Characterization of the Functional Binding Properties of Antibody Conjugated Quantum Dots

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Received November 19, 2006; Revised Manuscript Received May 7, 2007

ABSTRACT

Antibody conjugated quantum dots are an emerging technology for high-resolution labeling of biological systems. In this work we determined the number of functional antibodies (i.e., antibodies that are sterically available for functional binding to target proteins) conjugated to semiconductor quantum dots. This is critical for the interpretation of biological data labeled with these methods. We found that the number of available functional antibodies varied significantly for different conjugation methods and are lower than previously estimated. These results may suggest potential strategies for improving quantum dot labeling of biological preparations.

Semiconductor quantum dots have physical and optical properties that make them useful tools for high-resolution labeling and imaging of proteins in cells. They are brighter than organic fluorescent dyes, exhibit minimal photobleaching, and have narrow emission spectra which support multiplexing of signals (i.e., the use of multiple quantum dots of different colors) in the same preparation.1–5 By chemically conjugating antibodies and other peptides to their surface, quantum dots can specifically target cellular ligands of interest.6–15

One critical issue that has not been addressed is experimentally determining the number of antibodies bound to quantum dots which are functionally available for target protein binding. This is critical for the analysis and proper interpretation of biological data labeled using this method. For example, we have previously shown that immunoglobulin G (IgG) antibody functionalized quantum dots can be used for high-resolution imaging of fixed neurons and glial cells if conjugation and blocking conditions are optimized.7 However, we also demonstrated reproducible nonspecific artifact labeling, which could be mistaken for specific labeling, if antibody−quantum dot conditions are less than optimal (Figure 1). While other groups have qualitatively characterized antibody-functionalized quantum dots using transmission electron microscopy, atomic force microscopy, UV spectroscopy, and gel electrophoresis,16–19 and in some cases have suggested estimates of the putative
number of total antibodies bound to quantum dots. No calculations of the number of functional antibodies bound to quantum dots based on quantitative experimental results have been reported. In the present work we derived the number of functional IgG antibodies conjugated to quantum dots based on calculations of quantitative electrophoresis experiments using two different conjugation schemes: a common direct covalent conjugation using a reduced disulfide maleimide reaction, and biotinylated antibodies bound to streptavidin-functionalized quantum dots (see Methods in the Supporting Information). Antibody–quantum dot complexes were run in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate the functional component of conjugated antibodies from the quantum dots. We then blotted the antibody fragments onto a membrane to determine the identity and amount of the antibodies and quantitatively compared the amount of transferred antibody to known antibody concentrations to derive the number of bound antibody.

The number of functional antibodies covalently bound to commercially available quantum dots was on average much less than one functional IgG molecule per quantum dot (0.076 ± 0.014) and therefore of potential limited utility for biological experiments. In contrast, antibodies bound to quantum dots via the streptavidin–biotin system resulted in higher numbers of functional antibodies, with 0.60 ± 0.14 IgG molecules per quantum dot for a 1:1 IgG : quantum dot molar ratio and 1.3 ± 0.35 IgG molecules per quantum dot for a 2:1 ratio, thereby supporting biological labeling. In addition to these specific results, our methods may be of broader interest because our approach is easily extendable for experimentally deriving the number of functional antibodies or peptides bound to other classes of nanoparticles (e.g., magnetic nanoparticles).

We begin by considering the covalent conjugation of antibodies to quantum dots. Using a commercially available direct conjugation kit (Invitrogen) and following published protocols, antibodies were reduced using dithiothreitol (DTT), which generates three distinct fragments identifiable by their molecular weights: A 25 kD light chain, which importantly includes half of the specific antigen binding site for a particular IgG molecule, a 50 kD heavy chain, which includes the other half of the binding site, and a 75 kD partially cleaved chain consisting of a heavy chain and a light chain held together by an unreduced disulfide bond (Figure 2a). Following this, individual fragments were covalently bound to quantum dots via an SMCC linkage bond which cannot be broken by DTT treatment, an important consideration for the interpretation of the experimental results that follow. This gives rise to three possible antibody fragment binding scenarios to quantum dots (Figure 2b): covalently bound light chains, covalently bound heavy chains, and covalently bound heavy–light chain partial fragments, of which only the latter can undergo further DTT reduction to remove the light chain fragment from heavy chains that remain bound to quantum dots, or heavy chains removed from light chains bound to quantum dots.

We first confirmed that antibodies were indeed covalently bound to the quantum dots by running IgG–quantum dot complexes though SDS-PAGE with and without DTT. For DTT reduced conditions we observed light chains cleaved from covalently bound partial fragments (Figure 3a, lanes 4–6). As expected, this separation occurred minimally in lanes without DTT (Figure 3a, lanes 2–3). The presence of a weak band at the 25 kD position in nonreduced lanes (Figure 3a, lanes 2 and 3) was due to low concentrations of reducing agents in the gel and running buffers. Interestingly, we saw no heavy chains being dissociated from light chain bound partial fragments. It is unclear why this was the case, although we hypothesize that the probability of the heavy chain portion of a partial fragment binding to a quantum dot is considerably higher than the light chain portion because there is twice the surface area for heavy chain binding and it is a condition that may be sterically favored (since the bend in the partial chain may tend to hide the light chain from the quantum dot). Another potential explanation for the lack of heavy chain band is methodological. Given the intensity of other bands in the membranes, small amounts of free heavy chain may have gone undetected given the exposure time we used to develop the membrane, which if it had been longer may have shown the presence of heavy chains but would have overexposed the other darker bands,
resulting in uninterpretable smearing. Additional evidence that heavy chains covalently bound to quantum dots remained bound to the quantum dots is inferred by a nonspecific colloidal blue protein stain which labels any protein in the gel that did not transfer to the membrane (Figure 3b). Since blue bands appeared at the position in the gels that corresponded to the quantum dots, some amount of residual protein did remain on their surface. Given that most of the light chains were cleaved, since they transferred strongly to the membrane, this residual protein is mostly heavy chain. Regardless, for the purpose of calculating the amount of functional antibody on quantum dot surfaces this is of minimal importance, since it is the amount of available light chain that we are interested in. For an antibody to be functional, both the light chain and the heavy chain must be present. More specifically, it is the light chain in combination with the heavy chain that allows target binding. Molecularly, roughly the first 110 amino acids at the amino terminal end of both heavy and light chains form the variable, or V, regions which contain highly variable segments called complementary determining regions.21,22 The pairwise association of V regions from both heavy and light chains is what actually forms the antigen binding site.21,22 Another important consideration to note is that the amount of partial fragments initially available for binding to quantum dots following the initial DTT reduction was very low, as evident

Figure 2. Antibody reduction and conjugation to quantum dots. (a) Schematic of antibody cleavage sites by DTT at disulfide linkages. The fragments that can result from DTT reduction include the light chain, heavy chain, and partially cleaved fragments due to incomplete reduction. (b) Schematic of direct SMCC covalent conjugation of antibodies to quantum dots. Further reduction with DTT following the primary reduction associated with the conjugation reaction yields the light chains which are counted in the derivation of the average number of functional IgG molecules originally on quantum dots. (c) Similar schematic for biotinlated antibodies conjugated to streptavidin-coated quantum dots.
in the reduced unconjugated IgG controls (Figure 3a, lanes 7–9). This point is an important consideration for why the number of available functional antibody in the covalently conjugated condition was calculated to be so low. (Note that no partial fragments were visible for the quantum dot lanes because the entire partial chain cannot be cleaved intact from the quantum dot since the SMCC linkage cannot be broken by DTT.) Furthermore, additional evidence that antibodies were covalently attached, not electrostatically attached, comes from the fact that several bands would have shown up in nonreduced lanes if they were electrostatically attached (Figure 3a, lanes 2 and 3) because the gel would have separated the antibodies from the quantum dots according to their molecular size and weight. Further indirect evidence that antibodies were covalently bound is implied by the fact that quantum dots in nonreduced lanes did not travel through

Figure 3. Separation of IgG antibodies into fragments using SDS-PAGE and membrane transfer under different experimental and control conditions. (a) Covalently conjugated IgG to quantum dots via an SMCC linker and controls. (b) Colloidal Blue nonspecific stain for proteins in gels for the direct conjugation method. (c) Biotinylated IgG bound to streptavidin-coated quantum dots and controls.
the gels but remained in the loading wells due to the large size of the unreduced complex (visible as intense signals in the loading wells for lanes 2 and 3 of the SDS PAGE in Figure 3a).

We ran the same experiments with biotinylated antibodies and streptavidin-coated quantum dots at 2:1 and 1:1 antibody to quantum dot molar ratios. Biotinylated antibodies have four–eight biotin molecules attached at random locations throughout the entire antibody, which results in the IgG molecules being conjugated to quantum dots presumably in all possible spatial arrangements (Figure 2c). Importantly and very differently from the direct covalent conjugation reaction, using the biotin–streptavidin system, the entire antibody molecule is conjugated to the quantum dot; it is not reduced into light chain and heavy chain fragments prior to binding. Similar to the covalent antibody conjugation method, non-reduced conditions resulted in quantum dots remaining in the loading wells (Figure 3c, lane 4) while reduced conditions allowed quantum dots to run through the gels (Figure 3c, lanes 2, 3, and 5). Some amount of antibody did transfer in nonreduced conditions for biotin–streptavidin IgG–quantum dot complexes because of the reducing agents in the running buffers and the gel, causing the light chain to dissociate in the same manner as for the covalent conjugation. Since all bands were much stronger in the biotin–streptavidin method in general, bands for the nonreduced condition were correspondingly stronger. Bands in non-DTT treated antibody lanes (i.e., Figure 3c, lanes 7, 9, and 10) show the reduction process in greater detail since reduction agents in the running buffers reduced the antibodies less efficiently than DTT-treated conditions (Figure 4a, lanes 2, 3, 5, 6, and 8). Additional controls (supplementary figure) are discussed in the Supporting Information.

On the basis of these data and the qualitative models introduced above that describe the different putative binding scenarios for antibodies directly covalently conjugated to quantum dots and for antibodies bound to quantum dots via biotin and streptavidin (Figure 2), we derived the average number of functional IgG conjugated to quantum dots. We use the term “functional antibody” to describe the amount of $F_{ab}$ light chain, which includes a part of the target protein binding epitope, that is physically oriented outward from a quantum dot and presumably able to interact with its ligand. As such, only a partial fragment bound to the quantum dot would be functional since it contains both the light and heavy chains required to bind to the target protein. Furthermore, because of the structure of the antigen binding site, a partial fragment covalently bound to the quantum dot oriented with the light chain facing the nanoparticle would almost surely prevent ligand binding. Since it is the $F_{ab}$ light chain portion of the antibody that actively binds to proteins, quantifying the amount of light chain fragments not directly bound to quantum dots and oriented outward gives a good approximation of the functional activity of antibody–quantum dot complexes.

To determine the number of functional IgG bound to quantum dots, we measured the density of the 25 kDa light chain bands and compared them to controls of known antibody concentrations. Using image analysis software that measures the band density of electrophoresis gels (ImageQuant TL, GE Healthcare; see Methods in the Supporting Information), we fitted curves to known concentrations of unconjugated IgG to obtain standard curves of IgG band densities (Figure 4a,b; all $r^2 \geq 0.89$). Using these curves, we then determined the concentration of IgG bands associated with covalently bound IgG and 2:1 and 1:1 IgG:quantum dot molar ratio streptavidin-biotin conjugation conditions (Figure 4c,d). Finally, we calculated the number of functional antibodies bound to the quantum dots for each condition (Figure 4e; see Supporting Information for detailed calculations).

For covalently conjugated IgG we calculated that on average there is much less than one antibody molecule (0.076 ± 0.014) per quantum dot. In other words, adding 10 $\mu$L of antibodies directly conjugated to quantum dots is equivalent to adding 0.455 $\mu$L from a 0.5 mg/mL stock. This suggests that covalently conjugated antibodies have low amounts of functionally available antibodies and are of potentially inadequate sensitivity for reliable specific labeling of target proteins (see Figure 1). In contrast, the number of antibodies bound to quantum dots via the streptavidin–biotin system resulted in a more biologically reasonable 0.60 ± 0.14 IgG molecules per quantum dot for a 1:1 IgG:quantum dot molar ratio and, as would be expected, 1.3 ± 0.35 IgG molecules per quantum dot for a 2:1 ratio. This is equivalent to a functional volume of 0.943 $\mu$L of antibody for a 2:1 molar ratio or 0.53 $\mu$L for 1:1 molar ratio starting from 4 $\mu$L from a 0.5 mg/mL stock concentration. We acknowledge that these numbers are an approximation, since light chains near the quantum dot surface attached to a heavy chain bound to the quantum dot as part of a partial fragment would be sterically unavailable for antigen binding but could still dissociate following DTT reduction. However, we suspect this represents a small source of error because it is likely sterically difficult for bound heavy–light chain domains to bind to the quantum dot; it thermodynamically favors the functional partial fragment orientation (see Figure 2b). In any case, this error would contribute to an overestimation of the number of functional antibodies conjugated to a quantum dot. Therefore it represents an estimation of an upper bound on the number of putative functional antibodies, further emphasizing the significance of these results.

These results are significantly less than suggested estimates of between 2–10 antibodies conjugated per quantum dot. To the best of our knowledge no conjugation reaction can control the binding orientation of IgG molecules. Consequently, due to Brownian motion the number of bound functional antibodies is almost certainly less than the number of total bound IgG. An important question is: Why did covalent conjugations result in lower numbers of functional antibodies compared to streptavidin–biotin conjugations? One explanation is that DTT-reduced antibody conjugations attaching to the surface of quantum dots leave few opportunities for light chain fragments to be properly oriented outward and available for protein binding. Only partial fragments result in functional antibodies, and even then the
orientation of the partial fragment binding to the quantum dot surface must be correct to allow the light chain fragment to point outward in order to interact with its ligand. In biotin–streptavidin conjugations the antibody is never cleaved, leaving the whole molecule bound to the quantum dot surface and structurally offering more opportunities for light chain fragments to bind their targets. It is plausible that other covalent conjugation chemistries result in higher yields of functional antibodies, comparable to those we report for streptavidin–biotin conjugates or even higher, but it cannot simply be assumed so since, as we show here, at least one well established and commonly used covalent conjugation reaction results in low numbers of functional antibody on quantum dots. We propose that functionalized quantum dot labeling of biological preparations need to be preceded by the experimental determination of the number of functionalized antibodies per quantum dot, especially given the variability in conjugation methods between different labs. These considerations have a direct impact on the quality, interpretation, and relevance of biological or physiological results obtained using quantum dot labeling nanotechnologies.

Figure 4. Derivation of the average number of functional antibodies on both covalently conjugated and streptavidin–biotin conjugated quantum dots based on measurements of the bound density for different concentrations. (a and b) Fitted linear log control curves (ln y = ax – b) for known volumes of unconjugated IgG antibody band densities in SDS-PAGE gels. Note that the data for each gel were fitted with its own curve in order to control for inter-gel variability. Each symbol represents a different gel (n = 6 gels for covalently conjugated IgG conditions containing a total of 32 unconjugated IgG controls and 13 IgG–quantum dot complexes, and 7 gels for streptavidin–biotin IgG–quantum dot complexes containing a total of 35 unconjugated IgG controls and 28 IgG–quantum dot complexes). (c and d) Corresponding derived volumes from SDS-PAGE band densities for conjugated and streptavidin–biotin conjugated antibody–quantum dot complexes using the curves plotted in panels a and b, respectively. (e) Calculated values for the average number of antibodies conjugated to quantum dots for both conditions based on the derived measurements of functional antibody volumes (* and ** p < 0.01). See Supporting Information for detailed calculations.
Acknowledgment. We thank Invitrogen (formerly Quantum Dot Corp.) for the quantum dots. We also thank Craig Sharp for assistance with ImageQuant analysis and Diana Yu for assistance with Western blots. This work was supported by funds from NIH Grant R01 NS054736-01.

Note Added after ASAP Publication. The figure showing a schematic of antibody–quantum dot cross-linking and additional control conditions was omitted from the Supporting Information file posted May 31, 2007; the corrected version was published ASAP June 27, 2007.

Supporting Information Available: Descriptions of materials and methods including quantum dot conjugation and membrane transfer, additional information regarding additional sources of functional antibody loss, additional controls, and calculations of equivalent quantum dot conjugated antibody concentrations, and a figure showing antibody–quantum dot cross-linking and additional control conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

References


NL062706I
Characterization of the Functional Binding Properties of Antibody Conjugated Quantum Dots: Supplemental Materials

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Supplemental Materials

Materials and Methods

Quantum Dot Conjugation

605 nm quantum dots were conjugated to anti-GFAP and anti-CD90 IgG (BD PharMingen) using Quantum Dot Corporation’s 605 antibody conjugation kit (catalog #2200-1). Briefly, quantum dots were activated using the SMCC crosslinker, which resulted in a maleimide functional group on the surface of the particles. Antibodies were simultaneously reduced with DTT to cleave the disulfide bonds and make –SH groups available for conjugation. Quantum dots were then added to the reduced antibody solution where covalent coupling occurred. The reaction was then quenched with β-mercaptoethanol. Excess antibody was removed with size exclusion chromatography. After conjugation, quantum dots were stored at 4°C.

Biotin-streptavidin-quantum dot complexes were synthesized using modifications of published protocols. Briefly, biotin-streptavidin conjugates were formed by gentle vortexing followed by incubation of the biotinylated IgG with streptavidin coated 605 quantum dots (Invitrogen Corporation) for 30 minutes. Two reactions were performed: 1:1 and 2:1 molar ratios of biotinylated IgG to streptavidin coated quantum dots.

SDS-PAGE.

NuPAGE gels, 4-12% Bis-Tris (Invitrogen Catalogue # NP0321BOX), and 1M DTT in 1.0 mm X 10 mm wells were used for reduction of quantum dot conjugates. Briefly, 4X NuPAGE LDS sample buffer (Invitrogen Catalogue # NP0007), 1X NuPAGE sample buffer, DTT (in reduced samples only), and each sample were combined in a centrifuge tube according to standard
Western protocols. They were heated to 89°C for 15 minutes, centrifuged briefly, mixed gently, and loaded into the gels. Gels were run for 1.5 hours at 60 mA and 200 V in running buffer (760 mL of DI water and 40 mL NuPage MOPS SDS running buffer). Quantum dots were visualized in gels using a standard UV gel transilluminator. We ran 6 gels with covalently conjugated IgG containing a total of 32 unconjugated IgG controls and 13 IgG-quantum dot complexes. We ran 7 gels of streptavidin-biotin IgG-quantum dot complexes containing a total of 35 unconjugated IgG controls and 28 IgG-quantum dot complexes.

**Membrane Transfer**

Sponges, filter paper, and nitrocellulose membranes were soaked in transfer buffer (for one gel: 120 mL 100% methanol, 1020 mL DI water, and 60 mL NuPage transfer buffer concentrate (20X); for two gels: 240 mL 100% methanol, 900 mL DI water, and 60mL NuPage transfer buffer concentrate). After removing gels from their casings they were placed on soaked filter papers which were placed on top of two soaked sponges. Nitrocellulose membranes were cut, placed on the gels and covered with another piece of soaked filter paper. Two more sponges were placed on top of the second piece of filter paper and the entire sandwich was enclosed in the transfer apparatus. Transfer buffer was poured into the casings and run for 2 hours at 30 V.

**Visualization**

Membranes were blocked with 1.25 g of evaporated milk in 25 μl TBS (1 packet of Trizma Set Crystals (Sigma) in 2 L DI water, 17.6 g NaCl (200 mM), and 2 mL Tween-20) for 1 hour at room temperature. Secondary anti-mouse HRP conjugate was added and incubated for 1 hour at room temperature. Membranes were rinsed 3 X with TBS for 5 minutes each. SuperSignal West
Pico Chemiluminescent Substrate (Pierce Product #34080) was added for detection of HRP and incubated for 1 minute with the membranes. Visualization took place 10 minutes later with films pressed against the membrane blots for 1 sec, 30 sec, or 1 min development points. The films were processed in a standard film developer.

**Colloidal Blue Labeling**

Colloidal Blue (Invitrogen Catalogue # LC6025) labeling was performed in some gels instead of the transfer step. Briefly, gels were fixed for 10 minutes in fixing solution (40 ml DI water, 50 ml methanol, 10 ml acetic acid), incubated for 3 hours with Colloidal Blue dye, and rinsed for 7 hours with DI water. Images were taken with a digital camera. Note that smaller proteins (light chains especially) diffused out of the gels at longer incubation times.

**ImageQuant Analysis**

Membranes were scanned with an HP PSC 2175 scanner and loaded into ImageQuant software (Amersham BioSciences), which calculated the size and density of each band, and plotted them against the known concentration of the controls. The data in Fig. 4 were fit to linear log curves given by \( \ln y = ax - b \), where the parameters \( a \) and \( b \) were determined by ImageQuant. For quantification, gels were run with standards of 5 controls and 4 samples along with 1 lane of MagicMark Protein standard (Invitrogen LC5602). The 5 control lanes consisted of 1 μl, 0.75 μl, 0.5 μl, 0.25μl μl, and 0.1 μl of antibody from stock (BD PharMingin, 0.5mg/ml) to form the standard curves and 2 samples (4 μl and 2 μl) each of 1:1 and 2:1 IgG : quantum dot molar ratios for the biotin-streptavidin system.
**Supporting Information**

*Additional sources of functional antibody loss*

As described in the main text, any light chain that is covalently bound to the quantum dot surfaces will be unavailable for binding with target proteins due to steric considerations. Another potential source of antibody loss is free antibodies cross linking to other antibodies during the conjugation process (see supplementary figure panel a). The dark bands produced at the top of the membrane in Fig. 3a lanes 4-6 represent a significant fraction of antibodies that are cross-linked with each other. There is no fluorescence in the corresponding region of the gel, indicating that quantum dots were not present. Furthermore, non-reduced lanes (Fig 3a, lanes 2-3) do not have strong bands since quantum dots do not transfer to the membrane. It is only unbound networks of antibodies that are too large to run through the gel that remain in lanes 4-6. These cross-linked antibodies are also visible in the colloidal blue stain in the loading wells of Fig. 3b. This provides strong evidence that only cross-linked antibodies remained in the loading wells, and not antibodies conjugated to quantum dots. Cross linking does not occur in the biotin-streptavidin conditions (Figure 3c, lanes 2,3,5) creating another source of antibody loss in direct but not in biotin-strep- further increasing the difference in functionally available antibodies in the two methods.

*Additional controls*

Additional controls included non-functionalized quantum dots and partially conjugated quantum dots in order to ensure bands did not appear on membranes due to non-specific binding or other experimental artifacts (Fig. supplementary figure panel b). In lanes 2 and 4 of the supplementary figure, non-functionalized amino coated quantum dots were added; no bands were present on the corresponding membrane. Bare streptavidin quantum dots were also run with the same results:
no bands present on the corresponding membrane. Also, even though the concentration of antibody conjugated quantum dots was too low to show up in gels, protein bands were transferred to membranes (lanes 3 and 5). Other controls included using partially conjugated quantum dots. In particular, “excess IgG” quantum dots and “excess SMCC” (4-(N-maleimidomethyl)-cyclohexanecarboxylic acid N-hydroxsuccinimide ester) quantum dots (SMCC is used to cross-link amino and sulfhydryl groups) were collected just after the filtration cut off step for the collection of the functionalized quantum dots. No antibody was present in either of these lanes, demonstrating that only conjugated IgG quantum dot complexes result in enough light chain dissociations to be detected.

**NuPage versus NativeGel characterization of the number of bound antibodies**

Since different numbers of antibodies bind to different quantum dots, in theory, quantum dot bands should separate according to the number of antibodies bound due to differences in molecular weight. We tried to detect the differences in molecular weight for different antibody-quantum dot complexes using NativeGel. The NativeGel prevents antibody reduction, leaving all antibodies attached to the quantum dot. Unfortunately, due to excessive smearing, we were unable to discern any differences in molecular weight. With the NuPage, antibody dissociation from quantum dots prevented any differences in molecular weight to appear for different numbers of conjugated antibodies. Our method therefore presents an average number of functional antibodies per quantum dot.

**Calculations of equivalent quantum dot conjugated antibody concentrations**

The total number of quantum dot nanoparticles added to each well was calculated by multiplying the concentration from stock by the volume added. For direct conjugations, we used 2 μM and
for streptavidin quantum dots we used 1 μM. The resulting number (in moles) was then
multiplied by Avogadro’s number to obtain the total number of particles in the solution. Next, to
find the total number of antibodies in solution, the equivalent antibody concentration in μl
(obtained from ImageQuant) was converted to the equivalent antibody concentration in
milligrams by multiplying by the antibody stock solution concentration (0.5 mg/ml). Taking the
molecular weight of a single antibody to be 150kD (BD PharMingen) and converting it to grams
(i.e. multiplying by 1.650e-24) yielded 2.475e-19 grams per antibody. Using Avogadro’s number
gives a similar value with no change in the final result. The antibody concentration in
milligrams divided by the molecular weight of a single antibody in grams gives the total number
of antibodies in solution. Finally, dividing the number of antibodies by the number of quantum
dots in solution gives the number of antibodies per quantum dot.

For the direct conjugation method, 10μl of quantum dots were used at a 2μM concentration. To
obtain the total number of quantum dots in 10μl:

\[
\frac{2 \mu mol}{L} \cdot \frac{L}{10^6 \mu L} = 2e^{-6} \mu mol \cdot 10\mu L = 2e^{-5} \mu mol \cdot \frac{mol}{10^6 \mu mol} \cdot 6.022e^{23} \text{ particles/mole} = 1.2e^{13} \text{ quantum dots/sample}
\]

The average antibody equivalent concentration is 0.455 μl for a 0.5mg/ml stock solution.

\[
\frac{0.5 mg}{ml} \cdot \frac{0.455 \mu l}{1000 \mu l} \cdot \frac{ml}{mg} \cdot \frac{1}{2.475e^{-19} g/\text{antibody}} = 9.19e^{11} \text{ antibodies}
\]

\[
\frac{9.19e^{11} \text{ antibodies}}{1.2e^{13} \text{ quantum dots}} = 0.076 \text{ antibodies per quantum dot}
\]

For biotin-streptavidin conjugations:

\[
\frac{1 \mu mol}{L} \cdot \frac{L}{10e^6 \mu l} \cdot 4 \mu l \cdot \frac{5 \text{ mol}}{8 \cdot 10e^6 \mu mol} \cdot 6.022e^{23} = 1.505e^{12} \text{ quantum dots per sample}
\]
5/8 is used because 30 μl of antibody was reacted with 50 μl of quantum dots, so that

$$\left( \frac{50}{80} \cdot \text{total volume} \right)$$
yields the fraction in 1 μmol of quantum dots.

$$\frac{0.5 \text{mg}}{\text{ml}} \cdot \frac{0.943 \mu l}{1000 \mu l} \cdot \frac{0.0001 \text{g}}{\text{mg}} \cdot \frac{1}{2.475 \times 10^{-19} \text{g/antibody}} = 1.9 \times 10^{12} \text{ antibodies}$$

$$\frac{1.9 \times 10^{12} \text{ antibodies}}{1.5 \times 10^{12} \text{ quantum dots}} = 1.3 \text{ antibodies per quantum dot}$$

References
Supplementary Figure.  
a. Schematic showing antibody-quantum dot cross-linking, which prevents antibody-quantum dot conjugates from running through the gel and is a source of antibody loss.  
b. Additional control conditions (see text).