Quantum dots in microscopy and cytometry: immunostaining applications

N. Barteneva¹,² and I. Vorobjev³,⁴
¹ Immune Disease Institute and Program in Cellular and Molecular Medicine at Children's Hospital, D-239, 200 Longwood Avenue, 02115, Boston, MA, USA
² Department of Pathology, Harvard Medical School, D-239, 200 Longwood Avenue, 02115, Boston, MA, USA
³ Hematology Scientific Center, 4 Novyi Zykovsky Proezd, 125167, Moscow, Russia
⁴ A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119992, Moscow, Russia

Semiconductor quantum dots (QDots) have unique narrow emission spectra, high fluorescence efficiency, minimal photobleaching, and simultaneous excitation by multiple light sources. They have been successfully established for imaging and for monitoring a variety of biological functions including cell tracking, live-cell imaging, and in situ hybridization. However, intracellular QDot applications have just begun to materialize. Focusing on QDot nanoparticles, this review attempts to illustrate interconnectivity between imaging applications of QDot nanocrystals and the development of immunostaining applications of quantum dots in immunohistochemistry, and flow and imaging cytometry. The relative novelty of QDots presents problems in their practical use, and advice for transfer experiments between the microscope and the flow cytometer will be discussed.

Keywords: Quantum dots, microscopy, flow cytometry, imaging cytometry, intracellular

1. Introduction. QDots general characteristics.

QDots represent a new generation of fluorochromes. They are semiconductor nanocrystals that exhibit bright, photostable fluorescence with high extinction coefficients, have narrow emission spectra with peak emission wavelengths that depend upon their size and composition [1, 2]. Generally, QDots consist of a heavy metal core, coated by a semiconductor shell, and outer layers of a cap. In terms of their chemical composition, QDots synthesized for biological applications are most commonly built from cadmium, and either selenium or tellurium (CdSe and CdTe), and their shell is frequently comprised of zinc sulphide (ZnS). QDots may also have biopolymeric coatings that allow conjugation with antibodies, mRNA, DNA and other biomolecules by using linkers such as streptavidin, maltose-binding protein, protein A and others [3-5]. QDots can be discriminated by their different sizes and shapes (QDots 525, 565 and 585 nm Cd/Zn are spherical and QDots 605 and 655 nm are oblong) [6]. The dimension of the core defines the band gap and the spectral region of the emission. The emission wavelength and quantum efficiency depends on the quantum confinement of electrons within the core and can be tuned during QDots fabrication. Thus, an increase in particle sizes induces a shift to a red region in the emission spectrum of QDots (tuned from blue to red with an increase in core diameter from 1.5 to 6.0 nm). The hydrodynamic radius of functionalized QDots is 15-20 nm, which is larger than their actual core size. Size-tunable properties of different types of QDots may show considerable variations in signal brightness (measured by the absorption coefficient and fluorescence quantum yield) at different emission wavelengths [7]. For example, integrated signal intensity of green CdSe QDots (525 nm emission) is 17 times lower than that of the red QDots (655 nm). Another observed phenomenon was QDot "blinking" which affects single-molecule applications by the saturation of signal. During blinking the QDots alternate between emitting and non-emitting state [8,9]. QDots comparison with organic fluorophores is summarized in Table 1.

Table 1. Comparison of QDots with organic fluorochromes

<table>
<thead>
<tr>
<th>Property</th>
<th>QDots</th>
<th>Organic fluorochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar extinction coefficients (at 488 nm)</td>
<td>≈10⁵ – 10⁶ L mol⁻¹ cm⁻¹ (Invitrogen Inc., Bioprobes 2007)</td>
<td>FITC=80 000 PE ≈ 700 000</td>
</tr>
<tr>
<td>Absorption spectra</td>
<td>Broad spectra with a first size-dependent excitation peak</td>
<td>Similar with their emission spectra</td>
</tr>
<tr>
<td>Emission spectra</td>
<td>Symmetric, ≤40 nm for (CdSe)ZnS with λ=500-660</td>
<td>Broad (&gt;40 nm), asymmetric</td>
</tr>
<tr>
<td>Photobleaching</td>
<td>High-resistant</td>
<td>Low-resistant</td>
</tr>
<tr>
<td>Stokes shift</td>
<td>Large, 100-500 nm</td>
<td>Cy3-20 nm; Cy5-21 nm; FITC-24 nm</td>
</tr>
<tr>
<td>Size</td>
<td>10-20 nm (functionalized)</td>
<td>FITC – 1 nm; R-PE – 20 nm, 240KDa;</td>
</tr>
<tr>
<td>Blinking interval</td>
<td>500 ms</td>
<td>0.5 ms [10-11]</td>
</tr>
</tbody>
</table>
All QDots can be efficiently excited by a single laser in the UV to the blue region of spectra [12]. QDots emitting in red-infrared regions also can be excited by a red laser. Figure 1 compares the emission spectra of some QDots of different sizes. However, in our hands the emission spectrum of some commercially available QDots is relatively wide (QDot 705) which is similar in conclusion with Ioannou et al [13]. QDots can serve as multi-functional probes with unique advantages for both imaging and cytometry studies, and may have potential for use both in in vivo imaging and in vitro assays and single-molecule studies [14-16] eliminating several of the major problems of dye-based fluorescent detection technologies. Their ability to be excited by single wavelength and their large Stokes shift make QDots particularly well suited for multiplex applications [17]. Besides, due to sufficient electron density of QDots cores they can be directly visualized by electron microscopy, which opens a number of additional imaging opportunities[18-19].

![Figure 1](image.png)

**Figure 1.** Fluorescence of four QDots conjugated to monoclonal antibodies: emission spectrum. Antibody conjugates were taken at the same concentration. Almost no overlap exists between emission spectra. Numbers represent individual QDots. Excitation wavelength – 440 nm; slit width – 4 nm.

### 2. QDots in immunohistochemistry

Multiplexed immunohistochemical studies are currently limited in their applicability [20]. Two basic strategies are currently employed to detect multiple antigens in tissue sections: 1) chromogenic and immunoenzymatic detection systems which can define up to four antigens from different cell populations or different compartments of the same cell [21], but are practically limited to the detection of only two antigens; 2) and combinations of different antibodies conjugated with fluorochromes of distinct emission maxima [22-23]. With adequate filter combinations and the fluorochrome-conjugates panel, up to four antigens can be immunostained side by side in the tissue sections. Conventional organic fluorochromes have wide emission spectra and are prone to photobleaching. They have small Stokes shift (wavelength distance between excitation and emission peaks), and therefore require that the corresponding excitation and emission filters have close wavelengths, which results in reduced sensitivity [24]. Simultaneous staining of specimens for more than four proteins is problematic due to wide emission peaks and the need of multiple laser lines for excitation. The ability to repeatedly image the specimen is often required and this is almost impossible with organic fluorochromes because of their fading. A chronic problem in fluorescence-based immunohistochemistry is the photobleaching of organic fluorochrome. Photobleaching decreases the signal-to-noise ratio and even reliability of z-stack data in confocal microscopy. QDots are more stable to photobleaching than organic dyes and successful immunostaining with QDot conjugates overcomes the problem of photostability of the specimens [25-27].

The large size of QDots (nanometer range) defines the combining of numerous copies of antibodies with one QDot. On the other hand, in case of organic dyes coupled with antibodies, one antibody is coupled with many dye molecules. Therefore, the conjugate of QDots with antibodies has a larger size as well as a free ligand-binding site which may lead to different behavior for intracellular staining applications. In many applications with QDots, the labeling strategy utilizes a three-layer approach: the primary antibody, the biotinylated secondary antibody, and the streptavidin-conjugated QDot. Since primary and secondary antibodies are divalent, this strategy could lead to the formation of molecular aggregates and cross-linking of target proteins [28]. Therefore, controlling the number of the linkers on the QDots surface may be necessary. Initially, use of QDots was focused on visualizing cell surface molecules and endocytosis-related processes [29-30]. Although different approaches for QDots delivery in cytoplasm were explored (liposomes, cell-penetrating peptides, electroporation, nanotube needles etc), all of these methods may result in non-homogenous distribution of the QDots in the cytoplasm and aggregation in punctae or the trapping QDots inside endosomes [31]. Delivery of nanoparticles into unspecified locations of the cytoplasm and aggregation of QDots along with other consequences can create undesirable fluorescent background.

Much of the challenge of using QDots in immunohistochemistry and intracellular staining comes from sensitivity to fixation, the relatively large size of biomolecule-conjugated nanoparticles (15-20 nm) and the difficulty of delivering nanoparticles into the cytoplasm and the nucleus.
No universal protocol exists for detecting all proteins with QDot-antibody conjugates, and the three main parameters should be considered in the development of new protocol, namely: type and duration of fixation; type and duration of detergent permeabilization, and temperature. Each protocol is a compromise between good labeling and good cell/tissue structure preservation. The nature of cells of interest is important; cells with a relatively dense cytoplasm (muscle) allow less penetration of QDots conjugates compared with others (kidney, liver) and may require stronger permeabilization.

Several groups have reported single and multiplex immunohistochemical research using QDots [17, 20, 26, 32]. The feasibility of using QDots for antigen detection in fixed cells was first described by Bruchez et al in 1998 [3]. Shortly, nuclear antigens and F-actin filaments were labeled with green and red QDots and simultaneously detected in fixed mouse fibroblasts. The increased photostability is especially helpful in 3-dimensional (3D) analysis, where photobleaching of traditional fluorochromes may affect correct reconstruction of 3D images [26]. In this research, a detailed cytoskeleton structure was imaged using a confocal microscope, and use of QDots demonstrated optimization of acquisition comparable to conventional Alexa dyes.

Recently, Kingeter and Schaefer [33] combined a panel of three QDots (QDots 655, 705 and 800) in a mixed panel with DAPI and Alexa 555 fluorochromes and GFP in 6-color combination. Ness et al [34] developed an immunohistochemical protocol that combines QDot-conjugate labeling with enzymatic signal amplification for detection of intracellular antigens in rat and mouse brain tissue. Akhtar et al [35] described a multilabeling protocol with QDots allowing a combination of QDots conjugates with tyramide signal amplification to accomplish sensitive simultaneous triple labeling.

Most studies on QDot immunofluorescent labeling has been carried out with cell lines or freshly obtained tissues. However, the majority of available clinical samples are formalin fixed, paraffin-embedded (FFPE) tissues that can be a few or more years old. Xing et al [36] successfully developed simultaneous staining of up to four different biomarkers associated with the epithelial-mesenchymal transition, namely N-cadherin, elongation factor-alpha, E-cadherin and vimentin, using antibodies conjugated with QDots 565, 605, 655 and 705.

Quantitative labeling of neurons and glia with QDot 605 antibody-conjugates to anti-beta-tubulin III and antiglial fibrillar acidic protein antibodies was described by Pathak et al [37]. One critical issue that was addressed by this group in the next publication was determining the number of antibodies which are functionally available for the target protein binding using the quantitative electrophoresis approach [38]. The approximate estimated amount of functionally available antibodies was 1.3±0.35 IgG molecules per QDot for a 2:1 ratio, which is significantly less than estimates suggested after TEM imaging of -2-10 antibodies conjugated per QDot.

Bodo et al [39] successfully applied QDots 605 and 655 for immunofluorescent quantification of phosphoproteins (PP) in fixed tissues using the Kinetworks PP screen and a panel of various human cell lines. They were able to show semi-quantitatively downregulation of phosphoproteins in a murine colon cancer xenograft model using the PKC-beta inhibitor enzastaurin. This type of phosphoproteins determination has an advantage of morphological spatial evaluation over western blotting or other tissue extraction-based methods because it allows specific analysis of tumor cells and subcellular components (nuclear vs cytoplasmic). Authors report that dehydration in ethanol and xylene with subsequent embedding is important to obtain stable QDot staining.

Another attractive potential application of QDots is immunolabeling of targets in tissue sections with high level of autofluorescence [40] like the heart, where accumulated lipofuscin and blood-derived pigments during heart injury can lead to an increase of autofluorescence. Autofluorescence of live cells originates mostly from mitochondria and lysosomes [41] and may increase after fixation of tissue samples.

Tissue arrays allow one to investigate multiple samples simultaneously using immunohistochemistry. Caldwell et al [42] used two QDots, 605 and 705, for protein targets MDM-2 and β-actin to quantify a level of staining in renal cell carcinoma (RCC) tissue arrays on a cohort of 25 RCC samples. Authors conclude that their results support the multiplex staining capacity of QDots, albeit on a model with only two classifiers. Chen et al [43] used lung cancer tissue microarray to evaluate the accuracy and sensitivity of using QDots in immunohistochemical tissue array applications comparing with conventional immunohistochemistry techniques. Using caveolin-1 and PCNA as model antigens they came to the conclusion that QDot-based immunohistochemistry is superior to the conventional one and provides a level of sensitivity and specificity necessary for high-throughput applications.

Similarly Chen et al. [44] using QDots 605 and 545 streptavidin conjugates for the assessment of HER2 status in breast cancer found this approach to be more sensitive and more accurate than the use of organic fluorochromes, especially for identification of low-expressed HER2 (2+ on IHC scale) and its precursor the ER protein. They also demonstrated fluorescent imaging of QDots on the standard hematoxylin and eosin stained tissue sections.

Because of the unique optical properties of QDots the standard fluorescent microscope is not convenient for rapid sequential detection of different QDots. To optimize detection of QDots signal under a conventional fluorescent microscope we modified it by the following: filter cube for excitation use the BP filter 435/40 (Semrock Inc., Rochester, USA). Beamsplitter has the cutoff wavelength of 510 nm and the emission filter in the cube is LP 500. This cube allows the observation of fluorescence from different QDots. To select the individual signal from each QDot we put the filter wheel with a set of bandpass filters in front of the CCD camera. The filter wheel contains several band pass (BP) filters centered for each QDot. Use of the narrow band-pass filters gave sufficient signal from each QDot (acquisition time
was about 1 s) and results in very low bleed through neighboring filters. In direct measurements the spillover for QDot 655 was 5% for 605/15 BP filter and 1.4% for 712/20 BP filter, and similar results were obtained for QDot 565 (Table 2).

Table 2. Sections stained with QDots – spectral compensation

<table>
<thead>
<tr>
<th>Intensity, relative units</th>
<th>Filter</th>
<th>Spillover</th>
<th>QDot/specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>82.86</td>
<td>712/20</td>
<td>1.40%</td>
<td>QDot 655, frozen section, CD45</td>
</tr>
<tr>
<td>5911.1</td>
<td>655/15</td>
<td>100%</td>
<td>direct staining</td>
</tr>
<tr>
<td>299.21</td>
<td>605/15</td>
<td>5.06%</td>
<td>QDot 565, paraffin section, CD20</td>
</tr>
<tr>
<td>716.06</td>
<td>605/15</td>
<td>7.75%</td>
<td>indirect staining</td>
</tr>
<tr>
<td>9239.27</td>
<td>565/24</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>773.68</td>
<td>525/20</td>
<td>8.37%</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Double staining of the lymph node with CLL. A. CD20/CD5 staining. Expression of both antigens on the cell surface results in yellow emission. B. Same specimen stained with CD20/CD3. Green staining shows rare T-cells in the section.

Primary antibodies:
- mouse anti-human antibodies CD20, (Dako), 1:100
- rabbit anti-human antibodies CD5, (Labvision), 1:100
- rabbit anti-human antibodies CD3, (Dako), 1:100

Secondary antibodies:
- QDot® 605 conjugated goat anti-mouse IgG, Invitrogen, 1:100
- QDot® 565 conjugated goat anti-rabbit IgG, Invitrogen, 1:50

3. QDots : cytometry and quantitative approach

Organic fluorochromes (e.g. fluorescein isothiocyanate (FITC), R-phycoerythrin (R-PE), and fluorescent proteins are intensively used in flow cytometry and fluorescent microscopy, including quantitative measurements. Unfortunately, QDots currently lack commercially available reliable standards for quantitation. Typical quantitative approaches rely on the development of calibration beads conjugated with the fluorochrome of interest for the conversion of the mean fluorescence intensity data to MESF (Molecules of Equivalent Soluble Fluorochromes) [45]. However, QDots provide several obstacles to the development of these standards, namely, lack of stability and size uniformity of QDots. QDots cell surface modifications lead to significant changes in nanoparticles binding over unmodified QDots. Some groups made attempts to develop suitable methods of QDots standardization [46-47]. Thus, Smith, Giorgio [47] used conversion of fluorescent intensity of QDot 585 to the mean intensity of equivalent fluorochrome R-PE. However, at the time of the publication no commercial QDot-conjugated standard beads were available.

QDot staining reagents can be combined with fluorescent proteins or conjugates with conventional dyes to expand the number of multicolor parameters registered in the sample - up to 17 or more as reported by scientists from Roederer laboratory [48-50]. They described a panel consisted from 7 QDots and 10 organic and tandem fluorochromes and used cytometer with separate “violet octagon” to acquire fluorescent signals from QDot-conjugated antibodies. Besides
increase in number of colours, the major advantage of adding QDots in multi-color panel is low spreading error in QDot-channels.

While antibody conjugates with QDots became more available on the market, the markers for intracellular flow cytometry labeling are still in development. In our experiments multiplexed detection of intracellularly stained Zap-70 and 4 other antigens (namely, CD3, CD19, CD5 and CD38) was successfully achieved with anti-Zap-70 antibody conjugated with QD 655 and antibodies conjugated with four conventional fluorochromes [52]. QDot 655 can be successfully incorporated in 8-color panel of antibodies conjugated with organic fluorochromes developed for chronic lymphoid leukemia characterization [53].

**Figure 3.** Representative scheme of gating for B-cells Zap-70 expression on B-CLL patient samples (D, E). The B-CLL population was defined by lymphocyte gating (A) followed by gating of CD19^+^CD5^+^ cells (B) and using Zap-70 expression on T-cells (CD3^+^CD5^+^) as internal control (C, F). Histograms show Zap-70 expression on CD5^+^subpopulation of B-cells (E) and T-cells (F) vs isotype control.

When using QDots for immunophenotyping only a few high-frequency cell populations can be detected in a single blood sample. Hadrup et al [51] offered a combinatorial approach allowing PMHCI multimers to be coupled with a unique combination of distinct fluorochromes (six QDots and two conventional fluorochromes), enabling detection of up to 25 specificities. The group demonstrated that this approach can detect more populations with frequency of ≥0.02%. This method could be applied to screen for low frequency CTL subpopulations in autoimmune response, and infectious and cancer diseases. The use of combinations of QDots and organic fluorochromes led to a reduction in signal in the individual fluorescent channel but still allowed the detection of 25 out of 28 possible two-dimensional combinations. Using of combinations of dyes also removed background problems attributed to a random interaction with single fluorescent marker.

QDot-conjugated antibodies have been shown as a good alternative to organic fluorochromes when using laser scanning cytometry [54]. In this study a 405 nm violet diode was applied to excite three QDots for image cytometry analysis of rat pancreas tissue: QDot 525 was detected with green filter cube, QDot 585 with the orange, and QDot 655 in long red channel (Figure 4). LSC technology was developed in the late 1990s and allows quantification of fluorescent and chromatic events in cells and tissue sections by scanning cell populations in situ (on slides and plates). Because of the need for sequential scanning with different laser sources, photostability of QDots have a significant advantage for this technology over traditional dyes.
4. QDots and FISH

One of the first studies to use QDot-conjugates for standard fluorescence in situ hybridization (FISH) was done by Pathak et al. [55]. However, the QDot-conjugates used in this study showed poor stability and high non-specific binding. Improvement of bioconjugation technique and water solubility of QDots lead to successful FISH quantification on human and mouse chromosomes with a help of streptavidin-conjugated QDots. Xiao and Barker [56] using biotinylated DNA probes and QDot 605 streptavidin conjugate had demonstrated superior intensity and stability of QDot probes compared to FITC and Texas Red fluorochromes. Intensive QDot staining was observed for 1q12 probe and HER2 gene probe. However, staining with QDot probes for centromeric regions of some chromosomes failed. Besides the authors observed unstable fluorescence of QDots-blinking with a period about 300 ms.

Chan et al. [57] was the first to demonstrate duplex FISH of two nucleic acid targets by directly labeled QDot-conjugated oligonucleotide probes in frozen mouse brain tissue. Tholouli et al. [4] published a protocol of FISH with QDot-conjugates on formalin-fixed, paraffin-embedded tissue samples. Byers et al. [58] demonstrated a feasibility of duplex FISH in combination with spectral imaging for high-throughput applications.

QDots have better temperature stability than organic fluorochromes, which allow them to better tolerate hybridization temperatures required for telomere labeling [59]. Practically all traditional fluorescent probes including phycobiliproteins are lost or damaged during high heat treatment. In this study the authors determined that QDot conjugates largely retain fluorescence intensity, following high temperature hybridization, in contrast with traditional fluorochromes [59]. Data obtained with FISH-flow cytometry performed with QDots 605, 655 and 705 labeling was very close to previously reported values obtained using Southern blotting and traditional FISH. To check the validity of the simultaneous analysis authors tested telomere length for human PBMC CD3^+CD4^+ and CD3^+CD4^- and CD14^- cell populations and came to conclusion that the suggested modification of FISH-method allows simultaneous fluorescent immunophenotyping and telomere length measurement.

In spite of advantages of the nanocrystals like multiplexing and photostability, the FISH with QDots did not become a routine protocol replacing FISH with traditional fluorochromes. Though many groups published successful FISH protocols, FISH with QDot-conjugates still provides a lot of technical challenges. In fact, Ioannou et al. [13] published a study that described running parallel QDot-conjugate and Cy-3 based FISH experiments. They used commercially available streptavidin conjugates with QDot 525, 585 and reported difficulty in reproducibility of published protocols.
They observed increased background compared to traditional fluorochrome Cy3, false-positive staining with QDot probes and had difficulties in achieving reproducible QDot-FISH staining.

The reason for this discrepancy could be that many successful groups synthesized and used for FISH their custommade QDot conjugates [56, 60-61]. For example, Ma et al [61] used smaller size of QDots to prevent steric hindrance influence on hybridization ability.

5. QDots-conjugates with antibodies - other applications

Kaul et al [62] used QDots conjugated with a unique internalizing antibody against a heat shock protein-70 (hsp70) (mortalin) (i-QDots), which is upregulated on the surface of proliferating cells, to track the long-term fate of cancer cells in the culture. i-QDot 655 was used for stable, functionally inert labeling of mesenchymal stem cells [63]. i-QDots and their future modified forms can potentially be used to study cell progeny in development and gene therapy settings, complementing current methods that use the lentiviral vectors or thymidine analogs for cell tracking.

Doak et al [64] developed fixation and staining sample preparation methodology for coupled atomic force microscopy and confocal laser scanning microscopy (AFM-CLSM) with QDot conjugates. They applied a combination of fixation with ultra-pure methanol-free formaldehyde and QDot nanocrystal labeling with QDot-655 and QDot-525 streptavidin- and antibody conjugates for filopodia imaging. Utilizing QDots in place of conventional fluorochromes labeling allowed this group to overcome the photobleaching problem typical for procedures requiring multiple scans. Authors found that fixing the cells in ultra-pure methanol-free formaldehyde increased fluorescent signal comparing with standard formaldehyde (conventional formaldehyde usually contains 10-15% methanol to prevent its oxidation to formic acid).

The Zhou group [65] used fluorescence multiplexing to determine profiles for four subclasses of cells including T-cells, macrophages and cancer cells. QDot 525 staining was difficult to distinguish from autofluorescence on cancer cells, and similar problems had weakly expressing cells stained with QDot 585 and QD605. The best results were observed with QDot 705 and 800 conjugates or traditional fluorochromes (R-PE and Alexa 647). The authors considered a few reasons for the low sensitivity of QDot conjugates: 1) using 488 nm laser gives lower fluorescence than excitation 405 nm [66]; 2) the reduction in affinity of monoclonal antibodies after direct conjugation with QDots [67]; 3) QDots to antibody ratio is very low because of multiple molecules of antibody binding per QDot particle [68].

QDot-conjugated anti-myeloperoxidase (MPO) was used to detect activated neutrophils after stimulation with proinflammatory cytokines [69]. In the case of immunostaining of living neutrophils with anti-MPO antibody conjugated with conventional fluorochromes, the antibody lost their fluorescence due to quenching by reactive oxygen species produced by MPO. Staining with QDot-conjugated antibody stayed photostable in the activated neutrophils and did not react with inactivated neutrophils and can therefore be helpful for flow cytometric analysis of neutrophils in vitro.

QDots are widely used in immune detection of pathogenic bacteria [70-72]. As a result of their small sizes bacterial cells require extremely bright fluorochromes for positive identification of pathogenic microbes at low concentrations in cell mixtures. The QDots are superior fluorochromes for bacterial cell labeling and detection due to their brightness and narrow emission spectra.

6. Practical advices on QDots immunostaining

QDots are prone to form aggregates or precipitate. It is important to remove aggregates from staining cocktails with a short centrifugation step [50]. Glass vials are preferential for QDot-conjugates storage. Some QDot conjugates show significant loss of specific fluorescence over time. QDot batches of conjugated antibodies have a limited shelf-time (in our observation 6-12 months). One of the biggest difficulties associated with QDots is their nonspecific binding that can produce false positive results, most prominent in methanol fixed samples [73] and with self-made conjugates. Non-specific binding QDots to the glass can be minimized by pre-coating glass with poly-lysine [74]. Presence of trace amounts of bovine serum albumin in buffers improves signal-to-noise ratio for QDots antibody conjugates [75].

The fixation procedure and choice of fixatives can significantly change the fluorescent characteristics of QDots [52,76]. Since fixatives may negatively affect QDot fluorescence intensity, care should be taken about choosing appropriate fixative. The paraformaldehyde (PFA) was suggested for cells stained with QDots with different ranges of concentration (1-4%) and time exposure [31,52,77]. PFA and methanol taken at high concentrations decrease fluorescence of QDots-conjugates [52,76]. Glutaraldehyde increases autofluorescence mainly in 550-700 nm region of the spectrum and therefore should not be used for fixation of QDots-conjugates emitting in this part of the spectrum. QDot staining is very sensitive to overfixation, and formaldehyde/PFA concentrations higher than 2% or a long time of fixation (greater than 10-15 min) drastically decreases the QDot fluorescent signal. Optimization of fixation and permeabilization conditions for QDot-conjugated antibodies must be performed for each primary antibody and cell type.

QDots are relatively large particles (15-20 nm radius of functionalized QDots with molecular weight (MW) larger than 150 KDa), therefore staining protocol with QDot-conjugated antibodies requires more stringent tissue penetration.
conditions compared with organic fluorochromes. Staining of FFPE specimens with QDot conjugates requires longer incubation time (overnight at 4°C vs 1 hour at room temperature) and a higher QDot-antibody concentration than that required for freshly fixed cells [7].

The standard flow cytometer analysers and sorters currently available on the market are built for traditional fluorophores and include a set of lasers that are also capable to excite all QDots. Some special order instruments (FACSAria and LSR2, BD Biosciences) may include "violet octagon" that allows up to 7 channels for QDots acquisition and a violet-excitable organic fluorochrome and equipped with QDot-optimized filters [48]. Standard filters supplied for usage with organic fluorochromes are compatible with variety of QDots, though suboptimal for their acquisition. Ideally, QDots should be acquired with narrow filter (20 nm) centered around emission maximum (585/20, 605/20, 655/20 etc). QDots can be incorporated in multicolor panel built from antibody conjugates with organic fluorophores or can be a part of panel built exclusively for QDots.

Table 3 QDots acquisition on standard 3-laser cytometer (488 nm, 633 nm, 405 nm laser lines), FACScanto II (BD Biosciences, San Jose, CA)

<table>
<thead>
<tr>
<th>Excitation line</th>
<th>QDots excited by laser</th>
<th>Emission channels (standard filters)</th>
<th>QDots acquisition</th>
</tr>
</thead>
<tbody>
<tr>
<td>488 nm</td>
<td>QDot 525,565,585,605, 655,705, 800</td>
<td>FITC(530/30) PE (585/42 ) PERCP (670LP) PE-Cy7 (780/60)</td>
<td>QDot 525 QDot 565, 585, 605 QDot 655,705, 800 QDot 800</td>
</tr>
<tr>
<td>633 nm</td>
<td>QDots 655, 705, 800</td>
<td>APC (660/20) Alexa700 (720/40) or APC/Cy7 (780/60)</td>
<td>QDot 655 QDot 705 or QDot 800</td>
</tr>
<tr>
<td>405 nm</td>
<td>QDot 525, 565, 585, 605, 655,705, 800</td>
<td>AmCyan 450/50 PacOrange 585/40</td>
<td>- QDot 565,585</td>
</tr>
</tbody>
</table>

However, QDot excitation from 488 nm and 633 nm is less efficient than from 405 nm laser. From our experience, the best results on conventional 3-laser cytometer could be obtained from including QDot 655 in multicolor panels.

Despite the careful selection of organic fluorochrome, overlap of multiple dye fluorescence spectra is common due to a wide and asymmetric spectra of conventional fluorochromes, therefore requiring multicolor compensation and creating problems associated with using combinations of bright and dim fluorochromes. Narrow emission spectra of QDots results in little overlap between detectors and in advantages for resolving cell subpopulations during immunophenotyping. Including only one-two QDots in multi-color panel may lead to the significant loss of QDot brightness due to spreading error in QDots channels from organic fluorochrome (for example, for QDot 585 etc), therefore optimization of fluorescent acquisition with narrow bandpass filter is of particular importance.

Standard fluorescein or rhodamine microscopy filters can be used for the QDot 525 and QDot 605 with either xenon or mercury lamps as light source [6]. However, the optimal excitation filter for QDots is 425/40 nm [6] or 435/40 according to our studies and the emission (blocking) filter should be centered around the emission peak with a bandwidth of 15-20 nm for QDots from 525 to 655 and a bit larger for QDot 705. To expand multiparameter abilities of standard confocal instrument Kingeter and Schaefer [33] added two band-pass filters and one long-pass filter to a three-laser Zeiss Pascal confocal microscope. Specifically, they added 655/40 nm and 705/50 nm band pass filters and a 745 long pass filter to enable detection of QDots 655, 705 and 800 nm cover the under-utilized red and near-infrared (IR) regions of emission.

In confocal microscopy, the common argon and krypton-argon lasers are not optimal for QDots. It is preferential to use for QDots excitation a blue diode laser with a 442 nm line or violet with a 405 nm line. To compensate for differences in QDot brightness for microscopy, Lagerholm [78] suggested using QDot-conjugates mixture with 4:1:2 molar ratio of QDots 565, 605, and 655, respectively. Because of QDots excellent photostability, there is no need to use antifade reagents or exposure correction during acquisition of z-stacks. QDots possess advantages for multiphoton excitation, because of their greater 2-photon absorbtion compared with traditional fluorochromes [79].

Current filters available for fluorescent microscopy are summarized in Table 4.
Table 4. Optical filters for QDots, commercially available for fluorescent microscopy

<table>
<thead>
<tr>
<th>QDots</th>
<th>Dichroic filter</th>
<th>Bandpass emission filter</th>
<th>Maximum emission</th>
<th>Color/visibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>760</td>
<td>770 LP</td>
<td>800</td>
<td>Infrared (invisible)</td>
</tr>
<tr>
<td>705</td>
<td>685</td>
<td>710/40</td>
<td>705</td>
<td>Far red – invisible</td>
</tr>
<tr>
<td>655</td>
<td>630</td>
<td>655/15</td>
<td>655</td>
<td>Far red – poorly visible</td>
</tr>
<tr>
<td>605</td>
<td>595</td>
<td>605/15</td>
<td>605</td>
<td>Orange-Red</td>
</tr>
<tr>
<td>585</td>
<td>575</td>
<td>585/15</td>
<td>585</td>
<td>Yellow-Orange</td>
</tr>
<tr>
<td>565</td>
<td>550</td>
<td>565/24</td>
<td>565</td>
<td>Yellow</td>
</tr>
<tr>
<td>545</td>
<td>535</td>
<td>550/25</td>
<td>545</td>
<td>Green</td>
</tr>
<tr>
<td>525</td>
<td>505</td>
<td>525/20</td>
<td>525</td>
<td>Cyan-Green</td>
</tr>
</tbody>
</table>

7. Conclusive remarks

The influence of QDots nanocrystals on the status of fluorescent microscopy is already tremendous. QDots are brighter and more stable than organic dye molecules and thus suitable for fluorescent microscopy applications, especially for confocal microscopy. However, optimization of QDot application requires special filter sets that are different from those used with conventional fluorochromes.

Problems in using QDot conjugates could be summarized as follows: (i) a lack of uniformity attributed to different sizes, compositions and characteristics as well as a lack of standardization in model systems complicates the results of the studies; (ii) commercially available QDot conjugates should be used cautiously since not all of them give reproducible results; (iii) critical remains the surface-controlled reproducibility (i.e. batch-to-batch comparability of optical properties).

In cytometry QDots are a better perspective for multiplexed immunostaining panels due to narrow emission filters, limited need in spectral compensation and low spreading error. QDots can be used in addition to organic dyes as a part of multicolor panels for surface immunostaining. However, intracellular staining with QDot antibody conjugates requires determination of precise fixation-permeabilization protocol and can be influenced by large size of functionalized QDots. Optimization of detection of QDot signal may require changes in the filter sets.

Advances in nanocrystal synthesis will help to introduce new, perspective types of QDots. Another breakthrough in the applications of QDots in bioimaging and intracellular cytometry is anticipated with development of surface functionalization techniques that maintain an overall small size of QDots and resistance to fixation.

Supplement: Protocol for indirect staining with QDots conjugated with secondary antibodies used for paraffin sections.

<table>
<thead>
<tr>
<th>Steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation in 0.05% TBS (Tris-HCl buffer) with 2 % BSA (pH 7.4-7.6) 20 min at room temperature.</td>
</tr>
<tr>
<td>Mouse monoclonal antibodies (1:100) in 0.05% TBS (Tris-HCl buffer) with 2 % BSA (pH 7.4-7.6) 40 min at room temperature.</td>
</tr>
<tr>
<td>Rinse in Tris-HCl buffer with c Tween 20, 3 times for 5 min (Labvision, cat# TA-125-TT).</td>
</tr>
<tr>
<td>Incubation in 0.05% TBS (Tris-HCl buffer) with 2 % BSA (pH 7.4-7.6) 20 min at room temperature.</td>
</tr>
<tr>
<td>QDot-conjugated antibodies against mouse IgG – incubation for 50 min at room temperature.</td>
</tr>
<tr>
<td>Rinse in Tris-HCl buffer with c Tween 20, 3 times for 5 min (Labvision, cat# TA-125-TT).</td>
</tr>
<tr>
<td>Incubation in 0.05% TBS (Tris-HCl buffer) with 2 % BSA (pH 7.4-7.6) 20 min at room temperature.</td>
</tr>
<tr>
<td>Rabbit monoclonal antibodies (1:100) in 0.05% TBS (Tris-HCl buffer) with 2 % BSA (pH 7.4-7.6) 40 min at room temperature.</td>
</tr>
<tr>
<td>Rinse in Tris-HCl buffer with c Tween 20, 3 times for 5 min (Labvision, cat# TA-125-TT).</td>
</tr>
</tbody>
</table>

Acknowledgements The support of RFBR, Moscow Government research program and Russian Ministry of Science and Education for I.Vorobjev and support of internal 1025c grant from Immune Disease Institute for N. Barteneva is gratefully acknowledged. Authors are thankful to E. Korneva for preparing immunohistochemistry figure, David Krull for laser scanning cytometry picture, and to S. Jaron and I.Sotnikov for help with QDots conjugates.
References


