EXPERIMENTAL SECTION

Materials

The expression vector for the amber suppressor tRNA and the evolved tRNA synthetase were described previously.[1, 2] We introduced the Y102 amber mutation into pMT4 that carries a synthetic gene encoding wt bovine Rho [3] by QuikChange mutagenesis (Agilent). The HEK293F cell lines, the transfection reagents (FreeStyle MAX), and expression media were obtained from Invitrogen/Thermo Fisher Scientific. Sepharose 2B resin was purchased from Sigma. 1D4-Sepharose 2B was prepared from 1D4 mAb and CNBr-activated Sepharose 2B (2 mg IgG per mL packed beads) as described before.[4] BCN-POE3-NH-DY549 (BCN-DY549) and BCN-POE3-NH_2 were obtained from SynAffix as dry powder, dissolved in DMSO and stored at –20°C. Fluorescein-5-maleimide (FL-5-ML) was obtained from Thermo Scientific and dissolved in DMSO (23 mM) and stored at –20°C. N-ethyl-maleimide (NEM) was purchased from Fluka. Iodoacetamide (IAM), and L-glutathione (GSH, reduced form) were purchased from Sigma-Aldrich.

Heterologous expression of azF-tagged Rho in mammalian cell culture

In this study, the wt Rho was obtained from bovine rod outer segment as described before.[5] Y102azF Rho was expressed in HEK293F suspension cell culture. The culture and transfection conditions have been described in full detail before. [6] Briefly, transfecting 30-mL culture requires 38.6 μg plasmid DNA for amber codon suppresion (18.4 μg of pMT4.Rho containing the amber codon, 18.4 μg of pSVB.Yam, and 1.84 μg pcDNA.RS-azF were mixed together). The transfected cells were cultured in serum-free FreeStyle 293 expression medium supplemented with 1 mM azF. The cells were

harvested 96 hours post-transfection. The harvested cells were resuspended in DPBS (Gibco, supplemented with leupeptin and aprotinin, Sigma) at a density of 10^7 cells/mL in a 15-mL conical, polypropylene tube (Falcon). In the dark room, 11-*cis*-retinal ethanolic solution was added into the cell suspension to a final concentration of 5 μM. After overnight regeneration at 4°C, the excess 11-*cis*-retinal was removed by spinning down the cells and discarding the supernatant fraction. The regenerated cells can be immediately used, or may be stored at −20°C for several months.

Bioorthogonal labeling of azF-tagged Rho

To facilitate purification, the reaction was performed by immobilizing wt or azF-tagged Rho to 1D4-mAb-sepharose 2B that specifically captured the C-terminus of the receptor. Similar procedures have been described in full detail before.[6] Briefly, the 11-*cis*-retinal regenerated cells expressing Y102azF-Rho 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 washed three times for 30 min each with 0.5 mL reaction buffer (0.1% DM in DPBS, pH 7.2). Then the reaction buffer (200 μL) was mixed with the resin (100 μL) to give 300-μL slurry. The BCN-DY549 stock solution (5 mM in DMSO) was directly added to into the reaction mixture to give the appropriate final concentration. The reaction was agitated with a thermomixer at 25 °C. After 18-19 h, or otherwise specified duration, the reaction was stopped by centrifugation and removal of the supernatant fraction. The resin was then transferred into a microporous centrifugal filtering unit (Microcon-MC pore size

0.48 μm, Millipore). The resin was first washed with the reaction/wash buffer three times (30 min incubation each time) to deplete the unreacted dyes, and then with a low-salt buffer (0.1% (w/v) DM, 2 mM sodium phosphate buffer, pH 6.0). The receptor was eluted with elution buffer (100 μL, no less than the volume of the resin; 0.33 mg/mL C9 peptide in 0.1% (w/v) DM, 2 mM sodium phosphate buffer, pH 6.0). The resin was incubated with the elution buffer on ice for at least 1 h. The purified receptor was collected in a clean 1.5-mL Eppendorf tube. The elution was repeated a second time. The combined elutions were supplemented with 150 mM NaCl before subsequent characterizations.

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 absorbance at 500 nm was used to calculate the concentration of Rho using an extinction coefficient of 40,600 $M^{-1}cm^{-1}$. The DY549 concentration was calculated from 561-nm absorbance in the light spectrum using the extinction coefficient 150,000 $M^{-1}\,cm^{-1}$.

In-gel fluorescence to detect covalent Rho labeling

The DY549-labeled and purified Rho samples (50~100 ng per lane) were separated on 4%–12% SDS-PAGE gel 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 532-nm laser excitation and 560/20 nm band-pass for the DY549 emission. For fluorescein-labeled samples, the excitation wavelength was 488 nm and the emission filter band-pass 510-520 nm.

Silver staining for quantifying protein concentration

After in-gel fluorescence, the SDS-PAGE gel was washed with the fixative buffer (40% water, 50% methanol, 10% acetic acid) for overnight with two or three changes of buffer. Then the gel was immersed in 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. The brown precipitates clear up quickly. Then ultrapure water was added to make a total volume of 50 mL. 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 by mixing 30% formaldehyde (50 μL) with 1% citric acid (0.5 mL) and then adding water to a total volume of 100 mL. The time needed for achieving best contrast might vary with sampling loading. Staining was stopped when the background turned yellow 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.

Evaluating the effects of βME on the functional integrity of Rho

ROS Rho captured by 1D4 mAb-derivatized sepharose 2B resin (1 μg Rho/1 mL resin) was incubated at 25 ºC with various concentrations of βME (0.1 mM to 100 mM) added to the reaction buffer (0.1% DM in DPBS, pH 7.2). In each sample 20 μL of resin

(packed volume) was resuspended in 80 μL of buffer to make a total volume of 100 μL. After 24 h, βME was removed by repeated washing and Rho was specifically eluted with the C9 peptide (50 μL × 2; 0.1% DM in DPBS, pH 7.2, 0.33 mg/mL C9 peptide). The eluted product was characterized by UV-Vis spectroscopy, and then separated by SDS-PAGE gel (~1μg/lane) followed by silver staining. The sample was also analyzed by Western blot to examine whether the additional bands observed in the 100 mM βME-treated sample was due to the denaturation and dissociation of 1D4 mAb (derived from mouse). The blot was probed with goat-anti-mouse IgG 800CW secondary antibody (LI-COR) and scanned on a LI-COR Odyssey Sa Infrared imager.

To evaluate whether βME forms disulfide bond with the free cysteine, ROS Rho bound to 1D4 resin (20 μL) was first incubated with βME (10 mM) at 25 ºC for 24 h. Then βME was thoroughly removed by washing (0.1% DM in DPBS, pH 7.2, 50× resin volume three times). Then the total volume was adjusted to 100 μL and supplemented with FL-5-ML (50 μM final concentration). The reaction was allowed to proceed at 25 ºC for 24 h in the thermomixer. In the end the labeled sample was washed and eluted (50 μL × 2; 0.1% DM in DPBS, pH 7.2, 0.33 mg/mL C9 peptide). The eluted product was characterized by UV-Vis spectroscopy. The concentration of fluorescein was calculated from the A495nm in the light spectra (ε_{495nm} = 68,000 $M^{-1}cm^{-1}$). The samples separated on SDS-PAGE gel were analyzed by in-gel fluorescence (~1μg/lane) and then by silver staining.

The formation of adduct product between BCN and βME

The reaction of BCN-POE3-NH_2 (100 μM) and βME (10 mM) were allowed to proceed in DPBS for 24 h and 48 h (in duplicate). At each time point, the reaction was

quenched with N-ethyl-maleimide (100 mM) to inactivate the excess βME. The sample was analyzed by LC-MS. The concentration of BCN and BCN-βME was evaluated from the integrated area of the mass peaks.

The reduction of azF under reducing conditions

AzF solution (water, 0.9 mM, 100 μL) was supplemented with 10 mM of the following reducing reagents: βME, dithiothreitol (DTT), glutathione (GSH, reduced form). Each condition was tested in duplicate. After 24- and 46-h reaction at room temperature, the reduction product was diluted by 10-fold and analyzed by LC-MS.

The reaction between cell lysates and BCN-DY549

The cell lysates were prepared by lysing HEK293F cells in 1% DM DPBS buffer (pH 7.2). The protein concentration of lysates were determined by Bio-Rad total protein assay and normalized to 100 mg/mL. In a different sample, the cell lysates were reacted with N-ethylmaleimide (20 mM) or iodoacetamide (20 mM) at 25 ºC for 24 h to block the free thiols. Then the cell lysates were reacted with BCN-DY549 (50 mM) at 25 ºC for 18 h under the indicated conditions (Figure S8) and analyzed by in-gel fluorescence and Coomassie staining,

Determination of the partition coefficient of BCN-DY549 between micelle and water

The experiment is based on the different molecular weight of BCN-DY549 (MW = 1179.37) 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 BCN-DY549 partitioning into them, are expected to remain in the retentate fraction, while the free BCN-DY549 will flow through the membrane (filtrate). The critical micelle concentration (CMC) of DM (0.17 mM, 0.009%) is well below the tested concentrations

of DM in the filtration experiment (≥ 0.05%) the filtration of DM monomers through the membrane is negligible in these experiments.

Approximately 3 μM BCN-DY549 solution was prepared in DPBS buffer containing various concentrations of DM micelles (up to ~ 1% (w/v)). 200 μL of the solution was placed in an ultrafiltration spin filter (Amicon Ultra 0.5 mL centrifugal filters, 10 kDa MWCO) and centrifuged at 14,000×g for 5~7 min until the volume of the filtrate was approximately 100 μL. The volumes of the filtrate and the retentate were measured with an adjustable pipette. The concentrations of DY549-BCN in the filtrate and retentate were quantified by UV–Vis spectroscopy (ε_{555nm} = 150, 000 M^{-1} cm^{-1}).

Analysis of the partition experiment

The effective concentrations of BCN-DY549 in the micelle (c^B) and in the buffer (c^F) are given by

$$c^{B} = \frac{P}{1+Pf}\frac{n}{V} = \frac{P}{1+Pf}c \qquad \text{Eq.(1)} \qquad \text{and} \qquad c^{F} = \frac{1}{1+Pf}\frac{n}{V} \qquad \text{Eq.(2)}$$

The partition coefficient (P) from the filtration experiment is given by

$$P = \frac{\left(c_{2,t} - c_{1,t}\right)V_{2,t}}{c_{1,t}\left(V_{1,t} + V_{2,t}\right)f_0} \qquad \text{Eq.(3)}$$

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

Superscript: B, bound; F, free.

Subscripts: 0, the initial condition; 1, filtrate; 2, retentate.

The complete derivation of the equations was given in our earlier report.[7]

Table S1. The concentrations of BCN-DY549 and the volumes of filtrate and retentate

f_0 of DM (%)	c_1 (μM)	V_1 (μL)	c_2 (μM)	V_2 (μL)	c_0 (μM)	Recovery* (%)
1	1.65	95	5.65	90	3.59	100
1	1.72	90	5.5	95	3.59	102
0.5	1.8	105	5.61	105	3.68	101
0.5	1.68	90	5.83	90	3.68	102
0.25	1.86	95	5.37	100	3.64	101
0.25	1.92	85	5.34	105	3.64	105
0.1	2.28	85	6.51	40	3.7	98
0.1	2.31	95	6.03	80	3.7	108
0.05	2.02	100	4.32	100	3.2	99
0.05	2.2	110	4.3	100	3.2	100

*Note: The recovery of BCN-DY549 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 of BCN-DY549 at different DM concentrations

f_0 of DM (%)	P	c_F/c_0	C_B/c_0
1	1024 ± 162	0.464 ± 0.008	54 ± 1
0.5	665 ± 101	0.467 ± 0.027	107 ± 5
0.25	390 ± 5	0.506 ± 0.003	198 ± 1
0.10	229 ± 25	0.602 ± 0.036	398 ± 36
0.05	115 ± 4	0.636 ± 0.036	675 ± 71

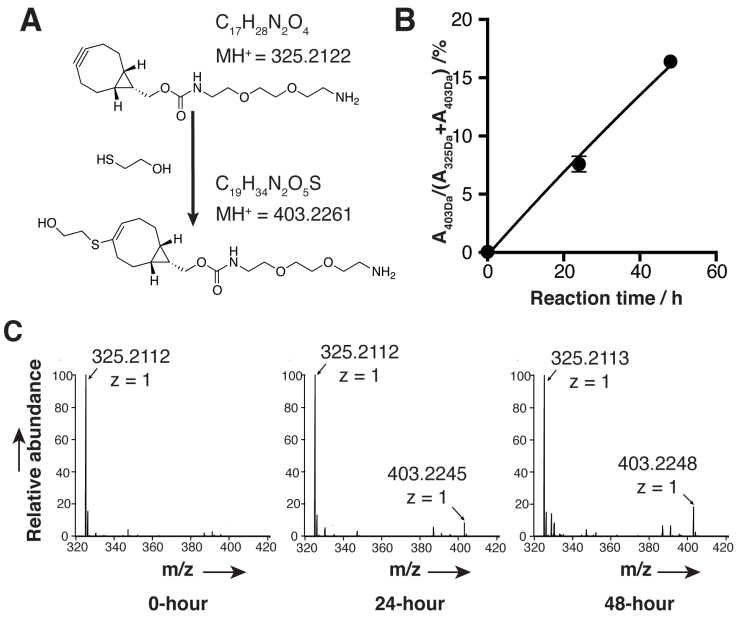

Figure S1. The formation of BCN-βME adduct. (A) The reaction between βME (100 mM) and BCN-POE_3-NH_2 (100 μM) results in an increase of molecular weight (+78.0139 Da). (B) The kinetics of adduct formation as measured by mass spectroscopy. The k_2 was calculated to be 10^{-4} $M^{-1}s^{-1}$. (C) The representative mass chromatograms. At $t = 0$, no adduct product was found. At 24 h and 48 h, the formation of the adduct product could be clearly detected.

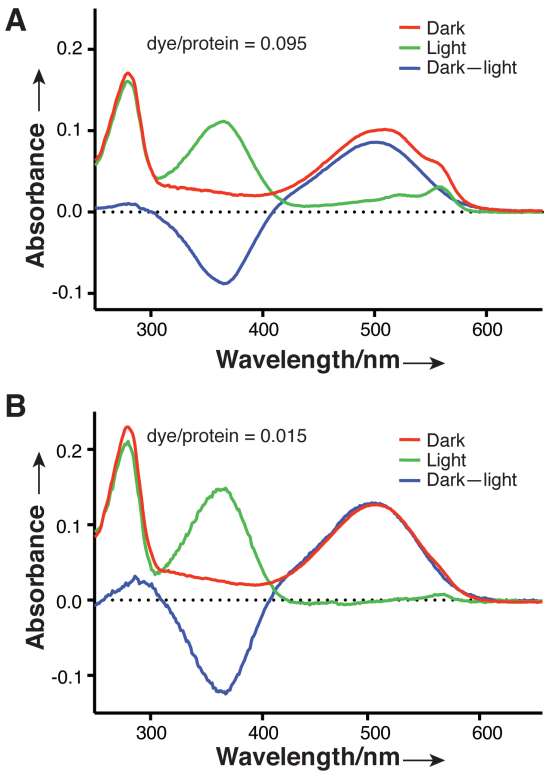

Figure S2. Labeling of wt Rho with BCN-DY549 (50 μM) in the presence of βME (14 mM). (A) The dark, light and difference (dark−light) spectra of the purified samples labeled in the presence of βME. (B) The spectra for Rho labeled in the absence of βME.

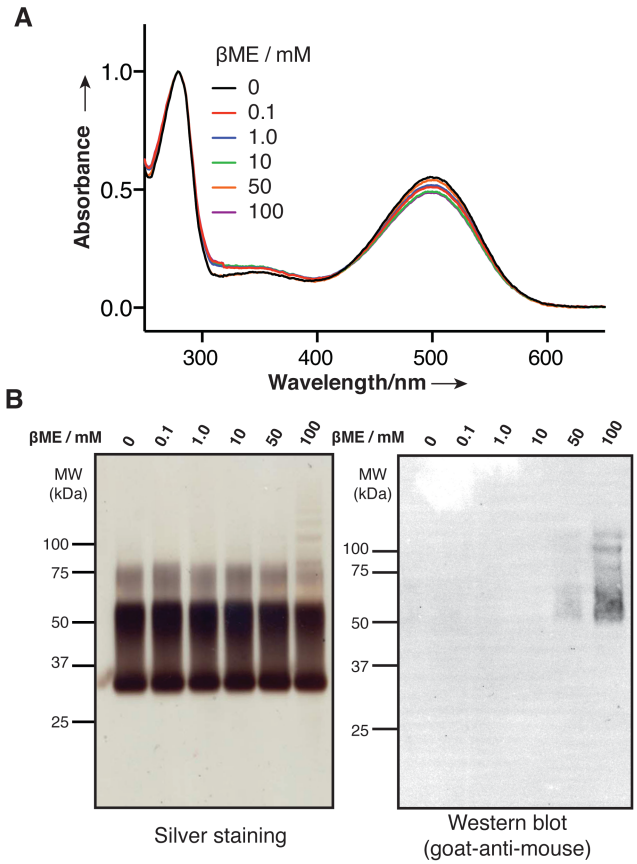

Figure S3. Incubation Rho with various concentrations of βME. (A) The UV-Vis spectra (normalized based on A_{280}) of purified Rho after incubation with the indicated concentration of βME. Rho was immobilized to 1D4-mAb sepharose resin during the incubation. (B) Left: the purified samples (~1 μg) were separated by 4–12% SDS-PAGE and visualized by silver staining. Additional bands were observed in the sample treated with 100 mM βME. Right: the purified samples were probed by goat-anti-mouse IgG secondary antibody, confirming that the additional bands observed in the 100 mM βME-treated sample was due to the dissociation of 1D4 mAb (derived from mouse) from sepharose 2B matrix. Minor dissociation of 1D4 mAb could be found in the 50 mM βME-treated sample as well.

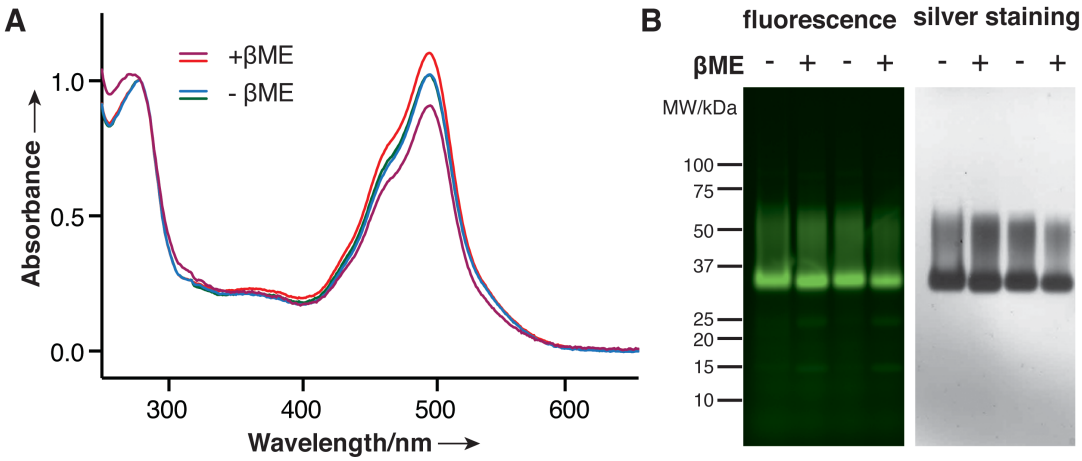

Figure S4. The accessibility of the free cysteines in Rho after βME treatment as probed by fluorescein-5-maleimide (FL-5-ML). (A) The spectra of purified Rho labeled with FL-5-ML. In the test group (+βME), Rho bound with 1D4-mAb resin was first incubated with βME (10 mM, 25 °C, 24 h) before reacting with FL-5-ML (50 μM). In the control group (−βME), Rho was directly reacted with FL-5-ML without the initial incubation with βME. The FL/Rho ratios for the test group and control group are 1.28 ± 0.08 and 1.01 ± 0.11, respectively. (B) The corresponding SDS-PAGE gel for the samples in **A**), first analyzed by in-gel fluorescence and then by silver staining. βME treatment causes slightly more dissociation of the antibody-light chain as judged from the in-gel fluorescence. Note that the efficiency of silver staining is sequence-specific and that the antibody light chain did not appear in the silver-staining image.

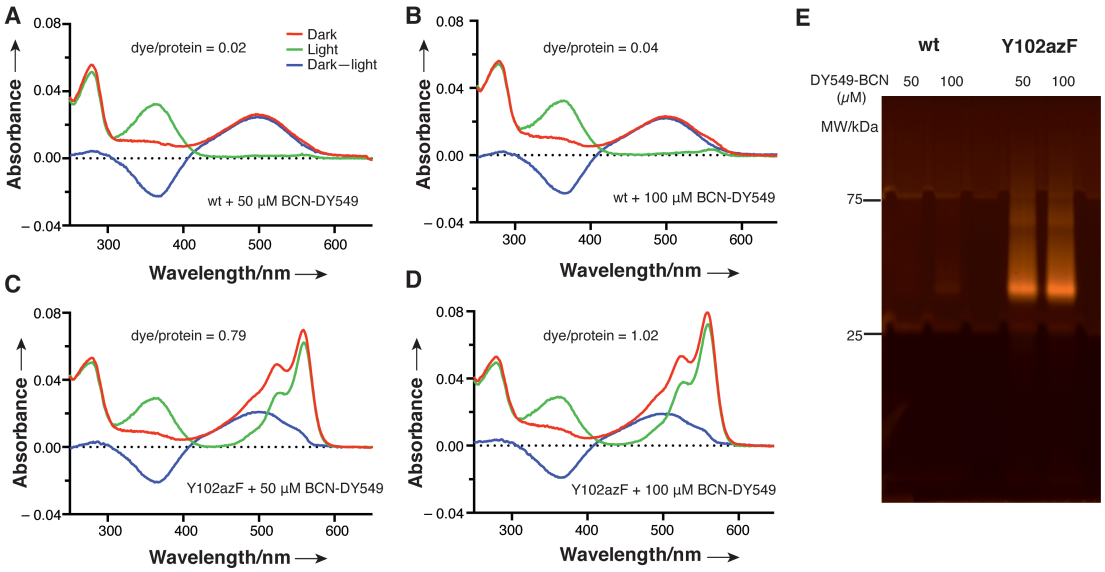

Figure S5. Labeling of wt and Y102azF Rho using different concentrations of BCN-DY549 in the presence of βME (14 mM). (A-D) The dark, light and difference (dark−light) spectra of the purified samples after treatment by BCN-DY549 (concentration indicated in the figure). All the reactions lasted 18 h at 25 ºC. (E) The corresponding in-gel fluorescence

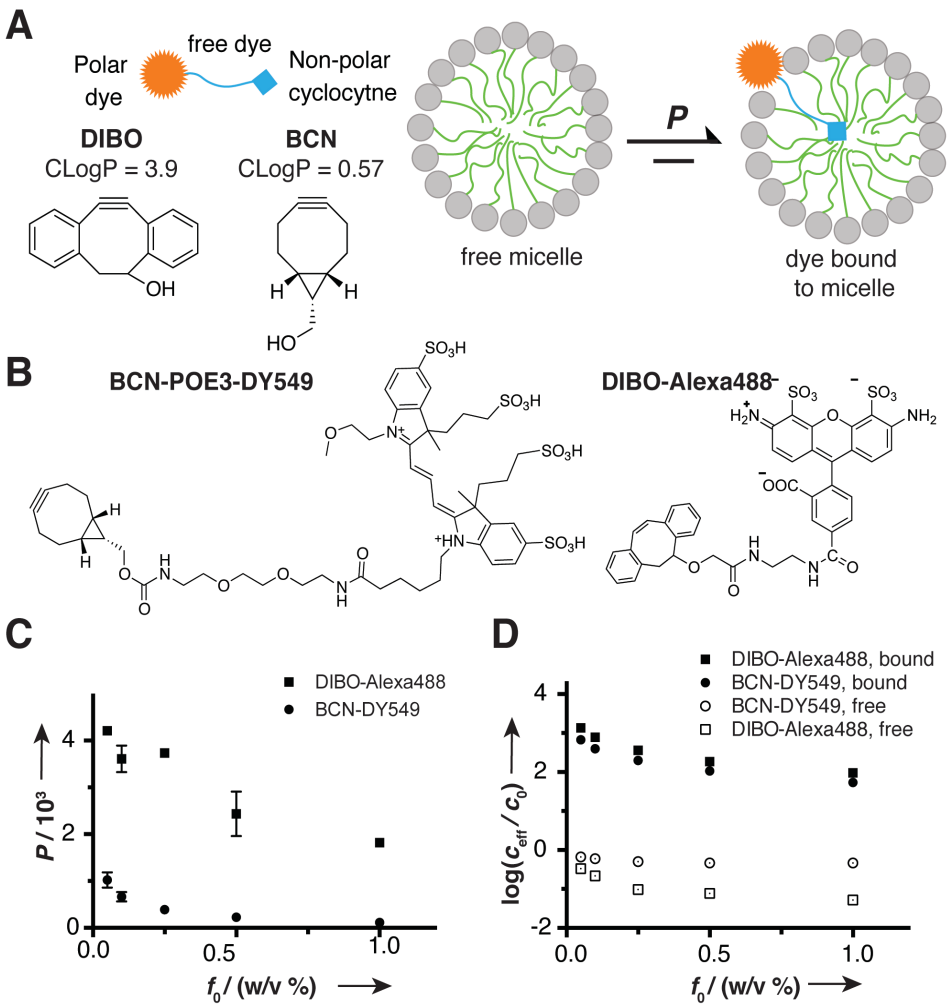

Figure S6. Comparing the partitioning behaviors of two cyclooctyne reagents between water and detergent (DM) micelles. (A) Both DIBO and BCN derivatives partition into the detergent micelles due to the hydrophobicity of the cyclooctyne moiety. DIBO is more hydrophobic than BCN by 3.3 unit of ^{c}LogP (Calculated by ChemDraw). (B) The molecular structures of BCN-DY549 and DIBO-Alexa488. (C) The partition coefficients of DIBO-Alexa488 and BCN-DY549 measured at various DM concentrations. (D) The effective concentrations (c_{eff}) of DIBO-Alexa488 and BCN-DY549 in the micelles or buffers relative to the apparent concentration (c_0).

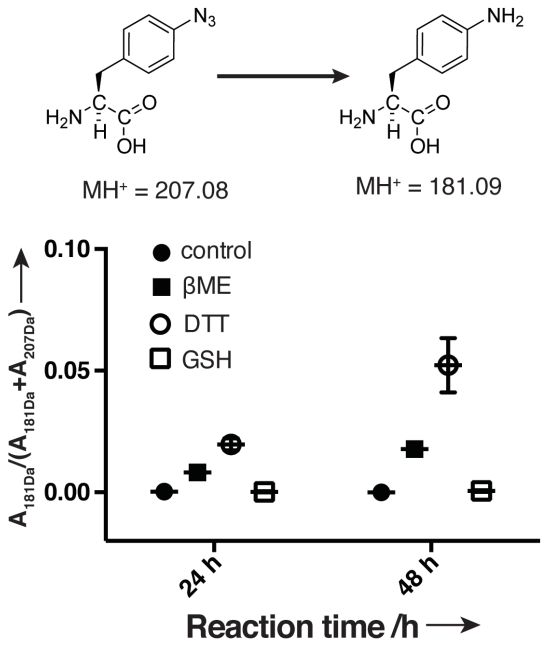

Figure S7. The stability of azF under reducing conditions. The reduction of azF to *p*-amino-L-phenylalanine by10 mM βME, dithiothreitol (DTT), or glutathione (GSH) was detected by mass spectroscopy. In the control group no reducing reagent was added. The relative reduction was calculated from the integrated areas of the 181.09Da and 207.08Da peaks ($A_{181.09}/(A_{181.09}+A_{207.08})$). No reduction of azido group to amino group was observed or in control group or in the presence of GSH. DTT is known to have a higher reducing capability than βME and caused a greater extent of reduction. Even under the strongest reducing condition (DTT, 48 h) the samples still contain predominantly azF.

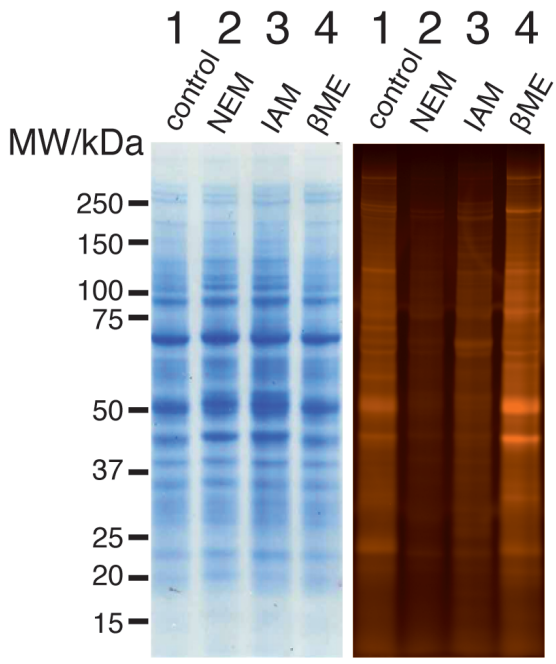

Figure S8. The non-specific labeling of cell lysates by BCN-DY549. Left: Coomassie staining. Right: in-gel fluorescence. In Lane 1 (control), the cell lysates were directly reacted with BCN-DY549. In Lane 2 and Lane 3, the lysates were first treated with N-ethyl-maleimide (NEM) or iodoacetamide (IAM) before the reaction with BCN-DY549. In both cases, blocking the cysteine thiols resulted in a clear reduction of non-specific labeling. The addition of βME (10 mM) into the cell lysates without blocking the free thiols, on the contrary to the observations from Rho, led to an increased level of thiol-yne labeling.

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