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Calibration of Flow Cytometry for Quantitative Quantum Dot Measurements

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Abstract

Observations of quantum dot (QD) labeled cells in biomedical research are mainly qualitative in nature, which limits the ability of researchers to compare results experiment-to-experiment and lab-to-lab to improve the state-of-the-art. Labeled cells are useful in a range of *in vitro* and *in vivo* assays where tracking behavior of administered cells is integral for answering research questions in areas such as tissue engineering and stem cell therapy. Before the full potential of QD based toolsets can be realized in the clinic, uptake of QDs by cells must be quantified and standardized. This unit describes a novel, simple method to assess the number of QDs per cell using flow cytometry and commercially available standards. This quick and easy method can be used by all researchers to calibrate their flow cytometry instruments and settings, and quantify QD uptake by cells for *in vitro* and *in vivo* experimentation for comparable results across QD conjugate types, cell types, research groups, lots of commercial QDs, and homemade QDs

Keywords

quantitative; quantum dot; cell uptake; flow cytometry; number of quantum dots per cell; QD uptake

INTRODUCTION

Without reliable, standardized quantitative information on quantum dot (QD) uptake by cells, researchers will not be able to conduct the dosage studies required to optimize QDs for biomedical and clinical applications, and most importantly, minimize QD toxicity if any — an area still under investigation. (Dubertret, Skourides et al. 2002; Medintz, Uyeda et al. 2005; Michalet, Pinaud et al. 2005; Hardman 2006; Jamieson, Bakhshi et al. 2007) A standardized, quantitative characterization of QD uptake will also facilitate optimization of QD conjugate chemistry to decrease batch-to-batch or lot-to-lot variance and resultant experiment-to-experiment variance currently plaguing the field. (Wu, Campos et al. 2007) This unit describes a novel, quick and easy method which can become the standard for determining a quantitative value for QD uptake by cells in units of number of QDs per cell.

Flow cytometry, unlike other methods to measure QD uptake such as elemental methods (inductively coupled plasma mass spectroscopy (ICP-MS), atomic emission mass spectroscopy (AES-MS)), or radiolabelling ((micro positron emission tomography (microPET))), is routinely used in cell biology and clinical labs and is nondestructive to samples. Commercially available fluorescent calibration beads are a standard for quantitative flow cytometry allowing direct and objective comparison of data between laboratories. (Wu, Campos et al. 2007) First, this unit will describe a protocol for developing a calibration curve for the flow cytometry instrument by titrating commercially available calibration beads with commercially available QDs. Second, this unit will describe the method by which one can use the calibration curve to determine the approximate number of QDs per cell for a given cell-labeling study.

BASIC PROTOCOL 1

FLOW CYTOMETR CALIBRATION

In this protocol, the method for calibrating the flow cytometer to obtain quantitative information on QD uptake by cells is described.

Materials

Quantum Simply Cellular (QSC) anti-Mouse IgG Beads (BangsLaboratories, Inc., 8 μ m)

Mouse IgG conjugated QDs (IgG QDs) with desired emission wavelength (Invitrogen)

0.05 M Tris phosphate buffered saline (TPS) pH 8.0 (Sigma)

0.1 % w/v BSA/0.05 M TPS buffer pH 8.0

Mouse IgG (Sigma)

Bovine serum albumin (BSA) (Sigma)

Deionized water

0.22 μ m filter unit (Nalgene)

Reference standard beads for desired emission wavelength

Flow Cytometer with 488 nm or 400 nm laser and appropriate filter sets for the emission wavelength of the QD under study

1. Make fresh 0.1 % BSA/TPS buffer pH 8.0 and filter buffer through 0.22 μ m filter.
2. Specific binding series: Vortex IgG QDs and carry out a 4 fold serial dilution in 0.1 % BSA/TPS buffer pH 8.0 from 80 nM to 20 pM in eppendorf tubes (about 7 concentration values or titration points) with final volume of 50 μ L. Makeup three of these dilution series and label each series appropriately (each series is a specific binding titration of QSC beads ABC4, ABC3, and ABC2).
3. Include a "0 nM" titration point at the end of each of the three dilution series with 50 μ L of 0.1 % BSA/TPS buffer pH 8.0.
4. Nonspecific binding series: Carry out a 10 fold serial dilution in 0.1 % BSA/TPS buffer pH 8.0 from 80 nM to 10 pM (about 3 concentration values or titration points) with a final volume of 30 μ L. Makeup three of these dilution series and label tubes appropriately (each series is a nonspecific binding titration of QSC beads ABC4, ABC3, and ABC2).

5. Prepare enough 30 μ M IgG solution in 0.1 % BSA/TPS buffer pH 8.0 to have 20 μ L per nonspecific binding sample. (For example, if there are 3 nonspecific titration points per ABC, then 180 μ L is required.) This will give a final blocking concentration of 10 μ M IgG per sample.
6. For each of the following beads ABC4, ABC3, and ABC2, of the QSC set, vortex provided vial and transfer about 5 drops into a labeled screw cap eppendorf tube. This will allow for precise measurement of bead suspension volume for titration. Unused beads can be stored for later use.
7. Vortex beads in eppendorf tube and distribute 10 μ L of beads to each specific binding titration tube, and immediately vortex to mix. Continue this distribution for each titration point in each series ABC4, ABC3, and ABC2.
8. Nonspecific binding series: Label a new set of tubes and distribute 10 μ L of beads to each nonspecific binding titration sample for each ABC.
9. Distribute 20 μ L of 30 μ M IgG to each nonspecific binding titration sample and immediately vortex to mix.
10. Place both specific and nonspecific titration samples on a rotator and rotate at room temperature for 30 minutes.
11. Wash beads by adding 1 mL of buffer to each specific binding titration sample, vortexing, and spinning down beads. For the beads used in the development of this protocol (bead diameter of 8 μ m), beads are spun at 5000 rcf for 5 minutes.
12. While specific binding samples are spinning down, add nonspecific binding serial dilution QDs to the nonspecific binding tubes and vortex immediately to mix. Place tubes on rotator and rotate at room temperature for 30 minutes.
13. Aspirate buffer from specific binding samples carefully as to not disturb bead pellet. Resuspend pellet in 1 mL buffer and repeat wash step 2 more times.
14. When nonspecific binding samples are finished incubating, wash 3 times with the same wash procedure.
15. Resuspend all samples in 350 μ L of buffer for flow cytometry analysis.
16. Collect at least 5,000 - 10,000 events for each sample.

Data analysis

17. Obtain the median signal of the fluorescence value for each sample using flow cytometry data analysis software such as FlowJo. Fit these median values for each ABC set of data to a single binding site, specific with nonspecific, binding curve in curve-fitting software such as Graphpad Prism.
18. Obtain the $B_{\max}$ value for the binding curves, making sure the fits are good with an R^2 value close to 1.0. The value, $B_{\max}$, for each ABC population represents the true value of the beads' fluorescence intensity at saturation binding.
19. Divide the $B_{\max}$ values by the median value of the reference standard to get $B_{\max}$ in terms of "reference standard units." This step normalizes data to variances in operation of the flow cytometer from experiment-to-experiment, and day-to-day. Alternatively, the flow cytometer settings could be adjusted experiment-to-experiment and day-to-day such that the reference standard exhibits consistent magnitude of fluorescence with prior experiments.

20. Plot $B_{\max}$ for each ABC against the reported binding capacity of each ABC population of beads as provided by the commercial source.
21. Fit the curve to a linear fit and obtain an equation for the line. This is the calibration curve for the given flow cytometer at the given settings.

BASIC PROTOCOL 2

QUANTITATIVE MEASUREMENT OF QD UPTAKE BY CELLS USING CALIBRATION CURVE

This protocol goes through the steps required to quantify the approximate number of QDs taken up by cells in a given cell study using the calibration curve derived above. Cells can be labeled by QDs conjugated with polyArginine (polyArg), cholera toxin B (CTB), transferrin (TF), or other reported methods. (Lagerholm, Wang et al. 2004; Mattheakis, Dias et al. 2004; Chakraborty, Fitzpatrick et al. 2007)

Materials

Labeled cells with QDs of emission wavelength calibrated on flow cytometer

Unlabelled cells without QDs (negative control)

1x phosphate buffered saline (PBS)

1. Trypsinize, count, and wash cells once in PBS by centrifugation. Resuspend pellet in 300 – 500 μ L PBS and transfer suspension to labeled flow cytometry tubes for flow cytometry analysis.
2. Run the reference standard beads at the same settings used for calibration and collect 5,000 – 10,000 events.
3. Run cell samples and collect 5,000 – 10,000 events. Forward scatter (FSC) will need to be turned down to gate for the cell population.
4. Obtain the median value of peak fluorescence for each sample using flow cytometry data analysis software such as FlowJo.

Data Analysis

5. Divide sample median values by the median signal for the reference standard. This will give signal in terms of reference standard units.
6. Use the calibration curve linear fit equation to find x , the number of QDs per cell, for the given y , or median signal in terms of reference standard units. See equation (1) in *Anticipated Results*.

REAGENTS AND SOLUTIONS

0.1% BSA/0.05 M TPS Buffer pH 8.0

Dissolve 1 TPS packet in 1 L deionized water. Dissolve appropriate mass of BSA in appropriate volume of TPS buffer by mixing with stir bar for 10 minutes. Filter solution through 0.22 μ m filter unit. Store buffer for no more than one day at 4°C.

COMMENTARY

Background

Over the past 10 years since quantum dots (QDs) were rendered biochemically stable for biological applications, (Bruchez, Moronne et al. 1998; Chan and Nie 1998) many groups

have been working to optimize — or have already applied — QD conjugates to answer biological, biomedical, and clinical research questions. (Medintz, Uyeda et al. 2005; Michalet, Pinaud et al. 2005; Jamieson, Bakhshi et al. 2007) QDs are semiconductor nanoparticles that have size and composition adjustable fluorescence emission wavelengths, narrow emission bands, and very high levels of brightness and photostability. (Cai, Chen et al. 2007) In biomedical research, QDs have potential as a tool for cell labeling, fluorescence in situ hybridization, cell tracking *in vitro* and *in vivo*, fluorescence resonance energy transfer, cancer diagnosis, and tumor targeting in vivo and more. (Gao, Cui et al. 2004; Lagerholm, Wang et al. 2004; Medintz, Uyeda et al. 2005; Michalet, Pinaud et al. 2005; Cai, Shin et al. 2006; Fischer, Liu et al. 2006; Cai, Chen et al. 2007; Chakraborty, Fitzpatrick et al. 2007; Jamieson, Bakhshi et al. 2007; Yezhelyev, Al-Hajj et al. 2007) However, one of the most significant obstacles to advancement of the technology in the particular case of cell tagging and tracking is that there is no clear standard by which researchers can compare results of QD uptake by cells *in vitro* and between labs. (Hardman 2006)

Literature often reports cellular uptake of QDs by microscopy in qualitative terms such as dimmed, low, moderate, or high fluorescence, brightness, percentage, or presence of QDs in few, some, or many cells. Many studies simply state the initial loading concentration of QDs per cell, which is not reflective of the number of QDs taken up by cells. Experimental conditions which affect cellular uptake vary from lab-to-lab. Only one study by Dubertret et al. has reported a measured yet approximate, quantitative value of QD uptake in terms of number of QDs per cell. However, their work involved the use of injection as a delivery method rather than internalization by QD conjugates. (Dubertret, Skourides et al. 2002; Hardman 2006) Another group reports an observation of 1 million QDs per cell after 1 hr incubation with QD conjugates and 3 million QDs per cell with no supporting data, methodology or reference as to how these values were obtained. (Gao, Cui et al. 2004)

The method presented here is simple, quantitative and reliable. We believe that this method will allow direct comparison of methods and materials developed in distinct laboratories, provided an analogous material is available, or can be prepared as a mouse antibody conjugate.

Critical Parameters

Prior to flow cytometer calibration and cell studies, measure the absorbance and fluorescence of QD stock materials. Absorbance readings can be used to calculate a more precise concentration of QD stock solutions from the commercial source which will give a more accurate quantification of the number of QDs per cell based on these stock concentrations. In our experience, fluorescence values will show the extent of fluorescence emission variance between QD conjugates and batches. Since the calibration curve is created with one conjugate and is used to characterize another conjugate, it is important to factor in variability in fluorescence emission or brightness (which is related to quantum yield and extinction coefficient) between conjugates and batches of QDs.

QSC bead kits contain five populations of polystyrene microbeads that have a predetermined, progressively increasing number of ligands on each bead (bead diameter of 8 μm). Lot-to-lot, kits vary in the binding capacity of given populations, therefore signal at saturation or B_{max} will also vary lot-to-lot.

Bead populations “ABC0” and “ABC1” should not be used for calibration as they both exhibited nonspecific binding behavior in protocol development studies regardless of efforts to optimize buffer composition. Instead, a nonspecific binding assay was developed for each bead population where beads are blocked with free IgG prior to titration with IgG QDs.

Troubleshooting

For successful calibration, the flow cytometer voltage settings must be adjusted such that cells loaded with reasonable concentrations of QDs are visible at the same settings used for the titration of QSC beads (settings used for the calibration).

Another variance by batch of beads is the number of beads in suspension provided by the commercial source. This concentration of particles will affect how much volume of beads you will need to titrate in order to read at least 5,000 events on your flow cytometer.

If there is a lot of visible, non-bead events seen in the scatter plot during flow cytometric analysis of QD IgG bound beads, this means that there is a significant amount of junk in stock QD materials. Spin down the QD stock from the commercial source at 5,000 rcf for 5 minutes and avoid bottom of vial, or set a threshold FSC on events to decrease the amount of debris counted as events in flow cytometry.

Anticipated Results

An example calibration curve generated for 705 nm emitting QDs is shown in Figure 1 and includes two batches of QSC bead sets. The R^2 value for the linear regression of the data was good at 0.9775. For this example calibration curve, any signal from cells run on the flow cytometer, *signal*, in terms of reference standard units (signal of the sample divided by the signal of the reference standard) can be related to number of QDs per cell, *No.QDs/Cell*, according to the following equation,

$$\text{No.QDs/Cell} = \frac{\text{signal} + 0.0922}{7 \times 10^{-6}} \quad (1)$$

The calibration curve generated in this protocol remains linear and holds true through the limit of detectable signal on the cytometer being used. For example, the limit of the cytometer used in development of this protocol is 100,000 arbitrary units, corresponding to approximately 1,000 reference standard units or 200 million QDs. In studies conducted during the development of this protocol, the largest average number of QDs detected per cell was 2.4 million, with some events as high as 100 million QDs per cell, in a dendritic cell line for a loading concentration of 8 nM QDs. In literature, cells are typically stained with QDs at a loading concentration of 1,000 pM to 10 nM. Therefore, it is reasonable that a sensitivity limit of 200 million QDs per cell using this calibration curve and cytometer settings falls far beyond the physical capacity of cells for QDs. In fact, this value is near the capacity of mammalian cells for QDs assuming the cell's entire volume is completely filled with QDs.

Time Considerations

The binding titration assay for calibration curve development takes about 2.5 hours. Flow cytometry analysis of these titrated samples takes about 4 hours. Data analysis of flow data in software such as FlowJo takes about 1 hour and fitting resultant data to curves in Graphpad Prism software takes about 1 hour for the experienced user. Deriving the calibration curve in spreadsheet software such as Microsoft Excel takes a few minutes.

Preparing cells for flow cytometry analysis takes about 1.5 hours and actual analysis on the flow cytometer takes 1 hour. Analysis of cell flow data in flow analysis software and quantification of number of QDs per cell in spreadsheet software using the developed calibration curve takes about 1 to a few hours depending on the number of samples of the cell study.

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Figure 1. Calibration curve for 705 nm emitting QDs to determine number of QDs per cell by flow cytometry. Error bars represent standard deviation of the mean of measurements in triplicate.