

# Probing cell surface interactions using atomic force microscope cantilevers functionalized for quantum dot-enabled Förster resonance energy transfer

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**Abstract.** Förster resonance energy transfer (FRET) between quantum dot (QD) donors and red fluorescent protein (RFP)-tagged integrin acceptors in live cells is reported for the first time. A silica microsphere was coated with CdSe/ZnS QDs and mounted to the cantilever of an atomic force microscope (AFM). The QD microsphere is then conjugated with fibronectin to bind with RFP- $\alpha v$  integrins expressed on the surface of HeLa cells. Following AFM-controlled cell contact with the QD-microsphere structure, FRET is observed between the QD-RFP pair using a photobleaching measurement technique. This FRET probe technique provides a novel tool for studying the cell surface receptor-ligand interactions in biomedical and biological research. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3174429]

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## 1 Introduction

Integrins are cell adhesion molecules that play a central role in cell interaction with the extracellular-matrix (ECM). Integrin-ECM interactions determine many fundamental processes, such as cell adhesion, spreading, migration, and growth. The initial integrins binding with ECM will form small dot-like clusters, which could then either mature into focal adhesions or disassemble and disappear in minutes,<sup>1</sup> suggesting rapid regulation of integrins by chemical and/or physical signaling.<sup>2,3</sup> Novel optical techniques are needed to address the temporal and spatial aspect of integrin-ligand interactions in lifetime with improved resolution.

Development in Förster resonance energy transfer (FRET) techniques have facilitated the study of protein-protein interactions in recent years.<sup>4</sup> This energy transfer is the result of long-range dipole-dipole interactions and takes place only when the two molecules are within 20–100 Å.<sup>5</sup> This distance constraint allows FRET techniques to provide extremely high spatial resolution for resolving molecular interactions.

Semiconductor quantum dots (QDs) have been effectively applied as FRET donors both as free particles<sup>6–9</sup> as well as when attached to a larger structure, such as an atomic force microscope (AFM) tip<sup>10</sup> or a microsphere.<sup>11</sup> QDs have broad excitation spectra that increase to shorter wavelengths and narrow, symmetric photoluminescence emission spectra (25–45 nm)<sup>12</sup> that can be size-tuned through the visible and into the near infrared.<sup>13</sup> These unique properties enable optimization of FRET with any chosen acceptor fluorophore by minimizing both acceptor excitation and overlap between donor and acceptor emission. Finally, QDs possess relatively high quantum yield and high resistance to photobleaching. This feature makes QDs optimal for use as the FRET donor for multiple measurements. Here, we report a QD-based FRET technique that enables the induction of focal contacts/adhesions and detection of fibronectin (FN)-integrin interactions on the cell membrane of a live cell.

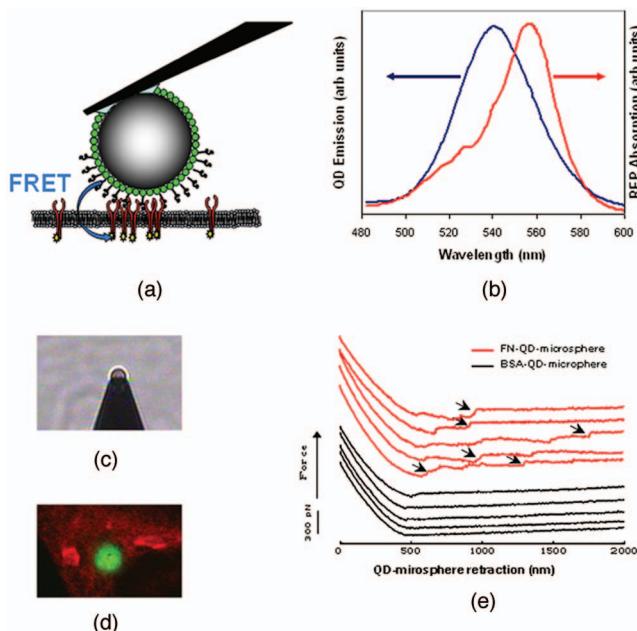
## 2 Experimental

CdSe QDs were fabricated using methods described by Peng and Peng.<sup>14</sup> Trioctylphosphine oxide, cadmium oxide, and a stabilizer were heated to 340 °C under argon for 40 min in a two-neck flask, then cooled to 280 °C. A Se-trioctylphosphine stock solution was injected to start QD synthesis. At a time point corresponding to the desired emission wavelength, the reaction was stopped by removal from heat. To make the QDs soluble in water, QDs in chloroform at 2.5 gm/L were mixed in a 1:1 ratio with butanol and 1 mol/L dihydrolipoic acid, and incubated at room temperature for 24 h. To coat silica microspheres with a layer of QDs, 3-APS-functionalized silica microspheres (diameter ~5 μm) are mixed with the DHLA-coated QDs in butanol under agitation. Free QDs are removed by gravity sedimentation procedure.

QD-coated microspheres (QD-microspheres) are attached to AFM cantilevers (spring constant 0.01–0.015 N/m, Veeco Metrology, Inc.) using epoxy (Progressive Epoxy Polymers Inc.). A drop of the microsphere-butanol solution and a drop of epoxy are applied side-by-side to a thoroughly cleaned slide, and the butanol evaporated. An AFM cantilever is first dipped into the epoxy and then lowered onto a QD-microsphere. On withdrawal of the AFM probe, the QD-microsphere sticks to the cantilever and the epoxy cures in 24 h. Immediately before the FRET experiment, the QD-microsphere on AFM cantilever is immersed in 10 μL of FN or bovine serum albumin (BSA) solution (0.5 mg/mL) for 8 min, then washed 4 times with phosphate buffered saline.

HeLa cells are cultured in minimal essential medium (MEM) with 10% fetal bovine serum in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. All culture reagents are purchased from Invitrogen (Carlsbad, CA). For FRET experiments, cells are transfected with construct encoding integrin  $\alpha v$ -red fluorescence protein (RFP) (Evrogen Inc., Russia) in 50-mm

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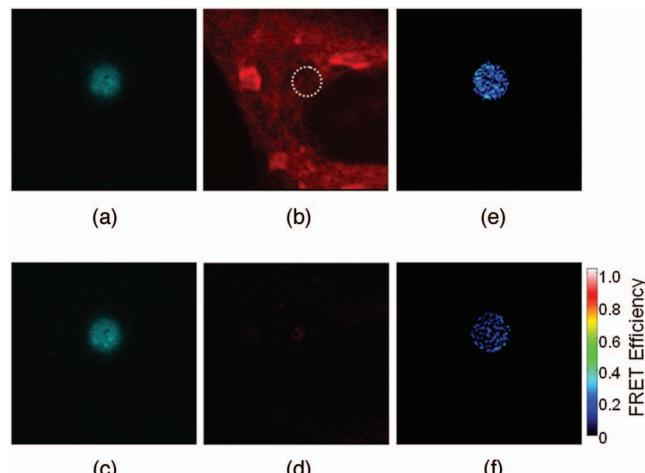
**Fig. 1** Illustration of the experimental concept. (a) Schematics of the QD-based FRET technique for detecting the cell surface integrin-ligand interactions. DHLA-coated QD is coated on a silica microsphere. The microsphere is mounted on an AFM cantilever and coated with FN. The FN-QD-microsphere is brought into contact with the cell surface to induce FN-integrin bindings. This brought QD close to acceptor fluorophores tagged on integrins to induce FRET. (b) Spectral overlap between the QD emission centered at 540 nm (blue) and the absorption of monomeric RFP (red). (c) Image of a QD-microsphere mounted on an AFM cantilever. (d) Overlay confocal image of QD-microsphere (green) in contact with a HeLa cell expressing integrin  $\alpha v$ -RFP (red). (e) Typical retraction force curves (tip velocity, 2  $\mu\text{m}/\text{s}$ ) obtained by interacting AFM probe with FN-QD-microsphere and the cell surface (upper traces). Arrows indicate adhesions between the AFM probe and the cell. Force curves obtained with the BSA-QD-microsphere tip did not show adhesions (lower traces).

dishes with a number 1 glass coverslip bottom (World Precision Instruments, Inc., Sarasota, FA). For transfection, HeLa cells are plated at 50–70% confluence overnight. 6  $\mu\text{L}$  Turbofectin reagents (Origene, Inc.) are first mixed with 100  $\mu\text{L}$  MEM at 25 °C for 5 min. 2  $\mu\text{g}$  DNA are then added to the mixture, and incubated for 20 min. The mixtures are then added to the cells. The expression of integrin  $\alpha v$ -RFP is usually observed within 24–48 h.

FRET measurements were performed with an Olympus Fluoview1000 confocal microscope. The FRET donor, QDs are excited at 440 nm and the emission collected at 520–550 nm, ~53% of the QD emission spectra [Fig. 1(b)]. There was no significant photobleaching of QDs over the data acquisition period. The FRET acceptor, RFP is excited at 561 nm, and the emission collected 580–680 nm [Fig. 1(b)]. There was minimal donor breakthrough into the acceptor channel [Fig. 2(d)].

### 3 Results

In this assay, the AFM lowers the FN-QD-microsphere onto the cell surface in order to interact with integrin  $\alpha v$ -RFP expressed on cell surface. Figure 1(a) is a concept sketch of the technique. Figure 1(b) illustrates donor emission and acceptor absorption spectra used in this experiment. The calculated



**Fig. 2** Typical photobleaching FRET measurement between a QD-microsphere and integrin  $\alpha v$ -RFP on a HeLa cell surface. (a) Luminescence from QD-microsphere in contact with the cell surface before photobleaching of the RFP. (b) Fluorescence from integrin  $\alpha v$ -RFP before photobleaching. (c) Luminescence from QD-microsphere after photobleaching of the RFP. (d) Fluorescence from integrin  $\alpha v$ -RFP after photobleaching. (e) FRET efficiency image calculated between QD-microsphere and integrin  $\alpha v$ -RFP on HeLa cell surface. (f) FRET efficiency image from control FRET measurement after acceptor already bleached.

Förster distance,  $R_0$ , at the FRET efficiency of 50% is 5.1 nm. The QD layer on the microsphere was easily detectable under fluorescent microscopy [Fig. 1(d)]. As opposed to embedded QDs, the surface layer of QDs minimized the background noise signal of donor emission. As a proof of concept, FRET with an efficiency of 25–40% was previously observed between QD-microsphere structures and  $\beta 3$ -integrins immunolabeled with Alexa546 on fixed cells.<sup>15</sup> In the current study, the RFP is tagged on the c-terminal of  $\alpha v$  integrins and resides on the inner leaflet of the cell membrane. Thus, the cell membrane separates the donor and acceptor by approximately 3.5–5 nm.<sup>16</sup> However, we demonstrate that the FRET signal is still experimentally observable.

The stability of the QD coating on the microsphere was confirmed by bringing the QD-microsphere into contact with the cell surface followed by retraction using AFM. No QDs were observed on the cell surface after microsphere retraction. For the FRET experiments, the FN-QD-microsphere was controlled by the AFM to stably engage with cell surface (contact force approximately 500–750 pN) for 20 min to induce FN-integrin interactions. We have previously shown that 20 min is sufficient to induce the formation of a focal adhesion site.<sup>17</sup> A photobleaching technique was applied for measurement of the FRET efficiency. FRET signal manifested as an increase in donor emission following photobleaching of the acceptor. Eight initial images of the donor and acceptor were acquired sequentially and averaged [Figs. 2(a) and 2(b)]. The acceptor was then photobleached over a period of ~3 min. Eight final images of the donor and acceptor were acquired and averaged [Figs. 2(c) and 2(d)]. After photobleaching, the RFP no longer shows fluorescence and the absence of a donor breakthrough in Fig. 2(d) is noted. A comparison of Fig. 2(a)–2(c) shows increased luminescence from the QD-microsphere. The FRET efficiency,  $E$ , is calculated from this increased luminescence using

$$E = (F_{DA} - F_{DB})/F_{DA},$$

where  $F_{DA}$  is donor emission after photobleaching and  $F_{DB}$  is donor emission before photobleaching. Over a number of measurement sites on multiple cells, average FRET efficiencies were generally observed to be in the 10–20% range. A typical result is shown in Fig. 2(e). In this case, the average FRET efficiency across the QD-microsphere is 12%, with individual pixels ranged from 0 to 36% with a standard deviation of 10%. As anticipated, FRET was not observed on the surface areas of the microsphere that was not in intimate contact with the cell surface. Figure 2(f) shows the result of a control FRET measurement performed with the acceptor already photobleached. This demonstrates a background noise level of ~4% with a standard deviation of 6.8%. Thus, the observed FRET signal is significantly above the noise level. Comparing to averaged FRET signal observed with FN-QD-microsphere (8–10% above the background noise level), the averaged FRET signal observed with BSA-QD-microsphere was significantly lower (2–5% above the background level). We interpret the FRET signal with the BSA as representing background plus free integrin on the cell surface. The approximately twofold higher signal with the FN indicates that integrin is accumulating at the bead site as a result of integrin binding to the FN.

These results demonstrate that this QD-based FRET technique can be applied to study the integrin-ECM interactions in live cells. In an *in vitro* study, Dennis and Bao have described FRET between QD and several fluorescent proteins, in which the QD was separated from RFP proteins by a layer of PEG lipid.<sup>9</sup> They calculated the FRET distance between QD and fluorescent proteins to be 5–6 nm, with the FRET efficiency to be 26–50% for a QD:fluorescent protein ratio of 1. We measured a lower FRET efficiency, suggesting that the QD-RFP distance is larger than their system. Because the system is dynamic and the FRET pair may or may not be physically tied together, there will be a range of donor/acceptor distances sampled in this experiment. The standard relationship between FRET efficiency and an apparent or most likely distance is given as

$$E = 1/[1 + (R/R_0)^6],$$

where  $R$  is the separation distance. For the FRET pair used in these experiments, efficiencies of ~15% suggest a most likely separation of ~7 nm. This is consistent with our projected QD-RFP distance of 6–10 nm, considering that cell membrane lipid-bilayer is ~4 nm thick. In addition to the QD-RFP distance, the ratio of RFP:QD pairs at the local spot would also affect the FRET measurement, with higher ratio corresponding to greater FRET efficiency.

In conclusion, we have developed a QD-based FRET technique that is integrated with an AFM for detection of the cell surface integrin-ECM interactions in live cells. By employing the FN-QD-microsphere as the FRET donor, we have demonstrated the detection of integrin-FN interactions on cell surface. Because QDs are resistant to photobleaching, this novel technique also enables multiple FRET measurements. Combination of the FRET approach with the AFM has the benefits of allowing precise positioning of the QDs on the cell surface for measurement of FRET and enabling the measurement and/or application of force to the cell simultaneously with the mea-

surement of FRET. This flexible FRET method should be applicable to the detection of other cell surface proteins and/or receptor-ligand interactions. In addition, it is relatively easy to coat the QD-microsphere with proteins through adsorption. This eliminates the need to construct a donor fluorophore-conjugated protein and thus makes the method more accessible for biological studies.

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#### References

1. A. Bhatt, I. Kaverina, C. Otey, and A. Huttenlocher, "Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain," *J. Cell. Sci.* **115**(Pt 17), 3415–3425 (2002).
2. K. Hodivala-Dilke, " $\alpha\beta\beta$  integrin and angiogenesis: a moody integrin in a changing environment," *Curr. Opin. Cell Biol.* **20**(5), 514–519 (2008).
3. C. M. Brown, B. Hebert, D. L. Kolin, J. Zareno, L. Whitmore, A. R. Horwitz, and P. W. Wiseman, "Probing the integrin-actin linkage using high-resolution protein velocity mapping," *J. Cell. Sci.* **119**(Pt 24), 5204–5214 (2006).
4. G. W. Gordon, G. Berry, X. H. Liang, B. Levine, and B. Herman, "Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy," *Biophys. J.* **74**(5), 2702–2713 (1998).
5. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum, New York (1999).
6. R. Patolsky, R. Gill, Y. Weizmann, T. Mokari, U. Banin, and I. Willner, "Lighting-up the dynamics of telomerization and DNA replication by CdSe-ZnS quantum dots," *J. Am. Chem. Soc.* **125**, 13918–13919 (2004).
7. I. L. Medintz, J. H. Konnert, A. R. Clapp, I. Stanish, M. E. Twigg, H. Mattooussi, J. M. Mauro, and J. R. Deschamps, "A fluorescence resonance energy transfer-derived structure of a quantum dot-protein bioconjugate nanoassembly," *Proc. Natl. Acad. Sci. U.S.A.* **101**(26), 9612–9617 (2004).
8. D. M. Willard, L. L. Carillo, J. Jung, and A. V. Orden, "CdSe-ZnS quantum dots as resonance energy transfer donors in a model protein-protein binding assay," *Nano Lett.* **1**(9), 469–474 (2001).
9. A. M. Dennis and G. Bao, "Quantum dot-fluorescent protein pairs as novel fluorescence resonance energy transfer probes," *Nano Lett.* **8**(5), 1439–1445 (2008).
10. Y. Ebenstein, T. Mokari, and U. Banin, "Quantum-dot-functionalized scanning probes for fluorescence-energy-transfer-based microscopy," *J. Phys. Chem. B* **108**, 93–99 (2004).
11. A. Sukhanova, A. S. Susha, A. Bek, S. Mayilo, A. L. Rogach, J. Feldmann, V. Oleinikov, B. Reveil, B. Donvito, J. H. M. Cohen, and I. Nabiev, "Nanocrystal-encoded fluorescent microbeads for proteomics; antibody profiling and diagnostics of autoimmune diseases," *Nano Lett.* **7**(8), 2322–2327 (2007).
12. A. P. Alivisatos, "Semiconductor clusters, nanocrystals, and quantum dots," *Science* **271**(5251), 933–937 (1996).
13. N. Gaponik, I. L. Radchenko, G. B. Sukhorukov, H. Weller, and A. L. Rogach, "Toward encoding combinatorial libraries: charge-driven microencapsulation of semiconductor nanocrystals luminescing in the visible and near IR," *Adv. Mater.* **14**(12), 879–882 (2002).
14. Z. A. Peng and X. Peng, "Formation of high-quality CdTe, CdSe, and CdS nanocrystals using CdO as precursor," *J. Am. Chem. Soc.* **123**(1), 183–184 (2001).
15. K. E. Meissner, Z. Sun, B. Nathwani, C. Needham, R. E. Beckham, W. N. Everett, X. Fan, G. L. Cote, and G. A. Meiminger, "Combining AFM and FRET for studies at the cellular level," *Proc. SPIE* **6863**, 686301 (2008).
16. V. I. P. Tibor Hianik, *Bilayer Lipid Membranes: Structure and Mechanical Properties*, Springer, Berlin (1995).
17. Z. Sun, L. A. Martinez-Lemus, M. A. Hill, and G. A. Meiminger, "Extracellular matrix-specific focal adhesions in vascular smooth muscle produce mechanically active adhesion sites," *Am. J. Physiol. Heart Circ. Physiol.* **295**(1), C268–278 (2008).