Supporting Information

No-wash Protein Labeling with Designed Fluorogenic Probes and Application to Real-Time Pulse-Chase Analysis

Shin Mizukami,[†,‡] Shuji Watanabe,[†] Yuri Akimoto,[†] and Kazuya Kikuchi[*,†,‡]

[†] Division of Advanced Science and Biotechnology, Graduate School of Engineering, Osaka University.

[‡] Immunology Frontier Research Center, Osaka University.

* To whom correspondence should be addressed: kkikuchi@mls.eng.osaka-u.ac.jp

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1. Materials and Instruments

Materials. General chemicals for organic synthesis were of the highest grade available supplied by Tokyo Chemical Industries, Wako Pure Chemical, and Aldrich Chemical Co, and were used without further purification. 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE·HCl) was obtained from Otsuka Chemical Co. Ltd. pcDNA 3.1(+) vector were purchased from invitrogen (21083-027). Restriction endonucleases and PrimeSTAR® HS DNA Polymerase were purchased from Takara Bio Inc. Plasmid DNA was isolated with a QIAprep Spin Miniprep kit (Qiagen Inc.). 5(6)-Carboxytetramethylrhodamine succinimidyl ester was purchased from Invitrogen (C1171). All labeling probes were dissolved in DMSO (biochemical grade, Wako) before fluorescence measurements in order to facilitate solubilization in aqueous solvents. Compound **1**, **FA**, and **RA** were synthesized as described elsewhere.[S1,S2]

Instruments. NMR spectra were recorded on a JEOL JNM-AL400 instrument at 400 MHz for ^{1}H and at 100.4 MHz for ^{13}C NMR, using tetramethylsilane as an internal standard. Mass spectra were measured on a Waters LCT-Premier XE mass spectrometer for ESI or on a JEOL JMS-700 for FAB. Fluorescence spectra were measured using a Hitachi F4500 spectrometer. The slit width was 2.5 nm for both excitation and emission, and the photomultiplier voltage was 700 V. HPLC analyses were performed with an Inertsil ODS-3 (4.6 mm×250 mm) column (GL Sciences Inc.) using an HPLC system that comprised a pump (PU-2080, JASCO) and a detector (MD-2010 or FP-2020, JASCO). Preparative HPLC was performed with an Inertsil ODS-3 (10.0 mm × 250 mm) column (GL Sciences Inc.) using an HPLC system that comprised a pump (PU-2087, JASCO) and a detector (UV-2075, JASCO). UV-visible absorbance spectra were measured using a Shimadzu UV1650PC spectrometer. Fluorescence images of the gels were visualized with AE-6935B VISIRAYS-B (ATTO) and captured with a digital camera. Kinetic analysis was performed using a SPEX NanoLog spectrometer (HORIBA Jobin Yvon). Fluorescence microscopic images were acquired using an Olympus

FV10i-LIV confocal laser scanning microscope equipped with a ×60 lens. The emission filter sets used were Olympus BA490–540 for **FA**, **FCAPO2**, and **FCAPO2-DA,** and Olympus BA570–620 for **RA**. All the captured microscopic images were analyzed with ImageJ software.

2. Syntheses of Compounds

Synthesis of CAP1. A mixture of compound **1** (560 mg, 1.1 mmol) and sodium iodide (1.6 g, 11 mmol) was stirred in acetone for 1 h at RT. The reaction mixture was concentrated under reduced pressure and diluted with ethyl acetate. The organic layer was washed with water and then dried with brine and sodium sulfate. The light-orange amorphous residue was used without further purification for the next reaction. The iodide derivative (100 mg, 162 μmol) was dissolved in acetonitrile (3 mL), following which pyridine-2-azo-*p*-dimethylpyridine (55 mg, 243 μmol) was added to the reaction mixture. The mixture was stirred for 16 h at RT under Ar. It was then diluted with diethyl ether, and the precipitate was collected and washed with ether to yield compound **2**. The crude compound **2** was used without further purification for the next reaction. Compound **2** (30 mg, 36 μmol) was dissolved in anhydrous CH_2Cl_2 (4 mL), following which thioanisole (600 μL) and trifluoroacetic acid (TFA) (2 mL) were added at 0 °C. The mixture was stirred for 4 h at 0 °C and then poured into cold ether (15 mL). The precipitate was collected and washed with ether. The residue was purified with reversed-phase HPLC and eluted with H_2O/acetonitrile containing 0.1% formic acid to yield **CAP1** (11 mg, 29% yield). HRMS (FAB^+) *m/z*: 496.2039 (calcd for $[M+H]^+$: 496.1761).

Synthesis of 3. 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE·HCl) (1.0 g, 2.5 mmol), HOBt (681 mg, 4.9 mmol), and Boc-β-alanine (513 mg, 2.7 mmol) were dissolved in DMF (5 mL), following which triethylamine (TEA) (250 mg, 2.5 mmol) and WSCD·HCl (570 mg, 3.0 mmol) were added at 0 °C. The mixture was stirred for 3 h at 0 °C

under Ar. It was then diluted with ethyl acetate; washed with saturated aqueous $NaHCO_3$, 10% aqueous citric acid, and water; and then dried with brine and sodium sulfate. After evaporation, the residue was purified with silica gel chromatography and eluted with hexane/ethyl acetate (1:1) to yield compound **3** (1.002 g, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 9H), 2.47 (t, *J* = 6.6 Hz, 2H), 3.37 (q, *J* = 6.6 Hz, 2H), 3.45 (d, *J* = 18.2 Hz, 1H), 3.63 (d, *J* = 18.2 Hz, 1H), 3.78 (s, 3H), 4.41 (d, *J* = 12.4 Hz, 1H), 4.51 (d, *J* = 12.4 Hz, 1H), 4.93 (d, *J* = 5.0 Hz, 1H), 5.06 (brs, 1H), 5.21 (s, 2H), 5.80 (dd, *J* = 5.0, 8.3 Hz, 1H), 6.56 (brs, 1H), 6.87 (d, *J* = 8.3 Hz, 2H), 7.31 (d, *J* = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 27.1, 28.4, 36.0, 43.2, 55.3, 57.6, 68.2, 68.3, 77.2, 79.6, 114.0, 125.7, 126.2, 126.6, 130.7, 156.0, 160.0, 161.1, 164.6; HRMS (FAB^+) *m/z*: 540.1566 (calcd for $[M+H]^+$: 540.1571).

Synthesis of CAP2. A mixture of compound **3** (200 mg, 370 μmol) and sodium iodide (555 mg, 3.7 mmol) was stirred in acetone for 1 h at RT. The reaction mixture was concentrated under reduced pressure and diluted with ethyl acetate. The organic layer was washed with water and then dried with brine and sodium sulfate. The light-orange amorphous residue was used without further purification for the next reaction. The iodide derivative (220 mg, 340 μmol) was dissolved in acetonitrile (3 mL), following which pyridine-2-azo-*p*-dimethylpyridine (95 mg, 420 μmol) was added to the reaction mixture. The mixture was stirred for 7 h at RT under Ar. It was then diluted with diethyl ether, and the precipitate was collected and washed with ether to yield compound **4**. The crude compound **4** was used without further purification for the next reaction. Compound **4** (200 mg, 233 μmol) was dissolved in anhydrous CH_2Cl_2 (30 mL), following which thioanisole (3 mL) and TFA (10 mL) were added at 0 °C. The mixture was stirred for 4 h at 0 °C and then poured into cold ether (15 mL). The precipitate was collected and washed with ether to yield **CAP2** (130 mg, quant.) as the TFA salt. HRMS (FAB^+) *m/z*: 510.1929 (calcd for $[M+H]^+$: 510.1918).

Synthesis of FCAP2. **CAP2** (15 mg, 32 μmol) was dissolved in DMF (500 μL), following which

TEA (10 mg, 95 μmol) and 6-carboxyfluorescein-succinimidyl ester (30 mg, 48 μmol) were added at 0 °C. The mixture was stirred for 2 h, and the solvent was lyophilized. The residue was purified with reversed-phase HPLC and eluted with H_2O/acetonitrile containing 0.1% formic acid to yield **FCAP2** (11 mg, 53% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.41 (t, J = 6.6 Hz, 2H), 3.05 (d, J = 6.6 Hz, 2H), 3.27 (s, 6H), 3.31 (d, J = 18.1 Hz, 1H), 3.51 (d, J = 18.1 Hz, 1H), 4.89 (d, J = 5.0 Hz, 2H), 5.41 (dd, J = 5.0, 8.3 Hz, 1H), 5.50 (d, J = 13.2 Hz, 1H), 6.07 (d, J = 13.2 Hz, 1H), 6.55 (m, 4H), 6.68 (s, 2H), 7.00 (d, J = 7.4 Hz, 2H), 7.63 (s, 1H), 7.83 (m, 1H), 8.02 (d, J = 7.4 Hz, 2H), 8.11 (m, 3H), 8.40 (t, J = 7.4 Hz, 1H), 8.72 (t, J = 5.0 Hz, 1H), 8.81 (d, J = 7.4 Hz, 1H), 9.53 (d, J = 5.0 Hz, 1H), 10.4 (brs, 2H); HRMS (FAB$^+$) *m/z*: 868.2415 (calcd for $[M+H]^+$: 868.2401).

Synthesis of 5. A mixture of compound **3** (400 mg, 740 μmol) and *m*-chloroperbenzoic acid (mCPBA) (555 mg, 740 μmol) was stirred in acetone for 15 min at 0 °C. The reaction mixture was then diluted with CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$ and water and then dried with brine and sodium sulfate. After evaporation, the residue was purified with silica gel chromatography and eluted with 5% MeOH/CH_2Cl_2 to yield compound **5** (377 mg, 92% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 9H), 2.48 (t, J = 6.6 Hz, 2H), 3.39 (m, 3H), 3.79 (m, 3H), 4.21 (d, J = 12.4 Hz, 1H), 4.47 (d, J = 5.0 Hz, 1H), 5.01 (d, J = 12.4 Hz, 1H), 5.07 (brs, 1H), 5.22 (s, 2H), 6.06 (dd, J = 5.0, 9.9 Hz, 1H), 6.70 (d, J = 9.9 Hz, 2H), 6.88 (d, J = 8.3 Hz, 2H), 7.33 (d, J = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.2, 35.0, 36.6, 44.1, 46.1, 55.1, 58.2, 66.5, 67.6, 77.6, 113.8, 120.2, 124.9, 126.8, 130.47, 155.4, 159.4, 160.5, 164.4, 171.3; HRMS (FAB$^+$) *m/z*: 556.1503 (calcd for $[M+H]^+$: 556.1520).

Synthesis of FCAPO2. A mixture of compound **5** (300 mg, 540 μmol) and sodium iodide (809 mg, 5.4 mmol) was stirred in acetone for 1 h at RT. The reaction mixture was concentrated under reduced pressure and diluted with ethyl acetate. The organic layer was washed with water and then dried with

brine and sodium sulfate. The light-orange amorphous residue was used without further purification for the next reaction. The iodide derivative (150 mg, 232 μmol) was dissolved in acetonitrile (15 mL), following which pyridine-2-azo-*p*-dimethylpyridine (79 mg, 347 μmol) was added to the reaction mixture. The mixture was stirred for 6 h at RT under Ar. It was then diluted with diethyl ether, and the precipitate was collected and washed with ether to yield compound **6**. The crude compound **6** was used without further purification for the next reaction. Compound **6** (150 mg, 171 μmol) was dissolved in anhydrous CH_2Cl_2 (30 mL), following which thioanisole (2.4 mL) and TFA (8 mL) were added at 0 °C. The mixture was stirred for 4 h at 0 °C and then poured into cold ether (50 mL). The precipitate was collected and washed with ether to yield **CAPO2** as the TFA salt. The crude compound **CAPO2** was used without further purification for the next reaction. **CAPO2** (15 mg, 32 μmol) was dissolved in DMF (500 μL), following which TEA (10 mg, 95 μmol) and 6-carboxyfluorescein-succinimidyl ester (26 mg, 41 μmol) were added at 0 °C. The mixture was stirred for 2 h, and the solvent was lyophilized. The residue was purified with reversed-phase HPLC and eluted with H_2O/acetonitrile containing 0.1% formic acid to yield **FCAPO2** (12 mg, 58% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.45 (t, J = 6.6 Hz, 2H), 3.27 (s, 6H), 3.45 (m, 4H), 4.71 (d, J = 3.3 Hz, 1H), 5.51 (m, 2H), 6.05 (d, J = 13.2 Hz, 1H), 6.56 (m, 4H), 6.68 (s, 2H), 7.00 (d, J = 7.4 Hz, 2H), 7.64 (s, 1H), 7.76 (m, 1H), 8.10 (m, 6H), 8.39 (m, 1H), 8.71 (t, J = 5.8 Hz, 1H), 9.23 (d, J = 5.8 Hz, 1H), 10.5 (brs, 2H); HRMS (FAB$^+$) *m/z*: 884.2361 (calcd for $[M+H]^+$: 884.2350).

Synthesis of FCAPO2-DA. A mixture of **FCAPO2** (25 mg, 28 μmol) and cesium carbonate (92 mg, 280 μmol) was prepared. Thereafter, acetic anhydride (29 mg, 280 μmol) was added to the reaction mixture, and the mixture was stirred for 30 min at 0 °C. It was then acidified with acetic acid (34 mg, 566 μmol), and the solvent was lyophilized. The residue was purified with reversed-phase HPLC and eluted with H_2O/acetonitrile containing 0.1% formic acid to yield **FCAPO2-DA** (8 mg, 29% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.22 (s, 6H), 2.40 (t, J = 6.8 Hz, 2H), 3.21 (s, 6H), 3.36 (m, 4H),

4.65 (d, J = 3.9 Hz, 1H), 5.46 (d, J = 13.4 Hz, 1H), 5.56 (m, 1H), 5.95 (d, J = 13.4 Hz, 1H), 6.88 (m, 6H), 7.23 (s, 2H), 7.72 (s, 2H), 7.97 (m, 3H), 8.05 (m, 1H), 8.14 (m, 2H), 8.33 (m, 1H), 8.69 (m, 1H), 9.21 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 20.9, 34.3, 36.1, 39.9, 40.6, 41.3, 58.3, 66.2, 81.2, 110.5, 111.6, 112.4, 115.7, 118.8, 122.4, 124.1, 125.3, 125.5, 127.6, 129.3, 129.4 (2 carbons), 129.9 (2 carbons), 138.4, 141.0, 142.5, 149.0, 150.8, 152.1, 152.2, 153.2, 162.0, 163.6, 164.4, 167.7, 168.9, 171.1; HRMS (FAB+) *m/z*: 968.2568 (calcd for $[M+H]^+$: 968.2561).

3. Experimental Procedures

***In vitro* fluorometric analysis.** All fluorescent probes were dissolved in DMSO to obtain 10 mM stock solutions, and the solutions were diluted to the desired final concentrations with an appropriate aqueous buffer solution. Relative fluorescence quantum yields of the compounds were obtained by comparing the area under the emission spectrum of the sample with that of a 0.5 μM fluorescein in 100 mM NaOH aqueous solution (λ = 0.85 when excited at 492 nm)[S3] for fluorescein derivatives. Enzyme assays with WT TEM and BL-tag were performed in 100 mM HEPES buffer (pH 7.4) at room temperature. A total of 0.3 μL of purified WT TEM (40 nM) or 7.5 μL of purified BL-tag[S4] (1 μM) was added to 300 μL of buffer containing 500 nM probes. The samples were excited at 487 nm for **FCAP2** or at 490 nm for **FCAPO2** and **FCAPO2-DA**, and fluorescence was monitored at 518 nm.

Detection of protein labeling by SDS-PAGE. In this experiment, 4 μM purified BL-tag was added to a solution of 6 μM **FCAPO2** in 100 mM HEPES buffer (pH 7.4) at 25 °C. After 15 min, the labeled protein was solubilized in 2× SDS gel loading buffer (100 mM Tris-HCl buffer (pH 6.8), 2.5% SDS, 20% glycerol, and 10% mercaptoethanol) and resolved by SDS-PAGE. The gels were then stained with Coomassie Brilliant Blue and photographed. Next, 4 μM purified BL-tag was mixed

with HEK293T cell lysate, which was prepared by a freeze-thaw method, and the mixture was added to a solution of 6 μM **FCAPO2** in 100 mM HEPES buffer (pH 7.4) at 25 °C. After 15 min, the labeled proteins were analyzed as described above.

Kinetic study of BL-tag labeling with FCAPO2. Purified BL-tag (125 nM) was added to a solution of **FCAPO2** (1.25–25 nM) in 100 mM HEPES buffer (pH 7.4) at 25 °C. Fluorescence (λ_{ex} = 490nm, λ_{em} = 518 nm) of the labeled protein was detected with a SPEX NanoLog spectrophotometer (Figure S4). The data were fitted to the equation $v_0 = k$[BL-tag][**FCAPO2**], where v_0 is the initial reaction rate and k is the bimolecular kinetic constant.

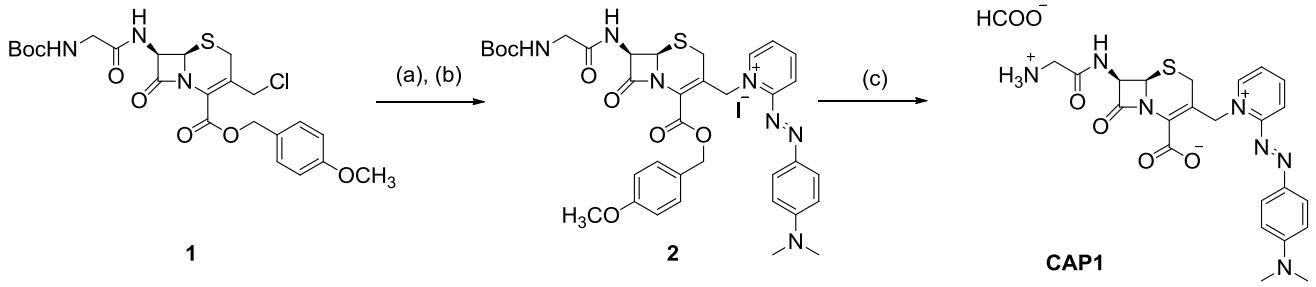
No-wash and time-lapse labeling of cell surface BL-EGFR with FA and FCAPO2. A plasmid coding BL-EGFR was transfected into HEK293T cells as described elsewhere.[S2] After 24 h, the cells were washed once with Hank's balanced salt solution (HBSS) and incubated with 500 nM **FA** or **FCAPO2** in HBSS at 37 °C for 10 min. Fluorescence images were captured using appropriate filter sets, without the washing procedure. For time-lapse imaging of fluorogenic labeling, 10 nM **FCAPO2** was used, and fluorescence images were captured every 1 min. Averaged fluorescence intensity values (n = 4) were calculated from data collected from the whole field of view, and plotted against time.

Real-time imaging of BL-EGFR trafficking with RA and FCAPO2. HEK293T cells expressing BL-EGFR were incubated with 100 nM **RA** in HBSS at 37 °C for 15 min. They were then washed three times with HBSS, and the culture medium was replaced by DMEM containing 20 nM **FCAPO2** and 10% FBS. Fluorescence images were captured using appropriate filter sets every 30 min under in a 5% CO_2 atmosphere.

No-wash labeling of BL-NLS-expressing cells with FCAPO2-DA. A plasmid coding BL-NLS was transfected into HEK293T cells as described elsewhere.[S5] After 24 h, the cells were washed once with HBSS and incubated with 5 μM **FCAPO2-DA** in HBSS at 37 °C for 2 h. The cell nuclei were co-stained with 100 ng mL^{-1} **Hoechst 33342**. Fluorescence images were captured using appropriate filter sets without the washing procedure.

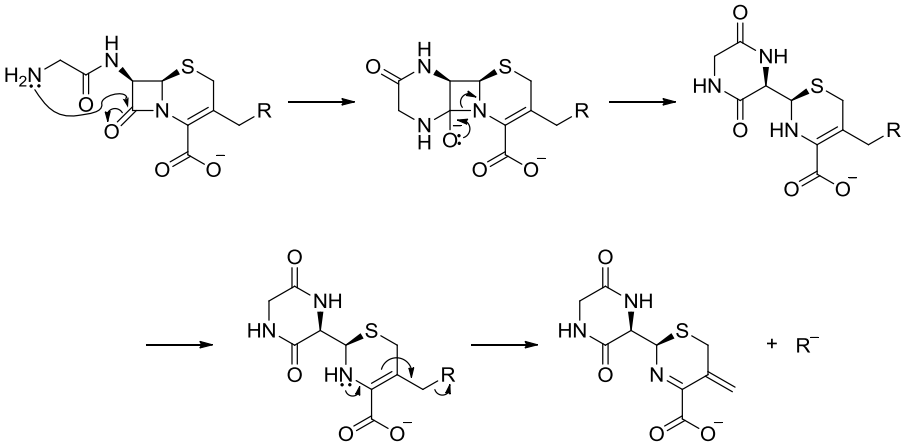
4. Supplementary Schemes,Figures, and Table

Scheme S1 Synthetic route to **CAP1**

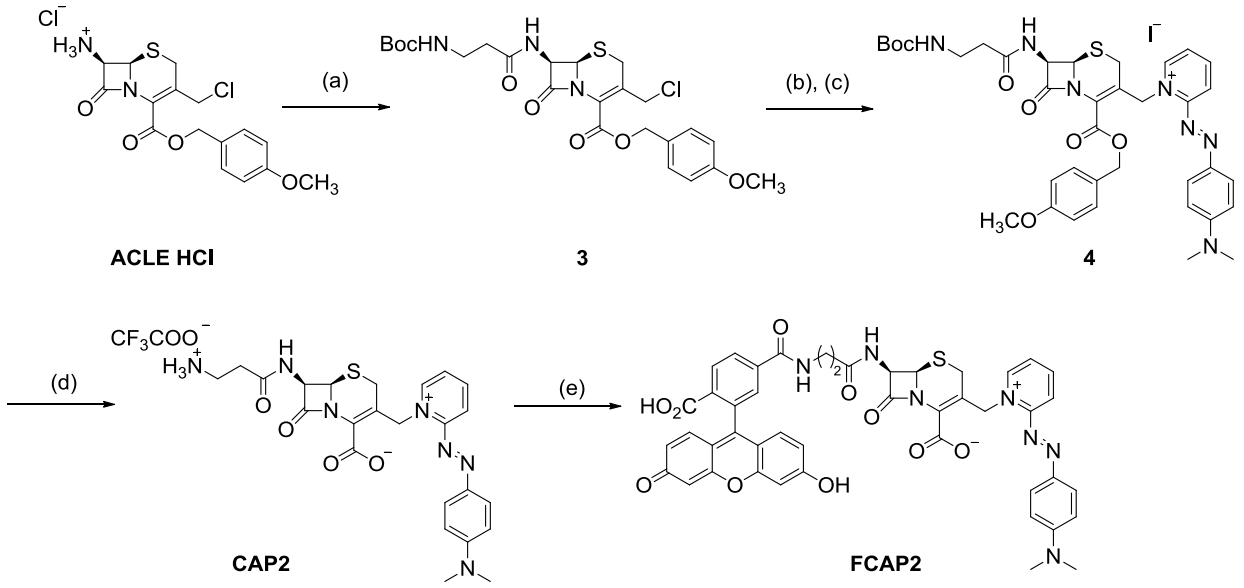


(a) NaI, acetone, RT. (b) Acetonitrile, 2-(4-dimethylaminophenylazo)pyridine, RT. (c) TFA, thioanisole, CH_2Cl_2, 0 ºC.

Scheme S2 Proposed mechanism of **CAP1** degradation via intramolecular cyclization reaction

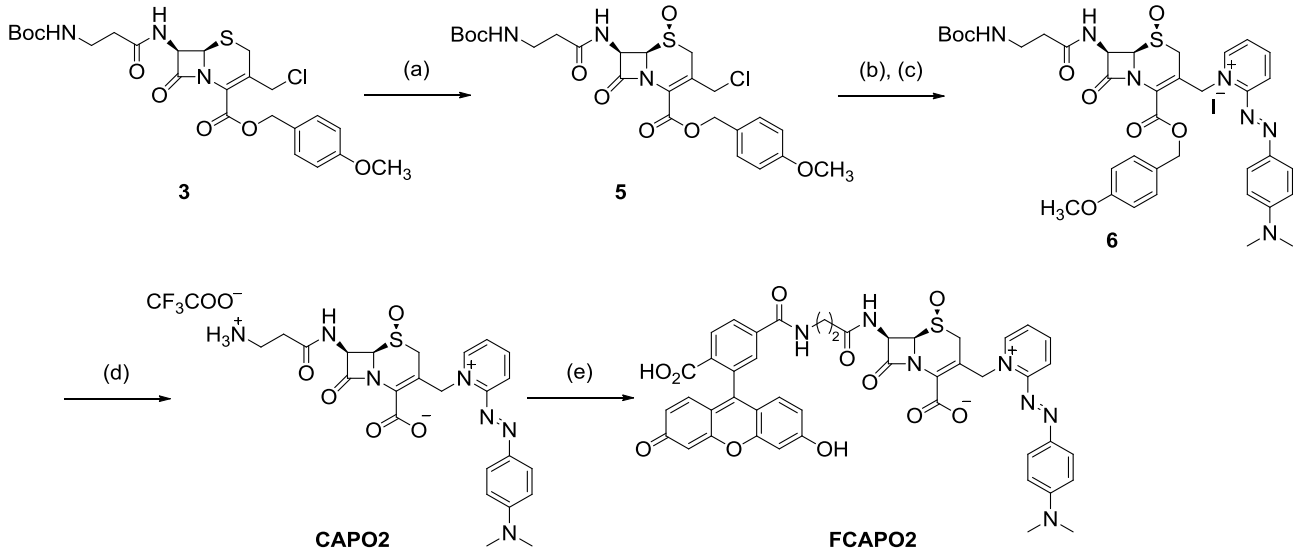


Scheme S3 Synthetic route to **FCAP2**



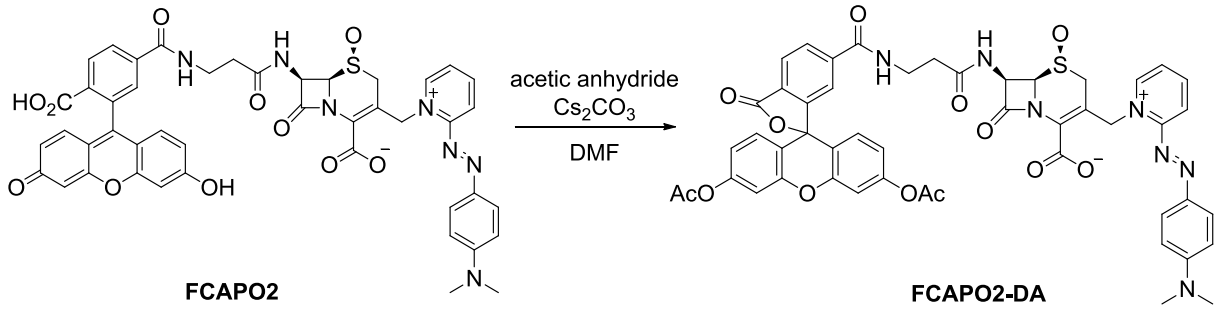
(a) Boc-β-alanine, HOBt, WSCD·HCl, TEA, DMF, 0 ºC. (b) NaI, acetone, RT. (c) 2-(4-Dimethylaminophenylazo)pyridine, acetonitrile, RT. (d) TFA, thioanisole, CH_2Cl_2, 0 ºC. (e) 6-Carboxyfluorescein-succinimidyl ester, TEA, DMF, 0 ºC.

Scheme S4 Synthetic route to **FCAPO2**



(a) mCPBA, CH_2Cl_2, 0 ºC. (b) NaI, acetone, RT. (c) 2-(4-Dimethylaminophenylazo)pyridine, acetonitrile, RT. (d) TFA, thioanisole, CH_2Cl_2, 0 ºC. (e) 6-Carboxyfluorescein-succinimidyl ester, TEA, DMF, 0 ºC.

Scheme S5 Synthetic route to **FCAPO2-DA**



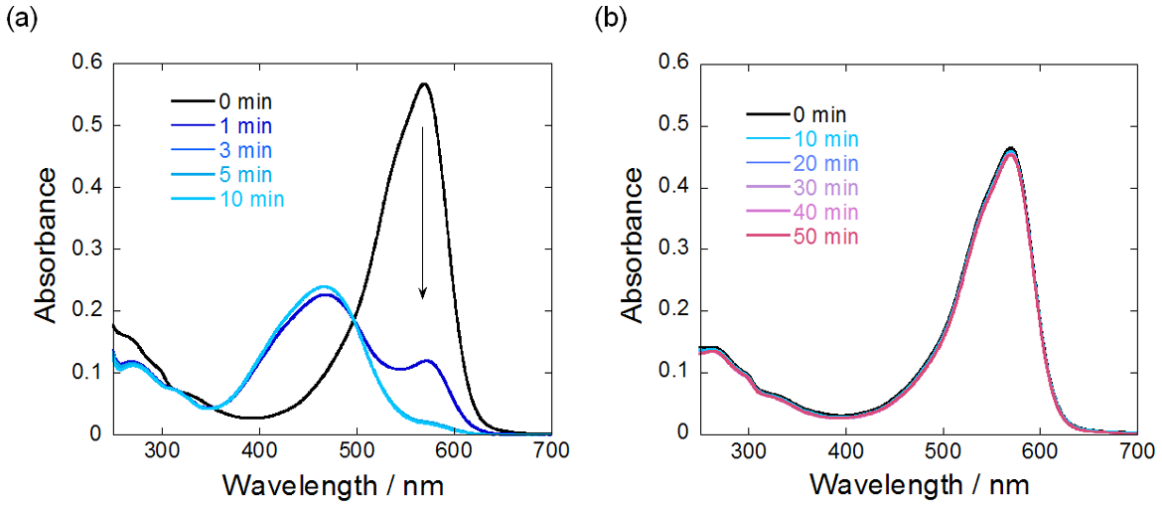


Figure S1 Time-dependent absorption spectra of 10 μM **CAP2** in the presence (**a**) and absence (**b**) of 40 nM WT TEM in 100 mM HEPES buffer (pH 7.4) at 25 °C.

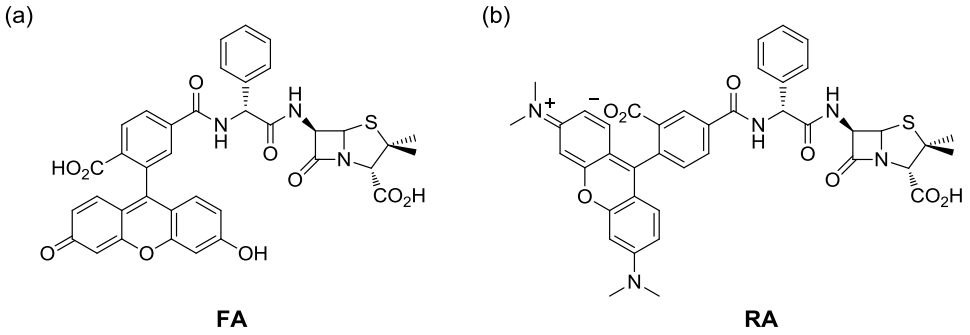


Figure S2 Structures of **FA** (**a**) and **RA**[S2] (**b**)

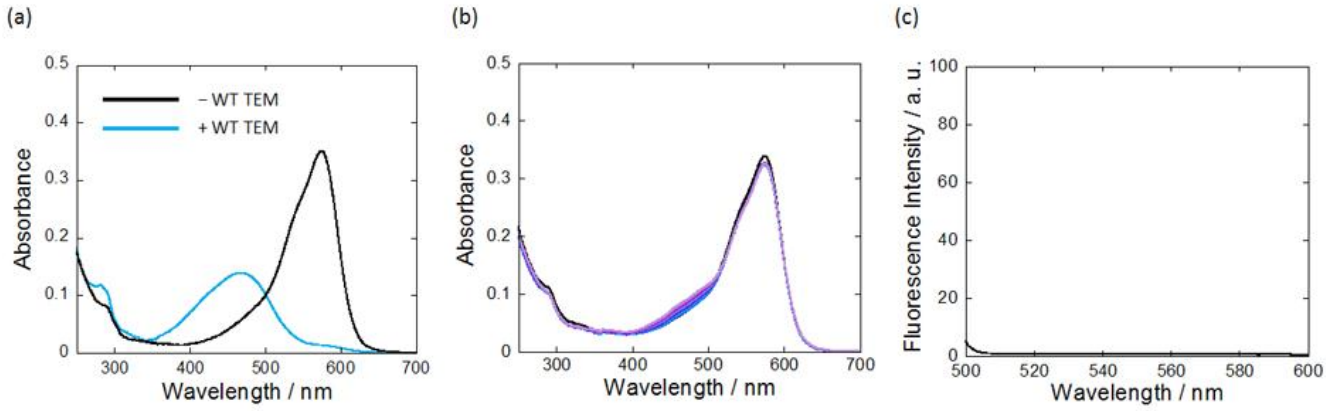


Figure S3 (**a**) Absorption spectra of 5 μM **FCAPO2-DA** before and 5 min after incubation with 40 nM WT TEM in 100 mM HEPES buffer (pH 7.4) at 25 °C. (**b**) Time-dependent absorption spectra (0–240 min) of 5 μM **FCAPO2-DA** in 100 mM HEPES buffer (pH 7.4) at 25 °C. (**c**) Emission spectrum (λ_{ex} = 492 nm) of 500 nM **FCAPO2-DA** in 100 mM HEPES buffer (pH 7.4) at 25 °C.

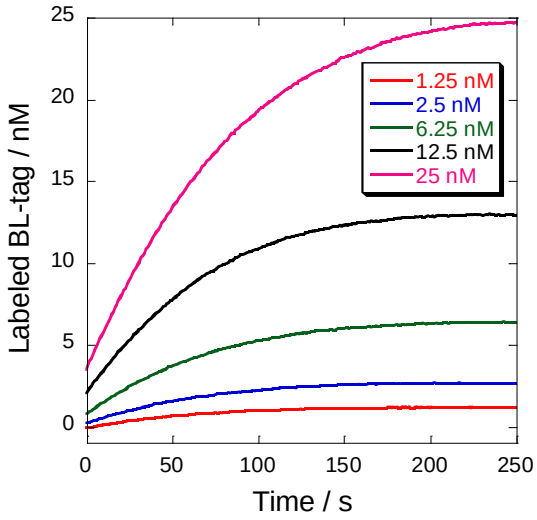


Figure S4 Kinetic study of fluorogenic labeling reaction of BL-tag with **FCAPO2** at the various concentration (1.25–25 nM). The concentration of labeled BL-tag was estimated from the fluorescence intensity (λ_{ex} = 490nm, λ_{em} = 518 nm). Reactions were performed in 100 mM HEPES buffer (pH 7.4) at 25 °C. [BL-tag] =125 nM.

Table S1. Bimolecular labeling rate constants for highly selective labeling tags and their specific probes

Labeling method	Substrate	k (M^{-1} s^{-1})	Fluorogenic property	Reference
BL-tag	**FCAPO2**	7.8×10^4	Yes	
SNAP-tag	**BGFL**	2.8×10^4	No	S6
	DRBG-488	7.9×10^3 [a]	Yes	S7
	CBG-549-TQ2	8.3×10^2 [b]	Yes	S8
CLIP-tag	**BCFL**	1.1×10^3	No	S6
Halo Tag	**GST-HaloTag**	2.7×10^6	No	S9

[a] The value was determined from the kinetic data in the reference. [b] A fast labeling variant of SNAP-tag, $SNAP_f$, was used.

5. References

S1) Mizukami, S.; Watanabe, S.; Hori, Y.; Kikuchi, K. *J. Am. Chem. Soc.* **2009**, *131*, 5016–5017.

S2) Watanabe, S.; Mizukami, S.; Hori, Y.; Kikuchi, K. *Bioconjug. Chem.* **2010**, *21*, 2320–2326.

S3) Parker, C. A.; Rees, W. T. *Analyst* **1960**, *85*, 587–600.

S4) Stroffekova, K.; Proenza, C.; Beam, K. G. *Pflugers Arch. Eur. J. Physiol.* **2001**, *442*, 859–886.

S5) Watanabe, S.; Mizukami, S.; Akimoto, Y.; Hori Y.; Kikuchi K. *Chem. Eur. J.* **2011**, *17*, 8342–8349.

S6) Gautier, A.; Juillerat, A.; Heinis, C.; Reis Correa, I., Jr.; Kindermann, M.; Beaufils, F.; Johnsson, K. *Chem. Biol.* **2008**, *15*, 128–136.

S7) Komatsu, T.; Johnsson, K.; Okuno, H.; Bito, H.; Inoue, T.; Nagano, T.; Urano, Y. *J. Am. Chem. Soc.* **2011**, *133*, 6745–6751.

S8) Sun, X.; Zhang, A.; Baker, B.; Sun, L.; Howard, A.; Buswell, J.; Maurel, D.; Masharina, A.; Johnsson, K.; Noren, C. J.; Xu, M. Q.; Correa, Jr, I. R. *ChemBioChem.* **2011**, *12*, 2217–2226.

S9) Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, M. G.; Learish, R.; Ohana, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *ACS Chem. Biol.* **2008**, *3*, 373–382.