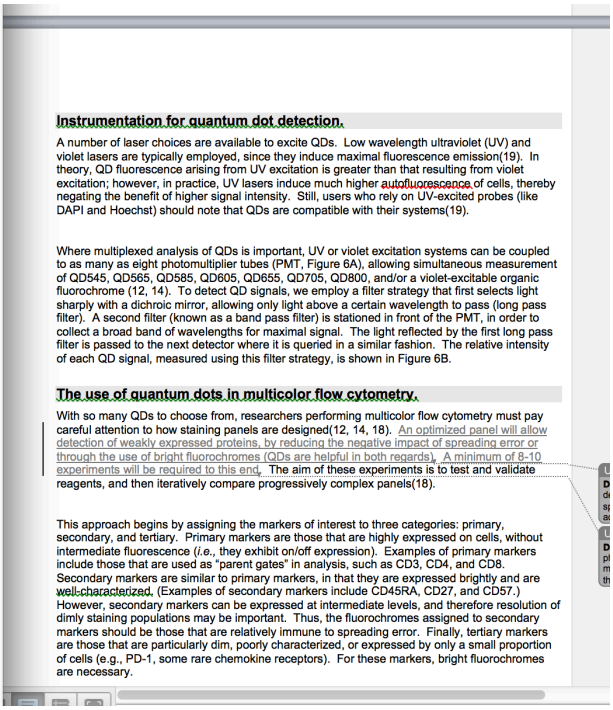
A number of laser choices are available to excite QDs. Low wavelength ultraviolet (UV) and violet lasers are typically employed, since they induce maximal fluorescence emission. In theory, QD fluorescence arising from UV excitation is greater than that resulting from violet excitation; however, in practice, 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 violet-excitable 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



Instrumentation for quantum dot detection.

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(19). 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(19).

Where multiplexed analysis of QDs is important, UV or violet excitation systems can be coupled to as many as eight photomultiplier tubes (PMT, Figure 6A), allowing simultaneous measurement of QD545, QD565, QD585, QD605, QD655, QD705, QD800, and/or a violet-excitable organic fluorochrome (12, 14). 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. The relative intensity of each QD signal, measured using this filter strategy, is shown in Figure 6B.

The use of quantum dots in multicolor flow cytometry.

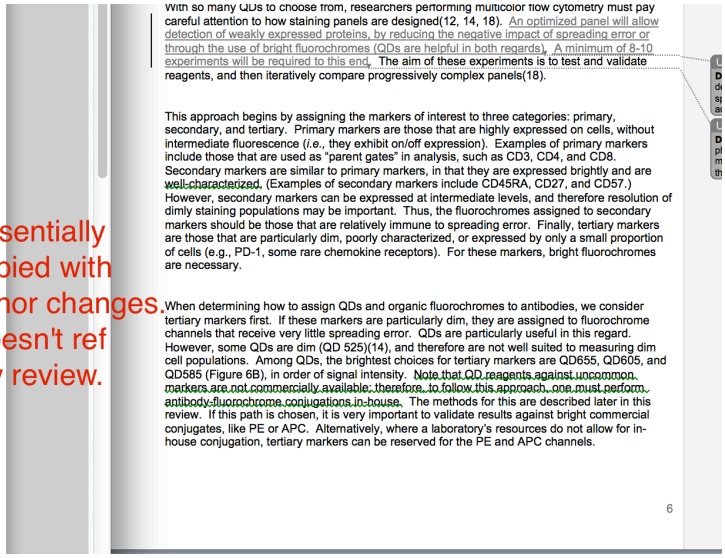
With so many QDs to choose from, researchers performing multicolor flow cytometry must pay careful attention to how staining panels are designed(12, 14, 18). An optimized panel will allow detection of weakly expressed proteins, by reducing the negative impact of spreading error or through the use of bright fluorochromes (QDs are helpful in both regards). A minimum of 8-10 experiments will be required to this end. The aim of these experiments is to test and validate reagents, and then iteratively compare progressively complex panels(18).

This approach begins by assigning the markers of interest to three categories: primary, secondary, and tertiary. Primary markers are those that are highly expressed on cells, without intermediate fluorescence (*i.e.*, they exhibit on/off expression). Examples of primary markers include those that are used as "parent gates" in analysis, such as CD3, CD4, and CD8. Secondary markers are similar to primary markers, in that they are expressed brightly and are well-characterized. (Examples of secondary markers include CD45RA, CD27, and CD57.) However, secondary markers 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 that are relatively immune to spreading error. Finally, tertiary markers are those that are particularly dim, poorly characterized, or expressed by only a small proportion of cells (e.g., PD-1, some rare chemokine receptors). For these markers, bright fluorochromes are necessary.

(pMHC) 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.

Essentially copied with minor changes. Doesn't ref my review.



With so many QDs to choose from, researchers performing multicolor flow cytometry must pay careful attention to how staining panels are designed(12, 14, 18). An optimized panel will allow detection of weakly expressed proteins, by reducing the negative impact of spreading error or through the use of bright fluorochromes (QDs are helpful in both regards). A minimum of 8-10 experiments will be required to this end. The aim of these experiments is to test and validate reagents, and then iteratively compare progressively complex panels(18).

This approach begins by assigning the markers of interest to three categories: primary, secondary, and tertiary. Primary markers are those that are highly expressed on cells, without intermediate fluorescence (*i.e.*, they exhibit on/off expression). Examples of primary markers include those that are used as "parent gates" in analysis, such as CD3, CD4, and CD8. Secondary markers are similar to primary markers, in that they are expressed brightly and are well-characterized. (Examples of secondary markers include CD45RA, CD27, and CD57.) However, secondary markers 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 that are relatively immune to spreading error. Finally, tertiary markers are those that are particularly dim, poorly characterized, or expressed by only a small proportion of cells (e.g., PD-1, some rare chemokine receptors). For these markers, bright fluorochromes are necessary.

When determining how to assign QDs and organic fluorochromes to antibodies, we consider tertiary markers 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)(14), and therefore are not well suited to measuring dim cell populations. Among QDs, the brightest choices for tertiary markers are QD655, QD605, and QD585 (Figure 6B), in order of signal intensity. Note that QD reagents against uncommon markers are not commercially available; therefore, to follow this approach, one must perform antibody-fluorochrome conjugations in-house. The methods for this are described later in this review. If this path is chosen, it is very important to validate results against bright commercial conjugates, like PE or APC. Alternatively, where a laboratory's resources do not allow for in-house conjugation, tertiary markers can be reserved for the PE and APC channels.

6

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.

Copied almost verbatim

potential for toxicity; for example, aerosolized QDs become lodged in bronchial spaces when inhaled(37). Readers are referred to a thoughtful review on the topic by Hoet, *et al.*(38)

Quantum dot applications.

In recent years, a wide variety of applications have emerged from QD technology. Our group published an early example demonstrating the utility of QDs in multicolor flow cytometry in 2006(14). In that study, we analyzed the maturity of various antigen-specific T-cell populations using a 17-color staining panel. This panel consisted of seven QDs and ten 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. The work demonstrated the power of a multiplexed approach; by identifying multiple phenotypically distinct subsets within each antigen-specific T-cell population, the remarkable intricacy of T-cell immunity can be appreciated (Figure 9). QDs also allowed us to measure many antigen-specific populations simultaneously, an important factor when sample availability is limited(14).

Aside from the successful multiplexing of QDs and organic fluorochromes, our work showed that QDs with SA groups can be used to produce pMHCI multimers (commonly called "tetramers"). Previously, 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 also display higher valency than PE or APC, so more pMHCI molecules could be bound to a QD than to a PE- or APC-SAV. This allows for brighter signals and better staining resolution (Figure 9). Along

AUTHOR DETAILS | CHAPTER STATISTICS | HOW TO LINK AND REFERENCE

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, QDs may bind plastic, precipitate, and lose activity, especially at low volumes.

Concepts repeated straight from my paper without attribution to me.

Implication that QD conjugations are performed in their facility, direct copying of my text.

Quantum dot caveats.

Clearly, QDs are useful tools in multicolor flow cytometry. However, since they are comparatively new technology, 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. There are a few potential reasons. First, the reagents we produce contain excess, unconjugated QD, because we do not employ a final antibody purification step (*e.g.*, using protein A or G columns, which can be labor intensive and lower yield significantly). In surface staining experiments, this free fluorochrome is easily washed away; however, in intracellular staining, it can be difficult to eliminate and can introduce non-specific signal. Second, the size of the QD conjugate may introduce steric problems that limit access to intracellular compartments(20). This is unlikely, in principle, because QD molecules are no larger than PE, a common fluorochrome used successfully in intracellular staining. However, in practice, QD-antibody pairs may form much larger, higher-order complexes during the

8

conjugation process. Furthermore, QDs may not disperse evenly throughout the intracellular environment(28, 29). Finally, QDs may be sensitive to certain chemicals used in the fixation and permeabilization process associated with intracellular staining. We have observed this even when QD reagents are used to stain surface markers, as subsequent permeabilization steps lower the fluorescence of the surface reagents by 10-fold (Figure 8). For bright QDs, this does not present major problems (as staining resolution is maintained); however, for dim QDs, positive staining can be obliterated. This phenomenon does not occur predictably; there is lot-to-lot variation in how most manufacturers' permeabilization kits will affect QDs. Thus, we recommend testing new lots of kits for this effect before they are used routinely.

Variation within the QDs themselves is also important to consider. The manufacturing process is often difficult to control across production runs, and the production history of a QD batch is not

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

Lightly Paraphrased, Not Referenced

to conjugate 1mg of antibody. However, this provides conjugated reagent for 8000 tests, and results in a cost of 19 cents per test. Thus, combined with the cost of the antibody, the total expense for an in-house conjugate is 25 cents per test. In contrast, the limited commercial conjugates available cost as much as $4.50 per test.

The procedure we employ for in-house conjugations is similar to that previously described for PE; there are only slight variations in the chemicals used and the ratio of antibodies to fluorescent molecules(24). The process relies on the coupling of maleimide groups on the QDs to free thiol groups on the antibody, after these groups are generated by treatment of the QDs with sulfo-SMCC and antibody with DTT. This activates the QD and reduces the antibody, which are exchanged (using columns) into the buffer needed for the conjugation reaction. Next, the SMCC-QD and DTT-antibody are mixed and incubated for one hour. The reaction is then quenched and the newly produced conjugate is exchanged into a storage buffer. The entire process takes about 3-4 hours(24).

Though our success rate for QD conjugations is high, it ought to be noted that some antibodies (about 5-10%) do not conjugate to QDs well, for reasons that remain unclear. For antibody conjugations that are successful, reproducibility is excellent: over 95% of our repeat conjugations give the same results.

The maleimide-thiol conjugation reaction presented here (also known as reductive cross-linking) is not the only available method for conjugating qots to biomolecules. Depending on the functional groups available, a variety of other conjugation chemistries can be employed(13, 20). When QDs with carboxylic acid groups are used, these can be coupled to primary amines to form stable amide bonds. With mercapto-coated QDs, an overnight adsorption procedure allows conjugation to thiolated proteins or oligonucleotides(26). Similarly, through electrostatic interactions, negatively charged nanocrystals can be adsorbed to polylysine chains that have been added previously to the biomolecule(16, 27). These adsorption methods share important disadvantages: significant portions of unreacted material remain(6), and adsorbed material is not