Supplementary Information

Labeling Thiols on Proteins, Living Cells, and Tissues with Enhanced Emission Induced by FRET

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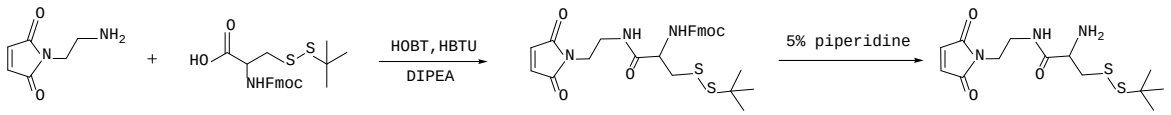
Contents:

1. Chemical syntheses and characterizations of Mal-Cys, CBT-GGG-FITC, and Luciferin-GGG-FITC

The preparations of compound **Mal-Cys**, **CBT-GGG-FITC**, and **Luciferin-GGG-FITC** were described as below; 2-cyano-6-aminobenzothiazole (CBT) was purchased from Shanghai Chemical Pharm-Intermediate Tech. Co. Ltd. (Shanghai, China).

*Synthesis of **Mal-Cys*:**

Scheme S1. Synthetic route for **Mal-Cys**.



A **Mal-Cys**

The mixture of N-(2-Aminoethyl)maleimide trifluoroacetate salt (25.4 mg, 0.1 mmol), HBTU (41.7 mg, 0.1 mmol), HOBT (13.5 mg, 0.1 mmol) in DMF (2 mL) was stirred for 30 min in presence of

DIPEA (11.1 mg, 0.1 mmol), then Fmoc-Cys(StBu)-OH (51.7 mg, 0.12 mmol) that dissolved in 1 mL of DMF was added into the mixture dropwise. After an overnight stirring, compound **A** (17 mg, yield: 76%) was obtained after HPLC purification. ^{1}HNMR of compound Maleimide-Cys(Fmoc)(StBu) (**A**) (d_6-DMSO, 300 MHz, Figure S1): 8.17 (t, J = 5.61 Hz, 1 H), 7.89 (d, J = 7.44 Hz, 2 H), 7.74 (d, J = 7.42 Hz, 2 H), 7.63 (d, J = 8.30 Hz, 1 H), 7.44 (t, J = 7.36 Hz, 2 H), 7.32 (t, J = 7.36 Hz, 2 H), 6.96 (s, 2 H), 4.32 (t, J = 11.31 Hz, 1 H), 4.22 (dd, J_1 = 6.38 Hz, J_2 = 4.93 Hz, 2 H), 4.13 (m, 1 H), 3.47 (s, 2 H), 3.15 (m, 2 H) , 2.91 (m, 2 H) , 1.29 (s, 9 H). MS: calculated for $C_{28}H_{32}N_3O_5S_2$ [$(M+H)^+$]: 554.17834; obsvd. HR-MALDI-TOF/MS: m/z 554.17051 (Figure S2). Deprotection of **A** with 10% piperidine in DMF (4 mL) at 0 °C for 20 min yielded compound **Mal-Cys** after HPLC preparation (10 mg, yield: 75 %). MS: calculated for $C_{13}H_{22}N_3O_3S_2$ [$(M+H)^+$]: 332.11026; obsvd. HR-GCT/MS: m/z 332.11029.

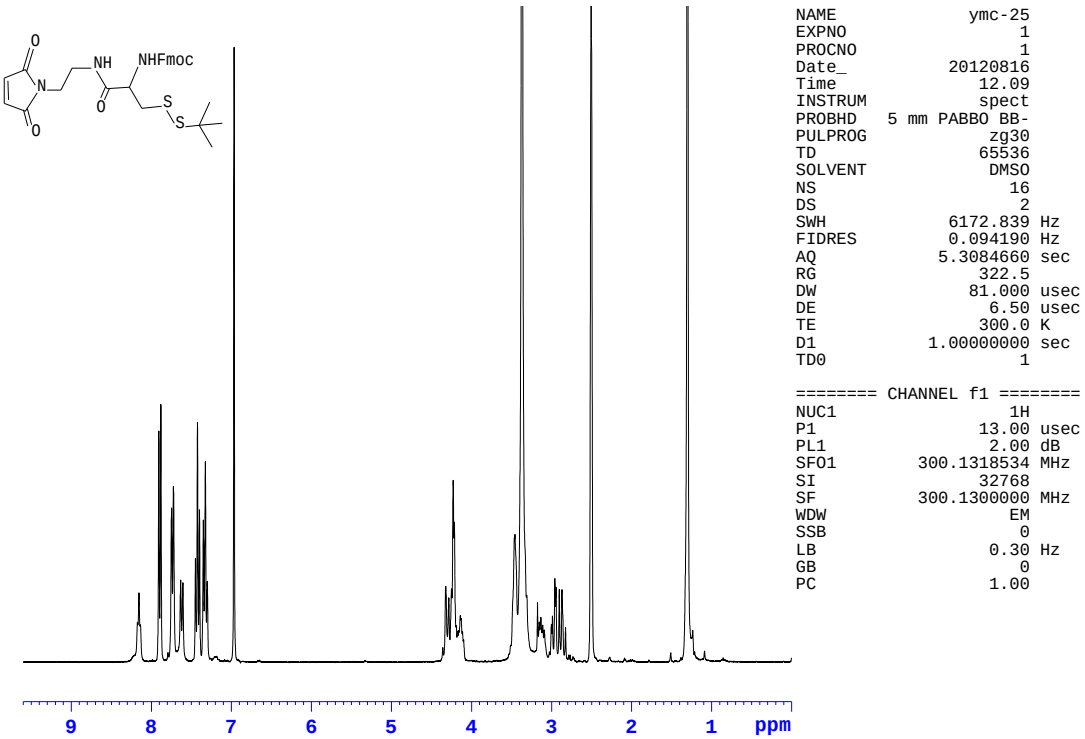



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Figure S1. ^{1}HNMR spectrum of compound Maleimide-Cys(Fmoc)(StBu) (**A**).

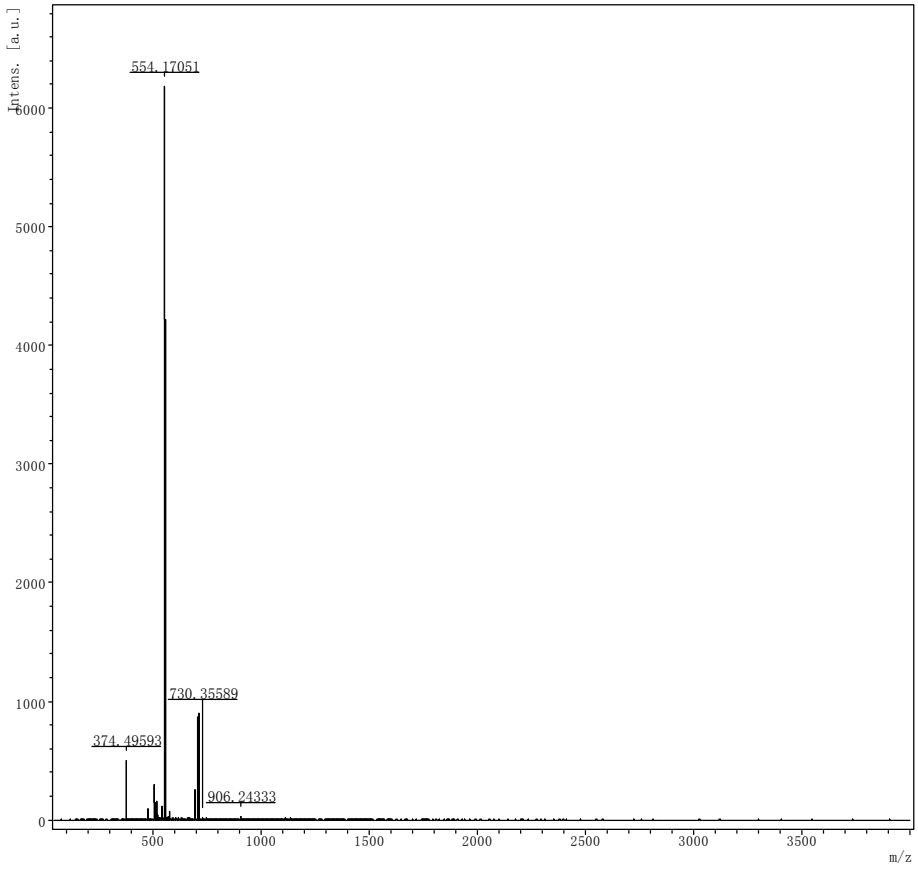
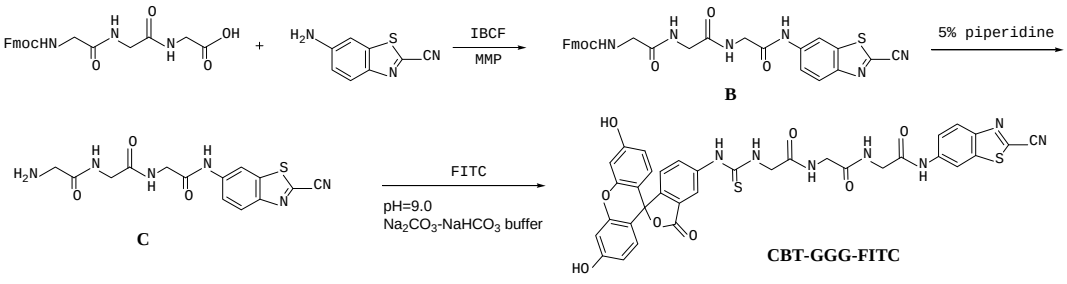


Figure S2. HR-MALDI-TOF/MS spectrum of Maleimide-Cys(Fmoc)(StBu) (**A**).

Synthesis of **CBT-GGG-FITC:**

Scheme S2. Synthetic route for **CBT-GGG-FITC**.



Peptide Fmoc- Gly-Gly-Gly-OH was prepared with solid phase peptide synthesis (SPPS). The

isobutyl chloroformate (ICBF, 19 mg, 0.14 mmol) was added to a mixture of Fmoc-Gly-Gly-Gly-OH (60 mg, 0.14 mmol) and 4-methylmorpholine (MMP, 74 mg, 0.7 mmol) in THF (1.5 mL) at 0 °C under N_2 and the reaction mixture was stirred for 20 min. The solution of 2-cyano-6-aminobenzothiazole (CBT, 28 mg, 0.16 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C then overnight at room temperature, and then purified by HPLC to yield compound **B** (30 mg, yield: 35%). The Fmoc protecting group of **B** was cleaved with 5% piperidine in DMF (4 mL) for 5 min at 0 °C, then 200 μL TFA was added to neutralize the alkaline, thus the compound **C** (25 mg, yield: 90%) was obtained after HPLC purification. Fluorescein isothiocyanate (FITC, 41 mg, 0.11 mmol) was dissolved in 100 μL of DMSO. Then the solution was dropped into the 2.5 mL Na_2CO_3-$NaHCO_3$ buffer in the presence of compound **C**. The desired compound **CBT-GGG-FITC** was obtained after HPLC purification (15 mg, yield: 50%). ^{1}HNMR of compound **CBT-GGG-FITC** (d_4-CH_3OH, 300 MHz, Figure S3): 8.66 (s, 1 H), 8.08 (s, 1 H), 8.01 (d, J = 9.2 Hz, 1H), 7.86 (d, J = 8.85 Hz, 1 H), 7.76 (d, J = 8.17 Hz, 1 H), 6.99 (d, J = 8.17 Hz, 1 H), 6.85 (s, 2 H), 6.78 (t, J = 10.44 Hz, 2 H), 6.69 (d, J = 8.12 Hz, 2 H), 4.12 (s, 2 H), 3.99 (s, 2 H), 3.67 (s, 2 H). MS: calculated for $C_{35}H_{26}N_7O_8S_2$ $[(M+H)^+]$: 736.12843; obsvd. HR-MALDI-TOF/MS: m/z 736.12060.

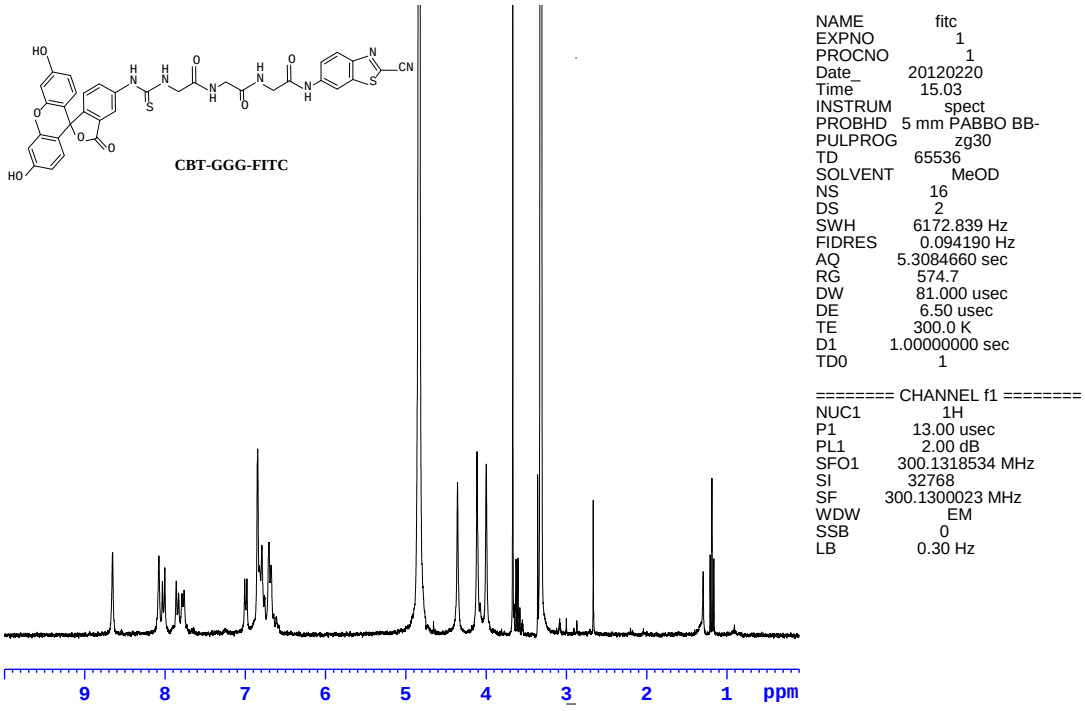
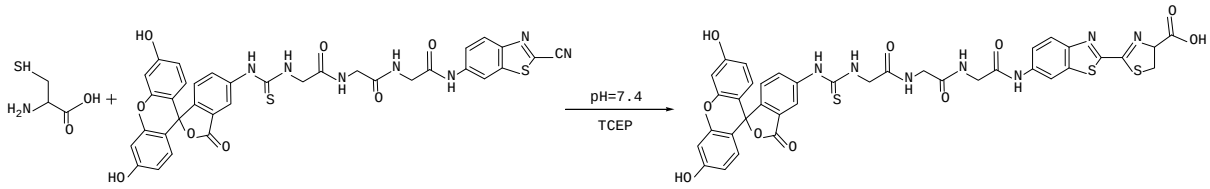


Figure S3. ^{1}HNMR spectrum of compound **CBT-GGG-FITC.**

Synthesis of ***Luciferin-GGG-FITC :***

Scheme S3. Synthetic route for **Luciferin-GGG-FITC.**



CBT-GGG-FITC **Luciferin-GGG-FITC**

The mixture of Cys (8 mM), **CBT-GGG-FITC** (8 mM, dissolved in 100 μL DMSO), and TCEP (16 mM) was dissolved in 3 mL PBS. Then the solution was stirred for 3 h at room temperature after its pH was adjusted to 7.4 by the addition of Na_2CO_3. The desired compound **Luciferin-GGG-FITC** was obtained after HPLC purification and sent for high-resolution mass analysis. MS: calculated for $C_{38}H_{29}N_7O_{10}S_3$ [M^+]: 839.11380; obsvd. HR-MALDI-TOF/MS: m/z 839.11380 (Figure S4).

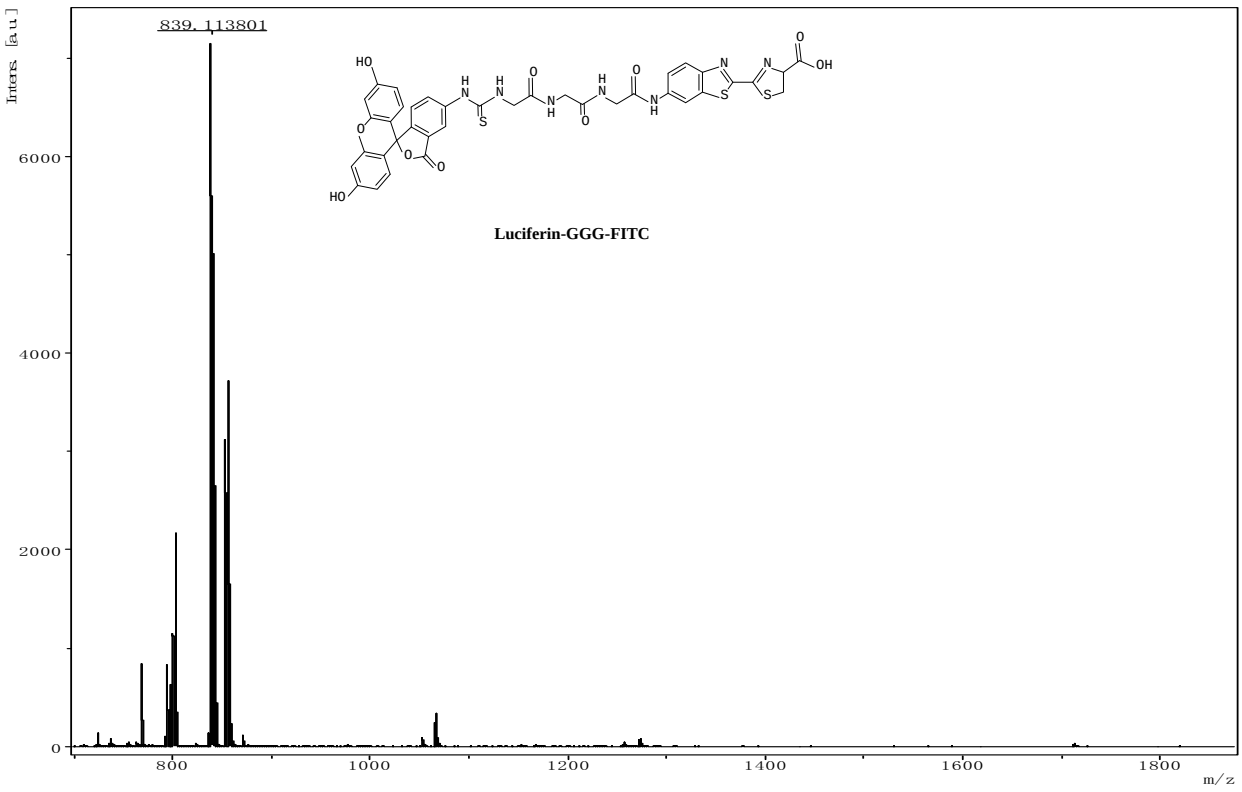
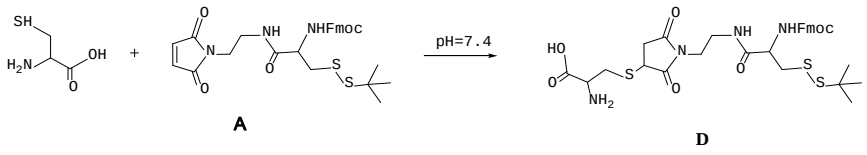


Figure S4. HR-MALDI-TOF/MS spectrum of **Luciferin-GGG-FITC.**

2. Model reaction of Cysteine with Maleimide-Cys(Fmoc)(StBu) (A)

Scheme S4. Model reaction of cysteine with **A.**



The mixture of L-Cysteine at 0.5 mM and Maleimide-Cys(Fmoc)(StBu) (**A**) at 1 mM (5% DMSO in PBS, pH 7.4) was stirred for 2 h at room temperature. Then the reaction mixture was injected into a HPLC system for purification and analysis (Figure S5). ^{1}HNMR of compound Cys-Maleimide-Cys(Fmoc)(StBu) (**D**) (d_6-DMSO, 300 MHz, Figure S6): 8.43 (s, 3 H), 8.18 (t, J =

5.53 Hz, 1 H), 7.89 (d, J = 7.41 Hz, 2 H), 7.75 (d, J = 7.29 Hz, 2 H), 7.63 (d, J = 8.25 Hz, 1 H), 7.43 (t, J = 7.44 Hz, 2 H), 7.32 (t, J = 7.35 Hz, 2 H), 4.35 (t, J = 11.25 Hz, 1 H), 4.24 (dd, J_1 = 6.36 Hz, J_2 = 4.25 Hz, 2 H), 4.05 (m, 1 H), 3.47 (t, J = 4.63 Hz, 2 H), 3.35 (m, 2 H), 3.25 (t, J = 4.93 Hz, 2 H), 3.14 (m, 2 H), 2.96 (m, 2 H), 2.55 (s, 2 H), 1.29 (s, 9 H). MS: calculated for $C_{31}H_{39}N_4O_7S_3$ $[(M+H)^+]$: 675.19809; obsvd. HR-MALDI-TOF/MS: m/z 675.19026 (Figure S7).

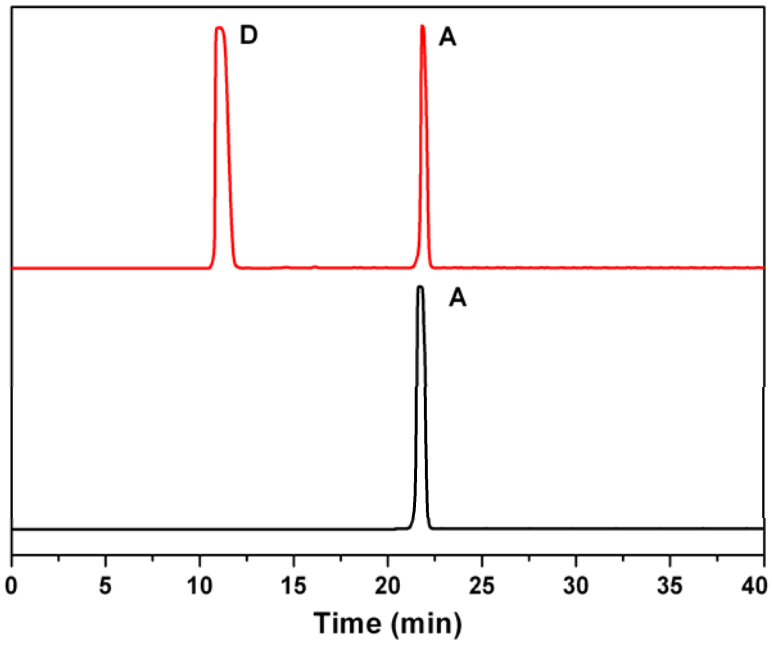


Figure S5. Lower: HPLC trace of Maleimide-Cys(Fmoc)(StBu) (**A**). Upper: HPLC trace of the reaction mixture of 1 mM of **A** with 0.5 mM of L-cysteine in PBS with 5% DMSO (pH 7.4) for 2 h at RT.

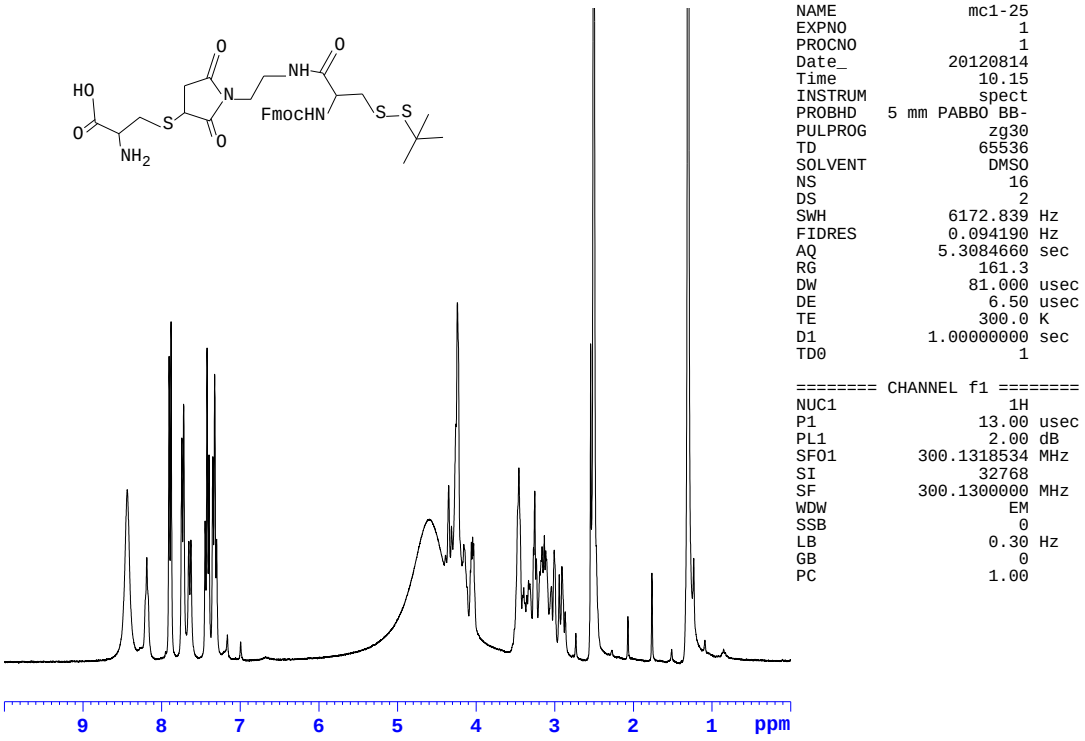



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Figure S6. ^{1}HNMR spectrum of compound Cys-Maleimide-Cys(Fmoc)(StBu) (**D**).

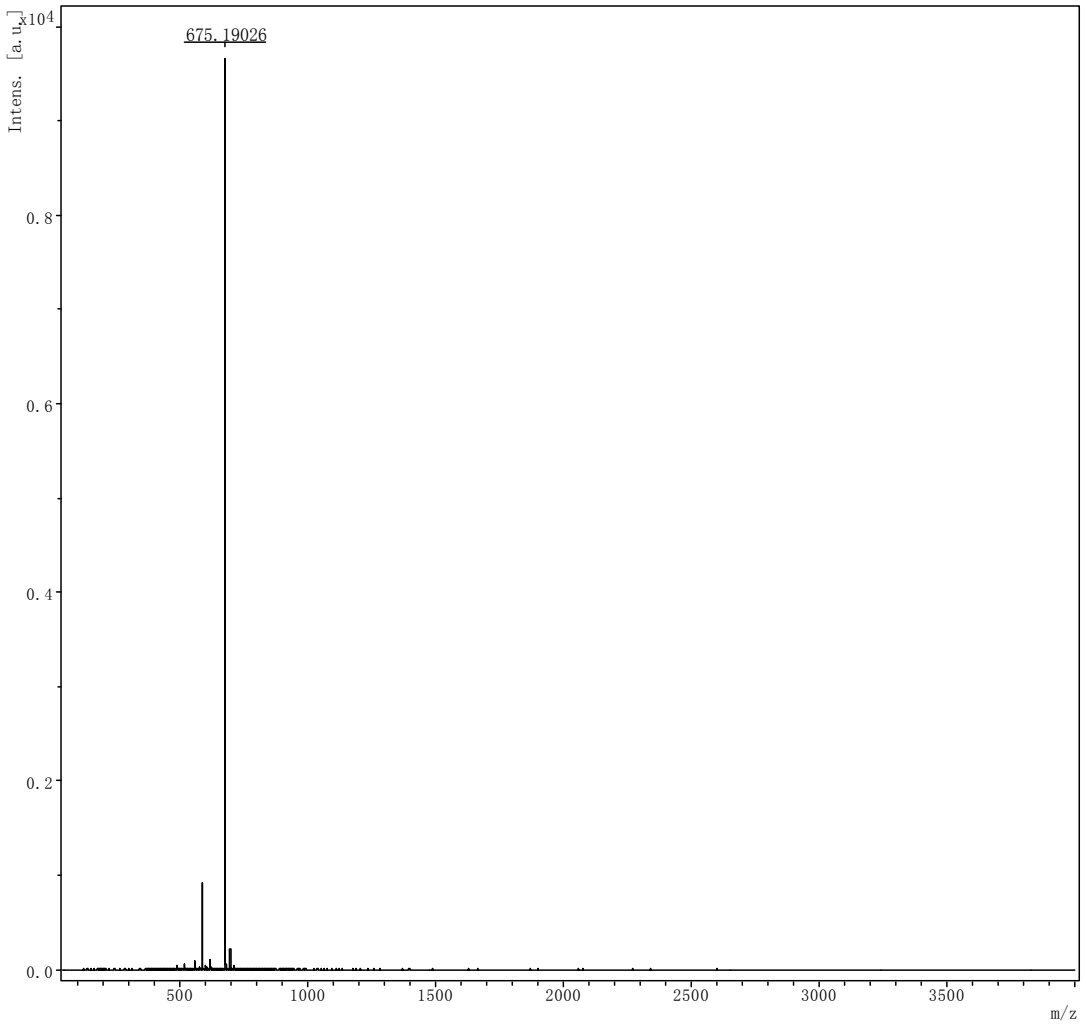
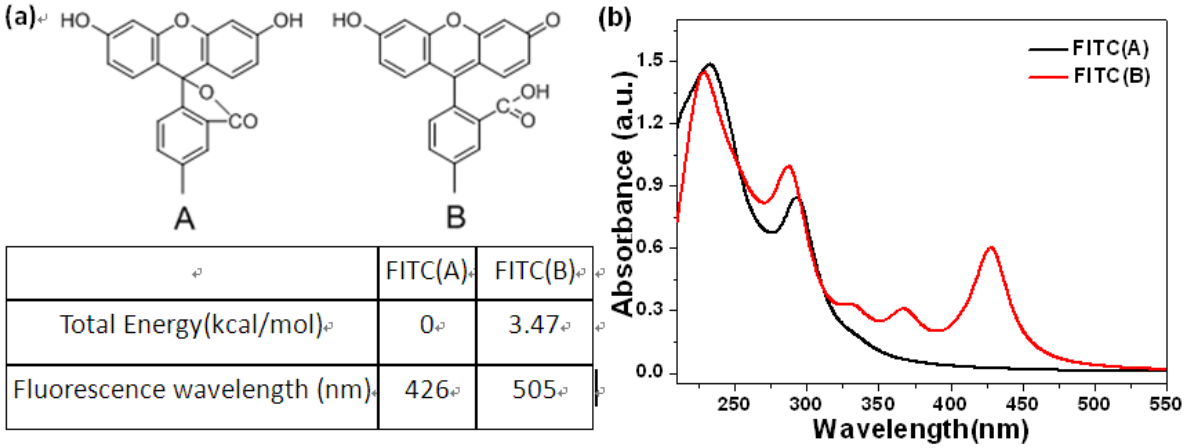


Figure S7. HR-MALDI-TOF/MS spectrum of Cys-Maleimide-Cys(Fmoc)(StBu) (**D**).

3. FRET mechanism



	FITC(A)	FITC(B)
Total Energy(kcal/mol)	0	3.47
Fluorescence wavelength (nm)	426	505

Figure S8. (a) The molecular structures, calculated total energies, and central fluorescence wavelengths for the two isomeric structures of FITC (i.e., FITC(A) and FITC(B)). (b) Calculated photon-absorption spectra of FITC(A) and FITC(B).

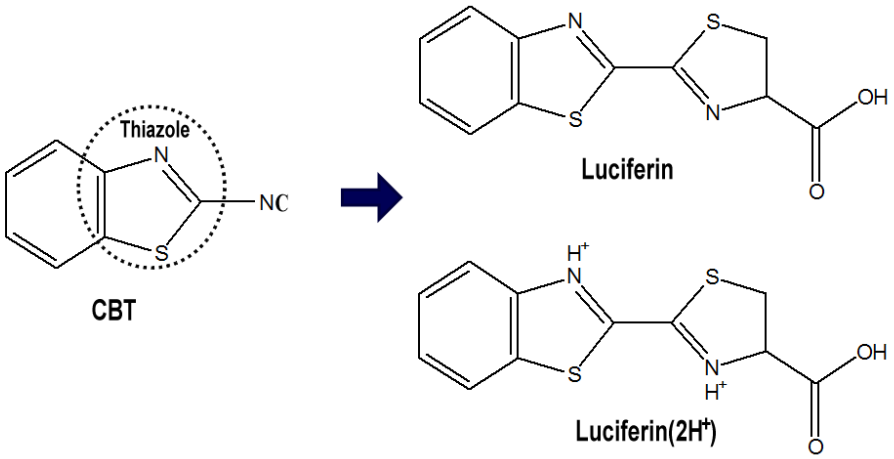


Figure S9. Molecular structures of CBT, Luciferin, and Luciferin($2H^+$).

Electronic Transitions:

From the electronic transition diagram (Figure S10 and S11), we can see the electronic wavefunction

distributions of CBT, Luciferin, and Luciferin($2H^+$) are almost identical. The energy differences of their excited states are responsible for their shifts of absorption and fluorescence wavelength.

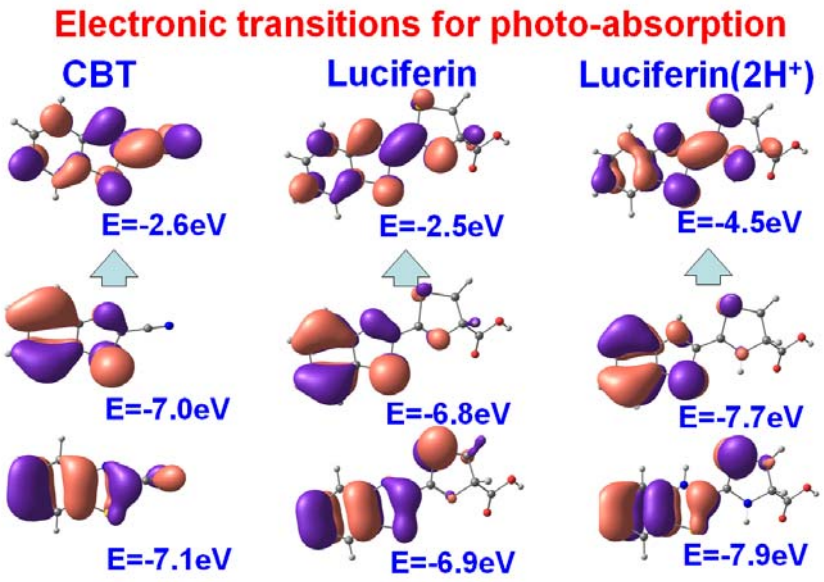


Figure S10. The computed wavefunction of frontier molecular orbits contributing to the photo-absorption of CBT, Luciferin, Luciferin($2H^+$) structures.

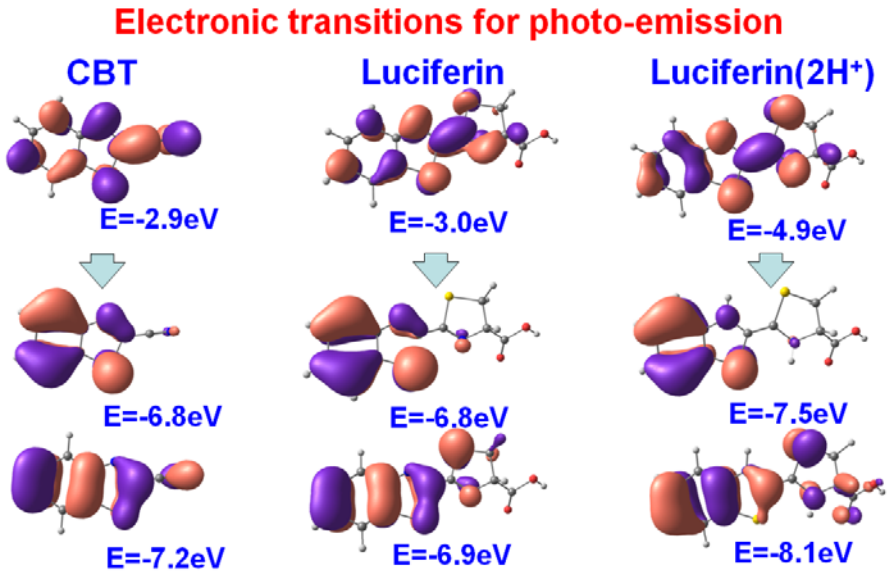


Figure S11. The computed wavefunction of frontier molecular orbits contributing to the

photo-emission of CBT, Luciferin, Luciferin($2H^+$) structures.

FRET formula:

Applying Fermi's golden rule, we may write the FRET rate (probability per unit time) from donor to acceptor:

$$K_{DA}=\sum_{i,j}\frac{2\pi}{\hbar}|W_{D_iA_j}|^2\int P(D_i^{'})P(A_j^{'})f_{D_i}^2 f_{Aj}^2\frac{\Gamma}{\pi\left[\left(E-E_{D_i}^{'}\right)^2+\Gamma^2\right]}\frac{\Gamma}{\pi\left[\left(E-E_{A_j}^{'}\right)^2+\Gamma^2\right]}f(x)dE \quad \text{(S1)}$$

where W_{DA} WDA is the purely electronic dipole-dipole matrix element, involving only electronic wavefunctions.

$$W_{DA}=\frac{\mu_m\cdot\mu_n}{|R_{mn}|^3}-3\frac{(\mu_m\cdot R_{mn})(\mu_n\cdot R_{mn})}{|R_{mn}|^5} \quad \text{(S2)}$$

Where P(D'i) P(A'j)=1, $\Gamma=\frac{1240}{430-15}-\frac{1240}{430+15}=0.2014eV=0.0074HF$, and probability density function $f(x)=\frac{1}{\sigma\sqrt{2\pi}}e^{-\frac{(x-\mu)^2}{2\sigma^2}}$, where σ is the beam width which describes the scope of excitation light's wavelength and μ is the beam center which is the wave crest of excitation light.

Above all, we can get a formula to calculate the rate of FRET:

$$K_{DA}=\sum_{i,j}\frac{4}{h}|W_{D_iA_j}|^2\int f_{D_i}^2 f_{Aj}^2\frac{\Gamma}{\left[\left(E-E_{D_i}^{'}\right)^2+\Gamma^2\right]}\frac{\Gamma}{\left[\left(E-E_{A_j}^{'}\right)^2+\Gamma^2\right]}f(x)dE \quad \text{(S3)}$$

As the excitation light whose wavelength is 470 nm~490 nm in experiment, we set

$$\mu_1=\frac{\frac{1240}{490}}{27.2114}=0.092998,\mu_2=\frac{\frac{1240}{470}}{27.2114}=0.0969556 \quad \text{(S4)}$$

,

then we can get a range of KDA:

$$K_{DA}\left(\text{CBT-GGG-FITC(B)}\right) = 8.1341*10^{10} \sim 5.2027*10^{10}$$
$$K_{DA}\left(\text{Luciferin-GGG-FITC(B)}\right) = 1.1929*10^{11} \sim 8.6479*10^{10}$$
$$K_{DA}\left(\text{Luciferin}(2H^{+})\text{-GGG-FITC(B)}\right) = 8.8896*10^{11} \sim 7.6620*10^{11} \quad \text{(S5)}$$

When excitation light's wavelength is 465nm,

$$K_{DA}\left(\text{CBT-GGG-FITC(B)}\right) = 9.134714*10^{10}$$
$$K_{DA}\left(\text{Luciferin-GGG-FITC(B)}\right) = 1.294885*10^{11}$$
$$K_{DA}\left(\text{Luciferin}(2H^{+})\text{-GGG-FITC(B)}\right) = 9.186197*10^{11} \quad \text{(S6)}$$

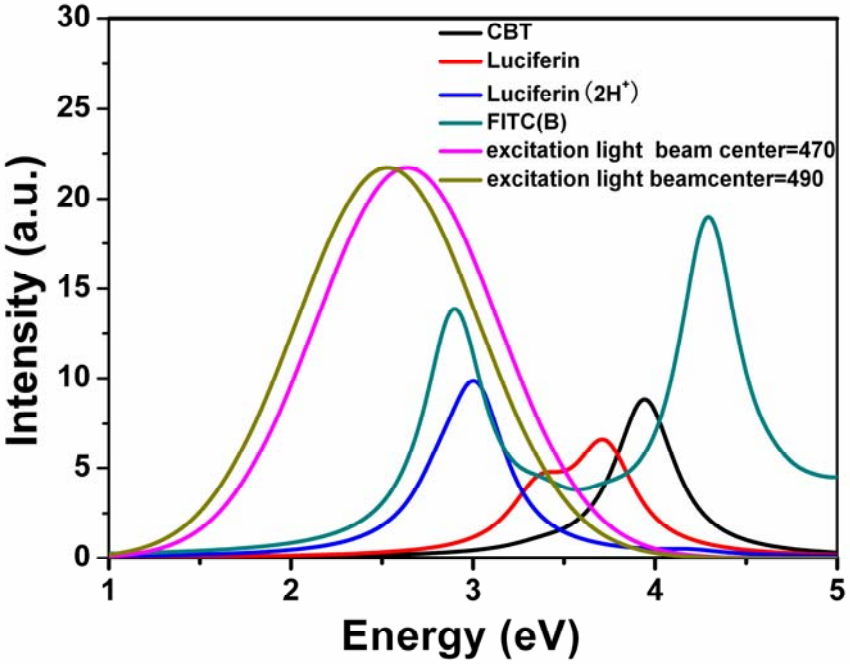


Figure S12. Transition probabilities as a function of electron energy for three donor groups of CBT, Luciferin, and Luciferin($2H^{+}$), and the acceptor group of FITC(B) for the FRET process at room temperature.

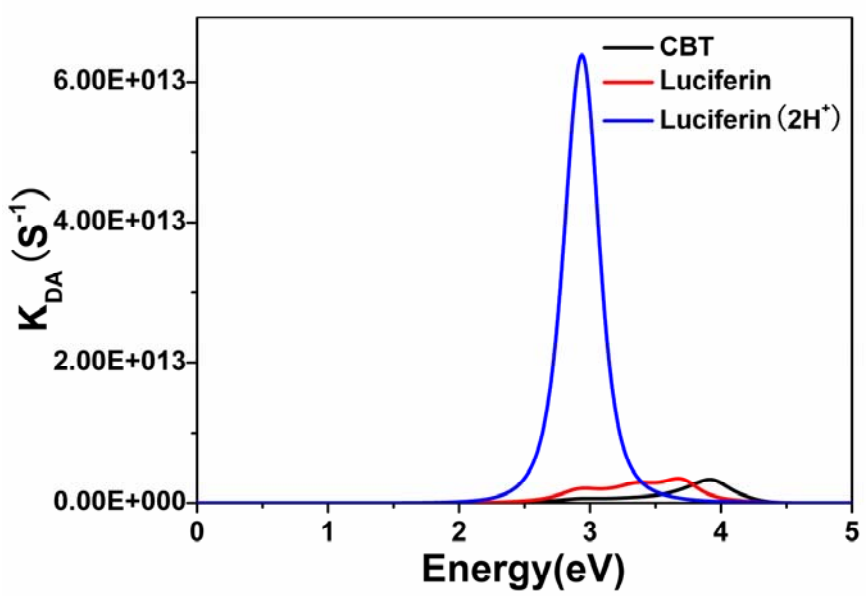


Figure S13. KDA's distributions as a function of the electron's energy for three donor groups of CBT, Luciferin, and Luciferin($2H^+$).

Figure S13 shows KDA's distribution along with the electron's energy change, the areas under the curves are $8.13*10^{10}$, $1.19*10^{11}$, $8.89*10^{11}$ for CBT, Luciferin, Luciferin($2H^+$) respectively, which fit the calculation above perfectly.

As was mentioned above, the percentage of **Luciferin-GGG-FITC(B)** and Luciferin($2H^+$)-GGG-FITC(B) is 35.6% and 64.4% respectively, we can get the increased ratio of FRET from CBT-GGG-FITC(B) to the mixture of **Luciferin-GGG-FITC(B)** and Luciferin($2H^+$)-GGG-FITC(B):

$$N = \frac{K_{DA}(\text{Luciferin-GGG-FITC(B)}) \times 0.356 + K_{DA}(\text{Luciferin}(2H^+)\text{-GGG-FITC(B)}) \times 0.644}{K_{DA}(\text{CBT-GGG-FITC(B)})}$$
$$= 7.56 \sim 10.08 \qquad \text{(S7)}$$

When excitation light's wavelength is 465 nm, N = 6.98, which means the FRET intensity

increases 6.98 times after the condensation reaction.

4. SDS-PAGE and gel imaging protocols

10 μL of above reaction mixture was mixed with 10 μL of sample buffer (3.55 mL de-ionized water, 1.25 mL of 0.5 M Tris-HCl (pH 6.8), 2.5 mL Glycerol, 2.0 mL 10 % (w/v) SDS, and 0.2 mL 0.5 % (w/v) bromophenol blue), heated at 100 ºC for 5 min. Then the mixture was applied to a 10% SDS-PAGE minigel and run at 100 mV for 40 min in stacking gel and 120 mV in running gel for 2 h until the blue front is at the bottom of the gel. Then the gel was removed from the cassette and the fluorescence images were taken under a gel imaging system with an excitation of 365 nm. Protocols of silver staining of above SDS-PAGE gels were from internet (e.g., Coachman, K., Ranish, J., Hanhn, S. Non-kit based Silver staining Method, http://labs.fhcrc.org/hahn/Methods/biochem_meth/silver_stain.html. Last modified 5/13/02). After being stained with silver, the gel was photographed under white light.

5. Protocol of outer cellular membrane imaging on living HepG-2 cells

We denoted step 1 as the incubation of 100 μM TCEP with cells for 15 min, step 2 as the incubation of 200 μM **Mal-Cys** with cells in culture medium without serum for 3 h at 37 °C, step 3 as the incubation of 100 μM TCEP and 15 μM **CBT-GGG-FITC** with cells in culture medium without serum for 1 h at 37 °C. The cells were washed with PBS for three times prior to imaging.

Optimum concentration of TCEP at step 3 for cellular outer membrane imaging

At step 3, the incubation time for incubation of TCEP and **CBT-GGG-FITC** with cells was 1 h at 37 ºC. To avoid the breakage of cell membrane, an appropriate concentration of TCEP for incubation was studied. If the amount of TCEP is too low, the disulfide bond of **Mal-Cys** would not be reduced sufficiently, inducing the condensation reaction incompleted. As shown in Figure S14, when the concentration of TCEP was increased from 10 μM to 100 μM, the intensity of cellular membrane labeling was enhanced. At 150 μM, fluorescence emission of **CBT-GGG-FITC** from the interior of cells was observed, indicating the breakage of cell membrane by high concentration of TCEP. Therefore, 100 μM is chosen as the optimized concentration for TCEP at step 3.

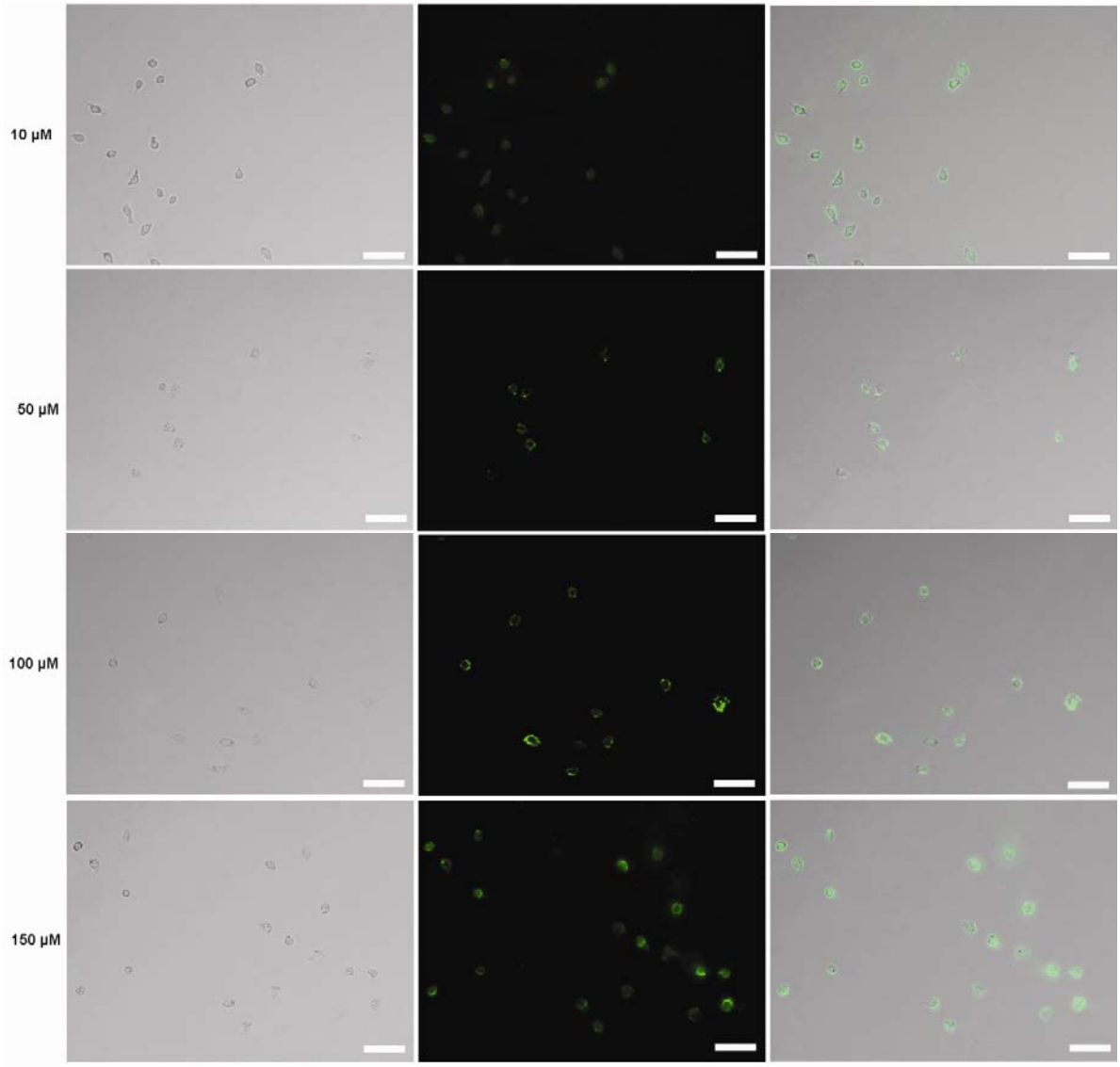


Figure S14. Microscopic images of HepG-2 cells after treatment of step 1, step 2, and at step 3 incubated with different concentrations of TCEP and 15 μM **CBT-GGG-FITC** with cells in culture medium without serum for 1 h at 37 °C. Left: DIC image. Middle: fluorescence image (EGFP channel). Right: an overlay of DIC and fluorescence images. Scale bar: 80 μm.

Optimum concentration of CBT-GGG-FITC at step 3 for cellular outer membrane imaging

An appropriate concentration of **CBT-GGG-FITC** is crucial for cellular outer membrane labeling. If

the concentration is too low, the outer membrane could not be stained clearly. If it is too high, excessive **CBT-GGG-FITC** will penetrate into the cells. In our study, four different concentrations of **CBT-GGG-FITC** (5, 10, 15, and 20 μM) were used at step 3, following step 2 that the cells were treated with 200 μM of **Mal-Cys**. As shown in Figure S15, when the concentration of **CBT-GGG-FITC** was lowered down to 5 μM, there was not enough probe for conjugating with the reduced **Mal-Cys** at step 2, consequently the staining was too unconspicuous to clearly display a circular shape of the outer membrane of a cell. From 10 to 15 μM, fluorescence imaging of the cells showed an obvious outer membrane staining and the intensity of fluorescence increases as the concentration of **CBT-GGG-FITC** increases, suggesting the reduced **Mal-Cys** at step 2 is excessive for conjugating with the probe. However, when the concentration of **CBT-GGG-FITC** was increased at 20 μM, we observed a uniform staining of the cells and the circular fluorescence emission disappeared, suggesting this concentration is too high for **Mal-Cys** conjugation. Therefore 15 μM was selected as the optimized concentration of **CBT-GGG-FITC** for staining.

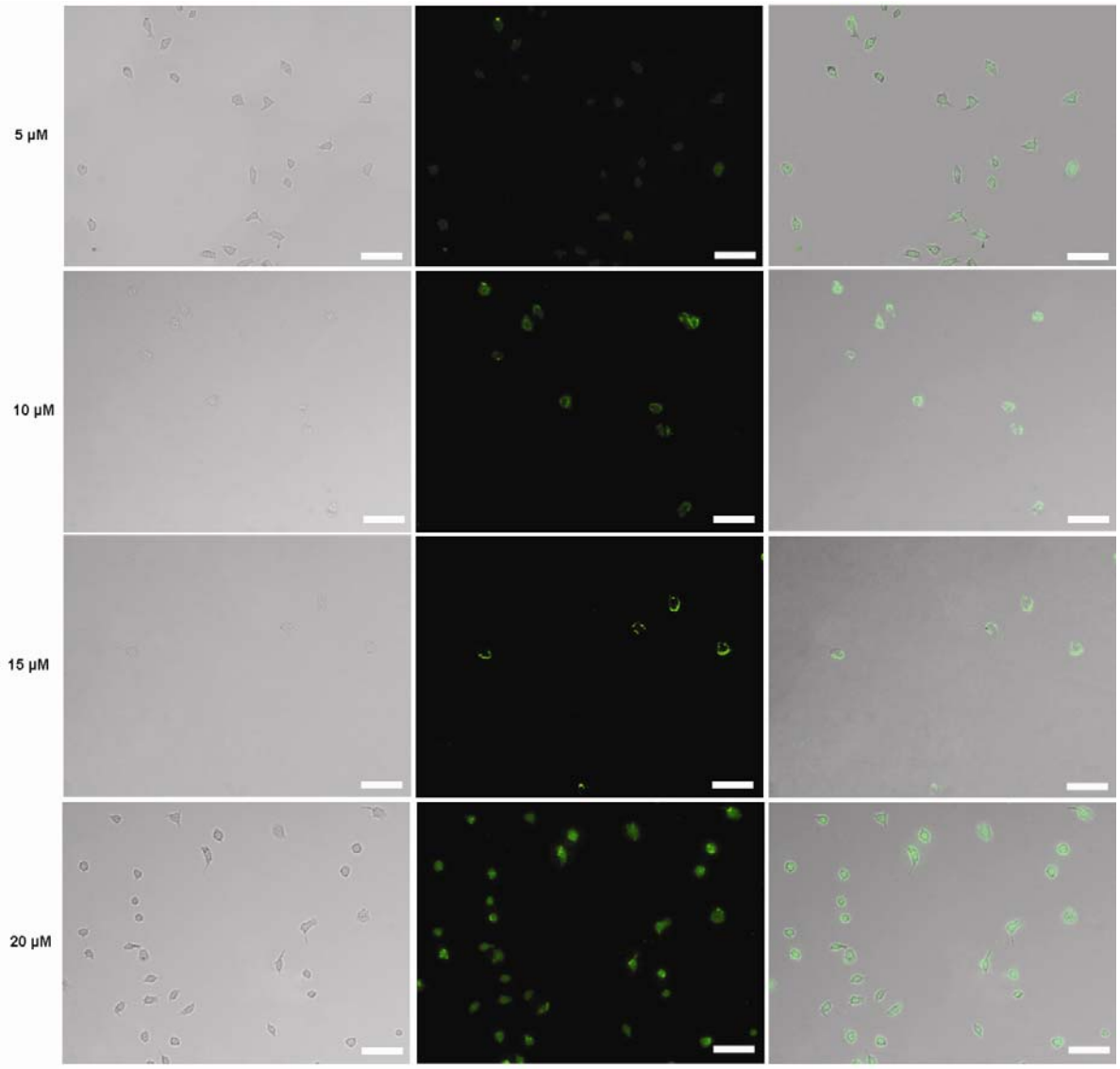


Figure S15. Microscopic images of HepG-2 cells after treatment of step 1, step 2, and at step 3 incubated with 100 μM TCEP and different concentrations **CBT-GGG-FITC** in culture medium without serum for 1 h at 37 °C. Left: DIC image. Middle: fluorescence image (EGFP channel). Right: an overlay of DIC and fluorescence images. Scale bar: 80 μm.

Optimum incubation time of CBT-GGG-FITC at step 3 for cellular outer membrane imaging

To obtain appropriate incubation time for outer membrane imaging of living HepG-2 cells at step 3, we studied four different time slots (0.5, 1, 2, and 4 h) for incubating 15 μM **CBT-GGG-FITC** and 100 μM TCEP with cells. Results indicated the condensation reaction between CBT motif and N-terminal Cys motif can be realized within 0.5 h (Figure S16). With the prolongation of incubation time (1 h and 2 h), an obvious enhancement of the fluorescence emission from the outer membranes of cells was observed. But when the incubation time was increased to 4 h, fluorescence emission from the interior of the cells was observed, indicating the endocytosis of **CBT-GGG-FITC** by the cells. Herein, 1 h was selected as the optimal incubation time at step 3.

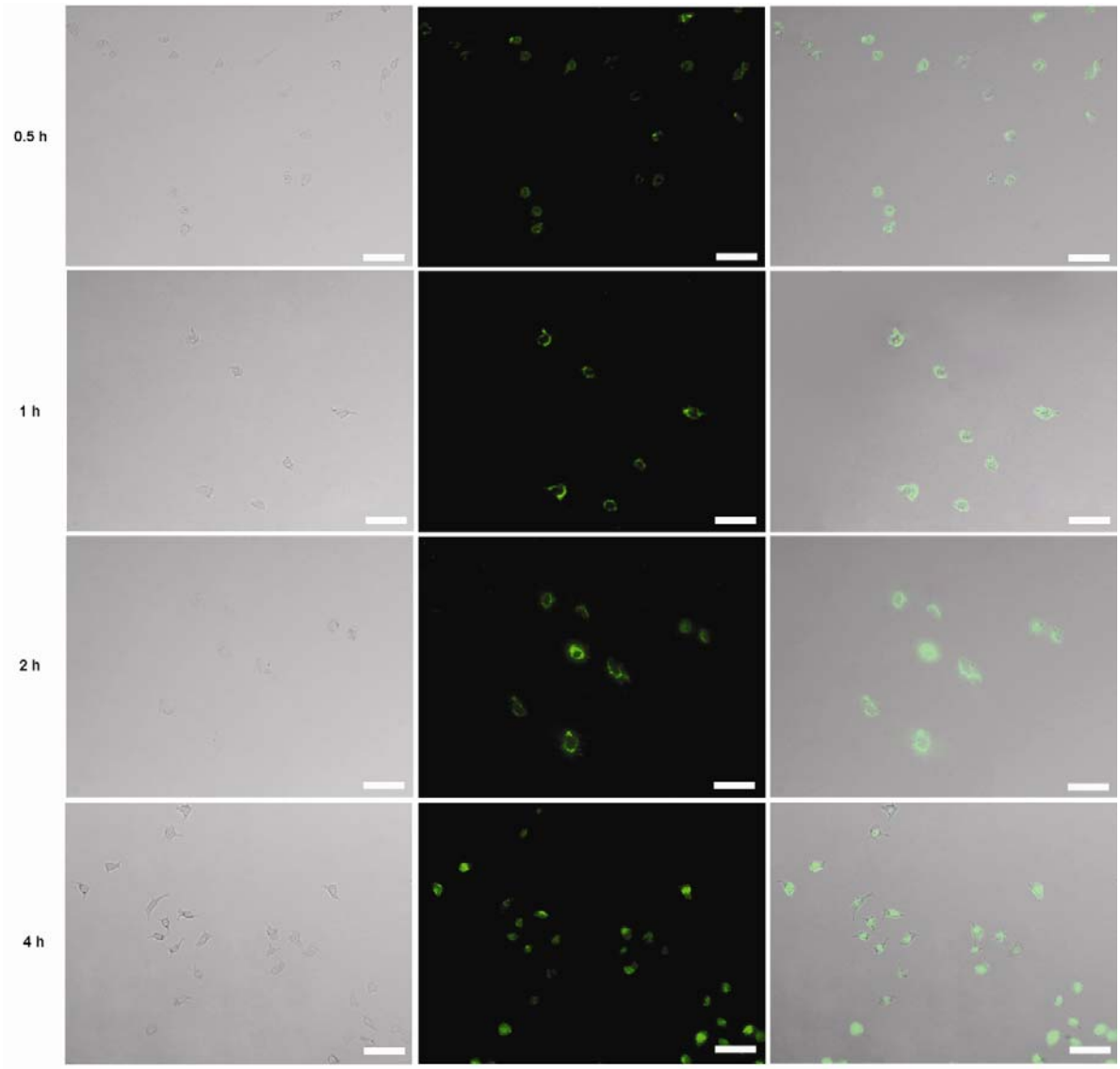


Figure S16. Microscopic images of HepG-2 cells after treatment of step 1, step 2, and at step 3 with 100 μM TCEP and 15 μM **CBT-GGG-FITC** in culture medium without serum for different incubating time at 37 °C. Left: DIC image. Middle: fluorescence image (EGFP channel). Right: an overlay of DIC and fluorescence images. Scale bar: 80 μm.

Optimum temperature for cellular outer membrane imaging at step 3

As the temperature is an important factor for cellular imaging. Herein we used three temperatures (20, 28, and 37 ºC) for the incubation at step 3 to study the temperature-dependent effect on cellular outer membrane imaging (Figure S17). At 20 ºC, besides the staining of cellular outer membrane, a faint staining of the interior of cells could be observed, indicating that the endocytosis of the probe **CBT-GGG-FITC** by cells is comparative to the condensation reaction between the probe and **Mal-Cys** at step 2. With the rising of the temperature (28 ºC and 37 ºC), cellular outer membrane imaging becomes clearer, suggesting the condensation reaction dominate the labeling of the probe and the endocytosis of the probe is neglectable. Since 37 ºC is the temperature widely used for cellular imaging and *in vivo* study, we chose 37 ºC for this study.

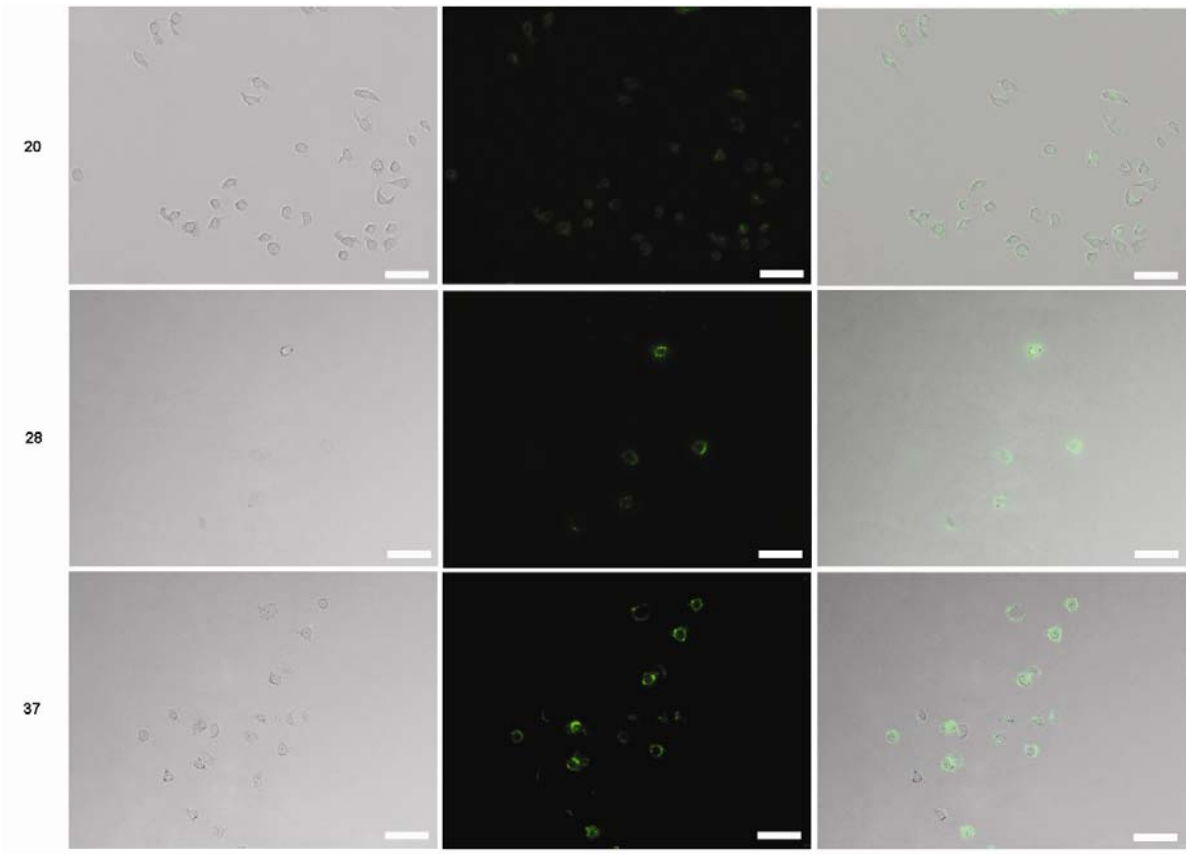


Figure S17. Microscopic images of HepG-2 cells after treatment of step 1, step 2, and at step 3 with 100 μM TCEP and 15 μM **CBT-GGG-FITC** in culture medium without serum for 1 h at different temperature (in °C). Left: DIC image. Middle: fluorescence image (EGFP channel). Right: an overlay of DIC and fluorescence images. Scale bar: 80 μm.

Optimum concentration of Mal-Cys for cellular outer membrane imaging at step 2

As a medium, the concentration of **Mal-Cys** is important for converting the thiols on the cell membrane into N-terminal cysteines and trapping the probe **CBT-GGG-FITC** on the cell membrane thereafter. Four concentrations of **Mal-Cys** (2 μM, 20 μM, 200 μM, and 1 mM) were chosen for study at step 2. As shown in Figure S18, when the concentration of **Mal-Cys** was 2 μM, smaller than the concentration of **CBT-GGG-FITC** used at step 3 (i.e., 15 μM), we observed uniform cellular imaging probably due to the excessive **CBT-GGG-FITC** entering into the cells. When the concentration of **Mal-Cys** was increased to 20 μM and 200 μM respectively, clear and bright cellular membrane staining was observed due to the **CBT-GGG-FITC** at step 3 (10 μM) being successfully trapped by the **Mal-Cys** modified on the cell membrane. Interestingly, when the concentration of **Mal-Cys** at step 2 reaches 1 mM, bright fluorescence emissions were observed not only from outer cellular membrane but also from the location of the nuclear. This might be explained as the high concentration of **Mal-Cys** leads to the property change of the cellular membrane (probably more leaky) and the absorption of **CBT-GGG-FITC** on the nuclear thereafter. Therefore, we choose 200 μM as the concentration of **Mal-Cys** used at step 2 for a promising cellular outer membrane labeling.

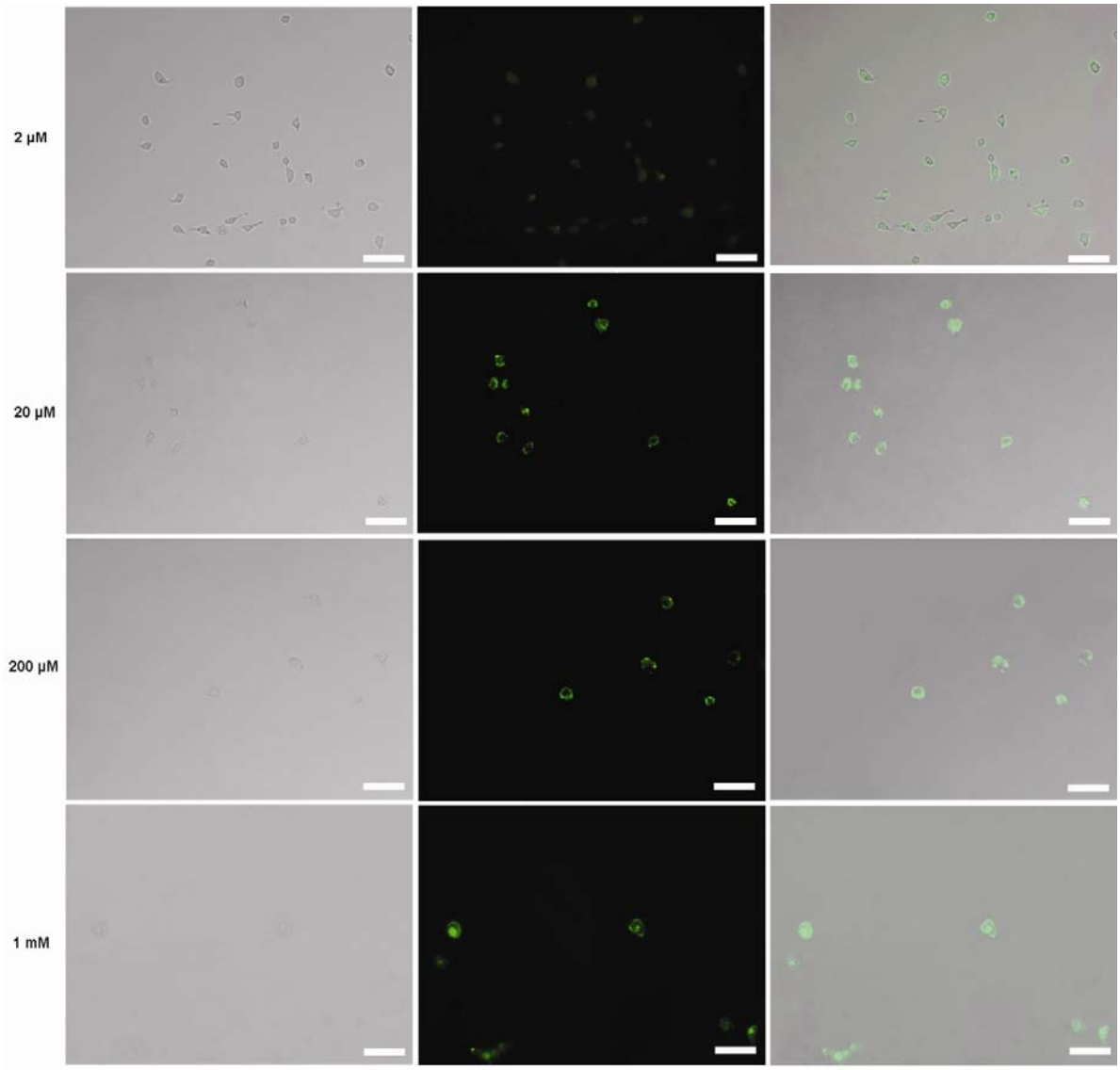


Figure S18. Microscopic images of HepG-2 cells after treatment of step 1, incubation of different concentrations of **Mal-Cys** with cells in culture medium without serum for 3 h at 37 °C at step 2, and at step 3 incubation with 100 μM TCEP and 15 μM **CBT-GGG-FITC** with cells in culture medium without serum for 1 h at 37 °C. Left: DIC image. Middle: fluorescence image (EGFP channel). Right: an overlay of DIC and fluorescence images. Scale bar: 80 μm.

Background interference test

Living HepG-2 cells were treated with step 1, step 2, and step 3 but without **CBT-GGG-FITC,** and then the cells were washed with PBS three times prior to imaging under a fluorescence microscope. As shown in Figure S19, no fluorescence signal was observed which suggests the background did not cause any interference.

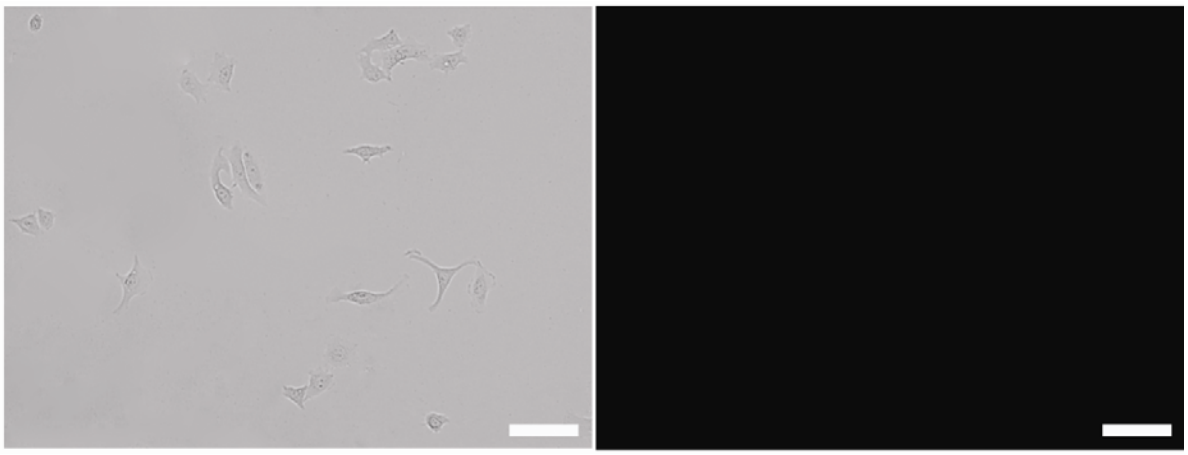


Figure S19. Left: differential interference contrast (DIC) image of HepG-2 cells treated with step 1, step 2, and step 3 but without **CBT-GGG-FITC**. Right: fluorescence image (EGFP channel) of HepG-2 cells in left. Scale bar: 80 μm.

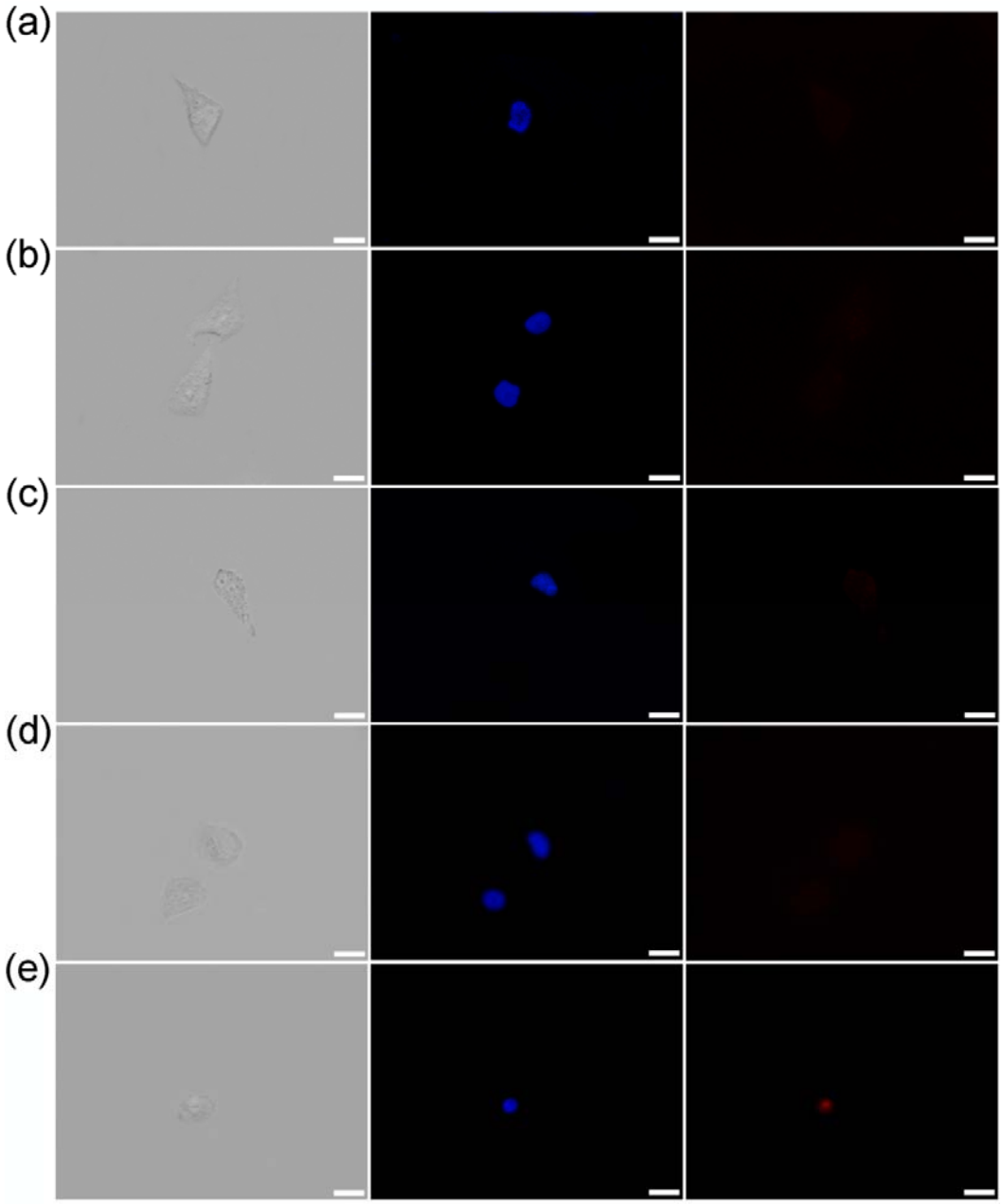


Figure S20. (a-d) Left: DIC images corresponding to Figures 4a-d. Middle: fluorescence images (DAPI channel). Right: fluorescence images (Texas Red channel). Scale bar: 20 μm. (e) Microscopic images of HepG-2 cells after treating with 3 mM H_2O_2 in culture medium without serum for 1 h at 37 °C. Left: DIC image. Middle: fluorescence image (DAPI channel). Right: fluorescence image (Texas Red channel). Scale bar: 20 μm.

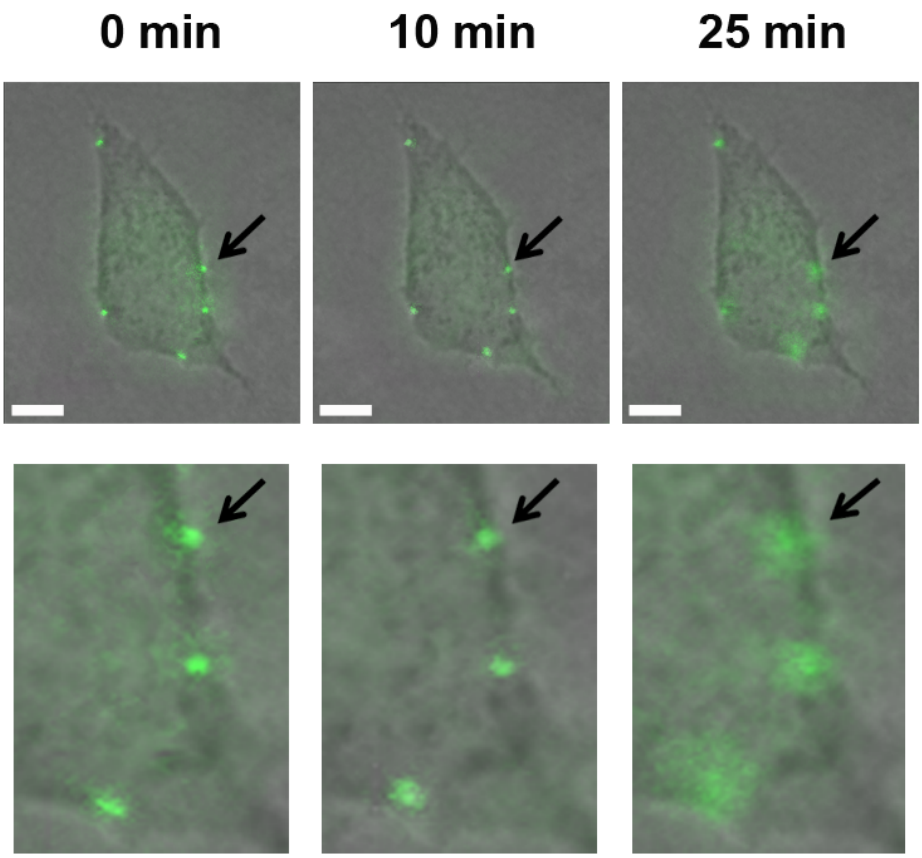


Figure S21. Labeling thiols on the outer membrane of living HepG-2 cells and time course of the translocation of membrane proteins. Arrows indicate the cell membrane translocation of the labeled proteins. Scale bar: 5 μm.

6. Supplementary tables

Table S1. Calculated FRET rate K_{DA} of three Donor-Acceptor systems: **CBT-GGG-FITC**(B), **Luciferin-GGG-FITC**(B), and Luciferin($2H^+$)-GGG-FITC(B).

	CBT-GGG-FITC(B)	Luciferin-GGG-FITC(B)	Luciferin($2H^+$)-GGG-FITC(B)
K_{DA} (10^9 1/s)	91.35	129.49	918.62

Table S2. HPLC condition for the purification of compound **Mal-Cys**.

Time (minute)	Flow (mL/min.)	H_2O %	CH_3OH %
0	3.0	70	30
3	3.0	70	30
35	3.0	10	90
37	3.0	10	90
38	3.0	70	30
40	3.0	70	30

Table S3. HPLC condition for the purification of compound **B** and **C**.

Time (minute)	Flow (mL/min.)	H_2O %	CH_3OH %
0	7.0	30	70
3	7.0	30	70
35	7.0	0	100
37	7.0	0	100
38	7.0	30	70
40	7.0	30	70

Table S4. HPLC condition for the purification of **CBT-GGG-FITC** and **Luciferin-GGG-FITC**.

Time (minute)	Flow (mL/min.)	H_2O %	CH_3OH %
0	3.0	50	50
3	3.0	50	50
35	3.0	5	95
37	3.0	5	95
38	3.0	50	50
40	3.0	50	50

Table S5. HPLC condition for the analysis of model reaction of **A**.

Time (minute)	Flow (mL/min.)	H_2O %	CH_3CN %
0	3.0	60	40
3	3.0	60	40
35	3.0	10	90
37	3.0	10	90
38	3.0	60	40
40	3.0	60	40