Supporting information for

A Cu-free clickable fluorescent probe for intracellular targeting of small biomolecules

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Supplementary Materials and Methods

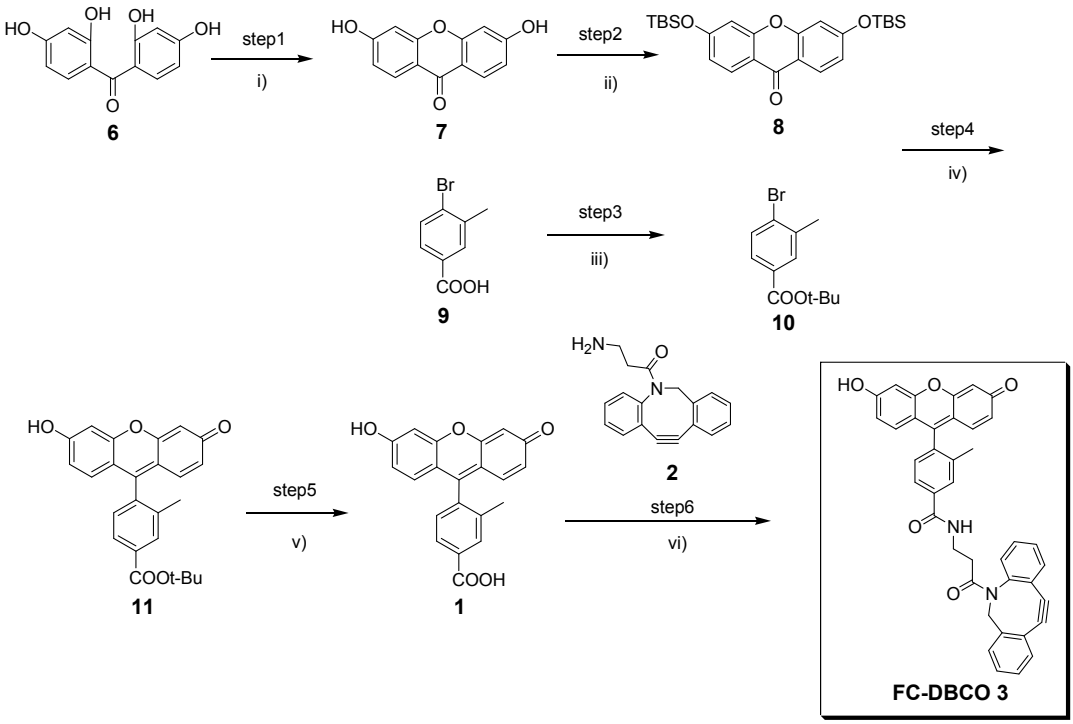
1. Synthesis and characterization of FC-DBCO

Materials

2,2',4,4'-Tetrahydroxybenzophenone, 4-bromo-3-methylbenzoic acid were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan) and DBCO-amine was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). All the other organic reagents were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan) and Sigma-Aldrich Japan K.K. (Tokyo, Japan) and used without further purification. All solvents were purchased from Kanto Chemical Industry Co. (Tokyo, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used as purchased.

Synthesis and characterization of FC-DBCO

Synthetic scheme for a novel intracellular Cu-free clickable fluorescent probe, FC-DBCO **3** is composed of 6 steps (**Scheme S1**). Synthesized compounds were characterized by ^{1}H-NMR. The NMR spectra were obtained using samples prepared in a deuterated solvent and recorded using an OXFORD NMR AS400 (400 MHz) spectrometer. ESI-MS spectra were recorded using a LCQ Fleet (Thermo Fisher Scientific) mass spectrometer. LC/high resolution (HR)-ESI-MS spectra were obtained using using the AB SCIEX TripleTOF™ 4600 System. IR spectra were recorded using FT-IR410 (JASCO Co.) spectrometer. Plastic sheets coated with 0.2 mm silica gel 60 (Merck & Co.) were used for thin-layer chromatography (TLC) to monitor the reactions.



i) no solvent, ii) TBSCl, imidazole, DMF, DMF, iii) oxalyl chloride, DMF, CH_2Cl_2, KO*t*-Bu, iv) *t*-BuLi, HCl, THF, v) TFA, CH_2Cl_2, vi) DMF, TEA, PyBOP

Scheme S1 Synthesis route of FC-DBCO.

Step 1&2

2,7- Dihydroxyxanthone (7) Slurry of **6** (3.12 g, 12.7 mmol) in a flask was heated for 10 hours at 220°C to yield **7**, which was dried *in vacuo* and used for the next step without any purification.

Xanthone diTBS ether (8) Slurry of **7** was added TBSCl (10.5 g, 76.2 mmol), imidazole (8.00 g, 127 mmol) and DMF (150 mL). The solution was stirred for 3 hours at room temperature (r.t.) and subsequently diluted with toluene, washed with H_2O (x3) and dried over anhydrous Na_2SO_4. Evaporation *in vacuo* gave brown solid, which was recrystallized from ethanol to give off white crystals **8** (3.53 g, 7.72 mmol, yield 61%). **^{1}H-NMR** (δ ppm in $CDCl_3$): 8.19-8.21 (m, 2H, ArH), 6.83-6.86 (m, 4H, ArH), 1.02 (s, 18H, t-Bu), 0.29 (s, 12H, Si-CH_3). **^{13}C-NMR** (δ ppm in $CDCl_3$): 180.1, 165.7, 162.1, 132.6, 121.9, 120.8, 111.7, 29.9, 22.6. **IR (cm^{-1})**: 2927, 1611, 1277, 843, 771. **ESI-MS (m/z)**: 457.50 ([M+H$^+$]$^+$, found), 456.22 (calcd) .

Step 3

4-Bromo-3-methylbenzoic acid tert-butyl ester (10) Under N_2 atmosphere, oxalyl chloride and a droplet of DMF were added to a suspension of 4-bromo-3-methylbenzoic acid **9** (10.0 mmol, 2.15 g) in CH_2Cl_2 (20 mL). The reaction proceeded for 2 hours. (The reaction mixture became a clear solution.) After removal of the solvent by a rotary evaporator and dried *in vacuo*, a solution of KO*t*Bu (15.0 mmol, 1.68 g) in dry THF (50 mL) was added at 0°C. Subsequently, the reaction was allowed to reach r.t. for 2 hours, after which H_2O was added and product extracted with EtOAc. The organic layer was dried over $MgSO_4$ and the solvent was removed by rotary evaporator and *in vacuo*, yielding the product as a yellow liquid **10** (1.86 g, 6.85 mmol, yield 69%).

^{1}H-NMR (δ ppm in CD_2Cl_2): 7.68 (d, 1H, ArH), 7.59 (dd, 1H, ArH), 7.36 (d, 1H, ArH), 2.26 (s, 3H, -CH_3), 1.47 (s, 9H, t-Bu). **^{13}C-NMR** (δ ppm in CD_2Cl_2): 165.0, 137.9, 132.3, 131.6, 131.2, 129.8, 128.3, 81.1, 28.2, 22.9.

Step 4

4-Carboxy-2-Me TokyoGreen tert-butyl ester (11) The three-necked flask was prepared and added to THF (10 mL). The solution of *t*-BuLi (6.65 mL, 8.65 mmol) and *N, N, N', N'*-tetramethyl-ethylenediamine (390 µl, 2.63 mmol) were added in sequence and finally the 4-bromo-3-methylbenzoic acid *t*-butyl ester **10** (429.0 mg, 1.58 mmol) in dry THF (15 mL) was slowly added at -78°C under N_2 flow, and the solution was stirred for 10 seconds at the same temperature. To this solution xanthone di-TBS ester **8** (600.0 mg, 1.31 mmol) and lanthanum (III) chloride bis(lithium chloride) complex solution 0.6 M in THF (2.20 mL, 1.31 mmol) in dry THF (15 mL) was added in a dropwise manner at -78°C under N_2 flow. The flask was stirred for 1 hour. The mixture was further treated with 2 N HCl (10 mL) and stirred for another 10 min. The reaction mixture was extracted with EtOAc (4 x 30 mL). The combined

organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation. Silica gel chromatography using CH_2Cl_2/MeOH (9/1). **ESI-MS** (m/z): 403.50 ([M+H$^+$]$^+$, found), 402.15 (calcd).

Step 5

4-Carboxy-2-Me TokyoGreen (1) Compound **11** (173.7 mg, 0.432 mmol) was treated with TFA/CH_2Cl_2 (1/1, 14.5 mL) at r.t. for 1 hour. The mixture was concentrated with a acid-resistant pump. Silica gel chromatography using $CHCl_3$/MeOH (12/1) gave an orange solid **1** (52.15 mg, 0.15 mmol 35%). **^{1}H NMR** (DMSO-d_6): δ 7.87 (s, 1H, ArH), 7.81 (d, 1H, ArH), 7.25(d, 1H, ArH), 6.75 (d, 2H, ArH), 6.44 (s, 2H, ArH), 6.42 (s, 2H, ArH), 1.91 (s, 3H, -CH_3). **ESI-MS (m/z)**: 346.08 ([M+H]$^+$, found), 347.33(calcd).

Step 6

FC-DBCO (3) To a solution of 4-carboxy-2-Me TokyoGreen **1** (15 mg, 0.0434 mmol) in DMF (1.5 mL) was added (benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (22.59 mg, 0.0434 mmol), TEA (6 μl, 0.0434 mmol), and dibenzylcyclooctyl amine (DBCO-amine) **2** (12 mg, 0.0434 mmol). The mixture was stirred at r.t. for 1 hour. The solvent was removed. Silica gel chromatography using $CHCl_3$/MeOH (40/1) gave an orange solid **3** (8.35 mg, 0.014 mmol, yield 29%). **^{1}H-NMR** (δ ppm in CD_3OD): 7.70-7.64 (t,2H,ArH), 7.57 (d,1H,ArH), 7.51-7.46 (m,1H,ArH), 7.34 (t,1H,ArH), 7.25 (m,2H,ArH), 7.11 (d,1H,ArH), 7.02-6.97 (d,2H,ArH), 6.73-6.66 (m,4H,ArH), 5.10-5.18 (d,1H,CH_2), 3.72-3.64 (d,1H,CH_2), 3.52-3.31 (m,2H,CH_2), 2.52-2.26 (m,1H,CH_2), 2.34-2.24 (m,1H,CH_2), 2.60(s,3H,CH_3). **ESI-MS** (m/z): 605.58 ([M+H]$^+$, found), 604.20 (calcd). ^{1}H-NMR and ESI-MS spectra of FC-DBCO **3** are shown in **Fig. S1** and **S2**.

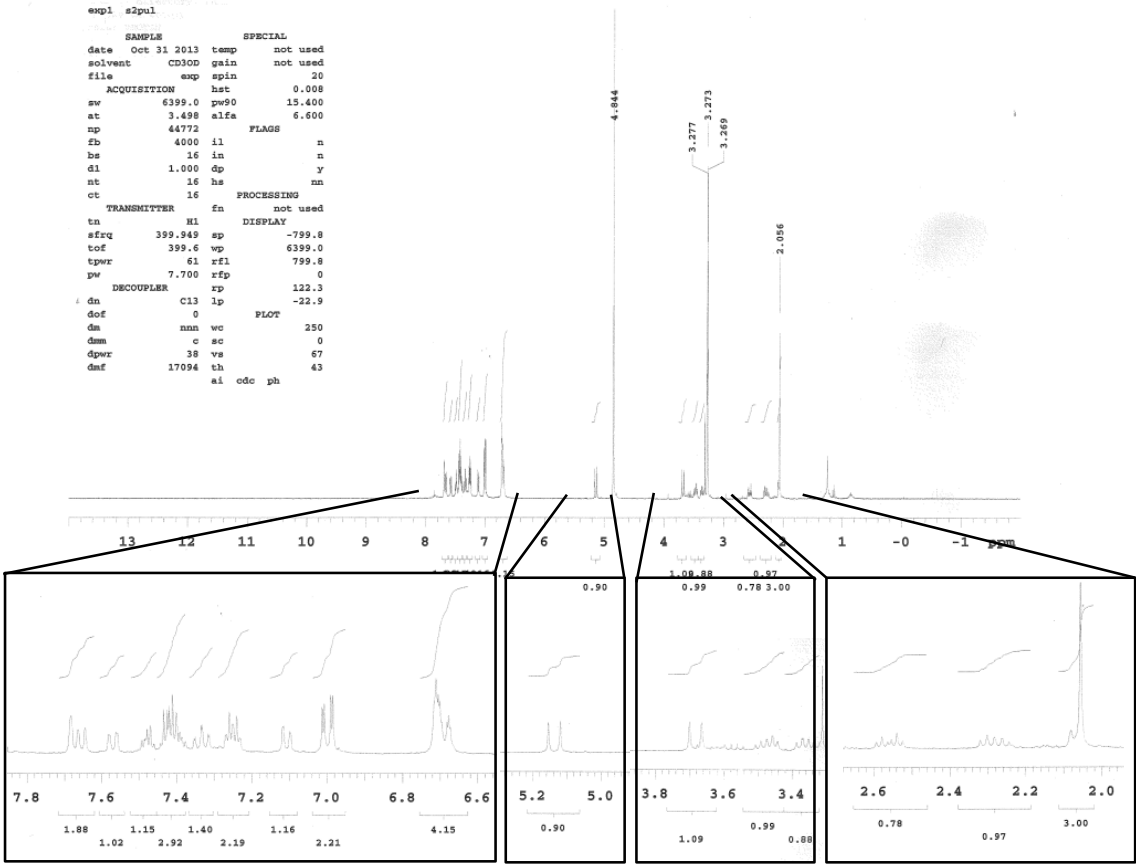


Fig. S1 ^{1}H-NMR spectrum of FC-DBCO **3**.

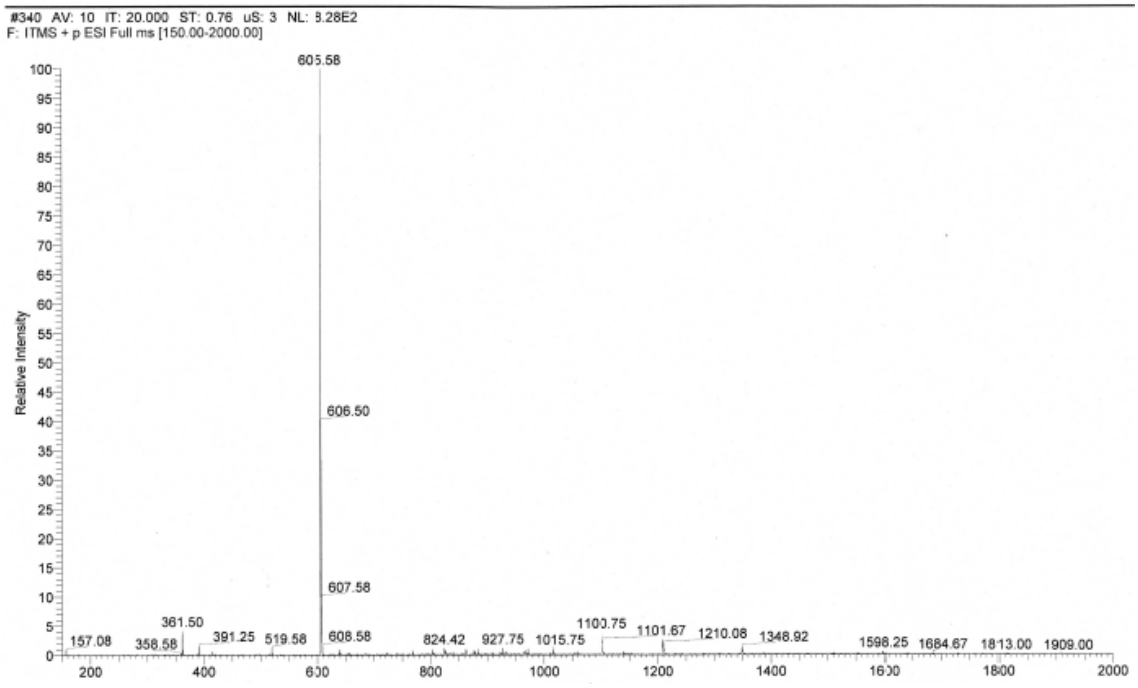


Fig. S2 ESI-MS spectrum of FC-DBCO **3**.

Excitation and Emission wave length of FC-DBCO

A 1 mM stock solution of FC-DBCO in DMSO was diluted 1000 times with pH 7.4 HEPES (final concentration: 1 μM). The solution was added in a quartz cuvette and measured by a spectrofluorophotometer (RF-5300PC, Shimadzu, Kyoto, Japan). Emission spectrum was obtained under excitation at 494 nm and the excitation spectrum was obtained at the maximum emission wavelength of 520 nm.

2. Evaluation of click reaction in cuvette

The reaction mixture was prepared as shown in **Table S1**. The mixture was kept at 37°C and UV-vis absorbance was measured by an UV/VIS/NIR spectrophotometer V-570 (JASCO) every 10 min (from 0 to 120 min) and 60 min (from 120 to 300 min).

Table S1 The click reaction mixture of *in vitro* evaluation.

Contents	Volume / μl	Final concentration / μM
1 mM FC-DBCO in DMSO	30	10
10 mM Ac_4ManNAz in DMSO	12	40
HEPES buffer (pH 7.4)	2958	—

LC/high resolution (HR)-ESI-MS measurement for the solution

Compound **3** and **4** in a DMSO solution (final concentration: 1 mM and 2 mM, respectively) was reacted for 24 hours. The MS spectrum and the UV-vis absorbance was measured for the solution.

3. Evaluation of cell membrane permeability of FC-DBCO

HeLa cells were incubated for 120 min on 35 mm / 27φglass base dishes (Iwaki, Tokyo, Japan) in culture medium (89% DMEM, (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen)) and a solution of FC-DBCO (1 mM stock solution in DMSO was diluted 100 times with medium, final concentration was 10 μM) at 37°C in the presence of 5% CO_2. The medium was gently aspirated, and the cells were washed twice with 1 mL of DPBS (Invitrogen). Replaced the fresh medium and HeLa cells were imaged with confocal microscope (Olympus, FV1000). After that, HeLa cells were incubated for 15, 30 min in culture medium and were washed two times with 1 mL of DPBS. Replaced the fresh medium and HeLa cells were imaged with confocal microscope.

4. Intracellular azide-mannose labeling in living HeLa cells

HeLa cells were incubated for 3, 6, 12 and 24 hours on glass base dishes in culture medium containing 40 μM Ac_4ManNAz (Invitrogen) at 37°C. The medium was gently aspirated, and the cells were washed twice with 1mL DPBS. The cells then were incubated with a solution of FC-DBCO (1 mM stock solution in DMSO was diluted 100 times with medium, the final concentration was 10μM, 1% DMSO) for 2 hours at 37°C. The medium containing FC-DBCO was gently aspirated, and the cells were washed three times with 1 mL culture medium. HeLa cells were incubated for 30 min in culture medium. The medium was gently aspirated, and the cells were washed three times with 1 mL culture medium. The medium was replaced with the fresh medium and HeLa cells were imaged with a confocal microscope.

LC/HR-ESI-MS measurement

Ac_4ManNAz (200 μM) was applied into the cells (2×10^5 cells) on 10-mm dish culture plates, and the cells were incubated for 4 hours before applying FC-DBCO. FC-DBCO (20 μM) was applied and the

cells were incubated for 2 hours for intracellular Cu-free click reaction. The unreacted probes were washed away by incubating cells under the fresh medium for 30 min. 0.25% Trypsin-EDTA (3 mL) was added and cell were incubated for 5 min. Then, fresh DMEM (7 mL) was added and the solution was pipetted. After centrifugation (1000 rpm, 2 min), the DMEM was removed and cells were treated with a 2% Triton X-100 solution (300 μL). The solution was decanted into an organic solvent-tolerant microtube, then added methanol (300 μL) and chloroform (300 μL). The solution was vortexed for about 5 min and centrifuged at 1500 rpm for 10 min. The MS spectrum was measured for the aqueous layer.

Each organelle tracker staining

Goigi Tracker (BODIPY; TR ceramide complexed to BSA, Molecular Probes)

HeLa cells were incubated for 30 min at 4°C with 5 μM ceramide BSA in HEPES. Cells were rinsed three times with ice-cold medium and incubated in fresh medium at 37°C for 30 min. The medium was replaced with the fresh medium and cells were observed with a confocal microscope.

Mito Tracker (MitoTarcker Deep Red FM, Molecular Probes)

HeLa cells were incubated for 30 min at 37°C with a 100 nM prewarmed staining solution in medium. Cells were rinsed three times with fresh medium and incubated in fresh medium at 37°C for 30 min. The medium was replaced with the fresh medium and cells were observed with a confocal microscope.

ER Tracker (ER-Tracker Blue-White DPX, Molecular Probes)

HeLa cells were incubated with 300 nM staining solution in medium at 37°C for 30 min. Then cells were washed with fresh medium twice. The medium was replaced with the fresh medium and cells were observed with a confocal microscope.

Lyso Tracker (LysoTracker Red DND-99, Molecular Probes)

HeLa cells were incubated with 75 nM staining solution in medium at 37°C for 30 min. Then cells

were washed with fresh medium twice. Then replaced with the fresh medium and observed with a confocal microscope.

Supplementary Results

5. Evaluation of Cu-free click reaction in cuvette

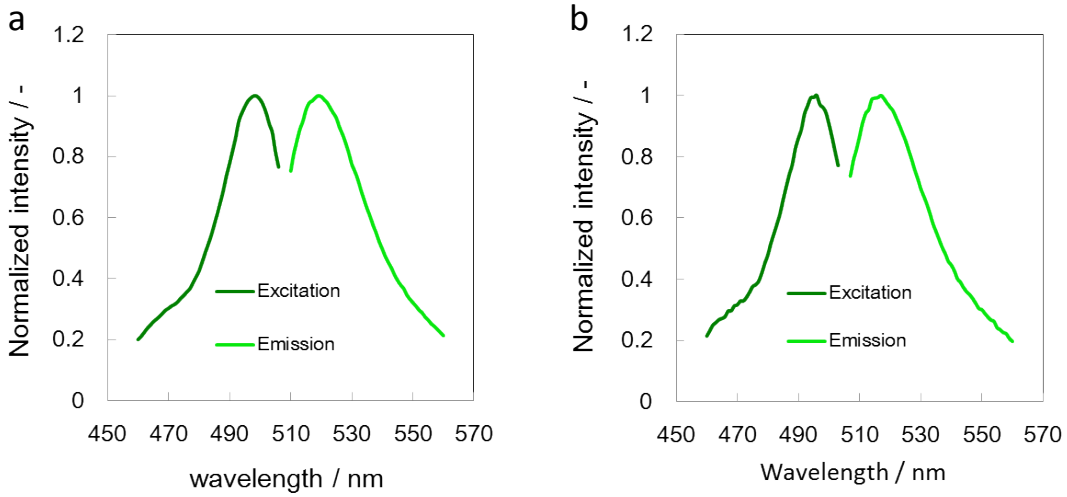


Fig. S3 Excitation and emission spectra of FC-DBCO before **(a)** and after **(b)** Cu-free click reaction.

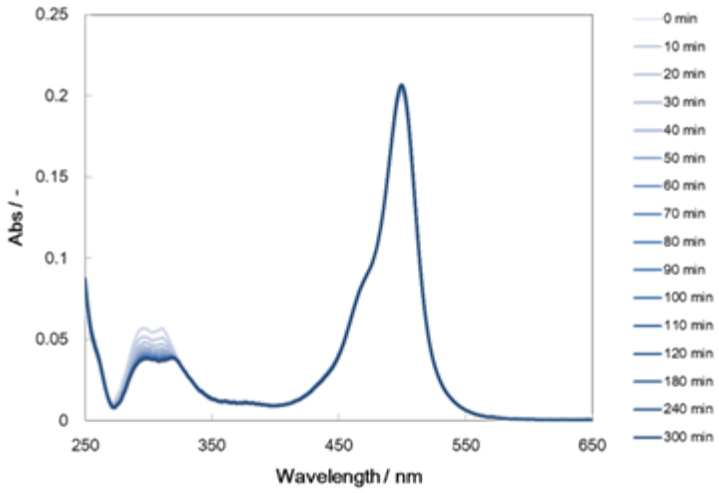


Fig. S4 UV-vis spectra of the reaction mixture during Cu-free click reaction between **3** and **4** (pH 7.4).

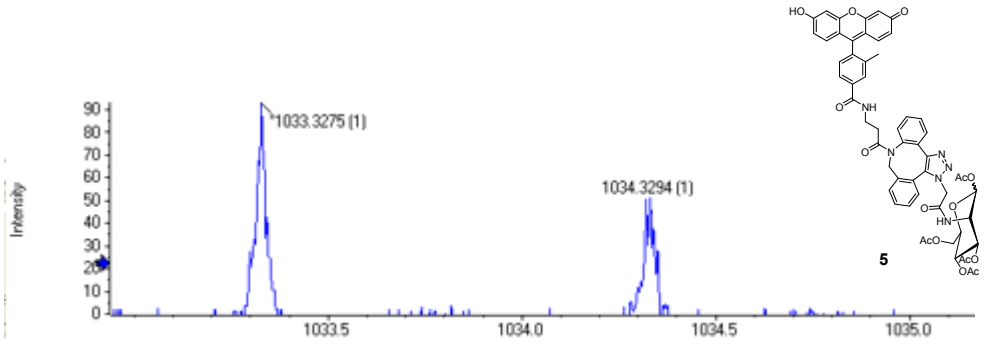


Fig. S5 HR-ESI-MS spectrum of **5** (m/z: 1033.3275 ([M-H]$^-$, obsd.), 1033.32504 (calcd. for $C_{55}H_{50}N_6O_{15}$, [M-H]$^-$), error: 1.1 ppm).

6. Evaluation of cell membrane permeability of FC-DBCO

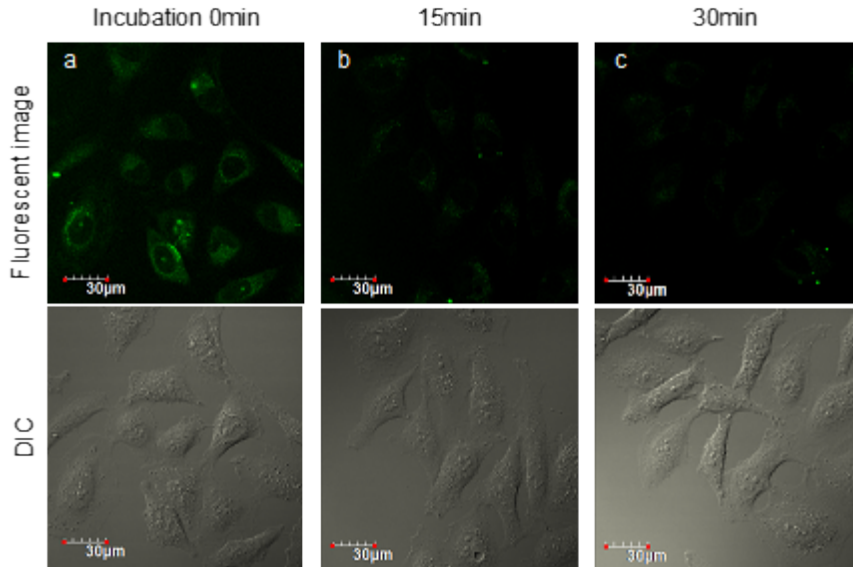


Fig. S6 Evaluation of cell membrane permeability of FC-DBCO (scale bar: 30 μm, upper: fluorescence images, bottom: DIC images). HeLa cells were incubated with 10 μM FC-DBCO at 37°C for 2 hours. Then, the culture medium was replaced. After 0 **(a)**, 15 **(b)**, and 30 min **(c)** incubation, cells were washed with DPBS and images were observed.

7. Intracellular azide-mannose labeling in living HeLa cells

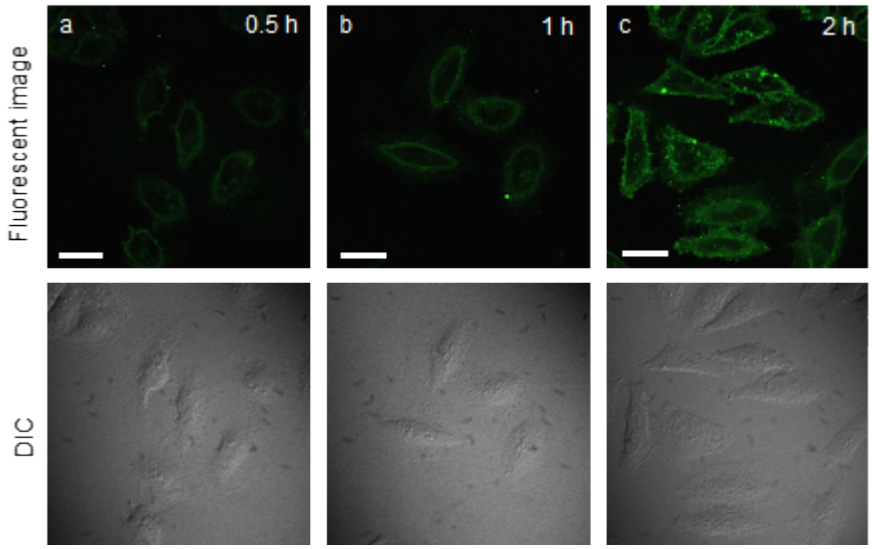


Fig. S7 Evaluation of FC-DBCO loading time with azide-mannose (scale bar: 30 μm, upper: fluorescence images, bottom: DIC images). HeLa cells were incubated with Ac_4ManNAz (40 μM) for 24 hours at 37°C, and then FC-DBCO (10 μM) was applied for the cells. After 0.5 **(a)**, 1 **(b)**, and 2 hours **(c)** incubation, cells were washed with DPBS and images were observed.

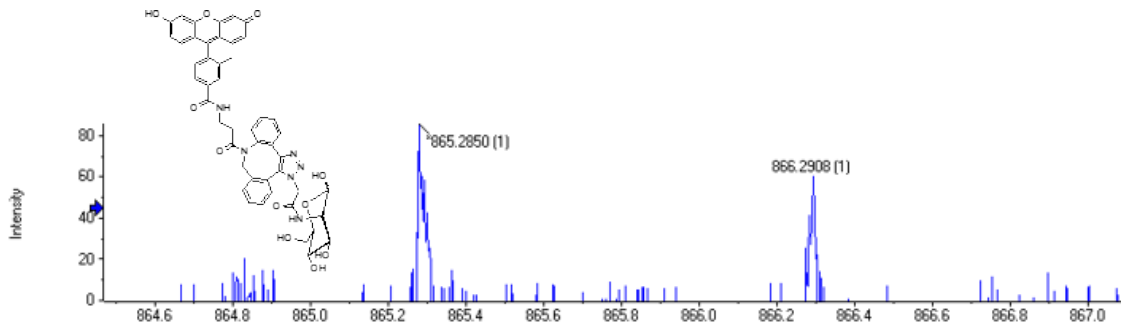


Fig. S8 HR-ESI-MS spectrum of FC-DBCO-conjugated ManNAz (m/z: 865.2850 ([M-H]$^-$, obsd.), 865.2839 (calcd. for $C_{39}H_{28}N_2O5$, [M-H]$^-$), error: 1.3 ppm).

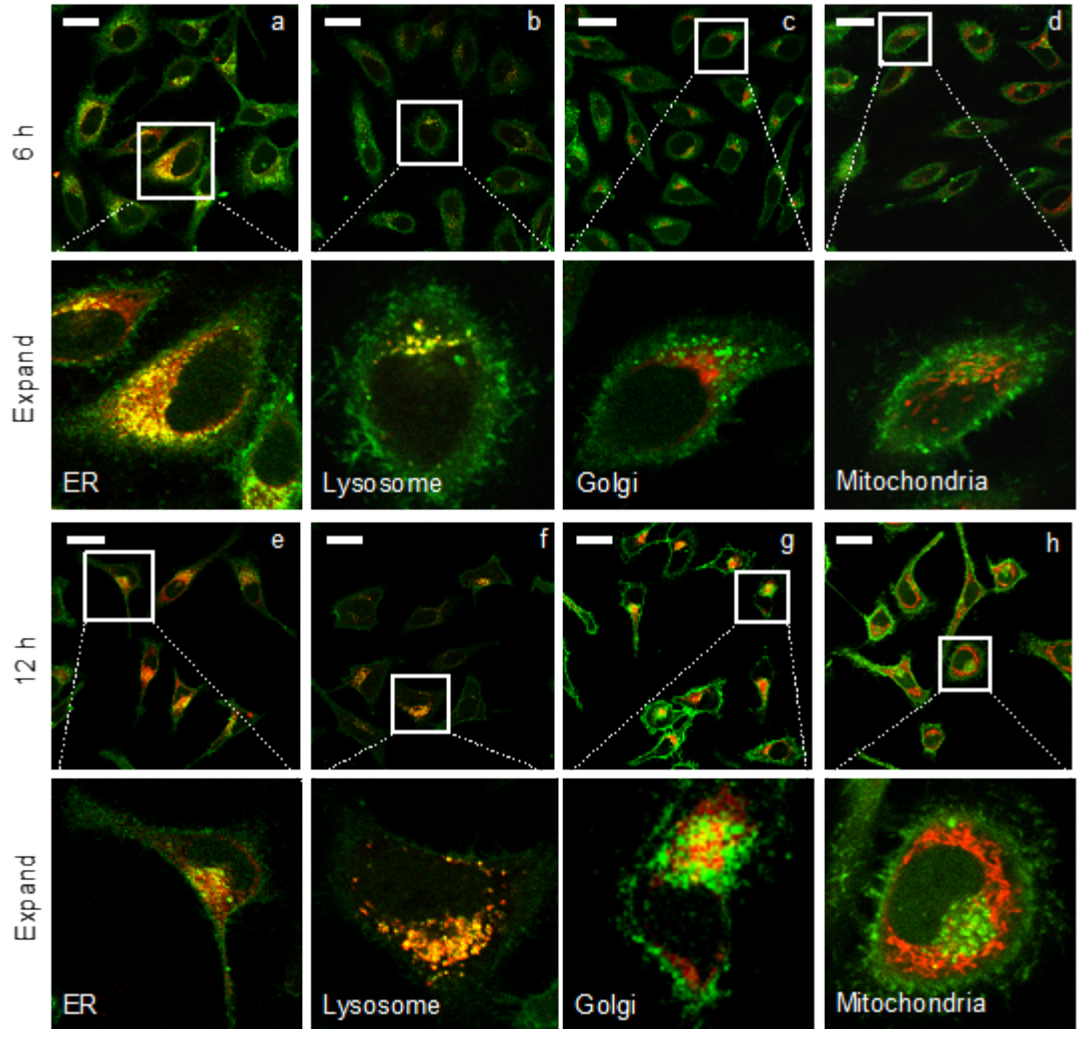


Fig. S9 Localization of azide-mannose and mannoglycoprotein in HeLa cells (scale bar: 30 μm). **Fig. 4 b** and **c** are expand images of **(a-h)**.