Electronic Supplementary Material (ESI) for RSC Advances.

Genetically encoded phenyl azide photochemistry drives positive and negative functional modulation of a red fluorescent protein

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Supporting Information.

Detailed Methods.

Site-directed mutagenesis

The mCherry gene (with a C-terminal hexahistidine tag) was cloned into the pBad/HisA vector (Invitrogen) using *Nco*I and *Xho*I restriction sites. Mutations were introduced by whole-plasmid inverse PCR using Phusion® DNA polymerase (NEB) [1] and the primer pairs in Table S2. The introduced amber stop codon (TAG) mutations were incorporated in the forward primer. Mutations were confirmed by DNA sequencing.

Protein production

Protein production was performed in *E. coli* TOP10 cells (Invitrogen) using ZYM5052 auto-induction medium. ZYM-5052 medium [2] was composed of 1 % (w/v) Tryptone, 0.5 % (w/v) Yeast Extract, 0.5 % (v/v) glycerol, 0.05 % (w/v) glucose, 0.2 % (w/v) lactose, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 1 × trace metals and 0.05 % (w/v) L-arabinose in ultra-pure water. 1 × trace metal mixture contained 4 µM $CaCl_2$, 2 µM $MnCl_2$, 2 µM $ZnSO_4$, 0.4 µM $CoCl_2$, 0.4 µM $CuCl_2$, 0.4 µM $NiCl_2$, 0.4 µM Na_2MoO_4, 0.4 µM H_3BO_3, and 10 µM $FeCl_3$ in ultra-pure water. *E. coli* cells were transformed with both pBAD-mCherry (native or TAG mutants) and pDULEazidoRS, which contains an engineered *Methanocaldococcus jannascii* tyrosyl-tRNA-synthetase and $tRNA^{CUA}$ orthogonal pair that can recognise azF [1]. For incorporation of amF, pDULEaminoRS was used that contains an engineered *Methanocaldococcus jannascii* tyrosyl-tRNA-synthetase for amF [1]. *p*-azido-L-phenylalanine (azF) and *p*-amino-L-phenylalanine (amF) (Bachem) were prepared prior to use by dissolving the appropriate weight of powdered amino acid in ultra-pure water and titrating in 1 M NaOH. AzF and amF were added to growth media to reach final concentrations of 1 and 10 mM, respectively. Single colonies were chosen and transferred to 10 mL LB containing ampicillin and tetracycline [1]. Expression cultures of ZYM5052 (+ Amp, + Tet) were inoculated with the 10 mL starter culture (1/200 dilution) and grown at 37°C for 20 hours. Protein production and solubility was confirmed using SDS-PAGE analysis.

Cells were harvested by centrifugation and lysed in sodium phosphate buffer (100 mM, 300 mM NaCl;pH 8) containing 200 µL BugBuster® (Novagen), 0.1 mg/mL lysozyme (Sigma-Aldrich), 1mM PMSF (Merck Millipore) and ~25 U Benzonase Nuclease (Sigma-Aldrich) by French Press. Protein samples were handled under

predominantly red-spectrum light to prevent premature azF photochemical breakdown.

Protein purification

Proteins were purified by Ni-affinity chromatography using HisTrap^{TM} HP column (GE Life Sciences) attached to an ÄKTApurifier (GE Life Sciences). Samples in PBS buffer (50 mM sodium phosphate, 100 mM NaCl, pH 8.0) were resolved at a flow rate of 1 mL/min. Protein elution was initiated by gradual increase in concentration of imidazole (0-250 mM) over 20 column volumes at a flow rate of 3 mL/min. Fractions containing mCherry protein were pooled and desalted in PBS buffer using Vivaspin 20 Sample Concentrator Unit 10,000 MWCO (GE Life Sciences). Proteins were further purified by size exclusion chromatography using Superdex 7510/300 GL column (GE Life Sciences) in PBS buffer at flow rate of 0.5 mL/min. Eluted fractions containing native or mutant mCherry were pooled were pooled and collected to a final volume of 1 mL. Elution was monitored by absorption at 280 nm (general protein absorbance) and 587 nm (mCherry specific absorbance). Protein purity was analysed by SDS-PAGE.

Fluorescence and Absorbance Spectroscopy

Fluorescence. Fluorescence excitation and emission spectra measured in a 5 × 5 mm QS quartz cuvette (Hellma). Excitation and emission spectra were recorded at 20°C and scan rate of 600 nm/min with a slit width of 10 nm (5 nm for wild-type mCherry) using Cary Eclipse fluorescence spectrophotometer (Varian). UV irradiation was performed in the QS cuvette using a 6 W UVM-57 mid-range UV handheld lamp (UVP) with a peak emission at 310 nm with the lamp at a distance of 1 cm from the sample. Live cell and cell lysate samples containing mCherry azF variants were diluted with PBS (100 mM sodium phosphate, 300 mM NaCl, pH 8) in a 1:1 volume ratio. Wild-type mCherry samples were diluted with PBS in a 1:6 ratio. Purified proteins were measured at a concentration of 1 µM in PBS. Cell lysates containing PAmCHerry^{197Y}, PEmCherry^{67amF} or PEmCherry^{67Y} were diluted at 1:2 ratio in PBS and placed in a 5 × 5 mm QS quartz cuvette. Quantum yields for each mCherry variant were calculated using wild-type mCherry as a reference (known φ = 0.22). Pure protein sample were diluted to a final absorbance of 0.05 at their respective λ_{max} and fluorescence emission spectra were recorded. Integrated emission intensity was calculated and used in the following formula to generate quantum yield values

$$\varphi x = \varphi st \,.\, (Area x/Area st).(\eta 2x/\eta 2st)$$

where the φ*x* and φst refer to the fluorescence quantum yield of the sample and fluorescein standard, respectively. Area*x* and Area*st* are the integrated emission intensities for the sample and fluorescein standard, respectively. ηx and ηst is the refractive index of the solvent for the sample and fluorescein standard, respectively. The refractive index correction here was negligible as 0.1 M NaOH and aqueous buffers differ in refractive index by <1% [1].

Absorbance. Purified proteins samples were diluted in PBS buffer to a final concentration of 20µM and placed in a 10 × 10 mm QS quartz cuvette. UV-vis absorbance spectra were recorded at 20°C using HP 8452A Diode Array Spectrophotometer (Hewlett Packard). Protein irradiation was performed as described above. Brightness was calculated by multiplying ε by quantum yield.

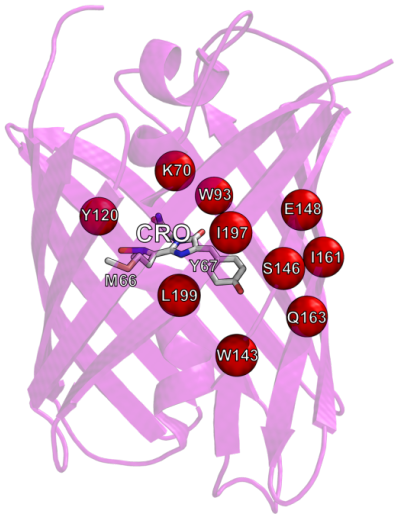
Widefield fluorescence microscopy

Widefield fluorescence microscopy was performed essentially as described previously[1]. *E. coli* TOP 10 cells producing PDmCherry^{W143azF} or PAmCherry^{I197azF} were immobilised within 2% low melting point agarose and mounted onto microscope slides. Samples were imaged using an Olympus IX73 inverted microscope utilising the ET-Sedat quad filter set (#89000 from Chroma), coupled to an ORCA Flash 4.0 camera (Hamamatsu). Photoactivation and photodeactivation experiments were performed using a Procan220 (Prior) 200W metal halide light source transmitted through a ET555/25x excitation filter for 500 ms, followed by irradiation through an AT350/50x excitation filter for 30 ms. Each imaging cycle (of visualisation and irradiation) took 2 s. Photocontrol experiments were performed using the same cycle of visualisation and irradiation. The cells were visualised using 100x oil immersion objective at 4x binning and imaged utilising the HCImage Live (Hamamatsu) software. Data was analysed with ImageJ software [3].

X-ray Crystallography

PDmCherry^{143azF} was produced and purified in the dark and diluted in 50 mM Tris-HCl (pH 8) to 10 mg/mL. Crystal screens were set up using the sitting drop vapour diffusion method with incubation at 4 °C. The screening conditions were based on 50 mM Tris-HCl with varying pH (7.9 – 8.9) and PEG4000 concentration (25 – 32%). Drops were set up with 0.2 µL of protein and reservoir solutions and dispensed with an Art Robins Phoenix robot (AlphaBiotech, UK). Crystals were transferred to mother liquor supplemented with 13% (w/v) ethylene glycol as a cryoprotectant and vitrified. Data were collected at beamline I02 (dark) and I03 (irradiated) of the Diamond Light Source (Harwell, UK). Data were reduced with the XIA2 package [4], space group assignment by POINTLESS [5], scaling and merging were completed with AIMLESS [6] and TRUNCATE [7]. Structures were solved by molecular replacement with the structure of mCherry (PDB 2H5Q) using PHASER [8]. Structures were manually adjusted to the electron density using COOT [9] and refined by TLS restrained refinement using RefMac [10]. All non-protein atoms were refined isotropically. The above routines were used as the CCP4 package (www.ccp4.ac.uk). Graphical representations were made with PyMOL Molecular Graphics System, Schrödinger, LLC.

Crystals of the dark state of PDmCherry^{143azF} formed at 27-28% PEG4000, pH 7.9-8.3, and the best crystal diffracted to 1.7 Å. The irradiated state of PDmCherry^{143azF} was obtained by irradiating the crystal tray containing remnant dark state PDmCherry^{143azF} crystals for 1 hour. The best data for the irradiated state of PDmCherry^{143azF} diffracted to 2.0 Å. Both the dark and irradiated forms of PDmCherry^{143azF} crystallized in the orthorhombic space group P2(1)2(1)2(1) and contained three molecules in the asymmetric unit. Final refinement statistics and model geometry are summarized in Table S3.



Residue	Rationale
M66	Within CRO. Residue was mutated to M from original Q in mRFP1 to produce mCherry. Residue mutated to W to produce mOrange and mStrawberry [1]
Y67	Within CRO (comprises phenol moiety). Residue mutated to W to produce mHoneydew [1] Equivalent to sfGFP^{Y66azF} [2]
K70	Close to Gly-moiety of CRO. K70N was a key substitution in mCherry to generate PAmCherry [3]
W93	Close to Gly-moiety of CRO.
Y120	Close to Met and Gly moieties of CRO
W143	Tryptophan has a similar size to AzF. Could take conformation pointing towards Tyr-moiety of CRO. Equivalent to sfGFP^{F145azF} [2]
S146	Directly interacts with CRO phenol OH group. Equivalent to sfGFP^{H148azF} [2]
E148	Side chain points toward CRO and lies approximately parallel to CRO plane. Important structural residue.
I161	In close proximity to Tyr-moiety of CRO.
Q163	Residue was mutated from M in mRFP1.4 to produce mCherry.
I197	In close proximity to Tyr-moiety of CRO; lies above plane close to K70. I197E substitution in mTangerine produced mBanana [1]
L199	Close to M and Y moieties of CRO.

Fig S1. Rational design of mCherry for incorporation of *p*-azido-L-phenylalanine (azF). The residues in mCherry chosen for replacement with azF are shown as red spheres. The right table details the rationale for selecting the residues. Reference: [1] N. C. Shaner, et al, *Nat Biotech*, 2004, 22, 1567-1572.; [2] S. C. Reddington et al , *Angew Chem Int Ed Engl*, 2013, 52, 5974-5977.; [3] F. V. Subach, et al, *Proc Natl Acad Sci USA*, 2009, 106, 21097-21102.

Table S1. Properties of azF-containing mCherry variants. NF refers to no observable fluorescence; NE no observable expression.

Variant	Soluble	Dark		Photosensitive	Result of Irradiation	Irradiated		Fold change in Flourescence after UV
		λ_{EX}	λ_{EM}			λ_{EX}	λ_{EM}	
mCherry (wt)	Yes	587	610	Y	Photobleaching	587	610	-27%
M66	NE	NF	NF					
Y67	Yes	541	580	Y	Activation	541	577	+336%(0-30min)
K70	Yes	NF	NF					
W93	Yes	585	609	Y	Reduction	587	609	-47%(0-90min)
Y120	Yes	580	602	Y	Reduction/Small shift in λ_{EX} and λ_{EM}	585	605	-
W143	Yes	585	608	Y	Reduction	580	608	-84%(0-150min)
S146	Yes	580	610	Y	Activation	580	610	+140%(0-10min)
E148	No	NF	NF					
I161	Yes	589	612	Y	Reduction	585	611	-56%(0-60min)
Q163	Yes	593	620	Y	Activation, shift in λ_{EX} and λ_{EM}	588	615	+56%(0-30min)
I197	Yes	587	610	Y	Activation	ND	ND	+830%(0-30min)
L199	Yes	NF	NF					

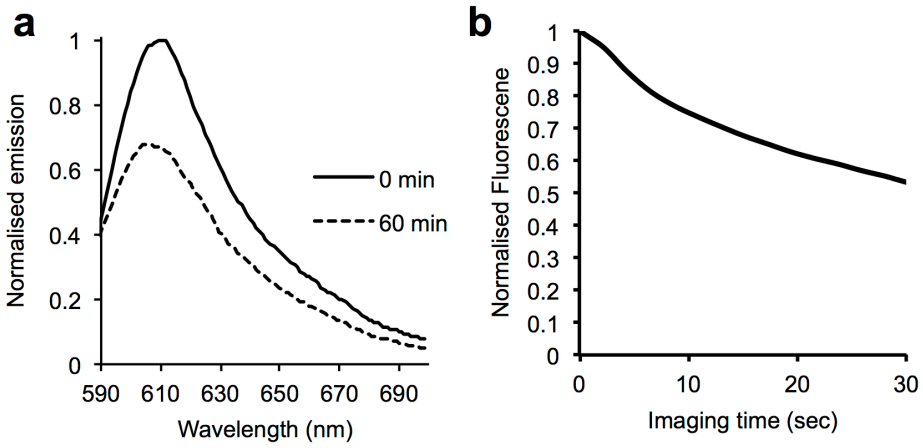


Fig S2. Response of wild type mCherry to irradiation. (a) Change in fluorescence emission (excitation at 585 nm) after 1 hour UV irradiation. (b) Change in emission under cell imaging conditions. Cells were imaged at 555 nm (AlexaA) for 500 ms, followed by irradiation at 350 nm (DAPI) for 30 ms, cycled every 2 seconds.

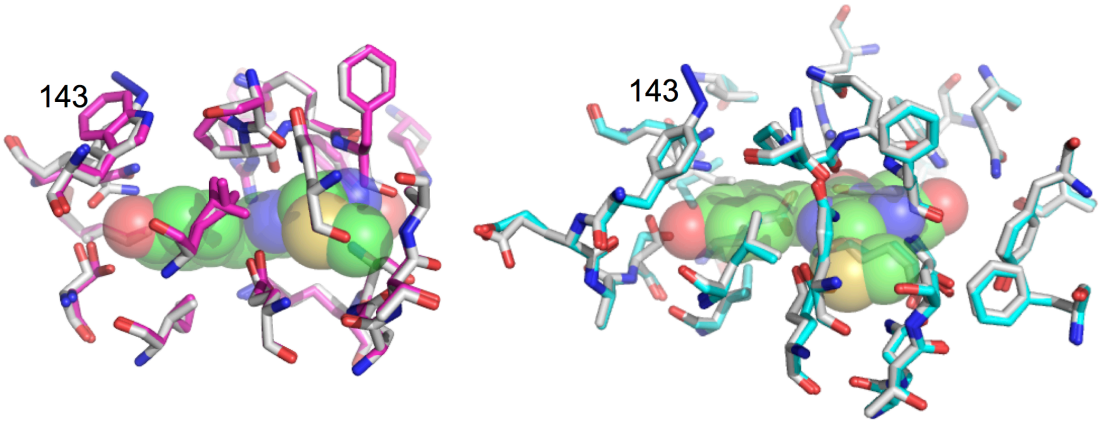


Fig S3. Effect of azF incorporation at residue 143 on chromophore environment. Substitution of tryptophan in the wild type (magenta) to azF (grey) results in no changes in the side chain orientation of residues 4 Å around the chromophore (shown as green spheres). Similarly, irradiation of azF (cyan) causes few major changes in structure but the electron density for several residues is poorly defined (see main text).

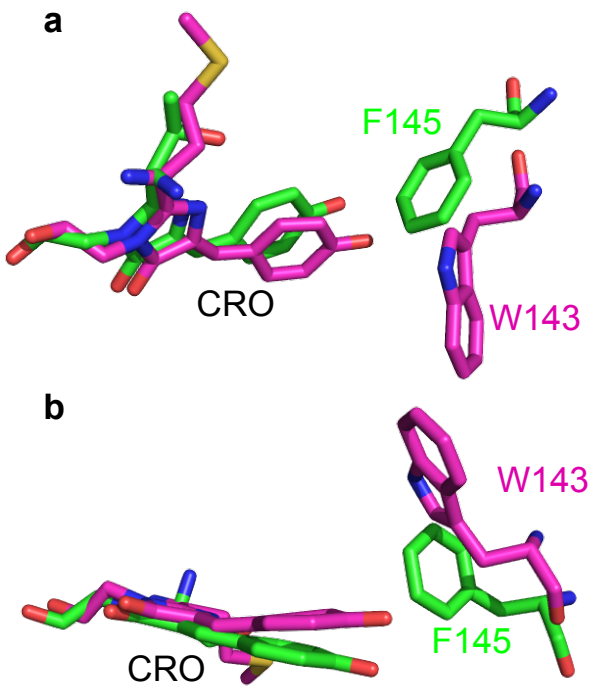


Fig S4. Structural comparison between mCherry (PDB H5Q; magenta) and superfolder GFP (PDB 2B3P; green) of the chromophore (CRO) and the residues equivalent to W143 in mCherry. A and B show 2 different views rotated by about 45° about the x axis.

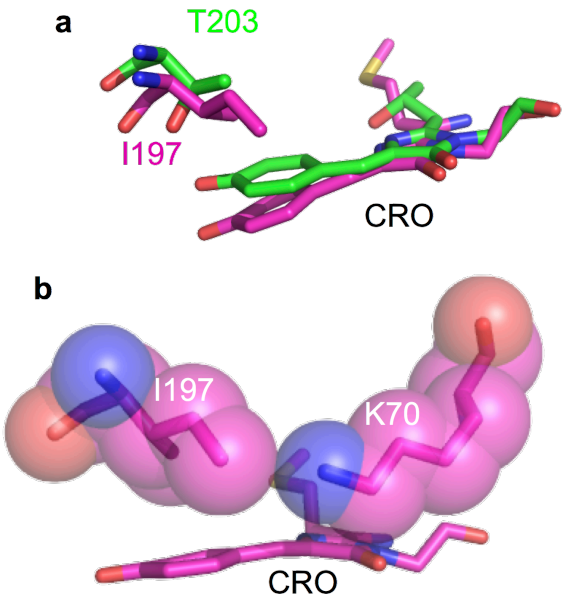


Fig S5. The position of residue 197 in mCherry. (a) Comparison of I197 in mCherry (magenta) with the equivalent residue in sfGFP (T203; green) and their structural positioning relative to the chromophore (CRO). (b) The structural relationship between I197 and K70 relative to the chromophore in mCherry.

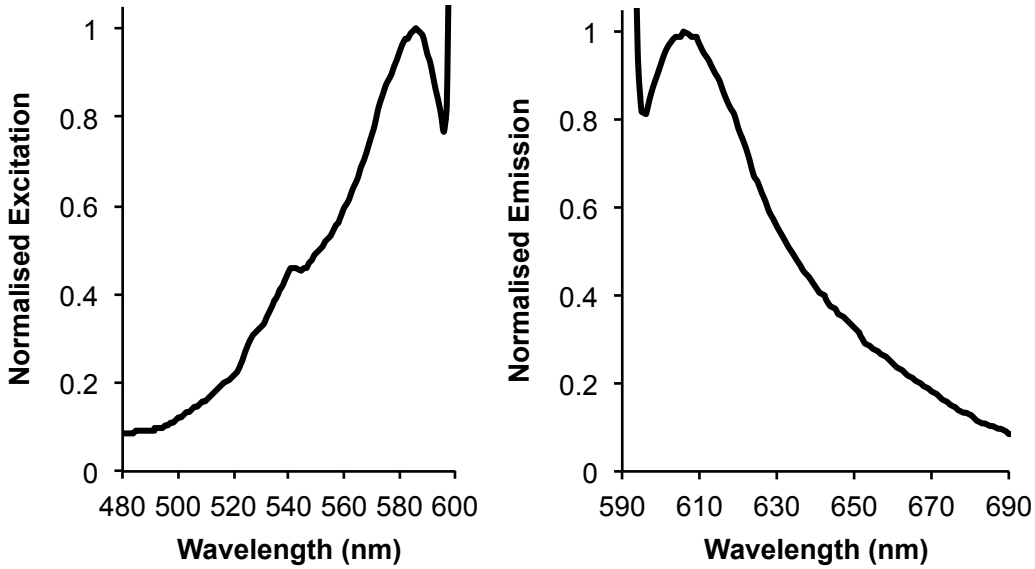
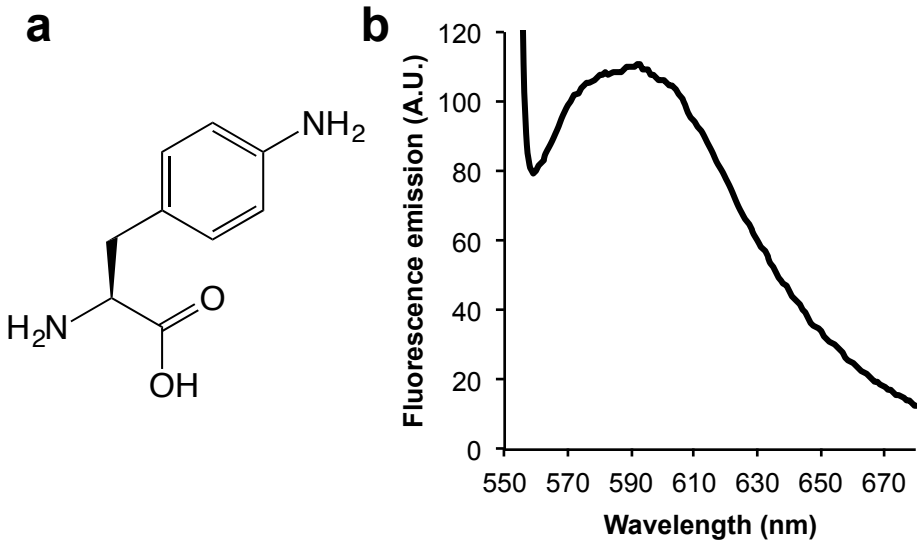


Fig S6. The excitation (left) and emission (right) spectra of mCherry I197Y mutant. Excitation spectrum was recorded by monitoring emission at 606 nm and the emission spectra recorded on excitation at 585 nm.



c

Fluorescence (nm)						Change in λ_{EM} of Fluorescence	
azF UV		amF		Y (wt)		**azF to amF**	**azF to Y**
λ_{EX}	λ_{EM}	λ_{EX}	λ_{EM}	λ_{EX}	λ_{EM}		
535	580	540	585	587	610	+5nm	+30nm

Fig S7. The effect of replacing residue Y67 with *p*-amino-L-phenylalanine (amF). (a) Chemical structure of amF. (b) Emission spectrum of the mCherry^{Y67amF} variant. (c) Comparison of the fluorescent properties of mCherry with different amino acids occupying residue 67.

Table S2. Primers used in this study. The mutations introduced are underlined.

Residue	Forward Primer (5' > 3')	Reverse Primer (5' > 3')
M66	CCCCTCAGTTCTAGTACGGCTCC	ACAGGATGTCCCAGGCGAAGG
Y67	CCTCAGTTCATGTAGGGCTCCAAGGC	GGACAGGATGTCCCAGGCG
K70	CCTCAGTTCATGTACGGCTCCTAGG	GGACAGGATGTCCCAGGCG
W93	GGCTTCAAGTAGGAGCGCGT	CTCGGGGAAGGACAGCTTCAAGTAGT
Y120	GACGGCGAGTTCATCTAGAAGGTGAA	CTGCAGGGAGGAGTCCTGGGT
W143	GACCATGGGCTAGGAGGCCT	TTCTTCTGCATTACGGGGCCG
S146	GGGAGGCCTAGTCCGAGCG	AGCCCATGGTCTTCTTCTGCATTAC
E148	GGCCTCCTCCTAGCGGATGTAC	TCCCAGCCCATGGTCTTCTTCT
I161	CCTGAAGGGCGAGTAGAAGCAGAG	GCGCCGTCCTCGGGG
Q163	CGAGATCAAGTAGAGGCTGAAGCTG	CCCTTCAGGGCGCCGTC
I197	CTACAACGTCAACTAGAAGTTGGACATCACC	GCGCCGGGCAGCTGC
L199	TCAACATCAAGTAGGACATCACCTCC	CGTTGTAGGCGCCGGG

Table S3. Crystal diffraction statistics

	PDmCherry^{143AzF} (Dark)	PDmCherry^{143AzF} (UV)
Data collection		
Wavelength	0.98	0.98
Beamline	Diamond I0-2	Diamond I0-3
Space group	$P2_12_12_1$	$P2_12_12_1$
a (Å)	43.28	43.11
b (Å)	103.43	103.17
c (Å)	150.72	149.94
Resolution range	51.72 - 1.67	84.99 - 2.00
Total reflections measured (outer shell)	567722 (41458)	336284 (23867)
Unique reflections (outer shell)	79561 (5770)	46254 (3402)
Completeness (%) (outer shell)	100 (100)	100 (100)
I/σ (outer shell)	13.6 (2.4)	9.5 (1.5)
R(merge)[1] (%) (outer shell)	7.4 (81.4)	11.5 (123.8)
B(iso) from Wilson (Å^2)	29.43	49.58
Refinement statistics		
Protein atoms excluding H	5669	5478
Solvent molecules	406	204
R-factor[2] (%)	20.8	20.3
R-free[3] (%)	24.8	26.3
Rmsd bond lengths (Å)	0.019	0.016
Rmsd angles (°)	2.12	1.911
Ramachandran plot statistics		
Core region (%)	98.4	98.27
Allowed region (%)	1.6	1.73
Additionally allowed region (%)	0	0
Disallowed region (%)	0	0

References

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