ShadowY: a dark yellow fluorescent protein for FLIM-based FRET measurement

Hideji Murakoshi1,2,3 & Akihiro C. E. Shibata1

Fluorescence lifetime imaging microscopy (FLIM)-based Förster resonance energy transfer (FRET) measurement (FLIM-FRET) is one of the powerful methods for imaging of intracellular protein activities such as protein–protein interactions and conformational changes. Here, using saturation mutagenesis, we developed a dark yellow fluorescent protein named ShadowY that can serve as an acceptor for FLIM-FRET. ShadowY is spectrally similar to the previously reported dark YFP but has a much smaller quantum yield, greater extinction coefficient, and superior folding property. When ShadowY was paired with mEGFP or a Clover mutant (CloverT153M/F223R) and applied to a single-molecule FRET sensor to monitor a light-dependent conformational change of the light-oxygen-voltage domain 2 (LOV2) in HeLa cells, we observed a large FRET signal change with low cell-to-cell variability, allowing for precise measurement of individual cell responses. In addition, an application of ShadowY to a separate-type Ras FRET sensor revealed an EGF-dependent large FRET signal increase. Thus, ShadowY in combination with mEGFP or CloverT153M/F223R is a promising FLIM-FRET acceptor.

Protein conformational changes and protein–protein interactions form the basis of intracellular biochemical signal transduction. Fluorescence lifetime imaging microscopy (FLIM)-based Förster resonance energy transfer (FRET) measurement (FLIM-FRET) is one of the powerful methods for imaging of intracellular protein activities such as protein–protein interactions and conformational changes1–3. As a pair of fluorescent proteins for FLIM-FRET, enhanced green fluorescent protein (EGFP) as an energy donor and red fluorescent protein (RFP) as an energy acceptor are frequently used4, 5. Because this pair has well-separated emission spectra, this combination prevents spectral contamination due to the bleed-through of RFP fluorescence to the EGFP channel. In contrast to this advantage, because the spectral overlap between the EGFP emission and RFP excitation spectra is relatively small, Förster distance is also relatively short6. Furthermore, these fluorescent proteins occupy a wide range of wavelengths (500–650 nm), which makes it difficult to use additional fluorescent dyes for multi-color imaging.

FLIM-FRET requires only donor fluorescence (not acceptor fluorescence) for the detection of FRET7. By means of this feature, a fluorescent protein with a low quantum yield called resonance energy-accepting chromoprotein (REACh) was developed and applied to an acceptor of FRET7. Because this protein has significant absorption properties, it can be used as an acceptor of FRET. When REACh is paired with EGFP, there are three advantages over the EGFP–RFP pair. First, because the spectral overlap of EGFP emission and REACh absorption is larger, the Förster distance is longer (5.6–6.2 nm)7, 8 than those of the EGFP–mRFP1/DsRed/mCherry pairs (~4.7–5.3 nm)9–11 allowing us to detect the FRET signal in a long range. Second, because REACh has only weak fluorescence, the spectral separation between EGFP and REACh emission is not required, enabling us to utilize the whole wavelength range of EGFP fluorescence (500–600 nm) as a signal. Third, because only EGFP fluorescence is present, multicolor imaging using another fluorescent protein such as RFP is possible7.

Almost a decade ago, an improved version of REACh called super REACh (sREACh) was designed by introducing several mutations, and the pairing of this protein with A206K-mutated monomeric EGFP (mEGFP) successfully improved the FRET signal because of enhanced maturation efficiency of sREACh in cells12. Although mEGFP–sREACh pair yields a superior FRET signal, the spectral contamination of basal sREACh fluorescence owing to residual quantum efficiency (0.07) can produce unexpected artifacts, limiting applications of this pair7.

1Supportive Center for Brain Research, National Institute for Physiological Sciences, Okazaki, Aichi, 444-8585, Japan. 2Department of Physiological Sciences, Sokendai (The Graduate University for Advanced Studies), Okazaki, Aichi, 444-8585, Japan. 3Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency (JST), Kawaguchi, Saitama, 332-0012, Japan. Correspondence and requests for materials should be addressed to H.M. (email: murakosh@nips.ac.jp)
and R168Y. ND: not determined. Following mutations in sREACh: N144A, N146P, S147V, A206K, and R223F. sREACh#2 contains the following mutations in addition to the above: N144A, N146P, S147V, A206K, and R223F. After the insertion of these mutations, colonies were confirmed to have no fluorescence under blue light by a method similar to the one described elsewhere. Vividly colored colonies under daylight, confirming that the mutants have high absorption. Subsequently, the pairing of the mutants with dark yellow fluorescent proteins that have a high extinction coefficient but a low quantum yield, we first identified sREACh#1, a mutant that shows improved darkness compared with sREACh. Furthermore, we confirmed that the pairing of ShadowY with mEGFP, Clover, or its mutant (Clover T153M/F223R) shows improved FRET signals with reduced cell-to-cell variability. Thus, mEGFP–ShadowY, Clover mutant–ShadowY are good FLIM-FRET pairs.

### Results

To create a dark yellow fluorescent protein, we applied saturation mutagenesis to amino acid positions N144, N146, S147, and V148 surrounding the chromophore in the previously reported dark yellow fluorescent protein, sREACh (Fig. 1). Because position W145 is crucial for reduction of quantum efficiency, we avoided introducing a mutation at this position. We introduced the A206K monomeric mutation, while F223R was reversed (Fig. 1a); F223R increases the dissociation constant more than F223R does. The PCR products with saturated mutations were ligated into a bacterial expression vector, and we thus constructed a genetic library. To screen the library for fluorescent proteins, we used fluorescence microscopy and fluorescence lifetime imaging microscopy (FLIM). The fluorescence lifetime of each protein was measured by FLIM, and the results were compared with those of sREACh. Furthermore, we confirmed that the pairing of ShadowY with mEGFP, Clover, or its mutant (Clover T153M/F223R) shows improved FRET signals with reduced cell-to-cell variability. Thus, mEGFP–ShadowY, Clover mutant–ShadowY are good FLIM-FRET pairs.

### Tabular Data

| Protein     | EC (M⁻¹·cm⁻¹) | QE | Ex (nm) | Em (nm) | Folding t₁/₂ (sec) | Oxidation t₁/₂ (min) | Fluorescence lifetime (ns) |
|-------------|---------------|----|---------|---------|------------------|---------------------|---------------------------|
| ShadowG     | 89,000*       | 0.005* | 486* | 510* | 37* | 76* | 0.16* ND |
| sREACh      | 115,000*      | 0.07* | 517 | 531* | 267 | 130 | 0.67 0.54 |
| sREACh (#1) | 114,000       | 0.03 | 518 | 530 | 149 | ND | 0.37 0.33 |
| sREACh (#2) | 130,000       | 0.02 | 519 | 531 | 75  | ND | 0.25 0.22 |
| ShadowY     | 136,000       | 0.01 | 519 | 531 | 73  | 140 | 0.19 0.17 |

### Table 1

Characteristics of ShadowY. EC: extinction coefficient, QE: quantum efficiency, Ex: excitation maximum, Em: emission maximum. *Values obtained from previously published data. sREACh#1 contains the following mutations in sREACh: N144A, N146P, S147V, A206K, and R223F. sREACh#2 contains the following mutations in sREACh#1: Q204S and S205A. ShadowY contains the following mutations in sREACh#2: K166S and R168Y. ND: not determined.
and absorption relative to sREACh2 (Table 1). We named this mutant ShadowY where Y stands for “yellow”, and decided to pursue further analyses of this protein.

Spectral analysis of purified ShadowY confirmed that it has an excitation peak at 519 nm and an emission peak at 531 nm (Fig. 2a, Table 1), similar to those of sREACh (Table 1). Further analysis revealed that the molar extinction coefficient of ShadowY is 136,000 M$^{-1}$ cm$^{-1}$: a 1.2-fold greater extinction coefficient than that of sREACh (Table 1). Quantum efficiency of ShadowY is 0.01, which is 7-fold smaller than that of sREACh (QE, 0.07; Table 1). Consistent with these results, two-photon excitation spectrum of ShadowY exhibited the low fluorescence compared with those of mEGFP and Clover (Fig. 2c), and the fluorescence lifetime of ShadowY (0.19 ns) is much shorter than that of sREACh (0.67 ns; Table 1, Fig. 2d).

We next characterized the folding and maturation kinetics of ShadowY by the urea-denaturation method as described previously. The fluorescence of denatured ShadowY recovered in 73 sec: faster than recovery of sREACh (267 sec; Table 1, Fig. 2e), suggesting that ShadowY has superior folding properties. Next, chromophores
of the urea-denatured ShadowY were reduced with dithionite, and reoxidation time and recovery were monitored after dilution in urea-free buffer. Reoxidation time of ShadowY (140 min) is comparable to that of sREACh (130 min; Fig. 2f, Table 1).

Next, we tested the performance of ShadowY as an energy acceptor for 2-photon FLIM-FRET via comparison with sREACh in HeLa cells. We used 2-photon excitation for imaging because of the reduced phototoxicity compared with 1-photon excitation. We chose mEGFP or Clover as an energy donor, because the emission spectra of these proteins significantly overlap with the excitation spectrum of ShadowY (Fig. 3a,b). To quantify the performance of mEGFP–ShadowY and Clover–ShadowY pairs in comparison with mEGFP–sREACh and Clover–sREACh pairs, we fused these fluorescent proteins to the N and C termini of a light-sensitive LOV2-Jα helix domain from Phototropin 11,18,19, respectively, creating mEGFP-LOV2-ShadowY, mEGFP-LOV2-sREACh, Clover-LOV2-ShadowY, and Clover-sREACh as LOV2 FRET sensors (Fig. 4a), and monitored the blue-light-dependent structural change in HeLa cells by means of 2-photon FLIM-FRET (Fig. 4a,b). HeLa cells expressing the LOV2 FRET sensor were illuminated with blue light at 35 mW/cm² for 2 sec (Fig. 4b–d). Right after illumination, the fluorescence lifetime of mEGFP in LOV2 FRET sensors increased, i.e., FRET decreased, and returned in ~60 sec, consistent with another study.20 The quantitative analysis indicated a significant increase in the fluorescence lifetime change of mEGFP-LOV2-ShadowY relative to mEGFP-LOV2-sREACh (Fig. 4e). Furthermore, we compared the cell-to-cell variability of FRET signals (Fig. 4g,h), and found that the variability of mEGFP-LOV2-ShadowY in both the basal state and after light illumination (before, 1.94 ± 0.05 ns; after, 2.13 ± 0.04 ns) is smaller than that of mEGFP-LOV2-sREACh (before, 1.96 ± 0.06 ns; after, 2.12 ± 0.05 ns). Taken together, these results suggest that ShadowY is superior FLIM-FRET acceptor.

Next, we compared Clover-LOV2-ShadowY and Clover-LOV2-sREACh and found that Clover-LOV2-ShadowY shows larger lifetime change and smaller cell-to-cell variability (before, 2.03 ± 0.05 ns; after, 2.21 ± 0.08 ns) than Clover-LOV2-sREACh does (before, 1.99 ± 0.08 ns; after, 2.13 ± 0.09 ns; Fig. 4d,f,i,j). Nevertheless, when Clover-LOV2-ShadowY was compared with mEGFP-LOV2-ShadowY, the cell-to-cell variability of cells expressing Clover-LOV2-ShadowY, especially after light illumination (after, 2.21 ± 0.08 ns; Fig. 4i), was larger than that of mEGFP-LOV2-ShadowY (after, 2.13 ± 0.04 ns; Fig. 4b). Therefore, we attempted to improve cell-to-cell variability by introducing mutations into Clover. We speculated that the difference in cell-to-cell variability originates from the difference in amino acid sequence between mEGFP and Clover, especially the amino acids whose side chains are outward-directed. First, using EYFP crystal structure (Fig. 1b), we confirmed that the side chains of amino acids located at R30, N39, S99, T105, T153, and A206 in Clover are outward-directed and are different from those of mEGFP. Next, each of the above amino acid residues was reverted to the residue corresponding to mEGFP, i.e., R30S, N39Y, S99F, T105N, T153M, and A206K, respectively. Among these mutations, T153M improved the lifetime change and reduced the cell-to-cell variability (before, 2.10 ± 0.05 ns; after, 2.32 ± 0.05 ns; Fig. 4k), compared with Clover/ShadowY pair. Furthermore, to increase monomericity, we introduced the F223R monomeric mutation into CloverT153M.21 Resultant construct CloverT153M/F223R–LOV2-ShadowY showed performance that was similar to that of CloverT153M–LOV2-ShadowY (before, 2.04 ± 0.06 ns; after, 2.27 ± 0.05 ns; Fig. 4l). Spectral analysis of purified Clover mutants confirmed that CloverT153M/F223R has a comparable extinction coefficient and quantum efficiency with those of Clover and CloverT153M (Table 2). Moreover, Förster distance between CloverT153M/F223R and ShadowY was 6.4 nm, comparable with that of mEGFP–ShadowY and Clover–ShadowY pairs (Table 2).

To further characterize ShadowY, we measured FRET efficiency and maturation efficiency using tandem fluorescent proteins in HeLa cells as described previously. We expressed tandem constructs (Fig. 5a), and the fluorescence lifetime of mEGFP, Clover, or CloverT153M/F223R was measured by 2-photon FLIM-FRET (Fig. 5b–g). Because the fluorescence lifetime decay curves are convolution of both the FRET efficiency and maturity of an acceptor, we measured these parameters separately, as described earlier.12 Although FRET efficiencies of all the compared pairs showed comparable values (Fig. 5b,d,f), the maturity of ShadowY was found to be slightly better than that of sREACh in all pairs (Fig. 5c,e,g).

Next, mEGFP–ShadowY, Clover–ShadowY, and CloverT153M/F223R–ShadowY pairs were applied to a separate-type H-Ras FRET sensor3,20, and their FRET signals were compared (Fig. 6). We did not compare with sREACh because it has a bleed-through effect (Fig. 5l). As a FRET donor, H-Ras was fused to mEGFP, Clover, or CloverT153M/F223R, and as an acceptor, the Ras-binding domain of Raf1 was fused to ShadowY (Fig. 6a). The donor

Figure 3. The spectral overlap of fluorescent proteins. (a,b) The spectral overlap (yellow region) between ShadowY’s excitation spectrum and mEGFP’s (a) or Clover’s (b) emission spectra.
and acceptor were fused via the P2A sequence to ensure equal expression of these molecules and to minimize the response variability due to the imbalanced expression of the donor and acceptor. We transfected HeLa cells with these FRET sensors and compared their response signals as a binding fraction change (Fig. 6b–g). After stimulation with epidermal growth factor (EGF), H-Ras was rapidly activated (within a few minutes; Fig. 6b,c). When the FRET response signals of Ras sensors were compared, all the three FRET sensors showed a similar signal change (Fig. 6d–g), with the values of $21.34 \pm 1.05$ (mEGFP–ShadowY), $18.39 \pm 0.77$ (Clover–ShadowY), and $20.62 \pm 0.86$ (Clover T153M/F223R–ShadowY), respectively (Fig. 6d).

Figure 4. Performance of ShadowY in LOV2 FRET sensors in HeLa cells. (a) A schematic of a conformational change of the light-sensitive LOV2 FRET sensors. (b) Representative fluorescence lifetime images of mEGFP-LOV2-ShadowY after blue light illumination for 2 seconds at 35 mW/cm². The scale bar is 50 µm. (c) An averaged time course of fluorescence lifetime changes in response to blue light illumination. The number of cells analyzed is 70 for mEGFP–sREACh and 73 for mEGFP–ShadowY. The data are presented as mean ± SEM. (d) An averaged time course of fluorescence lifetime changes in response to blue light illumination. The number of cells analyzed is 79 for Clover–sREACh, 76 for Clover–ShadowY, 74 for Clover T153M–ShadowY, and 73 for Clover T153M/F223R–ShadowY. The data are presented as mean ± SEM. (e,f) The fluorescence lifetime changes at 20 sec after blue light illumination were quantified using the data presented in (c,d), respectively. The data are presented as mean ± SEM. Asterisks denote statistical significance ($p < 0.05$, analysis of variance [ANOVA] followed by Scheffé’s post hoc test). N.S. = not statistically significant. (g–l) The lifetime changes in individual HeLa cells before and after light illumination (the same dataset as in panels c and d). The basal fluorescence lifetime (averaged over −1.3 to 0 min) of individual cells is plotted in the descending order (black) along with the corresponding fluorescence lifetime values (at 20 sec) after blue-light illumination (red). The data are also presented as mean ± SD on the right. The number of samples (n) is indicated in respective panels.
Plasmid construction. In all DNA construction procedures described below, a modified pEGFP-C1 plasmid (Clontech) served as a backbone vector. For construction of the EGFP or Clover with the CAAX motif of K-Ras (corresponding to amino acid residues 173–188), EGFP or Clover fused to CAAX via a linker encoding the peptide ASM was inserted into the vector by replacing EGFP. To create cytosolic mCherry, the peptide SGLRSRAQASNSAV was inserted into the vector by replacing EGFP. For construction of cytosolic mCherry, a donor fluorescent protein was fused to the N terminus of the LOV2 domain (DNA sequence corresponding to amino acid residues 404–546 in Phototropin 1) via a linker encoding the peptide ASK. The acceptor fluorescent protein was fused to the C terminus of the LOV2 domain via the linker peptide KLGS.

For construction of the H-Ras FRET sensors, we fused an acceptor fluorescent protein to the C terminus of the Ras-binding domain of Raf1 (amino acid residues 50–131 with two mutations: K65E and K108A) via the linker peptide GSG. Subsequently, H-Ras fused to a donor fluorescent protein via the linker peptide SGLRSG was fused to the C terminus of the acceptor protein via the P2A sequence11 so that the Ras-binding domain and H-Ras parts were translated into different polypeptides within the cell.

Fluorescent properties of the fluorescent proteins. His-tagged fluorescent proteins were over-expressed in Escherichia coli DH5α cells using a modified pRSET vector (Invitrogen) and purified on a Ni²⁺-nitrilotriacetate column (HiTrap, GE Healthcare). Excitation and emission spectra of the fluorescent proteins were measured under the two-photon fluorescence microscope (FVMPE-RS; Olympus). An Insight Ti:Sapphire laser (Spectra-Physics) with the power of 3.4–4.5 mW at the respective

| Protein      | EC (M⁻¹·cm⁻¹) | QE | Ex (nm) | Em (nm) | Fluorescence lifetime (ns) | Förster distance with mEGFP (nm) |
|--------------|---------------|----|---------|---------|---------------------------|----------------------------------|
| mEGFP       | 58000*        | 0.73* | 488*   | 507*   | 2.73 2.60 6.2 6.2*         | sREACh (nm)                      |
| Clover       | 111000*       | 0.76* | 505*   | 515*   | 3.29 3.11 6.3 6.3         |                                   |
| CloverΔN354  | 112000        | 0.80  | 505    | 515    | 3.34 3.11 6.4 6.4         |                                   |
| CloverΔN354ΔN99 | 122000      | 0.81  | 505    | 515    | 3.31 3.08 6.4 6.4         |                                   |

Table 2. Characteristics of Clover mutants. EC: extinction coefficient, QE: quantum efficiency, Ex: excitation maximum, Em: emission maximum. *Values obtained from previously published data8,13, respectively. For the calculation of Förster distance, random interferophore orientation were assumed30. ND: not determined.

Discussion
Here, we successfully developed a new dark yellow fluorescent protein, ShadowY, as a FLIM-FRET acceptor for pairing with mEGFP or the Clover mutant. ShadowY has superior properties in terms of absorption and folding kinetics relative to sREACh (Fig. 2, Table 1). These factors most likely contribute to the increased FRET signals and the reduced cell-to-cell variability, compared with those of sREACh (Figs 4 and 5). Furthermore, although sREACh is difficult to apply to a separate-type FRET sensor because of bleed-through fluorescence contamination8, ShadowY does not have this problem because of the superior darkness relative to sREACh (Fig. S1). An application of ShadowY to an LOV2 and H-Ras FRET sensors yielded a large FRET change (Figs 4c,d and 6c), which is larger than that of the previously reported mCherry or ShadowG version of sensors8.

In the past decade, several types of dark fluorescent proteins have been identified and applied to FRET imaging, photoacoustic imaging, and structural analysis7,8,12,22–25. We believe that ShadowY will be an additional useful tool for these studies, especially for FLIM-FRET.

Materials and Methods
Saturation mutagenesis. The sREACh gene in a customized pRSET vector (Invitrogen) served as an initial template for construction of genetic libraries. First, a XhoI restriction site was silently introduced at the positions corresponding to amino acid residues L141 and E142 in sREACh (Fig. 1a). Saturated mutagenesis was performed by PCR amplification of sREACh (the fragment corresponding to amino acid positions 141–238) with degenerate primers. These primers are as follows: For sREACh#1, FW 5′-gagactcgagctacNNBttgcgtatatcatggccgacaagcagaagaacggcatcaaggtgaacttcNNKatcNN-3′, RV 5′-gagaggatcccttgtacagctcgtccat-3′, RV 5′-gagaggatcccttgtacagctcgtccat-3′, and XhoI and BsrGI are used for subcloning into the custom pRSET vector; for sREACh#2, FW 5′- gagagccccgtctgctgccgacaaacactactgtacgtagctacNNBNNBaNaaagNNBqcagaacgcccccaac-3′, RV 5′-gagaggtcctttgacatcgctgc-3′, and Apal and BsrGI sites were used for subcloning; for ShadowY, FW 5′-gagactcgagctacNNBttgcgtatatcatggccgacaagcagaagaacggcatcaaggtgaacttcNNKatcNN-3′, RV 5′-gagaggtcctttgacatcgctgc-3′, and XhoI and BsrGI sites were used for subcloning. The plasmid was then introduced into electrocompetent cells, and the cells were grown for 18–20 h at 34°C on LB agar plates supplemented with antibiotics.

Plasmid construction. In all DNA construction procedures described below, a modified pEGFP-C1 plasmid (Clontech) served as a backbone vector. For construction of the EGFP or Clover with the CAAX motif of K-Ras (corresponding to amino acid residues 173–188), EGFP or Clover fused to CAAX via a linker encoding the peptide SGLRSRAQASNSAV was inserted into the vector by replacing EGFP. To create cytosolic mCherry, the peptide SGLRSRAQASNSAV was inserted into the vector by replacing EGFP. For construction of the LOV2 FRET sensor, a donor fluorescent protein was fused to the N terminus of the LOV2 domain (DNA sequence corresponding to amino acid residues 404–546 in Phototropin 1) via a linker encoding the peptide ASK. The acceptor fluorescent protein was fused to the C terminus of the LOV2 domain via the linker peptide KLGS.

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Fluorescent properties of the fluorescent proteins. His-tagged fluorescent proteins were over-expressed in Escherichia coli DH5α cells using a modified pRSET vector (Invitrogen) and purified on a Ni²⁺-nitrilotriacetate column (HiTrap, GE Healthcare). Excitation and emission spectra of the fluorescent proteins were diluted in PBS were recorded on a spectrophotometer (RF-6000; Shimadzu). Matured-protein concentrations were calculated from the extinction coefficient of the chromophore after denaturation in 0.1 N NaOH (40,000 M⁻¹·cm⁻¹ at 446 nm)30. The extinction coefficients of fluorescent proteins were determined by dividing the peak optical density by the molar concentration of matured proteins. Quantum efficiency of the proteins was determined by a comparison with that of Clover (0.76) as described elsewhere13.

Two-photon excitation spectra were measured under the two-photon fluorescence microscope (FVMPE-RS; Olympus). An Insight Ti:Sapphire laser (Spectra-Physics) with the power of 3.4–4.5 mW at the respective
Figure 5. FRET efficiency and maturity of ShadowY in tandem fluorescent proteins. (a) A schematic drawing of the tandem fluorescent protein used to evaluate the FRET efficiency and fraction of the donor fluorescent protein undergoing FRET (chromophore maturation efficiency) for ShadowY. (b,d,f) Comparison of FRET efficiency of the tandem fluorescent proteins. The fluorescence lifetime decay curve averaged over the whole image was used for the analysis (See Materials and Methods). The number of images used for the analysis is 10–13. Each image contains 8–15 cells, and the data are presented as mean ± SEM. Asterisks denote statistical significance (* t test, **P < 0.05, ***P < 0.01, ****P < 0.001, N.S. = not significant). (c,e,g) A comparison of the fraction of donor fluorescent protein undergoing FRET analyzed in individual cells and data was plotted in the descending order. The FRET fraction is directly related to the maturation efficiency of an acceptor, i.e., sREAC or ShadowY. Means ± SD are also plotted on the right (* t test, **P < 0.05, ***P < 0.01, ****P < 0.001, N.S. = not significant). The number of samples (n) and mean ± SD are also indicated in figures.

wavelength under the objective lens was used to excite the purified fluorescent proteins. Raw fluorescence intensity values were corrected by dividing them by squared laser power used for each wavelength.

Refolding and reoxidation. To measure the refolding time of ShadowY after denaturation, the proteins were dissolved in denaturation buffer (8 M urea, 1 mM dithiothreitol) and heated at 95 °C for 5 min as described previously. The refolding was initiated by diluting the denatured protein with a 100-fold volume of renaturation buffer (5 mM KCl, 2 mM MgCl₂, 50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol) at room temperature. For the reoxidation experiment, 5 mM dithionite was added into the denaturation buffer to reduce the chromophore. The respective fluorescent protein was excited at 517 nm with 5 nm bandwidth, and its fluorescence recovery was monitored at 531 nm with 5 nm bandwidth in a spectrofluorometer (RF-6000; Shimadzu).

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; supplemented with 10% of fetal bovine serum) at 37 °C and 5% of CO₂. The cells were transfected with the plasmids by means of Lipofectamine 3000 (Invitrogen), followed by incubation for 16–20 h. FLIM-FRET imaging was conducted in a solution containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 30 mM, pH 7.3)-buffered artificial cerebrospinal fluid (130 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1.25 mM NaH₂PO₄, 25 mM glucose) at room temperature.

Two-photon fluorescence lifetime imaging. Details of the 2-photon FLIM-FRET imaging were described elsewhere. Briefly, mEGFP or Clover in the FRET sensor was excited with a Ti-sapphire laser (Mai Tai; Spectra-Physics) tuned to 920 nm. The scanning mirror was controlled with the ScanImage software. The green fluorescent photon signals were collected by an objective lens (60×, 0.9 NA; Olympus) and a photomultiplier tube (H7422-40p; Hamamatsu) placed after a dichroic mirror (565DCLP; Chroma) and emission filter (FF01-510/84 or FF03-510/20 in Fig. S1; Semrock). Measurement of fluorescence lifetime was conducted using a time-correlated single-photon counting board (SPC-150; Becker & Hickl) controlled with custom software.
construction of a fluorescence lifetime image, the mean fluorescence lifetime in each pixel was translated into a color-coded image. Analysis of the lifetime change and binding-fraction change was conducted as described elsewhere. In Fig. 4, blue LED (244-87-470-50E-40; CoolLED) with a band pass filter (FF01-469/35-25; Chroma) was used for illumination to induce the structural change of LOV2 FRET sensors.

Analysis of the fluorescence lifetime image. To generate fluorescence lifetime images, we acquired the mean fluorescence lifetime in each pixel by calculating the mean photon arrival time \( \langle t \rangle \) as

\[
\langle t \rangle = \frac{\int t F(t) dt}{\int F(t) dt} - t_0
\]

where \( t_0 \) is obtained by fitting the whole image with single exponential or double exponential functions convolved with an instrument response function as described previously. After that, the mean fluorescence lifetime in each pixel was converted to the corresponding color. FRET efficiency and the fraction of the donor fluorescent protein undergoing FRET were calculated as in other studies.

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Author Contributions
H.M. conceived and designed the experiments. H.M. and A.S. conducted the experiments and data analysis. H.M. wrote the paper.

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