# Applications of Quantum Dots in Flow Cytometry

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## 1. Introduction

Among several applications of flow cytometry is the identification of cell populations, which is a demanding and often daunting task, given the multitude and the often intercalating pattern of protein expression between different cell types. This complexity nescessitated the use of multicolor flow cytometry, a technique that has been given new perspectives with the emergence of Quandum Dot (QD) technology, which permitted overcoming obstacles, such as limited fluorochrome availability or limited sensitivity of combining multiple organic fluorochromes. The first systematic studies of size-dependent optical properties of semiconductor crystals in colloidal solutions were performed at early 1980s (Henglein, 1982; Brus, 1983). Later, Spanhel et al (1987) performed one of the first core-shell syntheses, a major advance in increasing the quantum yield. Major improvements leading to highly fluorescent QDs were made in the mid-1990s (Hines MA & Guyot-Sionnest P, 1996; Dabbousi RO et al, 1997; Peng et al, 1997). Subsequently, CdSe crystals with silane-modified hydrophilic surfaces were introduced for biological applications (Bruchez et al, 1998; Chan, WCW & Nie S, 1998). Even more recent developments include the encapsulation of CdSe/ZnS core-hell nanocrystals into carboxylated polymer, followed by chemical modification of the surface with long-chain polyethylene glycol (PEG) (Quantum Dot Corporation, Hayward, CA).

### 2. Properties of Quantum Dots

QDs are inorganic fluorochromes manufactured with the use of semiconductor materials (cadmium selenide for QDs emitting light in the 525- to 655-nm range or cadmium telluride for QDs emitting higher wavelength light) that assemble into nanometer-scale crystals (Chan et al, 2002; Bruchez, 2005). The tiny size of the QD nanocrystals gives these materials unique physical properties which seem tailor-made for multicolour flow cytometry compared to typical semiconductors or other fluorochromes. The most important of them is their broad excitation spectra (Bruchez M, 2005). Actually, QDs can be excited over the entire visual wavelength range as well as far into the ultraviolet. Because of their exceptionally large Stokes shifts (up to 400 nm), QDs can potentially be used for the multicolor detection even by a single laser flow cytometer, whereas organic dyes require

multiple lasers for excitation in order to be used in multiplexed analysis (Bruchez M, 2005; Perfetto et al, 2004; Chattopadhyay et al, 2006). The light that the flow cytometer detects is the light which the electrons in ODs, after having been excited (excitons) by light absorption, emit as they return back from their conduction to their valence bands. What differentiates QDs from the typical fluorochromes is the so called "quantum confinement" phenomenon (Andersen et al, 2002), i.e. the phenomenon whereby, in contrast with the typical semiconductor materials where the distances between the bands are intimisimal (continuous), the excitons in QDs jump a discrete distance (known as the band gap) between bands, as a result of the very small size of the QD nanocrystal core. The narrow emission spectra of QDs usually overcomes the need for compensation, a standard process used in organic fluorochromes, which subtracts spillover fluorescence by estimating its magnitude as a fraction of the measured fluorescence in the primary detector (Roederer M, 2001) (see Fig. 1). In practice, it is reported that most ODs can be used simultaneously with only minimal (<10%) compensation between channels (Roederer et al, 2004). Moreover, when OD reagents are used with common fluorochromes excited by 488, 532, or 633 lasers (e.g., fluoroscein isothiacyanate, phycoerythrin [PE], or allophycocyanin), almost no spillover signal from other fluorochromes in the QD channels is found. Thus, in instruments with two or more lasers, QDs can be multiplexed with other fluorochromes to successfully measure even more colors (Chattopadhyay et al, 2007; 2010). In addition, the signals produced can be extremely bright, such as when an ultraviolet (350 nm) or violet (408 nm) laser is used to excite longer wavelength QDs (like QD605 andQD655), because of their high absorbance and low background levels (Hotz CZ, 2005; Wu et al, 2003). Finally, the emission properties of QDs also offer advantages over organic fluorochromes, albeit to a lesser extent.

Whereas organic fluorochromes of different colors come from a wide variety of source materials, each with distinct (and complex) physical, chemical, and biological properties which may not be compatible with each other or with staining conditions, this becomes less of a concern in QDs since QDs of different colors can be synthesized from the same starting materials (Chan et al, 2002), and thus multiplexed analysis is easier. However, the large surface-to-volume ratio in a nanosized crystal (about 50% of all atoms are on the surface) affects the emission of photons. Photochemical oxidation and surface defects in a crystal with no shell may lead to a broad emission and lower quantum yields. Indeed, early QD nanocrystals did not give stable or bright signals, exhibited poor solubility, and could not be attached to biologic probes (Riegler J & Nann T, 2004). These challenges were overcome by coating the QD nanocrystal with various materials such as inorganic zinc-sulfide, which is in turn coated with organic polymers (Bruchez et al, 1998). These organic polymers increase solubility and provide a platform of functional groups (such as amines, NH3) for conjugation to antibodies, streptavidin, and nucleic acids. Because they have similar coatings, QDs of various colors share uniform biophysical properties and a common conjugation procedure. The final QD product is about the size of PE, and can be linked to antibodies using a very similar conjugation chemistry. The shell helps to confine the excitation to the CdSe core and prevent the non-radiative relaxation.

The fluorescent properties of QDs are derived from their nanocrystal cores and not from the overall size of QD, which is actually similar in all QDs as a result of the fact that the cores are coated with various materials as mentioned above. Each QD has its characteristic emission peak, as long as the excitonic energy levels and quantum yields of fluorescence depend on exciton-photon interaction in the crystal and the size of the crystalline core. The

primary determinant of the emission spectrum of each particle is its size so that smaller nanocrystals have different quantum confinement properties than bigger ones, as a result of the fact that the distance jumped by the exciton differs (the band gap is larger), and light is emitted at a different wavelength upon return to resting state. Increasing crystal size (from 2-3 to 10-12 nm) results in shift of the emission maximum from 500 to 800 nm. However, although they fluoresce at different wavelengths, they are excited at the same wavelength, allowing detection of multiple QD colors from just one laser. Thus, QDs with the smallest nanocrystal cores (3 nm) emit light in the blue region of the spectrum, whereas QDs with the largest cores (~6 nm) emit far red light (Bruchez et al, 1998). The most commonly used QDs in multicolor flow cytometry emit light at 525 (referred to as QD525), 545, 565, 585, 605, 655, 705, and 800 nm (Perfetto et al, 2004); their nanocrystal cores range in size from 2 to 6 nm (Biju et al, 2008). Other QDs emitting light at intermediate wavelengths (like 625 nm) are also commercially available. Thus, by 'tuning' the size of the nanocrystal core with various procedures (Peng et al, 1998; Smet et al, 1999), the description of which is beyond the scope of this chapter, ODs of different colors can be produced from the same starting material (see Fig. 1).

The core-shell nanocrystals have large extinction coefficients and high quantum yields. These parameters describe the capacity of the system to capture and subsequently rerelease light. Although quantum yields of QD conjugates in aqueous buffers (20–50%) are comparable with those of conventional fluorophores, the excitation efficiency of QD conjugates is much higher, making them about two orders of magnitude more efficient at absorbing excitation light than organic dyes and fluorescent proteins. QDs have a fluorescence lifetime of 20–30 ns – about 10 times longer than the background autofluorescence of proteins. Thus, fluorescence from single CdSe crystals has been observed much longer than from other fluorophores, resulting in high turnover rates and a large number of emitted photons (Doose, 2003).

The procedure for conjugation of antibodies to QDs is similar to conjugation of antibodies to PE, with slight variations in the reagents used and ratio of antibodies to fluorescent molecules. Successful conjugation relies on the coupling of malemide groups on the QDs to thiol groups on the antibody. These groups are generated during the initial steps of the procedure, as amine groups on the QDs are activated with a heterobifunctional crosslinker (sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate, sulfo-SMCC) to generate the malemide moieties, and disulfide bonds in the antibody are reduced to thiol groups using dithiothreitol (DTT). Before conjugation, the DTT-reduced antibody is then mixed with two dye-labeled markers, Cyanin-3 (Cy3) and dextran blue, which track the monomeric fraction of antibodies as it passes through purification columns. Activated QDs and reduced antibody are subsequently purified over columns and mixed for conjugation.

A number of laser choices are available to excite QDs. Low wavelength ultraviolet (UV) and violet lasers are typically employed, since they induce maximal fluorescence emission. In theory, QD fluorescence arising from UV excitation is greater than that resulting from violet excitation; however, in practice, UV lasers induce much higher autofluorescence of cells, thereby negating the benefit of higher signal intensity. Still, users who rely on UV-excited probes (like DAPI and Hoechst) should note that QDs are compatible with their systems (Telford WG, 2004). Wheremultiplexed analysis of QDs is important, UV or violet excitation systems can be coupled to as many as eight photomultiplier tubes, allowing simultaneous

measurement of QD545, QD565, QD585, QD605, QD655, QD705, QD800, and/or a violetexcitable organic fluorochrome (Chattopadhyay et al, 2010). To detect QD signals, we employ a filter strategy that first selects light sharply with a dichroic mirror, allowing only light above a certain wavelength to pass (long-pass filter). A second filter (known as a band pass filter) is stationed in front of the PMT, in order to collect a broad band of wavelengths for maximal signal. The light reflected by the first long-pass filter is passed to the next detector where it is queried in a similar fashion.



Fig. 1. Emission spectra of fluorochromes. The colored bars represent the wavelength range of filters used for the detection of each fluorochrome. The grey squares note the overlap of neighboring fluorochromes and signify the need for compensation. A significant overlap of phycoerythrin (PE) and PE-based tandems is apparent, which necessitates high compensation values, whereas their long tails of emission can induce significant spreading error (A). In contrast, such an overlap is avoided in QDs, whose emission spectra are narrow and symmetrical, their sensitivity better and the need for compensation less (B)

## 3. Quantum Dot applications

*Multicolor Flow Cytometry*: The utility of QDs in multicolor flow cytometry has been documented by several studies. Chattopadhyay et al (2006) in their interesting study analyzed the maturity of various antigenspecific T-cell populations using a 17-color staining panel. This panel consisted of 7 QDs and 10 organic fluorochromes, which were measured simultaneously in the same sample. The QD reagents used were conjugates with conventional antibodies (against CD4, CD45RA, and CD57), as well as peptide MHC Class I (pMHCI) multimers designed to detect those antigen-specific T-cells directed against HIV, EBV, and CMV epitopes. By identifying multiple phenotypically distinct subsets within each antigenspecific T-cell population, the remarkable intricacy of T-cell immunity as well as the power of a multiplexed approach was shown. QDs also allowed the reasearchers to measure many antigen-specific populations simultaneously, an important factor when sample availability is limited.

Markers of interest for use in multicolor flow cytometry are assigned to three categories: primary, secondary, and tertiary (Chattopadhyay et al, 2006, 2010; Perfetto et al, 2004; Mahnke YD & Roederer M, 2007). Primary markers are those that are highly expressed on cells, without intermediate fluorescence (i.e., they exhibit on/off expression). Secondary markers alike are expressed brightly and are well-characterized, but can be expressed at intermediate levels, and therefore resolution of dimly staining populations may be important. Thus, the fluorochromes assigned to secondary markers should be those with the less spreading error. Finally, tertiary markers are particularly dim, poorly characterized, or expressed by only a small proportion of cells. For the latter, bright fluorochromes are necessary. In practice, tertiary markers must be considered first. If these markers are particularly dim, they are assigned to fluorochrome channels that receive very little spreading error. QDs are particularly useful in this regard. However, some QDs are dim (QD 525) (Chattopadhyay et al, 2006), and therefore are not suitable for the measurement of dim cell populations. Among QDs, the brightest choices for tertiary markers are QD655, QD605, and QD585, in order of signal intensity. Secondary markers are ideal candidates for conjugation to QDs, especially for slightly dimmer channels, such as QD545, QD565, or QD800, as long as these are often brightly expressed. Finally, primary markers can be assigned to dim channels or to fluorochrome pairs with significant spectral overlap and spreading error.

*Intracellular staining*: Although QDs are not always compatible with intracellular staining, there have been recent advances in the ability to stain intracellularly with QDs. One approach, designed to avoid steric issues or intracellular degradation, is to target the QD (with or without conjugated antibody) into a cell using enzymes, such as matrix metalloproteinases (Zhang et al, 2006; Tekle et al, 2008), or nuclear or mitochondrial signal peptides (Hoshino et al, 2004). When coupled to antibodies, QDs bound to delivery molecules might allow organelle directed, specific intracellular staining without fixation/permeabilization.

*Tetramer production*: In the past, only FITC-, PE-, APC-tetramers were available, which limited panel design because many novel or dimly staining antibodies are only found on these fluorochromes. QDs with SA groups can be used to produce pMHCI multimers (commonly called 'tetramers') (Chattopadhyay et al, 2006), displaying higher valency than PE or APC and, thus, allowing brighter signals and better staining resolution (Chattopadhyay et al, 2008).

*Pathogen detection*: Efforts to detect whole pathogens have been considerably more successful with the introduction of QDs. When applied to a mixture of pathogenic and harmless *Escherichia coli* strains, QDs conjugated to antibodies against *E. coli* can detect one pathogenic bacterium among 99 harmless ones (Hahn et al, 2008). These detection limits are comparable to current assays that use FITC, but QDs are 10-fold brighter and give more accurate results.

*Fluorescence resonance energy transfer (FRET) assays*: Another interesting potential application of QDs is for new QD-based FRET assays. In particular, recent studies have reported on efforts to achieve FRET with QDs as the donor or acceptor fluorochrome (Willard DM & VanOrden A, 2003). This might not be so difficult to do, since QDs may be available in a wide variety of colors but share similar biochemistry and, thus, it is easy to find an acceptor dye that emits fluorescence at the desired wavelength. Furthermore, signal from the acceptor and the donor are well discrete and easily recognised, because of their narrow emission spectra. Similarly, donors and acceptors can be chosen such that spectral overlap is minimized; this reduces background emission and increases sensitivity. These advantages are not available in traditional FRET assays using organic fluorochromes.

# 4. Caveats, safety & toxicity

Although QDs are emerging as useful tools in multicolor flow cytometry, they are not fully characterized and occasionally exhibit peculiar properties. As mentioned above, not all antibodies will successfully conjugate to QDs. In particular, markers for intracellular flow cytometry (e.g., reagents for intracellular cytokine detection) have been problematic to conjugate in our facility, owing in part to the presence of excessive quantities of unconjugated QDs, to limited access to intracellular compartments due to QD size-related steric problems, to uneven dispersion of QDs throughout the intracellular environment, or to high sensitivity of QDs to chemicals used in the fixation and permeabilization process associated with intracellular staining (Riegler J & Nann T, 2004; Jaiswal et al, 2004b; Tekle et al, 2008). Variation within the QDs themselves occasionally might also be considerable, due to difficulties in the control of their production process. Thus, subtle differences in incubation time or injection of precursor solutions can cause differences in size distribution, shape, and surface defects among QDs (Dabbousi et al, 1997). These can potentially impact basic properties like fluorescence. As a rule of thumb, when using QDs in multicolor flow cytometry it might be useful to engage compensation controls using exactly the same reagent as the experimental panel. Another matter of potential concern with QDs is storage method and stability, as long as QDs are prone to form aggregates or precipitate out of solution, albeit the organic coating surrounding QDs has significantly improved solubility (Jaiswal J & Simon S, 2004) and any precipitation does not actually result in loss of activity, nor does it affect staining patterns (since these aggregates stain very brightly in all channels and are easily gated out of analyses). Manufacturers typically recommend storage in glass vials or in specially coated, non-adherent plastic tubes, since in standard microcentrifuge tubes, ODs may bind plastic, precipitate, and lose activity, especially at low volumes.

Two important obstacles to biological applications of commercially available QDs until recently are low quantum yields in aqueous buffers and strong aggregation of conjugates, both determined by the surface chemistry. For the use of QDs as antibody labeled probes, their outer layer must insulate the CdSe/ZnS core structure from the aqueous environment, prevent the nonspecific adsorption of QDs to cells, as well as provide the functional groups

necessary for covalent attachment of antibodies. Lately, improvements on both nanocrystal core and shell technologies have enabled production of QD conjugates with exceptional brightness and low nonspecific adsorption (Larson DR et al, 2003). Recently, a new generation of QD nanocrystals was introduced with the application of a novel surface chemistry with the use of polymeric shell modified with long-chain, amino-functionalized PEGs. This new generation of QD nanocrystals has low nonspecific binding to cells and can be directly conjugated to antibodies through the introduced amino groups, using bisfunctional cross-linkers.

Since QDs are a new technology, their safety and toxicity are still a matter of concern. Although preliminary data from literature employing QDs for *in vivo* imaging of mice suggested that QDs are both safe and nontoxic (Voura et al, 2004; Shiohara et al, 2004; Bruchez, 2005; Gao et al, 2007), recent *in vitro* toxicology studies have questioned this assumption (Shiohara et al, 2004; Male et al, 2008). It appears that QDs coated with organic shells are relatively nontoxic for short incubation periods, but their degradation products (in particular Cd and Se), principally as a result of their oxidation and photolysis, may be toxic. Since QD size, charge, and composition of the outer shell are the main factors determinig oxidation and photolysis, toxicity likely differs by QD color and preparation. Regarding their risk on human health, data suggest that this is rather minimal, as long as QDs cannot enter the skin. However, this might not be true upon inhalation or ingestion as well, where there seems to be some potential for toxicity (Oberdorster et al, 2005; Hoet et al, 2004)

# 5. Conclusion

Although specific applications for QDs are still emerging, the basic technology has matured to the point that it can be relatively easily employed in multicolour flow cytometry. Unfortunately, just as applications for QDs are still nascent, so too is the commercial market for QD reagents. Therefore, to maximize the utility of QDs researchers must turn to in-house conjugations. Once implemented, the powerful potential of QD technology becomes evident. The remarkable spectral properties of QDs allow easy multiplexing, and therefore more information can be acquired from fewer samples. These properties make QDs useful in studying complex biologic systems.

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## Flow Cytometry - Recent Perspectives

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