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Use of quantum dots for live cell imaging

Jyoti K Jaiswal[1], Ellen R Goldman[2], Hedi Mattoussi[3] & Sanford M Simon[1]

[1]The Rockefeller University, Laboratory of Cellular Biophysics, 1230 York Avenue, New York, New York 10021, USA. [2]Center for Bio/Molecular Science and Engineering and [3]Division of Optical Sciences, United States Naval Research Laboratory, Washington, DC 20375, USA.

Monitoring interactions within and among cells as they grow and differentiate is a key to understanding organismal development. Fluorescence microscopy is among the most widely used approaches for high-resolution, noninvasive imaging of live organisms[1,2], and organic fluorophores are the most commonly used tags for fluorescence-based imaging[3]. Despite their considerable advantages in live cell imaging, organic fluorophores are subject to certain limitations. Fluorescent quantum dots (QDs) are inorganic fluorescent nanocrystals that overcome many of these limitations and provide a useful alternative for studies that require long-term and multicolor imaging of cellular and molecular interactions[4,5]. For labeling specific cellular proteins, QDs must be conjugated to biomolecules that provide binding specificity. Bioconjugation approaches vary with the surface properties of the hydrophilic QD used. The mixed surface self-assembly approach is recommended for conjugating biomolecules to QDs capped with negatively charged dihydroxylipoic acid (DHLA) (Fig. 1). In this approach DHLA-capped QDs are conjugated to proteins using positively charged adaptors[6–8], for example, a naturally charged protein (e.g., avidin[9]), a protein fused to positively charged leucine zipper peptide (zb)[8] or a protein fused to pentahistidine peptide (5× His)[10]. The use of avidin permits stable conjugation of the QDs to ligands, antibodies or other molecules that can be biotinylated, whereas the use of proteins fused to a positively charged peptide or oligohistidine peptide obviates the need for biotinylating the target molecule. This procedure describes the bioconjugation of QDs and specific labeling of both intracellular and cell-surface proteins[4,5]. For generalized cellular labeling, QDs not conjugated to a specific biomolecule may be used; various strategies are presented in Box 1, Generalized Labeling of Live Cells.

MATERIALS

REAGENTS

Quantum dots (QDs; e.g., Quantum Dot Corporation or Evident Technology)

Cells or tissue for labeling, prepared appropriately depending on the application (QDs can be used to tag live cells, label cell-surface proteins, or label fixed cells or tissue sections)

Amylose resin (New England Biolabs)

Antibodies of interest

Avidin (Sigma Chemicals)

Bovine serum albumin (BSA), 1% in PBS

Lipid-based transfection reagent (e.g., Lipofectamine 2000 (Invitrogen) or Fugene 6 (Roche))

Maltose (Sigma Chemicals)

Maltose-binding protein fused to the basic leucine zipper domain (MBP-zb) and protein G fused to the basic leucine zipper domain (PG-zb) expressed and purified from bacteria as described elsewhere[7].

Phosphate-buffered saline (PBS; Sigma Chemicals)

Sodium tetraborate (Sigma Chemicals)

Sulfo-NHS-SS biotin (Pierce Biotechnology)

Tris-buffered saline (TBS; Sigma Chemicals)

EQUIPMENT

Hand-held UV lamp

Fluorescence microscope (for details, see the section Imaging the Labeled Cells)

Bioconjugation of the QDs

PROCEDURE

1| Prepare the QD mix: combine 200 pmol avidin and 600 pmol MBP-zb; to this mixture add 100 pmol QD and bring the final volume to 200 µl with 10 mM sodium tetraborate buffer (pH 9.0).
QDs are often synthesized and conjugated to specific biomolecules in the investigator's laboratory. We use CdSe-ZnS QDs, which are rendered water soluble by capping with DHLA as described elsewhere[7].
Quantum Dot Corporation provides QDs conjugated to avidin for use with biotinylated proteins and antibodies. Evident Technology offers biotin-conjugated QDs and QDs that can be conjugated to the N or C terminus of a protein. Both suppliers provide QDs conjugated to specific antibodies as well as protocols for conjugating proteins to their QDs.
▲CRITICAL STEPS

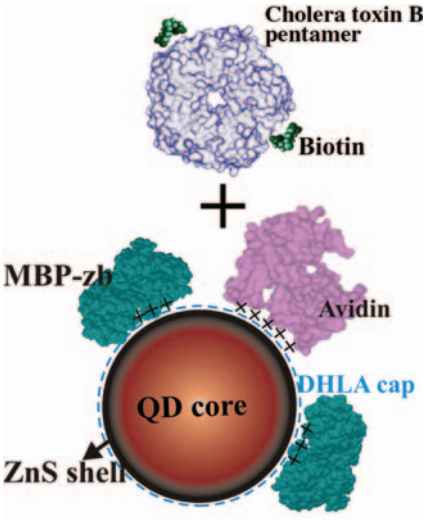

Figure 1 | The mixed surface approach for conjugating biomolecules to DHLA-capped QDs using avidin as the linker molecule. The positive charge on MBP-zb and avidin allow self-assembly of these molecules on the negatively charged surface of DHLA-capped QDs. Use of MBP-zb in molar excess to the avidin makes it possible to regulate the number of avidin molecules present on each QD. This, in turn, permits regulating the number of biomolecules (cholera toxin B subunit in this case) on each QD.

2| Allow the mixture to stand at 20–25 °C for 15 min.

3| Add an additional 150 pmol MBP-zb to the mixture and let it react at 20–25 °C for a further 15 min.

4| Set up a 500 µl amylose column, equilibrated with 10 mM sodium tetraborate buffer.

5| Load the entire preparation of the QD bioconjugate mix (from step 3) onto the column and wash the column twice with sodium tetraborate buffer.

6| Add 200 pmol of the biotinylated molecule of interest (in the case of avidin-conjugated QDs) or specific antibody (in the case of specific antigen-conjugated QDs) to the column and let it react at 20–25 °C for 30–60 min.

7| Wash the column twice with sodium tetraborate buffer and elute using 10 mM maltose (in PBS or sodium tetraborate buffer) until all the QDs are eluted from the column (QD elution can be easily monitored by placing the column in UV light and monitoring the QD fluorescence of the eluant)[7].
This approach provides a pure population of conjugated QDs, free of unbound QDs and of the unbound biomolecules.
➨TROUBLESHOOTING

8| To label live cells, follow **option A, Labeling of cell-surface proteins in live cells**; for labeling fixed cells, proceed to **option B, Labeling of proteins in fixed cells**.
Bioconjugated QDs are used for the specific labeling of both intracellular and cell-surface proteins[4,5]. In live cells, however, these approaches permit labeling only of the cell-surface proteins[4].

Option A. Labeling of cell-surface proteins in live cells

1| Wash the cells with fresh growth medium.

2| Incubate the cells for 30 min, at either 37 °C or 4 °C, in growth medium containing the appropriate amount of QDs bioconjugated to biotinylated ligand or antibody (from step 7 above).
Incubating the cells at 4 °C will help to minimize endocytic uptake of ligands, antibodies and QD bioconjugates.

3| Remove excess QD bioconjugates by washing the cells two or three times with growth medium or PBS.
When using biotinylated ligand or antibodies, continue with step 4.

4| Incubate the cells for 10–15 min (at 37 °C or 4 °C) with avidin-conjugated QDs.

5| Remove the excess unbound QDs by washing the cells two or three times with growth medium or PBS.
These cells can be monitored live or subsequently fixed using chemical fixatives such as 4% paraformaldehyde without affecting the QD fluorescence[11].

6| To visualize results, proceed to the section **Imaging the Labeled Cells**.
➡**TROUBLESHOOTING**

Option B. Labeling of proteins in fixed cells

1| Fix the cells using appropriate chemical fixative and wash the cells two or three times with PBS.

2| Incubate the cells for 30 min at 20–25 °C in 1% BSA in PBS.

3| Replace the 1% BSA solution with an appropriate amount of QD bioconjugates or biotinylated antibody or ligand prepared in 1% BSA solution and incubate at 20–25 °C for 30–45 min.

4| Wash the cells two or three times with PBS.
When using biotinylated ligand or antibodies continue with step 5.

5| Incubate the cells for 10–15 min at 20–25 °C with QD avidin, and then wash two or three times with PBS to remove excess unbound QD avidin.

6| To visualize results, proceed to the section **Imaging the Labeled Cells**.

Imaging the labeled cells

QDs can be imaged using any type of fluorescence microscope, including epifluorescence, confocal and multiphoton. However, unlike with conventional fluorophores, a single wavelength of light can be used to excite several different color QDs. Because most commercially available QDs emit in the green to red region of the visible spectrum, a microscope capable of providing an excitation beam (from lamp or laser) in the UV to blue region of the spectrum and capable of resolving multiple emission wavelengths could be used. As QDs are better excited by UV light, fixed cells can be imaged using a UV light source. To minimize UV-induced photodamage, live cells should be imaged using a blue (wavelength >400 nm) excitation light. For two-photon imaging, excitation at 800 nm is optimal, but any wavelength of light between 700 and 1,000 nm could be used[12]. The choice of emission filter will depend on the emission spectrum of the QD in use.
▲**CRITICAL STEPS**

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
Bioconjugation of QDs, Step 7 In following the protocol for bioconjugation, we find the QD bioconjugates do not elute from the column.	Use a fresh preparation of MBP-zb, and if the problem persists try a fresh batch of QDs.
Option A: Labeling of cell-surface proteins in live cells, Step 6 When labeling cell-surface proteins in live cells, we find the QD bioconjugates cause aggregation of labeled proteins on the surface of live cells.	This problem can be overcome by using the mixed surface conjugation approach (**Fig. 1**). See also **Critical Steps** [Bioconjugation of QDs, Step 1].

▲CRITICAL STEPS

Bioconjugation of QDs, Step 1. To regulate the number of linker molecules (e.g., avidin) on each QD, the molar ratio of MBP-zb to the linker molecule should be altered. Because of their net positive charge, these proteins compete with each other to bind the negatively charged DHLA coat on the surface of the QD. Altering the ratio of these proteins in the mixture facilitates regulating their relative numbers on each QD. Because these proteins bind to QDs in a competitive manner, it is critical that both proteins be mixed thoroughly before addition of QDs. Nonhomogeneous mixing could result in greater variation in the ratio of the two molecules on each QD. The ratio presented here results in an average of one avidin molecule present for three MBP-zb molecules bound to each QD. To optimize the number of linker molecules, a series of QD bioconjugates should be prepared with ratio of linker to MBP-zb above and below the suggested ratio of 1:3. These ratios should be individually tested for specificity and affinity of binding before deciding upon the best QD bioconjugate for cellular labeling.

Imaging the labeled cells. During the course of imaging for all live cell studies, it is recommended that the cells be maintained at 37 °C and not at room temperature (15–25 °C). Although QDs are highly photostable, long exposures to an excitation light source or exposure to UV light can lead to photodamage to the labeled cells. Thus, during long-term imaging, attempts should be made to minimize the length of exposure to excitation light and avoid the use of UV excitation. For multicolor imaging, instead of taking sequential images for each color QD, the emission from each color QD should be acquired simultaneously (if possible) using devices, such as dual view, quad view (Optical Insights), META detector (Zeiss) or AOBS (Leica), that allow simultaneous resolution of different-color QDs.

COMMENTS

The approaches described here have been found to be nontoxic both for DHLA-capped QDs and for some commercially available QDs; nevertheless, it is advisable to assess each new system or new QD formulation being used for live cell labeling. Despite the several advantages of QDs, such as their enhanced brightness[12] and resistance to metabolic degradation and photodamage, there are a few impediments to their successful use. Two of these are the tendency of QDs to aggregate in the cytosol and the tendency of single QDs to bind multiple molecules[13]. As these features limit the application of QDs for imaging molecules in the cytosol of live cells, there is great interest in overcoming them. Other difficulties arising from the physical and chemical properties of currently available QDs are also the focus of ongoing research aimed at developing QDs as routine tools for bioimaging[13].

Example of Application

GM1 gangliosides are a group of galactose-containing cerebrosides found in the plasma membranes of neurons and other cells. Cholera toxin B (CTxB) binds these gangliosides, which exist in lipid microdomains called rafts. Fluorescently labeled cholera toxin is thus frequently used to visualize these membrane microdomains. To specifically label the GM1 ganglioside, we used biotinylated CTxB in conjunction with QD-avidin conjugates[9], prepared as described in **Bioconjugation of the QDs**. Live HeLa cells were labeled by a first incubation with biotinylated CTxB followed by incubation with QD-avidin and Hoechst 3342. **Figure 2** shows an image of the lateral membrane staining for GM1 using QDs (in red) and nuclear staining using Hoechst (in blue). Punctuate labeling of the cell surface by QD bioconjugate is typical for molecules such as GM1 that are present in membrane rafts.

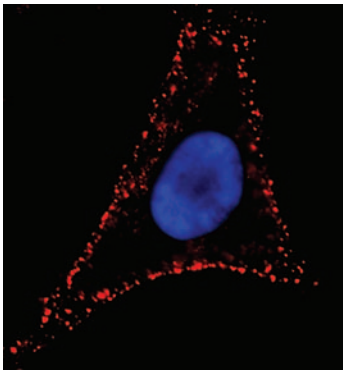

Figure 2 | Distribution of GM1 gangliosides found in the plasma membrane. Live HeLa cells growing on a glass coverslip were incubated for 15 min with biotinylated CTxB and then for 10 min with QD avidin (610 nm) and Hoechst 3342. The fluorescence image through the middle of a cell shows lateral membrane staining for GM1 using QDs (in red) and nuclear staining using Hoechst (in blue). The cells were imaged sequentially using 370/30-nm excitation and 420/40-nm emission filters for Hoechst and 480/40-nm excitation and 570-nm long-pass emission filters for QDs.

BOX 1 - GENERALIZED LABELING OF LIVE CELLS

Unmodified hydrophilic QDs can be used for applications that involve nonspecifically tagging cells for long-term or multicolor imaging. To tag cells at their surface, QD avidin bioconjugates can be used[4]. Because QDs are membrane impermeant, depending on the cells under study, one of the following alternative approaches can be used to tag cells with QDs. In all cases, the labeled cells may be observed as described in **Imaging the Labeled Cells**.

ENDOCYTIC LABELING

This approach leads to localization of the QDs to endosomes[4].

Incubate cells capable of endocytosis with 1 μM DHLA-capped QDs for 2–3 h in the appropriate growth medium. Remove the excess QDs by washing the cells several times with growth medium or an appropriate buffer.

Because different cells have different rates of endocytosis, the optimal time for endocytic loading should be determined for each cell type and cell line being used. Although DHLA-capped QDs are not toxic to cells when delivered by endocytosis[4] or by lipid-based reagent[11], QDs with different chemical coats may cause toxicity. Therefore, when used for the first time, QDs must be tested for any deleterious effects.

LABELING WITH CATIONIC LIPID–BASED REAGENTS

This approach also allows efficient and rapid delivery of QDs into the cytosol[11].

Incubate 100 pmol of DHLA-capped QDs in 100 μl of serum-free medium containing a lipid-based transfection reagent appropriate for transfecting 3 μg of plasmid DNA. Add this mixture to cells growing in serum-free or complete medium and incubate for 1–2 h.

QDs that do not have a negatively charged surface coating will give poor loading efficiency. Trying different transfection reagents and different ratios of mixing for the transfection reagent and QDs may improve the loading efficiency and reduce cell death.

LABELING USING AN AMPHIPATHIC PEPTIDE

The use of a carrier peptide can facilitate the uptake of cell-impermeant macromolecules. The peptide Pep1 (KETWWETWWTEWSQPKKKRKV) has been used to transport proteins[14] and QDs into cells (ref. 15; J.K. Jaiswal and S.M. Simon, unpublished observations).

Incubate cells for 1 h in serum-free medium containing a preformed Pep1-QD complex (10 μM peptide and 100–500 nM QDs in 100 μl of serum-free medium). Wash the cells two or three times with PBS or serum-free medium to remove extracellular QD-peptide complex.

Although the use of this approach allows cellular labeling using fewer QDs, it is still dependent on the endocytic ability of cells, as labeling is abrogated in cells incubated at 4 °C (J.K. Jaiswal and S.M. Simon, unpublished observations). This mode of QD delivery by Pep1 is consistent with TAT and polyarginine peptide–mediated protein delivery, which also occurs by endocytosis[16].

LABELING OF THE CELL SURFACE

The use of this approach allows labeling of both prokaryotic and eukaryotic cells.

Wash the cells free of growth medium using PBS, then incubate them in a solution of 1 mg/ml Sulfo-NHS-SS biotin in PBS either for 30 min at 4 °C or for 5 min at 20–25 °C. Quench the excess biotin by washing the preparation with TBS (pH 7.4), then incubate the cells for 10 min in a serum-free medium containing 0.5–1 μM avidin-conjugated QDs. Remove the unbound QDs by repeated washing with PBS.

Biotinylation of cell membrane and subsequent binding of avidin-conjugated QDs could affect the cell-surface properties, such as adhesion, cell-cell interaction and signaling. This concern must be taken into consideration when this approach is employed for functional studies using these cells.

MICROINJECTION

Microinjection is also useful for localized labeling of neurons or other cells *in situ*. This approach has been used to label isolated *Xenopus* eggs, which subsequently undergo normal development[17].

Resuspend QDs at a concentration of 1–10 pmol/μl in the appropriate buffer (for example, for mammalian cells, nuclear injection buffer can be used). Inject the desired amount of QDs into the cells. Allow the cells to recover before imagining.

SOURCE

This protocol was adapted from Imaging with Quantum Dots, in *Imaging in Neuroscience and Development* Chapter 66 (eds. Yuste, R. & Konnerth, A.) 511–516 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, in the press).

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