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Supplemental Information

Molecular Basis of Spectral Diversity in Near-Infrared Phytochrome-Based Fluorescent Proteins

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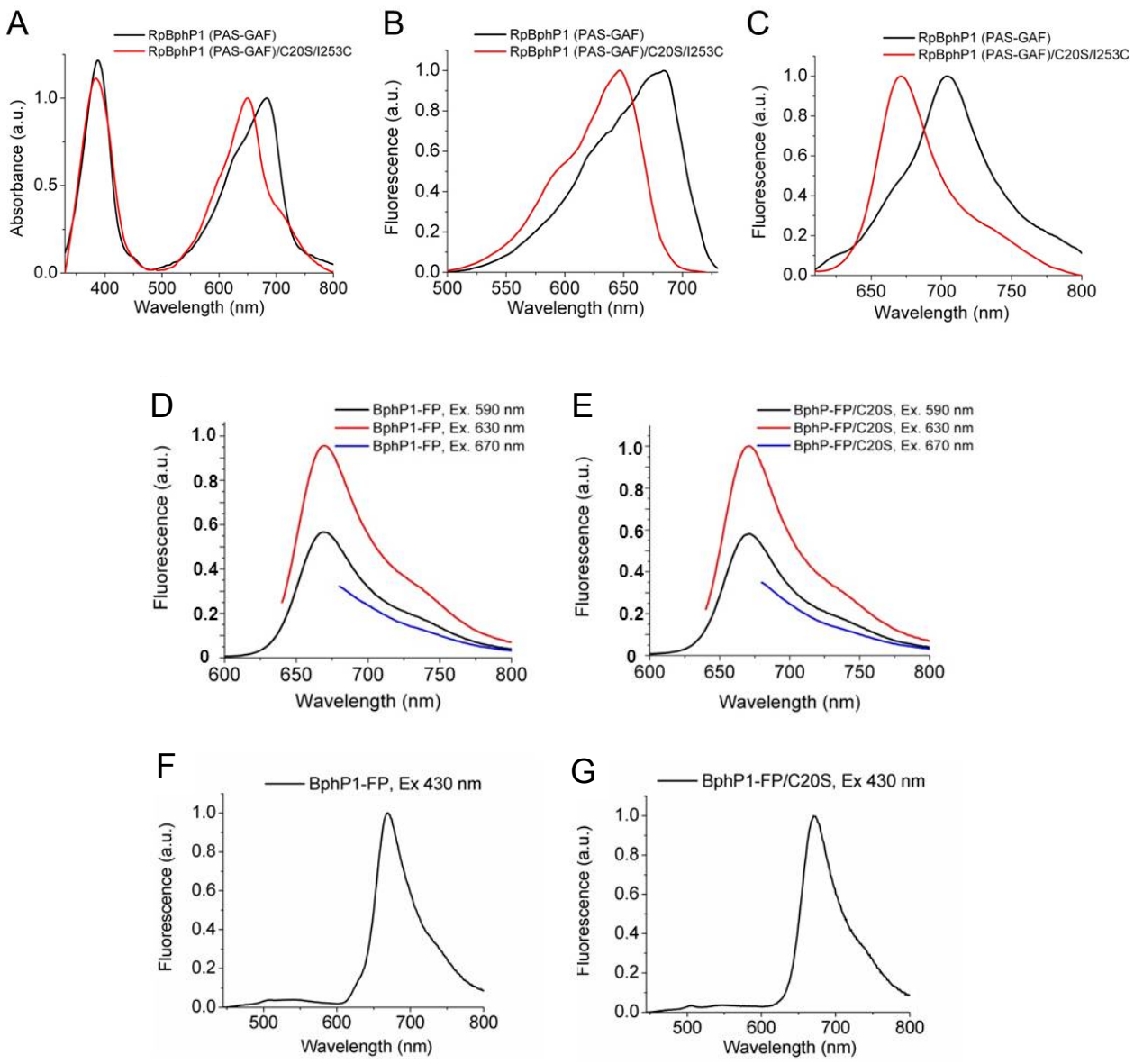

Figure S1, related to Figure 1. (A-C) Spectral properties of the PAS-GAF domains of wild-type *Rp*BphP1 and its C20S/I253C mutant. Overlays of (A) absorbance, (B) excitation and (C) emission spectra of these two proteins are shown. **(D-G)** Fluorescence emission spectra recorded at different excitation wavelengths for (D, F) BphP1-FP and (E, G) BphP1-FP/C20S. The fluorescence excitation wavelengths are indicated in the panels.

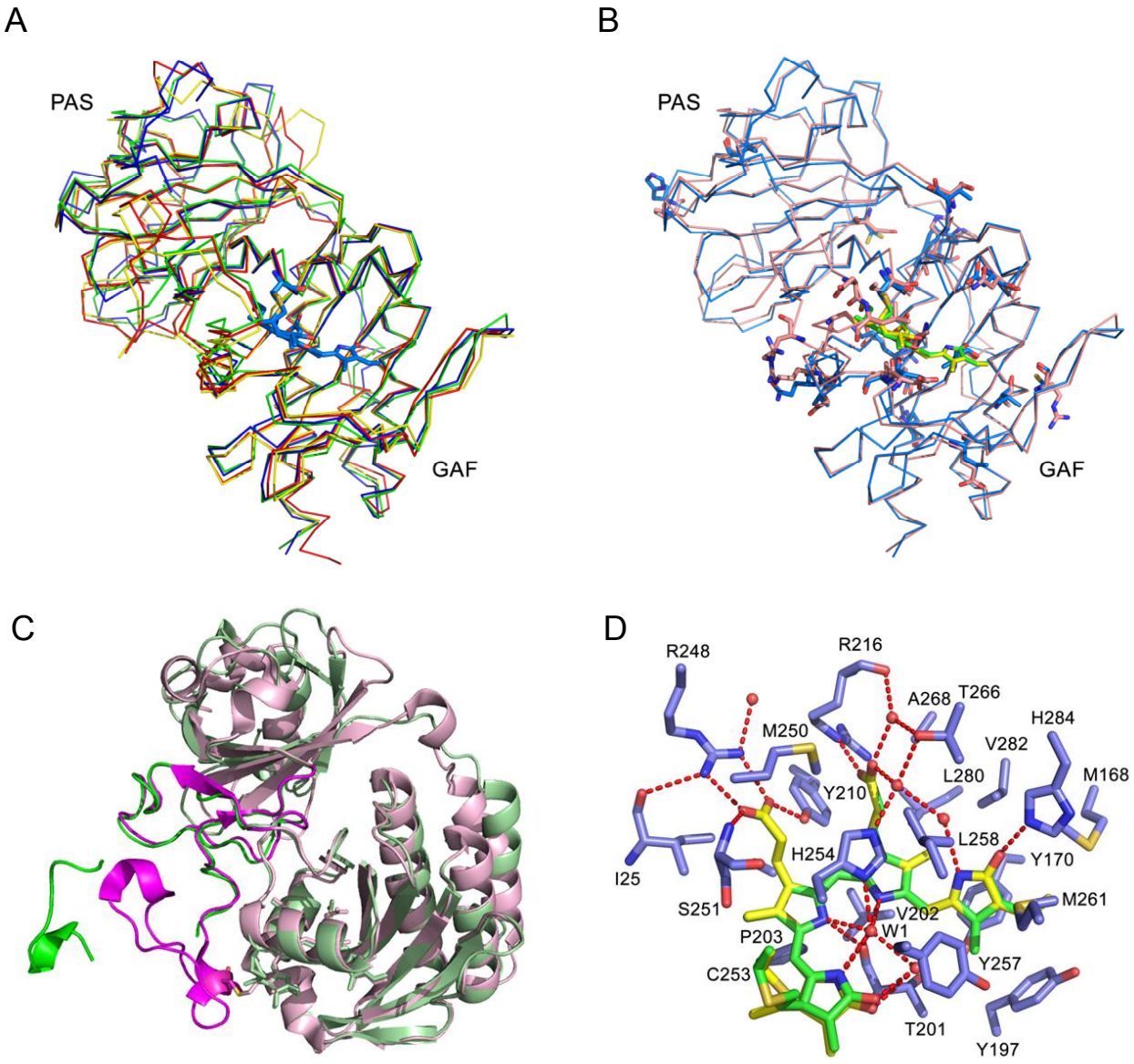

Figure S2, related to Figure 2. (A) Superposition of 3D structures of BphP1-FP/C20S (blue), PAS and GAF domains of *Rp*BphP1 (PDB: 4GW9; in green), Wi-Phy (PDB: 3S7Q; in red), and IFP2.0 (PDB: 4CQH; in yellow). **(B)** Positions of amino acid residues mutated in BphP1-FP/C20S (blue) relative to PAS and GAF domains of parental *Rp*BphP1 (PDB: 4GW9; pink). Non-identical residues are shown as sticks. Out of twenty-four amino acid residues, which differ in BphP1-FP/C20S compared to PAS-GAF of *Rp*BphP1, five residues (201, 202, 253, 286 and 282) are positioned in the vicinity of the chromophore, and the other nineteen reside on the protein surface. The chromophores of BphP1-FP/C20S connected to Cys253 via $C3^1$ and $C3^2$ atoms are shown in yellow and green, respectively. **(C)** Superimposed structures of BphP1-FP/C20S (PDB: 4XTQ; in green) and *Deinococcus radiodurans Dr*BphP PAS-GAF domains (PDB: 2O9C; in magenta. N-terminal extension and the knot lasso in both structures are highlighted with brighter color than the rest of the protein. **(D)** The immediate chromophore environment. The system of hydrogen bonds is shown as red dashed lines. Water molecules are shown as red spheres.

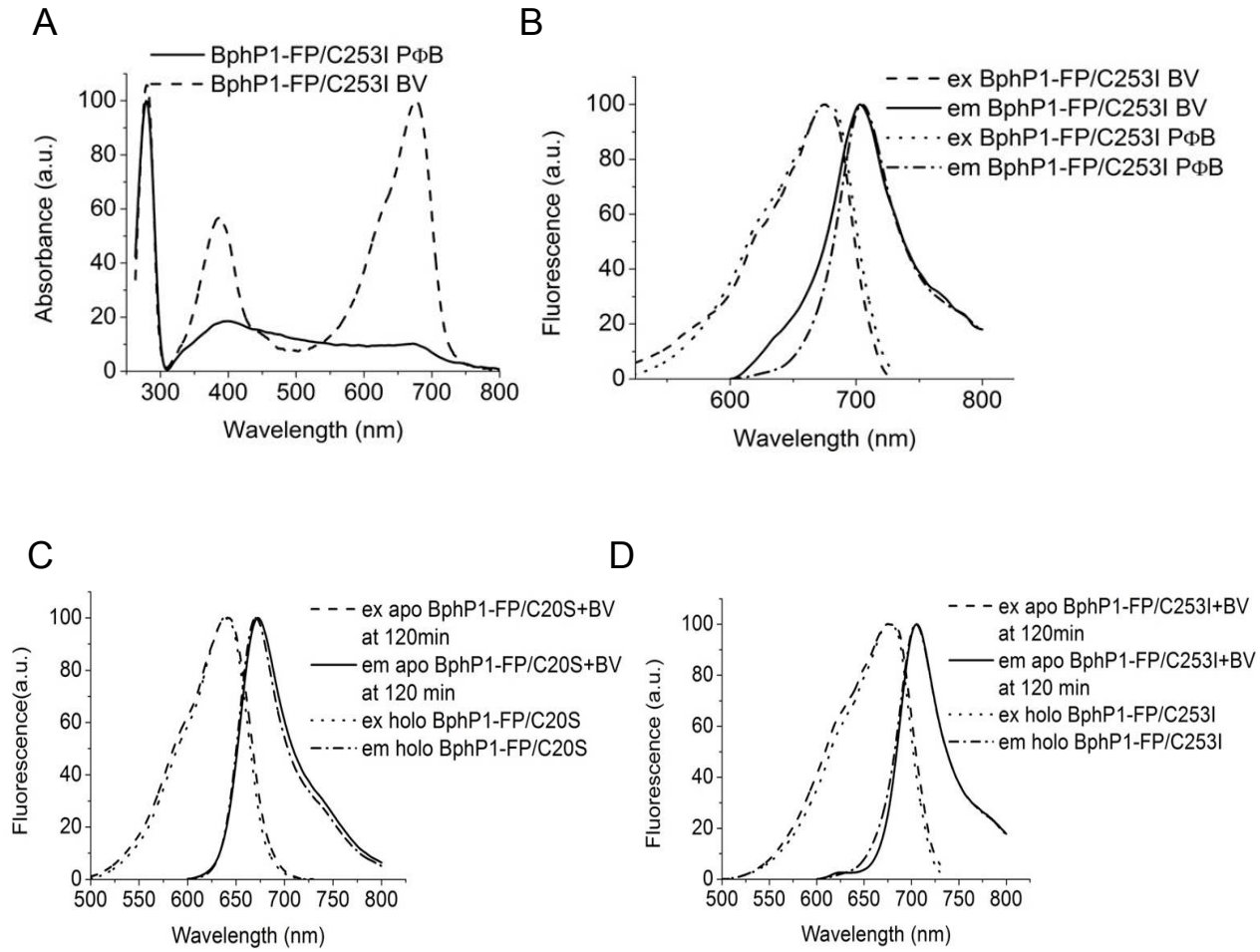

Figure S3, related to Figure 3. (A, B) Spectral properties of BphP1 variants assembled with BV and PΦB. (A) Overlay of absorbance spectra of BphP1-FP/C253I assembled with BV and PΦB. (B) Overlay of excitation and emission spectra of BphP1-FP/C253I assembled with BV and PΦB. **(C, D)** Fluorescence of BphP1 variants assembled with BV in bacteria and *in vitro.* (C) Overlay of excitation and emission spectra of BphP1-FP/C20S with BV in bacteria and *in vitro*. (D) Same as in (C) for BphP1-FP/C253I mutant.

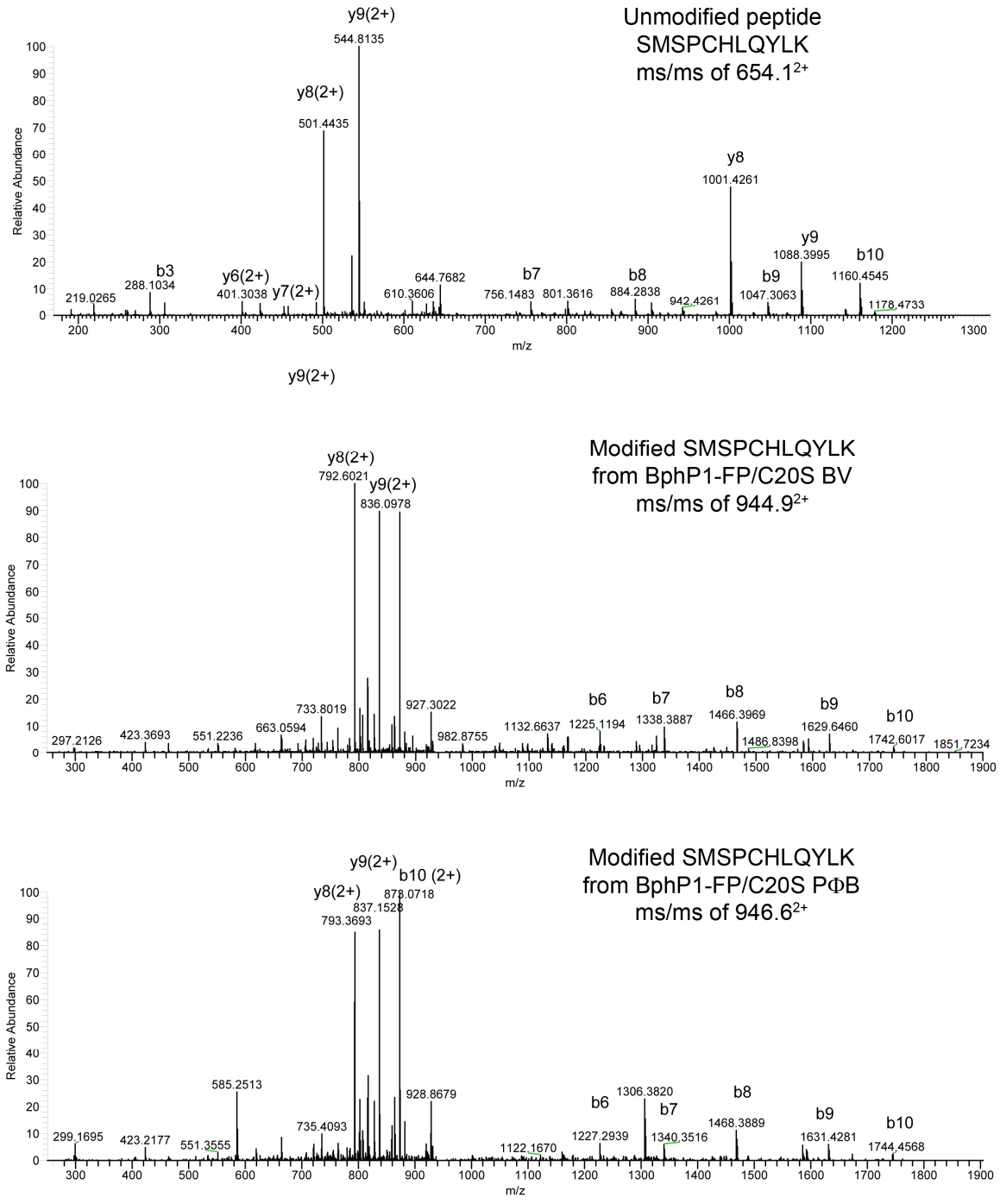

Figure S4, related to Figure 3. The MS/MS spectra of the S249 – K259 peptide isolated from the trypsinized samples of the purified BphP1-FP/C20S expressed with BV and BphP1-FP/C20S expressed with PΦB. The BV-bound and PΦB-bound peptides elute at the similar retention time, one at 48.48 min and another at 47.20 min.

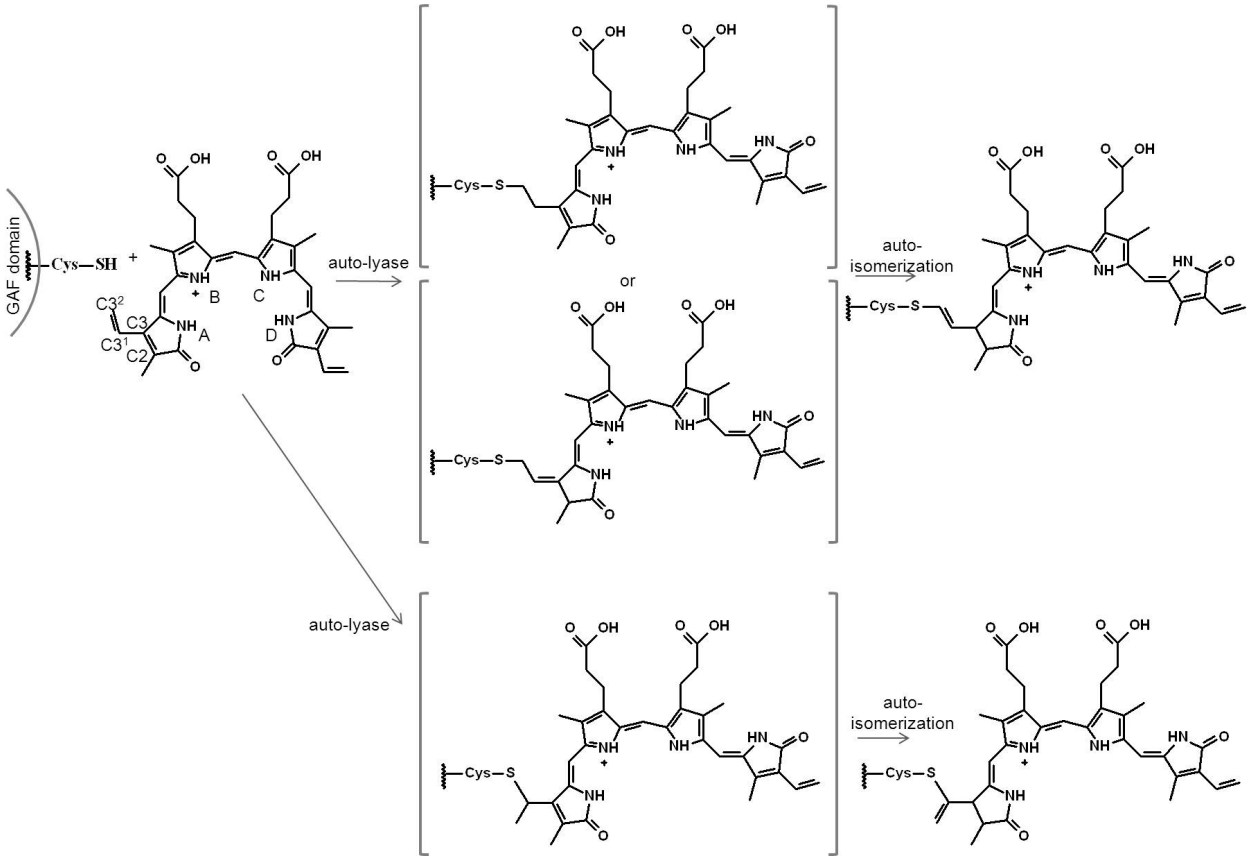

Figure S5, related to Figure 4. Proposed formation of the blue-shifted BV chromophores in BphP1-FP/C20S. The hypothetical intermediates are shown in parentheses. To release steric constraint in these intermediates caused by location of the Cys in the GAF domain above the BV chromophore plane, an autocatalytic chromophore isomerization occurs. This results in the spectral blue-shift. Pyrrole rings and carbon atoms in A ring are labeled in unbound BV.

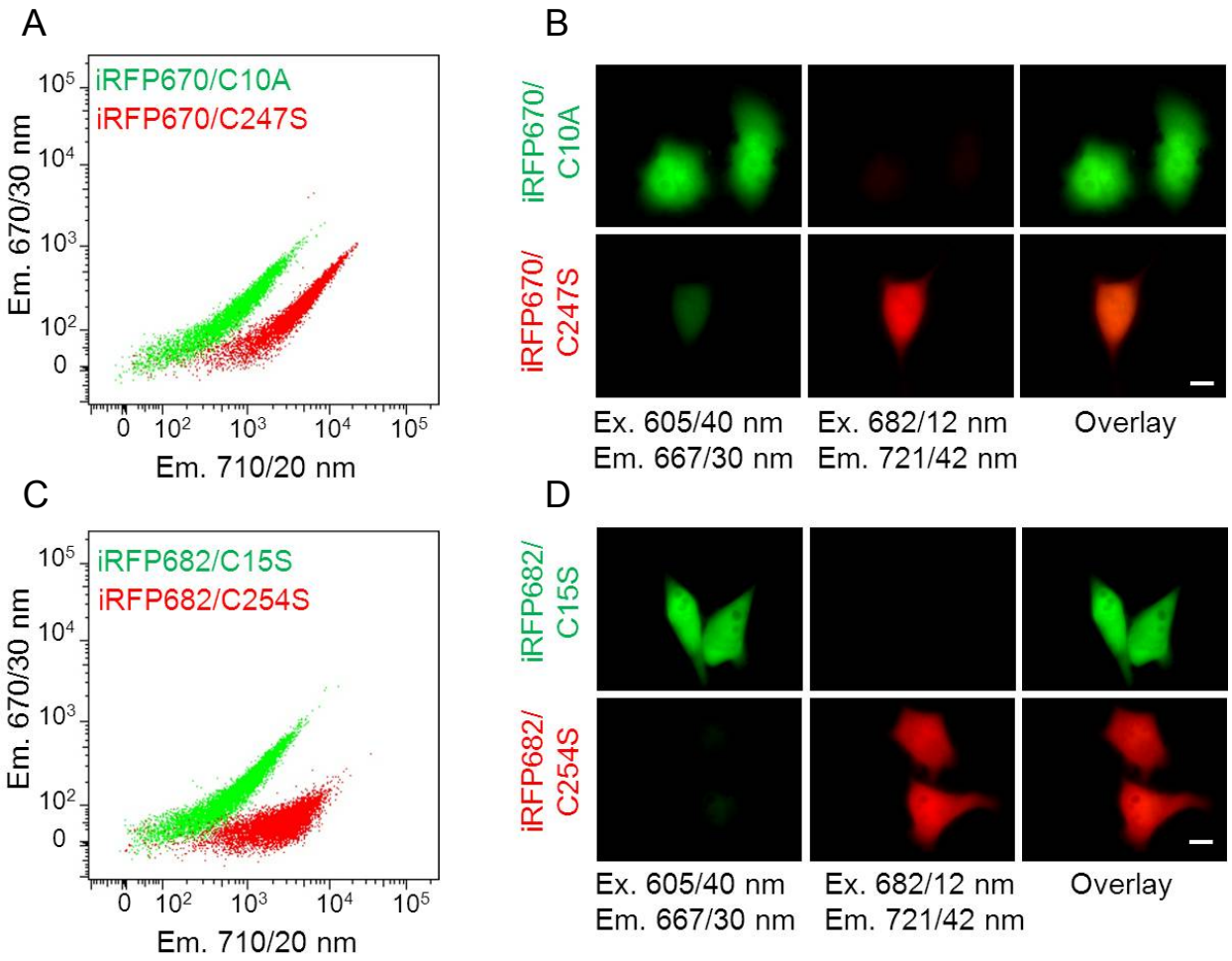

Figure S6, related to Figure 5. Two-color cell labeling using single Cys point mutants of iRFP670 and iRFP682 proteins. **(A)** Flow cytometry analysis of live HeLa cells expressing iRFP670/C10A and iRFP670/C247S. **(B)** Representative images of live HeLa cells expressing iRFP670/C10A and iRFP670/C247S. **(C)** Flow cytometry analysis of live HeLa cells expressing iRFP682/C15S and iRFP682/C254S. A 640 nm laser and a combination of two indicated emission filters were used for analysis of 20,000 events in each sample (A, C). **(D)** Representative images of live HeLa cells expressing iRFP682/C15S and iRFP682/C254S. The images in the 605/40 nm excitation and 667/30 nm emission channel are presented in a green pseudocolor, and images in the 682/12 nm excitation and 721/42 nm emission channel are presented in a red pseudocolor. Scale bar is 10 μm (B, D).

Table S1, related to Figure 1. Properties of the PAS-GAF domains of wild-type *Rp*BphP1 and its C20S/I253C mutant.

Protein	Absorbance maximum, nm	Excitation maximum, nm	Emission maximum, nm	Quantum yield, %
*Rp*BphP1 PAS-GAF	684	684	704	3.5
*Rp*BphP1 PAS-GAF C20S/I253C	650	647	671	4.1

Table S2, related to Figure 2. BphP1-FP/C20S data collection statistics.

Protein	**BphP1-FP/C20S**
Space group	$P2_12_12_1$
Unit cell parameters (Å)	a = 52.7, b = 53.1, c = 107.1
Temperature (K)	100
Wavelength (Å)	1.00
Resolution (Å)	50.0 - 1.64
Total reflections	267,748
Unique reflections	37,535
Completeness (%)	99.9 (99.9)
I/σ<I>	32.1 (2.2)
R-merge	0.058 (0.76)
Multiplicity	7.1 (6.5)

Data in parentheses are given for the outermost resolution shell, 1.70 – 1.64 Å.

Table S3, related to Figure 2. BphP1-FP/C20S refinement statistics.

Protein	**BphP1-FP/C20S**
No. of protein atoms	2,552
No. of solvent atoms	398
Resolution range (Å)	50.0 - 1.64
R-work	0.175
R-free	0.208
R.m.s.d. bond lengths (Å)	0.021
R.m.s.d. angles (º)	2.57
R.m.s.d. chirality (º)	0.16
R.m.s.d. planarity (º)	0.012
R.m.s.d. dihedral (º)	17.5
Mean B factors ($Å^2$)	
Protein atoms	
overall	17.7
main chain	16.0
side chain	19.4
chromophore	16.6
Ramachandran statistics (%)	
(for non-Gly/Pro residues)	
most favorable	94.5
additional allowed	5.1
generously allowed	0.4

Table S4, related to Figure 3. Masses of bound chromophores in BphP1-FP/C20S expressed with BV and with PΦB obtained by mass-spectrometry analysis.

Peptide fragments	**m/z in unmodified peptide (ms/ms of 654.1^{2+}), Da**	**m/z in modified peptide from BphP1-FP/C20S BV (ms/ms of 944.9^{2+}), Da**	**Chromophore mass in BphP1-FP/C20S BV, Da**	**m/z in modified peptide from BphP1-FP/C20S PΦB (ms/ms of 946.6^{2+}), Da**	**Chromophore mass in BphP1-FP/C20S PΦB, Da**
b7	756.1483	1338.3887	582.2404	1340.3516	584.2033
b8	884.2838	1466.3969	582.1131	1468.3889	584.1051
b9	1047.306	1629.646	582.3397	1631.4281	584.1218
b10	1160.455	1742.6017	582.1472	1744.4568	584.0023
$y8^{2+}$	501.4435	792.6021	582.3172	793.3693	583.8516
$y9^{2+}$	544.8135	836.0978	582.5686	837.1528	584.6786
Average chromophore mass Δm			582.2877± 0.164281		584.2033± 0.281166

Peptide fragments are extracted from MS/MS spectra corresponding to unmodified chromophore-binding peptide S249 – K259 and to the same peptides with bound chromophores (Figure S4). Chromophore masses were calculated as differences between mass-to-charge ratios (m/z) of peptide fragments with and without modification, multiplied by charge (z).

Table S5, related to Table 1. Spectral properties of iRFP670, iRFP682 and their mutants.

NIR FP	Natural BphP template	Absorbance maximum, nm	Excitation maximum, nm	Emission maximum, nm
iRFP670	*Rp*BphP6	643	642	670
iRFP670/C10A		641	639	669
iRFP670/C247S		675	673	704
iRFP682	*Rp*BphP2	663	660	682
iRFP682/C15S		659	657	683
iRFP682/C254S		694	692	714