Quantum Dots as Replacements for Tandem Dyes in Flow Cytometry

Gayle M. Buller, Yu-Zhong Zhang, William L. Godfrey

Invitrogen Corporation
29851 Willow Creek Road, Eugene, Oregon, 97402

ABSTRACT

Qdot® nanocrystals provide fluorescent labels that can be excited with UV or violet light sources, but can also be used with longer wavelength excitation. In flow cytometry, investigators achieve greater multiplexing of cellular markers by using tandem fluors, long-wavelength organic dyes coupled to fluorescent donor proteins, R-phycoerythrin (RPE) or allophycocyanin (APC), to enable far red emission using 488 and 635 nm laser excitation. Tandem fluors suffer from poor stability, batch variability, and donor dye spectral bleed-through. Qdot nanocrystals have long effective Stokes shifts and relatively narrow emission peaks. Even with sub-optimal excitation at 488 and 635 nm, they provide better population resolution than is achieved with most tandem fluors. For example, Qdot 705 and Qdot 800 secondary antibody reagents allow more effective resolution of CD4-positive populations in human blood than RPE and APC tandem fluors in far red emission regions. No emission is observed in the tandem fluor donor dye region, but fluorescence in the target emission region is observed with excitation off every laser. Nanocrystal reagents resolve tandem fluor issues with stability and donor bleed-through, but require cross-laser correction. Qdot nanocrystals can be used in place of tandem fluors in antibody staining, allowing detection of more targets over the same spectral range.

Keywords: quantum dot, flow cytometry, tandem dye, nanocrystal

1 INTRODUCTION

Flow cytometry is a powerful tool that is used to analyze complex antigen distributions on cell populations in research and clinical applications. The approach generally applies fluorescent antibody conjugates to quantify antigen marker expression on cell, one color per marker, and many users have been reluctant to move beyond 3 to 4 color analysis due to the complexities of correcting for multiple spectral overlaps between dyes. Qdot nanocrystals provide powerful tools that can easily add 2 to 5 colors to the antibody "palette." They have been demonstrated in a number of flow cytometry applications, including an 18-color panel for vaccine research [1].

2 METHODS

Reagents. Anti-human CD4 conjugates and quantum dot conjugates of streptavidin were acquired from Invitrogen (Carlsbad, CA), except for the RPE-Cy7 conjugate (BD Biosciences, San Jose, CA). Qdot 655 anti-human CD56 was prepared by the method used in Qdot antibody conjugation kits (Invitrogen).

Human blood was collected in CPT tubes (BD Biosciences) to prepare peripheral blood leukocytes. Cells were stained using standard protocols for direct and indirect staining, then analyzed on a BD LSR II flow cytometer equipped with violet, blue, green and red lasers. Table 1 shows the filters used for analysis. Signal to background (S/B) values were calculated as positive peak median fluorescence divided by negative peak median fluorescence.

Fluorophore	Excitation (nm)	Emission Filter
Qdot 655 nanocrystal	405	655/20
Qdot 705 nanocrystal	405	710/20
Qdot 800 nanocrystal	405	780/60
RPE	488	585/42
RPE-Alexa Fluor® 680 tandem	488	595/40
RPE-Cy5.5 tandem	488	695/40
RPE-Cy7 tandem	488	780/60
APC	635	660/20
APC-Cy7 tandem	635	780/60

Table 1: Primary excitation wavelengths and emission filters for the fluorochromes used.

3 RESULTS

3.1 Single Excitation, Narrow emissions

The excitation and emission profiles for the Qdot nanocrystals used in this paper are shown in Figure 1. Qdot nanocrystals can be excited by a wide range of wavelengths; excitation efficiency decreases with increasing wavelength. Optimal excitation is obtained with UV or violet (405-407 nm), but the nanocrystals can be efficiently excited with other common lasers on flow cytometers such as 488 nm, 532 nm, and 635 nm. On the other hand, Qdot nonocrystals have relatively narrow and symmetrical emission profiles.

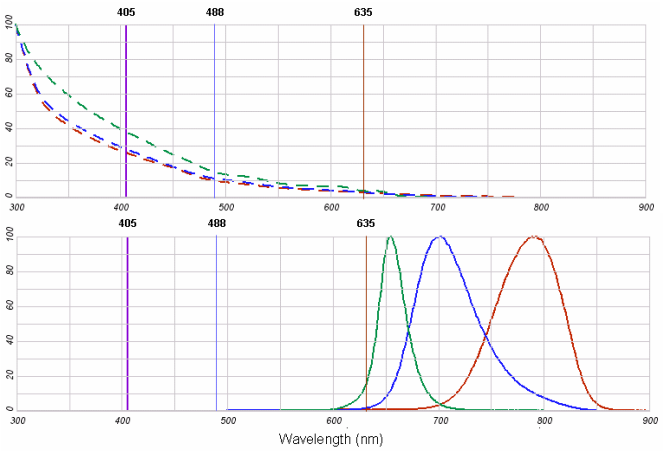

Figure 1: Excitation (top) and emission (bottom) characteristics of Qdot nanocrystals. Left to right: Qdot 655, Qdot 585, Qdot 705, and Qdot 800 nanocrystals.

3.2 Bright Emission, Sensitive Detection

Qdot nanocrystals offer the additional advantages of brightness and photostability. Because of their photophysical properties, Qdot nanocrystals are brighter than most common organic and phycobiliprotein-related fluorophores, allowing resolution of antigens that have low expression levels, such as CD56 (Figure 2). Flow cytometry has the ability to quantitate the amount of fluorescence being given off by a single cell or particle. In several clinical disease states, such as immunoglobulin light chain expression, changes in fluorescent intensity can help distinguish between different disease states [2]. Having a brighter fluorescent molecule will allow for better distinction between cells that express dim fluorescence and background.

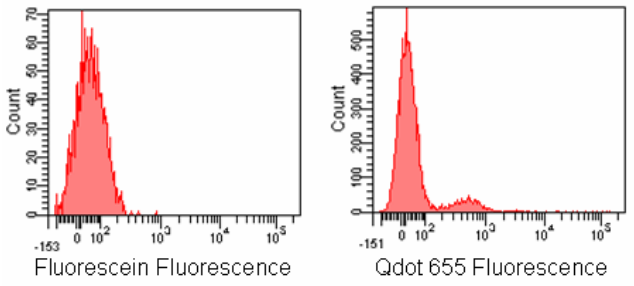

Figure 2: Human mononuclear cells stained with either fluorescein- or Qdot 655-conjugates of anti-human CD56.

3.3 Cross-Laser Excitation of Nanocrystals

Figure 3 compares Qdot 705 nanocrystal, RPE-Cy5.5 tandem and RPE- Alexa Fluor 680 tandem excitation by 405 nm, 488 nm, and 635 nm laser light. Qdot 705 conjugate-labeled cells showed strong separation of positive and negative populations when excited with 405 nm light, but also showed good resolution with 488 nm and 635 nm excitation. In contrast, the RPE-Alexa Fluor 680 conjugate showed better resolution than the RPE-Cy5.5 conjugate, but less resolution than obtained with the Qdot 705 conjugate. Both tandem dyes showed direct excitation of their acceptor dyes with 635 nm excitation, as well as weaker excitation of the RPE by 405 nm light.

Cells stained with Qdot 705 conjugates gave more than 100 times higher signal/noise ratio than seen with APC-Alexa Fluor 700 and APC-Cy5.5 conjugates under same experimental conditions using 635nm excitation (Figure 4). APC was also found to be weakly excited by 405 nm light (data not shown).

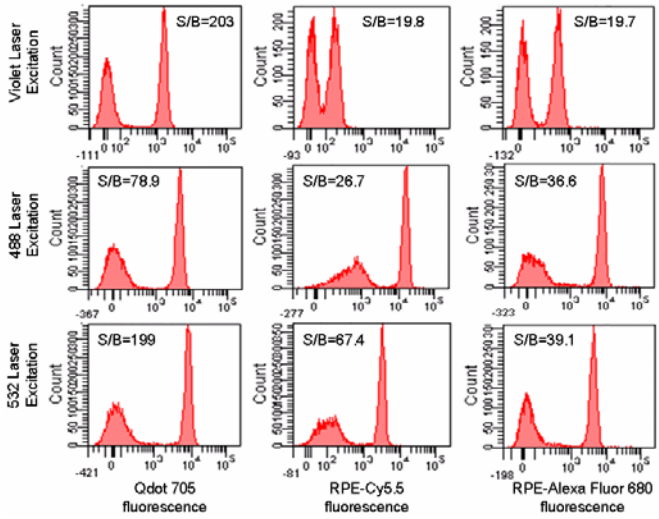

Figure 3: Comparison of Qdot 705, RPE-Cy5.5, and RPE-Alexa Fluor 680 conjugates. Cells were stained with biotin anti-CD4, followed by fluorophore-conjugated streptavidin.

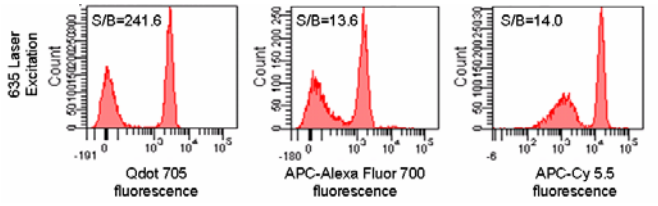

Figure 4: Comparison of Qdot 705, APC-Alexa Fluor 700, and APC-Cy5.5 conjugates. Cells were stained with biotin anti-CD4, followed by fluorophore-conjugated streptavidin.

3.4 Spectral Overlap Versus Tandem Dyes

Qdot nanocrystals show relatively narrow and symmetrical emission peaks (Figure 1). Nanocrystals can be selected to so that there is relatively little spectral overlap to be corrected. On the other hand, donor bleed-through is a common problem for tandem dye conjugates. In the case of RPE-Cy5.5 (Figure 5, top), the RPE signal that appears due to incomplete FRET will have to be subtracted, or “compensated” if RPE-labeled antibodies are

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to be used in the experiment. The Qdot 705 nanocrystal has no emission in the RPE channel (Figure 5, bottom).

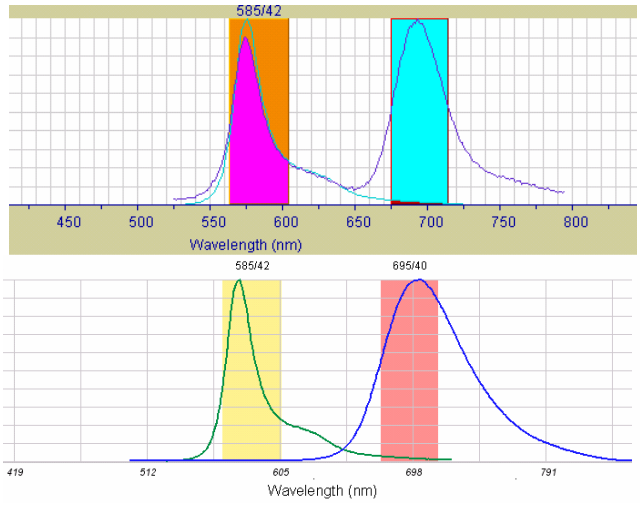

Figure 5: Spectral overlap in emissions from RPE and RPE-Cy5.5 (top) and from RPE and Qdot 705 nanocrystals (bottom).

Figure 6 shows the effect of spectral overlap on data analysis using Qdot 800 nanocrystals and RPE-Cy7 tandem dye with uncompensated data. With RPE-Cy7, the CD4-positive population is bright in the RPE-Cy7 channel, but also shows considerable RPE fluorescence as a vertical displacement (A). Qdot 800 conjugate staining shows a similarly bright CD4-positive population with 405 nm excitation, but shows no bleed-through into the RPE channel (B). Instead, there is emission in the RPE-Cy7 channel (C), as this captures the 780 nm wavelength range off the 488 nm laser. Both overlaps can be removed by compensation, but the tandem dye signal must be removed from the valuable RPE channel and other channels that RPE overlaps into; the Qdot 800 nanocrystal signal must only be removed from channels that capture the 780 nm range of light.

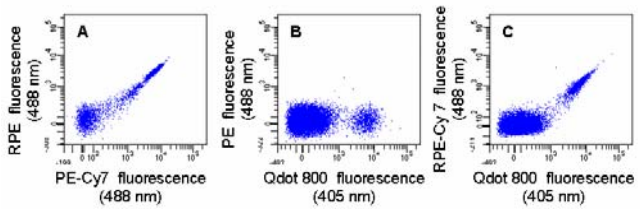

Figure 6: Comparison of RPE-Cy7 (A) and Qdot 800 (B,C) conjugates. Cells were stained with biotin anti-CD4, followed by fluorophore-conjugated streptavidin. Human mononuclear cells were stained biotin anti-CD4, followed fluorophore-conjugated streptavidin. For each conjugate dot plot, the x-axis shows emission in the primary fluorescence channel and the y-axis shows emission in potential overlap channels. The excitation wavelength is in parentheses. Data are shown without compensation for spectral overlap.

Similarly, APC-Cy7 overlaps into the APC channel (635 nm excitation, Figure 7A). Qdot 800 nanocrystals do not have any emission in the APC channel, but need to be corrected out of the 635 nm-excited APC-Cy7 channel (Figure 7B,C).

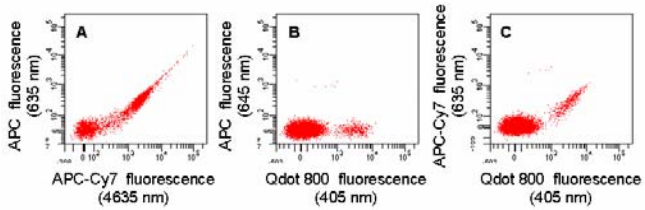

Figure 7: Comparison of APC-Cy7 (A) and Qdot 800 (B, C) conjugates. Human mononuclear cells were stained biotin anti-CD4, followed fluorophore-conjugated streptavidin. For each conjugate dot plot, the x-axis shows emission in the primary fluorescence channel and the y-axis shows emission in potential overlap channels. The excitation wavelength is in parentheses. Data are shown without compensation for spectral overlap.

4 DISCUSSION

Typical fluorescent dyes have excitation and emission spectra with relatively small Stokes shifts, which means that the optimal excitation wavelength is close to the emission peak.. Tandem dye conjugates have been developed and used in flow cytometry in order to get longer emissions from a single excitation source. These dyes take advantage of fluorescence resonance energy transfer (FRET) between a phycobiliprotein and a longer-wavelength acceptor dye to drive acceptor dye fluorescence with excitation of the donor fluorophore. However, tandem dyes suffer from incomplete energy transfer and from bleaching of acceptor dyes, particularly with longer wavelength acceptors like Cy7, both leading to donor dye bleed-through, stability problems and inconsistent performance between fluorophore batches.

Qdot nanocrystals are efficient fluorescent labels when excited with UV or violet light, allowing better resolution of even poorly-expressed antigens than obtained with conventional fluorophores. Qdot nanocrystals also provide bright fluorescence even when excited suboptimally with longer wavelengths, and can provide equivalent or better population resolution than seen with RPE or APC tandem dyes. When nanocrystals are used in combination with conventional dyes, the operator will have to apply *between laser* compensation to remove the nanocrystal signal from fluorochromes that are detected in the same wavelength range. However, Qdot nanocrystals can also be used with blue to red excitation *in place* of tandem dyes, thus avoiding the historic spectral overlap and stability problems of these dyes.

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