ShadowR: a novel chromoprotein with reduced non-specific binding and improved expression in living cells

Hideji Murakoshi1,2, Hiroshi Horiuchi2,4, Takahiro Kosugi3,5,6, Maki Onda1, Aiko Sato1, Nobuyasu Koga3,5,6 & Junichi Nabekura2,4

Here we developed an orange light-absorbing chromoprotein named ShadowR as a novel acceptor for performing fluorescence lifetime imaging microscopy-based Förster resonance energy transfer (FLIM-FRET) measurement in living cells. ShadowR was generated by replacing hydrophobic amino acids located at the surface of the chromoprotein Ultramarine with hydrophilic amino acids in order to reduce non-specific interactions with cytosolic proteins. Similar to Ultramarine, ShadowR shows high absorption capacity and no fluorescence. However, it exhibits reduced non-specific binding to cytosolic proteins and is highly expressed in HeLa cells. Using tandem constructs and a LOVTRAP system, we showed that ShadowR can be used as a FRET acceptor in combination with donor mRuby2 or mScarlet in HeLa cells. Thus, ShadowR is a useful, novel FLIM-FRET acceptor.

Fluorescent proteins are widely used to monitor the localization and dynamics of intracellular proteins in living cells. To detect protein-protein interactions and conformational changes in living cells, Förster resonance energy transfer (FRET) measurement is often used in combination with fluorescent proteins1–3. FRET can be monitored when a donor fluorescent molecule is excited and an acceptor molecule is in close proximity (<10 nm). The energy of the excited donor molecule is transferred to the acceptor molecule, and fluorescence is emitted from the acceptor molecule rather than from the donor molecule4,6–8. One of quantitative methods to measure and image FRET is fluorescence lifetime imaging microscopy (FLIM) where the fluorescence lifetime of the donor, the time spent in an excited state, is measured and used to quantify FRET8,9. As an example of FRET, green fluorescent protein (GFP) and red fluorescent protein (RFP) have often been used as energy donor and acceptor molecules, respectively10–12. When GFP is excited, it emits green fluorescence. But, if RFP is located within 10 nm of the GFP molecule, the excitation energy is transferred from GFP to RFP, leading to RFP emission1,3,5,7,10. Since GFP fluorescence decreases and RFP fluorescence increases under FRET, the fluorescence intensity changes of both the proteins are used for FRET imaging (i.e., ratiometric imaging) and analysis. When FRET occurs between GFP and RFP, the fluorescence lifetime of GFP is shortened10–12. This fluorescence lifetime change can be used as a readout of FRET in FLIM-FRET11. One of the characteristics of FLIM-FRET is that it measures only the donor fluorescence, but not the acceptor fluorescence13. This characteristic enables the use of a dim fluorescent protein as an acceptor for FRET14. Dim fluorescent proteins/chromoproteins, which have large extinction coefficients but low quantum yield have been developed and used for FLIM-FRET measurement15–21. The advantage of non-fluorescent acceptor proteins is that only donor fluorescence exists, and therefore spectral separation between donor and acceptor fluorescence is not required. Thus, FRET in combination with chromoproteins can be monitored in a narrow bandwidth, facilitating multicolor FRET imaging14,15,18,19,22.

1Supportive Center for Brain Research, National Institute for Physiological Sciences, Okazaki, Aichi, 444-8585, Japan. 2Department of Physiological Sciences, The Graduate University for Advanced Studies, Hayama, Kanagawa, 240-0193, Japan. 3Department of Structural Molecular Science, The Graduate University for Advanced Studies, Hayama, Kanagawa, 240-0193, Japan. 4Division of Homeostatic Development, National Institute for Physiological Sciences, Okazaki, Aichi, 444-8585, Japan. 5Exploratory Research Center on Life and Living Systems (ExCELLS), Okazaki, Aichi, 444-8585, Japan. 6Research Center of Integrative Molecular Systems, Institute for Molecular Science, Okazaki, Aichi, 444-8585, Japan. Hiroshi Horiuchi and Takahiro Kosugi contributed equally. Correspondence and requests for materials should be addressed to H.M. (email: murakosh@nips.ac.jp)
of mCherry (Fig. S1). We chose this chimera because it is slightly more hydrophilic than Ultramarine (Fig. 1b).

Many hydrophobic amino acids on its surface (Fig. 1a). We attempted to make Ultramarine more hydrophilic to replacing the Ultramarine amino acid sequences (1–51, 118–157, and 231–236) with the corresponding regions

identified 105 amino acids whose side chains are directed outward from the chimera. Among them, 32 hydrophobic amino acids indicated by green arrowheads in Fig. S1 were selected to be replaced by more hydrophilic ones

was used as a template to represent ShadowR. Relatively hydrophobic acids (I, V, L, F, C, M, A, G) are colored in

To create a novel chromoprotein, we chose Ultramarine as a template16. Ultramarine is a monomeric chromo-

Although fluorescent proteins are useful tools in monitoring cellular events, they may be toxic to cells due to non-specific binding to other cellular proteins and inhibition of their activity23–26. In addition, when florescent proteins are used to monitor the localization of a certain protein, non-specific interactions of the fluorescent protein could disturb proper localization of the labeled protein. Thus, it is crucial to develop fluorescent proteins or chromoproteins that do not non-specifically bind to cytosolic proteins. Since protein-protein interactions are

replacing the hydrophobic amino acids located at the surface of Ultramarine with hydrophilic ones to reduce non-specific binding to other cellular proteins and inhibition of their activity 23–26. In addition, when fluorescent proteins are used to monitor the localization of a certain protein, non-specific interactions of the fluorescent protein could disturb proper localization of the labeled protein. Thus, it is crucial to develop fluorescent proteins or chromoproteins that do not non-specifically bind to cytosolic proteins. Since protein-protein interactions are

To identify amino acids, we utilized the crystal structure (Protein Data Bank ID: 2H5Q) of mCherry which shares 64% homology to Ultramarine. Blue and Red squares indicate that the amino acids in Ultramarine were replaced to more hydrophilic and hydrophobic amino acids, respectively. Gray squares indicate that the hydrophobicity of the amino acids is identical between Ultramarine and ShadowR. The chromophore tripeptide is highlighted with a magenta box.

(a) Amino acid sequences of Ultramarine and ShadowR are shown in blue and black letters, respectively. Extra amino acids (gray characters) were added to the N and C termini of Ultramarine to match the molecular size of ShadowR. Squares (gray, blue, red) indicate that the amino acids (10–228 in Ultramarine/ShadowR) whose side chains are directed outward from the protein. To identify amino acids, we utilized the crystal structure (Protein Data Bank ID: 2H5Q) of mCherry which shares 64% homology to Ultramarine. Blue and Red squares indicate that the amino acids in Ultramarine were replaced to more hydrophilic and hydrophobic amino acids, respectively. Gray squares indicate that the hydrophobicity of the amino acids is identical between Ultramarine and ShadowR. The chromophore tripeptide is highlighted with a magenta box.

(b) Hydrophobicity index of respective proteins as calculated simply by summing the hydropathy index of amino acids31 is indicated by the squares. (c) A homology model of ShadowR created by SWISS-MODEL44. The X-ray crystal structure of mCherry mutant (PDB ID 2H5Q)43 was used as a template to represent ShadowR. Relatively hydrophobic acids (I, V, L, F, C, M, A, G) are colored in magenta. The rest of amino acids which are the relatively hydrophilic ones (T, S, W, Y, P, N, Q, D, E, H, K, R) are colored in blue and Red indicate the amino acids substituted to more hydrophilic or hydrophobic ones in Ultramarine.

Results

To create a novel chromoprotein, we chose Ultramarine as a template16. Ultramarine is a monomeric chromo-

protein with a relatively large extinction coefficient (64,000 M\(^{-1}\)cm\(^{-1}\)) and low quantum yield (~0.001), but has many hydrophobic amino acids on its surface (Fig. 1a). We attempted to make Ultramarine more hydrophilic to reduce its non-specific hydrophobic interaction with cytosolic proteins. To do that, we first created a chimera by replacing the Ultramarine amino acid sequences (1–51, 118–157, and 231–236) with the corresponding regions of mCherry (Fig. S1). We chose this chimera because it is slightly more hydrophilic than Ultramarine (Fig. 1b).

The rest of the sequence was not replaced because it surrounds the chromophore and is important for absorption and low quantum yield. Based on the crystal structure data of mCherry (Protein Data Bank ID: 2H5Q), we identified 105 amino acids whose side chains are directed outward from the chimera. Among them, 32 hydrophobic amino acids indicated by green arrowheads in Fig. S1 were selected to be replaced by more hydrophilic ones using single-amino acid saturation mutagenesis, or we replaced to either corresponding mCherry or Ultramarine amino acid (Fig. S1). When the outward directed amino acids are clustered, those are simultaneously subjected to

Figure 1. Sequence alignment of Ultramarine and ShadowR. (a) Amino acid sequences of Ultramarine and ShadowR are shown in blue and black letters, respectively. Extra amino acids (gray characters) were added to the N and C termini of Ultramarine to match the molecular size of ShadowR. Squares (gray, blue, red) indicate that the amino acids (10–228 in Ultramarine/ShadowR) whose side chains are directed outward from the protein. To identify amino acids, we utilized the crystal structure (Protein Data Bank ID: 2H5Q) of mCherry which shares 64% homology to Ultramarine. Blue and Red squares indicate that the amino acids in Ultramarine were replaced to more hydrophilic and hydrophobic amino acids, respectively. Gray squares indicate that the hydrophobicity of the amino acids is identical between Ultramarine and ShadowR. The chromophore tripeptide is highlighted with a magenta box. (b) Hydrophobicity index of respective proteins as calculated simply by summing the hydropathy index of amino acids31 is indicated by the squares. (c) A homology model of ShadowR created by SWISS-MODEL44. The X-ray crystal structure of mCherry mutant (PDB ID 2H5Q)43 was used as a template to represent ShadowR. Relatively hydrophobic acids (I, V, L, F, C, M, A, G) are colored in magenta. The rest of amino acids which are the relatively hydrophilic ones (T, S, W, Y, P, N, Q, D, E, H, K, R) are colored in blue and Red indicate the amino acids substituted to more hydrophilic or hydrophobic ones in Ultramarine, respectively. For the electrostatic surface potential of Ultramarine and ShadowR, see Supporting Fig. 2S.
be due to differences in experimental conditions or operational differences.

Since EC and QE measurements could be operation sensitive, we carried measurable because of the lack of fluorescence. Since the absorption spectrum of ShadowR significantly over -

with those of ShadowR and Ultramarine proteins, as described previously (Fig. 4a)18. We expressed tandem con-

ured FRET efficiency and maturation efficiency using fusion proteins in HeLa cells and compared the results

ShadowR may serve as FRET pairs.

by 2-photon FLIM-FRET (Fig. 4a–f). We used 2-photon excitation for imaging because of its low phototoxic-

structs by lipofection, and measured the fluorescence lifetime of mRuby2 or mScarlet in the living HeLa cells

binding of ShadowR to cytosolic proteins than that of Ultramarine (Fig. 2c,d).

proteins were pulled down by centrifugation. Subsequent silver staining indicated lower levels of non-specific

at 585 nm, and molar extinction coefficient of 97,100 M

Table 1. Characteristics of ShadowR. EC: extinction coefficient, QE: quantum efficiency, Abs: absorption

maximum, Ex: excitation maximum, Em: emission maximum, ND: not determined. *†Values obtained from

previously published data16,19, respectively. Extinction coefficients were measured by the alkaline denaturation

method (See Materials and Methods). Since EC and QE measurements could be operation sensitive, we carried

out the side-by-side measurement of Ultramarine as a control. The differences in EC values of Ultramarine may

due to differences in experimental conditions or operational differences.

saturation mutagenesis. The PCR products with saturated mutations were ligated into a bacterial expression vec-
tor and a genetic library was constructed. To screen the library for hydrophilic chromoproteins, we first identified
vivid purple colonies under ambient light, confirming that the mutants have high absorption. We also confirmed
that the colonies identified are not fluorescent under blue light illumination. Subsequently, we sequenced the
identified colonies and picked mutants with more hydrophobic amino acids than the original sequence. When
single-amino acid mutagenesis failed to produce purple colonies, the surrounding amino acids were simultane-
ously subjected to saturation mutagenesis or replaced to the corresponding Ultramarine or mCherry amino acids.
We sequentially repeated this process for the 32 positions (Fig. S1), and finally identified a mutant that shows high
absorption and non-fluorescence comparable to Ultramarine (Table 1), but with greater hydrophilicity calculated
by using the reported hydropathy index of amino acids31 (Fig. 1). As a result of these processes, 19 and 3 amino
acids among 32 positions in the Ultramarine/mCherry chimera were replaced to more hydrophilic and hydro-
phobic ones, respectively (Figs 1c, S1). The three amino acids were replaced to hydrophobic ones, because the
replacement of these amino acids to hydrophobic ones resulted in the loss of absorption. Eight amino acids were
unchangeable because of the loss of absorption, and 2 amino acids were replaced to the different amino acids, but
with the same hydropathy index31. The value of surface hydrophobicity of ShadowR is greatly reduced compared
to that of Ultramarine or the chimera (Fig. 1b). Furthermore, electrostatic surface potential maps revealed that
the electrostatic charge is increased at the surface of ShadowR compared with that Ultramarine (Fig. S2). We
named this hydrophilic mutant ShadowR, where R stands for “red”, since the absorption peak (585 nm) is similar
to that of red fluorescent proteins.

Using size-exclusion chromatography, we first confirmed that ShadowR is monomeric similar to Ultramarine
(Fig. 2a). To detect the non-specifically bound cytosolic proteins of HEK293 cells to Ultramarine/ShadowR, we
developed a new method called the non-specific binding assay (NSB assay). Ni³’-nitrilotriacetate beads saturated
with His-tagged Ultramarine or ShadowR were incubated with cell lysate (Fig. 2b), and non-specifically bound
proteins were pulled down by centrifugation. Subsequent silver staining indicated lower levels of non-specific
binding of ShadowR to cytosolic proteins than that of Ultramarine (Fig. 2c,d).

Spectral analysis of purified ShadowR confirmed that, similar to Ultramarine, ShadowR has an excitation peak
at 585 nm, and molar extinction coefficient of 97,100 M⁻¹·cm⁻¹ (Fig. 3a and Table 1). Quantum efficiency was not
measurable because of the lack of fluorescence. Since the absorption spectrum of ShadowR significantly overl-
laps with the emission spectrum of mRuby2 (Fig. 3b) and mScarlet (Fig. 3c), mRuby2/ShadowR and mScarlet/ShadowR
may serve as FRET pairs.

To quantify the performance of mRuby2/ShadowR and mScarlet/ShadowR pairs as FRET pairs, we mea-
ured FRET efficiency and maturation efficiency using fusion proteins in HeLa cells and compared the results
with those of ShadowR and Ultramarine proteins, as described previously (Fig. 4a)18. We expressed tandem con-
structs by lipofection, and measured the fluorescence lifetime of mRuby2 or mScarlet in the living HeLa cells
by 2-photon FLIM-FRET (Fig. 4a–f). We used 2-photon excitation for imaging because of its low phototoxic-
ity compared with single-photon excitation 32. Because of the low levels of expression of Ultramarine fusions
expression as fusion protein. The fluorescence level from individual cells was quantified by epifluorescence
microscopy (Fig. 5). The cells expressing mEGFP/ShadowR showed higher fluorescence intensities compared
with those expressing mEGFP/Ultramarine (Fig. 5a,b). Similar results were obtained with mRuby2 and mScarlet
fusion proteins (Fig. 5a,c,d). Since there is spectral overlap between mScarlet/mRuby2 emission and ShadowR
absorption (Fig. 3b,c), the increased brightness of mScarlet/mRuby2 could be due to the lower levels of complete

| Protein                          | EC (M⁻¹·cm⁻¹) | QE   | Abs (nm) | Ex (nm) | Em (nm) |
|----------------------------------|---------------|------|----------|--------|--------|
| Ultramarine                      | 64,000†       | 0.001†| 586      | —      | 626†   |
| Ultramarine (this study)         | 97,700        | ND   | 586      | —      | —      |
| mCherryI202Y                      | 32,000†       | 0.02†| 590      | 592†   | 620†   |
| ShadowR (this study)             | 97,100        | ND   | 585      | —      | —      |

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previously published data16,19, respectively. Extinction coefficients were measured by the alkaline denaturation
method (See Materials and Methods). Since EC and QE measurements could be operation sensitive, we carried
out the side-by-side measurement of Ultramarine as a control. The differences in EC values of Ultramarine may
due to differences in experimental conditions or operational differences.
that Zdk1 binds to mScarlet-LOV2 (Fig. 7c). Next, HeLa cells expressing the LOVTRAP FRET construct were illuminated with blue light at 35 mW/cm² for 2 s. Immediately after illumination, the fluorescence lifetime of mScarlet bound to Ultramarine/ShadowR-Zdk1 in cells, but not Ultramarine/ShadowR alone, suggesting that ShadowR is a superior chromoprotein in the LOVTRAP system. As a control experiment, we only expressed mScarlet-LOV2 and found that there was no binding fraction change after light illumination, suggesting that the change in binding fraction was due to the dissociation of Zdk1 (Fig. 7d–f).

**Figure 2.** Oligomeric property and non-specific binding of ShadowR to intracellular proteins. (a) Size-exclusion chromatography traces of dUltramarine2 (blue), Ultramarine (black), and ShadowR (red). Oligomeric status of ShadowR and Ultramarine was compared. To identify dimer position, dUltramarine220 was used. Molecular weight of dUltramarine2, Ultramarine, and ShadowR are 30913, 30913, and 31365, respectively. Slight difference of the peak positions between ShadowR and Ultramarine may be due to the difference of molecular weight and surface amino acids. (b) Schematic drawing of non-specific binding assay. To pulldown the non-specifically bound proteins with Ultramarine or ShadowR, saturated Ultramarine or ShadowR beads were mixed with cell lysate and washed three times (See Materials and Methods). (c) Non-specifically bound proteins were separated by SDS-polyacrylamide gel and identified by silver staining. Ultramarine-beads and ShadowR-beads (lane 1, 2) and their diluted sample were also loaded, respectively (lane 3, 4). The green and gray arrowheads indicate His-tagged chromoproteins and their cleaved products, respectively. (d) Intensity profiles of band patterns were analyzed. Since the signals of lane 1 and 2 were saturated, lane 3 and 4 in c were inverted, and line profiles (background subtracted) were measured and plotted. Integrated area (I. A.) of profiles are also indicated. Since the region indicated by yellow rectangle is the signal of chromoproteins, the regions were not counted for I.A.

Maturation of ShadowR compared with Ultramarine. However, this possibility is excluded since the maturation and FRET efficiency of ShadowR is comparable to that of Ultramarine (Fig. 4c–f).

Since the tandem constructs with ShadowR exhibited brighter fluorescence (Fig. 5), we next performed western blotting and real time PCR to determine if the increased fluorescence observed with ShadowR was due to increased protein or mRNA expression (Fig. 6). Western blotting revealed increased expression of mEGFP/ShadowR and mRuby2/ShadowR fusion proteins (Fig. 6a–c). Non-fused ShadowR also showed increased expression compared to Ultramarine (Fig. 6a,d). Slight band shift compared with Ultramarine fusion was observed for mEGFP/ShadowR and ShadowR. We rigorously checked their DNA sequences and confirmed that there is no unwanted insertion. Most likely, the band shift was due to the replacement of hydrophobic amino acids with charged hydrophilic amino acids or increased molecular weight (See Fig. 2a legend). Next, we quantified the mRNA levels of each construct, and found higher mRNA expression levels of ShadowR, mEGFP/ShadowR, and mRuby2/ShadowR than those of Ultramarine and its fusions (Fig. 6e). These results suggested that ShadowR exhibits enhanced mRNA and protein expression.

We further tested the performance of ShadowR using a genetically encoded optogenetic tool, the LOVTRAP system34. The LOVTRAP system consists of Zdk1 and LOV2 domains35,36, and their dissociation and association can be controlled by blue light. We fused mScarlet with LOV2 and ShadowR or Ultramarine with Zdk1, creating a LOVTRAP FRET construct (Fig. 7a). These pairs were expressed in HeLa cells and their blue light-dependent association and dissociation were imaged and quantified by 2pFLIM-FRET (Fig. 7b). We only tested mScarlet-LOV2, not mRuby2-LOV2, because mScarlet is much brighter in cells than mRuby2. In the absence of blue light, mScarlet-LOV2 bound to Ultramarine/ShadowR-Zdk1 in cells, but not Ultramarine/ShadowR alone, suggesting that Zdk1 binds to mScarlet-LOV2 (Fig. 7c). Next, HeLa cells expressing the LOVTRAP FRET construct were illuminated with blue light at 35 mW/cm² for 2 s. Immediately after illumination, the fluorescence lifetime of mScarlet in LOV2 increased by decreased FRET, and returned to basal levels in approximately 60 s, consistent with results of another study34. The binding fraction change (i.e., the fraction of mScarlet bound to Zdk1, see also Materials and Methods) of mScarlet-LOV2/ShadowR-Zdk1 was larger than that of mScarlet-LOV2/Ultramarine-Zdk1, suggesting that ShadowR is a superior chromoprotein in the LOVTRAP system. As a control experiment, we only expressed mScarlet-LOV2 and found that there was no binding fraction change after light illumination, suggesting that the change in binding fraction was due to the dissociation of Zdk1 (Fig. 7d–f).
Discussion
Here, we successfully developed a new chromoprotein, ShadowR, as a FLIM-FRET acceptor for pairing with mScarlet or mRuby2. Compared with the previously reported chromoprotein, Ultramarine, ShadowR has superior property in terms of reduced non-specific binding to cellular proteins (Fig. 2b–d). The observed reduced non-specific binding of ShadowR is most likely due to the increased hydrophilic property compared with Ultramarine. Another feature of ShadowR is its increased protein expression in HeLa cells (Figs 5, 6), that facilitates imaging with lower laser power for reduced photodamage. It is not currently known if there is the

Figure 3. Spectral properties of Ultramarine and ShadowR. (a) Normalized absorption spectra of Ultramarine and ShadowR. (b,c) The spectral overlap (yellow region) between ShadowR absorption spectrum and mRuby2 (b) and mScarlet (c) emission spectra.

Figure 4. FRET efficiency and maturity of ShadowR in tandem fluorescent proteins. (a) A schematic drawing of the constructs used to evaluate the FRET efficiency and fraction of the mRuby2 or mScarlet undergoing FRET. (b) Representative fluorescence lifetime images of the tandem proteins in HeLa cells; the images were taken at 1000-nm two-photon excitation. Because the expression level of mRuby2-Ultramarine and mScarlet-Ultramarine was low, we used different laser powers for each condition (5 mW for mRuby2-Ultramarine, 4 mW for mRuby2-ShadowR, 3 mW for mScarlet-Ultramarine, 2 mW for mScarlet-ShadowR). Scale bar, 50 μm. (c,e) Quantification of FRET efficiency of the tandem proteins. The fluorescence lifetime over the whole image was used for the analysis (See Materials and Methods). The number of images used for the analysis are indicated in the figure. Each image contains 4–12 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). (d,f) A comparison of the fraction of mRuby2 or mScarlet fluorescent protein undergoing FRET (chromophore maturation efficiency of Ultramarine or ShadowR) analyzed in individual cells; data were plotted in the descending order. The FRET fraction is directly related to the maturation efficiency of an acceptor, i.e., Ultramarine (d) or ShadowR (f). 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. (g) Comparison of E. coli expressing Ultramarine, ShadowR, or dUltramarine2. Purple colonies indicate that the E. coli cells express the respective chromoproteins. After transformation, the cells were incubated for 16 hr at 32 °C and imaged.
causal association between the increased expression and the surface hydrophilicity (Fig. 6a–d). Since the protein expression tends to correlate with mRNA expression37,38, the increased protein may be due to the increased levels of mRNA (Fig. 6e). A few possibilities should be considered regarding the mechanism underlying increased mRNA expression: 1) the expressed Ultramarine proteins inhibit the transcription machinery of mRNA (negative feedback) or 2) the transfected Ultramarine DNA adopts a structure that leads to inefficient transcription. However, the precise mechanism underlying increased mRNA levels is unknown and difficult to deduce from our experiments.

We previously reported the use of dark mCherry, mCherry I202Y, as a FRET acceptor19. The advantage of ShadowR over dark mCherry is its darkness. While the quantum efficiency of the dark mCherry is 0.02, the quantum efficiency of ShadowR is undetectable. This superior darkness may prevent artificial FRET signals due to fluorescence contamination18. The application of ShadowR to a LOVTRAP system yielded a large FRET
The data are presented as mean ± SEM (t test, *P < 0.05, **P < 0.01, ***P < 0.001, N.S. = not significant). (d) An averaged time course of binding fraction changes in response to blue light (blue; mScarlet-LOV2/Ultramarine-Zdk1, orange; mScarlet-LOV2/ShadowR-Zdk1, gray; mScarlet-LOV2 alone). The number of cells (n) analyzed are indicated. The data are presented as mean ± SEM (t test, *P < 0.05, **P < 0.01, ***P < 0.001, N.S. = not significant). (e,f) The lifetime changes in individual HeLa cells before and after light illumination (the same dataset as in panels c). 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.

changes, compared with those of the Ultramarine version of constructs (Fig. 7). The reason of the enhanced FRET change could be due to reduced non-specific interactions of ShadowR with mScarlet or LOV2 compared with Ultramarine. In the dark state, mScarlet-LOV2 binds to ShadowR-Zdk1 via LOV2 and Zdk1. However, if additional non-specific interactions such as the binding of ShadowR to mScarlet or LOV2 exists and are weaker than those of Ultramarine due to the surface amino acid difference, the light-dependent separation of ShadowR-Zdk1 from mScarlet-LOV2 compared with that of Ultramarine fusions is facilitated. While ShadowR constructs exhibit the significant FRET signals, they show quite large cell-to-cell variability (Figs 4d, f, 7e, f). One of the future directions for improving ShadowR is to minimize this variability for more accurate measurement.

Taken together, we believe that ShadowR will be an additional useful tool for these studies, especially for FLIM-FRET.

Materials and Methods
Saturation mutagenesis. The synthesized gene encoding Ultramarine was purchased from FASMAC (Kanagawa, Japan). This gene in a customized pRSET vector (Invitrogen) was used as a template for constructing genetic libraries for ShadowR development. Sequential saturation mutagenesis to the targeted positions was performed by PCR amplification with degenerate primers in combination with overlapping PCR. Subsequently, the amplicons were subcloned into the customized pRSET vector. For making a library, the plasmid library was introduced into electro- or chemically competent cells, and the cells were grown for 18–20 h at 34 °C on LB agar plates supplemented with antibiotics.

Plasmid construction for mammalian expression. For all DNA construction described below, a modified pEGFP-C1 plasmid (Clontech), where a kanamycin resistance gene was replaced with an ampicillin resistance gene, was used as a backbone vector. The synthesized LOV2, Zdk1, and mScarlet genes were purchased from FASMAC (Kanagawa, Japan). The mRuby2 gene construct was a gift from Michael Lin (Addgene plasmid #40255). For Ultramarine and ShadowR, the respective genes were inserted into the vector by replacing EGFP. Extra sequences encoding amino acid sequences MVSKGGEEDN and SDEMYK were fused to the N and C termini of Ultramarine, respectively, so it would match the molecular weight of ShadowR for reasonable comparison during experiments (Fig. 1a). To construct tandem protein plasmids, the Ultramarine (DNA sequence encoding amino acid residues 1–214) or ShadowR (1–214) gene was ligated with FLAG-tagged mEGFP (1–232, A206K-mutated monomeric EGFP), mRuby2 (1–229), or mScarlet plasmid (1–224) with a linker encoding the peptide VDGTAAGPGSG. These tandem plasmids were used for experiments shown in Figs 4–6.
To construct LOVTRAP system-based FRET constructs, we fused FLAG-tagged mScarlet2 (DNA sequence corresponding to amino acid residues 1–232) with the N terminus of the LOV2 domain (DNA sequence corresponding to amino acid residues 404–546 in phototropin) with a linker encoding the peptide SGLRS and used this as a donor for FRET. As an acceptor, FLAG-tagged Ultramarine (1–214) or ShadowR (1–214) genes were fused to the N terminus of Zdk1 with no linker.

**Spectral properties of the chromoproteins.** His-tagged chromoproteins were overexpressed in *Escherichia coli* DH5α cells and purified on an Ni⁺-nitrotriacetate column (HiTrap, GE Healthcare). Mature protein concentrations were calculated from the extinction coefficient of the chromophore after denaturation in 0.1 N NaOH (44,000 M⁻¹·cm⁻¹ at 452 nm)⁴⁸. Absorption spectra of the proteins diluted in PBS were recorded on a spectrophotometer (UV-1800; Shimadzu). The extinction coefficients of fluorescent proteins were determined by dividing the peak optical density by the molar concentration of matured proteins.

**Cell culture and transfection.** HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37 °C and 5% CO₂. The cells were transfected with the plasmids using Lipofectamine 3000 (Invitrogen), followed by incubation for 16–20 hr in the absence of serum. Two-photon PLIM-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 (23–35 °C).

**Non-specific binding assay.** A saturable amount of purified Ultramarine or ShadowR was bound to Ni⁺-nitrotriacetate beads (HiTrap, GE Healthcare), respectively, and the beads were washed three times with phosphate buffered saline (PBS) to wash out free proteins. To prepare HEK293 lysate, cells cultured in a 15 cm dish were trypsinized and suspended in DMEM supplemented with 5% FBS. Subsequently, cells were precipitated by centrifugation and suspended in 10 ml of PBS. The suspended cells were disrupted by sonicating on ice for 10 s at 30 W using an ultrasonic disruptor (UD-211, TOMY) and centrifuged. Then, supernatant was filtered with a 0.22 μm filter membrane. For the non-specific binding assay, 20 μl of ShadowR-bead slurry was mixed and incubated with 500 μl of HEK293 lysate for 20 min at room temperature. The beads were washed three times with PBS. A brief sonication was performed during each wash. Samples were dissolved in SDS sample buffer. Then, silver staining was performed using a silver stain reagent kit (Cosmo Bio).

**Size-exclusion chromatography.** Size-exclusion chromatography was carried out using a Superdex 200 Increase 10/300 GL column (GE Healthcare) on an AKTA pure 25 chromatography system (GE Healthcare). Proteins purified Ni-NTA column (500 μl with concentration of 40 μM) were subjected to gel filtration chromatography at the flow rate of 0.5 ml/min with PBS. To detect chromoproteins, 280 nm was used.

**RT-PCR.** Total RNAs from HeLa cells were isolated with an RNEasy Mini Kit (Qiagen, Valencia, CA, USA), and were reverse-transcribed with the Transcripter First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). Expression levels of mRNAs encoding FLAG were assessed by quantitative PCR using FastStart Essential DNA Green Master (Roche) on Step One real-time PCR system (Life Technologies, Carlsbad, CA, USA). All primers were obtained from FASMAC (Kanagawa, Japan), their sequences were as follows:

- Forward for flag: 5’-CGGCCGCGACACTAGATCA-3’;
- Reverse for flag: 5’-ATGTGTTCAGTTGAGGGAG-3’;
- Forward for β-actin: 5’-CCGGCGGCCCTTAAACCCA-3’;
- Reverse for β-actin: 5’-ATCATCATTGCGAAGGCGC-3’;

Gene expression values were calculated by the delta-delta Ct method. Assays were carried out in five independent trials.

**Epifluorescence imaging.** Hoechst 33342 was purchased from Dojindo. HeLa cells expressing fluorescent proteins were incubated with 1 μg/ml Hoechst for 10 min. Subsequently, the cells were observed under an epifluorescence microscope. For excitation, a blue light (475 nm LED; CoolLED) for mEGFP, a green light (565 nm LED; CoolLED) for mRuby2 and mScarlet, or a purple light (365 nm LED; CoolLED) for Hoechst was used. The fluorescence images were taken with a sCMOS camera (ZYLA 4.2; Andor) mounted on a microscope (BX51WI; Olympus) through a 20x objective lens.

**Two-photon fluorescence lifetime imaging.** A custom-made two-photon fluorescence lifetime imaging microscope was used. Briefly, mRuby2 or mScarlet as the FRET construct was excited with a Ti-sapphire laser (Mai Tai; Spectra-Physics) tuned to 1000 nm. The X/Y scan mirrors (6210H; Cambridge Technology) were controlled with ScanImage software⁴⁹. The fluorescence photon signals from cells were collected with an objective lens (60x, 1.0 NA; Olympus) and a photomultiplier tube (H7422-40p; Hamamatsu) placed after a dichroic mirror (FF553-SD101; Semrock) and emission filter (FF01-625/90; Semrock). For blue light illumination in the LOVTRAP experiment (Fig. 7), blue LED (244-870-50E-40; CoolLED) with a band pass filter (FF01-469/35-25; Chroma) was used. A fluorescence lifetime curve was recorded by a time-correlated single-photon-counting board (SPC-150; Becker & Hickl) controlled with custom software⁵¹. To construct a fluorescence lifetime image, the mean fluorescence lifetime values <τ> in each pixel were calculated using Eq. (1) and translated into a color-coded image⁵¹.
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 in the following section.

**Quantification of FRET efficiency and maturity.** To compare FRET efficiency between mScarlet/mRuby2 and ShadowR/Ulframarin in HeLa cells, we fitted the fluorescence lifetime curve with a double exponential function convolved with an instrument response function, \( G(t) \), assuming that two fractions exist in the cells: (1) mature donor fluorescent protein (i.e., mScarlet or mRuby2) fused to an immature acceptor fluorescent protein (i.e., ShadowR or Ulframarin); (2) mature donor fused to a mature acceptor where FRET occurs and the fluorescence lifetime of the donor gets shorter:

\[
F(t) = P_{\text{free}} \exp \left( \frac{\sigma_G^2}{2 \tau_{\text{free}}} \left( \frac{t - t_0}{\tau_G} \right) \right) \text{erfc} \left( \frac{\sigma_G^2 - \tau_{\text{free}}(t - t_0)}{\sqrt{2} \tau_{\text{free}} \sigma_G} \right) + P_{\text{FRET}} \exp \left( \frac{\sigma_G^2}{2 \tau_{\text{FRET}}} \left( \frac{t - t_0}{\tau_G} \right) \right) \text{erfc} \left( \frac{\sigma_G^2 - \tau_{\text{FRET}}(t - t_0)}{\sqrt{2} \tau_{\text{FRET}} \sigma_G} \right)
\]

(2)

In Eq. (2), \text{erfc} is a complementary error function, \( t_0 \) is the time offset, \( \sigma_G \) is the standard deviation of the IRF, and \( P_{\text{free}} \) and \( P_{\text{FRET}} \) are the populations of the free donor (i.e., donor fused to immature acceptor) and donor with FRET (i.e., donor fused to matured acceptor), respectively. \( \tau_{\text{free}} \) and \( \tau_{\text{FRET}} \) are the fluorescence lifetime of free donor and donor with FRET, respectively. \( \tau_{\text{free}} \) can be independently measured (mScarlet (3.69 ns), mRuby2 (2.45 ns)). By fixing these values in Eq. (2), we obtained \( \tau_{\text{FRET}} \) values for mRuby2-Ulframarin (0.84 ns), mRuby2-ShadowR (0.97 ns), mScarlet-Ulframarin (1.60 ns), and mScarlet-ShadowR (1.79 ns). The mean FRET efficiency \( (Y_{\text{FRET}}) \) between the donor and the mature acceptor was calculated as follows:

\[
Y_{\text{FRET}} = 1 - \frac{\tau_{\text{FRET}}}{\tau_{\text{free}}}
\]

(3)

Using the obtained \( \tau_{\text{free}} \) and \( \tau_{\text{FRET}} \) values, we calculated the fraction of the mature acceptor or the binding fraction (donor undergoing FRET) in individual cells using the following formula as described elsewhere:

\[
P_{\text{FRET}} = \frac{\tau_{\text{free}}(\tau_{\text{free}} - \tau_m)}{(\tau_{\text{free}} - \tau_{\text{FRET}})(\tau_{\text{free}} + \tau_{\text{FRET}} - \tau_m)}
\]

(4)

**Data Availability**

The data generated and analyzed during the current study are included in this published article and its supplementary information files. Datasets are available from the corresponding author on request.

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