# Supporting Information

# Small Quantum Dots Conjugated to Nanobodies as Immunofluorescence Probes for Nanometric Microscopy

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## Contents

## Co-localization

Each color is imaged on one half of an EMCCD. The two halves must be brought together (i.e. co-localization) and also corrected for any chromatic aberration of the detection system. We used nanoholes (SI Fig. 2a) to obtain the mapping transformation between the two channels, as well as to correct achromatic aberration[1,2]. Two metrics[3–5] were used to estimate the error associated with the mapping transformation $f_{LWM}\{x\}$. One was fiducial registration error (FRE),

$$FRE = \left(\frac{1}{N}\sum_{i=1}^{N}\left(x_{i,1} - f_{LWM}\{x_{i,2}\}\right)^2\right)^{1/2}$$

and the other was target registration error (TRE),

$$TRE = \left(\frac{1}{N}\sum_{i=1}^{N}\left(x_{i,1} - f_{i,LWM}\{x_{i,2}\}\right)^2\right)^{1/2}$$

where N is the number of nanoholes used for mapping. Both FRE and TRE are common measures of fiducial misalignment[3,5]. However, TRE also depends on the fiducial configuration (i.e. the arrangement of fiducial points) and thus is expected to be a better metric[3–5]. As shown in SI Fig. 2b and c, the FRE is ~ 0.4 nm and the TRE is ~ 1.1 nm, three times better than that obtained by using a fluorescent bead and moving the microscope stage [4], or using non-uniform nanoholes [2]. (Presumably the improvement is due to the uniformity of the nanoholes used in this study).

## Localization Precision

Instead of localizing the spots using FIONA directly, we performed super-resolution analysis using an algorithm based on QD-blinking for both channels[6,7]. When a QD blinks (on→off, or off→on), subtraction between adjacent frames (before and after the transition) gives the point spread function (PSF) of the blinking QD. The isolated PSF can then be used to localize the blinking QD precisely. An advantage is that the super-resolution algorithm allows us to have multiple localization events for the same sQD-GBP and thus to reduce errors in distance calculations. We characterized the precision of localization events during the super-resolution analysis by $p_{x,y} = \sqrt{\frac{\sigma_{x,y}^2}{N} + \frac{a^2}{12\,N} + \frac{8\pi\sigma_{x,y}^4 b^2}{a^2\,N^2}}$, where N is the photon count, $\sigma$ is the standard deviation of the fluorescent spot, a is the pixel size of the imaging detector, and b is the standard deviation of the background[8,9], and found that the localization precisions in x and y directions reached ~ 2.0 ± 0.5 nm (Mean ± SD, Fig. 3b and c). We note that a more theoretical accurate formula[2,10], $p_{x,y} = \sqrt{\frac{16}{9}\,\frac{\sigma_{x,y}^2 + a^2/12}{N} + \frac{8\,\pi\left(\sigma_{x,y}^2 + \frac{a^2}{12}\right)^2 b^2}{N^2 a^2}}$, gave ~ 2.7 nm for the localization precisions.

## Experimental Details

**Preparation of sQD-GBP conjugates.**

*Expression and purification of GBP.* GFP binding protein (GBP) was cloned into a pET28a (generous gift from V.I. Gelfand) and expressed and purified as described by Rothbauer et al.[11]. In brief, GBP was expressed in E. coli BL21 cells for 3 h at 37 ℃. Cell lysis was performed using lysozyme (1mg/ml) followed by sonication. Cell lysates was cleared by centrifugation. GBP was purified from the supernatant by nickel affinity chromatography.

*Preparation of carboxylated sQD.* The 580 nm and 620 nm organic CdSe/ZnS QDs were purchased from NN-Labs (Fayettiville, AR). The QDs were first mixed with a mixture of 97.5% PEGylated alkanethiol (HSC11(EG)4-OH) and 2.5% carboxyl PEGylated alkanethiol (HSC11(EG)4-COOH) (commercially available from Sigma-Aldrich [St. Louis, MO] and ProChimia Surfaces [Gdansk, Poland]) in $H_2O$ (degassed, 400 μL)/toluene (400 μL) with tetraethylammonium hydroxide (TEAH; 20% wt in $H_2O$) as base. The mixture was heated to 60 ℃ for 4 hours under nitrogen ($N_2$). During the process, the transfer of QDs from organic phase into aqueous phase was observed by examining the phases for fluorescence under UV light. The mixture was washed with chloroform ($CHCl_3$) three times (400 μL, 200 μL, 200 μL). The aqueous solution was then passed through a self-packed DEAE anion exchange column (GE Healthcare, 17-0709-10). The neutral QDs were primarily washed off with water first. The negatively charged QDs were washed off with 30 mM PBS to get the COOH functionalized QDs (QD-COOH) in aqueous solution.

*Conjugation of sQD to GBP*. Carboxylated sQDs can then be conjugated to streptavidin or GBP nanobodies via EDC-coupling as described previously[12]. In brief, 150 μL of QD-COOH solution (400 nM) was mixed with 15 μL of streptavidin solution and 3 μL of a fresh EDC solution and stirred for 2 h at room temperature. Free streptavidin was removed by a 100 kDa cutoff centrifugal filter unit (Amicon Ultra UFC510024). All sqdot solutions were filtered from aggregates using centrifugal filter units (0.2 μm, Pall Life Sciences, ODM02C34) and stored in PBS buffer at 4 ℃.

*Gel electrophoresis of sQDs and sQD-GBPs*. Both sQD-GBP and unconjugated (carboxylated) sQD samples were first mixed with 6X loading buffer and then loaded to a 1% agarose gel. The gel was run for 30 minutes at a constant voltage (100V), with running buffer of 10mM sodium phosphate at pH 8.0. The gel was illuminated with a UV illuminator (Spectronics Corp, New York, USA) and documented with a CCD camera (Apple, California, USA).

*Characterization of the size and morphology of sQD-GBPs by TEM*. High TEM was used to characterize the size and morphology of sQD-GBP conjugates. The sQD-GBP conjugates were loaded on an ultrathin carbon film TEM grid (Ted Pella Inc, California, USA) and imaged on a JEOL 2010 LaB6 high resolution TEM (JEOL USA Inc, Massachusetts, USA) operating at 200kV.

*Size measurement of sQDs and sQD-GBPs.* Dynamic light scattering (DLS) was employed to measure the sizes of sQDs and sQD-GBP conjugates. Measurements were carried out with concentrated samples (>100 nM), after filtering through an Antontop 10 (0.02 μm, Whatman GmbH, 6809-1102) syringe filter before measurements on a Marvin Instrument Ltd. nano-ZS Zetasizer. Each trace for autocorrelation was acquired for 15 s, and averaged over 12-15 runs per measurement. The autocorrelation function was analyzed using Zetasizer software (Ver. 7.02, Malvern Instruments Ltd.). Each DLS measurement resulted in an average QD diameter with a standard error of the mean. Finally, the size of sQDs was obtained by fitting the 25-30 DLS measurements to a Gaussian function.

**Application of sQD-GBP to observe the walking of kinesin-1.**

*Labeling of K560GFP and preparation for imaging.* K560GFP was labeled with a five-fold excess of the GBP coated sQDs for 30 minutes on ice and used without subsequent purification. Flow chambers were prepared by sticking several pieces of double sided scotch tape on a glass slide, which was then sandwiched with a PEG/PEG-biotin coated glass cover slip on top. The resulting chamber had the approximate dimensions of 20 mm x 5 mm x 0.2 mm and an approximate volume of 20 µl. A solution of 0.5 mg/ml neutravidin in BRB-10 containing 10 mg/ml BSA was flown into the chamber and incubated for five minutes. After a subsequent wash step with BRB-10 BSA, a praclitaxel-stabilized solution of biotin-microtubules (approx. 25 nM for the final concentration of tubulin) was flown into the chamber and microtubules were allowed to bind for up to ten minutes. Unbound microtubules were washed out with buffer containing 20 µM praclitaxel.

*Image acquisition and data analysis.* The final imaging buffer contained 10 mg/ml BSA, 1 mM ATP (Adenosine 5′ -triphosphate magnesium salt, Sigma), 10 mM bMPA (Beta-mercapto propionic acid, Sigma), 20 µM paclitaxel and an oxygen scavenging system consisting of 2.5 mM PCA (3,4-Dihydroxybenzoic acid, Fluka) and 50 nM PCD (Protocatechuate 3,4-Dioxygenase, Sigma). Images were recorded using an inverted Olympus Ixon 70 microscope with a 100× 1.45 numerical aperture objective (PlanApo 100×1.45 NA ∞/0.17) plus an extra 1.5× magnification coupled to an Andor EM-CCD camera (DV-897E-CS0). Micro quantum dots excited using a 532 diode (World Star Tech, 30 mW, model #TECGL-30) laser. Laser light was reflected into the objective by a triple band pass dichroic mirror (z488/532/633rpc, Chroma) and the emission light was filtered by a z488/532/635m (Chroma) triple bandpass filter. Movies of a total duration of 110 s with an integration time of 0.05 s were recorded. Motor-coated beads were tracked using custom written IDT software. Steps were detected by students t-test as described previously[13].

**Application of sQD-GBP to measuring the size of Piezo1 channels.**

*Plasmid construction.* The HumanPiezo1 was inserted into multiple cloning region of the vectors CGFP-EU and NGFP-EU[14] which adds a his tag to either the C-terminal (CGFP_EU) or to the N-terminal (NGFP_EU). To insert human PIEZO1 into CGFP_EU, we amplified the gene from the vector IRES2-EGFP HEK Piezo1[15] using the following primers:

```
CGFPHP15'Hind III   gagctctcgagaagctatggagccgcacgtgttgg
CGFPHP13'EcoRI      cgtcgatgcagaattctgcagaattctgctccttctcacg
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For insertion into the vector NGFP-EU the following primers were used:

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NGFPHP15′HindIII   cgcgcggctctcgagaagctatggagccgcacgtgttgg
NGFPHP13′BamHI     tatctagatccggtggatcctacctccttctcacgagtcc
```

PCR was done using Primestar GXL polymerase (Clonetech). 1ng of IRES2-EGFP HEK Piezo1 template was amplified with respective primers with the following protocol: 98 $C^o$ for 10 sec, 55 $C^o$ 15 sec., and 68 $C^o$ 8 min. PCR products were gel-purified by QIAkit (QIAGEN) according to manufacturer's specification. Vectors CGFP-EU and NGFP-EU were digested with HindIII and EcoRI (CGFP-EU) and HindIII and BamHI (NGFP-EU), respectively with restriction endonuleases (New England Biolabs) and then gel purified. The vectors and inserts were combined using Infusion HD enzyme (Clontech) according to their protocol. 2.5ul of Infusion reaction was transformed into 50ul Stellar Competent Cells (Clonetech) and grown overnight at 37C. Colonies were screened for the insertion by restriction digest. Plasmids of Piezo1 with GFP and his-tag fused at the N- or C- terminals were denoted by pHEKP1-TS-CGFP-His, pHis-NGFP-TS-HEKP1.

*Preparation and labeling of Piezo1 channels.* HEK293 cells were transfected with plasmids pHEKP1-TS-CGFP-His or pHis-NGFP-TS-HEKP1, and allowed for expression of Piezo1

proteins for 18 hours. Cells were then harvested into lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 10 μg/ml leupeptin, 6 mM n-dodecyl-B-D-maltoside (DDM)) and incubated for 30 minutes. The cell lysis was then spun at 600g for 10 minutes and supernatants were collected. The cell lysis was mixed with sQD580-GBP and sQD620-GBP on ice for 1 hour. The mixture was diluted 10 times in lysis buffer and ready for use.

*SimPull of Piezo1 channels.* The flow chambers were prepared on PEG passivated glass coverslips doped with 5% biotinylated PEG. Neutravidin (Thermo Scientific) at 0.5mg/ml in T50 (10mM Tris-HCl pH 8.0, 50mM NaCl) was introduced and incubated for 5 minutes, followed by washing the flow chamber by adequate T50 buffer. Biotinylated anti-his antibody (Penta-His Biotin Conjugate, Qiagen) at 2 μg/ml in T50 was added and incubated for 10 minutes. After washing the chamber with adequate lysis buffer, the Piezo1 sample prepared above was added and incubated for 5 minutes, followed by another thorough wash using T50 buffer.

*Super-resolution and SHREC analysis of sQD-GBP on Piezo1 channels.* An objective-type total internal reflection fluorescence (TIRF) microscope was used to acquire single molecule data, with a 532 nm laser (TECGL-30, World Star Technologies) for excitation. In the emission path, an Optosplit (Cairn Research) with an appropriate dichroic mirror (FF593-Di02-25x36, Semrock) and filters (HQ625/30 and D585/20, Chroma Technology) was inserted to observe both sQDs of different colors simultaneously in two parallel channels. Piezo1 channels prepared as described above were imaged for 2000 frames. Before performing imaging experiments on Piezo1 channels, nanoholes of 100nm in diameter and 1.5 μm apart from each other were used as fiduciary markers to map the green channel (~580nm) onto the red channel (~620nm) and a mapping matrix was generated by localization of every spot on the illumination area according to the algorithm in Goshtasby et al. The acquired images were analyzed using a super-resolution technique, gSHRImP [6], to get the localizations of sQDs. The localizations of sQDs in the red channel were then transformed to the green channel.

*Calculation of the sizes of Piezo1 channels.* As a quantum dot might be localized for several times, the center of mass (CM) of the multiple localizations from the same sQD was calculated and used as the position of the sQD. The distance ($\Delta_{NN}$, or $\Delta_{CC}$) between the CM of sQD580-GBP localizations and the CM of sQD620-GBP localizations in the same cluster (with diameter of < 200 nm) was calculated. The distribution of $\Delta_{NN}$, or $\Delta_{CC}$, was plotted and fitted with two Gaussians (one corresponding to the side length of Piezo1 tetramers and the other corresponding to the diagonal length): $f(x) = A_1 e^{-\frac{(x-\mu)^2}{2\sigma_1^2}} + A_2 e^{-\frac{(x-\sqrt{2}\mu)^2}{2\sigma_2^2}}$.

**Application of sQD-GBP to measuring AMPAR on neurons.**

*Neuron culture and labeling.* Primary cortical cultures were prepared from E18-19 rats according to university guidelines as previous described[16] with the following modifications. Neurons were dissociated in 3 mg/mL protease and plated on 18 mm coverslips coated with 0.1 mg/mL poly-L-Lysine. Neurons were cultured at 37 ℃ with 5% $CO_2$ in neurobasal media with B-27 supplement, 2 $\mu$M glutmax and 50 unit/mL penicillin and 50 unit/ml streptomycin. On 11-13 days in vitro (DIV), neurons were transfected with GluA2-pHluorin (0.4 μg/coverslip) by Lipofectamine 2000 transfection reagent (Life technologies). At 24 - 48 hours after transfection, the coverslips were transferred to warm imaging buffer (HBSS supplemented with 10 mM Hepes, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 2 mM D-glucose) for 5 min incubation and mounted onto an imaging dish (Warner RC-41LP). In the imaging dish, neurons were incubated for 5 min at 37 ℃ in imaging buffer containing QDs (1-2 nM final concentration) and casein (~80 times dilution, stock solution purchased from Vector labs, SP-5020) following by washing with 10 ml of imaging buffer. The

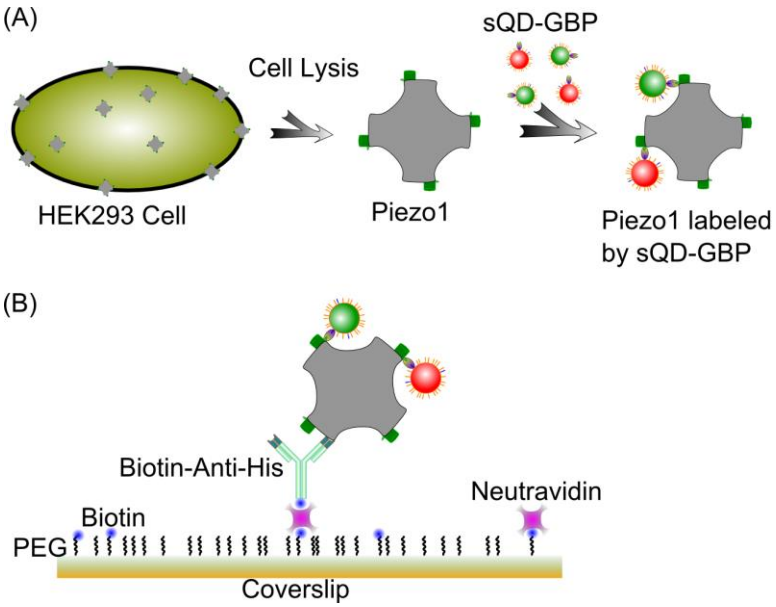
imaging dish was subsequently mounted on the sample stage of the microscope with 1 mL of imaging buffer.

*Optics and imaging.* A Nikon Ti Eclipse microscope with a Nikon APO 100 X objective (N.A. 1.49) was used for fluorescence imaging. The microscope has the Perfect Focus System, which stabilizes the sample in z-axis. An Agilent laser system MLC400B with 4 fiber-coupled lasers (405 nm, 488 nm, 561 nm and 640 nm) was used for illumination. A back illuminated EMCCD (Andor DU897) was used for recording. For fluorescence imaging, a quad-band dichroic (Chroma, ZT405-488-561-640RPC) was used and band-pass emission filters (525/50 and 641/75) were used.

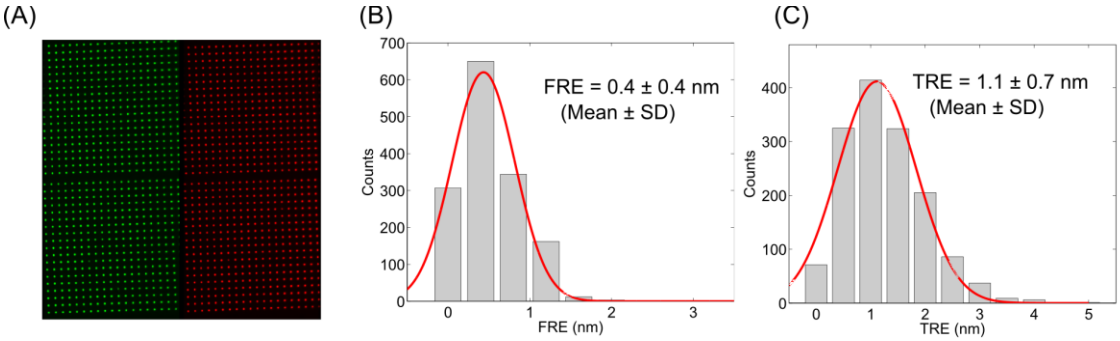
*Tracking AMPAR receptors.* The sample was first examined under bright-field and only coverslips with healthy neurons were used for experiments. After focusing on the sample, the Perfect Focus System was activated to minimize the sample drift in z direction. The samples were then scanned in the GFP channel (488 excitation, 525/50 emission) to locate transfected cells. A fluorescent image of the cells was taken for reference. To track the QD labeled receptors, 561 nm lasers was used for excitation in the Epi-fluorescence mode[17] with an band-pass filter (641/75) for collecting the fluorescence. Movies of 1000 frames were acquired with 50 ms exposure time.

*Data analysis.* QuickPalm[18], an ImageJ plug-in was used for analyzing single particle tracking data. Centroids of the all the QDs in x- and y- direction were localized in all the frames and a map of all the places QDs visited were obtained. A Matlab code was used to recover the trajectories of the QDs. In brief, the code finds locations of QDs in time t, and searches for nearby QDs in time t+1 as the next point on the trajectory. In the single particle tracking experiment, the maximum displacement of a QD in one time step is set to be 1 μm. The diffusion coefficients from the trajectories were calculated in Matlab by fitting the first 4 points of the mean-square-displacement curve of the trajectory.

## SI Figures and Figure Legends




SI Figure 1. Measurement of the size of Piezo1 using sQD-GBP by SimPull and SHREC. (A) HEK293 cells were transfected with plasmids for GFP-fused Piezo1 proteins, and allowed for expression of Piezo1 overnight. The cells were lysed with lysis buffer contain DDM, producing a solution of Piezo1 proteins. The solution is then mixed with μQD-GBP of two colors to allow labeling of Piezo1 channels. (B) Labeled Piezo1 proteins are immobilized on a coverslip through a lay-by-layer fashion. The surface of the coverslip is functionalized with PEG and biotinylated PEG to facilitate the binding of neutravidin, on top of which biotinylated anti-his antibodies bind. The anti-his antibodies bind to Piezo1 (with a his-tag) and immobilize the channels on the surface for later imaging.




SI Figure 2. Mapping for co-localization. (A) Nanoholes are 100 nm in diameter and 1.5 μm apart from each other. They are utilized as fiduciary markers to map the green channel (~580 nm) onto the red channel (~620 nm). A mapping matrix was generated by localization of every spot on the illumination area. (B) Distribution of the fiducial registration error from the mapping matrix gives a mean value of 0.4 ± 0.4 nm (Mean ± SD). (C) Distribution of the target registration error from the mapping matrix gives a mean value of 1.1 ± 0.7 nm (Mean ± SD).

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