Toolbox

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Quantum Dot Applications to Neuroscience: New Tools for Probing Neurons and Glia

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Introduction

Semiconductor fluorescent quantum dots are nanometer-sized functionalized particles that display unique physical properties that make them particularly well suited for visualizing and tracking molecular processes in cells using standard fluorescence microscopy (Jaiswal et al., 2003; Watson et al., 2003; Michalet et al., 2005). They are readily excitable and have broad absorption spectra with very narrow emission spectra, allowing multiplexing of many different colored quantum dots; they display minimal photobleaching, thereby allowing molecular tracking over prolonged periods; they also display a blinking property that allows the identification of individual quantum dots. 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. Neuroscience-specific applications of quantum dots are starting to emerge. Some work has focused on using this nanotechnology to address cellular and molecular questions of interest, although other work is pushing the development of the technology forward.

Quantum dots are nanometer-sized particles composed of a heavy metal core, such as cadmium selenium or cadmium telluride with an intermediate unreactive zinc sulfide shell and a customized outer coating of different bioactive molecules tailored to a specific application (Fig. 1). The composition and very small size of quantum dots (5–8 nm) gives them unique and very stable fluorescent optical properties that are readily tunable by changing their physical composition or size. The photochemical properties of quantum dots allow selective fluorescent tagging of proteins similar to classical immunocytochemistry. Additionally, the use of quantum dots is associated with minimal photobleaching and a much higher signal-to-noise ratio. Their broad absorption spectra but very narrow emission spectra allows multiplexing of many quantum dots of different colors in the same sample, something that cannot be achieved with traditional fluorophores. The physics responsible for these effects are beyond the scope of this brief introduction, but the small size of quantum dot particles results in large but specific energy jumps between the energy band gaps of excited electron–hole pairs in the semiconductor core. This effect results in scaled changes of absorption and emission wavelengths as a function of particle size, so that small changes in the radius of quantum dots translate into very distinct changes in color (Arya et al., 2005; Vanmaekelbergh and Liljeroth, 2005). This physical property represents another major advantage over traditional organic fluorophores that in general require distinct chemistries to produce different colors. For biological applications, quantum dots can be chemically functionalized to target proteins 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 nontoxic (Chan et al., 2002; Murphy, 2002; Jain, 2003; Watson et al., 2003; West and Halas, 2003).

Quantum dots and neuroscience

Quantum dots represent a new tool of significant potential in neuroscience research. In addition to offering an alternative to traditional immunocytochemistry, they are particularly valuable for studies of neurons and glia. Quantum dots can be used to visualize, measure, and track individual molecular events using fluorescence microscopy, and they provide the ability to visualize and track dynamic molecular processes over extended periods (e.g., from seconds to many minutes). These properties are difficult to achieve using other techniques or approaches. For example, quantum dots are useful for experiments that are limited by the restricted anatomy of neuronal and glial interactions, such as the small size of the synaptic cleft, or between an astrocyte process and a neuron. Because of their ex-

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tremely small size and optical resolution, they are also well suited for tracking the molecular dynamics of intracellular and/or intercellular molecular processes over long time scales. However, it should be appreciated that the hydrodynamic radius of functionalized quantum dots is larger (15–20 nm) than their actual size of 5–8 nm (Larson et al., 2003). Recent studies using quantum dots in neuroscience illustrate the potential of this technology. Triller and colleagues (Dahan et al., 2003) used antibody functionalized quantum dots to track the lateral diffusion of glycine receptors in cultures of primary spinal cord neurons. They were able to track the trajectory of individual glycine receptors for tens of minutes at spatial resolutions of 5–10 nm, demonstrating that the diffusion dynamics varied depending on whether the receptors were synaptic, perisynaptic, or extrasynaptic. Vu et al. (2005) tagged nerve growth factor (βNGF) to quantum dots and used them to promote neuronal-like differentiation in cultured pheochromocytoma 12 (PC12) cells. Ultimately, these approaches could be used to visualize and track functional responses in neurons. However, as with any new technology, there are caveats. For example, Vu et al. (2005) reported that βNGF conjugated to quantum dots had reduced activity compared with free βNGF. Other groups are pushing the technology forward and providing new quantum-dot-based tools. Brinker and colleagues (Fan et al., 2005) developed a technique to produce biocompatible water-soluble quantum dot micelles that retain the optical properties of individual quantum dots. These micelles showed uptake and intracellular dispersion in cultured hippocampal neurons. Ting and colleagues (Howarth et al., 2005) are developing a modified quantum dot labeling approach that addresses the relatively large size of antibody–quantum-dot conjugates and the instability of some quantum-dot–ligand interactions. Their technique tags cell surface proteins with a specific peptide (a 15 aa polypeptide called acceptor protein; GLNDIFEAQKIEVWHE) that can be directly biotinylated as a target for streptavidin-conjugated quantum dots. Using this approach, they were able to specifically label and track AMPA receptors on cultured hippocampal neurons.

Ultimately, quantum dot nanotechnologies will require easy-to-use approaches that can be straightforwardly replicated in a typical neurobiology lab. Unfortunately, in our experience, most quantum dot protocols intended for non-neural mammalian cells in the peer-reviewed literature and in instructions from commercial sources do not always readily work with primary neurons, glia, and related cell lines (Fig. 2*I*). In the supplemental data, we present a very simple and straightforward protocol for specific labeling of both neurons and glia (Fig. 2 and supplemental Table 1, available at www.jneurosci.org as supplemental material). Of particular note, as far as we are aware, these results represent the first use of quantum dots in glial cells. Given the unique properties that quantum dots have to offer neuroscience research, their future looks pretty bright (and stable).

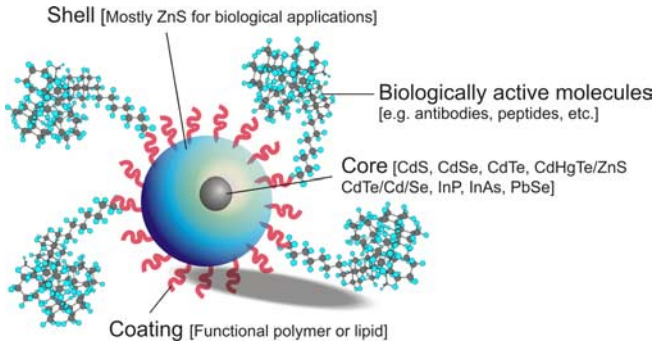

Figure 1. Structure of a semiconductor fluorescent quantum dot nanocrystal. The heavy metal core is responsible for the fluorescence properties of the quantum dot. The nonemissive shell stabilizes the core, whereas the coating layer provides anchor sites to organic and biological ligands such as antibodies, peptides, and other organic molecules.

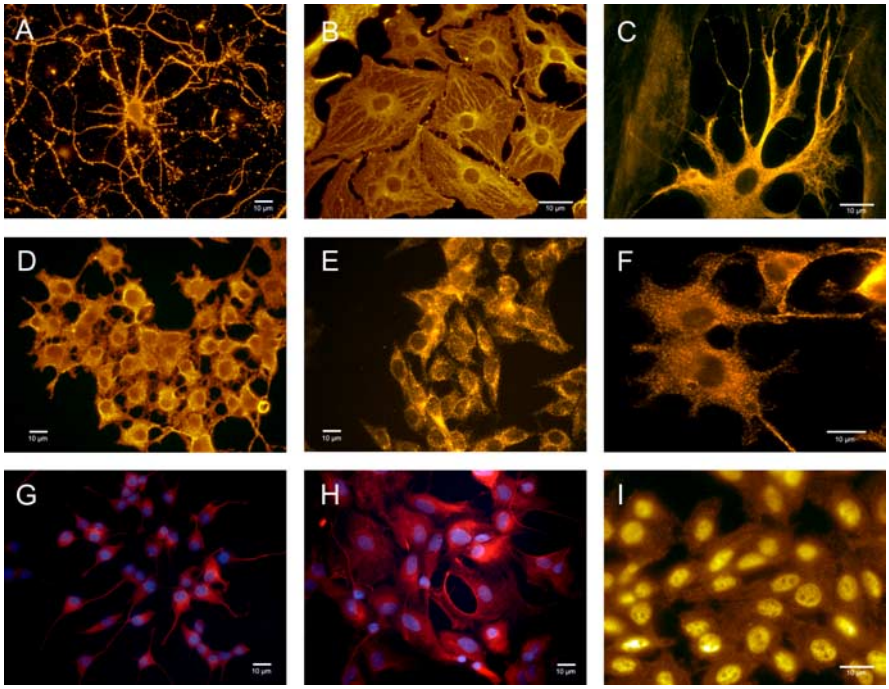

Figure 2. Fluorescent labeling of neurons and glia with antibody-conjugated 605 nm quantum dots. ***A***, Primary cortical neurons specifically labeled for β-tubulin. ***B***, ***C***, Primary cortical astrocytes specifically labeled for glial fibrillary acidic protein (GFAP). ***D***, ***F***, PC12 cells labeled for β-tubulin. ***E***, r-MC1 neural retinal Muller glial cells specifically labeled for GFAP. ***G***, PC12 cells labeled for β-tubulin using standard immunocytochemistry. ***H***, Primary spinal cord astrocytes labeled for GFAP using standard immunocytochemistry. ***I***, An example of artifactual nonspecific labeling in r-MC1 Muller cells with anti-GFAP-conjugated 605 nm quantum dots. In this case, putative nonspecific electrostatic interactions between quantum dots and cellular proteins led to intense nuclear staining and mild cytoplasmic staining using other quantum dot conjugation protocols described for mammalian cells. All imaging parameters were constant for the different experimental conditions, with an acquisition/exposure time of 30 ms for all panels, except for ***I***, which was taken with an acquisition time of 100 ms.

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Tool Box

Quantum dot applications to neuroscience: New tools for probing neurons and glia- Supplementary Information

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Quantum Dot Labeling Protocol for Neurons and Glia

Here we describe in detail our quantum dot labeling protocol for labeling neurons and glia. 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 should label any protein of interest. Table I summarizes the detailed methods described below. 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.

Materials and methods

Lysine coated glass cover slips were prepared by incubating 12mm glass cover slips in 0.1% wt/vol poly-D-lysine (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 streptavidin/biotin 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 A-C; Fig. 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.

All images were acquired using an Olympus IX81 inverted fluorescent confocal microscope (Olympus Optical, Tokyo, Japan) that included epifluoresence, 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.

Results and Discussion

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 in main text). 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 fluorophore tagged secondary antibodies (Fig. 2G 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 non-specific 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. 2I). Non-specific binding was also observed when using other published protocols for non-neural cells (Wu et al., 2003). Blocking conditions also 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). Another advantage to labeling with quantum dots is that each individually visualized dot in a fluorescence micrograph represents 1-3 individual quantum dots, based on our own calculations and those of others (Chan and Nie, 1998). This means that 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 (West and Halas, 2003; Michalet et al., 2005) that cannot be done with standard ICC (see (Dahan et al., 2003) 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.

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Table 1. Summary of quantum dot labeling protocol for neurons and glia

Pre-processing and Fixing
Remove media from wells by gently aspirating.
Wash cells with warmed PBS.
Fix cells with 4% paraformaldehyde (Electron Microscopy Sciences, catalog #157 15-S) in PBS for 10 minutes at room temperature.
Wash cells 3X with PBS.
Permeabilize cells with 0.2% Triton X-100 (Fisher Scientific, catalog #BP151-100) in PBS for 5 minutes.
Wash cells 3X for 5 minutes with PBS.
Incubate with 10% Horse Serum in PBS for 30 minutes at room temperature.
Rinse with PBS.
Apply Streptavidin/Biotin blocking kit (Vector Labs, catalog #SP-2002).
Primary Incubation
Rinse with PBS.
Add biotinylated molecule of interest. (E.g. antibodies; use ProtOn Biotin Labeling Kit or similar for biotinylation; Vector Labs, catalog #PLK-1202).
Incubate 2 hours at room temperature.
[Biotinylated secondary antibody for one hour- alternative 3 step labeling protocol]
Remove antibodies by gently aspiration and rinse 3X with PBS.
Quantum dot incubation
Add Streptavidin conjugated quantum dots (We used Quantum Dot Corporation's 605 nm quantum dots here, catalog #1010-1) in 10% Horse Serum.
Incubate 1 hour at room temperature.
Rinse 3X with PBS.
Mount with 90% glycerol (Sigma, catalog #G-6279) in PBS.