Supplementary Materials for **"Monomerizing the ultrabright AausFP1 yields mBiyu for advanced imaging across challenging bacterial and mammalian cells"**

This PDF file includes:

Figures S1 to S10

Supplementary Table 1 to 4

Captions for Movies S1 to S2

References

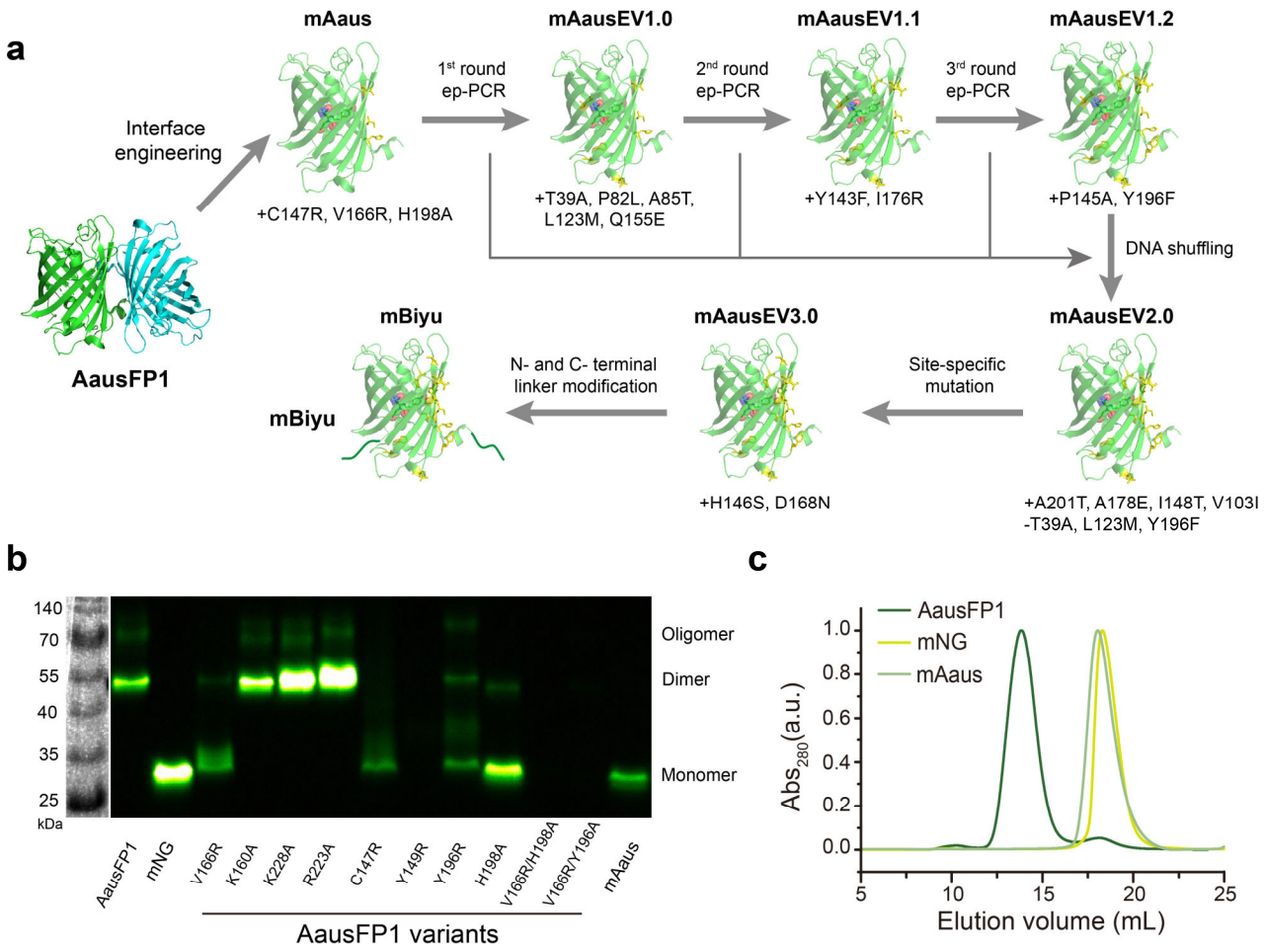


Figure S1 | General scheme of mBiyu engineering and characterization of interface residues. (a) Schematic diagram of engineering process and mutated residues to develop mBiyu. **(b)** Pseudonative SDS-PAGE of the dimer interface disrupted variants of AausFP1. mAaus exhibits monomeric size and fluorescence. **(c)** Size-exclusion chromatography of purified mAaus. AausFP1 and mNG serve as oligomeric and monomeric control, respectively.

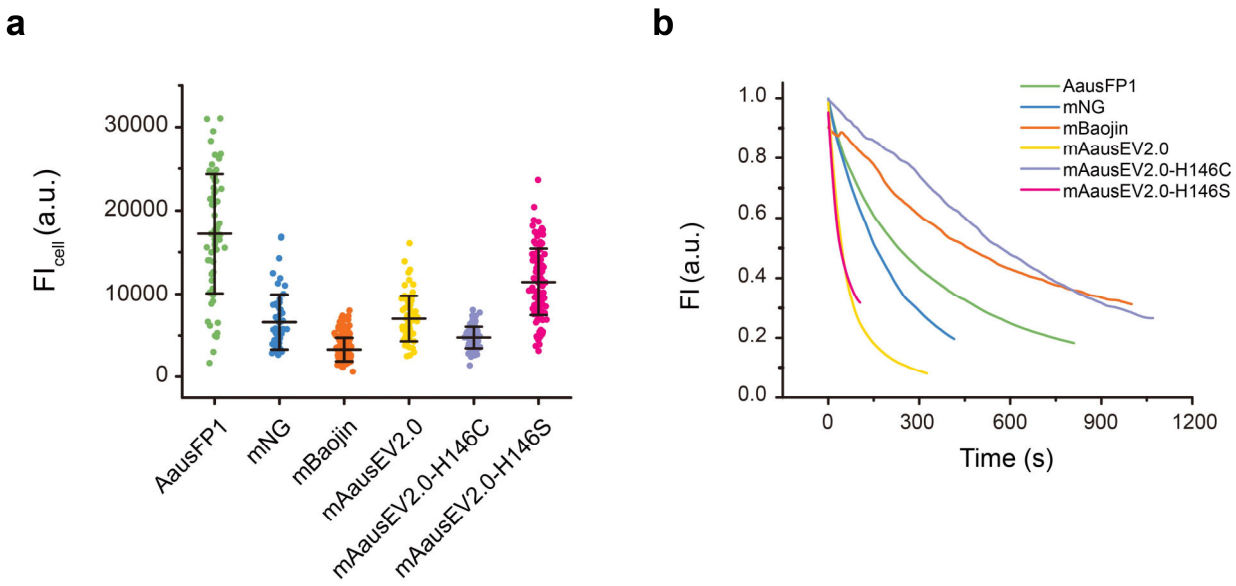
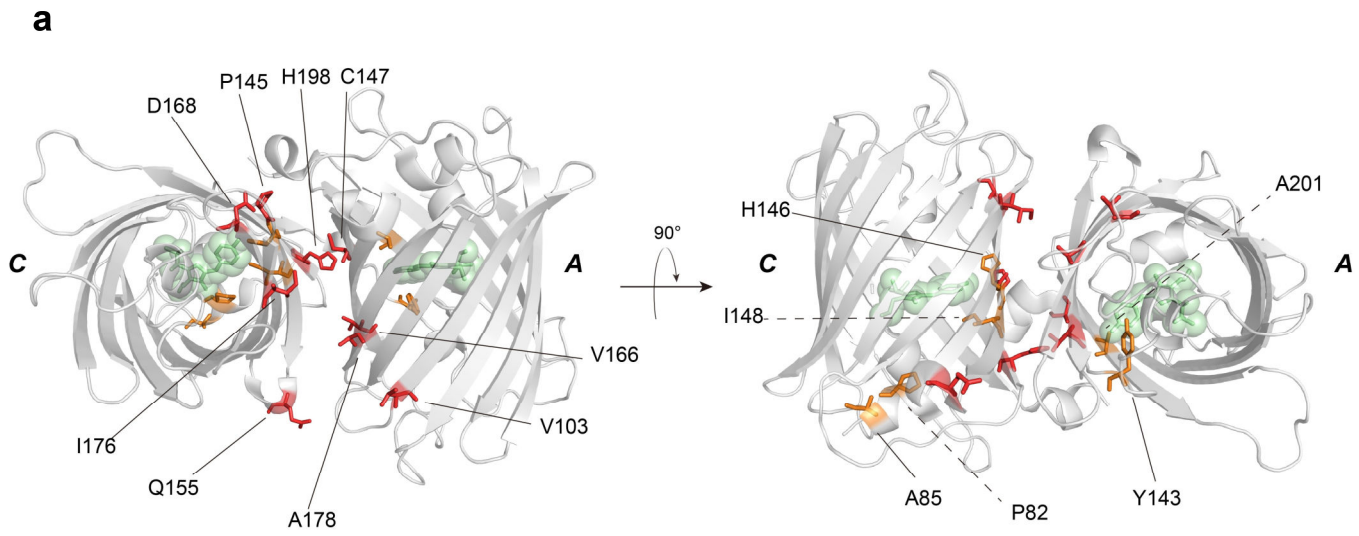


Figure S2 | Trade-off between brightness and photostability of the H146 position. (a) Single-cell fluorescence intensity (FI) of mAausEV2.0 and two variants of the histidine at 146 replaced by cystine or serine. AausFP1, mNG, and mBaojin serve as comparison. Squares indicate mean and boxes indicate s.d. n > 60 cells for each FP. **(b)** Decay of FI of the FPs with 1.6 W·cm^{-2} laser excitation. FIs are averaged from more than 15 cells for each FP.



b

```
AausFP1  M - - - - - - - - - - S Y G A L L F R E K I P Y V V E M E G D V E G M K F S V R G K G H G  35
mBiyu    M V S K G E E D N M A S Y G A L L F R E K I P Y V V E M E G D V E G M K F S V R G K G H G  45

AausFP1  D A N T G K I E A S F I C T T G E L P V P W S S I L T T V T Y G A Q C F A K Y P N D I K D  80
mBiyu    D A N T G K I E A S F I C T T G E L P V P W S S I L T T V T Y G A Q C F A K Y P N D I K D  90

AausFP1  Y P K S A M P E G Y V Q E R T I T F E N D G V Y K T R A E V T Y E K G S V Y N R V T L N G  125
mBiyu    Y L K S T M P E G Y V Q E R T I T F E N D G I Y K T R A E V T Y E K G S V Y N R V T L N G  135

AausFP1  S G F K K G G N I L G K K L E F N Y N P H C I Y V L P D V Q N N G I K C Y I N I V H D V I  170
mBiyu    S G F K K G G N I L G K K L E F N F N A S R T Y V L P D V E N N G I K C Y I N I R H N V I  180

AausFP1  G G G Q I I A A H Q Q L N T P L G G G P V D I P H Y H H I Q A H T I L S K D P K E T R D H  215
mBiyu    G G G Q I R A E H Q Q L N T P L G G G P V D I P H Y H A I Q T H T I L S K D P K E T R D H  225

AausFP1  M N V V E V F R A I D C K T A Y A - - - - - - -  232
         M N V V E V F R A I D C K T A Y A G M D E L Y K  249
```

Figure S3 | Mutations in AausFP1 generating mBiyu. (a) The dimer of AausFP1 with residues mutated in mBiyu highlighted: external residues are shown in red, and residues internal to the β-barrel are shown in orange. **(b)** Sequence alignment of mBiyu and AausFP1.

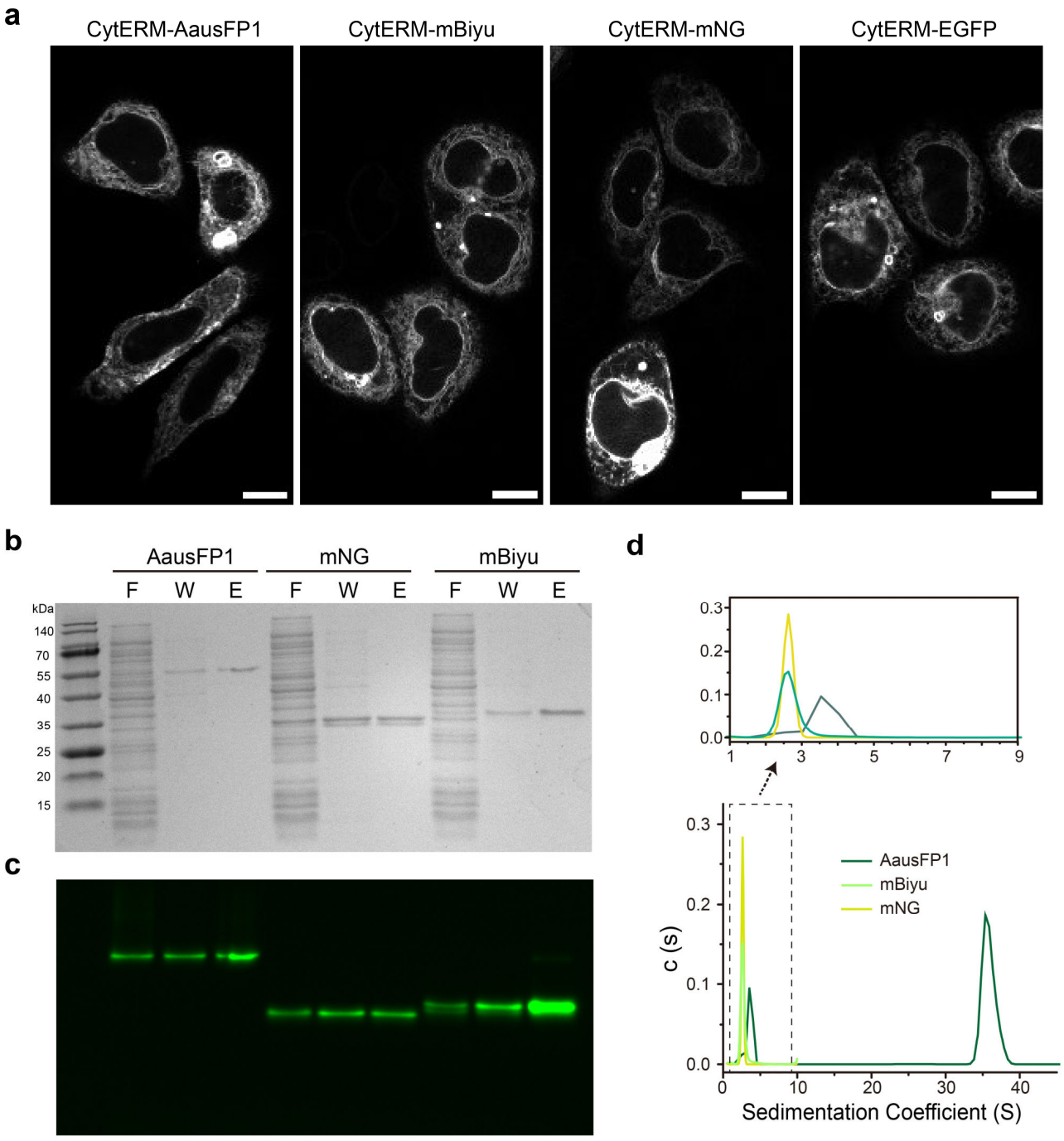


Figure S4 | Additional results showing monomericity of mBiyu. (a) Additional fluorescence images of OSER assay of AausFP1, mBiyu, mNG, and EGFP. Scale bars, 10 μm. **(b)** and **(c)** Pseudonative SDS-PAGE separation of the flow-through (F), wash (W), and elution (E) from purification process of AausFP1, mNG, and mBiyu. **(b)** The proteins were stained by Coomassie brilliant blue. **(c)** The green fluorescence image of the FPs. mBiyu migrates similar to the monomeric FP, mNG. **(d)** Analytical ultracentrifugation results showed mBiyu with molecule size (sedimentation) comparable with mNG, whereas AausFP1 exhibits multiple molecule sizes.

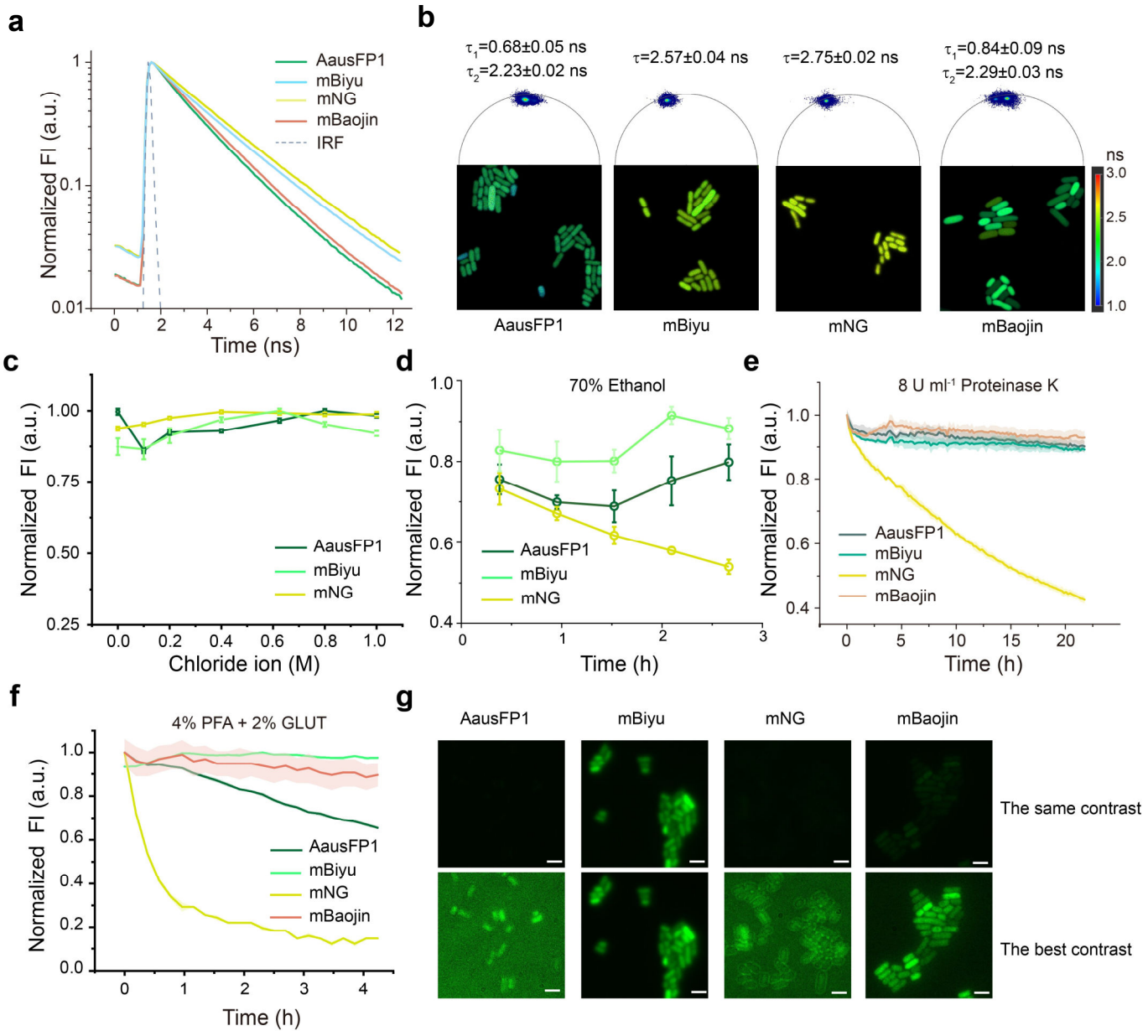


Figure S5 | Fluorescence lifetime and chemical stability of mBiyu. (a) Fluorescence intensity (FI) decay of AausFP1, mBiyu, mNG, and mBaojin. Lifetime measured in *E. coli* cells expressing FPs. **(b)** Representative FLIM images and lifetimes fitted for each FP. The phasor diagrams are shown above each image. **(c)** FI of AausFP1, mBiyu, and mNG at different concentration of sodium chloride. **(d)** Change of FI of AausFP1, mBiyu, and mNG after 70% Ethanol treatment. **(e)** Change of FI of AausFP1, mBiyu, mNG, and mBaojin after 8 U ml^{-1} protease treatment. **(f)** Change of FI of AausFP1, mBiyu, mNG, and mBaojin after 4% PFA + 2% GLUT treatment. **(g)** Representative images of *E. coli* cells before and after treatment with 1% OsO_4 for 4 h at 25 °C. Prior to OsO_4 treatment, cells were first fixed with 4% PFA + 5% GLUT at 25 °C for 2 h followed by incubation at 4 °C for 12 h. Scale bars, 2 μm.

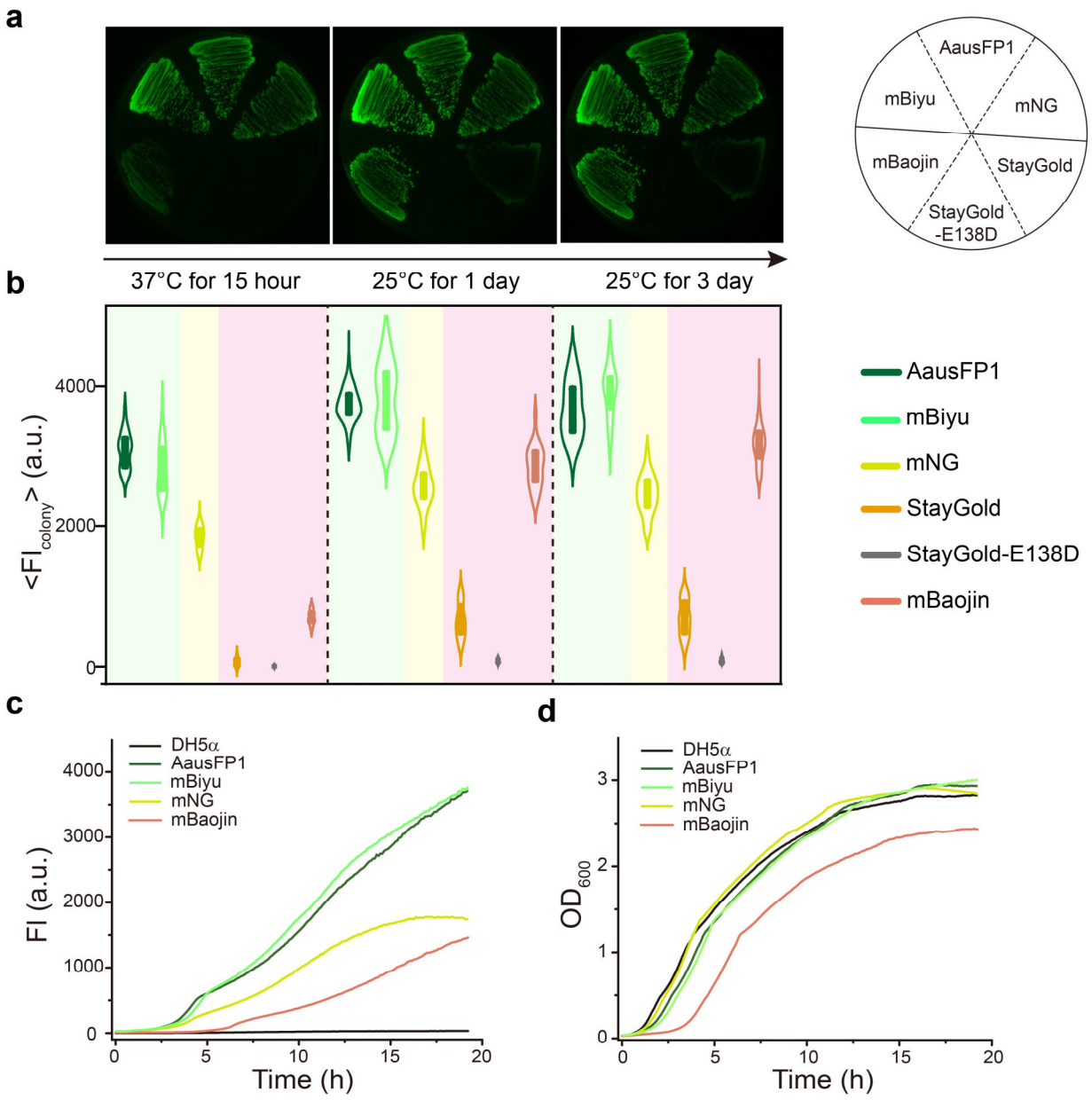


Figure S6 | Fast maturation of mBiyu in *E. coli* cells. (a) Representative plate images show green fluorescence of *E. coli* colonies express AausFP1, mNG, mBiyu, StayGold, mBaojin, and StayGold-E138D, positioned indicated on the right. The plate was incubated for 15 h at 37 ºC (left panel), then 1 d at 25 ºC (middle), and another 2 d at 25 ºC (right). **(b)** Colony FIs in **(a)** are quantified. AausFP1, mNG, and StayGold scaffold are indicated by green, yellow, and pink shade, respectively. The box indicates percentile 25 and 75. **(c)** FI and **(d)** OD_{600} of DH5α cell cultures. The cells express no FP, AausFP1, mBiyu, mNG, and mBaojin. mNG and mBaojin showed lower fluorescence and mBaojin slightly inhibited the cell growth.

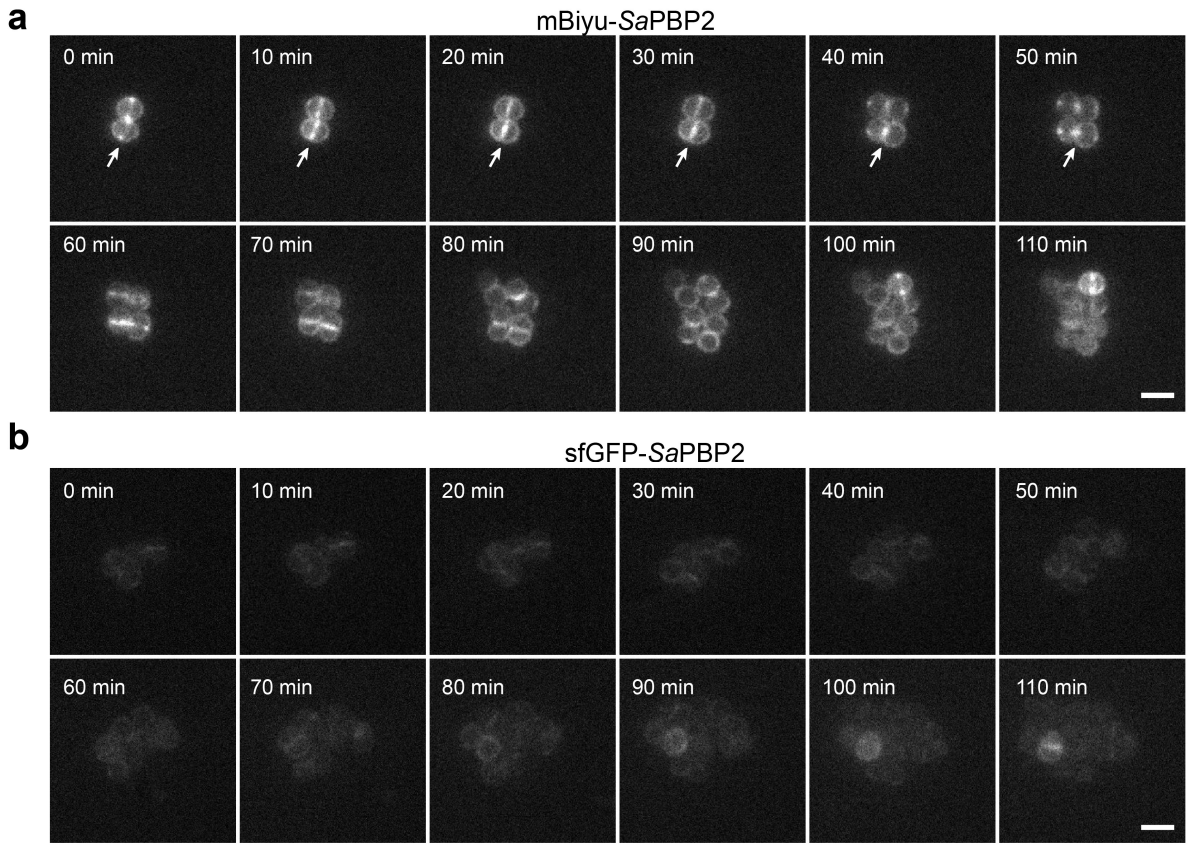


Figure S7 | Representative time-lapse images of *S. aureus* PBP2. (a) Representative time-lapse images of *S. aureus* cells expressing mBiyu-*Sa*PBP2. Cells are imaged average 10 min. The distribution of *Sa*PBP2 at the septum during cell division are labeled by white arrows. Scale bar, 2 μm. **(b)** Representative time-lapse images of *S. aureus* cells expressing sfGFP-*Sa*PBP2. Images were acquired every 10 min, and the contrast was set as in **(a)**, demonstrating dim signal of sfGFP-*Sa*PBP2. Scale bar, 2 μm.

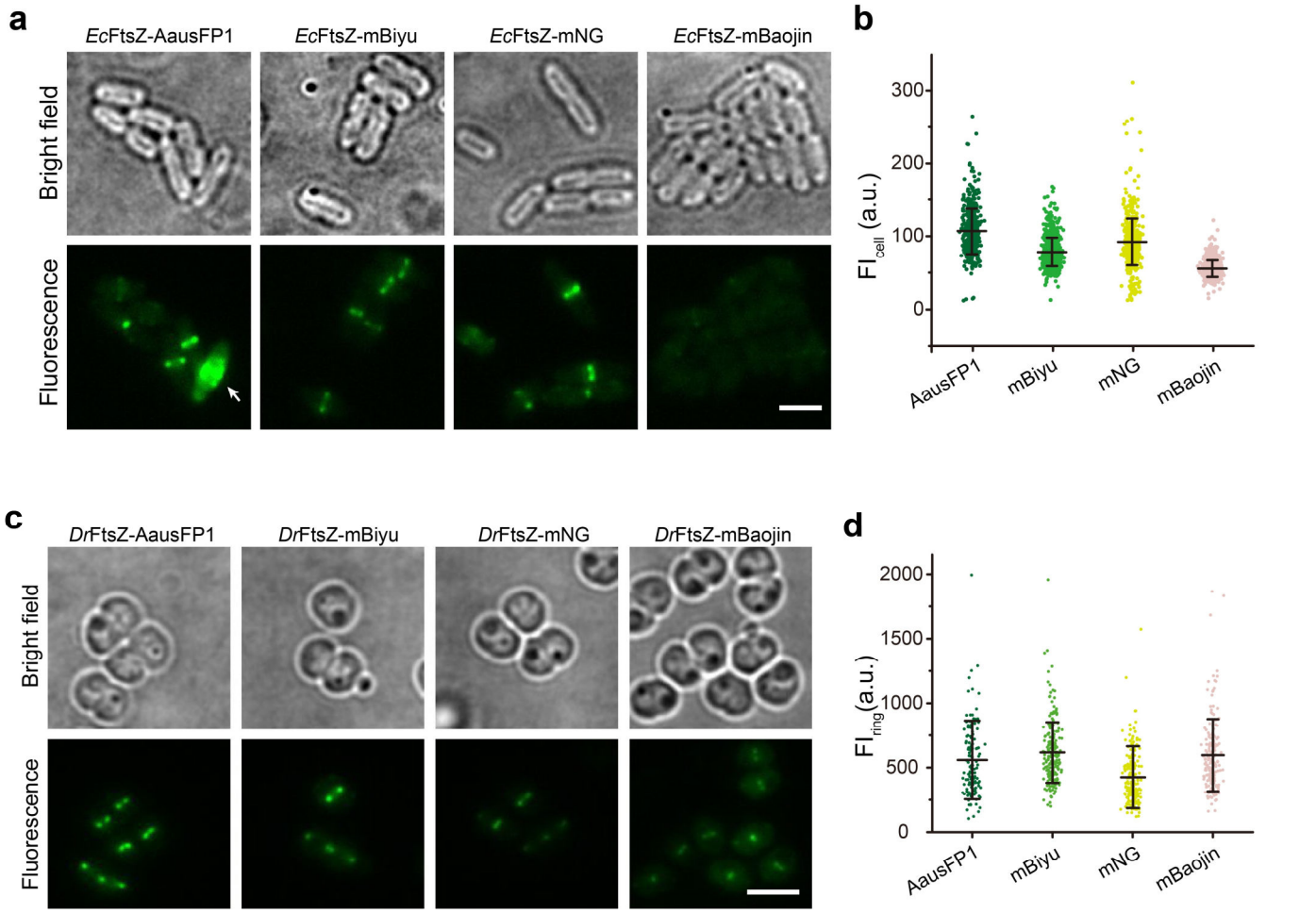
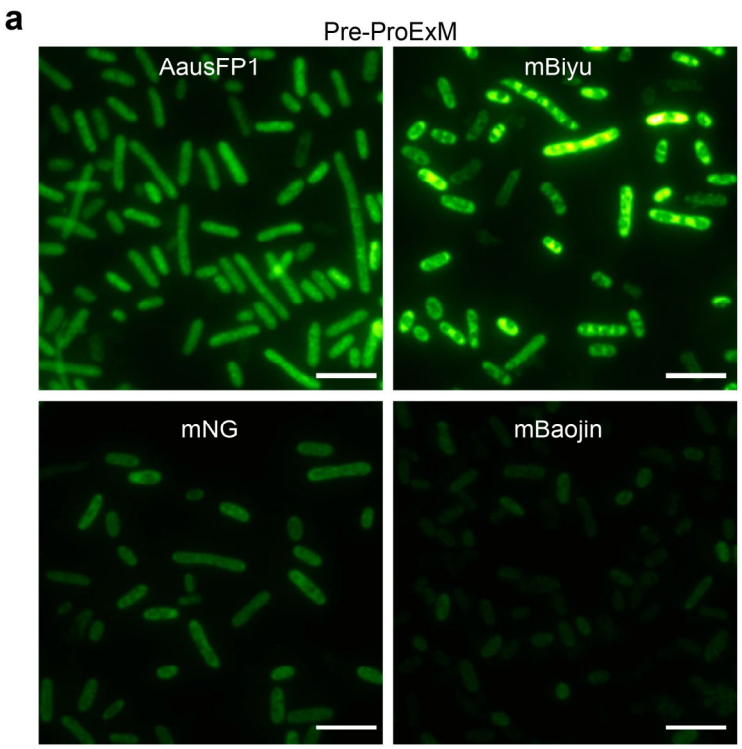
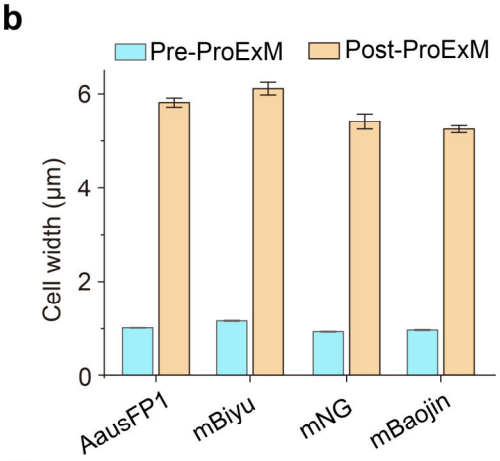


Figure S8 | mBiyu fusions in other bacterial cells. (a) Representative bright-field (top) and fluorescence (bottom) images of *E. coli* cells expressing *Ec*FtsZ-FP fusions. The contrast of fluorescence images is set to be same, demonstrating dim signal of *Ec*FtsZ-mBaojin. Arrow indicates abnormal Z-rings. **(b)** Quantification of single-cell FI from **a** (n = 495, 847, 554, 804 cells for AausFP1, mBiyu, mNG, and mBaojin). **(c)** Representative bright-field (top) and fluorescence (bottom) images of *D. radiodurans* cells expressing *Dr*FtsZ-FP fusions. **(d)** Quantification of Z-ring FI from **c** (n = 144, 224, 200, 177 cells for AausFP1, mBiyu, mNG, and mBaojin, respectively). **a** and **c**, scale bars, 2 μm. Data in **b** and **d** are presented as mean (lines) ± s.d. (whiskers).





c

Protein	Expansion fold	N_{cell}(pre)	N_{cell}(post)	FI % (pre)	FI % (post)
AausFP1	5.71 ± 0.08	1386	217	100	100
mBiyu	5.25 ± 0.11	1299	212	132.6 ± 2.5	84.0 ± 2.6
mNG	5.77 ± 0.15	807	136	49.3± 0.8	17.2 ± 0.7
mBaojin	5.40 ± 0.08	799	217	20.8 ± 0.5	19.3 ± 0.7

Figure S9 | mBiyu retains high fluorescence in bacterial expansion microscopy. (a) Representative images of *E. coli* cells expressing AausFP1, mBiyu, mNG, and mBaojin before ProExM process; the contrast of images is set to be same. Scale bars, 5 μm. **(b)** Average cell widths before and after expansion, data from three independent replica (mean ± 1.5 s.e.m.). **(c)** Quantification of expansion fold from **(b)** and single-cell fluorescence intensity from **Fig 6a**, normalized to AausFP1.

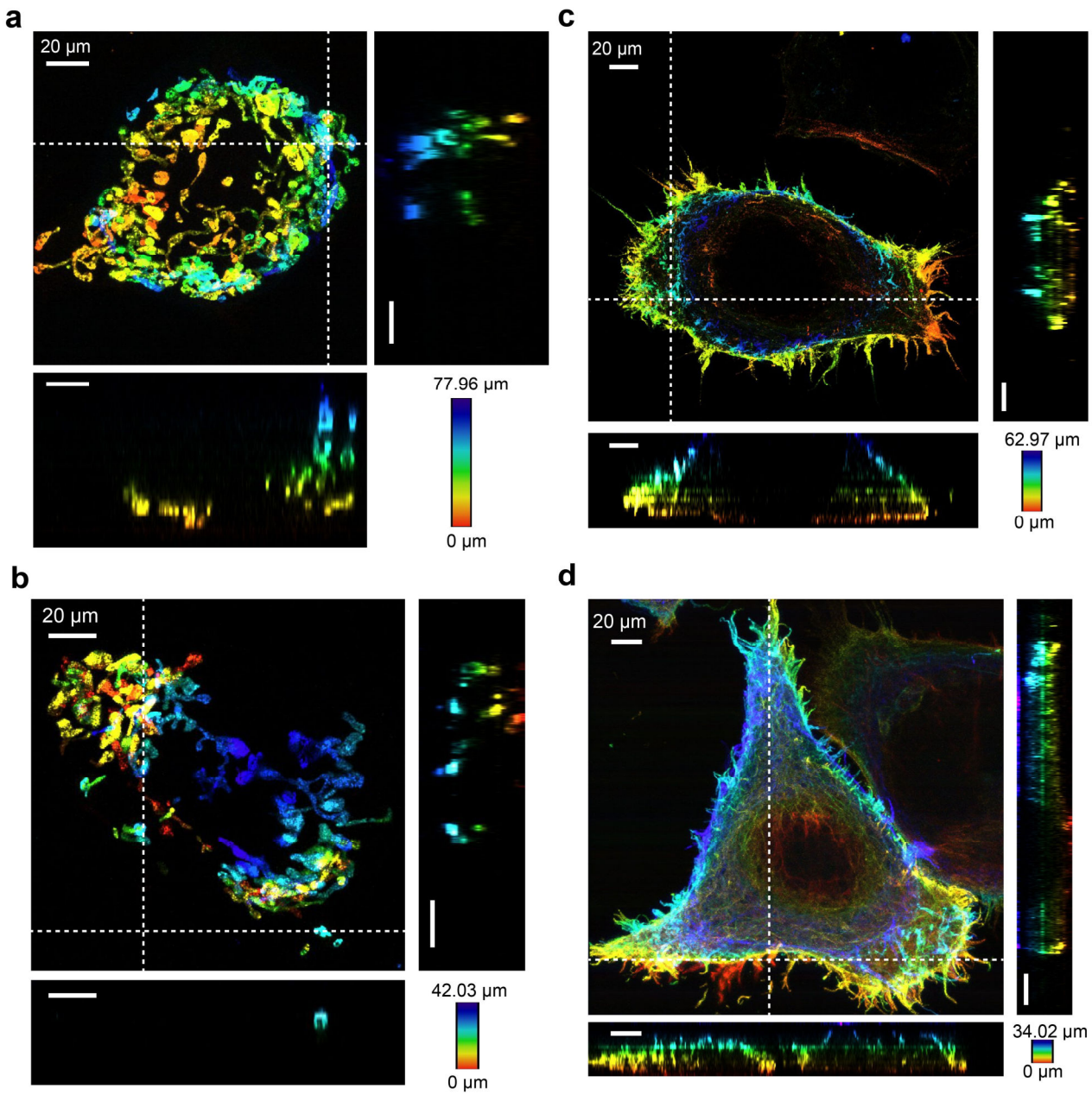


Figure S10 | Additional ProExM images of HeLa cells expressing COX8 (a, b) or LifeAct (c, d) fused mBiyu. Scale bars and color bars are indicated in each image.

Supplementary Table 1: Interface interactions at 4.0 Å and below between the Chain A and B of AausFP1 (PDB: 6S67).

Residue name (Atom name)	Residue name (Atom name)	Distance (Å)[a]
C147 (SG)	C147 (SG)	3.8
Y149 (OH)	N164 (O)	2.9
Y149 (OH)	H146 (CD2)	3.7
Y149 (CD2)	V166 (CG2)	3.8
Y196 (OH)	V166 (CG2)	3.9
Y196 (OH)	P145 (O)	2.8
Q174 (O)	K160 (NZ)	3.3
I176 (CG1)	Y162 (CZ)	3.7
N164 (ND2)	N164 (ND2)	3.6
H198 (NE2)	P145 (O)	3.9
Q174 (NE2)	Y196 (CB)	4.0
A232 (OXT)	R223 (NH1)	3.0
A232 (CA)	H202 (CD2)	3.7

[a] Distances measured in PyMOL Version 4.6.

Supplementary Table 2: Photophysical properties.

FP	Abs_{max} (nm)	Ex_{max} (nm)	Em_{max} (nm)	QY	EC ($M^{-1}cm^{-1}$)	Molecular brightness	pK_a[c]	t_{50}[d] (min)
mBiyu	498	498	511	0.99±0.03[a]	103128 ± 9998[a]	102±13	4.6±0.04	9.4±0.5
AausFP1	502	502	509	0.97[b]	170000[b]	165[b]	4.8±0.10	5.5±0.3
mNG	505	504	517	0.8[b]	116000[b]	93[b]	5.3±0.08	9.7±0.7

[a] Quantum yield (QY) and extinction coefficient (EC) measured in this study, referring to fluorescein in 0.1 M NaOH, for which QY = 0.95 with excitation at 460 nm (Methods);
[b] Data from FPbase.org[1];
[c] *pKa* measured in presence of 150 mM NaCl (Methods);
[d] Maturation half-time (t_{50}) measured using a translation-arrest assay[2].

Supplementary Table 3: Strains and plasmids.

Strain	**Genotype**	**Reference/source**
E. coli DH5α	supE44, Δ lacU(Φ80lacZ1 M15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1	Laboratory stock
E. coli BW25113	Δ *(araD-araB)567*, Δ *lacZ4787(::rrnB-3)*, *rph-1*, Δ *(rhaD-rhaB)568*, *hsdR514*	Laboratory stock
S. aureus RN4220	Restriction-deficient derivative of NCTC8325-4	Laboratory stock
M. smegmatis	Electroporation-proficient mutant of *M. smegmatis* mc^{2}6	Laboratory stock
D. radiodurans R1	*Deinococcus radiodurans* wild-type ATCC 13939	Laboratory stock
Plasmid	**Genotype**	**Reference/source**
pNCSY	p15A, *bla*, $P_{T7\text{-}Syn}$::*aausfp1*	This study
pYD028	p15A, *bla*, $P_{LtetO\text{-}1}$::*EcftsW-mng*	Laboratory stock, derived from pZH509[3]
pXY027	ColE1, *cat*, *LacI*, P_{Lac}::*EcftsZ-gfp*	[4]
pCN5	ColE1, pT181cop-wt repC (*S. aureus*), *bla* (*E. coli*), *cat* (*S. aureus*), *LacI*, Pspac::	Laboratory stock
pLY528	ColE1 (*E. coli*), OriM (*Mycobacteria*), *hyg (E. coli* and *Mycobacterium*), P_{TetRO}::	Laboratory stock
pJWK	ColE1, DrOri (*D. radiodurans*), *aph*, *repU*, AT-rich box*, $P_{LtetO\text{-}1}$::	[5]
pJW430	pJWK, PLtetO-1::*DrftsZ-mcherry*	Laboratory stock
pXY421	p15A, *cat*, P_{tac}:: *murJ-sgRNA*	[6]
pYY197	pNCSY, P_{T7}::*mng*	This study
pYY270	pNCSY, P_{T7}::*mbaojin*	This study
pYY408	pNCSY, P_{T7}::*mbiyu*	This study
pYY260	pZH509, $P_{LtetO\text{-}1}$::*EcftsZ-mbaojin*	This study
pYY298	pZH509, $P_{LtetO\text{-}1}$::*EcftsZ-mng*	This study
pYY299	pZH509, $P_{LtetO\text{-}1}$::*EcftsZ-aausfp1*	This study
pYY414	pZH509, $P_{LtetO\text{-}1}$::*EcftsZ-mbiyu*	This study
pYY391	pZH509, $P_{LtetO\text{-}1}$::*clpP-aausfp1*	This study
pYY392	pZH509, $P_{LtetO\text{-}1}$::*clpP-mng*	This study
pYY393	pZH509, $P_{LtetO\text{-}1}$::*clpP-mbaojin*	This study
pYY415	pZH509, $P_{LtetO\text{-}1}$::*clpP-mbiyu*	This study
pYY451	pCN5, Pspac::*mbaojin-Sapbp2*	This study
pYY452	pCN5, Pspac::*mbiyu-Sapbp2*	This study
pSJ197	pCN5, Pspac::*sfgfp-Sapbp2*	Laboratory stock
pWH406	pCN5, Pspac::*mng-Sapbp2*	Laboratory stock
pLX177	pLY528, P_{TetRO}::*MssepF-mbaojin*	This study
pLX178	pLY528, P_{TetRO}:: *MssepF -mbiyu*	This study
pLX179	pLY528, P_{TetRO}:: *MssepF -mng*	This study
pLX190	pLY528, P_{TetRO}:: *MssepF-aausfp1*	This study
pYY457	pJWK, $P_{LtetO\text{-}1}$::*DrftsZ-aausfp1*	This study
pYY458	pJWK, $P_{LtetO\text{-}1}$::*DrftsZ-mng*	This study
pYY459	pJWK, $P_{LtetO\text{-}1}$::*DrftsZ-mbiyu*	This study
pYY460	pJWK, $P_{LtetO\text{-}1}$::*DrftsZ-mbaojin*	This study
pYY443	pN1, *aph*, P_{CMV}::*CytERM-aausfp1*	This study
pYY444	pN1, *aph*, P_{CMV}::*CytERM-mng*	This study
pYY445	pN1, *aph*, P_{CMV}::*CytERM-egfp*	This study
pYY446	pN1, *aph*, P_{CMV}::*CytERM-mbiyu*	This study
pYY482	pcDNA, *bla*, P_{CMV}::*mbiyu-h2b*	This study
pYY485	pcDNA, *bla*, P_{CMV}::*aaus-h2b*	This study
pYY489	pcDNA, *bla*, P_{CMV}::*mbaojin-tubulin*	WikiGene #0000260[7]
pYY490	pN1, *aph*, P_{CMV}::*emtb-mbaojin*	WikiGene #0000268[7]
pYY491	pAAV, *bla*, P_{CAG}::*dmito2-mbaojin*	WikiGene #0000269[7]
pYY492	pN1, *aph*, P_{CMV}::*mbaojin-actin*	WikiGene #0000303[7]
pYY493	pN1, *aph*, P_{CMV}::*keratin-mbaojin*	WikiGene #0000737[7]
pYY494	pcDNA, *bla*, P_{CMV}::*mbiyu-tubulin*	This study
pYY495	pN1, *aph*, P_{CMV}::*emtb-mbiyu*	This study
pYY496	pAAV, *bla*, P_{CAG}::*dmito2-mbiyu*	This study
pYY497	pN1, *aph*, P_{CMV}::*mbiyu-actin*	This study
pYY498	pN1, *aph*, P_{CMV}::*keratin-mbiyu*	This study

Supplementary Table 4: DNA oligos.

Primer	Sequence	Purpose
1	GAAGATCCTTTGATCTTTTCTACG	Amplification of vector backbone for pYY196
2	AACGCCAGCAACGCGGC	
3	AGATCAAAGGATCTTCTTGAGATCGTTTTGGTCTGCG	Amplification of p15A for pYY196
4	GGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCC	
5	CATACCCTGGAAGTACAGGTTTT	Amplification of vector backbone for pYY197 and pYY270
6	TGACGTTTGATCCGGCTGCT	
7	TACTTCCAGGGTATGGTGAGCAAAGGCGAAGAAGATAA	Amplification of *mng* for pYY197
8	CCGGATCAAACGTCATTTATACAGTTCATCCATGCCCAT	
9	CTGTACTTCCAGGGTATGGTCTCAAAGGGAGAGGAGGA	Amplification of *mbaojin* for pYY270
10	CCGGATCAAACGTCACTTATACAGCTCGTCCATGCC	
11	GTGAGCAAAGGCGAAGAAGATAAC	Amplification of vector backbone and *mng* for pYY298
12	GCGGTACCTTTCTCCTCTTTAATG	
13	GAGGAGAAAGGTACCGCATGTTTGAACCAATGGAACTTAC	Amplification of *ftsZ* for pYY298
14	TTCGCCTTTGCTCACCCTTCGAATGTGTATCTTGCCTATTCTGCCATCAGCTTGCTTACGC	
15	GGGAGCAGGGGCCGGGCTCCCTCCTCCACCATCAGCTTGCTTACGCAGGAAt	Swapping *linke5* with *linker0* for pYY298
16	CCGGCCCCTGCTCCCGGAGGCGGGGGCAGCGTGAGCAAAGGCGAAGAAGATAA	
17	CGCCATGTTATCTTCTTCGCCTTTGC	Amplification of vector backbone for pYY299
18	GGCATGGATGAACTGTATAAATAATC	
19	AGAAGATAACATGGCGAGTTACGGAGCACTTTTGTTCAG	Amplification of *aausfp1* for pYY299
20	ACAGTTCATCCATGCCTGCATATGCGGTTTTGCAATCG	
21	AGAAGATAACATGGCGGTCTCAAAGGGAGAGGAGGAAAACAT	Amplification of *mbaojin* for pYY260
22	CCTCCTGCTAGATTACTTATACAGCTCGTCCATGCC	
23	CAGTTCATCCATGCCTGCATATGCGGTTTTGCAATCGA	Amplification of *mbiyu* for pYY414
24	GCGGTACCTTTCTCCTCTTTA	Amplification of pZH509 backbone
25	AGCGGTGGAGGAGGGAGTTACGGAGCACTTTTGTTCA	Amplification of *aausfp1* for pYY391
26	AGCGGTGGAGGAGGGGTGAGCAAAGGCGAAGAAGATAACATGG	Amplification of *mng* and *mbiyu* for pYY392 and pYY415
27	AGCGGTGGAGGAGGGGTCTCAAAGGGAGAGGAGGAAAACAT	Amplification of *mbaojin* for pYY393
28	GGAGAAAGGTACCGCATGTCATACAGCGGCGAACG	Amplification of *clpP*
29	CCCTCCTCCACCGCTATTACGATGGGTCAGAATCGAATCGAC	
30	GCTACCGCCACCGCCCGG	Amplification of pJWK vector
31	TAATCTAGCGGGCAAAACCCAG	
32	CCGGGCGGTGGCGGTAGCAGTTACGGAGCACTTTTGTTCA	Amplification of *aausfp1* for pYY457
33	GTTTTGCCCGCTAGATTATGCATATGCGGTTTTGCAATCG	
34	CCGGGCGGTGGCGGTAGCGTGAGCAAAGGCGAAGAAGATAA	Amplification of *mng* for pYY458
35	GTTTTGCCCGCTAGATTATTTATACAGTTCATCCATGCCCAT	
36	CCGGGCGGTGGCGGTAGCGTGAGCAAAGGCGAAGAAGATA	Amplification of *mbiyu* for pYY459
37	GTTTTGCCCGCTAGATTATTTATACAGTTCATCCATGCCTG	
38	CCGGGCGGTGGCGGTAGCGTCTCAAAGGGAGAGGAGGAA	Amplification of *mbaojin* for pYY460
39	GTTTTGCCCGCTAGATTACTTATACAGCTCGTCCATGCC	
40	TAAAGCGGCCGCGACTCTAGA	Amplification of p*CytERM* vector
41	GGTGGCGACCGGTGGATC	
42	ATCCACCGGTCGCCACCATGAGCTACGGCGCTCTGCT	Amplification of *aausfp1* for pYY443
43	TCTAGAGTCGCGGCCGCTTTAGGCGTAGGCGGTCTTGCAG	
44	ATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGA	Amplification of *mng* for pYY444
45	TCTAGAGTCGCGGCCGCTTTACTTGTACAGCTCGTCCATGCC	
46	ATCCACCGGTCGCCACCATGAGCAAGGGCGAGGAGCT	Amplification of *egfp* for pYY445
47	TCTAGAGTCGCGGCCGCTTTACTTGTACAGCTCGTCCATGCC	
48	ATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGA	Amplification of *mbiyu* for pYY446
49	TCTAGAGTCGCGGCCGCTTTACTTGTACAGCTCATCCATGCCG	
50	GGATCCATGCCTGAACCTACCA	Amplification of pcDNA-*h2b*
51	GGTGGCGCTAGCCAGCTTG	
52	CTGGCTAGCGCCACCATGGTGAGCAAGGGCGAGGA	Amplification of *mbiyu* for pYY482
53	TTCAGGCATGGATCCCTTGTACAGCTCATCCATGCCG	
54	CTGGCTAGCGCCACCATGAGCTACGGC	Amplification of *aausfp1* for pYY485
55	TTCAGGCATGGATCCGGCGTAGGCGGTCTTGCAG	
56	GGAGGTGGTGGATCAGGAGGAGGT	Amplification of pcDNA vector for pYY494
57	GGTGGCGGTACCAAGCTTGGGT	
58	AAGCTTGGTACCGCCACCATGGTGAGCAAG	Amplification of *mbiyu* for pYY494
59	TGATCCACCACCTCCCTTGTACAGCTCATCCATGCC	
60	TAGGCGGCCGCGACTCT	Amplification of pN1 vector for pYY495
61	CGAAGAGCCCTCAGGTGG	
62	CCTGAGGGCTCTTCGGATCCACCGGTCGCCA	Amplification of *mbiyu* for pYY495

63	AGTCGCGGCCGCCTACTTGTACAGCTCATCCATGCCG	
64	TAGGAATTCGATATCAAGCTTATCGA	Amplification of pAAV vector for pYY496
65	CTCGAGGCTAGCAGATCTAGC	
66	TCTGCTAGCCTCGAGATGGTGAGCAAGGGCGAGG	Amplification of *mbiyu* for pYY496
67	GATATCGAATTCCTACTTGTACAGCTCATCCATGC	
68	TCCGGACTCAGATCTGGCAG	Amplification of pN1 vector for pYY497
69	GGTGGCGACCGGTAGCG	
70	GCTACCGGTCGCCACCATGGTGAGCAAG	Amplification of *mbiyu* for pYY497
71	AGATCTGAGTCCGGACTTGTACAGCTCATCCATGCC	
72	TAAGATATCCAGCACAGTGGC	Amplification of pN1 vector for pYY498
73	GGGTACCGTCGACTGCAG	
74	CAGTCGACGGTACCCATGGTGAGCAAGGGCGAGG	Amplification of *mbiyu* for pYY498
75	CACTGTGCTGGATATCTTACTTGTACAGCTCATCCA	
76	ACCCTCATCGTATCTATGTTCTTCCA	Site-mutagenesis of C147R
77	ACATAGATACGATGAGGGTTGTAGTT	
78	ATTGCATCCGTGTTCTTCCAGATGTA	Site-mutagenesis of Y149R
79	GAAGAACACGGATGCAATGAGGGTT	
80	TGGAATCGCGTGCTACATCAACATTGTTCATGACG	Site-mutagenesis of K160A
81	TGTAGCACGCGATTCCATTATTTTGTACATCTGGAAG	
82	CAACATTCGTCATGACGTAATTGGAGGAGGACA	Site-mutagenesis of V166R
83	CGTCATGACGAATGTTGATGTAGCATTTGATTCCA	
84	TTCCACACCGTCATCATATACAAGCG	Site-mutagenesis of Y196R
85	ATATGATGACGGTGTGGAATGTCGAC	
86	CTACCATGCGATACAAGCGCACACAATACTTTCTAA	Site-mutagenesis of H198A
87	CTTGTATCGCATGGTAGTGTGGAATGTCGACTGG	
88	TGTTCGCGGCTATCGATTGCAAAACCGCATAT	Site-mutagenesis of R223A
89	ATCGATAGCCGCGAACACCTCTACTACGTTCATATGATCTC	
90	ATTGCGCGACCGCATATGCATGACGTTTGATC	Site-mutagenesis of K228A
91	ATATGCGGTCGCGCAATCGATAGCTCTGAACACCT	
92	AAACCTGTACTTCCAGGGTATG	Error-prone PCR of random mutagenesis of *aausfp1*
93	AGCCGGATCAAACGTCATGC	
94	ACAAATACGTGCTGCGCACCAACAA	Site-mutagenesis of I176R
95	TGCGCAGCACGTATTTGTCCTCCT	
96	CTTCAACNNKCATCGCATCTATGTTCTTCCAGAT	Saturation mutagenesis of P145
97	GTTGAAGTTAAATTCCAATTTTTTGCC	
98	CAGCCATNNKATCTATGTTCTTCCAGATGTA	Saturation mutagenesis of C147
99	TTCCATTATTTTCTACATCTGGAAGAACATAG	
100	CGACGGCNNKTATAAGACACGCGC	Saturation mutagenesis of V103
101	GTACGTGACTTCAGCGCGTGTCTTATA	
102	ACGAGCTNNKCACCAACAATTAAATA	Saturation mutagenesis of A178
103	AGCTCGTATTTGTCCTCCTCCA	
104	GCGATACAANNKCACACAATACTTT	Saturation mutagenesis of A201
105	TTGTATCGCATGGTAGTGTGGAA	
106	TTTAACTTCAACGCTNNKCGCNNKTATGTTCTTCCAGAT	Saturation mutagenesis of H146 and I148
107	AGCGTTGAAGTTAAATTCCAATTTTTTG	
108	CGGGTAGCGGTGCGGGTGCGGGTAGCATGGTCTCAAAGGGAGAGGAGGAA	Amplification of *mbaojin* for pLX177
109	AAGAATTCGAGCTCGGTACCTCACTTATACAGCTCGTCCATGCCC	
110	CGGGTAGCGGTGCGGGTGCGGGTAGCATGGTGAGCAAAGGCGAAGAAGAT	Amplification of *mbiyu* for pLX178
111	AAGAATTCGAGCTCGGTACCTTTATACAGTTCATCCATGCCTGCA	
112	CGGGTAGCGGTGCGGGTGCGGGTAGCATGGTGAGCAAAGGCGAAGAAGAT	Amplification of *mng* for pLX179
113	TAATTAAGAATTCGAGCTCGGTACCTCATTTATACAGTTCATCCATGCCCATCAC	
114	GTGCGGGTAGCGGTGCGGGTGCGGGTAGCAGTTACGGAGCACTTTTGTTC	Amplification of *aausfp1* for pLX190
115	AAGAATTCGAGCTCGGTACCTCATGCATATGCGGTTTTGC	
116	GTGGAATCCTGACAGGATCCGGTCAGCAGAAGGGTCGCCCGATGA	Amplification of *MssepF*
117	CACCGCTACCCGCACCCGCACCACGGTAGGAGTAGAAGCCCGCCT	

Movie Captions

Supplementary Video 1

Fluorescence time-lapse video of mBiyu-PBP2 (*S. aureus* RN4220) cells growing on 1.5% agarose gel pad with tryptic soy broth (TSB) at 37 ℃, corresponding to Fig S7a. The video was recorded every 10 min. Scale bar, 2 μm.

Supplementary Video 2

Fluorescence time-lapse video of sfGFP-PBP2 (*S. aureus* RN4220) cells growing on 1.5% agarose gel pad with tryptic soy broth (TSB) at 37 ℃, corresponding to Fig S7b. The video was recorded every 10 min. Scale bar, 2 μm.

Supplementary Video 3

3D reconstruction the representative HeLa cell with mitochondrial labeled with mBiyu, corresponding to Fig 6c. Z stacks with 3 μm voxel size were reconstructed and animated in Imaris. Scale bar, 20 μm. Color bar is the same as Fig. 6c.

Supplementary Video 4

3D reconstruction the representative HeLa cell with F-actin labeled with LifeAct-mBiyu, corresponding to Fig 6d. Z stacks with 3 μm voxel size were reconstructed and animated in Imaris. Scale bar, 30 μm. Color bar is the same as Fig. 6d

References

1 Lambert, T. J. FPbase: a community-editable fluorescent protein database. *Nature Methods* **16**, 277-278 (2019).

2 Balleza, E., Kim, J. M. & Cluzel, P. Systematic characterization of maturation time of fluorescent proteins in living cells. *Nature Methods* **15**, 47-51 (2018).

3 Hensel, Z. A plasmid-based *Escherichia coli* gene expression system with cell-to-cell variation below the extrinsic noise limit. *PLoS One* **12**, e0187259 (2017).

4 Buss, J. *et al.* A multi-layered protein network stabilizes the *Escherichia coli* FtsZ-ring and modulates constriction dynamics. *PLoS Genet* **11**, e1005128 (2015).

5 Wang, J. *et al.* Class A PBPs reinforce the septal cell wall following initial synthesis by SEDS–bPBP pairs during bacterial cytokinesis. Preprint at https://biorxiv.org/content/10.1101 (2025).

6 Yang, X. *et al.* A two-track model for the spatiotemporal coordination of bacterial septal cell wall synthesis revealed by single-molecule imaging of FtsW. *Nature Microbiology* **6**, 584-593 (2021).

7 Zhang, H. *et al.* Bright and stable monomeric green fluorescent protein derived from StayGold. *Nat Methods* **21**, 657-665 (2024).