Supplementary Information for

Exclusive formation of monovalent quantum dot imaging probes by steric exclusion

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I. Supplementary Notes

1. **Phosphorothioate sequence length required for quantitative formation of mQD:** We found that Steric Exclusion was maximally efficient using 50mer or longer phosphorothioate sequences. Shorter polyadenosine ptDNA sequences, including 20mers and 35mers, generated small amounts of unfunctionalized and divalent QDs depending upon the reaction conditions (Supplementary Fig. 2).

2. **Surface passivation of mQDs for optimal target specificity:**
Live cell imaging agents must exhibit low background binding to cells or their culture substrates at concentrations necessary to generate a strong fluorescence signal. To reduce background binding to cells, QDs are typically passivated with ligands containing polyethylene glycols. We evaluated the non-specific binding to cells of ptDNA-mQDs passivated with several thiolated PEG ligands (Supplementary Table 1). Jurkat cell membranes displaying complementary oligonucleotide targets were incubated with passivated 5 nM ptDNA-QDs. All passivated ptDNA-QDs showed reduced background binding to cell surfaces as judged by
flow cytometry (Supplementary Fig. 13) and fluorescence microscopy (Supplementary Fig. 12). We chose a C_{11}-PEG-COOH as a passivating ligand for subsequent experiments as it is a commercially available reagent that may provide extra stability to the PEG-QD interaction by forming a hydrophobic shell internal to the PEG functionality. All mQDs wrapped with ptDNA used in experiments were passivated with PEG. For biological experiments involving live cells the mQDs were further passivated with either BSA or casein (Supplementary Fig 7).

3. Determination of QD valency via agarose gel electrophoresis:

QDs were coated with mPEG-SH during-phase-transfer and were neutrally charged. DNA attachment to the QDs results in significant increase of the particle charge density accompanying slight increase of their hydrodynamic size (Fig. 1e, Supplementary reference 1). Thus, in a low percentage agarose gel (< 1%) where the size-sieving effect is minimized, QD-DNA conjugates migrate further as the number of attached DNA molecules increases. This principle has been shown to be effective to isolate nanoparticles (e.g. Au, QDs) bearing different numbers of oligonucleotides, particularly for small nanoparticles (<25 nm) in their hydrodynamic size [see reference 8 in main text]. We employed this method in determining the valency of QDs after DNA conjugation. Note that the hydrodynamic size of QDs coated with mPEG-SH (10 nm) used for this study is significantly smaller than that of QDs (22 nm) used in previous study reporting successful agarose gel electrophoretic isolation of QD-DNAs with similar DNA length (50-100 mer) [see reference 8 in main text], and thus better separation resolution between QD bearing different numbers of DNAs is expected.

4. Specificity of pt-DNA wrapped mQDs and Streptavidin QDots:

The Au nanoparticle hybridization and SNAP diffusion studies are valid only when non-specific interactions of QDs to the target are negligible. To validate that QD-Au nanoparticle dimers were assembled through specific interactions, we carried out control experiments with nanoparticles lacking specific targeting functionality. Both ptDNA-wrapped mQDs and Streptavidin QDots showed new bands corresponding the hybridized products in agarose gel electrophoresis only in the presence of targeting functionality (Supplementary Fig. 14), indicating the interactions between QDs and Au nanoparticles were specific. Similarly, these QDs also showed excellent specificity to SNAP proteins. Both electrophoretic analyses (Supplementary Fig. 17) and TIRF imaging (Supplementary Fig. 18) showed no noticeable non-specific interactions of QDs to SNAP proteins or to supported lipid bilayers.
Supplementary Fig. 1. Titration of QDs with increasing concentration of either a 5'-trithiolated 70mer ssDNA (ttDNA) (a) or 70mer ptDNA comprising an A₈₅₀ domain and a 20 nucleotide ssDNA tail (b). For ttDNA, significant amounts of unlabeled and multivalent products were produced along with desired monovalent QDs at any given concentrations (a). In contrast, QDs treated with ptDNA were converted to monovalent QDs exclusively at stoichiometric or higher ptDNA ratios. (b). To provide a more accurate analysis of the yield of monovalent QDs extracted from these images, we performed multi-peak fitting of the gel band intensities to Gaussians (c). We found that a quadruple Gaussian provides the best fit with a minimum residual, with individual Gaussians corresponding to unlabeled, monovalent, divalent and higher valent species. Using this method, we estimate a minimum yield of the monovalent species of 99%. In contrast, the ttDNA gel trace reveals a maximum yield of monovalent species of 39% at a 30:1 ttDNA to QD ratio using the same quadruple Gaussian approximation, consistent with the value expected from an underdispersed Poisson Statistics (c, top).

λ values (average DNA/QD ratio in products) were determined by a following equation, \( \lambda = 1 \times \frac{I_1}{I_t} + 2 \times \frac{I_2}{I_t} + 3 \times \frac{I_3}{I_t} \), where \( I_n \) is intensity of the \( n \)th band and \( I_t = I_0 + I_1 + I_2 + I_3 \).
Supplementary Fig. 2. 50mer or longer phosphorothioate sequences are required to ensure QD monovalency. QDs were treated with sequences bearing 20mer (lane #1), 35mer (#2), 50mer (#3), and 70mer (#4) poly-A\textsuperscript{S} ptDNA. Shorter poly-A\textsuperscript{S} ptDNA sequences including 20mers and 35mers, generated small amounts of unfunctionalized and divalent QDs depending upon the reaction conditions, while 50mer and longer sequences produced monovalent products exclusively. DNA lengths for lanes #1-3 were made identical (70mer total) by adding additional DNA nucleotides to the 3’ end of the shorter ptDNA sequences. A slightly longer DNA length (90mer total) was used for lane #4, resulting in increased band migration.

Supplementary Fig. 3. Preparative scale synthesis of mQD. Bright-field (a) and fluorescence (b) photographs of a 100 mL scale reaction. Only a single band with an increased migration in agarose gel electrophoresis (c) indicates complete conversion of bare QDs to monovalent QD products.

Supplementary Fig. 4. Sequence-dependent affinity of ptDNA and phosphodiester DNA on QDs. QDs were treated with sequences comprising A\textsuperscript{S}\textsubscript{50-m1}, T\textsuperscript{S}\textsubscript{50-m1}, or C\textsuperscript{S}\textsubscript{50-m1} DNA at one-to-one stoichiometric ratio. Sequences comprising polyadenosine ptDNA (poly-A\textsuperscript{S}) had the strongest binding to the QDs when compared to poly-T\textsuperscript{S}, poly-C\textsuperscript{S}, and phosphodiester poly-A sequences. Only A\textsuperscript{S}\textsubscript{50-m1} treated QDs produced mQDs quantitatively while poly-T\textsuperscript{S} and poly-C\textsuperscript{S} sequences generated mQDs at lower than 10% yield under identical conditions, indicating that the adenine base also contributes to the interaction with the nanoparticle surface. Poly-adenosine phosphohates (A\textsubscript{50-m1}) or hybridization sequences ((CT)\textsubscript{10}(ACTG)\textsubscript{5}) comprising phosphodiester linkages exhibited very weak (2%) or no affinity to QDs, respectively. These data indicate that both the phosphorothioate group and the DNA bases contribute to the interaction with the nanoparticle surface. We
were unable to characterize polyguanosine phosphodiester or phosphorothioate sequences due to competing secondary structures that confounded purification by HPLC and analysis by agarose gel electrophoresis.

Supplementary Fig. 5. ptDNA binding of QDs does not significantly alter QD fluorescence quantum yields. Absorption (black line) and emission spectra (blue line) of QD605-mPEG (a), and monovalent QD605-50xAS-m1 (b) at a same concentration. We found that the brightness and photostability of the mQDs were not significantly changed upon binding to DNA. The quantum efficiency of the mQDs was observed to be 31.3%, nearly identical to that (31.5%) of the bare QDs.

Supplementary Fig. 6. mQDs are photostable. Time traces in fluorescence intensity of monovalent QD-m1 (red), commercial QD (blue, streptavidin QDs from Life Technology), and Fluorescein isothiocyanate (black, FITC). Both mQDs and commercial Streptavidin QDs showed stable fluorescence, with slight increase in emission intensity (10%), under continuous illumination of excitation light source (Lambda-LS/OF30R Xe Arc lamp, 300W) over 10 minutes. Similarly, in some cases of single particle tracking experiments, we saw a more profound "turn-on" effect that levels out after 5-10 seconds of laser illumination. The enhancement of fluorescence intensity might be explained by a reduction in the number of surface electron traps [Supplementary reference 3].

Supplementary Fig. 7. Passivation scheme of mQDs. Bare QDs (0) are phase transfered with mPEG thiol (1) and subsequently bound to ptDNA and carboxylated PEG-thiols to produce the mQDs used throughout this study (2). These mQDs were further passivated with either BSA
or casein for experiments in which the mQDs would be exposed to live cells.

| Time          | Mean size     |
|---------------|---------------|
| 0 hr          | 12.2 ± 1.8 nm |
| 20 hr         | 12.8 ± 1.8 nm |
| 30 hr         | 12.6 ± 2.6 nm |
| 8 month       | 12.0 ± 2.2 nm |
| 6hr in L-15   | 12.8 ± 2.0 nm |

Supplementary Fig. 8. ptDNA-wrapped mQDs are colloidally and chemically stable. DLS analyses showed no sign of significant aggregation or DNA detachment of mQDs in PBS for at least for 30 hr (a, top three graphs). Moreover, mQDs were stable in Tris buffer (10 mM Tris, 30 mM NaCl) at 4 °C after over 8 months (a, b) and in serum-free culture media for 6 hr (a, c). Treatment of ptDNA-QD with an excess amount of mPEG-SH (10⁵ times, 10 mM) for 30 min resulted in no noticeable detachment of QDs.

Supplementary Fig. 9. Colloidal stability as a function of pH, salt concentration, and QD concentration. Colloidal stability of mQDs was assessed by electrophoresis and/or DLS. mQDs are stable in pH ranges of 5-
11 (a, d), slight shifts in dispersion at low pH are indicated with arrows in the DLS traces. mQDs are stable in NaCl salt up to 300 mM (b, d), with slight evidence of aggregation or tailing indicated with red arrows on the DLS and gel images, respectively. mQD concentration of ≤1 µM are also stable (c).
Supplementary Fig. 10. Representative single particle blinking trajectories of mQDs in culture media containing fetal bovine serum (FBS). To test colloidal stability of mQDs in culture media containing 10% serum for 30min, mQDs were immobilized on glass after hour-long incubation with complete media (McCoy’s 5A with 10% FBS). Intensity trajectories of single mQDs show characteristic single step on/off blinking, indicating little to no aggregation of mQDs in media containing FBS. Some spikes were also observed due to fast QD blinking (top zoom).

Supplementary Fig. 11. Modular mQDs for bioconjugation chemistry. Agarose gel electrophoresis analyses showed reduced migration of the target protein-treated QDs revealed that specific binding of monovalent QDs to the target protein with one-to-one stoichiometry. (a) Biotin-Streptavidin, (b) Benzylguanine-SNAP, (c) Benzylcytosine-CLIP chemistries.
Supplementary Fig. 12. Specific cell labeling with mQDs. Jurkat cell membranes displaying either non-complementary (b) or complementary (c) sequences treated with mQDs and imaged using confocal fluorescence microscopy. Cross sections through the center of the cells revealed intense fluorescence only at membranes targeted via hybridization, confirming the high specificity and low background of mQDs. Scale bar is 10 μm.

Supplementary Fig. 13. Evaluation of mQD specificity on live cells via flow cytometry. At least 30,000 cells from Supplementary Fig. 17 were analyzed by flow cytometry. Similar low background and high-specific labeling were observed for the mQDs.

Supplementary Fig. 14. mQDs are monovalent. Treatment of mQDs with 5 nm gold nanocrystals bearing a single complementary sequence of ssDNA yielded mQD-Au heterodimers exclusively (a, b). Au-cDNA nanoparticles used here were coated with either mPEG-SH (a) or COOH-PEG-SH (b). Reaction of Streptavidin QDs with the gold nanocrystals bearing biotinylated ssDNA resulted in multiple bands in agarose gel electrophoresis, indicating the formation of multivalent products as well as heterodimers (c).
**Supplementary Fig. 15.** Representative TEM Images of mQD-Au heterodimers. Most QDs were paired with Au nanoparticles at a one-to-one ratio. Note that some free gold nanoparticles and QDs were observed due to contamination during the gel slicing or detachment of heterodimers during sample preparation for TEM. We also found a very small percentage of Au nanoparticles surrounded by more than one QD. Since these events do not report on mQD valency, we interpreted them as having been formed as a result of a small fraction of Au NP bearing more than one DNA strand or non-specific interaction between Au NPs and free QDs during sample preparation for TEM.

| # Au partners per QD | 0       | 1       | 2       |
|----------------------|---------|---------|---------|
| total # of events observed (n = 545) | not counted | 533     | 12      |

**Supplementary Fig 16.** The distribution of QD diffusion constants change as a function of SNAP protein density on SLBs. Three serial 10-fold dilutions of SNAP protein were incubated in the presence of identically assembled lipid bilayers and subsequently linked to either mQDs or streptavidin QDots. After incubation for 30 minutes the bilayers were imaged via TIRF microscopy. The rate of diffusion of the SNAP protein as measured by either mQD or NHS-Atto488 labeling were invariant with respect to the concentration of added SNAP protein. The mean rate of diffusion of the SNAP protein as measured by streptavidin QDots, however, decreased with increasing added protein concentration and was significantly lower than that measured by mQDs or the organic dye. Solid lines conducted at equal “1x” concentration of SNAP protein incubated on SLBs, with dashed lines at equal 10x dilution, and dotted lines at 100x dilutions of SNAP protein. Each condition contains at least 180 measured diffusion coefficients.
**Supplementary Fig. 17.** Streptavidin-Qdots bind to biotinylated SNAP proteins specifically. To exclude the possibility that Streptavidin-Qdots crosslink SNAP proteins through non-specific interactions, the QDs were treated with SNAP-DNA conjugates with or without pendent biotin functionality. A broad new band with increased migration was observed only for biotinylated SNAP-DNA conjugates, indicating that cross-linking of SNAP proteins were a consequence of the multivalent nature of Streptavidin-Qdots.

**Supplementary Fig. 18.** Signal-to-background ratio of mQD-labeling of SNAP-labeled supported lipid bilayers. Supported lipid bilayers containing NTA-his-tag linked SNAP proteins were incubated with mQDs (or streptavidin QDots with bound biotin-DNA) in the presence or absence of benzylguanine-DNA. In the absence of benzylguanine-DNA complementary to the mQDs, mQDs did not bind to the bilayer or the SNAP protein. Shown are representative single locations of 4637μm² each, at single-molecule QD concentrations (~50pM). An average of 10.7 mobile mQDs or 15.3 Streptavidin QDots were found per location across 30 locations, with no mobile QDs found in any of the 30 locations lacking the targeting BG-DNA strands. Scale bar is 10 μm.

**Supplementary Fig. 19.** The SNAP-Notch construct is capable of activating a Notch reporter when expressed in U2OS cells. U2OS cells expressing either Notch-Gal4 (blue bars) or Snap-Notch-Gal4 (red bars) were transduced with virus containing H2B-mCherry driven by a UAS promoter. These cells were induced with doxycycline upon coculture with either U2OS cells expressing GFP alone (U2OS-Ø) or U2OS cells expressing Dll1 & GFP (U2OS-Dll1). After 48hrs, integrated intensity of all mCherry nuclei were divided by the total number of mCherry pixels to give total fluorescence/pixel signal. Similar results were obtained using the same reporter cells cultured on glass plated with Fc-Dll1 (R&D Systems).
Supplementary Fig. 20. Signal-to-background ratio of mQD-labeling of SNAP-Notch on cells. Average numbers of particles were separately counted on co-cultured cells expressing either SNAP-Notch or Notch-GFP proteins. These experiments were repeated four times at single molecule mQD concentrations (<1 nM). The mean signal-to-background ratio was 40:1.

Supplementary Fig. 21. Benzylguanine labeling specificity on live U2OS cells. To confirm the specificity of BG-mQD targeting SNAP-Notch, we prepared monolayer co-cultures of two populations of U2OS cells: one expressing SNAP-Notch and another identical cell line expressing a Notch construct lacking the SNAP-tag but fused at its C-terminal to eGFP (Notch-GFP) (a). Incubation of cocultures with 10 nM BG-mQDs exclusively labeled SNAP-Notch expressing U20S cells similarly to control experiments using BG-Alexafluor647 (b). Under these high density labeling conditions we observed a 125:1 signal to background ratio for mQDs on cells. Scale bar is 20 μm.
Supplementary Fig. 22. Steric Exclusion strategy is generally applicable for CdSe:ZnS QDs from a variety of commercial sources. Bare (lanes 1, 3, & 5) QDs were completely converted to monovalent QDs after stoichiometric treatment with $A_{50}^{S}$-m1 ptDNA. Monovalent QDs showed slightly different migrations and broadening in electrophoresis, depending on the commercial source, probably due to different sizes and shapes. Very recently, Life Technology has launched a new version of the QDs called QD605 VIVID dots. Our DNA-conjugation method also worked for this new QD, but we observed significant loss of its fluoroscence after the phase transfer step. We have not tried to optimize this method for these new QDs.

Supplementary Fig. 23. Dropwise addition of ptDNA is critical for exclusive formation of mQDs. In contrast to the exclusive formation of mQDs shown in Figure 1 and Supplementary Fig. 3, rapid addition of ptDNA to QDs at high reaction concentration (1 µM) yielded a mixture of unconjugated, monovalent, and multivalent QDs. Steric Exclusion effects, however, were clearly seen, where use of DNAs having longer phosphorothioate domain length (a: $A_{20}^{S}$-(m1)$_{2.5}$, b: $A_{35}^{S}$-(m1)$_{1.75}$, c: $A_{50}^{S}$-m1 ptDNA) resulted in higher yield of monovalent QDs.
Supplementary Fig. 24. ptDNA wrapping does not induce QD aggregation. TEM images of organic, phase-transferred, and ptDNA-wrapped mQDs showed no significant difference in size, shape, and assembly states of QDs. Measured sizes for nanoparticles are shown below each image (N = 49, 38, and 36 for organic, phase transferred, and ptDNA-wrapped, respectively). Scale bar: 50 nm.
Supplementary Table 1. Surface passivation of mQDs with a variety of PEG ligands. To improve signal-to-background and signal-to-noise ratios, we treated as-synthesized mQDs with various PEG molecule listed below. We evaluated specific-binding of these mQDs on cells via flow cytometry, where lipid-ssDNA anchored CHO cells were treated with either complementary or non-complementary mQDs. Mean fluorescence signals for non-treated (control, c), non-complementary mQD treated (negative control, n), and complementary mQD treated (experiment, e) cells were measured. Mean-background-increase (n/c) and mean-signal-increase (e/n) values of mQDs with various ligands are shown below. Although the dihydrolipoic acid-PEG-succinate molecule behaved the best (third entry), it required a two step synthesis, and did no perform significantly better than the commercially available alkanethiol PEG acid (last entry).

| Ligand                      | Mean Background Increase (n/c) | Mean Signal Increase (e/n) |
|-----------------------------|--------------------------------|----------------------------|
| Dihydrolipoic acid-PEG-succinate | 20.0                           | 4.3                        |
| Alkanethiol PEG acid        | 5.3                            | 2.8                        |
| Third entry                 | 1.1                            | 9.6                        |
| Last entry                  | 22.0                           | 4.1                        |
| Mean-background increase     | 5.3                            | 11.7                       |
| Mean-signal increase         | 3.7                            | 8.3                        |

Supplementary Table 2. DNA sequences used for conjugation with QDs

| Conjugation experiments | Sequence of QD conjugated DNA | Total length of oligonucleotides | Sequence of complementary DNA |
|-------------------------|-------------------------------|---------------------------------|------------------------------|
| QD-ttDNA                | 5’-trithiol-T<sub>50</sub><sup>m1</sup>-3’<br>m<sub>1</sub>=AGT GAC AGC TGG ATC GTT AC | 70 mer                          | -                            |
| QD-poly-A<sub>S</sub><sup>ptDNAs</sup> | 5’-A<sub>S</sub><sup>20(m1)</sup>-3’<br>5’-A<sub>S</sub><sup>35(m1)</sup>-3’<br>5’-A<sub>S</sub><sup>50m1</sup>-3’<br>5’-A<sub>S</sub><sup>70m1</sup>-3’ | 70 mer                          | -                            |
| QD-Au                   | 5’-A<sub>S</sub><sup>50</sup>(CAGT)<sub>5</sub>-3’ | 70 mer                          | 5’-Au nanoparticle-(CT)<sub>10</sub>(ACTG)<sub>5</sub>-3’ |
| QD-streptavidin         | 5’-A<sub>S</sub><sup>50m1</sup>-biotin-3’ | 70 mer                          | -                            |
| QD-SNAP                 | 5’-A<sub>S</sub><sup>50</sup>(ACTG)<sub>5</sub>-3’ | 70 mer                          | 5’-benzylguanine-(CAGT)<sub>5</sub>-3’ |
| QD-CLIP                 | 5’-A<sub>S</sub><sup>50</sup>(ACTG)<sub>5</sub>-3’ | 70 mer                          | 5’-benzylcytocine-(CAGT)<sub>5</sub>-3’ |
| Notch imaging           | 5’-A<sub>S</sub><sup>50</sup>(CT)<sub>10</sub>(ACTG)<sub>5</sub>-3’ | 90 mer                          | -                            |
Supplementary Video 1. Timelapse of SNAP proteins on supported lipid bilayers. Timelapse TIRF movie taken of SNAP protein embedded in a supported lipid bilayer labeled with either Streptavidin QDots linked via BG-DNA-biotin, or mQDs. SNAP protein labeled with NHS-Atto488 shown to the right. Images taken at 20 Hz; scale bar is 10 μm.

Supplementary Video 2. AF647 & mQD labeled SNAP-Notch on a live U2OS cell. Timelapse TIRF movie taken of SNAP.hN1 on a single cell. The cell’s AF647 stained receptors were imaged for 25 seconds and immediately followed by imaging the mQD stained receptors. The AF647 & mQD regions are identical, separated by ~30 s in time. The white boxed region is shown in Figure 4c. Images taken at 20 Hz; scale bar is 1 μm.

References:
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