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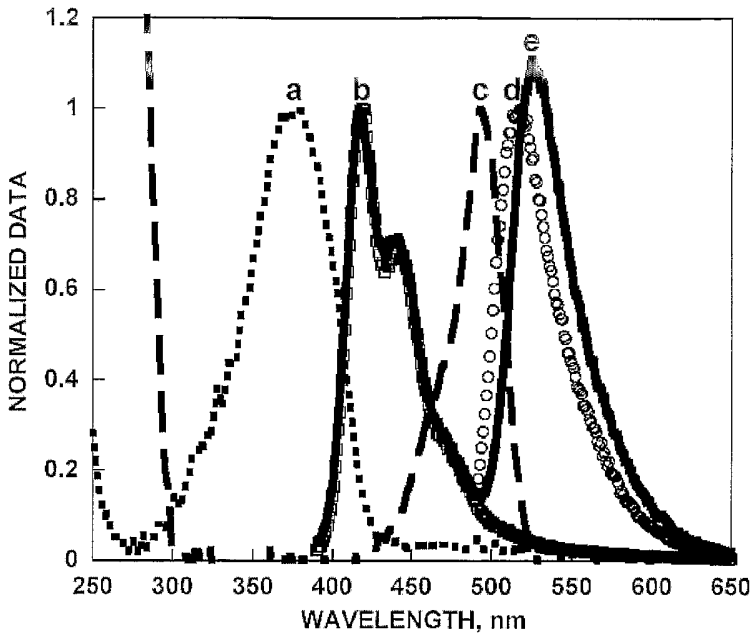
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(54) Title: METHODS AND COMPOSITIONS FOR DETECTION AND ANALYSIS OF POLYNUCLEOTIDES USING LIGHT HARVESTING MULTICHROMOPHORES



(57) Abstract: Methods, compositions and articles of manufacture for assaying a sample for a target polynucleotide are provided. A sample suspected of containing the target polynucleotide is contacted with a polycationic multichromophore and a sensor polynucleotide complementary to the target polynucleotide. The sensor polynucleotide comprises a signaling chromophore to receive energy from the excited multichromophore and increase emission in the presence of the target polynucleotide. The methods can be used in multiplex form. Kits comprising reagents for performing such methods are also provided.

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METHODS AND COMPOSITIONS FOR DETECTION AND ANALYSIS OF POLYNUCLEOTIDES USING LIGHT HARVESTING MULTICHROMOPHORES

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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TECHNICAL FIELD

This invention relates to methods, articles and compositions for the detection and analysis of polynucleotides in a sample.

BACKGROUND OF THE INVENTION

Methods permitting DNA sequence detection in real time and with high sensitivity are of great scientific and economic interest.[1,2,3] Their applications include medical diagnostics, identification of genetic mutations, gene delivery monitoring and specific genomic techniques.[4] Cationic organic dyes, such as ethidium bromide and thiazole orange, emit when intercalated into the grooves of double strand DNA (dsDNA), and serve as direct DNA hybridization probes, but lack sequence specificity.[5,6] Energy/electron transfer chromophore pairs for strand specific assays exist, but require chemical labeling of two nucleic acids, or dual modification of the same altered strand (for example, molecular beacons).[7,8] Difficulties in labeling two DNA sites result in low yields, high costs and singly labeled impurities, which lower detection sensitivity.[9]

There is a need in the art for methods of detecting and analyzing particular polynucleotides in a sample, and for compositions and articles of manufacture useful in such methods.

SUMMARY OF THE INVENTION

Methods, compositions and articles of manufacture for detecting and assaying a target polynucleotide in a sample are provided.

A sample suspected of containing the target polynucleotide is contacted with a polycationic multichromophore and a sensor polynucleotide complementary to the target polynucleotide. The sensor polynucleotide comprises an anionic backbone, such as a typical sugar phosphate backbone, and is conjugated to a signaling chromophore. In the presence of target polynucleotide in the sample, the signaling chromophore can acquire energy more efficiently from the excited polycationic multichromophore and emit increased amounts of light or signal which can be detected. The target polynucleotide can be analyzed as it occurs in the sample, or can be amplified prior to or in conjunction with analysis.

Although the anionic sensor can associate with the cationic multichromophore in the absence of target, a surprising increase in signal has been found to occur upon binding of the target polynucleotide to form a double-stranded complex relative to the signal produced from a mixture of non-complementary sequences. This increase in signal can be exploited for use in detection methods for assaying for a target polynucleotide in a sample.

Solutions are provided comprising reagents useful for performing the methods of the invention, as are kits containing such reagents. Also provided are sensing or detection complexes formed from the multichromophore and sensor polynucleotide, and signaling complexes further comprising the target polynucleotide. The methods can be used in multiplex settings where a plurality of different sensor polynucleotides are used to assay for a plurality of different target polynucleotides. The methods can optionally be performed on a surface, for example using a surface-associated polycationic multichromophore; the surface can be a sensor. The methods can also be provided in homogeneous formats. The methods and articles described herein can be used as alternatives to other techniques for detecting polynucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the absorption (**a** (dotted line) and **c** (dashed line)) and emission (**b** (squares) and **d** (circles)) spectra of polymer **1** and a fluorescein-

conjugated sensor polynucleotide. Excitation was done at 380 and 480 nm for polymer **1** and sensor polynucleotide, respectively. The energy transfer complex of polymer **1** and sensor polynucleotide excited at 380 nm is also shown in black (e, solid line).

Figure 2 presents the emission spectra of the sensor system containing hybridized (solid line) and non-hybridized (dotted line) sensor polynucleotides in 10 mmol Sodium Citrate and 100 mmol sodium chloride buffer at pH=8. Spectra are normalized relative to the emission of polymer **1**.

Figure 3 depicts the emission spectra of the sensor system containing hybridized (solid line) and non-hybridized (dotted line) sensor polynucleotides resulting from excitation of the multichromophore (polymer **1**) and energy transfer to the sensor polynucleotide followed by subsequent energy transfer to a polynucleotide specific dye (Ethidum Bromide). Measurements are in potassium phosphate-sodium hydroxide buffer solution (50 mM, pH=7.40). *See* Example 4.

Figure 4 depicts the amplified emission spectra of a polynucleotide-specific dye (Ethidium Bromide, EB) in the sensor system containing a hybridized (solid line) sensor polynucleotide. The amplified emission signal is a result of excitation of the multichromophore (polymer **1**) and energy transfer to a sensor polynucleotide followed by subsequent energy transfer to EB. Direct EB emission in double stranded DNA (dotted line) and the emission resulting from sensor polynucleotide excitation followed by energy transfer to EB (dashed line) is shown to demonstrate the increased signal provided by the polycationic multichromophore. Measurements are in potassium phosphate-sodium hydroxide buffer solution (50 mM, pH=7.40). *See* Example 5.

Figure 5 presents the emission spectra of EB upon excitation of polymer **1** in the presence of EB and both hybridized (solid line) and non-hybridized (dotted line) sensor polynucleotides in potassium phosphate-sodium hydroxide buffer solution (50 mM, pH=7.40). *See* Example 6. The emission is a result of energy transfer from the polycationic multichromophore to the intercalated EB only in the case of the hybridized, double stranded DNA.

DETAILED DESCRIPTION OF THE INVENTION

Present technologies for DNA and RNA sensors (including "gene-chips" and "DNA-chips") depend on the covalent attachment of fluorescent tags (lumophores) to single strands of DNA, relying on labeling of the sample or a sample component, with unavoidable problems resulting from variations in the efficiency of the labeling reaction from sample to sample, requiring complex cross-calibrations. Other systems rely on multiply labeled probes (e.g., molecular beacons,Taqman®, Scorpion® probesEclipse® probes), requiring the multiple attachment of lumophores and quenchers to precisely engineered sequences.

The method of the invention comprises contacting a sample with an aqueous solution comprising at least two components; (a) a light harvesting, polycationic, luminescent multichromophore system such as, for example, a conjugated polymer, semiconductor quantum dot or dendritic structure that is water soluble, and (b) a sensor polynucleotide conjugated to a luminescent signaling chromophore (referred to as "Oligo-C^*"). The emission of light with wavelength characteristic of the signaling chromophore indicates the presence in solution of a target polynucleotide with a base sequence complementary to that of the sensor polynucleotide. By using multiple sensor polynucleotides, each with a different base sequence and a different signaling chromophore ($Oligo_1$-C_1^*, $Oligo_2$-C_2^*, etc), multiple polynucleotides each with specific base sequences can be independently detected. A third component such as a polynucleotide-specific dye may be introduced to improve selectivity by further transferring energy from the sensor polynucleotide to the polynucleotide-specific dye.

The light harvesting chromophore and the signaling chromophore (C^*) are chosen so that the absorption bands of the two species have minimal overlap and so that the luminescent emission spectra of the two species are at different wavelengths. When prepared in aqueous solution, the light harvesting and luminescent multi-chromophore systems are positively charged (for example positively charged conjugated polyelectrolytes). The proximity between a negatively charged sensor polynucleotide and a positively charged light harvesting and luminescent multi-chromophore system is ensured by electrostatic attraction. When exposed to incident radiation with wavelength in the absorption band of the light harvesting chromophore, there is emission from the signaling chromophore, for example via the Förster energy transfer mechanism. Upon addition of a target polynucleotide such as a single

stranded DNA (ssDNA) with a base sequence that is complementary to the sequence of the sensor polynucleotide, the ssDNA hybridizes with the sensor polynucleotide resulting in an increase in negative charge density along the DNA strand.[10,11] Under these circumstances, and considering strictly electrostatic forces, the interaction between the sensor polynucleotide and the positively charged light harvesting multi-chromophore system will be favorable, leading to efficient energy transfer and intense emission from the signaling chromophore. When ssDNA with a base sequence that is not complementary to that of the sensor polynucleotide is added, base pair hybridization does not take place. Electrostatic complexation between a light harvesting multi-chromophore system and a sensor polynucleotide is screened by competition with the non-complementary ssDNA. Thus, the average distance between the light harvesting multi-chromophore system and the Oligo-C* is larger in the presence of a noncomplementary sequence, resulting in less effective energy transfer to the signaling chromophore. The emission of the signaling Oligo-C* is stronger when in the presence of its complementary target than when a non-complementary strand is present. The overall scheme provides a sensor for the presence of a target polynucleotide with a specific base sequence in the test solution. By using multiple sensor polynucleotides, each with a different base sequence and a different signaling chromophore, multiple targets can be separately detected.

In addition to the described method, the invention provides a predominantly aqueous solution comprising at least two components; (a) a cationic multichromophore, and (b) a "sensor polynucleotide" (Oligo-C*) comprising an anionic polynucleotide conjugated to a signaling chromophore.

As demonstrated in the Examples, the optical amplification provided by a water soluble multichromophore such as a conjugated polymer can be used to detect polynucleotide hybridization to a sensor polynucleotide. The amplification can be enhanced by using higher molecular weight water soluble conjugated polymers or other structures as the polycationic multichromophore as described herein. The invention can be provided in a homogeneous format that utilizes the ease of fluorescence detection methods. The invention can be used to detect amplified target polynucleotides or, because of the large signal amplification, as a stand alone assay, without need for polynucleotide amplification.

Unlike gene-chip technology, the present invention does not necessarily require labeling of each sample to be analyzed by covalent coupling of lumophores or chromophores to the polynucleotides contained in or derived from the sample prior to analysis. Those coupling methods have inherent difficulties in reproducibility of coupling efficiency and result in the need for cross-calibration from sample to sample.

The inventions described herein are useful for any assay in which a sample can be interrogated regarding a target polynucleotide. Typical assays involve determining the presence of the target polynucleotide in the sample or its relative amount, or the assays may be quantitative or semi-quantitative.

The methods of the invention can all be performed in multiplex formats. A plurality of different sensor polynucleotides can be used to detect corresponding different target polynucleotides in a sample through the use of different signaling chromophores conjugated to the respective sensor polynucleotides. Multiplex methods are provided employing 2, 3, 4, 5, 10, 15, 20, 25, 50, 100, 200, 400 or more different sensor polynucleotides which can be used simultaneously to assay for corresponding different target polynucleotides.

The methods can be performed on a substrate, as well as in solution, although the solution format is expected to be more rapid due to diffusion issues. Thus the assay can be performed, for example, in an array format on a substrate, which can be a sensor. This can be achieved by anchoring or otherwise incorporating an assay component onto the substrate, for example the sensor polynucleotide, the polycationic multichromophore, or both. These substrates may be surfaces of glass, silicon, paper, plastic, or the surfaces of optoelectronic semiconductors (such as, but not confined to, indium-doped gallium nitride or polymeric polyanilines, etc.) employed as optoelectronic transducers. The location of a given sensor polynucleotide may be known or determinable in an array format, and the array format may be microaddressable or nanoaddressable. In one variation, one or more samples, which may contain an amplification product, can be attached to the substrate, and the substrate can be contacted with one or more labeled sensor polynucleotides and the polycationic multichromophore.

Before the present invention is described in further detail, it is to be understood that this invention is not limited to the particular methodology, devices, solutions or apparatuses described, as such methods, devices, solutions or apparatuses can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

Use of the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, reference to "a target polynucleotide" includes a plurality of target polynucleotides, reference to "a signaling chromophore" includes a plurality of such chromophores, reference to "a sensor polynucleotide" includes a plurality of sensor polynucleotides, and the like. Additionally, use of specific plural references, such as "two," "three," etc., read on larger numbers of the same subject less the context clearly dictates otherwise.

Terms such as "connected," "attached," and "linked" are used interchangeably herein and encompass direct as well as indirect connection, attachment, linkage or conjugation unless the context clearly dictates otherwise. Where a range of values is recited, it is to be understood that each intervening integer value, and each fraction thereof, between the recited upper and lower limits of that range is also specifically disclosed, along with each subrange between such values. The upper and lower limits of any range can independently be included in or excluded from the range, and each range where either, neither or both limits are included is also encompassed within the invention. Where a value being discussed has inherent limits, for example where a component can be present at a concentration of from 0 to 100%, or where the pH of an aqueous solution can range from 1 to 14, those inherent limits are specifically disclosed. Where a value is explicitly recited, it is to be understood that values which are about the same quantity or amount as the recited value are also within the scope of the invention, as are ranges based thereon. Where a combination is disclosed, each subcombination of the elements of that combination is also specifically disclosed and is within the scope of the invention. Conversely, where different elements or groups of elements are disclosed, combinations thereof are also disclosed. Where any element of an invention is disclosed as having a plurality of alternatives, examples of

that invention in which each alternative is excluded singly or in any combination with the other alternatives are also hereby disclosed; more than one element of an invention can have such exclusions, and all combinations of elements having such exclusions are hereby disclosed.

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

All publications mentioned herein are hereby incorporated by reference for the purpose of disclosing and describing the particular materials and methodologies for which the reference was cited. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

DEFINITIONS

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule" are used interchangeably herein to refer to a polymeric form of nucleotides of any length, and may comprise ribonucleotides, deoxyribonucleotides, analogs thereof, or mixtures thereof. These terms refer only to the primary structure of the molecule. Thus, the terms includes triple-, double- and single-stranded deoxyribonucleic acid ("DNA"), as well as triple-, double- and single-stranded ribonucleic acid ("RNA"). It also includes modified, for example by alkylation, and/or by capping, and unmodified forms of the polynucleotide.

Whether modified or unmodified, the sensor polynucleotide is anionic and can interact with the cationic multichromophore in the absence of target polynucleotide.

The target polynucleotide can in principle be charged or uncharged, although typically it is expected to be anionic, for example RNA or DNA.

More particularly, the terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule" include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), including tRNA, rRNA, hRNA, and mRNA, whether spliced or unspliced, any other type of polynucleotide which is an N- or C-glycoside of a purine or pyrimidine base, and other polymers containing a phosphate or other polyanionic backbone, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. There is no intended distinction in length between the terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule," and these terms are used interchangeably herein. These terms refer only to the primary structure of the molecule. Thus, these terms include, for example, 3'-deoxy-2',5'-DNA, oligodeoxyribonucleotide N3' P5' phosphoramidates, 2'-O-alkyl-substituted RNA, double- and single-stranded DNA, as well as double- and single-stranded RNA, and hybrids thereof including for example hybrids between DNA and RNA, and also include known types of modifications, for example, labels, alkylation, "caps," substitution of one or more of the nucleotides with an analog, internucleotide modifications such as, for example, those with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (including enzymes (e.g. nucleases), toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelates (of, e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide or oligonucleotide.

It will be appreciated that, as used herein, the terms "nucleoside" and "nucleotide" will include those moieties which contain not only the known purine and pyrimidine bases, but also other heterocyclic bases which have been modified. Such modifications include methylated purines or pyrimidines, acylated purines or

pyrimidines, or other heterocycles. Modified nucleosides or nucleotides can also include modifications on the sugar moiety, e.g., wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or are functionalized as ethers, amines, or the like. The term "nucleotidic unit" is intended to encompass nucleosides and nucleotides.

Furthermore, modifications to nucleotidic units include rearranging, appending, substituting for or otherwise altering functional groups on the purine or pyrimidine base which form hydrogen bonds to a respective complementary pyrimidine or purine. The resultant modified nucleotidic unit optionally may form a base pair with other such modified nucleotidic units but not with A, T, C, G or U. Abasic sites may be incorporated which do not prevent the function of the polynucleotide; preferably the polynucleotide does not comprise abasic sites. Some or all of the residues in the polynucleotide can optionally be modified in one or more ways.

Standard A-T and G-C base pairs form under conditions which allow the formation of hydrogen bonds between the N3-H and C4-oxy of thymidine and the Nl and C6-NH2, respectively, of adenosine and between the C2-oxy, N3 and C4-NH2, of cytidine and the C2-NH2, N'-H and C6-oxy, respectively, of guanosine. Thus, for example, guanosine (2-amino-6-oxy-9-β-D-ribofuranosyl-purine) may be modified to form isoguanosine (2-oxy-6-amino-9-β-D-ribofuranosyl-purine). Such modification results in a nucleoside base which will no longer effectively form a standard base pair with cytosine. However, modification of cytosine (1-β-D-ribofuranosyl-2-oxy-4-amino-pyrimidine) to form isocytosine (l-β-D-ribofuranosyl-2-amino-4-oxy-pyrimidine) results in a modified nucleotide which will not effectively base pair with guanosine but will form a base pair with isoguanosine. Isocytosine is available from Sigma Chemical Co. (St. Louis, MO); isocytidine may be prepared by the method described by Switzer et al. (1993) Biochemistry 32:10489-10496 and references cited therein; 2'-deoxy-5-methyl-isocytidine may be prepared by the method of Tor et al. (1993) J. Am. Chem. Soc. 115:4461-4467 and references cited therein; and isoguanine nucleotides may be prepared using the method described by Switzer et al. (1993), supra, and Mantsch et al. (1993) Biochem. 14:5593-5601, or by the method

described in U.S. Patent No. 5,780,610 to Collins et al. Other nonnatural base pairs may be synthesized by the method described in Piccirilli et al. (1990) Nature 343:33-37 for the synthesis of 2,6-diaminopyrimidine and its complement (1-methylpyrazolo-[4,3]pyrimidine-5,7-(4H,6H)-dione). Other such modified nucleotidic units which form unique base pairs are known, such as those described in Leach et al. (1992) J. Am. Chem. Soc. 114:3675-3683 and Switzer et al., supra.

"Preferential binding" or "preferential hybridization" refers to the increased propensity of one polynucleotide or PNA to bind to its complement in a sample as compared to a noncomplementary polymer in the sample.

Hybridization conditions will typically include salt concentrations of less than about 1M, more usually less than about 500 mM and preferably less than about 200 mM. In the case of hybridization between a peptide nucleic acid and a polynucleotide, the hybridization can be done in solutions containing little or no salt. Hybridization temperatures can be as low as 5°C, but are typically greater than 22°C, more typically greater than about 30°C, and preferably in excess of about 37°C. Longer fragments may require higher hybridization temperatures for specific hybridization. Other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents and extent of base mismatching, and the combination of parameters used is more important than the absolute measure of any one alone. Suitable hybridization conditions for a given assay format can be determined by one of skill in the art; nonlimiting parameters which may be adjusted include concentrations of assay components, salts used and their concentration, ionic strength, temperature, buffer type and concentration, solution pH, presence and concentration of blocking reagents to decrease background binding such as repeat sequences or blocking protein solutions, detergent type(s) and concentrations, molecules such as polymers which increase the relative concentration of the polynucleotides, metal ion(s) and their concentration(s), chelator(s) and their concentrations, and other conditions known in the art.

"Multiplexing" herein refers to an assay or other analytical method in which multiple analytes can be assayed simultaneously.

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not.

THE SAMPLE

The portion of the sample comprising or suspected of comprising the target polynucleotide can be any source of biological material which comprises polynucleotides that can be obtained from a living organism directly or indirectly, including cells, tissue or fluid, and the deposits left by that organism, including viruses, mycoplasma, and fossils. The sample may comprise a target polynucleotide prepared through synthetic means, in whole or in part. Typically, the sample is obtained as or dispersed in a predominantly aqueous medium. Nonlimiting examples of the sample include blood, urine, semen, milk, sputum, mucus, a buccal swab, a vaginal swab, a rectal swab, an aspirate, a needle biopsy, a section of tissue obtained for example by surgery or autopsy, plasma, serum, spinal fluid, lymph fluid, the external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, tumors, organs, samples of *in vitro* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components), and a recombinant library comprising polynucleotide sequences. The sample may be presented on a substrate as described herein. The substrate may be a slide comprising the sample, such as is used in fluorescence *in situ* hybridization (FISH).

The sample can be a positive control sample which is known to contain the target polynucleotide or a surrogate thereof. A negative control sample can also be used which, although not expected to contain the target polynucleotide, is suspected of containing it (via contamination of one or more of the reagents) or another component capable of producing a false positive, and is tested in order to confirm the lack of contamination by the target polynucleotide of the reagents used in a given assay, as well as to determine whether a given set of assay conditions produces false positives (a positive signal even in the absence of target polynucleotide in the sample).

The sample can be diluted, dissolved, suspended, extracted or otherwise treated to solubilize and/or purify any target polynucleotide present or to render it accessible to reagents which are used in an amplification scheme or to detection reagents. Where the sample contains cells, the cells can be lysed or permeabilized to release the polynucleotides within the cells. One step permeabilization buffers can be used to lyse cells which allow further steps to be performed directly after lysis, for example a polymerase chain reaction.

THE TARGET POLYNUCLEOTIDE AND AMPLIFICATION PRODUCTS PRODUCED THEREFROM

The target polynucleotide can be single-stranded, double-stranded, or higher order, and can be linear or circular. Exemplary single-stranded target polynucleotides include mRNA, rRNA, tRNA, hnRNA, ssRNA or ssDNA viral genomes, although these polynucleotides may contain internally complementary sequences and significant secondary structure. Exemplary double-stranded target polynucleotides include genomic DNA, mitochondrial DNA, chloroplast DNA, dsRNA or dsDNA viral genomes, plasmids, phage, and viroids. The target polynucleotide can be prepared synthetically or purified from a biological source. The target polynucleotide may be purified to remove or diminish one or more undesired components of the sample or to concentrate the target polynucleotide. Conversely, where the target polynucleotide is too concentrated for the particular assay, the target polynucleotide may be diluted.

Following sample collection and optional nucleic acid extraction, the nucleic acid portion of the sample comprising the target polynucleotide can be subjected to one or more preparative reactions. These preparative reactions can include *in vitro* transcription (IVT), labeling, fragmentation, amplification and other reactions. mRNA can first be treated with reverse transcriptase and a primer to create cDNA prior to detection and/or amplification; this can be done *in vitro* with purified mRNA or in situ, e.g. in cells or tissues affixed to a slide. Nucleic acid amplification increases the copy number of sequences of interest such as the target polynucleotide. A variety of amplification methods are suitable for use; nonlimiting examples of

suitable amplification reactions include the polymerase chain reaction method (PCR), the ligase chain reaction (LCR), self sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), the use of Q Beta replicase, reverse transcription, nick translation, and the like.

Where the target polynucleotide is single-stranded, the first cycle of amplification forms a primer extension product complementary to the target polynucleotide. If the target polynucleotide is single-stranded RNA, a polymerase with reverse transcriptase activity is used in the first amplification to reverse transcribe the RNA to DNA, and additional amplification cycles can be performed to copy the primer extension products. The primers for a PCR must, of course, be designed to hybridize to regions in their corresponding template that will produce an amplifiable segment; thus, each primer must hybridize so that its 3' nucleotide is paired to a nucleotide in its complementary template strand that is located 3' from the 3' nucleotide of the primer used to replicate that complementary template strand in the PCR.

The target polynucleotide is typically amplified by contacting one or more strands of the target polynucleotide with a primer and a polymerase having suitable activity to extend the primer and copy the target polynucleotide to produce a full-length complementary polynucleotide or a smaller portion thereof. Any enzyme having a polymerase activity which can copy the target polynucleotide can be used, including DNA polymerases, RNA polymerases, reverse transcriptases, enzymes having more than one type of polymerase activity, and the enzyme can be thermolabile or thermostable. Mixtures of enzymes can also be used. Exemplary enzymes include: DNA polymerases such as DNA Polymerase I ("Pol I"), the Klenow fragment of Pol I, T4, T7, Sequenase® T7, Sequenase® Version 2.0 T7, *Tub*, *Taq, Tth, Pfx, Pfu, Tsp, Tfl, Tli* and *Pyrococcus sp* GB-D DNA polymerases; RNA polymerases such as *E. coli*, SP6, T3 and T7 RNA polymerases; and reverse transcriptases such as AMV, M-MuLV, MMLV, RNAse H⁻ MMLV (SuperScript®), SuperScript® II, ThermoScript®, HIV-1, and RAV2 reverse transcriptases. All of these enzymes are commercially available. Exemplary polymerases with multiple specificities include RAV2 and *Tli* (exo-) polymerases. Exemplary thermostable

polymerases include *Tub*, *Taq*, *Tth*, *Pfx*, *Pfu*, *Tsp*, *Tfl*, *Tli* and *Pyrococcus sp.* GB-D DNA polymerases.

Suitable reaction conditions are chosen to permit amplification of the target polynucleotide, including pH, buffer, ionic strength, presence and concentration of one or more salts, presence and concentration of reactants and cofactors such as nucleotides and magnesium and/or other metal ions (e.g., manganese), optional cosolvents, temperature, thermal cycling profile for amplification schemes comprising a polymerase chain reaction, and may depend in part on the polymerase being used as well as the nature of the sample. Cosolvents include formamide (typically at from about 2 to about 10 %), glycerol (typically at from about 5 to about 10 %), and DMSO (typically at from about 0.9 to about 10 %). Techniques may be used in the amplification scheme in order to minimize the production of false positives or artifacts produced during amplification. These include "touchdown" PCR, hot-start techniques, use of nested primers, or designing PCR primers so that they form stem-loop structures in the event of primer-dimer formation and thus are not amplified. Techniques to accelerate PCR can be used, for example centrifugal PCR, which allows for greater convection within the sample, and comprising infrared heating steps for rapid heating and cooling of the sample. One or more cycles of amplification can be performed. An excess of one primer can be used to produce an excess of one primer extension product during PCR; preferably, the primer extension product produced in excess is the amplification product to be detected. A plurality of different primers may be used to amplify different target polynucleotides or different regions of a particular target polynucleotide within the sample.

Amplified target polynucleotides may be subjected to post amplification treatments. For example, in some cases, it may be desirable to fragment the target polynucleotide prior to hybridization in order to provide segments which are more readily accessible. Fragmentation of the nucleic acids can be carried out by any method producing fragments of a size useful in the assay being performed; suitable physical, chemical and enzymatic methods are known in the art.

An amplification reaction can be performed under conditions which allow the sensor polynucleotide to hybridize to the amplification product during at least part of

an amplification cycle. When the assay is performed in this manner, real-time detection of this hybridization event can take place by monitoring for a change in light emission from the signaling chromophore that occurs upon such hybridization during the amplification scheme.

THE POLYCATIONIC MULTICHROMOPHORE

Light harvesting multichromophore systems have been demonstrated to be efficient light absorbers by virtue of the multiple chromophores they comprise. Examples include, but are not limited to, conjugated polymers, aggregates of conjugated molecules, luminescent dyes attached to saturated polymers, semiconductor quantum dots and dendritic structures. For example, each repeat unit on a conjugated polymer can be considered as a contributing chromophore, quantum dots are made up of many atoms, a saturated polymer can be functionalized with many luminescent dye molecules on side chains, and dendrimers can be synthesized containing many covalently bonded individual chromophores. Attachment of chromophore assemblies onto solid supports, such as polymer beads or surfaces, can also be used for light harvesting.

Light harvesting multichromophore systems can efficiently transfer energy to nearby luminescent species (e.g., "signaling chromophores"). Mechanisms for energy transfer include, for example, resonant energy transfer (Förster (or fluorescence) resonance energy transfer, FRET), quantum charge exchange (Dexter energy transfer) and the like. Typically, however, these energy transfer mechanisms are relatively short range; that is, close proximity of the light harvesting multichromophore system to the signaling chromophore is required for efficient energy transfer. Under conditions for efficient energy transfer, amplification of the emission from the signaling chromophore occurs when the number of individual chromophores in the light harvesting multichromophore system is large; that is, the emission from the signaling chromophore is more intense when the incident light (the "pump light") is at a wavelength which is absorbed by the light harvesting multichromophore system than when the signaling chromophore is directly excited by the pump light.

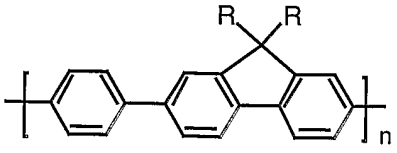
Conjugated polymers (CPs) are characterized by a delocalized electronic structure and can be used as highly responsive optical reporters for chemical and biological targets.[12,13] Because the effective conjugation length is substantially shorter than the length of the

polymer chain, the backbone contains a large number of conjugated segments in close proximity. Thus, conjugated polymers are efficient for light harvesting and enable optical amplification via Förster energy transfer.[14]

Spontaneous interpolymer complexation between cationic polyelectrolytes and DNA has been described and is largely the result of cooperative electrostatic forces.[15,16,17] Hydrophobic interactions between aromatic polymer units and DNA bases were also recently recognized.[18,19] The free energy of polyelectrolyte/DNA interactions is controlled by the structure of the participating species used in conjunction with solution variables such as pH, ionic strength, and temperature.[20] The strength and specificity of these interactions has recently been coordinated to recognize the tertiary structure of plasmid DNA.[21]

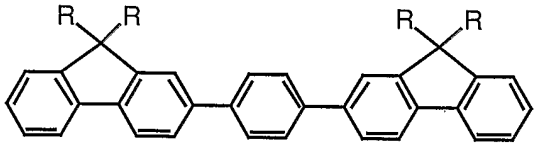
The multichromophores used in the present invention are polycationic and can interact with a sensor polynucleotide electrostatically. Any polycationic multichromophore that can absorb light and transfer energy to a signaling chromophore on a sensor polynucleotide can be used in the methods described. Exemplary multichromophores which can be used include conjugated polymers, saturated polymers or dendrimers incorporating multiple chromophores in any viable manner, and semiconductor nanocrystals (SCNCs). The conjugated polymers, saturated polymers and dendrimers can be prepared to incorporate multiple cationic species or can be derivatized to render them polycationic after synthesis; semiconductor nanocrystals can be rendered polycationic by addition of cationic species to their surface.

In a preferred embodiment, a conjugated polymer is used as the polycationic multichromophore. A specific example is shown in structure **1** where the cationic water soluble conjugated polymer is poly((9,9-bis(6'-N,N,N-trimethylammonium)-hexyl)-fluorene phenylene) with iodide counteranions (denoted in the following as polymer **1**).[22] The particular size of this polymer is not critical, so long as it is able to absorb light and transfer energy to signaling chromophores brought into proximity. Typical values of "n" fall within the range of two to about 100,000. This specific molecular structure is not critical; any water soluble cationic conjugated polymer with adequate luminescence quantum efficiency can be used.



1 -R = -$(CH_2)_6NMe_3^{\oplus}I^{\ominus}$

Water soluble conjugated oligomers can also be used as the polycationic multichromophore. An example of such a water soluble, cationic, luminescent conjugated oligomer with iodide counterions is shown below (denoted herein as oligomer **2**):



2 -R = -$(CH_2)_6NMe_3^{\oplus}I^{\ominus}$

Although the smaller oligomer **2** does not display the large signal amplification characteristic of a high molecular weight polymer, such smaller molecules are useful to deconvolute structure property relationships, which are difficult to determine with the inherent polydispersity and batch-to-batch variations found in polymers. Further, in aqueous media oligomers such as **2** are more soluble than their polymeric counterparts, and hydrophobic interactions are expected to be less important for **2** than for polymer structures. Assemblies of oligomers may thus be desirably used for specific applications. Addition of organic solvents, for example a water miscible organic solvent such as ethanol, can result in a decrease in background C* emission. The presence of the organic solvent can decrease hydrophobic interactions and reduce background C* emission.

THE SENSOR POLYNUCLEOTIDE

A sensor polynucleotide is provided that is anionic and is complementary to the target polynucleotide to be assayed, and has a predetermined sequence. The sensor polynucleotide can be branched, multimeric or circular, but is typically linear, and can contain nonnatural bases. The sensor polynucleotide can be prepared with any desired sequence of bases. Chemical methods for attaching the signaling

chromophore to the sensor polynucleotide are known in the art.[23] Specific sensor polynucleotide structures, including structures conjugated to chromophores, can be custom-made using commercial sources or chemically synthesized.

THE SIGNALING CHROMOPHORE

Chromophores useful in the inventions described herein include any substance which can absorb energy from a polycationic multichromophore in an appropriate solution and emit light. For multiplexed assays, a plurality of different signaling chromophores can be used with detectably different emission spectra. The chromophore can be a lumophore or a fluorophore. Typical fluorophores include fluorescent dyes, semiconductor nanocrystals, lanthanide chelates, polynucleotide-specific dyes and green fluorescent protein.

Exemplary fluorescent dyes include fluorescein, 6-FAM, rhodamine, Texas Red, tetramethylrhodamine, carboxyrhodamine, carboxyrhodamine 6G, carboxyrhodol, carboxyrhodamine 110, Cascade Blue, Cascade Yellow, coumarin, Cy2®, Cy3®, Cy3.5®, Cy5®, Cy5.5®, Cy-Chrome, phycoerythrin, PerCP (peridinin chlorophyll-a Protein), PerCP-Cy5.5, JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein), NED, ROX (5-(and-6)-carboxy-X-rhodamine), HEX, Lucifer Yellow, Marina Blue, Oregon Green 488, Oregon Green 500, Oregon Green 514, Alexa Fluor® 350, Alexa Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 633, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, 7-amino-4-methylcoumarin-3-acetic acid, BODIPY® FL, BODIPY® FL-Br_2, BODIPY® 530/550, BODIPY® 558/568, BODIPY® 564/570, BODIPY® 576/589, BODIPY® 581/591, BODIPY® 630/650, BODIPY® 650/665, BODIPY® R6G, BODIPY® TMR, BODIPY® TR, conjugates thereof, and combinations thereof. Exemplary lanthanide chelates include europium chelates, terbium chelates and samarium chelates.

A wide variety of fluorescent semiconductor nanocrystals ("SCNCs") are known in the art; methods of producing and utilizing semiconductor nanocrystals are described in: PCT Publ. No. WO 99/26299 published May 27, 1999, inventors Bawendi et al.; USPN 5,990,479 issued Nov. 23, 1999 to Weiss et al.; and Bruchez et

al., Science 281:2013, 1998. Semiconductor nanocrystals can be obtained with very narrow emission bands with well-defined peak emission wavelengths, allowing for a large number of different SCNCs to be used as signaling chromophores in the same assay, optionally in combination with other non-SCNC types of signaling chromophores.

Exemplary polynucleotide-specific dyes include acridine orange, acridine homodimer, actinomycin D, 7-aminoactinomycin D (7-AAD), 9-amino-6-chloro-2-methoxyacridine (ACMA), BOBO™-1 iodide (462/481), BOBO™-3 iodide (570/602), BO-PRO™-1 iodide (462/481), BO-PRO™-3 iodide (575/599), 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), 4',6-diamidino-2-phenylindole, dilactate (DAPI, dilactate), dihydroethidium (hydroethidine), dihydroethidium (hydroethidine), dihydroethidium (hydroethidine), ethidium bromide, ethidium diazide chloride, ethidium homodimer-1 (EthD-1), ethidium homodimer-2 (EthD-2), ethidium monoazide bromide (EMA), hexidium iodide, Hoechst 33258, Hoechst 33342, Hoechst 34580, Hoechst S769121, hydroxystilbamidine, methanesulfonate, JOJO™-1 iodide (529/545), JO-PRO™-1 iodide (530/546), LOLO™-1 iodide (565/579), LO-PRO™-1 iodide (567/580), NeuroTrace™ 435/455, NeuroTrace™ 500/525, NeuroTrace™ 515/535, NeuroTrace™ 530/615, NeuroTrace™ 640/660, OliGreen, PicoGreen® ssDNA, PicoGreen® dsDNA, POPO™-1 iodide (434/456), POPO™-3 iodide (534/570), PO-PRO™-1 iodide (435/455), PO-PRO™-3 iodide (539/567), propidium iodide, RiboGreen®, *SlowFade*®, *SlowFade*® *Light*, SYBR® Green I, SYBR® Green II, SYBR® Gold, SYBR® 101, SYBR® 102, SYBR® 103, SYBR® DX, TO-PRO®-1, TO-PRO®-3, TO-PRO®-5, TOTO®-1, TOTO®-3, YO-PRO®-1 (oxazole yellow), YO-PRO®-3, YOYO®-1, YOYO®-3, TO, SYTOX® Blue, SYTOX® Green, SYTOX® Orange, SYTO® 9, SYTO® BC, SYTO® 40, SYTO® 41, SYTO® 42, SYTO® 43, SYTO® 44, SYTO® 45, SYTO® Blue, SYTO® 11, SYTO® 12, SYTO® 13, SYTO® 14, SYTO® 15, SYTO® 16, SYTO® 20, SYTO® 21, SYTO® 22, SYTO® 23, SYTO® 24, SYTO® 25, SYTO® Green, SYTO® 80, SYTO® 81, SYTO® 82, SYTO® 83, SYTO® 84, SYTO® 85, SYTO® Orange, SYTO® 17, SYTO® 59, SYTO® 60, SYTO® 61, SYTO® 62, SYTO® 63, SYTO® 64, SYTO® Red, netropsin, distamycin, acridine orange, 3,4-benzopyrene, thiazole orange,

TOMEHE, daunomycin, acridine, pentyl-TOTAB, and butyl-TOTIN. Asymmetric cyanine dyes may be used as the polynucleotide-specific dye. Other dyes of interest include those described by Geierstanger, B.H. and Wemmer, D.E., Annu. Rev. Vioshys. Biomol. Struct. 1995, 24, 463-493, by Larson, C.J. and Verdine, G.L., Bioorganic Chemistry: Nucleic Acids, Hecht, S.M., Ed., Oxford University Press: New York, 1996; pp 324-346, and by Glumoff, T. and Goldman, A. Nucleic Acids in Chemistry and Biology, 2nd ed., Blackburn, G.M. and Gait, M.J., Eds., Oxford University Press: Oxford, 1996, pp375-441. The polynucleotide-specific dye may be an intercalating dye, and may be specific for double-stranded polynucleotides. Other dyes and fluorophores are described at www.probes.com (Molecular Probes, Inc.).

The term "green fluorescent protein" refers to both native *Aequorea* green fluorescent protein and mutated versions that have been identified as exhibiting altered fluorescence characteristics, including altered excitation and emission maxima, as well as excitation and emission spectra of different shapes (Delagrave, S. et al. (1995) Bio/Technology 13:151-154; Heim, R. et al. (1994) Proc. Natl. Acad. Sci. USA 91:12501-12504; Heim, R. et al. (1995) Nature 373:663-664). Delgrave et al. isolated mutants of cloned *Aequorea victoria* GFP that had red-shifted excitation spectra. Bio/Technology 13:151-154 (1995). Heim, R. et al. reported a mutant (Tyr66 to His) having a blue fluorescence (Proc. Natl. Acad. Sci. (1994) USA 91:12501-12504).

In one variation, a second signaling chromophore, which may be directly or indirectly attached to another of the assay components and/or to a substrate, is used to receive energy from the initial signaling chromophore. In particular applications, this can provide for significant additional selectivity. For example, a polynucleotide-specific dye can be used as either the initial or second signaling chromophore, and may be specific for double-stranded sequences. Energy can then be transferred from the excited cationic multichromophore to the initial signaling chromophore, which subsequently transfers energy to the second signaling chromophore, in an overall format that is selective for the target. This cascade of signaling chromophores can, in principle, be extended to use any number of signaling chromophores with compatible absorption and emission profiles. In one embodiment of this variation, an

intercalating dye that is specific for double-stranded polynucleotides is used as the second signaling chromophore, and an initial signaling chromophore that is capable of transferring energy to the second signaling chromophore is conjugated to the sensor polynucleotide. The intercalating dye provides the added selective requirement that the sensor and target polynucleotides hybridize before it is recruited to the detection complex. In the presence of target, the duplex is formed, the dye is recruited, and excitation of the multichromophore leads to signaling from the second signaling chromophore.

THE SUBSTRATE

The methods described herein can be performed on a substrate in any of a variety of formats. The substrate can comprise a wide range of material, either biological, nonbiological, organic, inorganic, or a combination of any of these. For example, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO_2, SiN_4, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, cross-linked polystyrene, polyacrylic, polylactic acid, polyglycolic acid, poly(lactide coglycolide), polyanhydrides, poly(methyl methacrylate), poly(ethylene-co-vinyl acetate), polysiloxanes, polymeric silica, latexes, dextran polymers, epoxies, polycarbonates, agarose, poly(acrylamide) or combinations thereof. Conducting polymers and photoconductive materials can be used.

Substrates can be planar crystalline substrates such as silica based substrates (e.g. glass, quartz, or the like), or crystalline substrates used in, e.g., the semiconductor and microprocessor industries, such as silicon, gallium arsenide, indium doped GaN and the like, and includes semiconductor nanocrystals.

The substrate can take the form of a photodiode, an optoelectronic sensor such as an optoelectronic semiconductor chip or optoelectronic thin-film semiconductor, or a biochip. The location(s) of the individual sensor polynucleotide(s) on the substrate can be addressable; this can be done in highly dense formats, and the location(s) can be microaddressable or nanoaddressable.

Silica aerogels can also be used as substrates, and can be prepared by methods known in the art. Aerogel substrates may be used as free standing substrates or as a surface coating for another substrate material.

The substrate can take any form and typically is a plate, slide, bead, pellet, disk, particle, microparticle, nanoparticle, strand, precipitate, optionally porous gel, sheets, tube, sphere, container, capillary, pad, slice, film, chip, multiwell plate or dish, optical fiber, etc. The substrate can be any form that is rigid or semi-rigid. The substrate may contain raised or depressed regions on which a sensor polynucleotide or other assay component is located. The surface of the substrate can be etched using well known techniques to provide for desired surface features, for example trenches, v-grooves, mesa structures, or the like.

Surfaces on the substrate can be composed of the same material as the substrate or can be made from a different material, and can be coupled to the substrate by chemical or physical means. Such coupled surfaces may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. The surface can be optically transparent and can have surface Si-OH functionalities, such as those found on silica surfaces.

The substrate and/or its optional surface are chosen to provide appropriate optical characteristics for the synthetic and/or detection methods used. The substrate and/or surface can be transparent to allow the exposure of the substrate by light applied from multiple directions. The substrate and/or surface may be provided with reflective "mirror" structures to increase the recovery of light.

The substrate and/or its surface is generally resistant to, or is treated to resist, the conditions to which it is to be exposed in use, and can be optionally treated to remove any resistant material after exposure to such conditions.

Sensor polynucleotides can be fabricated on or attached to the substrate by any suitable method, for example the methods described in U.S. Pat. No. 5,143,854, PCT Publ. No. WO 92/10092, U.S. Patent Application Ser. No. 07/624,120, filed Dec. 6, 1990 (now abandoned), Fodor et al., Science, 251: 767-777 (1991), and PCT Publ.

No. WO 90/15070). Techniques for the synthesis of these arrays using mechanical synthesis strategies are described in, e.g., PCT Publication No. WO 93/09668 and U.S. Pat. No. 5,384,261.

Still further techniques include bead based techniques such as those described in PCT Appl. No. PCT/US93/04145 and pin based methods such as those described in U.S. Pat. No. 5,288,514.

Additional flow channel or spotting methods applicable to attachment of sensor polynucleotides to the substrate are described in U. S. Patent Application Ser. No. 07/980,523, filed Nov. 20, 1992, and U.S. Pat. No. 5,384,261. Reagents are delivered to the substrate by either (1) flowing within a channel defined on predefined regions or (2) "spotting" on predefined regions. A protective coating such as a hydrophilic or hydrophobic coating (depending upon the nature of the solvent) can be used over portions of the substrate to be protected, sometimes in combination with materials that facilitate wetting by the reactant solution in other regions. In this manner, the flowing solutions are further prevented from passing outside of their designated flow paths.

Typical dispensers include a micropipette optionally robotically controlled, an ink-jet printer, a series of tubes, a manifold, an array of pipettes, or the like so that various reagents can be delivered to the reaction regions sequentially or simultaneously.

EXCITATION AND DETECTION OF THE CHROMOPHORES

Any instrument that provides a wavelength that can excite the polycationic multichromophore and is shorter than the emission wavelength(s) to be detected can be used for excitation. The excitation source preferably does not significantly excite the signaling chromophore directly. The source may be: a broadband UV light source such as a deuterium lamp with an appropriate filter, the output of a white light source such as a xenon lamp or a deuterium lamp after passing through a monochromator to extract out the desired wavelengths, a continuous wave (cw) gas laser, a solid state diode laser, or any of the pulsed lasers. The emitted light from the signaling chromophore can be detected through any suitable device or technique;

many suitable approaches are known in the art. For example, a fluorometer or spectrophotometer may be used to detect whether the test sample emits light of a wavelength characteristic of the signaling chromophore upon excitation of the multichromophore.

KITS

Kits comprising reagents useful for performing the methods of the invention are also provided. In one embodiment, a kit comprises a single-stranded sensor polynucleotide that is complementary to a target polynucleotide of interest and a polycationic multichromophore. The sensor polynucleotide is conjugated to a signaling chromophore. In the presence of the target polynucleotide in the sample, the sensor polynucleotide hybridizes to the target, resulting in increased emission of energy from the signaling chromophore, which can be detected.

The components of the kit are retained by a housing. Instructions for using the kit to perform a method of the invention can be provided with the housing, and can be provided in any fixed medium. The instructions may be located inside the housing or outside the housing, and may be printed on the interior or exterior of any surface forming the housing which renders the instructions legible. The kit may be in multiplex form, containing pluralities of one or more different sensor polynucleotides which can hybridize to corresponding different target polynucleotides.

EXAMPLES

The following examples are set forth so as to provide those of ordinary skill in the art with a complete description of how to make and use the present invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental error and deviation should be accounted for. Unless otherwise indicated, parts are parts by weight, temperature is degree centigrade and pressure is at or near atmospheric, and all materials are commercially available.

Example 1. Identification of a FRET Scheme

Hybridization tests using energy transfer from the light harvesting multi-chromophore system to the signaling chromophore was demonstrated using the cationic water soluble conjugated polymer poly(9,9-bis(6'-N,N,N-trimethylammonium)-hexyl)-fluorene phenylene), polymer **1** with iodide counteranions. The sensor polynucleotide sequence was 5'-GTAAATGGTGTTAGGGTTGC-3', corresponding to the anthrax (Bacillus anthracis) spore encapsulation plasmid, pX02, with fluorescein at the 5' position, forming an example of oligo-C*.[24] The absorption and emission spectra of the polymer and the signaling chromophore are shown in Figure 1. The data show an optical window for the excitation of polymer **1**, between the absorption of DNA and fluorescein. Direct excitation of polymer **1** results in energy transfer (ET) to fluorescein, as shown in Figure 1. The absorbance overlap of fluorescein with the emission of polymer **1** was selected to ensure FRET.[25] The extent of ET can be evaluated by the ratio of integrated acceptor to donor emission.

Example 2. Demonstration of FRET in the Presence of Target Polynucleotide

Hybridization of the Oligo-C* probe leads to changes in the ET ratio. The sensor polynucleotide ([Oligo-C*]=2.1×10^{-8} M) was annealed at 2°C below its T_m (58.4°C) in the presence of an equal molar amount of a 40 base pair strand containing a complementary 20 base pair sequence, 5'-CATCTGTAAATCCAAGAGTAGCAACCCTAACACCATTTAC-3', and in an identical fashion with a non-complementary 40 base strand with the sequence 5'-AAAATATTGTGTATCAAAATGTAAATGGTGTTAGGGTTGC-3'. Direct comparison of the resulting fluorescence reveals an ET ratio greater than 6 fold higher for the hybridized DNA. *See* Figure 2. It is also highly significant that these optical differences are observed in the presence of a 10 mmol Sodium Citrate and 100 mmol Sodium Chloride buffer. Buffer ions screen like charges on complementary DNA strands which facilitates hybridization but weakens electrostatic interactions between CPs and fluorescence quenchers of opposite charge.[26] Using a Xenon lamp fluorometer, equipped with a standard PMT, the hybridized DNA provided over 3 fold greater ET ratios, at [sensor polynucleotide]=2.8×10^{-9} M, than did the non-hybridized DNA.

Example 3. Optimization of Energy Transfer

Energy transfer was optimized by varying the ratio of polymer **1** to Oligo-C*. At a concentration of [Oligo-C*]=2.1×10^{-8} M, initial additions of polymer cause an immediate rise in the ET ratio, which drops off as the amount of polymer **1** begins to far exceed that of Oligo-C*. The maximum in the ET ratio corresponds to a near 1:1 ratio of polymer chains to Oligo-C*. At high polymer concentrations not every chain is as tightly complexed to Oligo-C* and the donor emission rises faster than that of the signaling chromophore. Such a relationship is expected, since when [polymer **1**]/[Oligo-C*] < 1, not all sensor polynucleotide strands can be complexed efficiently to independent polymer chains. Conversely, in the [polymer **1**]/[Oligo-C*] > 1 regime, not all the photons harnessed by polymer **1** (the donor) can be transferred to the Oligo-C* (the acceptor). Once the repeat unit of polymer to Oligo-C* reaches approximately 100, the photoluminescence of the reporting chromophore no longer increases, indicating acceptor saturation. Integrated fluorescence emission at this ratio was ~ 4 fold greater than that of the directly excited (480 nm) probe in the absence of polymer **1**, giving further evidence of signal amplification.

Example 4. FRET Transfer from a Multichromophore Through a Sensor Polynucleotide to a Polynucleotide Dye for Improved Selectivity

Hybridization of the Oligo-C* probe (5'-Fl-ATCTTGACTATGTGGGTGCT-3') leads to differences in the ET ratio to two different sensor chromophores. The sensor polynucleotide ([Oligo-C*]=1×10^{-8} M) was annealed at 2°C below its T_m (58.5°C) in the presence of an equal molar amount of a 20 base pair strand containing a complementary 20 base pair sequence, (5'-AGCACCCACATAGTCAAGAT-3'), and in an identical fashion with a non-complementary 20 base pair strand with the sequence (5'-CGTATCACTGGACTGATTGG-3'). The two DNA mixtures were mixed with Ethidum Bromide ([EB]=1.1×10^{-6} M) at room temperature in potassium phosphate monobasic-sodium hydroxide buffer solution (50 mM, pH=7.40) where the intercalation of the EB occurred within the duplex structure of the hybridized DNA pair. Addition of polymer **1** in water ([**1**]=1.6×10^{-7} M) and subsequent excitation of **1** (380 nm) resulted in energy transfer from **1** to the sensor polynucleotide and then energy transfer from the sensor polynucleotide to EB. Comparison of the resulting

fluorescence reveals that the sensor solution only exhibited emission from the intercalating dye EB in the presence of the hybridized DNA. *See* Figure 3. This result demonstrates that the DNA sequence sensor of this invention can detect the presence of target single stranded DNA with a specific base sequence complementary to that of the sensor polynucleotide by detecting the emission from a polynucleotide-specific dye upon excitation of a polycationic multichromophore. The chromophores used in this example were chosen for the proper overlaps in energy between the multichromophore and the two signaling chromophores.

Example 5. Demonstration of Optical Amplification of Polynucleotide Dyes Using FRET from Multichromophore Systems

Excitation of **1** in a solution of **1**, sensor polynucleotide and polynucleotide specific dye (Ethidium Bromide, EB) resulted in emission intensities of EB that were ~ 8 fold greater than that of the directly excited (500 nm) EB contained in the same double stranded DNA sequence which lacked the signaling chromophore on the sensor polynucleotide. Direct excitation of the signaling Oligo-C* (480 nm), in the absence of polymer **1**, only provided an approximate 4 fold sensitization of the intercalated EB. Altogether, this example shows evidence of signal amplification by FRET from the polycationic multichromophore (conjugated polymer **1**) to the diagnostic EB reporter. *See* Figure 4.

Example 6. FRET Transfer to a Polynucleotide Dye

Experiments using **1** and ethidium bromide ("EB") as a signaling chromophore demonstrated that direct energy transfer from **1** to EB could be shown in the presence of double-stranded DNA. The sensor polynucleotide (5'-ATCTTGACTATGTGGGTGCT-3') lacking the signaling chromophore ([Oligo]=1×10^{-8} M) was annealed at 2°C below its T_m (58.5°C) in the presence of an equal molar amount of a 20 base pair strand containing a complementary 20 base pair sequence, (5'-AGCACCCACATAGTCAAGAT-3'), and in an identical fashion with a non-complementary 20 base pair strand with the sequence (5'-CGTATCACTGGACTGATTGG-3'). The two DNA mixtures were mixed with

Ethidum Bromide ([EB]=1.1×10^{-6} M) at room temperature in potassium phosphate monobasic-sodium hydroxide buffer solution (50 mM, pH=7.40) where the intercalation of the EB occurred within the duplex structure of the hybridized DNA pair. Addition of polymer **1** in water ([**1**]=1.6×10^{-7} M) and subsequent excitation of **1** (380 nm) resulted in energy transfer from **1** to the intercalated EB only in the case of hybridized or double stranded DNA. Emission from the EB was detected upon excitation of polymer 1 for only the hybridized sequences. *See* Figure 5.

Although the invention has been described in some detail with reference to the preferred embodiments, those of skill in the art will realize, in light of the teachings herein, that certain changes and modifications can be made without departing from the spirit and scope of the invention. Accordingly, the invention is limited only by the claims.

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CLAIMS

What is claimed is:

1. An assay method comprising:

providing a sample that is suspected of containing a target polynucleotide;

providing a polycationic multichromophore that upon excitation is capable of transferring energy to a signaling chromophore;

providing an anionic sensor polynucleotide that is single-stranded and is complementary to the target polynucleotide, said sensor polynucleotide conjugated to the signaling chromophore, wherein said sensor polynucleotide interacts with the multichromophore and emitted light can be produced from the signaling chromophore upon excitation of the multichromophore in the absence of target polynucleotide, and wherein a greater amount of emitted light is produced from the signaling chromophore upon excitation of the multichromophore in the presence of target polynucleotide;

contacting the sample with the sensor polynucleotide and the multichromophore in a solution under conditions in which the sensor polynucleotide can hybridize to the target polynucleotide, if present;

applying a light source to the solution that can excite the multichromophore; and

detecting whether the light emitted from the signaling chromophore is increased in the presence of sample.

2. The method of claim 1, wherein the multichromophore comprises a structure that is selected from the group consisting of a saturated polymer, a conjugated polymer, an aggregate of conjugated molecules, a dendrimer, and a semiconductor nanocrystal.

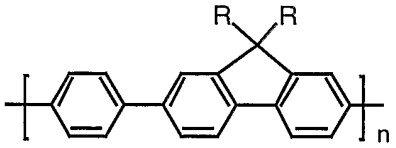
3. The method of claim 2, wherein the multichromophore comprises a saturated polymer.

4. The method of claim 2, wherein the multichromophore comprises a dendrimer.

5. The method of claim 2, wherein the multichromophore comprises a semiconductor nanocrystal.

6. The method of claim 2, wherein the multichromophore comprises a conjugated molecule.

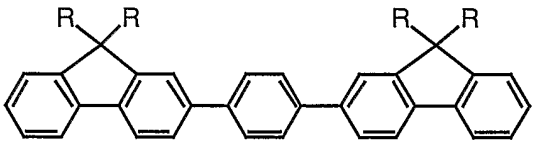
7. The method of claim 6, wherein the conjugated polymer has the structure



1 -R = -$(CH_2)_6NMe_3^{\oplus}I^{\ominus}$

8. The method of claim 2, wherein the multichromophore is an aggregate of conjugated molecules.

9. The method of claim 8, wherein the aggregate comprises molecules having the structure



2 -R = -$(CH_2)_6NMe_3^{\oplus}I^{\ominus}$

10. The method of claim 1, wherein the sample is contacted with the sensor polynucleotide and the multichromophore in the presence of a sufficient amount of an organic solvent to decrease hydrophobic interactions between the sensor polynucleotide and the multichromophore.

11. The method of claim 1, wherein the sample is contacted with a plurality of different sensor polynucleotides having corresponding different sequences, said different sensor polynucleotides comprising a corresponding different signaling chromophore, wherein each of said different sensor polynucleotides can selectively hybridize to a corresponding different target polynucleotide.

12. The method of claim 1, wherein the signaling chromophore is a fluorophore.

13. The method of claim 12, wherein the fluorophore is selected from a semiconductor nanocrystal, a fluorescent dye, and a lanthanide chelate.

14. The method of claim 13, wherein the fluorophore is a semiconductor nanocrystal.

15. The method of claim 13, wherein the fluorophore is a fluorescent dye.

16. The method of claim 15, wherein the fluorescent dye is fluorescein.

17. The method of claim 13, wherein the fluorophore is a lanthanide chelate.

18. The method of claim 1, wherein the target polynucleotide is DNA.

19. The method of claim 1, wherein the target polynucleotide is RNA.

20. The method of claim 1, wherein the sample comprises single-stranded target polynucleotide.

21. The method of claim 1, wherein the sample comprises double-stranded target polynucleotide.

22. The method of claim 1, wherein the target polynucleotide is produced via an amplification reaction.

23. A polynucleotide sensing solution comprising:

a signaling chromophore; and

a polycationic multichromophore that is capable of transferring energy to the signaling chromophore upon excitation when brought into proximity thereto, wherein a greater amount of energy can be produced from the signaling chromophore in the presence of the polynucleotide being sensed when the multichromophore is excited.

24. A kit for assaying a sample for a target polynucleotide comprising:

a sensor polynucleotide that is single-stranded and is complementary to the target polynucleotide;

a signaling chromophore;

a polycationic multichromophore that is capable of transferring energy to the signaling chromophore upon excitation when brought into proximity thereto, wherein said sensor polynucleotide interacts with the multichromophore and a detectibly greater amount of emitted light is produced from the signaling chromophore upon excitation of the multichromophore in the presence of target polynucleotide; and

a housing for retaining the reagents of the kit.

25. The method of claim 1, wherein light emitted from the signaling chromophore above a threshold level indicates that the target polynucleotide is present in the sample.

26. The method of claim 1, wherein the amount of light emitted from the signaling chromophore is quantitated and used to determine the amount of the target polynucleotide in the sample.

27. The method of claim 12, wherein the fluorophore is a green fluorescent protein.

28. The method of claim 1, wherein the target polynucleotide is not amplified.

29. The method of claim 1, wherein the method is performed on a substrate.

30. The method of claim 29, wherein the substrate is selected from the group consisting of a microsphere, a chip, a slide, a multiwell plate, an optical fiber, an optionally porous gel matrix, a photodiode, and an optoelectronic device.

31. The method of claim 30, wherein the substrate is a slide.

32. The method of claim 30, wherein the substrate is an optoelectronic device.

33. The method of claim 30, wherein the substrate is an optionally porous gel matrix.

34. The method of claim 33, wherein the substrate is a sol-gel.

35. The method of claim 30, wherein the substrate is nanoaddressable.

36. The method of claim 30, wherein the substrate is microaddressable.

37. The method of claim 30, wherein the substrate is conjugated to a plurality of different sensor polynucleotides having corresponding different sequences, wherein each of said different sensor polynucleotides can selectively hybridize to a corresponding different target polynucleotide.

38. The method of claim 1, wherein the method comprises performing fluorescence *in situ* hybridization (FISH).

39. The method of claim 22, wherein the amplification reaction comprises a polymerase chain reaction.

40. The method of claim 1, further comprising contacting the sample in a solution with the sensor polynucleotide, the multichromophore and a second signaling

chromophore that can absorb energy from the signaling chromophore in the presence of target and emit light, and wherein detecting whether the light emitted from the signaling chromophore is increased in the presence of sample comprises detecting whether the light emitted from the second signaling chromophore is increased in the presence of sample.

41. The method of claim 40, wherein the second signaling chromophore is a polynucleotide-specific dye.

42. A signaling complex formed by the method of claim 1.

43. The complex of claim 42, further comprising a second signaling chromophore.

44. The polynucleotide sensing solution of claim 23, further comprising a second signaling chromophore that can absorb energy from the signaling chromophore in the presence of target and emit light.

45. The polynucleotide sensing solution of claim 44, wherein the second signaling chromophore is a polynucleotide-specific dye.

46. A substrate-bound complex formed by the method of claim 29.

47. The substrate-bound complex of claim 46, further comprising a second signaling chromophore.

48. The kit of claim 24, further comprising a second signaling chromophore that can absorb energy from the signaling chromophore in the presence of target and emit light.

49. The kit of claim 48, wherein the second signaling chromophore is a polynucleotide-specific dye.

50. The kit of claim 24, further comprising instructions provided with said housing that describe how to use the components of the kit to assay the sample for the target polynucleotide.

51. A sensing complex comprising:

a sensor polynucleotide that is single-stranded and is complementary to a target polynucleotide, said sensor polynucleotide attached to a signaling chromophore;

a polycationic multichromophore that is capable of transferring energy to the signaling chromophore upon excitation when brought into proximity thereto, wherein said sensor polynucleotide interacts with the multichromophore and emitted light can be produced from the signaling chromophore upon excitation of the multichromophore in the absence of target polynucleotide, and wherein a greater amount of emitted light is produced from the signaling chromophore upon excitation of the multichromophore in the presence of target polynucleotide.

52. The polynucleotide sensing solution of claim 23, further comprising a sensor polynucleotide that is single-stranded and is complementary to the target polynucleotide.

53. The polynucleotide sensing solution of claim 23, wherein the signaling chromophore is a polynucleotide-specific dye that can absorb energy from the multichromophore in the presence of target and emit light.

54. The polynucleotide sensing solution of claim 23, wherein the signaling chromophore is a polynucleotide-specific dye that can absorb energy from the multichromophore in the presence of target and transfer energy to a second signaling chromophore conjugated to a sensor polynucleotide which then emits light.

55. The method of claim 3, wherein the saturated polymer is attached to luminescent dyes.

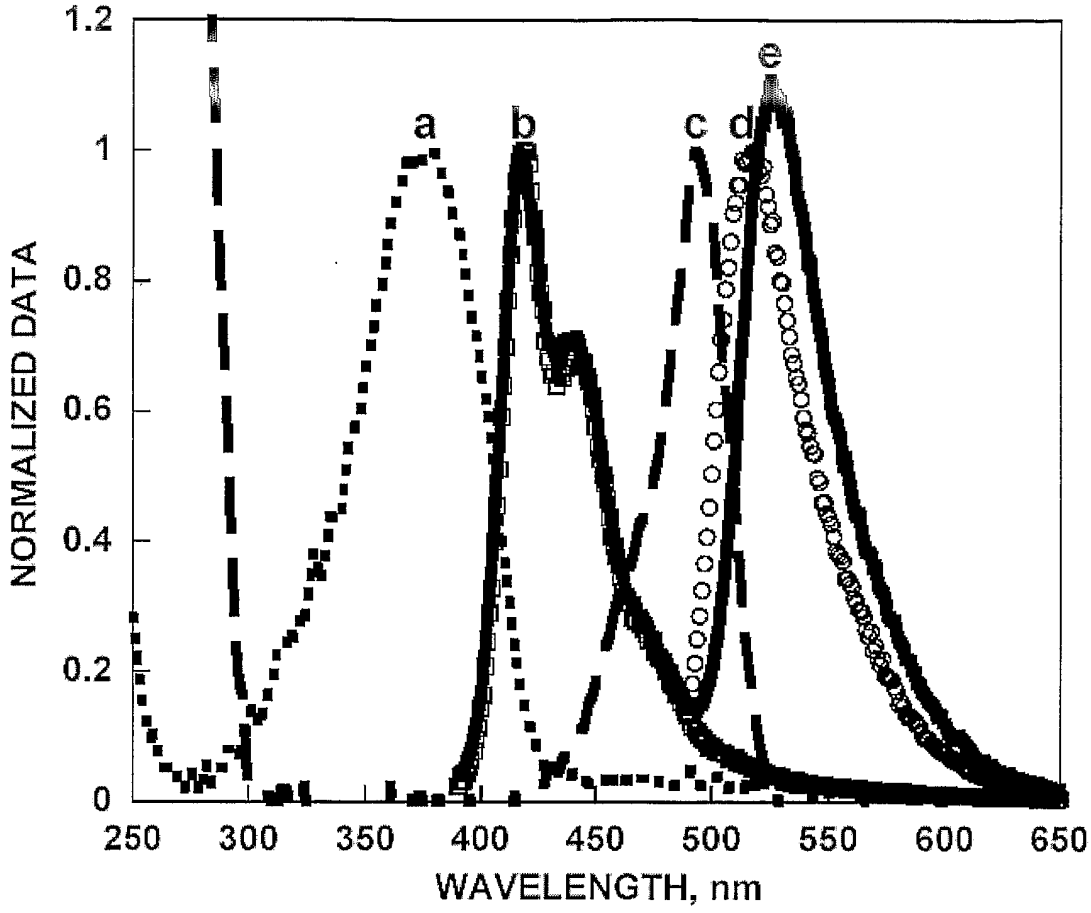

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Fig. 1

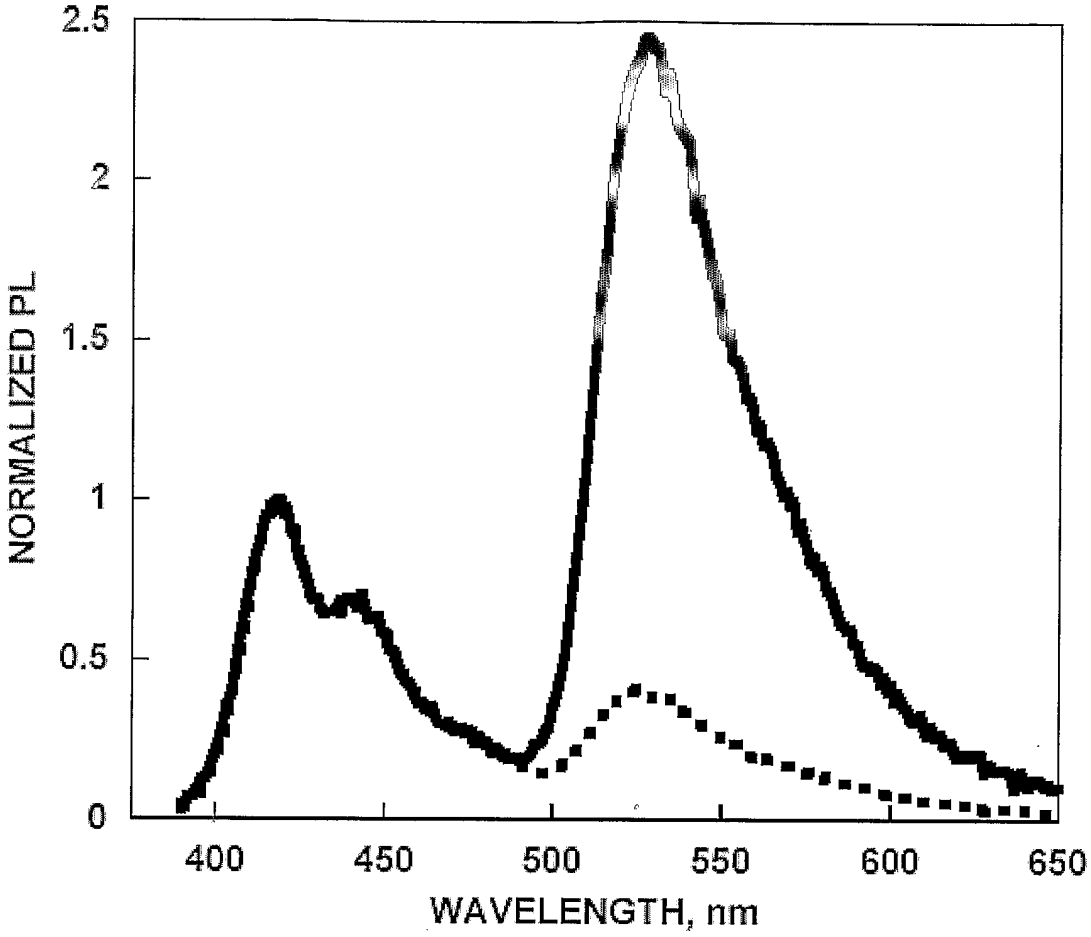

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Fig. 2

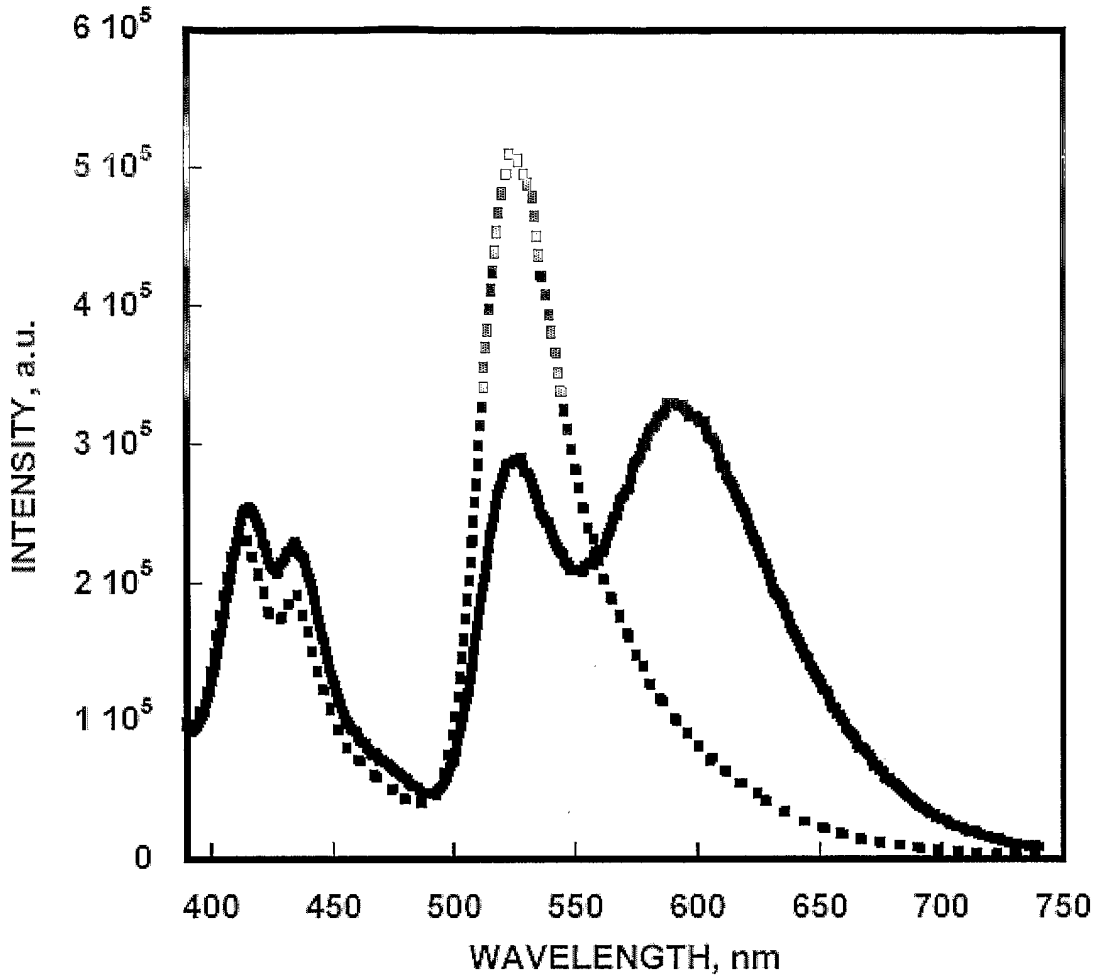

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Fig. 3

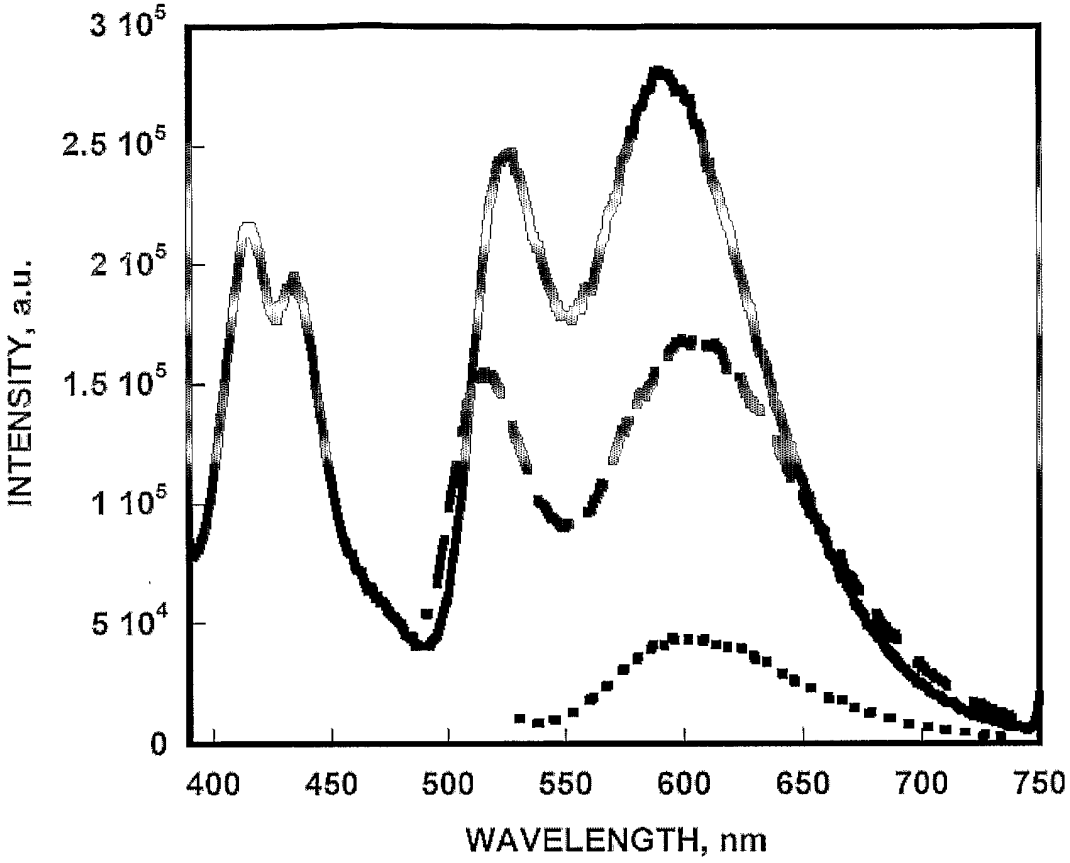

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Fig. 4

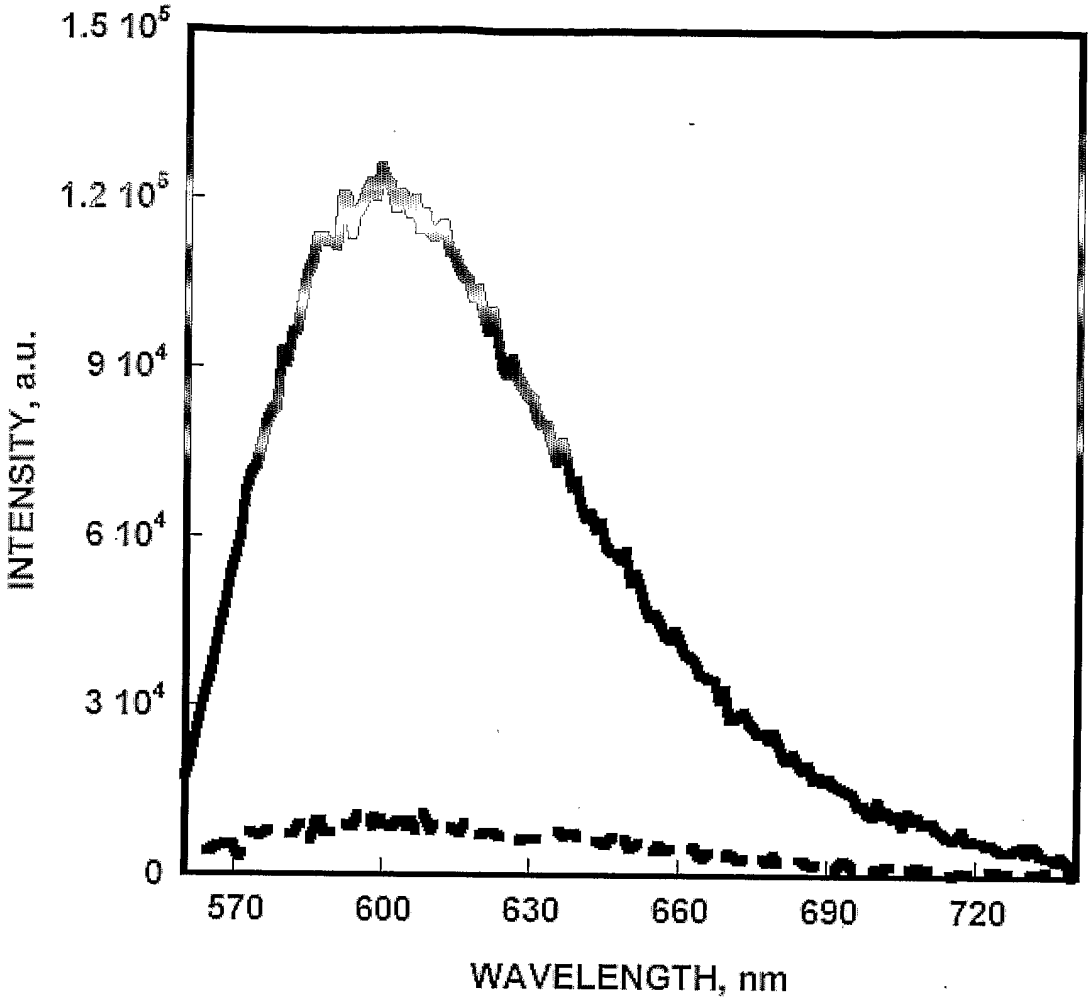

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WAVELENGTH, nm

Fig. 5