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Supporting Information

Live-Cell Imaging of Cellular Proteins by a Strain-Promoted Azide–Alkyne Cycloaddition

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Experimental Protocols

Cell Maintenance

Rat-1 fibroblasts cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were maintained in a 37 °C, 5% CO_2 humidified incubator chamber. Near-confluent cells were passaged with 0.05% trypsin in 0.52 mM EDTA.

Preparation of Cells for Fluorescence Microscopy

Lab-Tek chambered coverglass slides (8-well, Nalge Nunc International) were prepared by treatment with fibronectin solution (10 μg/mL). The wells were rinsed twice with PBS, blocked with a 2 mg/mL solution of heat-inactivated BSA at room temperature, and rinsed with PBS.

Near-confluent cells in 100 mm Petri dishes were rinsed twice with warm phosphate-buffered saline (PBS). Cells were detached with trypsin in EDTA and added to the appropriate media. The cells were pelleted via centrifugation (200 *g*, 3 min) and counted. Cells were added at a density of 1 x 10^4 cells per well to prepared slides. Cells were grown in media overnight.

After growth overnight in DMEM++, each well was washed twice (200 μL) with warm PBS. Cells were incubated for 5 min in serum-free medium lacking Met [SFM: DMEM, with 1 mg/mL bovine serum albumin (BSA, fraction V, Sigma-Aldrich), with 2 mM Glutamax I (Invitrogen), without Met], followed by 30 min in fresh SFM to deplete intracellular Met stores. Anisomycin (40 μM, Sigma-Aldrich) was added to cells during this time to inhibit protein synthesis. After incubation, either 1 mM Met or 1 mM Aha was added to the medium. After 4 h, wells were rinsed twice with DMEM++ before adding the dye-labeling mixture.

Cells were exposed to coumarin-cyclooctyne in the labeling media DMEM-Imaging [DMEM lacking phenol red, with HEPES (Invitrogen), supplemented with 10% FBS and 1 mg/mL BSA]. Each coumarin-cyclooctyne was added to the labeling medium as a dilution from a 10 mM stock solution in DMSO. Labeling was allowed to proceed 10 min at 37 °C in the incubator chamber. After labeling, cells were washed twice before counterstaining. Cells were counterstained for 10 min with 300 nM MitoTracker Red CMXRos (Invitrogen). After treatment, cells were washed thrice with DMEM-Imaging and then imaged in DMEM-Imaging. Cells were kept in an incubator until they could be imaged (up to 3 h).

For counterstaining with propidium iodide, cells were washed twice with DMEM++ before the addition of a 1:1000 dilution of propidium iodide (1.0 mg/mL; Invitrogen) in DMEM-Imaging for 10 min. Cells were washed thrice before imaging. Fixed (3.7% paraformaldehyde, 10 min) and permeabilized (0.1% Triton X-100 in PBS, 3 min) cells were also imaged as a control. Fixed cells stained with propidium iodide were not treated with coumarin-cyclooctyne.

Live cells were imaged on a confocal microscope (Zeiss LSM 510 Meta NLO) at California Institute of Technology's Biological Imaging Center. A heated chamber was placed around the microscope to image the cells at ~37 °C. MitoTracker Red or propidium iodide fluorescence was obtained by excitation at 543 nm with emission collected between 565 and 615 nm. Transmitted light images were also collected to differentiate individual cells. Coumarin fluorescence was obtained by twophoton excitation at 800 nm (Ti:sapphire laser) with emission collected between 376 and 494 nm. The set of images for each

dye was obtained with identical conditions to capture coumarin fluorescence. Individual optical slices of coumarin fluorescence were collected at 0.5 μm intervals in order to create an extended focus image (i.e., projection). Images were acquired with a Plan-Apochromat 63x/1.4 oil objective (Zeiss) and analyzed with Zeiss LSM and ImageJ[1] software.

For MitoTracker Red, propidium iodide, and coumarin images in **Figures 1, S1**, **S2** and **S7**, the brightness and contrast were manually adjusted for the images corresponding to each dye using ImageJ Software. Then, the minimum and maximum pixel values were applied to each image from samples treated with that dye. Stacks of coumarin fluorescence images were made into a projection using ImageJ software. A maximum intensity projection was created by taking the maximum value at each pixel along the z-axis of the image plane through a stack of ten 0.5 μm-thick slices. Again, the brightness and contrast were manually adjusted and applied to each image in the set (Aha, Met, and Aha+anisomycin).

Preparation of Cells for Flow Cytometry

Pulse-labeling was performed directly in the 6-well tissue culture dishes in which cells were grown. Each well was washed twice with warm PBS, followed by a 30 min incubation in SFM to deplete intracellular Met stores. Cells were then exposed to 1 mM Aha or 1 mM Met for 4 h. In addition to examining a 4 h pulse-length, we also examined 0.8, 0.25, 0.5, and 1 h pulses. Then, cells were washed twice with PBS. Coumarin-cyclooctyne dye in DMEM-Imaging was added to each well for coumarin-labeling. Dye concentrations of 0.5 μM to 50 μM were examined. To examine alternative dye-labeling times, 10 μM dye was added for 6-60 min in DMEM-Imaging. After labeling, cells were washed twice with warm PBS and detached using 250 μL of 0.05% trypsin in EDTA. Cells were added to 750 μL DMEM++, and 100 μL FBS was added to the bottom of the Eppendorf tube to improve cell pelleting. Cells were pelleted by centrifugation (200*g*, 3 min) and washed once with 1 mL DMEM-Imaging, again with a cushion of FBS added to the bottom of the tube. Finally, cells were resuspended in 400 μL DMEM-Imaging before filtering through a 50 μm Nytex nylon mesh screen (Sefar). Cells were stored on ice until analysis.

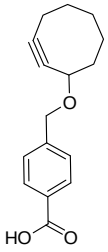
Flow Cytometry

Cells were analyzed on a FACSAria flow cytometer (BD Biosciences Immunocytometry Systems) at Caltech's Flow Cytometry Facility. Coumarin fluorescence was excited by a 407 nm laser and detected after passage through a 450/40 bandpass filter. Forward- and side-scatter properties were used to exclude doublets, dead cells, and debris from analysis. 7-aminoactinomycin D (7-AAD; Beckman Coulter) was used to exclude dead cells from analysis. 7-AAD was excited by a 488 nm laser and detected after passage through a 695/40 filter. Unlabeled cells, 7-AAD labeled cells, and coumarin labeled cells were analyzed to ensure minimal cross-over fluorescence in each channel. When necessary, compensation was applied to reduce cross-over fluorescence. Data was analyzed using FloJo7 software (Tree Star).

Synthesis of Coumarin-Cyclooctyne Dyes

All chemicals were purchased from Aldrich and used as received unless otherwise noted. Dry solvents were obtained from commercial suppliers and used as received. Silica chromatography was performed using 230-400 mesh silica gel 60 (EMD). TLC was performed using Baker-flex silica gel IB-F plates; R_f values are reported under the same solvent conditions as those used for chromatography unless otherwise noted. TLC plates were examined under UV light for fluorescent compounds or alternatively stained with $KMnO_4$, ceric ammonium molybdenate, or *p*-anisaldehyde. NMR spectra were recorded on Varian spectrometers (300 MHz for ^1H) and processed with NUTS NMR software. NMR spectra were referenced to internal standards; proton and carbon spectra were referenced to tetramethylsilane. Fluorine spectra were referenced to hexafluorobenzene. All coupling constants are reported in hertz. FAB mass spectrometry was performed at the California Institute of Technology Mass Spectrometry Facility.

Cyclooctyne acids **5** - **7** were prepared as described [2].



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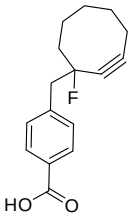

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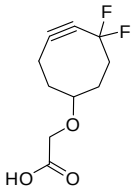

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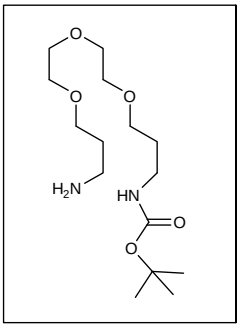
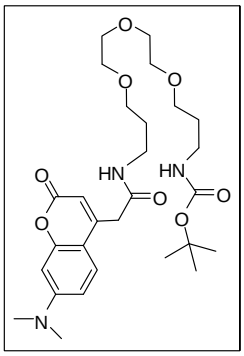
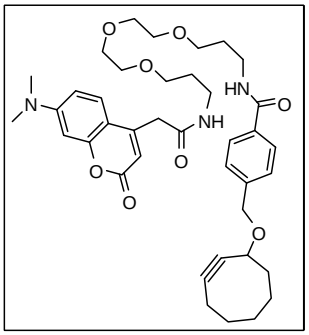


*1-Boc-amino-13-amino-4,7,10-trioxotridecane, **8***. A solution of ditertbutyldicarbonate (5.0 g, 23 mmol) in 100 mL of CH_2Cl_2 was added via a dropping funnel to a rapidly stirred solution of 1,13-diamino-4,7,10-trioxotridecane (10.1 g, 46 mmol) in 200 mL CH_2Cl_2 over a period of ~1 h. The resulting clear solution was stirred 16 h then transferred to a separatory funnel and washed twice with 100 mL of 0.1 M HCl and the aqueous layer was back-extracted 5 times with 50 mL of CH_2Cl_2. The combined organics were dried over $NaSO_4$ and evaporated to give 6.6 g (90%) of an opaque viscous oil, which was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 9 H), 1.55 (br s, 2H), 1.68-1.84 (m, 4H), 2.81 (t, 2H, *J* = 6.8), 3.23 (br q, 2H, *J* = 6.2), 3.51-3.72 (m, 12H), 5.02-5.29 (2x br t, rotomers, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.46, 29.61, 33.26, 38.48, 39.62, 69.47, 69.56, 70.18, 70.22, 70.58, 70.60, 78.80, 156.05, 156.10; FAB MS calc for $C_{15}H_{33}N_2O_5$ $(M+H)^+$ 321.2389, observed 321.2386.

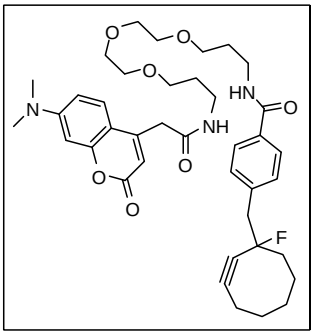


*Boc-Linker-Coumarin **9***. To a solution of 1-Boc-amino-13-amino-4,7,10-trioxotridecane, **8**, (0.027g, 80 μmol) and triethylamine (0.015 g, 140 μmol) in 2 mL of CH_2Cl_2 in a foil-wrapped flask was added 7-dimethylaminocoumarin-4-acetic acid succinimidyl ester (0.025 g, 73 μmol, Anaspec) in 1 mL of CH_2Cl_2. The reaction was stirred for 12 h and then the solvent was removed and the product purified by silica chromatography (1:19 ethanol:ethyl acetate, R_f = 0.25) yielding 40 mg ~100%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9 H), 1.73 (app quintet, 4 H, *J* = 6.2), 3.06 (s, 6 H), 3.19 (br q, 2 H, *J* = 6.3), 3.36 (q, 2 H, *J* = 6.0), 3.45-3.65 (m, 14 H), 5.01 (br, t, 1 H), 6.07 (s, 1 H), 6.50 (d, 1 H, *J* = 2.8), 6.62 (dd, 1 H, *J* = 2.5, 9.0), 6.75 (br t, 1 H), 7.53 (d 1 H, *J* = 9.0); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.44, 28.61, 29.66, 38.52, 40.12, 40.62, 69.42, 70.02, 70.08, 70.14, 70.24, 70.39, 78.89, 98.14, 108.50, 109.13, 110.24, 125.84, 150.18, 153.04, 155.98, 161.86, 168.01; FAB MS calc for $C_{28}H_{43}N_3O_8$ $(M+H)^+$ 549.3050, observed 549.3027.

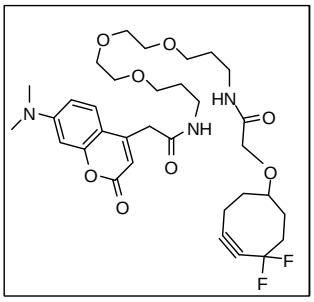


1. Cyclooctyne acid **5** (0.0245 g, 0.095 mmol) was dissolved in 2 mL of DMF in a foil wrapped flask and placed under argon. Pyridine (18 μL, 0.11 mmol) was added, and the solution was cooled in an ice bath. Pentafluorophenyl trifluoroacetate (18 μl, 0.11 mmol) was added, and the solution was stirred for 2 h. The solvent was diluted with ethyl acetate (20 mL), extracted twice with 1 M HCl, extracted once with saturated $NaHCO_3$, dried over Na_2SO_4, and evaporated to give a clear oil that was resuspended in 2 ml of CH_2Cl_2. This solution was added to a solution of **9** (0.043 g, 0.072 mmol) that had been deprotected by

treatment with 1:1 TFA:CH_2Cl_2 for 2 h, followed by solvent removal and resuspension in 2 mL CH_2Cl_2 and 22 µl (0.160 mmol) triethylamine. The combined solution was stirred under argon and monitored by TLC. After 6 h, the reaction mixture was applied directly to a silica column and eluted with 2:9:9 ethanol:CH_2Cl_2:ethyl acetate (R_f = 0.24 in 1:19 ethanol:CH_2Cl_2) to give 0.044 g (0.064 mmol, 88%) of a yellow-white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (m, 1 H), 1.59-1.76 (m, 4 H), 1.79-1.93 (m, 5 H), 1.93-2.39 (m, 4 H), 3.04 (s, 6 H), 3.27 (app q, 2 H, *J* = 5.9), 3.41-3.47 (m, 4 H), 3.51-3.66 (m, 12 H), 4.22 (m, 1 H), 4.16 (d, 1 H, *J* = 12.5), 4.69 (d, 1 H, *J* = 12.5), 6.06 (s, 1 H), 6.46 (d, 1 H, *J* = 2.4), 6.59 (dd, 1 H, *J* = 9.1, 2.5), 6.79 (br t, 1 H), 7.27 (br t, 1 H), 7.38 (d, 2 H, *J* = 8.1), 7.49 (d, 1 H, *J* = 9.1), 7.77 (d, 2 H, *J* = 8.1);^{13}C NMR (75 MHz, $CDCl_3$) δ 20.70, 26.37, 28.53, 28.85, 29.72, 34.30, 38.63, 38.89, 40.09, 40.49, 42.32, 53.46, 69.92, 70.06, 70.17, 70.28, 70.42, 70.46, 72.04, 92.50, 98.04, 100.66, 108.38, 109.20, 110.06, 125.76, 127.11, 127.70, 133.53, 141.81, 150.19, 153.07, 155.94, 162.12, 167.58, 168.39; FAB MS calculated for $C_{39}H_{51}N_3O_8$ $(M+H)^+$ 690.3754, observed 690.3720.



2. Cyclooctyne acid **6** (0.005 g, 0.019 mmol) was dissolved in 0.2 mL of DMF in a foil wrapped flask and placed under argon. Pyridine (6 µL, 0.08 mmol) was added, and the solution was cooled in an ice bath. Pentafluorophenyl trifluoroacetate (4 µL, 0.02 mmol) was added, and the solution was stirred for 3 h. The solvent was diluted with ethyl acetate (20 mL), extracted twice with 1 M HCl, extracted once with saturated $NaHCO_3$, dried over Na_2SO_4, and evaporated to give a clear oil that was resuspended in 2 mL of CH_2Cl_2. This solution was added to a solution of **9** (0.020 g, 0.04 mmol) that had been deprotected by treatment with 2 mL of 1:1 TFA:CH_2Cl_2 for 2 h, followed by solvent removal and resuspension in 2 mL CH_2Cl_2 and 22 µL (0.160 mmol) triethylamine. The combined solution was stirred under argon and monitored by TLC. After 8 h the reaction mixture was applied directly to a silica column and eluted with 1:19 ethanol:CH_2Cl_2 (R_f = 0.24) to give 0.006 g (0.009 mmol, 45%) of oily yellow product. ^{1}H NMR (300 MHz, $CDCl_3$) δ δ 1.29-1.45 (m, 1H), 1.60-1.76 (m, 4 H), 1.77-2.10 (m, 6 H), 2.13-2.36 (m, 3 H), 3.03 (s, 1 H), 3.04 (s, 6H) 3.07 (d, 1H), 3.25-.3.37 (app q, 2 H), 3.38-3.46 (m, 4 H), 3.52-3.68 (m, 12 H), 6.06 (s, 1 H), 6.49 (d, 1 H, *J* = 2.5), 6.60 (dd, 1 H, *J* = 2.5, 9.0), 6.64 (br t, 1 H), 7.16 (br t, 1H), 7.34 (d, 2 H, *J* = 7.9), 7.51 (d, 1H, *J* = 9.0), 7.74 (d, 2 H, *J* = 8.4); FAB MS calculated for $C_{39}H_{51}N_3O_7F$ $(M+H)^+$ 692.3711, observed 692.3726.



3. Cyclooctyne acid **7** (0.0028 g, 0.014 mmol) was dissolved in 200 mL of CH_2Cl_2 in a foil-wrapped flask and placed under argon. Triethylamine (8 µL, 0.06 mmol) was added, and the solution was cooled in an ice bath. Pentafluorophenyl trifluoroacetate (3 µL, 0.02 mmol) was added, and the solution was stirred for 1 h. The solvent was removed under reduced pressure, and the resulting oil was resuspended in 3 mL of 1:1 ethyl acetate: hexane and passed through a short plug of silica (in a pasteur pipette). The solvent was removed under reduced pressure, and the material was resuspended in 1 mL of CH_2Cl_2. This solution was added to a solution of **9** (0.0125 g, 0.023 mmol) that had been deprotected by treatment with 1.5 mL of 1:1 TFA:CH_2Cl_2 for 2 h, followed by solvent removal, azeotropic removal of TFA with toluene and resuspension in 0.2 mL CH_2Cl_2 and 30 µL (0.21 mmol) triethylamine. The combined solution was stirred under argon and monitored by TLC. After 3 h the reaction mixture was applied directly to a silica column and eluted with 1:19 ethanol:CH_2Cl_2 (R_f = 0.22) to give 5.3 mg (0.0082 mmol, 64%) of a yellow glassy solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.68-1.87 (m, 4H), 1.98-2.08 (m, 2H), 2.13-2.31 (m, 3H), 2.39-2.63 (m, 3H), 3.06 (s, 6H), 3.31-3.45 (m, 4H), 3.45-3.69 (m, 15H), 3.92 (s, 2H), 6.07 (s, 1H), 6.48 (d, 1H, *J* =

2.6), 6.61 (dd, 1H, *J* = 2.9, 8.8), 6.79 (br t, 1H), 6.98 (br t, 1H), 7.51 (d, 2H, *J* = 9.3); FAB MS calculated for $C_{33}H_{46}N_3O_8F_2$ $(M+H)^+$ 650.3253, observed 650.3233.

Caco-2 Permeability Analysis

Each of the three coumarin cyclooctynes **1-3** was submitted to Apredica (Watertown, MA) for Caco-2 (human colon carcinoma cell) permeability analysis. Each probe was supplied as a 10 mM stock solution in DMSO. A test concentration of 10 μM probe was applied to the apical side of a monolayer of differentiated Caco-2 cells. Each probe was applied in transport buffer (1.98 g/L glucose in 10 mM HEPES, 1x Hank's Balanced Salt Solution, pH 6.5) supplemented with 100 μM of the impermeable dye Lucifer Yellow. For comparison, low permeability controls (Ranitidine, Vinblastine), Quinidine, and a high permeability control (Warfarin) were also applied to the cells. After 2 h, the amount of permeation for each probe was determined by LC/MS/MS analysis of the transport buffer removed from the basolateral side of the monolayer. The value obtained is the permeability of each compound (P_{app}), which is defined as:

$$P_{app} = \frac{dQ/dt}{C_0 A}$$

where *dQ/dt* is the rate of permeation, C_0 is the initial concentration of the test agent, and A is the area of the monolayer. The P_{app} value for **1-3** and the standards are given in **Table S1**. From the P_{app} values, we can conclude that each of the probes is membrane permeable and able to pass through the Caco-2 monolayer, although the assay also predicts that the bioavailability would be low.

Table S1: Caco-2 Permeability Assay

Compound	**Mean apical → basolateral permeability: P_{app} (10^{-6} cm s^{-1})**
Ranitidine	0.8
Warfarin	48.4
Quinidine	7.4
Vinblastine	0.7
1	3.2
2	0.8
3	0.7

Fractionation of Proteins from Live Cells

Pulse-labeling was performed directly in the 10 cm tissue culture dishes in which cells were grown. Each plate was washed twice with warm PBS and incubated 30 min in SFM to deplete intracellular Met stores. Anisomycin (40 μM) was added at this time. Cells were exposed to 1 mM Aha, 1 mM Aha with anisomycin, or 1 mM Met for pulse-labeling (4 h). Then plates were washed twice with warm PBS. Coumarin-cyclooctyne dyes (10 μM) in DMEM++ was added to each plate for fluorophore-labeling. After labeling for 30 min, cells were washed twice with warm PBS and detached using 0.05% trypsin in 0.52 mM EDTA. Plates were scraped using a cell scraper and cells were collected and added to 4 mL DMEM++. Cells were pelleted by centrifugation (500 *g*, 10 min) and washed once with ice-cold PBS. Cells were transferred to a clean eppendorf tube and fractionated using the QProteome cell compartment kit (Qiagen). Each cell pellet was gently resuspended in 1 mL ice-cold extraction buffer CE1 and incubated at 4°C in a lab rotator for 10 min. The lysates were centrifuged at 1000 *g* for 10 min at 4°C. The supernatants (fraction 1, F1) were carefully collected and stored on ice; this fraction contains primarily cytoplasmic proteins. The pellets were then resuspended in ice-cold 1 mL extraction buffer CE2 and incubated at 4°C in a lab rotator for 30 min. The suspensions were then centrifuged at 6000 *g* for 10 min at 4°C. The supernatants (fraction 2, F2) were carefully collected and stored on ice; this fraction contains primarily membrane proteins and proteins from the lumen of organelles. The pellets were then treated with Benzonase nuclease for 15 min at room temperature. Then 500 μL of ice-cold extraction buffer CE3 was added to each tube. The tubes were incubated at 4°C in a lab rotator for 10 min. The suspensions were then pelleted by centrifugation (6800 *g*, 4°C, 10 min). The supernatants were carefully collected (fraction 3, F3); this fraction primarily contains nuclear proteins. The pelleted material was resuspended in 100 μL of room temperature buffer CE4 (fraction 4, F4); this final fraction contains the remaining proteins, most of which are cytoskeletal. Fractionated

proteins were precipitated in ice-cold acetone. Pelleted protein was resuspended in RIPA buffer (Sigma) and the total amount of protein in each fraction was determined by BCA assay (Pierce).

Protein samples isolated by the Qproteome kit were analyzed by western blot with antibodies appropriate for detecting proteins specific to different cellular fractions. Detection of β-tubulin, a microtubule component found primarily in the cytoplasm[3], was used to confirm that fraction 1 contained primarily cytosolic proteins. Glucose regulated protein (GRP78), a membrane protein found in the endoplasmic reticulum[4], was used to confirm that fraction 2 contained primarily membrane proteins and proteins from the lumen of organelles. Fibrallarin, a nucleolar protein[5], was used to confirm that fraction 3 contained primarily nuclear proteins. For western blot analysis, equal amounts of fractionated protein samples were separated on a 12% SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare) using a semi-dry transfer system (Amersham Biosciences). The membrane was blocked at room temperature for 1 h before incubation with primary antibodies for 2 h at room temperature [anti-GRP78 (BD Biosciences), 1:250 or anti-fibrillarin (Abcam), 1:1000]. Washed membranes were then probed with a secondary antibody, goat anti-mouse IgG-HRP conjugated (Chemicon, 1:10000). HRP activity was detected on a Typhoon molecular imager (GE Healthcare) following incubation with reagents from the ECL+ kit (GE Healthcare). For β-tubulin detection, the blot was incubated with anti-β-tubulin-HRP antibody (Abcam, 1:1000). Representative western blots of fractionated protein samples are shown in **Figure S3**.

Fluorescence from each fraction was measured on a Safire II fluorescence plate reader (Tecan). Raw fluorescence data were normalized either by cell number (**Table S2**) or by total amount of protein (**Table S3**) to yield the percentage of total fluorescence associated with each fraction.

	F1	F2	F3	F4
1	25.5± 0.8%	61.0± 2.7%	6.7± 1.7%	6.8± 0.1%
2	25.4± 2.4%	63.6± 1.1%	5.4± 2.3%	5.6± 0.9%
3	40.0± 3.4%	38.5± 2.6%	11.7± 1.3%	10.0± 0.5%

Table S2. Percent fluorescence normalized by cell number in Aha treated samples. F1, fraction F1, is primarily cytosolic proteins. F2 is primarily membrane proteins and proteins from the lumen of organelles. F3 is primarily nuclear proteins, while F4 is the insoluble fraction, which contains primarily cytoskeletal proteins.

	F1	F2	F3	F4
1	13.7± 0.8%	55.8± 2.7%	11.3± 2.9%	19.2± 0.7%
2	15.1± 1.1%	58.1± 3.0%	9.6± 4.3%	17.2± 0.2%
3	21.4± 1.4%	36.5± 0.5%	19.0± 1.6%	23.1± 0.7%

Table S3. Percent fluorescence normalized by protein concentration in Aha treated samples. F1, fraction F1, is primarily cytosolic proteins. F2 is primarily membrane proteins and proteins from the lumen of organelles. F3 is primarily nuclear proteins, while F4 is the insoluble fraction, which contains primarily cytoskeletal proteins.

In Vitro Reactions

Model reactions between 1 μM **3** and 1 mM amino acid were performed in PBS (pH 7.4). Reactions proceeded at 37 °C until analysis. Liquid chromatography was performed on a Waters HPLC system with a Microsorb C18 column (Varian) The buffers used for separation of the reaction components were 0.1% trifluoroacetic acid in water (Eluent A) and 100% Acetonitrile (Eluent B). The gradient was 0-2 min, 100% A; 2-4 min, 100-70% A; 4-14 min, 70-40% A; 14-28 min, 40-0% A.

Dual detection at 280 and 350 nm was used to identify the substrate and product peaks, which were collected for analysis by electrospray ionization mass spectrometry at California Institute of Technology's Mass Spectrometry Facility[6]. The peak corresponding to unreacted **3** was confirmed [calculated $C_{33}H_{46}N_3O_8F_2$ $(M+H)^+$ 650.33, observed 650.1]. The peak corresponding to the Aha-product was collected and confirmed [calculated $C_{37}H_{53}F_2N_7O_{10}$ $(M+H)^+$ 794.38, observed 794.3]. No product was observed for reactions of **3** with Met after 24 h.

The reaction of **3** with Cys resulted in a complex mixture of products that could not be characterized by electrospray mass spectrometry. Instead, the overnight reaction of **3** (50 μM) with Cys (1 mM) or glutathione (1 mM) was characterized by tandem mass spectrometry. The reaction mixture was subjected to reversed-phase capillary chromatography with an Agilent 1200 LC system using a 100-μm X 1-cm (5-μm, 100 Å Magic C18 resin; Michrom Bioresources) fritted capillary precolumn (Integrafit, New Objective) and a 100-μm X 10-cm self-packed C18 column (5-μm, 100 Å Magic C18 resin; Michrom Bioresources). A flow rate of 500 nl/min was accomplished through a splitter. The components were separated with a gradient between solvent A (0.1% formic acid) and solvent B (ACN, 0.1% formic acid). The gradient was from 2% up to 60% solvent B within 30 min. The species were identified by CID (collision induced dissociation) on a Thermo-Finnigan LTQ ion trap mass spectrometer. Six dependent scans followed each survey scan. Data were searched manually and tandem mass spectra peaks assigned, as shown in **Figure S6**.

The data from the HPLC analysis were fitted to a single-exponential decay function using Origin software (OriginLab). The peak area (y) for **3** over time (t) was fitted to the equation:

$$y = y_o + Ae^{(-t/\tau)}$$

The time constant (τ) was converted to the half-life ($t_{1/2}$) using the equation:

$$t_{1/2} = \tau \ln(2)$$

Four experiments were averaged to obtain the half-life for Aha (16 ± 3 min) and Cys (27 ± 5 min).

The model reactions of Aha, Cys, and Met with coumarins **1** and **2** were also examined. The reaction mixtures contained 5 μM **1** or **2** with 1 mM amino acid in PBS (pH 7.4). Reactions proceeded at 37 °C until analysis by HPLC as described above. Both **1** and **2** reacted with Aha, but did not form a product with Cys or Met after 24 h. The replacement of the fluorine on **2** with a hydroxyl occurred under the reaction conditions, as previously reported[2a].

Supporting Data:

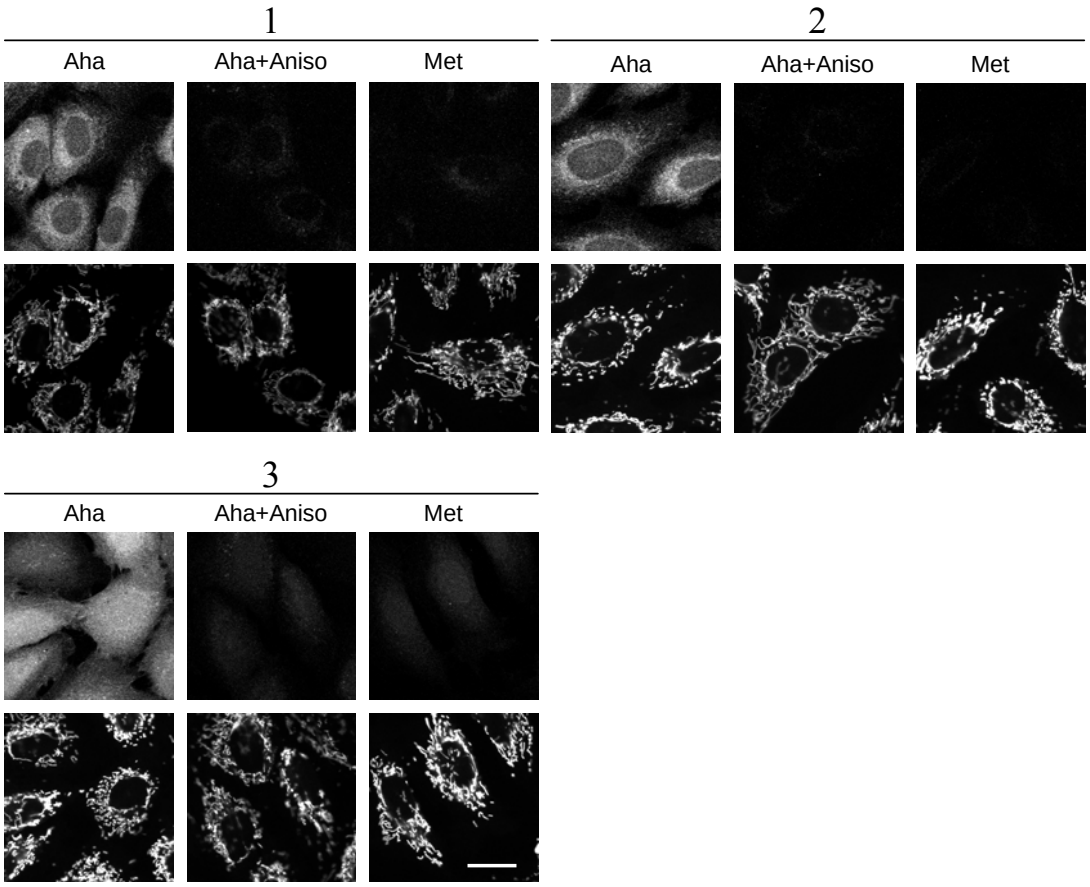


Figure S1. Projections of coumarin fluorescence from labeled proteins in Rat-1 Fibroblasts. The coumarin projections correspond to the single slice coumarin images shown in **Figure 1**. Single slice images of the mitochondria are included to distinguish individual cells and the cytoplasmic space and to ensure correct mitochondrial morphology. The scale bar represents 20 μm.

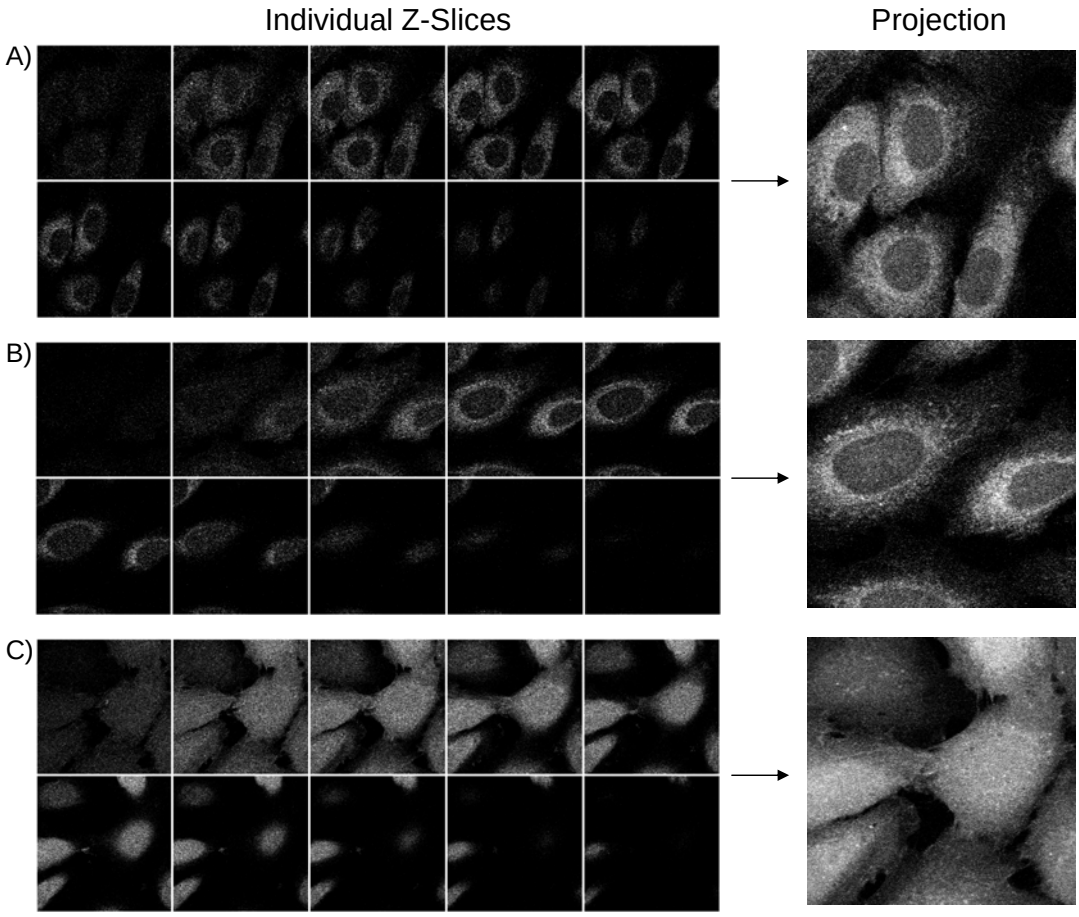


Figure S2. Individual optical slices of coumarin fluorescence acquired using confocal microscopy and two-photon excitation. Slices were collected at 0.5 μm intervals in order to create an extended focus image (i.e., projection) shown on the right. Cells were dye-labeled with fluorophores **1** (a), **2** (b), or **3** (c) as described in **Figure 1**.

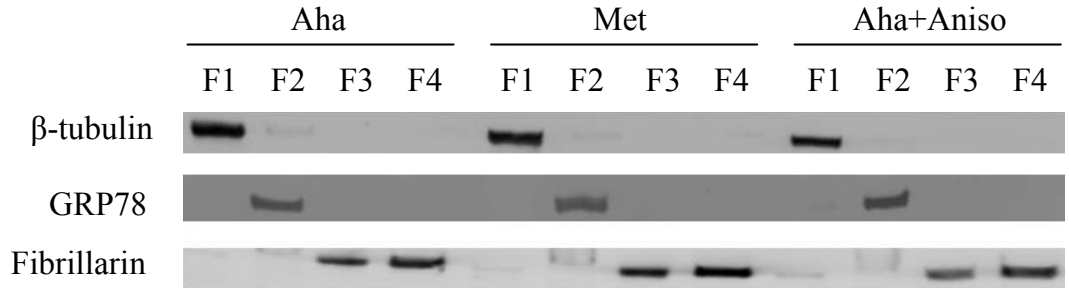


Figure S3. Western blot detection of specific marker proteins found within SDS-PAGE separated proteins from isolated cellular fractions. For each lane, 5 μg of total proteins was loaded. Top: β-tubulin, a microtubule protein residing primarily in the cytosol, was detected in fraction 1 (F1) samples. Middle: Glucose regulated protein (GRP78), a membrane protein residing within the endoplasmic reticulum, was detected in fraction 2 (F2). Bottom: Fibrillarin, a nucleolar marker protein, was detected in fractions 3 and 4.

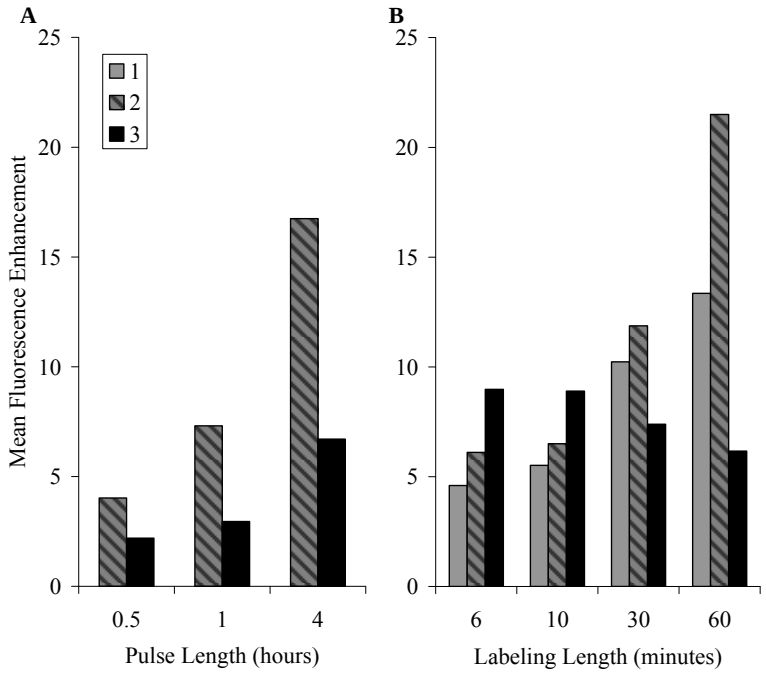


Figure S4. Flow cytometric analysis of pulse-labeling length and dye-labeling length. **A.** Mean fluorescence enhancement of cells pulse-labeled for 0.5, 1, or 4 h. Cells were dye-labeled with 10 μM **3** or 50 μM **2** for 10 min before analysis. **B.** Mean fluorescence enhancement of cells labeled with 10 μM **1**, **2** or **3** for different times after a 4 h pulse. For each sample, 30,000 events were collected.

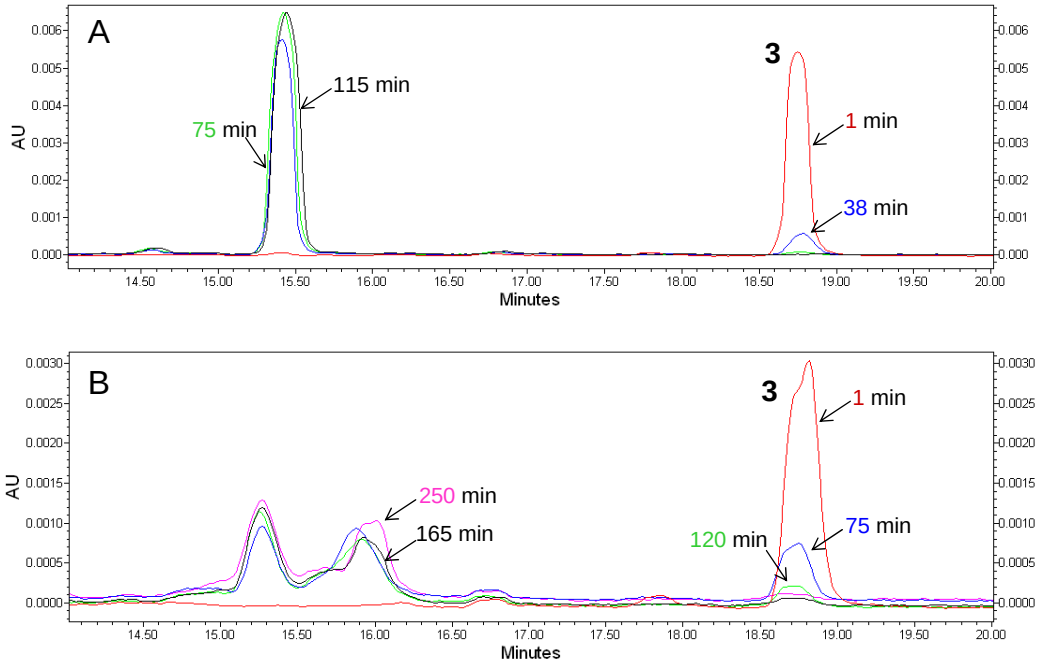


Figure S5. Chromatograms of the *in vitro* reaction of **3** with Aha or Cys. Reaction mixtures contained 1 μM **3** with 1 mM Aha (**A**) or 1 mM Cys (**B**) in PBS (pH 7.4). The reactions proceeded at 37 °C until analysis by HPLC. Detection at 350 nm was used to observe changes in the starting material (**3**: 18.5-19 min) and product peaks (Aha: 15-16 min, Cys: 14-17 min) at different time points.

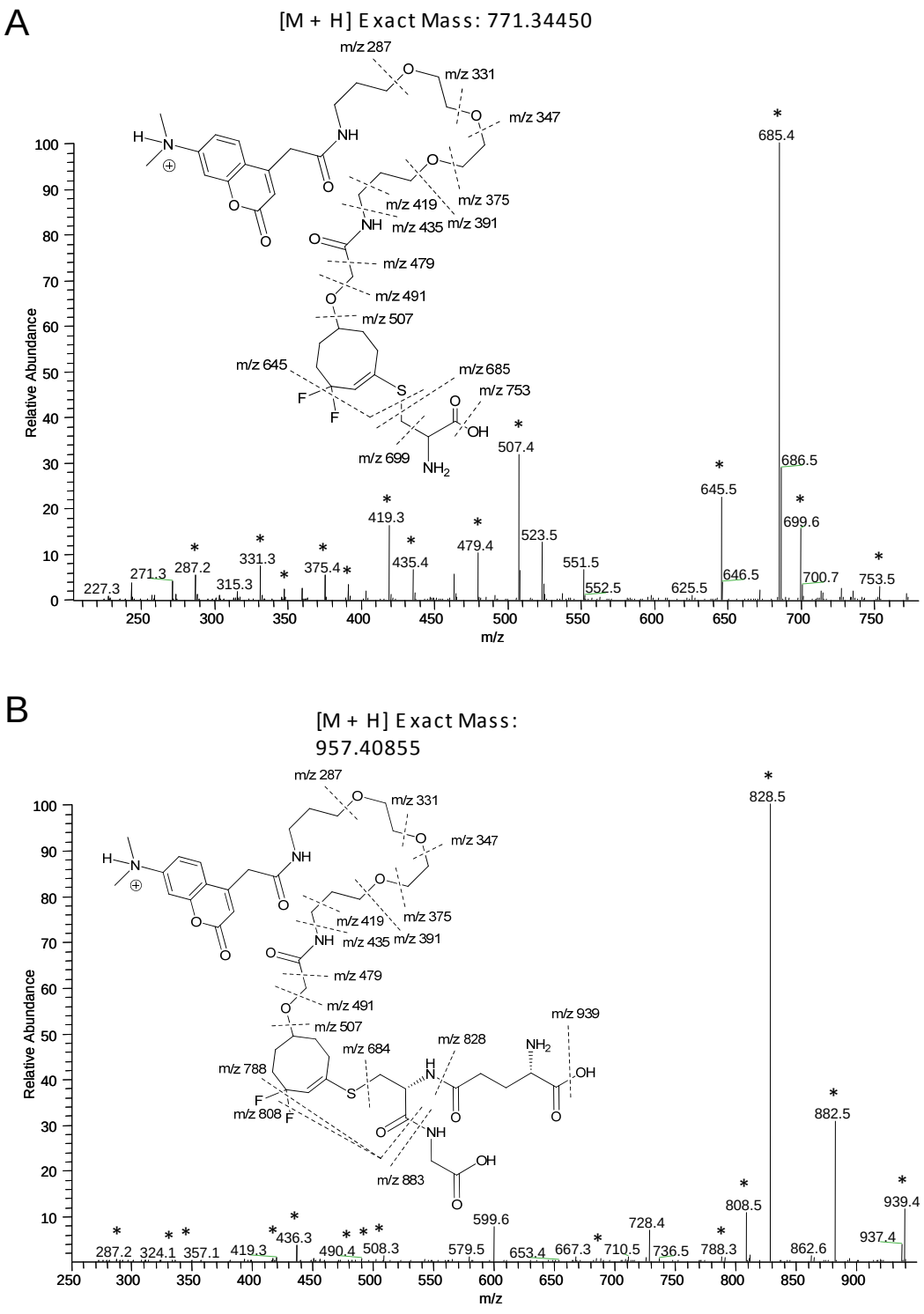


Figure S6. Tandem mass spectrum of compound **3** reacted with A) cysteine or B) glutathione. An asterisk denotes a peak found for the corresponding bond cleavages shown.

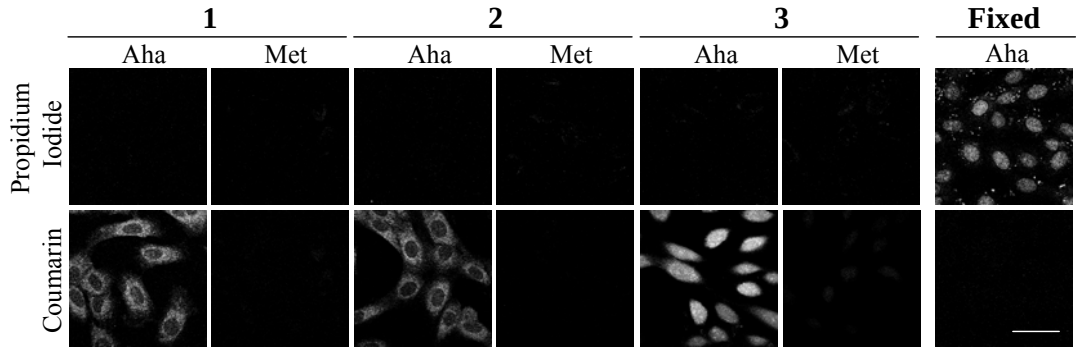


Figure S7. Viability of cells dye-labeled with coumarin-cyclooctynes. Cells were pulse-labeled 4 h in media supplemented with 1 mM Met or 1 mM Aha. Cells were dye-labeled 10 min with 50 μM **1**, 50 μM **2**, or 10 μM **3**. Cells were washed, treated with 1 μg/mL propidium iodide for 10 min, washed, and then imaged. Fixed cells were treated with 3.7% paraformaldehyde for 10 min instead of staining with coumarin cyclooctyne. After fixation, cells were permeabilized and stained with propidium iodide. The scale bar represents 50 μm.

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