SUPPLEMENTARY INFORMATION

A far-red fluorescent protein evolved from a cyanobacterial phycobiliprotein

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Supplementary Figure 1 | TeAPCα aligned to closest relatives and protein used for homology modeling.

	Sequence Identity Full-length	Relative TeAPCα Minus 1st 29 aa
TeAPCα		
MvAPCα	68.3%	83.3%
OnAPCα	68.3%	83.3%
CyAPCα	67.7%	82.6%
LaAPCα	67.1%	81.8%
LyAPCα	66.5%	81.1%
PyAPCα	41.0%	50.0%

TeAPCα, *Trichodesmium erythraeum* allophycocyanin α-subunit; MvAPCα, *Microcoleus vaginatus* APCα; OnAPCα, *Oscillatoria nigro-viridis* APCα; CyAPCα, *Cyanothece* sp. PCC 7425 APCα; LaAPCα, *Lynbya aestuarii* BL J APCα; LyAPCα, *Lyngbya* sp. PCC 8106 APCα; PyAPCα, *Pyropia yezoensis* APCα. TeAPCα is the only protein lacking the first 29 amino acids. Five closest relatives are shown below TeAPCα and the sequence of the structure (1KN1.pdb, PyAPCα) used for homology modeling is last. Changes in amino acids are shown in red. Yellow highlight, conserved C52 for covalent attachment to PCB.

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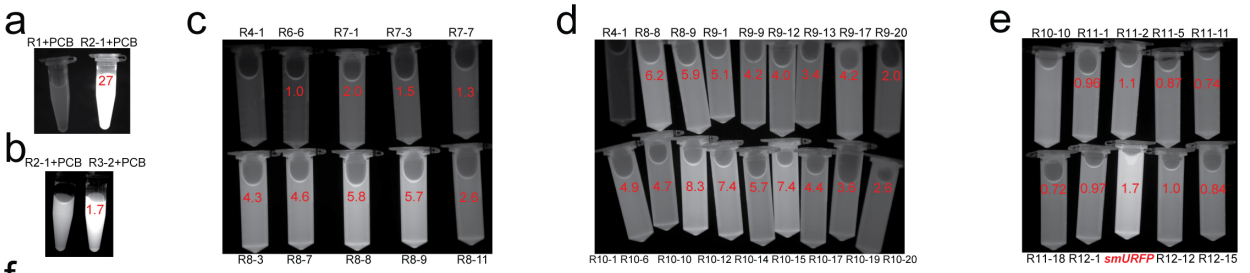
Supplementary Figure 2 | Evolving new APCα FPs.



f

	1	11	21	31	41
Consensus	M K T C E Q R V K I	A T L L S E N K K K	I V D K A S Q D L W	R R R P D L I A P G	G I A F S Q R E R A
Conservation	* * * * * * * * : *	* * * * * : * * : * *	* * * * * * * * * *	* * : * * : * * * *	* * * . * : * *
Charge variation					
TeAPCα	M K T G E Q R V K I	A T L L S E N E K K	I V D K A S Q D L W	R R R P D F I A P G	G N A F G Q R E R A
R1+PCB	M K T G E Q R V K I	A T L L S E N E K K	I V D K A S Q D L W	R R R P D F I A P G	G I A F G Q R E R A
R2-1+PCB	M K T G E Q R V K I	A T L L S E N E K K	I V D K A S Q D L W	R R R P D F I A P G	G I A F G Q R E R A
R3-2+PCB	M K T G E Q R V K I	A T L L S E N E K K	I V D K A S Q D L W	R R R P D F I A P G	G I A F G Q R E R A
R4-1	M K T G E Q R V K I	A T L L S E N E K K	I V D K A S Q D L W	R R R P D F I A P G	G I A F S Q R E R A
R5-2	M K T C E Q R V K I	A T L L S E N E K K	I V D K A S Q D L W	R R R P D F I A P G	G I A F S Q R E R A
R6-6	M K T C E Q R V K I	A T L L S E N E K K	I V D K A S Q D L W	R R R P D L I A P G	G I A F S Q R E R A
R7-7	M K T C E Q R V K I	A T L L S E N K K K	I V D K A S Q D L W	R R H P D L I A P G	G I A F S Q R E R A
R8-8	M K T C E Q R V K I	A T L L S E N K K K	I V D K A S Q D L W	R R H P D L I A P G	G I A F S Q R D R A
R8-9	M K T C E Q R V N I	A T L L S E N K K K	I V D K A S Q D L W	R R R P D L I A P G	G I A F S Q R D R A
R10-10	M K T C E Q R V N I	A T L L T E N K K K	I V D K A S Q D L W	R R H P D L I A P G	G I A F S Q R D R A
R11-2	M K T C E Q R V N I	A T L L T E N K K K	I V D K A S Q D L W	R R H P D L I A P G	G I A F S Q R D R A
smURFP	M K T S E Q R V N I	A T L L T E N K K K	I V D K A S Q D L W	R R H P D L I A P G	G I A F S Q R D R A

	51	61	71	81	91
Consensus	L C L R D Y G W Y L	H L I T F C L L A G	D K G P I E S I G L	I G I R E M Y N S L	G V P V P G M M E S
Conservation	* * * * * : * : *	: * * * : * * * *	* * . * * * * * * *	* . : * * * * * * *	* * * * * * : * *
Charge variation					
TeAPCα	L C L R D Y G W Y L	R L I T Y G L L A G	D K D P I E S I G L	I G V R E M Y N S L	G V P V P G M V E S
R1+PCB	L C L R D Y G W Y L	R L I T Y G L L A G	D K D P I E S I G L	I G V R E M Y N S L	G V P V P G M V E S
R2-1+PCB	L C L R D Y G W Y L	R L I T F C L L A G	D K D P I E S I G L	I G V R E M Y N S L	G V P V P G M V E S
R3-2+PCB	L C L R D Y G W Y L	R L I T F C L L A G	D K D P I E S I G L	I G I R E M Y N S L	G V P V P G M M E S
R4-1	L C L R D Y R W Y L	H L I T F C L L A G	D K D P I E S I G L	I G I R E M Y N S L	G V P V P G M M E S
R5-2	L C L R D H R W Y L	H L I T F C L L A G	D K D P I E S I G L	I G I R E M Y N S L	G V P V P G M M E S
R6-6	L C L R D Y G W Y L	H L I T F C L L A G	D K G P I E S I G L	I G I R E M Y N S L	G V P V P G M M E S
R7-7	L C L R D Y G W Y L	H L I T F C L L A G	D K G P I E S I G L	I G I R E M Y N S L	G V P V P G M M E S
R8-8	L C L R D Y G W Y L	H L I T F C L L A G	D K G P I E S I G L	I G I R E M Y N S L	G V P V P A M M E S
R8-9	L C L R D Y G W Y L	H L I T F C L L A G	D K G P I E S I G L	I G I R E M Y N S L	G V P V P D M M E S
R10-10	L C L R D Y G W Y L	H L I T F C L L A G	D K G P I E S I G L	I G I R E M Y N S L	G V P V P A M M E S
R11-2	L C L R D Y G W Y L	H L I T F C L L A G	D K G P I E S I G L	I S I R E M Y N S L	G V P V P A M M E S
smURFP	L C L R D Y G W F L	H L I T F C L L A G	D K G P I E S I G L	I S I R E M Y N S L	G V P V P A M M E S

	101	111	121	131
Consensus	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I K A	M S
Conservation	* * * * * * * * * *	* * : * * * * * : * *	* * * * * * * * * : *	: *
Charge variation				
TeAPCα	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I Q A	M S
R1+PCB	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I Q A	M S
R2-1+PCB	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I Q A	M S
R3-2+PCB	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I Q A	M S
R4-1	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I K A	M S
R5-2	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I K A	M S
R6-6	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I K A	M S
R7-7	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I K A	I S
R8-8	I R C L K E A S L S	L L D E E D A N E T	A P Y F D Y I I K A	M S
R8-9	I R C L K E A S L S	L L D E E D A K E T	A P Y F D Y I I K A	M S
R10-10	I R C L K E A S L S	L L D E E D A N E T	A P Y F D Y I I K A	M S
R11-2	I R C L K E A S L S	L L E E E D A N E T	A P Y F D Y I I K A	M S
smURFP	I R C L K E A S L S	L L D E E D A N E T	A P Y F D Y I I K A	M S

FPs are named after Round #-Clone # (R#-#), PCB denotes evolution with PCB, and no chromophore label denotes BV. (**a**,**b**) Evolving self-incorporation, covalent attachment of PCB produced in *Escherichia coli* (HO-1 + PcyA). EX / EM = 628 / 690 nm, respectively. (**c-e**) Evolving covalent attachment of BV produced in *Escherichia coli* (HO-1). EX / EM = 650 / 690 nm, respectively. R4-1/R5-2 were evolved with BV, EX / EM = 685 / 710 nm, respectively. Rounds 6-12 were evolved with BV, EX / EM = 650 / 690 nm, respectively. (**a-e**) Red #s are the fold increase in fluorescence relative to left/upper left FP. *Escherichia coli* was grown in LB + 0.2% arabinose at 37 ˚C for ~72 h (**a**,**b**) and ~18 h (**c-e**). (**f**) Sequence alignment of characterized APCαFPs. TeAPCα is the parent protein and nonfluorescent (**Fig. 2a**). Green highlight shows the N42I mutation, which allows autocatalytic, covalent attachment of PCB; yellow highlight shows early mutations that occurred in R3-2+PCB and are conserved in all APCαFPs+BV; red highlight shows mutations that cause red-shifted fluorescence; blue highlight shows mutations that are present only in BV binding FPs; purple highlight shows mutations present only in APCαFPs+BV with EX = 650 nm; and gray highlight shows mutations that occur only with BV.

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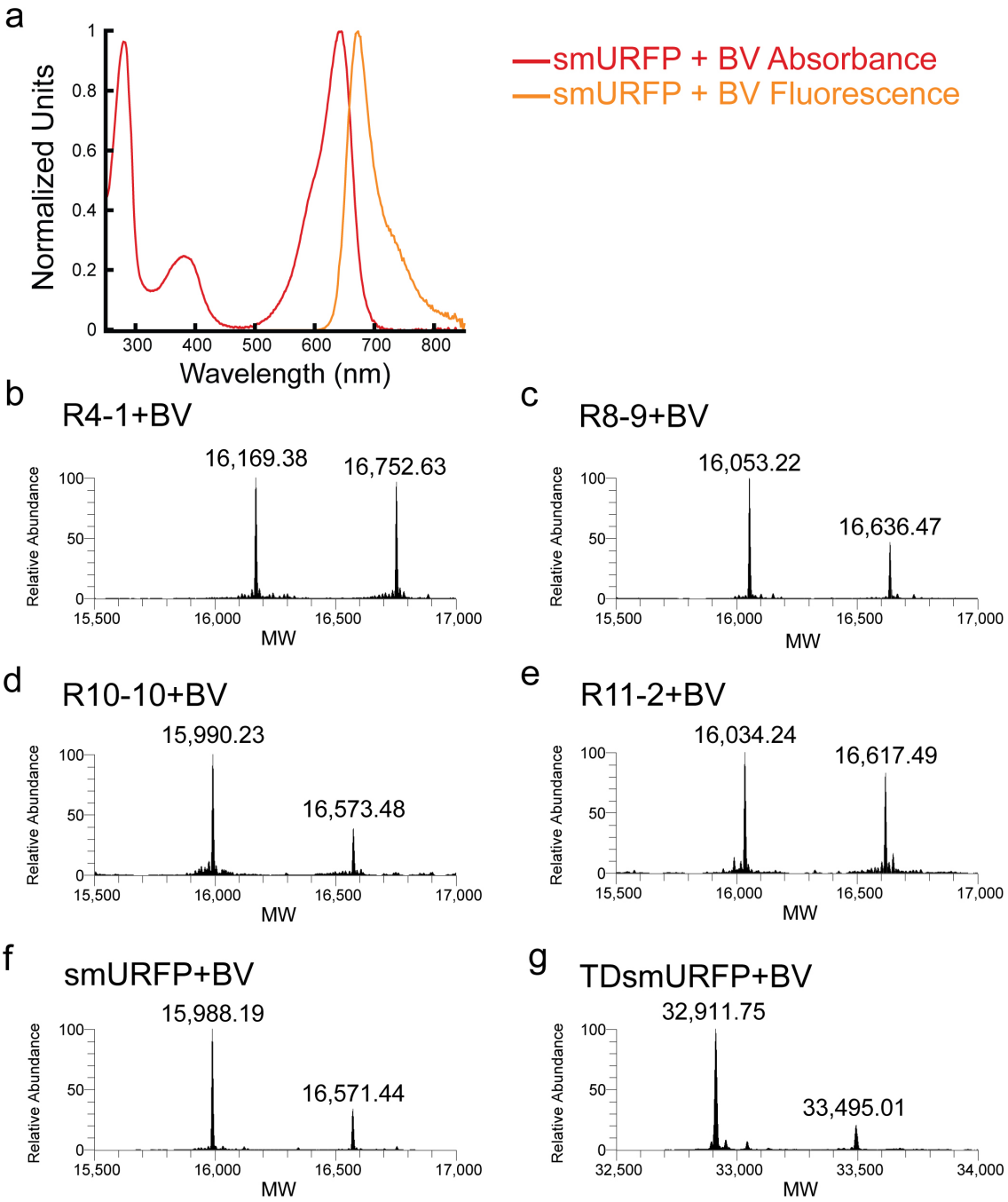
Supplementary Figure 3 | Full absorbance and fluorescence spectrum of smURFP and electrospray mass spectrometry of selected, evolved APCα FPs.



FPs + HO-1 were expressed in *Escherichia coli* for 18 h at 37 ˚C and purified as described in the **Online Methods**. (**a**) Full absorbance and fluorescence spectra of smURFP + BV purified from *Escherichia coli*. (**b**) R4-1+BV. Free protein: calculated = 16,170.66 D and found = 16,169.38 D. FP+BV: calculated = 16,753.31 D and found = 16,752.63 D. (**c**) R8-9+BV. Free protein: calculated = 16,054.55 D and found = 16,053.22 D. FP+BV: calculated = 16,637.20 D and found = 16,636.47 D. (**d**) R10-10+BV. Free protein: calculated = 15,991.45 D and found = 15,990.23 D. FP+BV: calculated = 16,574.10 D and found = 16,573.48 D. (**e**) R11-2+BV. Free protein: calculated = 16,035.51 D and found = 16,034.24 D. FP+BV: calculated = 16,618.16 D and found = 16,617.49 D. (**f**) smURFP+BV. Free protein: calculated = 15,989.42 D and found = 15,988.19 D. FP+BV: calculated = 16,572.07 D and found = 16,571.44 D. (**g**) TDsmURFP+BV. Free protein: calculated = 32,912.69 D and found = 32,911.75 D. TDFP+BV: calculated = 33,495.34 D and found = 33,495.01 D. (**b-g**) Average mass calculated by mass spectrometry program Xtract.

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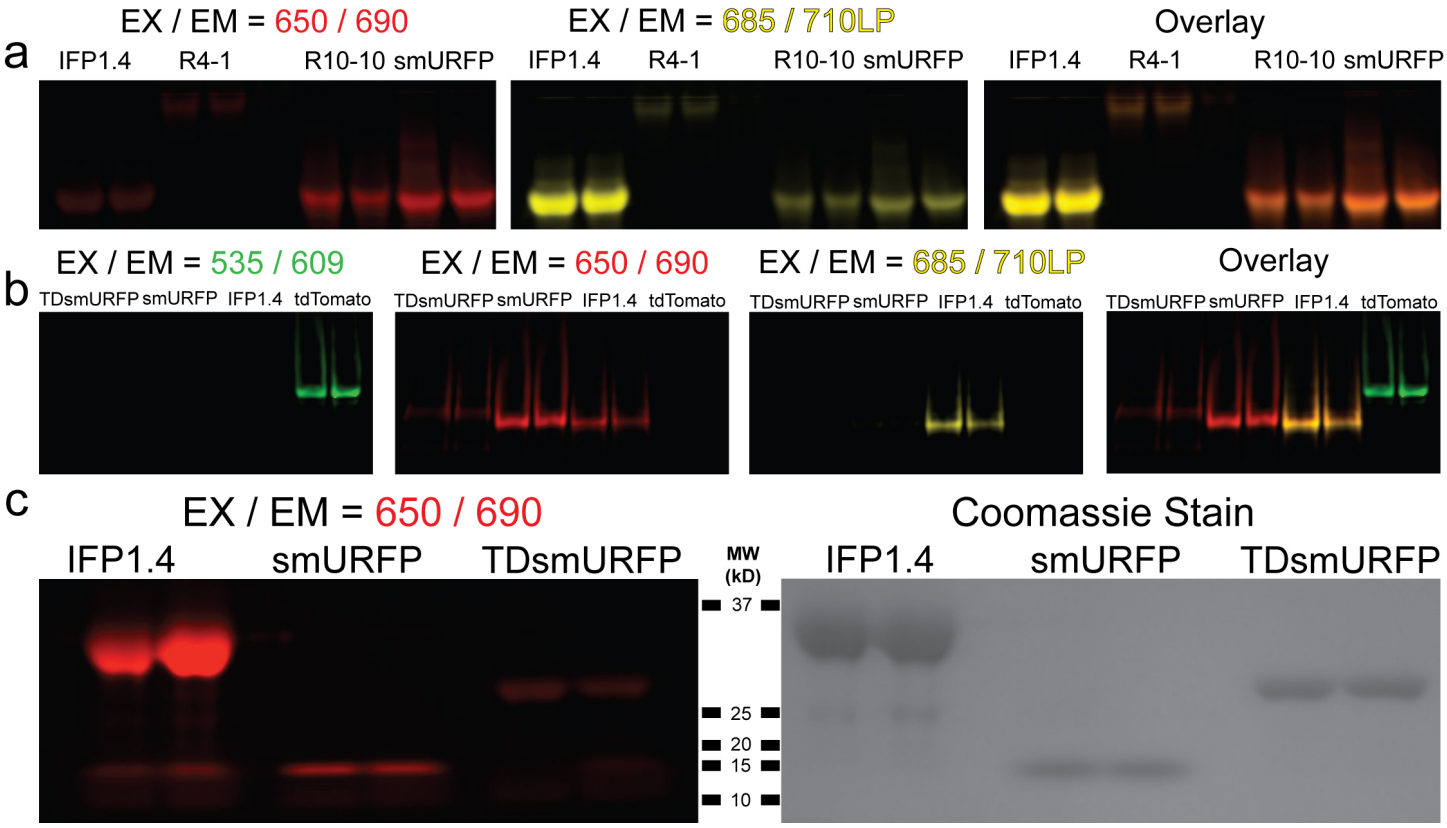
Supplementary Figure 4 | Quaternary structure characterization of smURFP and TDsmURFP.



(**a**) Native PAGE gel of APCαFPs. Monomeric IFP1.4 (36.5 kD) was ran as a reference. R4-1 runs as a tetramer, while R10-10 and smURFP run as a homodimer (32 kD). (**b**) Native PAGE gel of TDsmURFP. Monomeric IFP1.4 (36.5 kD) and tandem dimer Tomato (tdTomato, 54.2 kD) were ran as references. TDsmURFP (32.9 kD) runs slightly higher than smURFP due to 23 amino acid linker. (**c**) SDS denaturing PAGE gel of smURFP and TDsmURFP. BV was imaged by addition of zinc. All FPs show correct MW and covalently attached BV. TDsmURFP shows correct MW (32.9 kD) for intact tandem dimer. (**a-c**) Each FP was run on two lanes. EX is excitation maximum and EM is emission maximum.

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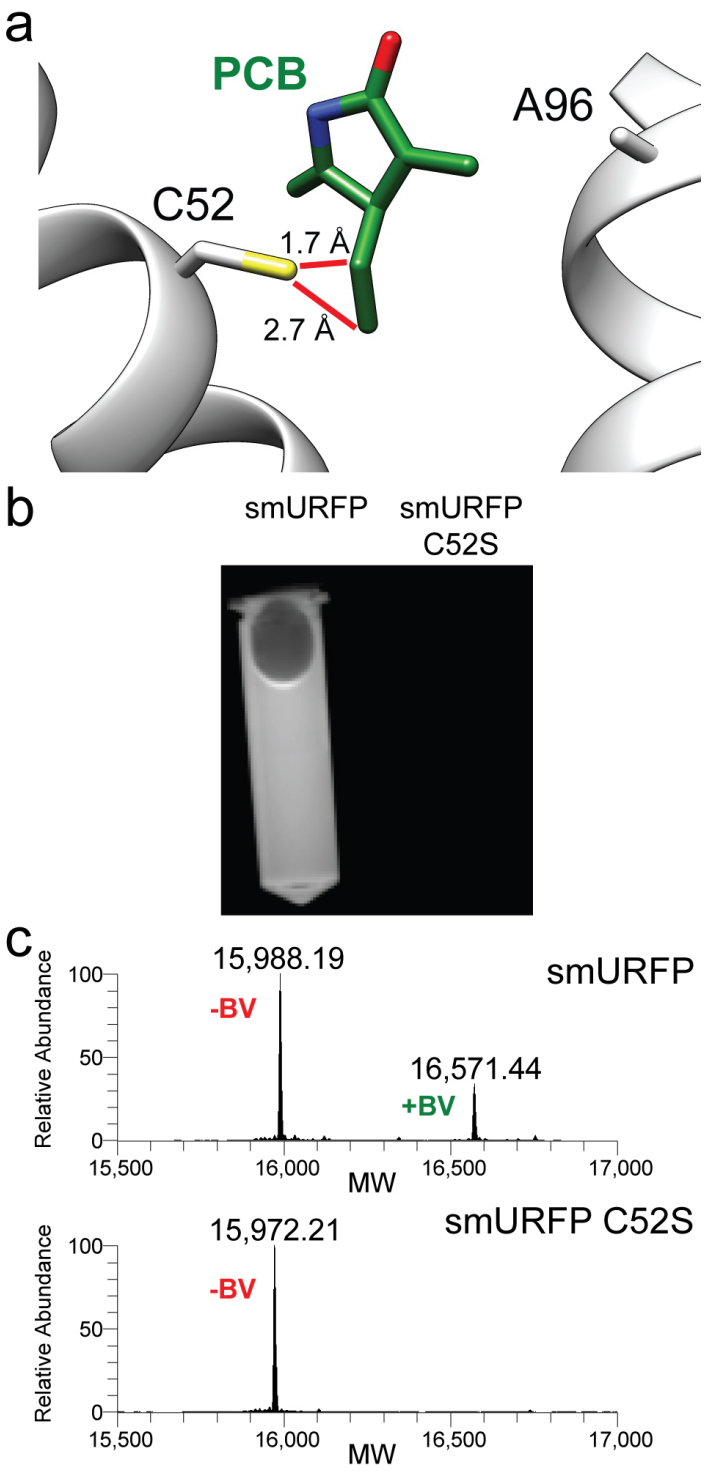
Supplementary Figure 5 | SmURFP C52 covalently attaches BV.



(**a**) smURFP homology model with C52 distances from PCB shown. (**b**) smURFP C52S eliminates fluorescence. *Escherichia coli* was grown in LB + 0.02% arabinose at 37 ˚C for 16 h. EX / EM = 650 / 690 nm. (**c**) Electrospray mass spectrometry, average mass of FPs was determined. smURFP. Free protein: calculated = 15,989.42 D and found = 15,988.19 D. FP+BV: calculated = 16,572.07 D and found = 16,571.44 D. smURFP C52S. Free protein: calculated = 15,973.36 D and found = 15,972.21 D. smURFP C52S+BV was not found in the mass spectra.

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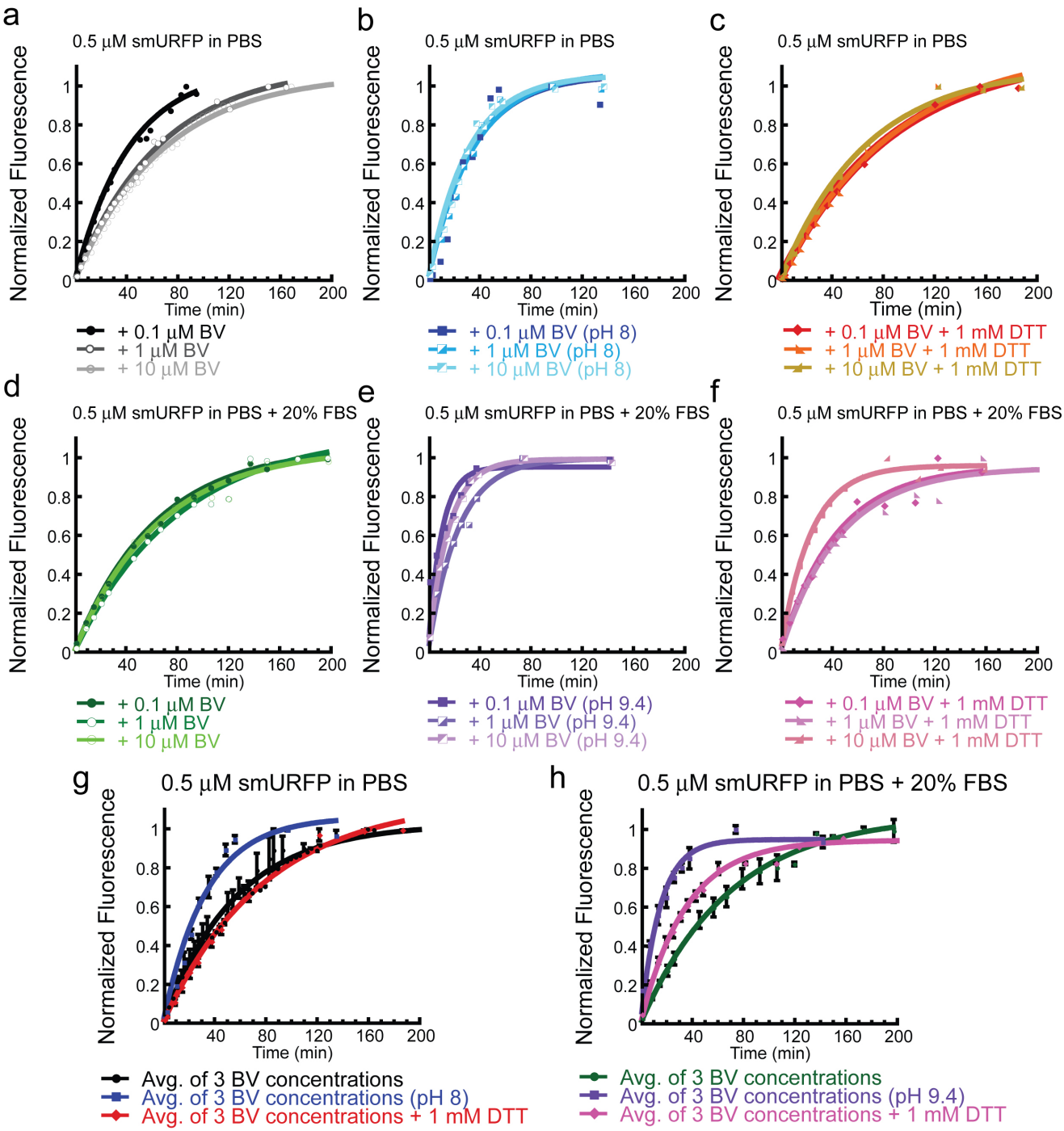
Supplementary Figure 6 | Incorporation rates *in vitro* of BV on initially chromophore-free smURFP.



Fluorescence activation of BV by smURFP involves two steps: binding and covalent attachment. 0.5 μM smURFP (1 μM chromophore sites) was incubated with 0.1, 1, and 10 μM BV in PBS alone (**a**-**c**) or PBS with 20% FBS (**d**-**f**). Increase in fluorescence in (**b**) PBS at pH 8 or (**e**) PBS with 20% FBS at pH 9.4. (**c**,**f**) Increase in fluorescence in the presence of 1 mM DTT. Fluorescence was monitored and fit to a 1st order increase in fluorescence ($F = A [1 - \exp^{-kt}]$, data in **Supplementary Table 2**). (**g**,**h**) The rate of BV incorporation was the similar for all three [BV] and the data was averaged (n = 3). Lack of rate change suggests high affinity binding for BV and the rate-limiting step is covalent attachment by C52. Solutions were made basic to increase nucleophilicity of C52. The rate of BV incorporation is increased and proves C52 covalent attachment is the rate-limiting step. SmURFP as the only protein (PBS) shows no significant rate change with 1 mM DTT, but with other proteins (20% FBS) the rate is significantly increased with 1 mM DTT. Error bars are s.e.m..

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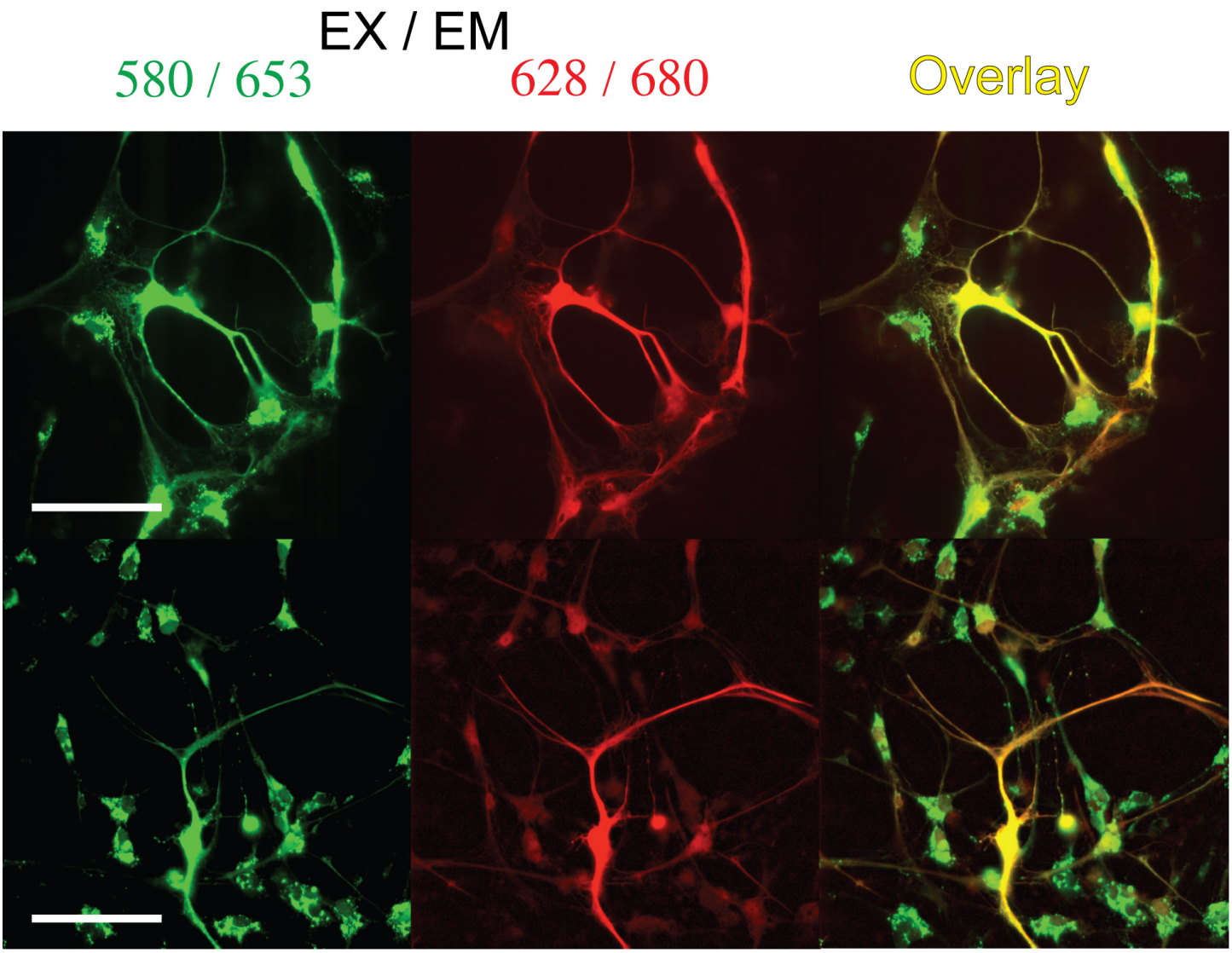
Supplementary Figure 7 | Rat primary neuronal cultures transduced with smURFP T2A mCherry lentivirus.



Representative neuronal culture images (32 images, ~160 neurons) 15 d after transduction and incubated with 25 μM BV for 10 min. Exposure time = 30 ms and 100 ms for EX / EM of 580 / 653 nm and 628 / 680 nm, respectively. Overlay shows extensive aggregation of mCherry in lysosomes relative to smURFP. EX is excitation maximum and EM is emission maximum. Scale bar = 100 μm.

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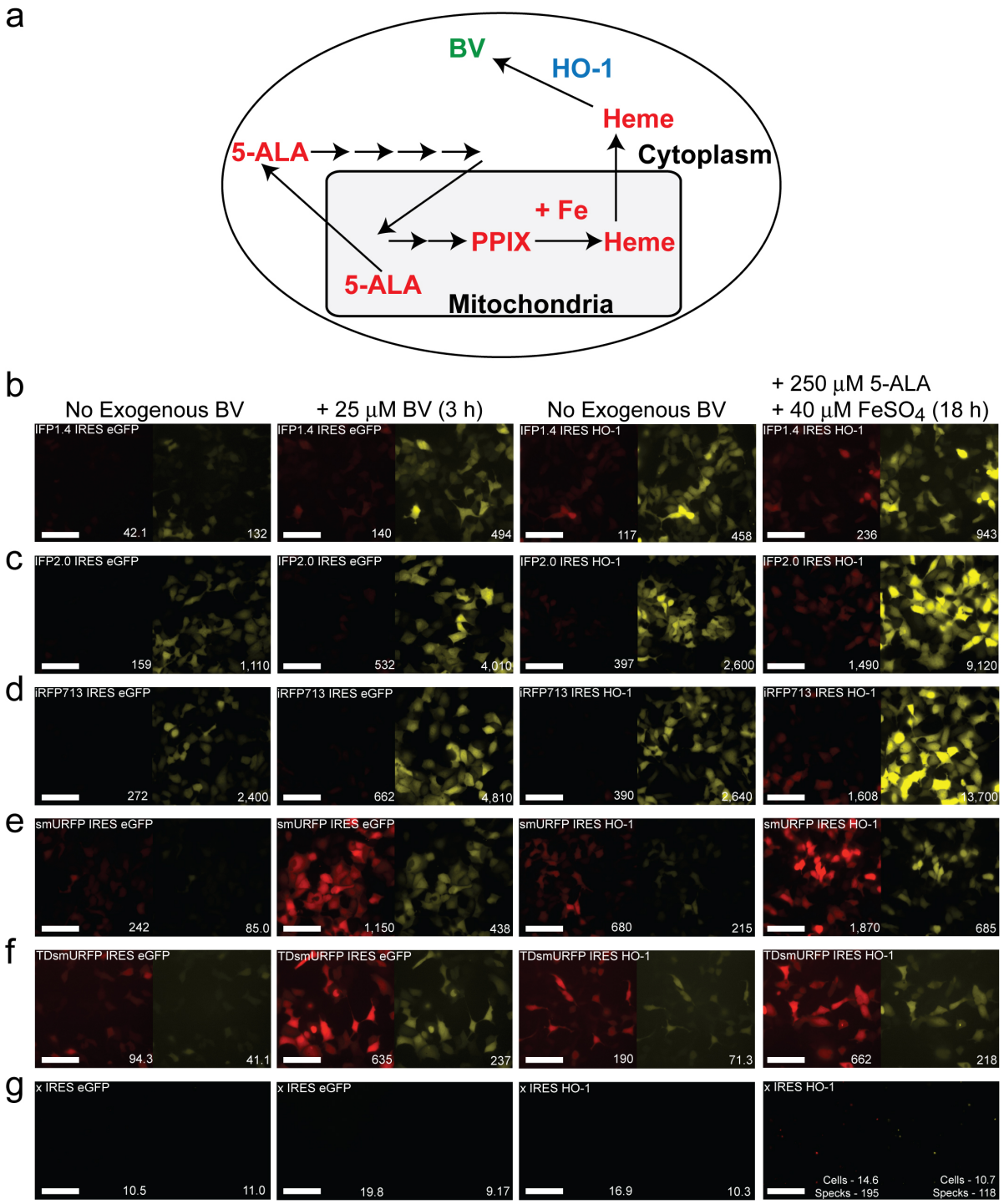
Supplementary Figure 8 | BV production by HO-1 and representative images of HO-1 experiments quantified in **Fig. 3a**,**b**.



(**a**) Schematic illustrating BV production in cells. Precursors to BV are shown in red. Arrows indicate movement from subcellular compartments and/or intermediates in the pathway. 5-ALA production is the rate-limiting step in the formation of heme. Representative images of (**b**) IFP1.4 IRES eGFP/HO-1, (**c**) IFP2.0 IRES eGFP/HO-1. (**d**) iRFP713 IRES eGFP/HO-1, (**e**) smURFP IRES eGFP/HO-1, (**f**) TDsmURFP IRES eGFP/HO-1, (**g**) Control for HO-1 expression, non-fluorescent iRFP713 fragment (x) IRES eGFP/HO-1. (**b-g**) For each sample, 5 images were collected and experimental conditions are listed on top of each column. White numbers are mean fluorescence intensity (n = 30). Red (left) is EX / EM = 628 / 680 nm and yellow (right) is EX / EM = 665 / 725 nm. Exposure time = 200 ms. Scale bar = 100 μm.

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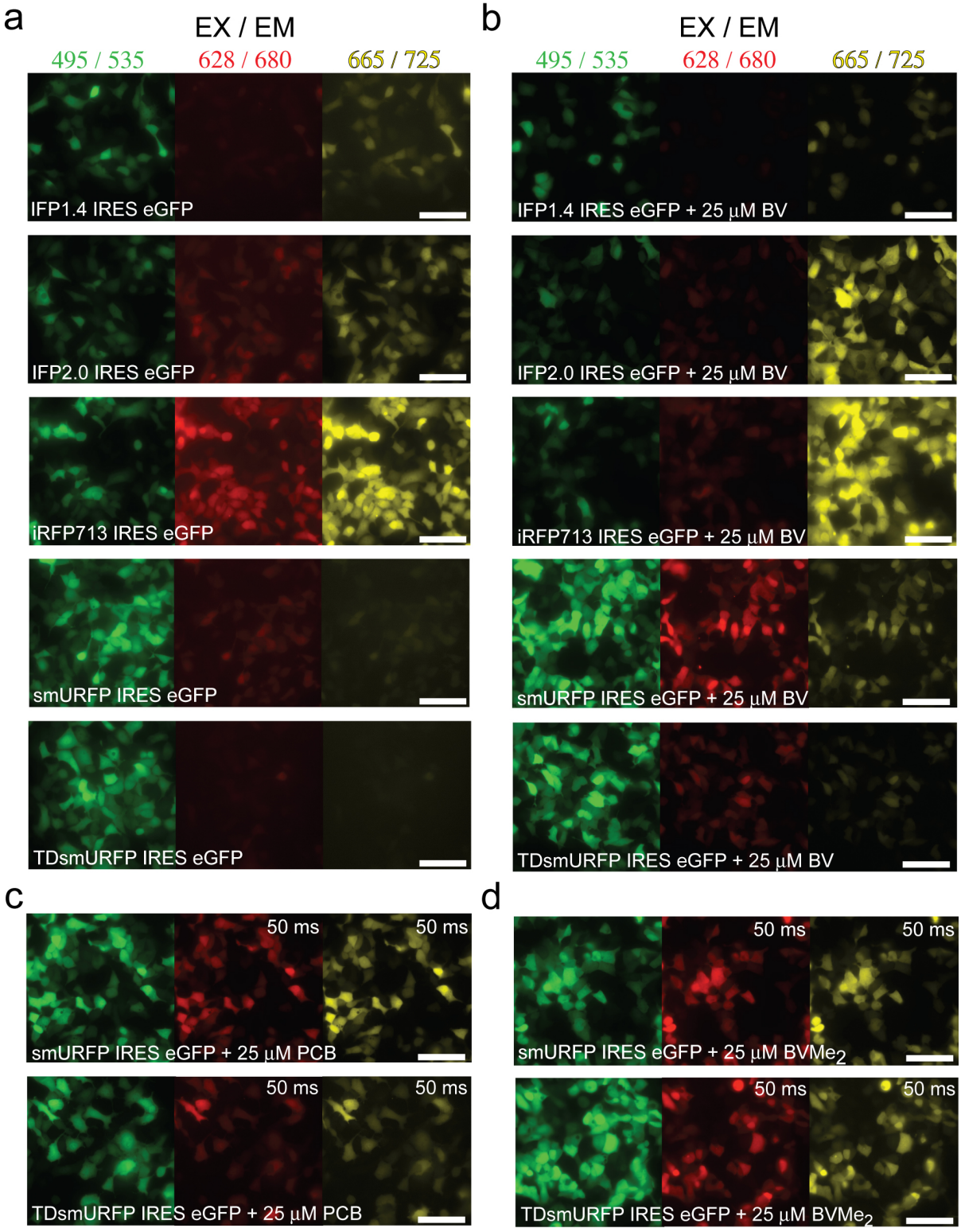
Supplementary Figure 9 | Representative images of FPs with and without exogenous chromophore addition.



FPs were expressed without HO-1. Representative images used for quantitation in **Fig. 3e**. Five total images were taken for each sample. (**a**) Representative images of FPs without exogenous chromophore. EX / EM = 628 / 680 (red) and 665 / 725 (yellow) nm images are brightened 4X relative to **b**-**d** to show dim fluorescent cells. FPs + 25 μM BV (**b**), 25 μM PCB (**c**), or 25 μM $BVMe_2$ (**d**). Incubation time of chromophore is 3 h. (**a**,**b**) Exposure = 250 ms and (**c**,**d**) Exposure = 50 ms. (**b**-**d**) Images are adjusted the same. Scale bar = 100 μm.

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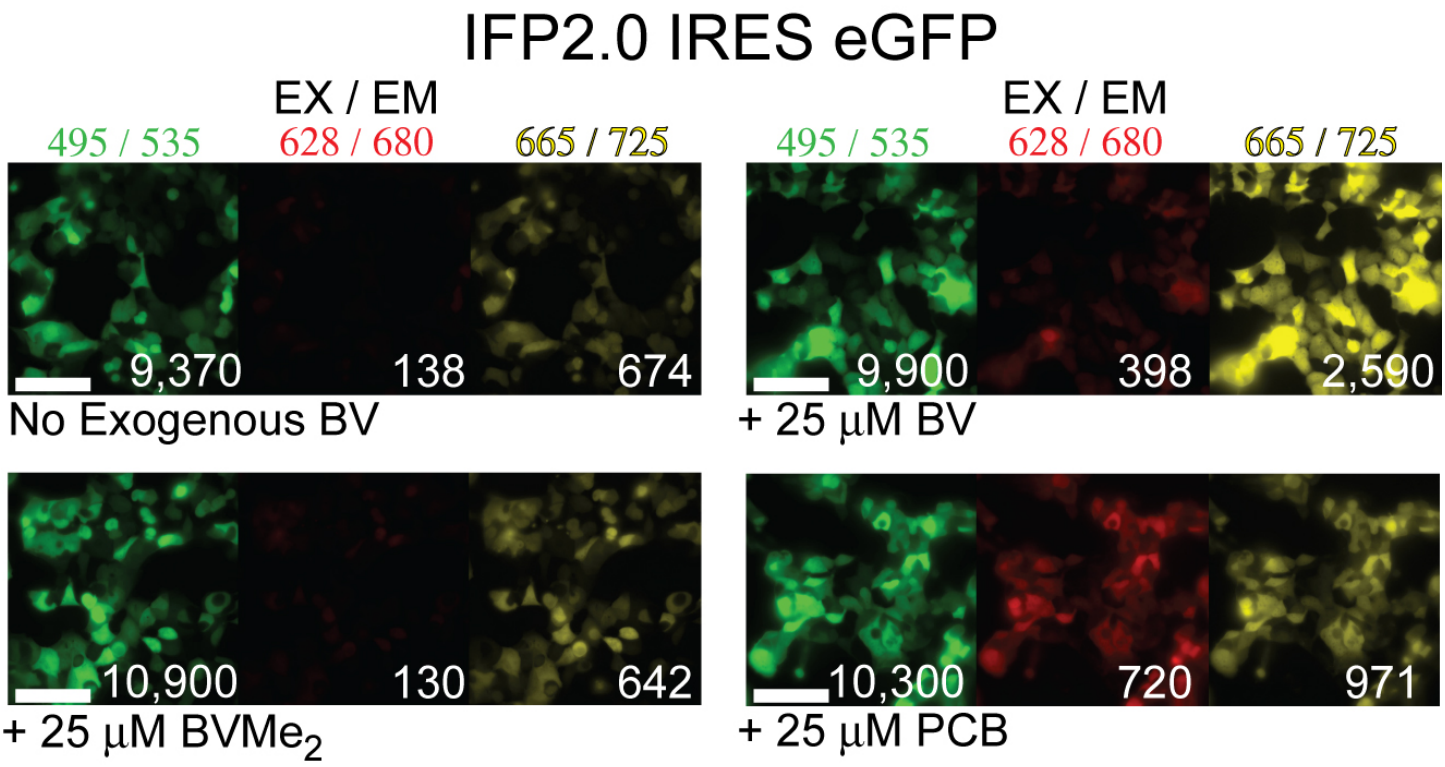
Supplementary Figure 10 | IFP2.0 does not bind $BVMe_2$ and PCB binding does not significantly increase fluorescence.



IFP2.0 IRES eGFP was expressed in HEK293A cells. Chromophore was incubated for 5 h. Exposure = 50 ms and 200 ms for EX / EM = 495 / 535 nm and 628 / 680 and 665 / 725 nm, respectively. Representative fluorescent images are shown from 6 images per sample. White numbers are mean fluorescence intensity (*n* = 30). Addition of BV to IFP2.0 causes a 3.8-fold increase in fluorescence. $BVMe_2$ shows no increase in fluorescence relative to no exogenous BV (some BV present in fetal bovine serum). IFP2.0+PCB results in blue-shifted fluorescence that is not significantly increased (as seen with smURFP and TDsmURFP; **Fig. 3e**). Scale bar = 100 μm.

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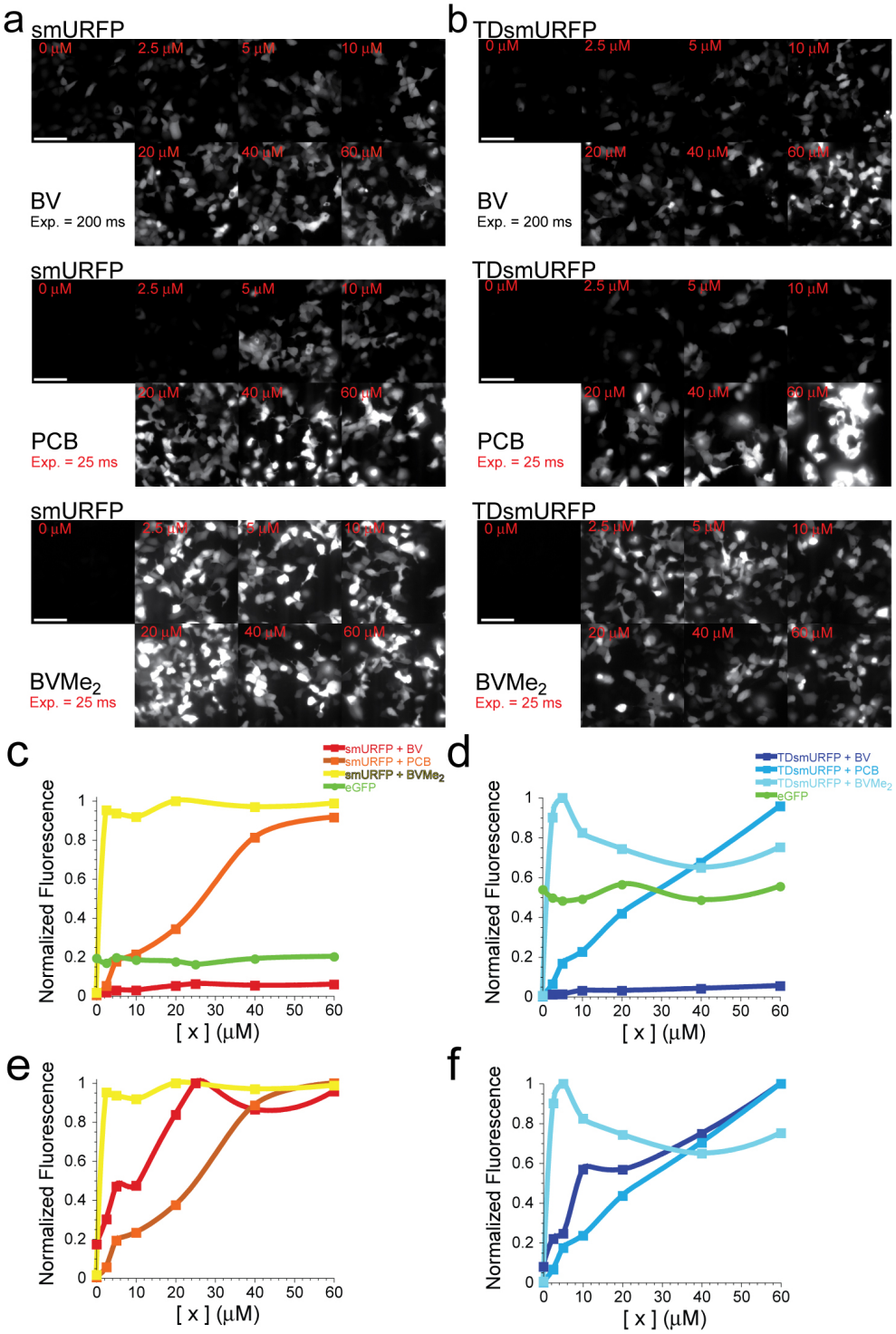
Supplementary Figure 11 | Chromophore concentration dependence of smURFP and TDsmURFP expressed in HEK293A cells.



(**a**,**b**) Representative images (6 images total for each sample) of smURFP and TDsmURFP + x μM BV/PCB/$BVMe_2$. Images are adjusted the same only within each set of chromophore and FP. EX / EM = 628 / 680 nm. Exposure time is under each chromophore. Exp. is exposure. Scale bar = 100 μm. (**c**,**d**) Normalized fluorescence of FP + x μM chromophore. The mean fluorescent intensity (*n* = 30) was determined for each concentration and normalized to brightest FP + x μM chromophore fluorescence. FP is expressed as smURFP/ TDsmURFP IRES eGFP. eGFP was measured with $BVMe_2$ addition and also normalized. SmURFP and TDsmURFP are brightest with $BVMe_2$ and PCB is brighter than BV. SmURFP and TDsmURFP+$BVMe_2$/≥40 μM PCB are brighter than eGFP. (**e**,**f**) Normalized fluorescence to brightest chromophore concentration for each chromophore set.

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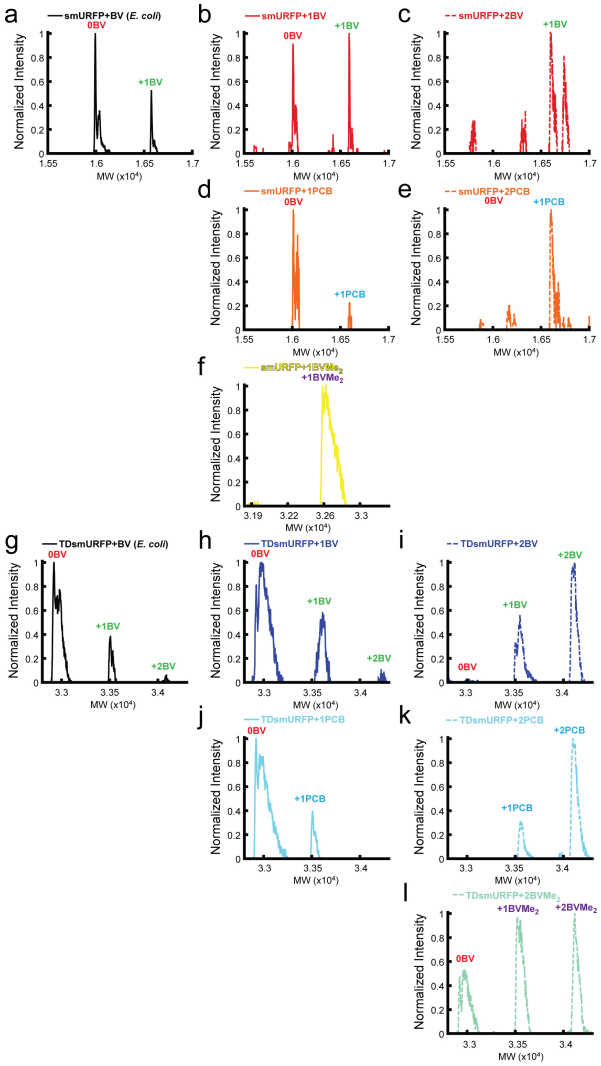
Supplementary Figure 12 | Electrospray mass spectrometry characterization of smURFP/TDsmURFP + 0, 1, or 2 chromophores.



SmURFP+BV (*Escherichia coli*) and TDsmURFP+BV (*Escherichia coli*) were purified with the expression of HO-1 under limited BV conditions. SmURFP and TDsmURFP without chromophore were purified lacking HO-1 from *Escherichia coli* and chromophore was added *in vitro* as described in the **Online Methods**. Samples are named after predominate fluorescent species (+1 or 2 chromophore). (**a**) smURFP+BV (*Escherichia coli*). Free protein: calculated = 15,989 D and found = 15,992 D. FP+BV: calculated = 16,572 D and found = 16,574 D. (**b**) smURFP+1BV. Free protein: calculated = 15,989 D and found = 16,002 D. FP+BV: calculated = 16,572 D and found = 16,588 D. (**c**) smURFP+2BV. FP+BV: calculated = 16,572 D and found = 16,603 D. (**d**) smURFP+1PCB. Free protein: calculated = 15,989 D and found = 16,008 D. FP+PCB: calculated = 16,576 D and found = 16,591 D. (**e**) smURFP+2PCB. FP+PCB: calculated = 16,576 D and found = 16,603 D. (**f**) smURFP+1BVMe_2. MS shows FP is ionized as the homodimer+BVMe_2. FP_2+BVMe_2: calculated = 32,590 D and found = 32,612 D. (**g**) TDsmURFP+BV (*Escherichia coli*). Free protein: calculated = 32,913 D and found = 32,919 D. TDFP+BV: calculated = 33,495 D and found = 33,505 D. TDFP+2BV: calculated = 34,078 D and found = 34,086 D. (**h**) TDsmURFP+1BV. Free protein: calculated = 32,913 D and found = 32,977 D. TDFP+BV: calculated = 33,495 D and found = 33,610 D. TDFP+2BV: calculated = 34,078 D and found = 34,219 D. (**i**) TDsmURFP+2BV. Free protein: calculated = 32,913 D and found = 32,993 D. TDFP+BV: calculated = 33,495 D and found = 33,546 D. TDFP+2BV: calculated = 34,078 D and found = 34,112 D. (**j**) TDsmURFP+1PCB. Free protein: calculated = 32,913 D and found = 32,921 D. TDFP+1PCB: calculated = 33,499 D and found = 33,505 D. (**k**) TDsmURFP+2PCB. TDFP+1PCB: calculated = 33,499 D and found = 33,549 D. TDFP+2PCB: calculated = 34,086 D and found = 34,097 D. (**l**) TDsmURFP+2BVMe_2. Free protein: calculated = 32,913 D and found = 32,978 D. TDFP+BVMe_2: calculated = 33,523 D and found = 33,521 D. TDFP+2BV: calculated = 34,134 D and found = 34,116 D. (**a**-**l**) Average mass calculated by program ProMass Deconvolution.

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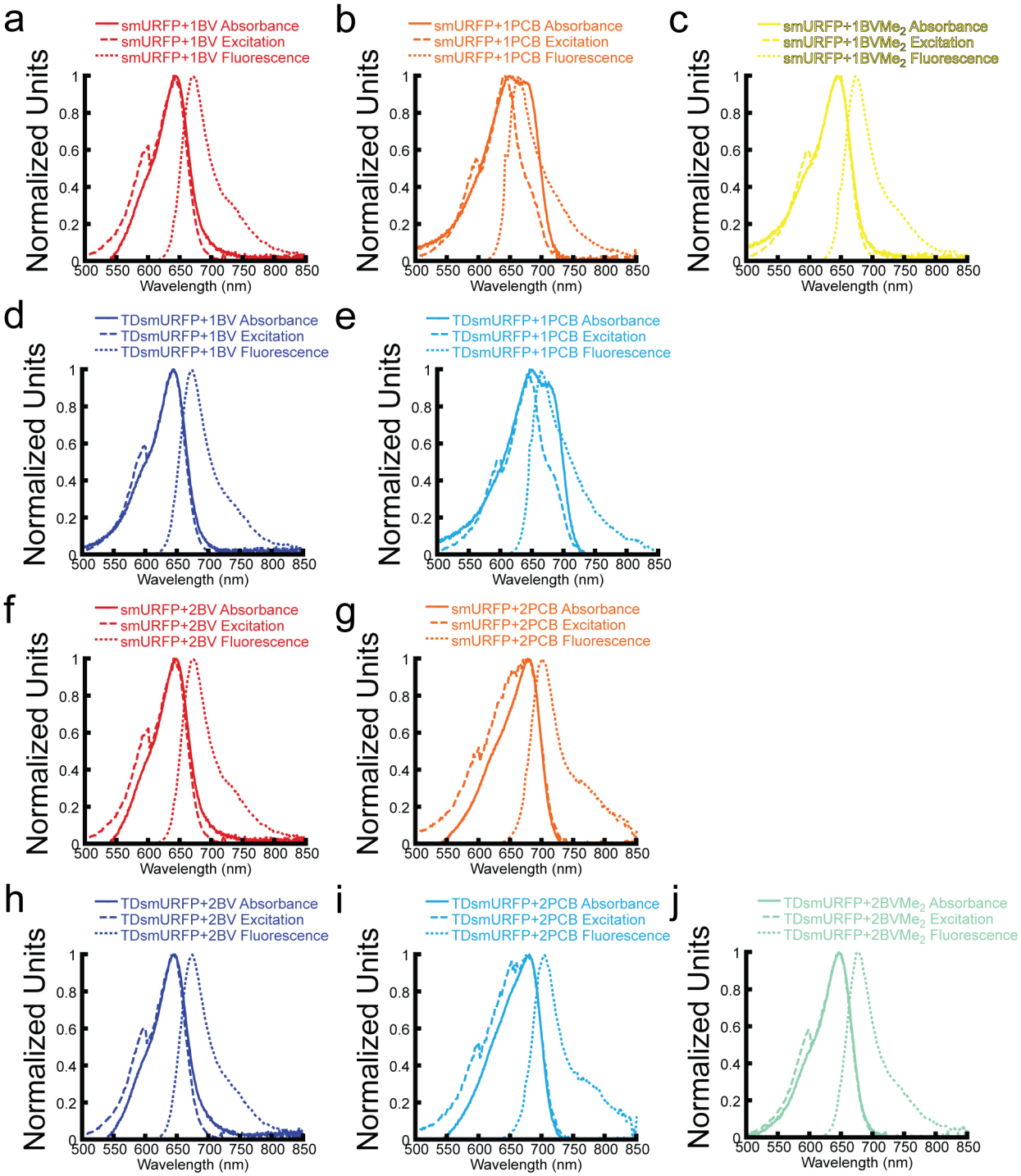
Supplementary Figure 13 | Absorbance, excitation, and fluorescence spectra of smURFP/TDsmURFP + 0, 1, or 2 chromophores.



Spectra were determined from samples in **Supplementary Figure 12**. (**a**-**e**) SmURFP and TDsmURFP+1 chromophore (BV/PCB/$BVMe_2$). SmURFP/ TDsmURFP+1BV and $1BVMe_2$ are identical spectrally. SmURFP/TDsmURFP+1PCB shows absorption broadening, but excitation is similar to BV/$BVMe_2$. SmURFP/TDsmURFP+1PCB fluorescence is blue-shifted by ~6 and ~10 nm, respectively. (**f**-**j**) SmURFP and TDsmURFP+2 chromophore (BV/PCB). SmURFP/TDsmURFP+2BV are spectrally identical to 1BV, but there is ~2.4-fold reduction in QY (**Supplementary Table 3**). SmURFP/TDsmURFP+2PCB shows a red-shifted excitation maximum (674 nm). SmURFP/TDsmURFP+2PCB fluorescence is red-shifted by 34 and 40 nm, respectively, and results in a 5.4- and 4.4-fold, respectively, reduction in QY (**Supplementary Table 3**). SmURFP+$2BVMe_2$ was not observed by changes in spectral properties and by MS (**Supplementary Table 3**, **Supplementary Fig. 12**). TDsmURFP+$2BVMe_2$ showed no change in spectra or QY, but had reduced EC and was identified by MS (**Supplementary Table 3**, **Supplementary Fig. 12**).

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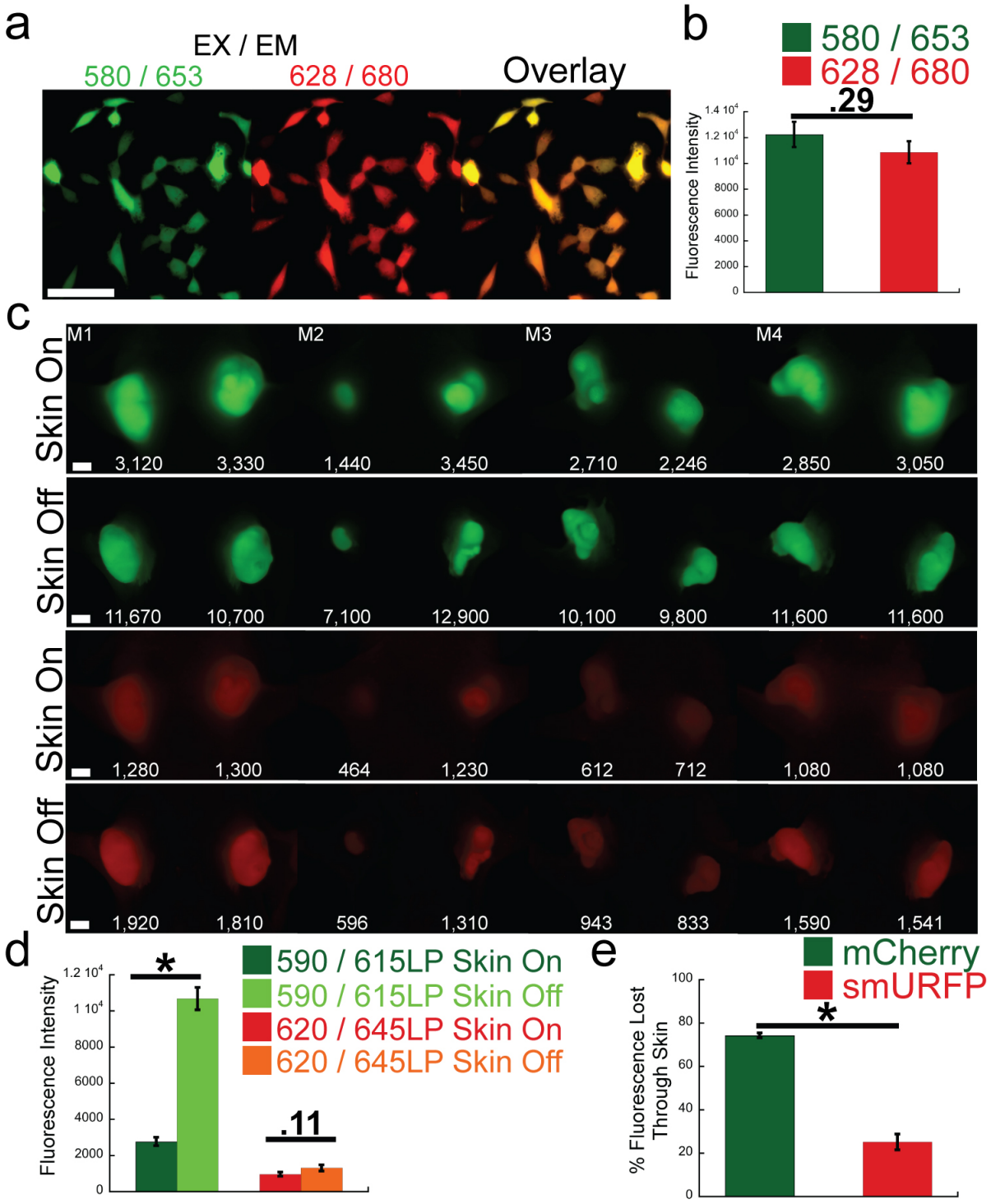
Supplementary Figure 14 | Comparing smURFP and mCherry fluorescence in mouse HT1080 tumor xenografts.



(**a**) Representative fluorescence images (10 images) of FPs expressed in HT108 cells *in vitro*. 12.5 μM BV was incubated for 3 h. Exposure = 200 ms and scale bar = 100 μm. (**b**) Average fluorescent intensity (*n* = 40) of image in **a** and six other images. (**c**) Four mice expressing two HT1080 tumors each. Green and red images are EX / EM = 590 / 615LP and 620 / 645LP nm, respectively. Injection of 250 nmol BV showed no increase in fluorescence after 2 h for all 8 tumors. White # (below tumor) is mean fluorescent intensity. Scale bar = 0.5 cm. (**d**) Mean fluorescent intensity (*n* = 8) of tumors in **c**. (**e**) Quantitation of the percent mean fluorescent intensity lost through skin (calculated for each specific tumor). EX is excitation maximum; EM is emission maximum; M# is mouse#; error bars are s.e.m., and * is *P* <0.0001.

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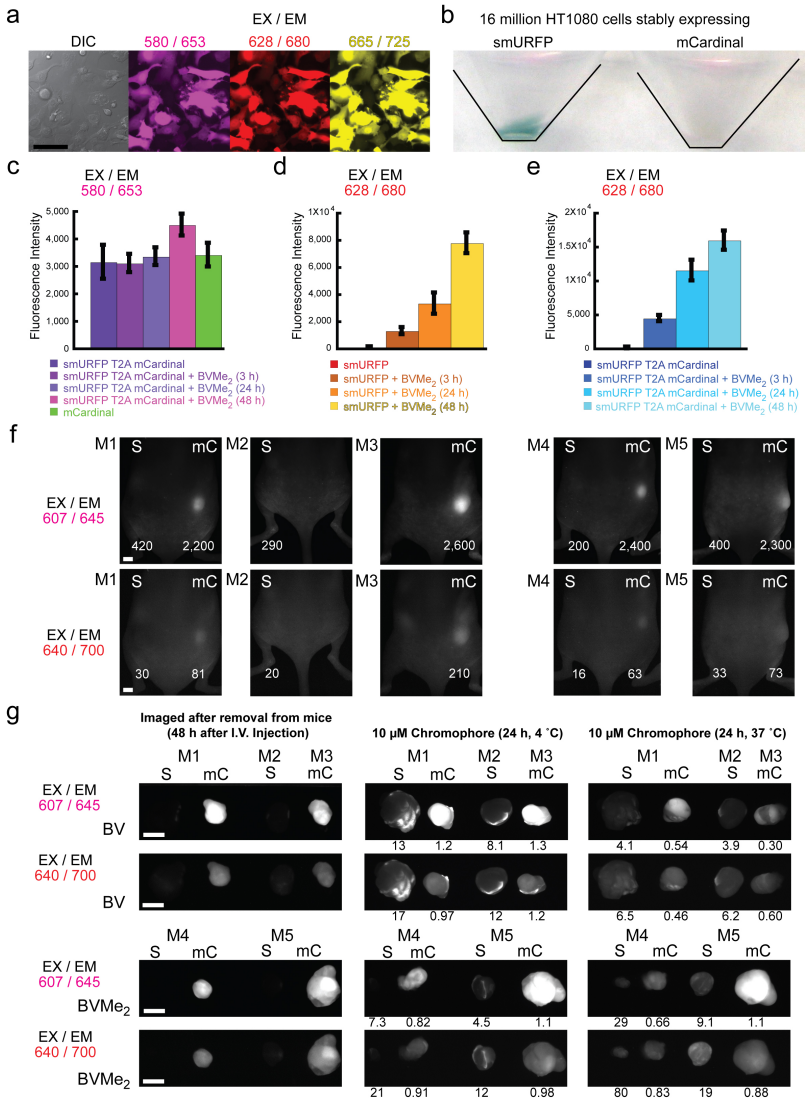
Supplementary Figure 15 | Comparing smURFP and mCardinal fluorescence in mouse HT1080 tumor xenografts.



(**a**) Representative fluorescence images (10 images) of smURFP T2A mCardinal stably expressed in HT108 cells *in vitro,* showing > 95% transduction efficiency. smURFP and mCardinal fluorescence spectrally overlap and lentivirus and stable cell lines expressing each FP separately was necessary. Similar efficiency was obtained for smURFP and mCardinal stably expressing HT1080 cells. 5 µM $BVMe_2$ was incubated for 3 h. Exposure = 50 ms and scale bar = 100 µm. (**b**) White light image of 16 million HT1080 cells stably expressing smURFP (left) and mCardinal (right) before injection into mice. smURFP is visible due to BV present in the fetal bovine serum. (**c**) Average fluorescent intensity (*n* = 30) of three images. mCardinal fluorescence is not altered by the addition of $BVMe_2$ and expression of mCardinal is the same in the two stable cell lines. (**d**,**e**) Average fluorescent intensity (*n* = 30) of 3 images. Addition of $BVMe_2$ for prolonged periods of time stabilizes smURFP and causes increased fluorescence. (**d**) SmURFP + $BVMe_2$ fluorescence is 2-fold greater than mCardinal, as expected from biophysical properties (**Table 1**). (**e**) smURFP fluorescence is 4-fold greater than mCardinal due to enhanced FP expression of the T2A construct (seen also in transient transfection). (**f**) Five mice with HT1080 tumors. White # (below tumor) is mean fluorescent intensity. The mean fluorescence was calculated by a ROI around the tumor and mouse autofluorescence (left knee) and black background next to mouse was subtracted to correct for background fluorescence. Images are adjusted the same in each row and 640 / 700 images were brightened 4X to show mC tumors. Scale bar = 0.5 cm. (**g**) Tumor imaging *ex vivo* 48 h after chromophore i.v. injection. 10 µm chromophore was added *in vitro* in DMEM + 10% FBS. Increase in smURFP fluorescence indicates the gene is expressed and chromophore is not entering cells *in vivo*. BV and $BVMe_2$ do not non-specifically increase fluorescence in mCardinal expressing tumors. Scale bar = 0.5 cm. S is smURFP stably expressing tumor; mC is mCardinal stably expressing tumor; EX is excitation maximum; EM is emission maximum; M# is mouse#; and error bars are s.e.m..

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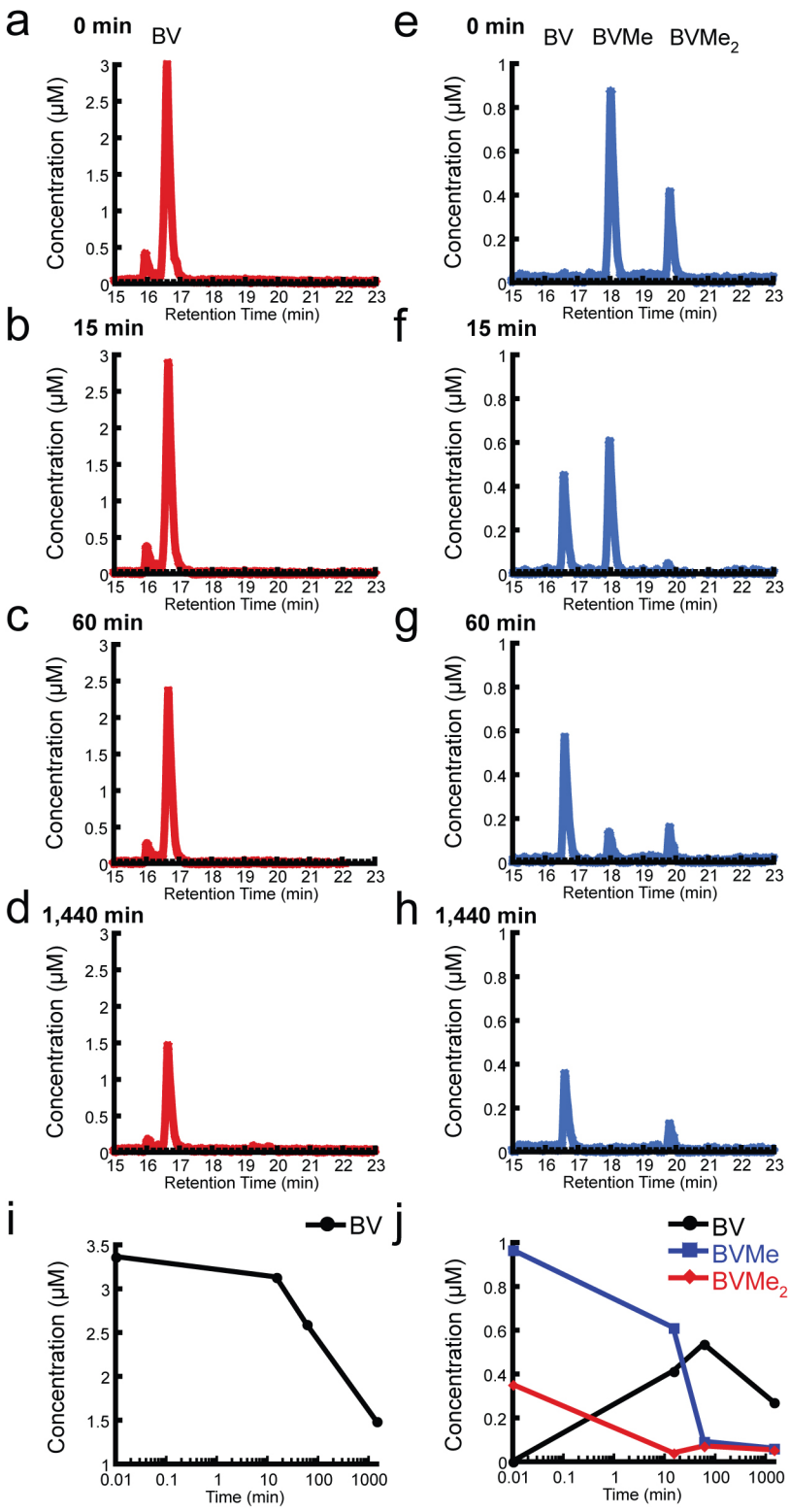
Supplementary Figure 16 | Concentration of BV or $BVMe_2$ in plasma, *in vitro*.



10 μM BV or $BVMe_2$ in plasma was incubated for 0 min at 4 ˚C and 15, 60, and 1,440 min at 37 ˚C. Reactions were stopped by freezing at -80 ˚C. (**a-e**) 10 μM BV and (**f-h**) 10 μM $BVMe_2$ reverse phase HPLC traces showing concentration of chromophore. Concentration of BV (**i**) or $BVMe_2$, BVMe, and BV (**j**) as a function of time. $BVMe_2$ is rapidly cleaved by esterases and BV is significantly degraded without the liver and spleen, which converts BV to bilirubin. BV analogues will be screened in the developed, *in vitro* plasma assay for enhanced stability and/or lack of modification removal.

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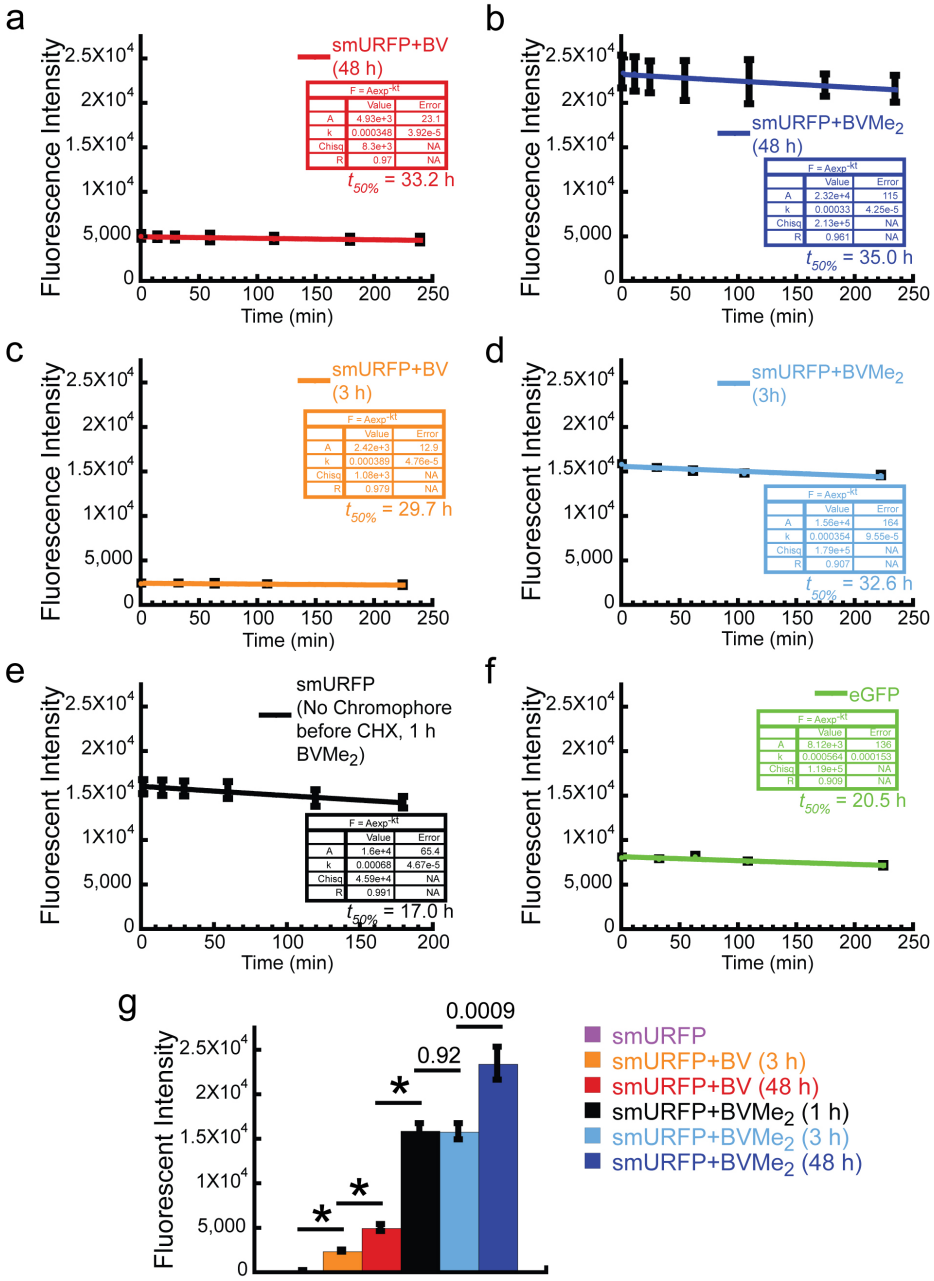
Supplementary Figure 17 | SmURFP stability with and without BV/$BVMe_2$.



HEK293A cells were transfected with smURFP IRES eGFP and protein was detected 48 h later. 25 μM BV (**a**) or 5 μM $BVMe_2$ (**b**) were incubated for 48 h to determine smURFP protein stability in the presence of chromophore. 25 μM BV (**c**) or 5 μM $BVMe_2$ (**d**) were incubated for 3 h to determine smURFP protein stability in the presence of a short burst of chromophore. (**e**) SmURFP stability without chromophore. (**f**) EGFP stability was measured and $t_{50\%}$ = 20.5 h ($t_{50\%}$ ≈ 24 h). 50 μg/ml cycloheximide was added with 25 μM BV (**a**), 5 μM $BVMe_2$ (**b**), or no chromophore (**c-f**). The mean fluorescent intensity (n = 50) was determined for each time point from 5 images and fit to a single exponential decay ($F = A\ exp^{-kt}$). SmURFP shows increased protein stability in the presence of chromophore and is comparable to eGFP. SmURFP protein stability is >7-fold relative to the BPH FPS (iRFP713 and IFP1.4, **Table 1**) (**g**) Mean fluorescent intensity (n = 50, 1 min time point) was plotted in order of increasing fluorescence. Incubation of smURFP with 25 μM BV increases fluorescence by 13- and 27-fold with 3 and 48 h, respectively. Incubation of smURFP with 5 μM $BVMe_2$ increases fluorescence by 85-, 85-, and 125-fold with 1, 3, and 48 h, respectively. Increasing membrane permeability with $BVMe_2$ significantly increases fluorescence. Increased incubation time also increases fluorescence by stabilizing smURFP. Error bars are s.e.m. and * is P <0.0001.

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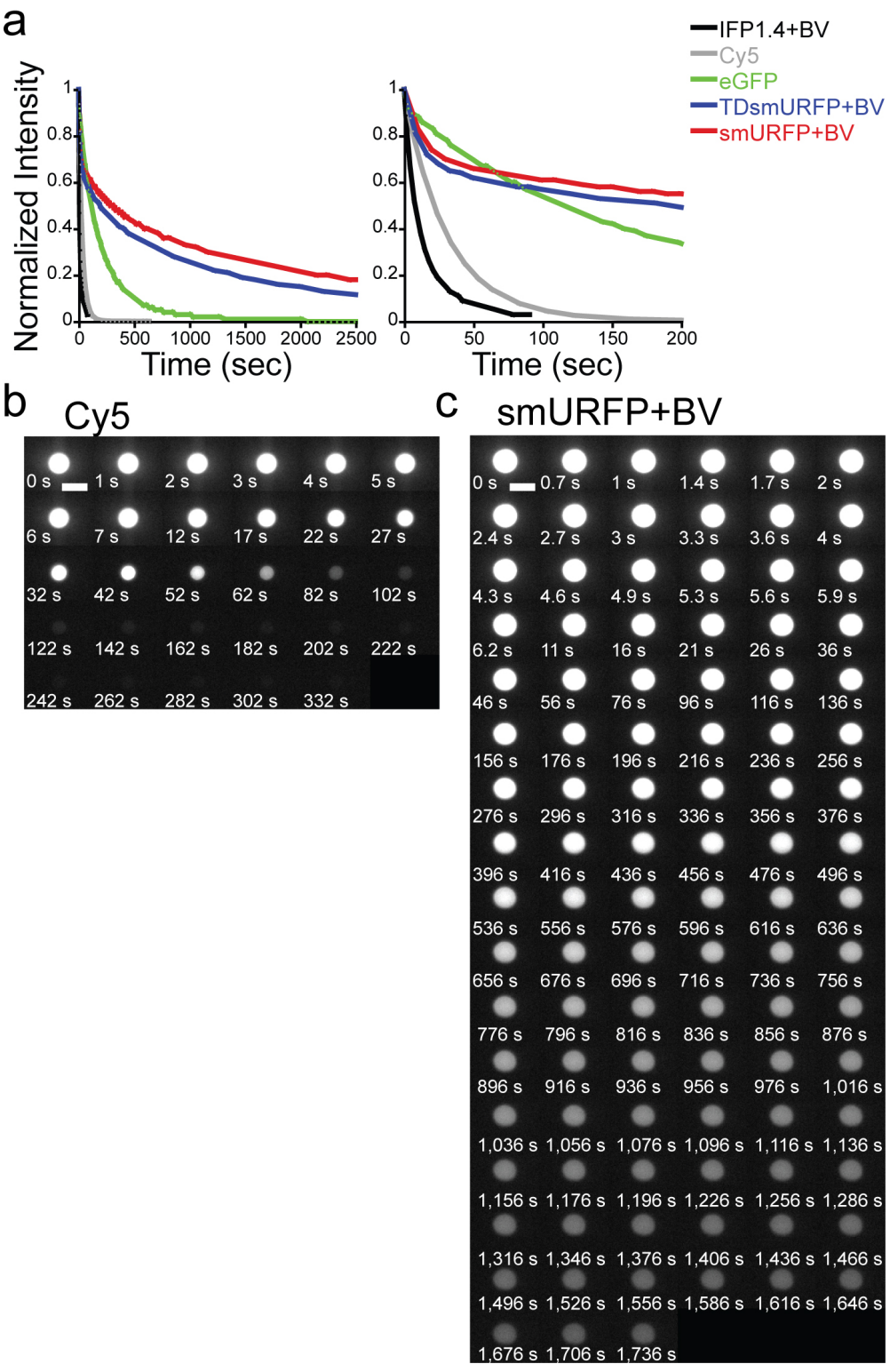
Supplementary Figure 18 | SmURFP+BV and TDsmURFP+BV are photostable *in vitro*.



Photobleaching experiments were performed as described in the **Online Methods**. (**a**) Photobleaching curves of Cy5 and FPs (Left, full time course and Right, magnified view). IFP1.4+BV has $t_{50\%}$ = 8.4 s, is not photostable, and BV alone does not confer photostability. Cy5 has a $t_{50\%}$ = 22 s. eGFP has $t_{50\%}$ = 110 s. TDsmURFP/smURFP+BV have $t_{50\%}$ = 190 s and 300 s, respectively, and are very photostable. (**b**,**c**) Representative photobleaching of Cy5 and smURFP+BV bubbles. Fluorescent images are adjusted the same and the entire photobleaching series was not included. Scale bar = 40 μm.

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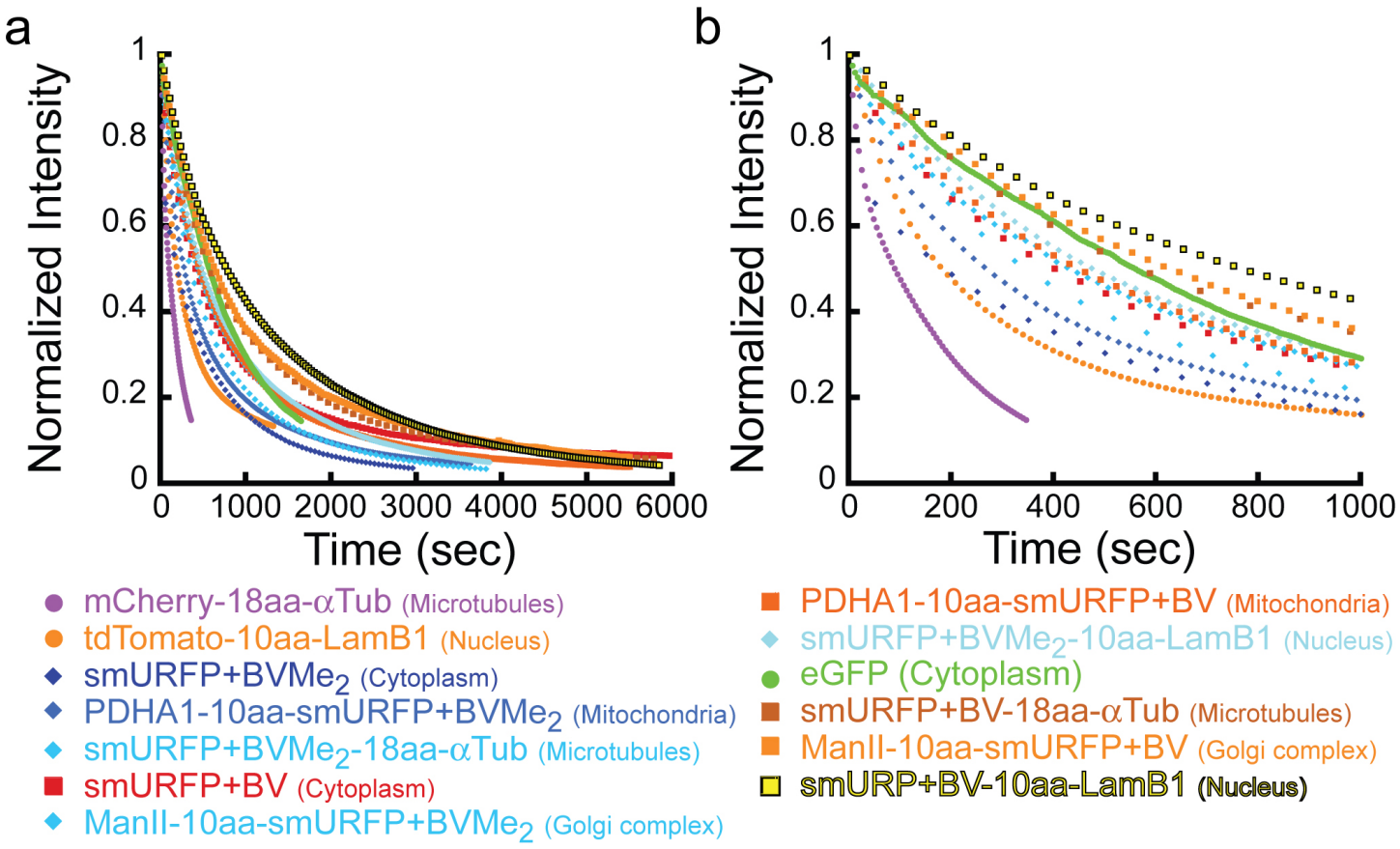
Supplementary Figure 19 | Photobleaching of FP or FP fusions in mammalian cells.



PC3 cells were transfected with DNA and protein was detected 48 h later. 25 μM BV or 1 μM $BVMe_2$ was incubated for 4 h. Mean fluorescent intensity (*n* in **Supplementary Table 4**) was normalized and averaged for all cells. The time axis represents normalized imaging time for FP initial emission rate of 1,000 photons/sec per chromophore. Full (**a**) or zoomed in (**b**) view of photobleaching curves for FP or FP fusions. Curves were fit to a single exponential decay and double exponential decay (data in **Supplementary Table 4**). SmURFP average time to bleach 50% emission (*n* = 5) is $t_{50\%}$ = 340 and 570 sec for $BVMe_2$ and BV, respectively. mCherry is commonly used for superresolution imaging, but is less photostable than smURFP+BV/$BVMe_2$. SmURFP+BV average $t_{50\%}$ is comparable to that of eGFP.

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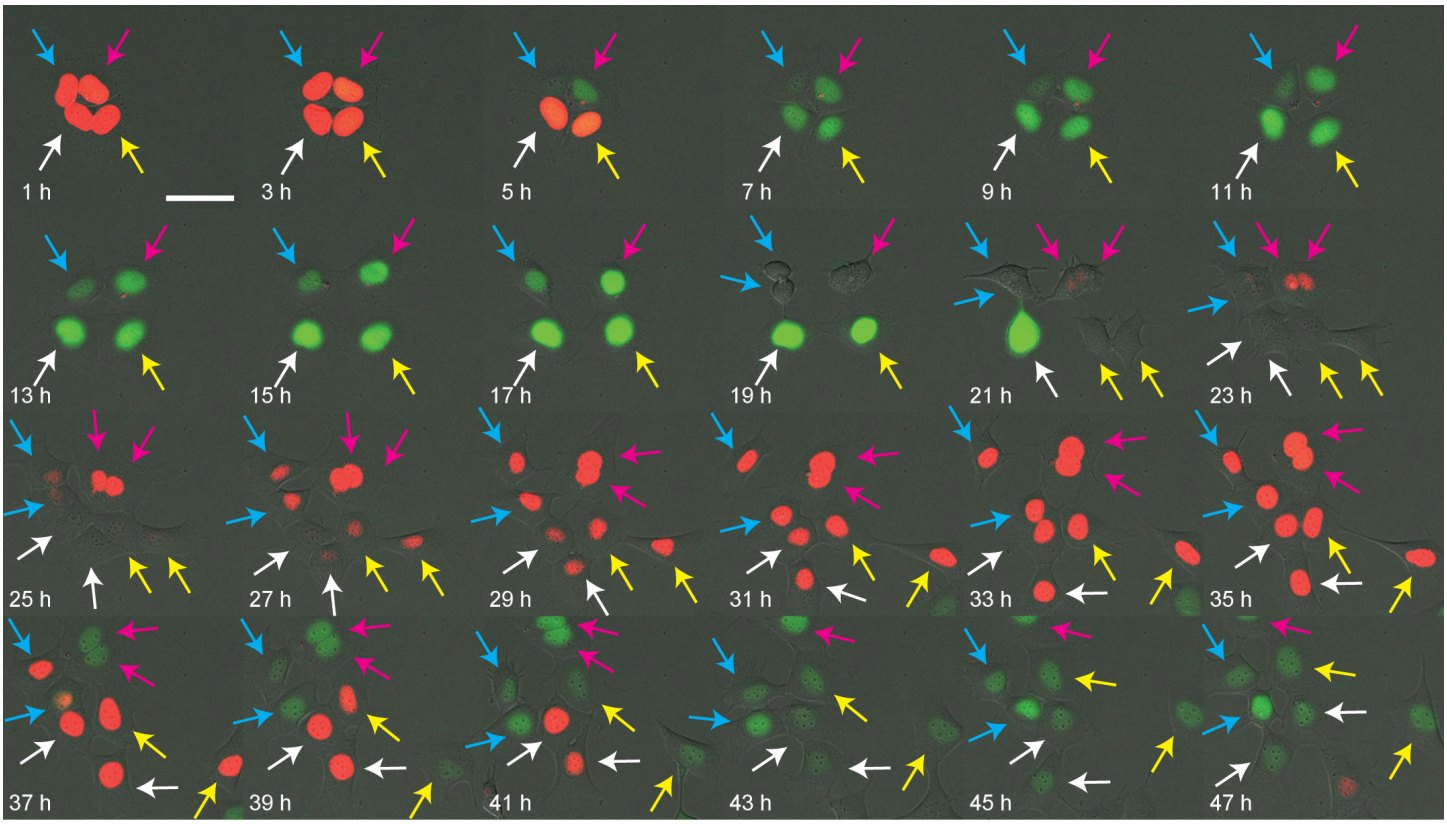
Supplementary Figure 20 | Time-lapse microscopy of mAG-hGem(1/110) and smURFP-hCdtI(30/120) FUCCI expressed in HEK293A cells.



MAG-hGem(1/110) and smURFP-hCdtI(30/120) fluorescence are shown in pseudocolor green and red, respectively. Cyan, magenta, white, and yellow arrows label the original four cells and their descendants. HEK293A cell division occurs with a doubling time of ~34 h. Green is EX / EM = 495(10) / 535(25) nm and red is EX / EX = 628(40) / 680(30) nm. EX is excitation; EM is emission; and scale bar = 50 μm.

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Supplementary Table 1 | Characteristics of evolved APCα FPs.

Fluorescent Protein	Excitation Maximum (nm)	Emission Maximum (nm)	Extinction Coefficient (M^{-1} cm^{-1})	Quantum Yield (%)	Net Charge[a]	Molecular Brightnes Relative to smURFP (%)
TeAPCα	NA	NA	NA	NA	-2.1	NA
R1+PCB	ND	ND	ND	ND	-2.1	ND
R2-1+PCB	626	648	65,000	7.2	-2.1	14
R3-2+PCB	620	648	74,000	13	-2.1	30
R4-1	647	674	93,000	9.0	-1	26
R5-2	671	696	71,000	5.3	-1	12
R6-6	648	676	190,000	9.6	-2.1	56
R7-7	648	676	250,000	16	0	123
R8-8	648	672	260,000	13	-1	104
R8-9	644	672	175,000	12	-1.1	65
R10-10	646	672	200,000	15	-2	93
R11-2	642	672	190,000	12	-2	70
smURFP	642	670	180,000	18	-1.9	100

NA, not applicable; ND, not determined. FPs named as in **Supplementary Fig. 2**. [a]Calculated using Innovagen protein calculator (http://pepcalc.com/protein-calculator.php).

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Supplementary Table 2 | SmURFP incorporation rates of BV.

0.5 μM smURFP in	A	k (min^{-1})	R	$t_{50\%}$ (min)
PBS, + 0.1 μM BV	1.1	0.024	1.0	29
PBS, + 1 μM BV	1.1	0.015	1.0	46
PBS, + 10 μM BV	1.1	0.015	1.0	46
PBS, + 0.1 μM BV (pH 8)	1.1	0.029	0.97	24
PBS, + 1 μM BV (pH 8)	1.1	0.029	0.99	24
PBS, + 10 μM BV (pH 8)	1.1	0.032	0.99	22
PBS, + 0.1 μM BV + 1 mM DTT	1.1	0.012	1.0	58
PBS, + 1 μM BV + 1 mM DTT	1.1	0.011	1.0	63
PBS, + 10 μM BV + 1 mM DTT	1.1	0.015	1.0	46
PBS + 20% FBS, + 0.1 μM BV	1.1	0.016	1.0	43
PBS + 20% FBS, + 1 μM BV	1.1	0.012	1.0	58
PBS + 20% FBS, + 10 μM BV	1.1	0.015	1.0	46
PBS + 20% FBS, + 0.1 μM BV (pH 9.4)	0.95	0.097	0.91	7.1
PBS + 20% FBS, + 1 μM BV (pH 9.4)	0.99	0.043	0.99	16
PBS + 20% FBS, + 10 μM BV (pH 9.4)	0.99	0.063	1.0	11
PBS + 20% FBS, + 0.1 μM BV + 1 mM DTT	0.95	0.023	0.99	30
PBS + 20% FBS, + 1 μM BV + 1 mM DTT	0.95	0.022	0.99	32
PBS + 20% FBS, + 10 μM BV + 1 mM DTT	0.96	0.043	1.0	16
PBS, Avg. 3 Conc.	1.0	0.018	0.99	39
PBS, Avg. 3 Conc. (pH 8)	1.1	0.030	0.99	23
PBS, Avg. 3 Conc. + 1 mM DTT	1.1	0.013	1.0	53
PBS + 20% FBS, Avg. 3 Conc.	1.1	0.014	1.0	50
PBS + 20% FBS, Avg. 3 Conc. (pH 9.4)	0.98	0.066	0.98	11
PBS + 20% FBS, Avg. 3 Conc. + 1 mM DTT	0.95	0.029	0.99	24

Data fit to 1st order increase in fluorescence: $F = A [1 - exp^{-kt}]$.
Avg., average; Conc., concentrations; $t_{50\%}$, time to reach 50% maximal fluorescence intensity.

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Supplementary Table 3 | Characteristics of smURFP/TDsmURFP + 1 or 2 chromophore.

Fluorescent Protein	Excitation Maximum (nm)	Emission Maximum (nm)	Extinction Coefficient (M^{-1} cm^{-1})	Quantum Yield (%)
smURFP+BV (*E. coli)*	642	670	180,000	18
smURFP+1BV	642	672	160,000	16
smURFP+2BV	640	672	2 X 170,000	6.7
smURFP+1PCB	642	666	65,000	7.0
smURFP+2PCB	***674***	***700***	2 X 120,000	1.3
smURFP+1BVMe_2	646	672	65,000	12
TDsmURFP+BV (*E. coli)*	642	670	170,000	18
TDsmURFP+1BV	644	674	150,000	16
TDsmURFP+2BV	644	674	2 X 190,000	6.5
TDsmURFP+1PCB	646	664	66,000	6.2
TDsmURFP+2PCB	***674***	***704***	2 X 120,000	1.4
TDsmURFP+1BVMe_2	646	672	64,000	12
TDsmURFP+2BVMe_2	646	674	2 X 18,000	12

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Supplementary Table 4 | Photobleaching kinetics of FPs or FP fusions in mammalian cells (**Supplementary Figure 19**).

	Single Exponential Decay Fit			Double Exponential Decay Fit						
Fluorescent Protein	A	k (s^{-1})	R	A_1	k_1 (s^{-1})	A_2	k_2 (s^{-1})	R	$t_{50\%}$ (s)	Cells (*n*)
mCherry-18aa-αTub	0.85	0.0054	0.99	0.24	0.066	0.76	0.0047	1.0	89	5
tdTomato-10aa-LamB1	0.79	0.0019	0.96	0.34	0.00074	0.63	0.0063	1.0	180	7
smURFP+$BVMe_2$	0.73	0.0015	0.97	0.42	0.020	0.57	0.0012	1.0	190	16
PDHA1-10aa-smURFP+$BVMe_2$	0.77	0.0013	0.97	0.32	0.00058	0.62	0.0038	0.99	270	11
smURFP+$BVMe_2$-18aa-αTub	0.87	0.0013	0.98	0.36	0.00065	0.63	0.0031	1.0	350	6
smURFP+BV	0.75	0.00080	0.95	0.23	0.00024	0.72	0.0022	1.0	410	4
ManII-10aa-smURFP+$BVMe_2$	0.83	0.00099	0.98	0.36	0.00051	0.59	0.0023	1.0	430	16
PDHA1-10aa-smURFP+BV	0.84	0.00097	0.98	0.26	0.00037	0.71	0.0020	1.0	450	11
smURFP+$BVMe_2$-10aa-LamB1	0.87	0.000099	0.99	0.44	0.00058	0.56	0.0025	1.0	480	7
eGFP	0.97	0.0012	1.0						560	12
smURFP+BV-18aa-αTub	0.85	0.00075	0.98	0.32	0.00031	0.67	0.0018	1.0	570	6
ManII-10aa-smURFP+BV	0.844	0.00069	0.98	0.34	0.0017	0.66	0.0017	1.0	630	9
smURFP+BV-10aa-LamB1	0.89	0.00066	0.99	0.47	0.00044	0.52	0.00044	1.0	770	15

Data fit to single exponential decay fit: $F = A \exp^{-kt}$.
Data fit to double exponential decay fit: $F = A_1 \exp^{-k_1 t} + A_2 \exp^{-k_2 t}$.
$t_{50\%}$, time to bleach for 50% emission intensity.

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Supplementary Video 1 | Time-lapse microscopy of mAG-hGem(1/110) and smURFP-hCdtI(30/120) FUCCI expressed in HEK293A cells. Video is full field, 40X objective view of **Supplementary Fig. 20** and total time is 70 h. MAG-hGem(1/110) and smURFP-hCdtI(30/120) fluorescence are shown in green and red, respectively. Green is EX / EM = 495(10) / 535(25) nm and red is EX / EX = 628(40) / 680(30) nm. EX is excitation and EM is emission.

Supplementary Video 2 | Time-lapse microscopy of FR/NIR FUCCI expressed in HEK293A cells. Video is full field, 40X objective view of **Fig. 5** and total time is 49 h. IFP2.0-hGem(1/110) and smURFP-hCdtI(30/120) fluorescence are shown in green and red, respectively. Green is EX / EM = 665(45) / 725(50) nm and red is EX / EX = 628(40) / 680(30) nm. EX is excitation and EM is emission.

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