Supplementary Information for

Construction of a FRET-based ratiometric fluorescent thiol probe

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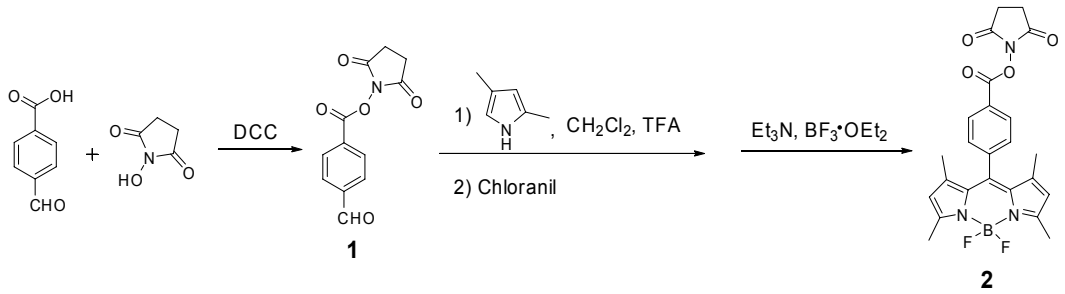
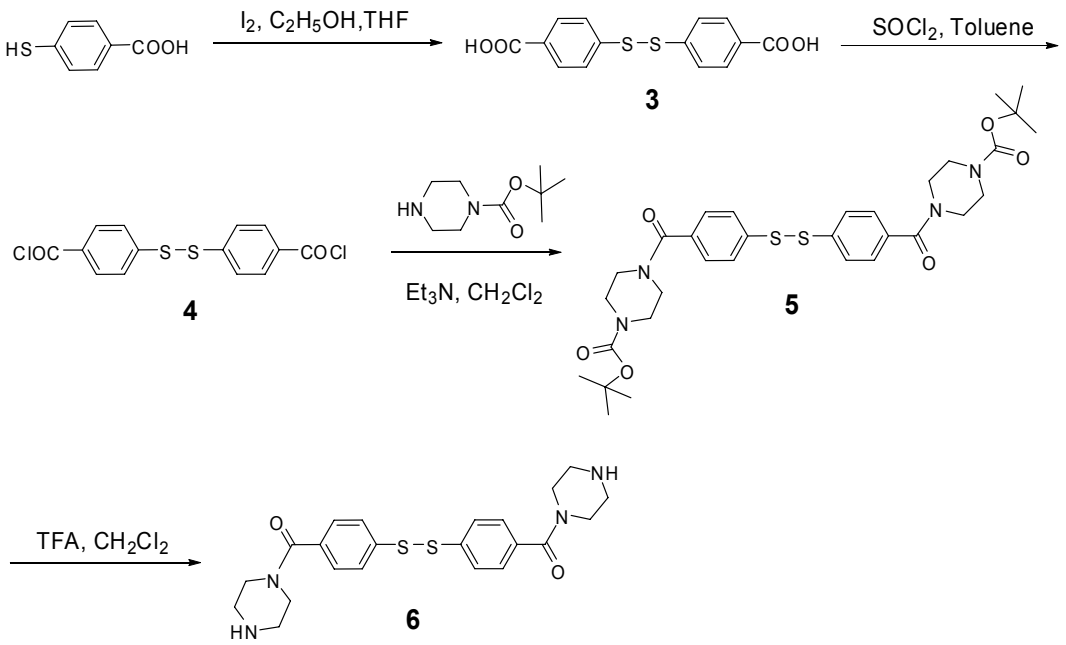
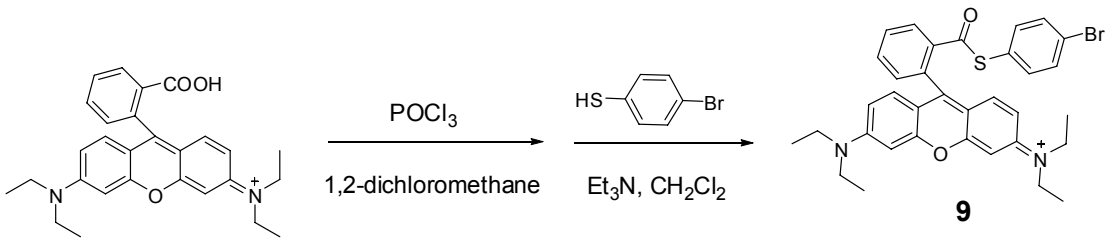
Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, all melting points were uncorrected; Low resolution mass spectra were performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or Agilent 1100 HPLC/MSD spectrometer; High resolution mass spectrometric (HRMS) analyses were measured on a Finnigan MAT 95 XP spectrometer. NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a LabTech UV Power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer, with the excitation and emission slit widths at 5.0 nm; Cells imaging were performed with an Olympus FV500 confocal microscope; The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; TLC analyses were performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.



Scheme S1. Synthesis of the Bodipy donor building block **2**.



Scheme S2. Synthesis of the linker building block **6**.



Scheme S3. Synthesis of the reference compound **9**.

Synthesis

Synthesis of 4-formylbenzoic succinimidyl ester 1

Compound **1** was synthesized according to the reported procedure (J. A. Phillips, E. L. Morgan, Y. Dong, G. T. Cole, C. McMahan, C.-Y. Hung, S. D. Sanderson, *Bioconjugate Chem*. 2009, **20**, 1950-1957.). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 10.15 (s, 1H, RC*H*O), 8.32 (d, J = 8.4 Hz, 2H, RC_6H_4R'), 8.04 (d, J = 8.4 Hz, 2H, RC_6H_4R'), 2.95 (d, J = 4.4 Hz, 4H, CH_2); MS (EI): m/z = 247.1 $(M)^+$.

Synthesis of Bodipy compound 2

4-formylbenzoic succinimidyl ester **1** (494.4 mg, 2.0 mmol) and 2,4-dimethylpyrrole (380.6 mg, 4.0 mmol) were dissolved in 300 mL dry CH_2Cl_2 (N_2 was bubbled through CH_2Cl_2 for 30 min), and five drops of TFA were added dropwise. The resulting red solution was stirred at room temperature in the dark overnight. After chloranil (495.8 mg, 2.0 mmol) was added, the reaction mixture was stirred for additional four hour, followed by the addition of 3mL Et_3N and 3mL BF_3•OEt_2. After further stirring for 30 min, the reaction mixture was washed three times with water and dried over Na_2SO_4. The solvent was then evaporated and the resulting residue was purified by flash column chromatography (silica gel, CH_2Cl_2: petroleum ether = 1:1, V/V) to give the orange solid **2** (85.0 mg, 9.1%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.28 (d, J = 8.8 Hz, 2H, RC_6H_4R'), 7.50 (d, J = 8.4 Hz, 2H, RC_6H_4R'), 6.01 (s, 2H, pyrrole-H), 2.96 (d, J = 7.2 Hz, 4H, CH_2), 2.57 (s, 6H, CH_3), 1.38 (s, 6H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 170.2, 161.3, 155.5, 144.0, 142.5, 141.0, 139.6, 130.8, 129.5, 125.1, 120.6, 30.5, 25.5, 14.2; MS (EI): m/z = 465.2 $(M)^+$; $C_{24}H_{22}BF_2N_3O_4$: calcd. C 61.96, H 4.77, N 9.03; found C 61.67, H 4.91, N 9.31.

Synthesis of 4,4'-dithiodibenzoic acid 3

Compound **3** was synthesized according to the reported procedure (K. Takeda, A. Kuwahara, K. Ohmori, T. Takeuchi, *J. Am. Chem. Soc*. **2009**, *131*, 8833-8838; A. Alagic, A. Koprianiuk, and R. Kluger, *J. Am. Chem. Soc*. **2005**, *127*, 8036-8043.). ^{1}H NMR (400 MHz, DMSO) δ ppm 7.94 (d, J = 8.4 Hz, 4H, RC_6H_4R'), 7.64 (s, J = 8.4 Hz, 4H, RC_6H_4R'); MS (ESI): m/z = 305 $(M-H)^-$.

Synthesis of 4,4'-Dithiodibenzoyl chloride 4

Compound **4** was synthesized according to the reported procedure (A. Alagic, A. Koprianiuk, and R. Kluger, *J. Am. Chem. Soc*. **2005**, *127*, 8036-8043.). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.06 (d, J = 8.8 Hz, 4H, RC_6H_4R'), 7.59 (d, J = 8.8 Hz, 4H, RC_6H_4R'); MS (EI): m/z = 341.9 $(M)^+$.

Synthesis of compound 5.

To a solution of 4,4'-Dithiodibenzoyl chloride (2.0 g, 5.9 mmol) and *mono*-Boc-piperazine (2.2 g, 11.7 mmol) in dried CH_2Cl_2 (10 mL) was dropwise added Et_3N (1.5 mL) in dried CH_2Cl_2 (20 mL) over 30 min with stirring at room temperature, and the reaction mixture was further stirred for 30

min. The solvent was evaporated under reduced pressure, and the resulting white solid was recrystallized in ethanol to obtain the pure product (3.5 g) in 92% yield. mp 162-164^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.53 (d, J = 8.4 Hz, 4H, RC_6H_4R'), 7.35 (d, J = 8.4 Hz, 4H, RC_6H_4R'), 3.40-3.73 (m, 16H, CH_2), 1.47 (s, 18H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 169.8, 154.5, 138.7, 134.1, 128.0, 126.8, 80.4, 45.8, 28.3. MS (ESI): m/z = 643.0 $(M+H)^+$. $C_{32}H_{42}N_4O_6S_2$: calcd. C 59.79, H 6.59, N 8.72; found C 59.42, H 6.72, N 8.55.

Synthesis of compound 6

CF_3COOH (1.5 mL) was added to a solution of compound **5** (2.0 g, 3.1 mmol) in CH_2Cl_2 (3 ml), and the reaction mixture was stirred at room temperature overnight. The solvent was evaporated under vacuum, and the resulting residue was dissolved in CH_2Cl_2 (30 mL) and neutralized by 1 M NaOH aqueous solution. The organic phase was separated and dried with $MgSO_4$. The solvent was then removed under reduced pressure, and the residue was purified by flash column chromatography (silica gel, methanol: CH_2Cl_2 = 1: 1) to give the white solid **6** (0.7 g, 52 %). Mp 54-56^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.42 (s, 2H, -NH-), 7.62 (d, J = 8.4 Hz, 4H, RC_6H_4R'), 7.50 (d, J = 8.4 Hz, 4H, RC_6H_4R'), 3.60-3.75 (m, 8H, CH_2), 3.11-3.17 (m, 8H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 168.4, 137.5, 133.9, 128.4, 126.5, 42.4. MS (ESI): m/z = 443.1 $(M+H)^+$; MS (EI): m/z = 442.1 (M); HRMS (EI): $(M)^+$ calcd. for $C_{22}H_{26}N_4O_2S_2$, 442.1497; found, 442.1503.

Synthesis of Bodipy amide 7

Compound **6** (300.0 mg, 0.7 mmol) and Bodipy succinimidyl ester **2** (200.0 mg, 0.4 mmol) were dissolved in dry CH_2Cl_2 (10 mL), and the reaction mixture was refluxed for 24 hours. The solvent was then evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (silica gel, CH_2Cl_2: ethanol = 25:1, V/V) to give the orange solid **7** (110.0 mg, 32.3%). Melting point: 150-152^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.52-7.58 (m, 6H, ArH), 7.35-7.40 (m, 6H, ArH), 6.00 (s, 2H, pyrrole-H), 2.89-3.78 (m, 16H, CH_2), 2.56 (s, 6H, CH_3), 1.37 (s, 6H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 169.9, 169.5, 156.0, 142.7, 140.0, 139.3, 138.3, 137.2, 135.6, 134.5, 133.6, 131.1, 128.6, 128.1, 126.9, 121.5, 62.1, 29.7, 14.6; MS (ESI): m/z = 793.1 $(M+H)^+$. $C_{42}H_{43}BF_2N_6O_3S_2$: calcd. C 63.63, H 5.47, N 10.60; found C 63.38, H

5.77, N 10.75.

Synthesis of compound 8

$NaBH_4$ (12.5 mg, 0.3 mmol) was added to a solution of compound **7** (26.0 mg, 0.03 mmol) in THF (8 mL), and the reaction was stirred at room temperature for 10 min (The reduction reaction was essentially complete within 10 min by the TLC analysis). The reaction was then quenched by addition of saturated NH_4Cl aqueous solution (40 mL), and the solution was extracted with CH_2Cl_2 (3 × 40 mL). The organic layer was combined, washed with water (50 mL), and dried with $MgSO_4$. The solvent was evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (silica gel, CH_2Cl_2: ethanol = 100:1, V/V) to give orange solid **8** (14.2 mg, 75%). Mp160-163^oC; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 7.54-7.58 (m, 4H, ArH), 7.39 (d, J = 6.8 Hz, 4H, ArH), 6.00 (s, 2H, pyrrole-H), 3.53-3.74 (m, 8H, CH_2), 2.56 (s, 6H, CH_3), 1.38 (s, 6H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 169.1, 158.3, 155.6, 143.1, 142.3, 142.0, 141.8, 137.3, 134.5, 130.8, 130.5, 128.8, 128.3, 127.0, 121.9, 63.5, 31.2, 14.5; MS (ESI): m/z = 571.2 $(M-H)^-$. $C_{31}H_{31}BF_2N_4O_2S$: calcd. C 65.04, H 5.46, N 9.79; found C 64.69, H 5.70, N 9.88.

Synthesis of probe NRFTP

A solution of rhodamine B (150.0 mg, 0.3 mmol) and phosphorus oxychloride (1 mL) in 1,2-dichloromethane (20 mL) was refluxed for 4 h. After cooling, the reaction mixture was concentrated under reduced pressure to obtain crude rhodamine acid chloride, which was then added to a solution of compound **8** (149.0 mg, 0.26 mmol) in dry CH_2Cl_2 (5 mL). After the reaction mixture was stirred for 1 min, Et_3N (0.3 mL) was then added, and the reaction mixture was further stirred at room temperature for 2h. The solvent was evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (silica gel, CH_2Cl_2: ethanol : petroleum ether = 15:1:5, V/V) to give the compound **NRFTP** as a purple solid (76.0 mg, 29.5%). ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.35 (d, J = 7.6 Hz, 1H, ArH), 7.83-7.88 (m, 2H, ArH), 7.55 (d, J = 8.0 Hz, 2H, ArH), 7.38-7.40 (m, 5H, ArH), 7.32 (d, J = 7.6 Hz, 2H, ArH), 7.12 (d, J = 9.2 Hz, 2H, ArH), 6.89 (dd, J = 9.6, 2 Hz, 2H, ArH), 6.81 (d, J = 2.4 Hz, 2H, ArH), 5.99 (s, 2H, pyrrole-H), 3.60-3.65 (m, 16H, CH_2), 2.56 (s, 6H, CH_3), 1.36 (s, 6H, CH_3), 1.31 (t, J = 7.2 Hz,

12H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 173.3, 172.9, 167.8, 157.8, 155.75, 155.71, 139.1, 137.1, 136.8, 132.5, 131.2, 131.0, 130.9, 130.1, 129.8, 128.9, 128.8, 128.5, 128.3, 127.1, 126.8, 121.5, 121.4, 114.0, 96.3, 62.1, 46.2, 32.0, 14.2, 12.7; MS (ESI): m/z = 997 $(M)^+$; HRMS (FAB): $(M)^+$ calcd. for $C_{59}H_{60}BF_2N_6O_4S^+$ 997.4452; found, 997.4456.

Synthesis of the reference compound 9

Compound **9** was prepared by employing the same procedure for the synthesis of compound **NRFTP** except that 4-bromobenzenethiol (228.0 mg, 1.1 mmol) in dry CH_2Cl_2 (10 mL) was used. The residue was purified by flash column chromatography (silica gel, acetone) to give compound **9** as a purple solid (385.0 mg, 53%). ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.35 (dd, *J* = 7.6, 0.8 Hz, 1 H, ArH), 7.90 (t, *J* = 7.2, 1H, ArH), 7.83 (t, *J* = 8.0 Hz, 1H, ArH), 7.45 (d, *J* = 8.8 Hz, 2H, ArH), 7.39 (d, *J* = 7.2 Hz, 1H, ArH), 7.11 (d, *J* = 9.2Hz, 4H, ArH), 6.93 (dd, *J* = 7.2, 2.4 Hz, 2H, ArH), 6.77 (d, *J* = 2.4Hz, 2H, ArH), 3.64 (q, *J* = 7.2Hz, 8H, CH_2), 1.32 (t , *J* = 7.2Hz, 12H, CH_3). MS (ESI): m/z = 613.2 $(M)^+$. HRMS (EI): $(M)^+$ calcd. for $C_{34}H_{34}BrN_2O_2S$, 613.1519; found, 613.1515.

Preparation of the test solution.

A stock solution of probe **NRFTP** (1.3×10^{-3} M) was prepared in CH_3CN. The test solution of the probe (1.3 μM) in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent) was prepared by placing 0.002 mL of the probe stock solution, 0.9 mL CH_3CN, and 1.1 mL of 45.5 mM sodium phosphate buffer (pH = 7.4). The solutions of various testing species were prepared from Cys, Phe, Ala, Gly, Glu, Arg, Lys, Tys, Leu, Ser, Val, glucose, KCl, $CaCl_2$, $ZnCl_2$, $FeCl_3$, H_2O_2, NADH, respectively. The resulting solution was shaken well and incubated for 20 min at room temperature before recording the spectra.

Determination of fluorescence quantum yield: [1-4]

Fluorescence quantum yield was determined using optically matching solutions of rhodamine 6G ($\Phi_{F(S)}$ = 0.9 in ethanol [5]) or fluorescein ($\Phi_{F(S)}$ = 0.93 in H_2O [6]) as a standard. The quantum yield was calculated using the following equation:

$$\Phi_{F(X)} = \Phi_{F(S)} (A_S F_X / A_X F_S) (n_X / n_S)^2$$

Where Φ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used. Subscripts $_S$ and $_X$ refer to the standard and to the unknown, respectively.

Calculation of energy transfer efficiency [7-10]

Energy transfer efficiency (E) was calculated using the following equation as reported. [7-10]

$$E = 1 - F_{DA} / F_D$$

Where, F_{DA} and F_D denote the donor fluorescence intensity with and without an acceptor, respectively.

Cell culture and fluorescence imaging.

Hela cells were seeded in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum for 24 h. Hela cells were then incubated with probe **NRFTP** (2 μM) in the culture medium for 30 min at 37^{o}C. After washing with PBS three times to remove the remaining probe, the confocal fluorescence images were acquired with an OLYMPUS FV500 confocal microscope. Excitation wavelength of laser was 488 nm, and emissions were centered at 515 ± 10 nm and 610 ± 10 nm (double channels). For the N-ethylmaleimide control experiment, the cell were pretreated with N-ethylmaleimide (1 mM) for 30 min at 37^{o}C followed by washing with PBS three times, and then incubated with probe **NRFTP** (2 μM) for 30 min at 37^{o}C. Fluorescence imaging was then carried out after washing the cells with PBS buffer three times.

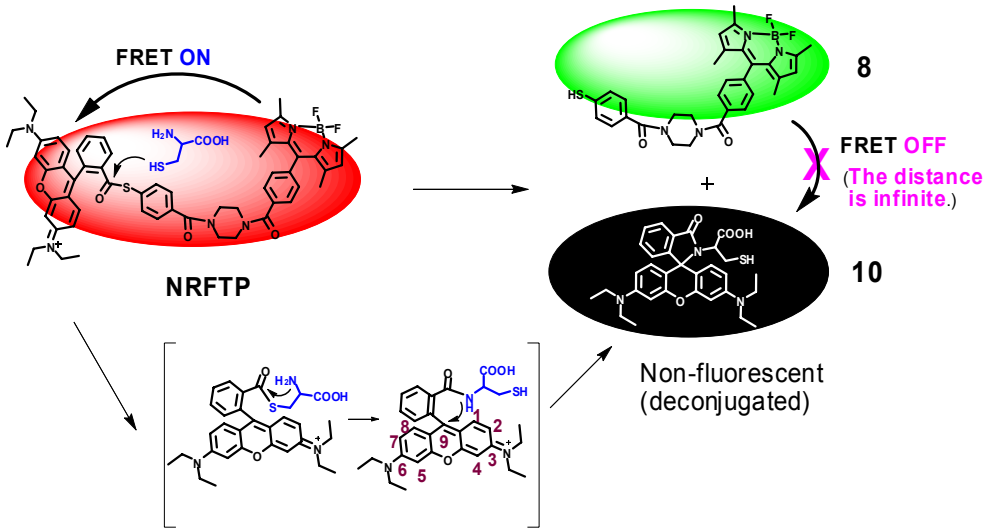


Figure S1. NCL mechanism-based design of ratiometric fluorescent thiol probe **NRFTP**.

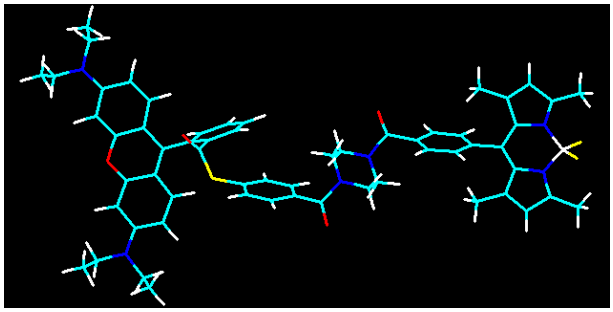


Figure S2. Energy minimized (HyperChem v. 7.5, PM3 semi-empirical module) structure of probe **NRFTP**. Calculated B-O distance 22.88 Å.

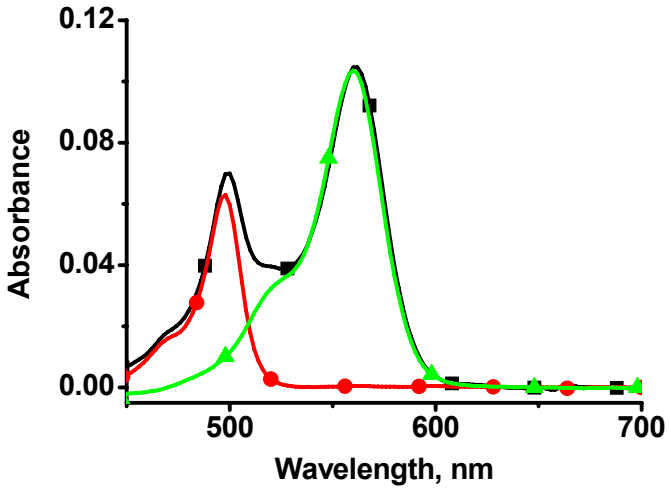


Figure S3. The absorption spectra of probe **NRFTP** (1.3 μM) (■), reference Bodipy donor **8** (1.3 μM) (●), and reference rhodamine acceptor **9** (1.3 μM) (▲) in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent).

Table S1. The spectroscopic data for compounds **NRFTP** and **8-10**.

compound	λ_{abs} (nm)	Logε ($M^{-1}cm^{-1}$)	λ_{em} (nm)	Φ_f
NRFTP	500	4.73	510	0.005 [a]
	562	4.91	590	0.162 [a]
8	498	4.69	511	0.464 [a]
9	562	4.90	590	0.186 [b]
10	--	--	--	0.003 [b]

[a] λ_{ex} = 470 nm. [b] λ_{ex} = 540 nm

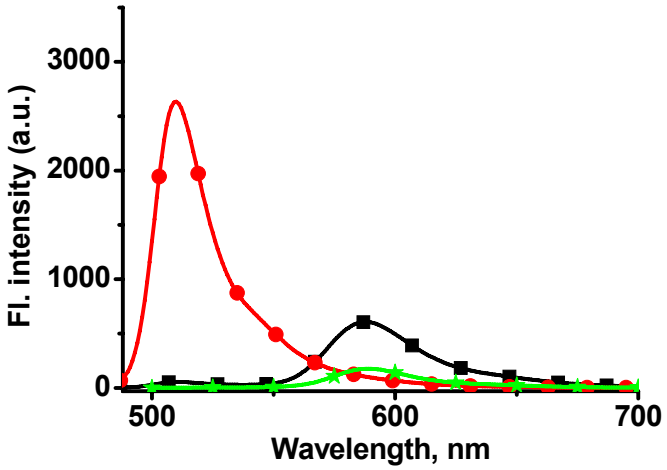


Figure S4. The fluorescence emission spectra of the probe **NRFTP** (1.3 μM) (■), the reference Bodipy donor **8** (1.3 μM) (●), and the reference rhodamine acceptor **9** (1.3 μM) (★) in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent). The excitation wavelength was 470 nm.

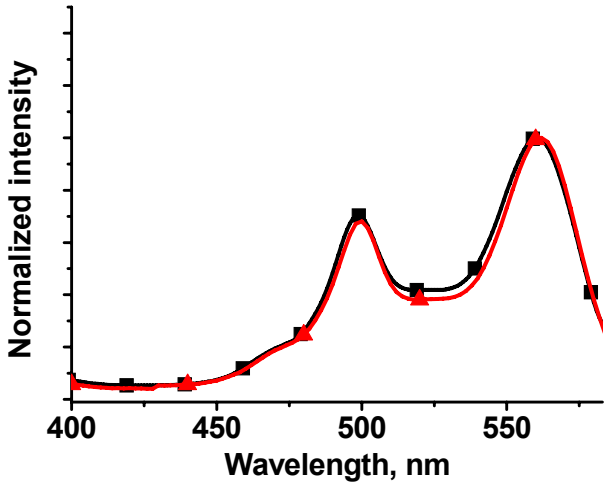


Figure S5. The absorption (▲) and corrected excitation (■) spectra of probe **NRFTP** in phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent). The excitation spectrum was monitored at 590 nm.

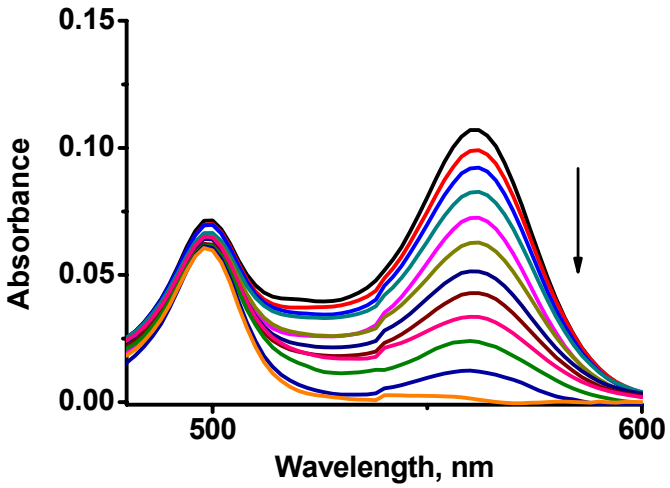


Figure S6. Absorption spectral changes of probe **NRFTP** (1.3 μM) upon addition of Cys (0 – 100 μM) in phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent). The spectral were recorded after mixing Cys with probe **NRFTP** for 20 minutes.

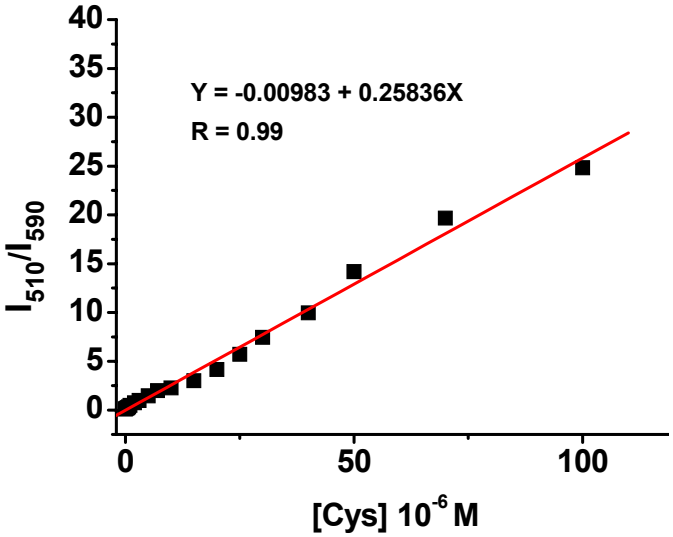


Figure S7. Plot of the fluorescence intensity ratios at 510 and 590 nm (I_{510}/I_{590}) as a function of the Cys concentration.

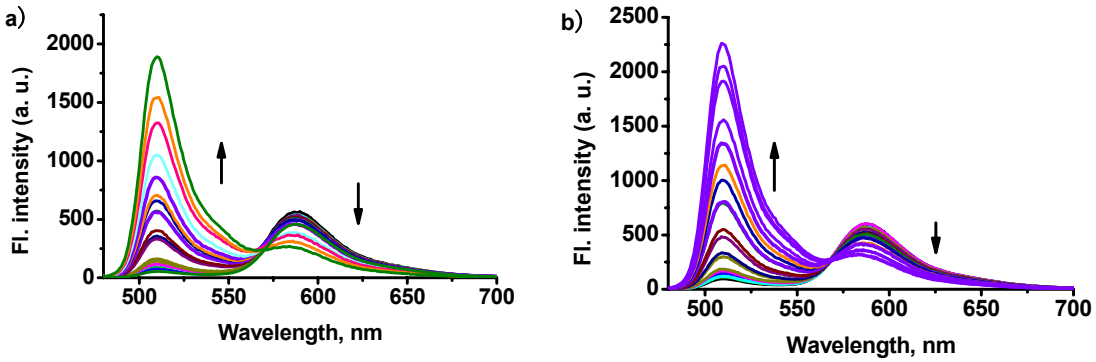


Figure S8. Fluorescence spectra (with excitation at 470 nm) of probe **NRFTP** (1.3 μM) upon addition of increasing concentrations (0 - 200 μM) of homocysteine (a) or glutathione (b) in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent).

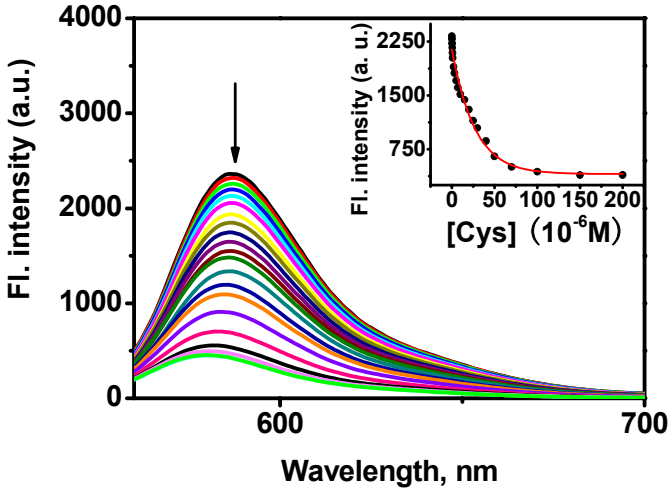


Figure S9. Fluorescence spectral changes (with excitation at 550 nm) of probe **NRFTP** (1.3 μM) upon addition of increasing concentrations (0 - 200 μM) of Cys. The inset shows the changes of fluorescent intensity at 590 nm (λ_{ex} = 550 nm) to increasing concentration of Cys.

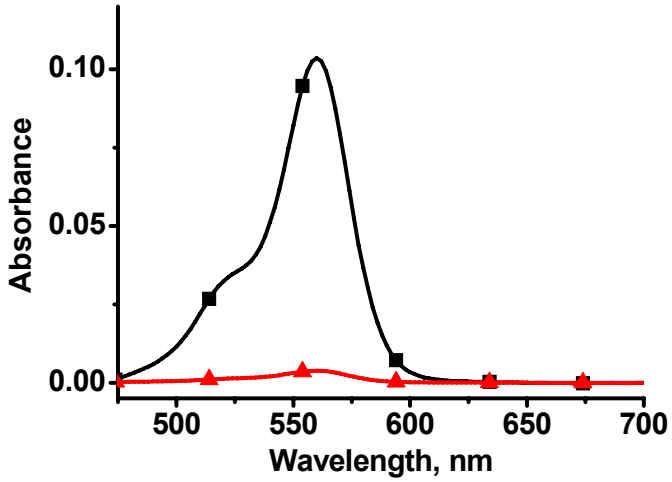


Figure S10. The absorption spectra of the reference rhodamine acceptor **9** (1.3 μM) in the absence (■) or presence (▲) of Cys (100 μM) in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent).

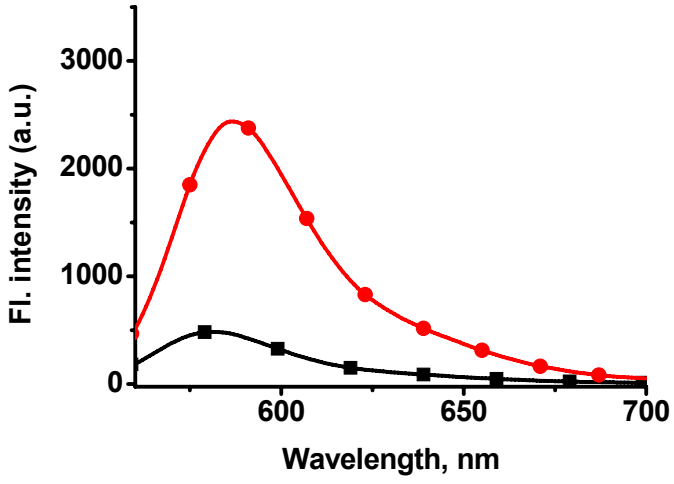


Figure S11. The fluorescence emission spectra (λ_{ex} = 550 nm) of the reference rhodamine acceptor **9** (1.3 μM) in the absence (●) or presence (■) of Cys (100 μM) in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent).

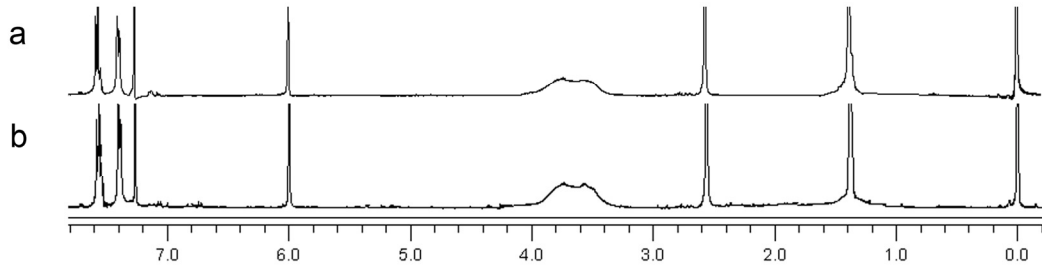


Figure S12. Partial ^{1}H NMR (400 MHz) spectra of (a) the separated product **8** of probe **NRFTP** + Cys, and (b) the standard compound **8**.

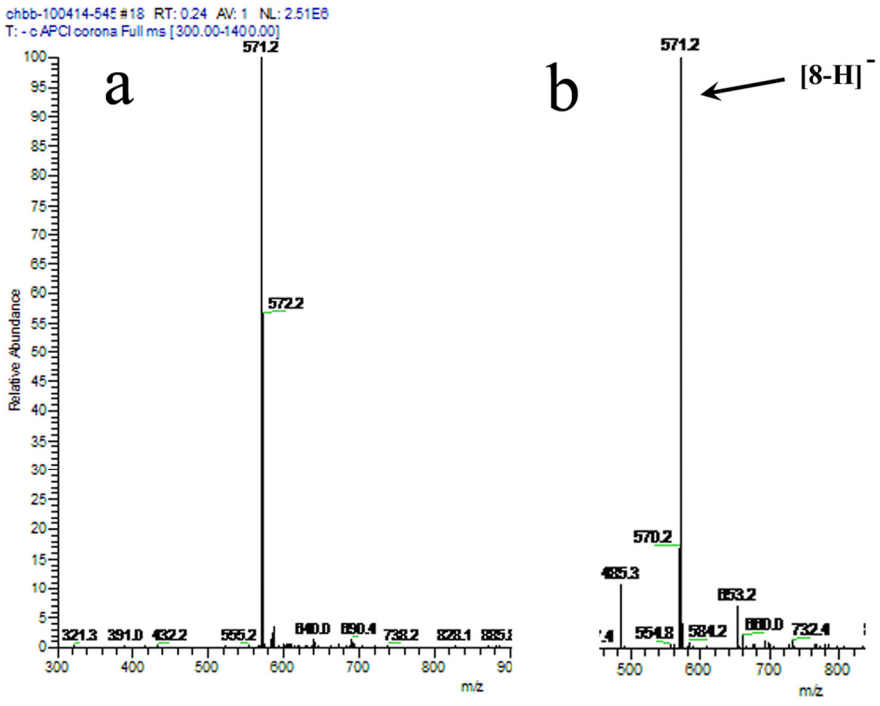


Figure S13. Partial Mass spectra of (a) the separated product **8** of probe **NRFTP** + Cys, and (b) the standard compound **8**.

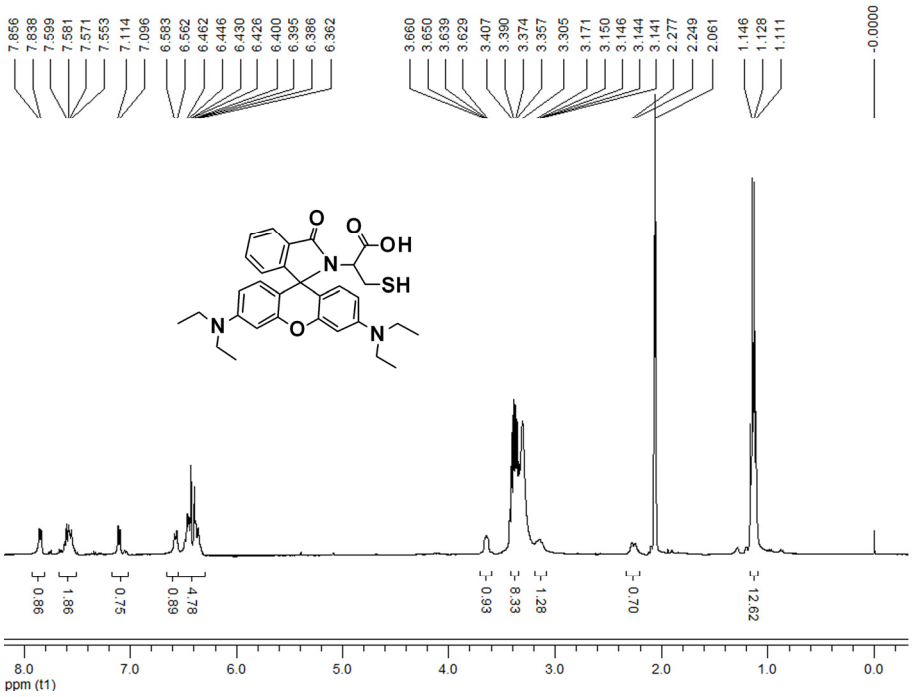


Figure S14. ^{1}H NMR (400 MHz) spectrum of the isolated product **10** of probe **NRFTP** + Cys.

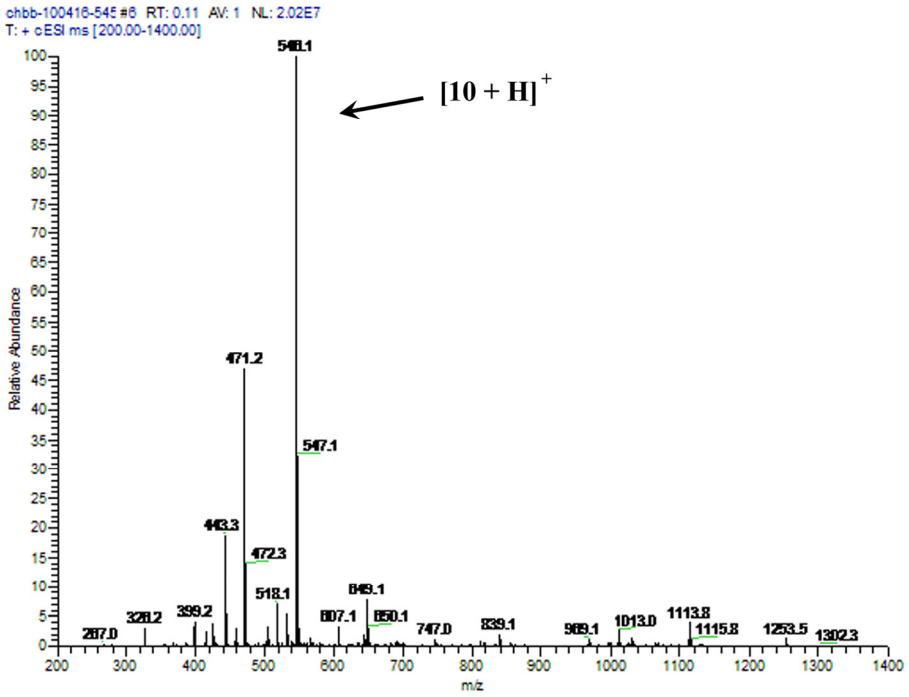


Figure S15. Mass spectrum of the isolated product **10** of probe **NRFTP** + Cys.

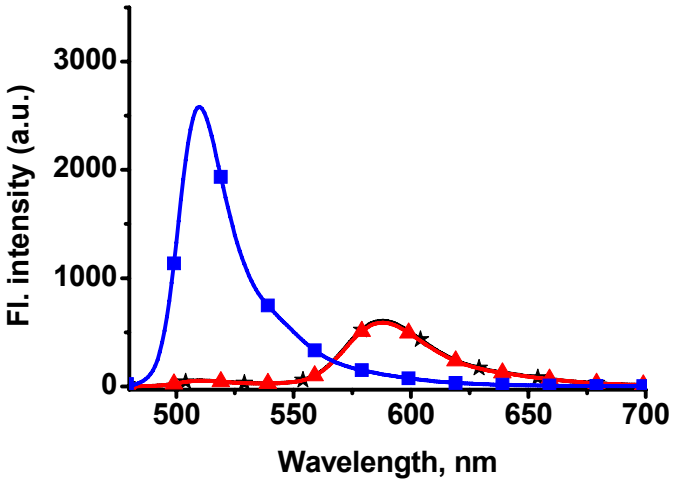


Figure S16. The fluorescence spectra (with excitation at 470 nm) of **NRFTP** (1.3 μM) in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent) kept for 0.5 hour (▲) and for 3 day (★). For comparison, the fluorescence spectrum of **NRFTP** (λ_{ex} = 470 nm) with addition of Cys (100 μM) for 20 min was also showed (■).

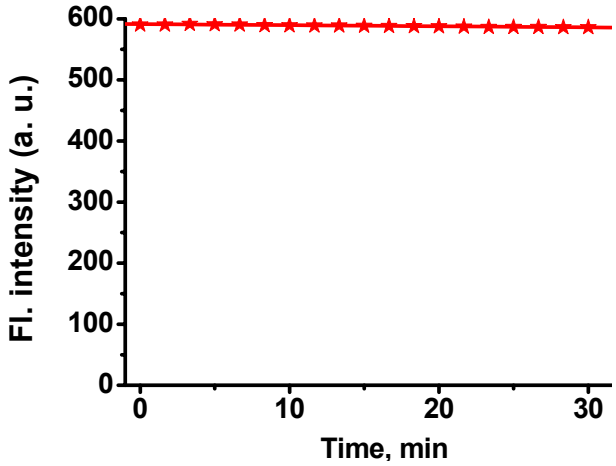


Figure S17. Fluorescence intensity at 590 nm for the probe **NRFTP** in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent) after excitation at 470 nm for 0-30 minutes.

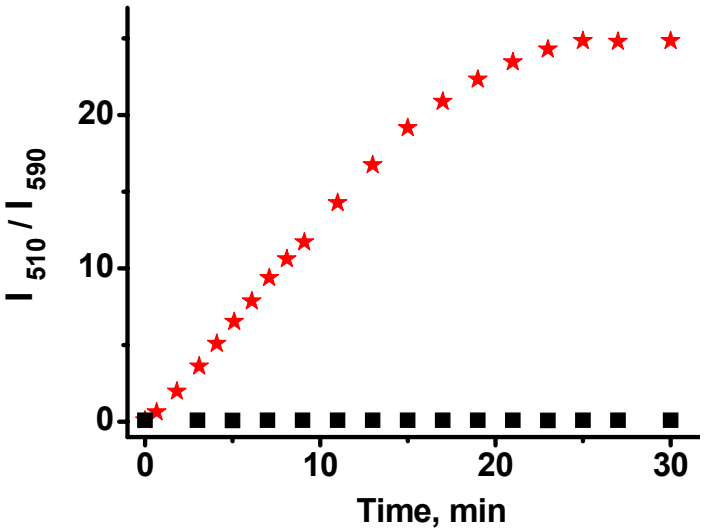


Figure S18. Time dependent fluorescence ratio changes of probe **NRFTP** (1.3 μM) in the absence (■) or presence of 100 μM Cys (★).

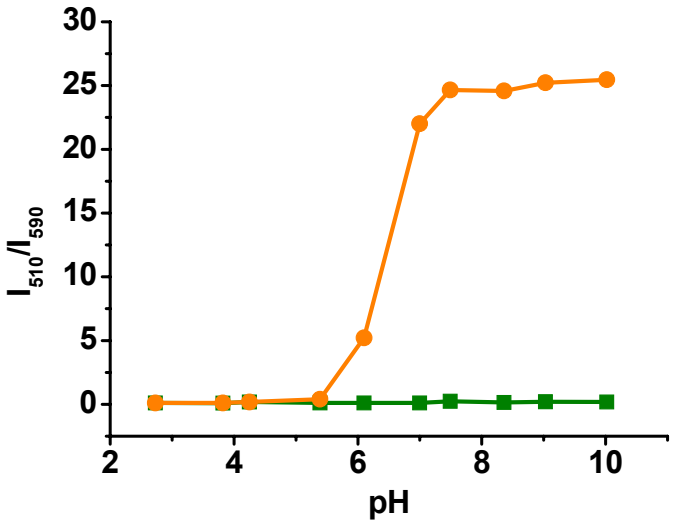


Figure S19. The fluorescence intensity ratios at 510 and 590 nm (I_{510}/I_{590}) for probe **NRFTP** (1.3 μ M) in the absence (■) or presence (●) of Cys (100 μ M) at various pH values. The excitation wavelength was 470 nm.

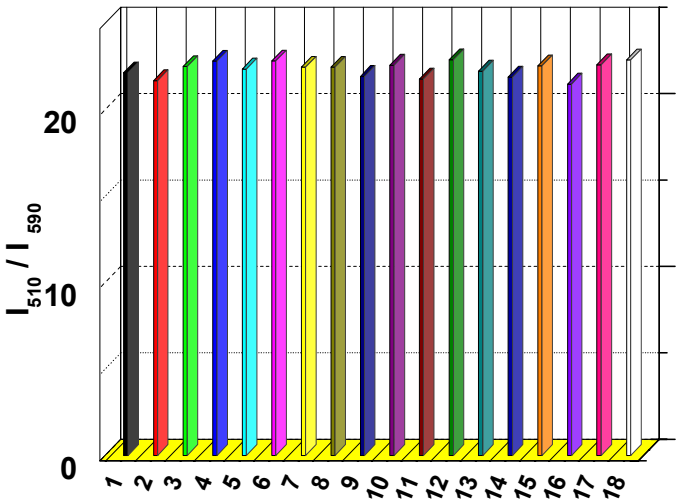


Figure S20. Ratiometric fluorescence (I_{510} / I_{590}) response of probe **NRFTP** (1.3 μM) to 100 μM of Cys in the presence of 100 μM of various species: (1) Free; (2) KCl; (3) $CaCl_2$; (4) $ZnCl_2$; (5) $FeCl_3$; (6) glucose; (7) NaDH; (8) H_2O_2; (9) Phe; (10) Ala; (11) Gly; (12) Glu; (13) Arg; (14) Lys; (15) Tys; (16) Leu; (17) Ser; (18) Val. λ_{ex} = 470 nm.

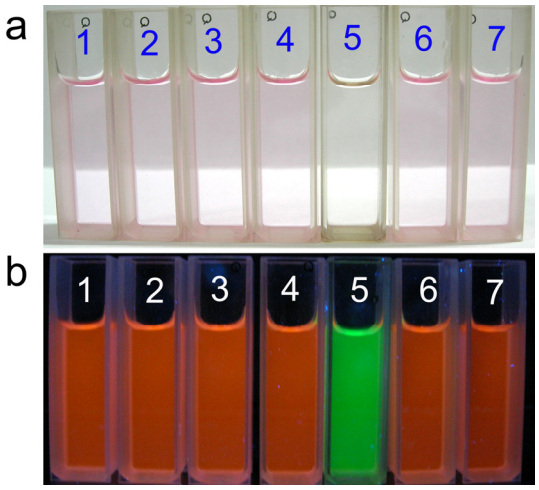


Figure S21. Color changes of probe **NRFTP** (1.3 μM) in the presence of 100 μM various species. 1: free, 2: Phe, 3: Ala, 4: Tys, 5: Cys, 6: Ser, 7: glucose. (a) visible color and (b) visual fluorescence color on excitation at 365 nm using a handheld UV lamp.

Detection of the thiols in biological fluids.

For the thiol assay in newborn-calf serum, the serum typically requires the reduction of disulfides to free thiols. This can be accomplished by using an excess of triphenylphosphine at room temperature [11-13]. 2 mL newborn-calf serum sample was firstly diluted with 1 mL distilled water, then treated with 1 mL triphenylphosphine solution in CH_3CN (1.5×10^{-3} M) for 30 min, after filtration, the reduced serum was directly used for the thiol assay. For the thiol assay in human urine, the sample was taken from a healthy volunteer. 1 mL urine sample was treated with 1 mL triphenylphosphine solution in CH_3CN (1.5×10^{-3} M) for 30 min, after filtration, the reduced sample was directly used for the thiol assay. Aliquots of the reduced newborn-calf serum solution or the reduced human urine samples were then added directly to a solution of probe **NRFTP** (1.3 μM, the total volume was 2 mL) in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent). Then the fluorescent intensity ratio at 510 and 590 nm (I_{510}/I_{590}) (λ_{ex} = 470 nm) was recorded. The unknown amount of thiols in the human urine sample was estimated by using the standard addition method with Cys as the standard.[12, 14] The total content of thiols in the urine sample was analyzed to be 40 ± 3 μM, which is well within the reported thiol concentration range for the urine samples from the healthy individuals ((a) T. Inoue, J. R. Kirchhoff, *Anal. Chem.* 2002, **74**, 1349; (b) M. P. Brigham, W. H. Stein, S. Moore, *J. Clin. Invest.* 1960, **39**, 1633.). The result is in good agreement with the value (36 ± 4 μM) obtained for our sample by employing a fluorescent thiol probe reported by Peng et al. (*Chem Commun*., 2009, 5904-5906.).

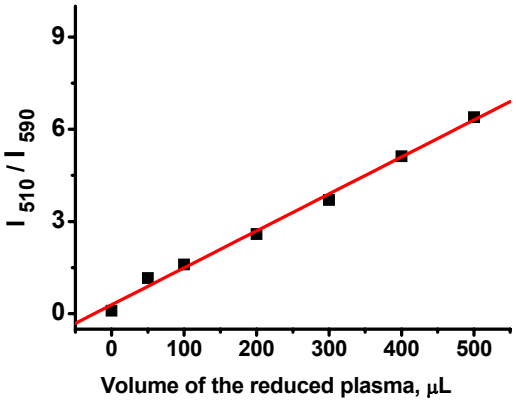


Figure S22. Linear relationship between the emission ratios and the volume of reduced newborn-calf serum added. The data were obtained by addition of different amounts of reduced new born-calf serum sample (0, 50, 100, 200, 300, 400 or 500 μ L) to probe **NRFTP** (1.3 μ M). Excitation at 470 nm.

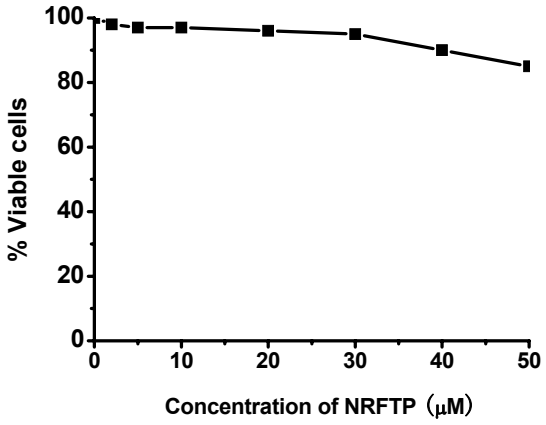


Figure S23. Cytotoxicity of **NRFTP** in cultured Hela cells. The cells were incubated with the probe at different concentrations for 24 h. The cell viability was measured by the MTT assay, and the data are reported as the percentage relative to the untreated cells.

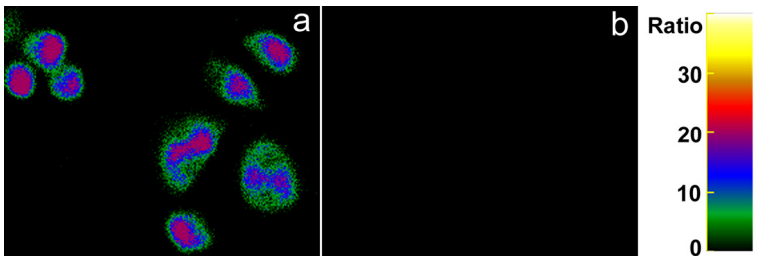


Figure 24. (a) Ratio fluorescence (I_{515} / I_{610}) image of Hela cells incubated with probe **NRFTP** (2 μM). (b) Ratio fluorescence (I_{515} / I_{610}) image of Hela cells pretreated with the thiol-blocking reagent *N*-ethylmaleimide (1 mM), then incubated with probe **NRFTP** (2 μM). The ratio imaging data were obtained using commercial software (Fluoview version 4.3).

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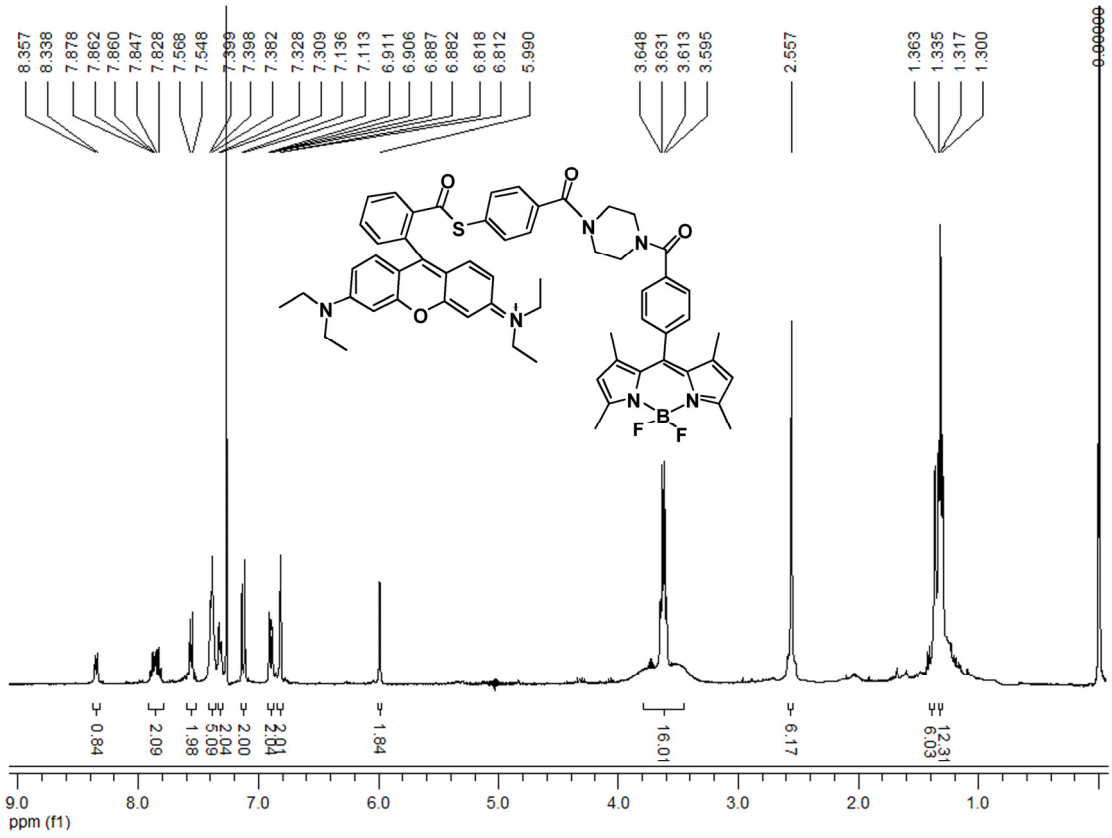
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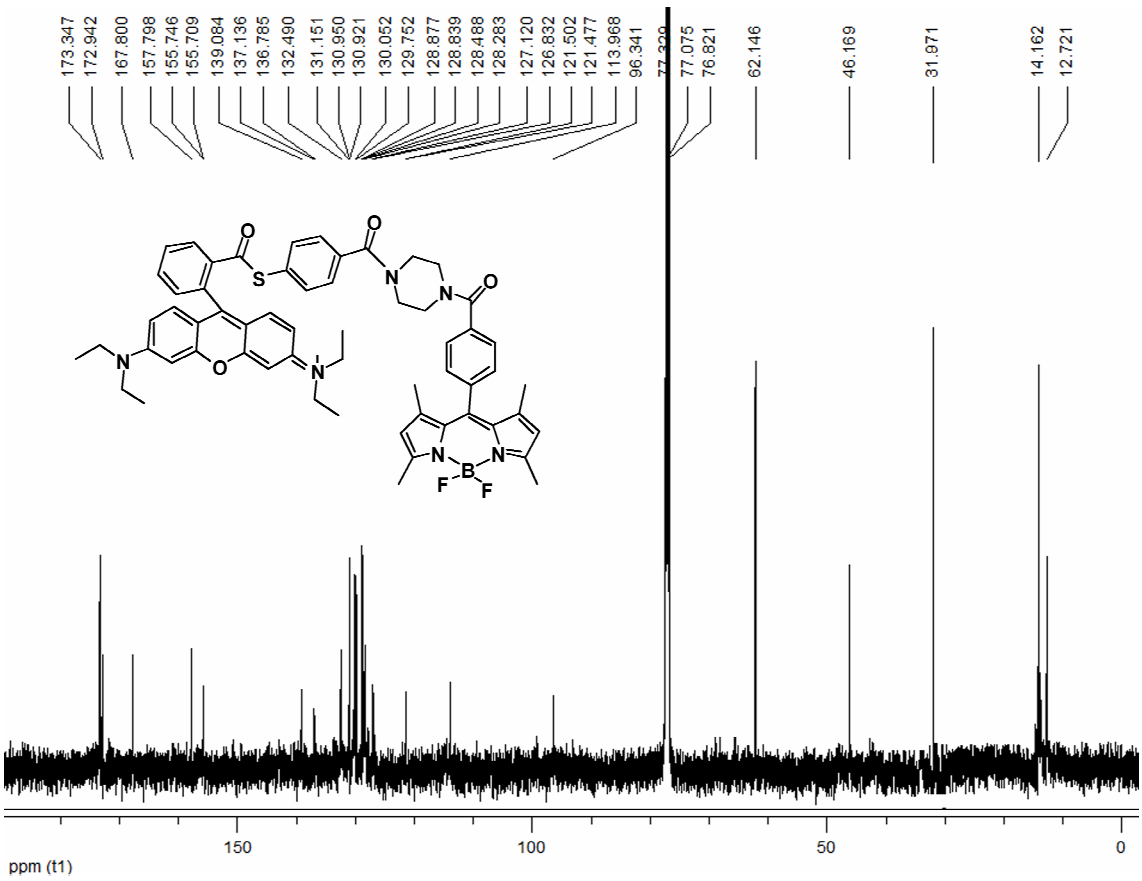
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