Electronic Supplementary Information (ESI) for

Rapid no wash labeling of PYP-tag proteins with reactive fluorogenic ligands affords stable fluorescent protein conjugates for long-term cell imaging studies

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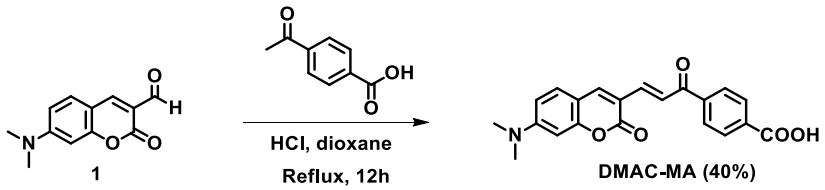
Supplementary Index

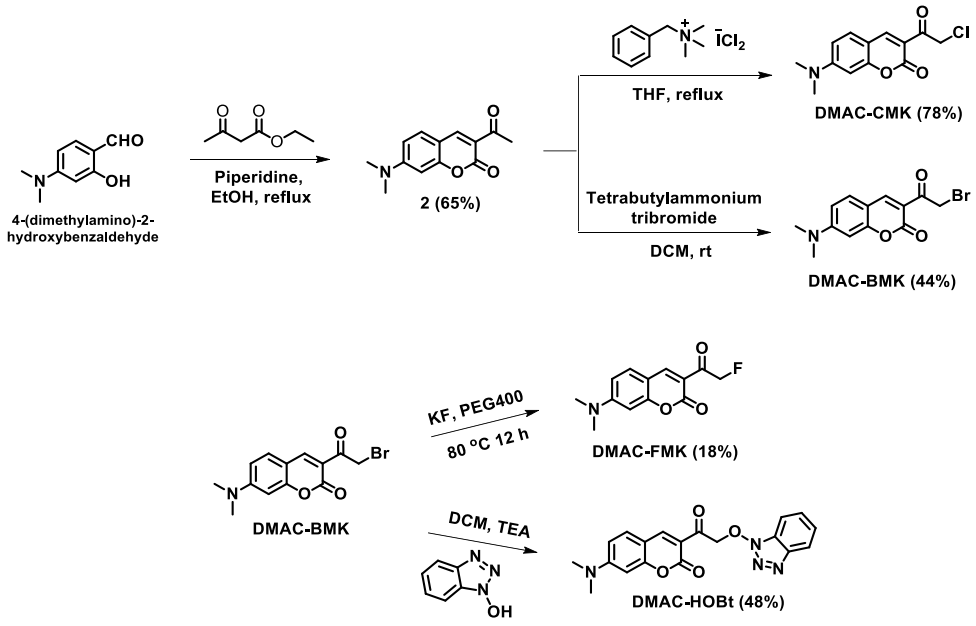
Synthesis detail and structural characterization



Scheme S1. Synthesis of Michael acceptor based ligand.

Compound **1** was synthesized as described previously.[S1]

Synthesis of compound DMAC-MA: A mixture of compound **1** (0.13 g; 0.61 mmol) and 4-acetylbenzoic acid (0.11 g; 0.67 mmol) in dioxane was added to conc. HCl (400 µL) under a nitrogen atmosphere The resulting mixture was then refluxed for 1 hour, 400 µL of conc. HCl then added and the reaction mixture refluxed overnight. The mixture was then cooled, evaporated to dryness and the residue purified by silica chromatography to obtain **DMAC-MA** in 40% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ = 8.55 (s, 1 H, ArH), 8.03-8.11 (m, 5 H, Ar-H and CH), 7.70 (d, *J* = 15 Hz, 1 H, Ar-H), 7.54 (d, *J* = 10 Hz, 1 H, Ar-H), 6.85 (s, 1 H, Ar-H), 6.65 (s, 1 H, Ar-H) and 3.11 (s, 6H, CH_3) ppm. ^{13}C NMR (DMSO-d6, 125 MHz): *δ* = 189.27, 167.11, 160.38, 156.59, 154.64, 146.67, 140.66, 134.68, 130.88, 130.25, 128.67, 121.47, 113.99, 110.68, 109.10, 97.20 and 41.76 ppm. HRMS (FAB+): Calcd for $C_{21}H_{17}NO_5^+$: $[M+H]^+$ = 364.1179; found 364.1176.



Scheme S2. Synthesis of dimethylamino coumarin (DMAC) based methyl ketone ligands.

Synthesis of compound 2: Piperidine (500 µL) was added to a stirred solution of 4-(dimethylamino)-2-hydroxybenzaldehyde (0.8 g; 4.86 mmol) and ethyl acetoacetate (0.75 g; 5. 83 mmol) in ethanol (10 mL) under a nitrogen atmosphere. The resultant mixture was refluxed for 4 hours, and the resultant orange precipitate filtered, washed with cold ethanol (10 mL) and dried to obtain compound **2** as an orange colored solid in 65% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 8.45 (s, 1 H, Ar-H), 7.42 (d, *J* = 10 Hz, 1 H, Ar-H), 6.65 (d, *J* = 10 Hz, 1 H, Ar-H), 6.48 (s, 1 H, Ar-H), 3.13 (s, 6 H, CH_3) and 2.69 (s, 3 H, CH_3) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* = 195.80, 160.80, 158.39, 154.92, 148.04, 131.63, 116.81, 110.02, 108.49, 97.06, 40.29 and 30.64 ppm. HRMS (FAB+): Calcd for $C_{13}H_{14}NO_3^+$: $[M+H]^+$ = 232.0968; found 232.0977.

Synthesis of DMAC-BMK: Compound **2** (0.1 g; 0.43 mmol) and tetrabutylammonium tribromide (0.22 g; 0.47 mmol) in dry DCM (6 mL) under a nitrogen atmosphere were stirred at room temperature for 12 hours. The solvent was removed and the crude residue purified by silica chromatography (CH_2Cl_2) to obtain **DMAC-BMK** as a yellow colored solid in 44% yield. ^{1}H NMR (Acetone-d_6, 500 MHz): δ = 8.57 (s, 1 H, Ar-H), 7.69 (d, *J* = 10 Hz, 1 H, Ar-H), 6.88 (d, *J* = 10 Hz, 1 H, Ar-H), 6.57 (s, 1 H, Ar-H), 4.76 (s, 2 H, CH_2) and 3.22 (s, 6 H, CH_3) ppm. ^{13}C NMR (Acetone-d6, 125 MHz): *δ* = 187.71, 159.84, 158.62, 155.82, 149.11, 132.18, 113.51, 110.52, 108.30, 96.53,

39.55 and 36.31 ppm. HRMS (FAB+): Calcd for $C_{13}H_{13}BrNO_3^+$: $[M+H]^+ = 310.0073$; found 310.0084.

Synthesis of DMAC-CMK: A mixture of compound **2** (0.05 g; 0.21 mmol) and benzyltrimethylammonium dichloroiodate (0.15 g; 0.43 mmol) in THF (5 mL) was refluxed for 4 hours under a nitrogen atmosphere. The reaction mixture was cooled down, then dissolved in ethyl acetate (20 mL) and washed with water. The organic layer was then dried (Na_2SO_4) and concentrated under vacuum to afford a crude residue that was purified by chromatography (CH_2Cl_2) to afford DMAC-CMK as a yellow solid in 78% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 8.57 (s, 1 H, Ar-H), 7.46 (d, *J* = 5 Hz, 1 H, Ar-H), 6.68 (d, *J* = 10 Hz, 1 H, Ar-H), 6.48 (s, 1 H, Ar-H), 4.95 (s, 2 H, CH_2) and 3.16 (s, 6 H, CH_3) ppm. ^{13}C NMR ($CDCl_3$,125 MHz): *δ* = 188.64, 160.63, 158.55, 155.47, 149.52, 132.04, 114.04, 110.43, 108.59, 97.05, 50.68 and 40.37 ppm. HRMS (FAB+): Calcd for $C_{13}H_{13}ClNO_3^+$: $[M+H]^+ = 266.0578$; found 266.0579.

Synthesis of DMAC-FMK: Potassium fluoride (0.02 g; 0.48 mmol) and PEG-400 (1 mL) in dry CH_3CN (5 mL) were heated at 80 °C under a nitrogen atmosphere for 1 h. DMAC-BMK (0.05 g; 0.16 mmol) in CH_3CN (5 mL) was then added drop wise to the reaction mixture, followed by heating at 80 °C for 12 hours. The solvent was removed and the resultant solid residue purified by silica chromatography (1% MeOH in CH_2Cl_2) to afford **DMAC-FMK** as a yellow solid in 18% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 8.60 (s, 1 H, Ar-H), 7.47 (d, *J* = 5 Hz, 1 H, Ar-H), 6.67-6.70 (m, 1 H, Ar-H), 6.49 (s, 1 H, Ar-H), 5.64 (s, 1 H, HCH), 5.54 (s, 1 H, HCH) and 3.16 (s, 6 H, CH_3) ppm. ^{13}C NMR ($CDCl_3$,125 MHz): *δ* = 190.99, 160.67, 158.50, 155.50, 148.97, 148.95, 132.11, 113.35, 110.48, 108.53, 97.10 and 40.37 ppm. HRMS (FAB+): Calcd for $C_{13}H_{13}FNO_3^+$: $[M+H]^+ = 250.0874$; found 250.0881.

Synthesis of DMAC-HOBt: TEA (1 equivalent) was added to a stirred solution of DMAC-BMK (0.02 g; 0.06 mmol) and hydroxybenzotriazole (8 mg; 0.06 mmol) in dry DCM (5 mL) and the resulting mixture stirred at room temperature for 12 h. Solvent was evaporated and the crude residue purified by silica chromatography (2% MeOH in CH_2Cl_2) to afford **DMAC-HOBt** in 48% yield. ^{1}H NMR ($CDCl_3$; 500 MHz): δ = 8.52 (s, 1 H, Ar-H), 7.94-7.99 (m, 2 H, Ar-H), 7.55 (t, *J* = 5 Hz, 1 H, Ar-H), 7.38-7.42 (m, 2 H, Ar-H), 6.66 (d, *J* = 10 Hz, 1 H, Ar-H), 6.47 (s, 1 H, Ar-H), 5.96 (s, 2 H, CH2) and 3.15 (s, 6 H, CH3) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 189.54, 160.65, 158.59, 155.62, 149.09, 143.51, 132.15, 127.84, 124.53, 119.87, 113.51, 110.53, 110.43, 108.55, 97.12, 83.09 and 40.38 ppm. HRMS (FAB+): Calcd for $C_{19}H_{17}N_4O_4^+$: $[M+H]^+ = 365.1244$; found 365.1246.

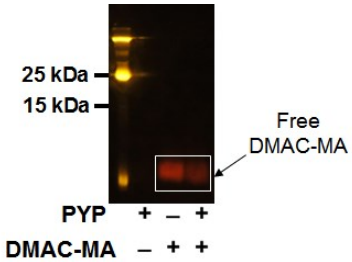


Figure S1. Fluorescence SDS-PAGE analysis of the reaction product of DMAC-MA and PYP-tag. Reaction conditions: DMAC-MA 10 μM, PYP-tag 20 μM in 20 mM HEPES buffer, 150 mM NaCl, pH 7.4 for 1 h at 37 ºC; λ_{ex} 365 nm.

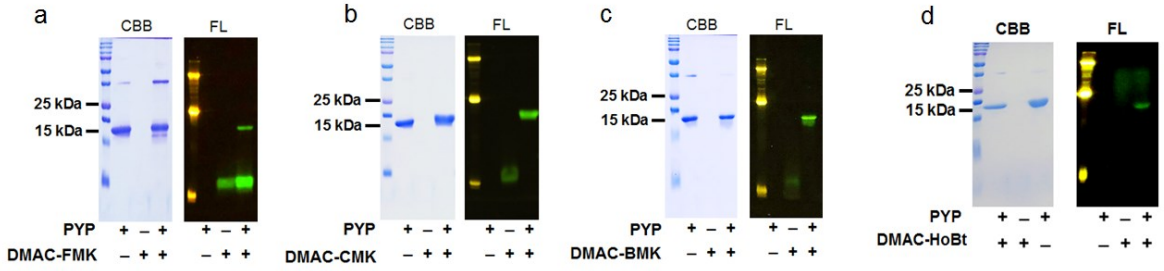


Figure S2. CBB-stained and fluorescence (FL) SDS-PAGE gels of products from labeling reactions of PYP-tag for: (a) DMAC-FMK; (b) DMAC-CMK; (c) DMAC-BMK and (d) DMAC-HOBt. Reaction conditions: (a) 40 μM DMAC-FMK, 30 μM PYP-tag, 24 h; (b) 5 μM DMAC-CMK, 10 μM PYP-tag, 1 h; (c) 5 μM DMAC-BMK, 10 μM PYP-tag, 1 h; (d) 20 μM DMAC-HOBt, 20 μM PYP-tag, 1 h, in 20 mM HEPES buffer, 150 mM NaCl, pH 7.4 (1.5% DMSO) at 37 ºC; irradiation carried out at λ_{ex} 365 nm.

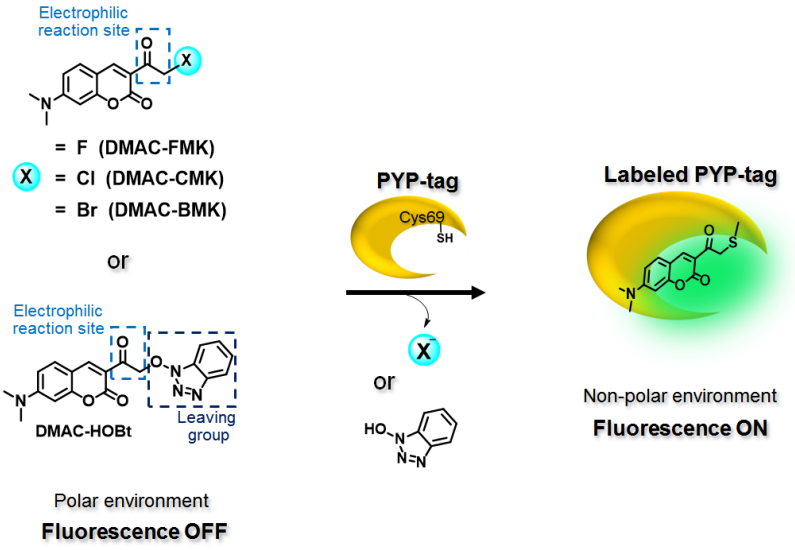


Figure S3. Protocol used for labeling PYP-tag with fluorogenic methyl ketone based ligands.

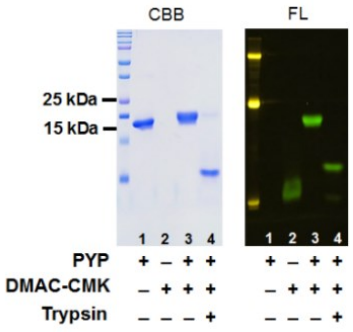


Figure S4. SDS-PAGE analysis of DMAC-CMK labeled PYP-tag after trypsin digestion. Reaction of PYP-tag (20 μM) with DMAC-CMK (10 μM) was performed for 1 h followed by addition of trypsin (4 μM), incubation for 2 h in 20 mM HEPES buffer, 150 mM NaCl, pH 7.4 (1.5% DMSO) at 37 ºC; irradiation carried out at λ_{ex} 365 nm.

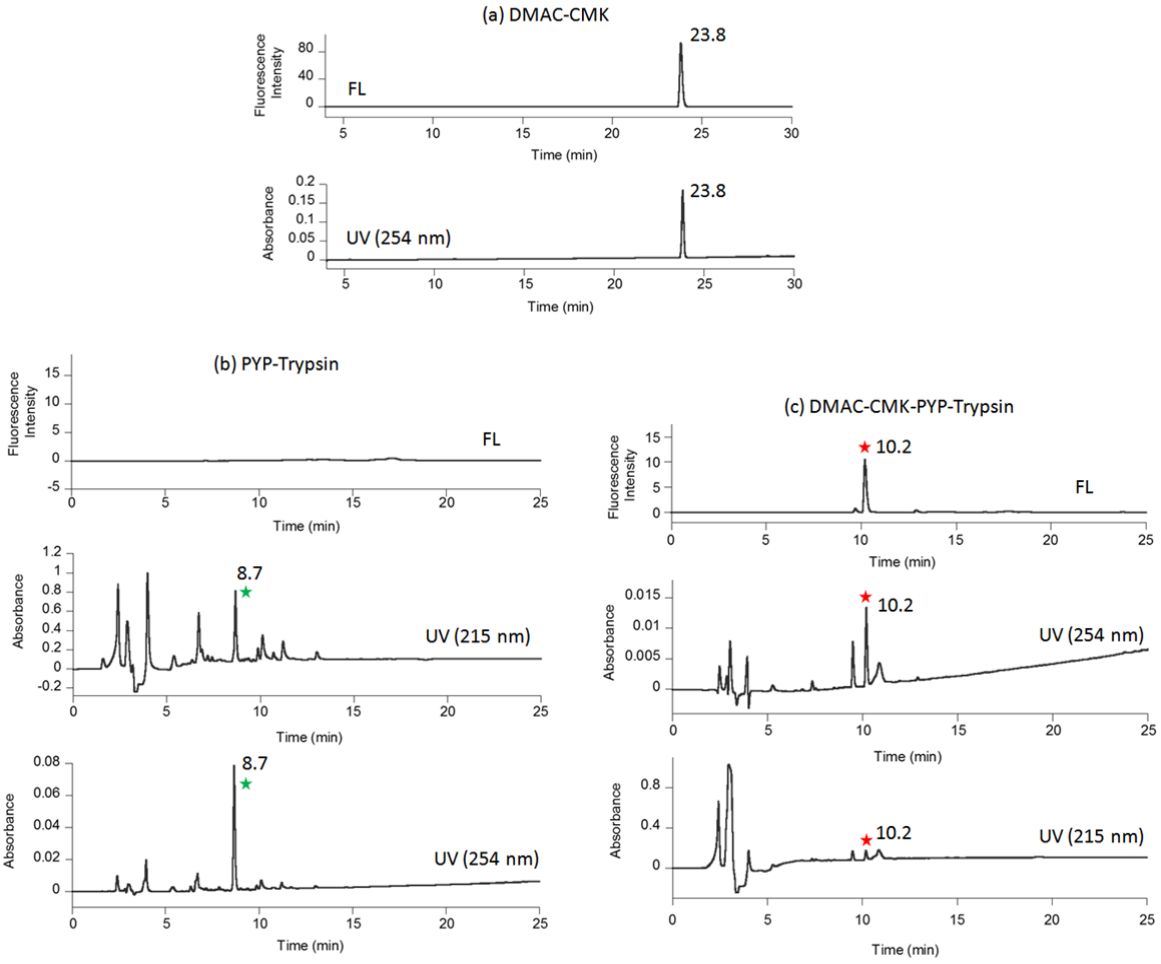
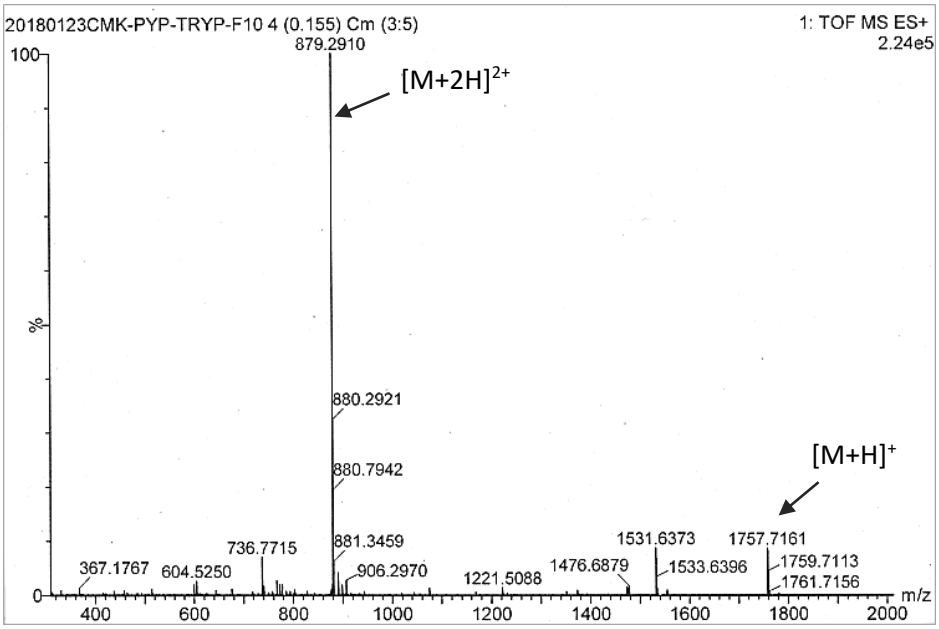
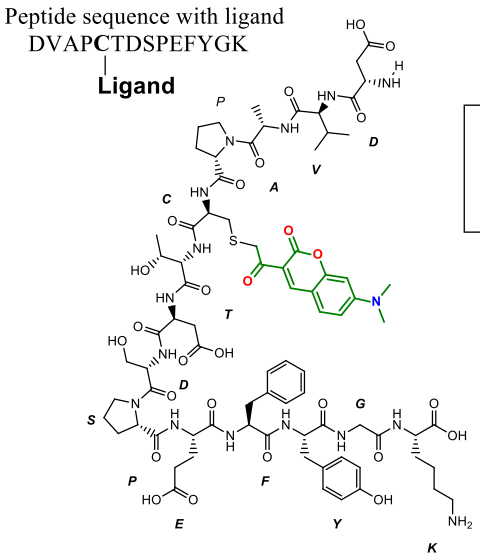


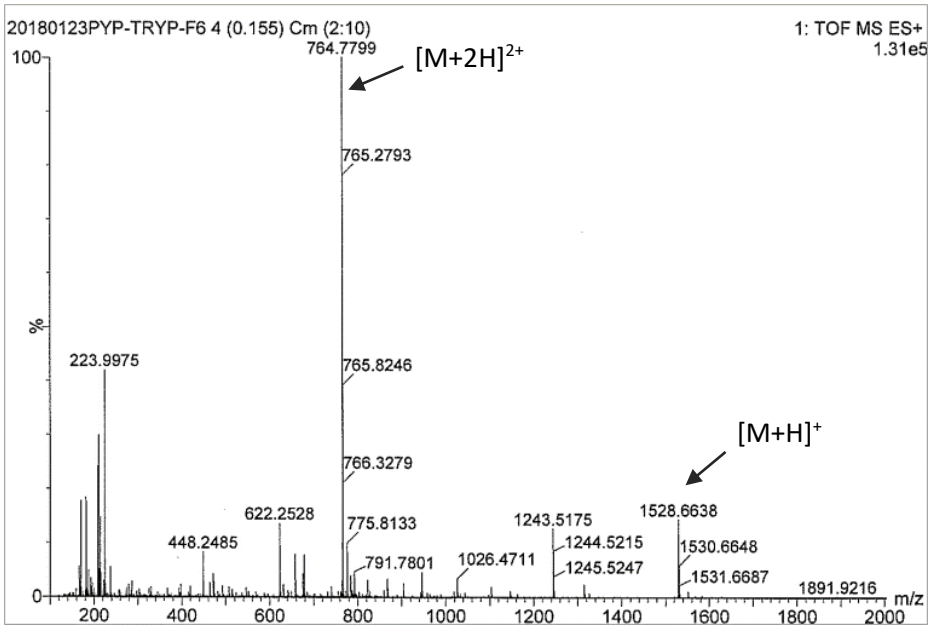
Figure S5. HPLC analysis: (a) Free DMAC-CMK; (b) PYP-tag digested with trypsin; (c) DAMC-CMK labeled PYP-tag after trypsin digestion. Reaction conditions for: (b) PYP-tag (200 μM) incubated with trypsin (10 μM) for 1 h; (c) PYP-tag (15 μM) reacted with DMAC-CMK (10 μM) for 1 h and the resultant complex digested with trypsin (1.5 μM, 1 h) in 20 mM HEPES buffer, 150 mM NaCl, pH 7.4 (1.5% DMSO) at 37 ºC. HPLC conditions: Buffer 0.1% HCOOH in (a) H_2O; (b) CH_3CN. Buffer gradient: 10%-90% (0-30 min). λ_{ex} 400 nm; λ_{em} 500 nm.

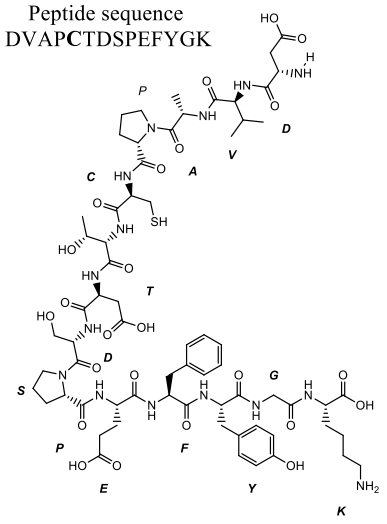




MS ES+ Calcd for $C_{80}H_{109}N_{16}O_{27}S$: $[M+H]^+$ = 1757.7363; found 1757.7161

Figure S6. ESI-MS spectrum of HPLC peak (10.2 min) obtained for trypsin digested DMAC-CMK labeled PYP-tag (Figure S5c). Mass analysis confirms correct molecular ion for peptide HO_2C-DVAPCTDSPEFYGK-NH_2 labelled with DMAC ligand.





MS ES+ Calcd for $C_{67}H_{98}N_{15}O_{24}S$:$[M+H]^+$= 1528.6624; found 1528.6638.

Figure S7. ESI-MS spectrum of HPLC peak (8.7 min) obtained for PYP-tag digest with trypsin (Figure S5b). Mass analysis confirms correct molecular ion for peptide sequence DVAPCTDSPEFYGK.

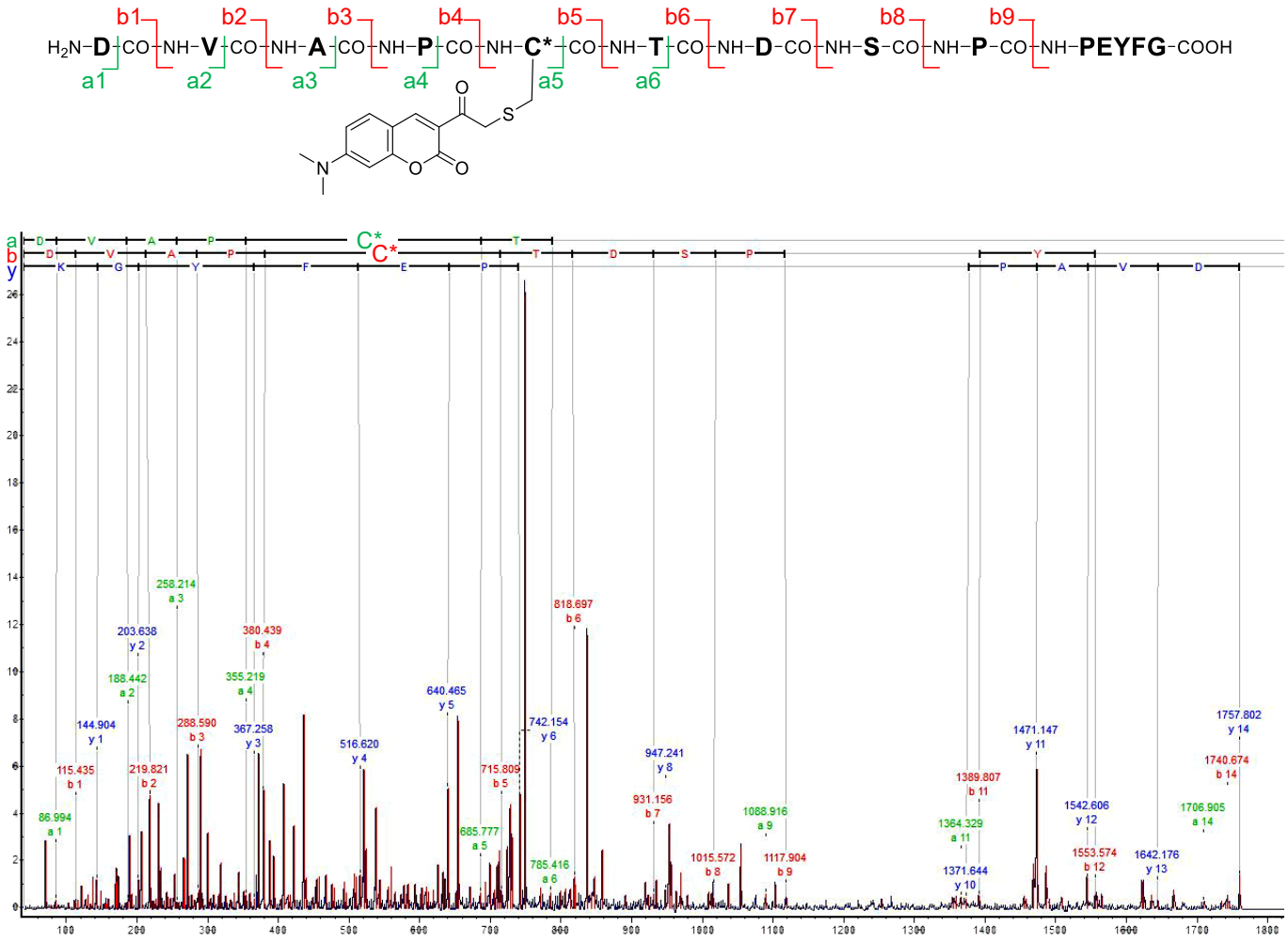


Figure S8. MALDI-LIFT-TOF/TOF MS analysis of the trypsin-digested peptide fragment shown in Fig. S7. PYP-tag labeled with DMAC-CMK was digested with trypsin and the extract purified using HPLC. The Cys69 residue conjugated to DMAC-CMK is shown as C*. The peptide fragment containing the modified Cys69 gave a-type and b-type ions in the MS/MS spectrum.

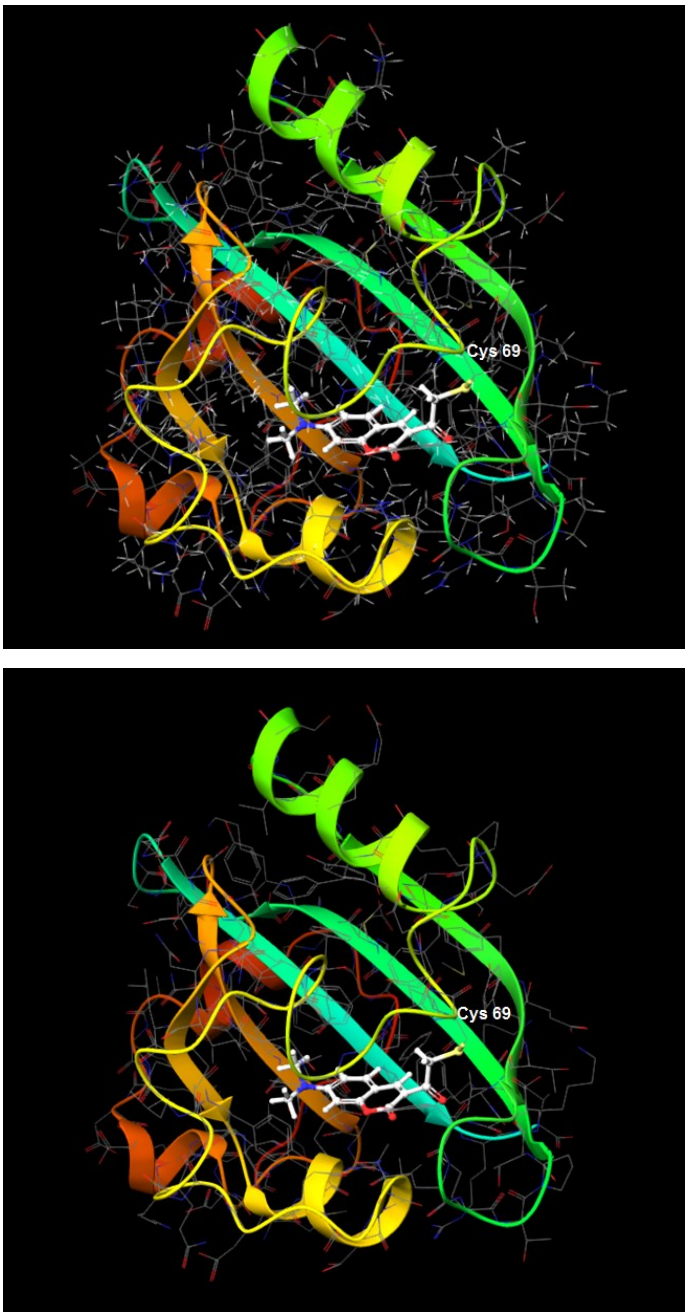


Figure S9. Theoretical structural model of PYP-tag-ligand complex. PYP-tag covalently attached to the ligand molecule left after labeling reaction with electrophilic methyl ketone ligands was employed to generate the predicted structure using MacroModel (Schrödinger K. K.). The structures of the whole complex, both with and without the H-atoms of the PYP-tag ligand, are shown in the top and bottom images, respectively.

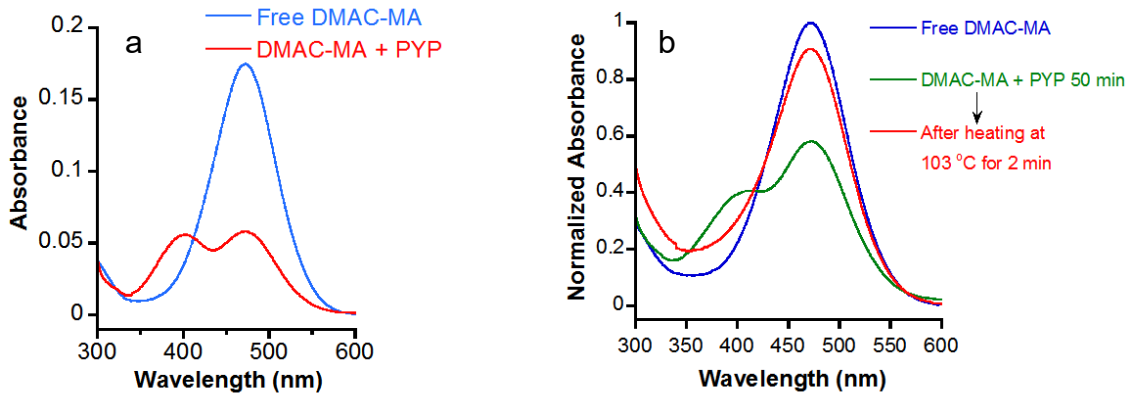


Figure S10. (a) Absorption spectra of DMAC-MA (10 µM, blue line) and DMAC-MA with PYP-tag (20 µM, red line). (b) Normalized absorption spectra of DMAC-MA (15 µM, blue line), DMAC with PYP-tag (10 µM, green line) and after heating the DMAC-MA-PYP-tag complex at 103 ºC for 2 min (red line). Reaction conditions: 20 mM HEPES buffer (pH 7.4), 150 mM NaCl at 37 ºC.

Analysis of DMAC-MA interactions with PYP-tag using gel filtration chromatography and analysis of equilibrium and kinetic data

Gel filtration analysis of PYP-tag incubated with DMAC-MA showed two absorptions (280 and 380 nm) for an elution volume of ~12.5 mL indicating the successful labeling of PYP-tag with DMAC-MA (Figure S11a, S11b). This also implies that there is no free DMAC-MA present as there no eluent gave absorption at 470 nm. The fraction showing absorption at 280 and 380 nm was then monitored by UV/vis spectroscopy (Figure S11c). Initially, it showed an absorbance at ~398 nm indicating DMAC-MA labeled PYP-tag. However, over a period the absorption band at ~398 nm decreased whilst the absorbance at ~472 nm corresponding to free DMAC-MA increased. This supports the reversible nature of DMAC-MA interaction with PYP-tag. The labeling reaction of DMAC-MA with PYP-tag was also analyzed using equilibrium and kinetic measurements (Figure S12). The calculated equilibrium constant was in the micromolar range (K_d = 7.4 µM), with the rates of the forward (k_{on}) and backward (k_{off}) reactions found to be 40.15 M^{-1} S^{-1} and 3.212 × 10^{-4} s^{-1}, respectively. These studies indicate that the reaction of DMAC-MA with PYP-tag is reversible in nature, which is consistent with

DMAC-MA binding to the hydrophobic pocket of PYP-tag through a Michael addition reaction, with a retro-Michael reaction (via an E1cb type elimination process) responsible for generating free DMAC-MA.

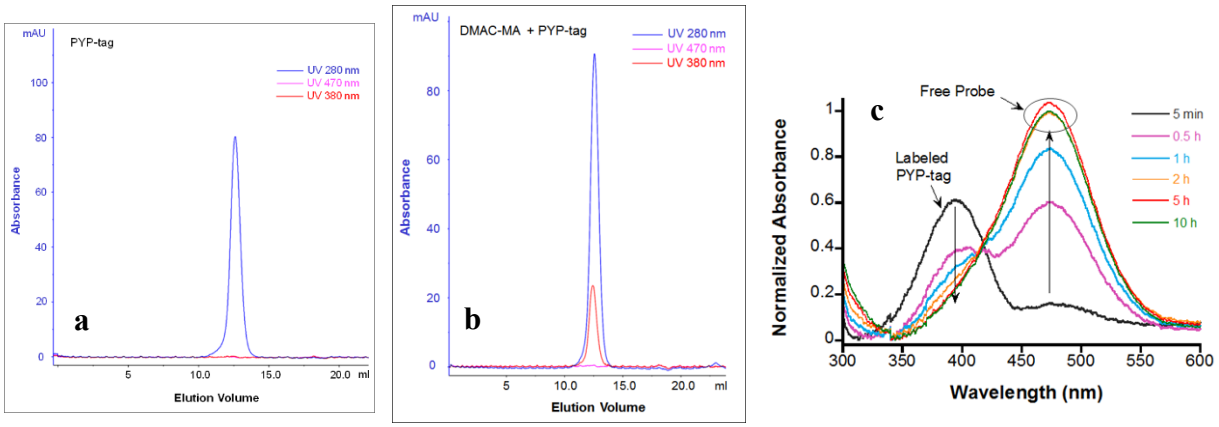


Figure S11. Gel permeation chromatographic (GPC) analyses of: (a) PYP-tag; (b) DMAC-MA (10 µM) + PYP-tag DMAC-MA (20 µM). Both samples incubated for 1 h before analysis. GPC solvent: 20 mM HEPES buffer (pH 7.4), 150 mM NaCl at 4 ºC. (c) UV/Vis analysis of fractions eluting at ~12.5 mL in trace (b), conditions: 20 mM HEPES buffer (pH 7.4), 150 mM NaCl at 37 ºC.

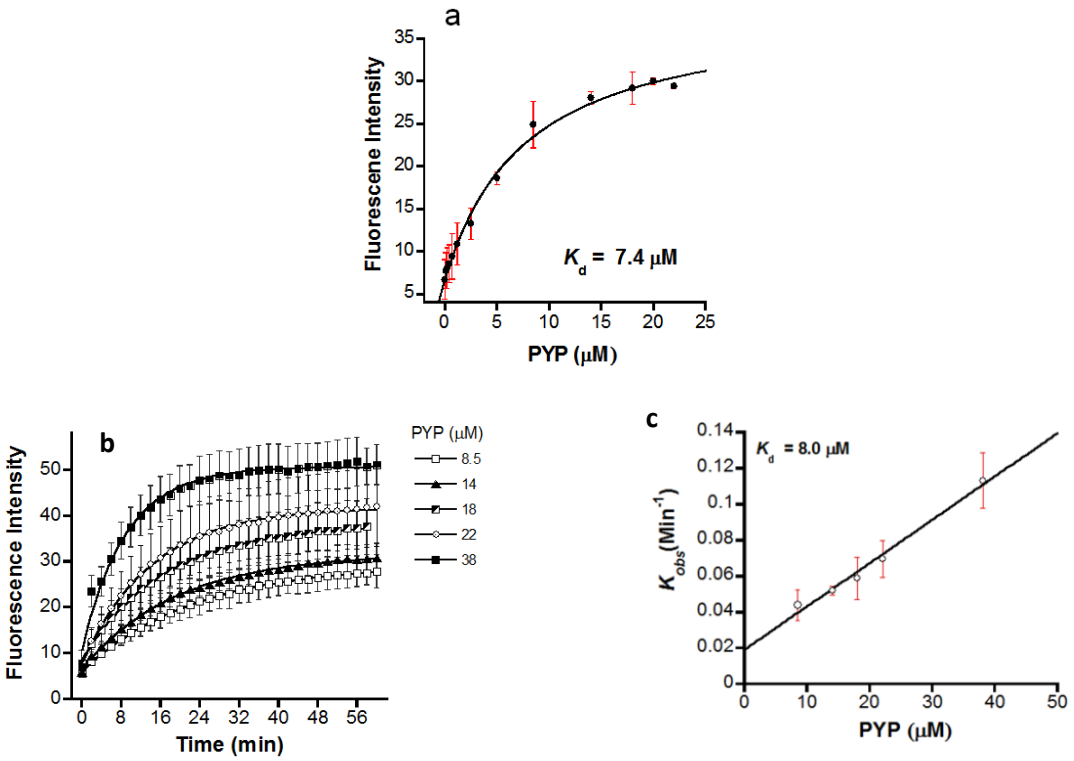


Figure S12. (a) Fluorescence titration experiments used to determination the equilibrium constant for labeling of PYP-tag with DMAC-MA. Fluorescence intensity of DMAC-MA (0.5 μM) plotted against PYP-tag concentration (0.1-22 μM). Error bars denote SD, $n = 3$. (b) Fluorescence monitoring of time course of labeling reaction of DMAC-MA (0. 5 μM) with different concentrations of PYP-tag; λ_{ex} 398 nm; λ_{em} 456 nm; (c) Plot of observed rate constants (K_{obs}) as a function of PYP-tag concentrations. Error bars denote SD, $n = 3$. (See Experimental Procedures for calculation details).

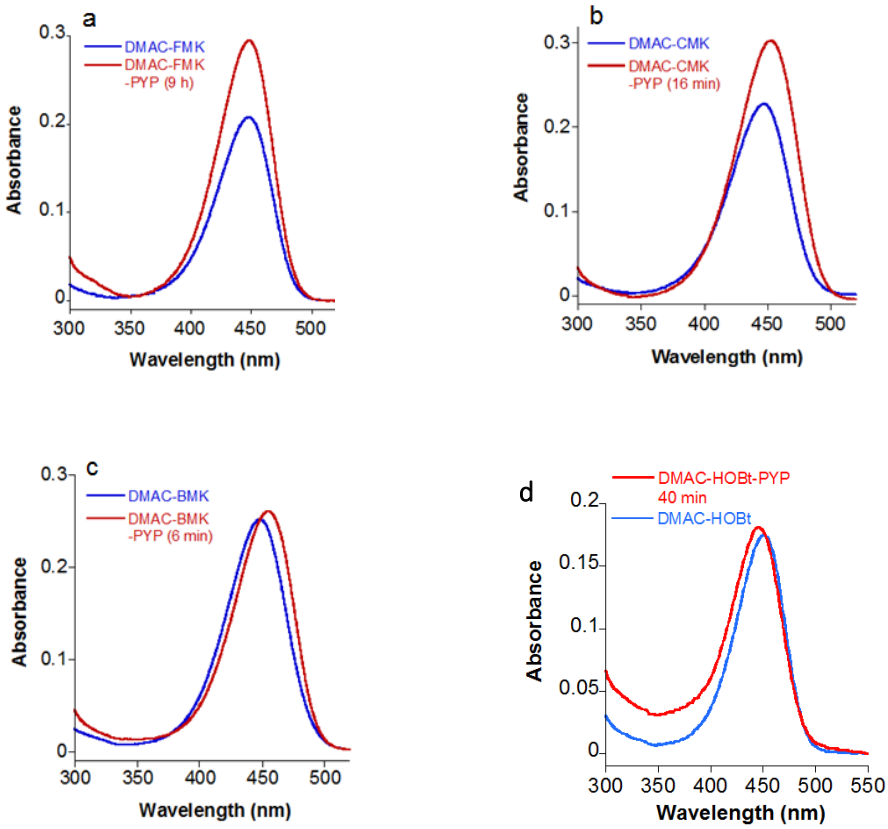


Figure S13. Absorption spectra of (a) DMAC-FMK; (b) DMAC-CMK; (c) DMAC-BMK; (d) DMAC-HOBt, with and without PYP-tag. Reaction conditions: Probe 5 μM; PYP-tag 10 μM in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl (1.5% DMSO except DMAC-HOBt where 5% DMSO was used) at 37 ºC.

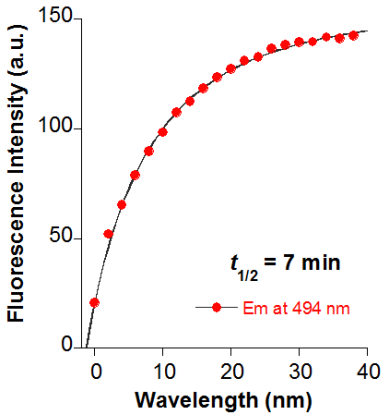


Figure S14. Time course of fluorescence intensity of DMAC-HOBt (5 µM) at 494 nm (λ_{ex} 450 nm) in the presence of PYP-tag (10 µM).

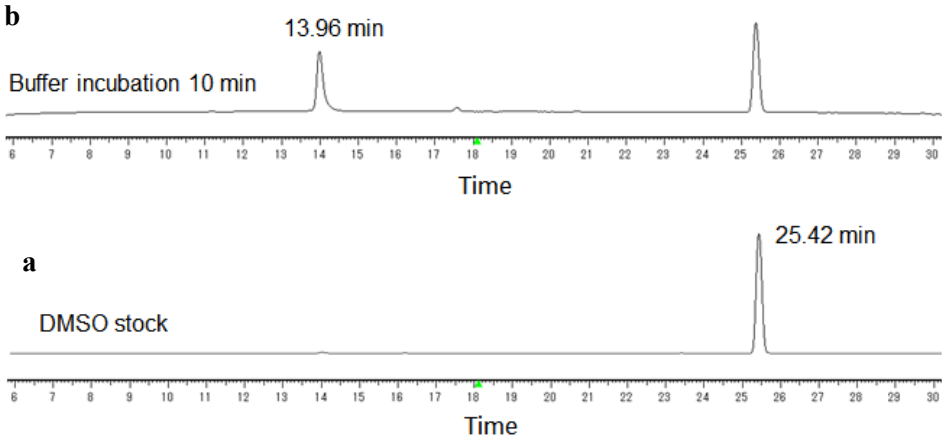


Figure S15. HPLC analyses of DMAC-HOBt. (a) DMSO stock of HOBt; (b) HOBt incubated for 10 min in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl (5% DMSO) at 37 ºC. Running conditions: Buffer: 0.1% HCOOH in (a) H_2O; (b) CH_3CN. Gradient: buffer b, 10-90% (0-30 min). λ_{ex} 450 nm; λ_{em} 500 nm.

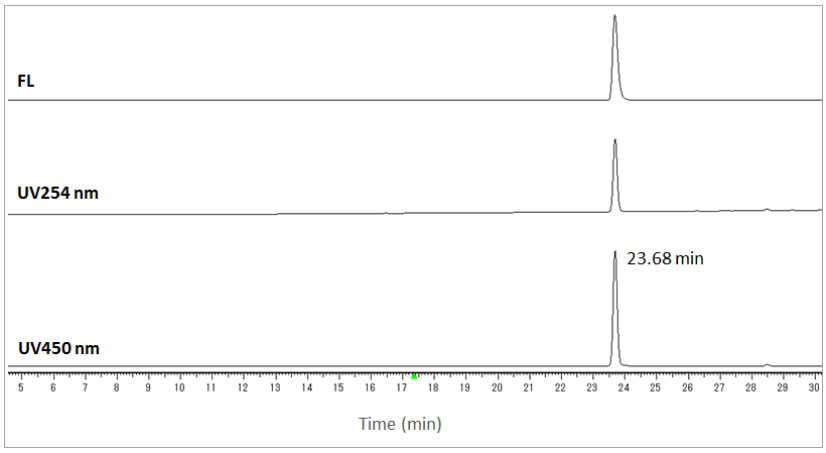


Figure S16. HPLC analysis of DMAC-CMK in DMSO. Buffer: 0.1% HCOOH in (a) H_2O; (b) CH_3CN. Gradient: buffer b, 10%-90% (0-30 min). λ_{ex} 450 nm; λ_{em} 500 nm.

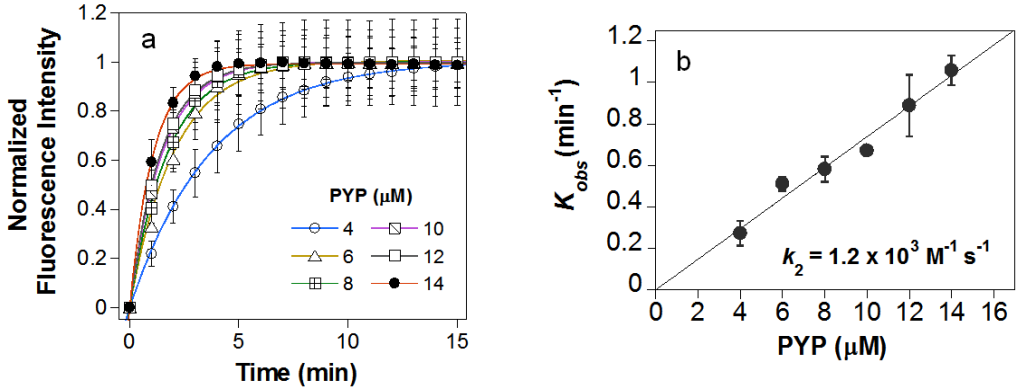
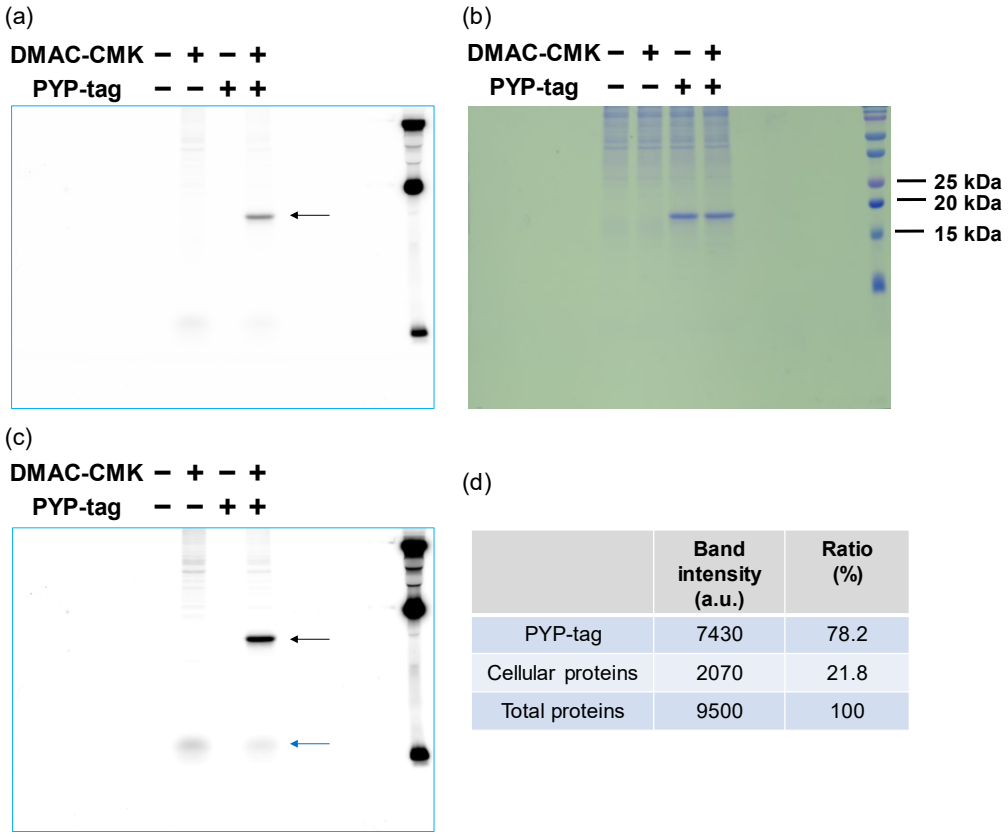


Figure S17. Rate constant evaluation. (a) Time course of labeling reaction of DMAC-CMK (0.2 μM) using different concentrations of PYP-tag in 20 mM HEPES buffer (pH 7.4), 150 mM NaCl (1.5% DMSO) at 37 ºC; $\lambda_{ex/em}$ 450/494 nm. (b) Plot of observed rate constant (K_{obs}) as a function of PYP-tag concentrations. Error bars denote SD, n = 3. (See Experimental Procedures for rate constant calculation details).



	Band intensity (a.u.)	Ratio (%)
PYP-tag	7430	78.2
Cellular proteins	2070	21.8
Total proteins	9500	100

Figure S18. Labeling reactions of PYP-tag in cell lysate using DMAC-CMK. Fluorescence and CBB images are shown in (a) and (b), respectively. (c) Contrast of fluorescence image (a) is enhanced by 70%. The black arrows in the fluorescence images identifies a band corresponding to the PYP-tag/DMAC-CMK complex, while the blue arrow represents a band derived from a free probe. (d) Quantification of band intensity of PYP-tag and cellular proteins in image (a). DMAC-CMK (10 μM) was reacted with PYP-tag (10 μM) in the presence of lysate prepared from NIH3T3 cells. The concentration of protein in the lysate was determined to be 1.2 mg/mL, using a Braford protein assay kit (Bio-rad). The fluorescence image was obtained using a Typhoon FLA9500 laser scanner (GE healthcare).

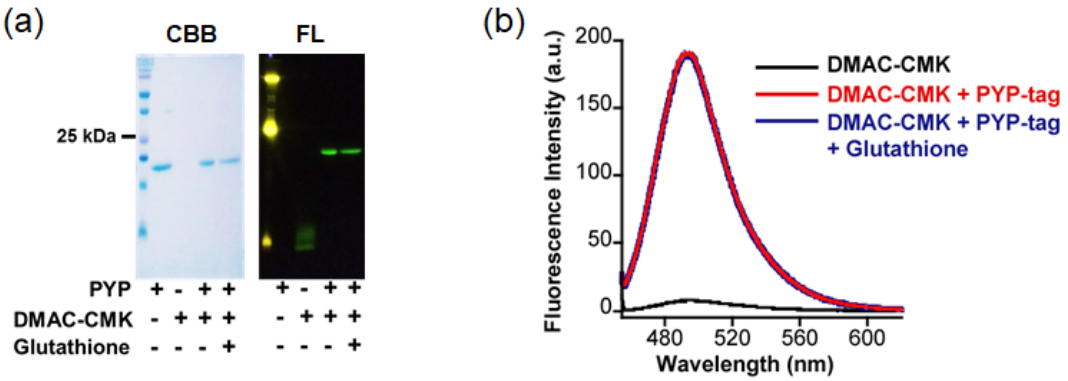


Figure S19. Resistance of PYP-tag/DMAC-CMK complex to cleavage by glutathione. (a) SDS-PAGE analysis of PYP-tag/DMAC-CMK complex in the presence of 10 mM glutathione. λ_{ex} 365 nm. (b) Fluorescence spectra of PYP-tag/DMAC-CMK complex in the presence and absence of 10 mM glutathione (λ_{ex} 450 nm). For both experiments, the complex was prepared by mixing 10 μM PYP-tag and 5 μM DMAC-CMK in 20 mM HEPES buffer, 150 mM NaCl, pH 7.4 (1.5% DMSO) at 37 ºC. In the SDS-PAGE and fluorescence analyses, the complex was incubated with 10 mM glutathione for 1 h and 26 min, respectively.

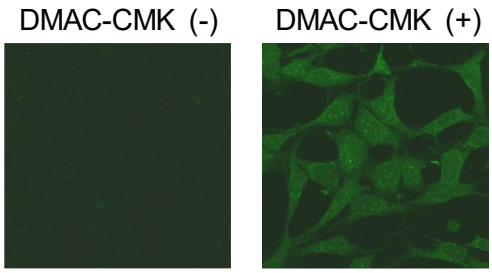


Figure S20. Intracellular accumulation of DMAC-CMK. Cells were incubated with 2 μM DMAC-CMK for 3 min and were imaged without washing. The fluorescence images of cells treated or untreated with DMAC-CMK were shown by enhancing the detection sensitivity of the microscope and increasing the contrast of the images.

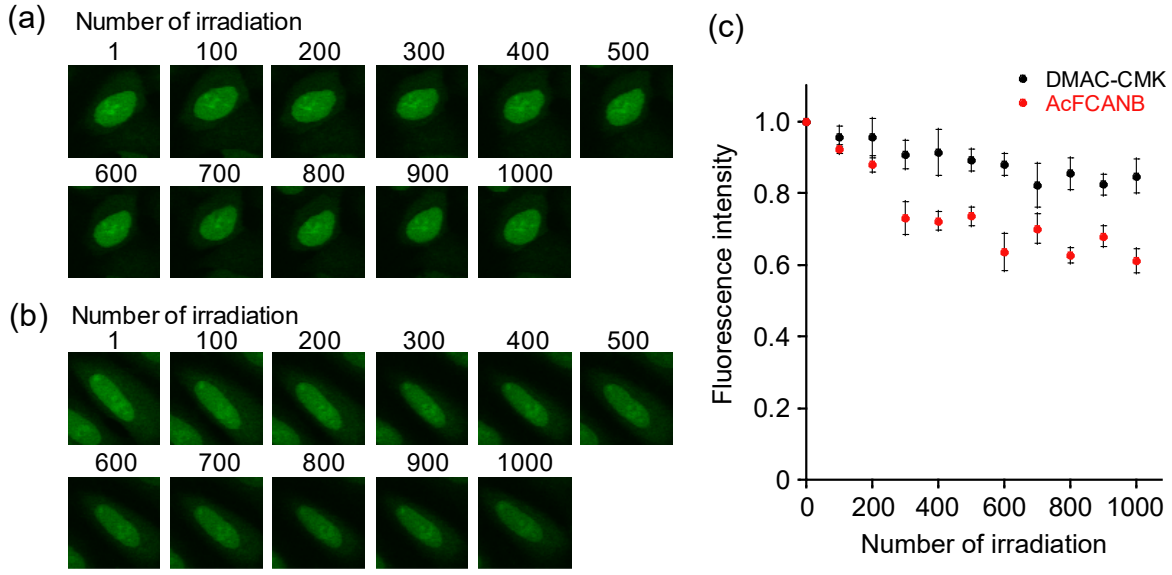


Figure S21. Photostability analyses of DMAC-CMK and AcFCANB. Representative live-cell images of (a) DMAC-CMK and (b) AcFCANB bound to PYP-NLS in HEK293T cells after repeated irradiations using a confocal green laser at 473 nm. The laser intensity was measured using a HIOKI 3664 optical power meter and Hioki 9742-10 optical sensor and found to be 12.5 mW/cm^2, with images obtained using a sampling speed of 2.0 μs/pixel. Cells were transfected with plasmid encoding PYP-NLS and incubated with DMAC-CMK (2.0 μM) for 15 min, or AcFCANB (2.0 μM) for 1 h, before irradiation experiments were then carried out. 1000 cellular images were acquired consecutively. (c) Plot of normalized fluorescence intensities of DMAC-CMK and AcFCANB bound to PYP-NLS. Fluorescence intensities quantified after every 100 rounds of laser irradiation, using ImageJ. Error bars denote SD; n = 3.

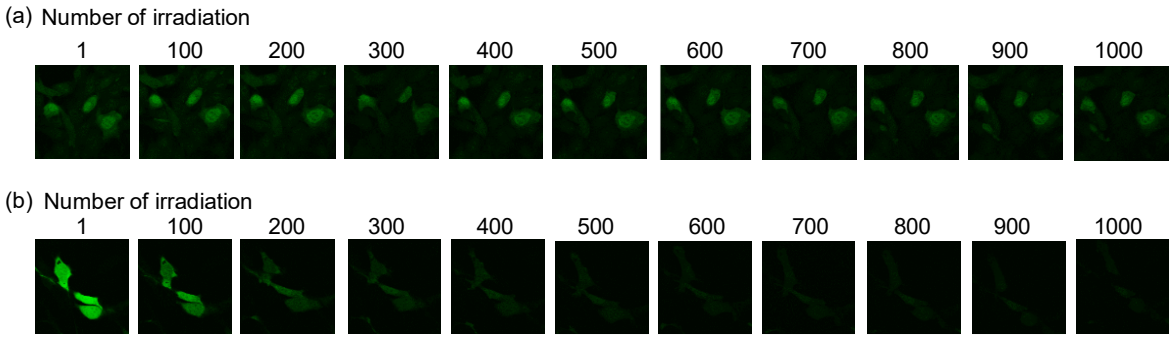


Figure S22. Photostability analyses of DMAC-CMK and EGFP. Representative live-cell images of (a) DMAC-CMK-bound PYP and (b) EGFP in NIH-3T3 cells after repeated irradiations using a confocal green laser at 473 nm. The laser intensity was measured using a HIOKI 3664 optical power meter and Hioki 9742-10 optical sensor and found to be 6.1 mW/cm^2, with images obtained using a sampling speed of 2.0 μs/pixel. Cells were transfected with plasmid encoding PYP or EGFP. PYP-expressing cells were incubated with DMAC-CMK (2.0 μM) for 15 min. 1000 cellular images were acquired consecutively.

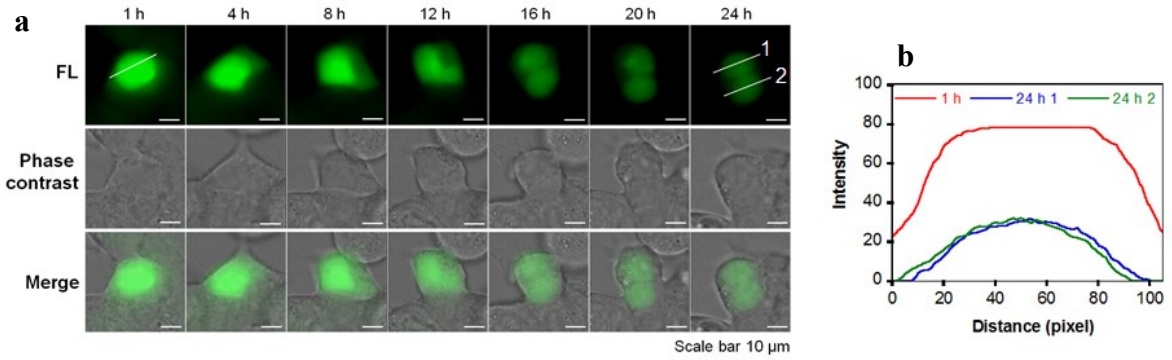


Figure S23. (a) 24-hour live-cell imaging of PYP-NLS using DMAC-CMK. HEK293T cells transfected with plasmid encoding PYP-NLS were incubated with DMAC-CMK (200 nM) and then imaged for 24 h at an interval of every 1 h. Fluorescence (FL), phase contrast and overlay (merge) images are shown in the top, middle and lower rows, respectively. λ_{ex} = 470/40 nm; λ_{em} = 525/50 nm. (b) Quantification of fluorescence intensity after 1 and 24 h of imaging (evaluated by ImageJ). The fluorescence intensity of the nuclei was reduced because of cell division.

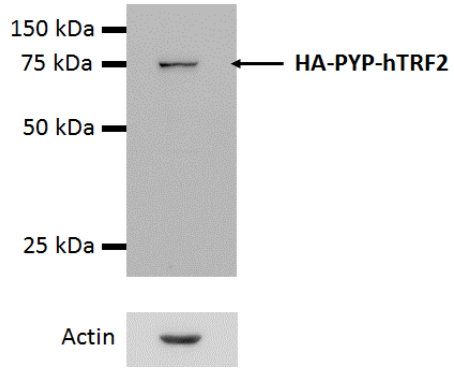


Figure S24. Western blot analysis of HA-PYP-hTRF2 expressed in U2OS cells. 1st antibody: anti-HA antibody; 2nd antibody: ECL peroxide labeled anti-mouse antibody. Exposure time: 3 min for both anti-HA and anti-actin.

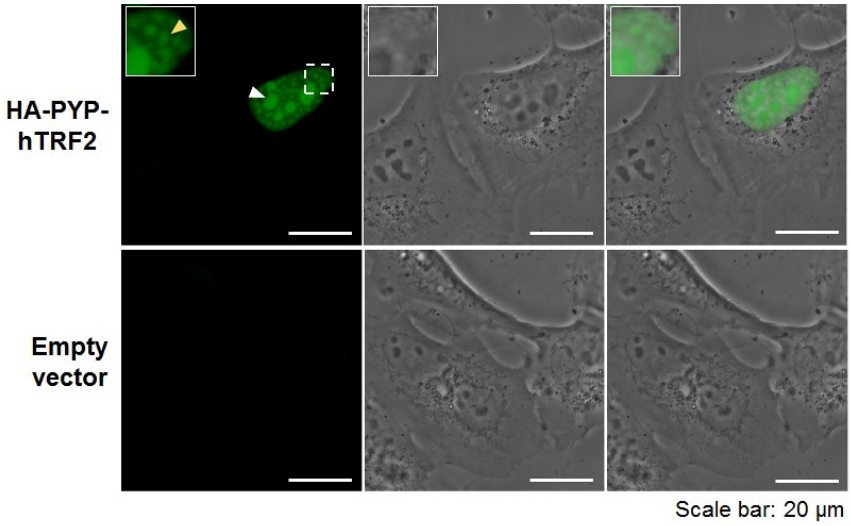


Figure S25. Live-cell imaging of HA-PYP-hTRF2 (top row) or empty vector (lower row) using DMAC-CMK in U2OS cells. Images were recorded after addition of DMAC-CMK (200 nM; incubation 10 min). Fluorescence (FL) images and their overlays of phase contrast images are shown in the left and right columns, respectively. Inset shows the area covered by dashed square. $\lambda_{ex/em}$ = 473/490-590 nm.

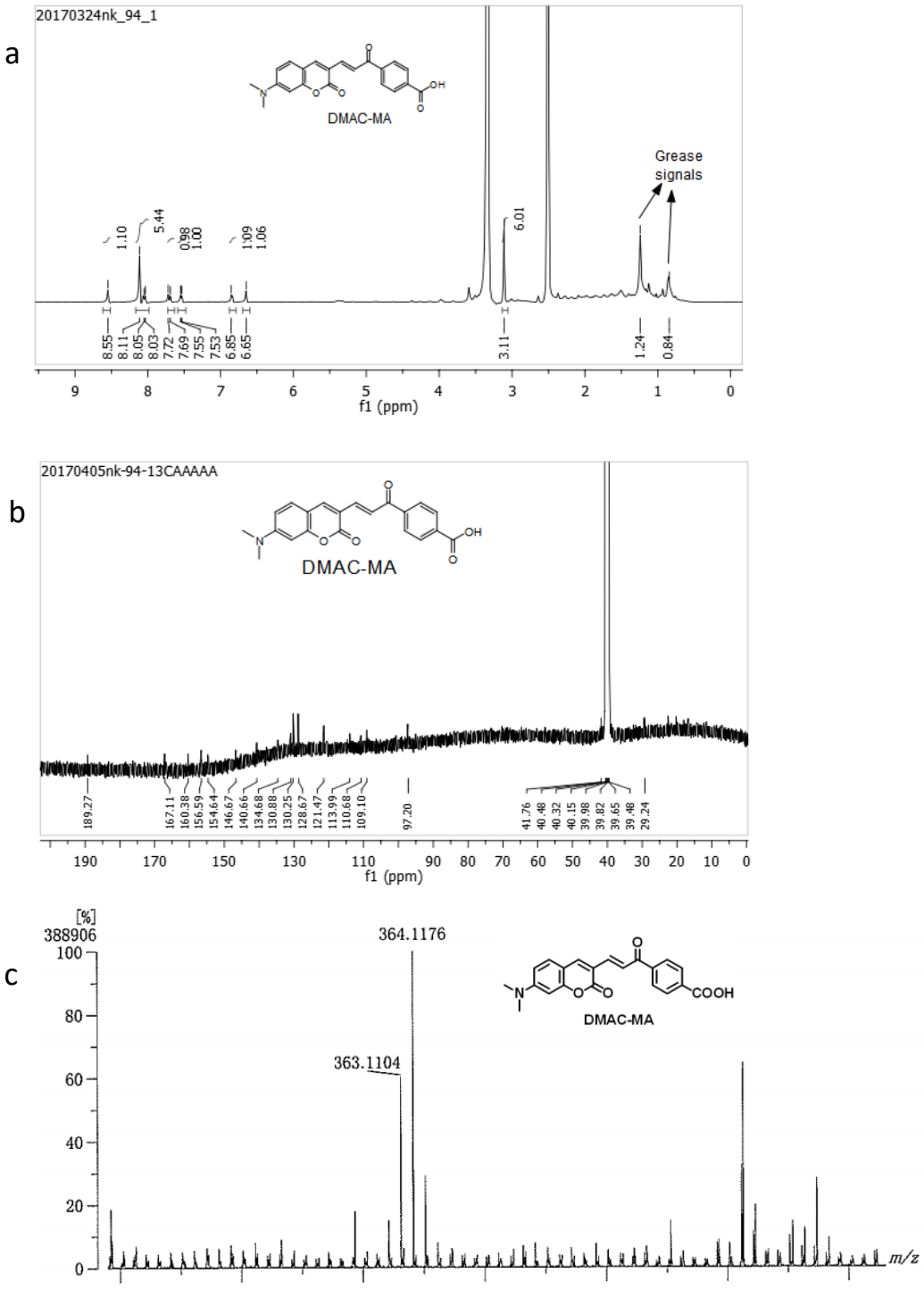


Figure S26. (a) ^{1}H NMR spectra of DMAC-MA in DMSO-d_6; (b) ^{13}C NMR spectra of DMAC-MA in DMSO-d_6; (c) HRMS (FAB+) spectrum of DMAC-MA.

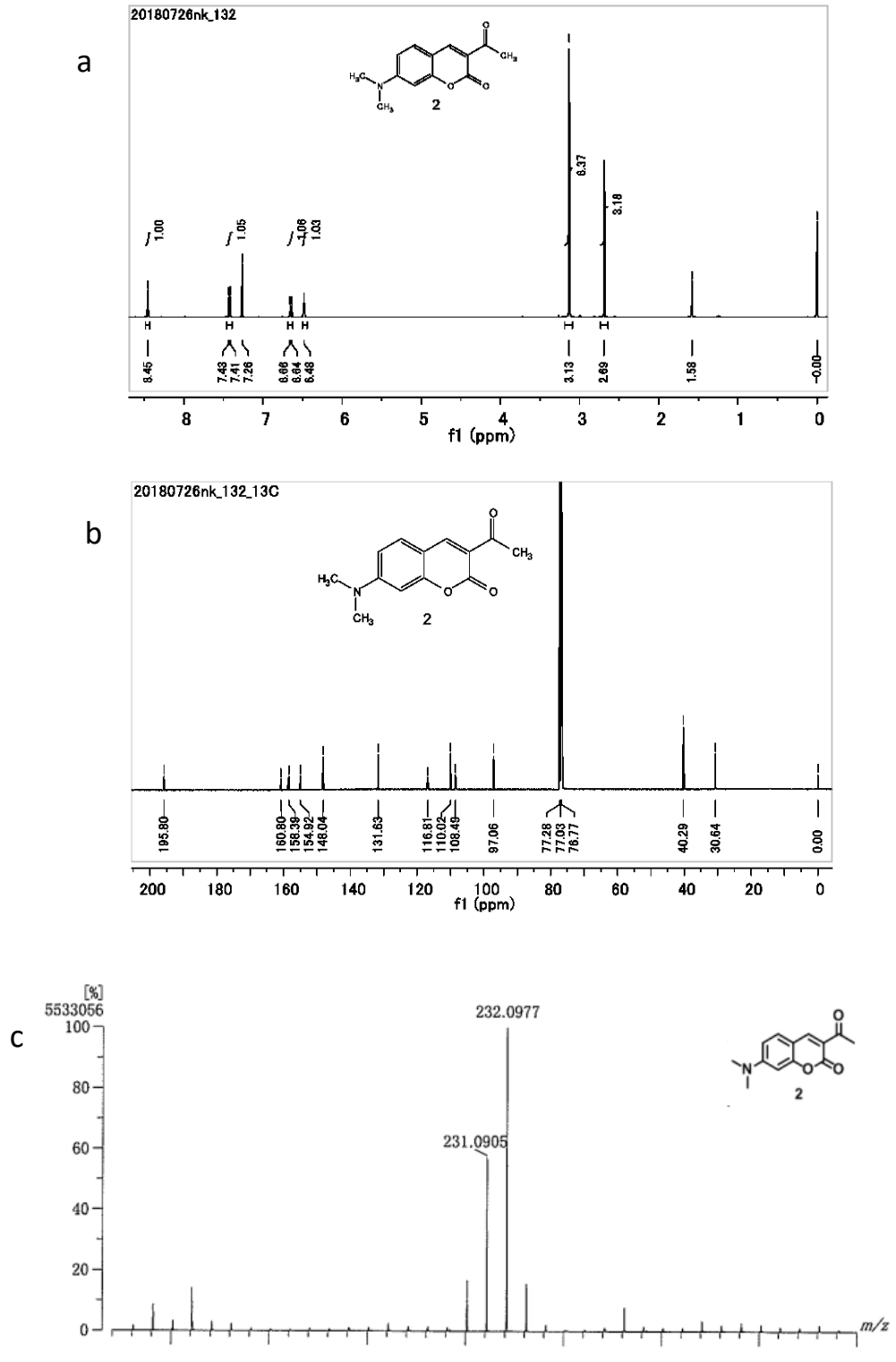


Figure S27. (a) ^{1}H NMR spectra of **2** in $CDCl_3$; (b) ^{13}C NMR spectra of **2** in $CDCl_3$; (c) HRMS (FAB+) spectrum of **2**.

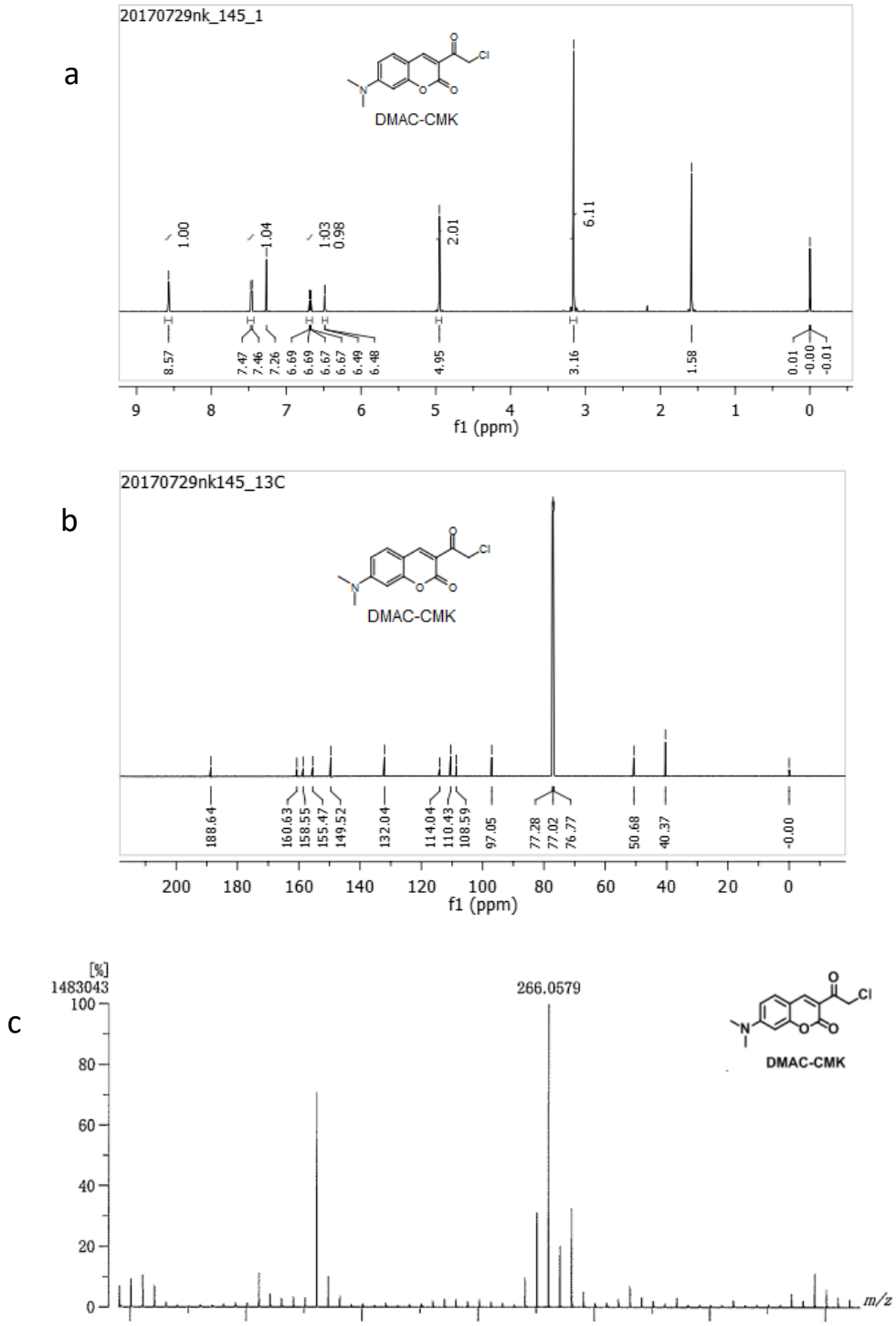


Figure S28. (a) ^{1}H NMR spectra of DMAC-CMK in $CDCl_3$; (b) ^{13}C NMR spectra of DMAC-CMK in $CDCl_3$; (c) HRMS (FAB+) spectrum of DMAC-CMK.

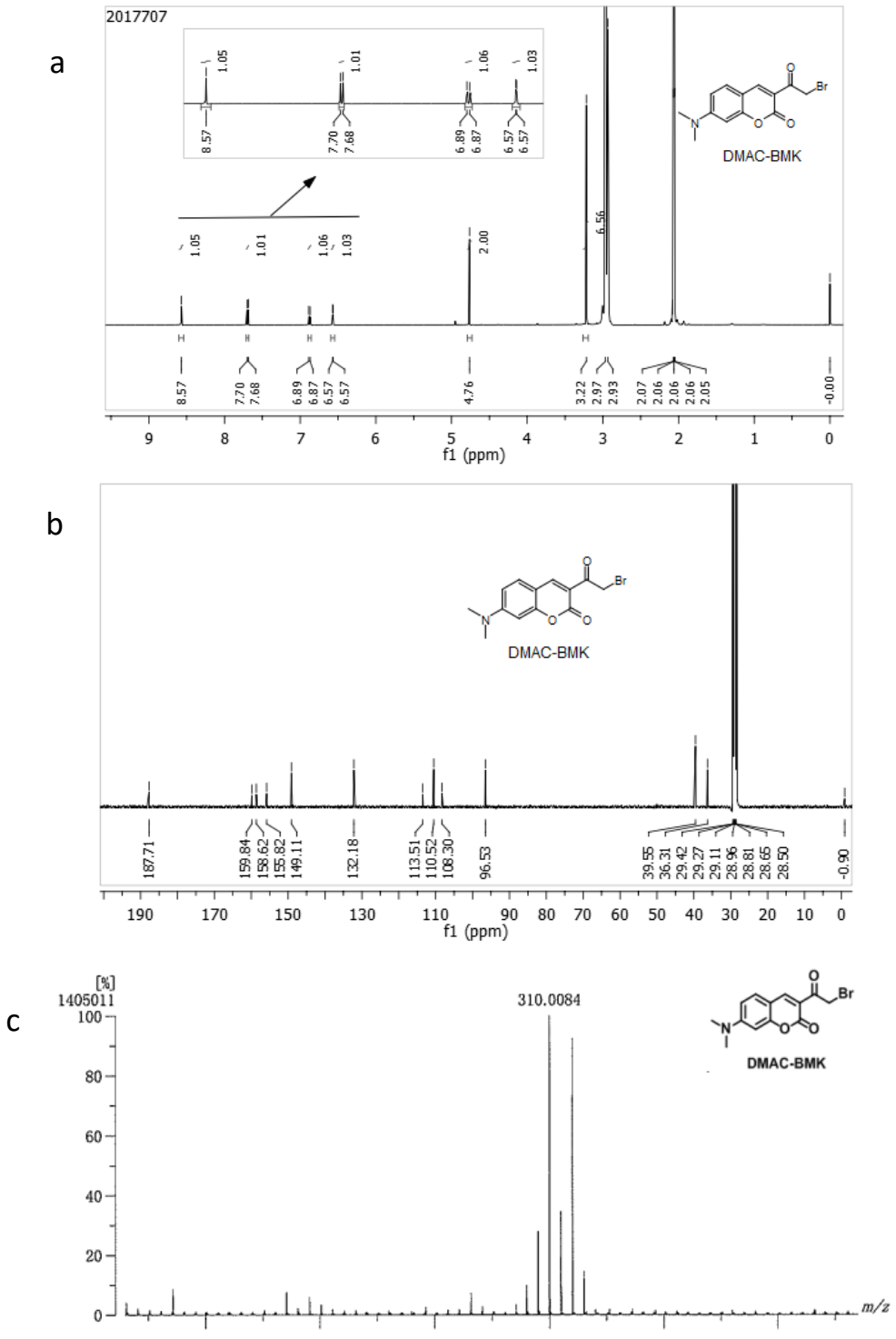


Figure S29. (a) ^{1}H NMR spectra of DMAC-BMK in acetone-d_6; (b) ^{13}C NMR spectra of DMAC-BMK in acetone-d_6; (c) HRMS (FAB+) spectrum of DMAC-BMK.

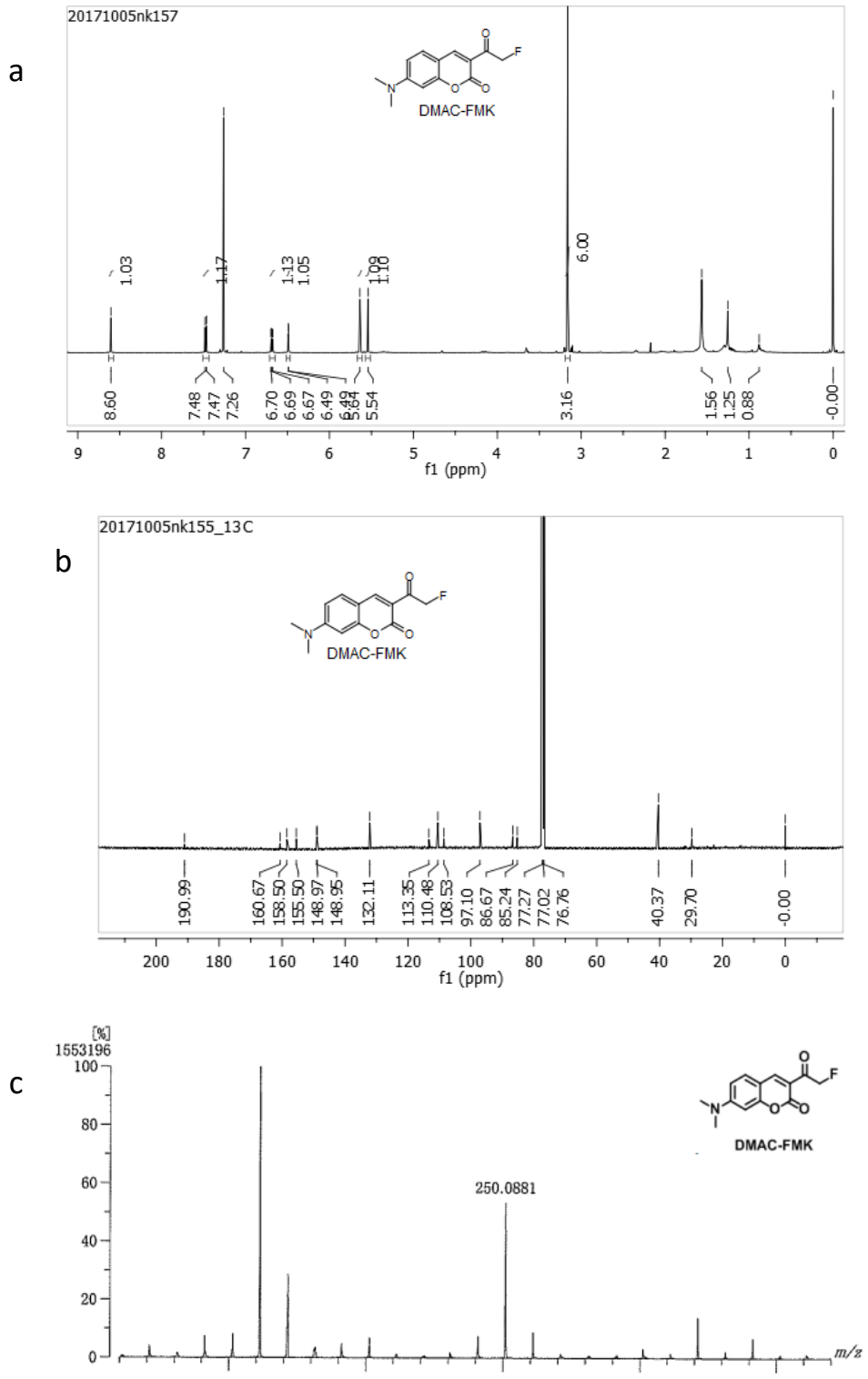


Figure S30. (a) ^{1}H NMR spectra of DMAC-FMK in $CDCl_3$; (b) ^{13}C NMR spectra of DMAC-FMK in $CDCl_3$; (c) HRMS (FAB+) spectrum of DMAC-FMK.

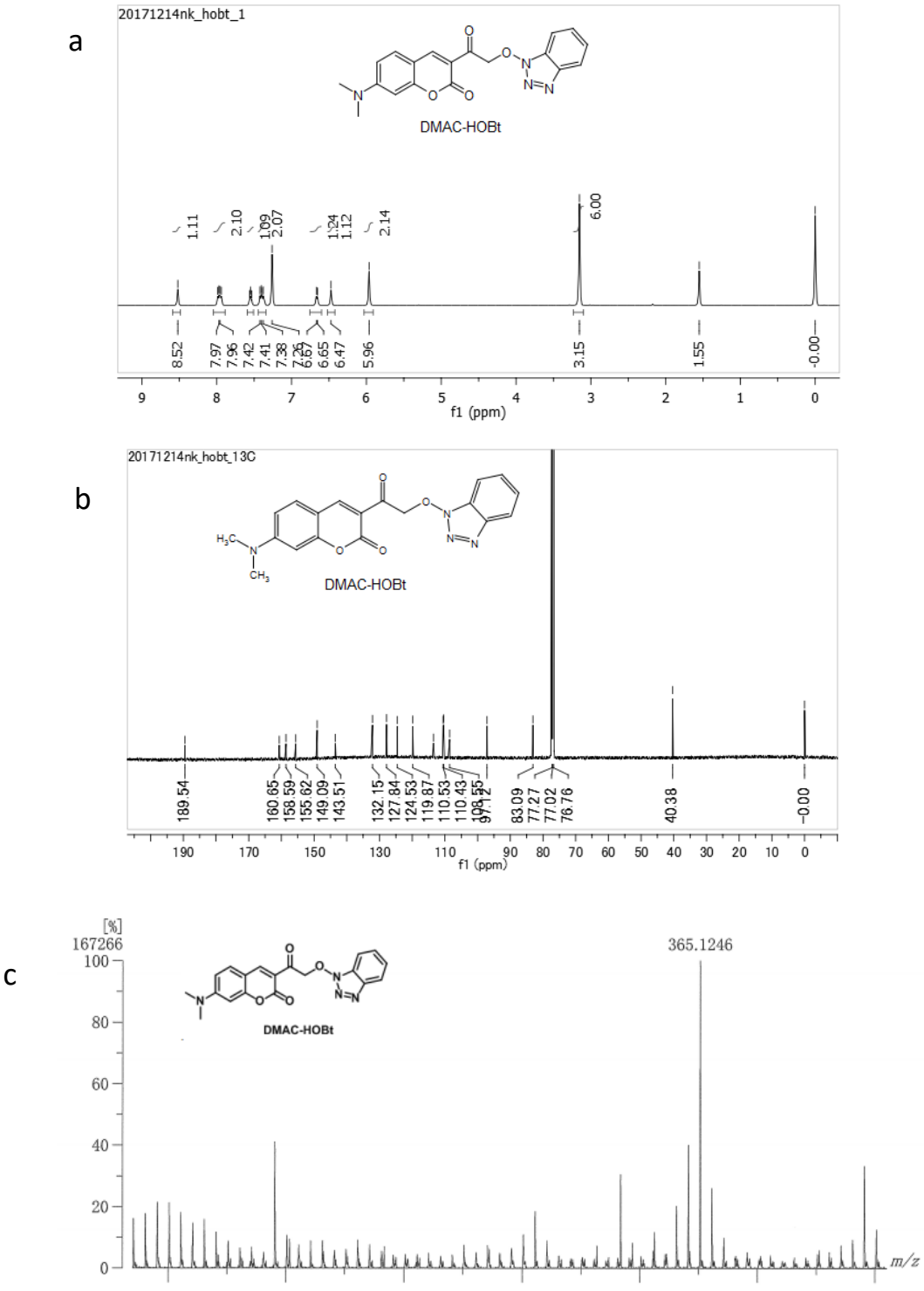


Figure S31. (a) ^{1}H NMR spectra of DMAC-HOBt in $CDCl_3$; (b) ^{13}C NMR spectra of DMAC-HOBt in $CDCl_3$; (c) HRMS (FAB+) spectrum of DMAC-HOBt.

Table S1. Labeling and optical properties of protein tags with covalent fluorogenic probes

Protein tag	Covalent fluorogenic probe	Ligand	Fluorophore	Second-order rate constant k_2 (M^{-1} s^{-1})	Incubation time for intracellular imaging (min)	λ_{abs} / λ_{em} (nm)	ε [free / tag-bound] (M^{-1} cm^{-1})	Φ_f [free / tag-bound]	Brightness [free / tag-bound] (ε Φ_f)	Fold increase in fluorescence upon binding to protein-tag	Ref.
PYP-tag	DMAC-CMK	7-Dimethyl-aminocoumarin (DMAC)	7-Dimethyl-aminocoumarin (DMAC)	1.2×10^3	5	450 / 494	45,000 / 60,000	0.008 / 0.18	360 / 10,800	33	This study
PYP-tag	TAMRA-DNB2	7-hydroxy-cinnamic acid	TAMRA	3.3×10^3	60	560 / 572	38,000 / 70,000	0.06 / 0.89	2280 / 62300	27	S2
SNAP-tag	DRBGFL	Benzylguanine	Fluorescein	4×10^2	180[a]	488 / 520	ND[b]	ND[b]	ND[b]	150	S3
SNAP-tag	SiR-SNAP	Benzylguanine	SiR	2.0×10^4	30-60	650 / 668	ND[b]	ND[b] / 0.30	ND[b]	ND[b]	S4
CLIP-tag	SiR-CLIP	Benzylcytosine	SiR	1.0×10^3	30-60	652 / 668	ND[b]	ND[b] / 0.46	ND[b]	ND[b]	S4
HaloTag	SiR-Halo	Chloroalkane	SiR	$>2.5 \times 10^5$	30-60	648 / 668	ND[b]	ND[b] / 0.39	ND[b]	ND[b]	S4
HaloTag	MaP618-Halo	Chloroalkane	MaP618	ND[b]	5	618 / 635	260 / 107,000	ND[b] / 0.61	ND[b] / 65,270	1000	S5
TMP-tag	TMP-Q-Atto520	Trimethoprim	ATTO 520	53	180	~520 / ~550	ND[b]	ND[b]	ND[b]	20	S6
dC10a	YC23	Dimaleimide	BODIPY	15	60	~510 / ~525	ND[b]	ND[b] / 0.27	ND[b]	800	S7

[a]DRBGFL is cell-membrane impermeable, so the fluorescein derivative of DRBGFL was diacetylated to generate DRBGFL-DA, which was then used for intracellular imaging.
[b]ND, not determined.

References

S1. X. Liu, D. Mao, J. M. Cole and Z. Xu, *Chem. Commun*., 2014, **50**, 9329-9332.

S2. S. Hirayama, Y. Hori, Z. Benedek, T. Suzuki and K. Kikuchi, *Nat. Chem. Biol.*, 2016, **12**, 853-859.

S3. T. Komatsu, K. Johnsson, H. Okuno, H. Bito, T. Inoue, T. Nagano and Y. Urano, *J. Am. Chem. Soc.*, 2011, **133**, 17, 6745-6751

S4. G. Lukinavičius, K. Umezawa, N. Olivier, A. Honigmann, G. Yang, T. Plass, V. Mueller, L. Reymond, I. R. Jr. Corrêa, Z. G. Luo, C. Schultz, E. A. Lemke, P. Heppenstall, C. Eggeling, S. Manley and K. Johnsson, *Nat. Chem.*, 2013, **5**, 132-139.

S5. L. Wang, M. Tran, E. D'Este, J. Roberti, B. Koch, L. Xue and K. Johnsson, *Nat. Chem.*, 2019, DOI: 10.1038/s41557-019-0371-1.

S6. C. Jing and V. W. Cornish, *ACS Chem.Biol.*, **2013**, 8, 1704-1712.

S7. Y. Chen, K. Tsao, S. L. Acton and J. W. Keillor, *Angew. Chem. Int. Ed.* 2018, **57**, 12390-12394.