Quantum Dot-Fluorescent Protein Pairs as Novel Fluorescent Resonance Energy Transfer Probes

Supporting Information

Materials. Carboxyl functionalized T2-MP EviTags emitting at 520, 540, and 560 nm were purchased from Evident Technologies (Troy, NY). Luria Bertani (LB) agar, LB broth, Ampicillin (Amp), Chloramphenicol (Cam), and isopropyl-ß-D-thiogalactoside (IPTG) were purchased from US Biologicals (Swampscott, Massachusetts). Phosphate buffered saline (PBS), bovine serum albumin (BSA), and TE buffer were procured from Sigma Aldrich (St. Louis, MO). A HHHHHHHHHH peptide (His10) was custom synthesized by AnaSpec (San Jose, CA).

Mutagenesis, Expression, and Purification of mCherry proteins. pRSET-B plasmids containing inserts for the fluorescent proteins (FP) mOrange, tdTomato, and mCherry were kindly provided by Roger Tsien's laboratory at the University of California, San Diego. These plasmids were modified using PCR mutagenesis to produce the control FPs, which lack a terminal polyhistidine sequence, and His6-FPs, which contain a His6-tag linked to the fluorescent protein with three glycines. The forward primer 5'-Phos-GTG AGC AAG GGC GAG GAG-3' was used to produce the control proteins, while the forward primer 5'-Phos-CAT CAC CAT CAC CAT CAC GGA GGT GGA GTG AGC AAG GGC GAG GAG-3' was used to create the experimental proteins. In both cases, the reverse primer 5'-Phos-ACC CCG CAT ATG TAT ATC TCC TTC TTA AAG-3' was used, thereby removing an unnecessary multiple cloning site, a His6-tag that was present in the original plasmid, an Xpress Epitope, and an enterokinase cleavage site from the N-terminus region of the original plasmid, but in order to maintain a reasonable melting temperature for the PCR reaction, it was necessary to retain two non-critical amino acids at the N-terminus, specifically an arginine and a glycine. All primers were synthesized by Integrated DNA Technologies, Inc. (IDT; Coralville, IA); the forward primer containing the insert was HPLC purified, while the other two primers underwent standard desalting. The Phusion High-Fidelity Master Mix (New England Biolabs, Ipswich, MA) was used for all the PCR reactions with 10 pg template DNA and 25 pmol of each primer per reaction. Thermocycler conditions were chosen as directed in the Phusion manual with an annealing temperature of 69.6˚C being used for each of the reactions.

Plasmids coding for the FPs were transformed into the Rosetta 2(DE3) strain of *E.coli* (Novagen, Madison, WI) per the manufacturer's protocol and plated on LB/Amp/Cam agar plates for selection. Single colonies were plucked from the plates to start overnight cultures in 10 mL LB/Amp/Cam broth at 30˚C and 200 rpm. The overnight cultures were used to inoculate 500 mL cultures, which were incubated at 37˚C and 200 rpm. After 3-4 hours, the cultures were induced with 0.5 mL of 1 M IPTG. After culturing another 5-6 hours at 37˚C or overnight at 30˚C, the *E.coli* were pelleted at 5,000 rpm in 500 mL jars in a Beckman J2-21 centrifuge. The cell pellets were frozen at -20˚C until they were needed.

The cell pellets containing expressed fluorescent protein were thawed and resuspended in 25 mL TE buffer. 25 mg of lysozyme was added to digest the cells at 4˚C for one hour. While kept on ice, the cells were further disrupted with five cycles of ten seconds on, ten seconds off with the Branson Sonifier 150 sonicator probe at level four. The cell slurry was centrifuged for fifteen minutes at 15,000 rpm in 50 mL tubes in the Beckman JA-21 centrifuge to produce a cleared cell lysate. Ammonium sulfate precipitation isolated the protein from the rest of the cell lysate. His6-tagged protein precipitates were resuspended in HisTrap Binding Buffer (20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, pH 7.4) and centrifuged to remove any insoluble fractions. The mCherry control proteins were resuspended in TE Buffer containing 30% ammonium sulfate.

The resolubolized proteins were purified using an ÄKTAprime plus (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ). The His-tagged proteins were purified on a 1 mL HisTrap HF column, while the untagged proteins were purified with on a hydrophobic column (HiPrep 16/10 Butyl FF) followed by size-exclusion chromatography (HiPrep 16/60 Sephacryl S-300 HR). The purified proteins were concentrated and buffer-exchanged into PBS using centrifugal filtration devices (Centricon Plus-20; Millipore, Billerica, MA). The proteins were aliquoted into PCR tubes and snap frozen in liquid nitrogen as previously described[1]. The protein purity was checked with SDS-PAGE and the protein concentrations were determined using a BCA Assay (Pierce, Rockford, IL).

Quantum Dot and Fluorescent Protein Spectra. The QD absorbance spectra of the stock solutions were taken using a NanoDrop ND-1000 with a stepsize of 3 nm. The QD emission spectra as well as the excitation and emission scans of the fluorescent proteins were all measured on a Tecan Safire multiplate reader (Durham, NC) using 1 nm stepsizes, excitation bandwidths of 12 nm, and emission bandwidths of 5 nm. Non-binding black flat-bottomed 384-well plates from Corning were used for all of the measurements on the Tecan Safire. A 100 nM solution of the EviTags were used to take emission spectra with an excitation at 400 nm. The fluorescent proteins were diluted to 5 μM for the spectral measurements. The excitation spectrum of mOrange was taken from 450 – 590 nm with a fixed emission wavelength of 605 nm. The same protein was excited at 500 nm for the measurement of its emission. tdTomato was excited at 510 nm for the emission measurement, and the emission was fixed at 620 nm while the excitation spectrum was taken. The excitation spectrum of mCherry was taken with a fixed emission wavelength of 650 nm, while the excitation wavelength was static at 550 nm during the emission scan. Spectra were graphed in Igor (v5.05A, Wavemetrics Inc., Lake Oswego, OR).

Direct Excitation of FPs During FRET Assays. Some direct excitation of the FRET acceptor is inevitable in the FRET assays, although in this case the direct excitation was minimal due to the brightness of the QDs (allowing for low concentrations of the donor, and therefore low acceptor concentrations) and the low absorption of the FPs at 400 nm, where the QDs were excited. By looking at the spectra in Figure S1, one can see that the majority of the FP emission in the FRET assays was indeed sensitized emission. The small peak at 500 nm seen in all of the samples was not due to the direct excitation of any of the FPs, but rather was consistent from well to well and was due to either a low amount of fluorescence from the BSA or another component of the buffer or was background from the multi-well plate or the detector.

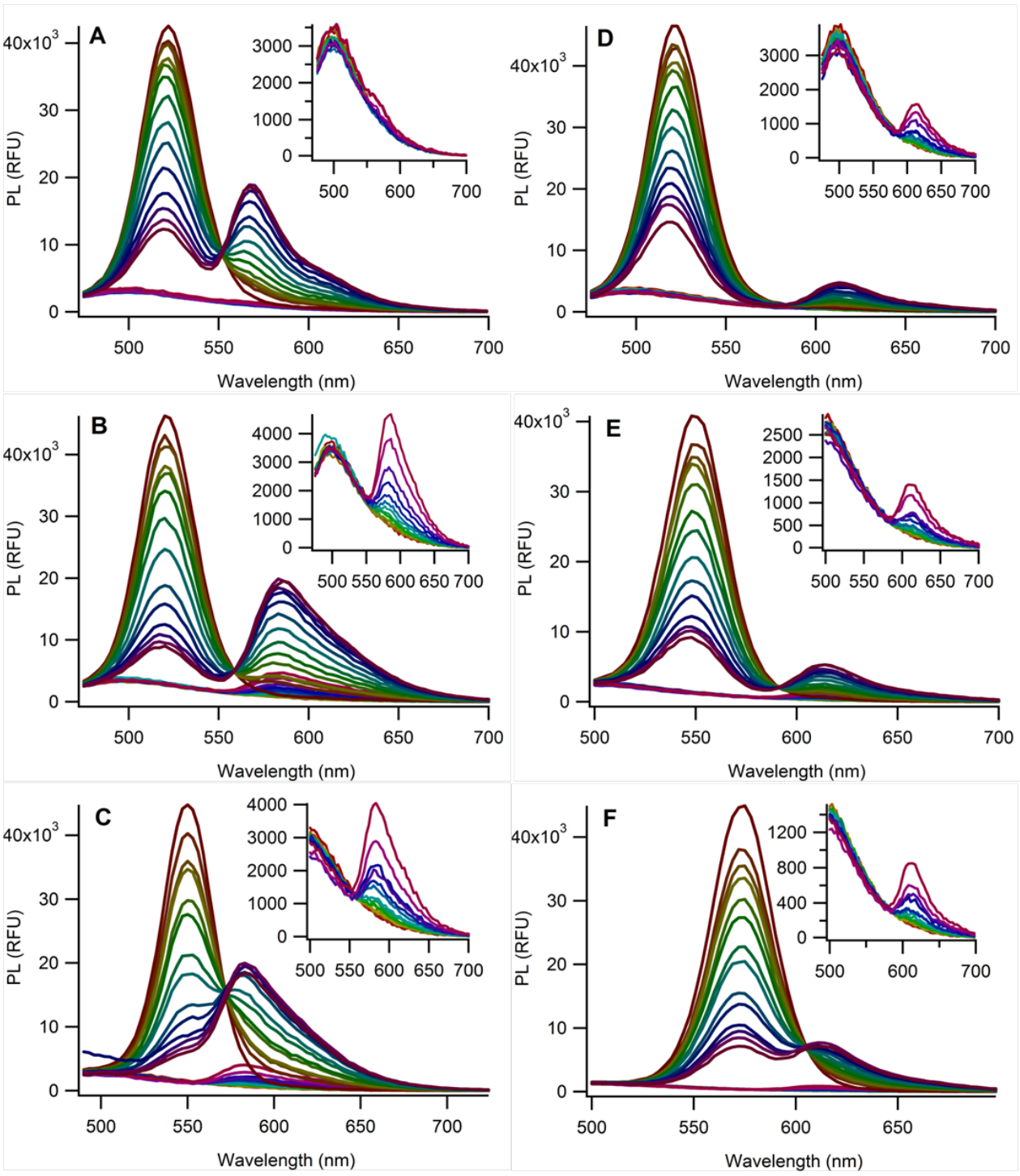

Figure S1: Fluorescence spectra of the QD-FP FRET interactions and the direct excitation of the His6-FP in the absence of the QD. Insets: The background emission from the His6-FPs scaled for clarity. A) QD520 + His6-mOrange; B) QD520 + His6-tdTomato; C) QD540 + His6-tdTomato; D) QD520 + His6-mCherry; E) QD540 + His6-mCherry; and F) QD560 + His6-mCherry.

Curvefitting. A modified Hill equation was used to extract useful information about the QD-FP interaction from the change in QD fluorescence. The traditional Hill equation[2]:

$$\theta = \frac{1}{1+\left(\frac{K_D}{c}\right)^h}$$

relates the fraction of ligand binding sites filled (θ) to the ligand concentration (c), the dissociation constant or ligand concentration at which 50% of the binding sites are filled (K_D), and the Hill coefficient (h). Several assumptions are made to adapt this equation for the current application. First, the number of ligand binding sites filled is not being measured, but rather the fluorescence emission of the QD in the presence of the ligand, i.e. the fluorescent protein. Because the fluorescence does not change uniformly regardless of the ligand used, but rather depends on the effectiveness of the donor-acceptor pair being studied, the FRET efficiency (E_{max}) of the pair is included in the expression. Also, because the fluorescence decreases with increased binding, the expression is subtracted from the maximum possible value, which in the case of the normalized fluorescence, is unity. These changes result in the expression:

$$\frac{F_{DA}}{F_D} = 1 - E_{\max}\left[\frac{1}{1+\left(\frac{K_D}{c}\right)^h}\right]$$

where F_{DA} is the fluorescence of the donor in the presence of the acceptor, F_D is the fluorescence of the donor in the absence of the acceptor, and E_{max} is the maximum FRET efficiency The His6-FP-mediated quenching data were fitted to the modified Hill equation, as described in the manuscript, but the small amount of quenching that arises from free FP in solution is better described by the Stern-Volmer equation[3]:

$$\frac{F_D}{F_{DA}} = 1 + K_{SV}[Q]$$

where [Q] is the quencher concentration and K_{SV} is the Stern-Volmer quenching coefficient. Figure S2 depicts the Stern-Volmer analysis of one of the FRET pairs.

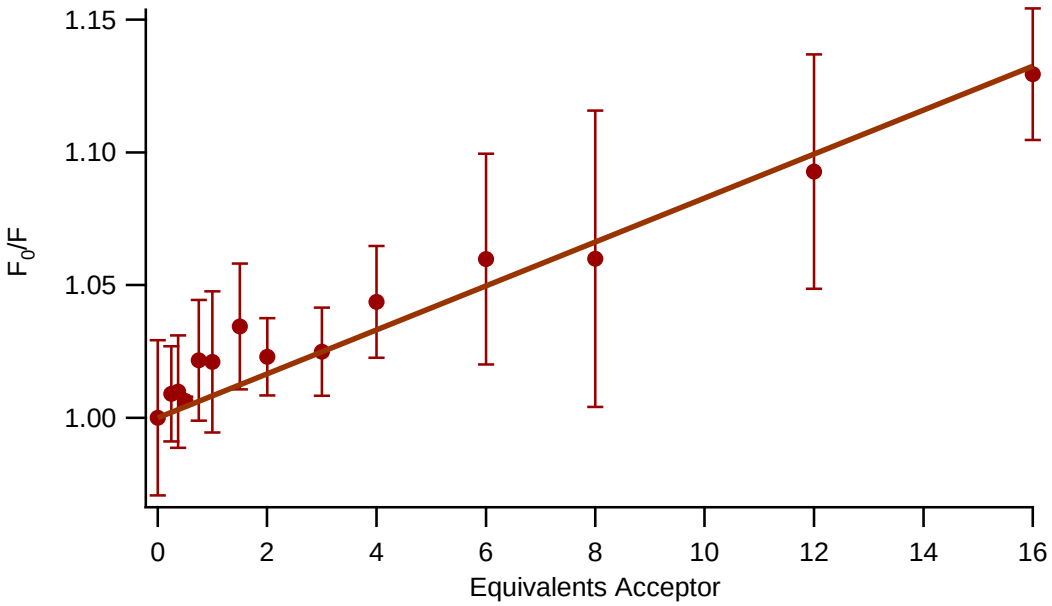

Figure S2: QD560 + mCherry (no polyhistidine insert) fit to the Stern-Volmer equation.

The Stern-Volmer quenching coefficients for the QD interactions with the non-his-tagged FPs are listed in Table S1. Also found in that table are the fit coefficients resulting from the curvefits for the His6-FP interactions with the EviTags shown in Figures 3 B,D, and F and 4 B, D, and F. Specifically, the fitted maximum quenching efficiencies (E_{max}), Hill coefficients (h), and nominal dissociation constants (K_D) are listed in Table S1. In all cases, the assays were performed in triplicate and the curves were fit to the means of the three trials. The standard deviations are fitting errors. The FRET efficiencies correspond well with what we expect from the max *E*s seen in Table 2 and the Hill coefficients are all very close to 1, indicating that there was no cooperative binding of the FPs to the QDs.

Table S1: Summary of Curvefit Coefficients

	Coefficients from Hill Equation fitting of His6-FP/QD data			Stern-Volmer Coefficient from FP/QD data
FRET Pair	E_{max}	K_D (nM)	**h**	K_{SV}
QD520-mOrange	0.827 ± 0.037	92.7 ± 9.6	1.256 ± 0.103	0.0129 ± 0.0012
QD520-tdTomato	0.960 ± 0.040	61.2 ± 6.6	1.085 ± 0.090	0.0097 ± 0.0022
QD540-tdTomato	0.920 ± 0.011	43.2 ± 1.3	1.301 ± 0.044	0.0392 ± 0.0068
QD520-mCherry	0.755 ± 0.028	72.0 ± 6.7	1.115 ± 0.077	0.0174 ± 0.0021
QD540-mCherry	0.852 ± 0.014	69.7 ± 3.0	1.101 ± 0.040	0.0104 ± 0.0015
QD560-mCherry	0.892 ± 0.016	59.8 ± 2.9	1.059 ± 0.044	0.0083 ± 0.0004

1. Deng, J.; Davies, D. R.; Wisedchaisri, G.; Wu, M.; Hol, W. G.; Mehlin, C., An improved protocol for rapid freezing of protein samples for long-term storage. *Acta Crystallogr D Biol Crystallogr* **2004,** 60, (Pt 1), 203-4.
2. Voet, D.; Voet, J. G., *Biochemistry*. 3rd ed.; J. Wiley & Sons: New York, 2004.
3. Lakowicz, J. R., *Principles of fluorescence spectroscopy*. 3rd ed.; Springer: New York, 2006.