Supporting Information

A Visible and Near-infrared Dual-Fluorescence Probe for Discrimination of Cys/Hcy and GSH and Its Application in Bioimaging

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Experiments

1 Reagents and instruments

4-Chloro-7-nitro-1, 2, 3-benzoxadiazole (NBD-Cl) and 1, 2-Ethanedithiol were purchased from TCI Development Co. Ltd. (Shanghai, China). Amino acids were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Hoechst 33342 and Mito-Tracker Red were purchased from KeyGen BioTech (Jiangsu, China). 3-(4, 5-Dimethylthialzol-2-yl)-2, 5-diphe-nyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-EDTA were purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS (China). All other chemicals were supplied by J&K Scientific Ltd. (Beijing, China) or Sinopharm chemical reagent Co. Ltd. (Shanghai, China) and utilized without further purification.

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker Advance Ⅲ-300 instrument (Bruker, Billerica, MA, USA) at 400 MHz for ^{1}H NMR and at 600 MHz for ^{13}C NMR, and TMS was utilized as an internal reference. Mass spectra were collected on tandem quadrupole mass spectrometer (Waters, Milford, MA, USA) with ESI resource respectively. Absorption spectra were recorded on Hitachi U-3310 spectrophotometer (Hitachi Tokyo, Japan), and fluorescence spectra were obtained by using Hitachi F-4700 spectrophotometer (Hitachi Tokyo, Japan) and PerkinElmer-LS55 spectrophotometer (PerkinElmer, USA).

2 Synthesis of Cy1, Cy2

Synthesis of Cy1:

Cy7-Cl (668 mg, 1 mmol, 1 equiv.) was dissolved in anhydrous methanol (40 mL), and sodium sulfide (118 mg, 1.5 mmol, 1.5 equiv.) were added in nitrogen protected environment. Then the solution was stirred at 30 °C for 15 min in the dark. NBD-Cl (200 mg, 1 mmol, 1 equiv.) in MeOH (20 mL) was then added dropwise within 10 min in nitrogen protected environment. The mixture was stirred at r.t. for another 30 min in dark. The reaction was monitored by thin-layer chromatography (TLC). After the reaction, the solvent was removed and the obtained crude product was purified by

the column chromatography (SiO_2, CH_2Cl_2:CH_3OH = 60:1, v/v) to acquire the desired green solid powder (322 mg, 39 % yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.61 (d, *J* = 8.0 Hz, 1 H, ArH), 8.48 (d, *J* = 12.0 Hz, 2 H, ArH), 7.54 (d, *J* = 8.0 Hz, 1 H, ArH), 7.35 (t, *J* = 4 Hz, 2 H, ArH), 7.21 - 7.24 (m, 4 H, ArH), 7.10 (d, *J* = 8.0 Hz, 2 H, ArH), 6.38 (d, *J* = 12.0 Hz, 2 H, –CH=CH–), 4.15 (d, *J* = 32.0 Hz, 4 H), 3.16 (s, 2 H), 2.83 (s, 2 H), 1.89 (dt, *J* = 14.5, 7.3 Hz, 6 H), 1.62 (s, 12 H), 1.06 (t, *J* = 8.0 Hz, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 172.36, 148.89, 143.96, 143.38, 142.38, 141.94, 140.76, 137.98, 132.61, 132.38, 128.73, 125.50, 125.18, 122.10, 111.17, 102.74, 49.09, 46.37, 26.86, 20.90, 20.47, 11.62. ESI-MS calculated for $[M\text{-}I^-]^+$ 700.3316, found 700.03 (Figures S1 - S3).

Synthesis of Cy2:

Cy7-Cl (268 mg, 0.4 mmol, 1 equiv.) was dissolved in anhydrous N, N-dimethylformamide (3 mL), 1, 2-Ethanedithiol (40 mg, 0.32 mmol, 0.8 equiv.) and Et_3N were added in nitrogen protected environment in order. Then the solution was stirred at room temperature for 0.5 h in the dark. NBD-Cl (80 mg, 0.4 mmol, 1 equiv.) in DMF (2 mL) was then added slowly in nitrogen protected environment. The mixture was reacted for another 4 h at 60 °C in dark. The reaction was monitored by thin-layer chromatography (TLC). After the reaction, the solvent was removed and the obtained crude product was purified by the column chromatography (SiO_2, CH_2Cl_2:CH_3OH = 50:1, v/v) to acquire the desired green solid powder (96 mg, 27 % yield). ^1H NMR (400 MHz, $CDCl_3$) *δ* 8.79 (d, *J* = 12.0 Hz, 2 H, ArH), 8.59 (d, *J* = 4.0 Hz, 1 H, ArH), 7.94 (d, *J* = 8.0 Hz, 1 H, ArH), 7.38 (t, *J* = 8.0 Hz, 4 H, ArH), 7.23 (d, *J* = 8.0 Hz, 2 H, ArH), 7.11 (d, *J* = 8.0 Hz, 2 H, ArH), 6.16 (d, *J* = 12.0 Hz, 2 H, -CH=CH-), 4.06 (t, *J* = 8.0 Hz, 4 H), 3.70 (t, *J* = 12.0 Hz, 2 H), 3.44-3.37 (m, 2 H), 2.67 (s, 4 H), 1.93 (dd, *J* = 8.0 Hz, 8.0 Hz, 6 H), 1.75 (s, 12H), 1.07 (t, *J* = 4.0 Hz, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 172.34, 155.35, 149.12, 145.44, 142.19, 140.86, 139.01, 133.63, 132.14, 128.61, 125.14, 123.77, 122.32, 110.64, 101.19, 49.31, 46.04, 35.59, 32.04, 28.19, 26.47, 20.80, 11.68. ESI-MS calculated for $[M\text{-}I^-]^+$ 760.3350, found 760.04 (Figures S4-S6).

3 General procedure for *in vitro* spectrum measurement

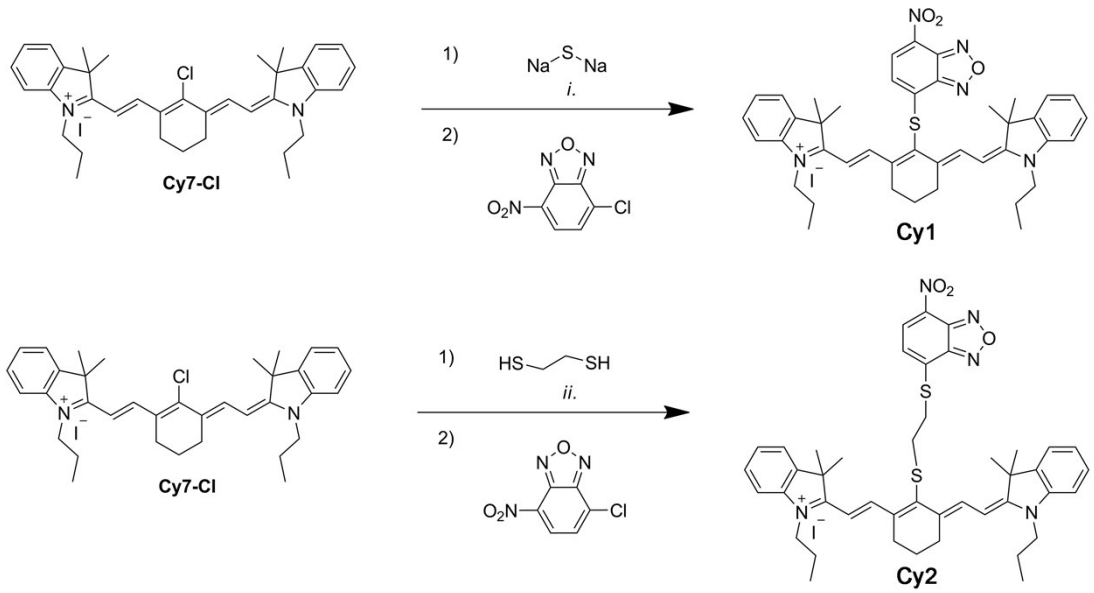
All UV-vis and fluorescence measurements were carried out in PBS solution containing 10 % MeCN, pH 7.4. Stock solution of probe (1 mM) in dimethyl sulfoxide and analytes (10 mM) in distilled water were initially prepared. In a 3.0 mL tube, PBS buffer (10 mM, pH 7.4, 2 mL) and probe (30 μL) solutions were mixed, then various analytes (30 μL) added to the above solution. The final solution volume was adjusted to 3.0 mL with PBS buffer. After rapid mixing of the solution, it was transferred to a 10 × 10 mm quartz cell and incubated at 37 °C for *in vitro* detection. Fluorescence spectra were recorded in the range from 490 to 680 nm and 740 to 860 nm with excitation at 470 nm or 720 nm, respectively.

4 Cell culture

U87 cell (human malignant glioma cell) and MCF-7 cell (human breast cancer cell), were obtained from American Type Culture Collection (ATCC, USA). The cell lines were cultivated on glass-bottom culture dishes with an atmosphere of 5 % CO_2 at 37 °C in DMEM supplemented with 10 % FBS and 1 % (v/v) penicillin-streptomycin.

5 Cytotoxicity assays

The *in vitro* cytotoxicity was evaluated by an MTT assay in U87 and MCF-7 cell lines. The cells were seeded into 96-well cell culture plate (1×10^4/well) and subsequently incubated for 24 h in CO_2 culture box. The cells were further maintained at 37 °C for 24 h under 5 % CO_2 after treatment probes (100 μL/well) at a wide concentration range from 0 to 14 μM. Each well was washed three times with PBS before addition of MTT solution (20 μL, 5.0 mg mL^{-1}) and the cells were incubated for another 4 h. The medium containing MTT was then carefully removed and 150 μL of DMSO was added into each well. The plates were gently shaken for 30 min at room temperature before the absorbance measurement. Three independent experiments were carried out for *in vitro* cytotoxicity analysis.



Scheme S1: Synthesis routine of Cy1, Cy2. Reagents and conditions: *i.*) MeOH, r.t. to 30 °C, 45 min, N_2; *ii.*) DMF, Et_3N, 60 °C, 4h, N_2.

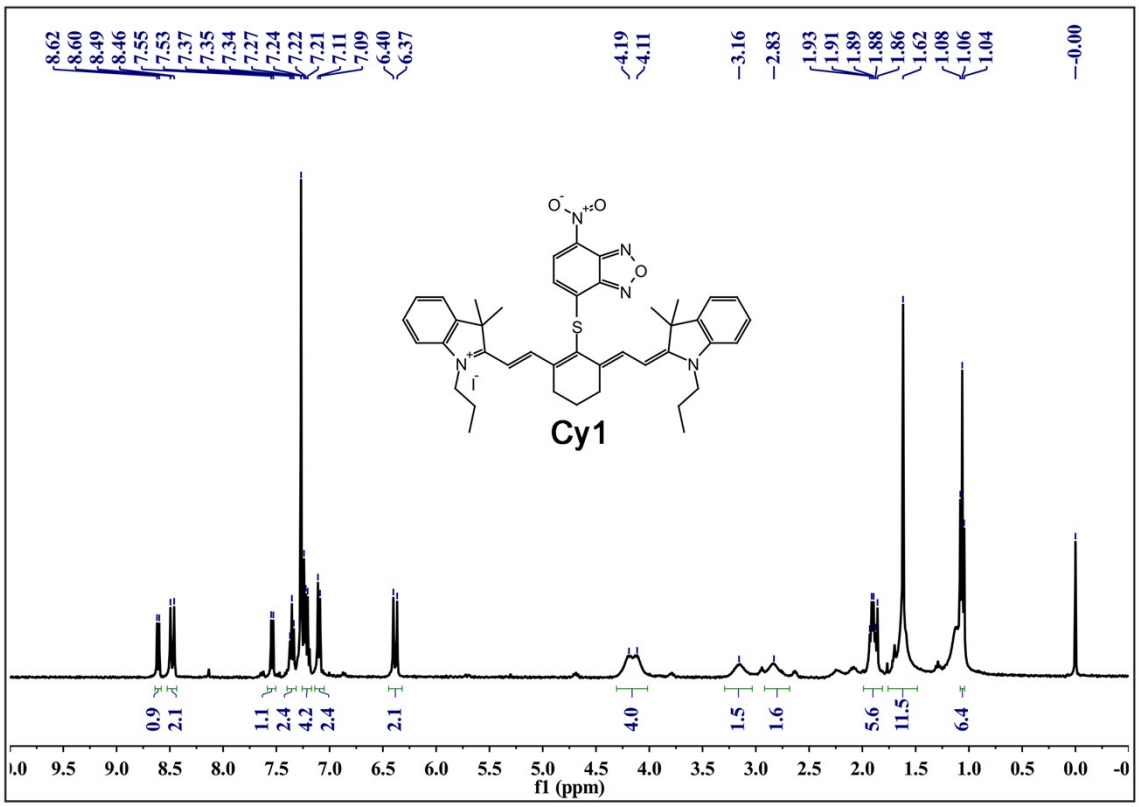


Figure S1. ^1H NMR spectrum of Cy1 in $CDCl_3$.

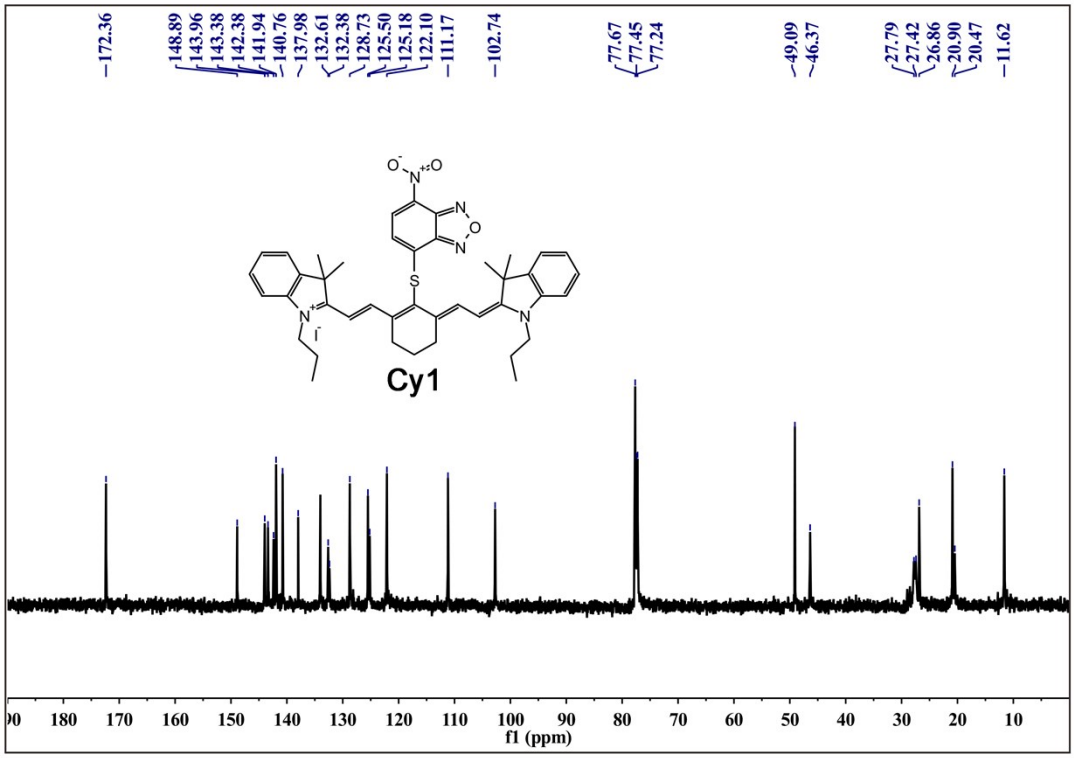


Figure S2. ^{13}C NMR spectrum of Cy1 in $CDCl_3$.

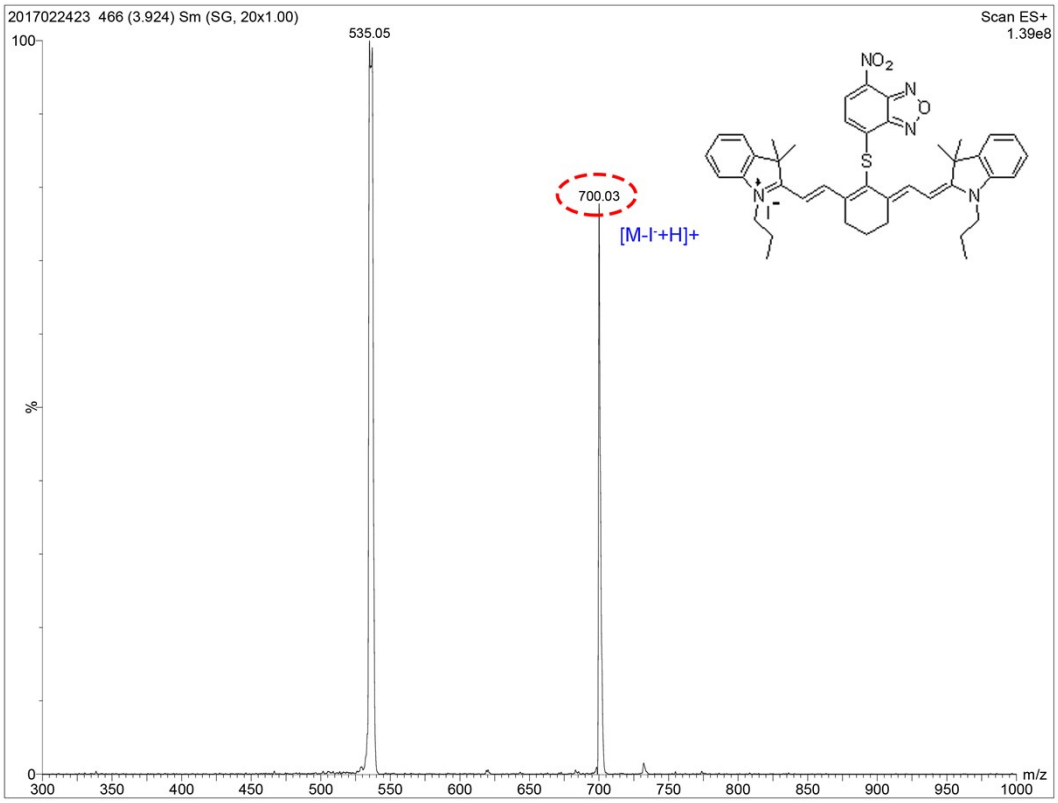


Figure S3. ESI-MS spectrum of Cy1.

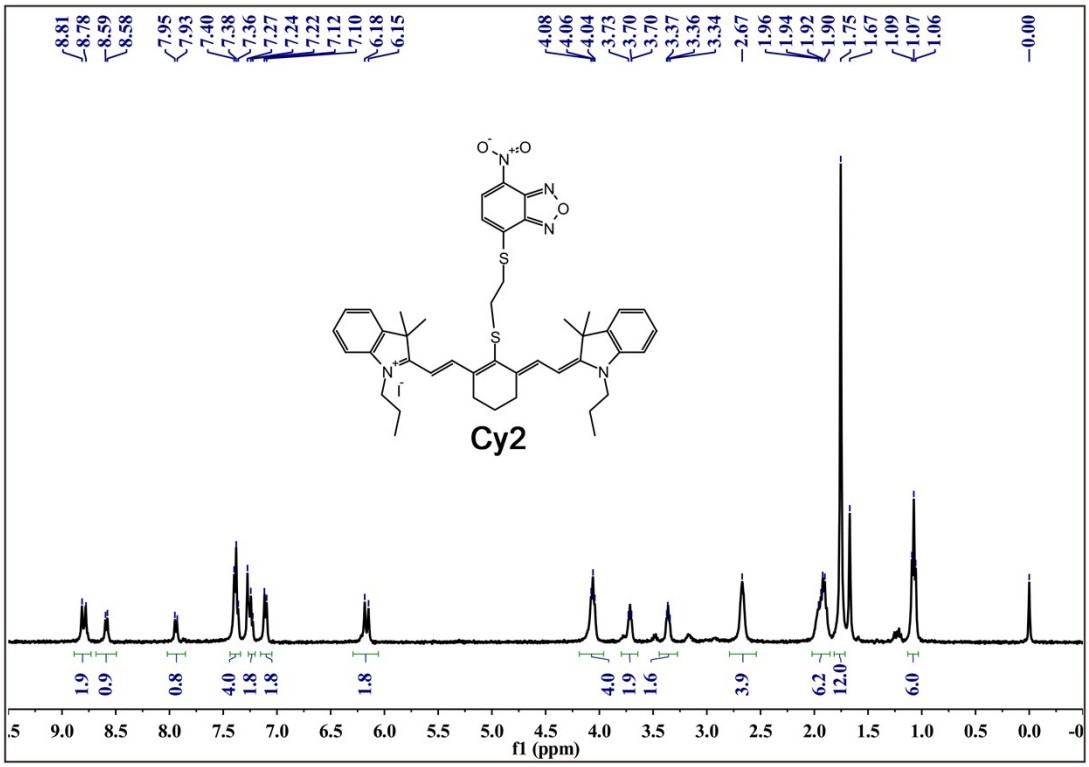


Figure S4. ^{1}H NMR spectrum of Cy2 in $CDCl_3$.

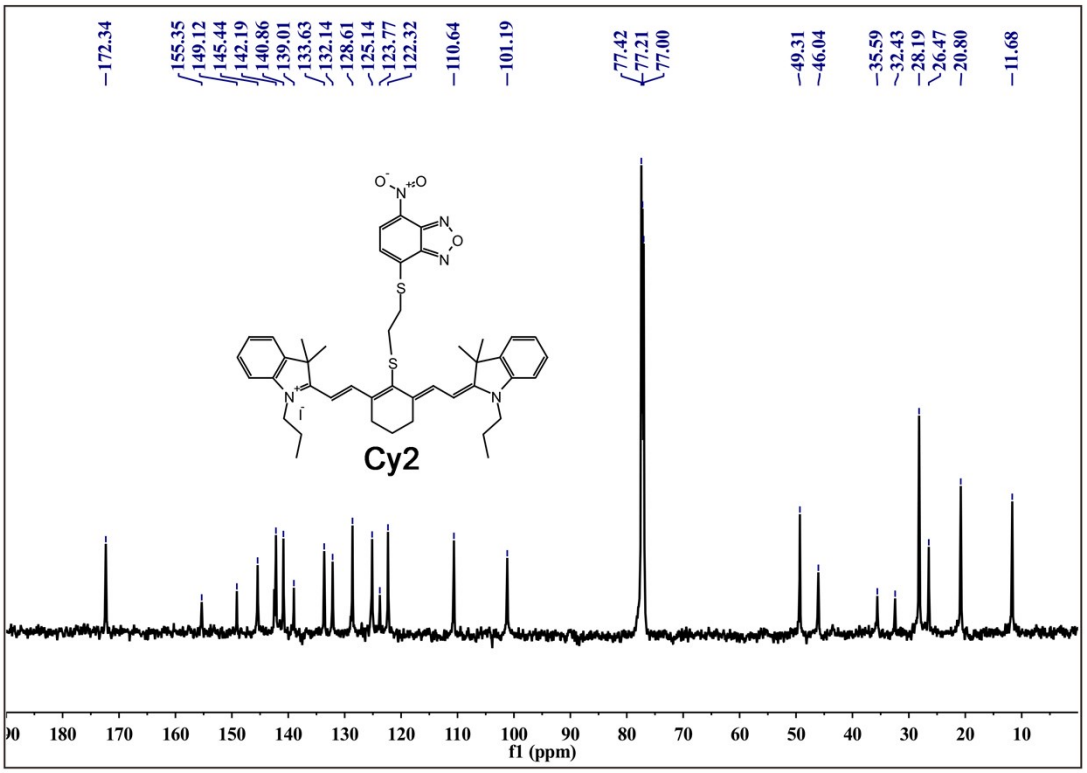


Figure S5. ^{13}C NMR spectrum of Cy2 in $CDCl_3$.

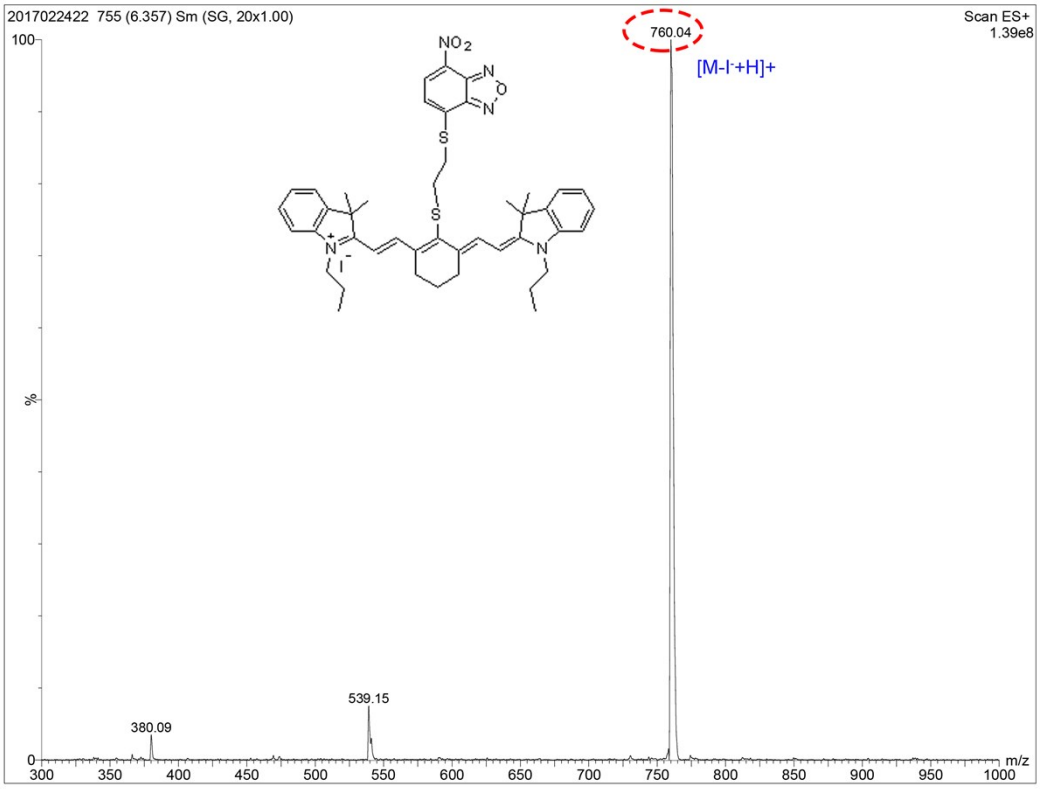


Figure S6. ESI-MS spectrum of Cy2.

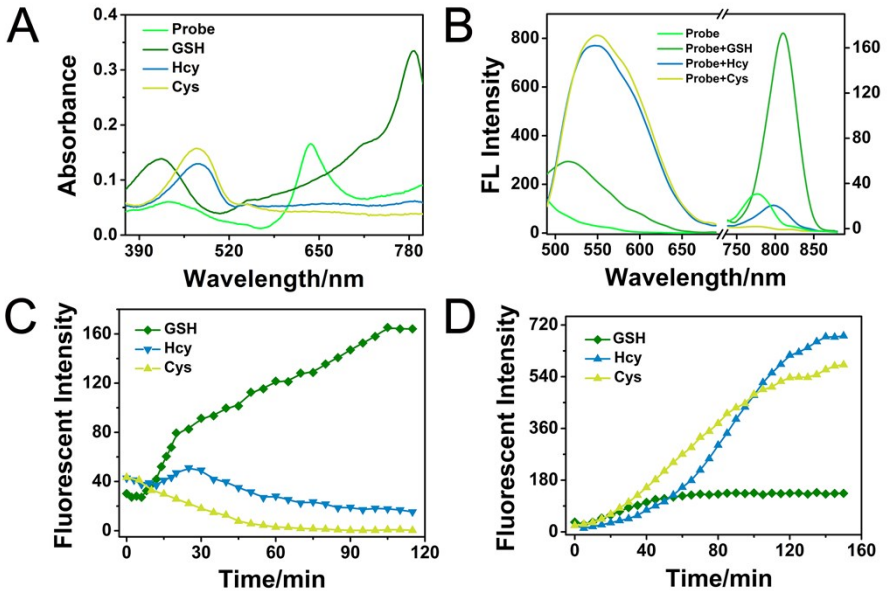


Figure S7. Absorption spectra (A) and fluorescence spectra (B) of Cy1 (10 μM) in the absence or presence of GSH, Hcy and Cys (100 μM) in PBS/DMSO solution (99/1, 1.0 mM, pH 7.4). The emission spectra were acquired with excitation at 470 and 720 nm for the left and right, respectively. Time-dependent fluorescent changes at

550 nm (C) and 810 nm (D) of Cy1 (10 μM) with the addition of GSH, Hcy and Cys (100 μM) in PBS/DMSO solution respectively.

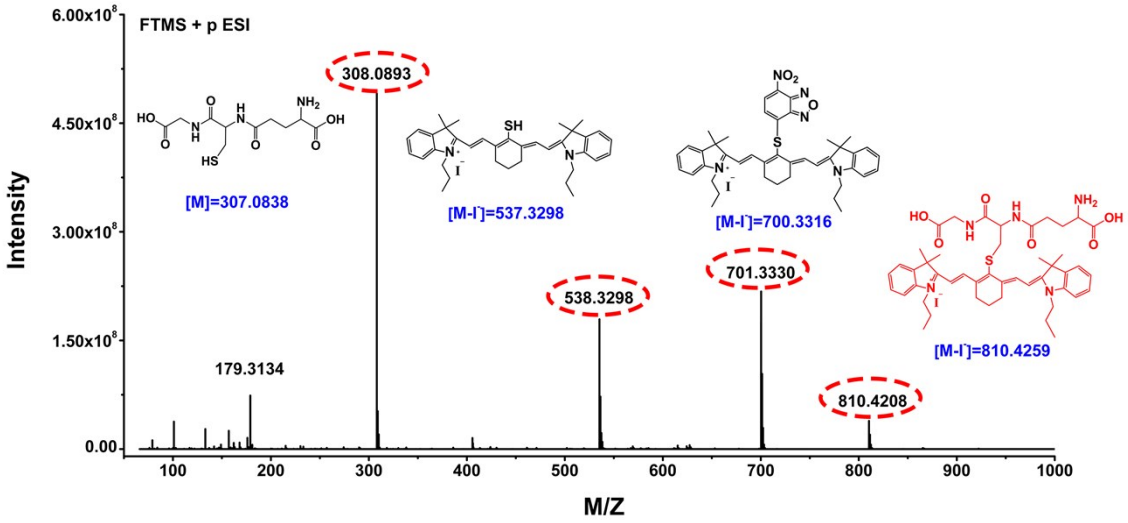


Figure S8. ESI-MS spectrum of Cy1 (10 μM) reacted with GSH (10 equiv.).

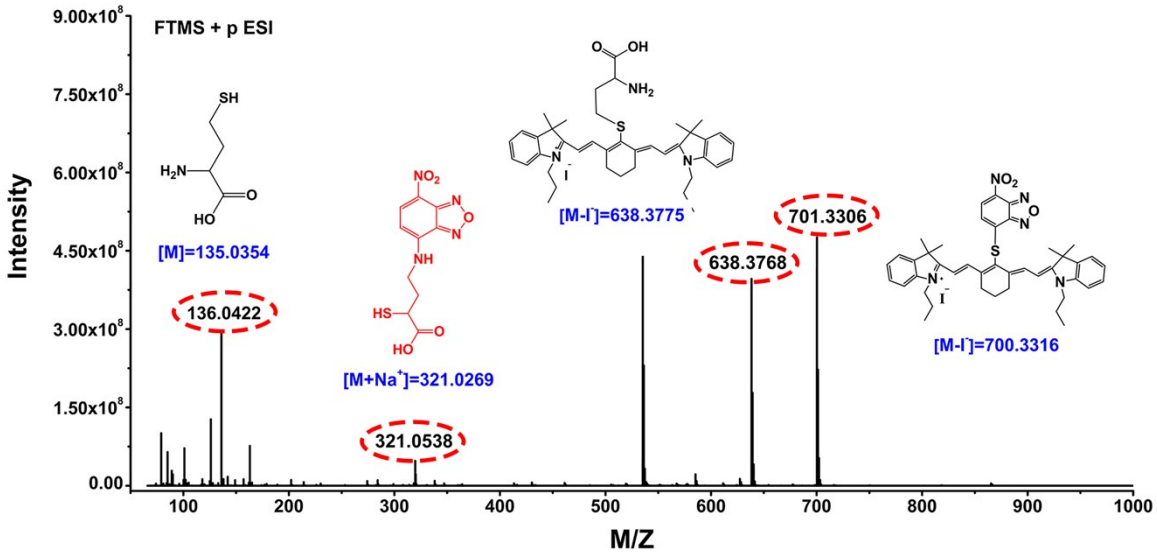


Figure S9. ESI-MS spectrum of Cy1 (10 μM) reacted with Hcy (10 equiv.).

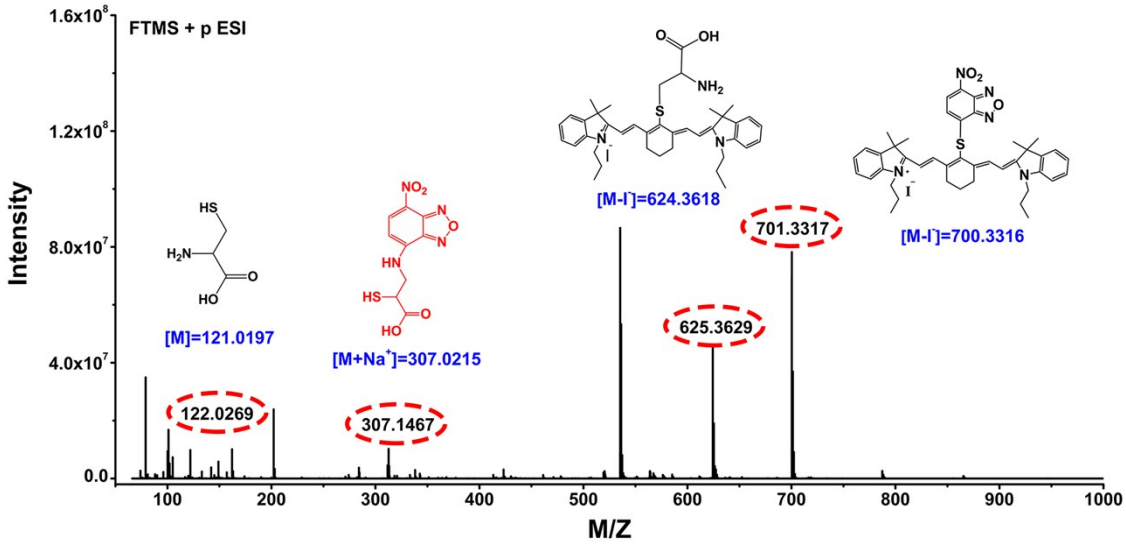


Figure S10. ESI-MS spectrum of Cy1 (10 μM) reacted with Cys (10 equiv.).

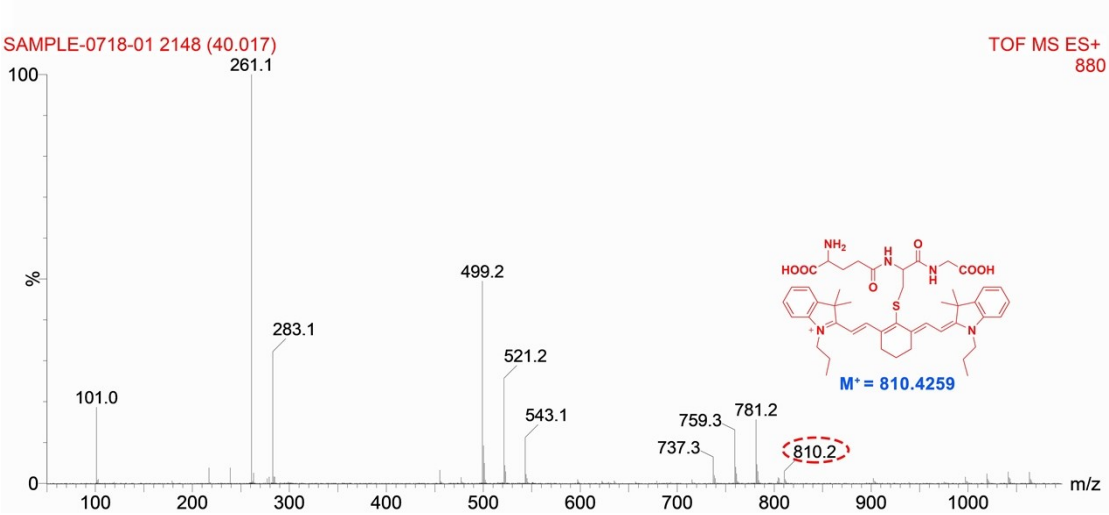


Figure S11. ES-MS spectrum of Cy1 (10 μM) reacted with GSH (10 equiv.).

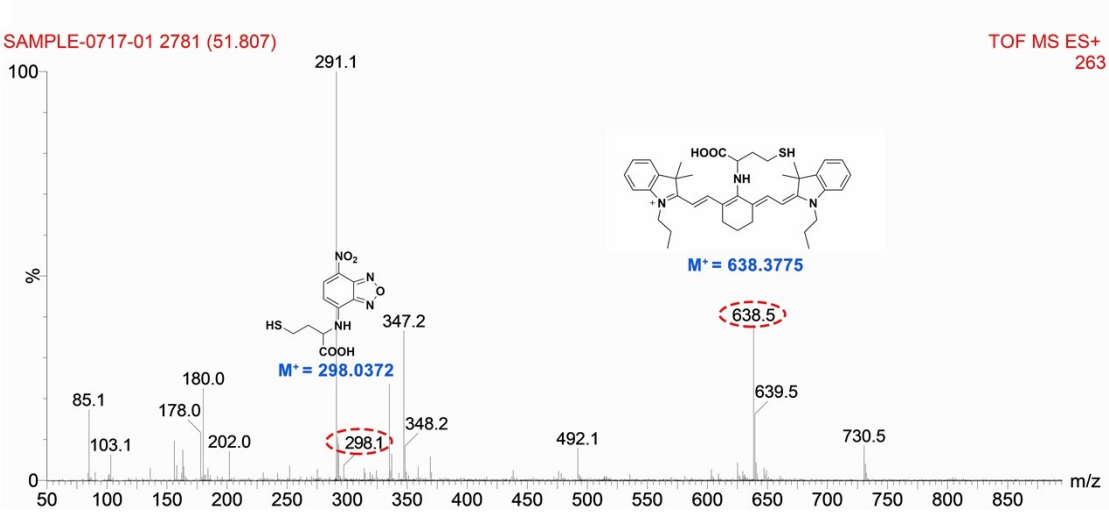


Figure S12. ES-MS spectrum of Cy1 (10 μM) reacted with Hcy (10 equiv.).

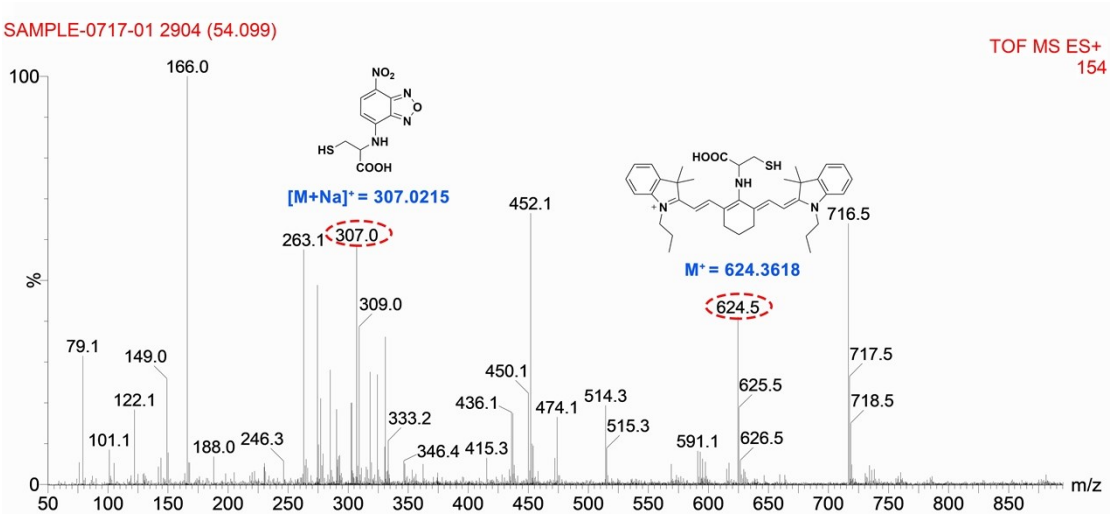


Figure S13. ES-MS spectrum of Cy1 (10 μM) reacted with Cys (10 equiv.).

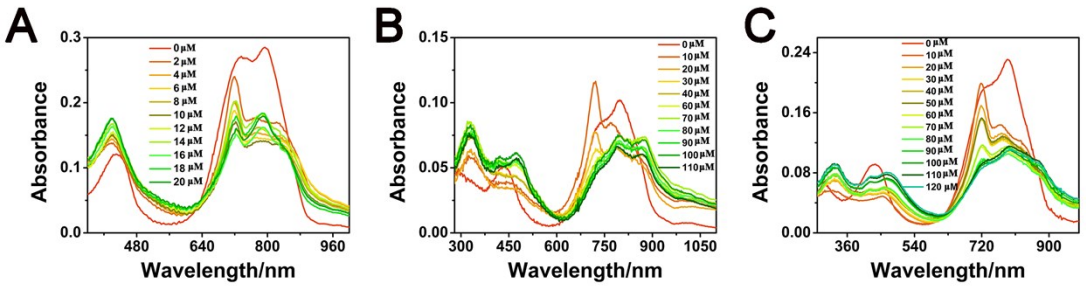


Figure S14. Absorption spectra of Cy2 (10 μM) in the presence of various concentrations of GSH (A), Hcy (B) and Cys (C) in PBS/MeCN solution (9/1, 1.0 mM, pH 7.4).

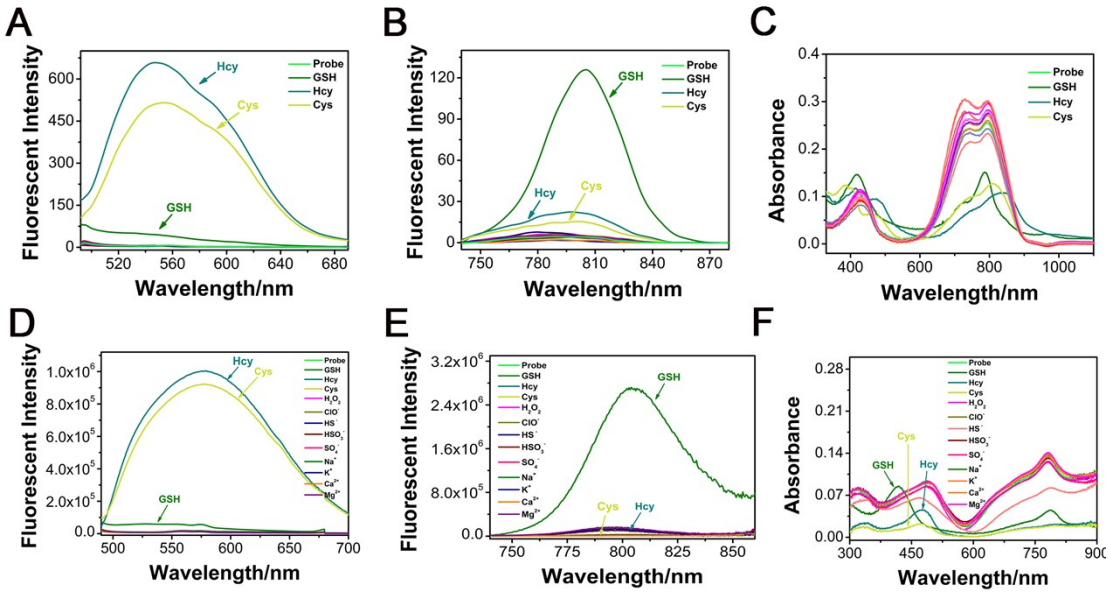


Figure S15. Fluorescence spectra of Cy2 (10 μM) in the presence of various agents with excitation at 470 nm (A and D), and 7200 nm (B and E) in PBS/MeCN solution (9/1, 1.0 mM, pH 7.4). Absorption spectra (C and F) of Cy2 (10 μM) in the presence of various agents in PBS/ MeCN solution (9/1, 1.0 mM, pH 7.4).

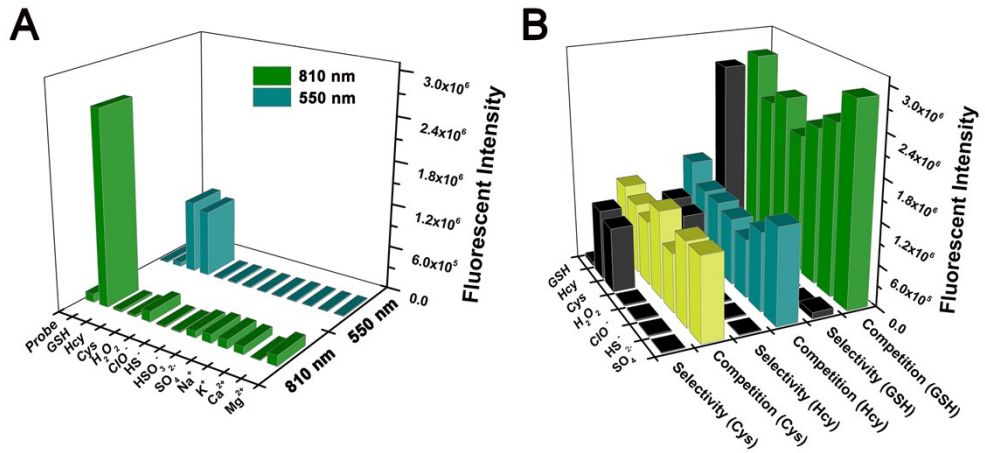


Figure S16. (A) Fluorescence intensity of Cy2 (10 μM) in the presence of different agents at 550 nm (λ_{ex} = 470 nm) and 810 nm (λ_{ex} = 720 nm) respectively. (B) Fluorescence intensity of Cy2 (10 μM) for different agents in the absence and presence of GSH, Hcy and Cys respectively.

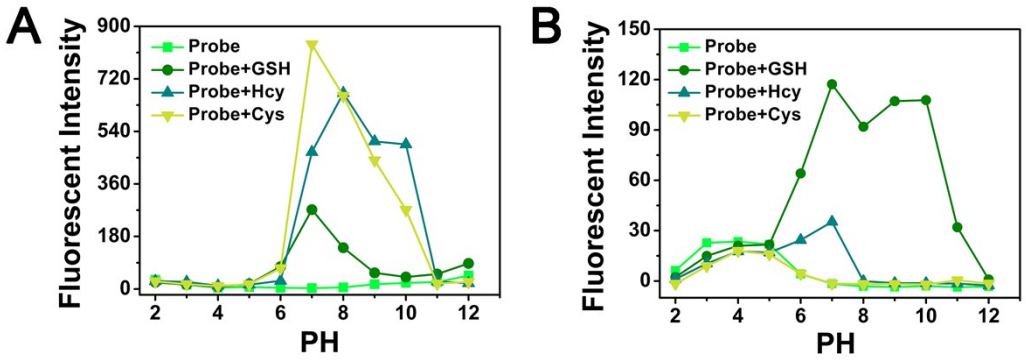


Figure S17. Effect of pH value on the fluorescence intensity at 550 nm (A) and 810 nm (B) of Cy2 (10 μM) in the absence or presence of GSH, Hcy and Cys (1.0 mM) in PBS/DMSO solution (99/1, 1.0 mM, pH 7.4).

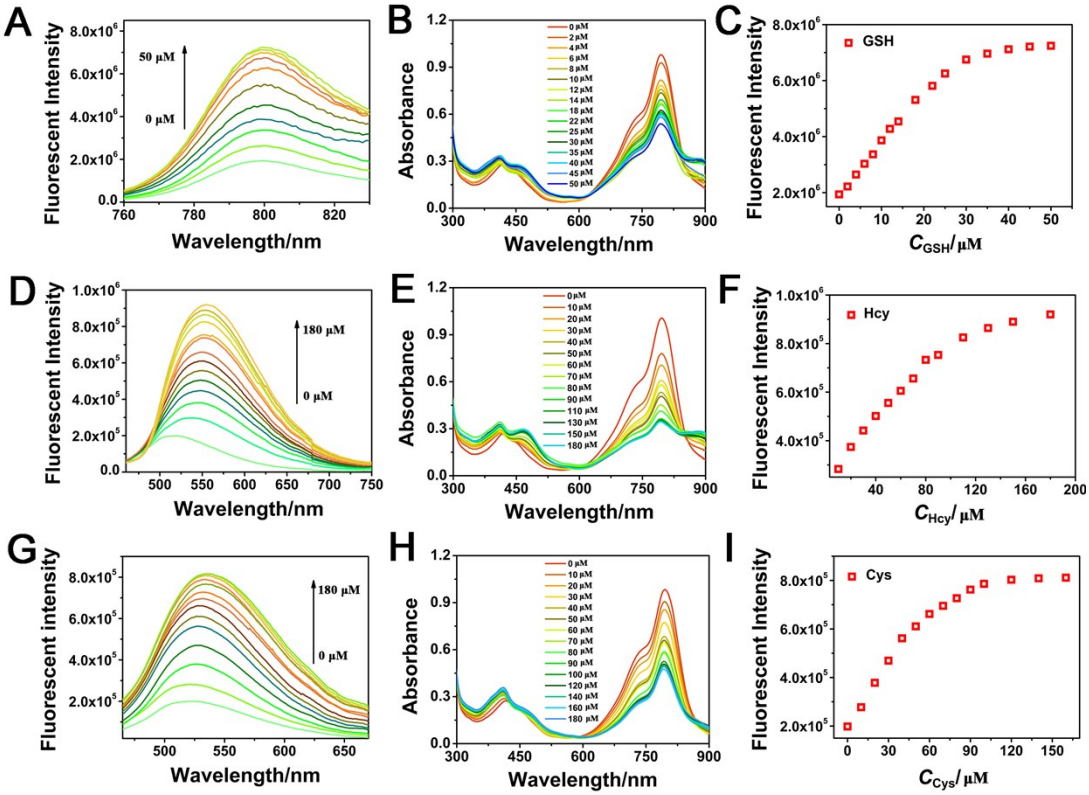


Figure S18. Fluorescence spectra of Cy2 (10 μM) in the presence of various concentrations of GSH (A) with excitation at 720 nm, Hcy (D) and Cys (G) with excitation at 470 nm in PBS/MeCN (9/1, 1.0 mM, pH 7.4) solution containing 5 % fetal bovine serum. Absorption spectra of Cy2 (10 μM) in the presence of various concentrations of GSH (B), Hcy (E) and Cys (H) in PBS/MeCN (9 / 1, 1.0 mM, pH 7.4) solution containing 5 % fetal bovine serum. Correlation between emission intensities at (C) 810 nm in (A), (F) 550 nm in (D) and (I) 550 nm in (G) and concentration of GSH,Hcy and Cys, respectively.

Table S1 Analytical results and recovery tests of GSH in serum samples

Spike (μM)	This work	Ellman method (μM)	Recovery (%)	RSD (%)
3	2.76	3.2	92.0	6.24
6	5.98	6.8	99.6	2.03
9	8.95	8.8	99.4	7.61
12	12.43	13.9	103.5	2.88
15	15.31	16.7	102.0	3.68

Table S2 Analytical results and recovery tests of Hcy in serum samples

Spike (μM)	This work	Ellman method (μM)	Recovery (%)	RSD (%)
5	4.56	7.25	91.2	4.44
15	16.50	18.37	110.0	5.50
25	27.66	22.62	110.6	6.90
45	47.65	46.37	105.8	8.27
75	83.33	74.25	111.1	5.45

Table S3 Analytical results and recovery tests of Cys in serum samples

Spike (μM)	This work	Ellman method (μM)	Recovery (%)	RSD (%)
45	41.48	50	92.1	5.26
55	54.73	59.5	99.5	3.45
65	68.51	62.5	105.4	7.80
75	77.70	79	103.6	5.68
95	95.81	100.5	100.8	10.03

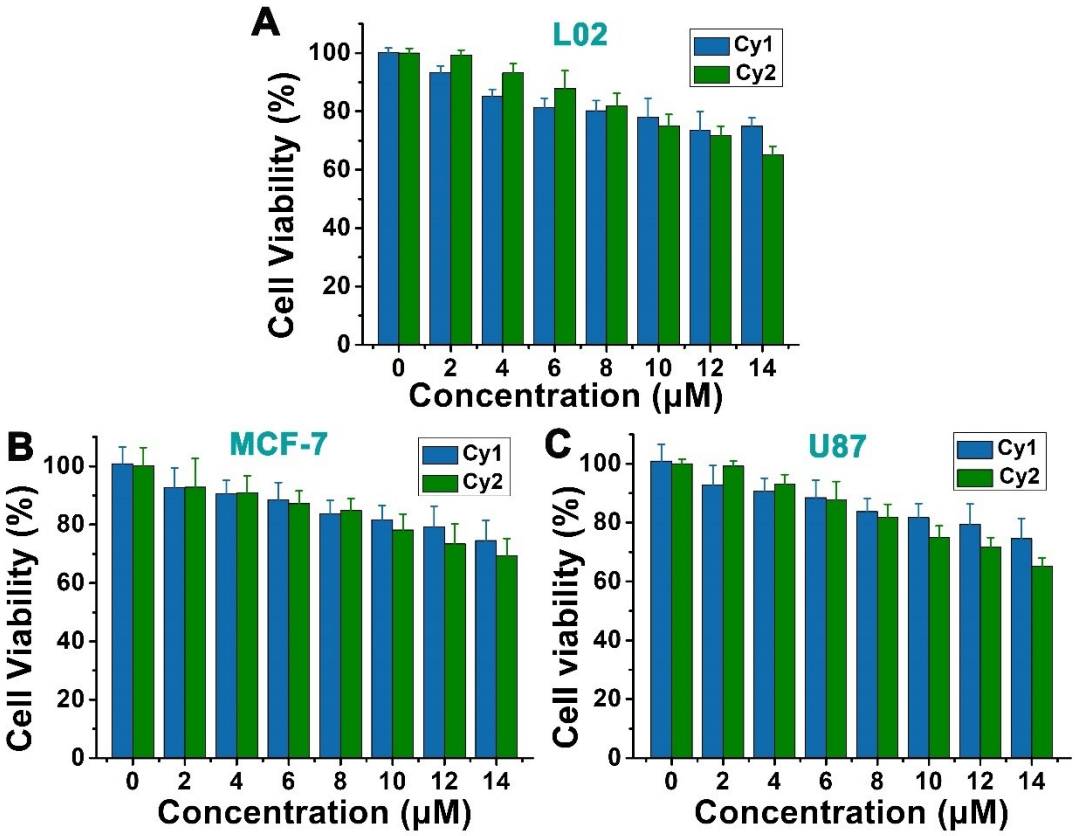


Figure S19. Cell viabilities (%) of Cy1 and Cy2 estimated by MTT proliferation tests. L02、U87 and MCF-7 cells were incubated with 0 - 14 μM probe at 37 °C.

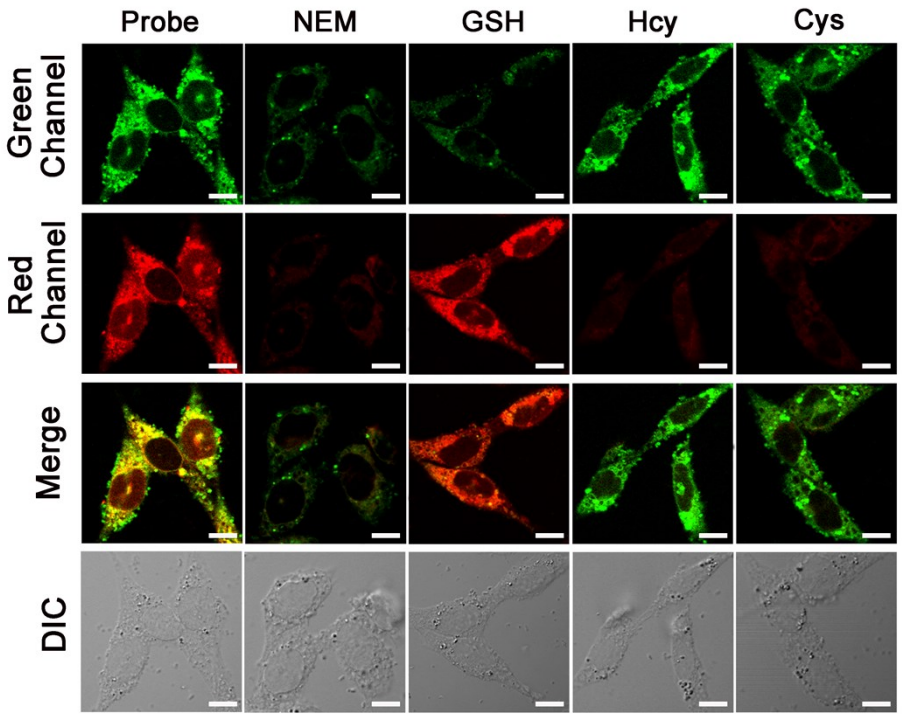


Figure S20. Confocal fluorescence microscope images of (a) MCF-7 cells incubated with Cy2 (10 μM) for 1 h. (b) MCF-7 cells pretreated with NEM (0.5 mM) for 0.5 h, and then incubated with Cy2 (10 μM) for 1 h. Confocal fluorescence microscope images of MCF-7 cells pretreated with NEM (0.5 mM) for 0.5 h, incubated with (c) GSH (250 μM), (d) Hcy (250 μM), and (e) Cys (250 μM) for 0.5 h, and then treated with Cy2 (10 μM) for 1 h. The fluorescence signals were collected at the Red channel (790 ± 30 nm, λ_{ex} = 639 nm), green fluorescence channel (530 ± 30 nm, λ_{ex} = 490 nm). Scale bar = 20 μm.

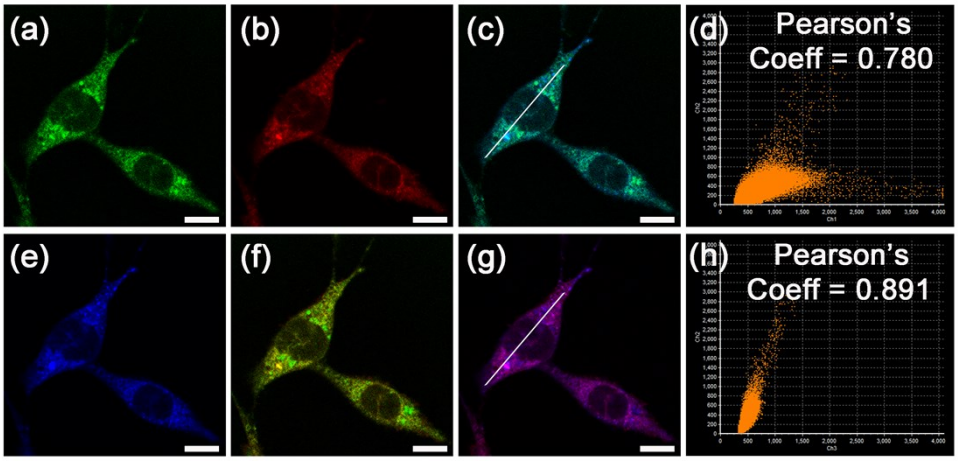


Figure S21. Fluorescence emission from Cy2 colocalized to mitochondria in live U87 cells. Cells were stained with Cy2 (10 μM, 1 h) and MitoTracker Red FM (1 μM, 0.5 h). (a) The green emission channel (λ_{ex} = 490 nm, λ_{em} = 505-550 nm). (b) The red emission channel (λ_{ex} = 639 nm, λ_{em}= 760-830 nm); (e) The blue emission channel (λ_{ex} = 579 nm, λ_{em}= 590-630 nm); (c) Merged image of a and e. (f) Merged image of a and b. (g) Merged image of b and e. (d) Correlation plot of a and e. (h) Correlation plot of b and e. Scale bar = 20 μm.

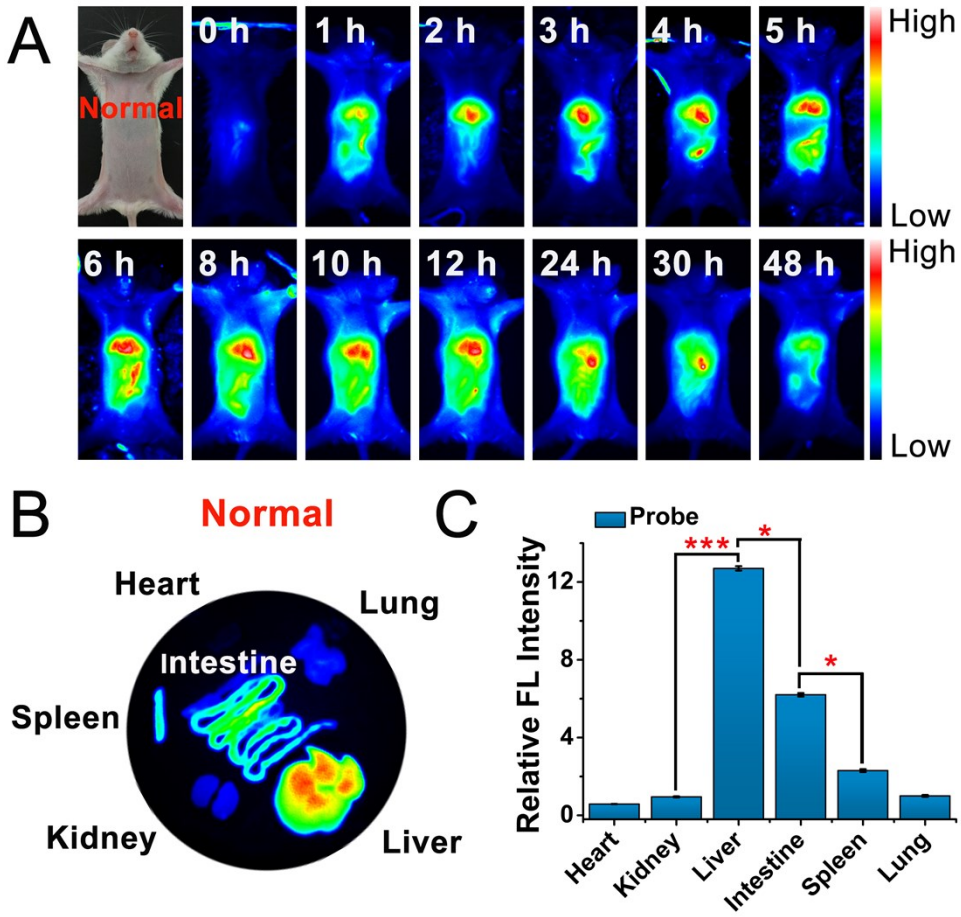


Figure S22. (A) Dynamic distribution of Cy2 in normal mice monitored by NIR fluorescence imaging system. (B) The *ex-vivo* fluorescence images of isolated organs (heart, liver, spleen, lung, kidney, and intestine) from the normal mice at 8 h post-injection of Cy2. (C) Relative fluorescence intensity of isolated organs as shown in B.