Supporting Information

A biocompatible condensation reaction for labeling terminal cysteine on proteins

Hongjun Ren, Fei Xiao, Ke Zhan, Young-Pil Kim, Hexin Xie, Zuyong Xia, and Jianghong Rao*

Biophysics, Cancer Biology, and Molecular Imaging Programs, Departments of Radiology and Chemistry, Stanford University, 1201 Welch Road, Stanford, California 94305

1. General Procedures and Materials

All chemicals were purchased from commercial sources (such as Aldrich, Fluka, ANASPECK and Novabiochem). Analytical TLC was performed with 0.25 mm silica gel 60F plates with fluorescent indicator (254 nm). Plates were visualized by ultraviolet light. The ^{1}H and ^{13}C NMR spectra were taken on Bruker 400 MHz magnetic resonance spectrometer. Data for ^{1}H NMR spectra are reported as follows: chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-d (δ 7.26, s); multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broadened); coupling constants are reported as a J value in Hertz (Hz); the number of protons (n) for a given resonance is indicated nH, and based on the spectral integration values. MALDI-MS spectrometric analyses were performed at the Mass Spectrometry Facility of Stanford University.

Short N-terminal cysteine peptides were synthesized using standard solid phase Fmoc chemistry. After coupling, the peptide was cleaved from the resin by incubating with TFA: TIS: H_2O (95:2.5:2.5) for 2 hours. The peptide was precipitated from the cleavage solution using cold diethyl ether and washed with cold diethyl ether for 3 times. The crude fresh peptides were used for the reaction directly. HPLC was performed on a Dionex HPLC System (Dionex Corporation) equipped with a GP50 gradient pump and an inline diode array UV-Vis detector. A reversed-phase C18 (Phenomenax, 5 μm, 10 x 150 mm) column was used with a MeCN/H_2O gradient mobile phase containing 0.1% trifluoroacetic acid at a flow of 3 ml/min.

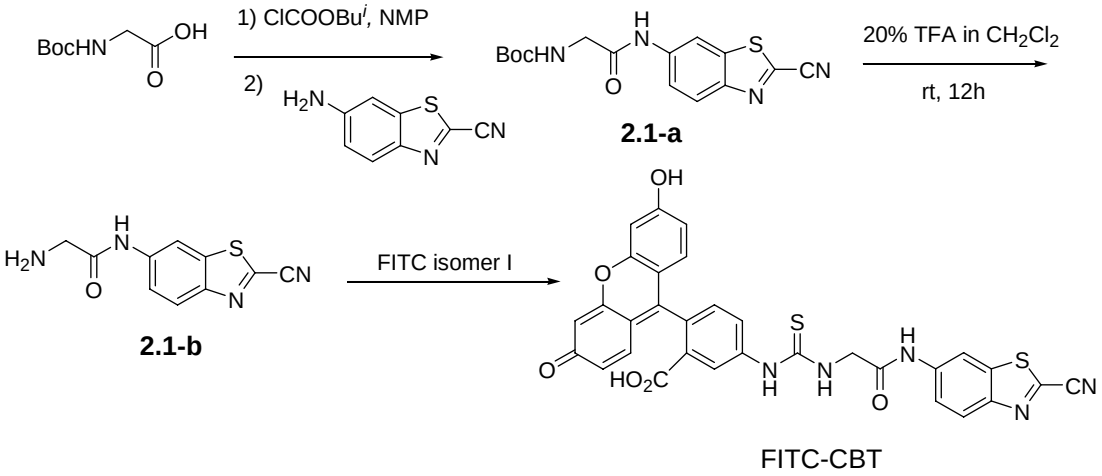
2. Preparation of probes

The 2-cyano-6-aminobenzothiazole (amino-CBT) was prepared according the published procedure [1,2]. ^{1}HNMR (^{6}d-DMSO, 400 MHz): 7.84 (d, J = 8.8 Hz, 1 H), 7.140 (s, 1 H), 6.95 (d, *J* = 8.8 Hz, 1 H), 6.12 (s, 2 H); ^{13}CNMR (^{6}d-DMSO, 100 MHz): 150.6, 143.2, 138.4, 127.8, 125.2, 117.4, 114.4, 102.3.

2.1. FITC-CBT

The isobutyl chlorformate (20 mg, 0.15 mmol) was added to the mixture of Boc-glycine (35 mg, 0.2 mol) and N-methyl morpholine (NMP) (30mg, 0.3 mmol) in THF (4.0 mL) at 0 °C under N_2 and the reaction mixture was stirred at this temperature for 20 min. The solution of 2-cyano-6-aminobenzothiazole (17.5 mg, 0.1 mmol) was added to the

reaction mixture and stirred for further 2 h at 0 °C then overnight at room temperature. Saturated $NaHCO_3$ was added and the reaction mixture was extracted with ethyl acetate (2 x 30 mL). The combined organic phase was dried by Na_2SO_4 and then evaporated. Pure product **2.1-a** (80%) was obtained after normal flash chromatography. ^{1}H NMR ($CDCl_3$, 400 Hz): 1.48 (s, 9 H), 3.96 (d, J = 6.0 Hz, 2 H), 5.20-5.40 (bs, 1 H), 7.40 (d, J = 9.0 Hz, 1 H), 8.07 (d, J = 9.0 Hz, 1 H), 8.60 (s, 1 H), 8.80-9.00 (bs, 1H).



2.1-a

2.1-b

FITC-CBT

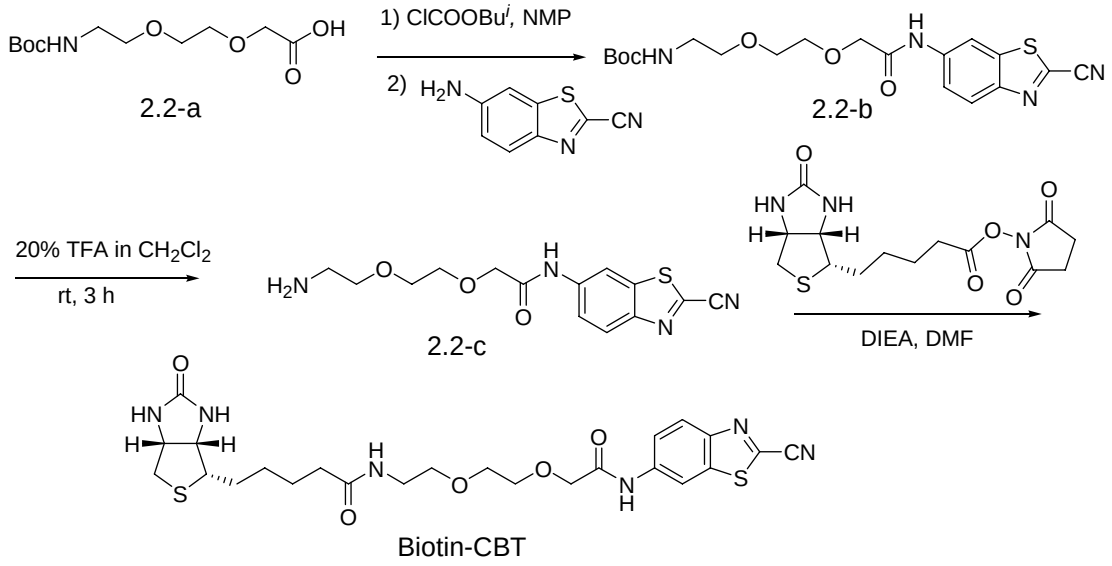
The protecting group Boc was cleaved in 20% TFA in CH_2Cl_2 for 12 h. Precipitated from the cleavage solution using cold diethyl ether, the amino compound **2.1-b** was obtained in good yield which can directly use for next step reaction.^{1}HNMR (CD3OD): 3.86 (s, 2 H), 7.62 (d, J = 8.8 Hz, 1 H), 8.08 (d, J = 8.8 Hz, 1 H), 8.62 (s, 1 H); IR (KBr): 2231.5 cm^{-1}.

The mixture of **2.1-b** (0.2 mg), fluorescein isothiocyanate (FITC) isomer I (0.1 mg) and DIEA in DMF was stirred for 2 h at room temperature. After preparative HPLC, pure FITC-CBT was obtained. HRMS (m/z): calculated for $C_{31}H_{20}N_5O_6S_2$ (M^+): 622.0855, found: 622.0858 (M^+).

2.2. Biotin-CBT

The isobutyl chlorformate (20 mg, 0.15 mmol) was added to the mixture of carboxyl acid **2.2-a** (33 mg, 0.2 mol) and N-methyl morpholine (NMP) (30 mg, 0.3 mmol) in THF (4.0 mL) at 0 °C under N_2 and the reaction mixture was stirred at this temperature for 20 min. The solution of 2-cyano-6-aminobenzothiazole (17.5 mg, 0.1 mmol) was added to the reaction mixture and stirred for further 2 h at 0oC then overnight at room temperature. Saturated $NaHCO_3$ was added and the reaction mixture was extracted with ethyl acetate (2 x 30 mL). The combined organic phase was dried by Na_2SO_4 and then evaporated. The pure product **2.2-b** (yield: 70 %) was obtained after normal flash chromatography. The protecting group Boc was cleaved in 20% TFA in CH_2Cl_2 for 12 h. Precipitated from the cleavage solution using cold diethyl ether, the deprotected amino compound **2.2-c** was obtained in a good yield which was directly used for next step reaction. ^{1}HNMR (CD3OD): 3.02-3.20 (m, 2 H), 3.60-3.90 (m, 6 H), 4.19-4.31 (m, 2 H), 7.42 (d, J = 8.8 Hz, 1 H), 8.12 (d, J = 8.8 Hz, 1 H), 8.67 (s, 1 H); IR (KBr): 2231.9 cm^{-1}; MS (ESI, M^++H): 321.1; cal: 320.1 (M^+).

The mixture of **2.2-c** (48 mg, 0.15 mmol), Biotin-NHS (33 mg, 0.1 mmol) and DIEA (20 mg, 0.20 mmol) in DMF (0.5 mL) was stirred for 3 h at room temperature. After preparative HPLC, pure Biotin-CBT was obtained (yield: 80 %). HRMS (m/z): calculated for $C_{24}H_{30}N_6O_5NaS_2$ (M^++Na): 569.1617, found: 569.1614 (M^++Na).



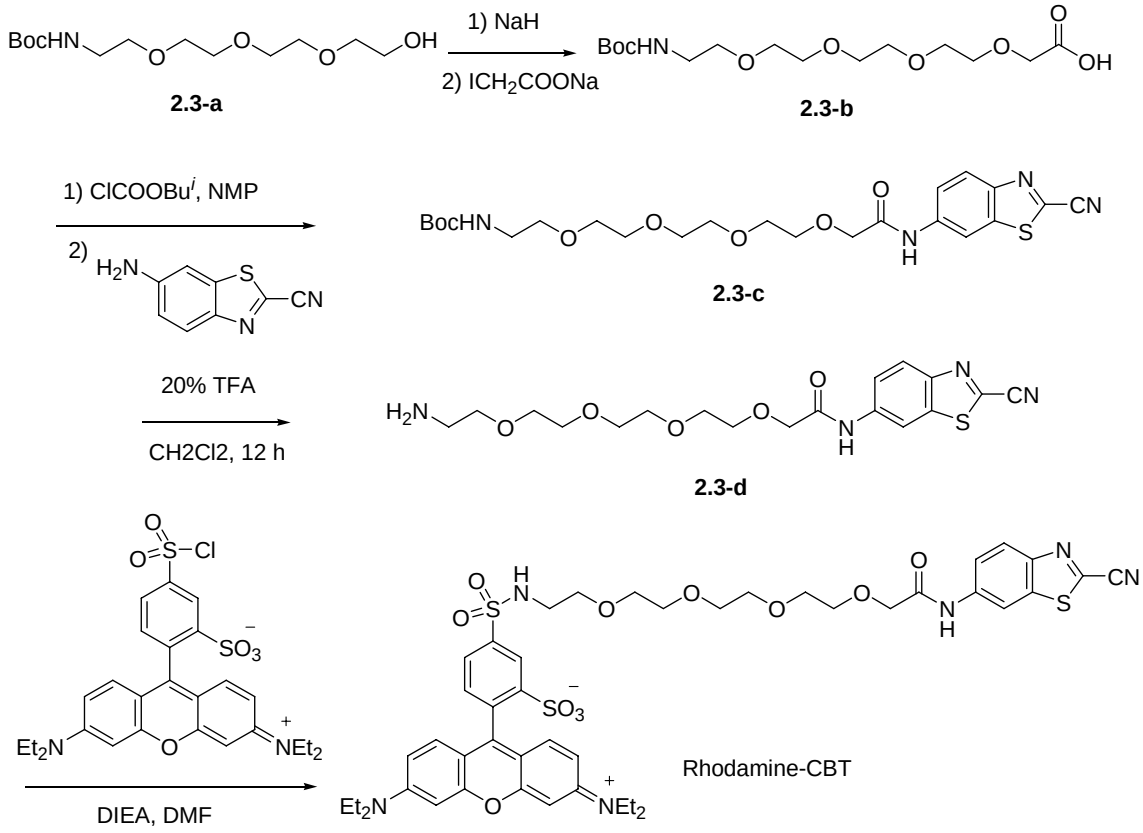
2.3. Rhodamine-CBT

NaH (80 %, 160 mg, 5mmol) was added to a solution of Boc protected alcohol **2.3-a** (1.46 g, 5 mmol) in anhydrous DMF (5 mL) at 0 °C under N_2. After stirring at 0 °C for 30 min, ICH_2COONa (1.1 g, 5 mmol) was added to the reaction mixture. The reaction mixture was stirred for 4 h and quenched with 1 M NaOH. The mixture was extracted with ether (3X30 mL) and the organic layer was discarded. The water phase was acidified to pH 3 with 1 M HCl and then extracted with ethyl acetate (3 x 30 mL). The combined organic phase was washed with brine and dried over Na_2SO_4. After evaporated the solvent, the free acid **2.3-b** was obtained (yield: 50 %). ^{1}H NMR ($CDCl_3$, 400 Hz): 1.42 (s, 9 H), 3.31 (s, 2 H), 3.40-3.80 (m, 14 H), 4.15 (s, 2 H), 8.20-9.20 (bs, COOH, 1 H).

The isobutyl chlorformate (20 mg, 0.15 mmol) was added to the mixture of carboxyl acid **2.3-b** (70 mg, 0.2 mol) and NMP (4-methylmorpholine, 30 mg, 0.3 mmol) in THF (4.0 mL) at 0 °C under N_2 and the reaction mixture was stirred at this temperature for 20 min. The solution of 2-cyano-6-aminobenzothiazole (17.5 mg, 0.1 mmol) was added to the reaction mixture and stirred for further 2 h at 0 °C then overnight at room temperature. Saturated $NaHCO_3$ was added and the reaction mixture was extracted with ethyl acetate (2 x 30 mL). The combined organic phase was dried by Na_2SO_4 and then evaporated. The pure product **2.3-c** (yield: 62 %) was obtained after flash chromatography. ^{1}H NMR ($CDCl_3$, 400 Hz): 1.41 (s, 9 H), 3.2-3.85 (m, 16 H), 4.16 (s, 2 H), 5.00 (bs, 1 H), 7.55 (dd, J_1= 9.2 Hz, J_2 = 2.0 Hz, 1 H), 8.09 (d, J = 9.2 Hz, 1 H), 8.72 (d, J = 2.0 Hz, 1 H), 9.24 (bs,1 H); ^{13}C NMR ($CDCl_3$, 100 MHz): 168.9, 155.9,

148.7, 138.1, 136.7, 135.2, 125.2, 120.9, 113.0, 111.5, 71.2, 70.6, 70.42, 70.36, 70.28, 70.21, 70.15, 70.13, 70.0, 40.2, 28.3.

The Boc group was deprotected using 30% TFA in CH_2Cl_2 for 12 h. Precipitated from the cleavage solution using cold diethyl ether, the amino compound **2.3-d** was obtained in good yield which can directly use for next step reaction. ^{1}H NMR: 3.15 (s, 2 H), 3.40-4.20 (m, 12 H), 4.23 (s, 1 H), 7.70 (d, J = 8.8 Hz, 1 H), 7.99 (bs, 1 H), 8.02 (d, J = 8.8 Hz, 1 H), 8.50 (s, 1H), 10.14 (s, 1 H); MS (ESI, M_++H): 409.2; cal: 408.2 (M_+); IR (oil): 2231.9 cm^{-1}.



The mixture of **2.3-d** (0.2 mg), Lissamine rhodamine B sulfonyl chloride (0.4 mg) and DIEA (5 μL) in DMF (0.2 mL) was stirred for 2 h at room temperature. After preparative HPLC, pure rhodamine-CBT was obtained. HRMS (m/z): calculated for $C_{45}H_{52}N_6O_{11}NaS_3$ (M^+ + Na): 971.2754, found: 971.2743 (M^+ + Na).

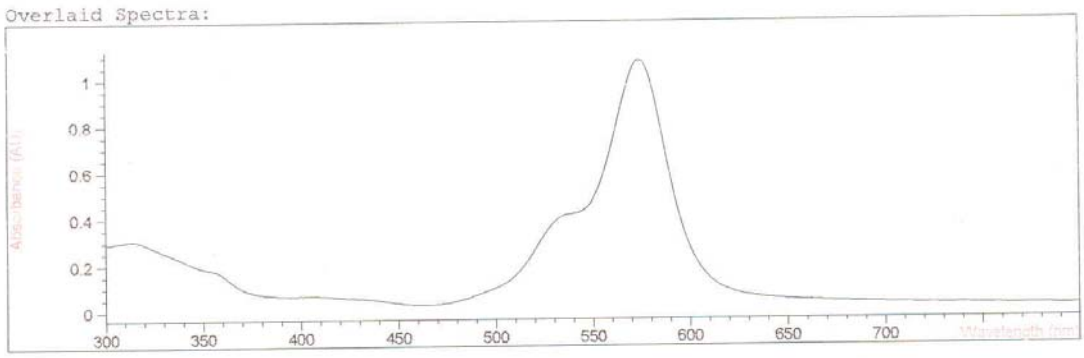
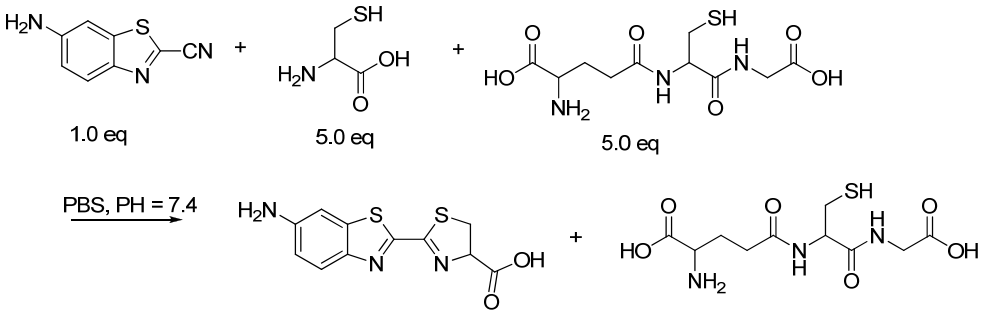


Figure S1. The UV spectroscopy of the rhodamine-CBT probe in PBS.

3. HPLC analysis of CBT reaction with a mixture of free cysteine and glutathione

To investigate the selectivity, the condensation reaction between CBT and cysteine was carried out in presence of glutathione, and monitored by HPLC. Only the cyclized product from the coupling of CBT and cysteine, amino-luciferin was detected (Figure S2).



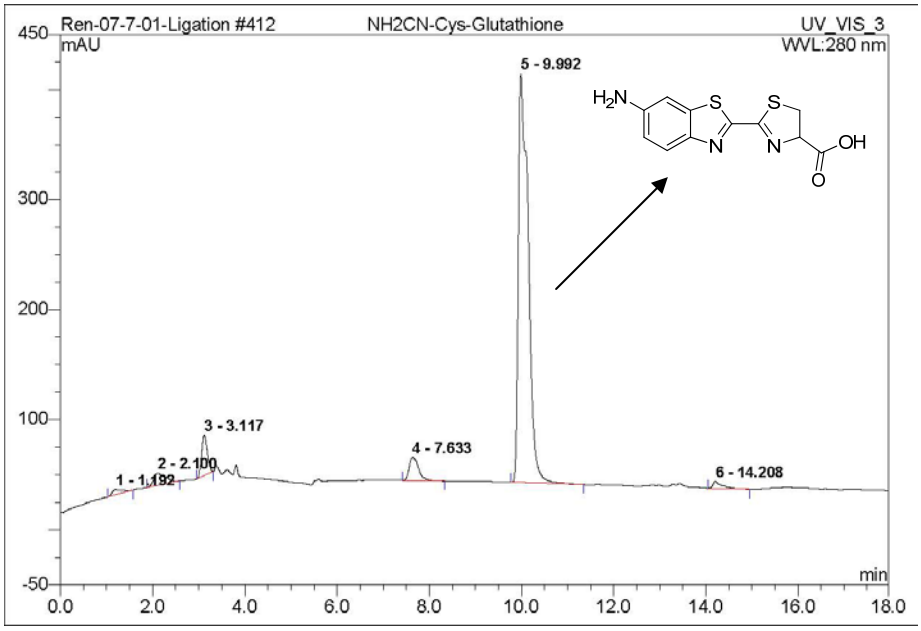
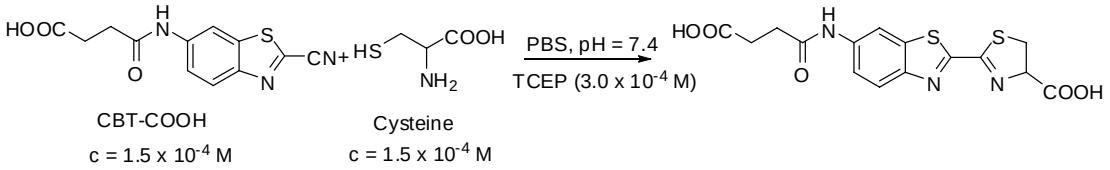


Figure S2. HPLC analysis of the condensation reaction of amino-CBT and free L-cysteine in the presence of glutathione; detection wavelength: 280 nm.

4. Kinetic Study for the reaction of CBT-COOH and free cysteine

We measured the condensation reaction kinetics of the following substrates:



To a solution of CBT-COOH in phosphate buffered saline (PBS) (1x, pH = 7.4) was added a solution of free cysteine and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in PBS (freshly prepared; small amount of sodium bicarbonate aqueous solution was added to adjust the pH of the solution to 7.4), affording the final concentration of CBT-COOH, cysteine and TCEP to 1.5 x 10^{-4} M, 1.5 x 10^{-4} M, 3.0 x 10^{-4} M, respectively. The mixture was stirred at room temperature (23 ^{o}C) and monitored by HPLC at different time points.

The conversion rate at each time point was calculated and plotted out vs. reaction time (**Figure S3**). Applying the similar kinetic analysis reported for the Click reaction [3], we plotted 1/[CBT-COOH] vs. reaction time (**Figure S4**) and the linear regression analysis of 1/[CBT-COOH] vs. time gives the second-order rate constant the value of 9.19 $M^{-1}\cdot S^{-1}$. For comparison, the rate constant of the copper-free click reaction was reported to be 7.6 x 10^{-2} $M^{-1}\cdot S^{-1}$ [3], which is 120-fold smaller.

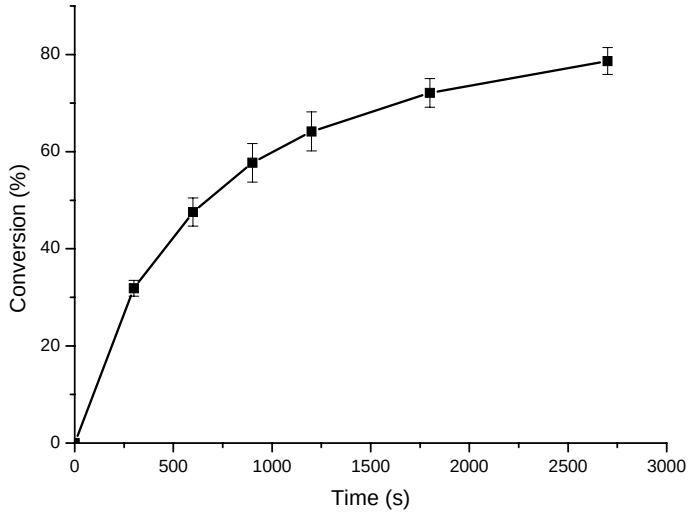


Figure S3. The conversion rate of the condensation reaction vs. time. Error bars indicate the standard deviations of two replicate experiments.

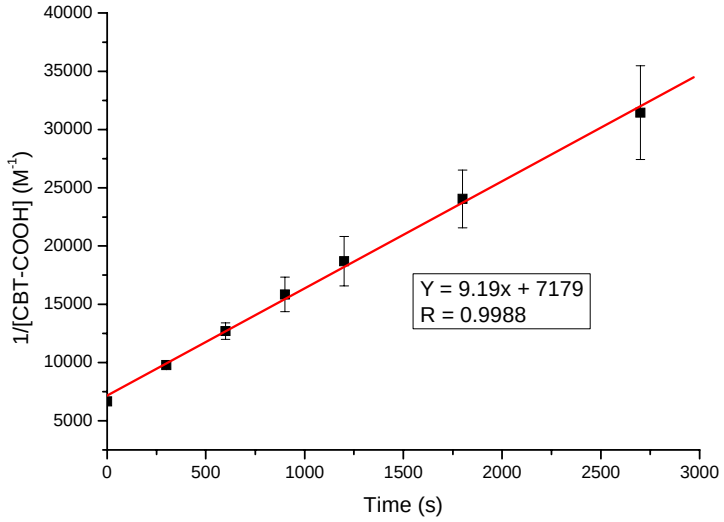


Figure S4. Linear regression analysis of 1/[CBT-COOH] vs. time of the CBT condensation reaction. Error bars indicate the standard deviations of two replicate experiments.

5. The reaction of N-terminal Cys peptides with CBT

General procedure for the reaction of N-terminal cysteine peptides with 2-cyano-6-aminobenzothiazole: The crude N-terminal cysteine peptides (~200 μM) and the 2-cyano-6-aminobenzothiazole (~400 μM) was incubated for 30 min in PBS buffer (PH = 7.4, 10 % DMSO) at room temperature and monitored by HPLC for the completion of reaction. All the desired products were confirmed by MS. Table S1 summarizes the results. Judging from the HPLC chromatograms, the conversion rates for all the peptides were at least 90% within 30 mins, and no major side product was detected.

Table S1. Reaction of CBT with cysteine-containing peptides.

Entry	Peptide sequence	Calculated MS of product	Observed MS of product (M+1)
1	Cys-Gly	336.0	337.1
2	Cys-Asp	394.0	395.0
3	Cys-Gly-Gly	393.0	394.1
4	Cys-Glu-Gly-Gly	522.1	523.1
5	Cys-Asp-Asp-Gly	566.1	567.3
6	Cys-Ile-Cys-Gly-Gly	609.1	610.2

6. In vitro protein labeling

6.1. Preparation of N-terminal cysteine protein rLuc

A His-tag followed by the TEV protease cleavage sequence with a cysteine mutation at the P1′ position was fused to the N-terminus of a *Renila* luciferase mutant Luc8. The Luc8 gene with desired insertion was amplified from pBAD-Luc8 with 5’-primer (5’-CC ATG GCT CAT CAT CAT CAT CAT CAT GAA ACC TGT ATT TTC AGT

GCG CTT CCA AGG TGT-3') and 3'-primer (5'- AAG CTT TTA CTG CTC GTT CTT CAG CAC-3'). The amplified DNA fragments were inserted into pBAD vector between Nco I and HindIII. The plasmid was transformed into Top 10 cell for expression. The transformed cells were grown in 1L of LB media at 37 °C, and induced with 0.2% arabinose at the OD_{600} of ~0.6. After 2 hours induction at 37 °C, the cells were harvested by centrifugation and frozen at -80 °C. The cells were thawed and lysed in 20 mM Tris pH 7.4. The cell lysates were applied on Ni-NTA agarose (Qiagen) column to purify the His-tagged proteins.

TEV protease cleavage of the His-tagged proteins was carried out under dialysis conditions. A 400 µl solution containing 2 mg of purified proteins and 200 units TEV protease was injected into a 3000 MWCO dialysis cassette in PBS buffer. The cleavage was initiated by adding β-mercaptoethanol to the dialysis buffer to a final concentration of 1 mM. The reaction was gently stirred and incubated at room temperature for 20 h. The cleavage by TEV protease not only generated N-terminal cysteine but also removed the N-terminal His-tag. The N-terminal cysteine proteins were purified by the addition of Ni-NTA agarose to the reaction solution to remove uncleaved His-tagged proteins.

6.2. Preparation of C-terminal cysteine proteins rLuc-Cys and rLuc-diCys

To fuse intein GyrA into the C-terminus of rLuc, two additional restriction sites EcoR I and Hind III were introduced into pBAD-Luc8 plasmid [4]. The intein GyrA mutant (N198A) gene was amplified from pTWIN-MBP1 (New England Biolabs) with 5'-primer (5'- A ATT GAA TTC TGC ATC ACG GGA GAT GCT) and 3'-primer (5'- A GCT AAG CTT GGT GAG GCC AGT AGC GTG-3'). The PCR product was digested by EcoR I and Hind III, and ligated into the same enzyme-digested pBAD-Luc8 to give pBAD-Luc8-GyrA plasmid. A sequence of peptide (Val-Pro-Leu-Ser-Leu-Thr-Met-Gly) was introduced between rLuc and GyrA. The rLuc gene was amplified from pBAD-Luc8 with 5'-primer (5'-A TGC CCA TGG CTT CCA AGG TGT AC-3'), and the 3'-primer (5'- ATGC GAA TTC ACC ACC CAT TGT CAG TGA CAG AGG TAC TCC TCC CTG CTC GTT CTT CAG-3'). The PCR products were digested with Nco I and EcoR I, and ligated into the same enzyme-digested pBAD-rLuc-GyrA to give plasmids containing different protease cleavage sites.

The expression plasmid was transformed into LMG194 cell, an *E. coli* strain deficient of arabinose. The transformed cells were grown in 1 L of LB media at 37 °C, and induced with 0.2% arabinose at the OD_{600} of ~0.6. After 4 hours induction at 30 °C, the cells were harvested by centrifugation and frozen at -80 °C. The cells were thawed in 10 ml of 20 mM Tris pH 7.4, 20 mM imidazole, 300 mM NaCl containing 1 mg/ml lysozyme, 5 µg/ml DNAse I, and 10 µg/ml RNAse A. The resuspended cells were incubated for 30 min at room temperature, and sonicated for <1 min. The lysates were clarified by centrifugation at 15,000 rpm for 30 min at 4 °C. The clarified supernatant containing expressed proteins was incubated with 2 ml of Ni-NTA agarose (Qiagen) at 4 °C for 1 h with gently shaking. The Ni-NTA agarose beads were washed with 100 mL of 20 mM Tris pH 7.4, 20 mM imidazole, 300 mM NaCl. His-tagged proteins were

eluted with 5 ml of the same buffer containing 250 mM of imidazole. The eluted fusion proteins were further purified using FPLC on Source 15Q anion exchange column. The luciferase activity of the Luc8-GyrA and other fusion proteins containing protease substrates was assessed by a calibrated luminometer with coelenterazine as the substrate.

The fusion protein (50 μM) was incubated with L-Cys or diCys (10 mM) in the presence of 2-mercaptoethanesulfonic acid (MESA) (20 mM) for 15 h at 4 °C in PBS buffers (pH 8.0). The reaction products were analyzed by gel electrophoresis (Figure 2).

6.3 In vitro protein labeling conditions

The labeling solution typically contained 10 μM of protein and 50 μM of CBT probe in PBS buffer (PH = 7.4) in the presence of 2 mM of glutathione or TECP in a volume of 20 μL. The reaction was incubated for 2 h at r.t, and quenched with cysteine to remove the excess probe before gel loading.

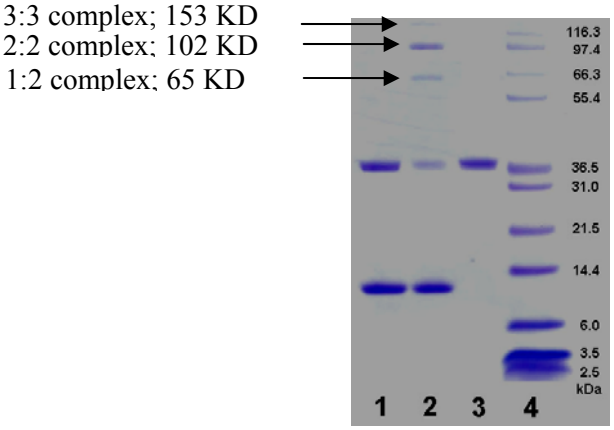


Figure S5. Gel analysis of Biotin-CBT labeled Cys-rLuc. Cys-rLuc (18 μM, 13 μL) was mixed with Biotin-CBT (500 μM, 7 μL) in PBS buffers in the presence of TCEP for 3 h, and then the reaction was quenched with free cysteine solution. Centrifuge filtration removed the excess ligand before mixing with streptavidin solution (at 1.5:1 ratio). After 30 min, the mixture was analyzed on the Nu PAGE gel. Lane 1: mixture of unlabeled Cys-rLuc (36.5 KD) and streptavidin (14.4 KD); Lane 2: streptavidin + labeled Cys-rLuc; Lane 3: labeled Cys-rLuc; lane 4: marker. Lane 2 shows the formation of 1:2, 2:2, and 3:3 complexes between biotinylated rLuc and streptavidin, as indicated by the arrows.

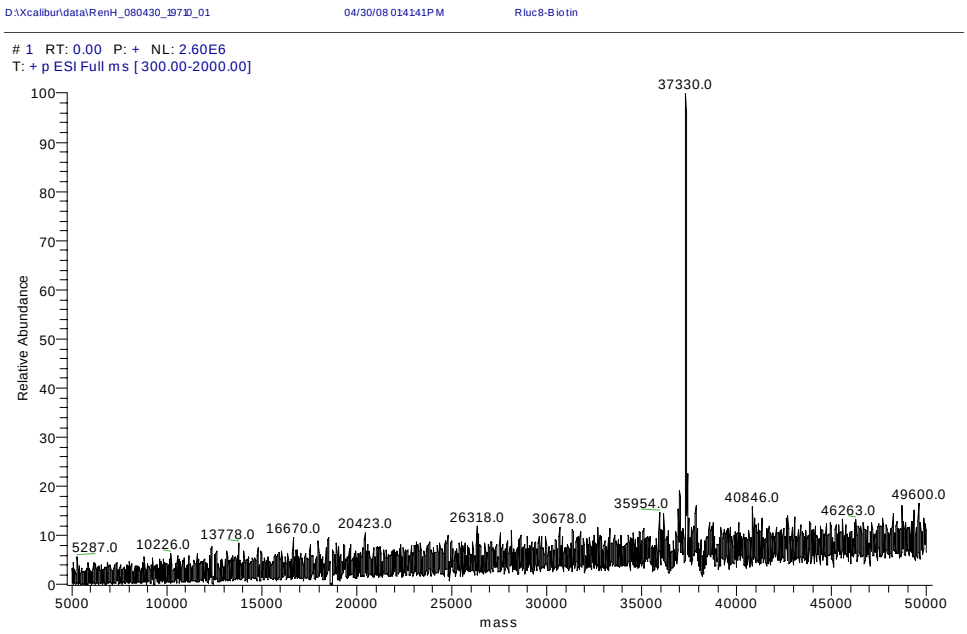
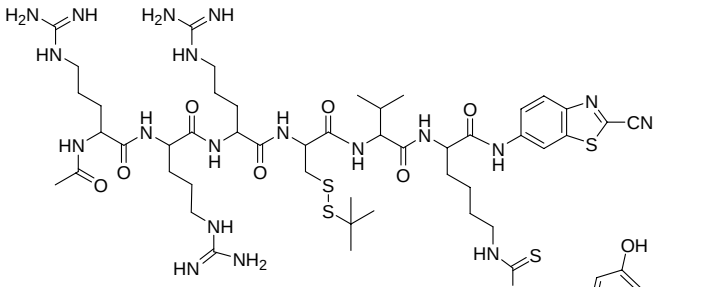


Figure S6. MS analysis of Biotin-CBT labeled Cys-rLuc.

In the case of C-terminus labeling, peptide-FITC-CBT was used. This ligation product rLuc-diCys-CBT showed different mobility on the gel from rLuc-diCys due to the addition of the short peptide sequence (**Figure S7**).

a



b Peptide-FITC-CBT

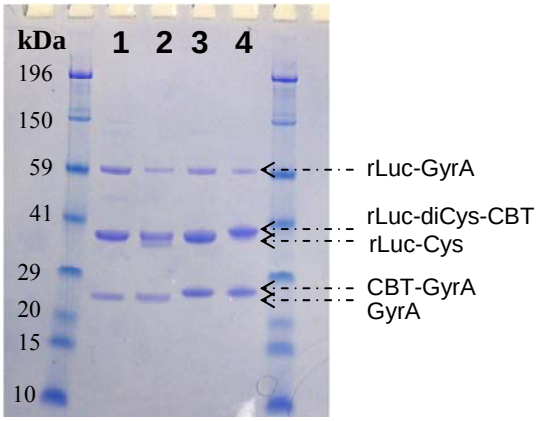


Figure S7. In vitro labeling of cysteine introduced at the C-terminus of proteins with CBT probes in PBS buffer at room temperature. (**a**) Structure of peptide-FITC-CBT. The addition of the tetrapeptide results in a different gel mobility for the ligation product. (**b**) White light image of a gel loaded with reaction solutions (indicated below) and stained with Coomassie Blue. 1: rLuc-Cys; 2: rLuc-diCys; 3: rLuc-Cys + Peptide-FITC-CBT; 4: rLuc-diCys + Peptide-FITC-CBT. The arrows indicate the corresponding proteins.

The labeling efficiency by CBT of protein containing terminal cysteine was examined with the labeling of rLuc-diCys by peptide-FITC-CBT. The fusion protein (rLuc-GyrA, 50 μM) was incubated with diCys (10 mM) in the presence of MESA (20 mM) for 15 h at 4 °C in PBS buffer (pH 8.0). The rLuc-diCys was then purified after removing His-tagged proteins by using Ni-NTA agarose. The purified rLuc-diCys (5 μM in PBS pH 8.0) was treated with different concentrations of peptide-FITC-CBT in the presence of TCEP (100 μM) at RT for 1h in 16 μL, and then analyzed by the NuPAGE gel (**Figure S8**) Both of unlabeled and labeled proteins can be resolved on the NuPAGE gel, and quantification of the reaction products indicated that with 2 equivalents of CBT, the labeling reaction could proceed to more than 90% completion in 1 hour, and nearly 100% in 1 hour with 5 equivalents of the CBT probes.

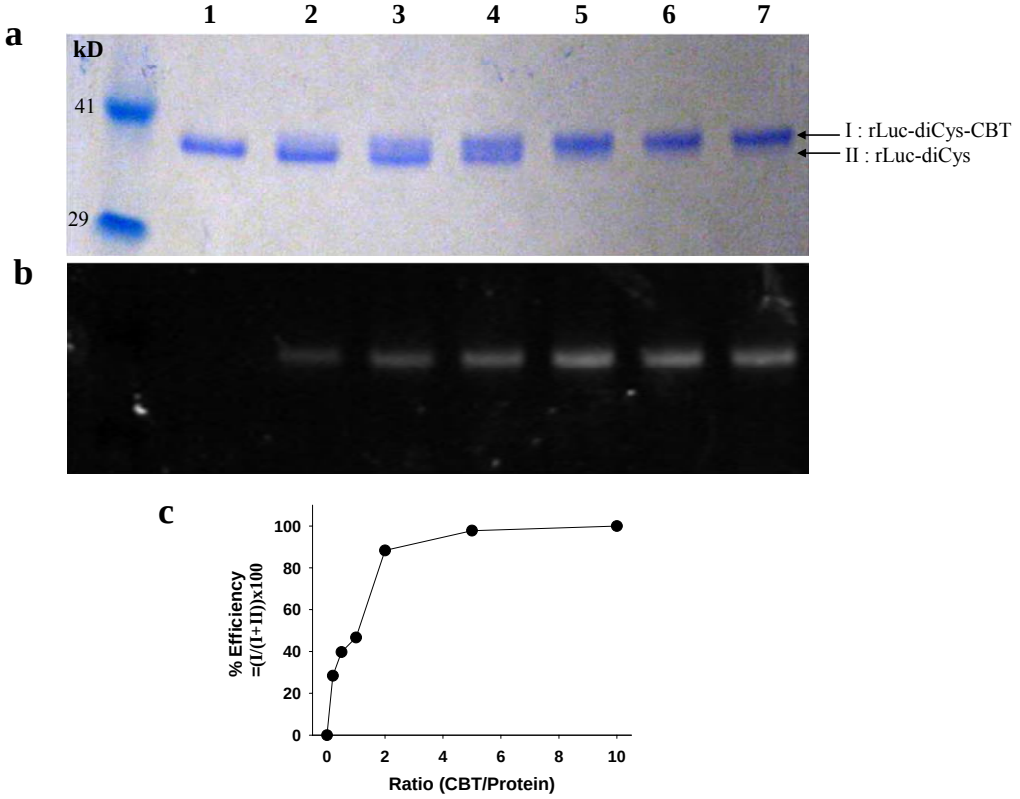
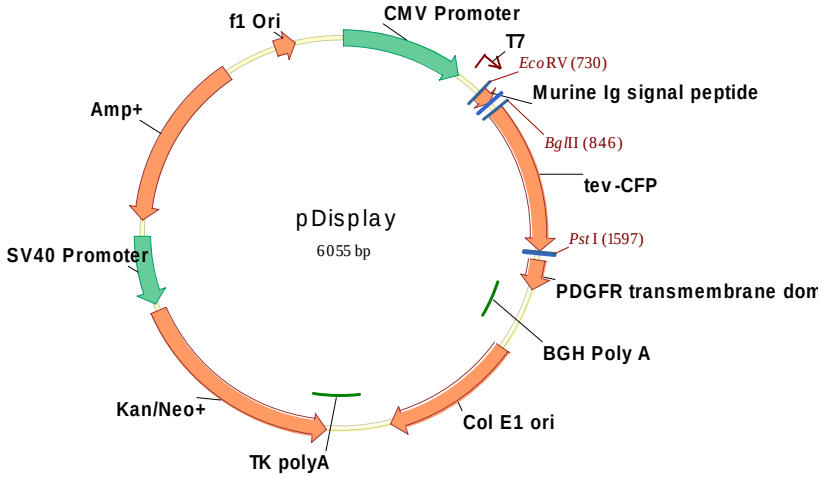


Figure S8. White light (**a**) and fluorescence (**b**) images of a gel loaded with solutions containing different ratios of fusion protein rLuc-diCys and CBT probe (peptide-FITC-CBT) and stained with Coomassie Blue. The purified rLuc-diCys (5 μM in PBS pH 8.0) was treated with different concentrations of peptide-FITC-CBT in the presence of TCEP (100 μM) at RT for 1 h in 16 μL (lane 1: 0 μM; lane 2: 1 μM; lane 3: 2.5 μM; lane 4: 5 μM; lane 5: 10 μM; lane 6: 25 μM; lane 7: 50 μM). (**c**) The efficiency of CBT reaction was calculated from the intensities of two bands in a gel (I: rLuc-diCys-CBT and II: rLuc-diCys). Fluorescence intensities of the bands were quantified by the gel analysis function in NIH ImageJ software.

7. In vivo protein labeling

7.1. Preparation of CFP-pDisplay and cell surface staining

The CFP-display fusion protein ENLYFQ↓C-CFP, contained TEV protease cleavage site (underlined) between N-terminus of the murine Ig κ-chain leader sequence and CFP, the C-terminus was fused with the platelet derived growth factor receptor (PDGFR) transmembrane domain. Both N- and C- termini domain ensured TEVP-CFP fusion protein was targeted on the extracellular side of the cell membrane. Upon TEV protease cleavage of the fusion protein, the N-terminus of the murine Ig κ-chain leader sequence in front of the cleavage site as indicated by the arrow will be released, and the CFP with N-terminus cysteine will be exposed. The CFP-display encoding fragment was amplified from plasmid CFP-pCDNA3.1 by PCR. The upstream primer, 5'-G GCC AGA TCT GAA AAC CTG TAT TTT CAG TGT GGT ACC GTG AGC AAG GGC G-3', contained a TEV protease cleavage site (underlined) between N-terminus of the murine Ig κ-chain leader sequence and CFP and provided a *Bgl*II site for cloning. The downstream primer, 5'-TC GTC GAC CTG CAG CTT GTA CAG CTC GTC CAT-3', contained a *Pst*I site for cloning. The PCR was carried out for 30 cycles at 94 °C for 40 sec, 57 °C for 40 sec, and 72 °C for 60 sec, followed by a final extension at 72 °C for 5 min. Amplified PCR fragments were purified with the Qiaquick PCR purification kit, restricted with BglII and PstI, and ligated into pDisplay™ (Invitrogen) vector.



7.2. Cell culturing and staining

Human epitheloid HeLa cells were cultured as monolayers in fresh growth medium (Dulbecco's Modified Eagle's Medium with 10% FBS and 1% penicillin /streptomycin) and grown at 37 °C in 5% CO_2. Cells were split on the poly-D-lysine coated coverslips one day before transfection and then transfected with CFP-pDisplay using Lipofectamine 2000 (4μg DNA/well of a 6-well plate). After 48 h at 37 °C, the media were removed and the cells were treated with 500 μL PBS containing 10 U of AcTEVTM protease and 2 μM of the rhodamine-CBT probe for 30 min at 37°C.

Thereafter, the cells were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) pH 7.4, once with growth medium.

7.3. Gel analysis of labeled cell lysates

The HeLa cells were harvested after 48 hours transfection by scraping into 1.5 mL of PBS. The cells were spun down at 300g for 5 min and were lysed by adding 50 µL of cell lysis buffer and incubating on ice for 30 min. After centrifuging at 10,000 g for 10 min (at 4 °C), the supernatant was collected and the total protein concentration of the cell lysate was quantified using the Bradford reagent assay. Lysate containing about 40 µg of protein were incubated with 10 µM of the probe in the presence or absence of TEV protease, and the excess of dye was removed with free cysteine before gel loading. Proteins were run on a 4-12% Bis-Tris gradient denaturing gel. The gel was stained with Coomassie Blue after the fluorescence image was taken.

7.4. Fluorescence imaging

Labeled cells were imaged in the same buffer on a Zeiss Axiovert 200M inverted epifluorescence microscope using a 60x oil-immersion lens. CFP (420/20excitation, 450 dichroic, 475/40 emission), rhodamine (545/30 excitation, 570 dichroic, 620/60 emission) and differential interference contrast (DIC) images were collected and analyzed using MetaMorph version 7.4 software (Molecular Devices). Fluorescence images were normalized to the same intensity range. Acquisition times ranged from 10-200 ms.

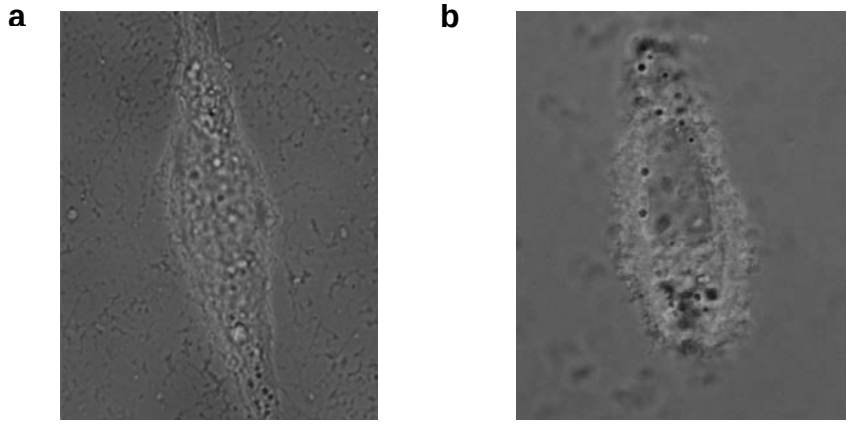


Figure S9. Differential Interference Contrast (DIC) images of cells (**a**) shown in Figure 4c and (**b**) of cells shown in Figure 4d.

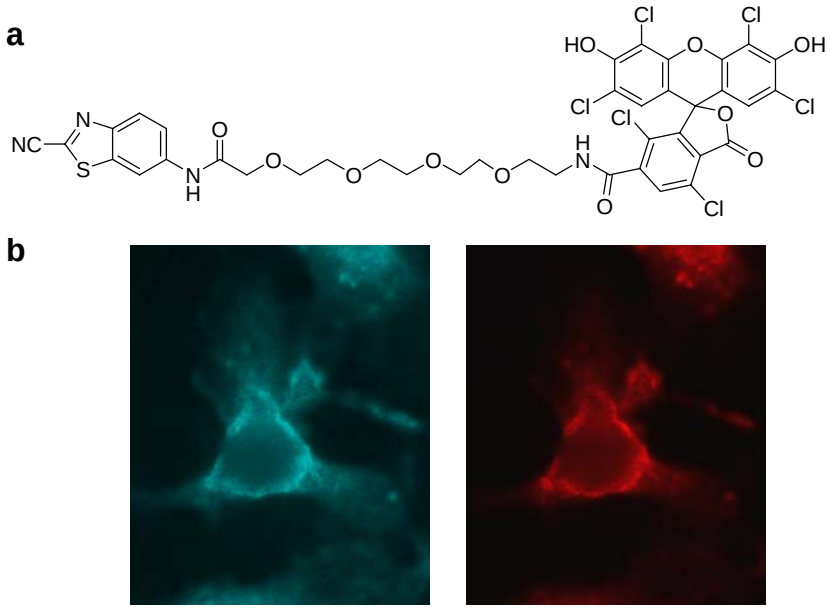


Figure S10. Specific labeling of membrane CFP in living COS-7 cells with the hexachloro FICT-CBT probe. (**a**) The structure of hexachloro FITC-CBT. (**b**) COS-7 cells transfected with tagged CFP and labeled with TEV protease and hexachloro FITC-CBT. Left: CFP fluorescence; Right: hexachloro FITC fluorescence.

8. Cytotoxicity of CBT

Cytotoxicity of CBT was determined by the MTT assay. HeLa cells were seeded into a 96-well plate at a concentration of $3x10^3$cells/well in 100 μL MEM medium with 10% FBS. Amino-CBT at different concentrations respectively, was added when cells were plated. Then, the cell cultures were incubated for 24, 48 hours at 37 °C and 5% CO_2. For dark cell survival assay, MTT assays were carried out to measure the proliferation of Hela cells. The optical density (OD) of the dissolved formazan crystals was measured at the wavelengths of 595 and 690 nm. The percentage of cell viability was calculated using the following equation: viability (%) = $OD_{\text{treatment group}} \times 100/OD_{\text{control group}}$, where OD = $OD_{595\text{-}690\ nm}$.

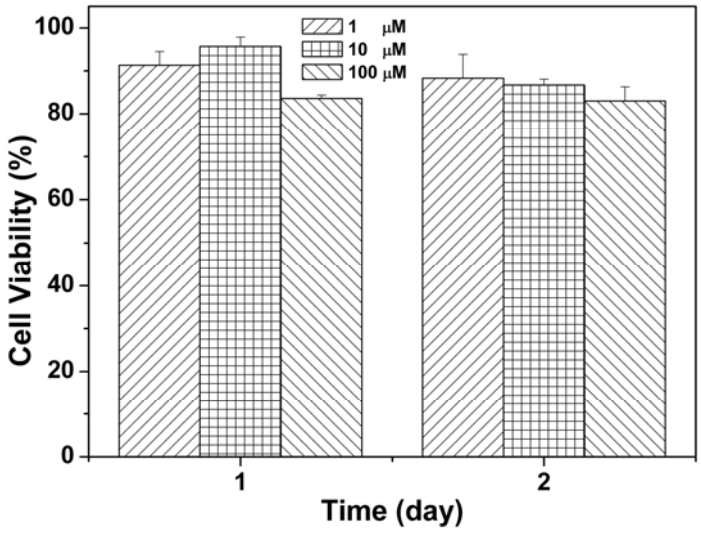


Figure S11. Cytotoxicity of amino-CBT in HeLa cells.

References

1. E. H. White, H. Wörther, H. H. Seliger, W. D. McElroy. *J. Am. Chem. Soc.* **1966**, *88*, 2015.
2. R. Shinde, J. Perkins, C. Contag. *Biochemistry* **2006**, *45*, 11103.
3. J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, C. R. Bertozzi. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16793.
4. A. M. Loening, T. D. Fenn, A. M. Wu, S. S. Gambhir, *Protein Eng. Des. Sel.* **2006**, *19*, 391.