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Labeling Cell-Surface Proteins Via Antibody Quantum Dot Streptavidin Conjugates

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Summary

The quantum dot is a novel fluorescent platform that has the potential to become an alternative to conventional organic dyes used to label biological probes such as antibodies or ligands. Compared to typical fluorescent organic dyes, cadmium selenide/zinc sulfide core-shell nanocrystals, or quantum dots, have greater photostability, resist metabolic and chemical degradation, are nontoxic, and display broad emission and narrow excitation bands. When conjugated to generic adaptor molecules such as streptavidin, quantum dots can be used to label different biotinylated antibodies or ligands without having to customize the quantum dot surface chemistry for each antibody or ligand. In this chapter, we outline the methodology for using streptavidin quantum dots to label biotinylated antibodies that target cell-surface ectodomain proteins on both living and fixed cells.

Key Words

Streptavidin; quantum dots; biotinylation; cell-surface proteins; imaging.

1. Introduction

The conjugation of highly luminescent semiconductor cadmium selenide/zinc sulfide core-shell nanocrystals (quantum dots) conjugated to biorecognition molecules has produced a new class of fluorescent labels that has the potential to revolutionize biological imaging. Compared with conventional fluorescent organic dyes, quantum dots display a narrow emission band that is tunable, have a broad excitation spectrum, are resistant to chemical and metabolic degradation, display photostablility, and offer versatility in attachment of biorecognition molecules ***(1–4)***. In this chapter, we demonstrate that streptavidin can serve as a bridge for binding biotinylated antibodies when conjugated to the surface of quantum dots. We also illustrate that the streptavidin quantum dots

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(SA-quantum dots) can be used to detect biotinylated antibodies used to label specific neuronal surface proteins on living cells.

2. Materials

1. Papain (Worthington, Lakewood, NJ).
2. Bovine serum albumin (BSA) (Sigma, St. Louis, MO).
3. DNase I (Sigma).
4. Hank's buffer (Gibco-BRL, Rockville, MD).
5. Nembutal (Abbott, North Chicago, IL).
6. Trypsin (Sigma).
7. Penicillin-streptomycin (Invitrogen, Carlsbad, CA).
8. Poly-D-lysine (Sigma-Aldrich, St. Louis, MO).
9. Laminin-coated cover slips (BD Biosciences, Bedford, MA).
10. Dulbecco's modified Eagle's medium (Gibco-BRL).
11. Basal medium (F-12 nutrient mixture, HAM) (Gibco-BRL).
12. Dialyzed fetal bovine serum (FBS) (Sigma-Aldrich).
13. Glutamine (Sigma-Aldrich).
14. Supplement B27 (Gibco-BRL).
15. CO_2-controlled incubator.
16. 70% Alcohol.
17. Angle-neck flasks (40 mL) (Nalgen Nunc, Rochester, NY).
18. Hemacytometer (Improved Neubauer) (BD Biosciences).
19. Inverted light microscope.
20. Phosphate-buffered saline (PBS) (1X solution): 0.1 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.5 m*M* Na_2HPO_4, 1 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$; pH to 7.4.
21. EZ-Link™ sulfo-NHS-Biotin (Pierce, Rockford, IL).
22. Nonenzymatic dissociation solution (Sigma-Aldrich).
23. Glycine (Sigma-Aldrich).
24. Microcentrifuge tube with screw cap (2 mL) (Fisher brand no. 05-669-23; Fisher, Pittsburg, PA).
25. Stir bar (0.25 in.) (Fisher).
26. Halothane (Abbott).
27. Dimethyl sulfoxide (DMSO), anhydrous (Fisher).
28. Sodium bicarbonate (Sigma-Aldrich).
29. MicroSpin G-25 spin columns (Amersham Biosciences, Piscataway, NJ).
30. G-25 purification resin (Amersham Biosciences).
31. Hank's balanced salt solution (HBSS), Ca^{+2} + Mg^{+2} free, 10X (no. 14186-012; Gibco-BRL).
32. Excitation/emission filter: 485/520 and 580–630.
33. Glass collection tubes (13 × 100 mm) (Fisher).
34. Swinging-bucket centrifuge.
35. KRH buffer: 60 m*M* NaCl, 2 m*M* KCl, 0.6 m*M* KH_2PO_4, 4 m*M* HEPES.
36. Rotating shaker (Stoval, Greensboro, NC).
37. Biotinylated secondary anti-rabbit IgG (Vector, Burlingame, CA).

38. SA-quantum dots (Quantum Dot, Hayward, CA).
39. SA-Alexa Red™ (Molecular Probes, Eugene, OR).
40. Borate buffer (Polysciences, Warrington, PA).
41. Mercury lamp HBO 100 (Zeiss, Oberkochen, Germany).
42. Charge-coupled device (CCD) digital video camera (Roper, Tucson, AZ).
43. Human norepinephrine transporter (hNET)-expressing HEK293 cell line (Dr. Randy Blakely, Vanderbilt University, Nashville, TN).
44. Monoclonal antibody (MAb) to limbic-associated membrane protein (LAMP) (Dr. Pat Levitt, Vanderbilt University).
45. Polyclonal antibody to hNET ectodomain (Dr. Randy Blakely, Vanderbilt University).
46. HEK293 parental cells (American Type Tissue Collection [ATTC], Manassas, VA).
47. Pregnant rat (Sprague-Dawley®; Harlan, Indianapolis, IN).
48. Polypropylene conical tubes (50 mL) (Becton Dickinson, Franklin lakes, NJ).
49. Glass-bottomed microwell dishes (MatTek, Ashland, MA).

3. Methods

The following methods describe how to grow dispersed primary neurons and a continuous cell line culture, biotinylation of cell-surface proteins and MAbs, cell-surface protein immunocytochemistry using a biotinylated monoclonal and an anti-rabbit IgG, detection of biotinylated antibody-labeled proteins with SA-quantum dots and SA-Alexa flurophores™, and fluorescent imaging of SA-quantum dots and SA-Alexa flurophores.

3.1. Tissue Culture

The growth and maintenance of a primary neuronal (hippocampal) culture and a continuous cell line (HEK293) culture are described next. Each procedure can be adapted for use with other tissues (*see* **Note 1**).

3.1.1. Culture of Dissociated Primary Hippocampal Neuron Monolayer

1. Euthanize an 18-d pregnant rat (Sprague-Dawley) with halothane, nembutal, or other approved means.
2. Surgically remove the pups from the mother.
3. Perform each of the following steps using aseptic technique in a laminar flow hood (*see* **Note 2**). Remove the brain from the skull and immerse in HBSS to prevent the tissue from drying. Place the brain on a glass culture dish on a dissecting scope illuminated from below.
4. The following briefly describes how to dissect the hippocampus; for a more detailed discussion *see* **ref. *5***. Dissect the hippocampus and incubate for 30 min at 37°C in 20 U/mL of papain and 100 U/mL of DNase I in Ca^{+2}/Mg^{+2}-free Hank's buffer. Briefly rinse the tissue twice with HBSS containing 1 mg/mL of trypsin inhibitor, 100 U/mL of DNase, and 1 mg/mL of BSA; allow to dissociate in growth medium consisting of basal medium, with supplement B27, 10% FBS, 0.5 m*M* glutamine, and 0.5 m*M* penicillin-streptomycin.

5. Plate the dissociated cells on a poly-D-lysine and laminin-coated cover glass of a MatTek culture dish at a density of 20,000 cells and incubate in a 5% CO_2 incubator at 37°C. After 3 h, flood the plates with 1 mL of growth medium. After 3 d of culturing, feed the cells with growth medium containing FBS, and culture the cells for another 5 d.

3.1.2. Culture of HEK Cell Line Monolayer

Human embryonic kidney (HEK293) cells, a parental line and a stable line expressing hNET (Dr. Randy Blakely, Vanderbilt University), were frozen on dry ice in cryogenic vials at a concentration of 1×10^8/mL. The vials were stored immediately in liquid nitrogen for later use. At the time of use, the vials were removed from the liquid nitrogen and freeze-thawed on ice. While thawing, flasks were labeled as either HEK293/parental or HEK293/hNET. Cell medium, previously stored at 4°C, was removed and placed in a 37°C water bath until needed.

In the remainder of this section, each step was performed using aseptic technique inside a laminar flow hood (*see* **Note 2**). Ten milliliters of medium was transferred by pipet into each labeled flask. Thawed vials were then sterilized by swabbing with alcohol. The vials were slowly opened, and the medium with the cells was transferred by pipet into the appropriately labeled flask. The flasks were next placed in a 37°C incubator at 5% CO_2, and the cells were allowed to grow until confluent.

Once confluent, the medium was aspirated off and the cells well rinsed with PBS, and then 3 mL of 0.1% trypsin was added. After approx 1 to 2 min, or when the cells began to round up and detach, 3 mL of medium was added to disperse the cells and to inhibit the trypsin (*see* **Note 3**). Cells from each flask were then transferred to an appropriately labeled 50-mL conical tube and centrifuged at 500*g* for 5 min. The supernatant was aspirated off, and the cells were resuspended in fresh medium.

Using a hemacytometer, the cells were counted and their concentration was determined (*see* **Note 4**). The cell solution was next diluted to 1×10^5 cells/mL and 150 µL was added to the glass cover slip of poly-D-lysine-coated sterile MatTek microwell dishes. The cells were incubated at 37°C with 5% CO_2 for 3 h to allow the cells to attach. Once the cells had attached, 2 mL of additional medium was added to the dish. The dishes were then returned to the incubator and allowed to grow for 3 d or until 50% confluent.

3.2. Biotinylation

The following protocols describe procedures for conjugating cell-surface proteins and antibodies to biotin. Once conjugated, the biotin acts as a bridge for attaching SA-quantum dots or SA-fluorophores. Streptavidin has an

exceptionally high affinity for biotin ($Ka = 10^{14}$ mol^{-1}) and therefore binds essentially irreversibly ***(6)***.

Because each cell line may vary in its nonspecific adsorption of SA-quantum dots, and because we have observed that SA-quantum dot integrity is compromised after long periods of storage, we recommend first conducting a control experiment using biotinylated cells to confirm the integrity of SA-quantum dots and to determine their signal over background for the cell line being used (*see* **Fig. 1**). **Subheadings 3.2.1.** and **3.2.2.** describe a biotinylation procedure for both dispersed primary neuronal and continuous cell line cultures.

Subheading 3.2.3. describes the step-by-step process for covalent coupling of biotinyl groups to the MAb LAMP. This procedure may also be used with other antibodies (IgG and different Ig classes) (*see* **Note 5**). Most secondary antibodies are commercially available with biotin already attached (i.e., Vector).

3.2.1. Cell-Surface Biotinylation

1. Seed poly-D-lysine-coated microwell dishes with parental HEK-293 cells at 50,000 cells/well, and incubate for 48 h as described in **Subheading 3.1.2.**
2. Remove the dishes from the incubator and wash quickly with 37°C KRH.
3. Treat the cells with EZ-Link sulfo-NHS-Biotin at 1.5 mg/mL at 4°C for 1 h in PBS/Ca^{+2} + Mg^{+2}.
4. After incubation, remove the biotinylating reagents and quench by washing twice with ice-cold 100 m*M* glycine in PBS/Ca^{+2} + Mg^{+2}.
5. Quench the reaction further by incubating with 100 m*M* ice-cold glycine for 30 min.
6. Rapidly wash the cells three times with PBS/Ca^{+2} + Mg^{+2}.

3.2.2. Primary Cultures

1. Culture primary hippocampal neurons as described in **Subheading 3.1.1.**
2. After neuronal growth has reached confluence and/or 8 d in age (*see* **Note 6**), perform **steps 3–6** in **Subheading 3.2.1.**.

3.2.3. Antibody Biotinylation

1. Transfer 200 µL of a 1.0 mg/mL antibody solution to a 2-mL tube containing a stir bar, place on a stir plate, and set the magnetic stirrer at 50 rpm (*see* **Note 7**).
2. To this tube add 20 µL of 1 *M* sodium bicarbonate (freshly made).
3. To a 2-mL reaction tube add 200 µg of DSB-X biotin succinimidyl ester (*see* **Note 8**).
4. To this tube add 40 µL of DMSO and mix by pipetting up and down (*see* **Note 9**).
5. Add 3 µL of this mix to the 2-mL tube with antibody that is stirring in **step 1**.
6. Allow the reaction mix to stir for 1 to 1.5 h at room temperature.
7. While the reaction mixture is stirring, perform **steps 8–13**.

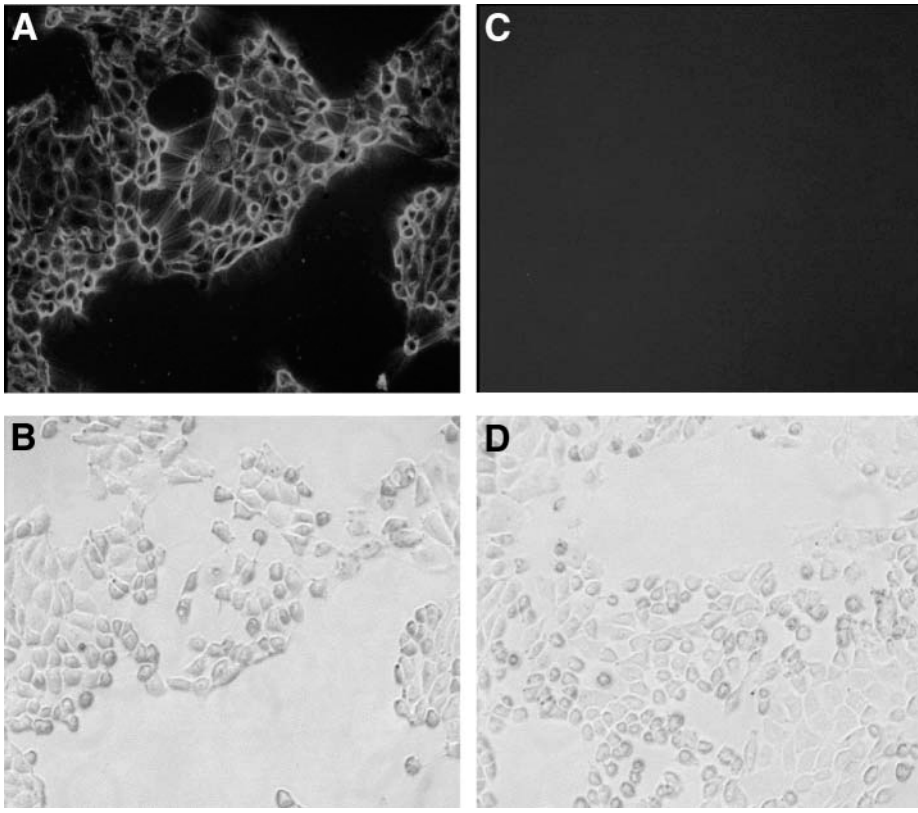

Fig. 1. Labeling of biotinylated HEK293 cells with SA-quantum dots (magnification: ×10). Three-day-old HEK293 cells cultured on poly-D-lysine glass-bottomed microwell dishes were biotinylated as described in **Subheading 3.2.1. (A,B)** Biotinylated HEK293 cells incubated with SA-quantum dots (50 n*M*); **(C,D)** biotinylated HEK293 cells labeled with SA-quantum dots after preincubating with 2 mg/mL of biotin.

8. Place a spin column into a 13 × 100 mm glass tube.
9. Stir the purification resin using a clean plastic tube.
10. Add 1 mL of the purification resin to the spin column.
11. Allow the resin to settle, and then add more resin until the bed volume is approx 1.5 m while allowing the column buffer to drain into the 13 × 100 mm glass tube.
12. Place the spin column into a collection tube.
13. Centrifuge the spin column with the collection tube for 3 min at 1100*g* using a swinging-bucket rotor.
14. Load the reaction mixture from **step 6** (~200 µL) onto the center of the spin column and allow it to be absorbed (*see* **Note 10**).

15. Place the spin column into an empty collection tube and centrifuge for 5 min at 1100*g*. The eluate in the bottom of the tube contains the biotinylated antibody in 20 m*M* PBS, pH 7.4. We determined the concentration of biotinylated antibody to be 800 µg/mL (*see* **Note 11**).

3.3. Immunocytochemistry

In **Subheading 3.3.1.** and **3.3.2.**, we describe respectively how to label a specific cell-surface protein by using a biotinylated MAb for direct detection and by indirect labeling using a nonbiotinylated polyclonal antibody followed by a secondary biotinylated anti-rabbit IgG.

3.3.1. Detection with Biotinylated Primary Antibody

1. Quickly wash with room temperature KRH dissociated primary hippocampal neurons (*see* **Note 12**) grown to confluency on microwell dishes (*see* **Subheading 3.1.1.**).
2. Remove the excess KRH present on the cover glass surface by aspiration.
3. Add 100 µL of biotinylated LAMP primary antibody (diluted to a concentration of 8 µg/mL) to the cells attached to the cover slip glass of the microwell dishes.
4. Incubate the dishes at room temperature for 1 h on a rotating shaker at 50 rpm.
5. After 1 h, wash the dishes three times for 5 min with KRH while rotating on a shaker at 50 rpm.
6. Aspirate the excess KRH from the cover glass, and label the attached cells using either SA-quantum dots or SA-Alexa Red (*see* **Subheadings 3.4.1.** and **3.4.2.**).

3.3.2. hNET Detection in HEK-293 Cell Line With Biotinylated Secondary Antibody

1. Quickly wash with room temperature KRH HEK-293 cells cultured on the cover slip glass of each microwell dish (*see* **Subheading 3.1.2.**).
2. Remove the excess KRH present on the cover glass surface by aspiration.
3. Add 100 µL of polyclonal anti-hNET (5 µg/mL) to the cells covering the microwell dish cover slip glass.
4. Incubate the cells for 1 h on a rotating shaker at 50 rpm.
5. Wash the dishes three times for 5 min with KRH while rotating on a shaker at 50 rpm.
6. Add 1.5 µg/mL of biotinylated anti-rabbit.
7. Incubate the dishes at room temperature for 1 h on a rotating shaker at 50 rpm.
8. Wash the dishes three times for 5 min with KRH while rotating at 50 rpm on the shaker. The cells on the cover slip glass are now ready to be labeled using either SA–quantum dots or SA–Alexa Red (*see* **Subheadings 3.4.1** and **3.4.2.**).

3.4. Detection Using SA-Quantum Dots and SA-Alexa Fluor™

Biotinylated antibodies are detected using quantum dots conjugated to streptavidin (SA-quantum dots) and the streptavidin-labeled fluorophore Alexa Red™

594 as a control. **Subheadings 3.4.1.** and **3.4.2.** describe a procedure that was developed for detecting biotinylated antibodies with either SA-quantum dots or SA-Alexa Red. Depending on the cell type, it may be necessary first to pre-incubate cells with a blocking solution when labeling with SA-quantum dots (*see* **Note 13**).

3.4.1. SA-Quantum Dots

1. Aspirate the excess KRH buffer on the cells covering the cover slip glass of the microwell dishes from **Subheadings 3.3.1.** and/or **3.3.2.**, being careful to avoid removing any of the attached cells.
2. Add 50 µL of SA-quantum dots (50 n*M*) in borate buffer (pH 8.0) to the cover slip glass of the microwell dishes.
3. Incubate the dishes at room temperature for 10 min with no agitation (*see* **Note 14**).
4. Quickly rinse the dishes three times with KRH.
5. Add 100 µL of KRH to the cells covering the cover slip glass of the microwell dishes.
6. Immediately image the SA-quantum dot-labeled cells (*see* **Subheading 3.5.**).

3.4.2. SA-Fluorophore (Alexa Red)

1. Aspirate off the excess KRH buffer from the cells covering the cover slip glass of the microwell dishes from **Subheadings 3.3.1.** and/or **3.3.2.**, being careful to avoid removing any of the attached cells.
2. Add 50 µL of SA-Alexa Red (300 µg/mL) in KRH buffer (pH 7.4) to the cover slip glass of the microwell dishes.
3. Incubate the dishes at room temperature for 10 min with no agitation.
4. Quickly rinse the dishes three times with KRH.
5. Add 100 µL of KRH to the cells on the cover slip glass well of the microwell dishes.
6. Immediately image the SA-Alexa Red-labeled cells (*see* **Subheading 3.5.**).

3.5. Fluorescent Imaging

To fluorescently image labeled cells on the cover glass of microwell dishes after incubation with SA-quantum dots or SA-Alexa Red requires an inverted fluorescent microscope equipped with a transmitted-light illumination system, for the initial bright-field examination; an incident-light illuminator, for the excitation of fluorophores; and a filter cube with the appropriate filter set (*see* **Fig. 2**). For convenience, the microscope should also have a phototube for mounting either a conventional 35-mm or an electronic CCD digital video camera that can be interfaced with a computer. The steps for imaging SA-quantum dot- and SA-Alexa Red-labeled cells from **Subheadings 3.4.1.** and **3.4.2.** using an Axiovert 110 (Zeiss) equipped with an HBO 100 mercury lamp (Zeiss) and a

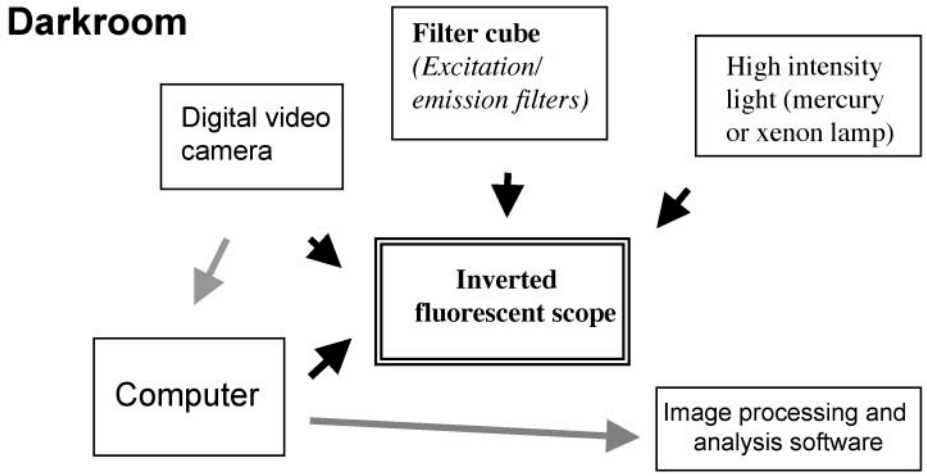

Fig. 2. Components required for fluorescent imaging. An inverted fluorescent microscope equipped with the appropriate excitation/emission filter(s) and camera insertion site also should include a mercury or xenon lamp for fluorophore excitation and a digital camera. The computer selected should have sufficient memory to process and to analyze digital images using analysis software. All equipment shown should be set up in a room in which lighting can be shut off without inconveniencing other laboratory operations.

Photometric Cool Snap™ digital CCD camera (Roper) are described next. However, these steps can be repeated using a comparable imaging system.

3.5.1. Fluorescent Imaging

1. Position cells on the cover glass of microwell dishes labeled with either SA-quantum dots or SA-Alexa Red on the stage of an inverted fluorescent microscope.
2. Using transmitted light, bring into focus the cells on the cover glass and capture a bright-field image using a CCD digital video camera.
3. Using 488 nm of incident light, excite the cells labeled by SA-quantum dots or SA-Alexa Red, and observe the fluorescence produced though a 485/20 and 580–630 band-pass filter.
4. Before capturing an image, illuminate the SA-quantum dot-labeled cells on the cover glass of the dishes with incident light for a minimum of 2 min to induce maximal fluorescence (*see* **Note 15**). Immediately capture images of SA-Alexa Red-labeled dishes to avoid bleaching.
5. To ensure that SA-quantum dots used to label cells have not aggregated while labeling, examine imaging fields for SA-quantum dot clusters that are not colocalized with cells (*see* **Fig. 4C**). When aggregates or clusters are present, it is assumed that SA-quantum dots did not remain in the solution while labeling and

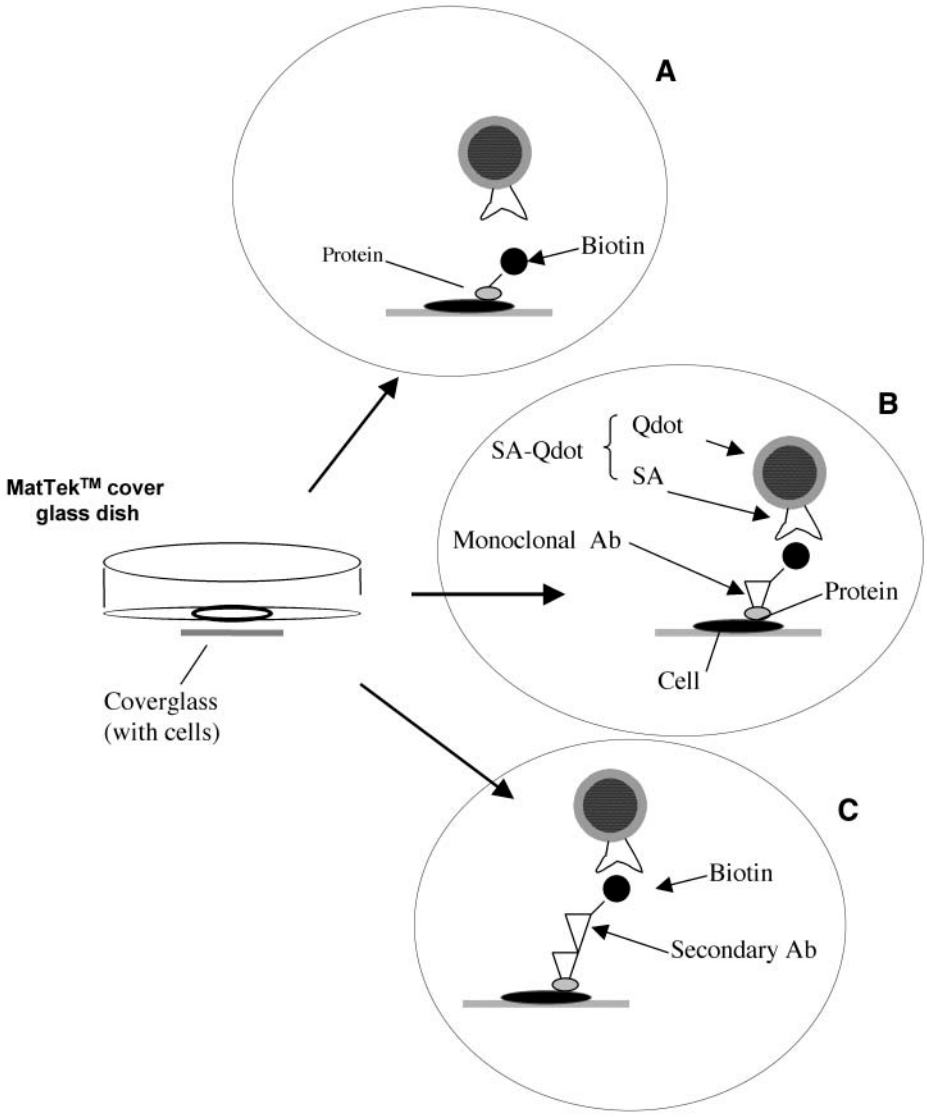

Fig. 3. Direct and indirect labeling of live cells by SA-quantum dots (SA-Qdot). **(A)** Biotinylated cell-surface proteins are labeled directly by SA-quantum dots. **(B)** Biotinylated MAb used to detect cell-surface protein is labeled by SA-quantum dots. **(C)** Biotinylated secondary antibody used to detect polyclonal antibody attached to the cell-surface protein is labeled by SA-quantum dot.

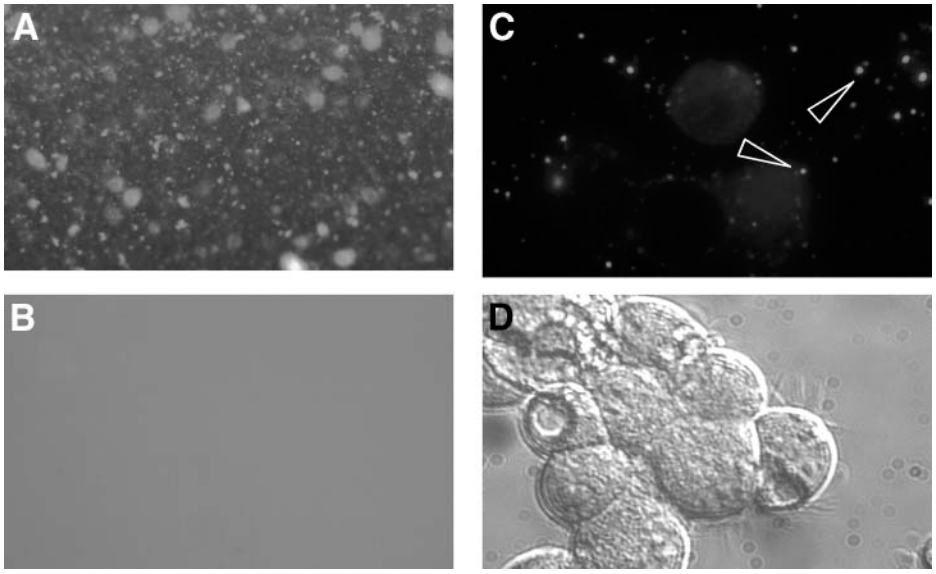

Fig. 4. Evidence for quantum dot aggregation (magnification: ×40): **(A)** fluorescent image of aggregated SA-quantum dots in absence of cells; **(B)** image of nonaggregated SA-quantum dots in absence of cells; **(C)** image of cells surrounded by aggregated SA-quantum dots (arrowheads); **(D)** bright-field image.

that labeling of the biotin-modified targets with SA-quantum dots was compromised (*see* **Note 16**).

4. Notes

1. Depending on which type of cell line is cultured, the growth medium used may vary. Whether or not a cell-line type expresses a particular protein in culture can be controlled by the nutritional constitution of the medium, such as hormones and other inducer or repressor substances, as well as the interaction between cells and their substrate. Most media are based on balanced salt solutions to which are added amino acids, vitamins, and other nutrients at concentrations roughly similar to those found in serum. The composition of the media commonly used in cell culture is provided in standard texts and in catalogs from companies that sell supplies for tissue culture ***(7,8)***. Suggested growth media that is commercially available for most cell culture lines is also listed with the ATTC ***(9)***. Because the requirements for dissociated primary tissue growth media can be specific, it is best to obtain media requirement details that optimize growth by referring to the materials and methods sections of publication(s) in which the tissue you are interested in has already been cultured. Reviews of culture techniques for specific cell types are available ***(10,11)***.

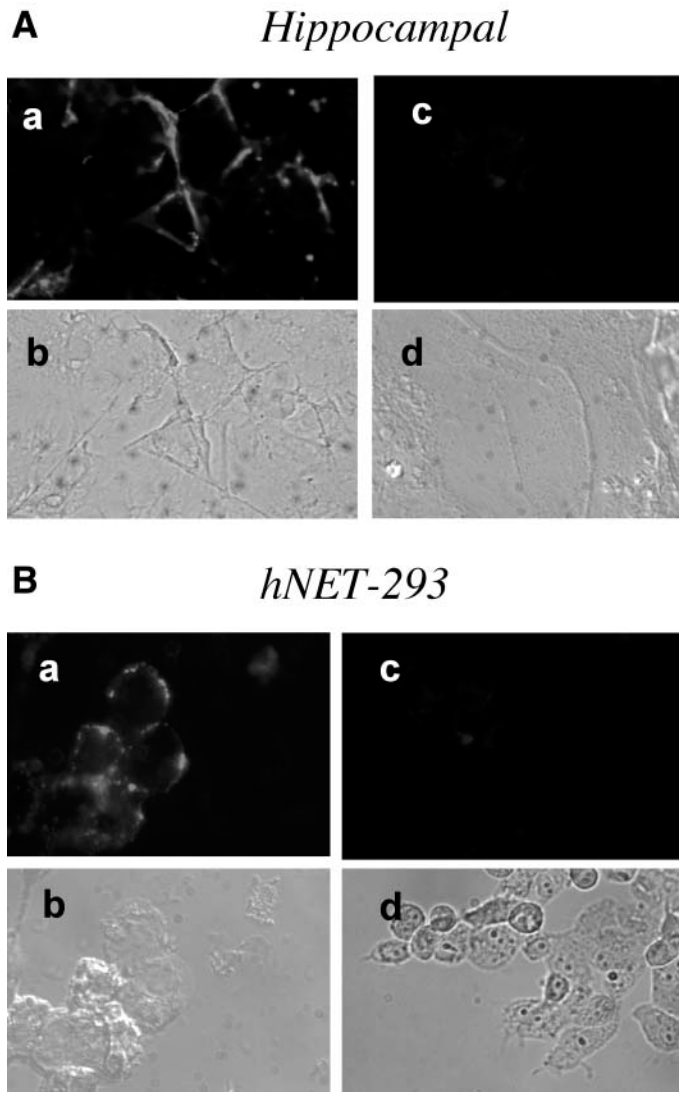

Fig. 5. SA-quantum dots and SA-Alexa Red recognize biotin-modified antibody-labeled cultures. **(A)** Eight-day-old *fixed* primary hippocampal cultures incubated with anti-LAMP followed by SA-quantum dots (a, b) or SA-quantum dots alone (c, d); **(B)** labeling of live hNET-293 cells with anti-hNET + biotinylated anti-rabbit IgG followed by SA-quantum dots (a, b) or SA-quantum dots alone (c, d).

2. In spite of the introduction of antibiotics, contamination by microorganisms remains a major problem in tissue culture. Correct aseptic technique should provide a barrier between microorganisms in the environment outside the culture. The procedure used for equipping and practicing aseptic technique in a laboratory has been described in a number of sources ***(7,11)***. Inability to follow steps in aseptic technique will likely result in bacterial contamination that may alter the biochemical behavior and cell-surface protein expression of the cell line. Taking time to obtain advice from personnel of other laboratories experienced in aseptic technique can greatly facilitate success in this area.
3. Although some cell lines can be dislodged from the bottom of the flask by gently pipetting the medium over the cells repeatedly, trypsinizing cells has the advantage of producing a single cell suspension. However, overtrypsinizing may reduce viability, and undertrypsinizing can result in cell clumps. Furthermore, cells treated with trypsin may require one or more days after plating before reexpression of surface proteins. If viability is a concern, commercially available nonenzymatic dissociation solutions can be used (e.g., from Sigma-Aldrich). These dissociation solutions are calcium free.
4. The concentration of a cell suspension may be determined by placing cells in an optically flat chamber, known as a hemacytometer, which is placed under a microscope. The cell number within a defined area of the hemacytometer can then be counted and the cell concentration derived from this count. Detailed instructions on how to use a hemacytometer (Improved Neubauer) are described elsewhere ***(12)***. An alternative approach to the hemacytometer is electronic particle counting. This method requires the use of an automated system called a Coulter Counter. In this system, cells in suspension are drawn though a fine orifice. As each cell passes though the orifice, it causes a change in current. This change produces a series of pulses, which are then counted ***(5)***.
5. Because the coupling conditions are mild, biotinylation normally does not have any adverse effect on the antibody. However, if a free amino group forms at a position on the protein that is essential for activity (i.e., the antigen-combining site), biotinylation will lower or destroy the activity of the antibody ***(13,14)***.
6. Dissociated primary tissue neurons are typically fastidious in their growth requirements. Failure to provide the appropriate growth factor or combination of factors can cause the neurons to die within a few days. Altering the composition of the medium can determine whether a protein is or is not expressed. One approach is to allow the cultured cells to produce their own growth factors. Most types of glia cells and neurons secrete growth factors in vitro. Thus, if appropriate mixtures of the cells are grown at high density, adequate amounts of the needed growth factor may accumulate. Another approach is to add growth factors directly to the medium. Various classes of neuronal growth factors have been used in culture. For a more complete discussion of required growth factors and their sources, *see* **refs. *5*** and ***7***.
7. If the purified antibody is in dilute buffer, such as 10–20 m*M* PBS, it may be used directly in the protocol. However, if the antibody is in a buffer containing

extraneous primary amines (i.e., Tris or glycine) or ammonium ions, then it must be desalted by dialyzing against PBS, or by using a spin column ***(15)***.

8. In our experiments, we had success when using the biotin succinimide ester DSB-X™ (Molecular Probes). Many biotinylated succinimide esters are now available. Most of these variations alter the size and characteristic of the spacer arm between the succinimide-coupling group and the biotin. However, all the esters are handled in a manner similar to that described in this protocol ***(14)***.
9. To ensure the reactivity of the succinimidyl ester, the solution in DMSO should be made immediately before use. This is to prevent precipitation of the biotin ester, which will occur if the final concentration of DMSO drops below 5%.
10. If any precipitation has occurred during the reaction, the sample must be centrifuged for 5 min to pellet the precipitate before loading. Only the supernatant should be loaded onto the column ***(15)***.
11. Typically, about 80–90% of the antibody in the reaction is recovered as a biotin conjugate.
12. It is necessary to separate out all nonneuronal cells in a neuronal preparation. Nonneuronal cells have a tendency to proliferate, displacing the neuronal cells. If nonneuronal proliferation impedes the detection of the neuronal cells, an antimitotic drug such as cytosine arabinofuranoside can be added (1–5 μ*M*). However, this may result in injury to the neuronal cells and should, therefore, be used at its minimum concentration ***(16)***.
13. Our experience is that the adsorbtion of quantum dots by cells can be cell-type specific. Therefore, preincubating cells and quantum dots with a blocking reagent before application may be necessary ***(17)***. Some reagents that we and others have found to be successful include BSA ***(18)***, 5% normal goat serum (Jackson Immunoresearch), and 0.1% cold-water fish skin gelatin (Amersham Life Bioscience) ***(4)***.
14. Depending on the cell type, rapid agitation may cause cells to detach; therefore, we recommend gently rotating dishes manually. Because borate buffer is harsh on cells, we incubated cells for no more than 15 min to prevent dissociation. However, this will vary with the cell type and number of days that they are cultured. Cells tend to dissociate most readily if cultured less than 2 d and/or grown past confluence.
15. When imaging quantum dots, it is necessary to illuminate for 2 to 3 min before maximum fluorescent intensity will be reached. The rate of quantum dot bleaching is approx 2%/min when illuminated continuously after reaching maximum fluorescence intensity. This is in contrast to conventional fluorophores, which are typically bleached entirely within minutes of illuminating ***(19)***.
16. When imaging, it is important to recognize when aggregates have formed. Our experience is that aggregates can occur unpredictably. Before adding quantum dots for labeling, we advise placing SA-quantum dots on the cover glass of a clean microwell dish and imaging at ×40 to determine whether aggregates have already formed (*see* **Fig. 4A,B**). This assay will confirm whether the SA-quantum dots should be used for labeling. If SA-quantum dots are not aggregated before

adding but aggregate after incubation with cells (*see* **Fig. 4C,D**) then the problem resides with the incubation step. Note that quantum dots when conjugated to certain proteins, such as transferrin, can be internalized by the cell though activation of transferrin receptors. Thus, proper controls should be included to distinguish surface labeling from potential internalization ***(20)***.

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