

Supporting Information for
Red fluorescent proteins engineered from green fluorescent proteins.

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Movies S1

AzamiGreen	MVS---VIKPEMKIKL C MRGTVN <u>GHN</u> FVIEGE <u>G</u> KGNP <u>Y</u> EGTQ I LDLN <u>V</u> TEGA <u>P</u> LPFAYDI	56
AzamiRed0.1	MVS---VIKEEMKIKLRMEGTVN <u>GHN</u> FVIEGE <u>G</u> E <u>G</u> N <u>P</u> YEGTQTAKLK V TEGG <u>P</u> LPFAYDI	56
DsRed	MRSSKNVIKEFMRFKVRM <u>EGT</u> VN <u>GHE</u> FE <u>I</u> EGE <u>G</u> EGR <u>P</u> YEGHNTVKLK V TKGG <u>P</u> LPFA <u>W</u> DI	60
eqFP578	MSE---LIKE <u>N</u> MHM K L <u>Y</u> MEGTVN <u>NNHH</u> FKCT <u>E</u> GE <u>G</u> K <u>P</u> YEGTQT <u>M</u> KIKVV <u>E</u> GG <u>P</u> LP <u>F</u> A <u>D</u> I	57
eqFP611	MNS---LIKE <u>N</u> M R MM <u>V</u> MEGS <u>V</u> NG <u>Y</u> QFKCT <u>E</u> GD <u>G</u> N <u>P</u> Y <u>M</u> GT <u>Q</u> T <u>M</u> R <u>I</u> KVV <u>E</u> GG <u>P</u> LP <u>F</u> A <u>D</u> I	57
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AzamiGreen	Q TTVFQYGNRAFT K YPADIQDYFKQT <u>F</u> PEG <u>H</u> WERSMTYED <u>Q</u> GICTATSNISMRGDCFFY	116
AzamiRed0.1	LSPQFMYGSKA <u>F</u> IKHPADIPDYFKQS <u>F</u> PEG <u>F</u> HW <u>V</u> MTYEDGG <u>V</u> T <u>C</u> ATQNTSLRGGCFFY	116
DsRed	LSPQFQYGSKVYV <u>K</u> HPADIPDYKKLS <u>F</u> PEG <u>F</u> KW <u>V</u> RM <u>N</u> FEDGG <u>V</u> V <u>T</u> QDSS <u>L</u> QDGCFIY	120
eqFP578	LATS <u>F</u> MYGS <u>K</u> TFINHT <u>Q</u> GIP <u>D</u> FFKQS <u>F</u> PEG <u>F</u> T <u>W</u> ERITTYEDGG <u>V</u> L <u>T</u> ATQDTSLQN <u>G</u> CIY	117
eqFP611	LATS <u>F</u> MYGS <u>K</u> TFIKHT <u>K</u> GIP <u>D</u> FFKQS <u>F</u> PEG <u>F</u> T <u>W</u> ERVTRYEDGG <u>V</u> F <u>T</u> VMQDTSL <u>E</u> DGCLVY	117
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AzamiGreen	D IRFDGVNFPPNGPVMQKKTL <u>K</u> WE <u>P</u> STE <u>K</u> MYVR <u>D</u> GVL <u>K</u> GD <u>V</u> N <u>M</u> ALL <u>L</u> EGGGHYRCDFKTT	176
AzamiRed0.1	DIKFDGVNFPSNGPVMQKKTL <u>G</u> WE <u>P</u> STE <u>K</u> LYPR <u>D</u> GVL <u>K</u> GD <u>V</u> N <u>M</u> ALL <u>L</u> EGGGHYRCDFKTT	176
DsRed	KVKFIGVNFP <u>S</u> DGPVMQKK <u>T</u> M <u>W</u> ESTERLYPR <u>D</u> GVL <u>K</u> GE <u>I</u> H <u>K</u> ALK <u>L</u> KDG <u>H</u> YL <u>V</u> E <u>F</u> KSI	180
eqFP578	NVKINGVNFP <u>S</u> NGSVMQKKTL <u>G</u> WE <u>AN</u> TE <u>M</u> LYPAD <u>G</u> GLR <u>G</u> H <u>S</u> Q <u>M</u> ALK <u>L</u> V <u>G</u> GG <u>L</u> H <u>C</u> S <u>F</u> KTT	177
eqFP611	HAKVTGVNFPSNGAVM <u>Q</u> KK <u>T</u> K <u>G</u> WE <u>P</u> NT <u>E</u> MLYPAD <u>G</u> GLR <u>G</u> Y <u>S</u> Q <u>M</u> AL <u>N</u> V <u>D</u> GG <u>G</u> L <u>S</u> CS <u>F</u> ETT	177
	. : . ****. :*.***** **.** : * ** *: * : ** : .*: .	
AzamiGreen	YKAKKD <u>V</u> ---RLPDYHF <u>D</u> HRI <u>E</u> ILKHD <u>D</u> YN <u>K</u> V <u>K</u> LY <u>E</u> NA <u>V</u> ARY <u>S</u> ML <u>P</u> SQA---K	225
AzamiRed0.1	YKAKKD <u>V</u> ---RLPGYHF <u>D</u> HRI <u>E</u> ILKHD <u>D</u> YN <u>K</u> V <u>K</u> Q <u>E</u> NA <u>V</u> ARY <u>S</u> ML <u>P</u> SQA---K	225
DsRed	YMAKKPV---QLPGYYV <u>D</u> SKLDITSHN <u>E</u> DT <u>I</u> V <u>E</u> Q <u>Y</u> ER <u>T</u> E <u>G</u> R <u>H</u> LF-----L	225
eqFP578	YRSKKPA <u>N</u> L <u>K</u> M <u>P</u> G <u>F</u> H <u>F</u> D <u>H</u> R <u>L</u> E <u>R</u> I <u>E</u> AD <u>K</u> E <u>T</u> Y <u>V</u> Q <u>H</u> E <u>M</u> A <u>V</u> A <u>K</u> Y <u>C</u> D <u>L</u> P <u>S</u> KL <u>G</u> H <u>R</u>	231
eqFP611	YRSKKTV <u>E</u> FK <u>M</u> P <u>G</u> F <u>H</u> F <u>D</u> H <u>R</u> L <u>R</u> LE <u>E</u> SD <u>K</u> E <u>M</u> F <u>V</u> V <u>Q</u> H <u>E</u> H <u>A</u> V <u>A</u> K <u>F</u> C <u>D</u> L <u>P</u> SK <u>L</u> G <u>R</u>	231
	* :** . :*: .*** :: . . . * :* : . . :	

Fig. S1. Sequence comparison of AzamiGreen and natural DsRed-type RFPs. Amino acid sequence alignment of AzamiGreen, AzamiRed0.1, DsRed, eqFP578, and eqFP611, generated using MAFFT(1). Black dots indicate positions that were mutated to generate AzamiRed0.1 from AzamiGreen. The second residue (Val) in AzamiGreen and AzamiRed0.1 was not counted because it was artificially inserted for efficient expression in mammalian cells.

AzamiRed0.1	MVSVIKEEMKIKL RMEGT VNGHNFVIEGE G GNPYEGT Q TAKLKVT EG G PLPFAYDIL SP	59
AzamiRed0.6	MVSVIKEEMKIKL RMEGT VNGHNFVIEGE G GNPYEGT Q TADLKVT EG G PLPFAYDIL SP	
AzamiRed1.0	MVSVIKEEMKIKL RMEGT VNGHNFVIEGE G GNPYEGT Q TMDLKVT EG G PLPFAYDIL SP	
AzamiRed1.1	MVSVIKPEMKIKL RMRGT VNGHNFVIEGE G GNPYEGT Q TMDLKVT EG G PLPFAYDIL SP	
AzamiRed1.2	MVSVIKPEMKIKL C MRGTVNGHNFVIEGE G GNPYEGT Q TMDLNVT E GA P LPFAYDIL SP	
AzamiRed1.3	MVSVIKPEMKIKL C MRGTVNGHNFVIEGE G GNPYEGT Q IMDLNVT E GA P LPFAYDIL SP	
AzamiRed1.4	MVSVIKPEMKIKL C MRGTVNGHNFVIEGE G GNPYEGT Q IMDLNVTEGA P LPFAYDIL SP	
AzamiRed1.5	MVSVIKPEMKIKL C MRGTVNGHNFVIEGE G GNPYEGT Q IMDLNVTEGA P LPFAYDIL TP	
AzamiRed1.6	MVSVIKPEMKIKL C MRGTVNGHNFVIEGE G GNPYEGT Q I L DLNVTEGA P LPFAYDIL TP	
AzamiGreen	MVSVIKPEMKIKL C MRGTVNGHNFVIEGE G GNPYEGT Q I L DLNVTEGA P LPFAYDIL TT	
AzamiRed0.1	Q F MYGSKAF IK H PAPIPDYF KQS FPEGFH W ER V MTYED GGV CTAT QNT SLRG GCF YDI K	119
AzamiRed0.6	Q F MYGSKAF IK K YPADIPDYF KQS FPEGFH W ER V MTYED GGV CTAT QNT SLRG D CFFYD IR	
AzamiRed1.0	Q F MYGSKAF IK K YPADIPDYF KQS FPEGFH W ER V MTYED GGV CTAT QNT SLRG D CFFYD VR	
AzamiRed1.1	Q F MYGSKAF IK K YPADIPDYF KQS FPEGFH W ER V MTYED GGV CTAT QNT SMRG D CFFYD VR	
AzamiRed1.2	Q F MYGSKAF IK K YPADIPDYF KQS FPEGFH W ER V MTYED GGV CTAT QNT SMRG D CFFYD VR	
AzamiRed1.3	Q F MYGSKAF IK K YPADIPDYF KQT FPEGFH W ER V MTYED GGV CTAT QNT SMRG D CFFYD VR	
AzamiRed1.4	Q F MYGSKAF IK K YPADIPDYF KQT FPEGFH W ERSMTYED DQGIC TAT QNT SMRG D CFFYD VR	
AzamiRed1.5	Q F MYGSKAF IK K YPADIPDYF KQT FPEGFH W ERSMTYED DQGIC TAT QNT SMRG D CFFYD VR	
AzamiRed1.6	Q F MYGSKAF IK K YPADIPDYF KQT FPEGFH W ERSMTYED DQGIC TAT QNT SMRG D CFFYD VR	
AzamiGreen	V F QYGNRAFT KYPADI QD YF KQT FPEGY H WERSMTYED DQGIC TAT SN ISM RGD CFFYD IR	
AzamiRed0.1	FDGVNF P SNGGPVM Q KKTL G WEP S TEK L Y P RD GVL K G D V N M ALL L EGGG H Y R CD F K T TY K A	179
AzamiRed0.6	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiRed1.0	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiRed1.1	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiRed1.2	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiRed1.3	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiRed1.4	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiRed1.5	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiRed1.6	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiGreen	FDGVNF P PNNGPVM Q KKTL G WEP S TEK M Y V RD GVL K G D V I K ALL L EGGG H Y R CD F K T TY K A	
AzamiRed0.1	K KDVRLP G YHF V D H R L E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	225
AzamiRed0.6	K KDVRLP G YHF V D H R L E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	
AzamiRed1.0	K KDVRLP G YHF V D H R I E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	
AzamiRed1.1	K KDVRLP D YHF V D H R I E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	
AzamiRed1.2	K KDVRLP D YHF V D H R I E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	
AzamiRed1.3	K KDVRLP D YHF V D H R I E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	
AzamiRed1.4	K KDVRLP D YHF V D H R I E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	
AzamiRed1.5	K KDVRLP D YHF V D H R I E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	
AzamiRed1.6	K KDVRLP D YHF V D H R I E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	
AzamiGreen	K KDVRLP D YHF V D H R I E I L K HD K D Y N K V K Q Y E N A V A R Y S M L P S Q A K	

Fig. S2. Amino acid sequence alignment of AzamiGreen and AzamiRed proteins. Residues different from AzamiGreen are highlighted in red.

AzamiGreen	MVSVI K PEMKIKLCMRGTVN G HNFVIE E GE G KGNP Y EGT Q I L D L N V T E GA P LP F A Y D I L T T	59
AzamiRed1.0	MVSVI E EMKIKL R ME G TVN G HNFVIE E GE G KGNP Y EGT Q T MDL K VTEGG PL P F A Y D I L S P	59
mcavGFP	-MSVI K PDMKIKL R ME G A V N G H K FVIE E GD G K P FE G K Q T M DL T V E GA P LP F A Y D I L T T	59
mcavRed0.1	-MSVI E DMKIKL R ME G A V N G H K FVIE E GD G K P FE G K Q T M DL K V E GG PL P F A Y D I L S P	59
mcavRed1.0	-MSVI E DMKIKL R ME G A V N G H K FVIE E GD G K R PF E G K Q T M DL K V E GG PL P F A Y D I L S P	59
AzamiGreen	VF Q YGNRAFT K YPAD I Q D Y F Q T PEG Y HW E RS M TY E D Q GI C TAT S N I SMRG---DCFFY	116
AzamiRed1.0	Q MY G SK A F I KYPAD I P D Y F K Q S F PEG F HW E RV M TY E D GG VC T AT Q NT S LRG---DCFFY	116
mcavGFP	VFDYG N RV F AK P KD I P D Y F K Q T PEG Y SW E RS M TY E D Q GI C IAT N DI T MM K GV V DD C FVY	119
mcavRed0.1	Q MY G SK V F I KYP K D I P D Y F K Q S F PEG F SW E RV M TY E D GG VC I AT Q DT T LM K GV V DD C FVY	119
mcavRed1.0	Q MY G SK V F I T Y P K D I P D Y F K Q S F PEG F SW E RV M TY E D GG VC I AT Q DT T LM E GV V DD C LVY	119
AzamiGreen	DIRFDGVNFPPNGPVM Q KKTL K WE P STE K MY V RD G V L K G D V N M ALL L EG GG HY R CDF K TT	176
AzamiRed1.0	D VRFDGVNFPPNGPVM Q KKTL G WE P STE K MY V RD G V L K G D V I K A LL L EG GG HY R CDF K TT	176
mcavGFP	KIRFDGVNF P ANGPVM Q RKTL K WE P STE K MY V RD G V L K G D V N M ALL L EG GG HY R CDF K TT	179
mcavRed0.1	K VRFDGVNF P ANGPVM Q RKTL G WE P STE K MY V RD G V L K G D V I K A LL L EG GG HY R CDF K TT	179
mcavRed1.0	K VRF Y GVNF P ANGPVM Q RKTL G WE P STE K MY V RD G V L K G D V I K A LL M EG GG HY R CDF K TT	179
AzamiGreen	YKAKKD V R L PD Y HF V D H R I E I L K H D K D YN K V K LY E NA V AR Y SM L PS Q AK	225
AzamiRed1.0	YKAKKD V R L PG Y HF V D H R I E I L K H D K D YN K V K Q Y EN A VA R Y S ML P SQ AK	225
mcavGFP	YKAKKV V QL P DY Y HF V D H R I E I V S H D K D YN K V K LY E HA E AH-S G LP R Q AK	227
mcavRed0.1	YKAKKV V QL P GY Y HF V D H R I E I V S H D K D YN K V K Q Y EH E AH-S G LP R Q AK	227
mcavRed1.0	Y K S KK V QL P GY Y HF V D H R I E I V S H D K D YN K V K LY E HA E AH-S G LP R Q AK	227

Fig. S3. Amino acid residues mutated to create RFPs from mcavGFP. Amino acid sequence alignment of AzamiGreen, AzamiRed1.0, mcavGFP, mcavRed0.1, and mcavRed1.0, generated using ClustalW(2). Residues in AzamiRed1.0, which are different from AzamiGreen, and those in mcavRed0.1 and mcavRed1.0, which are different from mcavGFP, are highlighted in red.

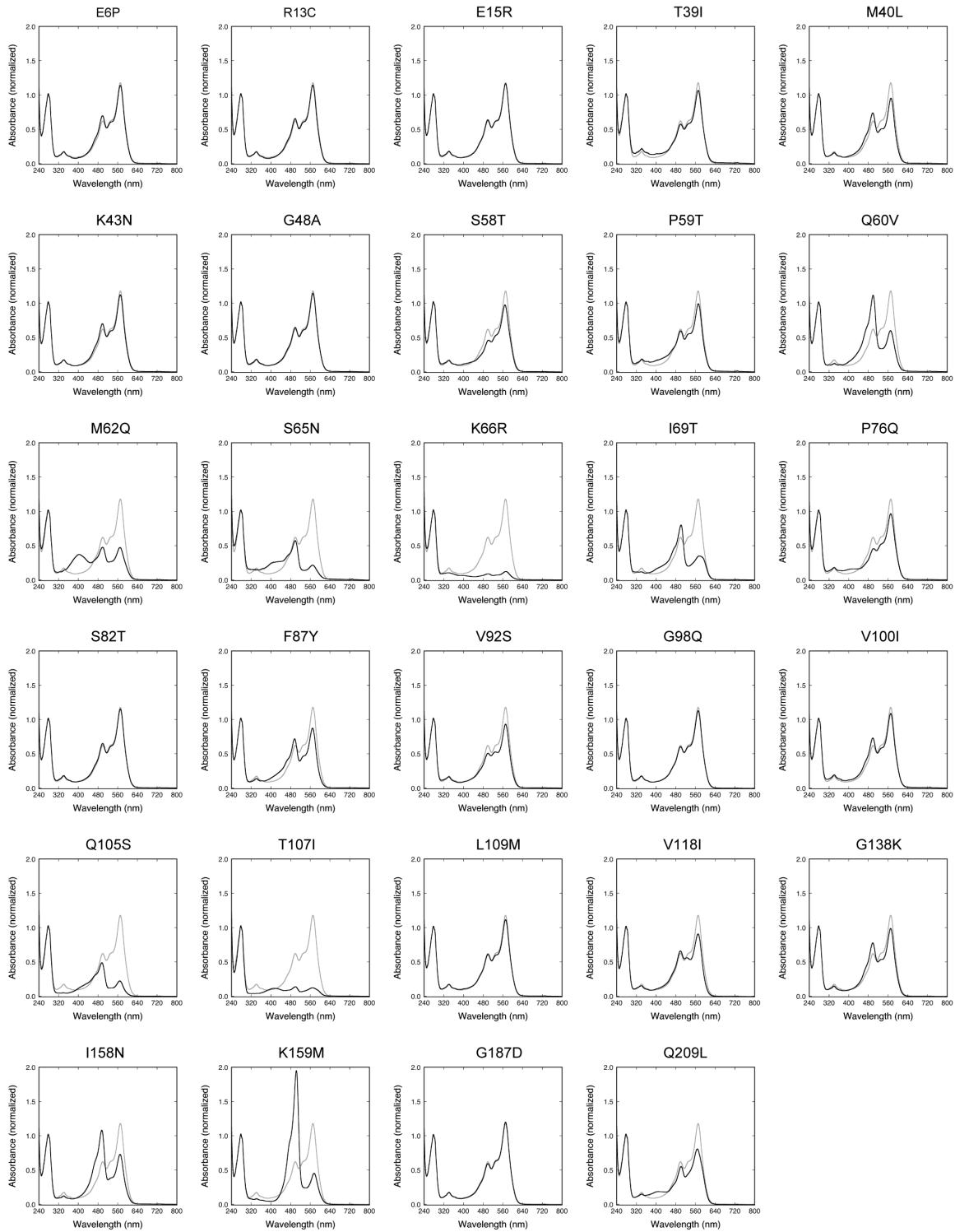


Fig. S4. Absorption spectra of single-reversion variants of AzamiRed1.0. Spectra are normalized by absorption at 280 nm. Grey line is the absorption spectrum of AzamiRed1.0.

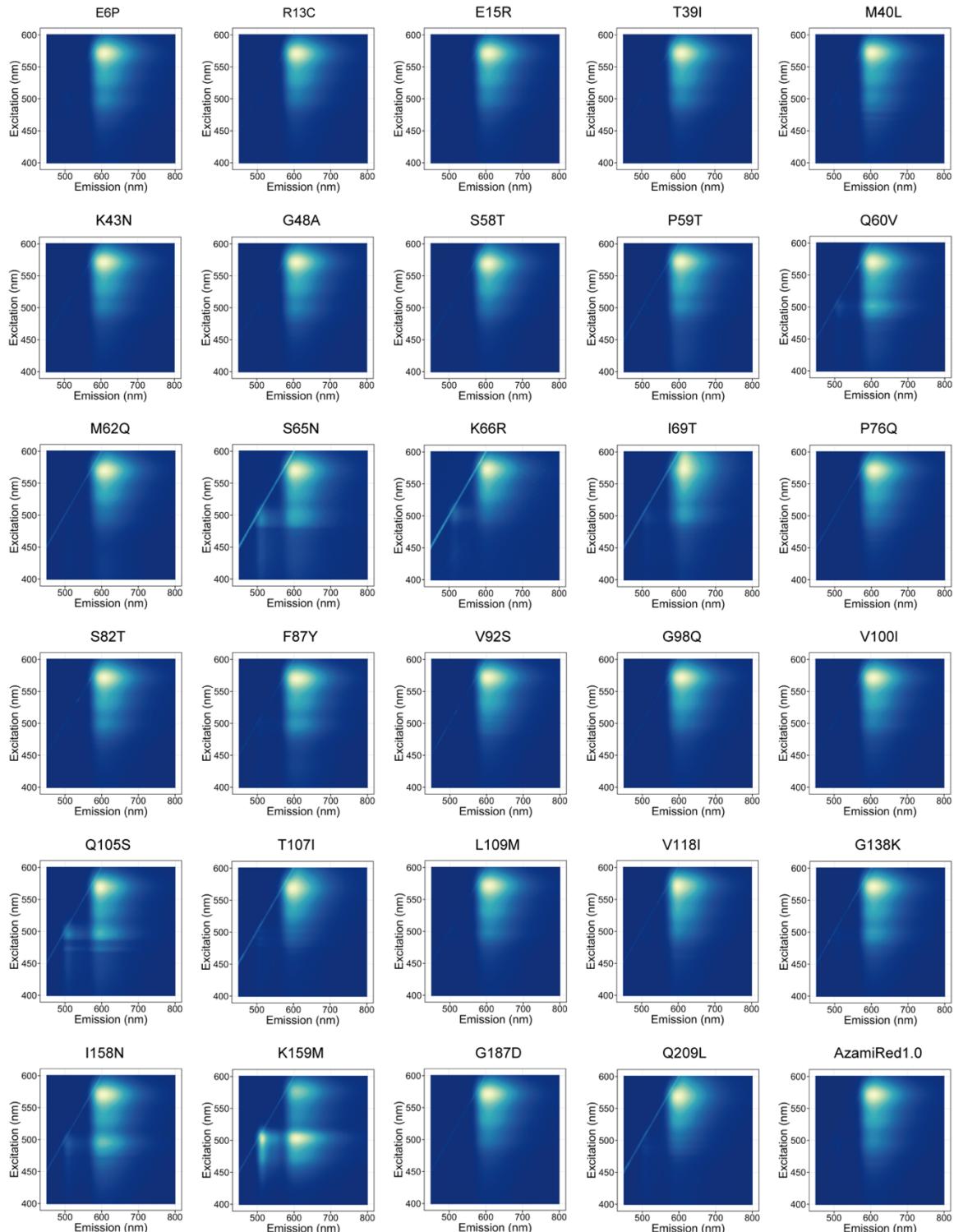


Fig. S5. Two-dimensional fluorescence spectra of single-reversion variants of AzamiRed1.0.

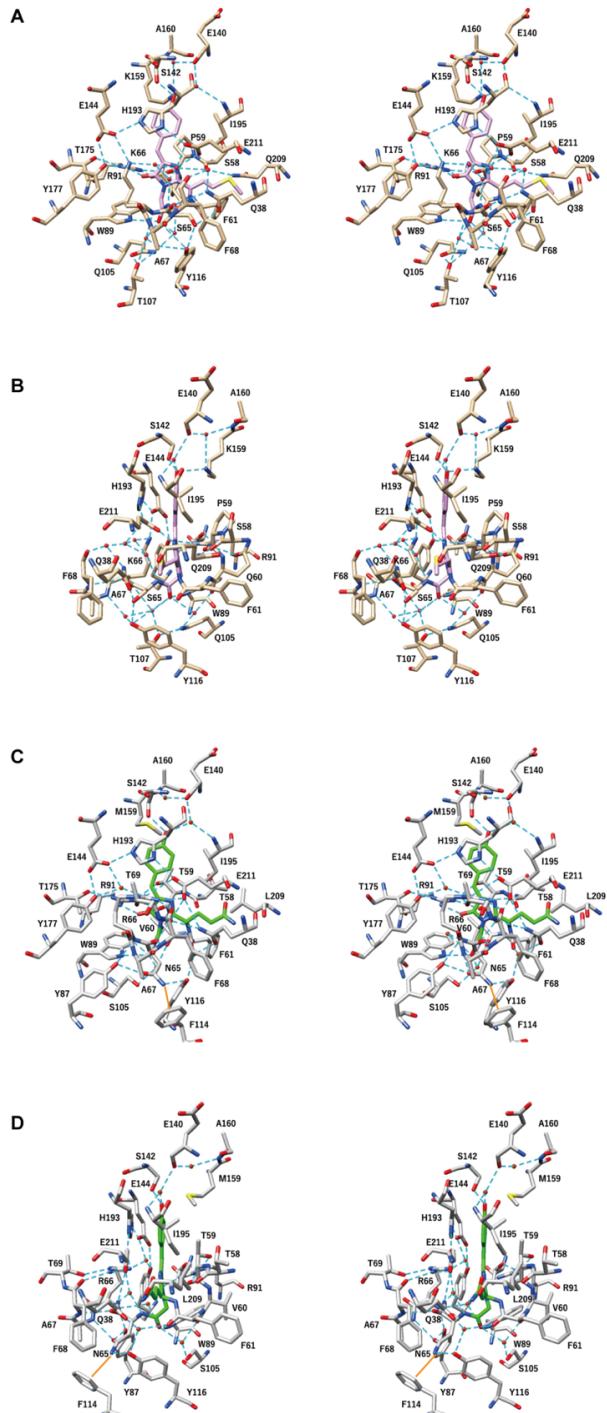


Fig. S6. Stereo diagrams around the fluorophore. Stereo diagrams (can be viewed by crossing one's eyes) of the hydrophilic interaction network around the fluorophore of AR1.0 (A, B) and AG (C, D). The fluorophores of AR1.0 and AG are colored with pink and green, respectively. The nitrogen, oxygen, and sulfur atoms are colored in blue, red, and yellow, respectively. Possible hydrogen bonds are indicated by cyan broken lines. The cation-π interaction in AG is shown by a solid orange line.

A

AzamiRed1.0 mARs1	MVSVIKEEMKIKLRMEGTVNNGHNFVIEGEKGKGNPYEGTQTMDLKVTTEGGPLPFAYDILSP	59
AzamiRed1.0 mARs1	QFMYGSKA I KYPA D IPDYFKQSFP E GFHW R EVMTYEDGGVCTATQNTSLRGDCFFYDVR	119
AzamiRed1.0 mARs1	QFMYGSKP F IKYPA D IPDYFKQS Y PEG Q H R EVMTYEDGGVCTATQNSLRGD C FFYDVR	119
AzamiRed1.0 mARs1	FDGVNFPPNGPVMQKKTLGWEPS T EKM V RDGVLKGD V I K ALLLEGGGHYRCDFKTTYKA	179
AzamiRed1.0 mARs1	FDGTNFPPNGPVMQKKTLG W PS S EKM V RDGVLKGD V S K ALLLEGGGHYRCDFKTTYKA	179
AzamiRed1.0 mARs1	KKDVR ^L PGYHFVDHR I EILKHD D DYNKV K QYEAVARYSMLPSQAK	225
AzamiRed1.0 mARs1	KKDVR ^L PG A H KVDHR I EILKHD D DYN N N V K L E IAVARYS-----	218

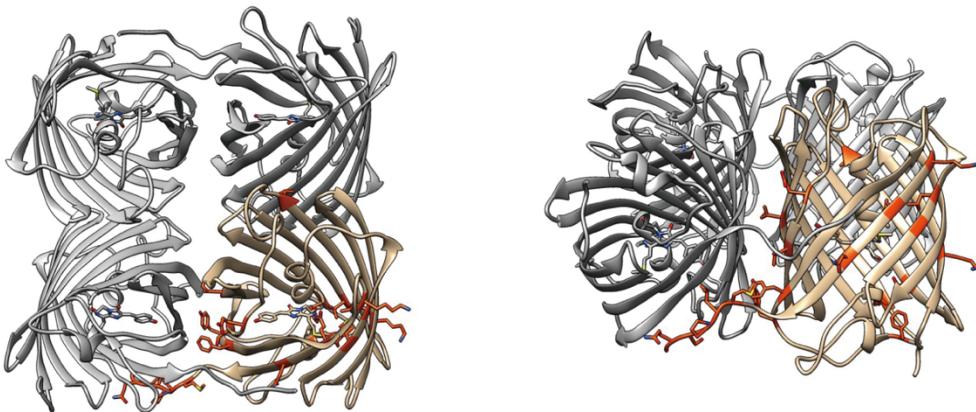
B

Fig. S7. Amino acid residues mutated to create mARs1 from AzamiRed1.0. (A) Amino acid sequence alignment of AR1.0 and mARs1. The mutated residues to produce mARs1 are highlighted in red in the mARs1 sequence. (B) Positions of the mutated residues mapped in the crystal structure of the AR1.0 tetramer. The fluorophore is shown by a stick representation. One subunit in the tetramer is colored with light orange. The residues mutated to produce mARs1 are indicated by a stick representation colored with red.

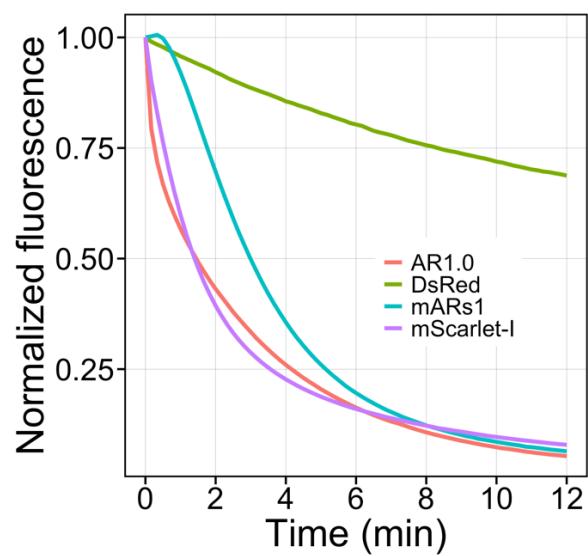


Fig. S8. Photostability of RFPs. HeLa cells expressing RFPs were illuminated with 562 nm LED light (3.1 W cm^{-2}) under a microscope. Fluorescence intensity was taken every 10 s. The lines are the averages of multiple cell data (AR1.0, 29 cells; mARs1, 24 cells; DsRed, 21 cells; mScarlet-I, 28 cells).

Table S1. Summary of X-ray data collection and refinement statistics

	Azami Red1.0	Azami Green
Data collection		
Space group	$I2_1$	$P2_12_12_1$
Cell dimensions		
a, b, c (Å)	83.13, 72.77, 213.84	64.79, 71.35, 196.05
α, β, γ (°)	90.00, 99.48, 90.00	90.00, 90.00, 90.00
Resolution (Å)	72.50-1.84 (1.87-1.84) *	67.05-1.62(1.65-1.62)
R_{sym} or R_{merge}	0.103 (0.397)	0.090 (0.484)
$I / \sigma I$	6.7 (2.5)	11.9 (3.9)
Completeness (%)	99.9 (99.9)	100.0 (100.0)
Redundancy	3.3 (3.4)	5.9 (5.8)
Refinement		
Resolution (Å)	72.50-1.84 (1.86-1.84) *	67.05-1.62 (1.65-1.62)
No. reflections	109126	116190
$R_{\text{work}} / R_{\text{free}}$	0.176/0.217	0.145/0.171
No. atoms		
Protein	10809	7273
Ligand/ion	3	0
Water	1099	1340
B -factors		
Protein	21.5	14.2
Ligand/ion	35.2	-
Water	27.5	27.3
R.m.s. deviations		
Bond lengths (Å)	0.008	0.014
Bond angles (°)	1.16	1.39

Movie S1 (separate file). Visualization of microtubule tips with mARs1-tagged EB3 in a HeLa cell.

SI References

1. K. Katoh, J. Rozewicki, K. D. Yamada, MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* **20**, 1160–1166 (2019).
2. J. D. Thompson, D. G. Higgins, T. J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680 (1994).