Supporting Information

A Two-Photon Fluorescent Probe with Near-infrared Emission for Hydrogen Sulfide Imaging in Biosystems

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1. Materials and instruments

All solvents used were of analytical grade without further purification. ¹H NMR and ¹³C NMR spectra were recorded on a VARIAN INOVA-400 spectrometer. UV-visible spectra were collected on a Perkin Elmer Lambda 35 UV-Vis spectrophotometer. Fluorescence measurements were performed on a VAEIAN CARY Eclipse Fluorescence Spectrophotometer (Serial No. FL1109-M018).

2. Photostability experiment

1-NH₂ and Mitotracker Deep Red were dissolved in PBS buffer (0.1 M, pH 7.4, 50% DMSO) at a concentration of 5 μ M. The solutions were irradiated under a 500W iodine-tungsten lamp for 4 h at a distance of 300 mm away. A saturated sodium nitrite aqueous solution was placed between the samples and the lamp as a light filter (to cut off the light shorter than 400 nm) and heat filter.

3. Determination of the detection limit

The detection limit was calculated based on the method reported in the previous literature ^[S1]. The fluorescence emission spectrum of **1** was measured by three times and the standard deviation of blank measurement was achieved. The fluorescence intensity at 670 nm was plotted as a concentration of H₂S. The detection limit was calculated by using detection limit = $3\sigma/k$: Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity versus H₂S concentration.

4. Cell incubation and fluorescence imaging

MCF-7 and HeLa cells were seeded onto the cover slips at a concentration of 2×10^4 cells•mL⁻¹ and cultured in DMEM in an incubator (37 °C, 5% CO₂ and 20% O₂). After 24 hours, the cover slips were rinsed slightly 3 times with PBS to remove the media and then cultured in PBS for later use. In respect to the verification procedure, 5 μ M of probe **1** was added to above cellular samples and incubated for 30 min, then the samples were slightly rinsed 3 times with PBS and observed under a Olympus FV1000-IX81 confocal fluorescence microscope, confocal fluorescence image 60 ×

objective lens. And then the cells were incubated with H_2S (250 μ M) in the medium for 30 and 60 minutes, then the samples were slightly rinsed 3 times with PBS and observed under the confocal fluorescent microscope to get pictures with white light and fluorescence, respectively.

5. Cytotoxicity test

Measurement of cell viability cell viability was evaluated by the reduction of MTT (3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazoliumromide) to formazan crystals by mitochondrial dehydrogenases (Mosmann,1983). Briefly, MCF-7 cells were seeded in 96-well microplates (Nunc, Denmark) at a density of 1×10^5 cells/mL in 100 µL medium containing 10% FBS. After 24 h of cell attachment, cells were cultured in medium with 5 µM of 1 or 1-NH₂ for 6 h and 24 h, respectively. Cells in culture medium without 1 and 1-NH₂ were used as the control. Six replicate wells were used for each control and test concentration. Plates were then washed with 100 µL/well PBS before 10 µL of MTT (5 mg/mL) prepared in PBS was added to each well and the plates were incubated at 37°C for another 4 h in a 5% CO₂ humidified incubator. The medium was then carefully removed, and the purple products were lysed in 200 µL DMSO. The plate was shaken for 10 min and the absorbance was measured at 570 nm and 630 nm using a microplate reader (Thermo Fisher Scientific). Cell viability was expressed as a percent of the control culture value.

6. Preparation of tumors slices

Tumor slices were prepared from nude mice which were seeded BEL7402 cells. Slices were incubated with 20 μ M of probe **1** in artificial cerebrospinal fluid (ACSF; 138.6 mM NaCl, 3.5 mM KCl, 21 mM NaHCO₃, 0.6 mM NaH₂PO₄, 9.9 mM D-glucose, 1 mM CaCl₂, and 3 mM MgCl₂) bubbled with 95% O₂ and 5% CO₂ for 60 min at 37 °C. Slices were next washed three times with ACSF and observed under the confocal fluorescence microscope. And then the tissue was incubated with H₂S (25 equiv.) in the above conditions for 60 minutes, then the samples were observed under the confocal fluorescent microscope to get pictures.

7. Fluorescent imaging in living mice

ICR mice, 5-10 g, were selected and divided into two groups. One group was given an s.p. (skin-pop) injection of probe **1** (40 μ M, in 25 μ L DMSO) on the back of ICR mice as the control experiment. The other group was then given an s.p. injection of 25 equiv. of NaHS (25 μ L, 0.1 mM PBS) after the same disposal of the control mice. Images were taken after incubation for different time by using a NightOWL II LB983 small animal *in vivo* imaging system containing a sensitive CCD camera, with an excitation laser of 530 nm and an emission filter of 655±20 nm.

8. Synthetic procedures

Scheme S1. Synthesis of probe 1



Synthesis

1-(2-hydroxyphenyl)butane-1,3-dione (4)



1-(2-hydroxyphenyl)ethanone (10.0 g, 73.5 mmol) was dissolved in 200 mL ethyl acetate, and then sodium (8.00 g, 0.34 mmol) was added into the solution. The grayish-green solid was filtered after violently stirring for 4h at ambient temperature. The solid was dissolved in 100 mL deionized water, followed by the adjustment of pH

of the solution to neutral. The aqueous solution was extracted with 200 mL EtOAc and the organic layers were dried over Na_2SO_4 , filtered, and concentrated to yield the final crude product as a brown solid (6.95 g, 53%) which was directly used in the next reaction without further purification.

2-methyl-4H-chromen-4-one (3)



Sulfuric acid (4.6 mL) was slowly added to a AcOH solution (70 mL) containing 1-(2-hydroxy phenyl)butane-1,3-dione (6.95 g, 38.9 mmol) . The mixture was refluxed for about 30 min and then was poured into 800 mL ice water, followed by the adjustment of pH of the solution to neutral with Na₂CO₃. The aqueous solution was extracted with methylene dichloride twice and the organic layers were dried over Na₂SO₄, filtered, and concentrated to yield the final crude product as an acicular gray solid (4.78 g, 76.9%). The crude product was directly used in the next reaction without further purification.

2-(2-methyl-4H-chromen-4-ylidene)malononitrile (2)



2-methyl-4H-chromen-4-one (4.78 g, 29.9 mmol) and malononitrile (2.40 g, 36.2 mmol) were dissolved in 25 mL acetic anhydride. The solution was refluxed for 14 h and then the solvent was evaporated in vacuo. Deionized water (80 mL) was added to the residue and the mixture was refluxed for anther 0.5 h, followed by extraction with methylene dichloride. The organic layers were dried over Na_2SO_4 , filtered, and

concentrated. The obtained crude product was purified by silica column chromatography to yield compound **2** as an orange solid (2.02 g, 32.5 %).¹H NMR (400 MHz, CDCl₃), δ (ppm): 8.92 (d, 1H, J = 8.0 Hz), 7.72 (t, 1H, J = 8.0 Hz), 7.46 (d, 1H, J = 8.0 Hz) 7.45 (t, 2H, J = 8.0 Hz), 6.72 (s, 1H), 2.44 (s, 3H); ¹³C NMR (100 MHz, CDCl₃), δ 161.68, 153.24, 152.91, 134.58, 126.04, 125.82, 118.64, 117.57, 116.57, 115.45, 105.48, 62.40, 20.47; HPLC-MS (API-ES): [M+H]⁺ 209.1, found 209.1; [M+Na]⁺ 231.1, found 231.1.

(E)-2-(2-(4-aminostyryl)-4H-chromen-4-ylidene)malononitrile (1-NH₂)



[S2] This step was synthesized according to previous reported method 2-(2-methyl-4H-chromen-4-ylidene)malononitrile (63.0 mg, 0.30 mmol). and N-(4-formyl- phenyl)acetamide (45.0 mg, 0.28 mmol) were dissolved in toluene (10 mL) with piperidine (0.15 mL) and acetic acid (0.15 mL) under argon protection at room temperature. Then the mixture was refluxed for 3 h to give an orange precipitate. After filtration, the orange solid was refluxed in a solution of conc. HCl and ethanol (2:1, 30 mL) for another 2 h before the pH of the solution was adjusted to neutral. The aqueous solution was extracted with ethyl acetate and then the organic layers were dried over Na₂SO₄, filtered, and concentrated to obtain the crude product which was purified by silica column chromatography to yield **1-NH**₂ as a crimson solid (40.0 mg, 42.9%, mp. 243.8-245.2°C). ¹H NMR (400 MHz, DMSO), δ (ppm): 8.73 (d, 1H, J = 8.0 Hz), 7.89 (t, 1H, J = 8.0 Hz), 7.77 (d, 1H, J = 8.0 Hz), 7.64 (d, 1H, J = 16.0 Hz), 7.58 (t, 1H, J = 8.0 Hz), 7.49 (d, 1H, J = 8.0 Hz), 7.08 (d, 1H, J = 16.0 Hz), 6.87 (s, 1H), 6.61 (d, 2H, J = 8.0 Hz), 5.99 (s, 2H); 13 C NMR (100 MHz, CDCl₃) δ 160.23, 153.04, 152.56, 152.52, 141.13, 135.46, 131.14, 126.35, 125.01, 122.79, 119.35, 118.24, 117.68, 116.89, 114. 28, 112.88, 105.16, 57.85; HPLC-MS (API-ES): [M+H]⁺

312.1, found 312.0; [M+Na]⁺ 334.1, found 334.0.





A solution of NaNO₂ (80 mg, 1.16 mmol) in 2 mL of water was added dropwise to a solution of (E)-2-(2-(4-aminostyryl)-4H-chromen-4-ylidene)malononitrile (40.0 mg, 0.13 mmol) in 4 M HCl (5 mL) at 0-5 °C. After stirring the mixture at this temperature for 45 min, a solution of NaN₃ (180 mg, 8.0 mmol) in water (2 mL) was added slowly to the mixture at the same temperature. Stirring was continued for 1 h below 5 °C and then at room temperature for another 1h. The yellow precipitate obtained was filtered and air-dried. The crude product was purified by silica gel column chromatography to yield **1** as a yellow solid (19.1 mg, 47.2%). ¹H NMR (400 MHz, Acetone-d₆), δ (ppm): 8.87 (d, 1H, J = 8.0 Hz), 7.93 (t, 1H, J = 8.0 Hz), 7.85 (d, 2H, J = 8.0 Hz), 7.84 (d, 1H, J = 16.0 Hz), 7.77 (d, 1H, J = 8.0 Hz), 7.62 (t, 1H, J = 8.0 Hz), 7.41 (d, 1H, J = 16.0 Hz), 7.20 (d, 2H, J = 8.0 Hz), 6.99 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 157.65, 152.94, 152.49, 142.39, 137.82, 134.87, 131. 63, 129.65, 126.06, 119.96, 118.75, 118.45, 118.03, 116.89, 115.83, 107.14, 63.18; HPLC-MS (API-ES): [M+H]⁺ 338.1, found 337.9; [M+Na]⁺ 360.1, found 360.0; [2M+Na]⁺ 697.1, found 697.1.

9. Reference

[S1] B. Zhu, C. Gao, Y. Zhao, C. Liu, Y. Li, Q. Wei, Z. Ma, B. Du, X. Zhang, *Chem. Commun.* **2011**, 47, 8656-8658.

[S2] W. Zhu, X. Huang, Z. Guo, X. Wu, H. Yu and H. Tan, *Chem. Commun.*, 2010, 48, 1784.

10. UV/Fluorescence studies of compounds

Dyes	Solvents	$\lambda^{OP}_{abs} (nm)^{a}$	$\lambda^{OP}_{em}(nm)^{a}$	$\Delta \lambda c (nm)$	$\Phi \delta / \mathrm{GM}^{\mathrm{b}}$ $(\lambda^{\mathrm{TP}}_{\mathrm{abs}} / \mathrm{nm})^{\mathrm{c}}$
	DMSO	515	660	145	50 (820)
1 NH	EtOH	495	645	150	10 (820)
1-1N H ₂	THF	488	614	126	2 (820)
	1,4-Dioxane	468	574	106	

Table S1. Photophysical parameters of 1-NH₂ in various solvents.

a. λ_{max} of the absorption and emission spectra in nm.

b. Two-photon action cross section of $1-NH_2$ referenced to Rhodamine B in MeOH. The samples were dissolved in solvents at a concentrations of 1.0×10^{-4} mol·L⁻¹. The errors of $1-NH_2$ measurement are $\pm 15\%$.

c. λ_{max} of the two-photon excitation spectra in nm.



Figure S1. a) Fluorescence responses of **1** (5 μ M) and **1-NH**₂ (5 μ M) to various pH in PBS buffer (0.1 M, pH 7.4, 50% DMSO). b) Normalized fluorescence changes of **1-NH**₂ and a commercial Cy5 NIR dye (Mitotracker Deep Red, $\lambda_{ex} = 644$ nm, $\lambda_{em} = 668$ nm) in PBS buffer (0.1 M, pH 7.4, 50% DMSO) were measured during light irradiation for 4 h using a 500 W iodine-tungsten lamp as the light source. $\lambda_{ex} = 520$ nm, points showed the fluorescence intensity at 670 nm. Blue points: **1**, Red points: **1-NH**₂.



Figure S2. Normalized absorption spectrum of **1**, **1-NH**₂ and **1** after reacted with H₂S in PBS buffer (0.1 M, pH 7.4, 50% DMSO). Black line: **1**, Red line: **1-NH**₂, Blue line: **1**+H₂S. The inset shows the color change before and after reaction with H₂S (probe = 5 μ M).



Figure S3. Fluorescence responses of 1 (5 μ M), 1-NH₂ (5 μ M) and 1 (5 μ M) after reacted with 50 equiv. of H₂S in PBS buffer (0.1 M, pH 7.4, 50% DMSO). Red line: 1-NH₂, Blue line: 1+H₂S, purple line: 1.



Figure S4. Fluorescence change of **1** (5 μ M) after incubation with 50 equiv. of H₂S for 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60 min. Conditions: PBS buffer (0.1 M, pH 7.4, 50% DMSO) at 37 °C, $\lambda_{ex} = 520$ nm.



Figure S5. The fluorescence intensity at 670 nm of probe **1** (5 μ M) was linearly related to the concentrations of H₂S (5-50 equiv.), each spectrum was obtained after 60 min of reaction. Conditions: PBS buffer (0.1 M, pH 7.4, 50% DMSO) at 37 °C, $\lambda_{ex} = 520$ nm.



Figure S6. a) Fluorescence change of **1** (5 μ M) after incubation with 40 equiv. of H₂S for 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 min. b) The fluorescence intensity change of **1** (5 μ M) after incubation with different concentrations of H₂S (5, 10, 20, and 40 equiv.) for 40 min, Y= 0.33987*X + 15.7352. Conditions: bovine serum at 37 °C, $\lambda ex = 520$ nm.



Figure S7. HPLC-MS of compound 1 after reaction with H_2S . $[M+H]^+$ 312.1; found 312. 0. The result indicates that the formula of the corresponding compound is $C_{20}H_{13}N_3O$.

11. Fluorescence images in biosystems



Figure S8. Fluorescence imaging of H₂S in HeLa cells by OPM. Cells incubated with probe 1 (5 μ M) for 0.5 h (top); image of cells after treatment with probe 1 (5 μ M) for 0.5 h and subsequent treatment of the cells with 50 equiv. of H₂S for 1 h (bottom). (a), (d) Bright-field images of the HeLa cells in samples; (b), (c) red emission (655-750 nm); (c) overlay image of (a) and (b); (f) overlay image of (d) and (e). $\lambda_{ex} = 515$ nm.



Figure S9. Fluorescence imaging of H_2S in MCF-7 cells by TPM. Cells incubated with probe **1** (5 μ M) for 0.5 h (top); image of cells after treatment with probe **1** (5 μ M) for 0.5 h, then treatment of the cells with 50 equiv. of H_2S for 0.5 h (middle) and 1 h (bottom). (a), (d), (g) Bright-field images

of the MCF-7 cells in samples; (b), (e), (h) red emission (575-630 nm); (c) overlay image of (a) and (b); (f) overlay image of (d) and (e); (i) overlay image of (g) and (h). $\lambda_{ex} = 800$ nm.



Figure S10. Fluorescence images of MCF-7 cells loaded with 5 μ M probe 1 exposed to normoxia or hypoxia. Cells were incubated under normoxia for 0.5 h (top); Cells were incubated under a cover glass (hypoxic condition) for 0.5 h (bottom). (a), (d) Bright-field images of the MCF-7 cells in samples; (b), (e) red emission (655-750 nm); (c) overlay image of (a) and (b); overlay image of (d) and (e); $\lambda ex = 515$ nm. Scale bar: 30 μ m.



Figure S11. The cytotoxicity of **1** and **1-NH**₂ with a concentration of 5 μ M in living MCF-7 cells for 6 h and 12 h. Blank bars: 6 h, Red bars: 24 h. The figure shows that after 24 h of cellular internalization of the compounds at a concentration of 5 μ M, more that 95% cells were viable, demonstrating the non-cttotoxicity of **1** and **1-NH**₂ to cells at our experimental conditions.



Figure S12. Images of rat liver cancer slices stained with 20 μ M probe 1 at 140 μ m depth for 60 min which was (b) not treated and treated with (c) 25 equiv. of NaHS for 60 min. (a) Brightfield image of the tissue slice. The TP fluorescence emission was collected at 575-630 nm upon excitation at 800 nm with a femtosecond pulse. Scale bar: 60 μ m.



Figure S13. 3D depth imaging in tissues. Depth fluorescence imaging of ANQ-IMC-6 in tissues were obtained with spectral confocal multiphoton microscopes (Olympus, FV1000) with a high-performance mode-locked titanium-sapphire laser source (MaiTai, Spectra-Physice, USA). And then, the changes of fluorescence intensity with scan depth were determined by spectral confocal multiphoton microscopes (Olympus, FV1000) in the z-scan mode from 0 μ m to 400 μ m (step size = 1 μ m).



Figure S14. a) Fluorescence image of mice (pseudo-color) s.p. injected with probe 1 (40 μ M, in 25 μ L DMSO) as the control experiment. b) Representative fluorescence images of mice given an s.p. injection of probe 1 (40 μ M, in 25 μ L DMSO) and then injected with 25 equiv. of NaHS (25 μ L, 0.1 mM PBS). Images were taken after incubation of NaHS for different time (0, 0.5, 1, 2, 3 and 4 h).

12. NMR and MS data for compounds



Figure S15. ¹H NMR spectrum of compound 2 in CDCl₃



Figure S16. ¹³C NMR spectrum of compound 2 in CDCl₃





Figure S17. ¹H NMR spectrum of compound 1-NH₂ in DMSO-d₆.



Figure S18. ¹³C NMR spectrum of compound 1-NH₂ in DMSO-d₆.

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Figure S19. 1 H NMR spectrum of compound 1 in acetone-d₆.



Figure S20. ¹³C NMR spectrum of compound 1 in acetone-d.