Electronic Supplementary Information for:

A simple fluorescence off-on probe for the discrimination of cysteine from glutathione

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1. Apparatus and reagents

^{1}H NMR and ^{13}C NMR spectra were measured with a Bruker DMX-400 spectrometer in DMSO-D_6. Electron impact time-of-flight mass spectra (EI-TOF MS) and high resolution EI-TOF mass spectra (HR-EI-TOF MS) were recorded with a GCT mass spectrometer (Micromass, Manchester, UK). Electrospray ionization (ESI) mass spectra were measured on LCQ Fleet mass spectrometer (ThermoFisher). Fluorescence measurements were made on a Hitachi F-4600 spectrophotometer in 10 mm × 10 mm quartz cells (Tokyo, Japan). Fluorescence quantum yield (Φ) was determined by using resorufin (Φ = 0.74 in water) as a standard. Absorption spectra were recorded in 1-cm quartz cells with a TU-1900 spectrophotometer (Beijing, China). A model HI-98128 pH-meter (Hanna Instruments Inc., USA) was used for pH measurements.

Resorufin sodium salts, 4-chloro-7-nitrobenzofurazan (NBD-Cl), glutathione (GSH) and cysteine (Cys) were purchased from J&K. Phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) of pH 7.4 was obtained from Invitrogen Co. All other chemicals used were of analytical grade. Ultrapure water (over 18 MΩ·cm) was used throughout. The stock solution (1.0 mM) of probe **1** was prepared in deoxygenated DMSO. Human plasma samples were obtained from Xijing Hospital, and an informed consent was obtained from each donor.

2. Synthesis of probe 1

A solution of 4-chloro-7-nitrobenzofurazan (102 mg, 0.51 mmol), resorufin sodium salts (90 mg, 0.38 mmol) and triethylamine (69 μL, 0.51 mmol) in DMF (15 mL) was stirred at room temperature. After 3 h, the mixture was diluted with EtOAc (50 mL). The organic layer was separated, washed three times with water (50 mL × 3), and then dried over Na_2SO_4. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography eluted with CH_2Cl_2/EtOAc (v/v, 7:3), affording 7-((7-nitro-benzo[c][1,2,5]oxadiazol-4-yl)oxy)-3H-phenoxazin-3-one (probe **1**) as an orange solid (33.3 mg, yield 23.3%). EI-TOF MS: *m/z* = 376 $[M]^+$. HR-EI-TOF MS: calcd for $[M]^+$, *m/z* = 376.0444; found, *m/z* = 376.0450. ^{1}H NMR (400 MHz, DMSO-D_6): δ = 8.68-8.66 (d, J = 8.0 Hz, 1H), 8.00-7.98 (d, J = 8.0 Hz, 1H), 7.62 (s, 1H), 7.57-7.55 (d, J = 8.0 Hz, 1H), 7.47-7.44 (t, J = 8.0 Hz, 1H), 7.07-7.05 (d, J = 8.0 Hz, 1H), 6.84-6.82 (d, J = 8.0 Hz, 1H), 6.28 (s, 1H). ^{13}C NMR (400 MHz, DMSO-D_6): δ 186.3, 156.9, 151.9, 150.0, 149.0, 146.1, 145.5, 145.2, 135.8, 135.7, 135.5, 132.7, 132.2, 132.0, 118.4, 113.4, 108.9, 106.9.

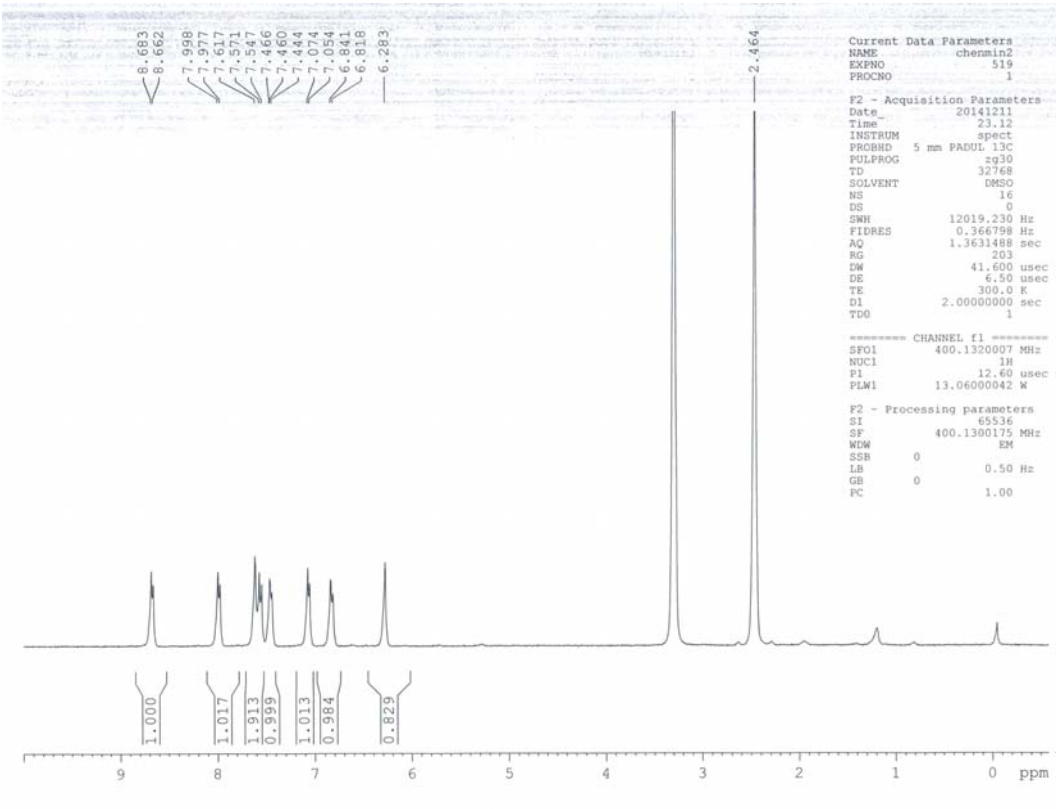

Fig. S1 ^{1}H NMR spectrum of **1** (400 MHz, DMSO-D_6, 298 K).

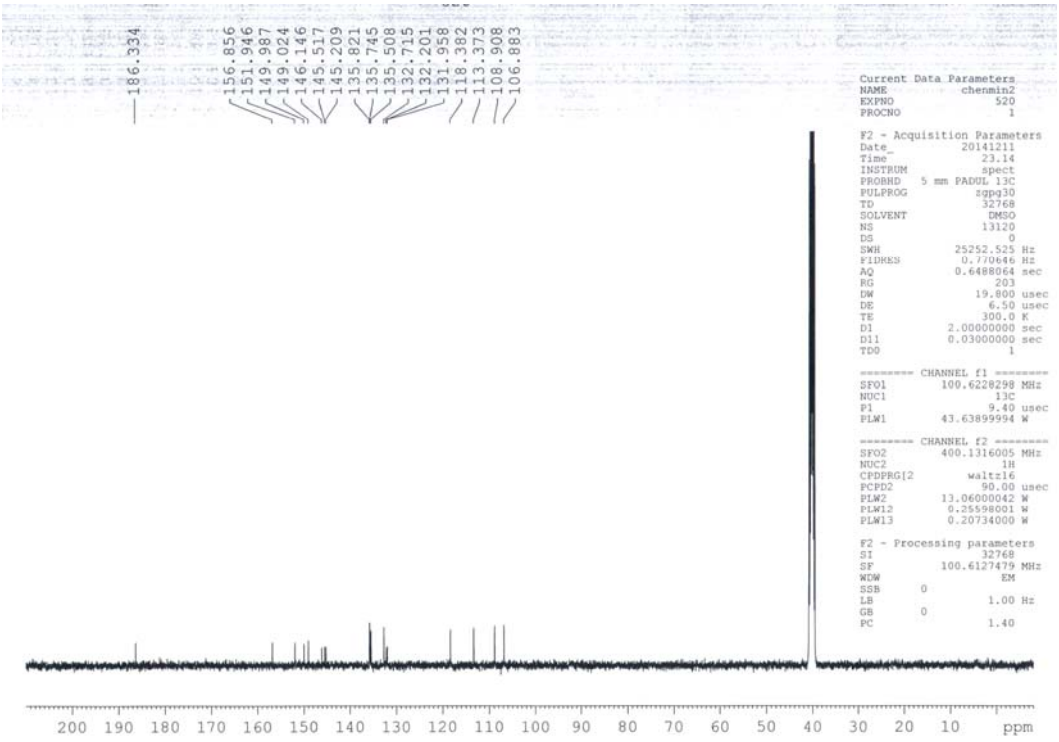

Fig. S2 ^{13}C NMR spectrum of **1** (400 MHz, DMSO-D_6, 298K).

3. General procedure for Cys and GSH detection

Unless otherwise noted, all the measurements were made in 10 mM PBS (pH 7.4) according to the following procedure. In a 2-mL tube, 1 mL of PBS and 10 μL of 1 mM probe were mixed. After incubation at 25 ^{o}C for 10 min in a thermostat, the reaction solution was transferred to a quartz cell of 1-cm optical length to measure absorbance or fluorescence with an excitation wavelength of λ_{ex} = 470 nm and emission wavelengths of λ_{em} = 540 and 585 nm; both excitation and emission slit widths were 5 nm. In the meantime, a blank solution containing no Cys or GSH (control) was prepared and measured under the same conditions for comparison.

Human plasma (1 mL) was deproteinized using acetonitrile (3 mL) and centrifuging at 8,000 rpm for 30 min. The supernatant was diluted in PBS buffer (pH 7.4, 10 mM). The Cys and GSH contents in the plasma sample were determined using the same procedure above and the standard calibration curves.

4. Fluorescence excitation spectra of reaction products

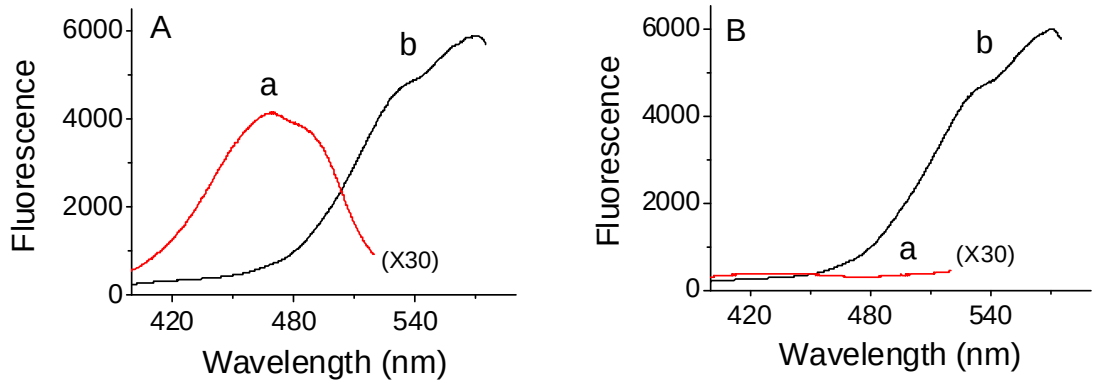

Fig. S3 Fluorescence excitation spectra of **1** (10 μM) reacting with 150 μM of Cys (A) and GSH (B) at λ_{em} = 540 nm (a) and λ_{em} = 585 nm (b), respectively. Note that curve a is magnified by 30 times.

5. Mass spectral analyses of reaction products

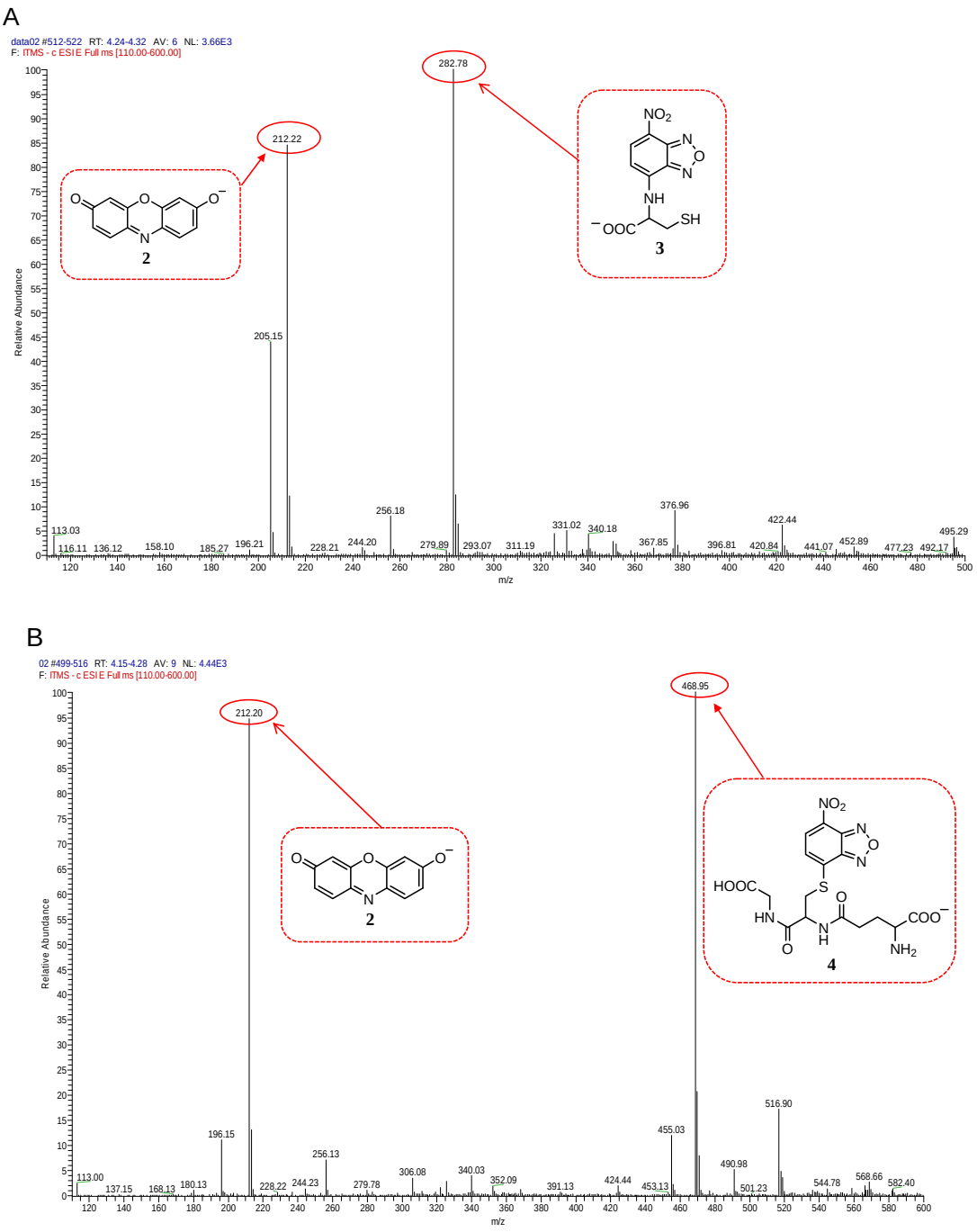

Fig. S4 ESI mass spectra of reaction products of the probe with Cys (A) and GSH (B). The spectra were recorded in negative mode with an LCQ Fleet mass spectrometer (ThermoFisher, USA). In Fig. S4A, the peak at *m/z* = 212 $[M]^-$ was characterized to be resorufin, and the peak at *m/z* = 283 $[M]^-$ was the product **3** (see Fig. 1 in text); similarly, in Fig. S4B the peak at *m/z* = 212 $[M]^-$ was characterized to be resorufin, and the peak at *m/z* = 469 $[M]^-$ was the product **4**.

6. Structure characterization of reaction products

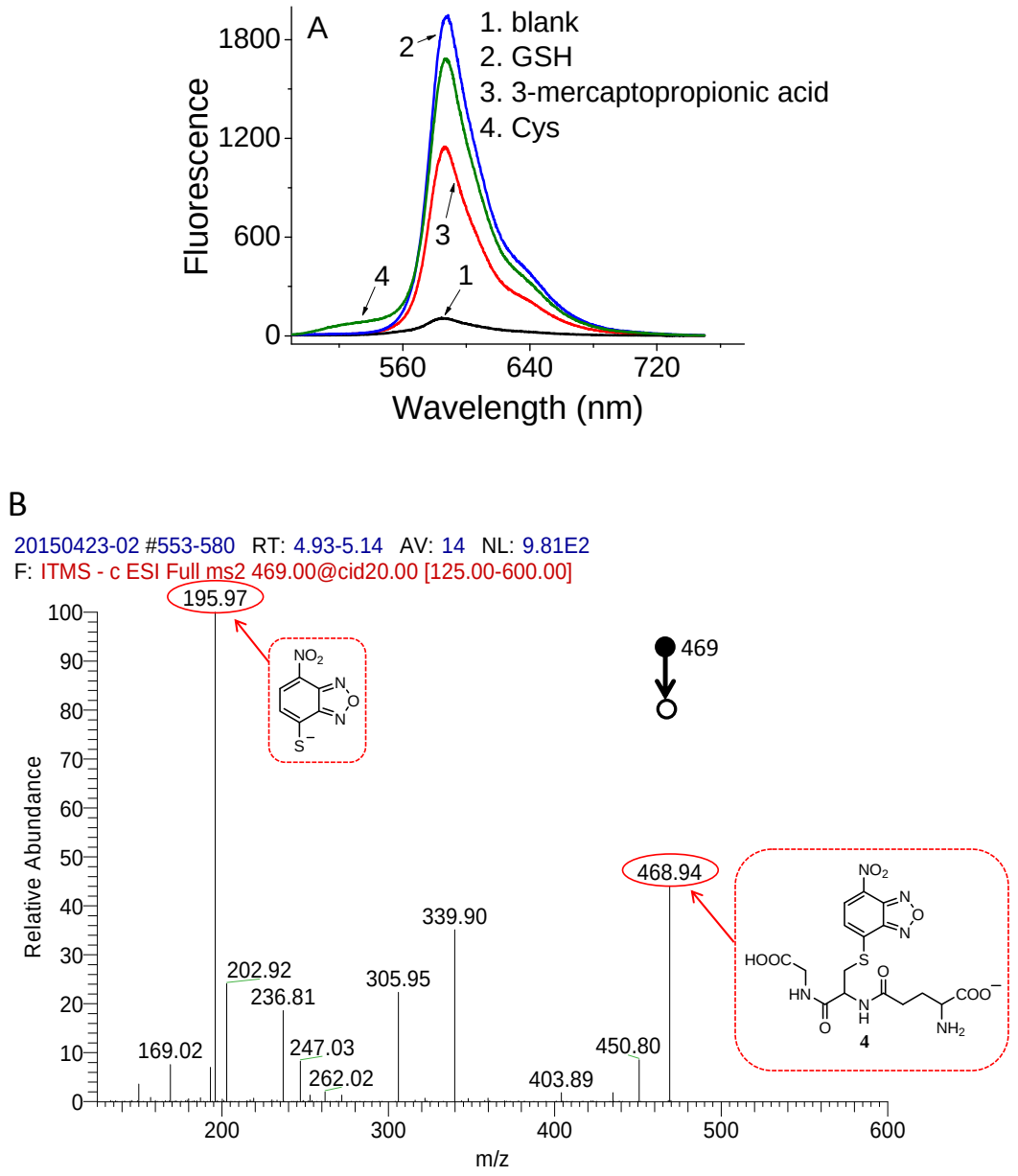

Fig. S5 (A) The fluorescence responses of **1** (10 μM) to Cys (10 μM), GSH (10 μM) and 3-mercaptopropionic acid (10 μM), respectively. As is seen, the reaction product from 3-mercaptopropionic acid produces fluorescence at 585 nm only; whereas Cys, unlike 3-mercaptopropionic acid, shows fluorescence at both 585 nm and 540 nm. The different fluorescence at 540 nm supports that Cys forms an amino-substitution product of NBD. (B) The MS/MS spectrum of the reaction product with *m/z* = 469 from GSH. The spectrum was recorded in negative mode with an LCQ Fleet mass spectrometer (ThermoFisher, USA). The peak at *m/z* = 196 $[M]^-$ was characterized to be the sulfur-substitution product of NBD from GSH.

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

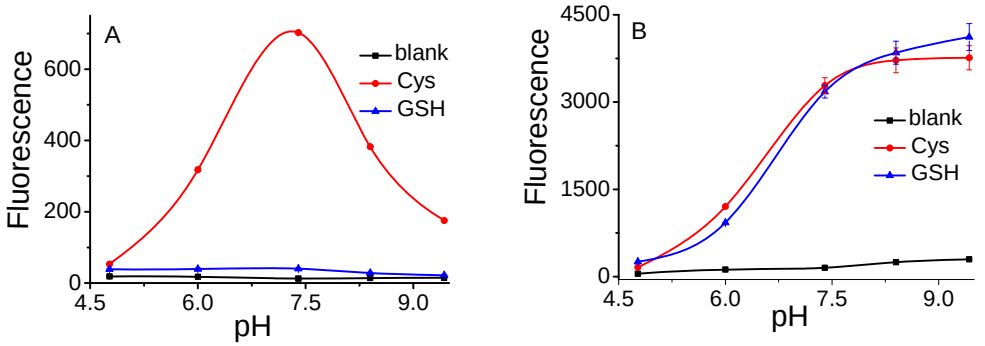

Fig. S6 Effects of pH on the fluorescence of **1** (10 μM) reacting with Cys (150 μM) and GSH (150 μM) at λ_{em} = 540 nm (A) and λ_{em} = 585 nm (B), respectively. The blank denotes the probe solution alone. λ_{ex} = 470 nm.

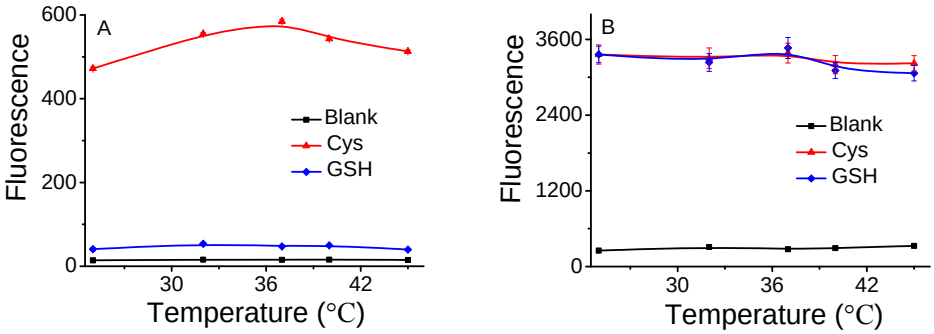

Fig. S7 Effects of temperature on the fluorescence of **1** (10 μM) reacting with Cys (150 μM) and GSH (150 μM) at λ_{em} = 540 nm (A) and λ_{em} = 585 nm (B), respectively. The blank denotes the probe solution alone. λ_{ex} = 470 nm.

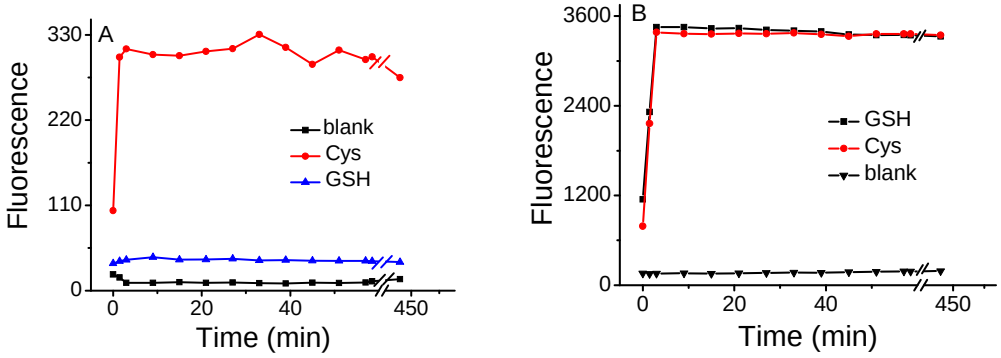

Fig. S8 Effects of reaction time on the fluorescence of **1** (10 μM) reacting with Cys (150 μM) and GSH (150 μM) at λ_{em} = 540 nm (A) and λ_{em} = 585 nm (B), respectively. The blank denotes the probe solution alone. λ_{ex} = 470 nm.

8. Working curves of probe 1 to Cys and GSH

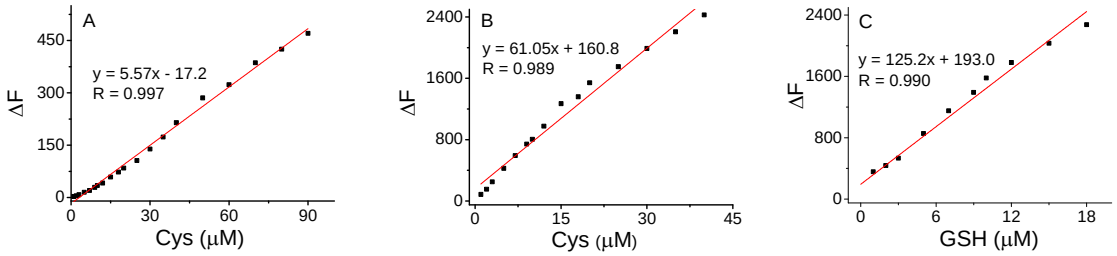

Fig. S9 Working curves of probe **1** (10 μM) for Cys at 540 nm (A) and 585 nm (B), and for GSH at 585 nm (C), respectively.

9. Selectivity study

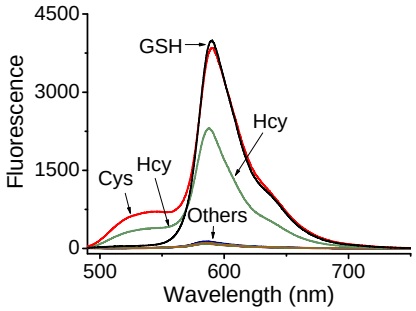

Fig. S10 Fluorescence response of **1** (10 μM) to different potential interfering substances (15 equiv.). The reaction was conducted for 10 min in PBS (10 mM, pH 7.4) at 25 °C. Others contain: control (**1** only), Gln, Ala, Asp, Ser, Arg, $MgCl_2$, $CaCl_2$, $CuSO_4$, $NaNO_2$, $NaNO_3$, creatinine, uric acid, glucose, and ascorbic acid. λ_{ex} = 470 nm.

10. Effect of Hcy on the detection of Cys

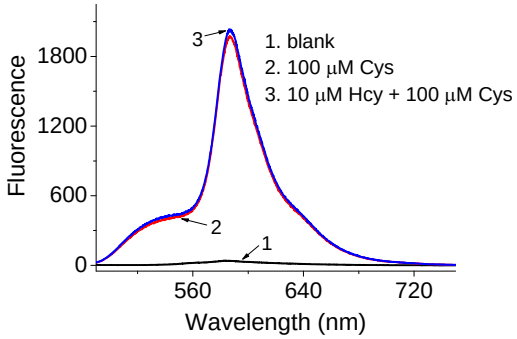

Fig. S11 Fluorescence emission spectra of **1** (10 μM) in different systems. Curve 1: blank (probe alone); curve 2: probe + 100 μM Cys; curve 3: probe + 10 μM Hcy + 100 μM Cys. The measurements were made at λ_{ex} = 470 nm in 10 mM pH 7.4 PBS. As is seen, Hcy at a concentration ratio of 1:10 (Hcy to Cys) shows no significant interference (error <10%).

11. Studies on fluorescence additivity in the linear range

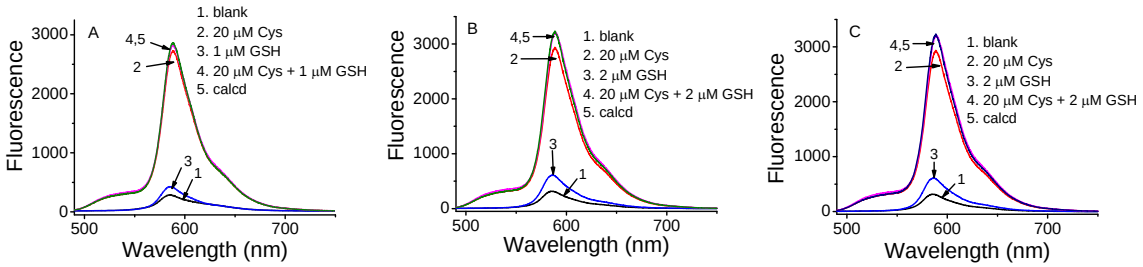

Fig. S12 Fluorescence emission spectra of **1** (10 μM) in the presence of 20 μM Cys with different concentrations of GSH: 1 μM (A), 2 μM (B) and 4 μM (C). Curve 1: blank (probe solution alone; control); curve 2: probe + 20 μM Cys; curve 3: probe + GSH with different concentrations; curve 4: probe + 20 μM Cys + GSH with different concentrations; curve 5: the additive fluorescence spectra obtained through the theoretical calculation. The measurements were made at λ_{ex} = 470 nm and 25 ^{o}C in 10 mM PBS (pH 7.4)