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Topic Introduction

Imaging Live Cells Using Quantum Dots

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Quantum dots (QDs) are nanoparticles with fluorescent properties that offer advantages over organic fluorophores. As a result, QDs have found wide application in biological imaging. In this introduction we discuss the approaches for using QDs for labeling and imaging individual cells and cellular processes in live cells both in vivo and in culture.

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INTRODUCTION

Quantum dots (QDs) are nanometer-scale particles that, like other fluorescent molecules, absorb photons of light at one wavelength and emit photons at a different wavelength. However, the physicochemical characteristic of QDs is very different from conventional fluorophores. A QD consists of a core made of two or more semiconductors with several layers of coating (usually zinc sulfide). The semiconductor materials are elements paired from Groups II and VI, III and V, or IV and VI in the chemical periodic table and frequently include cadmium, selenium, or tellurium. QDs are semiconductors whose electronic structure is closely related to the size and shape of the individual crystal because of quantum confinement. Simply put, quantum confinement describes the condition in which, because of the small size of the QD crystal, the distance an electron can move on being excited is smaller than the Bohr radii. Photo absorption results in promotion of an electron to an excited state, which initially undergoes rapid relaxation, losing some energy. At longer timescales, the excited state decays back to the ground state by emitting a photon (fluorescence). The photon energies required for absorption and emission are critically dependent on the size of the crystals produced, so it is possible to have very precise control over the fluorescent properties of the material.

The material difference between QDs and conventional fluorophores results in different physical and fluorescent properties. Some of these properties make QDs a better choice for certain biological applications, whereas other properties limit their usefulness compared with conventional fluorophores for different biological applications. The following section outlines some properties that distinguish QDs from other fluorophores. Whether a particular feature is advantageous will often depend on the intended use.

- *Brightness:* Each individual QD is several orders of magnitude brighter than most individual organic fluorophores (Wu et al. 2003).
- *Spectral characteristics:* QDs differ from conventional fluorophores in both their emission spectra and their excitation spectra. The emission spectra of QDs are narrow compared with conventional fluorophores (full width at half-maximal intensity of <30 nm vs. ~100 nm) (Jaiswal and Simon 2007). This is advantageous when simultaneously monitoring the emission of multiple fluorophores. The excitation spectrum of a QD is considerably broader than that of conventional

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fluorophores. As a result, it is possible to use an excitation wavelength that is well separated from the emission wavelength. Also, one excitation wavelength can be used to excite multiple QDs having different emission spectra (Jaiswal and Simon 2007). In some circumstances—specifically, when there is a need to use a minimal part of the spectrum for excitation to maximize the wavelengths available for collecting emission data—this feature of QDs is advantageous. In other circumstances, such as when there is a need to use separate excitation lines to selectively excite different fluorophores, using organic fluorophores is advantageous.

- *Photostability and resistance to metabolic degradation:* QDs are orders of magnitude more photostable than conventional fluorophores (Jaiswal et al. 2003). Additionally, because they are inorganic, they are resistant to metabolic degradation for periods ranging from weeks to months. This is a significant advantage when tracking cells. However, for some other applications, such as FRAP (fluorescence recovery after photobleaching) and superresolution techniques that depend on the ability to photobleach the fluorophores, this feature is disadvantageous.
- *Universal approaches for conjugating biomolecules:* QDs differ from each other with respect to the size of their core. However, their surface chemistry is the same, which allows biomolecules of interest to be conjugated to any QD using the same approach (Michalet et al. 2005). This can be advantageous when there is a need to change the fluorophore conjugated to a protein of interest without having to be concerned about altering the specificity. However, this interferes with the ability to simultaneously tag different biomolecules each with a different fluorophore.
- *Size:* The core of a QD ranges from 3 to 10 nm in size. Although QDs that emit light below 585 nm are round, those emitting at higher wavelengths appear rod-shaped (Deerinck et al. 2007). However, the coatings that are applied to make QDs biofunctional yield commercial QDs all being >20 nm in size. This size is not a limitation if the goal is to tag tissues (e.g., sentinel lymph nodes; Kim et al. 2004) or track cells (e.g., detect tumor cells in tissue; Voura et al. 2004) in vivo. However, if the goal is to label individual biomolecules, then the size of the QDs becomes a significant impediment.
- *Valency:* It has been difficult to develop QD surface chemistry so that the QDs can be linked monovalently to biomolecules. Multivalency simplifies the construction of a fluorescent probe having many molecules on the surface of the QD, such as a biosensor; but multivalency is clearly a problem if the goal is to label single molecules.
- *Delivery into cells:* The size and inorganic nature of QDs makes it difficult to deliver them into the cytosol. The use of "cell penetrating peptides" leaves a large percentage of the QDs in the endocytic system. Additionally, QDs tend to aggregate in the cytosol, limiting their use for various live cell-based applications.

In the sections below, we present approaches for using QDs for imaging live cells and discuss considerations in using QDs for these purposes.

APPLICATION 1: LABELING LIVE CELLS WITH QDs

Labeling Methods

QDs are particularly useful for long-term imaging of live cells in situ because of their photostability, the large spectral shift between excitation and emission (which helps reduce the contribution of autofluorescence), and their large two-photon action cross section, which greatly facilitates imaging with multiphoton excitation. There are multiple methods for labeling live cells with QDs by directly delivering them into cells. Many of these approaches rely on the endocytic ability of the cells. Labeled cells can remain fluorescent for weeks, and the label does not produce any detectable adverse effects on the physiology of the cell (Jaiswal et al. 2003). Other labeling approaches involve breaching the cell membrane to enable delivery of QDs directly into the cytoplasm.

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Direct QD Endocytosis

Cells are incubated for a few hours with water-soluble QDs in the appropriate growth medium. Excess QDs are washed away with growth medium or an appropriate buffer. The remaining QDs enter the cells inside endosomes and remain there for extended periods of time (Jaiswal et al. 2003).

Inclusion of Cationic Lipid-Based Reagents

Like direct QD endocytosis, this approach also allows efficient and rapid labeling of cells using the endocytic ability of the cell. Anionic QDs (e.g., with COOH groups at the surface) are incubated in serum-free medium containing a lipid-based transfection reagent, such as Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche). This method works well for labeling tumor cells. Labeled cells show no obvious differences in their physiology compared with unlabeled cells (Voura et al. 2004).

Inclusion of Carrier Peptides

Peptides are included to enhance endocytic uptake of QDs. One such peptide is Pep-1, which enhances transport of protein molecules into the cell (Morris et al. 2001). Cells are incubated for 1 h in serum-free medium containing a preformed Pep1-QD complex (Jaiswal et al. 2004). Other carrier peptides include polyarginine peptide and HIV-TAT peptide.

Microinjection

Glass capillaries having submicron-sized tips are used to deliver QDs locally and directly into the cell of interest, either in vitro or in situ. This requires the use of very small amount of QDs (1–10 pmol) and is reported to have no effect on cell physiology and development (Dubertret et al. 2002).

Scrape-Loading

Instead of microinjecting QDs into individual cells, cells growing on a substrate are scraped in the presence of 10–100 pmol of QDs (Uyeda et al. 2005). The cells are then transferred to a fresh cell culture dish, allowing cells to reseal the damage to their cell membranes and recover. Compared with microinjection, scrape-loading cannot be used for in situ cell labeling and a greater number of cells die during the process, but it does allow cytoplasmic delivery of QDs into a larger population of cells.

Considerations

As with any live cell reporter, a key requirement for the use of QDs for live cell imaging is ensuring that the labeling process has little to no impact on the cells or molecules being monitored. Features of QDs that need to be considered in this respect are the following.

Surface Coating

For cell biological applications QDs must be stable in aqueous medium. The QD core itself is not stable in water, so QDs are coated with polyacrylic acid polymer, phospholipid micelles, or similar reagents to make them water stable (Michalet et al. 2005). These coatings contribute significantly to the final size of the QD, and reducing its thickness often increases nonspecific interactions of QDs with cells and other QDs, as well as reducing quantum yield (Pinaud et al. 2004). Many of the properties of water-stable QDs in solution and inside cells depend on the nature of the surface coating. Certain coatings can cause the QDs to reduce cell viability, whereas other coatings can cause QDs to aggregate or to bind nonspecifically to other biomolecules. QDs should be carefully evaluated for their toxicity to cells and the efficiency of labeling. Two commercial suppliers, Invitrogen and Evident Technology, offer water-stable QDs with their patented, standardized surface coatings.

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Effect of QD Labeling on the Physiology and Viability of Cells

A large number of studies using QDs report that under typical cell growth conditions, QDs are inert and safe to use for studying QD-labeled live cells over long periods of time (Jaiswal and Simon 2004). However, some studies have also reported deleterious effects of QD labeling on specific cell types or cellular properties. Whenever QDs are used to label live cells, establish that the QD delivery method and extended labeling with QDs have no effect on cell growth and other physiological parameters even after prolonged periods of time (i.e., at least 1 d) (Jaiswal and Simon 2007).

Excitation Light

QDs can be excited by any wavelength of light that is lower than the emission wavelength, with the efficiency of excitation increasing with lower wavelengths. Thus for many in vitro experiments UV light is used for exciting QDs and some cellular studies have also been performed using UV excitation. Moreover, many suppliers provide excitation filters for use with QDs that transmit 350–400 nm light. Aside from regular UV-induced damage to live cells, UV excitation also causes increased degradation of QDs, causing leaching of cadmium from the QD core, which then causes additional damage to cells. Thus, despite the reduced effectiveness of QD excitation with visible light (compared with UV light), light above 400 nm should be used for imaging QD-labeled live cells. With multiphoton excitation, it is possible to access states similar to those directly populated with UV light, but with much less photodamage to the cells (Larson et al. 2003).

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APPLICATION 2: SPECIFIC LABELING OF PROTEINS

Labeling Methods

In addition to making QDs stable in aqueous medium, surface coatings also provide functional groups for conjugating biomolecules to QDs.

Labeling the Cell Surface

In this approach QDs are conjugated to biomolecules such as lectin for targeting glycoproteins or to streptavidin for binding to proteins present on the cell surface that can be conjugated to biotin. Streptavidin-QD conjugates are available commercially, and lectins can be conjugated to commercially available QDs that have been designed for that purpose. Suppliers of such QDs provide optimized protocols for conjugating lectins and other biomolecules to their QDs.

Labeling Specific Cell Proteins

Bioconjugated QDs can be used to label specific cellular proteins (Jaiswal et al. 2003) or subsets of cells using either of two different methods. In one approach, QDs are conjugated with target-protein-specific ligand or antibody, and then incubated with the cells (Fig. 1A). In the second approach, cells are incubated with a biotinylated primary antibody or ligand of interest and then, after washing, avidin-conjugated QDs are allowed to bind these molecules (Fig. 1B). Both approaches can be used in vitro to label specific intracellular and cell surface proteins (Jaiswal and Simon 2004; Koeppel et al. 2007). In live cells, these methods permit labeling of cell surface proteins only.

Labeling Single Molecules

Several QD features, including brightness, high photobleaching threshold, and high Stoke's shift, facilitate using QDs to track single fluorophores at a very high signal-to-noise ratio, often for extended periods of time (Dahan et al. 2003). To label single molecules, the approaches described in the previous paragraph are followed, except that lower QD concentrations should be used to minimize QD aggregation and nonspecific labeling.

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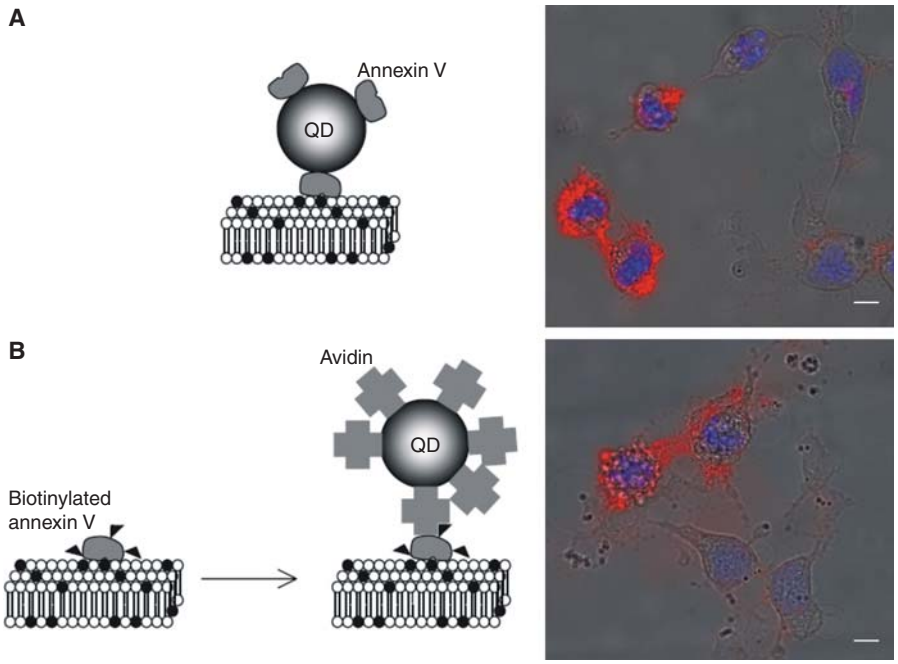

FIGURE 1. Labeling apoptotic cells using QDs. Phosphatidylserine (PS) externalized in cells triggered to apoptose by staurosporine treatment is labeled using (*A*) AnV-QD655 conjugate or (*B*) AnV-biotin followed by streptavidin-QD655 conjugate. The *left* panels show this schematically and the *right* panels show corresponding images of cells. In *A*, cells were incubated with 4 nM AnV-QD655 and, in *B*, cells were incubated with 4 nm QD655 conjugated to streptavidin. The images show an overlay of bright field (gray), Hoechst (blue), and QD (red) channels. Scale bar, 10 µm. For further details, see Koeppel et al. (2007).

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Considerations

Reducing Nonspecific Binding

In addition to making QDs hydrophilic, the molecules used to coat the QD surface often include additional polymers, such as PEG (polyethylene glycol), that further suppress the tendency of QDs to bind nonspecifically to other QDs and to proteins in cell culture medium or on the cell surface. This reduces aggregation of QDs in solution and nonspecific binding on or inside the cell (Uyeda et al. 2005).

Labeling Single Molecules

Monovalent labeling: Commercially available streptavidin- or antibody-functionalized QDs contain up to 10 functional molecules per QD, causing multivalent binding of biotinylated biomolecules to a single QD (Fig. 1) (Jaiswal and Simon 2004). This is a big impediment to using QD to study single molecules, because QD multivalency can cause cross-linking of surface proteins, which is known to alter normal cellular physiology by activating unwanted signaling pathways, and reducing mobility (Howarth et al. 2008) and normal interactions of the protein thus labeled with QD (Saxton and Jacobson 1997). Use of the F_{ab} fragment in place of antibodies can reduce the QD valency. Using a recently developed monomeric streptavidin or adopting a scheme for electrophoretic purification of monovalent QDs are two other approaches that can reduce the valency of QDs (Howarth et al. 2008).

Establishing singularity of the fluorophores: Many commonly used approaches to establish that fluorescence is from a single fluorophore cannot be used with QDs; for example, single-step photobleaching cannot be applied to QDs. Another diagnostic approach, fluorescence blinking from a single

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fluorophore under continuous wave illumination, is hindered by the sensitivity of QD blinking to changes in excitation intensity (Kagan et al. 1996), temperature (Banin et al. 1999), and the surrounding environment (Wang 2001). These factors alter QD blinking or under certain conditions eliminate it (Hohng and Ha 2004). As the cellular environment is highly reducing, it could make blinking a poor criterion for identifying single QDs in live cells. Similarly, it has been reported that for commercially available avidin-conjugated QDs, this interval could be as much as 100 sec (Hohng and Ha 2004), which is too long to track single QD-labeled molecules in the cytoplasm of live cells where they diffuse rapidly.

Size: When labeling individual biomolecules, the size of the biofunctional QDs (>20 nm) can contribute significantly to—perhaps even dominate—the observed properties of the molecule being studied. Improved coatings have made it possible to reduce the size of the biofunctional QDs (Howarth et al. 2008), and use of such QDs should be considered for single-molecule imaging.

DISCUSSION AND FUTURE DEVELOPMENTS

The utility and use of QDs have been steadily increasing. As described above, certain limitations remain in the use of QDs for live cell imaging. Thus, it is advisable to consider the strengths and weaknesses of QDs versus other fluorescent probes for your desired application. Despite the significant advantages offered by QDs, several improvements would increase their utility as probes within living cells. Important among these are the following:

- Improved methods for conjugating QDs to biomolecules, particularly in a monomeric fashion (this would help minimize the impact of QD labeling on the functioning of the labeled protein)
- Improved approaches to allow efficient delivery of QDs into the cytosol of live cells
- Modifications of QD coatings that would reduce QD aggregation in the cytosol

The latter two developments would enable more widespread use of QDs for imaging intracellular molecules in live cells. There are also ongoing efforts to use QDs to assess the function of labeled biomolecules—for example, using QDs as FRET (Förster resonance energy transfer)-based reporters of enzyme activity. These developments together with the advantages that QDs already offer for live cell imaging will continue to increase the utility of QDs for live cell imaging applications.

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