NANOREPORTERS AND METHODS OF MANUFACTURING AND USE THEREOF

This application claims the benefit of U.S. Provisional Application No. 60/753,758, filed on December 23, 2005, and U.S. Provisional Application No. 843,528, filed on September 8, 2006, the disclosures of each of which is incorporated herein by reference in their entireties.

1. FIELD OF THE INVENTION

The present invention relates to compositions and methods for detection and quantification of individual target molecules in biomolecular samples. In particular, the invention relates to coded, labeled reporter molecules, referred to herein as labeled "nanoreporters," that are capable of binding individual target molecules. Through the nanoreporters' label codes, the binding of the nanoreporters to target molecules results in the identification of the target molecules. Methods of making and using such nanoreporters are also provided. The nanoreporters can be used in diagnostic, prognostic, quality control and screening applications.

2. BACKGROUND OF THE INVENTION

This invention relates generally to the field of detection, identification, and quantification of target molecules in mixtures.

Although all cells in the human body contain the same genetic material, the same genes are not active in all of those cells. Alterations in gene expression patterns can have profound effects on biological functions. These variations in gene expression are at the core of altered physiologic and pathologic processes. Therefore, identifying and quantifying the expression of genes in normal cells compared to diseased cells can aid the discovery of new drug and diagnostic targets.

Nucleic acids can be detected and quantified based on their specific polynucleotide sequences. The basic principle underlying existing methods of detection and quantification is the hybridization of a labeled complementary probe sequence to a target sequence of interest in a sample. The formation of a duplex indicates the presence of the target sequence

in the sample and the degree of duplex formation, as measured by the amount of label incorporated in it, is proportional to the amount of the target sequence.

This technique, called molecular hybridization, has been a useful tool for identifying and analyzing specific nucleic acid sequences in complex mixtures. This technique has been used in diagnostics, for example, to detect nucleic acid sequences of various microbes in biological samples. In addition, hybridization techniques have been used to map genetic differences or polymorphisms between individuals. Furthermore, these techniques have been used to monitor changes in gene expression in different populations of cells or in cells treated with different agents.

In the past, only a few genes could be detected in a complex sample at one time. Within the past decade, several technologies have made it possible to monitor the expression level of a large number of transcripts within a cell at any one time (see, e.g., Schena et al., 1995, Science 270: 467-470; Lockhart et al., 1996, Nature Biotechnology 14: 1675-1680; Blanchard et al., 1996, Nature Biotechnology 14:1649). In organisms for which most or all of the genome is known, it is possible to analyze the transcripts of large numbers of the genes within the cell. Most of these technologies employ, DNA microarrays, devices that consist of thousands of immobilized DNA sequences present on a miniaturized surface that have made this process more efficient. Using a microarray, it is possible in a single experiment to detect the presence or absence of thousands of genes in a biological sample. This allows researchers to simultaneously perform several diagnostic tests on one sample, or to observe expression level changes in thousands of genes in one experiment. Generally, microarrays are prepared by binding DNA sequences to a surface such as a nylon membrane or glass slide at precisely defined locations on a grid. Then nucleic acids in a biological sample are labeled and hybridized to the array. The labeled sample DNA marks the exact position on the array where hybridization occurs, allowing automatic detection.

Unfortunately, despite the miniaturization of array formats, this method still requires significant amounts of the biological sample. However, in several cases, such as biopsies of diseased tissues or samples of a discrete cell type, the biological sample is in limited supply. In addition, the kinetics of hybridization on the surface of a microarray is less efficient than hybridization in small amounts of aqueous solution. Moreover, while methods exist to estimate the amount of nucleic acid present in a sample based on microarray hybridization result, microarray technology thus far does not allow for detection of target molecules on an

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individual level, nor are there microarray-based methods for directly quantifying the amount of target molecule in a given sample.

Thus, there exists a need for accurate and sensitive detection, identification and quantification of target molecules in complex mixtures.

Discussion or citation of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to methods for the generation of a diverse population of uniquely-labeled molecules, preferably synthetic molecules, referred to herein as nanoreporters, that can be used for the detection, identification, and direct quantification of a wide variety of target molecules. The methods are advantageous in that they generate large numbers of distinctly labeled reporter molecules, each capable of detecting a single target molecule, starting from just a small number of different types of label monomers.

In certain embodiments, the present invention provides a dual nanoreporter, or a "probe pair," comprising two components: a first probe and a second probe. In specific embodiments, the first probe is a complex comprising: (a) a first molecule, or a scaffold, comprising: (i) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached (directly or indirectly) one or more label monomers that emit light constituting a second signal; and (b) a first target-specific sequence attached to the first molecule. The second probe is a second molecule or a complex comprising a second molecule, said second molecule comprising (i) a second target-specific sequence; (ii) optionally, a third label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a third signal; and (iii) optionally, an affinity tag attached to said second molecule; wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

In alternative embodiments, the present invention provides a dual nanoreporter, or a "probe pair," comprising two components: a first probe and a second probe. In specific

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embodiments, the first probe is a complex comprising: (a) a first molecule, or a scaffold, comprising: (i) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a second signal; and (b) a first target-specific sequence attached to the first molecule. The second probe is a second molecule or a complex comprising a second molecule, said second molecule comprising (i) a second target-specific sequence; (ii) optionally, a third label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a third signal; and (iii) optionally, an affinity tag attached to said second molecule; wherein the first signal and the second signal are spatially or spectrally distinguishable; wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

The code can consist of the first and second signals and their locations relative to each other, or can consist of the first and second signals and additional signals similarly generated from additional label attachment regions (on the first or second molecule) and the locations of the signals relative to each other.

Generally, the target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA. In other embodiments, the target molecule is a variant of a naturally occurring molecule, for example a protein whose activity, stability and/or biodistribution has been improved or optimized, or a nucleic acid encoding such a protein.

In a first aspect of the dual nanoreporters of the invention in which the target attachment regions are nucleotide sequences, a first DNA molecule is hybridized to the first label attachment region, to which first DNA molecule are bound (directly or indirectly) said one or more label monomers that emit light constituting said first signal; and wherein a second DNA molecule is hybridized to the second label attachment region, to which second DNA molecule are bound (directly or indirectly) said one or more label monomers that emit light constituting said second signal.

In a second aspect of the dual nanoreporters of the invention in which the target attachment regions are nucleotide sequences, a first RNA molecule is hybridized to the first

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label attachment region, to which first RNA molecule are bound (directly or indirectly) said one or more label monomers that emit light constituting said first signal; and wherein a second DNA molecule is hybridized to the second label attachment region, to which second DNA molecule are bound (directly or indirectly) said one or more label monomers that emit light constituting said second signal.

In a third aspect of the dual nanoreporters of the invention in which the target attachment regions are nucleotide sequences, a plurality of first DNA molecules are hybridized to the first label attachment region, to which DNA molecules are bound (directly or indirectly) said one or more label monomers that emit light constituting said first signal; and wherein a plurality of second DNA molecules are hybridized to the second label attachment region, to which second DNA molecules are bound (directly or indirectly) said one or more label monomers that emit light constituting said second signal.

In a fourth aspect of the dual nanoreporters of the invention in which the target attachment regions are nucleotide sequences, a plurality of first RNA molecules are hybridized to the first label attachment region, to which RNA molecules are bound (directly or indirectly) said one or more label monomers that emit light constituting said first signal; and wherein a plurality of second RNA molecules are hybridized to the second label attachment region, to which second RNA molecules are bound (directly or indirectly) said one or more label monomers that emit light constituting said second signal.

In certain embodiments of the dual nanoreporters of the invention, the second probe is a nucleic acid complex comprising: (a) the second nucleic acid (scaffold) molecule, wherein the second nucleic acid molecule comprises a third label attachment region to which is hybridized a third RNA molecule, to which third RNA molecule are bound (directly or indirectly) one or more label monomers that emit light constituting a third signal; and (b) the second target-specific sequence covalently attached to the second nucleic acid molecule, wherein the code comprises the identity of the first, second and third signals and their locations relative to each other. In certain embodiments, the label monomers attached to the first label attachment regions emit light at the same wavelength, which light constitutes said first signal, and the label monomers attached to the second label attachment region emit light at the same wavelength, which light constitutes the second signal, and the label monomers attached to the third label attachment region emit light at the same wavelength, which light constitutes the third signal. In other embodiments, at least one of the first signal, the second signal and the third signal comprises light at a plurality of

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different wavelengths. The first, second and third signals may be spectrally distinguishable. Alternatively, the first and third signals emit at the same wavelength or wavelengths.

In certain embodiments, the first probe and/or the second probe comprises an affinity tag. Also, in specific embodiments, the second probe comprises a third label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a third signal. Preferably, the first and second molecules are nucleic acid molecules; more preferably, the label attachment regions and target-specific sequences are predetermined nucleotide sequences. In certain embodiments of the dual nanoreporters of the invention, the one or more label monomers attached to one or both of the label attachment regions are covalently bound to nucleic acids hybridized to their respective attachment regions, or are covalently bound to nucleic acids that are bound to the respective label attachment regions indirectly through one or more bridging nucleic acids.

In certain embodiments of the dual nanoreporters of the invention, the first and second target-specific sequences are unlabeled with any one or more of said label monomers; however, at least one target-specific sequence in a nanoreporter is attached to a scaffold that is directly or indirectly attached to one or more label monomers.

In other embodiments of the dual nanoreporters of the invention, the second molecule further comprises a fourth label attachment region to which is attached one or more label monomers that emit light constituting a fourth signal. Preferably, when such a dual nanoreporter is bound to its target molecule, the code comprises the identity of the first signal, second signal, third signal and fourth signal and their locations relative to each other.

However, the second nucleic acid need not comprise a third label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a third signal. Such probes are referred to herein as "ghost probes." Ghost probes contain a target-specific sequence, which improves the hybridization kinetics of a nanoreporter to its target molecule, and, optionally, an affinity tag that can be used to immobilize and stretch the dual nanoreporter. In nanoreporter embodiments employing a ghost probe, the first molecule (to which the label monomers are attached and is sometimes referred to herein as a "reporter probe") is preferably a nucleic acid molecule of about 2,000 to about 10,000 bases in length, more preferably about 5,000 to about 8,000 bases in length, and the second molecule (the "ghost probe") is preferably a nucleic acid molecule of about 40 to about 250 bases in length, more preferably about 50 to 100 bases in length. In a specific embodiment, both the first molecule and the second molecule are DNA molecules.

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As used herein, the use of the terms "about" and "approximately" before a number or range means that number or range plus or minus 5%.

An affinity tag can be indirectly attached to the first probe or second probe. For example an affinity tag can be covalently attached to an oligonucleotide hybridized to the first or second probe, or it can be covalently attached to an oligonucleotide hybridized to a "patch" that is hybridized to the scaffold of the first and/or second probe. Alternatively, the affinity tag is covalently attached to the first nucleic acid molecule and/or to the second nucleic acid molecule of the first and second probes.

The signals emitted by the label monomers attached to a given label attachment region can be the same, or different. Thus, in one embodiment, the label monomers attached to a specific (*e.g.*, first or second) label attachment region emit light at the same wavelength, which light constitutes the signal corresponding to that label attachment region. Alternatively, in another embodiment, at least one of the first signal and the second signal comprises light at a plurality of different wavelengths.

Preferably, in certain embodiments, signals emitted by label monomers attached to adjacent label attachment regions are spectrally distinguishable. Thus, in specific embodiments, the first and second signals are spectrally distinguishable.

The present invention further provides a probe, which can be a singular nanoreporter or one or both of the two components of a dual nanoreporter, said probe being a nucleic acid complex comprising (a) a nucleic acid molecule comprising (i) a first label attachment region to which is hybridized a first RNA molecule, to which first RNA molecule are bound (directly or indirectly) one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is hybridized a second RNA molecule, to which second RNA molecule are bound (directly or indirectly) one or more label monomers that emit light constituting a second signal; and (b) a target-specific sequence covalently attached to the nucleic acid molecule, wherein the first and second RNA molecules preferably are each at least 20 nucleotides, wherein the target-specific sequence binds to a target molecule, which target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA, and wherein when said probe is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule. In specific embodiments, the first and second RNA molecules preferably are each

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at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 nucleotides.

In other aspects, the present invention provides a probe, said probe being a nucleic acid complex comprising (a) a synthetic nucleic acid molecule comprising (i) a first label attachment region to which is hybridized a first RNA molecule, to which first RNA molecule are attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is hybridized a second RNA molecule, to which second RNA molecule are attached one or more label monomers that emit light constituting a second signal; and (b) a target-specific sequence covalently attached to the synthetic nucleic acid molecule, wherein the first and second RNA molecules are each at least 50 nucleotides, wherein the target-specific sequence binds to a target molecule, which target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA, and wherein when said probe is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

In yet other aspects, the present invention provides a probe, said probe being a nucleic acid complex comprising (a) a nucleic acid molecule comprising (i) a first label attachment region to which is hybridized a first RNA molecule, to which first RNA molecule are covalently attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is hybridized a second RNA molecule, to which second RNA molecule are covalently attached one or more label monomers that emit light constituting a second signal; and (b) a target-specific sequence covalently attached to the nucleic acid molecule, wherein the first and second RNA molecules are each at least 50 nucleotides, wherein the target-specific sequence binds to a target molecule, which target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA, and wherein when said probe is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

In certain aspects of the present invention, the label monomers are attached to the label attachment regions of the nanoreporters of the invention by way of one or more RNA patches, or RNA molecules. Such RNA patches are preferably about (+/- 5%) 100 to about 3,000 nucleotides each, more preferably about 500 to about 1,500 nucleotides each.

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Where a nanoreporter comprises an affinity tag, the affinity tag can be attached to a patch and, in a dual nanoreporter, the affinity tag can be attached to one or more patches on one or both components of the probe pair.

The affinity tag can be attached by being covalently attached to an oligonucleotide hybridized to one or more patches on a nanoreporter. Alternatively, the affinity tag can be attached to the nucleic acid molecule that is the scaffold of a nanoreporter.

In certain aspects of the invention, nanoreporter-target molecule complexes are provided. Optionally, one or more components of the nanoreporter complexes are covalently attached to an affinity tag for purification and/or for immobilization (either before or after stretching, as described in Section 5.12 below). In specific embodiments, the target molecule itself is attached to an affinity tag. For example, where the target molecule is a nucleic acid, the affinity tag can be biotin that is incorporated into the nucleic acid as biotin-modified nucleotides. The target molecule can be immobilized through the affinity tag prior to or after formation of the nanoreporter-target molecule complex. In embodiments in which the affinity tag is a biotin moiety, the target molecule can be immobilized on a solid surface that is coated with avidin or streptavidin.

One or all of the components (*e.g.*, scaffold, target-specific sequence) of a nanoreporter can be (or have the sequence of) a naturally-occurring molecule. However, the fully assembled and labeled nanoreporter is generally a synthetic molecule, for example a chimeric molecule made by joining naturally-occurring sequences (*e.g.*, a viral or plasmid-based scaffold and a mammalian target-specific sequence) and/or manmade sequences.

The nanoreporters of the invention, when complexed to their target molecules, are preferably imaged when immobilized and stretched. The nanoreporters and complexes comprising the nanoreporters and target molecules can be immobilized by any method known in the art. Preferably, the nanoreporter is attached to an affinity tag (*e.g.*, biotin or digoxigenin) that can be used to tether the nanoreporter to a surface containing a ligand for the affinity tag (*e.g.*, streptavidin or an anti-digoxigenin antibody, respectively). The immobilized nanoreporter can be stretched by any method known in the art, including but not limited to flow-stretching, a receding meniscus technique, electrostretching, constriction in the flow of a liquid containing the nanoreporter in conjunction with an oscillating electric field.

The present invention further provides populations of ten or more nanoreporters, or probes, each probe comprising a synthetic molecule, said synthetic molecule comprising,

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sequentially in the following order: (a) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (b) a second label attachment region to which is attached one or more label monomers that emit light constituting a second signal; (c) a third label attachment region to which is attached one or more label monomers that emit light constituting a third signal; and (d) a target-specific sequence that binds to a target molecule; wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable; wherein the first and second signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the second and third signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the first and third signals are spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein each probe comprises a target-specific sequence that binds to a different target molecule; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies each target molecule.

Preferably, the target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA.

Also preferably, the label attachment regions and target-specific sequences are predetermined nucleotide sequences.

In certain aspects of the invention, the probes, probe pairs, and/or synthetic nucleic acid molecules of the invention are purified.

In specific embodiments, the label monomers attached to the first label attachment region emit light at the same wavelength, which light constitutes said first signal; wherein the label monomers attached to the second label attachment region emit light at the same wavelength, which light constitutes the second signal; and the label monomers attached to the third label attachment region emit light at the same wavelength, which light constitutes the third signal. Thus, in such embodiments, the label monomers attached to a particular label attachment region emit light at the same wavelength(s).

In other specific embodiments, at least one of the first signal, second signal, and third signal comprises light at a plurality of different wavelengths. In such embodiments, two or more monomers attached to at least one label attachment region emit light at different wavelengths.

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The present invention further provides a population of ten or more nanoreporter-target molecule complexes, each complex comprising a synthetic molecule bound to a target molecule, said synthetic molecule comprising, sequentially in the following order: (a) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (b) a second label attachment region to which is attached one or more label monomers that emit light constituting a second signal; (c) a third label attachment region to which is attached one or more label monomers that emit light constituting a third signal; and (d) a target-specific sequence that binds to said target molecule; wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable; wherein the first and second signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the second and third signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the first and third signals are spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein each target-specific sequence (or, in a dual nanoreporter, pair of target-specific sequences) binds to a different said target molecule; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies each target molecule.

In specific embodiments, the population is immobilized to a solid surface, and is preferably stretched. For example the population can be subjected to flow-stretching, a receding meniscus technique, electrostretching, or constriction in the flow of a liquid containing said complexes in conjunction with an oscillating electric field.

The present invention yet further provides methods of detecting a target molecule, said methods comprising: (a) contacting a biomolecular sample with a probe under conditions that permit binding of the probe to the target molecule, wherein said probe is a synthetic molecule comprising, sequentially in the following order: (i) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is attached one or more label monomers that emit light constituting a second signal; (iii) a third label attachment region to which is attached one or more label monomers that emit light constituting a third signal; and (iv) a target-specific sequence that binds to a target molecule; wherein the target-specific sequence binds to a target molecule that may be present in said

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sample, wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable; (b) detecting said first, second and third signals under conditions: wherein the first and second signals are not spatially resolvable; wherein the second and third signals are not spatially resolvable; wherein the first and third signals are spatially resolvable under said conditions; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies a target molecule; and (c) determining whether said code is produced, thereby detecting said target molecule.

Preferably, the probe and the target molecule are nucleic acids.

In certain specific embodiments, the label monomers attached to the first label attachment region emit light at the same wavelength, the label monomers attached to the second label attachment region emit light at the same wavelength, and the label monomers attached to the third label attachment region emit light at the same wavelength. In such embodiment, each of the first signal, second signal, and third signal consists of light at a single wavelength.

In other embodiments, at least one of the first signal, second signal, and third signal comprises light at a plurality of different wavelengths.

The present invention further provides an artificial nucleic acid molecule comprising one or more synthetic regions (hereinafter, "Region"), each Region being at least 50 nucleotides and comprising a regularly repeated base, said regularly repeated base having a periodicity of four to twenty five, such that said type of base occurs at regular intervals of every fourth to every twenty fifth position in said region. In a specific embodiment, not more than 5% of said base in said Region appears at other than said regular intervals. The artificial nucleic acid can be a single-stranded nucleic acid (*e.g.*, useful as a nanoreporter scaffold) or at least partially (or fully) double-stranged (*e.g.*, a nanoreporter scaffold to which patches are hybridized). The artificial nucleic acid can be RNA, DNA, or a DNA/RNA hybrid. Preferably the regularly repeated base is a thymidine or a uracil. In specific embodiments, the periodicity of the regularly repeated base in said one or more Regions is about 6 to about 15; in other embodiments, the periodicity of the regularly repeated base in said one or more Regions is about 8 to about 12.

Preferably, the artificial nucleic acid comprises a plurality of Regions, for example about 2 to about 50 Regions, or about 3 to about 10 Regions.

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The plurality of Regions can be immediately adjacent to one another, or separated by one or more spacer regions. Spacer regions can include, for example, one or more restriction endonuclease recognition sequences (or the complement thereof) and/or one or more RNA polymerase promoter sequences (or the complement thereof).

In specific embodiments, the plurality of Regions differ from one another in sequence.

The artificial nucleic acid may further comprise an origin of replication.

Preferably, the GC content of the one or more Regions is about 25% to about 75%, more preferably about 40 to about 60%, and most preferably is about 50% (+/- 5%).

Each of said one or more Region in the artificial nucleic acids of the invention is preferably about 50 to about 50,000 bases in length, and is more preferably about 200 to about 2,000 bases in length.

The artificial nucleic acids of the invention are preferably about 50 to about 100,000 bases in length, and are more preferably about 200 to about 10,000 or about 500 to about 20,000 bases in length.

The artificial nucleic acids of the invention may comprise one or a plurality of Regions, which are useful as label attachment regions. Where an artificial nucleic acid comprises a plurality of Regions, the type of regularly repeated base can be the same in the plurality of Regions, or different. Also, the periodicity of the regularly repeated base can be the same in the plurality of Regions, or different. In a specific embodiment, the present invention provides an artificial nucleic acid comprising a plurality of Regions, in which both the type and the periodicity of the regularly repeated base are the same in the plurality of Regions.

The artificial nucleic acids of the invention may further comprise a target-specific sequence.

Preferably, the Regions do not comprise a recognition site for at least one six-cutter restriction endonucleotides, such as one of the following restriction endonucleases: BamH1, EcoRI, PstI, SmaI, XbaI, NotI, SacI, EcoRV, HindIII, ClaI, SalI, XhoI, ApaI, or KpnI. In a specific embodiment, the Regions do not comprise any recognition site for 6-cutter enzymes, or at least 6-cutter enzymes that are used for cloning within the artificial nucleic acid, for example 6-cutter enzymes present in the polylinker of a plasmid or in regions flanking the Regions.

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The artificial nucleic acids of the invention, or at least the Regions therein, preferably are not predicted by the MFOLD program to contain hairpin loops.

The artificial nucleic acids of the invention, or at least the Regions therein, preferably do not have direct or inverted repeats that are greater than 12 bases in length. In other embodiments, the artificial nucleic acids and/or Regions do not have direct or inverted repeats that are greater than about 11, about 10 or about 9 bases in length.

The artificial nucleic acids of the invention preferably comprises a promoter sequence (such as T7, T3 or SP6) or its complement.

The artificial nucleic acids of the invention may further comprise a phage encapsidation sequence.

The Regions in the artificial nucleic acids of the invention may be flanked by restriction endonuclease recognition sites.

The artificial nucleic acids can be used as nanoreporter scaffolds, in which the Regions are useful as label attachment regions. Accordingly, the present invention provides an artificial nucleic acid comprising one or more Regions, the regularly repeated base of said one or more Regions being attached to at least one light-emitting label monomer. At least one regularly-repeated base can be covalently attached to the label monomer, or it can be attached to the label monomer via hybridization to another nucleic acid to which the label monomer is covalently attached. In a specific embodiment, at least two different Regions are attached to a different light-emitting labeling monomer.

The artificial nucleic acids of the inventions may further comprise an affinity tag and/or an origin of replication.

In one aspect of the invention, an artificial nucleic acid is a partially or fully double-stranded molecule in which one strand is DNA to which is hybridized a plurality of RNAs, each RNA comprising a single region in which the regularly-repeated base is aminoallyl-modified uracil. In a specific embodiment, at least 50% of occurrences of said aminoallyl-modified uracil is covalently attached to at least one light-emitting label monomer; in other embodiments, the percentage of occurrences is at least 60%, at least 70%, at least 80% , at least 90%, at least 95% or at least 98%. This percentage of occurrences can be measured by any means known in the art.

As discussed elsewhere, the nanoreporters of the invention may comprise nucleic acid scaffolds that are attached to patches and, optionally, flaps. Details of such structures

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are discussed in Section 5.3, *infra*, and exemplary patch and flap structures are depicted in Figure 7.

The present invention further provides methods of detecting a target molecule, said methods comprising: (a) contacting a biomolecular sample that may contain the target molecule with any of the nanoreporter structures described herein under conditions that permit binding of the synthetic molecule to the target molecule, (b) subjecting the conditions that permit formation and/or detection of the nanoreporter code and (c) determining whether or not the nanoreporter code is produced or present, thereby detecting said target molecule.

In certain aspects of the invention, the methods nanoreporter is a synthetic molecule comprising: (i) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal, all said first signals being the same; (ii) a second label attachment region to which is attached one or more label monomers that emit light constituting a second signal, all said second signals being the same; (iii) a third label attachment region to which is attached one or more label monomers that emit light constituting a third signal, all said third signals being the same; wherein each attachment region comprises a plurality of patch pairs; wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable, and determining whether or not the nanoreporter code is present and/or produced comprises (b) detecting said first, second and third signals under conditions: wherein the first and second signals are not spatially resolvable; wherein the second and third signals are not spatially resolvable; wherein the first and third signals are spatially resolvable under said conditions; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies a target molecule. Thereby, the target molecule is detected.

4. BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1A-1F: Figure 1A illustrates a dual nanoreporter with a 16-position nanoreporter code, using two 8-position nanoreporter components. Figure 1B illustrates a dual nanoreporter with a 9-position nanoreporter code, using one 8-position nanoreporter component and one single-position nanoreporter component. Figure 1C illustrates a dual nanoreporter with an 8-position nanoreporter code, using one ghost probe and one 8-position nanoreporter component. Figure 1D illustrates a single nanoreporter with an 8-position nanoreporter code. In Figures 1A-1D, the star shape (depicted with an arrow) is illustrative

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of an affinity tag, which can be used to purify the nanoreporter or immobilize the nanoreporter (or nanoreporter-target molecule complex) for the purpose of imaging. The numbered region in Figure 1A-1D refer to separate label attachment regions. All except for position 12 of Figure 1A are labeled with one of four types of label monomers, depicted as grey, white, hatched or stripe "sun" diagrams. Position 12 of Figure 1A is an unlabeled "dark spot." Figures 1E and 1F represent variations on the nanoreporters of Figures 1B and 1D, respectively, in which the target molecule to which the nanoreporters are bound comprises biotin moieties (shown as small asterisks), for example biotin-modified nucleotides randomly incorporated into a target nucleic acid. The nanoreporters themselves further optionally comprise an affinity tag (not shown).

FIGURE 2A-2C: Figure 2A shows an illustration of a label unit of a nanoreporter, containing a scaffold with patch units and corresponding split flaps disposed along its length. Figure 2B illustrates the components of a single patch pair and its corresponding flap, containing: 1: a portion of a nanoreporter scaffold (e.g., M13 single-stranded DNA); 2: A patch pair; 3: a split flap pair; and 4: labeled oligonucleotides, each with a label monomer incorporated, hybridized to the split flap. Figure 2C shows a nanoreporter with 4 "spots," each spot designed to contain 9 patch pairs of 60-65 nucleotides, each attached to a split flap pair of 95-100 nucleotides. Each split flap pair had binding site for 12 oligonucleotides each attached to a single label monomer. Each spot therefore had binding sites for 108 label monomers.

FIGURE 3: A nanoreporter in which the patches are RNA segments can be used with (Figure 3A) and without registers (Figure 3B). Both Figure 3A and 3B depict a **(1)** nanoreporter scaffold (heavy black line) to which are attached **(2)** 8 RNA segments (heavy grey lines 1-8), **(3)** a target-specific sequence (dotted line "T") and **(4)** an oligonucleotide (checkered line "0") that is partly complementary to the nanoreporter scaffold and partly complementary to the target-specific sequence. This oligonucleotide is referred to as a "ligator" oligonucleotide. In Figure 3A, only one register, *i.e.*, every alternate RNA segment is labeled. The second register positions serve as "spacers," making it possible to generate a nanoreporter code in which consecutive positions in the code are the same "color," or spectrally indistinguishable. In Figure 3B, both registers, *i.e.*, adjacent RNA segments with no intervening spacers, are labeled, with no nearest neighbor of the same "color."

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FIGURE 4: Is an image of a dual nanoreporter hybridized to a target molecule. Here, both registers are labeled. The nanoreporters are labeled with three different colors, Alexa 488, Cy3 and Alexa 647 (labeled 1, 2 and 3, respectively). The left brackets show one probe of the dual nanoreporter and the right brackets show the other probe of the dual nanoreporter. Colors 1, 2 and 3 were each acquired in different channels and the first and second registers, seen as rows of spots, were shifted up by several pixels to be able to show each register individually.

FIGURE 5A-5D: This figure illustrates the various components of the dual nanoreporters shown in Figure 4. Figure 5A illustrates one color (here, Alexa 488, depicted in the left column as open circles), which is spectrally distinguishable from Cy3 (shown in Figure 5B, depicted in the left column as vertically striped circles) and Alexa 647 (shown in Figure 5C as diagonally striped circles). The images obtained from each were superimposed to generate Figure 5D.

FIGURE 6A-6E: Figure 6A is a schematic illustration of the experiment shown in Figures 6B and 6C. In this case, the star represents biotin that was used to attach the complex by one end to the surface prior to stretching. Figures 6B and 6C show images from experiments in which S2-A ghost probe, S2-B labeled nanoreporter and S2 target DNA (Figure 6B) or S2 target RNA (Figure 6C) were hybridized. Figure 6E shows a close-up of a nanoreporter complexes from Figure 6B, each containing S2-A ghost probe, S2-B labeled nanoreporter and S2 target DNA. Figure 6D shows an image of a negative control experiment, in which S2-A ghost probe, S2-B labeled nanoreporter and no S2 target RNA were hybridized.

FIGURE 7A-G. Figures 7A, 7B, 7C and 7D depict different permutations of patches on a nanoreporter scaffold, Figures 7E and 7F depict different permutations of split flaps on a nanoreporter scaffold, optionally hybridized to one or more oligonucleotides, as in Figure 7G. In Figure 7A-G, α refers to a 5′ or 3′ molecule or end of a molecule, and β refers to a corresponding 3′ or 5′ molecule or end of a molecule.

FIGURE 8: Figure 8 depicts a scheme in which single-stranded M13 phage is linearized for use as a nanoreporter scaffold. The circular M13 phage is annealed to a five-fold excess of BamH1 cutter oligonucleotide (hatched lines) (1), and the resulting partially double-stranded M13 digested with the restriction endonuclease BamH1 (2), resulting in a linearized M13 in which BamH1 cutter oligonucleotide is still attached (3). This M13-oligonucleotide complex is heated in the presence of an excess oligonucleotide

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complementary to the BamH1 cutter oligonucleotide (an "anti-BamH1 oligonucleotide") (grey lines) (4). The BamH1 cutter oligonucleotide anneals to the excess of anti-BamH1 oligonucleotide, and the M13 molecule is purified from the oligonucleotide, for example by using size exclusion columns, to yield M13 scaffold.

FIGURE 9A-9B: Shows a labeled nanoreporter with an affinity tag at each end, A1 and A2. In Figure 9, the labeled nanoreporter is immobilized through the binding of A1 to an immobilized affinity partner. In the absence of an affinity binding partner for A2, the A2 end of the nanoreporter remains in solution (Figure 9A), but in the presence of an affinity binding partner (A2'), the A2 end of the nanoreporter is also immobilized (Figure 9B). Upon immobilization, the nanoreporter can be stretched, or "elongated" as depicted in Figure 9B, for example by electrostretching, for separation of the label attachment regions in a manner that permits detection of the nanoreporter code.

FIGURE 10A-10C: Figure 10A shows a labeled nanoreporter containing a single affinity tag, A1. Another affinity tag, A2, can be attached to the nanoreporter by direct binding of the nanoreporter to a molecule containing A2 (*e.g.*, if the nanoreporter is or comprises a nucleic acid, it can hybridize directly with another nucleic acid to which A2 is attached), as depicted in Figure 10B. Alternatively, the second affinity tag, A2, can be attached to the labeled nanoreporter via a bridging molecule, such as the bridging nucleic acid ("X") depicted in Figure 10C.

FIGURE 11A-11B: Shows a labeled (nucleic acid-based) nanoreporter with an affinity tag, A1, at one end. In Figure 11, the labeled nanoreporter is immobilized through the binding of A1 to an immobilized affinity partner. The other end of the nanoreporter is in solution (Figure 11A), but can be immobilized by hybridization to a complementary oligonucleotide which contains another affinity tag (A2) used to immobilize the nanoreporter (Figure 11B). A1 and A2 can be the same, for example biotin, for immobilization on an avidin- or streptavidin-coated surface. Upon immobilization of A1, the nanoreporter can be stretched, or "elongated" as depicted in Figure 11, for example by electrostretching, for separation of the label attachment regions in a manner that permits detection of the nanoreporter code. Optionally, while the nanoreporter is in an elongated state, A2 is introduced and binds the end of the nanoreporter that is complementary to A2 down to the surface.

FIGURE 12A-12B. Figure 12A provides an illustration of a nanoreporter comprising an immobilized first portion F1; and Figure 12B provides an illustration of a

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nanoreporter extended in an electrical field and comprising immobilized first portion F1 and immobilized second portion F2, wherein F2 is immobilized via a complex with molecule F3.

FIGURE 13A-13C. Figure 13A provides an illustration of a three-member complex for immobilization of an extended nanoreporter; FIG. 13B provides an illustration of a two-member complex for immobilization of an extended nanoreporter; and FIG. 13C provides an illustration of an incomplete complex for immobilization of an extended nanoreporter.

FIGURE 14A-14D. Figure 14A provides an illustration of a nanoreporter comprising an immobilized first portion F1; FIG. 14B provides an illustration of an extended nanoreporter immobilized at first portion F1 and at a second portion via complexes with F2; FIG. 14C provides an illustration of a nanoreporter comprising a first portion immobilized to an avidin surface via biotin; and FIG. 14D provides an illustration of an extended nanoreporter immobilized at a first portion and at a second portion via selective binding of biotin to an avidin surface.

FIGURE 15A-15C. Figure 15A illustrates immobilization of one terminus of a DNA molecule in a microfluidic device; Figure 15B illustrates extension of the DNA in an electric field; and Figure 15C illustrates selective immobilization of a second terminus of the extended DNA molecule.

FIGURE 16 provides an image of extended nanoreporters selectively immobilized by the methods of the present invention.

FIGURE 17 depicts the relationship between the number of label attachment regions to the calculated entanglement threshold for nanoreporters for label attachment region sizes of 900 bp and 1100 bp

FIGURE 18 is a scatter plot showing normalized and average $\log_2$ signal values from each positive sample (n=3) for all 509 genes whose expression was measured in a nanoreporter multiplex assay as described in Example 9 (Section 14) below.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention pertains to nanoreporters, and their manufacture and use. A fully assembled and labeled nanoreporter comprises two main portions, a target-specific sequence that is capable of binding to a target molecule, and a labeled region which emits a "code" of signals (the "nanoreporter code") associated with the target-specific sequence. Upon binding of the nanoreporter to the target molecule, the nanoreporter code identifies the target molecule to which the nanoreporter is bound.

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Nanoreporters are modular structures. Generally, a nanoreporter is a molecular entity containing three basic elements: a scaffold containing two or more label attachment regions, one or more patches attached to the scaffold, and a target-specific sequence, also attached to the scaffold. The elements of a nanoreporter can be found in a single molecular entity (a "singular" nanoreporter), or two distinct molecular entities (a "dual" nanoreporter). Each molecular entity may be composed of one molecule or more than one molecule attached to one another by covalent or non-covalent means. Generally, each component of a dual nanoreporter has a target-specific sequence that binds to a different site on the same target molecule. This allows for smaller nanoreporter components with more efficient kinetics of binding of the nanoreporter to the target molecule and better signal:noise ratios resulting from the greater binding specificity.

The patches attached to a nanoreporter scaffold serve to attach label monomers to a nanoreporter scaffold. Patches may be directly labeled, for example by covalent incorporation of one or more label monomers into nucleic acid patches. Alternatively, patches may be attached to flaps, which maybe labeled directly, for example by covalent incorporation of one or more label monomers into a nucleic acid flap, or indirectly, for example by hybridization of a nucleic acid flap to an oligonucleotide which is covalently attached to one or more label monomers. Where the label monomers attached to a label attachment region are not directly incorporated into a patch or flap, the patch or flap serves as a "bridge" between the label monomer and the label attachment region, and may be referred to as a "bridging molecule," *e.g.*, a bridging nucleic acid.

Additionally, nanoreporters may have affinity tags for purification and/or for immobilization (for example to a solid surface). Nanoreporters, or nanoreporter-target molecule complexes, are preferably purified in two or more affinity selection steps. For example, in a dual nanoreporter, one probe can comprise a first affinity tag and the other probe can comprise a second (different) affinity tag. The probes are mixed with target molecules, and complexes comprising the two probes of the dual nanoreporter are separated from unbound materials (*e.g.*, the target or the individual probes of the nanoreporter) by affinity purification against one or both individual affinity tags. In the first step, the mixture can be bound to an affinity reagent for the first affinity tag, so that only probes comprising the first affinity tag and the desired complexes are purified. The bound materials are released from the first affinity reagent and optionally bound to an affinity reagent for the second affinity tag, allowing the separation of complexes from probes comprising the first

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affinity tag. At this point only full complexes would be bound. The complexes are finally released from the affinity reagent for the second affinity tag and then preferably stretched and imaged. The affinity reagent can be any solid surface coated with a binding partner for the affinity tag, such as a column, bead (*e.g.*, latex or magnetic bead) or slide coated with the binding partner. Immobilizing and stretching nanoreporters using affinity reagents is fully described in U.S. provisional application no. 60/753,816 by Sean M. Ferree and Dwayne L. Dunaway, entitled "Compositions Comprising Oriented, Immobilized Macromolecules and Methods for Their Preparation," attorney docket no. 11616-014-888 filed on December 23, 2005, which is incorporated by reference herein in its entirety.

Nanoreporter and nanoreporter-target complexes which are or comprise nucleic acids may be affinity-purified or immobilized using a nucleic acid, such as an oligonucleotide, that is complementary to at least part of the nanoreporter or target. In a specific application where the target includes a poly A or poly dA stretch, the nanoreporter-target complex can be purified or immobilized by an affinity reagent coated with a poly dT oligonucleotide.

The sequence of signals emitted by the label monomers associated with the various label attachment regions of the scaffold of a given nanoreporter allows for the unique identification of the nanoreporter. A nanoreporter having a unique identity or unique spectral signature is associated with a target-specific sequence that recognizes a specific target molecule or a portion thereof. When a nanoreporter is exposed to a mixture containing the target molecule under conditions that permit binding of the target-specific sequence(s) of the nanoreporter to the target molecule, the target-specific sequence(s) preferentially bind(s) to the target molecule. Detection of the spectral code associated with the nanoreporter allows detection of the presence of the target molecule in the mixture (qualitative analysis). Counting all the label monomers associated with a given spectral code or signature allows the counting of all the molecules in the mixture associated with the target-specific sequence coupled to the nanoreporter (quantitative analysis). Nanoreporters are thus useful for the diagnosis or prognosis of different biological states (*e.g.*, disease vs. healthy) by quantitative analysis of known biological markers. Moreover, the exquisite sensitivity of single molecule detection and quantification provided by the nanoreporters of the invention allows for the identification of new diagnostic and prognostic markers, including those whose fluctuations among the different biological states is too slight detect a correlation with a particular biological state using traditional molecular methods. The

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sensitivity of nanoreporter-based molecular detection permits detailed pharmacokinetic analysis of therapeutic and diagnostic agents in small biological samples.

Many nanoreporters, referred to as singular nanoreporters, are composed of one molecular entity, as depicted in Figure 1D. However, to increase the specificity of a nanoreporter and/or to improve the kinetics of its binding to a target molecule, a preferred nanoreporter is a dual nanoreporter composed of two molecular entities, each containing a different target-specific sequence that binds to a different region of the same target molecule. Various embodiments of dual nanoreporters are depicted in Figures 1A-1C. In a dual nanoreporter, at least one of the two molecular entities is labeled. The other molecular entity is not necessarily labeled. Such unlabeled components of dual nanoreporters are referred to herein as "ghost probes" (see Figure 1C) and often have affinity tags attached, which are useful to immobilize and/or stretch the complex containing the dual nanoreporter and the target molecule to allow visualization and/or imaging of the complex.

Because of their modular structures, nanoreporters may be assembled and labeled in a variety of different ways. For example, a nanoreporter scaffold can be attached to a target-specific sequence (for example by hybridization and, optionally, ligation), and the structure comprising the scaffold and target-specific sequence attached to one or more patches and, where desired, flaps. Alternatively, the nanoreporter scaffold can first be attached to one or more patches (and, optionally, flaps), and the scaffold/patch structure then attached to a target specific sequence. Thus, unless stated otherwise, a discussion or listing of steps in nanoreporter assembly does not imply that a specific route of assembly must be followed.

Nanoreporter assembly and use is exemplified herein largely by way of description of a variety of nucleic acid-based nanoreporters; however, one of skill in the art would recognize that the methods described herein are applicable to an amino acid-based (or hybrid nucleic acid-/amino acid-based) nanoreporter. Illustrative embodiments of partially and fully assembled nanoreporters are listed below.

At its simplest, the invention provides a scaffold having at least two label attachment regions capable of being labeled and resolved. The scaffold can be any molecular entity that allows the formation of label attachment regions on the scaffold that can be separately labeled and resolved. The number of label attachment regions to be formed on a scaffold is based on the length and nature of the scaffold, the means of labeling the nanoreporter, as well as the type of label monomers emitting a signal to be attached to the label attachment regions of the scaffold. A nanoreporter according to the invention may have a scaffold

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including two or more label attachment regions. Suitable scaffold structures include DNA-based scaffolds.

The invention also provides labeled nanoreporters wherein one or more label attachment regions are attached to corresponding label monomers, each label monomer emitting a signal. For example a labeled nanoreporter according to the invention is obtained when at least two label monomers are attached to two corresponding label attachment regions of the scaffold such that these labeled label attachment regions, or "spots," are distinguishable. Label monomers emitting a signal associated with different label attachment regions of the scaffold can emit signals that are spectrally indistinguishable under the detections conditions ("like" signals), or can emit signals that are spectrally distinguishable, at least under the detection conditions (*e.g.*, when the nanoreporter is immobilized, stretched and observed under a microscope).

The invention also provides a nanoreporter wherein two or more label monomers are attached to a label attachment region. The signal emitted by the label monomers associated with said label attachment region produces an aggregate signal that is detected. The aggregate signal produced may be made up of like signals or made up of at least two spectrally distinguishable signals.

In one embodiment, the invention provides a nanoreporter wherein at least two label monomers emitting like signals are attached to two corresponding label attachment regions of the scaffold and said two label monomers are spatially distinguishable. In another embodiment, the invention provides a nanoreporter wherein at least two label monomers emitting two distinguishable signals are attached to two neighboring label attachment regions, for example two adjacent label attachment regions, whereby said at least two label monomers are spectrally distinguishable.

The invention provides a nanoreporter wherein two spots emitting like signals are separated by a spacer region, whereby interposing the spacer region allows resolution or better resolution of said like signals emitted by label monomers attached to said two spots. In one embodiment, the spacer regions have a length determined by the resolution of an instrument employed in detecting the nanoreporter.

The invention provides a nanoreporter with one or more "double spots." Each double spot contains two or more (*e.g.*, three, four or five) adjacent spots that emit like signals without being separated by a spacer region. Double spots can be identified by their sizes.

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A label monomer emitting a signal according to the invention may be attached covalently or non-covalently (*e.g.*, via hybrization) to a patch that is attached to the label attachment region. The label monomers may also be attached covalently or non-covalently (*e.g.*, via hybrization) to a flap attached to a patch that is in turn attached to the scaffold. The flap can be formed by one molecule or two or more molecules ("flap pieces") that form a split flap.

The invention also provides a nanoreporter associated with a spectral code determined by the sequence of signals emitted by the label monomers attached (*e.g.*, indirectly via a patch) to label attachment regions on the scaffold of the nanoreporter, whereby detection of the spectral code allows identification of the nanoreporter.

In one embodiment, the invention provides a nanoreporter further comprising an affinity tag attached to the nanoreporter scaffold, such that attachment of the affinity tag to a support allows scaffold stretching and resolution of signals emitted by label monomers corresponding to different label attachment regions on the scaffold. Nanoreporter stretching may involve any stretching means known in the art including but not limited to, means involving physical, hydrodynamic or electrical means.

In yet another embodiment, the invention provides a nanoreporter further comprising flaps attached to label attachment regions of the scaffold, wherein a flap attached to a label attachment region of the scaffold attaches the label monomer corresponding to said label attachment region, thereby indirectly attaching label monomers to corresponding label attachment regions on said scaffold. In a further embodiment, each label monomer comprises a signal emitting portion and an oligonucleotide portion of a predetermined sequence, and the flaps comprise repeats of a flap sequence complementary to the oligonucleotide portion of a corresponding label, whereby one or more label monomers attach to a corresponding label attachment region through hybridization of said oligonucleotide portions of said label monomers to said repeats of said flap sequence thereby producing a labeled nanoreporter.

A nanoreporter according to the invention can further include a target-specific sequence coupled to the scaffold. The target-specific sequence is selected to allow the nanoreporter to recognize, bind or attach to a target molecule. The nanoreporters of the invention are suitable for identification of target molecules of all types. For example, appropriate target-specific sequences can be coupled to the scaffold of the nanoreporter to

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allow detection of a target molecule. Preferably the target molecule is DNA (including cDNA), RNA (including mRNA and cRNA), a peptide, a polypeptide, or a protein.

One embodiment of the invention provides increased flexibility in target molecule detection with label monomers according to the invention. In this embodiment, a dual nanoreporter comprising two different molecular entities, each with a separate target-specific region, at least one of which is labeled, bind to the same target molecule. Thus, the target-specific sequences of the two components of the dual nanoreporter bind to different portions of a selected target molecule, whereby detection of the spectral code associated with the dual nanoreporter provides detection of the selected target molecule in a biomolecular sample contacted with said dual nanoreporter.

The invention also provides a method of detecting the presence of a specific target molecule in a biomolecular sample comprising: (i) contacting said sample with a dual nanoreporter under conditions that allow binding of the target-specific sequences in the dual nanoreporter to the target molecule and (ii) detecting the spectral code associated with the dual nanoreporter. Depending on the nanoreporter architecture, the dual nanoreporter may be labeled before or after binding to the target molecule.

In certain embodiments, the methods of detection are performed in multiplex assays, whereby a plurality of target molecules are detected in the same assay (a single reaction mixture). In a preferred embodiment, the assay is a hybridization assay in which the plurality of target molecules are detected simultaneously. In certain embodiments, the plurality of target molecules detected in the same assay is at least 5 different target molecules, at least 10 different target molecules, at least 20 different target molecules, at least 50 different target molecules, at least 75 different target molecules, at least 100 different target molecules, at least 200 different target molecules, at least 500 different target molecules, or at least 750 different target molecules, or at least 1000 different target molecules. In other embodiments, the plurality of target molecules detected in the same assay is up to 50 different target molecules, up to 100 different target molecules, up to 150 different target molecules, up to 200 different target molecules, up to 300 different target molecules, up to 500 different target molecules, up to 750 different target molecules, up to 1000 different target molecules, up to 2000 different target molecules, or up to 5000 different target molecules. In yet other embodiments, the plurality of target molecules detected is any range in between the foregoing numbers of different target molecules, such as, but not limited to, from 20 to 50 different target molecules, from 50 to 200 different

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target molecules, from 100 to 1000 different target molecules, from 500 to 5000 different target molecules, and so on and so forth.

In certain embodiments, the invention is directed to detecting different splice forms of the same RNA. The different splice forms can be detected using a plurality of nanoreporter probes, each with a different target-specific sequence complementary to a different exon of the same gene.

Structural stability of a nanoreporter can be increased through ligation of the patches and, optionally, ligation of the split flaps and/or the labeled oligonucleotides hybridized to the split flaps.

In addition to the qualitative analytical capabilities provided by the nanoreporters of the invention and the analytical techniques based thereon, the nanoreporters of the invention are uniquely suitable for conducting quantitative analyses. By providing a one to one binding between the nanoreporters (whether singular or dual nanoreporters) of the invention and their target molecules in a biomolecular sample, all or a representative portion of the target molecules present in the sample can be identified and counted. This individual counting of the various molecular species provides an accurate and direct method for determining the absolute or relative concentration of the target molecule in the biomolecular sample. Moreover, the ability to address each molecule in a mixture individually leverages benefits of miniaturization including high sensitivity, minimal sample quantity requirements, high reaction rates which are afforded by solution phase kinetics in a small volume, and ultimately very low reagent costs.

As will be appreciated from the description and examples provided below, the present invention provides numerous advantages. For example, the complex modularity in forming nanoreporters according to the invention allows for systematic creation of libraries of unique nanoreporters having a very high degree of diversity (*e.g.*, millions of uniquely recognizable nanoreporters). This modularity allows flexibility in customizing nanoreporter populations to specific applications which in turn provides significant manufacturing efficiencies. Another advantage that will be appreciated through the following description stems from the flexibility in assembling the nanoreporters of the invention. That is, due to their modular structure, the nanoreporters of the invention can be assembled prior to shipment to a point of use or assembled at the point of use.

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5.1 NANOREPORTER NOMENCLATURE

NANOREPORTER: The term "nanoreporter" refers to a molecular entity that has (i) a molecule ("scaffold") containing at least two label attachment regions; (ii) at least one patch attached to at least one label attachment region; and (iii) a target-specific sequence. As described in detail below, nanoreporters can be singular nanoreporters (all components being in a single molecular entity) or dual nanoreporters (all the components being in two separate molecular entities). Nanoreporters are preferably synthetic, *i.e.*, non-naturally-occurring molecules, for example are chimeric molecules made by joining two or more manmade and/or naturally occurring sequences that normally exist on more than one molecule (*e.g.*, plasmid, chromosome, viral genome, protein, etc.).

LABELED NANOREPORTER: A labeled nanoreporter is a nanoreporter in which at least one patch of the nanoreporter is attached to one or more label monomers that generate(s) a signal that forms at least part of the nanoreporter code.

LABEL UNIT: The term "label unit" refers to the non-target-specific portions of a labeled nanoreporter.

PROBE: This refers to a molecule that has a target-specific sequence. In the context of a singular nanoreporter, the term "probe" refers to the nanoreporter itself; in the context of a dual nanoreporter, the term "probe" refers to one or both of the two components of the nanoreporter.

PROBE PAIR: This refers to a dual nanoreporter.

PATCH: The term "patch" refers to a molecular entity attached to the label attachment region of the nanoreporter scaffold, generally for the purpose of labeling the nanoreporter. The patch can have one or more label monomers either directly (covalently or noncovalently) or indirectly attached to it, either prior to or after its attachment to the scaffold.

FLAP: The term "flap" as used herein refers to a molecular entity attached to a patch or patch pair attached to a label attachment region. The flap is one or more molecule containing label monomers or capable of binding one or more molecules containing label monomers. By providing indirect labeling of the regions, the flaps provide more flexibility in controlling the number of signal emitting monomers associated with a region as well as the nature of those monomers. Flaps may be formed by a single molecular piece or several molecular pieces (*e.g.*, two pieces) forming a "split flap" (see, *e.g.*, Figure 7)

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TARGET-SPECIFIC SEQUENCE: The term "target-specific sequence" refers to a molecular entity that is capable of binding a target molecule. In the context of a nanoreporter, the target-specific sequence is attached to the nanoreporter scaffold. The target molecule is preferably (but not necessarily) a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA.

GHOST PROBE: A molecule comprising a target-specific sequence, but which is not labeled with a label monomer that emits a signal that contributes to the nanoreporter code.

REPORTER PROBE: A molecule comprising a target-specific sequence that is labeled with at least one label monomer that emits a signal that contributes to the nanoreporter code. A singular nanoreporter is a reporter probe, as is a labeled component of a dual nanoreporter.

F-HOOK and G-HOOK: In the context of a dual nanoreporter, F- and G-hooks are each an affinity tag that is capable of being selectively bound to one of the probes. In preferred embodiments, the F-hook and G-hook are biotinylated oligonucleotides that are hybridizable to respective complementary sequences present in (*e.g.*, via ligation) or attached to (*e.g.*, via hybridization) the respective nanoreporter probes in a dual nanoreporter. Thus, the F-hooks and G-hooks can be used for purification, immobilization and stretching of the nanoreporter. Generally, where a dual nanoreporter contains one reporter probe and one ghost probe, the G-hook becomes attached to the reporter probe and the F-hook becomes attached to the ghost probe. F-hooks and G- hooks can be biotinylated on either end or internally. They can also be amine-modified to allow for attachment to a solid substrate for affinity purification.

F-TAG and G-TAG: Tandemly-repeated sequences of about 10 to about 25 nucleotides that are complementary to the F-hook and G-hook, respectively. G-tags and F-tags are attached to the nanoreporter probes. Generally, an F-tag is present in or attached to a ghost probe via a ligator sequence and a G-tag is present in or attached the reporter probe scaffold via a ligator sequence.

SPOT: A spot, in the context of nanoreporter detection, is the aggregate signal detected from the label monomers attached to a single label attachment site on a nanoreporter, and which, depending on the size of the label attachment region and the nature (*e.g.*, primary emission wavelength) of the label monomer, may appear as a single point source of light when visualized under a microscope. Spots from a nanoreporter may be

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overlapping or non-overlapping. The nanoreporter code that identifies that target molecule can comprise any permutation of the length of a spot, its position relative to other spots, and/or the nature (*e.g.*, primary emission wavelength(s)) of its signal. Generally, for each probe or probe pair of the invention, adjacent label attachment regions are non-overlapping, and/or the spots from adjacent label attachment regions are spatially and/or spectrally distinguishable, at least under the detection conditions (*e.g.*, when the nanoreporter is immobilized, stretched and observed under a microscope, as described herein).

Occasionally, reference is made to a spot "size" as a certain number of bases or nucleotides. As would be readily understood by one of skill in the art, this refers to the number of bases or nucleotides in the corresponding label attachment region.

NANOREPORTER CODE: The order and nature (*e.g.*, primary emission wavelength(s), optionally also length) of spots from a nanoreporter serve as a nanoreporter code that identifies the target molecule capable of being bound by the nanoreporter through the nanoreporter's target specific sequence(s). When the nanoreporter is bound to a target molecule, the nanoreporter code also identifies the target molecule. Optionally, the length of a spot can be a component of the nanoreporter code.

DARK SPOT: The term "dark spot" refers to a lack of signal, or "spot," from a label attachment site on a nanoreporter. Dark spots can be incorporated into the nanoreporter code to add more coding permutations and generate greater nanoreporter diversity in a nanoreporter population.

REGISTER: The term "register" refers to a set of alternating label attachment regions.

5.2 THE NANOREPORTER SCAFFOLD

The nanoreporter scaffold can be any molecular entity, more preferably a nucleic acid molecule, containing label attachment regions to which label monomers can be directly or indirectly attached. In one embodiment, the nanoreporter scaffold is a protein scaffold; in a preferred embodiment, the nanoreporter scaffold is a nucleic acid scaffold in which the label attachment regions are single-stranded regions to which other nucleic acids, such as oligonucleotide patches, RNA patches, or DNA patches, can attach by hybridization. In specific embodiments, the nanoreporter scaffold is a nucleic acid molecule.

There are no particular limitations on the types of scaffolds that are suitable for forming the nanoreporters of the invention. A scaffold according to the invention can

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essentially have any structure including, for example, single stranded linear scaffold, double stranded linear scaffold, single stranded circular scaffold or double stranded circular scaffold. Examples of scaffold structures include, for example, a scaffold made of one molecular entity such as polypeptides, nucleic acids or carbohydrates. A scaffold may also include a combination of structures, for example, a scaffold may be made of one or more polypeptide stretches coupled to one or more carbohydrate stretches.

Suitable molecular entities for scaffolds according to the invention include polymeric structures particularly nucleic acid based polymeric structures such as DNA. DNA based structures offer numerous advantages in the context of the present invention due at least in part to the vast universe of existing techniques and methodologies that allow manipulation of DNA constructs.

As indicated above, the scaffold may be single stranded or double stranded. Double stranded scaffold can be either conventional double stranded DNA or a double strand that is composed of a linear single stranded stretch of nucleic acid with patch units or flat-patches attached.

A scaffold can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21-100 label attachment regions or more.

The label attachment regions of a nanoreporter scaffold will vary in size depending on the method of labeling. In various embodiments, a label attachment region can have a length anywhere from 10 nm to 10,000 nm, but is more preferably from 50 nm to 5,000 nm, and is more preferably from 100 nm to 1,000 nm. In various embodiments, the label attachment region is from about 100 nm to about 500 nm, from about 150 nm to about 450 nm, from about 200 nm to about 400 nm, or from 250 to about 350 nm. In a preferred embodiment, the label attachment region corresponds closely to the size of a diffraction-limited spot, *i.e.*, the smallest spot that can be detected with standard optics, which is about 300 nm.

Where the scaffold is a nucleic acid, 1 nm corresponds to approximately 3 nucleotides; thus, an approximately 300 nm-label attachment region corresponds to approximately 900 bases. In other preferred embodiments, the label attachment region is from about 300 nucleotides to about 1.5kb, from about 450 nucleotides to about 1.35 kb, from about 0.6 kb to about 1.2 kb, or from 0.75 kb to about 1.05 kb.

An illustrative example of a molecular entity for a nanoreporter scaffold according to the invention is M13 DNA, which is single-stranded. In one embodiment, the nanoreporter

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scaffold is circular at least partially single stranded DNA, such as circular M13. In a more preferred embodiment, the nanoreporter scaffold is linear at least partially single stranded DNA, such as linear M13. In a specific embodiment, the M13 single-stranded DNA obtained by operating a cut at the BamH1 site of circular M13 DNA.

It should be noted that within the context of the present invention, linear DNA provides additional advantages compared to circular DNA. One advantage of using linear DNA in forming a scaffold according to the invention relates to the significantly reduced torsional stress associated with linear DNA. The added torsional stress associated with circular DNA may interfere with the structural integrity of the scaffold upon the addition to the scaffold of other components of the nanoreporter, such as patch units. Severe torsional stress may lead to the breaking of the structure of the scaffold. It should be noted however that the nanoreporters where only a few, short label attachment sites are labeled, circular DNA may be suitable.

5.2.1 NOVEL SYNTHETIC NANOREPORTER SCAFFOLD SEQUENCES

The present invention provides nanoreporter scaffold that are artificial nucleic acid molecules (DNA, RNA, or DNA/RNA hybrids) designed to have features that optimize labeling and detection of the nanoreporter. In these aspects of the invention, a nanoreporter scaffold is an artificial nucleic acids comprising one or more synthetic sequences from 50 to 50,000 bases long. Accordingly, the nanoreporter scaffold, which is preferably a DNA, is designed to have one or more Regions, useful as label attachment regions, comprising a regular pattern of a particular base (the "regularly-repeated base"). In such regions, the regularly-repeated base occurs with a periodicity of every nth residue, where n is any number, and preferably from 4 to 25.

Preferably, not more than 25% of the regularly-repeated base in a Region appears at other than said regular intervals. For example, if in a Region of 100 nucleotides there are 12 thymidine bases, and thymidine is the regularly-repeated base, in this aspect of the invention not more than 25% of these, *i.e.*, 3 thymidine bases, appear outside the regular pattern of thymidines. In specific embodiments, not more than 20%, not more than 15%, not more than 10%, not more than 9%, not more than 8%, not more than 7%, not more than 6%, not more than 5%, not more than 4%, not more than 3%, not more than 2% or not more than 1% of said base appears at other than said regular intervals in said region.

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The regularly-repeated base in the Regions in a nanoreporter scaffold, or its complementary regularly-repeated base in an annealed patch (or segment) can be used to attach label monomers, preferably light emitting label monomers, to the nanoreporter in a regular, evenly spaced pattern for better distribution of the nanoreporter signal. Preferably, where a Region is labeled, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% of occurrences of the regularly-repeated base is attached to at least one light-emitting label monomer, either by covalent attachment of a label monomer to a base, or by hybridization to a nucleic acid in which the complements of the regularly-repeated base are so-labeled.

This percentage of occurrences can be measured by any means known in the art. In one method, the amount of nucleic acid produced in a labeling reaction is purified (for example, RNA can be purified using a Qiagen RNeasy kit) and subjected to UV spectrophotometry. The absorbance ("A") at the appropriate wavelengths is measured for each of the nucleic acid (260 nm) and the label monomer whose occurrence is to be measured (*e.g.*, 495 nm for Alexa Fluor 488; 590 nm for Alexa Fluor 594; 650 for Alexa Fluor 647; and 550 nm for Cy3). The absorbance of the nucleic acid is corrected by adjusting the value of the absorbance at 260 nm ("A260") to remove the "noise" contribution from the label monomerby subtracting the absorbance at the peak wavelength for the label monomer (A_{LM}) minus the correction factor for that label monomer. Where the nucleic acid is RNA, the number of label monomers per one thousand nucleotides is calculated according to the formula:

$$\frac{\text{no. of label monomers}}{\text{1000 nucleotides}} = \frac{A260}{A_{LM}} \times \frac{9010}{EC_{LM}} \times 1000$$

where EC_{LM} is the extinction coefficient for the label monomer. From this formula, the percentage of occurrences of the regularly-repeated base that are attached to a light-emitting label monomer can be calculated.

Generally, the preferred regularly-repeating base in a label attachment region is thymidine, so that the region can be labeled by hybridization to one or more complementary patches (*e.g.*, RNA segments) in which the regularly-repeated base is uridine. This permits the use of amino-allyl-modified UTPs, which are readily commercially available, as label monomer attachment sites, in an otherwise random sequence. Preferably, in addition to the regular periodicity of the Regions, the regions (and the nucleic acid comprising them) contain minimal secondary structure. The overall GC-content is preferably maintained close

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to 50%, and is preferably consistent over relatively short stretches to make local Tm's similar.

The artificial nucleic acids of the invention, or at least the Regions therein, preferably do not have direct or inverted repeats that are greater than 12 bases in length. In other embodiments, the artificial nucleic acids and/or Regions do not have direct or inverted repeats that are greater than about 11, about 10 or about 9 bases in length.

In an exemplary Region in which the regularly-repeated nucleotide is a thymidine and a GC content of approximately 50%, excess adenines would make up the loss in abundance of T's. To generate the selected sequence, random sequences with fixed patterns of T's ranging from every 4th base to every 25th base are created and screened to minimize the presence of inverted and direct repeats.

Sequences are also screened preferably to avoid common six-base-cutter restriction enzyme recognition sites. Selected sequences are additionally subjected to predicted secondary structure analysis, and those with the least secondary structure are chosen for further evaluation. Any program known in the art can be used to predict secondary structure, such as the MFOLD program (Zuker, 2003, Nucleic Acids Res. 31 (13):3406-15; Mathews *et al.*, 1999, J. Mol. Biol. 288:911-940).

An appropriate sequence is divided into label attachment regions ranging from 50 bases to 2 kilobases long (could be longer). Each label attachment region is a unique sequence, but contains a consistent number and spacing of T's in relation to the other label attachment regions in a given reporter sequence. These label attachment regions can interspersed with other regions whose sequence does not matter. The synthetic label attachment regions in a nanoreporter scaffold can be of different lengths and/or have different regularly-repeated bases. An optimized start sequence for transcription by RNA polymerase T7, T3, or SP6 (beginning at position +1 of the transcript) can be added to the 5′ end of each label attachment region. Restriction sites are optionally added at the boundaries of each label attachment region to allow specific addition or deletion of individual label attachment regions to the sequence using conventional cloning techniques. The number of synthetic label attachment regions in a nanoreporter preferably ranges from 1 to 50. In yet other embodiments, the number of synthetic label attachment regions in a nanoreporter ranges from 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 synthetic label attachment regions to 15, 20, 30, 40, or 50 synthetic label attachment regions, or any range in between.

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An example of such a novel synthetic label attachment region is given below. In this sequence, shown 5′ to 3′, the T's are placed in every 8th position and the region is bounded by a 5′ Sac I restriction site and a 3′ Kpn I restriction site. An optimized transcript start site for T7 polymerase (GGGAGA) is included at the 5′ end of the region, downstream of the 5′ restriction site. The complement of this sequence, when generated as a single-stranded molecule, forms the scaffold for the RNA molecule transcribed from this label attachment region.

GAGCTCGGGAGATGGCGAGCTGGAAGCATCAGAAAGTAGGAAGATGACAAAAT
AGGGCCATAGAAGCATGAAGAACTGAACGCATGAGACAATAGGAAGCTACGCC
ACTAGGGACCTGAGAAGCTGAGCGGCTCAGCGGGTCCGAGCGTCAAAAAATAA
AAGAGTGAAACAATAGACGAATGACGCGGTAAAACCATCCAGAAGTAAACGGG
TACAAACATACAGAGATAGCCACCTGGACCAATAGGCACGTACAAACGTACAA
GCCTGGCGCGATGAGGCAATCCACACGTGCAGAGCTGGAACAATGGAAAGATG
CAAGAATAAACCGATACCGGGATCGAGGGCTCAGCGAATAAAGCAGTCAACAA
CTGGAAAGATCCACACATACCGGCGTAACCGAGTCCAAACATACAGACCTGCA
AGACTCGCGACATGGGACGGTAAAACCATCCGACCGTAAACCGGTAACCAGGT
AGCCGGGTAAAAACATAGCAGGGTGGAGACCTCAGAACGTAAAGACGTCCAAG
GGTCGCCGGATAGCGAACTACGCGCATCGCCCAATGGGCCAATCAACAGATAA
ACGAGTAGAAAAGTCAGAAAATAAGAAACTAACGAAATACGAGGGTCCAAGG
ATGCAAGACTGAGGCCCTAAGGAGATAAGGAAATAGGCCGATGCAGACCTGAA
ACGATGCACCGATCCGACGGTAAAAGACTAGACACGTAGCCGGATCAGGGCCT
GGGAGGCTGGAACCGTGAGCACATAGCAAAGTCGCAGCGTCGGCAGATGCGCC
GGTAAAAAAGTAGAGGCATGACCGGATGGGCAAATAGCGACGTACAGCAGTGA
AGCACTAAAAGCATCCAAGGGTAGGAGACTAGGCGCCTCGACGGGTAGGTACC

The synthetic nucleic acids of the present invention can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the label attachment region and the annealed patches or segments, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the synthetic nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-

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isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine.

Alternatively, the synthetic nucleic acid can be produced biologically using a vector into which a nucleic acid has been subcloned.

In various embodiments, the synthetic nucleic acid molecules of the invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup *et al.*, 1996, Bioorganic & Medicinal Chemistry 4(1):5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup *et al.*, 1996, Bioorganic & Medicinal Chemistry 4(1): 5-23; Perry-O'Keefe *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93: 14670-675.

In an exemplary embodiment, the selected novel synthetic sequence can be constructed synthetically as double-stranded DNA by a commercial gene synthesis company and cloned in an oriented fashion into a "phagemid", a plasmid vector containing an M13 or f1 phage intergenic (IG) region which contains the *cis*-acting sequences necessary for DNA replication and phage encapsidation, such as pUC119. The appropriate orientation of the cloned insert relative to the phage origin of replication allows for the generation of a single-stranded DNA scaffold which is the reverse complement of the RNA molecules generated by *in vitro* transcription for each label attachment region.

In order to generate the single-stranded DNA scaffold of the novel reporter, the phagemid is transformed into an *E. coli* strain containing an F' episome. Subsequent infection of the transformed bacteria with a helper phage such as the M13 mutant K07 results in the secretion of the phagemid carrying the novel reporter sequence as a single-stranded, packaged phage from which the circular, single-stranded DNA is prepared using a standard protocol. This DNA is linearized and the vector portion is excised by annealing

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short, complementary oligonucleotides to either end of the novel reporter sequence to generate double-stranded restriction sites, followed by treatment with the appropriate restriction enzymes.

To make the RNA molecules (patches or "segments") for each label attachment region, polymerase chain reaction ("PCR") primers are designed to generate a double-stranded template beginning with an RNA polymerase promoter (T7, T3, or SP6) directly upstream (5') of the transcription start site and ending following the 3' restriction enzyme site. Using this template, *in vitro* transcription of RNA molecules is performed in the presence of amino-allyl modified regularly-repeated base in the RNA (*e.g.*, UTP) and unmodified other bases (*e.g.*, ATP, CTP and GTP). This leads to an RNA product in which every regulalry-repeated base (*e.g.*, U) is modified to allow covalent coupling of a label monomer at that position in the RNA molecule.

Coupling of light-emitting label monomers to the RNA molecules and annealing of the labeled RNA molecules to the scaffold are carried out as described below.

Some design considerations for the de novo sequence are listed in Table 1 below.

Feature Of Synthetic Scaffold	**Advantages**
Novel synthetic sequence	Can be of any length and be designed to incorporate any desired sequence feature including but not limited to those listed in this table.
Minimal secondary structure (select against inverted repeats)	Allows for consistent transcription of full-length RNA molecules. Allows for consistent annealing of RNA molecules to scaffold at predictable temperatures. Minimizes self-annealing and/or cross-annealing between RNA molecules or scaffolds.
Minimal repeated sequences	Avoids mis-annealing between RNA molecules and inappropriate regions of the scaffold.
Unique restriction sites at borders of label attachment regions	Allows addition and deletion of individual label attachment regions using standard molecular cloning techniques.
Defined, even spacing of T's and transcription with amino-allyl-modified UTP (no unmodified UTP)	Controls number of coupling sites for monomers in each label attachment region, allowing for consistent brightness of individual labeled RNA molecules. Controls distance between monomers: spacing can be optimized to avoid stearic hindrance and fluorescence quenching.
Optimized start sequence for transcription by RNA polymerase T7, T3, or SP6	Promotes efficient in vitro transcription of each label attachment region.

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5.3 PATCHES

Label monomers that emit signals which constitute all or part of the nanoreporter code are attached to label attachment region(s) of the nanoreporter scaffold through a structure referred to herein as a "patch." The label monomers can be directly (*e.g.*, covalently or noncovalently) attached to a patch, or indirectly attached to a patch (*e.g.*, through hybridization).

Nucleic acid patches can by anywhere from 25 nucleotides to several kilobases (*e.g.*, 5kb) in length, and are preferably 50 nucleotides to 2kb in length. In specific embodiments, nucleic acid patches are approximately 25 to 250, 50 to 200, 50 to 150, or 50 to 100 nucleotides in length. In other embodiments, nucleic acid patches are approximately 500 to 2,000, 500 to 1,500, 500 to 1,000, 750 to 1,250, or 750 to 1,000 nucleotides in length. Nucleic acid patches can be RNA patches or DNA patches.

A label monomer can be covalently attached to a patch before or after the patch is attached to the label attachment region of a nanoreporter scaffold. For example, where the patch is a nucleic acid molecule, the label can be covalently attached by incorporation of a nucleotide containing a label monomer into the nucleic acid during its synthesis but before it is attached, *e.g.*, via hybridization, to the label attachment region of the scaffold. Alternatively, during the synthesis of a nucleic acid patch, a nucleotide containing a label monomer acceptor group be included, and the label monomer added to the nucleic acid patch after its synthesis, either before or after it is attached to the label attachment region of the scaffold. Alternatively, the label monomer can be indirectly attached to the patch, for example by hybridization of the patch to a "flap" that serves as a basis for attachment of the label monomer to the nanoreporter.

Thus, where a patch is a nucleic acid, it can range anywhere from 20 nucleotides to more than 5 kb in length, depending on the method of assembly of the nanoreporter.

For example, where a patch has covalently incorporated into it one or more label monomers that emit signals that are part of the nanoreporter code in the context of the labeled nanoreporter, the patch is preferably about 100 to about 10,000 bases, more preferably 200 to about 2000 bases, and yet more preferably 700 to about 1200 nucleotides in length, and is generally referred to herein as a "segment," a "dark "segment being the patch prior to the incorporation of the label monomer (but, in a preferred embodiment, containing label monomer acceptor sites, such as amino allyl nucleotides), and a "colored" segment being one containing the desired label monomer or label monomers. The Tm of a

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segment when hybridized to its label attachment region preferably is >80°C, more preferably >90°C, in 825 mM Na^+ (5X SSC).

Where a patch merely serves as a template for flap attachment to the nanoreporter, then it is preferably smaller in size, for example about 25-250 nucleotides in length, an is most preferably about 50-100 nucleotides in length. Such patches are referred to herein as "oligonucleotide patches." As detailed in Section below, an oligonucleotide is preferably partially complimentary in sequence to a scaffold, such that when it is annealed to the scaffold, an overhang is generated that is complementary to all or portion of a flap.

The terms "segment" and "oligonucleotide patch" are used herein merely for convenience of description; however, there is no size cutoff to distinguish a "segment" from an "oligonucleotide patch." The purpose of both types of structures is to maximize the labeling --and thus signal intensity-- from the nanoreporter, thereby allowing for single target molecule detection by a nanoreporter.

In certain aspects, the present invention provides a synthetic molecule, whose configuration is illustrated by reference to **Figure 7A**, comprising a strand of a nucleic acid **(scaffold)** and a plurality of patch pairs hybridized to said strand, wherein each patch pair comprises an "A" patch and a "B" patch, wherein, for each patch pair, (a) each "A" patch is an oligonucleotide comprising a first region **(1P)** and a second region **(2P)**, said first region being (i) at the alpha end of said "A" patch, and (ii) hybridized to a first portion of said strand, said second region being (ii) at the beta end of said "A" patch; (b) each "B" patch is an oligonucleotide comprising a third region **(3P)** and a fourth region **(4P)**, said third region being (i) at the alpha end of said "B" patch, and (ii) hybridized to said second region of said "A" patch, said fourth region being (i) at the beta end of said "B" patch and (ii) hybridized to a second portion of said strand, said second portion of said strand being to the beta end of said first portion of said strand, wherein said second region or said third region further comprises at its beta end or alpha end, respectively, a hybridizable region that is not hybridized to said "B" patch or "A" patch, respectively.

In the synthetic molecule of **Figure 7A**, the second region may further comprise at its beta end a hybridizable region that is not hybridized to said "B" patch, as depicted in **Figure 7B**, or the third region further comprises at its alpha end a hybridizable region that is not hybridized to said "A" patch, as depicted in **Figure 7C**.

The present invention further provides a synthetic molecule, whose configuration is illustrated by reference to **Figure 7D**, comprising a strand of a nucleic acid **(scaffold)** and a

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plurality of patch pairs hybridized to said strand, wherein each patch pair comprises an "A" patch and a "B" patch, wherein, for each patch pair, (a) each "A" patch is an oligonucleotide comprising a first region **(1P)** and a second region **(2P)**, said first region being (i) at the alpha end of said "A" patch, and (ii) hybridized to a first portion of said strand, said second region being (ii) at the beta end of said "A" patch; (b) each "B" patch is an oligonucleotide comprising a third region **(3P)** and a fourth region **(4P)**, said third region being (i) at the alpha end of said "B" patch, and (ii) hybridized to said second region of said "A" patch, said fourth region being (i) at the beta end of said "B" patch and (ii) hybridized to a second portion of said strand, said second portion of said strand being to the first of said first portion of said strand, wherein said second region further comprises at its beta end a first hybridizable region that is not hybridized to said "B" patch, and wherein said third region further comprises at its alpha end a second hybridizable region that is not hybridized to said "A" patch.

In the synthetic molecule of **Figure 7B**, each patch pair can be attached to a flap pair, as depicted in **Figure 7F**, wherein each flap pair comprises an "A" flap and a "B" flap, wherein, for each flap pair, (a) each "A" flap is an oligonucleotide comprising a first flap region **(1F)** and a second flap region **(2F)**; said first flap region being at the alpha end of said "A" flap; said second flap region (i) being at the beta end of said "A" flap and (ii) comprising at its beta end a hybridizable region that is not hybridized to said "A" patch, "B" patch or "B" flap; and (b) each "B" flap is an oligonucleotide comprising a third flap region **(3F)**, a fourth flap region **(4F)**, and a fifth flap region **(5F)**; said third flap region being (i) being at the alpha end of said "B" flap and (ii) comprising at its alpha end a hybridizable region that is not hybridized to said "A" patch, "B" patch or "A" flap; said fourth flap region (i) being between the third flap region and the fifth flap region and (ii) hybridized to said first flap region of said "A" flap; said fifth flap region being (i) at the beta end of said "B" flap, and (ii) hybridized to said hybridizable region of said second region of said "A" patch.

In the synthetic molecule of **Figure 7C**, each patch pair can be attached to a flap pair, as depicted in **Figure 7E**, wherein each flap pair comprises an "A" flap and a "B" flap, wherein, for each flap pair, (a) each "A" flap is an oligonucleotide comprising an first flap region **(1F)**, a second flap region **(2F)**, and a third flap region **(3F)**; said "A" flap region being (i) at the alpha end of said "A" flap and (ii) hybridized to said hybridizable region of said third region of said "B" patch; said second flap region being between the first flap region and the third flap region; said third flap region (i) being at the beta end of said "A"

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flap and (ii) comprising at its beta end a hybridizable region that is not hybridized to said "A" patch, "B" patch or "B" flap, and (b) each "B" flap is an oligonucleotide comprising a fourth flap region **(4F)** and a fifth flap region **(5F)**; said fourth flap region being (i) being at the alpha end of said "B" flap and (ii) comprising at its alpha end a hybridizable region that is not hybridized to said "A" patch, "B" patch or "A" flap; said fifth flap region being (i) at the beta end of said "B" flap, and (ii) hybridized to said second flap region of said "A" flap.

In the synthetic molecule of **Figures 7D** and **7E**, the split flaps can be attached one (*e.g.*, **(1O)**), or more (*e.g.*, **(2O)** and **(3O)**) oligonucleotides, as depicted in **Figure 7G**. Thus, the one or more oligonucleotides can be attached to the all or a portion of the "A" flap individually (*e.g.*, **(1O)**), the "B" flap individually (*e.g.*, **(3O)**), or span all or a portion of each of the "A" flap and "B" flap (*e.g.*, **(2O)**). Such oligonucleotides are preferably covalently bound to one or more label monomers.

The hybridizable regions of said synthetic molecules may be hybridized to a plurality of oligonucleotides, each bound, preferably covalently bound, to at least one label monomer, more preferably to at least five label monomers. In certain embodiments, all the oligonucleotides attached to a single patch pair comprise the same label monomers, *e.g.*, comprise label monomers that emit light at the same wavelength(s); in specific embodiments, all the oligonucleotides attached to at least two, or at least four, adjacent patch pairs preferably comprise the same label monomers. One or more of the oligonucleotides may be bound to at least one affinity tag.

In certain preferred embodiments, the label monomers are fluorophores or quantum dots.

In the synthetic molecule described above, alpha can refers to either 5′or 3′, and the corresponding beta to either 3′ or 5′, respectively.

The region of complementary in each patch pair, or between a given patch and corresponding flap, is preferably about 20 to 5,000 nucleotides. In certain embodiments, the region of complementary is about 20 to 100 nucleotides, or about 5 to 50 nucleotides.

In the synthetic molecules described above, each flap is preferably about 50 to 5,000 nucleotides in length. In certain embodiments, each flap is about 50 to 150 nucleotides.

The synthetic molecules described above may further comprise a target-specific region which binds to a target molecule. The target-specific region can be attached to the beta or alpha end of said strand.

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In certain embodiments, the synthetic molecule described above comprise at least ten patch pairs, or at least fifty patch pairs.

In the synthetic molecules described above, the strand, or scaffold, can be a linearized vector, such as linearized M13.

The synthetic molecule described above may further comprise (a) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (b) a second label attachment region, which is non-overlapping with the first label attachment region, to which is attached one or more label monomers that emit light constituting a second signal; (c) a third label attachment region, which is non-overlapping with the first and second label attachment regions, to which is attached one or more label monomers that emit light constituting a third signal; wherein each attachment region comprises a plurality of patch pairs; wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable; wherein the first and second signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the second and third signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the first and third signals are spatially resolvable under conditions that can be used to detect said first, second and third signals; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies the target molecule.

5.4 LABEL MONOMERS

The nanoreporters of the present invention can be labeled with any of a variety of label monomers, such as a radioisotope, fluorochrome, dye, enzyme, nanoparticle, chemiluminescent marker, biotin, or other monomer known in the art that can be detected directly (*e.g.*, by light emission) or indirectly (*e.g.*, by binding of a fluorescently-labeled antibody). Generally, one or more of the label attachments regions in the nanoreporter is labeled with one or more label monomers, and the signals emitted by the label monomers attached to the label attachment regions of a nanoreporter constitute a code that identifies the target to which the target-specific region of the nanoreporter binds. In certain embodiments, the lack of a given signal from the label attachment region (*i.e.*, a "dark" spot)

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can also constitute part of the nanoreporter code. An example of a dark spot is depicted at position 12 of the nanoreporter in Figure 1A.

Radioisotopes are an example of label monomers that can be utilized by the invention. Several radioisotopes can be used as label monomers for labeling nucleotides or proteins, including, for example, ^{32}P, ^{33}P, ^{35}S, ^{3}H, and ^{125}I. These radioisotopes have different half-lives, types of decay, and levels of energy which can be tailored to match the needs of a particular experiment. For example, ^{3}H is a low energy emitter which results in low background levels, however this low energy also results in long time periods for autoradiography. Radioactively labeled ribonucleotides, deoxyribonucleotides and amino acids are commercially available. Nucleotides are available that are radioactively labeled at the first, or α, phosphate group, or the third, or γ, phosphate group. For example, both [α -^{32}P] dATP and [γ -^{32}P] dATP are commercially available. In addition, different specific activities for radioactively labeled nucleotides are also available commercially and can be tailored for different experiments.

Another example of label monomers that can be utilized by the invention are fluorophores. Several fluorophores can be used as label monomers for labeling nucleotides including, for example, fluorescein, tetramethylrhodamine, and Texas Red. Several different fluorophores are known, and more continue to be produced, that span the entire spectrum. Also, different formulations of the same fluorophore have been produced for different applications. For example, fluorescein, can be used in its isothiocynanate form (FITC), as mixed isomer or single isomer forms of carboxyfluorescein succinimidyl ester (FAM), or as isomeric dichlorotriazine forms of fluorescein (DTAF). These monomers are chemically distinct, but all emit light with a peak between 515-520 nm, thereby generating a similar signal. In addition to the chemical modifications of fluorescein, completely different fluorophores have been synthesized that have the same or very similar emission peaks as fluorescein. For example, the Oregon Green dye has virtually superimposable excitation and emission spectra compared to fluorescein. Other fluorophores such as Rhodol Green and Rhodamine Green are only slightly shifted in their emission peaks and so also serve functionally as substitutes for fluorescein. In addition, different formulations or related dyes have been developed around other fluorophores that emit light in other parts of the spectrum.

Non-radioactive and non-fluorescent label monomers are also available. For example, biotin can be attached directly to nucleotides and detected by specific and high

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affinity binding to avidin or streptavidin which has been chemically coupled to an enzyme catalyzing a colorimetric reaction (such as phosphatase, luciferase, or peroxidase). Digoxigenin labeled nucleotides can also similarly be used for non-isotopic detection of nucleic acids. Biotinylated and digoxigenin-labeled nucleotides are commercially available.

Very small particles, termed nanoparticles, also can be used as label monomers to label nucleic acids. These particles range from 1-1000 nm in size and include diverse chemical structures such as gold and silver particles and quantum dots.

When irradiated with angled incident white light, silver or gold nanoparticles ranging from 40-120 nm will scatter monochromatic light with high intensity. The wavelength of the scattered light is dependent on the size of the particle. Four to five different particles in close proximity will each scatter monochromatic light which when superimposed will give a specific, unique color. The particles are being manufactured by companies such as Genicon Sciences. Derivatized silver or gold particles can be attached to a broad array of molecules including, proteins, antibodies, small molecules, receptor ligands, and nucleic acids. For example, the surface of the particle can be chemically derivitized to allow attachment to a nucleotide.

Another type of nanoparticle that can be used as a label monomer are quantum dots. Quantum dots are fluorescing crystals 1-5 nm in diameter that are excitable by a large range of wavelengths of light. These crystals emit light, such as monochromatic light, with a wavelength dependent on their chemical composition and size. Quantum dots such as CdSe, ZnSe, InP, or InAs possess unique optical properties.

Many dozens of classes of particles can be created according to the number of size classes of the quantum dot crystals. The size classes of the crystals are created either 1) by tight control of crystal formation parameters to create each desired size class of particle, or 2) by creation of batches of crystals under loosely controlled crystal formation parameters, followed by sorting according to desired size and/or emission wavelengths. Use of quantum dots for labeling particles, in the context of the present invention, is new, but is old in the art of semiconductors. Two examples of earlier references in which quantum dots are embedded within intrinsic silicon epitaxial layers of semiconductor light emitting/detecting devices are United States Patent Nos. 5,293,050 and 5,354,707 to Chapple Sokol, *et al.*

In specific embodiments, one or more of the label attachments regions in the nanoreporter is labeled with one or more light-emitting dyes, each label attachment region containing, directly or indirectly, one or more label monomers. The light emitted by the

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dyes can be visible light or invisible light, such as ultraviolet or infra red light. In exemplary embodiments, the dye is a fluorescence resonance energy transfer (FRET) dye; a xanthene dye, such as fluorescein and rhodamine; a dye that has an amino group in the alpha or beta position (such as a naphthylamine dye, 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalende sulfonate and 2-p-touidinyl-6-naphthalene sulfonate); a dye that has 3-phenyl-7-isocyanatocoumarin; an acridine, such as 9-isothiocyanatoacridine and acridine orange; a pyrene, a bensoxadiazole and a stilbene; a dye that has 3-(ε-carboxypentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CYA); 6-carboxy fluorescein (FAM); 5&6-carboxyrhodamine-110 (R110); 6-carboxyrhodamine-6G (R6G); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); 6-carboxy-X-rhodamine (ROX); 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE); ALEXA Fluor™; Cy2; Texas Red and Rhodamine Red; 6-carboxy-2',4,7,7'-tetrachlorofluorescein (TET); 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein (HEX); 5-carboxy-2',4',5',7'-tetrachlorofluorescein (ZOE); NAN; NED; Cy3; Cy3.5; Cy5; Cy5.5; Cy7; and Cy7.5; Alexa Fluor 350; Alexa Fluor 488; Alexa Fluor 532; Alexa Fluor 546; Alexa Fluor 568; Alexa Fluor 594; or Alexa Fluor 647.

The label monomers can be incorporated into a nanoreporter at different stages of its assembly, or into a component (*e.g.*, a "flap" or of the nanoreporter prior to its assembly into the nanoreporter.

A label monomer can be directly attached to a nucleotide using methods well known in the art. Nucleotides can also be chemically modified or derivitized in order to attach a label monomer. For example, a fluorescent monomer such as a fluorescein molecule can be attached to dUTP (deoxyuridine-triphosphate) using a four-atom aminoalkynyl group. Each label monomer is attached to a nucleotide making a label monomer: nucleotide complex.

This label monomer: nucleotide complex can be incorporated into nucleic acids (for example, a DNA patch or a detection oligonucleotide) in a variety of ways. For example, a label monomer: nucleotide complex can be incorporated at only one location within a nucleic acid or at two or more locations within a nucleic acid.

Amine-reactive and thiol-reactive fluorophores are available and used for labeling nucleotides and biomolecules. Generally, nucleotides are fluorescently labeled during chemical synthesis, for example, incorporation of amines or thiols during nucleotide synthesis permit addition of fluorophores. Fluorescently labeled nucleotides are commercially available. For example, uridine and deoxyuridine triphosphates are available that are conjugated to ten different fluorophores that cover the spectrum.

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A nucleotide can be attached to a label monomer first and then be incorporated into a nucleic acid. Alternatively, an existing nucleic acid can be labeled by attaching a label monomer to a nucleotide within the nucleic acid. For example aminoallyl- ("AA-") modified UTP nucleotides can be incorporated into the RNA product during transcription. In various embodiments, 20% or more of UTP nucleotides in a transcription reaction to generate RNA patches are AA modified. In various embodiments, about 20% to 100%, 20% to 80%, 30 to 80%, 40 to 60% or 50% to 75% of UTPs in a transcription reaction are AA-modified, in a preferred embodiment, approximately 50% of UTPs in a transcription reaction are AA-modified.

In addition, for example, different types of label monomer: nucleotide complexes can be incorporated into a single acid nucleic acid, where one component of the nanoreporter code comprises more than one type of signal.

Fluorescent dyes that can be bound directly to nucleotides can also be utilized as label monomers. For example, FAM, JOE, TAMRA, and ROX are amine reactive fluorescent dyes that have been attached to nucleotides and are used in automated DNA sequencing. These fluorecently labeled nucleotides, for example, ROX-ddATP, ROX-ddCTP, ROX-ddGTP and ROX-ddUTP, are commercially available.

Other types of label monomers that may be used to label a nanoreporter are quantum dots. Due to their very small size the quantum dots can be coupled into oligonucleotides directly without affecting the solubility or use of the oligonucleotide. In a preferred embodiment, only one oligonucleotide molecule is coupled to each nanoparticle. To synthesize an oligonucleotide-nanoparticle complex in a 1:1 ratio by conventional batch chemistry, both the oligonucleotide and the nanoparticle require a single reactive group of different kinds that can be reacted with each other. For example, if an oligonucleotide has an amino group and a nanoparticle has an aldehyde group, these groups can react to form a Schiff base. An oligonucleotide can be derivitized to attach a single amino or other functional group using chemistry well known in the art. However, when a nanoparticle is derivatized, it is covered with a chemical reagent which results in coating the entire surface of the nanoparticle with several functional groups.

The invention provides a method of coupling one oligonucleotide to one nanoparticle by chemically coupling the oligonucleotide on a solid surface such as the glass support used for the oligonucleotide synthesis.

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For example, commercially available resins for oligonucleotide synthesis such as long chain alkylamino controlled pore glass (lcaa CPG) can be used.

Alternatively, a flat surface such as a derivitized microscope slide can be used. The surface density of the nascent oligonucleotide chains should be lower than the diameter of the nanoparticle. This can be achieved by either choosing a glass support with low surface density of the reactive groups, or by using diluted reagent for the first step of the oligonucleotide synthesis so that the surface is not saturated. Another point of consideration when using the standard glass matrices for oligonucleotide synthesis is to use a pore diameter higher than the nanoparticle diameter to ensure the flow of the reagents. For example, an oligonucleotide can be synthesized on a diluted basis relative to the solid support, for example one tenth of a normal synthesis, to ensure good spacing of the oligonucleotides on the glass support. After the oligonucleotide is synthesized with a reactive functional group, for example, an amino group, derivitized nanoparticles are passed over the glass support to react with the oligonucleotides. A sufficiently large pore size of the glass support can be chosen to prevent clogging with nanoparticles. For example, a pore size of about 200 nm can be used. After the reaction is complete, un-reacted groups on the nanoparticle can be blocked and the complexes can be uncoupled from the glass support.

5.5 THE NANOREPORTER CODE

5.5.1 DUAL NANOREPORTERS

A nanoreporter whose components exist in two molecular entities is referred to as a dual nanoreporter. In a dual nanoreporter, generally each component contains a target-specific sequence, which improves the specificity of and binding kinetics of the nanoreporter to its target. The two different target-specific sequences are designed or selected such that each recognizes a different portion of a target molecule.

Figures 1A-1C illustrates embodiments of the invention involving dual nanoreporters. In Figures 1A and 1B, each of the two component of the nanoreporter is labeled, such that the nanoreporter's spectral code is formed only when the two components of the nanoreporter come together upon binding of the dual nanoreporter to its target molecule. However, in a dual nanoreporter, it is not necessary that both components are labeled. For example, as depicted in Figure 1C, one component of a dual nanoreporter is labeled with the nanoreporter code; and the other component attached to an affinity tag (arrow) that is useful to immobilize the nanoreporter for stretching an visualization.

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5.5.2 REGISTERS

The term "register" refers to a set of alternating (every other) label attachment regions. Registers are useful where it is desirable to label adjacent label attachment regions without a spacer region, and where the signal emanating from adjacent label attachment regions cannot be spatially resolved using the desired method of detection. Thus, the signals detected with use of a register is that form by the alternating, rather than adjacent, label attachment regions. Signals detected from a plurality of registers (*e.g.*, that together are all the label attachment regions) can be combined to form a nanoregister code. Generally when using registers, adjacent label attachment regions are labeled with spectrally distinguishable label monomers.

Examples of registers are depicted in Figures 3 and 5. For example, in Figure 3A-3B, there are 8 label attachment regions 1-8. Alternating label attachment regions 1, 3, 5 and 7 form one register, and label attachment regions 2, 4, 6 and 8 form another register. In Figure 3A, only one of the registers (1, 3, 5 and 7) is labeled; in Figure 3B, both registers are labeled.

5.6 AFFINITY TAGS

A variety of affinity tags known in the art may be used to purify and/or immobilize nanoreporters.

Where an affinity tag is used to immobilize a nanoreporter for the purpose of detection or imaging, it may be referred to herein as an "anchor." In a preferred embodiment, a biotin anchor is attached to the nanoreporter, allowing immobilization of the nanoreporter on a streptavidin coated slide.

An affinity tag that can be used to used for attachment to beads or other matrixes for a variety of useful applications including but not limited to purification.

Non-limiting examples of suitable affinity tags are provided below. It should be understood that most affinity tags could serve dual purposes: both as anchors for immobilization of the nanoreporters and tags for purification of the nanoreporters (whether fully or only partially assembled) or their components.

In certain embodiments, the affinity tag is a protein monomer. Examples of protein monomers include, but are not limited to, the immunoglobulin constant regions (see Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology,

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Vol. 2, Ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan *et al.*, 1987, Gene 67:21-30), and various cellulose binding domains (U.S. Pat. Nos. 5,496,934; 5,202,247; 5,137,819; Tomme *et al.*, 1994, Protein Eng. 7:117-123), etc. Other affinity tags are recognized by specific binding partners and thus facilitate isolation and immobilization by affinity binding to the binding partner, which can be immobilized onto a solid support. For example, the affinity tag can be an epitope, and the binding partner an antibody. Examples of such epitopes include, but are not limited to, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemagglutinin (HA) epitope, or digoxigenin ("DIG"). In other embodiments, the affinity tag is a protein or amino acid sequence that is recognized by another protein or amino acid, for example the avidin/streptavidin and biotin.

In certain aspects of the invention, the affinity tag is a nucleotide sequence. A large variety of sequences of about 8 to about 30 bases, more preferably of about 10 to about 20 bases, can be used for purification and immobilization of nanoreporters, and the sequence can be tandemly repeated (*e.g.*, from 1 to 10 tandem repeats). Such a sequence is preferably not widely represented (that is, present in fewer than 5% of the genes, more preferably, present in fewer than 3% of the genes, and, most preferably, present in fewer than 1% of the genes) in the sample being assayed (for example, where the nanoreporter is used for detection of human cellular RNA, the sequence is preferably not widely represented in the human genome); have little or no secondary structure or self-complementarity either internally or with copies of itself when multimerized (that is, all secondary structures of the multimerized tag preferably have a Tm less than 25°C at 1 M NaCl); have no significant identity or complementarity with scaffold or segment sequences (that is, the Tm of complementary sequences is preferably less than 25°C at 0.2 M NaCl); and have a Tm of about 35-65°C, more preferably about 40-50°C, in 50 mM Na^+.

In certain embodiments, different sequences are used as purification and immobilization tags. In this case, for example, the purification tag can be as described above, but the immobilization tag can be in the range of 10 to 100 bases, with a Tm up to 95°C in 50 mM Na^+. An alternative embodiment would be to have the purification tag nested within the immobilization tag (*e.g.*, the affinity tag would comprise a 25-base sequence of which 15 bases are used as a purification tag and the entire 25 bases are used as the immobilization tag).

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In certain instances, the affinity tag can be used for labeling a nanoreporter in addition to purifying or immobilizing the nanoreporter.

As will be appreciated by those skilled in the art, many methods can be used to obtain the coding region of the affinity tags, including but not limited to, DNA cloning, DNA amplification, and synthetic methods. Some of the affinity tags and reagents for their detection and isolation are available commercially.

5.7 TARGET-SPECIFIC SEQUENCES

The term "target-specific sequence" refers to a molecular entity that is capable of binding a target molecule. In the context of a nanoreporter, the target-specific sequence is attached to the nanoreporter scaffold.

The target specific sequence is generally an amino acid sequence (*i.e.*, a polypeptide or peptide sequence) or a nucleic acid sequence.

In specific embodiments, where the target-specific sequence is an amino acid sequence, the target-specific sequence is an antibody fragment, such as an antibody Fab' fragment, a single chain Fv antibody.

The target-specific sequence is preferably a nucleic acid sequence, and is most preferably within an oligonucleotide that is either covalently attached (*e.g.*, by ligation) or noncovalently attached (*e.g.*, by hybridization) to the nanoreporter scaffold. A target-specific nucleic acid sequence is preferably at least 15 nucleotides in length, and more preferably is at least 20 nucleotides in length. In specific embodiments, the target-specific sequence is approximately 10 to 500, 20to 400, 30 to 300, 40 to 200, or 50 to 100 nucleotides in length. In other embodiments, the target-specific sequence is approximately 30 to 70, 40 to 80, 50 to 90, or 60 to 100, 30 to 120, 40 to 140, or 50 to 150 nucleotides in length.

A target-specific nucleotide sequence preferably has a Tm of about 65-90°C for each probe in 825 mM Na^+ (5X SSC), most preferably about 78-83°C.

In certain preferred embodiments, the target specific sequence of each probe of a dual nanoreporter is about 35 to 100 nucleotides (for a total target sequence of about 70 to 200 nucleotides, covered by 2 probes), most preferably about 40 to 50 nucleotides for each probe (for a total of about 80 to 100 nucleotides).

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5.8 TARGET MOLECULES

The term "target molecule" is the molecule detected or measured by binding of a labeled nanoreporter whose target-specific sequence(s) recognize (are specific binding partners thereto). Preferably, an target molecule can be, but is not limited to, any of the following: DNA, cDNA, RNA, mRNA, peptide, a polypeptide/protein (*e.g.*, a bacterial or viral protein or an antibody), a lipid, a carbohydrate, a glycoprotein, a glycolipid, a small molecule, an organic monomer, or a drug. Generally, a target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA.

A target molecule can be part of a biomolecular sample that contains other components or can be the sole or major component of the sample. A target molecule can be a component of a whole cell or tissue, a cell or tissue extract, a fractionated lysate thereof or a substantially purified molecule. The target molecule can be attached in solution or solid-phase, including, for example, to a solid surface such as a chip, microarray or bead. Also. the target molecule can have either a known or unknown structure or sequence.

In certain specific embodiments, that target molecule is not a chromosome. In other specific embodiments, the target molecule is no greater than 1,000 kb (or 1 mb) in size, no greater than 500 kb in size, no greater than 250 kb in size, no greater than 175 kb in size, no greater than 100 kb in size, no greater than 50 kb in size, no greater than 20 kb in size, or no greater than 10 kb in size. In yet other specific embodiments, the target molecule is isolated from its cellular milieu.

In specific, non-limiting embodiments, the target molecule is one of the following antibodies or an antigen recognized by one of the following antibodies: anti-estrogen receptor antibody, an anti-progesterone receptor antibody, an anti-p53 antibody, an anti-Her-2/neu antibody, an anti-EGFR antibody, an anti-cathepsin D antibody, an anti-Bcl-2 antibody, an anti-E-cadherin antibody, an anti-CA125 antibody, an anti-CA15-3 antibody, an anti-CA19-9 antibody, an anti-c-erbB-2 antibody, an anti-P-glycoprotein antibody, an anti-CEA antibody, an anti-retinoblastoma protein antibody, an anti-ras oncoprotein antibody, an anti-Lewis X antibody, an anti-Ki-67 antibody, an anti-PCNA antibody, an anti-CD3 antibody, an anti-CD4 antibody, an anti-CD5 antibody, an anti-CD7 antibody, an anti-CD8 antibody, an anti-CD9/p24 antibody, an anti-CD10 antibody, an anti-CD11c antibody, an anti-CD13 antibody, an anti-CD14 antibody, an anti-CD15 antibody, an anti-CD19 antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD23 antibody, an

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anti-CD30 antibody, an anti-CD31 antibody, an anti-CD33 antibody, an anti-CD34 antibody, an anti-CD35 antibody, an anti-CD38 antibody, an anti-CD41 antibody, an anti-LCA/CD45 antibody, an anti-CD45RO antibody, an anti-CD45RA antibody, an anti-CD39 antibody, an anti-CD100 antibody, an anti-CD95/Fas antibody, an anti-CD99 antibody, an anti-CD106 antibody, an anti-ubiquitin antibody, an anti-CD71 antibody, an anti-c-myc antibody, an anti-cytokeratins antibody, an anti-vimentins antibody, an anti-HPV proteins antibody, an anti-kappa light chains antibody, an anti-lambda light chain antibody, an anti-melanosome antibody, an anti-prostate specific antigen antibody, an anti-S-100 antibody, an anti-tau antigen antibody, an anti-fibrin antibody, an anti-keratins antibody, an anti-Tn-antigen antibody receptor protein, a lymphokine, an enzyme, a hormone, a growth factor, or a nucleic acid binding protein, a ligand for a cell adhesion receptor; a ligand for a signal transduction receptor; a hormone; a molecule that binds to a death domain family molecule; an antigen; a viral particle, a viral coating protein or fragment thereof, a toxic polypeptide selected from the group consisting of: (a) ricin, (b) Pseudomonas exotoxin (PE); (c) bryodin; (d) gelonin; (e) α-sarcin; (f) aspergillin; (g) restrictocin; (h) angiogenin; (i) saporin; (j) abrin; (k) pokeweed antiviral protein (PAP); and (l) a functional fragment of any of (a)-(k); a cytokine, or a soluble cytokine selected from the group consisting of erythropoietin, interleukins, interferons, fibroblast growth factors, transforming growth factors, tumor necrosis factors, colony stimulating factors and epidermal growth factor, Class I MHC antigens, class II MHC antigens, internalizing cell-surface receptors and/or viral receptors.

In specific, non-limiting embodiments, the target molecule is an antigen such as alpha fetoprotein, alpha-1 antitrypsin, α-2 macroglobulin, adiponectin, apoliprotein-A-1, apoliprotein-CIII, apoliprotein-H, BDNF, β-2 microglobulin, C reactive protein, calcitonin, cancer antigen 19-9, cancer antigen 125, CEA, CD 40, CD 40 ligand, complement 3, CK-MB, EGF, ENA-78, endothelin-1, enrage, eotaxin, erythropoietin, Factor VII, FABP, ferritin, FGF-basic, fibrinogen, G-CSF, GST, GM-CSF, growth hormone, haptoglobin, ICAM-1, IFN-gamma, IgA, IgE, IGF-1, IgM, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-16, insulin, leptin, lipoprotein (a), lymphotactin, MCP-1, MDC, MIP-1α, MIP-1β, MMP-2, MMP-3, MMP-9, myeloperoxidase, myoglobin, PAI-1, PAP, PAPP-A, SGOT, SHBG, PSA (free), RANTES, serum amyloid P, stem cell factor, TBG, thrombopoietin, TIMP-1, tissue factor, TNF-α, TNF-β, TNF RII, TSH, VCAM-1, VEGF, or vWF.

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In some embodiments, the target molecule is an autoimmune related molecule such as ASCA, β-2 glycoprotein, C1q, centromere Prot. B, collagen type 1, collagen type 2, collagen type 4, collagen type 6, Cyto P450, ds DNA, histone, histone H1, histone H2A, histone H2B, histone H3, histone H4, HSC-70, HSP-32, HSP-65, HSP-71, HSP-90α, HSP-90β, insulin, JO-1, mitochondrial, myeloperoxidase, pancreatic islet cells, PCNA, PM-1, PR3, ribosomal P, RNP-A, RNP-C, RNP, Sel-70, Smith, SSA, SSB, T3, T4, thyroglobulin, tTG, (celiac disease), or thyroid microsomal.

In some embodiments, the target molecule is a component isolated from an infectious agent, such as Cholera Toxin, Cholera Toxin β, *Campylobacter jejuni*, cytomegalovirus, Diptheria toxin, Epstein-Barr NA, Epstein-Barr EA, Epstein-Barr VCA, *Heliobacter pylori*, HBV core, HBV envelope, HBV surface (Ad), HBV surface (Ay), HCV core, HCV NS3, HCV NS4, HCV NS5, hepatitis A, hepatitis D, HEV orf2 3KD, HEV orf2 6 KD, HEV orf 3KD, HIV-1 p24, HIV-1 gp41, HIV-1 gp120, HPV, HSV-1/2, HSV-1 gD, HSV-2 gD, HTLV-1/2, influenza A, influenza A H3N2, influenza B, *Leishmania donorani*, Lyme disease, mumps, *M. pneumonia*, *M. tuberculosis*, parainfluenza 1, parainfluenza 2, parainfluenza 3, polio virus, RSV, Rubella, Rubeola, Streptolysin O, Tetanus Toxin, *T. pallidum* 15kD, *T. pallidum* p47, *T. cruzi*, Toxoplasma, Varicella zoster.

5.9 NANOREPORTER POPULATIONS

The present invention provides nanoreporter or nanoreporter label unit populations, for example nanoreporter or nanoreporter label unit libraries, that contain at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 75, at least 100, at least 200, at least 300, at least 400, at least 500, at least 750, or at least 1,000 unique nanoreporters or nanoreporter label units, respectively. As used herein, "unique" when used in reference to a nanoreporter or nanoreporter label units within a population is intended to mean a nanoreporter or label unit that has a code that distinguishes it from other nanoreporters or label units in the same population.

In specific embodiments, the present invention provides nanoreporter populations with at least 5,000, at least 10,000, at least 20,000 or at least 50,000 unique nanoreporters or nanoreporter label units.

The nanoreporters in a population of nanoreporters can be singular nanoreporters, dual nanoreporters, or a combination thereof. The nanoreporters can be labeled or unlabeled.

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The size of a nanoreporter population and the nature of the target-specific sequences of the nanoreporters within it will depend on the intended use of the nanoreporter. Nanoreporter populations can be made in which the target-specific sequences correspond to markers of a given cell type, including a diseased cell type. In certain embodiments, nanoreporters populations are generated in which the target-specific sequences represent at least 0.1%, at least 0.25%, at least 0.5%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, or at least 70% of the different type of transcripts in a cell. In certain embodiments, nanoreporters populations are generated in which the target-specific sequences represent at least 0.1%, at least 0.25%, at least 0.5%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, or at least 70% of the different genes in a cell. In yet other embodiments, nanoreporter populations are generated in which at least some of the target-specific sequences represent rare transcripts in a cell or tissue. Such nanoreporter populations preferably represent at least 5 rare transcripts. In specific embodiments, such nanoreporter populations represent at least 10, at least 20, at least 30, at least 40 or at least 50 rare transcripts.

In a specific embodiment, the cell or tissue is a mammalian cell or tissue, and more preferably is a human cell or tissue.

In certain embodiments, the nanoreporter population is a diagnostic or prognostic nanoreporter populations. For example, a diagnostic nanoreporter population can be generated that is useful for screening blood products, in which the target-specific sequences bind to the nucleic acids of contaminating viruses such the hepatitis B, hepatitis C, and the human immunodeficiency virus. Alternatively, the diagnostic nanoreporter population may contain target-specific sequences corresponding to cellular disease markers, such as tumor antigens. Prognostic nanoreporter populations generally include target-specific markers that represent different stages of a given disease such as cancer. By selecting appropriate target-specific sequences, a nanoreporter population can be used both to diagnose and prognose disease.

5.10 BIOMOLECULAR SAMPLES

The nanoreporter systems of the invention can be used to detect target molecule in any biomolecular sample. As will be appreciated by those in the art, the sample may

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comprise any number of things, including, but not limited to: cells (including both primary cells and cultured cell lines), cell lysates or extracts (incuding but not limited to RNA extracts; purified mRNA), tissues and tissue extracts (incuding but not limited to RNA extracts; purified mRNA); bodily fluids (including, but not limited to, blood, urine, serum, lymph, bile, cerebrospinal fluid, interstitial fluid, aqueous or vitreous humor, colostrum, sputum, amniotic fluid, saliva, anal and vaginal secretions, perspiration and semen, a transudate, an exudate (*e.g.*, fluid obtained from an abscess or any other site of infection or inflammation) or fluid obtained from a joint (*e.g.*, a normal joint or a joint affected by disease such as rheumatoid arthritis, osteoarthritis, gout or septic arthritis) of virtually any organism, with mammalian samples being preferred and human samples being particularly preferred; environmental samples (including, but not limited to, air, agricultural, water and soil samples); biological warfare agent samples; research samples including extracellular fluids, extracellular supernatants from cell cultures, inclusion bodies in bacteria, cellular compartments, cellular periplasm, mitochondria compartment, etc.

The biomolecular samples can be indirectly derived from biological specimens. For example, where the target molecule of interest is a cellular transcript, *e.g.*, a messenger RNA, the biomolecular sample of the invention can be a sample containing cDNA produced by a reverse transcription of messenger RNA. In another example, the biomolecular sample of the invention is generated by subjecting a biological specimen to fractionation, *e.g.*, size fractionation or membrane fractionation.

The biomolecular samples of the invention may be either "native," *i.e.*, not subject to manipulation or treatment, or "treated," which can include any number of treatments, including exposure to candidate agents including drugs, genetic engineering (e.g. the addition or deletion of a gene), etc.

5.11 SEPARATION OF LABEL MONOMERS

In addition to detecting an overall signal generated from a labeled nanoreporter, the invention provides for the determination of the spatial location of signals emanating from the label monomers (*i.e.*, spots) on a nanoreporter, each spot representing the aggregate signal from label monomers attached to a given label attachment region. A spot may contain signals of the same wavelength or of different wavelengths. Thus, the nature of the spots on a nanoreporter and their location constitutes the nanoreporter code code.

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Any of a variety of means can be used to "stretch" the nanoreporter to separate the individual spots. For example, a nanoreporter can be stretched using a flowstretch technique (Henegariu *et al.*, 2001, Biotechniques 31:246-250), a receding meniscus technique (Yokota *et al.*, 1997, Nuc. Acids Res. 25:1064-1070) or an electrostretching technique (Matsuura *et al.*, 2001, Nuc. Acids Res. 29: E79).

The use of flow-stretching, receding meniscus, or electro-stretching techniques allows for the separation of the label attachment regions within a nanoreporter so that one can determine spatially where a particular signal is positioned in the nanoreporter. Therefore, unique nanoreporters that have the same combination of label monomers and the same overall signal can be differentiated from one another based on the location of those label monomers within the nanoreporter.

This ability to locate the position of a label attachment region or spot within a nanoreporter allows for the position of the signal(s) emitted by the label monomers in each label attachment region to be used as a distinguishing characteristic when generating a set of unique nanoreporters. Hence, a complex set of nanoreporters can be generated using the same combination of starting label monomers by varying the positions of the label monomers within a nanoreporter.

Prior to stretching a nanoreporter, it is preferable to immobilize the nanoreporter to a solid surface using an affinity tag, as described in Section 5.6 above.

In certain aspects of the invention, one end of a nanoreporter is immobilized, either through specific or non-specific binding to a solid surface, the nanoreporter is stretched, and then the other end of the reporter is immobilized, also either through specific or non-specific binding to a solid surface. Accordingly, the nanoreporter is "frozen" in its stretched, or extended, state, to facilitate resolution of the nanoreporters code by detecting and/or imaging the signals emitted by the label monomers attached to a nanoreporter and their locations relative to one another. These aspects of the invention are described below in Section 5.12.

5.12 IMMOBILIZATION OF STRETCHED NANOREPORTERS

The present invention provides methods and compositions that facilitate the identification of primary structures of a variety of nanoreporters. In certain aspects, the present invention provides methods for the selective immobilization of nanoreporters in an extended state. According to the invention, a nanoreporter can be selectively immobilized

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while fully extended under whatever force is used for the extension. In addition, the methods of the invention facilitate the selective immobilization of extended nanoreporters that are oriented with respect to each other. In other words, according to the methods of the invention, a plurality of nanoreporters can readily be immobilized in the same orientation with respect to each other.

In one aspect, the present invention provides methods for selectively immobilizing a nanoreporter in an extended state. For the methods of this aspect of the invention, generally, a first portion of the nanoreporter is immobilized by any technique known to those of skill in the art. Indeed, the technique for immobilizing the first portion of the nanoreporter is not critical to many embodiments of the invention. In certain embodiments, the first portion of the nanoreporter can be immobilized selectively or non-selectively. In certain embodiments the first portion is immobilized by one or more covalent bonds. In certain embodiments, the first portion is immobilized by one or more non-covalent bonds. Exemplary immobilized first portions are described in the sections below.

With an immobilized first portion, the nanoreporter can be extended by any technique for extending a nanoreporter apparent to those of skill in the art. In certain embodiments, the technique for extending the nanoreporter is not critical for the methods of the invention. In certain embodiments, the technique for extending the nanoreporter appropriate for the class of nanoreporter according to the judgment of one of skill in the art. In certain embodiments, the nanoreporter is extended by application of a force capable of extending the nanoreporter. The force can be any force apparent to one of skill in the art for extending the nanoreporter. Exemplary forces include gravity, hydrodynamic force, electromagnetic force and combinations thereof. Specific techniques for extending the nanoreporter are described in the sections below.

The nanoreporter is in an extended state if it would be recognized as extended by one of skill in the art. In certain embodiments, the nanoreporter is in an extended state when it is in the field of a force capable of extending the nanoreporter. In certain embodiments, the nanoreporter is in an extended state when its average hydrodynamic radius is more than double the average hydrodynamic radius of the nanoreporter in its native state as recognized by those of skill in the art.

In this aspect of the invention, the methods generally comprise the step of selectively immobilizing a second portion of the nanoreporter while it is in an extended state. This can result in an immobilized nanoreporter that is extended between the first and the second

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portion. Remarkably, since the nanoreporter is selectively immobilized while extended, that extension can be preserved in the immobilized nanoreporter. Generally, the first portion and the second portion of the nanoreporter are not the same.

The selective immobilization can be according to any technique for selective immobilization of a portion of a nanoreporter apparent to those of skill in the art. The selective immobilization can be through, for example, the formation of one or more covalent bonds or one or more non-covalent bonds, or both. Particular examples of selective immobilization techniques are described in the sections below. In particular embodiments, one or more binding pairs are used to immobilize the second portion of the nanoreporter.

The second portion can be immobilized onto any substrate apparent to those of skill in the art. The substrate can be any substrate judged to be useful for immobilization known to those of skill in the art. In certain embodiments, the second portion can be immobilized to another molecule. Further useful substrates include surfaces, membranes, beads, porous materials, electrodes, arrays and any other substrate apparent to those of skill in the art.

In another aspect, the present invention provides a composition comprising a selectively immobilized, extended nanoreporter. The compositions generally comprise a substrate and an extended nanoreporter selectively immobilized onto the substrate. The substrate can be any substrate known to those of skill in the art. Exemplary substrates include those described in the sections below. At least two portions of the nanoreporter are immobilized onto the substrate, and the nanoreporter is in an extended state between the two portions. In certain embodiments, at least one portion of the nanoreporter is selectively immobilized onto the substrate. In certain embodiments, two or more portions of the nanoreporter are selectively immobilized onto the substrate. The nanoreporter can be extended and/or immobilized by any technique apparent to those of skill, including particularly the methods of the present invention.

In another aspect, the present invention provides methods for selectively immobilizing a nanoreporter in an oriented state. The nanoreporter can be any nanoreporter described above. In certain embodiments, the nanoreporter can be flexible, or in certain embodiments the nanoreporter can be rigid or semi-rigid. For the methods of this aspect of the invention, generally, a first portion of the nanoreporter is immobilized as described above. With an immobilized first portion, the nanoreporter can be oriented by any technique for extending a nanoreporter apparent to those of skill in the art. In certain embodiments, the technique for orienting the nanoreporter is not critical for the methods of

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the invention. In certain embodiments, the technique for orienting the nanoreporter appropriate for the class of nanoreporter according to the judgment of one of skill in the art. In certain embodiments, the nanoreporter is oriented by application of a force capable of orienting the nanoreporter. The force can be any force apparent to one of skill in the art for orienting the nanoreporter. Exemplary forces include gravity, hydrodynamic force, electromagnetic force and combinations thereof. Specific techniques for extending the nanoreporter are described in the subsections below.

The nanoreporter is in an oriented state if it would be recognized as oriented by one of skill in the art. In certain embodiments, the nanoreporter is in an oriented state when it is in the field of a force capable of orienting the nanoreporter. In certain embodiments, the nanoreporter is in an oriented state when its termini are arranged in parallel, as recognized by those of skill in the art, with the field of a force capable of orienting the nanoreporter. In certain embodiments, a plurality of nanoreporters is in an oriented state when the termini of the nanoreporters are arranged in parallel, as recognized by those of skill in the art,.

In this aspect of the invention, the methods generally comprise the step of selectively immobilizing a second portion of the nanoreporter while it is in an oriented state. This can result in an immobilized nanoreporter that is oriented between the first and the second portion. Remarkably, since the nanoreporter is selectively immobilized while extended, that orientation can be preserved in the immobilized nanoreporter. The selective immobilization can according to the methods described above.

In another aspect, the present invention provides a composition comprising a selectively immobilized, oriented nanoreporter. The compositions generally comprise a substrate and an oriented nanoreporter selectively immobilized onto the substrate. The substrate can be any substrate known to those of skill in the art. Exemplary substrates include those described in the sections below. At least two portions of the nanoreporter are immobilized onto the substrate, and the nanoreporter is in an oriented state between the two portions. In certain embodiments, at least one portion of the nanoreporter is selectively immobilized onto the substrate. In certain embodiments, both portions of the nanoreporter are selectively immobilized onto the substrate. The nanoreporter can be oriented and/or immobilized by any technique apparent to those of skill, including particularly the methods of the present invention.

The methods and compositions of the present invention can be used for any purpose apparent to those of skill in the art. For instance, the immobilized and extended and/or

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oriented nanoreporter can be used as a label for a substrate on which the nanoreporter is immobilized. The primary sequence of the immobilized and extended and/or oriented nanoreporter can be identified by any technique apparent to those of skill. Advantageously, immobilization of the extended and/or oriented nanoreporter can facilitate such techniques. In certain embodiments, the immobilized and extended and/or oriented nanoreporter can be used to guide the manufacture of nanopaths, for example to create nanowires or nanocircuits. Further uses for the immobilized and extended and/or oriented nanoreporters are described in the sections below.

All terms used herein have their ordinary meanings to those of skill in the art unless indicated otherwise. The following terms shall have the following meanings.

As used herein, the term "binding pair" refers to first and second molecules or moieties that are capable of selectively binding to each other, *i.e.* binding to each other with greater affinity than to other components in a composition. The binding between the members of the binding pair can be covalent or non-covalent. In certain embodiments, the binding is noncovalent. Exemplary binding pairs include immunological binding pairs (*e.g.*, any haptenic or antigenic compound in combination with a corresponding antibody or binding portion or fragment thereof, for example digoxigenin and anti-digoxigenin, fluorescein and anti-fluorescein, dinitrophenol and anti-dinitrophenol, bromodeoxyuridine and anti-bromodeoxyuridine, mouse immunoglobulin and goat anti-mouse immunoglobulin) and nonimmunological binding pairs (*e.g.*, biotin-avidin, biotin-streptavidin, hormone-hormone binding protein, receptor-receptor ligand (*e.g.*, acetylcholine receptor-acetylcholine or an analog thereof), IgG-protein A, lectin-carbohydrate, enzyme-enzyme cofactor, enzyme-enzyme inhibitor, complementary polynucleotide pairs capable of forming nucleic acid duplexes, and the like). For instance, immunoreactive binding members may include antigens, haptens, aptamers, antibodies (primary or secondary), and complexes thereof, including those formed by recombinant DNA methods or peptide synthesis. An antibody may be a monoclonal or polyclonal antibody, a recombinant protein or a mixture(s) or fragment(s) thereof, as well as a mixture of an antibody and other binding members. Other common binding pairs include but are not limited to, biotin and avidin (or derivatives thereof), biotin and streptavidin, carbohydrates and lectins, complementary nucleotide sequences (including probe and capture nucleic acid sequences), complementary peptide sequences including those formed by recombinant methods, effector and receptor molecules,

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hormone and hormone binding protein, enzyme cofactors and enzymes, enzyme inhibitors and enzymes, and so forth.

"Selective binding" refers to the any preferential binding of a pair of molecules or moieties for each other with respect to other molecules or moieties in a composition that would be recognized by one of skill in the art. In certain embodiments, a pair of molecules or moieties selectively binds when they preferentially bind each other compared to other molecules or moieties. Selective binding can include affinity or avidity, or both, of one molecule or moiety for another molecule or moiety. In particular embodiments, selective binding requires a dissociation constant (K_D) of less than about $1x10^{-5}$ M or less than about $1x10^{-6}$ M, $1x10^{-7}$ M, $1x10^{-8}$ M, $1x10^{-9}$ M, or $1x10^{-10}$ M. In contrast, in certain embodiments, non-selective binding has significantly less affinity, for example, a K_D greater than $1x10^{-3}$ M.

"Extended state" refers to a nanoreporter in a state that would be recognized as extended by one of skill in the art. In certain embodiments, a nanoreporter is in an extended state when it is extended relative to its native conformation in solution. In certain embodiments, a nanoreporter is in an extended state when it is in the field of a force capable of extending the nanoreporter. In certain embodiments, an extended state of a nanoreporter can be determined quantitatively. In such embodiments, those of skill in the art will recognize R as the end-to-end vector of the nanoreporter, *i.e.* the distance between two termini of the nanoreporter, and <R> as the average end-to-end vector such that 95% of R will be within 2<R> in a solution deemed appropriate to one of skill in the art. Exemplary solutions include, for example, a dilute solution of the nanoreporter in water or in a pH buffer. In particular embodiments, a nanoreporter is in an extended state when R is greater than 2.0<R>.

"Oriented state" refers to a nanoreporter in a state that would be recognized as oriented by one of skill in the art. In certain embodiments, a nanoreporter is in an oriented state when it is oriented relative to its native conformation in solution. In certain embodiments, the nanoreporter is oriented when it is arranged in parallel with the field of a force capable of orienting the nanoreporter. In certain embodiments, the nanoreporter is oriented when it is one of a plurality of nanoreporters that are arranged in parallel, as recognized by those of skill in the art.

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5.12.1 METHODS OF SELECTIVE IMMOBILIZATION

As described above, the present invention provides methods for the selective immobilization of a nanoreporter in an extended state. The nanoreporter, once selectively immobilized, can be used for any purpose apparent to those of skill in the art.

In certain embodiments, the nanoreporter is any polymer known to those of skill in the art. For instance, the nanoreporter can be a polysaccharide, a polypeptide or a polynucleotide. Useful polynucleotides include ribonucleic acids, deoxyribonucleic acids and other polynucleotides known to those of skill in the art.

The nanoreporter can be of any size that is sufficient to allow extension and immobilization of the nanoreporter according to the methods of the invention. In certain embodiments when the nanoreporter is a polynucleotide, the nanoreporter can have a length of greater than 500 bp, greater than 750 bp, greater than 1 kb, greater than 1.5 kb, greater than 2.0 kb, greater than 2.5 kb, greater than 3.0 kb, greater than 4.0 kb or greater than 5.0 kb. In certain embodiments, when the nanoreporter is a polypeptide, the nanoreporter can have a size of greater than 50 amino acids, greater than 100 amino acids, greater than 200 amino acids, greater than 300 amino acids, greater than 400 amino acids, greater than 500 amino acids, greater than 750 amino acids, greater than 1000 amino acids, greater than 1500 amino acids, greater than 2000 amino acids, greater than 2500 amino acids, greater than 3000 amino acids, greater than 4000 amino acids or greater than 5000 amino acids. In certain embodiments, when the nanoreporter is a polysaccharide, the nanoreporter can have a size of greater than 50 saccharides, greater than 100 saccharides, greater than 200 saccharides, greater than 300 saccharides, greater than 400 saccharides, greater than 500 saccharides, greater than 750 saccharides, greater than 1000 saccharides, greater than 1500 saccharides, greater than 2000 saccharides, greater than 2500 saccharides, greater than 3000 saccharides, greater than 4000 saccharides or greater than 5000 saccharides.

The nanoreporter can be a native nanoreporter as understood by those of skill in the art, or the nanoreporter can be a non-native nanoreporter. In certain embodiments, when the nanoreporter is a polypeptide, the nanoreporter can comprise only naturally occurring amino acids, or the nanoreporter can comprise naturally occurring amino acids and non-naturally occurring amino acids. The other amino acids can be any amino acids, or derivatives or analogs thereof, known to those of skill in the art. In certain embodiments, when the nanoreporter is a polynucleotide, the polynucleotide can comprise only naturally occurring nucleotides, or the polynucleotide can comprise naturally occurring nucleotides and non-

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naturally occurring nucleotides. In certain embodiments, when the nanoreporter is a polysaccharide, the polysaccharide can comprise only naturally occurring saccharides, or the polysaccharide can comprise naturally occurring saccharides and non-naturally occurring saccharides. In certain embodiments, the polymers can comprise only non-natural monomers. In further embodiments, the nanoreporter can comprise a plurality of classes of monomers, such as amino acids, nucleotides and/or saccharides.

In certain embodiments, the nanoreporter comprises only one primary, covalently linked chain of monomers. For instance, when the nanoreporter is a polypeptide, in certain embodiments, the nanoreporter comprises only one primary amino acid chain. When the nanoreporter is a polynucleotide, in certain embodiments, the nanoreporter is single stranded. In further embodiments, the nanoreporter comprises two primary, covalently linked chains of monomers. For instance, when the nanoreporter is a polypeptide, in certain embodiments, the nanoreporter comprises two primary amino acid chains. When the nanoreporter is a polynucleotide, in certain embodiments, the nanoreporter comprises two polynucleotide strands; in certain embodiments, the nanoreporter can be double stranded, in part or in whole. In further embodiments, the nanoreporter comprises three or more primary, covalently linked chains of monomers. For instance, when the nanoreporter is a polypeptide, in certain embodiments, the nanoreporter comprises three primary amino acid chains. When the nanoreporter is a polynucleotide, in certain embodiments, the nanoreporter comprises three polynucleotide strands. For instance, the nanoreporter can comprise three strands F1, X and F2 where a portion of strand X is complementary to strand F1 and a portion of strand X is complementary to strand F2. An example is illustrated in Figure 13A. In certain embodiments, the nanoreporter comprises more than three primary, covalently linked chains of monomers.

Advantageously, a nanoreporter of the invention can comprise one or more labels that facilitate the detection, imaging or identification of the nanoreporter by techniques known to those of skill in the art. The label can be any detectable moiety known to those of skill in the art. Exemplary labels for nanoreporters include detectable isotopes, radioisotopes, fluors, dyes, enzymes, ligands, receptors, antigens, antibodies, lectins, carbohydrates, nucleotide sequences, and any other detectable label apparent to those of skill in the art.

In certain embodiments, a polynucleotide is a polymer of natural (*e.g.* A, G, C, T, U) or synthetic nucleobases, or a combination of both. The backbone of the polynucleotide can

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be composed entirely of "native" phosphodiester linkages, or it may contain one or more modified linkages, such as one or more phosphorothioate, phosphorodithioate, phosphoramidate or other modified linkages. As a specific example, a polynucleotide may be a peptide nucleic acid (PNA), which contains amide interlinkages. Additional examples of synthetic bases and backbones that can be used in conjunction with the invention, as well as methods for their synthesis can be found, for example, in U.S. Patent No. 6,001,983; Uhlman & Peyman, 1990, *Chemical Review* 90(4):544 584; Goodchild, 1990, *Bioconjugate Chem.* 1(3):165 186; Egholm *et al.*, 1992, *J. Am. Chem. Soc.* 114:1895 1897; Gryaznov *et al.*, *J. Am. Chem. Soc.* 116:3143 3144, as well as the references cited in all of the above. Common synthetic nucleobases of which polynucleotides may be composed include 3-methlyuracil, 5,6-dihydrouracil, 4 thiouracil, 5 bromouracil, 5-thorouracil, 5-iodouracil, 6-dimethyl aminopurine, 6-methyl aminopurine, 2-aminopurine, 2,6-diamino purine, 6-amino-8-bromopurine, inosine, 5-methylcytosine, 7-deazaadenine, and 7-deazaguanosine. Additional non-limiting examples of synthetic nucleobases of which the target nucleic acid may be composed can be found in Fasman, *CRC Practical Handbook of Biochemistry and Molecular Biology*, 1985, pp. 385-392; *Beilstein's Handbuch der Organischen Chemie*, Springer Verlag, Berlin and Chemical Abstracts, all of which provide references to publications describing the structures, properties and preparation of such nucleobases.

The nanoreporter can be prepared according to any technique apparent to those of skill in the art. Advantageously, nanoreporters according to the invention can comprise labels and/or members of binding pairs, as described in the sections below, that can be used to facilitate preparation and/or purification of the nanoreporter. In addition, certain nanoreporters of the invention are capable of forming complexes with molecules that comprise members of binding pairs, as described below. These complexes can be used to facilitate preparation and/or purification of the nanoreporter or complex.

5.12.2 IMMOBILIZATION OF FIRST PORTION

In the methods of the invention, a first portion of the nanoreporter is immobilized. Generally, the first portion is immobilized if it would be recognized as immobilized by one of skill in the art. The first portion can be immobilized by any technique apparent to those of skill in the art. In certain embodiments, the technique for immobilization of the first portion of the nanoreporter is not critical for the methods of the invention.

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The first portion of the nanoreporter can be at any location in the nanoreporter. In certain embodiments, the first portion is at a terminus of the nanoreporter. For the purposes of the invention, a portion of a nanoreporter can be "at a terminus" when it is less than five, four, three, two, one or zero monomers from a terminus of the nanoreporter. Of course, although many nanoreporters have two termini, the methods of the invention are applicable to nanoreporters have more than two termini and to nanoreporters having one or zero termini, *e.g.* circular nanoreporters. In certain embodiments, the first portion is not at a terminus of the nanoreporter.

The nanoreporter can be immobilized onto any substrate apparent to those of skill in the art. The substrate can be any moiety to which the nanoreporter can be immobilized without limitation. In certain embodiments, the substrate is a surface, membrane, bead, porous material, electrode or array.

In certain embodiments, the first portion of the nanoreporter can be immobilized non-selectively. In further embodiments, the first portion of the nanoreporter can be immobilized selectively. In advantageous embodiments, after the first portion of the nanoreporter is immobilized, some portion of the nanoreporter should be free to move sufficiently so that the nanoreporter can be extended in the following steps of the method. In particular, in certain embodiments, when the first portion of the nanoreporter is immobilized non-selectively, it is important that the entire nanoreporter not be immobilized non-selectively to an extent that prevents extension of any portion of the nanoreporter.

The immobilization can be by any interaction with the substrate apparent to those of skill in the art. The immobilization can be via electrostatic or ionic interaction, via one or more covalent bonds, via one or more non-covalent bonds or combinations thereof. In certain embodiments, the immobilization can be via electrostatic interaction with an electrode. In further embodiments, the immobilization is via electrostatic interaction with a substrate other than the electrode.

In certain embodiments, the first portion of the nanoreporter comprises a first member of a binding pair. The first member of the binding pair can be covalently bound to the first portion of the nanoreporter, or they can be non-covalently bound. Useful covalent bonds and non-covalent bonds will be apparent to those of skill in the art. In useful embodiments, the substrate onto which the first portion of the nanoreporter is bound will comprise a second member of the binding pair. The substrate can be covalently bound to the second member, or they can be non-covalently bound. Figure 12A illustrates a

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nanoreporter that comprises a moiety F1 that is capable of selectively binding a moiety of the substrate. Moiety F1 can be, for example, biotin, capable of binding, for example, a substrate coated with avidin.

In certain embodiments, the first portion of the nanoreporter can comprise a member of a binding pair that is capable of binding with a member of a binding pair on the substrate to form one or more non-covalent bonds. Exemplary useful substrates include those that comprise a binding moiety selected from the group consisting of ligands, antigens, carbohydrates, nucleic acids, receptors, lectins, and antibodies. The first portion of the nanoreporter would comprise a binding moiety capable of binding with the binding moiety of the substrate. Exemplary useful substrates comprising reactive moieties include, but are not limited to, surfaces comprising epoxy, aldehyde, gold, hydrazide, sulfhydryl, NHS-ester, amine, thiol, carboxylate, maleimide, hydroxymethyl phosphine, imidoester, isocyanate, hydroxyl, pentafluorophenyl-ester, psoralen, pyridyl disulfide or vinyl sulfone, or mixtures thereof. Such surfaces can be obtained from commercial sources or prepared according to standard techniques.

In advantageous embodiments, the first portion of the nanoreporter can be immobilized to the substrate via an avidin-biotin binding pair. In certain embodiments, the nanoreporter can comprise a biotin moiety in its first portion. For instance, a polynucleotide nanoreporter can comprise a biotinylated nucleotide residue. Similarly, a polypeptide nanoreporter can comprise a biotinylated amino acid residue. The substrate comprising avidin can be any substrate comprising avidin known to those of skill in the art. Useful substrates comprising avidin are commercially available including TB0200 (Accelr8), SAD6, SAD20, SAD100, SAD500, SAD2000 (Xantec), SuperAvidin (Array-It), streptavidin slide (catalog #MPC 000, Xenopore) and STREPTAVIDINnslide (catalog #439003, Greiner Bio-one).

In certain embodiments, the first portion of the nanoreporter can comprise a nucleotide sequence that is capable of selectively binding a nucleotide sequence on the substrate.

In further embodiments, the first portion of the nanoreporter can comprise avidin, and the substrate can comprise biotin. Useful substrates comprising biotin are commercially available including Optiarray-biotin (Accler8), BD6, BD20, BD100, BD500 and BD2000 (Xantec).

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In further embodiments, the first portion of the nanoreporter is capable of forming a complex with one or more other molecules that, in turn, are capable of binding, covalently or non-covalently, a binding moiety of the substrate. For instance, a first portion of the nanoreporter can be capable of selectively binding another molecule that comprises, for instance, a biotin moiety that is capable of selectively binding, for instance, an avidin moiety of the substrate. Figure 13A illustrates a nanoreporter that is capable of selectively binding a second molecule X that is capable of selectively binding a third molecule that comprises F1. F1 is capable of selectively binding a moiety on a substrate. Figure 13B illustrates a nanoreporter that is capable of selectively binding a second molecule that comprises F1, and F1 is capable of selectively binding a moiety on a substrate.

In further embodiments, the first portion of the nanoreporter can comprise a member of a binding pair that is capable of reacting with a member of a binding pair on the substrate to form one or more covalent bonds. Exemplary useful substrates comprising reactive groups include those that comprise a reactive moiety selected from the group consisting of succinamides, amines, aldehydes, epoxies and thiols. The first portion of the nanoreporter would comprise a reactive moiety capable of reacting with the reactive moiety of the substrate. Exemplary useful substrates comprising reactive moieties include, but are not limited to, OptArray-DNA NHS group (Accler8), Nexterion Slide AL (Schott) and Nexterion Slide E (Schott).

In certain embodiments, the first portion of the nanoreporter can comprise a reactive moiety that is capable of being bound to the substrate by photoactivation. The substrate could comprise the photoreactive moiety, or the first portion of the nanoreporter could comprise the photoreactive moiety. Some examples of photoreactive moieties include aryl azides, such as *N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide; fluorinated aryl azides, such as 4-azido-2,3,5,6-tetrafluorobenzoic acid; benzophenone-based reagents, such as the succinimidyl ester of 4-benzoylbenzoic acid; and 5-Bromo-deoxyuridine.

In further embodiments, the first portion of the nanoreporter can be immobilized to the substrate via other binding pairs apparent to those of skill in the art.

5.12.3 EXTENSION OF THE NANOREPORTER

In certain methods of the invention, the nanoreporter is in an extended state. Generally, any nanoreporter is in an extended state if it would be recognized as such by one of skill in the art.

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In certain embodiments, the nanoreporter is in an extended state when it is in the field of a force capable of extending the nanoreporter under conditions suitable for extending the nanoreporter. Such forces and conditions should be apparent to those of skill in the art. For instance, many nanoreporters can be extended by hydrodynamic force or by gravity, and many charged nanoreporters can be extended by electromagnetic force. In certain embodiments, the force can be applied to the nanoreporter indirectly. For instance, the nanoreporter can comprise or can be linked, covalently or noncovalently, to a moiety capable of being moved by a force. In certain embodiments, the nanoreporter can be linked to a moiety

In certain embodiments, the force is an electromagnetic force. For instance, when the nanoreporter is charged, such as a polynucleotide, the nanoreporter can be extended in an electric or magnetic field. The field should be strong enough to extend the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for extending a nanoreporter in an electric or magnetic field are described in Matsuura *et al.*, 2002, *J Biomol Struct Dyn.* 20(3):429-36; Ferree & Blanch, 2003, *Biophys J.* 85(4):2539-46; Stigter & Bustamante, 1998, *Biophys J.* 1998 75(3):1197-210; Matsuura *et al.*, 2001, *Nucleic Acids Res.* 29(16); Ferree & Blanch, 2004, *Biophys J.* 87(1):468-75; the contents of which are hereby incorporated by reference in their entirety.

In certain embodiments, the force is a hydrodynamic force. For instance, many nanoreporters, including polysaccharides, polypeptides, and polynucleotides, can be extended in the field of a moving fluid. The hydrodynamic force should be strong enough to extend the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for extending a nanoreporter in hydrodynamic field are described in Bensimon *et al.*, 1994, *Science* 265:2096-2098; Henegariu *et al.*, 2001, *BioTechniques* 31: 246-250; Kraus *et al.*, 1997, *Human Genetics* 99:374 - 380; Michalet *et al.*, 1997, *Science* 277:1518-1523; Yokota *et al.*, 1997, *Nucleic Acids Res.* 25(5):1064-70; Otobe *et al.*, 2001, *Nucleic Acids Research* 29:109; Zimmerman & Cox, 1994, *Nucleic Acids Res.* 22(3):492-7, and U.S. Patent Nos. 6,548,255; 6,344,319; 6,303,296; 6,265,153; 6,225,055; 6,054,327; and 5,840,862, the contents of which are hereby incorporated by reference in their entirety.

In certain embodiments, the force is gravity. In advantageous embodiments, the force of gravity can be combined with, for example, hydrodynamic force to extend the nanoreporter. In certain embodiments, The force should be strong enough to extend the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for

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extending a nanoreporter with gravity are described in Michalet *et al.*, 1997, *Science* 277:1518-1523; Yokota *et al.*, 1997, *Nucleic Acids Res.* 25(5):1064-70; Kraus *et al.*, 1997, *Human Genetics* 99:374 - 380, the contents of which are hereby incorporated by reference in their entirety.

In particular embodiments, the force is applied through a moving meniscus. Those of skill in the art will recognize that a moving meniscus can apply various forces to a nanoreporter including hydrodynamic force, surface tension and any other force recognized by those of skill in the art. The meniscus can be moved by any technique apparent to those of skill in the art including evaporation and gravity. Exemplary techniques for extending a nanoreporter with a moving meniscus are described in, for example, U.S. Patent Nos. 6,548,255; 6,344,319; 6,303,296; 6,265,153; 6,225,055; 6,054,327; and 5,840,862, the contents of which are hereby incorporated by reference in their entireties.

In particular embodiments, the nanoreporter can be extended by an optical trap or optical tweezers. For instance, the nanoreporter can comprise or can be linked, covalently or noncovalently, to a particle capable of being trapped or moved by an appropriate source of optical force. Useful techniques for moving particles with optical traps or optical tweezers are described in Ashkin *et al.*, 1986, *Optics Letters* 11:288-290; Ashkin *et al.*, 1987, *Science* 235:1517–1520; Ashkin *et al.*, Nature 330:769–771; Perkins *et al.*, 1994, *Science* 264:822-826; Simmons *et al.*, 1996, *Biophysical Journal* 70:1813-1822; Block *et al.*, 1990, *Nature* 348:348 - 352; and Grier, 2003, *Nature* 424: 810-816; the contents of which are hereby incorporated by reference in their entireties.

In certain embodiments, the nanoreporter can be extended by combinations of the above forces that are apparent to those of skill in the art. In the examples, below, certain nanoreporters are extended by a combination of an electric field and hydrodynamic force.

The nanoreporter is extended when it would be recognized as extended by one of skill in the art according to standard criteria for extension of a nanoreporter. In certain embodiments, the nanoreporter is extended when it loses most of its tertiary structural features as recognized by those of skill in the art. In certain embodiments, the nanoreporter is extended when it loses most of its secondary structural features as recognized by those of skill in the art. In certain embodiments, the nanoreporter is extended when its primary structural features are detectable in sequence when imaged according to standard techniques. Exemplary imaging techniques are described in the examples below.

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In certain embodiments, an extended state of a nanoreporter can be recognized by comparing its hydrodynamic radius to its average hydrodynamic radius when free in dilute solution. For instance, in certain embodiments, a nanoreporter, or portion thereof, is extended when its hydrodynamic radius is more than about double its average hydrodynamic radius in dilute solution. More quantitatively, R represents the hydrodynamic radius of the nanoreporter, or portion thereof, and <R> represents the average hydrodynamic radius of the nanoreporter, or portion thereof, in dilute solution. The average <R> should be calculated such that R for the nanoreporter, or portion thereof, when unbound in dilute solution is less than 2<R> 95% of the time. In certain embodiments, a nanoreporter, or portion thereof, is in an extended state when R is greater than 1.5<R>, greater than 1.6<R>, greater than 1.7<R>, greater than 1.8<R>, greater than 1.9<R>, greater than 2.0<R>, greater than 2.1<R>, greater than 2.2<R>, greater than 2.3<R>, greater than 2.4<R>, greater than 2.5<R> or greater than 3.0<R>. In particular embodiments, a nanoreporter, or portion thereof, is in an extended state when R is greater than 2.0<R>.

5.12.4 ORIENTATION OF THE NANOREPORTER

In certain methods of the invention, the nanoreporter is in an oriented state. Generally, any nanoreporter is in an oriented state if it would be recognized as such by one of skill in the art.

In certain embodiments, the nanoreporter is in an oriented state when it is in the field of a force capable of orienting the nanoreporter under conditions suitable for orienting the nanoreporter. Such forces and conditions should be apparent to those of skill in the art.

In certain embodiments, the force is an electromagnetic force. For instance, when the nanoreporter is charged, such as a polynucleotide, the nanoreporter can be oriented in an electric or magnetic field. The field should be strong enough to orient the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for orienting a nanoreporter in an electric or magnetic field are described above.

In certain embodiments, the force is a hydrodynamic force. For instance, many nanoreporters, including polysaccharides, polypeptides, and polynucleotides, can be oriented in the field of a moving fluid. The hydrodynamic force should be strong enough to orient the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for orienting a nanoreporter in hydrodynamic field are described above.

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In certain embodiments, the force is gravity. In advantageous embodiments, the force of gravity can be combined with, for example, hydrodynamic force to orient the nanoreporter. In certain embodiments, The force should be strong enough to orient the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for orienting a nanoreporter with gravity are described above.

In certain embodiments, the nanoreporter can be oriented by combinations of the above forces that are apparent to those of skill in the art. In the examples, below, certain nanoreporters are oriented by a combination of an electric field and hydrodynamic force.

The nanoreporter is oriented when it would be recognized as oriented by one of skill in the art according to standard criteria for orientation of a nanoreporter. In certain embodiments, the nanoreporter is oriented when it is arranged in parallel, as recognized by those of skill in the art, with the field of a force capable of orienting the nanoreporter. In certain embodiments, the nanoreporter is oriented when it is one of a plurality of nanoreporters that are arranged in parallel, as recognized by those of skill in the art. For instance, a plurality of nanoreporters can be oriented when the vector from a first terminus to a second terminus of a nanoreporter is parallel, as recognized by those of skill in the art, to the vectors between corresponding termini of other nanoreporters in the plurality.

5.12.5 SELECTIVE IMMOBILIZATION OF SECOND PORTION OF NANOREPORTER

As discussed above, in the methods of the invention, a second portion of the nanoreporter is selectively immobilized. The second portion of the nanoreporter can be any portion of the nanoreporter that is not identical to the first portion of the nanoreporter. In some embodiments, the second portion of the nanoreporter does not overlap any part of the first portion of the nanoreporter.

In certain embodiments, the present invention provides methods that comprise the single step of selectively immobilizing a second portion of a nanoreporter while the nanoreporter is in an extended or oriented state, and while a first portion of the nanoreporter is immobilized. Exemplary methods for immobilization of the first portion of the nanoreporter, and for extension or orientation of the nanoreporter are described in detail in the sections above.

In certain embodiments, the present invention provides methods that comprise the step of extending a nanoreporter, while a first portion of the nanoreporter is immobilized, and the step of selectively immobilizing a second portion of a nanoreporter while the

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nanoreporter is in an extended state. Exemplary methods for immobilization of the first portion of the nanoreporter, and for extension of the nanoreporter are described in detail in the sections above.

In certain embodiments, the present invention provides methods that comprise the step of immobilizing a first portion of a nanoreporter, the step of extending the nanoreporter while the first portion is immobilized and the step of selectively immobilizing a second portion of a nanoreporter while the nanoreporter is in an extended state. Exemplary methods for immobilization of the first portion of the nanoreporter, and for extension of the nanoreporter are described in detail above.

In certain embodiments, the present invention provides methods that comprise the step of orienting a nanoreporter, while a first portion of the nanoreporter is immobilized, and the step of selectively immobilizing a second portion of a nanoreporter while the nanoreporter is in an oriented state. Exemplary methods for immobilization of the first portion of the nanoreporter, and for orienting the nanoreporter are described in detail in the sections above.

In certain embodiments, the present invention provides methods that comprise the step of immobilizing a first portion of a nanoreporter, the step of orienting the nanoreporter while the first portion is immobilized and the step of selectively immobilizing a second portion of a nanoreporter while the nanoreporter is in an oriented state. Exemplary methods for immobilization of the first portion of the nanoreporter, and for orienting the nanoreporter are described in detail above.

The selective immobilization of the second portion of the nanoreporter can follow any technique for selective immobilization of a nanoreporter apparent to those of skill in the art. Significantly, in advantageous embodiments of the invention, the second portion of the nanoreporter is not immobilized non-selectively. Selective immobilization can allow the nanoreporter to be immobilized while in a fully extended state or nearly fully extended state. Selective immobilization can also allow the nanoreporter to be immobilized in an oriented manner. In other words, the first portion and second portion of the nanoreporter can be immobilized along the direction of the field or fields used to extend the nanoreporter, with the first portion preceding the second portion in the field. When a plurality of nanoreporters are immobilized, the can be uniformly oriented along the field.

The second portion of the nanoreporter can be at any location in the nanoreporter. In certain embodiments, the second portion is at a terminus of the nanoreporter. In certain

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embodiments, the second portion is not at a terminus of the nanoreporter. In certain embodiments, the first portion, described in the sections above, is at one terminus of the nanoreporter, and the second portion is at another terminus of the nanoreporter.

As discussed above, the second portion of the nanoreporter is immobilized selectively. The immobilization can be by any selective interaction with the substrate apparent to those of skill in the art. The immobilization can be via electrostatic or ionic interaction, via one or more covalent bonds, via one or more non-covalent bonds or combinations thereof. In certain embodiments, the immobilization can be via electrostatic interaction with an electrode. In further embodiments, the immobilization is via electrostatic interaction with a substrate other than the electrode.

If the first portion and the second portion of the nanoreporter are selectively immobilized to the same substrate, the techniques of selective immobilization should of course be compatible with the substrate. In particular embodiments, the techniques of immobilization are the same. For instance, on a substrate coated with avidin, both the first and second portion of the nanoreporter can be immobilized selectively via biotin - avidin interactions. However, as will be apparent to those of skill in the art, the same interaction need not be used at both the first and second portions for immobilization on the same substrate. For instance, the substrate can comprise multiple moieties capable of selective binding, or the first portion can be immobilized non-selectively, or other techniques apparent to those of skill in the art.

In certain embodiments, the second portion of the nanoreporter comprises a first member of a binding pair. The second member of the binding pair can be covalently bound to the second portion of the nanoreporter, or they can be non-covalently bound. Useful covalent bonds and non-covalent bonds will be apparent to those of skill in the art. In useful embodiments, the substrate onto which the second portion of the nanoreporter is bound will comprise a second member of the binding pair. The substrate can be covalently bound to the second member, or they can be non-covalently bound.

In certain embodiments, the second portion of the nanoreporter can comprise a member of a binding pair that is capable of binding with a member of a binding pair on the substrate to form one or more non-covalent bonds. Exemplary useful substrates include those that comprise a binding moiety selected from the group consisting of ligands, antigens, carbohydrates, nucleic acids, receptors, lectins, and antibodies such as those described in the sections above.

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In advantageous embodiments, the second portion of the nanoreporter can be immobilized to the substrate via an avidin-biotin binding pair. In certain embodiments, the nanoreporter can comprise a biotin moiety in its first portion. For instance, a polynucleotide nanoreporter can comprise a biotinylated nucleotide residue. Similarly, a polypeptide nanoreporter can comprise a biotinylated amino acid residue. Useful substrates comprising avidin are described in the sections above.

In further embodiments, the second portion of the nanoreporter can comprise avidin, and the substrate can comprise biotin. Useful substrates comprising biotin are described in the sections above.

In further embodiments, the second portion of the nanoreporter can comprise a member of a binding pair that is capable of reacting with a member of a binding pair on the substrate to form one or more covalent bonds. Exemplary useful substrates comprising reactive groups are described in the sections above.

In certain embodiments, the second portion of the nanoreporter can comprise a reactive moiety that is capable of being bound to the substrate by photoactivation. The substrate could comprise the photoreactive moiety, or the second portion of the nanoreporter could comprise the photoreactive moiety. Some examples of photoreactive moieties include aryl azides, such as *N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide; fluorinated aryl azides, such as 4-azido-2,3,5,6-tetrafluorobenzoic acid; benzophenone-based reagents, such as the succinimidyl ester of 4-benzoylbenzoic acid; and 5-Bromo-deoxyuridine.

In further embodiments, the second portion of the nanoreporter can be immobilized to the substrate via other binding pairs described in the sections above.

In further embodiments, the second portion of the nanoreporter is capable of forming a complex with one or more other molecules that, in turn, are capable of binding, covalently or non-covalently, a binding moiety of the substrate. For instance, the second portion of the nanoreporter can be capable of selectively binding another molecule that comprises, for instance, a biotin moiety that is capable of selectively binding, for instance, an avidin moiety of the substrate. Figure 12B illustrates a nanoreporter of selectively binding a second molecule that comprises F3 that is, in turn, capable of selectively binding a moiety on a substrate. The interaction between the second portion of the nanoreporter and the molecule that comprises F3 can be mediated, for example, by an antigen-antibody interaction.

FIGS. 14A and 14B illustrate the selective immobilization of a nanoreporter according to methods of the present invention. In Figure 14A, a first portion of the

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nanoreporter comprises binding moiety F1 that is capable of selectively binding a moiety on the illustrated substrate S. Binding moiety F1 can be, for instance, biotin, and substrate S can be coated with, for instance, avidin. The nanoreporter of Figure 14A is extended by a force as described in the sections above. In Figure 14B, the force is an electrical potential. While extended, the nanoreporter is contacted with molecules comprising binding moiety F2 that is capable of selectively binding a moiety on the illustrated substrate S. Binding moiety F2 can be, for instance, biotin, and substrate S can be coated with, for instance, avidin. Significantly, up to three molecules comprising F2 are capable of selectively binding to a second portion of the nanoreporter to selectively immobilize it in its extended state. As illustrated, the molecules comprise a second binding moiety that selectively binds a repeated binding moiety of the nanoreporter. The binding moieties can be, for instance, complementary nucleic acid sequences, as illustrated in Figure 14B. The resulting nanoreporter is selectively immobilized in an extended state and should remain extended even when the force is removed. The selectively immobilized, extended nanoreporter can be used for any purpose apparent to those of skill in the art.

5.12.6 IMMOBILIZATION OF TWO PORTIONS OF AN EXTENDED OR ORIENTED NANOREPORTER

In certain embodiments, the present invention provides methods for selective immobilization of a first portion and a second portion of a nanoreporter that is in an extended or oriented state. Significantly, according to these methods of the invention, the nanoreporter need not be immobilized prior to application of a force capable of extending or orienting the nanoreporter.

In these methods, the nanoreporter is extended or oriented, or both, by a force capable of extending or orienting the nanoreporter. Such forces are described in detail in the sections above. In particular embodiments, the force is a force capable of extending or orienting the nanoreporter while maintaining the nanoreporter in one location, *i.e.* a force capable of extending or orienting without substantially moving the nanoreporter. Exemplary forces include oscillating electromagnetic fields and oscillating hydrodynamic fields. In a particular embodiment, the force is an oscillating electrical field. Exemplary techniques for extending or orienting a nanoreporter in an oscillating electric field are described in Asbury *et al.*, 2002, *Electrophoresis* 23(16):2658-66; Kabata *et al.*, 1993, *Science* 262(5139):1561-3; and Asbury and van den Engh, 1998, *Biophys J.* 74:1024-30, the contents of which are hereby incorporated by reference in their entirety.

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In the methods, the nanoreporter is immobilized at a first portion and at a second portion while extended or oriented. Both the first portion and the second portion can be immobilized non-selectively, both can be immobilized selectively, or one can be immobilized selectively and the other non-selectively. Techniques for immobilization of the first portion and second portion are described in detail in the sections above.

5.12.7 SUBSTRATE FOR IMMOBILIZATION

In the methods of the invention, the substrate for immobilization can be any substrate capable of selectively binding the nanoreporter apparent to those of skill in the art. Further, in certain aspects, the present invention provides compositions comprising a selectively immobilized nanoreporter in an extended state. The compositions comprise a substrate, as described herein, having immobilized thereto a nanoreporter in an extended state. The nanoreporter can be, of course, immobilized according to a method of the invention.

The only requirement of the substrate is that it be capable of selectively binding the second portion of the nanoreporter as described above. Thus, the substrate can be a filter or a membrane, such as a nitrocellulose or nylon, glass, a polymer such as polyacrylamide, a gel such as agarose, dextran, cellulose, polystyrene, latex, or any other material known to those of skill in the art to which capture compounds can be immobilized. The substrate can be composed of a porous material such as acrylic, styrene methyl methacrylate copolymer and ethylene/acrylic acid.

The substrate can take on any form so long as the form does not prevent selective immobilization of the second portion of the nanoreporter. For instance, the substrate can have the form of a disk, slab, strip, bead, submicron particle, coated magnetic bead, gel pad, microtiter well, slide, membrane, frit or other form known to those of skill in the art. The substrate is optionally disposed within a housing, such as a chromatography column, spin column, syringe barrel, pipette, pipette tip, 96 or 384 well plate, microchannel, capillary, etc., that aids the flow of liquid over or through the substrate.

The nanoreporter can be immobilized on a single substrate or on a plurality of substrates. For instance, in certain embodiments, the first and second portions of nanoreporter are immobilized on the same substrate, as recognized by those of skill in the art. In certain embodiments, the first portion of the nanoreporter can be immobilized on a

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first substrate while the second portion of the nanoreporter can be immobilized on a second substrate, distinct from the first.

The substrate can be prepared according to any method apparent to those of skill in the art. For a review of the myriad techniques that can be used to activate exemplary substrates of the invention with a sufficient density of reactive groups, see, the *Wiley Encyclopedia of Packaging Technology*, 2d Ed., Brody & Marsh, Ed., "Surface Treatment," pp. 867 874, John Wiley & Sons (1997), and the references cited therein. Chemical methods suitable for generating amino groups on silicon oxide substrates are described in Atkinson & Smith, "Solid Phase Synthesis of Oligodeoxyribonucleotides by the Phosphite Triester Method," In: *Oligonucleotide Synthesis: A Practical Approach*, M J Gait, Ed., 1984, IRL Press, Oxford, particularly at pp. 45 49 (and the references cited therein); chemical methods suitable for generating hydroxyl groups on silicon oxide substrates are described in Pease *et al.*, 1994, *Proc. Natl. Acad. Sci. USA 91*:5022 5026 (and the references cited therein); chemical methods for generating functional groups on polymers such as polystyrene, polyamides and grafted polystyrenes are described in Lloyd Williams *et al.*, 1997, Chemical Approaches to the Synthesis of Peptides and Proteins, Chapter 2, CRC Press, Boca Raton, FL (and the references cited therein).

Exemplary useful substrates include surfaces coated with streptavidin, *e.g.* Accelr8 TB0200. Further useful substrates include surfaces coated with N-hydroxysuccinamide that are capable of reacting with a portion of a nanoreporter that comprises an amine. One such surface is OptArray-DNA (Accelr8). Additional useful surfaces are coated with aldehyde (*e.g.* Nexterion Slide AL, Schott) and surfaces coated with epoxy (*e.g.* Nexterion Slide E, Schott). Another useful surface is a biotinylated BSA coated surface useful for selective immobilization of a portion of a nanoreporter that comprises avidin or streptavidin.

5.12.8 METHODS OF USING SELECTIVELY IMMOBILIZED, EXTENDED OR ORIENTED NANOREPORTERS

In certain embodiments, the selectively immobilized, elongated nanoreporters can be used to create macromolecular barcodes for the purposes of separation and sequential detection of labels. These labels spaced along the molecule provide a unique code that can be read when the nanoreporter is extended and immobilized. Extension and selective immobilization can facilitate the decoding of the macromolecular barcode.

The selectively immobilized, elongated nanoreporters can further be used for can be used in any context where detection or imaging of a nanoreporter might be useful. They can

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be used for diagnostic, prognostic therapeutic and screening purposes. For instance, they can be applied to the analysis of biomolecular samples obtained or derived from a patient so as to determine whether a diseased cell type is present in the sample and/or to stage the disease. They can be used to diagnose pathogen infections, for example infections by intracellular bacteria and viruses, by determining the presence and/or quantity of markers of bacterium or virus, respectively, in the sample. The compositions and methods of the invention can be used to quantitate target molecules whose abundance is indicative of a biological state or disease condition, for example, blood markers that are upregulated or downregulated as a result of a disease state. In addition, the compositions and methods of the invention can be used to provide prognostic information that assists in determining a course of treatment for a patient.

5.12.9 KITS COMPRISING SELECTIVELY IMMOBILIZED, EXTENDED OR ORIENTED NANOREPORTERS

The invention further provides kits comprising one or more components of the invention. The kits can comprise, for example, a substrate according to the invention and one or more extended or oriented, or both, nanoreporters selectively immobilized on the substrate. The kits can be used for any purpose apparent to those of skill in the art, including, those described above.

In certain embodiments, the present invention also provides kits useful for the extension and selective immobilization of nanoreporters. The kits can comprise a substrate for immobilization and one or more binding partners to facilitate extension or immobilization of a nanoreporter. The binding partners could, in certain embodiments, comprise a moiety useful for extension of the nanoreporter in an appropriate force. In certain embodiments, the binding partners could facilitate immobilization or selective immobilization of the nanoreporter to the surface. In further embodiments, the kit could comprise a nanoreporter for extension and immobilization. In further embodiments, the kit could comprise a device capable of extending the nanoreporter.

5.13 DETECTION OF NANOREPORTERS

Nanoreporters are detected by any means available in the art that is capable of detecting the specific signals on a given nanoreporter. Where the nanoreporter is fluorescently labeled, suitable consideration of appropriate excitation sources may be investigated. Possible sources may include but are not limited to arc lamp, xenon lamp,

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lasers, light emitting diodes or some combination thereof. The appropriate excitation source is used in conjunction with an appropriate optical detection system, for example an inverted fluorescent microscope, an epi-fluorescent microscope or a confocal microscope. Preferably, a microscope is used that can allow for detection with enough spatial resolution to determine the sequence of the spots on the nanoreporter.

5.13.1 MICROSCOPE AND OBJECTIVE LENS SELECTION.

The major consideration regarding the microscope objective lens is with the optical resolution, which is determined by its numerical aperture (NA). Generally, the larger the NA, the better the optical resolution. The required NA is preferably at least 1.07 based on the relationship of $\delta = 0.61\lambda/NA$ (δ = optical resolution and λ = wavelength). The amount of light that is collected by an objective is determined by NA^4/Mag^2 (Mag = magnification of the objective). Therefore, in order to collect as much light as possible, objectives with high NA and low magnifications should be selected.

5.13.2 CCD CAMERA SELECTION AND IMAGE CAPTURE TECHNIQUES.

When selecting a CCD camera, the first consideration is the pixel size, which partially determines the final resolution of the imaging system. Optimally the optical resolution should not be compromised by the CCD camera. For example, if the optical resolution is 210-300 nm, which corresponds to 12.6-18 μm on a CCD chip after a 60 x magnification, in order to resolve and maintain the optical resolution there should be at least two pixels to sample each spot. Or the pixel size of the CCD chip should be at most 6.3- 9 μm.

The second consideration is detection sensitivity which can be determined by many factors that include but are not limited to pixel size, quantum efficiency, readout noise and dark noise. To achieve high sensitivity, select a qualitative camera with big pixel size (which can give big collection area), high quantum efficiency and low noise. An exemplary camera with these criteria is the Orca-Ag camera from Hamamatsu Inc. The chip size is 1344 x 1024 pixels; when using the 60x objective, the field of view is 144 x 110 μm^2.

5.14 COMPUTER SYSTEMS

The invention provides computer systems that may be used to computerize nanoreporter image collection, nanoreporter identification and/or decoding of the

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nanoreporter code. Specifically, the invention provides various computer systems comprising a processor and a memory coupled to the processor and encoding one or more programs. The computer systems can be connected to the microscopes employed in imaging the nanoreporter, allowing imaging, identification and decoding the nanoreporter, as well as storing the nanoreporter image and associated information, by a single apparatus. The one or more programs encoded by the memory cause the processor to perform the methods of the invention.

In still other embodiments, the invention provides computer program products for use in conjunction with a computer system (*e.g.*, one of the above-described computer systems of the invention) having a processor and a memory connected to the processor. The computer program products of the invention comprise a computer readable storage medium having a computer program mechanism encoded or embedded thereon. The computer program mechanism can be loaded into the memory of the computer and cause the processor to execute the steps of the methods of the invention.

The methods described in the previous subsections can preferably be implemented by use of the following computer systems, and according to the following methods. An exemplary computer system suitable for implementation of the methods of this invention comprises internal components and being linked to external components. The internal components of this computer system include a processor element interconnected with main memory. For example, the computer system can be an Intel Pentium-based processor of 200 MHz or greater clock rate and with 32 MB or more of main memory.

The external components include mass storage. This mass storage can be one or more hard disks which are typically packaged together with the processor and memory. Such hard disks are typically of 1 GB or greater storage capacity. Other external components include user interface device, which can be a monitor and a keyboard, together with pointing device, which can be a "mouse", or other graphical input devices (not illustrated). Typically, the computer system is also linked to a network link, which can be part of an Ethernet link to other local computer systems, remote computer systems, or wide area communication networks, such as the Internet. This network link allows the computer system to share data and processing tasks with other computer systems.

Loaded into memory during operation of this system are several software components, which are both standard in the art and special to the instant invention. These software components collectively cause the computer system to function according to the

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methods of the invention. The software components are typically stored on mass storage. A first software component is an operating system, which is responsible for managing the computer system and its network interconnections. This operating system can be, for example, of the Microsoft Windows® family, such as Windows 95, Windows 2000, or Windows XP, or, alternatively, a Macintosh operating system, a Linux operating system or a Unix operating system. A second software component may include common languages and functions conveniently present in the system to assist programs implementing the methods specific to this invention. Languages that can be used to program the analytic methods of the invention include, for example, C, C++, JAVA, and, less preferably, FORTRAN, PASCAL, and BASIC. Another software component of the present invention comprises the analytic methods of this invention as programmed in a procedural language or symbolic package.

In an exemplary implementation, to practice the methods of the present invention, a nanoreporter code (*i.e.*, a correlation between the order and nature of spots on a nanoreporter and the identity of a target molecule to which such a nanoreporter binds) is first loaded in the computer system. Next the user causes execution of analysis software which performs the steps of determining the presence and, optionally, quantity of nanoreporters with a given nanoreporter code.

The analytical systems of the invention also include computer program products that contain one or more of the above-described software components such that the software components may be loaded into the memory of a computer system. Specifically, a computer program product of the invention includes a computer readable storage medium having one or more computer program mechanisms embedded or encoded thereon in a computer readable format. The computer program mechanisms encoded, *e.g.*, one or more of the analytical software components described above which can be loaded into the memory of a computer system and cause the processor of the computer system to execute the analytical methods of the present invention.

The computer program mechanisms or mechanisms are preferably stored or encoded on a computer readable storage medium. Exemplary computer readable storage media are discussed above and include, but are not limited to: a hard drive, which may be, e.g., an external or an internal hard drive of a computer system of the invention, or a removable hard drive; a floppy disk; a CD-ROM; or a tape such as a DAT tape. Other computer readable

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storage media will also be apparent to those skilled in the art that can be used in the computer program mechanisms of the present invention.

The present invention also provides databases useful for practicing the methods of the present invention. The databases may include reference nanoreporter codes for a large variety of target molecules. Preferably, such a database will be in an electronic form that can be loaded into a computer system. Such electronic forms include databases loaded into the main memory of a computer system used to implement the methods of this invention, or in the main memory of other computers linked by network connection, or embedded or encoded on mass storage media, or on removable storage media such as a CD-ROM or floppy disk.

Alternative systems and methods for implementing the methods of this invention are intended to be comprehended within the accompanying claims. In particular, the accompanying claims are intended to include the alternative program structures for implementing the methods of this invention that will be readily apparent to one of skill in the art.

5.15 APPLICATIONS OF NANOREPORTER TECHNOLOGY

The compositions and methods of the invention can be used for diagnostic, prognostic therapeutic and screening purposes. The present invention provides the advantage that many different target molecules can be analyzed at one time from a single biomolecular sample using the methods of the invention. This allows, for example, for several diagnostic tests to be performed on one sample.

5.15.1 DIAGNOSTIC/PROGNOSTIC METHODS

The present methods can be applied to the analysis of biomolecular samples obtained or derived from a patient so as to determine whether a diseased cell type is present in the sample and/or to stage the disease.

For example, a blood sample can be assayed according to any of the methods described herein to determine the presence and/or quantity of markers of a cancerous cell type in the sample, thereby diagnosing or staging the cancer.

Alternatively, the methods described herein can be used to diagnose pathogen infections, for example infections by intracellular bacteria and viruses, by determining the presence and/or quantity of markers of bacterium or virus, respectively, in the sample.

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Thus, the target molecules detected using the compositions and methods of the invention can be either patient markers (such as a cancer marker) or markers of infection with a foreign agent, such as bacterial or viral markers.

Because of the quantitative nature of nanoreporters, the compositions and methods of the invention can be used to quantitate target molecules whose abundance is indicative of a biological state or disease condition, for example, blood markers that are upregulated or downregulated as a result of a disease state.

In addition, the compositions and methods of the invention can be used to provide prognostic information that assists in determining a course of treatment for a patient. For example, the amount of a particular marker for a tumor can be accurately quantified from even a small sample from a patient. For certain diseases like breast cancer, overexpression of certain genes, such as Her2-neu, indicate a more aggressive course of treatment will be needed.

5.15.2 ANALYSIS OF PATHOLOGY SAMPLES

RNA extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples is typically poor in quality (fragmented) and low in yield. This makes gene expression analysis of low-expressing genes in histology samples or archival pathology tissues extremely difficult and often completely infeasible. The nanoreporter technology can fill this unmet need by allowing the analysis of very small quantities of low-quality total RNA.

To use nanoreporter technology in such an application, total RNA can be extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples (or similar) using commercially available kits such as RecoverAll Total Nucleic Acid Isolation Kit (Ambion) following manufacturer's protocols. RNA in such samples is frequently degraded to small fragments (200 to 500 nucleotides in length), and many paraffin-embedded histology samples only yield tens of nanograms of total RNA. Small amounts (5 to 100 ng) of this fragmented total RNA can be used directly as target material in a nanoreporter hybridization following the assay conditions described herein. As described in Example 6 in Section 11 below, nanoreporter analysis of approximately 3.3 ng cellular RNA permitted detection of transcripts present at approximately 0.5 copy/cell.

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5.15.3 SCREENING METHODS

The methods of the present invention can be used, *inter alia*, for determining the effect of a perturbation, including chemical compounds, mutations, temperature changes, growth hormones, growth factors, disease, or a change in culture conditions, on various target molecules, thereby identifying target molecules whose presence, absence or levels are indicative of a particular biological states. In a preferred embodiment, the present invention is used to elucidate and discover components and pathways of disease states. For example, the comparison of quantities of target molecules present in a disease tissue with "normal" tissue allows the elucidation of important target molecules involved in the disease, thereby identifying targets for the discovery/screening of new drug candidates that can be used to treat disease.

5.16 KITS

The invention further provides kits comprising one or more components of the invention. The kits can contained pre-labeled nanoreporters, or unlabeled nanoreporters with one or more components for labeling the nanoreporters. Moreover, the nanoreporters provided in a kit may or may not have target-specific sequences pre-attached. In one embodiment, the target sequences are provided in the kit unattached to the nanoreporter scaffold.

The kit can include other reagents as well, for example, buffers for performing hybridization reactions, linkers, restriction endonucleases, and DNA ligases.

The kit also will include instructions for using the components of the kit, and/or for making and/or using the labeled nanoreporters.

6. EXAMPLE 1: NANOREPORTER MANUFACTURING AND PROTOCOL

Herein is a step-by-step example of a method construction of a nanoreporter from various components.

It can be appreciated that various components can be constructed or added either at the same time, before or after other components. For example, annealing patch units or flaps to a scaffold can be done simultaneously or one after the other.

In this example the starting material is a circular M13mp18 viral vector. Using a single linear strand M13mp18, patch units are annealed to it to form a double stranded scaffold. Next, flaps are added then a target-specific sequence is ligated. Meanwhile

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purification steps aid to filter out excess, unattached patch units and flaps. Construction of labeled nucleic acids (patches and/or flaps and/or other labeled oligonucleotides) that bind the nanoreporter are also described.

Upon attachment (e.g., via hybridization) of a target molecule, the nanoreporter is attached to a surface and stretched. Finally the nanoreporters are imaged by a camera.

Nanoreporters were generated and successfully employed to detect target molecules using methods substantially as described in this example. An example of target detection using such this method is shown in Figure 4.

6.1 SCAFFOLD CONSTRUCTION

The oligonucleotide scaffold sequence selected was analyzed using Vector NTI® software. First, a single stranded nucleic acid was made from linearizing a circular M13mp18 single stranded DNA, which was commercially purchased from New England Biolabs. The circular M13mp18 was digested with BamH1 enzyme to linearize it. Materials used consisted of M13mp18 vector (250 ng/µl), Patch_1L_BamH1.02 (10 µM dilution of a 100 µM stock), 10X BamH1 Buffer, BamH1 enzyme. Protocol for making 0.8 pmol total of linear M13mp18 involve the following steps. 1) preheat heating block to 37 °C; 2) in a 0.65ml ependorff tube combine 40 µl of 250 ng/µl M13mp18 vector, 2 µl of 10 µM Patch_1L_BamH1.02, and 5 µl of 10X BamH1 Buffer; 3) place the ependorff tube in the 37°C heating block with foil over the top. Incubate the tube at 37°C for 15 minutes to allow the patch unit to hybridize to the M13mp18 scaffold; 4) after 15 minutes add 2 µl of BamH1 enzyme and let the reaction digest at 37 °C for 30 minutes, after which add an additional 2 µl of BamH1 enzyme and let the reaction continue to digest for another 30 minutes at 37 °C (final volume of BamH1 enzyme is 8%); and 5) aliquot 10 µl into 0.65ml ependorff tubes and store in freezer (final concentration of linear M13mp18 is 200 ng/µl).

6.2 PATCH UNIT PREPARATION OF THE BASE PATCH POOLS (BPP).

Second, patch units are prepared in pools. Patch oligonucleotide sequences were selected for optimal length and desired homology/non-homology to M13mp18 strand and the human genomic sequence. Patches were commercially manufactured oligonucleotides (purchased from Integrated DNA technologies) either 60 or 65 nucleotide bases in length. 50 nucleotide bases of each patch oligonucleotide are complementary to the M13mp18 single stranded DNA, 10 nucleotide bases are complementary to an adjacent patch, and 5

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nucleotides base pairs are complementary to a corresponding flap. The 10 nucleotide base match between patches forms a stem structure which stabilizes the structure and helps lift the flaps off the covered scaffold so they are more available to bind labeled oligonucleotides. Synthetic binding sites, the 5 nucleotide bases, on the patches for binding to the flaps make leveraging the power of a modular system possible.

The base patch pools contain nine patch units all corresponding to a specific letter grouping and position on the nanoreporter. For this example, there are four different fluorescent dyes (color) labeled A, B, C, and D and 8 different positions or regions where labeled nucleic acids can bind on a nanoreporter. For example, BPP A3 corresponds to all of the A patch units at position 3 (patch units 19-27) on the nanoreporter.

The nanoreporter positions are as follows:

Position 1: Patch units 1-9 (A or C)

Position 2: Patch units 10-18 (B or D)

Position 3: Patch units 19-27 (A or C)

Position 4: Patch units 28-36 (B or D)

Position 5: Patch units 37-45 (A or C)

Position 6: Patch units 46-54 (B or D)

Position 7: Patch units 55-63 (A or C)

Position 8: Patch units 64-72 (B or D)

Materials: right and left patches, pre-annealed to each other (each oligonucleotide is at a concentration of 10 μM). Materials for making 100 pmol of BPP 1: (In position 1, patch coordinate 1L is used for the BamH1 digest -this patch is not included in BPP 1): 10 μl each pre-annealed (10 μM/each) patch unit (coordinates 2-9), 5 μl [20 μM] Patch_1R (A or C). Final concentration of each patch is 1.18 pmol/ μl. Materials for making 100 pmol of BPP 2-8: 10 μl each pre-annealed (10 μM/each) appropriate patch unit. There are 9 patch units added to each, or 90 μl total. Final concentraion of each patch is 1.11 pmol/ μl.

Below is a table of all the patch unit pools made for this example, with 8 positions or regions for dye-labeled nucleic acids to bind on the nanoreporter. Positions 1, 3, 5, and 7 can bind to nucleic acid labeled with dye A or dye C, and a positions 2, 4, 6, and 8 can bind to nucleic acid labeled with dye B or dye D.

Table 2 of resulting Basic Patch Pools (correspond to labels on tubes)

BPP-A1 [Pre-Paired, Color=A, Coordinates 1-9]

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Patch_(1-9)R.A Patch_(2-9)L
BPP-B2 [Pre-Paired, Color=B, Coordinates 10-18] Patch_(10-18)R.B Patch_(10-18)L
BPP-A3 [Pre-Paired, Color=A, Coordinates 19-27] Patch_(19-27)R.A Patch_(19-27)L
BPP-B4 [Pre-Paired, Color=B, Coordinates 28-36] Patch_(28-36)R.B Patch_(28-36)L
BPP-A5 [Pre-Paired, Color=A, Coordinates 37-45] Patch_(37-45)R.A Patch_(37-45)L
BPP-B6 [Pre-Paired, Color=B, Coordinates 46-54] Patch_(46-54)R.B Patch_(46-54)L
BPP-A7 [Pre-Paired, Color=A, Coordinates 55-63] Patch_(55-63)R.A Patch_(55-63)L
BPP-B8 [Pre-Paired, Color=B, Coordinates 64-72] Patch_(64-72)R.B Patch_(64-72)L
BPP-C1 [Pre-Paired, Color=C, Coordinates 1-9] Patch_(1-9)R.C Patch_(2-9)L
BPP-D2 [Pre-Paired, Color=D, Coordinates 10-18] Patch_(10-18)R.D Patch_(10-18)L
BPP-C3 [Pre-Paired, Color=C, Coordinates 19-27] Patch_(19-27)R.C Patch_(19-27)L
BPP-D4 [Pre-Paired, Color=D, Coordinates 28-36] Patch_(28-36)R.D Patch_(28-36)L

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BPP-C5 [Pre-Paired, Color=C, Coordinates 37-45] Patch_(37-45)R.C Patch_(37-45)L
BPP-D6 [Pre-Paired, Color=D, Coordinates 46-54] Patch_(46-54)R.D Patch_(46-54)L
BPP-C7 [Pre-Paired, Color=C, Coordinates 55-63] Patch_(55-63)R.C Patch_(55-63)L
BPP-D8 [Pre-Paired, Color=D, Coordinates 64-72] Patch_(64-72)R.D Patch_(64-72)L

6.3 MATERIALS AND PREPARATION FOR ANNEALING THE SINGLE STRANDED OLIGONUCLEOTIDE WITH PATCH UNITS FOR A DOUBLE STRANDED SCAFFOLD.

Third, patch units are prepared to be annealed to the single stranded linear M13mp18, covering the strand in order to make a double stranded oligonucleotide scaffold. Conditions for annealing 60 and 65 nucleotide base patches to the M13mp18 need to occur at high salt concentrations so that binding will be very specific and patches will not anneal to an incorrect coordinate on the M13mp18 strand. For the annealing step, each patch unit is added at a 2:1 to 4:1 ratio with the single stranded M13mp18 sequence at 0.5 pmol total volume. Excess patches are removed before annealing flaps.

Materials used consisted of 20x SSC, linear M13mp18 (BamH1 digested at 0.08 pmol/μl or 200 ng/μl), appropriate base patch pools (BPP) (need 8 total at 1.11 pmol/μl – see above) and digital heat block set at 45°C. Annealing reaction make up is as follows. General guidelines: 2X each patch unit per M13mp18 molecule, pre-ligated flaps/patches (in position 1 or 8) added for purification later, and 5X SSC. Example (0.5 pmol of scaffold with F8 hook flaps) reaction consists of: 7.1 μl BamH1 Digested M13mp18 strand at 0.071 μM, 0.9 μl each new Base Patch Pools at 1.11 μM for first 7 positions: A1, B2, A3, B4, C5, B6 and A7:

1.7 μl A1 BPP (Pre-Annealed, 12/15; at 1.18 μM/each patch)

1.8 μl B2 BPP (Pre-Annealed, 12/15; at 1.11 μM/each patch)

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1.8 µl A3 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch)

1.8 µl B4 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch)

1.8 µl C5 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch)

1.8 µl B6 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch)

1.8 µl A7 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch),

2.4 µl BPP-D8 (pool of the first seven patch units – coordinates 64, 65, 66, 67, 68, 69, and 70 at position 8 – "D" specificity) with purification tags– F8 (FHF, which anneal to patch coordinates 71L, 71R, 72L, 72R, 73L making full split-flap/patch units that have "F" specificity for use as biotin linkers, at position F8) at 0.83 µM, and 7.3 µl 20X SSC. The final reaction volume will be 29.3 µl at 0.027 pmol/ µl.

Anti-Bam oligonucleotide is also added to anneal to region in M13 that is complementary to the (missing) 1L patch unit and to prevent recircularization of the M13 scaffold during ligation.

6.4 ANNEALING PATCH UNITS TO SINGLE STRANDED M13MP18 TO FORM A DOUBLE STRANDED SCAFFOLD.

The fourth step involves the protocol to anneal the patch units to the single stranded linear M13mp18, covering the strand in order to make a double stranded oligonucleotide scaffold, is performed in the following steps: 1) preheat heating block to 42°C, heat above reaction solution to 45°C in small PCR (or strip) tube(s) with foil over top for 15 minutes, turn heat block to 65°C and incubate for an additional 1 hour and 45 minutes and remove tubes, place on ice or freeze.

6.5 PURIFICATION OF NANOREPORTER SCAFFOLD USING BIOTIN AND MAGNETIC BEADS WITH STREPTAVIDIN.

The fifth step occurs before attaching the flaps, where excess patch units that have not annealed to the M13mp18 strand are separated from the double stranded oligonucleotide scaffold. A purification tag with a 5 nucleotide base homologous region to some of the patch units' complementary 5 nucleotide base overhang is annealed to 'hook' the scaffold. Biotinylated oligonucleotides are annealed to the 'purification tag' and magnetic beads with streptavidin are used to capture the scaffold using the biotinylated oligonucleotides. Excess patch units are removed with the supernatant. The scaffold melts off of the magnetic beads into solution for recovery.

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6.6 ANNEAL THE D-BIOTIN CATCHERS TO THE PURIFICATION TAGS

Anneal the D-Biotin catchers to the purification tags on the nanoreporter (making 2X to amount of D8-flap positions available in solution, which is 2X to M13, or 4X final): 0.5 pmol X 25 hook oligonucleotide positions (5 multiplied by 5), 4X makes 50 pmols translates to 0.50 µl of 100 pmol/ µl D-biotin, add 0.5 µl (D, E, F) – Biotin (at 100 µM) to sample, mix and incubate at room temperature for 30 minutes.

6.7 PURIFICATION PROTOCOL TO WASH OFF UNATTACHED PATCH UNITS FROM DOUBLE STRANDED SCAFFOLD.

Anneal F-hook oligonucleotides in a 25 fold excess to nanoreporters in 5X SSC for 30 min at room temperature. Pipet 200 µl DynaBead MyOne Streptavidin™ bead solution into 1.5 ml tubes, place on magnet and remove supernatant. Wash twice with 5X SSC by resuspending and clearing with magnet as in step above. Add 80 µl of sample in 5X SSC (80 fmoles of sample in this example). Resuspend well, by placing on vortex for 15 minutes. Clear solution with magnet and transfer supernatant to fresh tubes for later gel analysis. While on magnet, wash pellets (do not resuspend) with 80 µl TE by pipeting over pellet three times with the same 80 µl volume originally added. Remove wash, place in freshy "wash" tubes for analysis. Heat up TE buffer to 45°C, add 80 µl to each pellet and resuspend. Place tubes on 45°C heat block for 15 minutes, pipetting up/down once to insure beads remain suspended. Immediately clear product with magnet while warm and save. The majority of purified nanoreporters should be present in this product eluted at 45°C.

6.8 ANNEALING AND LIGATION OF FLAPS TO SCAFFOLD.

The sixth step involves split flap oligonucleotides which are annealed to the scaffold to make a 'covered scaffold.' Purification with magnetic beads is performed afterwards to remove excess split flaps. Ligation of the covered scaffold is done using T4 ligase to increase the stability of the structure. Only one type of flap is needed per fluorescent dye. Flaps are either 95 or 100 bases in length and have regions complementary to the patches, to labeled oligonucleotides and to each other. Each flap has 15 base repeating sequences for binding to labeled oligonucleotides. The repeat sequences are based on Lambda sequences that have been analyzed to remove any palindromes and hairpin structures.

Conditions for annealing the flaps are as follows. The sequence on the flaps that corresponds to the patch is 5 nucleotide base pairs long, and therefore the flaps anneal

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specifically to the patches even at high salt concentrations. The ratio of flaps to patches is 2:1. In order to increase stability at high temperatures, ligation of patches to each other and the flap to the patches may be carried out in the same reaction.

1) Quantify the purified scaffold using a spectrometer at A260 nm. Calculate the volume needed for appropriate amount of nanoreporter to prepare. For this example we used 110 ng or 0.023 pmol, reading at A260 nm shows 7.7 ng/ µl, or 14.3 µl for 110 ng. 2) Setup ligation reaction as follows (volume will vary, depending on the purification and scale). Currently using 1.5X flaps to patches, calculate accordingly. For this example, there are four different fluorescent dyes (color) labeled A, B, C, and D and 8 different positions or regions where dye-labeled nucleic acids can bind on a nanoreporter. The number of positions for each color (in this case 1-4) multiply by 9 multiply by 1.5 moles of scaffold = moles of flaps to use.

For the nanoreporter with fluorescent dye in the sequence/positions [ABABCBAD] :

ABABCBAD =

A:40.5 x .023 = .93 pmol; vol: .93 µl of SF (split flap)-AL at 1 µM

.93 µl of SF-AR at 1 µM

B:40.5 x .023 = .93 pmol; vol: .93 µl of SF-BL at 1 µM

.93 µl of SF-BR at 1 µM

C:13.5 x .023 = .31 pmol; vol: .31 µl of SF-CL at 1 µM

.31 µl of SF-CR at 1 µM

D:13.5 x .023 = .31 pmol; vol: .31 µl of SF-DL at 1 µM

.31 µl of SF-DR at 1 µM

Ligation reaction (25 µl total) consists of : Split Flaps (see above; 4.96 µl, or ~5 µl total), 14.3 µl of MODB-Scaffold at 0.0016 pmol/µl, 2.5 µl 10X T4 ligation Buffer, 2.2 µl NanoPure H2O and 1 µl T4 ligase. Incubate tubes 5 minutes at 45°C. Move to 37°C water bath, inc. for 5 minutes. Add 1 µl T4 ligase to samples. Incubate for additional 1hour at 37°C. Freeze immediately, or heat at 75°C for 5 minutes to kill T4 ligase.

6.9 LIGATION OF TARGET-SPECIFIC SEQUENCES TO NANOREPORTERS

The seventh step involves ligation of a target-specific sequence to the nanoreporter. A DNA target-specific sequence is designed to be complementary to the target molecule, which can be RNA (*e.g.*, mRNA) or DNA (*e.g.*, cDNA or genomic DNA). The target-specific sequence can be from 35, 60 or 70 nucleotide bases in length. The target-specific

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sequence can be ligated to the scaffold using a single stranded overhanging region on the covered scaffold. The scaffold with a single type of target-specific sequence can be manufactured separately and then mixed to form libraries.

6.10 NANOREPORTER CONSTRUCTION

Addition of oligonucleotides to a nanoreporter can be done at any point during the construction of a nanoreporter. In certain aspects of the present invention, a labeled oligonucleotide is 15 nucleotide bases long. On the 5' end, a single fluorophore dye is attached. Oligonucleotides with a particular fluorophore dye will generally have the same sequence. These labeled oligonucleotides bind to the repeat sequences of the split flaps. Fluorophores best suited for this example include but are not limited to Alexa 488, cy3, Alexa 594, and Alexa 647. The 15 nucleotide base length holds the fluorophores far enough apart so that they cannot quench each other and ensure that the labeled nucleic acids will be stable (will not melt off complementary strand) at conditions in the visualization process. Labeled oligonucleotides are stable at 40 °C. This short length also allows for packing a large number of fluorescent dyes onto the flaps. In certain aspects of the invention, labeled oligonucleotides are introduced during the target sample processing.

6.11 ATTACHMENT OF NANOREPORTERS TO TARGET MOLECULES

Nanoreporters can be attached to target molecules using any means known to one of skill in the art. In an exemplary embodiment, dual nanoreporters are hybridized to target molecules by mixing 250 pmols each of both the first probe and the second probe with 125 pmols of target. The total volume is adjusted to 4 µl and a final concentration of buffer of 5X SSC. This mixture is incubated in a covered PCR tube overnight at 42 degrees to allow hybridization to occur.

6.12 SURFACE ATTACHMENT

Once the nanoreporters are attached to both target molecule and corresponding labeled nucleic acids, *i.e.*, nucleic acids attached to label monomers, they are attached to a surface and stretched in resolve the order of signals emitted by the label monomers and thus identify the target molecule. In this example, the nanoreporters are stretched to spatially resolve their fluorescent dye codes which correspond to a particular target molecule. The nanoreporters are stretched by attaching one end to a surface (in this example – a coverslip,

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see preparations below). Two methods for surface attachment may be used: A) streptavidin coated slides from Accelr8 Corporation with the nanoreporters being biotinylated and B) biotin coated slides with the nanoreporters having streptavidin. In buffer, the nanoreporters are brought into contact with the active surface and allowed to incubate for a period of time. The reaction is performed in flow cells which were made from PDMS molded in etched silicon wafers to make the channels. Metal tubing is used to core wells at the ends of the channels for buffer and sample insertion. Channel dimensions are 0.5 mm or 1 mm wide and 54 μm high. Once the sample has been loaded into the flow cell lane and incubated, the nanoreporters should be attached. Nanoreporters can be stretched either by applying a voltage or by removing the liquid with a receding meniscus leaving the strings stretched and dry.

6.13 PREPARATION OF SURFACE AND ASSEMBLY OF DEVICE

The binding surfaces (Accelr8 brand Streptavidin-OptiChem, coated coverslips) are shipped in units of 5 surfaces per slide container, and each container is enclosed with a package of silica dessicant in a foil pouch. The pouches are stored at -20 °C until use.

To prepare the surface for binding, a pouch is first pulled from the freezer and allowed to come to room temperature over several minutes. If previously unopened, the pouch is then sliced along one edge to form a slit, and the container of surfaces is removed. Upon removal of the required surface, the container is replaced in the pouch with its dessicant, the slit is sealed closed with a strip of packaging tape, and the pouch is replaced in the freezer.

The surface is then lightly rinsed with a stream of Nanopure water (Barnstead Nanopure Diamond) and soaked for 10 minutes in 0.2 μm-filtered 1X PBS in a clean, slotted Coplin jar. After soaking, the surface is dipped in Nanopure water and dried by blowing filtered nitrogen across the surface edge.

The PDMS device used to mate with the surface and provide localization of the sample is cleaned just before use by applying cellophane tape to the PDMS surface and then peeling away dust or other particles which may have become attached during storage. The binding side of the Accelr8 surface is laid face-up, and the clean PDMS structure is centered, channel side down, on the surface. PDMS adheres readily to coated glass, and no further attachment mechanism is necessary.

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6.14 SAMPLE BINDING AND WASHING

The sample is bound to the surface by first applying a 5 μL drop of the sample (currently diluted in 100 mM sodium borate buffer, pH 9.8) in one well of the chosen lane. The drop should just touch the point at which the channel joins the well (some sample may wick into the channel at this point). The channel is filled, and binding is equalized throughout the channel, by pulling the droplet through the channel to the opposite well using a very weak vacuum (< 2 kPa). The process is repeated for the other samples in their respective lanes. Excess fluid is then removed from the wells, the wells are taped to reduce evaporation, and the device is incubated at room temperature in the dark for 20 minutes.

After binding, the tape is removed, and the top well of each lane is filled with 100 μL of the borate buffer described above. About 20 μL of that buffer is pulled through the channels to the other wells using the vacuum, and the process is repeated once. All borate buffer is then removed from all wells, and the top well is filled with 1X TAE, pH 8.3. About 50 μL TAE is pulled through the channel, then all TAE is removed and the well is refilled. The process is repeated three times, for a total of about 150 μL of TAE rinse. Finally, all wells are filled with 100 μL 1X TAE.

6.15 ELECTROSTRETCHING

The bottom of the coverslip/PDMS device is spotted with immersion oil and placed on the microscope. Electrodes are inserted into the wells on opposite ends of the first PDMS channel (negative electrode in top well, positive in bottom). The first image of the channel will be taken close to the bottom well; the microscope stage is adjusted so that the area of interest is in focus.

Voltage (200 V) is then applied across the channel. Voltage is supplied by a DC power supply (Agilent E3630A) and amplified 100X through a home-built amplifier. After the current is applied, focus is readjusted, and the imaging process begins.

The electrostretching and imaging process is then repeated with the remaining channels. Image the bindings.

6.16 LIGHT SOURCE FOR THE FLUORESCENT DYES ON THE NANOREPORTER

In using an arc lamp as a light source, the best fluorophore selection is the brightest types without leading to fluorescent overlap such as Alexa 488, Cy3, and Alexa 594. Weaker fluorescent dyes such as Alexa 647 and Cy5.5 may also be used.

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6.17 FILTERS TO IMAGE THE FLUORESCENT DYES ON THE NANOREPORTER

For the selected fluorophores Alexa 488, Cy3, Alexa 594 and Alexa 647 there maybe an overlap between the Cy3 and Alexa 594. However, custom ordering an emission filter with a bandwidth of 572-600 nm minimizes the overlap.

6.18 MICROSCOPE AND OBJECTIVE LENS TO IMAGE THE NANOREPORTERS

The microscope model used was the Nikon Eclipse TE2000E from Nikon Incorporation using the inverted fluorescence imaging station which has 6 filter cassettes that allow the selection of fluorescent emission from multiple fluorescent dye candidates. For the selected dyes, the optical resolution required is about 400 nm for all the wavelengths (500-700 nm). The selected objective lens is the Nikon Plan Apo TIRF lens which has a NA of 1.45 and magnification of 60. The optical resolution is ~210-300 nm for different wavelengths.

7. EXAMPLE 2: PATCH/FLAP NANOREPORTER MANUFACTURING PROTOCOL

This example demonstrates another way of making a nanoreporter which consists of a single stranded linear M13mp18 viral DNA, oligonucleotide patch units and long flaps.

Nanoreporter label units were successfully generated using methods substantially as described in this example.

Pre-phosphorylated patch units and flaps are added together with the M13mp18 DNA vector and ligated together. After the ligation of the flaps to the patch units which are ligated to the M13mp18 DNA, the BamH1 enzyme is introduced to linearize the vector.

Prepare a batch of nanoreporters starting with 5 µg of M13mp18 as a scaffold. The hybridization may be scaled up accordingly to the desired amount. This process will take about 1-2 days to complete.

Materials:

Qty	Item	Vendor
20	250 ug/µl M13mp18 viral ssDNA	New England Biolabs
27 µl	0.74 pmol/µl Oligonucleotide Patch Unit Mix	IDT
8 µl	Long Flap Oligonucleotide A 100 pmol/µl	IDT

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8 µl	Long Flap Oligonucleotide B 100 pmol/µl	IDT
0.5 µl	Flap patch Oligos at 100 pmol/µl from plates #529916 and #610591	IDT
31 µl	T4 Ligase 10x buffer	Fermentas
19 µl	T4 Ligase	Fermentas
15 µl	Optikinase 10x buffer	USB
4.2 µl	100 mM ATP	ANY
5 µl	Optikinase Enzyme 10 units/µl	USB
1 µl	BamH1 oligonucleotide 10 pmol/µl	IDT
20 µl	BamH1 10x buffer	Fermentas
3 µl	BamH1 Enzyme 10 units/µl	Fermentas

Preheat water bath to 37°C and 55°C °C before beginning protocol. Make sure buffers are all well mixed and thawed before using. A work plate should be available and labeled with the ordered oligos from IDT in plates #529916 and #610591. Take these two plates out and thaw at room temperature for 0.5-1 hours and spin down contents before removing the tape that covers the wells. Four separate reactions will be set up in 1.5 ml eppendorf tubes using specific oligonucleotides from these plates. To begin label these four separate tubes with roman numerals on their caps. Columns 5 and 6 A through H are for reaction I, Columns 7 and 8 A through H are for reaction ii are all found in plate #529916. Columns 1 and 2 are for reaction iv, and Columns 3 and 4 are for reaction iii.

Flap Ligations (Step A): Label four separate 1.5 ml tubes with roman numerals i through iv (mentioned above). Add the reagents below accordingly to each 50 µl reaction containing: 5 µl 10x ligase buffer, 0.5 µl/oligonucleotide from designated wells from plates #529916 and #610591, 4 µl Long Flap Oligo/reaction (A or B) for reactions I, ii and iv. 3 µl of LF for area iii, 29 H_20 for reactions I,ii and iv. 32 µl H_20 for reaction iii, and 4 µl T4 ligase. Preanneal oligos in this mix without the ligase at 37 °C for half an hour. Add ligase as last reagent and allow to ligate at room temperature for at least four hours. Product concentration is 1 pmol/flap/µl.

Flap Ligation Phosphorylation (Step B) Label four separate 1.5 ml tubes with roman numerals again, one through four with a P inside a circle to designate that the products are phosphorylated. Add the following reagents to the corresponding tube: 10 µl/Flap ligation. reaction (take 10 µl/flap ligation reaction above), 2.5 µl Optikinase buffer, 0.5 µl 100 mM

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ATP, 11.5 µl H_20, and 0.5 µl Optikinase enzyme. Incubate at 37 °C for 1 hour. Product concentration 0.4 pmol/flap/µl.

Oligonucleotide Patch Unit Phosphorylation (Step C) 27 µl Oligonucleotide Patch Unit mix 0.74 pmol/µl, 5 µl 10x buffer, 1 µl 100 mM ATP, 3 µl Optikinase enzyme, and14 µl H_20. Once reagents are all together gently mix the solution by flicking the tube a few times and spin down. Incubate at 37 °C for 1 hour.

Hybridization to M13mp18 scaffold (Step D) In a new 1.5 ml tube add the following reagents: 20 µl M13mp18 at 250 ng/µl, 27 µl Phosphorylated Oligonucleotide Patch Units 0.4 pmol/µl (Step C), 12.5 µl/ Phosph. Flap Ligation (Step B) preheat at 55 °C for 5 minutes and put on ice, 11 µl 10x ligase buffer and heat entire mixture at 55 °C for 1 minute. Hybridize mixture at 37 °C for at least 4 hours.

Ligation (Step E) Spin down eppendorf contents. Add 1.2 µl 100 mM ATP and 3 µl T4 ligase. Gently mix contents by flicking the tube, then spin down.

BamH1 Digest (Step F): 1 µl of 10 pmol BamH1 oligo, 20 µl 10x BamH1 buffer and hybride at 37 ~ 1 hour. Adjust volume to 200 µl. Add 3 µl BamH1 enzyme. Incubate at 37 °C for 1 hour.

First step: start by adding 20 µl of M13mp18 (NEB 250 µg/ml) to a clean 1.7 ml eppendorf tube. Take 5 µl of Phosphorylated Flap ligation reaction and preheat it at 70 for 2 minutes and immediately put on ice. Add the 5 µl of each Phosphorylated Flap Ligation reaction (1 pmol/flap/µl) to the tube and gently mix by pipetting a few times. Incubate the eppendorf tube at 37 °C for 1 hour.

Second step: put 13.5 µl Oligonucleotide Patch Unit Mix (0.74 pmol/µl) and 1 µl of Acrydite Mix (10 pmol/µl)in a new eppendorf 1.7 ml eppendorf tube. Add 5 µl 10x Optikinase buffer, 1 µl 100 mM ATP and 27.5 µl H_20. Mix gently by pipetting the solution. Add 2 µl Optikinase enzyme, gently mix by pipetting and incubate at 37 °C for 1 hr.

Third step: take the phosporylated oligos rxn and add it entirely to the contents of the M13mp18+Flaps Hybridization. The reaction is mixed gently by pipetting and it is allowed to incubate at 30 °C for 1 hour. After the hybridization is complete adjust the ATP by adding 1 µl (100 ATP) to the reaction.

Fourth step: spin down contents in eppendorf tube and add 4 µl T4 Ligase enzyme (5 units/µl), mix gently by pipetting. Incubate at room temperature for at least four hours. Add 1 µl BamH1 oligonucleotide (10 pmol/µl) to hybridize at room temperature while ligation is taking place.

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Fifth step: digest ligation reaction by adding 4 µl BamH1 enzyme (5 units/µl), mix gently by pipetting and incubate at 37 °C for 1 hour. Once the incubation period is over. Take an aliquot of 500 ng for QC.

Sixth step: treat with Psoralen, UV or DMPA light for 15 minutes.

Calculations include:

5 µg of M13 = 20 µl stock from New England Biolabs = 2 pmols

Oligonucleotide mix: 180-34 flap areas – 10 Acrydite modified Oligos = 0.74 pmol/oligo

10 pmols/oligonucleotide = 13.5 µl = 1350 pmols

Optikinase 1unit converts 1 nmol of phosphate to ends - use excess. 4 µl of Optikinase was used.

SEQ ID NO: 1 = M13mp18.

8. EXAMPLE 3: PROTOCOL FOR PRODUCTION OF RNA NANOREPORTERS

Nanoreporters were generated and successfully employed to detect target molecules using methods substantially as described in this example. An example of target detection using such this method is shown in Figure 6.

8.1 SCAFFOLD PRODUCTION

Single-stranded circular M13mp18 DNA (USB Corporation) is annealed to a 10-fold molar excess of an oligonucleotide complementary to the Bam HI recognition site (Bam Cutter oligo) and cut with Bam HI restriction enzyme to yield a linear single-stranded DNA backbone. An oligonucleotide complementary to the Bam Cutter oligonucleotide (anti-Bam oligonucleotide) is subsequently added in 50-fold excess to the Bam Cutter oligonucleotide to sequester free Bam Cutter oligonucleotide and thus prevent recircularization of the M13 during later steps.

The linear M13 molecule serves as a scaffold onto which RNA patches, or RNA segments, with incorporated fluorophores can be annealed.

8.2 PCR TO FORM DOUBLE-STRANDED POSITIONS ON THE M13 SCAFFOLD

Ten sets of oligonucleotide primer pairs were designed to create 10 different regions along the M13 scaffold. Each pair contains one primer which has a T7 RNA polymerase

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promoter at the 5′ end. Regions 2-7 are designed to be 900 bases (approximately 300 nm) long, as this is the approximate size of a diffraction-limited spot (the smallest spot that can be achieved with standard optics). Regions 1 and 8 have both long and short versions: the long versions cover the whole 900-base region, while the short versions cover only a portion of the 900-base region to allow a target-specific sequence to be ligated. Thus a target-specific sequence can be attached to either end. The ends can also be used for attachment of anchors or tags.

PCR is performed using Taq polymerase and 0.5 ng of double-stranded M13mp18 (USB Corporation) as a template. Reactions are cleaned up using a Qiaquick purification kit from Qiagen. Each PCR reaction yields a double-stranded fragment corresponding to one specific segment as illustrated below. These fragments are used as templates for the *in vitro* transcription of the RNA segments.

8.3 *IN VITRO* TRANSCRIPTION TO PRODUCE DARK RNA SEGMENTS

Using the PCR products described above as double-stranded templates, RNA segments are generated using an *in vitro* transcription kit from Ambion (Megascript T7 kit). The products of the transcription reactions are purified (including treatment with DNAse I to remove template) using a RNeasy Kit from Qiagen.

8.4 *IN VITRO* TRANSCRIPTION TO PRODUCE RNA SEGMENTS MODIFIED WITH AMINOALLYL GROUPS

Using the PCR products described above as double-stranded templates, RNA segments for later dye-coupling are generated using an *in vitro* transcription kit from Ambion (MessageAmp aRNA kit). Aminoallyl-modified UTP nucleotides are incorporated into the RNA segments during transcription. The products of the transcription reactions are purified (including treatment with DNAse I to remove template) using a RNeasy Kit from Qiagen.

8.5 DYE COUPLING OF AMINOALLYL RNA SEGMENTS TO PRODUCE COLORED RNA SEGMENTS

20-100 μg of aminoallyl-modified RNA segment is coupled with NHS-ester dyes using Ambion Aminoallyl Labeling Kit. Dyes used include Alexa 488, Alexa 594 and Alexa 647 (Invitrogen/Molecular Probes) as well as Cy3 (Amersham).

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Each segment is made separately in 4 colors so that each position on the scaffold can be filled with a segment in any of the four colors; thus different colors can be added at different positions to create many unique color combinations.

In this particular embodiment, adjacent segments must be of different colors or there may be dark segments interspersed so that each segment is detected as an individual 'spot'. Dark segments may be used as part of the nanoreporter code.

8.6 ASSEMBLY OF THE LABEL MOLECULE

Segments for each position are annealed in a 2:1 ratio of segment to M13 scaffold in 1X SSPE buffer at 70°C for 2 hours.

An assembled nanoreporter with labeled RNA segments is depicted in Figure 3A-3B. Figure 3A depicts a nanoreporter in which only alternate "spots" (1, 3, 5 and 7) are labeled, and Figure 3B depicts a nanoreporter in which every spot is labeled.

9. EXAMPLE 4: DETECTION OF TARGET (S2) RNA AND DNA MOLECULES USING AN RNA NANOREPORTER/GHOST PROBE COMBINATION

9.1 SYNTHESIS OF PROBE AND TARGET OLIGONUCLEOTIDES

S2 DNA target oligonucleotide was synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies). S2 RNA target molecules were generated by *in vitro* transcription of PCR products corresponding to region of cloned SARS coronavirus gene (Invitrogen) using an Ambion Megascript™ kit per manufacturer's instructions. The S2 ghost probe (Figure 6A (i)) was complementary to a specific 50-base region of the S2 target sequence (S2-*a*) and was synthesized with a biotin-TEG monomer at the 5′ end and purified by high performance liquid chromatograpy (Integrated DNA Technologies). A second oligonucleotide with 50 bps complementary to the S2 target (S2-*b*) plus 9 bp of a additional sequence used for ligation to the M13 scaffold (59bp total) was synthesized and purified by HPLC (Integrated DNA Technologies). Note that S2-*a* and S2-*b* target regions were not overlapping.

9.2 NANOREPORTER SYNTHESIS

Oligonucleotide S2-*b* was ligated to the 5′ end of linearized M13 [Figure 6A (iii)], and the resulting product was purified away from residual unligated oligonucleotide by size-exclusion filtration through a YM100 filter (Millipore) per manufacturer's instructions.

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Amino-allyl-modified RNA segments complementary to M13 is positions 2, 4, 6, and 8 (SEQ ID NOs:) (Figure 1C) were generated from *in vitro*-transcription of DNA templates (PCR products) via the Ambion Megascript™ kit per manufacturer's instructions. The segments were then coupled to NHS-ester-modified Alexa 647 dye (Invitrogen) per Ambion's instructions (amino allyl MessageAmp™ II aRNA kit). RNA segments corresponding to positions 1, 3, 5, and 7 of the M13 scaffold (Figure 1C) were generated as unmodified *in vitro*-transcribed RNAs from DNA templates as described above. Assembly of the nanoreporter was carried out by annealing 10 fmol/µl of each of the eight segments to 5 fmol/µl of the M13-S1-*b* scaffold for 2 hours at 70°C in 1X SSPE buffer (150 mM sodium chloride, 10 mM sodium phosphate, 1mM EDTA). The final product was a nanoreporter with 4 segments labeled with A647 (red) interspersed with dark segments.

9.3 HYBRIDIZATION CONDITIONS

Hybridization of nanoreporters and ghost probes to target were carried out under the following conditions: 5X SSPE (750 mM sodium chloride, 50 mM sodium phosphate, 5 mM disodium EDTA), 40 pM ghost probe (attachment oligonucleotide S2-*a*), 40 pM Nanoreporter S2-*b*, 100 ng/µl sheared salmon sperm DNA, 5X Denhardt's solution and 0.1% Tween. Final target concentrations were 20 pM S2 DNA target (Figure 6B) and 1pM S2 RNA target (Figure 6C). No target was added to the negative control (Figure 6D). The hybridization reaction was incubated at 65°C for at least 16h.

Hybridization reactions were diluted 1:2 with 100 mM Borate buffer solution (pH 9.8) and introduced into a flow cell channel and bound to a streptavidin-coated coverslip forming the bottom of the channel (Streptavidin-OptiChem coverslips from Accelr8). Attachment to the slide by one end of the nanorerporter/target/ghost probe complex was achieved via interaction of the biotinylated ghost probe with the streptavidin surface. After rinsing the channel with additional borate buffer to remove excess reporters not bound to the surface, the buffer was exchanged with 1X TAE (40 mM Tris-acetate, 1 mM EDTA) and a current of 200V was applied to stretch out the nanoreporter/target complexes during image capture.

Images were obtained using a Leica DMI 6000B microscope with a 63X oil immersion objective (1.4 NA), Xcite-120 light source (Exfo), customized filter sets (Chroma Technologies), an Orca-ER CCD camera (Hamamatsu) and Metamorph data acquisition software (Molecular Devices).

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As predicted, when the correct target molecule S2 hybridizes [Figure 6A (ii)] to both ghost probe [Figure 6A (i), S2-*a*] and S2-*b* target-specific nanoreporter [Figure 6A (iii)], the ghost probe/target/nanoreporter complex forms a single species that attaches to the slide and was visualized as 4 spots when exposed to 647 nm wavelength light (Figure 6B, 6C, and 6E). The amount of binding was dependent on the target concentration. There was no significant binding in absence of S2 target sequence (Figure 6D).

10. EXAMPLE 5: NANOREPORTER COMPRISING A MONOVALENT OR BIVALENT ANTIBODY FRAGMENT

Where a target molecule is a protein or polypeptide, a nanoreporter can be generated in which the nanoreporter scaffold is a nucleic acid and the target-specific sequence is a monovalent or bivalent antibody fragment.

Using routine methods, an antibody that recognizes a target molecule of interest is optinally digested with pepsin to generate F(ab')2 fragments. The two parts of the antibody or the two F(ab')2 fragments generated by the pepsin digestion are separated by mild reduction, for example with 2-mercaptoethylamine. This reduction separates either the antibody or the two F(ab')2 fragments into two monovalent fragments with two sulfhydryl groups that can be functionalized.

A heterobifunctional crosslinking reagent (*e.g.*, m-Maleimidobenzoyl-N-hydroxysuccinimide ester from Pierce Biotechnology Inc.) is used to attach a maleimide to an oligonucleotide with an amine modification (which can be ordered from many sources, such as Integrated DNA Technologies). The NHS on the cross-linking reagent is reacted with the amine on the oligonucleotides to produce a maleimide-conjugated oligonucleotide.

This maleimide conjugated oligonucleotide is then reacted with one of the sulfhydryl groups on the antibody fragment. Due to steric limitations, it is preferable that only one oligonucleotide be attached to each fragment.

This monovalent or bivalent antibody fragment attached to an oligonucleotide can then be hybridized to a complementary sequence on a nanoreporter scaffold, to generate a reporter probe in which the target-specific sequence is an antibody sequence. Such a reporter probe can be used alone to detect the target molecule, or in conjunction with a ghost probe or another reporter probe whose target-specific sequence is a monovalent or bivalent antibody or antibody fragment that binds to a different portion of the same target molecule.

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11. EXAMPLE 6: HYBRIDIZATION OF 25 CELLULAR GENES TO 100 NG OF PLACENTAL TOTAL RNA USING NANOSTRING REPORTER SYSTEM

Detection and quantitation of 25 endogenous cellular genes was carried out in a single multiplexed hybridization reaction. In addition, three non-human control sequences were spiked into each reaction that corresponded to approximately 10, 100 and 300 copies per cell, respectively. A negative control hybridization was also performed in the absence of cellular RNA.

11.1 HYBRIDIZATION REACTION

Each sample was hybridized in triplicate. Final concentrations of the hybridization reagents were as follows: 1.12nM total Nanoreporters (28 individual Nanoreporters at 40 pM each), 1.12nM total ghost probe (28 individual ghost probes), 5X SSPE (pH 7.5), 5X Denhardt's reagent, 100 ng/µl sheared salmon sperm DNA, 0.1% tween 20, 150 fM S3 spike DNA, 50 fM S4 spike, and 5 fM S6 spike. The final concentration of total placental RNA was 33 ng/µl. No total placental RNA was added to the negative control hybridizations. The final volume of the reaction was 30 µl. Reagents were mixed and incubated at 65°C in thermocycler block with heated lid for 20 hours.

Master mix	(1 Reaction)	(6 reactions)
1.8X hybridization mix*	16.7 µl	100 µl
25 endogenous gene reporters (0.6 nM each)	2 µl	12 µl
25 endogenous gene ghost probes (0.6 nM each)	2 µl	12 µl
Control reporters (0.6 nM each)	2 µl	12 µl
Control ghost probes (0.6 nM each)	2 µl	12 µl
10X control target mix	3 µl	18 µl
H_20	1.3 µl	8 µl
Total	29 µl	174 µl

*Hybridization mix (9X SSPE, 9X Denhardt's reagent, 180 ng salmon sperm DNA, 0.18% tween 20)

Reactions	1	2	3	4	5	6
Master mix	29 µl	29 µl	29 µl	29 µl	29 µl	29 µl
100 ng/µl placental RNA	1 µl	1 µl	1 µl	0 µl	0 µl	0 µl
H_20	0 µl	0 µl	0 µl	1 µl	1 µl	1 µl
Total Rxn volume	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl

Incubate reactions in thermocycler with heated lid overnight (18hours).

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11.2 POST-HYBRIDIZATION PURIFICATION

Hybridization reactions were purified to remove unhybridized reporters using an oligonucleotide complimentary to ghost probe attached to magnetic beads (F-bead). Hybridization reactions were diluted 5 fold in 0.1% tween 20 to bring the final salt concentration to 1X SSPE and the solution added to 30 µl of F-beads (prewashed 2 times in 150 µl of 1X SSPE/0.1% tween 20). Hybridized complexes were allowed to bind to the beads at room temperature for 15 minutes with continuous rotation, washed once in 150 µl of 0.5X SSPE, and eluted in 25 µl of 0.1X SSPE for 15 minutes at 45°C.

11.3 BINDING, STRETCHING, AND IMMOBILIZATION

The samples was prepared for binding by addition of 1 µl of 1/1000 dilution of 0.1 uM Tetraspec™ fluorescent microspheres (product # T7279, Molecular Probes) and 3 µl of 1M bis-tris propane (pH 9.0). Samples were loaded into a Nanostring fluidic device for attachment to Accerl8 Optichem® slide coated with streptavidin (product #TB0200). After loading, slide surface was washed once with 1X TAE and prepared for electrostretching by addition of 40 µl of TAE to each well. Attached complexes were stretch by applying 200V across the fluidic channel. After 1 minute the samples were immobilized in the stretched position by adding 60 µl of 500 mM of G-hook oligo solution to the well containing the negatively charged electrode while continuing to apply voltage for 5 minutes. After immobilization the TAE solution is removed and replaced with anti-photobleaching reagent for imaging.

11.4 IMAGING

Slides were imaged on Nikon Eclipse TE2000E equipped with a metal halide light source (X-cite 120, Exfo Corporation) and a 60X oil immersion lens (1.4 NA Plan Apo VC, Nikon). For each field of view, 4 images at different excitation wavelengths (480, 545, 580 and 622) were acquired with an Orca Ag CCD camera (Hamamatsu) under control of Metamorph software (Universal Imaging Corporation). Images were processed with custom image processing software.

11.5 DATA ANALYSIS

Raw data were extracted from processed images using custom software. Data were normalized to the average counts for control spikes in each sample. To determine if a gene was "detected" by the system, the counts obtained for each gene from hybridizations

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containing RNA were compared to counts obtained in hybridizations without RNA using a Student's ttest. Genes with p values <0.05 were determined to be detected. After background subtraction, the concentrations of cellular mRNA were estimated from the linear regression of the spike controls. These concentrations were converted to copies per cell using the following assumptions: 1 cell contains 10 pg total RNA; each cell contains 300,000 mRNA molecules; final volume of the reaction is 30 µl.

11.6 RESULTS AND CONCLUSION

Table 3 below shows the results of the data analysis described above. These results show that using the nanoreporter technology described herein, it was possible to detect transcripts, such as CASP3, that are present at a concentration of less than 1 transcript/cell. Thus, the nanoreporter technology provides an exquisitely sensitive means of detecting and quantifying gene expression.

Table 3: Transcript Concentration and Abundances

Gene	Avg counts*	error (counts)	concentration (fM)	error (conc.)	calculated copies/cell	error (copy/cell)	Detected/ Not Detected (p<0.05)
GM2A	149	17	3.39	0.39	6.12	0.07	D
ATF4	68	2	1.55	0.06	2.80	0.01	D
CTNNB1	792	50	17.95	1.19	32.44	0.22	D
IRF1	221	20	5.01	0.47	9.05	0.09	D
STAT5A	120	11	2.72	0.25	4.91	0.05	D
CREG1	409	17	9.28	0.44	16.76	0.08	D
CASP3	13	1	0.30	0.03	0.54	0.00	D
CCL20	2	1	0.04	0.03	0.07	0.01	ND
NMI	115	2	2.61	0.07	4.72	0.01	D
XBP1	719	46	16.30	1.10	29.45	0.20	D
PCGF4	75	18	1.70	0.40	3.08	0.07	D
IFI27	747	41	16.94	1.00	30.61	0.18	D
TAF7	185	11	4.19	0.26	7.57	0.05	D
OAS3	74	9	1.68	0.20	3.03	0.04	D
C2	850	49	19.28	1.19	34.83	0.21	D
IL6	8	3	0.19	0.07	0.34	0.01	D
MyD88	94	6	2.13	0.14	3.85	0.03	D
HIF1A	130	7	2.95	0.17	5.33	0.03	D
APOA2	-1	2	-0.01	-0.05	-0.03	-0.01	ND
KISS	6825	130	154.79	4.52	279.65	0.82	D
ELK3	55	4	1.25	0.09	2.27	0.02	D
CBF2	72	3	1.64	0.07	2.96	0.01	D
IFI30	625	47	14.16	1.10	25.59	0.20	D
RELB	35	5	0.78	0.11	1.42	0.02	D
CTCF	103	3	2.35	0.09	4.24	0.02	D

*Normalized and background subtracted.

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The hybridization methods described herein have been performed in single multiplexed reactions containing up to 120 different reporters with similar hybridization efficiencies and results.

12. EXAMPLE 7: CONSIDERATIONS REGARDING NANOREPORTER HYBRIDIZATION KINETICS

12.1 BACKGROUND

Solution hybridizations with a large excess of probe over target follow pseudo-first order kinetics. In this regime the speed of the reaction depends only on the probe concentration and not on the target concentration. For a two-probe, one-target strategy to provide accurate information on the concentration of a target in solution, the probes should both be present in excess of the target. The possible concentration range is preferably therefore bounded on the lower end by the concentration of the target. However, the useful concentration range for the nanoreporter technology described herein is practically bounded on the lower end by the amount of time needed to perform the hybridization.

12.2 HYBRIDIZATION KINETICS

In preferred embodiments, target detection and quantification assays are performed in which the target (T) must hybridize to both a reporter probe (R) and a ghost probe (G) to be detected (for example by affinity selection and detection of complexes comprising only (R) and (G), which in turn only form complexes in the presence of (T)) . Assuming that these reactions are irreversible, there are four possible elementary reactions that occur.

$$R + T \xrightarrow{k_1} RT$$

$$T + G \xrightarrow{k_2} TG$$

$$RT + G \xrightarrow{k_3} RTG$$

$$R + TG \xrightarrow{k_4} RTG$$

Because RT and TG are intermediate complexes of two out of the three species, these four reactions can be simplified to

$$R + T + G \rightarrow RTG$$

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However, to quantitatively calculate the rate of production of RTG (the reporter-target-ghost probe complex), all four reactions must be considered. The differential equations describing the system are:

$$\frac{dC_G}{dt} = -k_2 C_G C_T - k_3 C_G C_{RT} \quad \frac{dC_R}{dt} = -k_1 C_R C_T - k_4 C_R C_{TG} \quad \frac{dC_T}{dt} = -k_2 C_G C_T - k_2 C_R C_T$$

$$\frac{dC_{TG}}{dt} = k_2 C_G C_T - k_4 C_R C_{TG} \quad \frac{dC_{RT}}{dt} = k_1 C_R C_T - k_3 C_G C_{RT}$$

$$\frac{dC_{RTG}}{dt} = k_4 C_R C_{TG} + k_3 C_G C_{RT}$$

where C_R, C_T, C_G, C_{RT}, C_{TG}, and C_{RTG} are the concentrations of the various species, and k_1-k_4 are the kinetic constants for the four elementary reactions. Values for these kinetic constants when the probes and targets are complementary single-stranded molecules (i.e. when there is no purification tag on the ghost probe and no reporter) can be calculated from data available in the literature (Wetmur, J. *Annu. Rev. Biophys. Bioeng.* 1976.5:337-361).

$$k = k_N \frac{\sqrt{L}}{N} \frac{\alpha_{salt}}{\alpha_{ref}}$$

In the above equation, k_N is the nucleation rate constant, L is the nucleic acid length (in base pairs), N is the nucleic acid complexity (equal to L for non-repetitive sequences) and a_{salt} and a_{ref} are corrections for salt concentration (Britten *et al.*, 1974, *Methods in Enzymology* 29E:363-406). In the nanoreporter systems described herein, the kinetic constants will depend on the sizes of the attached ghost probe tags and reporter probe. Without being bound by any theory, it is the inventors' belief that the kinetic constants will have the same dependence on length that an elementary reaction has on the diffusion constants of the reactants.

$$k = k_N \frac{\sqrt{L}}{N} \frac{\alpha_{salt}}{\alpha_{ref}} \frac{D_1 + D_2}{2D_{50}}$$

In the above equation D_1 and D_2 are the diffusion constants of the two reacting species (see the reactions above) and D_{50} is the diffusion constant of a 50-mer single-stranded DNA molecule. Assuming a 100-base single-stranded target, 100-base single-stranded ghost probe, and 7200-base double stranded reporter, the relevant kinetic constants are

$k_1 = 2.64 \times 10^5$ L/mol/s
$k_2 = 6.55 \times 10^5$ L/mol/s
$k_3 = 3.99 \times 10^5$ L/mol/s

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$k_4 = 1.91 \times 10^5$ L/mol/s

Numerically solving the system of differential equations with these kinetic constants (assuming at least a 10-fold excess of probes over target) yields the prediction that 5 pM reporter and 5 pM ghost probe will drive hybridization to 10% of completion in an overnight reaction (16-18 hours). At concentrations lower than 5 pM, the amount of completely hybridized molecules is likely impractical to measure. Thus, in a preferred embodiment, the lower concentration of a nanoreporter component (ghost probe and/or reporter probe) is 5pM.

12.3 ENTANGLEMENT OF REPORTERS

As probe concentrations increase, theory predicts that hybridization kinetics speed up without bound – the only limit being the solubility of the probes. However, the reporter probe can be very large compared to the target-specific sequence in the nanoreporter systems of the invention. Without being bound by any theory, the inventors believe that by its attachment to the reporter probe the kinetics of the target-specific sequence are altered from classical solution hybridization kinetics. Because the reporter probe is a large, polymeric molecule, it can have long-lived interactions (entanglements) with other nanoreporters when they come into contact. At low concentration the probability of two polymers becoming entangled is small, but as the concentration and/or size of a polymer in solution increases, these interactions become more and more common. In the extreme case of very long molecules at very high concentration the polymers form a permanent network, or gel, in solution. For solution hybridization to occur, a probe (*e.g.*, a nanoreporter probe)/target pair must diffuse through solution until they contact one another and a hybridization nucleus forms. Classically, hybridization reactions are not diffusion limited because the translational diffusion of the molecules is faster than the nucleation of the hybridization (*i.e.*, the probe and target diffuse together and interact many times before a nucleation occurs). In dilute solution its large size will slow the translational diffusion of the reporter probe, but may not significantly affect the kinetics. At some intermediate concentration, the reporter probes take up almost all of the space in the solution, effectively forming a permanently entangled gel, and can no longer diffuse in solution. However, the ghost probe and the targets are smaller molecules that are believed to still diffuse through the entangled reporter probes, allowing hybridization to take place (although possibly at a slower rate). The inventors also believe that at some higher concentration the reporter probe

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in solution will also hinder the movement of the ghost probe and the targets to the point that the reaction becomes diffusion limited. This concentration (which is not quantitatively known and depends upon the reporter probe structure, the ghost probe structure, and the target size) is the upper limit of the useful concentration range in the nanoreporter system, and can be emprically determined by one of skill in the art guided by the principles described herein.

12.4 LENGTH DEPENDENCE OF KINETICS

Since the limiting upper concentration for hybridization depends upon both the reporter structure and ghost probe structure (of which there are many possible variations), a theoretical framework to predict the permutations of useful concentration ranges is useful in the practice of the invention Classical theory predicts that hybridization kinetics depend only on the size of the smaller probe. Theory would therefore predict that the size of the reporter will not play a role in the hybridization kinetics as long as both the target molecule and the ghost probe are significantly smaller. Theory then predicts that the rate of hybridization (for a constant target length) depends on $1/L^{1/2}$, where L is the length of the ghost probe, due to steric inhibition of hybridization. Consequently, the kinetics of hybridization will be faster with smaller ghost probes. As the ghost probe length increases, the hybridization rate should decrease as $1/L^{1/2}$. If a constant ghost probe length is assumed, then the range of reporter lengths and concentrations that will result in a measurable mount of hybridization events can be defined. Once a reporter size has been defined, then the approximate range of ghost probe sizes can be determined. This is an iterative process, but may give good starting points from which to gather data to generate detailed empirical guidelines, given that the theories that the inventors' rationale is based upon were generated from hybridization data in systems that do not employ a reporter probe.

12.5 ENTANGLEMENT THRESHOLD

A reporter probe is essentially a polymer in free solution, which behaves as a random coil. The volume occupied by a single reporter, V_p, can be calculated from polymer physics theories according to the Freely-Jointed Chain model (FJC, for a flexible polymer, such as single-stranded DNA or RNA) or the Worm-Like Chain model (WLC, for a stiff polymer such as double-stranded DNA or a reporter). For either model

$$V_p = \frac{4}{3}\pi R_g^{\ 3}$$

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where R_g is the radius of gyration. For the FJC

$$R_g = b\left(\frac{N}{6}\right)^{0.6}$$

Where b is the segment length and N is the number of segments in the chain. For the WLC

$$R_g = \sqrt{\frac{1}{6}Nb^2 - \frac{b^2}{4} + \frac{b^2}{4N}\left(1 + \frac{1}{2N}\left(e^{-2N} - 1\right)\right)}$$

The entanglement threshold concentration is defined as the concentration where the entire volume of the solution is occupied by the reporters.

$$C^* = \frac{3}{4\pi R_g^3 N_A}$$

where N_A is Avogadro's number. Above this concentration it is assumed that the translational diffusion of the reporters is severely restricted. The entanglement threshold concentration varies with the reporter structure. As the reporter length increases, the entanglement threshold decreases (as $1/L^{1.5}$). From the equations above, the theoretical entanglement threshold for reporter probes with different spot sizes and different lengths can be calculated. The result of such calculations is shown in Figure 17, which shows that for a 7200 bp RNA/DNA hybrid reporter probe with 8 label attachment regions of about 900 bp each, the entanglement threshold is about 70 nM.

If both the target and the ghost probe are much smaller than the reporters, then they will most likely be free to diffuse through the solution even at these high concentrations of reporters. Initial data indicates that hybridization kinetics do not slow appreciably up to a concentration of 80 nM with a 7200-bp reporter probe, a 100-base target, and a 100-base ghost probe.

12.6 EFFECT OF ENTANGLEMENT THRESHOLD ON MULTIPLEXING

Assuming that the maximum concentration for reporters in a hybridization reaction is C^*, then the concentration of each reporter (specific to a particular target) is equal to C^*/M, where M is the multiplex of the reaction (number of different targets being addressed simultaneously). Conversely, the possible multiplex level for a particular reporter structure can be calculated from the lower limit of probe concentration (C_p from kinetics ~ 10 nM) and the entanglement threshold

$$M = \frac{C^*}{C_p}$$

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If the number of nanoreporter codes available does not depend on reporter probe size, then the multiplexing of the nanoreporter depends primarily on the reporter probe size and concentration (since it is much larger than the ghost probe). Because the ghost probe makes an insignificant contribution to entanglement during hybridiziation, it is the inventors' belief that the concentration of the ghost probe can be increased far above the concentration of the reporter probe. In Table 4 below, the maximum total ghost probe concentration ([G]) is set to 1000 nM for all reporter concentrations. This difference in concentration of ghost probe and reporter probe is an adjustable parameter. Preliminary experiments show that in a multiplex hybridization reaction with a 7200 bp reporter and 100b ghost, 40pM of each reporter probe and 200 pM of each ghost probe results in near complete hybridization in an overnight reaction.

12.7 OPTIMAL SIZE AND CONCENTRATION RANGES

Below in Table 4 is a summary of the optimal useful size and concentration ranges of the ghost probe and reporter probe at different multiplexing as approximated by the above theories. It is the inventors' belief that ghost probes up to about 200 bases will be practical for most applications.

Reporter Length (bp)	Ghost Length (b)	Minimum [R] (pM)	Minimum [G] (pM)	Maximum [R](nM)	Maximum [G](nM)	Max Multiplex
2000	100	5	5	603	1000	114417
2000	50	4	4	603	1000	161811
2000	200	7	7	603	1000	80905
3000	100	6	6	292	1000	45182
3000	50	5	5	292	1000	63897
3000	200	9	9	292	1000	31948
4000	100	7	7	178	1000	23912
4000	50	5	5	178	1000	33817
4000	200	11	11	178	1000	16908
5000	100	8	8	123	1000	14746
5000	50	6	6	123	1000	20854
5000	200	12	12	123	1000	10427
6000	100	9	9	91	1000	9988
6000	50	6	6	91	1000	14125
6000	200	13	13	91	1000	7062
7200	100	10	10	68	1000	6792
7200	50	7	10	68	1000	6792
7200	200	14	10	68	1000	6792
8000	100	11	11	57	1000	5444
8000	50	7	7	57	1000	7699

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8000	200	15	15	57	1000	3850
10000	100	12	12	40	1000	3419
10000	50	8	8	40	1000	4835
10000	200	17	17	40	1000	2417

Table 4: Optimal size and concentration ranges of reporter probe, ghost probe and target, as well as multiplicity of probes, in the nanoreporter systems of the invention.

13. EXAMPLE 8: EXEMPLARY EMBODIMENTS FOR DUAL NANOREPORTER ASSEMBLY

This section describes an embodiment for assembly of a dual nanoreporter in which one probe is a ghost probe and the other probe is a reporter probe comprising color RNA segments assembled on an M13 backbone. The ghost probe is attached to a biotinylated F-hook and the reporter probe is attached to a biotinylated G-hook. The dual nanoreporter is hybridized to a biomolecular sample to detect and quantify a target molecule. The steps below do not have to be performed in the order presented. Moreover, each particular step represents a specific embodiment that may be combined with embodiments other than those presented below.

13.1 PREPARATION OF THE M13 SCAFFOLD

Single-stranded circular M13mp18 DNA (USB Corporation) is annealed to a 5-fold molar excess of an oligonucleotide complementary to the Bam HI recognition site (Bam Cutter oligo) and cut with Bam HI restriction enzyme to yield a linear single-stranded DNA backbone. An oligonucleotide complementary to the Bam Cutter oligonucleotide (anti-Bam oligonucleotide) is subsequently added in 50-fold excess to sequester free Bam Cutter oligonucleotide and thus prevent recircularization of the M13 during later steps.

The linear M13 molecule serves as a scaffold onto which RNA patches, or RNA segments, with incorporated fluorophores can be annealed.

13.2 ATTACHMENT OF A TARGET-SPECIFIC SEQUENCE TO THE SCAFFOLD

An oligonucleotide comprising a sequence (of, *e.g.*, 30-70 nucleotides) complementary to the target nucleic acid of interest, plus 9 bp of additional sequence used for ligation to the M13 scaffold, is generated and ligated to the 3′ end of the linearized M13 scaffold.

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13.3 ATTACHMENT OF G-TAGS TO THE SCAFFOLD

A G-tag (*e.g.*, an oligonucleotide having the sequence 5′-AACATCACACAGACC AACATCACACAGACC AACATCACACAGACC AACATCACACAGACC AGCCCTTTG-3′, which includes 4 copies of the complement of the G-hook 5′-GGTCTGTGTGATGTT-3′, followed by 9 bases of ligator sequence, and which is complementary to the G-hook) is attached to the 5′ end of the linearized single-stranded M13 backbone to allow for (1) purification of the reporter following ligation and/or annealing of segments; and (2) immobilization of the reporter once it is "stretched" on a solid surface. The sequence of the ligator for attaching G-tag to the 5′ end of single-stranded M13 which has been linearized at the BamHI site can be 5′-CTCTAGAGGATCCAAAGGGCT-3′. The ligation reaction can be performed according to the following protocol to produce approximately 80 pmol of G-tag/M13 ligation product:

Materials:

[100uM] anti-G4 tag oligo
[100uM] anti-G4 tag ligator oligo
[80 nM] Linear single-stranded M13
10X T4 DNA Ligase Buffer (Fermentas)
T4 DNA Ligase (Fermentas)
20X SSC (Ambion)
DEPC H2O (Ambion)

Method:

1. Pre-anneal the G-tag and ligator:
   25uM 2:1 G/Glig in 1X SSC
   20 µl [100uM] G-tag Ligator
   40 µl [100uM] G-tag
   4 µl 20X SSC
   16 µl DEPC H2O
   * Anneal on the MJ Thermocycler
   95°C, 3min; 72°C, 30sec, -1°C/cycle, x 68 cycles; hold at 4°C
2. Ligate the G-tag to the linear M13:
   64nM M13-G4 in 1X Lig Buffer
   1000 µl [80 nM] Linear M13
   80 µl [25uM] 2:1 G/Glig in 1X SSC

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124 µl 10X T4 DNA Ligase Buffer
40 µl T4 DNA Ligase

* Ligate in an aluminum heat block covered with foil at 37°C for 2hr then at 65°C for 15 minutes to inactivate the enzyme.

13.4 PREPARATION OF RNA SEGMENTS

Ten sets of oligonucleotide primer pairs are designed to create 10 different regions along the M13 scaffold. Each pair contains one primer which has a T7 RNA polymerase promoter at the 5′ end. Regions 2-7 are designed to be 900 bases (approximately 300 nm) long, as this is the approximate size of a diffraction-limited spot (the smallest spot that can be achieved with standard optics). Regions 1 and 8 have both long and short versions: the long versions cover the whole 900-base region, while the short versions cover only a portion of the 900-base region to allow a target-specific sequence to be ligated. Thus a target-specific sequence can be attached to either end. The ends can also be used for attachment of anchors or tags.

PCR is performed using Taq polymerase and 0.5 ng of double-stranded M13mp18 (USB Corporation) as a template. Reactions are cleaned up using a Qiaquick purification kit from Qiagen. Each PCR reaction yields a double-stranded fragment corresponding to one specific segment as illustrated below. These fragments are used as templates for the in vitro transcription of the RNA segments.

Using the PCR products described above as double-stranded templates, RNA segments are generated using an in vitro transcription kit from Ambion (Megascript T7 kit). The products of the transcription reactions are purified (including treatment with DNAse I to remove template) using a RNeasy Kit from Qiagen.

13.5 LABELING OF THE RNA SEGMENTS

Using the PCR products described above as double-stranded templates, RNA segments for later dye-coupling are generated using an in vitro transcription kit from Ambion (MessageAmp aRNA kit). Aminoallyl-modified UTP nucleotides are incorporated into the RNA segments during transcription. The products of the transcription reactions are purified (including treatment with DNAse I to remove template) using a RNeasy Kit from Qiagen.

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20-100 μg of aminoallyl-modified RNA segment is coupled with NHS-ester dyes using Ambion Aminoallyl Labeling Kit. Dyes used include Alexa 488, Alexa 594 and Alexa 647 (Invitrogen/Molecular Probes) as well as Cy3 (Amersham).

Each segment is made separately in 4 colors so that each position on the scaffold can be filled with a segment in any of the four colors; thus different colors can be added at different positions to create many unique color combinations.

In this particular embodiment, adjacent segments are of different colors or there may be dark segments interspersed so that each segment is detected as an individual 'spot'. Dark segments may be used as part of the nanoreporter code.

13.6 ANNEALING OF THE RNA SEGMENTS TO THE SCAFFOLD

Segments for each position are annealed in a 2:1 ratio of segment to M13 scaffold in 1X SSPE buffer at 70°C for 2 hours. An assembled nanoreporter with labeled RNA segments is depicted in Figure 3A-3B. Figure 3A depicts a nanoreporter in which only alternate "spots" (1, 3, 5 and 7) are labeled, and Figure 3B depicts a nanoreporter in which every spot is labeled.

13.7 PREPARATION OF THE GHOST PROBE

One or more oligonucleotides comprising sequences (of, *e.g.*, 30-70 nucleotides) complementary to different regions of the target nucleic acid(s) of interest than those to which the target-specific sequences of the reporter probe are complementary, are generated. Optionally, F-tags for F-hook attachment are ligated to the 5′ end of the ghost probe using a a ligator oligonucleotide that is complementary to a short sequence on the 3' end of the F-hook as well as a short sequence on the 5′ end of the ghost probe. The sequences that are complementary to the ligator oligonucleotide are not part of the F-hook sequence or the probe sequence, but are additional nucleotides added to those oligos in order to facilitate ligation.

13.8 ATTACHMENT OF F-TAGS TO THE GHOST PROBE

An F-tag (*e.g.*, an oligonucleotide having the sequence 5′-GATGGAGAC GTCTATCATCACAGC GTCTATCATCACAGC-biotin -3′, which includes 2 copies of the complement of the F-hook 5′-GCTGTGATGATAGAC -3′, followed by 9 bases of ligator sequence and is complementary to the F-hook) is attached to the 3′ end of the ghost probe to allow for (1) purification of the ghost-probe-target-reporter hybridization complex; and

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(2) attachment of the hybridization complex on the slide via the biotin moiety. The sequence of the ligator for attaching F-tag to the 3′ end of the ghost probe can be 5′-GTCTCCATCTTCCGACAG-3′.

Materials:

100uM F-biotin tag

100uM F ghost probe ligator

Fermentas 10X T4 DNA Ligase Buffer

1uM ghost probes

Fermentas T4 DNA Ligase

Method:

1. Pre-anneal the hook and ligator:

   5uM F-biotin tag /ligator mix

   5 µl [100uM] F-biotin tag

   5 µl [100uM] F-ghost probe ligator

   10 µl 10X T4 DNA Ligase Buffer

   80 µl DEPC H_2O

Anneal on the MJ Thermocycler (95°C, 3min; 72°C, 30sec, -1°C/cycle x 68 cycles; hold at 4°C).

2. Set up the following ghost probe ligation:

   300 nM anti-F2-biotin -GP

   6.0 µl [1uM] Ghost Probe

   4.8 µl [5uM] anti-F2-biotin tag /ligator mix

   1.52 µl 10X T4 DNA Ligase Buffer

   3.68 µl DEPC H_2O

   4.0 µl T4 DNA Ligase

Ligate on the MJ Thermocycler (37°C, 18hr; 65°C, 15 minutes; hold at 4°C)

3. QC the ligation on a 15% Novex TBE-Urea gel:

Prepare the following loading solutions:

Ligation	Neg Control-Ghost Probe
3.33 µl [300 nM] ligation	1 µl [1uM] ghost probe
1.67 µl DEPC H_2O	0.33 µl 10X T4 DNA Ligase Buffer
5 µl 2X Loading Buffer	3.67 µl DEPC H_2O
	5 µl 2X Loading Buffer

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Neg Control- F-biotin tag /ligator mix

2 µl [0.5uM] F-biotin tag /ligator mix

0.33 µl 10X T4 DNA Ligase Buffer

2.67 µl DEPC H_2O

5 µl 2X Loading Buffer

50 bp Oligo Ladder

4 µl Ladder

6 µl 2X loading buffer

Run of a 15% Novex TBE-Urea gel at 180V for 50 minutes.

Stain with SYBR Gold for 30 minutes.

13.8.1 ALTERNATIVE EMBODIMENTS

Rather than covalently coupling biotin to the single-stranded F-tag, the biotinylation of the ghost probe can also be accomplished by annealing a biotinylated oligonucleotide (DNA or RNA) with a sequence complementary to the common portion of the ghost probe. Such a sequence could be the F sequence itself, or another sequence which is added to the ghost probe in addition to the F sequence. If such an additional sequence is added, it could be from 10-100 bases long, from 1-10 copies, with the preferred configuration being a single copy from 50-100 bases long.

13.9 BIOTINYLATION OF TARGET MRNA

There are a number of commercially available kits available for the direct labeling of an mRNA sample including *Label* IT® $\mu Array_{TM}^{Biotin}$ Mirus #MIR 8010) and Biotin-Chem-Link (Roche (1 812 149). Following manufacturer's procedures biotin labeled mRNA is added to the hybridization reaction as described in Section 3d (below) with the following modifications: Since most protocols suggest the use of poly A+ mRNA, the amount of RNA used could be reduced below the 100 ng total RNA in a typical hybridization to 10 ng and possibly 1ng. No ghost probe should be added to this reaction. F bead post-hybridization purification is no longer required. G-bead post-hybridization purification should be used to remove unhybridized biotinylated mRNA that might compete for binding to the slide. Depending on the amount of RNA used, this may or may not be required. Alternatively, total RNA could be biotinylated without the need for purification of the poly A+ fraction. In this case, the original amount of total RNA should be used (100 ng). The

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use of total RNA might require modifications of the manufacturer's protocol to increase labeling efficiency.

An alternative approach would be to enzymatically generate biotinylated 1st strand cDNA or biotinylated amplified RNA (aRNA) using commercially available kits and use these in place of total or mRNA. This approach would require a redesign of the reporter probes to be in the sense orientation. Both ghost probe and F-bead post-hybridization reactions would be omitted while G-bead purification would remain for removal of non-hybridized RNA.

13.10 HYBRIDIZATION OF DUAL NANOREPORTER TO TARGET

Many hybridization conditions are sufficient for achieving gene expression data. To shorten hybridization times while maintaining reasonable hybridization efficiency, several parameters can be altered: i) increasing ghost probe and reporter concentrations, ii) fragmenting of total RNA to average size range of 200-500 bp while lowering the pH of hybridization to 6.5, iii) using more total RNA in same hybridization volume, iv) lowering hybridization volume to approximately 10 µl. Blocking reagents such as Denhardt's and ssDNA can be removed without deleterious effects on hybridization efficiency or cross hybridization to mRNAs from different species.

The following protocol has been performed successfully with multiplexing from 1 to >500 nanoreporters with ghost probes (an example demonstrating a nanoreporter assay utilizing 25 nanoreporters is described in Example 6 above, and another example demonstrating a nanoreporter assay utilizing 509 nanoreporters is described in Example 9 below). The final concentration of all nanoreporters varies depending on 1) the concentration of each reporter and 2) the number of genes being multiplexed.

Typical total nanoreporter concentrations range from 40pM (1 gene @ 40pM) to 20nM (500 genes @40pM). Ghost probe concentrations also vary from 200pM (1 gene @ 200pM) to 100nM (500 genes @ 200pM). The example that follows describes a single multiplexed hybridization containing approximately 500 endogenous genes with positive and negative controls. Add, 11.1 µl of 2.7X hybridization mix [13.5X SSPE pH 7.5 (USB #75890), 0.27 µg/ µl sheared salmon sperm DNA (Sigma #D-7656), 0.27% tween 20 (Sigma #P-1379), and 13.5X Denhardt's reagent (Sigma D-2532)], 5 µl ofgene Nanoreporter mix (0.24nM each or 123nM total, includes 509 endogenous genes and 8 hybridization controls), 4.6 µl 513 gene ghost probe mix (1.3nM each or 667 nM total, includes 509

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endogenous genes and 8 hybridization controls), 1 µl of purification control reporter mix (0.5pM), 1 µl of total cellular RNA (100ng/ul), 1 µl of 30X spike target mix (1.5nM-3fM) and 6.3 µl of DEPC treated water (Ambion #9922) to a 0.2ml thin wall tube (final volume 30 µl).

Final concentration of hybridization reagents should be 5X SSPE, 0.1% tween 20, 100ng/µl sheared salmon sperm DNA, 5X Denhardt's reagent, 40 pM each Nanoreporter (~20nM total), 200pM each ghost probe (~100nM total) and 33ng/µl of total cellular RNA. Control spike targets typically vary in range from 50 fM down to 0.1 fM in a single reaction. All reagents are most preferably free of all nuclease activity. For optimal results, all reagents should be free of nuclease activity.

Mix reagents well and incubate in temperature block with heated lid for 20 hours. After hybridization purify the nanoreporters with affinity reagents for both the ghost probe and the reporter probe.

13.10.1 ALTERNATIVE EMBODIMENT: HYBRIDIZATION PROTOCOL FOR WITHOUT SSDNA AND DENHARDT'S REAGENT

This protocol has been performed successfully with multiplexing from 1-500 nanoreporters and ghost probes. Removal of ssDNA and Denhardt's reagent from hybridizations performed with human reagents (Nanoreporters and ghost probes) had no effect on cross hybridization with mouse total RNA when compared to a hybridization containing ssDNA and Denhardt's. In addition, removal of ssDNA and Denhardt's does not result in an increased background signal (based on negative hybridization controls). Finally, there is no significant loss (or gain) of signal for endogenous genes hybridized in the presence or absence of ssDNA and Denhardt's (509 genes, R^2 value=0.998).

13.10.2 ALTERNATIVE EMBODIMENT: HYBRIDIZATION CONDITIONS FOR FRAGMENTED CELLULAR MRNA

Fragmentation of cellular RNA has been achieved by both thermal and cation catalyzed protocols. These protocols were designed to obtain fragment lengths between 100 and 700 bp (on average). Thermal fragmentation: Dilute total RNA sample to 200 ng/µl in RNAse free water. Heat sample to 95°C in temperature block with heated lid. Stop fragmentation by placing sample on ice. Use immediately or store at -80°C until use. Fragmentation via cation catalyzed reaction modified from manufacturer's protocol (Ambion). Bring volume of RNA sample up to 9 µl with RNAse free water. Final

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concentration of total RNA should be between 0.2 and 2 μg/ml. Add 1 μl of 10X fragmentation buffer (Ambion10X fragmentation buffer). Incubate at 70°C for 5 minutes in temperature block. Longer times will result in smaller fragment size on average. Stop reaction by addition of 1 μl 200 mM EDTA. Use immediately or store at -80°C until use.

Fragmented RNA samples are hybridized as described herein except for the following modifications: i) pH of SSPE is reduced to 6.5 and ii) the time of reaction is reduced to 6hours (for hybridization reactions in which reporter probe and ghost probe concentrations are 200 pM).

13.11 PURIFICATION OF NANOREPORTER-TARGET COMPLEXES

Post-hybridization purification is preferred when the total reporter probe concentration is above 1 nM. Purification significantly decreases non-specific binding and increases specific binding efficiency to the slide at higher reporter and ghost probe concentrations. In the example provided above, a single F-bead purification is described (purifies hybridized complexes from the ghost-probe end). As described in Example 9 below, optimal results at high ghost probe concentrations (>5nM total) are obtained via a subsequent G-bead purification which purifies the hybridization complexes from the 5'end of the reporter effectively removing excess non-hybridized ghost probes. The preferred order of purification is F-bead, then G-bead but the order can be reversed and the protocols optimized accordingly. The exact sequences used in these affinity purifications can likely be changed and optimized in alternative embodimentsof the technology. These affinity purification steps and reagents are currently nucleic acid based but could theoretically be any sort of binding pairs that exhibit specific binding to one another and can be released by chemical treatment or alteration of binding conditions such that the interaction is disrupted and released. For example, an antibody/antigen pair, a protein/metal interaction, or ligand/receptor interaction, *etc.*

One example of purification is provided below.

After hybridization is complete, the salt of a hybridization sample (30 μl, starting at 5x SSPE = 825mM Na^+) is adjusted to a final concentration of approximately 1x SSPE. The diluted sample is added to 30 μl F-hook MyOne Dynabeads (F-MODB) and bound for 15 minutes at room temperature while rotating. The beads are sequestered with a magnet and the supernatant removed. The beads are washed twice with 150 μl 0.1xSSPE + 0.1% Tween at room temperature for 15 minutes w/ rotation and discarded. The purified reporters

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are eluted in 30 µl 0.1x SSPE at 45 °C for 15 minutes w/ rotation. At this point the hybridized reporters are purified from the contaminating un-hybridized reporters. The elution still contains contaminating un-hybridized ghost probes which will compete with the reporters for biotin-binding sites on the streptavidin coated slide. The 30 µl is added to 130 µl of 1xSSPE + 0.1% Tween to increase salt concentration. The sample (150 µl) is then loaded onto 30 µl of G-MODB and bound for 15 min at room temperature. The supernatant is discarded and the beads washed with 150 µl 0.1xSSPE + 0.1% Tween at room temperature for 15 minutes w/ rotation. The wash is discarded and the fully purified reporters eluted with 25 µl 0.1x SSPE at 45 °C for 15 minutes w/ rotation. At this point only targets molecules that are hybridized to both a ghost probe (containing the anti-F sequence) and a reporter (containing the anti-G sequence) will remain in solution.

13.12 IMMOBILIZATION AND STRETCHING AND IMAGING OF NANOREPORTER-TARGET COMPLEXES

Attachment to the slide and immobilization of the stretched complex may be achieved via a biotin-streptavidin interaction. In alternative embodiments, immobilization and stretching are achieved with other interaction pairs provided one of the two could be immobilized on the slide and the other attached to either the ghost probe or the reporter. Stretching does not have to be achieved via electrophoresis but can be done mechanically. The addition of bis-tris propane to the sample before binding is not required. The technology is not limited to the use of particular label monomers exemplified herein as long as the different label monomers can be separated by image processing.

One example of an immobilization and stretching protocol is provided below.

After purification, the hybridization products are loaded directly into an open well of a microfluidic device. The liquid is pulled into a microfluidic channel by capillary action where the hybridized molecules bind to the streptavidin-coated slide through the biotinylated ghost probe. The microfluidic device then intermittently tilts along the axis perpendicular to the length of the channels in alternating directions in order to force the reaction mixture to repeatedly pass through the channel and increase the binding efficiency.

After binding the hybridization reaction, the channel is washed with 1x TAE for 5 minutes by tilting the device at an angle. Fresh TAE is then added to each well to a level sufficient to contact platinum electrodes which are inserted in the wells (30 microliters in our current geometry). An electrical potential of 200V is then applied between the two wells connected by the microfluidic channel, stretching the reporters. After one minute of

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pre-electrophoresis to remove any remaining contaminating un-bound reporter molecules in the channel, a solution of 0.5 μM G-hooks in 1x TAE is added to the cathodic well (60 microliters of this solution). The electrical potential draws the G-hooks through the channel toward the anodic well. As they pass through the channel, the hooks hybridize with the free G-tag sequences on the free-end of the reporters which are bound to the surface and stretched. The streptavidin on the surface then binds the biotin on the G-hook and immobilizes the free end. When the potential is removed, the reporters remain stretched for imaging.

14. EXAMPLE 9: HYBRIDIZATION OF 509 CELLULAR GENES TO 100NG TOTAL RNA FROM A549 CELLS USING NANOSTRING REPORTER SYSTEM.

14.1 HYBRIDIZATION REACTION

Detection of 509 endogenous cellular genes was carried out in single multiplexed hybridization reaction. Eight non-human control sequences were spiked into each reaction that corresponded to approximately 0.1, 0.5, 1, 5, 10, 50, and 100 copies per cell as well as two reporters with no target (negative controls). There were also 4 reporters added that served as positive (3) and negative (1) controls for the post-hybridization purification process. A set of negative control hybridization was also performed containing the entire Nanostring reporter library but lacking cellular RNA.

Each sample was hybridized in triplicate. Final concentrations of the hybridization reagents were as follows: 20.8 nM total Nanoreporters (521 individual Nanoreporters at 40pM each), 103 nM total ghost probe (517 individual ghost probes @ 200pM each), 5X SSPE (pH 7.5), 5X Denhardt's reagent, 100ng/ul sheared salmon sperm DNA, 0.1% tween 20, 50fM S11 spike target DNA, 10fM S10 spike target DNA, 5fM S9 spike target DNA, 1fM S8 spike target DNA, 0.5 fM S7 spike target DNA, 0.1fM S6 spike target DNA. S3 and S4 were added as negative controls. RNA was obtained from A549 lung epithelial cells under two different conditions. The final concentration of total RNA per hybridization was 33ng/ul. No total RNA was added to the negative control hybridizations. The final volume of the reaction was 30ul. Reagents were mixed and incubated at 65°C in thermocycler block with heated lid for 20 hours.

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Master mix	(1 Reaction)	(9.3 reactions)
2.7 X hybridization mix*	11.1 µl	103.2 µl
513 endogenous gene reporters (0.24nM each)	5 µl	46.5 µl
513 endogenous gene ghost probes (1.3nM each)	4.6 µl	42.9 µl
Purification Control reporters (0.6nM each)	1 µl	9.3 µl
30X control target mix	1 µl	9.3 µl
Total	22.7 µl	211.2 µl

*Hybridization mix (13.5X SSPE, 13.5X Denhardt's reagent, 270 ng salmon sperm DNA, 0.27% tween 20)

Reactions	1	2	3	4	5	6	7	8	9
Master mix	22.7	22.7	22.7	22.7	22.7	22.7	22.7	22.7	22.7
48.5ng/µl RNA #1	2.1	2.1	2.1	0	0	0	0	0	0
48.4ng/µl RNA #2	0	0	0	2.1	2.1	2.1	0	0	0
H_20	5.2	5.2	5.2	5.2	5.2	5.2	7.3	7.3	7.3
Total Rxn volume	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl

Incubate reactions in thermocycler with heated lid overnight (20hours).

14.2 POST-HYBRIDIZATION PURIFICATION

Hybridization reactions were purified to remove unhybridized reporters using an oligonucleotide complimentary to ghost probe attached to magnetic beads (F-bead). Hybridization reactions were diluted 5 fold in 0.1% Tween-20/TE to bring the final salt concentration to 1X SSPE. The diluted hybridization solution was then added to 100ul of F-beads (in 0.1% Tween-20) and allowed to bind to the beads at room temperature for 30min with continuous rotation. The beads were then washed three times in 150ul of 0.1X SSPE/0.1% Tween-20 and eluted in 100ul of 0.1X SSPE/0.1% Tween-20 for 15min at 45°C.

After F-bead elution, samples were purified from the opposite end of the hybridized complex using G-beads. Elutions were brought to a final concentration of 1X SSPE by the addition of 50ul of 3X SSPE/0.1% Tween-20 and bound to 30ul of G-beads (in 0.1% Tween-20) for 15min at room temperature with rotation. Beads were then washed as above and eluted in 30ul of 0.1X SSPE/Tween-20 and prepared for binding as described below.

14.3 BINDING, STRETCHING, AND IMMOBILIZATION

The samples was prepared for binding by addition of 1ul of 1/5000 dilution of 0.1 uM Tetraspec™ fluorescent microspheres (product # T7279, Molecular Probes). Samples were loaded into a Nanostring fluidic device and attached to Accerl8 Optichem® slide

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coated with strepavidin (product #TB0200) by tilting the device 45deg for 15min and repeated a total of 4 times. After loading, slide surface was washed once with 90ul of 1X TAE. After wash buffer is removed the sample is prepared for electrostretching by addition of 40ul of TAE to each well. Attached complexes were stretched by applying 200V across the fluidic channel. After 1 minute the samples were immobilized in the stretched position by adding 60ul of 500nM of G-hook oligo solution to the well containing the negatively charged electrode while continuing to apply voltage for 5 minutes. After immobilization the TAE solution is removed and replaced with anti-photobleaching reagent for imaging.

14.4 IMAGING

Slides were imaged on Nikon Eclipse TE2000E equipped with a metal halide light source (X-cite 120, Exfo Corporation) and a 60X oil immersion lens (1.4 NA Plan Apo VC, Nikon). For each field of view, 4 images at different excitation wavelengths (480, 545, 580 and 622) were acquired with an Orca Ag CCD camera (Hamamatsu) under control of either Metamorph (Universal Imaging Corporation) or custom software. Images were processed with custom image processing software.

14.5 DATA ANALYSIS

Raw data was extracted from processed images using custom software. Data was normalized to the average counts for control spikes in each sample. To determine if a gene was "detected" by the system, the counts obtained for each gene from hybridizations containing RNA were compared to average counts of the two negative controls using a Student's ttest. The number of genes detected was 441 (87%) and 445 (88%) in sample #1 and #2, respectively.

A scatter plot (Figure shows normalized and average $\log_2$ signal values from each positive sample (n=3) for all 509 genes. The genes that were significantly different in the two samples were identified by a T-test of signal values in sample #2 against sample #1. In the graph below, the solid lines indicate the 2 fold upregulated threshold (black line) and 2-fold downregulated threshold (gray line) relative to sample #1. Genes with significant fold changes (p-value < 0.05) are shown in solid black diamonds. Genes whose fold change p-values were above this threshold are shown in open black squares.

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15. EXAMPLE 10: DETECTION OF SMALL SPOTS

As mentioned above, the label attachment regions of a nanoreporter scaffold region have a length anywhere from 10 nm to 10,000 nm, but preferably corresponds closely to the smallest spot that can be detected with standard optics, which is about 300 nm. Spots of different color (spectrally distinguishable) are spatially resolvable at closer spacing than spots of the same color. It is possible to fit one, two, three or four spots of different colors between two spots of the same color, and yet spectrally and spatially resolve all the spots. It is also possible to significantly reduce the distance between two spots of the same color.

The limits of spatial resolution, *i.e.*, differentiating closely spaced spots of the same color, are often thought of as hard limits, *i.e.*, the Rayleigh Criteria (Inoue, S., Spring,. Video Microscopy (Plenum Press, 1997), p 30.) There are many techniques to drive beyond these limits that involve different imaging and/or image processing techniques. On the imaging side, structured illumination is one method to resolve spots of the same color that are spaced closer together. 50 nm has been demonstrated but, in theory, resolution with structured illumination is unlimited (Gustafsson, 2005, Proc. Nat'l. Acad. Sci. U.S.A. 102:13081-13086). On the image processing side, mixture modeling is an effective technique to push beyond commonly accepted limits (Thomann *et al.*, 2002, J. Microsc. 211:230-248). The combination of these techniques allows for drastically smaller nanoreporters with smaller spots, corresponding to label attachment regions of less than 50 nm.

These smaller spot spacings could allow for drastically shorter and more stable reporters, a larger number of codes, as well as a higher degree of multiplexing before the entanglement threshold is passed (for an explanation of entanglement thresholds, see Example 9 (described in Section 14) above.

The tradeoff of making the spots much smaller and the reporters much shorter would be decreased signal and slower scan times. However, other technical advances, such as brighter light sources, and more efficient CCDs may offset the increased scan times making these approaches reasonable.

16. REFERENCES CITED

All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent

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application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

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WHAT IS CLAIMED IS:

1. A probe pair comprising a first probe and a second probe,
said first probe being a complex comprising:

(a) a first molecule, comprising: (i) a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal; and

(b) a first target-specific sequence attached to the first molecule,

said second probe being a second molecule, comprising (i) a second target-specific sequence; (ii) optionally, a third label attachment region to which are attached one or more label monomers that emit light constituting a third signal; and (iii) optionally, an affinity tag attached to said second molecule;

wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule,

wherein the target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA,

and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule.

2. A probe pair comprising a first probe and a second probe,
said first probe being a complex comprising:

(a) a first molecule, comprising: (i) a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which are attached one or more label monomers that emit light constituting a second signal; and

(b) a first target-specific sequence attached to the first molecule,

said second probe being a second molecule, comprising (i) a second target-specific sequence; (ii) optionally, a third label attachment region to which are attached one or more

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label monomers that emit light constituting a third signal; and (iii) optionally, an affinity tag attached to said second molecule;

wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule,

wherein the first signal and the second signal are spatially or spectrally distinguishable;

wherein the target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA,

and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule.

3. The probe pair of claim 1 or 2, wherein said first and second label attachment regions are predetermined nucleotide sequences.

4. The probe pair of claim 3, wherein a first DNA molecule is hybridized to the first label attachment region, to which first DNA molecule are bound said one or more label monomers that emit light constituting said first signal; and wherein a second DNA molecule is hybridized to the second label attachment region, to which second DNA molecule are bound said one or more label monomers that emit light constituting said second signal.

5. The probe pair of claim 3, wherein a first RNA molecule is hybridized to the first label attachment region, to which first RNA molecule are bound said one or more label monomers that emit light constituting said first signal; and wherein a second RNA molecule is hybridized to the second label attachment region, to which second RNA molecule are bound said one or more label monomers that emit light constituting said second signal.

6. The probe pair of claim 3, wherein a plurality of first DNA molecules are hybridized to the first label attachment region, to which DNA molecules are bound said one or more label monomers that emit light constituting said first signal; and wherein a plurality of second DNA molecules are hybridized to the second label attachment region, to which second DNA molecules are bound said one or more label monomers that emit light constituting said second signal.

7. The probe pair of claim 3, wherein a plurality of first RNA molecules are hybridized to the first label attachment region, to which RNA molecules are bound said one

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or more label monomers that emit light constituting said first signal; and wherein a plurality of second RNA molecules are hybridized to the second label attachment region, to which second RNA molecules are bound said one or more label monomers that emit light constituting said second signal.

8. The probe pair of any one of claims 1-7, wherein the first probe further comprises an affinity tag.

9. The probe pair of claim 1-7, wherein the second probe comprises an affinity tag.

10. The probe pair of claim 1-7 wherein the second probe comprises a third label attachment region to which are attached one or more label monomers that emit light constituting a third signal.

11. The probe pair of claim 10, wherein said third label attachment region is a predetermined nucleotide sequence, and wherein a third RNA molecule is hybridized to the third label attachment region, to which third RNA molecule are attached said one or more label monomers that emit light constituting said third signal.

12. The probe pair of claim 11, wherein when the probe pair is bound to its target molecule, the code comprises the identity of the first signal, second signal and third signal and their locations relative to each other.

13. The probe pair of claim 1 or 2, wherein the code comprises the identity of the first and second signals, their locations relative to each other, and the size of the spot resulting from at least one of said signals.

14. The probe pair of claim 9, wherein the first and second molecules are nucleic acid molecules.

15. The probe pair of claim 14, wherein the label attachment regions and target-specific sequences are predetermined nucleotide sequences.

16. The probe pair of claim 15 in which said one or more label monomers are covalently bound to nucleic acids hybridized to their respective attachment regions.

17. The probe pair of claim 16, wherein said nucleic acids are hybridized to the

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respective attachment regions by way of one or more non-covalently bound bridging nucleic acids.

18. The probe pair of claim 1 or 2 in which the first and second target-specific sequences are unlabeled with any one or more of said label monomers.

19. The probe pair of claim 9, wherein the second molecule further comprises a fourth label attachment region, which is non-overlapping with the third label attachment region, to which is attached one or more label monomers that emit light constituting a fourth signal.

20. The probe pair of claim 19, wherein when the probe pair is bound to its target molecule, the code comprises the identity of the first signal, second signal, third signal and fourth signal and their locations relative to each other.

21. The probe pair of claim 8, wherein the second probe further comprises an affinity tag.

22. The probe pair of claim 8, wherein a first RNA molecule is hybridized to the first label attachment region, to which first RNA molecule are bound said one or more label monomers that emit light constituting said first signal; and wherein a second RNA molecule is hybridized to the second label attachment region, to which second RNA molecule are bound said one or more label monomers that emit light constituting said second signal and wherein the first probe and/or the second probe further comprises an affinity tag.

23. The probe pair of claim 8, wherein the affinity tag is covalently attached to the first molecule or to the second molecule.

24. The probe pair of claim 1, wherein the label monomers attached to the first label attachment region emit light at the same wavelength, which light constitutes said first signal, and wherein the label monomers attached to the second label attachment region emit light at the same wavelength, which light constitutes the second signal.

25. The probe pair of claim 1, wherein at least one of the first signal and the second signal comprises light at a plurality of different wavelengths.

26. The probe pair of claim 1, wherein first and second signals are spectrally distinguishable.

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27. The probe pair of claim 5, wherein the first and second RNA molecules are each 100 to 3,000 nucleotides in length.

28. The probe pair of claim 5, wherein the first and second RNA molecules are each 500 to 1,500 nucleotides in length.

29. The probe pair of claim 5, wherein the second probe is a nucleic acid complex comprising:

(a) the second molecule, wherein the second molecule comprises a third label attachment region to which is hybridized a third RNA molecule, to which third RNA molecule are bound one or more label monomers that emit light constituting a third signal; and

(b) the second target-specific sequence attached to the second molecule,

wherein the code comprises the identity of the first, second and third signals and their locations relative to each other.

30. The probe pair of claim 29, wherein the label monomers attached to the first label attachment regions emit light at the same wavelength, which light constitutes said first signal; wherein the label monomers attached to the second label attachment region emit light at the same wavelength, which light constitutes the second signal; and wherein the label monomers attached to the third label attachment region emit light at the same wavelength, which light constitutes the third signal.

31. The probe pair of claim 29, wherein at least one of the first signal, the second signal and the third signal comprises light at a plurality of different wavelengths.

32. The probe pair of claim 29, in which the first target-specific sequence and the second target-specific sequence are unlabeled with any one or more of said label monomers.

33. The probe pair of claim 29, wherein first, second and third signals are spectrally distinguishable.

34. The probe pair of claim 29, wherein first and third signals emit at the same wavelength or wavelengths.

35. The probe pair of claim 29, wherein the first probe further comprises an affinity tag.

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36. The probe pair of claim 29, wherein the second probe further comprises an affinity tag.

37. The probe pair of claim 35, wherein the second probe further comprises an affinity tag.

38. The probe pair of claim 35, wherein the affinity tag is covalently attached to the first molecule.

39. The probe pair of claim 36, wherein the affinity tag is covalently attached to the second molecule.

40. The probe pair of claim 29, wherein the first, second and third RNA molecules are each 100 to 3,000 nucleotides.

41. The probe pair of claim 29, wherein the first, second and third RNA molecules are each 500 to 1,500 nucleotides.

42. A method of detecting a target molecule in a biomolecular sample comprising: (i) contacting said sample with a probe pair according to any one of claims 1-41 under conditions that allow binding of the first target-specific sequence and the second target-specific sequence to the target molecule and (ii) detecting the code that identifies the target molecule.

43. The method of claim 42, further comprising quantitating the amount of said target molecule in said biomolecular sample.

44. The method of claim 42 or 43, wherein the concentration of each of said first and second target-specific sequences is in excess of the concentration of said target molecule.

45. A method of detecting a plurality of target molecules in a biomolecular sample comprising: (i) contacting said sample with a population of probe pairs according to any one of claims 1-41, under conditions that allow binding of the first target-specific sequence and the second target-specific sequence of each probe pair to their respective target molecule, wherein each probe pair in said population when bound to its respective target molecule is associated with a distinguishable code, and (ii) detecting the codes that identify the plurality of target molecule6.

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46. The method of claim 45, further comprising quantitating the amount of each of said plurality of target molecules in said biomolecular sample.

47. The method of claim 45 or 46, wherein the concentration of each of said first and second target-specific sequences of each probe pair of said population is in excess of the concentration of its respective target molecule.

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FIGURE 1A-1C

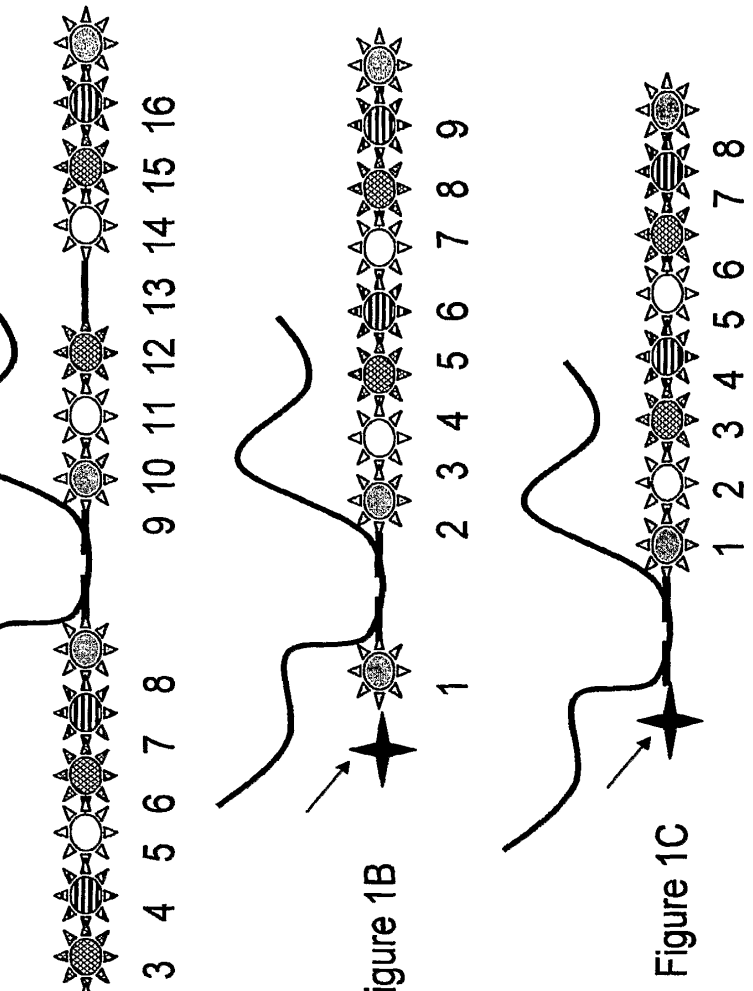

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Figure 1A
1 2 3 4 5 6 7 8 9
Figure 1B
1 2 3 4 5 6 7 8
Figure 1C

FIGURE 1D-1F

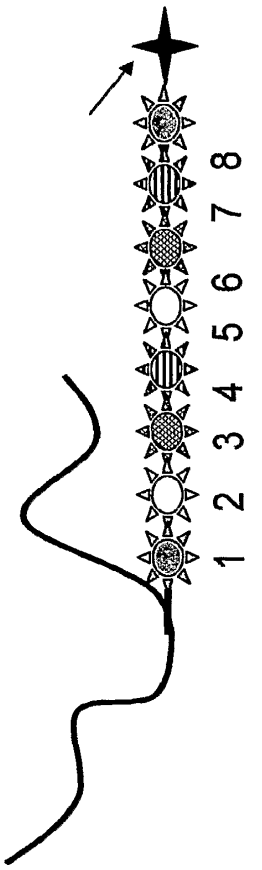


Figure 1D

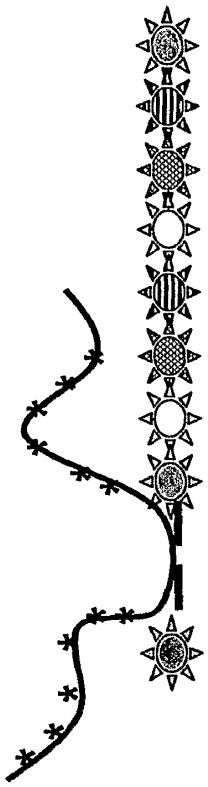


Figure 1E

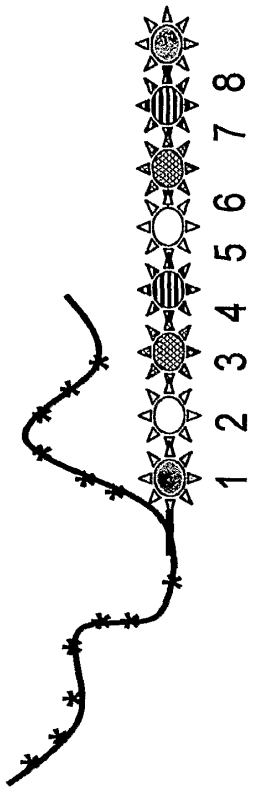


Figure 1F

FIGURE 2

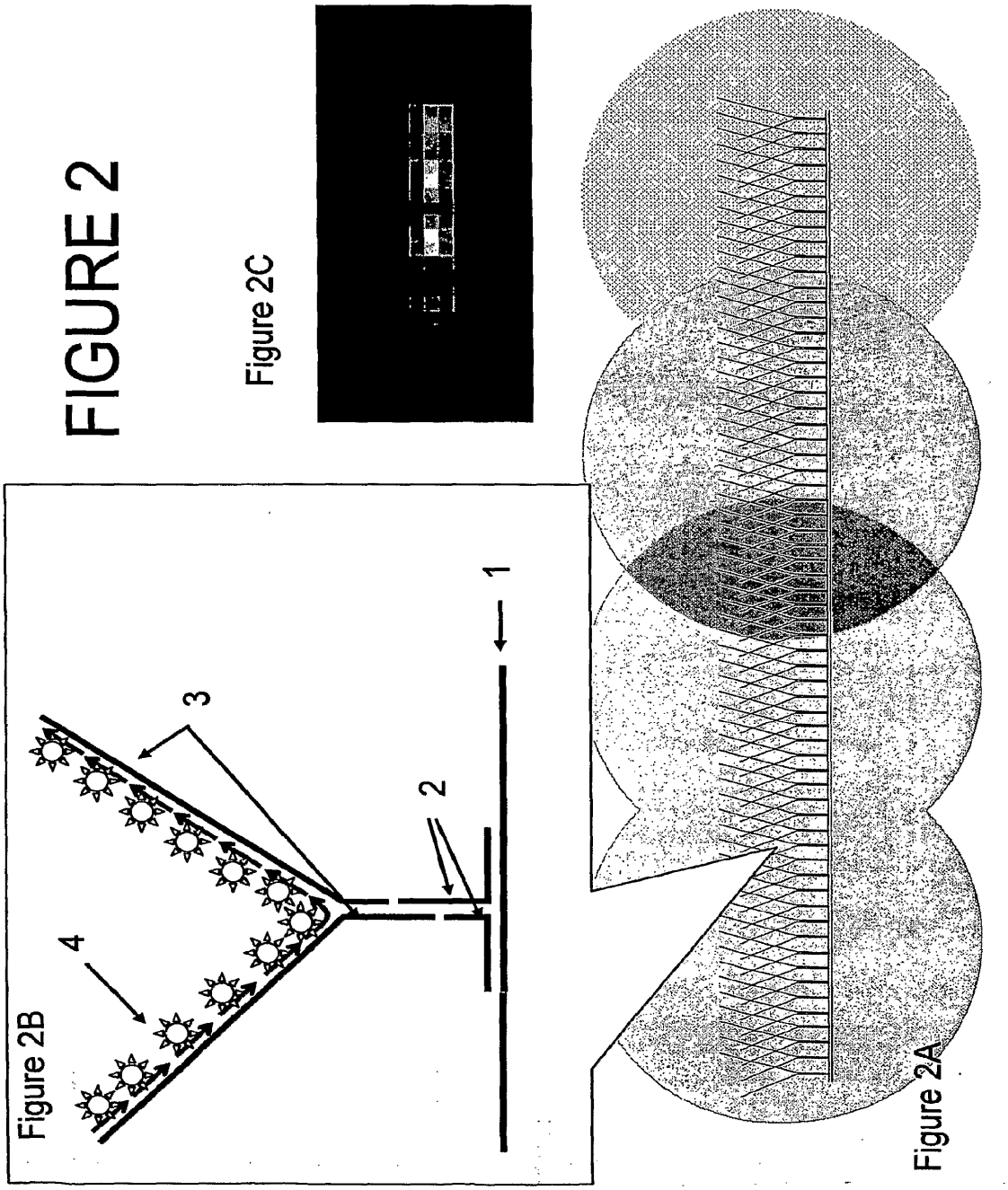


FIGURE 3

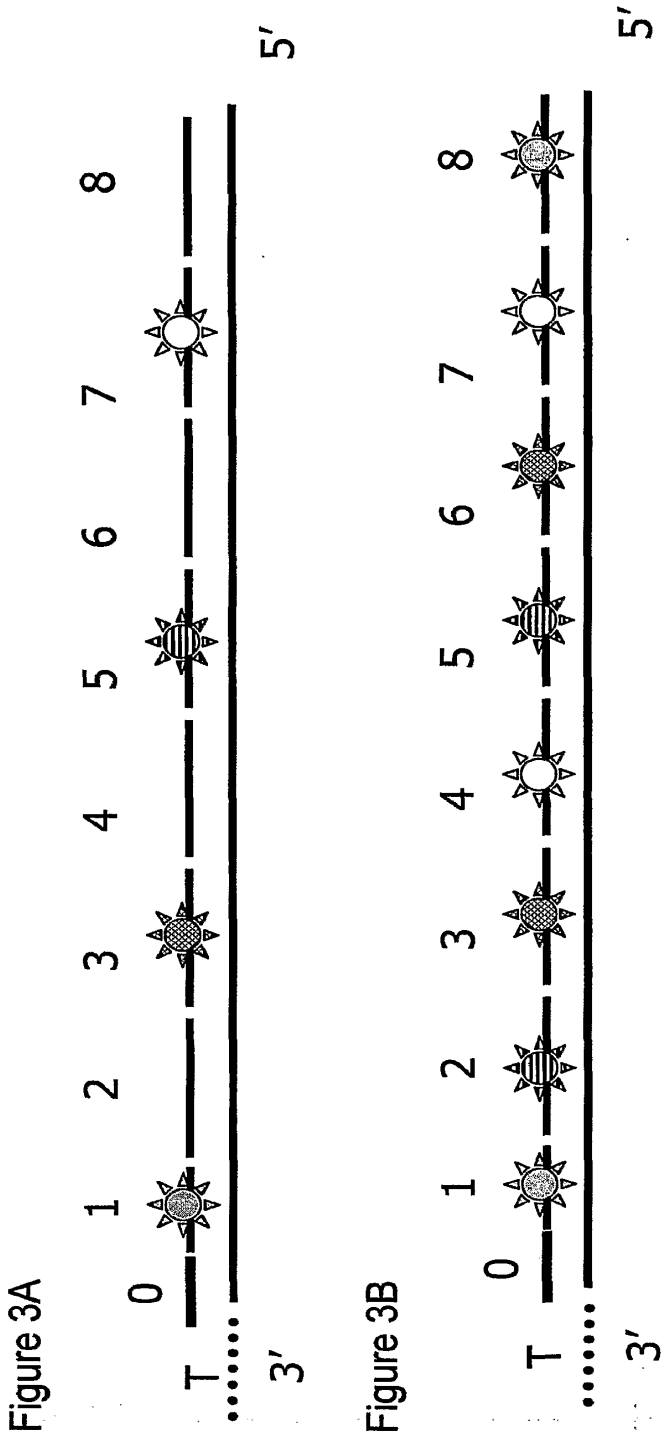
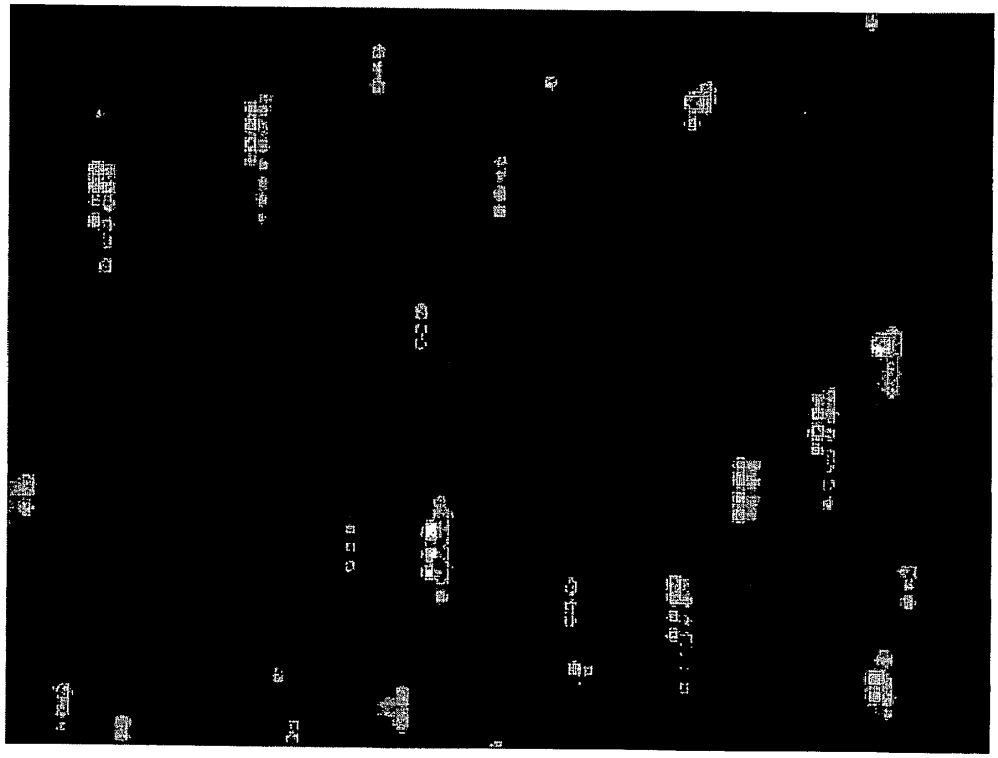


FIGURE 4



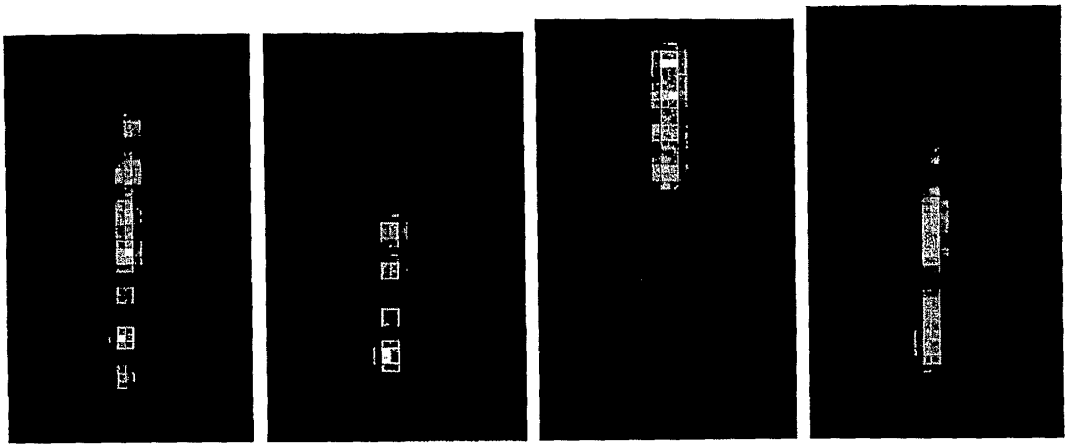


FIGURE 5

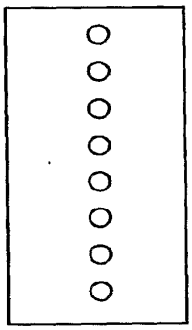


Figure 5A

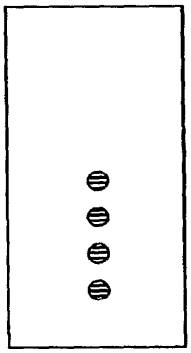


Figure 5B

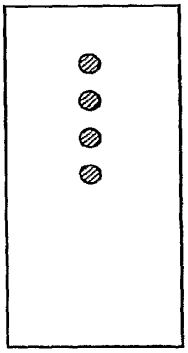


Figure 5C

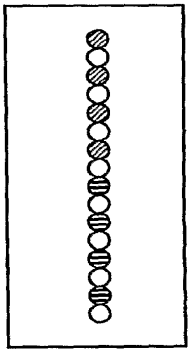


Figure 5D

FIGURE 6

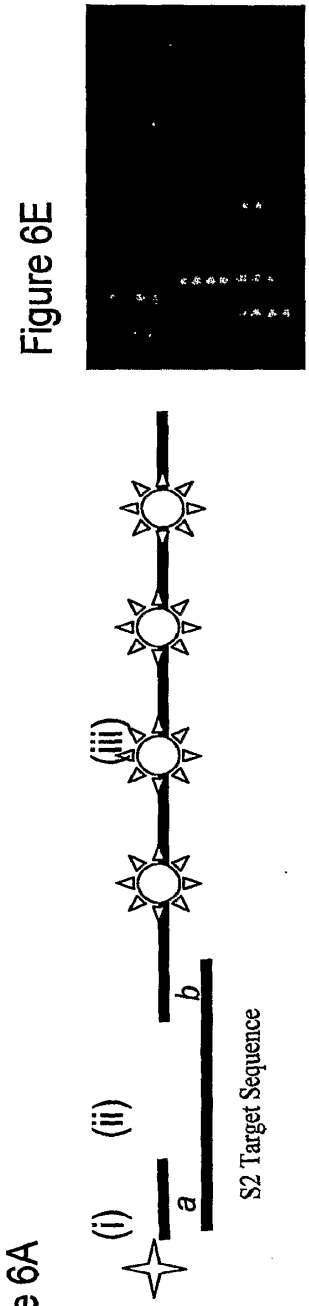


Figure 6A

Figure 6E

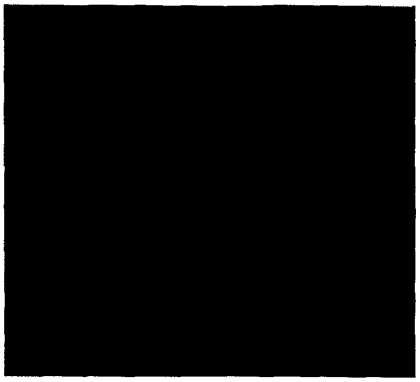


Figure 6D

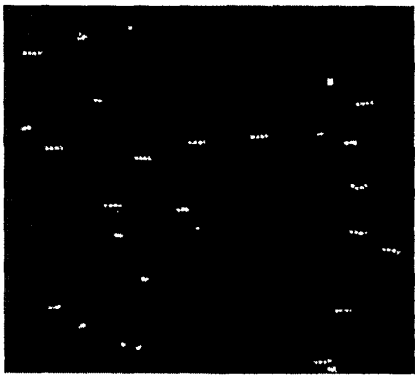


Figure 6C

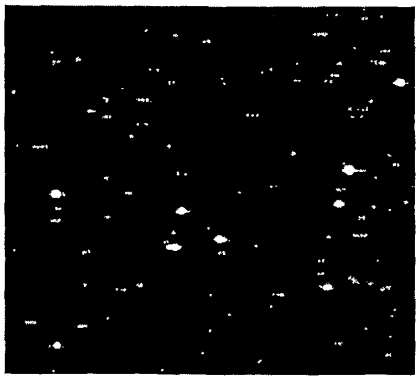


Figure 6B

FIGURE 7A-D

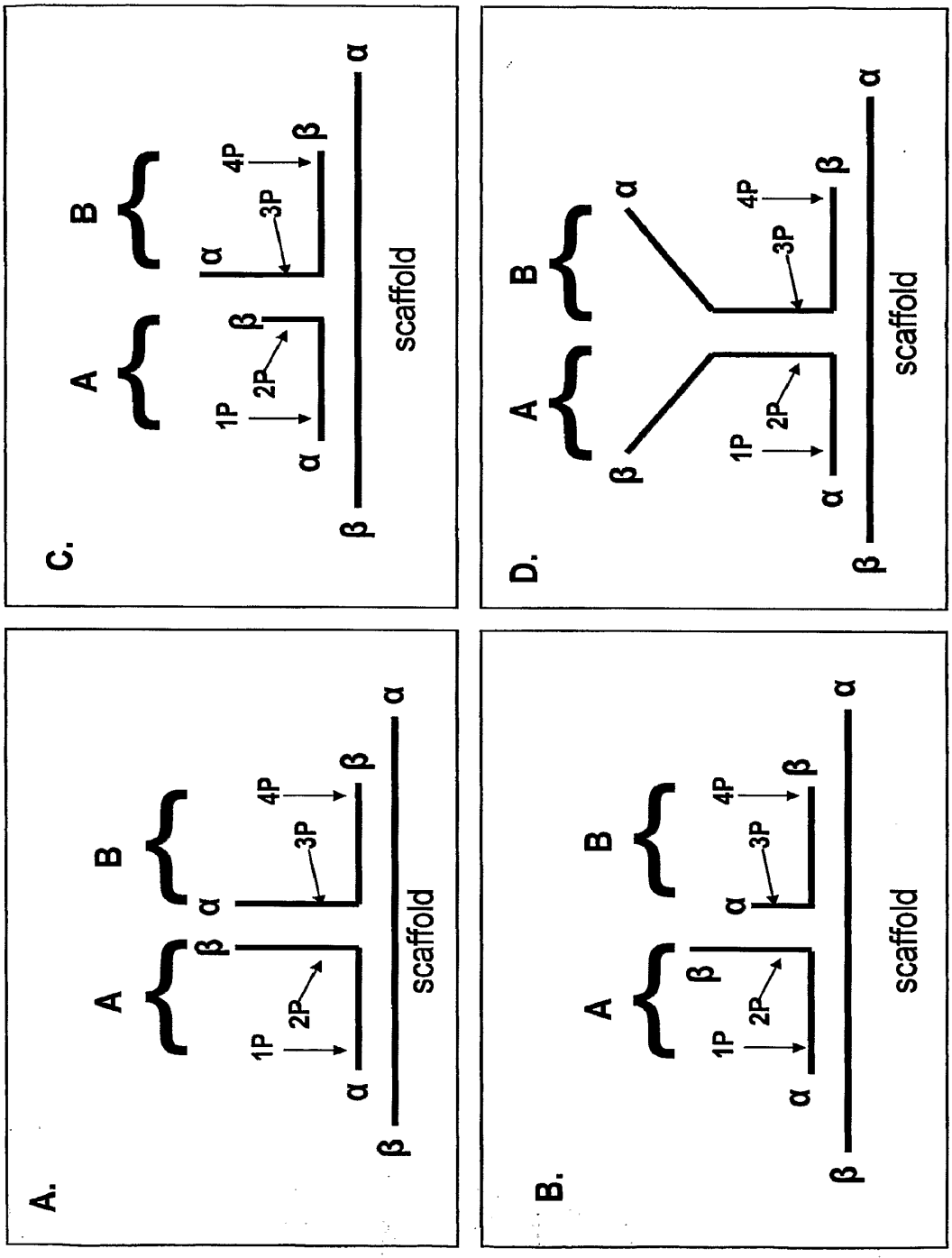
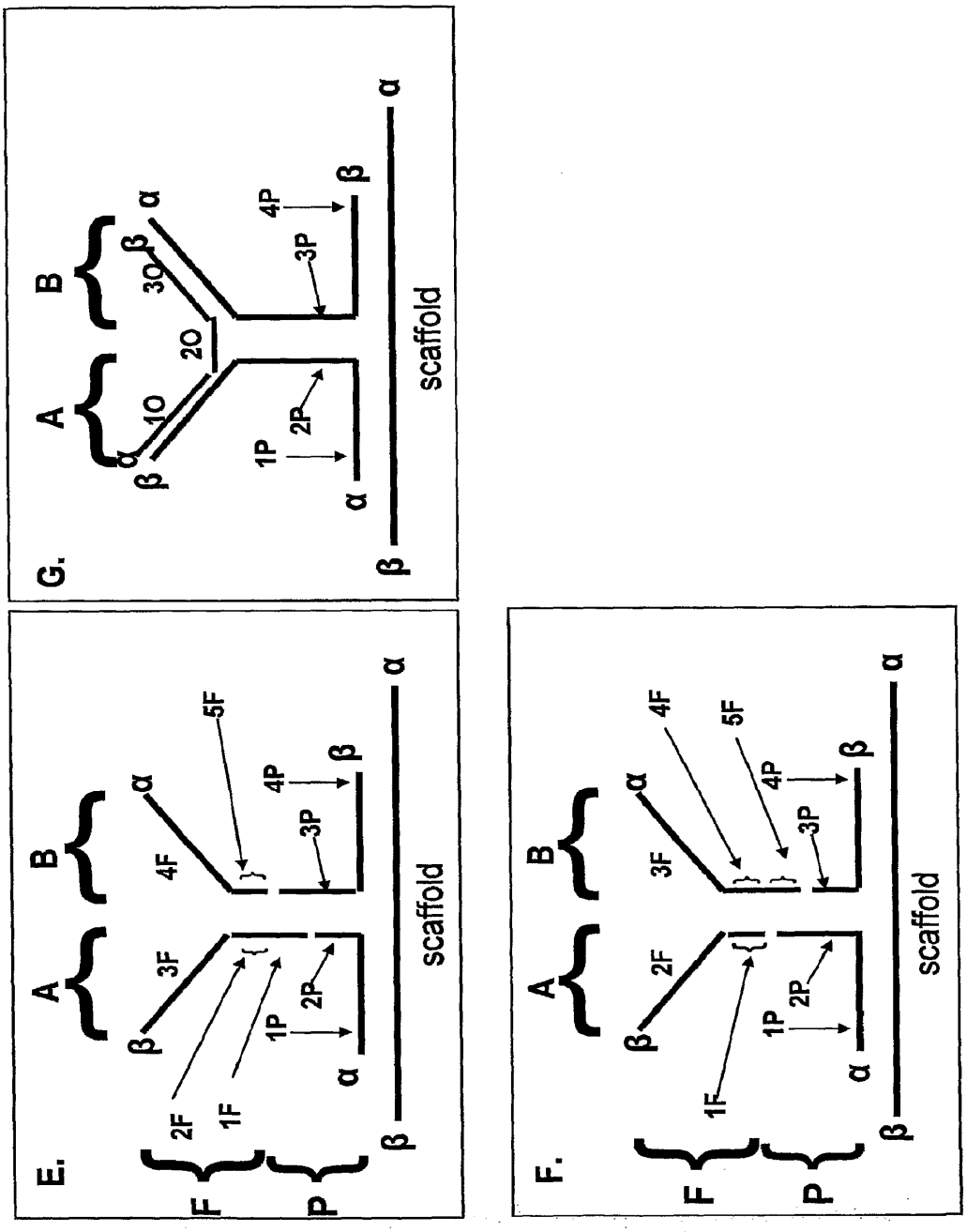


FIGURE 7E-G



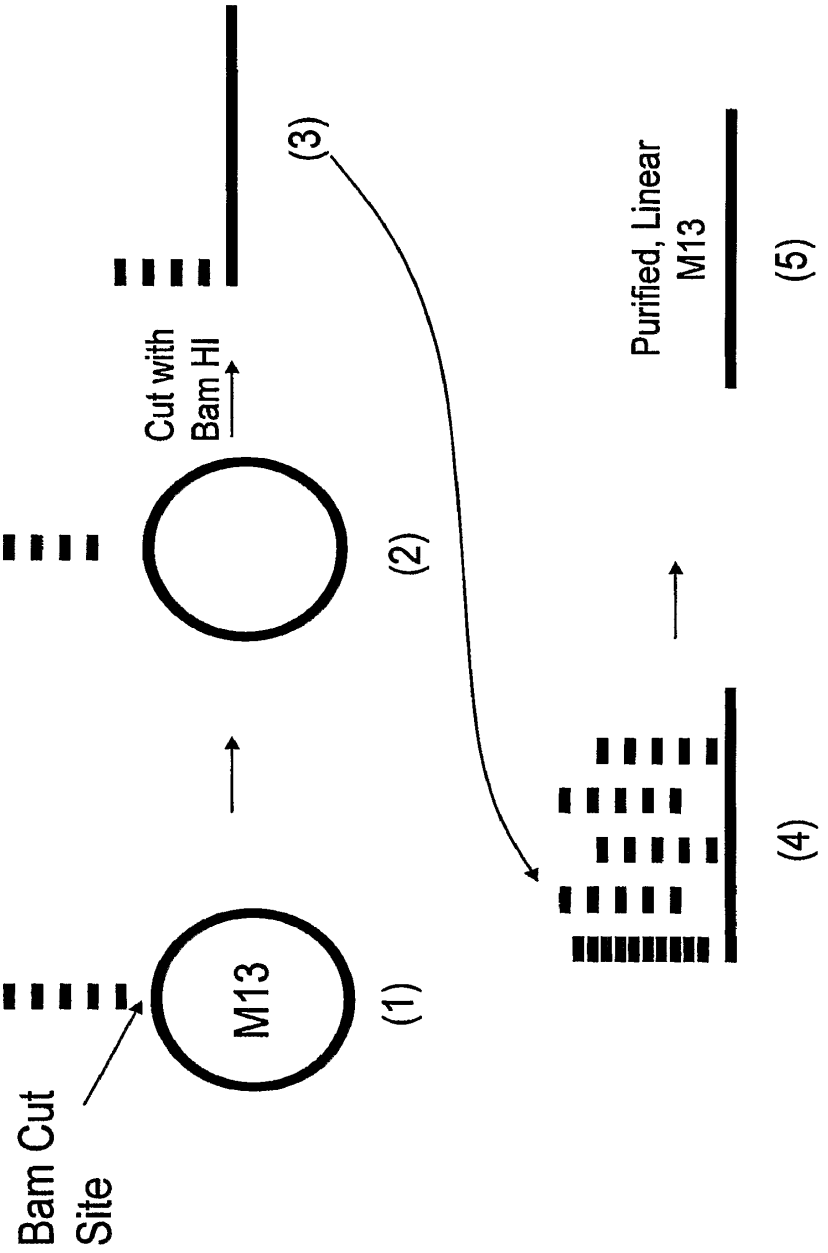


FIGURE 8

FIGURE 9

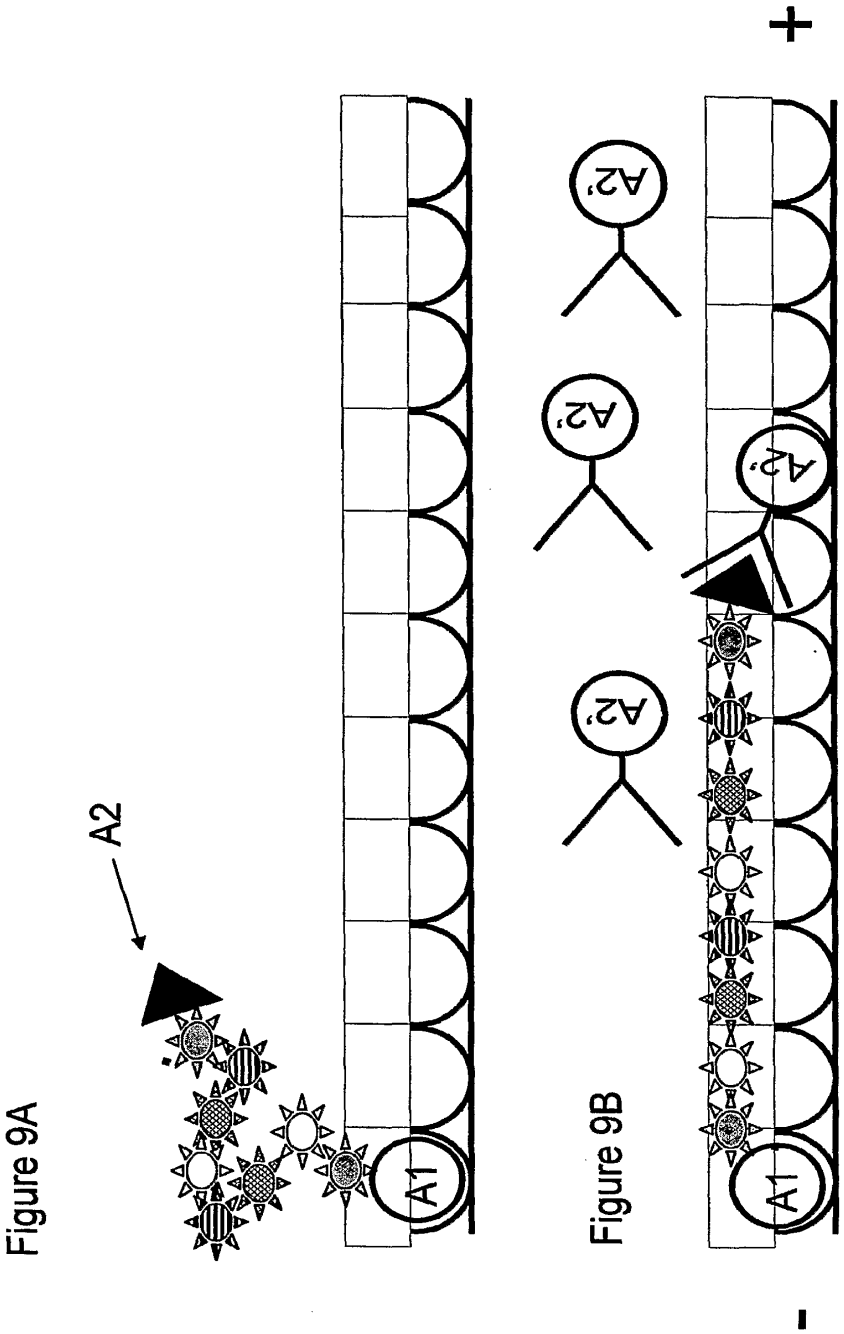


FIGURE 10

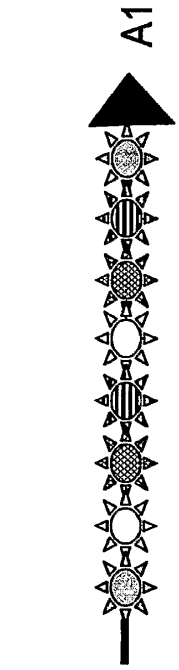


Figure 10A

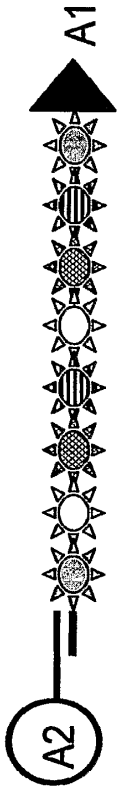


Figure 10B

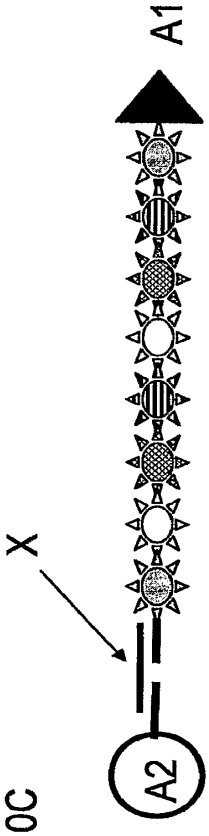


Figure 10C

FIGURE 11

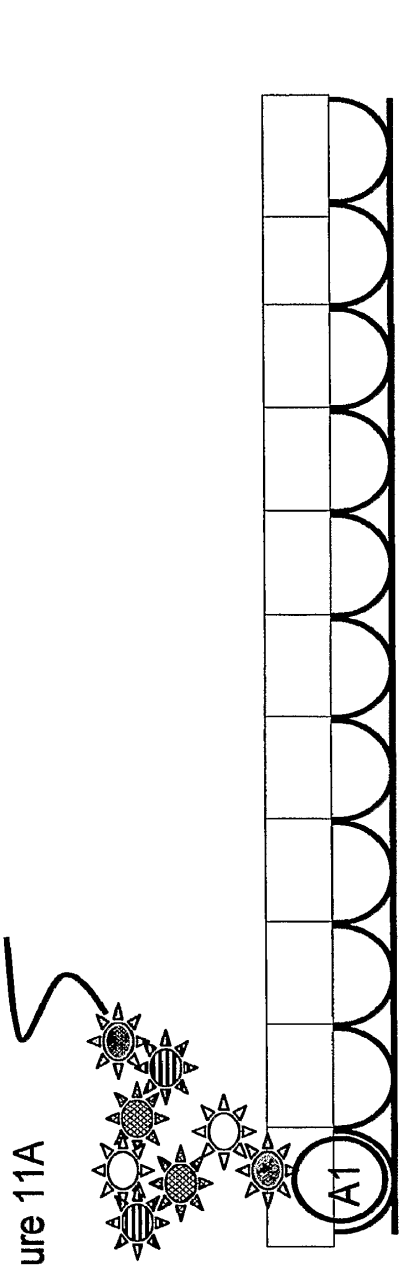


Figure 11A

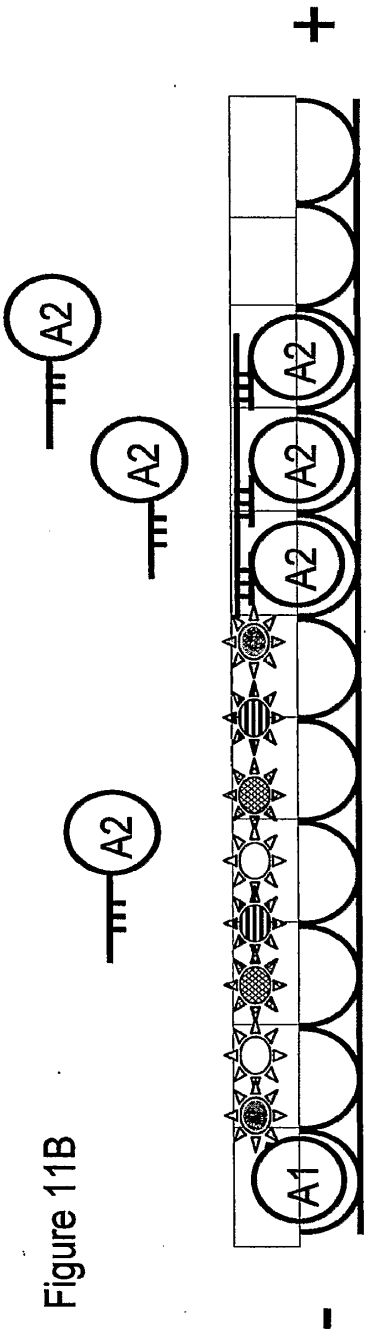


Figure 11B

FIGURE 12A-12B

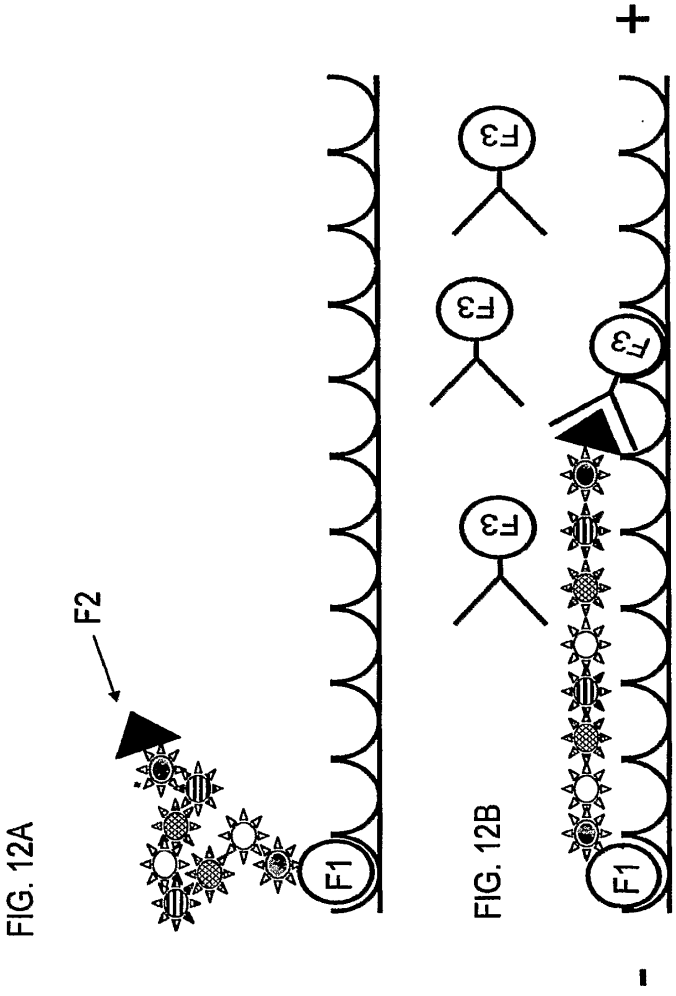


FIG. 12A

FIG. 12B

FIGURE 13A-13C

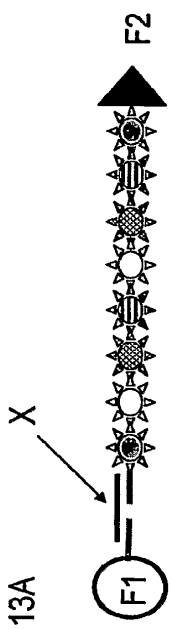


FIG. 13A

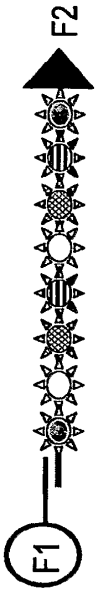


FIG. 13B



FIG. 13C

FIGURE 14A-14B

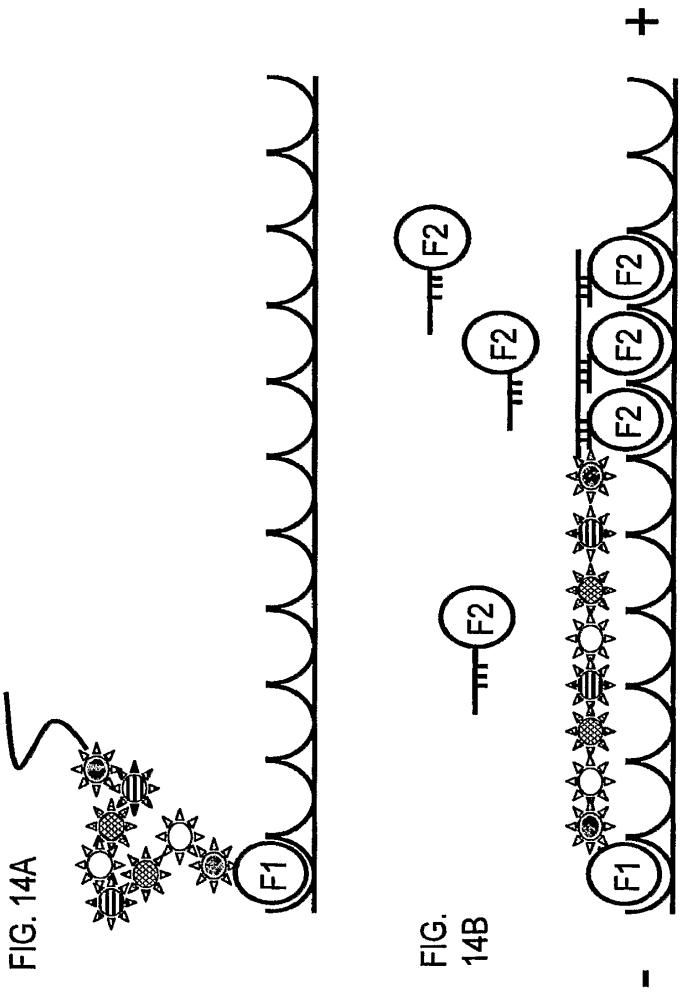
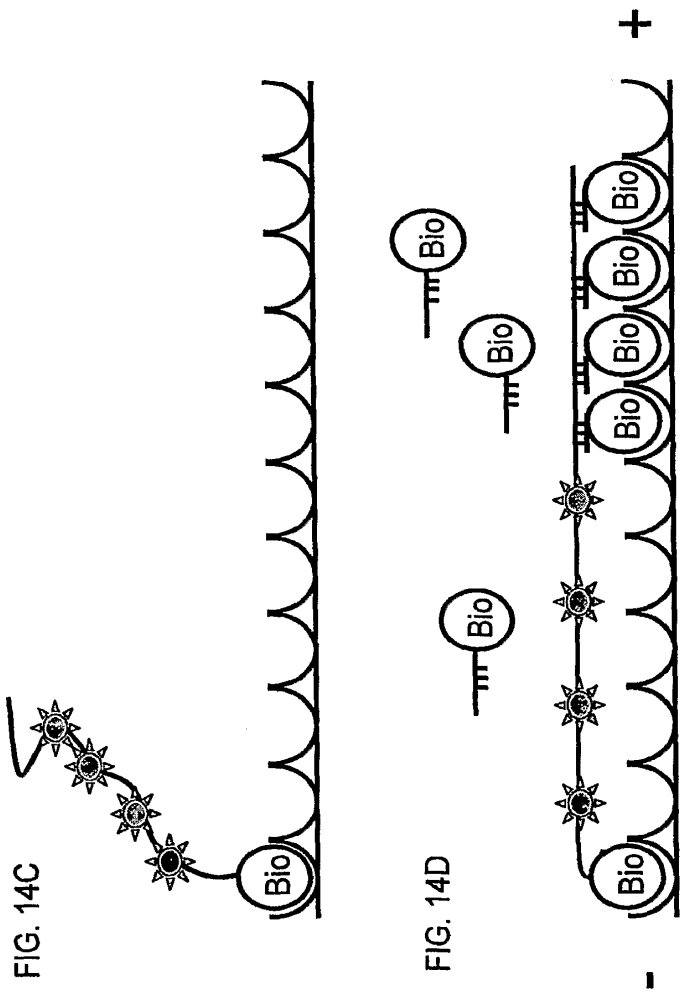


FIGURE 14C-14D



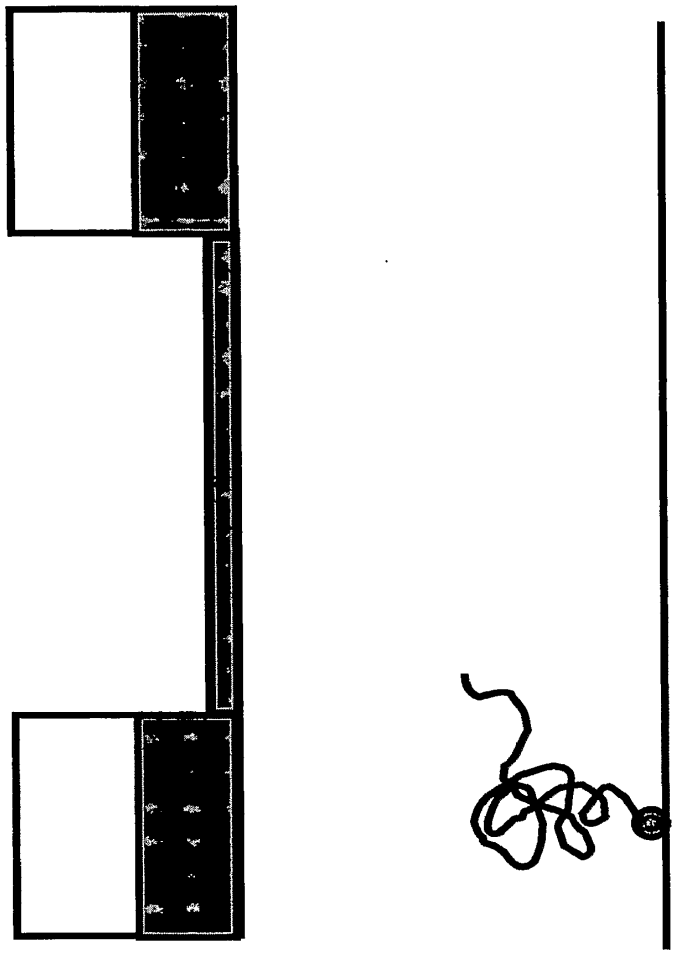


FIGURE 15A

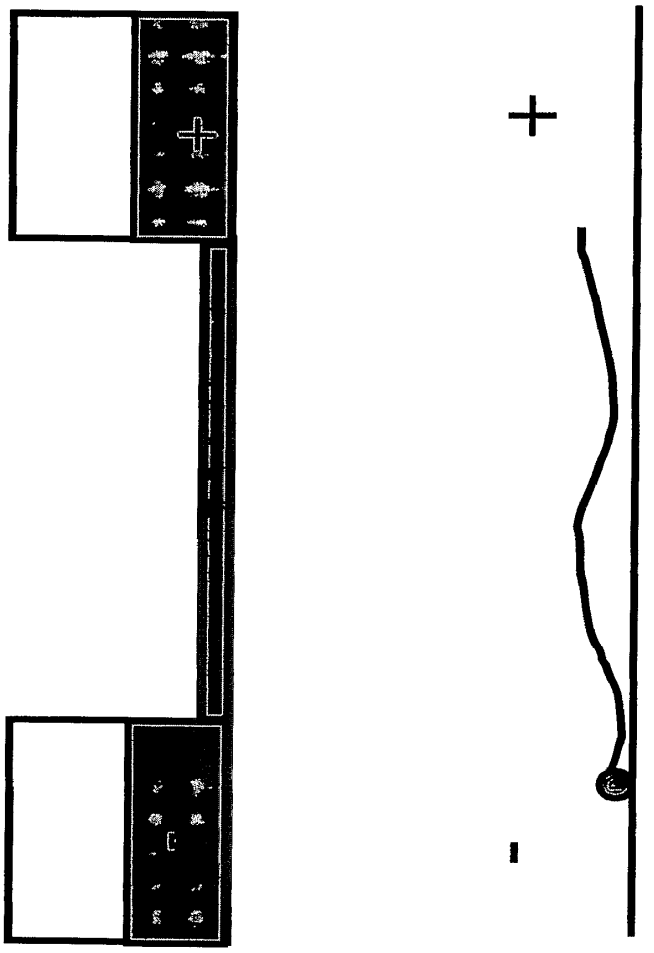


FIGURE 15B

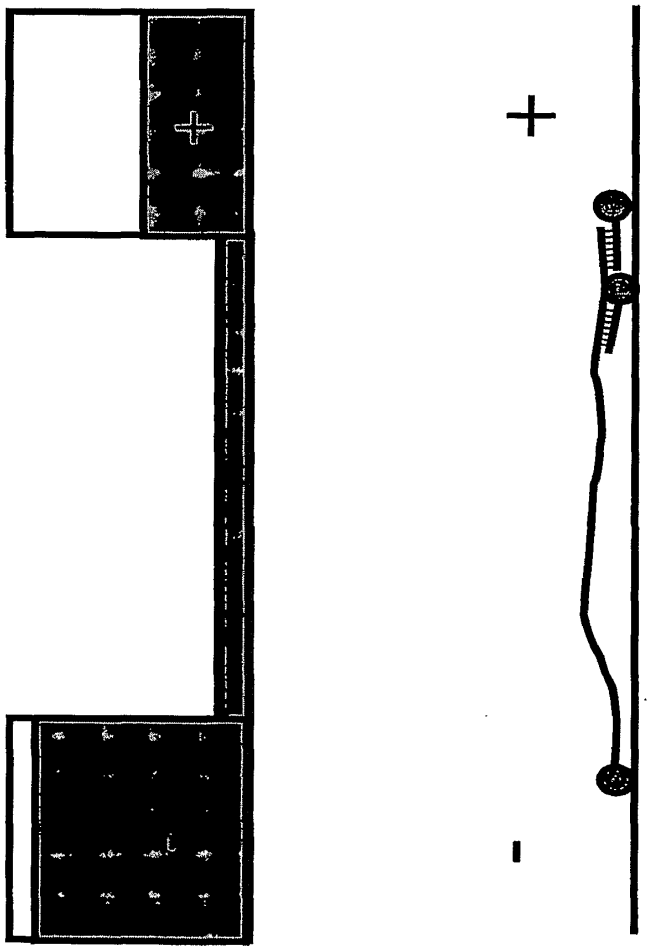


FIGURE 15C

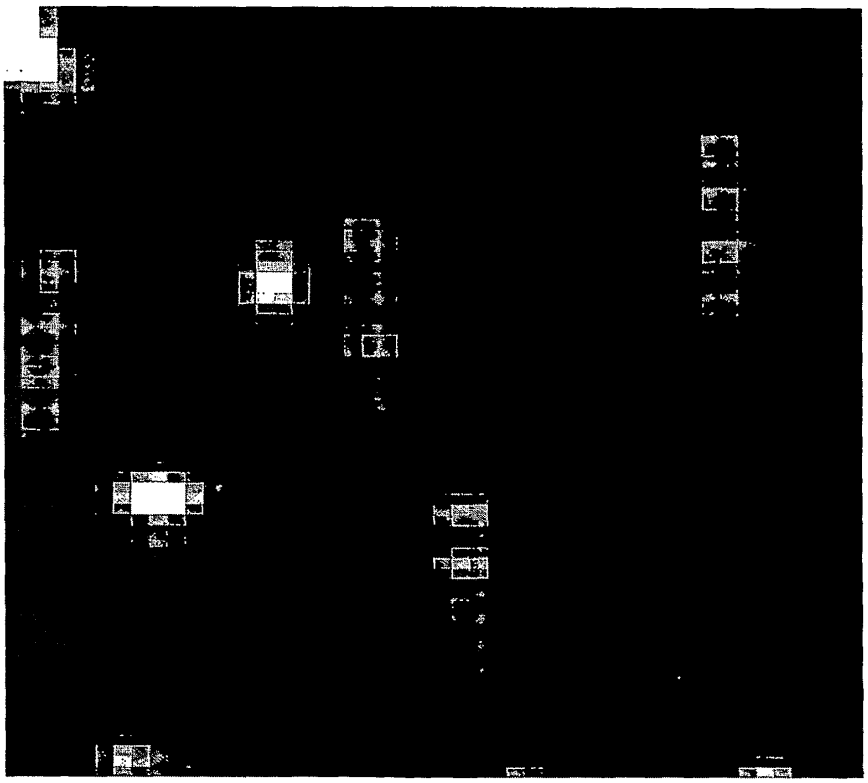


FIGURE 16

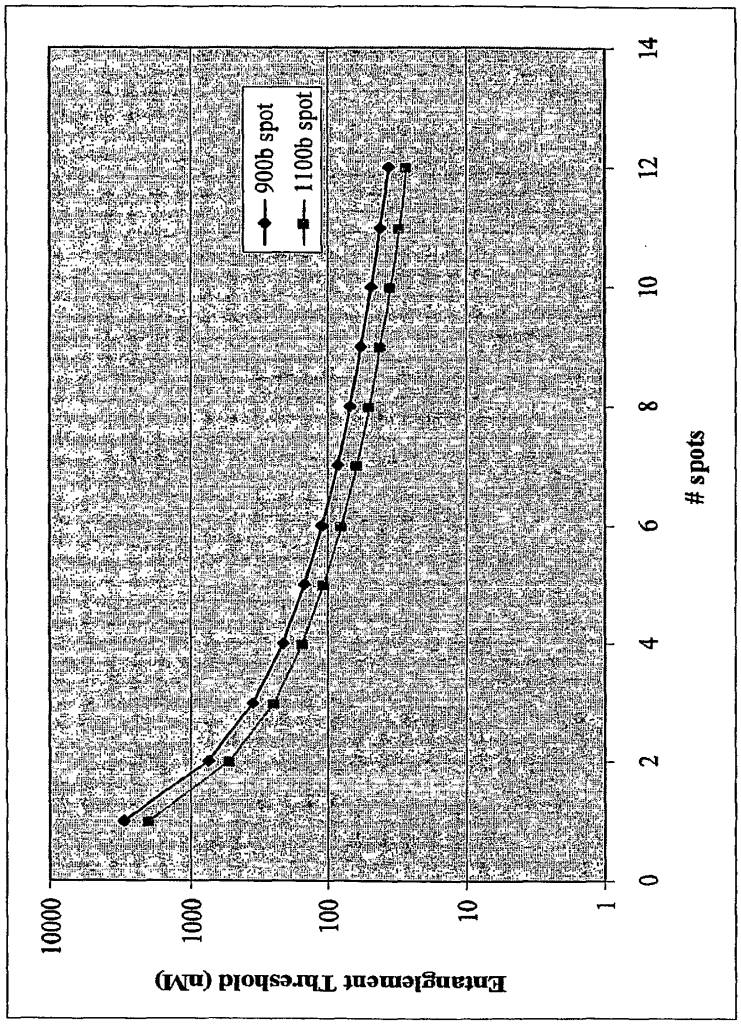
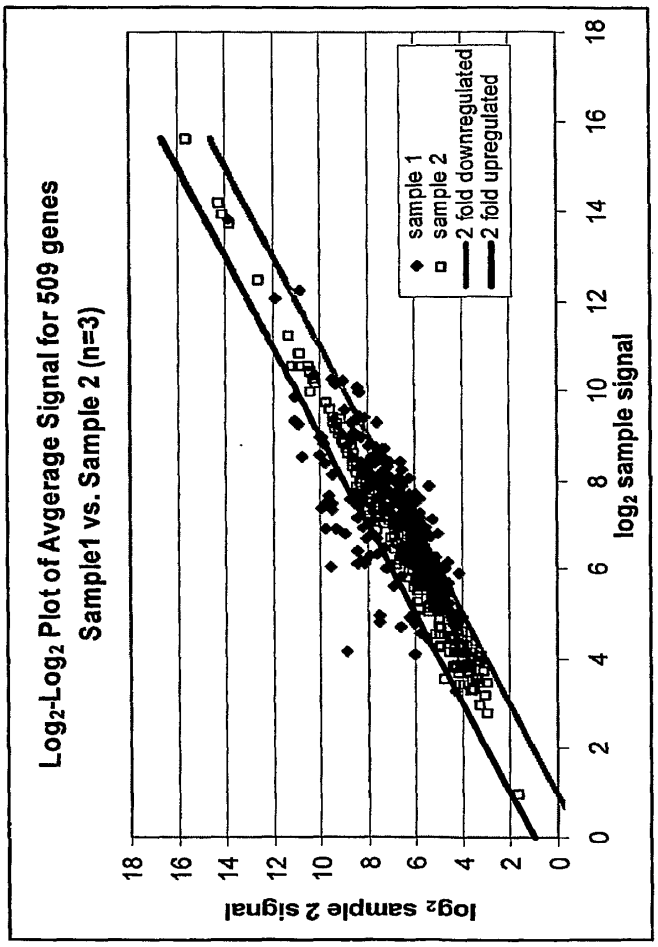

Entanglement Threshold (nM)
10000
1000
100
10
1
0
2
4
6
8
10
12
14
spots
900b spot
1100b spot

FIGURE 17

FIGURE 18


Log2-Log2 Plot of Avgerage Signal for 509 genes
Sample1 vs. Sample 2 (n=3)
log2 sample 2 signal
log2 sample signal
sample 1
sample 2
2 fold downregulated
2 fold upregulated
0
2
4
6
8
10
12
14
16
18

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(75) Inventors/Applicants *(for US only)*: **GEISS, Gary, K.** [US/US]; 647 Nw 78th Street, Seattle, WA 98117 (US). **FERREE, Sean, M.** [US/US]; 7018 3rd Avenue Nw, Seattle, WA 98117 (US). **WEBSTER, Philippa, J.** [US/US]; 4317 Densmore Avenue N, Seattle, WA 98103 (US). **DIMITROV, Krassen, M.** [BG/US]; 8804 Ravenna Avenue NE, Seattle, WA 98115 (US).

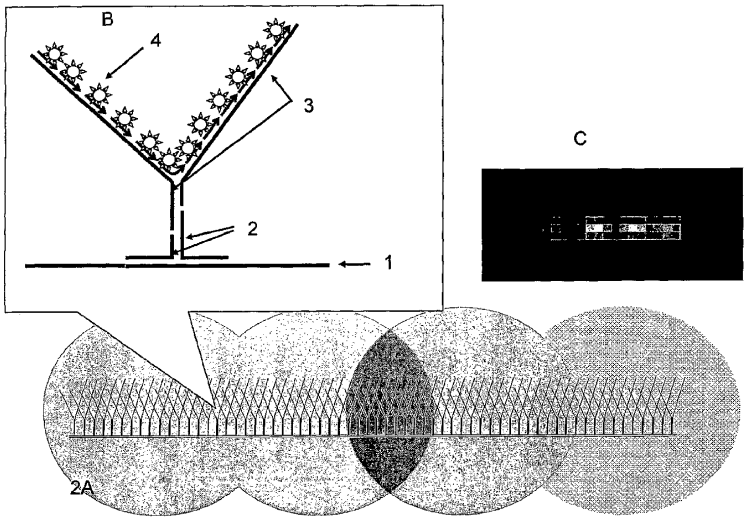
(74) Agents: **ANTLER, Adriane, M.** et al.; Jones Day, 222 East 41st Street, New York, NY 10017-6702 (US).

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[Continued on next page]

(54) Title: NANOREPORTERS AND METHODS OF MANUFACTURING AND USE THEREOF



(57) Abstract: The present invention relates to compositions and methods for detection and quantification of individual target molecules in biomolecular samples. In particular, the invention relates to coded, labeled probes that are capable of binding to and identifying target molecules based on the probes' label codes. Methods of making and using such probes are also provided. The probes can be used in diagnostic, prognostic, quality control and screening applications.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 06/49274

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8)- C12Q 1/68, C12P 19/34 (2007.01)
USPC- 435/6, 91.2
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)
USPC: 435/6, 91.2
IPC: C12Q 1/68, C12P 19/34 (2007.01)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (USPT, PGPB, EPAB, JPAB); DialogPRO; Google Scholar
Search terms: Molecular, reporter, hybrid$7, label, target , flap, fluorescent, probe, stretch$5, DNA, RNA, nano$9, patch

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 2004/0091864 A1 (FRENCH et al.) 13 May 2004 (13.05.2004), entire document especially claims 1, 7 and 11, FIG. 1 and para [0004]-[0006], [0009]-[0031], [0041], [0081] and [0119]-[0120].	1-7, 13, 24-26, 29-31, 33-34 --- 18, 27-28, 32, 35-41
Y	US 2002/0137036 A1 (SORGE et al.) 26 September 2002 (26.09.2002), especially, para [0022]-[0028],[0033],[0057]-[0059], FIG. 1, 4 and 9.	27-28, 35-41
Y	US 2002/0039732 A1 (BRUCHEZ et al.) 04 April 2002 (04.04.2002), especially, claims 1 and 8, FIG. 1 and 11, para [0013]-[0017].	18, 32

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"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
29 May 2007 (29.05.2007)	12 SEP 2007
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 06/49274

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.: 8-12, 14-17, 19-23 and 42-47
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.

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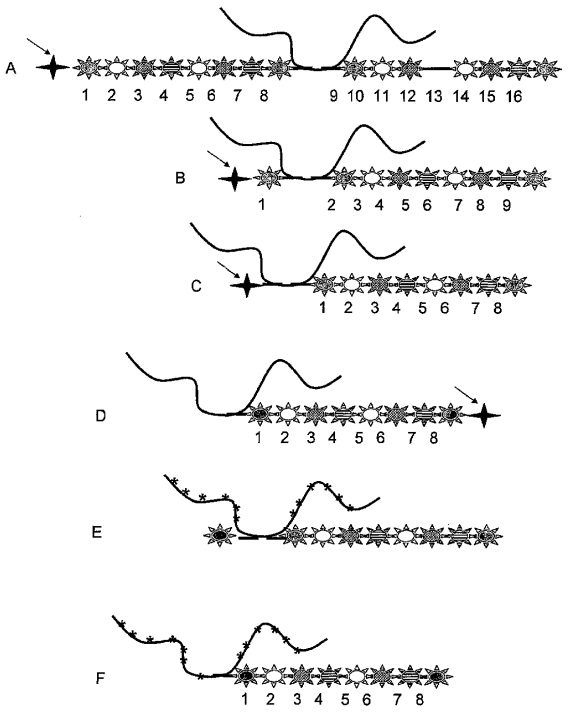
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[Continued on next page]

(54) Title: NANOREPORTERS AND METHODS OF MANUFACTURING AND USE THEREOF



(57) Abstract: The present invention relates to compositions and methods for detection and quantification of individual target molecules in biomolecular samples. In particular, the invention relates to coded, labeled probes that are capable of binding to and identifying target molecules based on the probes' label codes. Methods of making and using such probes are also provided. The probes can be used in diagnostic, prognostic, quality control and screening applications.

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FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

NANOREPORTERS AND METHODS OF MANUFACTURING AND USE THEREOF

This application claims the benefit of U.S. Provisional Application No. 60/753,758, filed on December 23, 2005, and U.S. Provisional Application No. 843,528, filed on September 8, 2006, the disclosures of each of which is incorporated herein by reference in their entireties.

1. FIELD OF THE INVENTION

The present invention relates to compositions and methods for detection and quantification of individual target molecules in biomolecular samples. In particular, the invention relates to coded, labeled reporter molecules, referred to herein as labeled "nanoreporters," that are capable of binding individual target molecules. Through the nanoreporters' label codes, the binding of the nanoreporters to target molecules results in the identification of the target molecules. Methods of making and using such nanoreporters are also provided. The nanoreporters can be used in diagnostic, prognostic, quality control and screening applications.

2. BACKGROUND OF THE INVENTION

This invention relates generally to the field of detection, identification, and quantification of target molecules in mixtures.

Although all cells in the human body contain the same genetic material, the same genes are not active in all of those cells. Alterations in gene expression patterns can have profound effects on biological functions. These variations in gene expression are at the core of altered physiologic and pathologic processes. Therefore, identifying and quantifying the expression of genes in normal cells compared to diseased cells can aid the discovery of new drug and diagnostic targets.

Nucleic acids can be detected and quantified based on their specific polynucleotide sequences. The basic principle underlying existing methods of detection and quantification is the hybridization of a labeled complementary probe sequence to a target sequence of interest in a sample. The formation of a duplex indicates the presence of the target sequence

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in the sample and the degree of duplex formation, as measured by the amount of label incorporated in it, is proportional to the amount of the target sequence.

This technique, called molecular hybridization, has been a useful tool for identifying and analyzing specific nucleic acid sequences in complex mixtures. This technique has been used in diagnostics, for example, to detect nucleic acid sequences of various microbes in biological samples. In addition, hybridization techniques have been used to map genetic differences or polymorphisms between individuals. Furthermore, these techniques have been used to monitor changes in gene expression in different populations of cells or in cells treated with different agents.

In the past, only a few genes could be detected in a complex sample at one time. Within the past decade, several technologies have made it possible to monitor the expression level of a large number of transcripts within a cell at any one time (see, e.g., Schena et al., 1995, Science 270: 467-470; Lockhart et al., 1996, Nature Biotechnology 14: 1675-1680; Blanchard et al., 1996, Nature Biotechnology 14:1649). In organisms for which most or all of the genome is known, it is possible to analyze the transcripts of large numbers of the genes within the cell. Most of these technologies employ, DNA microarrays, devices that consist of thousands of immobilized DNA sequences present on a miniaturized surface that have made this process more efficient. Using a microarray, it is possible in a single experiment to detect the presence or absence of thousands of genes in a biological sample. This allows researchers to simultaneously perform several diagnostic tests on one sample, or to observe expression level changes in thousands of genes in one experiment. Generally, microarrays are prepared by binding DNA sequences to a surface such as a nylon membrane or glass slide at precisely defined locations on a grid. Then nucleic acids in a biological sample are labeled and hybridized to the array. The labeled sample DNA marks the exact position on the array where hybridization occurs, allowing automatic detection.

Unfortunately, despite the miniaturization of array formats, this method still requires significant amounts of the biological sample. However, in several cases, such as biopsies of diseased tissues or samples of a discrete cell type, the biological sample is in limited supply. In addition, the kinetics of hybridization on the surface of a microarray is less efficient than hybridization in small amounts of aqueous solution. Moreover, while methods exist to estimate the amount of nucleic acid present in a sample based on microarray hybridization result, microarray technology thus far does not allow for detection of target molecules on an

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individual level, nor are there microarray-based methods for directly quantifying the amount of target molecule in a given sample.

Thus, there exists a need for accurate and sensitive detection, identification and quantification of target molecules in complex mixtures.

Discussion or citation of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to methods for the generation of a diverse population of uniquely-labeled molecules, preferably synthetic molecules, referred to herein as nanoreporters, that can be used for the detection, identification, and direct quantification of a wide variety of target molecules. The methods are advantageous in that they generate large numbers of distinctly labeled reporter molecules, each capable of detecting a single target molecule, starting from just a small number of different types of label monomers.

In certain embodiments, the present invention provides a dual nanoreporter, or a "probe pair," comprising two components: a first probe and a second probe. In specific embodiments, the first probe is a complex comprising: (a) a first molecule, or a scaffold, comprising: (i) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached (directly or indirectly) one or more label monomers that emit light constituting a second signal; and (b) a first target-specific sequence attached to the first molecule. The second probe is a second molecule or a complex comprising a second molecule, said second molecule comprising (i) a second target-specific sequence; (ii) optionally, a third label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a third signal; and (iii) optionally, an affinity tag attached to said second molecule; wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

In alternative embodiments, the present invention provides a dual nanoreporter, or a "probe pair," comprising two components: a first probe and a second probe. In specific

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embodiments, the first probe is a complex comprising: (a) a first molecule, or a scaffold, comprising: (i) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a second signal; and (b) a first target-specific sequence attached to the first molecule. The second probe is a second molecule or a complex comprising a second molecule, said second molecule comprising (i) a second target-specific sequence; (ii) optionally, a third label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a third signal; and (iii) optionally, an affinity tag attached to said second molecule; wherein the first signal and the second signal are spatially or spectrally distinguishable; wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule and wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

The code can consist of the first and second signals and their locations relative to each other, or can consist of the first and second signals and additional signals similarly generated from additional label attachment regions (on the first or second molecule) and the locations of the signals relative to each other.

Generally, the target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA. In other embodiments, the target molecule is a variant of a naturally occurring molecule, for example a protein whose activity, stability and/or biodistribution has been improved or optimized, or a nucleic acid encoding such a protein.

In a first aspect of the dual nanoreporters of the invention in which the target attachment regions are nucleotide sequences, a first DNA molecule is hybridized to the first label attachment region, to which first DNA molecule are bound (directly or indirectly) said one or more label monomers that emit light constituting said first signal; and wherein a second DNA molecule is hybridized to the second label attachment region, to which second DNA molecule are bound (directly or indirectly) said one or more label monomers that emit light constituting said second signal.

In a second aspect of the dual nanoreporters of the invention in which the target attachment regions are nucleotide sequences, a first RNA molecule is hybridized to the first

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label attachment region, to which first RNA molecule are bound (directly or indirectly) said one or more label monomers that emit light constituting said first signal; and wherein a second DNA molecule is hybridized to the second label attachment region, to which second DNA molecule are bound (directly or indirectly) said one or more label monomers that emit light constituting said second signal.

In a third aspect of the dual nanoreporters of the invention in which the target attachment regions are nucleotide sequences, a plurality of first DNA molecules are hybridized to the first label attachment region, to which DNA molecules are bound (directly or indirectly) said one or more label monomers that emit light constituting said first signal; and wherein a plurality of second DNA molecules are hybridized to the second label attachment region, to which second DNA molecules are bound (directly or indirectly) said one or more label monomers that emit light constituting said second signal.

In a fourth aspect of the dual nanoreporters of the invention in which the target attachment regions are nucleotide sequences, a plurality of first RNA molecules are hybridized to the first label attachment region, to which RNA molecules are bound (directly or indirectly) said one or more label monomers that emit light constituting said first signal; and wherein a plurality of second RNA molecules are hybridized to the second label attachment region, to which second RNA molecules are bound (directly or indirectly) said one or more label monomers that emit light constituting said second signal.

In certain embodiments of the dual nanoreporters of the invention, the second probe is a nucleic acid complex comprising: (a) the second nucleic acid (scaffold) molecule, wherein the second nucleic acid molecule comprises a third label attachment region to which is hybridized a third RNA molecule, to which third RNA molecule are bound (directly or indirectly) one or more label monomers that emit light constituting a third signal; and (b) the second target-specific sequence covalently attached to the second nucleic acid molecule, wherein the code comprises the identity of the first, second and third signals and their locations relative to each other. In certain embodiments, the label monomers attached to the first label attachment regions emit light at the same wavelength, which light constitutes said first signal, and the label monomers attached to the second label attachment region emit light at the same wavelength, which light constitutes the second signal, and the label monomers attached to the third label attachment region emit light at the same wavelength, which light constitutes the third signal. In other embodiments, at least one of the first signal, the second signal and the third signal comprises light at a plurality of

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different wavelengths. The first, second and third signals may be spectrally distinguishable. Alternatively, the first and third signals emit at the same wavelength or wavelengths.

In certain embodiments, the first probe and/or the second probe comprises an affinity tag. Also, in specific embodiments, the second probe comprises a third label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a third signal. Preferably, the first and second molecules are nucleic acid molecules; more preferably, the label attachment regions and target-specific sequences are predetermined nucleotide sequences. In certain embodiments of the dual nanoreporters of the invention, the one or more label monomers attached to one or both of the label attachment regions are covalently bound to nucleic acids hybridized to their respective attachment regions, or are covalently bound to nucleic acids that are bound to the respective label attachment regions indirectly through one or more bridging nucleic acids.

In certain embodiments of the dual nanoreporters of the invention, the first and second target-specific sequences are unlabeled with any one or more of said label monomers; however, at least one target-specific sequence in a nanoreporter is attached to a scaffold that is directly or indirectly attached to one or more label monomers.

In other embodiments of the dual nanoreporters of the invention, the second molecule further comprises a fourth label attachment region to which is attached one or more label monomers that emit light constituting a fourth signal. Preferably, when such a dual nanoreporter is bound to its target molecule, the code comprises the identity of the first signal, second signal, third signal and fourth signal and their locations relative to each other.

However, the second nucleic acid need not comprise a third label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a third signal. Such probes are referred to herein as "ghost probes." Ghost probes contain a target-specific sequence, which improves the hybridization kinetics of a nanoreporter to its target molecule, and, optionally, an affinity tag that can be used to immobilize and stretch the dual nanoreporter. In nanoreporter embodiments employing a ghost probe, the first molecule (to which the label monomers are attached and is sometimes referred to herein as a "reporter probe") is preferably a nucleic acid molecule of about 2,000 to about 10,000 bases in length, more preferably about 5,000 to about 8,000 bases in length, and the second molecule (the "ghost probe") is preferably a nucleic acid molecule of about 40 to about 250 bases in length, more preferably about 50 to 100 bases in length. In a specific embodiment, both the first molecule and the second molecule are DNA molecules.

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As used herein, the use of the terms "about" and "approximately" before a number or range means that number or range plus or minus 5%.

An affinity tag can be indirectly attached to the first probe or second probe. For example an affinity tag can be covalently attached to an oligonucleotide hybridized to the first or second probe, or it can be covalently attached to an oligonucleotide hybridized to a "patch" that is hybridized to the scaffold of the first and/or second probe. Alternatively, the affinity tag is covalently attached to the first nucleic acid molecule and/or to the second nucleic acid molecule of the first and second probes.

The signals emitted by the label monomers attached to a given label attachment region can be the same, or different. Thus, in one embodiment, the label monomers attached to a specific (*e.g.*, first or second) label attachment region emit light at the same wavelength, which light constitutes the signal corresponding to that label attachment region. Alternatively, in another embodiment, at least one of the first signal and the second signal comprises light at a plurality of different wavelengths.

Preferably, in certain embodiments, signals emitted by label monomers attached to adjacent label attachment regions are spectrally distinguishable. Thus, in specific embodiments, the first and second signals are spectrally distinguishable.

The present invention further provides a probe, which can be a singular nanoreporter or one or both of the two components of a dual nanoreporter, said probe being a nucleic acid complex comprising (a) a nucleic acid molecule comprising (i) a first label attachment region to which is hybridized a first RNA molecule, to which first RNA molecule are bound (directly or indirectly) one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is hybridized a second RNA molecule, to which second RNA molecule are bound (directly or indirectly) one or more label monomers that emit light constituting a second signal; and (b) a target-specific sequence covalently attached to the nucleic acid molecule, wherein the first and second RNA molecules preferably are each at least 20 nucleotides, wherein the target-specific sequence binds to a target molecule, which target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA, and wherein when said probe is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule. In specific embodiments, the first and second RNA molecules preferably are each

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at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 nucleotides.

In other aspects, the present invention provides a probe, said probe being a nucleic acid complex comprising (a) a synthetic nucleic acid molecule comprising (i) a first label attachment region to which is hybridized a first RNA molecule, to which first RNA molecule are attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is hybridized a second RNA molecule, to which second RNA molecule are attached one or more label monomers that emit light constituting a second signal; and (b) a target-specific sequence covalently attached to the synthetic nucleic acid molecule, wherein the first and second RNA molecules are each at least 50 nucleotides, wherein the target-specific sequence binds to a target molecule, which target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA, and wherein when said probe is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

In yet other aspects, the present invention provides a probe, said probe being a nucleic acid complex comprising (a) a nucleic acid molecule comprising (i) a first label attachment region to which is hybridized a first RNA molecule, to which first RNA molecule are covalently attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is hybridized a second RNA molecule, to which second RNA molecule are covalently attached one or more label monomers that emit light constituting a second signal; and (b) a target-specific sequence covalently attached to the nucleic acid molecule, wherein the first and second RNA molecules are each at least 50 nucleotides, wherein the target-specific sequence binds to a target molecule, which target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA, and wherein when said probe is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

In certain aspects of the present invention, the label monomers are attached to the label attachment regions of the nanoreporters of the invention by way of one or more RNA patches, or RNA molecules. Such RNA patches are preferably about (+/- 5%) 100 to about 3,000 nucleotides each, more preferably about 500 to about 1,500 nucleotides each.

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Where a nanoreporter comprises an affinity tag, the affinity tag can be attached to a patch and, in a dual nanoreporter, the affinity tag can be attached to one or more patches on one or both components of the probe pair.

The affinity tag can be attached by being covalently attached to an oligonucleotide hybridized to one or more patches on a nanoreporter. Alternatively, the affinity tag can be attached to the nucleic acid molecule that is the scaffold of a nanoreporter.

In certain aspects of the invention, nanoreporter-target molecule complexes are provided. Optionally, one or more components of the nanoreporter complexes are covalently attached to an affinity tag for purification and/or for immobilization (either before or after stretching, as described in Section 5.12 below). In specific embodiments, the target molecule itself is attached to an affinity tag. For example, where the target molecule is a nucleic acid, the affinity tag can be biotin that is incorporated into the nucleic acid as biotin-modified nucleotides. The target molecule can be immobilized through the affinity tag prior to or after formation of the nanoreporter-target molecule complex. In embodiments in which the affinity tag is a biotin moiety, the target molecule can be immobilized on a solid surface that is coated with avidin or streptavidin.

One or all of the components (*e.g.*, scaffold, target-specific sequence) of a nanoreporter can be (or have the sequence of) a naturally-occurring molecule. However, the fully assembled and labeled nanoreporter is generally a synthetic molecule, for example a chimeric molecule made by joining naturally-occurring sequences (*e.g.*, a viral or plasmid-based scaffold and a mammalian target-specific sequence) and/or manmade sequences.

The nanoreporters of the invention, when complexed to their target molecules, are preferably imaged when immobilized and stretched. The nanoreporters and complexes comprising the nanoreporters and target molecules can be immobilized by any method known in the art. Preferably, the nanoreporter is attached to an affinity tag (*e.g.*, biotin or digoxigenin) that can be used to tether the nanoreporter to a surface containing a ligand for the affinity tag (*e.g.*, streptavidin or an anti-digoxigenin antibody, respectively). The immobilized nanoreporter can be stretched by any method known in the art, including but not limited to flow-stretching, a receding meniscus technique, electrostretching, constriction in the flow of a liquid containing the nanoreporter in conjunction with an oscillating electric field.

The present invention further provides populations of ten or more nanoreporters, or probes, each probe comprising a synthetic molecule, said synthetic molecule comprising,

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sequentially in the following order: (a) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (b) a second label attachment region to which is attached one or more label monomers that emit light constituting a second signal; (c) a third label attachment region to which is attached one or more label monomers that emit light constituting a third signal; and (d) a target-specific sequence that binds to a target molecule; wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable; wherein the first and second signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the second and third signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the first and third signals are spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein each probe comprises a target-specific sequence that binds to a different target molecule; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies each target molecule.

Preferably, the target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA.

Also preferably, the label attachment regions and target-specific sequences are predetermined nucleotide sequences.

In certain aspects of the invention, the probes, probe pairs, and/or synthetic nucleic acid molecules of the invention are purified.

In specific embodiments, the label monomers attached to the first label attachment region emit light at the same wavelength, which light constitutes said first signal; wherein the label monomers attached to the second label attachment region emit light at the same wavelength, which light constitutes the second signal; and the label monomers attached to the third label attachment region emit light at the same wavelength, which light constitutes the third signal. Thus, in such embodiments, the label monomers attached to a particular label attachment region emit light at the same wavelength(s).

In other specific embodiments, at least one of the first signal, second signal, and third signal comprises light at a plurality of different wavelengths. In such embodiments, two or more monomers attached to at least one label attachment region emit light at different wavelengths.

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The present invention further provides a population of ten or more nanoreporter-target molecule complexes, each complex comprising a synthetic molecule bound to a target molecule, said synthetic molecule comprising, sequentially in the following order: (a) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (b) a second label attachment region to which is attached one or more label monomers that emit light constituting a second signal; (c) a third label attachment region to which is attached one or more label monomers that emit light constituting a third signal; and (d) a target-specific sequence that binds to said target molecule; wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable; wherein the first and second signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the second and third signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the first and third signals are spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein each target-specific sequence (or, in a dual nanoreporter, pair of target-specific sequences) binds to a different said target molecule; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies each target molecule.

In specific embodiments, the population is immobilized to a solid surface, and is preferably stretched. For example the population can be subjected to flow-stretching, a receding meniscus technique, electrostretching, or constriction in the flow of a liquid containing said complexes in conjunction with an oscillating electric field.

The present invention yet further provides methods of detecting a target molecule, said methods comprising: (a) contacting a biomolecular sample with a probe under conditions that permit binding of the probe to the target molecule, wherein said probe is a synthetic molecule comprising, sequentially in the following order: (i) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is attached one or more label monomers that emit light constituting a second signal; (iii) a third label attachment region to which is attached one or more label monomers that emit light constituting a third signal; and (iv) a target-specific sequence that binds to a target molecule; wherein the target-specific sequence binds to a target molecule that may be present in said

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sample, wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable; (b) detecting said first, second and third signals under conditions: wherein the first and second signals are not spatially resolvable; wherein the second and third signals are not spatially resolvable; wherein the first and third signals are spatially resolvable under said conditions; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies a target molecule; and (c) determining whether said code is produced, thereby detecting said target molecule.

Preferably, the probe and the target molecule are nucleic acids.

In certain specific embodiments, the label monomers attached to the first label attachment region emit light at the same wavelength, the label monomers attached to the second label attachment region emit light at the same wavelength, and the label monomers attached to the third label attachment region emit light at the same wavelength. In such embodiment, each of the first signal, second signal, and third signal consists of light at a single wavelength.

In other embodiments, at least one of the first signal, second signal, and third signal comprises light at a plurality of different wavelengths.

The present invention further provides an artificial nucleic acid molecule comprising one or more synthetic regions (hereinafter, "Region"), each Region being at least 50 nucleotides and comprising a regularly repeated base, said regularly repeated base having a periodicity of four to twenty five, such that said type of base occurs at regular intervals of every fourth to every twenty fifth position in said region. In a specific embodiment, not more than 5% of said base in said Region appears at other than said regular intervals. The artificial nucleic acid can be a single-stranded nucleic acid (*e.g.*, useful as a nanoreporter scaffold) or at least partially (or fully) double-stranged (*e.g.*, a nanoreporter scaffold to which patches are hybridized). The artificial nucleic acid can be RNA, DNA, or a DNA/RNA hybrid. Preferably the regularly repeated base is a thymidine or a uracil. In specific embodiments, the periodicity of the regularly repeated base in said one or more Regions is about 6 to about 15; in other embodiments, the periodicity of the regularly repeated base in said one or more Regions is about 8 to about 12.

Preferably, the artificial nucleic acid comprises a plurality of Regions, for example about 2 to about 50 Regions, or about 3 to about 10 Regions.

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The plurality of Regions can be immediately adjacent to one another, or separated by one or more spacer regions. Spacer regions can include, for example, one or more restriction endonuclease recognition sequences (or the complement thereof) and/or one or more RNA polymerase promoter sequences (or the complement thereof).

In specific embodiments, the plurality of Regions differ from one another in sequence.

The artificial nucleic acid may further comprise an origin of replication.

Preferably, the GC content of the one or more Regions is about 25% to about 75%, more preferably about 40 to about 60%, and most preferably is about 50% (+/- 5%).

Each of said one or more Region in the artificial nucleic acids of the invention is preferably about 50 to about 50,000 bases in length, and is more preferably about 200 to about 2,000 bases in length.

The artificial nucleic acids of the invention are preferably about 50 to about 100,000 bases in length, and are more preferably about 200 to about 10,000 or about 500 to about 20,000 bases in length.

The artificial nucleic acids of the invention may comprise one or a plurality of Regions, which are useful as label attachment regions. Where an artificial nucleic acid comprises a plurality of Regions, the type of regularly repeated base can be the same in the plurality of Regions, or different. Also, the periodicity of the regularly repeated base can be the same in the plurality of Regions, or different. In a specific embodiment, the present invention provides an artificial nucleic acid comprising a plurality of Regions, in which both the type and the periodicity of the regularly repeated base are the same in the plurality of Regions.

The artificial nucleic acids of the invention may further comprise a target-specific sequence.

Preferably, the Regions do not comprise a recognition site for at least one six-cutter restriction endonucleotides, such as one of the following restriction endonucleases: BamH1, EcoRI, PstI, SmaI, XbaI, NotI, SacI, EcoRV, HindIII, ClaI, SalI, XhoI, ApaI, or KpnI. In a specific embodiment, the Regions do not comprise any recognition site for 6-cutter enzymes, or at least 6-cutter enzymes that are used for cloning within the artificial nucleic acid, for example 6-cutter enzymes present in the polylinker of a plasmid or in regions flanking the Regions.

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The artificial nucleic acids of the invention, or at least the Regions therein, preferably are not predicted by the MFOLD program to contain hairpin loops.

The artificial nucleic acids of the invention, or at least the Regions therein, preferably do not have direct or inverted repeats that are greater than 12 bases in length. In other embodiments, the artificial nucleic acids and/or Regions do not have direct or inverted repeats that are greater than about 11, about 10 or about 9 bases in length.

The artificial nucleic acids of the invention preferably comprises a promoter sequence (such as T7, T3 or SP6) or its complement.

The artificial nucleic acids of the invention may further comprise a phage encapsidation sequence.

The Regions in the artificial nucleic acids of the invention may be flanked by restriction endonuclease recognition sites.

The artificial nucleic acids can be used as nanoreporter scaffolds, in which the Regions are useful as label attachment regions. Accordingly, the present invention provides an artificial nucleic acid comprising one or more Regions, the regularly repeated base of said one or more Regions being attached to at least one light-emitting label monomer. At least one regularly-repeated base can be covalently attached to the label monomer, or it can be attached to the label monomer via hybridization to another nucleic acid to which the label monomer is covalently attached. In a specific embodiment, at least two different Regions are attached to a different light-emitting labeling monomer.

The artificial nucleic acids of the inventions may further comprise an affinity tag and/or an origin of replication.

In one aspect of the invention, an artificial nucleic acid is a partially or fully double-stranded molecule in which one strand is DNA to which is hybridized a plurality of RNAs, each RNA comprising a single region in which the regularly-repeated base is aminoallyl-modified uracil. In a specific embodiment, at least 50% of occurrences of said aminoallyl-modified uracil is covalently attached to at least one light-emitting label monomer; in other embodiments, the percentage of occurrences is at least 60%, at least 70%, at least 80% , at least 90%, at least 95% or at least 98%. This percentage of occurrences can be measured by any means known in the art.

As discussed elsewhere, the nanoreporters of the invention may comprise nucleic acid scaffolds that are attached to patches and, optionally, flaps. Details of such structures

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are discussed in Section 5.3, *infra*, and exemplary patch and flap structures are depicted in Figure 7.

The present invention further provides methods of detecting a target molecule, said methods comprising: (a) contacting a biomolecular sample that may contain the target molecule with any of the nanoreporter structures described herein under conditions that permit binding of the synthetic molecule to the target molecule, (b) subjecting the conditions that permit formation and/or detection of the nanoreporter code and (c) determining whether or not the nanoreporter code is produced or present, thereby detecting said target molecule.

In certain aspects of the invention, the methods nanoreporter is a synthetic molecule comprising: (i) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal, all said first signals being the same; (ii) a second label attachment region to which is attached one or more label monomers that emit light constituting a second signal, all said second signals being the same; (iii) a third label attachment region to which is attached one or more label monomers that emit light constituting a third signal, all said third signals being the same; wherein each attachment region comprises a plurality of patch pairs; wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable, and determining whether or not the nanoreporter code is present and/or produced comprises (b) detecting said first, second and third signals under conditions: wherein the first and second signals are not spatially resolvable; wherein the second and third signals are not spatially resolvable; wherein the first and third signals are spatially resolvable under said conditions; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies a target molecule. Thereby, the target molecule is detected.

4. BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1A-1F: Figure 1A illustrates a dual nanoreporter with a 16-position nanoreporter code, using two 8-position nanoreporter components. Figure 1B illustrates a dual nanoreporter with a 9-position nanoreporter code, using one 8-position nanoreporter component and one single-position nanoreporter component. Figure 1C illustrates a dual nanoreporter with an 8-position nanoreporter code, using one ghost probe and one 8-position nanoreporter component. Figure 1D illustrates a single nanoreporter with an 8-position nanoreporter code. In Figures 1A-1D, the star shape (depicted with an arrow) is illustrative

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of an affinity tag, which can be used to purify the nanoreporter or immobilize the nanoreporter (or nanoreporter-target molecule complex) for the purpose of imaging. The numbered region in Figure 1A-1D refer to separate label attachment regions. All except for position 12 of Figure 1A are labeled with one of four types of label monomers, depicted as grey, white, hatched or stripe "sun" diagrams. Position 12 of Figure 1A is an unlabeled "dark spot." Figures 1E and 1F represent variations on the nanoreporters of Figures 1B and 1D, respectively, in which the target molecule to which the nanoreporters are bound comprises biotin moieties (shown as small asterisks), for example biotin-modified nucleotides randomly incorporated into a target nucleic acid. The nanoreporters themselves further optionally comprise an affinity tag (not shown).

FIGURE 2A-2C: Figure 2A shows an illustration of a label unit of a nanoreporter, containing a scaffold with patch units and corresponding split flaps disposed along its length. Figure 2B illustrates the components of a single patch pair and its corresponding flap, containing: 1: a portion of a nanoreporter scaffold (e.g., M13 single-stranded DNA); 2: A patch pair; 3: a split flap pair; and 4: labeled oligonucleotides, each with a label monomer incorporated, hybridized to the split flap. Figure 2C shows a nanoreporter with 4 "spots," each spot designed to contain 9 patch pairs of 60-65 nucleotides, each attached to a split flap pair of 95-100 nucleotides. Each split flap pair had binding site for 12 oligonucleotides each attached to a single label monomer. Each spot therefore had binding sites for 108 label monomers.

FIGURE 3: A nanoreporter in which the patches are RNA segments can be used with (Figure 3A) and without registers (Figure 3B). Both Figure 3A and 3B depict a **(1)** nanoreporter scaffold (heavy black line) to which are attached **(2)** 8 RNA segments (heavy grey lines 1-8), **(3)** a target-specific sequence (dotted line "T") and **(4)** an oligonucleotide (checkered line "0") that is partly complementary to the nanoreporter scaffold and partly complementary to the target-specific sequence. This oligonucleotide is referred to as a "ligator" oligonucleotide. In Figure 3A, only one register, *i.e.*, every alternate RNA segment is labeled. The second register positions serve as "spacers," making it possible to generate a nanoreporter code in which consecutive positions in the code are the same "color," or spectrally indistinguishable. In Figure 3B, both registers, *i.e.*, adjacent RNA segments with no intervening spacers, are labeled, with no nearest neighbor of the same "color."

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FIGURE 4: Is an image of a dual nanoreporter hybridized to a target molecule. Here, both registers are labeled. The nanoreporters are labeled with three different colors, Alexa 488, Cy3 and Alexa 647 (labeled 1, 2 and 3, respectively). The left brackets show one probe of the dual nanoreporter and the right brackets show the other probe of the dual nanoreporter. Colors 1, 2 and 3 were each acquired in different channels and the first and second registers, seen as rows of spots, were shifted up by several pixels to be able to show each register individually.

FIGURE 5A-5D: This figure illustrates the various components of the dual nanoreporters shown in Figure 4. Figure 5A illustrates one color (here, Alexa 488, depicted in the left column as open circles), which is spectrally distinguishable from Cy3 (shown in Figure 5B, depicted in the left column as vertically striped circles) and Alexa 647 (shown in Figure 5C as diagonally striped circles). The images obtained from each were superimposed to generate Figure 5D.

FIGURE 6A-6E: Figure 6A is a schematic illustration of the experiment shown in Figures 6B and 6C. In this case, the star represents biotin that was used to attach the complex by one end to the surface prior to stretching. Figures 6B and 6C show images from experiments in which S2-A ghost probe, S2-B labeled nanoreporter and S2 target DNA (Figure 6B) or S2 target RNA (Figure 6C) were hybridized. Figure 6E shows a close-up of a nanoreporter complexes from Figure 6B, each containing S2-A ghost probe, S2-B labeled nanoreporter and S2 target DNA. Figure 6D shows an image of a negative control experiment, in which S2-A ghost probe, S2-B labeled nanoreporter and no S2 target RNA were hybridized.

FIGURE 7A-G. Figures 7A, 7B, 7C and 7D depict different permutations of patches on a nanoreporter scaffold, Figures 7E and 7F depict different permutations of split flaps on a nanoreporter scaffold, optionally hybridized to one or more oligonucleotides, as in Figure 7G. In Figure 7A-G, α refers to a 5′ or 3′ molecule or end of a molecule, and β refers to a corresponding 3′ or 5′ molecule or end of a molecule.

FIGURE 8: Figure 8 depicts a scheme in which single-stranded M13 phage is linearized for use as a nanoreporter scaffold. The circular M13 phage is annealed to a five-fold excess of BamH1 cutter oligonucleotide (hatched lines) (1), and the resulting partially double-stranded M13 digested with the restriction endonuclease BamH1 (2), resulting in a linearized M13 in which BamH1 cutter oligonucleotide is still attached (3). This M13-oligonucleotide complex is heated in the presence of an excess oligonucleotide

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complementary to the BamH1 cutter oligonucleotide (an "anti-BamH1 oligonucleotide") (grey lines) (4). The BamH1 cutter oligonucleotide anneals to the excess of anti-BamH1 oligonucleotide, and the M13 molecule is purified from the oligonucleotide, for example by using size exclusion columns, to yield M13 scaffold.

FIGURE 9A-9B: Shows a labeled nanoreporter with an affinity tag at each end, A1 and A2. In Figure 9, the labeled nanoreporter is immobilized through the binding of A1 to an immobilized affinity partner. In the absence of an affinity binding partner for A2, the A2 end of the nanoreporter remains in solution (Figure 9A), but in the presence of an affinity binding partner (A2'), the A2 end of the nanoreporter is also immobilized (Figure 9B). Upon immobilization, the nanoreporter can be stretched, or "elongated" as depicted in Figure 9B, for example by electrostretching, for separation of the label attachment regions in a manner that permits detection of the nanoreporter code.

FIGURE 10A-10C: Figure 10A shows a labeled nanoreporter containing a single affinity tag, A1. Another affinity tag, A2, can be attached to the nanoreporter by direct binding of the nanoreporter to a molecule containing A2 (*e.g.*, if the nanoreporter is or comprises a nucleic acid, it can hybridize directly with another nucleic acid to which A2 is attached), as depicted in Figure 10B. Alternatively, the second affinity tag, A2, can be attached to the labeled nanoreporter via a bridging molecule, such as the bridging nucleic acid ("X") depicted in Figure 10C.

FIGURE 11A-11B: Shows a labeled (nucleic acid-based) nanoreporter with an affinity tag, A1, at one end. In Figure 11, the labeled nanoreporter is immobilized through the binding of A1 to an immobilized affinity partner. The other end of the nanoreporter is in solution (Figure 11A), but can be immobilized by hybridization to a complementary oligonucleotide which contains another affinity tag (A2) used to immobilize the nanoreporter (Figure 11B). A1 and A2 can be the same, for example biotin, for immobilization on an avidin- or streptavidin-coated surface. Upon immobilization of A1, the nanoreporter can be stretched, or "elongated" as depicted in Figure 11, for example by electrostretching, for separation of the label attachment regions in a manner that permits detection of the nanoreporter code. Optionally, while the nanoreporter is in an elongated state, A2 is introduced and binds the end of the nanoreporter that is complementary to A2 down to the surface.

FIGURE 12A-12B. Figure 12A provides an illustration of a nanoreporter comprising an immobilized first portion F1; and Figure 12B provides an illustration of a

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nanoreporter extended in an electrical field and comprising immobilized first portion F1 and immobilized second portion F2, wherein F2 is immobilized via a complex with molecule F3.

FIGURE 13A-13C. Figure 13A provides an illustration of a three-member complex for immobilization of an extended nanoreporter; FIG. 13B provides an illustration of a two-member complex for immobilization of an extended nanoreporter; and FIG. 13C provides an illustration of an incomplete complex for immobilization of an extended nanoreporter.

FIGURE 14A-14D. Figure 14A provides an illustration of a nanoreporter comprising an immobilized first portion F1; FIG. 14B provides an illustration of an extended nanoreporter immobilized at first portion F1 and at a second portion via complexes with F2; FIG. 14C provides an illustration of a nanoreporter comprising a first portion immobilized to an avidin surface via biotin; and FIG. 14D provides an illustration of an extended nanoreporter immobilized at a first portion and at a second portion via selective binding of biotin to an avidin surface.

FIGURE 15A-15C. Figure 15A illustrates immobilization of one terminus of a DNA molecule in a microfluidic device; Figure 15B illustrates extension of the DNA in an electric field; and Figure 15C illustrates selective immobilization of a second terminus of the extended DNA molecule.

FIGURE 16 provides an image of extended nanoreporters selectively immobilized by the methods of the present invention.

FIGURE 17 depicts the relationship between the number of label attachment regions to the calculated entanglement threshold for nanoreporters for label attachment region sizes of 900 bp and 1100 bp

FIGURE 18 is a scatter plot showing normalized and average $\log_2$ signal values from each positive sample (n=3) for all 509 genes whose expression was measured in a nanoreporter multiplex assay as described in Example 9 (Section 14) below.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention pertains to nanoreporters, and their manufacture and use. A fully assembled and labeled nanoreporter comprises two main portions, a target-specific sequence that is capable of binding to a target molecule, and a labeled region which emits a "code" of signals (the "nanoreporter code") associated with the target-specific sequence. Upon binding of the nanoreporter to the target molecule, the nanoreporter code identifies the target molecule to which the nanoreporter is bound.

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Nanoreporters are modular structures. Generally, a nanoreporter is a molecular entity containing three basic elements: a scaffold containing two or more label attachment regions, one or more patches attached to the scaffold, and a target-specific sequence, also attached to the scaffold. The elements of a nanoreporter can be found in a single molecular entity (a "singular" nanoreporter), or two distinct molecular entities (a "dual" nanoreporter). Each molecular entity may be composed of one molecule or more than one molecule attached to one another by covalent or non-covalent means. Generally, each component of a dual nanoreporter has a target-specific sequence that binds to a different site on the same target molecule. This allows for smaller nanoreporter components with more efficient kinetics of binding of the nanoreporter to the target molecule and better signal:noise ratios resulting from the greater binding specificity.

The patches attached to a nanoreporter scaffold serve to attach label monomers to a nanoreporter scaffold. Patches may be directly labeled, for example by covalent incorporation of one or more label monomers into nucleic acid patches. Alternatively, patches may be attached to flaps, which maybe labeled directly, for example by covalent incorporation of one or more label monomers into a nucleic acid flap, or indirectly, for example by hybridization of a nucleic acid flap to an oligonucleotide which is covalently attached to one or more label monomers. Where the label monomers attached to a label attachment region are not directly incorporated into a patch or flap, the patch or flap serves as a "bridge" between the label monomer and the label attachment region, and may be referred to as a "bridging molecule," *e.g.*, a bridging nucleic acid.

Additionally, nanoreporters may have affinity tags for purification and/or for immobilization (for example to a solid surface). Nanoreporters, or nanoreporter-target molecule complexes, are preferably purified in two or more affinity selection steps. For example, in a dual nanoreporter, one probe can comprise a first affinity tag and the other probe can comprise a second (different) affinity tag. The probes are mixed with target molecules, and complexes comprising the two probes of the dual nanoreporter are separated from unbound materials (*e.g.*, the target or the individual probes of the nanoreporter) by affinity purification against one or both individual affinity tags. In the first step, the mixture can be bound to an affinity reagent for the first affinity tag, so that only probes comprising the first affinity tag and the desired complexes are purified. The bound materials are released from the first affinity reagent and optionally bound to an affinity reagent for the second affinity tag, allowing the separation of complexes from probes comprising the first

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affinity tag. At this point only full complexes would be bound. The complexes are finally released from the affinity reagent for the second affinity tag and then preferably stretched and imaged. The affinity reagent can be any solid surface coated with a binding partner for the affinity tag, such as a column, bead (*e.g.*, latex or magnetic bead) or slide coated with the binding partner. Immobilizing and stretching nanoreporters using affinity reagents is fully described in U.S. provisional application no. 60/753,816 by Sean M. Ferree and Dwayne L. Dunaway, entitled "Compositions Comprising Oriented, Immobilized Macromolecules and Methods for Their Preparation," attorney docket no. 11616-014-888 filed on December 23, 2005, which is incorporated by reference herein in its entirety.

Nanoreporter and nanoreporter-target complexes which are or comprise nucleic acids may be affinity-purified or immobilized using a nucleic acid, such as an oligonucleotide, that is complementary to at least part of the nanoreporter or target. In a specific application where the target includes a poly A or poly dA stretch, the nanoreporter-target complex can be purified or immobilized by an affinity reagent coated with a poly dT oligonucleotide.

The sequence of signals emitted by the label monomers associated with the various label attachment regions of the scaffold of a given nanoreporter allows for the unique identification of the nanoreporter. A nanoreporter having a unique identity or unique spectral signature is associated with a target-specific sequence that recognizes a specific target molecule or a portion thereof. When a nanoreporter is exposed to a mixture containing the target molecule under conditions that permit binding of the target-specific sequence(s) of the nanoreporter to the target molecule, the target-specific sequence(s) preferentially bind(s) to the target molecule. Detection of the spectral code associated with the nanoreporter allows detection of the presence of the target molecule in the mixture (qualitative analysis). Counting all the label monomers associated with a given spectral code or signature allows the counting of all the molecules in the mixture associated with the target-specific sequence coupled to the nanoreporter (quantitative analysis). Nanoreporters are thus useful for the diagnosis or prognosis of different biological states (*e.g.*, disease vs. healthy) by quantitative analysis of known biological markers. Moreover, the exquisite sensitivity of single molecule detection and quantification provided by the nanoreporters of the invention allows for the identification of new diagnostic and prognostic markers, including those whose fluctuations among the different biological states is too slight detect a correlation with a particular biological state using traditional molecular methods. The

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sensitivity of nanoreporter-based molecular detection permits detailed pharmacokinetic analysis of therapeutic and diagnostic agents in small biological samples.

Many nanoreporters, referred to as singular nanoreporters, are composed of one molecular entity, as depicted in Figure 1D. However, to increase the specificity of a nanoreporter and/or to improve the kinetics of its binding to a target molecule, a preferred nanoreporter is a dual nanoreporter composed of two molecular entities, each containing a different target-specific sequence that binds to a different region of the same target molecule. Various embodiments of dual nanoreporters are depicted in Figures 1A-1C. In a dual nanoreporter, at least one of the two molecular entities is labeled. The other molecular entity is not necessarily labeled. Such unlabeled components of dual nanoreporters are referred to herein as "ghost probes" (see Figure 1C) and often have affinity tags attached, which are useful to immobilize and/or stretch the complex containing the dual nanoreporter and the target molecule to allow visualization and/or imaging of the complex.

Because of their modular structures, nanoreporters may be assembled and labeled in a variety of different ways. For example, a nanoreporter scaffold can be attached to a target-specific sequence (for example by hybridization and, optionally, ligation), and the structure comprising the scaffold and target-specific sequence attached to one or more patches and, where desired, flaps. Alternatively, the nanoreporter scaffold can first be attached to one or more patches (and, optionally, flaps), and the scaffold/patch structure then attached to a target specific sequence. Thus, unless stated otherwise, a discussion or listing of steps in nanoreporter assembly does not imply that a specific route of assembly must be followed.

Nanoreporter assembly and use is exemplified herein largely by way of description of a variety of nucleic acid-based nanoreporters; however, one of skill in the art would recognize that the methods described herein are applicable to an amino acid-based (or hybrid nucleic acid-/amino acid-based) nanoreporter. Illustrative embodiments of partially and fully assembled nanoreporters are listed below.

At its simplest, the invention provides a scaffold having at least two label attachment regions capable of being labeled and resolved. The scaffold can be any molecular entity that allows the formation of label attachment regions on the scaffold that can be separately labeled and resolved. The number of label attachment regions to be formed on a scaffold is based on the length and nature of the scaffold, the means of labeling the nanoreporter, as well as the type of label monomers emitting a signal to be attached to the label attachment regions of the scaffold. A nanoreporter according to the invention may have a scaffold

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including two or more label attachment regions. Suitable scaffold structures include DNA-based scaffolds.

The invention also provides labeled nanoreporters wherein one or more label attachment regions are attached to corresponding label monomers, each label monomer emitting a signal. For example a labeled nanoreporter according to the invention is obtained when at least two label monomers are attached to two corresponding label attachment regions of the scaffold such that these labeled label attachment regions, or "spots," are distinguishable. Label monomers emitting a signal associated with different label attachment regions of the scaffold can emit signals that are spectrally indistinguishable under the detections conditions ("like" signals), or can emit signals that are spectrally distinguishable, at least under the detection conditions (*e.g.*, when the nanoreporter is immobilized, stretched and observed under a microscope).

The invention also provides a nanoreporter wherein two or more label monomers are attached to a label attachment region. The signal emitted by the label monomers associated with said label attachment region produces an aggregate signal that is detected. The aggregate signal produced may be made up of like signals or made up of at least two spectrally distinguishable signals.

In one embodiment, the invention provides a nanoreporter wherein at least two label monomers emitting like signals are attached to two corresponding label attachment regions of the scaffold and said two label monomers are spatially distinguishable. In another embodiment, the invention provides a nanoreporter wherein at least two label monomers emitting two distinguishable signals are attached to two neighboring label attachment regions, for example two adjacent label attachment regions, whereby said at least two label monomers are spectrally distinguishable.

The invention provides a nanoreporter wherein two spots emitting like signals are separated by a spacer region, whereby interposing the spacer region allows resolution or better resolution of said like signals emitted by label monomers attached to said two spots. In one embodiment, the spacer regions have a length determined by the resolution of an instrument employed in detecting the nanoreporter.

The invention provides a nanoreporter with one or more "double spots." Each double spot contains two or more (*e.g.*, three, four or five) adjacent spots that emit like signals without being separated by a spacer region. Double spots can be identified by their sizes.

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A label monomer emitting a signal according to the invention may be attached covalently or non-covalently (*e.g.*, via hybrization) to a patch that is attached to the label attachment region. The label monomers may also be attached covalently or non-covalently (*e.g.*, via hybrization) to a flap attached to a patch that is in turn attached to the scaffold. The flap can be formed by one molecule or two or more molecules ("flap pieces") that form a split flap.

The invention also provides a nanoreporter associated with a spectral code determined by the sequence of signals emitted by the label monomers attached (*e.g.*, indirectly via a patch) to label attachment regions on the scaffold of the nanoreporter, whereby detection of the spectral code allows identification of the nanoreporter.

In one embodiment, the invention provides a nanoreporter further comprising an affinity tag attached to the nanoreporter scaffold, such that attachment of the affinity tag to a support allows scaffold stretching and resolution of signals emitted by label monomers corresponding to different label attachment regions on the scaffold. Nanoreporter stretching may involve any stretching means known in the art including but not limited to, means involving physical, hydrodynamic or electrical means.

In yet another embodiment, the invention provides a nanoreporter further comprising flaps attached to label attachment regions of the scaffold, wherein a flap attached to a label attachment region of the scaffold attaches the label monomer corresponding to said label attachment region, thereby indirectly attaching label monomers to corresponding label attachment regions on said scaffold. In a further embodiment, each label monomer comprises a signal emitting portion and an oligonucleotide portion of a predetermined sequence, and the flaps comprise repeats of a flap sequence complementary to the oligonucleotide portion of a corresponding label, whereby one or more label monomers attach to a corresponding label attachment region through hybridization of said oligonucleotide portions of said label monomers to said repeats of said flap sequence thereby producing a labeled nanoreporter.

A nanoreporter according to the invention can further include a target-specific sequence coupled to the scaffold. The target-specific sequence is selected to allow the nanoreporter to recognize, bind or attach to a target molecule. The nanoreporters of the invention are suitable for identification of target molecules of all types. For example, appropriate target-specific sequences can be coupled to the scaffold of the nanoreporter to

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allow detection of a target molecule. Preferably the target molecule is DNA (including cDNA), RNA (including mRNA and cRNA), a peptide, a polypeptide, or a protein.

One embodiment of the invention provides increased flexibility in target molecule detection with label monomers according to the invention. In this embodiment, a dual nanoreporter comprising two different molecular entities, each with a separate target-specific region, at least one of which is labeled, bind to the same target molecule. Thus, the target-specific sequences of the two components of the dual nanoreporter bind to different portions of a selected target molecule, whereby detection of the spectral code associated with the dual nanoreporter provides detection of the selected target molecule in a biomolecular sample contacted with said dual nanoreporter.

The invention also provides a method of detecting the presence of a specific target molecule in a biomolecular sample comprising: (i) contacting said sample with a dual nanoreporter under conditions that allow binding of the target-specific sequences in the dual nanoreporter to the target molecule and (ii) detecting the spectral code associated with the dual nanoreporter. Depending on the nanoreporter architecture, the dual nanoreporter may be labeled before or after binding to the target molecule.

In certain embodiments, the methods of detection are performed in multiplex assays, whereby a plurality of target molecules are detected in the same assay (a single reaction mixture). In a preferred embodiment, the assay is a hybridization assay in which the plurality of target molecules are detected simultaneously. In certain embodiments, the plurality of target molecules detected in the same assay is at least 5 different target molecules, at least 10 different target molecules, at least 20 different target molecules, at least 50 different target molecules, at least 75 different target molecules, at least 100 different target molecules, at least 200 different target molecules, at least 500 different target molecules, or at least 750 different target molecules, or at least 1000 different target molecules. In other embodiments, the plurality of target molecules detected in the same assay is up to 50 different target molecules, up to 100 different target molecules, up to 150 different target molecules, up to 200 different target molecules, up to 300 different target molecules, up to 500 different target molecules, up to 750 different target molecules, up to 1000 different target molecules, up to 2000 different target molecules, or up to 5000 different target molecules. In yet other embodiments, the plurality of target molecules detected is any range in between the foregoing numbers of different target molecules, such as, but not limited to, from 20 to 50 different target molecules, from 50 to 200 different

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target molecules, from 100 to 1000 different target molecules, from 500 to 5000 different target molecules, and so on and so forth.

In certain embodiments, the invention is directed to detecting different splice forms of the same RNA. The different splice forms can be detected using a plurality of nanoreporter probes, each with a different target-specific sequence complementary to a different exon of the same gene.

Structural stability of a nanoreporter can be increased through ligation of the patches and, optionally, ligation of the split flaps and/or the labeled oligonucleotides hybridized to the split flaps.

In addition to the qualitative analytical capabilities provided by the nanoreporters of the invention and the analytical techniques based thereon, the nanoreporters of the invention are uniquely suitable for conducting quantitative analyses. By providing a one to one binding between the nanoreporters (whether singular or dual nanoreporters) of the invention and their target molecules in a biomolecular sample, all or a representative portion of the target molecules present in the sample can be identified and counted. This individual counting of the various molecular species provides an accurate and direct method for determining the absolute or relative concentration of the target molecule in the biomolecular sample. Moreover, the ability to address each molecule in a mixture individually leverages benefits of miniaturization including high sensitivity, minimal sample quantity requirements, high reaction rates which are afforded by solution phase kinetics in a small volume, and ultimately very low reagent costs.

As will be appreciated from the description and examples provided below, the present invention provides numerous advantages. For example, the complex modularity in forming nanoreporters according to the invention allows for systematic creation of libraries of unique nanoreporters having a very high degree of diversity (*e.g.*, millions of uniquely recognizable nanoreporters). This modularity allows flexibility in customizing nanoreporter populations to specific applications which in turn provides significant manufacturing efficiencies. Another advantage that will be appreciated through the following description stems from the flexibility in assembling the nanoreporters of the invention. That is, due to their modular structure, the nanoreporters of the invention can be assembled prior to shipment to a point of use or assembled at the point of use.

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5.1 NANOREPORTER NOMENCLATURE

NANOREPORTER: The term "nanoreporter" refers to a molecular entity that has (i) a molecule ("scaffold") containing at least two label attachment regions; (ii) at least one patch attached to at least one label attachment region; and (iii) a target-specific sequence. As described in detail below, nanoreporters can be singular nanoreporters (all components being in a single molecular entity) or dual nanoreporters (all the components being in two separate molecular entities). Nanoreporters are preferably synthetic, *i.e.*, non-naturally-occurring molecules, for example are chimeric molecules made by joining two or more manmade and/or naturally occurring sequences that normally exist on more than one molecule (*e.g.*, plasmid, chromosome, viral genome, protein, etc.).

LABELED NANOREPORTER: A labeled nanoreporter is a nanoreporter in which at least one patch of the nanoreporter is attached to one or more label monomers that generate(s) a signal that forms at least part of the nanoreporter code.

LABEL UNIT: The term "label unit" refers to the non-target-specific portions of a labeled nanoreporter.

PROBE: This refers to a molecule that has a target-specific sequence. In the context of a singular nanoreporter, the term "probe" refers to the nanoreporter itself; in the context of a dual nanoreporter, the term "probe" refers to one or both of the two components of the nanoreporter.

PROBE PAIR: This refers to a dual nanoreporter.

PATCH: The term "patch" refers to a molecular entity attached to the label attachment region of the nanoreporter scaffold, generally for the purpose of labeling the nanoreporter. The patch can have one or more label monomers either directly (covalently or noncovalently) or indirectly attached to it, either prior to or after its attachment to the scaffold.

FLAP: The term "flap" as used herein refers to a molecular entity attached to a patch or patch pair attached to a label attachment region. The flap is one or more molecule containing label monomers or capable of binding one or more molecules containing label monomers. By providing indirect labeling of the regions, the flaps provide more flexibility in controlling the number of signal emitting monomers associated with a region as well as the nature of those monomers. Flaps may be formed by a single molecular piece or several molecular pieces (*e.g.*, two pieces) forming a "split flap" (see, *e.g.*, Figure 7)

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TARGET-SPECIFIC SEQUENCE: The term "target-specific sequence" refers to a molecular entity that is capable of binding a target molecule. In the context of a nanoreporter, the target-specific sequence is attached to the nanoreporter scaffold. The target molecule is preferably (but not necessarily) a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA.

GHOST PROBE: A molecule comprising a target-specific sequence, but which is not labeled with a label monomer that emits a signal that contributes to the nanoreporter code.

REPORTER PROBE: A molecule comprising a target-specific sequence that is labeled with at least one label monomer that emits a signal that contributes to the nanoreporter code. A singular nanoreporter is a reporter probe, as is a labeled component of a dual nanoreporter.

F-HOOK and G-HOOK: In the context of a dual nanoreporter, F- and G-hooks are each an affinity tag that is capable of being selectively bound to one of the probes. In preferred embodiments, the F-hook and G-hook are biotinylated oligonucleotides that are hybridizable to respective complementary sequences present in (*e.g.*, via ligation) or attached to (*e.g.*, via hybridization) the respective nanoreporter probes in a dual nanoreporter. Thus, the F-hooks and G-hooks can be used for purification, immobilization and stretching of the nanoreporter. Generally, where a dual nanoreporter contains one reporter probe and one ghost probe, the G-hook becomes attached to the reporter probe and the F-hook becomes attached to the ghost probe. F-hooks and G- hooks can be biotinylated on either end or internally. They can also be amine-modified to allow for attachment to a solid substrate for affinity purification.

F-TAG and G-TAG: Tandemly-repeated sequences of about 10 to about 25 nucleotides that are complementary to the F-hook and G-hook, respectively. G-tags and F-tags are attached to the nanoreporter probes. Generally, an F-tag is present in or attached to a ghost probe via a ligator sequence and a G-tag is present in or attached the reporter probe scaffold via a ligator sequence.

SPOT: A spot, in the context of nanoreporter detection, is the aggregate signal detected from the label monomers attached to a single label attachment site on a nanoreporter, and which, depending on the size of the label attachment region and the nature (*e.g.*, primary emission wavelength) of the label monomer, may appear as a single point source of light when visualized under a microscope. Spots from a nanoreporter may be

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overlapping or non-overlapping. The nanoreporter code that identifies that target molecule can comprise any permutation of the length of a spot, its position relative to other spots, and/or the nature (*e.g.*, primary emission wavelength(s)) of its signal. Generally, for each probe or probe pair of the invention, adjacent label attachment regions are non-overlapping, and/or the spots from adjacent label attachment regions are spatially and/or spectrally distinguishable, at least under the detection conditions (*e.g.*, when the nanoreporter is immobilized, stretched and observed under a microscope, as described herein).

Occasionally, reference is made to a spot "size" as a certain number of bases or nucleotides. As would be readily understood by one of skill in the art, this refers to the number of bases or nucleotides in the corresponding label attachment region.

NANOREPORTER CODE: The order and nature (*e.g.*, primary emission wavelength(s), optionally also length) of spots from a nanoreporter serve as a nanoreporter code that identifies the target molecule capable of being bound by the nanoreporter through the nanoreporter's target specific sequence(s). When the nanoreporter is bound to a target molecule, the nanoreporter code also identifies the target molecule. Optionally, the length of a spot can be a component of the nanoreporter code.

DARK SPOT: The term "dark spot" refers to a lack of signal, or "spot," from a label attachment site on a nanoreporter. Dark spots can be incorporated into the nanoreporter code to add more coding permutations and generate greater nanoreporter diversity in a nanoreporter population.

REGISTER: The term "register" refers to a set of alternating label attachment regions.

5.2 THE NANOREPORTER SCAFFOLD

The nanoreporter scaffold can be any molecular entity, more preferably a nucleic acid molecule, containing label attachment regions to which label monomers can be directly or indirectly attached. In one embodiment, the nanoreporter scaffold is a protein scaffold; in a preferred embodiment, the nanoreporter scaffold is a nucleic acid scaffold in which the label attachment regions are single-stranded regions to which other nucleic acids, such as oligonucleotide patches, RNA patches, or DNA patches, can attach by hybridization. In specific embodiments, the nanoreporter scaffold is a nucleic acid molecule.

There are no particular limitations on the types of scaffolds that are suitable for forming the nanoreporters of the invention. A scaffold according to the invention can

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essentially have any structure including, for example, single stranded linear scaffold, double stranded linear scaffold, single stranded circular scaffold or double stranded circular scaffold. Examples of scaffold structures include, for example, a scaffold made of one molecular entity such as polypeptides, nucleic acids or carbohydrates. A scaffold may also include a combination of structures, for example, a scaffold may be made of one or more polypeptide stretches coupled to one or more carbohydrate stretches.

Suitable molecular entities for scaffolds according to the invention include polymeric structures particularly nucleic acid based polymeric structures such as DNA. DNA based structures offer numerous advantages in the context of the present invention due at least in part to the vast universe of existing techniques and methodologies that allow manipulation of DNA constructs.

As indicated above, the scaffold may be single stranded or double stranded. Double stranded scaffold can be either conventional double stranded DNA or a double strand that is composed of a linear single stranded stretch of nucleic acid with patch units or flat-patches attached.

A scaffold can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21-100 label attachment regions or more.

The label attachment regions of a nanoreporter scaffold will vary in size depending on the method of labeling. In various embodiments, a label attachment region can have a length anywhere from 10 nm to 10,000 nm, but is more preferably from 50 nm to 5,000 nm, and is more preferably from 100 nm to 1,000 nm. In various embodiments, the label attachment region is from about 100 nm to about 500 nm, from about 150 nm to about 450 nm, from about 200 nm to about 400 nm, or from 250 to about 350 nm. In a preferred embodiment, the label attachment region corresponds closely to the size of a diffraction-limited spot, *i.e.*, the smallest spot that can be detected with standard optics, which is about 300 nm.

Where the scaffold is a nucleic acid, 1 nm corresponds to approximately 3 nucleotides; thus, an approximately 300 nm-label attachment region corresponds to approximately 900 bases. In other preferred embodiments, the label attachment region is from about 300 nucleotides to about 1.5kb, from about 450 nucleotides to about 1.35 kb, from about 0.6 kb to about 1.2 kb, or from 0.75 kb to about 1.05 kb.

An illustrative example of a molecular entity for a nanoreporter scaffold according to the invention is M13 DNA, which is single-stranded. In one embodiment, the nanoreporter

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scaffold is circular at least partially single stranded DNA, such as circular M13. In a more preferred embodiment, the nanoreporter scaffold is linear at least partially single stranded DNA, such as linear M13. In a specific embodiment, the M13 single-stranded DNA obtained by operating a cut at the BamH1 site of circular M13 DNA.

It should be noted that within the context of the present invention, linear DNA provides additional advantages compared to circular DNA. One advantage of using linear DNA in forming a scaffold according to the invention relates to the significantly reduced torsional stress associated with linear DNA. The added torsional stress associated with circular DNA may interfere with the structural integrity of the scaffold upon the addition to the scaffold of other components of the nanoreporter, such as patch units. Severe torsional stress may lead to the breaking of the structure of the scaffold. It should be noted however that the nanoreporters where only a few, short label attachment sites are labeled, circular DNA may be suitable.

5.2.1 NOVEL SYNTHETIC NANOREPORTER SCAFFOLD SEQUENCES

The present invention provides nanoreporter scaffold that are artificial nucleic acid molecules (DNA, RNA, or DNA/RNA hybrids) designed to have features that optimize labeling and detection of the nanoreporter. In these aspects of the invention, a nanoreporter scaffold is an artificial nucleic acids comprising one or more synthetic sequences from 50 to 50,000 bases long. Accordingly, the nanoreporter scaffold, which is preferably a DNA, is designed to have one or more Regions, useful as label attachment regions, comprising a regular pattern of a particular base (the "regularly-repeated base"). In such regions, the regularly-repeated base occurs with a periodicity of every nth residue, where n is any number, and preferably from 4 to 25.

Preferably, not more than 25% of the regularly-repeated base in a Region appears at other than said regular intervals. For example, if in a Region of 100 nucleotides there are 12 thymidine bases, and thymidine is the regularly-repeated base, in this aspect of the invention not more than 25% of these, *i.e.*, 3 thymidine bases, appear outside the regular pattern of thymidines. In specific embodiments, not more than 20%, not more than 15%, not more than 10%, not more than 9%, not more than 8%, not more than 7%, not more than 6%, not more than 5%, not more than 4%, not more than 3%, not more than 2% or not more than 1% of said base appears at other than said regular intervals in said region.

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The regularly-repeated base in the Regions in a nanoreporter scaffold, or its complementary regularly-repeated base in an annealed patch (or segment) can be used to attach label monomers, preferably light emitting label monomers, to the nanoreporter in a regular, evenly spaced pattern for better distribution of the nanoreporter signal. Preferably, where a Region is labeled, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% of occurrences of the regularly-repeated base is attached to at least one light-emitting label monomer, either by covalent attachment of a label monomer to a base, or by hybridization to a nucleic acid in which the complements of the regularly-repeated base are so-labeled.

This percentage of occurrences can be measured by any means known in the art. In one method, the amount of nucleic acid produced in a labeling reaction is purified (for example, RNA can be purified using a Qiagen RNeasy kit) and subjected to UV spectrophotometry. The absorbance ("A") at the appropriate wavelengths is measured for each of the nucleic acid (260 nm) and the label monomer whose occurrence is to be measured (*e.g.*, 495 nm for Alexa Fluor 488; 590 nm for Alexa Fluor 594; 650 for Alexa Fluor 647; and 550 nm for Cy3). The absorbance of the nucleic acid is corrected by adjusting the value of the absorbance at 260 nm ("A260") to remove the "noise" contribution from the label monomerby subtracting the absorbance at the peak wavelength for the label monomer (A_{LM}) minus the correction factor for that label monomer. Where the nucleic acid is RNA, the number of label monomers per one thousand nucleotides is calculated according to the formula:

$$\frac{\text{no. of label monomers}}{\text{1000 nucleotides}} = \frac{A260}{A_{LM}} \times \frac{9010}{EC_{LM}} \times 1000$$

where EC_{LM} is the extinction coefficient for the label monomer. From this formula, the percentage of occurrences of the regularly-repeated base that are attached to a light-emitting label monomer can be calculated.

Generally, the preferred regularly-repeating base in a label attachment region is thymidine, so that the region can be labeled by hybridization to one or more complementary patches (*e.g.*, RNA segments) in which the regularly-repeated base is uridine. This permits the use of amino-allyl-modified UTPs, which are readily commercially available, as label monomer attachment sites, in an otherwise random sequence. Preferably, in addition to the regular periodicity of the Regions, the regions (and the nucleic acid comprising them) contain minimal secondary structure. The overall GC-content is preferably maintained close

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to 50%, and is preferably consistent over relatively short stretches to make local Tm's similar.

The artificial nucleic acids of the invention, or at least the Regions therein, preferably do not have direct or inverted repeats that are greater than 12 bases in length. In other embodiments, the artificial nucleic acids and/or Regions do not have direct or inverted repeats that are greater than about 11, about 10 or about 9 bases in length.

In an exemplary Region in which the regularly-repeated nucleotide is a thymidine and a GC content of approximately 50%, excess adenines would make up the loss in abundance of T's. To generate the selected sequence, random sequences with fixed patterns of T's ranging from every 4th base to every 25th base are created and screened to minimize the presence of inverted and direct repeats.

Sequences are also screened preferably to avoid common six-base-cutter restriction enzyme recognition sites. Selected sequences are additionally subjected to predicted secondary structure analysis, and those with the least secondary structure are chosen for further evaluation. Any program known in the art can be used to predict secondary structure, such as the MFOLD program (Zuker, 2003, Nucleic Acids Res. 31 (13):3406-15; Mathews *et al.*, 1999, J. Mol. Biol. 288:911-940).

An appropriate sequence is divided into label attachment regions ranging from 50 bases to 2 kilobases long (could be longer). Each label attachment region is a unique sequence, but contains a consistent number and spacing of T's in relation to the other label attachment regions in a given reporter sequence. These label attachment regions can interspersed with other regions whose sequence does not matter. The synthetic label attachment regions in a nanoreporter scaffold can be of different lengths and/or have different regularly-repeated bases. An optimized start sequence for transcription by RNA polymerase T7, T3, or SP6 (beginning at position +1 of the transcript) can be added to the 5' end of each label attachment region. Restriction sites are optionally added at the boundaries of each label attachment region to allow specific addition or deletion of individual label attachment regions to the sequence using conventional cloning techniques. The number of synthetic label attachment regions in a nanoreporter preferably ranges from 1 to 50. In yet other embodiments, the number of synthetic label attachment regions in a nanoreporter ranges from 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 synthetic label attachment regions to 15, 20, 30, 40, or 50 synthetic label attachment regions, or any range in between.

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An example of such a novel synthetic label attachment region is given below. In this sequence, shown 5′ to 3′, the T's are placed in every 8th position and the region is bounded by a 5′ Sac I restriction site and a 3′ Kpn I restriction site. An optimized transcript start site for T7 polymerase (GGGAGA) is included at the 5′ end of the region, downstream of the 5′ restriction site. The complement of this sequence, when generated as a single-stranded molecule, forms the scaffold for the RNA molecule transcribed from this label attachment region.

GAGCTCGGGAGATGGCGAGCTGGAAGCATCAGAAAGTAGGAAGATGACAAAAT
AGGGCCATAGAAGCATGAAGAACTGAACGCATGAGACAATAGGAAGCTACGCC
ACTAGGGACCTGAGAAGCTGAGCGGCTCAGCGGGTCCGAGCGTCAAAAAATAA
AAGAGTGAAACAATAGACGAATGACGCGGTAAAACCATCCAGAAGTAAACGGG
TACAAACATACAGAGATAGCCACCTGGACCAATAGGCACGTACAAACGTACAA
GCCTGGCGCGATGAGGCAATCCACACGTGCAGAGCTGGAACAATGGAAAGATG
CAAGAATAAACCGATACCGGGATCGAGGGCTCAGCGAATAAAGCAGTCAACAA
CTGGAAAGATCCACACATACCGGCGTAACCGAGTCCAAACATACAGACCTGCA
AGACTCGCGACATGGGACGGTAAAACCATCCGACCGTAAACCGGTAACCAGGT
AGCCGGGTAAAAACATAGCAGGGTGGAGACCTCAGAACGTAAAGACGTCCAAG
GGTCGCCGGATAGCGAACTACGCGCATCGCCCAATGGGCCAATCAACAGATAA
ACGAGTAGAAAAGTCAGAAAATAAGAAACTAACGAAATACGAGGGTCCAAGG
ATGCAAGACTGAGGCCCTAAGGAGATAAGGAAATAGGCCGATGCAGACCTGAA
ACGATGCACCGATCCGACGGTAAAAGACTAGACACGTAGCCGGATCAGGGCCT
GGGAGGCTGGAACCGTGAGCACATAGCAAAGTCGCAGCGTCGGCAGATGCGCC
GGTAAAAAAGTAGAGGCATGACCGGATGGGCAAATAGCGACGTACAGCAGTGA
AGCACTAAAAGCATCCAAGGGTAGGAGACTAGGCGCCTCGACGGGTAGGTACC

The synthetic nucleic acids of the present invention can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the label attachment region and the annealed patches or segments, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the synthetic nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-

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isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine.

Alternatively, the synthetic nucleic acid can be produced biologically using a vector into which a nucleic acid has been subcloned.

In various embodiments, the synthetic nucleic acid molecules of the invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup *et al.*, 1996, Bioorganic & Medicinal Chemistry 4(1):5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup *et al.*, 1996, Bioorganic & Medicinal Chemistry 4(1): 5-23; Perry-O'Keefe *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93: 14670-675.

In an exemplary embodiment, the selected novel synthetic sequence can be constructed synthetically as double-stranded DNA by a commercial gene synthesis company and cloned in an oriented fashion into a "phagemid", a plasmid vector containing an M13 or f1 phage intergenic (IG) region which contains the *cis*-acting sequences necessary for DNA replication and phage encapsidation, such as pUC119. The appropriate orientation of the cloned insert relative to the phage origin of replication allows for the generation of a single-stranded DNA scaffold which is the reverse complement of the RNA molecules generated by *in vitro* transcription for each label attachment region.

In order to generate the single-stranded DNA scaffold of the novel reporter, the phagemid is transformed into an *E. coli* strain containing an F' episome. Subsequent infection of the transformed bacteria with a helper phage such as the M13 mutant K07 results in the secretion of the phagemid carrying the novel reporter sequence as a single-stranded, packaged phage from which the circular, single-stranded DNA is prepared using a standard protocol. This DNA is linearized and the vector portion is excised by annealing

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short, complementary oligonucleotides to either end of the novel reporter sequence to generate double-stranded restriction sites, followed by treatment with the appropriate restriction enzymes.

To make the RNA molecules (patches or "segments") for each label attachment region, polymerase chain reaction ("PCR") primers are designed to generate a double-stranded template beginning with an RNA polymerase promoter (T7, T3, or SP6) directly upstream (5′) of the transcription start site and ending following the 3′ restriction enzyme site. Using this template, *in vitro* transcription of RNA molecules is performed in the presence of amino-allyl modified regularly-repeated base in the RNA (*e.g.*, UTP) and unmodified other bases (*e.g.*, ATP, CTP and GTP). This leads to an RNA product in which every regulalry-repeated base (*e.g.*, U) is modified to allow covalent coupling of a label monomer at that position in the RNA molecule.

Coupling of light-emitting label monomers to the RNA molecules and annealing of the labeled RNA molecules to the scaffold are carried out as described below.

Some design considerations for the de novo sequence are listed in Table 1 below.

Feature Of Synthetic Scaffold	**Advantages**
Novel synthetic sequence	Can be of any length and be designed to incorporate any desired sequence feature including but not limited to those listed in this table.
Minimal secondary structure (select against inverted repeats)	Allows for consistent transcription of full-length RNA molecules. Allows for consistent annealing of RNA molecules to scaffold at predictable temperatures. Minimizes self-annealing and/or cross-annealing between RNA molecules or scaffolds.
Minimal repeated sequences	Avoids mis-annealing between RNA molecules and inappropriate regions of the scaffold.
Unique restriction sites at borders of label attachment regions	Allows addition and deletion of individual label attachment regions using standard molecular cloning techniques.
Defined, even spacing of T's and transcription with amino-allyl-modified UTP (no unmodified UTP)	Controls number of coupling sites for monomers in each label attachment region, allowing for consistent brightness of individual labeled RNA molecules. Controls distance between monomers: spacing can be optimized to avoid stearic hindrance and fluorescence quenching.
Optimized start sequence for transcription by RNA polymerase T7, T3, or SP6	Promotes efficient in vitro transcription of each label attachment region.

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5.3 PATCHES

Label monomers that emit signals which constitute all or part of the nanoreporter code are attached to label attachment region(s) of the nanoreporter scaffold through a structure referred to herein as a "patch." The label monomers can be directly (*e.g.*, covalently or noncovalently) attached to a patch, or indirectly attached to a patch (*e.g.*, through hybridization).

Nucleic acid patches can by anywhere from 25 nucleotides to several kilobases (*e.g.*, 5kb) in length, and are preferably 50 nucleotides to 2kb in length. In specific embodiments, nucleic acid patches are approximately 25 to 250, 50 to 200, 50 to 150, or 50 to 100 nucleotides in length. In other embodiments, nucleic acid patches are approximately 500 to 2,000, 500 to 1,500, 500 to 1,000, 750 to 1,250, or 750 to 1,000 nucleotides in length. Nucleic acid patches can be RNA patches or DNA patches.

A label monomer can be covalently attached to a patch before or after the patch is attached to the label attachment region of a nanoreporter scaffold. For example, where the patch is a nucleic acid molecule, the label can be covalently attached by incorporation of a nucleotide containing a label monomer into the nucleic acid during its synthesis but before it is attached, *e.g.*, via hybridization, to the label attachment region of the scaffold. Alternatively, during the synthesis of a nucleic acid patch, a nucleotide containing a label monomer acceptor group be included, and the label monomer added to the nucleic acid patch after its synthesis, either before or after it is attached to the label attachment region of the scaffold. Alternatively, the label monomer can be indirectly attached to the patch, for example by hybridization of the patch to a "flap" that serves as a basis for attachment of the label monomer to the nanoreporter.

Thus, where a patch is a nucleic acid, it can range anywhere from 20 nucleotides to more than 5 kb in length, depending on the method of assembly of the nanoreporter.

For example, where a patch has covalently incorporated into it one or more label monomers that emit signals that are part of the nanoreporter code in the context of the labeled nanoreporter, the patch is preferably about 100 to about 10,000 bases, more preferably 200 to about 2000 bases, and yet more preferably 700 to about 1200 nucleotides in length, and is generally referred to herein as a "segment," a "dark "segment being the patch prior to the incorporation of the label monomer (but, in a preferred embodiment, containing label monomer acceptor sites, such as amino allyl nucleotides), and a "colored" segment being one containing the desired label monomer or label monomers. The Tm of a

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segment when hybridized to its label attachment region preferably is >80°C, more preferably >90°C, in 825 mM Na^+ (5X SSC).

Where a patch merely serves as a template for flap attachment to the nanoreporter, then it is preferably smaller in size, for example about 25-250 nucleotides in length, an is most preferably about 50-100 nucleotides in length. Such patches are referred to herein as "oligonucleotide patches." As detailed in Section below, an oligonucleotide is preferably partially complimentary in sequence to a scaffold, such that when it is annealed to the scaffold, an overhang is generated that is complementary to all or portion of a flap.

The terms "segment" and "oligonucleotide patch" are used herein merely for convenience of description; however, there is no size cutoff to distinguish a "segment" from an "oligonucleotide patch." The purpose of both types of structures is to maximize the labeling --and thus signal intensity-- from the nanoreporter, thereby allowing for single target molecule detection by a nanoreporter.

In certain aspects, the present invention provides a synthetic molecule, whose configuration is illustrated by reference to **Figure 7A**, comprising a strand of a nucleic acid **(scaffold)** and a plurality of patch pairs hybridized to said strand, wherein each patch pair comprises an "A" patch and a "B" patch, wherein, for each patch pair, (a) each "A" patch is an oligonucleotide comprising a first region **(1P)** and a second region **(2P)**, said first region being (i) at the alpha end of said "A" patch, and (ii) hybridized to a first portion of said strand, said second region being (ii) at the beta end of said "A" patch; (b) each "B" patch is an oligonucleotide comprising a third region **(3P)** and a fourth region **(4P)**, said third region being (i) at the alpha end of said "B" patch, and (ii) hybridized to said second region of said "A" patch, said fourth region being (i) at the beta end of said "B" patch and (ii) hybridized to a second portion of said strand, said second portion of said strand being to the beta end of said first portion of said strand, wherein said second region or said third region further comprises at its beta end or alpha end, respectively, a hybridizable region that is not hybridized to said "B" patch or "A" patch, respectively.

In the synthetic molecule of **Figure 7A**, the second region may further comprise at its beta end a hybridizable region that is not hybridized to said "B" patch, as depicted in **Figure 7B**, or the third region further comprises at its alpha end a hybridizable region that is not hybridized to said "A" patch, as depicted in **Figure 7C**.

The present invention further provides a synthetic molecule, whose configuration is illustrated by reference to **Figure 7D**, comprising a strand of a nucleic acid **(scaffold)** and a

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plurality of patch pairs hybridized to said strand, wherein each patch pair comprises an "A" patch and a "B" patch, wherein, for each patch pair, (a) each "A" patch is an oligonucleotide comprising a first region **(1P)** and a second region **(2P)**, said first region being (i) at the alpha end of said "A" patch, and (ii) hybridized to a first portion of said strand, said second region being (ii) at the beta end of said "A" patch; (b) each "B" patch is an oligonucleotide comprising a third region **(3P)** and a fourth region **(4P)**, said third region being (i) at the alpha end of said "B" patch, and (ii) hybridized to said second region of said "A" patch, said fourth region being (i) at the beta end of said "B" patch and (ii) hybridized to a second portion of said strand, said second portion of said strand being to the first of said first portion of said strand, wherein said second region further comprises at its beta end a first hybridizable region that is not hybridized to said "B" patch, and wherein said third region further comprises at its alpha end a second hybridizable region that is not hybridized to said "A" patch.

In the synthetic molecule of **Figure 7B**, each patch pair can be attached to a flap pair, as depicted in **Figure 7F**, wherein each flap pair comprises an "A" flap and a "B" flap, wherein, for each flap pair, (a) each "A" flap is an oligonucleotide comprising a first flap region **(1F)** and a second flap region **(2F)**; said first flap region being at the alpha end of said "A" flap; said second flap region (i) being at the beta end of said "A" flap and (ii) comprising at its beta end a hybridizable region that is not hybridized to said "A" patch, "B" patch or "B" flap; and (b) each "B" flap is an oligonucleotide comprising a third flap region **(3F)**, a fourth flap region **(4F)**, and a fifth flap region **(5F)**; said third flap region being (i) being at the alpha end of said "B" flap and (ii) comprising at its alpha end a hybridizable region that is not hybridized to said "A" patch, "B" patch or "A" flap; said fourth flap region (i) being between the third flap region and the fifth flap region and (ii) hybridized to said first flap region of said "A" flap; said fifth flap region being (i) at the beta end of said "B" flap, and (ii) hybridized to said hybridizable region of said second region of said "A" patch.

In the synthetic molecule of **Figure 7C**, each patch pair can be attached to a flap pair, as depicted in **Figure 7E**, wherein each flap pair comprises an "A" flap and a "B" flap, wherein, for each flap pair, (a) each "A" flap is an oligonucleotide comprising an first flap region **(1F)**, a second flap region **(2F)**, and a third flap region **(3F)**; said "A" flap region being (i) at the alpha end of said "A" flap and (ii) hybridized to said hybridizable region of said third region of said "B" patch; said second flap region being between the first flap region and the third flap region; said third flap region (i) being at the beta end of said "A"

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flap and (ii) comprising at its beta end a hybridizable region that is not hybridized to said "A" patch, "B" patch or "B" flap, and (b) each "B" flap is an oligonucleotide comprising a fourth flap region **(4F)** and a fifth flap region **(5F)**; said fourth flap region being (i) being at the alpha end of said "B" flap and (ii) comprising at its alpha end a hybridizable region that is not hybridized to said "A" patch, "B" patch or "A" flap; said fifth flap region being (i) at the beta end of said "B" flap, and (ii) hybridized to said second flap region of said "A" flap.

In the synthetic molecule of **Figures 7D** and **7E**, the split flaps can be attached one (*e.g.*, **(1O)**), or more (*e.g.*, **(2O)** and **(3O)**) oligonucleotides, as depicted in **Figure 7G.** Thus, the one or more oligonucleotides can be attached to the all or a portion of the "A" flap individually (*e.g.*, **(1O)**), the "B" flap individually (*e.g.*, **(3O)**), or span all or a portion of each of the "A" flap and "B" flap (*e.g.*, **(2O)**). Such oligonucleotides are preferably covalently bound to one or more label monomers.

The hybridizable regions of said synthetic molecules may be hybridized to a plurality of oligonucleotides, each bound, preferably covalently bound, to at least one label monomer, more preferably to at least five label monomers. In certain embodiments, all the oligonucleotides attached to a single patch pair comprise the same label monomers, *e.g.*, comprise label monomers that emit light at the same wavelength(s); in specific embodiments, all the oligonucleotides attached to at least two, or at least four, adjacent patch pairs preferably comprise the same label monomers. One or more of the oligonucleotides may be bound to at least one affinity tag.

In certain preferred embodiments, the label monomers are fluorophores or quantum dots.

In the synthetic molecule described above, alpha can refers to either 5′or 3′, and the corresponding beta to either 3′ or 5′, respectively.

The region of complementary in each patch pair, or between a given patch and corresponding flap, is preferably about 20 to 5,000 nucleotides. In certain embodiments, the region of complementary is about 20 to 100 nucleotides, or about 5 to 50 nucleotides.

In the synthetic molecules described above, each flap is preferably about 50 to 5,000 nucleotides in length. In certain embodiments, each flap is about 50 to 150 nucleotides.

The synthetic molecules described above may further comprise a target-specific region which binds to a target molecule. The target-specific region can be attached to the beta or alpha end of said strand.

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In certain embodiments, the synthetic molecule described above comprise at least ten patch pairs, or at least fifty patch pairs.

In the synthetic molecules described above, the strand, or scaffold, can be a linearized vector, such as linearized M13.

The synthetic molecule described above may further comprise (a) a first label attachment region to which are attached (directly or indirectly) one or more label monomers that emit light constituting a first signal; (b) a second label attachment region, which is non-overlapping with the first label attachment region, to which is attached one or more label monomers that emit light constituting a second signal; (c) a third label attachment region, which is non-overlapping with the first and second label attachment regions, to which is attached one or more label monomers that emit light constituting a third signal; wherein each attachment region comprises a plurality of patch pairs; wherein the first and second signals are spectrally distinguishable; wherein the second and third signals are spectrally distinguishable; wherein the first and second signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the second and third signals are not spatially resolvable under conditions that can be used to detect said first, second and third signals; wherein the first and third signals are spatially resolvable under conditions that can be used to detect said first, second and third signals; and wherein the identities of the first, second and third signals and the locations of the first and third signal relative to each other constitute at least part of a code that identifies the target molecule.

5.4 LABEL MONOMERS

The nanoreporters of the present invention can be labeled with any of a variety of label monomers, such as a radioisotope, fluorochrome, dye, enzyme, nanoparticle, chemiluminescent marker, biotin, or other monomer known in the art that can be detected directly (*e.g.*, by light emission) or indirectly (*e.g.*, by binding of a fluorescently-labeled antibody). Generally, one or more of the label attachments regions in the nanoreporter is labeled with one or more label monomers, and the signals emitted by the label monomers attached to the label attachment regions of a nanoreporter constitute a code that identifies the target to which the target-specific region of the nanoreporter binds. In certain embodiments, the lack of a given signal from the label attachment region (*i.e.*, a "dark" spot)

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can also constitute part of the nanoreporter code. An example of a dark spot is depicted at position 12 of the nanoreporter in Figure 1A.

Radioisotopes are an example of label monomers that can be utilized by the invention. Several radioisotopes can be used as label monomers for labeling nucleotides or proteins, including, for example, ^{32}P, ^{33}P, ^{35}S, ^{3}H, and ^{125}I. These radioisotopes have different half-lives, types of decay, and levels of energy which can be tailored to match the needs of a particular experiment. For example, ^{3}H is a low energy emitter which results in low background levels, however this low energy also results in long time periods for autoradiography. Radioactively labeled ribonucleotides, deoxyribonucleotides and amino acids are commercially available. Nucleotides are available that are radioactively labeled at the first, or α, phosphate group, or the third, or γ, phosphate group. For example, both [α -^{32}P] dATP and [γ -^{32}P] dATP are commercially available. In addition, different specific activities for radioactively labeled nucleotides are also available commercially and can be tailored for different experiments.

Another example of label monomers that can be utilized by the invention are fluorophores. Several fluorophores can be used as label monomers for labeling nucleotides including, for example, fluorescein, tetramethylrhodamine, and Texas Red. Several different fluorophores are known, and more continue to be produced, that span the entire spectrum. Also, different formulations of the same fluorophore have been produced for different applications. For example, fluorescein, can be used in its isothiocynanate form (FITC), as mixed isomer or single isomer forms of carboxyfluorescein succinimidyl ester (FAM), or as isomeric dichlorotriazine forms of fluorescein (DTAF). These monomers are chemically distinct, but all emit light with a peak between 515-520 nm, thereby generating a similar signal. In addition to the chemical modifications of fluorescein, completely different fluorophores have been synthesized that have the same or very similar emission peaks as fluorescein. For example, the Oregon Green dye has virtually superimposable excitation and emission spectra compared to fluorescein. Other fluorophores such as Rhodol Green and Rhodamine Green are only slightly shifted in their emission peaks and so also serve functionally as substitutes for fluorescein. In addition, different formulations or related dyes have been developed around other fluorophores that emit light in other parts of the spectrum.

Non-radioactive and non-fluorescent label monomers are also available. For example, biotin can be attached directly to nucleotides and detected by specific and high

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affinity binding to avidin or streptavidin which has been chemically coupled to an enzyme catalyzing a colorimetric reaction (such as phosphatase, luciferase, or peroxidase). Digoxigenin labeled nucleotides can also similarly be used for non-isotopic detection of nucleic acids. Biotinylated and digoxigenin-labeled nucleotides are commercially available.

Very small particles, termed nanoparticles, also can be used as label monomers to label nucleic acids. These particles range from 1-1000 nm in size and include diverse chemical structures such as gold and silver particles and quantum dots.

When irradiated with angled incident white light, silver or gold nanoparticles ranging from 40-120 nm will scatter monochromatic light with high intensity. The wavelength of the scattered light is dependent on the size of the particle. Four to five different particles in close proximity will each scatter monochromatic light which when superimposed will give a specific, unique color. The particles are being manufactured by companies such as Genicon Sciences. Derivatized silver or gold particles can be attached to a broad array of molecules including, proteins, antibodies, small molecules, receptor ligands, and nucleic acids. For example, the surface of the particle can be chemically derivitized to allow attachment to a nucleotide.

Another type of nanoparticle that can be used as a label monomer are quantum dots. Quantum dots are fluorescing crystals 1-5 nm in diameter that are excitable by a large range of wavelengths of light. These crystals emit light, such as monochromatic light, with a wavelength dependent on their chemical composition and size. Quantum dots such as CdSe, ZnSe, InP, or InAs possess unique optical properties.

Many dozens of classes of particles can be created according to the number of size classes of the quantum dot crystals. The size classes of the crystals are created either 1) by tight control of crystal formation parameters to create each desired size class of particle, or 2) by creation of batches of crystals under loosely controlled crystal formation parameters, followed by sorting according to desired size and/or emission wavelengths. Use of quantum dots for labeling particles, in the context of the present invention, is new, but is old in the art of semiconductors. Two examples of earlier references in which quantum dots are embedded within intrinsic silicon epitaxial layers of semiconductor light emitting/detecting devices are United States Patent Nos. 5,293,050 and 5,354,707 to Chapple Sokol, *et al.*

In specific embodiments, one or more of the label attachments regions in the nanoreporter is labeled with one or more light-emitting dyes, each label attachment region containing, directly or indirectly, one or more label monomers. The light emitted by the

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dyes can be visible light or invisible light, such as ultraviolet or infra red light. In exemplary embodiments, the dye is a fluorescence resonance energy transfer (FRET) dye; a xanthene dye, such as fluorescein and rhodamine; a dye that has an amino group in the alpha or beta position (such as a naphthylamine dye, 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalende sulfonate and 2-p-touidinyl-6-naphthalene sulfonate); a dye that has 3-phenyl-7-isocyanatocoumarin; an acridine, such as 9-isothiocyanatoacridine and acridine orange; a pyrene, a bensoxadiazole and a stilbene; a dye that has 3-(ε-carboxypentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CYA); 6-carboxy fluorescein (FAM); 5&6-carboxyrhodamine-110 (R110); 6-carboxyrhodamine-6G (R6G); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); 6-carboxy-X-rhodamine (ROX); 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE); ALEXA Fluor™; Cy2; Texas Red and Rhodamine Red; 6-carboxy-2',4,7,7'-tetrachlorofluorescein (TET); 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein (HEX); 5-carboxy-2',4',5',7'-tetrachlorofluorescein (ZOE); NAN; NED; Cy3; Cy3.5; Cy5; Cy5.5; Cy7; and Cy7.5; Alexa Fluor 350; Alexa Fluor 488; Alexa Fluor 532; Alexa Fluor 546; Alexa Fluor 568; Alexa Fluor 594; or Alexa Fluor 647.

The label monomers can be incorporated into a nanoreporter at different stages of its assembly, or into a component (*e.g.*, a "flap" or of the nanoreporter prior to its assembly into the nanoreporter.

A label monomer can be directly attached to a nucleotide using methods well known in the art. Nucleotides can also be chemically modified or derivitized in order to attach a label monomer. For example, a fluorescent monomer such as a fluorescein molecule can be attached to dUTP (deoxyuridine-triphosphate) using a four-atom aminoalkynyl group. Each label monomer is attached to a nucleotide making a label monomer: nucleotide complex.

This label monomer: nucleotide complex can be incorporated into nucleic acids (for example, a DNA patch or a detection oligonucleotide) in a variety of ways. For example, a label monomer: nucleotide complex can be incorporated at only one location within a nucleic acid or at two or more locations within a nucleic acid.

Amine-reactive and thiol-reactive fluorophores are available and used for labeling nucleotides and biomolecules. Generally, nucleotides are fluorescently labeled during chemical synthesis, for example, incorporation of amines or thiols during nucleotide synthesis permit addition of fluorophores. Fluorescently labeled nucleotides are commercially available. For example, uridine and deoxyuridine triphosphates are available that are conjugated to ten different fluorophores that cover the spectrum.

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A nucleotide can be attached to a label monomer first and then be incorporated into a nucleic acid. Alternatively, an existing nucleic acid can be labeled by attaching a label monomer to a nucleotide within the nucleic acid. For example aminoallyl- ("AA-") modified UTP nucleotides can be incorporated into the RNA product during transcription. In various embodiments, 20% or more of UTP nucleotides in a transcription reaction to generate RNA patches are AA modified. In various embodiments, about 20% to 100%, 20% to 80%, 30 to 80%, 40 to 60% or 50% to 75% of UTPs in a transcription reaction are AA-modified, in a preferred embodiment, approximately 50% of UTPs in a transcription reaction are AA-modified.

In addition, for example, different types of label monomer: nucleotide complexes can be incorporated into a single acid nucleic acid, where one component of the nanoreporter code comprises more than one type of signal.

Fluorescent dyes that can be bound directly to nucleotides can also be utilized as label monomers. For example, FAM, JOE, TAMRA, and ROX are amine reactive fluorescent dyes that have been attached to nucleotides and are used in automated DNA sequencing. These fluorecently labeled nucleotides, for example, ROX-ddATP, ROX-ddCTP, ROX-ddGTP and ROX-ddUTP, are commercially available.

Other types of label monomers that may be used to label a nanoreporter are quantum dots. Due to their very small size the quantum dots can be coupled into oligonucleotides directly without affecting the solubility or use of the oligonucleotide. In a preferred embodiment, only one oligonucleotide molecule is coupled to each nanoparticle. To synthesize an oligonucleotide-nanoparticle complex in a 1:1 ratio by conventional batch chemistry, both the oligonucleotide and the nanoparticle require a single reactive group of different kinds that can be reacted with each other. For example, if an oligonucleotide has an amino group and a nanoparticle has an aldehyde group, these groups can react to form a Schiff base. An oligonucleotide can be derivitized to attach a single amino or other functional group using chemistry well known in the art. However, when a nanoparticle is derivatized, it is covered with a chemical reagent which results in coating the entire surface of the nanoparticle with several functional groups.

The invention provides a method of coupling one oligonucleotide to one nanoparticle by chemically coupling the oligonucleotide on a solid surface such as the glass support used for the oligonucleotide synthesis.

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For example, commercially available resins for oligonucleotide synthesis such as long chain alkylamino controlled pore glass (lcaa CPG) can be used.

Alternatively, a flat surface such as a derivitized microscope slide can be used. The surface density of the nascent oligonucleotide chains should be lower than the diameter of the nanoparticle. This can be achieved by either choosing a glass support with low surface density of the reactive groups, or by using diluted reagent for the first step of the oligonucleotide synthesis so that the surface is not saturated. Another point of consideration when using the standard glass matrices for oligonucleotide synthesis is to use a pore diameter higher than the nanoparticle diameter to ensure the flow of the reagents. For example, an oligonucleotide can be synthesized on a diluted basis relative to the solid support, for example one tenth of a normal synthesis, to ensure good spacing of the oligonucleotides on the glass support. After the oligonucleotide is synthesized with a reactive functional group, for example, an amino group, derivitized nanoparticles are passed over the glass support to react with the oligonucleotides. A sufficiently large pore size of the glass support can be chosen to prevent clogging with nanoparticles. For example, a pore size of about 200 nm can be used. After the reaction is complete, un-reacted groups on the nanoparticle can be blocked and the complexes can be uncoupled from the glass support.

5.5 THE NANOREPORTER CODE

5.5.1 DUAL NANOREPORTERS

A nanoreporter whose components exist in two molecular entities is referred to as a dual nanoreporter. In a dual nanoreporter, generally each component contains a target-specific sequence, which improves the specificity of and binding kinetics of the nanoreporter to its target. The two different target-specific sequences are designed or selected such that each recognizes a different portion of a target molecule.

Figures 1A-1C illustrates embodiments of the invention involving dual nanoreporters. In Figures 1A and 1B, each of the two component of the nanoreporter is labeled, such that the nanoreporter's spectral code is formed only when the two components of the nanoreporter come together upon binding of the dual nanoreporter to its target molecule. However, in a dual nanoreporter, it is not necessary that both components are labeled. For example, as depicted in Figure 1C, one component of a dual nanoreporter is labeled with the nanoreporter code, and the other component attached to an affinity tag (arrow) that is useful to immobilize the nanoreporter for stretching an visualization.

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5.5.2 REGISTERS

The term "register" refers to a set of alternating (every other) label attachment regions. Registers are useful where it is desirable to label adjacent label attachment regions without a spacer region, and where the signal emanating from adjacent label attachment regions cannot be spatially resolved using the desired method of detection. Thus, the signals detected with use of a register is that form by the alternating, rather than adjacent, label attachment regions. Signals detected from a plurality of registers (*e.g.*, that together are all the label attachment regions) can be combined to form a nanoregister code. Generally when using registers, adjacent label attachment regions are labeled with spectrally distinguishable label monomers.

Examples of registers are depicted in Figures 3 and 5. For example, in Figure 3A-3B, there are 8 label attachment regions 1-8. Alternating label attachment regions 1, 3, 5 and 7 form one register, and label attachment regions 2, 4, 6 and 8 form another register. In Figure 3A, only one of the registers (1, 3, 5 and 7) is labeled; in Figure 3B, both registers are labeled.

5.6 AFFINITY TAGS

A variety of affinity tags known in the art may be used to purify and/or immobilize nanoreporters.

Where an affinity tag is used to immobilize a nanoreporter for the purpose of detection or imaging, it may be referred to herein as an "anchor." In a preferred embodiment, a biotin anchor is attached to the nanoreporter, allowing immobilization of the nanoreporter on a streptavidin coated slide.

An affinity tag that can be used to used for attachment to beads or other matrixes for a variety of useful applications including but not limited to purification.

Non-limiting examples of suitable affinity tags are provided below. It should be understood that most affinity tags could serve dual purposes: both as anchors for immobilization of the nanoreporters and tags for purification of the nanoreporters (whether fully or only partially assembled) or their components.

In certain embodiments, the affinity tag is a protein monomer. Examples of protein monomers include, but are not limited to, the immunoglobulin constant regions (see Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology,

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Vol. 2, Ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan *et al.*, 1987, Gene 67:21-30), and various cellulose binding domains (U.S. Pat. Nos. 5,496,934; 5,202,247; 5,137,819; Tomme *et al.*, 1994, Protein Eng. 7:117-123), etc. Other affinity tags are recognized by specific binding partners and thus facilitate isolation and immobilization by affinity binding to the binding partner, which can be immobilized onto a solid support. For example, the affinity tag can be an epitope, and the binding partner an antibody. Examples of such epitopes include, but are not limited to, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemagglutinin (HA) epitope, or digoxigenin ("DIG"). In other embodiments, the affinity tag is a protein or amino acid sequence that is recognized by another protein or amino acid, for example the avidin/streptavidin and biotin.

In certain aspects of the invention, the affinity tag is a nucleotide sequence. A large variety of sequences of about 8 to about 30 bases, more preferably of about 10 to about 20 bases, can be used for purification and immobilization of nanoreporters, and the sequence can be tandemly repeated (*e.g.*, from 1 to 10 tandem repeats). Such a sequence is preferably not widely represented (that is, present in fewer than 5% of the genes, more preferably, present in fewer than 3% of the genes, and, most preferably, present in fewer than 1% of the genes) in the sample being assayed (for example, where the nanoreporter is used for detection of human cellular RNA, the sequence is preferably not widely represented in the human genome); have little or no secondary structure or self-complementarity either internally or with copies of itself when multimerized (that is, all secondary structures of the multimerized tag preferably have a Tm less than 25°C at 1 M NaCl); have no significant identity or complementarity with scaffold or segment sequences (that is, the Tm of complementary sequences is preferably less than 25°C at 0.2 M NaCl); and have a Tm of about 35-65°C, more preferably about 40-50°C, in 50 mM Na^+.

In certain embodiments, different sequences are used as purification and immobilization tags. In this case, for example, the purification tag can be as described above, but the immobilization tag can be in the range of 10 to 100 bases, with a Tm up to 95°C in 50 mM Na^+. An alternative embodiment would be to have the purification tag nested within the immobilization tag (*e.g.*, the affinity tag would comprise a 25-base sequence of which 15 bases are used as a purification tag and the entire 25 bases are used as the immobilization tag).

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In certain instances, the affinity tag can be used for labeling a nanoreporter in addition to purifying or immobilizing the nanoreporter.

As will be appreciated by those skilled in the art, many methods can be used to obtain the coding region of the affinity tags, including but not limited to, DNA cloning, DNA amplification, and synthetic methods. Some of the affinity tags and reagents for their detection and isolation are available commercially.

5.7 TARGET-SPECIFIC SEQUENCES

The term "target-specific sequence" refers to a molecular entity that is capable of binding a target molecule. In the context of a nanoreporter, the target-specific sequence is attached to the nanoreporter scaffold.

The target specific sequence is generally an amino acid sequence (*i.e.*, a polypeptide or peptide sequence) or a nucleic acid sequence.

In specific embodiments, where the target-specific sequence is an amino acid sequence, the target-specific sequence is an antibody fragment, such as an antibody Fab' fragment, a single chain Fv antibody.

The target-specific sequence is preferably a nucleic acid sequence, and is most preferably within an oligonucleotide that is either covalently attached (*e.g.*, by ligation) or noncovalently attached (*e.g.*, by hybridization) to the nanoreporter scaffold. A target-specific nucleic acid sequence is preferably at least 15 nucleotides in length, and more preferably is at least 20 nucleotides in length. In specific embodiments, the target-specific sequence is approximately 10 to 500, 20to 400, 30 to 300, 40 to 200, or 50 to 100 nucleotides in length. In other embodiments, the target-specific sequence is approximately 30 to 70, 40 to 80, 50 to 90, or 60 to 100, 30 to 120, 40 to 140, or 50 to 150 nucleotides in length.

A target-specific nucleotide sequence preferably has a Tm of about 65-90°C for each probe in 825 mM Na^+ (5X SSC), most preferably about 78-83°C.

In certain preferred embodiments, the target specific sequence of each probe of a dual nanoreporter is about 35 to 100 nucleotides (for a total target sequence of about 70 to 200 nucleotides, covered by 2 probes), most preferably about 40 to 50 nucleotides for each probe (for a total of about 80 to 100 nucleotides).

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5.8 TARGET MOLECULES

The term "target molecule" is the molecule detected or measured by binding of a labeled nanoreporter whose target-specific sequence(s) recognize (are specific binding partners thereto). Preferably, an target molecule can be, but is not limited to, any of the following: DNA, cDNA, RNA, mRNA, peptide, a polypeptide/protein (*e.g.*, a bacterial or viral protein or an antibody), a lipid, a carbohydrate, a glycoprotein, a glycolipid, a small molecule, an organic monomer, or a drug. Generally, a target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA.

A target molecule can be part of a biomolecular sample that contains other components or can be the sole or major component of the sample. A target molecule can be a component of a whole cell or tissue, a cell or tissue extract, a fractionated lysate thereof or a substantially purified molecule. The target molecule can be attached in solution or solid-phase, including, for example, to a solid surface such as a chip, microarray or bead. Also. the target molecule can have either a known or unknown structure or sequence.

In certain specific embodiments, that target molecule is not a chromosome. In other specific embodiments, the target molecule is no greater than 1,000 kb (or 1 mb) in size, no greater than 500 kb in size, no greater than 250 kb in size, no greater than 175 kb in size, no greater than 100 kb in size, no greater than 50 kb in size, no greater than 20 kb in size, or no greater than 10 kb in size. In yet other specific embodiments, the target molecule is isolated from its cellular milieu.

In specific, non-limiting embodiments, the target molecule is one of the following antibodies or an antigen recognized by one of the following antibodies: anti-estrogen receptor antibody, an anti-progesterone receptor antibody, an anti-p53 antibody, an anti-Her-2/neu antibody, an anti-EGFR antibody, an anti-cathepsin D antibody, an anti-Bcl-2 antibody, an anti-E-cadherin antibody, an anti-CA125 antibody, an anti-CA15-3 antibody, an anti-CA19-9 antibody, an anti-c-erbB-2 antibody, an anti-P-glycoprotein antibody, an anti-CEA antibody, an anti-retinoblastoma protein antibody, an anti-ras oncoprotein antibody, an anti-Lewis X antibody, an anti-Ki-67 antibody, an anti-PCNA antibody, an anti-CD3 antibody, an anti-CD4 antibody, an anti-CD5 antibody, an anti-CD7 antibody, an anti-CD8 antibody, an anti-CD9/p24 antibody, an anti-CD10 antibody, an anti-CD11c antibody, an anti-CD13 antibody, an anti-CD14 antibody, an anti-CD15 antibody, an anti-CD19 antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD23 antibody, an

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anti-CD30 antibody, an anti-CD31 antibody, an anti-CD33 antibody, an anti-CD34 antibody, an anti-CD35 antibody, an anti-CD38 antibody, an anti-CD41 antibody, an anti-LCA/CD45 antibody, an anti-CD45RO antibody, an anti-CD45RA antibody, an anti-CD39 antibody, an anti-CD100 antibody, an anti-CD95/Fas antibody, an anti-CD99 antibody, an anti-CD106 antibody, an anti-ubiquitin antibody, an anti-CD71 antibody, an anti-c-myc antibody, an anti-cytokeratins antibody, an anti-vimentins antibody, an anti-HPV proteins antibody, an anti-kappa light chains antibody, an anti-lambda light chain antibody, an anti-melanosome antibody, an anti-prostate specific antigen antibody, an anti-S-100 antibody, an anti-tau antigen antibody, an anti-fibrin antibody, an anti-keratins antibody, an anti-Tn-antigen antibody receptor protein, a lymphokine, an enzyme, a hormone, a growth factor, or a nucleic acid binding protein, a ligand for a cell adhesion receptor; a ligand for a signal transduction receptor; a hormone; a molecule that binds to a death domain family molecule; an antigen; a viral particle, a viral coating protein or fragment thereof, a toxic polypeptide selected from the group consisting of: (a) ricin, (b) Pseudomonas exotoxin (PE); (c) bryodin; (d) gelonin; (e) α-sarcin; (f) aspergillin; (g) restrictocin; (h) angiogenin; (i) saporin; (j) abrin; (k) pokeweed antiviral protein (PAP); and (l) a functional fragment of any of (a)-(k); a cytokine, or a soluble cytokine selected from the group consisting of erythropoietin, interleukins, interferons, fibroblast growth factors, transforming growth factors, tumor necrosis factors, colony stimulating factors and epidermal growth factor, Class I MHC antigens, class II MHC antigens, internalizing cell-surface receptors and/or viral receptors.

In specific, non-limiting embodiments, the target molecule is an antigen such as alpha fetoprotein, alpha-1 antitrypsin, α-2 macroglobulin, adiponectin, apoliprotein-A-1, apoliprotein-CIII, apoliprotein-H, BDNF, β-2 microglobulin, C reactive protein, calcitonin, cancer antigen 19-9, cancer antigen 125, CEA, CD 40, CD 40 ligand, complement 3, CK-MB, EGF, ENA-78, endothelin-1, enrage, eotaxin, erythropoietin, Factor VII, FABP, ferritin, FGF-basic, fibrinogen, G-CSF, GST, GM-CSF, growth hormone, haptoglobin, ICAM-1, IFN-gamma, IgA, IgE, IGF-1, IgM, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-16, insulin, leptin, lipoprotein (a), lymphotactin, MCP-1, MDC, MIP-1α, MIP-1β, MMP-2, MMP-3, MMP-9, myeloperoxidase, myoglobin, PAI-1, PAP, PAPP-A, SGOT, SHBG, PSA (free), RANTES, serum amyloid P, stem cell factor, TBG, thrombopoietin, TIMP-1, tissue factor, TNF-α, TNF-β, TNF RII, TSH, VCAM-1, VEGF, or vWF.

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In some embodiments, the target molecule is an autoimmune related molecule such as ASCA, β-2 glycoprotein, C1q, centromere Prot. B, collagen type 1, collagen type 2, collagen type 4, collagen type 6, Cyto P450, ds DNA, histone, histone H1, histone H2A, histone H2B, histone H3, histone H4, HSC-70, HSP-32, HSP-65, HSP-71, HSP-90α, HSP-90β, insulin, JO-1, mitochondrial, myeloperoxidase, pancreatic islet cells, PCNA, PM-1, PR3, ribosomal P, RNP-A, RNP-C, RNP, Sel-70, Smith, SSA, SSB, T3, T4, thyroglobulin, tTG, (celiac disease), or thyroid microsomal.

In some embodiments, the target molecule is a component isolated from an infectious agent, such as Cholera Toxin, Cholera Toxin β, *Campylobacter jejuni*, cytomegalovirus, Diptheria toxin, Epstein-Barr NA, Epstein-Barr EA, Epstein-Barr VCA, *Heliobacter pylori*, HBV core, HBV envelope, HBV surface (Ad), HBV surface (Ay), HCV core, HCV NS3, HCV NS4, HCV NS5, hepatitis A, hepatitis D, HEV orf2 3KD, HEV orf2 6 KD, HEV orf 3KD, HIV-1 p24, HIV-1 gp41, HIV-1 gp120, HPV, HSV-1/2, HSV-1 gD, HSV-2 gD, HTLV-1/2, influenza A, influenza A H3N2, influenza B, *Leishmania donorani*, Lyme disease, mumps, *M. pneumonia*, *M. tuberculosis*, parainfluenza 1, parainfluenza 2, parainfluenza 3, polio virus, RSV, Rubella, Rubeola, Streptolysin O, Tetanus Toxin, *T. pallidum* 15kD, *T. pallidum* p47, *T. cruzi*, Toxoplasma, Varicella zoster.

5.9 NANOREPORTER POPULATIONS

The present invention provides nanoreporter or nanoreporter label unit populations, for example nanoreporter or nanoreporter label unit libraries, that contain at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 75, at least 100, at least 200, at least 300, at least 400, at least 500, at least 750, or at least 1,000 unique nanoreporters or nanoreporter label units, respectively. As used herein, "unique" when used in reference to a nanoreporter or nanoreporter label units within a population is intended to mean a nanoreporter or label unit that has a code that distinguishes it from other nanoreporters or label units in the same population.

In specific embodiments, the present invention provides nanoreporter populations with at least 5,000, at least 10,000, at least 20,000 or at least 50,000 unique nanoreporters or nanoreporter label units.

The nanoreporters in a population of nanoreporters can be singular nanoreporters, dual nanoreporters, or a combination thereof. The nanoreporters can be labeled or unlabeled.

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The size of a nanoreporter population and the nature of the target-specific sequences of the nanoreporters within it will depend on the intended use of the nanoreporter. Nanoreporter populations can be made in which the target-specific sequences correspond to markers of a given cell type, including a diseased cell type. In certain embodiments, nanoreporters populations are generated in which the target-specific sequences represent at least 0.1%, at least 0.25%, at least 0.5%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, or at least 70% of the different type of transcripts in a cell. In certain embodiments, nanoreporters populations are generated in which the target-specific sequences represent at least 0.1%, at least 0.25%, at least 0.5%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, or at least 70% of the different genes in a cell. In yet other embodiments, nanoreporter populations are generated in which at least some of the target-specific sequences represent rare transcripts in a cell or tissue. Such nanoreporter populations preferably represent at least 5 rare transcripts. In specific embodiments, such nanoreporter populations represent at least 10, at least 20, at least 30, at least 40 or at least 50 rare transcripts.

In a specific embodiment, the cell or tissue is a mammalian cell or tissue, and more preferably is a human cell or tissue.

In certain embodiments, the nanoreporter population is a diagnostic or prognostic nanoreporter populations. For example, a diagnostic nanoreporter population can be generated that is useful for screening blood products, in which the target-specific sequences bind to the nucleic acids of contaminating viruses such the hepatitis B, hepatitis C, and the human immunodeficiency virus. Alternatively, the diagnostic nanoreporter population may contain target-specific sequences corresponding to cellular disease markers, such as tumor antigens. Prognostic nanoreporter populations generally include target-specific markers that represent different stages of a given disease such as cancer. By selecting appropriate target-specific sequences, a nanoreporter population can be used both to diagnose and prognose disease.

5.10 BIOMOLECULAR SAMPLES

The nanoreporter systems of the invention can be used to detect target molecule in any biomolecular sample. As will be appreciated by those in the art, the sample may

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comprise any number of things, including, but not limited to: cells (including both primary cells and cultured cell lines), cell lysates or extracts (incuding but not limited to RNA extracts; purified mRNA), tissues and tissue extracts (incuding but not limited to RNA extracts; purified mRNA); bodily fluids (including, but not limited to, blood, urine, serum, lymph, bile, cerebrospinal fluid, interstitial fluid, aqueous or vitreous humor, colostrum, sputum, amniotic fluid, saliva, anal and vaginal secretions, perspiration and semen, a transudate, an exudate (*e.g.*, fluid obtained from an abscess or any other site of infection or inflammation) or fluid obtained from a joint (*e.g.*, a normal joint or a joint affected by disease such as rheumatoid arthritis, osteoarthritis, gout or septic arthritis) of virtually any organism, with mammalian samples being preferred and human samples being particularly preferred; environmental samples (including, but not limited to, air, agricultural, water and soil samples); biological warfare agent samples; research samples including extracellular fluids, extracellular supernatants from cell cultures, inclusion bodies in bacteria, cellular compartments, cellular periplasm, mitochondria compartment, etc.

The biomolecular samples can be indirectly derived from biological specimens. For example, where the target molecule of interest is a cellular transcript, *e.g.*, a messenger RNA, the biomolecular sample of the invention can be a sample containing cDNA produced by a reverse transcription of messenger RNA. In another example, the biomolecular sample of the invention is generated by subjecting a biological specimen to fractionation, *e.g.*, size fractionation or membrane fractionation.

The biomolecular samples of the invention may be either "native," *i.e.*, not subject to manipulation or treatment, or "treated," which can include any number of treatments, including exposure to candidate agents including drugs, genetic engineering (e.g. the addition or deletion of a gene), etc.

5.11 SEPARATION OF LABEL MONOMERS

In addition to detecting an overall signal generated from a labeled nanoreporter, the invention provides for the determination of the spatial location of signals emanating from the label monomers (*i.e.*, spots) on a nanoreporter, each spot representing the aggregate signal from label monomers attached to a given label attachment region. A spot may contain signals of the same wavelength or of different wavelengths. Thus, the nature of the spots on a nanoreporter and their location constitutes the nanoreporter code code.

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Any of a variety of means can be used to "stretch" the nanoreporter to separate the individual spots. For example, a nanoreporter can be stretched using a flowstretch technique (Henegariu *et al.*, 2001, Biotechniques 31:246-250), a receding meniscus technique (Yokota *et al.*, 1997, Nuc. Acids Res. 25:1064-1070) or an electrostretching technique (Matsuura *et al.*, 2001, Nuc. Acids Res. 29: E79).

The use of flow-stretching, receding meniscus, or electro-stretching techniques allows for the separation of the label attachment regions within a nanoreporter so that one can determine spatially where a particular signal is positioned in the nanoreporter. Therefore, unique nanoreporters that have the same combination of label monomers and the same overall signal can be differentiated from one another based on the location of those label monomers within the nanoreporter.

This ability to locate the position of a label attachment region or spot within a nanoreporter allows for the position of the signal(s) emitted by the label monomers in each label attachment region to be used as a distinguishing characteristic when generating a set of unique nanoreporters. Hence, a complex set of nanoreporters can be generated using the same combination of starting label monomers by varying the positions of the label monomers within a nanoreporter.

Prior to stretching a nanoreporter, it is preferable to immobilize the nanoreporter to a solid surface using an affinity tag, as described in Section 5.6 above.

In certain aspects of the invention, one end of a nanoreporter is immobilized, either through specific or non-specific binding to a solid surface, the nanoreporter is stretched, and then the other end of the reporter is immobilized, also either through specific or non-specific binding to a solid surface. Accordingly, the nanoreporter is "frozen" in its stretched, or extended, state, to facilitate resolution of the nanoreporters code by detecting and/or imaging the signals emitted by the label monomers attached to a nanoreporter and their locations relative to one another. These aspects of the invention are described below in Section 5.12.

5.12 IMMOBILIZATION OF STRETCHED NANOREPORTERS

The present invention provides methods and compositions that facilitate the identification of primary structures of a variety of nanoreporters. In certain aspects, the present invention provides methods for the selective immobilization of nanoreporters in an extended state. According to the invention, a nanoreporter can be selectively immobilized

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while fully extended under whatever force is used for the extension. In addition, the methods of the invention facilitate the selective immobilization of extended nanoreporters that are oriented with respect to each other. In other words, according to the methods of the invention, a plurality of nanoreporters can readily be immobilized in the same orientation with respect to each other.

In one aspect, the present invention provides methods for selectively immobilizing a nanoreporter in an extended state. For the methods of this aspect of the invention, generally, a first portion of the nanoreporter is immobilized by any technique known to those of skill in the art. Indeed, the technique for immobilizing the first portion of the nanoreporter is not critical to many embodiments of the invention. In certain embodiments, the first portion of the nanoreporter can be immobilized selectively or non-selectively. In certain embodiments the first portion is immobilized by one or more covalent bonds. In certain embodiments, the first portion is immobilized by one or more non-covalent bonds. Exemplary immobilized first portions are described in the sections below.

With an immobilized first portion, the nanoreporter can be extended by any technique for extending a nanoreporter apparent to those of skill in the art. In certain embodiments, the technique for extending the nanoreporter is not critical for the methods of the invention. In certain embodiments, the technique for extending the nanoreporter appropriate for the class of nanoreporter according to the judgment of one of skill in the art. In certain embodiments, the nanoreporter is extended by application of a force capable of extending the nanoreporter. The force can be any force apparent to one of skill in the art for extending the nanoreporter. Exemplary forces include gravity, hydrodynamic force, electromagnetic force and combinations thereof. Specific techniques for extending the nanoreporter are described in the sections below.

The nanoreporter is in an extended state if it would be recognized as extended by one of skill in the art. In certain embodiments, the nanoreporter is in an extended state when it is in the field of a force capable of extending the nanoreporter. In certain embodiments, the nanoreporter is in an extended state when its average hydrodynamic radius is more than double the average hydrodynamic radius of the nanoreporter in its native state as recognized by those of skill in the art.

In this aspect of the invention, the methods generally comprise the step of selectively immobilizing a second portion of the nanoreporter while it is in an extended state. This can result in an immobilized nanoreporter that is extended between the first and the second

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portion. Remarkably, since the nanoreporter is selectively immobilized while extended, that extension can be preserved in the immobilized nanoreporter. Generally, the first portion and the second portion of the nanoreporter are not the same.

The selective immobilization can be according to any technique for selective immobilization of a portion of a nanoreporter apparent to those of skill in the art. The selective immobilization can be through, for example, the formation of one or more covalent bonds or one or more non-covalent bonds, or both. Particular examples of selective immobilization techniques are described in the sections below. In particular embodiments, one or more binding pairs are used to immobilize the second portion of the nanoreporter.

The second portion can be immobilized onto any substrate apparent to those of skill in the art. The substrate can be any substrate judged to be useful for immobilization known to those of skill in the art. In certain embodiments, the second portion can be immobilized to another molecule. Further useful substrates include surfaces, membranes, beads, porous materials, electrodes, arrays and any other substrate apparent to those of skill in the art.

In another aspect, the present invention provides a composition comprising a selectively immobilized, extended nanoreporter. The compositions generally comprise a substrate and an extended nanoreporter selectively immobilized onto the substrate. The substrate can be any substrate known to those of skill in the art. Exemplary substrates include those described in the sections below. At least two portions of the nanoreporter are immobilized onto the substrate, and the nanoreporter is in an extended state between the two portions. In certain embodiments, at least one portion of the nanoreporter is selectively immobilized onto the substrate. In certain embodiments, two or more portions of the nanoreporter are selectively immobilized onto the substrate. The nanoreporter can be extended and/or immobilized by any technique apparent to those of skill, including particularly the methods of the present invention.

In another aspect, the present invention provides methods for selectively immobilizing a nanoreporter in an oriented state. The nanoreporter can be any nanoreporter described above. In certain embodiments, the nanoreporter can be flexible, or in certain embodiments the nanoreporter can be rigid or semi-rigid. For the methods of this aspect of the invention, generally, a first portion of the nanoreporter is immobilized as described above. With an immobilized first portion, the nanoreporter can be oriented by any technique for extending a nanoreporter apparent to those of skill in the art. In certain embodiments, the technique for orienting the nanoreporter is not critical for the methods of

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the invention. In certain embodiments, the technique for orienting the nanoreporter appropriate for the class of nanoreporter according to the judgment of one of skill in the art. In certain embodiments, the nanoreporter is oriented by application of a force capable of orienting the nanoreporter. The force can be any force apparent to one of skill in the art for orienting the nanoreporter. Exemplary forces include gravity, hydrodynamic force, electromagnetic force and combinations thereof. Specific techniques for extending the nanoreporter are described in the subsections below.

The nanoreporter is in an oriented state if it would be recognized as oriented by one of skill in the art. In certain embodiments, the nanoreporter is in an oriented state when it is in the field of a force capable of orienting the nanoreporter. In certain embodiments, the nanoreporter is in an oriented state when its termini are arranged in parallel, as recognized by those of skill in the art, with the field of a force capable of orienting the nanoreporter. In certain embodiments, a plurality of nanoreporters is in an oriented state when the termini of the nanoreporters are arranged in parallel, as recognized by those of skill in the art,.

In this aspect of the invention, the methods generally comprise the step of selectively immobilizing a second portion of the nanoreporter while it is in an oriented state. This can result in an immobilized nanoreporter that is oriented between the first and the second portion. Remarkably, since the nanoreporter is selectively immobilized while extended, that orientation can be preserved in the immobilized nanoreporter. The selective immobilization can according to the methods described above.

In another aspect, the present invention provides a composition comprising a selectively immobilized, oriented nanoreporter. The compositions generally comprise a substrate and an oriented nanoreporter selectively immobilized onto the substrate. The substrate can be any substrate known to those of skill in the art. Exemplary substrates include those described in the sections below. At least two portions of the nanoreporter are immobilized onto the substrate, and the nanoreporter is in an oriented state between the two portions. In certain embodiments, at least one portion of the nanoreporter is selectively immobilized onto the substrate. In certain embodiments, both portions of the nanoreporter are selectively immobilized onto the substrate. The nanoreporter can be oriented and/or immobilized by any technique apparent to those of skill, including particularly the methods of the present invention.

The methods and compositions of the present invention can be used for any purpose apparent to those of skill in the art. For instance, the immobilized and extended and/or

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oriented nanoreporter can be used as a label for a substrate on which the nanoreporter is immobilized. The primary sequence of the immobilized and extended and/or oriented nanoreporter can be identified by any technique apparent to those of skill. Advantageously, immobilization of the extended and/or oriented nanoreporter can facilitate such techniques. In certain embodiments, the immobilized and extended and/or oriented nanoreporter can be used to guide the manufacture of nanopaths, for example to create nanowires or nanocircuits. Further uses for the immobilized and extended and/or oriented nanoreporters are described in the sections below.

All terms used herein have their ordinary meanings to those of skill in the art unless indicated otherwise. The following terms shall have the following meanings.

As used herein, the term "binding pair" refers to first and second molecules or moieties that are capable of selectively binding to each other, *i.e.* binding to each other with greater affinity than to other components in a composition. The binding between the members of the binding pair can be covalent or non-covalent. In certain embodiments, the binding is noncovalent. Exemplary binding pairs include immunological binding pairs (*e.g.*, any haptenic or antigenic compound in combination with a corresponding antibody or binding portion or fragment thereof, for example digoxigenin and anti-digoxigenin, fluorescein and anti-fluorescein, dinitrophenol and anti-dinitrophenol, bromodeoxyuridine and anti-bromodeoxyuridine, mouse immunoglobulin and goat anti-mouse immunoglobulin) and nonimmunological binding pairs (*e.g.*, biotin-avidin, biotin-streptavidin, hormone-hormone binding protein, receptor-receptor ligand (*e.g.*, acetylcholine receptor-acetylcholine or an analog thereof), IgG-protein A, lectin-carbohydrate, enzyme-enzyme cofactor, enzyme-enzyme inhibitor, complementary polynucleotide pairs capable of forming nucleic acid duplexes, and the like). For instance, immunoreactive binding members may include antigens, haptens, aptamers, antibodies (primary or secondary), and complexes thereof, including those formed by recombinant DNA methods or peptide synthesis. An antibody may be a monoclonal or polyclonal antibody, a recombinant protein or a mixture(s) or fragment(s) thereof, as well as a mixture of an antibody and other binding members. Other common binding pairs include but are not limited to, biotin and avidin (or derivatives thereof), biotin and streptavidin, carbohydrates and lectins, complementary nucleotide sequences (including probe and capture nucleic acid sequences), complementary peptide sequences including those formed by recombinant methods, effector and receptor molecules,

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hormone and hormone binding protein, enzyme cofactors and enzymes, enzyme inhibitors and enzymes, and so forth.

"Selective binding" refers to the any preferential binding of a pair of molecules or moieties for each other with respect to other molecules or moieties in a composition that would be recognized by one of skill in the art. In certain embodiments, a pair of molecules or moieties selectively binds when they preferentially bind each other compared to other molecules or moieties. Selective binding can include affinity or avidity, or both, of one molecule or moiety for another molecule or moiety. In particular embodiments, selective binding requires a dissociation constant (K_D) of less than about $1x10^{-5}$ M or less than about $1x10^{-6}$ M, $1x10^{-7}$ M, $1x10^{-8}$ M, $1x10^{-9}$ M, or $1x10^{-10}$ M. In contrast, in certain embodiments, non-selective binding has significantly less affinity, for example, a K_D greater than $1x10^{-3}$ M.

"Extended state" refers to a nanoreporter in a state that would be recognized as extended by one of skill in the art. In certain embodiments, a nanoreporter is in an extended state when it is extended relative to its native conformation in solution. In certain embodiments, a nanoreporter is in an extended state when it is in the field of a force capable of extending the nanoreporter. In certain embodiments, an extended state of a nanoreporter can be determined quantitatively. In such embodiments, those of skill in the art will recognize R as the end-to-end vector of the nanoreporter, *i.e.* the distance between two termini of the nanoreporter, and <R> as the average end-to-end vector such that 95% of R will be within 2<R> in a solution deemed appropriate to one of skill in the art. Exemplary solutions include, for example, a dilute solution of the nanoreporter in water or in a pH buffer. In particular embodiments, a nanoreporter is in an extended state when R is greater than 2.0<R>.

"Oriented state" refers to a nanoreporter in a state that would be recognized as oriented by one of skill in the art. In certain embodiments, a nanoreporter is in an oriented state when it is oriented relative to its native conformation in solution. In certain embodiments, the nanoreporter is oriented when it is arranged in parallel with the field of a force capable of orienting the nanoreporter. In certain embodiments, the nanoreporter is oriented when it is one of a plurality of nanoreporters that are arranged in parallel, as recognized by those of skill in the art.

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5.12.1 METHODS OF SELECTIVE IMMOBILIZATION

As described above, the present invention provides methods for the selective immobilization of a nanoreporter in an extended state. The nanoreporter, once selectively immobilized, can be used for any purpose apparent to those of skill in the art.

In certain embodiments, the nanoreporter is any polymer known to those of skill in the art. For instance, the nanoreporter can be a polysaccharide, a polypeptide or a polynucleotide. Useful polynucleotides include ribonucleic acids, deoxyribonucleic acids and other polynucleotides known to those of skill in the art.

The nanoreporter can be of any size that is sufficient to allow extension and immobilization of the nanoreporter according to the methods of the invention. In certain embodiments when the nanoreporter is a polynucleotide, the nanoreporter can have a length of greater than 500 bp, greater than 750 bp, greater than 1 kb, greater than 1.5 kb, greater than 2.0 kb, greater than 2.5 kb, greater than 3.0 kb, greater than 4.0 kb or greater than 5.0 kb. In certain embodiments, when the nanoreporter is a polypeptide, the nanoreporter can have a size of greater than 50 amino acids, greater than 100 amino acids, greater than 200 amino acids, greater than 300 amino acids, greater than 400 amino acids, greater than 500 amino acids, greater than 750 amino acids, greater than 1000 amino acids, greater than 1500 amino acids, greater than 2000 amino acids, greater than 2500 amino acids, greater than 3000 amino acids, greater than 4000 amino acids or greater than 5000 amino acids. In certain embodiments, when the nanoreporter is a polysaccharide, the nanoreporter can have a size of greater than 50 saccharides, greater than 100 saccharides, greater than 200 saccharides, greater than 300 saccharides, greater than 400 saccharides, greater than 500 saccharides, greater than 750 saccharides, greater than 1000 saccharides, greater than 1500 saccharides, greater than 2000 saccharides, greater than 2500 saccharides, greater than 3000 saccharides, greater than 4000 saccharides or greater than 5000 saccharides.

The nanoreporter can be a native nanoreporter as understood by those of skill in the art, or the nanoreporter can be a non-native nanoreporter. In certain embodiments, when the nanoreporter is a polypeptide, the nanoreporter can comprise only naturally occurring amino acids, or the nanoreporter can comprise naturally occurring amino acids and non-naturally occurring amino acids. The other amino acids can be any amino acids, or derivatives or analogs thereof, known to those of skill in the art. In certain embodiments, when the nanoreporter is a polynucleotide, the polynucleotide can comprise only naturally occurring nucleotides, or the polynucleotide can comprise naturally occurring nucleotides and non-

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naturally occurring nucleotides. In certain embodiments, when the nanoreporter is a polysaccharide, the polysaccharide can comprise only naturally occurring saccharides, or the polysaccharide can comprise naturally occurring saccharides and non-naturally occurring saccharides. In certain embodiments, the polymers can comprise only non-natural monomers. In further embodiments, the nanoreporter can comprise a plurality of classes of monomers, such as amino acids, nucleotides and/or saccharides.

In certain embodiments, the nanoreporter comprises only one primary, covalently linked chain of monomers. For instance, when the nanoreporter is a polypeptide, in certain embodiments, the nanoreporter comprises only one primary amino acid chain. When the nanoreporter is a polynucleotide, in certain embodiments, the nanoreporter is single stranded. In further embodiments, the nanoreporter comprises two primary, covalently linked chains of monomers. For instance, when the nanoreporter is a polypeptide, in certain embodiments, the nanoreporter comprises two primary amino acid chains. When the nanoreporter is a polynucleotide, in certain embodiments, the nanoreporter comprises two polynucleotide strands; in certain embodiments, the nanoreporter can be double stranded, in part or in whole. In further embodiments, the nanoreporter comprises three or more primary, covalently linked chains of monomers. For instance, when the nanoreporter is a polypeptide, in certain embodiments, the nanoreporter comprises three primary amino acid chains. When the nanoreporter is a polynucleotide, in certain embodiments, the nanoreporter comprises three polynucleotide strands. For instance, the nanoreporter can comprise three strands F1, X and F2 where a portion of strand X is complementary to strand F1 and a portion of strand X is complementary to strand F2. An example is illustrated in Figure 13A. In certain embodiments, the nanoreporter comprises more than three primary, covalently linked chains of monomers.

Advantageously, a nanoreporter of the invention can comprise one or more labels that facilitate the detection, imaging or identification of the nanoreporter by techniques known to those of skill in the art. The label can be any detectable moiety known to those of skill in the art. Exemplary labels for nanoreporters include detectable isotopes, radioisotopes, fluors, dyes, enzymes, ligands, receptors, antigens, antibodies, lectins, carbohydrates, nucleotide sequences, and any other detectable label apparent to those of skill in the art.

In certain embodiments, a polynucleotide is a polymer of natural (*e.g.* A, G, C, T, U) or synthetic nucleobases, or a combination of both. The backbone of the polynucleotide can

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be composed entirely of "native" phosphodiester linkages, or it may contain one or more modified linkages, such as one or more phosphorothioate, phosphorodithioate, phosphoramidate or other modified linkages. As a specific example, a polynucleotide may be a peptide nucleic acid (PNA), which contains amide interlinkages. Additional examples of synthetic bases and backbones that can be used in conjunction with the invention, as well as methods for their synthesis can be found, for example, in U.S. Patent No. 6,001,983; Uhlman & Peyman, 1990, *Chemical Review* 90(4):544 584; Goodchild, 1990, *Bioconjugate Chem.* 1(3):165 186; Egholm *et al.*, 1992, *J. Am. Chem. Soc.* 114:1895 1897; Gryaznov *et al.*, *J. Am. Chem. Soc.* 116:3143 3144, as well as the references cited in all of the above. Common synthetic nucleobases of which polynucleotides may be composed include 3-methlyuracil, 5,6-dihydrouracil, 4 thiouracil, 5 bromouracil, 5-thorouracil, 5-iodouracil, 6-dimethyl aminopurine, 6-methyl aminopurine, 2-aminopurine, 2,6-diamino purine, 6-amino-8-bromopurine, inosine, 5-methylcytosine, 7-deazaadenine, and 7-deazaguanosine. Additional non-limiting examples of synthetic nucleobases of which the target nucleic acid may be composed can be found in Fasman, *CRC Practical Handbook of Biochemistry and Molecular Biology*, 1985, pp. 385-392; *Beilstein's Handbuch der Organischen Chemie*, Springer Verlag, Berlin and Chemical Abstracts, all of which provide references to publications describing the structures, properties and preparation of such nucleobases.

The nanoreporter can be prepared according to any technique apparent to those of skill in the art. Advantageously, nanoreporters according to the invention can comprise labels and/or members of binding pairs, as described in the sections below, that can be used to facilitate preparation and/or purification of the nanoreporter. In addition, certain nanoreporters of the invention are capable of forming complexes with molecules that comprise members of binding pairs, as described below. These complexes can be used to facilitate preparation and/or purification of the nanoreporter or complex.

5.12.2 IMMOBILIZATION OF FIRST PORTION

In the methods of the invention, a first portion of the nanoreporter is immobilized. Generally, the first portion is immobilized if it would be recognized as immobilized by one of skill in the art. The first portion can be immobilized by any technique apparent to those of skill in the art. In certain embodiments, the technique for immobilization of the first portion of the nanoreporter is not critical for the methods of the invention.

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The first portion of the nanoreporter can be at any location in the nanoreporter. In certain embodiments, the first portion is at a terminus of the nanoreporter. For the purposes of the invention, a portion of a nanoreporter can be "at a terminus" when it is less than five, four, three, two, one or zero monomers from a terminus of the nanoreporter. Of course, although many nanoreporters have two termini, the methods of the invention are applicable to nanoreporters have more than two termini and to nanoreporters having one or zero termini, *e.g.* circular nanoreporters. In certain embodiments, the first portion is not at a terminus of the nanoreporter.

The nanoreporter can be immobilized onto any substrate apparent to those of skill in the art. The substrate can be any moiety to which the nanoreporter can be immobilized without limitation. In certain embodiments, the substrate is a surface, membrane, bead, porous material, electrode or array.

In certain embodiments, the first portion of the nanoreporter can be immobilized non-selectively. In further embodiments, the first portion of the nanoreporter can be immobilized selectively. In advantageous embodiments, after the first portion of the nanoreporter is immobilized, some portion of the nanoreporter should be free to move sufficiently so that the nanoreporter can be extended in the following steps of the method. In particular, in certain embodiments, when the first portion of the nanoreporter is immobilized non-selectively, it is important that the entire nanoreporter not be immobilized non-selectively to an extent that prevents extension of any portion of the nanoreporter.

The immobilization can be by any interaction with the substrate apparent to those of skill in the art. The immobilization can be via electrostatic or ionic interaction, via one or more covalent bonds, via one or more non-covalent bonds or combinations thereof. In certain embodiments, the immobilization can be via electrostatic interaction with an electrode. In further embodiments, the immobilization is via electrostatic interaction with a substrate other than the electrode.

In certain embodiments, the first portion of the nanoreporter comprises a first member of a binding pair. The first member of the binding pair can be covalently bound to the first portion of the nanoreporter, or they can be non-covalently bound. Useful covalent bonds and non-covalent bonds will be apparent to those of skill in the art. In useful embodiments, the substrate onto which the first portion of the nanoreporter is bound will comprise a second member of the binding pair. The substrate can be covalently bound to the second member, or they can be non-covalently bound. Figure 12A illustrates a

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nanoreporter that comprises a moiety F1 that is capable of selectively binding a moiety of the substrate. Moiety F1 can be, for example, biotin, capable of binding, for example, a substrate coated with avidin.

In certain embodiments, the first portion of the nanoreporter can comprise a member of a binding pair that is capable of binding with a member of a binding pair on the substrate to form one or more non-covalent bonds. Exemplary useful substrates include those that comprise a binding moiety selected from the group consisting of ligands, antigens, carbohydrates, nucleic acids, receptors, lectins, and antibodies. The first portion of the nanoreporter would comprise a binding moiety capable of binding with the binding moiety of the substrate. Exemplary useful substrates comprising reactive moieties include, but are not limited to, surfaces comprising epoxy, aldehyde, gold, hydrazide, sulfhydryl, NHS-ester, amine, thiol, carboxylate, maleimide, hydroxymethyl phosphine, imidoester, isocyanate, hydroxyl, pentafluorophenyl-ester, psoralen, pyridyl disulfide or vinyl sulfone, or mixtures thereof. Such surfaces can be obtained from commercial sources or prepared according to standard techniques.

In advantageous embodiments, the first portion of the nanoreporter can be immobilized to the substrate via an avidin-biotin binding pair. In certain embodiments, the nanoreporter can comprise a biotin moiety in its first portion. For instance, a polynucleotide nanoreporter can comprise a biotinylated nucleotide residue. Similarly, a polypeptide nanoreporter can comprise a biotinylated amino acid residue. The substrate comprising avidin can be any substrate comprising avidin known to those of skill in the art. Useful substrates comprising avidin are commercially available including TB0200 (Accelr8), SAD6, SAD20, SAD100, SAD500, SAD2000 (Xantec), SuperAvidin (Array-It), streptavidin slide (catalog #MPC 000, Xenopore) and STREPTAVIDINnslide (catalog #439003, Greiner Bio-one).

In certain embodiments, the first portion of the nanoreporter can comprise a nucleotide sequence that is capable of selectively binding a nucleotide sequence on the substrate.

In further embodiments, the first portion of the nanoreporter can comprise avidin, and the substrate can comprise biotin. Useful substrates comprising biotin are commercially available including Optiarray-biotin (Accler8), BD6, BD20, BD100, BD500 and BD2000 (Xantec).

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In further embodiments, the first portion of the nanoreporter is capable of forming a complex with one or more other molecules that, in turn, are capable of binding, covalently or non-covalently, a binding moiety of the substrate. For instance, a first portion of the nanoreporter can be capable of selectively binding another molecule that comprises, for instance, a biotin moiety that is capable of selectively binding, for instance, an avidin moiety of the substrate. Figure 13A illustrates a nanoreporter that is capable of selectively binding a second molecule X that is capable of selectively binding a third molecule that comprises F1. F1 is capable of selectively binding a moiety on a substrate. Figure 13B illustrates a nanoreporter that is capable of selectively binding a second molecule that comprises F1, and F1 is capable of selectively binding a moiety on a substrate.

In further embodiments, the first portion of the nanoreporter can comprise a member of a binding pair that is capable of reacting with a member of a binding pair on the substrate to form one or more covalent bonds. Exemplary useful substrates comprising reactive groups include those that comprise a reactive moiety selected from the group consisting of succinamides, amines, aldehydes, epoxies and thiols. The first portion of the nanoreporter would comprise a reactive moiety capable of reacting with the reactive moiety of the substrate. Exemplary useful substrates comprising reactive moieties include, but are not limited to, OptArray-DNA NHS group (Accler8), Nexterion Slide AL (Schott) and Nexterion Slide E (Schott).

In certain embodiments, the first portion of the nanoreporter can comprise a reactive moiety that is capable of being bound to the substrate by photoactivation. The substrate could comprise the photoreactive moiety, or the first portion of the nanoreporter could comprise the photoreactive moiety. Some examples of photoreactive moieties include aryl azides, such as *N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide; fluorinated aryl azides, such as 4-azido-2,3,5,6-tetrafluorobenzoic acid; benzophenone-based reagents, such as the succinimidyl ester of 4-benzoylbenzoic acid; and 5-Bromo-deoxyuridine.

In further embodiments, the first portion of the nanoreporter can be immobilized to the substrate via other binding pairs apparent to those of skill in the art.

5.12.3 EXTENSION OF THE NANOREPORTER

In certain methods of the invention, the nanoreporter is in an extended state. Generally, any nanoreporter is in an extended state if it would be recognized as such by one of skill in the art.

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In certain embodiments, the nanoreporter is in an extended state when it is in the field of a force capable of extending the nanoreporter under conditions suitable for extending the nanoreporter. Such forces and conditions should be apparent to those of skill in the art. For instance, many nanoreporters can be extended by hydrodynamic force or by gravity, and many charged nanoreporters can be extended by electromagnetic force. In certain embodiments, the force can be applied to the nanoreporter indirectly. For instance, the nanoreporter can comprise or can be linked, covalently or noncovalently, to a moiety capable of being moved by a force. In certain embodiments, the nanoreporter can be linked to a moiety

In certain embodiments, the force is an electromagnetic force. For instance, when the nanoreporter is charged, such as a polynucleotide, the nanoreporter can be extended in an electric or magnetic field. The field should be strong enough to extend the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for extending a nanoreporter in an electric or magnetic field are described in Matsuura *et al.*, 2002, *J Biomol Struct Dyn.* 20(3):429-36; Ferree & Blanch, 2003, *Biophys J.* 85(4):2539-46; Stigter & Bustamante, 1998, *Biophys J.* 1998 75(3):1197-210; Matsuura *et al.*, 2001, *Nucleic Acids Res.* 29(16); Ferree & Blanch, 2004, *Biophys J.* 87(1):468-75; the contents of which are hereby incorporated by reference in their entirety.

In certain embodiments, the force is a hydrodynamic force. For instance, many nanoreporters, including polysaccharides, polypeptides, and polynucleotides, can be extended in the field of a moving fluid. The hydrodynamic force should be strong enough to extend the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for extending a nanoreporter in hydrodynamic field are described in Bensimon *et al.*, 1994, *Science* 265:2096-2098; Henegariu *et al.*, 2001, *BioTechniques* 31: 246-250; Kraus *et al.*, 1997, *Human Genetics* 99:374 - 380; Michalet *et al.*, 1997, *Science* 277:1518-1523; Yokota *et al.*, 1997, *Nucleic Acids Res.* 25(5):1064-70; Otobe *et al.*, 2001, *Nucleic Acids Research* 29:109; Zimmerman & Cox, 1994, *Nucleic Acids Res.* 22(3):492-7, and U.S. Patent Nos. 6,548,255; 6,344,319; 6,303,296; 6,265,153; 6,225,055; 6,054,327; and 5,840,862, the contents of which are hereby incorporated by reference in their entirety.

In certain embodiments, the force is gravity. In advantageous embodiments, the force of gravity can be combined with, for example, hydrodynamic force to extend the nanoreporter. In certain embodiments, The force should be strong enough to extend the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for

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extending a nanoreporter with gravity are described in Michalet *et al.*, 1997, *Science* 277:1518-1523; Yokota *et al.*, 1997, *Nucleic Acids Res.* 25(5):1064-70; Kraus *et al.*, 1997, *Human Genetics* 99:374 - 380, the contents of which are hereby incorporated by reference in their entirety.

In particular embodiments, the force is applied through a moving meniscus. Those of skill in the art will recognize that a moving meniscus can apply various forces to a nanoreporter including hydrodynamic force, surface tension and any other force recognized by those of skill in the art. The meniscus can be moved by any technique apparent to those of skill in the art including evaporation and gravity. Exemplary techniques for extending a nanoreporter with a moving meniscus are described in, for example, U.S. Patent Nos. 6,548,255; 6,344,319; 6,303,296; 6,265,153; 6,225,055; 6,054,327; and 5,840,862, the contents of which are hereby incorporated by reference in their entireties.

In particular embodiments, the nanoreporter can be extended by an optical trap or optical tweezers. For instance, the nanoreporter can comprise or can be linked, covalently or noncovalently, to a particle capable of being trapped or moved by an appropriate source of optical force. Useful techniques for moving particles with optical traps or optical tweezers are described in Ashkin *et al.*, 1986, *Optics Letters* 11:288-290; Ashkin *et al.*, 1987, *Science* 235:1517–1520; Ashkin *et al.*, Nature 330:769–771; Perkins *et al.*, 1994, *Science* 264:822-826; Simmons *et al.*, 1996, *Biophysical Journal* 70:1813-1822; Block *et al.*, 1990, *Nature* 348:348 - 352; and Grier, 2003, *Nature* 424: 810-816; the contents of which are hereby incorporated by reference in their entireties.

In certain embodiments, the nanoreporter can be extended by combinations of the above forces that are apparent to those of skill in the art. In the examples, below, certain nanoreporters are extended by a combination of an electric field and hydrodynamic force.

The nanoreporter is extended when it would be recognized as extended by one of skill in the art according to standard criteria for extension of a nanoreporter. In certain embodiments, the nanoreporter is extended when it loses most of its tertiary structural features as recognized by those of skill in the art. In certain embodiments, the nanoreporter is extended when it loses most of its secondary structural features as recognized by those of skill in the art. In certain embodiments, the nanoreporter is extended when its primary structural features are detectable in sequence when imaged according to standard techniques. Exemplary imaging techniques are described in the examples below.

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In certain embodiments, an extended state of a nanoreporter can be recognized by comparing its hydrodynamic radius to its average hydrodynamic radius when free in dilute solution. For instance, in certain embodiments, a nanoreporter, or portion thereof, is extended when its hydrodynamic radius is more than about double its average hydrodynamic radius in dilute solution. More quantitatively, R represents the hydrodynamic radius of the nanoreporter, or portion thereof, and <R> represents the average hydrodynamic radius of the nanoreporter, or portion thereof, in dilute solution. The average <R> should be calculated such that R for the nanoreporter, or portion thereof, when unbound in dilute solution is less than 2<R> 95% of the time. In certain embodiments, a nanoreporter, or portion thereof, is in an extended state when R is greater than 1.5<R>, greater than 1.6<R>, greater than 1.7<R>, greater than 1.8<R>, greater than 1.9<R>, greater than 2.0<R>, greater than 2.1<R>, greater than 2.2<R>, greater than 2.3<R>, greater than 2.4<R>, greater than 2.5<R> or greater than 3.0<R>. In particular embodiments, a nanoreporter, or portion thereof, is in an extended state when R is greater than 2.0<R>.

5.12.4 ORIENTATION OF THE NANOREPORTER

In certain methods of the invention, the nanoreporter is in an oriented state. Generally, any nanoreporter is in an oriented state if it would be recognized as such by one of skill in the art.

In certain embodiments, the nanoreporter is in an oriented state when it is in the field of a force capable of orienting the nanoreporter under conditions suitable for orienting the nanoreporter. Such forces and conditions should be apparent to those of skill in the art.

In certain embodiments, the force is an electromagnetic force. For instance, when the nanoreporter is charged, such as a polynucleotide, the nanoreporter can be oriented in an electric or magnetic field. The field should be strong enough to orient the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for orienting a nanoreporter in an electric or magnetic field are described above.

In certain embodiments, the force is a hydrodynamic force. For instance, many nanoreporters, including polysaccharides, polypeptides, and polynucleotides, can be oriented in the field of a moving fluid. The hydrodynamic force should be strong enough to orient the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for orienting a nanoreporter in hydrodynamic field are described above.

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In certain embodiments, the force is gravity. In advantageous embodiments, the force of gravity can be combined with, for example, hydrodynamic force to orient the nanoreporter. In certain embodiments, The force should be strong enough to orient the nanoreporter according to the judgment of one of skill in the art. Exemplary techniques for orienting a nanoreporter with gravity are described above.

In certain embodiments, the nanoreporter can be oriented by combinations of the above forces that are apparent to those of skill in the art. In the examples, below, certain nanoreporters are oriented by a combination of an electric field and hydrodynamic force.

The nanoreporter is oriented when it would be recognized as oriented by one of skill in the art according to standard criteria for orientation of a nanoreporter. In certain embodiments, the nanoreporter is oriented when it is arranged in parallel, as recognized by those of skill in the art, with the field of a force capable of orienting the nanoreporter. In certain embodiments, the nanoreporter is oriented when it is one of a plurality of nanoreporters that are arranged in parallel, as recognized by those of skill in the art. For instance, a plurality of nanoreporters can be oriented when the vector from a first terminus to a second terminus of a nanoreporter is parallel, as recognized by those of skill in the art, to the vectors between corresponding termini of other nanoreporters in the plurality.

5.12.5 SELECTIVE IMMOBILIZATION OF SECOND PORTION OF NANOREPORTER

As discussed above, in the methods of the invention, a second portion of the nanoreporter is selectively immobilized. The second portion of the nanoreporter can be any portion of the nanoreporter that is not identical to the first portion of the nanoreporter. In some embodiments, the second portion of the nanoreporter does not overlap any part of the first portion of the nanoreporter.

In certain embodiments, the present invention provides methods that comprise the single step of selectively immobilizing a second portion of a nanoreporter while the nanoreporter is in an extended or oriented state, and while a first portion of the nanoreporter is immobilized. Exemplary methods for immobilization of the first portion of the nanoreporter, and for extension or orientation of the nanoreporter are described in detail in the sections above.

In certain embodiments, the present invention provides methods that comprise the step of extending a nanoreporter, while a first portion of the nanoreporter is immobilized, and the step of selectively immobilizing a second portion of a nanoreporter while the

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nanoreporter is in an extended state. Exemplary methods for immobilization of the first portion of the nanoreporter, and for extension of the nanoreporter are described in detail in the sections above.

In certain embodiments, the present invention provides methods that comprise the step of immobilizing a first portion of a nanoreporter, the step of extending the nanoreporter while the first portion is immobilized and the step of selectively immobilizing a second portion of a nanoreporter while the nanoreporter is in an extended state. Exemplary methods for immobilization of the first portion of the nanoreporter, and for extension of the nanoreporter are described in detail above.

In certain embodiments, the present invention provides methods that comprise the step of orienting a nanoreporter, while a first portion of the nanoreporter is immobilized, and the step of selectively immobilizing a second portion of a nanoreporter while the nanoreporter is in an oriented state. Exemplary methods for immobilization of the first portion of the nanoreporter, and for orienting the nanoreporter are described in detail in the sections above.

In certain embodiments, the present invention provides methods that comprise the step of immobilizing a first portion of a nanoreporter, the step of orienting the nanoreporter while the first portion is immobilized and the step of selectively immobilizing a second portion of a nanoreporter while the nanoreporter is in an oriented state. Exemplary methods for immobilization of the first portion of the nanoreporter, and for orienting the nanoreporter are described in detail above.

The selective immobilization of the second portion of the nanoreporter can follow any technique for selective immobilization of a nanoreporter apparent to those of skill in the art. Significantly, in advantageous embodiments of the invention, the second portion of the nanoreporter is not immobilized non-selectively. Selective immobilization can allow the nanoreporter to be immobilized while in a fully extended state or nearly fully extended state. Selective immobilization can also allow the nanoreporter to be immobilized in an oriented manner. In other words, the first portion and second portion of the nanoreporter can be immobilized along the direction of the field or fields used to extend the nanoreporter, with the first portion preceding the second portion in the field. When a plurality of nanoreporters are immobilized, the can be uniformly oriented along the field.

The second portion of the nanoreporter can be at any location in the nanoreporter. In certain embodiments, the second portion is at a terminus of the nanoreporter. In certain

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embodiments, the second portion is not at a terminus of the nanoreporter. In certain embodiments, the first portion, described in the sections above, is at one terminus of the nanoreporter, and the second portion is at another terminus of the nanoreporter.

As discussed above, the second portion of the nanoreporter is immobilized selectively. The immobilization can be by any selective interaction with the substrate apparent to those of skill in the art. The immobilization can be via electrostatic or ionic interaction, via one or more covalent bonds, via one or more non-covalent bonds or combinations thereof. In certain embodiments, the immobilization can be via electrostatic interaction with an electrode. In further embodiments, the immobilization is via electrostatic interaction with a substrate other than the electrode.

If the first portion and the second portion of the nanoreporter are selectively immobilized to the same substrate, the techniques of selective immobilization should of course be compatible with the substrate. In particular embodiments, the techniques of immobilization are the same. For instance, on a substrate coated with avidin, both the first and second portion of the nanoreporter can be immobilized selectively via biotin - avidin interactions. However, as will be apparent to those of skill in the art, the same interaction need not be used at both the first and second portions for immobilization on the same substrate. For instance, the substrate can comprise multiple moieties capable of selective binding, or the first portion can be immobilized non-selectively, or other techniques apparent to those of skill in the art.

In certain embodiments, the second portion of the nanoreporter comprises a first member of a binding pair. The second member of the binding pair can be covalently bound to the second portion of the nanoreporter, or they can be non-covalently bound. Useful covalent bonds and non-covalent bonds will be apparent to those of skill in the art. In useful embodiments, the substrate onto which the second portion of the nanoreporter is bound will comprise a second member of the binding pair. The substrate can be covalently bound to the second member, or they can be non-covalently bound.

In certain embodiments, the second portion of the nanoreporter can comprise a member of a binding pair that is capable of binding with a member of a binding pair on the substrate to form one or more non-covalent bonds. Exemplary useful substrates include those that comprise a binding moiety selected from the group consisting of ligands, antigens, carbohydrates, nucleic acids, receptors, lectins, and antibodies such as those described in the sections above.

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In advantageous embodiments, the second portion of the nanoreporter can be immobilized to the substrate via an avidin-biotin binding pair. In certain embodiments, the nanoreporter can comprise a biotin moiety in its first portion. For instance, a polynucleotide nanoreporter can comprise a biotinylated nucleotide residue. Similarly, a polypeptide nanoreporter can comprise a biotinylated amino acid residue. Useful substrates comprising avidin are described in the sections above.

In further embodiments, the second portion of the nanoreporter can comprise avidin, and the substrate can comprise biotin. Useful substrates comprising biotin are described in the sections above.

In further embodiments, the second portion of the nanoreporter can comprise a member of a binding pair that is capable of reacting with a member of a binding pair on the substrate to form one or more covalent bonds. Exemplary useful substrates comprising reactive groups are described in the sections above.

In certain embodiments, the second portion of the nanoreporter can comprise a reactive moiety that is capable of being bound to the substrate by photoactivation. The substrate could comprise the photoreactive moiety, or the second portion of the nanoreporter could comprise the photoreactive moiety. Some examples of photoreactive moieties include aryl azides, such as *N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide; fluorinated aryl azides, such as 4-azido-2,3,5,6-tetrafluorobenzoic acid; benzophenone-based reagents, such as the succinimidyl ester of 4-benzoylbenzoic acid; and 5-Bromo-deoxyuridine.

In further embodiments, the second portion of the nanoreporter can be immobilized to the substrate via other binding pairs described in the sections above.

In further embodiments, the second portion of the nanoreporter is capable of forming a complex with one or more other molecules that, in turn, are capable of binding, covalently or non-covalently, a binding moiety of the substrate. For instance, the second portion of the nanoreporter can be capable of selectively binding another molecule that comprises, for instance, a biotin moiety that is capable of selectively binding, for instance, an avidin moiety of the substrate. Figure 12B illustrates a nanoreporter of selectively binding a second molecule that comprises F3 that is, in turn, capable of selectively binding a moiety on a substrate. The interaction between the second portion of the nanoreporter and the molecule that comprises F3 can be mediated, for example, by an antigen-antibody interaction.

FIGS. 14A and 14B illustrate the selective immobilization of a nanoreporter according to methods of the present invention. In Figure 14A, a first portion of the

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nanoreporter comprises binding moiety F1 that is capable of selectively binding a moiety on the illustrated substrate S. Binding moiety F1 can be, for instance, biotin, and substrate S can be coated with, for instance, avidin. The nanoreporter of Figure 14A is extended by a force as described in the sections above. In Figure 14B, the force is an electrical potential. While extended, the nanoreporter is contacted with molecules comprising binding moiety F2 that is capable of selectively binding a moiety on the illustrated substrate S. Binding moiety F2 can be, for instance, biotin, and substrate S can be coated with, for instance, avidin. Significantly, up to three molecules comprising F2 are capable of selectively binding to a second portion of the nanoreporter to selectively immobilize it in its extended state. As illustrated, the molecules comprise a second binding moiety that selectively binds a repeated binding moiety of the nanoreporter. The binding moieties can be, for instance, complementary nucleic acid sequences, as illustrated in Figure 14B. The resulting nanoreporter is selectively immobilized in an extended state and should remain extended even when the force is removed. The selectively immobilized, extended nanoreporter can be used for any purpose apparent to those of skill in the art.

5.12.6 IMMOBILIZATION OF TWO PORTIONS OF AN EXTENDED OR ORIENTED NANOREPORTER

In certain embodiments, the present invention provides methods for selective immobilization of a first portion and a second portion of a nanoreporter that is in an extended or oriented state. Significantly, according to these methods of the invention, the nanoreporter need not be immobilized prior to application of a force capable of extending or orienting the nanoreporter.

In these methods, the nanoreporter is extended or oriented, or both, by a force capable of extending or orienting the nanoreporter. Such forces are described in detail in the sections above. In particular embodiments, the force is a force capable of extending or orienting the nanoreporter while maintaining the nanoreporter in one location, *i.e.* a force capable of extending or orienting without substantially moving the nanoreporter. Exemplary forces include oscillating electromagnetic fields and oscillating hydrodynamic fields. In a particular embodiment, the force is an oscillating electrical field. Exemplary techniques for extending or orienting a nanoreporter in an oscillating electric field are described in Asbury *et al.*, 2002, *Electrophoresis* 23(16):2658-66; Kabata *et al.*, 1993, *Science* 262(5139):1561-3; and Asbury and van den Engh, 1998, *Biophys J.* 74:1024-30, the contents of which are hereby incorporated by reference in their entirety.

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In the methods, the nanoreporter is immobilized at a first portion and at a second portion while extended or oriented. Both the first portion and the second portion can be immobilized non-selectively, both can be immobilized selectively, or one can be immobilized selectively and the other non-selectively. Techniques for immobilization of the first portion and second portion are described in detail in the sections above.

5.12.7 SUBSTRATE FOR IMMOBILIZATION

In the methods of the invention, the substrate for immobilization can be any substrate capable of selectively binding the nanoreporter apparent to those of skill in the art. Further, in certain aspects, the present invention provides compositions comprising a selectively immobilized nanoreporter in an extended state. The compositions comprise a substrate, as described herein, having immobilized thereto a nanoreporter in an extended state. The nanoreporter can be, of course, immobilized according to a method of the invention.

The only requirement of the substrate is that it be capable of selectively binding the second portion of the nanoreporter as described above. Thus, the substrate can be a filter or a membrane, such as a nitrocellulose or nylon, glass, a polymer such as polyacrylamide, a gel such as agarose, dextran, cellulose, polystyrene, latex, or any other material known to those of skill in the art to which capture compounds can be immobilized. The substrate can be composed of a porous material such as acrylic, styrene methyl methacrylate copolymer and ethylene/acrylic acid.

The substrate can take on any form so long as the form does not prevent selective immobilization of the second portion of the nanoreporter. For instance, the substrate can have the form of a disk, slab, strip, bead, submicron particle, coated magnetic bead, gel pad, microtiter well, slide, membrane, frit or other form known to those of skill in the art. The substrate is optionally disposed within a housing, such as a chromatography column, spin column, syringe barrel, pipette, pipette tip, 96 or 384 well plate, microchannel, capillary, etc., that aids the flow of liquid over or through the substrate.

The nanoreporter can be immobilized on a single substrate or on a plurality of substrates. For instance, in certain embodiments, the first and second portions of nanoreporter are immobilized on the same substrate, as recognized by those of skill in the art. In certain embodiments, the first portion of the nanoreporter can be immobilized on a

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first substrate while the second portion of the nanoreporter can be immobilized on a second substrate, distinct from the first.

The substrate can be prepared according to any method apparent to those of skill in the art. For a review of the myriad techniques that can be used to activate exemplary substrates of the invention with a sufficient density of reactive groups, see, the *Wiley Encyclopedia of Packaging Technology*, 2d Ed., Brody & Marsh, Ed., "Surface Treatment," pp. 867 874, John Wiley & Sons (1997), and the references cited therein. Chemical methods suitable for generating amino groups on silicon oxide substrates are described in Atkinson & Smith, "Solid Phase Synthesis of Oligodeoxyribonucleotides by the Phosphite Triester Method," In: *Oligonucleotide Synthesis: A Practical Approach*, M J Gait, Ed., 1984, IRL Press, Oxford, particularly at pp. 45 49 (and the references cited therein); chemical methods suitable for generating hydroxyl groups on silicon oxide substrates are described in Pease *et al.*, 1994, *Proc. Natl. Acad. Sci. USA 91*:5022 5026 (and the references cited therein); chemical methods for generating functional groups on polymers such as polystyrene, polyamides and grafted polystyrenes are described in Lloyd Williams *et al.*, 1997, Chemical Approaches to the Synthesis of Peptides and Proteins, Chapter 2, CRC Press, Boca Raton, FL (and the references cited therein).

Exemplary useful substrates include surfaces coated with streptavidin, *e.g.* Accelr8 TB0200. Further useful substrates include surfaces coated with N-hydroxysuccinamide that are capable of reacting with a portion of a nanoreporter that comprises an amine. One such surface is OptArray-DNA (Accelr8). Additional useful surfaces are coated with aldehyde (*e.g.* Nexterion Slide AL, Schott) and surfaces coated with epoxy (*e.g.* Nexterion Slide E, Schott). Another useful surface is a biotinylated BSA coated surface useful for selective immobilization of a portion of a nanoreporter that comprises avidin or streptavidin.

5.12.8 METHODS OF USING SELECTIVELY IMMOBILIZED, EXTENDED OR ORIENTED NANOREPORTERS

In certain embodiments, the selectively immobilized, elongated nanoreporters can be used to create macromolecular barcodes for the purposes of separation and sequential detection of labels. These labels spaced along the molecule provide a unique code that can be read when the nanoreporter is extended and immobilized. Extension and selective immobilization can facilitate the decoding of the macromolecular barcode.

The selectively immobilized, elongated nanoreporters can further be used for can be used in any context where detection or imaging of a nanoreporter might be useful. They can

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be used for diagnostic, prognostic therapeutic and screening purposes. For instance, they can be applied to the analysis of biomolecular samples obtained or derived from a patient so as to determine whether a diseased cell type is present in the sample and/or to stage the disease. They can be used to diagnose pathogen infections, for example infections by intracellular bacteria and viruses, by determining the presence and/or quantity of markers of bacterium or virus, respectively, in the sample. The compositions and methods of the invention can be used to quantitate target molecules whose abundance is indicative of a biological state or disease condition, for example, blood markers that are upregulated or downregulated as a result of a disease state. In addition, the compositions and methods of the invention can be used to provide prognostic information that assists in determining a course of treatment for a patient.

5.12.9 KITS COMPRISING SELECTIVELY IMMOBILIZED, EXTENDED OR ORIENTED NANOREPORTERS

The invention further provides kits comprising one or more components of the invention. The kits can comprise, for example, a substrate according to the invention and one or more extended or oriented, or both, nanoreporters selectively immobilized on the substrate. The kits can be used for any purpose apparent to those of skill in the art, including, those described above.

In certain embodiments, the present invention also provides kits useful for the extension and selective immobilization of nanoreporters. The kits can comprise a substrate for immobilization and one or more binding partners to facilitate extension or immobilization of a nanoreporter. The binding partners could, in certain embodiments, comprise a moiety useful for extension of the nanoreporter in an appropriate force. In certain embodiments, the binding partners could facilitate immobilization or selective immobilization of the nanoreporter to the surface. In further embodiments, the kit could comprise a nanoreporter for extension and immobilization. In further embodiments, the kit could comprise a device capable of extending the nanoreporter.

5.13 DETECTION OF NANOREPORTERS

Nanoreporters are detected by any means available in the art that is capable of detecting the specific signals on a given nanoreporter. Where the nanoreporter is fluorescently labeled, suitable consideration of appropriate excitation sources may be investigated. Possible sources may include but are not limited to arc lamp, xenon lamp,

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lasers, light emitting diodes or some combination thereof. The appropriate excitation source is used in conjunction with an appropriate optical detection system, for example an inverted fluorescent microscope, an epi-fluorescent microscope or a confocal microscope. Preferably, a microscope is used that can allow for detection with enough spatial resolution to determine the sequence of the spots on the nanoreporter.

5.13.1 MICROSCOPE AND OBJECTIVE LENS SELECTION.

The major consideration regarding the microscope objective lens is with the optical resolution, which is determined by its numerical aperture (NA). Generally, the larger the NA, the better the optical resolution. The required NA is preferably at least 1.07 based on the relationship of $\delta = 0.61\lambda/NA$ (δ = optical resolution and λ = wavelength). The amount of light that is collected by an objective is determined by NA^4/Mag^2 (Mag = magnification of the objective). Therefore, in order to collect as much light as possible, objectives with high NA and low magnifications should be selected.

5.13.2 CCD CAMERA SELECTION AND IMAGE CAPTURE TECHNIQUES.

When selecting a CCD camera, the first consideration is the pixel size, which partially determines the final resolution of the imaging system. Optimally the optical resolution should not be compromised by the CCD camera. For example, if the optical resolution is 210-300 nm, which corresponds to 12.6-18 μm on a CCD chip after a 60 x magnification, in order to resolve and maintain the optical resolution there should be at least two pixels to sample each spot. Or the pixel size of the CCD chip should be at most 6.3- 9 μm.

The second consideration is detection sensitivity which can be determined by many factors that include but are not limited to pixel size, quantum efficiency, readout noise and dark noise. To achieve high sensitivity, select a qualitative camera with big pixel size (which can give big collection area), high quantum efficiency and low noise. An exemplary camera with these criteria is the Orca-Ag camera from Hamamatsu Inc. The chip size is 1344 x 1024 pixels; when using the 60x objective, the field of view is 144 x 110 μm^2.

5.14 COMPUTER SYSTEMS

The invention provides computer systems that may be used to computerize nanoreporter image collection, nanoreporter identification and/or decoding of the

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nanoreporter code. Specifically, the invention provides various computer systems comprising a processor and a memory coupled to the processor and encoding one or more programs. The computer systems can be connected to the microscopes employed in imaging the nanoreporter, allowing imaging, identification and decoding the nanoreporter, as well as storing the nanoreporter image and associated information, by a single apparatus. The one or more programs encoded by the memory cause the processor to perform the methods of the invention.

In still other embodiments, the invention provides computer program products for use in conjunction with a computer system (*e.g.*, one of the above-described computer systems of the invention) having a processor and a memory connected to the processor. The computer program products of the invention comprise a computer readable storage medium having a computer program mechanism encoded or embedded thereon. The computer program mechanism can be loaded into the memory of the computer and cause the processor to execute the steps of the methods of the invention.

The methods described in the previous subsections can preferably be implemented by use of the following computer systems, and according to the following methods. An exemplary computer system suitable for implementation of the methods of this invention comprises internal components and being linked to external components. The internal components of this computer system include a processor element interconnected with main memory. For example, the computer system can be an Intel Pentium-based processor of 200 MHz or greater clock rate and with 32 MB or more of main memory.

The external components include mass storage. This mass storage can be one or more hard disks which are typically packaged together with the processor and memory. Such hard disks are typically of 1 GB or greater storage capacity. Other external components include user interface device, which can be a monitor and a keyboard, together with pointing device, which can be a "mouse", or other graphical input devices (not illustrated). Typically, the computer system is also linked to a network link, which can be part of an Ethernet link to other local computer systems, remote computer systems, or wide area communication networks, such as the Internet. This network link allows the computer system to share data and processing tasks with other computer systems.

Loaded into memory during operation of this system are several software components, which are both standard in the art and special to the instant invention. These software components collectively cause the computer system to function according to the

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methods of the invention. The software components are typically stored on mass storage. A first software component is an operating system, which is responsible for managing the computer system and its network interconnections. This operating system can be, for example, of the Microsoft Windows® family, such as Windows 95, Windows 2000, or Windows XP, or, alternatively, a Macintosh operating system, a Linux operating system or a Unix operating system. A second software component may include common languages and functions conveniently present in the system to assist programs implementing the methods specific to this invention. Languages that can be used to program the analytic methods of the invention include, for example, C, C++, JAVA, and, less preferably, FORTRAN, PASCAL, and BASIC. Another software component of the present invention comprises the analytic methods of this invention as programmed in a procedural language or symbolic package.

In an exemplary implementation, to practice the methods of the present invention, a nanoreporter code (*i.e.*, a correlation between the order and nature of spots on a nanoreporter and the identity of a target molecule to which such a nanoreporter binds) is first loaded in the computer system. Next the user causes execution of analysis software which performs the steps of determining the presence and, optionally, quantity of nanoreporters with a given nanoreporter code.

The analytical systems of the invention also include computer program products that contain one or more of the above-described software components such that the software components may be loaded into the memory of a computer system. Specifically, a computer program product of the invention includes a computer readable storage medium having one or more computer program mechanisms embedded or encoded thereon in a computer readable format. The computer program mechanisms encoded, *e.g.*, one or more of the analytical software components described above which can be loaded into the memory of a computer system and cause the processor of the computer system to execute the analytical methods of the present invention.

The computer program mechanisms or mechanisms are preferably stored or encoded on a computer readable storage medium. Exemplary computer readable storage media are discussed above and include, but are not limited to: a hard drive, which may be, e.g., an external or an internal hard drive of a computer system of the invention, or a removable hard drive; a floppy disk; a CD-ROM; or a tape such as a DAT tape. Other computer readable

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storage media will also be apparent to those skilled in the art that can be used in the computer program mechanisms of the present invention.

The present invention also provides databases useful for practicing the methods of the present invention. The databases may include reference nanoreporter codes for a large variety of target molecules. Preferably, such a database will be in an electronic form that can be loaded into a computer system. Such electronic forms include databases loaded into the main memory of a computer system used to implement the methods of this invention, or in the main memory of other computers linked by network connection, or embedded or encoded on mass storage media, or on removable storage media such as a CD-ROM or floppy disk.

Alternative systems and methods for implementing the methods of this invention are intended to be comprehended within the accompanying claims. In particular, the accompanying claims are intended to include the alternative program structures for implementing the methods of this invention that will be readily apparent to one of skill in the art.

5.15 APPLICATIONS OF NANOREPORTER TECHNOLOGY

The compositions and methods of the invention can be used for diagnostic, prognostic therapeutic and screening purposes. The present invention provides the advantage that many different target molecules can be analyzed at one time from a single biomolecular sample using the methods of the invention. This allows, for example, for several diagnostic tests to be performed on one sample.

5.15.1 DIAGNOSTIC/PROGNOSTIC METHODS

The present methods can be applied to the analysis of biomolecular samples obtained or derived from a patient so as to determine whether a diseased cell type is present in the sample and/or to stage the disease.

For example, a blood sample can be assayed according to any of the methods described herein to determine the presence and/or quantity of markers of a cancerous cell type in the sample, thereby diagnosing or staging the cancer.

Alternatively, the methods described herein can be used to diagnose pathogen infections, for example infections by intracellular bacteria and viruses, by determining the presence and/or quantity of markers of bacterium or virus, respectively, in the sample.

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Thus, the target molecules detected using the compositions and methods of the invention can be either patient markers (such as a cancer marker) or markers of infection with a foreign agent, such as bacterial or viral markers.

Because of the quantitative nature of nanoreporters, the compositions and methods of the invention can be used to quantitate target molecules whose abundance is indicative of a biological state or disease condition, for example, blood markers that are upregulated or downregulated as a result of a disease state.

In addition, the compositions and methods of the invention can be used to provide prognostic information that assists in determining a course of treatment for a patient. For example, the amount of a particular marker for a tumor can be accurately quantified from even a small sample from a patient. For certain diseases like breast cancer, overexpression of certain genes, such as Her2-neu, indicate a more aggressive course of treatment will be needed.

5.15.2 ANALYSIS OF PATHOLOGY SAMPLES

RNA extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples is typically poor in quality (fragmented) and low in yield. This makes gene expression analysis of low-expressing genes in histology samples or archival pathology tissues extremely difficult and often completely infeasible. The nanoreporter technology can fill this unmet need by allowing the analysis of very small quantities of low-quality total RNA.

To use nanoreporter technology in such an application, total RNA can be extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples (or similar) using commercially available kits such as RecoverAll Total Nucleic Acid Isolation Kit (Ambion) following manufacturer's protocols. RNA in such samples is frequently degraded to small fragments (200 to 500 nucleotides in length), and many paraffin-embedded histology samples only yield tens of nanograms of total RNA. Small amounts (5 to 100 ng) of this fragmented total RNA can be used directly as target material in a nanoreporter hybridization following the assay conditions described herein. As described in Example 6 in Section 11 below, nanoreporter analysis of approximately 3.3 ng cellular RNA permitted detection of transcripts present at approximately 0.5 copy/cell.

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5.15.3 SCREENING METHODS

The methods of the present invention can be used, *inter alia*, for determining the effect of a perturbation, including chemical compounds, mutations, temperature changes, growth hormones, growth factors, disease, or a change in culture conditions, on various target molecules, thereby identifying target molecules whose presence, absence or levels are indicative of a particular biological states. In a preferred embodiment, the present invention is used to elucidate and discover components and pathways of disease states. For example, the comparison of quantities of target molecules present in a disease tissue with "normal" tissue allows the elucidation of important target molecules involved in the disease, thereby identifying targets for the discovery/screening of new drug candidates that can be used to treat disease.

5.16 KITS

The invention further provides kits comprising one or more components of the invention. The kits can contained pre-labeled nanoreporters, or unlabeled nanoreporters with one or more components for labeling the nanoreporters. Moreover, the nanoreporters provided in a kit may or may not have target-specific sequences pre-attached. In one embodiment, the target sequences are provided in the kit unattached to the nanoreporter scaffold.

The kit can include other reagents as well, for example, buffers for performing hybridization reactions, linkers, restriction endonucleases, and DNA ligases.

The kit also will include instructions for using the components of the kit, and/or for making and/or using the labeled nanoreporters.

6. EXAMPLE 1: NANOREPORTER MANUFACTURING AND PROTOCOL

Herein is a step-by-step example of a method construction of a nanoreporter from various components.

It can be appreciated that various components can be constructed or added either at the same time, before or after other components. For example, annealing patch units or flaps to a scaffold can be done simultaneously or one after the other.

In this example the starting material is a circular M13mp18 viral vector. Using a single linear strand M13mp18, patch units are annealed to it to form a double stranded scaffold. Next, flaps are added then a target-specific sequence is ligated. Meanwhile

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purification steps aid to filter out excess, unattached patch units and flaps. Construction of labeled nucleic acids (patches and/or flaps and/or other labeled oligonucleotides) that bind the nanoreporter are also described.

Upon attachment (e.g., via hybridization) of a target molecule, the nanoreporter is attached to a surface and stretched. Finally the nanoreporters are imaged by a camera.

Nanoreporters were generated and successfully employed to detect target molecules using methods substantially as described in this example. An example of target detection using such this method is shown in Figure 4.

6.1 SCAFFOLD CONSTRUCTION

The oligonucleotide scaffold sequence selected was analyzed using Vector NTI® software. First, a single stranded nucleic acid was made from linearizing a circular M13mp18 single stranded DNA, which was commercially purchased from New England Biolabs. The circular M13mp18 was digested with BamH1 enzyme to linearize it. Materials used consisted of M13mp18 vector (250 ng/µl), Patch_1L_BamH1.02 (10 µM dilution of a 100 µM stock), 10X BamH1 Buffer, BamH1 enzyme. Protocol for making 0.8 pmol total of linear M13mp18 involve the following steps. 1) preheat heating block to 37 °C; 2) in a 0.65ml ependorff tube combine 40 µl of 250 ng/µl M13mp18 vector, 2 µl of 10 µM Patch_1L_BamH1.02, and 5 µl of 10X BamH1 Buffer; 3) place the ependorff tube in the 37°C heating block with foil over the top. Incubate the tube at 37°C for 15 minutes to allow the patch unit to hybridize to the M13mp18 scaffold; 4) after 15 minutes add 2 µl of BamH1 enzyme and let the reaction digest at 37 °C for 30 minutes, after which add an additional 2 µl of BamH1 enzyme and let the reaction continue to digest for another 30 minutes at 37 °C (final volume of BamH1 enzyme is 8%); and 5) aliquot 10 µl into 0.65ml ependorff tubes and store in freezer (final concentration of linear M13mp18 is 200 ng/µl).

6.2 PATCH UNIT PREPARATION OF THE BASE PATCH POOLS (BPP).

Second, patch units are prepared in pools. Patch oligonucleotide sequences were selected for optimal length and desired homology/non-homology to M13mp18 strand and the human genomic sequence. Patches were commercially manufactured oligonucleotides (purchased from Integrated DNA technologies) either 60 or 65 nucleotide bases in length. 50 nucleotide bases of each patch oligonucleotide are complementary to the M13mp18 single stranded DNA, 10 nucleotide bases are complementary to an adjacent patch, and 5

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nucleotides base pairs are complementary to a corresponding flap. The 10 nucleotide base match between patches forms a stem structure which stabilizes the structure and helps lift the flaps off the covered scaffold so they are more available to bind labeled oligonucleotides. Synthetic binding sites, the 5 nucleotide bases, on the patches for binding to the flaps make leveraging the power of a modular system possible.

The base patch pools contain nine patch units all corresponding to a specific letter grouping and position on the nanoreporter. For this example, there are four different fluorescent dyes (color) labeled A, B, C, and D and 8 different positions or regions where labeled nucleic acids can bind on a nanoreporter. For example, BPP A3 corresponds to all of the A patch units at position 3 (patch units 19-27) on the nanoreporter.

The nanoreporter positions are as follows:

Position 1: Patch units 1-9 (A or C)

Position 2: Patch units 10-18 (B or D)

Position 3: Patch units 19-27 (A or C)

Position 4: Patch units 28-36 (B or D)

Position 5: Patch units 37-45 (A or C)

Position 6: Patch units 46-54 (B or D)

Position 7: Patch units 55-63 (A or C)

Position 8: Patch units 64-72 (B or D)

Materials: right and left patches, pre-annealed to each other (each oligonucleotide is at a concentration of 10 μM). Materials for making 100 pmol of BPP 1: (In position 1, patch coordinate 1L is used for the BamH1 digest -this patch is not included in BPP 1): 10 μl each pre-annealed (10 μM/each) patch unit (coordinates 2-9), 5 μl [20 μM] Patch_1R (A or C). Final concentration of each patch is 1.18 pmol/ μl. Materials for making 100 pmol of BPP 2-8: 10 μl each pre-annealed (10 μM/each) appropriate patch unit. There are 9 patch units added to each, or 90 μl total. Final concentraion of each patch is 1.11 pmol/ μl.

Below is a table of all the patch unit pools made for this example, with 8 positions or regions for dye-labeled nucleic acids to bind on the nanoreporter. Positions 1, 3, 5, and 7 can bind to nucleic acid labeled with dye A or dye C, and a positions 2, 4, 6, and 8 can bind to nucleic acid labeled with dye B or dye D.

Table 2 of resulting Basic Patch Pools (correspond to labels on tubes)

BPP-A1 [Pre-Paired, Color=A, Coordinates 1-9]

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Patch_(1-9)R.A Patch_(2-9)L
BPP-B2 [Pre-Paired, Color=B, Coordinates 10-18] Patch_(10-18)R.B Patch_(10-18)L
BPP-A3 [Pre-Paired, Color=A, Coordinates 19-27] Patch_(19-27)R.A Patch_(19-27)L
BPP-B4 [Pre-Paired, Color=B, Coordinates 28-36] Patch_(28-36)R.B Patch_(28-36)L
BPP-A5 [Pre-Paired, Color=A, Coordinates 37-45] Patch_(37-45)R.A Patch_(37-45)L
BPP-B6 [Pre-Paired, Color=B, Coordinates 46-54] Patch_(46-54)R.B Patch_(46-54)L
BPP-A7 [Pre-Paired, Color=A, Coordinates 55-63] Patch_(55-63)R.A Patch_(55-63)L
BPP-B8 [Pre-Paired, Color=B, Coordinates 64-72] Patch_(64-72)R.B Patch_(64-72)L
BPP-C1 [Pre-Paired, Color=C, Coordinates 1-9] Patch_(1-9)R.C Patch_(2-9)L
BPP-D2 [Pre-Paired, Color=D, Coordinates 10-18] Patch_(10-18)R.D Patch_(10-18)L
BPP-C3 [Pre-Paired, Color=C, Coordinates 19-27] Patch_(19-27)R.C Patch_(19-27)L
BPP-D4 [Pre-Paired, Color=D, Coordinates 28-36] Patch_(28-36)R.D Patch_(28-36)L

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BPP-C5 [Pre-Paired, Color=C, Coordinates 37-45] Patch_(37-45)R.C Patch_(37-45)L
BPP-D6 [Pre-Paired, Color=D, Coordinates 46-54] Patch_(46-54)R.D Patch_(46-54)L
BPP-C7 [Pre-Paired, Color=C, Coordinates 55-63] Patch_(55-63)R.C Patch_(55-63)L
BPP-D8 [Pre-Paired, Color=D, Coordinates 64-72] Patch_(64-72)R.D Patch_(64-72)L

6.3 MATERIALS AND PREPARATION FOR ANNEALING THE SINGLE STRANDED OLIGONUCLEOTIDE WITH PATCH UNITS FOR A DOUBLE STRANDED SCAFFOLD.

Third, patch units are prepared to be annealed to the single stranded linear M13mp18, covering the strand in order to make a double stranded oligonucleotide scaffold. Conditions for annealing 60 and 65 nucleotide base patches to the M13mp18 need to occur at high salt concentrations so that binding will be very specific and patches will not anneal to an incorrect coordinate on the M13mp18 strand. For the annealing step, each patch unit is added at a 2:1 to 4:1 ratio with the single stranded M13mp18 sequence at 0.5 pmol total volume. Excess patches are removed before annealing flaps.

Materials used consisted of 20x SSC, linear M13mp18 (BamH1 digested at 0.08 pmol/μl or 200 ng/μl), appropriate base patch pools (BPP) (need 8 total at 1.11 pmol/μl – see above) and digital heat block set at 45°C. Annealing reaction make up is as follows. General guidelines: 2X each patch unit per M13mp18 molecule, pre-ligated flaps/patches (in position 1 or 8) added for purification later, and 5X SSC. Example (0.5 pmol of scaffold with F8 hook flaps) reaction consists of: 7.1 μl BamH1 Digested M13mp18 strand at 0.071 μM, 0.9 μl each new Base Patch Pools at 1.11 μM for first 7 positions: A1, B2, A3, B4, C5, B6 and A7:

1.7 μl A1 BPP (Pre-Annealed, 12/15; at 1.18 μM/each patch)

1.8 μl B2 BPP (Pre-Annealed, 12/15; at 1.11 μM/each patch)

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1.8 µl A3 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch)

1.8 µl B4 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch)

1.8 µl C5 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch)

1.8 µl B6 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch)

1.8 µl A7 BPP (Pre-Annealed, 12/15; at 1.11 µM/each patch),

2.4 µl BPP-D8 (pool of the first seven patch units – coordinates 64, 65, 66, 67, 68, 69, and 70 at position 8 – "D" specificity) with purification tags– F8 (FHF, which anneal to patch coordinates 71L, 71R, 72L, 72R, 73L making full split-flap/patch units that have "F" specificity for use as biotin linkers, at position F8) at 0.83 µM, and 7.3 µl 20X SSC. The final reaction volume will be 29.3 µl at 0.027 pmol/ µl.

Anti-Bam oligonucleotide is also added to anneal to region in M13 that is complementary to the (missing) 1L patch unit and to prevent recircularization of the M13 scaffold during ligation.

6.4 ANNEALING PATCH UNITS TO SINGLE STRANDED M13MP18 TO FORM A DOUBLE STRANDED SCAFFOLD.

The fourth step involves the protocol to anneal the patch units to the single stranded linear M13mp18, covering the strand in order to make a double stranded oligonucleotide scaffold, is performed in the following steps: 1) preheat heating block to 42°C, heat above reaction solution to 45°C in small PCR (or strip) tube(s) with foil over top for 15 minutes, turn heat block to 65°C and incubate for an additional 1 hour and 45 minutes and remove tubes, place on ice or freeze.

6.5 PURIFICATION OF NANOREPORTER SCAFFOLD USING BIOTIN AND MAGNETIC BEADS WITH STREPTAVIDIN.

The fifth step occurs before attaching the flaps, where excess patch units that have not annealed to the M13mp18 strand are separated from the double stranded oligonucleotide scaffold. A purification tag with a 5 nucleotide base homologous region to some of the patch units' complementary 5 nucleotide base overhang is annealed to 'hook' the scaffold. Biotinylated oligonucleotides are annealed to the 'purification tag' and magnetic beads with streptavidin are used to capture the scaffold using the biotinylated oligonucleotides. Excess patch units are removed with the supernatant. The scaffold melts off of the magnetic beads into solution for recovery.

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6.6 ANNEAL THE D-BIOTIN CATCHERS TO THE PURIFICATION TAGS

Anneal the D-Biotin catchers to the purification tags on the nanoreporter (making 2X to amount of D8-flap positions available in solution, which is 2X to M13, or 4X final): 0.5 pmol X 25 hook oligonucleotide positions (5 multiplied by 5), 4X makes 50 pmols translates to 0.50 µl of 100 pmol/ µl D-biotin, add 0.5 µl (D, E, F) – Biotin (at 100 µM) to sample, mix and incubate at room temperature for 30 minutes.

6.7 PURIFICATION PROTOCOL TO WASH OFF UNATTACHED PATCH UNITS FROM DOUBLE STRANDED SCAFFOLD.

Anneal F-hook oligonucleotides in a 25 fold excess to nanoreporters in 5X SSC for 30 min at room temperature. Pipet 200 µl DynaBead MyOne Streptavidin™ bead solution into 1.5 ml tubes, place on magnet and remove supernatant. Wash twice with 5X SSC by resuspending and clearing with magnet as in step above. Add 80 µl of sample in 5X SSC (80 fmoles of sample in this example). Resuspend well, by placing on vortex for 15 minutes. Clear solution with magnet and transfer supernatant to fresh tubes for later gel analysis. While on magnet, wash pellets (do not resuspend) with 80 µl TE by pipeting over pellet three times with the same 80 µl volume originally added. Remove wash, place in freshy "wash" tubes for analysis. Heat up TE buffer to 45°C, add 80 µl to each pellet and resuspend. Place tubes on 45°C heat block for 15 minutes, pipetting up/down once to insure beads remain suspended. Immediately clear product with magnet while warm and save. The majority of purified nanoreporters should be present in this product eluted at 45°C.

6.8 ANNEALING AND LIGATION OF FLAPS TO SCAFFOLD.

The sixth step involves split flap oligonucleotides which are annealed to the scaffold to make a 'covered scaffold.' Purification with magnetic beads is performed afterwards to remove excess split flaps. Ligation of the covered scaffold is done using T4 ligase to increase the stability of the structure. Only one type of flap is needed per fluorescent dye. Flaps are either 95 or 100 bases in length and have regions complementary to the patches, to labeled oligonucleotides and to each other. Each flap has 15 base repeating sequences for binding to labeled oligonucleotides. The repeat sequences are based on Lambda sequences that have been analyzed to remove any palindromes and hairpin structures.

Conditions for annealing the flaps are as follows. The sequence on the flaps that corresponds to the patch is 5 nucleotide base pairs long, and therefore the flaps anneal

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specifically to the patches even at high salt concentrations. The ratio of flaps to patches is 2:1. In order to increase stability at high temperatures, ligation of patches to each other and the flap to the patches may be carried out in the same reaction.

1) Quantify the purified scaffold using a spectrometer at A260 nm. Calculate the volume needed for appropriate amount of nanoreporter to prepare. For this example we used 110 ng or 0.023 pmol, reading at A260 nm shows 7.7 ng/ µl, or 14.3 µl for 110 ng. 2) Setup ligation reaction as follows (volume will vary, depending on the purification and scale). Currently using 1.5X flaps to patches, calculate accordingly. For this example, there are four different fluorescent dyes (color) labeled A, B, C, and D and 8 different positions or regions where dye-labeled nucleic acids can bind on a nanoreporter. The number of positions for each color (in this case 1-4) multiply by 9 multiply by 1.5 moles of scaffold = moles of flaps to use.

For the nanoreporter with fluorescent dye in the sequence/positions [ABABCBAD] :

ABABCBAD =

A:40.5 x .023 = .93 pmol; vol: .93 µl of SF (split flap)-AL at 1 µM

.93 µl of SF-AR at 1 µM

B:40.5 x .023 = .93 pmol; vol: .93 µl of SF-BL at 1 µM

.93 µl of SF-BR at 1 µM

C:13.5 x .023 = .31 pmol; vol: .31 µl of SF-CL at 1 µM

.31 µl of SF-CR at 1 µM

D:13.5 x .023 = .31 pmol; vol: .31 µl of SF-DL at 1 µM

.31 µl of SF-DR at 1 µM

Ligation reaction (25 µl total) consists of : Split Flaps (see above; 4.96 µl, or ~5 µl total), 14.3 µl of MODB-Scaffold at 0.0016 pmol/µl, 2.5 µl 10X T4 ligation Buffer, 2.2 µl NanoPure H2O and 1 µl T4 ligase. Incubate tubes 5 minutes at 45°C. Move to 37°C water bath, inc. for 5 minutes. Add 1 µl T4 ligase to samples. Incubate for additional 1hour at 37°C. Freeze immediately, or heat at 75°C for 5 minutes to kill T4 ligase.

6.9 LIGATION OF TARGET-SPECIFIC SEQUENCES TO NANOREPORTERS

The seventh step involves ligation of a target-specific sequence to the nanoreporter. A DNA target-specific sequence is designed to be complementary to the target molecule, which can be RNA (*e.g.*, mRNA) or DNA (*e.g.*, cDNA or genomic DNA). The target-specific sequence can be from 35, 60 or 70 nucleotide bases in length. The target-specific

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sequence can be ligated to the scaffold using a single stranded overhanging region on the covered scaffold. The scaffold with a single type of target-specific sequence can be manufactured separately and then mixed to form libraries.

6.10 NANOREPORTER CONSTRUCTION

Addition of oligonucleotides to a nanoreporter can be done at any point during the construction of a nanoreporter. In certain aspects of the present invention, a labeled oligonucleotide is 15 nucleotide bases long. On the 5′ end, a single fluorophore dye is attached. Oligonucleotides with a particular fluorophore dye will generally have the same sequence. These labeled oligonucleotides bind to the repeat sequences of the split flaps. Fluorophores best suited for this example include but are not limited to Alexa 488, cy3, Alexa 594, and Alexa 647. The 15 nucleotide base length holds the fluorophores far enough apart so that they cannot quench each other and ensure that the labeled nucleic acids will be stable (will not melt off complementary strand) at conditions in the visualization process. Labeled oligonucleotides are stable at 40 °C. This short length also allows for packing a large number of fluorescent dyes onto the flaps. In certain aspects of the invention, labeled oligonucleotides are introduced during the target sample processing.

6.11 ATTACHMENT OF NANOREPORTERS TO TARGET MOLECULES

Nanoreporters can be attached to target molecules using any means known to one of skill in the art. In an exemplary embodiment, dual nanoreporters are hybridized to target molecules by mixing 250 pmols each of both the first probe and the second probe with 125 pmols of target. The total volume is adjusted to 4 μl and a final concentration of buffer of 5X SSC. This mixture is incubated in a covered PCR tube overnight at 42 degrees to allow hybridization to occur.

6.12 SURFACE ATTACHMENT

Once the nanoreporters are attached to both target molecule and corresponding labeled nucleic acids, *i.e.*, nucleic acids attached to label monomers, they are attached to a surface and stretched in resolve the order of signals emitted by the label monomers and thus identify the target molecule. In this example, the nanoreporters are stretched to spatially resolve their fluorescent dye codes which correspond to a particular target molecule. The nanoreporters are stretched by attaching one end to a surface (in this example, a coverslip,

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see preparations below). Two methods for surface attachment may be used: A) streptavidin coated slides from Accelr8 Corporation with the nanoreporters being biotinylated and B) biotin coated slides with the nanoreporters having streptavidin. In buffer, the nanoreporters are brought into contact with the active surface and allowed to incubate for a period of time. The reaction is performed in flow cells which were made from PDMS molded in etched silicon wafers to make the channels. Metal tubing is used to core wells at the ends of the channels for buffer and sample insertion. Channel dimensions are 0.5 mm or 1 mm wide and 54 μm high. Once the sample has been loaded into the flow cell lane and incubated, the nanoreporters should be attached. Nanoreporters can be stretched either by applying a voltage or by removing the liquid with a receding meniscus leaving the strings stretched and dry.

6.13 PREPARATION OF SURFACE AND ASSEMBLY OF DEVICE

The binding surfaces (Accelr8 brand Streptavidin-OptiChem, coated coverslips) are shipped in units of 5 surfaces per slide container, and each container is enclosed with a package of silica dessicant in a foil pouch. The pouches are stored at -20 °C until use.

To prepare the surface for binding, a pouch is first pulled from the freezer and allowed to come to room temperature over several minutes. If previously unopened, the pouch is then sliced along one edge to form a slit, and the container of surfaces is removed. Upon removal of the required surface, the container is replaced in the pouch with its dessicant, the slit is sealed closed with a strip of packaging tape, and the pouch is replaced in the freezer.

The surface is then lightly rinsed with a stream of Nanopure water (Barnstead Nanopure Diamond) and soaked for 10 minutes in 0.2 μm-filtered 1X PBS in a clean, slotted Coplin jar. After soaking, the surface is dipped in Nanopure water and dried by blowing filtered nitrogen across the surface edge.

The PDMS device used to mate with the surface and provide localization of the sample is cleaned just before use by applying cellophane tape to the PDMS surface and then peeling away dust or other particles which may have become attached during storage. The binding side of the Accelr8 surface is laid face-up, and the clean PDMS structure is centered, channel side down, on the surface. PDMS adheres readily to coated glass, and no further attachment mechanism is necessary.

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6.14 SAMPLE BINDING AND WASHING

The sample is bound to the surface by first applying a 5 μL drop of the sample (currently diluted in 100 mM sodium borate buffer, pH 9.8) in one well of the chosen lane. The drop should just touch the point at which the channel joins the well (some sample may wick into the channel at this point). The channel is filled, and binding is equalized throughout the channel, by pulling the droplet through the channel to the opposite well using a very weak vacuum (< 2 kPa). The process is repeated for the other samples in their respective lanes. Excess fluid is then removed from the wells, the wells are taped to reduce evaporation, and the device is incubated at room temperature in the dark for 20 minutes.

After binding, the tape is removed, and the top well of each lane is filled with 100 μL of the borate buffer described above. About 20 μL of that buffer is pulled through the channels to the other wells using the vacuum, and the process is repeated once. All borate buffer is then removed from all wells, and the top well is filled with 1X TAE, pH 8.3. About 50 μL TAE is pulled through the channel, then all TAE is removed and the well is refilled. The process is repeated three times, for a total of about 150 μL of TAE rinse. Finally, all wells are filled with 100 μL 1X TAE.

6.15 ELECTROSTRETCHING

The bottom of the coverslip/PDMS device is spotted with immersion oil and placed on the microscope. Electrodes are inserted into the wells on opposite ends of the first PDMS channel (negative electrode in top well, positive in bottom). The first image of the channel will be taken close to the bottom well; the microscope stage is adjusted so that the area of interest is in focus.

Voltage (200 V) is then applied across the channel. Voltage is supplied by a DC power supply (Agilent E3630A) and amplified 100X through a home-built amplifier. After the current is applied, focus is readjusted, and the imaging process begins.

The electrostretching and imaging process is then repeated with the remaining channels. Image the bindings.

6.16 LIGHT SOURCE FOR THE FLUORESCENT DYES ON THE NANOREPORTER

In using an arc lamp as a light source, the best fluorophore selection is the brightest types without leading to fluorescent overlap such as Alexa 488, Cy3, and Alexa 594. Weaker fluorescent dyes such as Alexa 647 and Cy5.5 may also be used.

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6.17 FILTERS TO IMAGE THE FLUORESCENT DYES ON THE NANOREPORTER

For the selected fluorophores Alexa 488, Cy3, Alexa 594 and Alexa 647 there maybe an overlap between the Cy3 and Alexa 594. However, custom ordering an emission filter with a bandwidth of 572-600 nm minimizes the overlap.

6.18 MICROSCOPE AND OBJECTIVE LENS TO IMAGE THE NANOREPORTERS

The microscope model used was the Nikon Eclipse TE2000E from Nikon Incorporation using the inverted fluorescence imaging station which has 6 filter cassettes that allow the selection of fluorescent emission from multiple fluorescent dye candidates. For the selected dyes, the optical resolution required is about 400 nm for all the wavelengths (500-700 nm). The selected objective lens is the Nikon Plan Apo TIRF lens which has a NA of 1.45 and magnification of 60. The optical resolution is ~210-300 nm for different wavelengths.

7. EXAMPLE 2: PATCH/FLAP NANOREPORTER MANUFACTURING PROTOCOL

This example demonstrates another way of making a nanoreporter which consists of a single stranded linear M13mp18 viral DNA, oligonucleotide patch units and long flaps.

Nanoreporter label units were successfully generated using methods substantially as described in this example.

Pre-phosphorylated patch units and flaps are added together with the M13mp18 DNA vector and ligated together. After the ligation of the flaps to the patch units which are ligated to the M13mp18 DNA, the BamH1 enzyme is introduced to linearize the vector.

Prepare a batch of nanoreporters starting with 5 µg of M13mp18 as a scaffold. The hybridization may be scaled up accordingly to the desired amount. This process will take about 1-2 days to complete.

Materials:

Qty	Item	Vendor
20	250 ug/µl M13mp18 viral ssDNA	New England Biolabs
27 µl	0.74 pmol/µl Oligonucleotide Patch Unit Mix	IDT
8 µl	Long Flap Oligonucleotide A 100 pmol/µl	IDT

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8 µl	Long Flap Oligonucleotide B 100 pmol/µl	IDT
0.5 µl	Flap patch Oligos at 100 pmol/µl from plates #529916 and #610591	IDT
31 µl	T4 Ligase 10x buffer	Fermentas
19 µl	T4 Ligase	Fermentas
15 µl	Optikinase 10x buffer	USB
4.2 µl	100 mM ATP	ANY
5 µl	Optikinase Enzyme 10 units/µl	USB
1 µl	BamH1 oligonucleotide 10 pmol/µl	IDT
20 µl	BamH1 10x buffer	Fermentas
3 µl	BamH1 Enzyme 10 units/µl	Fermentas

Preheat water bath to 37°C and 55°C °C before beginning protocol. Make sure buffers are all well mixed and thawed before using. A work plate should be available and labeled with the ordered oligos from IDT in plates #529916 and #610591. Take these two plates out and thaw at room temperature for 0.5-1 hours and spin down contents before removing the tape that covers the wells. Four separate reactions will be set up in 1.5 ml eppendorf tubes using specific oligonucleotides from these plates. To begin label these four separate tubes with roman numerals on their caps. Columns 5 and 6 A through H are for reaction I, Columns 7 and 8 A through H are for reaction ii are all found in plate #529916. Columns 1 and 2 are for reaction iv, and Columns 3 and 4 are for reaction iii.

Flap Ligations (Step A): Label four separate 1.5 ml tubes with roman numerals i through iv (mentioned above). Add the reagents below accordingly to each 50 µl reaction containing: 5 µl 10x ligase buffer, 0.5 µl/oligonucleotide from designated wells from plates #529916 and #610591, 4 µl Long Flap Oligo/reaction (A or B) for reactions I, ii and iv. 3 µl of LF for area iii, 29 H_20 for reactions I,ii and iv. 32 µl H_20 for reaction iii, and 4 µl T4 ligase. Preanneal oligos in this mix without the ligase at 37 °C for half an hour. Add ligase as last reagent and allow to ligate at room temperature for at least four hours. Product concentration is 1 pmol/flap/µl.

Flap Ligation Phosphorylation (Step B) Label four separate 1.5 ml tubes with roman numerals again, one through four with a P inside a circle to designate that the products are phosphorylated. Add the following reagents to the corresponding tube: 10 µl/Flap ligation. reaction (take 10 µl/flap ligation reaction above), 2.5 µl Optikinase buffer, 0.5 µl 100 mM

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ATP, 11.5 µl H_20, and 0.5 µl Optikinase enzyme. Incubate at 37 °C for 1 hour. Product concentration 0.4 pmol/flap/µl.

Oligonucleotide Patch Unit Phosphorylation (Step C) 27 µl Oligonucleotide Patch Unit mix 0.74 pmol/µl, 5 µl 10x buffer, 1 µl 100 mM ATP, 3 µl Optikinase enzyme, and14 µl H_20. Once reagents are all together gently mix the solution by flicking the tube a few times and spin down. Incubate at 37 °C for 1 hour.

Hybridization to M13mp18 scaffold (Step D) In a new 1.5 ml tube add the following reagents: 20 µl M13mp18 at 250 ng/µl, 27 µl Phosphorylated Oligonucleotide Patch Units 0.4 pmol/µl (Step C), 12.5 µl/ Phosph. Flap Ligation (Step B) preheat at 55 °C for 5 minutes and put on ice, 11 µl 10x ligase buffer and heat entire mixture at 55 °C for 1 minute. Hybridize mixture at 37 °C for at least 4 hours.

Ligation (Step E) Spin down eppendorf contents. Add 1.2 µl 100 mM ATP and 3 µl T4 ligase. Gently mix contents by flicking the tube, then spin down.

BamH1 Digest (Step F): 1 µl of 10 pmol BamH1 oligo, 20 µl 10x BamH1 buffer and hybride at 37 ~ 1 hour. Adjust volume to 200 µl. Add 3 µl BamH1 enzyme. Incubate at 37 °C for 1 hour.

First step: start by adding 20 µl of M13mp18 (NEB 250 µg/ml) to a clean 1.7 ml eppendorf tube. Take 5 µl of Phosphorylated Flap ligation reaction and preheat it at 70 for 2 minutes and immediately put on ice. Add the 5 µl of each Phosphorylated Flap Ligation reaction (1 pmol/flap/µl) to the tube and gently mix by pipetting a few times. Incubate the eppendorf tube at 37 °C for 1 hour.

Second step: put 13.5 µl Oligonucleotide Patch Unit Mix (0.74 pmol/µl) and 1 µl of Acrydite Mix (10 pmol/µl)in a new eppendorf 1.7 ml eppendorf tube. Add 5 µl 10x Optikinase buffer, 1 µl 100 mM ATP and 27.5 µl H_20. Mix gently by pipetting the solution. Add 2 µl Optikinase enzyme, gently mix by pipetting and incubate at 37 °C for 1 hr.

Third step: take the phosporylated oligos rxn and add it entirely to the contents of the M13mp18+Flaps Hybridization. The reaction is mixed gently by pipetting and it is allowed to incubate at 30 °C for 1 hour. After the hybridization is complete adjust the ATP by adding 1 µl (100 ATP) to the reaction.

Fourth step: spin down contents in eppendorf tube and add 4 µl T4 Ligase enzyme (5 units/µl), mix gently by pipetting. Incubate at room temperature for at least four hours. Add 1 µl BamH1 oligonucleotide (10 pmol/µl) to hybridize at room temperature while ligation is taking place.

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Fifth step: digest ligation reaction by adding 4 µl BamH1 enzyme (5 units/µl), mix gently by pipetting and incubate at 37 °C for 1 hour. Once the incubation period is over. Take an aliquot of 500 ng for QC.

Sixth step: treat with Psoralen, UV or DMPA light for 15 minutes.

Calculations include:

5 µg of M13 = 20 µl stock from New England Biolabs = 2 pmols

Oligonucleotide mix: 180-34 flap areas – 10 Acrydite modified Oligos = 0.74 pmol/oligo

10 pmols/oligonucleotide = 13.5 µl = 1350 pmols

Optikinase 1unit converts 1 nmol of phosphate to ends - use excess. 4 µl of Optikinase was used.

SEQ ID NO: 1 = M13mp18.

8. EXAMPLE 3: PROTOCOL FOR PRODUCTION OF RNA NANOREPORTERS

Nanoreporters were generated and successfully employed to detect target molecules using methods substantially as described in this example. An example of target detection using such this method is shown in Figure 6.

8.1 SCAFFOLD PRODUCTION

Single-stranded circular M13mp18 DNA (USB Corporation) is annealed to a 10-fold molar excess of an oligonucleotide complementary to the Bam HI recognition site (Bam Cutter oligo) and cut with Bam HI restriction enzyme to yield a linear single-stranded DNA backbone. An oligonucleotide complementary to the Bam Cutter oligonucleotide (anti-Bam oligonucleotide) is subsequently added in 50-fold excess to the Bam Cutter oligonucleotide to sequester free Bam Cutter oligonucleotide and thus prevent recircularization of the M13 during later steps.

The linear M13 molecule serves as a scaffold onto which RNA patches, or RNA segments, with incorporated fluorophores can be annealed.

8.2 PCR TO FORM DOUBLE-STRANDED POSITIONS ON THE M13 SCAFFOLD

Ten sets of oligonucleotide primer pairs were designed to create 10 different regions along the M13 scaffold. Each pair contains one primer which has a T7 RNA polymerase

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promoter at the 5′ end. Regions 2-7 are designed to be 900 bases (approximately 300 nm) long, as this is the approximate size of a diffraction-limited spot (the smallest spot that can be achieved with standard optics). Regions 1 and 8 have both long and short versions: the long versions cover the whole 900-base region, while the short versions cover only a portion of the 900-base region to allow a target-specific sequence to be ligated. Thus a target-specific sequence can be attached to either end. The ends can also be used for attachment of anchors or tags.

PCR is performed using Taq polymerase and 0.5 ng of double-stranded M13mp18 (USB Corporation) as a template. Reactions are cleaned up using a Qiaquick purification kit from Qiagen. Each PCR reaction yields a double-stranded fragment corresponding to one specific segment as illustrated below. These fragments are used as templates for the *in vitro* transcription of the RNA segments.

8.3 *IN VITRO* TRANSCRIPTION TO PRODUCE DARK RNA SEGMENTS

Using the PCR products described above as double-stranded templates, RNA segments are generated using an *in vitro* transcription kit from Ambion (Megascript T7 kit). The products of the transcription reactions are purified (including treatment with DNAse I to remove template) using a RNeasy Kit from Qiagen.

8.4 *IN VITRO* TRANSCRIPTION TO PRODUCE RNA SEGMENTS MODIFIED WITH AMINOALLYL GROUPS

Using the PCR products described above as double-stranded templates, RNA segments for later dye-coupling are generated using an *in vitro* transcription kit from Ambion (MessageAmp aRNA kit). Aminoallyl-modified UTP nucleotides are incorporated into the RNA segments during transcription. The products of the transcription reactions are purified (including treatment with DNAse I to remove template) using a RNeasy Kit from Qiagen.

8.5 DYE COUPLING OF AMINOALLYL RNA SEGMENTS TO PRODUCE COLORED RNA SEGMENTS

20-100 μg of aminoallyl-modified RNA segment is coupled with NHS-ester dyes using Ambion Aminoallyl Labeling Kit. Dyes used include Alexa 488, Alexa 594 and Alexa 647 (Invitrogen/Molecular Probes) as well as Cy3 (Amersham).

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Each segment is made separately in 4 colors so that each position on the scaffold can be filled with a segment in any of the four colors; thus different colors can be added at different positions to create many unique color combinations.

In this particular embodiment, adjacent segments must be of different colors or there may be dark segments interspersed so that each segment is detected as an individual 'spot'. Dark segments may be used as part of the nanoreporter code.

8.6 ASSEMBLY OF THE LABEL MOLECULE

Segments for each position are annealed in a 2:1 ratio of segment to M13 scaffold in 1X SSPE buffer at 70°C for 2 hours.

An assembled nanoreporter with labeled RNA segments is depicted in Figure 3A-3B. Figure 3A depicts a nanoreporter in which only alternate "spots" (1, 3, 5 and 7) are labeled, and Figure 3B depicts a nanoreporter in which every spot is labeled.

9. EXAMPLE 4: DETECTION OF TARGET (S2) RNA AND DNA MOLECULES USING AN RNA NANOREPORTER/GHOST PROBE COMBINATION

9.1 SYNTHESIS OF PROBE AND TARGET OLIGONUCLEOTIDES

S2 DNA target oligonucleotide was synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies). S2 RNA target molecules were generated by *in vitro* transcription of PCR products corresponding to region of cloned SARS coronavirus gene (Invitrogen) using an Ambion Megascript™ kit per manufacturer's instructions. The S2 ghost probe (Figure 6A (i)) was complementary to a specific 50-base region of the S2 target sequence (S2-*a*) and was synthesized with a biotin-TEG monomer at the 5′ end and purified by high performance liquid chromatograpy (Integrated DNA Technologies). A second oligonucleotide with 50 bps complementary to the S2 target (S2-*b*) plus 9 bp of a additional sequence used for ligation to the M13 scaffold (59bp total) was synthesized and purified by HPLC (Integrated DNA Technologies). Note that S2-*a* and S2-*b* target regions were not overlapping.

9.2 NANOREPORTER SYNTHESIS

Oligonucleotide S2-*b* was ligated to the 5′ end of linearized M13 [Figure 6A (iii)], and the resulting product was purified away from residual unligated oligonucleotide by size-exclusion filtration through a YM100 filter (Millipore) per manufacturer's instructions.

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Amino-allyl-modified RNA segments complementary to M13 is positions 2, 4, 6, and 8 (SEQ ID NOs:) (Figure 1C) were generated from *in vitro*-transcription of DNA templates (PCR products) via the Ambion Megascript™ kit per manufacturer's instructions. The segments were then coupled to NHS-ester-modified Alexa 647 dye (Invitrogen) per Ambion's instructions (amino allyl MessageAmp™ II aRNA kit). RNA segments corresponding to positions 1, 3, 5, and 7 of the M13 scaffold (Figure 1C) were generated as unmodified *in vitro*-transcribed RNAs from DNA templates as described above. Assembly of the nanoreporter was carried out by annealing 10 fmol/µl of each of the eight segments to 5 fmol/µl of the M13-S1-*b* scaffold for 2 hours at 70°C in 1X SSPE buffer (150 mM sodium chloride, 10 mM sodium phosphate, 1mM EDTA). The final product was a nanoreporter with 4 segments labeled with A647 (red) interspersed with dark segments.

9.3 HYBRIDIZATION CONDITIONS

Hybridization of nanoreporters and ghost probes to target were carried out under the following conditions: 5X SSPE (750 mM sodium chloride, 50 mM sodium phosphate, 5 mM disodium EDTA), 40 pM ghost probe (attachment oligonucleotide S2-*a*), 40 pM Nanoreporter S2-*b*, 100 ng/µl sheared salmon sperm DNA, 5X Denhardt's solution and 0.1% Tween. Final target concentrations were 20 pM S2 DNA target (Figure 6B) and 1pM S2 RNA target (Figure 6C). No target was added to the negative control (Figure 6D). The hybridization reaction was incubated at 65°C for at least 16h.

Hybridization reactions were diluted 1:2 with 100 mM Borate buffer solution (pH 9.8) and introduced into a flow cell channel and bound to a streptavidin-coated coverslip forming the bottom of the channel (Streptavidin-OptiChem coverslips from Accelr8). Attachment to the slide by one end of the nanorerporter/target/ghost probe complex was achieved via interaction of the biotinylated ghost probe with the streptavidin surface. After rinsing the channel with additional borate buffer to remove excess reporters not bound to the surface, the buffer was exchanged with 1X TAE (40 mM Tris-acetate, 1 mM EDTA) and a current of 200V was applied to stretch out the nanoreporter/target complexes during image capture.

Images were obtained using a Leica DMI 6000B microscope with a 63X oil immersion objective (1.4 NA), Xcite-120 light source (Exfo), customized filter sets (Chroma Technologies), an Orca-ER CCD camera (Hamamatsu) and Metamorph data acquisition software (Molecular Devices).

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As predicted, when the correct target molecule S2 hybridizes [Figure 6A (ii)] to both ghost probe [Figure 6A (i), S2-*a*] and S2-*b* target-specific nanoreporter [Figure 6A (iii)], the ghost probe/target/nanoreporter complex forms a single species that attaches to the slide and was visualized as 4 spots when exposed to 647 nm wavelength light (Figure 6B, 6C, and 6E). The amount of binding was dependent on the target concentration. There was no significant binding in absence of S2 target sequence (Figure 6D).

10. EXAMPLE 5: NANOREPORTER COMPRISING A MONOVALENT OR BIVALENT ANTIBODY FRAGMENT

Where a target molecule is a protein or polypeptide, a nanoreporter can be generated in which the nanoreporter scaffold is a nucleic acid and the target-specific sequence is a monovalent or bivalent antibody fragment.

Using routine methods, an antibody that recognizes a target molecule of interest is optinally digested with pepsin to generate F(ab')2 fragments. The two parts of the antibody or the two F(ab')2 fragments generated by the pepsin digestion are separated by mild reduction, for example with 2-mercaptoethylamine. This reduction separates either the antibody or the two F(ab')2 fragments into two monovalent fragments with two sulfhydryl groups that can be functionalized.

A heterobifunctional crosslinking reagent (*e.g.*, m-Maleimidobenzoyl-N-hydroxysuccinimide ester from Pierce Biotechnology Inc.) is used to attach a maleimide to an oligonucleotide with an amine modification (which can be ordered from many sources, such as Integrated DNA Technologies). The NHS on the cross-linking reagent is reacted with the amine on the oligonucleotides to produce a maleimide-conjugated oligonucleotide.

This maleimide conjugated oligonucleotide is then reacted with one of the sulfhydryl groups on the antibody fragment. Due to steric limitations, it is preferable that only one oligonucleotide be attached to each fragment.

This monovalent or bivalent antibody fragment attached to an oligonucleotide can then be hybridized to a complementary sequence on a nanoreporter scaffold, to generate a reporter probe in which the target-specific sequence is an antibody sequence. Such a reporter probe can be used alone to detect the target molecule, or in conjunction with a ghost probe or another reporter probe whose target-specific sequence is a monovalent or bivalent antibody or antibody fragment that binds to a different portion of the same target molecule.

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11. EXAMPLE 6: HYBRIDIZATION OF 25 CELLULAR GENES TO 100 NG OF PLACENTAL TOTAL RNA USING NANOSTRING REPORTER SYSTEM

Detection and quantitation of 25 endogenous cellular genes was carried out in a single multiplexed hybridization reaction. In addition, three non-human control sequences were spiked into each reaction that corresponded to approximately 10, 100 and 300 copies per cell, respectively. A negative control hybridization was also performed in the absence of cellular RNA.

11.1 HYBRIDIZATION REACTION

Each sample was hybridized in triplicate. Final concentrations of the hybridization reagents were as follows: 1.12nM total Nanoreporters (28 individual Nanoreporters at 40 pM each), 1.12nM total ghost probe (28 individual ghost probes), 5X SSPE (pH 7.5), 5X Denhardt's reagent, 100 ng/µl sheared salmon sperm DNA, 0.1% tween 20, 150 fM S3 spike DNA, 50 fM S4 spike, and 5 fM S6 spike. The final concentration of total placental RNA was 33 ng/µl. No total placental RNA was added to the negative control hybridizations. The final volume of the reaction was 30 µl. Reagents were mixed and incubated at 65°C in thermocycler block with heated lid for 20 hours.

Master mix	(1 Reaction)	(6 reactions)
1.8X hybridization mix*	16.7 µl	100 µl
25 endogenous gene reporters (0.6 nM each)	2 µl	12 µl
25 endogenous gene ghost probes (0.6 nM each)	2 µl	12 µl
Control reporters (0.6 nM each)	2 µl	12 µl
Control ghost probes (0.6 nM each)	2 µl	12 µl
10X control target mix	3 µl	18 µl
H_2O	1.3 µl	8 µl
Total	29 µl	174 µl

*Hybridization mix (9X SSPE, 9X Denhardt's reagent, 180 ng salmon sperm DNA, 0.18% tween 20)

Reactions	1	2	3	4	5	6
Master mix	29 µl	29 µl	29 µl	29 µl	29 µl	29 µl
100 ng/µl placental RNA	1 µl	1 µl	1 µl	0 µl	0 µl	0 µl
H_2O	0 µl	0 µl	0 µl	1 µl	1 µl	1 µl
Total Rxn volume	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl

Incubate reactions in thermocycler with heated lid overnight (18hours).

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11.2 POST-HYBRIDIZATION PURIFICATION

Hybridization reactions were purified to remove unhybridized reporters using an oligonucleotide complimentary to ghost probe attached to magnetic beads (F-bead). Hybridization reactions were diluted 5 fold in 0.1% tween 20 to bring the final salt concentration to 1X SSPE and the solution added to 30 µl of F-beads (prewashed 2 times in 150 µl of 1X SSPE/0.1% tween 20). Hybridized complexes were allowed to bind to the beads at room temperature for 15 minutes with continuous rotation, washed once in 150 µl of 0.5X SSPE, and eluted in 25 µl of 0.1X SSPE for 15 minutes at 45°C.

11.3 BINDING, STRETCHING, AND IMMOBILIZATION

The samples was prepared for binding by addition of 1 µl of 1/1000 dilution of 0.1 uM Tetraspec™ fluorescent microspheres (product # T7279, Molecular Probes) and 3 µl of 1M bis-tris propane (pH 9.0). Samples were loaded into a Nanostring fluidic device for attachment to Accerl8 Optichem® slide coated with streptavidin (product #TB0200). After loading, slide surface was washed once with 1X TAE and prepared for electrostretching by addition of 40 µl of TAE to each well. Attached complexes were stretch by applying 200V across the fluidic channel. After 1 minute the samples were immobilized in the stretched position by adding 60 µl of 500 mM of G-hook oligo solution to the well containing the negatively charged electrode while continuing to apply voltage for 5 minutes. After immobilization the TAE solution is removed and replaced with anti-photobleaching reagent for imaging.

11.4 IMAGING

Slides were imaged on Nikon Eclipse TE2000E equipped with a metal halide light source (X-cite 120, Exfo Corporation) and a 60X oil immersion lens (1.4 NA Plan Apo VC, Nikon). For each field of view, 4 images at different excitation wavelengths (480, 545, 580 and 622) were acquired with an Orca Ag CCD camera (Hamamatsu) under control of Metamorph software (Universal Imaging Corporation). Images were processed with custom image processing software.

11.5 DATA ANALYSIS

Raw data were extracted from processed images using custom software. Data were normalized to the average counts for control spikes in each sample. To determine if a gene was "detected" by the system, the counts obtained for each gene from hybridizations

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containing RNA were compared to counts obtained in hybridizations without RNA using a Student's ttest. Genes with p values <0.05 were determined to be detected. After background subtraction, the concentrations of cellular mRNA were estimated from the linear regression of the spike controls. These concentrations were converted to copies per cell using the following assumptions: 1 cell contains 10 pg total RNA; each cell contains 300,000 mRNA molecules; final volume of the reaction is 30 µl.

11.6 RESULTS AND CONCLUSION

Table 3 below shows the results of the data analysis described above. These results show that using the nanoreporter technology described herein, it was possible to detect transcripts, such as CASP3, that are present at a concentration of less than 1 transcript/cell. Thus, the nanoreporter technology provides an exquisitely sensitive means of detecting and quantifying gene expression.

Table 3: Transcript Concentration and Abundances

Gene	Avg counts*	error (counts)	concentration (fM)	error (conc.)	calculated copies/cell	error (copy/cell)	Detected/ Not Detected (p<0.05)
GM2A	149	17	3.39	0.39	6.12	0.07	D
ATF4	68	2	1.55	0.06	2.80	0.01	D
CTNNB1	792	50	17.95	1.19	32.44	0.22	D
IRF1	221	20	5.01	0.47	9.05	0.09	D
STAT5A	120	11	2.72	0.25	4.91	0.05	D
CREG1	409	17	9.28	0.44	16.76	0.08	D
CASP3	13	1	0.30	0.03	0.54	0.00	D
CCL20	2	1	0.04	0.03	0.07	0.01	ND
NMI	115	2	2.61	0.07	4.72	0.01	D
XBP1	719	46	16.30	1.10	29.45	0.20	D
PCGF4	75	18	1.70	0.40	3.08	0.07	D
IFI27	747	41	16.94	1.00	30.61	0.18	D
TAF7	185	11	4.19	0.26	7.57	0.05	D
OAS3	74	9	1.68	0.20	3.03	0.04	D
C2	850	49	19.28	1.19	34.83	0.21	D
IL6	8	3	0.19	0.07	0.34	0.01	D
MyD88	94	6	2.13	0.14	3.85	0.03	D
HIF1A	130	7	2.95	0.17	5.33	0.03	D
APOA2	-1	2	-0.01	-0.05	-0.03	-0.01	ND
KISS	6825	130	154.79	4.52	279.65	0.82	D
ELK3	55	4	1.25	0.09	2.27	0.02	D
CBF2	72	3	1.64	0.07	2.96	0.01	D
IFI30	625	47	14.16	1.10	25.59	0.20	D
RELB	35	5	0.78	0.11	1.42	0.02	D
CTCF	103	3	2.35	0.09	4.24	0.02	D

*Normalized and background subtracted.

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The hybridization methods described herein have been performed in single multiplexed reactions containing up to 120 different reporters with similar hybridization efficiencies and results.

12. EXAMPLE 7: CONSIDERATIONS REGARDING NANOREPORTER HYBRIDIZATION KINETICS

12.1 BACKGROUND

Solution hybridizations with a large excess of probe over target follow pseudo-first order kinetics. In this regime the speed of the reaction depends only on the probe concentration and not on the target concentration. For a two-probe, one-target strategy to provide accurate information on the concentration of a target in solution, the probes should both be present in excess of the target. The possible concentration range is preferably therefore bounded on the lower end by the concentration of the target. However, the useful concentration range for the nanoreporter technology described herein is practically bounded on the lower end by the amount of time needed to perform the hybridization.

12.2 HYBRIDIZATION KINETICS

In preferred embodiments, target detection and quantification assays are performed in which the target (T) must hybridize to both a reporter probe (R) and a ghost probe (G) to be detected (for example by affinity selection and detection of complexes comprising only (R) and (G), which in turn only form complexes in the presence of (T)) . Assuming that these reactions are irreversible, there are four possible elementary reactions that occur.

$$R + T \xrightarrow{k_1} RT$$

$$T + G \xrightarrow{k_2} TG$$

$$RT + G \xrightarrow{k_3} RTG$$

$$R + TG \xrightarrow{k_4} RTG$$

Because RT and TG are intermediate complexes of two out of the three species, these four reactions can be simplified to

$$R + T + G \rightarrow RTG$$

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However, to quantitatively calculate the rate of production of RTG (the reporter-target-ghost probe complex), all four reactions must be considered. The differential equations describing the system are:

$$\frac{dC_G}{dt} = -k_2 C_G C_T - k_3 C_G C_{RT} \quad \frac{dC_R}{dt} = -k_1 C_R C_T - k_4 C_R C_{TG} \quad \frac{dC_T}{dt} = -k_2 C_G C_T - k_2 C_R C_T$$

$$\frac{dC_{TG}}{dt} = k_2 C_G C_T - k_4 C_R C_{TG} \quad \frac{dC_{RT}}{dt} = k_1 C_R C_T - k_3 C_G C_{RT}$$

$$\frac{dC_{RTG}}{dt} = k_4 C_R C_{TG} + k_3 C_G C_{RT}$$

where C_R, C_T, C_G, C_{RT}, C_{TG}, and C_{RTG} are the concentrations of the various species, and k_1-k_4 are the kinetic constants for the four elementary reactions. Values for these kinetic constants when the probes and targets are complementary single-stranded molecules (i.e. when there is no purification tag on the ghost probe and no reporter) can be calculated from data available in the literature (Wetmur, J. *Annu. Rev. Biophys. Bioeng.* 1976.5:337-361).

$$k = k_N \frac{\sqrt{L}}{N} \frac{\alpha_{salt}}{\alpha_{ref}}$$

In the above equation, k_N is the nucleation rate constant, L is the nucleic acid length (in base pairs), N is the nucleic acid complexity (equal to L for non-repetitive sequences) and a_{salt} and a_{ref} are corrections for salt concentration (Britten *et al.*, 1974, *Methods in Enzymology* 29E:363-406). In the nanoreporter systems described herein, the kinetic constants will depend on the sizes of the attached ghost probe tags and reporter probe. Without being bound by any theory, it is the inventors' belief that the kinetic constants will have the same dependence on length that an elementary reaction has on the diffusion constants of the reactants.

$$k = k_N \frac{\sqrt{L}}{N} \frac{\alpha_{salt}}{\alpha_{ref}} \frac{D_1 + D_2}{2D_{50}}$$

In the above equation D_1 and D_2 are the diffusion constants of the two reacting species (see the reactions above) and D_{50} is the diffusion constant of a 50-mer single-stranded DNA molecule. Assuming a 100-base single-stranded target, 100-base single-stranded ghost probe, and 7200-base double stranded reporter, the relevant kinetic constants are

$k_1 = 2.64 \times 10^5$ L/mol/s
$k_2 = 6.55 \times 10^5$ L/mol/s
$k_3 = 3.99 \times 10^5$ L/mol/s

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$k_4 = 1.91 \times 10^5$ L/mol/s

Numerically solving the system of differential equations with these kinetic constants (assuming at least a 10-fold excess of probes over target) yields the prediction that 5 pM reporter and 5 pM ghost probe will drive hybridization to 10% of completion in an overnight reaction (16-18 hours). At concentrations lower than 5 pM, the amount of completely hybridized molecules is likely impractical to measure. Thus, in a preferred embodiment, the lower concentration of a nanoreporter component (ghost probe and/or reporter probe) is 5pM.

12.3 ENTANGLEMENT OF REPORTERS

As probe concentrations increase, theory predicts that hybridization kinetics speed up without bound – the only limit being the solubility of the probes. However, the reporter probe can be very large compared to the target-specific sequence in the nanoreporter systems of the invention. Without being bound by any theory, the inventors believe that by its attachment to the reporter probe the kinetics of the target-specific sequence are altered from classical solution hybridization kinetics. Because the reporter probe is a large, polymeric molecule, it can have long-lived interactions (entanglements) with other nanoreporters when they come into contact. At low concentration the probability of two polymers becoming entangled is small, but as the concentration and/or size of a polymer in solution increases, these interactions become more and more common. In the extreme case of very long molecules at very high concentration the polymers form a permanent network, or gel, in solution. For solution hybridization to occur, a probe (*e.g.*, a nanoreporter probe)/target pair must diffuse through solution until they contact one another and a hybridization nucleus forms. Classically, hybridization reactions are not diffusion limited because the translational diffusion of the molecules is faster than the nucleation of the hybridization (*i.e.*, the probe and target diffuse together and interact many times before a nucleation occurs). In dilute solution its large size will slow the translational diffusion of the reporter probe, but may not significantly affect the kinetics. At some intermediate concentration, the reporter probes take up almost all of the space in the solution, effectively forming a permanently entangled gel, and can no longer diffuse in solution. However, the ghost probe and the targets are smaller molecules that are believed to still diffuse through the entangled reporter probes, allowing hybridization to take place (although possibly at a slower rate). The inventors also believe that at some higher concentration the reporter probe

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in solution will also hinder the movement of the ghost probe and the targets to the point that the reaction becomes diffusion limited. This concentration (which is not quantitatively known and depends upon the reporter probe structure, the ghost probe structure, and the target size) is the upper limit of the useful concentration range in the nanoreporter system, and can be emprically determined by one of skill in the art guided by the principles described herein.

12.4 LENGTH DEPENDENCE OF KINETICS

Since the limiting upper concentration for hybridization depends upon both the reporter structure and ghost probe structure (of which there are many possible variations), a theoretical framework to predict the permutations of useful concentration ranges is useful in the practice of the invention Classical theory predicts that hybridization kinetics depend only on the size of the smaller probe. Theory would therefore predict that the size of the reporter will not play a role in the hybridization kinetics as long as both the target molecule and the ghost probe are significantly smaller. Theory then predicts that the rate of hybridization (for a constant target length) depends on $1/L^{1/2}$, where L is the length of the ghost probe, due to steric inhibition of hybridization. Consequently, the kinetics of hybridization will be faster with smaller ghost probes. As the ghost probe length increases, the hybridization rate should decrease as $1/L^{1/2}$. If a constant ghost probe length is assumed, then the range of reporter lengths and concentrations that will result in a measurable mount of hybridization events can be defined. Once a reporter size has been defined, then the approximate range of ghost probe sizes can be determined. This is an iterative process, but may give good starting points from which to gather data to generate detailed empirical guidelines, given that the theories that the inventors' rationale is based upon were generated from hybridization data in systems that do not employ a reporter probe.

12.5 ENTANGLEMENT THRESHOLD

A reporter probe is essentially a polymer in free solution, which behaves as a random coil. The volume occupied by a single reporter, V_p, can be calculated from polymer physics theories according to the Freely-Jointed Chain model (FJC, for a flexible polymer, such as single-stranded DNA or RNA) or the Worm-Like Chain model (WLC, for a stiff polymer such as double-stranded DNA or a reporter). For either model

$$V_p = \frac{4}{3}\pi R_g^3$$

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where R_g is the radius of gyration. For the FJC

$$R_g = b\left(\frac{N}{6}\right)^{0.6}$$

Where b is the segment length and N is the number of segments in the chain. For the WLC

$$R_g = \sqrt{\frac{1}{6}Nb^2 - \frac{b^2}{4} + \frac{b^2}{4N}\left(1 + \frac{1}{2N}\left(e^{-2N} - 1\right)\right)}$$

The entanglement threshold concentration is defined as the concentration where the entire volume of the solution is occupied by the reporters.

$$C^* = \frac{3}{4\pi R_g^3 N_A}$$

where N_A is Avogadro's number. Above this concentration it is assumed that the translational diffusion of the reporters is severely restricted. The entanglement threshold concentration varies with the reporter structure. As the reporter length increases, the entanglement threshold decreases (as $1/L^{1.5}$). From the equations above, the theoretical entanglement threshold for reporter probes with different spot sizes and different lengths can be calculated. The result of such calculations is shown in Figure 17, which shows that for a 7200 bp RNA/DNA hybrid reporter probe with 8 label attachment regions of about 900 bp each, the entanglement threshold is about 70 nM.

If both the target and the ghost probe are much smaller than the reporters, then they will most likely be free to diffuse through the solution even at these high concentrations of reporters. Initial data indicates that hybridization kinetics do not slow appreciably up to a concentration of 80 nM with a 7200-bp reporter probe, a 100-base target, and a 100-base ghost probe.

12.6 EFFECT OF ENTANGLEMENT THRESHOLD ON MULTIPLEXING

Assuming that the maximum concentration for reporters in a hybridization reaction is C^*, then the concentration of each reporter (specific to a particular target) is equal to C^*/M, where M is the multiplex of the reaction (number of different targets being addressed simultaneously). Conversely, the possible multiplex level for a particular reporter structure can be calculated from the lower limit of probe concentration (C_p from kinetics ~ 10 nM) and the entanglement threshold

$$M = \frac{C^*}{C_p}$$

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If the number of nanoreporter codes available does not depend on reporter probe size, then the multiplexing of the nanoreporter depends primarily on the reporter probe size and concentration (since it is much larger than the ghost probe). Because the ghost probe makes an insignificant contribution to entanglement during hybridiziation, it is the inventors' belief that the concentration of the ghost probe can be increased far above the concentration of the reporter probe. In Table 4 below, the maximum total ghost probe concentration ([G]) is set to 1000 nM for all reporter concentrations. This difference in concentration of ghost probe and reporter probe is an adjustable parameter. Preliminary experiments show that in a multiplex hybridization reaction with a 7200 bp reporter and 100b ghost, 40pM of each reporter probe and 200 pM of each ghost probe results in near complete hybridization in an overnight reaction.

12.7 OPTIMAL SIZE AND CONCENTRATION RANGES

Below in Table 4 is a summary of the optimal useful size and concentration ranges of the ghost probe and reporter probe at different multiplexing as approximated by the above theories. It is the inventors' belief that ghost probes up to about 200 bases will be practical for most applications.

Reporter Length (bp)	Ghost Length (b)	Minimum [R] (pM)	Minimum [G] (pM)	Maximum [R](nM)	Maximum [G](nM)	Max Multiplex
2000	100	5	5	603	1000	114417
2000	50	4	4	603	1000	161811
2000	200	7	7	603	1000	80905
3000	100	6	6	292	1000	45182
3000	50	5	5	292	1000	63897
3000	200	9	9	292	1000	31948
4000	100	7	7	178	1000	23912
4000	50	5	5	178	1000	33817
4000	200	11	11	178	1000	16908
5000	100	8	8	123	1000	14746
5000	50	6	6	123	1000	20854
5000	200	12	12	123	1000	10427
6000	100	9	9	91	1000	9988
6000	50	6	6	91	1000	14125
6000	200	13	13	91	1000	7062
7200	100	10	10	68	1000	6792
7200	50	7	10	68	1000	6792
7200	200	14	10	68	1000	6792
8000	100	11	11	57	1000	5444
8000	50	7	7	57	1000	7699

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8000	200	15	15	57	1000	3850
10000	100	12	12	40	1000	3419
10000	50	8	8	40	1000	4835
10000	200	17	17	40	1000	2417

Table 4: Optimal size and concentration ranges of reporter probe, ghost probe and target, as well as multiplicity of probes, in the nanoreporter systems of the invention.

13. EXAMPLE 8: EXEMPLARY EMBODIMENTS FOR DUAL NANOREPORTER ASSEMBLY

This section describes an embodiment for assembly of a dual nanoreporter in which one probe is a ghost probe and the other probe is a reporter probe comprising color RNA segments assembled on an M13 backbone. The ghost probe is attached to a biotinylated F-hook and the reporter probe is attached to a biotinylated G-hook. The dual nanoreporter is hybridized to a biomolecular sample to detect and quantify a target molecule. The steps below do not have to be performed in the order presented. Moreover, each particular step represents a specific embodiment that may be combined with embodiments other than those presented below.

13.1 PREPARATION OF THE M13 SCAFFOLD

Single-stranded circular M13mp18 DNA (USB Corporation) is annealed to a 5-fold molar excess of an oligonucleotide complementary to the Bam HI recognition site (Bam Cutter oligo) and cut with Bam HI restriction enzyme to yield a linear single-stranded DNA backbone. An oligonucleotide complementary to the Bam Cutter oligonucleotide (anti-Bam oligonucleotide) is subsequently added in 50-fold excess to sequester free Bam Cutter oligonucleotide and thus prevent recircularization of the M13 during later steps.

The linear M13 molecule serves as a scaffold onto which RNA patches, or RNA segments, with incorporated fluorophores can be annealed.

13.2 ATTACHMENT OF A TARGET-SPECIFIC SEQUENCE TO THE SCAFFOLD

An oligonucleotide comprising a sequence (of, *e.g.*, 30-70 nucleotides) complementary to the target nucleic acid of interest, plus 9 bp of additional sequence used for ligation to the M13 scaffold, is generated and ligated to the 3′ end of the linearized M13 scaffold.

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13.3 ATTACHMENT OF G-TAGS TO THE SCAFFOLD

A G-tag (*e.g.*, an oligonucleotide having the sequence 5'-AACATCACACAGACC AACATCACACAGACC AACATCACACAGACC AACATCACACAGACC AGCCCTTTG-3', which includes 4 copies of the complement of the G-hook 5'-GGTCTGTGTGATGTT-3', followed by 9 bases of ligator sequence, and which is complementary to the G-hook) is attached to the 5' end of the linearized single-stranded M13 backbone to allow for (1) purification of the reporter following ligation and/or annealing of segments; and (2) immobilization of the reporter once it is "stretched" on a solid surface. The sequence of the ligator for attaching G-tag to the 5' end of single-stranded M13 which has been linearized at the BamHI site can be 5'-CTCTAGAGGATCCAAAGGGCT-3'. The ligation reaction can be performed according to the following protocol to produce approximately 80 pmol of G-tag/M13 ligation product:

Materials:

[100uM] anti-G4 tag oligo
[100uM] anti-G4 tag ligator oligo
[80 nM] Linear single-stranded M13
10X T4 DNA Ligase Buffer (Fermentas)
T4 DNA Ligase (Fermentas)
20X SSC (Ambion)
DEPC H2O (Ambion)

Method:

1. Pre-anneal the G-tag and ligator:
   25uM 2:1 G/Glig in 1X SSC
   20 µl [100uM] G-tag Ligator
   40 µl [100uM] G-tag
   4 µl 20X SSC
   16 µl DEPC H2O
   * Anneal on the MJ Thermocycler
   95°C, 3min; 72°C, 30sec, -1°C/cycle, x 68 cycles; hold at 4°C
2. Ligate the G-tag to the linear M13:
   64nM M13-G4 in 1X Lig Buffer
   1000 µl [80 nM] Linear M13
   80 µl [25uM] 2:1 G/Glig in 1X SSC

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124 µl 10X T4 DNA Ligase Buffer
40 µl T4 DNA Ligase

* Ligate in an aluminum heat block covered with foil at 37°C for 2hr then at 65°C for 15 minutes to inactivate the enzyme.

13.4 PREPARATION OF RNA SEGMENTS

Ten sets of oligonucleotide primer pairs are designed to create 10 different regions along the M13 scaffold. Each pair contains one primer which has a T7 RNA polymerase promoter at the 5' end. Regions 2-7 are designed to be 900 bases (approximately 300 nm) long, as this is the approximate size of a diffraction-limited spot (the smallest spot that can be achieved with standard optics). Regions 1 and 8 have both long and short versions: the long versions cover the whole 900-base region, while the short versions cover only a portion of the 900-base region to allow a target-specific sequence to be ligated. Thus a target-specific sequence can be attached to either end. The ends can also be used for attachment of anchors or tags.

PCR is performed using Taq polymerase and 0.5 ng of double-stranded M13mp18 (USB Corporation) as a template. Reactions are cleaned up using a Qiaquick purification kit from Qiagen. Each PCR reaction yields a double-stranded fragment corresponding to one specific segment as illustrated below. These fragments are used as templates for the in vitro transcription of the RNA segments.

Using the PCR products described above as double-stranded templates, RNA segments are generated using an in vitro transcription kit from Ambion (Megascript T7 kit). The products of the transcription reactions are purified (including treatment with DNAse I to remove template) using a RNeasy Kit from Qiagen.

13.5 LABELING OF THE RNA SEGMENTS

Using the PCR products described above as double-stranded templates, RNA segments for later dye-coupling are generated using an in vitro transcription kit from Ambion (MessageAmp aRNA kit). Aminoallyl-modified UTP nucleotides are incorporated into the RNA segments during transcription. The products of the transcription reactions are purified (including treatment with DNAse I to remove template) using a RNeasy Kit from Qiagen.

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20-100 μg of aminoallyl-modified RNA segment is coupled with NHS-ester dyes using Ambion Aminoallyl Labeling Kit. Dyes used include Alexa 488, Alexa 594 and Alexa 647 (Invitrogen/Molecular Probes) as well as Cy3 (Amersham).

Each segment is made separately in 4 colors so that each position on the scaffold can be filled with a segment in any of the four colors; thus different colors can be added at different positions to create many unique color combinations.

In this particular embodiment, adjacent segments are of different colors or there may be dark segments interspersed so that each segment is detected as an individual 'spot'. Dark segments may be used as part of the nanoreporter code.

13.6 ANNEALING OF THE RNA SEGMENTS TO THE SCAFFOLD

Segments for each position are annealed in a 2:1 ratio of segment to M13 scaffold in 1X SSPE buffer at 70°C for 2 hours. An assembled nanoreporter with labeled RNA segments is depicted in Figure 3A-3B. Figure 3A depicts a nanoreporter in which only alternate "spots" (1, 3, 5 and 7) are labeled, and Figure 3B depicts a nanoreporter in which every spot is labeled.

13.7 PREPARATION OF THE GHOST PROBE

One or more oligonucleotides comprising sequences (of, *e.g.*, 30-70 nucleotides) complementary to different regions of the target nucleic acid(s) of interest than those to which the target-specific sequences of the reporter probe are complementary, are generated. Optionally, F-tags for F-hook attachment are ligated to the 5′ end of the ghost probe using a a ligator oligonucleotide that is complementary to a short sequence on the 3' end of the F-hook as well as a short sequence on the 5′ end of the ghost probe. The sequences that are complementary to the ligator oligonucleotide are not part of the F-hook sequence or the probe sequence, but are additional nucleotides added to those oligos in order to facilitate ligation.

13.8 ATTACHMENT OF F-TAGS TO THE GHOST PROBE

An F-tag (*e.g.*, an oligonucleotide having the sequence 5′-GATGGAGAC GTCTATCATCACAGC GTCTATCATCACAGC-biotin -3′, which includes 2 copies of the complement of the F-hook 5′-GCTGTGATGATAGAC -3′, followed by 9 bases of ligator sequence and is complementary to the F-hook) is attached to the 3′ end of the ghost probe to allow for (1) purification of the ghost-probe-target-reporter hybridization complex; and

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(2) attachment of the hybridization complex on the slide via the biotin moiety. The sequence of the ligator for attaching F-tag to the 3′ end of the ghost probe can be 5′-GTCTCCATCTTCCGACAG-3′.

Materials:

100uM F-biotin tag

100uM F ghost probe ligator

Fermentas 10X T4 DNA Ligase Buffer

1uM ghost probes

Fermentas T4 DNA Ligase

Method:

1. Pre-anneal the hook and ligator:

5uM F-biotin tag /ligator mix

5 µl [100uM] F-biotin tag

5 µl [100uM] F-ghost probe ligator

10 µl 10X T4 DNA Ligase Buffer

80 µl DEPC H_2O

Anneal on the MJ Thermocycler (95°C, 3min; 72°C, 30sec, -1°C/cycle x 68 cycles; hold at 4°C).

2. Set up the following ghost probe ligation:

300 nM anti-F2-biotin -GP

6.0 µl [1uM] Ghost Probe

4.8 µl [5uM] anti-F2-biotin tag /ligator mix

1.52 µl 10X T4 DNA Ligase Buffer

3.68 µl DEPC H_2O

4.0 µl T4 DNA Ligase

Ligate on the MJ Thermocycler (37°C, 18hr; 65°C, 15 minutes; hold at 4°C)

3. QC the ligation on a 15% Novex TBE-Urea gel:

Prepare the following loading solutions:

Ligation	Neg Control-Ghost Probe
3.33 µl [300 nM] ligation	1 µl [1uM] ghost probe
1.67 µl DEPC H_2O	0.33 µl 10X T4 DNA Ligase Buffer
5 µl 2X Loading Buffer	3.67 µl DEPC H_2O
	5 µl 2X Loading Buffer

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Neg Control- F-biotin tag /ligator mix

2 µl [0.5uM] F-biotin tag /ligator mix

0.33 µl 10X T4 DNA Ligase Buffer

2.67 µl DEPC H_2O

5 µl 2X Loading Buffer

50 bp Oligo Ladder

4 µl Ladder

6 µl 2X loading buffer

Run of a 15% Novex TBE-Urea gel at 180V for 50 minutes.

Stain with SYBR Gold for 30 minutes.

13.8.1 ALTERNATIVE EMBODIMENTS

Rather than covalently coupling biotin to the single-stranded F-tag, the biotinylation of the ghost probe can also be accomplished by annealing a biotinylated oligonucleotide (DNA or RNA) with a sequence complementary to the common portion of the ghost probe. Such a sequence could be the F sequence itself, or another sequence which is added to the ghost probe in addition to the F sequence. If such an additional sequence is added, it could be from 10-100 bases long, from 1-10 copies, with the preferred configuration being a single copy from 50-100 bases long.

13.9 BIOTINYLATION OF TARGET MRNA

There are a number of commercially available kits available for the direct labeling of an mRNA sample including *Label* IT® $\mu Array_{TM}^{Biotin}$ Mirus #MIR 8010) and Biotin-Chem-Link (Roche (1 812 149). Following manufacturer's procedures biotin labeled mRNA is added to the hybridization reaction as described in Section 3d (below) with the following modifications: Since most protocols suggest the use of poly A+ mRNA, the amount of RNA used could be reduced below the 100 ng total RNA in a typical hybridization to 10 ng and possibly 1ng. No ghost probe should be added to this reaction. F bead post-hybridization purification is no longer required. G-bead post-hybridization purification should be used to remove unhybridized biotinylated mRNA that might compete for binding to the slide. Depending on the amount of RNA used, this may or may not be required. Alternatively, total RNA could be biotinylated without the need for purification of the poly A+ fraction. In this case, the original amount of total RNA should be used (100 ng). The

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use of total RNA might require modifications of the manufacturer's protocol to increase labeling efficiency.

An alternative approach would be to enzymatically generate biotinylated 1st strand cDNA or biotinylated amplified RNA (aRNA) using commercially available kits and use these in place of total or mRNA. This approach would require a redesign of the reporter probes to be in the sense orientation. Both ghost probe and F-bead post-hybridization reactions would be omitted while G-bead purification would remain for removal of non-hybridized RNA.

13.10 HYBRIDIZATION OF DUAL NANOREPORTER TO TARGET

Many hybridization conditions are sufficient for achieving gene expression data. To shorten hybridization times while maintaining reasonable hybridization efficiency, several parameters can be altered: i) increasing ghost probe and reporter concentrations, ii) fragmenting of total RNA to average size range of 200-500 bp while lowering the pH of hybridization to 6.5, iii) using more total RNA in same hybridization volume, iv) lowering hybridization volume to approximately 10 µl. Blocking reagents such as Denhardt's and ssDNA can be removed without deleterious effects on hybridization efficiency or cross hybridization to mRNAs from different species.

The following protocol has been performed successfully with multiplexing from 1 to >500 nanoreporters with ghost probes (an example demonstrating a nanoreporter assay utilizing 25 nanoreporters is described in Example 6 above, and another example demonstrating a nanoreporter assay utilizing 509 nanoreporters is described in Example 9 below). The final concentration of all nanoreporters varies depending on 1) the concentration of each reporter and 2) the number of genes being multiplexed.

Typical total nanoreporter concentrations range from 40pM (1 gene @ 40pM) to 20nM (500 genes @40pM). Ghost probe concentrations also vary from 200pM (1 gene @ 200pM) to 100nM (500 genes @ 200pM). The example that follows describes a single multiplexed hybridization containing approximately 500 endogenous genes with positive and negative controls. Add, 11.1 µl of 2.7X hybridization mix [13.5X SSPE pH 7.5 (USB #75890), 0.27 µg/ µl sheared salmon sperm DNA (Sigma #D-7656), 0.27% tween 20 (Sigma #P-1379), and 13.5X Denhardt's reagent (Sigma D-2532)], 5 µl ofgene Nanoreporter mix (0.24nM each or 123nM total, includes 509 endogenous genes and 8 hybridization controls), 4.6 µl 513 gene ghost probe mix (1.3nM each or 667 nM total, includes 509

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endogenous genes and 8 hybridization controls), 1 µl of purification control reporter mix (0.5pM), 1 µl of total cellular RNA (100ng/ul), 1 µl of 30X spike target mix (1.5nM-3fM) and 6.3 µl of DEPC treated water (Ambion #9922) to a 0.2ml thin wall tube (final volume 30 µl).

Final concentration of hybridization reagents should be 5X SSPE, 0.1% tween 20, 100ng/µl sheared salmon sperm DNA, 5X Denhardt's reagent, 40 pM each Nanoreporter (~20nM total), 200pM each ghost probe (~100nM total) and 33ng/µl of total cellular RNA. Control spike targets typically vary in range from 50 fM down to 0.1 fM in a single reaction. All reagents are most preferably free of all nuclease activity. For optimal results, all reagents should be free of nuclease activity.

Mix reagents well and incubate in temperature block with heated lid for 20 hours. After hybridization purify the nanoreporters with affinity reagents for both the ghost probe and the reporter probe.

13.10.1 ALTERNATIVE EMBODIMENT: HYBRIDIZATION PROTOCOL FOR WITHOUT SSDNA AND DENHARDT'S REAGENT

This protocol has been performed successfully with multiplexing from 1-500 nanoreporters and ghost probes. Removal of ssDNA and Denhardt's reagent from hybridizations performed with human reagents (Nanoreporters and ghost probes) had no effect on cross hybridization with mouse total RNA when compared to a hybridization containing ssDNA and Denhardt's. In addition, removal of ssDNA and Denhardt's does not result in an increased background signal (based on negative hybridization controls). Finally, there is no significant loss (or gain) of signal for endogenous genes hybridized in the presence or absence of ssDNA and Denhardt's (509 genes, R^2 value=0.998).

13.10.2 ALTERNATIVE EMBODIMENT: HYBRIDIZATION CONDITIONS FOR FRAGMENTED CELLULAR MRNA

Fragmentation of cellular RNA has been achieved by both thermal and cation catalyzed protocols. These protocols were designed to obtain fragment lengths between 100 and 700 bp (on average). Thermal fragmentation: Dilute total RNA sample to 200 ng/µl in RNAse free water. Heat sample to 95°C in temperature block with heated lid. Stop fragmentation by placing sample on ice. Use immediately or store at -80°C until use. Fragmentation via cation catalyzed reaction modified from manufacturer's protocol (Ambion). Bring volume of RNA sample up to 9 µl with RNAse free water. Final

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concentration of total RNA should be between 0.2 and 2 μg/ml. Add 1 μl of 10X fragmentation buffer (Ambion10X fragmentation buffer). Incubate at 70°C for 5 minutes in temperature block. Longer times will result in smaller fragment size on average. Stop reaction by addition of 1 μl 200 mM EDTA. Use immediately or store at -80°C until use.

Fragmented RNA samples are hybridized as described herein except for the following modifications: i) pH of SSPE is reduced to 6.5 and ii) the time of reaction is reduced to 6hours (for hybridization reactions in which reporter probe and ghost probe concentrations are 200 pM).

13.11 PURIFICATION OF NANOREPORTER-TARGET COMPLEXES

Post-hybridization purification is preferred when the total reporter probe concentration is above 1 nM. Purification significantly decreases non-specific binding and increases specific binding efficiency to the slide at higher reporter and ghost probe concentrations. In the example provided above, a single F-bead purification is described (purifies hybridized complexes from the ghost-probe end). As described in Example 9 below, optimal results at high ghost probe concentrations (>5nM total) are obtained via a subsequent G-bead purification which purifies the hybridization complexes from the 5'end of the reporter effectively removing excess non-hybridized ghost probes. The preferred order of purification is F-bead, then G-bead but the order can be reversed and the protocols optimized accordingly. The exact sequences used in these affinity purifications can likely be changed and optimized in alternative embodimentsof the technology. These affinity purification steps and reagents are currently nucleic acid based but could theoretically be any sort of binding pairs that exhibit specific binding to one another and can be released by chemical treatment or alteration of binding conditions such that the interaction is disrupted and released. For example, an antibody/antigen pair, a protein/metal interaction, or ligand/receptor interaction, *etc.*

One example of purification is provided below.

After hybridization is complete, the salt of a hybridization sample (30 μl, starting at 5x SSPE = 825mM Na^+) is adjusted to a final concentration of approximately 1x SSPE. The diluted sample is added to 30 μl F-hook MyOne Dynabeads (F-MODB) and bound for 15 minutes at room temperature while rotating. The beads are sequestered with a magnet and the supernatant removed. The beads are washed twice with 150 μl 0.1xSSPE + 0.1% Tween at room temperature for 15 minutes w/ rotation and discarded. The purified reporters

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are eluted in 30 µl 0.1x SSPE at 45 °C for 15 minutes w/ rotation. At this point the hybridized reporters are purified from the contaminating un-hybridized reporters. The elution still contains contaminating un-hybridized ghost probes which will compete with the reporters for biotin-binding sites on the streptavidin coated slide. The 30 µl is added to 130 µl of 1xSSPE + 0.1% Tween to increase salt concentration. The sample (150 µl) is then loaded onto 30 µl of G-MODB and bound for 15 min at room temperature. The supernatant is discarded and the beads washed with 150 µl 0.1xSSPE + 0.1% Tween at room temperature for 15 minutes w/ rotation. The wash is discarded and the fully purified reporters eluted with 25 µl 0.1x SSPE at 45 °C for 15 minutes w/ rotation. At this point only targets molecules that are hybridized to both a ghost probe (containing the anti-F sequence) and a reporter (containing the anti-G sequence) will remain in solution.

13.12 IMMOBILIZATION AND STRETCHING AND IMAGING OF NANOREPORTER-TARGET COMPLEXES

Attachment to the slide and immobilization of the stretched complex may be achieved via a biotin-streptavidin interaction. In alternative embodiments, immobilization and stretching are achieved with other interaction pairs provided one of the two could be immobilized on the slide and the other attached to either the ghost probe or the reporter. Stretching does not have to be achieved via electrophoresis but can be done mechanically. The addition of bis-tris propane to the sample before binding is not required. The technology is not limited to the use of particular label monomers exemplified herein as long as the different label monomers can be separated by image processing.

One example of an immobilization and stretching protocol is provided below.

After purification, the hybridization products are loaded directly into an open well of a microfluidic device. The liquid is pulled into a microfluidic channel by capillary action where the hybridized molecules bind to the streptavidin-coated slide through the biotinylated ghost probe. The microfluidic device then intermittently tilts along the axis perpendicular to the length of the channels in alternating directions in order to force the reaction mixture to repeatedly pass through the channel and increase the binding efficiency.

After binding the hybridization reaction, the channel is washed with 1x TAE for 5 minutes by tilting the device at an angle. Fresh TAE is then added to each well to a level sufficient to contact platinum electrodes which are inserted in the wells (30 microliters in our current geometry). An electrical potential of 200V is then applied between the two wells connected by the microfluidic channel, stretching the reporters. After one minute of

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pre-electrophoresis to remove any remaining contaminating un-bound reporter molecules in the channel, a solution of 0.5 μM G-hooks in 1x TAE is added to the cathodic well (60 microliters of this solution). The electrical potential draws the G-hooks through the channel toward the anodic well. As they pass through the channel, the hooks hybridize with the free G-tag sequences on the free-end of the reporters which are bound to the surface and stretched. The streptavidin on the surface then binds the biotin on the G-hook and immobilizes the free end. When the potential is removed, the reporters remain stretched for imaging.

14. EXAMPLE 9: HYBRIDIZATION OF 509 CELLULAR GENES TO 100NG TOTAL RNA FROM A549 CELLS USING NANOSTRING REPORTER SYSTEM.

14.1 HYBRIDIZATION REACTION

Detection of 509 endogenous cellular genes was carried out in single multiplexed hybridization reaction. Eight non-human control sequences were spiked into each reaction that corresponded to approximately 0.1, 0.5, 1, 5, 10, 50, and 100 copies per cell as well as two reporters with no target (negative controls). There were also 4 reporters added that served as positive (3) and negative (1) controls for the post-hybridization purification process. A set of negative control hybridization was also performed containing the entire Nanostring reporter library but lacking cellular RNA.

Each sample was hybridized in triplicate. Final concentrations of the hybridization reagents were as follows: 20.8 nM total Nanoreporters (521 individual Nanoreporters at 40pM each), 103 nM total ghost probe (517 individual ghost probes @ 200pM each), 5X SSPE (pH 7.5), 5X Denhardt's reagent, 100ng/ul sheared salmon sperm DNA, 0.1% tween 20, 50fM S11 spike target DNA, 10fM S10 spike target DNA, 5fM S9 spike target DNA, 1fM S8 spike target DNA, 0.5 fM S7 spike target DNA, 0.1fM S6 spike target DNA. S3 and S4 were added as negative controls. RNA was obtained from A549 lung epithelial cells under two different conditions. The final concentration of total RNA per hybridization was 33ng/ul. No total RNA was added to the negative control hybridizations. The final volume of the reaction was 30ul. Reagents were mixed and incubated at 65°C in thermocycler block with heated lid for 20 hours.

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Master mix	(1 Reaction)	(9.3 reactions)
2.7 X hybridization mix*	11.1 µl	103.2 µl
513 endogenous gene reporters (0.24nM each)	5 µl	46.5 µl
513 endogenous gene ghost probes (1.3nM each)	4.6 µl	42.9 µl
Purification Control reporters (0.6nM each)	1 µl	9.3 µl
30X control target mix	1 µl	9.3 µl
Total	22.7 µl	211.2 µl

*Hybridization mix (13.5X SSPE, 13.5X Denhardt's reagent, 270 ng salmon sperm DNA, 0.27% tween 20)

Reactions	1	2	3	4	5	6	7	8	9
Master mix	22.7	22.7	22.7	22.7	22.7	22.7	22.7	22.7	22.7
48.5ng/µl RNA #1	2.1	2.1	2.1	0	0	0	0	0	0
48.4ng/µl RNA #2	0	0	0	2.1	2.1	2.1	0	0	0
H_2O	5.2	5.2	5.2	5.2	5.2	5.2	7.3	7.3	7.3
Total Rxn volume	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl

Incubate reactions in thermocycler with heated lid overnight (20hours).

14.2 POST-HYBRIDIZATION PURIFICATION

Hybridization reactions were purified to remove unhybridized reporters using an oligonucleotide complimentary to ghost probe attached to magnetic beads (F-bead). Hybridization reactions were diluted 5 fold in 0.1% Tween-20/TE to bring the final salt concentration to 1X SSPE. The diluted hybridization solution was then added to 100ul of F-beads (in 0.1% Tween-20) and allowed to bind to the beads at room temperature for 30min with continuous rotation. The beads were then washed three times in 150ul of 0.1X SSPE/0.1% Tween-20 and eluted in 100ul of 0.1X SSPE/0.1% Tween-20 for 15min at 45°C.

After F-bead elution, samples were purified from the opposite end of the hybridized complex using G-beads. Elutions were brought to a final concentration of 1X SSPE by the addition of 50ul of 3X SSPE/0.1% Tween-20 and bound to 30ul of G-beads (in 0.1% Tween-20) for 15min at room temperature with rotation. Beads were then washed as above and eluted in 30ul of 0.1X SSPE/Tween-20 and prepared for binding as described below.

14.3 BINDING, STRETCHING, AND IMMOBILIZATION

The samples was prepared for binding by addition of 1ul of 1/5000 dilution of 0.1 uM Tetraspec™ fluorescent microspheres (product # T7279, Molecular Probes). Samples were loaded into a Nanostring fluidic device and attached to Accerl8 Optichem® slide

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coated with strepavidin (product #TB0200) by tilting the device 45deg for 15min and repeated a total of 4 times. After loading, slide surface was washed once with 90ul of 1X TAE. After wash buffer is removed the sample is prepared for electrostretching by addition of 40ul of TAE to each well. Attached complexes were stretched by applying 200V across the fluidic channel. After 1 minute the samples were immobilized in the stretched position by adding 60ul of 500nM of G-hook oligo solution to the well containing the negatively charged electrode while continuing to apply voltage for 5 minutes. After immobilization the TAE solution is removed and replaced with anti-photobleaching reagent for imaging.

14.4 IMAGING

Slides were imaged on Nikon Eclipse TE2000E equipped with a metal halide light source (X-cite 120, Exfo Corporation) and a 60X oil immersion lens (1.4 NA Plan Apo VC, Nikon). For each field of view, 4 images at different excitation wavelengths (480, 545, 580 and 622) were acquired with an Orca Ag CCD camera (Hamamatsu) under control of either Metamorph (Universal Imaging Corporation) or custom software. Images were processed with custom image processing software.

14.5 DATA ANALYSIS

Raw data was extracted from processed images using custom software. Data was normalized to the average counts for control spikes in each sample. To determine if a gene was "detected" by the system, the counts obtained for each gene from hybridizations containing RNA were compared to average counts of the two negative controls using a Student's ttest. The number of genes detected was 441 (87%) and 445 (88%) in sample #1 and #2, respectively.

A scatter plot (Figure shows normalized and average $\log_2$ signal values from each positive sample (n=3) for all 509 genes. The genes that were significantly different in the two samples were identified by a T-test of signal values in sample #2 against sample #1. In the graph below, the solid lines indicate the 2 fold upregulated threshold (black line) and 2-fold downregulated threshold (gray line) relative to sample #1. Genes with significant fold changes (p-value < 0.05) are shown in solid black diamonds. Genes whose fold change p-values were above this threshold are shown in open black squares.

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15. EXAMPLE 10: DETECTION OF SMALL SPOTS

As mentioned above, the label attachment regions of a nanoreporter scaffold region have a length anywhere from 10 nm to 10,000 nm, but preferably corresponds closely to the smallest spot that can be detected with standard optics, which is about 300 nm. Spots of different color (spectrally distinguishable) are spatially resolvable at closer spacing than spots of the same color. It is possible to fit one, two, three or four spots of different colors between two spots of the same color, and yet spectrally and spatially resolve all the spots. It is also possible to significantly reduce the distance between two spots of the same color.

The limits of spatial resolution, *i.e.*, differentiating closely spaced spots of the same color, are often thought of as hard limits, *i.e.*, the Rayleigh Criteria (Inoue, S., Spring,. Video Microscopy (Plenum Press, 1997), p 30.) There are many techniques to drive beyond these limits that involve different imaging and/or image processing techniques. On the imaging side, structured illumination is one method to resolve spots of the same color that are spaced closer together. 50 nm has been demonstrated but, in theory, resolution with structured illumination is unlimited (Gustafsson, 2005, Proc. Nat'l. Acad. Sci. U.S.A. 102:13081-13086). On the image processing side, mixture modeling is an effective technique to push beyond commonly accepted limits (Thomann *et al.*, 2002, J. Microsc. 211:230-248). The combination of these techniques allows for drastically smaller nanoreporters with smaller spots, corresponding to label attachment regions of less than 50 nm.

These smaller spot spacings could allow for drastically shorter and more stable reporters, a larger number of codes, as well as a higher degree of multiplexing before the entanglement threshold is passed (for an explanation of entanglement thresholds, see Example 9 (described in Section 14) above.

The tradeoff of making the spots much smaller and the reporters much shorter would be decreased signal and slower scan times. However, other technical advances, such as brighter light sources, and more efficient CCDs may offset the increased scan times making these approaches reasonable.

16. REFERENCES CITED

All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent

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application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

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WHAT IS CLAIMED IS:

1. A probe pair comprising a first probe and a second probe,

said first probe being a complex comprising:

(a) a first molecule, comprising: (i) a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region, which is non-overlapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal; and (iii) an affinity tag attached to said first molecule and

(b) a first target-specific sequence attached to the first molecule,

said second probe being a second molecule, comprising (i) a second target-specific sequence; and (ii) an affinity tag attached to said second molecule;

wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule,

wherein the target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA,

wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule; and

wherein the second molecule does not comprise a third label attachment region to which are attached one or more label monomers that emit light constituting a third signal.

2. A probe pair comprising a first probe and a second probe,

said first probe being a complex comprising:

(a) a first molecule, comprising: (i) a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which are attached one or more label monomers that emit light constituting a second signal; and (iii) an affinity tag attached to said first molecule; and

(b) a first target-specific sequence attached to the first molecule,

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said second probe being a second molecule, comprising (i) a second target-specific sequence; (ii) an affinity tag attached to said second molecule;

wherein the first target-specific sequence and the second target-specific sequence bind to different regions of the same target molecule,

wherein the first signal and the second signal are spatially or spectrally distinguishable;

wherein the target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA,

wherein when said probe pair is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitute at least part of a code that identifies the target molecule; and

wherein the second molecule does not comprise a third label attachment region to which are attached one or more label monomers that emit light constituting a third signal.

3. The probe pair of claim 1 or 2, wherein said first and second label attachment regions are predetermined nucleotide sequences.

4. The probe pair of claim 3, wherein a first DNA molecule is hybridized to the first label attachment region, to which first DNA molecule are bound said one or more label monomers that emit light constituting said first signal; and wherein a second DNA molecule is hybridized to the second label attachment region, to which second DNA molecule are bound said one or more label monomers that emit light constituting said second signal.

5. The probe pair of claim 3, wherein a first RNA molecule is hybridized to the first label attachment region, to which first RNA molecule are bound said one or more label monomers that emit light constituting said first signal; and wherein a second RNA molecule is hybridized to the second label attachment region, to which second RNA molecule are bound said one or more label monomers that emit light constituting said second signal.

6. The probe pair of claim 3, wherein a plurality of first DNA molecules are hybridized to the first label attachment region, to which DNA molecules are bound said one or more label monomers that emit light constituting said first signal; and wherein a plurality of second DNA molecules are hybridized to the second label attachment region, to which second DNA molecules are bound said one or more label monomers that emit light constituting said second signal.

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7. The probe pair of claim 3, wherein a plurality of first RNA molecules are hybridized to the first label attachment region, to which RNA molecules are bound said one or more label monomers that emit light constituting said first signal; and wherein a plurality of second RNA molecules are hybridized to the second label attachment region, to which second RNA molecules are bound said one or more label monomers that emit light constituting said second signal.

8. The probe pair of any one of claims 1 to 7, wherein the first molecule is a first nucleic acid that is 2,000 to 10,000 bases in length, and where the second molecule is a second nucleic acid that is 40 to 250 bases in length.

9. The probe pair of claim 8, wherein the first nucleic acid is 5,000 to 8,000 bases in length, and where the second nucleic acid is 50 to 100 bases in length.

10. The probe pair of claim 8 or 9 wherein the first nucleic acid and the second nucleic acid are DNA molecules.

11. A method of detecting a target molecule in a biomolecular sample comprising: (i) contacting said sample with a probe pair according to any one of claims 1-10 under conditions that allow binding of the first target-specific sequence and the second target-specific sequence to the target molecule and (ii) detecting the code that identifies the target molecule.

12. The method of claim 11, further comprising quantitating the amount of said target molecule in said biomolecular sample.

13. The method of claim 11 or 12, wherein the concentration of each of said first and second target-specific sequences is in excess of the concentration of said target molecule.

14. A method of detecting a plurality of target molecules in a biomolecular sample comprising: (i) contacting said sample with a population of probe pairs according to any one of claims 1-10 under conditions that allow binding of the first target-specific sequence and the second target-specific sequence of each probe pair to their respective target molecule, wherein each probe pair in said population when bound to its respective target molecule is associated with a distinguishable code, and (ii) detecting the codes that identify the plurality of target molecules.

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15. The method of claim 14, further comprising quantitating the amount of each of said plurality of target molecules in said biomolecular sample.

16. The method of claim 14 or 15, wherein the concentration of each of said first and second target-specific sequences of each probe pair of said population is in excess of the concentration of its respective target molecule.

17. A probe, said probe being a nucleic acid complex comprising

(a) a synthetic nucleic acid molecule comprising (i) a first label attachment region to which is hybridized a first RNA molecule, to which first RNA molecule are attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is hybridized a second RNA molecule, to which second RNA molecule are attached one or more label monomers that emit light constituting a second signal; and

(b) a target-specific sequence covalently attached to the synthetic nucleic acid molecule,

wherein the first and second RNA molecules are each at least 50 nucleotides,

wherein the target-specific sequence binds to a target molecule, which target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA,

and wherein when said probe is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

18. A probe, said probe being a nucleic acid complex comprising

(a) a nucleic acid molecule comprising (i) a first label attachment region to which is hybridized a first RNA molecule, to which first RNA molecule are covalently attached one or more label monomers that emit light constituting a first signal; (ii) a second label attachment region to which is hybridized a second RNA molecule, to which second RNA molecule are covalently attached one or more label monomers that emit light constituting a second signal; and

(b) a target-specific sequence covalently attached to the nucleic acid molecule,

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wherein the first and second RNA molecules are each at least 50 nucleotides,

wherein the target-specific sequence binds to a target molecule, which target molecule is a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA,

and wherein when said probe is bound to its target molecule, the identity of the first and second signals and their locations relative to each other constitutes at least part of a code that identifies the target molecule.

19. The probe of claim 17 or 18, wherein the probe further comprises an affinity tag.

20. The probe of claim 19, wherein the affinity tag is attached to said first RNA molecule or second RNA molecule.

21. The probe of claim 20, wherein the affinity tag is attached by being covalently bound to an oligonucleotide hybridized to the first RNA molecule or the second RNA molecule.

22. The probe of claim 19, wherein the affinity tag is covalently bound to the nucleic acid molecule.

23. The probe of claim 17 or 18, wherein at least one of the first signal and the second signal comprises light at a plurality of different wavelengths.

24. The probe of claim 17 or 18, wherein the label monomers attached to the first label attachment regions emit light at the same wavelength, which light constitutes said first signal, and wherein the label monomers attached to the second label attachment region emit light at the same wavelength, which light constitutes the second signal.

25. The probe of claim 17 or 18, in which the target-specific sequence is unlabeled with any one or more of said label monomers.

26. The probe of claim 17 or 18, wherein first and second signals are spectrally distinguishable.

27. The probe of claim 17 or 18, wherein the first and second RNA molecules are each 100 to 3,000 nucleotides.

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28. The probe of claim 17 or 18, wherein the first and second RNA molecules are each 500 to 1,500 nucleotides.

29. A nucleic acid comprising one or more non-overlapping regions, each said region being at least 50 nucleotides and comprising a regularly-repeated base, said regularly-repeated base having a periodicity of four to twenty five such that said base occurs at regular intervals of every fourth to every twenty fifth position in said region.

30. The nucleic acid of claim 29 wherein not more than 5% of said base appears at other than said regular intervals in said region.

31. The nucleic acid of claim 29 which is a single-stranded nucleic acid.

32. The nucleic acid of claim 29 which is a double-stranded nucleic acid.

33. The nucleic acid of claim 29 which is a RNA.

34. The nucleic acid of claim 29 which is a DNA.

35. The nucleic acid of claim 29, wherein the regularly-repeated base is a thymidine or a uracil.

36. The nucleic acid of claim 35, wherein the uracil is aminoallyl-modified.

37. The nucleic acid of claim 29, wherein the periodicity of the regularly-repeated base in said one or more regions is 6 to 15.

38. The nucleic acid of claim 37, wherein the periodicity of the regularly-repeated base in said one or more regions is 8 to 12.

39. The nucleic acid of claim 29, which comprises a plurality of said regions.

40. The nucleic acid of claim 39, wherein the nucleic acid comprises 2 to 50 regions.

41. The nucleic acid of claim 40, wherein the nucleic acid comprises 3 to 10 regions.

42. The nucleic acid of claim 39, wherein the plurality of regions are immediately adjacent to one another.

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43. The nucleic acid of claim 39, wherein the plurality of regions are separated by one or more spacer regions.

44. The nucleic acid of claim 43, in which the spacer region comprises a restriction endonuclease recognition sequence or its complement and/or an RNA polymerase promoter sequence.

45. The nucleic acid of claim 39, wherein each region in the plurality of regions differs from one another in sequence.

46. The nucleic acid of claim 29, which further comprises an origin of replication.

47. The nucleic acid of claim 29, wherein the GC content of the one or more regions is a GC content of 25% to 75%.

48. The nucleic acid of claim 47, wherein the GC content of the one or more regions is 40 to 60%.

49. The nucleic acid of claim 48, wherein the GC content of the one or more regions is 45% to 50%.

50. The nucleic acid of claim 29 in which each of said one or more regions is 50 to 50,000 bases in length.

51. The nucleic acid of claim 50 in which each of said one or more regions is 200 to 2,000 bases in length.

52. The nucleic acid of claim 50 which is 50 to 100,000 bases in length.

53. The nucleic acid of claim 51 which is 200 to 10,000 bases in length.

54. The nucleic acid of claim 50 which comprises no more than one region.

55. The nucleic acid of claim 50 which comprises a plurality of regions.

56. The nucleic acid of claim 55, wherein the regularly-repeated base is the same in at least two regions in the plurality of regions.

57. The nucleic acid of claim 55, wherein the periodicity of the

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regularly-repeated base is the same in at least two regions in the plurality of regions.

58. The nucleic acid of claim 55, wherein both the regularly-repeated base and its periodicity are the same in at least two regions in the plurality of regions.

59. The nucleic acid of claim 29 which further comprises a target-specific sequence.

60. The nucleic acid of claim 29 in which each of said one or more regions does not comprise a recognition site for at least one restriction endonuclease with a 6 base-pair recognition site.

61. The nucleic acid of claim 60, wherein the at least one restriction endonuclease is BamH1, EcoRI, PstI, SmaI, XbaI, NotI, SacI, EcoRV, HindIII, ClaI, SalI, XhoI, ApaI, or KpnI.

62. The nucleic acid of claim 29 in which said one or more regions are not predicted by the MFOLD algorithm to contain hairpin loops.

63. The nucleic acid of claim 29 in which said one or more regions do not have direct or inverted repeats that are greater than 10 bases in length.

64. The nucleic acid of claim 29 which further comprises a promoter sequence or its complement.

65. The nucleic acid of claim 64 in which the promoter is a T7, T3 or SP6 transcription promoter.

66. The nucleic acid of claim 29 which further comprises a phage encapsidation sequence.

67. The nucleic acid of claim 29 in which said one or more regions is flanked by restriction endonuclease recognition sites.

68. The nucleic acid of claim 29 in which at least 80% of occurrences of said regularly-repeated base of said one or more regions are attached to at least one light-emitting label monomer.

69. The nucleic acid of claim 68 in which at least 80% of said occurrences of

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said regularly-repeated base are covalently attached to the label monomer.

70. The nucleic acid of claim 68 in which at least 80% of said occurrences of said regularly-repeated base are indirectly attached to the label monomer via hybridization to another nucleic acid to which the label monomer is covalently attached.

71. The nucleic acid of claim 68 which comprises a plurality of different regions, wherein at least 80% of said occurrences of said regularly-repeated bases of each of the different regions are attached to a different light-emitting label monomer.

72. The nucleic acid of claim 29 which further comprises an affinity tag.

73. The nucleic acid of claim 29 which is a partially or fully double-stranded molecule, in which one strand is DNA to which is hybridized a plurality of RNAs, each RNA comprising not more than one region, the regularly-repeated base in said region being aminoallyl-modified uracil.

74. The nucleic acid of claim 73 in which 80% of occurrences of said aminoallyl-modified uracil are covalently attached to at least one light-emitting label monomer.

75. The nucleic acid of any of claims 29 to 74 which is purified.

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FIGURE 1A-1C

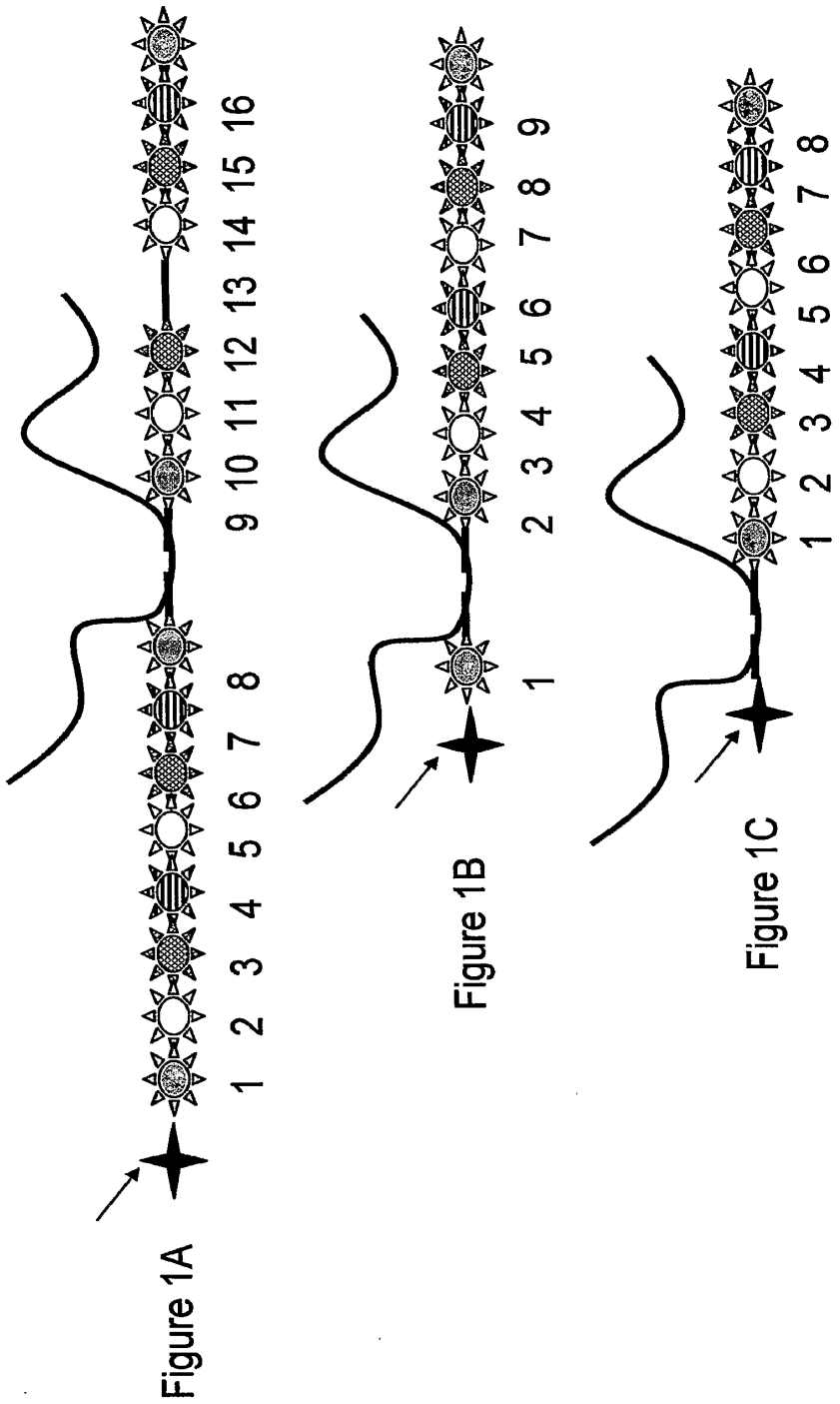


Figure 1A Figure 1B Figure 1C

FIGURE 1D-1F

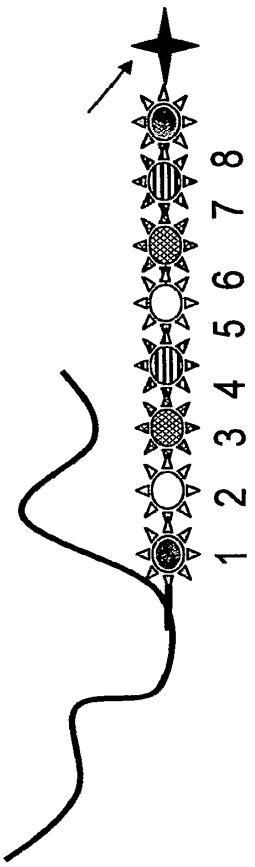


Figure 1D

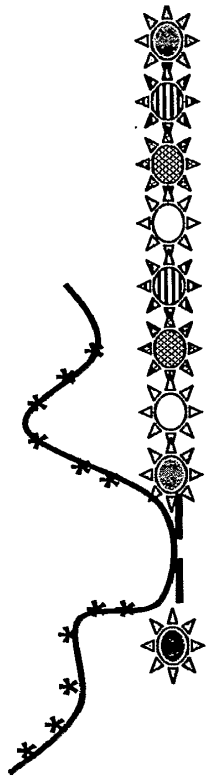


Figure 1E

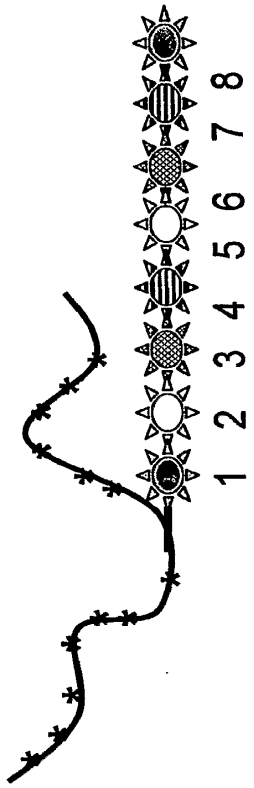


Figure 1F

FIGURE 2

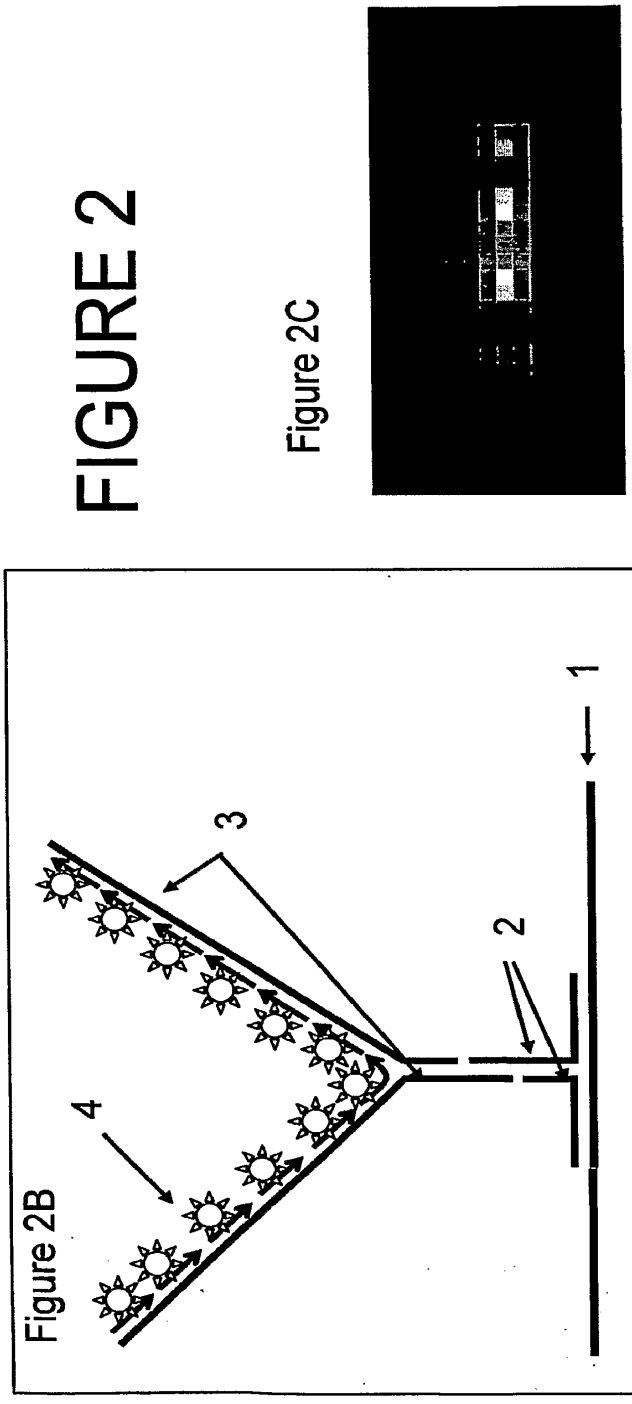


Figure 2B

Figure 2C

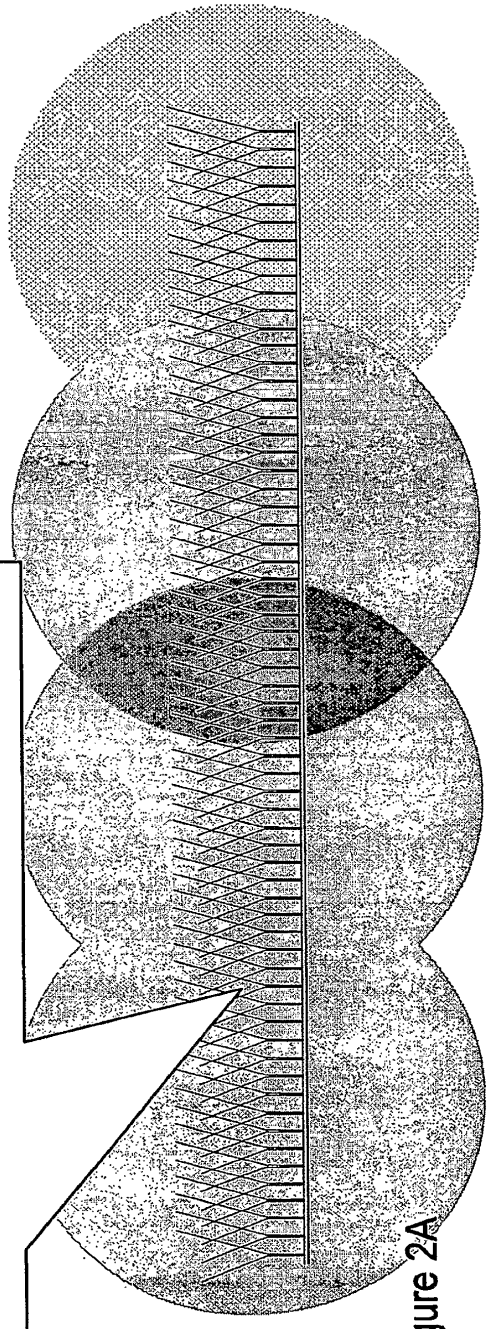


Figure 2A

FIGURE 3

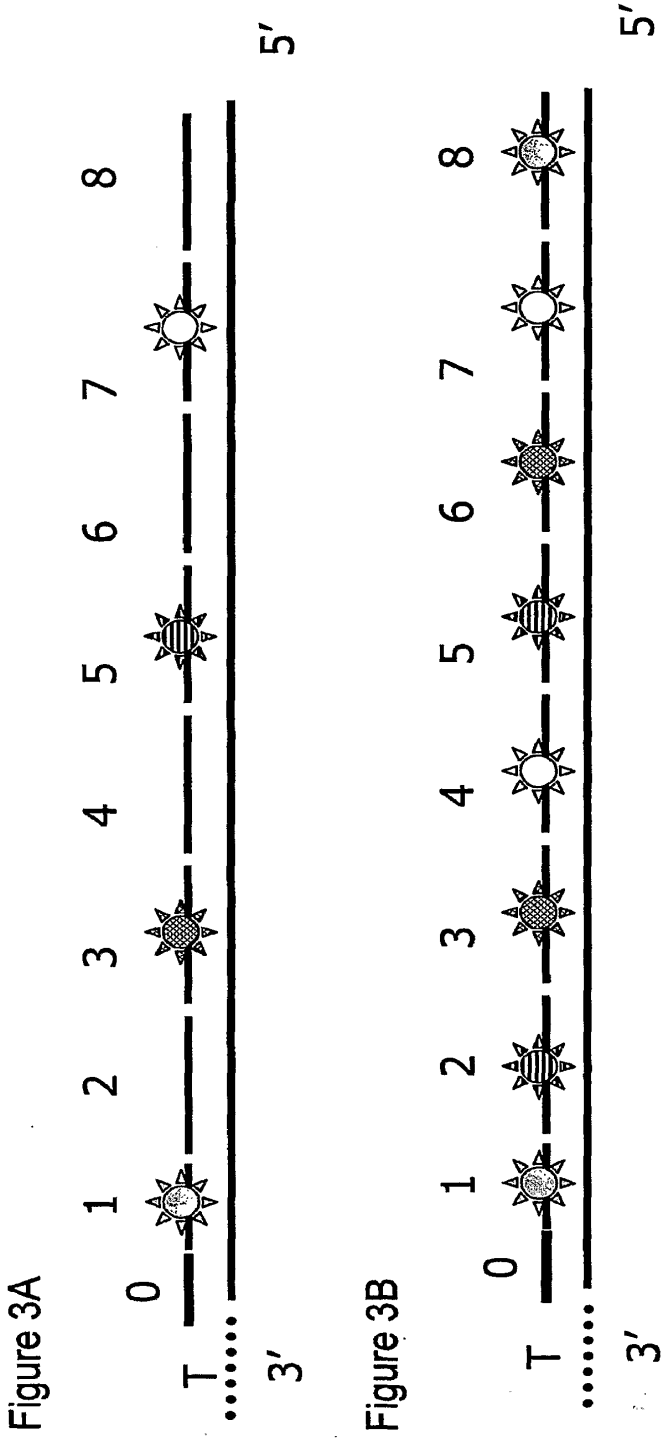
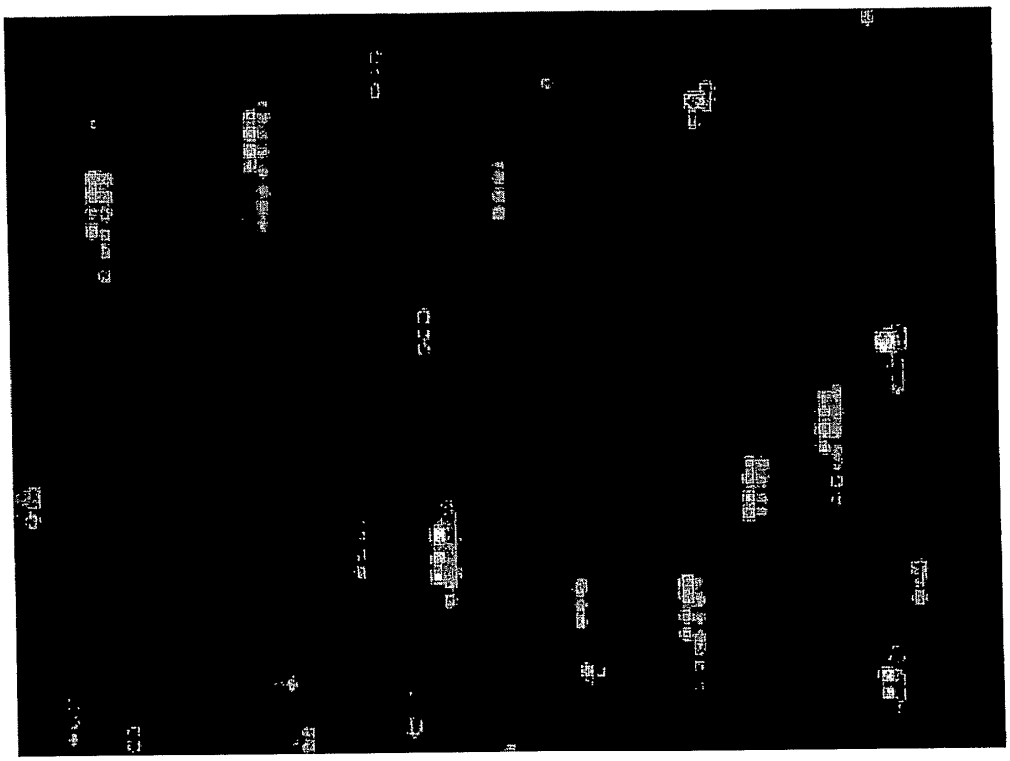


Figure 3A

Figure 3B

FIGURE 4



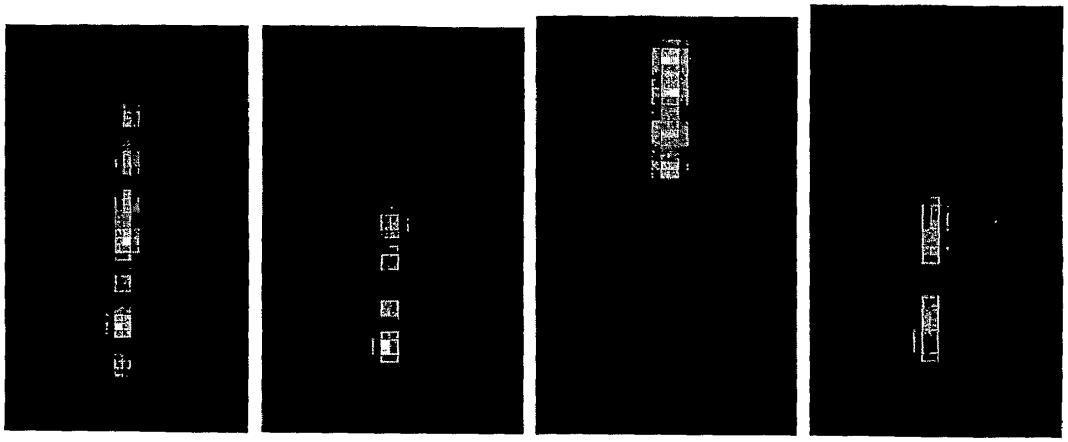


FIGURE 5

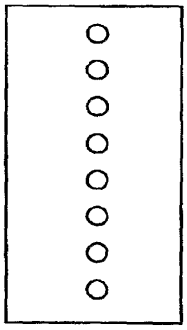


Figure 5A

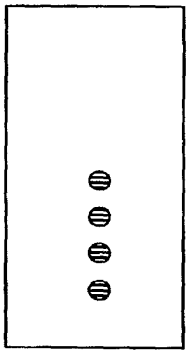


Figure 5B

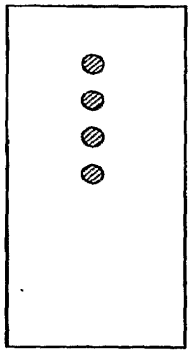


Figure 5C

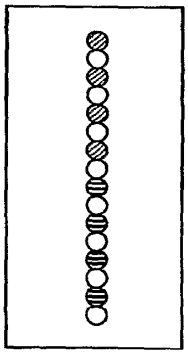


Figure 5D

FIGURE 6

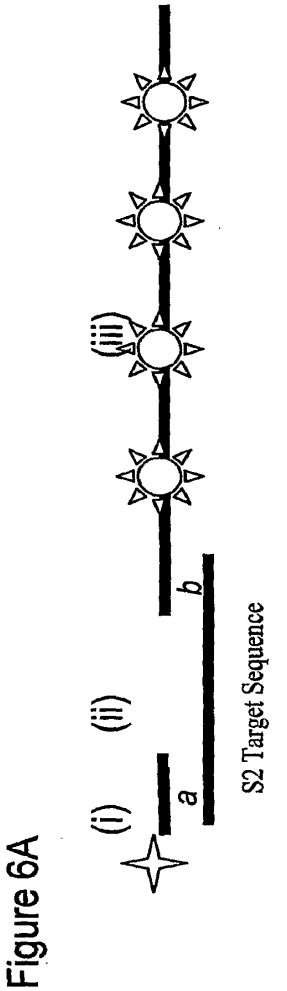


Figure 6A



Figure 6E

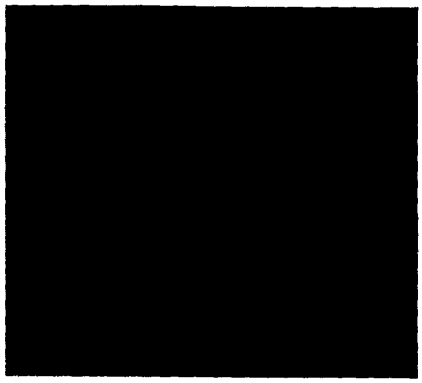


Figure 6D

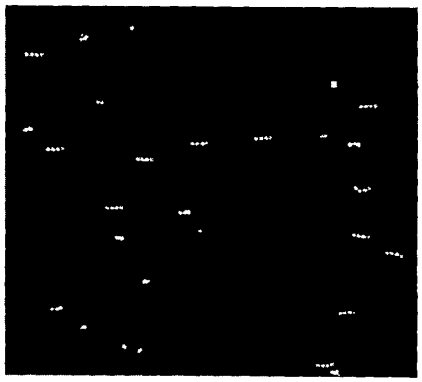


Figure 6C



Figure 6B

FIGURE 7A-D

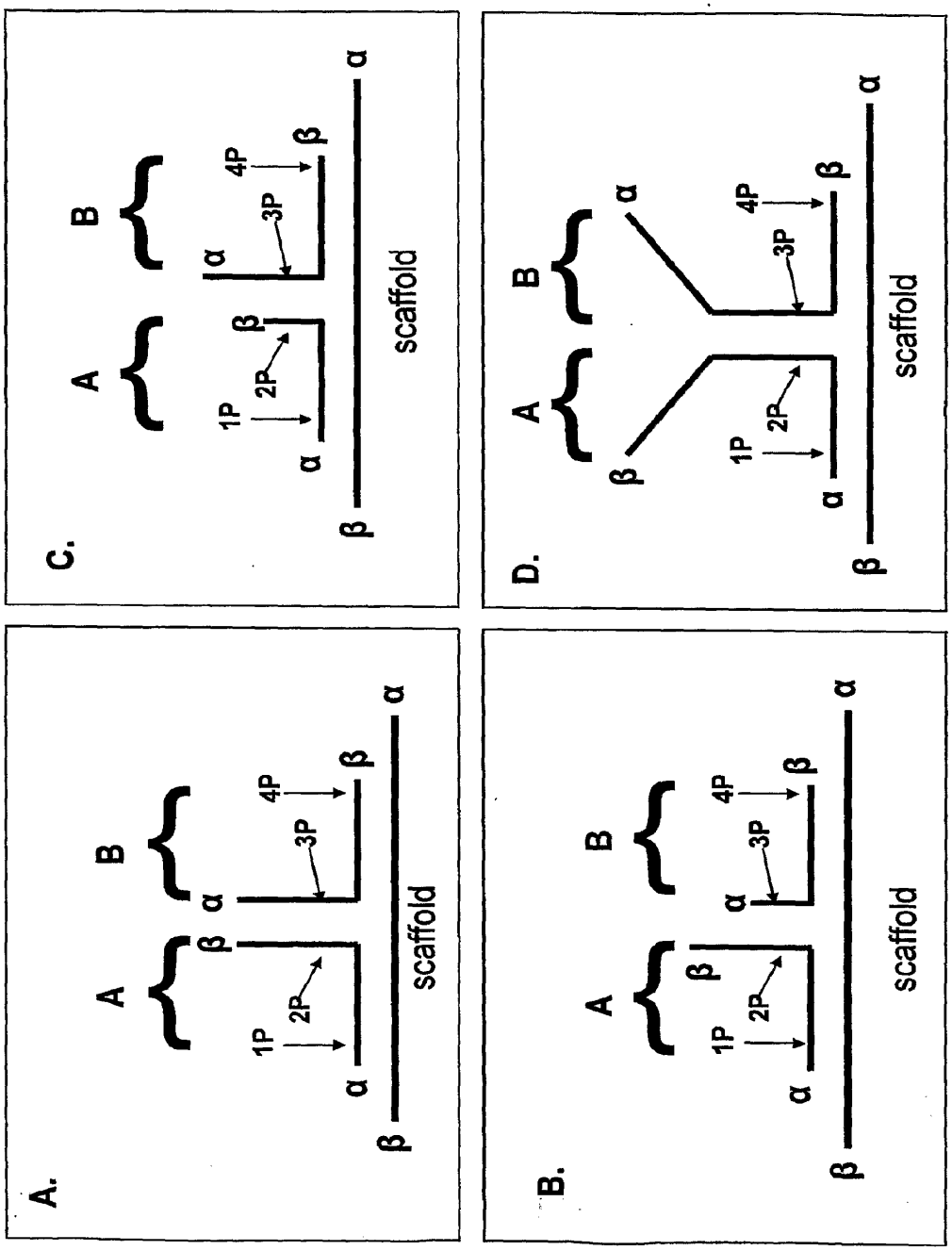
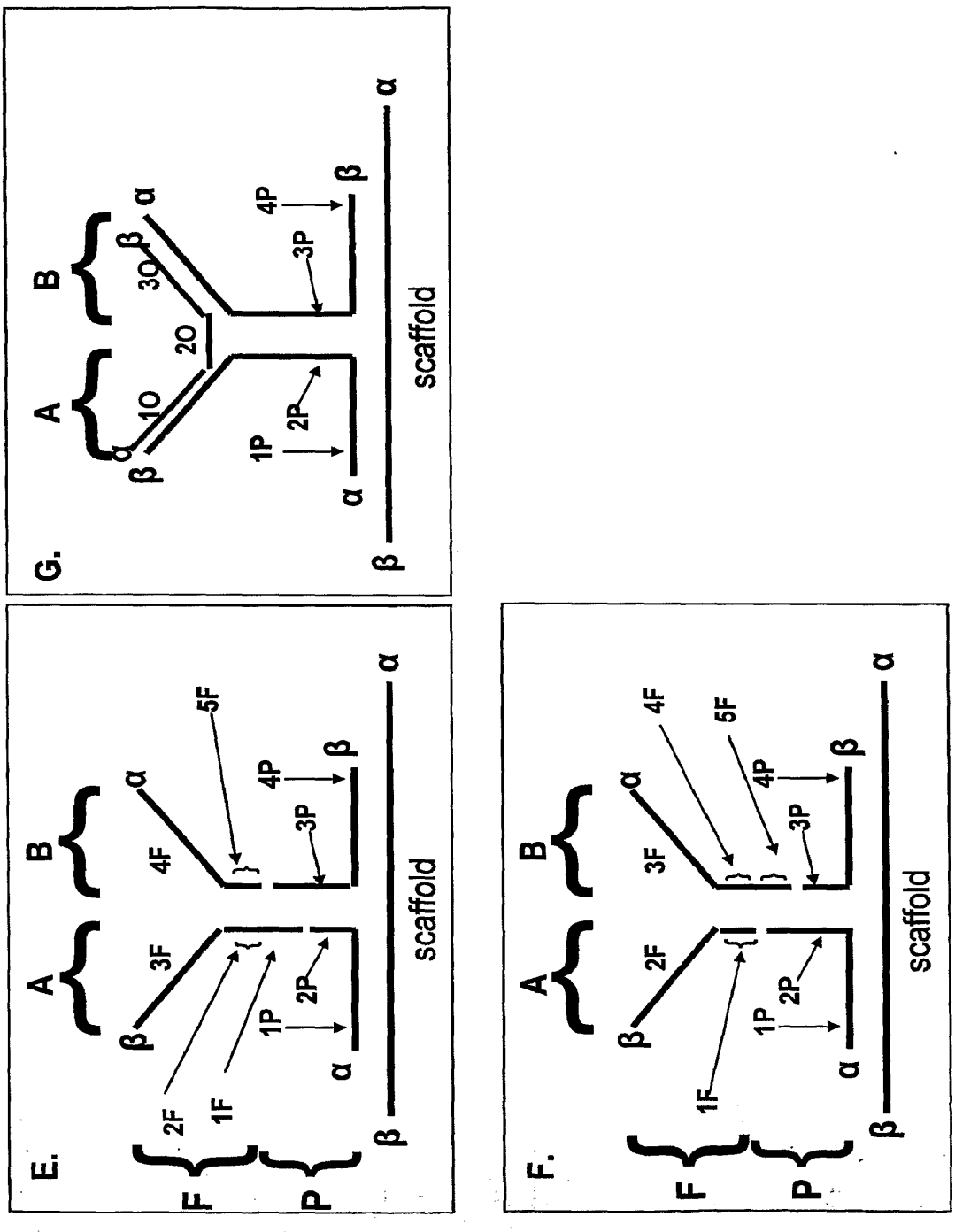


FIGURE 7E-G



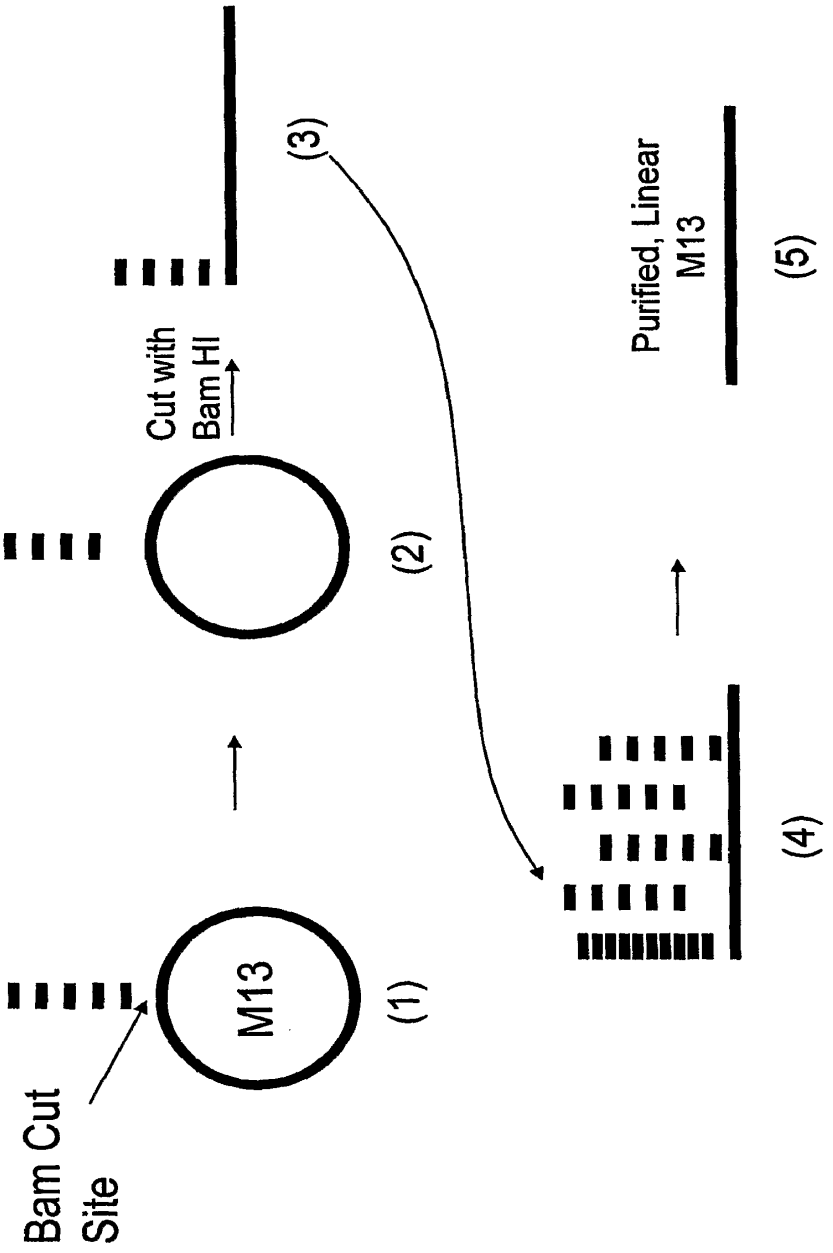


FIGURE 8

FIGURE 9

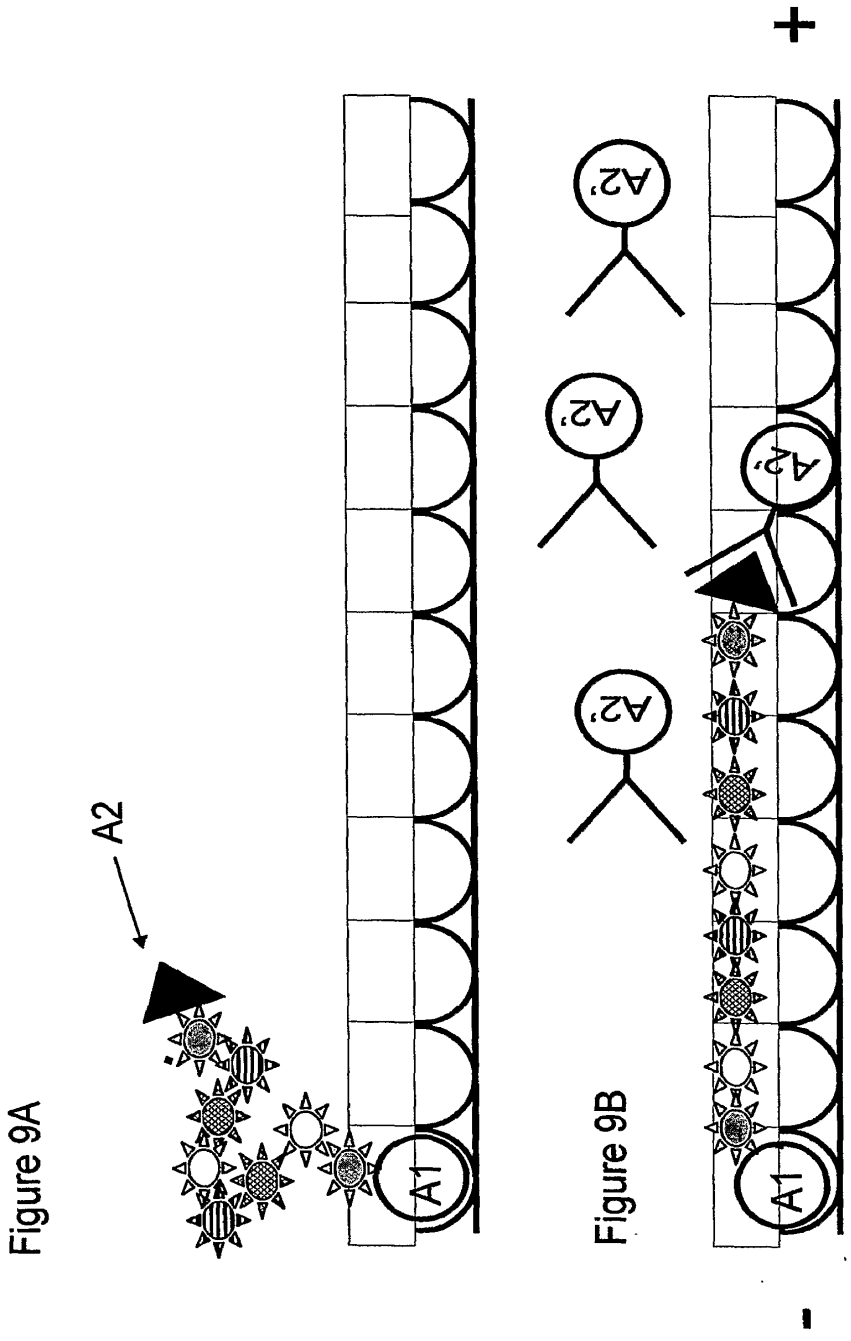


Figure 9A

Figure 9B

FIGURE 10



Figure 10A

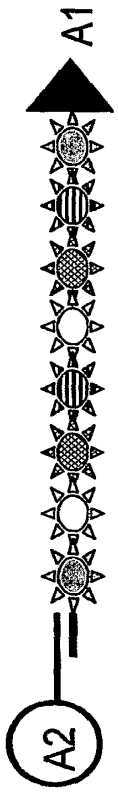


Figure 10B



Figure 10C

FIGURE 11

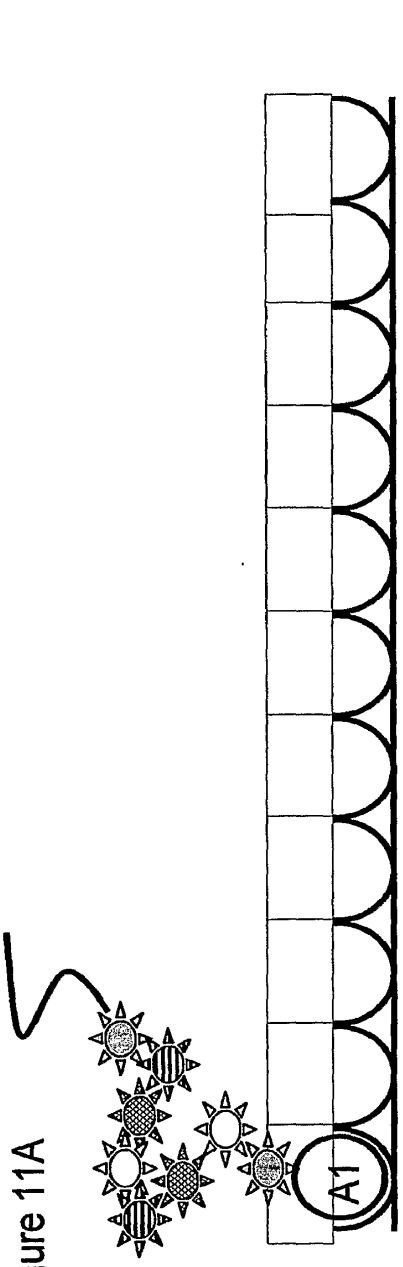


Figure 11A

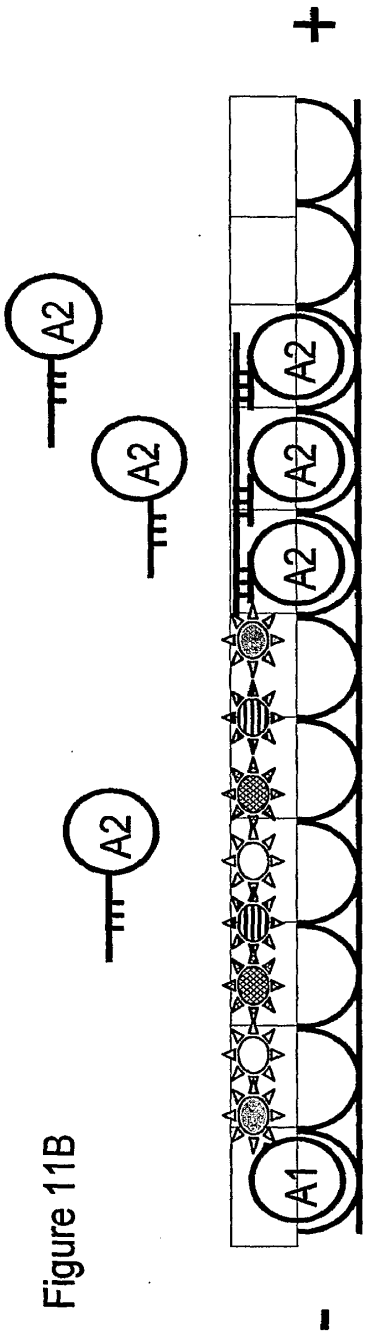


Figure 11B

FIGURE 12A-12B

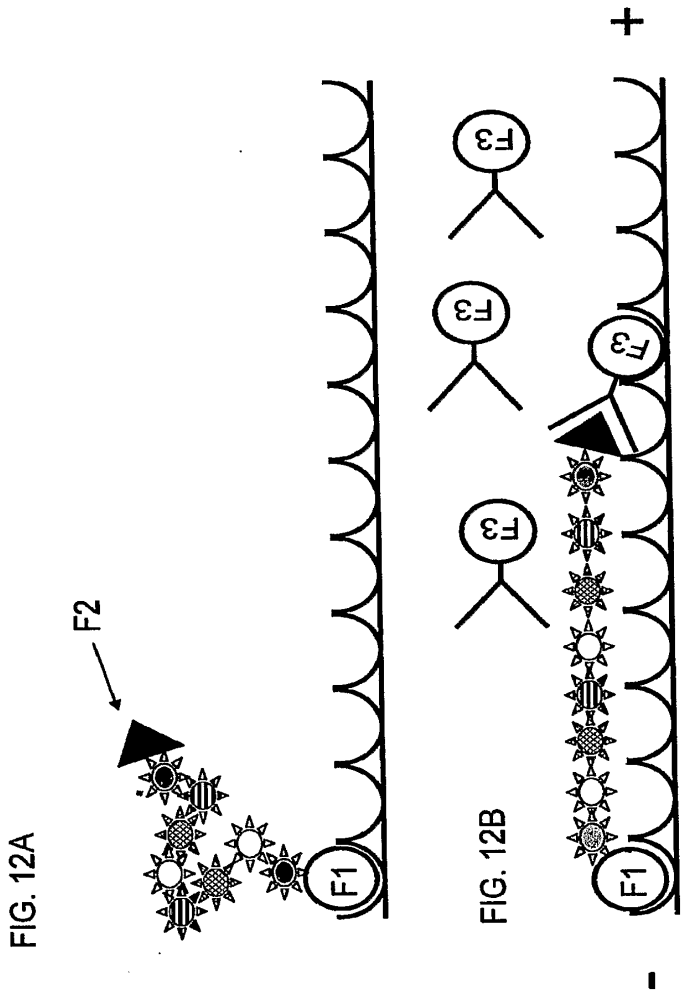


FIG. 12A FIG. 12B

FIGURE 13A-13C

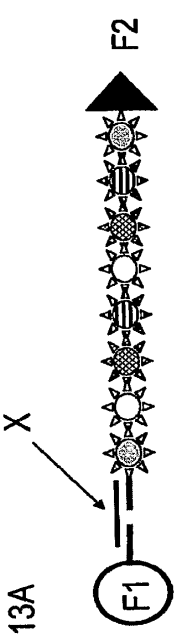


FIG. 13A



FIG. 13B



FIG. 13C

FIGURE 14A-14B

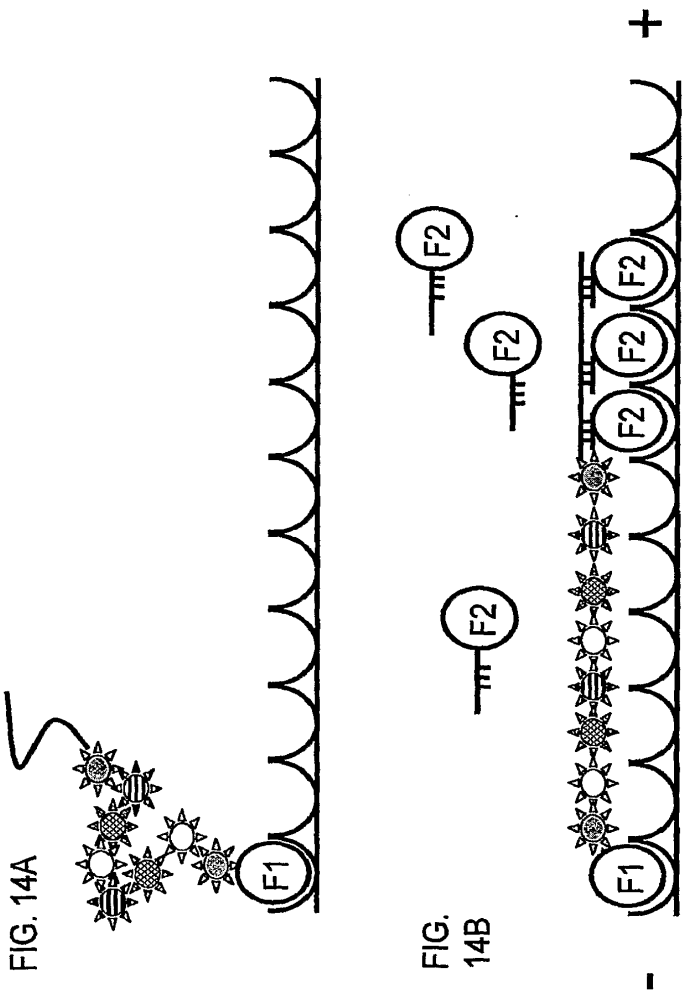


FIGURE 14C-14D

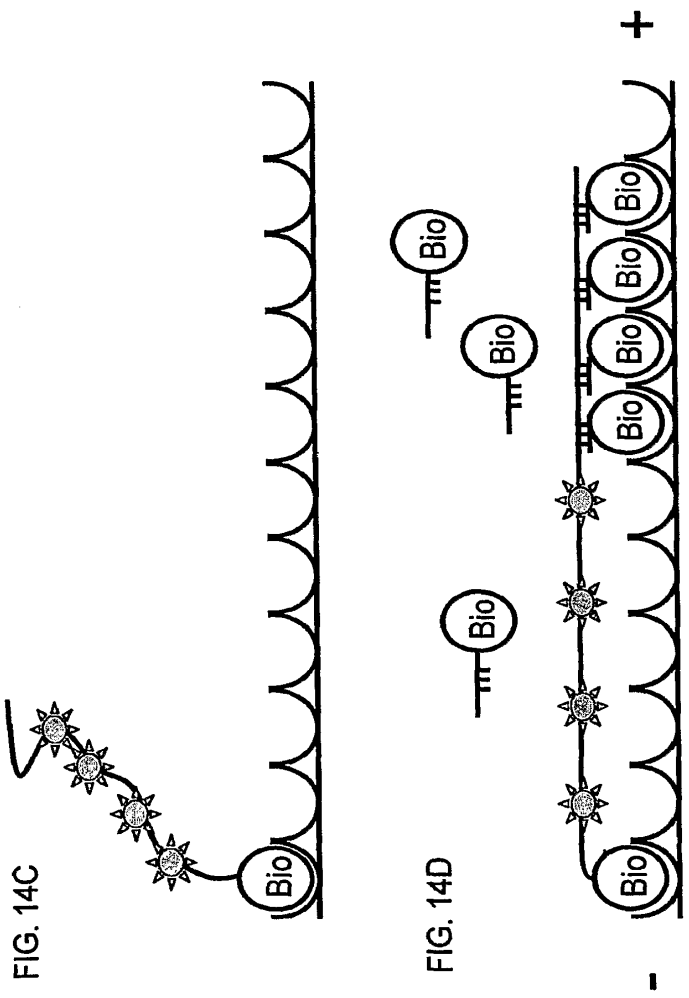


FIG. 14C

FIG. 14D

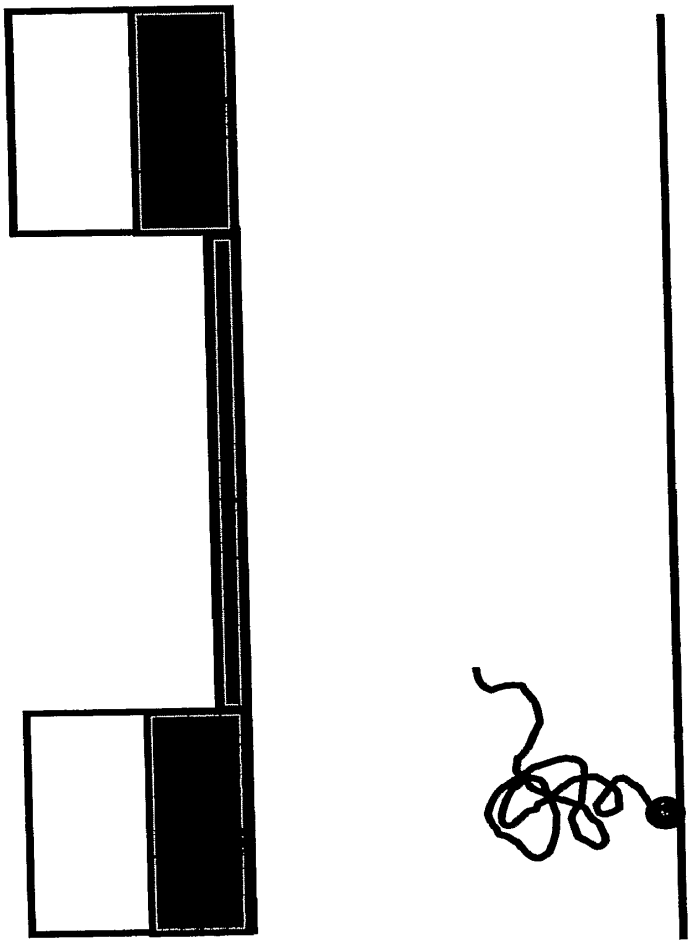


FIGURE 15A

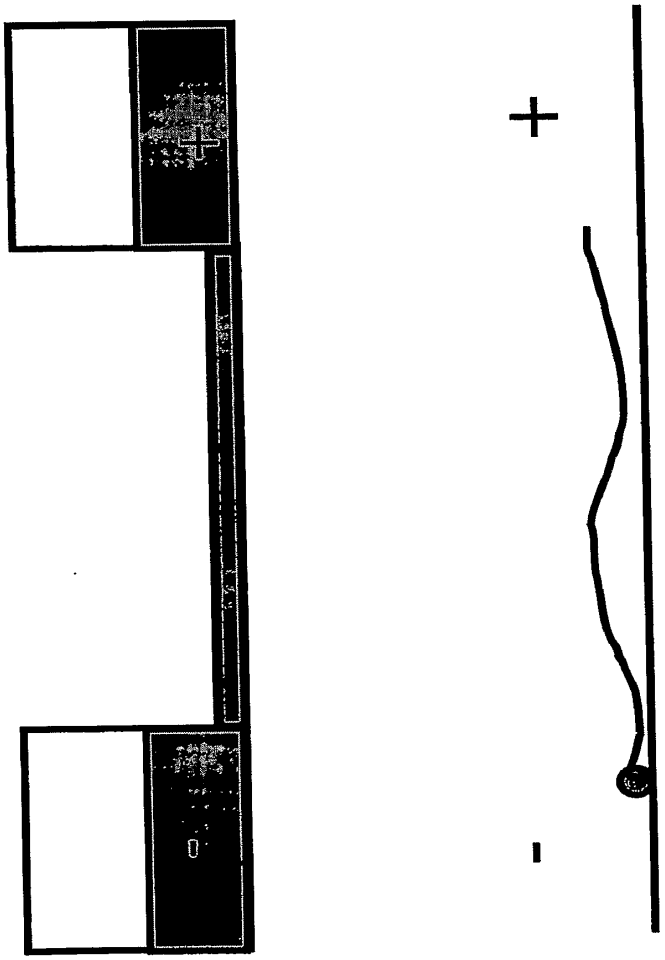


FIGURE 15B

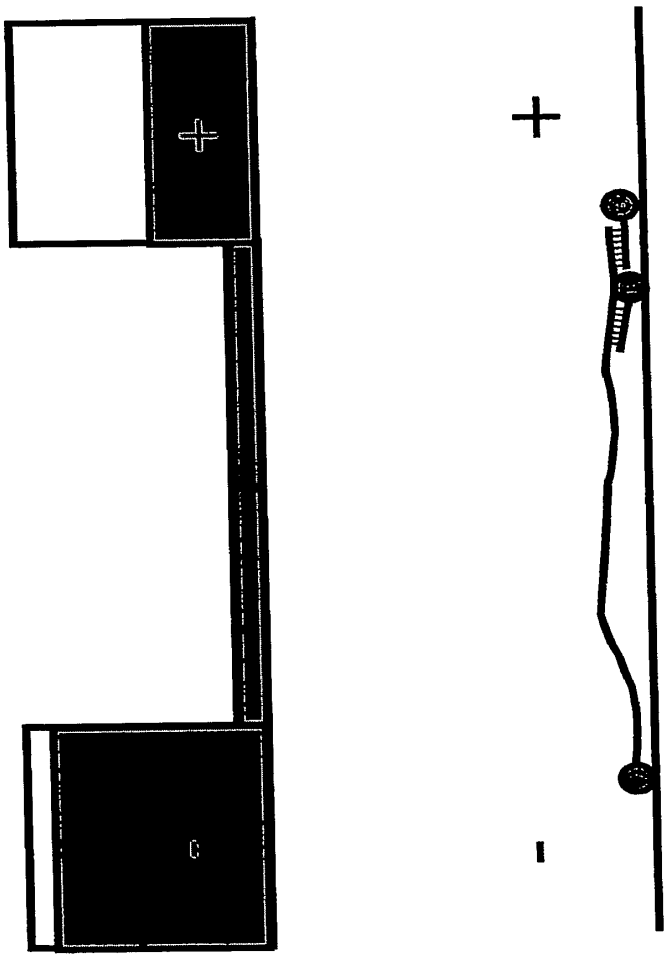


FIGURE 15C



FIGURE 16