

Supplementary Information for:

Non-endocytic delivery of functional engineered nanoparticles into the cytoplasm of live cells using a novel, high through-put microfluidic device.

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1. Materials and Methods

Materials

All chemicals were obtained from Sigma Aldrich and used as received unless indicated otherwise. Air sensitive materials were handled in an Omni-Lab VAC glovebox under dry nitrogen atmosphere with oxygen levels <0.2ppm. All solvents were Spectroscopic or reagent. Aromatic ring-bearing compounds were visualized on TLC using a hand-held UV lamp and KMnO₄. Amine-bearing compounds were visualized on TLC using a Ninhydrin stain. Flash column chromatography was performed on a Teledyne Isco Combi Flash Companion. HeLa cells were purchased from ATCC and all cell medium materials were purchased from Mediatech unless indicated otherwise.

Instrumentation

¹H NMR spectra were recorded on a Bruker DRX 401 NMR Spectrometer. MS-ESI was performed on a Bruker Daltonics APEXIV 4.7 FT-ICR-MS machine. UV-Vis absorbance spectra were taken using an HP 8453 diode array spectrophotometer. Photoluminescence and absorbance spectra were recorded with a BioTek Synergy 4 Microplate Reader. Polymer molecular weights were determined in DMF solution on an Agilent 1100 series HPLC/GPC system with three PLgel columns (103, 104, 105 Å) in series against narrow polystyrene standards. Dye derivatives were purified using Varian ProStar Prep HPLC system. Modified polymer was purified using GE Healthcare's PD-10 columns packed with Sephadex™ G-25M. Ligand exchanged QDs were purified by centrifugation dialysis with Millipore Amicon Ultra 30K cut-off centrifugal filters and by GFC on AKTAprime Plus chromatography system (Amersham Biosciences) equipped with a self-packed Superdex 200 10/100 glass column. Flow cytometry measurements were made on LSR Fortessa (BD Biosciences).

QD synthesis

CdSe cores with 478nm first absorption peak were synthesized using a previously reported method (1). To summarize, 0.4 mmol (54.1mg) of CdO, 0.8 mmol (0.2232g) of TDPA, 9.6mmol (3.72g) of TOPO were placed in 25mL round bottom flask. The solution was degassed for 1 hr at 160 °C and heated to 300 °C under argon until the CdO dissolved and formed a clear homogenous solution. This was followed by putting the solution under vacuum at 160 °C to remove evolved water. The solution was reheated to 360 °C under argon and a TOP-Se solution (1.5mL of 1.5M TOP-Se in 1.5mL of TOP) was rapidly added to give CdSe cores with the first absorption feature at 478nm.

CdS shells were deposited on CdSe cores via modification of previously reported procedures (2). Cores isolated by repeated precipitations from hexane with acetone were brought to 180 °C in a solvent mixture of oleylamine (3 mL) and octadecene (6 mL). Cd and S precursor solutions were then introduced continuously at a rate of 4 mL/hr. The Cd precursor consisted of 0.33 mmol Cd-oleate and 0.66 mmol oleylamine in a

solvent mixture of octadecene (1.5 mL) and TOP (3 mL). The S precursor consisted of 0.3 mmol hexamethyldisilathiane [(TMS)₂S] in 6 mL TOP. Addition of a total of 3 monolayers each of Cd and S yielded QDs with emission at 541 nm and a quantum yield of 60% when diluted in octane. The extinction coefficient of CdSe(CdS) was calculated using the extinction coefficient of CdSe cores from the literature (3) and assuming that 95% of the CdSe cores were retained during the overcoating step.

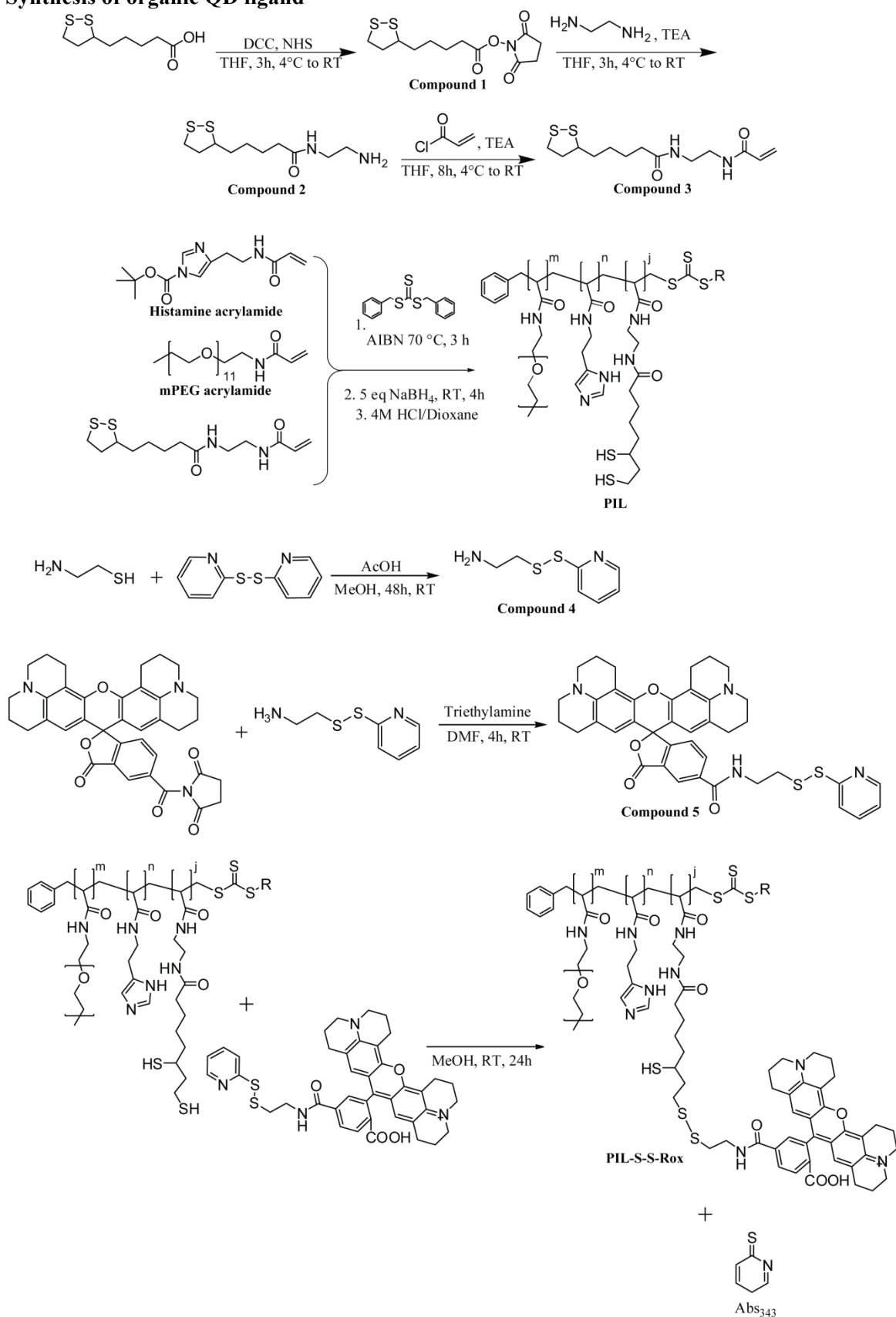
Device fabrication and mounting system

The silicon-based devices are fabricated at the MIT microfabrication facility using photolithography and deep reactive ion etching techniques. In this process, 6" silicon wafers with a 450um thickness are treated with Hexamethyldisilazane (HMDS), spin coated with photoresist (OCG934, FujiFilm) for 60s at 3000rpm, exposed to UV light (EV1- EVG) through a chrome mask with the constriction channel design, and developed in AZ405 (AZ Electronic Materials) solution for 100s. After 20min of baking at 90°C, the wafer is etched by deep reactive ion etching (SPTS Technologies) to the desired depth (typically 15um). Piranha treatment (H₂O₂ and H₂SO₄) is used to remove any remaining photoresist after the etching process is complete. To etch the access holes (i.e. inlet and outlet) the process is repeated on the opposite side of the wafer (i.e. the one not containing the etched channels) using a different mask, which contains the access hole patterns, and a thicker photoresist AZ9260 (AZ Electronic Materials).

Oxygen plasma and RCA cleaning are used to remove any remaining impurities. Wet oxidation is then used to grow 100-200nm of silicon oxide before the wafer is anodically bonded to a Pyrex wafer and diced into individual devices. Each device is individually inspected for defects prior to use.

Before each experiment, devices are mounted onto a holder with inlet and outlet reservoirs (all designed in-house and produced by Firstcut). These reservoirs interface with the device using Buna-N O-rings (McMaster-Carr) to provide proper sealing. The inlet reservoir is connected to a home-made pressure regulator system using Teflon tubing to provide the necessary driving force to push material through the device. The current system can only accommodate pressures up to 70psi.

Synthesis of organic QD ligand



Compound 1 To a solution of lipoic acid (5 g, 20 mmol) and dicyclohexylcarbodiimide (6 g, 30 mmol) in

THF at 4°C, a solution of N-hydroxysuccinimide (3.34 g, 30 mmol) in THF was added drop by drop. The solution was brought to room temperature and stirred for 3 h. The precipitate was removed by vacuum filtration and the solvent evaporated in vacuo, followed by a second precipitation in ethyl acetate and vacuum filtration. The crude product was recrystallized from a solution of hot ethyl acetate/hexane (1:1 v/v) as a pale-yellow solid. ¹H NMR (400MHz, CDCl₃): δ (ppm) 3.59 (1H, quin), 3.03-3.13 (2H, m), 2.85 (4H, t), 2.6 (2H, t), 2.45 (1H, quin), 1.91 (1H, quin), 1.63-1.8 (4H, m), 1.5-1.6 (3H, m)

Compound 2 A solution of lipoic acid NHS-ester (**1**, 2g, 6.6 mmol) in THF was added drop by drop to a solution of ethylene diamine (3.96 g, 70 mmol) and triethylamine (1.2 eq) in THF at 4°C. The reaction brought to room temperature and stirred until NMR of crude product in solution phase showed ethylene diamine peaks but no NHS peaks. The reaction was filtered and the solvent and excess ethylene diamine were removed in vacuo. Entire product was transferred to the next reaction to form compound **3**.

Compound 3 To a solution of lipoic ethylene amine (**2**) and triethylamine (0.8 g, 10 mmol) stirring in dry THF at 4°C, a solution of acryloyl chloride (0.72 g, 10 mmol) in THF was added drop by drop. The reaction was brought back to room temperature and stirred for 6 h. Disappearance of amine was monitored with TLC. The solution was filtered and the solvent evaporated in vacuo. The crude product was purified by silica column (4% to 10% methanol/dichloromethane gradient) to give the pure product. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 6.3 (1H, d), 6.19 (1H, q), 5.62 (1H, d), 3.5 (2H, sex), 3.43 (3H, m), 3.1 (2H, sex), 2.21 (1H, sex), 2.18 (2H, t), 1.83 (1H, sex), 1.61 (4H, m), 1.4 (1H, q)

Typical PIL synthesis

Methoxy terminated poly(ethylene glycol)₁₁ acrylamide and Histamine acrylamide were synthesized with slight modifications on established procedure (2).

Methoxy poly(ethylene glycol)₁₁ amine Neat methoxy poly(ethylene glycol) (mean MW 550, 8 g, 14.5 mmol), Ag₂O (5.06 g, 21.8 mmol), Tosyl chloride (3.05 g, 16 mmol), and KI (0.6 g, 3.6 mmol) were stirred in dichloromethane at RT overnight. Salts were filtered through celite followed by ethyl acetate wash of the crude solution. The solvent was evaporated in vacuo and the crude product purified by silica column (2% methanol/dichloromethane). The entire crude was dissolved in DMF and sodium azide (1.42 g, 21.8 mmol) was added. The reaction was stirred at 110°C for 3 h, then cooled to RT and the solvent evaporated in vacuo. Tosylate salts were crashed out in dichloromethane until NMR showed tosylate peaks had disappeared. To the entire product dissolved in dry THF at 4°C was added triphenylphosphine (4.2 g, 16 mmol). This reaction was brought to RT and stirred for 10 h, at which point 1 mL of water was added. After 3 h further stirring, the solvent was removed in vacuo and the crude product was purified with silica column (10% hydroxylamine_{0.1}-methanol_{0.9}/ethyl acetate).

Methoxy poly(ethylene glycol)₁₁ acrylamide The purified methoxy poly(ethylene glycol)₁₁ amine (1.3 g, 2.36 mmol) and triethylamine (0.36 g, 3.55 mmol) were dissolved in dry THF at 4°C. A solution of acryloyl chloride (0.32 g, 3.55 mmol) in THF was added drop by drop and the reaction brought back to room temperature and stirred for 6 h. Disappearance of amine was monitored with TLC. The solution was filtered and the solvent evaporated in vacuo. The crude product was purified by silica column (4% to 10% methanol/dichloromethane gradient) to give the pure product.

Histamine acrylamide To a solution of histamine free base (2.9 g, 26 mmol) and triethylamine (3.17 g, 31.3 mmol) in 2.2:1 DMF/THF mixture at 4°C was added a solution of acryloyl chloride (2.83 g, 31.3 mmol) in DMF/THF drop by drop. The reaction was raised to RT and stirred for 6 h. Precipitates were filtered and the solvent was evaporated in vacuo. The crude product was purified on silica column (10% hydroxylamine_{0.1}-methanol_{0.9}/dichloromethane).

Boc-protected histamine acrylamide To a solution of purified histamine acrylamide and triethylamine (2.90 g, 28.7 mmol) in DMF was added a solution of Boc-anhydride (6.26 g, 28.7 mmol) in DMF drop by drop. The solution was stirred at RT for 6 h and purified by silica column (50% to 80% ethyl acetate/hexane gradient).

Polymer synthesis All monomers were kept as a dilute stock solution in ethylacetate. RAFT-mediated polymerization has been reported elsewhere (2). To summarize, all monomers were weighed out volumetrically into an 8 mL vial. The solvent was removed in vacuo and RAFT reagent and AIBN were added along with dry DMF. The contents of the vial were mixed, centrifuged, and transferred to a 1 mL ampule. The ampule was freeze-pump-thawed 4x and sealed under vacuum. Polymerization proceeded in a 70°C oil bath for 6 h.

PIL deprotection and reduction Disulfide on PIL was deprotected with 3-4 eq NaBH₄ in 1:4 MeOH/water mixture for 4 hours followed by quenching of NaBH₄ and deprotection of Boc-protecting groups with 0.5 mL of 4 M HCl in dioxane. After 1h at RT, the HCl was removed in vacuo. The deprotected polymer was dissolved in MeOH, to which a solution of NaOH in water (4M) was added dropwise to adjust the pH to be between 8-9. The solvent was removed in vacuo, and then CHCl₃ was added to precipitate the salts. The solution was filtered through a 0.45 um PTFE filter and solvent removed in vacuo to yield the final polymer for Rox-S-S-pyr conjugation.

Compound 4 Aldrithiol-2 (4.98 g, 22.6 mmol) was dissolved in 40 ml of methanol and 1.8 ml of AcOH under N₂. A solution of 2-aminoethanethiol HCl (1.28 g, 11.30 mmol) in methanol was added drop by drop and the reaction was stirred at RT for 48 h. The solvent was evaporated in vacuo and the residual oil was washed 2x with 40 ml of diethyl ether. The crude compound was dissolved in 10 ml of methanol and the product was precipitated twice with cold diethyl ether to get the pure product as a white solid.

Compound 5 5(6)-Rox NHS ester from Invitrogen (100 mg, 0.16 mmol), triethylamine (22.4 mg, 0.22 mmol), and cysteamine thiopyridine (**4**, 41 mg, 0.22 mmol) were stirred in methanol for 4 hours. The product was extracted 2x in water/CHCl₃ and 4x in brine/CHCl₃. The crude product was purified with HPLC Varian column (45% to 100% ACN_{0.8}/water/TFA_{0.005} gradient). M/Z 703.2439 (100), 704.2483 (44.2), 705.2482 (18.4), 706.2492 (5.4).

PIL-S-S-Rox synthesis Varying equivalents of Rox-S-S-pyr was dissolved in methanol and added to a solution of PIL in methanol. The solution was stirred for 24 hours at room temperature, devoid of light. The mixture was precipitated with CHCl₃ and filtered with a 0.45 um PTFE filter, and solvent removed in vacuo to be dissolved in water. The aqueous solution of Rox-S-S-polymer was purified from excess Rox-S-S-pyr by PD-10 size exclusion column (GE-Healthcare). The solvent of the eluted product was removed in vacuo to yield the final PIL for QD ligand exchange.

Ligand exchange QDs (2 nmol) were precipitated using acetone and ethanol, and brought into 50 μL of CHCl₃. The QD stock solution was mixed with a solution of PIL-S-S-Rox (5 mg) in 30 μL, stirred for 15 min at RT, after which 30 μL of methanol was added. The solution was stirred for another 30 min, then precipitated by the addition of ethanol (30 μL) and CHCl₃ (30 μL) and excess hexanes. The sample was centrifuged at 4000xg for 2.5 min, clear supernatant discarded. The pellet redissolved in ethanol (50 μL) and CHCl₃ (50 μL) and precipitated by the addition of excess hexanes, centrifuged, and the supernatant discarded. The pellet was dried in vacuo and dissolved in PBS (500 μL, pH 7.4). The sample was purified by GFC with PBS (pH 7.4) as the mobile phase with a flow rate of 1.0 mL/min. Detection was achieved by measuring the absorption at 280 nm.

Characterization of glutathione and TCEP reduction QD-PIL-S-S-Rox was incubated at 37°C on a stirplate in a solution of varying concentrations of glutathione in PBS and varying concentrations of TCEP in PBS (pH adjusted to pH 7.4) for 30 minutes. Absorbance and emission were measured and recorded simultaneously on BioTek Synergy 4 Microplate Reader at 488 nm excitation.

Disulfide bond stability in full medium The construct was incubated on a 37°C stirplate in air and its emission spectrum measured at various hours. To measure the stability of disulfide bond in full medium at 37°C at 5% CO₂, the construct was incubated in a cell incubator and the emission intensity in QD541 and Rox channel was measured with a confocal microscope. The constructs were run on AKTA GFC column to measure any aggregation.

Cell culture HeLa cells were cultured in high glucose Dulbecco's modified essential medium supplemented with 10% FBS and 1% penicillin streptomycin, at 37 °C and 5% CO₂. When applicable, adherent cells were suspended in solution by treatment with 0.05% trypsin/EDTA for 5 minutes.

Confocal microscopy Confocal images were taken on live cells using the C1 confocal add-on unit on a Nikon TE2000-U inverted microscope with a 60x water-immersion lens. Fluorescence samples were excited at 488 nm from an argon-Ion laser. Emission was collected with a 585 Long-Pass dichroic mirror for QD541 fluorescence collection through 535/30 filter and Rox fluorescence collection through a 605/30 filter. A single image is an average from n=8 scans.

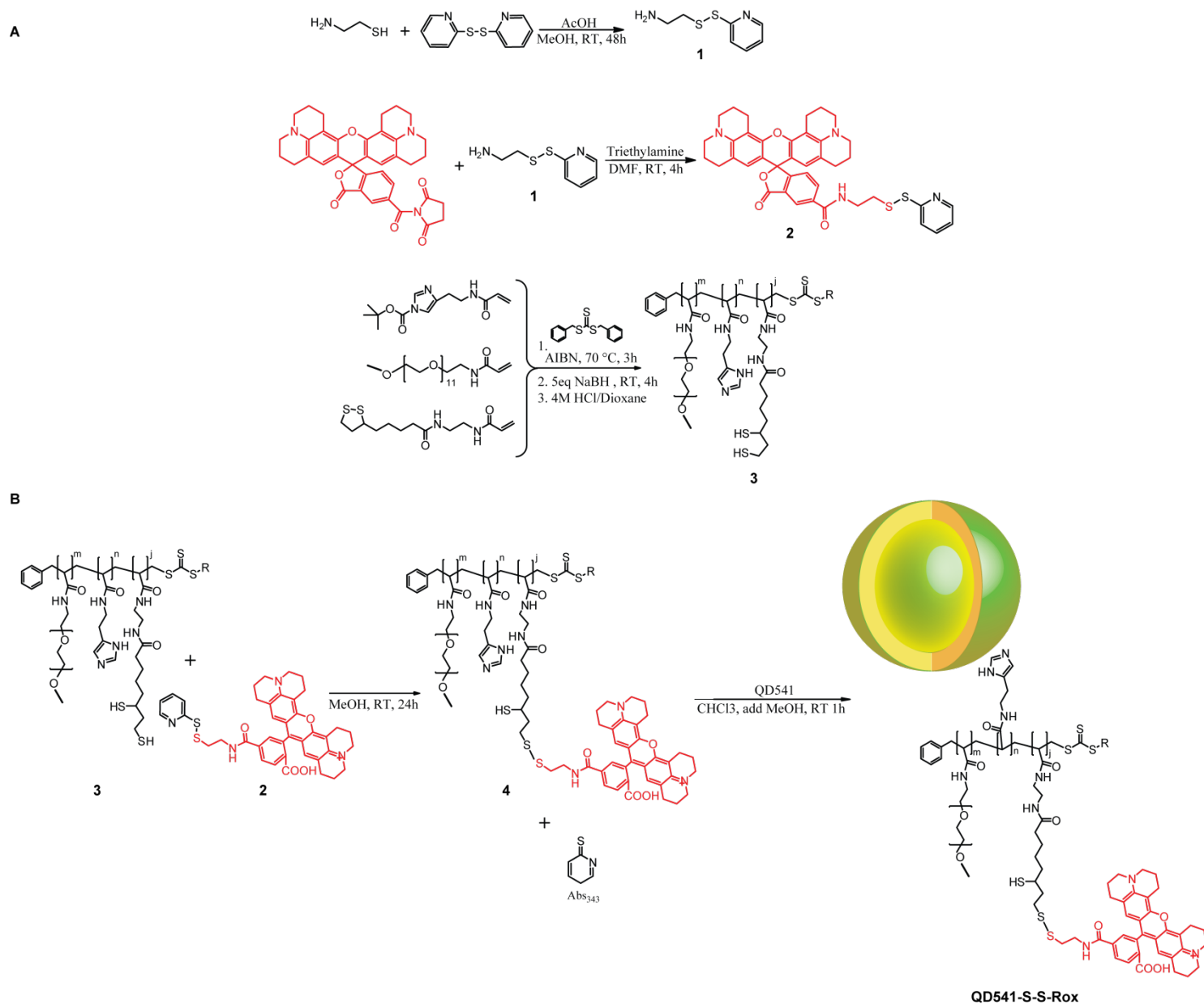
Epifluorescence microscopy Epifluorescence images were taken on live cells using a Nikon TE2000-U inverted microscope with a 60x water-immersion lens and a Princeton Instruments MicroMAX camera with an additional 1.5x magnification tube lens. Bright field images were collected using differential interference contrast and 10 ms exposure. Fluorescence samples were excited from an LED lamp source centered on 480 nm excitation and collected with 50-500 ms exposure. Collection of QD541 fluorescence was through a 460/30 nm excitation filter, 488 dichroic mirror, and 525/30 emission filter. Collection of Rox fluorescence was through a 488 dichroic mirror and 595/50 emission filter.

Flow cytometry Cells were centrifuged at 400 rcf and washed 2-3 times with PBS prior to being suspended in FACS buffer comprised of PBS supplemented with 3% FBS, 1% F-68 Pluronics (Sigma), and 10 µg/ml propidium iodide (Sigma). QD and QD-Rox fluorescence upon excitation at 405 nm excitation wavelength was analyzed. Dead cells were sorted out from the sample by discarding cells showing propidium iodide fluorescence excited at 488 nm and measured through a 695 Long-Pass filter. Emission for QD541 was collected using a 525/50 filter and emission for Rox fluorescence was collected using a 595 Long-Pass filter.

Delivery procedure To perform an experiment, cells are first suspended in PBS (although growth medium or other buffers have been found to work equally well) mixed with the desired delivery material and placed in the device's inlet reservoir. This reservoir is connected to a compressed air line controlled by a regulator and the selected pressure (0-70psi) is used to drive the fluid through the device. Treated cells are then collected from the outlet reservoir. Cells are incubated at room temperature in the delivery solution for 5min post-treatment to ensure pore closure before being subject to any further treatment. All experimental conditions were carried out in triplicate and the error bars represent two standard deviations.

- (1) Peng, Z. A.; Peng, X. J. *Am. Chem. Soc.* **2001**, *123*, 183-184.
- (2) Liu, W.; Greytak, A. B.; Lee, J.; Wong, C. R.; Park, J.; Marshall, L. F.; Jiang, W.; Curtin, P. N.; Ting, A. Y.; Nocera, D. G.; Fukumura, D.; Jain, R. K.; Bawendi, M. G. *J. Am. Chem. Soc.* **2010**, *132*, 472-483.
- (3) Leatherdale, C. A.; Woo, W. K.; Mikulec, F. V.; Bawendi, M. G. *J. Phys. Chem. B.* **2002**, *106*, 7619-7622.
- (4) Sharei, A., et al. A vector-free microfluidic platform for intracellular delivery. *Submitted*.

2. Ligand and QD-Rox construct synthesis scheme



Scheme S1. Synthesis of PIL ligand conjugated to Rox through a disulfide bond and attachment to QD.

3. Compound characterizations

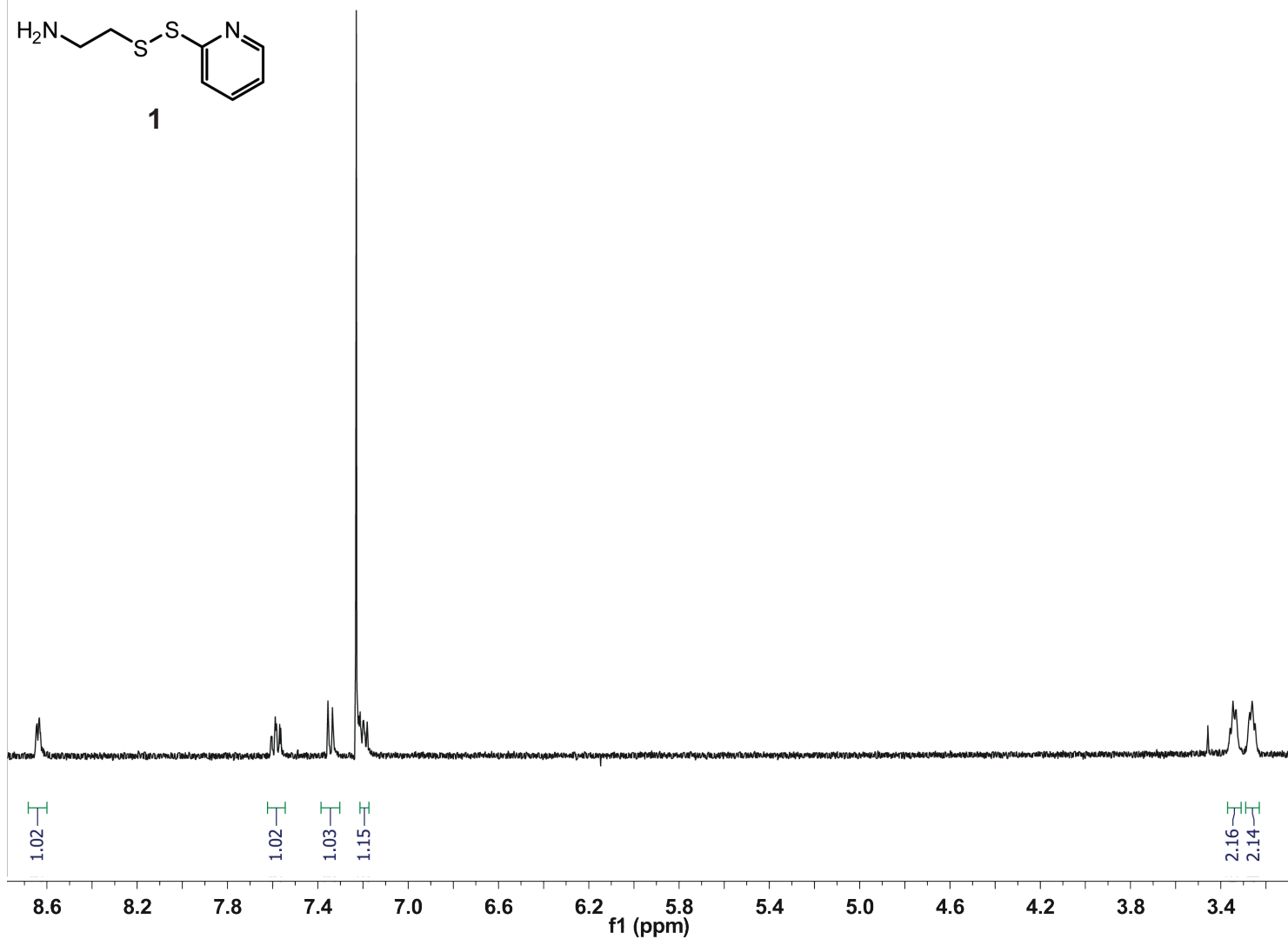


Figure S1. NMR characterization of compound 1.

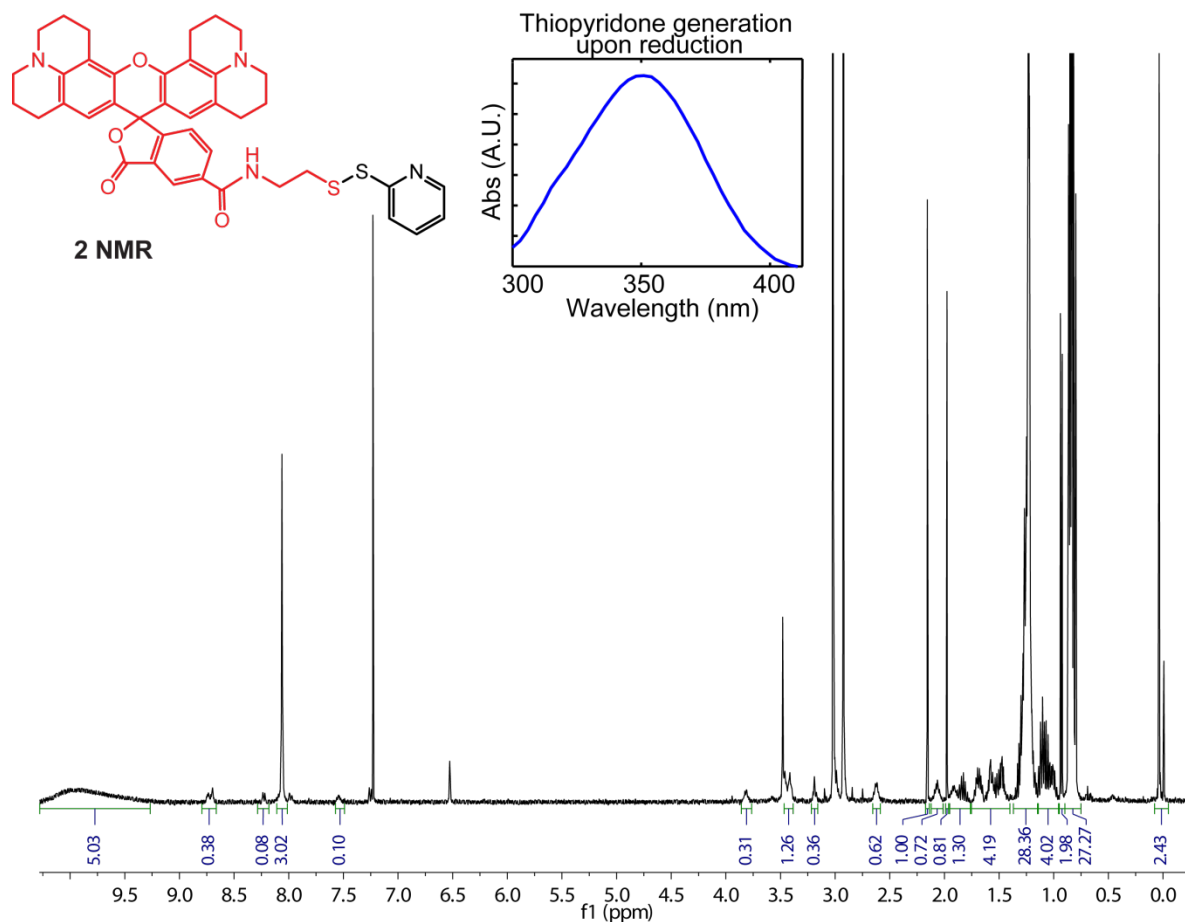


Figure S2A. NMR characterization of compound **2** and confirmation of thiopyridone formation when the compound is put in contact with a reductant, as measured by absorption at 350 nm.

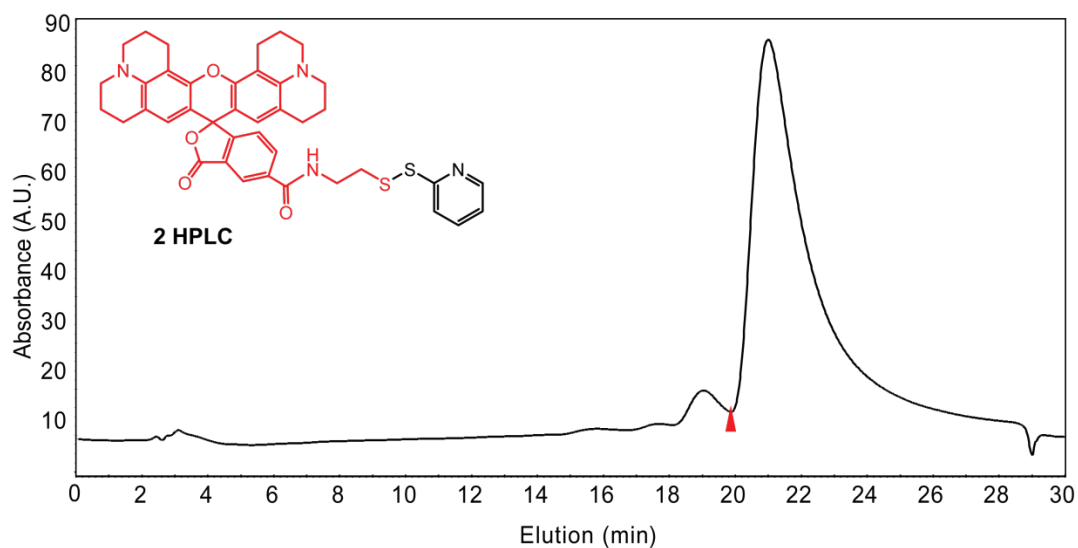


Figure S2B. Purity as measured by HPLC.

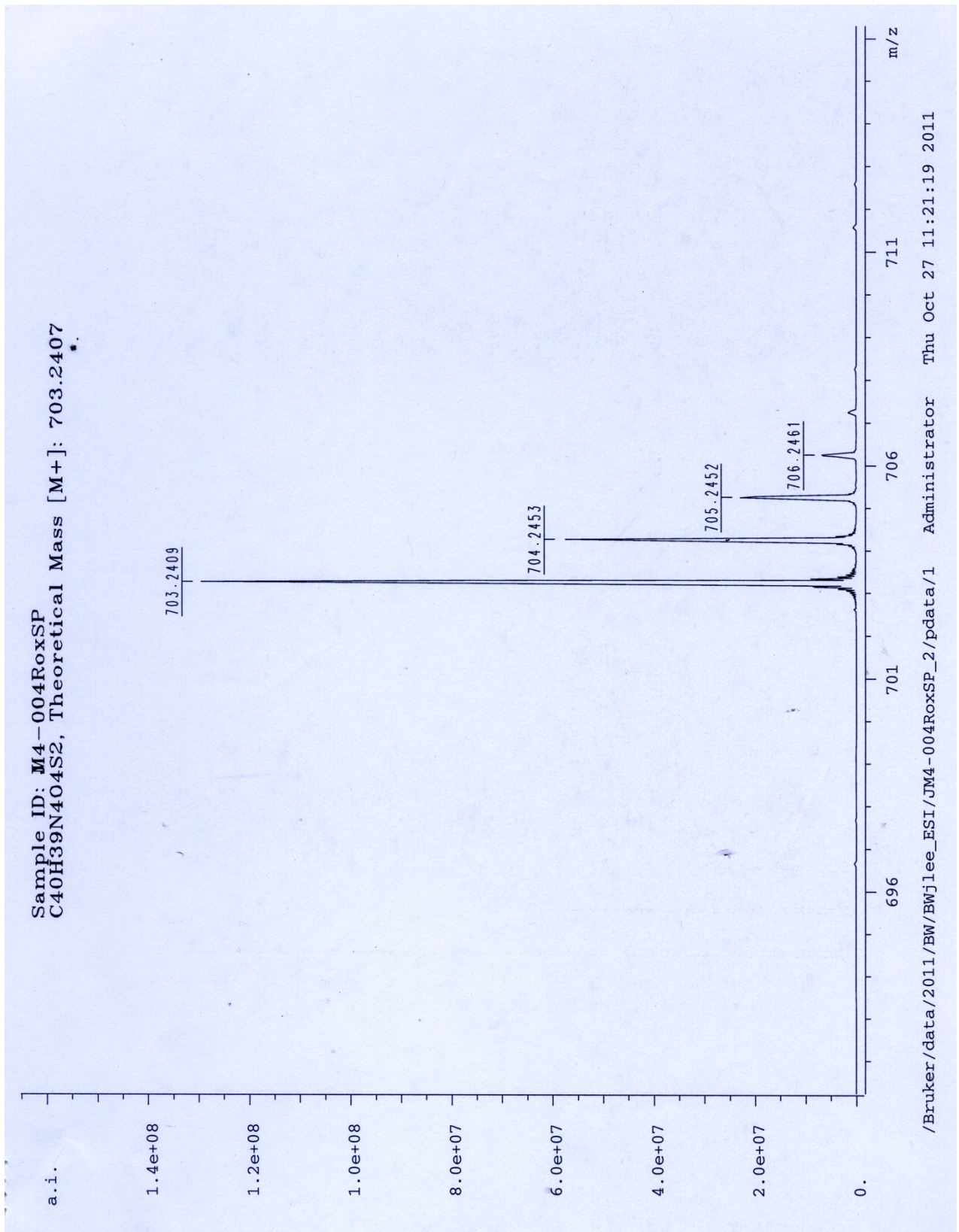


Figure S2C. M/Z spectrum of compound 2.

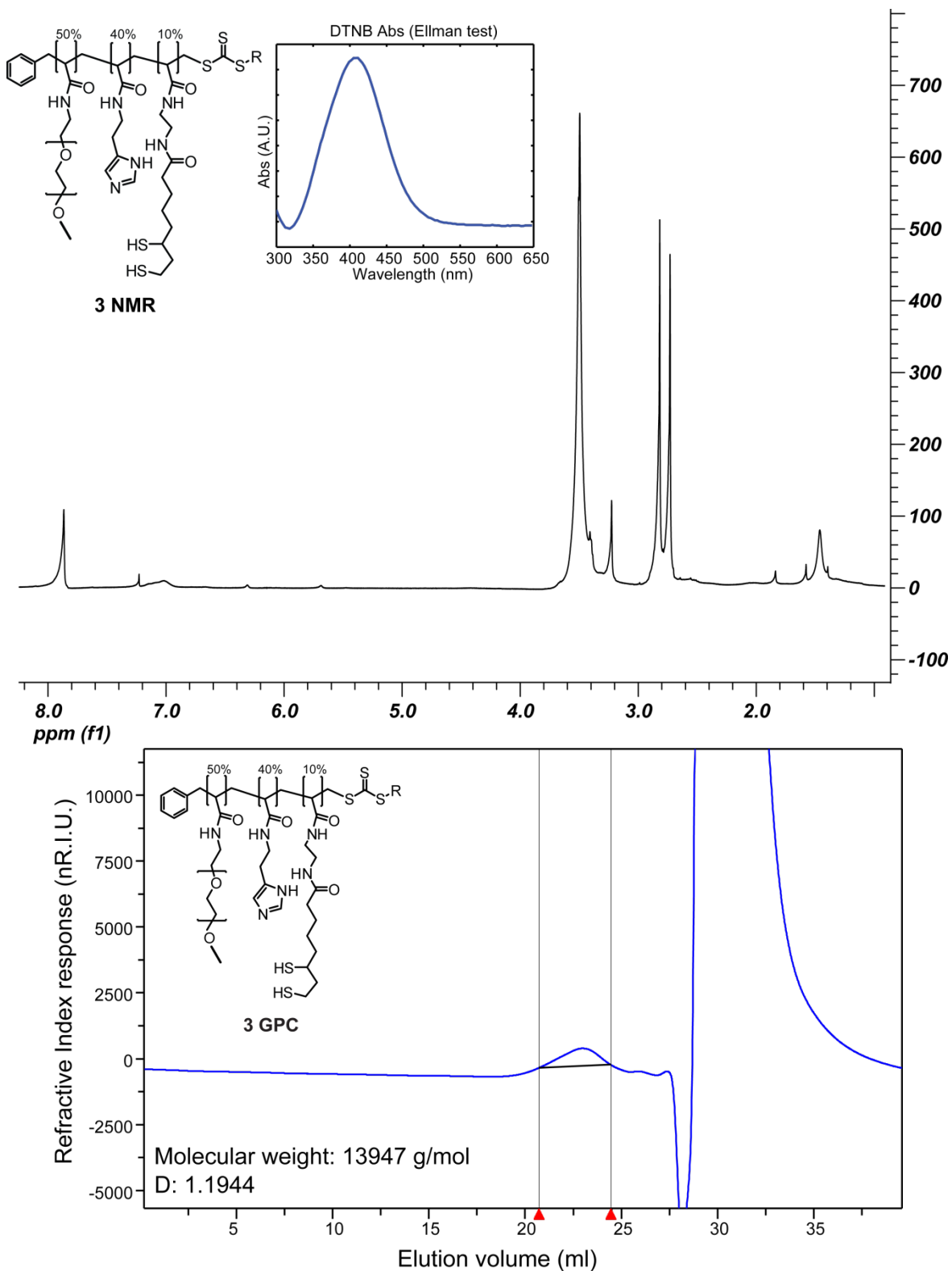


Figure S3. NMR (top) and GPC (bottom) identification and molecular weight measurement, respectively, of PIL 3.

Figure S4. Reproducibility and stoichiometric control of Rox per QD ratio and disulfide cleavage. **a**, Number of Rox per QD is consistent with reaction stoichiometry. **b** and **c**, absorbance spectrum showing both QD and Rox features, followed by *in vitro* reduction of the disulfide bond conjugating Rox to QD by glutathione (GSH) and non-thiol based reductant tris(2-carboxyethyl)phosphine (TCEP).

a

Sample	Equivalents Rox added per thiol on PIL	Number of Rox per QD
4-008	1.5x	8
4-014	3.5x	18
4-016	2x	13

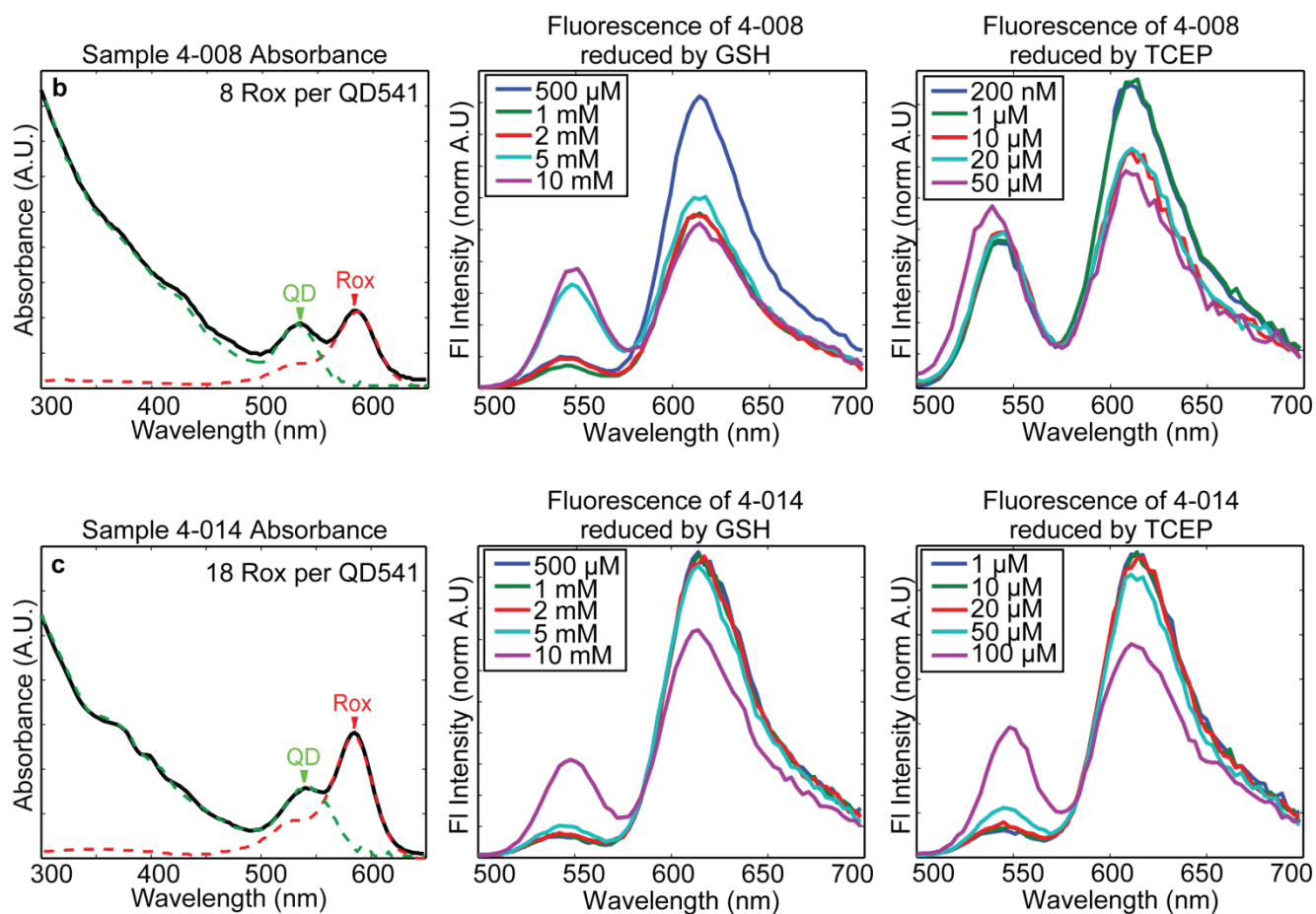


Figure S5. Stability of QD-S-S-Rox construct against **b**, aggregation in full medium at 37 °C and **c**, **d**, cleavage in both PBS and full medium at 37 °C.

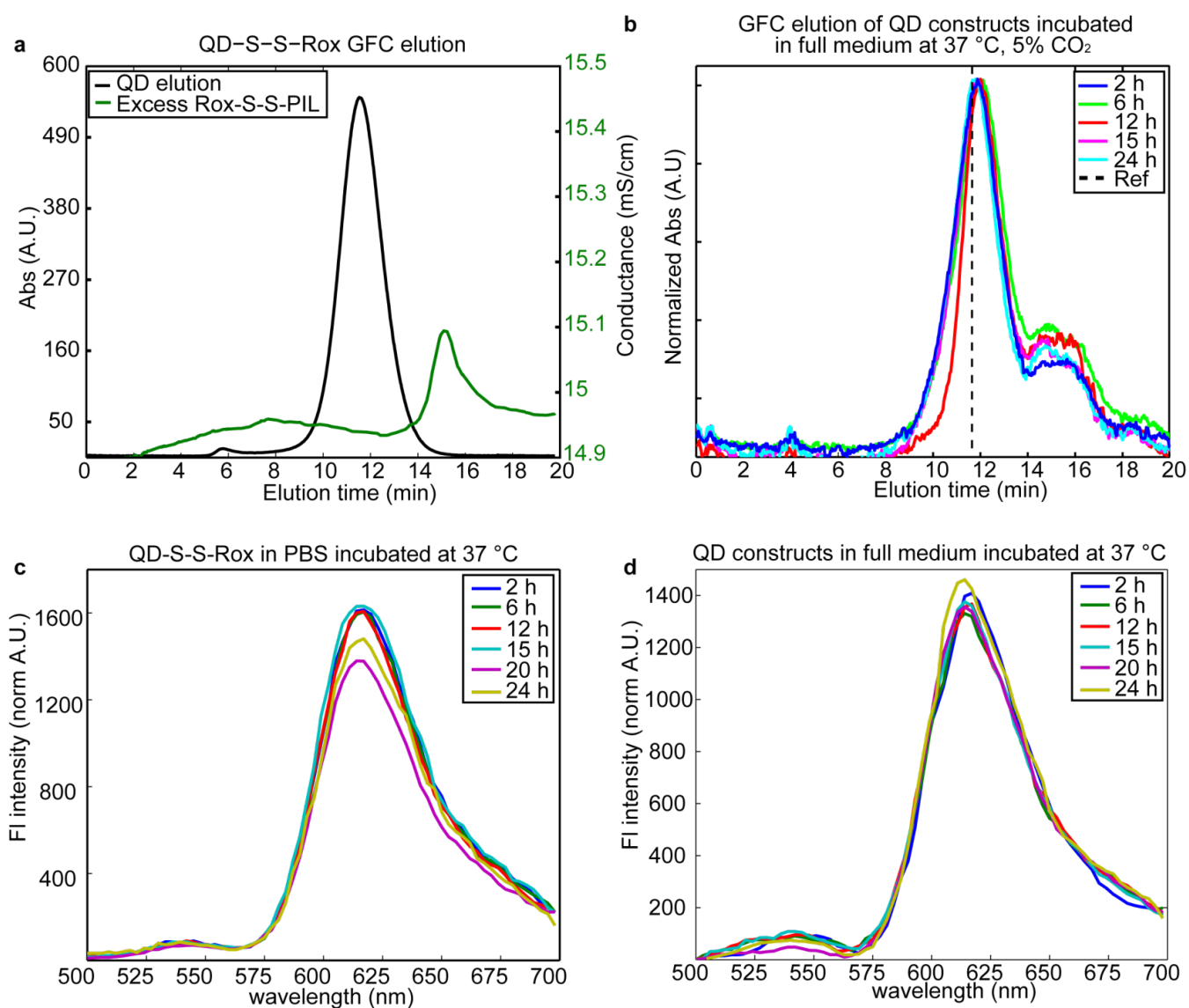


Figure S6A. Viability of HeLa cells upon delivery of plain QD535 by the device, as measured by propidium iodide staining and flow cytometry measurement.

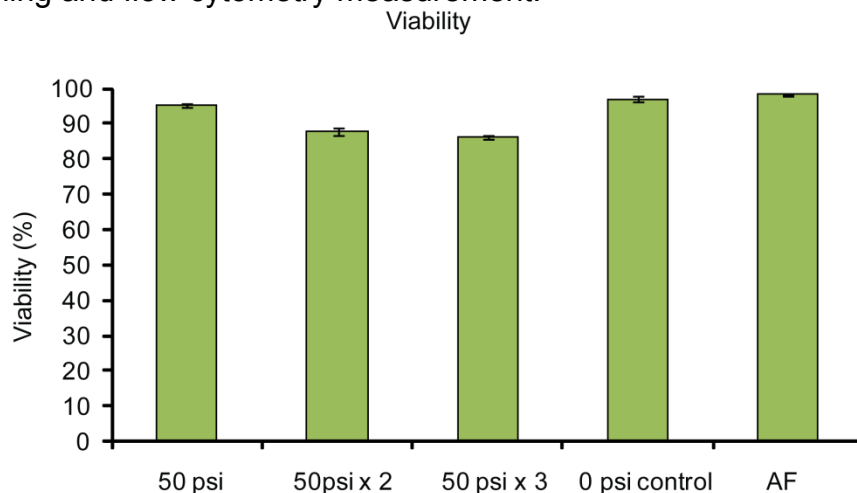


Figure S6B. Far-field view of plain QD535 treated HeLa cells showing increase in cell density from 0 h to 48 h after treatment, demonstrating cell proliferation.

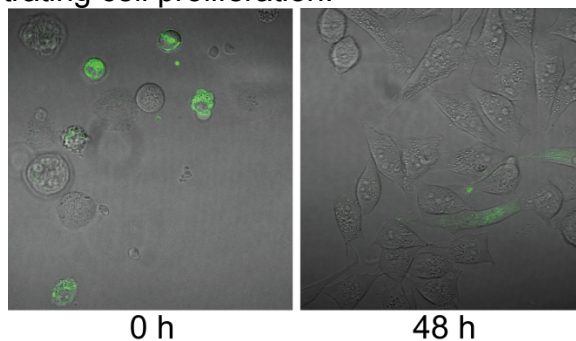


Figure S6C. Delivery efficiency into HeLa cell cytosol upon device treatment with QDs coated with PIL. For delivery efficiency calculation methodology, see figure S13.

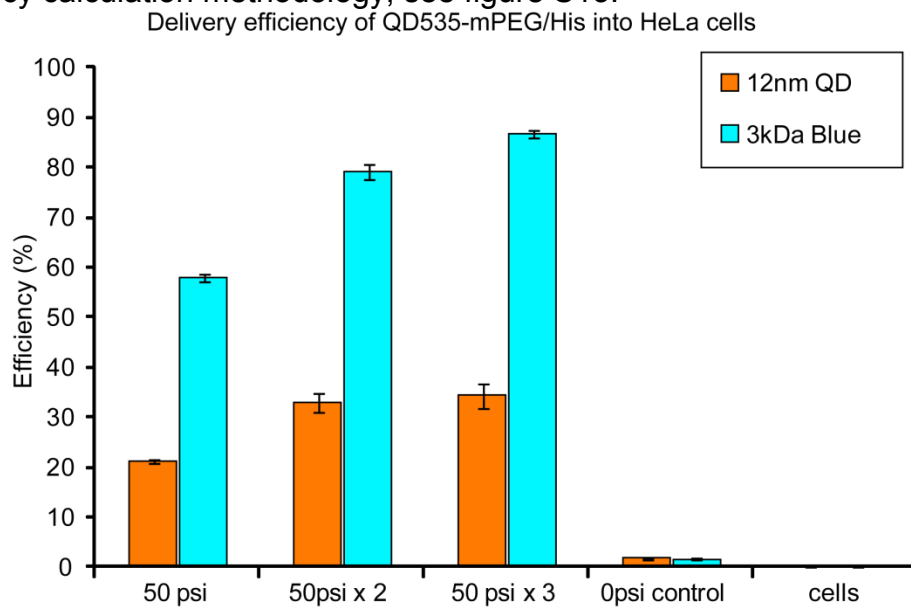
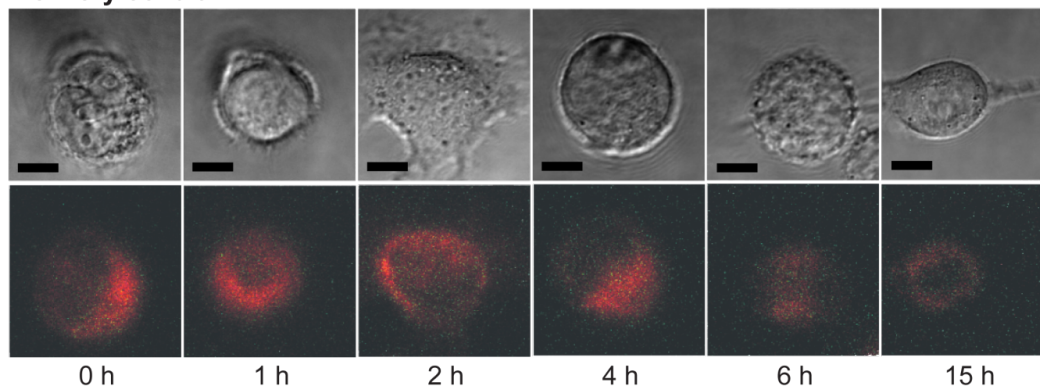
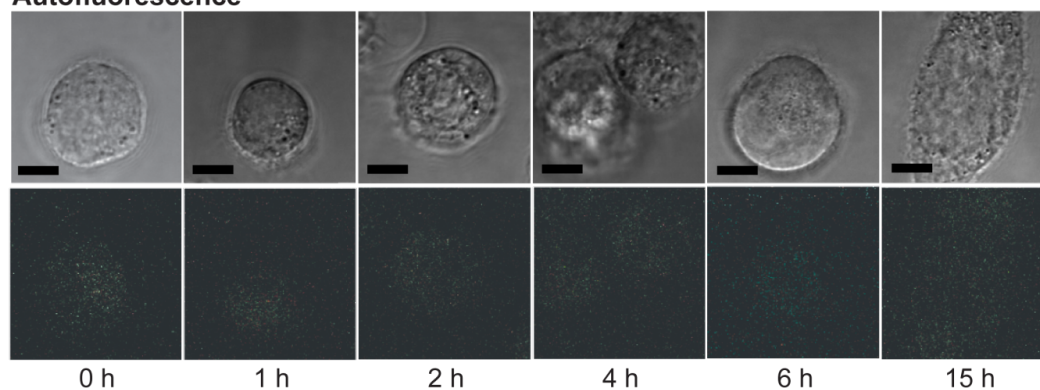


Figure S7. a, Example QD and Rox channel confocal overlay images in the of cells that were incubated, but not delivered, with QD-S-S-Rox (top) and autofluorescence from cells that were completely untreated (bottom). **b**, line graph of average absolute QD and Rox intensities in these cells. Scale bars are 10 μm .

a Delivery control



Autofluorescence



Absolute QD541 and Rox channel intensities in non-delivered controls and autofluorescence cells

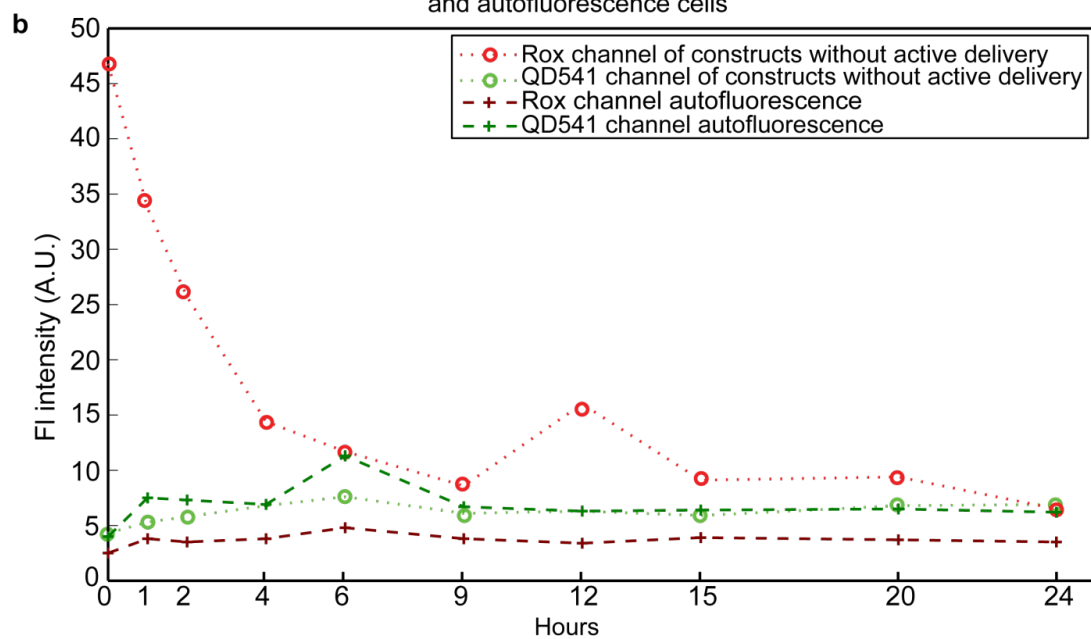


Figure S8. Fluorescence and transmission overlay images of QD-S-S-Rox constructs that were endocytosed into HeLa cells. Cells were trypsinized and incubated with QD-S-S-Rox for 40 minutes at 37 °C, 5% CO₂.

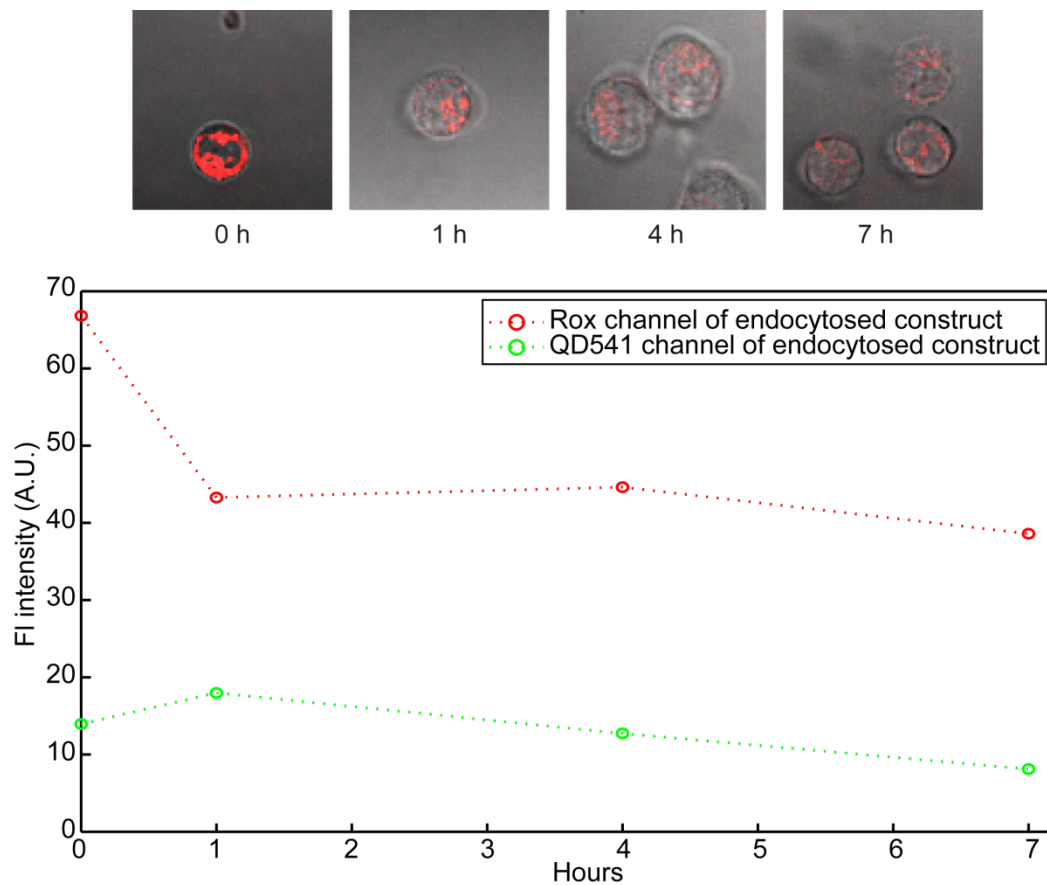


Figure S9. Reproducibility of cytoplasmic delivery as shown by flow cytometry measurements of different delivery instances of QD-S-S-Rox.

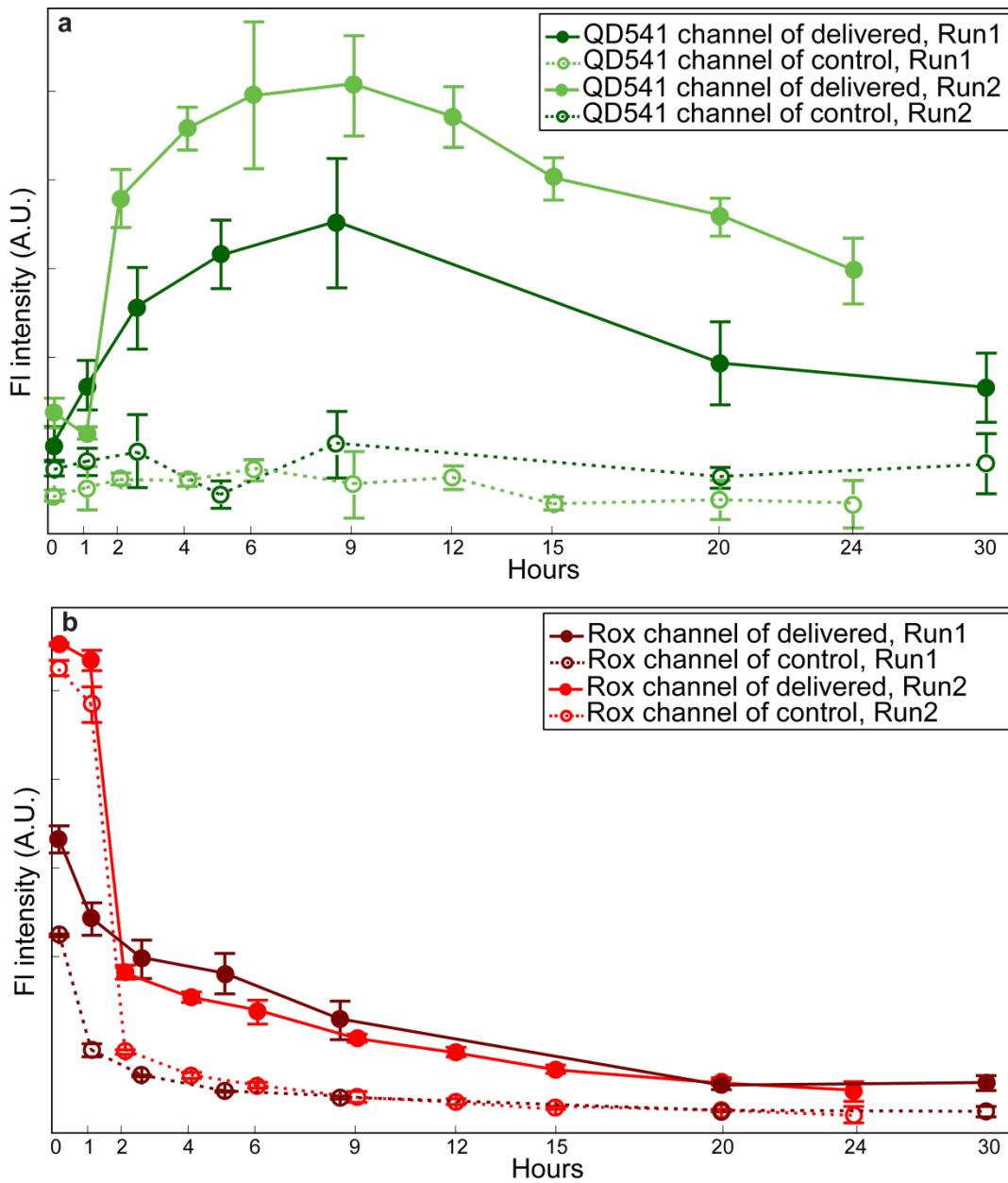


Figure S10. Additional QD and Rox channel overlay confocal images of HeLa cells that have been delivered with QD-S-S-Rox by the device. Scale bars are 20 μm

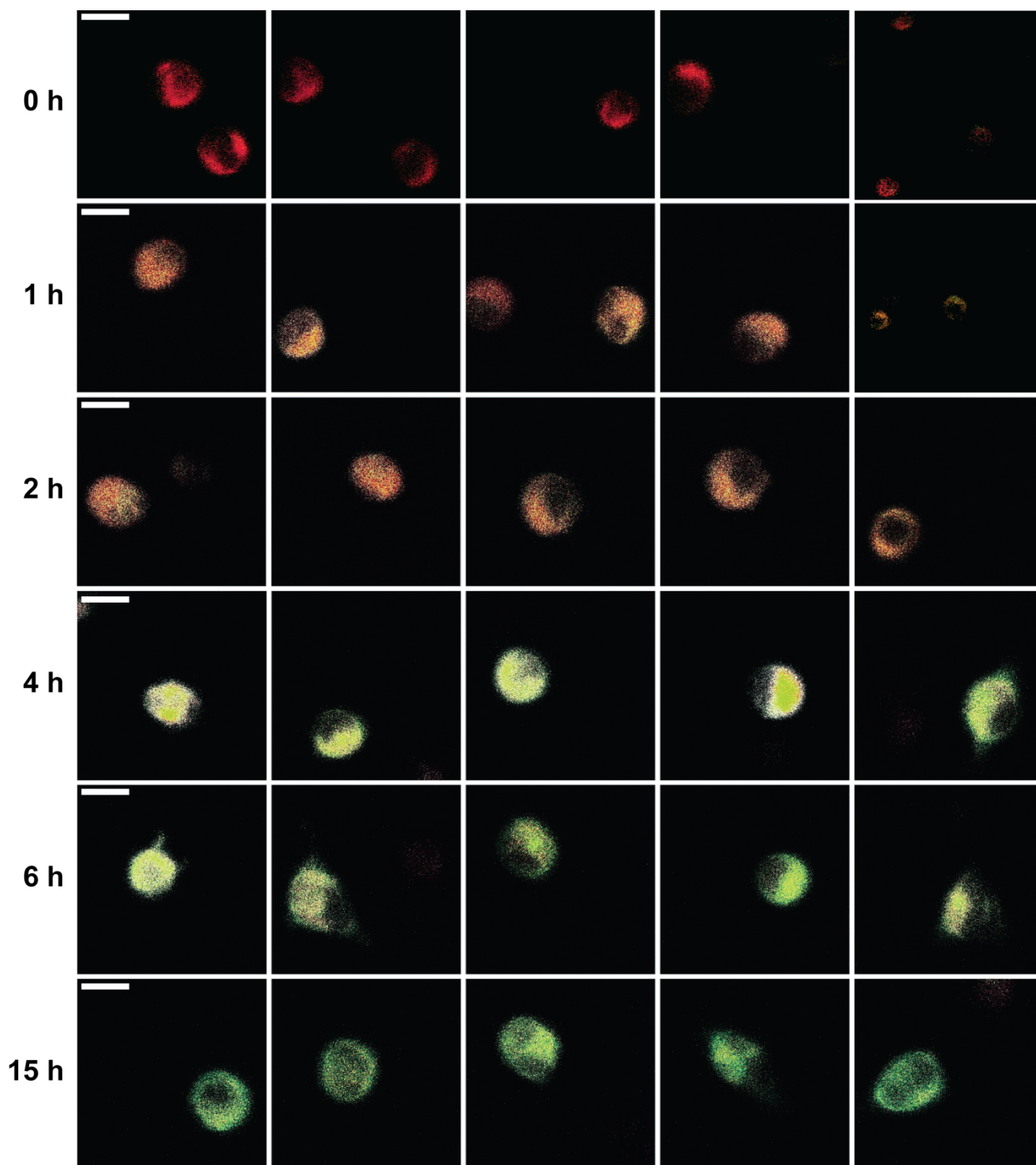


Figure S11. Additional QD and Rox channel overlay confocal images of HeLa cells that have only been incubated for 5 minutes at room temperature, not delivered, with QD-S-S-Rox. Scale bars are 20 μm .

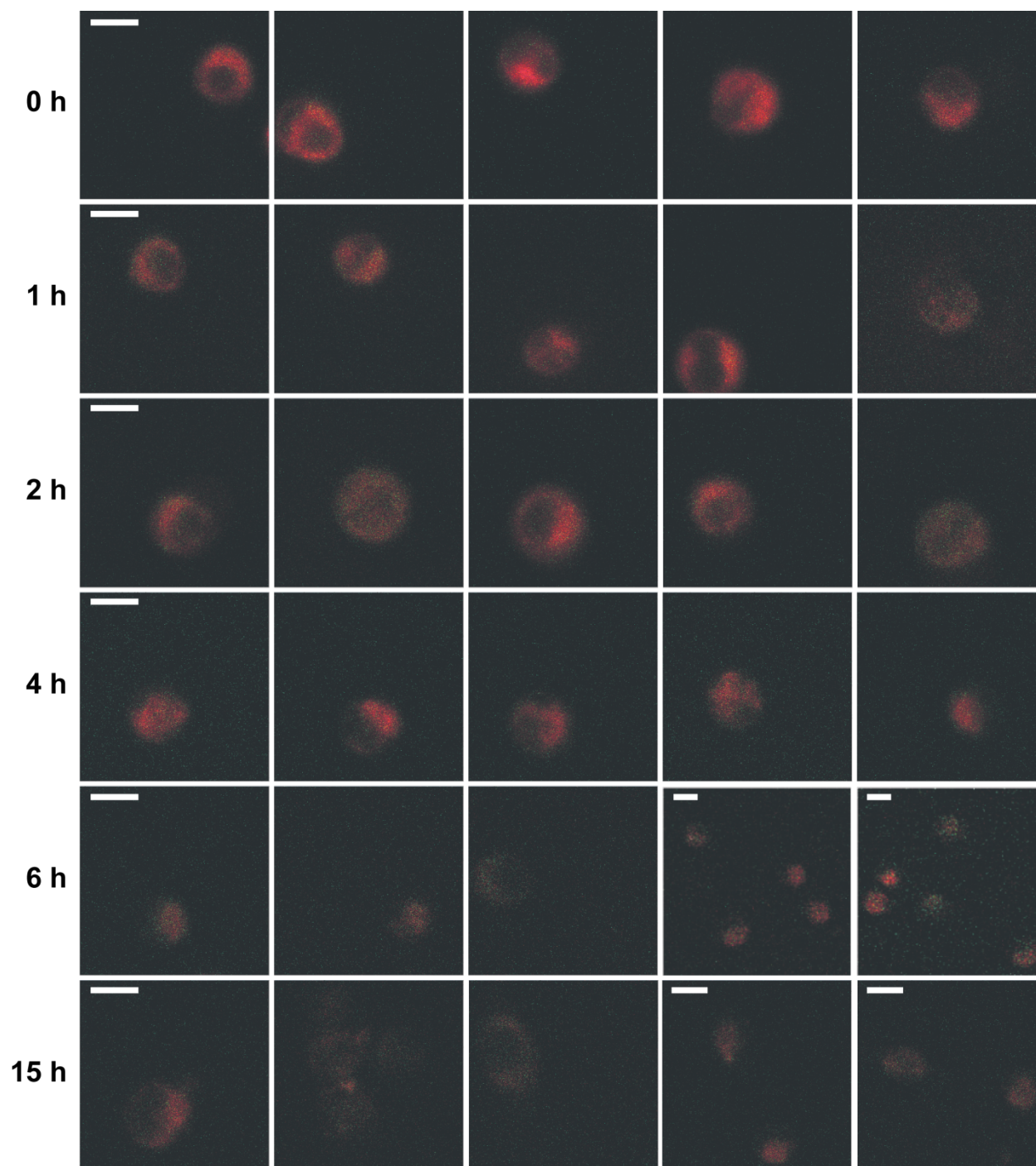


Figure S12A. Additional epifluorescence images of single QDs blinking inside the cell cytoplasm upon 10 nM QD delivery. Scale bars are 1 μ m.

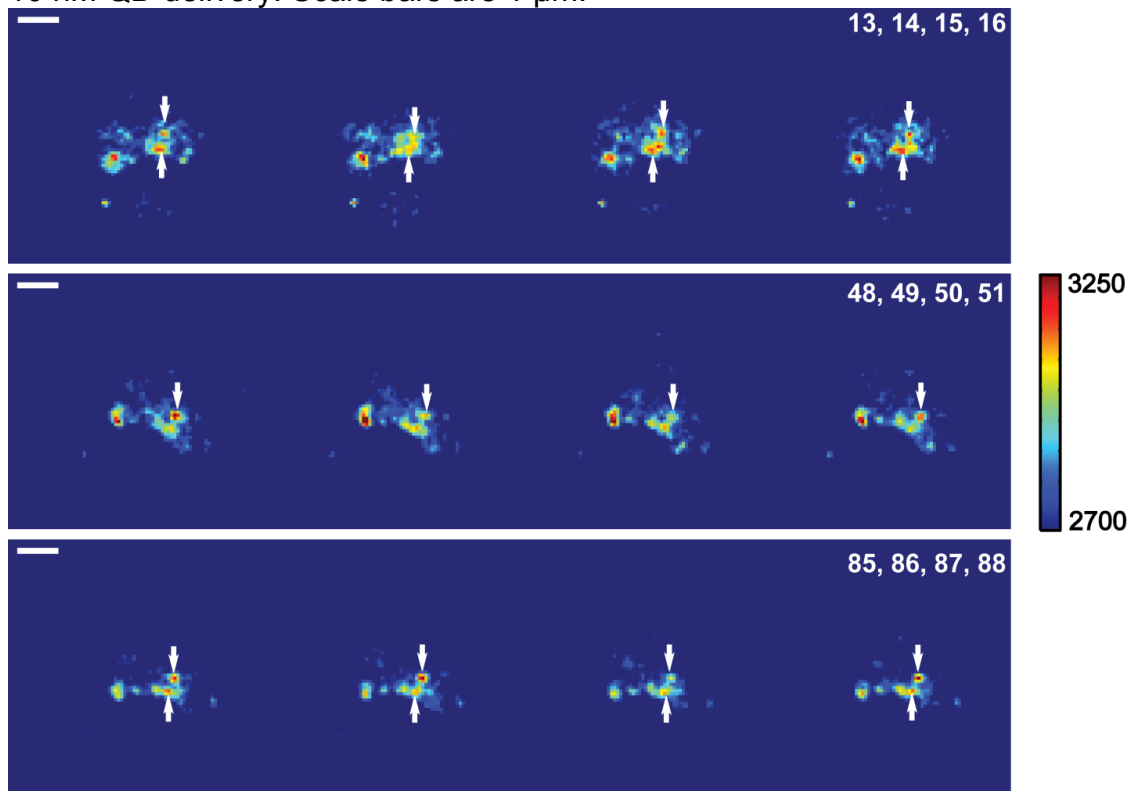
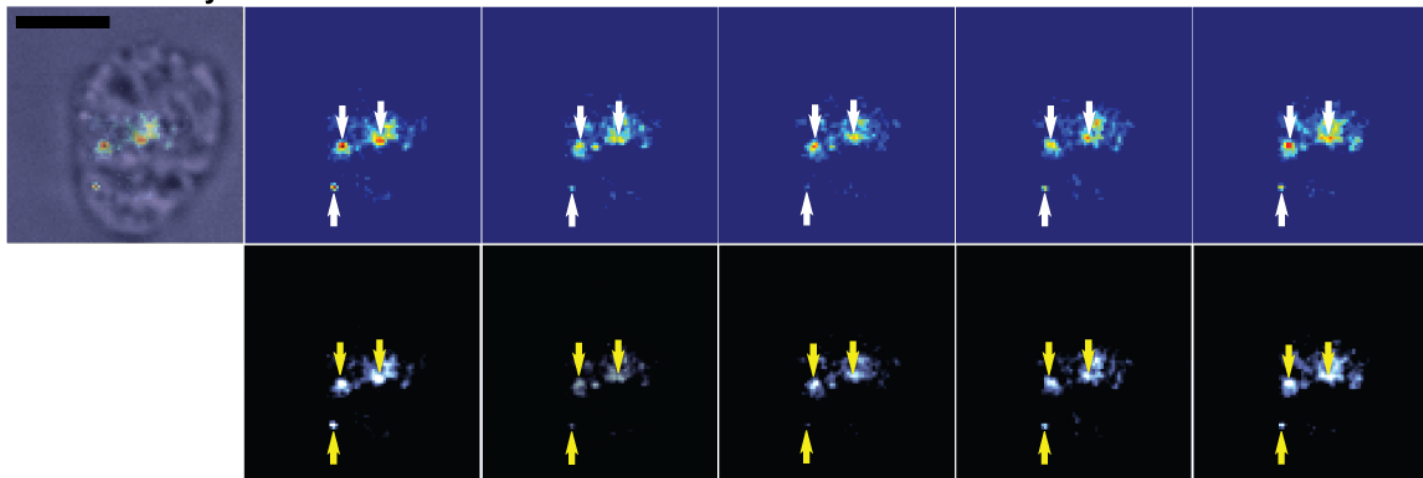


Figure S12B. Grayscale image and cells that have had 100 nM QD delivery, corroborating the lack of lateral diffusion for 10 nM QD delivery as well. Scale bars are 1 μ m.

10 nM delivery



100 nM delivery

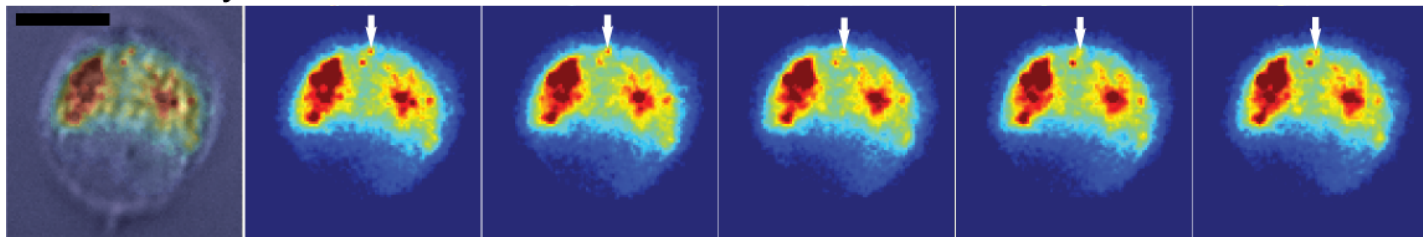


Figure S13. A demonstration of how we determine the fraction of treated cells amongst the population that have been delivered with fluorescence dyes. **a**, Flow cytometry measurement of the control cell population (incubated with pacific blue dye for 5 minutes at room temperature but untreated by device). **b**, Flow cytometry measurement of cells that have been treated with pacific blue using the device. The grey area represents the level of fluorescence that would not be considered as efficient delivery by the device.

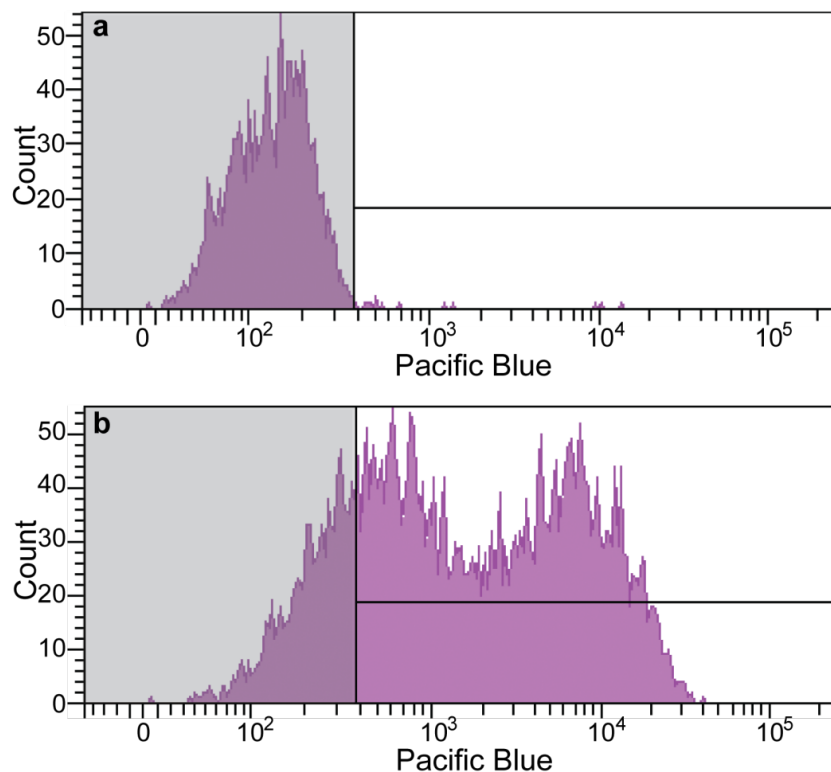


Figure S14. Delivery efficiency of QD-S-S-Rox construct as measured by tracing the QD fluorescence measured by flow cytometry. The ultimate percentage of the cell population that has QD-S-S-Rox inside the cytosol should be at least ~35% according to the plot. This method underestimates cells that have had QD-S-S-Rox delivered, since not all Rox dyes are cleaved from the QD surface.

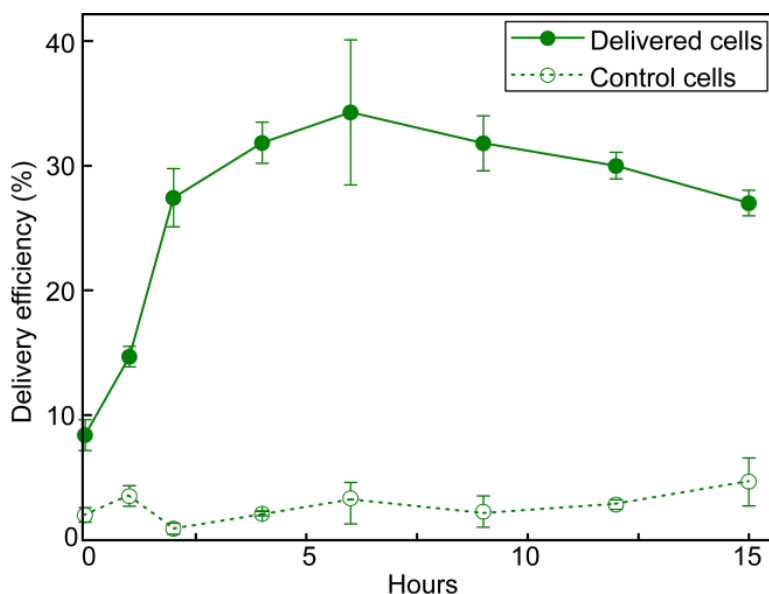


Figure S15. QDs are delivered into HeLa cells at 4 °C, when biological processes are arrested. Control group was incubated in PBS, and indicate autofluorescence levels. Inlet groups were allowed to sit inside the microfluidic device but were not flown through the channels. (a) Delivery efficiency of QD540 QD fluorescence measured by flow cytometry, and (b) viability of treated, control, and inlet group cells. In these figures, the first number indicates the temperature at which delivery was performed and the second indicates the temperature at which the cells were allowed to recover for 5 minutes prior to washing the QDs.

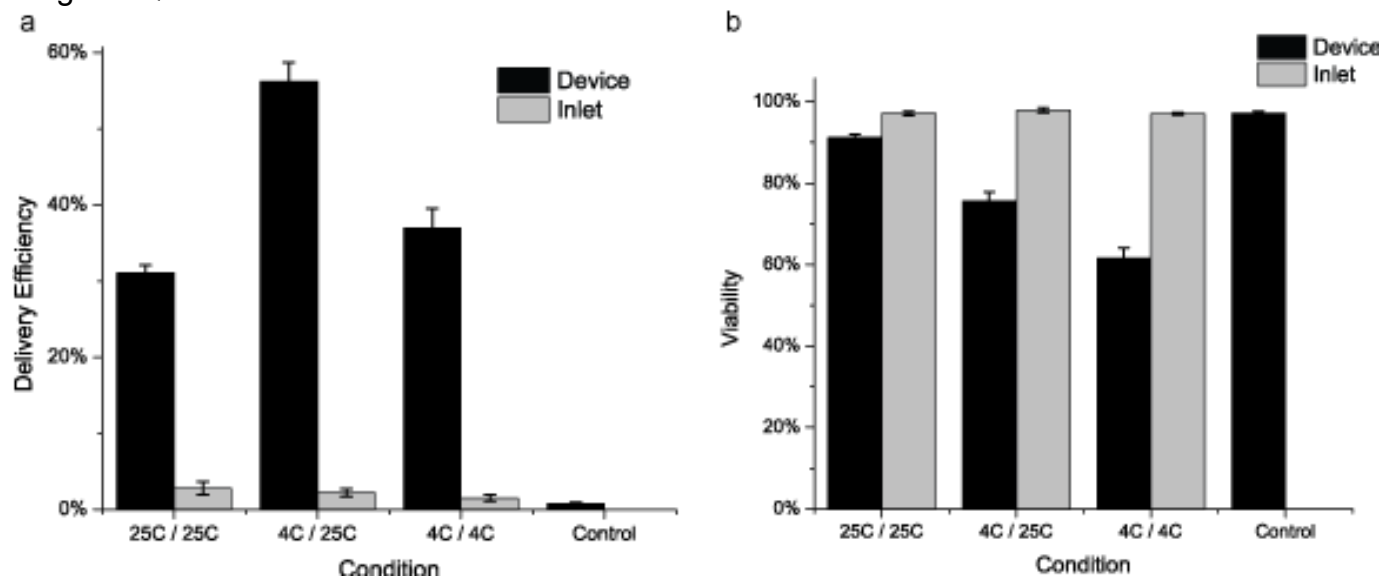


Figure S16. Delivery efficiency measured at 5uM, 1uM, 500nM and 100nM QD concentrations during HeLa cell treatment with the microfluidic device. Inlet groups were allowed to sit inside the microfluidic device but were not flown through the channels. Note that delivery concentrations as low as 10nM were detectable by microscopy, at low enough concentrations to be observed as single molecules.

