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Quantum Dots for quantitative flow cytometry

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Summary

In flow cytometry, the quantitation of fluorophore-tagged ligands and receptors on cells or at particulate surfaces is achieved by the use of standard beads of known calibration. To the best of our knowledge, only those calibration beads based on fluorescein, EGFP, phycoerythrin and allophycocyanine are readily available from commercial sources. Because fluorophore based standards are specific to the selected fluorophore tag, their applicability is limited to the spectral region of resonance. Since quantum dots can be photo-excited over a continuous and broad spectral range governed by their size, it is possible to match the spectral range and width (absorbance and emission) of a wide range of fluorophores with appropriate quantum dots. Accordingly, quantitation of site coverage of the target fluorophores can be readily achieved using quantum dots whose emission spectra overlaps with the target fluorophore.

This chapter will focus on the relevant spectroscopic concepts and molecular assembly of quantum dot fluorescence calibration beads. We will first examine the measurement and applicability of spectroscopic parameters, ϵ , ϕ , and %T to fluorescence calibration standards. Where, ϵ is the absorption coefficient of the fluorophore, ϕ is the quantum yield of the fluorophore and %T is the percent fraction of emitted light that is transmitted by the bandpass filter at the detector PMT. The modular construction of beads decorated with discrete quantities of quantum dots with defined spectroscopic parameters is presented in the context of a generalizable approach to calibrated measurements of fluorescence in flow cytometry.

Keywords

Quantum dots; fluorescence calibration beads; flow cytometry; extinction coefficient; spectroscopy

1. Introduction

1.1. General Considerations

The use of standard calibration beads to define the quantity of fluorophore-tagged ligands or proteins on cells or beads represents an essential element of quantitative flow cytometry that has enabled the direct comparison of inter-laboratory data as well as quality control in clinical flow cytometry (1-4). Commercial standards such as Quantum™ FITC MESF beads

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(www.bangslabs.com) are comprised of a mixture of five distinct populations of beads, where the subsets are functionalized with discrete titers of fluorescein conjugates. The equivalence of fluorescence radiance of each bead population in the mix is correlated to the fluorescence of soluble fluorescein. Thus the surface occupancy of fluorescein on the bead standards is known as the Molecules of Equivalent Soluble Fluorophores (MESF) where the MESF value is equal to the known number of molecules in solution (3,4). We have recently described a simple method of producing fluorescence calibration beads that bear discrete and defined quantities of quantum dots and demonstrated their applicability as standard calibration beads (5,6) useful for multiple color calibration. The measurement model is based on a simple ratiometric formula, Equation 1, for comparing the number of fluorophores of Sample 1 and Sample 2. Sample 1 is an analyte capable of emitting radiation of intensity I_1 that is equivalent to a single unit of Sample 2; Sample 2 is a calibration standard emitting radiation of intensity I_2 . For simplicity both samples must be excited at the same wavelength and emit in the same spectral region as defined by the same bandpass (BP) filter. Equation 1 is derived from spectroscopic parameters: extinction coefficient measured at the excitation wavelength (ϵ_λ), quantum yield (ϕ), and the BP transmittance (%T) of sample emission. It enables one to determine the number density (ρ_i) of sample fluorophores.

$$\frac{I_1}{I_2} = \frac{\rho_1}{\rho_2} = \frac{\epsilon_{\lambda_{ex},1}}{\epsilon_{\lambda_{ex},2}} \cdot \frac{\phi_1}{\phi_2} \cdot \frac{\%T_1}{\%T_2} \quad (1)$$

In Equation 1, ϵ can be measured from absorption spectra for most probes. The quantum yields ϕ_i can be determined by the end-user relative to known textbook standards (7,8).

The photophysical properties of qdots are not clearly defined like the intrinsic physical constants of molecular systems. Quantum dots are semiconductor nanoparticles with tunable optical properties that are strongly dependent on size (9). The spectroscopic properties of dots are dependent on their size dispersion, shape, and surface defects (10-23). Subtle differences in preparation can lead to batch-to-batch variation in basic spectroscopic properties such as quantum yield (10-23). To the best of our knowledge, the commercial dots are not spectroscopically standardized nor are they well characterized in the product literature for them to be used “as made” (10). In this chapter we refer to the previous characterization (6) of a pair of CdSe quantum dots with emission bands centered at 525 nm (QD525 purchased from Invitrogen, Carlsbad, CA; lot# 1005-0045) and 585 nm (QD585, Invitrogen lot# 0905-0031). The lot numbers are cited to emphasize the notion that the spectroscopic characteristics of these dots are strictly relevant to samples from the indicated lots (6). Our previous study of these samples showed an anomalous dependence of emission yield on excitation wavelength that was more pronounced in QD525 dots than in the QD585 quantum dots. This was attributed to size heterogeneity and surface defects in the QD525, consistent with characteristics previously described in the literature (10,22,23). It is therefore important to characterize new batches of dots before they can be used as calibration standards. Two lots of subsequently purchased QD585 dots (Invitrogen lot# 4526A and 49987A) were shown to have quantum yield values of 0.12 and 0.3 respectively.

1.2. Molecular Assembly of Qdot Fluorescence Calibration Beads

Qdots can be photo-excited at any wavelength that can be matched to a fluorophore of choice. Therefore, calibration standards can be assembled for any fluorophore by using qdots whose emission spectra overlap with the target fluorophore. The approach is based on the display of variable and discrete site densities of streptavidin-functionalized qdots on biotin functionalized beads. Streptavidin functionalized quantum dots are decorated by up to 8 streptavidin tetrameric units per dot. It is therefore difficult to control the valency with which each dot binds to biotinfunctionalized bead. The problem of unregulated multivalency has been shown to significantly limit the effort to consistently produce discrete molecular assemblies of qdots and beads at each titer of qdots (5). We have previously shown that an intervening layer of site directed biotin labeled antibodies attached to beads circumvents the problem of multivalency by limiting the capture of a single dot to a single antibody in a reproducible and theoretically predictable manner (*see Fig. 1*) (5). The molecular assembly on beads has been well characterized by us in terms of dissociation constants at each junction module (24-29). We have shown that titration of streptavidin functionalized qdot to antibody bearing beads (**Fig. 1B**) can yield discrete quantities of bound qdots in a manner consistent with the weakest equilibrium binding constant between the qdots and the antibody (25,28).

1.3. Principles and Practice: Applicability of Qdot Calibration Beads to Quantitative Flow Cytometry

Although calibration standards such as Quantum™ FITC MESF beads are strictly applicable to fluorescein based probes, it is useful to present a simple correction factor that extends their applicability to other probes operating in the same spectral range. These include most fluorescein conjugates that tend to have lower quantum yields than the parent molecule where simple derivitization of fluorescein typically attenuates the yield by at least 18% [e.g., fluorescein biotin (30)] or greater [e.g., 80% for FITC antibody conjugates (f/p ratio 4:1)]. The correction factor (cf) that is derived from Equation 1 is shown in Equation 2.

$$cf = \frac{(\varepsilon_{488} \phi_{\%T})_{\text{sample}}}{(\varepsilon_{488} \phi_{\%T})_{\text{std}}} \quad (2)$$

Correction factors for several dyes have been calculated in **Table 1**. In this way, Quantum FITC MESF or QD525 Quantum dot beads are shown to be comparable calibration standards for GFP, Alexa 488, BODIPY 505FL dyes, etc. (5). By extension this approach is applicable to all other calibration schemes, as long as standards and samples use the same detectors and excitation source (5). For the readers' conveniences, correction factors for octadecylrhodamine B (R18, a commonly used lipophilic fluorescent dye) as well as rhodamine B to two different batches of QD585 are also listed in **Table 1**.

2. Materials

2.1. Determination of the Relative Emission Yields of Quantum Dots

1. Streptavidin coated quantum dots QD525 and QD585, fluorescein, and rhodamine B (Invitrogen).
2. Quartz cuvettes for absorption and fluorescence measurements.
3. Probes are solubilized in any of the working buffers listed below, in dilute solutions where the optical density is kept below 0.1 at the highest absorption wavelength ($\lambda_{\max}$).
4. Phosphate-buffered saline (PBS) (Mediatech, Inc, Herndon, VA).
5. Tris buffer: 10 mM or 25 mM Tris-HCl, 150 mM NaCl, pH 7.5.
6. HHB buffer: 30 mM HEPES, 110 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 10 mM glucose, pH 7.4.

2.2. Preparation of Qdot-labeled Microbeads

1. Streptavidin coated polystyrene particles (6.7 μ m in diameter, 0.5% w/v) (Spherotech Inc., Libertyville, IL).
2. Streptavidin coated quantum dots QD525 and QD585 (Invitrogen).
3. FLAG peptide (DYKDDDDK), bioM2 anti-FLAG antibody (Sigma, St. Louis, MO). BioM2 antibody contains significant quantities of free biotin and requires purification prior to use (*see Note 1*).
4. Biotinylated FLAG (FLAG^{bio}) and FITC conjugated FLAG peptides (FLAG^{FITC}) synthesized at University of New Mexico (31).
5. PBS, Tris and HHB buffers as described in **Subheading 2.1**.
6. Tris and HHB buffers supplemented with 0.1% bovine serum albumin (BSA).
7. Soluble streptavidin; soluble biotin.

2.3. Equipment

1. Spectrophotometer such as Hitachi model U-3270 (Hitachi, San Jose, CA).

¹Commercial bioM2 is contaminated with high levels of free biotin, which is very likely to block binding of bioM2 to beads. This is removed by using a 100 kilodalton cutoff Microcon Centrifugal Filter Device (Millipore, Billerica, MA).

- a. Assuming a starting material of 500 μ L of anti-FLAG bioM2 antibody (in 50% glycerin, 1 mg/mL). Add to centrifugal device and centrifuge in cold room at 3,000g for 25 min (or till there are less than 50 μ L).
- b. Wash the antibody in 25 mM (or 50 mM) Tris buffer 3X by repeating **Step 1a**. It should be noted that glycerin is very hard to filter through, therefore longer centrifugation time and/or additional washing steps might be needed.
- c. Resuspend the purified and concentrated bioM2 in 100 μ L of 25 mM Tris buffer (5 mg bioM2/mL), make 20 μ L aliquots and keep frozen in -20°C freezer till needed.
- d. Assuming 4×10^{15} molecules of bioM2/mg: each aliquot of bioM2 (0.1 mg) can coat 25 million 6.7 μ m streptavidin bead (1×10^{14} biotin binding sites) with 2X excess of bioM2.

2. Spectrofluorometer such as QuantaMaster™ Model QM-4/2005 (Photon Technology International, Inc., Lawrenceville, NJ).
3. Flow cytometer such as BD FACScan (BD Biosciences, San Jose, CA).

3. Methods

3.1. Determination of the Relative Emission Yields of Quantum Dots

This protocol describes the measurement of relative emission quantum yields of quantum dots. The quantum yield of QD525 is measured relative to fluorescein and QD585 is measured relative to rhodamine B. These dots are selected because their spectral characteristics are most suitable for analysis on a standard BD FACScan. Several factors including size heterogeneity and potential impurities have been shown to cause anomalous dependence of the quantum yield on the excitation wavelength when dots are excited at different wavelengths, in apparent violation of Kasha's rule (32): that the energy and yield of the emission should be independent of the excitation wavelength. It is therefore useful to collect a photoexcitation spectrum of any new batch of quantum dots to determine characteristic emission behavior over the putative spectral range of analysis. The protocol described in this chapter is applicable to measurements made using instruments comparable to the ones utilized here.

1. Measure absorption spectra of the probes following standard procedures (*see Fig. 2A*). The molar absorptivity of all probe solutions should be at or below 0.1. In this limit, errors due to inner filter effects alone are on the order of 10% (7).
2. The slitwidths of the excitation and emission monochromators must both be set at 2 nm. Narrow slitwidths allow for better resolution of size dependent emission of poly disperse sized dots.
3. Use appropriate wavelength cutoff and BP filters for stray light rejection (5).
4. It is useful to make measurements of complete emission spectra of probes by off-resonance excitation (i.e., away from absorption maxima): at 450 nm for the green probes (fluorescein and QD525) and 540 nm for the yellow probes (Rhodamine B and QD585). Make sure that the molar absorptivity of the qdots and standard sample are matched at the excitation wavelengths.
5. Use Equation 3 to calculate the relative quantum yields (ϕ_s) of QD525 and QD585 using fluorescein and rhodamine B as standards.

$$\phi_s = \phi_{ref} \frac{I_s}{I_{ref}} \frac{OD_{ref}}{OD_s} \frac{n_s^2}{n_{ref}^2} \quad (3)$$

Use the integrated intensity of the emission spectra of sample relative to fluorescein ($\phi_{ref} = 0.93$) or rhodamine B [0.31 in water (33)]. I_s and I_{ref} are the integrated band intensities. The optical densities (OD) of the sample (s) and reference (ref) are taken at the excitation wavelength. n is the index of refraction of the solvent; 1.32 for water. For qdots, ϕ_s is accurate for the given lot number and excitation wavelength; therefore, lot number and source should always be identified (6).

6. Measure the emission intensity of all probes exciting at 488 nm (the primary excitation wavelength in a BD FACScan flow cytometer) and calculate relative quantum yields using Equation 3.
7. To collect photoexcitation spectra, set the wavelength for measuring the emission intensity at the peak (e.g., 520 nm for fluorescein) or along the red edge of the emission spectrum (e.g., 540 nm for fluorescein). Collection at the emission peak allows for greater sensitivity while collection along the red edge allows for a broader spectral window.
8. The excitation spectra of fluorescein and rhodamine B are expected to match their corresponding absorption spectra, provided the photoexcitation spectra have been corrected for the spectral distribution (L_λ) of the lamp (7). L_λ depends on the characteristics of the lamp and monochromators. Most modern instruments automatically perform this correction provided it is enabled and properly set up. In cases where a spectrofluorometer instrument lacks the capability to automatically perform a correction of L_λ of excitation spectra, this can be done manually (*see Note 2*). This is an important consideration as significant distortions can be observed in the uncorrected photoexcitation spectrum (*see Figs. 2B and 3*).
9. Photoexcitation spectra of qdots measured relative to standards are useful for determining the quantum yield per unit wavelength of qdots over a desired spectral window (*see Fig 4*).
10. Measure %T. The spectra of BP filters can be obtained from manufacturers' websites. Overlay the measured emission spectra with the appropriate BP filter and the calculated fraction of the emission band that overlaps with the BP filter is %T (*see Fig. 5*).

3.2. General Preparation of Qdot-labeled Microbeads

3.2.1. Preparation of M2 beads—The following protocol is summarized as a schematic in **Fig. 1**. Conceptual details can be found in the original literature (5,34). Much of the work involves the assembly of bioM2 antibodies on beads. The M2 antibodies are critical for achieving an *effective* monovalent (one antibody:one qdot) binding interaction. This is an empirical finding from centrifugation assays in which it has been shown that the number of antibody-captured qdots is equal to the site density of antibodies on the same beads (5,34). Based on earlier studies (5,34-37), this chapter makes the following assumptions about the

²Correcting excitation spectra for spectral distribution of the lamp. The excitation spectra are shown in **Fig. 2B**. Uneven features in spectra are due to the uneven spectral distribution (L_λ) of the lamp (7). To extract (L_λ) easily:

- a. Overlay the normalized absorption spectra and the excitation spectra as shown in **Fig. 3A**. Because emission almost always originates from the lowest vibrational level of the first excited state, the emission yield is independent of excitation wavelength for most organic fluorophores (8,32). Therefore, one expects a resemblance between the normalized absorption and photoexcitation spectra of fluorescein. The excitation lineshape of fluorescein retains the same basic features that mirror the absorption spectrum except for structural artifacts introduced by the uneven spectral distribution of the Xenon arc lamp.
- b. Divide the normalized excitation spectrum column with the normalized absorption spectrum column. The result, L_λ , is shown in **Fig. 3B**.
- c. Divide all of the uncorrected excitation spectra columns with L_λ . The corrected data are shown in **Fig. 3C**.

stock suspensions of 6.7 μm Spherotech beads: 1) There are typically 40,000 beads/ μL ; 2) There are ≈ 4 million saturable bioM2 binding sites on these beads as determined from centrifugation binding assays of a fluorescently labeled FLAG peptide (5,34). Nevertheless, the reader should always verify particle counts by using a hemocytometer or particle counter.

1. Use a pipette to extract the desired volume of beads from a vigorously shaken stock suspension. Centrifuge beads at 14,000g and resuspend in desired buffer (Tris or HHB) containing 0.1% BSA in a minimum volume of 100 μL . To minimize non-specific binding, the beads must be vortexed gently for 30 min to allow 0.1% BSA to coat beads.
2. **Step a in Fig. 1A.** Add bioM2 antibody at the desired stoichiometry: 2 bioM2:1 total streptavidin sites on all beads (*see Note 1*). Vortex or shake for an hour at room temperature.
3. Centrifuge and wash beads twice to remove free bioM2. The two footed binding assures that the bioM2 is tightly bound to beads ($K_d \ll nM$).
4. **Step b in Fig. 1A.** Commercially available biotinylated M2 antibodies have about 3-4 biotin conjugates on the *Fc* domain of the antibody according to manufacturer's data sheet. It is likely that two of the biotin groups are *cis*-bound to the streptavidin on the beads. To prevent the extra biotins from subsequent binding to streptavidin functionalized quantum dots, add a stoichiometric excess of soluble streptavidin to M2 beads, and allow to vortex mildly at room temperature for 15 min. Wash the streptavidin-saturated M2 beads once in "buffer containing 0.1% BSA and centrifuge at 14,000g for 2 min.
5. **Step c in Fig. 1A.** After removal of the supernatant in **Step b**, add 80 μL of 1 mM soluble biotin to the bead pellet, and spin down immediately after brief vortexing. Wash 5 times by repeated centrifugation and removal of supernatant, and finally resuspend in 100 [proportional]L of buffer. Soluble biotin is added in order to saturate excess biotin-binding sites on the bound streptavidin to prevent undesired binding of biotinylated FLAG peptide used to tether qdots to bioM2 (*see steps d and e in Fig. 1*).
6. Depending on the stock of bioM2 beads, aliquot samples into 25 μL volumes (or 1×10^6 beads) for long-term storage (*see Note 3*).

³Qdots and bioM2 antibodies have limited shelf lives. Although Invitrogen Qdots are suspended in a proprietary buffer environment that confers fluorescence, when qdots are diluted in a regular buffer, they gradually lose fluorescence intensity over time. It is therefore important to maintain qdots in this buffer for as long as possible. If calibration beads are not stored in this buffer, then it is important to frequently check the fluorescence intensity of the beads. This can be done by comparing the fluorescence intensity of dots left in regular buffer with a fluorophore of known concentration over time. The reader might also consider making minimal quantities of Qdot beads (**Fig. 1B**). In this way, long term storage of qdots outside their native buffer is avoided. BioM2 antibodies also lose activity - 30% over 6 months (5,35). The activity of bioM2 beads can be assayed using FITC labeled FLAG peptide (31) and commercial (QuantumTM FITC MESF) standard calibration beads to check on the integrity of the bioM2 beads over time. The typical assay involves periodic checking of the surface density of FITC labeled FLAG peptide on bioM2 beads using QuantumTM FITC MESF beads or a centrifugation assay (5). The narrow distribution of histograms (*see Fig. 6*) associated with each titer of qdots mixed with bioM2 beads can be used as an indicator of quality of calibration beads. Broad histograms associated with freshly made calibration beads usually indicate: a) Prevalence of non-specific binding, i.e., **Steps a-c in Fig. 1**. This can be checked by running a negative control, i.e., adding qdots to bioM2 beads without the biotinylated FLAG peptide; b) Heterogeneous binding of qdots due to loss of bioM2 activity.

3.2.2. Preparation of Quantum dot Calibration Beads

1. **Step d in Fig. 1B.** An aliquot of 1×10^6 bioM2 beads is first saturated with 100 nM biotinylated FLAG peptide (FLAG^{bio}) at room temperature for 1 h under mild vortexing, and washed 2 times in Tris-BSA buffer by repeated centrifugation and removal of supernatant. Resuspend in 100 μ L of buffer. It is important to note, that washes of the beads does not confer significant loss, by dissociation, of the peptide as one might expect. In cells or beads, where the density of receptors is relatively high (e.g., $>1 \times 10^5$ receptors/cell), the binding and dissociation of ligands is believed to occur in the “collisional limit” regime (38). In this limit the probability of dissociated ligand rebinding to an adjacent receptor is much higher in terms of normal bimolecular mass action kinetics, where the ligand would normally diffuse away. Therefore, the 8 million peptide binding sites on these beads are expected to show anomalous low loss of ligand (5,36).
2. **Step e in Fig. 1B.** Add 100 nM streptavidin-coated Qdots (final concentration) to the beads, and incubate at room temperature for an hour. The qdots bind to the biotin end of the FLAG peptide. Wash the beads, resuspend them in buffer, and analyze them at the flow cytometer. The attachment of qdots and beads is governed by the bivalent FLAG/M2 interaction $K_d = 2$ nM.

3.3. Discrete Labeling of Quantum Dots Calibration Beads from Mass Action Considerations

This protocol describes the application of mass action to the preparation of beads of known surface coverage. The quantitative display of streptavidin coated Qdots on beads is regulated by the weakest affinity constant in the assembly **Step e in Fig. 1B**. The quantity of bound qdots can be determined from a simple mass action model as shown in Equation 4 where Q or $[Q]_0$, and Ab or $[Ab]_0$ represent the initial concentrations of qdots and bioM2 sites on beads respectively. $[Q]_{\text{free}}$ is unbound qdots at equilibrium. v is the volume of buffer in which beads are suspended, n is the number of beads and A is Avogadro's number.

$$[Q \bullet Ab] = \frac{[Q]_{\text{free}}[Ab]_{\text{free}}}{K_D} \quad (4b)$$

$$qdots/bead = \frac{[Q \bullet Ab] v A}{n} \quad (4c)$$

In keeping with standard practice of generating fluorescence calibration beads, it is desirable to decorate 4 distinct populations of beads with surface concentrations of quantum dots that span a wide range of surface occupancy. For convenience, here we describe the synthesis of a four-component set of fluorescence calibration bead populations decorated with discrete qdots surface densities ranging from 6×10^4 to ≈ 2 million.

Using 1×10^6 bioM2 beads in 100 μL volumes yields 66.0 nM Ab. Use Equation 4b to establish the desired surface densities. The reader might find it useful to realize that, based on mass action considerations, the limiting reagent $[Q]_0$ is depleted by Ab, and in the concentration range of interest, $[Q \cdot Ab] \approx [Q]_0$. The reader can show that the error in this assumption is within 10% when $[Q]_0 = 0.7[Ab]_0$. To establish a good distribution in surface occupancy of qdots, one might consider to space $[Q]_0$ titer intervals at half log, i.e., $[Q]_0 = 1 \text{ nM}, 3 \text{ nM}, 10 \text{ nM}, 30 \text{ nM}$ to yield $[Q \cdot Ab] = 0.97 \text{ nM}, 2.9 \text{ nM}, 9.6 \text{ nM}, 28.0 \text{ nM}$ corresponding to $5.8 \times 10^4, 1.75 \times 10^5, 5.84 \times 10^5, 8.76 \times 10^5, 1.75 \times 10^6$ qdot/bead.

1. Add 25 μL of bioM2 beads (1×10^6 beads in total) to 25 μL of 20 μM FLAG^{bio} and 50 μL of Tris buffer, mildly vortex the mixture at room temperature for 1 h.
2. Wash the beads twice in Tris-BSA buffer by centrifugation and resuspension (at max. speed, 14,500g).
3. Resuspend the beads in *freshly* made (*see Note 3*) qdot solution in Tris-BSA buffer, mildly vortex at room temperature for at least an hour (*see Note 3*).
4. Centrifuge the QD-M2 calibration beads at max speed for 90 sec and remove 60 μL of the supernatant. Check beads on flow cytometer (*see Fig. 6*).
5. Discrete histograms of narrow distribution are a good indicator of a well-regulated assembly (*see Note 3*). Plot mean channel fluorescence (MCF) versus Qdot/Bead. A linear plot ($R^2 = 0.99$) is a good indicator of a well prepared set of calibration standards.
6. Store the beads at 4°C and use for up to one month (*see Note 3*).

3.4. Standardization of Flow Cytometry Data with Fluorescence Calibration Beads: Correction for Spectral Mismatch, Quantum Yield between Sample and Standard Calibration Beads

It is useful to demonstrate the applicability of qdot calibration beads relative to QuantumTM FITC MESF calibration beads. This is achieved through Equation 2, which establishes a correction factor that is used to account for the differences in the measurement parameters, ϵ , ϕ , and %T, between any given probe and the calibration standard. Correction factors for green wavelength fluorophores commonly used in our work are shown in **Table 1**.

3.5. Quantitative Measurement of Qdot Labeled Virus Particles Bound to Cells using Flow Cytometry and TEM

Virus particles are amenable for tagging with qdots. Once labeled, the degree of labeling can be resolved by transmission electron microscopy (TEM). In this way flow cytometry can be used to quantify bound viruses. Human papillomaviruses (HPVs) are etiologic agents of a number of benign and malignant tumors of the skin and mucosa (39). We have recently analyzed the binding of QD585 labeled HPV particles to A431 cells by flow cytometry. QD585 calibration beads were used to measure the total number of qdots per cell ($\approx 12,000$ QD585 per cell, *see ref. 5*) using Equation 1. In previously unpublished data from that study, TEM was used to estimate the number of qdots per virus particle, as shown in **Fig. 7**.

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Fig. 1. Summary of the molecular assembly of quantum dot fluorescence calibration beads on streptavidin coated beads. **(a)** Addition of biotinylated M2 antiFLAG antibodies (bioM2) to 6.7 μm beads. **(b)** Elimination of free biotin sites with soluble streptavidin prevents direct binding by of qdots. **(c)** Biotin is used to block streptavidin-biotinylated FLAG peptide interactions. **(d)** Addition and capture of biotinylated FLAG peptides by bioM2 antibodies. **(e)** Addition and capture of streptavidin qdots with biotinylated FLAG peptides tethered to bioM2.

Fig. 2.

Comparison of absorbance (**A**), and uncorrected excitation spectra (**B**) of quantum dots and fluorescein: (a) Fluorescein, (b) QD525 lot# 1005-0045, and (c) QD585 lot# 0905-0031. The samples' absorbances were matched at 488 nm by design. (**Fig. 2A** is reprinted from *Analytical Biochemistry*, Vol 364, Wu, Y., Lopez, G. P., Sklar, L. A. and Buranda, T. Spectroscopic characterization of streptavidin functionalized quantum dots. 193-203, (2007) with permission from Elsevier).

Fig. 3.

Correcting excitation spectra for the lamp's spectral distribution (L_λ). **(A)** Overlay of fluorescein's uncorrected excitation spectra with the absorption spectrum. **(B)** Intensity versus wavelength plots of: (a) the spectral distribution of the Xenon arc lamp used for sample excitation. The lamp profile was derived from **(A)**, by dividing the L_λ -uncorrected emission spectrum of fluorescein by its absorption spectrum; (b) L_λ -uncorrected excitation spectrum of QD525; (c) L_λ -QD525 excitation corrected spectrum. **(C)** Overlay of L_λ -corrected spectra of (a) fluorescein, (b) QD525, and (c) QD585. (**Fig. 3C** is reprinted from *Analytical Biochemistry*, Vol 364, Wu, Y., Lopez, G. P., Sklar, L. A. and Buranda, T. Spectroscopic characterization of streptavidin functionalized quantum dots. 193-203, (2007) with permission from Elsevier).

Fig. 4.

Plot of quantum yields of (a) fluorescein, (b) QD525 lot# 1005-0045, (c) QD585 lot# 0905-0031, and (d) QD605 versus excitation wavelength. The quantum yields were calculated as described Equation. 3. The lines are data derived from excitation spectra while data points represent data derived from integrated intensity. (Reprinted from *Analytical Biochemistry*, Vol 364, Wu, Y., Lopez, G. P., Sklar, L. A. and Buranda, T. Spectroscopic characterization of streptavidin functionalized quantum dots. 193-203, (2007) with permission from Elsevier).

Fig. 5.

Normalized emission spectra of fluorescein, rhodamine B and quantum dots. Intensity maxima of, QD525 lot# 1005-0045 and QD585 lot# 0905-0031 correspond to their quantum yields relative to fluorescein and rhodamine B (33). Dark and light (green and orange in the electronic version) bars represent BP filters used in a standard flow cytometer (530/30 BP for the FL1 channel, and 585/42 BP for the FL2 channel). The resonance overlap between the BP filters and emission spectra regulates the amount of light that is transmitted or rejected by the BP filter: 28% of fluorescein emission is transmitted to the FL1 channel compared to 38% of QD525, and 65% of QD585 emission is transmitted to the FL2 channel. (Reprinted from *Analytical Biochemistry*, Vol 364, Wu, Y., Lopez, G. P., Sklar, L. A. and Buranda, T. Spectroscopic characterization of streptavidin functionalized quantum dots. 193-203, (2007) with permission from Elsevier).

Fig. 6. (A) Flow cytometry histograms of calibration beads bearing a wide range of site densities of QD585 as a result of mixing 1 million bioM2beads with 0, 1 nM, 3 nM, 10 nM, and 30 nM QD585 dots (*see Subheading 3.3.*). (B) Linear plot of Qdot sites on calibration beads versus the corresponding mean channel fluorescence (MCF) reading. (Reprinted from Analytical Biochemistry, Vol 364, Wu, Y., Campos, S. K., Lopez, G. P., Ozbun, M. A., Sklar, L. A., and Buranda, T. The development of quantum dot calibration beads and quantitative multicolor bioassays in flow cytometry and microscopy. 180-192, (2007) with permission from Elsevier).

Fig. 7.

Simultaneous two-color measurement of eGFP/Qdot 585 nm (QD585) double-stained Human papillomavirus (HPV) particles on A431 cells using flow cytometry and microscopy. **(A)** Flow cytometry measurement of GFP fluorescence. Solid histogram (MCF ≈ 100) corresponds to cells bearing HPV particles while open histogram (MCF ≈ 50) represents negative control cells with no virus. **(B)** Fluorescence measurement of QD585. Solid histogram (MCF ≈ 1100) represents cells bearing HPV particles stained with QD585, and open histogram (MCF ≈ 100) represents negative control cells (no virus). **(C)** Confocal images of pseudovirion (PsV)-bearing A431 cells prepared under similar conditions to flow cytometry measurements as in **(A)**. **(D)** Confocal images of the same cells after 5 scans show the near complete photobleaching of eGFP. **(E)** TEM micrograph of QD585 dots on transparent biotinylated PsV particles, delineated by a random honeycomb grid scaled to fit the size of 60 nm PsV. Insert shows PsV negatively stained with uranyl acetate. The bar scale is 100 nm for both micrographs. **(F)** Frequency distribution histogram of Qdots/PsV-mean: 11 ± 5 . **(Fig. 7 A-D)** is reprinted from *Analytical Biochemistry*, Vol 364, Wu, Y., Campos, S. K., Lopez, G. P., Ozbun, M. A., Sklar, L. A., and Buranda, T. The development of quantum dot calibration beads and quantitative multicolor bioassays in flow cytometry and microscopy. 180-192, (2007) with permission from Elsevier).

Table 1

Some Correction Factors for “Fluorescein-like” Green Fluorophores

Probe	$\epsilon(\lambda_{\text{ex},488\text{nm}})^1$	ϕ^2	%T ³	cf(MESF) ⁴	cf(QD525) ⁵	cf(QD585a/QD585b) ⁶
Fluorescein (MESF beads)	78,000	0.93	28.	1	1.37	-
Fluorescein biotin	72,600	0.74	28	0.75	1.02	-
FLAG ^{FTTC}	72,600	0.15	28	0.16	0.21	-
BODIPY fl	44,000	0.9	20	0.40	0.53	-
EGFP	56,000	0.6	40	0.60	0.91	-
EGF ^{FTTC}	72,600	0.45	28	0.46	0.61	-
QD525	130,000	0.30	38	0.73	1	-
QD585a (Lot 4526A)	530,000	0.12	65	-	-	1/0.4
QD585b (Lot 49987A)	530,000	0.3	65	-	-	2.5/1
R18	4,750	0.3	51	-	-	0.017/0.007
Rhodamine B	13,000	0.31	51	-	-	0.046/0.019

¹ Extinction coefficient of probe at excitation wavelength.² Quantum yield measured relative to fluorescein.³ %T is the percent fraction of emitted light that is transmitted by the bandpass (BP) filter at detector (see **Fig. 5**). The BP filters are those on a standard model BD FACScan flow cytometer. The maximum transmittance of 80% by the BP filter is assumed for all samples. Spectral mismatch between fluorescein and derivatives is assumed to be negligible in this table.⁴ Correction factor $cf = \frac{(\epsilon_{488} \phi \% T)_{\text{sample}}}{(\epsilon_{488} \phi \% T)_{\text{standard}}}$ needed to normalize data to spectral characteristics of standard beads (e.g., cf × MESF value).⁵ Correction factor needed to normalize data to spectral characteristics of QD525 (Invitrogen lot# 1005-0045).⁶ Correction factors needed to normalize data to spectral characteristics of QD585 from two different batches (Invitrogen lot# 4526A and 49987A). These latter batches were purchased after QD585 lot# 0905-0031 was used up.