SUPPORTING INFORMATION

High-affinity labeling and tracking of individual histidine-tagged proteins in live cells using Ni^{2+} tris-nitrilotriacetic acid quantum dot conjugates

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SUPPORTING METHODS

Chemical Synthesis (follows scheme in Figure S1)

gallate-PEG-trisNTA-OtBu (4). The phosgenation of compound **1** (gallate-PEG-OH) was carried out under argon atmosphere at room temperature to yield compound **2**. Compound **1** (100 mg, 2128 g/mol), bis(trichloromethyl)carbonate (14 mg, 296 g/mol), *N*,*N*-Diisopropylethylamine (DIPEA, 10 μL, 129.24 g/mol, 0.742 g/mL) and anhydrous dichloromethane (DCM, 3 mL), were loaded into a 50 mL round bottom flask. Following argon purge, the reaction mixture was agitated for 4.5 hours and dried under vacuum (14 mbar, 1 hour). Compound **3** *(*trisNTA-OtBu*)* (67 mg, 3595 g/mol) was added with 3 mL DCM and DIPEA (10 μL, 129.24 g/mol, 0.742 g/mL) and a second reaction was carried out for 22 hours under argon and evaporated. For purification, a column chromatography was performed (Silica Gel, DCM/Methanol from 100/0 to 90/10) and compound **4** was obtained (79 mg, 47% yield).

^{1}H NMR (200MHz, $CDCl_3$): 7.3 ppm (s, 2H), 4.5 ppm (t, 2H),4.05 ppm (t, 6H), 3.85 ppm (t, 6H), 3.8-3.6 ppm (m, 134H), 3.5-3.25 (m, 28H), 2.9 (s, 1H), 2.85-2.75 (m, 6H), 2.05-1.95 (m, 2H), 1.8 ppm (m, 6H), 1.65-1.45 (m, 12H), 1.5-1.1 ppm (m, 48H), 1.45(s, 27H), 1.43 (s, 54H), 0.9 ppm (t, 9H).

gallate-PEG-trisNTA (5). Deprotection of the acidic functions of compound **4** (20 mg, 3595 g/mol) was performed in presence of trifluoroacetic acid (TFA, 0.483 mL, 1.535 g/mL, 114.02 g/mol) and 6 mL DCM in a 10 mL round bottom flask. After 22 hours of reaction the mixture was evaporated under vacuum (7 mbar, 2 hours) and compound **5** was obtained without any purification (17 mg, 98% yield).

^{1}H NMR (200MHz, $CDCl_3$): 7.3 ppm (s, 2H), 4.5 ppm (t, 2H),4.05 ppm (t, 6H), 3.85 ppm (t, 6H), 3.8-3.6 ppm (m, 134H), 3.5-3.25 (m, 28H), 2.9 (s, 1H), 2.85-2.75 (m, 6H), 2.05-1.95 (m, 2H), 1.8 ppm (m, 6H), 1.65-1.45 (m, 12H), 1.5-1.1 ppm (m, 48H), 0.9 ppm (t, 9H).

Preparation of QD-trisNTA. Biocompatible micelle-encapsulated QDs were prepared using a previously published procedure[1, 2]. Briefly, QD-trisNTA was prepared from hydrophobic CdSe/ZnS QDs (λ_{max}=595 nm, Evident Technologies) in toluene. Compounds **1** and **5** were mixed at the desired ratio (typically 85% and 15%, respectively). The mixture was added directly to a ~10 μM solution of QDs in toluene and the solvent was evaporated by drying under 70 mbar vacuum. The micellisation of the QDs in aqueous buffer was obtained by hydration of the mixture in ~ 1 mL of borate saline solution (20 mM boric acid, 150 mM NaCl, pH 8.5) by 5 minutes of warming at 50 °C and vortexing the sample for several minutes. Next, the crude suspension was centrifuged (20000 g, 10 minutes) to remove aggregates. The supernatant was extracted and purified on disposable SEC columns (NAP-10, GE Healthcare) to eliminate excess gallate-PEG molecules. For a final purification step, the sample was reconcentrated over a 10000 MW ultrafiltration device (Vivaspin 500, Sartorius). QD-trisNTA was loaded with Ni^{2+} by incubation with a 300 fold molar excess of $NiCl_2$ for 30 minutes in Hank's balanced salt solution (HBSS; 10 mM glucose, 20 mM Hepes, 1.2 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, pH 7.4) or Hepes buffered saline (HBS; 20 mM Hepes, 150 mM NaCl, pH 7.5)**.** Excess Ni^{2+} was removed by two cycles of purification using Sephadex G-10 Macrospin columns (Harvard Apparatus). Unless noted otherwise, all assays with QD-trisNTA were performed following Ni^{2+} loading.

QD-trisNTA characterization. Absorbance measurements were performed on a Cary 100 UV-VIS scan spectrophotometer (Varian, Australia). Steady-state fluorescent emission measurements were performed on a Fluorolog-3-22 fluorescence spectrometer (Horiba Jobin Yvon) using an excitation wavelength of 350 nm. Quantum yields (QY) were obtained by comparing the integrated relative emission values of the QDs to a standard dye, Rhodamine 6G (QY = 0.95) in water. The concentrations of QDs were estimated from the absorbance spectrum and published extinction coefficients derived for

the CdSe QD core material[3]. Dynamic light scattering (DLS) measurements were performed at a scattering angle of 173° at 25°C on a Zetasizer NanoZS (Malvern Instruments) after the samples were filtered through a 0.2 μm filter (Millex GV, Millipore) and the raw intensity distribution was converted to a volume distribution using the instrument software.

Gel electrophoresis was performed on 0.5% agarose gels in 0.5X TAE buffer (20 mM Tris, 20 mM Acetate, 0.5 mM EDTA, pH 8.3). QD samples were diluted to 100 nM in 3% glycerol loading buffer immediately before use. A 20 V/cm electric field was applied for 20 seconds followed by a 10 V/cm electric field for 30 minutes. QD fluorescence was detected directly on gels using ultraviolet (UV) excitation and imaged with a standard CCD camera (Kodak).

Fluorophores and proteins. ^{FEW646}trisNTA was prepared by conjugating the fluorophore FEW S0387 (FEW Chemical GmbH, Wolfen, Germany) to the primary amino group on trisNTA as described[4]. Ifnar2 was fused to a H10 tag (Ifnar2-H10) and IFNα2 S136C was site-specifically labeled with Oregon Green 488 (^{OG488}IFNα2) as previously published[5-7]. A labeling degree of ~0.8 was determined by photometric analysis. Maltose binding protein with a C-terminal H10 tag[8] was labeled with Cy5 through a cysteine residue, which was incorporated by site-directed mutagenesis (^{Cy5}MBP-H10). The protein was expressed and purified as the wild-type, reacted with Cy5-maleimide (GE Healthcare) by standard protocols and purified by size exclusion chromatography. The labeling degree as determined by photometric analysis was ~0.5.

For expression of a membrane-anchored CFP with an N-terminal H10 tag in HeLa cells, an oligonucleotide linker encoding for 10 consecutive histidines and suitable flanking bases was inserted into the *Bgl*II site of the vector pDisplay (Invitrogen) containing the gene for an AP-CFP fusion (obtained from Alice Ting, MIT). H10-Ifnar1 and AP-Ifnar2 with H10-tag and AP-tag, respectively, were co-expressed in HeLa cells using the vector pVITRO2 (InvivoGen), which contains two multiple cloning sites for expression under two different promoters (http://www.invivogen.com/PDF/pVITRO2-

neo-mcs_TDS.pdf). For this purpose, the genes of Ifnar1 and Ifnar2 lacking the cytosolic domains as well as the secretion sequence were first individually cloned into the vector pDisplay through the restriction sites *Bgl*II and *Pst*I. Subsequently, N-terminal AP-tag (Ifnar2) and H10-tag (Ifnar1) were inserted through the *Bgl*II site. These constructs including the secretion sequence provided by the pDisplay vector were sequentially cloned into the MCS of pVITRO through the restriction sites *BamH*I and *EcoR*V for Ifnar1 and *Xho*I and *Nhe*I for Ifnar2. All constructs were verified by DNA sequencing.

Surface binding assays using RIf and TIRFS. Common organic compounds and biochemicals were purchased from Sigma-Aldrich. Transducer slides were cleaned in piranha (a freshly prepared mixture of one part 30% hydrogen peroxide and two parts concentrated sulfuric acid). After rinsing with water and drying in a nitrogen stream, the surfaces were silanized with (3-glycidyloxypropyl) trimethoxysilane (GOPTS) and diamino poly(ethylene glycol) with an average molecular mass of 2000 g/mol was reacted with the epoxy groups on the surface as described previously[9]. To incorporate maleimide functionality, the slides were thoroughly rinsed with water, dried with nitrogen and 5 μL of maleoyl-β-alanine in dry DMF (200 mg/mL) was homogeneously applied onto the surface. The coupling reaction was initiated by adding 5 μL of diisopropylcarbodiimide (DIC) and incubating for 2 hours at 75 ºC by assembling the surfaces of two slides face to face. The excess reaction mixture was removed by washing with chloroform and the slides were dried under nitrogen stream. To incorporate polyhistidine onto the surface, 10 μL of 1 mM solution of the peptide $H_6(SGGG)_2C$ (EMC Microcollections) in Hepes buffered saline (HBS; 20 mM Hepes, 150 mM NaCl, pH 7.5) was incubated onto the maleimide surface for 1 hour at room temperature by assembling the surfaces of two slides face to face. After rinsing with water and drying with nitrogen, the transducer slides were stored under ambient conditions until use.

All surface binding assays were carried out with HBS as running buffer on a TIRFS-RIf setup[10]. First, the polyhistidine surfaces were treated with 100 mM HCl for 185 s followed by equilibration in HBS.

QD-trisNTA(1%, 5% and 15% densities) were injected at a concentration of 10 nM in HBS for 185 s to monitor the association. Control samples were preincubated with 1 mM EDTA for 30 minutes. The dissociation of QD-trisNTA from the surface was monitored by elution with 250 mM imidazole in HBS for 185 s. Residual QD-trisNTA could be removed by an injection of 1 M imidazole in HBS for 36 s or the surface was regenerated by injection of 100 mM HCl for 36 s. For the fluorescence-based detection by TIRFS, the excitation source was a 405 nm laser (145 μW). All surface binding assays were conducted on the same transducer to obtain the most reliable comparisons.

Solution based-assays using SEC. Analytical size-exclusion chromatography (SEC) was using to investigate solution-based binding of QD-trisNTA to proteins carrying a polyhistidine tag. QD-trisNTA was incubated with the protein(s) of interest (Ifnar2-H10, ^{OG488}IFNα2 or ^{Cy5}MBP-H10) in HBS for 30 minutes. Final concentrations of QD-trisNTA and proteins were 10 nM and 1 μM, respectively, in order to ensure there would be an excess amount of protein over QD-trisNTA. The sample was loaded onto a SEC column (Superdex 200 PC 3.2/30) in an Ettan LC system (GE Healthcare) and elution was monitored at 280, 350 and 488 nm for detection of ^{OG488}IFNα2 or 650 nm for detection of ^{Cy5}MBP-H10. The absorbance of the QDs was monitored at 350 nm. The absorbance at 280 nm represents the absorbance of the proteins, QD core material and the gallate-PEG-trisNTA coating.

To determine the relative amount of proteins of interest bound to QD-trisNTA in the solution-based experiments, the absorbance of QD-trisNTA at 350 nm and either ^{OG488}IFNα2 at 488 nm or ^{Cy5}MBP-H10 at 650 nm was corrected based on control measurements of QD-trisNTA alone and dye-labeled protein alone (using the method described in reference[11]). From the corrected absorbance of the chromatograms, we estimate that ^{Cy5}MBP-H10 binds to QD-trisNTA at a ratio of 11:1 and that ^{OG488}IFNα2 binds to QD-trisNTA through the Ifnar2-H10 bridge at a ratio of 8:1.

Cell culture and transfection. HeLa and COS7 cells were maintained at 37 °C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. One day prior to transfection, cells were plated on glass coverslips to achieve a confluency of 50-80%. Cells were transfected with the desired plasmid (H10-CFP-TM or H10-Ifnar1/AP-Ifnar2) using Fugene 6 (Roche Applied Science) reagent according to the manufacturer's protocol.

Cell labeling and imaging of H10-CFP-TM. HeLa cells were transfected with H10-CFP-TM 24 hours before labeling. Cell labeling was performed by placing the glass coverslips on a heating block at 37 °C. Cells were labeled with 100 nM ^{FEW646}trisNTA by incubation in PBS or Hank's balanced salt solution (HBSS; 10 mM glucose, 20 mM Hepes, 1.2 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, pH 7.4) (supplemented with 1% BSA) for 10 minutes followed by repeated washing with PBS. Cells were labeled with 7.5 nM QD-trisNTA by incubation in HBSS buffer (supplemented with 1% BSA) for 60 seconds followed by repeated washing in PBS. Cells were imaged at room temperature in OptiMem medium. When described, imidazole was added to the medium from a 2 M stock solution in PBS at the concentration and duration indicated (typically 200 mM, 2 minutes) and afterwards, the medium was replaced with fresh OptiMem.

Cell imaging was on an inverted wide-field epifluorescent microscope (Olympus, IX70) equipped with a 60X oil objective (NA=1.45, Olympus). Fluorophores were excited by a broad-spectrum mercury lamp source. Images were acquired using a back-illuminated CCD camera (Micromax 512EBFT, Roper Scientific) and MetaView software (Universal Imaging). H10-CFP-TM was imaged through a CFP filter set (excitation: 440±10 nm, dichroic: 455 nm, emission: 480±20 nm). ^{FEW646}trisNTA was imaged through a Cy5 filter set (excitation: 640±10 nm, dichroic: 660 nm, emission: 680±15 nm). QD-trisNTA was imaged through a custom filter set (excitation: 420±10 nm, dichroic: 470 nm, emission: 595±10 nm).

Two color labeling and imaging of Ifnar1 and Ifnar2. COS7 cells were transfected with H10-Ifnar1 and AP-Ifnar2 24 hours before labeling. Cell labeling was performed by placing the glass coverslips on a heating block at 37 °C. First, AP-Ifnar2 was biotinylated by incubating cells in the culture medium supplemented with 100 nM biotin ligase (BirA), 1 mM adenosine triphosphate (ATP) and 10 μM biotin for 30 minutes. For labeling with QDs, QD-trisNTA and QD-sav were simultaneously added to the cells at concentrations of ~5 nM in HBSS buffer (supplemented with 1% BSA) for 2 to 5 minutes followed by repeated washing in PBS.

Cell imaging was performed at 37 °C on an inverted wide-field epifluorescent microscope (Olympus, IX71) equipped with a 60X oil objective (NA=1.45, Olympus), a wide spectrum mercury lamp source and a back-illuminated CCD camera (Quantem or Photometrics 512B, Roper Scientific). Image acquisition was done using Metamorph software (Universal Imaging). Simultaneous excitation of QD-trisNTA(15%) emitting at 595 nm and Qdot 655 ITK Streptavidin conjugate (Invitrogen) (QD-sav) was performed with a 455BP70 excitation filter and a 500DCXR dichroic mirror. Simultaneous detection of QD-trisNTA and QD-sav was performed on a dual-view system (Optical Insights) configured with a 630DRLP dichroic mirror, a 595±30 nm emission filter and a 655±20 nm emission filter. The signal from QD-trisNTA and QD-sav were projected onto the two halves of the CCD camera and image sequences were acquired continuously using an integration time of 75 ms per frame.

Data Analysis and single particle tracking. The trajectories and diffusion coefficients of QDs were analyzed using a home-made tracking and analysis software written in labview (Pinaud *et al.*, submitted). Briefly, the position of single QDs was determined by locating the center of the fluorescence spots by a two-dimensional Gaussian fit with a spatial resolution of 10-30 nm. Trajectories were qualitatively revealed by superimposing all the frames from an image sequence. Only trajectories of single QD-trisNTA or QD-sav identified by their fluorescence intermittency were used for the

analysis. Diffusion coefficients for the trajectories were calculated by fitting the first 5 points of mean squared displacement curves (D_1-D_4) with the equation for a simple Brownian diffusion with position error: $4\sigma^2+4Dt$. Instantaneous diffusion coefficients at time t were determined using a sliding time window of 20 frames and fitting D_1-D_4 with $4\sigma^2+4Dt$. In Figure 4 and Movie S2, the background was filtered by wavelet decompostion[12] to facilitate visualization. However, particle tracking was performed on original data.

SUPPORTING MOVIES

Movie S1. Motion of QD-trisNTA on the surface of HeLa cells. Playback is in real-time, from a 30 second acquisition collected with an integration time of 100 ms. This movie corresponds to the trajectories shown in Figure S5A.

Movie S2. Two color QD-trisNTA and QD-sav movie showing the interaction of the Ifnar1 and Ifnar2 subunits on COS7 cell membranes. Playback is in real-time, from a 70 second acquisition collected with an integration time of 75 ms. This movie corresponds to the trajectories shown in Figure 4 of the main text.

SUPPORTING TABLES

Table S1. Parameters obtained from exponential fits of the imidazole-induced dissociation kinetics of trisNTA and QD-trisNTA from immobilized H6.

sample	k_{d1} [s^{-1}]	Relative Amplitude	k_{d2} [s^{-1}]	Relative Amplitude
trisNTA alone	0.4	1	X	X
QD-trisNTA(1%)	0.43	0.75	0.008	0.25
QD-trisNTA(5%)	0.093	0.54	0.007	0.46
QD-trisNTA(15%)	0.044	0.35	0.005	0.65

SUPPORTING FIGURES

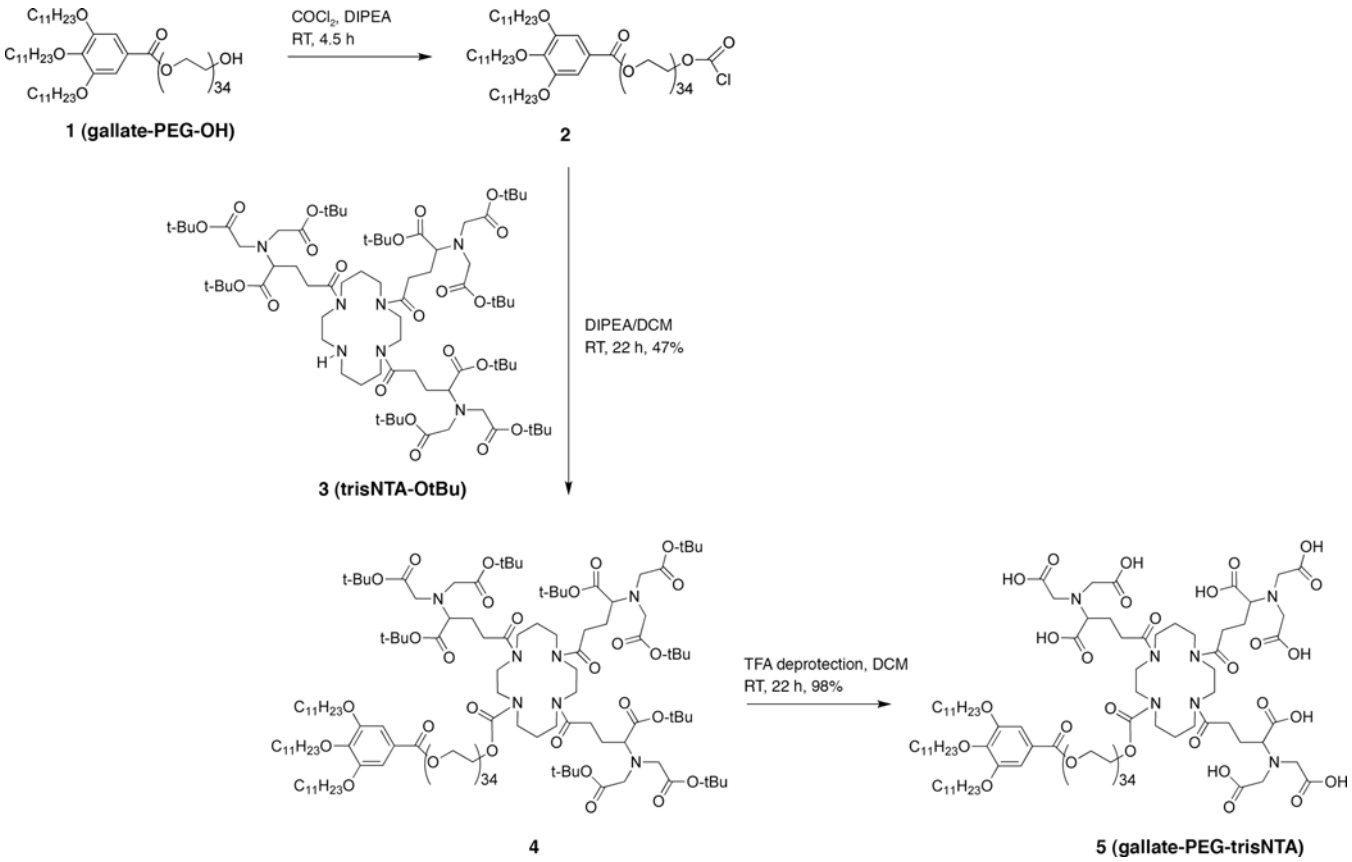

Figure S1. Scheme for the chemical synthesis of gallate-PEG-trisNTA.

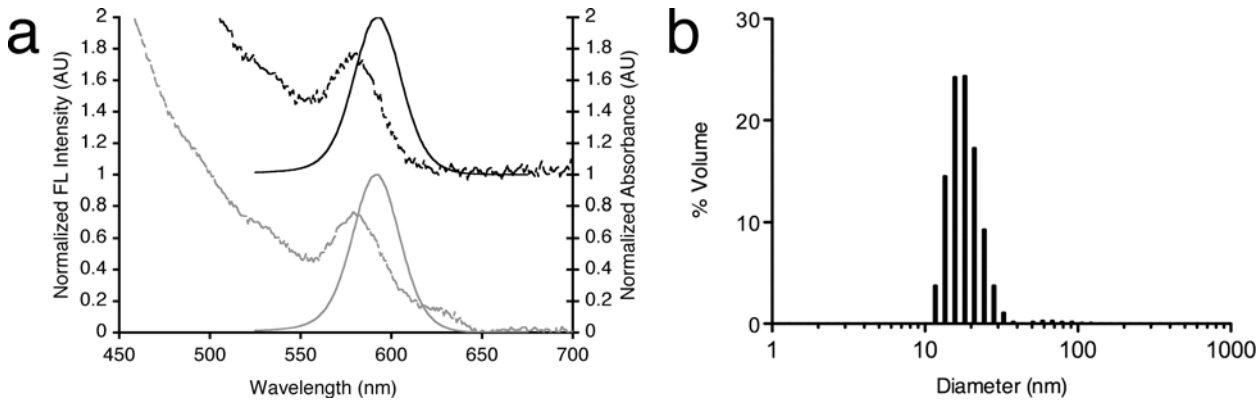

Figure S2. Characterization of QD-trisNTA spectra and size. Data is for QD-trisNTA(15%), however results were similar for other trisNTA densities (from 1% to 10%, data not shown). (a) Absorbance (dashed lines) and emission spectra (solid lines) of the hydrophobic QDs in toluene (gray) and QD-trisNTA (black) following encapsulation in the micelle and dispersion in aqueous buffer. (b) DLS measurement of the size distribution of QD-trisNTA in aqueous buffer following purification. The size distribution is represented as a percent volume; a primary peak centered at ~18.4 nm and representing greater than 99% of the volume distribution is assigned to QD-trisNTA.

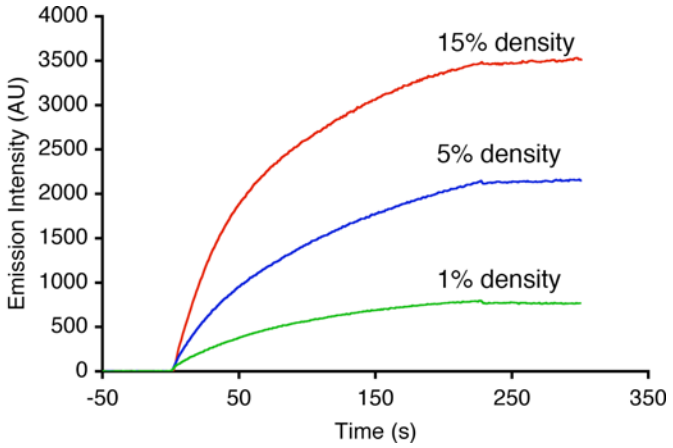

Figure S3. TIRFS-based binding assay of QD-trisNTA towards polyhistidine. The association of 10 nM QD-trisNTA containing 15% (red), 5% (blue) and 1% (green) trisNTA density to an H6 functionalized surface.

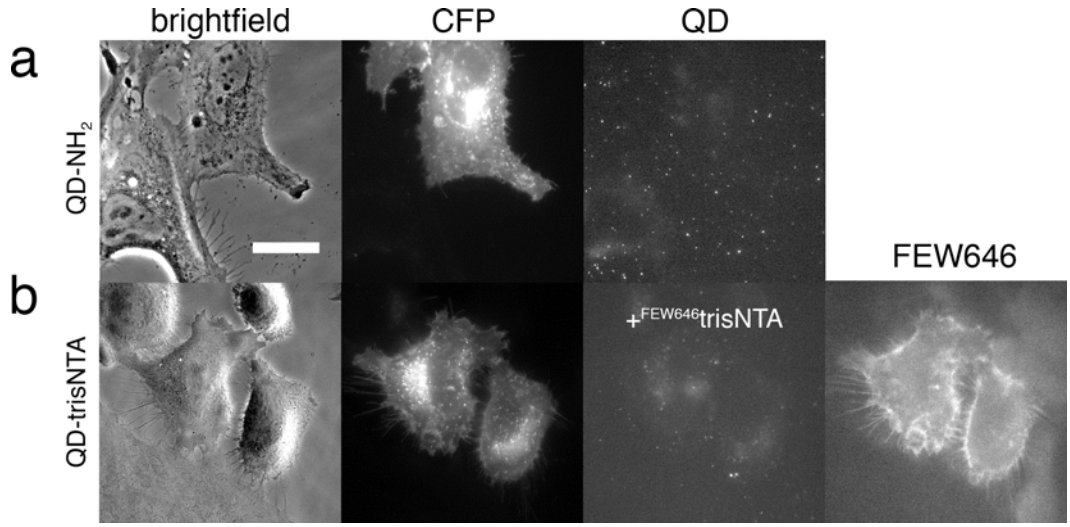

Figure S4. Control and competition experiments with QD-trisNTA and H10-CFP-TM expressed in HeLa cells. (a) Control experiment with QD-NH_2 functionalized with 10% NH_2 groups in lieu of trisNTA and loaded with Ni^{2+}. (b) Competition experiment with QD-trisNTA(15%) and a 1200 times molar excess of ^{FEW646}trisNTA. Scale bar=25 μm.

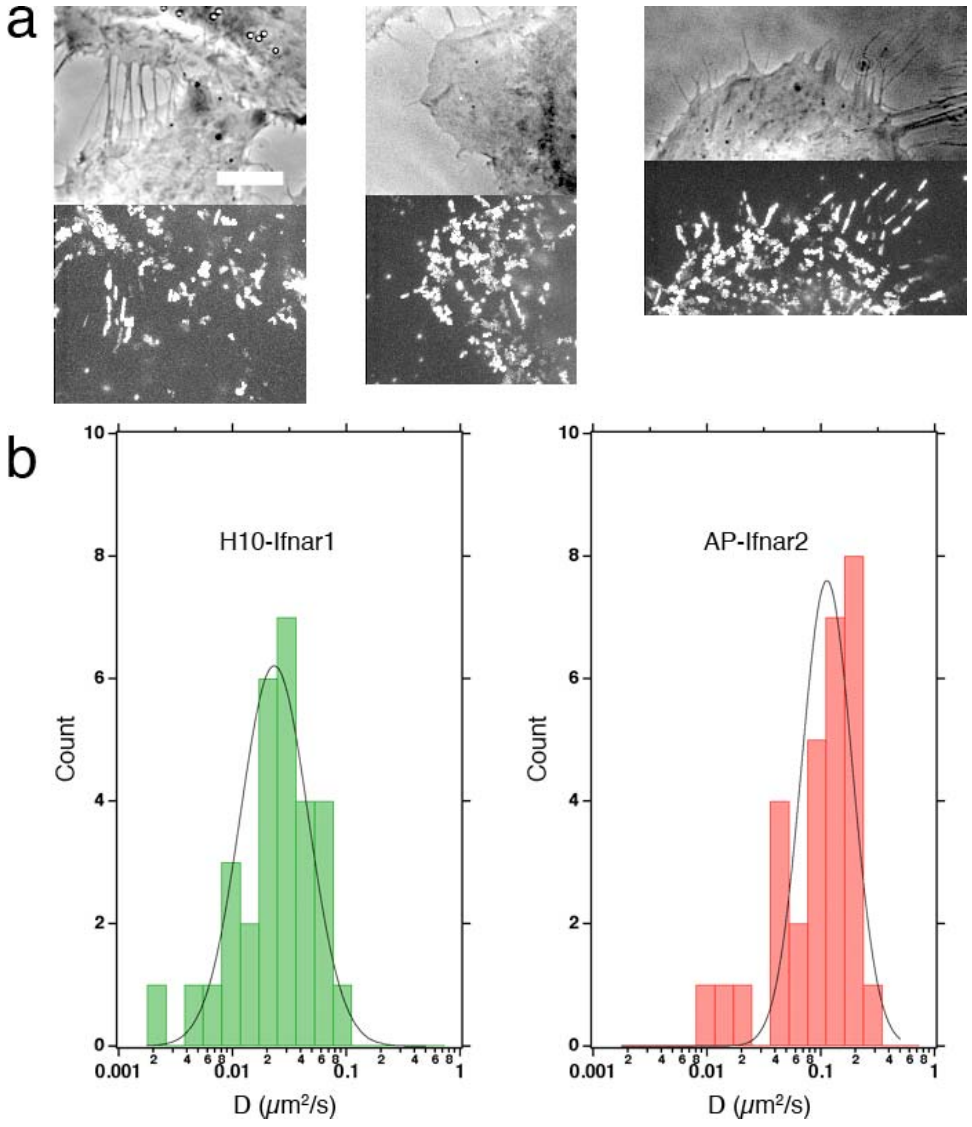

Figure S5. Tracking of membrane proteins using QDs. (a) Lateral motion of QD-trisNTA(15%) bound to H10-CFP-TM. Selected brightfield (top) and QD channel (bottom) images. In the QD channel, the maximum intensity of the image series has been projected over 30 seconds of acquisition (100 ms exposure) to shown the trajectories of the diffusing QD-trisNTA. Scale bar=10 μm. (b) Histograms of the diffusion coefficients (D, n=30) for H10-Ifnar1 (green) and AP-Ifnar2 (red). Diffusion values were estimated by fitting the histogram to a Gaussian function and standard errors (SE) were determined using a 1000 bootstrap replica of the distributions[13]. For Ifnar1, $D=2.32\times10^{-2}$ $\mu m^2/s$ (SE: 1.97×10^{-2} – 2.74×10^{-2}) and for Ifnar2, $D=1.13\times10^{-1}$ $\mu m^2/s$ (SE: 0.95×10^{-1} – 1.34×10^{-1}).

SUPPORTING REFERENCES

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