Qdot® nanocrystal conjugates

FOR FLOW CYTOMETRY

Take the easy route to multicolor flow cytometry. With applications across a wide range of biological investigations, Qdot® nanocrystals are powerful complements to traditional fluorophore conjugates.

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QDOT® NANOCRYSTALS ARE AN EXCITING and novel group of fluorophores that have applications across the universe of biological investigation, from detecting proteins in electrophoresis gels to *in vivo* imaging of cells. As an application area that relies on fluorescence for data acquisition, flow cytometry is poised to reap substantial benefits from Qdot® nanocrystal conjugates.

Flow cytometry is a research and diagnostic technology that interrogates thousands of cells per second to characterize the antigenic and functional parameters of a population of cells. Scientists are continually seeking ways to combine this rapid analysis capability with multiple fluorescent conjugates to maximize the information received from each flow cytometry run. For the design and execution of multiplexed flow cytometry experiments, Qdot® nanocrystals provide unsurpassed utility and are potent complements to traditional fluorophore conjugates. Invitrogen offers a growing selection of antibody conjugates, including monoclonal antibody conjugates,

that incorporate Qdot® 605, Qdot® 655, Qdot® 705, or Qdot® 800 nanocrystals. Qdot® nanocrystal conjugates are efficiently excited with commonly available flow cytometer light sources. For flow cytometry users to be comfortable incorporating these new conjugates in their experiments, however, they must understand how to use the reagents with common sample preparation and staining protocols, how to identify appropriate excitation lines and filters, and how to analyze the resulting data.

WHY QDOT® NANOCRYSTALS?

Qdot® nanocrystals are nanometer-scale semiconductor particles comprising a core, shell, and coating (Figure 1A). The core is made up 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 a sulfhydryl/maleimide chemistry.

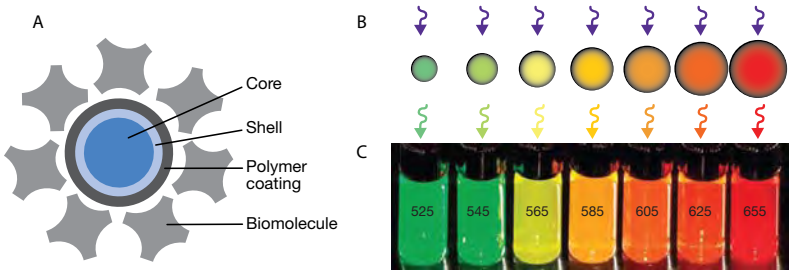

Figure 1— Composition and properties of Qdot® nanocrystals. (**A**) Schematic of the overall structure of a Qdot® conjugate. (**B**) Nanocrystals absorb light and then re-emit the light in a different color; the size of the nanocrystal determines the color. (**C**) Seven nanocrystal solutions are shown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

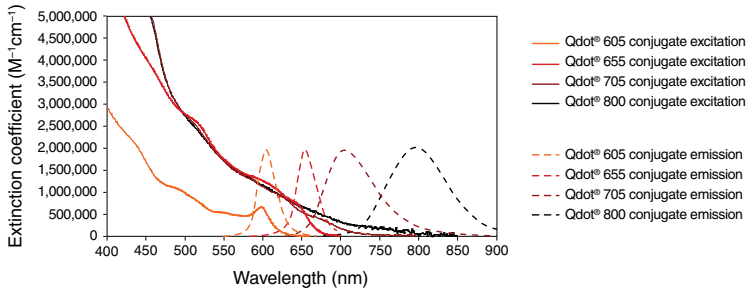

Figure 2—Extinction coefficients and emission profiles for selected Qdot® nanocrystals. Excitation is presented as extinction coefficient; emission is normalized to maximum peak height.

The fluorescence properties of Qdot® nanocrystals are different from 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 from green to red, all manufactured from the same underlying semiconductor material (Figures 1B and 1C). 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. Qdot® nanocrystals have broad spectra with absorption that increases dramatically at shorter excitation wavelengths. Their emission peaks are narrow and symmetrical, and do not change with variations in the excitation source (Figure 2). Qdot® nanocrystals are optimally excited by UV or violet (405–407 nm) laser light, although sufficient excitation can also be obtained using other excitation sources as discussed below.

BECAUSE OF THEIR SPECTRAL PROPERTIES, QDOT® NANOCRYSTALS ARE BRIGHTER THAN MOST ORGANIC FLUOROPHORES.

Qdot® nanocrystal conjugates are increasingly prevalent in multispectral flow cytometry.[1–5] Using these nanocrystal conjugates allows the addition of one to six colors, all excited by the violet laser. Qdot® nanocrystals provide the additional advantages of brightness and photostability. Because of their spectral properties, Qdot® nanocrystals are brighter than most organic fluorophores. When compared to conventional dyes, Qdot® conjugates remain fluorescent under constant illumination while conventional dyes photobleach to varying degrees (Figure 3). The fluorescence stability of a Qdot® nanocrystal translates to better stability of the reagent and consequently of the stained sample, and also permits additional analysis steps after sorting.

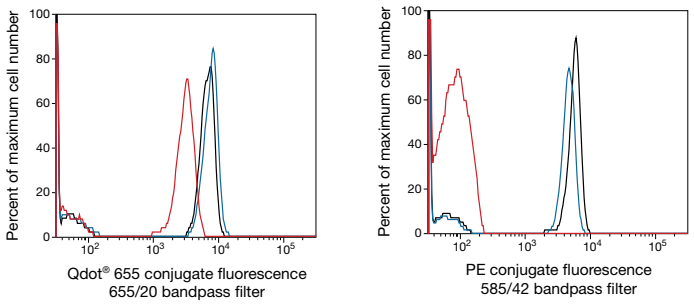

Figure 3—Photostability of conjugates. Anti–human CD4 conjugates were exposed to fluorescent light for a specified time before being used to stain human peripheral blood leukocytes (PBLs). Control (no light exposure) = black, 2 hour exposure = blue, 24 hour exposure = red. Samples were analyzed using an LSR II cytometer (BD Biosciences) with 405 nm or 488 nm excitation and the specified emission filters.

CONJUGATION AND CELL STAINING

Qdot® nanocrystal antibody conjugates are made using the same conjugation technology employed in the Qdot® antibody conjugation kits. The nanocrystals are modified with a thiol-reactive maleimide, and the antibodies are reduced to make hinge-region thiol groups available. When mixed, the antibody forms covalent thioethers with the nanocrystal, and the resulting conjugate is purified by size-exclusion chromatography (Figure 4).

Qdot® nanocrystal conjugates may be used in the same way as conventional conjugates. Conjugates are provided at a specific concentration of Qdot® nanocrystal, usually 1–2 µM. Because staining conditions may vary, reagents should be titered with samples to obtain optimal staining concentrations. Figure 5 shows typical staining patterns for a number of Qdot® antibody conjugates.

COMPATIBILITY WITH REAGENTS FOR SAMPLE PREPARATION

Samples used in flow cytometry, particularly blood samples, often require some level of preparation before, during, or after staining. Peripheral blood leukocyte (PBL) staining usually requires lysis of erythrocytes when whole blood is used. Intracellular staining requires that cells be permeabilized. Sample fixation is often required as part of intracellular staining protocols and/or before storing stained samples. Reagents used for these types of sample preparation steps can interfere with fluorophore performance.

Most conventional reagents used for erythrocyte lysis, including ammonium chloride and Cal-Lyse™ reagent, ›

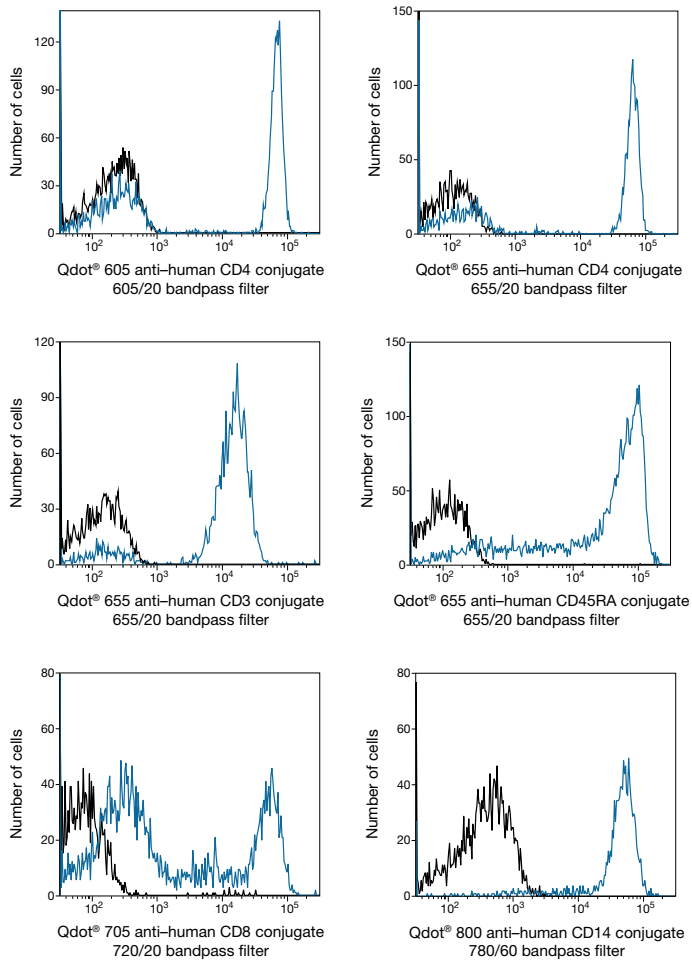

Figure 5—Staining profiles for characteristic Qdot® nanocrystal antibody conjugates. Human peripheral blood leukocytes (PBLs) were stained with the specified Qdot® nanocrystal antibody conjugates. Samples were analyzed using an LSR II cytometer (BD Biosciences) with 405 nm excitation and the specified emission filters. The blue peaks correspond to stained leukocytes, and the black peaks show the position of unstained cells in the histograms for anti-CD3, anti-CD4, anti-CD8, and anti-CD45RA. For anti-CD14, the blue peak corresponds to monocytes and the black peak corresponds to lymphocytes.

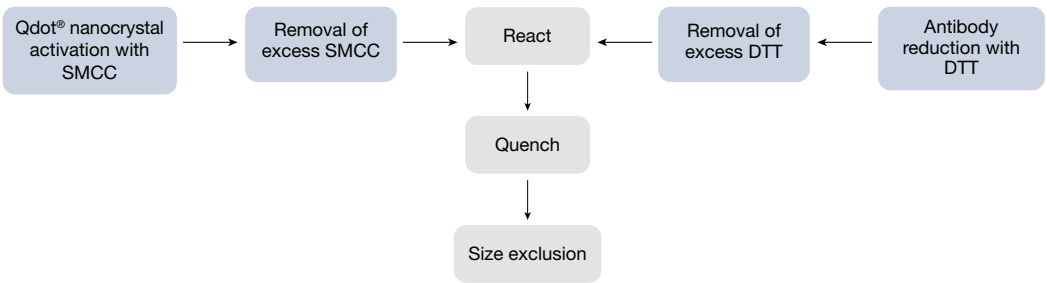

Figure 4—Schematic of Qdot® nanocrystal conjugation to an antibody. SMCC is used to derivatize the nanocrystals using a thiol-reactive maleimide group. The antibody is reduced with dithiothreitol (DTT) to expose hinge-region thiol groups. The components are reacted to form the nanocrystal–antibody conjugate, which is then purified by size-exclusion chromatography.

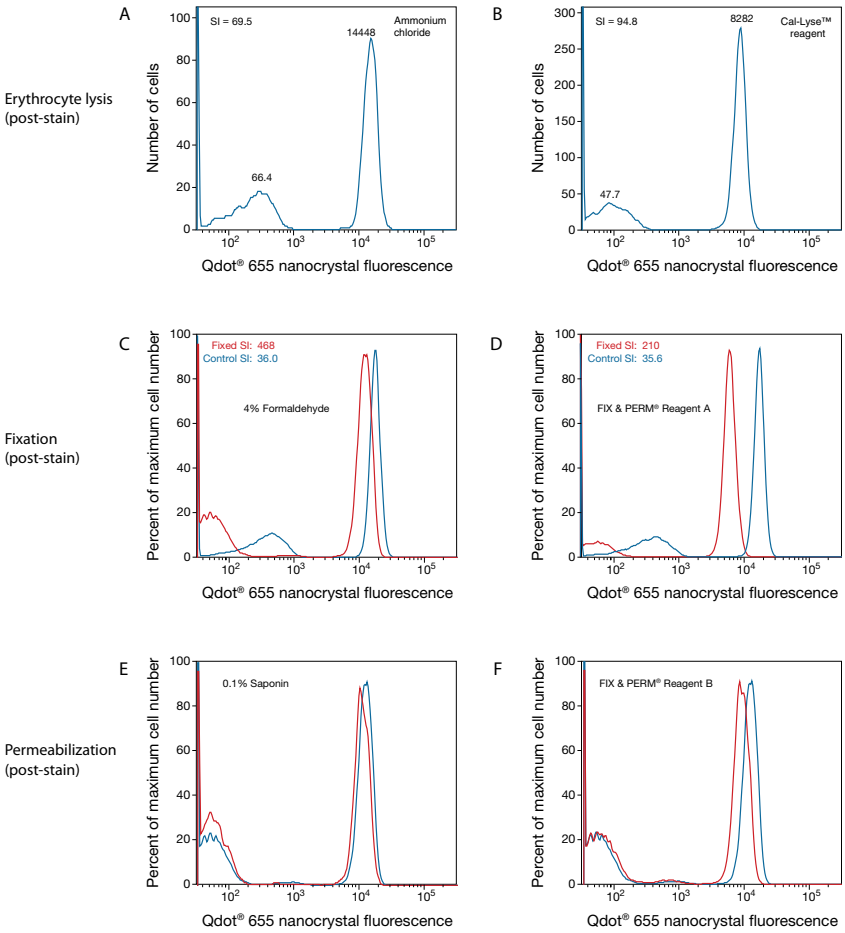

Figure 6—Representative results using common sample preparation reagents. (**A**,**B**) Human blood was stained with mouse anti–human CD4 Qdot® 655 conjugate before erythrocyte lysis with the specified reagents. Isolated human peripheral blood leukocytes (PBLs) were either stained with mouse anti–human CD4 Qdot® 655 conjugate before treatment with the specified fixatives (**C**,**D**; red = fixed, blue = unfixed control), or stained and fixed with formaldehyde before treatment with the specified permeabilizing reagents (**E**,**F**; red = fixed and permeabilized, blue = fixed only). Samples were analyzed using an LSR II cytometer (BD Biosciences) with 405 nm excitation and a 655/20 emission filter. Histograms are smoothed and labeled with geometric mean fluorescence values (**A**,**B**). 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 (**A**–**D**).

have minimal effect on the fluorescence intensity of cells stained with Qdot® conjugates (Figures 6A and 6B). FACS™ Lysing Solution (BD Biosciences) usually has minimal impact on Qdot® nanocrystal fluorescence in our experiments, although there are reports of occasional decreases in Qdot® nanocrystal fluorescence that may be related to particular batches of FACS™ Lysing Solution.

Aldehyde-based fixatives may cause a small change in Qdot® nanocrystal fluorescence. For example, Figure 6 shows a 2-fold reduction in fluorescence after fixation with formaldehyde, although negative peak fluorescence also decreased. This change in fluorescence is generally tolerable given the population resolution achieved with Qdot® conjugates.

Reagents commonly used to permeabilize cells after fixation have not been shown to damage Qdot® conjugate fluorescence. Reagents tested include FIX & PERM® Reagent B, BD Cytoperm, 0.1% saponin, 0.05% Triton® X-100 (Union Carbide Chemicals), and methanol solutions (Figures 6E and 6F).

INSTRUMENT SETUP: FILTER SELECTION

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

Table 1—LSR II filter combinations for detecting selected Qdot® nanocrystals.

Fluorophore	Emission maximum (nm)	Configuration A: narrow-bandpass filters		Configuration B: broad-bandpass filters [1,2]	
Qdot® 565 nanocrystal	565	555LP	565/20	557LP	560/40
Qdot® 605 nanocrystal	605	570LP	605/20	595LP	605/40
Qdot® 655 nanocrystal	655	640LP	655/20	640LP	660/40
Qdot® 705 nanocrystal	705	690LP	720/20	670LP	705/70
Qdot® 800 nanocrystal	800	750LP	780/60	750LP	780/60

nanocrystal. Therefore, samples stained with Qdot® nanocrystal conjugates can be analyzed on any cytometer that has an appropriate filter selection.

Because most nanocrystals have symmetrical and relatively narrow emission peaks (Figure 2), emission can be efficiently detected with a 20 nm wide filter centered on the emission maximum of a given nanocrystal. Users can minimize the need to correct for spectral overlap between Qdot® nanocrystals by selecting reagents with at least a 40 nm separation between maximum emissions. Table 1 shows two filter schemes that can be used with Qdot® nanocrystals on a LSR II cytometer (BD Biosciences). Configuration A uses narrow-bandpass filters to minimize effects of spectral overlap. These filter selections are shown graphically in Figure 7. Configuration B, developed in the lab of Mario Roederer, uses wider-bandpass filters to collect more photons, and longpass filters close to the emission maximum to minimize spectral overlap.[1]

In multicolor flow cytometry, spectral overlap from one fluorophore into the detector used to quantify another fluorophore must be corrected out of the channel used for the other fluorophore, a process known as compensation. Compensation values represent the percentage of the "wrong" dye signal that is subtracted from the "right" dye channel for each event. As stated above, Qdot® nanocrystals can be excited by multiple laser lines, and in some cases will show strong emissions in channels used for other fluorophores. Figure 8 shows an example of how filters are used to decrease the overlap of the Qdot® 605 nanocrystal into the R-phycoerythrin (R-PE)–Texas Red® channel. When using a conventional 610/20 nm filter for R-PE–Texas Red® dye, the nanocrystal is detected better in this channel than is the target dye, as seen from the 124% compensation value. By moving the R-PE–Texas Red® dye filter to a slightly longer wavelength (620/10 nm), less Qdot® nanocrystal fluorescence is collected relative to the organic dye, and the required compensation value decreases to 28%.

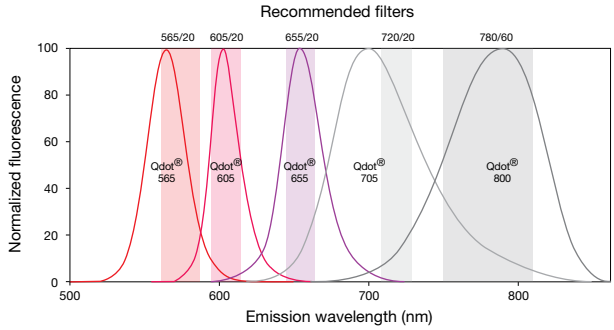

Figure 7—Filter configuration A (from Table 1) vs. emission profiles for selected Qdot® nanocrystals.

Figure 9 shows a six-color staining example that combines Qdot® 605 and Qdot® 655 nanocrystals with

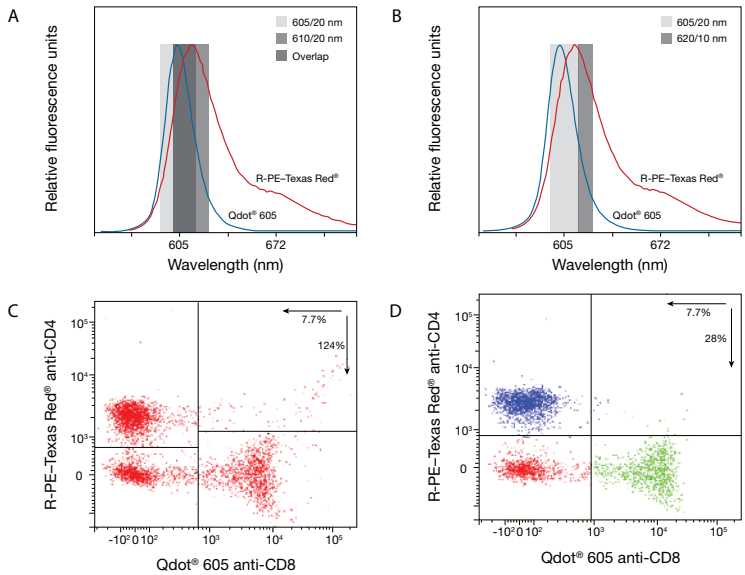

Figure 8—Filter optimization for Qdot® 605 nanocrystals. Human peripheral blood leukocytes (PBLs) were stained with Qdot® 605 anti-CD8 and R-phycoerythrin (R-PE)–Texas Red® anti-CD4. Samples were analyzed on an LSR II flow cytometer (BD Biosciences) with 405 nm and 488 nm excitation using the specified filter combinations. (**A**,**B**) The histograms show the filter combinations overlaid on emission profiles for the two fluorophores. (**C**,**D**) The two-color dot plots show PBL staining results gated on lymphocytes by scatter. Each dot plot is labeled with the compensation required for the filters used, and each axis is labeled with reagent and filter combination.

Table 2—Qdot® nanocrystal compatibility with specific fixed-configuration cytometers.			
Cytometer	**Parameters (filter specification)**		**Compatible Qdot® nanocrystals**
BD FACScan™ cytometer	**488 nm excitation**		
	FL1	(530/30)	Qdot® 525 nanocrystal
	FL2	(585/42)	Qdot® 565, Qdot® 585 nanocrystals
	FL3	(650 nm longpass)	Qdot® 655, Qdot® 705, Qdot® 800 nanocrystals
BD FACSCalibur™ cytometer (4-color)	**488 nm excitation**		
	FL1	(530/30)	Qdot® 525 nanocrystal
	FL2	(585/42)	Qdot® 565, Qdot® 585 nanocrystals
	FL3	(670 nm longpass)	Qdot® 705, Qdot® 800 nanocrystals
	635 nm excitation		
	FL4	(661/16)	Qdot® 655 nanocrystal

conventional dyes that involve significant spectral overlaps (R-PE–Texas Red® dye, allophycocyanin (APC)). The dot plots show staining of human PBLs for T cell markers (CD3, CD4, CD8), a B cell marker (CD19), and natural killer cell markers (CD16, CD56). In addition, leukocytes are gated using a pan-leukocyte marker CD45. The dot plots show good resolution of CD3 (Qdot® 655 nanocrystal) and CD8 (APC) populations. There is also good resolution of the small CD19 (R-PE–Texas Red® dye) from CD4 (Qdot® 605 nanocrystal).

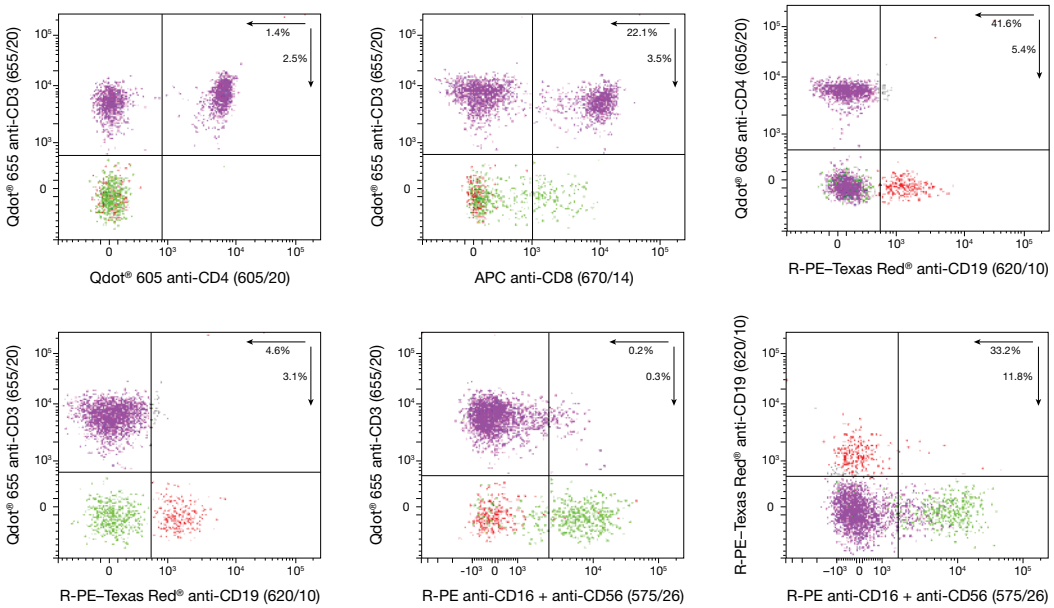

Figure 9—Six-color staining. Human peripheral blood leukocytes (PBLs) were stained with antibody conjugates against CD3, CD4, CD8, CD16, CD19, CD56, and CD45. Samples were analyzed on an LSR II flow cytometer (BD Biosciences) using 405 nm (for Qdot® nanocrystals), 488 nm (for R-PE and R-PE–Texas Red® dye), and 633 nm (for allophycocyanin (APC)) excitation. The two-color dot plots, gated on leukocytes by scatter and CD45 expression, are labeled with reagent, filter, and required compensation.

USE ON FIXED-FILTER FLOW CYTOMETERS

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 have 488 nm excitation sources. For instruments with fixed filter configurations, such as the BD FACScan™ cytometer (BD Biosciences), you can match specific nanocrystals to the filters installed on the instrument. Examples are shown in Table 2.

TAKE THE EASY ROUTE TO MULTICOLOR FLOW CYTOMETRY

Qdot® nanocrystal conjugates of monoclonal antibodies are a powerful way to extend the number of colors in your multicolor flow cytometry panels. These easy-to-use conjugates are compatible with standard sample preparation reagents and staining protocols, and can be used efficiently on cytometers with UV or violet excitation sources with appropriate filters. As with other fluorescent conjugates in multicolor work, care must be taken to design a reagent panel that minimizes spectral overlap, with particular attention to the cross-laser excitation of nanocrystals. Qdot® nanocrystals can be used efficiently on cytometers with 488 nm or longer excitation as long as the nanocrystals are matched to available emission filters. To learn more about Qdot® nanocrystals for flow cytometry, visit **www.invitrogen.com/qdotinflow**. □

REFERENCES

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2. Perfetto, S.P. et al. (2004) *Nat Rev Immunol* 4:648–655.
3. Telford, W.G. (2004) *Cytometry A* 61:9–17.
4. Chattopadhyay, P.K. et al. (2007) in *Quantum Dots, Applications in Biology*, Bruchez, M.P. and Hotz, C.Z. eds., Totowa, NJ: Humana Press, pp. 175–84.
5. Abrams, B. and Dubrovsky, T. (2007) in *Quantum Dots, Applications in Biology*, Bruchez, M.P. and Hotz, C.Z., eds., Totowa, NJ: Humana Press, pp. 185–206.

Product	Quantity	Cat. no.
CD4, mouse anti-human, Qdot® 655 conjugate	100 µl	Q10007
CD4, mouse anti-human, Qdot® 605 conjugate	100 µl	Q10008
CD8, mouse anti-human, Qdot® 605 conjugate	100 µl	Q10009
CD3, mouse anti-human, Qdot® 655 conjugate	100 µl	Q10012
CD14, mouse anti-human, Qdot® 605 conjugate	100 µl	Q10013
CD45RA, mouse anti-human, Qdot® 605 conjugate	100 µl	Q10047
CD45, mouse anti-human, Qdot® 605 conjugate	100 µl	Q10051
CD38, mouse anti-human, Qdot® 605 conjugate	100 µl	Q10053
CD3 (UCHT1), mouse anti-human, Qdot® 605 conjugate	100 µl	Q10054
CD8, mouse anti-human, Qdot® 655 conjugate	100 µl	Q10055
CD14, mouse anti-human, Qdot® 655 conjugate	100 µl	Q10056
CD38, mouse anti-human, Qdot® 655 conjugate	100 µl	Q10057
CD8, mouse anti-human, Qdot® 705 conjugate	100 µl	Q10059
CD4, mouse anti-human, Qdot® 705 conjugate	100 µl	Q10060
CD45, mouse anti-human, Qdot® 705 conjugate	100 µl	Q10062
CD14, mouse anti-human, Qdot® 800 conjugate	100 µl	Q10064
CD27, mouse anti-human, Qdot® 605 conjugate	100 µl	Q10065

Product	Quantity	Cat. no.
CD27, mouse anti-human, Qdot® 655 conjugate	100 µl	Q10066
CD45RA, mouse anti-human, Qdot® 655 conjugate	100 µl	Q10069
mouse IgG2a, Qdot® 605 conjugate	100 µl	Q10014
mouse IgG2a, Qdot® 655 conjugate	100 µl	Q10015
mouse IgG1, Qdot® 605 conjugate	100 µl	Q10073
mouse IgG2b, Qdot® 605 conjugate	100 µl	Q10074
mouse IgG2a, Qdot® 800 conjugate	100 µl	Q10075
mouse IgG2a, Qdot® 705 conjugate	100 µl	Q10076
Cal-Lyse™ solution	25 ml	GAS-010
Cal-Lyse™ solution	100 ml	GAS-010S-100
High-Yield Lyse	500 ml	HYL-250
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