Concatenation of Cyan and Yellow Fluorescent Proteins for Efficient Resonance Energy Transfer†

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ABSTRACT: Highly efficient fluorescence resonance energy transfer between cyan(CFP) and yellow fluorescent proteins (YFP), the cyan- and yellow-emitting variants of the *Aequorea* green fluorescent protein, respectively, was achieved by tightly concatenating the two proteins. After the C-terminus of CFP and the N-terminus of YFP were truncated by 11 and 5 amino acids, respectively, the proteins were fused through a leucine-glutamate dipeptide. The resulting chimeric protein, which we called Cy11.5, exhibited a simple emission spectrum that peaked at 527 nm when the protein was excited at 436 nm. The time-resolved emission of Cy11.5 was measured using a streak camera. After excitation of Cy11.5 with a 400 nm ultrashort pulse, a fast decay of the CFP emission and a concomitant rise of the YFP emission were observed with a lifetime of 66 ps. By contrast, the emission from CFP alone showed a decay component with a lifetime of 2.9 ns. We concluded that in fully folded Cy11.5 molecules, intramolecular FRET occurred with an efficiency of 98%. Importantly, most Cy11.5 molecules were properly folded, and the protein was highly resistant to all of the tested proteases. In living cells, therefore, Cy11.5 behaved as a single fluorescent protein with a broad excitation spectrum. Moreover, Cy11.5 was used as an optical highlighter after photobleaching of YFP. When HeLa cells expressing Cy11.5 were irradiated at 514.5 nm, a 10-fold increase in the 475 nm fluorescence intensity was observed. These features make Cy11.5 useful as an optical highlighter and a new-colored fluorescent protein for multicolor imaging.

Fluorescence resonance energy transfer (FRET)[1] is the radiationless transfer of excited-state energy from an initially excited donor to an acceptor (*1*). This transfer depends on the spectral overlap of the donor and the acceptor, the distance between them, and the relative orientation of the transition dipoles of the chromophores. In FRET experiments, the cyan- and yellow-emitting variants of *Aequorea* GFP (CFP and YFP, respectively) have often been used as the donor and the acceptor, respectively (*2*, *3*). A variety of fluorescent indicators have been developed by concatenating CFP and YFP with peptide linkers. In some cases, long, flexible linkers have been used to allow efficient folding and maturation of both CFP and YFP (*4*). Also, due to the weak dimerization propensity of *Aequorea* GFP (*5*), CFP and YFP can associate, resulting in moderate FRET. Furthermore, if the linker contains a sequence that is recognized by a protease, the chimeric protein can serve as an indicator for the activity of the protease.

In this study, we attempted to tightly concatenate CFP and YFP. Since the relative orientation of the two fluorescent proteins has a significant impact on FRET (*1*), we optimized the concatenation to maximize the FRET efficiency. One of the constructed chimeric proteins, Cy11.5, exhibited extremely high FRET efficiency and resistance to proteolysis. Because Cy11.5 can be used as a single fluorescent protein that absorbs at 436 nm and emits at 527 nm, it can be added to the palette of currently available fluorescent proteins. The relatively large Stokes shift of this protein will be advantageous for multicolor imaging. To explore the dynamics of the efficient FRET in Cy11.5, we measured time-resolved emission lifetimes and anisotropies after the protein sample was excited with a 400 nm pulse generated from the second harmonic of a titanium−sapphire laser.

MATERIALS AND METHODS

Gene Construction. To make an N-terminally truncated YFP, the Venus cDNA (*6*) was amplified using a sense primer containing an *Xho*I site and an antisense primer containing a termination codon followed by an *Eco*RI site. Using different sense primers, a series of N-terminal truncations was made. To make a C-terminally truncated CFP, ECFP cDNA (Clontech, Mountain View, CA) was amplified using a sense primer containing a *Bam*HI site in frame with

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[1] Abbreviations: CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; FRET, fluorescence resonance energy transfer.

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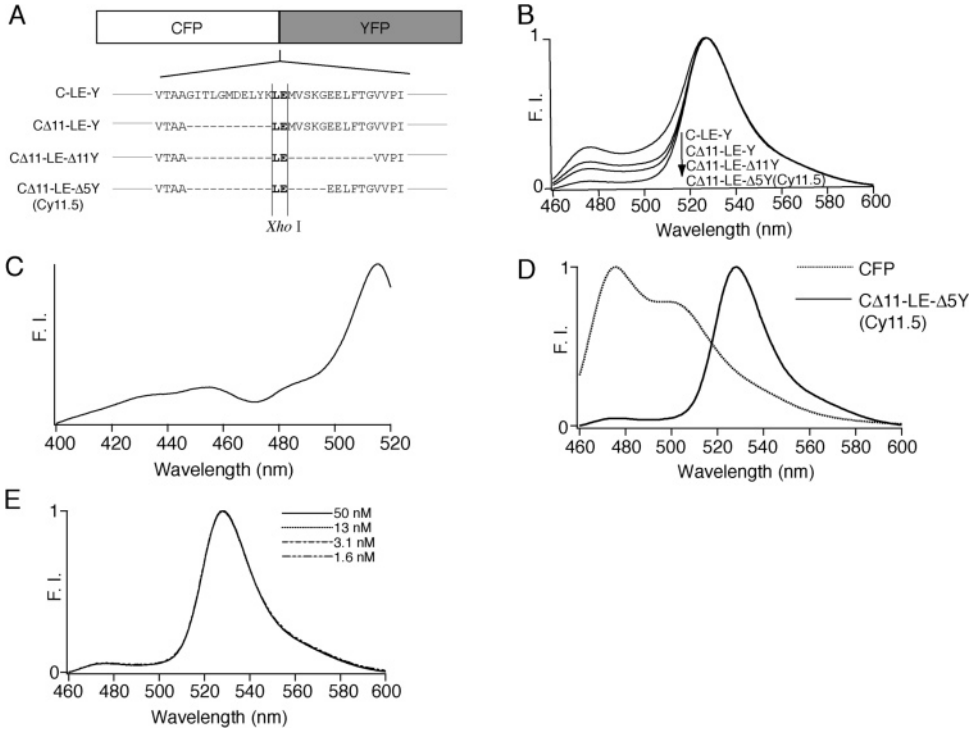

FIGURE 1: CFP−YFP fusion proteins including the Cy11.5 variant. (A) A diagram of the CFP−YFP fusion proteins. The amino acid sequences of the linking regions of four representative chimeric proteins are shown. (B) Normalized emission spectra of the four CFP−YFP fusion proteins shown in panel A. (C) Excitaion spectrum of Cy11.5 at 540 nm emission. (D) Emission spectra of Cy11.5 and CFP at 440 nm excitation. (E) The Cy11.5 emission spectrum does not change with changes in the concentration of Cy11.5: 50 (—), 13 (···), 3.1 (— - —), and 1.6 nM (— - - —).

a polyhistidine tag and an antisense primer containing an *Xho*I site. Using different antisense primers, a series of C-terminal truncations was made. For bacterial expression, the fragments encoding CFP and YFP were ligated into the *Bam*HI and *Eco*RI sites of $pRSET_B$ (Invitrogen, Carlsbad, CA). For mammalian expression, the *Bam*HI−*Eco*RI fragment of Cy11.5 was subcloned into pcDNA3 (Invitrogen).

Protein Expression and in Vitro Spectroscopy. Recombinant proteins with N-terminal polyhistidine tags were expressed in *Escherichia coli* [JM109(DE3)] at room temperature, purified, and spectroscopically characterized as described previously (*6*). Emission spectra of samples excited at 440 nm were measured using a fluorescence spectrophotometer (F-2500, Hitachi, Tokyo, Japan). The steady-state anisotropy of free CFP was measured using a fluorescence spectrophotometer (F-4500, Hitachi).

Proteolysis Assay. Protein concentrations were measured using a Bradford assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin serving as the standard. Each aliquot (0.6 μg of protein) was digested with trypsin (Sigma-Aldrich, St. Louis, MO), papain (Sigma-Aldrich), α-chymotrypsin (Wako Pure Chemical Industries, Osaka, Japan), or elastase (Wako Pure Chemical Industries) for 2 h at 37 °C.

Imaging. Between 2 and 4 days after transfection, HeLa cells expressing Cy11.5 in Hanks' balanced salt solution buffer (Invitrogen) were imaged. Wide-field fluorescence microscopy was performed on an IX-70 inverted microscope using a UApo/340 40×, 1.35 NA oil-immersion objective lens (Olympus, Tokyo, Japan). The imaging was performed with a 440DF20 excitation filter, a 455DRLP dichroic mirror, two emission filters (480DF30 for CFP and 535DF25 for YFP) in a filter changer (Lambda 10-2, Sutter Instruments, Novato, CA), and a cooled CCD camera (Cool SNAP fx, Roper Scientific, Tucson, AZ). Interference filters were obtained from Omega Optical. Image acquisition and analysis were performed using Metamorph/Metafluor 5.0 (Molecular Devices, Downingtown, PA). Acceptor photobleaching was performed using a 525AF45 excitation filter and a 560DCLP dichroic mirror. All the filters and dichroic mirrors were purchased from Omega Optical (Brattleboro, VT).

Picosecond Time-Resolved Fluorescence. Picosecond time-resolved fluorescence measurements were performed with the detection system of a streak camera (C4334, Hamamatsu, Shizuoka, Japan). A Ti−sapphire regenerative amplifier (Spitfire, Spectra-Physics, Mountain View, CA) seeded by a Ti−sapphire mode-locked oscillator laser (Tsunami, Spectra-Physics) was used as a light source, and the second harmonic of the amplified pulse (400 nm, 1 μW) was used for excitation. The polarization of the excitation and detection light was set at the magic angle. The measurements were taken with time resolutions of 20 and 120 ps (fwhm) for the sweep ranges of 1 and 10 ns, respectively. The fluorescence was collected with the backscattering geometry with respect to the excitation beam. All measurements were performed at room temperature. The sample solution (5 mg/mL) was contained in a cell with a thickness of 1 mm.

RESULTS AND DISCUSSION

Concatenation of CFP and YFP with Different C- and N-Terminal Truncations. The C-terminus of CFP and the N-terminus of YFP were truncated to various degrees before the variants were fused using a dipeptide (Leu-Glu: LE) encoded by an *Xho*I restriction site (Figure 1A). The chimeric proteins are termed $C\Delta X_1$-LE-ΔX_2Y, where X_1 and X_2 are the numbers of amino acids that were deleted from CFP and

YFP, respectively. The bacterially expressed proteins were purified and spectrally characterized by measuring the emission spectra resulting from excitation at 440 nm. Since the orientation factor is critical for the efficiency of FRET between CFP and YFP, numerous constructs with different truncations were examined (see Table S1 of the Supporting Information). The emission ratio of YFP (527 nm) to CFP (476 nm) increased irregularly as larger deletions were made (Figure 1B). Additionally, CFP and/or YFP were misfolded in some of the chimeric proteins. For example, N-terminal truncations of more than 10 amino acids appeared to prevent proper folding of YFP.

Highly Efficient FRET in CΔ11-LE-Δ5Y (Cy11.5). Among the chimeric proteins that were examined, CΔ11-LE-Δ5Y (Figure 1B) produced a bright signal and exhibited the highest emission ratio (527 nm to 476 nm), which reached 20. The emission signal at ∼480 nm, which was emitted from the CFP of CΔ11-LE-Δ5Y, was almost negligible. The excitation spectrum of CΔ11-LE-Δ5Y recorded at 540 nm emission was broad (Figure 1C). The emission spectra of CFP and CΔ11-LE-Δ5Y could be separated using linear unmixing technology when the proteins were excited at 440 nm (Figure 1D).

Because intermolecular FRET may occur when CΔ11-LE-Δ5Y is highly concentrated, we examined the dependence of the emission spectrum of CΔ11-LE-Δ5Y on its concentration. Within a range from 50 to 1.6 nM (a significantly low concentration range considering the dissociation constant for CFP and YFP is 100−300 μM), the shape of the emission spectra was constant (Figure 1E), indicating that FRET from CFP to YFP primarily takes place within a single CΔ11-LE-Δ5Y molecule. The protein was named "Cy11.5" and further characterized in comparison with a control protein in which neither CFP nor YFP was truncated (C-LE-Y).

Resistance of Cy11.5 to Proteases. When expressed in living cells, Cy11.5 may be exposed to a variety of proteases. Since proteolysis of Cy11.5 into CFP and YFP should give rise to a significant spectral change, we examined the effects of proteases on the chimeric protein in vitro. Cy11.5 was not affected by 2 h treatments at 37 °C with an excess amount of trypsin, α-chymotrypsin, papain, or elastase, whereas under the same conditions, the linker of C-LE-Y was cleaved, resulting in disruption of FRET (Figure 2).

High-Efficiency FRET (98%) in Cy11.5 Determined by Lifetime Measurements. For a CFP−YFP tandem construct in a cuvette, the increase in fluorescence intensity of CFP after proteolysis by trypsin allows us to obtain the efficiency of FRET from CFP to YFP (*7*). This approach, however, cannot be used for Cy11.5 because the chimeric protein is resistant to proteases. Thus, we measured the excited-state lifetime of CFP (*8*) in Cy11.5 using a streak camera. Cy11.5 was excited with a 400 nm pulse generated from the second harmonic of a titanium−sapphire laser. The emission spectrum changed with time as shown in Figure 3A. The time-resolved emission traces in a short (450−500 nm) and a long (550−600 nm) wavelength region are shown in panels B and C of Figure 3, respectively. The emission between 450 and 500 nm exhibited an initial fast-decay component with a lifetime of 66 ps (71%), which was followed by long-lived emission with two lifetimes of 490 ps (24%) and 2.9 ns (5%) (Figure 3B). The two long lifetime components were characteristic of unquenched CFP; when free CFP was used

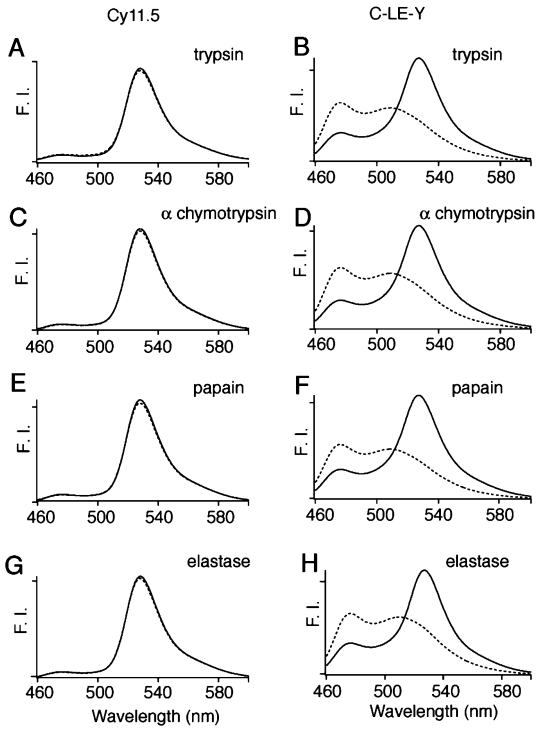

FIGURE 2: Protease treatment of Cy11.5 and C-LE-Y. The emission spectra of Cy11.5 (A, C, E, and G) and C-LE-Y (B, D, F, and H) before (—) and after (- - -) treatment with trypsin (A and B), α-chymotrypsin (C and D), papain (E and F), and elastase (G and H) are shown.

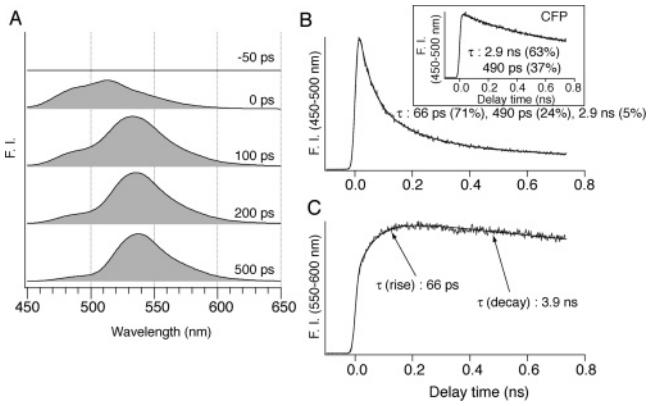

FIGURE 3: Time-resolved emission after excitation with a 400 nm pulse generated from the second harmonic of a titanium−sapphire laser. (A) Time-resolved emission spectra of Cy11.5. (B) Time-resolved emission trace between 450 and 500 nm from Cy11.5. The inset is the time-resolved emission trace between 450 and 500 nm from free CFP. (C) Time-resolved emission trace between 550 and 600 nm from Cy11.5.

under the same conditions, the emission decayed with average lifetimes of 490 ps (37%) and 2.9 ns (63%) (Figure 3B, inset). The lifetime of 2.9 ns reflects the radiative process to the ground state; the reported fluorescence lifetimes of CFP are ∼3 ns (*9*). By contrast, the lifetime of 490 ps may be related to a conversion to the distinct conformation proposed to exist in CFP (*10*). Since Cy11.5 and CFP exhibited similar fractions of the emissions in the 490 ps decay components (24% vs 37%), it is assumed that the deactivation process was not involved in the FRET. It is also likely that the decay of Cy11.5 characterized by the lifetime of 2.9 ns resulted from CFP molecules that included a misfolded YFP.

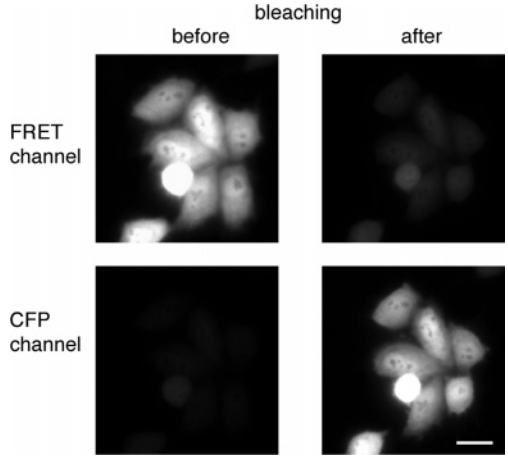

FIGURE 4: Fluorescence images of HeLa cells expressing Cy11.5 before and after photobleaching of YFP. The top panels are images taken through the FRET channel (excitation at 440 nm and emission at 535 nm) before (left) and after (right) photobleaching. The bottom panels are images taken through the CFP channel (excitation at 440 nm and emission at 480 nm) before (left) and after (right) photobleaching.

Despite the contamination of the samples with these partially mature products, it is possible to quantify the FRET efficiency (E) in fully mature Cy11.5 using the following equation:

$$E = 1 - \tau_{DA}/\tau_D \quad (1)$$

where τ_{DA} and τ_D are the excited-state lifetimes of the donor in the presence and absence of the acceptor, respectively. In this situation, τ_{DA} and τ_D are 66 ps and 2.9 ns, respectively. Thus, the FRET efficiency was calculated to be 98%.

There was a fast rise in the emission between 550 and 600 nm (Figure 3C), which occurred concomitantly with the fast decay of the emission between 450 and 500 nm (Figure 3B). Both the rise and decay shared the same lifetime of 66 ps, indicating that the lifetime indeed represented the FRET: the fast rise and decay are due to fluorescence of YFP and CFP in Cy11.5, respectively. The emission between 550 and 600 nm then decayed slowly with a lifetime of 3.9 ns, which is characteristic of YFP.

It should be noted that Cy11.5 folds efficiently in bacterial cells as evidenced by the fact that a large fraction of the prepared molecules were fully mature. Thus, the ratio of the fluorescence intensity emitted at 527 nm to that emitted at 476 nm was as high as 20 (Figure 1). The efficient folding of Cy11.5 was confirmed in mammalian cells. Cy11.5 expressed in HeLa cells was distributed uniformly in the cytosol and the nuclei. Spectral imaging revealed that the color of the emitted signals (or the fluorescence intensity ratio of YFP to CFP) was identical in the cytosol and the nucleus, and the signals were stable as long as the cells were alive, suggesting again that Cy11.5 was stable. After the cells were illuminated by intense green light from a xenon lamp (4.4 W/cm^2 at the specimen), no fluorescence signal was detected from YFP (Figure 4). On the other hand, the signal from CFP increased approximately 10-fold. The FRET efficiency was calculated to be ~90% using the following equation:

$$E = 1 - F_{DA}/F_D \quad (2)$$

where F_{DA} and F_D are the fluorescence intensities of the donor in the presence and absence of the acceptor, respectively.

Because the 527 nm to 476 nm emission ratio dramatically changes after the protein is illuminated by intense green light, Cy11.5 can be used as an optical highlighter. In the past few years, a variety of optical highlighters based on photoactivation and photoconversion have been developed, including PA-GFP (*11*), Kaede (*12*), PS-CFP (*13*), EosFP (*14*), kikGR (*15*), and PA-mRFP1 (*16*). There are also highlighters based on photobleaching. For example, a CFP−YFP fusion protein has been used for fluorescence localization after photobleaching (FLAP) (*17*). In this technique, YFP is photobleached and the ratio of YFP fluorescence to CFP fluorescence is monitored to track the movement of a tagged protein. CFP serves as an internal control because its fluorescence does not increase after irradiation with green light. In contrast, the CFP in Cy11.5 is significantly dequenched by photobleaching (Figure 4). Moreover, using Cy11.5 can provide a higher level of contrast between marked and unmarked molecules than the FLAP method. Also, the stability of Cy11.5 ensures minimal disruption of FRET and therefore low background signals. A recent paper by Valentin et al. (*18*) reported photoconversion of YFP variants, including Venus (*6*) and Citrine (*19*), into a CFP-like species during YFP photobleaching. The YFP photoconversion may yield false values for FRET efficiency obtained using eq 2. In our experiment that used cells expressing Venus, however, the photoconversion was not seen during the illumination by intense green light from a xenon lamp (4.4 W/cm^2 at the specimen) (see Figure S1 of the Supporting Information). In contrast with the wide-field illumination, Valentin et al. performed laser illumination at 514 nm in the point-scan mode, under which most LSCMs operate. It is therefore possible that the YFP photoconversion depends on the transient but high-power illumination of the laser line.

Anisotropy Measurements. To examine the structural basis of the high FRET efficiency of Cy11.5, we measured the time-resolved anisotropy of the FRET-mediated emission from YFP when CFP was selectively excited with a 400 nm pulse. The anisotropy of the emission between 550 and 600 nm quickly decayed in 0.2 ns and then gradually approached a steady-state level (Figure 5A). As shown in Figure 3C, the emission consisted of two components: a fast-rise component (τ = 66 ps) and a slow-decay component (τ = 3.9 ns). The anisotropy value just after excitation is 0.4, which arose from CFP (Figure 1C). To exclude the CFP-derived emission signal, we examined the anisotropy in the range of >0.4 ns, where the YFP-derived emission signal should dominate. Another factor to be considered was the angular displacement between the absorption and emission dipoles of CFP. Since this factor may change with the excitation wavelength, we measured the excitation anisotropy spectrum of CFP (Figure 5B). The anisotropy was almost constant when the excitation wavelength was greater than 390 nm. Considering the slow rotation of Cy11.5, the anisotropy value was estimated to approach 0.10. The relative angle (θ) between the two transition dipoles of CFP and YFP in Cy11.5 was calculated using the following equation:

$$r = 0.4[(3\cos^2\theta - 1)/2] \quad (3)$$

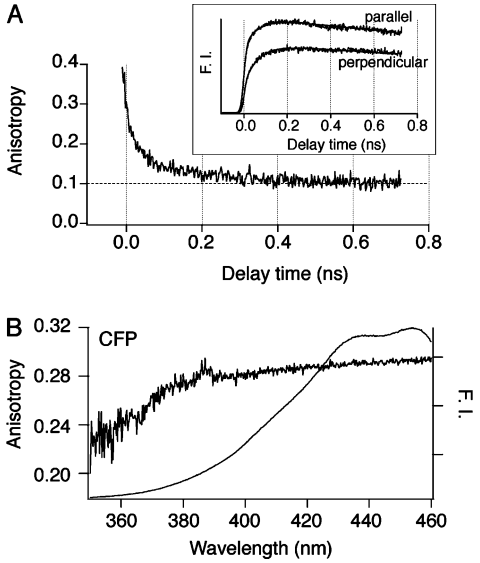

FIGURE 5: Anisotropy measurements. (A) Time-resolved anisotropy of the Cy11.5 emission between 550 and 600 nm after excitation with a 400 nm pulse generated from the second harmonic of a titanium−sapphire laser. The inset shows the parallel and perpendicular components of time-resolved emission. (B) Excitation spectra of the steady-state anisotropy (thick line) and intensity (thin line) of free CFP.

where r is the steady-state anisotropy. θ was determined to be 45°.

CONCLUSIONS

By concatenating CFP and YFP, we have developed a chimeric protein named Cy11.5, which exhibited the highest 527 nm to 476 nm emission ratio of the constructs that contain CFP and YFP. This is due to two characteristics of Cy11.5. First, highly efficient FRET occurs from CFP to YFP in the protein, which was demonstrated by time-resolved emission measurements. After excitation with a 400 nm pulse generated from the second harmonic of a titanium−sapphire laser, the emission from CFP decayed with a lifetime of 66 ps. Considering that the emission from free CFP decayed with a lifetime of 2.9 ns, the FRET efficiency was calculated to be 98%. Second, the integrity of Cy11.5 is high. Cy11.5 was found to fold well and was resistant to proteases in both bacterial and mammalian cells. Thus, Cy11.5 behaves as a stable fluorescent protein that displays highly efficient intramolecular FRET. It would be interesting to understand why the FRET in Cy11.5 is so efficient. Time-resolved anisotropy measurements determined the relative angle between the two transition dipoles of CFP and YFP in Cy11.5 to be 45°. Further studies including crystallography, however, will be required to reveal the structural basis for the efficient FRET.

SUPPORTING INFORMATION AVAILABLE

YFP (527 nm) to CFP (476 nm) emission ratios and folding efficiencies (Table S1) and changes in the emission spectrum during photobleaching by wide-field illumination (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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