SUPPORTING INFORMATION

EXPERIMENTAL SECTION

UV–Vis spectroscopy of the purified Rho

The dark-state absorption spectrum of Rho was recorded on a Lambda-800 spectrophotometer (PerkinElmer Life Sciences) in a 50-μL micro-cuvette with a 10-mm path length. The receptor was then photobleached by irradiating the sample for 30 s with a 335-mW 505-nm LED light source (Thorlabs) placed on top of the cuvette before acquiring the light spectrum. The dark−light difference spectrum at 500 nm was used to calculate the concentration of Rho using an extinction coefficient of 40,600 $M^{-1}cm^{-1}$. The Alexa-488 concentration was calculated from 495-nm absorbance in the light spectrum using the extinction coefficient 73,000 M^{-1} cm^{-1}. The labeling stoichiometry is the ratio of the Alexa-488 and Rho concentrations determined this way.

In-gel fluorescence to detect covalent Rho labeling

The labeled and purified Rho samples (50 ng per lane) were separated 4%–12% SDS-PAGE under reducing conditions. In order to minimize receptor aggregation, the samples were not heated prior to loading. Gels were briefly washed in ultrapure water and then visualized on a confocal Typhoon 9400 fluorescence scanner (GE) with 488-nm laser excitation and 510–520 nm band-pass for the Alexa488 emission.

Silver staining to quantify protein concentration.

After in-gel fluorescence, the SDS-PAGE gel was washed with the fixative buffer (40% water, 50% methanol, 10% acetic acid) for at least one hour with two or three changes of buffer. Then the gel was transferred into ultrapure water to remove the residual acid and methanol. The staining buffer was prepared fresh by adding solution A (0.4 g silver nitrate dissolved in 2 mL water) drop wise into solution B (0.7 mL 30% ammonium hydroxide added into 10.5 mL 0.36% sodium hydroxide solution) under constant stirring. Following the clearing of the brown precipitate, the total volume of the staining buffer was brought to 50 mL with ultrapure water. The gel was stained under

gentle agitation for 10 to 15 minutes, and briefly washed with ultrapure water to remove the staining buffer. Meanwhile, the developing buffer was prepared as follows: mix 30% formaldehyde (50 μL) with 1% citric acid (0.5 mL), and add water to 100 mL. The time needed for achieving best contrast may vary from one experiment to another. Typically, when the background began to turn yellow, we stopped development by transferring the gel into 1% acetic acid. After thorough washing in water, the silver-stained gel was scanned with an HP flat bed scanner and analyzed with ImageJ.

Determination of the partition coefficient of Alexa488-DIBO between micelle and water

Approximately 5 μM Alexa488-DIBO solution was prepared in 1× DPBS buffer containing various concentrations of DM micelles (up to ~ 1% (w/v)). 400 μL of the solution was placed in an ultrafiltration spin filter (Amicon Ultra 0.5 mL centrifugal filters, MWCO 10 kDa) and centrifuged at 14,000×g for 5~7 min until the volume of the filtrate was approximately 200 μL. The accurate volumes of the filtrate and the retentate were determined by pipetting. The concentrations of Alexa488-DIBO in the filtrate and retentate were quantified by UV-vis spectroscopy (ε_{495nm} = 73, 000 M^{-1} cm^{-1}).

SUPPLEMENTARY RESULTS

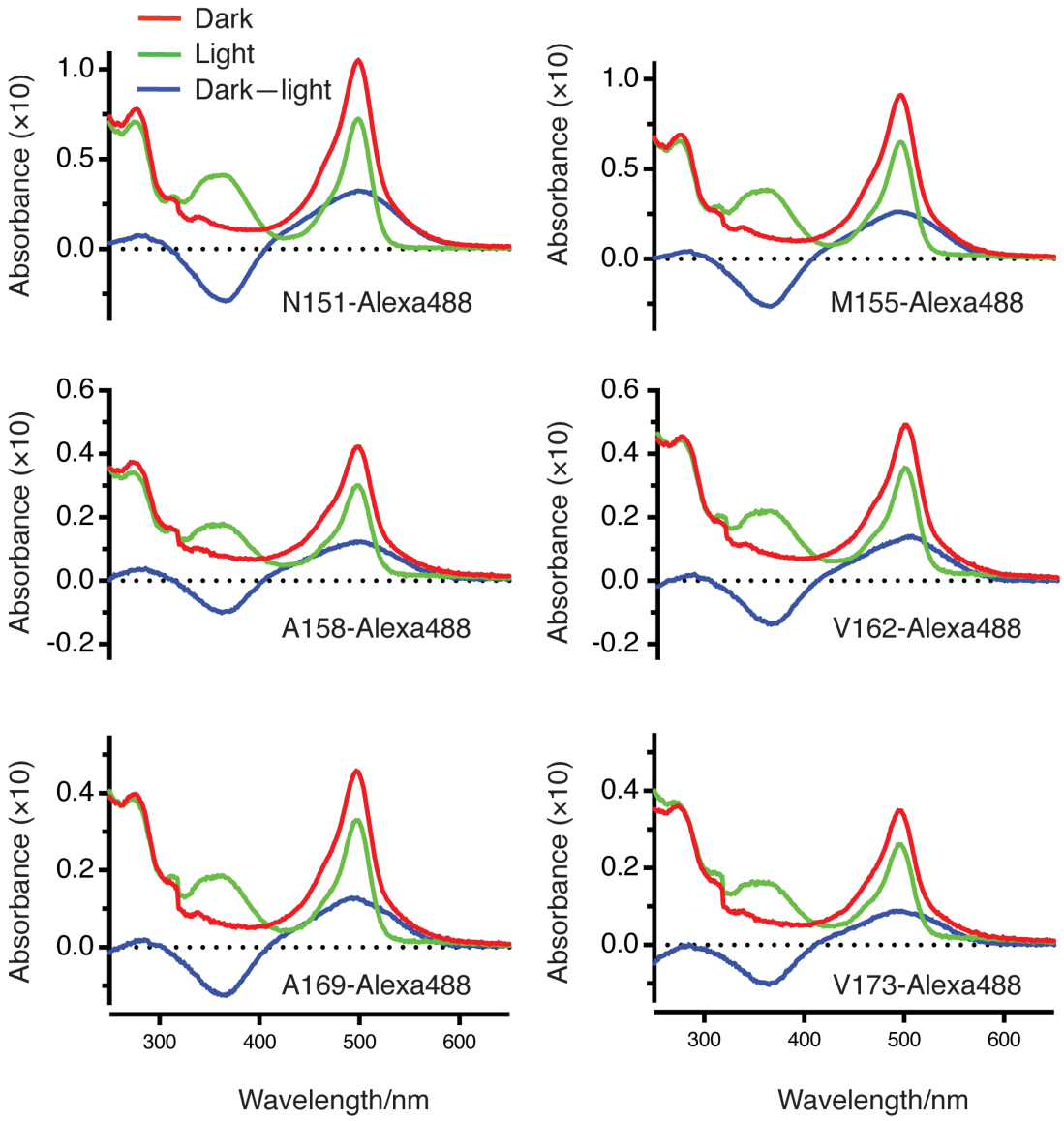


Figure S1. The UV-Vis spectra of the Alexa488-Rho variants labeled at TM4 sites (*cf.* Figure 3B). The 11-*cis*-retinal regenerated cells were lysed with the solubilization buffer (1 mL per 10^7 cells, 1% (w/v) DM, 50 mM HEPES or Tris-HCl, pH 6.8, 100 mM NaCl, 1 mM $CaCl_2$ with Complete EDTA-free Protease Inhibitor Cocktail, Roche) for at least 1 h at 4°C. The lysate was cleared by centrifugation at 100,000 × g for 30 min and incubated overnight at 4°C with 1D4-mAb-sepharose 2B (100 µL). The resin was transferred into a 1.5-mL Eppendorf tube and washed three times for 30 min each with reaction buffer (0.1% DM (w/v) DPBS, pH 7.2; 0.5 mL). Then reaction buffer (200 µL) was mixed with

the resin (100 μL) to give 300 μL slurry. Then Alexa488-DIBO (3 μL of 5 mM stock solution DMSO) was added to mixture to give a final concentration of 50 μM. The reaction was allowed to proceed at 25 °C for 18 h. After the reaction was complete, the resin was transferred into a microporous centrifugal filtering unit (Microcon-MC pore size 0.48 μm, Millipore) and washed to remove excess Alexa488-DIBO (5 times with each 0.4 mL; 0.1% DM (w/v) DPBS, pH 7.2). The labeled receptor was eluted by adding the C9 peptide (TETSQVAPA, 0.33 mg/mL, 2 mM sodium phosphate buffer, pH 6.0). *Red*: dark-state Rho; *green*: photobleached Rho; *blue*: the difference spectrum (dark–light). The dark−light difference spectrum at 500 nm was used to calculate the concentration of Rho (c_{Rho}) using an extinction coefficient of 40,600 M^{-1} cm^{-1}. The Alexa488 concentration (c_{Alexa488}) was calculated from 495-nm absorbance in the light spectrum using an extinction coefficient of 73,000 M^{-1} cm^{-1}. The label-to-protein ratio was calculated as $c_{\mathrm{Alexa488}}/c_{\mathrm{Rho}}$.

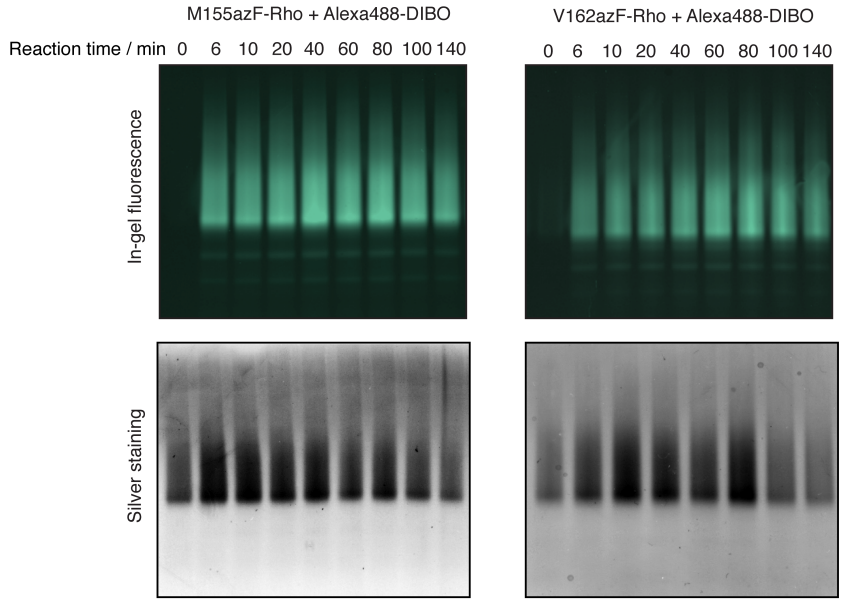
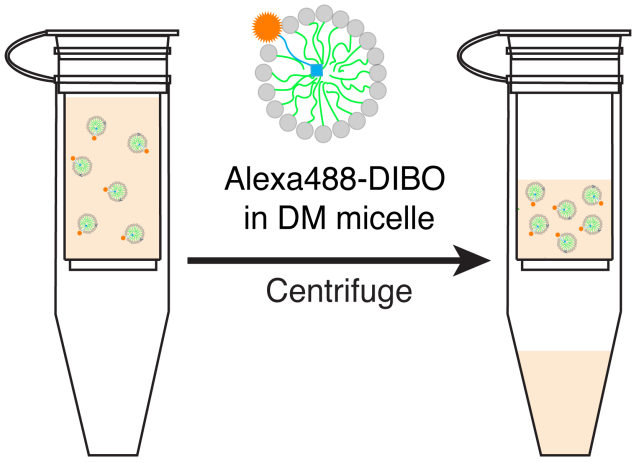


Figure S2. Kinetic study of the SpAAC reaction for M155azF-Rho and V162-azF Rho. The reaction was performed under the same conditions as described in Figure S1. The reaction mixture consisted of azF-Rho bound to 100 μL 1D4-sepharose resin and 200 μL reaction buffer. The initial concentration of Alexa488-DIBO (10 μM) was approximately 20-fold excess molarity. At the indicated time points, an aliquot of the resin/buffer mixture (30 μL) was taken out and added into a clean 1.5-mL Eppendorf tube. The reaction was quenched by adding pre-cooled wash buffer and centrifugation. The excess Alexa488-DIBO was washed away and the labeled receptor was eluted by adding the C9 peptide. Upper panel: the time course of the reaction shown by in-gel fluorescence. For each time point, 10 μL of sample was loaded to the SDS-PAGE gel and scanned with a Typhoon9400 scanner (ex: 488 nm, em: 520 ±10 nm). Lower panel: the amount of protein loading was obtained from silver staining of the same gel. The fluorescence intensity (I_F) and silver staining intensity (I_S) for each band were quantified with ImageJ. The relative extent of labeling (fluorescence signal corrected for protein loading) was calculated ratios of I_F/I_S. The normalized extent of reaction was plotted versus the reaction time (upon completion the extent of reaction is assigned to 1.0). The data was fitted using pseudo first-order kinetic model (*cf.* Figure 4A).

Filtration experiment to determine the partition coefficient of Alexa488-DIBO between DM micelles and PBS buffer

The experiment is based on the different molecular weight of Alexa488-DIBO (MW = 1039.23) and DM micelles (~50 kDa). The molecular weight cut-off of the microporous membrane is 10 kDa. Therefore, the heavier DM micelles, together with the Alexa488-DIBO partitioning into them, are expected to remain in the retentate fraction, while the free Alexa488-DIBO will flow through the membrane. The critical micelle concentration (cmc) of DM is low (0.17 mM, 0.009%) and consequently the filtration of DM monomers through the membrane is negligible in these experiments.



Scheme S1. The filtration experiment with spin filter. Alexa488-DIBO solution with DM was added into the spin filter (MWCO = 10 kDa). During centrifugation, the free dyes penetrate the membrane, while the dyes bound to DM micelles remain in the retentate.

Analysis of the experimental results

Symbols: c, concentration; n, amount of substance; V, volume; P, partition coefficient; f, weight/volume percent of DM.

Superscript: B, bound; F, free; mic, micelle; buf, buffer.

Subscripts: 0, the initial condition; t, the condition at time point t; 1, filtrate; 2, retentate.

The concentration of DM-micelle bound and free Alexa488-DIBO

The partition coefficient of Alexa488-DIBO between DM micelles and buffer is defined as:

$$P = \frac{c^{\mathrm{B}}}{c^{\mathrm{F}}} \qquad \text{Eq. (1)}$$

Here "bound" denotes the Alexa488-DIBO molecules in the DM micelle, and "free" the molecules in the buffer. It can be re-written as:

$$P = \frac{n^{\mathrm{B}} / V^{\mathrm{mic}}}{n^{\mathrm{F}} / V^{\mathrm{buf}}} \qquad \text{Eq.(2)}$$

The total volume of the solution equals the addition of the volume of DM micelle and the volume of buffer.

$$V = V^{\mathrm{mic}} + V^{\mathrm{buf}} \qquad \text{Eq.(3)}$$

Assuming the density of DM micelles is very close to that of water the mass concentration of DM (f) approximates the volume fraction:

$$V^{\mathrm{mic}} / V = f \qquad \text{Eq.(4)}$$

Because f is generally below 0.01, V^{mic} is neglegible compared to V^{buf}.

$$V^{\mathrm{buf}} \approx V \qquad \text{Eq.(5)}$$

The total moles of Alexa488-DIBO (n) is:

$$n = n^{\mathrm{B}} + n^{\mathrm{F}} \qquad \text{Eq.(6)}$$

The amount of Alexa488-DIBO in the micelles is:

$$n^{\mathrm{B}} = \frac{Pf}{1+Pf} n \qquad \text{Eq.(7)}$$

and

$$n^{\mathrm{F}} = \frac{1}{1+Pf} n \qquad \text{Eq.(8)}$$

The molar concentrations of Alexa488-DIBO bound in DM micelles and free in the buffer are:

$$c^{\mathrm{B}} = \frac{n^{\mathrm{B}}}{V^{\mathrm{mic}}} = \frac{Pf}{1+Pf} \frac{n}{V^{\mathrm{buf}}} \qquad \text{Eq.(9)}$$

and

$$c^{\mathrm{F}} = \frac{n^{\mathrm{F}}}{V^{\mathrm{buf}}} = \frac{1}{1+Pf}\frac{n}{V^{\mathrm{buf}}} \qquad \text{Eq.(10)}$$

Substitute Eq. (4) into Eq.(9), and Eq.(5) into Eq.(10) we have

$$c^{\mathrm{B}} = \frac{P}{1+Pf}\frac{n}{V} = \frac{P}{1+Pf}c \qquad \text{Eq.(11)}$$

and

$$c^{\mathrm{F}} = \frac{1}{1+Pf}\frac{n}{V} \qquad \text{Eq.(12)}$$

Because the apparent concentration (c) was used to calculate the reaction rate (k_2), the rate enhancement factor is $P/(1+Pf)$. It can be seen that for a given P, using lower concentration of DM (smaller f) gives higher effective concentration. Practically the concentration of DM must be above its critical micelle concentration (CMC), which is 0.17 mM, corresponding to 0.009% (*w*/*v*) to prevent precipitation of Rho. In our labeling and kinetics study we used 0.1% (*w*/*v*).

Derivation of the partition coefficient (P) from the filtration experiment

As the volume of the retentate decreases during centrifugation, the weight/volume percent of DM is:

$$f = \frac{V_{2,0}}{V_{2,\mathrm{t}}} f_0 \qquad \text{Eq.(13)}$$

The amount of free Alexa488-DIBO in the retentate is:

$$n_2^{\mathrm{F}} = \frac{1}{1+Pf} n_2 \qquad \text{Eq.(14)}$$

Substitute Eq.(13) into Eq.(14):

$$n_2^{\mathrm{F}} = \frac{1}{1+Pf_0V_{2,0}/V_{2,\mathrm{t}}} n_2 \qquad \text{Eq.(15)}$$

The concentration of free Alexa488-DIBO is:

$$c_2^{\mathrm{F}} = \frac{n_2^{\mathrm{F}}}{V_{2,\mathrm{t}}} = \frac{n_2}{V_{2,\mathrm{t}} + Pf_0V_{2,0}} \qquad \text{Eq.(16)}$$

Assuming fast equlibrium of Alexa488-DIBO between DM micelles and buffer, and only the Alexa488-DIBO in the buffer can flow through the membrane, thus the concentration of Alexa488-DIBO in the filtrate is equal to that of the free Alexa488-DIBO in the retentate:

$$c_{1,\mathrm{t}} = c_2^{\mathrm{F}} \qquad \text{Eq.(17)}$$

Substitute Eq.(17) into Eq. (18), we have:

$$c_{1,t} = \frac{c_{2,t} V_{2,\mathrm{t}}}{V_{2,\mathrm{t}} + P f_0 V_{2,0}} \qquad \text{Eq.(18)}$$

Note that

$$V_{2,0} = V_0 = V_{1,\mathrm{t}} + V_{2,\mathrm{t}} \qquad \text{Eq.(19)}$$

Substitute Eq. (19) into Eq.(18) and rearrange, we have:

$$P = \frac{\left(c_{2,\mathrm{t}} - c_{1,\mathrm{t}}\right) V_{2,t}}{c_{1,\mathrm{t}} \left(V_{1,t} + V_{2,t}\right) f_0} \qquad \text{Eq.(20)}$$

Table S1. The concentrations of Alexa488-DIBO and the volumes of filtrate and retentate (MWCO = 10 kDa)

f_0 of DM (%)	c_1 (μM)	V_1 (μL)	c_2 (μM)	V_2 (μL)	c_0 (μM)	Recovery* (%)
0	2.86	190	3.28	210	4.69	66
0	3.05	170	4.13	220	5.40	68
0.05	2.12	185	9.05	210	5.76	101
0.10	1.33	200	10.5	210	5.74	105
0.10	1.37	180	9.29	210	5.74	98
0.25	0.562	180	10.2	205	5.64	101
0.25	0.557	180	9.58	220	5.64	98
0.50	0.519	185	10.6	210	5.70	103
0.50	0.423	195	11.8	200	5.81	106
1.00	0.325	175	10.8	220	5.92	104

*Note: The recovery of Alexa488-DIBO was calculated as $\frac{c_1V_1 + c_2V_2}{c_0\left(V_1 + V_2\right)}$

Table S2. Calculation of the partition coefficient at different DM concentrations

f_0 of DM (%)	$\frac{\left(c_2 - c_1\right)V_2}{c_1\left(V_1 + V_2\right)}$	P ($\times 10^3$)	Rate enhancement
0	0.08	N.A.	N.A.
0	0.20	N.A.	N.A.
0.05	1.74	4.21	1.35×10^3
0.10	3.53	3.81	795
0.10	3.11	3.41	773
0.25	9.13	3.78	362
0.25	8.91	3.69	361
0.50	10.3	2.10	183
0.50	13.6	2.77	187
1.00	18.0	1.82	95

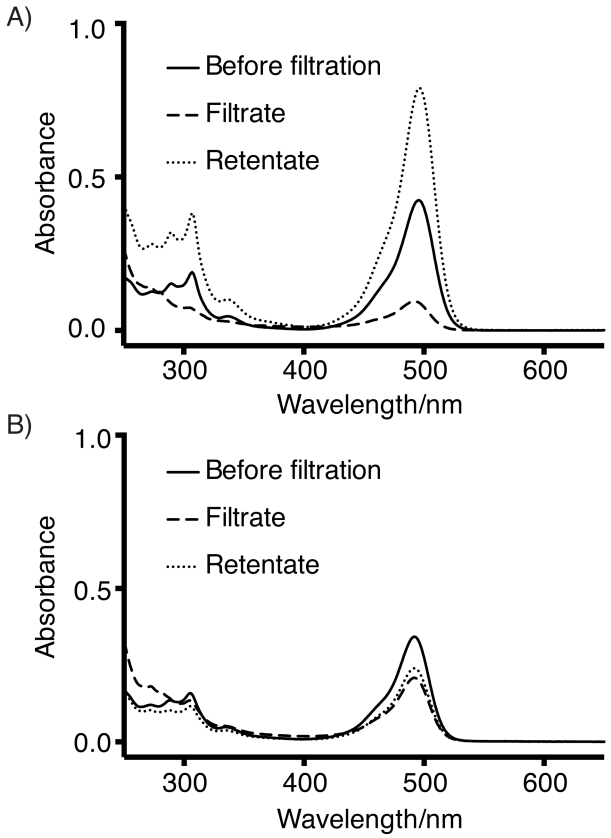


Figure S3. Representative spectra for the filtration experiment (10 kDa MWCO). A) Alexa488-DIBO solution (~5 μM) prepared in DPBS with 0.1% DM. *Solid*: the Alexa488-DIBO solution before filtration; *dash*: the filtrate; *dot*: the retentate. In the presence of DM, the recovery of Alexa488-DIBO was approximately 100%. B) Alexa488-DIBO solutions in DPBS. *solid*: before filtration; *dash*: the filtrate; *dot*: the retentate. In the absence of DM, the recovery of Alexa488-DIBO was lowered to approximately 67% likely due to adsorption to the ultrafiltration membrane. The concentration of Alexa488-DIBO was calculated from the 492-nm absorbance (ε_{495nm} = 73, 000 M^{-1} cm^{-1}).

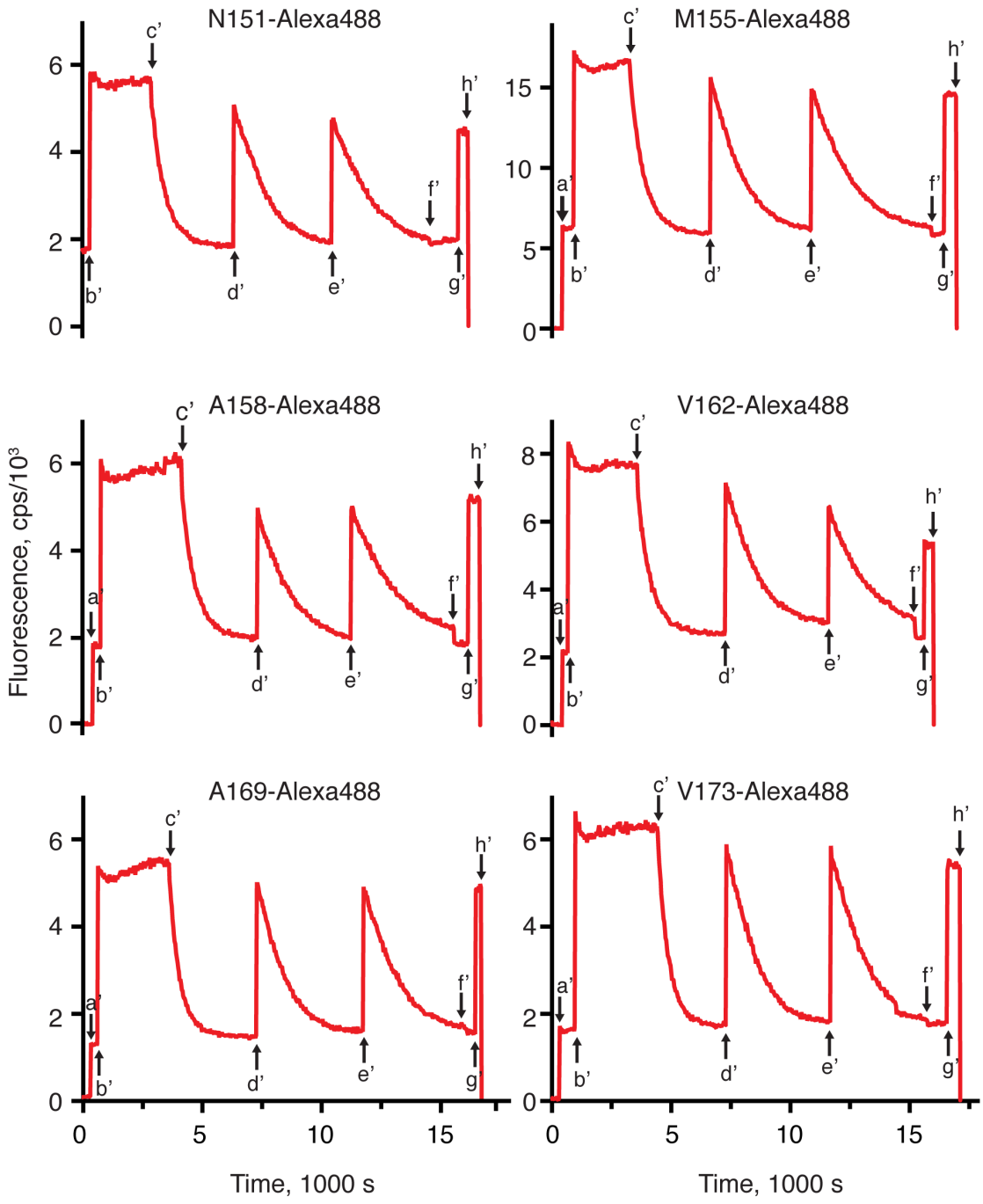


Figure S4. The fluorescence time course of the Alexa488-Rho variants in steady-state fluorescence quenching assay (*cf*. Figure 5). Fluorescence spectroscopy was performed at 28°C on a SPEX Fluorolog spectrofluorometer in photon counting mode. During time scan experiments the excitation wavelength was 480 nm with 0.2-nm band-pass to

minimize photobleaching, and the emission was measured at a wavelength of 525 nm with 15-nm band-pass. The Alexa488-labeled Rho elutions (30 µL) were added into the cuvette (indicated by a′) at a final concentration of 15–50 nM to the assay buffer (450 µL; 10 mg/mL POPC, 10 mg/mL CHAPS, 125 mM KCl, 25 mM MES, 25 mM HEPES, 12.5 mM KOH, pH 6.0) under constant stirring. Rho was photoactivated with green light (indicated by b′). The decay of the photoproducts to the apoprotein opsin was monitored for 45 min before 11-*cis*-retinal (final concentration ~1.5 µM) was added (indicated by c′). The exponential decay of the fluorescence back to the pre-illuminated levels indicated a pseudo first-order reaction regenerating rhodopsin from opsin. Repeated illumination (indicated by d′) resulted in a fluorescence change of similar magnitude but opposite sign as the regeneration reaction. Repeated bleaching (indicated by e′) resulted in a similar change of signal. The two additional illuminations at d′ and e′ illustrated the reversibility of the process. However, in this case opsin that forms by decay of the Meta-II state immediately regenerated to the dark state due to the presence of excess 11-*cis*-retinal. We added 50 mM hydroxylamine (NH_2OH) at pH 6 (indicated by f′) to inactivate retinal by oxime formation. Bleaching in presence of hydroxylamine (indicated by g′) resulted in fast decay of the photoproducts to yield opsin. The shutter was closed at the end of the experiment (indicated by h′).