

## Supporting Information

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## 1. General information

All of chemicals and solvents were purchased from commercial suppliers without any purification. Iodo-BODIPY **1**,<sup>1</sup> [Ir(ppy)<sub>2</sub>(phen-N<sub>3</sub>)]PF<sub>6</sub> (**3**),<sup>2</sup> 3-azido-7-hydroxycoumarin (**4**),<sup>3</sup> 7-ethynyl-4-methylcoumarin (**5**),<sup>4</sup> FI-DIBO **6**,<sup>5</sup> alkyne-tagged-BSA,<sup>6</sup> and amine **S1**<sup>7</sup> were synthesized by reported procedures. Azide-tagged BSA was synthesized adapting a reported procedure<sup>5</sup> to the aliphatic azide, 2-bromo-*N*-(3-azidopropyl)-acetamide<sup>8</sup>. Yes-MBP, Fyn SH3, and *E. coli* lysate (BL21) were prepared following a previously reported procedure.<sup>9</sup> Fluorescein isothiocyanate **S2** (isomer I, 90% pure) was purchased from ACROS Organics (#119252500). BSA (A2153), lysozyme (L6876), trypsin inhibitor (T9003), bicyclo[6.1.0]non-4-yn-9-ylmethyl *N*-succinimidyl carbonate (BCN NHS carbonate) (#744867) were purchased from Sigma-Aldrich. Transfer buffer was prepared following a protocol from Life Technologies; 20X transfer buffer (50 mL), which consists of 25-mM bicine, 25-mM bis-tris, 1-mM EDTA, and 0.05-mM chlorobutanol, was mixed with methanol (100 mL), water (849 mL), and antioxidant (1 mL, Invitrogen NP0005) to prepare 1X transfer buffer. Fluorescence measurement of membrane sample was performed on a Horiba-Jovin Yvon Fluorolog 3. Blotted membranes with **3**, **4**, **6** were excited at 370 nm and one with **5** was excited at 330 nm.

### Imaging of blot membrane

Fluorescence imaging was performed on Fujifilm LAS-4000 instrument using epi-UV light source (370 nm LED) and a longpass filter at 410, 515, or 605 nm.

### Chemical blotting with 3-azido-7-hydroxycoumarin (**4**)—a procedure in brief

Alkyne-labeled protein soln (typically 1–5 µL) was mixed with 2.5 µL of 4X LDS sample buffer (Life Technologies, NP0007), diluted to 10 µL with water, and loaded onto a 12% bis-tris gel (Life Technologies, NP0343BOX). Electrophoretic separation was performed with MOPS buffer (Life Technologies, NP0001) for 50 min at 200 V. PVDF membrane (Thermo Scientific #22860 or Azure Biosynthesis AC2108) was activated with methanol, rinsed twice with water, and soaked in cold 1X transfer buffer for 5 min. The separated proteins were transferred to the membrane at 24 V for 25–30 min using cold 1X transfer buffer. The transferred membrane was then soaked in methanol for 10 min, rinsed with water twice, and incubated with “click chemistry reaction soln” in a 150-mm diameter petri dish for 30 min. The click chemistry reagents were added from stock soln. The reaction soln contains sodium ascorbate (1.5 mM, 120 µL from 100 mM stock soln), THPTA (100 µM, 8 µL from 100 mM stock soln), **4** (10 µM, 4 µL from 20 mM stock soln), and copper sulfate (75 µM, 6 µL from 100 mM stock soln) in 1:1 H<sub>2</sub>O/dimethyl sulfoxide (20 mL). The membrane was washed with DMSO with shaking for 3 min. For a membrane from Azure Biosynthesis, 1:1 methanol/dimethyl sulfoxide was used instead of dimethyl sulfoxide to avoid overly brittle membranes prone to breakage. The membrane was rinsed with methanol after each wash step. The whole wash procedure was repeated three times, followed by a final wash with 70% aq ethanol. The membrane was imaged using an LAS-4000 imager. The membrane could be preserved for later analysis by storage in 70% aq ethanol (See below.).

### General Procedure for sample preparation

#### Protein sample with *E. coli* lysate

To the mixture of protein sample and the LDS sample buffer, *E. coli* lysate was added to make final 20% solution by volume for all of screening experiments or 10% solution by volume for double blotting experiments. (The final volume of the sample was adjusted to 10  $\mu$ L with water before injection.)

#### Preparation of stock chemical solutions

Freshly prepared 100-mM sodium ascorbate in water

100-mM triazole ligand THPTA (Click Chemistry Tools, #1010) in water or 100-mM TBTA (Click Chemistry Tools, #1061) in DMSO

100-mM copper (II) sulfate in water

10-mM **3** in DMSO (solution)

20-mM **4** in DMSO (solution)

20-mM **5** in DMSO (suspension)

2.5-mM **6** in DMSO (suspension)

#### **Preparation of azide-labeled BSA**

BSA (630  $\mu$ M) was incubated with 2-bromo-*N*-(3-azidopropyl)-acetamide (19 mM) in 4:1 PBS buffer/acetonitrile (2400  $\mu$ L) overnight at rt. The reaction mixture was diluted to 4 mL with PBS buffer and then dialyzed (MWCO: 3.5K) against PBS buffer. Protein concentration was determined using NanoDrop UV-vis spectroscopy.

#### **Preparation of alkyne-labeled BSA**

BSA (150  $\mu$ M) was incubated with 4-pentynoyl NHS ester (1.2 mM) in 9:1 PBS buffer/dimethyl sulfoxide (500  $\mu$ L) overnight at 4  $^{\circ}$ C. The reaction mixture was diluted to 10 mL with PBS buffer and then concentrated to 500  $\mu$ L using a centrifugal filter (MWCO: 30K). Protein concentration was determined using NanoDrop UV-vis spectroscopy.

#### **Preparation of cyclooctyne-labeled proteins**

Lysozyme or trypsin inhibitor (75  $\mu$ M) was incubated with BCN NHS carbonate (2.5 mM) and sodium dodecyl sulfate (0.75  $\mu$ M) in 1:1 PBS buffer/acetonitrile (300  $\mu$ L) for 2.5 h at 4  $^{\circ}$ C. The reaction mixture (10  $\mu$ L) was diluted with tris buffer (90  $\mu$ L, 20mM, pH = 8.5) and subject to the standard analysis fluorogenic analysis with azide **4**. The reaction mixture was diluted to 2,000  $\mu$ L with the tris buffer and then concentrated to 100  $\mu$ L using a centrifugal filter (MWCO: 3K, EMD Millipore, UFC500324). Protein concentration was determined using NanoDrop UV-vis spectroscopy.

#### **Sequential blotting of alkyne-labeled BSA and cyclooctyne-labeled lysozyme**

Soln of cyclooctyne-labeled lysozyme (20  $\mu$ M, 4.0  $\mu$ L) and alkyne-labeled BSA (20  $\mu$ M, 1.0  $\mu$ L) were mixed and subject to SDS-PAGE and transfer to blot membrane described above. The membrane was first incubated with iridium complex **3** (20  $\mu$ M), sodium ascorbate (1.5 mM), and THPTA (100  $\mu$ M) in 1:1 H<sub>2</sub>O/dimethyl sulfoxide (8 mL) for 1 h. After washed with 1:1 acetonitrile/dimethyl sulfoxide three times followed by 70% aq ethanol once,

the membrane was imaged with a LAS-4000 imager. The membrane was soaked in methanol for 5 min, rinsed with water, and incubated with second click chemistry soln: azide **4** (3.3  $\mu\text{M}$ ), sodium ascorbate (1.5 mM), THPTA (100  $\mu\text{M}$ ), and copper (II) sulfate (75  $\mu\text{M}$ ) in 1:1  $\text{H}_2\text{O}$ /dimethyl sulfoxide (8 mL) for 0.5 h. The membrane was imaged after wash with 1:1 methanol/dimethyl sulfoxide three times followed by 70% aq ethanol once.

## 2. Complete protocol for chemical blotting with 3-azido-7-hydroxycoumarin (4)

### *Materials and Equipment*

#### SDS-PAGE

4X LDS sample buffer (Life Technologies NP0007)

0.5 mL boilproof microtubes (Phenix Research Products MAX-805)

12% bis-tris gel (Life Technologies NP0343BOX)

MOPS buffer (Life Technologies NP0001)

Electrophoresis chamber (Life Technologies XCell SureLock™ Mini-Cell)

Power supply (Bio-Rad model 200/2.0)

#### Protein Transfer

1X transfer buffer (See “Preparation of materials”.)

Bicine (Fisher BioReagents BP2646)

Bis-tris (Chem-Impex Int'l Inc. #6976-37-0)

EDTA (Fisher BioReagents BP121)

Chlorobutanol (Sigma-Aldrich #112054)

Antioxidant (Life Technologies NP0005)

Extra thick blot filtrate paper (Bio-Rad #1703966)

Methanol (Fisher Chemical A412).

Low-fluorescent PVDF membrane (Thermo Scientific #22860 or Azure Biosynthesis AC2108)

Tweezers

Transfer cell (Bio-Rad Trans-Blot SD Semi-Dry)

Power supply (Bio-Rad model 200/2.0)

#### Chemical blotting

Methanol (Fisher Chemical A412)

100 × 15 mm petri dish (VWR 25384-302)

150 × 15 mm petri dish (VWR 25384-326)

0.5 mL boilproof microtubes (Phenix Research Products MAX-805)

1.5 mL microcentrifuge tubes (VWR #20170)

4.0 mL clear vials (Thermo Scientific B7999-2)

Sodium ascorbate (Sigma-Aldrich A7631)

THPTA (Click Chemistry Tools #1010) or TBTA (Click Chemistry Tools #1061)

Copper (II) sulfate (ACROS organics AC42361)

Azide **4** (Q-N<sub>3</sub>, See “Preparation of materials”.)

Dimethyl sulfoxide (Sigma-Aldrich #472301 or EMD Millipore MX1458)

Parafilm (4 inch × 125 ft.)

Aluminum foil  
200 Proof Ethanol (VWR V1001)  
70% aq ethanol  
50-mL centrifuge tubes (Thermo Scientific #339652)  
Shaker (New Brunswick Scientific C25KC)  
Vortex (Scientific Industries, Inc. S8223)

### Imaging

Imager (Fuji film LAS-4000)  
Tweezers

### ***Preparation of materials***

#### 1X transfer buffer

The preparation of the buffer follows a protocol provided by Life Technologies ([https://tools.lifetechnologies.com/content/sfs/manuals/blotmod\\_pro.pdf](https://tools.lifetechnologies.com/content/sfs/manuals/blotmod_pro.pdf)).

- 20X transfer buffer: Dissolve bicine (10.2 g, 25 mM), bis-tris (13.1 g, 25 mM), EDTA (0.75 g, 1 mM), chlorobutanol (0.025 g, 0.05 mM) in water (final volume 125 mL). Store the 20X buffer at rt.
- 1X transfer buffer (1000 mL): Mix the 20X buffer (50 mL), methanol (100 mL), water (849 mL), and antioxidant (1 mL). Store the 1X buffer at rt and cool it to below 10 °C before use.

#### Stock solution of chemicals

All of the stock solutions could be prepared by dissolving powder or crystal of chemical in a solvent using a microtube or a vial. Frozen stock solution stored in a freezer should be completely thawed and mixed well with vortex before use.

- Sodium ascorbate in water (100 mM). The solution can't be stored for long-term. Prepare it every time by dissolving the compound (20 mg) in water (1 mL).
- THPTA in water (100 mM). Storage: -20 °C freezer.
- TBTA in DMSO (100 mM). Storage: -20 °C freezer.
- Copper (II) sulfate in water (100 mM). Storage: rt.
- **4** in DMSO (10-mM solution). Storage: -20 °C freezer in the dark. (Note: See page 3 for stock soln concentration of other dyes.)

### ***Experimental procedure***

Typical methods for SDS-PAGE and protein transfer to a membrane are used. Follow a protocol of a vendor of a gel or electrophoresis system for a detailed procedure.

#### SDS-PAGE (~60–90 min)

1. Mix alkyne- (or azide-) labeled protein solution (0.002–0.2 mg/mL) with the sample loading buffer (2.5 µL)

and dilute to final volume (10  $\mu$ L) with water.

2. Boil the sample or reduce it with reducing agent if necessary. (Note: Azide-labeled protein should not be treated with reducing agent, especially phosphine-based one such as TCEP, because of potential decomposition of azide.)
3. Load the sample into a precast gel and conduct electrophoresis using MOPS buffer at 200 V for 50 min. (Note: Due to light-sensitivity of azide, the electrophoresis of azide-labeled protein in the dark is recommended.)
4. Remove the gel from the cassette and cut out the top (the comb) and bottom (below the dye) of the gel.

#### Protein Transfer (~30–40 min)

5. Place PVDF membrane in methanol to activate it for 5 min.
6. Rinse the membrane twice with water.
7. Soak the membrane, the gel, and extra thick filtrate papers in cold 1X transfer buffer for 5–10 min.
8. Sandwich the membrane and the gel with the filtrate paper in the transfer cell.
9. Remove air bubbles using a roller or a test tube.
10. Wet the sandwich by pipetting the cold transfer buffer. Wipe the overflowed buffer with a paper towel.
11. Conduct electrotransfer at 24 V for 30 min. (Note: Voltage and time for the transfer vary depending on the protein of interest.)

#### Chemical Blotting (~60 min–overnight)

12. Soak the membrane in methanol for 5 min (Cut the membrane with clean scissors before soaking if necessary). Discard the solvent and repeat this process again.
13. Rinse the membrane twice with water.
14. Put 1:1 water/dimethyl sulfoxide as solvent of the click reaction into a petri dish. (Note: A 150-mm diameter petri dish with 15–20-mL final volume of reaction solution for a 7  $\times$  9-cm membrane, or a 100-mm diameter petri dish with 5–8-mL final volume of reaction solution for a 7  $\times$  3–5-cm membrane is appropriate.)
15. Place the membrane into the petri dish.
16. Add stock solution of click reaction reagents: freshly prepared sodium ascorbate, THPTA (or TBTA), a fluorogenic dye, and copper (II) sulfate. Mix well each time after addition of reagents. (Note: See Table S1 for suitable concentration of reagents.).
17. Seal the petri dish with parafilm and cover the whole dish with aluminum foil.
18. Shake the petri dish using a shaker at 70–100 rpm. (See Table S1 for incubation time of each dye)

#### Membrane wash and imaging (~30–50 min)

19. Discard the reaction solution.
20. Rinse the membrane with methanol until the membrane becomes opaque. (Note: A membrane blotted with alkyne **5** and cyclooctyne **6** also should be rinsed with methanol. Due to a solubility issue, azide **3** is preferred to be rinsed with acetonitrile).
21. Put dimethyl sulfoxide and shake the dish for 5–10 min using a shaker. (Note 1: A membrane blotted with azide

**3** and alkyne **5** also should be washed with dimethyl sulfoxide. 1:1 dichloromethane/methanol should be used for cyclooctyne **6**. Note 2: Due to sensitivity of a membrane from Azure Biosynthesis toward high concentration of dimethyl sulfoxide, 1:1 dimethyl sulfoxide/methanol (**4**, **5**, **6**) or 1:1 dimethyl sulfoxide/acetonitrile (**3**) should be used. Note 3: A glass container has to be used when washing the membrane with dichloromethane. Note 4: Dichloromethane is extremely volatile and toxic, and it should be used in a well-ventilated fume hood. Close the container well before taking out from the hood for shaking.)

22. Repeat step 20–21 twice if dye concentration is less than 100  $\mu$ M. Repeat four times if not or if the wash solution still has color after three sets of wash.
23. Rinse the membrane with methanol followed by 70% aq ethanol.
24. Put 70% aq ethanol and shake for 5–10 min using a shaker.
25. Rinse the membrane with 70% aq ethanol.
26. Image the membrane or store in 70% aq ethanol or ethanol. (Note: Membrane can be dry or half-wet for imaging.)



### 3. Table of reaction conditions and filter sets employed

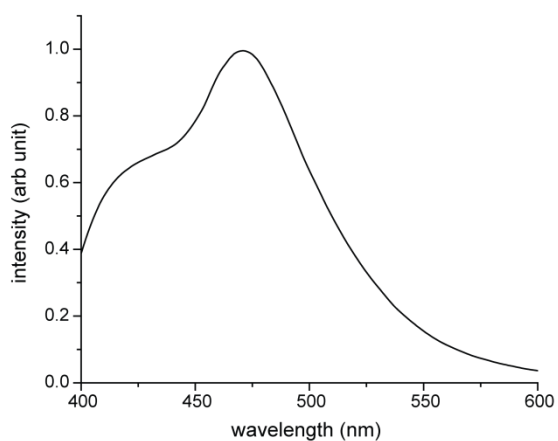
**Table S1.** Reaction condition and an imaging filter set of fluorogenic dyes.

	Na ascorbate <sup>a</sup> (mM)	THPTA <sup>b</sup> ( $\mu$ M)	dye ( $\mu$ M)	CuSO <sub>4</sub> ( $\mu$ M)	solvent	reaction time	longpass filter <sup>c</sup> (nm)
<b>3</b> (Ir-N3)	1.5	100	20–200	20–75	H <sub>2</sub> O/DMSO (1:1)	1 h–overnight	515 or 605
<b>4</b> (Q-N3)	1.5–5.0	25–100	0.01–200	75–500	H <sub>2</sub> O/DMSO (1:1)	0.5 h–overnight	410 or 515
<b>5</b> (Q-yne)	5.0	25	10–200	500	H <sub>2</sub> O/DMSO (1:1)	3 h–overnight	515 (or 410)
<b>6</b> (FI-DIBO)	-	-	50–125	-	<i>i</i> PrOH	3 h–overnight	410 (or 515)

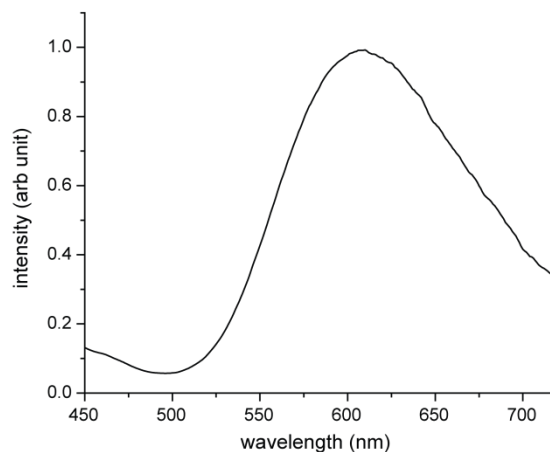
<sup>a</sup> Sodium ascorbate concentration should be 10–20 equivalent to copper (II) sulfate. <sup>b</sup> TBTA could be used instead of THPTA. <sup>c</sup> Excitation at 370 nm (DAPI excitation) is applicable for all four dyes.

## 4. Emission spectra of membrane dyes

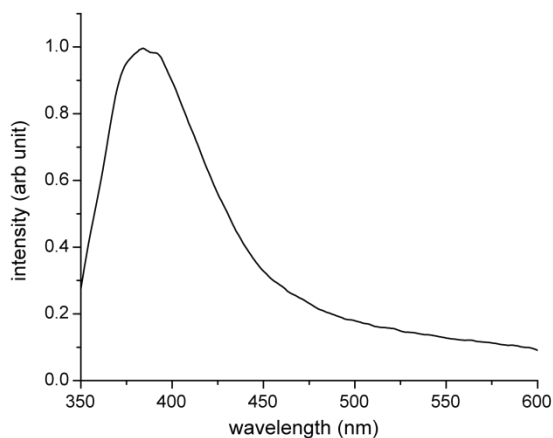
a) Q-N<sub>3</sub>



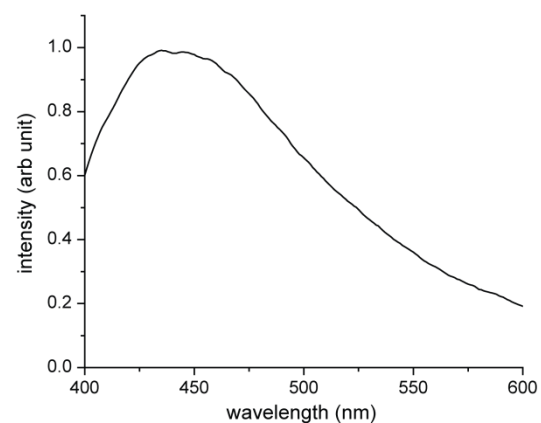
b) Ir-N<sub>3</sub>



c) Q-alkyne



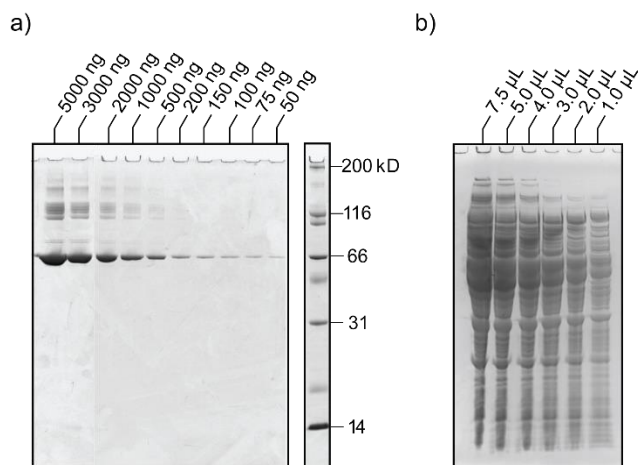
d) FI-DIBO



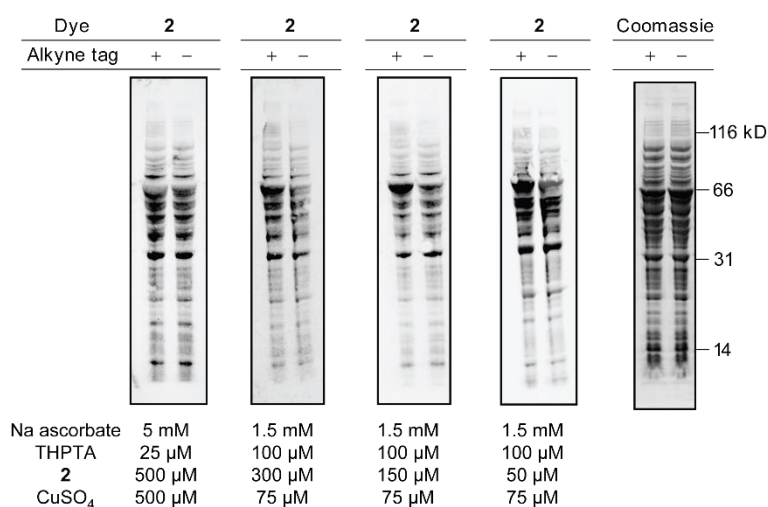
**Figure S1.** Emission spectra of triazole compounds on membrane: a) azide **4** with alkyne-tagged BSA, b) azide **3** with alkyne-tagged BSA, c) alkyne **5** with azide-tagged BSA, and d) cyclooctyne **5** with azide-tagged BSA.

## 5. Imaging optimization studies

### 5-1. Coomassie staining of commercial BSA and *E. coli* lysate, and screening of azido-fluorescein **2**



**Figure S2.** a) Coomassie staining of commercial BSA. Extra protein bands were observed at ~116 kD. b) Coomassie staining of *E. coli* lysate with differing amount injected into a gel (total injection volume: 10 µL). The 2.0-µL volume was used for detailed screening of probes **2-6**. The 1.0-µL was used for double blotting experiment considering resolution of smaller proteins.

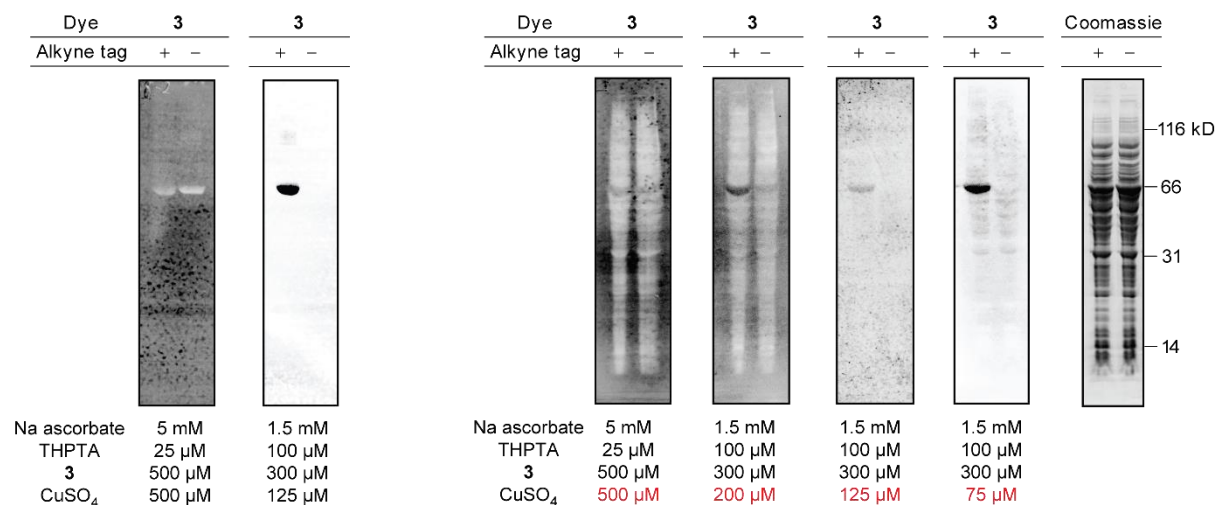


**Figure S3.** Additional attempts imaging with fluorescent azido-fluorescein **2**. Each lane contains BSA with or without alkyne label (10 µL, 0.3 mg/mL) with *E. coli* lysate. The membrane was imaged with a 515-nm longpass filter.

## 5-2. Screening of azido-iridium complex **3** (Ir-N<sub>3</sub>)

a) pure BSAs

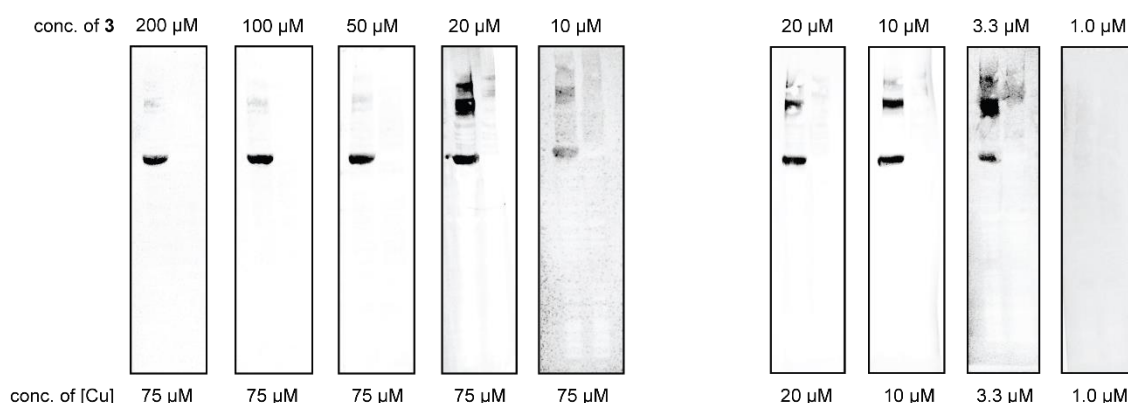
b) BSAs with *E. coli* lysate



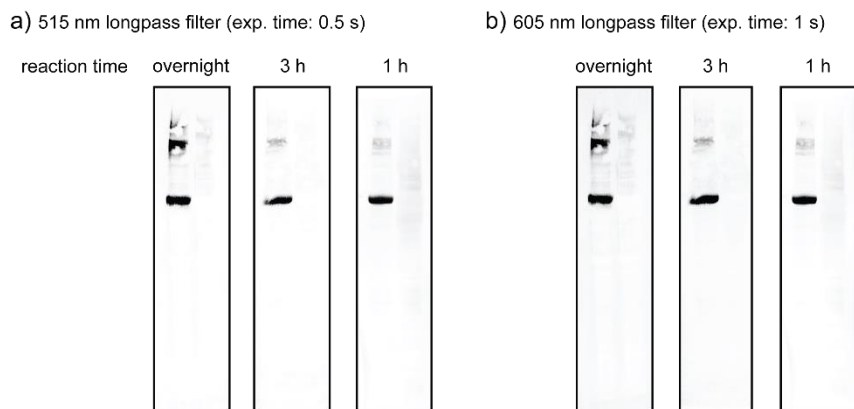
**Figure S4.** Initial screening of blotting with azide **3**. Each lane contains BSA with or without alkyne label (10  $\mu$ L, 0.3 mg/mL) without (a) or with (b) *E. coli* lysate. Less copper (II) sulfate produces better contrast of the BSA band from background. The membrane was imaged with a 515-nm longpass filter.

a) exposure time: 2.0 s (75  $\mu$ M Cu)

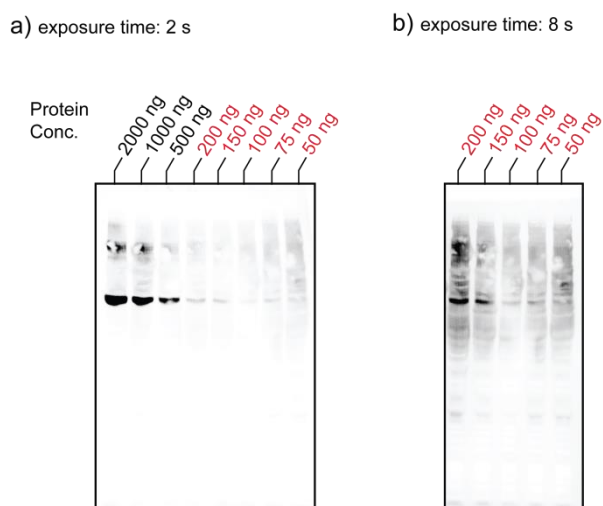
b) exposure time: 4.0 s (equimolar Cu)



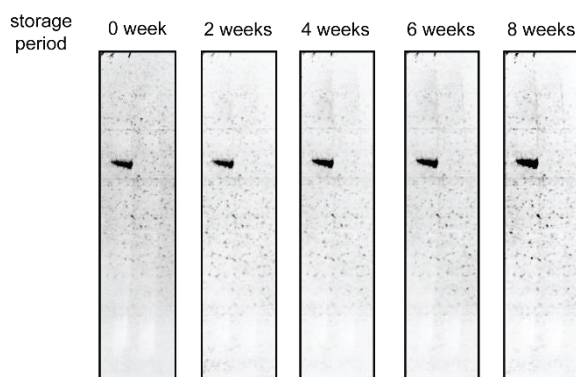
**Figure S5.** Optimization of the concentration of azide **3**. Each lane contains BSA with (left) or without (right) alkyne label (10  $\mu$ L, 0.3 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (1.5 mM), THPTA (100  $\mu$ M), copper (II) sulfate (75  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (8 mL) at rt overnight. The membrane was imaged with a 605-nm longpass filter.



**Figure S6.** Dependence of image quality on blotting time. Each lane contains BSA with (left) or without (right) alkyne label (10  $\mu$ L, 0.3 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (1.5 mM), THPTA (100  $\mu$ M), azide **3** (20  $\mu$ M), copper (II) sulfate (75  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (8 mL) at rt. Both 515- and 605-nm longpass filters are applicable for the probe **3**.



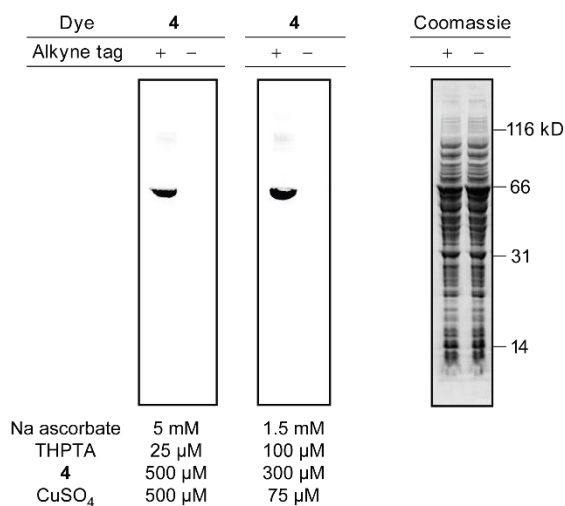
**Figure S7.** Sensitivity test of azide **3**. Each lane contains alkyne-labeled BSA with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (1.5 mM), THPTA (100  $\mu$ M), azide **3** (20  $\mu$ M), copper (II) sulfate (75  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (20 mL) at rt for 3 h. The membrane was imaged with a 605-nm longpass filter.



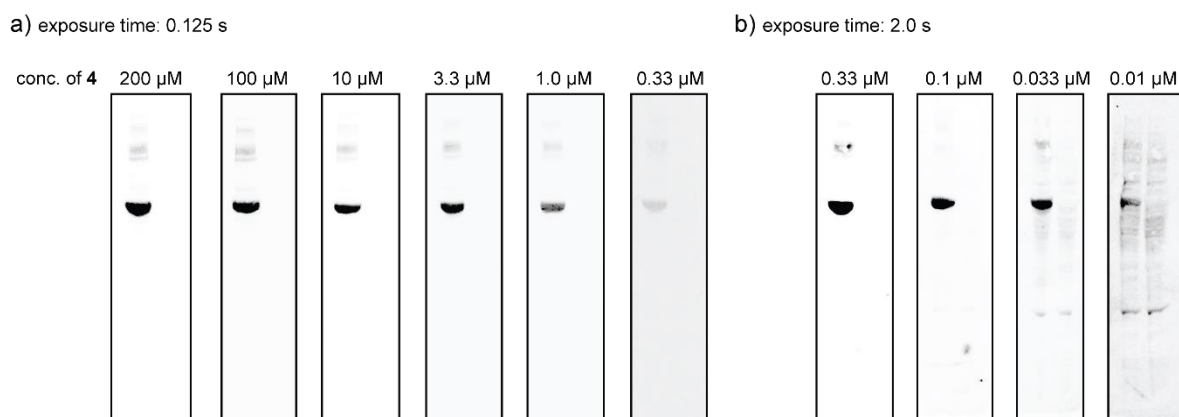
**Figure S8.** Long term stability of the fluorescence band after blotting. The membrane blotted with azide **3** was

stored in 70% aq ethanol and imaged with a 605-nm longpass filter after the storage. Each lane contains BSA with (left) or without (right) alkyne label (10  $\mu$ L, 0.13 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (1.5 mM), THPTA (100  $\mu$ M), azide **3** (300  $\mu$ M), copper (II) sulfate (75  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (8 mL) at rt overnight.

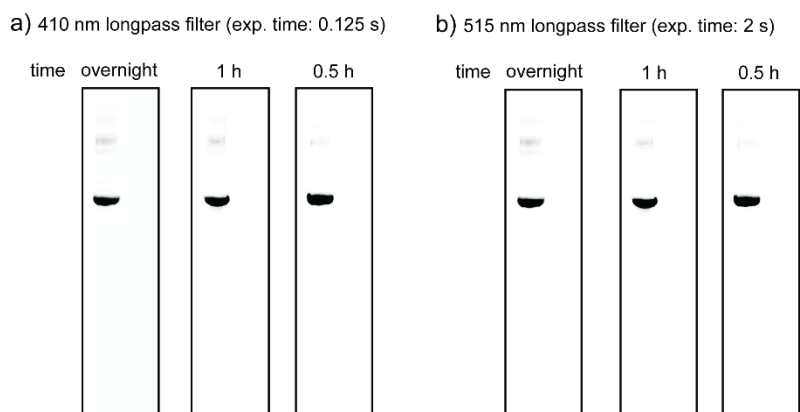
### 5-3. Screening of azido-coumarin 4 (Q-N<sub>3</sub>)



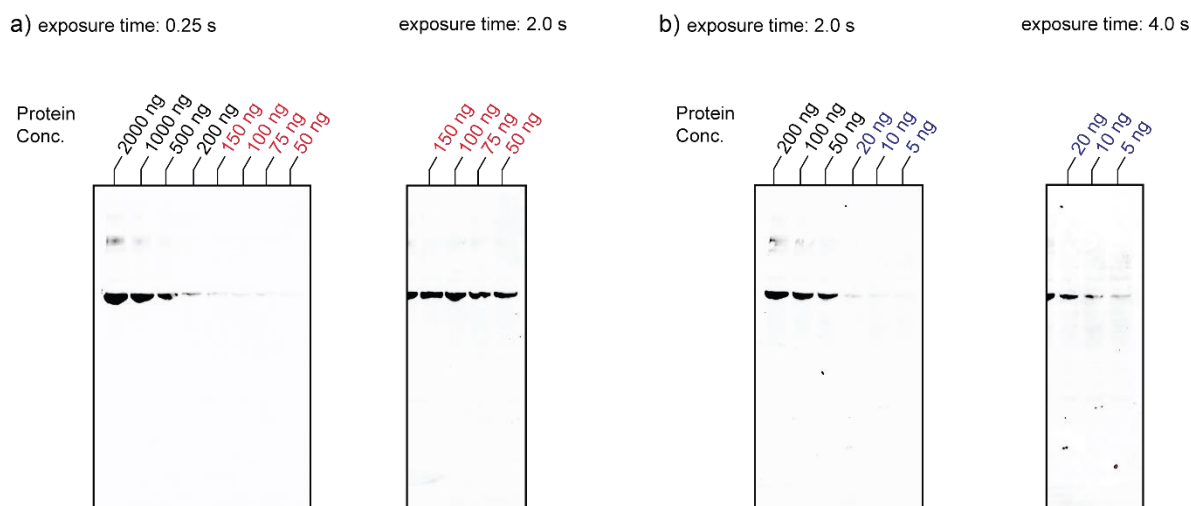
**Figure S9.** Initial screening of blotting with azide **4**. Each lane contains BSA with or without alkyne label (10 μL, 0.3 mg/mL) with *E. coli* lysate. Blotting with azide **4** is not sensitive to the concentration of reagents. The membrane was imaged with a 410-nm longpass filter.



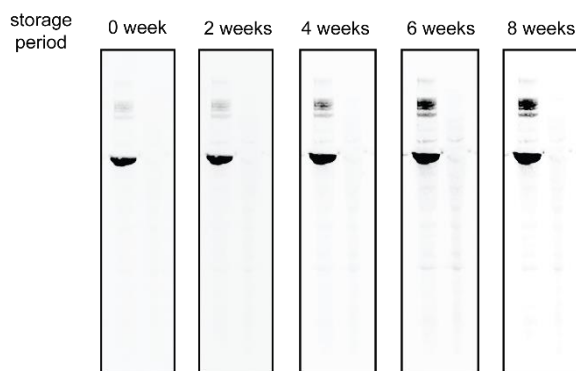
**Figure S10.** Optimization of the concentration of azide **4**. Each lane contains BSA with (left) or without (right) alkyne label (10 μL, 0.3 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (1.5 mM), THPTA (100 μM), copper (II) sulfate (75 μM) in 1:1 H<sub>2</sub>O/DMSO (8 mL) at rt overnight. The membrane was imaged with a 410-nm longpass filter.



**Figure S11.** Dependence of image quality on blotting time. Each lane contains BSA with (left) or without (right) alkyne label (10  $\mu$ L, 0.3 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (1.5 mM), THPTA (100  $\mu$ M), azide **4** (10  $\mu$ M), copper (II) sulfate (75  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (8 mL) at rt. Both 410- and 515-nm longpass filters are applicable for the probe **4**.



**Figure S12.** Sensitivity test of azide **4**. Each lane contains alkyne-labeled BSA with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (1.5 mM), THPTA (100  $\mu$ M), azide **4** (10  $\mu$ M), copper (II) sulfate (75  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (20 mL) at rt for 30 min. The membrane was imaged with a 410-nm longpass filter.

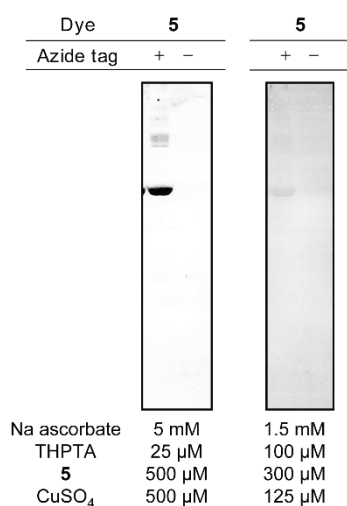


**Figure S13.** Long term stability of the fluorescence band after blotting. The membrane blotted with azide **4** was

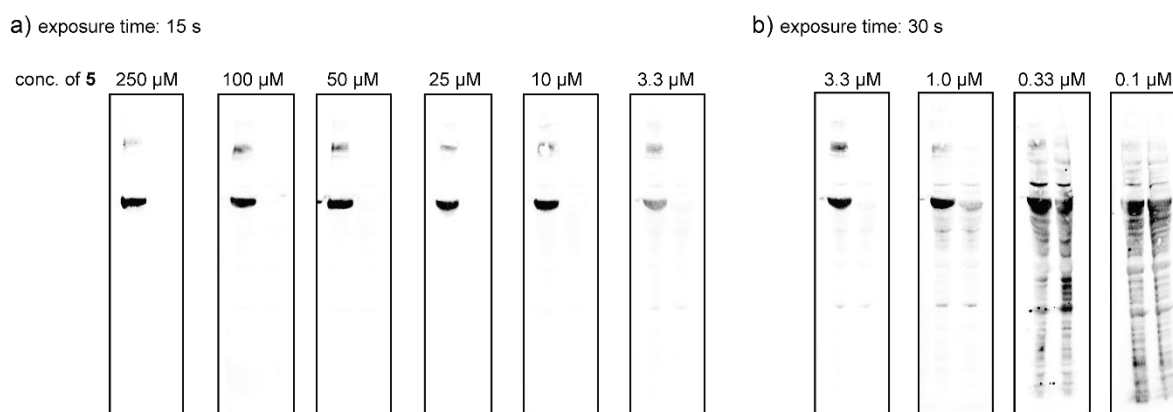


stored in 70% aq ethanol and imaged with a 515-nm longpass filter after the storage. Each lane contains BSA with (left) or without (right) alkyne label (10  $\mu$ L, 0.13 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (1.5 mM), THPTA (100  $\mu$ M), azide **4** (300  $\mu$ M), copper (II) sulfate (75  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (8 mL) at rt overnight.

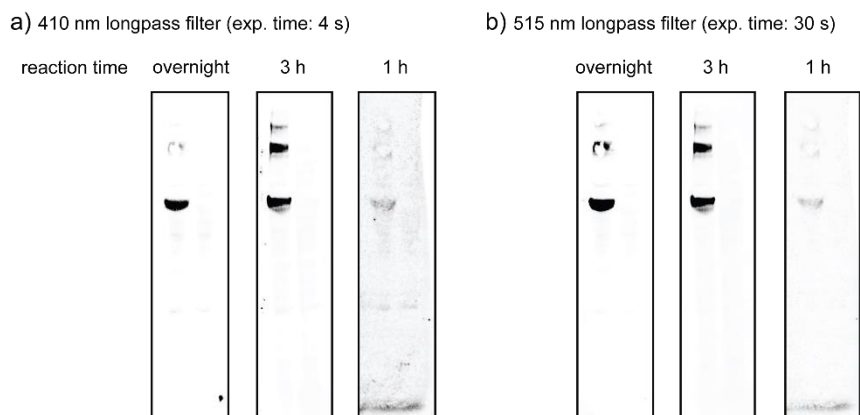
## 5-4. Screening of ethynyl coumarin 5 (Q-yne)



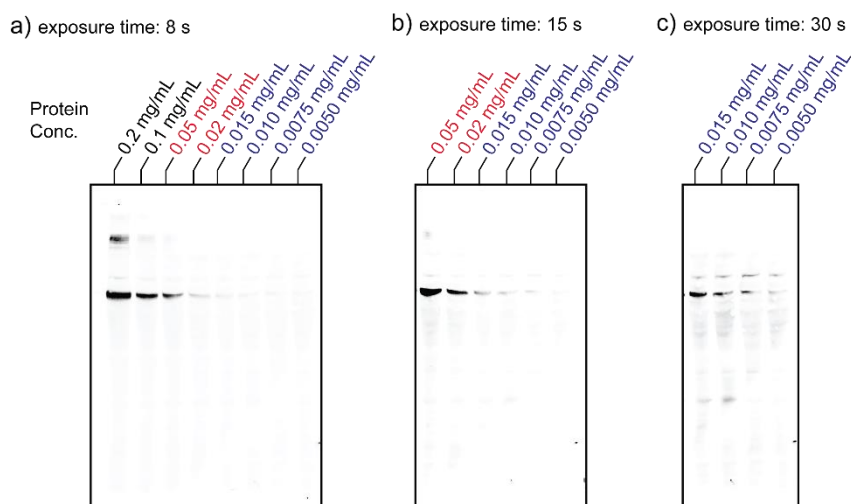
**Figure S14.** Initial screening of blotting with alkyne **5**. Each lane contains BSA with or without azide label (10  $\mu$ L, 0.3 mg/mL) in the absence of *E. coli* lysate. Excess of copper (II) sulfate is crucial for the probe **5**. The membrane was imaged with a 515-nm longpass filter.



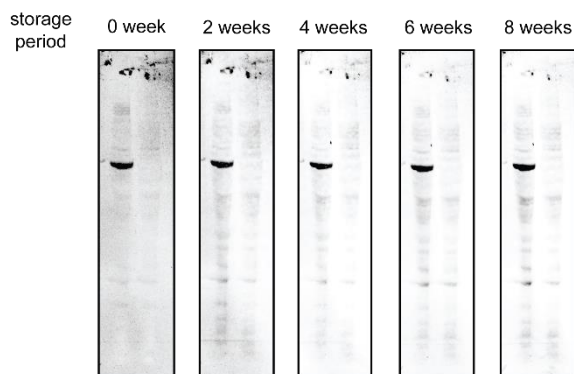
**Figure S15.** Optimization of the concentration of alkyne **5**. Each lane contains BSA with (left) or without (right) azide label (10  $\mu$ L, 0.3 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (5.0 mM), THPTA (25  $\mu$ M), copper (II) sulfate (500  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (8 mL) at rt overnight. The membrane was imaged with a 515-nm longpass filter.



**Figure S16.** Dependence of image quality on blotting time. Each lane contains BSA with (left) or without (right) azide label (10  $\mu$ L, 0.3 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (5.0 mM), THPTA (25  $\mu$ M), alkyne **5** (10  $\mu$ M), copper (II) sulfate (500  $\mu$ M) in 1:1  $H_2O$ /DMSO (8 mL) at rt. 515-nm longpass filter is better to minimize background signal for alkyne **5** than 410-nm one.



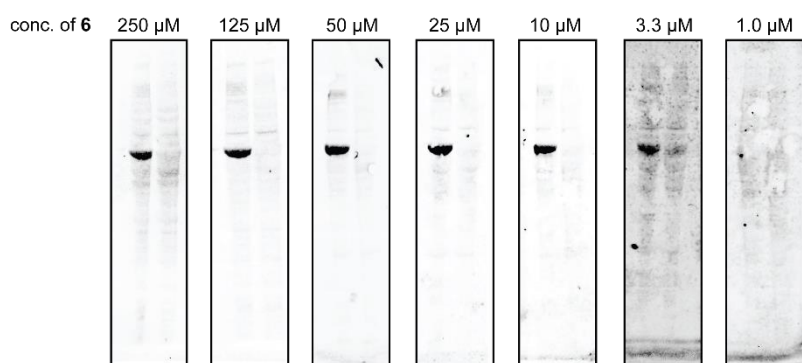
**Figure S17.** Sensitivity test of alkyne **5**. Each lane contains azide-labeled BSA with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (5.0 mM), THPTA (25  $\mu$ M), alkyne **5** (10  $\mu$ M), copper (II) sulfate (500  $\mu$ M) in 1:1  $H_2O$ /DMSO (20 mL) at rt overnight. The membrane was imaged with a 515-nm longpass filter.



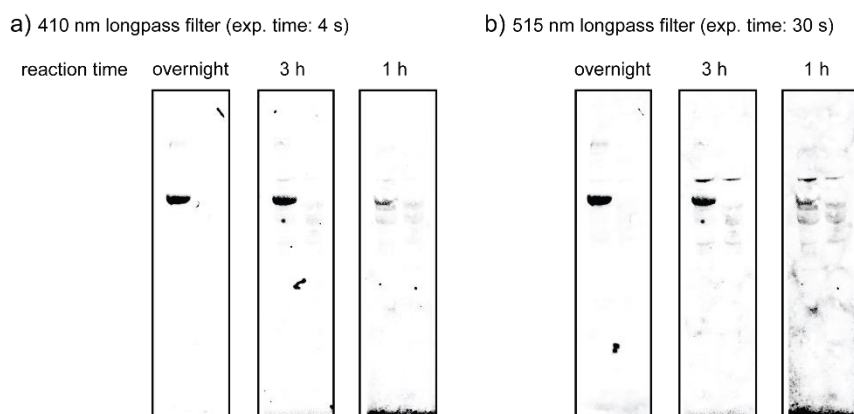
**Figure S18.** Long term stability of the fluorescence band after blotting. The membrane blotted with alkyne **5** was

stored in 70% aq ethanol and imaged with a 515-nm longpass filter after the storage. Each lane contains BSA with (left) or without (right) azide label (10  $\mu$ L, 0.13 mg/mL) with *E. coli* lysate. Composition of click reaction soln: sodium ascorbate (5.0 mM), THPTA (25  $\mu$ M), alkyne **5** (500  $\mu$ M), copper (II) sulfate (500  $\mu$ M) in 1:1 H<sub>2</sub>O/DMSO (8 mL) at rt overnight.

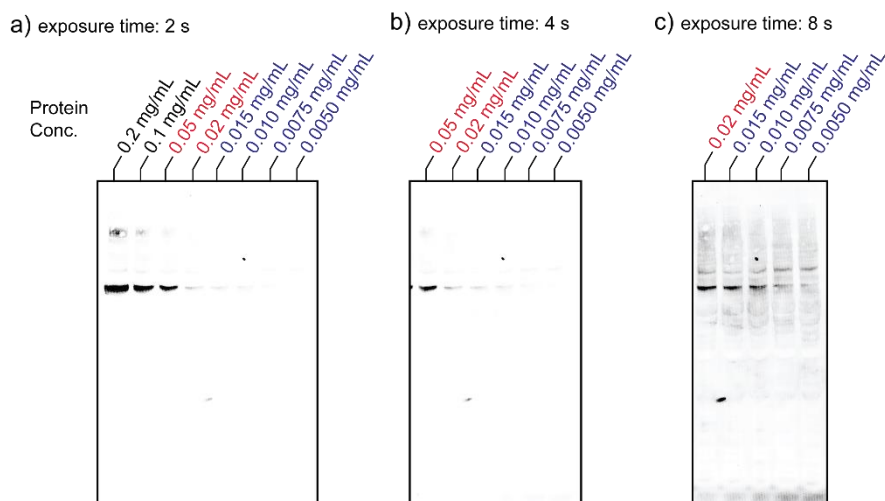
## 5-5. Screening of fluorogenic cyclooctyne **6** (FI-DIBO)



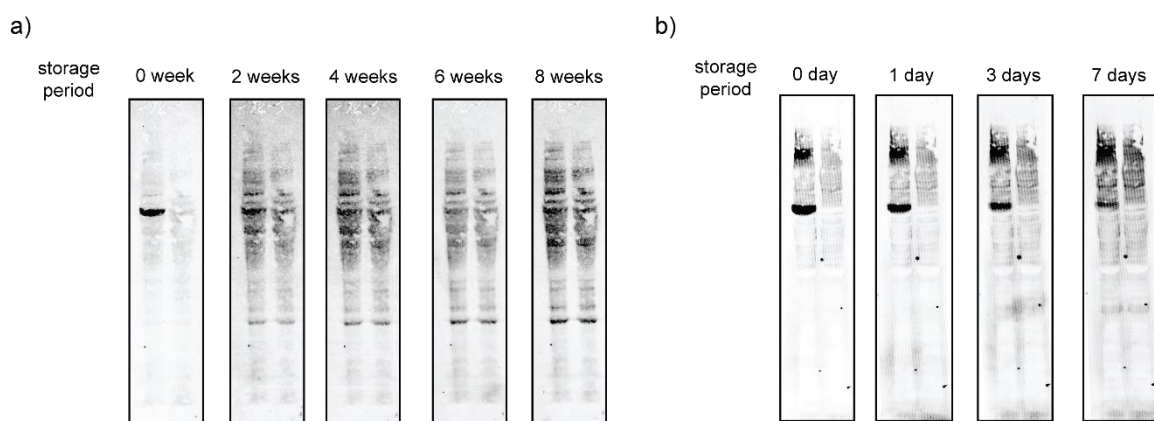
**Figure S19.** Optimization of the concentration of cyclooctyne **6**. Each lane contains BSA with or without azide label (10  $\mu$ L, 0.3 mg/mL) with *E. coli* lysate. Composition of click reaction soln:  $i$ PrOH (5 mL) at rt overnight. The membrane was imaged with a 410-nm longpass filter.



**Figure S20.** Dependence of image quality on blotting time. Each lane contains BSA with (left) or without (right) azide label (10  $\mu$ L, 0.3 mg/mL) with *E. coli* lysate. Composition of click reaction soln: cyclooctyne **6** (50  $\mu$ M) in  $i$ PrOH (5 mL) at rt. 410-nm longpass filter is better to minimize background signal for cyclooctyne **6** than 515-nm one.



**Figure S21.** Sensitivity test of cyclooctyne **6**. Each lane contains azide-labeled BSA with *E. coli* lysate. Composition of click reaction soln: cyclooctyne **6** (50  $\mu$ M) in *i*PrOH (15 mL) at rt overnight. The membrane was imaged with a 410-nm longpass filter.



**Figure S22.** Long term stability of the fluorescence band after blotting. The membrane blotted with cyclooctyne **6** was stored in 70% aq ethanol and imaged with a 410-nm longpass filter after the storage. Each lane contains BSA with (left) or without (right) azide label (10  $\mu$ L, 0.13 mg/mL) with *E. coli* lysate. Composition of click reaction soln: cyclooctyne **6** (125  $\mu$ M) in *i*PrOH (5 mL) at rt overnight.

**a)**

$N_3$   $\xrightarrow{3}$   $N_3$   $\xrightarrow{4}$

*Cu-free* *Cu-catalyzed*

filter (nm)	410	605
exp. (s)	0.25	1.0

200 kD  
116  
66  
31  
14

**b)**

$N_3$   $\xrightarrow{5}$   $N_3$   $\xrightarrow{4}$

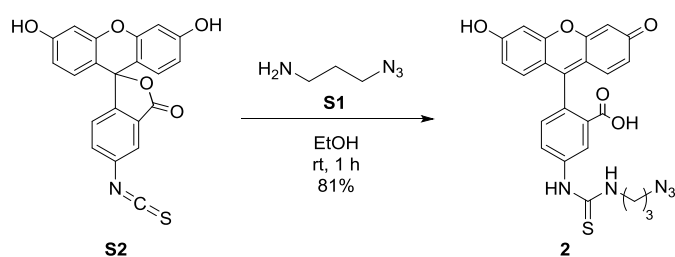
*Cu-catalyzed* *Cu-catalyzed*

410	515
2.0	8.0

2.0 8.0 2.0 8.0 2.0 8.0

23

## 6. Synthesis of fluorophore 2



### Azidofluorescein **2**

Fluorescein isothiocyanate **S2** (50 mg, 0.128 mmol) was suspended in ethanol (2.5 mL). Amine **S1** (120 mg, 0.120 mmol) was added to the solution, and the mixture became dark red soln. After stirred at rt for 1h, the mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> and poured into aq KHSO<sub>4</sub> (0.1 N, 10 mL) causing precipitation of a dark red solid. EtOAc (25 mL) was added to dissolve the precipitate. Organic layer was extracted, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, concentrated under vacuum, and purified by silica gel column chromatography (pure EtOAc) to afford a dark red solid (51 mg, 81%). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): δ 8.12 (d, *J* = 2.0 Hz, 1H), 7.75 (dd, *J* = 8.0, 2.0 Hz, 1H), 7.15 (dd, *J* = 8.0, 0.5 Hz, 1H), 6.68 (m, 4H), 6.54 (dd, *J* = 8.5, 2.5 Hz, 2H), 3.71 (m, 2H), 3.43 (t, *J* = 6.5 Hz, 2H), 1.93 (m, 2H). <sup>13</sup>C{<sup>1</sup>H} NMR (125 MHz, CD<sub>3</sub>OD): δ 183.2, 171.4, 161.8, 154.4, 149.7, 142.5, 132.2, 130.5, 129.4, 125.9, 120.4, 113.9, 111.7, 103.7, 50.5, 43.2, 29.5. ESI-MS: calcd for C<sub>24</sub>H<sub>18</sub>N<sub>3</sub>O<sub>5</sub>S [M-H]<sup>-</sup> 488.1, found *m/z* 487.9.



## 7. NMR spectra

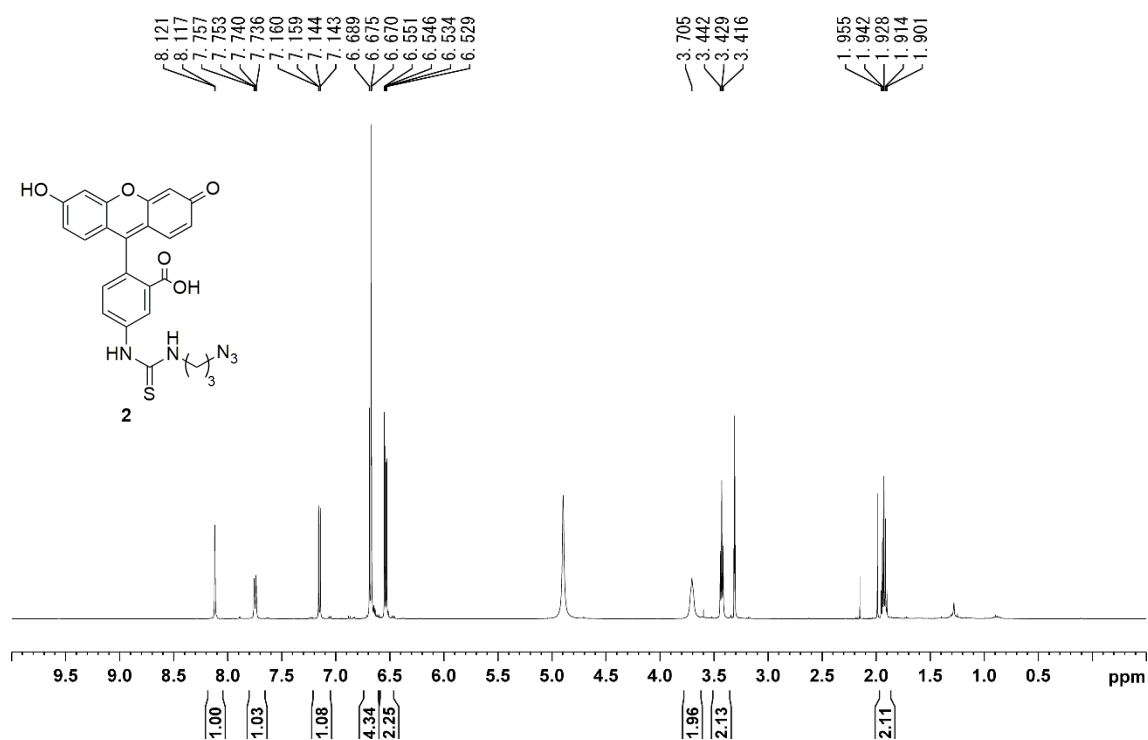


Figure S24.  $^1\text{H}$  NMR spectrum of **2** in  $\text{CDCl}_3$ .

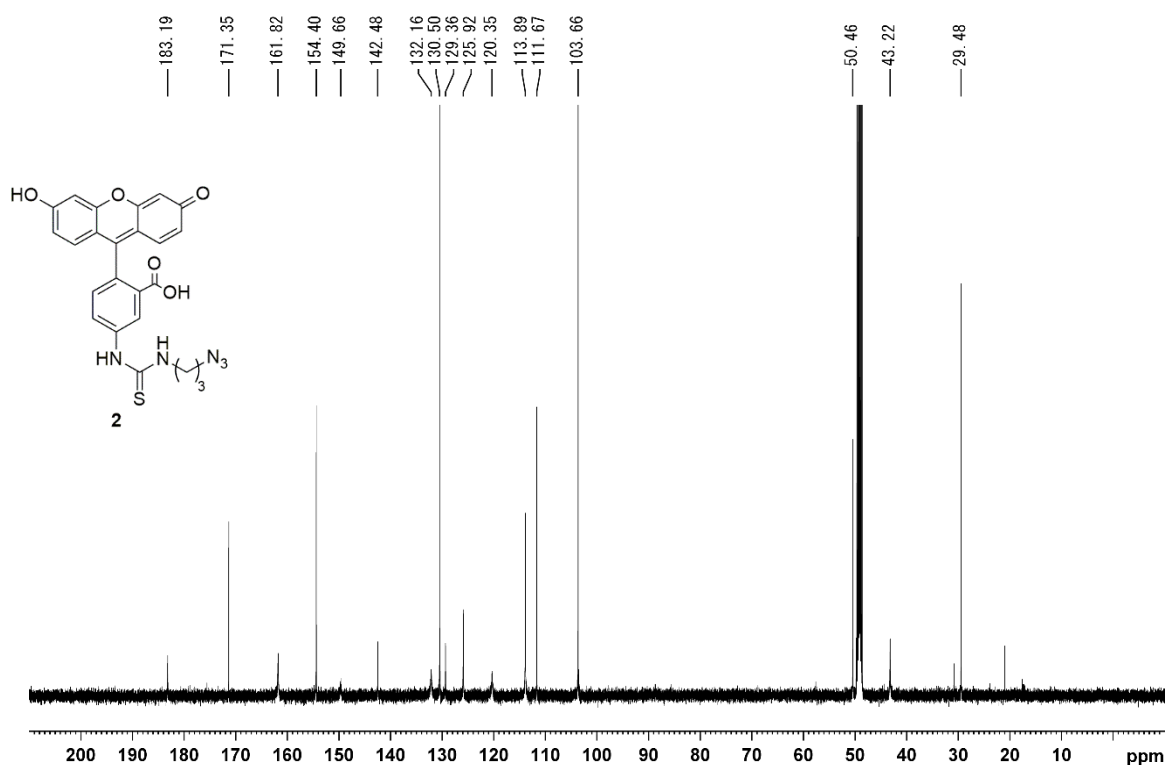


Figure S25.  $^{13}\text{C}\{^1\text{H}\}$  NMR spectrum of **2** in  $\text{CDCl}_3$ .

## 8. References

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