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Quantum Dot Nanoparticle Conjugation, Characterization, and Applications in Neuroscience

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Materials Science and Engineering

by

Smita Pathak

Committee in Charge:

Professor Gabriel Silva, Chair
Professor Mike Heller
Professor Xiaohua Huang
Professor Vlado Lubarda
Professor Sungho Jin

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Chair

University of California, San Diego

2008
Dedicated to

my family and friends.
There is nothing like looking,
if you want to find something.
You certainly usually find something,
    If you look,
But it is not always quite
The something you were after.

_J.R.R. Tolkien_
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Quantum dot are semiconducting nanoparticles that have been used for decades in a variety of applications such as solar cells, LEDs and medical imaging. Their use in the last area, however, has been extremely limited despite their potential as revolutionary new biological labeling tools.

Quantum dots are much brighter and more stable than conventional fluorophores, making them optimal for high resolution imaging and long term studies. Prior work in this area involves synthesizing and chemically conjugating quantum dots to molecules of interest in-house. However this method is both time consuming and prone to human error. Additionally, non-specific binding and nanoparticle aggregation currently prevent researchers from utilizing this system to its fullest capacity. Another critical issue that has not been addressed is determining the number of ligands bound to nanoparticles, which is crucial for proper interpretation of results.
In this work, methods to label fixed cells using two types of chemically modified quantum dots are studied. Reproducible non-specific artifact labeling is consistently demonstrated if antibody-quantum dot conditions are less than optimal. In order to explain this, antibodies bound to quantum dots were characterized and quantified. While other groups have qualitatively characterized antibody functionalized quantum dots using TEM, AFM, UV spectroscopy and gel electrophoresis, and in some cases have reported calculated estimates of the putative number of total antibodies bound to quantum dots, no quantitative experimental results had been reported prior to this work. The chemical functionalization and characterization of quantum dot nanocrystals achieved in this work elucidates binding mechanisms of ligands to nanoparticles and allows researchers to not only translate our tools to studies in their own areas of interest but also derive quantitative results from these studies. This research brings ease of use and increased reliability to nanoparticles in medical imaging.
CHAPTER 1: INTRODUCTION

Quantum dots are nanometer sized semiconducting nanoparticles that have unique optical properties which can be used for labeling targets of interest in biomedical sciences. Conventional fluorescent probes photobleach over time and thus cannot allow long term tracking of proteins or other molecules of interest. To date, quantum dots have had limited success in the biomedical sciences due to problems associated with non-specific binding, nanoparticle aggregation and the inability to delivery quantum dots to the cytoplasm of live cells.

The lack of characterization of functionalized nanoparticles is another hurdle that prevents researchers from properly interpreting the results of their data. The inability to probe the surface of quantum dots, combined with non-specific binding issues, prevents the use of standard techniques such as AFM, ELISA and gel electrophoresis. This work addresses these issues and presents novel methods for labeling cells and tissue sections using characterized functionalized quantum dot nanocrystals.
CHAPTER 2: QUANTUM DOT LABELING OF NEURONS AND GLIA

2.1 Introduction

Quantum dots are nanometer sized particles that have unique physical properties that make them well suited for visualizing and tracking molecular processes in cells using standard fluorescence microscopy [1-3]. They are readily excitable and have broad absorption spectra with very narrow emission spectra, allowing multiplexing of many different colored quantum dots. They also display minimal photobleaching, thereby allowing molecular tracking over prolonged periods. Additionally, they display a blinking property that allows the identification of individual quantum dots at the whole cell (i.e. micron) level. As a result, single molecule binding events can be identified and tracked using optical fluorescence microscopy, allowing the pursuit of experiments that are difficult or not possible given other experimental approaches.

2.2 Background

Quantum dots are composed of a heavy metal core such as cadmium selenide or cadmium telluride with an intermediate zinc sulfide shell or cap. This cap prevents the loss of fluorescence due to non-radiative emissive pathways. The particles can then be customized with an outer coating of different bioactive molecules tailored to a specific application (Fig. 2.1). The composition and very small size of quantum dots gives them
unique and very stable fluorescent optical properties that are readily tunable by changing the physical composition or size of the nanoparticles. Conjugating an antibody or targeting peptide to the surface of quantum dots allows selective fluorescent tagging of proteins similar to the tagging offered by classical immunocytochemistry but in a much more stable configuration (i.e. with negligible photobleaching). Another advantage of using quantum dot labeling over traditional immunocytochemistry is the higher signal to noise ratios, resulting in dramatically improved signal detection due to their broad absorption spectra but very narrow emission spectra.

![Figure 2.1 Structure of a semiconductor fluorescent quantum dot nanocrystal. The heavy metal core is responsible for the fluorescence properties of the quantum dot. The non-emissive shell stabilizes the core, while the external coating, made up of organic ligands, provides binding sites and an interface for application specific biologically active molecules.](image)

For biological applications quantum dots can be chemically functionalized to target proteins of interest at high ligand-receptor densities. Recent work has shown that, at least in some cellular systems, quantum dots conjugated with natural ligands are
readily internalized into cells, do not interfere with intracellular signaling, and are non-
toxic [1, 4-7].

2.3 Methods and Materials

We conjugated anti- β-tubulin III and anti-glial fibrilary acidic protein (GFAP) antibodies to 605 nm quantum dots and labeled primary cortical neurons, PC12 cells, primary cortical astrocytes, and r-MC1 retinal Muller glial cells. β-tubulin III and GFAP are ubiquitous cytoskeletal proteins specific to neurons and macroglia, respectively, but the protocols can be used to label any protein of interest. Where the use of specific products are indicated without further description on how to use them the reader can assume that the instructions that accompany that particular product work as indicated. We also list the vendor and catalog numbers for all products.

Lysine coated glass cover slips were prepared by incubating 12mm glass cover slips in 0.1% wt/vol poly-Dlysine (Sigma, catalog #P-7886) solution in double distilled water overnight. They were then washed 3 times with phosphate buffered saline (PBS, always at a corrected pH of 7.4; Gibco, catalog #14190-144) and allowed to dry. Cells were seeded onto the PDL coated glass cover slips in 24-well plates and incubated at 37°C for 24 hours in order to allow them to attach to the substrate. (For PC12 cells, 1.25μl of nerve growth factor (NGF; Invitrogen, catalog #13257-019) was added at a concentration of 20μg/ml to each well.) After incubation periods of 24-48 hours for PC12’s in NGF and induction media (induction media: 490 ml DMEM high glucose, 5
ml fetal bovine serum (FBS), 5 ml penicillin-streptomycin-neomycin) and 24-48 hours with regular growth media for Muller cells, the culture media was removed from the wells by gentle aspiration and the cells washed with warmed PBS. Primary cortical astrocytes, neurons, and growth media were obtained from Cambrex (catalog #R-CXAS-520 and R-CX-500 respectively) and the cells grown as indicated. Primary neurons and glia were also seeded onto PDL coated glass, although astrocytes will adhere to non-coated glass also.

All cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, catalog #157 15-S) in PBS for 10 minutes at room temperature, followed by three washes of 5 minutes each with PBS. The cells were permeabilized with 0.2% Triton X-100 (Fisher Scientific, catalog #BP151-100) in PBS for 5 minutes and washed again three times for five minutes in PBS. They were then incubated with 10% horse serum in PBS for 30 minutes at room temperature, followed by a another quick rinse in PBS. We then applied a biotin/streptavidin blocking kit (Vector Labs, catalog #SP-2002) in order to block endogenous biotin in the samples before treating with functionalized quantum dots. This was followed by another three times five minute washes in PBS.

At this point one would incubate with a biotinylated molecule of interest. In our case we used anti-β-tubulin antibody at a dilution of 1:100 (BD Pharmigen, catalog #556321) and anti-GFAP antibody at a dilution of 1:1000 (BD Pharmigen, catalog #556330) in PBS with 10% horse serum. Note that we used the ProtOn Biotin Labeling Kit in order to biotinylate the antibodies (Vector Labs, catalog #PLK-1202). Also note
that a GFAP dilution of 1:100 also works well if needed, but we found negligible
differences using a 1:1000 dilution. For controls, we incubated each antibody at their
respective dilutions in 10% horse serum without biotinylation, and incubated with 10%
horse serum in the absence of primary antibody in order to control for non-specific
binding of quantum dots or streptavidin (see below). All primary antibody and control
incubations were for 2 hours at room temperature. This was followed by three times
five minute washes with PBS.

At this point we added 605 nm streptavidin conjugated quantum dots (Quantum
Dot Corporation, catalog #1010-1) using their suggested dilution of 1:100 in 10% horse
serum. A 1:1000 dilution of quantum dots also labeled cells but with more punctuate
labeling. For controls, we used secondary antibody anti-mouse TRITC IgG at a dilution
of 1:100 (Sigma, catalog #T-7782) following primary antibody incubations without
biotinylation. Both quantum dots and controls were incubated for 1 hour at room
temperature, rinsed three times five minutes with PBS, and mounted with 90% glycerol
(Sigma, catalog #G-6279) in PBS. All experimental conditions within a given
experiments were replicates of at least five and all experiments were repeated in their
entirety three to five times.

An alternative labeling method involves doing three incubations instead of two
but provides a greater amount of specificity and cytoanatomical detail (Fig. 2.2 A-C;
Fig. 2.2 D-F reflects the 2 step process). After the biotin-streptavidin blocking step
described above, we incubated with primary antibody in PBS with 10% horse serum for
one hour. This was followed by three times five minute washes with PBS. A biotinylated secondary antibody in PBS with 10% horse serum was then added. In our case, we used anti-mouse IgG at a dilution of 1:200 (Sigma, catalog#B7151). After a one hour incubation, the cells were rinsed again three times for five minutes in PBS. Finally, 605 nm streptavidin conjugated quantum dots were added with 10% horse serum and the cover slips mounted with 90% glycerol in PBS. This was later found to quench quantum dot fluorescence over time. Currently Cytoseal 60 is being used to mount coverslips.

All images were acquired using an Olympus IX81 inverted fluorescent confocal microscope (Olympus Optical, Tokyo, Japan) that included epifluorescence, confocal, phase, brightfield, and Hoffman differential interference contrast (DIC) modalities. Our microscope was equipped with a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and Image-Pro Plus data acquisition and morphometric software (version 5.1.0.20, Media Cybernetics, Inc., Silver Spring, MD). Of particular note, although the 605 nm quantum dots could be visualized with our standard TRITC filter set, they appear much brighter and can be imaged at much shorter acquisition times using the XF304 Qdot605 filter set from Omega Optical.

2.4 Results

Using our protocols we were able to get excellent specific labeling of β-tubulin in neurons and PC12 cells and GFAP in astrocytes and Muller cells, with negligible
non-specific binding or background (see Fig. 2.2). Labeling with unconjugated or primary antibody omitted streptavidin conjugated quantum dots showed no labeling at all (data not shown). β-tubulin and GFAP labeling using functionalized quantum dots displayed similar labeling patterns to those expected using standard immunocytochemistry (ICC) controls visualized with fluorophor tagged secondary antibodies (Fig. 2.2 G and H).

For comparable imaging conditions, quantum dot labeled cells were brighter and displayed more detailed and sharper microstructural anatomy. The pattern of quantum dot labeling was typical for that observed in other cell types, displaying a dense punctuate pattern and fine details of both intracellular intermediate filaments and cellular processes, unlike traditional fluorophores which tend to have a diffused appearance due to the broad point spread function of their fluorescence signal. Non-specific artifact labeling using some quantum dot protocols may label neural cells incorrectly due to nonspecific putative electrostatic interactions. We observed this when conjugating antibodies directly to quantum dots, which resulted in unconjugated quantum dots non-specifically staining the nucleus of Muller cells (see Fig. 2.2 F). Non-specific binding was also observed when using other published protocols for non-neural cells[8].

Blocking conditions need to be carefully optimized since most standard blocking approaches did not work satisfactorily in our hands, including 1-5% bovine serum
albumin, 10% horse serum, and 10% fetal bovine serum among others, which resulted in a high level of non-specific quantum dot binding to the cells (data not shown).

Qualitative and potentially quantitative information can be measured for individual binding events between quantum dot conjugated molecules and their cellular molecular targets, a direct result of the underlying physics[3, 6] that cannot be done with standard ICC (see [9] for an example). The wide spread use of quantum dot nanotechnology in molecular and cellular neurobiology has the potential to open the door to new experiments that can not be achieved with other methods.
Figure 2.2. Targeted fluorescent quantum dot labeling of neurons and glia. A. PC12 neurons specifically labeled for β-tubulin using anti-β-tubulin antibody conjugated 605 nm quantum dots. B. Close up of two quantum dot labeled neurons. C. PC12 neurons labeled for β-tubulin using standard immunocytochemistry. Nuclei were stained with a non-specific Hoechst stain. D. r-MC1 retinal Muller glial cells specifically labeled for glial fibrillary acidic protein (GFAP) using anti-GFAP antibody conjugated 605 nm quantum dots. E. r-MC1 Muller cells labeled for GFAP using standard immunocytochemistry. F. An example of artifact non-specific labeling of r-MC1 Muller cells labeled with anti-GFAP conjugated 605 nm quantum dots. In this case putative non-specific electrostatic interactions between quantum dots and cellular proteins led to intense nuclear staining and mild cytoplasmic staining using quantum dots conjugated based on other conjugation protocols described for mammalian cells. Care must be taken to validate true labeling of target molecules of interest in the specific cell types being investigated. (All imaging parameters were kept constant for the different experimental conditions, with an acquisition/exposure time of 30 ms for all panels except F, which was taken with an acquisition time of 100 ms.)

2.5 Discussion

Quantum dots represent a new tool that has a variety of advantages over traditional labeling methods and can be used to identify previously undiscovered processes in neuroscience research. Besides offering an alternative to traditional immunocytochemical approaches that cannot compete with the ability of quantum dots
to last longer and label many different ligands at once (i.e. photobleaching stability and multiplexing, respectively), they are particularly adept at addressing some of the unique molecular challenges encountered when investigating neurons and glia. In particular, quantum dots provide the ability to visualize, measure, and track individual molecular events using optical (i.e. fluorescence) microscopy; and they provide the ability to visualize and track dynamic molecular processes over extended periods (e.g. tens of minutes at a time). These properties provide a unique set of experimentally attainable conditions that in general cannot be achieved using other techniques or approaches. In particular, quantum dots support experiments that are limited by the restricted anatomy of neuronal and glial interactions, such as the small size of the synaptic cleft [9]; or between an astrocyte process and a neuron. Another area that they are well suited for is tracking the molecular dynamics of intracellular and/or intercellular molecular processes over long time scales (e.g. where organic fluorophores would experience significant bleaching) or over very short physical dimensions that can take advantage of their extremely small size and optical resolution.

Emerging work using quantum dots targeted specifically to neuroscience research, although still few, illustrate the significant potential of this technology in the lab. Triller and colleagues used antibody functionalized quantum dots to track the diffusion dynamics of glycine receptors in cultures of primary spinal cord neurons [9]. They were able to track the trajectory of individual glycine receptors for tens of minutes at spatial resolutions of 5 to 10 nm, and were able to show that the diffusion dynamics of the receptors varied depending on the area in which they were found (defined as
synaptic, perisynaptic, or extrasynaptic). Vu, Desai and colleagues tagged nerve growth factor (βNGF) to quantum dots and used them to activate TrkA receptors to promote neuronal differentiation in cultured PC12 cells [10].

However, as with any new technology considerable work remains; for example Vu et al. measured reduced effects of βNGF conjugated to quantum dots compared to free βNGF, although significantly higher than negative controls. Other groups are pushing the technology forward and providing new quantum dot based tools for neuroscience research. For example, Brinker and colleagues have developed a technique to produce biocompatible water soluble quantum dot micelles that retain the optical properties of the individual quantum dots. These authors were able to demonstrate the uptake and intracellular dispersion of the quantum dot containing micelles by cultured hippocampal neurons [11]. Ting and colleagues are developing a modified quantum dot labeling approach that addresses the relatively large size of antibody-quantum dot conjugates and instability of some quantum dot-ligand interactions. Their technique tags cell surface proteins of interest with a specific peptide (a 15 amino acid polypeptide called acceptor protein that has the sequence GLNDIFEAQKIEVWHE) that can be directly biotinylated in order to act as a target for streptavidin-conjugated quantum dots [12]. Using this approach they were able to specifically label and track AMPA receptors on cultured hippocampal neurons.

Ultimately, the wide spread use of quantum dot nanotechnologies in neuroscience research will require easy to use approaches that can be either easily
replicated in a typical neurobiology lab or take advantage of the growing number of commercially available products. Of particular note, as far as we are aware these results represent the first application of quantum dots to glial cells. As with any new research tool only time will tell what its ultimate success will be, but given the unique properties that quantum dots have to offer neuroscience research their future looks pretty bright (and stable).

CHAPTER 3: FUNCTIONALIZED QUANTUM DOT CHARACTERIZATION AND QUANTIFICATION

3.1 Introduction

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. By chemically conjugating antibodies and other peptides to their surface, quantum dots can specifically target cellular ligands of interest. 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. However, we also demonstrated reproducible nonspecific artifact labeling, which could be mistaken for specific labeling, if antibody-quantum dot conditions are less than optimal.
3.2 Background

While other groups have qualitatively characterized antibody-functionalized quantum dots using transmission electron microscopy, atomic force microscopy, UV spectroscopy, and gel electrophoresis[13] and in some cases have suggested estimates of the putative number of total antibodies bound to quantum dots[14], 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.

3.3 Methods

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[10]. 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.

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, 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.

For visualization, membranes then 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 for1 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 to visualize protein
remaining on the quantum dot nanoparticles. 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.

For quantification of the proteins, 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 was 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 \( \mu l \), 0.75 \( \mu l \), 0.5 \( \mu l \), 0.25\( \mu l \), and 0.1 \( \mu l \) of antibody from stock (BD PharMingen, 0.5mg/ml) to form the standard curves and 2 samples (4 \( \mu l \) and 2 \( \mu l \)) each of 1:1 and 2:1 IgG : quantum dot molar ratios for the biotin-streptavidin system.

3.4 Results

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 (Figure 3.1).
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 (Figure 3.2). 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).

![Figure 3.2](image)

**Figure 3.2** Separation of IgG antibodies into fragments using SDS-PAGE and membrane transfer for biotinylated IgG bound to streptavidin-coated quantum dots and controls.

We begin the analysis of these results 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 3.4). 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.

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 3.1, lanes 4-6). As expected, this separation occurred minimally in lanes without DTT (Figure 3.1, lanes 2-3). The presence of a weak band at the 25 kD position in nonreduced lanes (Figure 3.1, 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.
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 3.3). 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. The association of V regions from both heavy and light chains is what actually forms the antigen binding site.

Another important consideration to note is that the amount of partial fragments (at 75 kD) initially available for binding to quantum dots following the initial DTT reduction was very low, as evident in the reduced unconjugated IgG controls (Figure 3.2, 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.)

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 3.1, 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 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 3.1).
This gives rise to three possible antibody fragment binding scenarios to quantum dots (Figure 3.4): covalently bound light chains, covalently bound heavy chains, and covalently bound heavy-light chain partial fragments. Only the partially fragments 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.
Figure 3.4. Antibody reduction and conjugation to quantum dots. (a) 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. (b) Similar schematic for biotinyalted antibodies conjugated to streptavidin-coated quantum dots. (c) 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.
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 3.4). 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, nonreduced conditions resulted in quantum dots remaining in the loading wells (Figure 3.2 B, lane 4) while reduced conditions allowed quantum dots to run through the gels (Figure 3.2 B, 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 3.2, 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 3.2, lanes 2, 3, 5, 6, and 8).

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 3.2), we derived the average number of functional IgG conjugated to quantum dots. We use the term “functional antibody” to describe the amount of Fab light chain, which includes a part of the target protein binding epitope, that is physically oriented outward from a quantum dot and 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 Fab light chain portion of the antibody that actively binds to proteins, quantifying the amount of light chain fragments not directly bound to the particle 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 kD light chain bands and compared them to controls of known antibody concentrations. Using image analysis software that measures the band density of electrophoresis gels (Image- Quant TL, GE Healthcare; see Methods in the Supporting Information), we fitted curves to known antibody concentrations to obtain standard curves of IgG band densities.

Using these curves, we then determined the concentration of IgG bands associated with covalently bound IgG, 2:1, and 1:1 IgG:quantum dot molar ratio
streptavidin-biotin conjugation conditions (Figure 3.4). We calculated the number of functional antibodies bound to the quantum dots for each condition. 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 uL of antibodies directly conjugated to quantum dots is equivalent to adding 0.455 uL 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. In contrast, the number of antibodies bound to quantum dots via the strepavidin-biotin system resulted in a more biologically reasonable 0.60 (0.6 IgG molecules per quantum dot for a 1:1 IgG/quantum dot molar ratio) and, as would be expected, 1.3 (1.3 IgG molecules per quantum dot for a 2:1 ratio). This is equivalent to a functional volume of 0.943 uL of antibody for a 2:1 molar ratio or 0.53 uL of antibody for 1:1 molar ratio for a 4 uL sample of quantum dot conjugated sample made from a 0.5 mg/mL antibody stock concentration.

We acknowledge that these numbers are an approximation, since light chains near the quantum dot surface attached to a heavy chain directly 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. In any case, this error would contribute to an overestimation of the number of functional antibodies conjugated to a quantum dot.
Figure 3.5 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 (c and d) Corresponding derived volumes from SDS-PAGE band densities for direct bound 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.
In Figure 3.5 also 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).

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 2-5 antibodies conjugated per quantum dot[14]. 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.

Additional sources of functional antibody loss include any light chain that is covalently bound to the quantum dot surfaces which 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 (Figure 3.6).
Figure 3.6 Additional sources of antibody loss, in particular, through antibody cross-linking to other quantum dots, resulting in aggregation of nanoparticle-antibody conjugates and low functionality of antibodies.

The dark bands produced at the top of the membrane in Fig. 3.1 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 3.1, 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. 3.3. 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 3.2, 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 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. 3.7)

Figure 3.7 Additional control conditions including bare quantum dots which did not transfer to the membrane (lanes 2 and 3).
In lanes 2 and 4 of Figure 3.7, non-functionalized amino coated quantum dots were added and no bands were present on the corresponding membrane. Bare streptavidin quantum dots were also run with the same results: no bands were 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-Nmaleimidomethyl)-cyclohexanecarboxylic acid N-hydroxysuccinimide 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 were performed 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. 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.
3.5 Discussion

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 fragments 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.

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4.1 Introduction

Semiconductor quantum dot nanoparticles have unique fluorescence optical properties that, when chemically functionalized with biological ligands, have the potential to provide high resolution labeling of biological preparations [2, 3, 15-18]. Despite their use and success with a variety of cell types and tissues, the application of functionalized quantum dots for imaging neural cells and tissues has been much more limited, with only a few examples described in the literature. This is the result of some of the unique challenges associated with working with neural cells, including their highly specialized and terminally differentiated phenotypes, issues associated with preserving complex and often delicate cellular and tissue cytoarchitectures, and the lack of neural specific optimized labeling protocols, since most published protocols for non-neural cell types do not immediately translate to neurons and glia. Still, progress in the application of quantum dot labeling and imaging techniques to neural cells is being made by both our group and others for high resolution cytoanatomical imaging, dynamic protein and receptor tracking, and induced functional responses [9, 10, 19], and it can be argued that the use of quantum dot nanotechnologies as a novel
neuroscience research tool will be very significant, with few other fields potentially benefiting as much.

4.2 Background

In particular, one area that has seen very little to no progress is the development and use of functionalized quantum dots for labeling neural tissue preparations. However, well validated labeling and imaging methods for neural tissues is crucial because of the very significant challenges associated with elucidating structural and anatomical relationships in neuronal and glial “wiring”, which ultimately have a direct bearing on imaging and measuring neural function at the cellular level. This is even more so when large volumes of neural tissue are considered in an attempt to visualize complex spatial network organizations. These challenges are the direct result of the morphological nature of neurons and glial cells (specifically astrocytes and oligodendrocytes) that can, for example, have multiple long processes and/or fantastic fractal like arborizations (e.g. the dendrites of hippocampal pyramidal cells). It is anticipated that the use of quantum dot specific labeling will significantly facilitate the imaging of sub-populations of different neural cell types in their native environment while preserving the cytoarchitecture, and will compliment emerging methods for imaging dynamic neural function[20].

Here, building on our previous work, we have optimized functionalized quantum dot labeling protocols targeting glial fibrillary acidic protein (GFAP) in glial cells in
intact mammalian neural retinal preparations, specifically astrocytes in the inner nuclear
layer of the neural sensory retina and Muller cells that span the thickness of the retina
up to the inner limiting membrane. We show that in a laser induced rat model of
choroidal neovascularization (CNV), which is a traumatic model of the vascular
changes associated with specific forms of human macular degeneration, the detection of
induced reactive gliosis based on an upregulation in GFAP intermediate filaments can
be visualized at high resolutions and with excellent signal to noise ratio (i.e. very little
non-specific background). Reactive gliosis is a ubiquitous physiological response to all
forms of traumatic and degenerative events in the central nervous system (CNS) which
although part of the CNS’s natural injury response produces biochemical responses and
scarring that act as barriers to functional neuronal regeneration[21-24]. In addition, we
labeled individual dissociated spinal cord astrocytes using anti-GFAP quantum dot
conjugates and identified networks of fine intermediate filament processes that
cytostucturally form intercellular bridges and link different cells. This data raises
questions about the structural and functional cellular continuity between cells in the
astrocyte synsition which would have been difficult to visualize using traditional
optical techniques.

In the neural retina, the upregulation of GFAP and associated gliosis directly
contribute to the apoptosis of photoreceptor neurons associated with macular
degeneration[25]. As such, being able to image the spatiotemporal evolution of the
gliosis response in detail at high resolution compliments other molecular and cellular
methods for investigating it and will provide new insights into its progression and
contribution to the disease process. Quantum dot labeling and imaging also provides the opportunity to investigate and address fundamental cellular questions where the use of conventional methods fall short. For example, the upregulation of GFAP in Muller cells starts in their endfeet processes closest to the retinal capillaries in the ganglion cell layer (GCL) where the mechanism of GFAP polymerization into intermediate filaments is still not clear. New GFAP filaments appear to be assembled at a site away from the nucleus because unpolymerized subunits can be found in the cytoplasm, resulting in intermediate filament outgrowth [26]. It has also been proposed that mRNA for GFAP is transported from the cell body to the end feet where new GFAP is synthesized [27]. Imaging gliosis and the glial scar using standard fluorescent dyes has been done [28], but the level of cellular detail and therefore tissue reconstruction is limited due to relatively high non-specific background and signal noise associated with the use of traditional fluorophores (e.g. the need for longer acquisition times due to low signals depending on conditions, photobleaching, etc.). All of these issues will benefit from the quantum dot protocols we introduce here.

Labeling protocols using anti-GFAP antibody functionalized semiconductor quantum dot nanocrystals were optimized and used for specific labeling of GFAP intermediate filaments in astrocyte and Muller glial cells in microtomed sections of intact rat neural sensory retina and dissociated primary spinal cord astrocytes. Quantum dot labeling resulted in stable and robust imaging of retinal astrocytes and Muller cells with very little non-specific background labeling and bright intense fluorescence resulting in a high signal to noise ratio. Quantum dots clearly and efficiently imaged
normal levels of GFAP in the retina and differentiated it from pathologically high levels of GFAP associated with a reactive gliotic response. The specificity of the labeling in combination with the very low non-specific background allowed the clear visualization of the gliosis boundary, and should facilitate quantitative area and volume measures of the spread and extent of gliosis not possible using other optical fluorescence methods. Labeling and imaging of dissociated astrocytes at high resolution demonstrated the presence of what appeared to be highly complex organizations of fine GFAP intermediate filaments that spanned between cells to form intricate networks of filamentous intercellular bridges. The identification of similar bridges in situ and ultimately in vivo, and the investigation of their functional roles will be the focus of future work. However, the discovery of such structures using optical imaging at micron scales was greatly facilitated by the quantum dot labeling.

4.3 Methods and Materials

Six Brown Norway rats weighing between 200 and 350 g were used for the experiments described. All procedures and methods were in accordance with the animal care and use guidelines of the University of California, San Diego Institutional Animal Care and Use Committee (IACUC), and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were anesthetized with an intraperitoneal injection of ketamine (21 mg/kg) and xylazine (5.25 mg/kg) and the pupils dilated with 0.5% tropicamide and 2.5% phenylephrine. The fundus was visualized with a 5.4 mm rat fundus laser lens
(Ocular Instruments, Bellevue, WA) with a 2.5% hydroxypropyl methylcellulose solution. A diode laser (Oculight SLx; Iridex Co., Mountain View, CA) with a wavelength of 810 nm was used to induce focal lesions on the retina. Laser parameters consisted of a 75 µm spot size, 100 ms exposure time, and 280-350 mW of power. A pattern of 5 to 7 spot lesions was concentrically placed around the optic disk in both eyes of each rat. The formation of a bubble indicated rupture of Bruch's membrane.

Rats were sacrificed 3 weeks after the laser procedure was performed. Eyes were enucleated and fixed in either 4% paraformaldehyde or in 70% ice cold methanol for 2 hours. They were then rinsed with phosphate-buffered saline (PBS). The eyes were then embedded in paraffin and sliced into 10 µm sections with a microtome. Slices were subsequently placed on coverslips and incubated at 65 °C overnight.

A variety of antigen retrieval methods were tested due to poor labeling following standard techniques. Heat treatment was used, both with the DAKO target retrieval buffer as well as with the standard 10 mM TBS-HCl solution. This method resulted in very low labeling both for quantum dots and for standard fluorescein isothiocyanate (FITC) fluorophore controls. Some labeling was achieved with the DAKO target retrieval buffer with quantum dots, but very minimal labeling was observed using standard immunocytochemistry. Proteinase K was also used to permeabilize the tissue, which resulted in good labeling for FITC, but was not sufficient to allow enough permeabilization of the tissue for quantum dot labeling to be achieved. The best results by far were achieved by incubating tissue sections with 0.1%
Trypsin for 30 minutes. Both FITC immunocytochemistry and quantum dot labeling results were bright and reproducible. After permeabilization, retinal sections were washed with PBS.

Single cell antibody functionalized quantum dot labeling was done using our previously published methods[29], while quantum dot labeling of retinal tissue sections was done using a modification of these methods. Specifically, retinal sections were blocked in 3% bovine serum albumin (BSA) for 30 minutes to prevent any non-specific binding of the antibody. This was followed by incubation of primary rat anti-GFAP (BD Pharmagen, San Diego, CA). Primary antibody was incubated with tissue sections in 3% BSA for 2 hours at room temperature. Samples were then subsequently washed three times in PBS, followed by treatment with a biotin-streptavidin blocking kit (Vector Labs, Burlingame, CA). A secondary biotinylated anti-rat antibody (BD Pharmagen, San Diego, CA) was incubated with the tissue sections for 1 hour at room temperature in 3% BSA followed by three washes in PBS. Finally, 605 nm streptavidin conjugated quantum dots from Invitrogen (Carlsbad, CA) were incubated for 30 minutes at room temperature followed by 3 washes in PBS. Samples were then mounted with Cytosol 60 and left overnight to dry. Note that incubation with glycerol, as recommended by Invitrogen, was found to quench quantum dot fluorescence over time and was not used.

Direct covalently bound anti-GFAP antibody quantum dot conjugates were also used to label retinal tissue sections. In particular, the same anti-mouse secondary 605
nm quantum dots from Invitrogen were used but without the biotin-streptavidin blocking kit. The results (data not shown) showed little to no labeling using all antigen retrieval methods. The larger size of covalently bound antibodies to the quantum dots may prevent their tissue penetration and therefore use in tissue labeling. Indeed, some labeling was observed on the surface of retinal tissue preparations, but none seemed to penetrate the tissue enough to allow labeling deeper into the sections. Streptavidin quantum dots on the other hand are much less bulky and in our hands proved to result in much more reproducible labeling with a low non-specific background and high signal to noise ratio, making the additional processing steps very worthwhile.

All images were obtained with an Olympus IX81 inverted fluorescent confocal microscope (Olympus Optical, Tokyo, Japan) equipped with a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and Image-Pro Plus data acquisition software. The XF304 Qdot605 filter set from Omega Optical (Brattleboro, VT) was used to optimize the collected quantum dot fluorescence.

4.4 Results

In the normal neural retina GFAP expression is associated with the astrocyte layer in the inner nuclear layer and the endfeet of Muller cells near the retinal capillaries. Quantum dot labeling of GFAP in control sections of rat retina showed only Muller cell endfeet and astrocytes were GFAP positive, with no GFAP upregulation and no non-specific binding (Fig. 4.1 a-b). The high specificity and signal to noise ratio of
our quantum dot labeling protocol is particularly emphasized in fig. 4.1 because the micrographs were taken in widefield non-confocal mode which collects light from the entire thickness of the tissue slice, unlike in confocal mode where stray light is physically excluded from the plane of focus. This is critical because, as we have previously shown, one of the biggest difficulties associated with immunospecific quantum dot labeling of neural cells is non-specific interactions and clumping between quantum dot particles that can produce false positive results[30].

Methodologically, non-specific binding proved to be more of an issue with the methanol fixed samples. All samples shown in the results were fixed using paraformaldehyde followed by Triton 100-X to permeabilize the tissue. It is essential for the bulkier quantum dots, compared to the size of traditional fluorophores, to experience a low amount of cross-linking in the tissue in order to avoid clumping-achieved in the target retrieval step.

It is a common mistake to assume that quantum dot nanoparticles are smaller than fluorescent dyes, when in fact they are 10-20 times larger (depending on the color) than FITC. Serially sectioned 10 μm slices throughout the thickness of the retina, of which two consecutive slices are shown in Fig. 4.1 A and B, showed no observable non-specific labeling. These results are similar to control retinal sections labeled using traditional immunocytochemistry with a primary antibody specific to the target antigen and a FITC tagged secondary antibody that binds to the primary antibody and acts as a fluorescent reporter (Fig. 4.1 C-D), although the FITC labeling was somewhat more
qualitatively diffuse and did show some degree of non-specific labeling despite our best attempts.

Figure 4.1: Control labeling of the non-injured rat neural sensory retina for glial fibrillary acidic protein (GFAP). (a-b) Anti-GFAP antibody functionalized quantum dot conjugates specifically label only Muller cell endfeet associated retinal capillaries and astrocytes associated in the inner nuclear layer associated with retinal ganglion cells. Two slices from a widefield non-confocal image stack are shown, and display no observable non-specific labeling despite the use of non-confocal mode. (c-d) Widefield non-confocal standard immunocytochemistry using an anti-GFAP conjugated primary antibody and FITC fluorophore tagged secondary antibody (green). A non-specific nuclear DAPI stain was used to visualize the other retinal layers. Note the more diffuse labeling using FITC compared to the quantum dots and the presence of some non-specific labeling in the distal layers of the retina. Panels (a), (b), and (d) were taken at 40x and 50 ms exposure times, while panel (c) was taken at 20x at a 50 ms exposure. All micrographs are 10 μm slices.
Upregulation of GFAP in Muller cells and astrocytes occurs only under pathological conditions and is considered the hallmark of the reactive glial response. GFAP upregulation in Muller cells is particularly apparent because it spans the length of their cell bodies throughout most of the thickness of the retina up to the inner limiting membrane. In the rat laser induced CNV model we used, gliosis and glial scarring occur as secondary processes and result in a strong upregulation of GFAP. Figure 4.2 shows a confocal z-stack of a 10 μm tissue section with an imaged slice thickness of 1 μm centered at a laser induced lesion site.
Figure 4.2: Specific labeling of GFAP upregulation in the rat neural retina at a laser induced lesion site imaged using anti-GFAP quantum dot conjugates. A serial cross-section of the retina 10 μm thick that encompassed one of the induced lesions was imaged at 1 μm thick optical slices using confocal microscopy using a 1.8 second exposure time.

To the best of our knowledge, these results represent the first successful specific labeling in situ of an intact neural tissue preparation. The intense upregulation in GFAP in both Muller cells and astrocytes indicated a strong reactive response to the induced trauma. Our quantum dot labeling protocol was optimized to ensure even tissue penetration, maximal non-specific antigen labeling, and maximal specific antigen retrieval. Given this, the fact that the upregulation in GFAP for all lesions we looked at
extended over a cross-sectional thickness of the retina of about 10 μm, as demonstrated in fig. 4.2 by the drop in fluorescence signal in the confocal stack by slice 10, suggested that the reactive volume of the neural retina in response to the laser induced injury parameters detailed in the experimental methods section is on average between 9-10 μm in cross-sectional width. The high signal to noise ratio of the quantum dot labeling procedure also putatively provides greater observable and therefore measurable cellular detail throughout the volume of the glial response. In the representative stack in fig. 4.2 the upregulation progresses from proximal Muller cell processes near the boundary of the lesion site (progressively from slice 1 to slice 3) to the entire length of the Muller cells and astrocyte layer near the center of the lesion (slices 4 to 6), followed by a progressive visible decrease in reactivity near the other side of the lesion boundary (progressively from slice 7 to 9).

Therefore, this labeling method should be amenable to quantitatively measuring the extent and thickness of glial scars and presumably other neuronal and glial specific markers in neural tissue preparations at high spatial resolutions due to the cellular specificity and low background of the procedure. Such an approach would conceivably allow better quantitative measurements and statistics of both physiologically normal and, as illustrated here, pathological cellular processes.
This quantum dot labeling procedure is considerably superior to non-confocal widefield epifluorescence microscopy of retinal sections for specific labeling and imaging of GFAP upregulation in gliosis due to diffuse labeling and higher non-specific background in the latter (Fig. 4.1), as introduced above. In our hands the quantum dot labeling procedure was subjectively less diffuse, more intense, had a noticeably lower non-specific background, and showed more cellular detail than the best optimized standard FITC immunochemistry labeling we could achieve (Fig. 4.3 c-d). This was especially true near the border of imaged lesions where GFAP upregulation gradually decreased and there was less signal intensity (Fig. 4.3 d). In these cases FITC labeling appeared to display considerably higher non-specific background, which makes this approach less than ideal for identifying the edges of GFAP upregulation and gliosis lesion boundaries, resulting in less confidence in any derived measurements of lesion volumes and the spread of the reactive glial response.
Figure 4.3: Specific labeling of GFAP upregulation in the rat neural retina at a laser induced lesion site imaged using standard FITC immunocytochemistry. (a-b) Widefield non-confocal standard immunocytochemistry using an anti-GFAP conjugated primary antibody and FITC fluorophore tagged secondary antibody (green). A non-specific nuclear DAPI stain was used to visualize the other retinal layers. (c-d) Confocal imaging of two different lesions with a 1.6 μm optical slice and an acquisition time of 2 seconds, comparable with the data shown in fig. 2. Panel (c) shows a slice near the center of a lesion, while panel (d) shows a slice closer to the boundary of a lesion. Note the particularly high background and diffuse labeling in (d).

Finally, in order to qualitatively investigate the expression patterns of GFAP in individual cells in more detail, we labeled and imaged dissociated astrocyte cultures at high resolution using functionalized quantum dots and epifluorescence microscopy. To
our surprise, the organization of GFAP intermediate filaments appeared much more complex and intricate than anticipated, and in many cases displayed fine web-like structures that branched from larger filament bundles (Fig. 4.4). Most interesting were what appeared to be intercellular GFAP intermediate filament bridges that connected neighboring cells. These filaments extended from the main processes themselves in type I stellate astrocytes (Fig. 4.4 a arrows), and directly connected the cell bodies of type II protoplasmic astrocytes (Fig. 4.4 b arrows). In addition, the quantum dot labeling revealed fine web-like networks between the processes of some cells that appeared to fill in the volume between them (Fig. 4.4 c).
Figure 4.4: High resolution epifluorescence specific labeling of GFAP intermediate filaments in dissociated primary spinal cord astrocyte cultures imaged using anti-GFAP quantum dot conjugates. Note the fine intercellular bridges that extend out from primary intermediate filament bundles and appear to join neighboring cells. These cellular bridges are apparent in type I stellate astrocytes (a, arrows), type II protoplasmic astrocytes (b, arrows), and appear to branch off and fill in the volume between processes (c). Panel (d) shows a composite micrograph of a GFAP positive field of view.

To the best of our knowledge such structures have not been previously reported in the literature, and at this point can only speculate on any mechanical and/or functional roles these intermediate filament bridges may have in astrocyte networks.

Microtuble cellular bridges between PC12 cells, an immortalized neuron-like cell line,
have been shown to transport organelles and vesicles between connected cells[31].

Similar microtubule bridges have been reported between astrocytes grown in culture although their function is not known[32], but in both cases microtubule self-assembly depends on actin polymerization. Interestingly, a more recent report suggests that actin is not required for the generation of nanotubular astrocyte protrusions grown on substrates with nanoscale features[33], although again the molecular nature and functional properties of these protrusions are not entirely clear. For both the data we present here and these other studies, the cells were grown under non-physiological \textit{in vitro} conditions and therefore the interpretation and extrapolation for the potential roles of these cellular bridges \textit{in vivo} are limited.

However, the functional role of the GFAP intermediate filaments we describe here both under normal physiological and pathophysiological conditions raises some potentially interesting possibilities because an upregulation in GFAP self-assembly is directly correlated with the reactive glial response. The functional processes associated with gliosis, on the other hand, (e.g. hypertrophy, leakiness, the reversal of membrane transporters, etc.) depend on the structure of the cytoskeleton. For example, speculatively it would be interesting to explore whether such intercellular GFAP bridges augment and/or propagate a gliosis signal through the astrocytic syntitium. Ultimately, the level of spatial resolution achieved using optical microscopy and functionalized quantum dot conjugates provided the initial experimental observation, providing an example of the unique capabilities and potential of these methods for neuroscience research. Future work will explore whether such GFAP intercellular
bridges form *in situ* in intact tissue preparations and ultimately whether they occur *in vivo*. Towards these goals, quantum dot and related imaging nanotechnologies may provide the neuroscientist with novel experimental capabilities not possible otherwise.

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CHAPTER 5: SUMMARY, CONCLUSIONS, AND FUTURE WORK

Quantum dot nanoparticles have been successfully characterized and used to label GFAP filaments in cells and in tissue sections. These techniques can be easily translated to other researchers’ tool box for probing the conjugation of other nanoparticles and for labeling targets of interest. While these tools have been developed for fixed cells, protocols for labeling live cells would be the next logical step for these studies. One key barrier that prevents quantum dots from internally labeling live cells is cellular uptake in an endosomal compartment, which does not allow antibodies to label targets inside the cell. This could be overcome using optoinjection, as has been demonstrated by Cyntellect Inc. These experiments are suggested as future work in this area. Antibody conjugated fluorophores have already been shown to specifically label actin so it is logical that antibody conjugated quantum dots would specifically bind to their targets of interest, as long as an excessive amount of particles was not added. There is no current method to turn off quantum dot fluorescence so free quantum dots would also fluoresce.

In terms of the use of quantum dot nanoparticles for in vivo tracking and delivery, toxicity issues and clearance from the body are both issues that need to be addressed. At this point, it is recommended that quantum dots are only be used as powerful tools for basic science research.
REFERENCES


