# Supporting Information

for

**Assessing the Structural Stability of Designed β-Hairpin Peptides in the Cytoplasm of Live Cells**

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## Experimental Section.

**General methods and materials**: The gene encoding the protein referred to as CFP was constructed by introducing the Cerulean[1] mutations (S72A, Y145A and H148D) into the gene for ECFP (*Aequorea* GFP with mutations K26R, F64L, S65T, Y66W, N146I, M153T, V163A, N164H, and H231L)[2] in the pBAD/His B vector (Invitrogen Corp., Carlsbad, CA) by a whole-plasmid amplification-based method using mismatched primers. This specific version of CFP has not been previously described in the literature but is at least as brightly fluorescent as Cerulean (Hui-wang Ai and REC, unpublished results). The gene encoding the protein referred to as YFP is the A206K mutant[3] of Citrine.[4] All synthetic DNA oligonucleotides were purchased from Sigma-Genosys Canada (Oakville, ON). PCR products and products of restriction digest were routinely purified using the QIAquick PCR purification kit according to the manufacturers protocols (Qiagen, Mississauga, Ontario). Restriction enzymes were purchased from either Invitrogen or New England Biolabs (Ipswich, MA). The identity of all cDNA constructs was confirmed by dye terminator cycle sequencing using the DYEnamic ET kit (Amersham Biosciences, Uppsala, Sweden). All sequencing reactions were analyzed at the University of Alberta Molecular Biology Service Unit. Unless otherwise noted, protein samples for all biochemical characterization experiments including FRET measurements, proteolysis, and CD spectroscopy were in 50 mM Tris, pH 7.5. To calculate CFP to YFP interchromophore distances ($r$) from FRET efficiencies ($E$), the following formula[5] was used with $R_O$ = 5 nm:[6]

$$r = \left( \frac{R_O^6}{E} - R_O^6 \right)^{\frac{1}{6}}$$

**Construction of CFP-peptide-YFP expression vectors**: The vector used for cloning and expression of all peptides flanked by CFP and YFP is referred to as pZC1 and was constructed as follows. The gene encoding YFP was PCR amplified with Pfu polymerase (Fermentas Canada Inc, Burlington, ON) and a 5' primer that appended Xho1/Kpn1/Pst1 restriction sites to the codon encoding Glu5. The 3' primer appended an EcoR1 site after the stop codon. The PCR product was digested with Xho1/EcoR1 and ligated with T4 DNA ligase (Invitrogen Corp.) into pBAD/His B (Invitrogen Corp.) that had been digested with the same two restriction enzymes. The ligated product was used to transform *E. coli* DH10B (Invitrogen Corp.) by electroporation. Appropriate dilutions of transformed bacteria were plated on LB/agar containing ampicillin (0.1 mg/ml) and arabinose (0.2%) and after overnight incubation at 37 °C, single colonies were picked and used to inoculate 2 ml of LB media containing ampicillin (0.1 mg/ml). Cultures were grown with shaking overnight at 37 °C before plasmids were isolated using the QIAprep spin miniprep kit according to the manufacturers protocols (Qiagen, Mississauga, Ontario). The gene encoding CFP was PCR amplified with a 5' primer that appended an Xho1 restriction site before the start codon. The 3' primer appended Spe1/Sac1/Kpn1 restriction sites immediately after the codon encoding Thr230. The PCR product was digested with Xho1/Kpn1 and ligated into the similarly digested pBAD/His B vector containing the gene for YFP. Bacterial transformation and plasmid isolation was performed as described above. The resulting vector (pZC1) has a convenient set of restriction sites (Spe1/Sac1/Kpn1/Pst1) flanked by a 5' gene for CFP and a 3' gene for YFP with minimal intervening linker sequence. Double stranded DNA encoding the target peptides and with appropriate sticky ends for ligation into the Sac1/Kpn1 sites of pZC1 were created by slow cooling pairs of complementary single stranded oligonucleotides from 95 °C to room temperature. The synthetic annealed dsDNA sequences were ligated into pZC1 that had been digested with Sac1 and Kpn1. Bacterial transformation and plasmid isolation was performed as described above. The sequence of the insert was confirmed by DNA sequencing with a forward primer (5'-CCCTCGTGACCACCCTGACCTGG-3') that anneals to the chromophore region of the gene for CFP but not YFP. The resulting plasmids encode proteins of the general structure Nterm-$His_6$-EK-CFP(1-230)-TSGAQ-peptide-GTSAE-YFP(5-238)-Cterm, where '$His_6$' represents six consecutive histidine residues that facilitate metal affinity

purification,[7] 'EK' is the recognition sequence for the protease enterokinase, and 'peptide' is a 16mer from the **tz** series.

To construct the CFP-**tz1**-CFP and YFP-**tz1**-YFP expression vectors, pZC1 containing the gene for full-length **tz1** was digested with either Xho1/Sac1 or Kpn1/EcoR1 to excise the gene for CFP or YFP, respectively. In place of the excised fluorescent protein gene was inserted the PCR amplified and appropriately digested gene for YFP or CFP, respectively.

**Construction of CFP-tz1-peptide expression vectors.** To construct the vector for expression of a CFP-fused protein precursor of **tz1-peptide**, pZC1 containing the gene for the full-length **tz1** protein was digested with Kpn1/EcoR1 to remove the YFP gene. The cut vector was ligated with a synthetic dsDNA that had appropriate sticky ends and a stop codon immediately after the Kpn1 site. The resulting vector was subject to whole plasmid PCR amplification with a 5' (sense) primer that annealed immediately 5' of the **tz1** sequence and appended a Sac1 restriction site. The 3' (antisense) primer annealed to the 3' of the gene encoding CFP and appended a sequence encoding an AcTEV protease recognition sequence (ENLYFQG) followed by a Sac1 restriction site. The resulting PCR product was digested with Sac1 and circularized by self-ligation. The resulting vector encodes proteins with the general structure $N_{term}$-$His_6$-EK-CFP(1-230)-AcTEV-AQ-peptide-GT-$C_{term}$, where 'AcTEV' is the protease recognition sequence, and 'peptide' is the peptide portion of protein **tz1**.

**Protein and peptide expression and purification**: The pBAD/His B vector used for all cloning and expression allows proteins to be expressed at high levels under control of the arabinose operon. A typical protein purification procedure started with the inoculation of 1 liter LB media containing ampicillin (0.1 mg/ml) and arabinose (0.2%) with a single colony of *E. coli* DH10B expressing the protein of interest. Cultures were grown overnight at 37 °C with shaking at 225 rpm. Cultures were cooled to 4 °C on ice and harvested by centrifugation (10 min, 5000 rpm). The cell pellet was resuspended in PBS buffer (130 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.6) and the cells lysed by a single passage through a French Press (Thermo Electron, Waltham, MA). Insoluble cell debris was pelleted by centrifugation at 4 °C (10 min, 10 000 rpm) and 1 ml of Ni-NTA resin (Qiagen) was added to the supernatant. Following one hour of gentle mixing at 4

°C, the supernatant was loaded onto a 6 ml polypropylene column, washed, and gravity eluted in 100 mM imidazole (pH 7.5) according to the manufacturers protocol. FRET constructs containing both CFP and YFP were further purified on an AKTAdesign chromatography system equipped with a Hiload 16/60 Superdex 75 prepgrade gel filtration column (Amersham Biosciences, Buckinghamshire, England) that was equilibrated with 50 mM Tris pH 7.5. Isolated proteins were concentrated with a Centricon centrifugal filter YM-30 (Millipore, Billerica, MA) and stored at 4 °C.

For the CFP-**tz1-peptide** protein, the Ni-NTA purified protein (7.5 mg, 0.25 μmol) was treated with 50 units of AcTEV protease (Invitrogen) and incubated at 30 °C for 4 days. Following complete digestion, the released peptide (sequence $N_{term}$-GAQKKWTWNPATGKWTWQEGT-$C_{term}$, designated **tz1-peptide**), was purified by reversed phase chromatography on an AKTAdesign chromatography system equipped with a Source 15RPC ST 4.6/100 column (Amersham Biosciences). A linear gradient of increasing acetonitrile in $H_2O$ with 0.1% trifluoroacetic acid was used to elute the target peptide. The fraction containing the target peptide was lyophilized to provide a fluffy white powder (0.5 mg, 80% yield). The identity of the peptide was confirmed by MALDI-TOF mass spectrometry (m/z calculated for $C_{114}H_{162}N_{31}O_{31}$ 2462.7 $[M+H]^+$, found 2462.3 $[M+H]^+$). A calculated extinction coefficient of 22,300 $M^{-1}cm^{-1}$ at 280 nm was used to determine the peptide concentration.

**Spectroscopy**: Steady-state fluorescence spectra for all **tz** proteins (0.04 μM) were recorded on a QuantaMaster spectrofluorometer (Photon Technology International, London, Ontario) equipped with a Xenon arc lamp. Relative rates of trypsinolysis were determined with a Safire2 monochromator-based 96-well platereader (Tecan, Salzburg, Austria). To a solution of each purified FRET construct (0.08 μM) was added a buffered solution of trypsin to a final concentration of 0.5 μg/ml, and the fluorescence intensity at 530 and 480 nm (excitation at 430 nm) recorded every 5 seconds. All CD spectra were obtained with 8 μM protein in a 1 mm path length cuvette on an Olis DSM 17 CD spectrometer (Olis, Bogart, CA).

**Description of the Bacterial Colony Imaging System**: The system for imaging the fluorescence of bacterial colonies grown on 10 cm Petri dish is a custom built device similar to one previously described.[8] Few details were provided in the previous

publication so here we provide sufficient description to allow other researchers to assemble a system with similar capabilities. The light from a 175W xenon-arc lamp (Sutter Instrument Company, Novato, CA) is passed through a filter wheel (Sutter) holding either a 426 nm to 446 nm bandpass filter (Chroma Technology Corp., Rockingham, VT) or a 490 nm to 510 nm bandpass filter for excitation of CFP or YFP respectively. The filtered light then enters a bifurcated fiber optic bundle (Newport Corporation, Stratford, CT) that is positioned to illuminate a 10 cm dish placed in a recessed holder on the bench top. The fluorescence emission is filtered through a second filter wheel that is directly above the plate and that is holding either a 460 nm to 500 nm bandpass filter for CFP or a 520 nm to 550 nm bandpass filter for YFP. Images are acquired with a Retiga 1300i 12-bit CCD camera (QImaging, Burnaby, BC) positioned immediately behind the emission filter. With the exception of the lamp, the whole system is shrouded in black fabric to eliminate stray room light. Image Pro Plus (Media Cybernetics Inc., Silver Spring, MD) is used for automated camera control and digital image processing with custom macros (available upon request). Typical exposure times are 1 second and all images are background corrected with identically acquired images of a Petri dish containing LB/agar but lacking bacterial colonies. Individual colonies are automatically identified within the digital images and the pixel intensities are summed in each of 3 images corresponding to the CFP channel, the FRET channel, and direct YFP channel. The measurement referred to as the YFP/CFP ratio in Figure 3 and in Figure S3 is the ratio of the average intensity in the FRET channel divided by the average intensity in the CFP channel for a single bacterial colony. The intensity of the YFP channel is directly proportional to the expression level of the fusion construct. For all imaging experiments bacterial colonies were grown on LB/agar supplemented with ampicillin (0.1 mg/ml) and arabinose (0.2%).

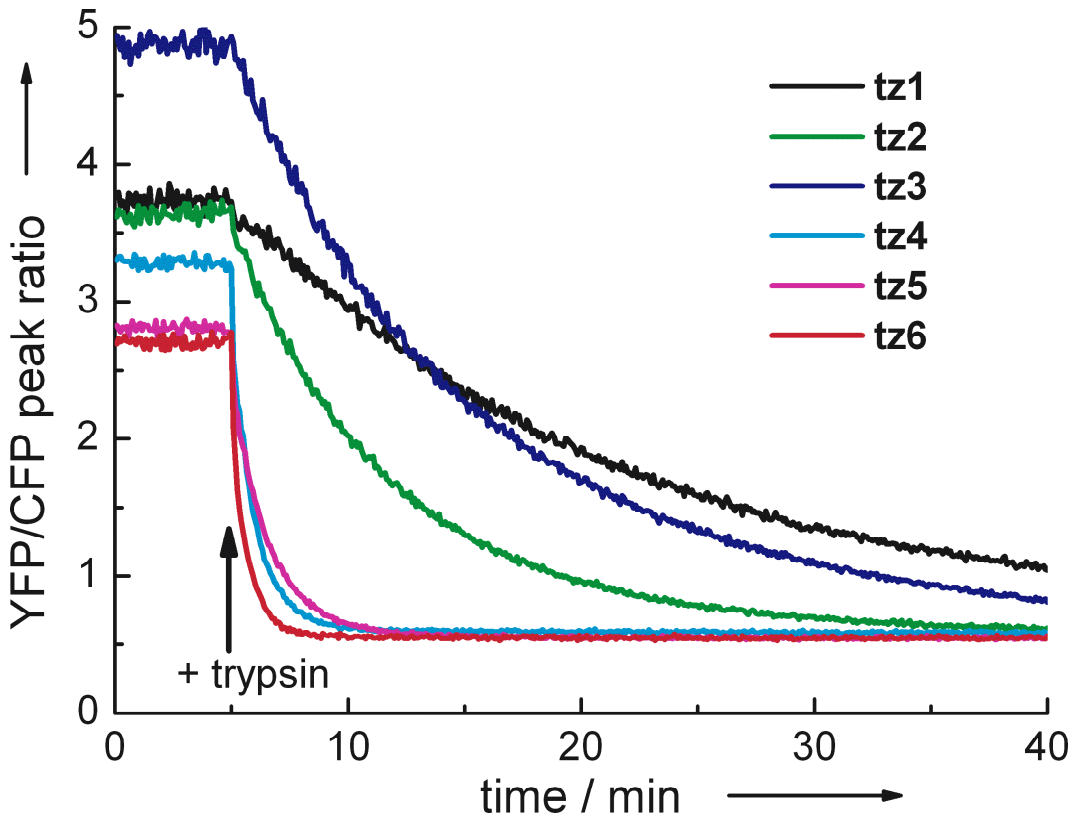


**Figure S1**. Ratio of YFP fluorescence at 530 nm to CFP fluorescence at 480 nm (with excitation at 430 nm) for purified proteins **tz1** through **tz6** upon treatment with trypsin. The initial ratios are determined by the FRET efficiencies of the intact proteins. The final ratio (~0.5) corresponds to the 530/480 nm ratio of CFP itself and is stable over many hours demonstrating that the fluorescent protein is not digested under the conditions of this experiment.

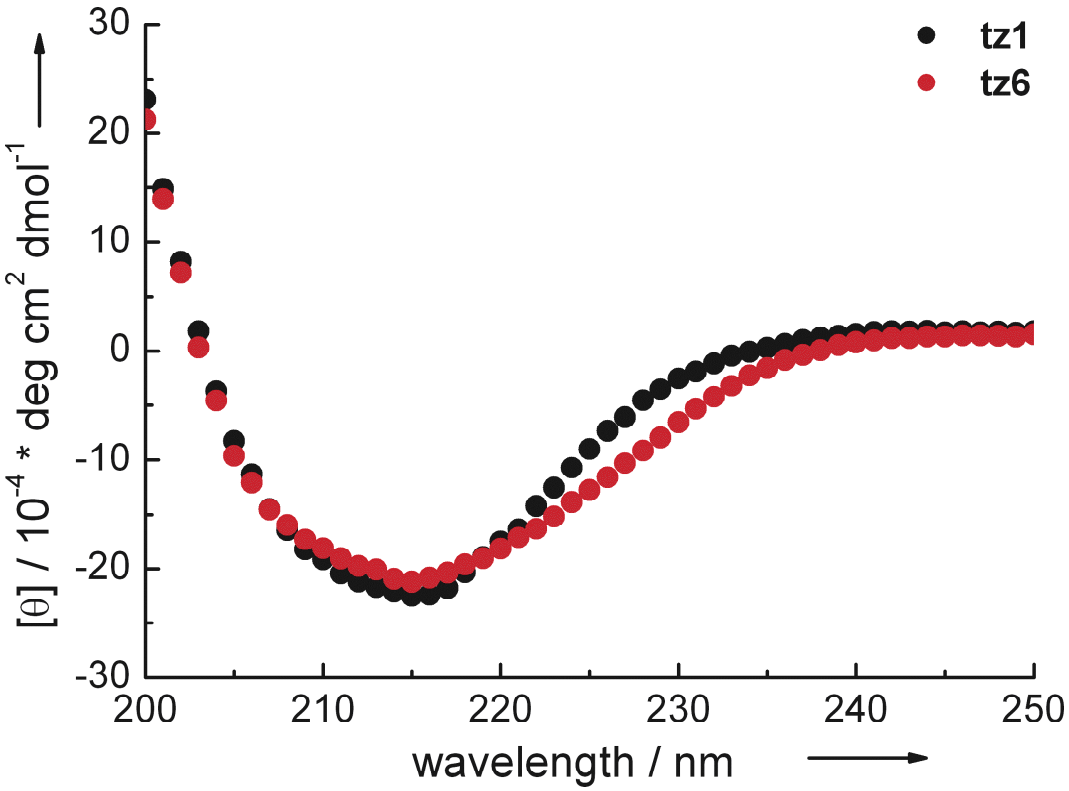


**Figure S2**. The far-UV CD spectra of identical concentrations of **tz1** and **tz6.**

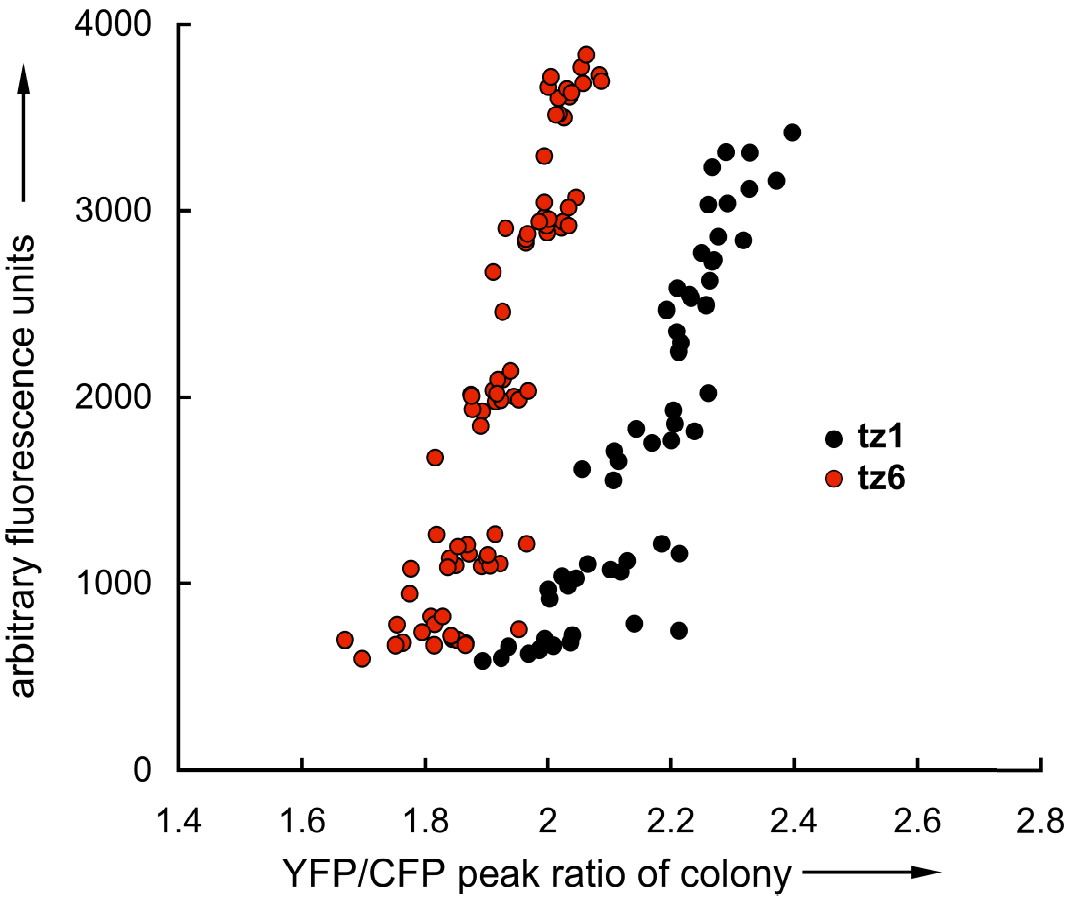


**Figure S3. Relationship between the YFP/CFP peak ratio and the protein expression level.** Shown in this chart are the YFP/CFP intensity ratios plotted against YFP fluorescence (direct excitation of YFP) for 16 individual colonies of bacteria expressing **tz6** (red circles) and 11 individual colonies bacteria expressing **tz1** (black circles) imaged at 5 different time points (20, 25, 35, 45, and 55 hours after plating). By imaging similarly sized drops of purified protein under otherwise identical conditions we have arrived at an estimate of 1100 fluorescence units for 5 μM protein at 1 s exposure time. Correspondingly, the range of intracellular concentrations for the bacteria represented in this chart is approximately 3 μM to 18 μM.

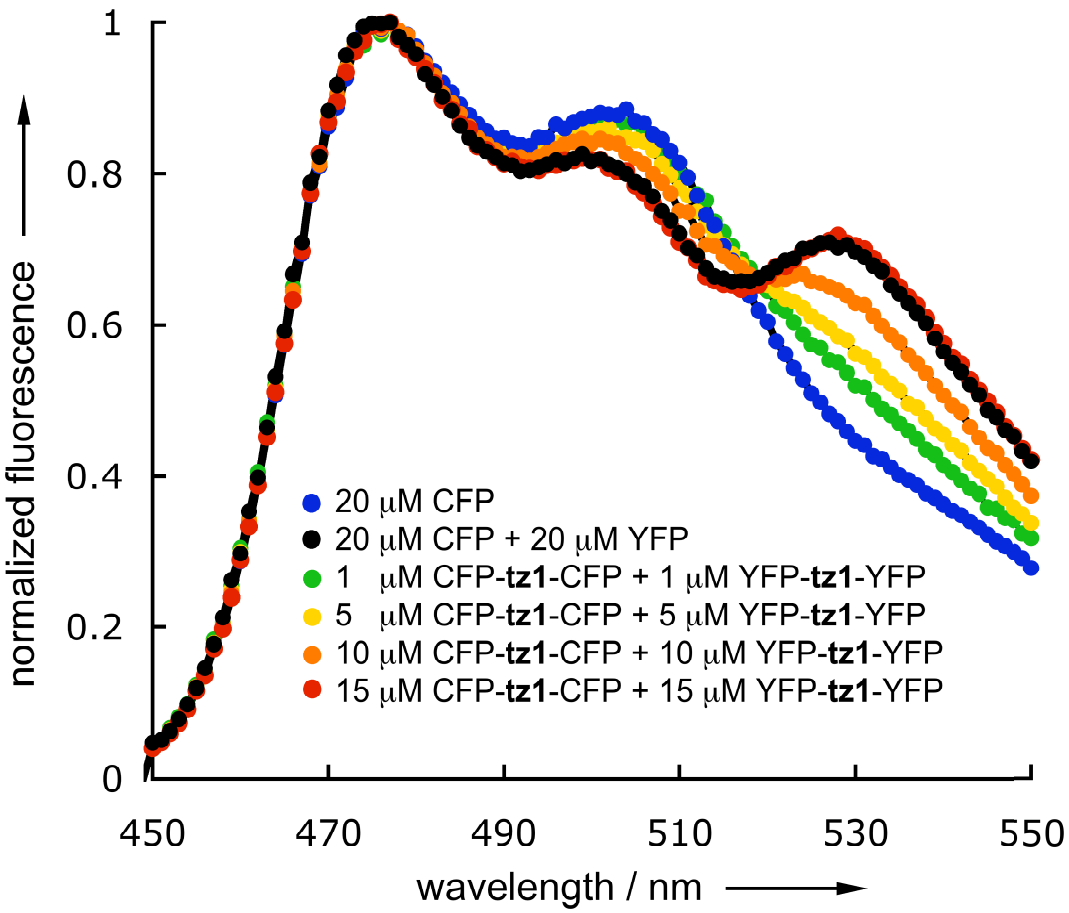


**Figure S4. The tz1 protein does not aggregate at typical intracellular concentrations.** Shown in the chart are the *in vitro* fluorescence emission spectra of the indicated protein constructs. The apparent energy transfer peak at 530 nm is observed only at concentrations greater than 1 μM and is not diminished by treatment with trypsin. The magnitude of the effect and the preferential quenching of the longer-wavelength 'hump' of the CFP emission is consistent with inner filtering of the CFP emission by the YFP chromophore.

## Supporting Information References


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