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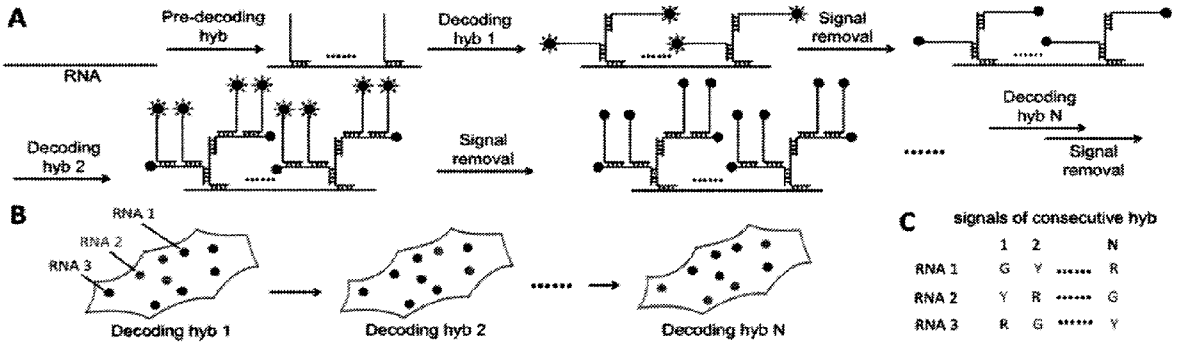
(54) **Title:** CONSECUTIVE HYBRIDIZATION FOR MULTIPLEXED ANALYSIS OF BIOLOGICAL SAMPLES

FIGS. 1A-1C



(57) **Abstract:** Provided herein are high-throughput, high-quality methods of consecutive in situ hybridization for analysis of the genome and/or transcriptome in an individual cell with single- molecule sensitivity. In particular, provided herein are methods comprising visualizing individual genomic loci or transcripts as single detectable signals (e.g., fluorescent spots) which remain in place during consecutive hybridization. In each cycle of consecutive hybridization, detectably labeled probes hybridize to the probe used in the previous cycle, and also introduce the binding sites for the probe of the following cycle. Through consecutive cycles of probe hybridization, imaging, and signal removal, different genomic loci or RNA species can be identified by unique detectable signal profiles (e.g., fluorescent spots with unique color sequences). The number of varied color sequences increases exponentially with the number of hybridization cycles, which enables the genome or transcriptome-wide analysis.

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CONSECUTIVE HYBRIDIZATION FOR MULTIPLEXED ANALYSIS OF BIOLOGICAL SAMPLES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/334,047, filed on May 10, 2016, which is incorporated by reference in its entirety as if fully set forth herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] Not applicable.

BACKGROUND

[0003] The ability to profile genome and transcriptome comprehensively in single cells *in situ* is crucial to understand the molecular mechanisms underlying cancer, neurobiology, and stem cell biology. Due to the inherent heterogeneity of these biological systems, conventional genomic technologies carried out on pools of cells mask gene expression variations and spatial complexity of different RNA species in a population. The differences between individual cells in complex biological systems may have significant consequences in the function and health of the entire systems. Thus, single cell analysis is required to explore such cell heterogeneity. The precise location of cells in a tissue and transcripts in a cell is critical for effective cell-cell interactions and gene expression regulation, which determine cell fates and functions. Therefore, to fully understand the organization, regulation and function of a heterogeneous biological system, the *in situ* analysis of the transcriptome of individual cells in the system is required. However, most of the existing methods for *in situ* RNA analysis can only quantify a small number of different transcripts in a biological sample. Other *in situ* sequencing based methods may miss transcripts present at low copy numbers. For example, single molecule fluorescence *in situ* hybridization (smFISH) has emerged as a powerful tool to quantify the copy number and spatial organization of transcripts in single cells, but only a handful of different RNA species in a biological sample can be simultaneously detected using smFISH. Although such technologies significantly advanced our ability to analyze transcripts in their natural contexts, many problems remain. For example, the multiplexing capacities of most current approaches are insufficient to

permit transcriptome-wide analysis, methods involving time-consuming photobleaching processes to remove fluorescence signals are limited by the small number of cells analyzed in a sample, and the cost of preparing multiple FISH probes, which must be synthesized individually, can be prohibitively expensive.

[0004] Accordingly, there remains a need in the art for improved methods for single-cell *in situ* genetic analysis. In particular, there remains a need for single-cell *in situ* genetic analysis methods that are comprehensive, low cost, and high-throughput that yield high quality data with single-molecule sensitivity.

SUMMARY

[0005] The present disclosure overcomes the aforementioned drawbacks by providing low-cost, high-throughput, rapid and high-quality methods of consecutive *in situ* hybridization capable of *in situ* analysis of genome and transcriptome in individual cells of intact tissues with single-molecule sensitivity. In this method, individual genomic loci or transcripts are visualized as single fluorescent spots, which remain in place during consecutive hybridization. In each cycle of consecutive hybridization, detectably labeled probes hybridize to the probe used in the previous cycle, and also introduce the binding sites for the probe of the following cycle. Through consecutive cycles of probe hybridization, imaging, and detectable signal removal, different genomic loci or RNA species can be identified (e.g., as fluorescent spots) with unique color sequences or other detectable signal profiles. The number of varied color sequences increases exponentially with the number of hybridization cycles, which enables the genome or transcriptome-wide analysis.

[0006] In a first aspect, provided herein is a method of *in situ* analysis of transcripts or DNA loci in a single cell. The method can comprise or consist essentially of the following steps: (a) performing a first contacting step that comprises contacting a cell comprising a plurality of transcripts or genomic loci to a plurality of pre-decoding oligonucleotides, where each pre-decoding oligonucleotide of the plurality comprises a targeting sequence that specifically hybridizes to a target transcript or genomic locus and further comprises one or more binding sites for specific hybridization to a decoding oligonucleotide in a subsequent hybridization step, wherein the first contacting step occurs under conditions that promote hybridization of the plurality of pre-decoding oligonucleotides to target transcripts or genomic loci; (b) performing a

second contacting step that comprises contacting the cell with a plurality of decoding oligonucleotides, wherein each decoding oligonucleotide comprises a detectable moiety and is configured to have one or more binding sites for specific hybridization to a pre-decoding oligonucleotide, wherein the second contacting step occurs under conditions that promote hybridization of the decoding oligonucleotides to the pre-decoding oligonucleotides; and (c) imaging the cell after the second contacting step whereby a detectable signal generated from hybridization of the decoding oligonucleotides to the pre-decoding oligonucleotides, whereby the detectable signal indicates hybridization to the target transcript or genomic locus. In some cases, the method further comprises (d) removing the detectable signal generated in step (c); and (e) optionally consecutively repeating the first and second contacting steps, imaging step, and removing step, each time with a new plurality of detectably labeled decoding oligonucleotides configured to have one or more binding sites for hybridization to detectably labeled decoding oligonucleotides of each subsequent cycle, wherein each new plurality of differs from detectably labeled decoding oligonucleotides of each subsequent cycle by at least one difference in detectable moiety labeling. Steps (a)-(e) can be consecutively performed at least 16 times. Two decoding probes can be used to detect each target transcript or genomic locus.

[0007] The detectable moiety can be selected from the group consisting of a fluorophore, radioactive isotope, and metal isotope. The fluorophore can be selected from the group consisting of TAMRA, ALEXA FLUOR™ 594, ATTO 647N, and ATTO 700. In some cases, the first plurality of oligonucleotides targets at least 10 different transcripts and/or DNA loci. Removing the detectable signal can comprise chemically cleaving the detectable moiety.

[0008] In some cases, the method further comprises washing to remove unhybridized oligonucleotides and non-specifically hybridized oligonucleotides following each contacting step.

[0009] In another aspect, provided herein is a kit for detecting target transcripts or DNA loci in a cell sample. The kit can comprise or consist essentially of a first plurality of oligonucleotide probes configured to hybridize to a target transcript or DNA locus; a second plurality of oligonucleotide probes, which second plurality includes oligonucleotides labeled with a detectable moiety and configured to have one or more binding sites for specific hybridization to oligonucleotides of the first plurality; and a written insert component comprising instructions for performing consecutive in situ analysis of target transcripts or DNA loci according to a method

provided herein. The detectable moiety can be selected from the group consisting of a fluorophore, radioactive isotope, and metal isotope. The oligonucleotide probes can be synthetic DNA oligonucleotide probes.

[0010] The foregoing and other advantages of the invention will appear from the following description. In the description, reference is made to the accompanying drawings which form a part hereof, and in which there is shown by way of illustration a preferred embodiment of the invention. Such embodiment does not necessarily represent the full scope of the invention, however, and reference is made therefore to the claims and herein for interpreting the scope of the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0011] FIGS. 1A-1C illustrate an exemplary protocol for single-cell *in situ* transcriptome analysis having single-molecule sensitivity. (A) Schematic diagram of consecutive cycles of probe hybridization, fluorescence imaging, and signal removal. (B) Schematic diagram of C-FISH images. In each cycle, individual transcript is visualized as a single spot with a specific color. (C) As transcripts remain in place during the whole C-FISH cycles, different RNA species can be distinguished by the unique color sequences.

[0012] FIGS. 2A-2C demonstrate single-cell *in situ* transcript analysis by C-FISH. (A) In the first cycle of C-FISH, GAPDH and Ki67 transcripts are detected as green and red spots, respectively. Through 16 cycles of C-FISH, individual transcripts are detected as fluorescent spots with unique color sequences (inset panels 1-16). (B) GAPDH and (C) Ki67 transcripts are detected by conventional smFISH. Scale bars, 5 μm.

[0013] FIGS. 3A-3B. (A) Mean copy number per cell (n = 30 cells) of GAPDH and Ki67 transcripts measured by C-FISH and smFISH. The y axis is on a logarithmic scale. (B) Percentage of spots accurately identified in C-FISH cycles.

[0014] FIG. 4 is a series of images (panels 1-9) collected for single-cell *in situ* genomic loci analysis by C-FISH. Through 9 cycles of C-FISH, genomic locus 4p16.1 is detected as fluorescent spots with unique color sequences. Scale bars, 5 μm.

[0015] FIG. 5 is a schematic illustrating an exemplary protocol for preparing C-FISH probes by enzymatic amplification of an array-derived oligonucleotide pool. Each sequence in the oligonucleotide pool contains a T7 promoter sequence, a variable probe sequence, and two

flanking index primers. First, templates from a library of probes are selected and amplified by indexed PCR (polymerase chain reaction). Next, the resulting PCR products are further amplified into RNA by in vitro transcription. Finally, the RNA library is reverse transcribed into cDNA. Natural (unlabeled) or fluorescently labeled oligonucleotides are used as reverse transcription to generate pre-decoding or decoding probes, respectively.

[0016] FIG. 6 is a schematic illustrating an exemplary protocol for preparing C-FISH decoding probes by chemical synthesis followed by enzymatic incorporation. Generated on a DNA synthesizer, decoding probes comprising unlabeled nucleotides are extended with NH_2-ddUTP (2',3'-dideoxyuridine-5'-triphosphate) by terminal transferase (TdT), which is a template independent polymerase that catalyzes the addition of deoxynucleotides to the 3' hydroxyl terminus of DNA molecules.

[0017] FIG. 7 illustrates chemical cleavage of fluorescent decoding probes for high-throughput and rapid C-FISH.

[0018] FIGS. 8A-8D. (A) Synthesis of NHS ester functionalized cleavable TAMRA. (B) Ki67 transcripts are detected by cleavable TAMRA labeled decoding probes. (C) Fluorescence signals are removed by chemical cleavage using TCEP. (D) Ki67 transcripts reappear after the second cycle of hybridization with Cy5 labeled decoding probes. Scale bars, 5 μm.

[0019] FIG. 9. Single-cell *in situ* transcripts analysis by the second-generation C-FISH. In the first cycle of C-FISH, GAPDH and Ki67 transcripts were detected as green and red spots, respectively. Through 16 cycles of C-FISH using only two decoding probes for each transcript, individual transcripts are detected as fluorescent spots with unique color sequences. Scale bars, 5 μm.

DETAILED DESCRIPTION

[0020] The present invention, in some embodiments thereof, relates to methods for high throughput transcriptome and genome analysis using consecutive fluorescence *in situ* hybridization (C-FISH) and probes specifically designed thereof. The methods and compositions provided herein are based at least in part on the inventors' discovery of a low-cost, high-throughput, and high-quality approach to consecutive fluorescence *in situ* hybridization (C-FISH). As described herein, the inventors' discovery provides for *in situ* analysis of transcriptome in individual cells of intact tissues with single-molecule sensitivity.

Accordingly, in a first aspect, provided herein is a method for analysis of transcripts or genomic (e.g., DNA) loci in a single cell. In exemplary embodiments, the method comprises (a) performing a first contacting step that comprises contacting a cell comprising a plurality of transcripts or genomic loci to a plurality of pre-decoding oligonucleotides, where each pre-decoding oligonucleotide of the plurality comprises a targeting sequence that specifically hybridizes to a target transcript or genomic locus and further comprises one or more binding sites for specific hybridization to a decoding oligonucleotide in a subsequent hybridization step, wherein the first contacting step occurs under conditions that promote hybridization of the plurality of pre-decoding oligonucleotides to target transcripts or genomic loci; (b) performing a second contacting step that comprises contacting the cell with a plurality of decoding oligonucleotides, wherein each decoding oligonucleotide comprises a detectable moiety and is configured to have one or more binding sites for specific hybridization to a pre-decoding oligonucleotide, wherein the second contacting step occurs under conditions that promote hybridization of the decoding oligonucleotides to the pre-decoding oligonucleotides; and (c) imaging the cell after the second contacting step whereby a detectable signal generated from hybridization of the decoding oligonucleotides to the pre-decoding oligonucleotides, whereby the detectable signal indicates hybridization to the target transcript or genomic locus. In some cases, the method further comprises (d) removing the detectable signal generated in step (c); and
(e) optionally consecutively repeating the first and second contacting steps, imaging step, and removing step, each time with a new plurality of detectably labeled decoding oligonucleotides configured to have one or more binding sites for hybridization to detectably labeled decoding oligonucleotides of each subsequent cycle, wherein each new plurality of differs from detectably labeled decoding oligonucleotides of each subsequent cycle by at least one difference in detectable moiety labeling.

[0021] As described herein, the methods of the present invention provide for visualization and analysis of individual transcripts as single fluorescent spots. When the contacted cell is fixed using a fixative such as paraformaldehyde or formaldehyde, the fluorescent spots remain in place during each consecutive hybridization cycle. For each cycle, fluorescently labeled decoding probes specifically hybridize to the probe used in the previous cycle (e.g., unlabeled pre-decoding probes or previously contacted plurality of decoding probes) and, in doing so, also introduce binding sites for probes of the following cycle. Through consecutive cycles of probe

hybridization, fluorescence imaging, and signal removal, different RNA species can be identified as fluorescent spots with unique color sequences. The number of varied color sequences increases exponentially with the number of hybridization cycles, which enables genome-wide or transcriptome-wide analysis.

[0022] Referring to FIG. 1, a *de novo* approach for single-cell *in situ* transcriptome analysis is presented. As shown in FIG. 1A, each transcript is hybridized with a set of pre-decoding probes. Preferably, the pre-decoding probes are not labeled with a detectable moiety. The pre-decoding probes have varied targeting sequences to bind to the different regions on the target transcript. In addition, the pre-decoding probes all share a common decoding sequence for specific hybridization to decoding probes. Subsequently, a plurality of detectably labeled decoding probes is contacted to the cell under conditions suitable for hybridization of detectable decoding probes to transcript-bound pre-decoding probes. As a result, an individual transcript can be visualized as a single spot when visualized using, for example, fluorescence microscope (FIG. 1B) to detect decoding probes comprising a fluorescent moiety. After detection of the detectable moiety by any appropriate means (e.g., fluorescence microscopy) and data storage, the detectable moiety is removed.

[0023] To maintain the signal to background ratio in subsequent consecutive cycles, each plurality of decoding probes is configured to have one or more binding sites to hybridize to the probe of the previous cycle (either the pre-decoding probes or the decoding probes of a previous hybridization/visualization/removal cycle). Each plurality of decoding probes is further configured to have multiple binding sites for hybridization to the probe of the following cycle. As cells or tissue samples are fixed, transcripts remain in place during the whole C-FISH process. Through consecutive cycles of probe hybridization, fluorescence imaging, and signal removal, each transcript is identified as a fluorescent spot with a unique color sequence (FIG. 1C).

[0024] The terms “hybridize” and “hybridization” as used herein refer to the association of two nucleic acids to form a stable duplex. Nucleic acids hybridize due to a variety of well characterized physico-chemical forces, such as hydrogen bonding, solvent exclusion, base stacking and the like. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, part I chapter 2, “Overview of principles of hybridization and the strategy

of nucleic acid probe assays" (Elsevier, N.Y.). One of skill in the art will understand that "hybridization" as used herein does not require a precise base-for-base complementarity. That is, a duplex can form, between two nucleic acids that contained mismatched base pairs. The conditions under which nucleic acids that are perfectly complementary or that contain mismatched base pairs will hybridize to form a duplex are well known in the art and are described, for example, in MOLECULAR CLONING: A LABORATORY MANUAL, 3rd ed., Sambrook et al., eds., Cold Spring Harbor Press, Cold Spring Harbor (2001) at Chapter 10, which is herein incorporated by reference. As used herein, the term "complementary" refers to a nucleic acid that forms a stable duplex with its "complement". For example, nucleotide sequences that are complementary to each other have mismatches at less than 20% of the bases, at less than about 10% of the bases, preferably at less than about 5% of the bases, and more preferably have no mismatches.

[0025] As used herein, the term "probe" refers to and encompasses any physical string or collection of monomer units (e.g., nucleotides) that can connect to form a string of nucleotides, including a polymer of nucleotides (e.g., DNA oligonucleotides, RNA oligonucleotides, peptide nucleic acid (PNA) oligonucleotides, locked nucleic acid (LNA) oligonucleotides), peptide nucleic acids (PNAs), modified oligonucleotides (e.g., oligonucleotides comprising nucleotides that are not typical to biological RNA or DNA, such as 2′-O-methylated oligonucleotides), and the like. The nucleotides of the oligonucleotide can be deoxyribonucleotides, ribonucleotides or nucleotide analogs, and can be natural or non-natural, and can be unsubstituted, unmodified, substituted or modified. The nucleotides can be linked by phosphodiester bonds, or by phosphorothioate linkages, methylphosphonate linkages, boranophosphate linkages, or the like. The oligonucleotide can additionally comprise non-nucleotide elements such as labels, quenchers, blocking groups, or the like. The oligonucleotide can be single-stranded or double-stranded.

[0026] As used herein, the terms "target nucleic acid" and "target transcript" include nucleic acids originating from one or more biological entities within a sample. The target nucleic acid of interest to be detected in a sample can be a sequence or a subsequence from DNA, such as nuclear or mitochondrial DNA, or cDNA that is reverse transcribed from RNA in the sample. In some cases, the target transcript is a RNA sequence of interest, such as mRNA, rRNA, tRNA, miRNA, siRNAs, antisense RNAs, or long noncoding RNAs. More generally, the target

transcripts and target nucleic acids sequences of interest can be selected from any combination of sequences or subsequences in the genome (e.g., a genomic locus or genomic loci) or transcriptome of a species or an environment. In some cases, a defined set of decoding probes are designed to hybridize to the plurality of sequences that would be expected in a sample, for example a genome or transcriptome, or a smaller set when the sequences are known and well-characterized, such as from an artificial source.

[0027] Oligonucleotide probes useful for the methods provided herein are of any length sufficient to permit probe penetration and to optimize hybridization of probes of subsequent C-FISH. Preferably, probe length is about 20 bases to about 500 bases. As probe length increases, so increases the number of binding sites that can be incorporated into a given probe for hybridization to the probe of the following cycle as well as the signal to noise ratio. However, longer than 500 bases, the probes may not efficiently penetrate the cellular membrane. The C-FISH hybridization oligonucleotide probes provided herein include probes consisting of between 20 and 500 nucleotides, 20 and 250, 50 and 250, 150 and 250 nucleotides, 20 and 150, or 50 and 150 nucleotides, inclusive.

[0028] Any detectable moiety can be used for the methods provided herein. As used herein, "detectable moiety" refers to a label molecule (isotopic or non-isotopic) possessing a property or function which can be used for detection purposes and include, without limitation, chromophore moieties, fluorescent moieties, phosphorescent moieties, chemiluminescent moieties, light absorbing moieties, radioactive isotopes, metal isotopes, and particle-based signals. Suitable fluorescent moieties are those known from the art of immunofluorescence technologies, e.g., flow cytometry or fluorescence microscopy. In some cases, the detectable moiety is a fluorophore. In other cases, the detectable moiety is a radioactive isotope (e.g., ^{13}C, ^{15}N) or metal isotope. Metal isotopes can detected by mass spectrometry imaging. Several methods are well described in the literature and are known to be used to render signals that are detectable by various means (e.g., microscopy (bright-field, fluorescent, electron, scanning probe)), flow cytometry (fluorescent, particle, magnetic) or a scanning device.

[0029] Fluorophores that can be used for attachment to oligonucleotides post-synthesis include, without limitation, TAMRA (labeled with tetramethylrhodamine or “TMR”), ALEXA FLUOR™ 594, and ATTO 647N and ATTO 700 fluorophores (ATTO-TEC, Germany). Other fluorophores appropriate for use according to the methods provided herein include, without

limitation, ALEXA FLUOR™ 350, ALEXA FLUOR™ 532, ALEXA FLUOR™ 546, ALEXA FLUOR™ 568, ALEXA FLUOR™ 647, BODIPY 493/503, BODIPY FL, BODIPY R6G, BODIPY 530/550, BODIPY TMR, BODIPY 558/568, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY 581/591, BODIPY 630/650, BODIPY 650/665, Cascade Blue, Cascade Yellow, Dansyl, lissamine rhodamine B, Marina Blue, Oregon Green 488, Oregon Green 514, Pacific Blue, rhodamine 6G, rhodamine green, rhodamine red, tetramethyl rhodamine, DYLIGHT™ DYES (e.g., DYLIGHT™ 405, DYLIGHT™ 488, DYLIGHT™ 549, DYLIGHT™ 594, DYLIGHT™ 633, DYLIGHT™ 649, DYLIGHT™ 680, DYLIGHT™ 750, DYLIGHT™ 800 and the like), Texas Red, and Cy2, Cy3.5, Cy5.5, and Cy7. In addition to the use of fluorophores as a detectable moiety, other luminescent labels such as chemiluminescent agents can be used.

[0030] When fluorescently labeled oligonucleotides are used, fluorescence photomicroscopy can be used to detect and record the results of consecutive *in situ* hybridization using routine methods known in the art. Alternatively, digital (computer implemented) fluorescence microscopy with image-processing capability may be used. Two well-known systems for imaging FISH of chromosomes having multiple colored labels bound thereto include multiplex-FISH (M-FISH) and spectral karyotyping (SKY). See Schrock et al. (1996) *Science* 273:494; Roberts et al. (1999) *Genes Chrom. Cancer* 25:241; Fransz et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:14584; Bayani et al. (2004) *Curr. Protocol. Cell Biol.* 22.5.1-22.5.25; Danilova et al. (2008) *Chromosoma* 117:345; U.S. Pat. No. 6,066,459; and FISH TAG™ DNA Multicolor Kit instructions (Molecular Probes) for a review of methods for painting chromosomes and detecting painted chromosomes.

[0031] Any appropriate means of removing a detectable signal or detectable moiety (e.g., a fluorophore) can be used according to the methods provided herein. Methods of removal can include without limitation photobleaching (see Example 1 below), chemical deactivation, chemical cleavage of the fluorophores (e.g., disulfide cleavage), enzymatic cleavage (using, for example, an exonuclease, endonuclease, protease, or USER™ (Uracil-Specific Excision Reagent) cleavage system), DNA/RNA strand displacement, chemical or heat denaturing of an intermediate fluorescent oligonucleotide, and the like. Removal of fluorescence can be performed by photobleaching or by chemical removal of the fluorescent moiety (or other detectable moiety). Since photobleaching can be a time-consuming step, in some cases the

methods provided herein comprise efficiently removing fluorescence signals by chemical deactivation or chemical or enzymatic cleavage of detectable moieties from decoding probes. After hybridization with cleavable fluorescent decoding probes, all of the different fluorophores in the whole sample can be chemically cleaved simultaneously in 30 minutes. To further reduce the assay time by decreasing the cycle numbers and increasing the number of fluorophores used in each cycle, C-FISH probes can be synthesized to be labeled with various cleavable fluorophores. Consequently, C-FISH performed with such cleavable fluorescent probes reduces the cycling time (minus imaging) from weeks to approximately 1.5-2.5 hours for thousands of single cells in a human tissue.

[0032] Using the reaction scheme set forth in FIG. 5A, NHS ester-functionalized cleavable fluorescent probes can be obtained comprising TAMRA (labeled with tetramethylrhodamine or "TMR"), ALEXA FLUOR™ 594, ATTO 647N (ATTO-TEC, Germany), and ATTO 700 fluorophores (ATTO-TEC, Germany). C-FISH probes comprising TAMRA, ALEXA FLUOR™ 594, ATTO 647N, and ATTO 700 can be imaged using filter sets SP102v1 (Chroma), a custom set5 (Omega), SP104v2 (Chroma) and SP105 (Chroma), respectively. With C-FISH probes labeled with these four fluorophores (TAMRA, ALEXA FLUOR™ 594, ATTO 647N, and ATTO 700), only 5 cycles (4^5 = 1,024) are required to analyze one thousand RNA species.

[0033] In some cases, the methods provided herein comprise chemical inactivation of fluorophores. For example, fluorophores can be inactivated by oxidation. Protocols for oxidation of dyes with hydrogen peroxide, which can be catalyzed using either acidic or basic conditions, or reactive oxygen species (ROS) are known to those practitioners in the art for changing the fluorescent properties of dyes and fluorescent proteins.

[0034] To minimize issues of autofluorescence or background signal, cleavable fluorescent decoding probes can be designed to include three or more probe binding sites. Thus, an increased number of probes will hybridize to each transcript to enhance signal to noise ratio. As used herein, the terms "binding," "to bind," "binds," "bound," or any derivation thereof refers to any stable, rather than transient, chemical bond between two or more molecules, including, but not limited to, covalent bonding, ionic bonding, and hydrogen bonding. Thus, this term also encompasses interaction between a nucleic acid molecule and another entity such as, a nucleic acid or probe element. Specifically, binding, in certain embodiments, includes the hybridization of nucleic acids.

[0035] Biological samples can be obtained from any biological entity containing genetic material (e.g., nucleic acid). Sources for nucleic acid-containing biological entities include, without limitation, an organism or organisms including a cell or cells, bacteria, yeast, fungi, *algae*, viruses, and mammals (e.g., humans) or other animal species. The methods and compositions described herein can be performed using a variety of biological or clinical samples comprising cells that are in any (or all) stage(s) of the cell cycle (e.g., mitosis, meiosis, interphase, G0, G1, S and/or G2). As used herein, the term "sample" include all types of cell culture, animal or plant tissue, peripheral blood lymphocytes, buccal smears, touch preparations prepared from uncultured primary tumors, cancer cells, bone marrow, cells obtained from biopsy or cells in bodily fluids (e.g., blood, urine, sputum and the like), cells from amniotic fluid, cells from maternal blood (e.g., fetal cells), cells from testis and ovary, and the like. These examples are not to be construed as limiting the sample types applicable to the methods and/or compositions described herein. Samples are prepared for assays of the invention using conventional techniques, which typically depend on the source from which a sample or specimen is taken. Preferably, the methods provided herein comprise a cell fixation step. For example, the cells of a biological sample (e.g., tissue sample) can be fixed (e.g., using formaldehyde or paraformaldehyde fixation techniques known to one of ordinary skill in the art). Any fixative that does not affect DNA or RNA can be utilized in according to the methods provided herein.

[0036] Kits

[0037] In another aspect, provided herein is a kit comprising reagents for performing in situ analysis of transcripts or DNA loci according to a method provided herein. In some cases, the kit comprises a first plurality of oligonucleotide probes configured to hybridize to a target transcript or DNA locus; a second plurality of oligonucleotide probes, which second plurality includes oligonucleotides labeled with a detectable moiety and configured to have one or more binding sites for specific hybridization to oligonucleotides of the first plurality; and a written insert component comprising instructions for performing consecutive *in situ* analysis of target transcripts or DNA loci according to a method provided herein. Preferably, the detectable moiety is a fluorophore. As described herein, the oligonucleotide probes can be synthetic DNA oligonucleotide probes. A kit will preferably include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

[0038] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the chemicals, cell lines, vectors, animals, instruments, statistical analysis and methodologies which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0039] As used herein and in the appended claims, the singular forms "a", "an" and "the" include plural reference, unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and equivalents thereof known to those skilled in the art, and so forth. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. In addition, the terms "comprising", "including" and "having" can be used interchangeably.

[0040] As used herein, “about” means within 5% of a stated concentration range or within 5% of a stated time frame.

[0041] Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

[0042] Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non-limiting fashion.

[0043] Materials & Methods

[0044] *Preparation of fluorescently labeled oligonucleotide probes:* Oligonucleotides (1 nmol) were dissolved in 1 µL of nuclease-free water. To this solution was added the sodium bicarbonate aqueous solution (1M, 3 µL) and Quasar 570 (BioSearch) or Cy5 (AAT Bioquest) in DMF (20 mM, 5 µL). The mixture was then diluted to a volume of 10 µL with nuclease-free water and incubated at RT for 2 hours. Subsequently, the

fluorescently labeled oligonucleotides were purified by nucleotide removal kit (Qiagen) and dried in a Savant SpeedVac Concentrator (Thermo Scientific).

[0045] *Cell culture:* HeLa CCL-2 cells (ATCC) were maintained in Dulbelcco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 10 U/mL penicillin and 100 g/mL streptomycin in a humidified atmosphere at 37°C with 5% CO2. Cells were plated on chambered coverglass (Thermo Scientific) and allowed to reach 60% confluency in 1-2 days.

[0046] *Cell fixation*: Cultured HeLa CCL-2 cells were washed with 1X PBS at RT for 5 minutes, fixed with fixation solution (4% formaldehyde (Polysciences) in 1X PBS) at RT for 10 minutes, and subsequently washed another 2 times with 1x PBS at RT, each for 5 minutes.

[0047] The dried fluorophore conjugated oligonucleotides were then further purified via an HPLC (Agilent) equipped with a C18 column (Agilent) and a dual wavelength detector set to detect DNA absorption (260 nm) as well as the absorption of the coupled fluorophore (548nm for Quasar 570, 650nm for Cy5). For the gradient, triethyl ammonium acetate (Buffer A) (0.1 M, pH 6.5) and acetonitrile (Buffer B) (pH 6.5) were used, ranging from 7% to 30% Buffer B over the course of 30 minutes, then at 70% Buffer B for 10 minutes followed with 7% Buffer B for another 10 minutes, all at a flow rate of 1 mL per minute. The collected fraction was subsequently dried in a Savant SpeedVac Concentrator and stored as the stock decoding probe solution at 4°C in 200 μL nuclease-free water to which 1X Tris EDTA (TE) (2 μL, pH 8.0) was added.

[0048] *Consecutive RNA FISH:* The pre-decoding hybridization solution was prepared by dissolving 10 pmol of GAPDH and Ki67 pre-decoding probes in 100 μL of pre-decoding hybridization buffer (100 mg/mL dextran sulfate, 1 mg/mL *Escherichia coli* tRNA, 2 mM vanadyl ribonucleoside complex, 20 μg/mL bovine serum albumin and 10% formamide in 2X SSC). The decoding hybridization solution was prepared by adding 2 μL of the Quasar 570 or Cy5 labeled stock decoding probe solution to 100 μL of decoding hybridization buffer (100 mg/mL dextran sulfate, 2 mM vanadyl ribonucleoside complex, and 10% formamide in 2X SSC).

[0049] The fixed HeLa CCL-2 cells were first permeabilized with 70% (v/v) EtOH at 4°C for at least overnight, and then washed once by wash buffer (10% formamide in 2X

SSC) for 5 minutes. Subsequently, the cells were incubated with pre-decoding hybridization solution at 37°C overnight, and washed 3 times with wash buffer, each at 37°C for 30 minutes. Then, the cells were incubated with decoding hybridization solution at 37°C for 30 minutes, and then with 5ng/mL DAPI in wash buffer at 37°C for 30 minutes. After incubated with GLOX buffer (0.4% glucose and 10mM Tris HCl in 2X SSC) for 1-2 min, the stained cells were imaged in GLOX solution (0.37 mg/mL glucose oxidase and 1% catalase in GLOX buffer). After imaging, each target cell in 1x PBS was photobleached individually with the Quasar 570 filter for 20 s or the Cy5 filter for 5 s at each z step. 1x PBS was changed every 3 minutes during photobleaching to remove the radicals. Following photobleaching, the HeLa cells were imaged again in GLOX solution, and then incubated with the next cycle decoding hybridization solution.

[0050] *Consecutive DNA FISH:* The fixed HeLa CCL-2 cells were incubated with 0.5% (v/v) Triton-X100 in 1X PBS at RT for 10 minutes, and then with 0.1% Tween-20 in 1X PBS at RT for 2 min. After incubated with 0.1 M HCl at RT for 5 min, the cells were washed three times with 2X SSCT (0.3 M NaCl, 0.03 M Sodium Citrate, 0.1% Tween-20) at RT for 2 minutes. The cells were incubated with 70% formamide in 2X SSCT at RT for 5 min, then at 78°C for 15 minutes, and then at 60°C for 20 minutes. Subsequently, the cells were incubated with 100 μL of predecoding hybridization solution composed of 80 pmol of the decoding probes, 2X SSCT, 50% formamide and 10% dextran sulfate (wt/vol) at 78°C for 5 minutes. After hybridized at 37°C overnight, the cells were washed in 2X SSCT at 60°C for 15 minutes, then in 2X SSCT at RT for 10 min, and then for 10 min in 0.2X SSC at RT. Subsequently, the cells were incubated with 100 μL of decoding hybridization solution composed of 2 μL of the Quasar 570 or Cy5 labeled stock decoding probe solution, 2X SSCT, 10% formamide and 10% dextran sulfate (wt/vol) at 37°C for 30 minutes. The cells were washed with 10% formamide in 2X SSCT at 37°C for 30 minutes, and then with 5 ng/mL DAPI and 10% formamide in 2X SSCT at 37°C for 30 minutes. After washed with GLOX buffer for 1-2 min at TR, the cells were imaged in GLOX solution. After imaging, each target cell in 1X PBS was photobleached individually with the Quasar 570 filter for 20 sseconds or the Cy5 filter for 5 seconds at each z step. 1X PBS was changed every 3 minutes during photobleaching to remove the radicals. Following photobleaching, the HeLa cells were imaged again in GLOX solution, and then incubated with the next cycle decoding hybridization solution.

[0051] *Imaging and data analysis:* Stained cells were imaged under a Nikon Ti-E epifluorescence microscope equipped with 100X objective, using a 5-μm z range and 0.3-μm z pacing. Images were captured using a CoolSNAP HQ2 camera and NIS-Elements Imaging software. Chroma filters 49004 and 49009 were used for Quasar 570 and Cy5, respectively.

[0052] Example 1: Single-cell *in situ* transcript analysis by C-FISH

[0053] To demonstrate the feasibility of applying C-FISH for single-cell *in situ* transcriptome analysis, we designed and synthesized pre-decoding probes and corresponding decoding probes for mRNA GAPDH and Ki67. The mRNA binding sequences on the pre-decoding probes were generated by using the well documented software[5] for RNA FISH probe design. And the binding sequences on decoding probes were selected from a set of orthogonal sequences, which have been established to minimize cross-hybridization[15]. We synthesized the pre-coding probes with natural nucleotides and amino modified decoding probes on a DNA synthesizer. Subsequently, the amino groups on the decoding probes were coupled to NHS ester modified Quasar 570 or Cy5. In each C-FISH cycle, two decoding probes labeled with different fluorophores were applied to stain GAPDH and Ki67 transcripts, respectively (FIG. 2A, top). Through 16 cycles of hybridization, imaging, and photobleaching, individual GAPDH and Ki67 transcripts were unambiguously detected as fluorescent spots with distinct color sequences (FIG. 2A, bottom). The copy numbers and spatial distributions of transcripts obtained by C-FISH are consistent with those in conventional smFISH (FIGS. 2B and 2C). The copy numbers per cell obtained by C-FISH and conventional smFISH closely resembled each other (FIG. 3A). Additionally, more than 95% of the spots identified by colocalization in the first two C-FISH cycles reoccurred throughout all the following 14 cycles (FIG. 3B). All the reappearing spots could be accurately and unambiguously identified by comparing the signal intensities in different fluorescence channels. These results suggest that the time-consuming error correction process in sequential hybridization and MER-FISH can be completely removed; and the entire transcriptome can be quantified by C-FISH using the $2^{16} = 65,536$ distinct color sequences at the single-molecule sensitivity.

[0054] Example 2: Single-cell *in situ* genomic loci analysis by C-FISH

[0055] To demonstrate the feasibility of applying C-FISH for single-cell *in situ* genome analysis, we designed and prepared 100 pre-decoding probes targeting human 4p16.1. The pre-

decoding probes for DNA C-FISH (Table 3) were designed and synthesized similarly as the ones for RNA C-FISH (see Table 1 and Table 2). For each DNA or RNA C-FISH cycle, two orthogonal decoding probes labeled with Quasar 570 or Cy5 (Tables 4 and 5) were incubated with HeLa cells. Only one of two probes has complimentary binding sequences to the probes used in the previous cycle. As shown in FIG. 4, nine cycles of hybridization, imaging, and photobleaching were performed to unambiguously detect individual genomic loci 4p16.1 as fluorescent spots with a unique color sequence.

Table 1. RNA C-FISH Pre-decoding Probes (5'-3')

GAPDH (5'-3')	SEQ ID NO:
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttaacggctgcccattcattt	1
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttggagaaatcgggccagctag	2
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttctaggaaaagcatcacccg	3
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcccaatacgaccaaatcaga	4
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcagagttaaaagcagccctg	5
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttgggtcattgatggcaacaat	6
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttaccatgtagttgaggtcaat	7
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcatgggtggaatcatattgg	8
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttttgacggtgccatggaattt	9
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcattgatgacaagcttcccg	10
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttctggaagatggtgatgggat	11
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcttgattttggagggatctc	12
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcagtggactccacgacgtac	13
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttttctccatggtggtgaagac	14
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttagagatgatgacccttttgg	15
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttatgacgaacatgggggcatc	16
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttgtcatacttctcatggttca	17
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttattgctgatgatcttgaggc	18
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttctaagcagttggtggtgcag	19
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttagttgtcatggatgaccttg	20
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcatgagtccttccacgatac	21
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcagtgatggcatggactgtg	22
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttagaggcagggatgatgttc	23
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcagctcagggatgaccttg	24
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcactgacacgttggcagtgg	25
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttttggcaggtttttctagacg	26
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcaccttcttgatgtcatcat	27
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttgctgttgaagtcagaggaga	28
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttcgtcaaaggtggaggagtgg	29
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttaaagtggtcgttgagggcaa	30
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttgtcataccaggaaatgagct	31
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttgttgctgtagccaaattcg	32
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tcatccgatatggtgatccatttttcatccgatatggtgatccatttttaactgtgaggaggggagatt	34

tcatccgatatggtgatccatttttcatccgatatggtgatccatttttctcttcaaggggtctacatg	35
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttatggtacatgacaaggtgcg	36
tcatccgatatggtgatccatttttcatccgatatggtgatccatttttttaactggttgagcacaggg	37

Table 2. RNA C-FISH Pre-decoding Probes (5'-3')

Ki67 (5'-3')	SEQ ID NO:
gtctttacagatgtctgcggtttttgtctttacagatgtctgcggtttttgcattaccagagactttct	38
gtctttacagatgtctgcggtttttgtctttacagatgtctgcggtttttggcttataaccaagctttgt	39
gtctttacagatgtctgcggtttttgtctttacagatgtctgcggtttttggagttttcctaggactag	40
gtctttacagatgtctgcggtttttgtctttacagatgtctgcggtttttaggaacctctgtctgagat	41
gtctttacagatgtctgcggtttttgtctttacagatgtctgcggtttttagacactctctttgaaggca	42
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gtctttacagatgtctgcggtttttgtctttacagatgtctgcggtttttttgcacgttgcttcaatacttt	73
gtctttacagatgtctgcggtttttgtctttacagatgtctgcggtttttttttgcagattccttcaatgc	74

Table 3. DNA C-FISH Pre-decoding Probes

5'->3'

5'-tcatccgatatggtgatccatttttctgttccttgctccttgctttctccatactc (SEQ ID NO:75)
5'-tcatccgatatggtgatccatttttcttgaacacctttttccaactcaccgtgctggt (SEQ ID NO:76)
5'-tcatccgatatggtgatccatttttggcattctcttcaaacctccaggtattgtgta (SEQ ID NO:77)
5'-tcatccgatatggtgatccatttttgttacagcataagatagaccgtaaaagccaac (SEQ ID NO:78)
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5'-tcatccgatatggtgatccatttttatgatggcaatcctggatgagtgttttagtct (SEQ ID NO:80)
5'-tcatccgatatggtgatccatttttttctgccattagctgtgggtcgatcgtgtccta (SEQ ID NO:81)
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5'-tcatccgatatggtgatccatttttgcttgtgtgcagggatgacttaaaggaagtct (SEQ ID NO:115)
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5'-tcatccgatatggtgatccatttttttccatctgtaaactaggatgagtagttttccc (SEQ ID NO:142)
5'-tcatccgatatggtgatccatttttggtttgagctgataagacatagaagtactaca (SEQ ID NO:143)
5'-tcatccgatatggtgatccatttttaggatgggagcctgagtcaactggttttgtaa (SEQ ID NO:144)
5'-tcatccgatatggtgatccatttttgctacatgctcttgcttccttcattgttcttg (SEQ ID NO:145)
5'-tcatccgatatggtgatccatttttagtgcttatgcctttgtggttctcacagcacc (SEQ ID NO:146)
5'-tcatccgatatggtgatccatttttttgagcagttctaaaccaagttcaagttactag (SEQ ID NO:147)
5'-tcatccgatatggtgatccatttttccatgagctcagtctctaaggcccggtaacat (SEQ ID NO:148)
5'-tcatccgatatggtgatccatttttgacttgggttaattagccttctagttgaaggt (SEQ ID NO:149)
5'-tcatccgatatggtgatccatttttggagtctgtagaacacctttcctatggagtct (SEQ ID NO:150)
5'-tcatccgatatggtgatccatttttttccacggcaccctcctaccaaggctcttgca (SEQ ID NO:151)
5'-tcatccgatatggtgatccatttttggcagcagcttagaaggccaagacttagctgc (SEQ ID NO:152)
5'-tcatccgatatggtgatccatttttcttgcagtagtcaagcatgacataccctattt (SEQ ID NO:153)

5'-tcatccgatatggtgatccatttttagctgtccatgaatttggaagtgatgtccctg (SEQ ID NO:154)
5'-tcatccgatatggtgatccatttttaagtaccatggatgtgtataggctcctgagtc (SEQ ID NO:155)
5'-tcatccgatatggtgatccatttttctccttccctctggcacataggtatgttcca (SEQ ID NO:156)
5'-tcatccgatatggtgatccatttttaatagtgacccaacgagatctcctgtgaaatg (SEQ ID NO:157)
5'-tcatccgatatggtgatccatttttagaccaggagagttaattaattacctgaggac (SEQ ID NO:158)
5'-tcatccgatatggtgatccatttttgtatacttctggccttctcaggagtaagcaga (SEQ ID NO:159)
5'-tcatccgatatggtgatccatttttaccctcgatgaggaggtttggaaagaatggat (SEQ ID NO:160)
5'-tcatccgatatggtgatccatttttgttgactctgtacttaatgtcagaagtggtgg (SEQ ID NO:161)
5'-tcatccgatatggtgatccatttttcaaagataccacctgtcatgcaacatatatgt (SEQ ID NO:162)
5'-tcatccgatatggtgatccatttttactgtcgtgatgtggaactccaaaaggtgaat (SEQ ID NO:163)
5'-tcatccgatatggtgatccatttttatgcagaatgtcatcccttccatacttggcag (SEQ ID NO:164)
5'-tcatccgatatggtgatccatttttttgatttcgttaccttgaaccagtggccaaag (SEQ ID NO:165)
5'-tcatccgatatggtgatccatttttttgacatttgtgctttaggaccatactaggat (SEQ ID NO:166)
5'-tcatccgatatggtgatccatttttcattaaacatgagcccttaaagcagcagttgc (SEQ ID NO:167)
5'-tcatccgatatggtgatccatttttgagcaggttatctttattggcacaaatgttac (SEQ ID NO:168)
5'-tcatccgatatggtgatccatttttgttgctctaggagaactggagtcctagcccag (SEQ ID NO:169)
5'-tcatccgatatggtgatccatttttacaggccaaggagttgtgacccattagctttg (SEQ ID NO:170)
5'-tcatccgatatggtgatccatttttgtgcatggctaagtcattagcttaaccattgc (SEQ ID NO:171)
5'-tcatccgatatggtgatccatttttgagttcccagcagcaaccacagggagggccag (SEQ ID NO:172)
5'-tcatccgatatggtgatccatttttttgtcgcatgaacatgccacatctgtccacca (SEQ ID NO:173)
5'-tcatccgatatggtgatccatttttttgagaattgtcttcccagaagggtaggtcctt (SEQ ID NO:174)

Table 4. DNA C-FISH Decoding Probes

		SEQ ID NO:
cycle1	5'-Quasar 570-tggatcaccatatcggatgatttttggctatgtccgtaacactccttttttggctatgtccgtaacactcc	175
cycle2	5'-Cy5-gaaccatggtacctgagatctttttgaaccatggtacctgagatctttttggagtgttacggacatagcc	176
cycle3	5'-Quasar 570-gatctcaggtaccatggttctttttcgtccagattatgtttctccttttttcgtccagattatgtttctcc	177
cycle4	5'-Cy5-cagcttagaatagaacattgtttttcagcttagaatagaacattgtttttggagaaacataatctggacg	178
cycle5	5'-Quasar 570-caatgttctattctaagctgtttttctggatgatgttctctcatcttttttctggatgatgttctctcatc	179
cycle6	5'-Cy5-gctcaagtgttggtgaaggtttttttgctcaagtgttggtgaaggtttttttgatgagagaacatcatccag	180
cycle7	5'-Quasar 570-accttcaccaacacttgagctttttatagccctggacatgaacgtttttttatagccctggacatgaacgt	181
cycle8	5'-Cy5-cgtacagaggtagcaagggatttttcgtacagaggtagcaagggatttttacgttcatgtccagggctat	182
cycle9	5'-Quasar 570-tcccttgctacctctgtacgtttttcgatgcatcaggtacccagtttttttcgatgcatcaggtacccagt	183

Table 5. RNA C-FISH Decoding Probes

		SEQ ID NO:
GAPDH		

cycle1	5'-Quasar 570-tggatcaccatatcggatgatttttggctatgtccgtaacactccttttggctatgtccgtaacactcc	184
cycle2	5'-Cy5-gaaccatggtacctgagatctttttgaaccatggtacctgagatctttttggagtgttacggacatagcc	185
cycle3	5'-Quasar 570-gatctcaggtaccatggttctttttcgtccagattatgtttctccttttcgtccagattatgtttctcc	186
cycle4	5'-Cy5-cagcttagaatagaacattgtttttcagcttagaatagaacattgtttttggagaaacataatctggacg	187
cycle5	5'-Quasar 570-caatgttctattctaagctgtttttctggatgatgttctctcatctttttctggatgatgttctctcatc	188
cycle6	5'-Cy5-gctcaagtgttggtgaaggtttttgctcaagtgttggtgaaggtttttgatgagagaacatcatccag	189
cycle7	5'-Quasar 570-accttcaccaacacttgagctttttatagccctggacatgaacgtttttatagccctggacatgaacgt	190
cycle8	5'-Cy5-cgtacagaggtagcaagggatttttcgtacagaggtagcaagggatttttacgttcatgtccagggctat	191
cycle9	5'-Quasar 570-tcccttgctacctctgtacgtttttcgatgcatcaggtacccagttttttcgatgcatcaggtacccagt	192
Cycle10	5'-Cy5-gatggtcgtagtgtggcacatttttgatggtcgtagtgtggcacatttttactgggtacctgatgcatcg	193
Cycle11	5'-Quasar 570-tgtgccacactacgaccatctttttatgagtcttccagtcgtagtttttatgagtcttccagtcgtagt	194
Cycle12	5'-Cy5-tcacgtaatgttctccacgatttttcacgtaatgttctccacgattttactacgactggaagactcat	195
Cycle13	5'-Quasar 570-tcgtggagaacattacgtgatttttcttgctatcttccagcgaagtttttcttgctatcttccagcgaag	196
Cycle14	5'-Cy5-tagttgaaggagtgctcgtgtttttttagttgaaggagtgctcgtgtttttcttcgctggaagatagcaag	197
Cycle15	5'-Quasar 570-cacgagcactccttcaactatttttcacttcgtcatggagcatgatttttcacttcgtcatggagcatga	198
Cycle16	5'-Cy5-tcatgctccatgacgaagtg	199
Ki67		
Cycle1	5'-Cy5-ccgcagacatctgtaaagacttttccgatgttgacggactaatctttttccgatgttgacggactaatc	200
Cycle2	5'-Quasar 570-tagtagttcagacgccgttatttttttagtagttcagacgccgttatttttgattagtccgtcaacatcgg	201
Cycle3	5'-Cy5-taacggcgtctgaactactatttttccgtacctagatacactcaatttttccgtacctagatacactcaa	202
Cycle4	5'-Quasar 570-ccaggcaatatggtggtacatttttccaggcaatatggtggtacatttttttgagtgtatctaggtacgg	203
Cycle5	5'-Cy5-tgtaccaccatattgcctggtttttcgtgaagcttgagtggaatctttttcgtgaagcttgagtggaatc	204
Cycle6	5'-Quasar 570-gtgtgaggcgctagagcatatttttgtgtgaggcgctagagcatatttttgattccactcaagcttcacg	205
Cycle7	5'-Cy5-tatgctctagcgcctcacacttttggtatggcacgcctaatctgtttttggtatggcacgcctaatctg	206
Cycle8	5'-Quasar 570-atctccagtggcatccttctttttatctccagtggcatccttctttttcagattaggcgtgccatacc	207
Cycle9	5'-Cy5-agaaggatgccactggagatttttttggtaactgcgcatagttggctttttggtaactgcgcatagttggc	208
Cycle10	5'-Quasar 570-ggtacagtaagtgagaatccttttggtacagtaagtgagaatccttttgccaactatgcgcagttacc	209
Cycle11	5'-Cy5-ggattctcacttactgtacctttttgccaccttaacacgcgatgatttttgccaccttaacacgcgatga	210
Cycle12	5'-Quasar 570-cattgatcttggtgctgctgtttttcattgatcttggtgctgctgtttttttcatcgcgtgttaaggtggc	211
Cycle13	5'-Cy5-cagcagcaccaagatcaatgtttttgctattacgagcgcttggatttttttgctattacgagcgcttggat	212
Cycle14	5'-Quasar 570-tatgttgtgccttacgcctctttttttatgttgtgccttacgcctctttttatccaagcgctcgtaatagc	213
Cycle15	5'-Cy5-gaggcgtaaggcacaacatatttttttaaccgaactgacggccatttttttttaaccgaactgacggccat	214
Cycle16	5'-Quasar 570-atggccgtcagttcggttaa	215

[0056] Example 3: Production of C-FISH probes

[0057] As shown in FIG. 5, C-FISH probes can be obtained by enzymatic amplification of an array-derived oligonucleotide pool. Each sequence in the oligonucleotide pool contains a T7 promoter sequence, a variable probe sequence, and two flanking index primers. First, templates from a library of probes are selected and amplified by indexed PCR (polymerase chain reaction). Next, the resulting PCR products are further amplified into RNA by *in vitro* transcription. Finally, the RNA library is reverse transcribed into cDNA. Natural (unlabeled) or fluorescently labeled oligonucleotides are used as reverse transcription to generate pre-decoding or decoding probes, respectively.

[0058] Using the protocol illustrated in FIG. 5, RNA C-FISH probes are designed and generated for 30 selected genes in mTOR, PI(3)K, Ras, Wnt, or Notch pathways, as the transcriptional profiles of these genes in HeLa cells are well documented by RNA-Seq. To make C-FISH signals detectable, at least 40 pre-decoding probes are required to hybridize to individual transcripts. To cost-effectively generate these large number of predecoding probes simultaneously, a well-established oligopaint protocol is used (see FIG. 5). This protocol has been successfully applied in our laboratory to prepare libraries of oligonucleotides for *in situ* DNA analysis.

[0059] Briefly, PCR reactions are performed to amplify the 1200 probe sequences from a library of array-derived oligonucleotides containing tens of thousands of custom sequences. After purification using a Zymo DNA purification column, the PCR products are further amplified by transcription into RNA using a T7 *in vitro* transcription kit. The T7 reaction is incubated at 37°C for 4 hours to maximize yield. Subsequently, the RNA products are purified using a RNeasy spin column and reverse-transcribed into single-stranded pre-coding probes using Maxima reverse transcription kit. To selectively hydrolyze the template RNA, 20 μL of 0.25 M EDTA and 0.5 N NaOH are added to 50 μL of the reaction mixture. After incubation at 95°C for 10 minutes, the library of the pre-coding probes is then purified using a Zymo DNA purification column. To analyze 30 different transcripts with decoding probes labeled with two different fluorophores, 5 cycles of C-FISH ($2^5 = 32$) and 150 (30 X 5) orthogonal decoding probes are required. A DNA synthesizer is used to prepare this relatively small number of amino modified oligonucleotides. For each cycle of C-FISH, 15 decoding probes are labeled with Quasar 570 and another 15 decoding probes are labeled with Cy5. To couple the 15 probes to

corresponding fluorophores simultaneously, a reaction mixture is prepared that contains 1 nmol of combined oligonucleotides, 100 nmol NHS ester modified fluorophores, 1 nL of 1 M NaHCO3 and 9 nL of nuclease free water. After incubation at room temperature overnight, the generated fluorescent decoding probes are purified using an HPLC equipped with a C18 column.

[0060] As shown in FIG. 6, C-FISH decoding probes can be obtained by chemical synthesis followed by enzymatic incorporation. Generated on a DNA synthesizer, decoding probes comprising unlabeled nucleotides are extended with NH_2-ddUTP (2',3'-dideoxyuridine-5'-triphosphate) by terminal transferase (TdT), which is a template independent polymerase that catalyzes the addition of deoxynucleotides to the 3' hydroxyl terminus of DNA molecules.

[0061] Using such a protocol, RNA C-FISH probes are designed and generated for 100 selected genes in mTOR, PI(3)K, Ras, Wnt, or Notch pathways using well documented transcriptional profiles in HeLa cells[16]. The large number (100 X 40 = 4, 000) of pre-decoding C-FISH probes for these 100 transcripts are prepared using the chemical synthesis followed by enzymatic incorporation approach as discussed above. To quantify 100 different transcripts using decoding probes labeled with two different fluorophores, 7 cycles of C-FISH (2^7 = 128) and 700 (100 X 7) orthogonal decoding probes will be required. To generate these decoding probes precisely and cost-effectively, a DNA synthesizer is used first to prepare all 700 decoding probes with natural, unlabeled nucleotides. Subsequently, each of the 50 probes that will be used in the same C-FISH cycle are combined and labeled with the same fluorophore. Then, a template-independent terminal transferase (TdT) is used to incorporate amino modified ddUTP at the 3' ends of all 50 probes simultaneously (see FIG. 6). To 1 mL of 1X TdT reaction buffer, 100 μL of 2.5 mM $CoCl_2$, 1 nmol of combined oligonucleotides, 5 nmol amino modified ddUTP, and 1 unit of TdT are added. After incubated at 37°C for 1 hour, the extended oligonucleotides are purified using a Qiagen nucleotide removal column. Finally, the amino modified decoding probes are coupled the specific fluorophores and purified by HPLC, using the same protocol as described above. The decoding probes will be precisely generated using this approach, as they are prepared one by one on a DNA synthesizer. Additionally, since 3' amino modifier phosphoramidite ($8/25 nmol) is much more expensive than natural nucleotides ($0.1/25 nmol), we will dramatically reduce the probe preparation cost by avoiding the direct chemical synthesis of amino modified probes.

[0062] Example 4: C-FISH probes labeled with cleavable fluorophores

[0063] As shown in FIG. 7, fluorophores are attached to the oligonucleotides through a chemically cleavable linker. After hybridization with these probes, it is possible to simultaneously chemically cleave all of the different fluorophores in the whole sample in a short time.

[0064] To assess the feasibility of this approach, we designed and synthesized an azido-based linker (FIG. 8A). The amino group on one end of the linker was coupled with TAMRA NHS ester. Then, the hydroxyl group on the other end of the linker was converted into a new NHS ester group. This group was used to conjugate cleavable TAMRA to amino groups on oligonucleotides. Using this scheme, we can prepared cleavable fluorescent C-FISH probes labeled with any commercially available NHS fluorophores. With the cleavable TAMRA labeled probes, we performed C-FISH to analyze Ki67 transcripts. After the first cycle of hybridization and imaging (FIG. 8B), the cells were incubated with 100 mM tris(2-carboxyethyl)phosphine (TCEP) at 37°C and pH 9.0 for 30 minutes. With the cleavage efficiency of more than 95%, this chemical cleavage reaction erased all the original fluorescent spots (FIG. 8C). After the second cycle of hybridization with Cy5 labeled probes, almost all the spots reappear (FIG. 8D), suggesting that the TCEP treatment does not damage RNA molecules. The very small number of non-colocalized spots may be generated by non-specific probe binding. Additionally, the color of all the spots were determined accurately, indicating the minimum leftover signals from the previous cycle does not interfere with the subsequent cycles. The copy numbers and spatial distributions of Ki67 transcripts obtained by cleavable C-FISH probes are consistent with those in conventional smFISH (FIG. 2C). These results demonstrate that the fluorophores on cleavable fluorescent C-FISH probes can be efficiently removed by a RNA compatible reaction within 30 minutes, which enables high-throughput and rapid RNA analysis in single cells *in situ*.

[0065] Example 5 - Two-Probe C-FISH Method

[0066] In first-generation C-FISH, each transcript or genomic locus requires a unique decoding probe per hybridization cycle. For *in situ* genome or transcriptome analysis, a large number of decoding probes must be designed and prepared. As a result, the potential cross-hybridization between these decoding probes may lead to false positive signals. Additionally, the cost to prepare this large number of probes can be relatively high.

[0067] To further minimize the possibility of probe cross-hybridization and reduce the assay cost, we developed the second-generation C-FISH with only two decoding probes for each transcript or genomic locus. In this approach, each transcript or genomic locus is hybridized with a set of pre-decoding probes. These probes have varied targeting sequences to bind to the different regions on the target, and the shared decoding sequence to bind to decoding probes. Subsequently, the fluorescently labeled decoding probe "A" hybridizes to the pre-decoding probes, and also introduces the binding sites for the probe used in the second cycle. After fluorescence imaging and data storage, the fluorescence signals are efficiently removed. Then, the fluorescently labeled decoding probe "B" hybridizes to the decoding probe "A", and also introduces the binding sites, which will be hybridized by decoding probe "A" again in the third hybridization cycle. Through consecutive hybridization of probes "A" and "B", each transcript or genomic loci is identified as a fluorescent spot with a unique color sequence. In this way, to quantify 30,000 transcripts or genomic loci with two colors, the number of required decoding probes is reduced about one order of magnitude from 480,000 to 60,000.

[0068] To demonstrate the feasibility of this approach, we stained two mRNA GAPDH and Ki67 with only 4 decoding probes by 16 cycles of consecutive hybridization. As shown in FIG. 9, each individual transcript is unambiguously detected. The results obtained by the second-generation C-FISH and the first-generation C-FISH closely resemble each other. These results suggest that transcripts and genomic loci can be quantitatively detected in single cells *in situ* by the second-generation C-FISH.

[0069] The experimental methods for the first-generation and second-generation C-FISH are similar. The predecoding probes used in the two approaches are the same. The sequences of the decoding probes used in the second-generation C-FISH are as follows:

GAPDH

Probe "A" 5'-Quasar 570-tggatcaccatatcggatgatttttggctatgtccgtaacactccttttttggctatgtccgtaacactcc (SEQ ID NO:216)

Probe "B" 5'-Cy5-tcatccgatatggtgatccatttttttcatccgatatggtgatccatttttggagtgttacggacatagcc (SEQ ID NO:217)

Ki67

Probe "A" 5'-Cy5-ccgcagacatctgtaaagacttttttccgatgttgacggactaatctttttttccgatgttgacggactaatc

(SEQ ID NO:218)

Probe "B" 5'-Quasar 570-gtctttacagatgtctgcggtttttgtctttacagatgtctgcggtttttgattagtccgtcaacatcgg (SEQ ID NO:219)

[0070] The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention.

CLAIMS

We claim:

1. A method of *in situ* analysis of transcripts or genomic loci in a single cell, the method comprising the following steps:

(a) performing a first contacting step that comprises contacting a cell comprising a plurality of transcripts or genomic loci to a plurality of pre-decoding oligonucleotides, wherein each pre-decoding oligonucleotide of the plurality comprises a targeting sequence that specifically hybridizes to a target transcript or genomic locus and further comprises one or more binding sites for specific hybridization to a decoding oligonucleotide in a subsequent hybridization step, wherein the first contacting step occurs under conditions that promote hybridization of the plurality of pre-decoding oligonucleotides to target transcripts or genomic loci;

(b) performing a second contacting step that comprises contacting the cell with a plurality of decoding oligonucleotides, wherein each decoding oligonucleotide comprises a detectable moiety and is configured to have one or more binding sites for specific hybridization to a pre-decoding oligonucleotide, wherein the second contacting step occurs under conditions that promote hybridization of the decoding oligonucleotides to the pre-decoding oligonucleotides; and

(c) imaging the cell after the second contacting step whereby a detectable signal generated from hybridization of the decoding oligonucleotides to the pre-decoding oligonucleotides, whereby the detectable signal indicates hybridization to the target transcript or genomic locus.

2. The method of claim 1, further comprises:

(d) removing the detectable signal generated in step (c); and

(e) optionally consecutively repeating the contacting, imaging, and removing steps, each time with a new plurality of detectably labeled decoding oligonucleotides configured to have one or more binding sites for hybridization to detectably labeled decoding oligonucleotides of each subsequent cycle, wherein each new plurality of differs from detectably labeled decoding oligonucleotides of each subsequent cycle by at least one difference in detectable moiety labeling.

3. The method of claim 2, wherein steps (a)-(e) are consecutively performed at least 16 times.

4. The method of claim 1, wherein two decoding probes are used to detect each target transcript or genomic locus.

5. The method of claim 1, wherein the detectable moiety is selected from the group consisting of a fluorophore, radioactive isotope, and metal isotope.

6. The method of claim 5, wherein the fluorophore is selected from the group consisting of TAMRA, ALEXA FLUOR™ 594, ATTO 647N, and ATTO 700.

7. The method of claim 1, wherein the pre-decoding oligonucleotides target at least 10 different transcripts or genomic loci.

8. The method of claim 1, wherein removing the detectable signal comprises chemically cleaving the detectable moiety.

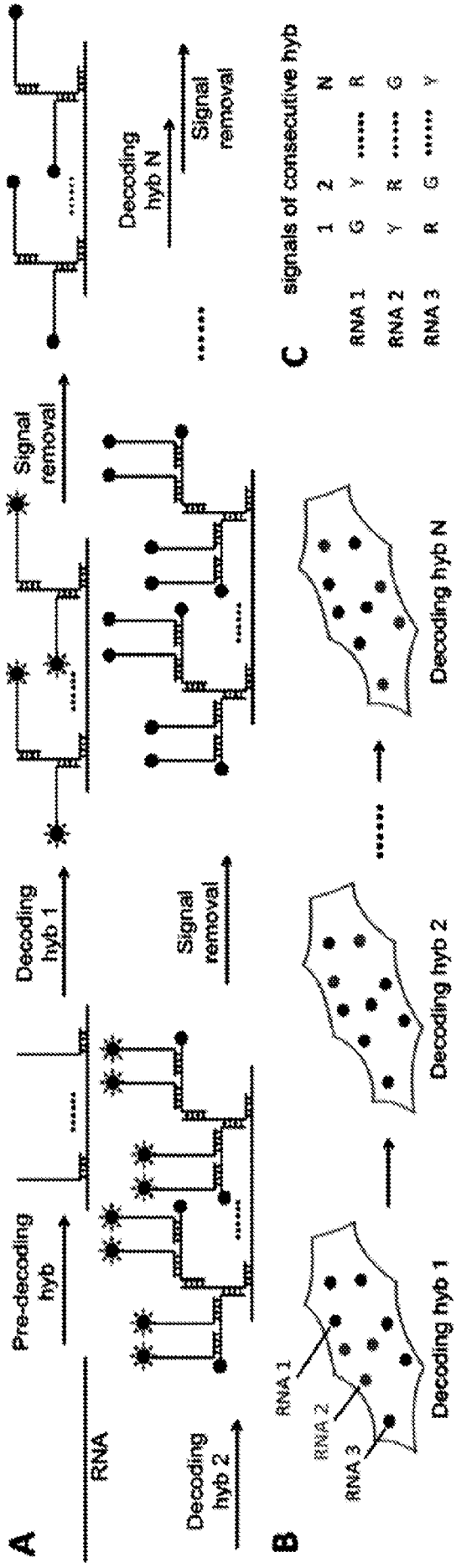
9. The method of claim 1, further comprising washing to remove unhybridized oligonucleotides and non-specifically hybridized oligonucleotides following each contacting step.

10. A kit for detecting target transcripts or genomic loci in a cell sample, the kit comprising a first plurality of oligonucleotide probes configured to hybridize to a target transcript or genomic locus; a second plurality of oligonucleotide probes, which second plurality includes oligonucleotides labeled with a detectable moiety and configured to have one or more binding sites for specific hybridization to oligonucleotides of the first plurality; and a written insert component comprising instructions for performing consecutive *in situ* analysis of target transcripts or genomic loci according to the method of claim 1.

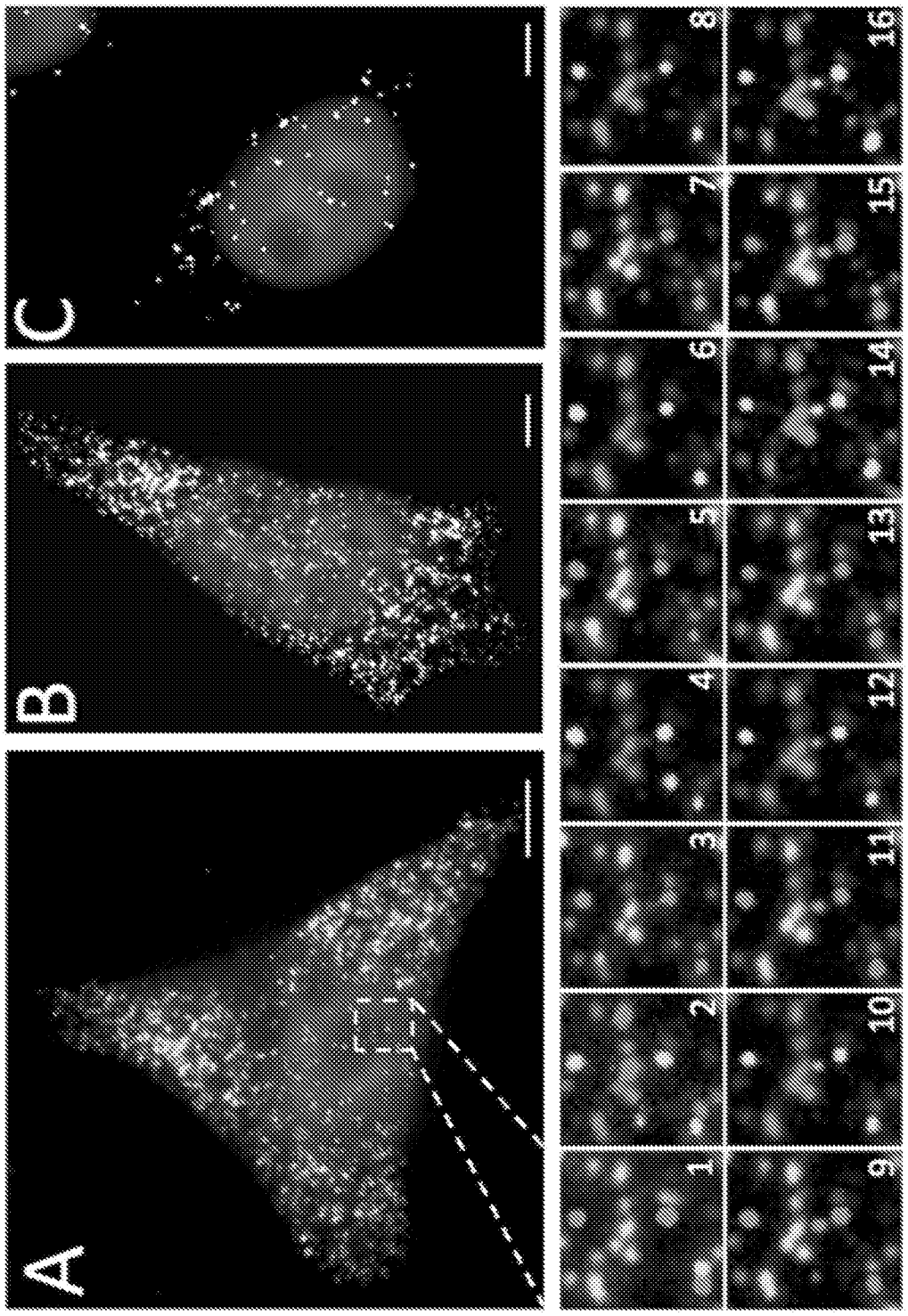
11. The kit of claim 10, wherein the detectable moiety is selected from the group consisting of a fluorophore, radioactive isotope, and metal isotope

12. The kit of claim 10, wherein oligonucleotide probes of the first or second plurality are selected from the group consisting of DNA oligonucleotides, RNA oligonucleotides, peptide nucleic acid (PNA) oligonucleotides, locked nucleic acid (LNA) oligonucleotides, peptide nucleic acids (PNAs), and modified oligonucleotides.

FIGS. 1A-1C


A
RNA
Pre-decoding hyb
Decoding hyb 1
Signal removal
Decoding hyb 2
Signal removal
Decoding hyb N
Signal removal
B
RNA 1
RNA 2
RNA 3
Decoding hyb 1
Decoding hyb 2
Decoding hyb N
C
signals of consecutive hyb
1 2 N
RNA 1 G Y ***** R
RNA 2 Y R ***** G
RNA 3 R G ***** Y

FIGS. 2A-2C



FIGS. 3A-3B

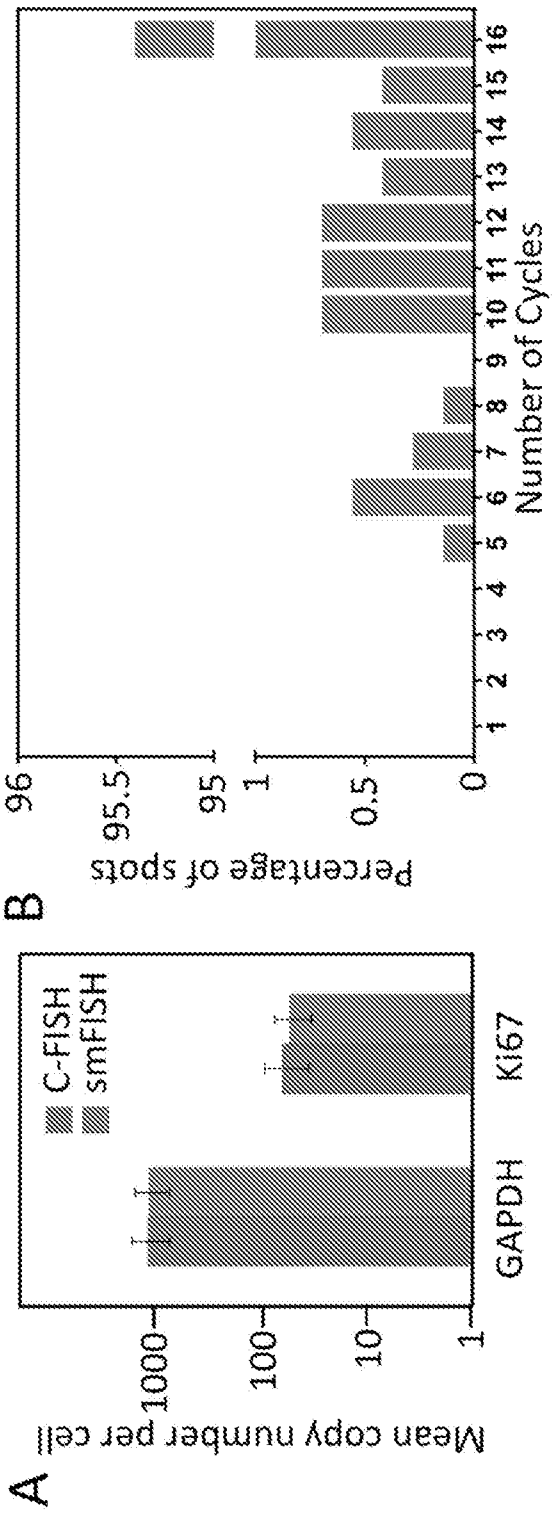

A
Mean copy number per cell
1000
100
10
1
C-FISH
smFISH
GAPDH
KI67
B
Percentage of spots
96
95.5
95
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Number of Cycles

FIG. 4

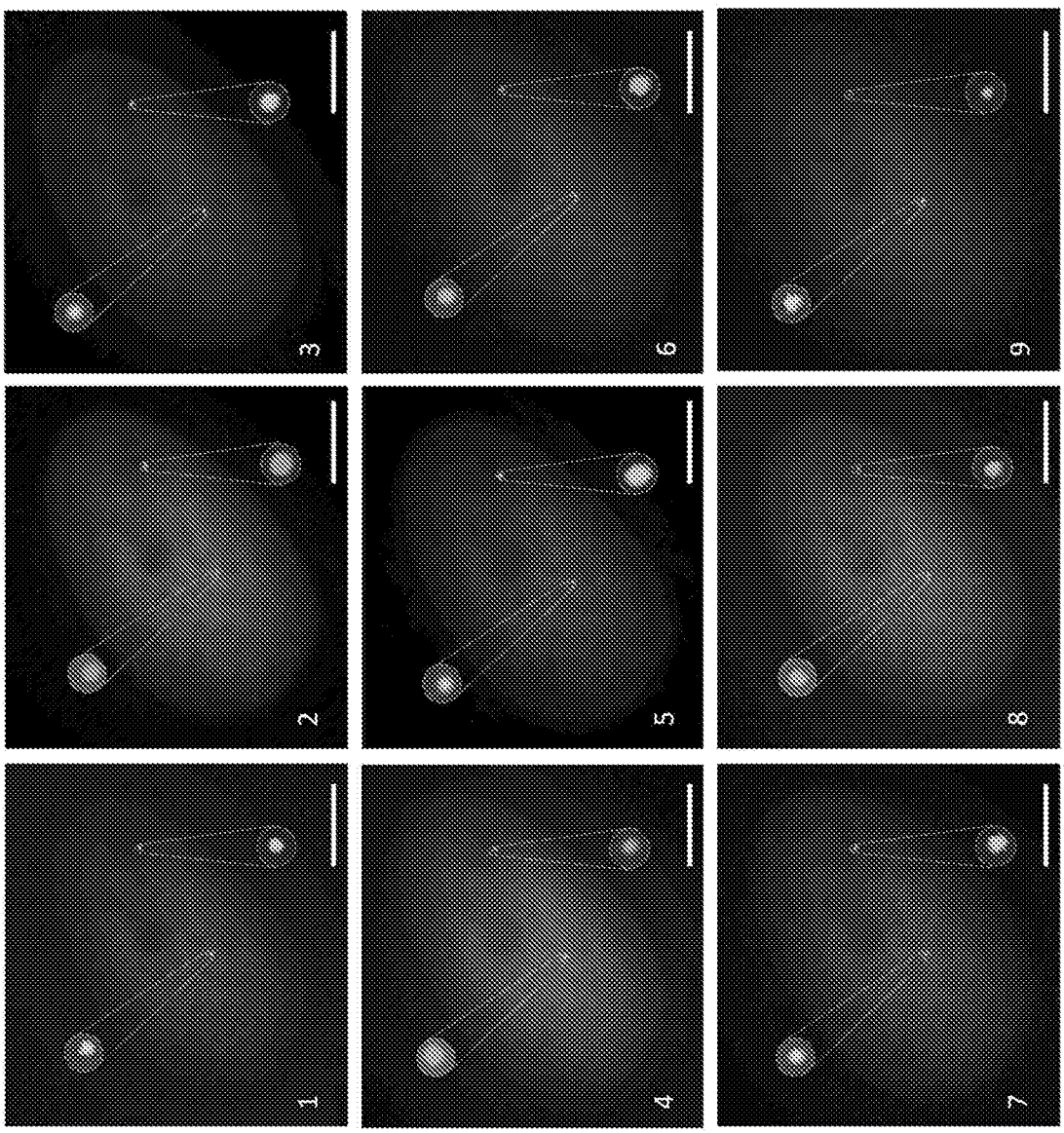


FIG. 5

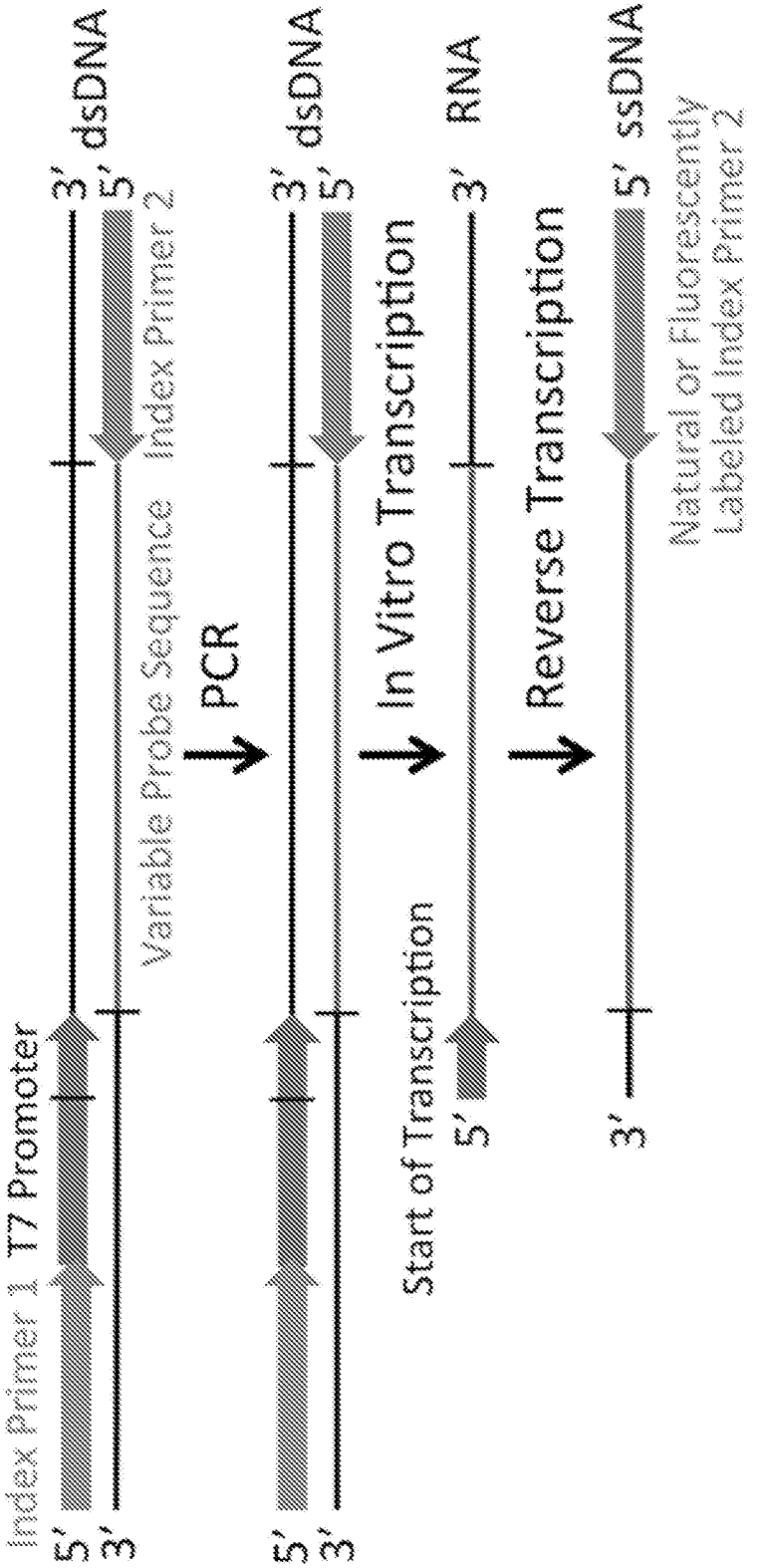

Index Primer 1
T7 Promoter
Variable Probe Sequence
Index Primer 2
5'
3'
3' dsDNA
5' dsDNA
PCR
In Vitro Transcription
Start of Transcription
3' RNA
5'
Reverse Transcription
5' ssDNA
3'
Natural or Fluorescently Labeled Index Primer 2

FIG. 6

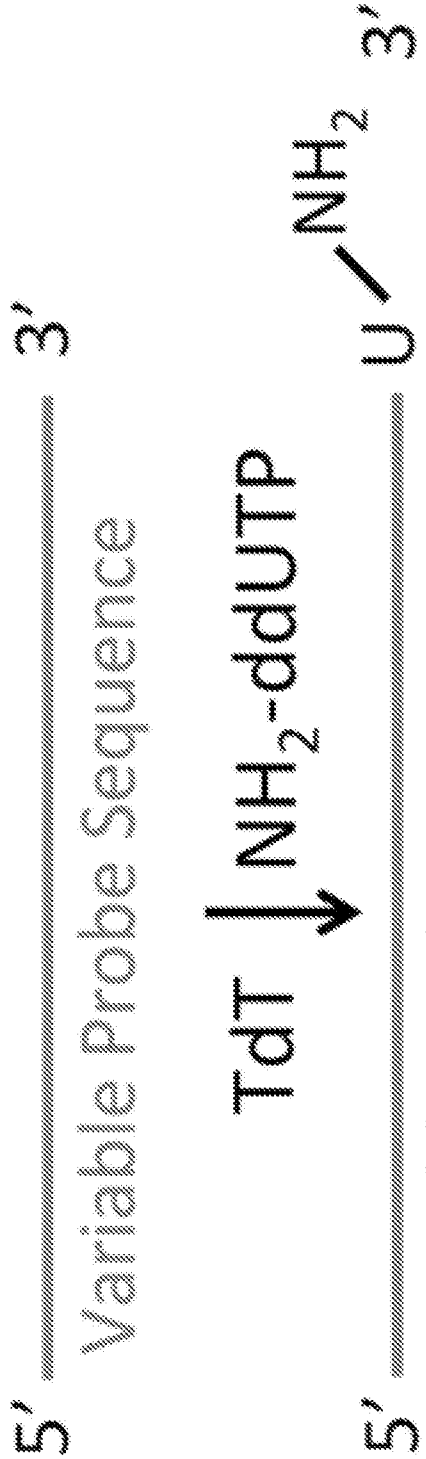
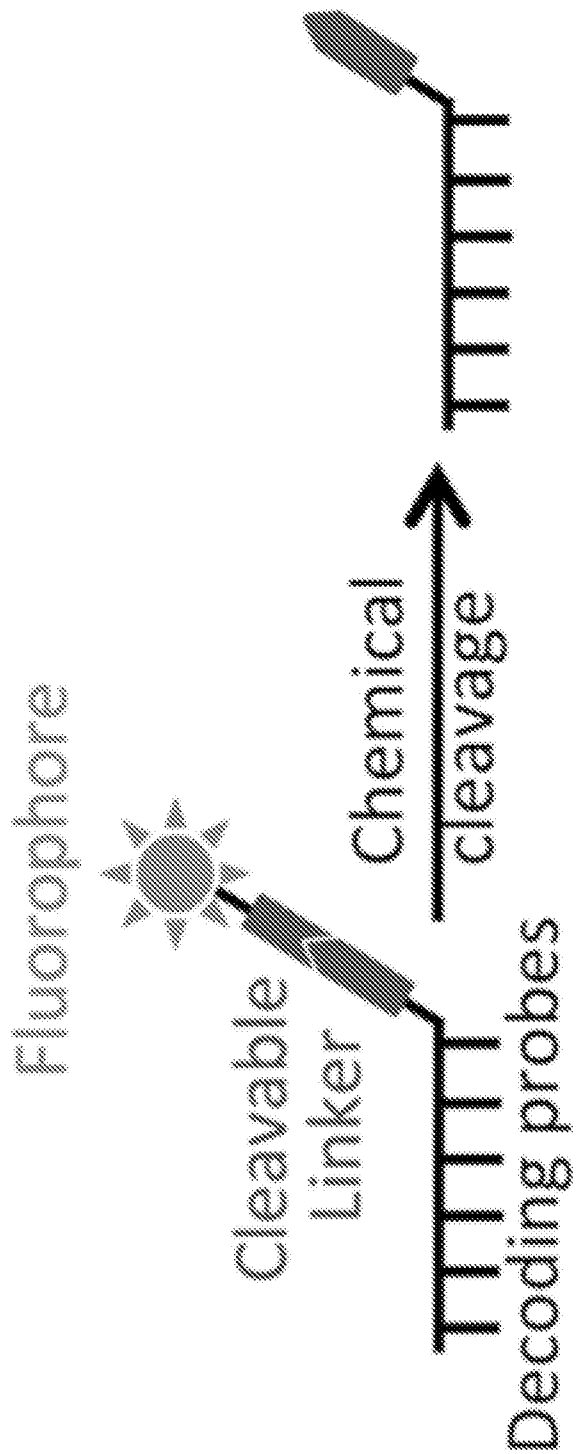

5'
3'
Variable Probe Sequence
TdT
NH2-ddUTP
U
NH2
5'
3'
Variable Probe Sequence

FIG. 7


Fluorophore
Cleavable Linker
Decoding probes
Chemical cleavage

FIGS. 8A-8D

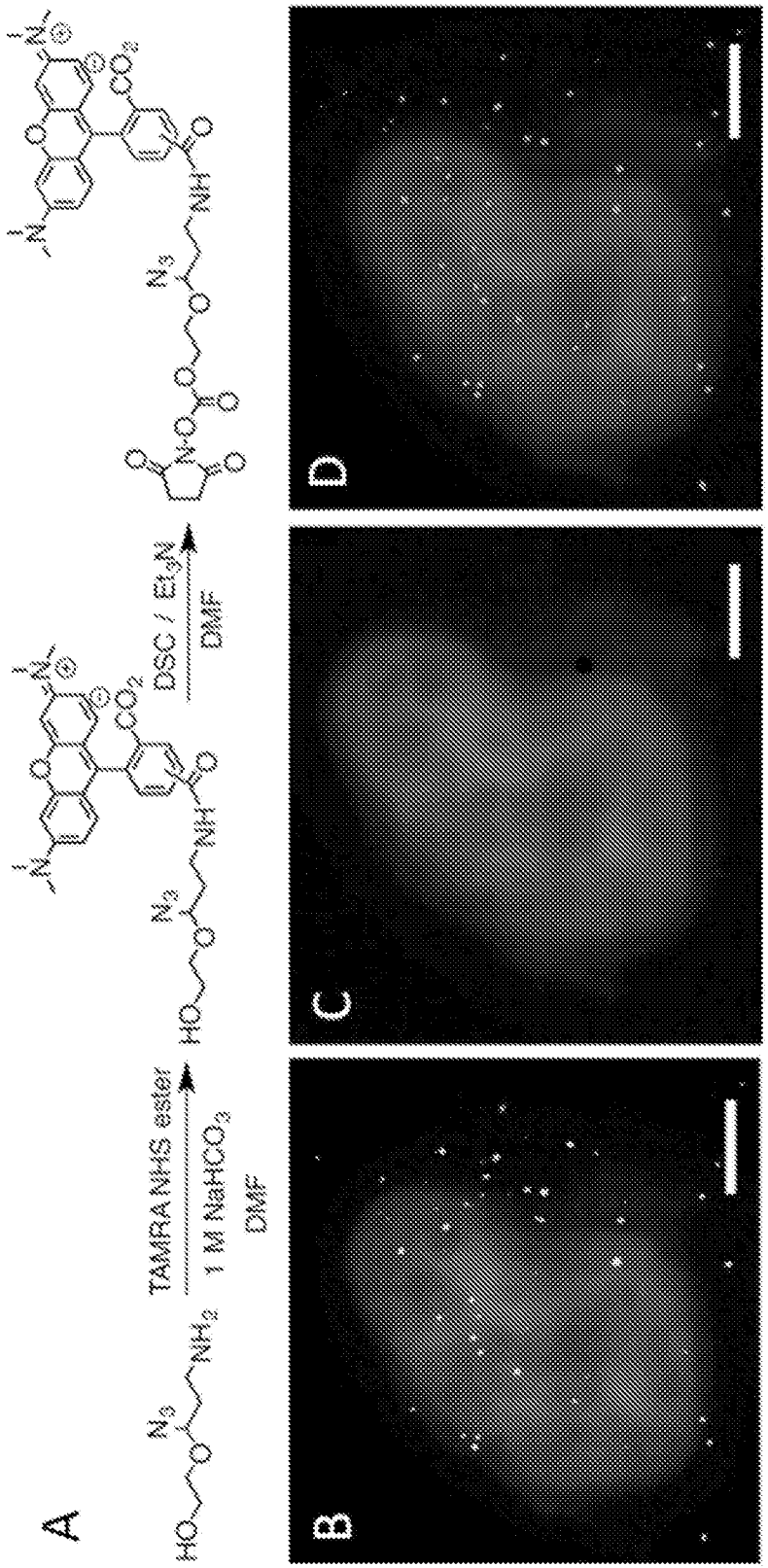
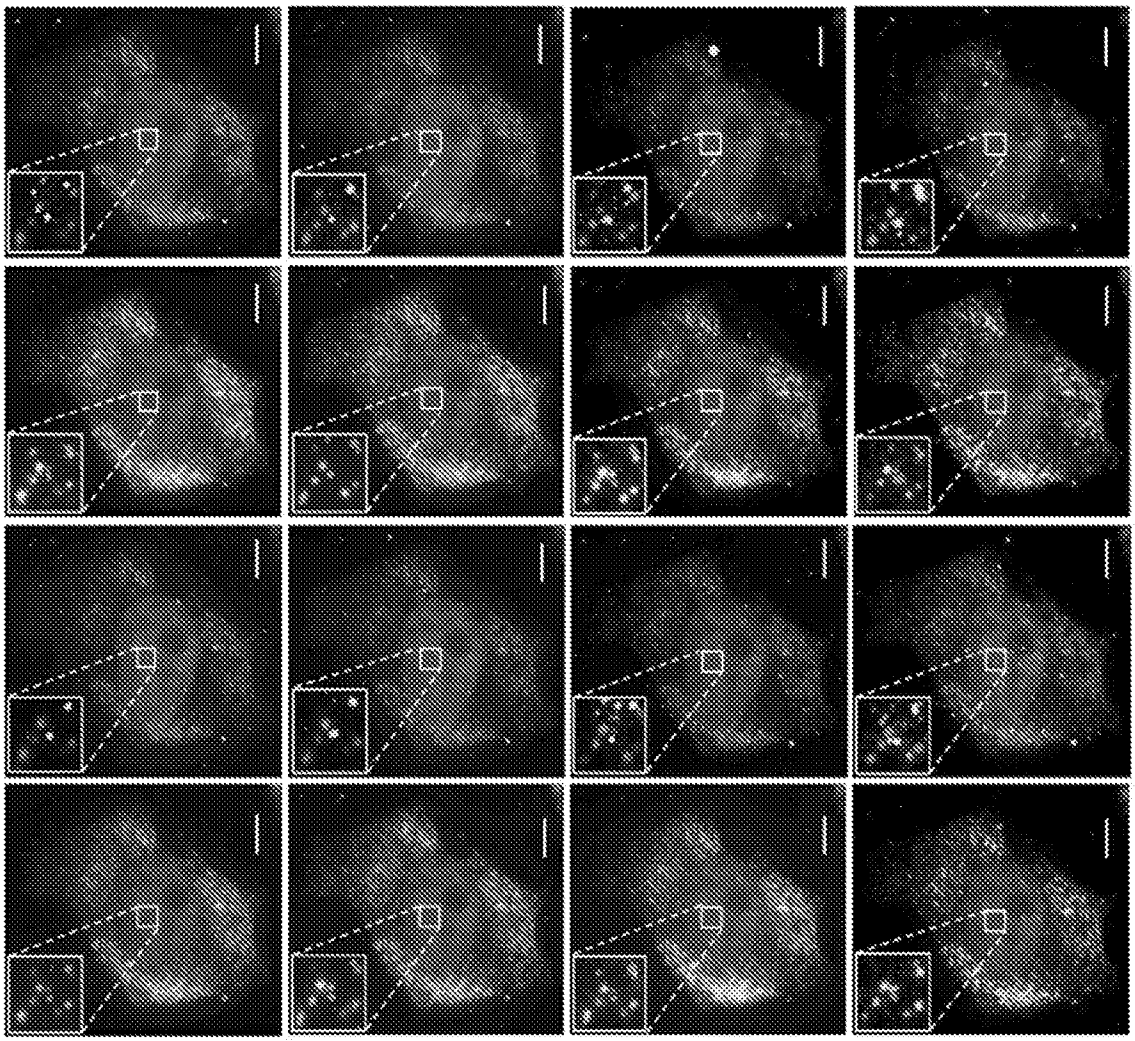


FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/29271

A. CLASSIFICATION OF SUBJECT MATTER

IPC - G01N 21/64; C12Q 1/68; C12P 19/34; C07H 21/02, 21/04 (2017.01)

CPC - C12Q 1/6841, 1/6876, 1/6816, 1/6809; C07H 21/02, 21/04; C12P 19/34; G01N 21/6428, 21/6458, 33/50

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.
X --- Y	US 2015/0267251 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) September 24, 2015; Paragraphs [0004], [0016], [0020], [0023], [0025], [0030], [0031], [0082], [0083], [0085], [0091], [0093], [0096], [0098], [0099], [0104]-[0111], [0123], [0147], [0155], [0193], [0218], [0225], [0240], [0258], [0266]; Claims 20, 43, 50, 53	1-5, 7-12 --- 6
Y	(HUANG, J et al.) Fluorescence resonance energy transfer-based hybridization chain reaction for in situ visualization of tumor-related mRNA. Chemical Science. 25 February 2016, Vol. 7; pages 3829-3835; page 3830, 1st column, 2nd paragraph; scheme 1; DOI: 10.1039/c6sc00377j	6
A	US 2016/0054308 A1 (GUO, J) February 25, 2016; entire document	1-12
A	US 2006/0228733 A1 US 2006/0228733 A1 (PIERCE, NA et al.) October 12, 2006; entire document	1-12

☐ 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

"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	Date of mailing of the international search report
07 July 2017 (07.07.2017)	20 JUL 2017
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774