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Supporting information for

A single point mutation converts a proton-pumping rhodopsin into a red-shifted, turn-on fluorescent sensor for chloride

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Methods

General. All reagents and chemicals were purchased from Sigma-Aldrich, Thermo Fisher Scientific, or VWR and were used as received unless otherwise stated.

Design and cloning of plasmids, site-saturation mutagenesis library, and point mutations. The DNA sequence encoding the *Gloeobacter violaceus* rhodopsin (GR, UniProt ID: Q7NP59) was used as previously described but with T121.[1] This gene was synthesized and cloned into the pET-21a(+) vector between the NdeI and NotI restriction sites (GenScript, Figure S1A). For the GR T121-CFP construct, the DNA sequence encoding the cyan fluorescent protein from *Aequorea victoria* (CFP, UniProt ID: P42212) was used as previously described.[1] This gene was synthesized and cloned into the GR T121 pET-21a(+) construct between the NotI and XhoI restriction sites (GenScript, Figure S1B).

The site-saturation mutagenesis (SSM) library was generated using the GR T121-CFP construct as previously described.[2,3] The forward primers were 37 bases long with the degenerate bases NDT, VHG, or TGG at position 121, and the reverse primer was 34 bases long that contained 18 overlapping bases with the forward primer (Sigma-Aldrich, Table S1). A solution of the three forward primers were combined in a 12:9:1 ratio. The forward and reverse primers were each diluted to a final concentration of 10 µM with autoclaved water. The polymerase chain reaction (PCR) was carried out with the Phusion High Fidelity PCR Kit (New England Biolabs) according to the manufacturer's instructions with 1 µL of template plasmid from a 10 ng/µL stock solution, 1.5 µL of the forward and reverse primers from 10 µM stock solutions, 0.5 µL of dNTPs from a 10 mM stock solution, 0.5 µL of dimethyl sulfoxide (DMSO), 5 µL of Phusion GC buffer, 0.25 µL of Phusion DNA polymerase, and 9.75 µL of autoclaved water to a final volume of 25 µL. The PCR conditions are outlined in Table S2. After the PCR, the reaction mixture was treated with 1 µL of DpnI (New England Biolabs) for 2 h at 37 °C to remove the template DNA. The PCR product was then purified with agarose gel electrophoresis and extracted with the Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions. Ten microliters of the purified PCR product were ligated using the Gibson Assembly Master Mix (New England Biolabs) followed by cleanup with the DNA Clean & Concentrator Kit (Zymo Research) according to the manufacturers' instructions. One microliter of the resulting DNA was used to transform *E. cloni* EXPRESS BL21(DE3) Competent Cells (Lucigen) by electroporation (Bio-Rad Laboratories). The constructs for wild-type (*wt*) GR (D121) and the GR1 variant (V121) were prepared with the pET-21a(+) GR T121 construct. The forward primers were 37 bases long with the mutation site, and the reverse primer described above was used (Table S1). The PCR conditions and downstream processing steps were carried out as described above (Table S3).

Following electroporation, *E. cloni* were plated on Luria Broth (LB) agar plates containing 100 µg/mL ampicillin. To verify the sequences of the point mutations, colonies were picked into 5 mL of LB containing 100 µg/mL ampicillin in 14-mL culture tubes (Corning) and incubated overnight (New Brunswick Innova 42R Shaker) at 37 °C with shaking at 250 rpm. The following day, cells were collected by centrifugation at 3,000*g* for 5 min (Allegra X-14R, Beckman Coulter) and stored at -20 °C. The plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen) and sequenced to identify the correct clone (Eurofins Scientific).

SSM library expression, screening, and validation. The methods described here were adapted from a previously reported procedure.[1] Eighty-eight single colonies of *E. cloni* transformed with the SSM library were picked into 300 µL of LB containing 100 µg/mL ampicillin in a 96-well deep well plate (Greiner Bio-One) and sealed with an Easy App microporous film (USA Scientific). The 96-well deep well plate was incubated overnight at 37 °C with shaking at 230 rpm. The following day, each well of a 96-well deep well plate was filled with 950 µL of LB containing 100 µg/mL ampicillin and inoculated with 50 µL of the overnight culture using a Biomek NX^P liquid handler (Beckman Coulter). After 2 h and 15 min of incubation at 30 °C with shaking at 230 rpm, protein expression was induced with the addition of 50 µL of LB containing 100 µg/mL ampicillin, 105 mM isopropyl beta-D-thiogalactopyranoside (IPTG, Gold Biotechnology), and 2.1 mM all-*trans*-retinal (ATR, Sigma-Aldrich) to each well for a final concentration of 500 µM IPTG and 10 µM ATR. Protein expression was carried out at 30 °C with shaking at 230 rpm. After 4 h, the cells were harvested by centrifugation at 4,000*g* for 5 min (Allegra X-14R, Beckman Coulter). Using the liquid handler, the cell pellets were resuspended in 300 µL of 50 mM sodium acetate buffer at pH 5, 50 mM sodium phosphate buffer at pH 6, or 50 mM sodium phosphate buffer at pH 7, and 175 µL of the cell suspension was transferred to a clear 96-well microtiter plate (Caplugs) for plate reader measurements. Excitation for the rhodopsin was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 580–760 nm with a 20-nm bandwidth, a 10-nm step size, 25 flashes, and gain of 100 (Spark 20M, Tecan). Excitation for CFP was provided at 425 nm with a 10-nm bandwidth, and the emission was collected from 470–510 nm with a 20-nm bandwidth, a 10-nm step size, 25 flashes, and gain of 55. Following these emission scans, 25 µL of a 3.2 M sodium chloride stock solution in water was added to each well using a multichannel pipette (Eppendorf) for a final concentration of 400 mM sodium chloride. Then, the 96-well plate was shaken for 5 s and processed for plate reader measurements as described above.

Variants that showed an increase in fluorescence intensity in the presence of sodium chloride were streaked onto fresh LB agar plates containing 100 µg/mL ampicillin to isolate single colonies for rescreening. Eight single colonies of each variant were picked into 5 mL of LB containing 100 µg/mL ampicillin in 14-mL culture tubes and incubated overnight at 37 °C with shaking at 250 rpm. The following day, the overnight cultures were diluted 1:20 (v/v) into 3 mL of LB containing 100 µg/mL ampicillin in 14-mL culture tubes and incubated at 30 °C with shaking at 250 rpm. After 2.5 h, protein expression was induced with 15 µL of 100 mM IPTG and 15 µL of 2 mM ATR for a final concentration of 500 µM IPTG and 10 µM ATR and incubated at 30 °C for 4 h with shaking at 250 rpm. For a negative control, three single colonies of GR1-CFP were grown as described above but not induced (Figure S2). The cell pellets were collected by centrifugation at 2,500*g* for 5 min (5810 R, Eppendorf) and resuspended in 200 µL of 50 mM sodium acetate buffer at pH 5. For the plate reader rescreen, 25 µL of the cell suspension for each biological replicate was transferred to two separate wells of a 96-well microtiter plate containing 175 µL of 50 mM sodium acetate buffer at pH 5 or buffer containing 457 mM sodium chloride for a final concentration of 400 mM sodium chloride. Excitation scans were acquired to determine the optimal excitation wavelength for the rhodopsin emission at 710 nm with a 20-nm bandwidth. Excitation spectra were collected from 500–650 nm with a 10-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100 (Spark 10M, Tecan). Consistent with the SSM library screening methods, the rhodopsin was excited at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100. The CFP was excited at 390 nm with a 10-nm bandwidth, and the emission was collected from 425–560 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 55. The rhodopsin

and CFP emissions were integrated from 600–800 nm (F_{GR}) and 425–560 nm (F_{CFP}), respectively, using the *trapz* function in MATLAB R2017a (MathWorks). For each variant, the average emission response of eight biological replicates with standard error of the mean is reported (Figures S4, S6–S9).

Rhodopsin family sequence alignment. The amino acid sequence for *wt*GR (UniProt ID: Q7NP59, residues 45–270) was used to search the UniProt Knowledgebase for homologous proteins in the rhodopsin family using the HMMER webserver v2.41.1 (www.ebi.ac.uk/Tools/hmmer).[4,5] The expectation value (E) threshold was set to 0.01 to filter and obtain 6,906 protein sequences that are similar to *wt*GR. Next, we excluded protein sequences containing consecutive gaps, in the alignment, longer than 20% of the protein length (> 45 consecutive gaps) to reduce the noise from partial sequences. The resulting 3,285 protein sequences were aligned using the Clustal Omega software v1.2.4. The amino acid diversity at the counterion position in *wt*GR (D121) was analyzed and is presented in Figure S4C.

***wt*GR and GR1 chloride titrations in live *Escherichia coli*.** Three 3-mL cultures of *E. cloni* containing the *wt*GR and GR1 only constructs were expressed at 30 °C as described above. After 4 h, the expression cultures were collected by centrifugation at 3,000*g* for 5 min (Allegra X-14R, Beckman Coulter) and resuspended in 200 µL of 50 mM sodium acetate buffer at pH 5. In a 96-well plate, 175 µL of the cell suspension was diluted with 25 µL of 0 M or 3.2 M sodium chloride in water to a final concentration of 0 mM and 400 mM sodium chloride. Excitation was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–760 nm with a 20-nm bandwidth, a 5 nm-step size, 25 flashes and a gain of 100 (Spark 10M, Tecan). The average of three technical replicates with standard error of the mean is reported (Figure S10).

***wt*GR-CFP and GR1-CFP pH profiles in live *E. coli*.** Three single colonies of *E. cloni* containing the *wt*GR-CFP and GR1-CFP plasmids were picked into 5 mL of LB containing 100 µg/mL ampicillin and incubated overnight at 37 °C as described above. The next day, the overnight cultures were diluted 1:20 (v/v) into 25 mL of LB containing 100 µg/mL ampicillin in 125 mL baffled flasks and incubated at 30 °C with shaking at 250 rpm. After 2.5 h, protein expression was induced with 119 µL of 105 mM IPTG and 20.8 µL of 12 mM ATR to a final concentration of 500 µM IPTG and 10 µM ATR and incubated at 30 °C with shaking at 250 rpm. After 4 h, 6 mL of each expression culture was collected by centrifugation at 2,500*g* for 5 min (5810 R, Eppendorf), washed with 3 mL of 50 mM sodium acetate buffer at pH 5, 50 mM sodium phosphate buffer at pH 6, or 50 mM sodium phosphate buffer at pH 7, and resuspended in 400 µL of the corresponding buffer. In a 96-well plate, 25 µL of the cell suspension was diluted with 175 µL of the corresponding buffer containing 0 mM or 457 mM sodium chloride to a final concentration of 0 mM and 400 mM sodium chloride. Excitation scans were acquired for the rhodopsin emission at 710 nm with a 20-nm bandwidth. Excitation spectra were collected from 500–650 nm with a 10-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100. The rhodopsin was excited at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100. The CFP was excited at 390 nm with a 10-nm bandwidth, and the emission was collected from 425–560 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 55 (Spark 10M, Tecan). The rhodopsin and CFP emissions were integrated from 600–800 nm (F_{GR}) and 425–560 nm (F_{CFP}), respectively, using the *trapz* function in MATLAB R2017a (MathWorks). The average of three biological replicates with standard error of the mean is reported (Figures 3, S3, S5).

Large-scale protein expression and purification. For large-scale protein expression, *E. cloni* were transformed as described above with plasmids encoding *wt*GR and GR1. We note the following describes a representative protocol. Single colonies were picked into 50 mL of 2xYT media containing 50 µg/mL ampicillin in 250 mL baffled flasks and incubated overnight at 37 °C with shaking at 230 rpm. The following day, cultures were diluted 1:40 (v/v) into 1 L of 2xYT containing 50 µg/mL ampicillin in 2.8-L baffled flasks and incubated at 37 °C until the OD_{600} reached ~0.6–0.8. The flasks were then cooled on ice for 30 min. Following this, protein expression was induced with the addition of 952 µL of 105 mM IPTG and 833 µL of 12 mM ATR to a final concentration of 100 µM IPTG and 10 µM ATR and incubated overnight at 18 °C with shaking at 230 rpm. The following day, the cells were collected by centrifugation at 3,000*g* (5810 R, Eppendorf) for 30 min at 4 °C and stored at -20 °C until further use.

The following steps were carried out at 4 °C. The frozen cell pellet was thawed and resuspended in a 1:5 (w/v) of pre-chilled 20 mM Tris buffer at pH 7.5 containing 200 mM sodium chloride, 5 mM magnesium chloride, 30 µg/mL deoxyribonuclease I, and two cOmplete Protease Inhibitor Tablets (Roche) and homogenized with stirring in a foiled beaker. Cell lysis was carried out with sonication at 40% amplitude, 15 s pulse on, and 45 s pulse off for 10 min (Q500, QSonica). The cell debris was collected by centrifugation at 3,000*g* for 30 min (5810 R, Eppendorf). The membrane fraction was isolated by ultracentrifugation at 159,200*g* (Sorvall WX 80+, Thermo Fisher Scientific) for 1 h, resuspended in 15 mL of 20 mM Tris buffer at pH 7.5 containing 200 mM sodium chloride and 10% glycerol, homogenized using a tissue grinder (Wheaton), and stored at -80 °C until further use.

The following day, the isolated cell membrane was thawed on ice for 30 min and solubilized with vigorous stirring in 35 mL of 20 mM Tris buffer at pH 7.5 containing 300 mM sodium chloride, 1% n-dodecyl-β-D-maltopyranoside (DDM, w/v, Anatrace), and one cOmplete Protease Inhibitor Tablet. Insoluble debris was collected by ultracentrifugation at 159,200*g* (Sorvall WX 80+, Thermo Fisher Scientific) for 20 min, and the supernatant was loaded onto a 90-mL sample loop (Bio-Rad Laboratories). Protein purification was carried out with the NGC Quest 10 Chromatography System (Bio-Rad Laboratories). A 5-mL nickel nitrilotriacetic acid (Ni-NTA) affinity column (HisTrap, GE Healthcare) was equilibrated with 5 column volumes (CV) of the 20 mM Tris running buffer at pH 7.5 containing 300 mM sodium chloride, 25 mM imidazole, and 0.02% DDM (w/v) at a flow rate of 5 mL/min. The protein sample was loaded onto the Ni-NTA column with a 0.5 mL/min flow rate, eluted with a 0–100% gradient of the running buffer and 20 mM Tris elution buffer at pH 7.5 containing 300 mM sodium chloride, 500 mM imidazole, and 0.02% DDM (w/v) over 20 CV, and washed with 5 CV of the elution buffer. Fractions with an absorbance at 570 nm were combined and loaded onto a HiPrep 26/10 desalting column (GE Healthcare) pre-equilibrated with 50 mM sodium acetate buffer at pH 5 containing 150 mM sodium gluconate and 0.05% DDM (w/v). Protein fractions were pooled and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit with a 10 kDa molecular weight cut-off (MilliporeSigma), aliquoted, flash frozen in powdered dry ice, and stored at -80 °C for assays described below.

SDS-PAGE and Coomassie staining. The purity of purified *wt*GR and GR1 was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a previously reported procedure with the modification that samples were heated in Laemmli sample buffer at 37 °C for 10 min (Figure S11).[2]

General spectroscopic materials and methods. Absorbance and fluorescence spectra were collected using the Agilent Cary 7000 Spectrophotometer and the Spark 10M plate reader (Tecan).[2] Measurements were acquired at room temperature (24–26 °C) with a 0.2-cm x 1-cm quartz cuvette (0.4 mL, Hellma USA) or 96-well half-area microtiter plate (Greiner Bio-One).

Extinction coefficient and quantum yield determination with purified proteins. Aliquots of *wt*GR and GR1 in 50 mM sodium acetate buffer with 0.05% DDM at pH 5 were first diluted to ~5 µM with 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate. Protein concentrations were determined using the Beer-Lambert law by dividing the *wt*GR and GR1 absorbance values at 550 nm and 565 nm, respectively, by their molar extinction coefficients and the pathlength of 1 cm. The molar extinction coefficients were determined using a previously reported procedure.[6] Briefly, a 2.7 M hydroxylamine stock solution was prepared fresh in deionized water, and 148 µL of this solution was added to 252 µL of the diluted protein stock solution for a final concentration of 1 M hydroxylamine and ~3 µM protein in a 0.4 mL quartz cuvette to displace the covalently bound Schiff base chromophore (SBC). Absorbance spectra were collected from 300–700 nm with a 2-nm bandwidth and a 5-nm step size every 10 min for 12 h. The change in absorbance of free ATR at 360 nm (ΔAbs_{ATR}) was plotted versus the change in absorbance of the rhodopsin at 550 nm for *wt*GR or 565 nm for GR1 (ΔAbs_{GR}) over time and fitted to a linear fit model: y = mx + b, where m is the slope variable and is equal to $\Delta Abs_{GR} / \Delta Abs_{ATR}$. The molar extinction coefficients for *wt*GR and GR1 (ε_{GR}) were determined using the following equation: $\varepsilon_{GR} = \Delta Abs_{GR} / \Delta Abs_{ATR} * \varepsilon_{ATR}$ where the molar extinction coefficient for ATR (ε_{ATR}) in ethanol is 43.4 x 10^3 M^{-1} x cm^{-1}.[7] The average of three technical replicates with standard error of the mean is reported (Figure S12).

The *wt*GR and GR1 quantum yield experiments were carried out in the absence and presence of 400 mM sodium chloride with respect to rhodamine 101 inner salt in methanol (Φ = 1.0) as a reference.[8] Serial dilutions of *wt*GR and GR1 were prepared in 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate, and serial dilutions of rhodamine 101 inner salt were prepared in methanol. Each sample was transferred to a 96-well half-area microtiter plate (Greiner Bio-One) for plate reader measurements. Absorbance spectra were collected from 400–750 nm with a 5-nm step size, and the absorbance values were kept between 0 and 0.3. Excitation was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 565–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 58 (Spark 10M, Tecan). The emission of the rhodopsins and rhodamine 101 inner salt were integrated from 565–800 nm using the *trapz* function in MATLAB. A standard curve was generated for each sample by plotting the absorbance value at 565 nm versus the integrated emission using the linear fit model: y = mx + b, where m is the slope variable ($R^2 > 0.99$). The quantum yields for *wt*GR and GR1 were calculated using the equation: $F_{GR} = (m_{GR} / m_{ref})(\eta_{GR}^2 / \eta_{ref}^2)$ where η is the refractive index values for water (η = 1.770) and methanol (η = 1.765) (Figure S14).[9,10] The average of three technical replicates with standard error of the mean is reported (Figure S14).

Chloride titrations with purified protein. Aliquots of purified *wt*GR and GR1 in 50 mM sodium acetate buffer at pH 5 with 150 mM sodium gluconate and 0.05% DDM were diluted into 50 mM sodium acetate buffer at pH 4 containing 600 mM sodium gluconate or 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate to a final concentration of ~6 µM protein. The protein stock solution was further diluted 1:1 (v/v) into the corresponding buffers containing 600 mM sodium gluconate and 0, 25, 50, 100, 200, 400, 600, and 800 mM sodium chloride for a final concentration

of ~3 μM protein and 0, 12.5, 25, 50, 100, 200, 300, and 400 mM sodium chloride. Excitation spectra were collected from 475–650 nm with a 10-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100 for the rhodopsin emission at 710 nm with a 20-nm bandwidth. The rhodopsin was excited at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100 (Spark 10M, Tecan). The rhodopsin emission was integrated from 600–800 nm using the *trapz* function in MATLAB. The integrated emission response of *wt*GR and GR1 defined as $F = (F_{obs} - F_{min}) / (F_{max} - F_{min})$ was plotted versus the $[Cl^-]$ in Kaleidagraph v4.5 (Synergy Software) to determine the apparent dissociation constant (K_d). The K_d was calculated using the following equation: $F = [Cl^-] / (K_d + [Cl^-])$, where F_{obs} is the observed fluorescence and F_{min} and F_{max} are the fluorescence intensities at 0 mM and 400 mM sodium chloride, respectively. The average of three technical replicates with standard error of the mean is reported (Figures 4, S13, S15, and S21–S22).

Anion selectivity with purified proteins. Aliquots of purified GR1 in 50 mM sodium acetate buffer at pH 5 with 150 mM sodium gluconate and 0.05% DDM were diluted into 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate to a final concentration of ~6 μM protein. The protein stock solution was further diluted 1:1 (v/v) into 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate and/or 800 mM sodium chloride, sodium bromide, sodium iodide, sodium nitrate, or sodium dihydrogen phosphate to a final concentration of ~3 μM protein and 0 mM or 400 mM of the anion. The excitation for GR1 was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100 (Spark 10M, Tecan). The GR1 emission was integrated from 600–800 nm using the *trapz* function in MATLAB. The average emission response for three technical replicates with standard error of the mean is reported (Figures 4C, S16).

Bromide and iodide titrations with purified GR1. Aliquots of purified GR1 in 50 mM sodium acetate buffer at pH 5 with 150 mM sodium gluconate and 0.05% DDM were diluted into 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate to a final concentration of ~6 μM protein. The protein stock solution was further diluted 1:1 (v/v) into 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate and 0, 25, 50, 100, 200, 400, 600, and 800 mM sodium bromide or sodium iodide for a final concentration of ~3 μM protein and 0, 12.5, 25, 50, 100, 200, 300, and 400 mM sodium bromide or sodium iodide. Excitation spectra were collected from 475–650 nm with a 10-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100 for the rhodopsin emission at 710 nm with a 20-nm bandwidth. The GR1 was excited at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100 (Spark 10M, Tecan). The GR1 emission was integrated from 600–800 nm using the *trapz* function in MATLAB, and the K_ds were determined as described above. The average of three technical replicates with standard error of the mean is reported (Figures S17–S18).

Determination of the Schiff base pK_a with purified proteins. Aliquots of purified *wt*GR and GR1 in 50 mM sodium acetate buffer at pH 5 with 150 mM sodium gluconate and 0.05% DDM were diluted into 50 mM sodium acetate buffer from pH 3–5, 50 mM sodium phosphate buffer from pH 6–8, or 50 mM N-cyclohexyl-3-aminopropanesulfonic acid buffer at pH 10 containing 600 mM sodium gluconate to a final concentration of ~3 μM protein. In a microtiter plate, the rhodopsin excitation was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 580–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100 (Spark 10M, Tecan). The rhodopsin

emission was integrated from 580–800 nm using the *trapz* function in MATLAB. The relative emission response was plotted versus pH in Kaleidagraph v4.5 and fitted to the Henderson-Hasselbach equation to determine the SBC pK_a. The average integrated emission for three technical replicates with standard error of the mean and a student's *t*-test are reported (Figures S19–S20).

General protein expression protocol and plate reader settings for assays with live *E. coli*. *E. cloni* were transformed with plasmids encoding *wt*GR-CFP and GR1-CFP as described above. Single colonies were picked into 5 mL of LB containing 100 µg/mL ampicillin in 14-mL culture tubes and incubated overnight at 37 °C with shaking at 250 rpm. The next day, overnight cultures were diluted 1:20 (v/v) in either 3 mL of LB containing 50 µg/mL ampicillin in 14-mL culture tubes or 25 mL LB containing 50 µg/mL ampicillin in 125-mL baffled flasks. The cultures were incubated at 30 °C with shaking at 250 rpm. After 2.5 h, protein expression was induced with the addition of 15 µL of 100 mM IPTG and 15 µL of 2 mM ATR in 3 mL of LB or 119 µL of 105 mM IPTG and 20.8 µL of 12 mM ATR in 25 mL LB to a final concentration of 500 µM IPTG and 10 µM ATR. Protein expression was carried out at 30 °C for 4 h with shaking at 250 rpm and processed for plate reader assays. For the rhodopsin, excitation was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5 nm-step size, 30 flashes and a gain of 100 (Spark 10M, Tecan). Excitation for CFP was provided at 390 nm with a 10-nm bandwidth, and the emission collected from 425–560 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and a gain of 55. For each variant, the rhodopsin emission was integrated from 600–800 nm (F_{GR}), and the CFP emission was integrated from 425–560 nm (F_{CFP}) using the *trapz* function in MATLAB. The normalized emission is reported as F_{GR}/F_{CFP}. All plate reader measurements were carried out at room temperature (24–26 °C) unless otherwise stated.

Chloride titration assays in live *E. coli*. Three 25-mL cultures of *E. cloni* containing the *wt*GR-CFP and GR1-CFP plasmids were expressed at 30 °C as described above. After 4 h, 6 mL of each expression culture was collected by centrifugation at 2,500*g* for 5 min (5810 R, Eppendorf), washed with 3 mL of 50 mM sodium acetate buffer at pH 5 and resuspended in 400 µL of 50 mM sodium acetate buffer at pH 5. In a 96-well plate, 25 µL of the cell suspension was diluted with 175 µL of 50 mM sodium acetate buffer at pH 5 containing 0, 14.3, 28.5, 57, 114.3, 228.5, or 457 mM sodium chloride to a final concentration of 0, 12.5, 25, 50, 100, 200, and 400 mM sodium chloride. The plate reader measurements were carried out as described above. The GR1-CFP apparent K_d for chloride were determined as described above. The average of three technical replicates with standard error of the mean is reported (Figures 5, S23–S24).

This assay was repeated in sodium phosphate buffer to test the effects of sodium acetate. Three 3-mL cultures of *E. cloni* containing the GR1-CFP plasmid were expressed at 30 °C as described above. After 4 h, the cells were collected by centrifugation at 2,500*g* for 5 min (5810 R, Eppendorf), washed with 3 mL of 50 mM sodium phosphate buffer at pH 5, and resuspended in 200 µL of 50 mM sodium phosphate buffer at pH 5. In a 96-well microtiter plate, 25 µL of the cell suspensions were diluted into 175 µL of 50 mM sodium phosphate buffer at pH 5 containing 0 mM, 457 mM sodium chloride, or 457 mM sodium acetate to a final concentration of 0 mM, 400 mM sodium chloride, or 400 mM sodium acetate. The plate reader measurements were carried out as described above. The average of three biological replicates with standard error of the mean is reported (Figure S25).

To test the reversibility of the chloride response, three 25-mL cultures of *E. cloni* containing the GR1-CFP plasmid were expressed at 30 °C as described above. After 4 h, 6 mL of each expression culture was collected by centrifugation at 2,500*g* for 5 min (5810 R, Eppendorf), washed with 3 mL of 50 mM sodium acetate buffer at pH 5, and resuspended in 400 µL of buffer. In four 2-mL centrifuge tubes (USA Scientific), 75 µL of the cell suspensions were diluted into 525 µL of 50 mM sodium acetate buffer at pH 5 or buffer containing 457 mM sodium chloride to a final concentration of 0 mM and 400 mM sodium chloride. The cell suspensions were incubated for 10 min at room temperature with shaking at 300 rpm (ThermoMixer C, Eppendorf). Then, 200 µL of the cell suspensions were transferred to a 96-well microtiter plate for plate reader measurements. The remaining cells were collected by centrifugation at 500*g* for 15 min (5424 R, Eppendorf), resuspended in 400 µL of 50 mM sodium acetate buffer at pH 5 or buffer containing 400 mM sodium chloride or 400 mM sodium gluconate, and incubated for 10 min at room temperature with shaking at 300 rpm. The plate reader measurements were repeated with 200 µL of the resuspended cells in a 96-well microtiter plate. The average of three biological replicates with standard error of the mean is reported (Figure 5C).

Live *E. coli* imaging and analysis. Three 3-mL cultures of *E. cloni* containing the *wt*GR-CFP and GR1-CFP plasmids were expressed at 30 °C as described above. After 4 h, the cells were collected by centrifugation at 2,500*g* for 5 min (5810 R, Eppendorf) and resuspended in 100 µL of 50 mM sodium acetate buffer at pH 5. Fifty microliters of each cell suspension were further diluted into 350 µL of 50 mM sodium acetate buffer at pH 5, and 3 µL of this solution was transferred to a 1.5% agarose pad and inverted onto a 35 mm imaging dish with a No. 1.5 20-mm glass coverslip (MatTek). The agarose pads (1.5%, w/v, Gold Biotechnology) were prepared in 50 mM sodium acetate buffer at pH 5 containing 0 mM or 400 mM sodium chloride. The agarose was solidified to an even thickness between two microscope slides and cut into 3 mm^2 pads.

Images were acquired at room temperature (24–26 °C) using a confocal laser scanning microscope equipped with a single motorized pinhole, a cooled GaAsP photomultiplier, a multi-alkali photomultiplier, a motorized volume phase holographic transmission diffraction grating, a motorized adjustable slit, an external fluorescence light source, and a LED lamp with an integrated external transmitted light photomultiplier detector (FV3000RS, Olympus). A UPLSAPO 100X silicone immersion objective with a numerical aperture of 1.35 and working distance of 0.2 mm was used for imaging. Z-stacks were acquired for each sample. First, the rhodopsin was excited at 561 nm (20 mW), and the emission was collected from 630–730 nm. Then, the CFP was excited at 405 nm (50 mW), and the emission collected from 450–550 nm. Images were analyzed using the software Fiji Is Just ImageJ (Fiji) v2.0.[11] Default threshold settings were applied to the CFP maximum intensity Z-projection to create masks. From the masks, regions of interest (ROIs) with pixels greater than 3 and circularity between 0 and 1 were selected using the Analyze Particles function in Fiji. Overlapping cells that could not be identified as single cells were grouped as one ROI. For each Z-stack, the ROIs were transferred to the maximum intensity Z-projection of both the rhodopsin and CFP channels, and the fluorescence median intensity was measured. For each biological replicate (n = 3), at least four different fields of cells were sampled. The median fluorescence intensity of the rhodopsin (F_{GR}) was normalized to the median fluorescence intensity of CFP (F_{CFP}) (Figures 6, S26).

Proton and chloride-pumping assays in live *E. coli*. Proton and chloride pumping activities were measured using modified literature procedures.[12] Three 25-mL cultures of *E. cloni* containing the *wt*GR-CFP and GR1-CFP plasmids were expressed at 30 °C as described above. After 4 h, the cells

were collected by centrifugation at 3,000*g* (Allegra X-14R, Beckman Coulter) for 5 min at 4 °C, washed 3 times with 10 mL of 400 mM sodium chloride, and resuspended in 4 mL of 400 mM sodium chloride to a final OD_{600} ~0.8. The cells were incubated in the dark for 1 h at 4 °C and then illuminated with a 90-W BR40 LED light bulb (EcoSmart, Home Depot) for 2 min. Light induced pH changes were measured using a pH electrode (Mettler Toledo) every 10 s for 300 s. The summation of the change in pH (∆pH) was calculated for each time point. Pumping assays were also carried out with cells treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma-Aldrich). A stock solution of 10 mM CCCP was prepared in DMSO and 12 µL of the stock solution was added to the 4 mL cell resuspension to a final concentration of 30 µM CCCP. The average ∆pH for three biological replicates with standard error of the mean is reported (Figure S27).

Chloride titrations in live *E. coli* with CCCP. To validate that the GR1-CFP turn-on response to chloride is independent of the membrane potential, chloride titrations were carried out with cells treated with 30 µM CCCP. Three 3-mL cultures of *E. cloni* containing the *wt*GR-CFP and GR1-CFP plasmids were expressed at 30 °C as described above. After 4 h, each expression culture was collected by centrifugation at 2,500*g* for 5 min (5810 R, Eppendorf) and resuspended in 400 µL of 50 mM sodium acetate buffer at pH 5. In a 96-well plate, 10 µL of the cell suspension was diluted into 175 µL of 50 mM sodium acetate buffer at pH 5 containing 0, 14.3, 28.5, 57, 114.3, 228.5, or 457 mM sodium chloride to a final concentration of 0, 12.5, 25, 50, 100, 200, and 400 mM sodium chloride. Each well was further diluted with 15 µL of 3.2% DMSO (v/v) in buffer as the control or 400 µM CCCP in buffer for a final concentration of 0 µM and 30 µM CCCP. The 96-well microtiter plate was incubated at 30 °C for 30 min with shaking for 1 min every 5 min in the plate reader. The plate reader measurements were carried using the settings described above. The average of three biological replicates with standard error of the mean is reported (Figure S28).

Membrane potential measurements with $DiOC_2(3)$. To confirm that CCCP treatment at pH 5 uncouples the membrane potential, experiments were carried out with the ratiometric dye 3-3'-diethlyoxacarbocyanine iodide ($DiOC_2(3)$, Alfa Aesar).[13] Three 5-mL cultures of *E. cloni* containing the GR1-CFP plasmid were incubated overnight at 37 °C with shaking at 250 rpm. The following day, 1 mL of the overnight cultures were harvested by centrifugation at 2,500*g* for 5 min (5810 R, Eppendorf) and resuspended with 1 mL of 50 mM sodium acetate buffer at pH 5. A portion of the cell resuspension (119 µL) was transferred to a 5-mL Falcon Round-Bottom Polystyrene Tube (Corning) and further diluted into 875 µL of sodium acetate buffer at pH 5 containing 0 mM, 457 mM sodium chloride, or 457 mM sodium gluconate to a final concentration of 0 mM, 400 mM sodium chloride, or 400 mM sodium gluconate. The samples were then treated with 1 µL of DMSO or 1 µL of a 30 mM CCCP stock solution in DMSO and 4 µL of an 11 mM $DiOC_2(3)$ stock solution in DMSO for a final concentration of 0 µM or 30 µM CCCP and 22 µM $DiOC_2(3)$. The samples were incubated in the dark at room temperature for 30 min before flow cytometry measurements (BD LSRFortessa). Cells were excited with the 488 nm laser (50 mW power). The green and red emissions were collected using the FITC emission at 530 nm with a 30-nm bandpass and the PerCP-Cy5-5 emission at 710 nm with a 50-nm bandpass, respectively. For each sample 50,000 events were recorded. Unstained and untreated *E. cloni* in 50 mM sodium acetate buffer at pH 5 were used as negative controls to gate cell populations using the software FlowJo v10.8. For each treatment, the average median emission intensity of three biological replicates with standard error of the mean is reported (Figure S29).

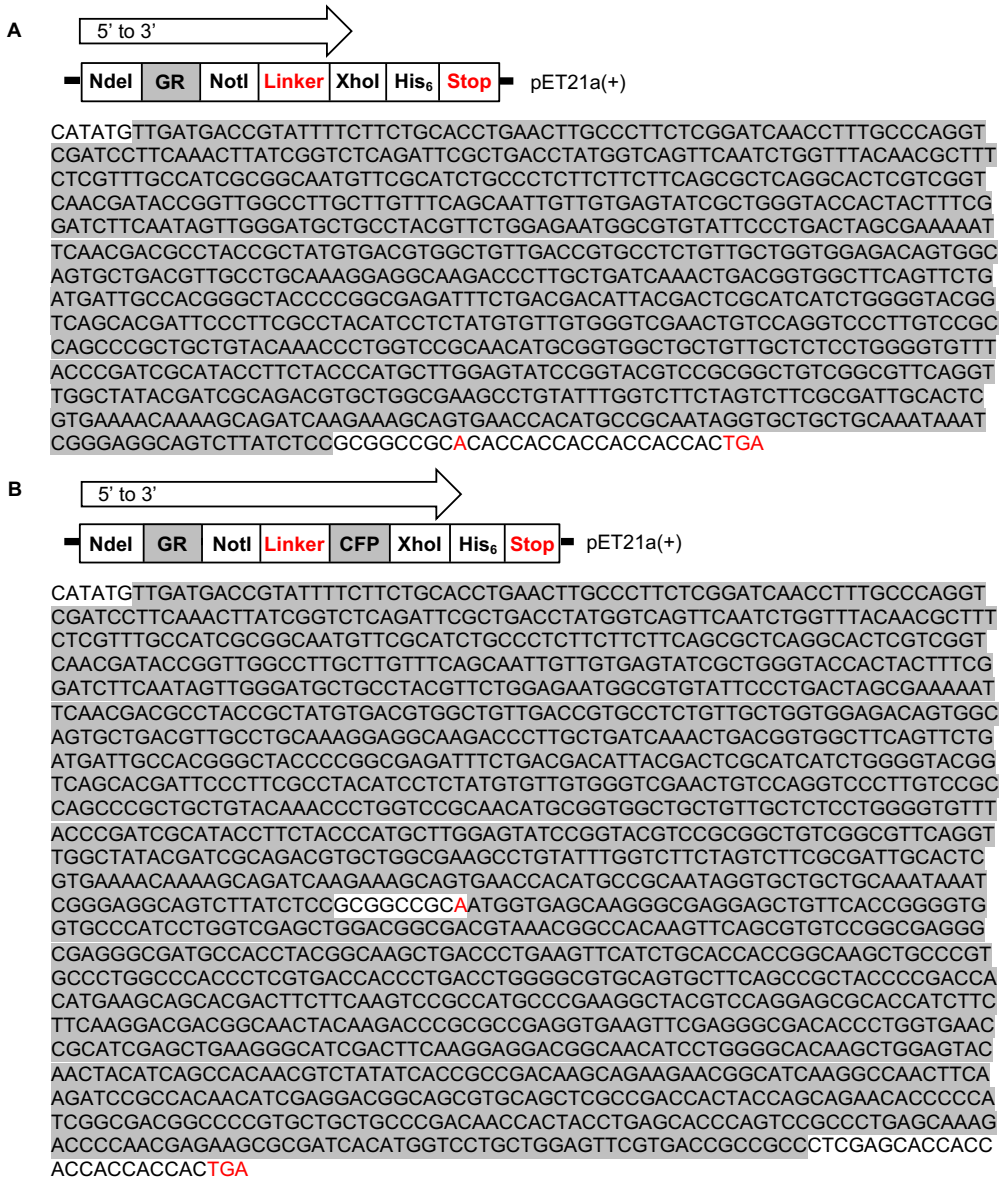

Figure S1. (A) The GR T121 plasmid used to generate the plasmids encoding *wt*GR (D121) and GR1 (V121). The nucleotide sequence for *Gloeobacter violaceus* rhodopsin (GR, UniProt ID: Q7NP59) with T121 was cloned into the pET-21a(+) vector between the NdeI and NotI restriction sites followed by a single adenine nucleotide linker, a C-terminal polyhistidine-tag (His_6), and a stop codon. (B) The GR T121-CFP plasmid used to generate the site-saturation mutagenesis library at position 121. Starting from the GR T121 plasmid in panel (A), the nucleotide sequence encoding cyan fluorescent protein (CFP, UniProt ID: P42212) was cloned between the NotI and XhoI restriction sites followed by a C-terminal His_6-tag and a stop codon.

Table S1. List of the primers used to generate the GR-CFP site-saturation mutagenesis library at position 121 and the plasmids for *wt*GR and GR1. The mutation site in each primer is highlighted red.

Description	Sequence (5' to 3')
121 SSM Forward Primer	GACGCCTACCGCTATGTGNDTTGGCTGTTGACCGTGC
	GACGCCTACCGCTATGTGVHGTGGCTGTTGACCGTGC
	GACGCCTACCGCTATGTGTGGTGGCTGTTGACCGTGC
*wt*GR Forward Primer	GACGCCTACCGCTATGTGGATTGGCTGTTGACCGTGC
GR1 Forward Primer	GACGCCTACCGCTATGTGGTTTGGCTGTTGACCGTGC
Reverse Primer	CACATAGCGGTAGGCGTCGTTGAATTTTTCGCTA

Table S2. Polymerase chain reaction conditions used to generate the GR-CFP site-saturation mutagenesis library at position 121.

Step	Temperature (ºC)	Time (s)	Cycle Number
Template Denaturation	95	30	1
	95	10	30
Annealing	57	30	
Short Extenstion	72	275	
Long Extension	72	600	1
Storage	10	∞	1

Table S3. Polymerase chain reaction conditions used to generate plasmids encoding *wt*GR and GR1.

Step	Temperature (ºC)	Time (s)	Cycle Number
Template Denaturation	98	30	1
	98	10	30
Annealing	57	30	
Short Extenstion	72	250	
Long Extension	72	600	1
Storage	10	∞	1

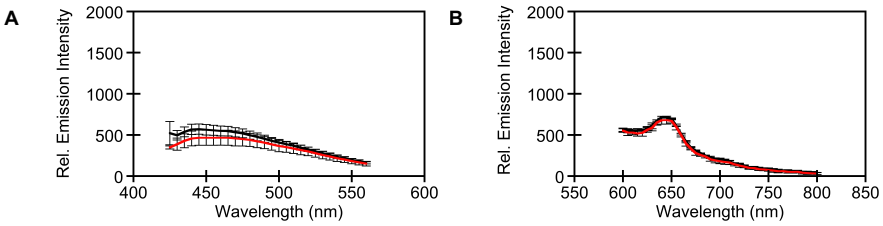

Figure S2. Spectroscopic characterization of non-induced *E. coli* in 50 mM sodium acetate buffer at pH 5 with 0 mM (black) and 400 mM (red) sodium chloride. Emission of (A) non-induced *E. coli* with excitation at 390 nm. The emission was collected from 425–560 nm. Emission of (B) non-induced *E. coli* with excitation at 530 nm. The emission was collected from 600–800 nm. The average of three biological replicates with standard error of the mean is reported.

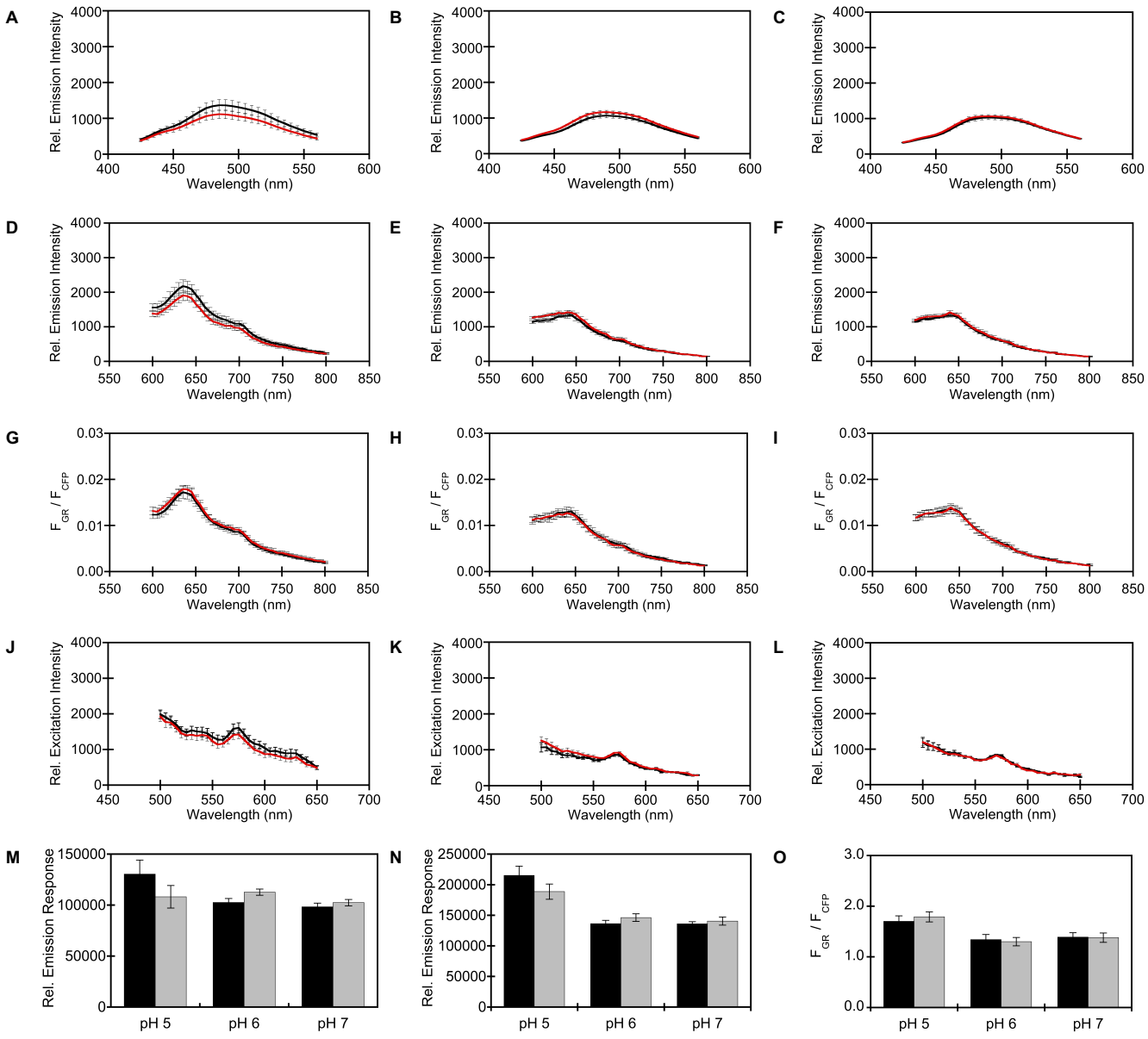

Figure S3. Spectroscopic characterization of *wt*GR-CFP in live *E. coli* in 50 mM sodium acetate buffer at pH 5, 50 mM sodium phosphate buffer at pH 6, and 50 mM sodium phosphate buffer at pH 7 with 0 mM (black) and 400 mM (red) sodium chloride. Emission spectra of CFP at (A) pH 5, (B) pH 6, and (C) pH 7. Excitation was provided at 390 nm, and the emission was collected from 425–560 nm. Emission spectra of *wt*GR at (D) pH 5, (E) pH 6, and (F) pH 7. Excitation was provided at 530 nm, and the emission was collected from 600–800 nm. Normalized emission spectra of *wt*GR-CFP at (G) pH 5, (H) pH 6, and (I) pH 7. For each pH, the emission spectra of *wt*GR-CFP (F_{GR}) were normalized by the integrated emission of the CFP from 425–560 nm (F_{CFP}). Excitation spectra of *wt*GR-CFP for the rhodopsin emission at 710 nm at (J) pH 5, (K) pH 6, and (L) pH 7. For each pH, the excitation maximum is at 570 nm. Integrated emission response of (M) CFP from 425–560 nm (F_{CFP}) and (N) *wt*GR from 600–800 nm (F_{GR}). (O) Normalized integrated emission response (F_{GR}/F_{CFP}) at pH 5, pH 6, and pH 7. Bars represent the integrated emission response in the absence (black bars) and presence of 400 mM sodium chloride (gray bars). The average of three biological replicates with standard error of the mean is reported.

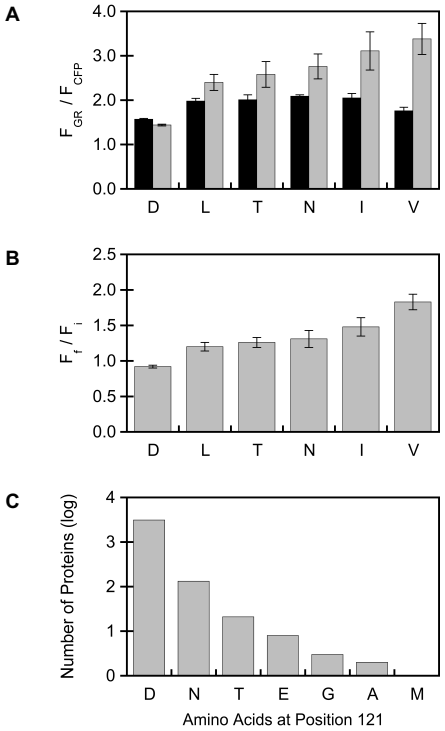

Figure S4. (A) Chloride-sensitive variants identified by site-saturation mutagenesis at position 121 in live *E. coli*. The single letter amino acid code is shown on the x-axis. Bars represent the integrated emission response of the rhodopsin (F_{GR}) normalized to the integrated emission of the CFP (F_{CFP}) in the absence (black) and presence of 400 mM (gray) sodium chloride. (B) Normalized emission response of *wt*GR-CFP and chloride-sensitive variants from (A). Bars represent the ratio of the normalized integrated emission response (F_{GR}/F_{CFP}) in the presence of 400 mM (F_f) sodium chloride over the normalized integrated emission response (F_{GR}/F_{CFP}) in the absence of sodium chloride (F_i). All spectra were acquired in 50 mM sodium acetate buffer at pH 5. Excitation for the rhodopsin was provided at 530 nm, and the emission was collected and integrated from 600–800 nm (F_{GR}). Excitation was provided at 390 nm for the CFP, and the emission was collected and integrated from 425–560 nm (F_{CFP}). The average of eight biological replicates with standard error of the mean is reported. (C) The diversity of amino acids in the rhodopsin family at the position corresponding to 121 in *wt*GR generated from a multiple sequence alignment. The single letter amino acid code is shown on the x-axis, and the log of the number of proteins identified is shown on the y-axis. Note: only one protein sequence was identified for M.

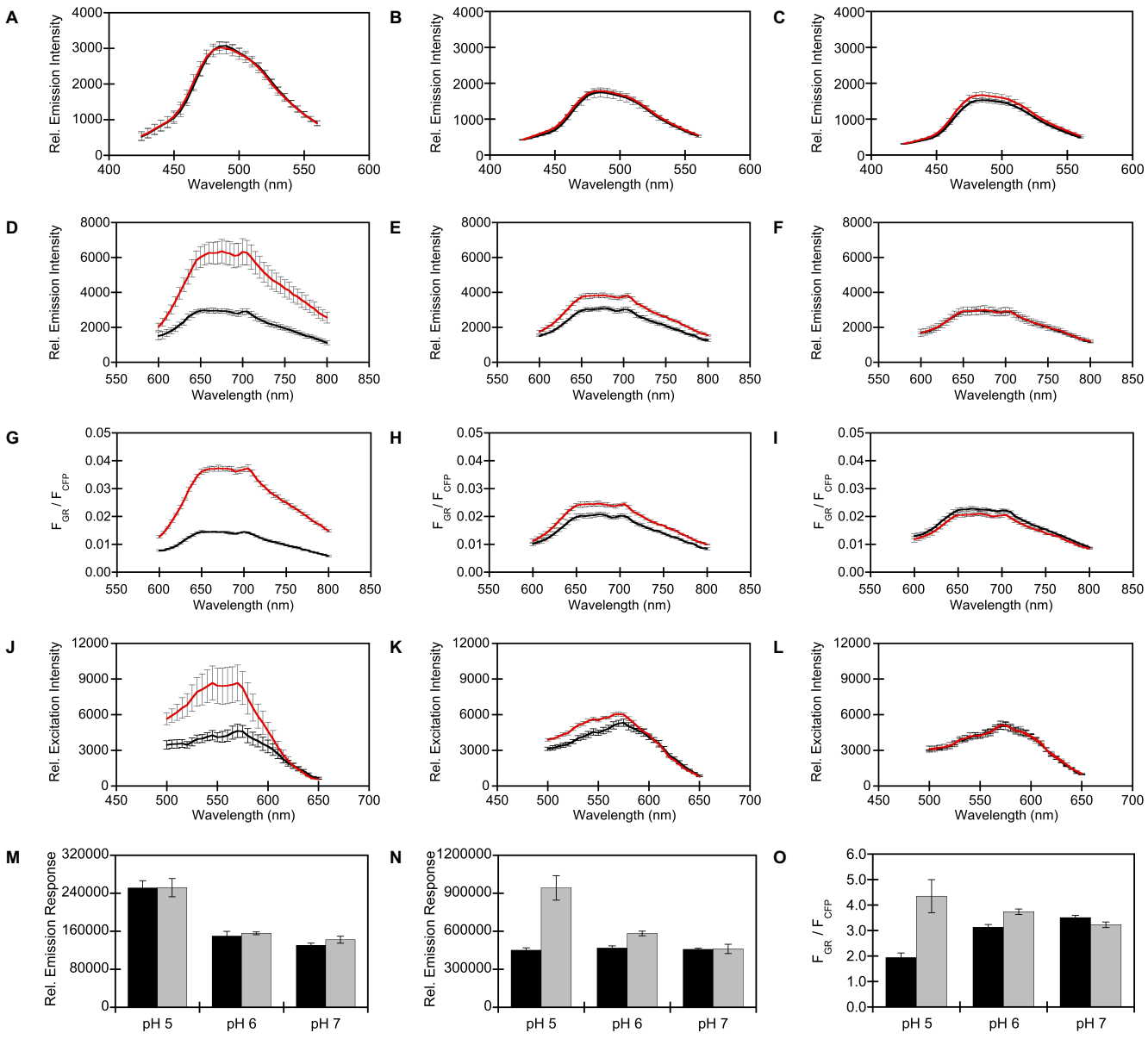

Figure S5. Spectroscopic characterization of GR1-CFP (GR D121V-CFP) in live *E. coli* in 50 mM sodium acetate buffer at pH 5, 50 mM sodium phosphate buffer at pH 6, and 50 mM sodium phosphate buffer at pH 7 with 0 mM (black) and 400 mM (red) sodium chloride. Emission spectra of CFP at (A) pH 5, (B) pH 6, and (C) pH 7. Excitation was provided at 390 nm, and the emission was collected from 425–560 nm. Emission spectra of GR1 at (D) pH 5, (E) pH 6, and (F) pH 7. Excitation was provided at 530 nm, and the emission was collected from 600–800 nm. Normalized emission spectra of GR1-CFP at (G) pH 5, (H) pH 6, and (I) pH 7. For each pH, the emission spectra of GR1-CFP (F_{GR}) were normalized by the integrated emission of the CFP from 425–560 nm (F_{CFP}). Excitation spectra of GR1-CFP for the rhodopsin emission at 710 nm at (J) pH 5, (K) pH 6, and (L) pH 7. For each pH, the excitation maximum is at 570 nm. Integrated emission response of (M) CFP from 425–560 nm (F_{CFP}) and (N) GR1 from 600–800 nm (F_{GR}). (O) Normalized integrated emission response (F_{GR}/F_{CFP}) at pH 5, pH 6, and pH 7. Bars represent the integrated emission response in the absence (black bars) and presence of 400 mM sodium chloride (gray bars). The average of three biological replicates with standard error of the mean is reported.

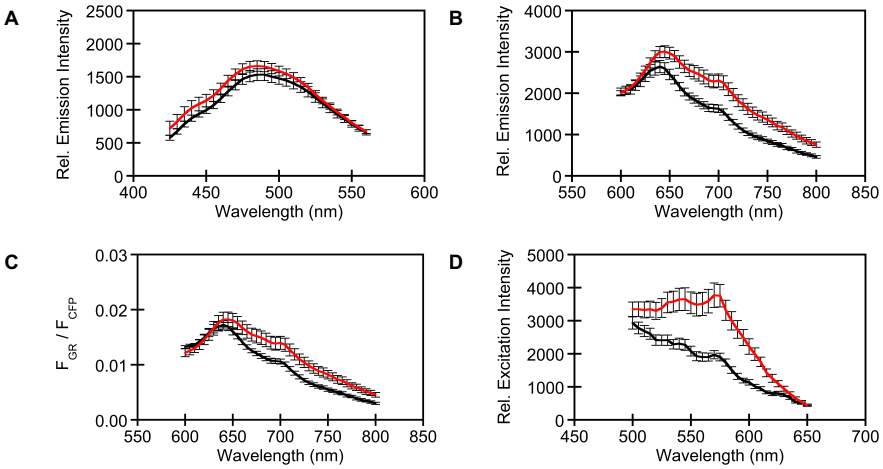

Figure S6. Spectroscopic characterization of GR D121L-CFP in live *E. coli* in 50 mM sodium acetate buffer at pH 5 with 0 mM (black) and 400 mM (red) sodium chloride. (A) Emission spectra of the CFP. Excitation was provided at 390 nm, and the emission was collected from 425–560 nm. (B) Emission spectra of GR D121L-CFP. Excitation was provided at 530 nm, and the emission was collected from 600–800 nm. (C) Emission spectra of GR D121L-CFP (F_{GR}) normalized by the integrated emission of the CFP from 425–560 nm (F_{CFP}). (D) Excitation spectra of GR D121L-CFP for the rhodopsin emission at 710 nm. The excitation maximum is at 570 nm. The average of eight biological replicates with standard error of the mean is reported.

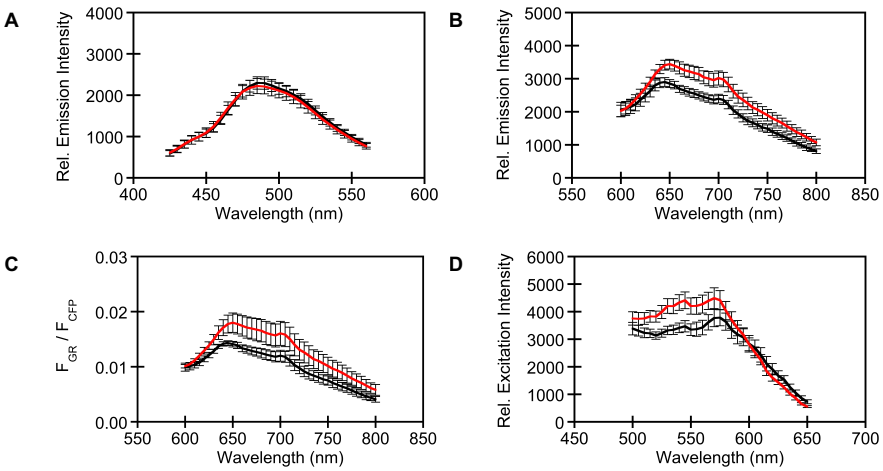

Figure S7. Spectroscopic characterization of GR D121T-CFP in live *E. coli* in 50 mM sodium acetate buffer at pH 5 with 0 mM (black) and 400 mM (red) sodium chloride. (A) Emission spectra of the CFP. Excitation was provided at 390 nm, and the emission was collected from 425–560 nm (F_{CFP}). (B) Emission spectra of GR D121T-CFP. Excitation was provided at 530 nm, and the emission was collected from 600–800 nm. (C) Emission spectra of GR D121T-CFP (F_{GR}) normalized by the integrated emission of the CFP from 425–560 nm (F_{CFP}). (D) Excitation spectra of GR D121T-CFP for the rhodopsin emission maximum is at 710 nm. The excitation maximum is at 575 nm. The average of eight biological replicates with standard error of the mean is reported.

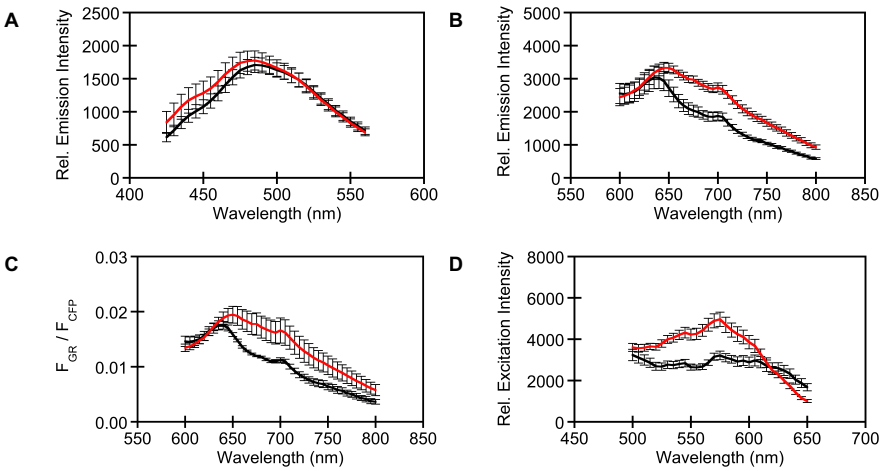

Figure S8. Spectroscopic characterization of GR D121N-CFP in live *E. coli* in 50 mM sodium acetate buffer at pH 5 with 0 mM (black) and 400 mM (red) sodium chloride. (A) Emission spectra of the CFP. Excitation was provided at 390 nm, and the emission was collected from 425–560 nm. (B) Emission spectra of GR D121N-CFP. Excitation was provided at 530 nm, and the emission was collected from 600–800 nm. (C) Emission spectra of GR D121N-CFP (F_{GR}) normalized by the integrated emission of the CFP from 425–560 nm (F_{CFP}). (D) Excitation spectra of GR D121N-CFP for the rhodopsin emission is at 710 nm. The excitation maximum is at 575 nm. The average of eight biological replicates with standard error of the mean is reported.

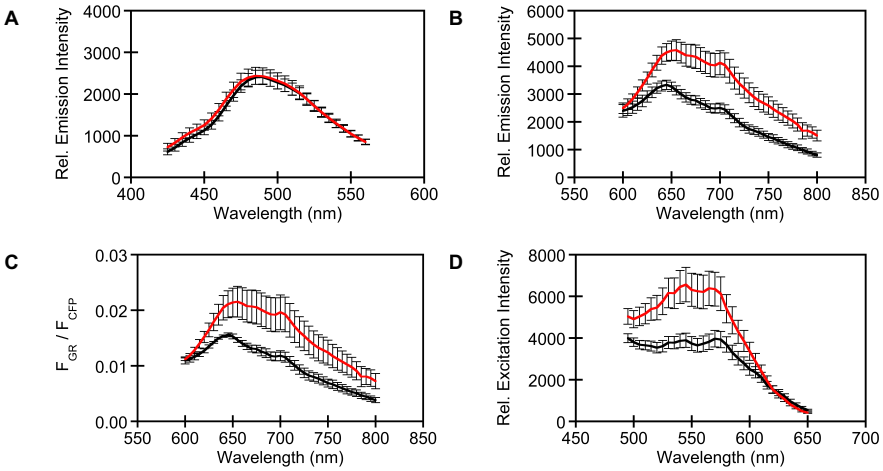

Figure S9. Spectroscopic characterization of GR D121I-CFP in live *E. coli* in 50 mM sodium acetate buffer with 0 mM (black) and 400 mM (red) sodium chloride. (A) Emission spectra of the CFP. Excitation was provided at 390 nm, and the emission was collected from 425–560 nm. (B) Emission spectra of GR D121I-CFP. Excitation was provided at 530 nm, and the emission was collected from 600–800 nm. (C) Emission spectra of GR D121I-CFP (F_{GR}) normalized by the integrated emission of the CFP from 425–560 nm (F_{CFP}). (D) Excitation spectra of GR D121I-CFP for the rhodopsin emission at 710 nm. The excitation maximum is at 570 nm. The average of eight biological replicates with standard error of the mean is reported.

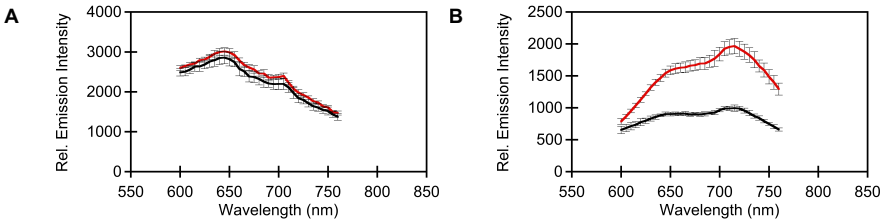

Figure S10. Emission spectra of (A) *wt*GR and (B) GR1 in live *E. coli* in 50 mM sodium acetate buffer at pH 5 with 0 (black) and 400 mM (red) sodium chloride. Excitation was provided at 530 nm, and the emission was collected and integrated from 600–760 nm. The average of three biological replicates with standard error of the mean is reported.

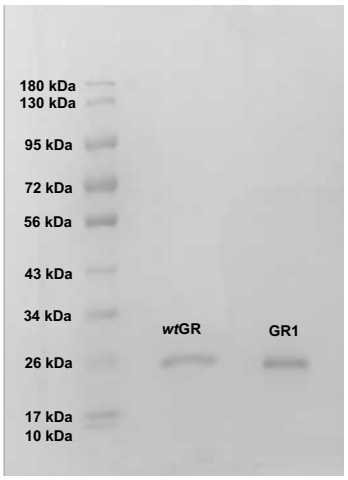

Figure S11. Coomassie stained SDS-PAGE gel of purified *wt*GR and GR1. Theoretical molecular weight is ~26 kDa.

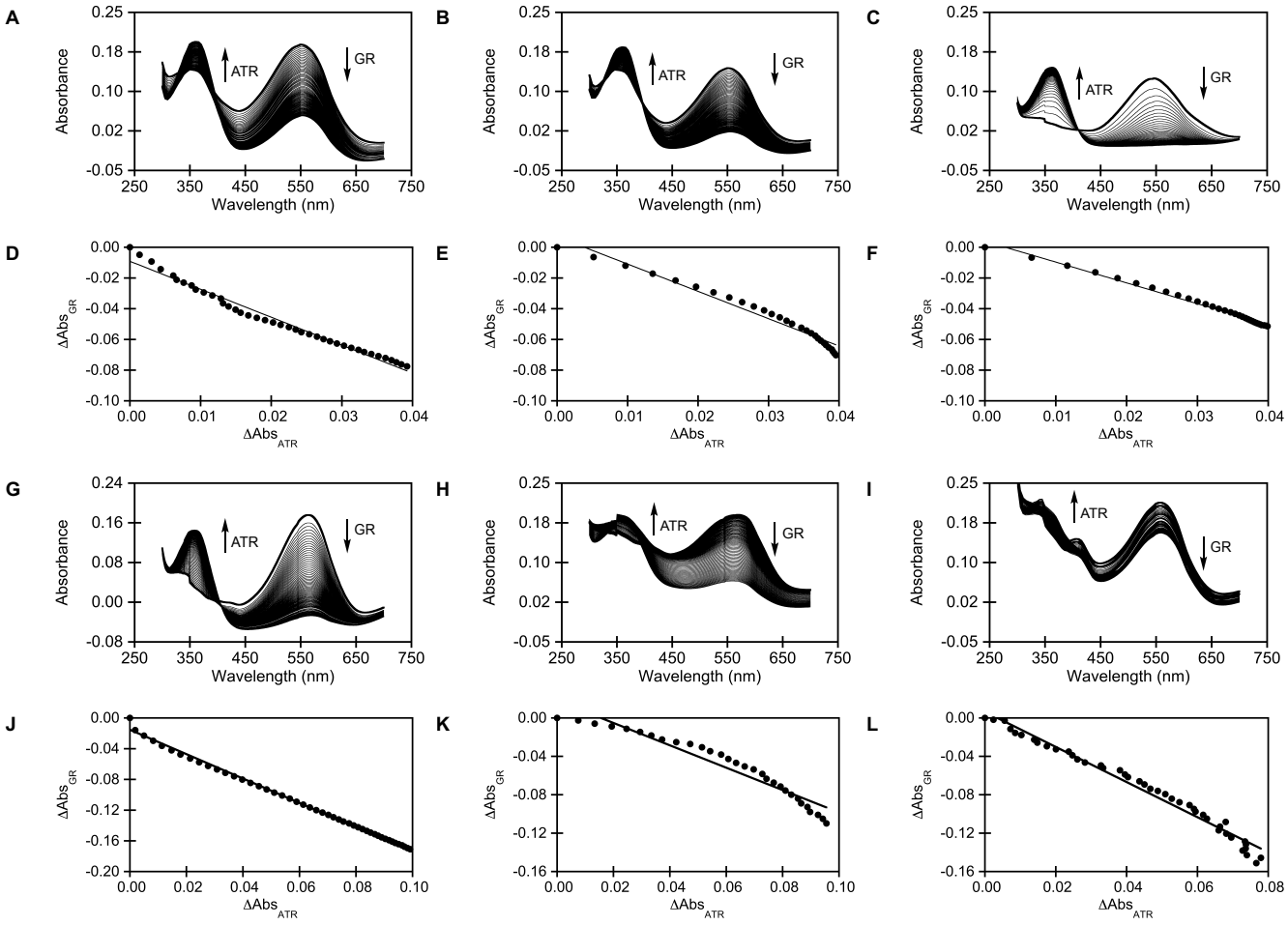

Figure S12. Absorbance spectra for all-*trans*-retinal (ATR) bleaching with 1 M hydroxylamine for three technical replicates of purified (A–C) *wt*GR and (G–I) GR1. Absorbance spectra were collected from 300–700 nm every 10 min for 12 h. The increase in intensity at 360 nm over time corresponds to unbound ATR. Standard curves for the change in absorbance of free ATR at 360 nm (ΔAbs_{ATR}) on the x-axis versus the change in absorbance for three technical replicates of (D–F) *wt*GR at 550 nm and (J–L) GR1 at 565 nm (ΔAbs_{GR}) on the y-axis. The extinction coefficient for *wt*GR and GR1 is 58.9 x 10^3 ± 1.4 x 10^3 M^{-1} • cm^{-1} and 63.9 x 10^3 ± 3.4 x 10^3 M^{-1} • cm^{-1}, respectively. The average of three technical replicates with standard error of the mean is reported.

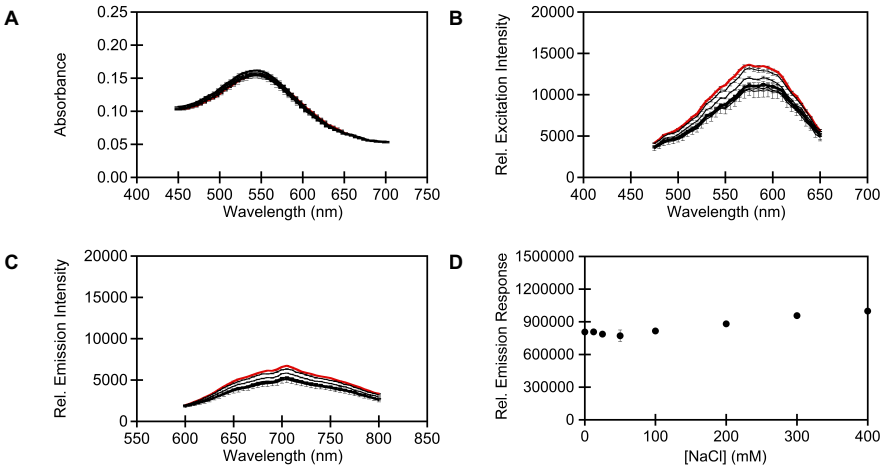

Figure S13. (A) Absorbance, (B) excitation, and (C) emission spectra of ~3 μM purified *wt*GR in the presence of 0 (bold black), 12.5, 25, 50, 100, 200, 300, and 400 mM (red) sodium chloride. (D) Integrated emission response of *wt*GR from panel (C). Spectra were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 5. Excitation for *wt*GR was provided at 530 nm, and the emission was integrated from 600–800 nm. The average of three technical replicates with standard error of the mean is reported.

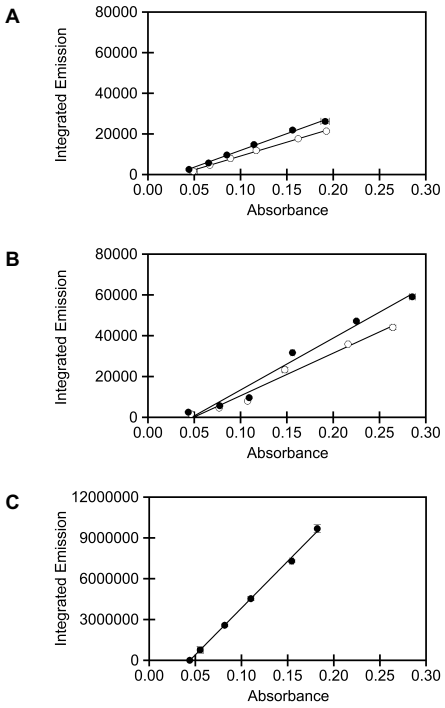

Figure S14. Quantum yield standard curves for (A) *wt*GR and (B) GR1 in the absence (open circles) and presence (black filled circles) of 400 mM sodium chloride in reference to (C) rhodamine 101 inner salt in methanol. Emission spectra for the rhodopsins were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 5. Excitation for the rhodopsins and rhodamine 101 inner salt was provided at 530 nm, and the emission was collected and integrated from 565–800 nm. Absorbance values at 565 nm were plotted versus the integrated emission ($R^2 > 0.99$). The *wt*GR quantum yield in the absence and presence of 400 mM chloride is $\Phi = 2.0 \times 10^{-3} \pm 4.8 \times 10^{-5}$ and $\Phi = 2.4 \times 10^{-3} \pm 5.6 \times 10^{-5}$, respectively. The GR1 quantum yield in the absence and presence of 400 mM chloride is $\Phi = 3.0 \times 10^{-3} \pm 1.4 \times 10^{-4}$ and $\Phi = 3.9 \times 10^{-3} \pm 6.7 \times 10^{-5}$, respectively. The average of three technical replicates with standard error of the mean is reported.

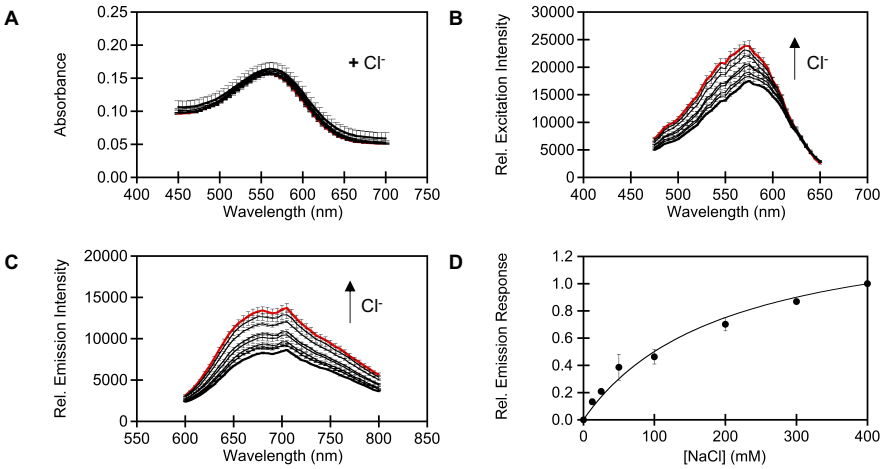

Figure S15. (A) Absorbance, (B) excitation, and (C) emission spectra of ~3 μM purified GR1 in the presence of 0 (bold black), 12.5, 25, 50, 100, 200, 300, and 400 mM (red) sodium chloride. Arrow direction corresponds to increasing sodium chloride concentrations. (D) Integrated emission response of GR1 to sodium chloride from panel (C) to determine the apparent dissociation constant (K_d). The K_d for chloride is 203 ± 41 mM. Excitation for GR1 was provided at 530 nm, and the emission was integrated from 600–800 nm. Spectra were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 5. The average of three technical replicates with standard error of the mean is reported.

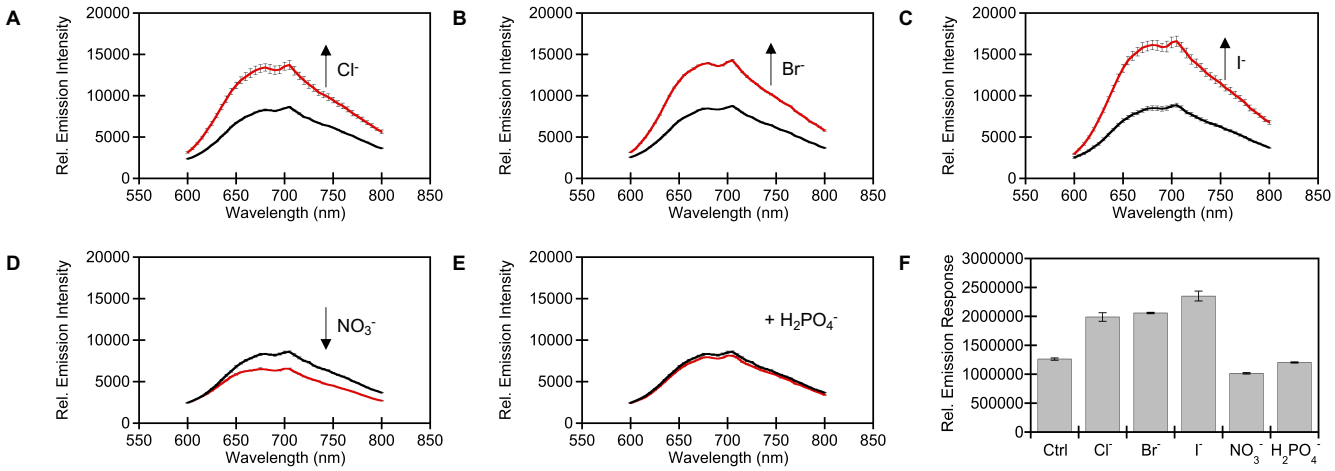

Figure S16. Purified GR1 is sensitive to halides and nitrate. Emission spectra of ~3 μM GR1 in the presence of 0 mM (bold black) and 400 mM (red) sodium (A) chloride, (B) bromide, (C) iodide, (D) nitrate, and (E) dihydrogen phosphate. Arrow direction corresponds to the response with increasing anion concentrations. (C) Integrated emission response of GR1 to 0 mM (Ctrl) or 400 mM anion. All spectra were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 5. Excitation for GR1 was provided at 530 nm, and the emission was collected and integrated from 600–800 nm. The average of three technical replicates with standard error of the mean is reported.

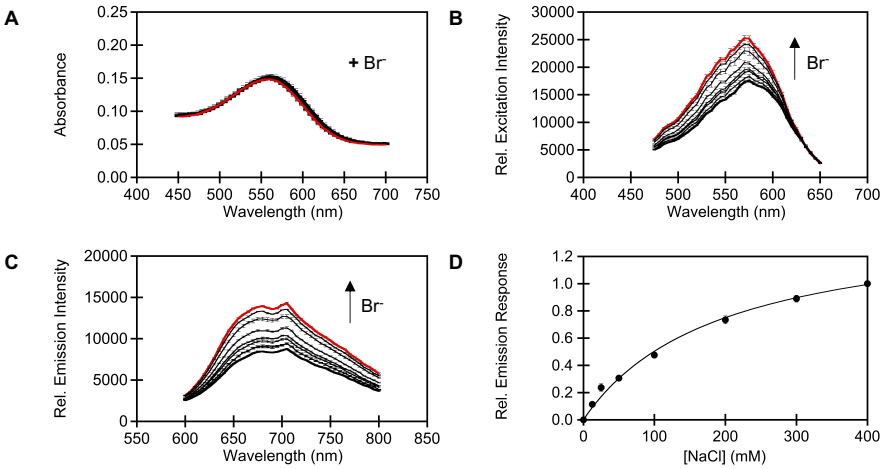

Figure S17. (A) Absorbance, (B) excitation, and (C) emission spectra of ~3 μM purified GR1 in the presence of 0 (bold black), 12.5, 25, 50, 100, 200, 300, and 400 mM (red) sodium bromide. Arrow direction corresponds to increasing sodium bromide concentrations. (D) Integrated emission response of GR1 to sodium bromide from panel (C) to determine the apparent dissociation constant (K_d). The K_d for bromide is 194 ± 14 mM. Excitation for GR1 was provided at 530 nm, and the emission was integrated from 600–800 nm. Spectra were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 5. The average of three technical replicates with standard error of the mean is reported.

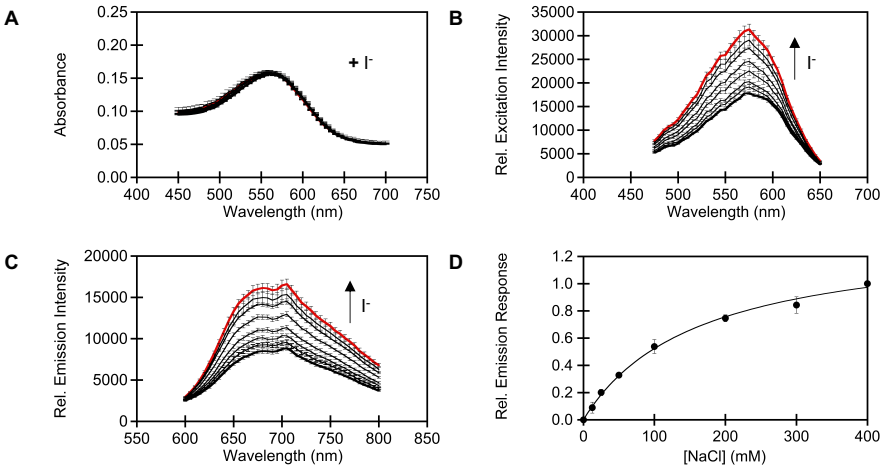

Figure S18. (A) Absorbance, (B) excitation, and (C) emission spectra of ~3 μM purified GR1 in the presence of 0 (bold black), 12.5, 25, 50, 100, 200, 300, and 400 mM (red) sodium iodide. Arrow direction corresponds to increasing sodium iodide concentrations. (D) Integrated emission response of GR1 to sodium iodide from panel (C) to determine the apparent dissociation constant (K_d). The K_d for iodide is 170 ± 34 mM. Excitation for GR1 was provided at 530 nm, and the emission was integrated from 600–800 nm. Spectra were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 5. The average of three technical replicates with standard error of the mean is reported.

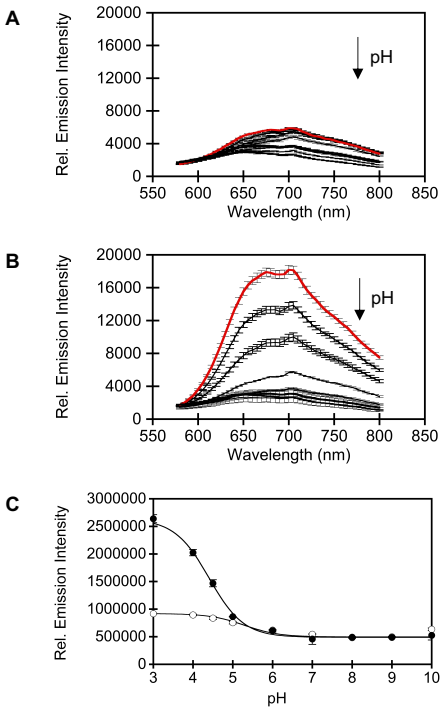

Figure S19. The pH titration of ~3 μM *wt*GR in (A) 0 mM sodium chloride and (B) 400 mM sodium chloride. Arrow direction corresponds to increasing pH. (C) Determination of the pK_a for the protonated retinylidene Schiff base chromophore (SBC) in the absence (open circles) and presence of 400 mM sodium chloride (filled circles). The pK_a of the SBC for *wt*GR is 4.9 ± 0.1 and 4.6 ± 0.1 in the absence and presence of 400 mM sodium chloride, respectively. Excitation was provided at 530 nm, and the emission was integrated from 580–800 nm. Spectra were acquired in 50 mM sodium acetate buffer from pH 3–5, 50 mM sodium phosphate buffer from pH 6–8, or 50 mM N-cyclohexyl-3-aminopropanesulfonic acid buffer at pH 10 containing 600 mM sodium gluconate. The average of three technical replicates with standard error of the mean is reported.

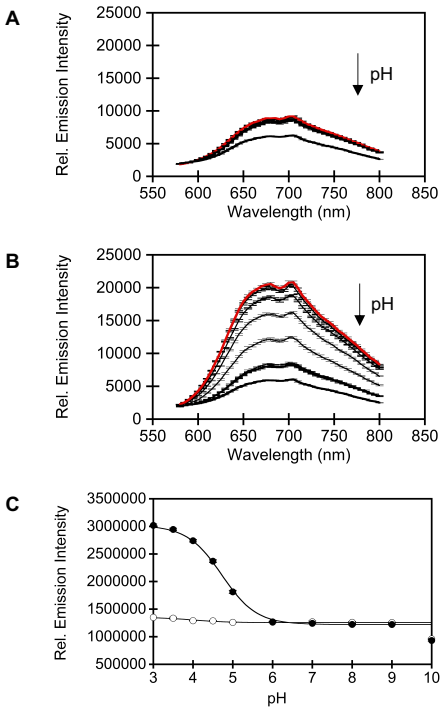

Figure S20. The pH titration of ~3 μM GR1 in (A) 0 mM sodium chloride and (B) 400 mM sodium chloride. Arrow direction corresponds to increasing pH. (C) Determination of the pK_a for the protonated SBC in the absence (open circles) and presence of 400 mM sodium chloride (filled circles). The pK_a of the SBC for GR1 is 3.1 ± 0.1 and 4.8 ± 0.1 in the absence and presence of 400 mM sodium chloride, respectively. Excitation was provided at 530 nm, and the emission was integrated from 580–800 nm. Spectra were acquired in 50 mM sodium acetate buffer from pH 3–5, 50 mM sodium phosphate buffer from pH 6–8, or 50 mM N-cyclohexyl-3-aminopropanesulfonic acid buffer at pH 10 containing 600 mM sodium gluconate. The average of three technical replicates with standard error of the mean is reported.

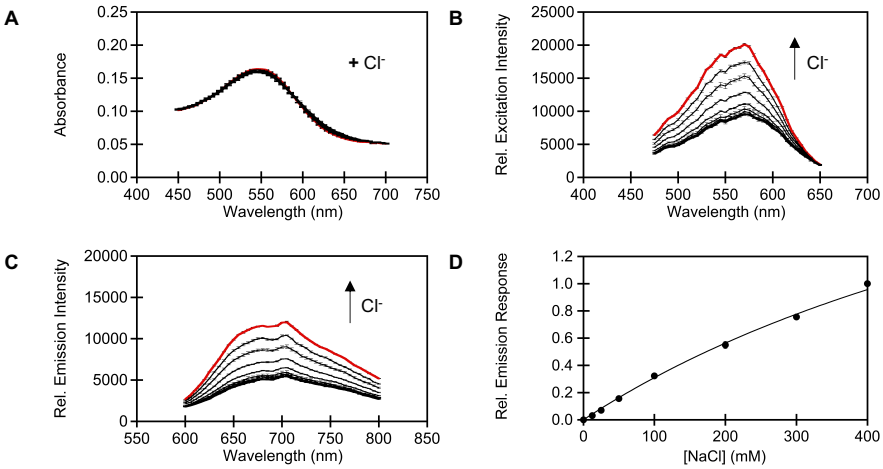

Figure S21. (A) Absorbance, (B) excitation, and (C) emission spectra of ~3 µM purified *wt*GR in the presence of 0 (bold black), 12.5, 25, 50, 100, 200, 300, and 400 mM (red) sodium chloride. Arrow direction corresponds to increasing sodium chloride concentrations. (D) Integrated emission response of *wt*GR to sodium chloride from panel (C) to determine the apparent dissociation constant (K_d). The K_d for chloride is 944 ± 123 mM. Excitation for *wt*GR was provided at 530 nm, and the emission was integrated from 600–800 nm. Spectra were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 4. The average of three technical replicates with standard error of the mean is reported.

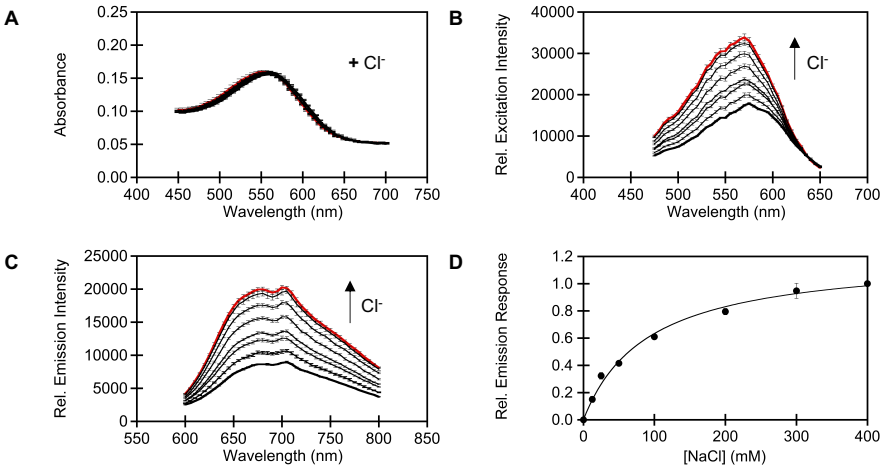

Figure S22. (A) Absorbance, (B) excitation, and (C) emission spectra of ~3 μM purified GR1 in the presence of 0 (bold black), 12.5, 25, 50, 100, 200, 300, and 400 mM (red) sodium chloride. Arrow direction corresponds to increasing sodium chloride concentrations. (D) Integrated emission response of GR1 to sodium chloride from panel (C) to determine the apparent dissociation constant (K_d). The K_d for chloride is 97 ± 6 mM. Excitation for GR1 was provided at 530 nm, and the emission was integrated from 600–800 nm. Spectra were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 4. The average of three technical replicates with standard error of the mean is reported.

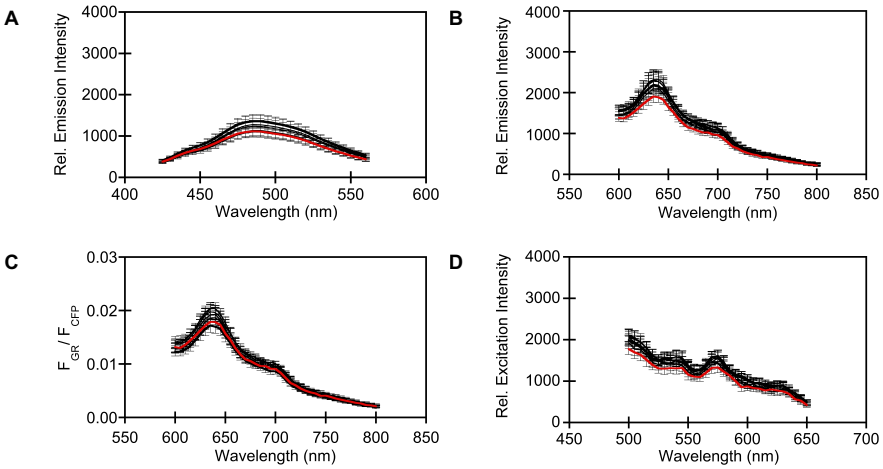

Figure S23. Spectroscopic characterization of *wt*GR-CFP in live *E. coli* in 50 mM sodium acetate buffer at pH 5 with 0 (bold black), 12.5, 25, 50, 100, 200, and 400 mM (red) sodium chloride. (A) Emission spectra of CFP. Excitation was provided at 390 nm, and the emission was collected from 425–560 nm. (B) Emission spectra of *wt*GR-CFP. Excitation was provided at 530 nm, and the emission was collected from 600–800 nm. (C) Emission spectra of *wt*GR-CFP (F_{GR}) normalized by the integrated emission of the CFP from 425–560 nm (F_{CFP}). (D) Excitation spectra of *wt*GR-CFP for the rhodopsin emission at 710 nm. The excitation maximum is at 570 nm. The average of three biological replicates with standard error of the mean is reported.

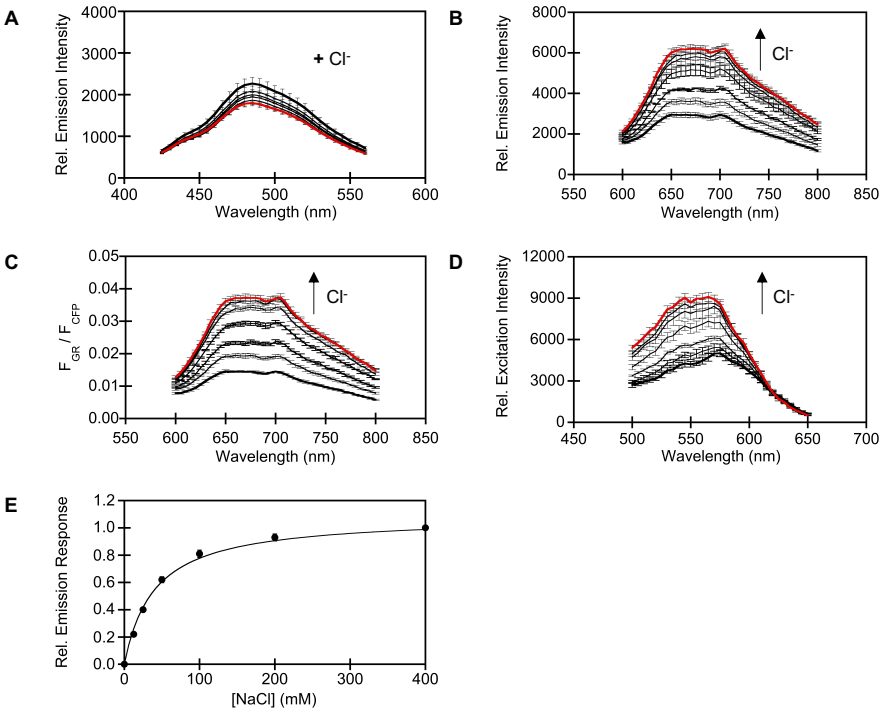

Figure S24. Spectroscopic characterization of GR1-CFP in live *E. coli* in 50 mM sodium acetate buffer at pH 5 with 0 (bold black), 12.5, 25, 50, 100, 200, and 400 mM (red) sodium chloride. (A) Emission spectra of CFP. Excitation was provided at 390 nm, and the emission was collected and integrated from 425–560 nm (F_{CFP}). (B) Emission spectra of GR1-CFP. Excitation was provided at 530 nm, and the emission was collected from 600–800 nm. (C) Emission spectra of GR1-CFP (F_{GR}) normalized by the integrated emission of the CFP from 425–560 nm (F_{CFP}). (D) Excitation spectra of GR1 for the rhodopsin emission at 710 nm. The excitation maximum is at 570 nm. Arrow direction corresponds to increasing chloride concentrations. (E) Normalized integrated emission response of GR1-CFP to chloride from panel (C) to determine the apparent dissociation constant (K_d). The K_d for chloride is 42 ± 1 mM. The data shown is for the rhodopsin emission integrated from 600–800 nm normalized by F_{CFP}. The average of three biological replicates with standard error of the mean is reported.

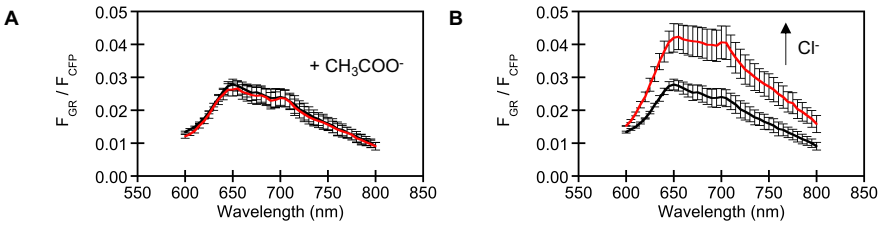

Figure S25. Normalized emission spectra of GR1-CFP in the presence of 0 (black) and 400 mM (red) sodium (A) acetate and (B) chloride in live *E. coli*. Arrow direction corresponds to the response with increasing anion concentrations. All spectra were acquired in 50 mM sodium phosphate buffer at pH 5. Excitation for GR1 was provided at 530 nm, and the emission was collected from 600–800 nm (F_{GR}). Excitation for CFP was provided at 390 nm, and the emission was collected and integrated from 425–560 nm (F_{CFP}). The GR1 emission (F_{GR}) was normalized to the integrated emission response of CFP (F_{CFP}). The average of three biological replicates with standard error of the mean is reported.

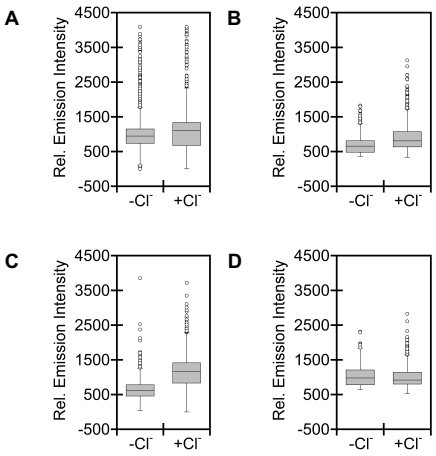

Figure S26. Single-cell analysis of the *wt*GR-CFP emission response to 400 mM sodium chloride for the (A) rhodopsin emission and (B) CFP emission (for 0 mM sodium chloride n = 2,829 regions of interest (ROIs); for 400 mM sodium chloride n = 3,818 ROIs). Single-cell analysis of the GR1-CFP emission response to 400 mM sodium chloride for the (C) rhodopsin emission and (B) CFP emission (for 0 mM sodium chloride n = 1,890 ROIs; for 400 mM sodium chloride n = 2,913 ROIs). Boxplots represent the analysis for all of the cells in the fields of view with the lower and upper quartile data enclosed by the gray box. The median values are indicated by the black line in the gray box, and the minimum and maximum values for each data set are indicated by the lines extending below and above the gray box. Data points that fall outside these parameters are considered outliers and are shown as open circles. At least four different fields were analyzed for each biological replicate (n = 3).

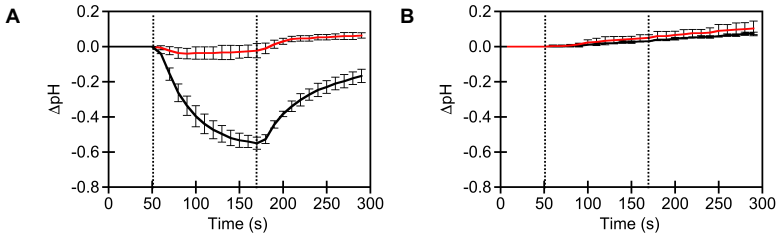

Figure S27. Light-induced pumping activity of (A) *wt*GR-CFP and (B) GR1-CFP in live *E. coli* measured in 400 mM sodium chloride (black) and 400 mM sodium chloride with 30 μM CCCP (red). The sample was illuminated at t = 50 s and t = 180 s as denoted by the vertical dotted lines. The average of three biological replicates with standard error of the mean is reported.

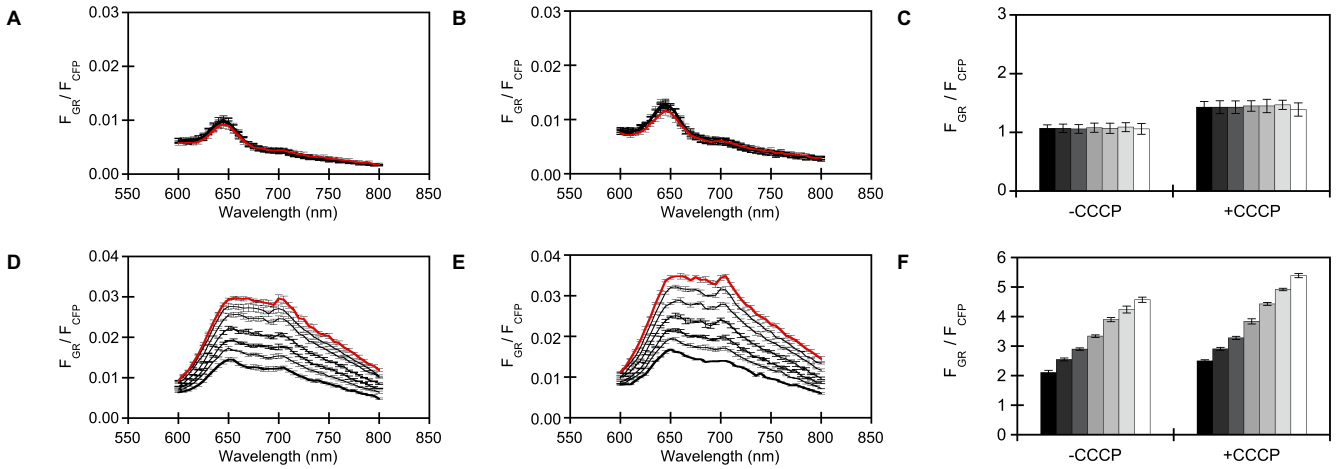

Figure S28. Normalized emission spectra of *wt*GR-CFP in live *E. coli* treated with (A) 0 μM or (B) 30 μM CCCP and in the presence of 0 (bold black), 12.5, 25, 50, 100, 200, and 400 mM (red) sodium chloride. Normalized emission spectra of GR1-CFP in live *E. coli* treated with (D) 0 μM or (E) 30 μM CCCP and in the presence of 0 (bold black), 12.5, 25, 50, 100, 200, and 400 mM (red) sodium chloride. Excitation for the rhodopsins was provided at 530 nm, and the emission was collected and integrated from 600–800 nm (F_{GR}). Excitation for CFP was provided at 390 nm, and the emission was collected and integrated from 425–560 nm (F_{CFP}). Normalized integrated emission response (F_{GR}/F_{CFP}) of (C) *wt*GR-CFP and (F) GR1-CFP to 0 mM (black bar), 12.5, 25, 50, 100, 200, and 400 mM (white bar) sodium chloride. All spectra were acquired in 50 mM sodium acetate buffer at pH 5. Cells were treated with 0 μM or 30 μM CCCP in DMSO for 30 min. The average of three biological replicates with standard error of the mean is reported.

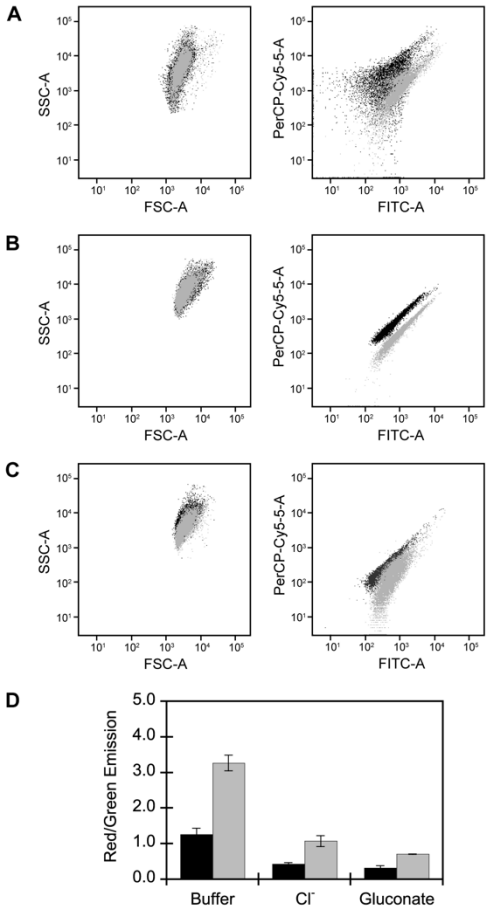

Figure S29. Analysis of the membrane potential with $DiOC_2(3)$ stained *E. coli* in 50 mM sodium acetate buffer at pH 5 using flow cytometry. Representative forward and side scatter (left) and fluorescence plots (right) of *E. coli* treated with 0 µM (black) or 30 µM CCCP (light gray) in the presence of (A) 0 mM, (B) 400 mM sodium chloride, and (C) 400 mM sodium gluconate. (D) Plot of the median fluorescence intensity of the red emission at 710 nm (PerCP-Cy5-5) normalized by the green emission at 530 nm (FITC) in the absence (black bars) or presence of CCCP (gray bars). The average of three biological replicates with standard error of the mean is reported.

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