Electronic Supplementary Information

Comparison of N-acetylcysteine and cysteine in their ability to replenish intracellular cysteine by a specific fluorescent probe

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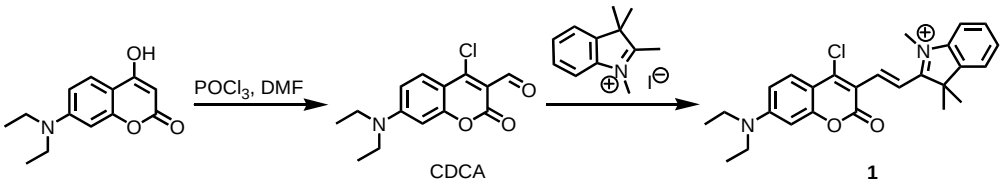
1. Apparatus and reagents

Fluorescence measurements were made on a Hitachi F-4600 spectrophotometer in 10 mm × 10 mm quartz cells (Tokyo, Japan). ^{1}H NMR and ^{13}C NMR spectra were measured with a Bruker DMX-400 spectrometer in CD_3OD-D_4. Electrospray ionization (ESI) mass spectra were measured on LCQ Fleet mass spectrometer (Thermo Fisher). High resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded on an APEX IV FTMS instrument (Bruker, Daltonics). Absorption spectra were recorded in 1-cm quartz cells with a TU-1900 spectrophotometer (Beijing, China). A Delta 320 pH-meter [Mettler-Toledo Instruments (Shanghai) Co., China] was used for pH measurements.

1,2,3,3-Tetramethyl-3H-indolium iodide, 3-diethylaminophenol, malonic acid were purchased from Alfa Aesar. Glutathione (GSH) and cysteine (Cys) were purchased from J &K. Dulbecco's modified eagle media (DMEM), fetal bovine serum, streptomycine, penicillin and phosphate buffer saline (PBS; 155 mM NaCl, 2.97 mM Na_2HPO_4, and 1.06 mM KH_2PO_4) of pH 7.4 were obtained from Thermo Fisher. All other chemicals used were of analytical grade. Ultrapure water (over 18 MΩ·cm) was used throughout. A mixed serum sample from healthy people was provided by Xijing Hospital and informed consent was obtained from each donor.

2. Synthesis of probe 1



Scheme S1. Synthesis of probe **1**.

4-Chloro-7-diethylaminocoumarin-3-aldehyde (CDCA) was synthesized starting from 7-(diethylamino)-4-hydroxy-2*H*-chromen-2-one following the procedure reported in literature (Liu et al, *J. Am. Chem. Soc.*, 2014, 136, 574).

Then, CDCA (1 mmol, 279 mg) and 1,2,3,3-tetramethyl-3*H*-indolium iodide (1.2 mmol, 361 mg) were dissolved in 20 mL ethanol. The mixture was refluxed for 4 h with stirring. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was purified by silica gel chromatography eluted with CH_2Cl_2/CH_3OH (v/v, 10:1), affording probe **1** as purple solid (153 mg, yield 35%). HR-ESI-MS: calcd for $[C_{26}H_{28}ClN_2O_2]^+$, *m/z* = 435.1839; found, *m/z* = 435.1834. ^{1}H NMR (400 MHz, CD_3OD-D_4): δ = 8.63-8.59 (d, J = 16.0 Hz, 1H), 8.34-8.30 (d, J = 16.0 Hz, 1H), 7.98-7.95 (d, J = 12.0 Hz, 1H), 7.83-7.79 (m, 2H), 7.69-7.63 (m, 2H), 7.05-7.03 (d, J = 8.0 Hz, 1H), 6.70 (s, 1H), 4.10 (s, 3H), 3.70-3.65 (q, J=6.7 Hz, 4H), 1.89 (s, 6H), 1.35-1.31 (t, J=8.0 Hz, 6H). ^{13}C NMR (400 MHz, CD_3OD-D_4): δ 182.2, 158.3, 156.1, 154.9, 154.3, 145.5, 143.2, 141.9, 129.3, 129.1, 122.5, 114.2, 112.5, 112.0, 109.8, 108.4, 96.2, 51.8, 45.2, 32.9, 25.8, 11.4.

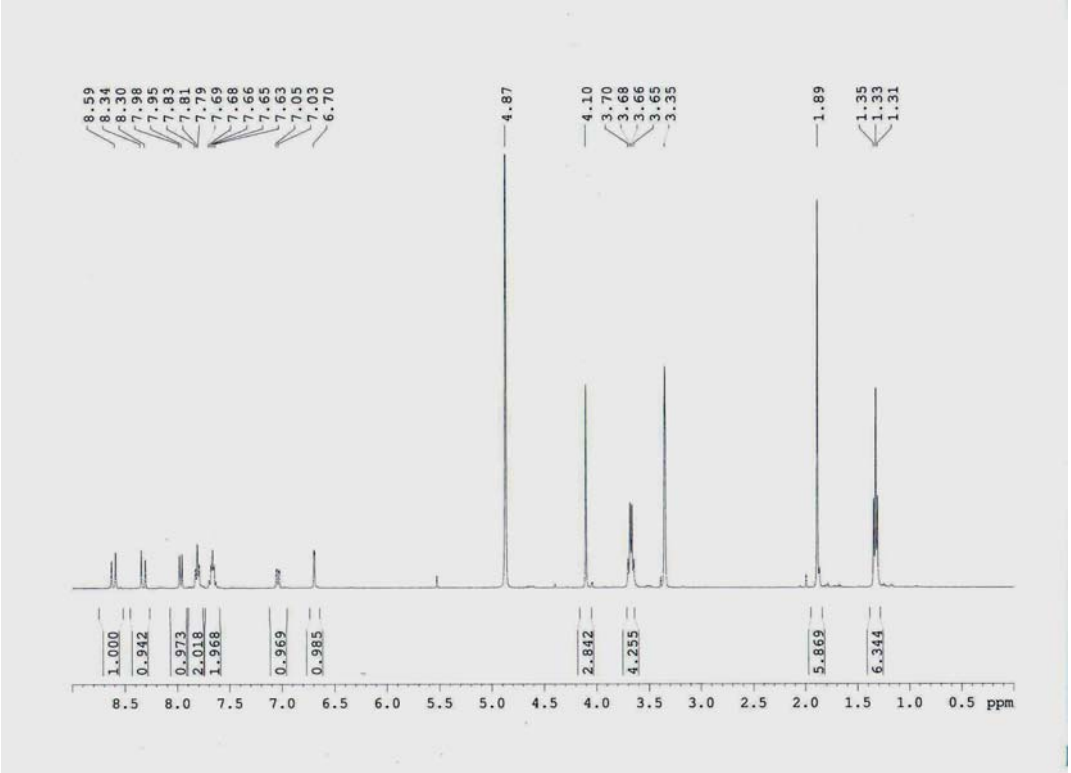


Fig. S1 ^{1}H NMR spectrum of probe **1** (400 MHz, CD_3OD-D_4, 298 K).

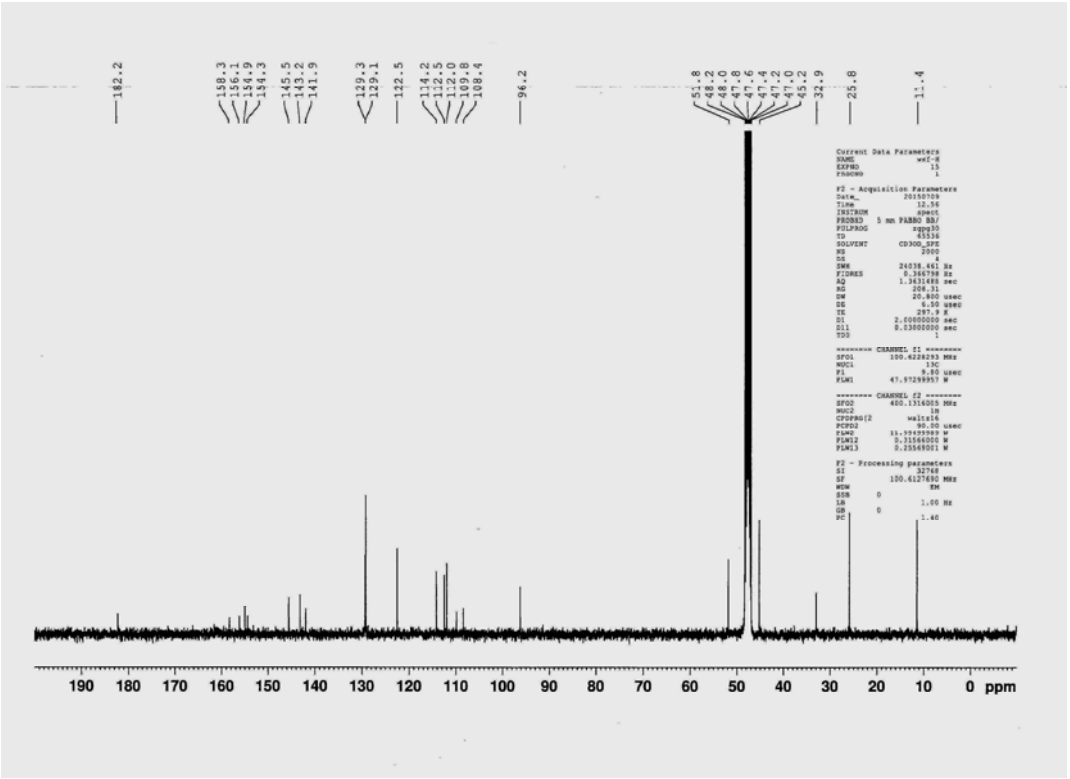


Fig. S2 ^{13}C NMR spectrum of probe **1** (400 MHz, CD_3OD-D_4, 298K).

3. General procedure for determining Cys

Unless otherwise noted, all the measurements were conducted in PBS (pH 7.4) in accordance with the following procedure. In a 2-mL tube, 1 mL of PBS and 10 μL of 1 mM probe **1** were mixed, followed by addition of appropriate volume of Cys solution. After incubation at 37 ^{o}C for 2 h in a thermostat, the reaction solution was transferred to a quartz cell of 1-cm optical length to measure absorbance or fluorescence with $\lambda_{ex/em}$ = 405/460 nm and both excitation and emission slit widths of 10 nm. In the meantime, a blank solution containing no Cys was prepared and measured under the same conditions for comparison.

4. Absorption spectra of probe 1 towards other substances

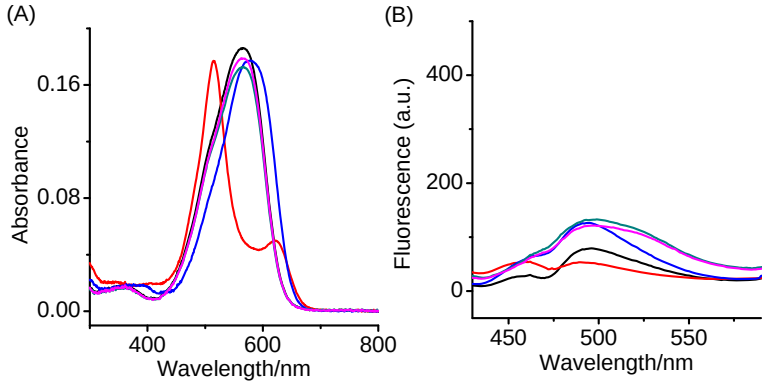
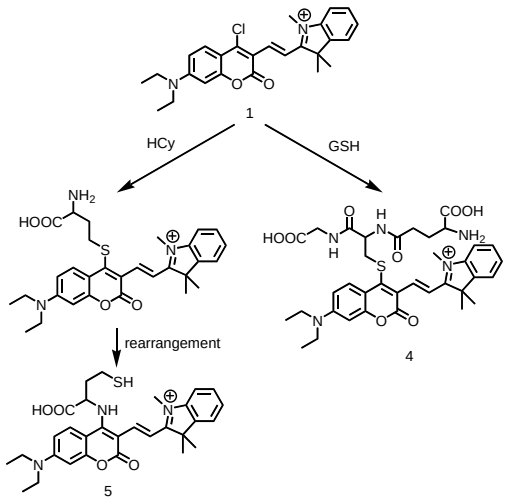


Fig. S3 (A) Absorption and (B) fluorescence emission spectra of probe **1** (10 μM) in the presence of various species: probe **1** itself (black); 100 μM Hcy (red); 100 μM GSH (blue); 100 μM glycine (dark cyan); 100 μM methionine (magenta).

5. Reaction mechanisms of probe 1 with Hcy and GSH



Scheme S2 Possible reaction mechanisms of probe **1** with Hcy and GSH.

6. Analyses of reaction products

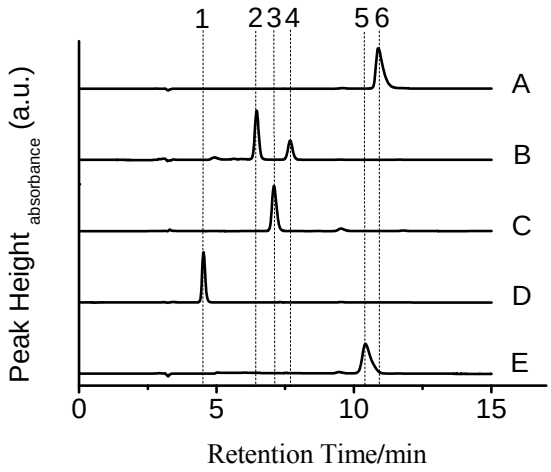


Fig. S4 Chromatographs of probe **1** (20 μM) in the presence of various substances: (A) probe **1** itself; (B) 100 μM Cys; (C) 100 μM NAC; (D) 100 μM GSH and (E) 100 μM Hcy. The assignments of the peaks: (1) 4.53 min, product **4**; (2) 6.47 min, product **2**; (3) 7.08 min, product **3**; (4) 7.60 min, product **2**'; (5) 10.42 min, product **5**; (6) 10.89 min, probe **1** (see Scheme 1, Scheme S2 and Fig. S5g below for the structures of these products).

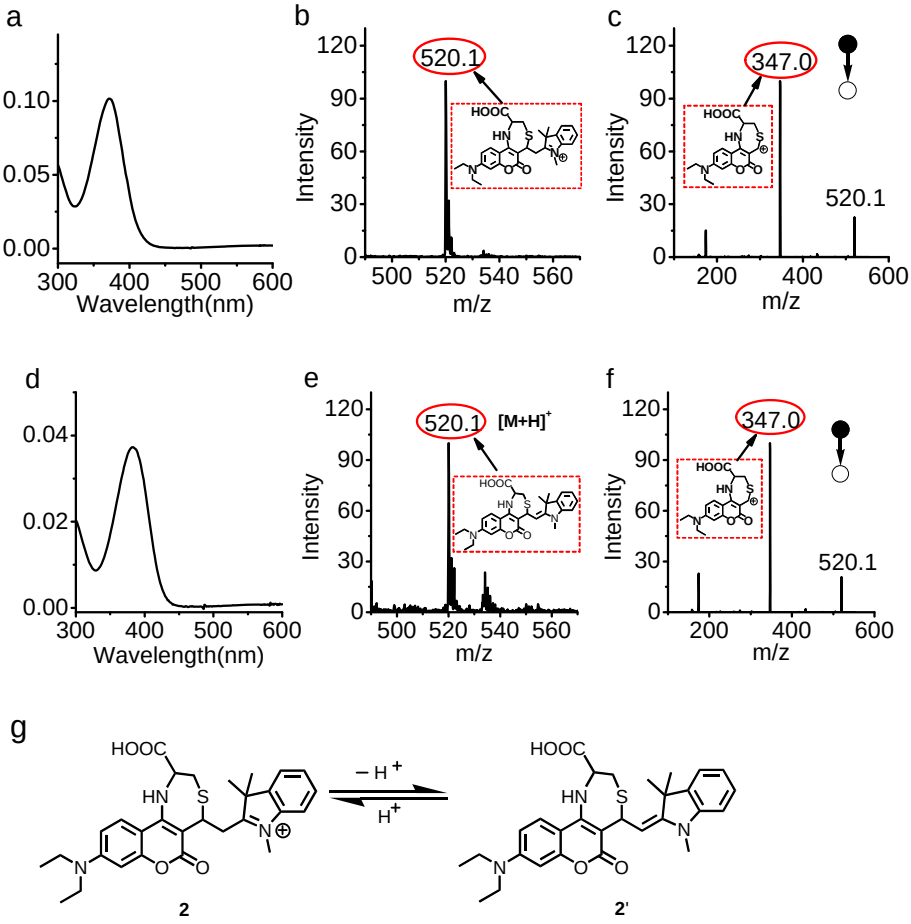


Fig. S5 Absorption spectra of (a) product **2** and (d) product **2**' (i.e., peak 2 and peak 4 in curve B of Fig S4). (b) ESI mass spectrum of the isolated product **2**. (c) The MS/MS

spectrum of the isolated product **2** with *m/z* = 520.1. (e) ESI mass spectrum of the isolated product **2**'. (f) The MS/MS spectrum of the isolated product **2**' with *m/z* = 520.1. (g) Proposed equilibrium of the two products **2** and **2**'.

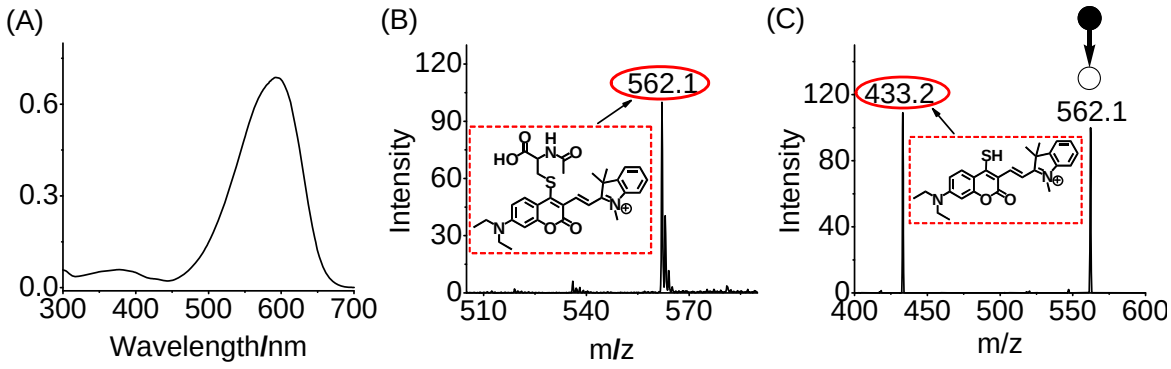


Fig. S6 (A) Absorption spectrum and (B) ESI mass spectrum of the isolated reaction product of probe **1** with NAC. (C) The MS/MS spectrum of the isolated reaction product with *m/z* = 562.1.

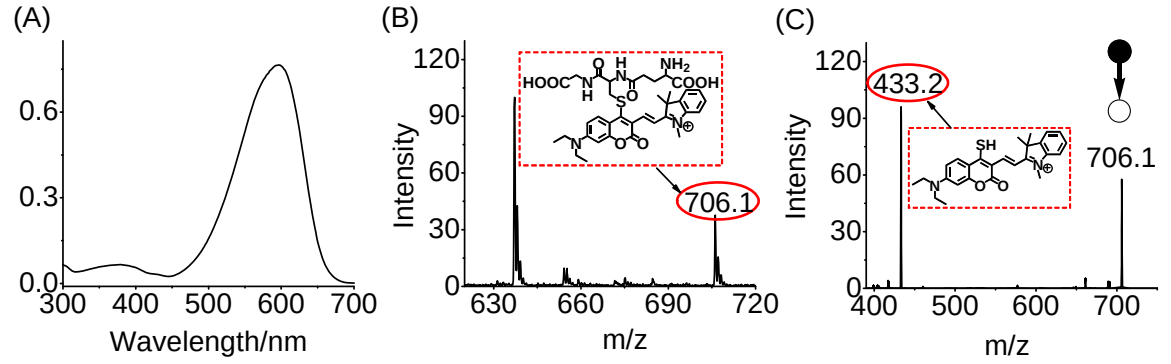


Fig. S7 (A) Absorption spectrum and (B) ESI mass spectrum of the isolated reaction product of probe **1** with GSH. (C) The MS/MS spectrum of the isolated reaction product with *m/z* = 706.1.

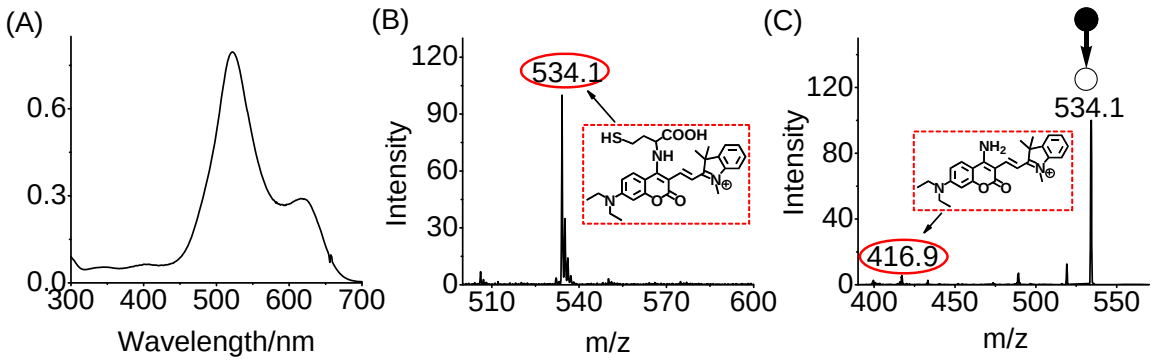


Fig. S8 (A) Absorption spectrum and (B) ESI mass spectrum of the isolated reaction product of probe **1** with Hcy. (C) The MS/MS spectrum of the isolated reaction product with *m/z* = 534.1.

7. Effects of pH, temperature and time on the reaction

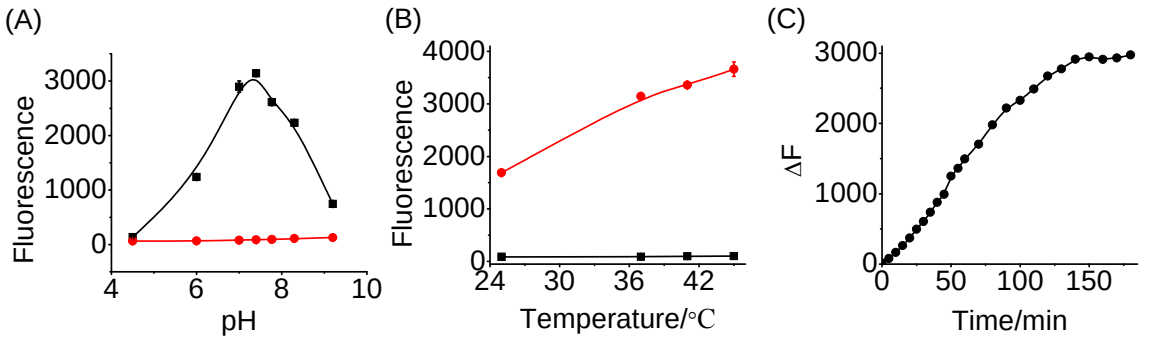


Fig. S9 Effects of (A) pH, (B) reaction temperature and (C) time on the fluorescence of probe **1** (10 μM) with Cys (100 μM). $\lambda_{ex}/\lambda_{em}$ = 405 nm/460 nm.

8. Selectivity study

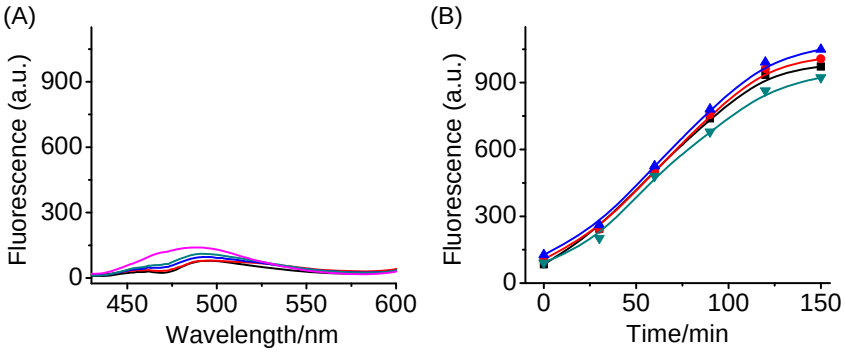


Fig. S10 (A) Fluorescence response of probe **1** (10 μM) to NAC at varied concentrations (from bottom to top: 0, 10, 20, 50 and 100 μM). (B) Time-dependent fluorescence response of probe **1** (10 μM) in the presence of different thiols: 20 μM Cys (black, control); 20 μM Cys + 20 μM NAC (red); 20 μM Cys + 1 mM GSH (blue); 20 μM Cys + 20 μM Hcy (dark cyan). $\lambda_{ex/em}$ = 405/460 nm.

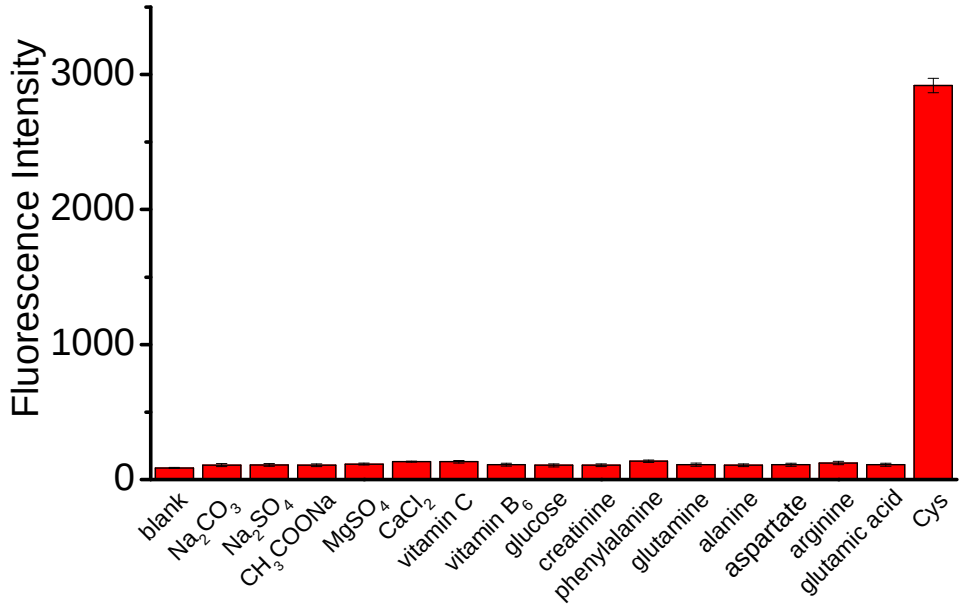


Fig. S11 Fluorescence response of probe **1** (10 μM) to various substances: (1) blank; (2) Na_2CO_3 (100 μM); (3) Na_2SO_4 (100 μM); (4) CH_3COONa (100 μM); (5) $MgSO_4$ (1 mM); (6) $CaCl_2$ (1 mM); (7) vitamin C (100 μM); (8) vitamin B_6 (100 μM); (9) glucose (100 μM); (10) creatinine (100 μM); (11) phenylalanine (100 μM); (12) glutamine (100 μM); (13) alanine (100 μM); (14) aspartate (100 μM); (15) arginine (100 μM); (16) glutamic acid (100 μM); (17) Cys (100 μM). $\lambda_{ex/em}$ = 405/460 nm.

9. Determination of Cys in human serum

Human serum sample (1 mL) was transferred to a 5-mL centrifuge tube. Then, 0.15 mL of a 66.7 g/L tris(2-carboxyethyl)phosphine solution (reducing reagent) at nearly neutral pH (ca. pH 6) was added to the sample. The resulting mixture was vigorously vortex-mixed at intervals and incubated for 30 min at room temperature. Afterward, 3 mL of acetonitrile was introduced to precipitate the protein, and the separated supernatant was blow-dried in a tube by a pure N_2 flow. Then, 1 mL of borate buffer solution (20 mM, pH 7.4, containing 2 mM EDTA) was added to the tube, vortex-mixed, and centrifuged at 12000 r/min for 5 min. The supernatant was collected. For fluorescence analysis, 100 μL of the supernatant was taken out and mixed with 900 μL of PBS (pH 7.4) containing 10 μM of probe **1**, and 2 h later fluorescence signal was recorded. For HPLC analysis (Liu et al, *Anal. Chem.*, 2015, **87**, 11475), 180 μL of the supernatant was taken out and mixed with 20 μL of a 5 g/L ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F) solution for the derivatization reaction. The reaction was performed at 60 °C in a water bath for 1 h. The final solution was filtered with a 0.22 μm Millipore membrane and kept at 4 °C for use. The HPLC separation of the Cys derivatives was carried out using isocratic elution method. The mobile phase consisted of 0.1 M acetate buffer (pH 4.5) and methanol, whose volume ratio is 97:3 (v/v).

Table S1 Determination of Cys in human serum

Analyte	added (μM)	found[a] (μM)	recovery[a] (%)
Cys	0	262 ± 4	—
	100	363 ± 7	100 ± 2
	200	456 ± 10	99 ± 2

[a] Mean of three determinations ± standard deviation

Table S2 Determination of Cys in human serum with added GSH or NAC

Substance added	concentration (μM)	Cys found[a] (μM)	recovery[a] (%)
GSH	100	268 ± 4	102 ± 1
	200	270 ± 6	103 ± 2
NAC	100	260 ± 7	99 ± 3
	200	265 ± 7	101 ± 3

[a] Mean of three determinations ± standard deviation

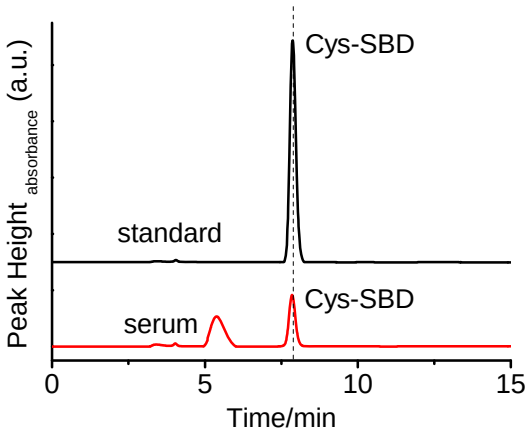


Fig. S12. HPLC chromatograms of the standard Cys sample (black) and the human serum sample (red). The concentration of Cys in human serum was determined to be 247 ± 13 μM.

10. Studies on the cytotoxicity of probe 1

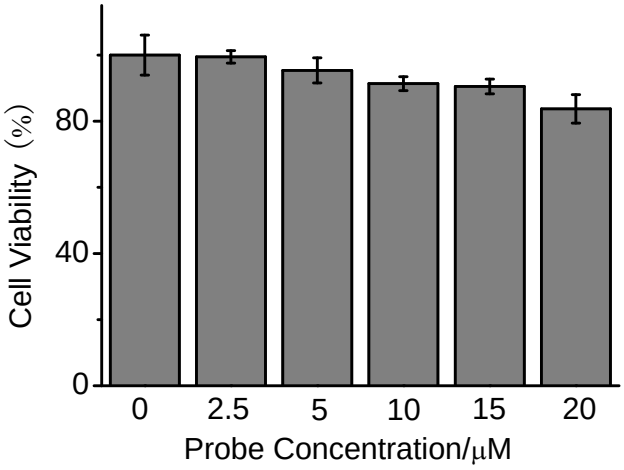


Fig. S13 Percentage of viable LO2 cells after treatment with indicated concentrations of probe **1** after 24 h.

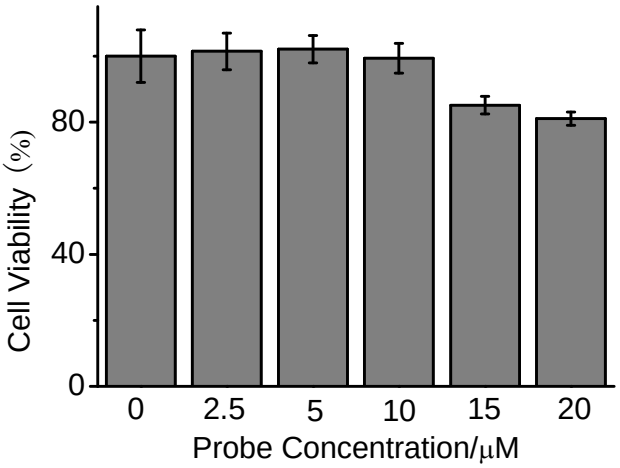


Fig. S14 Percentage of viable HepG2 cells after treatment with indicated concentrations of probe **1** after 24 h.

11. Cell imaging

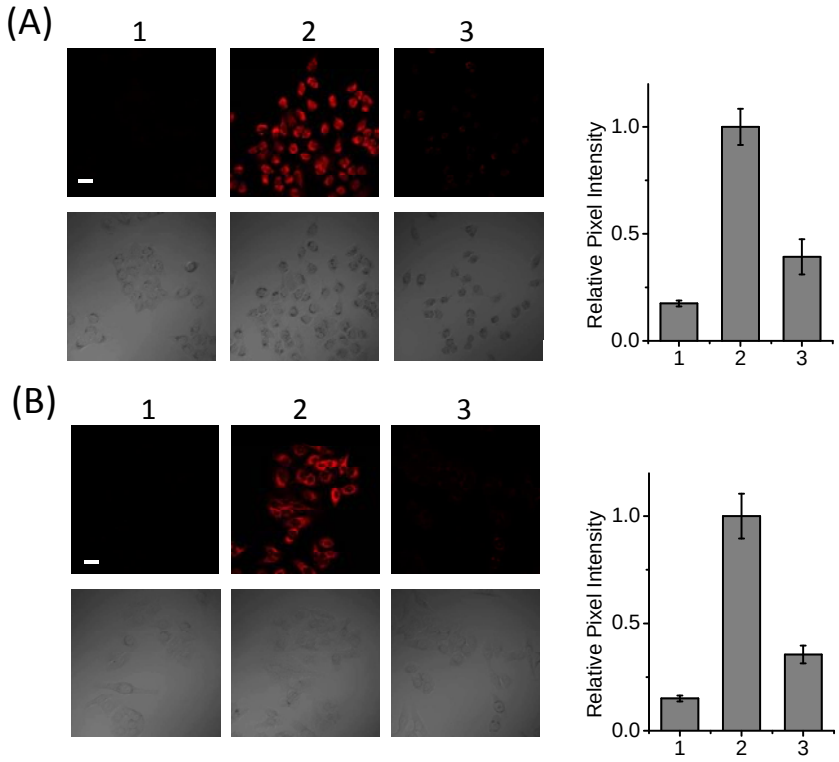


Fig. S15 Fluorescence images and relative pixel intensities of the corresponding fluorescence images of (A) LO2 and (B) HepG2 cells. (1) Cells only; (2) cells incubated with 10 μM of probe **1** for 1 h at 37 ^{o}C; (3) cells pretreated with 2 mM of NEM, and then incubated with 10 μM of probe **1** for 1 h at 37 ^{o}C. Scale bar, 20 μm.

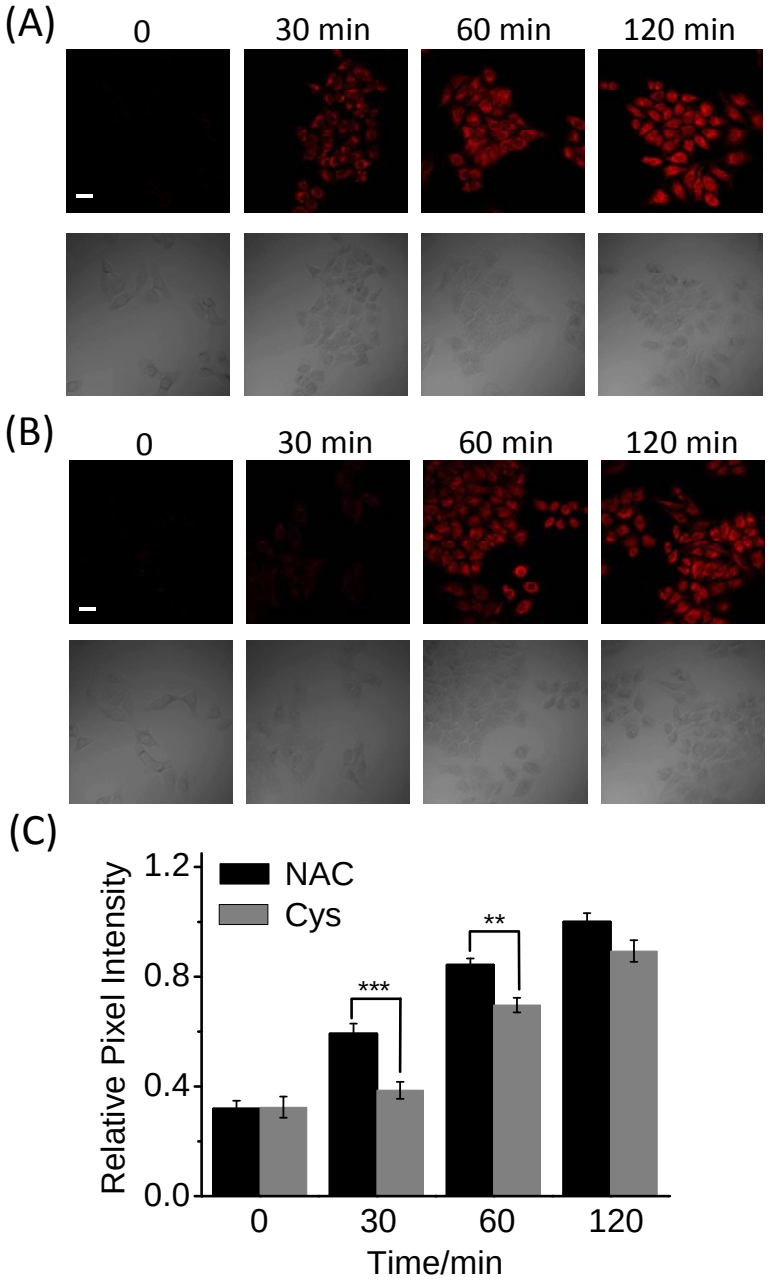


Fig. S16 Fluorescence images of HepG2 cells. HepG2 cells were pre-treated with NEM (2 mM), then incubated with 100 μM of NAC (A) or Cys (B) for different periods of time (0, 30, 60, 120 min), and finally incubated with probe **1** (10 μM). The differential interference contrast images are shown below the corresponding fluorescence images. (C) Relative pixel intensity of the corresponding fluorescence images. Statistical analyses are performed using the Student's t-test: ** $p < 0.01$, *** $p < 0.001$. Emission was collected at 430–490 nm with excitation at 405 nm. Scale bar, 20 μm.