SEQ ID NO: 3860, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3860, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3860.

67. The method of claim 66, wherein the PH2 oligonucleotide comprises a nucleotide sequence of
AATCCGGCGTACGTAAGGCAGCTTGCGTTATTCCACTCAACTTTAACCCG
(SEQ ID NO: 3861), the reverse complement of SEQ ID NO: 3861, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3861, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3861.
68. The method of any one of claims 62 to 67, wherein the enzymatic ligation reaction is catalyzed by a T4 DNA ligase, T7 DNA ligase, T3 DNA ligase, Taq DNA ligase, Ampligase, E. coli DNA ligase, or SplintR ligase.
69. The method of any one of claims 1 to 68, wherein the PH1 oligonucleotide, the PH2 oligonucleotide, and the H1 oligonucleotide each comprise DNA.
70. The method of any one of claims 1 to 69, wherein a signal generated by the third probe is amplified through branched amplification.
71. The method of any one of claims 1 to 70, wherein the antibody is biotinylated.
72. The method of any one of claims 1 to 71, wherein the PH2 oligonucleotide is biotinylated.
73. The method of any one of claims 1 to 72, wherein the cell is a mammalian cell.
74. The method of any one of claims 1 to 73, wherein the first probe does not comprise biotin.
75. The method of any one of claims 1 to 74, further comprising quantitating an epigenetic modification level of the genomic locus of interest.
76. The method of any one of claims 2 to 75, wherein the method further comprises identifying each of the plurality of first probes using a barcoding scheme.
77. The method of any one of claims 1 to 76 wherein step d) precedes step e).
78. The method of any one of claims 1 to 76, wherein steps d) and e) take place simultaneously.

79. The method of any one of claims 1 to 76, wherein step e) precedes step d).
80. The method of any one of claims 2 to 79, wherein steps f) and g) precede steps i) and j).
81. The method of any one of claims 2 to 79, wherein steps f) and g) take place simultaneously with steps i) and j).
82. The method of any one of claims 2 to 79, wherein steps i) and j) precede steps f) and g).
83. The method of any one of claims 1 to 82, wherein the method is carried out at a genomic locus within the cell.
84. The method of any one of claims 1 to 82, wherein the method is carried out at a genomic locus on a chromosome spread, extracted chromatin or extracted DNA outside of the cell or extra-cellular DNA.
85. A method for diagnosing, prognosing, and/or predicting treatment response of a disease in a subject, the method comprising *in situ* visualization of a chromatin modification of a cell of the subject according to the method of any one of claims 1 to 84.
86. The method of claim 85, wherein the disease is a cancer.
87. A method of *in situ* visualization of a chromatin modification at a genomic locus in a cell comprising the steps of:
 - a) providing at least one first probe comprising a first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein said first oligonucleotide binds to a genomic locus of interest, and wherein said PH1 oligonucleotide forms a first hairpin loop structure,
 - b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest, and wherein said PH2 oligonucleotide forms a second hairpin loop structure,
 - c) providing an activator oligonucleotide, wherein the activator oligonucleotide is capable of binding to either the PH1 oligonucleotide or the PH2 oligonucleotide, wherein binding of the activator oligonucleotide with the PH1 oligonucleotide causes the first hairpin loop structure to open, and wherein binding of the activator oligonucleotide with the PH2

- oligonucleotide causes the second hairpin loop structure to open,
- d) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is labeled,
 - e) contacting the cell with the first probe under conditions that allow binding of said first oligonucleotide of said first probe to said genomic locus of the cell,
 - f) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
 - g) contacting the cell with the activator oligonucleotide under conditions that allow binding of said activator oligonucleotide to said PH1 oligonucleotide or said PH2 oligonucleotide, wherein said binding of said activator causes PH1 and PH2 to hybridize,
 - h) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to a sequence made available when said PH1 and PH2 oligonucleotides have hybridized, and
 - i) detecting the label of the third probe.
88. The method of claim 87, wherein step a) comprises providing a plurality of first probes, each of which targets a genomic locus of interest; wherein step e) comprises contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell, and wherein the method further comprises the steps of:
- j) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,
 - k) contacting the cell with each labeled readout probe, and
 - l) detecting each label of each readout probe.
89. The method of claim 88, wherein the plurality of readout probes are labeled with a plurality of dyes.
90. The method of claim 88, wherein the plurality of readout probes are labeled with the same dye.

91. The method of claim 89 or 90, wherein the dye(s) are fluorescent dye(s).
92. The method of any one of claims 88 to 91, wherein each of said plurality of first oligonucleotides comprises one or more readout probe binding sites each selectively bound by one of the plurality of labeled readout probes.
93. The method of claim 92 wherein the one or more readout probe binding sites comprises a nucleotide sequence of
AATCGATCCACTACCGTCGATCCCGTGTCC (SEQ ID NO: 9), the reverse complement of SEQ ID NO: 9, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9.
94. The method of claim 92 or 93, wherein the one or more readout probe binding sites comprises a nucleotide sequence of
AACCGGTACATGACGCGGAACCTAAGGTCG (SEQ ID NO: 10), the reverse complement of SEQ ID NO: 10, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10.
95. The method of any one of claims 92 to 94, wherein the one or more readout probe binding sites comprises a nucleotide sequence of
TAGCAAAGCCGGTAGCGACAACCGTTTCCC (SEQ ID NO: 11), the reverse complement of SEQ ID NO: 11, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11.
96. The method of any one of claims 87 to 95, wherein the chromatin modification is a DNA modification, a DNA-binding protein, or a modification to a DNA-binding protein.
97. The method of claim 96, wherein the chromatin modification is a histone modification.
98. The method of any one of claims 87 to 97, wherein the cell is fixed.
99. The method of any one of claims 87 to 98, wherein the genomic locus of interest is disposed within the nucleus of the cell.
100. The method of any one of claims 87 to 99, wherein the method further comprises determining in three dimensions a location of the third probe.

101. The method of claim 100, wherein the method further comprises using the location of the third probe to analyze chromatin structure.
102. The method of any one of claims 88 to 101, wherein the method further comprises determining in three dimensions a location of the readout probe.
103. The method of claim 102, wherein the method further comprises using the location of the readout probe to analyze chromatin structure.
104. The method of any one of claims 87 to 103, wherein the antibody is coupled to the PH2 oligonucleotide by a biotin-streptavidin bridge.
105. The method of any one of claims 87 to 103, wherein the antibody is coupled to the PH2 oligonucleotide by a covalent bond.
106. The method of any one of claims 87 to 105, wherein coupling of the antibody to the PH2 oligonucleotide comprises nucleotide hybridization.
107. The method of any one of claims 87 to 106, wherein the H1 oligonucleotide is labeled with a first dye.
108. The method of claim 107 wherein the first dye is a fluorescent dye.
109. The method of claim 107 or claim 108, wherein the first dye is Alexa Fluor 647.
110. The method of any one of claims 87 to 109, wherein coupling of the first probe to the PH1 oligonucleotide comprises nucleotide hybridization.
111. The method of any one of claims 87 to 109, wherein coupling of the first probe to the PH1 oligonucleotide comprises a covalent bond.
112. The method of any one of claims 87 to 111, wherein the H1 oligonucleotide forms a hairpin loop structure.
113. The method of any one of claims 87 to 112, wherein a signal generated by the third probe is amplified through sequential hybridization comprising binding a fourth probe comprising a hybridization 2 (H2) oligonucleotide to the H1 oligonucleotide, wherein said H2 oligonucleotide is labeled.
114. The method of any one of claims 87 to 113, wherein the H1 oligonucleotide comprises a nucleotide sequence of
ACAGACGACTCCCACATTCTCCAGGTGGGAGTCGTCTGTAACATGAAGTA
(SEQ ID NO: 6), the reverse complement of SEQ ID NO: 6, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 6, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence

- identity to SEQ ID NO: 6.
115. The method of any one of claims 87 to 113, wherein the H1 oligonucleotide comprises a nucleotide sequence of
GCGAATCAGTCAGACGTACCTCATGTCTGACTGATTCGCAACTCCCTCTA
(SEQ ID NO: 3857), the reverse complement of SEQ ID NO: 3857, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3857, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3857.
116. The method of any one of claims 113 to 115, wherein the H2 oligonucleotide forms a hairpin loop structure.
117. The method of any one of claims 113 to 114 and 116, wherein the H2 oligonucleotide comprises a nucleotide sequence of
CTGGAGAATGTGGGAGTCGTCTGTTACTTCATGTTACAGACGACTCCCAC
(SEQ ID NO: 8), the reverse complement of SEQ ID NO: 8, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 8, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 8.
118. The method of any one of claims 113 and 115 to 116, wherein the H2 oligonucleotide comprises a nucleotide sequence of
ATGAGGTACGTCTGACTGATTCGCTAGAGGGAGTTGCGAATCAGTCAGAC
(SEQ ID NO: 3858), the reverse complement of SEQ ID NO: 3858, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3858, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3858.
119. The method of any one of claims 113 to 118, wherein the H2 oligonucleotide is labeled with a second dye.
120. The method of claim 119 wherein the second dye is a fluorescent dye.
121. The method of claim 119 or claim 120, wherein the second dye is Alexa Fluor 647.
122. The method of any one of claims 87 to 121, wherein the PH1 oligonucleotide and the PH2 oligonucleotide each form hairpin loop structures.
123. The method of any one of claims 87 to 114, 116-117, and 119-122, wherein the

- PH1 oligonucleotide comprises a nucleotide sequence of
AAAAATCGTCTGTGGCATGAAGGCCCGCTGTATTCAGTGAATGCGAGTCAG
ACGAATACAGCGGGCCTTCATGCCACAGACGA (SEQ ID NO: 4), the reverse
complement of SEQ ID NO: 4, a nucleotide sequence with at least 75%
sequence identity to SEQ ID NO: 4, or a nucleotide sequence complementary to
a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4.
124. The method of any one of claims 87 to 113, 115-116 and 119 to 122, wherein
the PH1 oligonucleotide comprises a nucleotide sequences of
AAAAATGATTCGCTTCTCCCTCAGAGTGGTGTGCAGAACTGTGCGAGAGTC
GATGCACACCACTCTGAGGGAGAAGCGAATCA (SEQ ID NO: 3854), the
reverse complement of SEQ ID NO: 3854, a nucleotide sequence with at least
75% sequence identity to SEQ ID NO: 3854, or a nucleotide sequence
complementary to a nucleotide sequence with at least 75% sequence identity to
SEQ ID NO: 3854.
125. The method of any one of claims 87 to 114, 116-117, and 119-123, wherein the
PH2 oligonucleotide comprises a nucleotide sequence of
AAAAAGTGGGAGTCGTCTGTAACATGAAGGCCCGCTGTATTCGTCTTACTT
CATGTTACAGACGACTCCAC (SEQ ID NO: 2), the reverse complement of
SEQ ID NO: 2, a nucleotide sequence with at least 75% sequence identity to
SEQ ID NO: 2, or a nucleotide sequence complementary to a nucleotide
sequence with at least 75% sequence identity to SEQ ID NO: 2.
126. The method of any one of claims 87 to 113, 115-116, 119 to 122 and 124,
wherein the PH2 oligonucleotide comprises a nucleotide sequence of
AAAAAGTCTGACTGATTCGCAACTCCCTCAGAGTGGTGTGCATCGATAGAG
GGAGTTGCGAATCAGTCAGAC (SEQ ID NO: 3855), the reverse complement
of SEQ ID NO: 3855, a nucleotide sequence with at least 75% sequence identity
to SEQ ID NO: 3855, or a nucleotide sequence complementary to a nucleotide
sequence with at least 75% sequence identity to SEQ ID NO: 3855.
127. The method of any one of claims 87 to 126, wherein a plurality of PH1
oligonucleotides are coupled to the first oligonucleotide through a linker
oligonucleotide.
128. The method of claim 127, wherein four PH1 oligonucleotides are coupled to the

first oligonucleotide.

129. The method of claim 127 or claim 128, wherein the linker oligonucleotide comprises a nucleotide sequence of
GCTATCGTTCGTTTCGAGGCCAGAGCATTTCGGCTATCGTTCGTTTCGAGGCCA
GAGCATTTCGGCTATCGTTCGTTTCGAGGCCAGAGCATTTCGGCTATCGTTCGT
TCGAGGCCAGAGCATTTCGCGCAACGCTTGGGACGGTTCCAATCGGATC
(SEQ ID NO: 12), the reverse complement of SEQ ID NO: 12, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 12, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 12.
130. The method of any one of claims 127 to 129, wherein the first oligonucleotide is coupled to the linker oligonucleotide through nucleotide hybridization.
131. The method of any one of claims 127 to 129, wherein the first oligonucleotide is coupled to the linker oligonucleotide through a covalent bond.
132. The method of any one of claims 87 to 131, wherein step g) further comprises, the activator oligonucleotide hybridizing with the PH1 oligonucleotide and causing the first hairpin loop structure to open, a portion of the PH1 oligonucleotide being made available by the opening of the first hairpin loop structure subsequently hybridizing to the PH2 oligonucleotide and causing the second hairpin loop structure to open and make available said sequence made available when said PH1 and PH2 oligonucleotides have hybridized.
133. The method of any one of claims 87 to 132, wherein the activator oligonucleotide comprises a nucleotide sequence of
GACTCGCATTCACTGAATACAGCGGGCCTTCATGCCACAGACGA (SEQ ID NO: 1), the reverse complement of SEQ ID NO: 1, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 1, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 1 when the PH1 oligonucleotide comprises a nucleotide sequence of
AAAAATCGTCTGTGGCATGAAGGCCCGCTGTATTCAGTGAATGCGAGTCAG
ACGAATACAGCGGGCCTTCATGCCACAGACGA (SEQ ID NO: 4), the reverse

- complement of SEQ ID NO: 4, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 4.
134. The method of any one of claims 87 to 132, wherein the activator oligonucleotide comprises a nucleotide sequence of
 CTCTCGCACAGTTCTGCACACCACTCTGAGGGAGAAGCGAATCA (SEQ ID NO: 3856), the reverse complement of SEQ ID NO: 3856, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3856, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3856 when the PH1 oligonucleotide comprises a nucleotide sequences of
 AAAAATGATTGCTTCTCCCTCAGAGTGGTGTGCAGAACTGTGCGAGAGTC
 GATGCACACCACTCTGAGGGAGAAGCGAATCA (SEQ ID NO: 3854), the reverse complement of SEQ ID NO: 3854, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3854.
135. The method of any one of claims 87 to 134, wherein
 step a) comprises providing a plurality of first probes each comprising a first oligonucleotide coupled to a unique proximity hybridization (PH1) oligonucleotide,
 step d) comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the unique PH1 oligonucleotides of said plurality of first probes,
 step h) comprises contacting the cell with each third probe of the plurality of third probes, and
 step i) comprises detecting each label of each third probe.
136. The method of claim 135, wherein the antibody is coupled to a plurality of unique PH2 oligonucleotides, wherein each unique PH2 oligonucleotide comprises a nucleotide sequence capable of binding to one of the unique PH1 oligonucleotides, thereby causing a first hairpin loop structure formed by the unique PH1 oligonucleotide to open.

137. The method of claim 136, wherein the antibody is coupled to the plurality of unique PH2 oligonucleotides through nucleotide hybridization of unique PH2 oligonucleotides to an antibody linker oligonucleotide that is covalently coupled to the antibody.
138. The method of claim 136 or 137, wherein the method further comprises providing a plurality of unique activator oligonucleotides, wherein each unique activator oligonucleotide is capable of binding to one of the unique PH2 oligonucleotide and thereby causing a second hairpin loop structure formed by the unique PH2 oligonucleotide to open and make available said nucleotide sequence capable of binding to one of the unique PH1 oligonucleotides.
139. The method of any one of claims 87 to 133, wherein
step b) comprises providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a unique chromatin modification or set of chromatin modifications of interest,
step d) comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes,
step h) comprises contacting the cell with each third probe of the plurality of third probes, and
step i) comprises detecting each label of each third probe.
140. The method of claim 139, wherein the first oligonucleotide is coupled to a plurality of unique PH1 oligonucleotides, wherein each unique PH1 oligonucleotide comprises a nucleotide sequence capable of binding to one of the unique PH2 oligonucleotides, thereby causing a second hairpin loop structure formed by the unique PH2 oligonucleotide to open.
141. The method of claim 140, wherein the first oligonucleotide is coupled to the plurality of unique PH1 oligonucleotides through nucleotide hybridization to a linker oligonucleotide.
142. The method of claim 140 or 141, wherein the method further comprises providing a plurality of unique activator oligonucleotides, wherein each unique activator oligonucleotide is capable of binding to one of the unique PH1 oligonucleotide

- and thereby causing a first hairpin loop structure formed by the unique PH1 oligonucleotide to open and make available said nucleotide sequence capable of binding to one of the unique PH2 oligonucleotides.
143. The method of any one of claims 139 to 142, wherein each of said plurality of second probes comprises a unique antibody covalently coupled to an antibody linker oligonucleotide, and wherein a unique PH2 oligonucleotide is coupled to the antibody through nucleotide hybridization to the antibody linker oligonucleotide.
144. The method of any one of claims 139 to 143, wherein the plurality of third probes is labeled with a plurality of dyes.
145. The method of any one of claims 139 to 143, wherein the plurality of third probes is labeled with the same dye.
146. The method of claim 144 or 145, wherein the dye(s) are fluorescent dye(s).
147. The method of any one of claims 87 to 146, wherein the first oligonucleotide is coupled to a plurality of PH1 oligonucleotides by branched amplification.
148. The method of any one of claims 87 to 147, wherein the antibody is coupled to a plurality of PH2 oligonucleotides by branched amplification.
149. The method of any one of claims 87 to 148, wherein the PH1 oligonucleotide, the PH2 oligonucleotide, and the H1 oligonucleotide each comprise DNA.
150. The method of any one of claims 87 to 149, wherein a signal generated by the third probe is amplified through branched amplification.
151. The method of any one of claims 87 to 150, wherein the antibody is biotinylated.
152. The method of any one of claims 87 to 151, wherein the PH2 oligonucleotide is biotinylated.
153. The method of any one of claims 87 to 152, wherein the cell is a mammalian cell.
154. The method of any one of claims 87 to 153, wherein the first probe does not comprise biotin.
155. The method of any one of claims 87 to 154, wherein the method further comprises quantitating an epigenetic modification level of the genomic locus of interest.
156. The method of any one of claims 87 to 155, wherein the method further comprises identifying each of the plurality of first probes using a barcoding

scheme.

157. The method of any one of claims 87 to 156, wherein step e) precedes step f).
158. The method of any one of claims 87 to 156, wherein steps e) and f) take place simultaneously.
159. The method of any one of claims 87 to 156, wherein step f) precedes step e).
160. The method of any one of claim 88 to 159, wherein steps g), h) and i) precede steps k) and l).
161. The method of any one of claim 88 to 159, wherein steps g), h) and i) take place simultaneously with steps k) and l).
162. The method of any one of claim 88 to 159, wherein steps k) and l) proceed steps g), h) and i).
163. The method of any one of claims 85 to 162, wherein the method is carried out at a genomic locus within the cell.
164. The method of any one of claims 85 to 162, wherein the method is carried out at a genomic locus on a chromosome spread, extracted chromatin or extracted DNA outside of the cell or extra-cellular DNA.
165. A kit for *in situ* visualization of a chromatin modification of a cell according to the method of any one of claims 87 to 164, the kit comprising the first probe, the second probe, the activator oligonucleotide, and the third probe.
166. The kit of claim 165, wherein the kit comprises one or more said labeled readout probes.
167. A method for *in situ* visualization of a chromatin modification at a genomic locus in a cell comprising the steps of:
 - a) providing at least one first probe comprising a first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein said first oligonucleotide binds to a genomic locus of interest,
 - b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest,
 - c) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is coupled to a label,
 - d) contacting the cell with the first probe under conditions that allow binding

- of said first oligonucleotide of said first probe to said genomic locus of the cell,
- e) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
 - f) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, wherein when the H1 oligonucleotide binds to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, the 3' end of the PH1 oligonucleotide is disposed proximal to the 5' end of the PH2 oligonucleotide or the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide, and
 - g) detecting the label coupled to the third probe.
168. The method of claim 167, wherein step a) comprises providing a plurality of first probes, each of which targets a genomic locus of interest; wherein step d) comprises contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell, and wherein the method further comprises the steps of:
- h) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,
 - i) contacting the cell with each labeled readout probe, and
 - j) detecting each label of each readout probe.
169. The method of claim 168, wherein the plurality of readout probes are labeled with a plurality of dyes.
170. The method of claim 168, wherein the plurality of readout probes are labeled with the same dye.
171. The method of claim 169 or 170, wherein the dye(s) are fluorescent dye(s).
172. The method of any one of claims 168 to 171, wherein each of said plurality of first oligonucleotides comprises one or more readout probe binding sites each

- selectively bound by one of the plurality of labeled readout probes.
173. The method of claim 172 wherein one of the readout probe binding sites comprises a nucleotide sequence of AATCGATCCACTACCGTCGATCCCGTGTCC (SEQ ID NO: 9), the reverse complement of SEQ ID NO: 9, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 9.
174. The method of claim 172 or 173, wherein one of the readout probe binding sites comprises a nucleotide sequence of AACCGGTACATGACGCGGAACCTAAGGTCG (SEQ ID NO: 10), the reverse complement of SEQ ID NO: 10, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 10.
175. The method of any one of claims 172 to 174, wherein one of the readout probe binding sites comprises a nucleotide sequence of TAGCAAAGCCGGTAGCGACAACCGTTTCCC (SEQ ID NO: 11), the reverse complement of SEQ ID NO: 11, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 11.
176. The method of any one of claims 167 to 175, wherein the chromatin modification is a DNA modification, a DNA-binding protein, or a modification to a DNA-binding protein.
177. The method of claim 176, wherein the chromatin modification is a histone modification.
178. The method of any one of claims 167 to 177, wherein the cell is fixed.
179. The method of any one of claims 167 to 178, wherein the genomic locus of interest is disposed within the nucleus of the cell.
180. The method of any one of claims 167 to 179, wherein the method further comprises determining in three dimensions a location of the third probe.
181. The method of claim 180, wherein the method further comprises using the location of the third probe to analyze chromatin structure.
182. The method of any one of claims 168 to 181, wherein the method further

- comprises determining in three dimensions a location of the readout probe.
183. The method of claim 182, wherein the method further comprises using the location of the readout probe to analyze chromatin structure.
184. The method of any one of claims 167 to 183, wherein the antibody is coupled to the PH2 oligonucleotide by a biotin-streptavidin bridge.
185. The method of any one of claims 167 to 183, wherein the antibody is coupled to the PH2 oligonucleotide by a covalent bond.
186. The method of any one of claims 167 to 185, wherein coupling of the antibody to the PH2 oligonucleotide comprises nucleotide hybridization.
187. The method of claim 185, wherein the antibody is coupled to the PH2 oligonucleotide through a DBCO-mediated copper-free click reaction.
188. The method of any one of claims 167 to 187, wherein the H1 oligonucleotide is coupled to a first dye.
189. The method of claim 188 wherein the first dye is a fluorescent dye.
190. The method of claim 188 or claim 189, wherein the first dye is Alexa Fluor 647.
191. The method of any one of claims 21 to 190, wherein the H1 oligonucleotide is labeled with or coupled to the first dye by being bound by a readout probe comprising the first dye.
192. The method of any one of claims 21 to 190, wherein the H1 oligonucleotide is labeled with or coupled to the first dye by a covalent bond.
193. The method of any one of claims 167 to 190, wherein coupling of the first probe to the PH1 oligonucleotide comprises nucleotide hybridization.
194. The method of any one of claims 167 to 190, wherein coupling of the first probe to the PH1 oligonucleotide comprises a covalent bond.
195. The method of any one of claims 167 to 194, wherein the H1 oligonucleotide forms a hairpin loop structure.
196. The method of any one of claims 167 to 195, wherein a signal generated by the third probe is amplified through sequential hybridization comprising binding a fourth probe comprising a hybridization 2 (H2) oligonucleotide to the H1 oligonucleotide, wherein said H2 oligonucleotide is labeled.
197. The method of claim 196, wherein the H2 oligonucleotide is labeled with a second dye.

198. The method of claim 197, wherein the second dye is a fluorescent dye.
199. The method of claim 197 or claim 198, wherein the second dye is Alexa Fluor 647.
200. The method of any one of claims 33 to 199, wherein the H2 oligonucleotide is labeled with the second dye by being bound by a readout probe comprising the second dye.
201. The method of any one of claims 33 to 199, wherein the H2 oligonucleotide is labeled with the second dye by a covalent bond.
202. The method of any one of claims 196 to 201, wherein the H2 oligonucleotide forms a hairpin loop structure.
203. The method of any one of claims 167 to 202, wherein the H1 oligonucleotide comprises a nucleotide sequence of
AGATATAGAGGCAGGGACCGGGTTAAAGTTGAGTGGCCAGTCTAATAACCA
CTCAACTTTAACCCGGTCCCT (SEQ ID NO: 7), the reverse complement of
SEQ ID NO: 7, a nucleotide sequence with at least 75% sequence identity to
SEQ ID NO: 7, or a nucleotide sequence complementary to a nucleotide
sequence with at least 75% sequence identity to SEQ ID NO: 7.
204. The method of any one of claims 167 to 203, wherein the PH1 oligonucleotide comprises a nucleotide sequence of
CGCAACGCTTGGGACGGTTCCAATCGGATCTTCCACTCAACTTTAACCCG
(SEQ ID NO: 5), the reverse complement of SEQ ID NO: 5, a nucleotide
sequence with at least 75% sequence identity to SEQ ID NO: 5, or a nucleotide
sequence complementary to a nucleotide sequence with at least 75% sequence
identity to SEQ ID NO: 5.
205. The method of any one of claims 167 to 204, wherein the PH2 oligonucleotide comprises a nucleotide sequence of
GTCCCTGCCTCTATATCTTTAATCCGGCGTACGTAAGGCAGCTTGCGTTA
(SEQ ID NO: 3), the reverse complement of SEQ ID NO: 3, a nucleotide
sequence with at least 75% sequence identity to SEQ ID NO: 3, or a nucleotide
sequence complementary to a nucleotide sequence with at least 75% sequence
identity to SEQ ID NO: 3.
206. The method of any one of claims 167 to 205, wherein the method further

- comprises covalently coupling the PH1 oligonucleotide to the PH2 oligonucleotide through a click reaction.
207. The method of claim 206, wherein the click reaction is copper-catalyzed.
208. The method of claim 207, wherein the PH1 oligonucleotide is azide-modified and the PH2 oligonucleotide is hexynyl-modified, or the PH1 oligonucleotide is hexynyl-modified and the PH2 oligonucleotide is azide-modified.
209. The method of any one of claims 167 to 205, wherein the method further comprises covalently coupling the PH1 oligonucleotide to the PH2 oligonucleotide through an enzymatic ligation reaction.
210. The method of claim 209, wherein the PH2 oligonucleotide comprises a phosphate modification located at its 5' end.
211. The method of claim 209 or claim 210, wherein the 5' phosphate-modified PH2 oligonucleotide comprises a nucleotide sequence of
GTCCCTGCCTCTATATCTTTAATCCGGCGTACGTAAGGCAGCTTGCGTTA
(SEQ ID NO: 3), the reverse complement of SEQ ID NO: 3, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3.
212. The method of claim 209, wherein the PH1 oligonucleotide comprises a phosphate modification located at its 5' end.
213. The method of claim 212, wherein the 5' phosphate-modified PH1 oligonucleotide comprises a nucleotide sequence of
GTCCCTGCCTCTATATCTTTGCAACGCTTGGGACGGTTCCAATCGGATC
(SEQ ID NO: 3860), the reverse complement of SEQ ID NO: 3860, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3860, or a nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3860.
214. The method of claim 213, wherein the PH2 oligonucleotide comprises a nucleotide sequence of
AATCCGGCGTACGTAAGGCAGCTTGCGTTATTCCACTCAACTTTAACCCG
(SEQ ID NO: 3861), the reverse complement of SEQ ID NO: 3861, a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3861, or a

- nucleotide sequence complementary to a nucleotide sequence with at least 75% sequence identity to SEQ ID NO: 3861.
215. The method of any one of claims 209 to 214, wherein the enzymatic ligation reaction is catalyzed by a T4 DNA ligase, T7 DNA ligase, T3 DNA ligase, Taq DNA ligase, Ampligase, *E. coli* DNA ligase, or SplintR ligase.
216. The method of any one of claims 167 to 215, wherein
step b) comprises providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a unique chromatin modification or set of chromatin modifications of interest,
step c) comprises providing a plurality of third probes, each of which comprises a unique H1 oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes,
step f) comprises contacting the cell with each third probe of the plurality of third probes, and
step i) comprises detecting each label coupled to each third probe.
217. The method of claim 216, wherein each third probe is coupled to a label by being bound by a labeled readout probe.
218. The method of claim 216 or 217, wherein the first oligonucleotide is coupled to a plurality of unique PH1 oligonucleotides, wherein each unique H1 oligonucleotide selectively binds to a sequence of each of one of the unique PH2 oligonucleotides and one of the unique PH1 oligonucleotides, wherein when each unique H1 oligonucleotide binds to a nucleotide sequence of each of said one of the unique PH1 and PH2 oligonucleotides, the 3' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 5' end of the one of the unique PH2 oligonucleotides or the 5' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 3' end of the one of the unique PH2 oligonucleotides.
219. The method of any one of claims 216 to 218, wherein the first oligonucleotide is coupled to the plurality of unique PH1 oligonucleotides through nucleotide hybridization to a linker oligonucleotide.
220. The method of any one of claims 216 to 219, wherein the plurality of third probes

- is coupled to a plurality of dyes.
221. The method of any one of claims 216 to 219, wherein the plurality of third probes is coupled to the same dye.
222. The method of claim 220 or 221, wherein the dye(s) are fluorescent dye(s).
223. The method of any one of claims 216 to 222, wherein each unique PH1 oligonucleotide is covalently coupled to a corresponding unique PH2 oligonucleotide by a click reaction.
224. The method of any one of claims 216 to 222, wherein each unique PH1 oligonucleotide is covalently coupled to a corresponding unique PH2 oligonucleotide by an enzymatic ligation reaction.
225. The method of any one of claims 167 to 224, wherein the PH1 oligonucleotide, the PH2 oligonucleotide, and the H1 oligonucleotide each comprise DNA.
226. The method of any one of claims 167 to 225, wherein a signal generated by the third probe is amplified through branched amplification.
227. The method of any one of claims 167 to 226, wherein the antibody is biotinylated.
228. The method of any one of claims 167 to 227, wherein the PH2 oligonucleotide is biotinylated.
229. The method of any one of claims 167 to 228, wherein the cell is a mammalian cell.
230. The method of any one of claims 167 to 229, wherein the first probe does not comprise biotin.
231. The method of any one of claims 167 to 230, wherein the method further comprises quantitating an epigenetic modification level of the genomic locus of interest.
232. The method of any one of claims 168 to 231, wherein the method further comprises identifying each of the plurality of first probes using a barcoding scheme.
233. The method of any one of claims 167 to 232, wherein step d) precedes step e).
234. The method of any one of claims 167 to 232, wherein steps d) and e) take place simultaneously.
235. The method of any one of claims 167 to 232, wherein step e) precedes step d).

236. The method of any one of claims 167 to 235, wherein steps f) and g) precede steps i) and j).
237. The method of any one of claims 167 to 235, wherein steps f) and g) take place simultaneously with steps i) and j).
238. The method of any one of claims 167 to 235, wherein steps i) and j) precede step f) and g).
239. The method of any one of claims 167 to 238, wherein the method is carried out at a genomic locus within the cell.
240. The method of any one of claims 167 to 238, wherein the method is carried out at a genomic locus on a chromosome spread, extracted chromatin or extracted DNA outside of the cell or extra-cellular DNA.
241. A kit for *in situ* visualization of a chromatin modification of a cell according to the method of any one of claims 167 to 240, the kit comprising the first probe, the second probe, and the third probe.
242. The kit of claim 241, wherein the kit comprises one or more said labeled readout probes.
243. A method of *in situ* visualization of a chromatin modification of a cell at a plurality of genomic loci comprising the steps of:
- a) providing a plurality of first probes each comprising a unique first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein each said unique first oligonucleotide binds to a genomic locus of interest, and wherein said PH1 oligonucleotide forms a first hairpin loop structure,
 - b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest, and wherein said PH2 oligonucleotide forms a second hairpin loop structure,
 - c) providing an activator oligonucleotide, wherein the activator oligonucleotide is capable of binding to either the PH1 oligonucleotide or the PH2 oligonucleotide, wherein binding of the activator oligonucleotide with the PH1 oligonucleotide causes the first hairpin loop structure to open, and wherein binding of the activator oligonucleotide with the PH2

- oligonucleotide causes the second hairpin loop structure to open,
 - d) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is labeled,
 - e) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said unique first oligonucleotides of the said plurality of first probes,
 - f) contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell,
 - g) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
 - h) contacting the cell with the activator oligonucleotide under conditions that allow binding of said activator oligonucleotide to said PH1 oligonucleotide or said PH2 oligonucleotide, wherein said binding of said activator causes PH1 and PH2 to hybridize,
 - i) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to a sequence made available when said PH1 and PH2 oligonucleotides have hybridized,
 - j) detecting the label of the third probe,
 - k) contacting the cell with each labeled readout probe, and
 - l) detecting each label of each readout probe.
244. A method of *in situ* visualization of a plurality of chromatin modifications at a genomic locus of a cell comprising the steps of:
- a) providing at least one first probe comprising a first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides, wherein said first oligonucleotide binds to a genomic locus of interest, and wherein each of said plurality of unique PH1 oligonucleotides forms a hairpin loop structure,
 - b) providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each of said unique antibodies recognizes a unique chromatin

- modification or set of chromatin modifications of interest, and wherein each of said PH2 oligonucleotides forms a hairpin loop structure,
- c) providing a plurality of unique activator oligonucleotides, wherein each unique activator oligonucleotide is capable of binding to a corresponding one of the plurality of unique PH1 or PH2 oligonucleotides, and wherein binding of one of the plurality of unique activator oligonucleotides with the corresponding one of the plurality of PH1 or PH2 oligonucleotides causes the hairpin loop structure formed by the one of the plurality of PH1 or PH2 oligonucleotides to open,
 - d) providing a plurality of third probes each comprising a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes or to one of the unique PH1 oligonucleotides, wherein each said unique H1 oligonucleotides is labeled,
 - e) contacting the cell with the first probe under conditions that allow binding of said first oligonucleotide of said first probe to said genomic locus of the cell,
 - f) contacting the cell with the plurality of second probes under conditions that allow each of said unique antibodies of said plurality of second probes to bind to said unique chromatin modification or set of chromatin modifications,
 - g) contacting the cell with each unique activator oligonucleotide under conditions that allow binding of each said unique activator oligonucleotide to said corresponding one of the plurality of PH1 or PH2 oligonucleotides, wherein said binding of said activator causes one of the unique PH1 oligonucleotides and one of the unique PH2 oligonucleotides to hybridize,
 - h) contacting the cell with each of the plurality of third probes under conditions that allow binding of the unique H1 oligonucleotide of each said third probe to a sequence made available when one of the plurality of unique PH1 oligonucleotides and one of the plurality of unique PH2 oligonucleotides have hybridized, wherein the one of the plurality of unique PH2 or PH1 oligonucleotides comprises said sequence made

available, and

- i) detecting the label of each of the third probes.

245. A method for *in situ* visualization of a plurality of chromatin modifications at a plurality of genomic loci in a cell comprising the steps of:

- a) providing a plurality of first probes each comprising a unique first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides, wherein each said unique first oligonucleotide binds to a genomic locus of interest, and wherein each of said plurality of unique PH1 oligonucleotides forms a hairpin loop structure,
- b) providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each of said unique antibodies recognizes a unique chromatin modification or set of chromatin modifications of interest, and wherein each of said PH2 oligonucleotides forms a hairpin loop structure,
- c) providing a plurality of unique activator oligonucleotides, wherein each unique activator oligonucleotide is capable of binding to a nucleotide sequence of one of the plurality of unique PH1 or PH2 oligonucleotides, and wherein binding of one of the plurality of unique activator oligonucleotides with a corresponding one of the plurality of unique PH1 or PH2 oligonucleotides causes the hairpin loop structure formed by the unique PH1 or PH2 oligonucleotide to open,
- d) providing a plurality of third probes each comprising a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes or to one of the unique PH1 oligonucleotides, wherein each said unique H1 oligonucleotides is labeled,
- e) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said unique first oligonucleotides of the said plurality of first probes,
- f) contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell,

- g) contacting the cell with the plurality of second probes under conditions that allow each of said unique antibodies of said plurality of second probes to bind to said unique chromatin modification or set of chromatin modifications,
 - h) contacting the cell with each unique activator oligonucleotide under conditions that allow binding of each said unique activator oligonucleotide to the corresponding one of the plurality of PH1 or PH2 oligonucleotides, wherein said binding of said activator causes one of the unique PH1 oligonucleotides and one of the unique PH2 oligonucleotides to hybridize,
 - i) contacting the cell with each of the plurality of third probes under conditions that allow binding of the unique H1 oligonucleotide of each said third probe to a sequence made available when one of the plurality of unique PH1 and one of the plurality of unique PH2 oligonucleotides have hybridized, wherein the one of the plurality of unique PH2 or PH1 oligonucleotides comprises said sequence made available,
 - j) detecting the label of each of the third probes,
 - k) contacting the cell with each labeled readout probe, and
 - l) detecting each label of each readout probe.
246. A method for *in situ* visualization of a chromatin modification at a plurality of genomic loci in a cell comprising the steps of:
- a) providing a plurality of first probes each comprising a unique first oligonucleotide coupled to a proximity hybridization 1 (PH1) oligonucleotide, wherein each said unique first oligonucleotide binds to a genomic locus of interest,
 - b) providing a second probe comprising an antibody coupled to a proximity hybridization 2 (PH2) oligonucleotide, wherein said antibody recognizes a chromatin modification or set of chromatin modifications of interest,
 - c) providing a third probe comprising a hybridization 1 (H1) oligonucleotide, wherein said H1 oligonucleotide is coupled to a label,
 - d) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,

- e) contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell,
 - f) contacting the cell with the second probe under conditions that allow binding of said antibody of said second probe to said chromatin modification or set of chromatin modifications,
 - g) contacting the cell with the third probe under conditions that allow binding of said H1 oligonucleotide of said third probe to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, wherein when the H1 oligonucleotide binds to a nucleotide sequence of each of said PH1 and PH2 oligonucleotides, the 3' end of the PH1 oligonucleotide is disposed proximal to the 5' end of the PH2 oligonucleotide or the 5' end of the PH1 oligonucleotide is disposed proximal to the 3' end of the PH2 oligonucleotide,
 - h) detecting the label coupled to each third probe,
 - i) contacting the cell with each labeled readout probe, and
 - j) detecting each label of each readout probe.
247. A method for *in situ* visualization of a plurality of chromatin modifications at a genomic locus in a cell comprising the steps of:
- a) providing at least one first probe comprising a first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides, wherein said first oligonucleotide binds to a genomic locus of interest,
 - b) providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a chromatin modification or set of chromatin modifications of interest,
 - c) providing a plurality of third probes, each of which comprises a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes and to one of the unique PH1 oligonucleotides, wherein said H1 oligonucleotide is coupled to a label,
 - d) contacting the cell with the first probe under conditions that allow binding

- of said first oligonucleotide of said first probe to said genomic locus of the cell,
- e) contacting the cell with each of the plurality of second probes under conditions that allow binding of said unique antibodies of said second probes to said chromatin modification or set of chromatin modifications,
 - f) contacting the cell with each third probe of the plurality of third probes under conditions that allow binding of each said unique H1 oligonucleotide of each of said plurality of third probes to a nucleotide sequence of each of one of the unique PH2 oligonucleotides and one of the unique PH1 oligonucleotides, wherein when the unique H1 oligonucleotide binds to a nucleotide sequence of each of said one of the unique PH1 and PH2 oligonucleotides, the 3' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 5' end of the one of the unique PH2 oligonucleotides or the 5' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 3' end of the one of the unique PH2 oligonucleotides, and
 - g) detecting each label coupled to each third probe.
248. A method for *in situ* visualization of a plurality of chromatin modifications at a plurality of genomic loci in a cell comprising the steps of:
- a) providing a plurality of first probes each comprising a unique first oligonucleotide coupled to a plurality of unique proximity hybridization 1 (PH1) oligonucleotides, wherein each said unique first oligonucleotide binds to a genomic locus of interest,
 - b) providing a plurality of second probes each comprising a unique antibody coupled to a unique proximity hybridization 2 (PH2) oligonucleotide, wherein each unique antibody recognizes a chromatin modification or set of chromatin modifications of interest,
 - c) providing a plurality of third probes, each of which comprises a unique hybridization 1 (H1) oligonucleotide that selectively binds to one of the unique PH2 oligonucleotides of said plurality of second probes and to one of the unique PH1 oligonucleotides, wherein said H1 oligonucleotide is coupled to a label,

- d) providing a plurality of labeled readout probes, each of which selectively binds to at least one of the plurality of said first oligonucleotides of the said plurality of first probes,
 - e) contacting the cell with each of the plurality of first probes under conditions that allow binding of said first oligonucleotides of said first probes to said genomic loci of the cell,
 - f) contacting the cell with each of the plurality of second probes under conditions that allow binding of said unique antibodies of said second probe to said chromatin modification or set of chromatin modifications,
 - g) contacting the cell with each third probe of the plurality of third probes under conditions that allow binding of each said unique H1 oligonucleotide of each of said plurality of third probes to a nucleotide sequence of each of one of the unique PH2 oligonucleotides and one of the unique PH1 oligonucleotides, wherein when the unique H1 oligonucleotide binds to a nucleotide sequence of each of said one of the unique PH1 and PH2 oligonucleotides, the 3' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 5' end of the one of the unique PH2 oligonucleotides or the 5' end of the one of the unique PH1 oligonucleotides is disposed proximal to the 3' end of the one of the unique PH2 oligonucleotides,
 - h) detecting each label coupled to each third probe,
 - i) contacting the cell with each labeled readout probe, and
 - j) detecting each label of each readout probe.
249. The method of any one of claims 243 to 248, wherein the method is carried out at a genomic locus within the cell.
250. The method of any one of claims 243 to 248, wherein the method is carried out at a genomic locus on a chromosome spread, extracted chromatin or extracted DNA outside of the cell or extra-cellular DNA.
251. The method of any one of claims 243 to 250, wherein the step of contacting the cell with the first probe(s) precedes the step of contacting the cell with the second probe(s).
252. The method of any one of claims 243 to 250, wherein the step of contacting the

cell with the first probe(s) and the step of contacting the cell with the second probe(s) take place simultaneously.

253. The method of any one of claims 243 to 250, wherein the step of contacting the cell with the second probe(s) precedes the step of contacting the cell with the first probe(s).
254. The method of any one of claims 243, 245, 246, 248 to 253, wherein the steps of contacting the cell with the third probe(s) and detecting the label of the third probe(s) precede the steps of contacting the cell with the readout probe(s) and detecting the label of the readout probe(s).
255. The method of any one of claims 243, 245, 246, 248 to 253, wherein the steps of contacting the cell with the third probe(s) and detecting the label of the third probe(s) take place simultaneously with the steps of contacting the cell with the readout probe(s) and detecting the label of the readout probe(s).
256. The method of any one of claims 243, 245, 246, 248 to 253, wherein the steps of contacting the cell with the readout probe(s) and detecting the label of the readout probe(s) precede the steps of contacting the cell with the third probe(s) and detecting the label of the third probe(s).

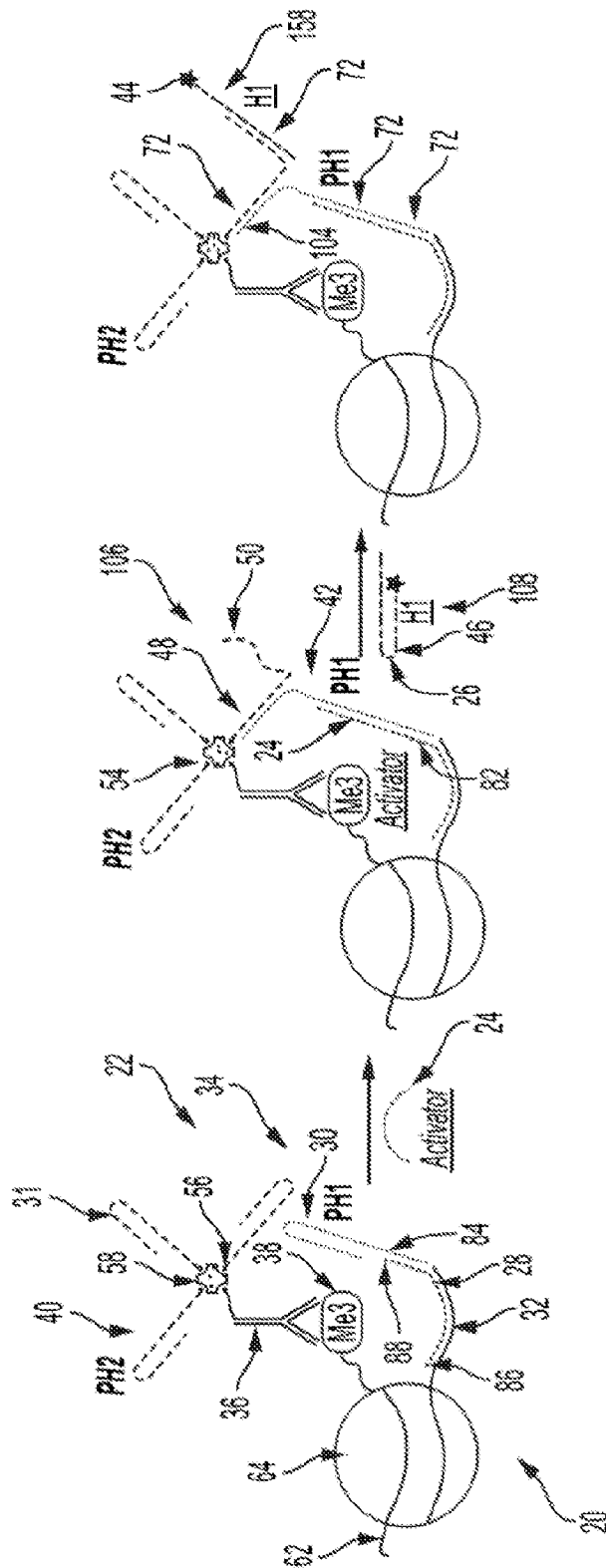


FIG. 1A

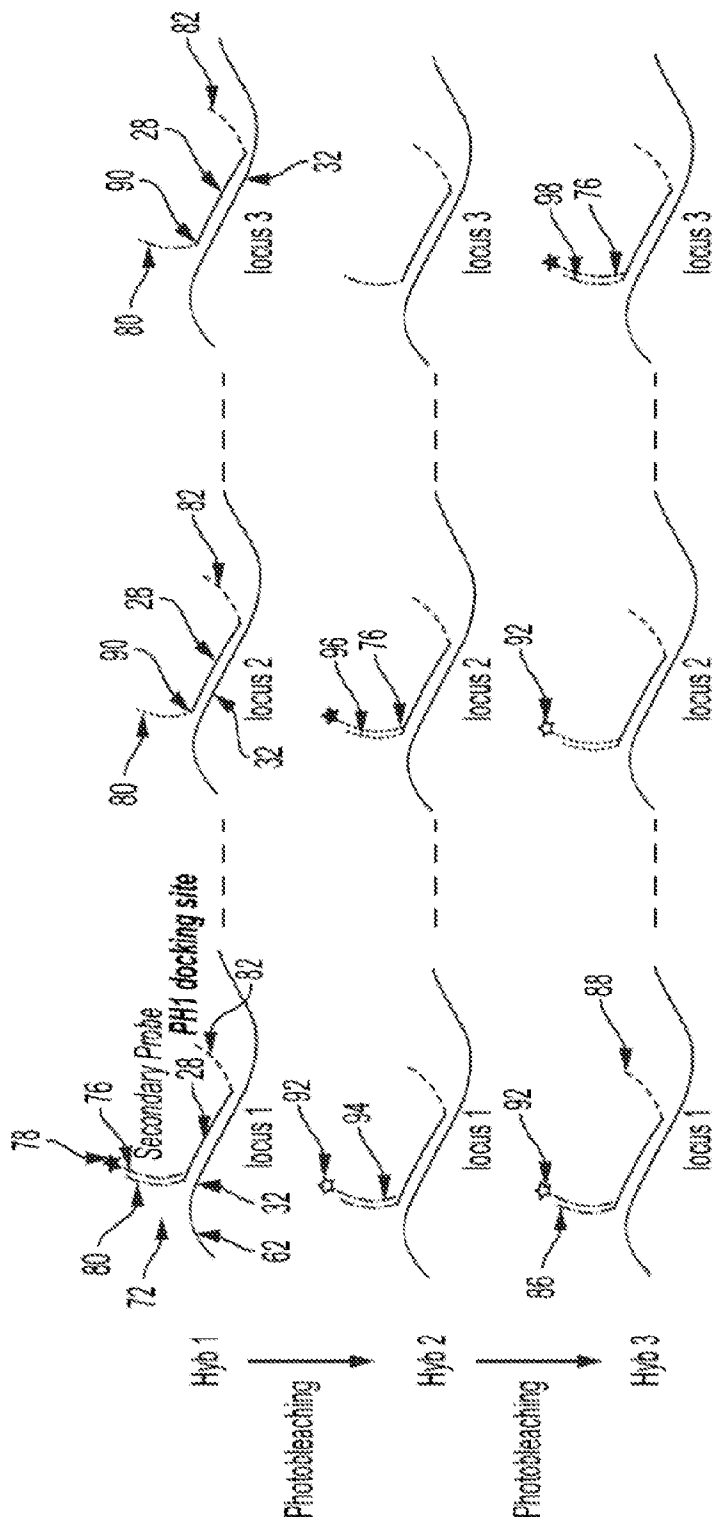


FIG. 1B

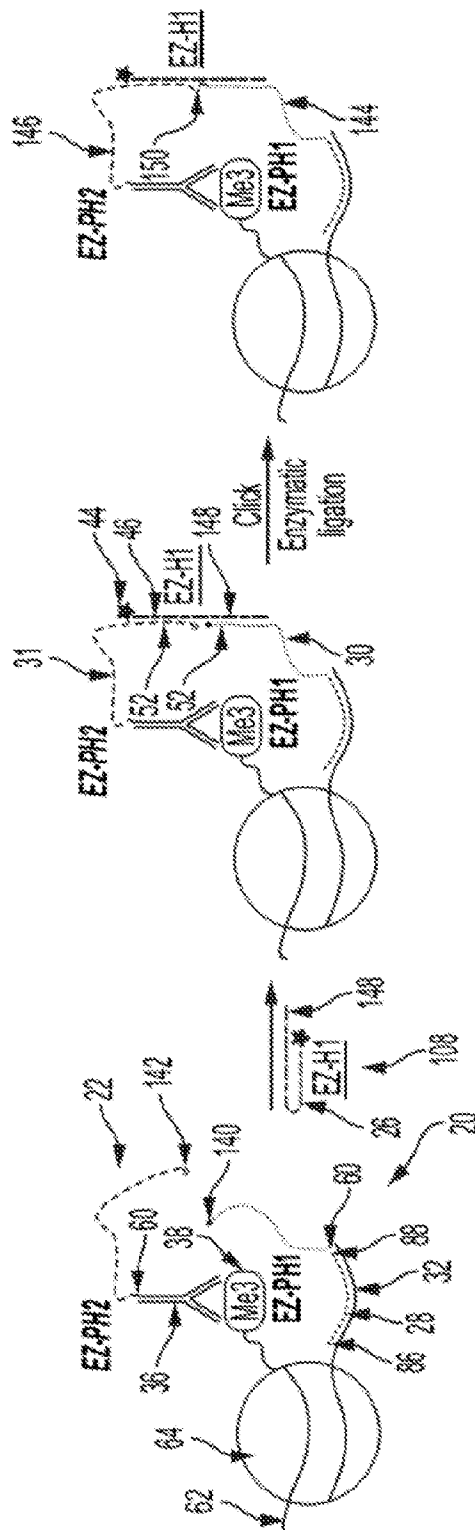


FIG. 1C

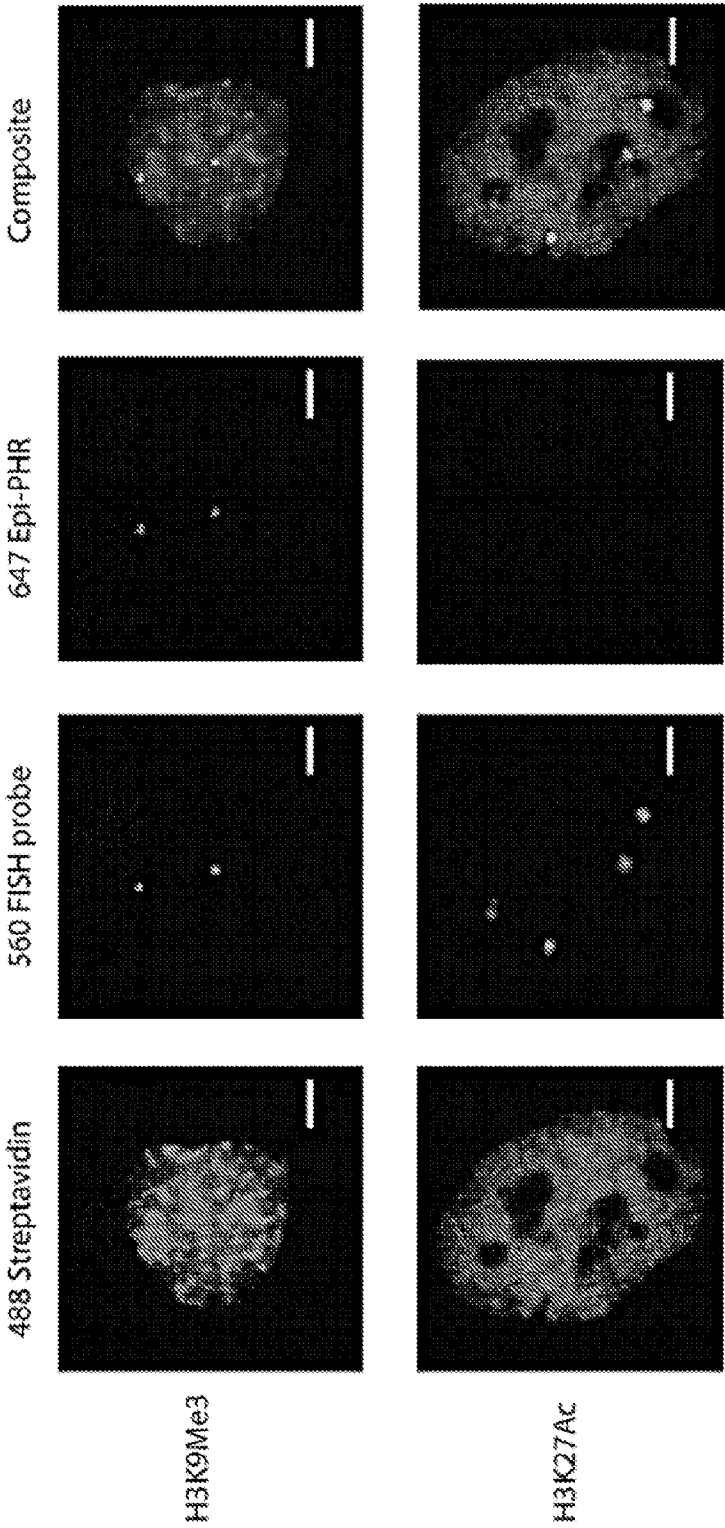


FIG. 2A

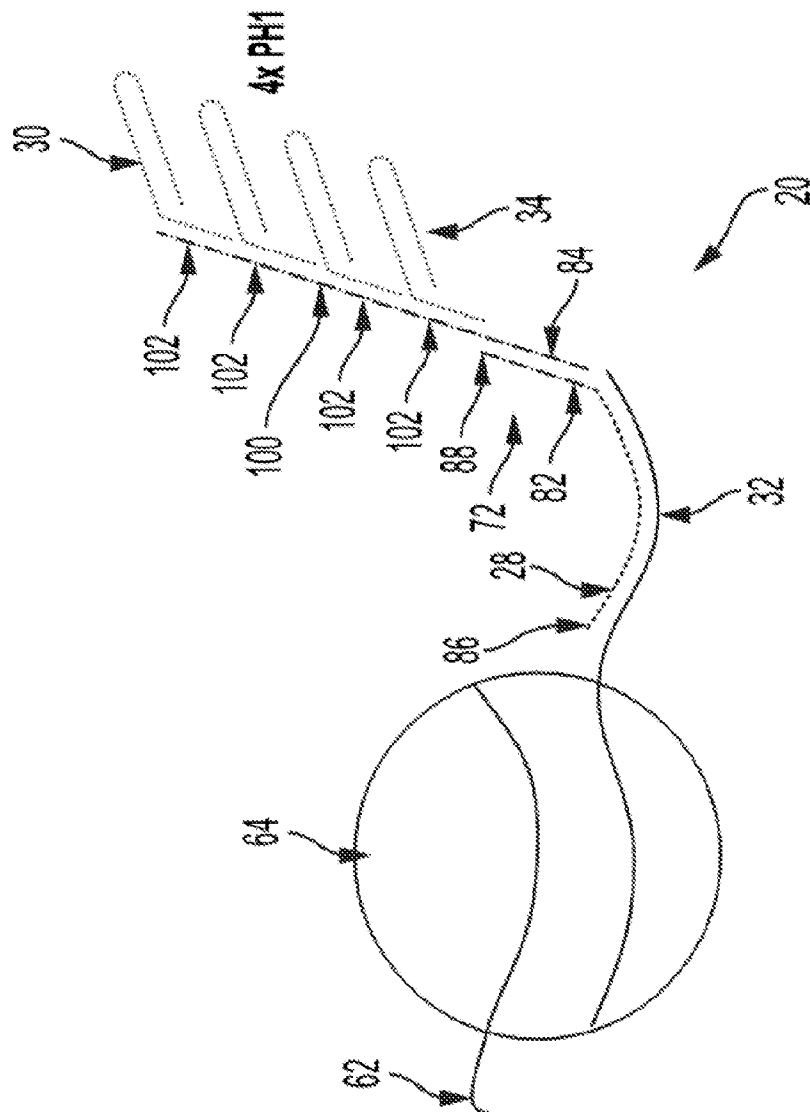


FIG. 2B

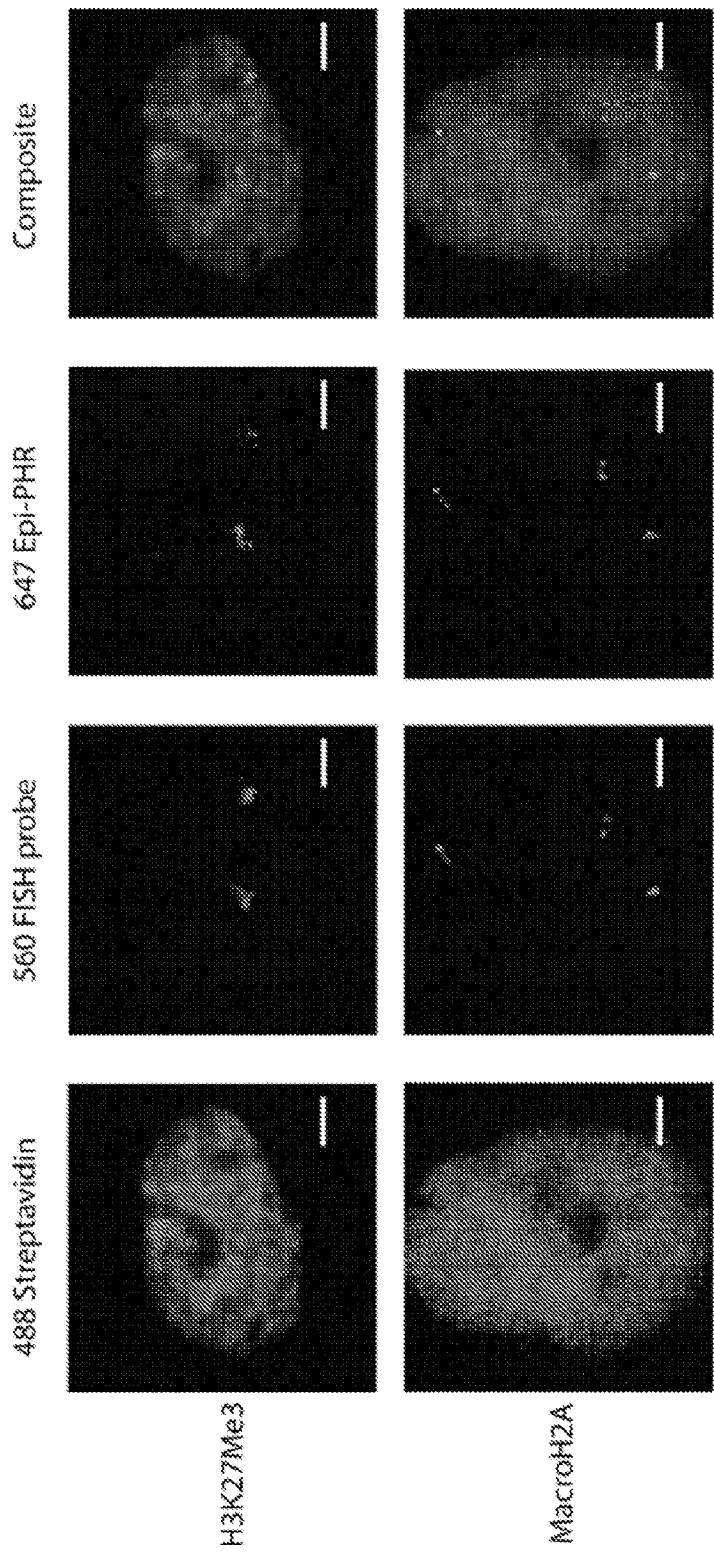


FIG. 2C

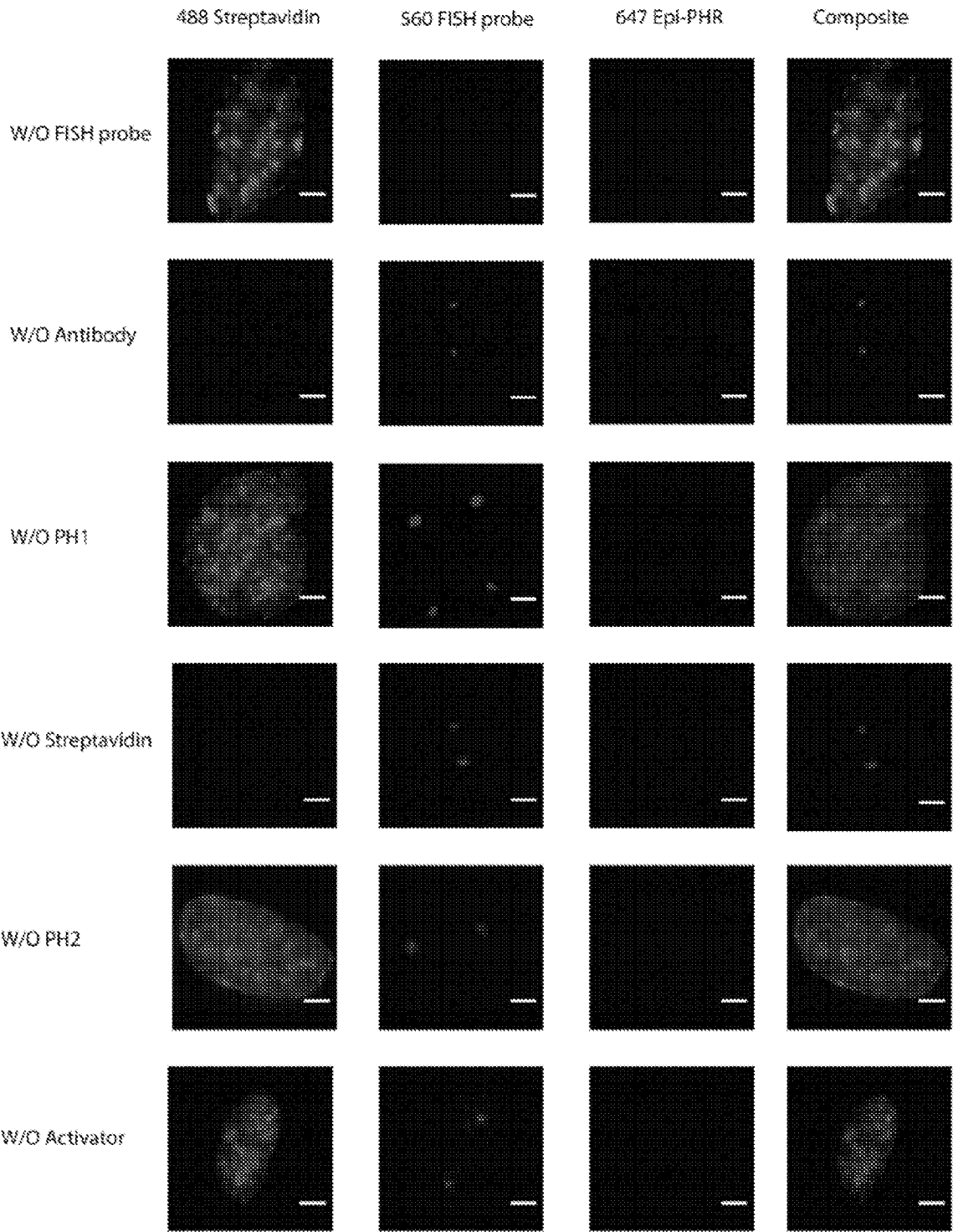


FIG. 3

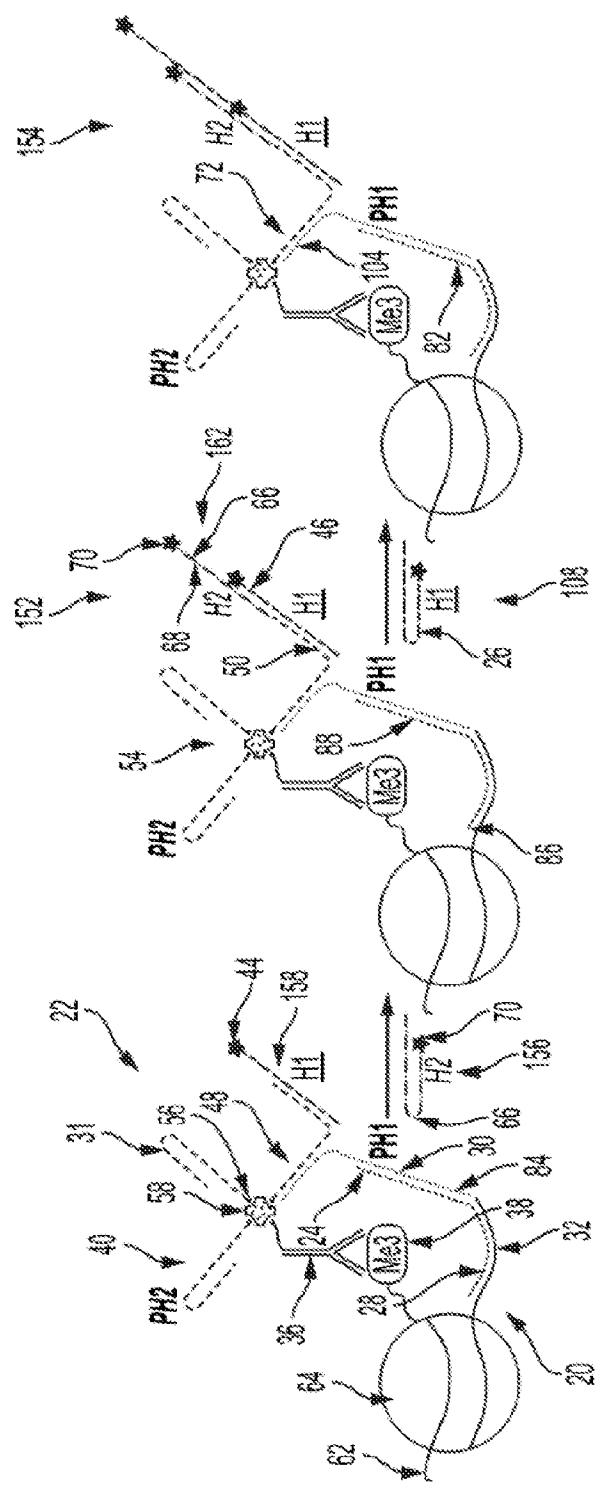


FIG. 4A

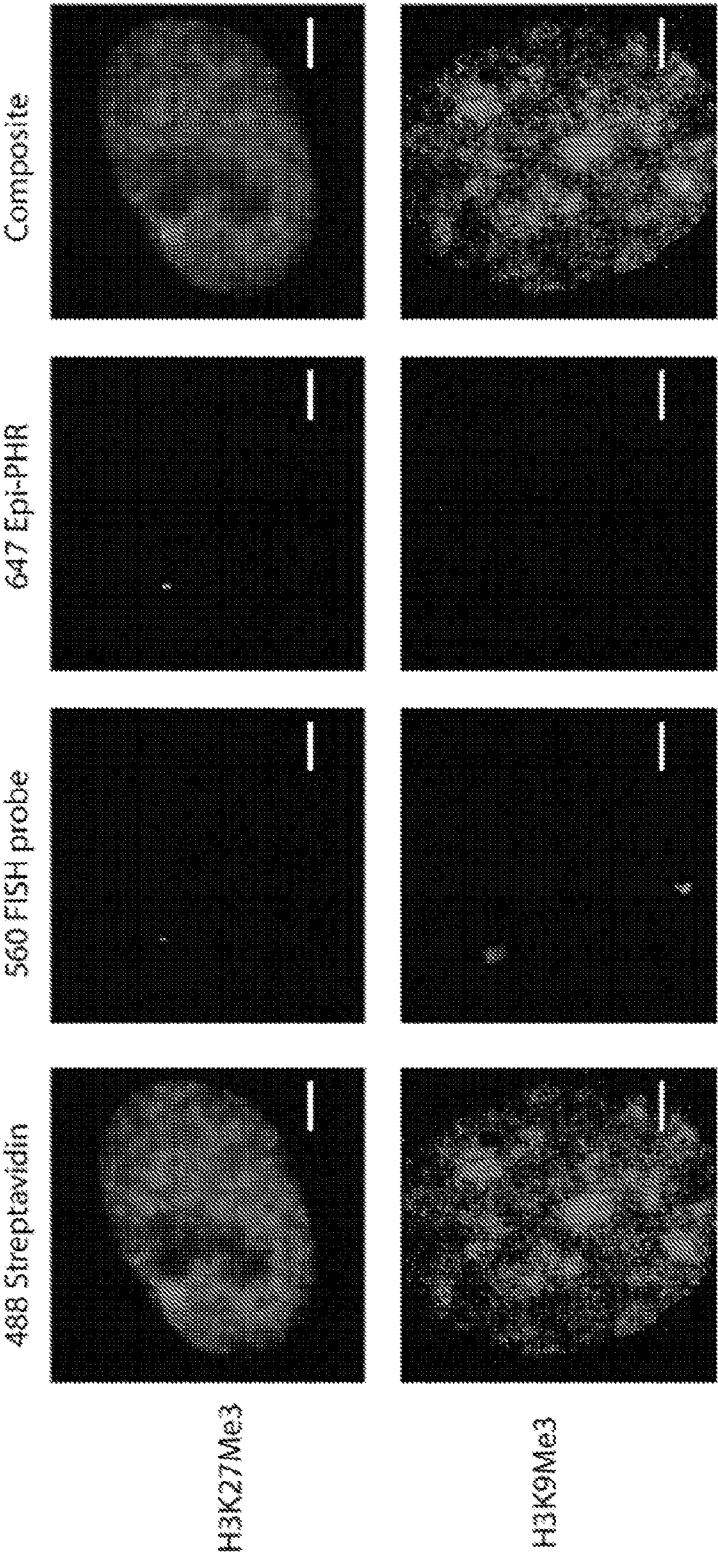


FIG. 4B

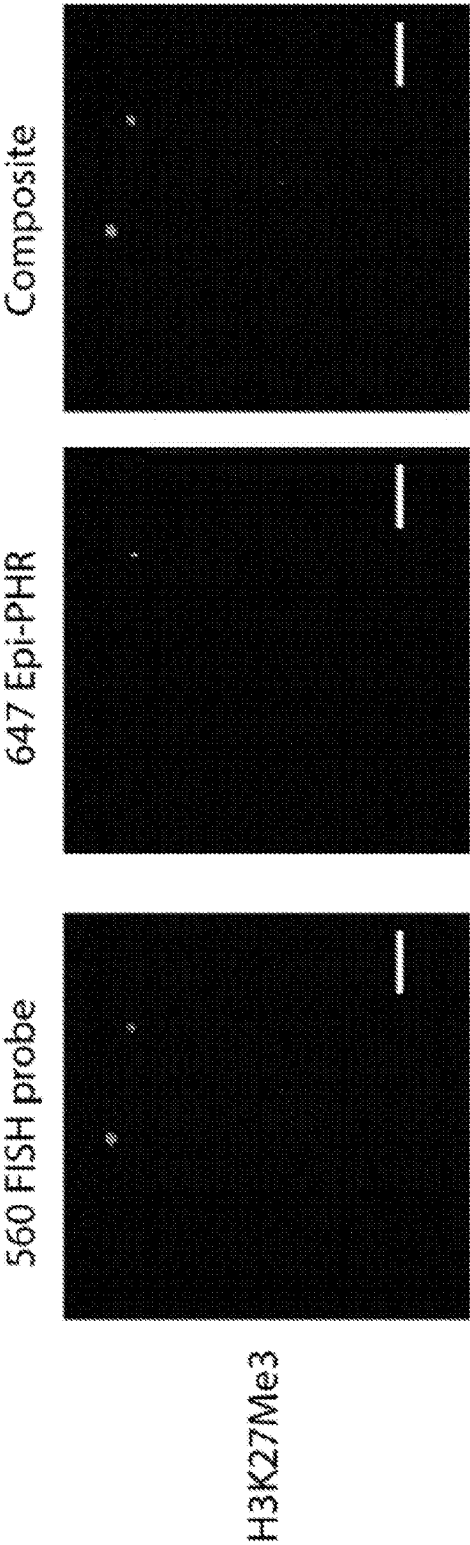


FIG. 4C

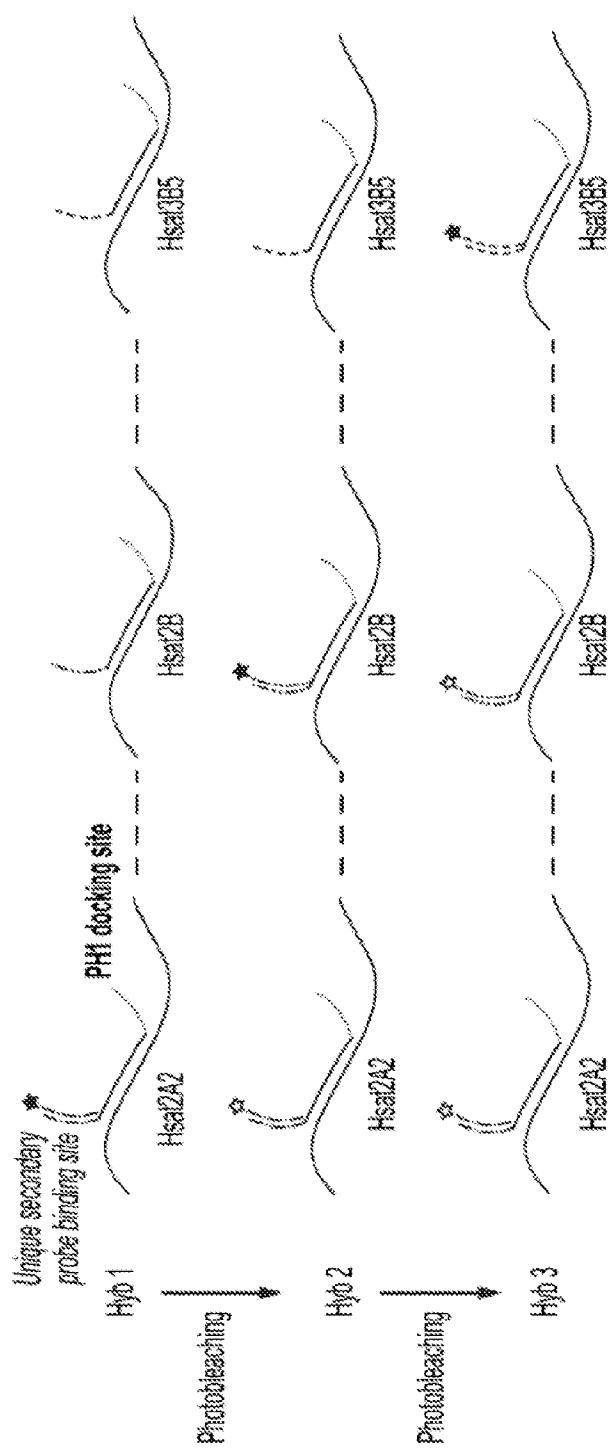


FIG. 5A

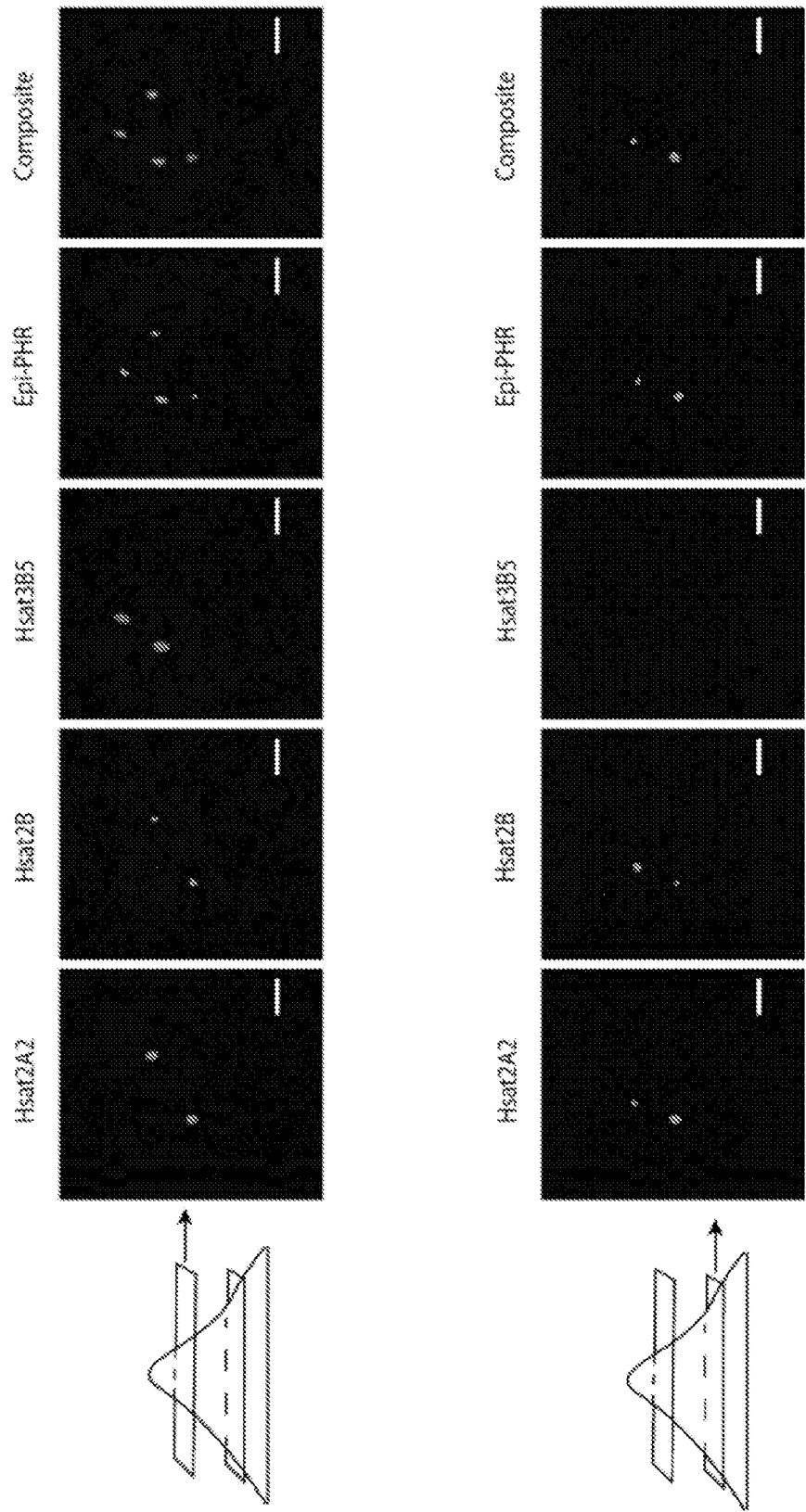


FIG. 5B

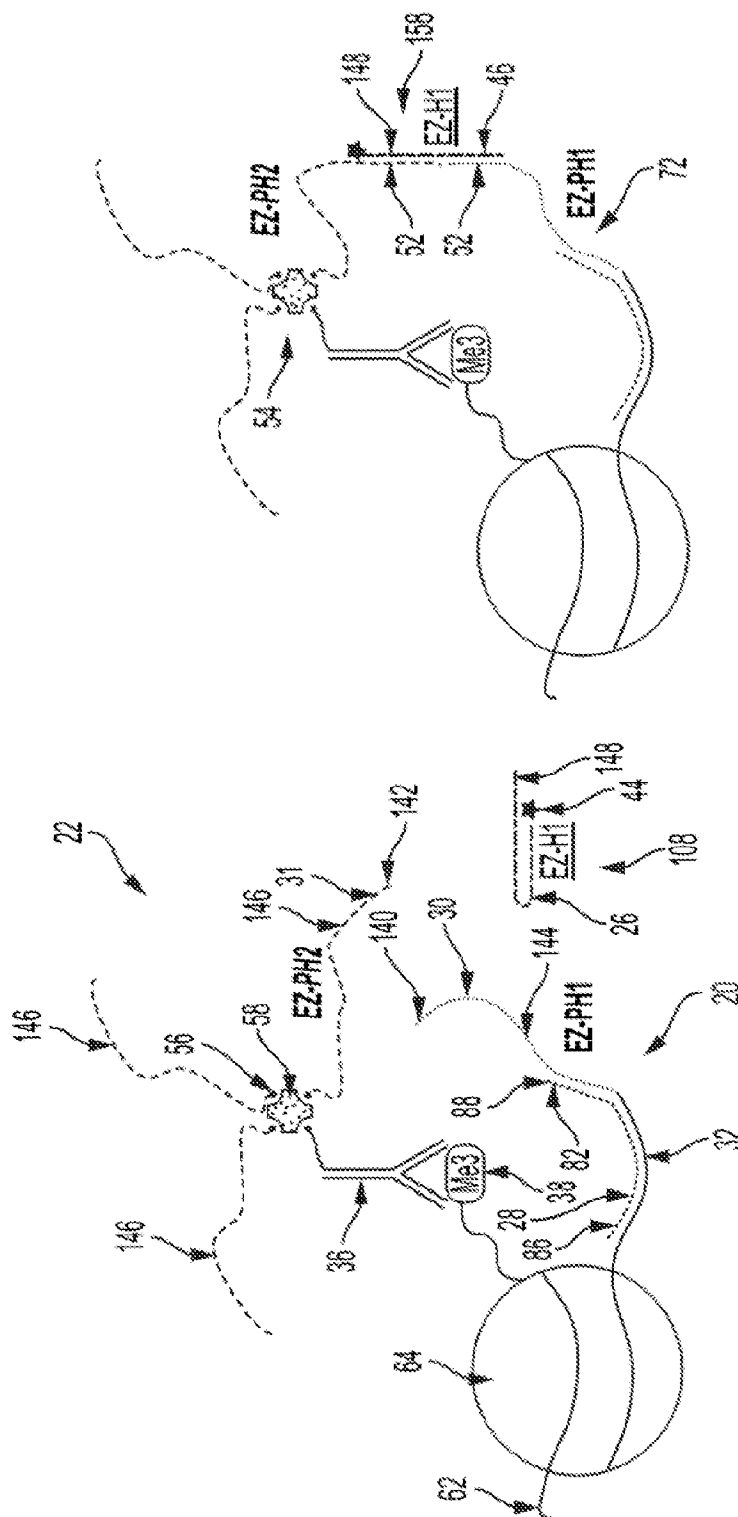


FIG. 6A

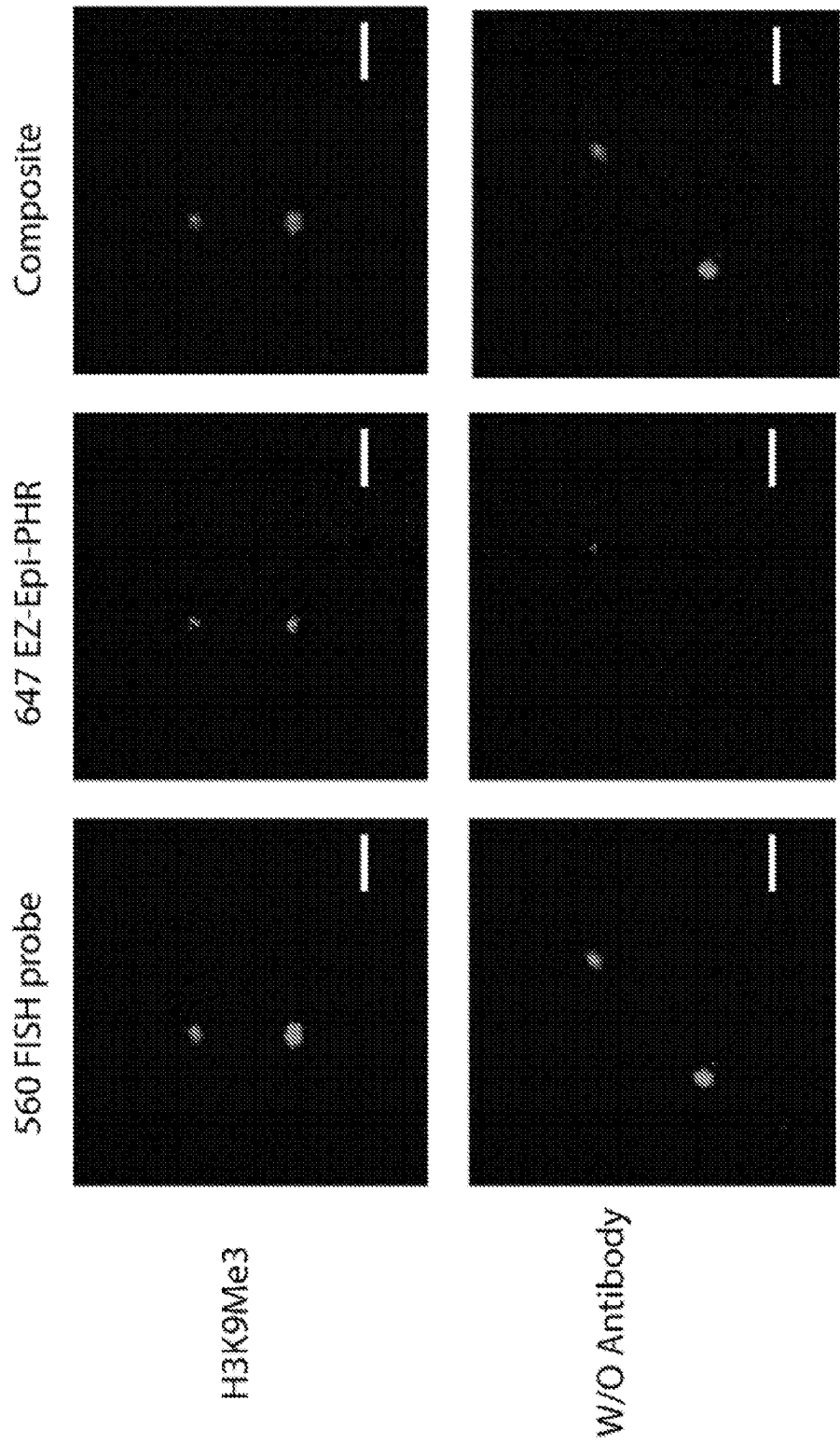


FIG. 6B

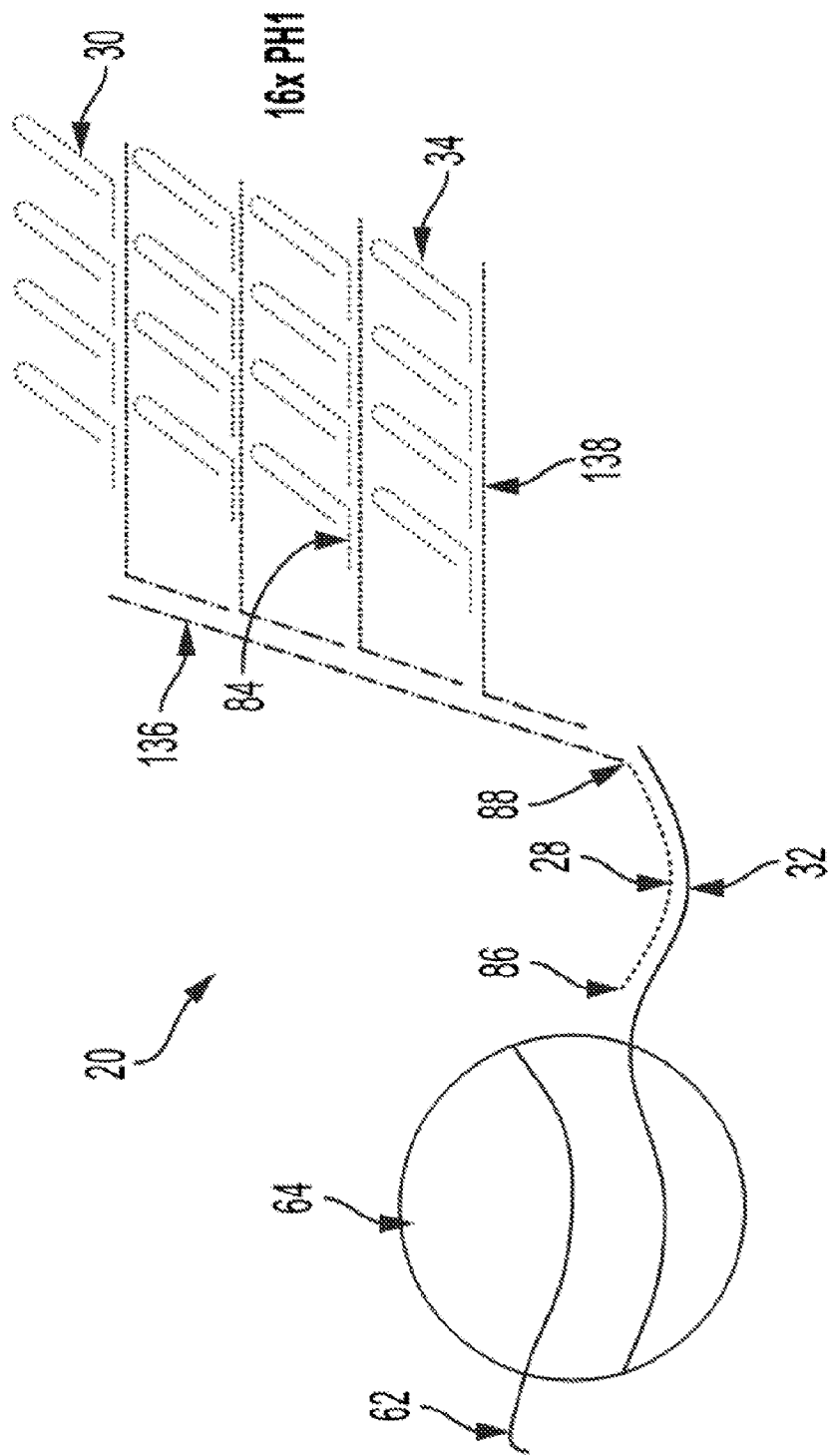


FIG. 7A

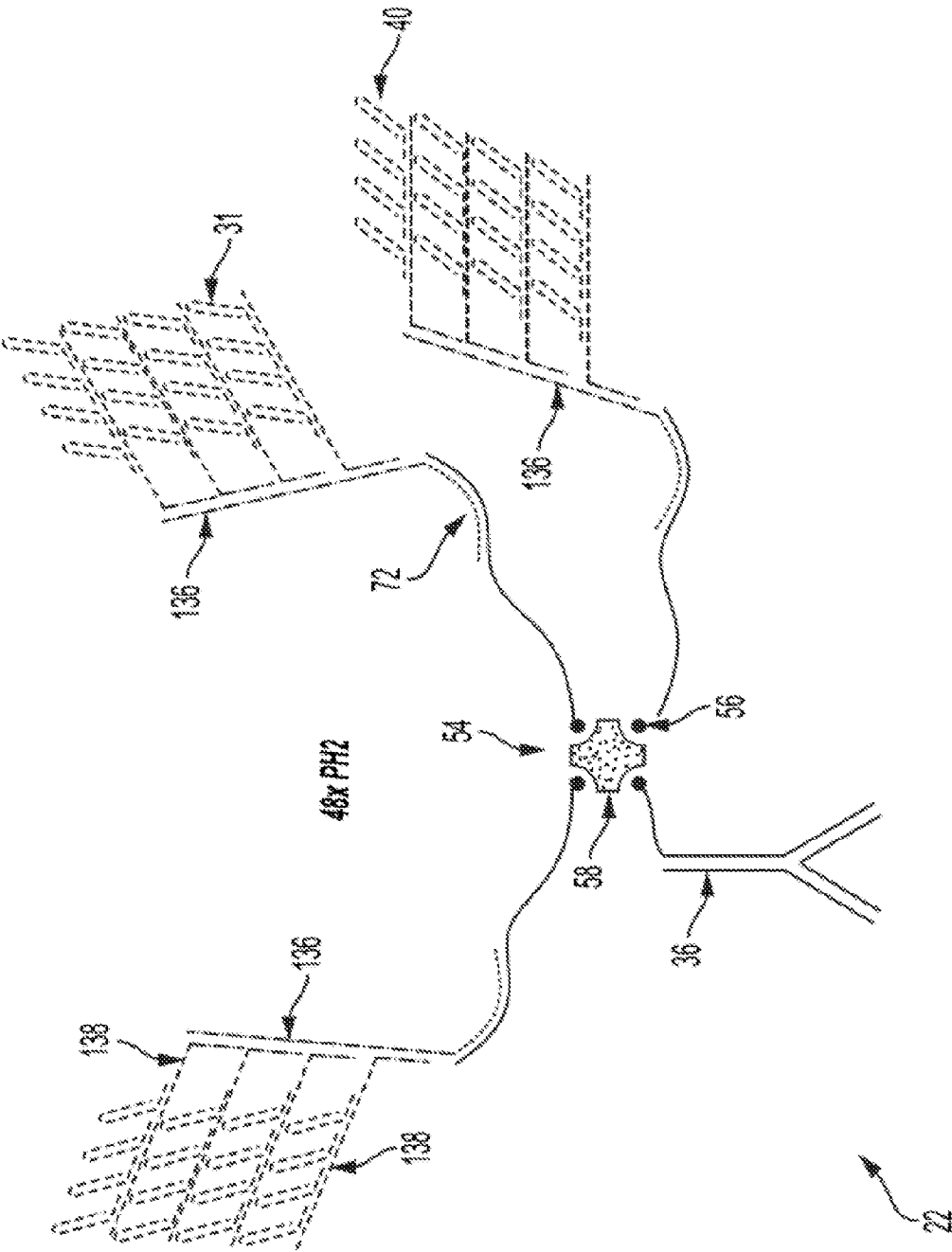


FIG. 7B

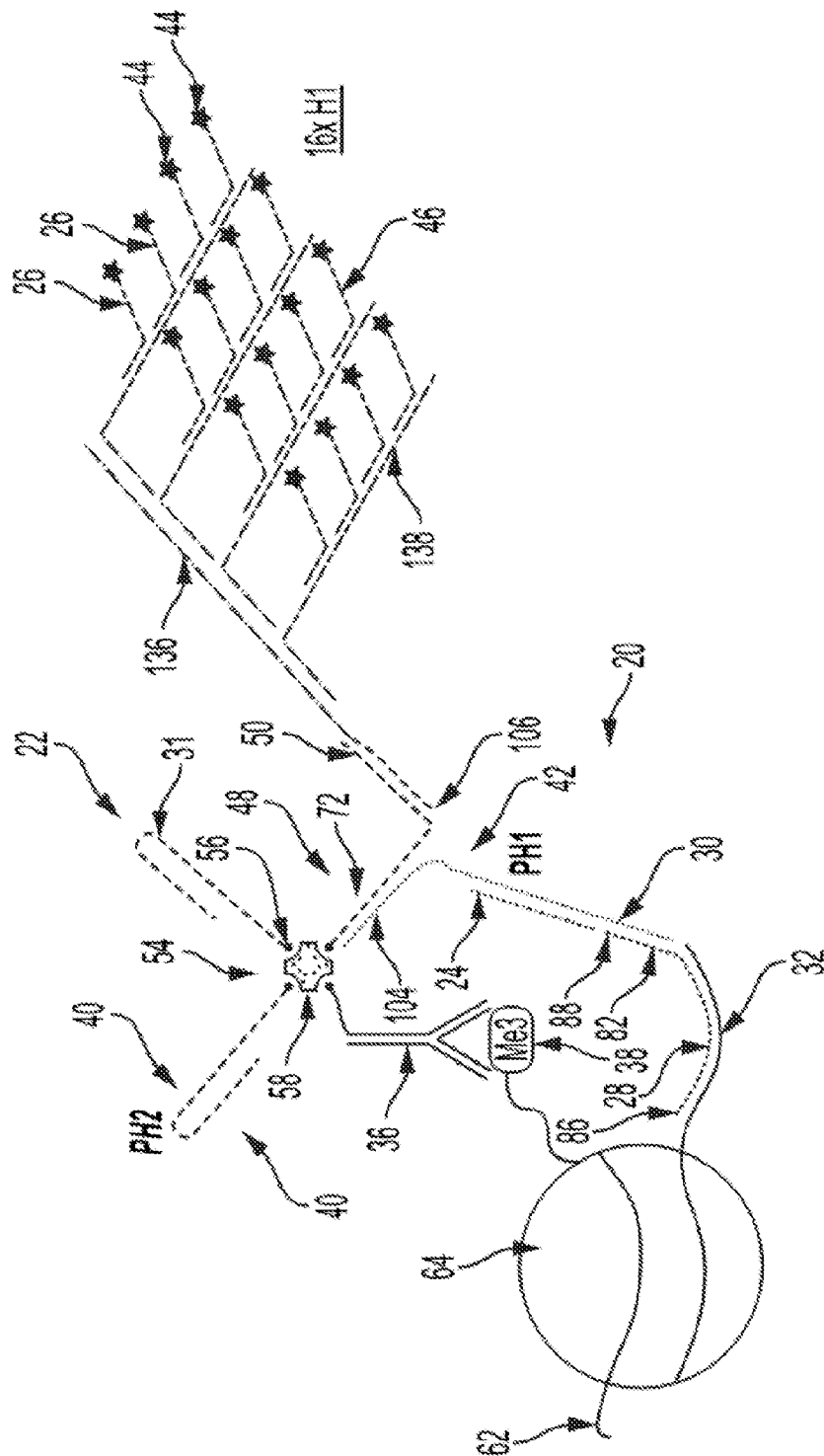


FIG. 7C

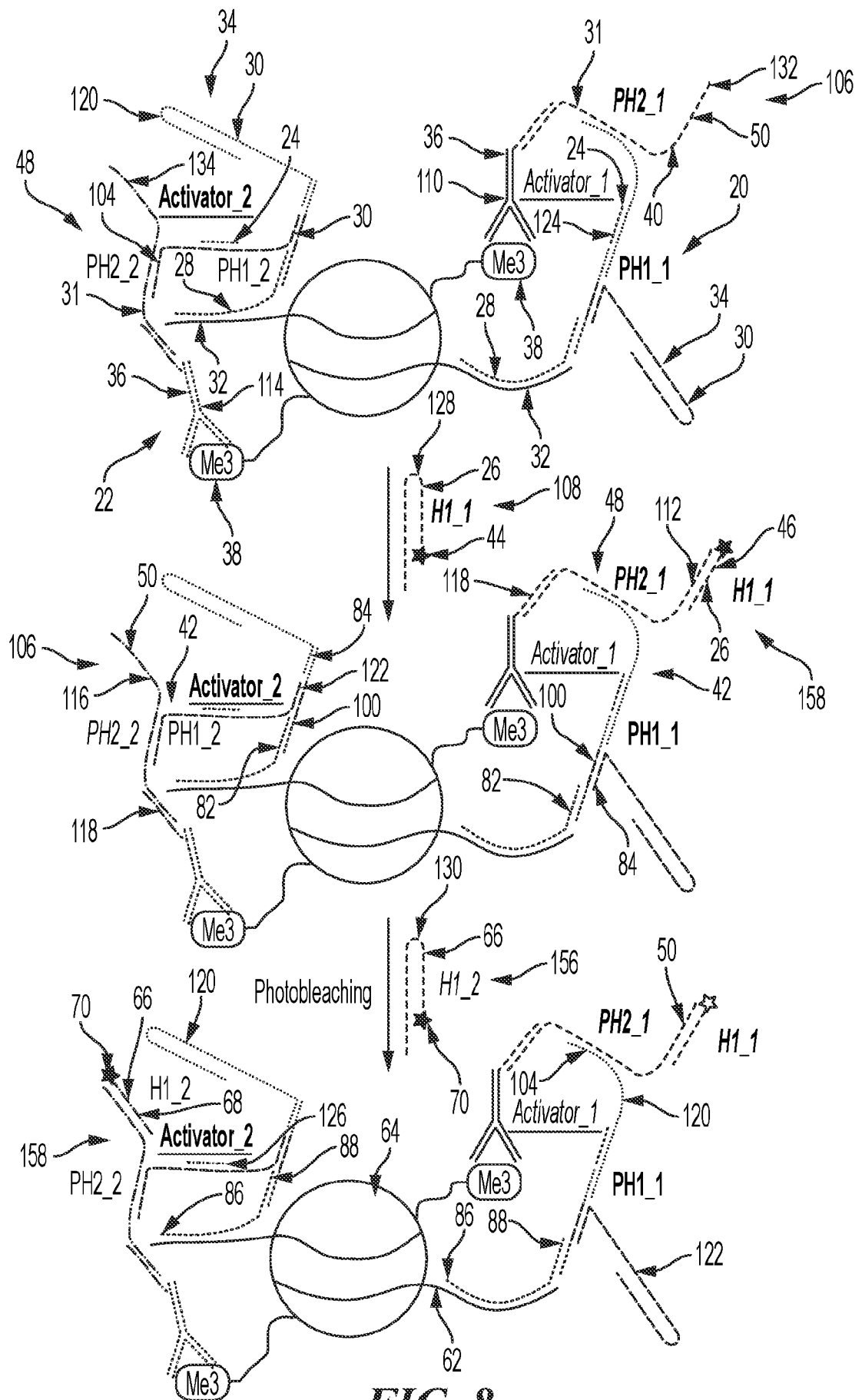


FIG. 8

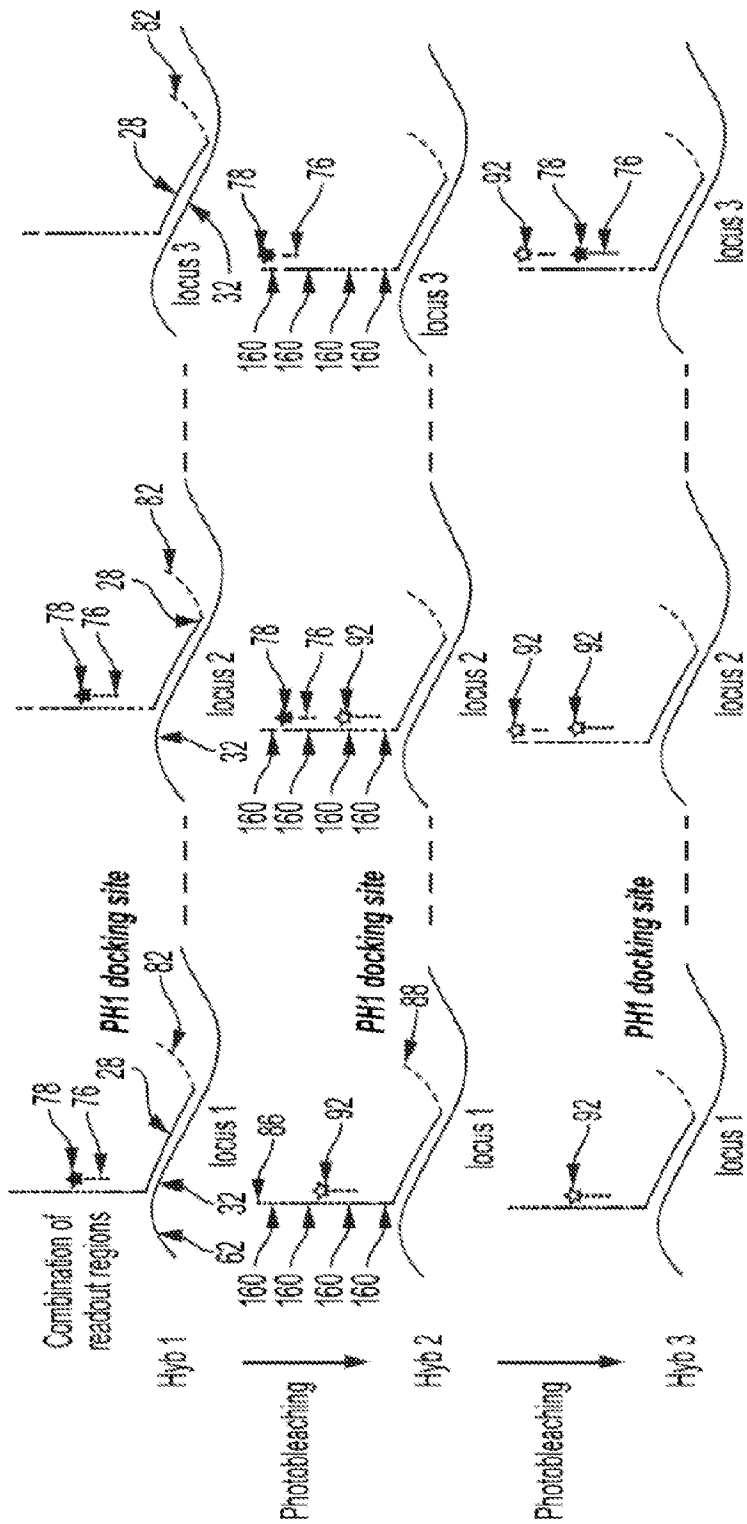
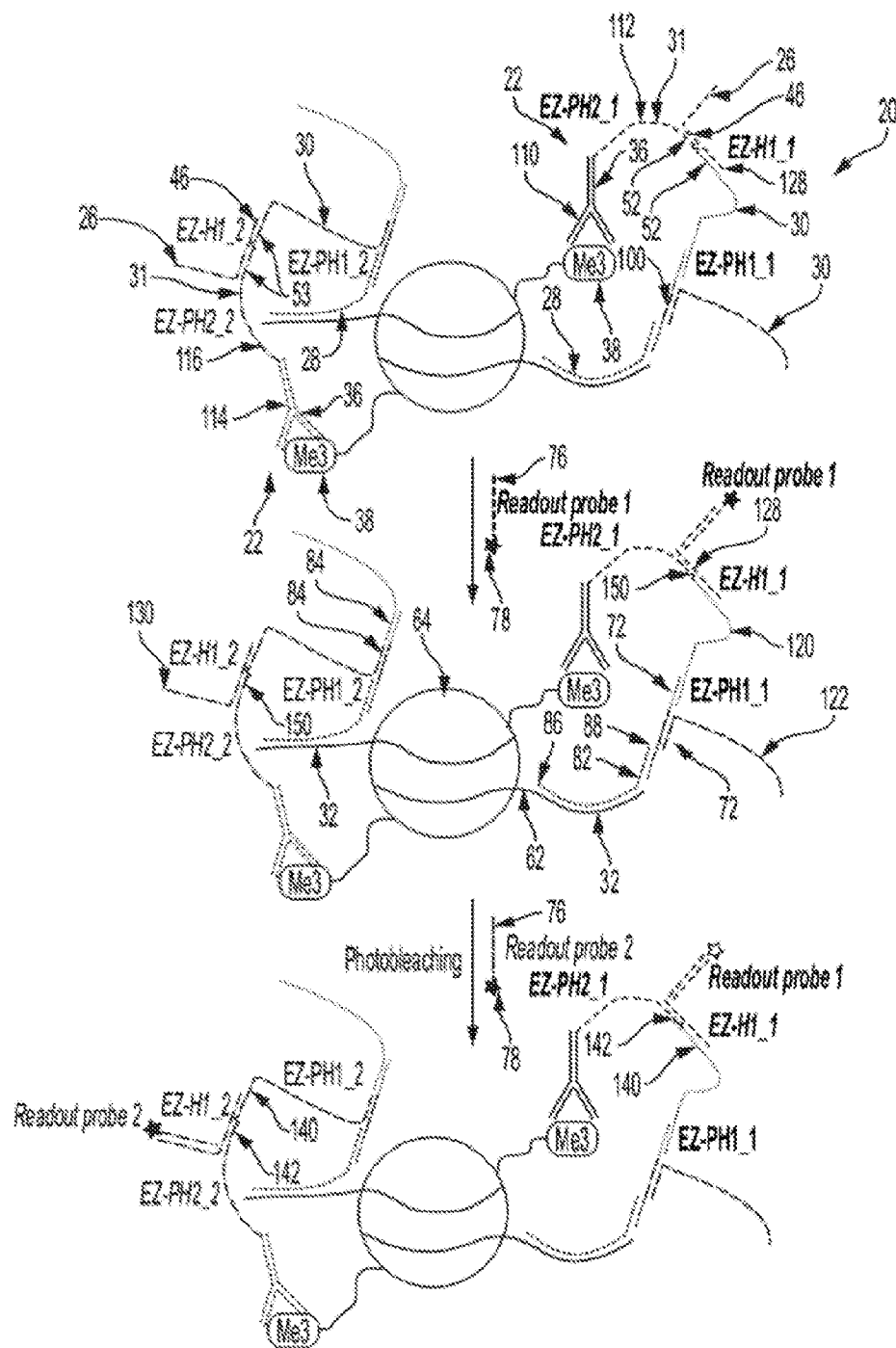


FIG. 9

**FIG. 10**

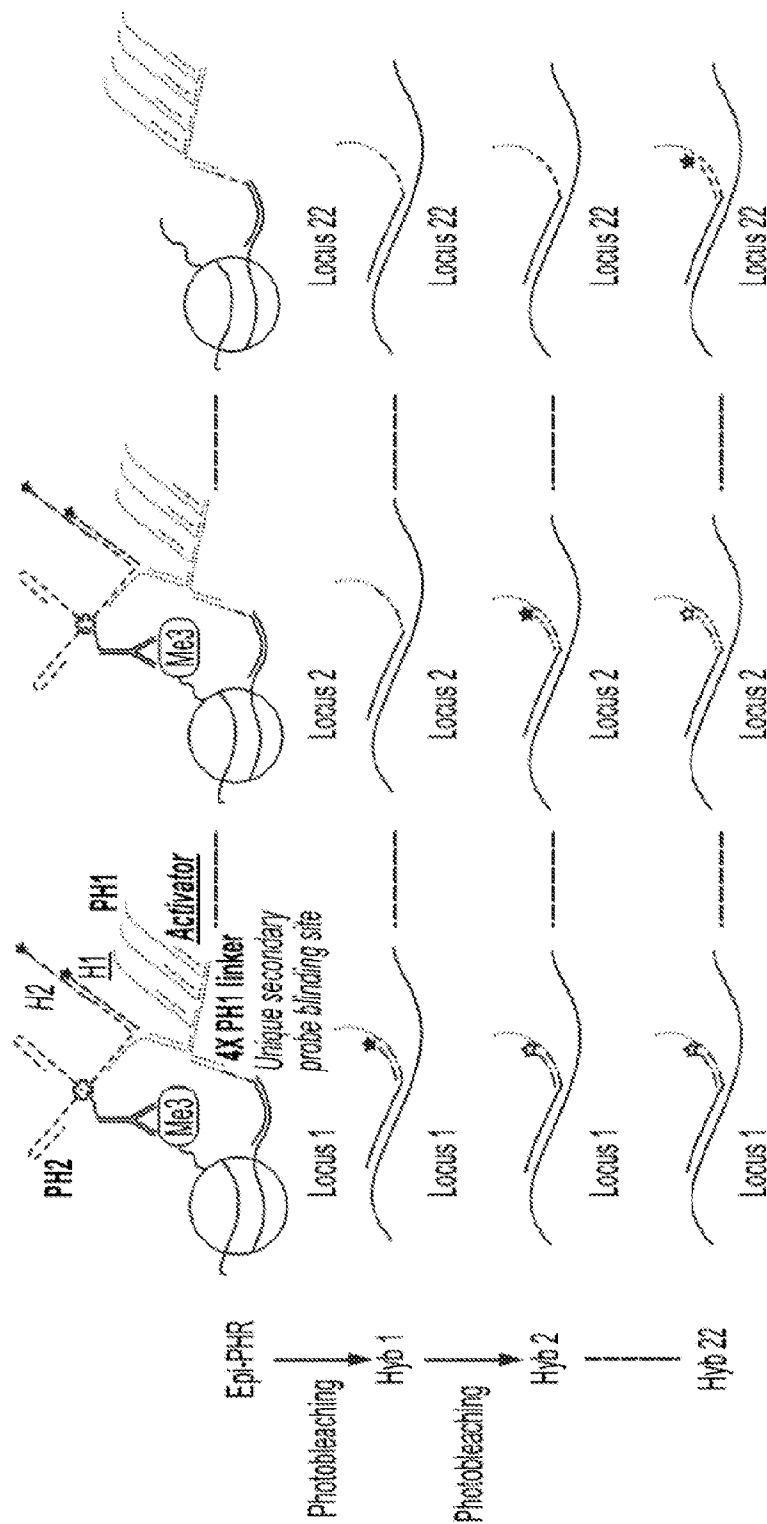


FIG. 11A

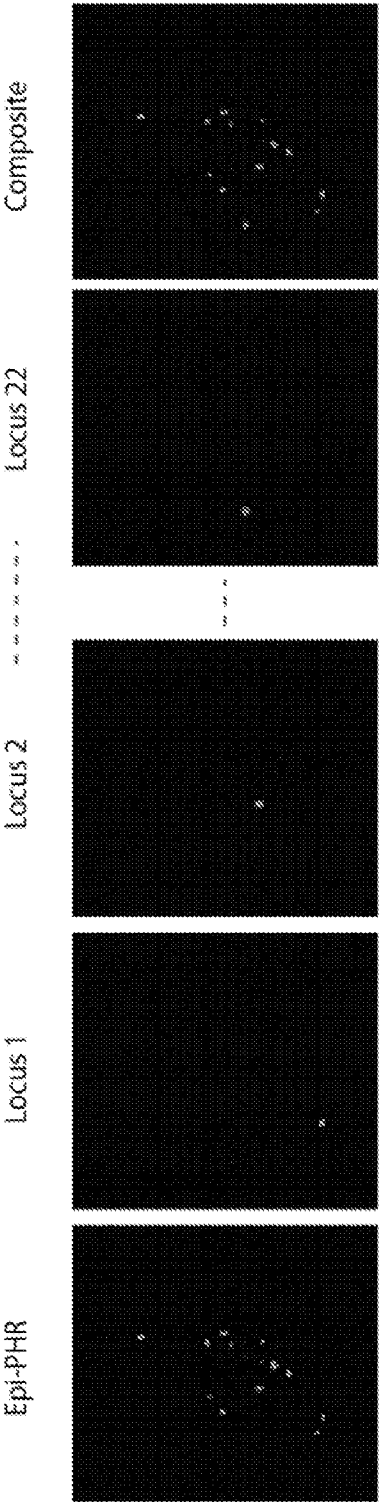


FIG. 11B

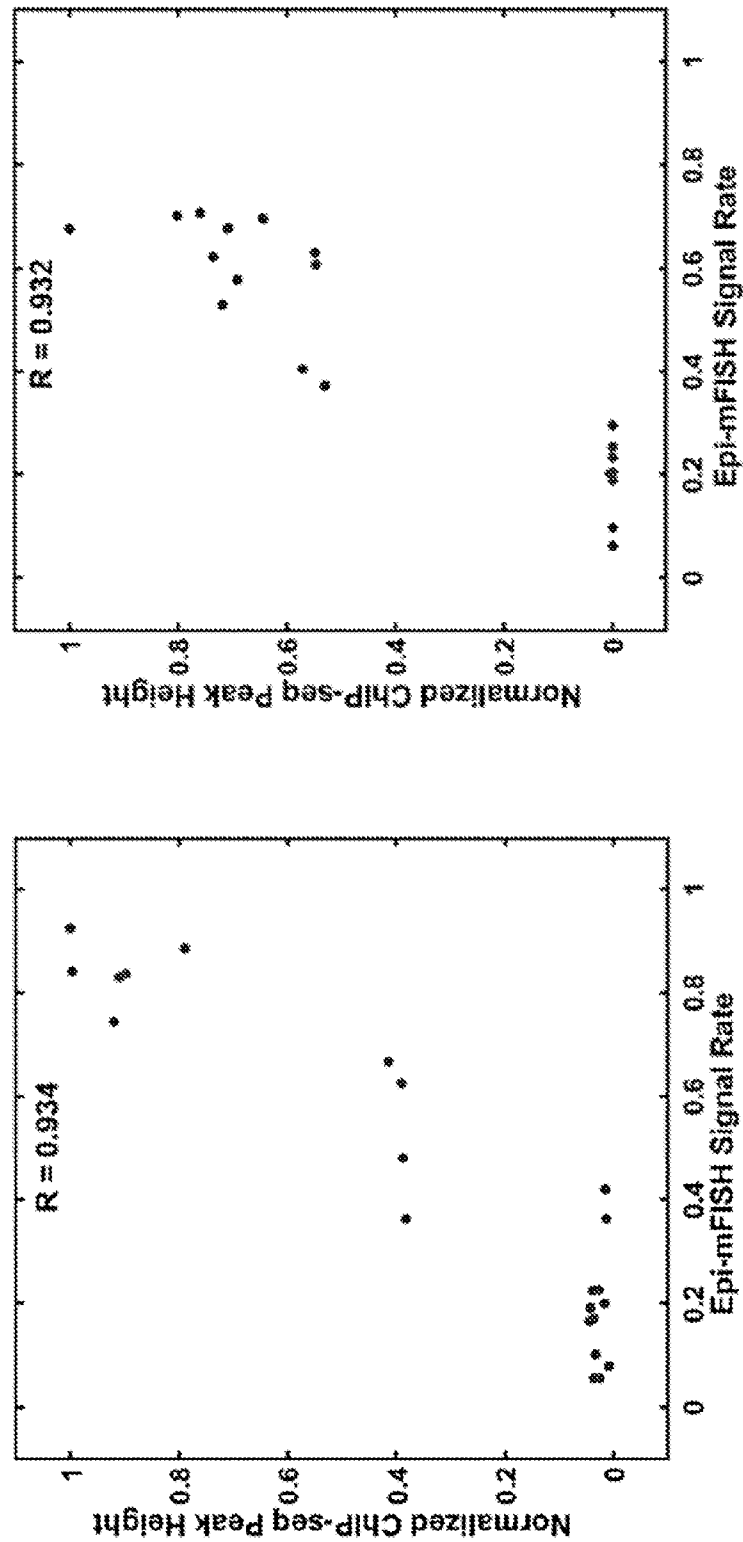


FIG. 11C

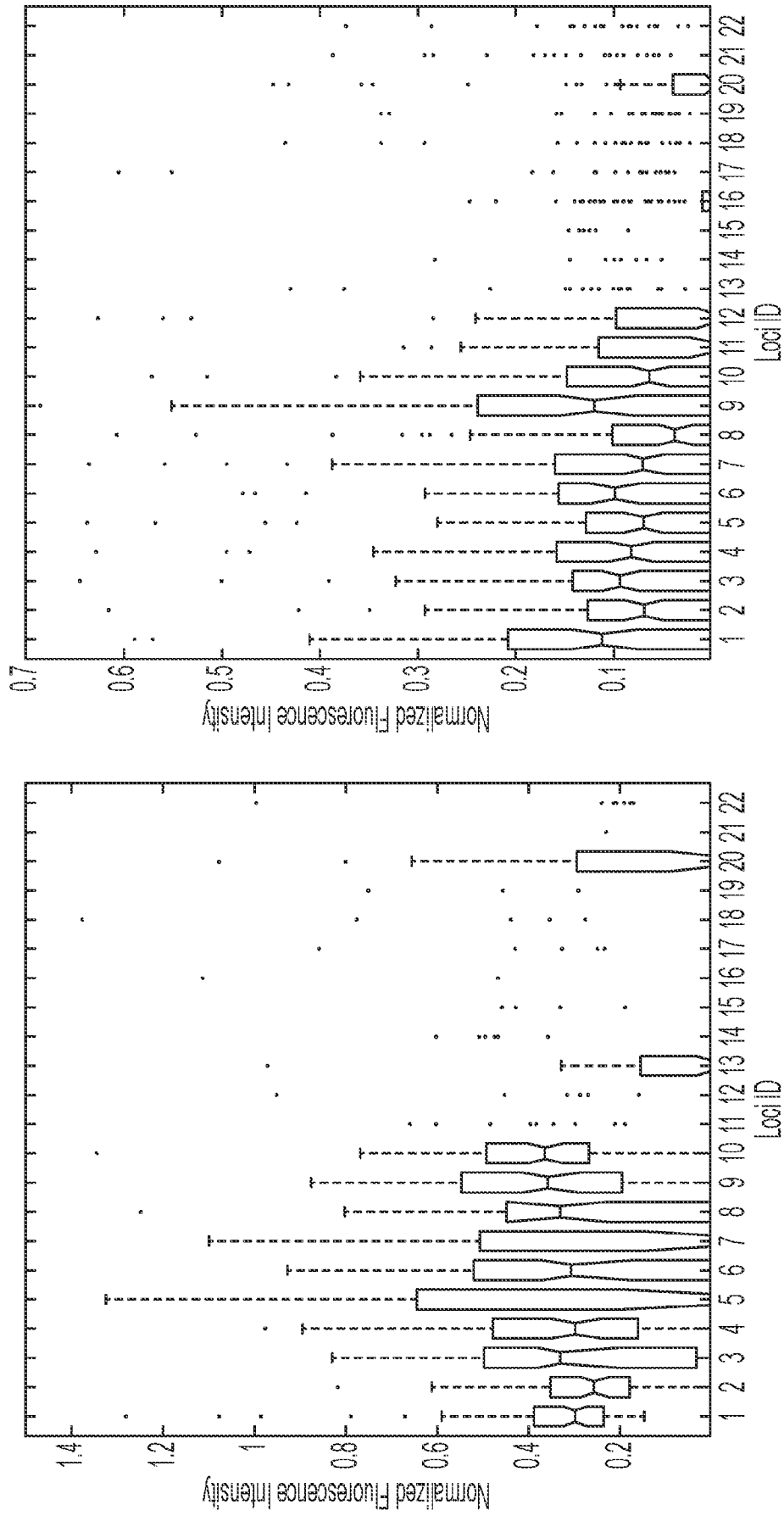


FIG. 11D

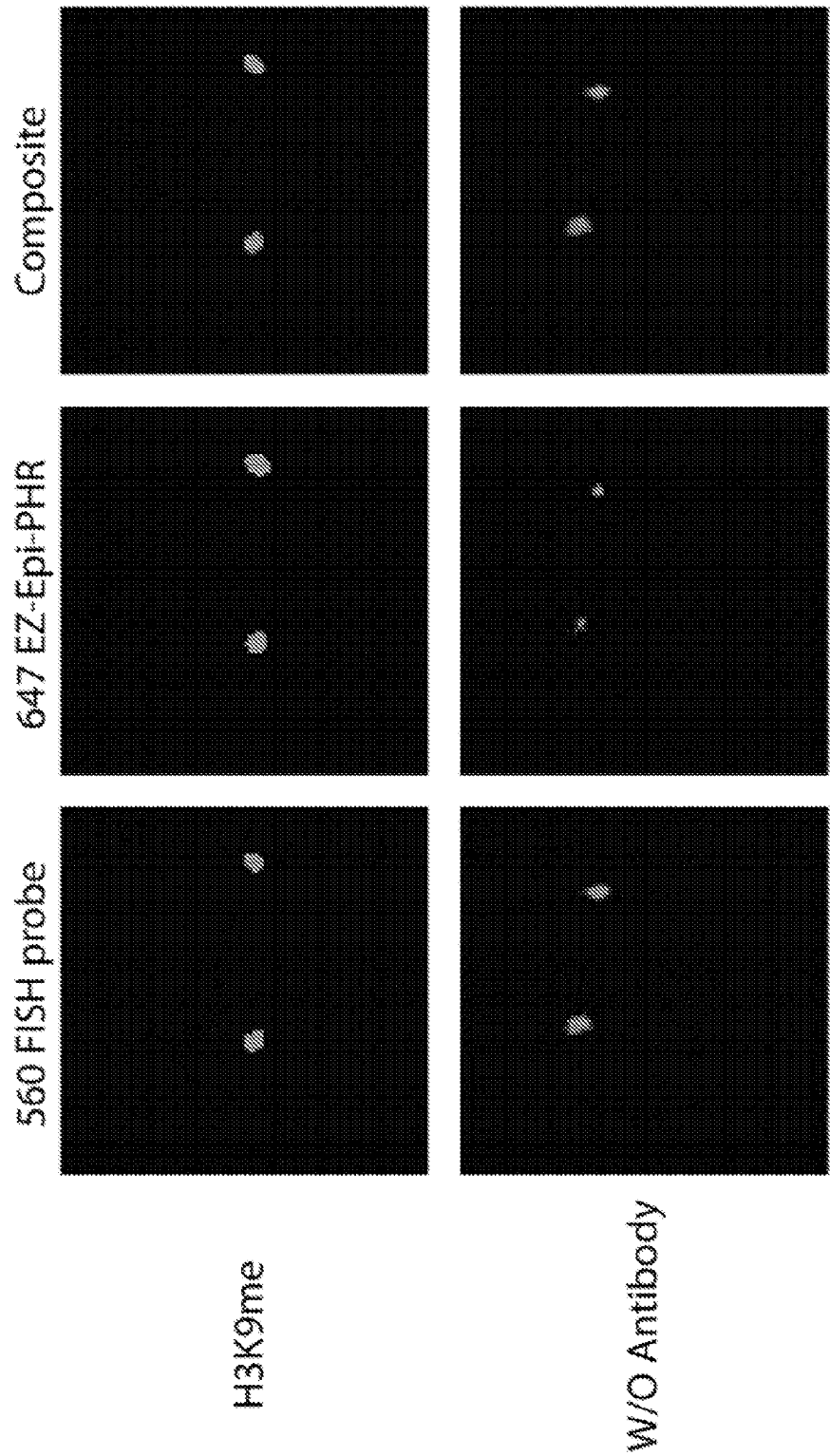


FIG. 12A

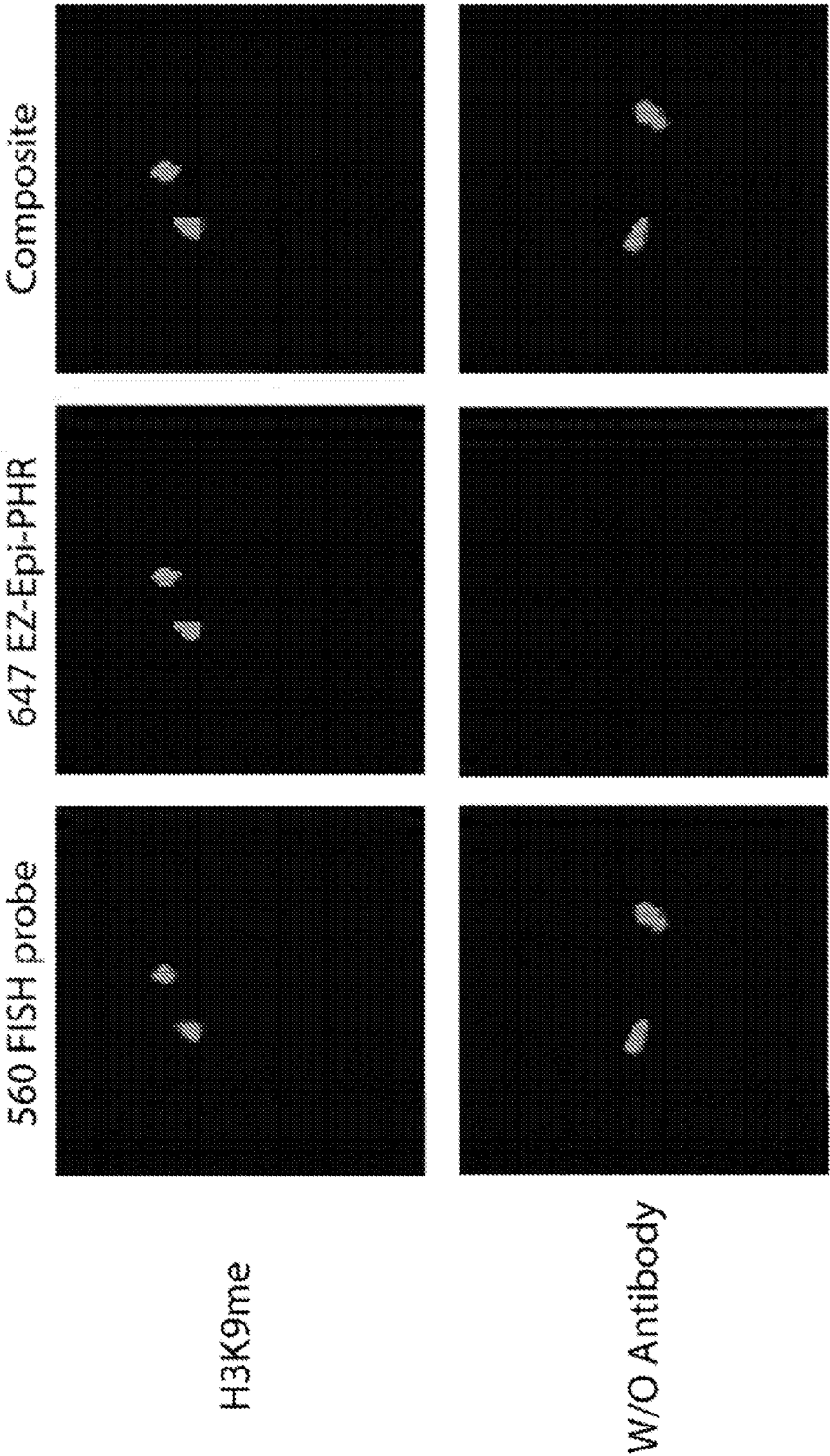


FIG. 12B

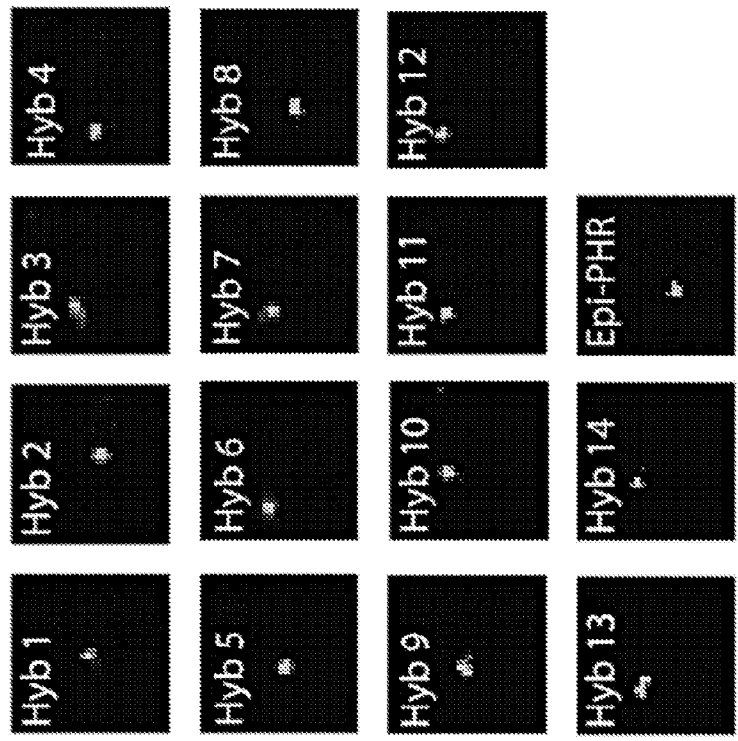
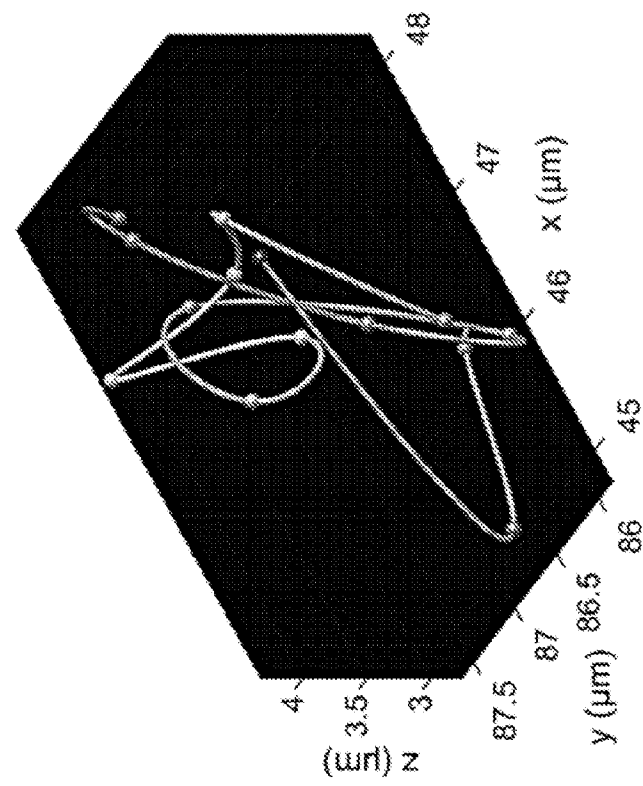


FIG. 13A

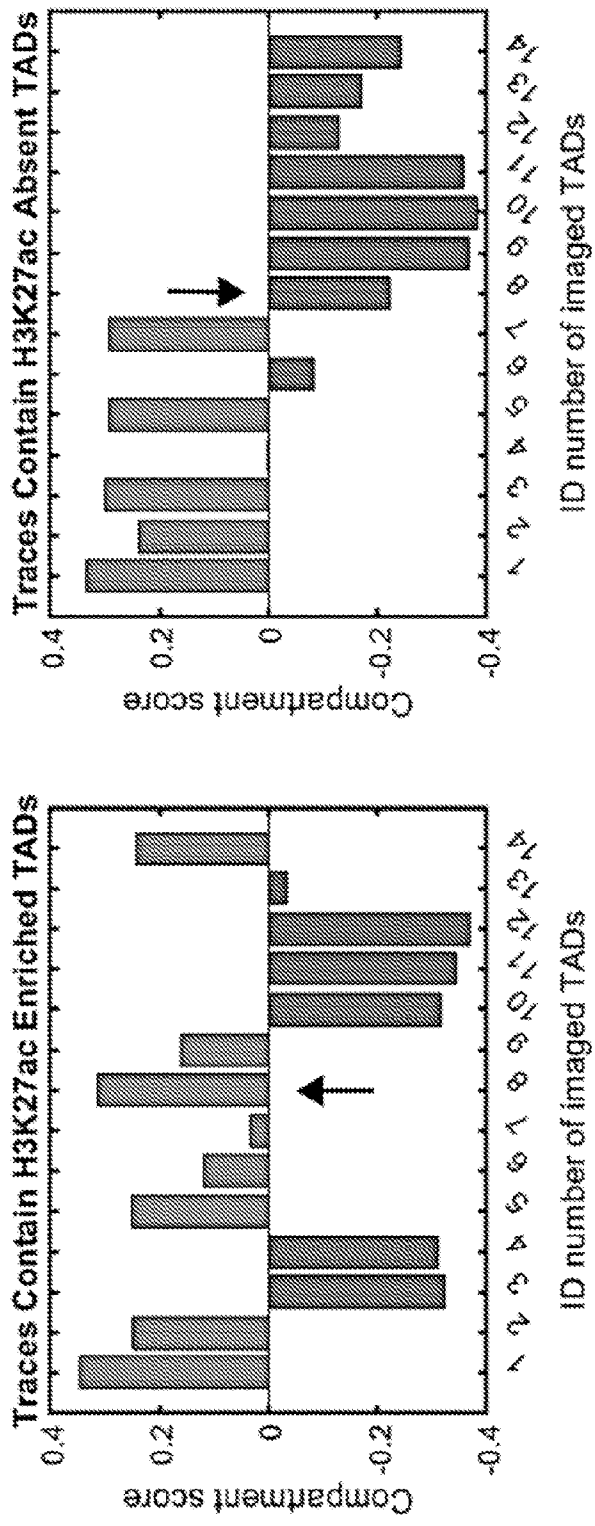


FIG. 13B

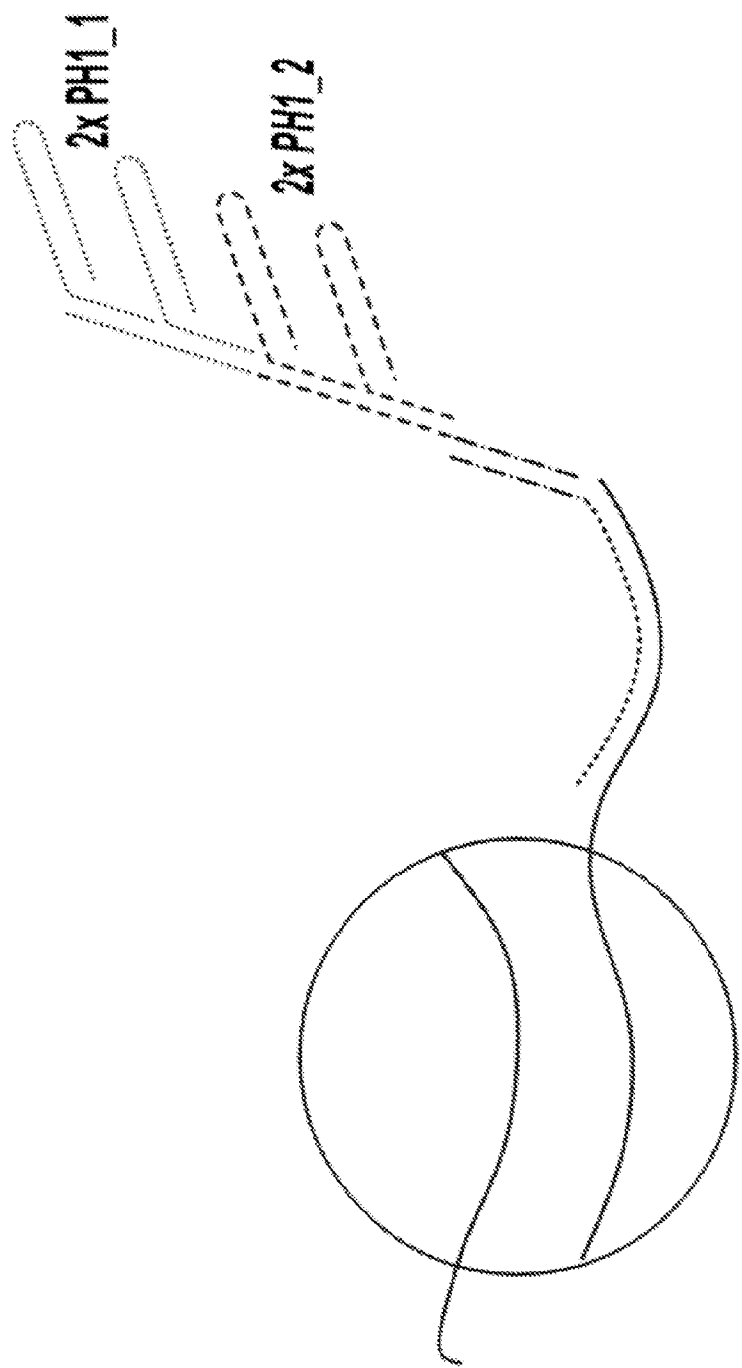


FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/044994

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 16/00; C07K 16/40; C12N 9/22; C12N 15/09; C12N 15/11 (2021.01)

CPC - C07K 16/40; C12N 9/22; C12N 15/11; C12N 2310/20; C12N 2800/80 (2021.08)

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)

see Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

see Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2018/0340221 A1 (SIGMA-ALDRICH CO. LLC et al) 29 November 2018 (29.11.2018) entire document	1-5, 87-91, 167-171, 243-250
Y	US 2010/0240062 A1 (BRAWLEY et al) 23 September 2010 (23.09.2010) entire document	1-5, 87-91, 167-171, 243-250
Y	WO 2019/113547 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 13 June 2019 (13.06.2019) entire document	2-5, 87-91, 168-171, 243-246, 248-250
A	US 2015/0191770 A1 (HTG MOLECULAR DIAGNOSTICS INC.) 09 July 2015 (09.07.2015) entire document	1-5, 87-91, 167-171, 243-250
A	US 2019/0139117 A1 (LIFE TECHNOLOGIES CORPORATION) 09 May 2019 (09.05.2019) entire document	1-5, 87-91, 167-171, 243-250

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"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

22 October 2021

Date of mailing of the international search report

DEC 14 2021

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Harry Kim

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/044994

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.: 6-86, 92-166, 172-242, 251-256
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:

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.:

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.