# Optimization of Qdot® nanocrystals for flow cytometry: filter selection and sample preparation

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Researchers today are trying to maximize the information that they get out of flow cytometry experiments by looking at more parameters in a single sample. Qdot® nanocrystals provide a powerful way to multiply fluorophore selection using commonly available excitation sources. Invitrogen currently offers a growing selection of antibody conjugates using Qdot® 565, Qdot® 605, Qdot® 655, Qdot® 705, and Qdot® 800 nanocrystals. This application note discusses:

- Advantages of using Qdot® nanocrystal primary antibody conjugates
- Compatibility of Qdot® conjugates with common reagents used for sample preparation
- Filter optimization and instrument setup for use of Qdot® nanocrystal conjugates

## Why Qdot® nanocrystals?

Qdot® nanocrystal conjugates are being used increasingly in multispectral flow cytometry [1–5]. These nanocrystal conjugates allow the addition of 1–6 colors excited by a UV or violet laser, and provide the opportunity to replace problematic fluorophores excited by lasers in the blue to red region. Qdot® nanocrystals provide the additional advantages of brightness and photostability.

Qdot® nanocrystals are nanometer-scale semiconductor particles comprising a core, shell, and coating (Figure 1A). The core is composed of a few hundred to a few thousand atoms of a semiconductor material, often cadmium mixed with sulfur, selenium, or tellurium. The core is coated with a semiconductor shell, typically ZnS, to improve the optical properties of the material. The core and shell are encased in an amphiphilic polymer coating to provide a water-soluble surface, which is covalently modified with a functionalized polyethylene glycol (PEG) outer coating. The PEG surface has been shown to reduce nonspecific binding in flow cytometry, thereby improving signal-to-noise ratios and providing clearer resolution of cell populations. Finally, antibodies are conjugated to the PEG layer using sulfhydryl/maleimide chemistry.

The fluorescence properties of Qdot® nanocrystals are different than those of typical dye molecules. The color of light that the Qdot® nanocrystal emits is strongly dependent on the particle size, creating a common platform of labels that ranges from green to red, all manufactured from the same underlying semiconductor material (Figure 1B). Conventional fluorophores such as fluorescein and R-phycoerythrin (R-PE) have excitation and emission spectra with relatively small Stokes shifts, which means that the emission maximum for the fluorophore is generally within 20–50 nm of the excitation maximum. Qdot® nanocrystals have symmetrical and relatively narrow emission peaks that can be 150 to

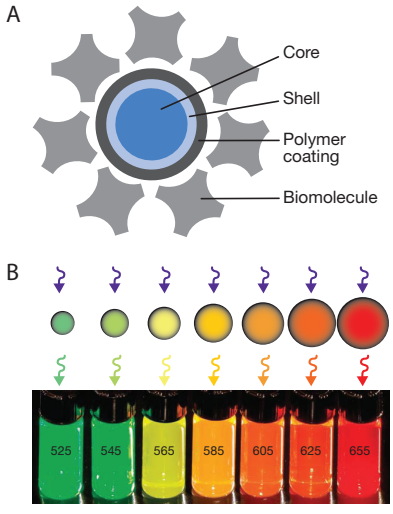


Figure 1. Qdot® nanocrystals. (**A**) Schematic of the overall structure of a Qdot ® nanocrystal. (**B**) Nanocrystals absorb light and then re-emit the light in a different color; the size of the nanocrystal determines the color. Five different nanocrystal solutions are shown excited with the same long-wavelength UV lamp.

400 nm above their excitation wavelengths (Figure 2). By using nanocrystals with emission peaks that are separated by at least 40 nm (Qdot® 565, Qdot® 605, Qdot® 655, Qdot® 705, and Qdot® 800 nanocrystals), conjugates can be made that have minimal spectral overlap.

Unlike conventional dyes, Qdot® conjugates remain fluorescent under constant illumination while conventional dyes photobleach to different extents (Figure 3). The high fluorescence stability of a Qdot® nanocrystal results in better reagent stability, and an increase in the fluorescence stability of stained samples facilitates re-analysis after sorting.

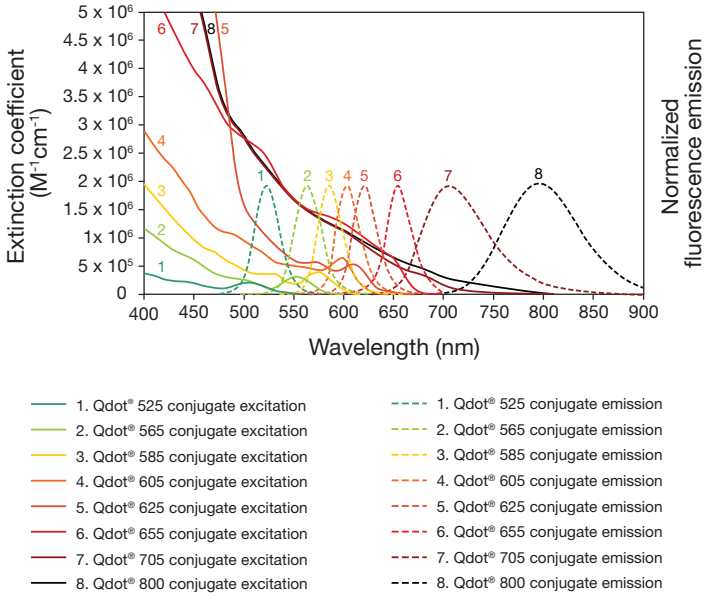


**Figure 2. Extinction coefficients and emission profiles for selected Qdot® nanocrystals.** Excitation is presented as extinction coefficient (left axis); emission is normalized to maximum peak height (right axis).

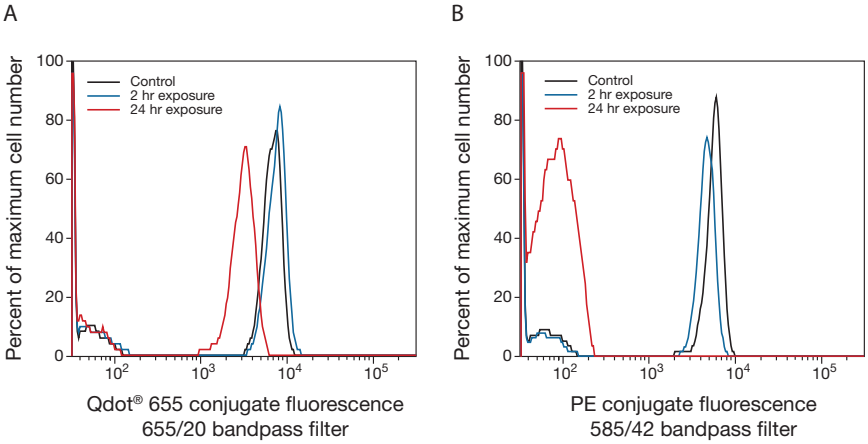


**Figure 3. Photostability of Qdot® nanocrystal conjugates.** Anti–human CD4 conjugates of Qdot® 655 (**A**) or PE (**B**) were exposed to light for the specified time before being used to stain human peripheral blood mononuclear cells (PBMC). Samples were analyzed using a BD™ LSR II flow cytometer with 405 nm or 488 nm excitation. Samples were gated on lymphocytes by scatter and collected with the identified emission filters.

## Using Qdot® nanocrystal conjugates to label surface antigens

Qdot® nanocrystal conjugates may be used in the same way as conventional antibody conjugates. Conjugates are provided at a specific concentration of Qdot® nanocrystal, usually 1–2 µM, and this concentration can be used to standardize experiments. Because staining conditions may vary, reagents should be titered with samples to obtain optimal staining concentrations. Figure 4 shows typical staining profiles for a number of Qdot® antibody conjugates.

### Materials

- Qdot® nanocrystal antibody conjugates
- Conventional fluorophore-antibody conjugates
- Sample: cells from blood, tissue culture, or singulated tissue
- Sample preparation reagents:
  - Cal-Lyse™ solution (Invitrogen), or equivalent red cell lysis reagent
  - IC Fixation Buffer (Invitrogen), fixative from FIX & PERM® (Invitrogen), 2% buffered formaldehyde, or equivalent cell fixation reagent
  - IC Permeabilization Buffer (Invitrogen), permeabilizer from FIX & PERM®, or equivalent cell permeabilizing reagent
- Labeling buffer: phosphate-buffered saline with 1% BSA (PBS/1% BSA), or equivalent

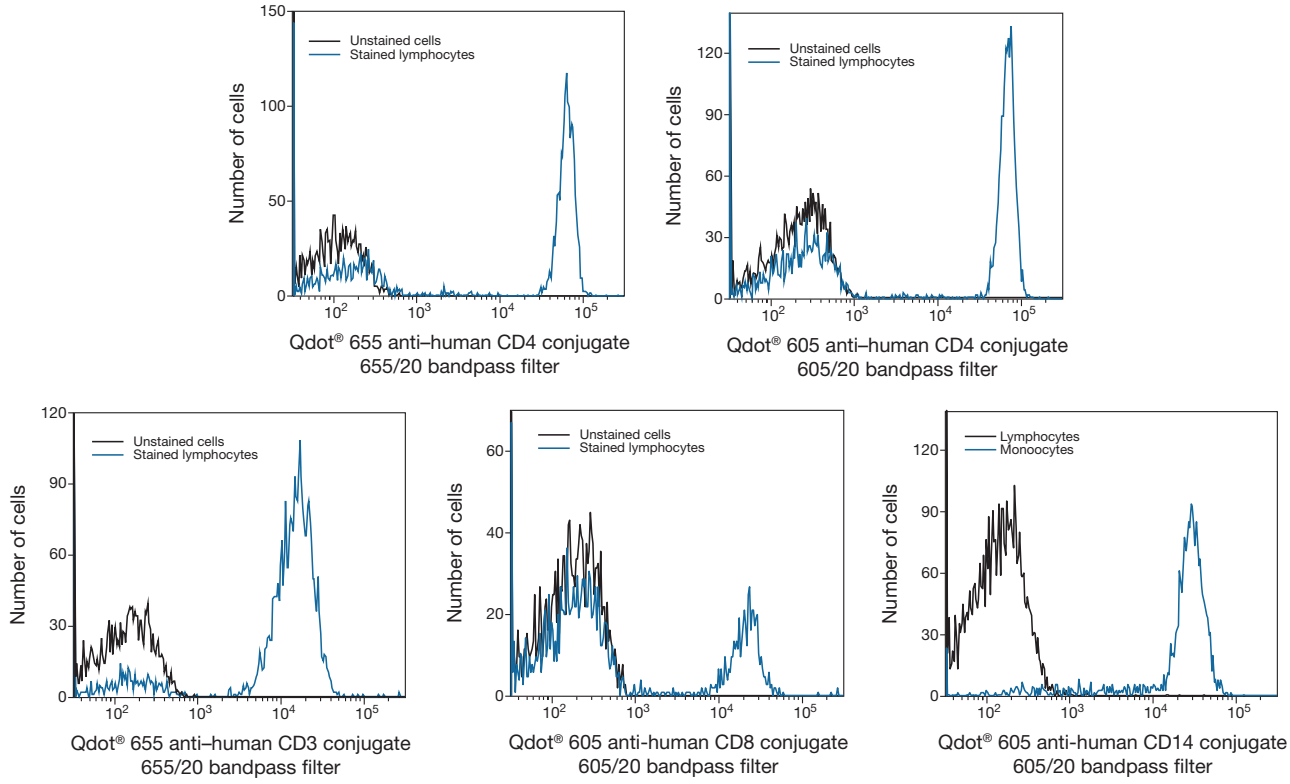


**Figure 4. Staining profiles for Qdot® nanocrystal–conjugated antibodies.** Human peripheral blood mononuclear cells (PBMC) were stained with the specified antibody-Qdot® conjugates. Samples were analyzed using a BD™ LSR II flow cytometer with 405 nm excitation and the specified emission filters. Samples were gated on lymphocytes and monocytes by scatter. The blue histograms represent stained lymphocytes; black histogram overlays represent unstained cells in the histograms for anti-CD3, anti-CD4, and anti-CD8. For anti-CD14, the blue histogram represents monocytes and the black histogram represents lymphocytes.

## Protocol

1. Collect whole blood or prepare a cell suspension in a suitable staining buffer such as PBS/1% BSA.
2. Add 100 µL cell suspension (at 1 x $10^6$ cells/mL) or 100 µL whole blood to a 12 x 75 mm flow cytometry tube.
3. Add the antibody conjugates together at optimal concentrations, including Qdot® antibody conjugate at optimal concentration, and incubate either at room temperature for 15 minutes or on ice for 30 minutes. Qdot® antibody conjugates may be added separately or mixed with other antibody conjugates.

Notes:

- Like any antibody conjugate, a Qdot® nanocrystal conjugate should be titered to determine its optimal staining concentration.
- Qdot® conjugates should be diluted in staining buffer, and diluted solutions should be used within the day of dilution. Any leftover diluted stock should be discarded.
- Antibody conjugates may be added separately or mixed and added as a cocktail. Cocktails containing Qdot® nanocrystal conjugates should be used within the day of dilution.

4. Wash the cells twice with staining buffer by centrifugation and resuspend in 100 µL of staining buffer for analysis.

Note:

- The sample may be fixed with IC Fixation Buffer for 15 minutes at room temperature. It is best to resuspend the sample in staining buffer after fixation, as long-term exposure to aldehyde-based fixatives can alter fluorochrome and cell scatter properties.

## Hints:

- **Titration:** Proper conjugate titration is critical for optimal results. In general, concentrations at or slightly below saturation should have the optimal signal/background ratio, while concentrations substantially higher than saturation will compromise the assay with higher background levels.
- **Light sensitivity:** Qdot® nanocrystals do not exhibit significant photobleaching. There is no need to protect the sample from light unless conventional fluorophores are also being used.
- **Fc receptor blocking:** If concerned about conjugate interactions with Fc receptors, cells may be blocked with either 100 µg of mouse or goat IgG and incubated on ice for 10 minutes prior to incubating with conjugates. For murine studies, anti–mouse CD16/CD32 (Cat. No. MCFR00) effectively blocks mouse Fc receptors.
- **Storage concentration:** Qdot® nanocrystal conjugates are provided at 1–2 µM. Conjugates may lose activity if stored for extended periods at lower concentrations. For this reason, working stocks should be made and used on the day of an experiment. Nanocrystal conjugates should not be stored for extended periods as part of an antibody cocktail.
- **Buffer compatibility:** Qdot® nanocrystal conjugates show stable emission characteristics in a number of different buffer salts and across a range of pH conditions. At working concentrations, the quantum yield and colloidal dispersion of these materials have been found to be remarkably stable across pH 6–9 in Tris, HEPES, phosphate, and borate buffers. Qdot® nanocrystal conjugates are stable and non-aggregated at working concentrations in buffered NaCl up to 500 mM. Higher salt concentrations result in microscopic precipitation, but do not appear to cause bulk precipitation of the materials at working dilutions. In addition, Qdot® nanocrystals have been shown to maintain fluorescence in a number of surfactants and additives at 0.05% concentrations, including Tween 20, Triton-X-100, Pluronic F68, non-detergent sulphobetaine (NDSB) 201, and ethylenediamine tetraacetic acid (EDTA). In contrast, gelatin and dextran sulfate have been found to promote aggregation of Qdot® nanocrystal conjugates, and should be avoided in labeling applications.
- **Disposal:** Qdot® conjugates contain cadmium and selenium in an inorganic crystalline form. The CdSe core is encapsulated in shells of ZnS and polymer, which may prevent dissolution of free Cd. We have not investigated the toxicity of Qdot® nanocrystals, but have demonstrated the utility of these materials in a variety of live-cell *in vitro* labeling experiments. Please dispose of the material in compliance with all applicable local, state, and federal regulations for disposal of these classes of material. For more information on the composition of these materials, consult the Material Safety Data Sheet.

# Effect of sample preparation reagents on Qdot® nanocrystal fluoresecence

## Reagents for erythrocyte lysis

Most conventional reagents used for erythrocyte lysis, including ammonium chloride and Cal-Lyse™ solution, have minimal effect on the staining index of a sample incubated with Qdot® conjugates, although treatment can cause a decrease in fluorescence intensity (Figure 5). FACS™ Lysing Solution (BD™ Biosciences, San Jose, CA) has had minimal impact on Qdot® nanocrystal fluorescence in our hands, although we have received reports that Qdot® nanocrystal fluorescence can be lost with particular batches of FACS™ Lysing Solution. PhosFlow™ Lyse reagent (BD™ Biosciences) caused a decrease in Qdot® nanocrystal fluorescence when used after staining.

In some cases, poor fluorescence intensity will be observed when cells are stained after erythrocyte lysis. This decrease can be related to fixatives present in some lysis reagents, such as PhosFlow™ Lyse, as a result of altering the antigenic determinants recognized by particular antibodies rather than a direct effect on Qdot® nanocrystal fluorescence.

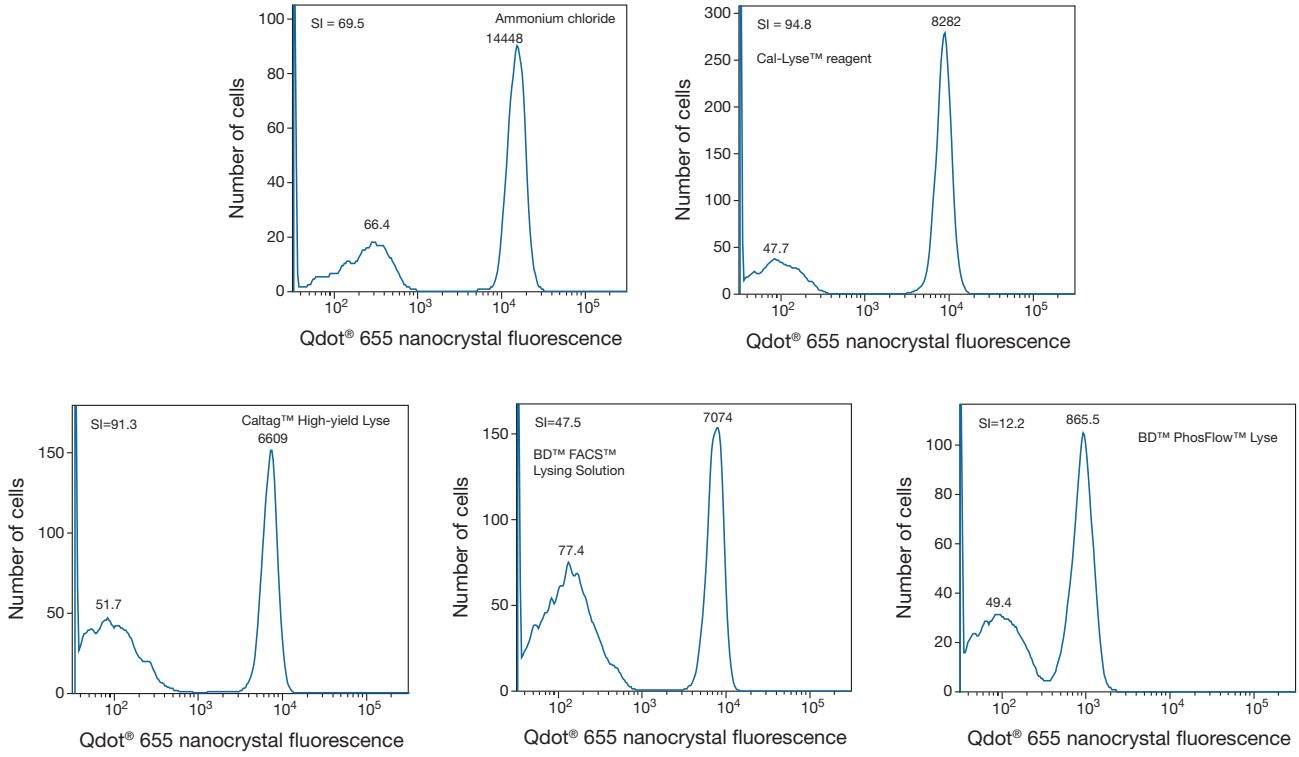


Figure 5. Effect of erythrocyte lysis reagents on Qdot® nanoctrystal fluorescence. Human blood was stained with mouse anti–human CD4 Qdot® 655 conjugate prior to erythrocyte lysis with the specified reagents. Samples were analyzed using a BD™ LSR II flow cytometer with 405 nm excitation and a 655/20 emission filter. Samples were gated on lymphocytes by scatter. Histograms are smoothed and labeled with geometric mean fluorescence values. Staining index (SI), to quantify population resolution, is calculated as the difference in population mean fluorescence values divided by twice the negative peak standard deviation.

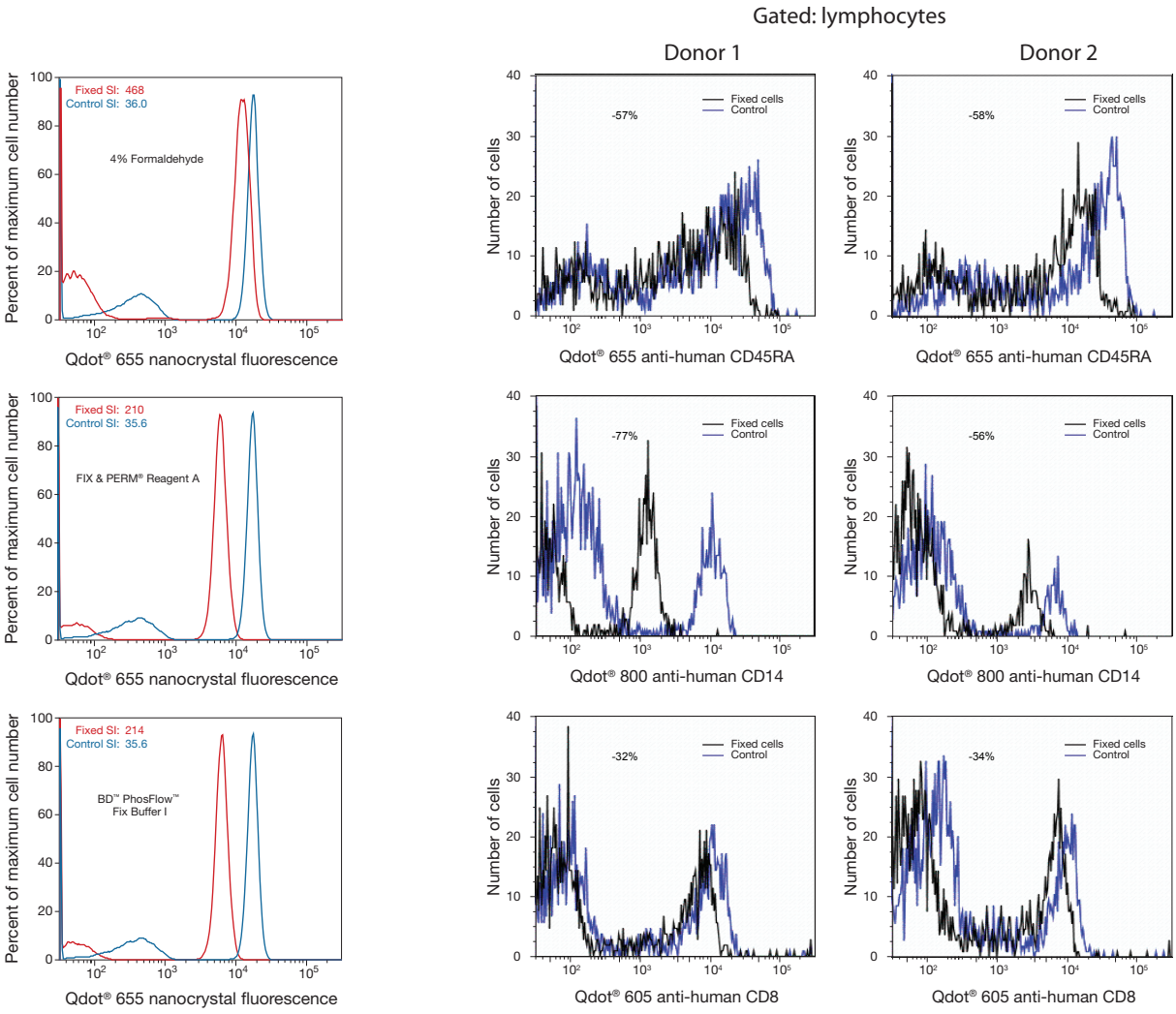


**Figure 6. Effect of fixatives on Qdot® nanoctrystal fluorescence.** Human peripheral blood mononuclear cells (PBMC) were stained with mouse anti–human CD4 Qdot® 655 conjugate before treatment with the specified fixatives. Samples were analyzed using a BD™ LSR II flow cytometer with 405 nm excitation and a 655/20 emission filter. Samples were gated on lymphocytes by scatter. Histograms are smoothed. Red histograms represent fixed cells; blue histograms represent cells that were not fixed (control). Staining index (SI) is calculated as in Figure 5.

**Figure 7. Effect of fixative on multiple Qdot® nanocrystal conjugates and donors.** Human peripheral blood mononuclear cells (PBMC) were stained with Qdot® nanocrystal conjugates before treatment with the specified fixatives. Samples were analyzed using a BD™ LSR II flow cytometer with 405 nm excitation and the specified emission filters. Samples were gated on lymphocytes by scatter. Black histograms represent fixed cells; blue histograms represent cells that were not fixed (control).

## Fixatives

Aldehyde-based fixatives will cause a decrease in Qdot® nanocrystal fluorescence. For example, Figure 6 shows a 2-fold reduction in fluorescence after fixation with three different formaldehyde-based fixative preparations, although negative peak fluorescence also decreased. Of the fixatives tested, IC Fixation Buffer provides the best preservation of Qdot® nanocrystal fluorescence. However, actual results vary with nanocrystal, antibody, and donor. Figure 7 shows staining results before and after fixation across four Qdot® nanocrystal conjugates and two donors. Fluorescence loss after fixation of a Qdot® nanocrystal stain (generally 30–60%) is greater than the loss observed with a phycoerythrin conjugate (30–40%). In at least the case of a CD14 conjugate, a higher loss was observed in one of two donors. Even with substantial loss of fluorescence, stained populations could be distinguished.

## Reagents for cell permeabilization

Reagents commonly used to permeabilize cells after fixation have not been shown to decrease Qdot® conjugate fluorescence. Reagents tested include CALTAG™ permeabilization solution, BD Cytoperm™ solution, 0.1% saponin, 0.05% Triton X-100, and methanol solutions (Figure 8).

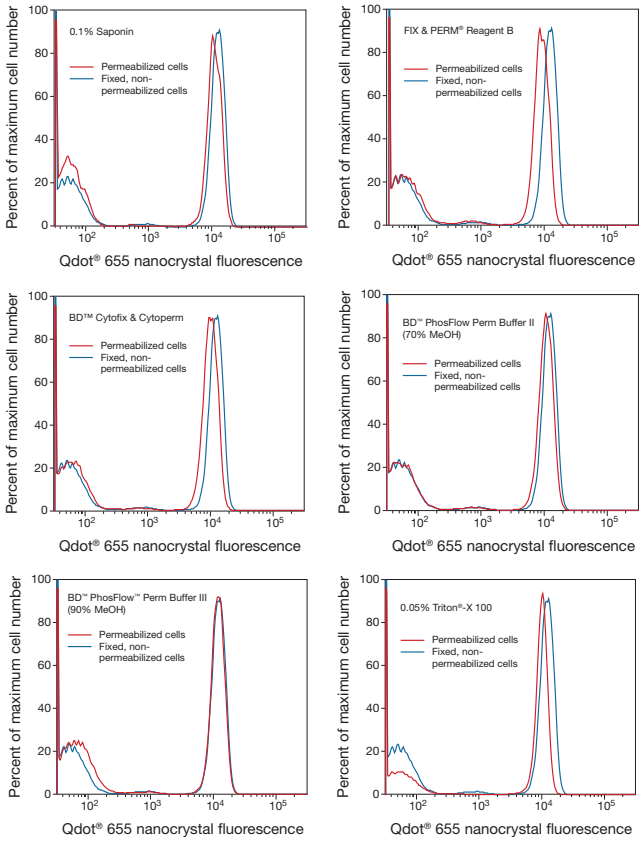


Figure 8. Effect of cell permeabilizing reagents on Qdot® nanocrystal fluorescence. Human peripheral blood mononuclear cells (PBMC) were stained with mouse anti–human CD4 Qdot® 655 conjugate, and fixed with formaldehyde before treatment with the specified permeabilizing reagents. Samples were analyzed using a BD™ LSR II flow cytometer with 405 nm excitation and a 655/20 emission filter. Samples were gated on lymphocytes by scatter. Histograms are smoothed. Red histograms represent permeabilized cells; blue histograms represent cells that were fixed but not permeabilized.

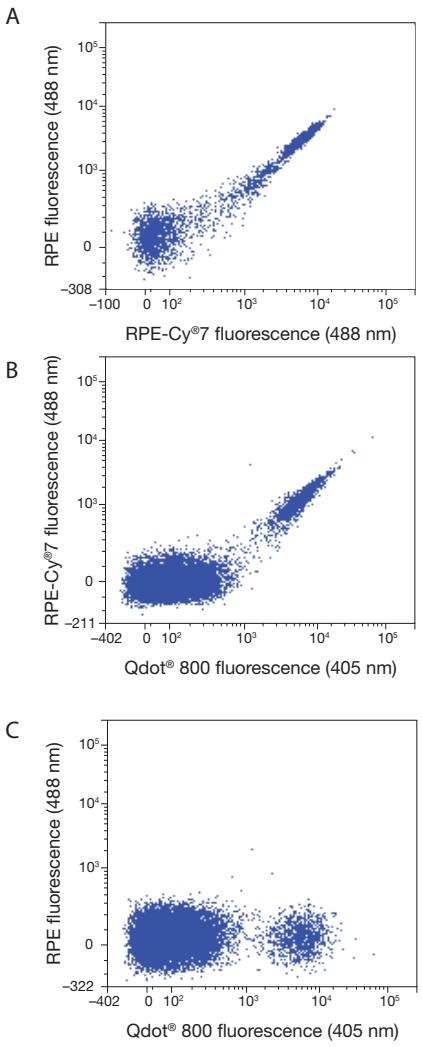


Figure 9. Example of cross-laser spectral overlap. The plots show uncompensated data from human peripheral blood mononuclear cells (PBMC) stained with biotin–anti-CD4 and either streptavidin–R-PE–Cy®7 (A) or streptavidin–Qdot® 800 nanocrystal (B, C). (**A**) Overlap of R-PE–Cy®7 into R-PE channel. The Qdot® 800 nanocrystal must be compensated out of the R-PE–Cy®7 channel (**B**) to use with this fluorophore, but it does not show spectral overlap into the R-PE channel (**C**). Samples were analyzed on a BD™ LSR II flow cytometer; axes show filters and excitation lines used. Samples were gated on lymphocytes by scatter.

## Filter optimization for Qdot® nanocrystals

Qdot® nanocrystals are optimally excited by a UV or violet (405–407 nm) laser. Unlike conventional fluorophores such as fluorescein and R-phycoerythrin (R-PE), a Qdot® nanocrystal is also excited, although with decreasing efficiency, by wavelengths up to its emission wavelength (Figure 2). Therefore, a researcher must pay attention to cross-laser (rather than within-laser) compensation for spectral overlap. In this section, we review the use of Qdot® nanocrystal conjugates in multicolor combinations with conventional fluorophores, with particular attention to filter selection to minimize the impact on fluorophore combinations that show significant spectral overlap with particular nanocrystals.

### Spectral overlap and compensation

Conventional fluorophores have relatively broad emission profiles that generally tail off towards higher wavelengths. When more than one fluorophore is detected using a single excitation source, the emission from one fluorophore can overlap into the wavelength range being detected for another fluorophore. As a result, some of the fluorescence detected in the "home channel" for a particular fluorophore on a flow cytometer will come from other fluorophores used in the experiment. The impact of spectral overlap is particularly severe with "tandem" fluorophores—fluorophores where a donor such as R-PE is used to drive emission from an acceptor dye such as Cy®7 by fluorescence resonance energy transfer (FRET). Because FRET can be inefficient, donor dye emission is often detected as well as the desired acceptor dye emission. Figure 9A shows how the emission from a cell population labeled with R-PE–Cy®7 is detected in both the R-PE–Cy®7 and R-PE channels.

To facilitate the analysis of antigen expression patterns on subpopulations of cells, flow cytometry data is corrected mathematically, or compensated, to remove contributions of other fluorophores from the home channel of a particular fluorophore. Compensation is applied to the emission values of each event by subtracting a percentage of the signal in a fluorophore's home channel from the signals in the other channels being detected, and a compensation matrix is generally established using single-color controls. Compensation comes with a cost—populations with low intensity show increasing spread as compensation increases, making it difficult to distinguish negative from positive populations. Researchers performing multispectral studies should try to minimize the impact of spectral overlap by careful selection of fluorophores, excitation wavelengths, and detector filters.

### Cross-laser spectral overlap

Because Qdot® nanocrystals are excited with multiple lasers, their fluorescence will be detected in any channel off one of these lasers that has a filter which passes the nanocrystal emission wavelength range. The result of this cross-laser spectral overlap can be seen in Figure 9B. Cells labeled with Qdot® 800 nanocrystal are detected in a 780/60 filter using violet excitation, but also are detected in the 488 nm–excited channel used for R-PE–Cy®7, which also uses a 780/60 filter (Figure 9B). This fluorescence is real, and would need to be compensated out of the R-PE–Cy®7 channel if the two fluorophores are being used together. On the other hand, Qdot® 800 nanocrystal does not show any of the emission in the R-PE channel, a problem that plagues the R-PE–Cy®7 tandem dye (Figure 9C).

Table 1 shows fluorophore combinations with potentially severe spectral overlap challenges. In the following studies, we explored filter combinations to minimize the detection of cross-laser spectral overlap, and therefore compensation, between nanocrystals and conventional fluorophores. Optimization can be accomplished through a variety of strategies. Filters are usually selected to capture a majority of the emission peak from a fluorophore to provide the brightest possible signal. If the emission profiles of two fluorophores show an offset, a filter can be selected for this region to decrease the detection of the unwanted fluorophore as long as the signal from the desired fluorophore can still be detected. Compensation can also be decreased by strengthening signal of the unwanted fluorophore in its home channel, the channel being used as the basis for compensation subtraction. A stronger signal can be achieved with either a wider bandpass filter on a fluorophore's home channel, or possibly by increasing excitation intensity.

In the following examples, Spectra Viewer plots illustrate emission profiles and the interaction between the emissions and selected band pass filters. All experiments were performed using standard staining protocols with available conjugates (Invitrogen) on human peripheral blood leukocytes, and were analyzed using a BD™ LSR II flow cytometer. Note that this flow cytometer configuration arranges detectors sequentially from longer to shorter wavelengths. Filter combinations will need to be adjusted for other cytometer architectures.

## Qdot® 655 nanocrystals

Qdot® 655 nanocrystals and allophycocyanin (APC) provide a good starting point to illustrate filter selection. Although the emission profiles of the Qdot® 655 nanocrystal and APC show considerable overlap, the nanocrystal is excited to a lesser extent by 633 nm light than APC. APC data collected with red laser excitation and a typical 660/20 filter requires relatively low compensation for the Qdot® 655 nanocrystal (Figure 10A). Improving the nanocrystal signal intensity by increasing laser power from 25 mW to 50 mW had little effect on compensation values. Using a 670/14 filter on APC to capture a shoulder on the red side of the APC emission peak slightly decreased compensation (Figure 10B). However, the conventional 660/20 filter for APC should provide adequate staining results for experiments with Qdot® 655 nanocrystal reagents. Note that APC is excited by violet light and the Qdot® 655 nanocrystal signal must be compensated for APC emission. The Qdot® 655 nanocrystal requires relatively little compensation versus adjacent nanocrystals: <5% for the Qdot® 605 nanocrystal and <10% for the Qdot® 705 nanocrystal.

Table 1. Fluorophores with potentially severe spectral overlaps compared to Qdot® nanocrystals.

| Violet excitation | Blue-green excitation | Red excitation |
|---|---|---|
| Qdot® 565 nanocrystal | R-PE | |
| Qdot® 605 nanocrystal | R-PE–Texas Red® dye | |
| Qdot® 655 nanocrystal | R-PE–Cy®5 | APC<br>Alexa Fluor® 647 dye |
| Qdot® 705 nanocrystal | PerCP, R-PE–Cy®5.5,<br>R-PE–Alexa Fluor® 700 dye | Alexa Fluor® 700 dye |
| Qdot® 800 nanocrystal | R-PE-Cy®7 | APC–<br>Alexa Fluor® 750 dye |

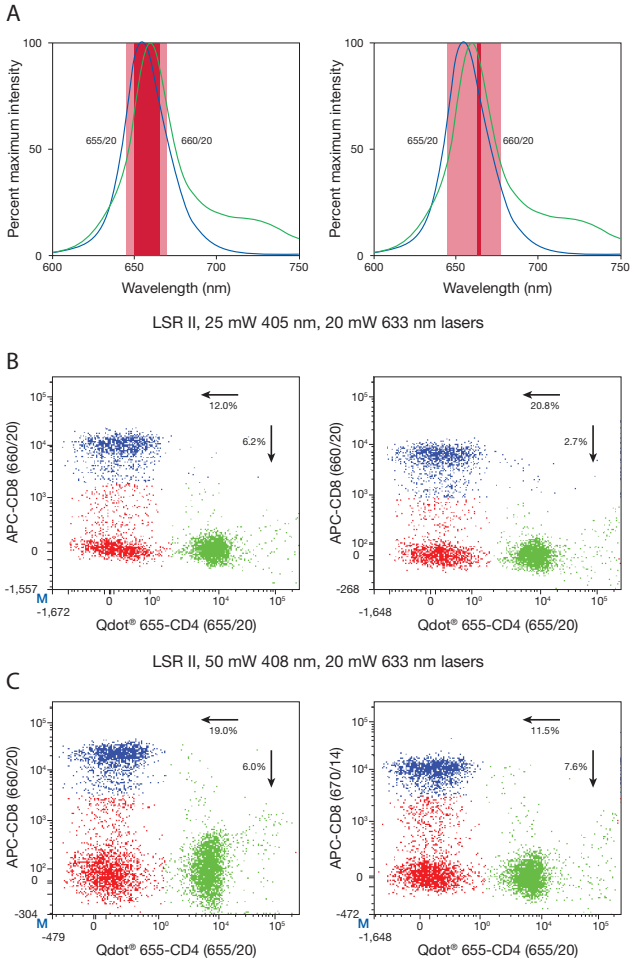


Figure 10. Filter selection for Qdot® 655 nanocrystal and APC. Human peripheral blood mononuclear cells (PBMC) were stained with Qdot® 655 anti-CD4 and APC anti-CD8 conjugates. Samples were gated on lymphocytes by scatter and collected with the identified filters on instruments equipped with either a 25 mW or a 50 mW violet laser. Compensation matrices were calculated with single-color controls.

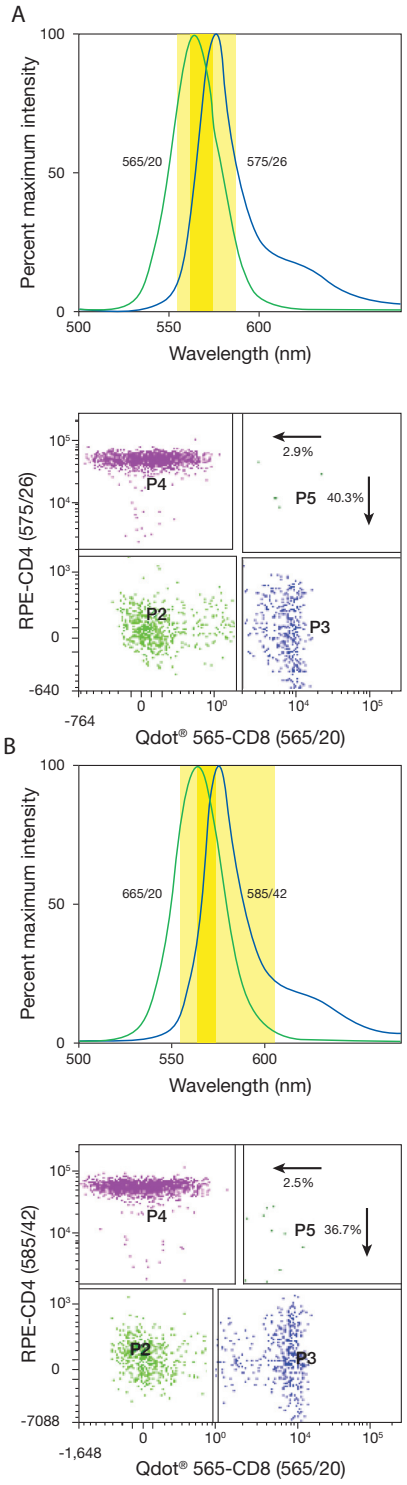


Figure 11. Filter selection for Qdot® 565 nanocrystal and R-PE. Human peripheral blood mononuclear cells (PBMC) were stained with Qdot® 565 anti-CD8 and R-PE anti-CD4 conjugates. Samples were gated on lymphocytes by scatter and collected with the identified filters. Compensation matrices were calculated with single-color controls.

## Qdot® 565 nanocrystals

The emission profiles of the Qdot® 565 nanocrystal and R-PE show considerable overlap (Figure 11), but the nanocrystal is excited with much less efficiency than R-PE by the 488 nm laser. R-PE data collected with a typical 575/26 filter required ~40% compensation for the Qdot® 565 nanocrystal (Figure 11A), and compensation could be decreased slightly using a 585/42 filter (Figure 11B). Conventional R-PE filters should provide adequate staining results for experiments with Qdot® 565 nanocrystal reagents. Note that R-PE is excited by violet light and the Qdot® 565 nanocrystal signal must be compensated for R-PE emission. The Qdot® 565 nanocrystal requires <5% versus the Qdot® 605 nanocrystal.

## Qdot® 605 nanocrystals

The Qdot® 605 nanocrystal and Texas Red® dye emission profiles overlap significantly, and the nanocrystal is excited efficiently by the 488 nm laser. As a result, Qdot® 605 emission is detected more strongly in the R-PE–Texas Red® channel than the tandem dye, and the channel requires >100% compensation to remove Qdot® 605 fluorescence (Figure 12A). The filter for the R-PE–Texas Red® channel can be moved to 620/10 to take advantage of the longer emission profile of the tandem dye relative to the nanocrystal. The 620/10 filter provides good population resolution with lower compensation, but a 630 longpass filter was included on the adjacent channel to avoid laser scatter in this detector from the red laser on the cytometer (Figure 12B). The Qdot® 605 nanocrystal requires <5% compensation for Qdot® 565 and Qdot® 655 nanocrystals.

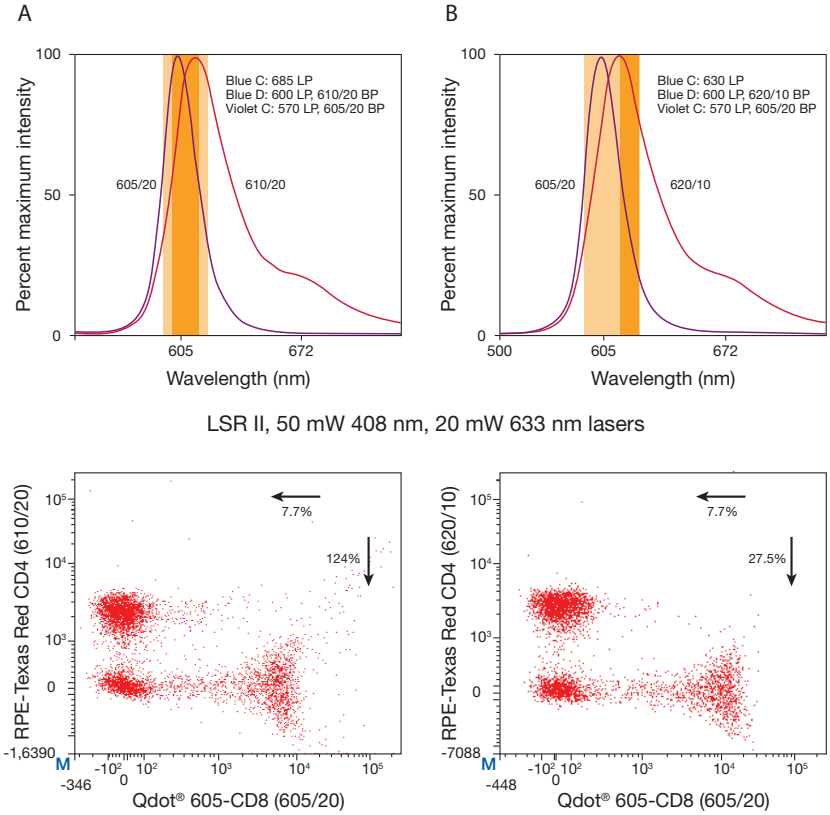


Figure 12. Filter selection for Qdot® 605 nanocrystal and R-PE-Texas Red® dye. Human peripheral blood mononuclear cells (PBMC) were stained with Qdot® 605 anti-CD8 and RPE-Texas Red® anti-CD4 conjugates. Samples were gated on lymphocytes by scatter and collected with the identified filters. Compensation matrices were calculated with single-color controls.

## Qdot® 705 nanocrystals

The Qdot® 705 nanocrystal shows significant spectral overlap with R-PE–Cy®5.5 conjugates, and the conventional 695/40 filter for R-PE–Cy®5.5 requires >300% compensation for the nanocrystal (Figure 13). Moving the R-PE–Cy®5.5 filter to a shorter wavelength reduces the amount of compensation required, but also results in unacceptable loss of intensity from the tandem dye. Therefore, these two fluorophores are not recommended to be used together.

On the other hand, the Qdot® 705 nanocrystal can be used with the R-PE–Alexa Fluor® 700 dye (Figure 14). Conventional filters require relatively high compensation of the nanocrystal from the tandem dye (Figure 14A); however, collecting more signal from the Qdot® 705 nanocrystal with either a 695/40 or 710/50 filter yields greater brightness from the nanocrystal and reduces compensation values versus the R-PE–Alexa Fluor® 700 tandem below 50%. Because of its emission profile, the Qdot® 705 nanocrystal can require >15% compensation for Qdot® 655 and Qdot® 800 nanocrystals.

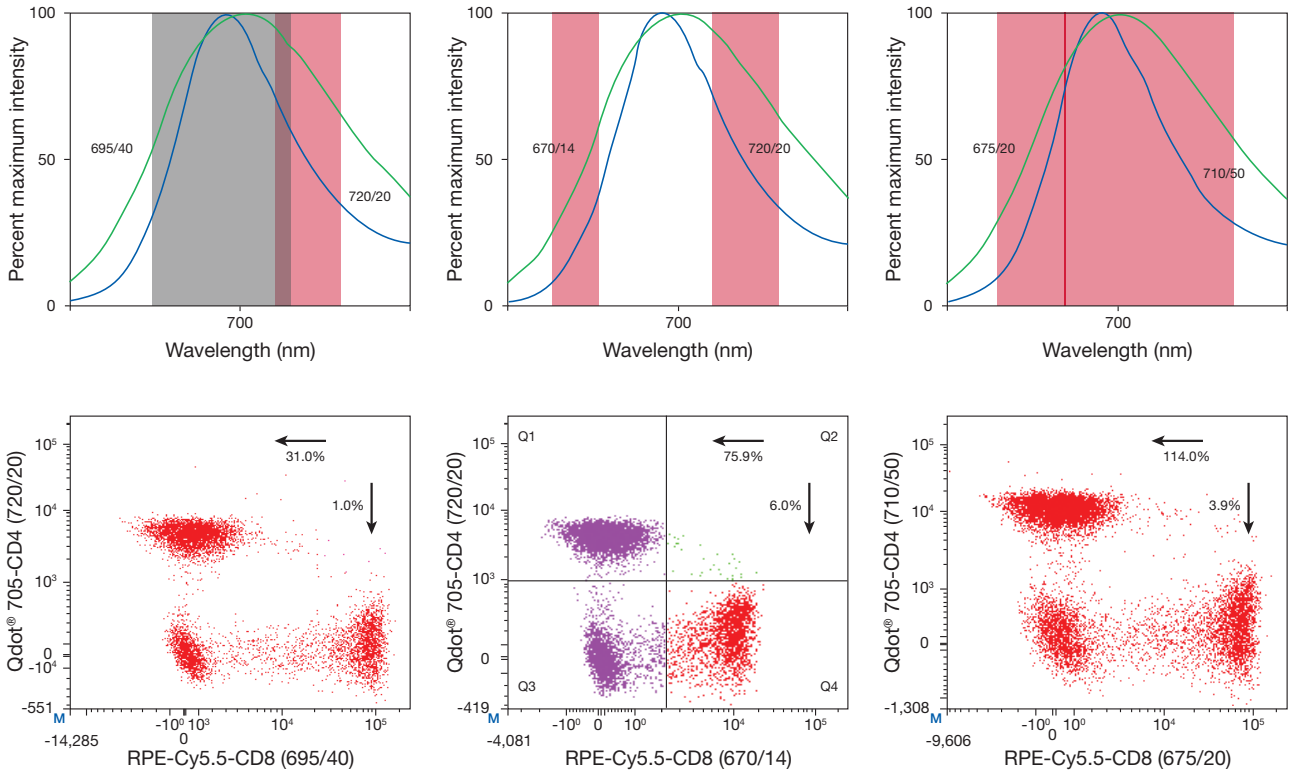


Figure 13. Filter selection for Qdot® 705 nanocrystal and R-PE–Cy®5.5. Human peripheral blood mononuclear cells (PBMC) were stained with Qdot® 705 anti-CD4 and R-PE–Cy®5.5 anti-CD8 conjugates. Samples were gated on lymphocytes by scatter and collected with the identified filters. Compensation matrices were calculated with single-color controls.

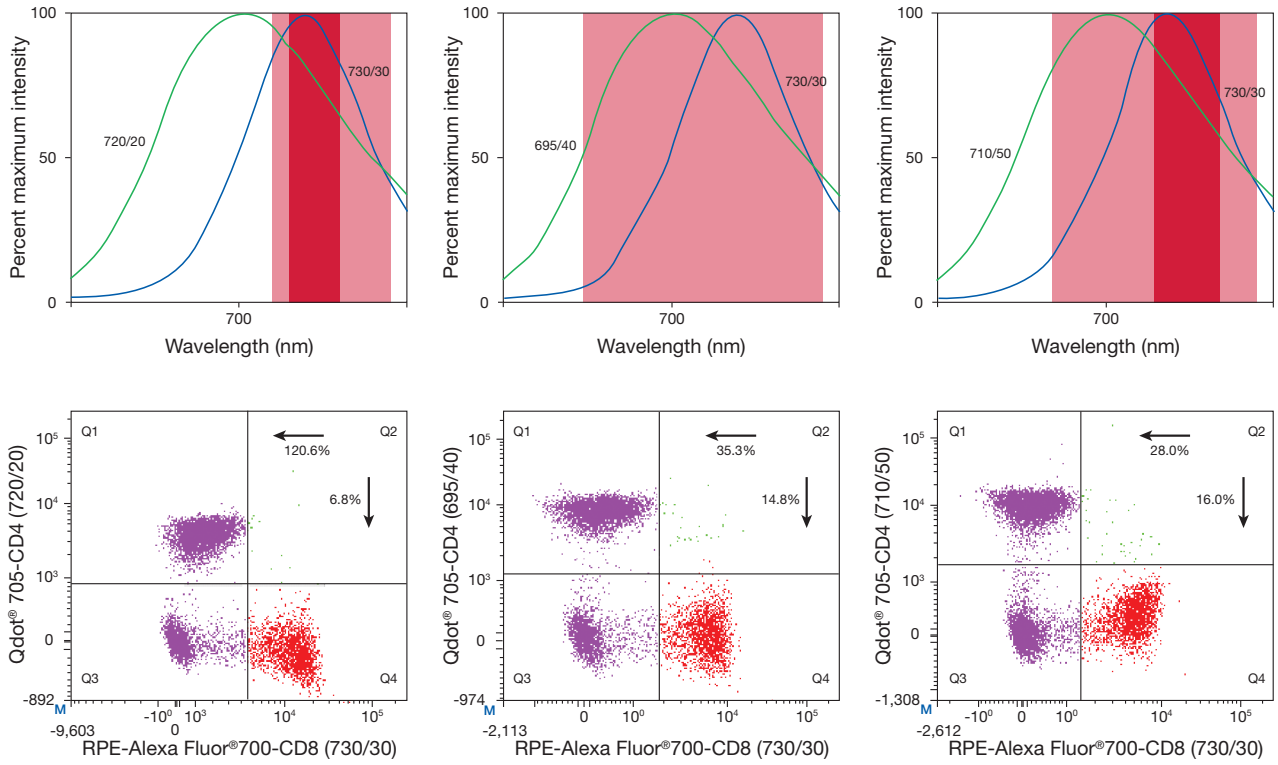


Figure 14. Filter selection for Qdot® 705 nanocrystal and R-PE–Alexa Fluor® 700 dye. Human peripheral blood mononuclear cells (PBMC) were stained with Qdot® 705 anti-CD4 and R-PE–Alexa Fluor® 700 anti-CD8 conjugates. Samples were gated on lymphocytes by scatter and collected with the identified filters. Compensation matrices were calculated with single-color controls.

### Qdot® 800 nanocrystals

The Qdot® 800 nanocrystal uses a 780/60 filter, which is at the far end of the detection range on most flow cytometers. The nanocrystal is excited well by both blue and red lasers, resulting in compensation values relative to R-PE–Cy®7 and APC–Alexa Fluor® 750 of well over 100% (Figure 15). There is little that can be done to improve filter combinations, so these fluorophores are difficult to use together. On the other hand, a Qdot® 800 conjugate can be substituted for an R-PE–Cy®7 or APC–Alexa Fluor® 750 conjugate and used with violet, blue, or red excitation. Because of its emission profile, the Qdot® 800 nanocrystal can require >15% compensation for the Qdot® 705 nanocrystal.

### Multispectral reagent combination

Figure 16 shows human peripheral blood leukocytes stained with an 8-color reagent combination containing Qdot® 605–anti-CD4, Qdot® 655–anti-CD3, Qdot® 705–anti-CD45, FITC–anti-CD2, R-PE–anti-CD16+CD56, R-PE–Cy®7-anti-CD19, APC–anti-CD14, and APC–Alexa Fluor® 750–anti-CD8. This set of fluorophores contains a number of combinations with potentially challenging spectral overlaps, including the Qdot® 655 nanocrystal versus APC; R-PE–Cy®7 and APC–Alexa Fluor® 750 versus the Qdot® 705 nanocrystal. However, through filter optimization and careful matching of antibodies to fluorophores, most compensation values could be maintained below 15%. The two exceptions were the Qdot® 655 nanocrystal correction out of APC (59.6%) and the Qdot® 705 nanocrystal correction out of R-PE–Cy®7 (74.2%).

## Instrument setup

Qdot® nanocrystals exhibit the brightest emission when excited with either a UV or a violet laser source, but acceptable fluorescence can be obtained from any excitation below the emission maximum of a given nanocrystal. Therefore, samples labeled with Qdot® nanocrystal conjugates can be analyzed on any flow cytometer that has an appropriate filter selection. Table 2 shows a filter configuration that can be used with Qdot® nanocrystals on a BD™ LSR II flow cytometer, and which employs narrow bandwidth filters to minimize effects of spectral overlap.

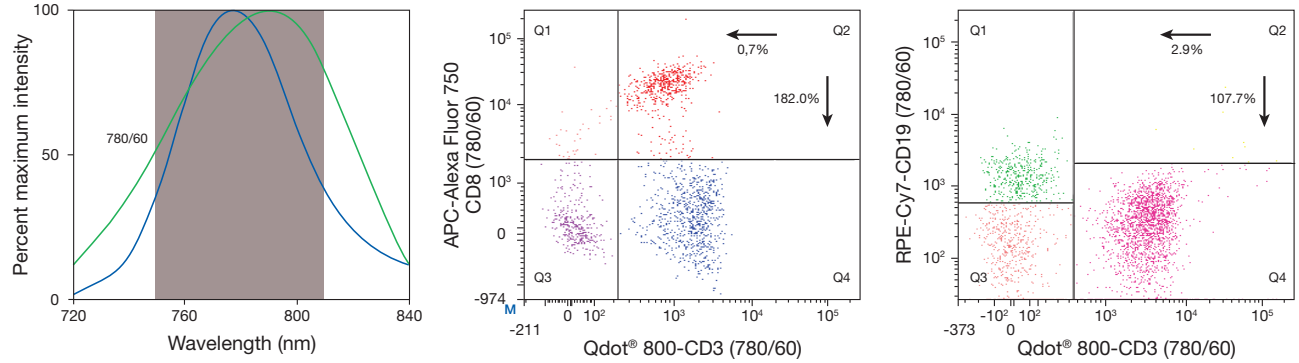


Figure 15. Filter selection for Qdot® 800 nanocrystal and infrared tandem dyes. Human peripheral blood mononuclear cells (PBMC) were stained with Qdot® 800 anti-CD3 and either APC–Alexa Fluor® 750 anti-CD8 or R-PE–Cy®7 anti-CD19 conjugates. Samples were gated on lymphocytes by scatter and collected with the identified filters. Compensation matrices were calculated with single-color controls.

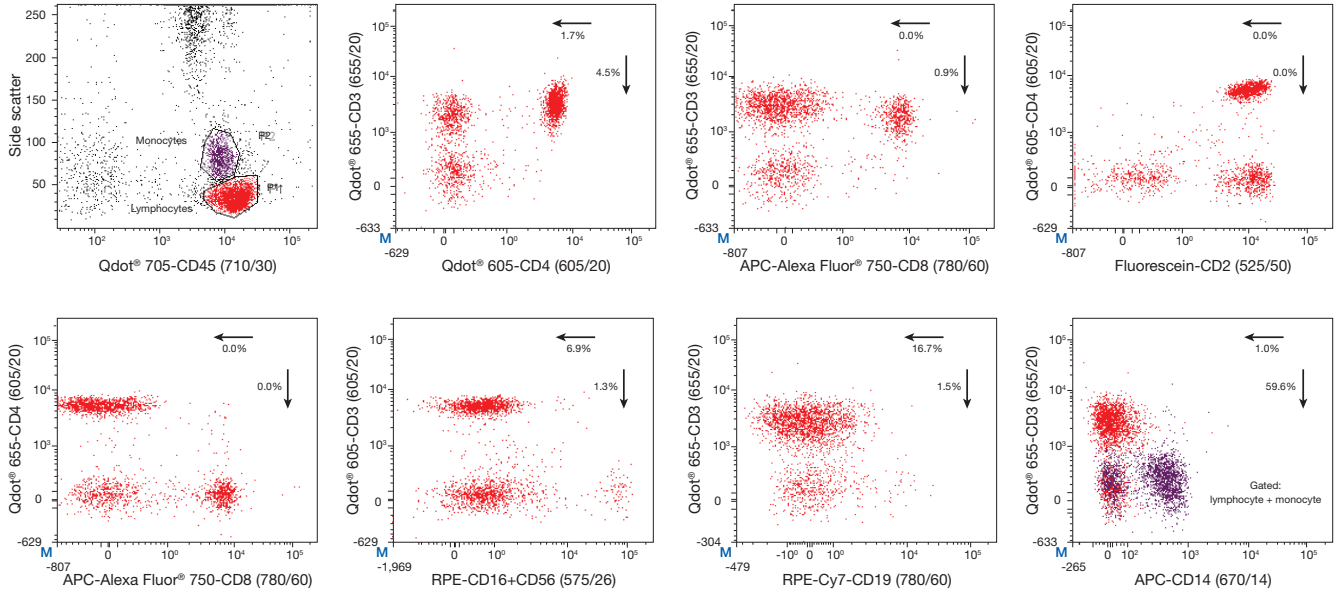


Figure 16. Eight-color immunolabeling combination with low compensation values. Human peripheral blood mononuclear cells (PBMC) were stained with Qdot® 605–anti-CD4, Qdot® 655–anti-CD3, Qdot® 705–anti-CD45, FITC–anti-CD2, R-PE–anti-CD16+CD56, R-PE–Cy®7-anti-CD19, APC–anti-CD14, and APC–Alexa Fluor® 750–anti-CD8. Samples were run on a BD™ LSR II flow cytometer. Plots are gated on lymphocytes by side scatter/CD45. Axes are labeled with the filters used; plots are labeled with compensation values.

**Other compensation values:**

Qdot® 705 CD45 – % APC–Alexa Fluor® 750 CD8 = 0.0%
APC–Alexa Fluor® 750 CD8 – % Qdot® 705 CD45 = 13.6%
Qdot® 705 CD45 – % R-PE–Cy®7 CD19 = 0.0%
R-PE–Cy®7 CD19 – % Qdot® 705 CD45 = 74.2%
APC–Alexa Fluor® 750 CD8 – % R-PE–Cy®7 CD19 = 1.4%
R-PE–Cy®7 CD19 – % APC–Alexa Fluor® 750 CD8 = 9.4%

Qdot® nanocrystals can be excited by wavelengths below their emission wavelength, but with decreasing efficiency at longer excitation wavelengths (Figure 2). Even so, nanocrystals can be used efficiently on instruments that only have 488 nm excitation sources. For instruments with fixed filter configurations such as the BD™ FACScan™ flow cytometer, you can match specific nanocrystals to the filters installed on the instrument. Examples are shown in Table 3.

Table 2. LSR II filter configuration to detect selected Qdot® nanocrystals and conventional fluorophores.

| Fluorophore | Excitation laser (nm) | Emission maximum (nm) | LSR II filter configuration |
|---|---|---|---|
| Pacific Blue™ dye | 405 | 565 | 555LP, 565/20 |
| Qdot® 565 nanocrystal<br>Pacific Orange™ dye* | 405 | 565<br>551 | 555LP, 565/20<br>555LP, 575/26 |
| Qdot® 605 nanocrystal | 405 | 605 | 570LP, 605/20 |
| Qdot® 655 nanocrystal | 405 | 655 | 640LP. 655/20 |
| Qdot® 705 nanocrystal | 405 | 705 | 690LP, 720/20 |
| Qdot® 800 nanocrystal* | 405 | 800 | 750LP, 780/60 |
| Alexa Fluor® 488 dye | 488 | | 525/50 |
| R-PE | 488 | | 575/26 |
| R-PE–Texas Red® dye* | 488 | | 620/10 |
| R-PE–Alexa Fluor® 700 dye* | 488 | | 730/30 |
| R-PE–Cy®7 | 488 | | 780/60 |
| Alexa Fluor® 647 dye or APC | 633/635 | | 660/20 or 670/14 |
| Alexa Fluor® 700 dye* | 633/635 | | 730/30 |
| APC–Alexa Fluor® 750 dye | 633/635 | | 780/60 |

* Fluorophores with spectral overlap challenges.

Table 3. Qdot® nanocrystal compatibility with specific fixed-configuration cytometers.

| Cytometer | Parameters (filter specification) | Typical fluorophores | Compatible Qdot® nanocrystals |
|---|---|---|---|
| BD™ FACScan™ flow cytometer | 488 nm excitation | | |
| | • FL1 (530/30) | Fluorescein | Qdot® 525 nanocrystal |
| | • FL2 (585/42) | R-PE | Qdot® 565, Qdot® 585 nanocrystals |
| | • FL3 (650 nm longpass) | PerCP | Qdot® 655, Qdot® 705, Qdot® 800 nanocrystals |
| BD™ FACS™ Calibur flow cytometer (4-color) | 488 nm excitation | | |
| | • FL1 (530/30) | Fluorescein | Qdot® 525 nanocrystal |
| | • FL2 (585/42) | R-PE | Qdot® 565, Qdot® 585 nanocrystals |
| | • FL3 (670 nm longpass) | PerCP | Qdot® 705, Qdot® 800 nanocrystals |
| | 635 nm excitation | | |
| | • FL4 (661/12) | APC | Qdot® 655 nanocrystal |
| Applied Biosystems® Attune™ Acoustic Focusing Cytometer (6-color) | 405 nm excitation | | |
| | • VL3 (603/48) | Pacific Orange™ | Qdot® 605 nanocrystal |
| | 488 nm excitation | | |
| | • BL3 (640 nm longpass) | PerCP | Qdot® 655 nanocrystal |
| | | PE Tandems | Qdot® 705 nanocrystal |
| | | | Qdot® 800 nanocrystal |

## Hints

- **Filter selection:** It is important to select the right filter when using Qdot® conjugates. Typically, bandpass filters should have at least a 20 nm bandwith centered on the emission maximum of the Qdot® nanocrystal (Table 2). Dichroic filters should be chosen so that the maximum amount of light is transmitted to detect the majority of the nanocrystal emission. Optimized filters are available from Chroma Technologies and Omega Optical.
- **Spectral overlap across lasers:** Qdot® nanocrystals can be excited with multiple laser lines used on flow cytometers, and emissions can overlap with conventional fluorophores. In most cases, standard approaches to setting compensation will provide reasonable compensation values, even for apparently difficult overlaps such as the Qdot® 655 nanocrystal with APC. Where compensation values are extreme, small changes in filter ranges as discussed above can provide usable compensation values.
- **Laser light exclusion:** Select filters that do not pass scattered laser light from other lasers on a multilaser flow cytometer. For example, reflected light from a 633 or 635 nm laser could contaminate the signals collected with a 630/20 filter.

# Summary

Qdot® nanocrystal conjugates of monoclonal antibodies provide powerful and easy-to-use tools for expanding the number of colors in your multicolor flow cytometry panels. Qdot® conjugates are compatible with standard sample preparation reagents and staining protocols, and can be used efficiently on flow cytometers with UV or violet excitation sources, with selection of appropriate filters. As with other fluorophore conjugates in multicolor work, care must be taken in designing a reagent panel to minimize spectral overlap, with particular attention paid to the cross-laser excitation of the nanocrystals. Qdot® nanocrystals can be used efficiently on flow cytometers with 488 nm or longer excitation as long as the nanocrystals are matched to the available emission filters, and can be substituted for problematic tandem fluorophore reagents.

## References


1. Chattopadhyah PK et al. (2006) *Nature Med* 12:972.
2. Perfetto SP et al. (2004) *Nat Rev Immunol* 4:648.
3. Telford WG (2004) *Cytometry A* 61A:9.
4. Chattopadhyay PK et al. (2007). In: Quantum Dots, Applications in Biology. Bruchez MP and Hotz CZ (ed) Humana Press, Totowa, NJ, pp 175–184.
5. Abrams B et al. (2007). In: Quantum Dots, Applications in Biology. Bruchez MP and Hotz CZ (ed) Humana Press, Totowa, NJ, pp 185–206.

## Related products

| Product | Quantity | Cat. No. |
|---|---|---|
| **Qdot® nanocrystal conjugates*** | | |
| CD4, mouse anti-human, Qdot® 655 conjugate | 100 µL | Q10007 |
| CD4, mouse anti-human, Qdot® 605 conjugate | 100 µL | Q10008 |
| CD4, mouse anti-human, Qdot® 705 conjugate | 100 µL | Q10060 |
| CD3, mouse anti-human, Qdot® 655 conjugate | 100 µL | Q10012 |
| CD8, mouse anti-human, Qdot® 565 conjugate | 100 µL | Q10152 |
| CD8, mouse anti-human, Qdot® 605 conjugate | 100 µL | Q10009 |
| CD45, mouse anti-human, Qdot® 705 conjugate | 100 µL | Q10062 |
| * For a complete list of Qdot® nanocrystal conjugates, visit: www.invitrogen.com/qdotinflow. | | |
| **Conventional fluorophore conjugates** | | |
| CD2, mouse anti-human, Fluorescein | 0.5 mL | CD0201 |
| CD4, mouse anti-human, R-PE-Texas Red® | 0.5 mL | MHCD0417 |
| CD8, mouse anti-human, R-PE-Cy®5.5 | 0.5 mL | MHCD0818 |
| CD8, mouse anti-human, R-PE-Alexa Fluor® 700 | 0.5 mL | MHCD0824 |
| CD8, mouse anti-human, APC | 0.5 mL | MHCD0805 |
| CD8, mouse anti-human, APC-Alexa Fluor® 750 | 0.5 mL | MHCD0827 |
| CD14, mouse anti-human, APC | 0.5 mL | MHCD1405 |
| CD16, mouse anti-human, R-PE | 0.5 mL | MHCD1604 |
| CD19, mouse anti-human, R-PE-Cy®7 | 0.5 mL | MHCD1912 |
| CD56, mouse anti-human, R-PE | 0.5 mL | MHCD5604 |
| **Auxilliary reagents** | | |
| Cal-Lyse™ solution | 25 mL | GAS-010 |
| Cal-Lyse™ solution | 100 mL | GAS-010S-100 |
| High-Yield Lyse | 500 mL | HYL-250 |
| IC Fixation Buffer | 100 mL | FB001 |
| IC Permeabilization Buffer | 2 x 125 mL | PB001 |
| FIX & PERM® Reagent A | 5 mL | GAS001S-5 |
| FIX & PERM® Reagent A | 100 mL | GAS001S-100 |
| FIX & PERM® Reagent B | 5 mL | GAS002S-5 |
| FIX & PERM® Reagent B | 100 mL | GAS002S-100 |
| FIX & PERM® Reagents | 50 mL | GAS003 |
| FIX & PERM® Reagents | 200 mL | GAS004 |
| CD16/CD32, rat anti-mouse, purified (blocks FcγRIII/II) | 250 µg | MFCR00 |