Supporting information for:
Thermodynamics, Kinetics, and Photochemistry of β-Strand Association and Dissociation in a Split-GFP System

Keunbong Do and Steven G. Boxer*
Department of Chemistry, Stanford University, Stanford, California 94305-5080, United States

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Protein preparation

Protein purification

Both s10:loop:GFP and s10:loop:GFP T203Y (sequences are given below) were expressed from the pET-15b vectors in BL21 (DE3) cells (Invitrogen). The cells were incubated in LB Broth, Miller (EMD), induced with IPTG (0.25 g/L) at OD 0.6, and incubated overnight (~20 hours) at 17 ^{o}C (note: this temperature was required to obtain high yields of these proteins). The culture was spun down, and the resulting pellet was resuspended in lysis buffer (50 mM HEPES, 300 mM NaCl, and 10 v% glycerol at pH 8.0; all lysis buffer is at pH 8.0 in this supporting information) and lysed with a homogenizer. The cell lysate was spun down, and the supernatant was poured onto a Ni-NTA column equilibrated with lysis buffer. One column volume of lysis buffer containing 20 mM imidazole was used for washing, and the same buffer with 200 mM imidazole was used to elute the protein from the column. The eluate was further purified with anion-exchange chromatography (HiTrap^{TM} 5 mL Q HP, GE). The buffer of the final sample was exchanged back into lysis buffer using a desalting column for further quantitative experiments.

Loop cleavage

Purified whole protein (s10:loop:GFP or s10:loop:GFP T203Y) was loaded onto a Ni-NTA column, and the column was equilibrated with cleavage buffer (50 mM Tris and 20 mM $CaCl_2$ at pH 8.0). Lyophilized trypsin (from bovine pancreas, Sigma) was dissolved in 1 mM HCl to make ~10 units/μL trypsin solution, and the solution was applied to the protein-loaded column to match ~100 units trypsin per 1 mg protein. The protein was digested on the column for 30 minutes while periodically rotating the column for gentle mixing. The column was washed with lysis buffer containing 20 mM imidazole and the digested protein (s10:~~loop~~:GFP or s10:~~loop~~:GFP T203Y) was eluted with the same buffer containing 200 mM imidazole. The eluate was further purified with anion-exchange chromatography.

Protein denaturation, size exclusion, and renaturation

Digested and purified protein was denatured in denaturing buffer (6 M guanidine hydrochloride, 50 mM HEPES, and 50 mM NaCl at pH 8.0). The denatured sample was applied to the size exclusion column (Superdex^{TM} 75 10/300) equilibrated with denaturing buffer to separate the truncated protein (~~s10:loop~~:GFP) from strand 10. The separated ~~s10:loop~~:GFP was partly refolded in lysis buffer by diluting the denaturing buffer by ~1000 fold. To completely remove guanidine hydrochloride and to isolate monomer ~~s10:loop~~:GFP from aggregates, 2 mL of the partly refolded sample (~10 μM) was applied to the size exclusion column equilibrated with lysis buffer. The monomer fraction was collected for further quantitative experiments. When the monomer fraction was diluted to 1.5 μM (OD ~0.04) right after elution, the sample stayed mostly monomeric for more than two days (Figure S1). We note that all quantitative experiments involving isolated ~~s10:loop~~:GFP were performed with less than 1.5 μM protein concentration within several hours after collecting the monomer fraction to avoid complications associated with protein aggregation as ~~s10:loop~~:GFP tends to precipitate at higher concentrations (>20 μM).

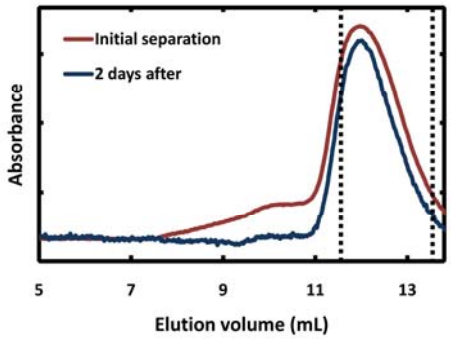

Figure S1. Absorbance at 280 nm monitored in size exclusion chromatography with column equilibrated with lysis buffer. The initial separation (red trace) of a partly refolded ~~s10:loop~~:GFP (~10 μM, 2 mL) was performed to isolate monomer (the fraction in between the two vertical dotted lines in the figure, 11.5~13.5 mL, was taken) from dimer (small peak around 10 mL) and higher order aggregates (in the void volume around 8 mL). The monomer eluate (~5 μM) was diluted down to 1.5 μM, kept in room temperature for two days, and re-applied onto the column giving the chromatogram in the blue trace. Note that the two traces do not share the same ordinate scale.

Synthetic peptides
Peptides were ordered from Elim Biopharmaceuticals Inc. with >80 % HPLC purity.

Instrumentation and Basic Methods

Fluorescence spectrometer
Kinetic measurements involving stopped-flow initiation were performed with a Fluorolog 3 from Jobin Yvon Horiba fluorimeter, and all other fluorescence measurements were done with a LS55 from Perkin Elmer. The excitation slit width of 3~4 nm was used for kinetic measurements, which typically results in 0.5~1 $\mu W \cdot mL^{-1}$ irradiation. Light-enhancement of the peptide exchange process was first observed by exciting the 1.3 μM $\underline{s10}^{203T}$•~~s10:loop~~:GFP and excess $\underline{s10}^{203Y}$ mixture with the maximum excitation slit width (15 nm) at 390 nm, which results in 10~15 $\mu W \cdot mL^{-1}$ irradiation and 70- to 80-fold rate enhancement. The enhanced rate did not depend on the excess peptide concentration; presence of 5 μM, 20 μM, and 100 μM $\underline{s10}^{203Y}$ resulted in a similar enhanced rate with the same excitation wavelength and intensity. For the equilibrium binding data in Figure 3, 1 % BSA was used to passivate all plastic surface.

UV-vis spectrometer
A Lambda 25 from Perkin Elmer was used for all absorbance measurements.

Isothermal titration calorimetry (ITC)
VP-ITC from MicroCal was used for all ITC measurements.

Mass spectrometer
A Micromass nanoESI APIUS Quadrupole Time-of-Flight mass spectrometer was used for all protein mass measurements. All samples had the buffer exchanged into double deionized water before being injected into the spectrometer.

Concentration determination
The relative absorbance at 447 nm before and after denaturing the protein in 0.1 M NaOH was used to estimate the extinction coefficient of the protein through the known 447 nm extinction coefficient of the GFP chromophore (44,100 $M^{-1}cm^{-1}$) in 0.1 M NaOH.[1] The estimated extinction coefficient was then used to determine concentrations. Extinction coefficients of synthetic peptides were estimated by titrating ~~s10:loop~~:GFP with a peptide to compare the absorbance with concentration at the extrapolated equivalence point. Extinction coefficients for $\underline{s10}^{203T}$ and $\underline{s10}^{203Y}$ were determined respectively as approximately 1,500 $M^{-1}cm^{-1}$ and 3,000 $M^{-1}cm^{-1}$ at 280 nm, which are similar to those obtained through calculation (1,490 $M^{-1}cm^{-1}$ and 2,980 $M^{-1}cm^{-1}$)[2].

Spectral comparison

Absorbance
Figure S2 shows the absorbance spectra of proteins purified with anion-exchange chromatography after different stages of processing. It can be seen that losing a covalent bond in the loop has minimal effect on the absorbance, while reassembling the truncated protein with a synthetic peptide slightly increases the intensity of the protonated peak (393 nm for GFP and 396 nm for YFP) relative to the deprotonated peak (467 nm for GFP and 503 nm for YFP) by 5~6 %. This small difference is likely because the native strand 10 carries 21 additional amino acids at the N-terminus including the polyhistidine tag (see sequence below).

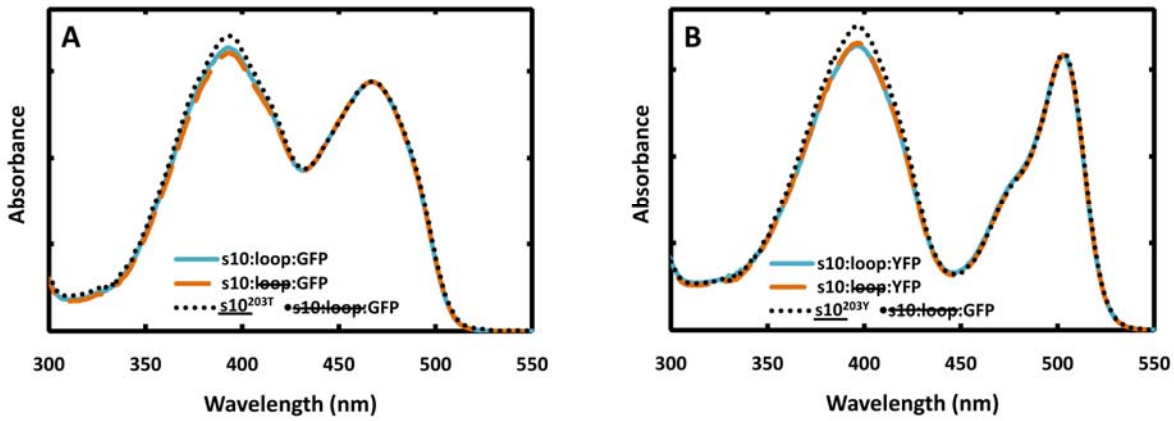

Figure S2. Absorbance spectra in lysis buffer. Spectra were normalized to the absorbance at (A) 465 nm or (B) 500 nm.

Fluorescence

Likewise, when the fluorescence excitation spectra are normalized by concentration, s10:loop:GFP and s10:~~loop~~:GFP are indistinguishable (data not shown). However, s10•~~s10:loop~~:GFP shows ~20 % decrease in fluorescence quantum yield (Figure S3) while the overall shape of the fluorescence spectra being indistinguishable. Again, this can be accounted for by the absence of 21 amino acids in the synthetic strand.

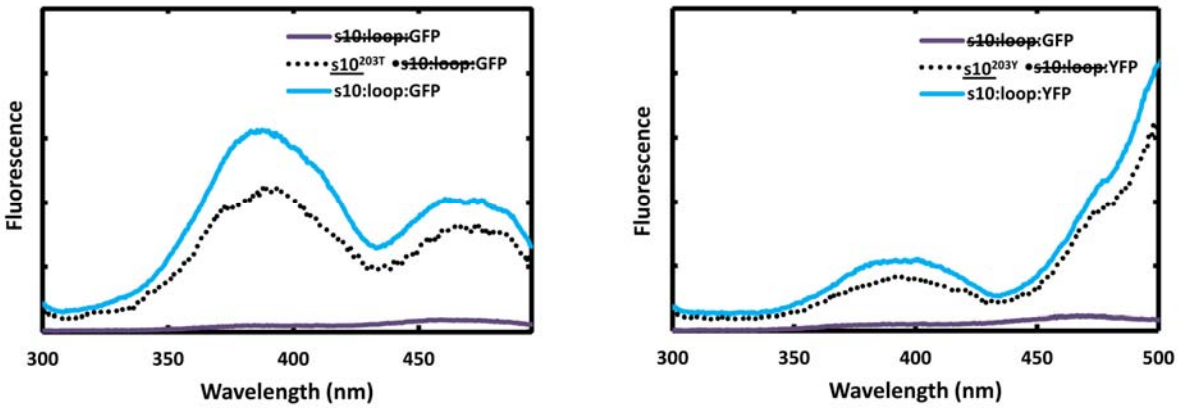

Figure S3. Fluorescence excitation spectra normalized by concentration. Excitation spectra were scanned while detecting emission at (A) 505 nm or (B) 520 nm.

Mass spectrometry results

s10:loop:GFP
Expected: 30571.9 Da
Measured: 30571.0 Da

~~s10:loop~~:GFP
Expected: 25296.2 Da
Measured: 25297.0 Da

s10^{203T}
Expected: 2171.3 Da
Measured: 2171.0 Da

$\underline{s10}^{203Y}$
Expected: 2233.4 Da
Measured: 2233.0 Da

Protein design criteria and amino acid sequences

Proteins were designed based on the sequence of GFP1-10OPT and GFP11M3.[3] The starting point of the s10:loop:GFP circular permutation (L195) was chosen referring to the results reported from studies of circularly permuted superfolder GFPs.[4]

s10:loop:GFP and ~~s10:loop~~:GFP

MGSSHHHHHHSSGLVPGGSHMLPDNHYLSTQTVLSKDPNEGTRGSGSIEGR▼HSGSGSKRDHMVLHEYVN
AAGITHGMDELYGGTGGSASQGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATIGKLTLKFISTTGKLPV
PWPTLVTTLSYGVQAFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGKYKTRAVVKFEGDTLVNRIELK
GTDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFTVRHNVEDGSVQLADHYQQNTPIGDGPVL

The proteolytic cleavage site is indicated by "▼" (observed by mass spec).

s10:loop:GFP T203Y and ~~s10:loop~~:GFP T203Y

MGSSHHHHHHSSGLVPGGSHMLPDNHYLSYQTVLSKDPNEGTRGSGSIEGR▼HSGSGSKRDHMVLHEYVN
AAGITHGMDELYGGTGGSASQGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATIGKLTLKFISTTGKLPV
PWPTLVTTLSYGVQAFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGKYKTRAVVKFEGDTLVNRIELK
GTDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFTVRHNVEDGSVQLADHYQQNTPIGDGPVL

$\underline{s10}^{203T}$

LPDNHYLSTQTVLSKDPNE

$\underline{s10}^{203Y}$

LPDNHYLSYQTVLSKDPNE

Kinetic fit of the peptide exchange process

For the pseudo 1st order exchange process, a single exponential function $c\cdot(1-e^{-kt})$ was fit to the data using c (the asymptotic parameter) and *k* (the unimolecular rate constant) as fit parameters. However, since the exchange rate in the dark is very slow and the data collection time is limited by protein aggregation and microbial contamination over days at room temperature, it was not possible to collect sufficient data points to be fit with both parameters (Figure S4A). Instead of using both c and *k* as fit parameters, c was fixed to the calculated equilibrium composition based on K_d values measured from the binding titration (~96 % YFP complex ~4 % GFP complex in this particular case), and only *k* was varied to fit the data. For all light-activated exchange processes, the rate was fast enough to acquire data

points that are sufficient to be fit by varying both parameters (Figure S4B). The agreement between the fit and the calculated c is good at low intensity light (less than 5 % different up to 17 mW), but the c value tends to be increasingly underestimated (by 11 % with 77 mW) by the fit as the light intensity is increased, probably because of increased photobleaching at higher light intensities. This may also account for the apparently incomplete saturation of the peptide-exchange rate constant in higher light intensity (Figure 5D).

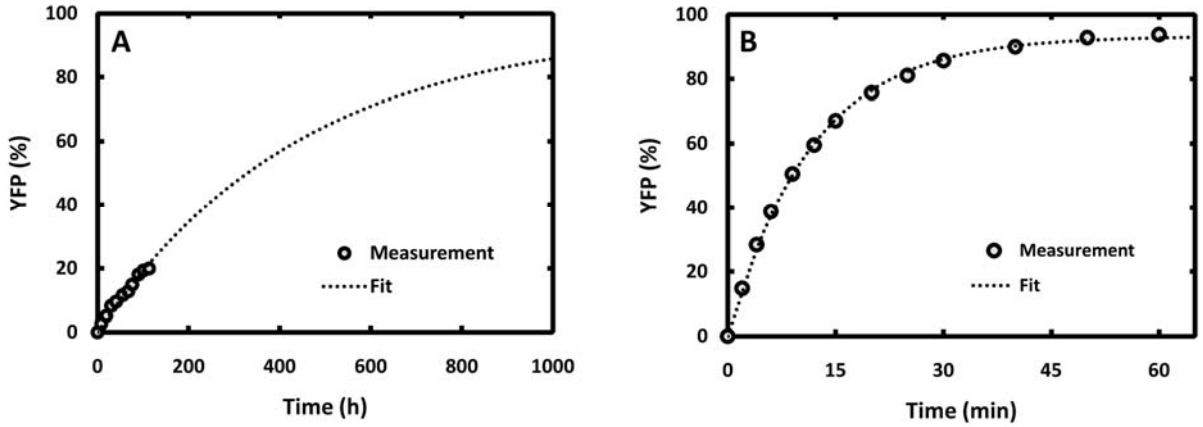

Figure S4. YFP complex ratio change over time; (A) starting from a 2.13 μM s10^{203T}•~~s10:loop~~:GFP and 31 μM s10^{203Y} mixture and left in the dark; and (B) starting from a 3 mL of 1.3 μM s10^{203T}•~~s10:loop~~:GFP and 30 μM s10^{203Y} mixture with 17 mW 405 nm light irradiation.

Quantum yield calculation

Light from a 405 nm laser was directed to the 3 mL mixture of 1.3 μM s10^{203T}•~~s10:loop~~:GFP and 30 μM s10^{203Y} inside a 1 cm pathlength quartz cuvette with constant stirring. The cuvette was placed between the laser and a metallic surface that reflects 67 % of the incident light. The quantum yield for the light enhanced strand exchange was estimated crudely as follows.

1) In 405 nm light, there are 2.04×10^{15} photons per mJ:

$$\frac{1mJ}{h\nu}=\frac{1mJ\times\lambda}{hc}=\frac{(1\times10^{-3}J)\times(4.05\times10^{-7}m)}{(6.626\times10^{-34}J\cdot s)\times(2.998\times10^{8}m\cdot s^{-1})}=2.04\times10^{15}\ photons/mJ$$

2) For a 1cm pathlength and for 1.3 μM s10^{203T}•~~s10:loop~~:GFP, 5.75 % of the 405 nm light is absorbed:

$$\text{Absorbance: } \varepsilon_{405nm}bc=(19770cm^{-1}M^{-1})\times(1cm)\times(1.30\times10^{-6}M)=0.0257$$
$$\%\text{ absorbed: } 1-T=1-10^{-0.0257}=0.0575=5.75\%\ .$$

3) Based on the transmittance of the cuvette filled with lysis buffer (T = 0.9226), it was estimated that approximately 4 % of the incident light is reflected off the cuvette before reaching the sample solution, and 8.87 % of the incident light is absorbed by s10^{203T}•~~s10:loop~~:GFP in total:

$$\text{The 1}^{st}\text{ 1cm path (incident light from the laser): } 0.96\times0.0575=0.0552=5.52\%$$
$$\text{The 2}^{nd}\text{ 1cm path (reflected light from the metal behind the sample):}$$
$$(0.96-0.0552)\times0.67\times0.96\times0.0575=0.0336=3.35\%$$

$$\text{Total absorbed} = \ 5.52\% + 3.35\% = 8.87\% .$$

4) With 3.8 mW (or 1.3 mW·mL^{-1}) of 405nm laser irradiation, a peptide exchange rate of 5.41×10^{-4} s^{-1} was measured, which means 1.27×10^{12} s10^{203T}•~~s10:loop~~:GFP complexes were converted into s10^{203Y}•~~s10:loop~~:GFP complex per second:

Total number of s10^{203T}•~~s10:loop~~:GFP complexes initially present:

$$cVN_A = (1.30 \times 10^{-6} M) \times (3ml) \times (6.022 \times 10^{23} mol^{-1}) = 2.35 \times 10^{15} complexes$$

Total number of s10^{203T}•~~s10:loop~~:GFP complexes converted into s10^{203Y}•~~s10:loop~~:GFP complexes initially per second:

$$N_{tot}(1 - e^{-k}) = (2.35 \times 10^{15} complexes) \times (1 - e^{-5.41 \times 10^{-4}}) = 1.27 \times 10^{12} complexes .$$

5) Assuming the conversion in the dark is negligible, consistent with the observed dark rate, and assuming that each s10^{203T}•~~s10:loop~~:GFP complex interacts with one photon at a time, the quantum yield of the peptide exchange process can be estimated as approximately 0.2 %:

$$\varphi_{GFP \to YFP} = \frac{N_{GFP \to YFP}}{N_{photons}} = \frac{1.27 \times 10^{12}}{(3.8mJ) \times (2.04 \times 10^{15} mJ^{-1}) \times 0.0887} = 0.00185 \approx 0.2\% .$$

Photodissociation

To determine whether s10^{203T}•~~s10:loop~~:GFP and s10^{203Y}•~~s10:loop~~:GFP undergo photodissociation in the absence of an added replacement strand, that is, whether photodissociation is the elementary step or whether the strand added in excess is contributing, each was irradiated at 405 nm. If photodissociation occurs, the resulting absorbance spectrum would be a linear combination of those of ~~s10:loop~~:GFP and the complex, which are sufficiently different (Figure 2), and once the irradiation is stopped, the absorbance and the fluorescence would return to those of the initial complex following the biomolecular association rate that was independently measured. As shown in Figure S5, the spectrum following irradiation could be expressed as a linear combination of the basis spectra of ~~s10:loop~~:GFP and the complex, and through the linear fit, the approximate composition of the two species could be obtained. For example, the absorbance spectrum of 550 nM s10^{203Y}•~~s10:loop~~:GFP converges to a certain absorbance spectrum after 15 minutes of 25 mW·mL^{-1} 405nm light irradiation (the spectrum taken after 15 minutes irradiation is nearly identical with the one taken after 30 minutes irradiation). The absorbance taken after 15 minutes of 25 mW·mL^{-1} 405 nm light irradiation could be fit with a linear combination of ~~s10:loop~~:GFP and s10^{203Y}•~~s10:loop~~:GFP basis spectra with 0.31 and 0.69 weighting factor on each. From the fit, it could be estimated that there is 378 nM (69 % of 550 nM) s10^{203Y}•~~s10:loop~~:GFP at equilibrium. From the peptide exchange rate measurement, the pseudo 1st order rate constant of YFP to GFP conversion with 25 mW·mL^{-1} 405 nm light irradiation was estimated as 5×10^{-4} s^{-1}, and dividing this by the measured association rate of s10^{203Y} (5658 M^{-1}s^{-1}), a K_d of 88 nM is obtained. Concentration of s10^{203Y}•~~s10:loop~~:GFP calculated using this K_d is 370 nM (see calculation below), which is only 2 % lower than the value evaluated from the spectral fit. Likewise, the calculation shows good agreement with the fit in the GFP complex dissociation.

[s10^{203Y}•~~s10:loop~~:GFP] at equilibrium ([A] ≡ [~~s10:loop~~:GFP]$_{total}$, [B] ≡ [s10^{203Y}]$_{total}$):

$$\frac{[A]+[B]+K_d - \sqrt{([A]+[B]+K_d)^2 - 4 \cdot [A] \cdot [B]}}{2} = \frac{550+550+88 - \sqrt{(550+550+88)^2 - 4 \times 550 \times 550}}{2} \approx 370 \quad \text{(nM)}$$

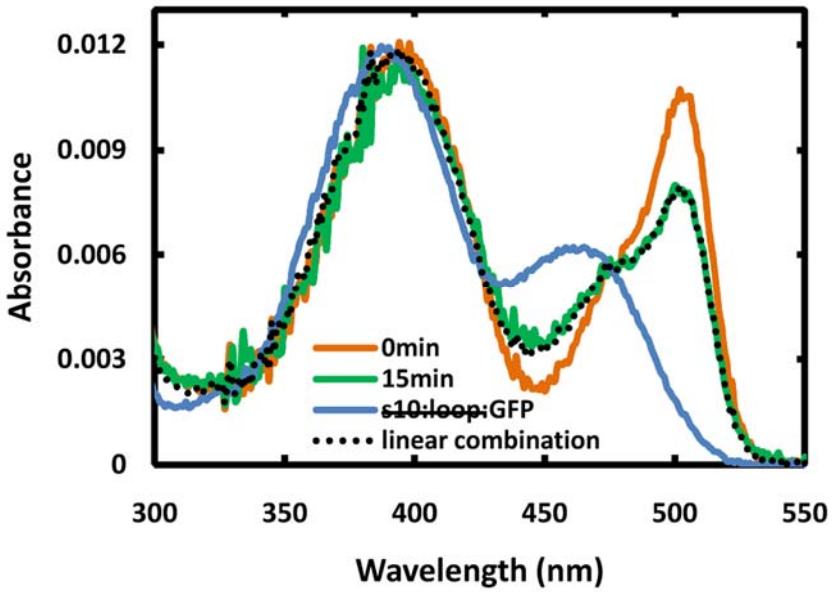

Figure S5. Light excitation of 550 nM $\underline{s10}^{203Y}$•~~s10:loop~~:GFP with 25 mW·mL^{-1} 405 nm light. The absorbance spectrum of the YFP complex is shown in the orange solid line, and the absorbance spectrum taken after 15 minutes of 25 mW·mL^{-1} 405 nm light irradiation is shown in green solid line. The basis spectrum of ~~s10:loop~~:GFP is normalized to the isosbestic point at 476 nm and presented as the blue solid line. The linear combination of the $\underline{s10}^{203Y}$•~~s10:loop~~:GFP and ~~s10:loop~~:GFP spectra with the weighting factor of 0.69 and 0.31 for each gives the black dotted line.

References

(1) Ward, W. W. *Bioluminescence and Chemiluminescence* (1981) De Luca, M. and McElroy, D. W., eds., Academic, New York, 235–242.
(2) ProtParam http://web.expasy.org/protparam/
(3) Cabantous, S.; Terwilliger, T. C.; Waldo, G. S. *Nat. Biotechnol.* (2005), 23, 102-107.
(4) Pedelacq, J.; Cabantous, S.; Tran, T.; Terwilliger, T. C.; Waldo, G. S. *Nat. Biotechnol*. (2006), 24, 79-88.