Supporting Information

Two-Photon 3D FIONA of Individual Quantum Dots in an Aqueous Environment

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Materials and Methods

Flow chamber and *in vitro* QD samples: A glass slide (Fisher Scientific) with two holes was drilled at 1.5 – 2 cm separation. A glass coverslip (Fisher Scientific) with two strips of double-stick tape was added to form a flow chamber, followed by 5 Minute Epoxy (ITW Devcon) to seal the side of the flow chamber. 100 μL of 1 mg/mL BSA-biotin in pH 7.0 Phosphate Buffered Saline (PBS) buffer was flowed over the coverslip and allowed to sit for 10 min at room temperature (RT), and then washed with 60 μL PBS buffer. 100 μL of 100 pM QDs (525/565/585/605/655 nm, Invitrogen) in PBS buffer was flowed into the chamber and incubated at RT for 10 min. To photostabilize the QDs, 100 μL 100 mM DTT (or 10% 2-*mercaptoethanol*, or other reductants at various concentrations as tested in experiments) was flowed through the chamber to wash away non-immobilized QDs. Otherwise, PBS buffer was used.

E. coli cells: *E. coli* cells picked from a single colony on an agar plate were grown at 265 rpm rotation and 37°C water bath overnight in 2 mL LB media in a 14 mL Falcon tube with 34 μg/mL antibiotics chloramphenicol. The next day 25 μL incubated cells were taken and added into 5 mL LB medium with antibiotics and 100 μM isopropyl-β-D-galactopyranoside (IPTG). It was then grown at 37°C in water bath and 265 rpm rotation until the OD of the cell media reached 0.5 – 0.6. Cells were centrifuged in a 15 mL Eppendorf tube at 1000 rcf for 5 minutes, and the pellet was resuspended in 1 mL pH 7.0 PBS buffer. 100 μL of suspended *E. coli* cells in PBS buffer were then added to an appropriate volume of QD-streptavidin conjugates (Invitrogen), and they were incubated in RT for 10 min. Different kinds of QDs (525/565/605/655 nm or a combination) were selected as needed. 10 nM QDs were used to get dense labeling, while 1 nM or 100 pM were used to obtain single QD labeling. Labeled cells were centrifuged at 1000 rcf for 5 minutes and then washed by resuspending the pellet with 200 μL of pH 7.0 PBS buffer and repeated 3 to 5 times.

Breast cancer cells: MDA-MB-468 cells were cultured in L-15 medium (PAA, Farnborough, UK) supplemented with 10% fetal calf serum. Cells were plated onto sterile glass cover slips, fixed with 4% (w/v) paraformaldehyde for 15 min at RT before being treated with sodium borohydride (1 mg/mL in PBS, pH 7.4) to reduce free aldehyde groups, and blocked with 1% bovine serum albumin containing 100 nM streptavidin for 30 min at RT. Subsequently, cells were stained for 45 min at RT in the dark with 1 nM pre-formed EGF-QD complexes, which were prepared using EGF-biotin and a streptavidin-QD655 conjugate (both from Invitrogen, Paisley, UK) as described earlier[26]. Cells were washed five times with PBS before being mounted in CyGEL (Biostatus Limited, Leicestershire, UK).

Wide-field, and single-point scanning 2-photon microscope: The 2-photon widefield microscope was basically the same as a 1-photon widefield microscope, except a wavelength-tunable IR laser, Mai Tai HP (SpectraPhysics), was used as an excitation source instead of visible lasers (see SM1). Briefly, the microscope was based on an Olympus IX-71 inverted microscope. The laser was directed by dielectric mirrors (BB1-E03 and BB2-E03, Thorlabs) and focused by a 300 mm focus length (fl) lens (AR-coated at 650-1050 nm, Thorlabs) into the back aperture of a 100× 1.45 numerical aperture (NA) achromatic objective (Olympus) and collimated through it. A 60×1.2 NA achromatic lens (Olympus) was also used for excitation beyond 900 nm due to its higher transparency to IR laser. For the single-point scanning setup, one 60 mm fl lens and one 300 mm fl lens (AR-coated at 650-1050 nm, Thorlabs) were used to expand and collimate the IR laser beam. The laser was focused to a diffraction limited excitation spot. A pair of motorized mirrors (ISS Inc., Champaign, IL) and a piezo stage (ISS) under the objective were used to scan the laser in x-y and z-axes, respectively; they were synchronized by an ISS dual clock module. The scan could run at various step sizes (typically at 50 nm, 64 nm or 100 nm), up to 100 μm scan range. A 725 nm short-pass or a 650 nm short-pass dichroic (Chroma) was used in the microscope. Emission filters such as HQ610/130M, HQ535/50M and ET750SP (Chroma) were selected according to QD emission spectra and inserted into the microscope to filter out excitation laser. For multicolor imaging, a QuadView tube or a DualView tube (Photometrics) was added after the microscope, and

FF01-525/565/605/655/15-25 single band emission filters (Semrock) were included in the tube for the four colors of QDs. Images were recorded with an EM gain CCD camera (iXon+, Andor) or a Photomultiplier Tube (H7421-40, Hamamatsu). Details can be found in the next section.

Holographic matrix scanning microscope: The excitation laser spots were generated by a diffractive beam splitter, here called the Holographic Matrix (HM) (Holo/Or Ltd.). The HM was able to split the beam to be a 9 × 9 matrix with 0.09° separation. The line separation could be adjusted to 1.5 μm by adding an extra pair of 4f lenses with 0.4× magnification to the scanning beam path. The matrix scan shared the same beam path with the single-point scan, though the images could only be taken by EMCCD camera. Since the matrix scan is a kind of parallel scan, the scan mirrors need only to move over 1.5 μm × 1.5 μm to cover a 13.5 μm × 13.5 μm area, an 80-fold increase in imaging rate. The beam splitter worked optimally with 785 nm laser.

Matrix uniformity and calibration: The power distribution of excitation matrix spots is quite uniform, with the s.d. equal to 6%. The corresponding maximum fluorescence variation is ~14%. The effect of intensity variation on the accuracy of FIONA is negligible: since the localization accuracy is approximately proportional to the reciprocal of the square root of the total photon number, at most 14% of intensity variation would result in 6% variation of localization accuracy, i.e. 0.6 angstrom. Nevertheless, if correction is desired, we can calibrate the intensity by the fluorescence of the matrix on a dense and uniform film of QDs (fig.1b). Similar images are taken multiple times at different sample positions and averaged to diminish any effect of sample inhomogeneity. Intensities of fluorescent spots in the data images is then corrected by the normalized factors from the fluorescence intensity of each corresponding focal point of the matrix.

Excitation Spectra: We took widefield images of 1 μM QDs which densely covered the imaging area of the glass surface. The sample was made by dropping 4 μL 1 μM QD onto a glass slide and then clamped by an 18 mm × 18 mm cover slip and sealed by 5 Minute Epoxy. We varied the excitation laser wavelength and recorded the excitation power and

the emission intensity. To correct the excitation spectra, we obtained the emission intensity per unit excitation power by $Em_0 = Em/Ex^2$, where the Em is the original emission intensity.

***E. coli* and breast cancer cells imaging.** Labeled living *E. coli* cells were self-adhesive to the glass surface, and sealed between a coverslip and a slide. Fixed labeled breast cancer cells were mounted in CyGel and sealed between coverslip and slide. The samples were excited by a diffraction-limited excitation spot (single-point scan) or a multi-point HM scan. Laser power was tuned to ensure enough emission intensity while keeping excitation outside the diffraction limited spot to minimum. The scan range and step size were predetermined according to the need of the experiment, but most used scan step sizes were 50 nm, 64 nm, or 100 nm. Dwell time on each scan pixel was from 0.02 msec to 1 msec. Brightfield images of cells were also taken to show the profile of the whole cells.

Myosin V stepping assay and analysis: Myosin V was labeled on its cargo-binding domain with a 655 nm QD via anti-GFP antibody. F-Actin was polymerized at 1:20 biotinylation ratio (1 biotin/20 actin monomers) and immobilization onto glass coverslip surface via BSA-biotin-NeutrAvidin (Invitrogen)[9]. Myosin V was then flowed into the sample chamber, excess myosin washed away, and a solution containing 1 μM ATP and 100 mM DTT was added. The sample was excited by 2-photon widefield, at 840 nm and 300 mW, and the fluorescence emission was imaged onto the EMCCD camera. The images were taken at 30 msec exposure time, 10 MHz readout rate, 5.2× pre-gain and 40 EM gain. With 250× total magnification (100× objective plus 2.5× additional tube lens), the effective pixel size was 64 nm. Detailed description about 2D-FIONA can be found in Yildiz et al.[9]. Localization accuracy is the standard error of the mean of the center of the Point Spread Function (PSF). The step size was determined by student's t-test.

3D FIONA data analysis: We obtain 3D FIONA either by 3D Gaussian fitting for the whole scan image PSF or by applying 2D Gaussian fitting in x-y image and virtual x-z/y-z images. 3D Gaussian fitting is described in the article. Getting x-y location (x_0, y_0) with

nanometer accuracy could also be done by applying standard 2D FIONA on the PSF in the brightest z slice. And, to accurately localize the z coordinate of the 3D spot via x-z or y-z PSF fitting, we established a virtual x-z plane by extracting intensities on all pixels at a constant y_0. Similarly, a y-z PSF could be drawn. Moreover, we established a z-only one-dimensional PSF by extracting all z pixels at the constant point (x_0, y_0). Then we fit x-z and y-z PSF to 2D Gaussian, or z-only PSF to 1D Gaussian. Residuals of 3D and 2D Gaussian fitting were plotted and calculated. They showed the fittings to be excellent. All analysis was run by in-lab written programs in IDL or MatLab. Analysis of variance (ANOVA) showed no significant difference in the z localizations determined via the three kinds of PSFs above, as expected from theory.

Supplementary Figures

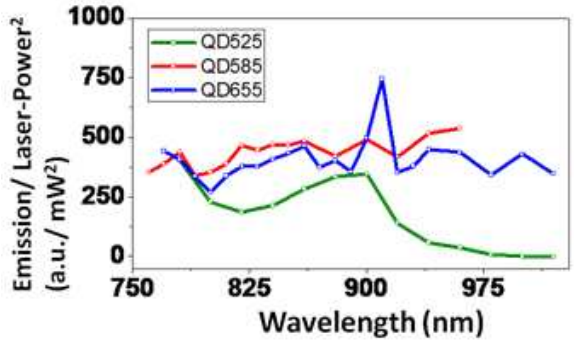

Figure S1. Excitation spectra of three kinds of QDs whose emission peaks are at 525 nm, 585 nm and 655 nm, respectively. For one-photon excitation, see Larson et al.

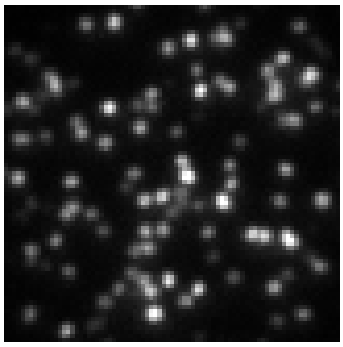

Figure S2. Individual QD655 excited by TIR 2P excitation. Taken with EMCCD at 29 ms and 100 EM gain. 50 mM DTT.

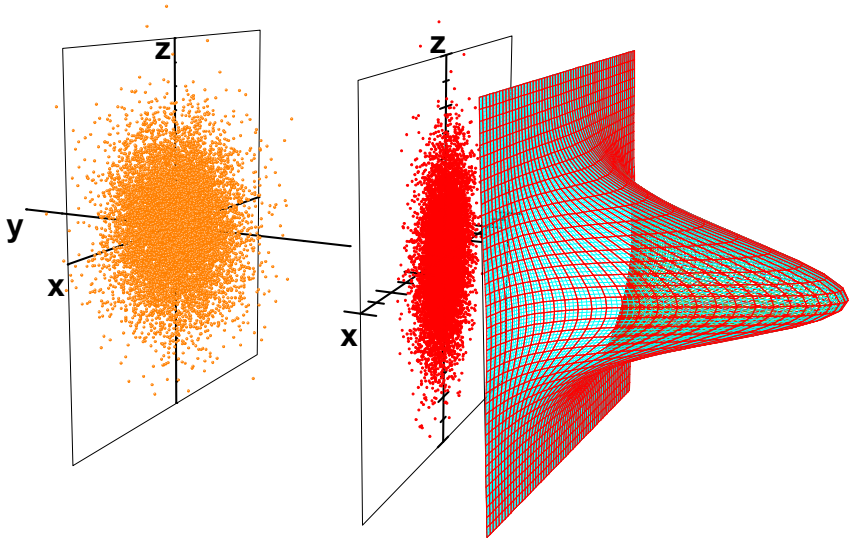

Figure S3. Illustration of emitted photon distribution of a single Qdot in 3D space (left), its projection on x-z plane (middle) and the Gaussian Fit of the drawn virtual 2D x-z image.

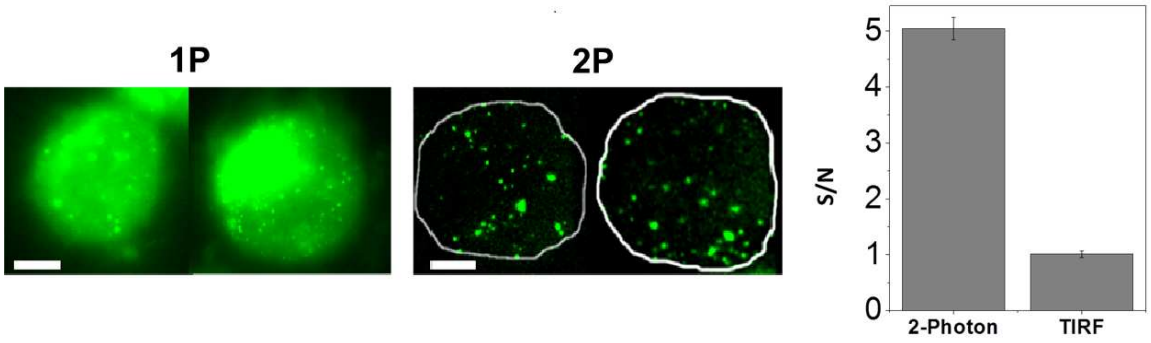

Figure S4. Comparison of the Signal to Noise (S/N) ratio of the same sample of breast cancer cells with EGFR labeled QDs, imaged using two excitation methods: 1P TIRF and 2P spot scanning. For 1P TIRF imaging the QDs emission was masked by cells' membrane auto-fluorescence, while with 2P imaging QDs were clearly visible with minimal to no auto-fluorescence. S/N was calculated as the ratio of the mean intensity of observed QDs on the cell to the mean intensity of the whole cells, calculated for ten cells.

Supplementary Movie 1. Widefield 2P excitation of single QD 585 nm. In PBS buffer. Average laser power 65 kW/cm^2 at 800nm. Movie taken at 30 msec/frame.

Supplementary Movie 2. Holographic 2P scan 3D image of a breast cancer cell. Cells were treated with 4 nM EGF-QD605nm conjugates and then fixed. EGFR endosomes are clearly seen. Scan pixel residence time 0.2 ms, frame time 45 ms.