Supplementary Information

A multi-signal mitochondria-targeted fluorescent probe for real-time visualizing cysteine metabolism in living cells and animals

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Experimental Section

1. Apparatus

Absorption spectra were measured on a Lambda 35 UV/VIS spectrometer, PerkinElmer precisely. Fluorescence spectra measurements were performed on a HITACHI F-4600 fluorescence spectrophotometer, and the excitation and emission wavelength band passes were both set at 5 nm, excitation voltage was 700V. NMR spectra were measured on a Bruker DTX-400 spectrometer in CDCl$_3$ with TMS as internal standard. Mass spectral determination was carried on a Q-Tof HR-MS spectrometer (Waters Micromass) by using methanol as mobile phase. MCF-7 cell fluorescence imaging were taken under a LEICA TCS SP8 laser scanning confocal microscope.

2. Materials

All chemicals and reagents were used as received from commercial sources without further purification. Solvents for chemical synthesis and analysis were purified according to standard procedures. Double distilled water was used throughout the experiment. Probe stock solution was prepared in DMSO. The solutions of anions and amino acid were prepared from corresponding salts.

3. Cell Culture and Imaging

MCF-7 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, at 37 °C, in 5% CO$_2$. Cells were plated on culture dish and allowed to adhere for 24 h. Before the experiments, cells were washed with PBS 3 times. To observe the subcellular distributions of the probe, the cells were treated with a mitochondria staining probe, Mito Tracker Green FM (500 nM) for additional 30 min. The media was removed and the cells were washed three times with PBS buffer (pH 7.4). Cells were imaged using Leica TCS SP8 confocal microscope.

4. Zebrafish Culture and Imaging

Wildtype zebrafish were obtained from the Nanjing Eze-Rinka Biotechnology Co., Ltd. Zebrafishes were fed in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 0.15 mM KH$_2$PO$_4$, 0.05 mM Na$_2$HPO$_4$, 0.7 mM NaHCO$_3$, 10-5% methylene blue; pH 7.5) at 28 °C. The 5-day-old zebrafish were incubated with NIR-Cys (10 μM) for 30 min, and then imaged after washing by PBS buffer, as the control group. Zebrafish were pretreated with N-ethylmaleimide (NEM) (0.5 mM) for 30 min, subsequently incubated with NIR-Cys for 30 min, and then imaged after washing by PBS buffer, as the negative control group. Zebrafish were pretreated with Cys (100 μM) for 30 min, subsequently incubated with NIR-Cys for 30 min, and then imaged after washing by PBS buffer, as the experimental group. Zebrafish were incubated with NaHSO$_3$ (100 μM) for 30 min, and then imaged. To monitor cysteine metabolism, Zebrafish were incubated with 10 mM of Cys for 30 min and then treated 10 μM probe for 2 h and washed with PBS buffer. Zebrafish were imaged using Leica TCS SP8 confocal microscope.
5. Mice imaging

Before imaging, the Kunming mice were fasted for 12 h to avoid possible food fluorescence interference to the dye fluorescence. The mice were divided into three groups. The first group was given an injection of PBS (100 μL) for 30 min and then given an injection of NIR-Cys (10 μM, 100 μL) and was imaged after 30 min. The second group was given an injection of NEM (1 mM, 100 μL) for 30 min, and followed by subcutaneously injection with NIR-Cys (10 μM, 100 μL) and was imaged after 30 min. The third group was successively treated with Cys (100 μM, 100 μL) and followed by subcutaneously injection with NIR-Cys (10 μM, 100 μL) and was imaged after 30 min. and then injection with NaHSO₃ (100 μM, 100 μL) for 30 min. To monitor metabolite SO₂ in mice, one group was injected with Cys (10 mM, 100 μL), As control, the other group was injected with PBS (100 μL), and both followed by i.p. injection with NIR-Cys (10 μM, 100 μL) and liver was imaged after 30 min. Before imaging, the mice were anesthetized with 4% chloral hydrate (15 mL/kg) by intraperitoneal injection. Whole body images of the mice were then acquired using an IVIS Lumina III system with two channel (channel 1: 480-nm excitation and 570-nm emission, channel 2: 560-nm excitation and 670-nm emission).

Note:

Procedures involving animals and their care were conducted in conformity with the guidelines of the Use and Care of Laboratory Animals of National Institutes of Health (NIH Pub.No. 85-23, revised 1996). Ethics committee approval was obtained from the Laboratory Animal Ethics Committee of College of Life Sciences, Shandong University, China.

6. Synthesis

Intermediates compound 1 and 2 were synthesized according to previous report¹².

Scheme S1. Synthetic route of probe NIR-Cys.
Synthesis of probe NIR-OH: Compound 1 (313 mg, 1 mmol) and compound 2 (204 mg, 1 mmol) were dissolved in methanesulfonic acid (3 ml) and stirred at 90°C overnight. After the reaction was completed, the reaction solution was cooled to room temperature, and the reaction solution was added dropwise to 25 ml of saturated saline. A large amount of solid was observed to be precipitated, filtered, and washed three times with 100 ml of ice water. The resulting solid was subjected to column chromatography (DCM : MeOH = 20 : 1, v/v) and 352 mg of a purple-black solid was obtained, yield: 80%. $^1$HNMR (CDCl$_3$, 400 MHz, ppm): δ : 1.26 (s, 6 H), 3.71 (d, 4 H, J = 6.8 Hz), 6.90 (d, 1 H, J = 2 Hz), 7.03 (d, 1 H, J = 8.8 Hz), 7.24 (d, 1 H, J = 8.4 Hz), 7.39 (s, 2 H), 7.53 (d, 1 H, J = 7.6 Hz), 7.79 (d, 1 H, J = 7.6 Hz), 7.81 (t, 1 H, J = 7.6 Hz), 7.89 (t, 2 H, J = 6.4 Hz), 8.02 (s, 1 H), 8.18 (t, 1 H, J = 7.6 Hz), 8.36 (s, 1 H), 11.83 (s, 1 H). $^{13}$CNMR(CDCl$_3$, 100 MHz, ppm): δ 13.04, 46.13, 96.74, 99.98, 102.62, 111.09, 112.09, 115.78, 130.25, 131.14, 133.19, 133.39, 147.63, 155.91, 157.32, 158.03, 158.81, 167.27; HR-MS: Calcd for [C$_{29}$H$_{24}$NO$_{6}$]$^+$. 482.1598, found 482.1401.

Synthesis of probe NIR-Cys:

The compound NIR-OH (154.3 mg, 0.32 mmol) was dissolved in 15 ml of anhydrous dichloromethane, 133 µl of triethylamine was added to the above solution, and dissolved in 10 ml of anhydrous dichlorohydrate in an ice bath. Acryloyl chloride was added dropwise to the solution (completely after about 20 minutes). After dropping, the ice bath was removed and the solution was stired at room temperature for 2 h. After the reaction monitored by TLC was finished, the solvent was evaporated under reduced pressure to give a purple crude product. Purified by column chromatography (DCM : MeOH = 60:1, v/v) to give 122.17 mg of a dark yellow solid with a yield of 73.24%. $^1$H NMR (CDCl$_3$, 400 MHz, ppm): 1.22 (t, 6 H, J = 7.2 Hz), 3.41 (q, 4 H, J = 7.2 Hz), 6.10 (d, 1 H, J = 0.8 Hz), 6.36 (m, 2H), 6.51 (d, 2 H, J = 8.8 Hz), 6.67 (m, 1H), 6.75 (s, 1H), 7.20 (t, 2 H, J = 8.4 Hz), 7.31 (d, 2 H, J = 9.2 Hz), $^{13}$CNMR (CDCl$_3$, 100 MHz, ppm): δ 12.57, 44.46, 97.38, 103.74, 109.26, 109.75, 116.65, 118.26, 118.72, 123.98, 125.06, 126.59, 127.28, 128.53, 129.47, 129.78, 133.81, 134.54, 139.35, 145.86, 149.44, 152.59, 153.45, 143.64, 153.93, 157.57, 163.69, 169.69. HR-MS: Calcd for [C$_{32}$H$_{26}$NO$_{7}$]$^+$. 536.1704; Found 536.1704.


7. Supplementary Spectra

**Figure S1** (A) The UV-vis spectra of probe NIR-Cys (10 µM) with Cys (10 equiv.) and other biologically relevant species (10 equiv.) in the HEPES buffer (10 mM, pH=7.4). (B) The UV-vis spectra of probe NIR-Cys (10 µM) in the presence of Cys (0–20 equiv.) in HEPES buffer (10 mM, pH=7.4).

**Figure S2** Fluorescence emission spectra of probe NIR-Cys (10.0 µM) in the presence of Cys (0–10 equiv.). Linear plot of the fluorescence emission intensity (656 nm) against Cys concentrations (λ<sub>ex</sub> = 580 nm).
Figure S3 The fluorescence emission intensity (656 nm) of probe NIR-Cys (10 µM) under different pH (1-12) in the absence and presence of Cys (10 equiv.). ($\lambda_{ex} = 580$ nm)

Figure S4 The fluorescence intensity of probe NIR-Cys (10 µM) with Cys (10 equiv.) and other amino acids (Arg, His, Glu, Tyr, Lys, Ser, Pro, Thr, Leu, Asp, Val, Gly, Ala, Phe, Gln, Hcy, GSH, HS-’) (10 equiv.) in the HEPES buffer (10 mM, pH=7.4) ($\lambda_{ex} = 580$ nm).
**Figure S5** The fluorescence intensity of probe NIR-Cys (10 µM) at 656 nm changes upon the addition of various analytes (100 µM) in the presence of Cys (100 µM) in the HEPES buffer (10 mM, pH=7.4). ($\lambda_{ex} = 580$ nm)

**Scheme S2** Proposed reaction mechanism of probe NIR-Cys with Cys.
Figure S6 HR-MS spectra of probe NIR-Cys in the presence of Cys (10 equiv.).

Figure S7 (A) Fluorescent emission spectra of NIR-Cys (10.0 µM) in presence of HSO₃⁻ (0–15 equiv.) in HEPES buffer (10 mM, pH=7.4). (B) Time-dependent fluorescent emission of NIR-Cys (10.0 µM) and 100 µM HSO₃⁻ at 560 nm. ($\lambda_{ex} = 460$ nm)

Figure S8 The UV-vis spectra of probe NIR-Cys (10 µM) in the presence of NaHSO₃ (0–15 equiv.) in the HEPES buffer (10 mM, pH=7.4).
Figure S9 Linear plot of the fluorescence emission intensity (560 nm) of probe NIR-Cys (10 µM) against NaHSO$_3$ concentrations ($\lambda_{ex} = 470$ nm).

Scheme S3 Proposed reaction mechanism of probe NIR-Cys with NaHSO$_3$.

Figure S10 HR-MS spectra of probe NIR-Cys in the presence of NaHSO$_3$ (10 equiv.).
Figure S11. (A) UV-vis spectra of NIR-OH (10 µM) with HSO$_3^-$ (10 equiv.) and other biologically relevant species (10 equiv.) in HEPES buffer (10 mM, pH=7.4). (B) Fluorescent emission spectra of NIR-OH (10.0 µM) in the presence of HSO$_3^-$ (0–10 equiv.). (B) The UV-vis spectra of probe NIR-OH (10 µM) in the presence of NaHSO$_3$ (0–15 equiv.) in HEPES buffer (10 mM, pH=7.4, containing 30% ethanol).

Figure S12. The fluorescence intensity of probe NIR-OH (10 µM) with HSO$_3^-$ increasing (0-15 equiv.). ($\lambda_{ex} = 470$ nm)
**Figure S13** Linear plot of the fluorescence emission ratios of probe NIR-OH (10 µM) (F550 nm/F664 nm) against NaHSO₃ concentrations. (λₑₓ = 470 nm)

**Figure S14** The fluorescence emission ratios (F550 nm/F664 nm) of probe NIR-OH (10 µM) under different pH (1-12) in the absence and presence of HSO⁻₃ (10 equiv.).
Figure S15 The fluorescence emission ratios (F550 nm/F664 nm) of NIR-OH (10 μM) upon the addition of various analytes (100 μM) in the presence of HSO– 3 (100 μM) in HEPES buffer (10 mM, pH=7.4, containing 30% ethanol). (λ<sub>ex</sub> = 470 nm)

Scheme S4 Proposed reaction mechanism of probe NIR-OH with NaHSO₃.
Figure S16 HR-MS spectra of probe NIR-OH in the presence of NaHSO$_3$ (10 equiv.).

Figure S17 $^1$H NMR spectra of probe NIR-OH in the presence of NaHSO$_3$ (0-3 equiv.).
Figure S18. Cell viability of cells treated with different concentrations of NIR-Cys (green column) for 24 h in fresh medium. The results are the mean standard deviation of three separate measurements.

Figure S19. Confocal fluorescence images of NIR-Cys colocalized to the mitochondria in MCF-7 cells. Cells were stained with (a) Bright field pattern of cells. (b) MitoTracker Green FM (500 nM, green channel). (c) NIR-Cys (10 μM, red channel) simultaneously. (d) The merged pattern of a, b and c. (e) Intensity profile of ROI across the MCF-7 cells costained with NIR-Cys and MitoTracker Green FM. (f) Intensity scatter plot of two channels. (green channel of 470-560 nm with excitation 488 nm, red channel of 610-700 nm at 552 nm)
**Figure S20** Two-photon fluorescence images of probe NIR-Cys responding to Cys and NaHSO₃ in living MCF-7 cells by confocal fluorescence imaging. (a) Cells were incubated with probe NIR-Cys (10 μM, 30 min), and then imaged. (b) Cells were treated with Cys (100 μM, 30 min) subsequently incubated with probe NIR-Cys (10 μM, 30 min), and then imaged. (c) Cells were pretreated with Cys (100 μM, 30 min) and probe NIR-Cys (10 μM, 30 min), then incubated with NaHSO₃ (100 μM, 30 min), and then imaged. The fluorescence images were captured from the green channel of 500-580 nm and red channel of 610-700 nm with excitation at 680 nm.

**Figure S21** Fluorescence images of NIR-OH responding to NaHSO₃ in living MGC-803 cells, (a-c) Cells were incubated with probe NIR-OH (10 μM, 30 min), and then imaged. (d-f) Cells were treated with NaHSO₃ (100 μM, 30 min) subsequently incubated with NIR-OH (10 μM, 30 min), and then imaged.
Figure S22. Time-dependent confocal images of exogenous HSO$_3^-$ of NIR-OH in MCF-7 cells. (green channel of 500-590 nm with excitation 488 nm, red channel of 610-700 nm at 552 nm)

Figure S23. Confocal fluorescence images of MCF-7 cells incubated with 10 mM of cysteine for 30 min and then treated with probe NIR-Cys (10 μM) for different times. From a to f, the time is 0, 15, 45, 90, 180, and 240 min, respectively. (green channel of 500-590 nm with excitation 488 nm, red channel of 610-700 nm at 552 nm)
Figure S24 Fluorescence images of probe NIR-Cys responding to Cys and NaHSO$_3$ in living zebrafish by confocal fluorescence imaging. (a1-4) Zebrafish were incubated with probe NIR-Cys (10 μM, 30 min), and then imaged. (b1-4) Zebrafish were pretreated with NEM (500 μM, 30 min) subsequently incubated with probe NIR-Cys (10 μM, 30 min), and then imaged. (c1-4) Zebrafish were treated with Cys (100 μM, 30 min) subsequently incubated with probe NIR-Cys (10 μM, 30 min), and then imaged. (d1-4) Zebrafish were pretreated with Cys (100 μM, 30 min) and probe NIR-Cys (10 μM, 30 min), then incubated with NaHSO$_3$ (100 μM, 30 min), and imaged. The fluorescence images were captured from the green channel of 500-580 nm with excitation 488 nm and red channel of 610-700 nm at 552 nm.
Figure S25 Fluorescent detection of NaHSO$_3$ in living mice injected with NIR-Cys in PBS. Mice treated with PBS buffer (100 μL, left) intraperitoneal injection for 30 min. subcutaneously injected probe NIR-Cys (100 μL, 10 μM), imaged (left). Then mice treated with NaHSO$_3$ (100 μL, 10 μM) for 30 min, imaged (right). (560 nm excitation and 670 nm emission)

Figure S26. Fluorescence images of liver. (a) channel 1: 480-nm excitation and 570-nm emission. (b) channel 2: 560-nm excitation and 670-nm emission.
Figure S27 $^1$H NMR spectrum of NIR-OH in CDCl$_3$.

Figure S28 $^{13}$C NMR spectrum of NIR-OH in CDCl$_3$. 
Figure S29 HR-MS spectra of NIR-OH.

Figure S30 $^1$H NMR spectrum of NIR-Cys in CDCl$_3$. 
Figure S31 $^{13}$C NMR spectrum of NIR-Cys in CDCl$_3$.

Figure S32 HR-MS spectra of NIR-Cys.