Novel Multistep BRET-FRET Energy Transfer using Nanoconjugates of Firefly Proteins, Quantum Dots, and Red Fluorescent Proteins

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Electronic Supplementary Information

Materials and Methods.

Chemicals & Materials: Cadmium oxide (CdO, 99.99%), trioctylphine (TOP, 90%), trioctylphine oxide (TOPO, 90%), octadecene (ODE, 90%), methylphosphonic acid (MPA, 98%), sulfur (S, 100 mesh), zinc acetate ($ZnAc_2$, 99.99%), octylamine (99%), olelyamine (90%), L-histidine (His, >99.8%), sodium borohydrate ($NaBH_4$, >96%), sodium tetraborate (99.5%), boric acid (>99.5%), toluene (≥99.5%), chloroform (>99.8%), methanol (>99.8%), acetone (99.5%) were purchased from Sigma Aldrich. Selenium (Se, 200 mesh 99.99%) was purchased from Alfa Aesar. Octadecylphosphonic acid (ODPA, 98%) and hexylphosphonic acid (HPA, 98%) were purchased from Strem Chemicals. Ultrapure water (18.2 MΩ) was provided from a Sartorius Stedim Arium 61316 reverse osmosis unit combined with an Arium 611DI polishing unit. The Mg-ATP (bacterial source) was purchased from Sigma-Aldrich, and restriction endonucleases from New England Biolabs (Beverly, MA). Firefly luciferin (LH_2) and benzothiophene luciferin ($BtLH_2$) were generous gifts from Promega (Madison, WI).

Synthesis of CdSe Quantum Dot: CdSe QDs were synthesized following traditional methods with slight modification *(S1)*. In a typical synthesis, CdO (0.06 g, 0.47 mmol), TOPO (3.00 g, 7.7 mmol), ODPA (0.28 g, 0.84 mmol) and 2 ml of ODE were mixed and heated to 150 °C under vacuum for 1 hour. Then in an inert atmosphere, the reaction mixture was heated to 330 °C in order to dissolve CdO. When the solution changed from red-brown to clear and colorless, the temperature was increased to 350 °C. Once the temperature stabilized an injection of Se (0.05 g, 0.63 mmol) and TOP (1.5 ml) was prepared in the glove box and injected into the reaction mixture, then immediately cooled to room temperature. In order to prevent solidification, a small amount of toluene was added at 60 °C. The QDs were then purified free of excess ligands via multiple methanol extraction and precipitations. This process was repeated twice, and the final QD product was dried and dispersed in toluene.

Synthesis of CdSe/ZnS Quantum Dot with dot-in-dot Morphology: Thin layer of ZnS was grown on CdSe QDs following the SILAR approach *(S2)*. This was achieved at 200 °C in a mixture of CdSe QDs, TOPO (1.0 g) and ODE (5.0 ml). The zinc precursor (0.2 M $ZnAc_2$ dissolved in octylamine) and sulfur precursor (0.2 M S dissolved in ODE) were injected slowly and sequentially, 100-200µl injections at a time waiting 10 minutes between injections. After the final injection, the reaction mixture was allowed to anneal for 30 minutes. The total volume of

precursor used was calculated based on the size of the QDs and the desired shell thickness. Finally the QDs were purified free of excess ligands via multiple methanol extraction and precipitations then dispersed in toluene.

Synthesis of CdSe/Cds Quantum Rod with dot-in-rod Morphology: CdSe QD cores were synthesized similarly to the CdSe QD described above. The selenium precursor was injected at 350 °C, then reaction mixture was taken off the heating mantle and cooled to room temperature as soon as a color change was observed, resulting in smaller QDs (d = 2.5 nm). The QD cores were then purified as described above, and used for CdS rod shell was grown. In a typical experiment, a 25 ml four neck flask was filled with CdO (0.06 g, .47 mmol), TOPO (3.00 g, 7.7 mmol), ODPA (0.28 g, 0.84 mmol), HPA (0.08 g, 0.48 mmol) and 2 ml of ODE and heated to 150 °C under vacuum for 1 hour. Then in an inert atmosphere, the reaction was heated to 330 °C until the solution turned clear and colorless, then the temperature was increased to 350 °C. Once the temperature stabilized a mixture of 8 x 10^{-8} moles of dried CdSe QDs and sulfur (0.12 g, 3.7 mmol) dissolved in 2.0 ml of TOP in the glove box and quickly injected into the reaction mixture and annealed for 10 min. The resulting CdSe/CdS QR(556) with *dot-in-rod* morphology. Finally the QRs were purified free of excess ligands via multiple methanol extraction and precipitations then dispersed in toluene.

Histidine-Mediated Phase transfer: In order to phase transfer the hydrophobic QDs and QRs into aqueous buffers we employed a phase transfer technique we recently developed *(S3)*. The organic ligands of the QD were directly exchanged with the molecule *L*-histidine (His), rendering them hydrophilic, and both colloidally and optically stable. This histidine-mediated phase transfer method was achieved by adding a ~5000-fold [His]:[QD] molar excess. Initially a histidine solution was prepared by dissolving histidine in a basic 3:1 MeOH/H_2O solution. Then ~5000-fold excess of the histidine solution was added to cleaned QDs dispersed in chloroform and vortexed for 1 minute. This resulted in the QDs being transferred to the aqueous layer, to 10 mM borate buffer (pH 8.3). Excess organic ligands were back-extracted by addition of fresh chloroform, vortexing, and decanting of the organic solution. This extraction procedure was repeated at least four times. Then excess histidine molecules were removed by rinsing the hydrophilic QRs with 10 mM borate buffer using a 100kDa molecular weight centrifugal filter (Millipore). Finally the QDs were dispersed in 10 mM borate buffer and refrigerated before use. The QD concentration was calculated as described below.

TagRFP Expression: TagRFP was cloned using the pPA-TagRFP-N vector as template DNA. The gene was ligated into pET15b for expression, as well as the addition a N-terminal hexa-histidine tag. The constructed vector was then transformed into BL21 Gold (DE3) *E. coli* and expressed overnight under 0.01 mM IPTG induction. The cells were then harvested, sonicated, and the soluble fractions was purified utilizing immobilized metal affinity chromatography (IMAC). The samples were analyzed using SDS-PAGE, western blotting, and concentrations were calculated using the extinction coefficient at 555 nm *(S4)*.

Ppy Expression: The Ppy WT was expressed as a GST-fusion protein and purified by affinity chromatography and stored as described (*S5*) in detail previously. The plasmids for 6xHis-Ppy GRTS was constructed by excising the corresponding genes for Ppy GRTS (*S5*) from the pGEX-6P-2 vector and ligating them into a modified pQE30 expression vector using

previously described procedures (*S6*). The His-tagged proteins were expressed, purified and stored using procedures described (*S6*) elsewhere. The found molecular masses (Da) of the proteins not previously reported were within the allowable experimental error (0.01%) of the calculated values (in parenthesis): 6xHis-Ppy GRTS, 61 996 (62 002). Protein concentrations were determined with the Bio-Rad Protein Assay system using BSA as the standard. DNA sequencing to verify the ligations was performed at the W. M. Keck Biotechnology Laboratory at Yale University. Specific activity and steady state kinetics measurements were determined as previously reported (*S6-8*) except that the final LH_2 concentration was 0.3 mM and integration times were 15 min. Bioluminescence emission spectra were obtained using methods and equipment previously described.(*S6*) Mass spectral analyses were performed by tandem HPLC-electrospray ionization mass spectrometry (LC/ESIMS) using a ThermoFinnigan Surveyor HPLC system and a ThermoFinnigan LCQ Advantage mass spectrometer and previously developed conditions for protein mass determinations (*S9*).

PpyGRTS-QD-RFP Conjugation: To construct the PpyGRTS-QD-RFP BRET-FRET nano-conjugates, the His-functionalized QDs were incubated with the hexa-histidine tagged PpyGRTS in 10 mM borate buffer at a 1:1 ratio on ice. Then RFP was added to the mixture at varying loading ratios (*L*) = [tagRFP]:[Ppy] and incubation was allowed to proceed for at least 15 minutes before BRET analysis.

Instrumentation:

UV–Vis spectrophotometry (UV–Vis): The UV–Vis measurements were collected on a Varian Cary100 Bio UV–Vis spectrophotometer between 200 and 900 nm. The instrument is equipped with an 8-cell automated holder with high precision Peltier heating controller.

Photoluminescence (PL) and Bioluminescence: The QR PL emission and Ppy bioluminescence was collected on a Fluoromax-4 photon counting spectrofluorometer (Horiba Jobin Yvon). The instrument is equipped with a 150 W xenon white light excitation source and computer-controlled monochromator. The detector is a R928P high sensitivity photon counting detector that is calibrated to emission wavelength. All PL emission and excitation spectra were collected using both wavelength correction of source intensity and detector sensitivity. The excitation wavelength for QR quantum yield calculations was 400 nm using 3 nm excitation and emission slits unless otherwise noted. The bioluminescence and BRET were collected on a Varian Cary-Eclipse spectrophotometer in bioluminescence /chemiluminescence mode using a 96-well plate reading accessory. The instrument was corrected for detector sensitivity by comparison of fluorescence standard emission intensities (500 – 800 nm) with the corrected detector on the Fluoromax-4 spectrophotometer (above).

Transmission electron microscopy (TEM): TEM measurements were performed on a JEOL 2000EX instrument operated at 100 kV with a tungsten filament (SUNY-ESF, N.C. Brown Center for Ultrastructure Studies). Negative staining was achieved using phosphotungstic acid. Particle size and aspect ratio were analyzed manually with statistical analysis per- formed using ImageJ software on populations of at least 100 counts.

BRET Measurement and Analysis: In a typical BRET experiment, a mixture of 100 μL of 500 μM $BtLH_2$ (modified firefly luciferin) and 30 μL of 8.66 mM Mg-ATP in 50 mM 2-Amino- 2- methyl- 1, 3- propane buffer (pH 9.1) or a mixture of 100 μL of 91 μM LH_2 (firefly luciferin) and 30 μL of 8.66 mM Mg-ATP in 25 mM gly-gly buffer (pH 7.8) is quickly added to the Ppy-QD conjugate solution in a 96-well plate and bioluminescence emission is immediately collected. White 96-well plates were employed, with volumes ranging from 50-200 μL. Bioluminescence spectra were collected every 15 seconds for 7.5 minutes. The presented BRET results are the average of the first five spectra collected over 1.5 min after addition of $BtLH_2$ or LH_2. Control experiments showed that the BRET ratio did not change over the course of the typical BRET decay. Finally, the BRET efficiencies of the systems were calculated as BRET ratio (*BR*), which is defined as the ratio of peak area of the acceptor and donor emission respectively. Peak area was calculated by spectral deconvolution of each spectrum using the data analysis package in Igor Pro (Wavemetrics Inc.).

Calculations:

QD and QR Concentration*:* The concentrations of the QD cores were calculated based on UV-vis optical absorption measurements of the QD first band edge absorption (1s-1s) intensity using QD size dependent optical extinction coefficients (ε) *(S11)*. The final concentrations were obtained using the Beer-Lambert equation, Abs = εbc; where ε is the estimated extinction coefficient (M^{-1} cm^{-1}), b is the path length, and c is concentration.

Quantum Yield (QY)*:* The QR photoluminescence quantum yields (QY) were calculated based on comparison to a reference dye using standard methods (equation 1) *(S12)*:

$$QY_{qdot}(\%) = QY_R\left(\frac{Abs_R}{Abs_{qdot}}\right)\left(\frac{PL_{qdot}}{PL_R}\right)\left(\frac{\eta^2{}_{qdot}}{\eta^2{}_R}\right) \quad (1)$$

where QY_R is the reference dye quantum yield (Rhodamine 6G = 95%), Abs_R and Abs_{QD} are the optical absorption at specific excitation for the reference dye and QR samples respectively. Here, careful attention was paid to prepare samples with optical absorption below 0.10 in order to limit inner filter effects. PL_R and PL_{QD} correspond to the total area of the PL emission after wavelength dependent calibration of both the excitation source, and photoluminescence detector, as well as after PL spectra baseline correction.

Förster Resonance Energy Transfer (FRET) Calculations: In this study, the bioluminescence resonance energy transfer (BRET) constants were calculated in the identical manner to FRET. In FRET, the Förster distance (R_0) is calculated using equation 2 *(S12-13)*:

$$R_0^{\,6} = 8.8\times10^{23}\,k_p^{\,2}\eta_D^{\,-4}Q_D J \quad (2)$$

where η_D refractive index of the medium (η_D = 1.33), k_p is the polarization parameter (k_p = 2/3), Q_D is the donor quantum yield QY(Ppy($BtLH_2$) ≈ 10 %, and J is the spectral overlap integral. The J value can be calculated using equation 3:

$$J = \int f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \quad (3)$$

where λ is the defined wavelengths of the donor-acceptor spectral overlap (λ = 450 – 650 nm), and $f_D(\lambda)$ is the integrated donor emission and $\varepsilon_A(\lambda)$ represents the integrated acceptor absorption using the acceptor extinction coefficient ($\varepsilon_A = 1.00\times10^5$ $M^{-1}cm^{-1}$). The values of R_0 = 4.2 nm, and $J = 3.2\times10^{-13}$ cm^6 were calculated using equations 2 and 3 as well as the software *PhotoChemCAD* for Ppy and RFP.

Using the R_0 values calculated above, the FRET efficiency, E, was calculated using equation 4 *(S13-14)*:

$$E = 1 - \frac{F_{DA}}{F_D} = \frac{R_0^{\,6}}{R_0^{\,6} + r^6} \quad (4)$$

where F_{DA} is donor fluorescence in the presence of acceptor, and F_D is fluorescence of the donor without acceptor.

Supporting Tables

Table S1: Optical and FRET parameters for BRET - FRET nanosystem using QD(556).

	Ppy(BTLH$_2$) – QD – RFP			
	Acceptor Absorption[1]		**FRET Calculation[2]**	
	λ_A (nm)	**ε ($M^{-1}cm^{-1}$)**	***J*** **(cm^6)**	***R_0*** **(nm)**
Ppy – tagRFP	555	1.00×10^5	3.2×10^{-13}	4.2
Ppy – QD	532	8.40×10^4	3.5×10^{-13}	4.3
QD – tagRFP	555	1.00×10^5	5.6×10^{-13}	4.7

[1)] First absorption maxima (λ_A), and calculated extinction coefficient (ε). [2)] Calculated using Ppy QY of 10 %, QD(556) QY of 10 %.

Table S2: Optical and FRET parameters for BRET - FRET nanosystem using QR(556).

	Ppy(BTLH$_2$) – QR – RFP			
	Acceptor Absorption[1]		**FRET Calculation[2]**	
	λ_A (nm)	**ε ($M^{-1}cm^{-1}$)**	***J*** **(cm^6)**	***R_0*** **(nm)**
Ppy – tagRFP	555	1.00×10^5	3.2×10^{-13}	4.2
Ppy – QR	531	8.20×10^4	1.7×10^{-12}	5.6
QR – tagRFP	555	1.00×10^5	5.1×10^{-13}	4.7

[1)] First absorption maxima (λ_A), and calculated extinction coefficient (ε). [2)] Calculated using Ppy QY of 10 %, QR(556) QY of 12 %.

Table S3: Optical and FRET parameters for BRET - FRET nanosystem using QD(530) with the $BtLH_2$ substrate.

	Ppy($BTLH_2$) – QD – RFP			
	Acceptor Absorption[1]		**FRET Calculation[2]**	
	λ_A (nm)	**ε ($M^{-1}cm^{-1}$)**	***J*** **(cm^6)**	***R_0*** **(nm)**
Ppy – tagRFP	555	1.00×10^5	3.2×10^{-13}	4.2
Ppy – QD	517	6.80×10^4	1.9×10^{-13}	3.9
QD – tagRFP	555	1.00×10^5	3.3×10^{-13}	5.0

[1)] First absorption maxima (λ_A), and calculated extinction coefficient (ε). [2)] Calculated using Ppy QY of 10 %, QD(530) QY of 27 %.

Table S4: Optical and FRET parameters for BRET - FRET nanosystem using QD(530) with the LH_2 substrate.

	Ppy(LH_2) – QD – RFP			
	Acceptor Absorption[1]		**FRET Calculation[2]**	
	λ_A (nm)	**ε ($M^{-1}cm^{-1}$)**	***J*** **(cm^6)**	***R_0*** **(nm)**
Ppy – tagRFP	555	1.00×10^5	4.0×10^{-13}	5.4
Ppy – QD	517	6.80×10^4	-[3]	-[3]
QD – tagRFP	555	1.00×10^5	3.3×10^{-13}	5.0

[1)] First absorption maxima (λ_A), and calculated extinction coefficient (ε). [2)] Calculated using Ppy QY of 32 %, QD(530) QY of 27 %. [3)] Since the QD(530) first band edge absorption at 517 nm is below that of Ppy+LH2 emission at 547 nm, energy transfer is prohibited.

Supporting Figures

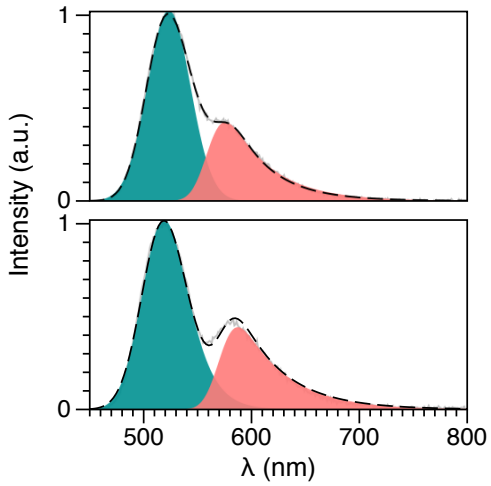

Figure S1: Representative emission spectra for a solution of free Ppy+$BtLH_2$ and tagRFP at molar ratios of [tagRFP] : [Ppy] = 1 (i), and 2 (ii), in the absence of QD linkers. Results indicate that a small degree of tagRFP emission occurs due to inner filter effects of Ppy+$BtLH_2$ emission. These values (BR ~ 0.4 – 0.5) are much lower however than when Ppy+BtLH2 and tagRFP are bound to QD(565) (Fig. 1), QR(565) (Fig. 2), QD(530) (Fig. 3), and QD(530) when LH_2 is used (Fig. 4).

Supporting References

S1. (a) L. Qu, X. Peng, *J. Am. Chem. Soc.* 2002, **124**, 2049. (b) (b) D. V. Talapin, J. H. Nelson, E. V. Shevchenko, S. Aloni, B. Sadtler, A. P. Alivisatos, *Nano letters* 2007, **7**, 2951. (c) R. Alam, M. M. Maye, *Inorganica Chimica Acta.* 2012, **380**, 114.

S2. J. J. Li, Y. A. Wang, W. Z. Guo, J. C. Keay, T. D. Mishima, M. B. Johnson, X. G. Peng, *J. Am. Chem. Soc.* 2003, **125**, 12567.

S3. (a) H. Han, J. Zylstra, M. M. Maye, *Chem. Mater.* 2011, **23**, 4975. (b) J. Zylstra, J. Amey, N. J. Miska, L. Pang, C. R. Hine, J. Langer, R. P. Doyle, M. M. Maye, *Langmuir* 2011, **27**, 4371. (c) J. Zylstra, R. Alam, H. Han, R. P. Doyle, M. M. Maye. *ACS Symposium Series*. 2012, **1112**, 59.

S4. E. M. Merzlyak , J. Goedhart, D. Shcherbo, M. E. Bulina, A. S. Shcheglov, A. F. Fradkov, A. Gaintzeva, K. A. Lukyanov, S. Lukyanov, T. W. Gadella, D. M. Chudakov, Nat. Methods, 2007, **4**, 555.

S5. B. R. Branchini, D. M. Ablamsky, J. M. Rosenman, L. Uzasci, T. L. Southworth, M. Zimmer, *Biochemistry* 2007, **46**,13847.

S6. B. R. Branchini, D. M. Ablamsky, J. C. Rosenberg, *Bioconjugate Chem.* 2010, **21**, 2023.

S7. B. R. Branchini, T. L. Southworth, N. F. Khattak, E. Michelini, A. Roda, *Anal. Biochem.* 2005, **345**, 140.

S8. B. R. Branchini, D. M. Ablamsky, J. M. Rosenman, L. Uzasci, T. L. Southworth, M. Zimmer, *Biochemistry* 2007, **46**, 13847.

S9. B. R. Branchini, J. C. Rosenberg, D. M. Fontaine, T. L. Southworth, C. E. Behney, L. Uzasci, *J. Am. Chem. Soc.* 2011, **133**, 11088.

S10. C. C. Woodroofe, P. L. Miesenheimer, D. H. Klaubert, Y. Kovic, J. C. Rosenberg, C. E. Behney, T. L. Southworth, B. R. Branchini. *Biochemistry* 2012, **51**, 9807.

S11. (a) W. W. Yu, L. H. Qu, W. Z. Guo, X. G. Peng, *Chem. Mater.* 2003, **15**, 2854. (b) Y. Liu, M. Kim, Y. Wang, Y. A. Wang, X. Peng, *Langmuir* 2006, **22**, 6341.

S12. J. R. Lakowicz, Principles of Fluorescence Spectroscopy (3rd ed.), Springer, New York, 2006. pp. 443-476.

S13 A. R. Clapp, I. L. Medintz, H. Mattoussi, *Chemphyschem* 2006**,** **7**, 47.