Supporting Information

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SI Materials and Methods

Arch-His_{6x} and Arch-CFP Nucleotide Sequences.

> Arch-His_{6x}

ATGGACCCGATAGCACTACAGGCGGGATACGACCTACTCGGGGACGGTCGCCCCGAGACGTTGTGGTTGGGTATCGGAACGTTACTAATGCTCATCGGGACCTTCTACTTCCTCGTCAG
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GCCATCCTCGGCGACACCGAGGCGCCGGAGCCCTCCGCGGGCGCCGACGTCTCCGCCGCGGACgcggccgcactcgagcaccaccaccaccaccactga

> Arch-CFP

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CCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCTAA

Analysis of Screening Data. Data from libraries were processed and analyzed using custom software in R. For the error-prone library, mutants with both significantly improved raw opsin fluorescence or normalized fluorescence over Arch(DETC) at neutral pH were restreaked for single colonies from the 200-μL preculture plates and rescreened in quadruplicate at pH 5, pH 7, and pH 9. The different pH buffers were used to assay the pH dependence of fluorescence of each variant. For site-saturation libraries, variants with both raw and normalized fluorescence >2 SDs above Arch (DETC) at neutral pH were rescreened in quadruplicate to confirm increased in vivo fluorescence followed by Sanger sequencing.

***Escherichia coli* Imaging.** Following ∼4 h of protein expression, *E. coli* were immobilized between an agar pad [1.5% (wt/vol) low-melt agar supplemented with Dulbecco's PBS (Invitrogen)] and a glass-bottom imaging dish (HBSt-5040; WillCo) (1). Images were obtained using a Nikon Ti-E inverted microscope equipped with the Nikon Perfect Focus System (PFS) autofocus module. Microscope operation was controlled by the Molecular Devices commercial software (Metamorph 7.5.6.0) driving a ZYLA sCMOS camera (Andor), motorized stage (ASI instruments), brightfield shutters (Sutter Instruments), and a SOLA Light Engine light source (Lumencor). Phase-contrast illumination was provided by a halogen bulb to allow verification of cell focus and cell shape. All experiments used a Nikon Phase 100× Plan Apo (N.A. 1.4) objective with additional magnification using a 1.5× optovar. Filter sets used were as follows: Chroma 31044v2 [cyan fluorescent protein (CFP)] and Semrock Cy5-4040C (opsin). The exposure time for brightfield, CFP, and opsin images was 309 ms, 1 s, and 6 s, respectively. Fluorescent images were background-subtracted with rolling ball radius of 50 pixels and false-colored in ImageJ.

Mammalian Cell Culture and Imaging. We replaced eYFP in the pLenti-CaMKIIa-eArch3.0-EYFP vector (35514; Addgene) with eGFP, to reduce cross-talk between the fluorescent tag and fluorescence of the opsin, and introduced the D95E and T99C mutations into Archaerhodopsin-3 (Arch). HEK 293FT cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FBS, 1% sodium bicarbonate, 1% sodium pyruvate, and penicillin-streptomycin. D95E/T99C mutant and wild-type Arch vectors were transfected with Fugene6 into HEK cells according to the

manufacturer's protocol, plated at a density of 5,000 cells per cm^2 onto 12-mm coverslips at 18 h posttransfection, and imaged at 48 h posttransfection. Imaging was performed on a Zeiss LSM 780 Confocal Microscope with a Zeiss W plan-Apochromat 63×/1.0 M27 objective. EGFP fluorescence was excited with 488 nm at 12.5 mW/mm^2 (3% of laser power) whereas Arch and Arch (DETC) fluorescence was excited at 633 nm at 250 mW/mm^2, respectively (100% of laser power). Fluorescence emission was imaged using the LSM 780's GaAsP detectors with a detection range of 496–535 for eGFP and 650–695 for Arch and Arch (DETC). Regions of interest were drawn around the membrane of each cell, for 12 cells, and total fluorescence of the membrane-localized opsin compared with eGFP was quantified and compared.

Quantum Yield Determination. Approximately 0.5 mg of Alexa Fluor 680 carboxylic acid, succinimidyl ester (part no. A-20008; Life Technologies) was resuspended in 2 mL of PBS (137 mM sodium chloride, 1.8 mM potassium dihydrogen phosphate, 10.2 mM disodium hydrogen phosphate, 2.7 mM potassium chloride, pH 7.4) and further diluted to enable accurate determination of the absorption and emission spectra. Both the dye standard Arch variants were diluted to an absorbance (A_{620}) of 0.04. The emission spectra of the dye and Arch variants were measured between 635 nm and 849 nm following excitation at 620 nm in a Tecan plate reader. Quantum yields of proteins (QY_P) were computed according to the formula $QY_P = QY_{DYE}(n_P/n_{DYE})^2(A_{DYE}/A_P)(I_P/I_{DYE})$, where Q_{DYE} equals 0.36. The refractive indices of PBS (n_{DYE}) and the buffer used for solubilizing proteins (n_P) were determined to be 1.3352 and 1.3357, respectively, using a refractometer (model no. 334620; Fisher Scientific); thus, $(n_P/n_{DYE})^2$ is ∼1. Because both the dye and proteins were diluted to A_{620} of 0.04, $A_{DYE}/A_P = 1$; thus, QY_P reduces to $0.36(I_P/I_{DYE})$, where I_{DYE} and I_P correspond to the integrated emission spectra of the dye and protein, respectively. Emission spectra were fit using third, fourth, and fifth order Gaussian models, the integrals of which were averaged to estimate I_{DYE} and I_P.

Estimation of Extinction Coefficients. We added 117 μL of 2.7 M hydroxylamine (pH 6.7) to 200 μL of purified protein in a 96-well plate (final concentration of 1 M hydroxylamine). Absorption spectra were monitored for Arch variants every 10 min between 300 nm and 800 nm. The extinction coefficients of Arch variants, ε_P, were calculated according to the formula, $\varepsilon_P = \varepsilon_R\langle\Delta A_P/\Delta A_R\rangle$, where ε_R is the extinction coefficient of *all-trans* retinal oxime, 51,600 $M^{-1}\cdot cm^{-1}$ (2), ΔA_P is the change in absorbance at the λ_{max} of Arch (which is different depending on the mutant) over time, and ΔA_R is the change in absorbance at 380 nm, from the formation of free retinal oxime, over time. $\langle\Delta A_P/\Delta A_R\rangle$ was averaged over the linear portion of the hydroxylamine reaction for each mutant.

Determination of Schiff-Base pK_a. Purified Arch variants [in 0.15% *n*-dodecyl-β-D-maltoside (DDM), 10 mM Tris, 200 mM NaCl, pH 6.5] were added to 2 mL of a six-buffer mix (pH 4.5) of 5 mM 3-(Cyclohexylamino)-1-propanesulfonic acid, 5 mM 2-(Cyclohexylamino) ethanesulfonic acid, 5 mM Bicine, 5 mM 3-(N-morpholino)propanesulfonic acid, 5 mM 2-(*N*-morpholino)ethanesulfonic acid, and 5 mM citric acid with 0.15% DDM and 200 mM NaCl. The six-buffer mix gives nearly constant buffering capacity from pH 2–11. To shift the pH, 3–4 μL of 1 M NaOH or 1 M HCl was added to 2.2 mL of protein-containing buffer in a 1 cm × 1 cm quartz cuvette. Absorption spectra were recorded using a Shimadzu UV-1601 spectrophotometer. The Schiff-base pK_a was determined using the Henderson–Hasselbalch equation.

Flow Cytometry. Five milliliters of *E. coli* containing opsin-CFP expression plasmids were induced for 4 h in the presence of 500 μM isopropyl β-D-1 thiogalactopyranoside (IPTG) and 10 μM retinal. Cells were harvested and resuspended in 180 mM $NaCl_2$ and 50 mM potassium phosphate buffer at pH 7. The opsin was excited with a 561-nm laser, and emission was detected with a 750-nm long-pass filter using a MACSQuant VYB flow cytometer (Miltenyi Biotec). Data were processed using custom MATLAB scripts (The MathWorks, Inc.), as described previously (3).

1. Young JW, et al. (2012) Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. *Nat Protoc* 7(1):80–88.
2. Trehan A, et al. (1990) On retention of chromophore configuration of rhodopsin isomers derived from three discis retinal isomers. *Bioorg Chem* 18:30–40.
3. McIsaac RS, Oakes BL, Botstein D, Noyes MB (2013) Rapid synthesis and screening of chemically activated transcription factors with GFP-based reporters. *J Vis Exp* (81):e51153.

GR	D121	W122	T125	V126	L129	M158	I159	G162	E166	G178	S181	T182	F185	W222	Y225	P226	Y229	D253	A256	K257
Arch	D95	W96	T99	T100	L103	M128	I129	G132	A136	W148	S151	T152	M155	W192	Y195	P196	W199	D222	A225	K226

Fig. S1. Structure-guided alignment of amino acid residues in the retinal binding pockets of Arch and *Gloeobacter violaceus* rhodopsin (GR).

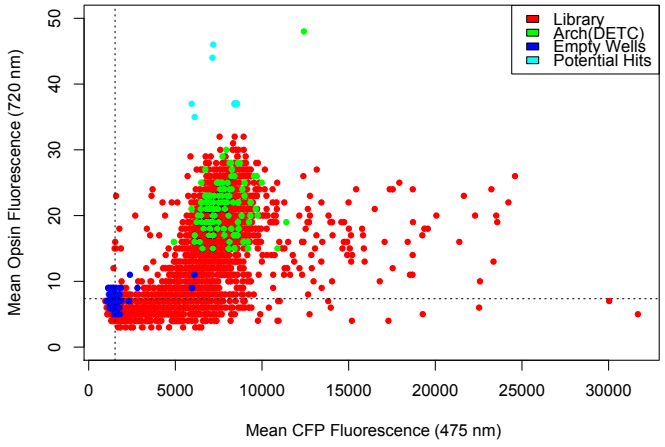

Fig. S2. Fluorescence results from error-prone PCR library of Arch(DETC).

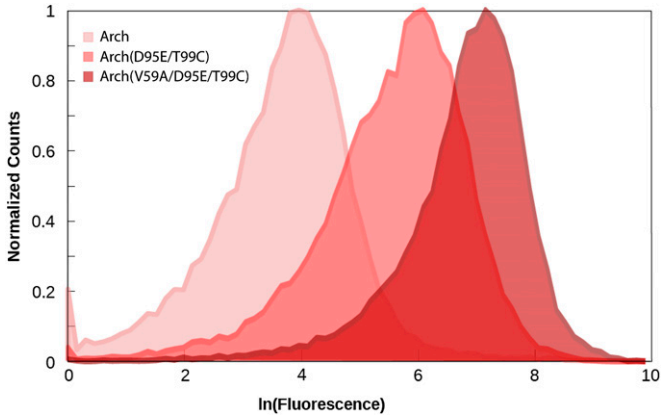

Fig. S3. Fluorescence of Arch, Arch(D95E/T99C), and Arch(V59A/D95E/T99C) measured in single *E. coli* cells using flow cytometry.

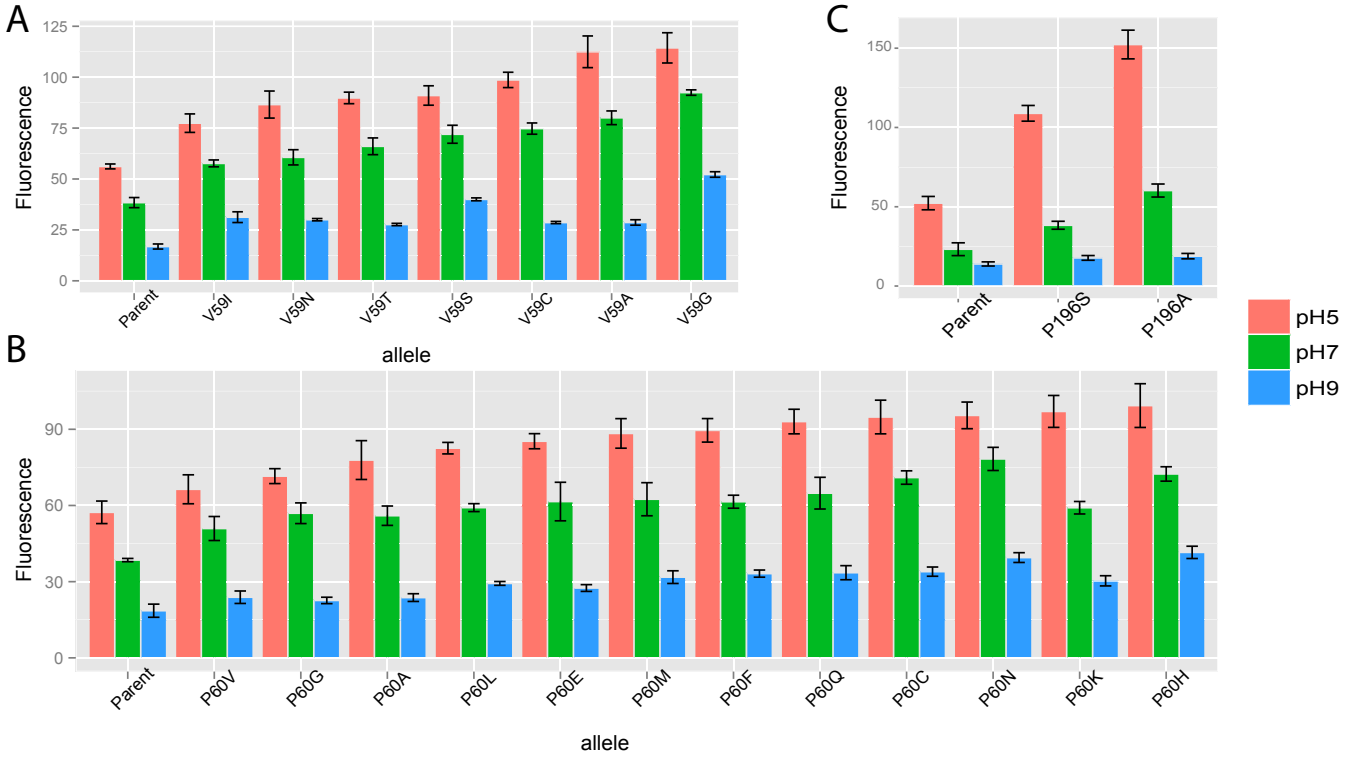

Fig. S4. Site-saturation mutants in Arch(DETC) at (*A*) V59, (*B*) P60, and (*C*) P196S rescreened for improved fluorescence at pH 5, pH 7, and pH 9.

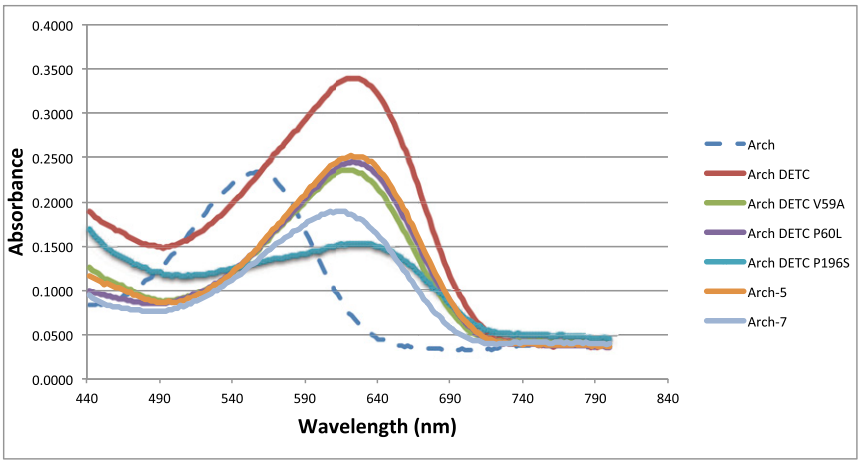

Fig. S5. Absorbance spectra of purified Arch variants in 10 mM Tris, 200 mM NaCl, and 0.15% DDM at pH 6.5.

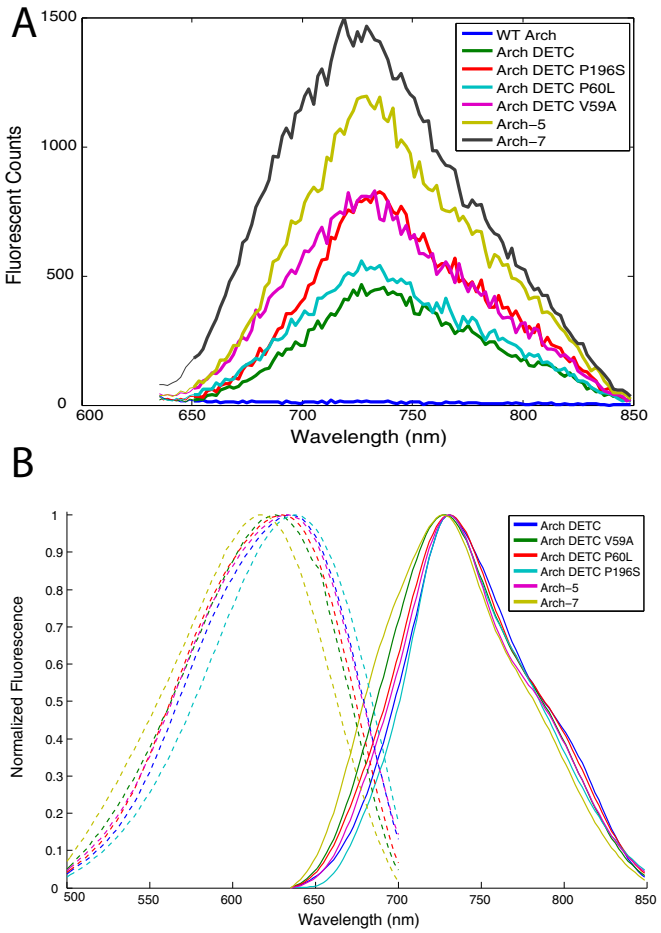

Fig. S6. Excitation/emission spectra of laboratory-evolved Arch variants. (*A*) Purified proteins were normalized to A_{620} = 0.04 and then excited at 620 nm. Emitted fluorescence was measured from 635 nm to 850 nm. (*B*) Normalized excitation/emission profiles for six Arch variants.

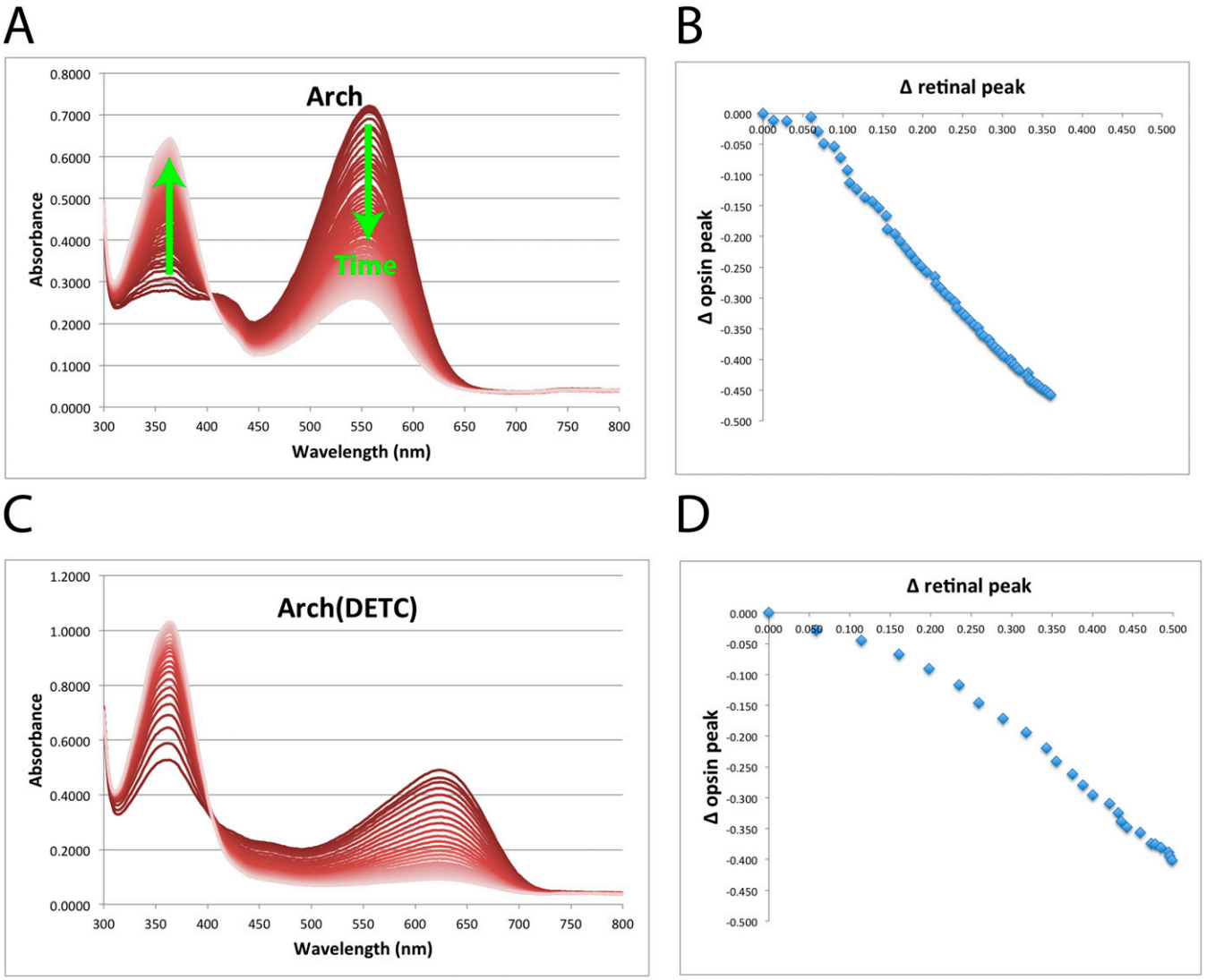

Fig. S7. Representative hydroxylamine-mediated bleaching reactions of retinal from Arch (*A* and *B*) and Arch(DETC) (*C* and *D*). Following addition of 1 M hydroxylamine to purified protein, absorbance spectra were measured every 10 min for up to 8 h. We calculated the change in peak heights at 360 nm and the rhodopsin maximum, which is mutant-dependent.

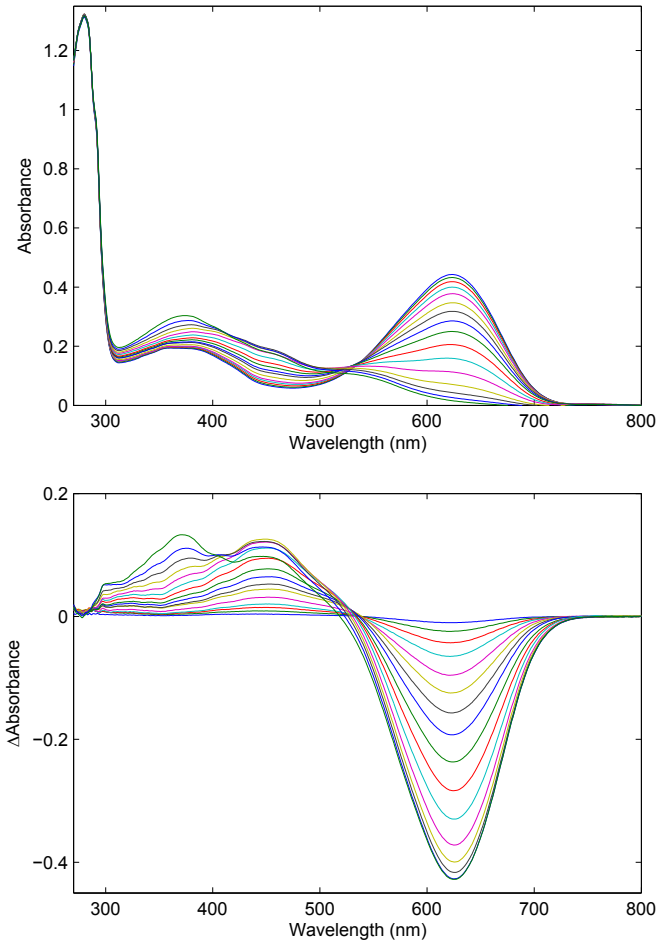

Fig. S8. pH titration of Arch(DETC). (*Upper*) Absorbance of Arch(DETC) at pH values ranging from 4.65 to 9.3. Note that, as pH increases, the pigment peak decreases, and absorbance in the UV (~360 nm to 380 nm) increases. (*Lower*) Difference spectra for Arch(DETC). Absorbance spectra at different pH values are subtracted from the absorbance spectrum at pH 4.65.

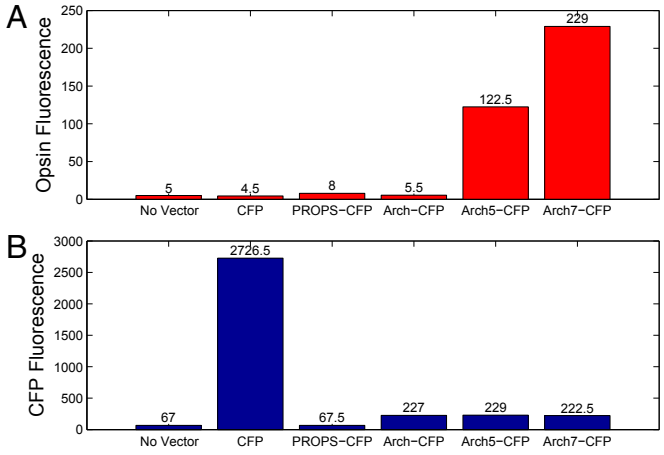

Fig. S9. Comparing the (*A*) opsin and (*B*) CFP fluorescence among PROPS-CFP, Arch-CFP, Arch5-CFP, and Arch7-CFP translational fusions. Protein expression was induced with 500 μM IPTG in the presence of 10 μM *all-trans* retinal. *E. coli* cells expressing only CFP or containing no vector were measured as controls. Fluorescence measurements were made using a Tecan plate reader, and the average of two technical replicates is indicated above each bar. (*A*) For measuring "Opsin Fluorescence," cells were excited at 620 nm, and the emission was measured at 720 nm. (*B*) For measuring "CFP Fluorescence," cells were excited at 425 nm, and the emission was measured at 475 nm.

Table S1. Summary of primers used in this manuscript

Primers No.	Name	Sequence (5′-3′)
Primers for cloning genes into expression plasmid		
1	Arch_pET21a_FW	TTTAAGAAGGAGATATACATATGGACCCGATAGCACTAC
2	Arch_pET21a_RV	GGTGGTGCTCGAGTGCGGCCGCGTCCGCGGCGG
Primers for cloning eCFP cotranslational fusion		
3	eCFP_FW	GCGGCCGCACTCGAGATGGTGAGCAAGGGC
4	eCFP_RV	TCAGTGGTGGTGGTGGTGGTGCTTAGGCGGCGGTC
Primers for Arch error-prone PCR and site-saturation mutagenesis		
5	Arch_error-prone_FW	GATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG
6	Arch_error-prone_RV	TTGCTCACCATCTCGAGTGCGGCCGC
7	V59A _NDT_FW	ACTACGCGGTCACGATCCTC**NDT**CCGGGGATCGC
8	V59A _VHG_FW	ACTACGCGGTCACGATCCTC**VHG**CCGGGGATCGC
9	V59A _TGG_FW	ACTACGCGGTCACGATCCTC**TGG**CCGGGGATCGC
10	V59A _RV	GAGGATCGTGACCGCGTAGTACTCGCGGGCG
11	P196S _NDT_FW	TGGTGCTCTGGACGGCCTAC**NDT**ATCCTGTGGATCATCG
12	P196S _VHG_FW	TGGTGCTCTGGACGGCCTAC**VHG**ATCCTGTGGATCATCG
13	P196S _TGG_FW	TGGTGCTCTGGACGGCCTAC**TGG**ATCCTGTGGATCATCG
14	P196S _RV	GTAGGCCGTCCAGAGCACCAGGACCAGCGCGG
15	P60L _NDT_FW	ACGCGGTCACGATCCTCGTG**NDT**GGGATCGCGTCG
16	P60L _VHG_FW	ACGCGGTCACGATCCTCGTG**VHG**GGGATCGCGTCG
17	P60L _TGG_FW	ACGCGGTCACGATCCTCGTG**TGG**GGGATCGCGTCG
18	P60L _RV	CACGAGGATCGTGACCGCGTAGTACTCGCGGGC
19	P60L_V59A_FW	ACGCGGTCACGATCCTC**GCGCTG**GGGATCGCGTCG
20	P60L_V59A_RV	**CGC**GAGGATCGTGACCGCGTAGTACTCGCGGGC
21	AR3-double-22c_FW1	CCCTCCTGTTCATGGTTCTC**NDT**GTGACG**NDT**AAGGTCGGC
22	AR3-double-22c_FW2	CCCTCCTGTTCATGGTTCTC**VHG**GTGACG**NDT**AAGGTCGGC
23	AR3-double-22c_FW3	CCCTCCTGTTCATGGTTCTC**TGG**GTGACG**NDT**AAGGTCGGC
24	AR3-double-22c_FW4	CCCTCCTGTTCATGGTTCTC**NDT**GTGACG**VHG**AAGGTCGGC
25	AR3-double-22c_FW5	CCCTCCTGTTCATGGTTCTC**VHG**GTGACG**VHG**AAGGTCGGC
26	AR3-double-22c_FW6	CCCTCCTGTTCATGGTTCTC**TGG**GTGACG**VHG**AAGGTCGGC
27	AR3-double-22c_FW7	CCCTCCTGTTCATGGTTCTC**NDT**GTGACG**TGG**AAGGTCGGC
28	AR3-double-22c_FW8	CCCTCCTGTTCATGGTTCTC**VHG**GTGACG**TGG**AAGGTCGGC
29	AR3-double-22c_FW9	CCCTCCTGTTCATGGTTCTC**TGG**GTGACG**TGG**AAGGTCGGC
30	AR3-double-22c_RV	GAGAACCATGAACAGGAGGGTCTCGATGCCGAGGC
Sequencing primers		
31	T7_forward	TAATACGACTCACTATAGGG
32	T7_reverse	GCTAGTTATTGCTCAGCGG

Sites that were targeted by site-saturation mutagenesis are shown in bold. Mixed-base codons are written according to the International Union of Pure and Applied Chemistry standard.