

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
13 April 2006 (13.04.2006)

PCT

(10) International Publication Number
WO 2006/037226 A1

- (51) International Patent Classification⁷: C12Q 1/04, G01N 33/52, 33/58, 21/64
- (21) International Application Number: PCT/CA2005/001527
- (22) International Filing Date: 5 October 2005 (05.10.2005)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/616,175 5 October 2004 (05.10.2004) US
- (71) Applicants (for all designated States except US):
MCGILL UNIVERSITY [CA/CA]; 845 Sherbrooke Street W., Montreal, Quebec H3A 2T5 (CA). UNIVERSITY OF LEEDS [GB/GB]; Woodhouse Lane, Leeds, Yorkshire LS2 9JY (GB).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): NADEAU, Jay, L. [CA/CA]; 604 de la Metairie, Ile des Soeurs, Quebec H3E 1T2 (CA). COHEN, Netta [GB/GB]; 14 Ingledew Crescent, Flat 2, Leeds, Yorkshire LS8 1BP (GB).
- (74) Agent: RIDOUT & MAYBEE LLP; 1 Queen Street East, Suite 2400, Toronto, Ontario M5C 3B1 (CA).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: USE OF QUANTUM DOTS FOR BIOLOGICAL LABELS AND SENSORS

(57) Abstract: A composition comprising a semiconductor nanocrystal (quantum dot) conjugated with one or more electron donors or acceptors is disclosed. Also disclosed are a method for fluorescence internal labeling a living cell or organism, and a method for detecting a metabolism abnormality or metabolically-active intracellular environment of a cell or organism, employing the composition. In addition, disclosed are a composition and an ion-, or voltage-, or ion and voltage-sensitive probe or label for a transmembrane or intramembrane process, each of which comprises an uncapped semiconductor nanocrystal embedded in a lipid membrane. A method for sensing a transmembrane or intramembrane process of a cell or organism and a method of labeling cells, employing an uncapped semiconductor nanocrystal embedded in a lipid membrane are also disclosed.



WO 2006/037226 A1

USE OF QUANTUM DOTS FOR BIOLOGICAL LABELS AND SENSORS

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/616,175 filed on October 5, 2005, the entire teachings of which are incorporated herein by reference.

BACKGROUND

The ability to label cells and cell membranes is of great importance in biological applications. For example, labeling cell membranes can provide tools for trafficking, visualizing nerve terminals, understanding membrane fusion, and other applications. Traditional methods for labeling cells rely on the use of radioactive markers or organic dyes. However, conventional radioactive detection systems use health hazardous radioactive materials, posing general safety issues. Also, inherently short half-lives with many commonly-used isotopes limit the use of radioactive detection systems. Conventional approaches with organic dyes have also drawbacks that include photobleaching, poor spectral separation, low fluorescence intensity, broad spectral line widths and emission spectra having long tails.

As alternatives to the conventional radioactive markers or organic dyes, quantum dots (QDs) have been recognized in the art. Quantum dots (QDs) are semiconductor nanocrystals with size-dependent optical and electronic properties. Generally, QDs include a core of at least one of a Group II-VI semiconductor material, III-V semiconductor material, a group IV semiconductor material, or a combination thereof. The quantum dots can further include a shell which passivates the core of the QDs to form core-shell QDs. The properties of QDs result from quantum-size confinement, which occurs when metal and semiconductor particles are smaller than their exciton Bohr radii.

Due to their size-dependent optical and electronic properties, QDs have high potential in various biological applications, including labeling cells and other biological components. Although QDs have high potential in various biological applications, there are still obstacles to the use of QDs, in particular, water-insoluble uncapped QDs, in biological applications. For example, QDs are generally prepared in organic solvents and are not suitable for biological application. Thus, the instability and/or insolubility of these QDs in aqueous media have limited their usefulness in biological applications. Water-soluble QDs can be prepared by capping water-insoluble QDs with polar capping compounds, for example, by treating water-insoluble QDs with mercaptocarboxylic acid or by silicizing the surface of water-insoluble QDs. However, the photoluminescence (PL) of such QDs after solubilization with thiol-terminated compounds, such as mercaptoacetic acid (MAA), is very weak, almost imperceptible.

In addition to labeling cells and other biological components, the spectral properties of QDs make them ideal fluorescence resonance energy transfer (FRET) donors/acceptors. However, the energy transfer is a dipole-dipole interaction and hence drops off with distance between the pair as r^{-6} . Biocompatible QDs are usually passivated by a polymer layer and a protein layer, creating stable, high quantum-yield photoluminescence (PL), but increasing the particle size by up to 10-15 nm and reducing or eliminating FRET.

Thus, there is still a need to develop methods to overcome the aforementioned problems in the use of QDs in various biological applications.

SUMMARY OF THE INVENTION

It has now been found that quantum dots conjugated to electron donors or electron acceptors can be used as "Off-On" biological labels in response to specific redox-dependent metabolic mechanisms. Also, it has now been found that uncapped QDs can be embedded within lipid membranes and act as FRET donors or acceptors, thereby labeling cell membranes. Based upon these discoveries, a composition comprising a semiconductor nanocrystal (quantum dot) conjugated with one or more electron donors or acceptors, and its use in labeling a living cell or organism and

- 3 -

detecting a metabolism abnormality or metabolically-active intracellular environment of a cell or organism are disclosed herein. Also, a composition comprising uncapped QDs embedded in a lipid membrane and its use in labeling cells and cell membranes are disclosed herein.

5 One aspect of the present invention relates to the use of a semiconductor nanocrystal (quantum dot) conjugated with one or more electron donors or acceptors.

 In one embodiment, the present invention provides a composition comprising a semiconductor nanocrystal and one or more electron donors or acceptors
10 conjugated with the nanocrystal. The conjugation of the nanocrystal and electron donors or acceptors quenches fluorescence emission of the nanocrystal.

 In another embodiment, the invention is directed to a method for fluorescence internal labeling a living cell or organism by the use of the conjugate of a semiconductor nanocrystal with one or more electron donors or acceptors. The
15 method comprises the steps of:

- (a) providing a conjugate of a semiconductor nanocrystal with one or more electron donors or acceptors, fluorescence emission of the nanocrystal being quenched by the conjugation; and
- (b) incubating a plurality of the conjugates with the living cell or
20 organism, whereby one or more of the conjugates are incorporated into the living cell or organism, wherein metabolism of the electron donors or acceptors by the living cell or organism causes the quenched fluorescence emission of the nanocrystal to be restored, thereby labeling the living cell or organism.

 In yet another embodiment, the invention is directed to a method for
25 detecting a metabolism abnormality or metabolically-active intracellular environment of a cell or organism by the use of the conjugate of the nanocrystal with one or more electron donors or acceptors. The method comprises the steps of:

- (a) providing a conjugate of a semiconductor nanocrystal with one or more electron donors or acceptors;
- (b) incubating a plurality of the conjugates with the cell or organism
30 under detection for a period of time;

- 4 -

(c) measuring nanocrystal-associated fluorescence of the cell or organism; and

(d) comparing the nanocrystal-associated fluorescence of the cell or organism under detection with nanocrystal-associated fluorescence of a control cell or organism having normal metabolism, thereby detecting a metabolism abnormality or a metabolically-active intracellular environment of the cell or organism under detection.

A second aspect of the present invention relates to the use of an uncapped semiconductor nanocrystal embedded in a lipid membrane, for example, as a fluorescence resonance energy transfer (FRET) donor or acceptor.

In one embodiment of the second aspect of the invention, the present invention provides a composition comprising an uncapped semiconductor nanocrystal and a lipid membrane, where the uncapped semiconductor nanocrystal is embedded in the lipid membrane.

In another embodiment of the second aspect of the invention, the present invention is directed to an ion-, or voltage-, or ion and voltage-sensitive probe or label for a transmembrane or intramembrane process, comprising an uncapped semiconductor nanocrystal and the lipid membrane, where the uncapped semiconductor nanocrystal is embedded in a lipid membrane. Fluorescence emission mediated by the uncapped nanocrystal changes with a voltage change or an ion-concentration change across the membrane or both a voltage change and an ion-concentration change across the membrane.

In yet another embodiment of the second aspect of the invention, the present invention is directed to a method for sensing a transmembrane or intramembrane process of a cell or organism by the use of an uncapped semiconductor nanocrystal embedded in a lipid membrane. The method comprises the steps of:

(a) embedding one or more uncapped semiconductor nanocrystals within a lipid membrane, wherein fluorescence emission spectrum or fluorescence emission intensity or both fluorescence emission spectrum and fluorescence emission intensity, mediated by the nanocrystals, change with a voltage change or an ion-concentration change across the membrane or a voltage change and an ion-concentration change across the membrane; and

(b) monitoring the change in the emission spectrum or intensity or both spectrum and intensity, thereby sensing the transmembrane or intramembrane process.

Also, the present invention provides a method of labeling cells by the use of uncapped nanocrystals. The method comprises embedding one or more uncapped semiconductor nanocrystals within one or more lipid membranes of the cells, and measuring the fluorescence emission spectrum or fluorescence emission intensity or both fluorescence emission spectrum and fluorescence emission intensity of the uncapped semiconductor nanocrystals, thereby labeling the cells.

As discussed above, photoluminescence (PL) of solubilized, capped QDs, for example, QDs solubilized with a thiol-terminated compound such as mercaptoacetic acid (MAA), is very weak, almost imperceptible. Thus, the use of a conjugate of MAA-solubilized QDs with one or more electron donors or acceptors in biological labeling can provide a very low-background labeling system.

In addition, many biological processes occurring within lipid membranes have been poorly understood because of the difficulty of probing an about 5 nm thick hydrophobic interface. The present invention utilizing uncapped QDs provides a powerful tool for probing, sensing or labeling the cell membranes. Also, the present invention utilizing uncapped QDs has advantages over currently-existing organic dyes. Although organic, voltage sensitive dyes that change their spectrum according to the potential change across a lipid bilayer have been used in the art, these organic dyes are greatly limited by the small size of their spectral changes with voltage and the lack of quantitative understanding of their properties. In contrast, in the Applicants' present invention utilizing uncapped QDs, a significantly large spectral shift can be generated. Also, uncapped, unsolubilized QDs are in general more stable, and their quantum yields are generally much higher than those of capped, water-soluble QDs.

SHORT DESCRIPTION OF FIGURES

FIGs. 1a-1c are schematics for QD fluorescence modulation by conjugation of QDs to an electron donor or an electron acceptor.

FIGs. 2A-2C are fluorescence emission spectra showing effects of adenine conjugation on fluorescence of three different colors of bare-core CdSe QDs: green (■), yellow (▲), and red (●): FIG. 2A, emission spectra of bare-core QDs after solubilization with MAA, showing very weak fluorescence; FIG. 2B, emission spectra of bare-core CdSe QDs after conjugation to adenine and subsequent exposure to light for 3 hours, showing weak fluorescence; FIG. 2C, emission spectra of bare-core QDs after conjugation to a non-electron donor, glucosamine, showing strong, bright fluorescence in all three different colors of bare-core CdSe QDs.

FIG. 3 is a graph showing time resolved emission data for unconjugated QDs (black open circle: ○), red QD-adenine (black solid line #2), and green QD-adenine (grey solid line #1), illustrating quantification of electron transfer/quenching by adenine.

FIG. 4a shows emission spectra (arbitrary units) of *B. subtilis* cultures after 3 hours' incubation with yellow QD-adenine for wild-type (●), *ade* (▲), *apt* (■), and *ade-apt* (+) mutants; and heat-killed wild type (— — -); and original spectrum of QD-adenine (----).

FIG. 4b shows fluorescence emission spectra (arbitrary units) of QD-AMP after 3 hours' incubation with *apt* mutant (■); *ade* mutant (▲); *ade-apt* double mutant (+); wild-type (●); and heat-killed (— — -); and original spectrum of QD-adenine (----).

FIGs. 4c-4h are epifluorescence images showing autofluorescence of: FIG. 4c, heat-killed, wild-type *B. subtilis* incubated with QD-AMP; FIG. 4d, heat-killed wild-type *B. subtilis* incubated with QD-wheat germ agglutinin (WGA) conjugates; FIG. 4e, wild-type *B. subtilis* incubated with QD-adenine; FIG. 4f, wild-type *B. subtilis* incubated with QD-AMP; FIG. 4g, *apt* mutant of *B. subtilis* incubated with QD-adenine; and FIG. 4h, *apt* mutant of *B. subtilis* incubated with QD-AMP.

FIG. 5a is an emission spectra of adenine auxotrophic mutant ATCC 23804 incubated with QD-adenine (●) and QD-AMP (■); wild-type with QD-adenine (○) and QD-AMP (□); and heat-killed auxotroph with QD-adenine (— — -).

FIGs. 5b and 5c are epifluorescence images of (scale bar = 5 μm): FIG. 5b, wild-type *E. coli* incubated with QD-adenine for 30 minutes in a minimal growth

medium; FIG. 5c, adenine auxotrophic *E. coli* incubated with QD-adenine for 30 minutes in a minimal growth medium.

FIGs. 6a-6d are TEM images of QDs in *B. subtilis*: FIG. 6a, a transverse thin section of *B. subtilis ade* mutant incubated with yellow QD-AMP, showing QDs inside and outside the cell: the cell wall (cw), cytoplasm (cyt) and cell exterior (ext); FIG. 6b, a closeup of a fraction of the cell shown in FIG. 6a; FIG. 6c, a CPD (critical point drying) image of *B. subtilis apt* mutant without QDs: nucleoid (light area); FIG. 6d, a CPD image of cell labeled with QD-AMP, showing many internalized QD-AMP particles.

FIGs. 7A-7B are fluorescence emission spectra of green QDs (left) and yellow QDs (right) in aqueous solution before conjugation (-----) and after conjugation to dopamine (-■-■-), showing effects of photo-oxidation (y-axes in arbitrary units, excitation at 400 nm): FIG. 7A, fluorescence emission spectra before photo-oxidation; and FIG. 7B, fluorescence emission spectra after exposure of quenched QD-dopamine to 60 seconds of high-intensity UV illumination (photo-oxidation).

FIGs. 8A-8C are fluorescence microscopic images showing dopamine-specific uptake and fluorescence in cell lines and neurons (scale bar = 20 μ m): FIG. 8A, fluorescence of mouse A9 cells stably transfected with human D2 dopamine receptors (ATCC CRL-10225), after 1 hour of exposure to QD-dopamine and 10 seconds of exposure to Hg bulb illumination; FIG. 8B, HEK 293 cells under the same conditions as those for FIG. 8A; and FIG. 8C, fluorescence of primary neuronal culture of mixed dopaminergic/GABAergic immunoreactivity after 2 hours of exposure to QD-dopamine.

FIG. 8D is a graph showing fluorescence emission spectra from individual cells in panels FIGs. 8A and 8B, showing fluorescence of a labeled A9 cell (A9) compared to that of a 293 cell (293) and that of the original unconjugated QDs (QD) and QD-dopamine (QD+DA, fully quenched).

FIG. 9A-9D are fluorescence microscopic images of mammalian cells after exposure to QD-dopamine in the presence and absence of antioxidants (scale bar = 10 μ m): FIG. 9A, phase-contrast image of A9 cells after incubation with green QD-dopamine in the absence of antioxidants; FIG. 9B, high-power brightfield image of

cells under the same conditions as FIG. 9A; FIG. 9C, phase contrast image of A9 cells after incubation with green QD-dopamine with the addition of 5.7 mM of β -mercaptoethanol; and FIG. 9D, high-power brightfield image of cells under the same conditions as FIG. 9C.

5 FIGs, 10a-10c are fluorescence microscopic images showing redox-sensitive labeling with QD-dopamine and comparison with commercial redox sensor dye RedoxSensor Red for cells labeled with RedoxSensor Red alone (left), QD-dopamine alone (center), and a high-power zoom of QD-dopamine with LysoTracker Red (right): FIG. 10a, cells under the most oxidizing conditions (10 mM BSO); FIG.
10 10b, untreated, actively proliferating cells; and FIG. 10c, cells in reducing environment (1 mM GSH-MEE).

 FIGs. 11A-11C are graphs showing absorbance and emission spectra of green-emitting QDs (absorbance: #1; emission: #2), red-emitting QDs (absorbance: #3; emission: #4) and dyes (absorbance: #5; emission: #6) (y-axes in arbitrary units):
15 FIG. 11A, DiD as a dye; FIG. 11B, Cy 3.5 as a dye; and FIG. 11C, di-4-ANEPPS as a dye.

 FIGs. 12A-12C are emission spectra in arbitrary units for: QDs alone (#1); then after addition of 3 μ L (#2), 6 μ L (#3), 9 μ L (#4), 12 μ L (#5), and 15 μ L (#6) of a stock solution containing 10 μ M of Cy 3.5 and 0.1 mM of DiD, showing FRET
20 between QDs in vesicles and dyes in lipid and in solution at an excitation wavelength of 400 nm: FIG. 12A, between green QDs and Cy 3.5; FIG. 12B, between red QDs and DiD; and FIG. 12C, between green QDs and DiD.

 FIG. 12D is a graph showing a fraction of QD quenching that was due to FRET (f , dimensionless) plotted against dye concentration in relative units: red QDs and DiD in vesicles (Red-DiD); green QDs and DiD in vesicles (Green-DiD); green
25 QD in vesicles with Cy3.5 in external solution (Green-Cy3.5); and a control with QDs acted as donors to tetramethylrhodamine in solution (dashed line).

 FIGs. 13A and 13B are TEM images of whole and thin sectioned vesicles, respectively: FIG. 13A, a negatively-stained whole vesicle at low resolution
30 showing dark spots "peppered" over the surface; and FIG. 13B, a thin section through a vesicle at the same magnification, showing dark areas associated with the lipid membrane.

FIG. 13C is a schematization of FIG. 13B, showing the location of QDs within the lipid bilayer.

FIGs. 13D and 13E are TEM images of whole and thin sectioned vesicles, respectively: FIG. 13D, a thin section through a multilamellar vesicle, showing multiple layers of lipid, all of which contained QDs material; and FIG. 13E, a closeup of FIG. 13D, showing QD material concentrated within the lipid (narrower layers) rather than in the aqueous solution between the lamellae (wider layers).

FIG. 13F is a Schematization of FIG. 13E, showing the multiple layers of lipid (black lines) containing QD material in aggregates of various sizes.

FIGs. 14A-14C are graphs showing excitation spectra at $\lambda_{Em} = 610$ nm of vesicles containing red QDs, ANEPPS (5 μ M), or both in the presence of valinomycin: FIG. 14A, ANEPPS alone; FIG. 14B, red QDs alone; and FIG. 14C, red QDs with ANEPPS, in different K^+ ion concentrations: low K^+ both inside (K_{in} (mM)) and outside (K_{out} (mM)) (1/1, ●); high K_{in} /low K_{out} (150/1, ■); high K_{in} /high K_{out} (150/150, +); and low K_{in} /high K_{out} (1/150, ▲).

FIGs. 14D-14G are graphs showing excitation spectra at $\lambda_{Em} = 610$ nm of vesicles containing: QDs alone (■), ANEPPS alone (●) and both QDs and ANEPPS (▲) in different K^+ ion concentrations: FIG. 14D, low K_{in} /low K_{out} (1/1); FIG. 14E, high K_{in} /low K_{out} (150/1); FIG. 14F, low K_{in} /high K_{out} (1/150); and FIG. 14G, high K_{in} /high K_{out} (150/150).

FIG. 15A is a graph showing excitation spectral differences ($\lambda_{Em} = 610$ nm, y axis in arbitrary unit, but scaled to show the position of zero) between vesicles containing both ANEPPS and red QDs and vesicles containing ANEPPS alone for: low K_{in} /low K_{out} (1/1, ●), high K_{in} /low K_{out} (150/1, ■), high K_{in} /high K_{out} (150/150, +), and low K_{in} /high K_{out} (1/150, ▲); and excitation spectra of QDs only (---) and ANEPPS only (— —).

FIG. 15B is a schematic drawing showing a structure of di-4-ANEPPS before (above) and after (below $h\nu$) photoexcitation.

FIG. 15C is a schematic representation showing a probable orientation of ANEPPS binding to lipid bilayers and presumed location of quantum dot.

FIG. 16A is a graph showing emission spectra ($\lambda_{\text{Ex}} = 440 \text{ nm}$) of vesicles containing both red QDs and di-4-ANEPPS in solutions containing different values of internal and external K^+ ($\text{K}_{\text{in}}/\text{K}_{\text{out}}$ (mM) (y axes in arbitrary units with numbers permitting comparison) in the presence of valinomycin: low K^+ both inside and
 5 outside (1/1, ●); high $\text{K}_{\text{in}}/\text{low K}_{\text{out}}$ (150/1, ■); high $\text{K}_{\text{in}}/\text{high K}_{\text{out}}$ (150/150, +); and low $\text{K}_{\text{in}}/\text{high K}_{\text{out}}$ (1/150, ▲); and in osmotically-balanced solutions with different values of $\text{K}_{\text{in}}/\text{K}_{\text{out}}$ (— “no Val”) in the absence of valinomycin.

FIG. 16B is a graph showing subtractions of the "no Val" of FIG. 16A from the emission spectra of FIG. 16A for the vesicles containing both red QDs and di-4-
 10 ANEPPS in the presence of valinomycin: low K^+ both inside and outside (1/1; ●); high $\text{K}_{\text{in}}/\text{low K}_{\text{out}}$ (150/1; ■); high $\text{K}_{\text{in}}/\text{high K}_{\text{out}}$ (150/150,+); and low $\text{K}_{\text{in}}/\text{high K}_{\text{out}}$ (1/150, ▲).

FIGs. 17A and 17B are fluorescent images of vesicles containing both red QDs and 5 μM di-4-ANEPPS before (FIG. 17A) and after valinomycin addition
 15 (FIG. 17B) (scale bar=5 μm).

FIG. 18A is a graph showing spectra of vesicles containing both QDs and ANEPPS, or ANEPPS alone, in the presence of valinomycin (y axes in arbitrary units with numbers permitting comparison): concentrations of ANEPPS ranging from 0 μM to 7 μM for vesicles containing QDs (—, concentrations on right hand
 20 side) or vesicles without QDs (— —, concentrations on left hand side).

FIG. 18B is a graph showing subtractions of a spectrum of directly-excited ANEPPS dye from the emission spectra of FIG. 18A for vesicles containing both QDs and ANEPPS, showing a concentration-dependent quenching.

FIG. 18C is a graph showing subtractions of a directly-excited QD peak from
 25 the subtractions of FIG. 18B.

FIG. 19A is a graph showing spectral changes seen with QDs and ANEPPS in symmetric 1 mM KCl solution in the absence of valinomycin.

FIG. 19B is a graph showing appearance of unknown spectrum for red QDs (original peak, 620 nm, —) and green QDs (original peak, 570 nm, ----).

FIGs. 20A and 20B are fluorescence microscopic images showing delivery
 30 of QDs and dyes to cell membranes and evaluation of fluorescence changes with

voltage: FIG. 20A, delivery of hydrophobic QDs to primary rat cortical neurons in culture (3 days *in vitro*); and FIG. 20B, mouse epithelial (A9) cell line containing both QDs and di-4-ANEPPS, showing fluorescence enhancement in a clamped cell in a depolarized state (holding +50 mV) (arrow; the bright spot is the recording electrode; the presence of the electrode also leads to a blurred image).

FIG. 20C is a graph showing spectral signals from the cell held at +50 mV (-----) and an unclamped neighbor cell (—).

FIGs. 21A and 21B are fluorescence microscopic images showing voltage-dependent effects of QD-dye FRET seen in primary rat cortical neurons in culture (12 days *in vitro*, scale bar = 20 μ m): FIG. 21A, image of a cell culture containing di-4-ANEPPS and green-emitting QDs (peak 540 nm); and FIG. 21B, the cell culture of FIG. 21A immediately after depolarization with 30 mM glutamate.

FIG. 21C is a graph showing spectra obtained from liquid crystal filter for selected cells within the culture: addition of di-4-ANEPPS alone ("dye"); addition of QDs alone ("QD"); addition of di-4-ANEPPS and QD ("QD+dye"); and addition of glutamate("+glu").

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "quantum dot" refers to a semiconductor nanocrystal that includes a core of at least one of a Group II-VI semiconductor material, III-V semiconductor material, a group IV semiconductor material, or a combination thereof. The terms "quantum dot (QD)" and "semiconductor nanocrystal" are used interchangeably herein. Examples of quantum dots (QDs) that can be used in the invention include nanocrystals of CdSe, ZnS, ZnSe, ZnTe, CdS, CdTe, GaN, GaP, GaAs, GaSb, InP, INAs, InSb, AlS, AlP, AlAs, AlSb, PbS, PbSe, Ge and Si, and ternary and quaternary mixtures thereof.

As used herein, the term "nanocrystal" means a single crystal particle having an average cross-section no larger than about 20 nanometers (nm) or 20×10^{-9} meters (200 angstroms), typically no larger than about 15 nm (150 angstroms) and a minimum average cross-section of about 0.5-1 nm. Preferably, the nanocrystal has an average cross-section ranging in size from about 1 nm (10 angstroms) to about 10 nm (100 angstroms).

The quantum dots can further include a dopant such as a rare earth metal or a transition metal. Alternatively, the quantum dots can further include a shell which passivates the core of the QDs. Materials for the shell are preferably semiconductors having a greater band gap than that of the core. For example, a core of CdSe, CdTe or CdS can be passivated with a shell of ZnS or ZnSe. One specific example is a core-shell CdSe QD passivated with a ZnS shell ("CdSe/ZnS").

In addition, quantum dots can be linked to a capping material. Examples of capping materials include compounds having the formula of $Q[(CRR')_nCO_2H]_m$ or a salt thereof, where: $n=1, 2, 3, 4$ or 5 ; Q is $HS-$, H_2N- , $O=P-$, or $P<$; m is 1 when Q is $HS-$, H_2N- , or $O=P-$ or m is 3 when Q is $P<$; and R and R' are independently $-H$, substituted or unsubstituted alkyl, or substituted or unsubstituted aryl. Specific examples of suitable capping materials for the invention include mercaptoacetic acid (MAA) and mercapto-functionalized amines, such as homocysteine, aminoethanethiol-HCl and 1-amino-2-methyl-2-propanethiol-HCl.

As used herein, the term "uncapped semiconductor nanocrystal" refers to a semiconductor nanocrystal that does not include any capping materials. It is understood that the term "uncapped semiconductor nanocrystal" encompasses both a core semiconductor nanocrystal that does not have any shells passivating the core of the semiconductor nanocrystal, and a core-shell semiconductor nanocrystal that has one or more shells around the core of the semiconductor nanocrystal.

QDs can fluoresce when excited by light. The fluorescence of QDs results from confinement of electronic excitations to the physical dimensions of the nanocrystals, and the band gap of the semiconductor nanocrystals depends on the size of the nanocrystals. Technology for controlling their sizes is known in the art (see, for example, quantumdot.com).

Nanometer crystals (QDs) of Group II-VI semiconductors can be formed by methods known in the art, for example, colloidal precipitation techniques (see, for example, U.S. Pat. Nos. 5,251,018; 5,505,928; and 5,262,357. The entire teachings of these patents are incorporated herein by reference).

In one such technique, a group II metal source, such as a Cd(II) or Zn(II) salt, is dissolved in water, and then this solution is suspended in an organic liquid,

such as hexane, heptane, octane, or the like, with a colloid former, such as deoctylsulfosuccinate. A suitable counterion (e.g., sulfide, selenide or telluride) source is dissolved in water and similarly suspended in an organic liquid. The two suspensions are then mixed together to yield a colloidal suspension of nanocrystals of the semiconductor compound. This resulting suspension is destabilized by addition of a capping material, for example, a thioacid (e.g., thiophenol or mercaptoacetic acid). This causes the nanocrystals to precipitate for recovery.

In another technique, a group III metal source, such as a Ga(III), In (III) or Al(III) salt, or a corresponding metal 1-6 carbon trialkyl, is reacted directly with a suitable counterion source, for example, arsine, phosphine or stibine, in liquid phase at an elevated temperature. In this technique, the metal and anion sources are mixed together in a nonaqueous liquid reaction medium which includes a crystal growth terminator and heated to a temperature of, for example, at least about 100°C for a prolonged period of, for example, at least 1 hour. Polar organic compounds, such as nitrogen-, and/or phosphorus-containing organic compounds, can serve as crystal growth terminators.

Typical metal Group II or III metal sources include corresponding metal halides or alkyls (e.g., mono-, di-, or tri-alkyls), such as GaCl₃, GaBr₃, GaI₃, InCl₃, InBr₃, AlCl₃, Ga(Me)₃, Ga(Et)₃, Ga(Bu)₃, CdCl₂, CdBr₂, CdI₂, Cd(1-6 carbon alkyl)₂, ZnCl₂, ZnBr₂, ZnI₂ and Zn(1-6 carbon alkyl)₂. Typical counterion sources include AsH₃, PH₃, AsH₂(1-6 carbon alkyl), As(1-4 carbon alkyl)₃, P(1-4 carbon alkyl)₃, As(Si(1-6 carbon alkyl)₃)₃, P(Si(1-6 carbon alkyl)₃)₃, Se(Si(1-4 carbon alkyl)₃)₃, alkali metal sulfides, alkali metal selenides and alkali metal tellurides, such as Na₂S, K₂S, Na₂Se, K₂Se, Na₂Te and K₂Te.

Other colloidal precipitation methods can also be used for forming nanocrystals of Group II-VI semiconductors. For example, Group II-VI nanocrystals can be precipitated out of acidic or basic media in the presence of one or more capping materials, such as mercaptoacetate ions, in the form of colloidal nanocrystals

1. Quantum Dots Conjugated To Electron Donors and/or Acceptors

One aspect of the invention relates to a composition comprising a QD conjugated with one or more electron donors and/or acceptors, and its use as "Off-On" biological labels in response to specific redox-dependent metabolic mechanisms.

5 In a preferred embodiment, the electron donors or acceptors are metabolizable by a living cell or organism. The fluorescence emission of QDs, such as CdSe and/or CdSe/ZnS QDs, is quenched by conjugation to electron donors or acceptors. When the quenched conjugates are added to populations of living cells or organisms that are able to metabolize these electron donors or acceptors,
10 fluorescence returns. When the quenched conjugates are added to dead cells or cells unable to utilize the donors, there is no fluorescence. The schematic for the principle behind this mechanism is shown in FIGs. 1a-1c. In FIGs. 1a-1c, visible fluorescence occurs when blue light ($h\nu$) excites an electron-hole pair across the semiconductor bandgap (CB = conduction band). If the QD is exposed to an
15 oxidizer O or reducer R, an electron will transfer to O (1) or from R (2), respectively (FIG. 1b). Analytes A and B are detected when they bind O or R, raising the redox levels above the band edge and thus preventing an electron transfer (FIG. 1c).

Because the PL of the quenched QD conjugate returns in a metabolism-dependent manner, as discussed above, a metabolism abnormality or a
20 metabolically-active intracellular environment of a cell or organism can be detected by the use of the QD conjugate. The detection can be done by comparing the QD-associated fluorescence of a cell or organism under detection with QD-associated fluorescence of a control cell or organism having normal metabolism. For example, CdSe QDs conjugated with electron donors, such as adenine or dopamine, are
25 exposed to a culture of living cells. The fluorescence of the QD conjugate quenched by conjugation to the electron donor, may fully or partially return, or alternatively may not return, depending upon the ability of the cell or organism under detection to take up and utilize the electron donors.

Examples of suitable electron donors include adenine, nicotinamide adenine
30 dinucleotide, dopamine, tryptophan and tyrosine. In a preferred embodiment, dopamine is employed for the invention.

Particular examples of the invention include: (1) adenine as the electron donor, and bacteria as the adenine metabolizer, and (2) dopamine as the electron donor, and cells bearing dopamine receptors as the metabolizer. Tryptophan, tyrosine or NADH can also be used as electron donors for the invention.

5 Alternatively, electron acceptors such as benzoquinone, flavin, flavin adenine dinucleotide, flavoproteins, hydroquinone and sulfur containing proteins can be used to quench the fluorescence, with their corresponding metabolizers in the invention.

 In one embodiment, the QD is conjugated with one or more electron donors.
10 In another embodiment, the QD is conjugated with one or more electron acceptors. Alternatively, the QD can be conjugated with both one or more electron donors and one or more electron acceptors.

 The conjugation can be formed, for example, by using an activator known in the art, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). In one
15 example, QDs solubilized with a capping material, such as mercaptoacetic acid (MAA) or a mercapto-functionalized amine, can be conjugated to dopamine, adenine or AMP in the presence of EDC.

 Suitable examples of QDs for this invention are as described above. CdSe QDs are preferred, including both CdSe core QDs and CdSe core-shell QDs, such as
20 CdSe/ZnS. In a preferred embodiment, the QD is capped by a capping material of the formula of $Q[(CRR')_nCO_2H]_m$ or a salt thereof, where n, Q, m, R and R' are as described above. A preferred capping material is a compound having the formula of $Q[(CRR')_nCO_2H]_m$ or a salt thereof, where n=1, 2 or 3; Q is HS-; m is 1; and R and R' are as described above. Mercaptoacetic acid is more preferred.

25

2. Uncapped QDs embedded in a lipid membrane

 Another aspect of the invention includes a composition comprising an uncapped QD embedded in a lipid membrane, and its application as an ion-, or voltage-, or ion and voltage-sensitive probe or label for a transmembrane or
30 intramembrane process, and in labeling cells and/or cell components (e.g., cell membranes). Typically, the uncapped QD is a fluorescence resonance energy transfer donor or acceptor.

In a preferred embodiment, the composition also includes an organic dye that has a lipophilic fluorophore. The organic dye is sensitive to a voltage change or an ion-concentration change across the membrane, or a voltage change and an ion-concentration change across the membrane. In these embodiments, the organic dye is used in conjunction with a membrane-embedded QD, but the organic dye and the uncapped QD is not covalently conjugated to each other. Fluorescence resonance energy transfer can occur between the uncapped QD and the organic dye.

In one embodiment, the organic dye is a lipid-soluble organic dye. The lipid-soluble organic dye is embedded in the membrane where the uncapped QD is embedded. Examples of such organic dyes include (aminonaphthylethenylpyridinium) dyes (ANEP dyes), such as 3-(4-(2-(6-(dibutylamino)-2-naphthyl)-trans-ethenyl)pyridinium)propanesulfonate (di-4-ANEPPS), 3-(4-(2-(6-(dioctylamino)-2-naphthalenyl)pyridinium)propanesulfonate (di-8-ANEPPS).

In another embodiment, the organic dye is a water-soluble organic dye. The water-soluble organic dye is in an aqueous environment near the membrane where the uncapped QD is embedded. Examples of the hydrophilic dyes include cyanine dyes, such as Cy 2.5, Cy3 or Cy 5. In general, cyanine dyes have the general formula of $R_2N[CH=CH]_nCH=N^+R_2$ in which the nitrogen and part of the conjugated chain usually form part of a heterocyclic system, such as imidazole, pyridine, pyrrole, quinoline, thiazole and the like.

In a preferred embodiment, the cell for under detection is an excitable cell. Herein, an "excitable" cell means a cell which is capable of firing action potentials. Examples of excitable cells include neurons and cardiac myocytes, specifically neuron or a cardiac cell in self-organization or in a development state.

Examples of QDs suitable for the invention are as described above. CdSe QDs are preferred, including both CdSe core QDs and CdSe core-shell QDs, such as CdSe/ZnS. Specific examples include red, green and yellow CdSe QDs.

Fluorescence emission spectrum or fluorescence emission intensity or both fluorescence emission spectrum and fluorescence emission intensity, mediated by the uncapped QD that is embedded in a lipid membrane, change with a voltage change or an ion-concentration (e.g., H^+ , K^+ , Na^+ , Ca^{2+} , Mg^{2+} or Cl^-) change across

the membrane, or both a voltage change and an ion-concentration change across the membrane. Particles embedded in a cell membrane can experience any voltage drop that exists across that membrane; in certain types of cells, this voltage can be greater than 100 mV/10 nm (10^5 V/cm). In particular, monitoring the change in the
5 emission spectrum or intensity or both spectrum and intensity provides a tool for labeling cells, and sensing/detecting a transmembrane or intramembrane process, such as activity in an excitable cell (e.g., neuron or a cardiac cell). In these embodiments, the uncapped QD typically acts as a fluorescence resonance energy transfer donor or acceptor.

10 Optionally, a pore forming peptide can further be employed in the invention. A "pore-forming peptide" refers to a natural or synthetic peptide that causes pore formation in a target cell membrane and behaves as a charge carrier, so that an ion, such as H^+ , K^+ , Na^+ , Ca^{2+} , Mg^{2+} and Cl^- , can be transported into or through the membrane. Examples include valinomycin, daptomycin and lactacin.

15 In a preferred embodiment of this invention, the quantum dot and organic dye are located within the membrane of a living cell. In this instance, charge carriers (ion channels) in the living cells, rather than pore-forming peptides, create the voltage change. In the case of "excitable" cells (e.g., neurons, cardiac myocytes), the voltage changes occur spontaneously and are indicated by
20 fluorescence changes of the quantum dot-organic dye complex.

 The uncapped QDs can be directly embedded in a lipid membrane under detection. Alternatively, the uncapped QDs can be embedded in a lipid membrane under detection *via* a lipid vesicle where the uncapped QDs are embedded in the membrane of the vesicle. When the lipid vesicle is used, optionally, a pore forming
25 peptide as described above can further be employed.

 In general, uncapped QDs are hydrophobic and insoluble in water. Advantages associated with the use of uncapped, hydrophobic QDs include that the quantum yields of the uncapped, hydrophobic QDs can be much higher than those of water-solubilized QDs. Also, uncapped, hydrophobic QDs are more stable than
30 water-solubilized QDs.

EXEMPLIFICATION

Example 1. Quantum Dots Conjugated To Electron Donors As Low Background,
“Off-On” Biological Labels

This Example illustrates low-background “off-on” labeling of adenine, adenosine monophosphate (AMP) or dopamine metabolism in living cells. The degree of uptake of the conjugates by the cells was shown to be dependent upon two factors: first, the presence of specific receptors and uptake mechanisms in the cells; and second, the degree of quenching of the QD fluorescence by the conjugate. In the first part of the example, uptake of QD-adenine conjugates by the bacteria *Bacillus subtilis* and *Escherichia coli* is demonstrated. Green and yellow QDs, which were quenched by conjugation, were taken up whereas unquenched red QDs were not. The second part of the example illustrates a similar set of phenomena in mammalian cells. QD-dopamine conjugates that were fully quenched were taken up by endocytosis in cells that had dopamine receptors; QDs that were only partially quenched were not taken up. Removal of quenching, and thus observation of the QDs, was effected by light exposure or exposure to oxidizing regions of the cell. This allowed for redox-sensitive labeling of subcellular structures in these labeled cells.

A1. Materials and methods

CdSe QDs were synthesized, characterized, and mercaptoacetic-acid solubilized follows: CdSe nanocrystals were synthesized using cadmium acetate and selenium metal as precursors. Elemental Se was incubated for at least 24 h in trioctylphosphine oxide (TOPO), generating TOP-Se, which was then injected with the Cd precursor into a TOPO solution at 250-350 °C and baked for 30s – 30 min. Resulting QDs were washed several times in methanol, in which they are insoluble, and finally suspended in dichloromethane. This resulted in CdSe particles passivated with TOPO (“passivation” refers to the elimination of crystal surface defects with an external coating; without passivation, fluorescence quantum yield was greatly reduced). To create CdSe/ZnS core-shell QDs, the hot reaction mixture remaining after CdSe crystal synthesis was allowed to cool to ~180 °C and 5 mL of ZnS precursor (hexamethyldisilathiane and dimethylzinc in trioctylphosphine [TOP]) was added drop-wise over the course of 10 to 15 minutes and baked for 1-2

hours. Physical characterization of the particles was carried out by transmission electron microscopy (TEM) with energy dispersive spectroscopy (EDS) and by time correlated single photon counting (TCSPC) (see, for example, J. Nadeau, *et. al.*, "Photo-physical properties of biologically compatible CdSe quantum dot structures", *J. Phys. Chem. B* **109**, 9996-10003 (2005). Physical characterization of the particles was carried out by transmission electron microscopy (TEM) with energy dispersive spectroscopy (EDS). "Cap exchange" using mercaptoacetic acid (MAA) was used to replace the TOPO with a water-soluble layer, allowing the QDs to be suspended in aqueous solution. 0.48 mL of MAA was added to 2.5 mL of TOPO-capped QDs in dichloromethane and rocked gently for 2 h in the dark. After the addition of 2.5 mL of phosphate buffered saline (PBS, pH 7.5), the solution was vigorously agitated and the layers then allowed to separate naturally. The pigmented layer was removed and washed 2-3 times in PBS by centrifugation (5 min at 14,000 rpm in a microcentrifuge) and removal of supernatant. Finally, the resulting colloid was suspended in 1-2 mL PBS, placed into a microdialysis chamber (Slide-A-Lyzer 10K dialysis cassette, Pierce Chemical Company, Rockford, IL), and dialyzed vs. 2 L PBS for 1 hour to remove residual MAA. The resulting water-solubilized QDs were stored at RT (room temperature) in the dark.

Solubilized QDs were conjugated to adenine, AMP, or dopamine using the activator EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl). Each 1 mL reaction in PBS (phosphate buffered saline) contained 1-2 mg EDC, 10 mM conjugate, and 100-200 mL of a standard QD solution with an optical density of 0.1 at the exciton peak. Conjugation was for 2h, and unbound conjugate was removed by dialysis or centrifugation and washing with H₂O. QDs and conjugates were stored in "dark" conditions (wrapped in aluminum foil or kept in a sealed drawer) unless stated otherwise. The pH of all solutions of adenine and AMP was adjusted to neutral (6.5 – 7.5) using NaOH before addition of EDC and quantum dots. Redox potentials of the solutions were measured vs. Ag/AgCl using an immersible multifunction electrode ("Pentrode," Thermo Orion).

For QD incubation with bacteria, 50-200 μ L conjugate was added to 250 μ L bacteria (logarithmic growth phase) and ~0.8 mL nutrient medium (final QD concentrations, ~ 10-40 nM for bare CdSe, 2-10 nM for core-shell CdSe/ZnS).

Control cultures received an identical volume of H₂O or a solution of unlabeled adenine or AMP for a final concentration of 2 mM. *E. coli* strains were purchased from the American Type Culture Collection (ATCC): wild-type ATCC 25922, and the adenine auxotroph ATCC 23804. *B. subtilis* strains were a gift from Per
5 Nygaard, Institute of Molecular Biology, University of Copenhagen. Bacteria were grown as clonal cultures. Growth media was Luria-Bertani (LB). To label bacteria, 100 µL QD-adenine conjugate was added to 0.2 mL bacterial culture and 0.4 mL nutrient medium. Growth media was Luria-Bertani (LB). For light exposure experiments, cultures were grown in translucent plastic tubes and exposed through a
10 clear plastic incubator cover to room light or a hand-held UV wand on the “long wavelength” setting (365 nm).

QD-dopamine uptake was studied in mammalian cell lines. Mouse epithelial A9 cells expressing the human D2 dopamine receptor were purchased from ATCC (ATCC CRL-10225). Negative controls included human embryonic kidney (HEK)
15 293 cells and mouse 3T3 fibroblasts. Cells were maintained in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum in a 5% CO₂ atmosphere at 37°C. For labeling with QD-dopamine, growth medium was removed by 2 washes in sterile PBS, and then replaced with 1 mL serum-free medium without phenol red. When used, β-mercaptoethanol (βME) was
20 added to a final concentration of 5 mM. 0.1 mL of the QD-dopamine conjugate was then added, the dishes were swirled for 30s, then returned to the incubator for a 1 hour incubation at 37 °C in the dark. Before imaging, the cells were rinsed twice and imaged in PBS.

Confocal images were taken of cells incubated with QDs as above, as well as
25 cells co-incubated with selected dyes. All dyes were purchased from Molecular Probes, and were added 30 min after the beginning of incubation with the QDs and allowed to remain on the cells for 30 min before washing. Dyes and final concentrations used were: the nuclear stain SYTO Red (5 µM), the lysosomal indicator LysoTracker Red or LysoTracker Blue (1 µM), and the redox sensitive dye
30 RedoxSensor Red (1 µM). In some cells, LysoTracker Blue and RedoxSensor Red were used together. Quenching of QD-fluorescence by RedoxSensor Red and LysoTracker Red was tested under Hg lamp illumination in order to unquench the

QD-dopamine conjugates. Varying amounts of dye (0.1 –5 μ M) were added to a given amount of QD-dopamine and fluorescence monitored under illumination for 2-5 minutes. The dye was considered to quench QD fluorescence if the QD emission peak remained more than five-fold weaker than an exposed spot containing

5 QD-dopamine without dye.

To alter intracellular redox potentials, cells were treated with 0.1, 1, or 10 mM glutathione monoethyl ester (GSH-MEE) or L-buthionine-sulfoximine (BSO) in DMEM for 4 hours. Following this treatment, the cells were washed, and QDs and dyes were added as above.

10 Confocal imaging of redox-labeled cells was performed on a Zeiss 510 LSM with a PlanApo 100 oil objective. QDs were excited with an Ar ion laser (458 nm line); SYTO Red, LysoTracker Red, and RedoxSensor Red were excited with a HeNe laser (543 nm line). Cells labeled with more than 1 probe were examined for channel bleed-through before imaging. When RedoxSensor was used, it was located

15 under brightfield and imaged first in order to avoid photo-oxidation and migration of the dye.

B1. QD Properties

Three distinct preparations of bare CdSe QDs were used in bacterial uptake experiments: “green” (emission peak at 575 nm; mean diameter $\pm 4.6 \pm 0.4$ nm);

20 “yellow” (emission peak at 587 nm; mean diameter 4.8 ± 0.4 nm); and “red” (emission peak at 615 nm; mean diameter 5.4 ± 0.4 nm). FIGs. 2A-2C show photoluminescence (PL) of all of these colors of QDs conjugated to adenine (“QD-adenine”) at neutral pH, where the conjugation was performed in the dark, and before measurements, all QDs were exposed to room light for 4 hours at 37 °C.

25 Also shown for comparison are conjugates to a molecule that was not an electron donor or acceptor (glucosamine). As shown in the spectra of FIGs. 2A-2C, the green QD-adenine was almost completely quenched, and the red QD-adenine conjugates were slightly brighter than the green QD-adenine or yellow QD-adenine conjugate. In contrast, the QD-glucosamine conjugates showed strong, bright

30 fluorescence for all three different colors of CdSe QDs (see FIG. 2C). These results indicate that conjugation of QDs to adenine, an electron donor, quenches the

fluorescence of QDs, but conjugation of QDs to glucosamine, a non-electron donor, does not quench the fluorescence of QDs.

Quenching of green- and yellow-emitting by adenine was quantified time-resolved emission spectroscopy. Time resolved emission data for unconjugated QDs (black open circle: O), red QD-adenine (solid line #2), and green QD-adenine (solid line #1) are shown in FIG. 3. The fast component of the green QD-adenine emission was shortened, leading to a smaller area under the curve which correlated with quenched fluorescence and supporting the electron transfer mechanism shown in FIGs. 1a-1c.

This observed quenching was correlated with uptake by bacteria. No uptake of red QD-adenine was ever observed, whereas uptake of both green- and yellow QD-adenine conjugates was efficient and reproducible. All data described below are the findings with yellow QD-adenine, as the yellow fluorescence was significantly different from bacterial autofluorescence, leading to extremely low backgrounds.

C1. Metabolism-dependent uptake of adenine and AMP conjugates by *Bacillus subtilis*

Tags, even large ones, on the amine group of adenine usually do not inhibit recognition and uptake by cells and organisms, so it is likely that specific enzymes will be able to recognize QD-adenine and QD-AMP. In these experiments, QDs-adenine and QDs-AMP conjugates were applied to living bacterial cultures to see whether metabolic processing would result in PL restoration, using *Bacillus subtilis*, a harmless soil bacterium often used as an anthrax simulant. Mutants of *B. subtilis* that were deficient in purine metabolizing enzymes, adenine deaminase (the *ade* mutant), adenine phosphoribosyltransferase (the *apt* mutant), or both (the *ade-apt* mutant) were also tested. Both single mutants were able to take up adenine, but at a lower rate than the wild type. The *ade* mutant was inhibited in the processing of adenine to hypoxanthine, and from there to ammonia or AMP. The *apt* mutant eliminated the direct pathway from adenine to AMP. The double mutant did not take up adenine, but actually excreted it. None of the strains has been known to import AMP, but could convert AMP to adenosine extracellularly. Adenosine

uptake was feedback regulated, being inhibited by abundant adenine in the medium in cells that could import adenine.

QDs-adenine and QDs-AMP conjugates were incubated for 3-5 hours under normal culture conditions with wild-type *B. subtilis* (ATCC 9372) and each of the 3 mutant strains. As a control, heat-killed wild-type was also incubated with QDs in the same fashion as the live cells. Cells were washed once with saline and subjected to epifluorescence microscopy and spectrofluorimetry. Typical results using CdSe QDs are shown in FIGs. 4a-4h. The scale bars of FIGs. 4c-4h for epifluorescence images were 5 μ m. In FIGs. 4a and 4b, all spectrofluorimetry points were averages of $n=3$ (error bars smaller than symbols).

The killed control cells demonstrated no QD-associated fluorescence with QD-adenine or QD-AMP (FIGs. 4a, 4b, 4c), even though these cells could be readily labeled with cell-wall targeted probes such as wheat germ agglutinin (WGA) (FIG. 4d). In contrast, a strong fluorescence signal was seen with wild-type incubated with QD-adenine (FIGs. 4a, 4e). None of the mutant strains were labeled with QD-adenine (FIGs. 4a, 4g). AMP-QDs, on the other hand, strongly labeled all mutant strains, especially the apt mutant (FIGs. 4b, 4h). Labeling was nearly absent in the wild type (FIGs. 4b, 4f). This indicates that the mutant strains were not simply inhibited or killed by the QDs, but that truly metabolism-specific labeling was occurring.

Under epifluorescence microscopy, labeling with QD-adenine and QD-AMP appeared throughout the cell, in contrast to what was seen with WGA-QDs, suggesting that the conjugates had been taken up by the bacteria. Labeling was associated only with cells and not the surrounding medium. These results were highly reproducible when conjugates were added to cells in logarithmic phase (48/50 cultures tested resulted in labeling). Labeling was weak or absent if conjugates were added to cells in death phase (2/20 cultures showed some labeling).

D1. Metabolism-dependent uptake of adenine and AMP conjugates by *E. coli*

Adenine auxotrophs of *E. coli* are available commercially; these strains are unable to grow without addition of adenine or AMP. Wild-type (ATCC 25922) and auxotroph (ATCC 23804) *E. coli* strains were used, and these bacteria were

incubated with QD-adenine and QD-AMP in the same fashion as described above for *B. subtilis*.

No labeling of wild-type was seen if incubated with adenine- or AMP-conjugated QDs. Similarly, labeling was not seen in the adenine auxotroph *E. coli* incubated in a LB medium. However, when incubated in a minimal medium, the auxotroph showed strong fluorescence beginning as early as 15 minutes after QD addition; labeling was similar with both adenine and AMP. Spectra and appearance did not differ significantly from those seen in *B. subtilis* except for the presence of a greater red tail in the fluorescence emission (FIGs. 5a-5c). FIG. 5a shows labeling of *E. coli* in minimal culture medium after 1 hour of incubation with yellow QDs conjugated to adenine (yellow QD-adenine) and AMP (yellow QDs-AMP): emission spectra of adenine auxotrophic mutant ATCC 23804 incubated with QDs-adenine (●) and QD-AMP (■); wild-type with QD-adenine (○) and QD-AMP (□); and heat-killed auxotroph with QD-adenine (— —). In FIG. 5a, background fluorescence was minimal, and no subtractions were done. FIG. 5b shows an epifluorescence image of wild-type *E. coli* incubated with QD-adenine for 30 minutes in a minimal growth medium. FIG. 5c shows an epifluorescence image of adenine auxotrophic *E. coli* under the same conditions as those for wild-type *E. coli*. Red-shifts were seen at cell poles (arrow). For both FIGs. 5b and 5c, scale bars were 5 μm.

E1. Confirmation of QD uptake by bacteria

Due to the small sizes of bacterial cells, confirming QD uptake was difficult, although the protoplast data were suggestive. Several different electron microscopic approaches were taken in order to observe internalized materials.

First, labeled cells were embedded into agar worms for thin sectioning and examined by low resolution TEM, resulting in images of the inside of the cells (see, for example, FIGs. 6a, 6b).

Energy-dispersive X-ray spectroscopy (EDS) was performed on 100 spots per imaged cell (spot size, 8.8 nm) to measure atomic % of Cd and Se. Cells that displayed no fluorescence (e.g., wild-type *B. subtilis* with AMP or the mutants with adenine) did not display any noticeable electron-dense areas or any measurable Cd

or Se content (less than 2% each for more than 100 spots tested per cell, more than 50 cells tested). In preparations that displayed fluorescent labeling, crystals consistent with whole QDs were seen in the cell membrane and throughout the cytoplasm. EDS analysis confirmed that these areas consisted of greater than 30 %
5 Cd and Se, with a Cd/Se ratio identical to that of original QDs exposed to culture medium (QDs-AMP exposed to medium: Cd/Se = $1.33 \pm .03$, $n = 50$; QDs-AMP internal to *apt* mutant, Cd/Se = 1.35 ± 0.02 , $n = 100$).

Another approach used was critical point drying (CPD). CPD rendered the cells electron-transparent and made internal contents visible. In control cells, the
10 separation between cell wall and plasma membrane was evident, as was the nucleoid, which appeared light in color (FIG. 6c). In QD-exposed cells, electron-dense particles were present between the cell wall and plasma membrane and inside the cytoplasm at remarkably high densities (FIG. 6d). These particles were confirmed by EDS to consist of Cd and Se at about 1:1 ratios.

15 FIGS. 6a-6d show TEM images of QDs in *B. subtilis*. All analyses shown were performed with *ade* and *apt* mutants exposed to QD-AMP. FIG. 6a shows a transverse thin section of *B. subtilis ade* mutant incubated with yellow QD-AMP, showing QDs inside and outside the cell. Visible in FIG. 6a are the cell wall (cw), cytoplasm (cyt), and cell exterior (ext). QDs were seen outside the cell (arrow) and
20 inside the cytoplasm (arrowheads). FIG. 6b is a closeup of a fraction of the cell in FIG. 6a, showing distinct particles inside the cytoplasm. In FIG. 6b, EDS values from these particles are given above, and white spot above scale bar shows size of EDS beam. FIG. 6c shows a CPD (critical point drying) image of *B. subtilis apt* mutant without QDs. FIG. 6d shows a CPD image of cell labeled with QD-AMP,
25 showing many internalized particles. In FIG. 6d, the two dark areas about 5 μm apart consisted of (atomic %): C = 47.3, O = 32.69, Mg = 1.28, P = 12.4, Ca = 4.82 (insignificant: Cd, Se, and S).

F1. Results with dopamine

30 Dopamine is generally a strong quencher of QD fluorescence, able to suppress fluorescence from both bare (CdSe) and core-shell (CdSe/ZnS) QDs. All dopamine experiments shown here were performed with core-shell QDs because the

initial fluorescence was very bright, making quenching easy to quantify. Two specific batches of core-shell QDs were used: “green” (emission peak 560 nm) and “yellow” (emission peak 590 nm). With these particles, the ability of dopamine to fully quench steady-state emission was correlated with cellular uptake by cells, consistent with what was seen with QD-adenine in bacteria.

FIGs. 7A-7B show quenching and unquenching of two colors of QDs conjugated to dopamine (DA). Yellow QDs show distinctly different responses to dopamine conjugation than green QDs, being only weakly quenched. No uptake of the yellow QD-conjugate into cells was ever seen (data not shown; $n > 50$ experiments). Shown in FIGs. 7A-7B are green QDs (left) and yellow QDs (right) in aqueous solution before conjugation (dotted) and after conjugation to dopamine (-■-■-). As shown in FIG. 7A, conjugates of green QDs to dopamine were fully quenched. In yellow-QD conjugates, quenching was much less efficient. After exposure of the quenched QD-dopamine to 60 seconds of high-intensity UV illumination (photo-oxidation), the original QDs retained their initial spectrum (see FIG. 7B) and intensity for at least several minutes under these conditions (not shown; all spectra overlapped the dashed line given). The green QD conjugate showed intense fluorescence, while yellow QD-dopamine showed weak fluorescence. This response to photo-oxidation was equivalent to the response seen in oxidizing compartments of cells (see FIGs. 10a-10c and associated text).

FIGs. 8A-8D show specific uptake of quenched green QD-dopamine in dopamine-receptor-bearing cells with fluorescence return. Return was a result of exposure to UV light on microscopic examination, but was not uniform throughout the cell. The pattern of intracellular labeling seen was consistent and reproducible. In FIG. 8A, mouse A9 cells stably transfected with human D2 dopamine receptors (ATCC CRL-10225) showed cell-related fluorescence after 1 hour of exposure to QD-dopamine and 10 seconds of exposure to Hg bulb illumination. In contrast, as shown in FIG. 8B, HEK 293 cells under the same conditions as those for mouse A9 cells stably transfected with human D2 dopamine receptors showed no fluorescence. Shown in FIG. 8C is primary neuronal culture of mixed dopaminergic/GABAergic immunoreactivity after 2 hours of exposure to QD-dopamine. In FIG. 8C, no labeling of the underlying glial layer was apparent. The large spots were aggregates

of QDs that had not been taken up by the cells. Spectra from individual cells in panels of FIG. 8A and 8B showing the fluorescence of a labeled A9 cell (A9) are compared to that of a 293 cell (293) and that of the original unconjugated QDs (QD) and QD-dopamine (QD+DA, fully quenched) in FIG. 8D.

5 FIGs. 9A-9D show cellular toxicity and its prevention with antioxidants. The addition of antioxidant drugs allowed QD-dopamine to be used as a tool for labeling cells without compromising their health or metabolism. FIG. 9A shows phase-contrast image of A9 cells after incubation with green QD-dopamine in the absence of antioxidants. Shown in FIG. 9A are membrane blebbing (rough edges)
10 and preponderance of round, very bright cells, which indicates detachment from the culture dish. FIG. 9B shows high-power brightfield image of cells under the same conditions as those for FIG. 9A. Under this high resolution, signs of cell death were apparent as blebbing (arrow) and nuclear extrusion (arrowhead). Phase contrast image of A9 cells after incubation with green QD-dopamine with the addition of 5.7
15 mM β -mercaptoethanol is shown in FIG. 9C. In FIG. 9C, smooth cell edges and absence of rounded cells were noted. FIG. 9D shows high-power brightfield image of cells under the same conditions as those for FIG. 9C. Cell health was indicated by smooth edges and lamellopodia strongly adhered to the dish.

 FIGs, 10a-10c show redox-sensitive labeling with QD-dopamine and
20 comparison with commercial redox sensor dye RedoxSensor Red. All experiments were performed with green QDs in the presence of the antioxidant β -mercaptoethanol, using A9 cells bearing dopamine receptors. Shown are cells labeled with RedoxSensor Red alone (left), QD-dopamine alone (center), and a high-power zoom of QD-dopamine with Lysotracker Red (right). Cells shown in FIG.
25 10a were under the most oxidizing conditions (10 mM BSO, a suppressor of glutathione). Both RedoxSensor Red and QDs were fluorescent throughout the cell, excluding the nucleus. Labeling for lysosomes revealed weak staining and very few punctae, suggesting lysosomal rupture. Cells shown in FIG. 10b were untreated, actively proliferating cells. RedoxSensor Red distributed to mitochondria and
30 lysosomes. With QD-dopamine, a large perinuclear "cap" was observed in all cells, with some staining throughout the rest of the cell, including structures suggestive of mitochondria. Co-labeling with Lysotracker showed that the bright cap corresponds

to lysosomes. Cells shown in FIG. 10c were in reducing environment (1 mM GSH-MEE, a cell-permeant glutathione). Labeling with RedoxSensor Red was weak and was restricted to lysosomes. QD-dopamine labeled cells had a similar appearance, and high-resolution inspection of Lysotracker-colabeled cells showed very little
5 fluorescence from QDs alone (green) and many bright co-labeled punctae (right): thus, essentially all of the QD fluorescence was lysosomal.

QDs have so far been used in the art as unreactive fluorescent labels. Herein, the ability of QDs change fluorescence in response to specific, redox-dependent metabolic mechanisms was demonstrated. The resultant background labeling was
10 extremely low. The utility of these conjugates in both bacteria and mammalian cells was demonstrated. This can allow QDs to replace radioactive labels of metabolism and allow for visual identification of the function of a single gene in a cell (e.g., adenine phosphoribosyltransferase or glutathione).

15 Example 2. Bare QDs Embedded in Biological Membranes As Ion and/or Voltage-Sensitive FRET Sensors

This Example illustrates that hydrophobic QDs act as FRET donors or acceptors, within a model membrane system using biomimetic lipid vesicles. Experiments herein show that hydrophobic QDs can associate within lipid
20 membranes without significant damage to or leakage of the membrane. Also demonstrated herein is delivery of hydrophobic QDs and membrane dyes to mammalian cells, including primary neurons in culture. Voltage-dependence of the fluorescence in these systems was observed using patch-clamp and pharmacological techniques.

25 Organic voltage-sensitive dyes, which are lipophilic fluorophores that change spectrum according to the potential drop across a lipid bilayer, have been used in the art to measure activity in neurons and cardiac cells. Their usefulness is, however, greatly limited by the small size of their spectral changes with voltage and the lack of quantitative understanding of their properties.

30 Herein, the possible utility of QDs as voltage-sensitive probes was tested in three ways. The model system made use of the same biomimetic vesicles as in the experiments using DiD (1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine, 4-

chlorobenzenesulfonate) and Cy 3.5; a Nernst potential was generated across the vesicle bilayer by varying the concentrations of K^+ ions internal and external to the vesicles, and creating charge separation using a pore-forming peptide that was selective for K^+ ions (valinomycin). QDs themselves, in this model system, showed insignificant spectral shifts with voltage, indicating that features of the semiconductor particles such as the Stark shift were too small to be measurable here.

A second approach was to embed both QDs and the most commonly used voltage-sensitive dyes. In a specific example, both QDs and (3-(4-(2-(6-(dibutylamino)-2-naphthyl)-trans-ethenyl)pyridinium) propanesulfonate [di-4-ANEPPS]) were embedded into the same vesicles. Very large shifts in emission spectra were seen upon addition of valinomycin; results depended strongly upon K^+ concentration. Although interpretation of results were generally difficult due to the extremely broad absorption and emission spectra of di-4-ANEPPS, the observed shifts appeared to result from direct interactions between K^+ , ANEPPS, and QDs rather than from the absolute value of the Nernst potential.

The QDs and di-4-ANEPPS were then delivered to cells in culture. In non-excitabile cell lines, voltage dependence of this combination of fluorescence probes was confirmed by altering membrane potential using patch-clamp. In excitable cells (neurons), application of pharmacologic agents that changed membrane potential led to rapid and reversible changes in fluorescence of the di-4-ANEPPS/QD combination.

A2. Experimental section

QDs in this study were synthesized as known in the art (see, for example, Wong, M. S.; Stucky, G. D. *Abstracts of Papers of the American Chemical Society* **2001**, 221, U572 and Kloepper, J. A.; Mielke, R. E.; Wong, M. S.; Neilson, K. H.; Stucky, G.; Nadeau, J. L. *Appl Environ Microbiol* **2003**, 69, 4205, the entire teachings of which are incorporated herein by reference). To form a ZnS shell, a hot reaction mixture containing Cd(II) and selenide sources was allowed to cool to about 180 °C and 5 mL of ZnS precursor (hexamethyldisilathiane and dimethylzinc in trioctylphosphine [TOP]) was added drop-wise over the course of 10 to 15 minutes and baked for 1-2 hours. Resulting QDs were washed several times in methanol and

dissolved in CH₂Cl₂. This yielded CdSe/ZnS nanocrystallites passivated with trioctylphosphine oxide (TOPO). TOPO was removed by 2 washes in hexane/pyridine. Fluorescence quantum yield was not changed significantly by the TOPO removal. Quantum yield was measured at an excitation wavelength of 450 nm using
 5 a standard with a published value of 1 (rhodamine 101 in ethanol). The formula:

$$QY = QY_R \frac{I}{I_R} \frac{OD_R}{OD} \frac{n^2}{n_R^2} \quad (1)$$

was used where “R” refers to rhodamine and n to the indices of refraction of the respective solutions (1.424 for CH₂Cl₂, 1.3605 for ethanol). Resulting values were 10% for “green” QDs, emission peak 570 nm; 12% for “red” QDs, emission peak
 10 620 nm). The stock solutions had a concentration of about 1 μM based upon mean particle size (3.5 nm, determined by high resolution electron microscopy) and assumed extinction coefficient ($10^5 \text{ cm}^{-1} \text{ M}^{-1}$ at the first exciton peak).

To form vesicles with QDs, 10 μL of stock QD solution was added to 100 μL of the lipid 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) in chloroform
 15 (Avanti Polar Lipids #850375, Alabaster, AL). The solution was dried under N₂, suspended in 1 mL buffer, and ultrasonicated until cloudy. The suspension was subjected to 3 rounds of freeze-thaw in liquid N₂, and resulting vesicles were inspected by epifluorescence microscopy and transmission electron microscopy (TEM). An Akashi EM-002B microscope operating at 100 kV was used to for TEM
 20 and also for energy dispersive X-ray spectroscopy (EDS). The area sampled by the Oxford spectrum analyzer is approximately 8.8 nm, and wt% of greater than 2% Cd or Se was taken as significant. Acquisition rates were maintained at 10-20% deadtime with 60 s of lifetime at 83 kx. The electron beam was defocused at the condenser lens to maintain counting rates below 1 kHz and live time efficiency
 25 greater than 95%.

Vesicle preparations were visualized three ways: unstained; negative stained; and thin-sectioned (to determine location of the particles within the membrane). Preparation of unstained mounts was performed by depositing 10-20 μL of fresh (collected within 2 hours) vesicles in buffer onto carbon-coated Cu grids. After 2

minutes, the excess solvent was wicked away with filter paper, leaving behind vesicles deposited on the grid surface. After being allowed to air dry, the grids were washed 2-3 times in deionized H₂O to remove any traces of buffer. Negatively-stained preparations were prepared by staining on the grid with 2% uranyl acetate for 30s after the sample was dry. In order to prepare thin sections, the vesicles were sedimented by centrifugation (2 minutes at 14,000 rpm in a tabletop microcentrifuge) and the pellet dispersed in Noble agar worms of desired length. The samples were stained by post fixing in osmium tetroxide and staining with uranyl acetate; they were then dehydrated in ethanol and acetone before embedding in EPON resin. Sample resin blocks were trimmed and sectioned (50-60 nm) on a MT-X Ultramicrotome with a 45° Diatome diamond knife. Ultrathin sections were placed on 200 mesh formvar/carbon coated copper grids. The pre-stained ultrathin section samples were subsequently post-stained with 2% uranyl acetate before final imaging.

Buffers used were “low K⁺”: 1 mM KCl, 0.2 mM EGTA, 20 mM HEPES, sucrose; and “high K⁺”: 150 mM KCl, 5 mM EGTA, 20 mM HEPES; osmolarity was adjusted to within ± 2 mOsm with sucrose. After the final round of freeze-thaw, vesicles were diluted 1:10 into the appropriate buffer in a 96-well plate with 300 μ L wells. Experiments using Cy 3.5 and DiD were performed using low K⁺ buffer inside and outside the vesicles; experiments using di-4-ANEPPS were performed at varying K_{in}/K_{out}. Emission spectra were taken on a Gemini EM plate reader in epifluorescence mode. Dyes were added to the wells as stock solutions: Cy3.5, 10 μ M in buffer; DiD, 10 mg/mL in DMSO diluted to 0.1 mg/mL (about 0.1 mM) in buffer; di-4-ANEPPS, 2 mM in ethanol, diluted 0.1 μ M in buffer. All dyes were added in 1 μ L increments to the experimental wells (DiD control wells received diluted DMSO only and ANEPPS controls received ethanol only). Controls for the lipid-soluble dyes were lipid vesicles containing no QDs. The Cy 3.5 used was conjugated to a DNA oligomer (22 base pairs in length), but is simply referred to as “Cy 3.5” herein.

For voltage-dependence experiments using di-4-ANEPPS, spectra were collected with and without 1 μ M valinomycin in DMSO. Spectra were stable for at least 5 h after addition of valinomycin. Spectra shown in Results reflect an

excitation wavelength of 440 nm; spectra were also taken at 360 nm, 400 nm, and 530 nm. Absorbance spectra were recorded with a Hewlett Packard 8453 UV-visible spectrophotometer. Excitation spectra were taken on a Gemini EM plate reader in Excitation mode using much lower concentrations of QDs than were used for
5 absorbance spectra (optical density of solutions less than 0.05). This avoided artifacts but resulted in excitation spectra in which typical absorbance peaks could not be seen (see the discussions below).

Experiments with cell lines were performed using mouse epithelial A9 cells bearing D2 dopamine receptors (ATCC CRL-10255), maintained at 37 °C/5% CO₂
10 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Primary rat cortical neurons were a gift of F. K. Bedford (McGill University). For delivery of hydrophobic QDs to cells, 5 µL of a highly concentrated (10-30 µM) QD solution in pyridine was dissolved in 1 mL dimethylsulfoxide (DMSO) containing 1 % Pluronic (Dupont). After a well-
15 dispersed, lightly pigmented solution was obtained, a concentration of 1-3 µL/mL was added to serum-free culture medium, applied to washed cells, and rotated gently for 1-2 minutes before returning the cells to the incubator. When di-4-ANEPPS was used, labeling with QDs in Pluronic was performed after labeling with di-4-ANEPPS. Di-4-ANEPPS was stored at 4 °C as a 2 mM stock solution in ethanol, and
20 was added to cells to a final concentration of 2 µM for 1-2 hours.

Path-clamp recordings from single cells were performed with a HEKA EPC-10 Double patch clamp amplifier using the acquisition software PatchMaster. For single-cell recordings, cells were visualized on an Olympus IX-71 inverted microscope enclosed in a Cu mesh Faraday cage and floated on an air table (Kinetic
25 Systems). The bathing solution consisted of (in mM) 150 NaCl, 5 KCl, 4 MgCl₂, 2 CaCl₂, 10 HEPES, pH 7.4, and the pipette solution 140 CsCl, 1 EGTA, 10 HEPES, pH 7.4. Pipettes were pulled on a Sutter P-97 puller and had a resistance of 4-6 MΩ when filled with recording solution. Data were acquired at 10 kHz and filtered at 2.9 kHz; simultaneous spectral data were obtained from a Nuance imaging system
30 (Cambridge Research Instruments).

B2. Spectra of Vesicle-Embedded QDs

Emission spectra of vesicle-embedded QDs were not changed from those of the original QDs, showing broad absorption to the band edge and narrow emission. Significant overlap was seen between the emission of green QDs and Cy 3.5, and of both red and green QDs and DiD (FIG. 11A, 11B). With the voltage-sensitive dye di-4-ANEPPS, it appeared that QDs functioned as acceptors rather than donors (FIG. 11C).

FIGs. 11A-11C show absorbance and emission spectra of green-emitting QDs (absorbance: #1; emission: #2), red-emitting QDs (absorbance: #3; emission: #4) and dyes (absorbance: #5; emission: #6), where y-axes are arbitrary units. As shown in FIG. 11A, green-emitting QDs were able to function as donors to DiD, but not so efficiently as red QDs. As shown FIG. 11B, only green-emitting QDs showed spectral overlap with Cy 3.5. The spectrum of di-4-ANEPPS at 0 transmembrane potential showed a potential role of QDs as acceptors but not as donors (FIG. 11C). The vertical lines indicated the excitation wavelengths used in this study: 400 nm, 440 nm, and 530 nm (not shown but also tested: 360 nm). Shifts in spectrum due to the membrane potentials used in this study (± 126 mV, see results) were not large enough to affect the overlap significantly.

C2. FRET to DiD and Cy3.5

No changes in emission spectrum were seen upon addition of any dye to QDs in vesicles when the QDs remained passivated with trioctylphosphine oxide. In addition, even after removal of this inorganic layer by pyridine wash, no quenching of QD emission was seen upon addition of Cy3.5 to red QD-vesicles, up to a concentration of 150 μ M. With washed QDs, significant quenching of QD fluorescence and associated increase in dye emission were seen with green QDs and Cy3.5, and with both red and green QDs and DiD (FIG. 12A-12C). FIGs. 12A-12D show FRET between QDs in vesicles and dyes in lipid and in solution at an excitation wavelength of 400 nm for: QDs alone (#1); then after addition of 3 (#2), 6 (#3), 9 (#4), 12 (#5), and 15 (#6) μ L of a stock solution (10 μ M Cy 3.5; 0.1 mM DiD). Background, represented by directly-excited dye and vesicle scatter, was subtracted from all traces. FIG. 12D shows a fraction of QD quenching that was due to FRET (f , dimensionless) plotted against dye concentration in relative units for red

QDs and DiD in vesicles (Red-DiD); green QDs and DiD in vesicles (Green-DiD); and green QD in vesicles with Cy3.5 in external solution (Green-Cy3.5). Shown for comparison (dashed line) were values from another study in which QDs acted as donors to tetramethylrhodamine in solution.

5 Using the formula

$$f = \frac{\Delta I_{FRET}^{dye} \phi^{QD}}{\Delta I_{quench}^{QD} \phi^{dye}} \quad (2)$$

yielded values of fluorescence quenching due to FRET of 22-30% for Green QD-Cy3.5, from 27-31% for Green QD-DiD, and from 67 to 98% for red QD-DiD across the dye concentration ranges, using a recently published value of 12% for the quantum yield of DiD in lipid, and a value for Cy 3.5 of 15% (Fig 12D). These data imply highly efficient energy transfer between QDs and the lipid-soluble dye DiD; the difference between the red and green QDs reflects the greater spectral overlap between the emission of the red QDs and the absorbance of the dye. There was no immediate large QD quench that was non-FRET-related. This probably reflects the lack of QD clumping and other nonspecific mechanisms that occurred when the QDs float free but not when they were embedded in vesicles.

D2. TEM Examination of Vesicles by Whole Mount and Thin section

The relatively low values of FRET for Cy3.5, that were relatively independent of concentration, suggested that only a small fraction of the QDs in vesicles were accessible to this water-soluble dye. Similar results were seen with the Cy3.5 inside rather than outside the vesicles, suggesting that most of the QDs were inside the lipid bilayer and did not contact the aqueous solution. In order to confirm this, vesicles were inspected by TEM as whole mounts, as well as embedded and sectioned into 50-100 nm thick sections for TEM examination. Resulting images showed dark particles, confirmed by EDS to consist of Cd and Se at an about 1:1 ratio, located primarily between vesicle leaflets (FIGs. 13A-13F).

FIGs. 13A, 13B, 13D and 13E show TEM images of whole and thin sectioned vesicles. In FIG. 13A, a negatively-stained whole vesicle at low resolution showed dark spots "peppered" over the surface. These spots had greater

than 30% each Cd and Se. FIG. 13B shows a thin section through the vesicle of FIG. 13A at the same magnification, showing dark areas associated with the lipid membrane. A schematization of FIG. 13B is shown in FIG. 13C, showing the location of QDs within the lipid bilayer. The dye DiD also localized within the lipid bilayer. The vesicle was immersed in an aqueous external solution that might contain Cy3.5. FIG. 13D shows a thin section through a multilamellar vesicle, showing multiple layers of lipid, all of which contained QD materials. FIG. 13E is a close-up of FIG. 13D, showing QD material concentrated within the lipid (narrower layers) rather than in the aqueous solution between the lamellae (wider layers). FIG. 13F shows a schematization of FIG. 13E, showing the multiple layers of lipid (black lines) containing QD material in aggregates of various sizes.

E2. FRET Between QDs and di-4-ANEPPS

For studies of energy transfer between QDs and di-4-ANEPPS, vesicles were prepared containing high- K^+ and low- K^+ solutions, and dissolved in either high K^+ solution, low K^+ solution, or a 50:50 mix, for a total of 6 sets of vesicles (low K_{in} /low K_{out} ; high K_{in} /high K_{out} ; low K_{in} /high K_{out} ; high K_{in} /low K_{out} ; high K_{in} /50:50 K_{out} ; and low K_{in} /50:50 K_{out}). In the absence of a charge carrier, e.g. a pore-forming peptide, the Nernst potential across the membrane of all of these vesicles was 0. However, if a K^+ ion channel was added to the membrane, a potential was generated according to the usual Gibbs free energy equation:

$$V = \frac{RT}{F} \ln \frac{K_{out}}{K_{in}} \quad (3)$$

The K^+ channel used in these studies was the peptide antibiotic valinomycin. While the $V = 0$ case, just before the addition of valinomycin, was used as a control, simply varying K^+ concentrations without charge separation. Vesicles containing ANEPPS would be sensitive to external ionic concentrations as well as to transmembrane potential.

Both ANEPPS and QDs were tested for function as energy acceptors, beginning by an examination of excitation spectra. At a wavelength where the

acceptor emitted but the donor did not, the magnitude of the excitation spectrum of the energy acceptor (A) could be determined by the expression $A = \varepsilon_A + f \varepsilon_D$, where f was the transfer efficiency and ε_A and ε_D were the extinction coefficients of the energy acceptor and donor. When there was no FRET, the excitation spectrum was
 5 identical to the absorption spectrum of the energy acceptor.

The hypothesis that QDs could act as energy donors to ANEPPS was tested first. Both excitation and emission spectra were examined. Excitation spectra were taken with emission wavelengths beyond the red edge of the QD emission: 650 nm (for red QDs) and 635 nm (for green QDs). Results revealed no energy transfer:
 10 excitation spectra were equivalent to those of ANEPPS alone. Spectra changed by less than 1 % upon addition of valinomycin (spectra available in Supplementary Information).

The possibility of QDs behaving as acceptors had not been eliminated. Results for this test are shown in FIGs. 14A-14G. FIGs. 14A-14G show excitation
 15 spectra at $\lambda_{Em} = 610$ nm of vesicles containing red QDs, ANEPPS, or both. In these spectra, the y axes are arbitrary units but relative strengths are preserved. Valinomycin was present; concentration of ANEPPS was 5 μ M. QDs were highly diluted so that their typical absorbance peaks were not seen. FIGs. 14A-14C show emission spectra for low K^+ (mM) both inside and outside (low K_{in} /low K_{out} : 1/1; ●);
 20 high K_{in} /low K_{out} (150/1; ■); high K_{in} /high K_{out} (150/150,+); and low K_{in} /high K_{out} (1/150, ▲): FIG. 14A, ANEPPS alone, FIG. 14B, red QDs alone, and FIG. 14C, red QDs+ANEPPS. FIGs. 14D-14G show comparison of spectra containing QDs alone (■), ANEPPS alone (●), and QDs+ANEPPS (▲): FIG. 14D, low K^+ both inside and outside (1/1); FIG. 14E, high K_{in} /low K_{out} (150/1); FIG. 14F, low K_{in} /high K_{out}
 25 (1/150); and FIG. 14G, high K_{in} /high K_{out} (150/150).

For excitation scans of FIGs. 14A-14G, the emission wavelength was chosen to be the red QD emission peak, 610 nm. At this wavelength, vesicles containing ANEPPS alone excited efficiently and showed a strong K^+ dependence: the excitation peak was at least 2-fold higher when external K^+ was high, irrespective of
 30 voltage (FIG. 14A). QDs showed somewhat of a similar effect, with strongest excitation when both voltage and external K^+ were high (FIG. 14B). It was notable

that the exciton peak visible in the QD absorbance spectrum (refer again to FIGs. 11A-11C) did not appear on these excitation scans.

When both QD and ANEPPS were in vesicles, the difference between high and low external K^+ was greatly lessened, with moderate qualitative changes to the shape of the spectra (FIG. 14C). Seen alternatively, this meant that at the emission wavelength of 610 nm, there was a large difference between the excitation spectra of ANEPPS alone and QD+ANEPPS only when external K^+ was low (FIG. 14D). When either internal or external K^+ was high, there was a moderate change to the ANEPPS spectrum when QDs were added: this could either be a spectral enhancement (FIG. 14E) or a suppression (FIG. 14F). However, when both internal and external K^+ were high, the absorbance of vesicles containing both QDs and ANEPPS (QD + ANEPPS) was almost indistinguishable from that of ANEPPS alone (FIG. 14G).

The most striking spectral differences between ANEPPS alone and QD + ANEPPS were seen with low K^+ both external and internal to the vesicles. The differences between the excitation spectra of QD + ANEPPS and ANEPPS alone revealed at least 2 processes that were occurring. In the low K_{in} /low K_{out} case, the difference was a sum of QD excitation and ANEPPS excitation. That is, the presence of the QDs appeared to mitigate the reduction in ANEPPS excitation due to low K^+ , returning the dye almost to its high K^+ performance. In addition, QDs did seem to act as energy acceptors to some extent, resulting in an increase in QD associated excitation; however, this effect was much smaller than that of ANEPPS enhancement. With high K_{in} and low K_{out} , only an increase in QD excitation was seen, with no apparent ANEPPS component. High K_{in} /high K_{out} appeared similar, but there was some degree of overall excitation suppression. In the low K_{in} /high K_{out} case, any possible QD component was swamped by a large ANEPPS suppression.

All of the subtraction spectra are shown in FIG. 15A, with the spectra of QD vesicles alone and ANEPPS alone included for comparison. FIG. 15A shows excitation spectral differences (y axis is arbitrarily scaled but included to show the position of 0) between vesicles with ANEPPS and red QDs and ANEPPS alone at $\lambda_{Em} = 610$ nm. Differences are shown for low K^+ both inside and outside (1/1; ●); high K_{in} /low K_{out} (150/1; ■); high K_{in} /high K_{out} (150/150,+); and low K_{in} /high K_{out}

(1/150, ▲). Shown for comparison are the excitation spectra of QDs only (---) and ANEPPS only (— — -). Also shown is a schematic of ANEPPS binding to lipid bilayers that explains its different response to ions inside vs. outside the vesicle (FIG. 15B, 15C). A probable orientation of ANEPPS binding to lipid bilayers and a presumed location of quantum dot are shown in FIG. 15C. In FIG. 15C, shown are the polar headgroups (ovals) and hydrophobic tails (wavy lines) of the lipid bilayers, the approximate relative size and position of a hydrophobic QD (grey circle), and a blown-up image (center) showing ANEPPS binding to both the polar headgroups and hydrophobic tails.

10 It thus appeared that while the presence of ANEPPS might cause increased QD-associated excitation, the predominant feature of the spectra was a decrease in K^+ sensitivity of ANEPPS in the presence of QDs. Emission spectra and epifluorescence microscopy were performed to confirm or deny these observations. Because of the broad QD absorption, choosing a wavelength at which only di-4-
15 ANEPPS was excited was not possible. Four wavelengths were investigated: three at which QDs excited efficiently and ANEPPS did not, 360 nm, 400 nm, and 530 nm; and one at which both excited efficiently, 440 nm. No significant changes with K^+ concentrations or valinomycin addition were seen with any excitation wavelength other than 440 nm.

20 Emission spectra at 440 nm excitation ($\lambda_{Ex} = 440$ nm) of vesicles containing both red QDs and di-4-ANEPPS in solutions containing different values of internal and external K^+ are shown in FIGs. 16A and 16B. In these spectra, the y axes were in arbitrary units with numbers included to permit comparison. The traces shown were averages of 3 independent experiments with error bars smaller than the
25 symbols; the numbers give K_{in}/K_{out} in mM. As shown in FIG. 16A, in the absence of valinomycin, vesicles in osmotically-balanced solutions with different values of K_{in}/K_{out} showed the same emission peak (— “no Val”). Upon valinomycin addition, the largest spectral change was seen in vesicles with low K^+ both inside and outside (1/1; ●); a smaller change in high $K_{in}/$ low K_{out} (150/1; ■); a very small
30 change in high $K_{in}/$ high K_{out} (150/150,+); and no significant change in low $K_{in}/$ high K_{out} (1/150, ▲). Subtraction of the “no Val” background showed that the

fluorescence change was broad and centered about 610 nm, or slightly bluer than the original QDs (original peak 620 nm) (FIG. 16B). *

At 440 nm excitation, vesicles with low K_{in} /low K_{out} exhibited visibly brighter fluorescence than any of the other vesicle preparations (FIG. 16 A).

5 Interestingly, because the Nernst potential was 0 in this case, the effect was dependent upon the presence of valinomycin: in the absence of valinomycin, the emission spectra of all of the vesicle preparations were indistinguishable. Corresponding with the excitation spectra, the vesicles with high external K^+ showed weak or imperceptible changes when valinomycin was added (FIG. 16 B).

10 The observed spectral variations differed quantitatively and qualitatively from the classic voltage-related spectral changes of ANEPPS; the latter were detectable by our fluorescence techniques, but only represented a about 5% shift in spectral peak. Vesicles containing ANEPPS only did not change visually upon epifluorescence inspection when valinomycin was added, irrespective of the
15 solutions inside or outside. In contrast, vesicles containing QD and ANEPPS in low K_{in} /low K_{out} demonstrated rapid visible brightening and spectral shift when valinomycin was added (FIGs. 17A and 17B).

FIGs. 17A and 17B show epifluorescence of vesicles with red QD and 5 μ M di-4-ANEPPS before and after valinomycin addition (scale bar=5 μ m). Internal and
20 external solutions were both 1 mM KCl: FIG. 17A, before addition of valinomycin. FIG. 17B, after addition of valinomycin. As shown in FIGs. 17A and 17B, after valinomycin was added, a substantial increase in fluorescent intensity and a blue-shift were observed. The images of FIGs. 17A and 17B were taken with identical gain and exposure times and unprocessed.

25 The excitation difference spectrum in FIG. 15A seemed to imply that the spectral changes seen in low K_{in} /low K_{out} represented energy transfer to QDs as well as changes in efficiency of ANEPPS excitation. If this were the case, the emission spectra should reflect the sum of ANEPPS and QD emission at varying intensities. In addition, the spectral changes seen should be dependent upon the concentrations
30 of ANEPPS and QDs.

To test this hypothesis, we measured spectra across a 32-fold range of ANEPPS concentrations, using low K_{in} /low K_{out} solutions only. Spectra of vesicles

containing QD with ANEPPS (QD + ANEPPS) or ANEPPS alone, in the presence of valinomycin are shown in FIGs. 18A-18C. In these spectra, the y axes are in arbitrary units with the positions of the zeroes shown. In FIG. 18A, concentrations of ANEPPS ranged from 0 μ M to 7 μ M for vesicles containing QDs (—, concentrations on right hand side) or vesicles without QDs (---, concentrations on left hand side). Subtraction of the directly-excited ANEPPS dye from the QD with ANEPPS vesicles showed a concentration-dependent quenching, as shown in FIG. 18B. The peak of the directly-excited QDs was apparent (arrow). As shown in FIG. 18C, subtraction of the directly-excited QD peak from FIG. 18B yielded a broad peak centered about 605 nm.

At all ANEPPS concentrations, vesicles containing both QDs and were dimmer than those containing ANEPPS alone (FIG. 18A). In addition, the spectrum in the presence of QDs showed a significantly redder component than the dye alone, one that was not accounted for by direct QD excitation (FIG. 18B, 18C).

The subtraction spectra of QD + ANEPPS shown in FIG. 18C could not be fit to the ANEPPS spectrum alone: that is, the dimming of vesicles containing QDs was not due simply to quenching of ANEPPS. A reasonable fit could only be obtained by introducing an “unknown” spectrum and solving the equation

$$Difference(\lambda) = \alpha[ANEPPS] + \beta[U] \quad (4)$$

where α , and β are amplitudes, [ANEPPS] represented the amount of ANEPPS quenching at a given concentration, and [U] the unknown spectrum. This provided a unique fit to FIG. 18C with a fraction of the ANEPPS quenching ranging from 20%-82% as the ANEPPS concentration rose. The “unknown” spectrum was enhanced as ANEPPS was quenched (FIG. 19A).

The interpretation of the meaning of this unknown spectrum is not entirely known. However, the use of green QDs in a series of identical experiments yielded a solution for an unknown that was different than that obtained with red QDs (FIG. 19B). Hence it is likely that this enhanced spectrum reflects some form of energy transfer between QDs and ANEPPS. Relatively few studies have been performed with QDs as energy acceptors; in most of these, the QDs were embedded in a thin

polymer film. Energy transfer was attributed both to dipole-dipole Förster mechanisms and to exciton diffusion; in the latter case, unexplained spectral shifts were also seen.

Herein was demonstrated that efficient FRET occurred between QDs
5 embedded in vesicles and a lipid-soluble dye (DiD). Less efficient but non-negligible FRET was seen to a water-soluble dye, Cy 3.5, which interacted with that fraction of the embedded QDs that were sufficiently close to the aqueous solution. Leakage of vesicles was not seen over a time course of several hours. This work opens a way towards using QDs within the membrane leaflets of biological cells,
10 where they can act as FRET donors either to naturally occurring molecules (e.g., proteins), to genetically engineered constructs (e.g., proteins linked to a fluorescent protein), or to organic dyes added to the membrane or to the inside or outside of the cell.

Intriguing spectral changes were seen with QDs in vesicles and the voltage-sensitive dye di-4-ANEPPS. Energy transfer occurred between the QDs and the dye
15 in the presence of valinomycin, presumably with the dye acting as a donor and the QDs as an acceptor. The spectrum that was enhanced with ANEPPS quenching was shifted from the original QD spectrum. The direction of the shift, but not its magnitude, was dependent upon the spectrum of the original QDs.

20 Also, It has been shown the magnitude of the spectral shift with valinomycin addition was dependent upon KCl concentration, but substantially not upon membrane potential. Negligible changes were seen upon valinomycin addition to vesicles in high K^+ (150 mM) external solution.

Excitation spectra were able to eliminate ANEPPS behaving as an energy
25 acceptor, since at wavelengths at which ANEPPS emitted but QDs did not, the excitation spectra were simply equivalent to those of ANEPPS. However, at a wavelength at which both emitted, excitation spectra were seen that were consistent with the observed changes in emission. Low external K^+ efficiently suppressed ANEPPS excitation; addition of QDs removed this suppression, which resulted in a
30 series of spectra that changed qualitatively based upon K^+ concentration.

F2. Delivery to Mammalian Cells

QDs capped with pyridine and dissolved in a solution of Pluronic were found to assemble spontaneously into cell membranes, including those of primary neurons (FIG. 20A). Membrane potential could be controlled with high spatial and temporal resolution by targeting of individual cells for patch-clamp analysis and simultaneous multispectral imaging. When QDs and ANEPPS were delivered to cells, strong, reversible, reproducible changes in emission spectrum with voltage were observed (FIGs. 20B, 20C). As shown in FIG. 20A, epifluorescence image of primary rat cortical neurons in culture (3 days *in vitro*), where hydrophobic QDs were delivered, showed strongest QD fluorescence from the cell edges and patchy labeling in the dendrites. As shown in FIG. 20B showing epifluorescence image of mouse epithelial (A9) cell line containing both QDs and di-4-ANEPPS, fluorescence was enhanced in clamped cell in a depolarized state (holding +50 mV) (arrow; the bright spot is the recording electrode; the presence of the electrode also leads to a blurred image). Spectral signals from the cell held at +50 mV (dotted) and an unclamped neighbor cell (solid) are shown in FIG. 20C. These signals were reproducible over at least ten cycles of polarization and depolarization.

In spontaneously excitable cells (neurons), the same spectral changes as observed in FIGs. 20A-20C could be evoked with agents that caused firing of action potentials (FIGs. 21A-C). Voltage-dependent effects of QD-dye FRET seen in primary rat cortical neurons in culture (12 days *in vitro*, scale bar = 20 μ m) is shown in FIGs. 21A and 21B. Spectra obtained from liquid crystal filter for selected cells within the culture are shown in FIG. 21C. As shown in FIG. 21C, addition of di-4-ANEPPS alone ("dye") or QDs alone ("QD") led to classic spectral peaks in the cell membrane. When both were present in a resting cell with 5 mM external KCl (expected membrane potentials $\sim$ -60 mV), a broad peak somewhat larger than the sum of the di-4-ANEPPS and QD peaks alone was seen ("QD+dye"). When the cell was depolarized with glutamate, a dramatic blue-shift and increase in intensity were observed on the edges of the cells ("+glu").

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled

- 43 -

in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A composition comprising:
 - 5 a) a semiconductor nanocrystal; and
 - b) one or more electron donors or acceptors conjugated with the nanocrystal, wherein the conjugation of the nanocrystal and electron donors or acceptors quenches fluorescence emission of the nanocrystal.
- 10 2. The composition of Claim 1, wherein the electron donors or acceptors are metabolizable by a living cell or organism.
3. The composition of Claim 2, wherein the semiconductor nanocrystal is
 - 15 capped by a capping material of the formula $Q[(CRR')_nCO_2H]_m$ or a salt thereof, where:

$n=1, 2, 3, 4$ or 5 ;

Q is $HS-$, H_2N- , $O=P-$, or $P<$;

R and R' are independently $-H$, substituted or unsubstituted alkyl, or substituted or unsubstituted aryl; and

m is 1 when Q is $HS-$, H_2N- , or $O=P-$ or m is 3 when Q is $P<$.
4. The composition of Claim 3, wherein the capping material is mercaptoacetic acid.
- 25 5. The composition of Claim 2, wherein the composition includes electron donors.
6. The composition of Claim 2, wherein the composition includes electron
 - 30 acceptors.

- 45 -

7. The composition of Claim 5, wherein the electron donors are selected from the group consisting of adenine, nicotinamide adenine dinucleotide, dopamine, tryptophan and tyrosine.
- 5 8. The composition of Claim 6, wherein the electron acceptors are selected from the group consisting of benzoquinone, flavin, flavin adenine dinucleotide, flavoproteins, hydroquinone and sulfur containing proteins.
9. The composition of Claim 2, wherein the semiconductor nanocrystal is
10 CdSe.
10. The composition of Claim 2, wherein the semiconductor nanocrystal is CdSe with a ZnS shell.
- 15 11. A composition comprising:
 - a) an uncapped semiconductor nanocrystal; and
 - b) a lipid membrane, wherein the uncapped semiconductor nanocrystal is embedded in the lipid membrane.
- 20 12. The composition of Claim 11, wherein the uncapped semiconductor nanocrystal is a fluorescence resonance energy transfer donor or acceptor.
13. The composition of Claim 12, further including an organic dye that has a lipophilic fluorophore.
- 25 14. The composition of Claim 13, wherein the fluorescence resonance energy transfer occurs between the uncapped nanocrystal and the organic dye.
15. The composition of Claim 11, wherein the semiconductor nanocrystal is
30 CdSe.

- 46 -

16. The composition of Claim 11, wherein the semiconductor nanocrystal is CdSe with a ZnS shell.
17. The composition of Claim 13, further comprising a lipid vesicle, wherein the semiconductor nanocrystal is embedded in the membrane of the vesicle.
18. The composition of Claim 17, further comprising a pore-forming peptide that is introduced into the vesicle.
19. The composition of Claim 18, wherein the pore-forming peptide is selective for potassium ion.
20. The composition of Claim 19, wherein the pore-forming peptide is valinomycin.
21. The composition of Claim 13, wherein the organic dye is a lipid-soluble dye, the lipid-soluble dye being embedded within the membrane.
22. The composition of Claim 21, wherein the organic dye is an (aminonaphthylethenyl pyridinium) dye.
23. The composition of Claim 13, wherein the organic dye is a water-soluble organic dye, the water-soluble dye being in an aqueous environment near the membrane.
24. The composition of Claim 23, wherein the organic dye is a cyanine dye.
25. An ion-, or voltage-, or ion and voltage-sensitive probe for a transmembrane or intramembrane process in a cell or an organism, comprising:
- a) an uncapped semiconductor nanocrystal; and
- b) a lipid membrane, the uncapped nanocrystal being embedded in the membrane, wherein fluorescence emission mediated by the uncapped

nanocrystal changes with a voltage change or an ion-concentration change across the membrane or both a voltage change and an ion-concentration change across the membrane.

- 5 26. The probe of Claim 25, wherein the uncapped semiconductor nanocrystal is a fluorescence resonance energy transfer donor or acceptor.
27. The probe of Claim 26, further including an organic dye that has a lipophilic fluorophore.
- 10 28. The probe of Claim 27, wherein the fluorescence resonance energy transfer occurs between the uncapped nanocrystal and the organic dye.
29. The probe of Claim 25, wherein the semiconductor nanocrystal is CdSe.
- 15 30. The probe of Claim 25, wherein the semiconductor nanocrystal is CdSe with a ZnS shell.
31. The probe of Claim 25, wherein said cell is an excitable cell.
- 20 32. The probe of Claim 31, wherein the excitable cell is neuron or a muscle cell.
33. The probe of Claim 27, wherein the organic dye is a lipid-soluble dye, the lipid-soluble dye being embedded in the membrane.
- 25 34. The probe of Claim 33, wherein the organic dye is an (aminonaphthylethenyl pyridinium) dye.
35. The probe of Claim 27, wherein the organic dye is a water-soluble organic dye, the water-soluble organic dye being in an aqueous environment near the
- 30 membrane.

36. The probe of Claim 35, wherein the organic dye is a cyanine dye.
37. A method for fluorescence internal labeling a living cell or organism, comprising the steps of:
- 5 (a) providing a conjugate of a semiconductor nanocrystal with one or more electron donors or acceptors, fluorescence emission of the nanocrystal being quenched by the conjugation; and
- (b) incubating a plurality of the conjugates with the living cell or organism, whereby one or more of the conjugates are incorporated
- 10 into the living cell or organism, wherein metabolism of the electron donors or acceptors by the living cell or organism causes the quenched fluorescence emission of the nanocrystal to be restored, thereby labeling the living cell or organism.
- 15 38. The method of Claim 37, wherein the semiconductor nanocrystal is capped by a capping material of the formula $Q[(CRR')_nCO_2H]_m$ or a salt thereof, where:
- $n=1, 2, 3, 4$ or 5 ;
- Q is $HS-$, H_2N- , $O=P-$, or $P<$;
- 20 R and R' are independently $-H$, substituted or unsubstituted alkyl, or substituted or unsubstituted aryl; and
- m is 1 when Q is $HS-$, H_2N- , or $O=P-$ or m is 3 when Q is $P<$.
- 25 39. The method of Claim 38, wherein the capping material is mercaptoacetic acid.
40. The method of Claim 37, wherein electron donors are conjugated with the nanocrystal.
- 30 41. The method of Claim 37, wherein electron acceptors are conjugated with the nanocrystal.

42. The method of Claim 40, wherein the electron donors are selected from the group consisting of adenine, nicotinamide adenine dinucleotide, dopamine, tryptophan and tyrosine.
- 5
43. The method of Claim 41, wherein the electron acceptors are selected from the group consisting of benzoquinone, flavin, flavin adenine dinucleotide, flavoproteins, hydroquinone and sulfur containing proteins.
- 10
44. The method of Claim 37, wherein the semiconductor nanocrystal is CdSe.
45. The method of Claim 37, wherein the semiconductor nanocrystal is CdSe with a ZnS shell.
- 15
46. A method for detecting a metabolism abnormality or a metabolically-active intracellular environment of a cell or organism where metabolites are electron donors or acceptors, comprising the steps of:
- (a) providing a conjugate of a semiconductor nanocrystal with one or more electron donors or acceptors;
- 20 (b) incubating a plurality of the conjugates with the cell or organism under detection for a period of time;
- (c) measuring semiconductor nanocrystal-associated fluorescence of the cell or organism; and
- (d) comparing the semiconductor nanocrystal-associated fluorescence of the cell or organism under detection with semiconductor nanocrystal-associated fluorescence of a control cell or organism having normal metabolism, thereby detecting a metabolism abnormality or a metabolically-active intracellular environment of the cell or organism under detection.
- 25
- 30

- 50 -

47. The method of Claim 46, wherein the semiconductor nanocrystal is capped by a capping material of the formula $Q[(CRR')_nCO_2H]_m$ or a salt thereof, where:
- $n=1, 2, 3, 4$ or 5 ;
- Q is $HS-$, H_2N- , $O=P-$, or $P<$;
- R and R' are independently $-H$, substituted or unsubstituted alkyl, or substituted or unsubstituted aryl; and
- m is 1 when Q is $HS-$, H_2N- , or $O=P-$ or m is 3 when Q is $P<$.
48. The method of Claim 47, wherein the capping material is mercaptoacetic acid.
49. The method of Claim 46, wherein electron donors are conjugated with the nanocrystal.
50. The method of Claim 46, wherein electron acceptors are conjugated with the nanocrystal.
51. The method of Claim 46, wherein both electron donors and acceptors are conjugated with the nanocrystal.
52. The method of Claim 46, wherein the semiconductor nanocrystal is $CdSe$.
53. The method of Claim 46, wherein the semiconductor nanocrystal is $CdSe$ with a ZnS shell.
54. A method for sensing a transmembrane or intramembrane process of a cell or organism, comprising:
- (a) embedding one or more uncapped semiconductor nanocrystals within a lipid membrane, wherein fluorescence emission spectrum or fluorescence emission intensity or both fluorescence emission spectrum and fluorescence emission intensity, mediated by the

- 51 -

- semiconductor nanocrystals, change with a voltage change or an ion-concentration change across the membrane or a voltage change and an ion-concentration change across the membrane; and
- (b) monitoring the change in the emission spectrum or intensity or both spectrum and intensity, thereby sensing the transmembrane or intramembrane process.
55. The method of Claim 54, wherein the uncapped semiconductor nanocrystal is a fluorescence resonance energy transfer donor or acceptor.
56. The method of Claim 55, further including embedding into the cell or organism an organic dye that has a lipophilic fluorophore.
57. The method of Claim 56, wherein the fluorescence resonance energy transfer occurs between the uncapped nanocrystal and the organic dye.
58. The method of Claim 54, wherein said cell is an excitable cell.
59. The method of Claim 58, wherein the excitable cell is neuron or a muscle cell.
60. The method of Claim 54, further including the step of embedding said plurality of uncapped semiconductor nanocrystals within the membrane *via* a lipid vesicle.
61. The method of Claim 55, wherein the organic dye is a lipid-soluble dye, the lipid-soluble dye being embedded in the membrane.
62. The method of Claim 55, wherein the organic dye is a water-soluble dye, the water-soluble organic dye being in an aqueous environment near the membrane.

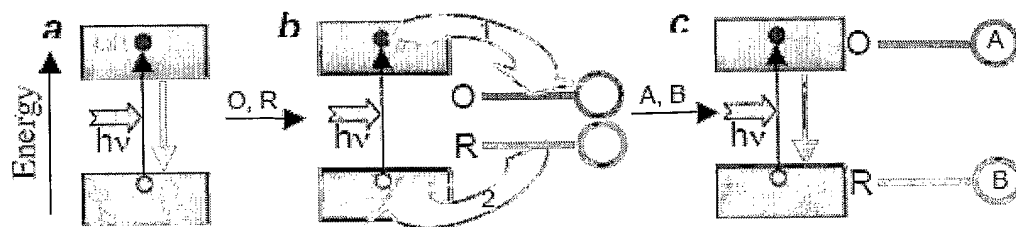
- 52 -

63. The method of Claim 54, wherein the semiconductor nanocrystal is CdSe.
64. The method of Claim 54, wherein the semiconductor nanocrystal is CdSe with a ZnS shell.
- 5
65. A method for labeling cells, comprising:
- (a) embedding one or more uncapped semiconductor nanocrystals within one or more lipid membranes of said cells, and
 - (b) measuring the fluorescence emission spectrum or fluorescence
- 10 emission intensity or both fluorescence emission spectrum and fluorescence emission intensity of said uncapped semiconductor nanocrystals, thereby detecting said cells.
66. The method of Claim 65, wherein the uncapped semiconductor nanocrystal is
- 15 a fluorescence resonance energy transfer donor or acceptor.
67. The method of Claim 66, further including embedding into the cell or organism an organic dye that has a lipophilic fluorophore.
- 20 68. The method of Claim 67, wherein the fluorescence resonance energy transfer occurs between the uncapped nanocrystal and the organic dye.
69. The method of Claim 68, wherein the organic dye is a lipid-soluble dye, the lipid-soluble dye being embedded in the membrane.
- 25 70. The method of Claim 68, wherein the organic dye is a water-soluble dye, the water-soluble organic dye being in an aqueous environment near the membrane.
- 30 71. The method of Claim 66, wherein said cell is an excitable cell.

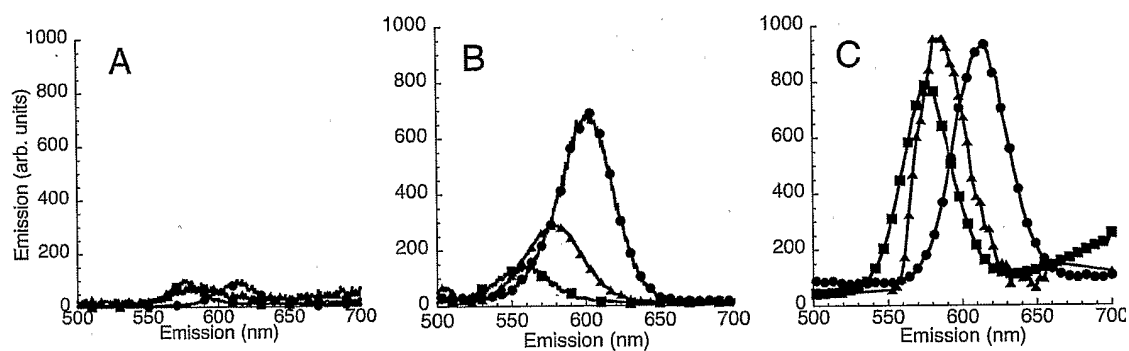
- 53 -

72. The method of Claim 71, wherein the excitable cell is neuron or a muscle cell.
73. The method of Claim 66, further including the step of embedding said
5 plurality of uncapped semiconductor nanocrystals within the membrane *via* a lipid vesicle.
74. The method of Claim 65, wherein the semiconductor nanocrystal is CdSe.
- 10 75. The method of Claim 65, wherein the semiconductor nanocrystal is CdSe with a ZnS shell.

1/12



FIGs. 1a-1c



FIGs. 2A-2C

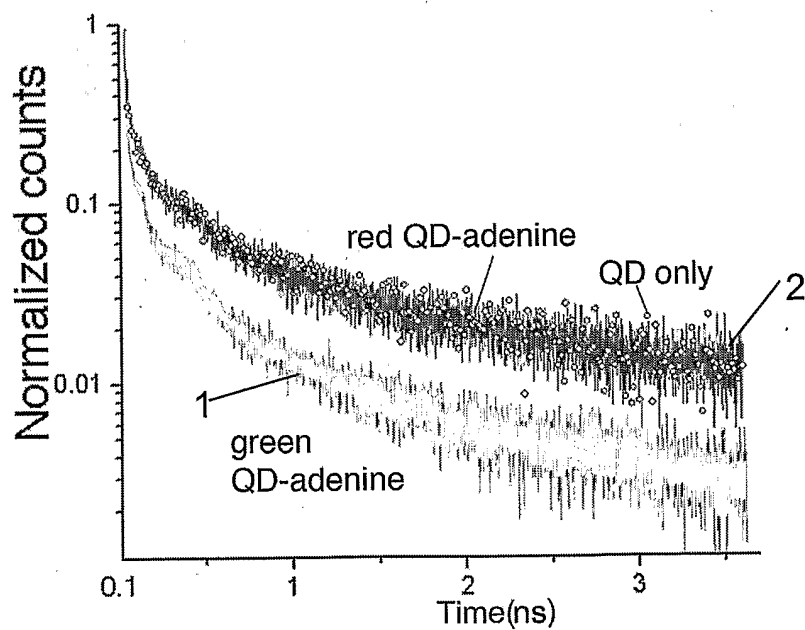
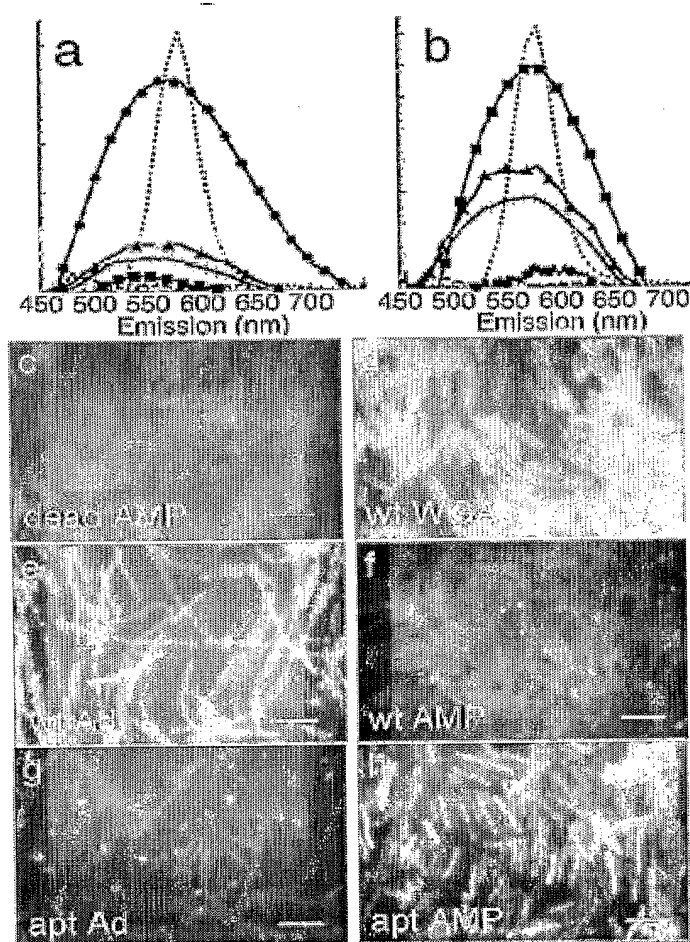
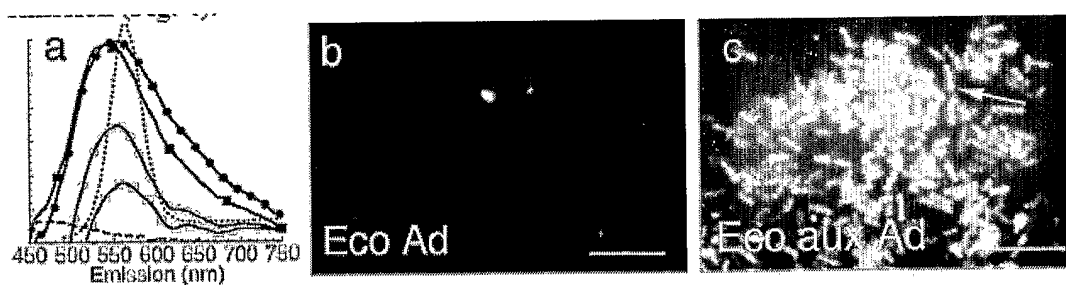


FIG. 3

2/12



FIGs. 4a-4h

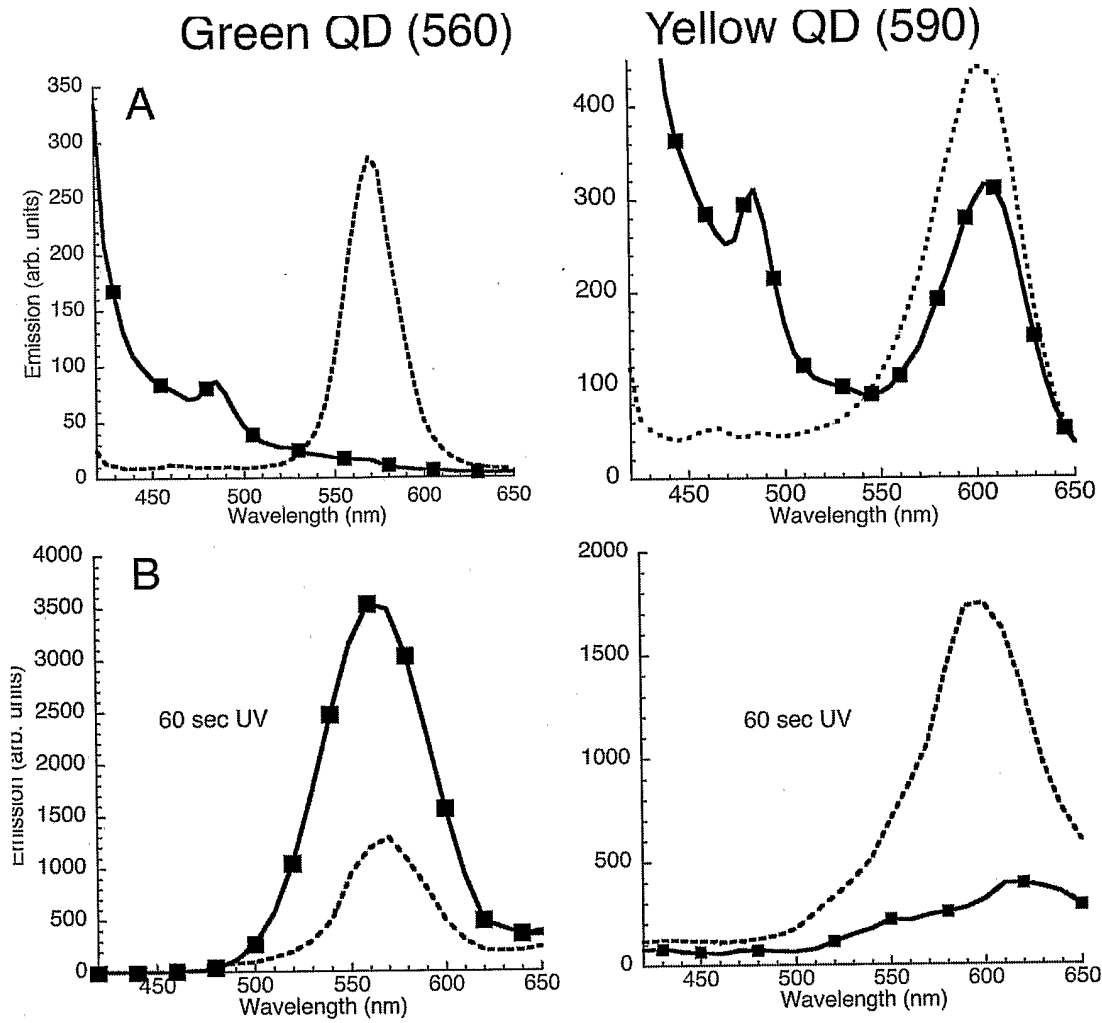


FIGs. 5a-5c

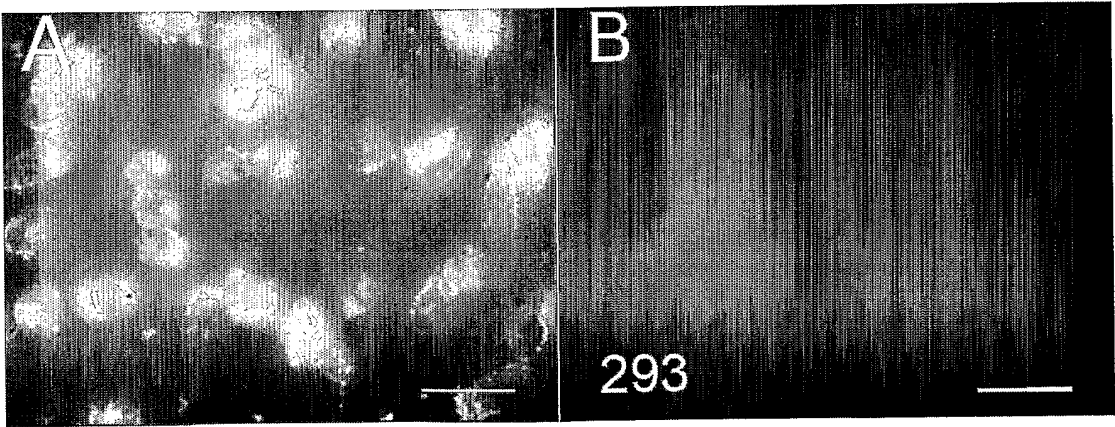


FIGs. 6a-6d

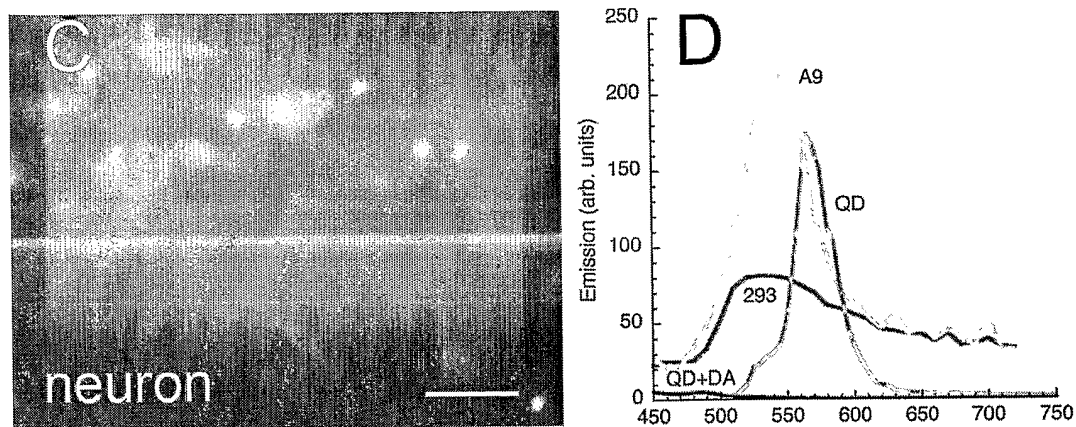
4/12



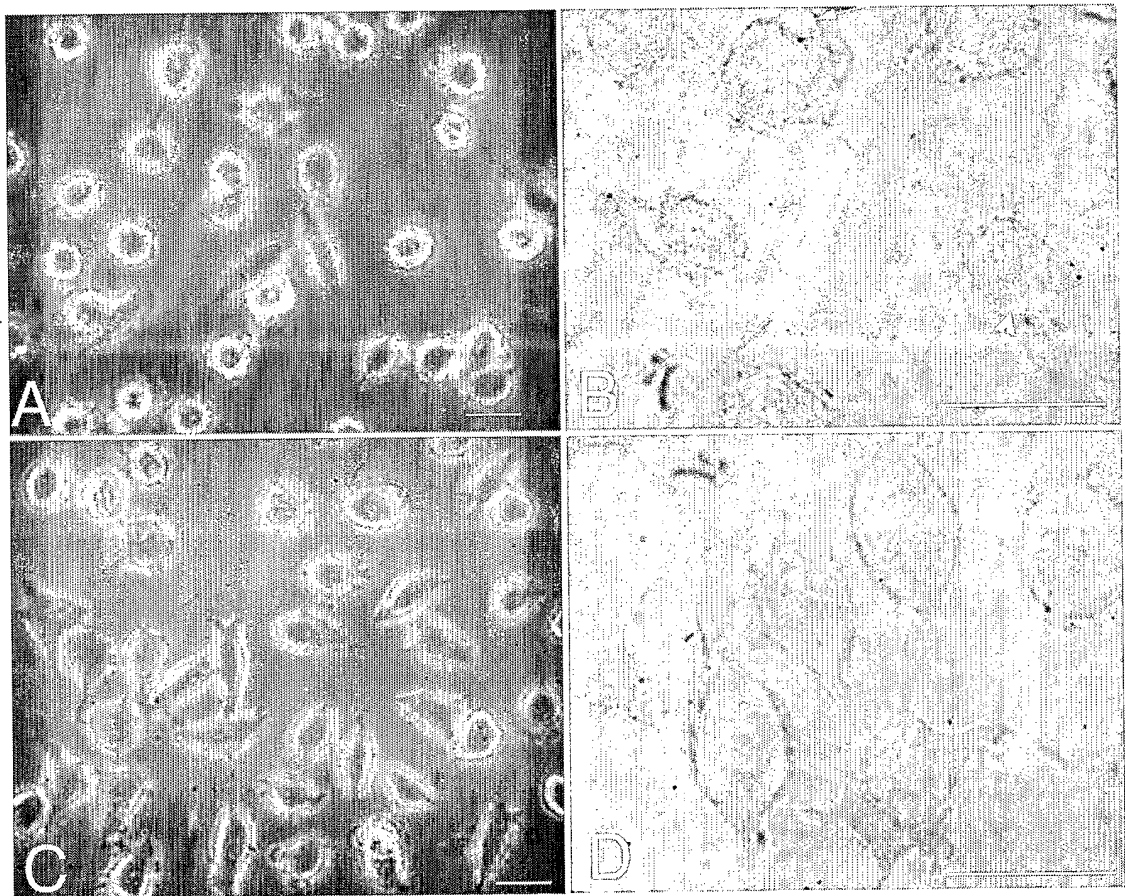
FIGs. 7A-7B



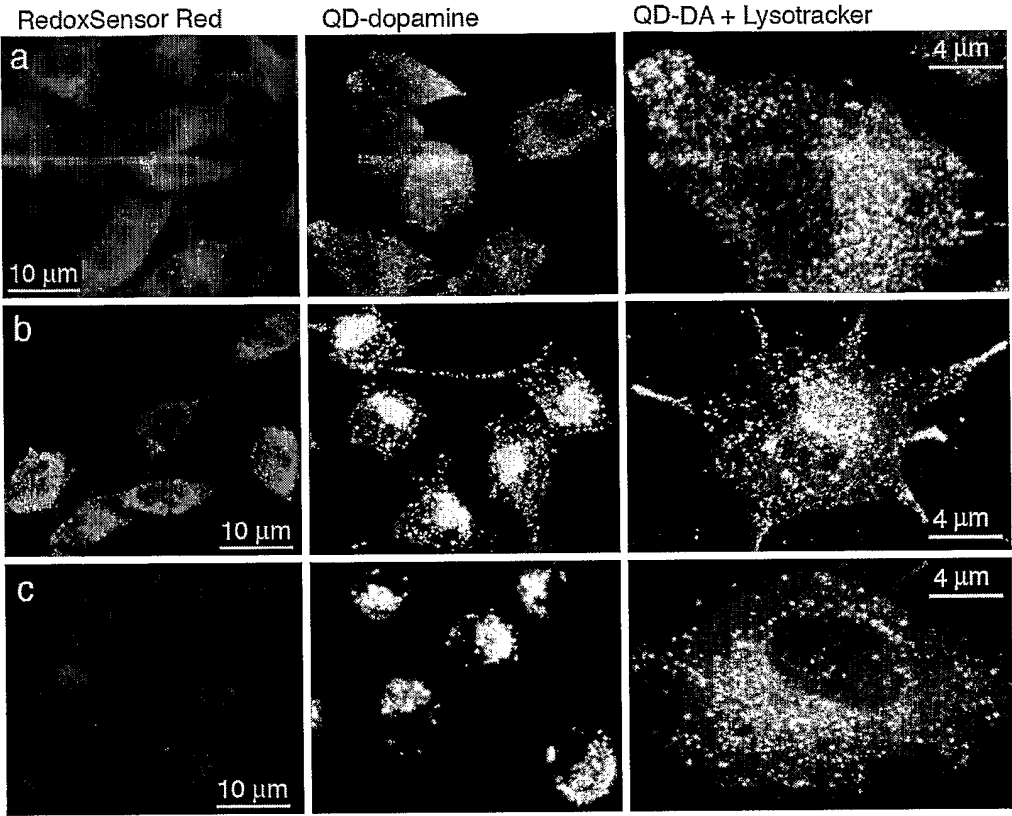
FIGs. 8A-8B



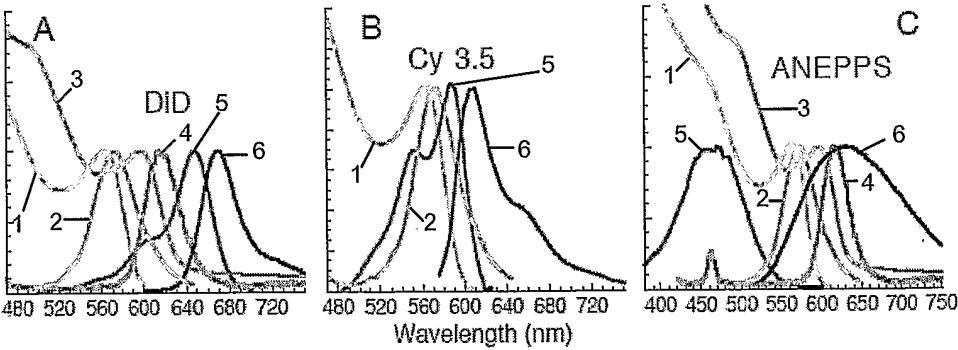
FIGs. 8C-8D



FIGs. 9A-9D

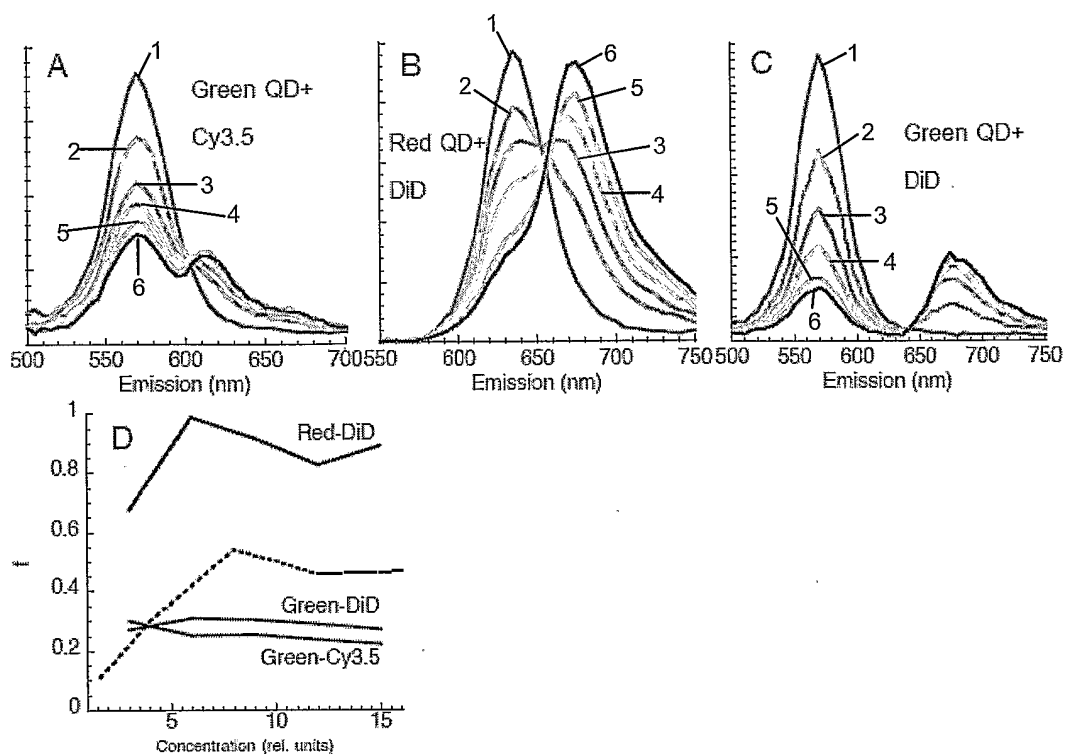


FIGs. 10a-10c

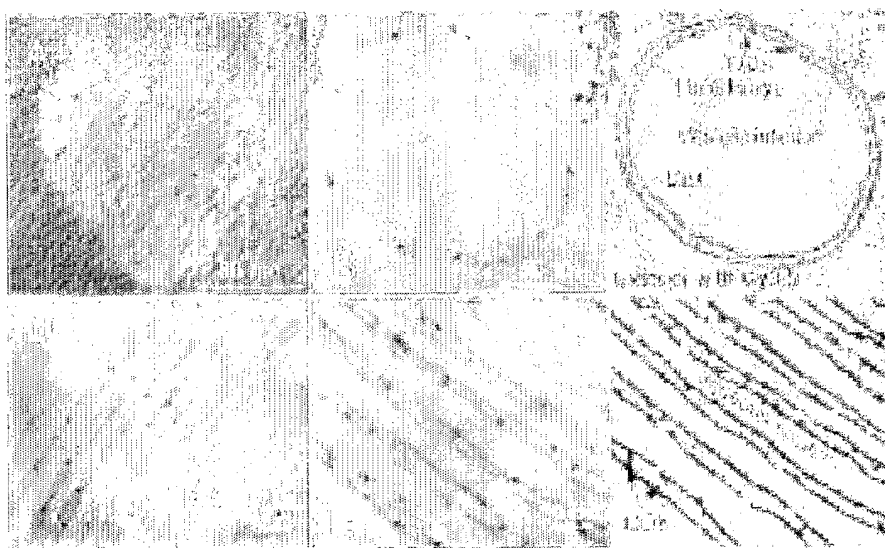


FIGs. 11A-11C

7/12

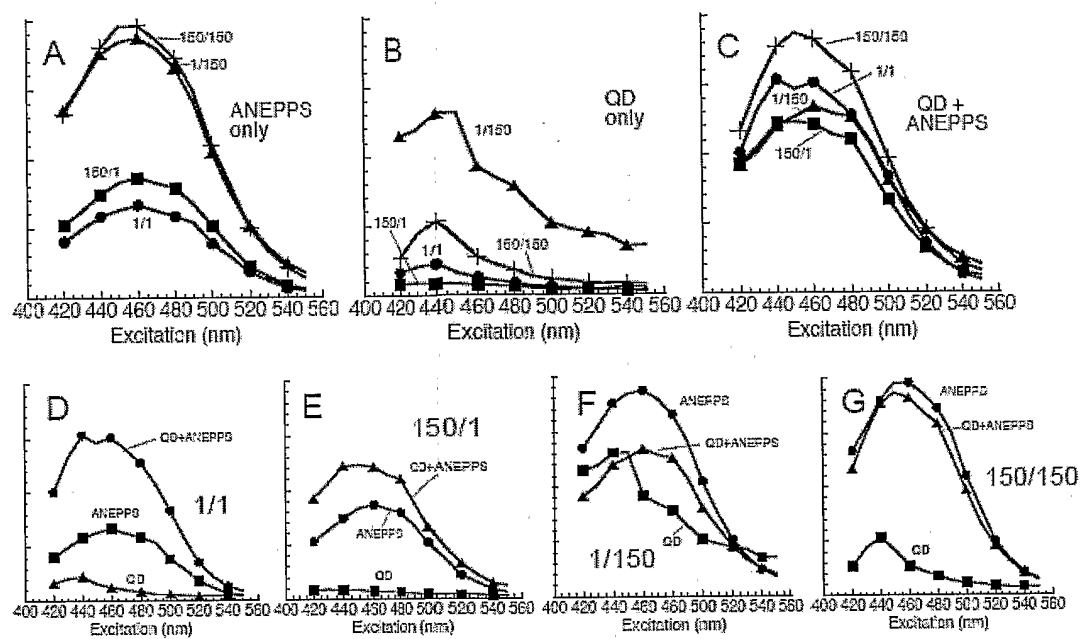


FIGs. 12A-12D



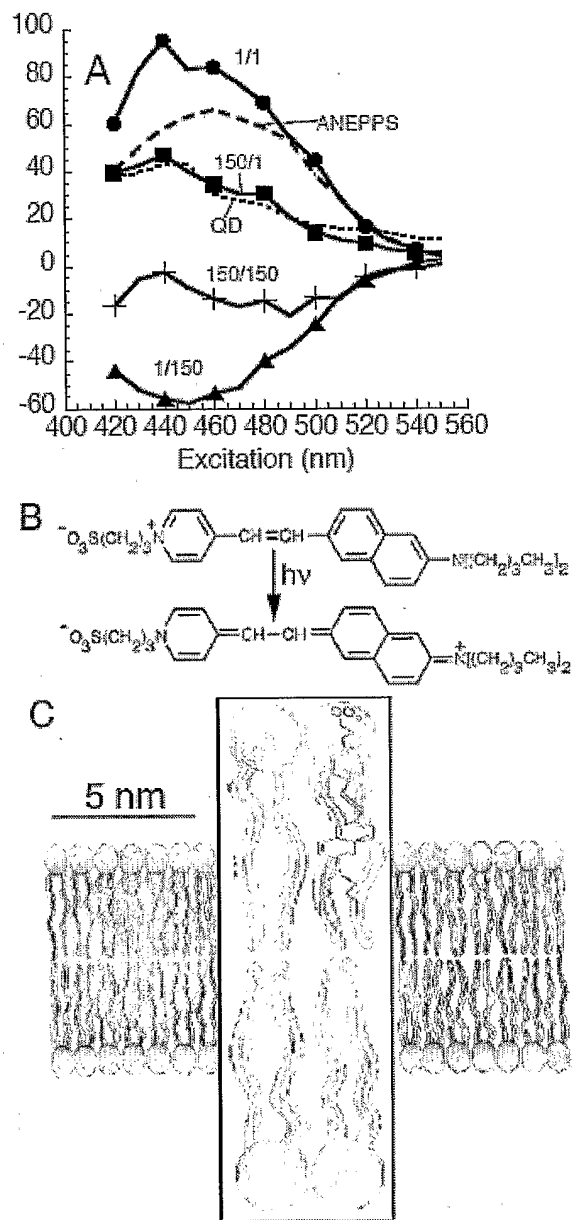
FIGs. 13A-13F

8/12



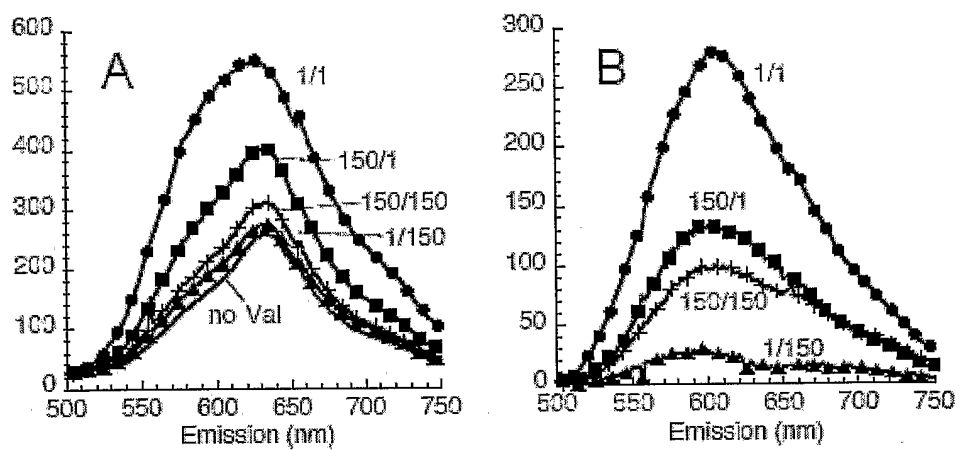
FIGS. 14A-14G

9/12

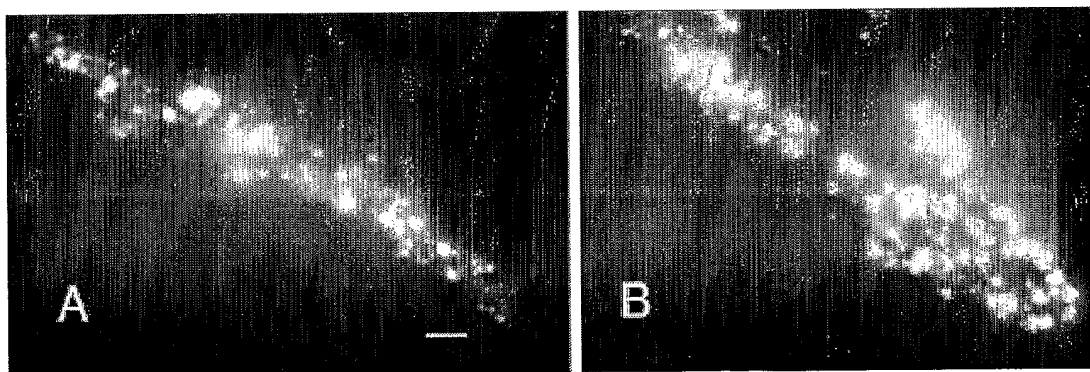


FIGs. 15A-15C

10/12

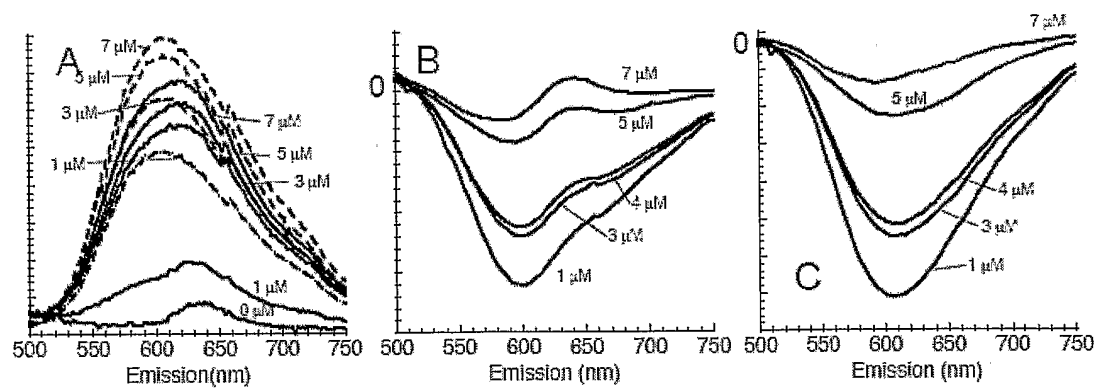


FIGs. 16A-16B

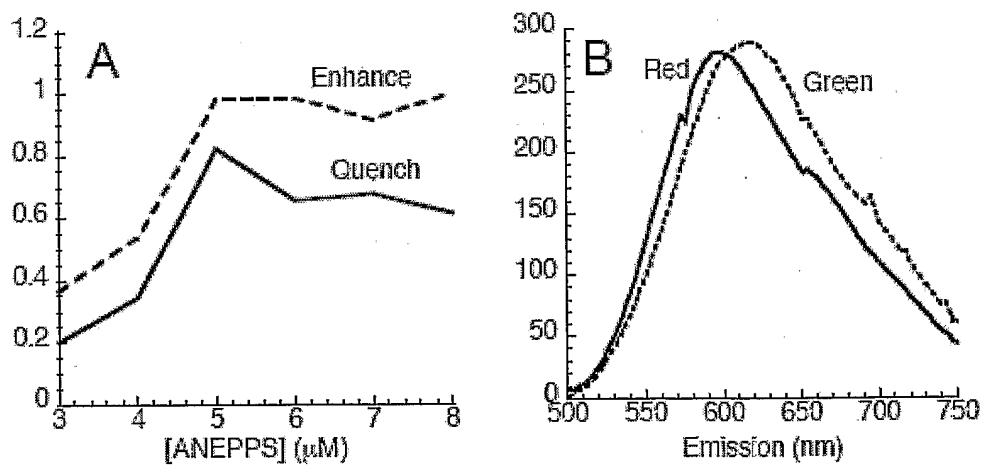


FIGs. 17A-17B

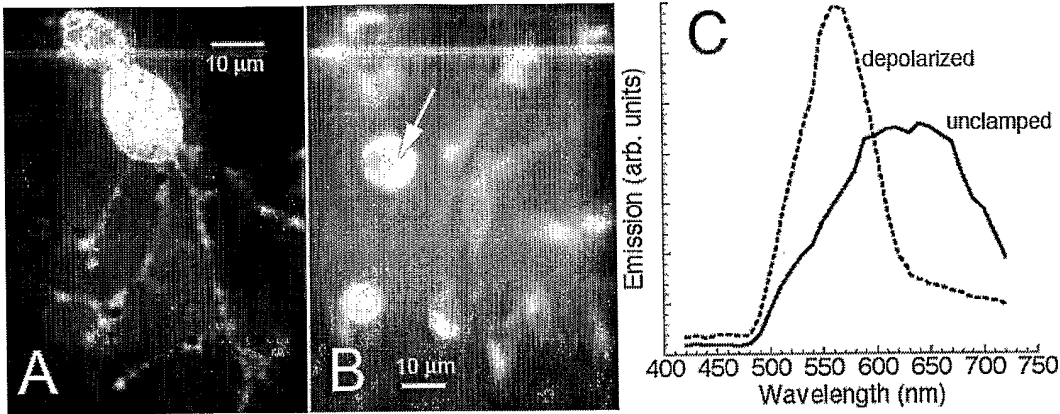
11/12



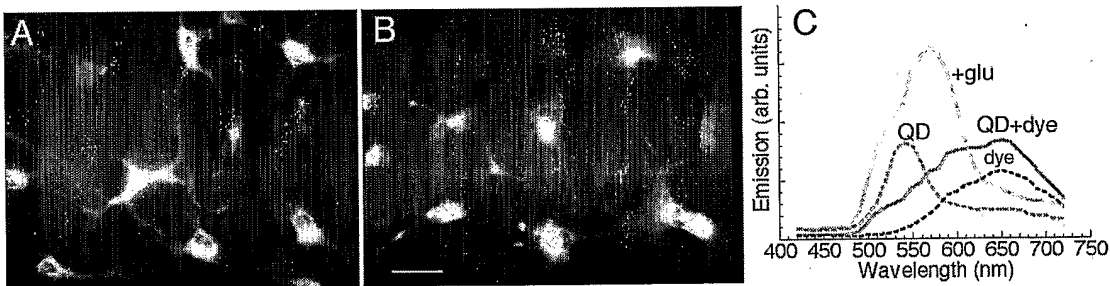
FIGs. 18A-18C



FIGs. 19A-19B



FIGs. 20A-20C



FIGs. 21A-21C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001527

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **C12Q 1/04** (2006.01), **G01N 33/52** (2006.01), **G01N 33/58** (2006.01), **G01N 21/64** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(7): C12Q 1/04, G01N 33/52, G01N 33/58, G01N 21/64

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

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

CAPLus, Delphion, Pubmed, Canadian Patent Database, Keywords: quantum dot, QD, nanocrystal, lipid, electron, FRET, fluorescence, CdSe, biosensor, conjugate, label, labeling

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2345376 A1 (ADVANCED RESEARCH AND TECHNOLOGY INSTITUTE, INC.,)	1-6, 9-10, 37-41 and 44-45
Y	25 May 2000. whole document	13-14, 21-24, 27-28, 31-36, 46-53, and 67-70
X	MEDINTZ IG., et al. "Self-assembled Nanoscale Biosensors Based on Quantum Dot FRET Donors", NATURE MATERIALS September 2003, published on-line 24 August 2003, vol. 2, pages 630-638 whole document	1-2, 5-6 and 9-10
X	MEDINTZ IG., et al. "A Fluorescence Resonance Energy Transfer-derived Structure of a Quantum Dot-protein Bioconjugate Nanoassembly", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, 29 June, 2004 vol. 101, no. 26, pages 9612-9617 whole document	1-2, 5-6 and 9-10

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

☒ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 December 2005 (22-12-2005)

Date of mailing of the international search report

18 January 2006 (18-01-2006)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001(819)953-2476

Authorized officer

Nicole Harris (819) 997-4541

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001527

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 37-75

because they relate to subject matter not required to be searched by this Authority, namely :

Although claims 37-75 encompass diagnostic methods practised on a human or animal which this Authority is not obliged to search under Rule 39.1(iv) of the PCT, the search has been carried out based on the use of the compounds referred to therein.

2. ☐ Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. ☐ Claim Nos. :

because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

Group 1 : Claims 1-10 and 37-53 are directed towards compositions comprising capped semiconductor nanocrystals and uses thereof in labelling cells and organisms.

Group 2: Claims 11-36 and 54-75 are directed towards compositions and probes comprising uncapped semiconductor nanocrystals and uses thereof in labelling lipid membranes.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001527

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KLOEPFER JA., et al. "CdSe Quantum Dot Internalization by <i>Bacillus subtilis</i> and <i>Escherichia coli</i> ", SPIE-THE INTERNATIONAL SOCIETY FOR OPTICAL ENGINEERING June 2004, vol 5361, pages 133-141 whole document	1-7, 9-10, 37-42, and 44-45
X	DUBERTRET B et al., "In Vivo Imaging of Quantum Dots Encapsulated in Phospholipid Micelles", SCIENCE, 29 November 2002, vol. 298, pages 1759-1762 whole document	11, 12, 15-17, 25-26, 29-30, 65-66 and 71-75
Y		13-14, 21-24, 27-28, 31-36, and 67-70
Y	KLOEPFER JA et al. "Quantum Dots as Strain- and Metabolism-Specific Microbiological Labels", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2003, vol. 69, no. 7, pages 4205-4213	46-53
P,X	KLOEPFER JA., et al. "Uptake of CdSe and CdSe/ZnS Quantum Dots into Bacteria via Purine-Dependent Mechanisms", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2005, vol. 71, no., 5, pages 2548-2557	1-2, 5-7, 9-10, 37, 40-42, 44-46 and 49-53
P,X	CLAPP AR et al. "Can Luminescent Quantum Dots Be Efficient Energy Acceptors with Organic Dye Donors ?", JOURNAL OF THE AMERICAN CHEMISTRY SOCIETY, 2005, published on-line 1 June 2005 vol. 127, pages 1242-1250	1-2, 5-6, 9-10, 37-38, 40-42, 44-45 and 49-53
P,X	ZHOU D, et al. "Fluorescence resonance energy transfer between a quantum dot donor and a dye acceptor attached to DNA". CHEMICAL COMMUNICATIONS 14 October 2005 published on line 6 September 2005 vol. 38, pages 4801-4809	1-3, 5-6 and 9-10
P,X	RAMACHANDRAN S et al. "Colloidal Quantum Dots Initiating Current Bursts in Lipid Bilayers. Biosens Bioelectron". 15 April 2005 vol. 20, no. 10, pages 2173-2176.	11-12, 15-16, 25-26 and 29-30
P,X	JAISWAL JK et al. "Use of Quantum Dots for Live Cell Imaging", NATURE METHODS, October 2004 vol. 1, no. 1, pages 72-78	1-2, 5-6, 9-10, 37, 40-41 and 44-45

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001527

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	KLOEPFER JA., et al. "FRET Between CdSe Quantum Dots in Lipid Vesicles and Water and Lipid-Soluble Dyes", JOURNAL OF PHYSICAL CHEMISTRY B, 2004, Web release 9 October 2004, vol. 108, pages 17042-17049 abstract	11-30, 33-36, 54-57 and 61-64 1-75
A	WO0142489 A1 (BIO-PIXELS LTD.,) 14 June 2001	1-75
P, A	GAO X, et al. "In Vivo Molecular and Cellular Imaging with Quantum Dots", CURRENT OPINIONS IN BIOTECHNOLOGY 16 February 2005 vol. 1, pages 63-72	1-75
P, A	MICHALET X et al. "Quantum Dots for Live Cells, in Vivo Imaging, and Diagnostics", SCIENCE 28 January 2005, vol. 307, no. 5709, pages 538-544 retrieved from NIH Public Access author manuscripts; deposited in PMC 15 June 2005	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/001527

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
CA2345376	25-05-2000	AU3468500 A	05-06-2000
		EP1115888 A2	18-07-2001
		JP2002530630T T	17-09-2002
		US6468808 B1	22-10-2002
		US2003008414 A1	09-01-2003
		WO0029617 A2	25-05-2000
WO0142489	14-06-2001	AU1953501 A	18-06-2001
		EP1240351 A1	18-09-2002
		US6194213 B1	27-02-2001
		WO0142489 A1	14-06-2001