Supporting Information

Reengineering the Optical Absorption Cross-section of Photosynthetic Reaction Centers

Palash K. Dutta,[1,2] Su Lin, [1,2] Andrey Loskutov,[2] Symon Levenberg, [1,2] Daniel Jun, [3] Rafael Saer,[3] J. Thomas Beatty,[3] Yan Liu,[1,2] Hao Yan,*[1,2] Neal Woodbury*[1,2]

[1]Department of Chemistry and Biochemistry and [2]The Biodesign Institute, Arizona State University, Tempe, AZ 85287, United States; [3]Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada.

Methods and experimental details.

Figures S1-S10.

Tables S1-S4.

References cited in the SI.

Reaction center mutations: The RC we used here contains a total of eight mutations relative to the wild type. Five of the mutations served to replace the five wild type cysteines with serine and alanine, and the remaining three mutations introduced cysteines at the points of interest. The eight mutations are as follows: (H)C156A, (H)C234S, (L)C92S, (L)C108S, (L)C247S, (L)E72C, (L)N274C and (M)E100C. The RC contains a six histidine tag at the C-terminus of the H subunit to facilitate purification with a Ni-sepharose affinity column.[1]

RC isolation and purification: RCs were isolated from a mutant which was derived from *R. sphaeroides 2.4.1* using a modification of a procedure previously published.[1,2] In short, the cells were grown at 30° C in 2 L of modified LB medium containing 810 μM $MgSO_4$, 510 μM $CaCl_2$, and 4 mM NaCl,[3] using 2 L Erlenmeyer flasks, shaken at 250 rpm. After 3.5 days the cells were centrifuged at 9000 *g* and resuspended overnight in 50 mM phosphate buffer (pH 8) containing 150 mM NaCl. The cells were then lysed using a French Press followed by addition of small amount of DNase. Unbroken cells were removed via centrifugation at 9000 *g* and the remaining supernatant was treated with imidazole (final concentration 5 mM) and N, N-Dimethyldodecylamine N-oxide (LDAO, final concentration 0.6% by weight). After 15 min of incubation, the solution was centrifuged at 14000 *g* followed by Ni-sepharose purification of the RC from the supernatant. The following paragraph lists the buffers used in the Ni-sepharose column purification.
Wash buffer: 50 mM phosphate buffer, pH 8, 0.1% LDAO, 150 mM NaCl, 5 mM imidazole
Elution buffer: 50 mM phosphate buffer, pH 8, 0.1% LDAO, 150 mM NaCl, 100 mM imidazole
Column wash buffer: 50 mM phosphate buffer, pH 8, 0.1% LDAO, 300 mM NaCl, 250 mM imidazole. The eluted protein was further purified by dialysis against 15 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA containing 0.025% LDAO) overnight to remove excess imidazole and LDAO. The concentration of the purified RC was measured using absorbance at 804 nm (extinction coefficient ~288000 $M^{-1}cm^{-1}$).[4]

Quinone removal procedure: A concentrated RC solution was diluted such that the OD_{804} was ~ 0.1 (corresponding to 0.37 μM) at a final volume of 200-250 mL in high-concentration LDAO buffer (10 mM Tris-HCl, 4% LDAO, 10 mM 1,10-phenanthroline, pH 8). This solution was stirred for 2 hrs at 25° C, and applied to a Di-Ethyl-Amino-Ethyl (DEAE) column at 4 mL/min loading speed, followed by washing with high LDAO buffer for 2 hrs and a flow rate 2 mL/min. The column was then washed with a buffer containing a low concentration of LDAO (15 mM Tris-HCl, 0.025% LDAO, 1 mM EDTA, pH 8) for 1 hr and then the RC was eluted with 15 mM Tris-HCl, 0.25 M NaCl, 0.1% was, 1 mM EDTA, pH 8 followed by overnight dialysis . The quinone-depleted RC (Q_{del}RC) was used in transient absorbance experiments to observe the trapped charge-separated state.

RC-dye conjugation procedure: 1 mg of dye (AF647, AF660 or AF750) functionalized with a maleimide group (Invitrogen) was dissolved in DMSO to make a 15 mM solution. A solution of 50-70 μM RC in 1× PBS (pH 7.4, containing 0.025% LDAO) was first treated with 10-fold excess of 50 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl; Thermo Scientific) to reduce possible disulfide bonds, and the protein was washed four times with the 1× PBS buffer mentioned above using an Amicon centrifugal filter (50 kD molecular weight cut-off). The TCEP-treated RC was added to the dye solution with a RC-dye molar ratio of 1:15 and incubated overnight at 4° C. The maleimide group of the dye is expected to link to thiol of

cysteines on the RC surface through a coupling reaction. Excess glutathione (1.5 fold to the dye) was added to consume the unreacted thiol-reactive dye molecules. The mixture was then washed three times using Amicon centrifugal filter (50 kD molecular weight cut-off) with 1× PBS buffer containing 0.025% LDAO to remove excess dye molecules. The sample was further purified by Ni-sepharose chromatography and dialyzed overnight against tris buffer (15 mM Tris-HCl, pH 8, 0.025% LDAO, 150 mM NaCl, 1 mM EDTA).

BSA-dye conjugation and purification: A 50 μM solution of BSA (Sigma) in 1×PBS (pH 7.4, 0.025% LDAO) was treated with 50 mM TCEP-HCl (5 fold molar excess) and washed with 1× PBS buffer mentioned above using an Amicon centrifugal filter (30 kD molecular weight cut-off), and then the RC was added to the dye solution with a BSA-dye molar ratio of 1:15. The reaction mixture was kept overnight at 4^{o} C. Excess glutathione was added to consume the unreacted dye molecules. The mixture was then washed three times using Amicon centrifugal tube (30 kD molecular weight cut-off filter) with 1×PBS buffer containing 0.025% LDAO to remove excess dye molecules. The sample was then dialyzed overnight against tris buffer (15 mM Tris-HCl, pH 8, 0.025% LDAO, 150 mM NaCl, 1 mM EDTA). The concentration of the purified BSA was measured using absorbance at 279 nm (extinction coefficient ~ 44308 $M^{-1}cm^{-1}$).[5]

MALDI-TOF procedure: A mixture of formic acid/water/isopropyl alcohol (IPA) (3:1:2) was prepared 4 hrs in advance prior to the sample preparation. A saturated solution of α-cyano-4-hydroxycinnamic acid (4HCCA) was then prepared in the above mixture and centrifuged at 14000 g for 6 min to remove any matrix micro-aggregates. The supernatant was transferred to a fresh tube and named Matrix 2. A 20 μM solution of RC was diluted to 5 μM with 0.1% trifluoroacetic acid (TFA) (v/v) followed by dilution in matrix 2 (1:5, v/v). Next a saturated solution of 4HCCA in 1:1 (v/v) mixture of TFA aq. 0.2%/acetonitrile was prepared and centrifuged to remove any matrix aggregates. The supernatant was transferred ito a new tube, diluted 4-fold with IPA, and named Matrix 1. 20 μL of Matrix 1 was applied over a clean sample plate (5 cm × 5 cm), and allowed to partially dry. When the plate was almost dry, the matrix was wiped off the plate using a Kimwipe tissue, leaving behind a faint layer of 4HCCA, which was only visible as a yellowish reflection when the plate was angled towards the light. Following this, 0.5 μL of protein diluted in Matrix 2 was deposited onto the thin layer of 4HCCA. After drying the plate, MALDI was carried out in an Applied Biosystem Voyager System 4320 with an accelerating voltage of 25000 V.

Absorption and fluorescence spectroscopy: Absorption spectra were measured using a quartz cuvette (1 cm path length) in a Jasco V-670 spectrophotometer. Steady state fluorescence spectra were obtained in a nanolog fluorometer (Horiba Jobin Yvon), using a quartz cell (1 cm path length), and all emission spectra were corrected for the wavelength dependence of the detection system response. Fluorescence life-time decay measurements were analyzed by time-correlated single-photon-counting as described below.

Light-minus-dark experiment: The light-minus-dark spectra were obtained by subtracting the absorbance spectrum of a sample taken in the dark from the spectrum of the same sample exposed to a continuous, actinic light source centered at 650 nm (bandwidth ~ 10 nm) that had illuminated the sample for 3 minutes prior to measurement. The path of the actinic beam was

perpendicular to the path of the probe light from the UV-Vis absorbance spectrophotomer. The excitation light at 650 nm was obtained using a white light source (Dolan-Jenner MH-100 Metal Halide Fiber Optic Illuminator) passed through two filters (RG610 and IF650; 10 nm band pass). For all measurements, samples contained a 50-fold excess of 1,10-phenanthroline compared to the RC concentration.

Time-correlated single-photon counting kinetic measurement: The excitation source was a fiber supercontinuum laser (Fianium SC450) operated at 20 MHz. The laser output was sent through an Acousto-Optical Tunable Filer (Fianium AOTF) to obtain excitation pulses at wavelengths of 600 nm and 710 nm. Fluorescence emission was collected at a 90° geometry setting and detected using a double-grating monochromator (Jobin-Yvon, Gemini-180) and a microchannel plate photomultiplier tube (Hamamatsu R3809U-50). The polarization of the emission was 54.7° relative to that of the excitation. Data acquisition was done using a single photon counting card (Becker-Hickl, SPC-830). The typical IRF (instrument response function) had a FWHM (full width half maxima) of 50 ps, measured from the light scattered from sample at the excitation wavelength. The data were fitted using a locally written software package, ASUFIT, to a sum of exponential decay terms either globally (at many wavelengths simultaneously) or at a single wavelength.

Calculation of FRET Efficiency and average lifetime of dye molecules: FRET efficiencies (E) were calculated according to the following equation:

$$E = 1 - \frac{I_{DA} / A_{DA}}{I_D / A_D}$$

Where I_{DA} and I_D are the integrated area of fluorescence from the donor with and without an acceptor. A_{DA} and A_D are the absorbance of the donor at excitation wavelength with and without an acceptor.

Average lifetimes were calculated using the following equation.

$$\tau_{ave} = \frac{\sum_i A_i \tau_i}{\sum_i A_i}$$

Where A_i and τ_i are the fitted amplitude and the lifetime components.

The energy transfer efficiency determined from life-time measurements was calculated using the following equation.

$$E_{lifetime} = 1 - {}^{\tau_{ave,DA}}/_{\tau_{ave,D}}$$

Where $\tau_{ave,DA}$ and $\tau_{ave,D}$ are the average lifetime of the donor with and without an acceptor obtained from the fluorescence time-resolved single photon counting measurements.

Energy transfer efficiency calculated from transient absorbance measurements was obtained using the following equation.

$$E_{TA} = 1 - {}^{\tau_{ave,DA}}/_{\tau_{ave,D}}$$

Where $\tau_{ave,DA}$ and $\tau_{ave,D}$ are the average lifetime of the donor with and without an acceptor obtained from the transient absorbance measurements.

Time-resolved ultrafast transient absorption spectroscopy: Transient absorbance measurements were performed using a broadband and narrowband pump-probe system as described previously.[6] Laser pulses of 100 fs at 800 nm were generated from a regenerative amplifier system (Tsunami and Spitfire, Spectra-Physics) operated at 1 kHz. Part of the beam was used to pump an optical parametric amplifier (IR OPA, Spectra-Physics) to generate excitation pulses at 650 nm. The white-light probe pulses were generated by focusing part of the 800-nm beam onto a 3 nm sapphire plate and detected using a CCD camera (DU420, Andor Technology) for probing kinetics over a 140-nm wavelength window simultaneously. The collected data had a 2.3 nm spectral resolution. In some measurements, kinetics at a specific wavelength were recorded using a photodiode for higher signal-to-noise ratio. All time-resolved experiments were performed at room temperature. The absorbance changes as a function of time (t) and probe wavelength (λ) were fit globally to a multiple exponential model using locally written software, ASUFIT. The instrument response time function was fitted to a Gaussian curve (FWHM of 150 fs).

Cytochrome *c* oxidation experiment: Cytochrome *c* oxidation kinetic spectra were obtained by measuring the absorbance change at 550 nm in the presence of a 650 nm or 800 nm excitation beam. The excitation light centered at 800 nm was generated by passing white light through an 800 nm band pass filter (FB800-40, FWHM 40 nm). Light intensity at 650 nm was 5.8 fold higher than that at 800 nm. The sample contained 1 μM dye-modified RC (RC-AF647 or RC-AF660), 100 μM decylubiquinone (Sigma-Aldrich) and 10 μM reduced bovine heart cytochrome *c* (Sigma-Aldrich) in tris buffer (15 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.025% LDAO, pH 8).[7,8] Cytochrome *c* was reduced by using a published procedure.[9] In short, 1 mL of cytochrome *c* solution (1.2 mM) was mixed with a 10-fold molar excess of sodium ascorbate (600 mM stock solution) in 10 mM sodium phosphate buffer (pH 6.9) and agitated for 3 hrs at 4° C. The solution was then desalted using a Nap-25 column (GE Healthcare) to remove excess sodium ascorbate by washing with 10 mM sodium phosphate (pH 6.9) buffer, followed by tris buffer. The concentration of the reduced cytochrome *c* was calculated by measuring the absorbance at 550 nm (extinction coefficient 28000 $M^{-1}cm^{-1}$). Two different excitation lights were used, 650 nm and 800 nm.

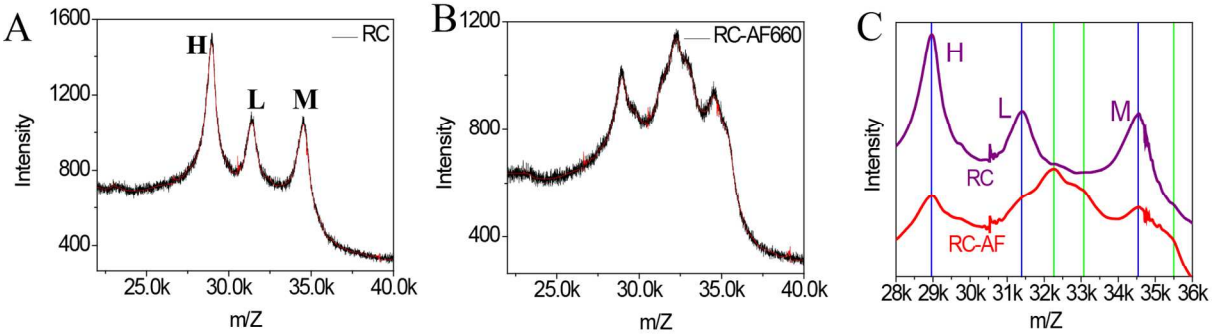

Figure S1. MALDI-TOF spectra of the RC (A), RC-AF660 (B) and together (smoothed) (C). The RC spectrum shows three peaks at 28960, 31395 and 34554 m/Z, corresponding to the H, L and M subunits, respectively. In contrast, the Alexa Fluor 660 conjugate (the center spectrum)

has two additional peaks (32260 and 33066 m/Z), roughly 865 and 1671 Daltons different from that of the L subunit, corresponding to L subunits bound to one and two dye molecules. The peak of the M subunit has a shoulder at 35474 m/Z, differing by roughly 900 Daltons from that of the M subunit, corresponding to one dye molecule per M subunit. This result confirms the selective conjugation of the dye to the mutated RC, in which the L subunit has two surface cysteine residues and the M subunit has one surface cysteine, while the H subunit lacks cysteine.

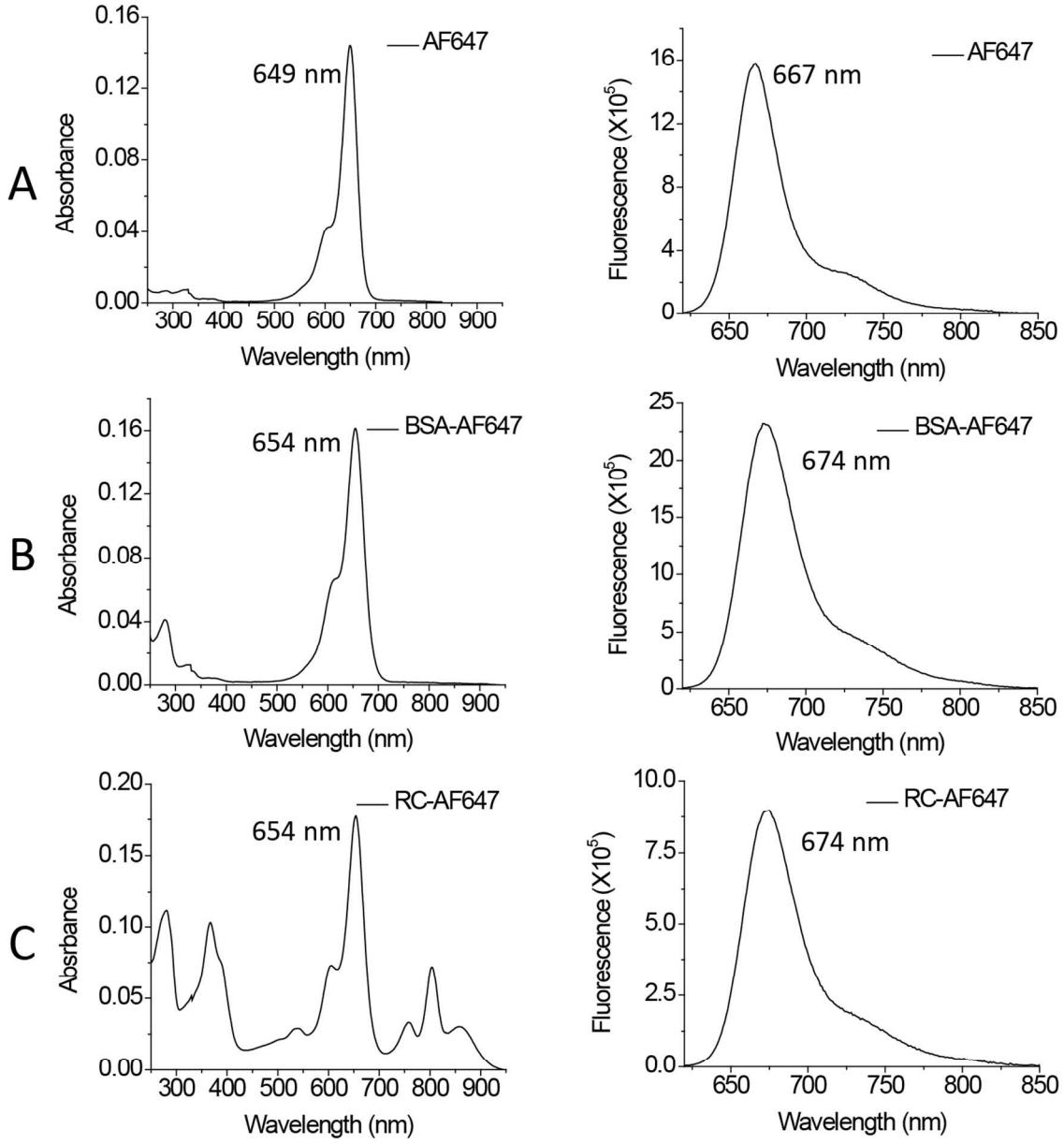

Figure S2. A, B and C are the absorption spectra (left) and fluorescence spectra (right) of AF647, BSA-AF647 and RC-AF647, respectively, with the corresponding absorbance and emission maxima marked.

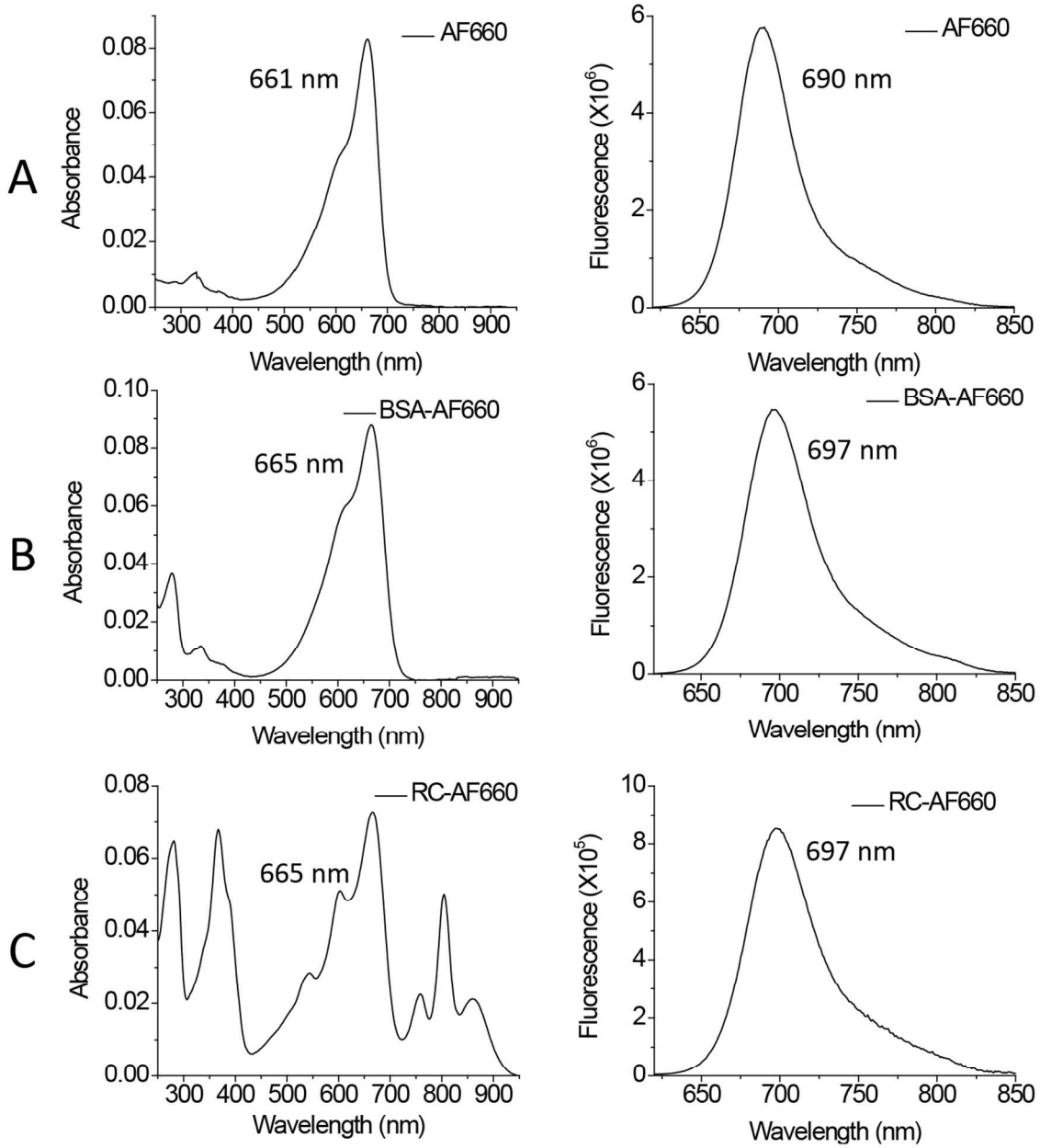

Figure S3. A, B and C are the absorption spectra (left) and fluorescence spectra (right) of AF660, BSA-AF660 and RC-AF660, respectively, with the corresponding absorbance and emission maxima marked.

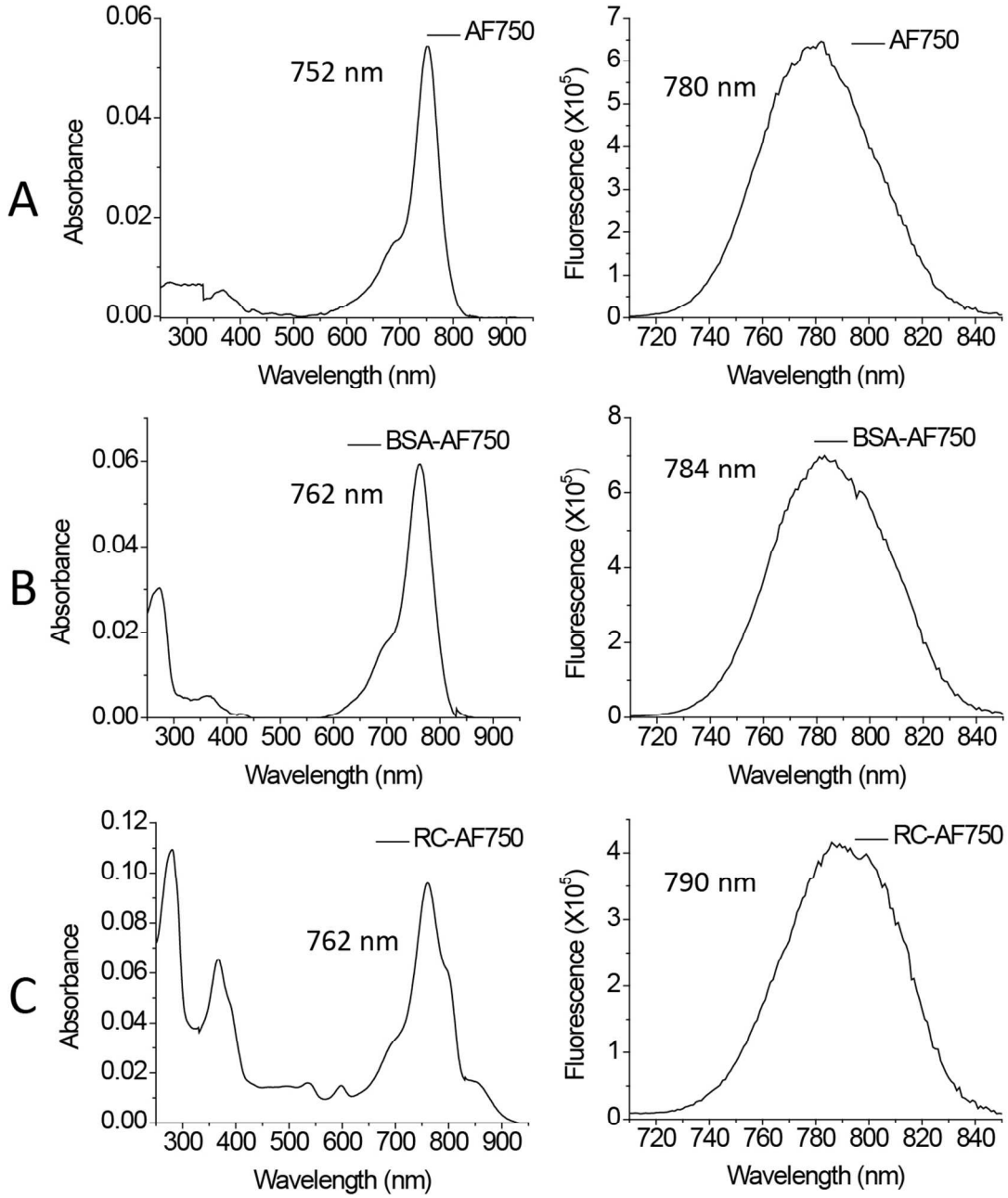

Figure S4. A, B and C are the absorption spectra (left) and fluorescence spectra (right) of AF750, BSA-AF750 and RC-AF750, respectively, with the corresponding absorbance and emission maxima marked.

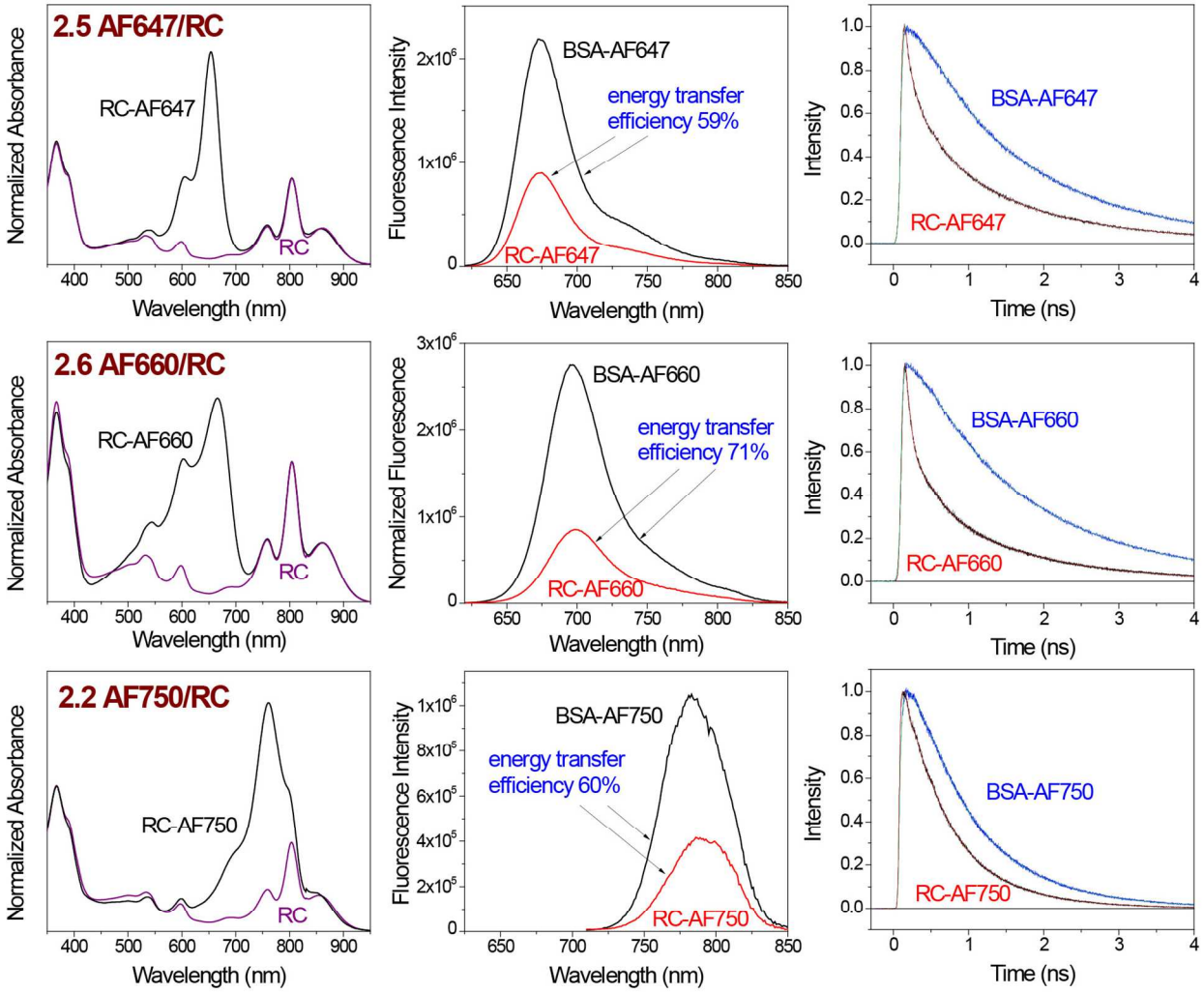

Figure S5. (Left to right) Absorption spectra of RC (purple) and RC conjugated with Alexa Fluor dye (black), fluorescence emission spectra of Alexa Fluor dye conjugated to BSA (black) and to RC (red), and time resolved fluorescence decay profile of Alexa Fluor dye conjugated to BSA (blue) and RC (purple) using time correlated single photon counting. The upper row data are for Alexa Fluor 647 (excitation wavelength 600 nm, decay kinetics monitored at 670 nm), the middle row data are for Alexa Fluor 660 (excitation wavelength 600 nm, decay kinetics monitored at 698 nm) and the bottom row data are for Alexa Fluor 750 (excitation wavelength 710 nm, decay kinetics monitored at 780 nm).

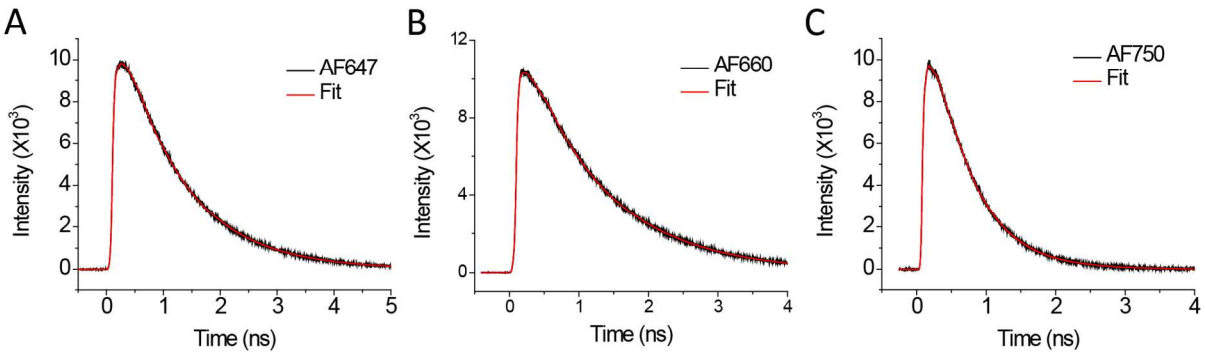

Figure S6. A, B and C are the fluorescence life-time decay profile of free dye AF647, AF660 and AF750, respectively, in tris buffer (15 mM tris, pH 8, 0.025% LDAO, 150 mM NaCl, 1 mM EDTA). Biexponential decay kinetics was used for fitting all of the three dyes. The fitting parameters are shown in Table S2.

Table S1. Absorption, and fluorescence maxima and quantum yields for all the samples

Sample		Absorption maxima (nm)	Emission Maxima (nm)	Quantum Yield
AF647	Free dye	649	667	0.33
	On BSA	654	674	0.43
	On RC	654	674	-
AF660	Free dye	661	690	0.37
	On BSA	665	697	0.32
	On RC	665	697	-
AF750	Free dye	752	780	0.12
	On BSA	762	784	0.12
	On RC	762	790	-

Table S2. Fitting parameters of the life-time data for all the samples.

Sample		τ1 ns (amplitude %)	τ2 ns (amplitude %)	τ3 ns (amplitude %)	Average lifetime (ns)	χ^2
AF647	Free	0.41 (6.9)	1.09 (93.1)	-	1.04	1.04
λ_{ex} = 600	BSA	0.05 (30.8)	0.64 (19.8)	1.73 (49.4)	0.99	1.03
nm	RC	0.07 (62.9)	0.46 (16.2)	1.61 (20.9)	0.45	1.04
AF660	Free	0.55 (19)	1.24 (81)	-	1.11	1.18
λ_{ex} = 600	BSA	0.10 (13.2)	0.81 (25.5)	1.82 (61.3)	1.33	1.04
nm	RC	0.04 (67.9)	0.31 (16.1)	1.46 (16.0)	0.31	1.08
	Q^-RC	0.05 (60.3)	0.47 (18.9)	1.60 (20.8)	0.45	1.10
AF750	Free	0.55 (98.6)	1.17 (1.4)	-	0.56	1.02
λ_{ex} = 710	BSA	0.12 (11.7)	0.73 (76.0)	1.63 (12.3)	0.77	1.16
nm	RC	0.06 (40.0)	0.38 (34.6)	0.85 (25.4)	0.37	1.13

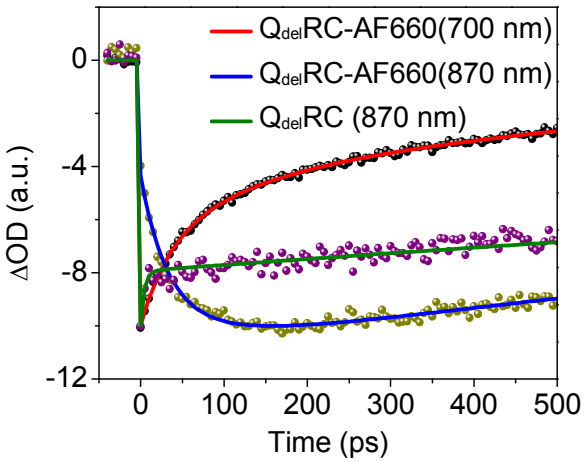

Figure S7. Normalized transient absorbance of AF660 (red) in Q_{del}RC-AF660, and P^+ in Q_{del}RC-AF660 (blue) and in the unconjugated Q_{del}RC (green). Excitation was at 650 nm for all samples.

Table S3. Fitting parameters for the pump-probe data (pump at 650 nm and probe at 700 nm).

Sample	τ1 ps (amplitude %)	τ2 ps (amplitude %)	τ3 ps (amplitude %)	Average (ps)
AF660	519 (30%)	1390 (70%)	-	1129
BSA-AF660	531 (18%)	1800 (82%)	-	1572
Q_{del}RC-AF660	25 (35%)	164 (29%)	1418 (36%)	567

Table S4. Comparison of energy transfer (ET) efficiency calculated from steady-state fluorescence, fluorescence lifetime decay and transient absorbance with corresponding dye on BSA as control.

Sample	ET from steady-state fluorescence[1] (%)	ET from fluorescence lifetime measurement[2] (%)	ET from transient absorbance[3] (%)
RC-AF647	59	55	NA
RC-AF660	71	77	NA
Q_{del}RC-AF660	70	66	64
RC-AF750	60	52	NA

[1] $E = 1 - \frac{I_{DA}/A_{DA}}{I_D/A_D}$, where I_{DA} and I_D are the integrated fluorescence emission from the donor with and without an acceptor. A_{DA} and A_D are the absorbance of donor at the excitation wavelength with and without an acceptor.

[2] $E_{lifetime} = 1 - {\tau_{ave,DA}}/{\tau_{ave,D}}$, where $\tau_{ave,DA}$ and $\tau_{ave,D}$ are the average lifetime of the donor with and without an acceptor obtained from the fluorescence time-resolved single photon counting measurements.

[3] $E_{TA} = 1 - {\tau_{ave,DA}}/{\tau_{ave,D}}$, where $\tau_{ave,DA}$ and $\tau_{ave,D}$ are the average lifetime of the donor with and without an acceptor obtained from the transient absorbance measurements.

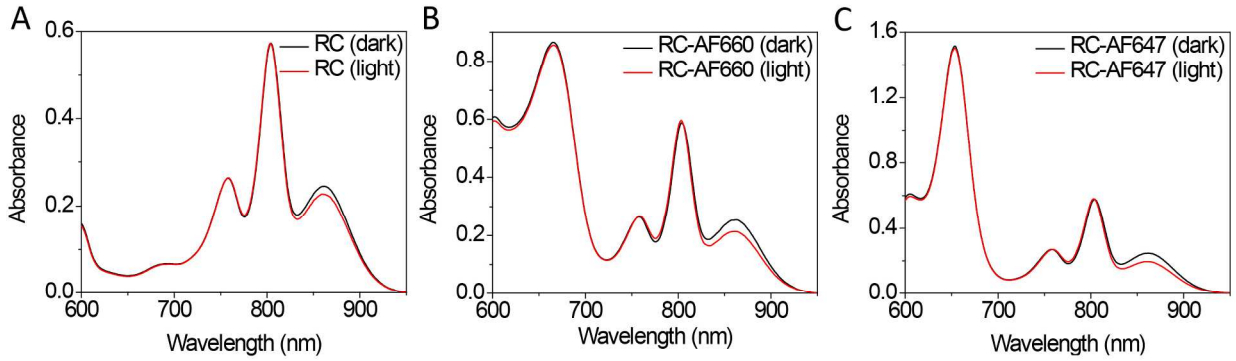

Figure S8. The absorbance in light (with excitation at 650 nm) and in dark for RC only (A), RC-AF660 conjugate (B) and RC-AF647 conjugate (C).

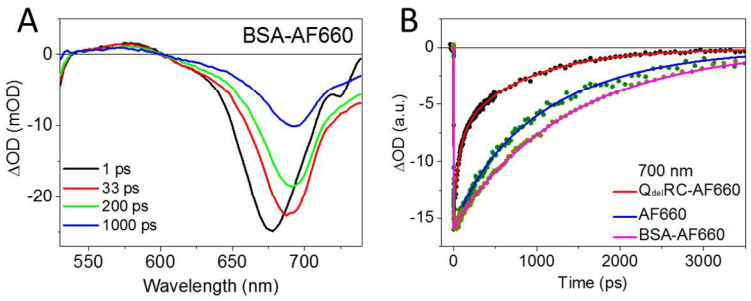

Figure S9. (A) Time-resolved transient absorption difference spectra of BSA-AF660 in the 530-730 nm region. (B) Transient absorbance kinetics at 700 nm for unconjugated AF660 dye in solution (AF660, blue), quinone-depleted RC-AF660 conjugates (Q_{del}RC-AF660, red) and BSA-AF660 conjugates (BSA-AF660, pink). For all samples, λ_{ex} = 650 nm.

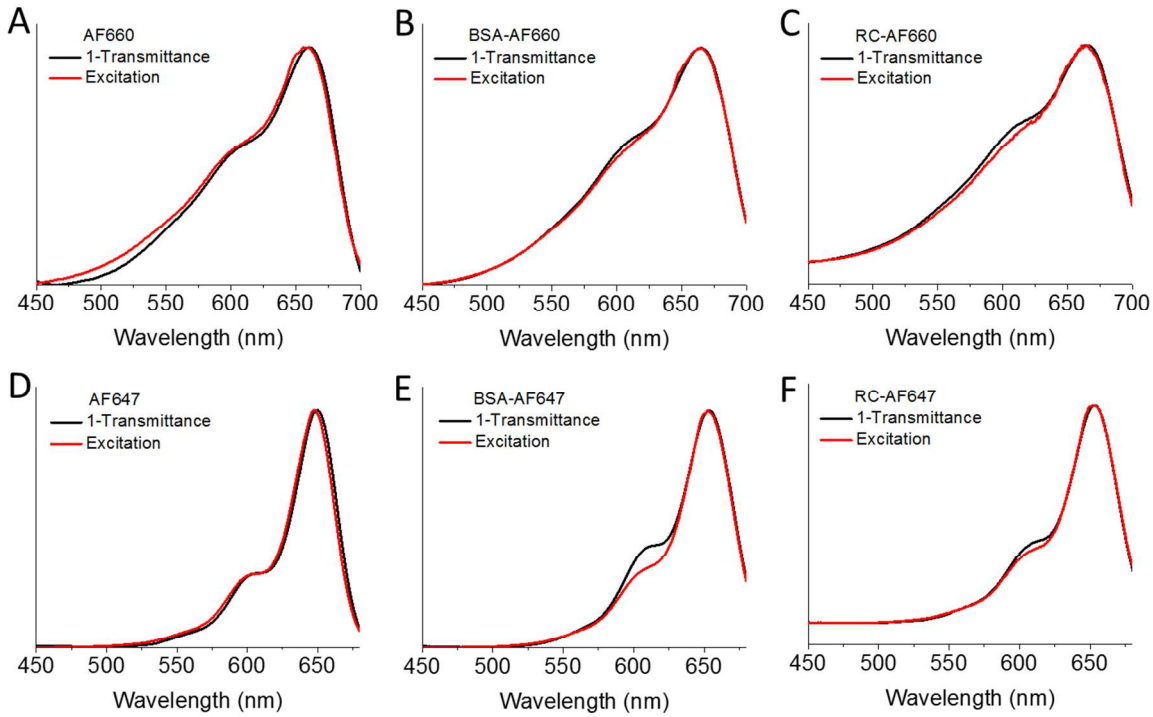

Figure S10. Comparison between 1-transmittance and excitation spectra of different samples. For AF660 dye series, the excitation was done from 450 nm to 700 nm and the emission was taken at 710 nm. Whereas for AF647 dye series, the excitation was done from 450 nm to 680 nm and the emission was taken at 690 nm.

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