Supplementary Material (ESI) for *PCCP*

Supporting Information

Enzyme Catalyzed Site-Specific Protein Labeling and Cell Imaging with Quantum Dots

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Supporting Figures

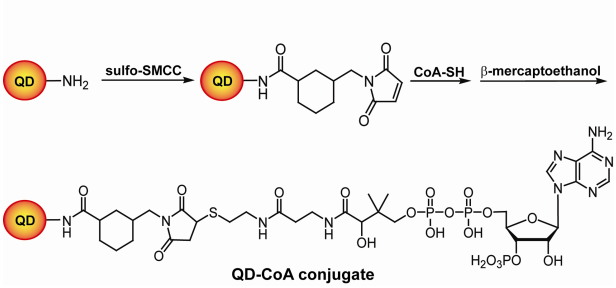

Figure S1. Synthesis of CoA-conjugated QDs. Sulfo-SMCC, sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate).

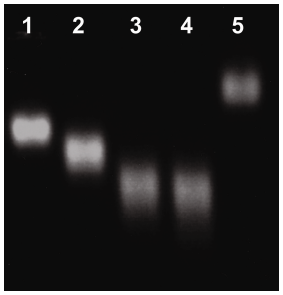

Figure S2. Electrophoresis of QD-CoA conjugates on 0.5% agarose gel with QDs migrating from top to bottom. QD-CoA conjugates were prepared by incubating different ratios of CoA with maleimide functionalized QDs. Lane 1, CoA/QD=3; Lane 2, CoA/QD=10; Lane 3, CoA/QD=25; Lane 4, CoA/QD=50; Lane 5, unfunctionalized QDs.

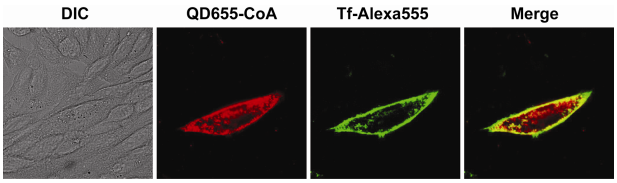

Figure S3. Confocal fluorescence microscope images of QD labeled representative TRVb cells transfected with TfR1-PCP. TRVb cells expressing TfR1-PCP were first labeled with QD655-CoA conjugate (red) in the presence of Sfp at 30 ^{o}C for 20 minutes, washed with PBS, and incubated with transferrin ligand Alexa555 conjugate (Tf-Alexa555, green) at 22 ^{o}C for 10 minutes. Cells were washed with PBS, fixed and imaged.

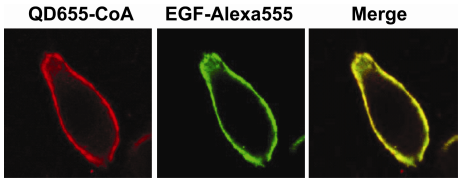

Figure S4. Confocal fluorescence microscope images of QD labeled representative HeLa cells transfected with S6-EGFR. HeLa cells expressing S6-EGFR were first labeled with QD655-CoA conjugate (red, CoA/QD >50) in the presence of Sfp at 37 ^{o}C for 20 minutes, washed with PBS, and incubated with epidermal growth factor ligand Alexa555 conjugate (EGF-Alexa555, green) at 22 ^{o}C for 10 minutes. Cells were washed with PBS, fixed and imaged.

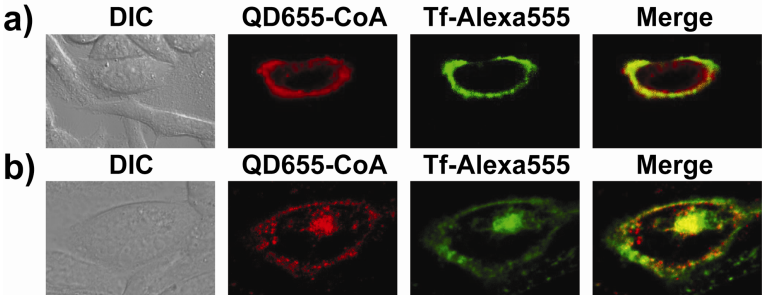

Figure S5. Stacked confocal images of TRVb cells transfected with TfR1-A1, showing internalization of QD labeled TfR1 and transferrin ligands in live cells. a) TRVb cells expressing TfR1-A1 were first labeled with QD655-CoA (red) in the presence of AcpS at 30 ^{o}C for 20 min, washed with PBS and incubated with Tf-Alexa555 ligand (green) at 22 ^{o}C for 10 minutes. Cells were then imaged live at 22 ^{o}C; b) TRVb cells expressing TfR1-A1 were first labeled with QD655-CoA (red) in the presence of AcpS at 30 ^{o}C for 20 min, washed with PBS and incubated with Tf-Alexa555 ligand (green) at 30 ^{o}C for 10 minutes. Cells were then imaged live at 22 ^{o}C.

Materials and Methods

a) Preparation of QD655-CoA and Alexa488-CoA Conjugates

QDot® 655 ITK™ Amino (PEG) quantum dots (QD655) were purchased from Invitrogen at a concentration of 8 μM in 50 mM borate buffer. These QDs had a CdSe-ZnS core and were coated with amino PEG modified triblock copolymer.[1] The hydrodynamic diameter of the QDs was about 20 nm. Buffer exchange was performed by suspending 75 μL of QD in ~4 mL of 25 mM borate buffer (pH 7.4) and transferring the solution into a 100 kDa Amicon Ultra centrifugal filter device (Millipore). The filter unit was centrifuged at a low speed (1250g) until the retentate volume was ~100 μL. Subsequently, the retentate was transferred into a 2 mL polypropylene microfuge tube and 500 equivalents of sulfo-SMCC was added (15 μL of 20 mM solution in water). The mixture was allowed to react for 2 hours at 28 ^{o}C with gentle shaking (200 rpm). Excess sulfo-SMCC was removed by exchanging the reaction mixture twice with fresh 25 mM borate buffer as described above. Following the second round of centrifugation, the retentate (~100 uL total volume) was transferred to a 2 mL microfuge tube and 10 equivalents of coenzyme A (CoA, 3 μL of 2 mM solution in water) were added to the mixture and allowed to react under the same condition as above. After 5 hours, 500 equivalents of β-mercaptoethanol (2 μL of a 150 mM solution in water) were added to cap the unreacted maleimide groups on the QD surface. Following an hour of reaction time, excess CoA and β-mercaptoethanol were removed by buffer exchange using the 100 kDa Amicon Ultra centrifugal filter device. QD solution was washed with 3× ~4 mL of 50 mM borate buffer (pH 8.3), and concentrated to a volume of ~100 μL. QD-CoA conjugate was filtered through a 0.2 μm Spin-X microfuge tube filter (Costar), and stored at 4 ^{o}C before use. In this procedure, we did not observe significant level of QD aggregation after CoA attachment. We found low background binding of QD to cell surface when QD-CoA conjugate was first filtered before being used for cell labeling experiments. The QD-CoA conjugate was stable for more than 3 months when stored at 4 ^{o}C without aggregation or significant changes in its luminescent property. The concentration of QD-CoA was calculated based on its absorbance spectrum using the equation reported in the literature.[2]

Synthesis of Alexa488-CoA conjugate was previously reported.[3]

b) Expression and Purification of PPTases and MBP Fusions

AcpS and Sfp were expressed and purified as previously described.[3] Expression vectors for A1-MBP, S6-MBP and PCP-MBP were constructed before with pET21 plasmids (Novagen).[4] These proteins were expressed as previously reported. [3] Briefly plasmids harbouring the genes of the C-terminal 6×His tagged fusion proteins were transformed into *E. coli* BL21(DE3)pLysS chemical competent cells (Invitrogen). Cells were grown at 37 °C in 1 L LB medium supplemented with 100 mg/mL ampicillin to an optical density at 600 nm of 0.6. Protein expression was then induced with the addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG) and the cell culture was grown at 15 °C overnight. The next day, the cells were harvested by centrifugation at 5000 rpm for 15 minutes and the cell pellets were resuspended in the lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl and 5 mM imidazole) supplemented with 1 unit/ml DNAse I. The resuspended cells were disrupted by French Press (Thermo Spectronic) with three

passages at 12,000 psi. Cell debris was removed by centrifugation at 16,000 rpm for 30 minutes. The clarified cell extract was incubated with 1 mL 50% suspension of Ni-NTA resin (Qiagen) for 3 hours at 4 °C in a batch-binding format. The suspension was then loaded into a gravity column and washed with 20 mL of the lysis buffer. Protein bound to the column was eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl and 250 mM imidazole). The purity of the fractions containing the protein of interest was checked by SDS-PAGE stained with Coomassie brilliant blue. Fractions with the desired purity were pooled and dialyzed against 2 × 1 L of 50 mM HEPES, pH 7.5, 100 mM NaCl and 10% glycerol. Protein solutions were then aliquoted and stored at -80 °C.

c) MBP labeling with QDs and the pull-down assay

MBP labeling reactions were performed in a total volume of 50 μL and allowed to react for 1 hour at 30 ^{o}C in a reaction buffer containing 10 mM $MgCl_2$ and 50 mM HEPES (pH 7.4). To the reaction mixture, 5 μM of peptide-tagged MBP, 100 nM QD655-CoA, and 0.5-1 μM of PPTase were added. AcpS was used to label A1-MBP and Sfp to label S6-MBP and PCP-MBP. Control reactions were carried out in the absence of PPTases.

For pull-down experiments, 100 μL of amylose resin (New England Biolabs) was added to the reaction mixture after the labeling reaction and continuously mixed by inversion for 30 minutes at room temperature. The resin was allowed to settle, and the reaction tubes were then exposed to 365 nm UV light to observe the fluorescence contained in the resin and in the supernatant.

d) Labeling of TfR1-A1 and TfR1-PCP with QDs in TRVb Cells

TRVb cells were maintained at 37 ^{o}C under 5% CO_2 in F12 medium supplemented with 10% FBS and 50 U/mL penicillin/streptomycin. For cell labeling, TRVb cells were seeded overnight in 8-well culture chambers (Nunc) with a cover slip bottom and were allowed to grow to 85-90% confluency. The cells were transfected with pcDNA3.1(+) plasmids carrying the genes of C-terminal tagged TfR1-A1 or TfR1-PCP using Fugene 6 (Roche) transfection reagent according to the manufacturer's protocol. After 36-48 hours at 37 ^{o}C to allow protein expression, TRVb cells were washed with PBS and incubated in serum-free medium for 2 hours prior to protein labeling. Cells in each well were then labeled at 30 ^{o}C in 100 μL F12 medium with the following composition: 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 250 nM QD655-CoA, 0.1% BSA, 2-4 μM AcpS (for TfR1-A1 labeling) or 1-2 μM Sfp (for TfR1-PCP labeling). The optimal labeling time was determined to be 15-20 minutes. Following QD labeling, cells were washed 4 times with PBS and incubated for 10 minutes at 22 ^{o}C in 100 μL F12 medium containing 10 μg/mL transferrin ligand-Alexa555 conjugate (Invitrogen Molecular Probes). For internalization experiments, the incubation was done at 30 ^{o}C for 10 minutes. Cells were then washed 3 times with PBS. Finally the cells were imaged alive or after being fixed with 4% paraformaldehyde in PBS. Labeling and imaging experiments were repeated and the results were confirmed on three occasions using distinct preparations of QDs and separate transfections.

e) Orthogonal Labeling of S6-EGFR and TfR1-A1 in HeLa Cells

HeLa cells were maintained at 37 ^{o}C under 5% CO_2 in DMEM medium supplemented with 10% FBS and 50 U/mL penicillin/streptomycin. For cell labeling, HeLa cells were seeded overnight in 8-well culture chambers (Nunc) with a cover slip bottom to obtain 85-90% confluency. The following day, the cells were co-transfected with pUSE plasmid carrying the gene of N-terminal tagged S6-EGFR and pcDNA3.1(+) plasmid carrying the gene of C-terminal tagged TfR1-A1 using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. After 24-36 hours at 37 ^{o}C for protein expression, HeLa cells were washed with PBS and incubated in serum-free medium for 2 hours prior to protein labeling. Cells were first labeled with QDs at 37 ^{o}C in 100 μL of culture medium with the following components: 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 250 nM QD655-CoA, 4 μM AcpS. The optimal incubation time was determined to be 15-20 minutes. Following QD labeling, cells were washed 4 times with PBS and the second labeling reaction was carried out in culture medium containing the following components: 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 μM Alexa488-CoA, 2 μM Sfp. Cells were then washed 4 more times with PBS and fixed with 4% paraformaldehyde in PBS. Labeling and imaging experiments were repeated and the results were confirmed on three occasions using different preparations of QDs and separate transfections.

f) Imaging

Cells were imaged using a Leica SP2 AOBS confocal microscope (University of Chicago Microscopy Core Facility) with either 63× or 100× oil objectives. QDs were excited using a 405 nm UV laser line, while a 514 nm line was used to excite Alexa555 and a 488 nm line was used to excite Alexa488 fluorophores. Images were background corrected and the two channels (green and red) were merged using ImageJ analysis software.

References:

[1] X. Gao, Y. Cui, R. M. Levenson, L. W. K. Chung, S. Nie, *Nat. Biotechnol.*, 2004, **22**, 969
[2] W. W. Yu, L. Qu, W. Guo, X. Peng, *Chem. Mater.*, 2003, **15**, 2854
[3] J. Yin, A. J. Lin, P. D. Buckett, M. Wessling-Resnick, D. E. Golan and C. T. Walsh, *Chem. Biol.*, 2005, **12**, 999
[4] Z. Zhou, P. Cironi, A. J. Lin, Y. Xu, S. Hrvatin, D. E. Golan, P. A. Silver, C. T. Walsh, J. Yin, *ACS Chem. Biol.* 2007, **2**, 337.