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(54) **DICHROMIC FLUORESCENT COMPOUNDS**

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**Publication Classification**

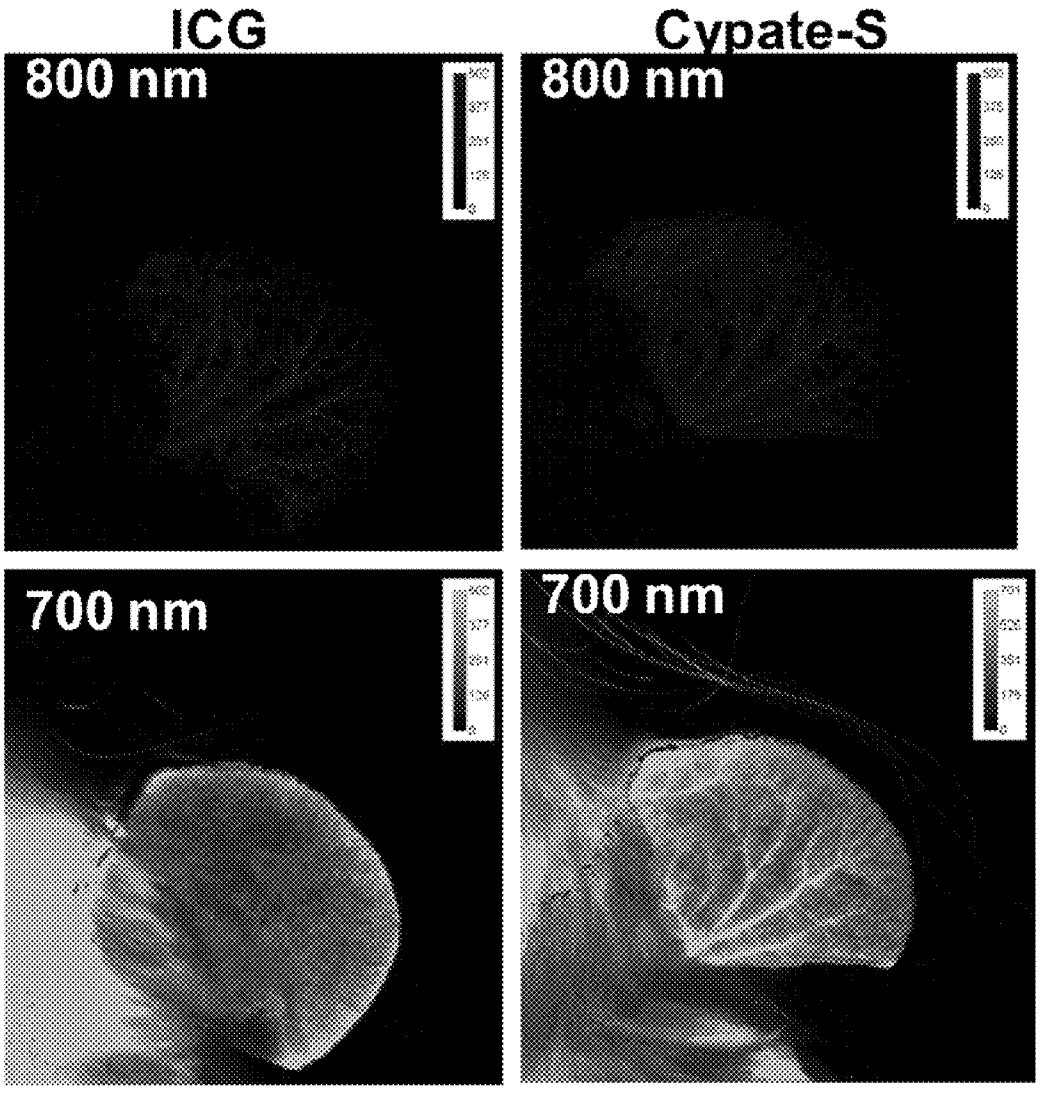
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| *C07K 7/02* | (2006.01) |
| *C07K 7/54* | (2006.01) |

(52) **U.S. Cl.** ..... **424/9.6**; 548/427; 530/329; 530/317

(57) **ABSTRACT**

The present invention provides dichromic fluorescent compounds, as well as processes for making and methods for using the dichromic fluorescent compounds.



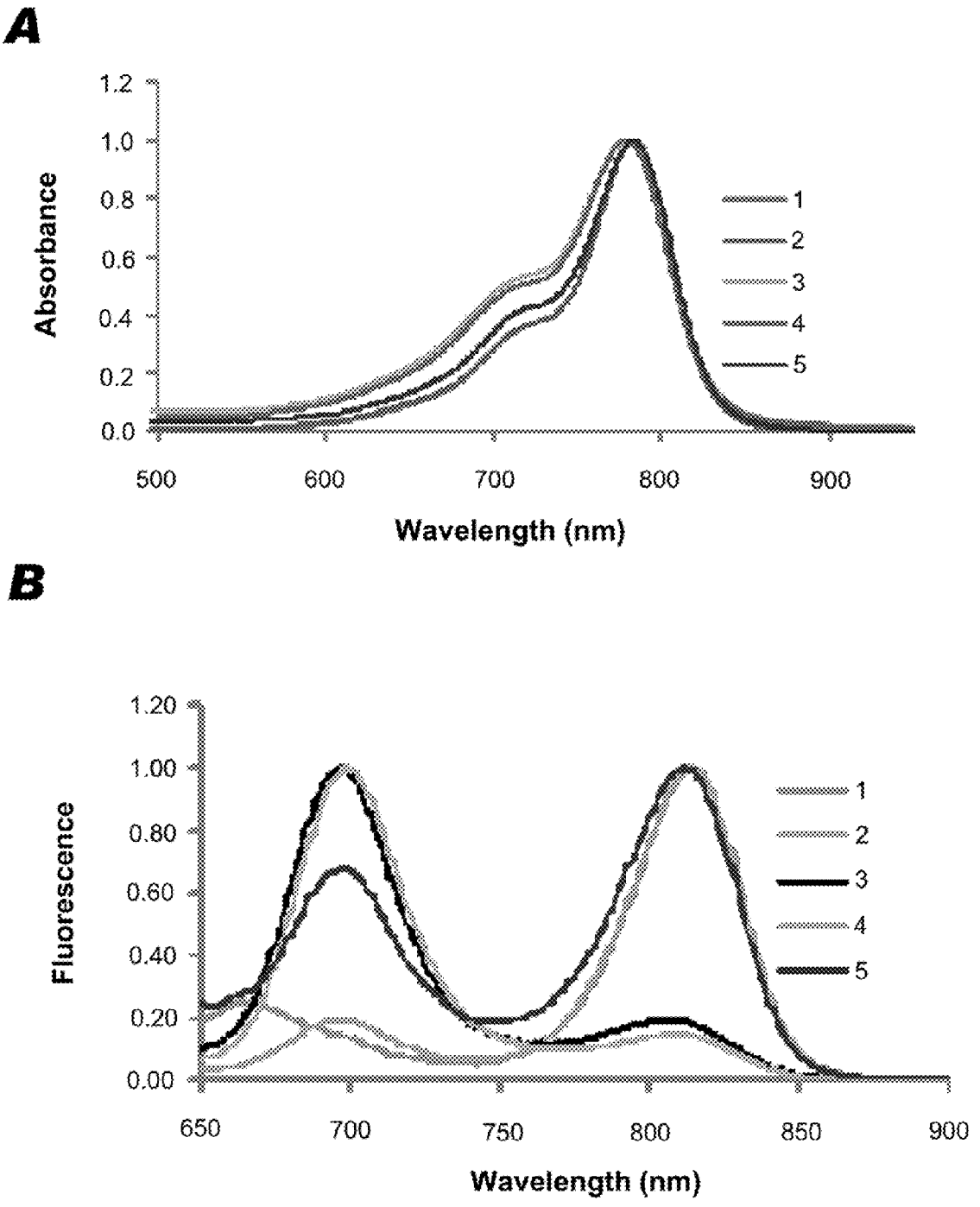

A
Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
500
600
700
800
900
Wavelength (nm)
1
2
3
4
5
B
Fluorescence
1.20
1.00
0.80
0.60
0.40
0.20
0.00
650
700
750
800
850
900
Wavelength (nm)
1
2
3
4
5

FIG. 1

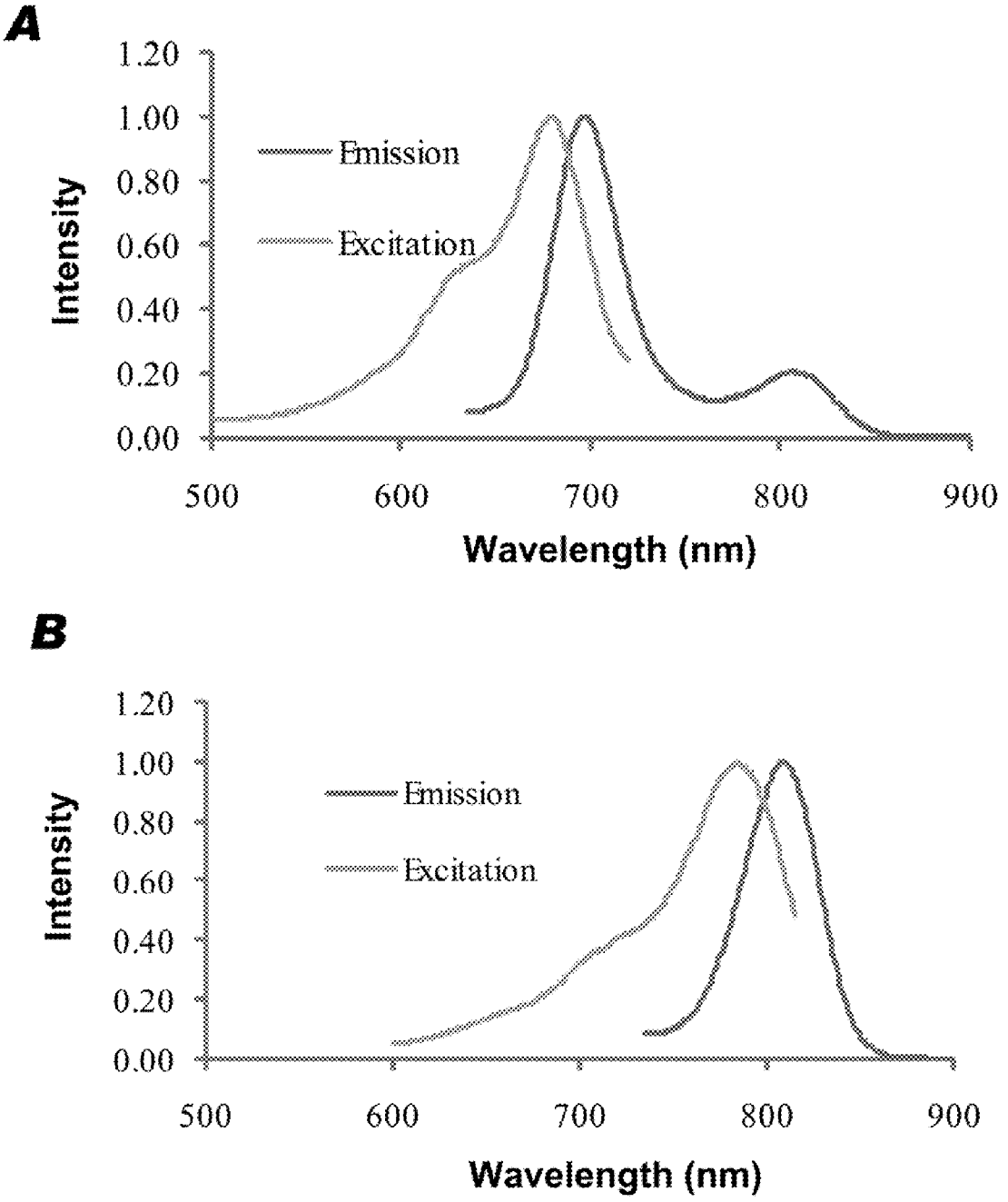

A
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Intensity
Emission
Excitation
500
600
700
800
900
Wavelength (nm)
B
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Intensity
Emission
Excitation
500
600
700
800
900
Wavelength (nm)

FIG. 2

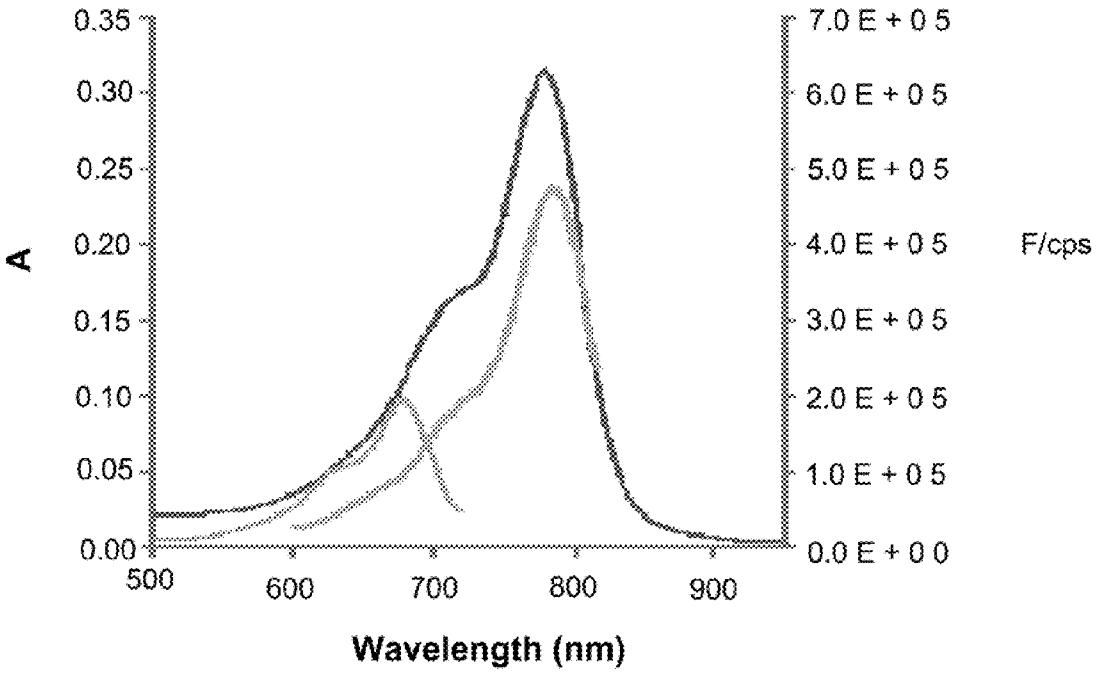
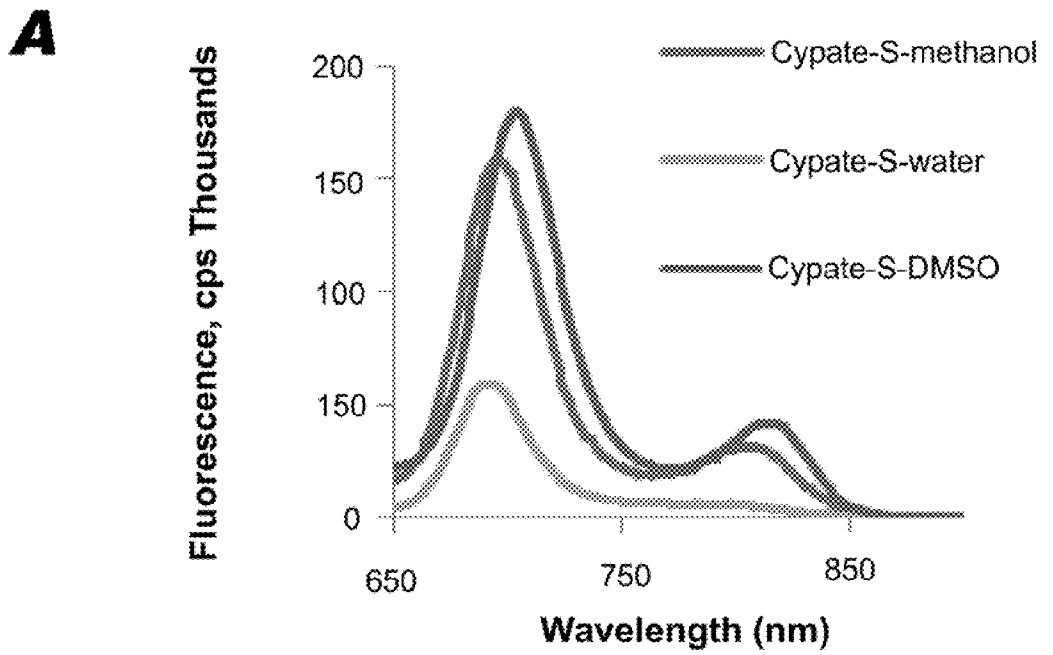

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A
7.0 E + 0 5
6.0 E + 0 5
5.0 E + 0 5
4.0 E + 0 5
3.0 E + 0 5
2.0 E + 0 5
1.0 E + 0 5
0.0 E + 0 0
F/cps
500
600
700
800
900
Wavelength (nm)

FIG. 3


A
Fluorescence, cps Thousands
200
150
100
150
0
650
750
850
Wavelength (nm)
Cypate-S-methanol
Cypate-S-water
Cypate-S-DMSO

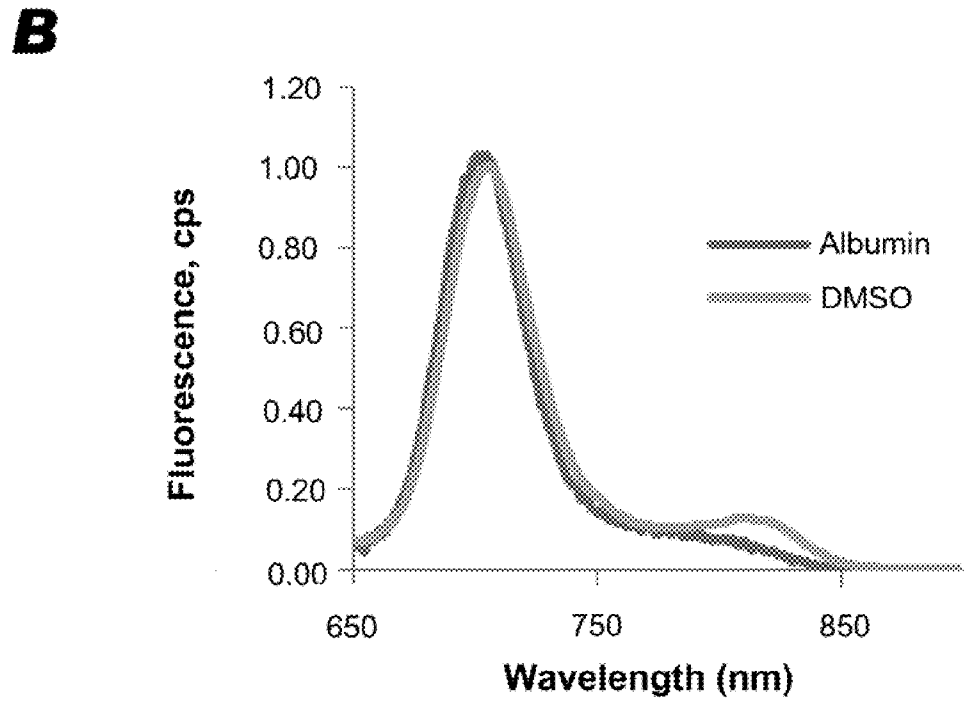

B
Fluorescence, cps
1.20
1.00
0.80
0.60
0.40
0.20
0.00
650
750
850
Wavelength (nm)
Albumin
DMSO

FIG. 4

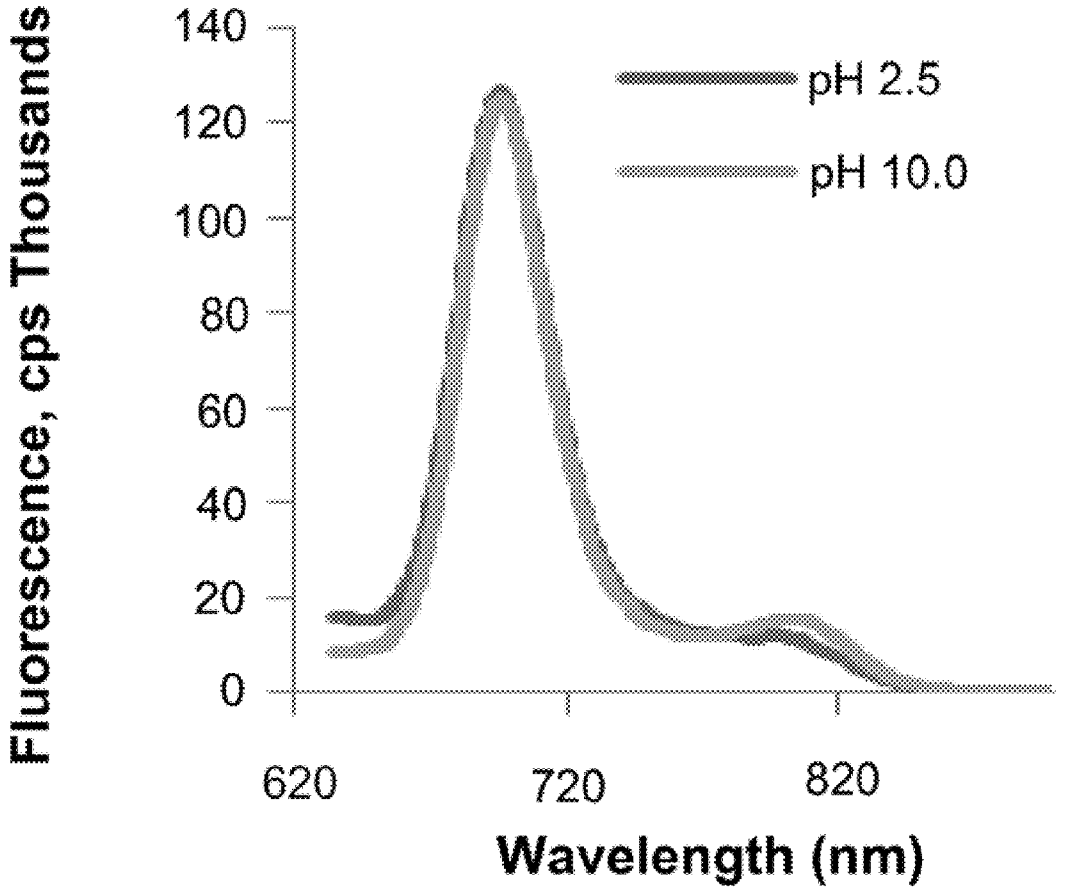
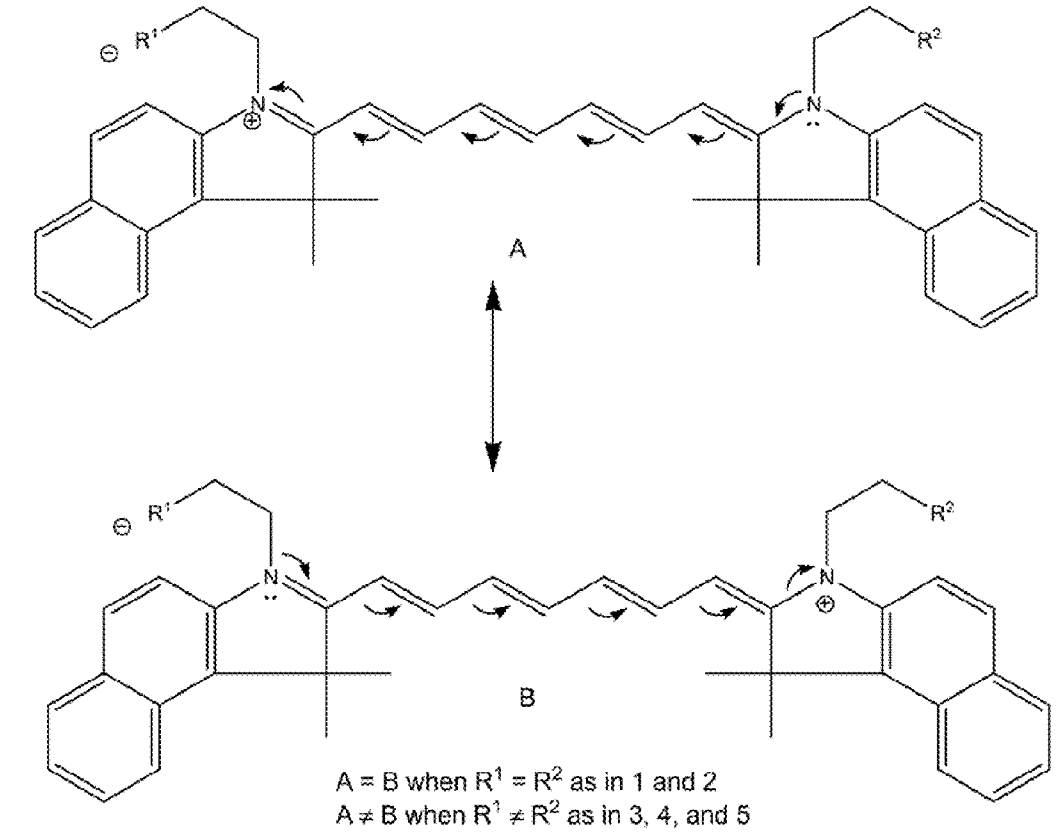

Fluorescence, cps Thousands
140
120
100
80
60
40
20
0
620
720
820
Wavelength (nm)
pH 2.5
pH 10.0

FIG. 4C



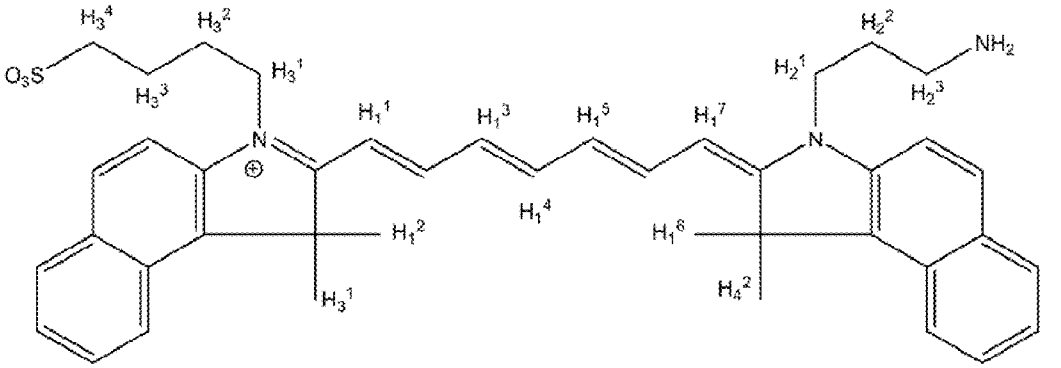



FIG. 5A

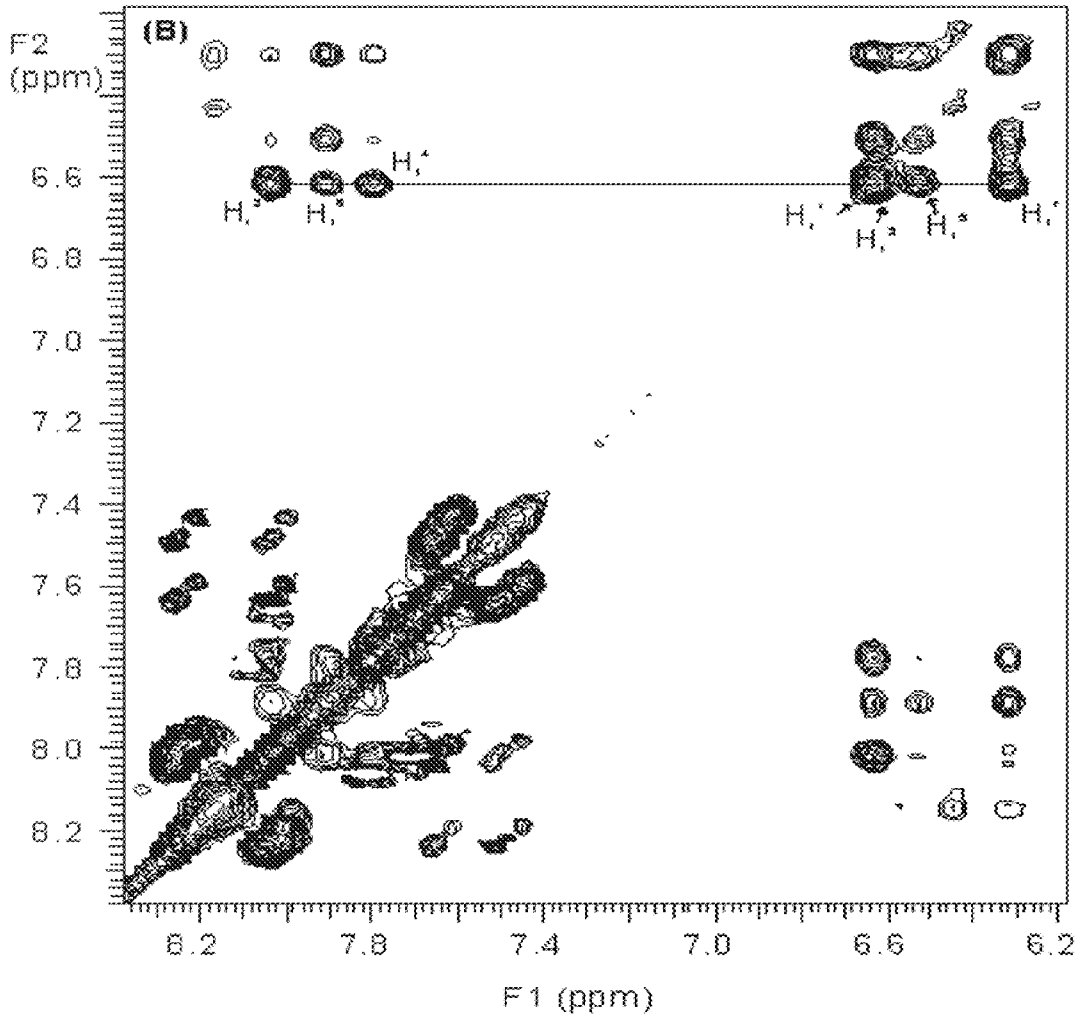

(B)
F2 (ppm)
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
H,⁴
H,³
H,⁵
H,'
H,²'
H,⁴'
H,⁵'
H,'
8.2
7.8
7.4
7.0
6.6
6.2
F1 (ppm)

FIG. 5B

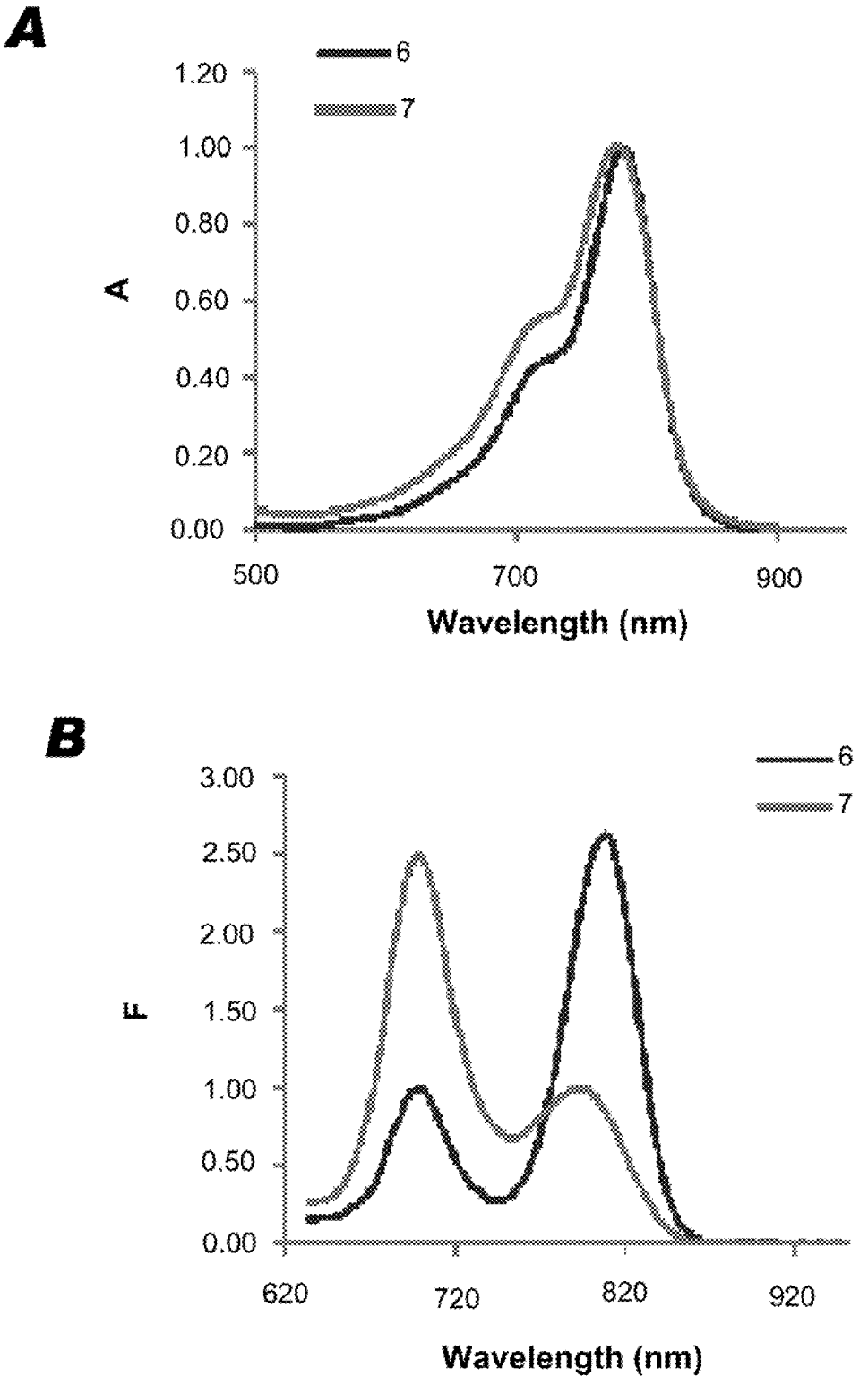

A
6
7
1.20
1.00
0.80
0.60
0.40
0.20
0.00
A
500
700
900
Wavelength (nm)
B
6
7
3.00
2.50
2.00
1.50
1.00
0.50
0.00
F
620
720
820
920
Wavelength (nm)

FIG. 6

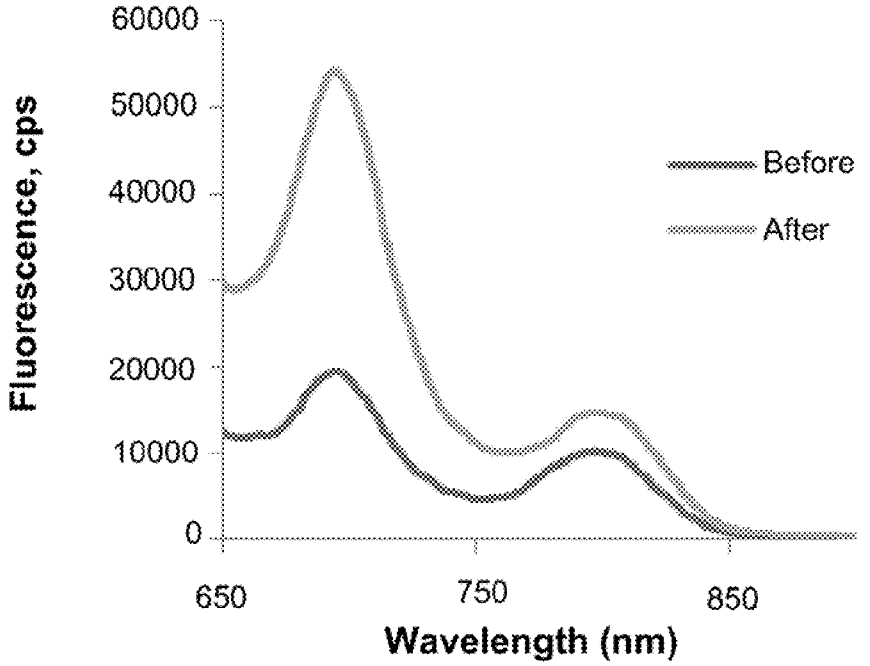

60000
50000
40000
30000
20000
10000
0
Fluorescence, cps
Before
After
650
750
850
Wavelength (nm)

FIG. 7

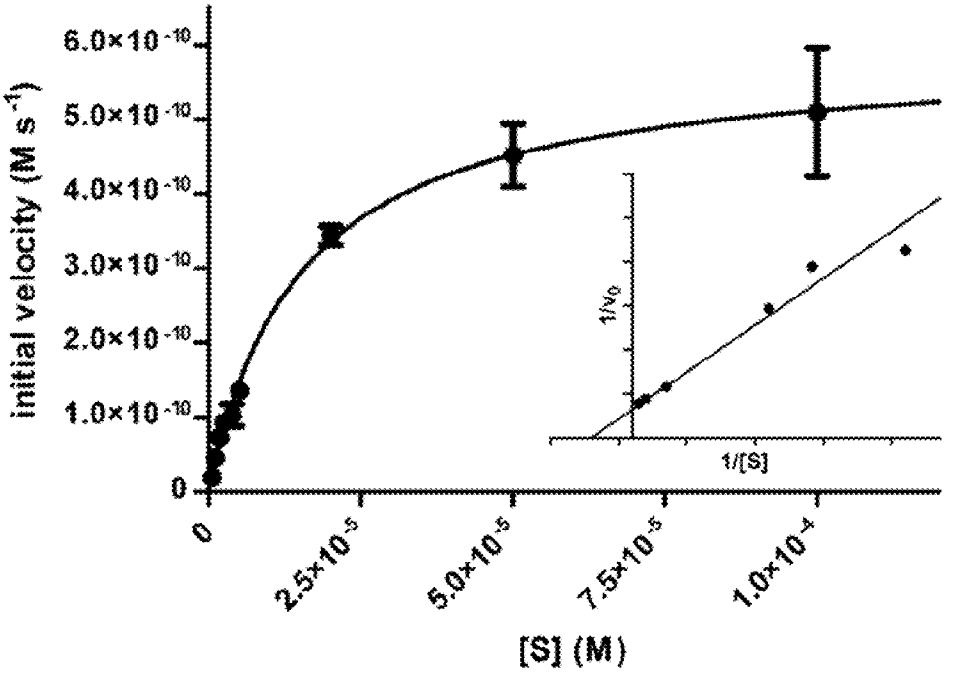
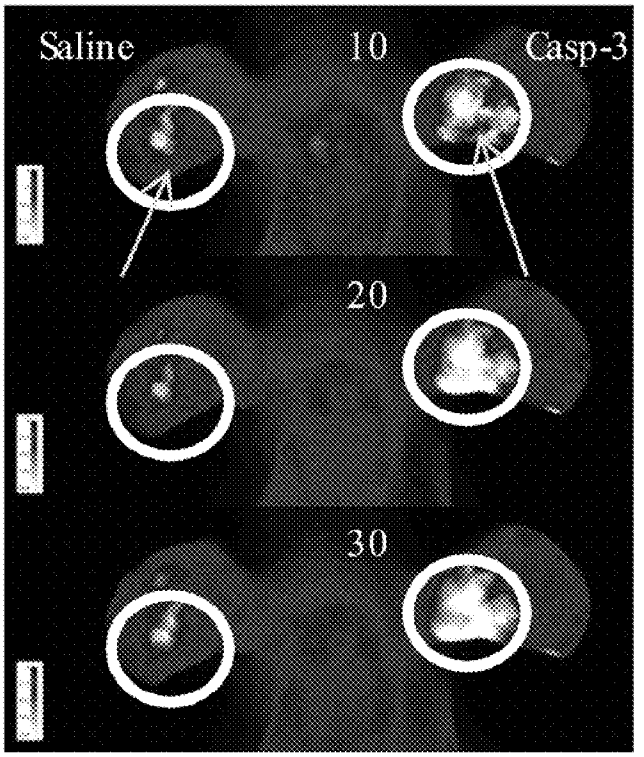

6.0×10⁻¹⁰
5.0×10⁻¹⁰
4.0×10⁻¹⁰
3.0×10⁻¹⁰
2.0×10⁻¹⁰
1.0×10⁻¹⁰
0
initial velocity (M s⁻¹)
1/v0
1/[S]
0
2.5×10⁻⁵
5.0×10⁻⁵
7.5×10⁻⁵
1.0×10⁻⁴
[S] (M)

FIG. 8

A


Saline
10
Casp-3
20
30

B

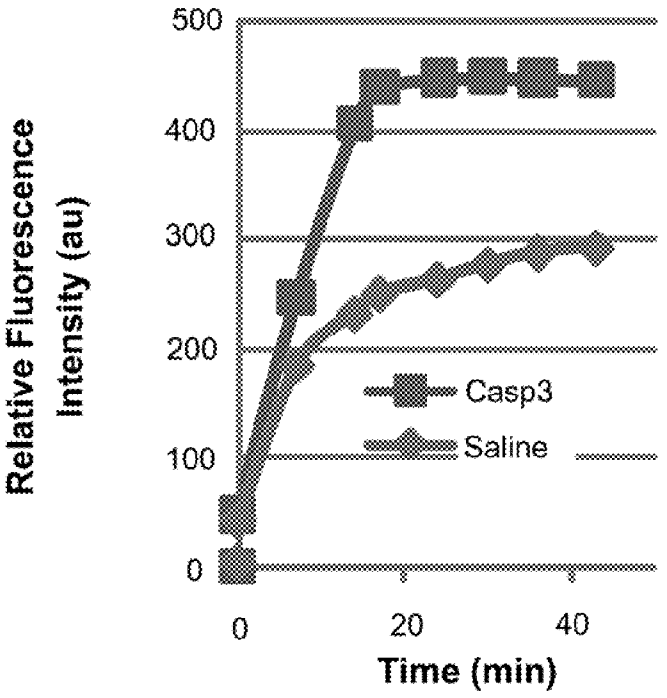
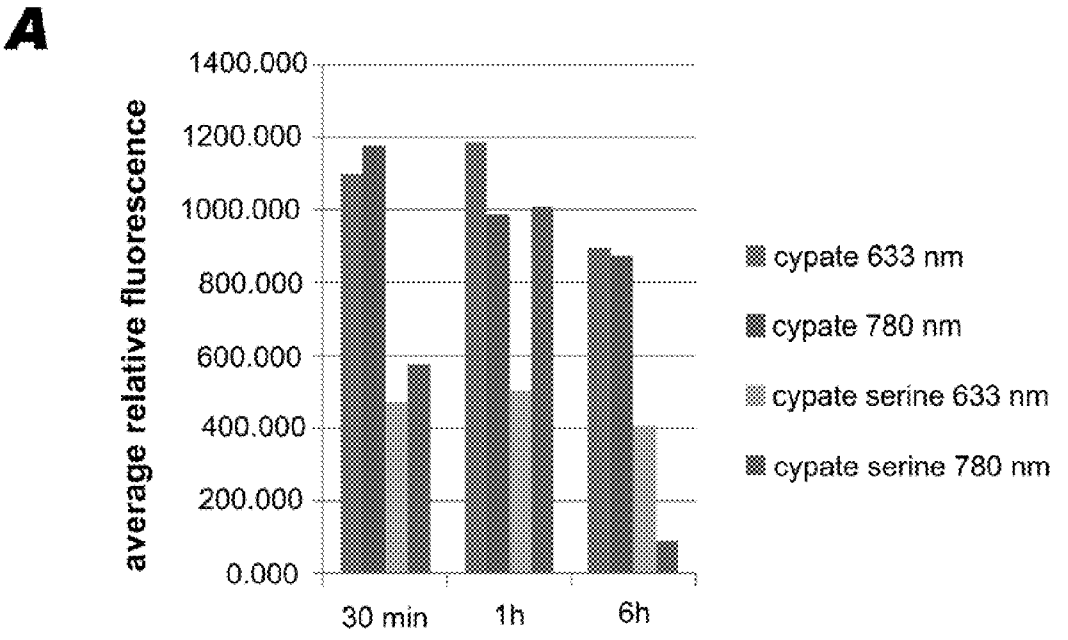

500
400
300
200
100
0
Relative Fluorescence Intensity (au)
Casp3
Saline
0
20
40
Time (min)

FIG. 9


A
1400.000
1200.000
1000.000
800.000
600.000
400.000
200.000
0.000
average relative fluorescence
30 min
1h
6h
cypate 633 nm
cypate 780 nm
cypate serine 633 nm
cypate serine 780 nm

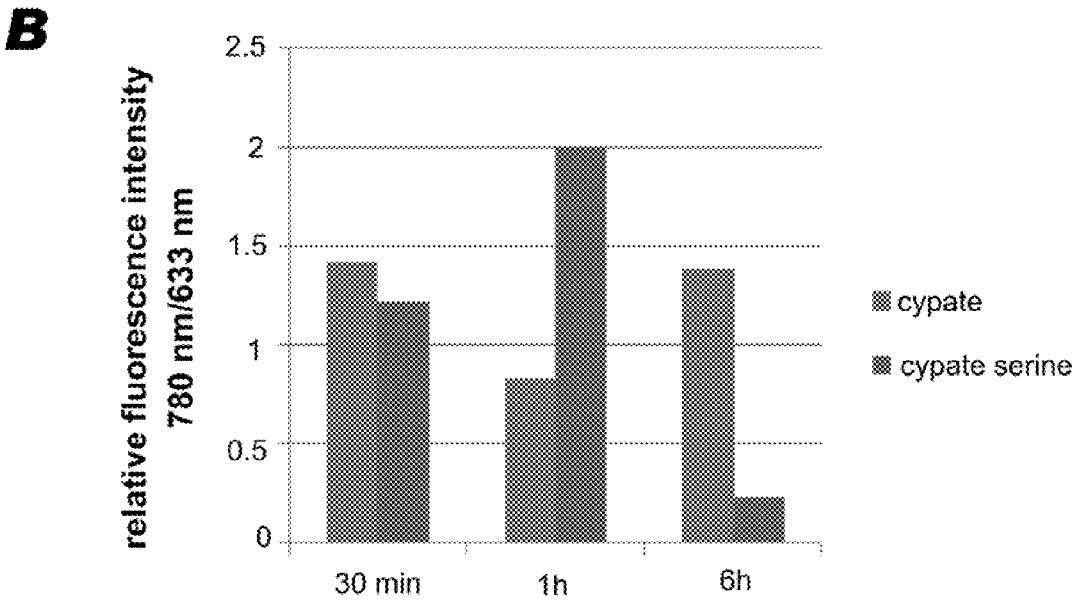

B
2.5
2
1.5
1
0.5
0
relative fluorescence intensity
780 nm/633 nm
30 min
1h
6h
cypate
cypate serine

FIG. 10

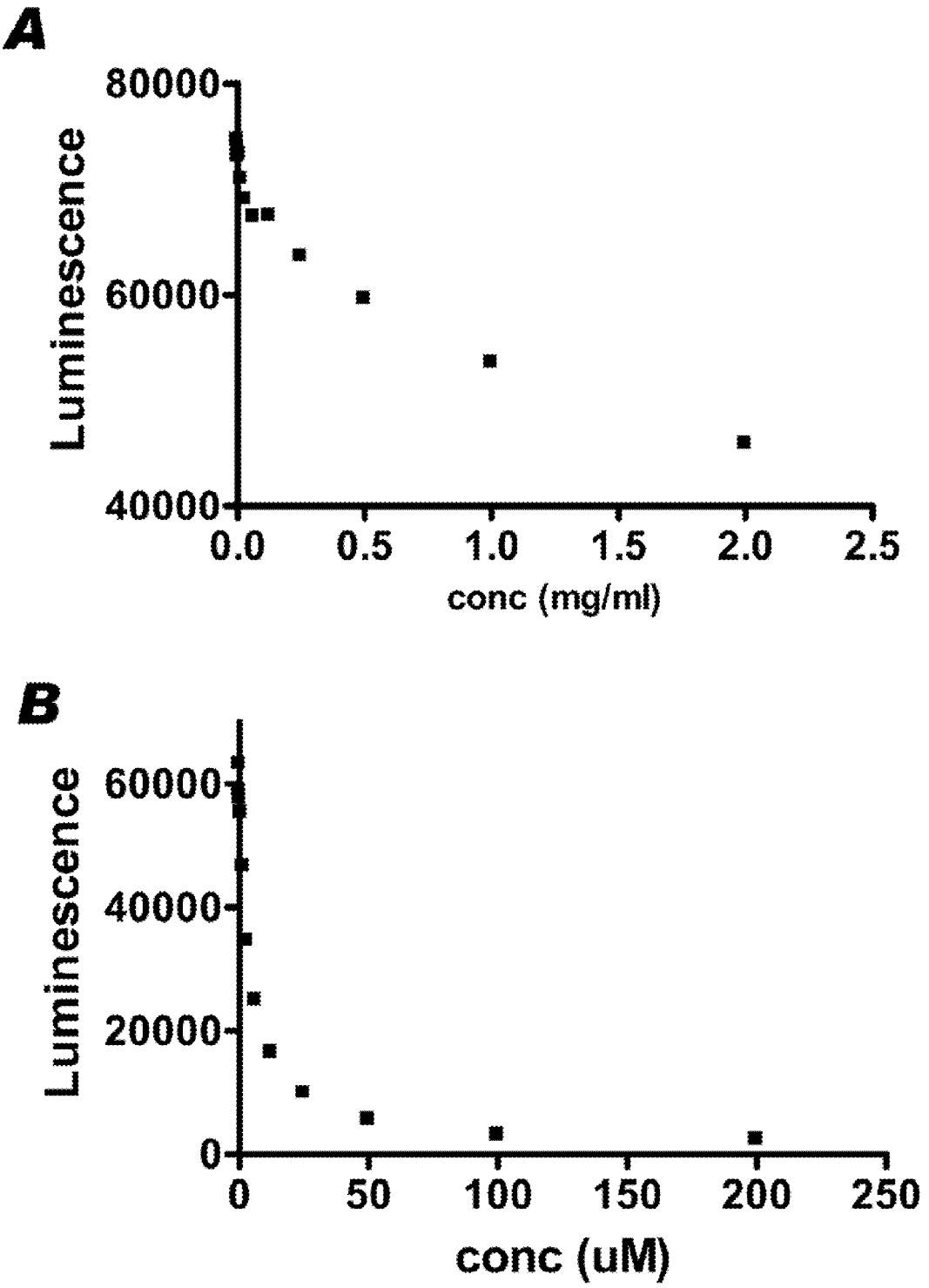

A
80000
60000
40000
Luminescence
0.0
0.5
1.0
1.5
2.0
2.5
conc (mg/ml)
B
60000
40000
20000
0
Luminescence
0
50
100
150
200
250
conc (uM)

FIG. 11

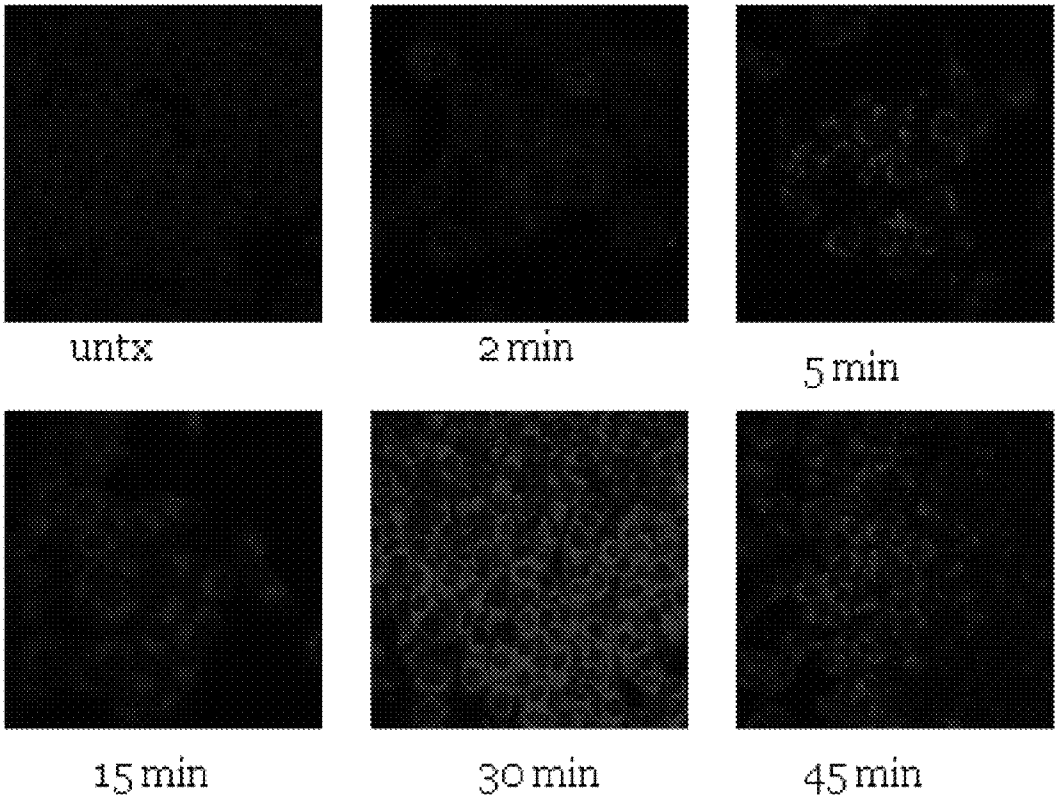



FIG. 12A

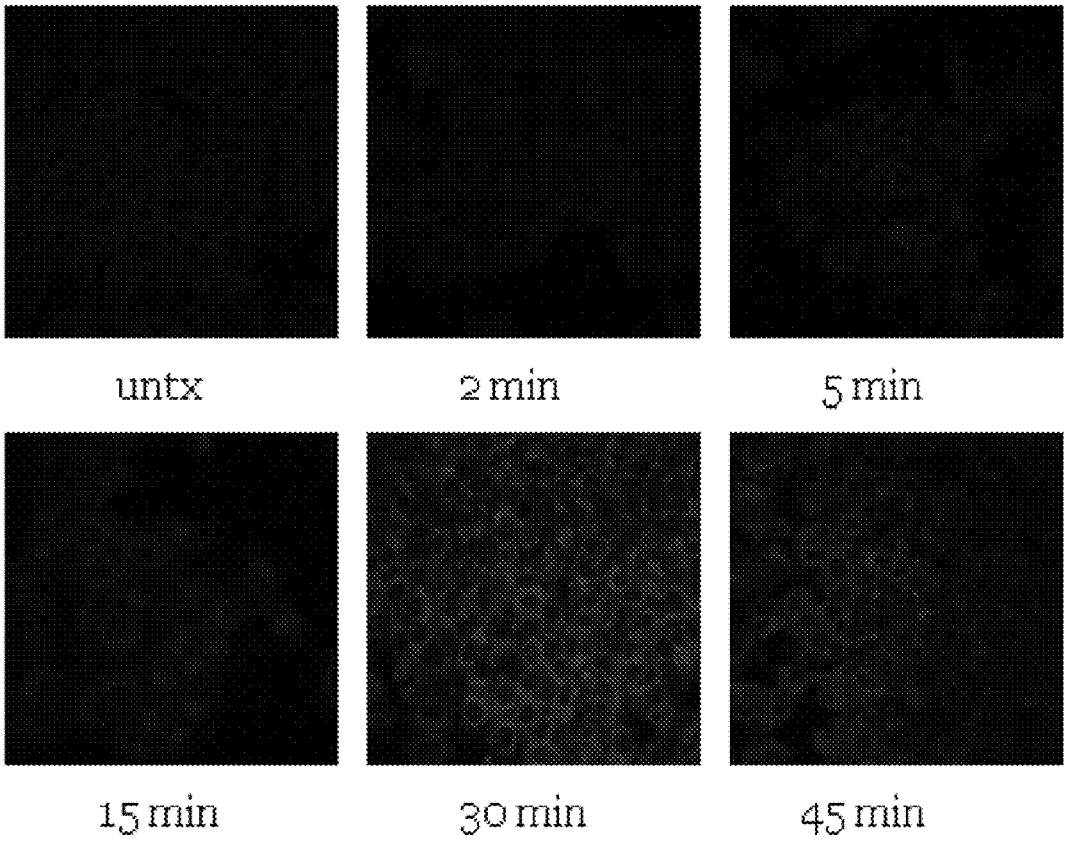



FIG. 12B

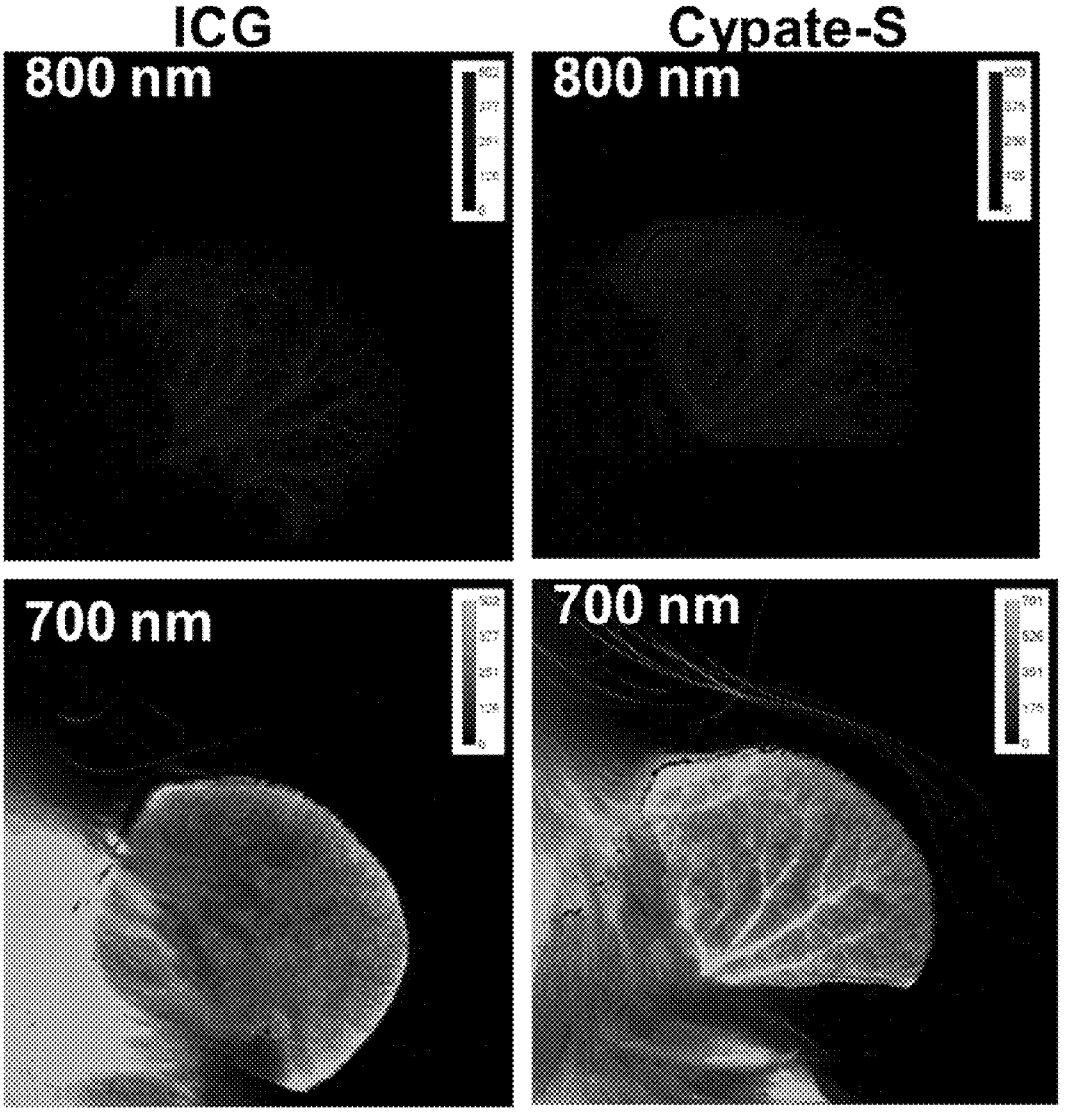



FIG. 13

# DICHROMIC FLUORESCENT COMPOUNDS

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims the priority of U.S. provisional application No. 61/029,412, filed Feb. 18, 2008, and U.S. provisional application No. 61/029,644, filed Feb. 19, 2008, each of which is hereby incorporated by reference in its entirety.

## GOVERNMENTAL RIGHTS

**[0002]** The present invention was supported by NIBIB Grant No. R01 EB1430, and NCI Grant Nos. R01 CA109754 and R21 CA12353. The United States Government has certain rights in this invention.

## FIELD OF THE INVENTION

**[0003]** The present invention generally relates to dichromic compounds. In particular, the invention provides dichromic fluorescent compounds, process for producing dichromic fluorescent compounds, and methods of using dichromic fluorescent compounds to monitor biological events.

## BACKGROUND OF THE INVENTION

**[0004]** Fluorescent materials that provide high detection sensitivity of molecular processes are central to advances in biochemical assays, molecular sensor technologies, and molecular optical imaging. In biological optical imaging, the low autofluorescence of tissues and the deep penetration of light at wavelengths between 650 and 900 nm allow the use of near infrared (NIR) fluorescent dyes as contrast agents in heterogeneous systems. The ease of synthesis, biocompatibility, tunable spectral properties, and exceptionally high molar absorptivity of NIR fluorescent carbocyanines provide added incentive for their use in cellular and in vivo imaging applications.

**[0005]** Despite the multitude of advantages, NIR fluorescent dyes are used for narrow applications in biological sciences and medicine because of poor sensitivity of NIR fluorescence intensity to changes in molecular processes. A traditional approach for using these dyes involves labeling biological molecules with the dyes along with the assumption that clearance from the nontarget process will allow detection of the targeted molecular event. For in vivo studies, this requires a method to remove nonspecific interactions, which is often difficult and cumbersome. Elimination of nonspecific targets in vivo, therefore, presents a major hurdle that can only be solved by sophisticated algorithms or via passive elimination that could take several days to achieve.

**[0006]** Another level of difficulty with fluorescence methods relates to quantification of the signal. Fluorescence intensity measurements are generally influenced by many factors that complicate quantitative analysis. An approach to solve this problem involves the use of ratiometric methods. Unfortunately, only a few molecules have the ability to generate two emission bands for this purpose, and there are no available NIR fluorescent probes with this capacity. Thus to overcome this challenge, two or more NIR dyes are typically attached to the same molecule for ratiometric analyses.

**[0007]** Yet another unsolved problem with NIR fluorescent imaging is the difficulty in detecting molecular interactions. Optical methods are particularly attractive for detecting molecular interactions because selective activation of light emission by fluorescence resonance energy transfer (FRET) or related methods using a variety of fluorescent dyes or proteins allows the detection of molecular interactions with high sensitivity and specificity. Unfortunately, most FRET or FRET-like methods rely on the use of two or more fluorophore systems to pre-quench the fluorescence efficiently before activation by the target biomolecule. In addition to the costs of developing two dyes and the potential cumulative toxicity, the approach also requires careful selection of the donor-acceptor dye pairs, stringent positioning of the dyes (because this may affect the interactions), and distance-dependent separation of the cleaved dye from cellular milieu to detect the fluorescence. Meeting these requirement is particularly challenging for developing NIR FRET probes that would be useful for deep tissue imaging. There is a need, therefore, for dual emitting NIR probes in which the ratio of the two fluorescence bands would change in response to biological events, such that the fluorescent signal could be quantified using a ratiometric analysis.

## SUMMARY OF THE INVENTION

**[0008]** Among the various aspects of the present invention is the provision of a dichromic compound comprising:

A-L-B

**[0009]** wherein:

**[0010]** A and B are each either structurally symmetrical moieties, or are each structurally asymmetrical moieties; and L is a linker comprising a chromophore.

The compound further comprises $R^1$ and $R^2$, wherein $R^1$ and $R^2$ are structurally distinct groups that are each either conjugated to the compound comprising structurally symmetrical A and B moieties in a manner that yields asymmetry such that the compound has two distinct fluorescence lifetimes at two different emission wavelengths; or $R^1$ and $R^2$ are each conjugated to the compound comprising structurally asymmetrical A and B moieties in a manner that yields symmetry such that the compound has two distinct fluorescence lifetimes at two different emission wavelengths.

**[0011]** Another aspect of the present invention encompasses a method for monitoring a biological event in a subject. The method comprises administering a dichromic compound to the subject and detecting whether the structural symmetry of the compound changes, thereby indicating the biological event. The dichromic compound comprises:

A-L-B

**[0012]** wherein:

**[0013]** A and B are each either structurally symmetrical moieties, or are each structurally asymmetrical moieties; and L is a linker comprising a chromophore.

The compound further comprises $R^1$ and $R^2$, wherein $R^1$ and $R^2$ are structurally distinct groups that are each either conjugated to the compound comprising structurally symmetrical A and B moieties in a manner that yields asymmetry such that the compound has two distinct fluorescence lifetimes at two different emission wavelengths; or $R^1$ and $R^2$ are each conjugated to the compound comprising structurally asymmetrical A and B moieties in a manner that yields symmetry such that the compound has two distinct fluorescence lifetimes at two different emission wavelengths.

**[0014]** A further aspect of the present invention provides a process for producing a dichromic compound comprising:

A-L-B

**[0015]** wherein:

**[0016]** A and B are either structurally symmetrical moieties or are each structurally asymmetrical moi-

eties; L is a linker comprising a chromophore; and the compound further comprises $R^1$ and $R^2$.

The process comprises conjugating $R^1$ and $R^2$, which are structurally distinct groups, to the compound comprising structurally symmetrical A and B moieties in a manner that yields asymmetry such that the compound has two distinct fluorescence lifetimes at two different emissions wavelengths; or conjugating $R^1$ and $R^2$ to the compound comprising structurally asymmetrical A and B moieties in a manner that yields symmetry such that the compound has two distinct fluorescence lifetimes at two different emissions wavelengths.

**[0017]** Other aspects and iterations of the invention are described more thoroughly below.

## REFERENCE TO COLOR FIGURES

**[0018]** The application file contains at least one photograph executed in color. Copies of this patent application publication with color photographs will be provided by the Office upon request and payment of the necessary fee.

## DESCRIPTION OF THE FIGURES

**[0019]** FIG. **1** illustrates the spectral properties of NIR fluorescent dyes. Absorbance (A) and dual fluorescence (B) spectra of representative dyes (compounds 1, 2, 3, 4) and peptide conjugate (compound 5). Excitation was corrected by lamp intensity (S/N). F, normalized fluorescence intensity.

**[0020]** FIG. **2** presents the spectral properties of compound 3. Fluorescence excitation and emission scans at 695 nm (A) and 806 nm (B) in methanol. Excitation was corrected by lamp intensity (S/N). I, normalized absorbance and fluorescence intensity.

**[0021]** FIG. **3** depicts the deconvolution of the broad absorbance spectra of compound 3 into two distinct bands.

**[0022]** FIG. **4** illustrates the effects of solvent (A), albumin (B), and pH (C) on the fluorescence spectra of compound 3.

**[0023]** FIG. **5** depicts the structural features of nonsymmetrical dichromic compounds (A) and a representative 2D TOSCY NMR (DMSO, 600 MHz) of compound 4 showing the seven vinyl protons in the polymethine bridge and their correlation (B).

**[0024]** FIG. **6** presents normalized absorption (A) and emission (B) spectra of diserine-cypate (6) and monophosphate diserine-cypate (7) in methanol.

**[0025]** FIG. **7** depicts the shifts in the fluorescence peaks of the cypate-DEVD probe before and after cleavage by caspase-3.

**[0026]** FIG. **8** presents enzyme kinetics of the cypate-DEVD probe. Shown is the nonlinear fit of the initial velocity with respect to substrate concentration and the corresponding Lineweaver-Burk plot (inset).

**[0027]** FIG. **9** illustrates in vivo imaging of capsase-3 activity. (A) 805 nm fluorescence intensity maps of mouse head and ears at indicated times (min) after intradermal injection of either saline or caspase-3 in left and right ears, respectively. (B) Plot of fluorescence intensity over time for the probe activation by caspase-3 starting from intravenous injection (0 min) and intradermal caspase-3 or saline injection (10 min).

**[0028]** FIG. **10** depicts the detection of phosphorylation in real time. (A) Plotted is the average relative fluorescence of cypate or cypate-serine upon excitation at 633 nm or 780 nm. (B) Plotted is the ratio of relative fluorescence intensity of cypate or cypate-serine.

**[0029]** FIG. **11** presents phosphorylation in A549 cells. Plotted is luminescence (which is inversely proportional to phosphorylation) as a function of concentration of casein kinase II peptide substrate (A) or cypate-diserine (6) (B).

**[0030]** FIG. **12** depicts two-channel imaging of fluorescence enhancement of A549 cells incubated with cypate-diserine (6). (A) 700 nm emission (excitation at 633 nm) of untreated cells (untx) and cells treated for 2-45 min. (B) 805 nm emission (excitation at 780 nm) of untreated cells (untx) and cells treated for 2-45 min.

**[0031]** FIG. **13** presents in vivo fluorescence intensity maps of mouse ears. Left panels depict ears of mice injected with indocyanine green (ICG) and right panels depict ears of mice injected with cypate-S (3).

## DETAILED DESCRIPTION OF THE INVENTION

**[0032]** It has been discovered that structural asymmetry of chromic compounds results in the generation of dichromic emissions that have distinct lifetimes. Because the fluorescent lifetimes of the two peaks are distinct, the information content may be multiplexed. The dichromic fluorescent compounds may advantageously be utilized for several applications including imaging, diagnostics, to monitor biological events, and for detecting and monitoring molecular processes.

### (I) Dichromic Fluorescent Compounds

**[0033]** The compounds of the invention are dichromic. Stated another way, a single compound has two distinct fluorescent lifetimes at two different emission wavelengths. This dichromic property of the compound may be generated by conjugating two structurally distinct groups to a symmetrical molecule to yield an asymmetrical compound. Alternatively, the dichomic property may be generated by conjugating two structurally distinct groups to an asymmetrical molecule to yield a symmetrical compound. In the context of the present invention the term "symmetry" means that a compound is bilaterally symmetrical with respect to both the type of atoms and their arrangement (i.e., straight chain, branched, or ring) within the compound.

**[0034]** In one embodiment, the dichromic compound comprises:

A-L-B

**[0035]** wherein:

**[0036]** A and B are each either structurally symmetrical moieties, or are each structurally asymmetrical moieties;

**[0037]** L is a linker comprising a chromophore; and

**[0038]** the compound further comprising $R^1$ and $R^2$, wherein $R^1$ and $R^2$ are structurally distinct groups that are each either conjugated to the compound comprising structurally symmetrical A and B moieties in a manner that yields asymmetry such that the compound has two distinct fluorescence lifetimes at two different emissions wavelengths; or $R^1$ and $R^2$ are each conjugated to the compound comprising structurally asymmetrical A and B moieties in a manner that yields symmetry such that the compound has two distinct fluorescence lifetimes at two different emissions wavelengths.

**[0039]** The A and B moieties, in one embodiment, are symmetrical. For this embodiment, A and B typically comprise the same group of atoms in the same arrangement. For example, if A comprises one halosubstituted benzene ring, then B comprises a halosubtituted benenze ring. Alternatively, the A and B moieties may be nonsymmetrical. For example, if A comprises a halosubstituted benzene ring, then B may comprise a benzene ring (i.e., that isn't substituted

with a halogen). Irrespective of the embodiment, the atoms forming A and B may be arranged linearly, they may be within a branched chain, they may comprise one or more rings or ring systems, or they may be combinations of any of the these. The atoms forming A and B may be one or more hydrocarbyls, substituted hydrocarbyls, heteroatoms, halogens or any other suitable atoms that may come together to form a stable compound. If A and B comprise rings or ring systems, the rings may be carbocyclic, heterocyclic, or mixtures of both with any suitable degree of saturation. In general, each member ring may comprise from 4 to 8 atoms. The rings may be substituted with any suitable group including hydrocarbyls, substituted hydrocarbyls, halogens, and heteroatoms.

**[0040]** The chromophore, L, is a linker that is disposed between the A and B moieties. Typically, L exists in one of two forms: a conjugated pi system or a metal complex. In conjugated pi systems, electron excitation occurs between pi orbitals spread across alternating single and double bonds. Non-limiting examples of conjugate pi chromophore moieties include acridine, anthracene, anthrapyridone, anthraquinone, aryl azo, azomethine, benzofuran, benzaopyrone, benzoxazole, benzidine, carbazole, indole, isoprenoid, naphthalene, naphthoquinone, oxazine, phenanthrene, phenazine, polymethine, pyrene, pyrenequinone, quinoline, quinazoline, stilbene, thioxanthene, triarylmethane, xanthene moieties, and derivatives thereof. Metal complex chromophores possess shared d-orbitals between transition metals (with incomplete d-shells) and ligands. Examples of metal complex chromophore moieties include bacteriochlorin, bilin (or bilane), chlorin, corphin, corrin, heme, phthalocyanine, phycobilin, porphyrin, and salen. Typically, the metal complex chromophore is complexed with an appropriate metal ion.

**[0041]** $R^1$ and $R^2$ can and will vary depending upon whether the A and B moieties are symmetrical or nonsymmetrical. Irrespective of the embodiment, $R^1$ and $R^2$ are distint groups. For example, $R^1$ may be an alkyl group substituted with a halogen and $R^2$ may be an alkyl group substituted with a heteroatom. If the A and B moieties are symmetrical, then $R^1$ and $R^2$ are selected and conjugated to the compound in a manner that yields an asymmetrical compound that has two distinct fluoresence lifetimes at two different emissions wavelengths. Alternatively, If the A and B moieties are asymmetrical, then $R^1$ and $R^2$ are selected and conjugated to the compound in a manner that yields a symmetrical compound that has two distinct fluoresence lifetimes at two different emissions wavelengths. $R^1$ and $R^2$ may comprise any group that results in the formation of a dichromic compound, as described herein. Suitable examples include a hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence. In one embodiment, $R^1$ and $R^2$ may both be conjugated to L. In another embodiment, one of $R^1$ or $R^2$ may be conjugated to A and the other to B. In an additional embodiment, one of $R^1$ or $R^2$ may be conjugated to A and the other to L. In another embodiment, one of $R^1$ or $R^2$ may be conjugated to L and the other to B.

**[0042]** In an additional embodiment, the dichromic compound comprises:

$$A(—CH═CH)_n—CH═B$$

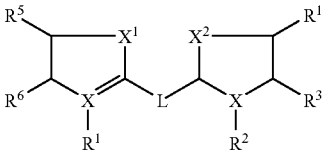
**[0043]** wherein:

**[0044]** A and B are as described above; and

**[0045]** n is an integer from 0 to 10. In an exemplary iteration, n is 3.

**[0046]** In still another embodiment, the dichromic compound comprises Formula (I):

(I)



**[0047]** wherein:

**[0048]** $R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

**[0049]** X is a heteroatom selected from the group consisting of nitrogen, phosphorus, silicon, and sulfur;

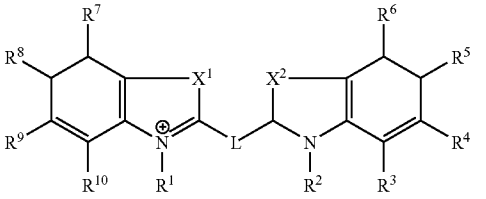
**[0050]** $X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

**[0051]** L is a linker comprising a chromophore; and

**[0052]** $R^3$, $R^4$, $R^5$, and $R^6$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl; provided that $R^3$ and $R^4$ and $R^5$ and $R^6$ may form a ring or a ring system.

**[0053]** In an additional embodiment, the dichromic compound comprises Formula (Ia):

(Ia)



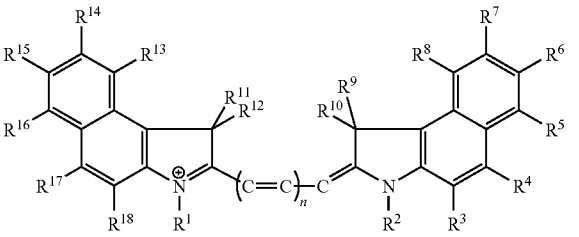
**[0054]** wherein:

**[0055]** $R^1$, $R^2$, L, $X^1$, and $X^2$ are as described for compounds comprising Formula (I);

**[0056]** $R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, and $R^{10}$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl; provided that any of $R^3$ and $R^4$, $R^4$ and $R^5$, $R^5$ and $R^6$, $R^7$ and $R^8$, $R^8$ and $R^9$, and $R^9$ and $R^{10}$ may form a ring or a ring system.

**[0057]** In certain embodiments, the dichromic compound comprises Formula (Ib):

(Ib)



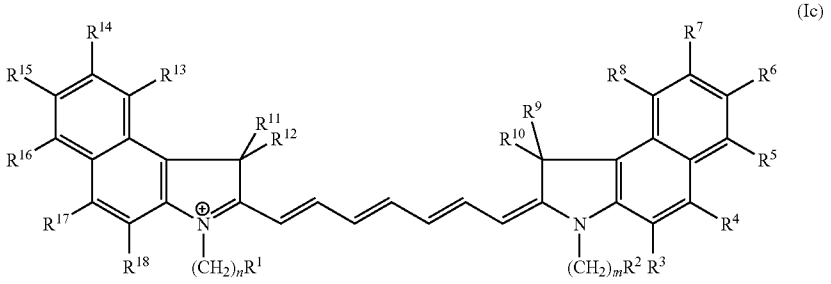
**[0058]** wherein:

**[0059]** $R^1$ and $R^2$ are as defined above for compounds comprising Formula (I);

**[0060]** $R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $R^{11}$, $R^{12}$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, and $R^{18}$ are each independently selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl; and

**[0061]** n is an integer from 0 to 10.

**[0062]** In one alternative embodiment, the compound comprises Formula (Ic):



(Ic)

**[0063]** wherein:

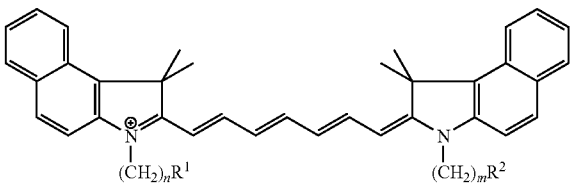
**[0064]** $R^1$ and $R^2$ are independently selected from the group consisting of hydrogen, amide, amino, carboxyl, halogen, hydroxy, phosphate, sulfonate, thiol, azido, alkyne, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

**[0065]** $R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $R^{11}$, $R^{12}$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, and $R^{18}$ are as described for compounds comprising Formula (Ib); and

**[0066]** m is an integer from 1 to 10; and

**[0067]** n is an integer from 1 to 10.

**[0068]** In another alternative embodiment, the compound comprises Formula (Id):

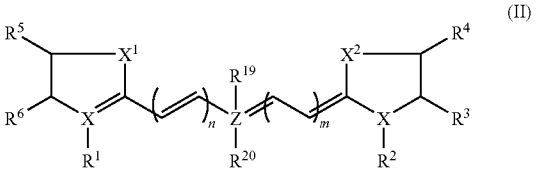


(Id)

**[0069]** wherein:

**[0070]** $R^1$, $R^2$, m, and n are as described for compounds comprising Formula (Ic).

**[0071]** In an additional embodiment, the dichromic compound comprises a polymethine chain that has a cyclic group that is centrally located within the chain. The cyclic ring may be derivatized with a hydrocarbyl or a substituted hydrocarbyl group at the meso position. The compound according to this embodiment may be a compound comprising Formula (II):



(II)

**[0072]** wherein:

**[0073]** $R^1$, $R^2$, $R^3$, $R^4$, $R^5$, $R^6$, X, $X^1$, and $X^2$ are as described for compounds comprising Formula (I);

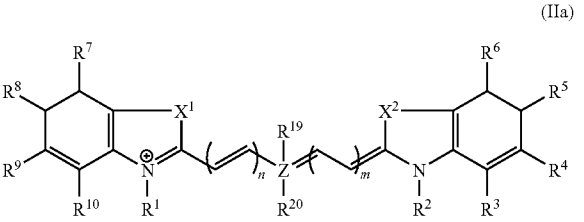
**[0074]** $R^{19}$ and $R^{20}$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence;

**[0075]** Z is a cyclic group selected from the group consisting of a carbocyclic ring and a heterocyclic ring;

**[0076]** n is an integer from 1 to 3; and

**[0077]** m is an integer from 1 to 3.

**[0078]** In another embodiment, the dichromic compound comprises Formula (IIa):



(IIa)

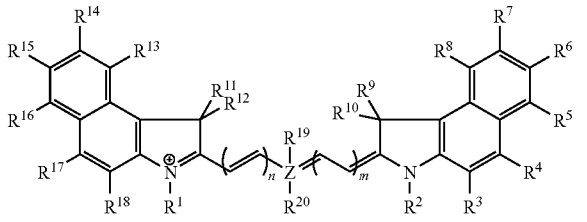
**[0079]** wherein:

**[0080]** $R^1$, $R^2$, $R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $X^1$, and $X^2$ are as described for compounds comprising Formula (Ia); and

**[0081]** Z, $R^{19}$, $R^{20}$, m, and n are as described for compounds having Formula (II).

**[0082]** In an additional embodiment, the dichromic compound comprises Formula (IIb):

(IIb)



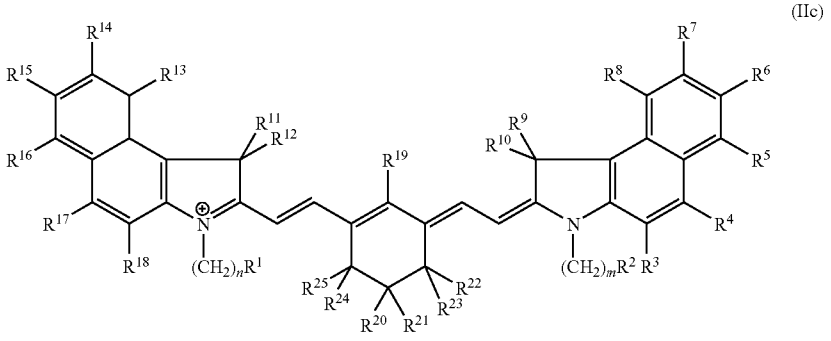
**[0083]** wherein:

**[0084]** $R^1$, $R^2$, $R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $R^{11}$, $R^{12}$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, and $R^{18}$ are as described for compounds comprising Formula (Ib); and

**[0085]** Z, $R^{19}$, $R^{20}$, m, and n are as described for compounds having Formula (II).

**[0086]** In an alternative of this embodiment, the dichromic compound may comprise Formula (IIc):

(IIc)



**[0087]** wherein:

**[0088]** $R^1$ and $R^2$ are independently selected from the group consisting of hydrogen, amide, amino, carboxyl, halogen, hydroxy, phosphate, sulfonate, thiol, azido, alkyne, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups

**[0089]** $R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $R^{11}$, $R^{12}$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, $R^{18}$, $R^{19}$, and $R^{20}$ are as described for compounds comprising Formula (IIb);

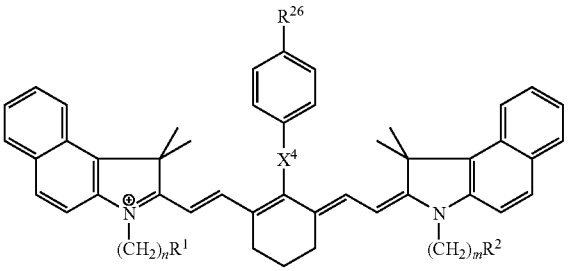
**[0090]** $R^{21}$, $R^{22}$, $R^{23}$, $R^{24}$, and $R^{25}$ are each independently selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl;

**[0091]** m is an integer from 1 to 10; and

**[0092]** n is an integer from 1 to 10.

**[0093]** In another alternative embodiment, the dichromic compound comprises Formula (IId):

(IId)



**[0094]** wherein:

**[0095]** $R^1$, $R^2$, m, and n are as described for compounds comprising Formula (IIc);

**[0096]** $R^{26}$ is selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; and

**[0097]** $X^4$ is a single bond or a heteroatom selected from the group consisting of sulfur, nitrogen, and oxygen;

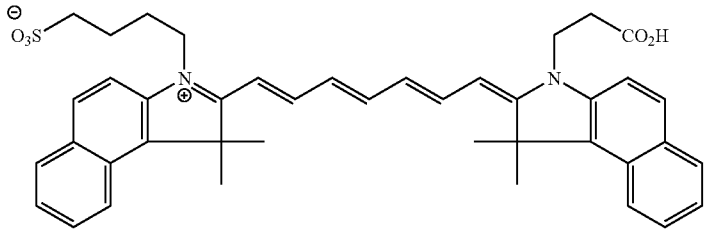
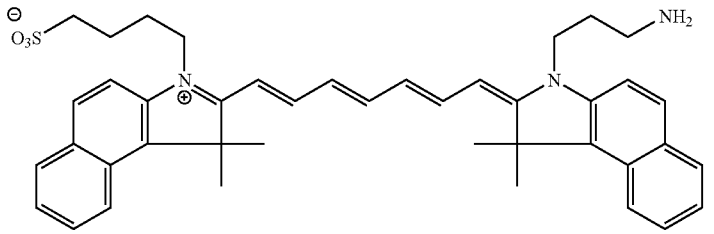
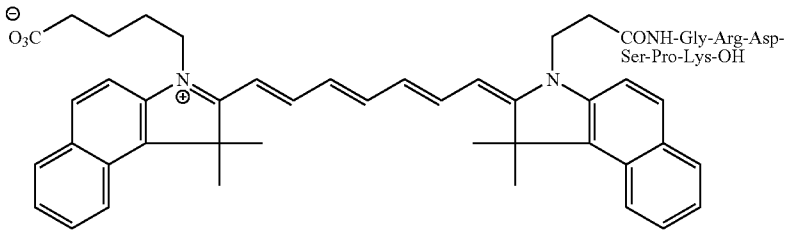
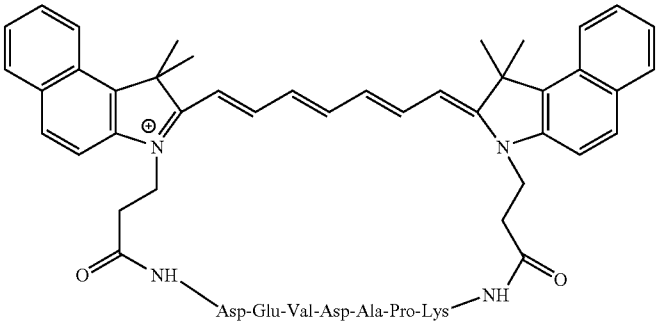
**[0098]** For each of the foregoing embodiments, the dichromic compounds of the invention may include one or more reactive groups for coupling the dye compound to a biomolecule. In one embodiment, one or both of the $R^1$ and $R^2$ groups may include a reactive group for coupling the dye compound to a biomolecule. In another embodiment, the $R^{19}$, $R^{20}$, or $R^{26}$ groups may include a reactive group for coupling the dye compound to a biomolecule. In still another embodiment, one or more of any of the aforementioned groups may include a reactive group for coupling the dye compound to a biomolecule. Suitable non-limiting examples of biomolecules include antigens, steroids, vitamins, drugs, haptens, metabolites, toxins, environmental pollutants, antibodies, DNA, RNA, peptides, proteins, siRNA, miRNA, carbohydrates, lipids, and small molecules. In an exemplary embodiment, the biomolecule may be a peptide. The biomolecule

may be coupled to the dye compound by methods generally known in the art or by methods described herein.

**[0099]** Alternatively, $R^1$, $R^2$, $R^{19}$, $R^{20}$, and $R^{26}$ may be selected to modify or optimize the physical and/or function properties of the dichromic compounds. $R^1$ and $R^2$ groups may be selected, for example, to modify a physical characteristic of the dye compound selected from the group consisting of water solubility, biodistribution, and spectral elongation.

**[0100]** Exemplary non-limiting examples of dichromic compounds of the invention are shown in Table A.

TABLE A

| Compound No. | Structure |
|---|---|
| A-1 |  |
| A-2 |  |
| A-3 |  |
| A-4 |  |

**[0101]** The compounds of the invention, as stated above, are dichromic. In this context, as shown in the examples, a single compound has two fluorescence lifetimes at two different emissions wavelengths. The two wavelengths generally have absorption spectra ranging from about 200 to about 1200 nm, depending upon the chromophore system (i.e., L) that is utilized. In an exemplary embodiment, the absorption spectrum of each wavelength is from about 700 nm to about 900 nm. In certain embodiments, the absorption spectra of each emission is above about 705 nm, 710 nm, 715 nm, 720 nm, 725 nm, 730 nm, 735 nm, 740 nm, 745 nm, 750 nm, 755 nm, 760 nm, 765 nm, 770 nm, 775 nm, 780 nm, 785 nm, 790 nm, 795 nm, 800 nm, 805 nm, 810 nm, 815 nm, 820 nm, 825 nm, 830 nm, 835 nm, 840 nm, 845 nm, 850 nm, 855 nm, 860 nm, 865 nm, 870 nm, 875 nm, 880 nm, 885 nm, 890 nm, 895 nm, 900 nm, or greater than 900 nm. The emissions of the two fluorescence bands may be separated from about 25 to about 400 nm. More typically, the emissions of the two fluorescence bands may be separated by from about 50 to about 300 nm. In one embodiment, the emissions of the two fluorescence bands may be separated by about 100 nm. By way of non-limiting

example, one emission may be at 700 nm and the other at 800 nm. Alternatively, one emission may be at 750 nm and the other at 850 nm.

**[0102]** Additionally, the dichromic compounds of the present invention may exist in tautomeric, geometric or stereoisomeric forms. The present invention contemplates all such compounds, including cis- and trans-geometric isomers, E- and Z-geometric isomers, R- and S-enantiomers, diastereomers, d-isomers, l-isomers, the racemic mixtures thereof and other mixtures thereof. Pharmaceutically acceptable salts of such tautomeric, geometric or stereoisomeric forms are also included within the invention. The terms "cis" and "trans", as used herein, denote a form of geometric isomerism in which two carbon atoms connected by a double bond will each have a hydrogen atom on the same side of the double bond ("cis") or on opposite sides of the double bond ("trans"). Some of the compounds described contain alkenyl groups, and are meant to include both cis and trans or "E" and "Z" geometric forms. Furthermore, some of the compounds described contain one or more stereocenters and are meant to include R, S, and mixtures of R and S forms for each stereocenter present.

(II) Conjugation of Dichromic Compounds to Biomolecules

**[0103]** The dichromic compounds may be attached to a biomolecule or a ligand to form a conjugated substrate. Attachment may be, for example, by covalent bonding, ionic bonding, dative bonding, hydrogen bonding, and other forms of molecular bonding.

**[0104]** Several types of biomolecules are suitable for conjugation to the dichromic compounds. For example, useful conjugated substrates of the invention include, but are not limited to, conjugates of antigens, small molecules, steroids, vitamins, drugs, haptens, metabolites, toxins, environmental pollutants, amino acids, peptides, proteins, photosensitizers, nucleotides, oligonucleotides, nucleic acids, carbohydrates, lipids, ion-complexing moieties, and non-biological polymers. In one exemplary embodiment, the conjugated substrate is a natural or synthetic amino acid; a natural or synthetic peptide or protein; or an ion-complexing moiety. Preferred peptides include, but are not limited to protease substrates, protein kinase substrates, phosphatase substrates, neuropeptides, cytokines, and toxins. Preferred protein conjugates include enzymes, antibodies, lectins, glycoproteins, histones, albumins, lipoproteins, avidin, streptavidins, protein A, protein G, casein, phycobiliproteins, other fluorescent proteins, hormones, toxins, growth factors, and the like.

**[0105]** The point of attachment of the biomolecule to the dichromic compound can and will vary depending upon the embodiment. In certain embodiments, the point of attachment may be at position $R^1$ of any of the compounds described herein. In another embodiment, the point of attachment may be at position $R^2$ of any of the compounds described herein. In yet another embodiment, the point of attachment may be at position $R^{19}$ of any of the compounds described herein. In yet another embodiment, the point of attachment may be at position $R^{20}$ of any of the compounds described herein. In yet another embodiment, the point of attachment may be at position $R^{26}$ of any of the compounds described herein. It is also envisioned that more than one biomolecule may be conjugated to the dichromic compounds. For example, two, three, or more than three biomolecules may be conjugated to the dichromic compound.

**[0106]** Several methods of linking dyes to various types of biomolecules are well known in the art. For example, methods for conjugating dyes to a biomolecule are described in R. Haughland, *The Handbook: A Guide to Fluorescent Probes and Labeling Technologies,* 9th Ed., 2002, Molecular Probes, Inc. and the references cited therein; and Brindley, 1992, *Bioconjugate Chem.* 3:2, which are all incorporated herein by reference. By way of example, a dichromic compound may be covalently attached to DNA or RNA via one or more purine or pyrimidine bases through an amide, ester, ether, or thioether bond; or may be attached to the phosphate or carbohydrate by a bond that is an ester, thioester, amide, ether, or thioether. Alternatively, a dichromic compound may be bound to the nucleic acid by chemical post-modification, such as with platinum reagents, or using a photoactivatable molecule such as a conjugated psoralen.

(III) Compositions Comprising Dichromic Compounds

**[0107]** A further aspect of the invention encompasses compositions comprising the dichromic compounds or conjugates thereof. The dichromic compounds may be in the form of free bases or pharmaceutically acceptable acid addition salts. The term "pharmaceutically-acceptable salts" refers to salts commonly used to form alkali metal salts and to form addition salts of free acids or free bases. The nature of the salt may vary, provided that it is pharmaceutically acceptable. Suitable pharmaceutically acceptable acid addition salts of the compounds may be prepared from an inorganic acid or from an organic acid. Examples of such inorganic acids include hydrochloric, hydrobromic, hydroiodic, nitric, carbonic, sulfuric, and phosphoric acid. Appropriate organic acids may be selected from aliphatic, cycloaliphatic, aromatic, araliphatic, heterocyclic, carboxylic, and sulfonic classes of organic acids, examples of which are formic, acetic, propionic, succinic, glycolic, gluconic, lactic, malic, tartaric, citric, ascorbic, glucuronic, maleic, fumaric, pyruvic, aspartic, glutamic, benzoic, anthranilic, mesylic, 4-hydroxybenzoic, phenylacetic, mandelic, embonic (pamoic), methanesulfonic, ethanesulfonic, benzenesulfonic, pantothenic, 2-hydroxyethanesulfonic, toluenesulfonic, sulfanilic, cyclohexylaminosulfonic, stearic, algenic, hydroxybutyric, salicylic, galactaric, and galacturonic acid. Suitable pharmaceutically-acceptable base addition salts of the compounds include metallic salts made from aluminum, calcium, lithium, magnesium, potassium, sodium and zinc or organic salts made from N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, meglumine-(N-methylglucamine), and procaine.

**[0108]** The composition comprising the dichromic compound may be administered to subject by a number of different means that will deliver an effective dose of the compound for either detection or treatment purposes. Such compositions may be administered orally, parenterally, by inhalation spray, rectally, intradermally, transdermally, or topically in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants, and vehicles as desired. Topical administration may also involve the use of transdermal administration such as transdermal patches or iontophoresis devices. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, or intrasternal injection, or infusion techniques. Formulation of pharmacologically active agents is discussed in, for example, Hoover, John E., Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa. (1975), and Liberman, H.

A. and Lachman, L., Eds., Pharmaceutical Dosage Forms, Marcel Decker, New York, N.Y. (1980).

**[0109]** Injectable preparations, e.g., sterile injectable aqueous or oleaginous suspensions, may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils may be employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may be useful in the preparation of injectables. Furthermore, dimethyl acetamide, surfactants including ionic and non-ionic detergents, and polyethylene glycols may be used. Mixtures of solvents and wetting agents such as those listed above also may be used.

**[0110]** Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the compound is ordinarily combined with one or more adjuvants appropriate to the indicated route of administration. If administered per os, the compound may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, cellulose alkyl esters, talc, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, gelatin, acacia gum, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and then tableted or encapsulated for convenient administration. Such capsules or tablets may contain a controlled-release formulation and as such may be provided in a dispersion of active compound in hydroxypropylmethyl cellulose. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents such as sodium citrate, or magnesium or calcium carbonate or bicarbonate. Tablets and pills may additionally be prepared with enteric coatings.

**[0111]** Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

**[0112]** Formulations for parenteral administration may be in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. These solutions and suspensions may be prepared from sterile powders or granules having one or more of the carriers or diluents mentioned above for use in the formulations for oral administration. The compounds may be dissolved in water, polyethylene glycol, propylene glycol, ethanol, corn oil, cottonseed oil, peanut oil, sesame oil, benzyl alcohol, sodium chloride, and/or various buffers. Other adjuvants and modes of administration are well and widely known in the pharmaceutical art.

(IV) Processes for the Production of Dichromic Compounds

**[0113]** The dichromic compounds of the invention may be made in accordance with methods generally known in the art. Non-limiting examples of suitable synthetic techniques are provided in the Examples herein.

**[0114]** In general, the dichromic compound comprising A-L-B, wherein A, B, and L are defined in section (I), is prepared by conjugating $R^1$ and $R^2$, which are structurally distinct groups, to the compound comprising structurally symmetrical A and B moieties in a manner that yields asymmetry such that the compound has two distinct fluorescence lifetimes at two different emissions wavelengths; or conjugating $R^1$ and $R^2$ to the compound comprising structurally asymmetrical A and B moieties in a manner that yields symmetry such that the compound has two distinct fluorescence lifetimes at two different emissions wavelengths.

(V) Uses of the Dichromic Compounds

**[0115]** The dichromic compounds of the invention are useful in many applications including those described for other fluorescent compounds in U.S. Pat. Nos. 7,172,907; 5,268,486; and U.S. Patent Publication Nos. 2004/0014981; and 2007/0042398, each of which is incorporated herein by reference. For example, fluorescent dyes may be used in imaging with techniques such as those based on fluorescence detection, including but not limited to fluorescence lifetime, anisotropy, photoinduced electron transfer, photobleaching recovery, and non-radioactive transfer. The fluorescent dichromic compounds, as such, may be utilized in all fluorescent-based imaging, microscopy, and spectroscopy techniques including variations on such. In addition, they may also be used for photodynamic therapy and in multimodal imaging. Exemplary fluorescence detection techniques include those that involve detecting fluorescence generated within a system. Such techniques include, but are not limited to, fluorescence microscopy, confocal microscopy, multiphoton microscropy, total internal reflection fluorescence microscopy (TIRF), fluorescence activated cell sorting (FACS), fluorescent flow cytometry, fluorescence correlation spectroscopy (FCS), fluorescence in situ hybridization (FISH), multiphoton imaging, diffuse optical tomography, molecular imaging in cells and tissue, fluorescence imaging with one nanometer accuracy (FIONA), free radical initiated peptide sequencing (FRIPs), and second harmonic retinal imaging of membrane potential (SHRIMP), as well as other methods known in the art.

**[0116]** Alternatively, the fluorescent dichromic compounds may be used as markers or tags to track dynamic behavior in living cells. In this regard, fluorescence recovery after photobleaching (FRAP) may be employed in combination with the fluorescent dichromic compounds of the invention to selectively destroy fluorescent molecules within a region of interest with a high-intensity laser, followed by monitoring the recovery of new fluorescent molecules into the bleached area over a period of time with low-intensity laser light. Variants of FRAP include, but are not limited to, polarizing FRAP (pFRAP), fluorescence loss in photo-bleaching (FLIP), fluorescence localization after photobleaching (FLAP). The resulting information from FRAP and variants of FRAP can be used to determine kinetic properties, including the diffusion coefficient, mobile fraction, and transport rate of the fluorescently labeled molecules. Methods for such photo-bleaching based techniques are described in Braeckmans, K. et al., Biophysical Journal 85: 2240-2252, 2003; Braga, J. et al., Molecular Biology of the Cell 15: 4749-4760, 2004; Haraguchi, T., Cell Structure and Function 27: 333-334, 2002; Gordon, G. W. et al., Biophysical Journal 68: 766-778, 1995, which are all incorporated herein by reference in their entirety.

**[0117]** In preferred embodiments, the dichromic compounds of the invention may be used to monitor biological events. In exemplary embodiments, the dichromic com-

pounds may be used to monitor molecular interactions or detect single molecules in cells. In general, the methods comprise administering a dichromic compound to a subject and detecting whether the structural symmetry of the compound changes, thereby indicating the biological event. The detection and imaging of biological or molecular interactions may be conducted in vitro or in vivo. Changes in the structural symmetry of the compound may be detected as detailed below.

**[0118]** The dichromic compounds generally have two peaks of emission that preferably differ by about 100 nm. Symmetrical dichromic compounds comprising benzoindole groups, which have essentially equivalent groups attached to the benzoindole N-heteroatoms, generally emit more light at the longer wavelength than the shorter wavelength. In contrast, nonsymmetrical fluorescent dichromic compounds comprising benzoindole groups, which have non-equivalent groups attached to the benzoindole N-heteroatoms, generally emit more light at the shorter wavelength that at the longer wavelength. Changes to the structural symmetry of a dye, i.e., changing a more symmetrical dye molecule into a less symmetrical dye molecule or vice versa, generally will alter the ratio of the dual emission fluorescence. Alterations in the ratio of fluorescence, therefore, provide a means to quantify the fluorescence signal by ratiometic imaging. Furthermore, the two emission peaks have distinct fluorescence lifetimes, providing another means to quantify the fluorescent signal.

**[0119]** In one embodiment, the structural symmetry of a dichromic compound may be altered by the activity of an enzyme. For example, if a kinase transfers a phosphate group to one of the $R^1$ or $R^2$ end-groups, then a symmetrical dichromic compound molecule may be converted into a nonsymmetrical dye molecule (see Example 7). Examples of enzymes whose activity may be monitored via changes to the structural symmetry of a dichromic compound molecule include kinases, phosphatases, hydrolases, capsases (e.g., see Example 8), proteases, nucleases, polymerases, ligases, transferases, synthetases, lipases, and so forth. Those of skill in the art will be familiar with appropriate end-groups to be attached to the N-heteroatoms of the dichromic compound molecules. Non-limiting examples of suitable end-groups include hydrocarbyl groups, alkyl groups, amino acids, peptides, nucleotides, oligonucleotides, monosaccharides, oligoscaccharides, lipids, small molecule enzyme inhibitors, receptor inhibitors, metals, and chelators. The amino acids may be D or L isomers; the nucleotides may be DNA or RNA, etc.

**[0120]** In one example, the end-groups of a symmetrical dye molecule (e.g., two N-linked end-groups of a molecule comprising symmetric indole or benzoindole groups) may be linked (cyclized) by an amino acid sequence such as -Lys-Asp-Glu-Val-Asp-Lys-, wherein cleavage of the peptide bond between Asp-Lys produces a nonsymmetrical dye molecule. In another example, an initially symmetric dye may comprise a first end-group comprising L amino acids and second end-group comprising the same sequence of D amino acids, wherein cleavage of the L amino acid chain produces a nonsymmetrical dye molecule. In a further example, an initially nonsymmetrical dye molecule may comprise a first end-group comprising one amino acid and a second end-group comprising five amino acids, wherein cleavage between the $1^{st}$ and $2^{nd}$ amino acid of the second end-group creates a symmetrical dye molecule. Those skilled in the art will appreciate that many other iterations are possible without departing from the scope of the invention.

**[0121]** Thus, changes in the structural symmetry and the concomitant ratio of fluorescence may be used to examine interactions between molecules in cells. Examples of molecular interactions include protein-protein interactions, protein-nucleic acid interactions, enzyme-substrate interactions, receptor-ligand interactions, cell signaling events, host-pathogen interactions, and so forth. In one application of this embodiment, the dichromic compound may be used to monitor tumor progression or tumor responsiveness to a therapeutic treatment. The dichromic compound may be targeted to tumor cells via addition of an Arg-Gly-Asp (RGD) peptide to another reactive group of the molecule. Targeting of RGD-tagged dichromic compounds to tumor cells was detailed in US Pat. Publication No. 2009/0028788, which is herein incorporated by reference in its entirety. In another embodiment, the dichromic compounds of the invention may be used as molecule beacons to detect or image target RNA or DNA molecules. In a further embodiments, the dichromic compounds may be used to detect and quantify metals.

**[0122]** In another embodiment, the structural symmetry of a dichromic compound may be used to track assembly of cellular structures in vitro or in vivo. The cellular structure may be a cytoskeletal structure, such as those formed by actin, tubulin, or intermediate filaments. Tracking cytoskeletal assembly permits the tracking of cell movement, cell motility, protein trafficking, vesicular transport, membrane vesicle transport, mitotic spindle assembly, cytokinesis, and other such cytoskeletal-mediated process. In one embodiment, the assembly of actin may be tracked by attaching an actin-binding site (such as the cap-binding protein or a fragment thereof) to one of the benzoindole N-heteroatoms and attaching a reactive group to the other benzoindole N-heteroatom of a dichromic compound comprising benzoindole groups. Upon binding of the dye to actin, the reactive group may form strong electrostatic interactions or hydrogen bonds with actin such that structural asymmetry is generated in the dichromic compound molecule. In another embodiment, the actin-binding site may be attached to another region of the dichromic compound molecule, and the benzoindole N-heteroatoms may be linked to equivalent reactive groups, wherein structural asymmetry is generated upon actin binding.

**[0123]** Besides being useful for monitoring, detecting, imaging, and/or treating in humans, the dichromic compounds of the invention also may be used in companion animals, research animals, exotic animals, and farm animals, including mammals, rodents, avians, and the like. More preferred animals include rodents, horses, dogs, cats, sheep, and pigs.

## DEFINITIONS

**[0124]** To facilitate understanding of the invention, several terms are defined below.

**[0125]** The term "acyl," as used herein alone or as part of another group, denotes the moiety formed by removal of the hydroxyl group from the group COOH of an organic carboxylic acid, e.g., RC(O)—, wherein R is $R^1$, $R^1O$—, $R^1R^2N$—, or $R^1S$—, $R^1$ is hydrocarbyl, heterosubstituted hydrocarbyl, or heterocyclo, and $R^2$ is hydrogen, hydrocarbyl, or substituted hydrocarbyl.

**[0126]** The term "acyloxy," as used herein alone or as part of another group, denotes an acyl group as described above bonded through an oxygen linkage (—O—), e.g., RC(O)O— wherein R is as defined in connection with the term "acyl."

**[0127]** Unless otherwise indicated, the alkyl groups described herein are preferably lower alkyl containing from one to eight carbon atoms in the principal chain and up to 20 carbon atoms. They may be straight or branched chain or cyclic and include methyl, ethyl, propyl, isopropyl, butyl, hexyl and the like.

**[0128]** Unless otherwise indicated, the alkenyl groups described herein are preferably lower alkenyl containing from two to eight carbon atoms in the principal chain and up to 20 carbon atoms. They may be straight or branched chain or cyclic and include ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, hexenyl, and the like.

**[0129]** Unless otherwise indicated, the alkynyl groups described herein are preferably lower alkynyl containing from two to eight carbon atoms in the principal chain and up to 20 carbon atoms. They may be straight or branched chain and include ethynyl, propynyl, butynyl, isobutynyl, hexynyl, and the like.

**[0130]** The terms "aryl" or "ar" as used herein alone or as part of another group denote optionally substituted homocyclic aromatic groups, preferably monocyclic or bicyclic groups containing from 6 to 12 carbons in the ring portion, such as phenyl, biphenyl, naphthyl, substituted phenyl, substituted biphenyl or substituted naphthyl. Phenyl and substituted phenyl are the more preferred aryl.

**[0131]** As used herein, the term "functional group" includes a group of atoms within a molecule that is responsible for certain properties of the molecule and/or reactions in which it takes part. Non-limiting examples of functional groups include, alkyl, carboxyl, hydroxyl, amino, sulfonate, phosphate, phosphonate, thiol, alkyne, azide, halogen, and the like.

**[0132]** The terms "halogen" or "halo" as used herein alone or as part of another group refer to chlorine, bromine, fluorine, and iodine.

**[0133]** The terms "heterocyclo" or "heterocyclic" as used herein alone or as part of another group denote optionally substituted, fully saturated or unsaturated, monocyclic or bicyclic, aromatic or nonaromatic groups having at least one heteroatom in at least one ring, and preferably 5 or 6 atoms in each ring. The heterocyclo group preferably has 1 or 2 oxygen atoms, 1 or 2 sulfur atoms, and/or 1 to 4 nitrogen atoms in the ring, and may be bonded to the remainder of the molecule through a carbon or heteroatom. Exemplary heterocyclo include heteroaromatics such as furyl, thienyl, pyridyl, oxazolyl, pyrrolyl, indolyl, quinolinyl, or isoquinolinyl and the like. Exemplary substituents include one or more of the following groups: hydrocarbyl, substituted hydrocarbyl, keto, hydroxy, protected hydroxy, acyl, acyloxy, alkoxy, alkenoxy, alkynoxyl, aryloxy, halogen, amido, amino, nitro, cyano, thiol, ketals, acetals, esters and ethers.

**[0134]** The term "heteroaromatic" as used herein alone or as part of another group denote optionally substituted aromatic groups having at least one heteroatom in at least one ring, and preferably 5 or 6 atoms in each ring. The heteroaromatic group preferably has 1 or 2 oxygen atoms, 1 or 2 sulfur atoms, and/or 1 to 4 nitrogen atoms in the ring, and may be bonded to the remainder of the molecule through a carbon or heteroatom. Exemplary heteroaromatics include furyl, thienyl, pyridyl, oxazolyl, pyrrolyl, indolyl, quinolinyl, or isoquinolinyl and the like. Exemplary substituents include one or more of the following groups: hydrocarbyl, substituted hydrocarbyl, keto, hydroxy, protected hydroxy, acyl, acyloxy, alkoxy, alkenoxy, alkynoxyl, aryloxy, halogen, amido, amino, nitro, cyano, thiol, ketals, acetals, esters and ethers.

**[0135]** The terms "hydrocarbon" and "hydrocarbyl" as used herein describe organic compounds or radicals consisting exclusively of the elements carbon and hydrogen. These moieties include alkyl, alkenyl, alkynyl, and aryl moieties. These moieties also include alkyl, alkenyl, alkynyl, and aryl moieties substituted with other aliphatic or cyclic hydrocarbon groups, such as alkaryl, alkenaryl and alkynaryl. Unless otherwise indicated, these moieties preferably comprise 1 to 20 carbon atoms.

**[0136]** The "substituted hydrocarbyl" moieties described herein are hydrocarbyl moieties which are substituted with at least one atom other than carbon, including moieties in which a carbon chain atom is substituted with a hetero atom such as nitrogen, oxygen, silicon, phosphorous, boron, sulfur, or a halogen atom. These substituents include halogen, carbocycle, aryl, heterocyclo, alkoxy, alkenoxy, alkynoxyl, aryloxy, hydroxy, protected hydroxy, keto, acyl, acyloxy, nitro, amino, amido, nitro, cyano, thiol, ketals, acetals, esters and ethers.

**[0137]** The term "linking group" includes a moiety on the compound that is capable of chemically reacting with a functional group on a different material (e.g., biomolecule) to form a linkage, such as a covalent linkage. See R. Haughland, *The Handbook—A Guide to Fluorescent Probes and Labeling Technologies, 9th* Edition, Molecular probes, Inc. (1992). Typically, the linking group is an electrophile or nucleophile that can form a covalent linkage through exposure to the corresponding functional group that is a nucleophile or electrophile, respectively. Alternatively, the linking group is a photoactivatable group, and becomes chemically reactive only after illumination with light of an appropriate wavelength. Typically, the conjugation reaction between the dye bearing the linking group and the material to be conjugated with the dye results in one or more atoms of the linking group being incorporated into a new linkage attaching the dye to the conjugated material.

**[0138]** The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention. Those of skill in the art should, however, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention, therefore all matter set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

## EXAMPLES

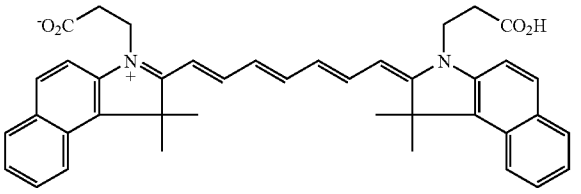
**[0139]** The following examples illustrate various embodiments of the invention.

### Example 1

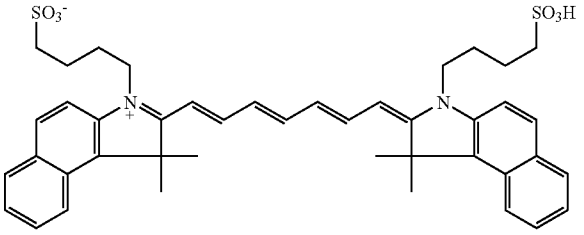
### Synthesis of NIR Dichromic Fluorescent Molecules

**[0140]** Carbodichromic compounds, such as cypate (compound 1) or indocyanine green (ICG) (compound 2), are generally safe for use in humans due to their established safety profile and known photophysical properties. The high absorption coefficient of carbocyanines in organic and aqueous solutions facilitates the enhanced detection sensitivity of optical imaging systems. Amongst several carbocyanines, heptamethine dichromic compounds are very attractive for in vivo imaging because they absorb and emit radiation in the NIR wavelengths that are suitable for imaging superficial and deep tissues. In addition, these dyes can be prepared in large quantities in pure form without HPLC purification.

1

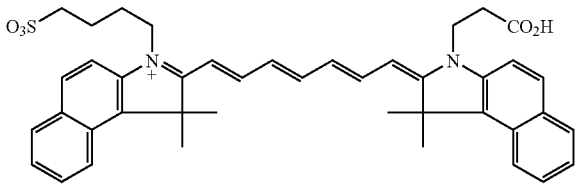


2

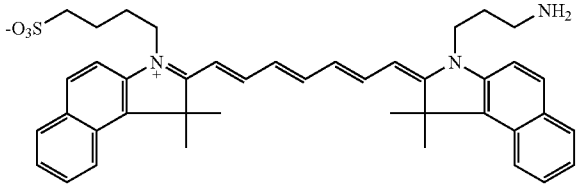


**[0141]** To improve the hydrophilicity of cypate (1) and minimize the potential side reactions caused by the presence of two carboxylic acid functions, a hybrid of 1 and 2 was synthesized. The new compound, cypate-S (compound 3), has only one reactive carboxylic acid and a water-solubilizing sulfonate group. Similarly, an analogous amine derivative, cypate-$NH_2$ or ICG-$NH_2$ (compound 4) was synthesized for labeling peptides or proteins via their activated carboxylic acid groups.

3

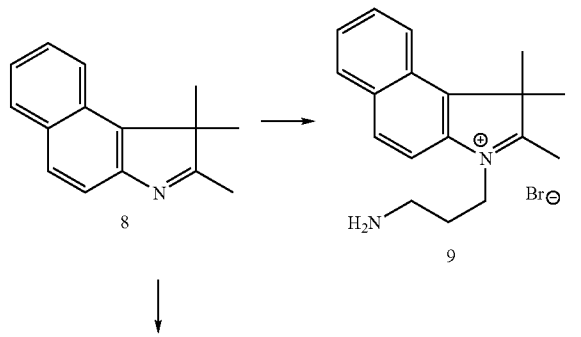


4



**[0142]** The synthesis of these nonsymmetrical compounds as represented by 4, is shown in reaction scheme 1.

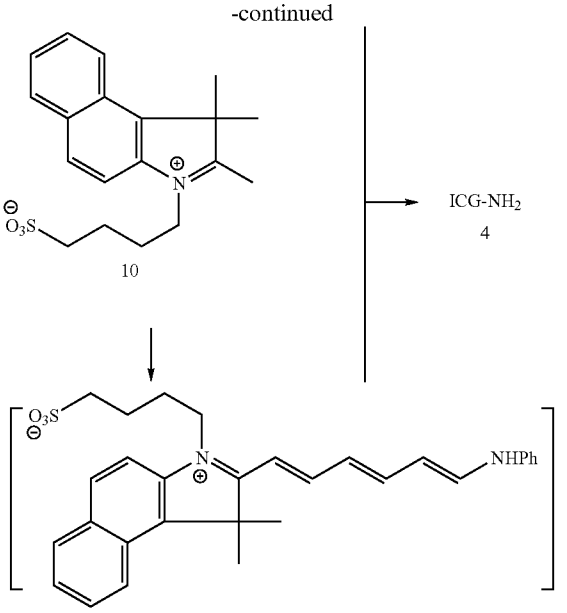
Scheme 1



8

9

-continued



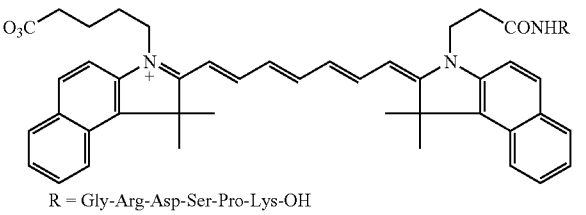
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11

**[0143]** A solution of 10 (0.35 g, 1 mmol) in MeCN was added to a refluxing mixture of glutaconic aldehyde dianiline hydrochloride (0.34 g, 1.20 mmol), $Ac_2O$ (120 μL), DIEA (600 μL) and NaOAc (78 mg) in a mixed solvent of MeCN/DCM. The resultant mixture was stirred for additional 2 h before adding excess of 9 (0.52 g, 1.50 mmol). The mixture was again refluxed for 2 h. The reaction was quenched by water and the solvent was removed by a rotary evaporator. The residue was washed sequentially with EtOAc, MeCN, EtOAc and water, and purified twice by column chromatography to afford 0.31 g (46%) of 4 compound as dark solid. $^1H$ NMR ($CD_3OD$, 500 MHz) δ 8.24-8.22 (m, 2H), 8.18 (d, J=8.5 Hz, 1H), 8.09-7.92 (m, 7H), 7.70-7.57 (m, 3H), 7.52 (t, J=7.5 Hz, 1H), (t, J=7.5 Hz, 1H), 6.67 (t, J=12.5 Hz, 1H), 6.57 (t, J=12.5 Hz, 1H), 6.46-6.38 (m, 2H), 4.32 (t, J=7.0 Hz, 2H), 4.22 (t, J=7.5 Hz, 2H), 4.22 (t, J=8.0 Hz, 2H), 2.94 (t, J=7.5 Hz, 2H), 2.18-2.13 (m, 2H), 2.10-2.04 (m, 2H), 2.02-1.98 (m, 2H), 1.96 (s, 6H), 1.95 (m, 6H). MS/EI: 675.2 ($MH_2^+$).

**[0144]** A nonsymmetrical cypate-peptide conjugate (compound 5) was also synthesized essentially as described by Achilefu et al., 2005, Proc. Natl. Acad. Sci. USA, 102, 7975, which is incorporated herein by reference in its entirety.

5



R = Gly-Arg-Asp-Ser-Pro-Lys-OH

Example 2

Steady-State Absorption and Fluorescence Properties

**[0145]** To further characterize these newly synthesized nonsymmetrical cypate dyes, their absorption and emission

spectra were evaluated and compared to those of cypate 1 and ICG 2. Cypate was synthesized according to the method of Achilefu et al., 2000, Invest. Radiol., 35, 479. ICG was purchased from a commercial source. All of the compounds, including ICG, were further purified on a Vydac C-18 column (250×4.6 mm) (Grace Davison Discovery Sciences, Deerfield, Ill.) using gradient aqueous MeCN (0.1% TFA) solvent system for multi-milligram production. Solutions of the samples in ACN/water were lyophilized to obtain about 10 mg in each case.

**[0146]** The polymethine dyes and their derivatives were dissolved in 150 mL of DMSO as stock solutions. To prevent inner effect in the fluorescent measurements, aliquots (10 mL) were diluted with the solvent of interest until the absorbance at the excitation wavelength was ≦0.15. UV-Vis spectra were obtained on Beckman Coulter DU 640 UV-Vis spectrometer (Beckman Coulter, Inc., Fullerton, Calif.). Emission spectra were recorded on a Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, N.J.).

**[0147]** Fluorescent spectra were recorded by using 620 nm and 720 nm excitation wavelengths. Fluorescent quantum yield was measured at 720 nm with slits width of 5 nm for both excitation and emission. The fluorescence quantum yield of ICG in DMSO ($\Psi$=0.12) was used as a reference standard. All measurements were conducted at room temperature. Excitation scans were performed in the range of 500-720 nm with monitoring at 735 nm fluorescence intensity, and 600-815 nm with monitoring at 830 nm. Excitation scans were corrected by lamp intensity using the equation F=S/R.

**[0148]** The absorption and emission spectra of the compounds are shown in FIGS. **1**A and **1**B, respectively. In addition to possessing the expected long wavelength fluorescence, the nonsymmetrical compounds 3 and 4 also exhibited a blue shifted second emission (700 nm) relative to the ~800 nm peak. Initially, the second peak was attributed to the presence of impurities or the half-dye. However, after careful purification and sample analysis, it was confirmed that both emissions originated from the same compound.

**[0149]** There was an excellent overlap of the absorbance spectra of the symmetrical (1 and 2) and nonsymmetrical (3, 4, and 5) compounds, with 3 and 4 exhibiting higher absorbance than other compounds at ~700 nm (FIG. **1**A). This trend persisted in different solvents, suggesting the peak was an inherent feature of the nonsymmetrical dyes and did not originate from H-aggregates (see solvent and pH effects below). In contrast to the absorption spectra, the emission spectral profiles of the symmetrical and nonsymmetrical compounds differed (FIG. **1**B). For example, the fluorescence of 3 at ~700 nm was much higher than the fluorescence at ~800 nm relative to 1 and 2 upon excitation at 645 nm. Fluorescence excitation scans of compound 3 at the two emission peaks (695 nm and 806 nm) resulted in calculated $S_0$-$S_0$ transitions corresponding to 686 nm and 796 nm, respectively (FIGS. **2**A and **2**B, respectively), suggesting the existence of inter-convertible isomers in the ground state of the molecules.

**[0150]** By combining the information from the fluorescence excitation spectra obtained at both wavelengths, the broad absorbance spectrum was deconvoluted into two distinct absorbance spectra of compound 3 (FIG. **3**). The result shows that each absorbance spectrum was characterized by a major peak and a shoulder. The striking similarity between the individual absorbance spectra indicate similar sets of $\pi \rightarrow \pi^*$ and other transitions.

## Example 3

### Lifetime of the Two Fluorescence Peaks

**[0151]** The 100 nm shift in the two emission spectra suggests that the excited state properties of the two fluorescence peaks may be different. Fluorescence lifetime (FLT) was measured using time correlated single photon counting (TCSPC) technique with excitation sources NanoLed® (HORIBA Jobin Yvon Inc.) at 700 nm and 773 nm and impulse repetition rate of 1 MHz at 900 to a R928P detector (Hamamatsu Photonics, Japan). The detector was set to either 750 nm (for Nanoled 700 nm) or for 820 nm (for Nanoled 773 nm) with a 20 nm bandpass. The electrical signal was amplified by TB-02 pulse amplifier (HORIBA Jobin Yvon Inc.) and the amplified signal was fed to the constant fraction discriminator CFD (Philips, Netherlands). The first detected photon represented the start signal by the time-to-amplitude converter (TAC) and the excitation pulse triggered the stop signal. The multi-channel analyzer (MCA) recorded repetitive start-stop signals from the TAC and generated a histogram of photons as a function of time-calibrated channels (6.878 ps/channel) until the peak signal reached 10,000 counts. The lifetime was recorded on a 50 ns scale. The instrument response function was obtained by using Rayleigh scatter of Ludox-40 (Sigma-Aldrich, St. Louis, Mo.) (0.03% in Milli-Q water) in a quartz cuvette at either 700 nm or 773 nm emission. DAS6 v6.1 decay analysis software (HORIBA Jobin Yvon Inc.) was used for lifetime calculations. The goodness of fit was judged by $X^2$ values and Durbin-Watson parameters and visual observations of fitted line, residuals and autocorrelation function

**[0152]** The fluorescence lifetime properties of compounds 1, 2, 3, and 4 are presented in Table 1. These results reveal that the fluorescence lifetime at 700 nm is >1.5 times longer than that at 800 nm.

TABLE 1

Dynamic optical properties of representative cyanine molecules in DMSO.

| Compound | Lifetime at 700 nm (ns)[a] | Fractional contribution (f %) | Lifetime at 800 nm (ns)[b] |
|---|---|---|---|
| 1 | Weak signal | 99 | 0.87[c] |
| 2 | 1.50[d] | 99 | 0.97[c] |
| 3 | 1.39 | 92 | 0.86 |
| 4 | 1.40 | 90 | 0.90 |

[a]Excitation 650 nm; emission 700 nm;
[b]Excitation 773 nm; emission 820 nm;
[c]See Berezin et al., 2007, Biophys. J. 93(8): 2892-2899;
[d]Average two exponential 1.26 ns (39%) and 1.59 ns (60%)

**[0153]** Although the 100 nm blue-shifted emission peak could indicate the elimination of a methine bond from the heptamethine structure, it is unlikely that the 700 nm emission resulted from the formation of lower homologues (tri- or penta-methines) of cypate or ICG, which typically have similar fluorescence lifetimes of cypate at 800 nm (0.86 ns in DMSO). Additionally, the steady state spectral properties of the half-dyes (a potential contaminant) used to prepare 3 and 4 is 35 nm shorter than the 700 nm peak, with a corresponding fluorescence lifetime of only 0.39 ns.

## Example 4

### Effect of Solvent and pH on Steady-State Fluorescence Properties

**[0154]** The ratios of the 700 nm and 800 nm emission peaks were examined in different environments and simulated dilute conditions (e.g., binding to albumin). For this, the fluorescence behavior of compound 3 was determined in different solvents (i.e., methanol, water, and DMSO), pH levels (i.e., 2.5 and 10), and in the presence of albumin (which binds

the dyes in its drug binding pocket). The spectral analysis was conducted as described above in Example 2. Spectra at pH 2.5 and 10.0 were obtained by titrating 20% DMSO/water solution with diluted aqueous HCl and NaOH.

**[0155]** As shown in FIG. **4**, the ratio of the fluorescence at 700 nm and 800 nm was only weakly dependent on solvent system or pH, and albumin does not significantly alter this ratio. Similarly, the absorption and fluorescence spectral profiles of the blue-shifted peaks were not affected by temperatures between 20° C. and 80° C., further confirming that aggregation was not responsible for the observed increase in the 700 nm absorption.

## Example 5

### Physical Characterization of the Nonsymmetrical Dichromic Compounds

**[0156]** The nonsymmetrical nature of these compounds was further examined by 2D NMR analysis, using 4 as a representative compound (see FIG. **5**). This analysis was made on a Varian Inova-600 spectrometer (Varian, Inc., Palo Alto, Calif.). COSY experiments were obtained with a 5200 Hz spectral width and an 8.5 μs π/2 pulse width. A data matrix with 2048 complex point in F2 and 512 real points in F1 dimension was collected with 16 transients per t1 increment. TOCSY spectra were recorded using an MELV-17 mixing sequence of 100 ms flanked by two 2 ms trim pulses. Phase-sensitive 2D spectra were obtained by employing the hypercomplex method. A total of 2×256×2048 data matrix with 16 scans per t1 increment were collected. Gaussian and sine-bell apodization functions were used in weighting the t2 and t1 dimensions, respectively. After two-dimensional Fourier transformation, the 2048×2048 frequency domain representation was phase and baseline corrected in both dimensions.

**[0157]** Resonances of the indole N-substituents ($H_2^1$-$H_2^3$ and $H_3^1$-$H_3^4$) and polymethine bridge ($H_1^1$-$H_1^7$) were identified by the through bond spin propagation at the fragment in TOCSY experiment. The correlated proton resonances at 4.20 and 4.26 ppm were unambiguously assigned to $H_2^1$ and $H_3^1$, respectively. Proton connectivities were confirmed by the vicinal J-coupling in COSY experiment. All the connections between adjacent protons were observed and a continuous path indicated the segment of $H_2^1$ and $H_3^1$, at the vinyl bridge as well as $H_2^1$-$H_2^3$, $H_3^1$-$H_3^4$ methylene chain in indole N-substituents. Assignments of $H_1^1$ resonance at 6.64 ppm and $H_1^7$ at 6.32 ppm were further confirmed by the observed $H_1^1$-$H_3^1$ and $H_1^7$-$H_2^1$ NOE cross peaks. In addition, the presence of the $H_1^6$-$H_4^2$ and $H_1^2$-$H_4^1$ NOE clearly indicated a 7.9 ppm and 8.01 ppm for the $H_1^6$ and $H_1^2$ resonances respectively, which are consistent with the assignment in COSY spectra.

**[0158]** In previous studies, the two gem-dimethyl groups in symmetrical cypate showed a singlet upfield (Ye et al., 2000, Photobiol., 72, 392). However, the present analysis revealed that the corresponding peak for the nonsymmetrical compound 4 was split into two singlets. In addition, the vinyl protons at δ 6-7 ppm, which correspond to $H_1^1$, $H_1^3$, $H_1^5$ and $H_1^7$, are doublets in cypate but are distorted in 4. The ensemble of these results suggest that one of the structures responsible for the 700 nm emission consists of a preferentially localized positive charge on one of the indole N atoms at ground state, which could further be stabilized by the neighboring sulfonate anion. It is therefore likely that the long wavelength emission arises from the conventional π-π* electronic transition when the charge is transiently even distributed. These two structural dispositions could be associated with different molecular orbital diagram, resulting in different HOMO and LUMO levels.

## Example 6

### Detecting Molecular Interactions with NIR Dichromic Fluorescent Probes

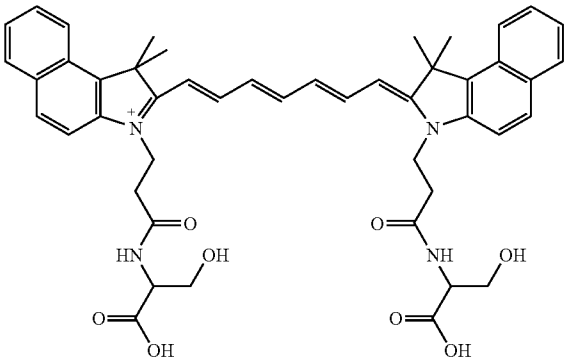
**[0159]** Both the symmetrical and nonsymmetrical dichromic compounds exhibited dual fluorescence at about 800 nm and 700 nm. The symmetrical dyes 1 and 2 have relatively small contributions of the 700 nm emission in the overall fluorescence spectra (18% and 24% respectively). The weak signal may account for the omission of the blue-shifted peaks in previous reports of the fluorescence properties of these compounds. Upon conjugation of the symmetrical dye cypate with a peptide (see Example 1), the resulting nonsymmetrical cypate-peptide conjugate 5 exhibited a 45% increase in the level of the secondary emission relative to cypate, albeit still lower than the 82% obtained with compounds 3 and 4. This result indicates that labeling peptide or protein with some of the NIR probes could induce structural asymmetry that results in a significant and detectable spectral change. A similar transformation could also be induced through electrostatic interactions to produce seemingly symmetrical dye molecules that are dislodged upon binding to the target.

## Example 7

### Generation of a Nonsymmetrical Cyanine Probe by Phosphorylation

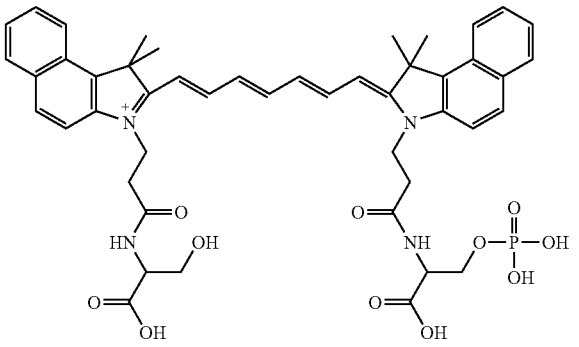
**[0160]** To demonstrate the potential of monitoring the activities of kinases involved in physiological process, the symmetrical di-serine cypate (6) and the nonsymmetrical monophosphoserine/serine cypate derivative (7) were prepared. The compounds were dissolved in methanol and the molar absorption coefficients for compounds 6 and 7 were determined to be about 220,000 $M^{-1}$ $cm^{-1}$ for both molecules, with fluorescence quantum yields of 0.059 (for 6) and 0.057 (for 7) relative to ICG in methanol (0.090).

6



-continued

7



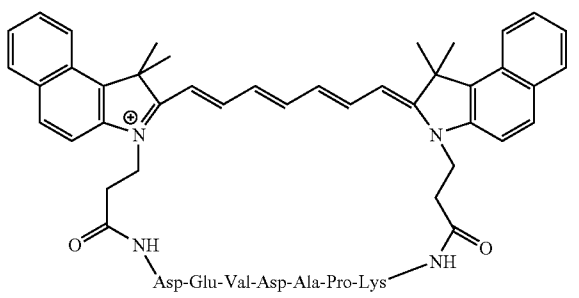
**[0161]** As shown in FIG. **6**, the ratio of the 700/800 nm emission peaks of the nonsymmetrical, phosphorylated compound 7 was increased relative to that of the symmetrical compound 6 (i.e., from 0.36 to 2.5, a 7-fold increase). Such a huge increase should allow the detection of kinase activity with high sensitivity, which is not available with conventional NIR fluorescent probes.

Example 8

Monitoring Caspase-3 Activity with Dichromic Fluorescent Probes

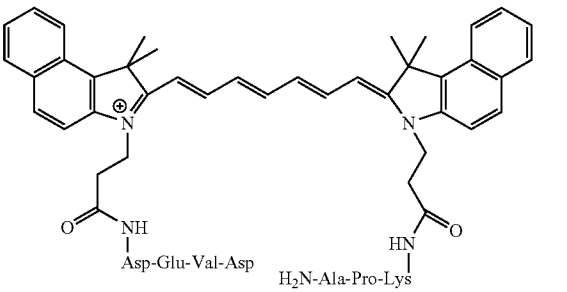
**[0162]** Another application of interest is monitoring the response of tumors to treatment by detecting the onset of apoptosis through the activity of caspase-3. To test the response of the dichromic fluorescent dyes to caspase-3 activity, a macrocyclic NIR dichromic compound (12) was prepared by linking the two carboxylic acid groups of cypate with a caspase-3 peptide substrate, $NH_2$-Asp-Glu-Val-Asp-Ala-Pro-Lys-COOH. The enzymatic hydrolysis by caspase-3 occurs at the C-terminal of aspartic acid producing a nonsymmetrical pendant molecule (13).

12



-continued

13



**[0163]** Hydrolysis of the more symmetrical compound 12 by recombinant caspase-3 (CalBiochem, San Diego, Calif.) after 16 hours of incubation at 37° C. to the less symmetrical compound 13 induced structural asymmetry that was monitored by fluorescence spectroscopy (FIG. **7**). This enzymatic reaction led to more than 300% increase in the 700 nm emission channel and only 45% increase in the 800 nm channel using 620 nm excitation (to obtain full emission spectra). The enhancement was independent of the excitation wavelength, in accordance with Kasha-Vavilov rule, which states that quantum yield and a position of fluorescence is independent of the excitation wavelength (Birks, 1970, Photophysics of Aromatic Molecules, Wiley, New York, N.Y.). Parallel measurements of 800 nm emission using 720 nm excitation showed 35% intensity increase. Under the same conditions, the control experiment (with no enzyme added), showed a small increase in the 700/800 nm ratio, which is attributable to a slow nonspecific hydrolysis. Thus, the observed increase in the emission ratio of 700/800 nm upon enzymatic hydrolysis confirmed that the change in fluorescence pattern was caused by the change in structural symmetry. However, moderate increase in the 700/800 nm ratio of compound 13 (2-fold increase) relative to the increase of cypate-monoserine phosphate compound 7 (7-fold increase) demonstrates the need to optimize the macrocyclic structure. These preliminary data, however, reveal that substrates with symmetrical structures as determined by the low 700/800 nm emission ratio are necessary to generate highly sensitive assays for monitoring molecular processes.

Example 9

Enzyme Kinetics of NIR Caspase-3 Probe

**[0164]** To examine the kinetics, a caspase-3 substrate was prepared using the standard FRET method where the tetrapeptide Asp-Glu-Val-Asp (DEVD) was flanked by two cypate dyes (cypate-DEVD probe). Enzyme assays were performed with a fluorometer by using commercially available recombinant caspase-3 (CalBiochem). An aliquot of the cypate-DEVD probe (substrate concentrations ranged from 70 nM to 100 μM) was dissolved in DMSO and added to a buffer comprising 10 mM PIPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT. Five units of the enzyme were added, and the assay was performed at 37° C. A control experiment under the same conditions but with no enzyme was conducted at the same time. The apparent $K_m$ for the cypate-DEVD probe for caspase-3 was calculated with the Cheng-Prusoff equation: $EC_{50}=K_i(1(+[S]/K_m)$, where [S] is the concentration of substrate and $K_i$ is the published inhibi-

tory constant of DEVD-CHO. To generate $EC_{50}$ values, initial rates were determined from the linear portion of substrate hydrolysis and were plotted against log-inhibitor concentration (DEVD-CHO). The apparent $k_{cat}$ values were calculated using the equation $k_{cat}=V(K_m+[S])/[E_t][S]$, where [S] is the concentration of substrate, [E] is the concentration of enzyme, and V is the initial rate of cleavage. To express V in units of micromolar per second, initial velocities were determined from plots of fluorescence intensity increase versus time (seconds), using only the linear portion of the curve (<40% of full hydrolysis). The slope was divided by the change in fluorescence intensity corresponding to complete hydrolysis (corrected for background fluorescence in the absence of the enzyme) and then multiplied by the substrate concentration to obtain initial velocity V (μM/s). $E_t$ was determined with known substrate (Ac-DEVD-AMC, BD Biosciences, Franklin Lakes, N.J.), wherein one unit of capsase activity equals 1 μmol substrate cleavage per minute per μg enzyme.

**[0165]** Cleavage of the cypate-DEVD probe by caspase-3 produced an increase in fluorescence intensity. A non-linear fit of the data produced values of 15±3 μM and 1.02±0.06 M $s^{-1}$ for μM and $k_{cat}$, respectively (FIG. **8**). These values compare favorably with those of the standard, Ac-DEVD-pNA ($K_M$=11 μM and $k_{cat}$=2.4 M $s^{-1}$).

## Example 10

### In Vivo Imaging of Capsase-3 Activity

**[0166]** To assess the NIR optical imaging of caspase-3 activity, 2 nmol of the pre-quenched (FRET) cypate-DEVD probe was injected intravenously in a nude mouse, followed by intradermal injection of caspase-3 solution (right pinna) or saline (left pinna). This approach provides positive and negative controls in one set of experiments. Mouse ears were imaged with Li-Cor Odyssey single-channel (805 nm) NIR imaging system (Li-Cor Biosciences, Lincoln, Nebr.). Images were captured before treatment, after injection of a probe and after intradermal injection of caspase-3 in the ear.

**[0167]** The results are shown in FIG. **9**. Measured fluorescence intensity increased in both ears at the sites of injection of saline or caspase-3 solution. The magnitude and area of increase, however, was significantly greater in the caspase-3-injected ear and increased with time relative to the saline-injected ear.

## Example 11

### Detecting the State of Phosphorylation in Real Time

**[0168]** To determine whether phosphorylation could be detected in real time, cells were incubated with cypate (1) or cypate diserine (6) and changes in the ratio of 700/800 nm were detected. Cypate diserine has two serine groups that can be phosphorylated by serine kinases, whereas, the structurally symmetrical cypate does not respond to phosphorylation.

**[0169]** The human non-small cell carcinoma cell line A549 was purchased from American Type Culture Collection (Manassas, Va.). A549 cells were maintained in Ham's F12K medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10% fetal calf serum, 100 units/mL penicillin, and 100 units/mL streptomycin.

**[0170]** Cells were grown on LAB-TEK™ slides (Nalgene Nunc International, Rochester, N.Y.). The medium was replaced and cells were incubated for 30 min, 1 h, or 6 h at 37° C. in the presence of 1 μM cypate (1) or cypate diserine (6). At the end of the incubation, the slides were rinsed with PBS, mounted with Prolong Gold mounting medium (Invitrogen Inc, Carlsbad, Calif.) and coverslipped. For confocal microscopy, cells were visualized with an Olympus FV1000 microscope using a 60×/1.20M, 0.13-0.21 NA water immersion objective. Fluorescence emission was detected from 645-745 nm and from 805-830 nm using a 633 nm laser and a 780 nm laser for excitation, respectively. For spectral imaging, fluorescence images were collected at 10 nm increments from 645-790 nm using a 633 nm laser and images of each group of slides were acquired with the same microscope settings during a single imaging session, allowing for qualitative comparison of the relative fluorescence. Relative fluorescence was quantitated with FV1000 software. Relative fluorescence of five areas of each image was determined and the results were averaged.

**[0171]** The results are presented in FIG. **10**. After 1 h, the ratio of the 700 to 800 emission was about 0.4:1 for cypate diserine (6), suggesting the phosphorylation of at least one of the serine groups. At 6 h, the ratio increased significantly to 3.5:1, suggesting the formation of di- or tri-phosphates on the serines. These results reveal that molecular processes may be monitored in real time using dichromic fluorescent dyes.

## Example 12

### Phosphorylation by Casein Kinase II

**[0172]** The following example was designed to further explore the use of cypate diserine (6) to monitor the activity of endogenous kinases. A549 cells (which express casein kinase II) were incubated with a standard casein kinase II peptide substrate or compound 6 (which is not specific for casein kinase II). Kinase activity was measured with the Kinase-Glo Luminescent Kinase Assay Platform (Promega Corp., Madison, Wis.) per the manufacturer's instructions. This assay measures free ATP in the reaction mixture. As phosphorylation increases, the levels of ATP decrease, and consequently, luminescence is inversely proportional to kinase activity.

**[0173]** The concentration-dependent decreases in luminescence (i.e., increased phosphorylation) for the casein kinase II peptide substrate and compound 6 are presented in FIGS. **11**A and **11**B, respectively. Fluorescence spectra of the cells incubated with compound 6 for 10 min showed a similar spectral change from compound 6 to the phosphorylated derivative as depicted in FIG. **6**B, suggesting substrate phosphorylation and the expected change in the structural symmetry of the dye.

**[0174]** It was hypothesized that since cypate diserine could be phosphorylated in cells, it is possible that the initial phosphorylation of one serine (as indicated by an increase in the 700 nm channel) could be followed by phosphorylation of the second serine. Phosphorylation of both serine would afford a more symmetrical molecule that would exhibit a corresponding increase in the 800 nm channel. To test this notion, A549 cells were incubated with of 1 μM of compound 6. It was found (see FIG. **12**) that the cells exhibited an early fluorescence increase in the 700 nm emission channel (i.e., 5 and 15 minutes) probably due to monophosphorylation, followed by a later (i.e., 30 and 45 minutes) fluorescence enhancement in both channels, which could correspond to diphosphorylation of the compound.

Example 13

In Vivo Dichromic Fluorescence Imaging

**[0175]** To assess the retention of dichromic fluorescence in vivo, 2 nmol of symmetrical ICG (compound 2) and nonsymmetrical cypate-S (compound 3) were each injected intravenously in a nude mouse. Mouse ears were imaged with a Li-Cor Odyssey system at two excitation (680 nm and 780 nm) and emission (700 and 800 nm) channels. The results are shown in FIG. **13**. The blood vessels were clearly seen in both channels for the nonsymmetrical compound (3). In contrast, ICG (2) showed the blood vessels only at the 800 nm channel, with autofluorescence dominating the 700 nm channel. Thus, the dual fluorescence contrast mechanism is tenable in vivo.

What is claimed is:

**1**. A dichromic compound, the compound comprising:

A-L-B

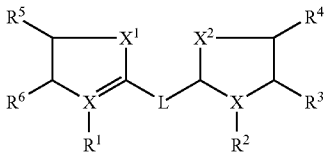
wherein:

A and B are each either structurally symmetrical moieties, or are each structurally asymmetrical moieties;

L is a linker comprising a chromophore; and

the compound further comprising $R^1$ and $R^2$, wherein $R^1$ and $R^2$ are structurally distinct groups that are each either conjugated to the compound comprising structurally symmetrical A and B moieties in a manner that yields asymmetry such that the compound has two distinct fluorescence lifetimes at two different emission wavelengths; or $R^1$ and $R^2$ are each conjugated to the compound comprising structurally asymmetrical A and B moieties in a manner that yields symmetry such that the compound has two distinct fluorescence lifetimes at two different emission wavelengths.

**2**. The dichromic compound of claim **1**, the compound comprising Formula (I):



(I)

wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

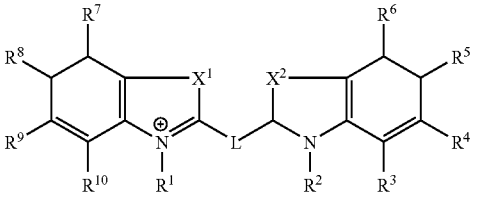
X is a heteroatom selected from the group consisting of nitrogen, phosphorus, silicon, and sulfur;

$X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

L is a linker comprising a chromophore; and

$R^3$, $R^4$, $R^5$, and $R^6$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl, and substituted hydrocarbyl; provided that $R^3$ and $R^4$ and $R^5$ and $R^6$ may form a ring or a ring system.

**3**. The dichromic compound of claim **2**, the compound comprising Formula (Ia):



(Ia)

wherein:

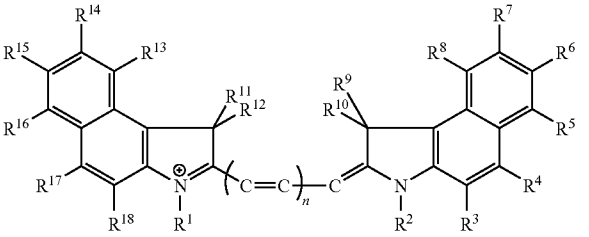
$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

L is a linker comprising a chromophore; and

$R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, and $R^{10}$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl; provided that any of $R^3$ and $R^4$, $R^4$ and $R^5$, $R^5$ and $R^6$, $R^7$ and $R^8$, $R^8$ and $R^9$, and $R^9$ and $R^{10}$ may form a ring or a ring system.

**4**. The dichromic compound of claim **3**, the compound comprising Formula (Ib):



(Ib)

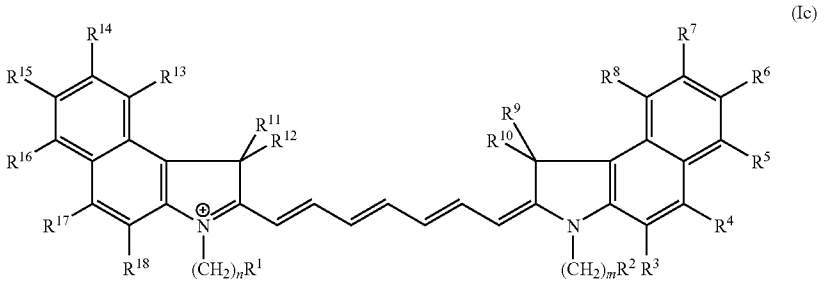
wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $R^{11}$, $R^{12}$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, and $R^{18}$ are each independently selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl; and

n is an integer from 0 to 10.

**5**. The compound of claim **4**, the compound comprising Formula (Ic):



(Ic)

wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrogen, amide, amino, carboxyl, halogen, hydroxy, phosphate, sulfonate, thiol, azido, alkyne, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $R^{11}$, $R^{12}$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, and $R^{18}$ are each independently selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl;

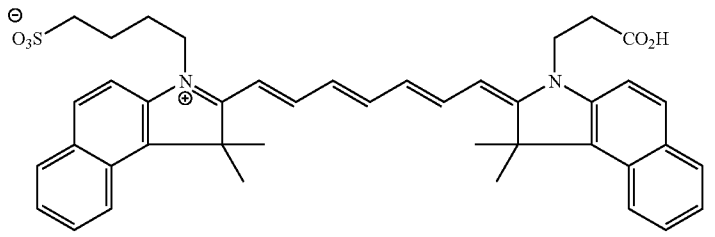
m is an integer from 1 to 10; and

n is an integer from 1 to 10.

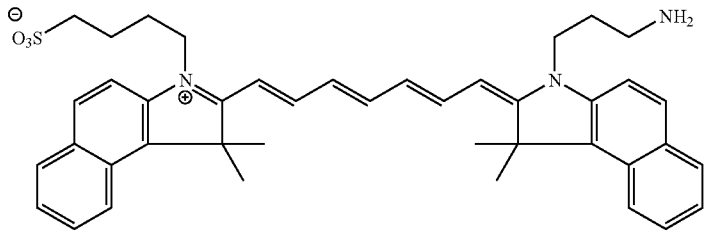
**6**. The dichromic compound of claim **5**, wherein $R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, and $R^{13}$ are each hydrogen; and $R^9$, $R^{10}$, $R^{11}$, and $R^{12}$ are each methyl.

**7**. The dichromic compound of claim **6**, wherein the compound is selected from the group consisting of A-1, A-2, A-3, and A-4:

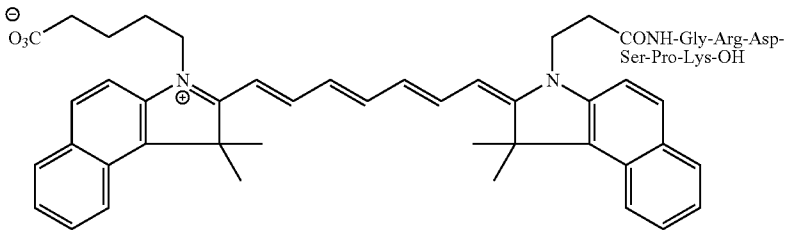
A-1



A-2

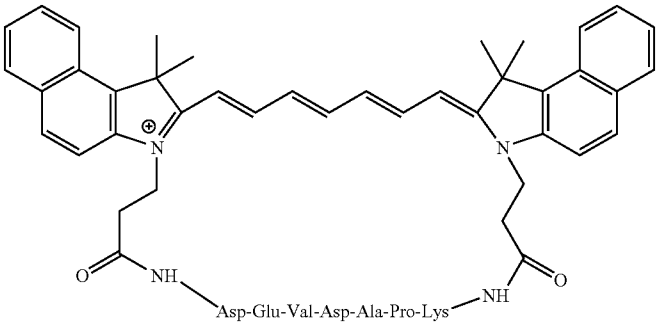


A-3



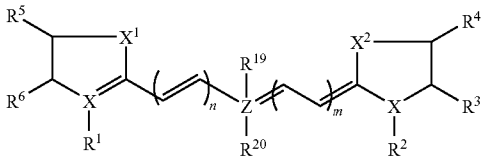
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A-4




**8**. The dichromic compound of claim **1**, the compound comprising Formula (II):

(II)



wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^3$, $R^4$, $R^5$, and $R^6$ are selected from the group consisting of halogen, hydrocarbyl and substituted hydrocarbyl; provided that $R^3$ and $R^4$ and $R^5$ and $R^6$ may form a ring or a ring system.

$R^{19}$ and $R^{20}$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence;

X is a heteroatom selected from the group consisting of nitrogen, phosphorus, silicon, and sulfur;

$X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

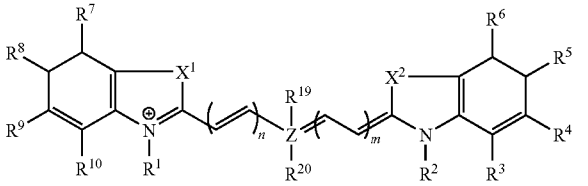
Z is a cyclic group selected from the group consisting of a carbocyclic ring and a heterocyclic ring;

m is an integer from 1 to 3; and

n is an integer from 1 to 3.

**9**. The dichromic compound of claim **8**, the compound comprising Formula (IIa):

(IIa)



wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, and $R^{10}$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl; provided that any of $R^3$ and $R^4$, $R^4$ and $R^5$, $R^5$ and $R^6$, $R^7$ and $R^8$, $R^8$ and $R^9$, and $R^9$ and $R^{10}$ may form a ring or a ring system;

$R^{19}$ and $R^{20}$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence;

$X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

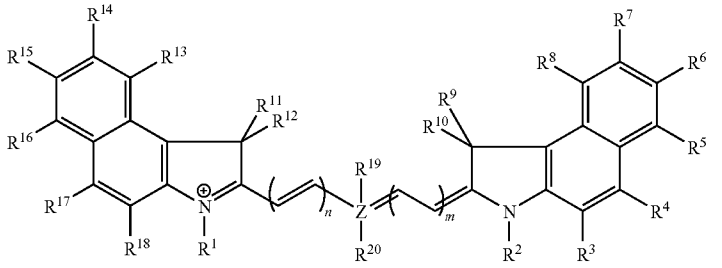
Z is a cyclic group selected from the group consisting of a carbocyclic ring and a heterocyclic ring;

n is an integer from 1 to 3; and

m is an integer from 1 to 3.

**10**. The dichromic compound of claim **9**, the compound comprising Formula (IIb):

(IIb)



wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $R^{11}$, $R^{12}$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, and $R^{18}$ are each independently selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl;

$R^{19}$ and $R^{20}$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence;

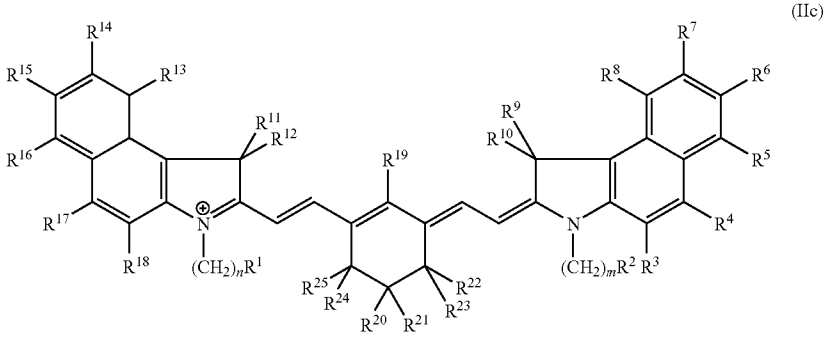
Z is a cyclic group selected from the group consisting of a carbocyclic ring and a heterocyclic ring;

m is an integer from 1 to 3; and

n is an integer from 1 to 3.

**11**. The dichromic compound of claim **10**, the compound comprising Formula (IIc):

(IIc)



wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrogen, amide, amino, carboxyl, halogen, hydroxy, phosphate, sulfonate, thiol, azido, alkyne, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^3$, $R^4$, $R^5$, $R^6$, $R^7$, $R^8$, $R^9$, $R^{10}$, $R^{11}$, $R^{12}$, $R^{13}$, $R^{14}$, $R^{15}$, $R^{16}$, $R^{17}$, $R^{18}$, $R^{21}$, $R^{22}$, $R^{23}$, $R^{24}$, and $R^{25}$ are each independently selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl;

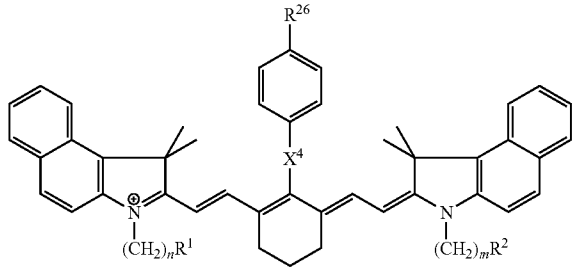
$R^{19}$ and $R^{20}$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence;

m is an integer from 1 to 10; and

n is an integer from 1 to 10.

**12**. The dichromic compound of claim **11**, the compound comprising Formula (IId):

(IId)



wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrogen, amide, amino, carboxyl, halogen, hydroxy, phosphate, sulfonate, thiol, azido, alkyne, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^{26}$ is selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence;

$X^4$ is a single bond or a heteroatom selected from the group consisting of sulfur, nitrogen, and oxygen; and

m is an integer from 1 to 10; and

n is an integer from 1 to 10.

**13**. The dichromic compound of claim **1**, wherein the two different emission wavelengths have absorption spectra from about 200 nm to about 1200 nm.

**14**. The dichromic compound of claim **1**, wherein the two different emission wavelengths are separated by about 25 nm to about 400 nm.

**15**. A method for monitoring a biological event in a subject, the method comprising administering a dichromic compound to the subject and detecting whether the structural symmetry of the compound changes, thereby indicating the biological event; the dichromic compound comprising:

A-L-B

wherein:

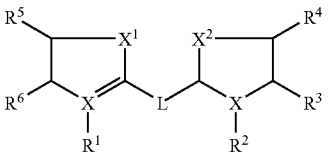
A and B are either structurally symmetrical moieties or are each structurally asymmetrical moieties;

L is a linker comprising a chromophore; and

the compound further comprising $R^1$ and $R^2$, wherein $R^1$ and $R^2$ are structurally distinct groups that are each either conjugated to the compound comprising structurally symmetrical A and B moieties in a manner that yields asymmetry such that the compound has two distinct fluorescence lifetimes at two different emission wavelengths; or $R^1$ and $R^2$ are each conjugated to the compound comprising structurally asymmetrical A and B moieties in a manner that yields symmetry such that the compound has two distinct fluorescence lifetimes at two different emission wavelengths.

**16**. The method of claim **15**, the dichromic compound comprising Formula (I):

(I)



wherein:

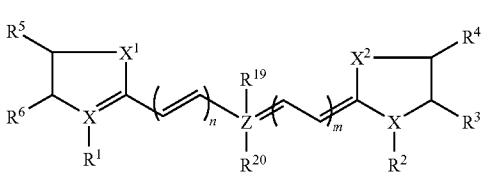
$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

X is a heteroatom selected from the group consisting of nitrogen, phosphorus, silicon, and sulfur;

$X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

$R^3$, $R^4$, $R^5$, and $R^6$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl, and substituted hydrocarbyl; provided that $R^3$ and $R^4$ and $R^5$ and $R^6$ may form a ring or a ring system.

**17**. The method of claim **15**, the dichromic compound comprising Formula (II):



(II)

wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^3$, $R^4$, $R^5$, and $R^6$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl; provided that $R^3$ and $R^4$ and $R^5$ and $R^6$ may form a ring or ring system.

$R^{19}$ and $R^{20}$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence;

X is a heteroatom selected from the group consisting of nitrogen, phosphorus, silicon, and sulfur;

$X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

Z is a cyclic group selected from the group consisting of a carbocyclic ring and a heterocyclic ring;

m is an integer from 1 to 3; and

n is an integer from 1 to 3.

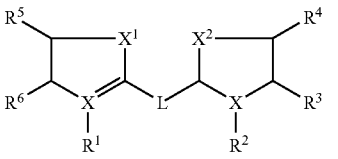
**18**. A process for producing a dichromic compound, the compound comprising:

A-L-B

wherein:

A and B are either structurally symmetrical moieties or are each structurally asymmetrical moieties; L is a linker comprising a chromophore; the compound further comprising $R^1$ and $R^2$; the process comprising conjugating $R^1$ and $R^2$, which are structurally distinct groups, to the compound comprising structurally symmetrical A and B moieties in a manner that yields asymmetry such that the compound has two distinct fluorescence lifetimes at two different emissions wavelengths; or conjugating $R^1$ and $R^2$ to the compound comprising structurally asymmetrical A and B moieties in a manner that yields symmetry such that the compound has two distinct fluorescence lifetimes at two different emissions wavelengths.

**19**. The process of claim **18**, the dichromic compound comprising Formula (I):



(I)

wherein:

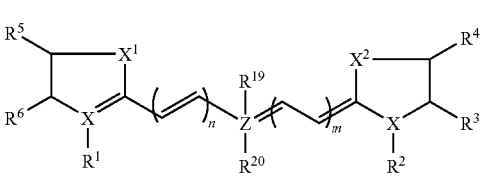
$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

X is a heteroatom selected from the group consisting of nitrogen, phosphorus, silicon, and sulfur;

$X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

$R^3$, $R^4$, $R^5$, and $R^6$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl, and substituted hydrocarbyl; provided that $R^3$ and $R^4$ and $R^5$ and $R^6$ may form a ring or ring system.

**20**. The process of claim **18**, the dichromic compound comprising Formula (II):



(II)

wherein:

$R^1$ and $R^2$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence; provided that $R^1$ and $R^2$ are different groups;

$R^3$, $R^4$, $R^5$, and $R^6$ are selected from the group consisting of hydrogen, halogen, hydrocarbyl and substituted hydrocarbyl; provided that $R^3$ and $R^4$ and $R^5$ and $R^6$ may form a ring or a ring system.

$R^{19}$ and $R^{20}$ are independently selected from the group consisting of hydrocarbyl, substituted hydrocarbyl, amino acid sequence, and nucleic acid sequence;

X is a heteroatom selected from the group consisting of nitrogen, phosphorus, silicon, and sulfur;

$X^1$ and $X^2$ are atoms independently selected from the group consisting of carbon, nitrogen, phosphorus, silicon, sulfur, and oxygen;

Z is a cyclic group selected from the group consisting of a carbocyclic ring and a heterocyclic ring;

m is an integer from 1 to 3; and

n is an integer from 1 to 3.

* * * * *