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Uptake and Processing of Semiconductor Quantum Dots in Living Cells Studied by Fluorescence Lifetime Imaging Microscopy (FLIM)

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Materials and Methods

QD synthesis and solubilization

Chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON). For synthesis of CdSe QDs capped with ZnS, 0.026 g of cadmium oxide (CdO) and 1 mL oleic acid (OA) were added to a three-necked flask containing 10 mL octadecene (ODE). This mixed was degassed and heated under N_2 gas to 260 °C. The mixture turned colorless around 150 °C. The selenium (Se) precursor was prepared by mixing 0.01 g of elemental Se with 0.5 mL trioctylphosphine (TOP) under an inert atmosphere and sonicating until the solution became transparent. The zinc sulfide (ZnS) precursor was prepared as follows: 0.5 mL of TOP was combined with 0.2 mL hexamethyldisilathiane ((TMS)2S) and 0.3 mL dimethylzinc (Zn(CH3)2) under an inert atmosphere and diluted to 5 mL with ODE. Once the CdO/OA/ODE mixture reached 260 °C, the heat was turned off, and the Se precursor was injected rapidly using a needled syringe. The ZnS precursor was injected over a time course of 5 min during the desired stage of QD growth. Afterwards, the temperature was allowed to drop to 100 °C and it was maintained at this temperature for several hours. The QDs were purified from the reaction side products by precipitation with acetone, anhydrous ethanol and chloroform, and resuspended in toluene. Mercaptopropionic acid (MPA) was used to replace the OA surfactant by a thiol -exchange reaction. 200 μL of concentrated QDs (optical density > 5) in toluene were added to 2 mL chloroform and 5 mL of methanol. 50 μL of MPA was added and the pH was adjusted to 9–10 with tetramethylammonium hydroxide pentahydrate (TMAH). This solution was left at room temperature in the dark for 24 h. The thiol-modified QDs were separated from excess MPA ligand by precipitation and washing with ethyl acetate. The QDs were dried at room temperature under air and resuspended in distilled H2O (Millipore). Absorbance spectra were measured on a SpectraMax Plus plate reader, and emission spectra on a SpectraMax Gemini (Molecular Devices, Sunnyvale, CA). Absorbance and emission spectra are shown Figure S1.

Conjugation to dopamine (DA) and incubation with cells

Dopamine hydrochloride was coupled to the QDs by a 1-ethyl-3-(dimethyaminopropyl) carbodiimide hydrochloride (EDC)-mediated reaction. QDs in H2O were dialyzed (membrane cutoff 10 kDa) against PBS for 1 h and diluted to a final concentration of 1 μM. EDC and DA were added to the reaction mixture at a ratio of 1000 : 1000 : 1 QD. The mixture was reacted for 30 min under gentle shaking and purified from excess side products by precipitation with THF and resuspension in PBS. The level of DA binding was quantified using the fluorescent indicator o-phthaldialdehyde (OPA) as described[1]. Their spectra relative to QDs alone showed a significant degree of fluorescence emission quenching, with almost no change in absorbance (Fig. S1). For imaging experiments, the conjugates were not purified or tested with OPA, but used immediately after preparation.

NIH 3T3 fibroblast cells were cultured in high-glucose DMEM (Invitrogen Canada, Burlington, ON) supplemented with L-glutamine (0.2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and FBS (10 %), and incubated in a 5 % CO2 atmosphere. Cells plated on glass bottom dishes (MatTek, Ashland, MA) were incubated with CdSe/ZnS and CdSe/ZnS:DA at 20 and 50 nM concentrations for 30

minutes. Prior to imaging, cells were washed with PBS and imaged in OptiMem (Invitrogen Canada, Burlington, ON). When MitoTracker or LysoTracker dye (Molecular Probes) was used, it was added to cells at a concentration of 1μM at least 30 min before the end of the QD incubation.

QD uptake by fibroblasts was confirmed using confocal imaging on a Zeiss 510 LSM with a PlanApo 100x oil objective. QDs were excited with an Ar ion laser 488 nm line. MitoTracker/LysoTracker Red were excited with a HeNe laser (543 nm line). Cells labeled with >1 probe were examined for channel bleed-through before imaging.

Lifetime measurements and data analysis

Fluorescence lifetime images were acquired on a Zeiss 710 NLO multiphoton microscope equipped with a 63x water-immersion objective. A Coherent ® Chameleon Vision II 2-photon excitation source was used; this mode-locked Ti:Sapphire laser produced 120 fs pulses (FWHM) at 80 MHz. A reference beam was sent to the SPC-150 TCSPC module (Becker and Hickl, Germany) and a 900 nm excitation wavelength was used to excite the sample (1980 mW, 9 % transmission). This wavelength was chosen after tuning from 750-950 nm and choosing the optimum signal. A 565-615 nm bandpass filter was placed before the detector to collect the QD emission. The fluorescence signal was transmitted to an HPM-100-40 GaAsP hybrid detector (Becker and Hickl, Germany) before reaching the TCSPC module. The hybrid PMT and its components were controlled via the DCC-100 detector controller of the FLIM system (see [2]for details). All scans lasted 60 s and images were recorded with 512 x 512 pixel resolution.

The bulk solution measurements were made using a custom TCSPC apparatus. 800 nm laser pulses out of a Coherent RegA 9050 Ti:sapphire regenerative amplifier operating at 250 kHz repetition rate were frequency doubled in a BBO (β-BaB_2O_4) crystal to produce 400 nm pulses for excitation of the samples. The temporal pulse width of the 400 nm pulses was approximately 100 femtoseconds at full width half maximum (FWHM) with approximately 3 nm in frequency bandwidth at FWHM, assuming a Gaussian beam profile. The beam was focused into the sample with the largest focal spot diameter of 0.785 mm. The excitation power was ~ 2.4 mW, with peak pulse intensities at the sample of 1 x 10^7 W/cm^2 with 1 x 10^{-6} J/cm^2 fluence after attenuation of the 400 nm excitation beam with neutral density filters placed before the focusing lens. The luminescence was collected with a 3.5 cm focal length lens placed perpendicular to the incident 400 nm beam and focused into a monochromator with a 10 cm focal length lens. The monochromator was a CVI CMSP112 double spectrograph with a 1/8 m total path length in negative dispersive mode with 600 groove/mm grating (overall *f* number 3.9). The slit widths were 0.66 mm, and based on a monochromator dispersion of 16 nm/mm, provided 10-20 nm resolution. The PMT was a Hamamatsu RU3809 micro-channel plate detector powered by a variable high voltage power supply at -3.0 kV and was mounted on the monochromator box exit slit. A Becker and Hickl SPC-630 photon counting board recorded the amplified emission signals. The reference signal was provided by a portion of the excitation beam sent to a fast photodiode.

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The instrument response function (IRF) was determined from scatter off a solution of dilute coffee creamer. When thiols (beta-mercaptoethanol or glutathione) were used, they were added to a final concentration of 1 mM immediately before measurement. QDs were dissolved in phosphate buffered saline, which has an index of refraction very similar to the index of refraction of NIH 3T3 fibroblasts (1.34 vs. 1.37)[3], although it was not possible to control for local variability of refractive index inside the cells.

FLIM decays were fit using the SPCImage software (Becker and Hickl, Germany). Image pixels were made to have a binning of 3 before performing analysis. Typically, 10 to 15 pixel traces were analyzed per image in regions of interest. This software was written to accommodate the fast repetition rate of the two-photon laser, which is necessary for mode locking and which cannot be adjusted. Typically, the interval between laser pulses should be 4-5 times that of the average fluorescence lifetime of the fluorophore, or a limit of 3 ns for an 80 MHz rep rate. When this is not the case, methods of data analysis must be used that take into account the incomplete decay of the fluorophores in between each laser pulse. These "incomplete decay" models have been implemented in the software for over 10 years (Wolfgang Becker, private communication), though the first publication appears in a conference proceeding in 2005[4]. A recent paper (2011) establishes the accuracy of this model for simulations of a variety of fluorophores using the exact parameters of our instrument[5]. In all our reported fits, we used the incomplete decay model. At least 1000, 10^4, or 10^5 photons at peak were required for a fit to a single, double, or triple exponential, respectively.

TCSPC data of QDs in bulk solution were analyzed using FluoFit (PicoQuant). In all cases, χ^2 values and weighted residuals were used to judge the goodness-of-fit.

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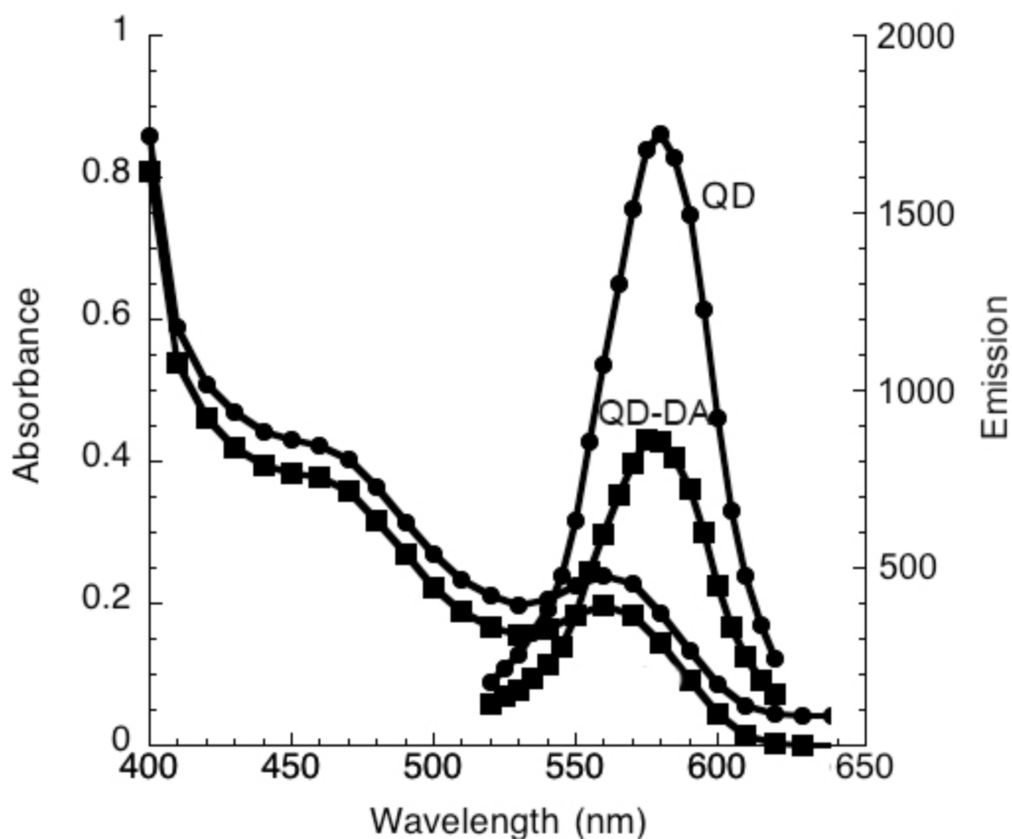

Figure S1. Absorbance and emission spectra of MPA-solubilized QDs (QD, circles) and QDs conjugated to dopamine at a ratio of 1000:1 (QD-DA, squares). The emission is in arbitrary units but the samples are at the same concentration.

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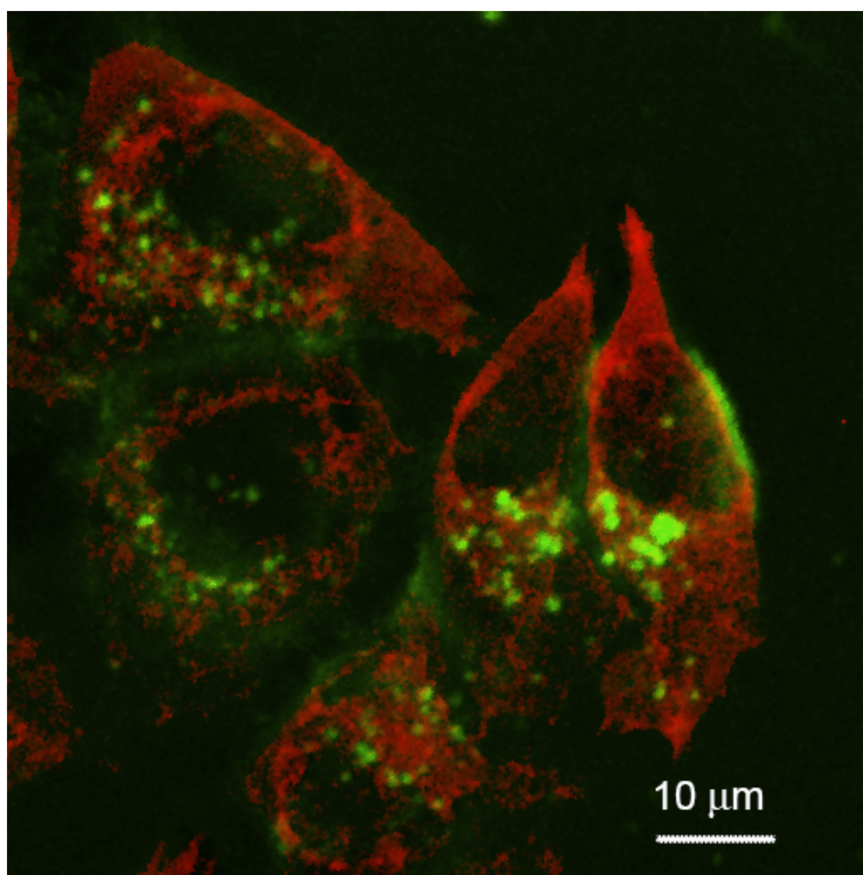

Figure S2. Confocal image of fibroblasts incubated with QDs (green) and MitoTracker Red (red), showing no overlap of the channels.

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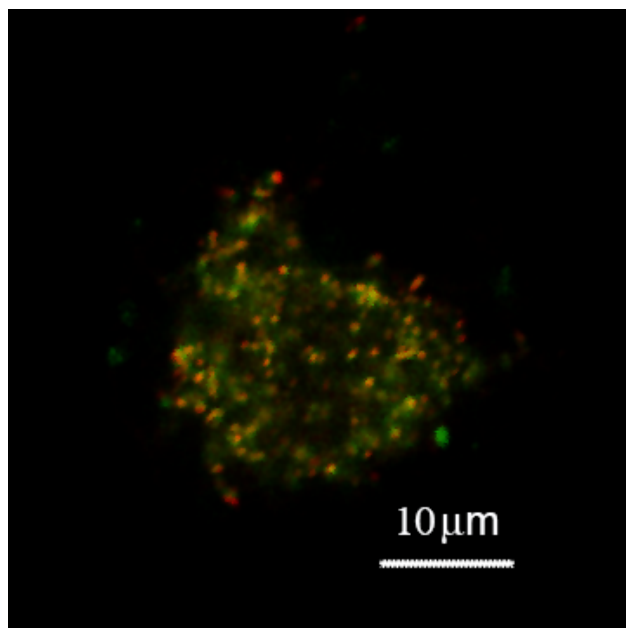

Figure S3. Confocal image of fibroblasts incubated with QDs (green) and LysoTracker Red (red), showing substantial overlap of the channels (yellow).

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