Quantum Dot-Fluorescent Protein FRET Probe for Monitoring Intracellular pH

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Supporting Information

Protein Engineering, Expression, and Purification. Four fluorescent proteins were used in this study: His6-mOrange, His6-mOrange M163K, His6-mOrange-Arg9, and His6-mCherry-Arg9. The N-terminal histidine tags were used for protein purification via immobilized metal affinity chromatography (IMAC). mOrange and mOrange M163K are proteins with pH sensitivity in a physiologically-relevant pH range. A polyarginine sequence was appended to the C-terminus of mOrange and mCherry to facilitate endosomal uptake of probes constructed with these proteins.[1] mCherry was introduced as a control for the degradation of the GFP-like proteins in the late endosome/lysosome because it maintains its fluorescence at a significantly lower pH than the mOrange variants.

The His6-tagged mOrange and His6-mCherry were derived from pRSET-b mOrange and pRSET-b mCherry plasmids kindly provided by Roger Tsien's lab as previously described.[2] C-terminal polyarginine variants of each of these were created via PCR insertion using Phusion High-Fidelity Master Mix (New England Biolabs (NEB), Ipswich, MA). The mOrange M163K point mutation was introduced into the His6-mOrange plasmid via PCR mutagenesis using the following primers: 5'-P-CGA GAT CAA GAA AAG GCT GAA GCT G-3' and 5'-P-CCC TTC AGG GCG CCG TC-3'. Each of the proteins was expressed in *E.coli* and purified via affinity chromatography as previously described.[2] All plasmids were sequenced at the Nevada Genomics Center (UNR, Reno, NV) to confirm that the desired plasmid product was achieved.

Characterization of mOrange and mOrange M163K. Purified samples of His6-mOrange and His6-mOrange-M163K were subjected to amino acid analysis at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University to precisely determine the protein concentration. These exact concentration values were critical to accurately assessing the molar extinction coefficient of the protein using the Beer-Lambert Law. Absorbance spectra of 10 μM solutions of mOrange and mOrange M163K in 20 mM PBS, pH 10.0, were measured during titration with 1 N HCl on an Ultrospec 2100 *pro* UV/Vis spectrophotometer (GE

Healthcare Life Sciences, Pittsburgh, PA). The maximum molar extinction coefficients at 547 nm of 61,000 $M^{-1}cm^{-1}$ and 46,000 $M^{-1}cm^{-1}$ were determined for mOrange and mOrange M163K, respectively, at pH 10 (Supplementary Figure S1).

Circular dichroism (CD) spectroscopy was used to ensure that the changes in the protein optical properties were not a result of significant changes in the characteristic barrel structure of the GFP-like fluorescent proteins. As with the optical spectra, the protein was diluted in 20 mM PBS, pH 10.0 (although without the BSA supplementation) and was titrated with 1 N HCl. The mean residue ellipticity (MRE) of the His6-mOrange M163K mutant did not vary significantly with pH (Supplementary Figure S2b). His6-mOrange, in contrast, showed a clear pH-dependent decrease in the negative peak at 218 nm (Supplementary Figure S2a). The plot of the magnitude of the MRE at 218 nm with respect to pH (Supplementary Figure S2a, inset) shows a clear similarity to the plots of other pH-dependent characteristics of mOrange like absorbance.

CD spectra of the two titrated proteins were examined to ensure that the change in the protein optical properties was not a result of protein degradation. mOrange M163K showed negligible change in MRE as the pH was titrated down, but mOrange showed a subtle, but pH-dependent decrease in the size of its peaks. Although it is clear that the general structure of the protein remains intact, it is possible that the mOrange secondary structure relaxes somewhat in response to the change in pH. The ancestral DsRed, like mOrange M163K, contains a lysine at amino acid residue 163. Analysis of the crystal structure of DsRed indicated that the lysine forms a salt bridge with the phenolate oxygen of the chromophore.[3] This interaction, absent in mOrange, may add the stability that prevents the mOrange M163K CD spectra from shifting in response to changes in pH.

Excitation and emission spectra of mOrange and mOrange M163K were measured during titration on a Horiba Jobin Yvon Fluorolog-3 Spectraphotometer (Supplementary Figure S3). The excitation spectra was taken with an emission wavelength of 585 nm, 1 nm excitation bandwidth, 3 nm emission bandwidth, and 5 nm stepsize. The emission spectra was measured with excitation at 515 nm, 1 nm excitation bandwidth, 3 nm emission bandwidth, and 5 nm stepsize. Following titration with 1 N HCl, a bolus of 1 N NaOH was added to raise the pH significantly to test for the reversibility of the pH sensitivity. None of the titrations added more than 6% volume to the starting sample.

Characterization of the Conjugated Probes. Absorbance spectroscopy was used to determine the conjugate concentration and composition. The absorbance spectra of both the conjugated probe and unconjugated Qdots were measured in the Ultrospec 2100 pro UV/Vis spectrophotometer. Qdot concentrations were determined using the Beer-Lambert law and a molar extinction coefficient of 360,000 M-1cm-1 at 405 nm (Invitrogen product data sheet). Following normalization of the spectra at 405 nm, the Qdot spectrum was subtracted from the conjugate spectrum to produce the absorbance spectrum of the conjugated protein (Figure 1b). The previously determined molar extinction coefficient of mOrange (Supplementary Figure S1) was similarly applied to calculate the protein concentration, thereby indicating the average number of proteins per particle.

Dynamic Light Scattering (DLS) Measurements of QD and FRET Probes. Dynamic light scattering (DLS) measurements were performed using the DynaPro Nanostar from Wyatt Technology to determine the hydrodynamic diameters of samples with QD alone (Invitrogen ITK QD with 525 nm peak emission) and with QD-FP FRET probes (ITK QD 525 nm and His6-mOrange). Samples were prepared in 10 mM borate buffer at pH 10.0 to a final concentration of 150 nM. The mean values of the hydrodynamic diameter were reported from 30 replicate measurements per sample with 5 s lag time between measurements. Laser intensity was autocorrected and all measurements were taken at 25°C.

As shown in Supplementary Figure S4, the DLS measurements indicated that the ITK QDs used in this study have a hydrodynamic diameter of 14.5 ± 1.5 nm, while the QD-FP FRET probes have a hydrodynamic diameter of 25.1 ± 2.3 nm. The size distribution of the QD-FP FRET probes is similar to that of the QDs, suggesting that the conjugation of the mOrange proteins to single QDs was quite uniform. The results in Supplementary Figure S2 further confirm that the mOrange proteins are bound to the surface of the QDs.

Titration of the FRET Probes. The spectral characteristics of the FRET probes, consisting of mOrange, mOrange M163K, or mCherry conjugated to 525 nm-emitting QDs, were measured over a range of pHs by diluting 15 pmol of the probe in 500 μL 20 mM phosphate buffered saline (PBS) + 1% (w/v) bovine serum albumin (BSA), pH 10.0, and titrating with 1 N HCl. Fluorescence emission spectra were measured with a Horiba Jobin Yvon Fluorolog-3 Spectrofluorimeter with 400 nm excitation, 1 nm excitation bandwidth, 3 nm emission bandwidth, and 5 nm stepsize. Following the titration with HCl, a bolus of 1 N NaOH was added

to demonstrate the reversibility of the pH probe. A spectral plot containing all of the titration pHs measured is shown in Supplementary Figure S5. The titrations of the mOrange and mOrange M163K probes were repeated in triplicate to generate the plot in Figure 1e.

A solution of QDs at the same concentration and under the same conditions was titrated to demonstrate the pH-insensitivity of the QDs in this environment (Supplementary Figure S6a). The photoluminescence of an unconjugated mixture of QDs and mOrange at the same concentrations as those found in the probe samples was measured at alkaline pH (pH 10.0) to control for the direct excitation of the protein. No mOrange emission was detectable following excitation at 400 nm (Supplementary Figure S6b).

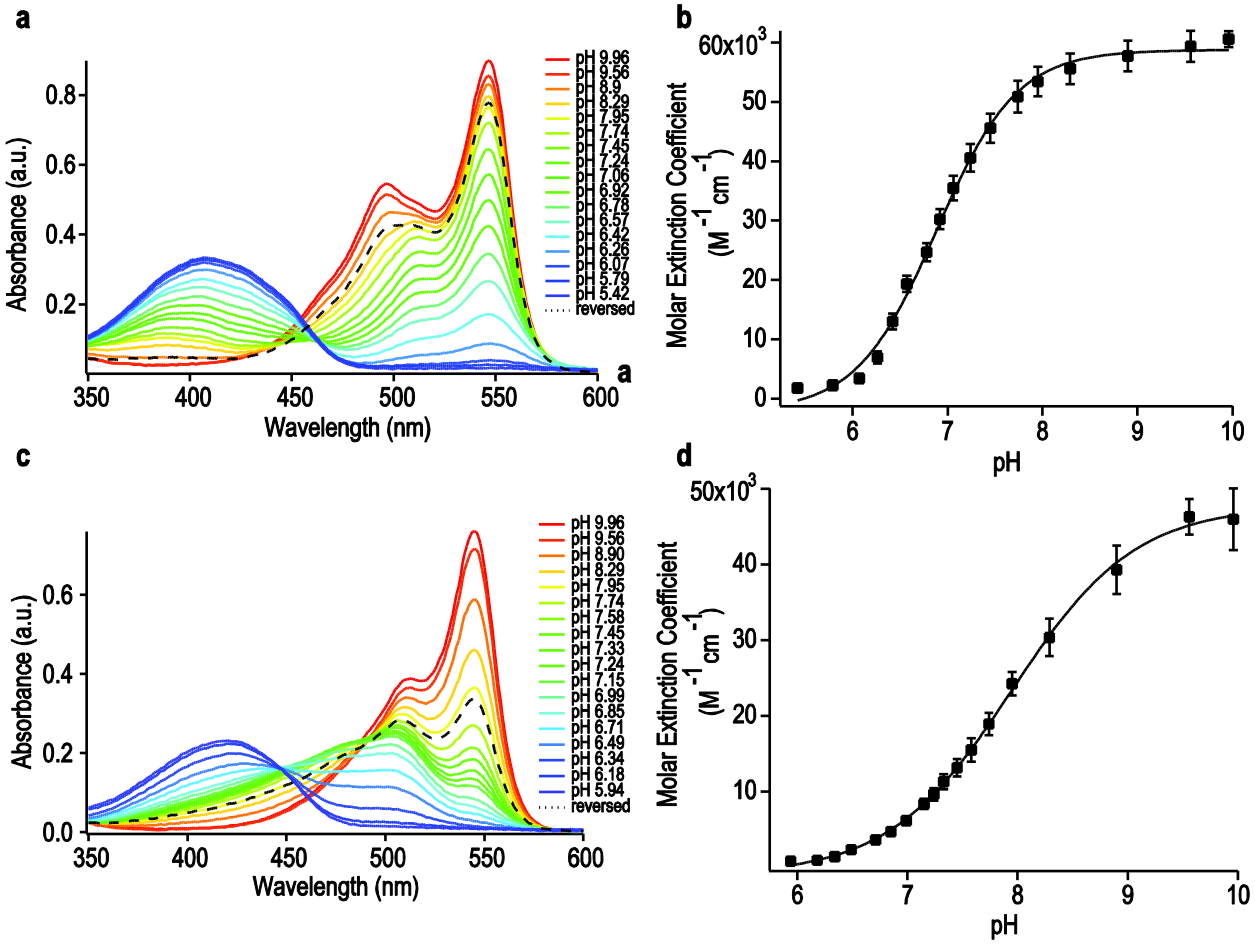
Titration of BCECF. The pH sensitivity of the pH-sensitive fluorophore BCECF was studied similarly to the FRET probe, through titration. Per the manufacturer instructions, the emission of BCECF at 525 nm was measured while the substrate was excited at its excitation peak and its excitation spectra isosbestic point, 490 nm and 440 nm, respectively. While the ratio of the emission at the two different excitation wavelengths shows a clear pH dependency (Supplementary Figure S7a), only a 4-fold change in the signal was seen over this pH range, compared to a 20-fold enhancement using the FRET probe (Figure 1f).

Titration of mCherry probe. A probe mCherry was titrated using the same protocol used for the mOrange and mOrange M163K probes, yielding a similar sigmoidal relationship between the acceptor-donor emission ratio and pH, but with a much lower ratio and a pK_a of 5.1 (Supplementary Figure S7b).

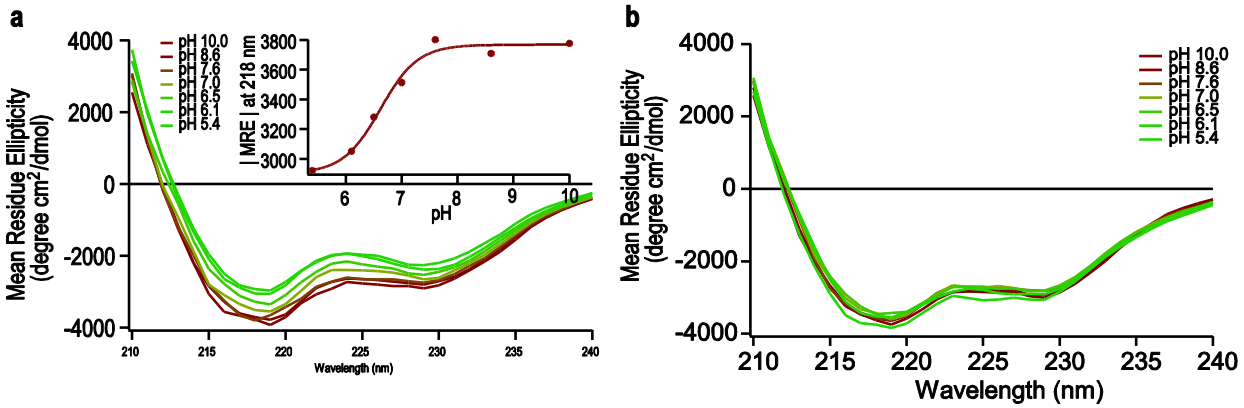
Cytotoxicity of QD-FP probes. A cytotoxicity study was carried out using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega) to determine the number of viable cells after probe internalization. QD-mOrange probes at concentrations of 50 nM, 100 nM, and 150 nM respectively were delivered into NIH/3T3 cells by incubation in Opti-MEM for 1 hour at 37˚C. The MTS/PMS solution mix was then added and cells were incubated for additional 2 hours at 37˚C. Absorbance at 490 nm was then measured which is directly proportional to the number of living cells in culture. As shown in Supplementary Figure S8, compared with control cells (without probe), cells with QD-FP probes internalized for at least 2 hours did not show any cytotoxicity even with relatively high probe concentrations.

References

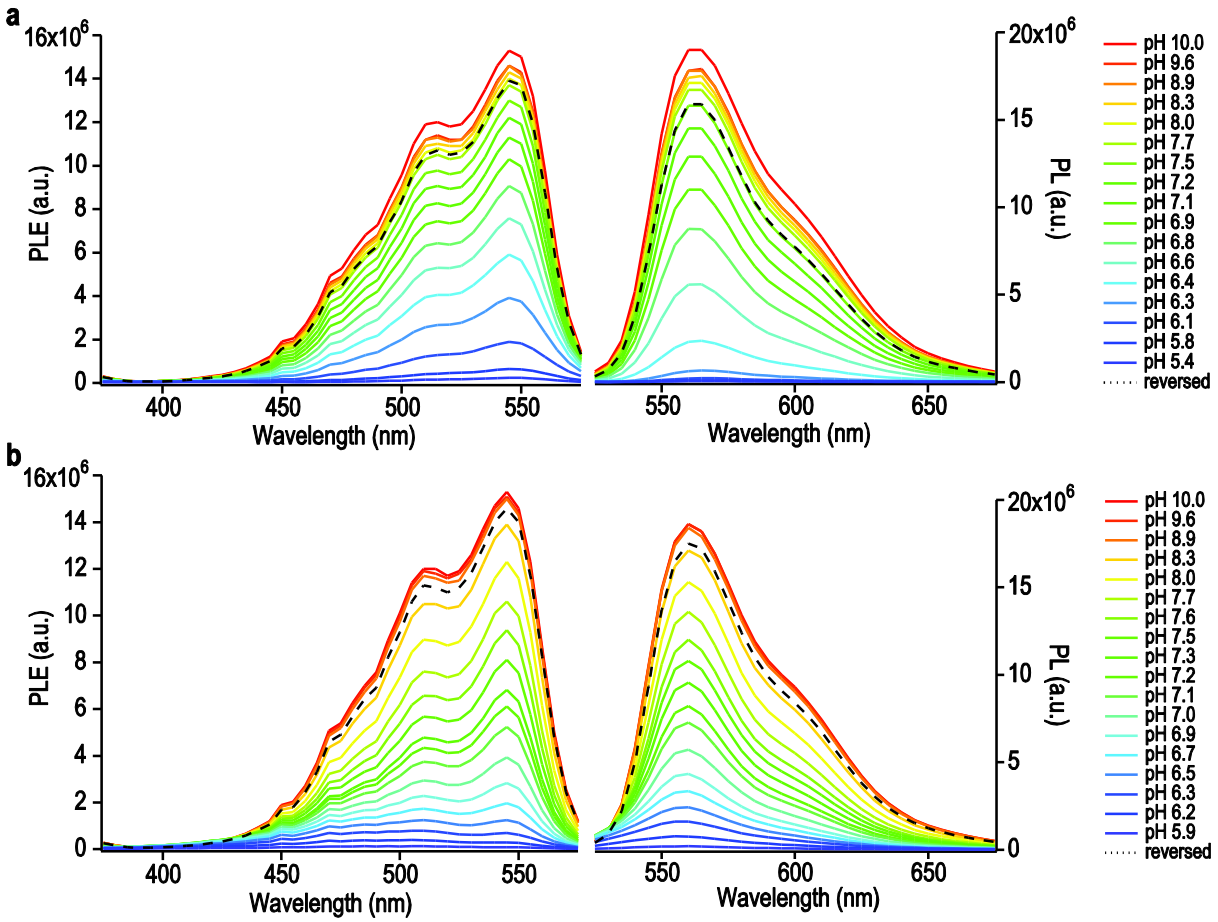
1. Medintz, I. L.; Pons, T.; Delehanty, J. B.; Susumu, K.; Brunel, F. M.; Dawson, P. E.; Mattoussi, H., Intracellular delivery of quantum dot-protein cargos mediated by cell penetrating peptides. 2008, 19, 1785-1795.
2. Dennis, A. M.; Bao, G., Quantum dot-fluorescent protein pairs as novel fluorescence resonance energy transfer probes. 2008, 8, 1439-45.
3. Yarbrough, D.; Wachter, R. M.; Kallio, K.; Matz, M. V.; Remington, S. J., Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-angstrom resolution. 2001, 98, 462-467.



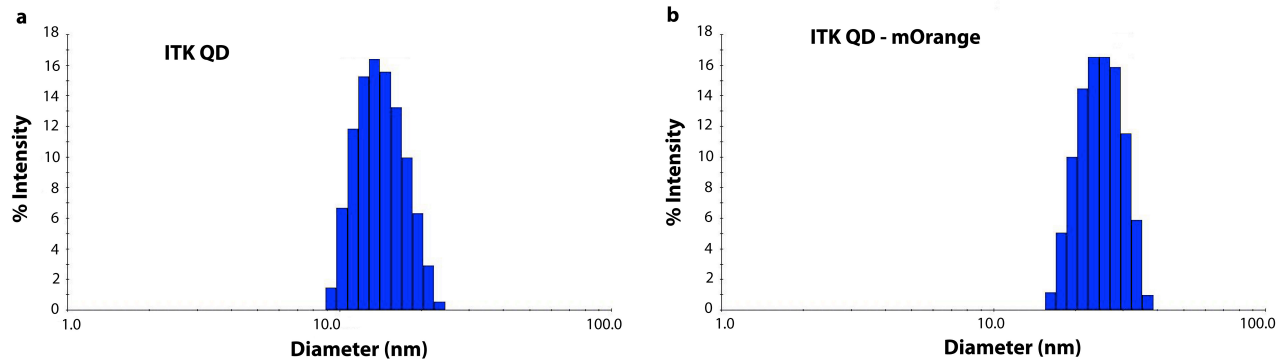
Supplementary Figure S1: pH-dependence of mOrange and mOrange M163K absorbance. The absorbance spectra of mOrange (a) and mOrange M163K (c) during titration with 0.1 M HCl. Bolus addition of NaOH demonstrated the reversibility of the spectral changes, indicated with the "reversed" spectra. Plots of the molar extinction coefficient versus pH of mOrange (b) and mOrange M163K (d) indicated pK_as of 6.9 and 7.9, respectively.



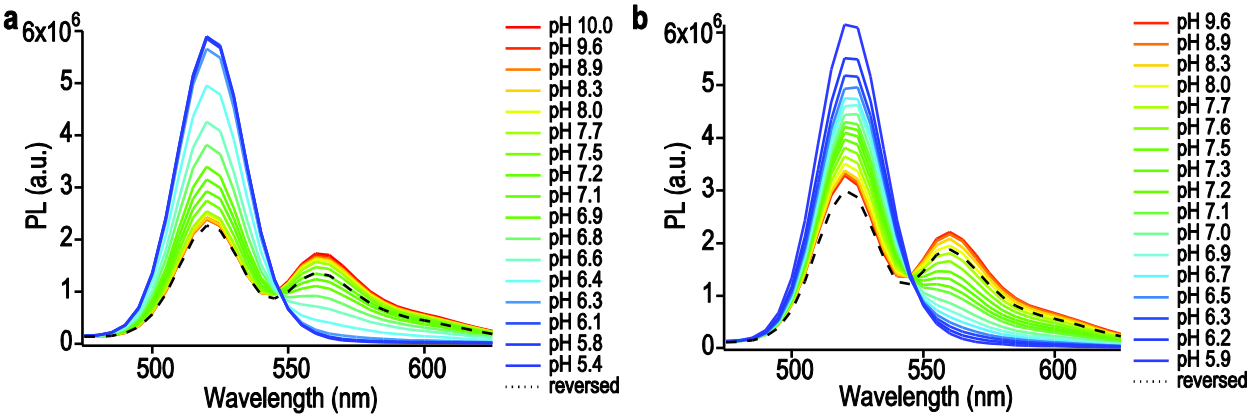
Supplementary Figure S2: pH-dependent CD spectra of mOrange and mOrange M163K. The CD spectra of His6-mOrange (a) and His6-mOrange M163K (b) were measured as the proteins were titrated with 1 N HCl. The magnitude of the mean residue ellipticity (MRE) of His6-mOrange at 218 nm is plotted against pH (a, inset).



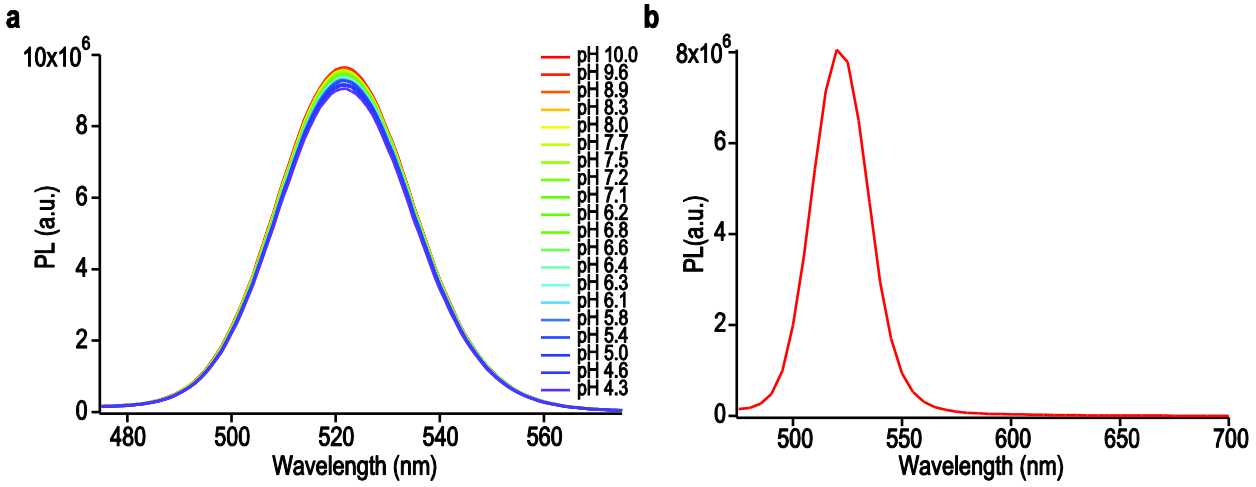
Supplementary Figure S3: Titration of the fluorescent proteins mOrange and mOrange M163K. The excitation and emission spectra of mOrange (a) and mOrange M163K (b) during titration with 0.1 M HCl. Bolus addition of NaOH demonstrated the reversibility of the spectral changes, indicated with the "reversed" spectra.



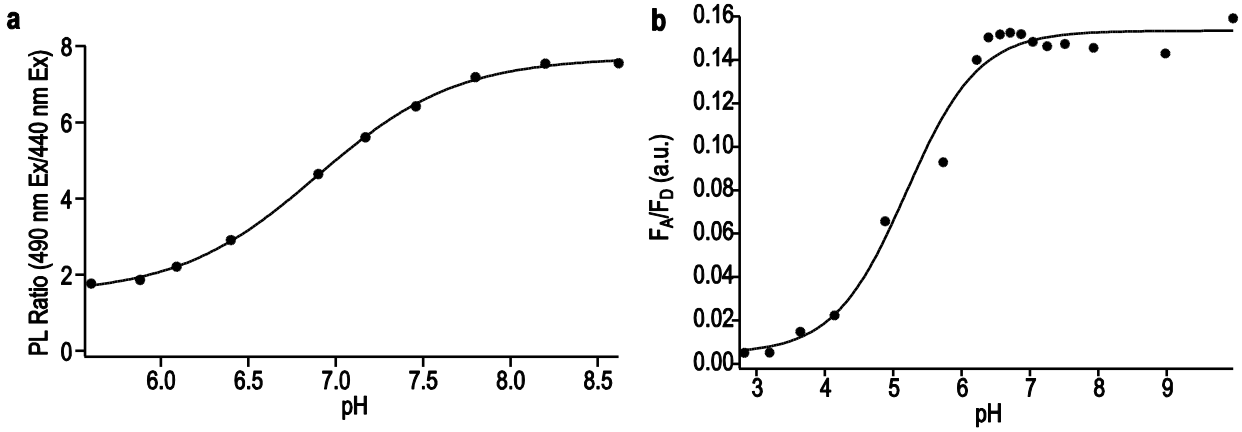
Supplementary Figure S4: Dynamic light scattering measurements of QD and QD-FP FRET probe. The hydrodynamic diameters of QDs only (a) and QD-mOrange FRET probes (b) were determined using dynamic light scattering (DLS). The QDs have a hydrodynamic diameter of 14.5 ± 1.5 nm, while the QD-FP FRET probes have a hydrodynamic diameter of 25.1 ± 2.3 nm.



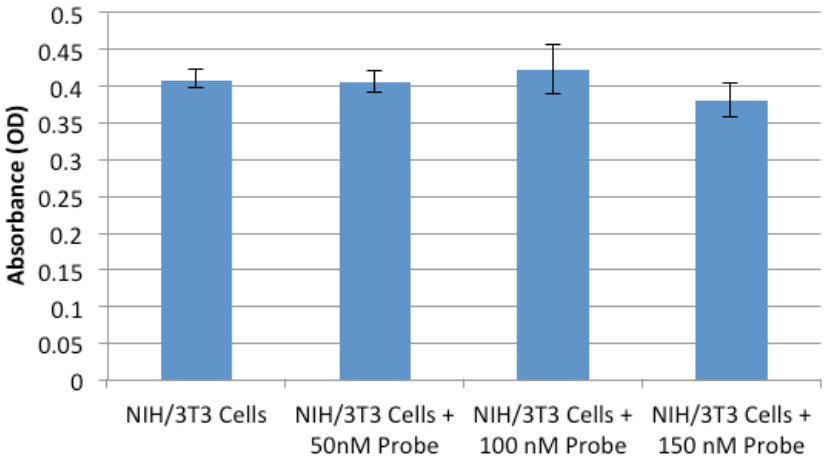
Supplementary Figure S5: Titration of the FRET probes. Full titration of the QD-FP FRET probes with 0.1 M HCl followed by bolus addition of NaOH ("reversed" spectra). (a) QD-mOrange; (b) QD-mOrange M163K.



Supplementary Figure S6: Fluorescence spectroscopy controls. (a) A 30 nM solution of 525 nm ITK Carboxyl Qdots in PBS + 1% (w/v) BSA, pH 10.0, was titrated with 1 N HCl to demonstrate the pH-insensitivity of the QDs in these conditions. (b) The PL of QDs and mOrange mixed but *not* conjugated (pH 10) shows that there is no significant direct excitation of the mOrange.



Supplementary Figure S7: Titrations of BCECF and mCherry-based FRET probe. (a) The pH-sensitive dye BCECF was titrated and the ratio of emission with excitation at the peak excitation wavelength (490 nm) and the isosbestic point of the excitation spectra (440 nm) plotted *versus* pH. A sigmoid fit of the plot indicated a pK_a of 6.9. (b) The ratio of the acceptor to donor emission of the mCherry-based FRET probe (605 nm emission/521 nm emission) versus pH. Sigmoidal curvefitting to the data indicates a pK_a of 5.1.



Supplementary Figure S8: Cytotoxicity of QD-FP probes. QD-mOrange probes at concentrations of 50 nM, 100 nM, and 150 nM respectively were delivered into NIH/3T3 cells by incubation in Opti-MEM for 1 hour at 37˚C. The MTS/PMS solution mix was then added and cells were incubated for additional 2 hours at 37˚C. Absorbance at 490 nm was measured which is directly proportional to the number of living cells in culture.