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Zwitterionic surface coating of quantum dots reduces protein adsorption and cellular uptake.

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I) Quantum dots synthesis and surface modification

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I.1) Materials and reagents

Oleic acid (tech, 90%, #364525), trioctylphosphine (TOP, tech, 90%, #117854), 1-octadecene (ODE, tech, 90%, #O806), oleylamine (tech, 70%, #O7805), 1,1-carbonyldiimidazole (97%, #21860), anhydrous chloroform (99%, #372978), (\pm)- α -lipoic acid (99%, #T5625), N,N-dimethylethylenediamine (98%, #39030), sodium borohydride (99%, #213462), sulfur (99.98%, #414980), and 1,3-propanesultone (98%, #P50706) were purchased from Sigma-Aldrich. Cadmium acetate dihydrate (99.999%, #11865) and selenium shots (99.99%, #93-3417) were purchased from Alfa Aesar and Strem, respectively. Anhydrous magnesium sulfate (99%, #M1387), chloroform (99.5%, #C0585), methanol (99.5%, #M0585), hexanes (85%, #H0109), and hydrochloric acid (2 N, #H0820) were purchased from Samchun chemicals. Water was triply distilled with a resistivity of 18.2 M Ω .cm using a Millipore filtration system. Amicon 50 kDa molecular weight cutoff (MWCO) centrifugal filters (#UFC905024) were purchased from Merck Millipore.

I.2) Synthesis of CdSe/CdS/ZnS core/shell/shell quantum dots

CdSe quantum dots (QDs) were prepared as described previously ¹. To obtain a cadmium precursor, cadmium acetate (1.2 mmol) was dissolved in oleic acid (6.0 mmol) at 100 °C under vacuum. The solution was cooled to room temperature, and the cadmium precursor solution was mixed with the selenium precursor prepared by dissolving selenium shots (6.0 mmol) in TOP (6 mL) in a glove box. ODE (40 mL) and oleylamine (6 mmol) were placed in a three-neck flask and heated to 300 °C under nitrogen gas flow. At this temperature, the mixture of cadmium and selenium precursors was quickly injected into the reaction flask and the temperature was maintained at 280 °C. The reaction mixture was kept stirring until CdSe QDs of the desired size (*i.e.* color of emission) were obtained. Upon completion, the mixture was cooled to room temperature and diluted by hexanes. For purification, the product mixture was precipitated by adding excess methanol, collected by centrifugation, and redispersed in a small amount of hexanes.

The diameter of the CdSe QD core, $d_c (= 2 \cdot r_c) = 3.5$ nm, was determined by transmission electron microscopy (TEM). We assumed that the QDs have a wurtzite crystal structure with

the following lattice parameters: $a = 0.43$ nm, $c = 0.702$ nm. Accordingly, the number of Cd atoms per CdSe core is 399 as calculated in the following way: The wurtzite lattice unit volume is $3 \cdot (\sqrt{3}/2) \cdot a^2 \cdot c = 3 \cdot (\sqrt{3}/2) \cdot (0.43 \text{ nm})^2 \cdot 0.702 \text{ nm} = 0.337 \text{ (nm)}^3$. Each wurtzite lattice unit has 6 atoms (both cations and anions). A spherical CdSe core with diameter $d_c = 3.5$ nm has a volume of $(4/3) \cdot \pi \cdot (d_c/2)^3 = (4/3) \cdot \pi \cdot (3.5 \text{ nm}/2)^3 = 22.4 \text{ nm}^3$. Thus, the number of Cd (or Se) atoms per CdSe QD core is $22.4 \text{ (nm)}^3 / 0.337 \text{ (nm)}^3 \cdot 6 = 399$. The concentration of CdSe core QDs was determined optically. For optical analysis, UV/vis absorption spectra were recorded and the QD concentration was determined from the absorption at 350 nm *via* the Beer Lambert law. An extinction coefficient of $\epsilon_{\text{QD}}(350 \text{ nm}) = 7.69 \cdot 10^5 \text{ M}^{-1}\text{cm}^{-1}$ was assumed.

For over-coating of the CdSe cores with a CdS shell to obtain CdSe/CdS (core/shell) QDs, a slightly modified successive ionic layer adsorption and reaction approach was used¹. 20 mL ODE, 7 mL oleylamine, and 500 nmol CdSe core QDs (the concentration was determined optically as described above) were loaded in a three-neck flask. Following 1 h of degassing under vacuum, the mixture was heated to 230 °C under nitrogen gas flow. To obtain the cadmium precursor, cadmium acetate (0.3 mmol) was dissolved in oleic acid (1.5 mmol) at 100 °C under vacuum. 0.1 M elemental sulfur dissolved in ODE, 0.1 M Cd-oleate in ODE was used as precursor for the growth of shell layers. A first injection of precursors was made after 10 min. For the 1st layer of the CdS shell on 500 nmol of CdSe core QDs, $500 \text{ nmol} \cdot 327 / 0.1 \text{ M} = 1.64 \text{ mL}$ of 0.1 M Cd-oleate in ODE was added to the CdSe core QD solution. After 10 min of reaction time, a 2nd injection of the Cd (and S) precursor solution was added to the CdSe core solution. The quantity of precursor used for the second (and third) monolayer of the shell was calculated for the successive increase in QD volume as a function of increasing shell thickness. The number of Cd (or S) atoms in the 2nd and 3rd CdS shell of each QD were 454 and 604, respectively. Therefore, 2.27 mL and 3.02 mL of the 0.1 M Cd (or S) precursor solution was added for each CdS shell overcoating on CdSe core QDs.

The contribution in Cd of the CdS shell was calculated in the following way. The thickness of each CdS monolayer was assumed to be 0.35 nm. With three CdS monolayers the thickness of the CdS shell is $3 \cdot 0.35 \text{ nm} = 1.05 \text{ nm}$. The diameter of the CdSe/CdS core/shell QDs thus is the sum of the CdSe core diameter and twice the thickness of the CdS shell: $d_c = 3.5 \text{ nm} + 2 \cdot 1.05 \text{ nm} = 5.6 \text{ nm}$. The volume of the CdS shell is the volume of the CdSe/CdS core/shell QD minus the volume of one CdSe core: $(4/3) \cdot \pi \cdot (d_c/2)^3 - 22.4 \text{ (nm)}^3 = (4/3) \cdot \pi \cdot (5.6/2)^3 - 22.4 \text{ (nm)}^3 = 69.5 \text{ (nm)}^3$. The unit volume of the CdS wurtzite lattice is $3 \cdot (\sqrt{3}/2) \cdot a^2 \cdot c = 3 \cdot (\sqrt{3}/2) \cdot (0.414 \text{ nm})^2 \cdot 0.675 \text{ nm} = 0.301 \text{ (nm)}^3$. In this way there is the following number of Cd (or S) atoms in the CdS shell of each QD: $69.5 \text{ (nm)}^3 / 0.301 \text{ (nm)}^3 \cdot 6 = 1390$.

For over-coating of the CdSe/CdS core/shell QDs with a ZnS shell to obtain CdSe/CdS/ZnS (core/shell/shell) QDs, 0.1 M elemental sulfur dissolved in ODE, and 0.1 M Zn-oleate were used as precursors for the growth of the shell layers. The quantities of precursors used for each monolayer of shells (3 monolayers for the ZnS shell) were calculated for the successive

increases in QD volume as a function of increasing shell thickness as specified below. Here, also the thickness of each ZnS monolayer in the ZnS shell (3 ZnS monolayers) was assumed to be 0.35 nm. The reaction temperature was 230 °C and the growth time was 10 min between each addition. The reaction mixture was cooled to room temperature, and diluted with hexanes. The product, *i.e.*, CdSe/CdS/ZnS (core/shell/shell) QDs was purified using the same methods used for the purification of CdSe core QDs. As a result, hydrophobic QDs dispersed in hexanes were obtained. The total diameter of a CdSe/CdS/ZnS core/shell/shell QD can be estimated as $d_c = 3.5 \text{ nm} + 2 \cdot 1.05 \text{ nm} + 2 \cdot 1.05 \text{ nm} = 7.7 \text{ nm}$. This corresponds exactly to the diameter determined with TEM shown in the main manuscript of $d_c = 2 \cdot r_c = (7.7 \pm 0.9) \text{ nm}$.

The diameter of the CdSe/CdS/ZnS core/shell/shell QDs with the 1st layer of ZnS is the sum of the CdSe/CdS core/shell diameter and two times the thickness of the ZnS monolayer shell: $d_c = 5.6 \text{ nm} + 2 \cdot 0.35 \text{ nm} = 6.3 \text{ nm}$. The volume of the ZnS shell is subtracted from the volume of the CdSe/CdS/ZnS core/shell/shell QD: $(4/3) \cdot \pi \cdot (d_c/2)^3 - 91.9 \text{ (nm)}^3 = (4/3) \cdot \pi \cdot (6.3/2)^3 - 91.9 \text{ (nm)}^3 = 39.0 \text{ (nm)}^3$. The unit volume of the ZnS wurtzite lattice is $3 \cdot (\sqrt{3}/2) \cdot a^2 \cdot c = 3 \cdot (\sqrt{3}/2) \cdot (0.382 \text{ nm})^2 \cdot 0.626 \text{ nm} = 0.237 \text{ (nm)}^3$. Therefore, the number of Zn (or S) atoms in the 1st ZnS shell of each QD is $39.0 \text{ (nm)}^3 / 0.237 \text{ (nm)}^3 \cdot 6 = 987$. For the 1st layer of the ZnS shell on 500 nmol of CdSe/CdS core/shell QDs, $500 \text{ nmol} \cdot 987 / 0.1 \text{ M} = 4.94 \text{ mL}$ of 0.1 M Zn-oleate in ODE was added to CdSe/CdS core/shell QD solution. After 10 min, the same amount of 0.1 M elemental sulfur in ODE was added to the solution. After 10 min, for the 2nd time Zn (or S) precursor solution was added to CdSe/CdS core/shell QD solution. The same calculation for the 2nd and 3rd layer of the ZnS shell on CdSe/CdS core/shell QDs was done. The number of Zn (or S) atoms in the 2nd and 3rd ZnS shell of each QD was 1228 and 1500, respectively. Therefore, 6.14 mL and 7.50 mL of the 0.1 M Zn (or S) precursor solution was added for each ZnS shell overcoating on CdSe/CdS core/shell QDs.

1.3) Surface modification leading to zwitterionic quantum dots

The hydrophobic ligands of the as-synthesized CdSe/CdS/ZnS core/shell/shell QDs were exchanged with hydrophilic zwitterionic ligands (*i.e.* sulfobetaine functionalized lipoic acid), in the following referred to as (+/-) ligands. A detailed procedure for synthesizing (+/-) ligands was published elsewhere¹. The oxidized form of the (+/-) ligand (10^5 molar excess with respect to the amount of the QDs to be coated) was dissolved in deionized water. Two equimolar amounts of sodium borohydride were added to the solution and vigorously stirred for 20 min under N₂ gas flow at room temperature. The reduced form of the (+/-) ligands was obtained in the solution. Crude hydrophobic QDs were purified by a methanol/hexanes anti-solvent/solvent system. The QDs were dispersed in chloroform. The QD solution (1 mL) was added to the reduced ligand solution and further stirred for 2 h under N₂ gas flow at room temperature. The QDs were transferred from the organic phase to the aqueous phase. To remove excess free ligands, the QD solution was dialyzed using Amicon 50 kDa MWCO centrifugal filters. The quantum yield of zwitterionic surface QDs in water was about 28%.

I.4) Surface modification leading to negatively charged quantum dots

Similar to the description in §1.4 the original hydrophobic ligand of the as-synthesized CdSe/CdS/ZnS core/shell/shell QDs was exchanged with hydrophilic lipoic acid, in the following termed as (-) ligands. For the detailed procedure for synthesizing the (-) ligands, we refer to a previous publication ¹. 10^5 equimolar amount of the oxidized form of (-) ligand to QDs was dissolved in deionized water. Two equimolar amounts of sodium borohydride to ligands was added to the solution and vigorously stirred for 20 min under N₂ gas flow at room temperature. The reduced form of (-) ligands were obtained in the solution. Crude hydrophobic QDs were purified by a methanol/hexanes anti-solvent/solvent system. The QDs were dispersed in chloroform. The QD solution (1 mL) was added to the reduced form of (-) ligand solution and further stirred for 2 h under N₂ gas flow at room temperature. The QDs were transferred from the organic phase to the aqueous phase. To remove excess free ligands, the QD solution was dialyzed using Amicon 50 kDa MWCO centrifugal filters. The quantum efficiency of the negatively charged QDs in water was about 27%.

I.5) Surface modification leading to positively charged quantum dots

The ligands of as-synthesized hydrophobic CdSe/CdS/ZnS core/shell/shell QDs were exchanged with tertiary amine decorated lipoic acid, in the following referred to as (+) ligands. A detailed procedure for synthesizing the (+) ligands had been published elsewhere ¹. 10^5 equimolar amount of the oxidized form of (+) ligand to QDs was dissolved in chloroform. 2 M HCl solution was added to the ligand solution and stirred vigorously. The ligand molecules were transferred to the acidic aqueous layer. The crude product in the aqueous phase was obtained by extraction. Two equimolar amounts of sodium borohydride to ligands was added to the solution and vigorously stirred for 20 min under N₂ gas flow at room temperature. The reduced form of (+) ligands were obtained in the solution. Crude hydrophobic QDs were purified by a methanol/hexanes anti-solvent/solvent system. The QDs were dispersed in chloroform. The QD solution (1 mL) was added to the reduced form of (+) ligand solution and further stirred for 2 h under N₂ gas flow at room temperature. The QDs were transferred from the organic phase to the aqueous phase. To remove excess free ligands, the QD solution was dialyzed using Amicon 50 kDa MWCO centrifugal filters. The quantum efficiency of QDs with positively charged surface was about 35%.

II) Photophysical and physicochemical characterization

II.1) Absorbance and photoluminescence spectrum of water soluble quantum dots

II.2) Dynamic light scattering and laser Doppler anemometry measurements

II.3) Concentration determination

II.1) Absorbance and photoluminescence spectra of water-soluble quantum dots

UV/vis absorption spectra were obtained using an Agilent 8453 spectrometer. Photoluminescence (PL) spectra were obtained using a HORIBA FluoroLog-3 spectrometer. The data are presented in Figure SI-II.1.

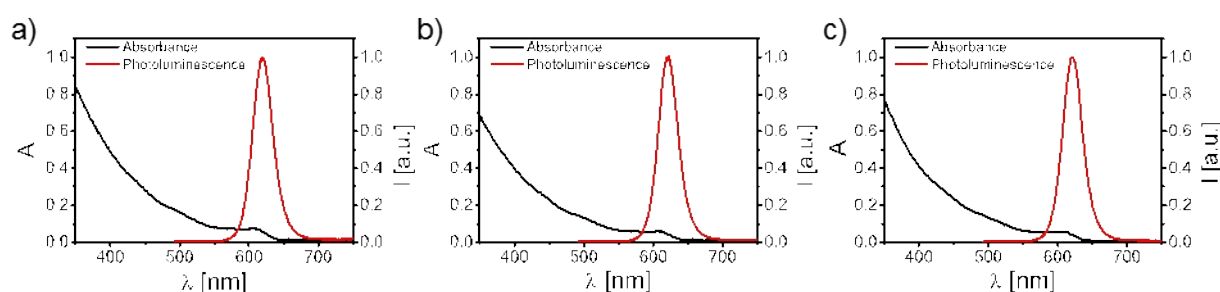


Figure SI-II.1: Absorption $A(\lambda)$ and photoluminescence $I(\lambda)$ spectra recorded in water of (a) negatively charged QDs, (b) QDs with a positive surface, and (c) zwitterionic QDs.

II.2) Dynamic light scattering and laser Doppler anemometry measurements

The hydrodynamic radii r_h and the zeta potentials ζ were measured in water by dynamic light scattering (DLS) and laser Doppler anemometry (LDA), respectively, using a Malvern zetasizer. Data are displayed in Figure SI-II.2.

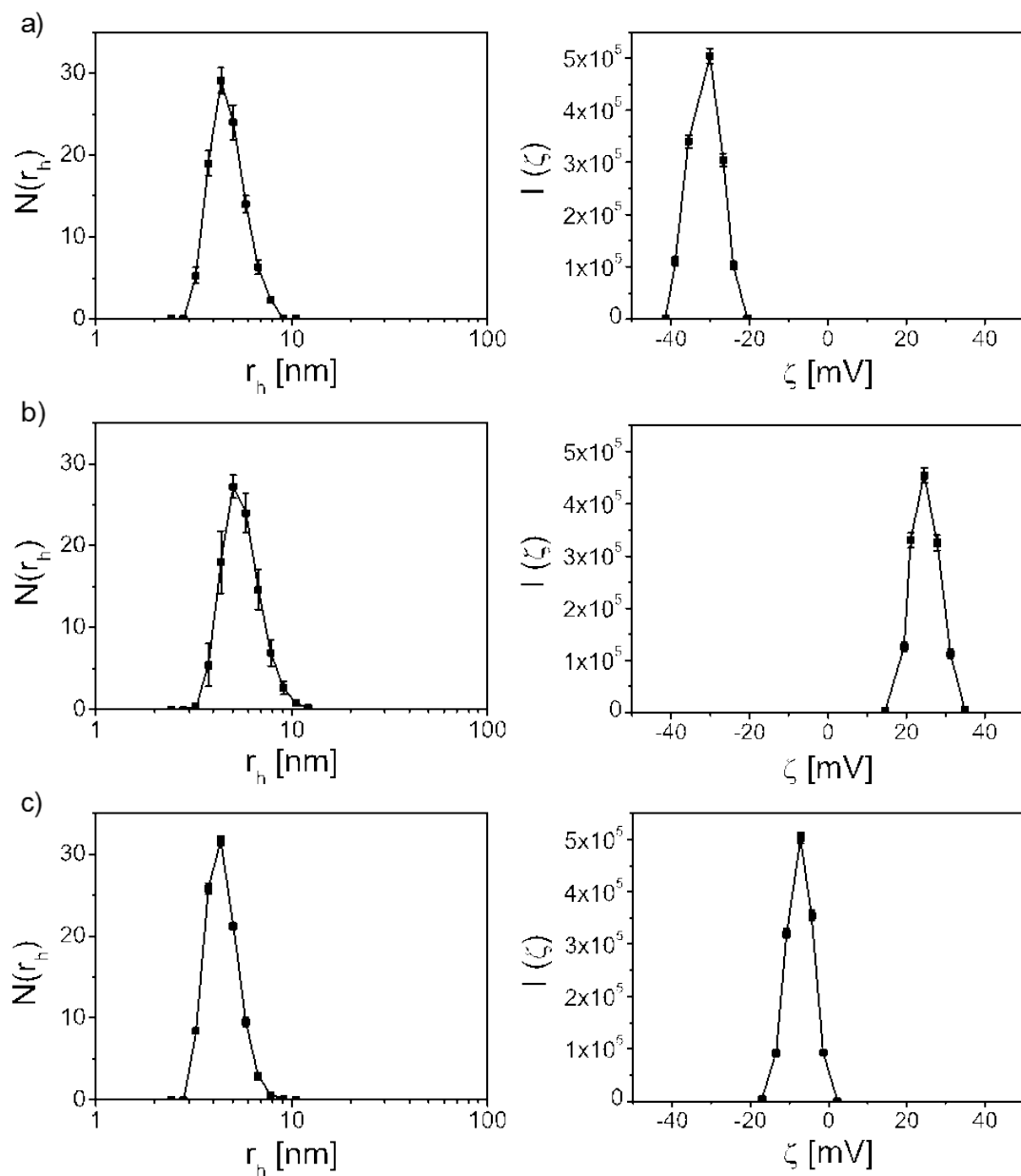


Figure SI-II.2. Graphs showing the number distributions of the hydrodynamic radii and zeta potential values of QDs with a) negatively, b) positively, and c) zwitterionic charged surface coatings, recorded in water. The mean values of the distributions are enlisted in the main manuscript.

II.3) Concentration determination

The QD concentration was determined either optically or by elemental analysis. For optical analysis, UV/vis absorption spectra were recorded and the QD concentration was determined from the absorption at 350 nm *via* the Beer Lambert law, with $\epsilon_{\text{QD}}(350 \text{ nm}) = 1.80 \cdot 10^6 \text{ M}^{-1}\text{cm}^{-1}$.

For concentration determination by elemental analysis, the amount of elemental Cd was measured with inductively coupled plasma mass spectrometry (ICP-MS). Scaling with the number of Cd atoms in each QD ($N_{\text{Cd/QD}}$) allowed for calculation of the QD concentration. The total number of Cd atoms in one QD was determined as $N_{\text{Cd/QD}} = 399 + 1390 = 1789 \approx 1800$ (see section I.2), with 399 atoms per core and 1390 in the shell.

III) Protein adsorption to the surface of quantum dots

Protein adsorption onto the surface of QDs was carried out on a confocal microscope (Microtime 200, PicoQuant, Berlin, Germany) equipped with pulsed laser excitation and time-correlated single-photon counting (TCSPC), as described earlier². Human serum albumin (HSA, A8763, Sigma-Aldrich, St. Louis, MO) was mixed with QDs in phosphate buffered saline (Dulbecco's PBS without Ca^{2+} and Mg^{2+} , PAA Labs, Cölbe, Germany). Solutions with HSA concentrations ranging from 450 μM to 10 nM were prepared by sequential dilution of a concentrated stock solution. To ensure excess protein at all HSA concentrations (so that one can assume that the concentration of QD-bound HSA can be neglected), the QDs were diluted to $c(\text{QD}) = (0.4 \pm 0.1) \text{ nM}$. FCS data were collected for 4 min at room temperature, keeping the solutions in a small borosilicate glass chamber. For thermal equilibration, each solution was kept for 10 min at room temperature prior to the measurement. The diffusion coefficient, D , was obtained from fits to the FCS data as described by Dertinger *et al.*³. The hydrodynamic radii, r_h , of the QDs were then calculated using the Stokes-Einstein equation:

$$r_h = k_B T / (6\pi\eta D)$$

Here, k_B is the Boltzmann constant, η the viscosity of the solution, and D the diffusion coefficient. The dependence of the hydrodynamic radius on the HSA concentration was fitted by using the equation⁴:

$$r_h(N) = r_h(0) \cdot \sqrt[3]{1 + \frac{V_P}{V_{QD}} N}$$

where $r_h(0)$ is the hydrodynamic radius of the QD without any proteins adsorbed, V_P and V_{QD} are the volumes of the HSA protein and the QD, respectively. The volume of HSA, V_P , was estimated as 100 nm³, assuming that the protein is shaped like an equilateral prism with 8 nm side length and 3 nm thickness⁵. The volume of the bare QD is $V_{QD} = (4\pi/3) r_h(0)^3$. N is the estimated number of HSA molecules bound to each QD, given by the Hill equation:

$$N = N_{\max} \frac{1}{1 + (K'_D / c(\text{HSA}))^n}$$

with N_{\max} denoting the maximum number of proteins binding to a QD; K'_D is the apparent equilibrium dissociation coefficient (HSA concentration at the midpoint of the isotherm), and n is the Hill coefficient.

IV) Elemental analysis of quantum dots internalized by cells

IV.1) Materials and reagents

IV.2) Incubation of cells with quantum dots and sample digestion

IV.3) Quantification of cells with the Lowry assay

IV.4) Quantification of quantum dots with elemental analysis

IV.5) Results

IV.6) Cells accumulate quantum dots

IV.1) Materials and reagents

Dulbecco's modified Eagle's medium (DMEM; #D6546), penicillin/streptomycin (P/S, #P4333), saponin (#S7900) and Hank's Balanced Salt Solution (HBSS, #H8264) were purchased from Sigma-Aldrich. GlutaMAX™ (#35050-038) and 0.05% Trypsin-EDTA (#25300) were purchased from GIBCO (life technologies). Glycin (#3908.1) was obtained from Roth. Human cervical carcinoma (HeLa) cells were obtained from the American type culture collection (ATCC). A Neubauer improved counting chamber (haemocytometer) by MARIENFELD Laboratory glassware was used for counting cells. μ -slide 8 well plates (Ibidi #80826) were purchased from ibidi. 6 well culture plates (# 83.1839.300) from Sarstedt were used for culturing cells. Cell culture flasks (25, 75, 150 cm²; #90025, #90075, #90150, respectively) from TPP were used to grow cells. Eppendorf (2 mL; # 72.695.500), and falcon tubes (15 and 50 mL; #62.554.502, #62.547.254, respectively) from Sarstedt were used. A Thermo electron corporation Varifuge 3.0 R by Fisher Scientific was used for filter purification of QDs using Amicon® Ultra ultracentrifugal membrane filters (MWCO 50 kDa) from Millipore. Double distilled deionized water with a resistivity of 18.2 M Ω .cm (Milli-Q) was used for washings and sample preparations. A UV/vis absorption spectrometer (8453 UV-visible spectrophotometer) from Agilent was used for measuring the absorption of the QD samples and the protein content in the Lowry tests. A total protein determination kit (micro-Lowry, Peterson's modification; TPO300-KT, batch# SLBF6513) was purchased from Sigma-Aldrich which contained: Lowry reagent powder (2 g; L3540-1VL, SLBD9543), and Folin and Ciocalteu's phenol reagent (F9252-1EA, Lot#SHBB8897V, Pcode: 1001449215). The inductively coupled plasma mass spectrometer (ICP-MS) from Agilent 7700 Series was used to determine the concentrations of cadmium and hence the QD concentrations. Hydrochloric acid (HCl; 35 wt%, ultra pure, #7647010), and nitric acid (HNO₃; 67 wt%, ultra pure, #7697372), were purchased from Fisher Chemicals.

IV.2) Incubation of cells with quantum dots and sample digestion

HeLa cells were grown in 150 cm² flasks and seeded for experiments in 6 well plates at a density of 200,000 cells per well (each well had a surface area of 8.95 cm² and was filled with

3 mL of medium). After 24 h, the growth medium was aspirated and fresh growth medium with freshly purified QDs at concentration $c(\text{QD})$ was added. QDs were purified directly before addition by means of centrifugation with ultracentrifugal membrane filters (MWCO 50 kDa) for 5 min at 3300 rpm followed by washing with double distilled deionized water. Uptake experiments were performed in the presence of two types of cell growth media: either complemented with 10% serum or serum free. QDs were added in two different concentrations: $c(\text{QD}) = 10 \text{ nM}$ and 80 nM . HeLa cells were incubated for 6, 12, and 24 h with the QDs inside an incubator set at 37°C with 5% CO_2 supply. After incubation of HeLa cells with QDs for defined time intervals, the cells were washed with phosphate buffered saline (PBS) and detached from the bottom of the plates using 0.05% trypsin-EDTA (2 min incubation). When cells with internalized QDs were detached, growth medium (twice the volume of the trypsin-EDTA solution) was added in order to minimize the effect of trypsin-EDTA. Detached cells were collected in sample vials. The sample vials were centrifuged at 1000 rpm for 5 min and the supernatant was removed. The pellets of cells were washed again with PBS, and as much supernatant as possible was removed (note that remaining trypsin in solution would interfere with determining protein concentrations as described later). Then 1x lysis buffer in water (100 μL per sample) was added to each cell pellet. Incubation at room temperature for 30 minutes resulted in dissolution of the pellets. The samples with the lysed cells were stored at -20°C for further processing, i.e., determination of the amount of proteins and elemental Cd in the lysates, as described in the following sections.

The number of QDs per cell that had been added, $N_{\text{QD,add}}$, can be calculated as follows. $V_{\text{medium}} = 1.2 \text{ mL}$ medium containing $c(\text{QD}) = 10 \text{ nM}$ or 80 nM QDs had been added to $N_{\text{cell}} = 200,000$ cells. Thus, $N_{\text{QD,add}} = (V_{\text{medium}} \cdot c(\text{QD}) \cdot N_A) / N_{\text{cell}}$, with N_A being the Avogadro number. For QD concentrations of 10 nM and 80 nM , $N_{\text{QD,add}} = 3.6 \cdot 10^7 \text{ QDs/cell}$ and $29 \cdot 10^7 \text{ QDs/cell}$, respectively.

IV.3) Quantification of cells with the Lowry assay

A commercial protein determination kit (Lowry assay; TPO300-KT) was used to quantify the cells by detecting the amount of proteins they contain. Lowry reagent solution was prepared by adding 40 mL of double distilled deionized water to 2 g of Lowry reagent powder, followed by shaking to dissolve all powder. The labeling solution was prepared by transferring 18 mL of Folin and Ciocalteu's phenol reagent into an amber glass, followed by rinsing the sides of the tube containing this reagent with 10 mL of water and adding this to the amber glass. Finally, 80 mL of additional water were added to the same amber glass in order to achieve the working concentration of labeling reagent.

First a calibration curve was obtained. HeLa cells were detached from cell culture flasks by adding 0.05% trypsin-EDTA (2 min incubation), twice the volume of the growth medium was added, cells were pelleted by centrifugation, PBS was added after removal of the supernatant,

cells were pelleted again and the supernatant was removed, as described in section IV.2. The cells were then suspended in a small volume of PBS and their density in terms of cells per volume of solution was determined by counting three times with a haemocytometer. Then 1x lysis buffer was added. After cell lysis, serial dilution of the cell lysates in lysis buffer was performed in order to achieve samples with subsequently smaller and smaller cell concentrations. For blank measurements, only lysis buffer was used without cell lysates. In sample tubes, 5 μL of cell lysates were added into 100 μL of Lowry reagent solution, mixed well, followed by waiting for 20 min for completion of complex formation between proteins of the cell lysates and the Lowry reagent solution. Then, 50 μL of labeling reagent solution were added. After waiting for 30 min, the absorption of samples was measured by UV/vis absorption spectrometry. Spectra were recorded from 550 - 800 nm, and from the spectra, the absorption A_{750} at 750 nm was determined, cf. Figure SI-IV.1. Note that the spectrum of the lysis buffer without cells has been subtracted as a blank. Then the absorption values (corresponding to the protein content of the samples) were plotted against the number of cells, leading to a calibration curve that correlated the absorption A_{750} to the number of cells, N_{cell} , cf. Figure SI-IV.1.

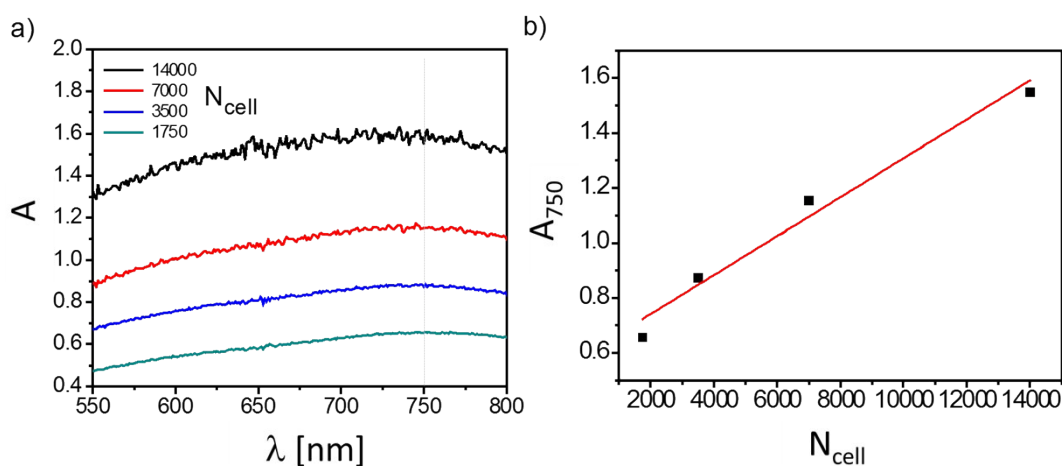


Figure SI-IV.1: (a) Absorption spectra $A(\lambda)$ of cell lysates recorded from 550-800 nm. (b) Absorption values A_{750} of cell lysates at 750 nm, plotted versus the number of cells, N_{cell} , in the sample. The slope of the fitted line was used for determining the amount of cells in unknown samples from their absorption values at 750 nm.

The number of cells in the lysates was then determined by the Lowry assays, and absorption of the lysates at 750 nm was converted into cell numbers using the calibration curve shown in Figure SI-IV.1.

IV.4) Quantification of quantum dots with elemental analysis

The amount of QDs in the cell lysates was determined in terms of elemental Cd as measured by inductively coupled plasma mass spectrometry (ICP-MS). The cell lysates containing the internalized QDs were first digested with aqua regia. For preparing the stock solution, 50 μ L of sample suspension was transferred into 6 mL perfluoroalkoxy alkane tubes (PFA) prefilled with freshly prepared 150 μ L aqua regia (consisting of 1 part HNO₃ and 3 parts HCl) and mixed for at least 8 h under constant agitation. During this period, the QDs as well as the remaining organic cell fragments were digested and broken down into small molecular /atomic components. In the second step, 4.6 mL of 2% HNO₃ solution as low matrix was introduced to each digested sample to prevent the *aqua regia* from digesting the ICP-MS machinery as well as to provide an ion stable environment with constant background conditions for all samples. ICP-MS measurements were performed using 5 repetitions per sample, 100 sweeps and a peak pattern of 3 peaks. The diluted samples were introduced to the ICP-MS set-up through an integrated autosampler coupled to a peltier cooling spray chamber where the samples were nebulized and taken up by the argon gas flow at a speed of ½ m/s. The concentration determination was performed using a calibration curve for Cd consisting of 9 measurement points (2500 to 0 μ g/L) of freshly prepared Cd concentrations derived from cadmium standard solutions from Agilent (1000 mg/L). Results are given as the mean of all five measurements and are presented in parts per billion or μ g/L (ppb = μ g/L; *i.e.* μ g Cd per L of sample solution). By knowing the volume of the sample solution the total mass of Cd in the sample can be determined.

IV.5) Results

Having determined the number of cells and the amount of Cd in each sample, the amount of Cd per cell was calculated. All data are summarized in Tables SI-IV.1 and SI-IV.2. In Figure SI-IV.2, the Cd⁺² uptake by HeLa cells [fg/cell] is shown at different time points. It can be seen that the internalization rate of positively charged QDs was higher than that of negatively charged QDs, whereas zwitterionic QDs showed almost negligible internalization. Uptake of QDs in serum free medium was higher than in serum containing medium.

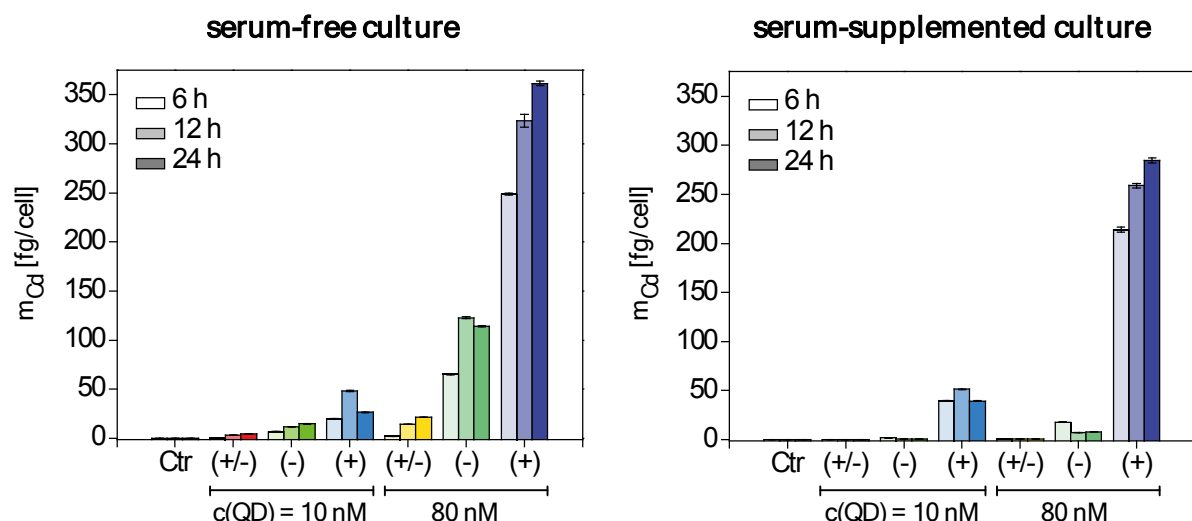


Figure SI-IV.2. Mass of Cd per cell (m_{Cd}), determined by ICP-MS after HeLa cells had been incubated for different times t with the 3 different QDs at different concentrations $c(QD)$. The same data are displayed in Figure 3 of the main manuscript in a different format. The data are enlisted in Tables SI-IV.1 and SI-IV.2. The gray levels as indicated in the legends refer to the incubation times.

t [h] \ c(QD)	c(QD) = 0	c(QD) = 10 nM			c(QD) = 80 nM		
	Control	(+/-)	(-)	(+)	(+/-)	(-)	(+)
6	$1.05 \cdot 10^{-3}$ $\pm 2.29 \cdot 10^{-5}$	0.733 ± 0.015	6.887 ± 0.109	19.79 ± 0.372	2.017 ± 0.008	65.31 ± 0.578	248.68 ± 0.979
12	$2.8 \cdot 10^{-3}$ $\pm 5.54 \cdot 10^{-5}$	3.22 ± 0.0345	12.118 ± 0.023	48.544 ± 0.665	14.655 ± 0.079	122.77 ± 1.185	323.45 ± 6.71
24	$5.2 \cdot 10^{-3}$ $\pm 2.34 \cdot 10^{-4}$	4.847 ± 0.056	14.827 ± 0.134	26.615 ± 0.225	21.78 ± 0.142	114.21 ± 0.768	361.45 ± 2.32

Table SI-IV.1. Mass of Cd per cell (m_{Cd}) in [fg/cell], determined by ICP-MS after HeLa cells had been incubated for different incubation times t with the 3 different QDs (+/-, -, +) having different QD concentration $c(QD)$ in serum-free culture. Data are presented as mean of 5 measurements \pm standard deviation.

t [h] \ c(QD)	c(QD) = 0	c(QD) = 10 nM			c(QD) = 80 nM		
	Control	(+/-)	(-)	(+)	(+/-)	(-)	(+)
6	$6 \cdot 10^{-4}$ $\pm 2.02 \cdot 10^{-5}$	0.19 ± 0.028	1.787 ± 0.08	40.08 ± 0.127	0.48 ± 0.005	17.86 ± 0.24	214.12 ± 2.37
12	$5.4 \cdot 10^{-4}$ $\pm 4.1 \cdot 10^{-5}$	0.125 ± 0.011	0.607 ± 0.006	51.404 ± 0.16	0.674 ± 0.015	7.104 ± 0.085	258.82 ± 2.095
24	$2.6 \cdot 10^{-4}$ $\pm 4.63 \cdot 10^{-6}$	0.192 ± 0.01	0.475 ± 0.018	39.481 ± 0.484	1.06 ± 0.005	8.054 ± 0.044	284.796 ± 2.65

Table SI-IV.2. Mass of Cd per cell (m_{Cd}) in [fg/cell], determined by ICP-MS after HeLa cells had been incubated for different times t with the 3 different QDs (+/-, -, +) at different QD concentrations $c(QD)$ in serum-supplemented culture. Data are presented as mean of 5 measurements \pm standard deviation.

As each QD comprises approximately $N_{Cd/QD} = 1800$ Cd atoms (*cf.* § II.3), the mass of Cd, m_{Cd} internalized by each cell can be estimated as $N_{QD,int} = (m_{Cd}/M_{Cd}) \cdot N_A / N_{Cd/QD}$ QDs, with the molar mass of Cd $M_{Cd} = 112$ g/mol and Avogadro's constant $N_A = 6.02 \cdot 10^{23}$ mol⁻¹. For positively charged QDs added at a concentration of $c(QD) = 80$ nM, *i.e.* $N_{QD,add} = 2.9 \cdot 10^8$ QD added per cell (*cf.* § IV.2) $m_{Cd} = 361$ fg/cell corresponds to $N_{QD,int} = 1.1 \cdot 10^6$ QDs internalized per cell.

IV.6) Cells accumulate quantum dots

ICP-MS measurements allow for estimating the number of internalized QDs per cell: $N_{QD,int}$. Knowing this number one can calculate the intracellular QD concentration $c_{int}(QD)$. For doing so, homogeneous distribution of the QDs along the cytoplasm is assumed, despite knowing that the QDs reside only inside endosomes/lysosomes. The volume of one HeLa cell is reported in databases ⁶ as (4400 - 5000) μm^3 ⁷ and (1200-4290) μm^3 ⁸. We here assume $V_{cell} = 4000 \mu m^3 = 4 \cdot 10^3 \cdot (10^{-5})^3 dm^3 = 4 \cdot 10^{-12} dm^3 = 4 \cdot 10^{-12} L$. The intracellular QD concentration thus is $c_{int}(QD) = (N_{QD,int}/N_A)/V_{cell}$. For positively charged QDs added at a concentration of $c(QD) = 80$ nM there are $N_{QD,int} = 1.1 \cdot 10^6$ QDs internalized per cell. This leads then to an intracellular QD concentration of $c_{int}(QD) = (1.1 \cdot 10^6 / 6.02 \cdot 10^{23} mol^{-1}) / 4 \cdot 10^{-12} L \approx 0.046 \cdot 10^{-5} mol/L = 0.46 \mu M = 460$ nM. Thus, inside cells the QDs are (460 nM/ 80 nM) ≈ 6 times more concentrated than the extracellular QD concentration to which cells have been exposed to.

V) Fluorescence analysis of quantum dots internalized by cells: microscope evaluation

V.1) Materials and reagents

V.2) Incubation of cells with quantum dots, immunostaining, and microscopy

V.3) Data analysis of microscopy images

V.4) Results

V.1) Materials and reagents

Paraformaldehyde (8%; #157-8-100) was purchased from Electron Microscopy Sciences. Phosphate buffered saline (PBS; Biochrom #L 1825) and fetal bovine serum (FBS; Biochrom, Germany, #S0615) were purchased from Merck Millipore. Bovine serum albumin (BSA; #001-000-161) and DyLight 649 donkey anti-mouse IgG (H+L, secondary antibody; #715-605-150) were purchased from Jackson ImmunoResearch Laboratories. Lysosomal-associated membrane protein 1 (LAMP 1; mouse anti-human IgG1; developmental studies hybridoma bank #H4A3, Supernatant) was obtained from the University of Iowa, Department of Biology, USA. Wheat germ agglutinin, Oregon green (WGA 488 #W6748) was purchased from Invitrogen (Molecular probes). Hoechst 33342, trihydrochloride, trihydrate (#H1399) was purchased from life technologies. Cell lysis buffer (5X reagent; #2018-02-12) was used from Promega Corporation.

A confocal laser scanning microscope (CLSM 510 Meta) from Zeiss was used for visualizing the fixed and immunostained cellular samples containing internalized QDs. For sample visualization and image acquisition, the CLSM was equipped with diode, argon and helium neon lasers emitting at 405, 488, 543 and 633 nm, respectively. Samples were observed through Plan-Apochromat 20X/0.8 M27 and 63X/1.40 oil-immersion DIC M27 objectives.

V.2) Incubation of cells with quantum dots, immunostaining, and microscopy

HeLa cells were initially grown in 150 cm³ flasks and seeded for QD uptake into 8 well μ -ibidi plates at a density of 20,000 cells per well. Each well had a surface area of 1 cm² and was filled with 0.3 mL of medium. After 24 h, the growth medium was changed and fresh growth medium with QDs was added. Directly before usage, the QDs were purified using ultracentrifugal membrane filters (MWCO 50 kDa) for 5 min at 3300 rpm, washed with double distilled deionized water, and their concentration was determined. Uptake experiments were performed with two types of growth media, either complemented with 10% FBS or without serum. QDs were added at two different final concentrations, *i.e.*, $c(\text{QD}) = 10 \text{ nM}$ and 80 nM , and cells were incubated with the QDs for different time intervals (6 h, 12 h, and 24 h) inside an incubator at 37 °C with 5% CO₂ supply. Control experiments were performed in parallel,

where cells were grown in the presence of serum containing and serum free medium (without QDs).

After incubating the HeLa cells with QDs for defined time intervals t , the cells were washed with PBS and fixed with 4% paraformaldehyde solution in PBS (20 min incubation at room temperature), followed by washing with PBS. Cell and nuclear membranes were stained with WGA 488 (20 $\mu\text{g}/\text{mL}$) and Hoechst reagent (5 $\mu\text{g}/\text{mL}$), respectively (15 min incubation in growth medium). The cells were washed three times with Hank's balanced salt solution (HBSS; actually PBS would have worked as well), and permeabilized by means of permeabilization solution (glycine 5 mg/mL and saponin 0.5 mg/mL , in PBS; 5 min incubation). Then the cells were incubated at 37 °C in an incubator under 5% CO_2 supply with blocking solution (= 20 mg/mL BSA in permeabilization solution) for 30 min. Subsequently, cells with QDs were immunostained using LAMP 1 (primary antibody in blocking solution; 5 $\mu\text{g}/\text{mL}$, 1 h incubation at 37 °C and 3 washing steps with blocking solution) and Dylight 649 (secondary antibody in PBS; 1.25 $\mu\text{g}/\text{mL}$, 1 h incubation at 37 °C followed by 3 times washing with PBS). Then, 300 μL of PBS was added per well of the 8 well μ -ibidi plates and samples were imaged with CLSM.

Fluorescence microscopic images of immunostained samples with internalized QDs and their corresponding controls (cells without QDs) were captured with CLSM using either a Plan-Apochromat 20x/0.8 M27 or a 63x/1.40 oil-immersion DIC M27 lens. For visualization of different stained cellular compartments and internalized QDs, the fixed samples were excited at 405, 488, 543, and 633 nm, respectively. Nuclei stained with Hoechst 44432 were excited at 405 nm, and emission of the dye was observed between 420 and 480 nm. Cellular membranes stained with WGA 488 were visualized by exciting the corresponding fluorophores at 488 nm, and emission was observed between 505 and 550 nm. The fluorescence of the QDs was excited at 543 nm, and emission was captured using a 560 nm long pass filter. Antibody-labeled lysosomes were excited at 633 nm, and their emission was observed using a 650 nm long pass filter. Control experiments demonstrated negligible cross-talk between the different channels.

25 images *per* sample were recorded, covering on average of 400 - 500 cells per condition. Some representative images of selected samples (incubated with 80 nM QDs) at different time points with corresponding controls are provided in Figures SI-V.1 to SI-V.4. As the positively charged QDs (at $c(\text{QD}) = 80 \text{ nM}$) showed the highest fluorescence intensity, the imaging conditions were fixed and set with respect to their fluorescence signals. Images of control samples were taken to obtain threshold values for data evaluation of treated samples in order to eliminate possible background noise (background fluorescence/auto fluorescence of cells). For determining the total QD uptake, cells were imaged using a 20x objective, while the QDs enrichment inside lysosomes was visualized with higher magnification using a 63x objective.

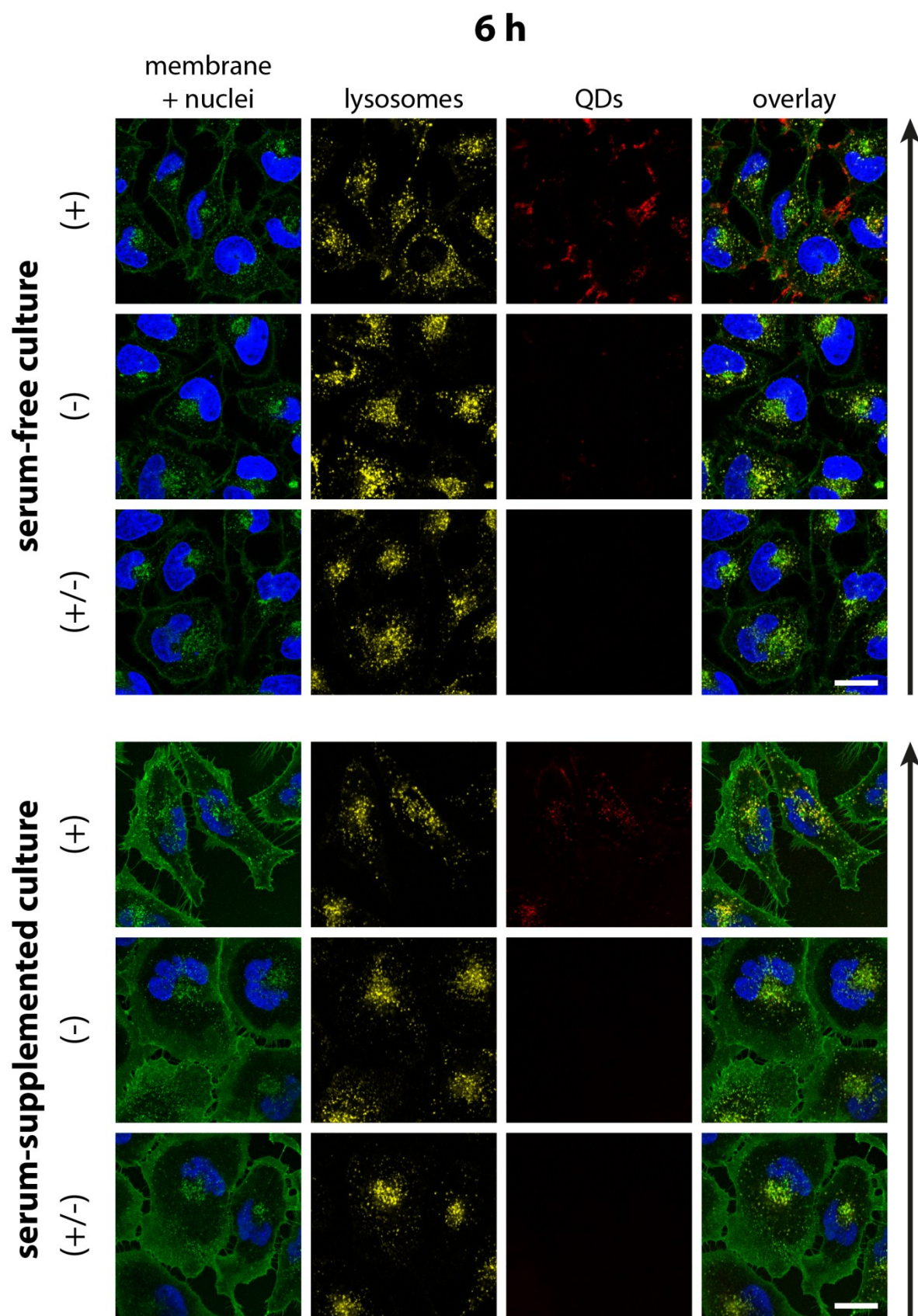


Figure SI-V.1. CLSM images of QDs ($c(QD) = 80 \text{ nM}$) internalized by HeLa cells after 6 h exposure, using a 63x objective. The scale bar corresponds to $25 \mu\text{m}$.

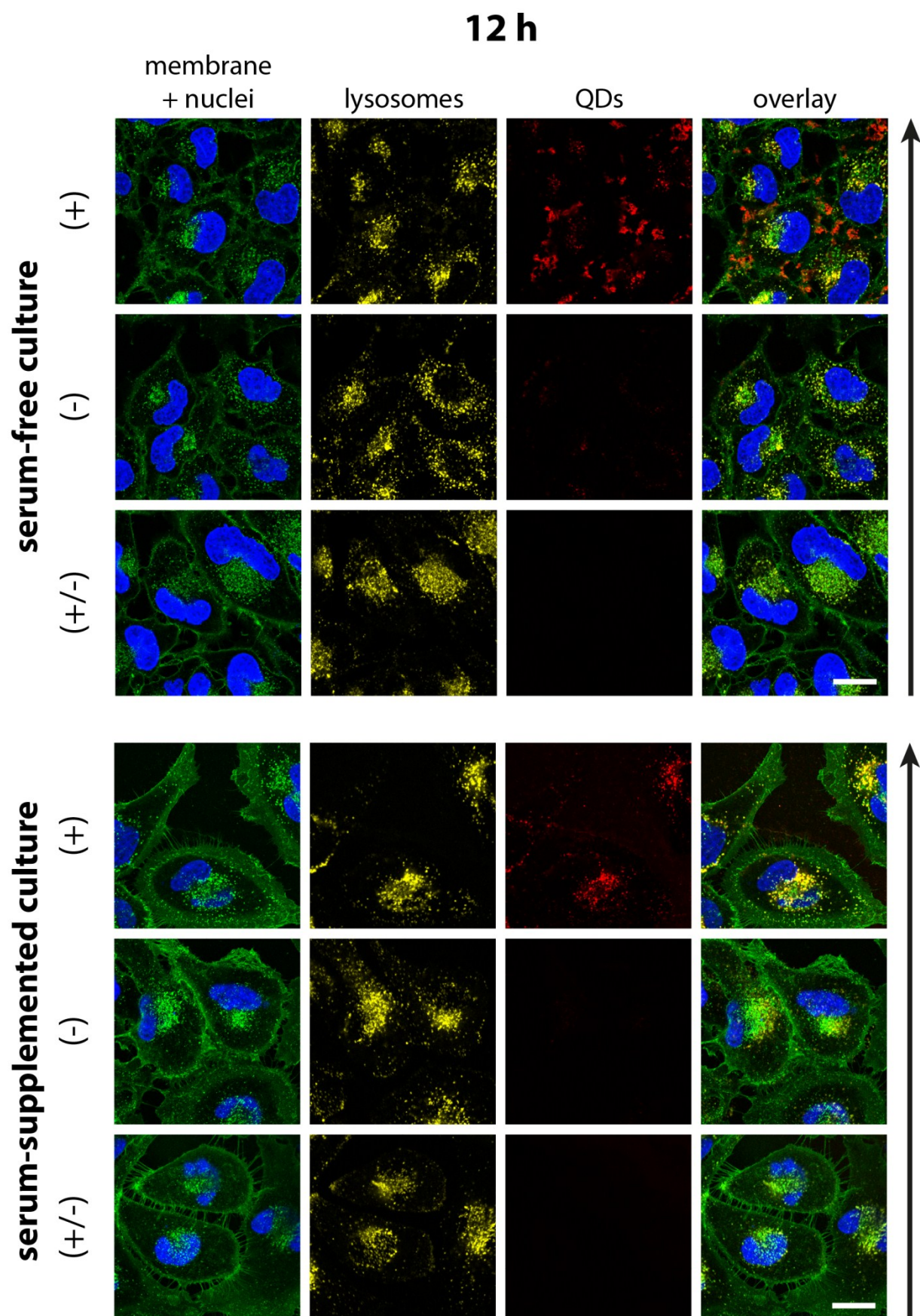


Figure SI-V.2. CLSM images of QDs ($c(\text{QD}) = 80 \text{ nM}$) internalized by HeLa cells after 12 h exposure, using a 63x objective. The scale bar corresponds to $25 \mu\text{m}$.

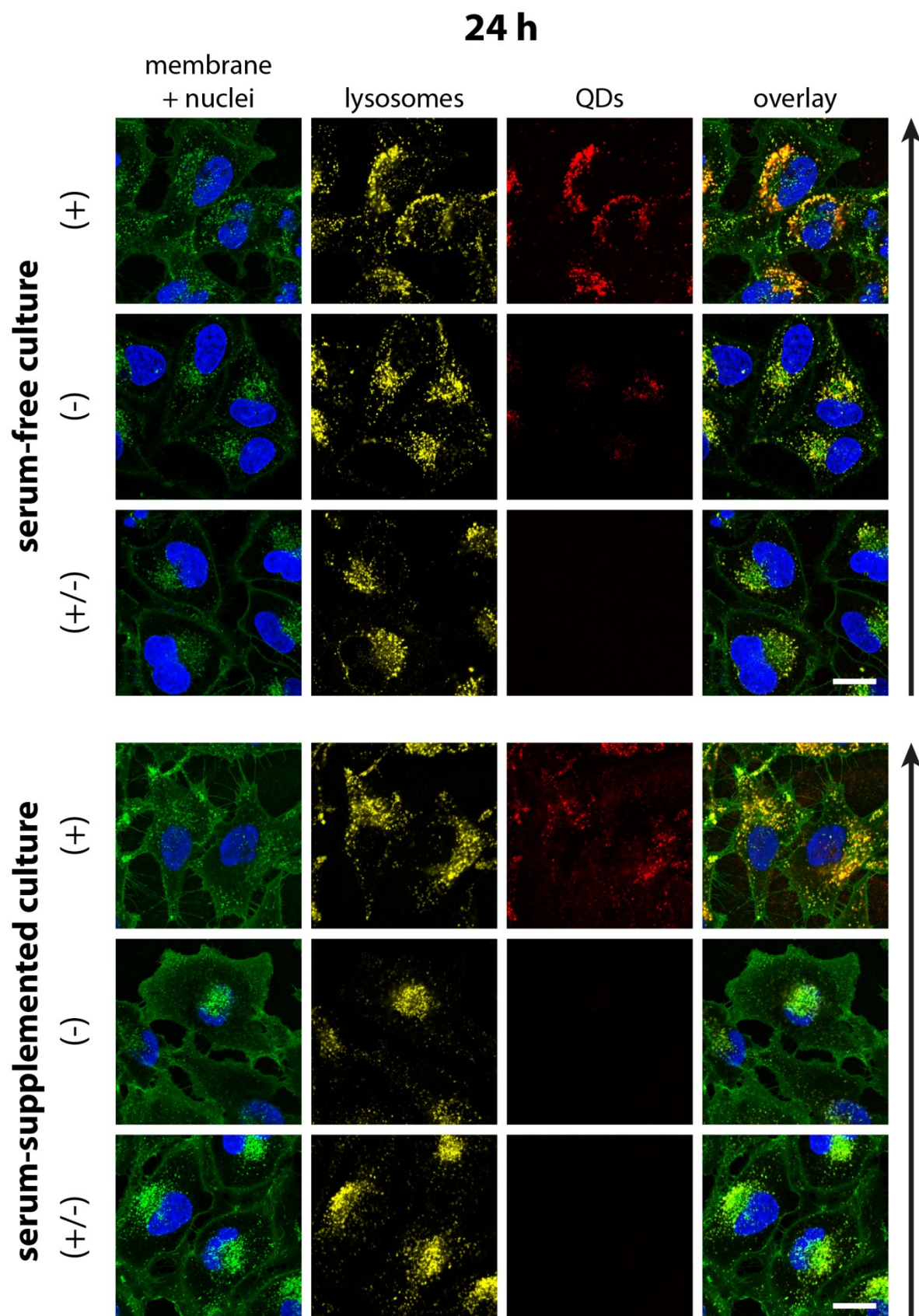


Figure SI-V.3. CLSM images of QDs ($c(\text{QD}) = 80 \text{ nM}$) internalized by HeLa cells after 24 h exposure, using a 63x objective. The scale bar corresponds to $25 \mu\text{m}$.

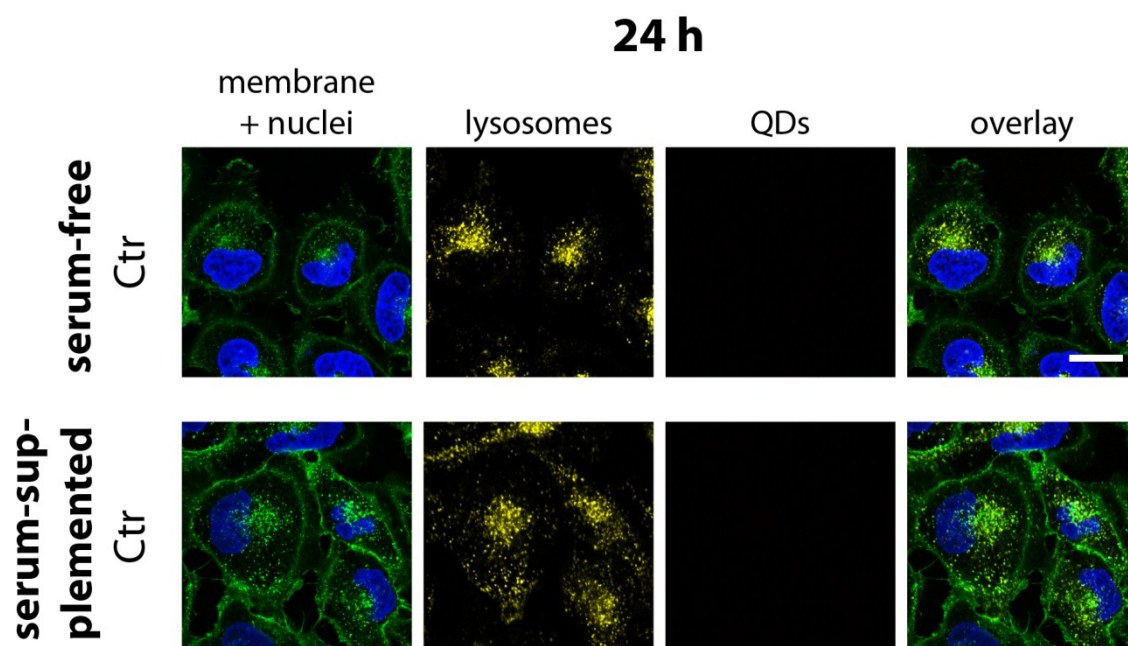


Figure SI-V.4. CLSM images of HeLa cells which had not been exposed to QDs, using a 63x objective. The scale bar corresponds to 25 μm .

It was observed that, in serum free medium, the positively charged QDs showed a tendency to agglomerate on the outer cell membrane. Even after washing with PBS before staining, the extracellular QDs could not be completely removed. They were sticking to the cell membranes and culture plates in the form of aggregates, *cf.* Figure SI-V.5. In contrast, in serum containing medium, positively charged QDs did not show the same agglomeration behavior, and after washing with PBS, most of the non-internalized QDs were removed.

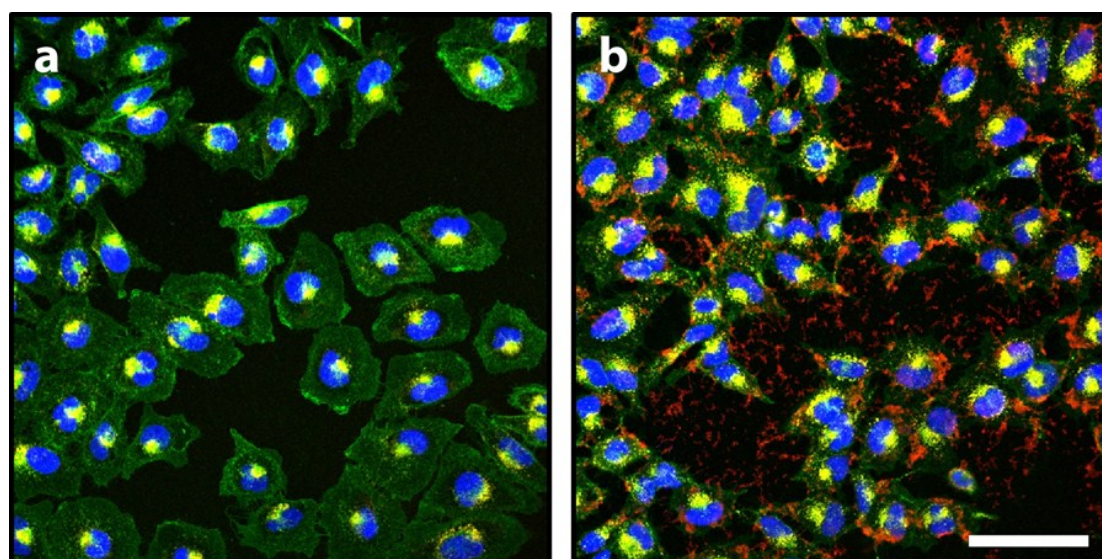


Figure SI-V.5. CLSM images of positively charged QDs uptake by HeLa cells after 6 h incubation in (a) serum containing and (b) in serum free cell growth media. In the serum free culture, precipitation of QDs (red fluorescence) to the bottom of the cell culture plate can be observed. The scale bar corresponds to 100 μm .

V.3) Data analysis of fluorescence microscopy images

For determining the total uptake of QDs by cells the fraction of QDs inside lysosomes from fluorescence micrographs, the fluorescence signal of intracellular QDs was quantified with CellProfiler and Matlab (Mathworks) ⁹. For measuring the total uptake, individual cells were identified based on their nuclear and cell membrane staining. In the first step, by setting appropriate threshold values, cell nuclei were identified and segmented as primary objects. In the second step, the outer boundaries/outlines of the cells were detected as secondary objects using the positions of the nuclei on the basis of stained cell membranes. For this purpose, the “Propagation” algorithm of CellProfiler was used ¹⁰. In the third step, lysosomes were identified on the basis of lysosomal staining by thresholding. Next, the shapes generated (nuclei, cell and lysosomal boundaries) as results of the above processing steps, were converted into masks. Then, the integrated fluorescence intensities, I_{cell} and I_{lyso} , of QDs internalized by individual cells and found in lysosomes was determined (defined by the masked areas). Finally, manual post-processing steps were performed with a graphical user interface written in Matlab to correct erroneous segmentation results and exclude dead cells and aggregates of QDs. A brief description of above mentioned evaluation process is given in Figure SI-V.6. A detailed description can be found in the following.

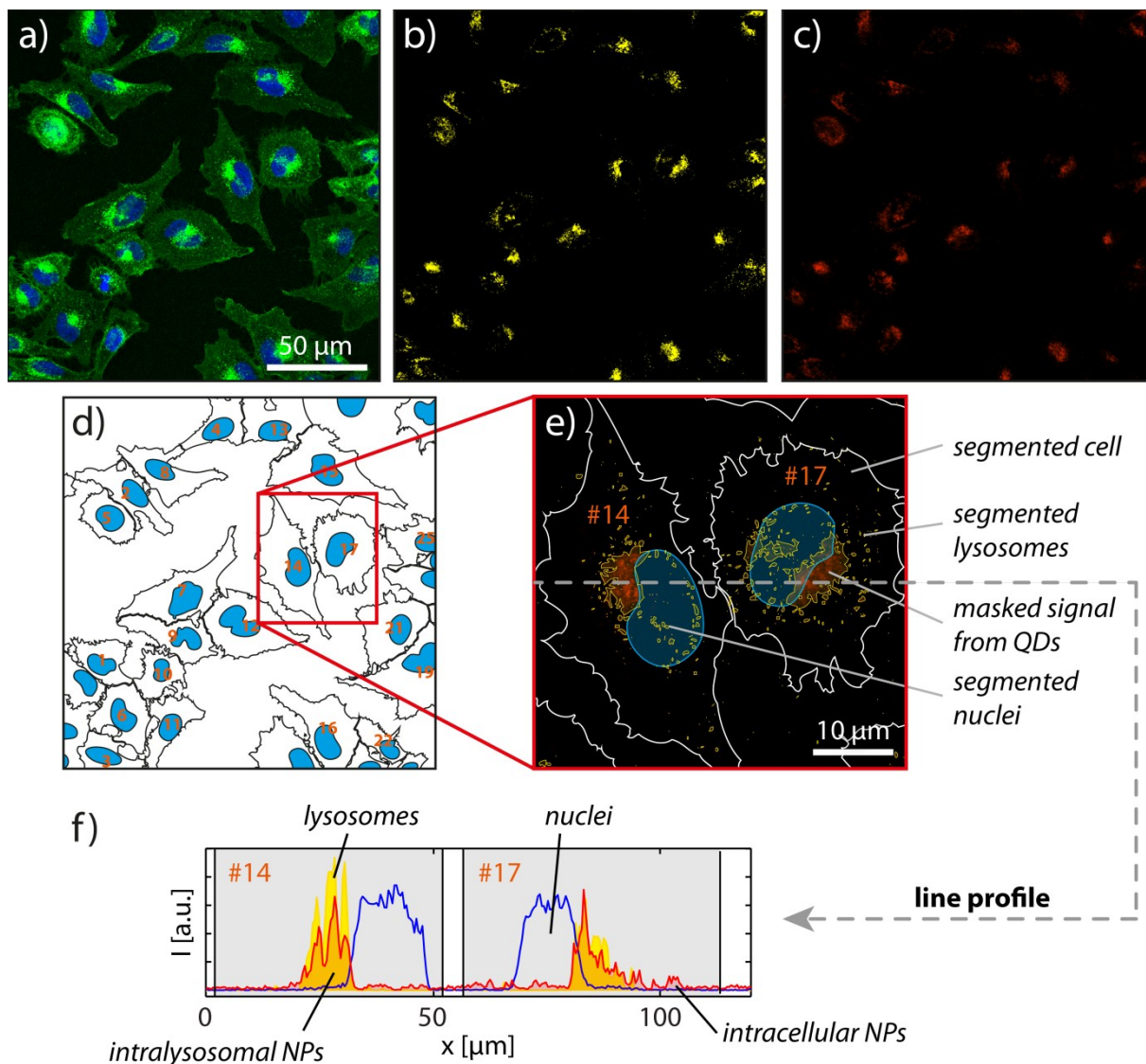


Figure SI-V.6: Illustration of image processing steps of QD uptake by HeLa cells. Uptake of QDs by HeLa cells was quantified by evaluating their fluorescence signal per cell based on CLSM micrographs. Multichannel CLSM micrographs (raw data) showing (a) the cell membrane (green) and nuclei (blue), (b) lysosomal membranes (yellow) and (c) QDs (red). In (d), segmented cellular compartments are shown. Positions of nuclei and the boundaries of cell membranes were used to identify the outlines of cells. The selected magnified area of (d) is shown in (e), showing segmented nuclei, cells, lysosomes, and masked fluorescence signals of QDs. In (f), an intensity line profile calculated along the dashed line in (e) is shown. The integrated intensity of the QD fluorescence per cell is considered to be proportional to the total relative uptake of QDs per cell. Partly imaged cells were not considered during image processing and were not used later for data evaluation. Due to variation in the sizes of different cells, the integrated fluorescence intensity I of QDs was determined. This means that inside each cell the mean pixel intensity of QDs was multiplied with the cross section area of the underlying cell. Unlike mean fluorescence intensity values of QDs per cell, which fluctuate with varying cell sizes, the integrated fluorescence intensity values remain the same if the same

amount of QDs is internalized by two cells being compared in case of assuming linear fluorescence response of QDs.

Image acquisition: Images were acquired using a confocal LSM 510 Meta from Zeiss operated by the software Zen 2009. Along each well of an 8-well μ -slide from Ibidi a grid of 16 positions was generated with Matlab by providing the coordinates of a focussed reference point on the slide and knowing the slide dimensions. The obtained position list was re-imported into Zen 2009. Imaging was performed either by using a 20x objective at a lateral sampling resolution of 0.44 μm or a 63x objective (lateral resolution 0.14 μm , pinhole aperture 1 Airy unit). For autofocusing a software-based routine was used. The boundary layer between substrate and cell medium was identified by the autofocus algorithm based on the increased level of back-scattered photons at this specific axial position. Imaging was performed at a pre-defined distance above this layer (typically 2 μm).

Image processing for the determination of QD uptake (from low-magnified images acquired with a 20x objective): The integrated fluorescence of QDs per cell and inside lysosomal structures was derived from fluorescence micrographs acquired as described above. Image files were stored as ".ism"-files containing multiple positions and spectral channels (nuclei, cell membranes, lysosomes, QDs) and imported into the Matlab workspace including metadata by using a modified version of the "tiffread29" function written by Nedelec *et al.*¹¹. Firstly, nuclei were identified. For this purpose, the image of the respective channel was median-filtered with a kernel size of 3 pixels (px) to remove photon-shot noise. In the next step, the resulting image was slightly blurred with a Gaussian lowpass filter (size: 10 px, standard deviation: 2) to avoid oversegmentation of nuclei. After rescaling the intensities of the image, the nuclei were identified by using the "IdentifyPrimAutomatic" function of the Matlab-based version of CellProfiler 1.0.5811⁹. As parameters, a global manually-set threshold was provided (strong background is usually not an issue in confocal fluorescence images) and intensity-based declumping was enabled to separate touching nuclei. Secondly, the cell outlines were reconstructed based on the fluorescence image representing the cell membranes, and the positions of the obtained nuclei which were used as seeds. For this purpose, the "IdentifySecondary" function of CellProfiler was used employing the "propagation" algorithm proposed by Jones *et al.*¹⁰ for cell segmentation. Cells touching the image boundary were not considered. The resulting cell objects were inspected visually, and potential segmentation errors were corrected manually by adjusting the cell outlines (dissecting, merging, expanding or shrinking cell objects using the "imfreehand" function of the Image Processing Toolbox) within a graphical user interface realized with Matlab. Thirdly, lysosomal structures were segmented from the corresponding fluorescence images by subtracting a manually set global threshold. Fourthly, for each cell object, the integrated fluorescence intensity of the QD-signal was determined (i) for the whole cell (I_{cell}) and (ii) for pixels associated with lysosomes (I_{lyso}). In (i), this was realized by masking the underlying image showing the QDs by each cell object successively, followed by summation of the remaining pixel intensity values. Similarly, for (ii), the masked images as obtained in (i) were only

evaluated where pixels were colocalizing with lysosomal structures. Finally, the obtained values for I_{cell} and I_{lyso} were averaged, and the standard deviation was calculated.

Image processing for determination of the lysosomal QD density (from images acquired with a 63x objective): For comparison with the results derived from low magnification images, the density of QDs inside lysosomes was estimated based on images acquired with a 63x objective. Image files were stored as “.lsm”-files containing multiple positions and spectral channels (lysosomes, QDs) and imported into Matlab as described above. Images of both channels were median-filtered with a kernel size of 3 pixels. Then, lysosomes were segmented by thresholding. Afterwards, the average lysosomal QD intensity was calculated by masking the image showing the fluorescence of the QDs with the segmented lysosomes and calculating the average intensity of the remaining pixels. The results are expressed as mean values \pm standard deviation between data obtained from different lateral positions.

Note, while the data evaluation is based on colocalization of QDs with cells and lysosomes, due to the limited lateral resolution of CLSM, QDs adherent to the outer cell membrane may be counted as false positives, *i.e.* internalized QDs.

V.4) Results

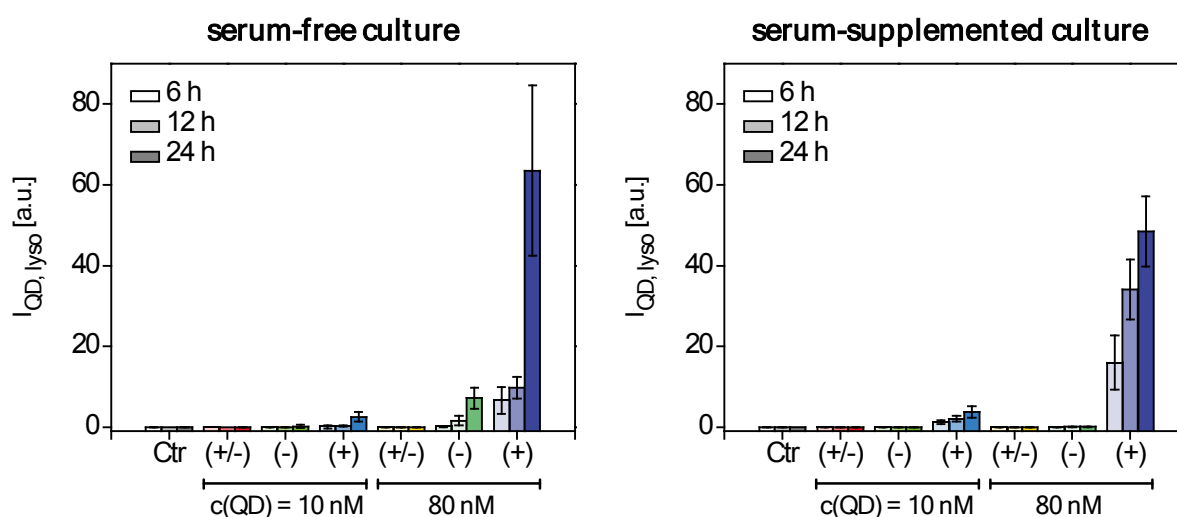


Figure SI-V.7. Intra-lysosomal QDs uptake $I_{\text{QD,lyso}}$ by HeLa cells at different time points determined by fluorescence microscopy, derived from higher magnification (63x objective) CLSM micrographs. These data correspond to the ones shown in other format in Figure 3 of the main manuscript. The data are enlisted in Tables SI-V.1 and SI-V.2.

t [h]	c(QD) = 0	c(QD) = 10 nM			c(QD) = 80 nM		
	Control	(+/-)	(-)	(+)	(+/-)	(-)	(+)
6	$5.8 \cdot 10^{-5}$ $\pm 6 \cdot 10^{-5}$	$1.1 \cdot 10^{-5}$ $\pm 0.0 \cdot 10^{-5}$	$2.8 \cdot 10^{-4}$ $\pm 3.2 \cdot 10^{-4}$	0.14 ± 0.3977	0.003 ± 0.005	0.097 ± 0.1	6.65 ± 3.38
12	$1.36 \cdot 10^{-5}$ $\pm 0.0 \cdot 10^{-5}$	$3.5 \cdot 10^{-3}$ $\pm 0.0 \cdot 10^{-5}$	$6.63 \cdot 10^{-3}$ ± 0.01	0.284 ± 0.203	0.003 ± 0.005	1.6 ± 1.167	9.82 ± 2.67
24	$4.75 \cdot 10^{-5}$ $\pm 5.74 \cdot 10^{-5}$	$9.95 \cdot 10^{-4}$ $\pm 1.45 \cdot 10^{-3}$	0.215 ± 0.385	2.59 ± 1.2	0.003 ± 0.005	7.19 ± 2.6	63.48 ± 21.06

Table SI-V.1. Integrated intensity $I_{QD,lyso}$ of QD fluorescence colocalizing with lysosomes, after HeLa cells had been incubated for different incubation times t with the 3 different QDs (+/-, -, +) at different concentrations $c(QD)$ in serum-free culture. Data are presented as mean value of 3 measurements \pm standard deviation and have been obtained from CLSM data with 63x objective.

t [h]	c(QD) = 0	c(QD) = 10 nM			c(QD) = 80 nM		
	Control	(+/-)	(-)	(+)	(+/-)	(-)	(+)
6	$3.37 \cdot 10^{-3}$ ± 0.005	$2.05 \cdot 10^{-3}$ $\pm 4.4 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$ $\pm 1.35 \cdot 10^{-3}$	1.29 ± 0.46	0.006 ± 0.012	0.005 ± 0.007	15.97 ± 6.72
12	$6.5 \cdot 10^{-5}$ $\pm 1.04 \cdot 10^{-4}$	$2.75 \cdot 10^{-4}$ $\pm 2.98 \cdot 10^{-4}$	$1.44 \cdot 10^{-3}$ $\pm 2.84 \cdot 10^{-3}$	2.1 ± 0.74	0.001 ± 0.001	0.145 ± 0.088	34.1 ± 7.37
24	$5.41 \cdot 10^{-5}$ $\pm 0.0 \cdot 10^{-5}$	$5.88 \cdot 10^{-4}$ $\pm 7.21 \cdot 10^{-4}$	$6.3 \cdot 10^{-5}$ $\pm 6.157 \cdot 10^{-5}$	3.73 ± 1.41	0.001 ± 0.001	0.14 ± 0.098	48.53 ± 8.7

Table SI-V.2. Integrated intensity $I_{QD,lyso}$ of QD fluorescence colocalizing with lysosomes, after HeLa cells had been incubated for different incubation times t with the 3 different QDs (+/-, -, +) with different QD concentration $c(QD)$ in serum-supplemented culture. Data are presented as mean value of 3 measurements \pm standard deviation and have been obtained from CLSM data with 63x objective.

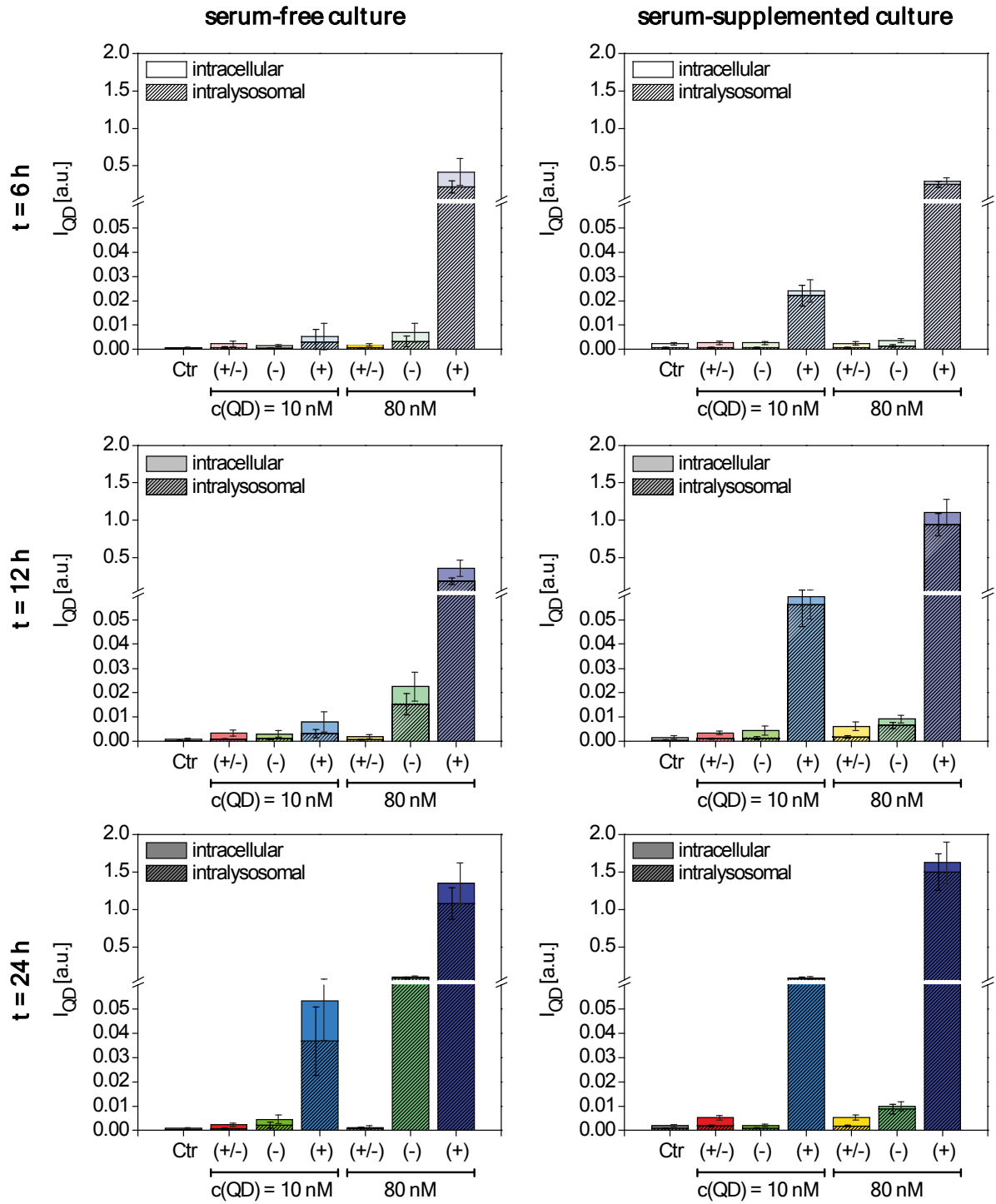


Figure SI-V.8: Total uptake I_{QD} and lysosomal fraction $I_{QD,lyso}$ as quantified by QD fluorescence inside HeLa cells and lysosomes, respectively, at different time points determined by fluorescence microscopy, using lower magnification (20x objective) CLSM micrographs. The same data are displayed in different format in Figure SI-V.9 and are enlisted in Tables SI-V.2 and SI-V.3.

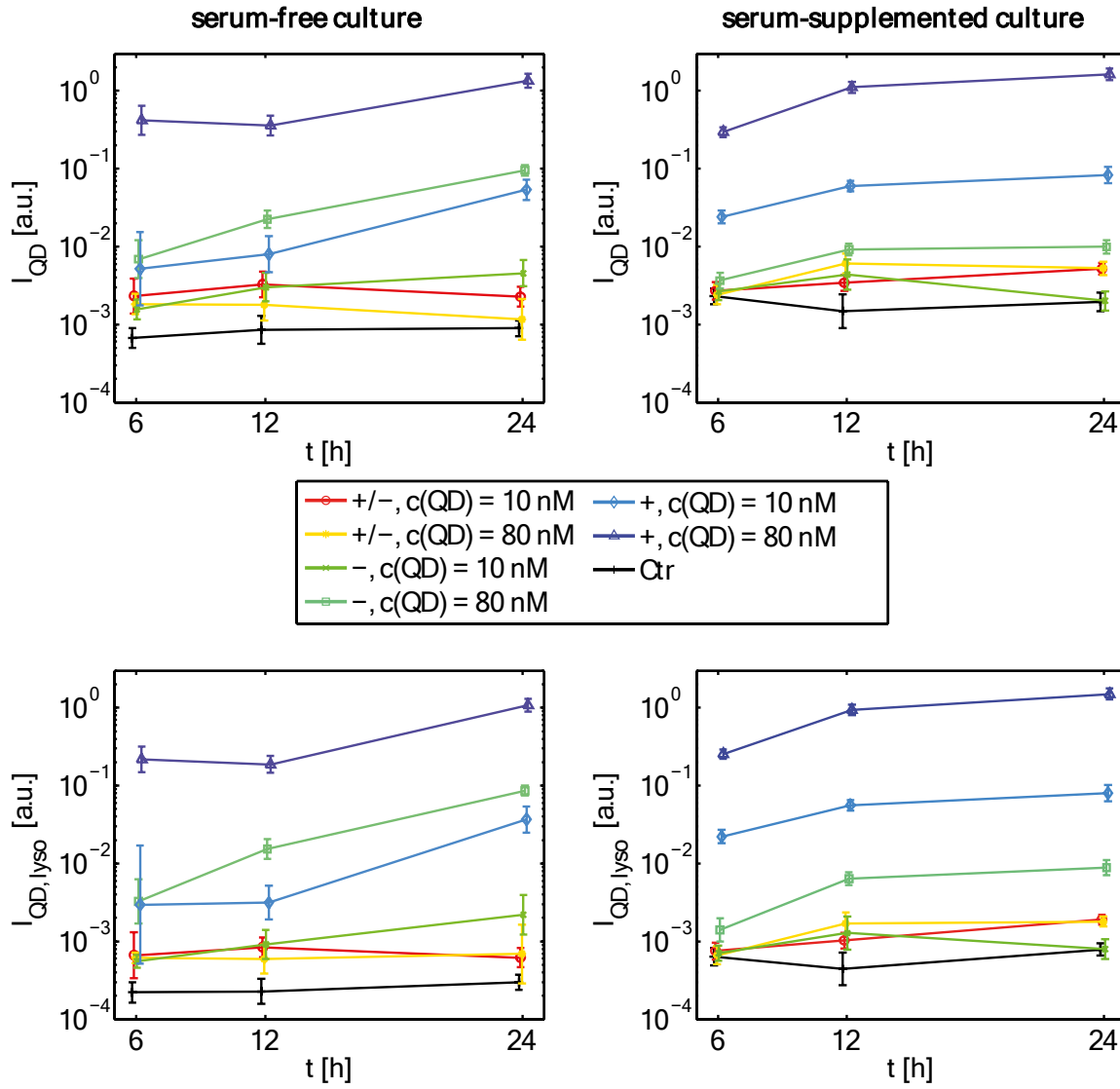


Figure SI-V.9: Total uptake I_{QD} and lysosomal fraction $I_{QD,lyso}$ as quantified by QD fluorescence inside HeLa cells and lysosomes, respectively, at different time points determined by fluorescence microscopy, using lower magnification (20x objective) CLSM micrographs. The same data are displayed in different format in Figure SI-V.8 and are enlisted in Tables SI-V.3 to SI-V.6.

t [h]	c(QD) = 0	c(QD) = 10 nM			c(QD) = 80 nM		
	Control	(+/-)	(-)	(+)	(+/-)	(-)	(+)
6	66.663 ± 19.72	230.935 ± 117.43	156.02 ± 45.381	516.734 ± 561.78	183.1 ± 55.01	689.645 ± 381.18	41808.573 ± 18123
12	85.364 ± 35.028	326.315 ± 123.54	300.88 ± 127.11	796.51 ± 423.56	179.36 ± 85.1	2248.3 ± 595.29	35794.35 ± 10671
24	89.364 ± 20.628	226.51 ± 66.65	454.855 ± 117.61	5341.1 ± 1628.4	116.4 ± 70.8	9585.64 ± 1496.4	134748.66 ± 27434

Table SI-V.3. Intensity I_{QD} of QD fluorescence colocalizing with cells, after HeLa cells had been incubated for different incubation times t with the 3 different QDs (+/-, -, +) with different QD concentration $c(QD)$ in serum-free culture. Data are presented as mean value of 3 measurements \pm standard deviation and have been obtained from CLSM data with a 20x objective.

t [h]	c(QD) = 0	c(QD) = 10 nM			c(QD) = 80 nM		
	Control	(+/-)	(-)	(+)	(+/-)	(-)	(+)
6	230.76 \pm 54.3	269.2 \pm 70.6	263.5 \pm 64.9	2411.8 \pm 456.86	241.6 \pm 70.23	366.55 \pm 86.85	29500.15 \pm 4419
12	148.65 \pm 74.29	342.76 \pm 78.68	436.87 \pm 196.06	5965.6 \pm 929.24	607.71 \pm 178.52	912.34 \pm 158	110462.9 \pm 17207
24	194.6 \pm 54.8	517.3 \pm 89.4	200.57 \pm 56.2	8273.4 \pm 1961.8	524.53 \pm 103.7	989 \pm 195	162653.2 \pm 27475

Table SI-V.4. Intensity I_{QD} of QD fluorescence colocalizing with cells, after HeLa cells had been incubated for different incubation times t with the 3 different QDs (+/-, -, +) with different QD concentration $c(QD)$ in serum-supplemented culture. Data are presented as mean value of 3 measurements \pm standard deviation and have been obtained from CLSM data with a 20x objective.

t [h]	c(QD) = 0	c(QD) = 10 nM			c(QD) = 80 nM		
	Control	(+/-)	(-)	(+)	(+/-)	(-)	(+)
6	22.161 \pm 6.69	66.484 \pm 44.99	55.26 \pm 10.64	295.89 \pm 517.95	61.432 \pm 9.67	327 \pm 214.28	21862.1 \pm 8290.3
12	22.87 \pm 8.54	84.1 \pm 24.97	91.725 \pm 39.72	313.54 \pm 157.19	59.25 \pm 25.8	1528.2 \pm 443.8	18687.1 \pm 4611.6
24	29.76 \pm 6.55	62.11 \pm 18	220.31 \pm 129.1	3681.5 \pm 1415.7	68.74 \pm 59.8	8629.43 \pm 1281.5	107711.8 \pm 20911

Table SI-V.5. Intensity $I_{QD,lyso}$ of QD fluorescence colocalizing with lysosomes, after HeLa cells had been incubated for different incubation times t with the 3 different QDs (+/-, -, +) with different QD concentration $c(QD)$ in serum-free culture. Data are presented as mean value of 3 measurements \pm standard deviation and have been obtained from CLSM data with a 20x objective.

t [h]	c(QD) = 0	c(QD) = 10 nM			c(QD) = 80 nM		
	Control	(+/-)	(-)	(+)	(+/-)	(-)	(+)
6	63.64 ± 16.1	75 ± 19	70.52 ± 15.44	2215.15 ± 430.88	66.53 ± 17.75	141.0 ± 47.86	25366.56 ± 3698
12	44.44 ± 21.35	102.42 ± 23.25	128 ± 62.34	5618 ± 891.7	171.51 ± 53.6	640.3 ± 126.4	94191 ± 14789
24	78.78 ± 14.38	189.9 ± 26.9	80.1 ± 23.3	7975.25 ± 1896.5	180.11 ± 26.0	883.2± 194.4	150107.6 ± 24334

Table SI-V.6. Intensity $I_{QD,lyso}$ of QD fluorescence colocalizing with lysosomes, after HeLa cells had been incubated for different incubation times t with the 3 different QDs (+/-, -, +) with different QD concentration $c(QD)$ in serum-supplemented culture. Data are presented as mean value of 3 measurements \pm standard deviation and have been obtained from CLSM data with a 20x objective.

VI) Fluorescence analysis of quantum dots internalized by cells: flow cytometer evaluation

VI.1) Incubation of cells with quantum dots, flow cytometer evaluation

VI.2) Results

VI.1) Incubation of cells with quantum dots, flow cytometer evaluation

To evaluate the uptake of QDs by HeLa cells, the fluorescence signal of cells exposed to QDs was quantified by a flow cytometer (BD LSR Fortessa Biosciences, Germany). Therefore, HeLa cells were seed on a 24 well plate at the density of 44.000 cells/well in 600 μ L and kept overnight. On the next day, the cells were exposed to QDs at concentrations of 5 nM - 80 nM for 6 h and 24 h in complete growth medium (DMEM supplemented with 10% FBS, 1% glutaMAXTM and 1% P/S) and an exposure volume of 600 μ L. After the desired time, cells were washed 3 times with 600 μ L PBS (Dulbecco's PBS without Ca^{2+} and Mg^{2+}). Then, cells were trypsinated, neutralized with complete cell medium (750 μ L), collected in BD Falcon Round-bottom tubs (5 mL), and centrifuged at 300 g for 5 minutes. After centrifugation, the supernatant was removed and cells were resuspended in 500 μ L PBS. The samples were kept on ice until analysis by flow cytometry. Samples were measured on a BD LSRFortessaTM cell analyzer flow cytometer (Becton, Dickinson and Company, USA). Data was analyzed with FlowJo, Single Cell Analysis Software (Ashland, OR, USA). The fluorescence of the QDs was excited with the Yellow-Green Laser (561 nm) and emission was captured using a PE(586/15 nm) filter.

VI.2) Results

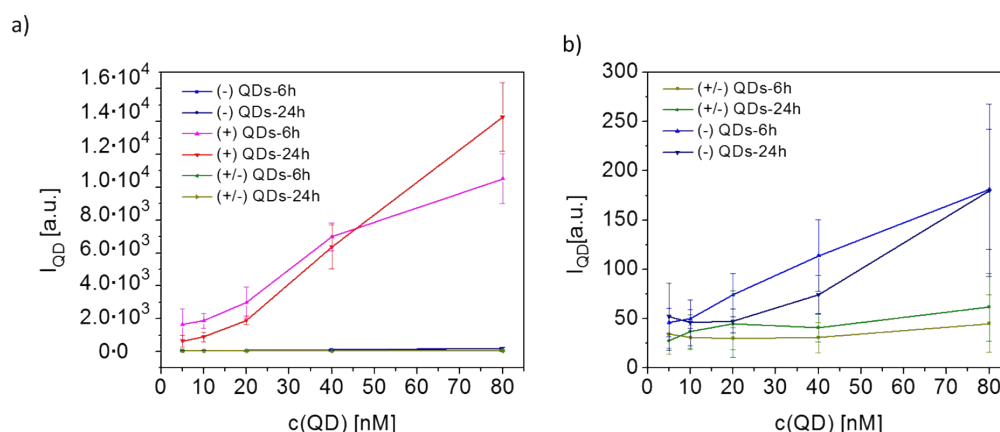


Figure SI-VI.1: QD uptake I_{QD} in HeLa cells cultured in serum supplemented medium, quantified by QD fluorescence at different time points, determined by Flow Cytometry analysis. a) Uptake I_{QD} of QDs (+/-, -, +) by HeLa cells. b) Close-up of panel a) representing the uptake I_{QD} of QD (+/-) and (-) in HeLa cells. The same data are displayed in different format in Table SI-IV.1 for

$c(QD)$ exposed to HeLa at doses of 10 nM and 80 nM for 6 h and 24 h, and the gating strategy is shown in Figure SI-VI.2.

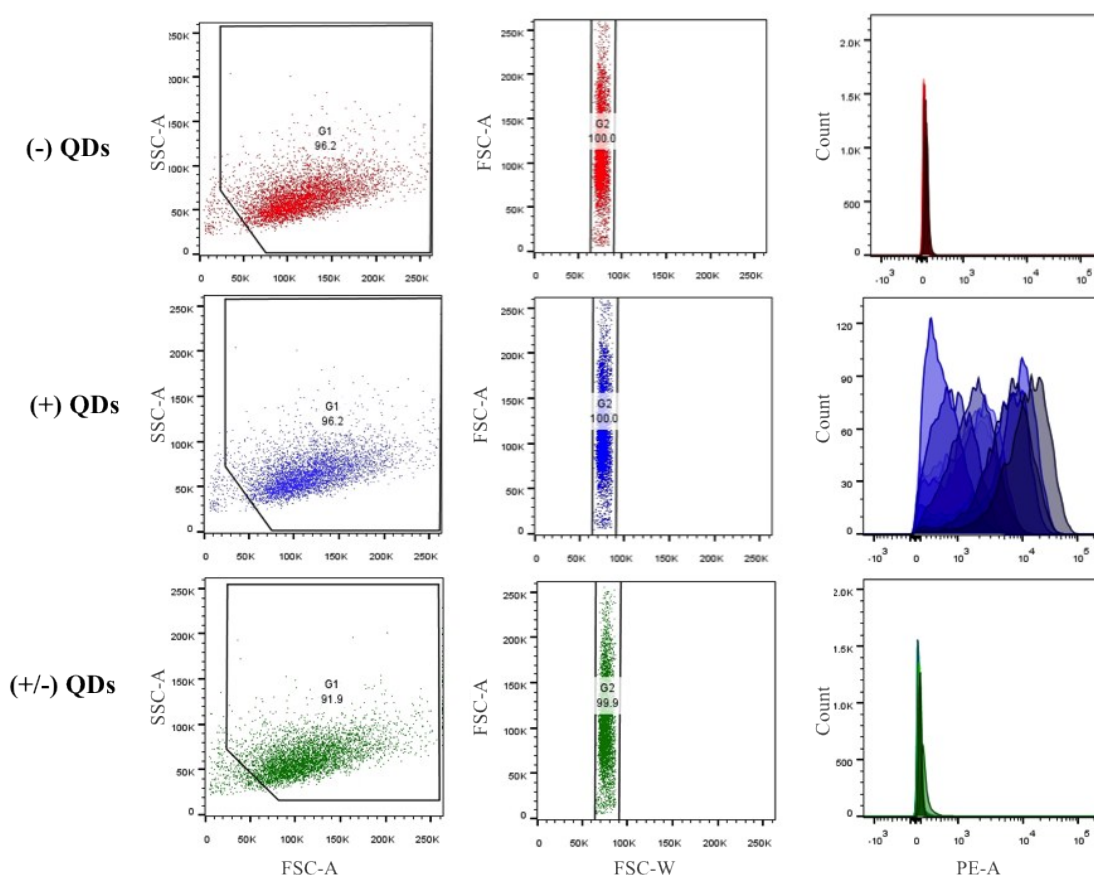


Figure SI-VI.2: Gating strategy of QD fluorescence measurements I_{QD} of HeLa cells exposed to the three types of QDs (+/-, -, +), at different times as determined by flow cytometry. One representative experiment is shown to illustrate the gating strategy used for the evaluation.

t [h]	$c(QD) = 0$			$c(QD) = 10 \text{ nM}$			$c(QD) = 80 \text{ nM}$		
	control			(-)	(+)	(+/-)	(-)	(+)	(+/-)
6	29.3 ± 10	31.8 ± 17	29.5 ± 12.4	49.8 ± 9.7	1870.0 ± 466.0	30.9 ± 12.3	181.3 ± 60.7	10526.0 ± 1516.4	45.0 ± 29.2
24	48.3 ± 38.4	44.3 ± 33.3	35.1 ± 18.6	46.0 ± 23.2	893.0 ± 289.5	36.9 ± 17.2	180.0 ± 87.5	14289.3 ± 2091.0	61.7 ± 34.5

Table SI-VI.1. Intensity I_{QD} of QD fluorescence following exposure to HeLa cells. For that, HeLa cells, were incubated with the three QDs (+/-, -, +) at different QD concentration $c(QD)$ in serum-supplemented culture medium for 6 h and 24 h. Data are presented as mean value of 3 independent measurements \pm standard deviation. Data have been obtained from the flow cytometry experiments.

VII) Viability measurements of cells exposed to quantum dots

VII.1) Incubation of cells with quantum dots, cell viability measurements

VI.2) Results

VII.1) Incubation of cells with quantum dots, cell viability measurements

Cell viability of HeLa cells exposed to the three types of QDs (+/-, -, +) and CdCl_2 was evaluated by the resazurin assay as previously reported (AlamarBlue® Cell Viability Reagent, ThermoFisher) ¹²⁻¹⁴. Briefly, 7500 HeLa cells were seeded in black 96-well transparent bottom plates (Corning # 3603, 7500 cells/well, area per well $\approx 0.32 \text{ cm}^2$) in 100 μL of complete growth medium (DMEM supplemented with 10% FBS, 1% glutaMAX and 1% P/S) and incubated overnight at 37 °C in 5% CO_2 . After the desired time, HeLa cells were exposed to QDs and CdCl_2 at the different doses in complete (*i.e.* serum supplemented) cell medium for 24 h. Then, the supernatant was removed and cells were washed with PBS (100 μL). 100 μL of 10% resazurin solution in complete cell medium was added into each well and incubated for 3 h at 37 °C. Note that HeLa cells exposed to complete medium were used as negative control. After incubation time, the fluorescence emission spectra of the solution were recorded with a fluorometer (Fluorolog-3, Horiba Jobin Yvon). Wells were excited at 560 nm, and fluorescence emission was recorded from 572 nm to 650 nm. The mean of the maximum fluorescence values for each well was determined, background correction was performed and values were normalized with respect to their maximum (negative control). Finally, results are expressed as % of cell viability, analyzed in triplicates from three or four independent experiments. An interference assay of QDs with the resazurin solution was performed as a control, no interference was found.

VII.2) Results

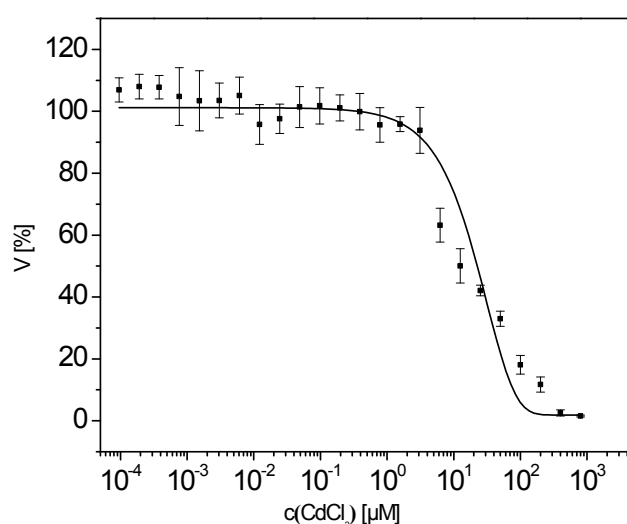


Figure SI-VII.1. Viability of HeLa cells exposed to CdCl₂. Cell viability V was assessed by the resazurin assay upon exposure CdCl₂ at different concentration $c(\text{CdCl}_2)$ in serum-supplemented medium for 24 h. Results are presented as mean value of cell viability (%) from three independent experiments \pm standard deviation.

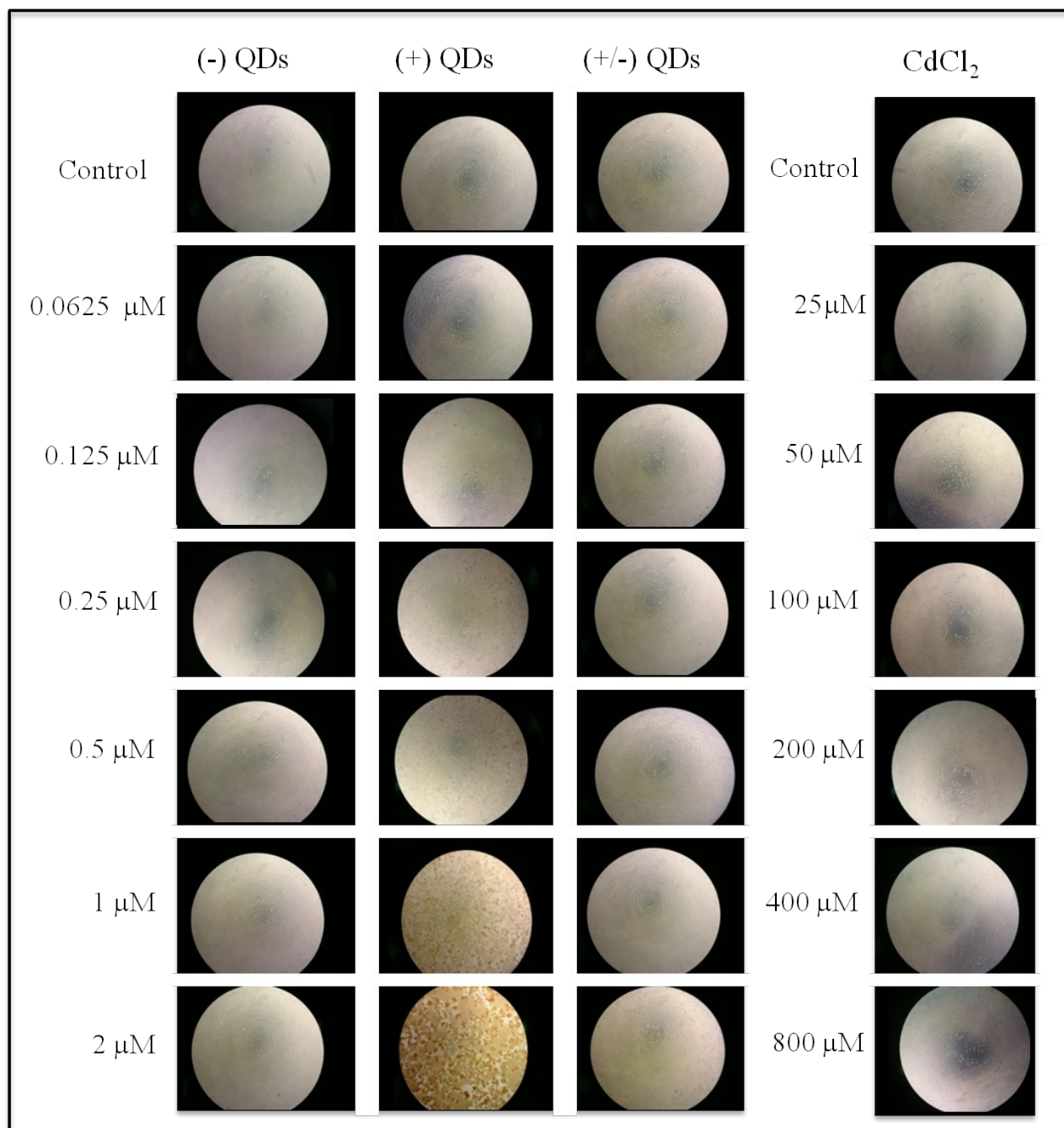


Figure SI-VII.2. Images of HeLa cells exposed to QDs (+/-, -, +) and CdCl₂ in serum-supplemented medium for 24 h, as recorded with the digital camera of a mobile phone through the ocular of a bright field microscope. Agglomeration of the positively charged QDs can be seen at high QD concentration.

VII) References

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