Supporting Information For

CdSe/ZnS Quantum Dot-Cytochrome *c* Bioconjugates for Selective Intracellular $O_2^{\bullet-}$ Sensing

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Experimental Section

Reagents. All reagents were of analytical grade and doubly distilled water was used for all experiments. Thioglycolic acid (TGA, >99%), KBH_4 (96%) and selenium powder (99.999%) were purchased from Sigma-Aldrich. Zinc sulfate ($ZnSO_4$, 99%), sodium sulfide (Na_2S, 99%), cadmium chloride hemi (pentahydrate) ($CdCl_2 \cdot 2.5H_2O$, 99%), sodium hydroxide (NaOH, 99%), sodium dithionite (80%) and ethanol (99%) were obtained from Aldrich (Milwaukee, WI) and used without further purification. N_2 (99.998%, pre-purified) was obtained from Cryogenic Gases (Detroit, MI). Cytochrome c (Cyt c, from horse heart), 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH), Phorbol Myristate Acetate (PMA), ascorbic acid (Vc) and L-glutathione (GSH) were purchased from Sigma-Aldrich. Dihydroethidium (HE) and ethidium bromide were purchased from Molecular Probes.

Apparatus. Fluorescence emission and excitation spectra were obtained with a Shimadzu Cary Eclipse (Varian) fluorometer and a Xenon lamp light source (LE-XM500, LEO Photonics Tech Co. Ltd. China) was used. The cell was irradiated with the Xenon lamp operated at 500 W through a homemade light chopper. The shutter chopping frequency was controlled manually. The pH value of a solution was measured by using a PHS-3C pH meter

(Switzerland Mettler Toledo Delta 320 pH meter). Desktop multifunction centrifugal ultrafilters were used (Eppendorf-5430, St. Co. Germany). A CHI660 electrochemical workstation (Shanghai Chenhua Co., Ltd., China) was equipped with a stirring machine (CH Instruments Inc.), the three-electrode system consisted of a glassy carbon working electrode, a saturated calomel electrode (SCE) as a reference electrode, and a platinum counter electrode was used for all electrochemical measurement, unless noted otherwise. Ultrapure water (18.2 MΩ) was obtained from a Water Pro water purification system (Labconco Corporation, Kansas City, MO, U.S.A.). All fluorescence images of cellular QDs were acquired with the same parameters using wide-field inverted fluorescence microscopy (Nikon-Ti, Co. Ltd. Japan) using a 60 × 1.2 NA objective and a matched electron multiplying charge-coupled device (EMCCD) (Roper).

Fluorescence Spectra of CdSe/ZnS QD-Protein Bioconjugates. Two set of oxidized and reduced 1.08×10^{-4} M Cyt *c* with Thioglycolic acid (TGA) and 2-(dimethylamino)ethanethiol hydrochloride (DMAET) modified CdSe/ZnS QDs were dissolved in 2 mL of Tris-HCl pH 8.0 solutions and incubated for several minutes. The samples were then deaerated for 5 min with nitrogen gas (Fig. S1). The fluorescence intensity of two kinds of QDs with oxidized and reduced Cyt *c* were recorded at $\lambda_{ex/em}$= 420/565 nm against a blank sample. The spectral properties of QD-reduced Cyt *c* bioconjugates were tested by their reaction with superoxide radicals under a nitrogen atmosphere.

Pyrogallol Assay for Superoxide Radical Anion ($O_2^{\bullet-}$): To examine the scavenging activity of free radicals, a pyrogallol autoxidation assay was performed. After incubation of 2.98 mL of Tris-HCl (50 mM, pH=8.0) in a 25 °C water bath for 20 min, the mixture was combined

with 0.1 mL of pyrogallol (1 mM), and then the reaction mixture were incubated in a 25 °C water bath Shaker for 5 min. It was immediately used for the experiment of scavenging activity of free radicals.

Electrochemical Measurements. A standard jacketed three-electrode cell was used for all electrochemical experiments. A Pt electrode and an SCE electrode were used as a counter electrode and a reference electrode, respectively. Electrochemistry measurements using approximately 1.08×10^{-4} M Cyt *c* were performed at the ITO electrode in Tris-HCl pH 8.0. The temperature was maintained at 25 °C using a recirculating water bath. All reported potentials are referenced to the formal potential of the ferrocene/ferrocenium (Fc/Fc^{+}) couple.

Cytochrome c Reduction. A stock solution of 1.08×10^{-4} M oxidized Cyt *c* was incubated with sodium dithionite for 5 min at room temperature (1 g of the salt/mmol protein). Excess salt was removed using a NAP-25 column (Amersham Biosciences). The concentration of reduced Cyt *c* was determined by UV–Vis spectroscopy, and absorption at 550 nm was evaluated prior to use. The solution was stored for a maximum of 24 h at 4 ^{o}C.

Interferences Assay. The concentrations of H_2O_2, NaClO, GSH, and Vc were all 1.0 μM. $^{1}O_2$ was generated by the reaction of H_2O_2 with NaClO. Ferrous ammonium sulfate (0.1 mM) and H_2O_2 (1 mM) were used to generate $^{\bullet}OH$. $ONOO^{-}$ was prepared by reacting KNO_2 and H_2O_2 in HCl at 0 ^{o}C, as previously reported,[26] and was frozen at less than -18 ^{o}C. $ROO^{\bullet}$ radicals were generated by thermal decomposition of 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH, 5 mM) at 37 °C. S-nitroso-N-acetylpenicillamine (SNAP, 1 mM) in phosphate buffer (100 mM, pH 7.4) at room temperature was used to generate nitric oxide (NO).

Cellular Assays. HeLa and HL-7702 cells were cultured to ~70% density and collected after trypsin digestion. The collected cells were washed twice using phosphate-buffered saline (PBS) and then suspended in Tris-HCl. Cell lysis (2×10^6 cells/mL) was performed by repeated cycles of freezing and thawing, and lysed cells were ready for assays.

Cellular Imaging. HeLa and HL-7702 cells were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL). Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were plated into a 24-well culture plate (200–300 cells/well) and allowed to adhere for 10 h before treatment. Culture medium containing 200 μg/mL QDs, CdSe/ZnS QD-oxidized Cyt *c* bioconjugates was added, and cells were incubated for 10 h. Next, the growth medium was removed, and the cells were washed several times with PBS. Then, the cells were fixed using a 10% methanol solution at room temperature for 20 min, followed by washing with PBS. The cover glass was then mounted on a microscopic glass slide and studied under a microscope. The conjugated QD-loaded cells were cultured for 4 h with and without the presence of PMA stimulation (400 ng/mL; a stimulator of cell respiratory burst to give rise to ROS) at 37 °C. Cells were washed three times with 0.2 M PBS buffer before imaging (excitation wavelength of QD-Cyt *c* bioconjugates was 420 nm). Moreover, HE was used to estimate intracellular $O_2^{\bullet-}$ production in HeLa cells. After treating HeLa cells with PMA, the medium was removed, and cells were washed with PBS and incubated in fresh culture medium with 2% FBS. A final concentration of 10 μM HE was added and incubated for 20 min. Cells were washed twice with PBS and maintained in fresh medium (excitation and emission at 510 and 590 nm, respectively). The

images were taken by using an inverted fluorescence scanning microscope with an objective lens (×60). All background parameters (laser intensity, exposure time and objective lens) were kept constant when the different fluorescence images were captured.

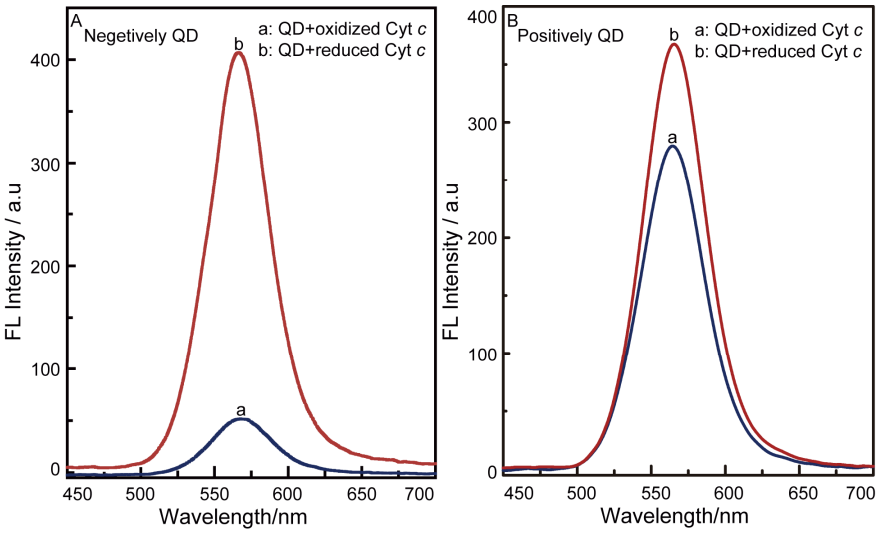


Fig. S1 A: (a) Emission spectra of oxidized Cyt *c* and (b) reduced Cyt *c* in the presence of negatively capped QDs with a constant concentration of 9.82×10^{-6} M. B: Emission spectra of (a) oxidized Cyt *c* and (b) reduced Cyt *c* in the presence of negatively capped QDs with a constant concentration of 9.82×10^{-6} M in Tris-HCl (pH 8.0).

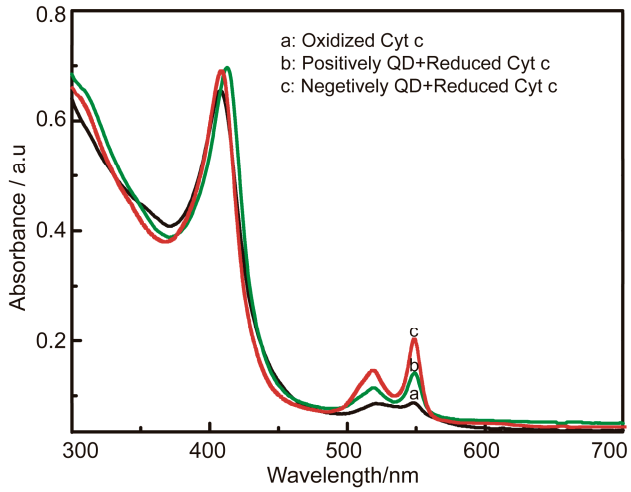
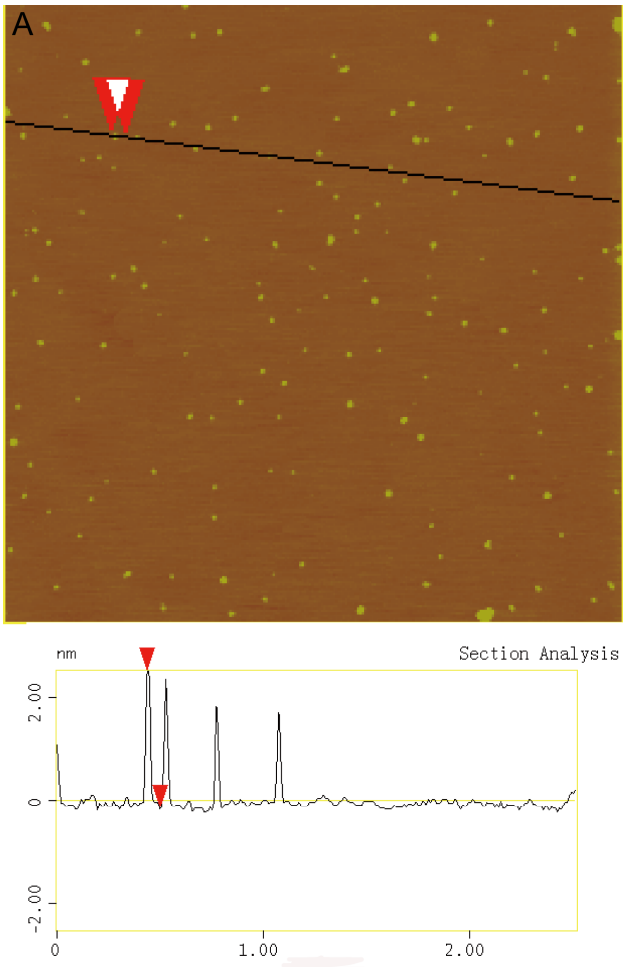


Fig. S2 Absorbance spectra of oxidized Cyt *c* (a) and corresponding spectra of reduced Cyt *c* with constant concentration (9.82×10^{-6} M) in the presence of positively (b) and negatively capped QDs (c).



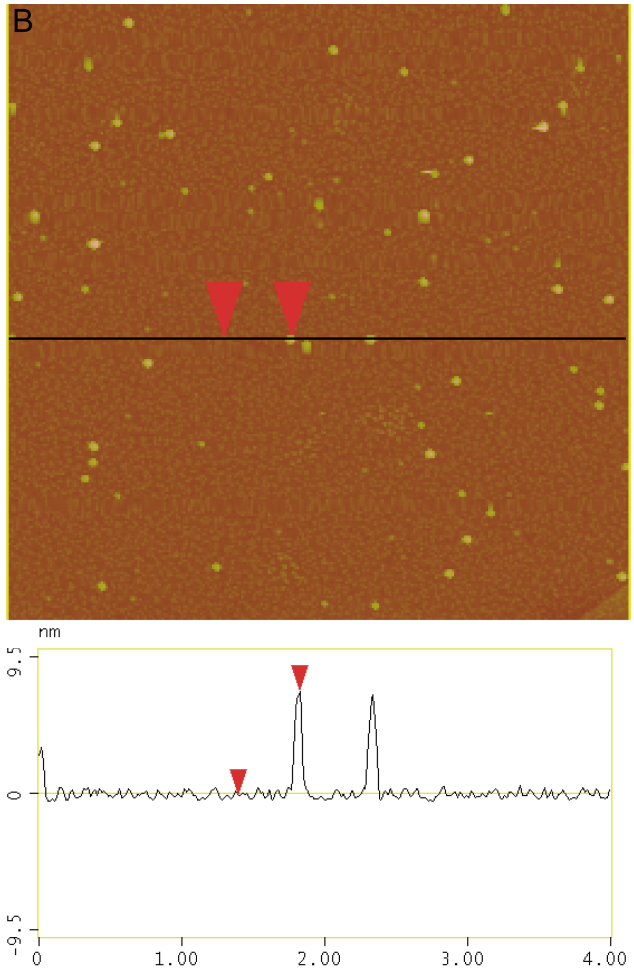


Fig. S3 AFM images of negatively capped CdSe/ZnS QDs (A) and negatively capped CdSe/ZnS-Cyt *c* bioconjugates (B).

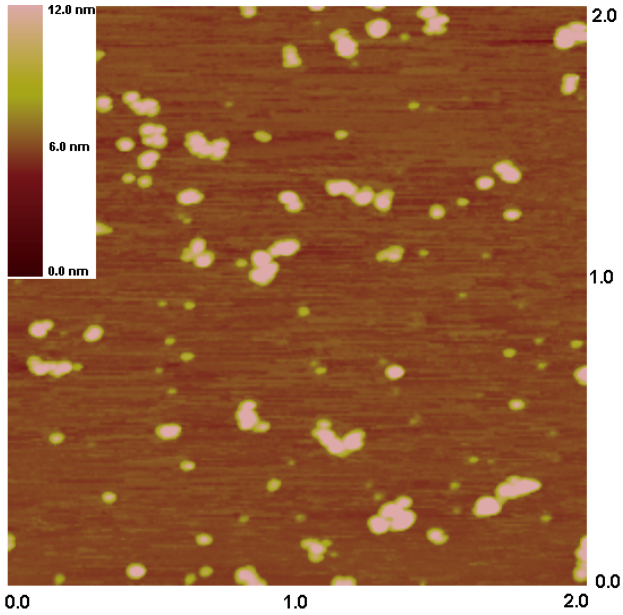


Fig. S4 AFM image of positively capped CdSe/ZnS-Cyt *c* bioconjugates.

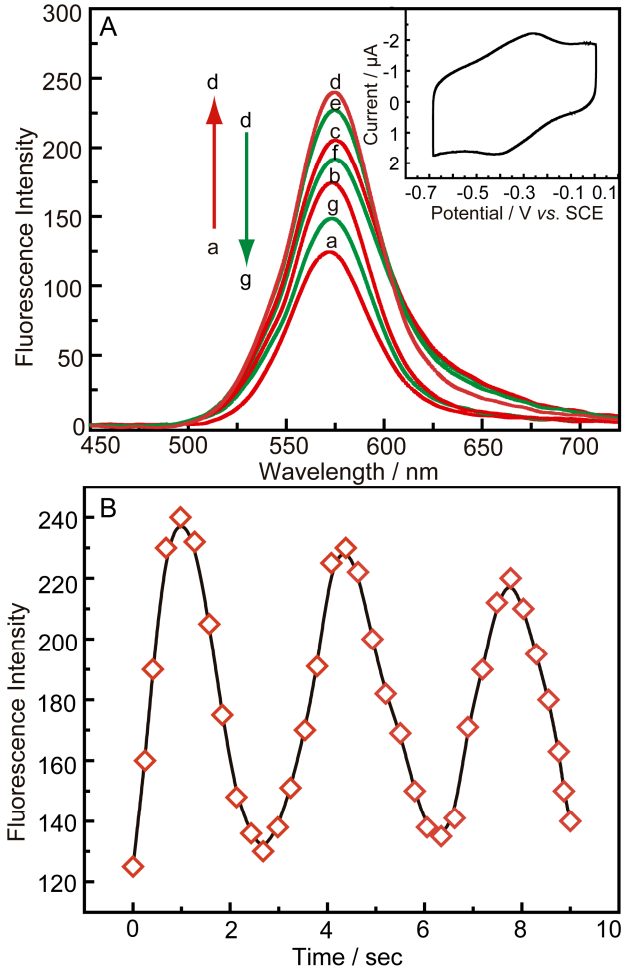


Fig. S5 The fluorescence emission spectra changes with two constant applied potential. A: fluorescence intensity of oxidized Cyt *c* in the presence of negatively capped QDs (**a**) enhanced by a -0.47 V voltage and stabilized for 60 s (**a**-**d**); fluorescence intensity of **d** quenched by giving -0.15 V voltage and stabilized for 90 s (**d**-**g**). B: time-dependent fluorescence intensity changes of oxidized Cyt *c* in the presence of negatively capped QDs with two constant applied potential.

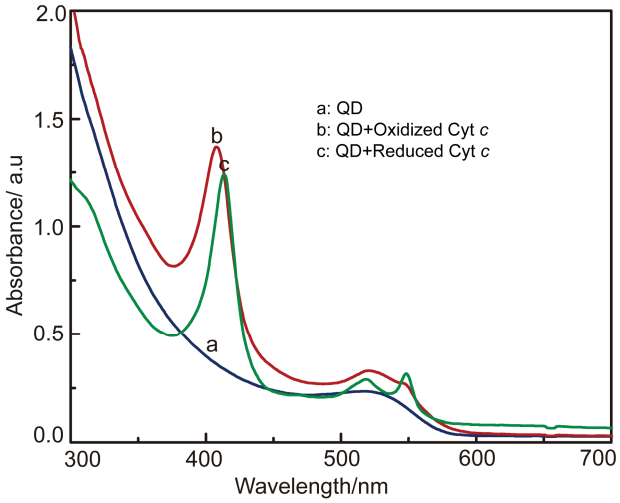


Fig. S6 The absorption spectra of three nanoparticle changes with constant applied potentials of -0.47 V and -0.15 V. (a) Negatively capped CdSe/ZnS QDs, (b) oxidized Cyt *c* (9.82×10^{-6} M) and (c) reduced Cyt *c* (9.82×10^{-6} M) in the presence of negatively capped QDs.

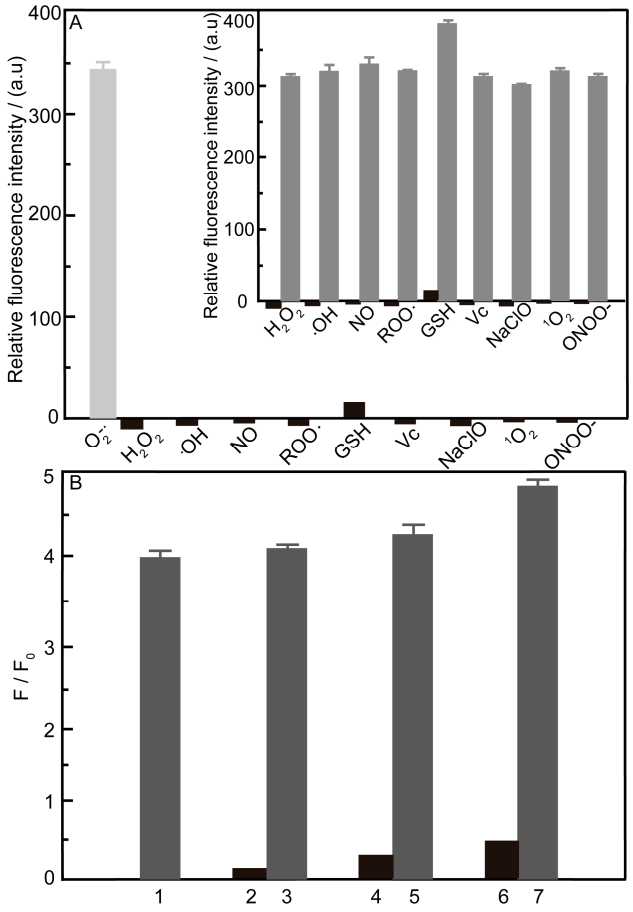


Fig. S7 (A) Relative fluorescence intensity observed upon reaction of QD-Cyt *c* with ROS and other biologically relevant compounds. Bars represent relative fluorescence responses to 1.0 μM of each compound. Insert: Two bars are shown for each compound representing reactions of QD-Cyt *c* with 1.0 μM of each compound in the absence of $O_2^{\bullet-}$ (black) and in the presence of 1.0 μM $O_2^{\bullet-}$ (gray), respectively. (B) The probe fluorescence response toward $O_2^{\bullet-}$ and GSH. Bars represent the final integrated fluorescence response (F) over the initial integrated emission (F_0). Conditions: 1) 1.0 μM $O_2^{\bullet-}$, 2) 1.0 μM GSH; 3) 1.0 μM GSH and 1.0 μM $O_2^{\bullet-}$, 4) 100 μM GSH; 5) 100 μM GSH and 1.0 μM $O_2^{\bullet-}$, 6) 1 mM GSH; 7) 1 mM GSH and 1.0 μM $O_2^{\bullet-}$, λ_{ex}=420 nm, λ_{em}=580 nm.

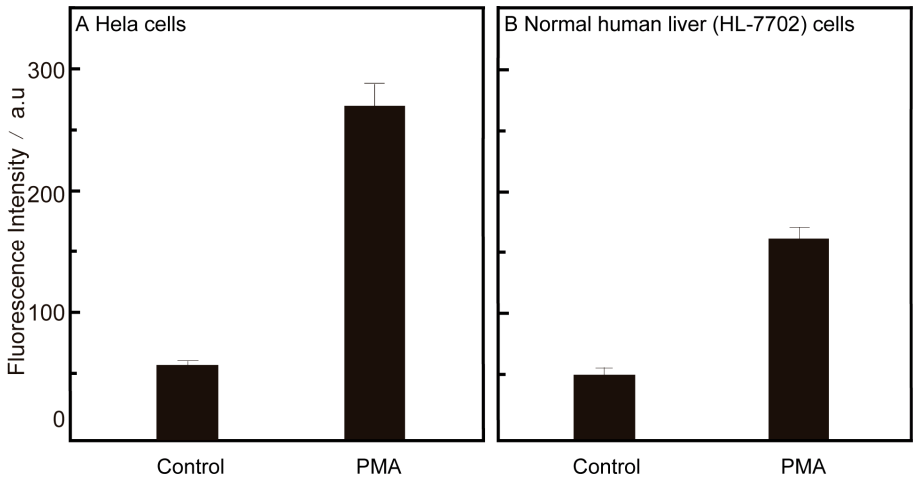


Fig. S8 Determination of HeLa cells (A) and HL-7702 cells (B) mediated superoxide flux with QD-oxidized Cyt *c* system. The assay mixture consisted of each cell type (2×10^6 cells/mL), PMA (400 ng/mL), QDs (50 μM), Cyt *c* (1.08×10^{-4} M) in buffer solution.

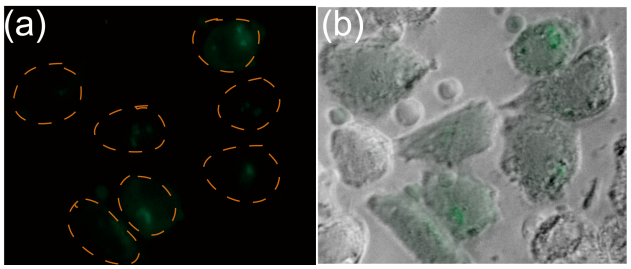


Fig. S9 (a) Fluorescence image of non-stimulated HeLa cells loaded by QD-Cyt *c* bioconjugates after an equivalent time of 4 h; (b) the merged images of phase-contrast of HeLa cells and fluorescence imaging.

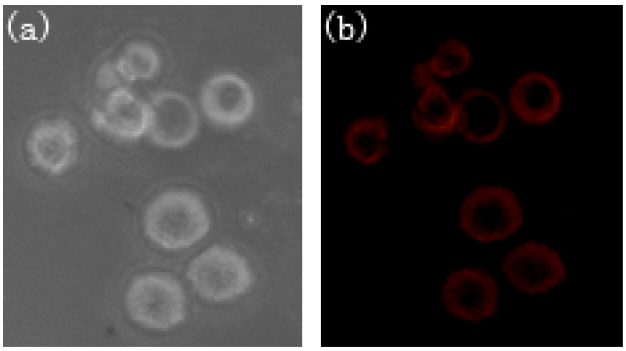


Fig. S10 PMA-induced HE fluorescence in HeLa cells. (a) Phase-contrast of HeLa cells; (b) fluorescence images of HeLa cells treated with PMA (400 ng/mL) for 30 min. After PMA treatment, HeLa cells were washed with PBS and incubated with HE (10 μM) for 20 min. Cells were washed twice with PBS and kept in the culture medium. The red fluorescence generated from HE was monitored by using fluorescence microscopy.