Supporting Information

for

High-Precision Tracking with Non-Blinking Quantum Dots Resolves Nanoscale Vertical Displacement

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ONLINE METHODS

SA-TIRF microscope. The apparatus was built around a Nikon Optihot-2 microscope utilizing a Plan Fluor 100×/NA 1.3 objective and Andor iXon^{EM}+ 897 camera (Belfast, Northern Ireland; 512 x 512 imaging array, 16 μm x 16 μm pixel size). The original microscope stage was removed and replaced with a Sutter MP-285 motorized high precision three-dimensional translational stage (Novato, CA). A homemade prism holder was attached to the Sutter stage, which held an equilateral Bk7 prism (Melles Griot, Albuquerque, NM). An adjustable 75-mW maximum 488-nm argon continuous wave (CW) laser (Uniphase, San Jose, CA) was used as the excitation source working at 20-mW output. A series of optics and motorized stages were set up to direct the laser path beneath the objective. First the laser beam was directed to a periscope and through a Uniblitz mechanical shutter (model LS2Z2, Vincent Associates, Rochester, NY). The beam then passed through a focusing lens (15-cm focal length) and on to the mirror of a galvanometer optical scanner (model 6220H, Cambridge Technology, Cambridge, MA). Two RazorEdge Long Pass 488 nm filters we placed in the optical path before the EMCCD.

The focusing lens was used to control the laser illumination size under the objective, which was adjusted to approximately 128 μm in diameter. This area corresponds to twice the width of the viewing area under the objective (64 μm x 64 μm) and allows for a less Gaussian energy distribution in the illumination area. The laser power at the surface of TIR was approximately 100 W/cm^2. The mirror galvanometer was use in conjunction with a high precision motorized linear stage (model MAA-PP, Newport, Irvine, CA) to direct the laser beam through the equilateral prism to the solid-liquid interface at different incident angles. An in-house software program was utilized to optimize the laser spot under the objective during various angles of incident light.

Non-blinking quantum dot (NBQD) and thin-shelled quantum dot (TSQD) synthesis[1]. **Materials.** Cadmium oxide (CdO, 99.998%), sulfur (S_8, 99.999%), trioctylphosphine (TOP, 90%) and oleic acid (90%) were purchased from Alfa Aesar; selenium (Se, pellets, ≥99.999%) and dioctylamine (98%) from Sigma-Aldrich; 1-octadecene (ODE) (90%) and oleylamine (80-90%) from Acros. Bis(2,2,4-trimethylpentyl)phosphinic acid (TMPPA) (CYANEX 272®) was obtained from Cytec Industries, Inc. Procedures were performed under dry inert gas atmosphere (N_2 or Ar) in a glove box or a Schlenk line. **Precursor solutions.** *0.16M TOPSe/ODE*. Se (71.4 mg, 904 μmol), TOP (577 mg, 1.56 mmol) and ODE (4.00 g, 15.84 mmol) were stirred and heated until optically clear. *2.1M TOPSe*. Se (144 mg, 1.82 mmol) and TOP (797 mg, 2.15 mmol) were stirred and heated until optically clear. *0.2M Cd(oleate)$_2$*. CdO (318 mg, 2.48 mmol), oleic acid (3.09 g, 10.94 mmol) and ODE (7.11 g, 28.16 mmol) were degassed under vacuum at 80°C for 60 min, refilled with Ar, and heated to 240°C until optically clear. *0.1M Cd(oleate)$_2$-amine.* Dioctyl-amine (12.5 mL, 41.36 mmol) was degassed under vacuum at 80°C for 30 min, refilled with Ar, and transferred into another flask containing 0.2M Cd(oleate)$_2$ (12.5 mL). The mixture was stirred at 60°C for 20 min. *0.1M S_8.* S_8 (79.0 mg, 2.47 mmol) and ODE (19.73 g, 78.13 mmol) were degassed under vacuum at 80°C for 30 min, refilled with Ar, heated to 180°C for 20 min until optically clear, and allowed to cool to room temperature. **CdSe cores.** *Small ca. 1.9nm±0.2nm CdSe cores.* CdO (15.0 mg, 117 μmol), TMPPA (304 mg, 1.05 mmol) and ODE (4.00 g, 15.84 mmol) were degassed under vacuum at 80°C for 30 min, refilled with Ar, and heated to 300°C for 6 h until optically clear. The solution was heated to 325°C and 0.16M TOPSe/ODE (5.7 mL, 904 μmol) swiftly injected. After ~5 s, the mixture was allowed to cool to room temperature. *Large ca. 4.7 nm ± 0.6 nm CdSe cores.* Dioctyl-amine (5 mL, 16.55

mmol) was degassed under vacuum at 80°C for 30 min, then refilled with Ar. 2.1M TOPSe (0.15 mL, 315 μmol) was added, and the temperature increased to 300°C. After 5 min, 0.2M $Cd(oleate)_2$ (1.5 mL, 300 μmol) was quickly injected and the temperature adjusted to 280°C. After 10 min, the mixture was allowed to cool to room temperature. Final CdSe cores were washed twice just prior to shell-growth by precipitation with 4:1 (v/v) acetone-methanol mixture and centrifugation at 4,200 rpm for 10 min. **CdSe/nCdS core/shells (n=1-20).** Freshly made CdSe was dissolved in hexane (5 mL, *ca.* 1.9nm cores) or toluene (5 mL, *ca.* 4.7nm cores). CdSe concentrations were determined from 1*S* peak at 490 nm (*ca.* 1.9nm cores) or 630 nm (*ca.* 4.7 nm cores) using updated extinction coefficients[2]. An aliquot containing 7.5×10^{-8} mol CdSe (10 μM initial concentration) was dried under vacuum at room temperature. ODE (4 mL, 12.5 mmol) and dioctyl-amine (3.8 mL, 12.57 mmol) were added. The mixture was degassed under vacuum at 80°C for 30 min, refilled with Ar, and heated to the necessary shell-growth temperature (*ca.* 1.9nm cores: 200°C 1-2 MLs, 230°C 3-6 MLs, 240°C 7-20 MLs; *ca.* 4.7 nm cores: 235°C 1-2 MLs, 245°C 3-20 MLs). Cd and S precursors were introduced in an alternating fashion using programmable syringe pumps, each followed by 15 min annealing or "wait" period. Cd precursor was injected first. The final mixture was allowed to cool to room temperature 15 min after last S injection. PL quantum yields (QY) were measured using well established procedures[3].

Non-blinking and thin-shelled QD modification. Crude CdSe(1.9nm)/5CdS (**TSQD**, diameter = 4.8nm±0.8nm, PL_{max} = 600nm, QY = 5%) and CdSe(4.7nm)/17CdS (**NBQD**, diameter = 14.5nm±1.6nm, PL_{max} = 660nm, QY = 8.2%) were transferred to deionized water via surface ligand exchange with dihydolipoic acid (DHLA) following published protocol[4], then washed once by precipitation with a 1:1 (v/v) acetone-methanol mixture and centrifugation at 4,200 rpm

for 10 min. DHLA modified NBQDs and TSQDs were diluted in 10 mM borate buffer and placed under sonication for up to 1 h to reduce clustering. Neutravidin was then added to the DHLA modified NBQD and TSQD borate buffer solutions and incubated at room temperature for 1 h.

Full-length kinesin motor proteins. Dr. Will Hancock from The Pennsylvania State University kindly provided us the BL21 (DE3) *Escherichia coli* bacteria with the full-length His-tagged kinesin plasmid. Isopropyl B-D-1-thiogalactopyranoside (IPTG) was used to express kinesin in the *E. coli.* Kinesin was purified on a Ni column according to the published protocol[5].

Microtubule preparation. Microtubules for NBQD studies were prepared in the following way. Taxol, GTP, and all tubulin were purchased from Cytoskeleton (Denver, CO). Tubulin aliquots were prepared with 86% natural bovine tubulin, 7% rhodamine tagged tubulin, and 7% biotinylated tubulin. Published protocol[6,7] was followed to produced microtubules biased with 12 protofilaments: 10 μl BRB80 solution containing 4 mM MgCl2, 0.5 mM GTP, 10 μM Taxol, and 9 μM tubulin was incubated for 30 min at 37 C to produced microtubules of approximately 20 μm in length. After 30 min, 100 μl of BRB80 buffer with 10 μM Taxol was added to stop microtubule nucleation. Microtubules were then resuspended in BRB0 solution supplemented with 10 μM Taxol.

Microtubules for TSQD studies were prepared in the same manner with the exception of using 7% fluorescein tagged tubulin instead of 7% rhodamine tubulin. The fast photobleaching of the fluorescein allowed for a better signal to noise ratio with the diminished fluorescent signal associated with the TSQDs.

Cover and microscope slide cleaning preparation. New Corning (Lowell, MA) No.1 22x22 mm glass slides were cleaned following the described procedure. Slides were sonicated first in a solution of 18MΩ water and Conrad 70 detergent, twice in a solution of 18MΩ, and finally in 200 proof ethanol. Each sonication session lasted a minimum of 30 minutes.

The quartz microscope slides (SPI, West Chester, PA) were used for multiple gliding assay chambers. The slides were sonicated while submersed in toluene for 1 hour. The cover slides would then be removed using tweezers. The slides were again sonicated for 1 hour in toluene followed by 1 hour in ethanol. Final preparation was performed following the procedure for cleaning the Corning cover slides.

***In vitro* microtubule transport.** Two strips of double-sided tape 50 μm thick (3M) were placed on a clean 25×51 mm quartz microscope slide (SPI, West Chester, PA) to use as spacers. A clean Corning (Lowell, MA) 22×22 mm glass slide was then place on top of the tape to complete the chamber. A solution of BRB80 with the appropriate dilutions of microtubules and neutravidin modified NBQDs was allowed to incubate for approximately 10 min, while simultaneously a series of solutions was then flowed through the chamber to prepare the gliding assay. First, two chamber volumes of BRB80 solution containing 1.0 mg/ml casein (Sigma, St. Louis, MO) and 0.1 M $CaCl_2$ (Fisher) was injected into the chamber and incubated at room temperature for 5 min. BRB80 solution containing 0.2 mg/ml casein, 0.2 mM MgATP, and kinesin displaced the previous solution in the chamber. After 5 min, BRB80 solution containing 0.2 mg/ml casein, 0.2 mM MgATP, 10 μm Taxol, and NBQD modified microtubules flowed into the chamber and kept at room temperature for 5 min. The chamber was then rinsed with BRB80 solution containing

0.2 mg/ml casein and 0.2 mM MgATP. Finally, the chamber was filled with BRB80 solution containing 0.2 mg/ml casein, 1 mM Mg ATP, 10 μm Taxol, and an oxygen scavenging system [50 μg/ml glucose oxidase (Sigma), 4 μg/ml catalase (Sigma), 1% (w/v) glucose (Sigma), and 0.1% (v/v) β-mercaptoethanol (Aldrich)] and placed under the microscope.

Absolute axial position determination. To prepare non-moving NBQD-modified microtubules in a quartz chamber the procedure described above was followed with the omission of the last solution. This allowed for the kinesin to hold the microtubules to the surface, but the microtubules were static due to the lack of ATP.

The localization precision in this experiment was dictated by the ability of our instrument to find and replicate the optimal incident illumination light angles and the consistency of the NBQDs to stay in the fluorescent emission state for prolonged periods of time. The automated calibration of the instrument was accomplished by using an in-house computer program that maximizes the signal to noise ratio of the region of interest (ROI) for each incident angle. More than 40 angles of incidence were imaged from subcritical angles to nearly 90° with intervals of approximately 0.25°. Before the data acquisition, the mirror position for each angle of incident light was set and both a rough-tune and a fine-tune position determination were performed. Each data point was the average of 5 measurements of the integrated fluorescence intensity of the entire particle taken with 50 ms exposure after subtracting the non-fluorescence background. The points were plotted against the incident angle of the laser. Non-linear least squares (NLLS) fitting determined the vertical center of the NBQDs.

A thorough explanation and study of the uncertainty in SA-TIRFM axial direction super-localization can be found in our previous work[8]. To find the confidence interval for the calculated axial positions of the NBQDs, a χ^2 test was performed when χ^2 is defined as

$$\chi^2 = \sum_1^m \frac{1}{\sigma_m^2}[F_{\text{exp}} - F_{\text{theor}}]^2$$

where σ_m is the standard deviation of m measurements of the fluorescent intensity taken at each incident angle; and the experimental and theoretical fluorescent intensities are defined as F_{exp} and F_{theor}, respectively. To find the standard deviation of axial position we varied z close to its fitted value where χ^2 reaches a minimum, χ^2(min). This is repeated for each fixed value of z, which reports another minimum χ^2 by floating the other parameters χ^2(par). F_χ statistics can estimate the confidence interval of z for the appropriate p parameters and v degrees of freedom:

$$F_\chi = \frac{\chi^2(\text{par})}{\chi^2(\text{min})} = 1 + \frac{v}{p} F(p, v, P)$$

where P is the probability that the value of F_χ is due to random error. When P is smaller than 0.32, there is a 68% probability that the corresponding F_χ consistent with the experimental values. This is the typical definition of one standard deviation.

F_χ, with our experimental fluorescence values, 2 parameters (p), 40 degrees of freedom (v), and a probability (P) of 0.32 is 1.16. This means that when χ^2(par) is 1.16 times χ^2(min), the corresponding z values are the standard deviation. If we take, for example, z_1 from Figure 1B, 1.16 times χ^2(min) leads to 11.5 nm and 26.5 nm resulting in a standard deviation of 7.5 nm.

Determination of blinking statistics. Unmodified NBQDs were diluted to an appropriate concentration in toluene and dispersed across a Corning 22x22 mm coverslip and sandwiched by a Corning 18x18 mm coverslip. The toluene was then allowed to evaporate and the chamber was

sealed with nail polish. DHLA-modified NBQDs were diluted in MilliQ water or 0.1% (v/v) β-mercaptoethanol and dispersed across a (3-aminopropyl)triethoxysilane (APTES) coated Corning 22 mm × 22 mm coverslip. The solution was then sandwiched by a Corning 18x18 mm coverslip and sealed with nail polish. Samples were viewed under TIRF conditions near the critical angle using an adjustable 75 mW maximum 488-nm argon continuous wave (CW) laser (Uniphase, San Jose, CA) as the excitation source. The sample was allowed to settle for 30 min under the objective to reduce sample drift before imaging. Data was recorded with 100 gain in frame transfer mode with a 50 ms exposure time for 6000 continuous frames (5 min). ImageJ was used to analyze the data. A region of interest (ROI) was drawn around each individual quantum dot. Using the Track2 plugin the intensity through all 6000 frames was recorded. If the signal of the ROI was higher than the background signal plus 3σ, the quantum dot was considered to be fluorescing or "on." Statistics of the various samples were calculated using Microsoft Excel.

A comprehensive statistical analysis of the fluorescence intermittency for NBQDs, thin-shelled quantum dots (TSQDs), and commercial QDs (EviTag T2) was performed for a large sample size of individual particles (**Supplementary Table 1**). Briefly, all samples were distributed across a microscope coverslip and excited by a 488 nm CW laser for 5 minutes. Data was recorded using an EMCCD with 50 ms exposure. Compared to the TSQDs and commercial QDs, unmodified NBQDs had vastly superior blinking statistics. A good comparison between traditional QDs and NBQDs can be held by looking at the sample fraction that emits for at least 80% of the acquisition time. A fraction of 37.5% of the commercial QDs emitted for at least 80% of the recorded time compared to unmodified NBQDs, of which 93.2% emitted. Even when the commercial QDs were analyzed in a solution of 1% (v/v) of β-mercaptoethanol (concentration appropriate for microtubule gliding assay), which is known to suppress fluorescent

intermittency[9], the fraction only increased to 60.1%. Without modification or a blinking suppressant environment, the NBQDs easily surpass the commercial QDs with regards to fluorescent intermittency. These statistics make the absolute positioning and dynamic tracking of NBQDs possible.

SUPPLEMENTARY REFERENCE

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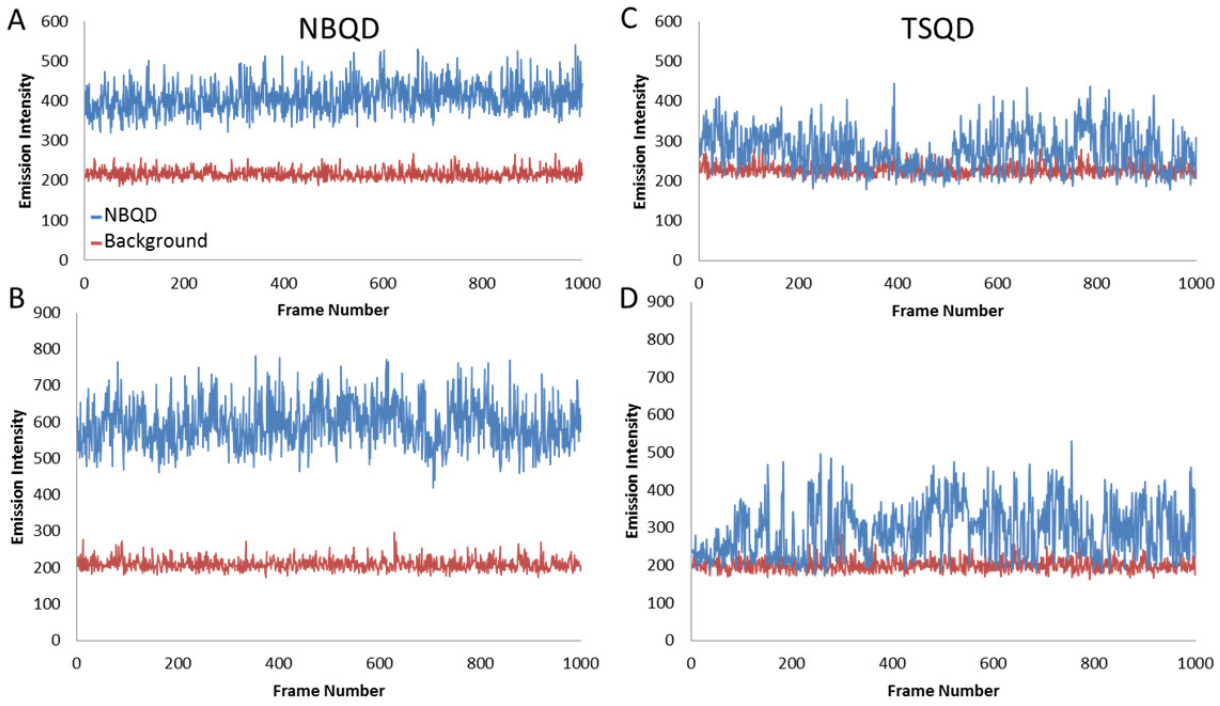
SUPPLEMENTARY TABLE 1

Fluorescent intermittency statistics of various batches of QDs and NBQDs. Emit percentage refers to the fraction of the particles that emitted light equal to or greater than that percentage of length of time. Average number of "blinks" refers to the number of times an average particles switched between the "on" and "off" fluorescence state. Average length of intermittency is the average length a particle stayed in the "off" fluorescence state. Parenthesis below column title describe solution sample was imaged in. MilliQ refers to a solution of 18Ω MiliQ water, βME refers to 0.1% (v/v) β-mercaptoethanol in MilliQ water, otherwise the sample was imaged in air.

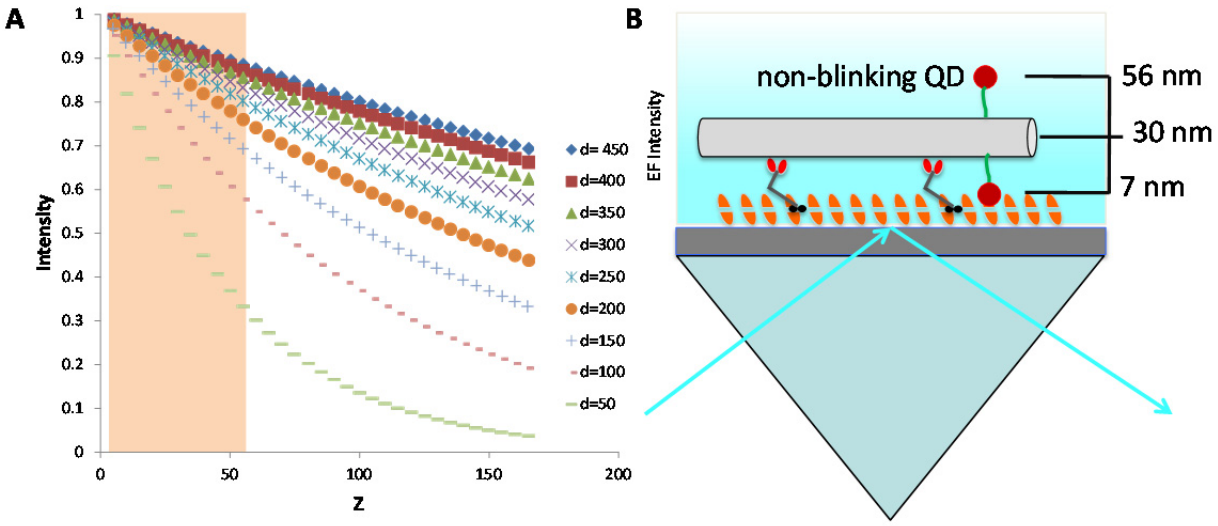
	TSQDs (MilliQ)	**EviTag** (MilliQ)	**EviTag** (βME)	**NBQDs**	**NBQDs** (MilliQ)	**NBQDs** (βME)
Particles Studied	291	293	248	192	291	254
Emit 98%	0%	11.3%	22.2%	58.3%	69.1%	89.8%
Emit 80%	11.0%	37.5%	60.1%	93.2%	87.3%	95.3%
Emit 60%	30.9%	58.0%	76.6%	96.9%	94.5%	98.0%
Average Number of "Blinks"	282 ± 159	289 ± 188	273 ± 225	91 ± 136	69 ± 128	44 ± 126
Average Length of Intermittency (seconds)	0.84 ± 0.95	0.53 ± 0.84	0.26 ± 0.47	0.13 ± 0.12	0.23 ± 0.25	0.13 ± 0.14

SUPPLEMENTARY FIGURE 1



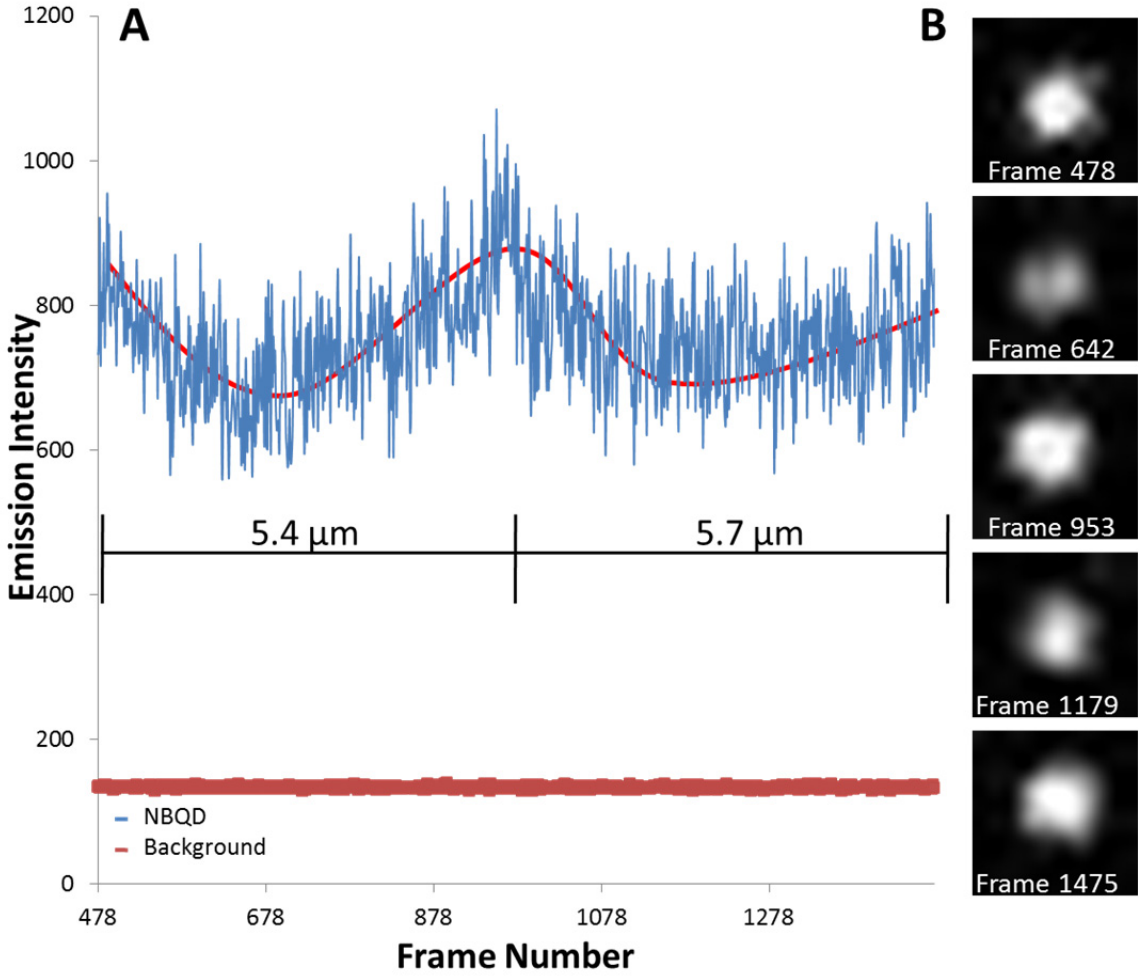
Supplementary Figure 1. Trace (A) corresponds to a NBQD attached to a kinesin-propelled microtubule. (B) Corresponds to a NBQD on the quartz surface in a gliding assay. (C) Corresponds to a TSQD attached to a kinesin-propelled microtubule, while the trace in (D) is a TSQD on quartz surface in a gliding assay. All traces were captured using 50 ms exposure time.

SUPPLEMENTARY FIGURE 2



Supplementary Figure 2. (A) Expected decay in signal intensity related to depth of EF. Shaded region relates to axial distance changes expected for microtubule system (7-56 nm). (B) Depiction of EF decay as the probe moves toward and away from the surface of TIR.

SUPPLEMENTARY FIGURE 3



Supplementary Figure 3. (A) Trace of NBQD attached to rotating microtubule followed for 2 full periods (50 ms exposure). (B) Images of NBQD at frames corresponding to maximums and minimums of (A).

MOVIES

All movies were recorded at 20 frames per second and playback at 100 frames per second.

Movie 1. NBQDs attached to a non-rotating microtubule. The red circle in Frame 1 indicates which NBQD corresponds to the trace in **Figure 2.**

Movie 2. NBQD attached to a rotating microtubule. The distance traveled by the NBQD between the maximums and minimums of intensity indicated a 12-protofilament microtubule. The intensity is plotted in **Figure 3.**

Movie 3. NBQD attached to a rotating microtubule. The distance traveled by the NBQD between the maximums and minimums of intensity indicated a 14-protofilament microtubule. The red circle in Frame 1 indicates which NBQD corresponds to the trace in **Supplementary Figure 3.**