In Vivo Imaging Using Quantum Dot–Conjugated Probes

Diane S. Lidke,[1] Peter Nagy,[2] and Donna J. Arndt-Jovin[3]

[1]University of New Mexico, Albuquerque, New Mexico
[2]University of Debrecen, Debrecen, Hungary
[3]Max Planck Institute for Biophysical Chemistry, Goettingen, Germany

ABSTRACT

This unit describes the use of quantum dots (QDs) for live-cell imaging and the use of QDs in flow cytometry for quantitative analysis of ligand binding constants and receptor density. Conventional fluorophores and visible fluorescent protein (VFP) constructs have allowed visualization of many cellular processes. However, organic and biomolecular fluorophores have limitations in their applications, due to their small Stokes' shift and tendency to photobleach during prolonged imaging. QDs have many advantages over conventional fluorophores, including high brightness and photostability, which make them an exceptional tool for live-cell imaging. There are a large variety of commercially available QDs with different surface reactivities and characteristics. The authors have limited the laboratory protocols presented here to the use of streptavidin-coupled QDs because this gives almost universal applicability to any cell surface receptor by coupling the ligand or antibody that recognizes the receptor to biotin and visualizing the complex by use of QDs. *Curr. Protoc. Cell Biol.* 36:25.1.1-25.1.18. © 2007 by John Wiley & Sons, Inc.

Keywords: quantum dots • live-cell imaging • binding constants • receptor number • biotinylated ligands

INTRODUCTION

The responses of a cell to its surrounding environment result largely from the transduction of signals from the outer cell surface to the cytoplasm and nucleus. These signals are initiated when a ligand binds to a membrane receptor, initiating signaling cascades that control numerous cellular processes such as gene expression, cell migration, and cell division. Macromolecular protein dynamics and localization, as well as signaling cascades, can be directly visualized in the living cell using fluorescence microscopy [see other units on FRAP (*UNIT 21.1*), ion imaging, and fluorescent imaging (Chapters 4 and 21)].

In this unit, the authors describe protocols for forming biotin-ligand (or biotinylated-'protein of interest') and streptavidin-QD (SAvQD) complexes for use in live-cell microscopy (see Support Protocol 1). In the case when the precoupling of the QD to the ligand inhibits function, an alternative two-step labeling protocol is provided. Support Protocol 2 discusses ways to avoid cross-linking of QD-ligand complexes if the ligand is labeled with multiple biotins. Basic Protocol 1 gives details for in vivo cell labeling with QD-ligand complexes and image acquisition, with special emphasis on temporal acquisition of image series to visualize cellular processes. Experiments to determine QD-ligand binding constants (Basic Protocol 2) and absolute number of receptors per cell (Basic Protocol 3) by complementary flow cytometry measurements are also described.

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STRATEGIC PLANNING

What are quantum dots and how are they useful for live-cell imaging?

Water-soluble, bio-functionalized semiconductor quantum dots (QDs) are fluorescent nanoparticles that provide advantages of much greater photostability compared to conventional fluorescent dyes, and as a consequence, single QDs can be easily detected in living cells and their localization monitored for minutes to hours to days. The core of the QD consists of a semiconductor nanocrystal, typically CdSe surrounded by a passivation shell of ZnS, as well as an outer shell to make them biocompatible (Alivisatos et al., 2005; Smith et al., 2006). Absorption of a photon anywhere in a continuum rising toward the UV generates an electron-hole pair, which upon recombination (in ~10 to 20 nsec) results in the emission of a less energetic photon. The emission wavelength is dependent on the size of the core (smaller core size = lower emission wavelength), which can be varied by controlled synthesis conditions (Fig. 25.1.1). These characteristics and the fact that QDs have a large absorption cross-section, particularly in low visible to UV wavelengths, allows for the simultaneous excitation of many spectrally distinct QD emitters with a single wavelength. (See later section on multispectral fluorescence imaging and analysis).

Many types of QDs are commercially available from Invitrogen (*http://probes.invitrogen.com/products/qdot*) and Evident Technologies (*http://www.evidenttech.com*). These include surface-accessible moieties such as amino, carboxyl, peptides, biotin, streptavidin, protein A, and IgGs. The manufacturers provide kits for coupling your favorite molecule to QDs with extensive protocols and the reader is referred to these sites for details. In addition, membrane-permeable QDs (having nona-arginine on the surface) and other methods of intracellular delivery are available so that QDs may be used to label cells for reintroduction into animals and long-term cell tracking. The larger CdSe QDs, emitting at >600 nm, have extended geometries with dimensions of ~40 nm. These large sizes make accessibility a problem in tight junctions between cells or in permeabilized tissues (Arndt-Jovin et al., 2006). Emission wavelengths of QDs have been extended into the near NIR using CdTe as cores (Kim et al., 2004; Gao et al., 2005; Smith et al., 2006), thus enhancing their use in live-animal studies. An exciting in vivo quantum dot

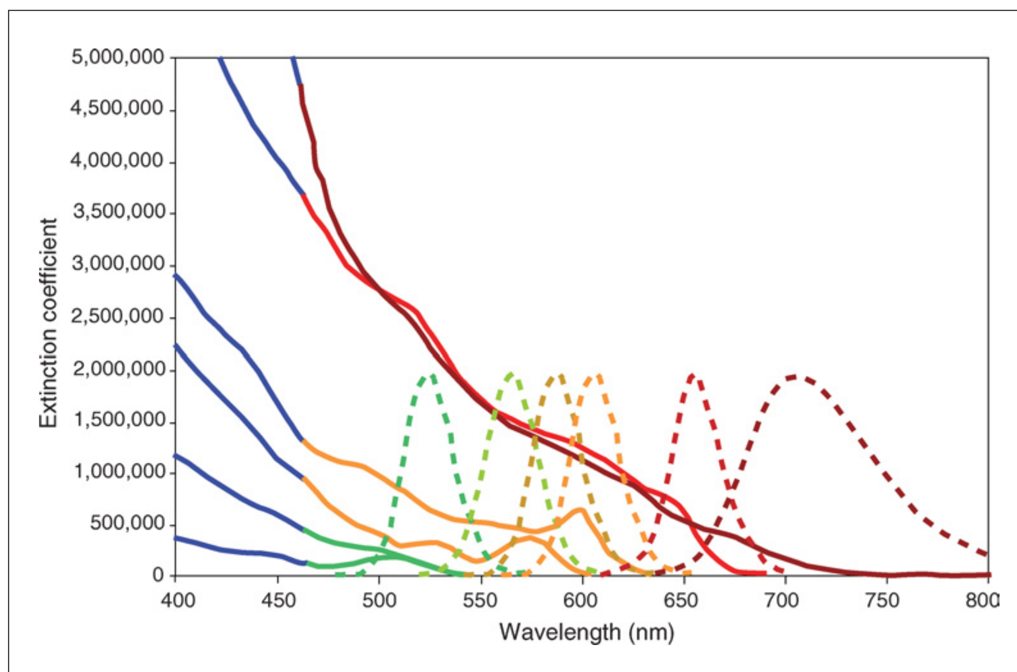

Figure 25.1.1 Excitation and emission spectra of CdSe core QDs. For color version of this figure see *http://www.currentprotocols.com*.

conjugate that emits by bioluminescence resonance energy transfer in the absence of external excitation has been prepared by coupling carboxylate-presenting quantum dots to a mutant of the bioluminescent protein *Renilla reniformis* luciferase (So et al., 2006).

Differences between QDs as probes and small or conventional fluorophores

Most conventional fluorophores have relatively broad absorption and emission bands with a small to medium Stokes' shift (the difference in wavelength between their excitation and emission maxima). For information on the excitation and emission maxima for many small molecule fluorophores see *http://probes.invitrogen.com/handbook*. Genetically encoded fluorophores (VFPs, visible fluorescent proteins) have small Stokes' shifts except for the recently described Keima (Chudakov et al., 2005; Shaner et al., 2005; Kogure et al., 2006). Thus, it is more difficult to excite several conventional fluorophores with a single wavelength. For excitations in the visible wavelengths many conventional fluorophores have higher extinction coefficients than QDs and with their shorter fluorescent lifetimes will appear 'brighter' to the observer. However, these dyes are much less photostable than QDs. This latter fact makes QDs the probe of choice for in vivo imaging over extended time periods.

Instrumentation

Microscopy

Macromolecular dynamics, localization, and aggregation state can be directly visualized in the living cell using fluorescence microscopy [see units on basic fluorescence microscopy (*UNIT 4.2*), FRAP (*UNIT 21.1*), FRET (*UNITS 17.1 & 17.9*) and fluorescence correlation spectroscopy (FCS)]. Commercial confocal microscopes allow for multi-laser excitation and the imaging of several different fluorophores simultaneously. Recent advances in multispectral fluorescence imaging, where the emission spectrum of the sample is measured in each sampled point, have made it possible to further increase the number of fluorophores that can be monitored (Fountaine et al., 2006). Commercial systems include the Zeiss LSM 510META and Live systems, Nikon Digital Eclipse C1si, Leica TCS SP5, Olympus Fluoview, and Olympus DSU spinning disk. QDs are particularly suited to simultaneous multi-color labeling of different macromolecules in the same sample due to their broad excitation spectra and narrow emission spectra, which allows for single line excitation of multiple QDs and separation of spectrally distinct QD emissions using filter-based or multispectral imaging.

In addition to confocal imaging, high spatial and time resolution can be achieved using a wide-field microscope and a sensitive CCD camera (see *UNIT 4.2*). The brightness and photostability of QDs make it possible to track individual protein dynamics in living cells at video rate or faster using the new generation electron multiplying (em) CCD cameras (Andor iXon, Roper Scientific Cascade, Hamamatsu C9100-02). The high quantum efficiency of CCD cameras extending into the NIR makes them far superior for detecting QDs with emissions >600 nm compared with PMT-based imaging systems.

Flow cytometry

Flow cytometry is a complementary technique to imaging microscopy. The authors include a discussion of it here to demonstrate its power for quantitative determination of binding constants, numbers of binding sites, as well as kinetic or dissociation constants with high statistical precision. Flow cytometry has both advantages and disadvantages compared to microscopy. It can record the fluorescence and light scattering of hundreds or thousands of cells in a second, but speed comes at the price of losing subcellular resolution. Although some flow cytometers are capable of multi-laser excitation, in the simplest and probably most wide-spread bench-top flow cytometers the number of excitation wavelengths is limited. Similarly as in the imaging microscope, the fact that several different types of QDs can be excited by a single laser line makes

multi-color labeling possible and easily detected in different fluorescence channels of the flow cytometer (Mattheakis et al., 2004; Chattopadhyay et al., 2006). The photostability of QDs is less valuable in flow cytometry due to the limited amount of time the cells are illuminated, but their relatively large extinction coefficient (especially with UV excitation) makes the labeling of cells with low numbers of binding sites more reliable.

BASIC PROTOCOL 1

IN VIVO, IN SITU LABELING OF CELLS WITH QD-LIGAND CONJUGATES FOR FLUORESCENCE MICROSCOPIC IMAGING

This method describes the general procedure for preparing cells for live imaging in order to follow the fate of the QD-ligands interacting with membrane-bound receptors (see Fig. 25.1.2). The methods can be used with a confocal laser scanning microscope or a wide-field microscope. The use of LabTek chambers (Nunc) requires the use of an inverted microscope. Other manufacturers such as Electron Microscopy Services (*http://www.emsdiasum.com/microscopy/products/preparation/dish.aspx*) make holders that accommodate coverslips of various dimensions that are equally suitable. With live-cell imaging, dynamic events (i.e., QD-ligand binding/internalization or endosomal trafficking) can be monitored by acquiring an image series over the time range of interest.

If quantitative analysis of data is desired care must be taken to acquire the images such that no pixels have intensities of 0 or less nor values above the range of the detector (8-bit, 12-bit, or 16-bit for example.)

Controls for the specificity of the QD-receptor interaction are important. It is absolutely essential to show that the signal that is acquired is specifically associated with the presumed receptor and that it reflects the behavior of the receptor without QDs attached. The researcher must do binding experiments with unconjugated QDs, and QD-ligand binding in the presence of competitive excess free ligand, determine if down-stream signaling is affected as expected (e.g. phosphorylation profiles are the same with QD-ligand compared with ligand alone), and demonstrate that there is no binding on cells that do not express the receptor.

If one wishes to perform single-particle tracking of individual receptors, it may be desirable to use the QD simply as a tracer of the molecule of interest. This may be done

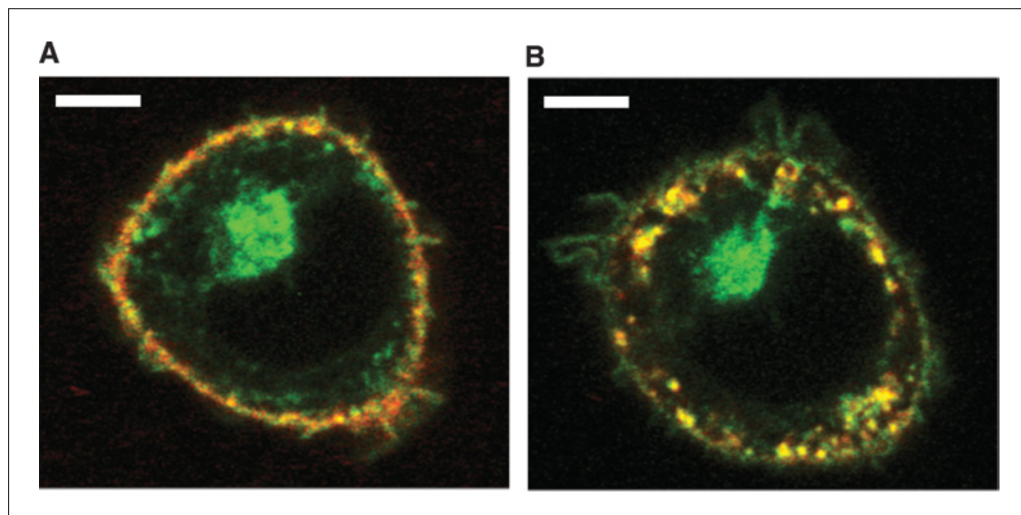

Figure 25.1.2 QD-EGF (red) bind to the surface of Chinese hamster ovary cells expressing EGFR-GFP (green) (**A**) and undergo endocytosis within minutes (**B**). Colocalization (yellow) of the QDs and receptor are seen at the membrane and after internalization in endosomes. Images acquired using a Zeiss LSM510-META system. Scale bar = 10 μm. For color version of this figure see *http://www.currentprotocols.com*.

by using a very low concentration of QD-ligand mixed with an excess of free ligand (Lidke et al., 2005a) or by binding the biotinylated-ligand (Alternate Protocol) followed by a tracer amount of SAvQDs (Echarte et al., 2007).

Materials

Logarithmically growing cells (*UNIT 1.1*)
Trypsin
Cell culture medium appropriate for the particular cell line
Tyrode's buffer plus (see recipe)
Preformed QD-ligands (Support Protocol 1 or 2)

LabTek 8-well coverslip chambers (Nunc)
Emission filters for specific QD emission wavelengths (Chroma or Omega, also available from the microscope manufacturers)

Additional reagents and equipment for culture of cells (*UNIT 1.1*)

Plate cells

1. One or two days before the experiment, trypsinize logarithmically growing cells (*UNIT 1.1*).

2. Plate the cells in LabTek 8-well coverslip chambers in complete culture medium at the appropriate density (one typically desires ~50% confluency on the day of the experiment).

   In the case of experiments with receptors that are sensitive to serum factors, the cells can be serum-starved for 4 to 16 hr prior to the experiment to reduce signaling induced by the serum in the medium.

Prepare sample for live-cell imaging with QD-ligand complexes

3. At the time of the experiment, wash the cells once with 200 µl Tyrode's buffer plus and maintain in this buffer.

   Culture medium without phenol red, containing 1% (w/v) BSA can be used for very long imaging periods (in place of Tyrode's buffer plus).

4. Place the chamber on the microscope stage and equilibrate to the desired temperature.

   The authors recommend an objective heating collar as the most effective way to keep the cells at the desired temperature during the duration of the experiment. An inexpensive and efficient setup can be purchased from Cell MicroControls (HLS8 × 0.8P and mTCII) and consists of an adjustable, flexible heating strip that can be secured around any objective with a strip of Velcro and a battery powered controller. For very long (hours to days) experiments a system having CO_2 input is necessary as well.

5. Dilute the QD-ligand complex (Support Protocol 1 or 2) to twice the desired final concentration in Tyrode's buffer plus.

   Dilute the ligand-SAvQD complex from PBS/1% BSA into Tyrode's plus just before use since the SAvQDs can aggregate with time in buffers containing divalent cations. Do not store this dilution for use on another day.

6. Add an equal volume (200 µl) of QD-ligand in Tyrode's buffer plus to the cells in the well under observation to a final concentration of 0.005 to 2 nM.

   The final concentration should be adjusted for the specific protein-ligand interaction and the known binding constants. Also, the concentration will depend on whether one wants to observe many or only a few of the receptors at one time (i.e., single-molecule tracking or measuring binding and uptake). The two-step labeling procedure is recommended if a maximal cellular response but single-molecule resolution are desired. This procedure allows using the QDs as tracers of the behavior of the macromolecule of interest.

Image acquisition

7. Initiate image acquisition either before or just after addition of the QD-ligand.

That is, when using preformed complexes, the QD-ligand can be added to the sample after images at several time points have already been acquired, taking care not to disturb the position of the sample during addition of the QD-ligand. Typically, addition of large volumes (e.g., addition of 200 μl of QD-ligand to 200 μl of buffer in chamber) results in rapid mixing without additional pipetting. The QD-ligand should also be kept at the same temperature as the imaging chamber to avoid focal drift due to temperature fluctuations. The time between images and duration of the time series acquisition is dependent on the events being monitored and must be determined by the researcher.

A 63× or 40× 1.2 NA water immersion objective is recommended.

In confocal imaging systems, simultaneous excitation of VFP and various QDs can be achieved by using different laser combinations. QDs having emissions of 605 nm and above can be excited well by 488 nm or higher wavelengths whereas lower wavelength QDs are more efficiently excited by 458-nm or 407-nm lasers. An imaging system with at least two detectors, allows the simultaneous collection of eGFP and QD signals with appropriate filters (eg., 520/20 bandpass and the appropriate QD emission bandpass filters). Single QD imaging requires high laser powers and pixel dwell times of about 6 μsec. Thus, whole 512 × 512 image acquisitions take seconds. Fast-tracking of single QDs requires more sensitive acquisition systems (see next section). For the most efficient separation of different emitting QDs in the same sample, selective QD emission filters or a spectral unmixing device should be used (Fountaine et al., 2006; Miskoski et al., 2006; see Fig 25.1.3).

Wide-field systems equipped with a mercury arc lamp most efficiently excite the QDs at 436 nm without cell damage. Image splitters and multispectral analysis systems can separate many QD and VFP labels simultaneously (Miskoski et al., 2006). EmCCD cameras can easily detect QD emission from single bound QDs with acquisition times of 50 ms or less (see Fig. 25.1.4).

Image analysis is specific to the particular question being addressed and therefore, must be developed by the researcher. There are many commercial and noncommercial resources available for image processing. Two of the most popular platforms are ImageJ, a stand-alone software available through NIH (http://rsb.info.nih.gov/ij/) and DIPimage, a

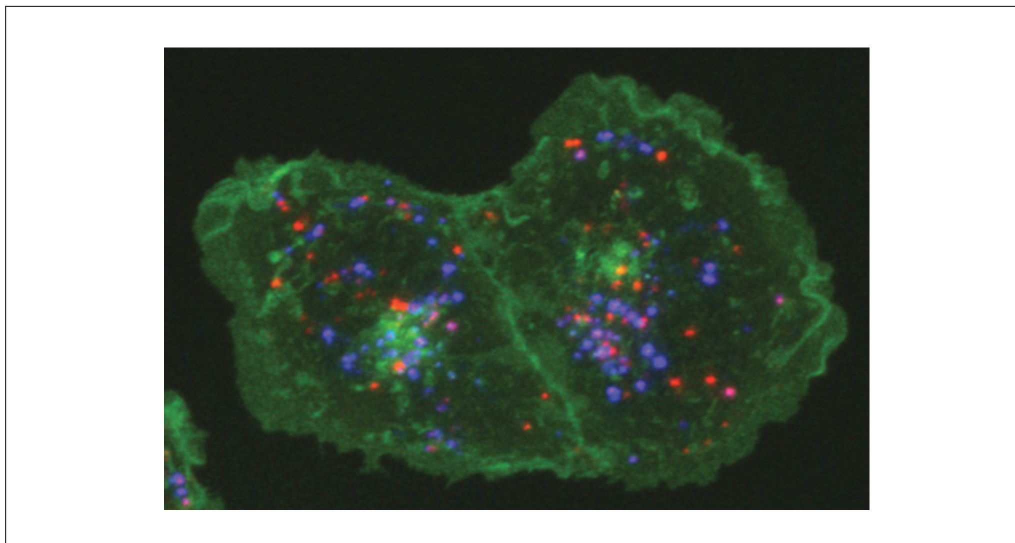

Figure 25.1.3 In a ‘pulse-chase’ experiment, two different colors of QD-EGF (red and blue) added to a cell expressing erbB1-eGFP (green) are used to monitor vesicle fusion. First, red QD-EGF is added and allowed to internalize. At a time point 15 min later, the blue QD-EGF is added. At a time point 22 min after addition of the blue QD-EGF, colocalization of red and blue QDs are seen in endosomes (purple), indicating that receptors activated later in time can ‘catch-up’ to the same compartment as those that were internalized earlier. Image acquired using a Zeiss LSM510-META system. For color version of this figure see *http://www.currentprotocols.com*.

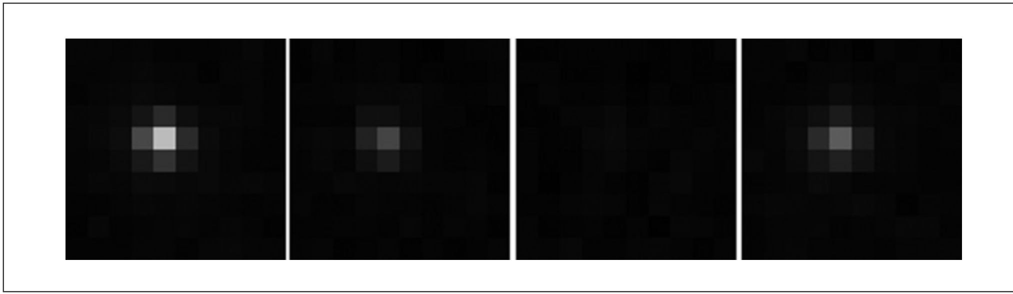

Figure 25.1.4 Single QD Blinking. Selected images from a time series showing the intermittent fluorescence of an individual QD. Time series acquired using an Olympus IX71 microscope with an Andor iXon emCCD camera. Excitation: 472-nm diode laser; emission: 655/40 QD filter. 60 msec between images, 20 msec integration time (see Lidke et al., 2005b).

scientific image processing toolbox for Matlab available from Delft University of Technology (http://www.ph.tn.tudelft.nl/DIPlib/). These two programs are free to University staff and students. A large number of plug-ins or scripts have been developed for both packages, such as masking operations, tracking algorithms, colocalization or 2-D histograms, and are freely available to the public.

LABELING CELL SURFACE RECEPTORS IN A TWO-STEP PROCEDURE

ALTERNATE PROTOCOL

In some cases, the presence of the QD may sterically hinder the QD-ligand binding to the cell surface membrane receptor. If one does not observe binding of the preformed complex, the following two-step protocol may be useful. The authors have successfully used this procedure with EGF, transferrin, and NGF receptors (Grecco et al., 2004; Lidke et al., 2004; Echarte et al., 2007).

Materials

Cells cultured in chambers or on coverslips (Basic Protocol 1)
Tyrode's buffer plus (see recipe), cold
Bio-ligand
Streptavidin-conjugated Quantum Dots (SAvQD; Invitrogen)
4% (w/v) paraformaldehyde
Tris-saline buffer
Phosphate-buffered saline (PBS; see recipe)
10° to 12°C water bath

1. Put chambers or coverslips containing cells on ice or float chamber in a water bath maintained at 10° to 12°C (recommended for cells of the immune system which are sensitive to extreme cold).
2. Wash cells once with cold Tyrode's plus and maintain in this buffer for the experiment.
3. Incubate cells with bio-ligand for 10 to 30 min.

   The concentration of bio-ligand and incubation times should be adjusted according to the binding constant of the protein-ligand interaction.

4. Wash with cold Tyrode's plus several times.
5. Add 0.05 to 2 nM cold SAvQD (diluted in Tyrode's plus) for 5 to 15 min.
6. Wash the cells several times with cold Tyrode's plus.
7. Mount for imaging.

   a. For live-cell imaging, immediately place cells on the microscope stage with appropriate temperature control and image (for more detail see Basic Protocol 1).

b. For snapshots of the behavior of the QD-ligand complex at different time points, incubate cells grown on coverslips at the appropriate temperatures and times and fix in 4% paraformaldehyde for 15 min on ice to prevent redistribution of the QDs. After fixation, wash the coverslips with Tris-saline buffer for 10 min several times and mount in PBS for imaging.

SUPPORT PROTOCOL 1

COUPLING MONOBIOTINYLATED LIGANDS TO STREPTAVIDIN-QDS

This method describes how to conjugate ligands to QDs via a biotin-streptavidin interaction. The authors have successfully used this protocol for several biotinylated ligands, including epidermal growth factor (EGF; Lidke et al., 2005a) and nerve growth factor (NGF; Echarte et al., 2007).

This protocol can be used with most ligands and proteins (such as antibodies) that have a single biotin moiety attached. The protocol uses QDs from Invitrogen that have 3 to 4 streptavidin molecules covalently bound to the QD bioconjugate shell (SAvQD). The authors recommend for most applications the PEG 2000 coated QDs since they show reduced nonspecific binding to cells and cell substrates such as polylysine or collagen.

The reader is reminded that each streptavidin can have up to four binding sites for biotin. The multi-valency of the SAvQDs can be an advantage in that it allows for the stochiometry of the QD-ligand complex to be varied by simply changing the ligand:QD ratio. A monovalent biotinylated ligand, where the biotin is conjugated to a single known residue or position, is preferred since the properties of the QD-ligand complex can be better controlled and potential cross-linking of the QDs via the ligand is avoided (see Support Protocol 2 for ligands with multiple biotins). By mixing SAvQDs, biotinylated ligand and free biotin at specific molar ratios, one can create QDs with the desired average number of attached ligands, assuming a Poisson distribution (Lidke et al., 2004).

After formation of the QD-ligand complex, unbound ligands that are smaller than the QDs can be removed from the conjugate by passing the mixture over a size exclusion column. For PEG-QDs cross-linked dextran size exclusion columns (eg, Sephadex G-25) are recommended, and for QDs without a PEG coating, polyacrylamide bead exclusion columns are most efficient (eg, Bio-gel P).

Materials

Streptavidin-conjugated Quantum Dots (SAvQD; Invitrogen)
Phosphate-buffered saline (PBS)/1% (w/v) BSA (see recipe)
Biotinylated-Ligand (bio-ligand)
PBS (see recipe)
1% (w/v) BSA

Shaker
Gel filtration spin columns, NAC-5

Prepare QD-ligand preformed complexes

1. Dilute SAvQD to 20 nM in 100 µl PBS/1% BSA.

2. Dilute bio-ligand to 20 to 60 nM in 100 µl PBS/1% BSA.

   The above concentration will yield a Poisson distribution of 1:1 to 3:1 ligands per QD. The reader should determine the valency of ligand desired for his/her particular experiment.

3. Add SAvQDs to ligand, mix with a micropipet, and vortex for 1 sec.

   Thoroughly mix the SAvQDs and ligand in equal volumes so that the stoichiometry does not vary throughout the solution at the time of mixing.

4. Incubate for at least 30 min at 4°C with gentle agitation or rotation.

Purify QD-ligand conjugates

5. Swell and pour the column material according to the manufacturer's instructions.

   For PEG-coated QDs coupled to small peptide ligands as described above, Sephadex G-25 medium-grade gel filtration is recommended; the QD-ligand complex will elute in the void volume. Use a 1:20 ratio of sample to column volume to ensure separation. Spin columns can be used for sample volumes of 50 µl or less. For non-PEG SAvQDs, Bio-gel P20 is recommended; the QDs appear in the void volume. To remove excess macromolecules from QD conjugations, ultra filtration units with 100-kDa or 200-kDa regenerated cellulose membranes are recommended (see Millipore Corporation for small volume ultracel or microcon units).

6. Equilibrate the column in PBS by washing with at least 3 column volumes.
7. Allow the buffer to just enter the top of the gel bed before adding the sample. Avoid air bubbles.
8. Add the sample to the top of the gel bed, allow the sample to enter the gel, and add PBS to elute the sample.
9. Collect the fractions containing the QDs. Add 1% (w/v) BSA (final concentration) to final product

   The purified QD-ligand complexes are stable for 4 weeks when stored at 4°C.

PREPARATION OF PREFORMED COMPLEXES OF QDS WITH LIGANDS WITH MULTIPLE BIOTINS

SUPPORT PROTOCOL 2

Some ligands must be multivalent for proper recognition by their receptors. The ligand may thus also be multi-biotinylated. The following protocol has been successfully used with such ligands and avoids cross-linking of QDs by the ligand by blocking excess biotin binding sites on SAv-QDs with free biotin shortly after addition of the ligand (see Cambi et al., 2007 for an example).

Materials

Streptavidin-conjugated Quantum Dots (SAvQD; Invitrogen)
PBS/1% (w/v) BSA (see recipe)
Bio-ligand

1. Dilute SAvQD to 20 nM in 100 µl PBS/1% BSA.
2. Dilute bio-ligand to the appropriate molarity in 100 µl PBS/1% BSA and mix with the QDs for 15 min with agitation.

   After addition of free biotin, the ligand-QD complexes can be purified as described above (Support Protocol 1, steps 3 and 4) or used directly if the free biotin and potentially free ligand do not interfere with receptor recognition.

   The absolute concentration of the ligand should be carefully determined. The number of biotins per ligand should also be determined.

   The authors recommend adding the ligand at a concentration such that the final dilution has a ratio of approximately three to six ligands to one QD and allowing binding to proceed with agitation for only 15 min followed by the addition of a 10-fold excess of free biotin to saturate any remaining streptavidin binding sites.

   The success of this procedure will depend to some extent upon the nature of the ligand. The authors assume that there are at least two available biotin binding sites for each streptavidin. Due to the geometries of the QDs, the nature of the ligand, as well as how the biotins are arrayed on its surface, not all biotins will be able to bind to the QD surface. The authors have found that polymeric ligands of ~30 to 40 kDa with approximately three biotins per polymer show no detectable cross-linking of QDs when added to the QDs at a polymer to QD ratio of 3:1 followed by excess biotin (Cambi et al., 2007).

BASIC PROTOCOL 2

FLOW CYTOMETRIC DETERMINATION OF THE BINDING CONSTANTS OF LIGANDS OR ANTIBODIES

Ligand binding to its receptor is the first step in transmembrane signaling. The easiest way to characterize this interaction is to determine the equilibrium binding curve, i.e., ligand binding as a function of ligand concentration. Fluorescence has largely replaced the use of radioactivity in such experiments. This protocol describes how fluorescent QDs can be used for the determination of dissociation constants.

Materials

Biotin-coupled ligand or antibody
Avidin-coated QD (Invitrogen or Evident Technologies) suitable for excitation with the flow cytometer available
Trypsin
Phosphate-buffered saline (PBS)/1% (w/v) BSA (see recipe)

Flow cytometry tube
Flow cytometer
Flow cytometric analysis software

Additional reagents and equipment for coupling the ligand or antibody of interest to QDs (Support Protocol 1) and culture of cells (*UNIT 1.1*)

1. Couple the ligand or antibody of interest to QDs (see Support Protocol 1).

2. Trypsinize cells (*UNIT 1.1*), resuspend them in PBS/1% BSA at a concentration of 5×10^6/ml.

3. Prepare a 2-fold dilution series of QD-ligand. Add 1000 µl of the highest concentration of QD-ligand made in PBS/1% BSA to a flow cytometry tube. Prepare dilutions starting from a concentration ~10× higher than the anticipated K_d. Mix thoroughly. Keep all the tubes on ice during the entire labeling procedure. Pipet 500 µl from this solution to another tube into which 500 µl of PBS/1% BSA is added yielding the second highest concentration. Repeat this procedure until the lowest concentration is reached.

   Be aware that in the presence of ligand concentration 9× higher than the K_d, 90% of the binding sites will be occupied, if binding is noncooperative.

   At the end of this step flow cytometry tubes will be filled with 500 µl of different concentrations of the QD-ligand on ice.

4. Add 20 µl of the cell suspension to each tube and incubate for 30 to 60 min on ice with regular mixing.

   The QD-ligand is diluted negligibly by the 20 µl cell suspension, since 500 µl >20 µl.

5. Measure the samples with a flow cytometer.

   Keep the cells on ice until measurement. Alternatively, samples can be fixed in 1% formaldehyde after removing unbound QD-ligand by washing twice with PBS.

   Measure a nonlabeled cell sample too, the fluorescence intensity of which will be subtracted from the mean fluorescence intensities of each sample. Pay attention to the fact that all cells in the brightest and darkest samples have to be on scale, otherwise the means will be distorted.

6. Using a flow cytometric analysis software, determine the mean fluorescence intensities of each sample gated on the FSC-SSC dot plot.

7. Plot the autofluorescence-corrected mean fluorescence intensities as a function of the total QD-ligand concentration (assumed to be equal to the free ligand concentration).

8. Fit the following, so-called Hill equation to the measured data points:

$$I = I_{\max} \frac{c^n}{c^n + K_d^n}$$

where I is the fluorescence intensity in the presence of QD-ligand concentration of c, K_d is the dissociation constant required to half-saturate the binding sites, and n is the Hill coefficient characterizing the cooperativity of binding.

If there is good reason to believe that there is no cooperativity (e.g., if one ligand binds to a monomeric receptor species), it can be assumed that $n = 1$, and a simpler equation can be used:

$$I = I_{\max} \frac{c}{c + K_d}$$

In this case K_d has real meaning characterizing the binding event, e.g., it is equal to the ratio of the dissociation and association rate constants. If $n \neq 1$, K_d lacks any direct molecular implications. Indeed, the Hill coefficient itself cannot be translated into a real molecular model, since the Hill equation is the result of a reductionist view of the molecular events taking place during complex formation.

FLOW CYTOMETRIC QUANTITATION OF THE NUMBER OF ANTIBODY BINDING SITES

BASIC PROTOCOL 3

The number of binding sites for a ligand or antibody, i.e., the total number of expressed antigens is a significant issue: for example, it has predictive value in cancer diagnosis. Flow cytometry has obvious advantages over cell suspension-based methods (e.g., radioactive binding analysis), since it is capable of resolving heterogeneity and eliminates the influence of dead cells and cell debris. The idea behind the calibration procedure is to run a series of reference beads with calibrated number of antibody binding sites or fluorescence intensities. Three approaches are widely used:

1. Beads with calibrated fluorescence intensities (e.g., Quantum FITC MESF beads from Bangs Laboratories). The kit contains several bead populations each corresponding to the fluorescence intensity of a calibrated number of fluorophores. The company provides a calibration software (QuickCal) for the calculations, which can also be easily carried out with a spreadsheet program. Different beads are available for different fluorophores. To the best of our knowledge, no such beads are available for the calibration of QD fluorescence intensities, therefore the authors will not provide a detailed protocol.

2. The Qifikit calibration kit (Dako) contains a series of beads coated with different, calibrated numbers of mouse monoclonal antibody molecules. The beads have to be labeled by the secondary antibody used for labeling of the cells. A plot of the fluorescence intensity of the beads against the number of binding sites will be constructed.

3. The Quantum Simply Cellular kit (Bangs Laboratories) contains subpopulations of beads with calibrated binding capacities for the Fc region of mouse or human antibodies. The beads have to be labeled with a primary antibody followed by a secondary, fluorochrome-conjugated antibody in the same way as the cells. A calibration plot of fluorescence intensity versus number of binding sites can be constructed.

Materials

Cells being investigated
Primary unlabeled antibody

Secondary QD-conjugated antibody against the primary antibody (Quantum Dot Corporation or Evident Technologies)
Phosphate-buffered saline (PBS)/1% (w/v) BSA (see recipe)

Qifikit calibration kit (Dako)
Vortex
Qifikit calibration beads (Dako-Cytomation, *www.dako.com*) or Quantum Simply Cellular beads (Bangs Laboratories, *www.bangslabs.com*)
Flow cytometer
Quantum Simply Cellular kit (Bangs Laboratories)

For Qifikit

1a. Label the cells under investigation with saturating concentration of the primary unlabeled antibody.

2a. After washing the cells twice with 2 ml PBS, label them with the secondary, QD-conjugated secondary $F(ab')_2$. Include a cell sample which is only labeled by the primary antibody for background correction.

Cells and beads should be labeled with secondary antibody at the same time (see step 4a).

3a. After vortexing remove 100 µl from the Setup and Calibration bead vials, centrifuge the beads 4 min at 1400 × g, 4°C, after adding 3 ml of PBS/1% BSA.

The Qifikit beads are coated with primary mouse antibodies, and can only be used for the calibration of secondary antibody binding to cells labeled by primary mouse antibodies.

4a. Remove the supernatant and label the bead pellet with the secondary QD-conjugated secondary $F(ab')_2$ under the same conditions as the cells.

This step should be done at the same time as step 2a.

5a. Run the cells and the two Qifikit bead samples (Setup and Calibration) on the flow cytometer.

6a. Subtract the fluorescence intensity of the sample labeled by the primary antibody only from the intensity of the sample labeled by both the primary and secondary antibodies (background-corrected intensity).

7a. Analyze the fluorescence of the unlabeled bead population in the Setup sample and the five subpopulations in the Calibration sample. Subtract the mean fluorescence intensity of the unlabeled bead population from the fluorescence intensities of the calibration beads. Plot the background-corrected fluorescence intensities of the calibration bead subpopulations as a function of the number of binding sites which is included in the manual for the kit. Fit a line to the data points.

8a. Find the number of antibody binding sites on the cells based on their background-corrected fluorescence intensity using the calibration line.

For Quantum Simply Cellular beads (secondary labeling)

1b. Combine one drop of each of the beads with graded number of binding sites for human or mouse antibodies.

The blank bead with no capacity for antibody binding can also be combined with the others, or can be processed separately.

The Quantum Simply Cellular beads can capture a calibrated number of mouse or human monoclonal antibodies by their Fc domain allowing them to be used for the calibration of both primarily or secondarily labeled cells. The authors have provided a protocol for the use of these beads with secondary labeling, but the principle can easily be adapted for primary labeling.

2b. After mixing the beads vortex them. Label both the cells and the beads with saturating concentration of the primary unlabeled antibody.

3b. Add 5 to 10 ml PBS, centrifuge 4 min at 1400 × g, 4°C, and repeat washing step once more. Label the cells and the beads with the secondary, QD-conjugated secondary $F(ab')_2$. Include a cell sample which is only labeled by the primary antibody for background correction.

4b. Add 5 to 10 ml PBS and centrifuge the samples 4 min at 1400 × g, 4°C. Repeat the washing step once more before running the samples on the flow cytometer.

5b. Subtract the mean fluorescence intensity of the blank bead population from the fluorescence intensities of the labeled beads. Plot the background-corrected fluorescence intensities of the calibration bead subpopulations as a function of the number of binding sites. Fit a line to the data points.

6b. Find the number of antibody binding sites on the cells based on their background-corrected fluorescence intensity using the calibration line.

The company provides an online program, QuickCal (www.bangslabs.com/flow/quickcal), which can perform the above calculations automatically.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A***; for suppliers, see* **SUPPLIERS APPENDIX***.*

Paraformaldehyde (PFA) fixative

Prepare a fresh 4% (w/v) solution of paraformaldehyde, analytical grade in PBS (see recipe). Use only for 1 day, keep on ice.

A 40% solution can be prepared and kept in small aliquots at −20°C. Thaw at 50°C and immediately dilute in PBS (see recipe) before use.

Phosphate-buffered saline (PBS)

137 mM NaCl
2.7 mM KCl
7.9 mM Na_2HPO_4
1.5 nM KH_2PO_4
Adjust pH to 7.3 using NaOH or HCl, if necessary
Autoclave

Store up to 6 months at room temperature

PBS/1% (w/v) BSA

Add 1% BSA to PBS (see recipe) just before use.

Tyrode's buffer

135 mM NaCl
10 mM KCl
0.4 mM $MgCl_2$
1 mM $CaCl_2$
10 mM HEPES
Adjust pH to 7.2 using NaOH
Autoclave

Store up to 6 months at room temperature

Tyrode's buffer plus

Add 20 mM glucose and 0.1% (w/v) BSA to Tyrode's buffer (see recipe) just before use.

COMMENTARY

Background Information

Published results of in-vivo QD imaging

QD are not "brighter" than conventional fluorophores since they have relatively long fluorescence emission lifetimes. Also, due to their large size (dictated by the necessity of several layers of material for passivation and biocompatibility beyond the emitting core), there may be steric hindrance to binding compared to small fluorophores in applications such as in situ hybridization or labeling to arrays like cytoskeletal components. They are most important for their extreme photostability and thus have been used most effectively as labels for in vivo imaging. In particular, their application to the study of the behavior of cell surface receptors has brought new insights at single-molecule resolution (Dahan et al., 2003; Lidke et al., 2005a; Arndt-Jovin et al., 2006; Courty et al., 2006; Echarte et al., 2007; Hagen et al., 2007). An area beyond the scope of these protocols is the application of QDs to tracking and homing in whole animal studies (see Lim et al., 2003; Ballou et al., 2004; Kim et al., 2004; Smith et al., 2006). A number of recent reviews refer to uses of QDs in imaging, flow cytometry, and immunoassays (Bruchez and Hotz, 2006; Fu et al., 2005; Michalet et al., 2005; Mulder et al., 2006; Pinaud et al., 2006; Portney and Ozkan, 2006; Prasad, 2006; Smith et al., 2006; Weng and Ren, 2006).

Special characteristics and behaviors of QDs

One of the most powerful ways to determine local proximity of biologically relevant molecules is through FRET (Förster resonance energy transfer; *UNITS 17.1 & 17.9*) between a donor and an acceptor fluorophore attached to the two molecules of interest. In the case of QDs, the large passivation and conjugation shells surrounding the fluorescent core make it difficult to use QDs for such measurements since the efficiency of transfer varies inversely with the 6th power of the distance between donor and acceptor. Additionally, the broad excitation spectrum of QDs prohibits their use as FRET acceptors since it is not possible to selectively excite the donor, resulting in a relatively small enhancement of acceptor fluorescence due to FRET. An exception is the use of a bioluminescent donor for QD-BRET as mentioned in the beginning of this unit (So et al., 2006). QDs can act as donors in a FRET pair when the acceptor lies in close proximity to the passivation shell (see Lidke et al., 2005a; Clapp et al., 2006; Pons et al., 2006). In this case the QD should not have a large bio-shell such as PEG conjugation.

Single QDs exhibit 'blinking' behavior or intermittent fluorescence (see Fig. 25.1.4). Although proper passivation of the semiconductor core reduces blinking, it is generally not possible to suppress this phenomenon completely. The blinking follows an inverse power law, as predicted for (1) an exponential distribution of trap depths or (2) a distribution of tunneling distances between QD core/interface state (Kuno et al., 2000; Shimizu et al., 2001). Hohng and Ha (2004) were able to reduce blinking of single QDs by treating them with high concentrations of thiols, a procedure that is incompatible with live-cell imaging. When tracking individual QDs attached to cell surface receptors or the cytoskeleton using high-speed acquisitions in the 20 to 50 msec range, correction for the blinking behavior must be made to avoid improper segmentation of the loci or jumping of the track between different QDs (Dahan et al., 2003; Arndt-Jovin et al., 2006). However, this blinking behavior can be used to advantage to localize individual QDs with super resolution (Lidke et al., 2005b; Hagen et al., 2007).

An advantageous property of QDs is the CdSe electron dense core that can be detected by transmission electron microscopy (Dahan et al., 2003; Nisman et al., 2004). This makes possible correlative dynamic fluorescence and high-resolution TEM studies on the same sample.

Critical Parameters and Troubleshooting

The "tail wagging the dog" problem: Quantum Dot size and valency considerations

The size of a QD is comparable to that of an antibody. Therefore, since the binding properties or biological activity of a ligand can be influenced by its conjugation to a QD, careful control experiments (such as flow cytometry

to characterize QD-ligand binding constants) must be made to establish that there is no steric influence of the QD.

QDs are multivalent, such that several ligands can be coupled to each QD. This can result in a single QDs binding multivalently to multiple receptors (Cambi et al., 2007). The researcher has to decide whether this is a concern for his or her particular problem. If so, the ligand should be coupled to the QD in a nearly 1:1 ratio (Lidke et al., 2004) and preblocking of excess binding sites with free biotin may be necessary (see Support Protocol 1).

Fixation

PFA (4%) is the preferred fixation solution for samples labeled with QDs. The QD fluorescence is destroyed by freezing or fixation in −20°C methanol. In addition, the use of various mounting mediums must be tested to ensure that the medium does not degrade the QD fluorescence. As QDs degrade, their emission gradually shifts to lower wavelength and this can be particularly precarious when several spectrally distinct QDs are present. To avoid such problems, store fixed samples in PBS at 4°C, mount fixed samples in PBS, and image within 24 hr of fixation.

Nonspecific binding

The authors recommend the PEG 2000-coated QDs for most cellular applications since they show reduced nonspecific binding to cells and cell substrates, such as polylysine or collagen. In addition, the presence of high concentrations of BSA and the use of a higher pH will help to reduce nonspecific binding. The reader is reminded that stock solutions of QDs are maintained by Invitrogen in 1 M trimethylglycine at pH 8.5 and dilution into buffers containing divalent cations will, over time, result in aggregation and precipitation of the QDs.

Controls

It is essential to prove that the QD signal is reflective of the uncoupled ligand's interaction with the receptor of interest and that the QDs do not interfere, when appropriate, with the ligand's physiological activity. Controls for such behavior include showing that (1) unconjugated QDs do not bind to the cell surface, (2) QD-ligands do not bind to cells not expressing the membrane protein of interest, (3) presaturation of the cell surface receptors with unlabeled ligand blocks QD-ligand binding, and (4) addition of QD-ligand mimics unconjugated ligand. In addition, flow cytometry (Basic Protocols 2 and 3) can be used to characterize QD-ligand binding constants and correlation with free ligand binding (Lidke et al., 2004).

Other bioconjugation techniques

As described above, many types of QDs with different bioconjugation are available. In addition, manufacturers sell kits for alternative bio-coupling methods. See the manufacturers' Web sites for more information (*http://probes.invitrogen.com/p1roducts/qdot* and *http://www.evidenttech.com*). For a review of other labeling methods see Smith et al. (2006).

Flow cytometric determination of binding constants

Antibodies and ligands typically induce the internalization of their receptor. In order to prevent this from happening, both the cell suspension and the ligand solutions have to be kept on ice for the duration of the experiment. If samples are not fixed before measurement, internalization can take place in the instrument as well. The sample can spend a significant amount of time at room temperature in flow cytometers into which multiple samples are loaded simultaneously (e.g., with a sample loader or on a 96-well plate). In such cases, the samples should be fixed before measurement. Before fixation the unbound ligand has to be removed by washing with excess PBS. Dissociation of bound ligands starts immediately after the ligand concentration drops in the medium. Therefore, washing can introduce errors into the measurement. Consequently, it is best to use flow cytometers into which single tubes are loaded one-by-one, and use unfixed cells without washing. This can be done since most flow cytometers are equipped with a constant background subtraction algorithm, therefore the contribution of extracellular fluorescence is reliably removed.

The authors strongly advise against using the Scatchard analysis, i.e., a plot of the ratio of bound ligand concentration to free ligand concentration as a function of bound ligand concentration, for fitting. In flow cytometric experiments the measured fluorescence intensities can be used instead of the bound ligand concentration. Before nonlinear regression programs became widely available, scientists transformed data to make linear plots. If the Scatchard plot can be fitted with a single line, the x intercept and the slope of the line correspond to the maximum number of binding sites (maximum fluorescence intensity) and the negative reciprocal value of the K_d, respectively. Although the shape of a Scatchard

plot reveals potentially important information about the nature of binding (positive or negative cooperativity in the case of concave down and concave up Scatchard plots, respectively), the fitting results will be distorted due to error propagation inherent in the transformation leading to the Scatchard plot.

If the fluorescence intensity does not saturate, the following possibilities have to be investigated:

1. Nonspecific binding: nonspecific binding sites are usually very difficult to saturate. Dead cells may have significant non-specific binding.

2. Ligand depletion: in most analyses it is assumed that the amount of bound ligand is negligible compared to the total amount available. If the number of binding sites is high, this assumption does not hold, and the free ligand concentration can be significantly lower than the total (ligand depletion). In such a case saturation of binding takes place at a much higher total ligand concentration than without ligand depletion. The ligand can also bind to the wall of the flow cytometric tube leading to a similar phenomenon.

3. Ligand binding can be compromised by coupling to QD.

Flow cytometric determination of the number of antibody binding sites

In the Quantum Simply Cellular kit the same primary antibody is used for labeling the beads and the cells whereas the Qifikit beads are coated with a mouse monoclonal antibody. Since secondary antibodies may show slight variation with regard to binding to primary antibodies, there is some advantage to the Quantum Simply Cellular in that the secondary antibody binds to the same primary antibody on both cells and beads. In most cases (especially if the same isotype is used for labeling the cells and coating the bead), this is unlikely to be a major source of error. On the other hand, the primary antibody is captured by its Fc domain by the Quantum Simply Cellular beads, a different orientation from when the Fab domain binds to its epitope. It is unknown to us whether the different orientation of the primary antibodies has any influence on the binding of the secondary antibody.

If the calibration line is prepared using the background-corrected fluorescence intensities of the calibration beads (as described in the above protocols), the calibration line should cross the origin of the plot, or have a *y* intercept very close to zero. The calibration curve is expected to be strictly linear, i.e., a correlation coefficient of >0.98 and no splaying at high concentrations. If the curve is nonlinear or it does not cross the *y* axis at zero, nonspecific binding of the antibody or incomplete labeling of the beads may have happened. Check the quality of the antibodies used and whether the beads were mixed regularly during labeling.

If the fluorescence intensity of the cells is higher than that of the brightest bead population, the calibration line can be extrapolated. But it has to be kept in mind that at very high densities antibody binding may not be strictly proportional to the number of antibody binding sites. Although the number of antibody binding sites on the different bead population may show batch-to-batch variation, the range of the Qifikit beads usually extends higher ($\sim 5 \times 10^5$) than that of the Quantum Simply Cellular beads ($\sim 2 \times 10^5$).

Anticipated Results

Support Protocols 1 and 2: Collection of eluted fractions from the size exclusion column or recovery of the QD fraction after filtration should result in a purified QD-ligand complex. The presence of QDs can be confirmed by recording an absorbance or emission spectrum. Concentration of QDs can be calculated from the peak absorbance value if one knows the extinction coefficient (available from the manufacturer) for that particular QD.

Basic Protocol 1: During the imaging times series, the researcher should observe a strong QD signal localized at the position of the receptor (either on the membrane or internalized in vesicles). Addition of unlabeled QDs should not result in QD binding to the cell surface. Large aggregates should not be seen in the images—this would indicate cross-linking of the QDs by ligand and the complex formation conditions should be adjusted as discussed in the Alternate Protocol.

For all of the basic protocols, detection limits depend on the imaging conditions. Under the right conditions and with sensitive emCCD cameras one can detect single QD binding as indicated in the Background Information section and literature cited therein. It is difficult to detect single QDs in raster scanning confocal microscopes in live samples, but possible in fixed material with long pixel dwell times and line averaging.

Time Considerations

Support Protocol 1. Preparation and mixing of reagents to form QD-ligand complexes should take 15 to 20 min. Complexes should incubate for at least 30 min. The time for

purification depends on whether spin columns, filters, or gravity columns are used, between 30 min to 2 hr is usual.

Basic Protocol 1. Replating of cells into imaging chambers takes ~30 min. The time for an imaging experiment is dependent on the processes being observed and the length of the time series acquired. Quantitative image analyses of image time series can be run as batch jobs once the conditions of the experiments have been standardized and macros or plug-ins to the basic image processing software have been concatenated. Tracking of individual QDs must normally be controlled by operator intervention to avoid analysis of false traces.

Basic Protocols 2 and 3. The labeling and the flow cytometric experiment can be carried out in ~2 hr and analysis typically takes ~1 hr.

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