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The Development of Quantum Dot Calibration Beads and Quantitative Multicolor Bioassays in Flow Cytometry and Microscopy¹

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Abstract

The use of fluorescence calibration beads has been the hallmark of quantitative flow cytometry. It has enabled the direct comparison of inter-laboratory data as well as quality control in clinical flow cytometry. In this paper we have described a simple method for producing color-generalizable calibration beads based on streptavidin functionalized quantum dots. Based on their broad absorption spectra and relatively narrow emission, that is tunable on the basis of dot-size, quantum dot calibration beads can be made for any fluorophore that matches their emission color. In an earlier publication (1) we characterized the spectroscopic properties of commercial streptavidin functionalized dots (Invitrogen). Here we describe the molecular assembly of these dots on biotinylated beads. The law of mass action is used to readily define the site densities of the dots on the beads. The applicability of these beads is tested against the industry standard, commercial fluorescein calibration beads. The utility of the calibration beads are also herein extended to the characterization surface densities of dot-labeled epidermal growth factor ligands as well as quantitative indicators of the binding of dot-labeled virus particles to cells.

Keywords

nanotechnology; quantum dots; calibration beads; flow cytometer; quantitation; fluorescence; multiplex; bioassays; virus particles; microscopy; spectroscopy; mass action

Introduction

Quantum dots are fluorescent semiconductor nanoparticles with tunable optical properties. They have very high absorption cross-sections and narrow emission bandwidths, relative to fluorescent dyes, that make them highly suitable for use as reporters for multiplexing assays

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on cells or beads(2). There has been growing interest in the use of quantum dots as fluorescent tags in biology(2,3). One popular method for attaching dots to biological molecules involves the use of streptavidin conjugated quantum dots that can be readily attached to biotinylated targets (4-8). Generally there are 8-10 streptavidin molecules per quantum dot, and the size is 10-15nm in diameter, which is similar to the size of an antibody (~14 nm) or a large protein (i.e. R-PE, 240 kDa, ~20nm) but much bigger than organic dyes (<3 nm) and small fluorescent proteins (i.e. GFP, ~5 nm)(2).

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(9-12). The utility of these standard beads is based on the correlation between the fluorescence intensity of a solution of known concentration and the intensity of a suspension of beads bearing the same fluorophores of indeterminate surface density. The equivalence of fluorescence radiance of the beads to the solution is known as the Molecules of Equivalent Soluble Fluorophores (MESF) where the MESF value is equal to the known number of molecules in solution (9,10). To the best of our knowledge the only commercially available sets of calibration beads are based on fluorescein, cyanine-5 (cy5) and phycoerythrin (PE) available from BangsLabs, Fiskers, IN) and EGFP from BD Biosciences.

There is a clear need for a generalizable approach to quantifying fluorophore sites on cells or molecular assemblies on beads. In this paper we have described a simple method for producing calibration beads based on quantum dots. Calibration beads can be made for any fluorophore, under the operational logic that the fluorescence intensity of any given molecule is proportional to $I_0 \epsilon \phi$, where I_0 is the *non-saturating* intensity of the light source. Irradiation of samples with saturating light intensities increases the potential for undesirable nonlinear effects on emission radiance as well as photobleaching of molecular probes(13-15). ϵ is the absorption coefficient and ϕ is the quantum yield of the fluorophore. Calibration standards can then work on the measurement model based on Equation 1 where subscripts 1 and 2 refer to the analyte and standard, respectively.

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

In the Equation, I_i are the respective intensities of the sample and standard beads excited by the same light source (I_0); ρ_i are the site densities of the fluorophores on the (1) sample bead and the calibration bead; %T is the percent fraction of fluorescence light transmitted through the bandpass filter and is used to account for the spectral mismatch between the sample fluorophore and the standard. The broad absorption continuum spectra, and tunability of emission energy suggests that a dot of a suitable color can be used as a calibration reporter standard on beads for a chosen fluorophore, provided all the spectroscopic parameters are known. This study is preceded by a systematic examination of some spectroscopic characteristics of commercially available quantum dots QD525, (Lot #1005-0045) and QD585, (Lot #0905-0031) (1). In that study, we determined the emission yields of the quantum dot samples over a wide range of excitation wavelengths (350-500 nm). We observed an anomalous dependence of quantum yields on the excitation wavelengths especially at wavelengths shorter than 420nm. This behavior is consistent with published literature on dots (16,17). Photo excitation of QD585 in the wavelength window between 420 and 500 nm yielded, on average, a uniform quantum efficiency of ~0.20. Within the same spectral window, anomalies associated with the QD525 photoluminescence spectra were more pronounced as the quantum yield varied from a high of ~0.4 at 450 nm to a low of 0.2 at 480 nm. Size heterogeneity in the dots was believed to be a significant contributor to the variation in quantum yield(1). The data from that work are included in Table 1. We carefully noted (1), from our reading of the

literature, that the behavior of the dots, unlike dye molecules, is susceptible to batch-to-batch variations and therefore the data shown in Table 1 is strictly characteristic of the samples belonging to the given lot numbers: i.e. QD525, (lot#1005-0045) and QD585, (Lot#0905-0031).

Here, we have extended our earlier work and describe a simple method for the assembly and characterization of calibration beads based on the characterized quantum dots. We show that they can be used to determine the surface coverage of fluorophores, including quantum dot-tagged molecular assemblies on beads or cells by flow cytometry. Our calibration beads based on QD525 dots yielded comparable results to Bangs Labs' QuantumTM FITC commercial beads. For the analysis of quantum dot labeled assemblies, we based our assays on the QD585 dots. We chose QD585 dots because of optimal spectroscopic characteristics for sensitive detection by our standard flow cytometer. We produced calibration beads of variable surface coverage spanning a range of a few hundred, up to 4 million dots per bead. We tested the applicability of the calibration beads on two ligand-receptor systems of interest in the study of several forms of cancer. First by measuring the surface density of QD585 dots attached to the ligand of the epidermal growth factor receptor (EGFR) on A431 cells. EGFR has been implicated in several forms of cancer as a result of mutations involving the over-expression or constant activation of EGFR(18). Second we established a conceptual framework for quantitative determination of the rate of viral entry into cells by using quantum dot labeled human pseudo-papillomavirus (HPV) particles. Human papillomavirus have been identified as mediators of a number of benign and malignant cancers of the skin and mucosa(19,20).

MATERIALS AND METHODS

Materials

Streptavidin coated and biotin coated polystyrene particles (6.7 μ m in diameter, 0.5% w/v) were purchased from Spherotech Inc. (Libertyville, IL). Streptavidin coated quantum dots QD525, QD585, QD605 were purchased from Invitrogen Corp (Carlsbad, CA). FLAG peptide (DYKDDDDK), M2 anti-FLAG antibody and paraformaldehyde (PFA), were purchased from Sigma (St. Louis, MO). Phosphate-buffered saline (PBS) was purchased from Mediatech, Inc, Herndon, VA). Biotinylated FLAG (FLAG^{bio}) and FITC conjugated FLAG peptides (FLAG^{FITC}) were synthesized at UNM as described elsewhere(21). TRIS (10 mM or 25 mM Tris, 150 mM NaCl, pH7.5) and HHB (30 mM HEPES, 110 mM NaCl, 10 mM KCl, 1 mM MgCl₂·6H₂O and 10 mM glucose, pH7.4) buffer were used in the presence or absence of 0.1% bovine serum albumin (BSA).

Cell Culture

A431 cells (ATCC) and HaCaT cells (a gift of N. Fusenig, DKFZ, Heidelberg) were maintained in sterile filtered Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated Fetal Bovine Serum (FBS), 100 units/mL penicillin, 100 μ g/mL streptomycin, 10mM HEPES, pH7.4, 20 μ g/mL ciprofloxacin, 2 mM L-glutamine, at 37°C in a CO₂ water jacketed incubator of 5% CO₂ and 95% air (Forma Scientific, Marietta, OH45750) in T75 tissue culture flasks or petri dishes (Greiner Bio-One GmbH, Frickenhausen, Germany). Cells were typically starved for 18 to 24 hrs in serum free DMEM before experiments.

Production of Biotinylated GFP labeled HPV16 PsV particles

HPV16 pseudovirion (PsV) particles were produced as described previously (22-24). Briefly, 293T cells were transfected with pXULL, an human papillomavirus type 16 (HPV16) L1/L2 capsid protein expression plasmid (a gift of J. Schiller, NIH) and a "pseudogenome" reporter plasmid encoding a histone H2B-GFP fusion (a gift of H. Kimura, Tokyo Medical and Dental University) (25). Cells were harvested 48 hours post-transfection, and incubated in lysis buffer

(PBS plus 9.5 mM NaCl, 0.25% Brij58, 0.3% benzonase, and [0.02 U/ μ l] exonuclease V) for 24 hours at 37° C. PsV particles were purified by CsCl density gradient centrifugation and dialyzed and in storage buffer (25 mM HEPES pH 7.0, 500 mM NaCl, 0.02% Brij58, 1 mM MgCl₂, 0.1 mM EDTA, 0.5% ethanol). Purity of the preparation was confirmed by SDS-PAGE and Coomassie staining and by negative stain transmission electron microscopy (TEM). Titer of the prep was determined by dot blot to quantify the number of nuclease resistant pseudogenomes as previously described (19) and is expressed in viral genome equivalents (vge). PsVs produced in this manner encapsidate the H2B-GFP fusion protein and can be directly visualized by laser scanning confocal microscopy (Campos and Ozbun, unpublished observations).

Biotinylation of PsV

8.3×10^8 vge of PsV was chemically biotinylated for 1 hour at room temperature by the addition of 1 μ g amine reactive N-hydroxysuccinimide ester biotin (NHS-biotin, Pierce) in 50 μ l storage buffer. Excess NHS-biotin was quenched by the addition of 450 μ l PBS + 50 mM glycine and the sample was concentrated to 100 μ l in storage buffer and frozen at -80 °C. Final concentration was 8.3×10^6 vge/ μ l.

Determination of the relative emission yields of quantum dots

Absorption and spectrofluorometric measurements were performed using a Hitachi model U-3270 spectrophotometer (San Jose, CA) and a Photon Technology International QuantaMaster™ Model QM-4/2005 spectrofluorometer (Lawrenceville, NJ) respectively. QD525, QD585, QD605, fluorescein, FITC biotin, FITC labeled FLAG peptide (FLAG^{FITC}) and Rhodamine B solutions were prepared in PBS (pH 7.4 or pH 8.0). The optical densities of all the samples were matched at either 405nm or 488nm. Excitation and emission spectra were collected for all samples. Fluorescence intensity data were collected at each fluorophore's emission maxima using appropriate bandpass filters (Corion Corp., Holliston, MA; CVI Laser, Albuquerque, NM).

Preparation of Qdot-labeled Microbeads

Biotinylated M2 anti-FLAG antibody coated streptavidin beads (M2 beads) were prepared as previously described (21). 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 block extra biotins from binding to added quantum dots, twenty microliters of 140 nM soluble streptavidin was added to 1×10^6 M2 beads, and the mixture was allowed to vortex mildly at room temperature for 15 minutes. The streptavidin-saturated M2 beads were then washed once in 25mM Tris-BSA, and centrifuged at $14,000 \times g$ for 2 minutes. After removal of the supernatant, 80 μ L of 1mM biotin was added to the bead pellet, and spun down immediately after brief vortexing in order to saturate excess biotin-binding sites on the bound streptavidin. The beads were washed 5 times by repeated centrifugation and removal of supernatant, and finally resuspended in 100 μ L before the addition of Qdots. Two interchangeable procedures were used to prepare Qdot-labeled M2 beads using a biotinylated FLAG peptide. An aliquot of 1×10^6 M2 beads was first saturated with 100nM biotinylated FLAG peptide (FLAG^{bio}) at room temperature for 1 hr under mild vortexing, and washed 3 times in Tris-BSA buffer by repeated centrifugation and removal of supernatant. After that, streptavidin coated Qdots were added to the beads, and incubated at room temperature for an hour. The beads were then washed and resuspended in Tris buffer, and analyzed of the flow cytometer. Alternatively, a 1 μ M sample of Qdots (32-40 μ M streptavidin sites) was mixed with 20 μ M aliquot of FLAG^{bio} and incubated in a 50 μ L volume for one hour at room temperature. The FLAG^{bio}-QD complex was then added to M2 beads and incubated for an hour before the beads were washed 3 and

analyzed. For negative control samples, the FLAG^{bio} peptide was excluded from the preparation.

For samples using biotin functionalized beads, biotin coated beads were simply mixed with the Qdots and incubated for 1hr at room temperature under mild vortexing, washed 3 times in Tris-BSA. For blocked samples, biotin coated beads were exposed to Qdot solutions that were pre blocked with soluble biotin.

Spectrofluorometric Centrifugation Assay

Spectrofluorimetric measurements were performed in single photon counting mode. The samples were excited at either 420 nm or 488 nm, with a 10 nm band pass interference filter (Corion Corp., Holliston, MA) used for line narrowing and stray light rejection. Fluorescence emission was monitored at 520 nm via a long-pass band filter (3-70 Kopp Glass, Pittsburgh, PA) and a 520 nm (10 nm bandpass) filter (Corion Corp). Neutral density filters were used to keep light intensities of the brightest samples within the dynamic range of the phototube. Samples containing 1×10^5 M2 beads or 2×10^5 biotin beads were incubated in 250 μ L of 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 nM QD585 solution in Eppendorf tubes for an hour at room temperature. The samples were then centrifuged and the residual supernatants were collected in cylindrical glass cuvettes (Sienco, Inc., Arvada, CO) for fluorescence intensity measurements. The beads were resuspended in buffer and used for flow cytometry analysis.

Equation 2a was used to determine the concentration of Qdots bound to beads ($[L]_b$). I_0 and I_r are the *background corrected* fluorescent intensities of Qdots before and after exposure to bead suspensions respectively, and $[L]_0$ is the initial concentration of Qdots.

$$[L]_b = \frac{I_0 - I_r}{I_0} \cdot [L]_0 \quad (2a)$$

$$\text{Sites} = \frac{[L]_b \cdot A}{n} \quad (2b)$$

The number of Qdots/bead was determined from Equation 2b, where A represents Avogadro's number and n is the number of beads per liter.

Flow Cytometry

Flow cytometric measurements were performed on a Becton-Dickinson FACScan flow cytometer (Sunnyvale, CA) interfaced to a G4 Macintosh using the CellQuest software package. The FACScan is equipped with a 15 mW air-cooled argon ion laser. The laser output is fixed at 488 nm. In general bead samples from the centrifugation assays were analyzed as previously described (26).

EGF and dot labeling of A431 cells

Cells were typically starved for 18-24 hrs in serum free DMEM before experiments. To remove the adherent cells from the flask, cells were rinsed once and allowed to soak in sterile 0.526 mM EDTA solution (Ca^{2+} and Mg^{2+} salt free, Irvine Scientific, Santa Ana, CA92605) at 37°C for up to 10 mins. Cells were then transferred into HHB buffer, and kept in suspension for subsequent labeling and analysis. Quantum dot labeled EGF ligands were formed by mixing biotinylated EGF (EGF^{bio}) with QD585 in PBS for 30mins at 7°C under mild vortexing. Experiments were performed at several EGF^{bio}/QD585 molar ratios, viz: 1:1, 4:1 and 12:1. For binding data, QD585-EGF^{bio} complexes ranging in concentration from 1 pM to 300 nM were added to A431 cell suspensions (20,000 cells per sample), and the cells were allowed to incubate either at 7°C for 1 to 5 hours under mild vortexing. Blocked samples were prepared by pre-incubating cells in buffers containing 0.1% BSA before exposure to QD585 (not

conjugated with EGF^{bio}). 140 μ L of HHB/HSA were added to cell suspensions immediately before flow cytometric analysis.

Fluorescein labeled EGF ligand were purchased from Invitrogen, Corp, and used without further purification. Fifty thousand suspension A431 cells were incubated in 20 μ L of 1 nM to 300 nM EGF^{fl} solution for an hour at 7°C under mild vortexing. Blocked samples were prepared by pre-incubation of A431 cells with at least 20 times excess of unlabeled EGF ligand (400 nM to 6 μ M) at 7°C for an hour before exposure to fluorescein EGF. 130 μ L of HHB/HSA buffer were added to the cell suspension immediately before flow cytometry analysis.

Binding of HPV16 PsV to A431 Cells

Adherent cells were removed and resuspended in HHB buffer as described above. Samples containing 100,000 cells in 100 μ L HHB buffer were mixed with 1×10^9 HPV16 PsV particles (1000 PsV/cell) at 37°C for 30mins. The cells were then washed once in HHB buffer containing 0.1% HSA before exposure to 1nM QD585 solution in HHB. After incubation at 37°C for 30 mins, the cells were washed and analyzed.

Confocal Microscopy Analysis

Pre-starved A431 cells were resuspended in HHB-BSA buffer ($3-5 \times 10^6$ cells/ml). For fixed cell measurements, 100 μ L of A431 cells were either labeled with EGF-QD585 or QD585/GFP-double tagged HPV16 PsV particles, and then added to 100 μ L freshly prepared cold 4% PFA in PBS. After fixing the cells in PFA for 30min at 0°C, the cells were washed twice in HHB-BSA buffer and spun down at $4,000 \times rpm$. The cell pellet was resuspended in 10 μ L Vectashield mounting medium (Vector Lab. Inc., Burlingame, CA) and mounted on a regular microscope slide. Confocal laser scanning microscopy was performed with a BioRad or Zeiss LSM 510 systems using a 60 $\times$ or 63 $\times$ 1.4 oil immersion objectives.

RESULTS AND DISCUSSION

The fluorescence signal measured by flow cytometry is usually expressed in arbitrary “mean channel fluorescence” (MCF) units that are dependent on the instrument settings used in taking the measurement. Running a sample of commercially available standard calibration beads in flow cytometry experiments allows normalization of multiple data sets, even if acquired with different detector settings or on different instruments (9,10). The utility of calibration beads lies in the application of the concept of MESF (9,10). MESF values rely on the equivalency of fluorescence intensity between a suspension of fluorophore bearing-beads and soluble fluorophores of the same species. The assignment of MESF values to a set of beads with a range of fluorophore intensities produces a calibration curve. The MESF values are based on the fluorescence properties of a native fluorophore, *e.g.* QuantumTM FITC beads are based on the properties of a NIST-calibrated fluorescein solution. Thus in order to be used accurately, the end user must have to account for the inevitable changes in quantum yields and spectral mismatches that occur when fluorescein is functionalized into a fluorescent tag (21,26).

In this study we have developed a simple assay for producing quantum dot calibration beads. The method relies on commercially available reagents, streptavidin functionalized beads, streptavidin-functionalized quantum dots and the law of mass action. Because of mass action it is possible to readily produce standard calibration beads whose site density of quantum dots span the range from few hundred dots/bead, up to 4 million. This flexibility allows the experimentalist to readily produce calibration beads with site densities of dots that are within a reasonable range of the samples under investigation. We demonstrate the utility of the beads: first in a comparative scheme to Bangslabs' QuantumTM FITC calibration beads; second in a quantitative determination of epidermal growth factor (EGF) receptor sites on A431 cells, as

well as in the visualization and quantification of HPV16 PsV particles tagged with Qdots on A431 cells.

Calibration and Application of QD585 labeled beads

We began this work by using two types of molecular assemblies to produce calibration beads, namely M2 beads and biotin beads. We intended to crosscheck the utility of one platform against the other, and eventually base the application solely on biotin beads based on the reasonable expectation that the latter beads would be generally easier to produce. The biotin beads turned out to be unsuitable because the valency of the dots (up to 8 streptavidin tetrameric units per dot) is unregulated. Because our analysis relies on mass action considerations, this problem was intractable. In addition the flow cytometry histograms were inconsistent. The molecular assembly, based on M2 beads circumvents the problem of multivalency by limiting the capture of a single dot to a single antibody in a reproducible and experimentally verifiable manner. The limiting stoichiometry is then simply reduced to the 1:1 interaction, between a known quantity of antibody binding sites on a known number of beads with a suspension of dots of known concentration (see schematic in Figure 1A). Conceptually, the premise of a limiting 1:1 M2:qdot binding stoichiometry is favored by the notion that the M2 antibodies and qdots are of similar size (10-15nm), thus one would expect steric hindrance to limit the binding stoichiometry. This notion is supported by experimental data as described below.

M2 Beads

For several years we have pursued methodologies concerned with the development of molecular assemblies suitable for quantitative analysis by flow cytometry. Through this effort we developed M2 antiFLAG-bearing beads as a platform to display and analyze, in quantitative fashion, FLAG epitope-tagged proteins on beads (21,27-31). From our previous work, we have shown that 6.7 micron streptavidin coated beads (Spherotech) can bear about 4 million biotinylated antibodies at saturation, when prepared under our experimental conditions (21, 30). The M2 antiFLAG antibody binds to the FLAG (DYKDDDDK) epitope-derived peptide with an affinity constant of ≈ 8 nM. In the absence of a competitor, this molecular assembly is very robust due to facile rebinding of the ligand (32-34), and remains wholly stable for days ((27)*vide infra*). Qdots were tethered to the beads using biotinylated FLAG peptides (Figure 1A). The results of a centrifugation assay are shown in Figure 1B where the site density of the quantum dots was derived from Equation 2. The maximum site coverage for QD585 was determined to be ≈ 4 million dots per bead. This value matches the site density of the antiFLAG M2 antibodies on each bead. This finding is consistent with the notion that the binding of quantum dots to M2 is most likely a 1:1 binding event (Figure 1A). The affinity constant of ≈ 2 nM derived from the centrifugation assay and the parallel flow cytometric analysis of binding data on the beads (Figure 1C) is higher than that (≈ 8 nM) which was measured for monovalent interactions between M2 and soluble peptides (21).

A simple law of mass action-based approach to producing calibration beads of known surface coverage

The quantitative display of streptavidin coated Qdots on beads is regulated by the weakest affinity constant in the assembly. Thus, for a given stoichiometry of quantum dots and beads the limiting site coverage of Qdots on the M2 beads depends on the M2/FLAG affinity constant (2 nM). Conceptually, the assembly of the calibration beads is based on simple mass action considerations as shown in Equation 3; where Q_0 and Q_{free} represent the initial and residual dot concentrations respectively; Ab , Ab_{total} represent the maximum concentration of saturable M2 sites on beads. Because a single dot occupies the bivalent M2 sites, "M2 sites" refers to the whole antibody in this case. Because the initial conditions are known $[Q]_{\text{total}}$ and K_d for

the M2 beads (35) and dots, a computer simulation using Equation 3b can be used as a template to produce a series of beads with the desired site densities of Qdots.

$$[Q]_0 - [Q]_{\text{free}} = [QAb] = \frac{[Ab] \times [Q]_{\text{free}}}{[Q]_{\text{free}} + K_D} \quad (3b)$$

$$\text{Sites} = \frac{[QAb] \times A}{n} \quad (3c)$$

In practice, M2 antiFLAG antibodies tend to lose activity over time. Therefore one would like to keep track of the changes in the FLAG peptide/M2 interaction. We have used FITC labeled FLAG peptide(21) and commercial (Quantum™ FITC MESF) standard calibration beads to periodically check on the integrity of the M2 beads over time. During the course of this work we noted a 30% drop in the activity of the M2 antibodies over a period of 6 months.

Spectroscopic Characteristics of Qdots: Sensitivity and detection limits

Our effort to develop calibration beads began with a study to characterize some spectroscopic properties of streptavidin functionalized quantum dots (1). An important finding from that work was the relatively low quantum yields of the Qdots relative to fluorescein. A significant point to be made from these results was the apparent fall in quantum yields at shorter wavelengths, thus neutralizing the potential gain in sensitivity one would hope to get by exciting a sample in the spectral region with the highest absorption cross-section. The results are summarized in Table 1. It is however worth noting that while the Qdots' low quantum yields negate the obvious advantage of large absorption cross-section their other advantages over organic dyes are realized in their large Stokes shifts and relatively narrow emission bandwidths. Typical organic dyes have overlapping excitation and emission spectra with very small Stokes Shifts (*e.g.* 24 nm for fluorescein). In order to avoid spillover of excitation light, bandpass filters are designed such that they have discrete narrow bands of transmission that tend to exclude a large cross-section of the emission spectrum (Figure 2). Because Qdots have relatively much larger Stokes shifts (up to >100 nm depending on excitation wavelength) and narrow bandwidths, emission filters can be designed to capture a large percentage of their integrated emission. As shown in Figure 2, the fluorescein spectrum has broad and asymmetric tails, to the extent that 6.4% of the red edge of fluorescein's emission overlaps with FL2 bandpass. In comparison, only 0.6% of QD525 emission has the potential to bleed into FL2. Recent advances in polychromatic flow cytometry have resulted in the design and implementation of instruments capable of measuring seventeen colors (36). However, because of spectral overlap between fluorescent dyes, spillover of light between detectors inhibits the simultaneous use of all the detectors (36). The relatively narrow band emission spectra associated with quantum dots are clearly more amenable for use in multicolor flow cytometry.

Using a flow cytometer to determine the relative detection sensitivity of a prototypical fluorescein labeled-ligand, fluorescein biotin, and the dot samples QD525 and QD585, we prepared samples of beads covered with fluorophores of known surface density, ranging from tens to thousands of fluorophores. The detection sensitivity (signal to noise ratio of 3:1) on our 15-year-old BD flow cytometer for fluorescein biotin was 5630 molecules/bead, for QD525 was 4100 units/bead and was 120 units for QD585. Details of the quantification are given in the notes to Table 1. Because the experimental measure of QD525 dots/bead and fluorescein biotin molecules/bead, was the product of the same 15mW laser excitation and detection in the

same spectral range (i.e. same bandpass filter), we could test the applicability of Equation 1. We have restated Equation 1 as Equation 4 to reflect the identity of the two fluorophores.

$$\frac{\rho_{\text{QD}}}{\rho_{\text{fl}}} = \frac{\epsilon_{488,\text{QD}}}{\epsilon_{488,\text{fl}}} \cdot \frac{\varphi_{\text{QD}}}{\varphi_{\text{fl}}} \cdot \frac{\%T_{\text{QD}}}{\%T_{\text{fl}}} \cdot \frac{\text{MCF}_{\text{QD}}}{\text{MCF}_{\text{fl}}} \quad (4)$$

We then calculated the ratio of the product of spectroscopic parameters (ϵ , φ , and $\%T$) of QD525 and fluorescein biotin as listed in Table 1. The MCF values were the same for the two samples. Therefore we calculated the number of QD525 units/bead (ρ_{QD}) by taking the product of the number of fluorescein biotin molecules/bead ($\rho_{\text{fl}}=5646$ in Table 1) and the spectroscopic parameter ratio (≈ 0.76); we obtained 4290. This value was within 4% of the 4140 units/bead estimated from mass action as listed in Table 1. The close agreement between independent semi-theoretical expectations (Equations 3 and 4) can be viewed as a performance validation of our quantum dot calibration beads measured against the industry yardstick QuantumTM FITC MESF beads. It is useful to note that workers at the National Institute of Standards and Technology (NIST) have published a detailed characterization of the applicability of the MESF beads in quantitative flow cytometry (9,10). A potential source of systematic error, which we have not seriously considered here, is the error introduced by the dichroic filter as a result of spectral mismatch between the emission spectrum of the sample (fluorescein biotin) and the spectrum of the standard calibration beads or our QD525 beads. This error can be as high as 18% for a spectral shift of up to 15nm, and it arises from changes in the transmission efficiency of the dichroic filter in a model flow cytometer in the 515-545 wavelength range delimited by the 530 bandpass filter (9,10). It is also instructive to note that the quantum yields of some fluorophores (e.g. fluorescein biotin) (37) or octadecyl rhodamine B (38)) depends on surface density due to self quenching at high surface occupancies (37). Thus, unless the characteristics of surface coverage-dependent changes in fluorophore quantum yield are known, calibration beads are generally most useful at low surface densities (9,10,37).

Applicability of Qdot calibration beads on cells: Determination of EGFR sites on A431 cells using calibration QD585-M2 beads

A431 cells are human vulvar (epidermoid) carcinoma cells, with high expression levels of epidermal growth factor receptors (EGFR). We chose this cell-receptor system to test our calibration beads because there is a commercially available biotinylated EGF ligand, and the receptor system has already been characterized with Qdots (39). In addition, A431 cells are a good model system to study the interaction of epidermal cells with a naturally occurring multivalent ligand, namely human papillomaviruses labeled with Qdots (*vide infra*, cf. Figure 6) (40,41).

In an earlier discussion in this paper, we used Equation 4 to validate the performance characteristics of our QD525 beads against those of standard MESF beads. Here we extend our characterization to cells. The issue of concern here is how the multivalent aspect of EGF ligand-bearing Qdots affects the quantitative assessment of site density and dissociation constant of the EGF ligand, relative to measurements performed with a univalent FITC labeled EGF ligand (EGF^{FITC}) characterized with MESF beads. In these experiments we turned to QD585 dots because of their superior signal to background sensitivity relative to QD525 (Table 1).

Figure 3 shows parabolic plots of EGF^{QD585}(1:1) and EGF^{FITC} sites per cell versus ligand concentration. It is worth noting that the signal (total bound) to background (nonspecific binding) for both data sets was $>10:1$ (raw data not shown, *c.f.* Figure 4). The site densities were determined from our calibration beads (*vide infra*) and Quantum FITCTM calibration beads respectively (*c.f.* Note 4 in Table 1). Curve *b* in Figure 3 corresponds to the binding of EGF^{bio}/QD585 complexes of nominal 1:1 stoichiometry. The data indicates that the parallel

incubation of equimolar amounts of EGF^{QD585}(1:1) or EGF^{FITC}, with cells produces comparable numbers of sites of bound ligand. Analysis of the parabolic data yielded the apparent binding constants (EC₅₀) of ≈ 58 nM (for EGF^{FITC}; curve *a* in Figure 3) and ≈ 43 nM (for EGF^{QD585}(1:1); curve *b* in Figure 3). The nominally lower B_{max} and K_d values associated with the EGF^{QD585} ligand maybe attributable to the contribution of multivalent EGF/QD585 ligand complexes e.g. EGF^{QD585}(1:1;2:1; 3:1; 4:1;.....). The close match between the site densities of QD585- and FITC tagged EGF at lower ligand concentrations suggests that the univalent EGF^{QD585}(1:1) species are dominant. The site density of the receptors is compatible with the literature value of $\approx 2.0 \times 10^6$ /cell for the A431 cell line (42). The characteristics of multivalent interactions between EGF^{QD585} and cells are examined below.

Figure 4A shows a parabolic plot of flow cytometry data (concentration of multivalent EGF^{QD585} (12:1) versus MCF). There is a two order of magnitude increase in the affinity of the binding of EGF^{QD585} (12:1) to cells. We used our M2 calibration beads to derive the site number of EGF-QD585 ligand- occupied receptors from the MCF values associated with the titration. To convert the MCF data to site density we produced a series of calibration beads bearing a wide range of known QD585 units. The beads were analyzed on the flow cytometer at the same detector settings for the A431 cells. The histograms are shown in Figure 4B. A calibration curve of site densities of QD585 versus fluorescence intensities in terms of MCF was obtained through linear fitting of the MCF for the 5 different populations of QD585 calibration beads. The maximum site density of QD585 tagged EGF was determined from the known MCF (1290) corresponding to the site density (cross hair in Figure 4A). The analysis gave a value of $\approx 1.02 \times 10^6$ sites. Once the site occupancy was determined for the binding curve, a sigmoidal plot of the site number versus free EGF ligand was used to derive an affinity constant of 0.2 nM. It is possible that the multivalent K_d is lower than 0.2nM due to ligand depletion.

The EGF receptor has long been known to display two distinct affinities for the EGF ligand (42-48). The high affinity form ($K_D < 1$ nM) of the receptors comprises 1-10% of the receptor population. The low affinity state has been revealed, from ¹²⁵I-EGF studies (43-45,47,48), to span the range of K_d values between 6-12nM and shown to be as low as 37 nM for EGF^{FITC} (42). Our apparent K_d values for EGF^{FITC} and monovalent EGF^{FITC} (43 nM and 58 nM respectively) are well within the 95% confidence interval range (30-70 nM; obtained from analysis of data in Figure 3) of literature measurements (42). The normal range for K_d's found in the literature are typically the product of 6 hour incubations at 4°C. Our primary interest was not to revisit the well characterized equilibrium binding determination of EGF but to determine the quantitative limits of ligand binding as measured with dots or fluorescein tags, which can be achieved by using higher than normal concentrations of ligand at shorter (2 hour) incubation times. We have succeeded in this regard.

It is worth noting that the binding of EGF ligand to its cognate receptor at ambient temperature (25°C $\leq$ temp $\leq$ 37°C), initiates a signaling cascade that involves the internalization of the activated receptor into endocytic vesicles of the cell (49,50). Figure 4D shows the confocal microscopy image of a QD585-M2 bead (160,000 Qdots/bead) next to a Qdot-EGF ligand activated A431 cell (100,000 Qdots per cell) (51). It would be useful to be able to attempt a correlation of relative intensity measurements of cells and calibration beads from microscopy to flow cytometry data. However at present we do not have the appropriate software to accurately quantify the relative intensities of the samples.

Application of QD585-M2 Calibration bead: parallel flow cytometry and fluorescence microscopy imaging of A431 Cells Bearing GFP and QD585 labeled HPV16 PsV Particles

Human papillomaviruses (HPVs) are etiologic agents of a number of benign and malignant tumors of the skin and mucosa. HPV-associated human cancers include malignancies progressing from anogenital cancers, such as penile, anal, and cervical carcinomas and adenocarcinomas and a subset of head-and-neck cancers. Cervical cancer is a significant cause of death and illness among women worldwide, and HPV infections are linked to greater than 99% of all cervical malignancies (52). The first act of viral infection involves the attachment of HPV and entry into cells. In order to elucidate the mechanisms of initial HPV entry and replication upon infection and the establishment of viral persistence, one would like to establish cell-based assays for quantifying HPV infectivity. An assay based on fluorescence labeling of virus particles allows one to monitor, in real time, by flow cytometry and microscopy, the early and late events of viral attachment and entry into cells. This study has begun to address some of the fundamental issues involved in establishing a quantitative Qdot based assay to probe viral infectivity of cells.

The HPV virion consists of the chromatinized 8 kB circular dsDNA genome packaged into a ≈ 60 nm icosahedral capsid built from 72 L1 pentamers with 12 monomers of L2 estimated at each of the vertex positions (53) (54). Figure 5A and B show the TEM image of purified HPV16 PsV particles and the western blot results of biotinylated PsV detected with streptavidin-HRP, respectively. We have recently synthesized HPV16 particles that contain histone-GFP proteins and maintain normal morphology and infectivity (see methods). In addition to the GFP tag, this system is amenable to chemical conjugation with amine reactive probes (fluorophores or biotin). Biotinylated HPV16 PsV particles were incubated with cells for 30 minutes. The cells were washed and stained with QD585 and then analyzed by flow cytometry and confocal microscopy (see methods).

The double labeling of the virus particles with GFP and Qdots allows us to make a qualitative comparison of the two probes using a single excitation source. The data shown in Figure 6 represents a simultaneous two-color measurement of GFP/QD585 dual stained virus particles on cells using flow cytometry and microscopy. Figure 6A shows an overlay of flow cytometry histograms of GFP fluorescence and autofluorescence from negative control cells. As shown the signal to background is barely above 2 to 1. In contrast, Figure 6B shows the measurement of QD585 units staining the same virus particle, where the fluorescence intensity of the virus particles is ten times above the negative control cells. We used calibration beads to quantify the levels of GFP ($\approx 13,800$ GFP per cell, Standard GFP FACS Calibration Beads (Clontech)) and QD585 ($\approx 12,000$ QD585 per cell) using our M2 calibration beads. From these calibration schemes it is worth noting that although the two probes have comparable site numbers on cells, the Qdots display a clear signal to background advantage over GFP. This advantage in sensitivity in the detection of QD585 follows the trend observed in the measurements on beads (Column 4 in Table 1).

We currently lack sufficient data to make reasonable estimates of the number of GFP molecules per particle, or the extent of biotinylation, and therefore do not know the precise number of HPV particles/cell. Current effort is underway to resolve this issue with biochemical and spectroscopic methods.

Figure 6C shows confocal images of PsV-bearing cells prepared under similar conditions to the flow cytometry measurements in Fig. 6A-B. The four panels represent measures of light intensity associated with GFP (green channel), QD585 (orange channel). Also shown are a differential interference contrast (DIC) picture and a merged image of the three panels. The colocalization of the GFP and QD585 signals are consistent with the double labeling of HPV16 PsV. Figure 6D, shows confocal images of the same cells after 5 scans, displaying the near

complete photobleaching of GFP to background levels while the orange channel signal is robust. The apparent change in intensity of the fluorescent image of QD585 is due to the diminution of detector crosstalk due to the ablation of GFP fluorescence.

Summary and Conclusions

In summary, this work has extended the spectroscopic characterization of Qdots described in a preceding paper(1) to practical development of functional calibration beads based on Qdots. The Qdot based-beads and the measurement model of Equation 1 have a singularly important advantage over MESF beads. Since MESF beads are based on a single fluorophore entity, fluorescein, (or EGFP), their utility is limited to the spectral range of fluorescein, and not applicable to the multitude of commercially available fluorophores. Because Qdots can be photo-excited at any wavelength that can be matched to a fluorophore of choice, quantitation of the target fluorophores' site coverage can be readily achieved by using Qdots whose emission spectra overlaps with the target fluorophore. Because the Qdots are all streptavidin functionalized, their production is governed by the same rules of mass action as described and demonstrated here for QD525 (using fluorescein biotin beads and Equation 3) and QD585 (EGF receptor site density characterization). Thus multiple calibration beads can be made to order, in accordance with experimental need.

References

1. Wu Y, Lopez G, Sklar L, Buranda T. Spectroscopic Characterization of Streptavidin functionalized Quantum dots. *Anal. Biochem.* 2006submitted
2. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science* 2005;28:538–544. [PubMed: 15681376]
3. Michalet X, Pinaud F, Lacoste TD, Dahan M, Bruchez MP, Alivisatos AP, Weiss S. Properties of fluorescent semiconductor nanocrystals and their application to biological labeling. *Single Molecules* 2001;2:261–276.
4. Chan WCW, Maxwell DJ, Gao XH, Bailey RE, Han MY, Nie SM. Luminescent quantum dots for multiplexed biological detection and imaging. *Current Opinion in Biotechnology* 2002;13:40–46. [PubMed: 11849956]
5. Giepmans BNG, Deerinck TJ, Smarr BL, Jones YZ, Ellisman MH. Correlated light and electron microscopic imaging of multiple endogenous proteins using Quantum dots. *Nature Methods* 2005;2:743–749. [PubMed: 16179920]
6. Medintz IL, Uyeda HT, Goldman ER, Mattoussi H. Quantum dot bioconjugates for imaging, labelling and sensing. *Nature Materials* 2005;4:435–446.
7. Parak WJ, Pellegrino T, Plank C. Labelling of cells with quantum dots. *Nanotechnology* 2005;16:R9–R25.
8. Lidke DS, Arndt-Jovin DJ. Imaging takes a quantum leap. *Physiology* 2004;19:322–325. [PubMed: 15546848]
9. Schwartz A, Wang A, Early E, Gaigalas AK, Zhang Y, Marti G, Vogt R. Quantitating Fluorescence Intensity from Fluorophore: the definition of MESF Assignment. *J. Res. Natl. Inst. Stand. Technol* 2002;107:83–91.
10. Wang L, Gaigalas AK, Abbasi F, Marti G, Vogt R, Schwartz A. Quantitating Fluorescence Intensity From Fluorophores: Practical Use of MESF Values. *J. Res. Natl. Inst. Stand. Technol* 2002;107:339–353.
11. Nolan JP, Sklar LA. The emergence of flow cytometry for sensitive, real-time measurements of molecular interactions. *Nat. Biotechnol* 1998;16:633–8. [PubMed: 9661195]
12. Sklar, L., editor. *Flow Cytometry for Biotechnology*. Oxford University Press; Oxford: 2005. Oxford University Press
13. Lakowicz, JR. *Principles of Fluorescence Spectroscopy*. Plenum Press; New York: 1999.
14. Shapiro, HM. *Practical Flow Cytometry*. Alan R. Liss, Inc; New York: 1985.

15. Parker, CA. Photoluminescence of solutions. Elsevier; Amsterdam: 1968.
16. Norris DJ, Nirmal M, Murray CB, Sacra A, Bawendi MG. Size-Dependent Optical Spectroscopy of II-VI Semiconductor Nanocrystallites (Quantum Dots). *Zeitschrift Fur Physik D Atoms Molecules And Clusters* 1993;26:1–4.
17. Hoheisel W, Colvin VL, Johnson CS, Alivisatos AP. Threshold for Quasi-Continuum Absorption and Reduced Luminescence Efficiency in CdSe Nanocrystals. *Journal Of Chemical Physics* 1994;15:8455–8460.
18. Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud O, Gjertsen BT, Nolan GP. Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell* 2004;118:217–28. [PubMed: 15260991]
19. Ozbun MA. Infectious human papillomavirus type 31b: purification and infection of an immortalized human keratinocyte cell line. *Journal Of General Virology* 2002;83:2753–2763. [PubMed: 12388811]
20. Lee JH, Yi SMP, Anderson ME, Berger KL, Welsh MJ, Klingelutz AJ, Ozbun MA. Propagation of infectious human papillomavirus type 16 by using an adenovirus and Cre/LoxP mechanism. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 2004;17:2094–2099. [PubMed: 14769917]
21. Buranda T, Lopez GP, Simons P, Pastuszyn A, Sklar LA. Detection of epitope-tagged proteins in flow cytometry: Fluorescence resonance energy transfer-based assays on beads with femtomole resolution. *Analytical Biochemistry* 2001;298:151–162. [PubMed: 11700971]
22. Buck CB, Pastrana DV, Lowy DR, Schiller JT. Efficient Intracellular Assembly of Papillomaviral Vectors. *J. Virol* 2004;78:751–757. [PubMed: 14694107]
23. Buck CB, Thompson CD, Pang YS, Lowy DR, Schiller JT. Maturation of Papillomavirus Capsids. *J. Virol* 2005;79:2839–2846. [PubMed: 15709003]
24. Pyeon D, Lambert PF, Ahlquist P. Production of infectious human papillomavirus independently of viral replication and epithelial cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 2005;102:9311–9316. [PubMed: 15958530]
25. Kimura H, Cook P. Kinetics of core histones in living Human cells: Little exchange of H3 and H4 and some rapid exchange of H2B. *Journal of Cell Biology* 2001;153:1341–1353. [PubMed: 11425866]
26. Buranda T, Jones GM, Nolan JP, Keij J, Lopez GP, Sklar LA. Ligand receptor dynamics at streptavidin-coated particle surfaces: A flow cytometric and spectrofluorimetric study. *Journal Of Physical Chemistry B* 1999;29:3399–3410.
27. Babbitt SE, Kiss A, Deffenbaugh AE, Chang YH, Bailly E, Erdjument-Bromage H, Tempst P, Buranda T, Sklar LA, Bauml J, Gogol E, Skowrya D. ATP hydrolysis-dependent disassembly of the 26S proteasome is part of the catalytic cycle. *Cell* 2005;20:553–565. [PubMed: 15907469]
28. Buranda T, Huang JM, Perez-Luna VH, Schreyer B, Sklar LA, Lopez GP. Biomolecular recognition on well-characterized beads packed in microfluidic channels. *Analytical Chemistry* 2002;1:1149. [PubMed: 11924977]
29. Piyasena ME, Buranda T, Wu Y, Huang JM, Sklar LA, Lopez GP. Near-simultaneous and real-time detection of multiple analytes in affinity microcolumns. *Analytical Chemistry* 2004;1:6266–6273. [PubMed: 15516117]
30. Simons PC, Shi M, Foutz T, Cimino DF, Lewis J, Buranda T, Lim WK, Neubig RR, McIntire WE, Garrison J, Prossnitz E, Sklar LA. Ligand-receptor-G-protein molecular assemblies on beads for mechanistic studies and screening by flow cytometry. *mol pharm* 2003;64:1227–38.
31. Simons, P.; Vines, CM.; Key, TA.; Potter, RM.; Shi, M.; Sklar, LA.; Prossnitz, ER., editors. *Analysis of GTP-binding Protein-coupled receptor Assemblies by Flow Cytometry*. Oxford University Press; New York City: 2005. Oxford University Press
32. Abbott AJ, Nelsestuen GL. The Collisional Limit - an Important Consideration for Membrane-Associated Enzymes and Receptors. *Faseb Journal* 1988;2:2858–2866. [PubMed: 2844615]
33. Shea LD, Omann GM, Linderman JJ. Calculation of diffusion-limited kinetics for the reactions in collision coupling and receptor cross-linking. *Biophys. J* 1997;73:2949–59. [PubMed: 9414209]
34. Christopoulos A, Kenakin T. G protein-coupled receptor allostery and complexing. *Pharmacol. Rev* 2002;54:323–74. [PubMed: 12037145]

35. Sun Q, Woodcock JM, Bagley CJ, Jones K, R DA, Lopez AF. A monoclonal antibody (MoAb) to the human IL-3 ; GM-CSF and IL-5 receptor common beta-chain (beta(c)) neutralises the binding and function of all three ligands. *Blood* 1996;88:2170–2170.
36. Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* 2004;4:648–55. [PubMed: 15286731]
37. Buranda T, Jones G, Nolan J, Keij J, Lopez GP, Sklar LA. Ligand Receptor Dynamics at Streptavidin Coated Particle Surfaces: A Flow Cytometric and Spectrofluorimetric Study. *J. Phys. Chem. B* 1999;103:3399–3410.
38. Johansson LB-Å, Niemi A. Electronic Energy Transfer in Anisotropic Systems. 1. Octadecylrhodamine B in Vesicles. *J. Phys. Chem* 1987;91:3020–3023.
39. Lidke DS, Nagy P, Heintzmann R, Arndt-Jovin DJ, Post JN, Grecco HE, Jares-Erijman EA, Jovin TM. Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. *Nature Biotechnology* 2004;22:198–203.
40. Culp TD, Christensen ND. Quantitative RT-PCR assay for HPV infection in cultured cells. *Journal Of Virological Methods* 2003;111:135–144. [PubMed: 12880929]
41. Culp TD, Christensen ND. Kinetics of in vitro adsorption and entry of papillomavirus virions. *Virology* 2004;5:152–161. [PubMed: 14967496]
42. Chung JC, Sciaky N, Gross DJ. Heterogeneity of Epidermal Growth Factor Binding Kinetics on Individual cells. *Biophysical Journal* 1997;73:1089–1102. [PubMed: 9251825]
43. Domagala T, Konstantopoulos N, Smyth F, Jorissen RN, Fabri L, Geleick D, Lax I, Schlessinger J, Sawyer W, Howlett GJ, Burgess AW, Nice EC. Stoichiometry, kinetic and binding analysis of the interaction between epidermal growth factor (EGF) and the extracellular domain of the EGF receptor. *Growth Factors* 2000;18:11–29. [PubMed: 10831070]
44. Mayawala K, Vlachos DG, Edwards JS. Heterogeneities in EGF receptor density at the cell surface can lead to concave up scatchard plot of EGF binding. *Febs Letters* 2005;6:3043–3047. [PubMed: 15896781]
45. Ozcan F, Klein P, Lemmon MA, Lax I, Schlessinger J. On the nature of low- and high-affinity EGF receptors on living cells. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 2006;11:5735–5740. [PubMed: 16571657]
46. Puddicombe SM, Wood L, Chamberlin SG, Davies DE. The interaction of an epidermal growth factor transforming growth factor alpha tail chimera with the human epidermal growth factor receptor reveals unexpected complexities. *Journal Of Biological Chemistry* 1996;29:30392–30397. [PubMed: 8940002]
47. Holbrook MR, Slakey LL, Gross DJ. Thermodynamic mixing of molecular states of the epidermal growth factor receptor modulates macroscopic ligand binding affinity. *Biochemical Journal* 2000;15:99–108. [PubMed: 11062062]
48. Blinov ML, Faeder JR, Goldstein B, Hlavacek WS. A network model of early events in epidermal growth factor receptor signaling that accounts for combinatorial complexity. *Biosystems* 2006;83:2–3.
49. Haigler HT, Mckanna JA, Cohen S. Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth-factor in human carcinoma-cells A-431. *Journal of Cell Biology* 1979;81:382–395. [PubMed: 313931]
50. Lidke DS, Lidke KA, Reiger B, Jovin TM, Arndt-Jovin DJ. Tracking erbB1 retrograde transport using quantum dot-labeled ligands. *Biophysical Journal* 2005;88:8A.
51. Lenzen C, Cool RH, Prinz H, Kuhlmann J, Wittinghofer A. Kinetic analysis by fluorescence of the interaction between Ras and the catalytic domain of the guanine nucleotide exchange factor Cdc25 (Mm). *Biochemistry* 1998;19:7420–7430. [PubMed: 9585556]
52. Walboomers JMM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJF, Peto J, Meijer CJLM, Muñoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology* 1999;189:12–19. [PubMed: 10451482]
53. Chen XS, Garcea RL, Goldberg I, Casini G, Harrison SC. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Molec. Cell* 2000;5:557–567. [PubMed: 10882140]

54. Favre M, Breitburd F, Croissant O, Orth G. Structural Polypeptides of Rabbit, Bovine, and Human Papillomaviruses. *Journal Of Virology* 1975;15:1239–1247. [PubMed: 167184]

Figure 1.

A. Limiting model schematic of a modular assembly of biotinylated M2 antiFLAG antibodies, biotinylated FLAG peptides, and Qdots on 6.7 μm streptavidin-coated beads. Typical quantum dots contain a nanoparticle core such as the CdSe, a ZnS shell for improving optical properties, and a polymer coating for isolation as well as attaching the functional group for further biological modification. The Qdot shown here is functionalized with streptavidin. The CdSe core nanocrystal is typically between 2-6 nm in size depending on the emission wavelength. A typical quantum dot is conjugated to 8-10 streptavidin molecules, and the final size of streptavidin conjugated quantum dots is between 10-15nm. **B.** Plot of sites/bead versus log Free [QD585], the analysis of the sigmoidal binding data from a centrifugation assay yielded

a K_d of ≈ 2 nM. At saturation, a site density of QD585 $\approx$ to $4.1 \pm 0.3 \times 10^6$ per bead was determined. **C.** MCF signal of M2-QD585 beads versus total QD585 concentration determined by flow cytometry using beads from the previous centrifugation assay. Closed and open circles represent total and nonspecific binding respectively. The sigmoidal binding curve of the cytometry data yielded a similar K_D to the centrifugation assay.

Figure 2.

Normalized emission spectra of (a) fluorescein, (b) QD525 dots and (c) QD585. Green and orange bars represent band pass filters used in a standard flow cytometer (530/30 BP for FL1, and 585/42 BP for FL2). The overlap between the bandpass filters and emission spectra regulates the amount of light that is transmitted or rejected by the BP filter: Less than 30% fluorescein emission is transmitted through the FL1 filter, compared to 38% of QD525, and 65% of QD585 emission that is transmitted to the FL2 channel. The long tails of fluorescein emission enables the spillover of 6% of its emission into the FL2 channel.

Figure 3.

Plot of (a) EGF^{FITC} and (b) $\text{EGF}^{\text{QD585}}$ binding sites per cell versus the initial concentration of the EGF ligands. The $\text{EGF}^{\text{QD585}}(1:1)$ ligand was formed from mixing a 1:1 molar ratio of the biotinylated EGF ligand, EGF^{bio} , and QD585 dots bearing up to 8 streptavidin units. (a) The number of sites per cell was determined using standard Quantum FITCTM calibration beads (c.f. Table 1) and (b) our Qdot calibration beads (c.f. Figure 4).

Figure 4.

Determination of the total number of epidermal growth factor receptor sites A431 cells by flow cytometry and Qdot calibration beads. **A.** Plot of MCF versus concentration of Qdot labeled EGF ligand (EGFQD585). Closed and open circles represent total and nonspecific binding respectively. Insert shows a Sigmoidal plot of the sites/cell versus log of Free ligand. The site densities were determined from the MCF data using Qdot calibration beads (*vide infra*). The analysis yielded a $K_d \approx 0.2$ nM. **B.** Flow cytometry histograms of calibration beads bearing a wide range of site densities of QD585. **C.** Determination of binding sites of Qdots on A431 cells using calibration beads. Linear plot of Qdot sites on calibration beads versus the corresponding MCF reading taken at the same settings as A431 cells. The intersection of the cross hairs corresponds to the number of sites at saturation on the cells-read at the y-intercept. **D.** Confocal microscopy image of a M2-QD585 calibration bead (top right) next to an A431

cell labeled with QD585 labeled EGF (EGFQD585; middle left) for 30mins at 23°C. The calibration bead has ~160,000 QD585 units while the A431 cell bears 100,000 Qdots.

Figure 5.

A. TEM micrograph of purified PsV preparation. Sample was negatively stained with 2% uranyl acetate. **B.** Western blot of biotinylated PsV probed with streptavidin-HRP. Major and minor bands correspond to biotinylated L1 and L2 respectively (see text for details).

Figure 6.

Simultaneous two color measurement of GFP/QD585 double stained virus particles on A431 cells using flow cytometry and microscopy. **A.** Flow cytometry measurement of GFP fluorescence. Solid histogram (MCF $\approx$ 100) corresponds to cells bearing HPV particles while open histogram (MCF $\approx$ 50) represents negative control cells. The GFP signal is weak, at the moderate voltage setting for FL2 (600 Volts), crossover from GFP is minimal (*cf.* Figure 8). **B.** Fluorescence measurement of QD585. Solid histogram (MCF $\approx$ 1100) represents cells bearing HPV particles stained with QD585, and open histogram (MCF $\approx$ 100) represents negative control cells. Calibration beads were used to determine the level of GFP ($\sim$ 13,800 GFP per cell, Standard GFP FACS Calibration Beads (Clontech)) and QD585 ($\sim$ 12,000 QD585 per cell) using our M2 calibration beads. **C.** Confocal images of PsV-bearing A431 cells prepared under similar conditions to flow cytometry measurements. **D.** Confocal images of the same cells after 5 scans shows the near complete photobleaching of GFP.

Table 1

Summary of spectroscopic properties of fluorescein conjugates and QD525, (lot#1005-0045) and QD585, (Lot#0905-0031) Quantum dots.

Fluors.	Bandwidth FWHM (nm)	$\lambda_{ex}(nm); \epsilon(M^{-1}cm^{-1})$	$\varphi_s^2 = \varphi_{ref} \frac{I_s}{I_{ref}} \frac{OD_{ref}}{OD_s} \frac{n_s^2}{n_{ref}^2}$	$\frac{I_s}{I_{ref}} \frac{OD_{ref}}{OD_s} \frac{n_s^2}{n_{ref}^2}$	% Trans. (BP Filter) ³	Sensitivity (# of fluors. at S/N=3:1) ⁴
Biotin ^{FITC} FLAG ^{FITC} EGF ^{FITC}	Asymmetric	488; 80,000 ^a	0.82 ^a 0.20 ^a 0.50 ^a		28(530/30) ^a	5646 ^a - -
QD525	33	488; 130,000 ^b 458; 216,000 405; 360,000 350; 710,000	0.30 ^a 0.40 ^a 0.27 ^b 0.15 ^b		38(530/30) ^b 48(530/30) ^c	4140 ^b
QD585	28	488; 530,000 ^c 458; 1,100,000 405; 2,200,000 350; 3,500,000	0.20 ^b , 0.67 ^c 0.20 ^d 0.14 ^d 0.10 ^d		65(585/42)	124 ^b

Notes:

- ¹ Extinction coefficients obtained from www.probes.invitrogen.com. (a). The extinction coefficient of fluorescein and some derivatives vary from 85,000 for the NIST fluorescein solution standard reference material SRM 1932 to 75,000 for some FITC derivatives, and values are for pH dependent. We have selected a median number here. (b-c) excitation wavelengths used in this work.
- ² Relative quantum yields (φ_s) were calculated using the integrated intensity of sample relative to: a). Fluorescein, $\varphi_{ref} = 0.95$; I_s and I_{ref} are the integrated band intensities. The optical densities (OD) of the sample (s) and reference (ref) were similar. n is the index of refraction of the solvent; 1.32 for water. (b). φ_s determined from photoexcitation spectra. (c). φ_s determined relative to Rhodamine B, $\varphi_{ref} = 0.31$ in water (Magde et. al., *Photochem. Photobiol.* 1999). (d). φ_s determined from photoexcitation spectra.
- ³ Bandpass filters on a standard model BD Faescan flow cytometer. The attenuation of the fluorescein/QD525 emission by the cytometer's dichroic filter is assumed to be roughly equal. (a) spectral mismatch between probes is negligible. (b) Emission from the excitation of QD525 at 488 nm (b) Excitation of QD525 at 450nm (see Wu *et al* (1)) Filter/probe combinations available at www.invitrogen.probes.com.
- ⁴ Measured using same 488 nm excitation. (a) fluorophore site density determined from Equation 4.1

$$Sites = \frac{MCF_S}{MCF_{std}} \cdot MESF \cdot \frac{1}{\varphi_s} \quad (4.1)$$

using MESF values of standard QuantumTM MESF FITC Beads. φ_s is the quantum yield of fluorescein biotin relative to fluorescein. The MESF values are based on the fluorescence intensity of the NIST Fluorescein Solution Standard Reference Material (SRM 1932); ($\varphi = 0.93$); (Data supplied by Bangs Beads upon request). (b) Derived from mass action.