Supporting Information

A new ratiometric fluorescent probe for rapid, sensitive and selective detection of endogenous hydrogen sulfide in mitochondria

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\# Equal contribution
Apparatus and chemicals

Synthesis

Preparation of the test solution

Determination of fluorescence quantum yield

Calculation of energy transfer efficiency

Cell culture and imaging of cells

Imaging of exogenous H₂S in living cells

Imaging of endogenous H₂S in living cells

Co-localization imaging of cells

Table S1. The spectroscopic data of compounds CPC, Donor and Acceptor

Fig. S1 – S14 Some spectra of the probe

Fig. S15 Thermo LCQ Fleet mass spectrum of the CPC (50 μM) solution in the presence of excessive Na₂S (500 μM).

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Fig. S18 – S27 IR, MS, ¹H NMR and ¹³C NMR spectra of intermediates and probes.

References
Apparatus and chemicals

$^1$H NMR (300 MHz) and $^{13}$C NMR (100 MHz) spectra were acquired on a Bruker Avance 300 or 400 spectrometer, with DMSO-$d_6$ used as a solvent and tetramethylsilane (TMS) as an internal standard. Mass spectrometry (MS) involved High-resolution mass spectrometry (HRMS) Q-TOF6510 spectrograph (Agilent) and Thermo LCQ Fleet mass spectrometer (Thermo). Melting points of compounds were measured on an XD-4 digital micro-melting-point apparatus. IR spectra were recorded with an IR spectrophotometer VERTEX 70 FT-IR (Bruker Optics). UV–vis spectra were measured by a Hitachi U-4100 spectrophotometer. Fluorescent measurements were performed on a Perkin-Elmer LS-55 luminescence spectrophotometer. Quartz cuvettes with a 1-cm path length and 3-mL volume were used for all measurements. The pH was determined with a model PHS-3C pH meter. Unless otherwise stated, all reagents were purchased from Aladdin, J&K or Sinopharm Chemical Reagent Co. and used without further purification. Twice-distilled water was used throughout all experiments. The salts used in stock aqueous solutions of metal ions were KNO₃, Ca(NO₃)₂$\cdot$4H₂O, NaNO₃, KCl, KBr, KI, NaClO, NaHCO₃, Na₂SO₄, CH₃COONa, Na₂SO₃.

Synthesis
Synthesis of compound 1

Piperazine (1.5 g, 17.41 mmol) was dissolved in H₂O (18 mL) and 2-methoxyethanol (20 mL). The mixture was heated to reflux, then the solution of 4-fluorobenzaldehyde (0.5 mL, 4.64 mmol) dissolved in 2-methoxyethanol (5 mL) was added dropwise over a period of 0.5 h. Then the mixture continued to reflux for 3 h. After cooling down to room temperature, the mixture was poured into H₂O (50 mL) to afford a yellow precipitate. The precipitate was filtered and dried to give a yellow solid 1 (0.59 g, 66.7%), m.p. 180–182 °C.

Compound 2 was synthesized according to the reported method.¹

Synthesis of compound 3

Scheme S1 Synthesis of probe CPC, Donor and Acceptor
Compound 1 (0.118 g, 0.618 mmol) was dissolved in dry DCM (20 mL) and NEt$_3$ (0.3 mL) under nitrogen atmosphere in ice-salt bath. Then compound 2 (0.157 g, 0.562 mmol) was added to the mixture in batches. Then the mixture reacted in ice-salt bath for 0.5 h further in room temperature for 3.5 h. The reaction completed as shown TLC analysis. The solvent was then evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (dichloromethane : ethyl acetate : methanol = 50 : 10 : 1, V/V/V) to afford target product 3 (0.140 g, 57.6%), m.p. 195-197 °C. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 9.73 (s, 1H), 8.02 (s, 1H), 7.74 (d, $J$ = 9 Hz, 2H), 7.51 (d, $J$ = 9 Hz, 1H), 7.06 (d, $J$ = 9 Hz, 2H), 6.76 (dd, $J$ = 9, 2.4 Hz, 1H), 6.57 (d, $J$ = 2.4 Hz, 1H), 3.72 - 3.46 (m, 12H), 1.14 (t, $J$ = 7.2 Hz, 6 H).

**Synthesis of compound 4**

2,3,3-Trimethyl-3H-indole (3.20 g, 20 mmol), iodomethane (5.68 g, 40 mmol) were mixed and dissolved in acetonitrile (10 mL). Then the mixture was refluxed for 11 h under nitrogen atmosphere. The mixture was cooled down to room temperature to afford a light pink precipitate. After filtered, the filter cake was washed with ethyl acetate (10 mL) for 3 times and dried in vacuum to give a light pink solid 4 (5.8 g, 96.0%).

**Synthesis of probe CPC**

Compound 3 (0.135 g, 0.311 mmol) and compound 4 (0.085 g, 0.282 mmol) were mixed and dissolved in ethanol (20 mL). Then the mixture was refluxed for 3 h. The solvent was then evaporated under reduced pressure, and the resulting residue was
purified by flash column chromatography (dichloromethane : ethyl acetate : methanol = 10 : 1 : 1, V/V/V) to afford a pure CPC (0.137 g, 68.0%). IR (KBr, cm\(^{-1}\)): 3449.5, 2973.2, 2935.4, 1713.6, 1577.9, 1522.5; \(^1\)H NMR (300 MHz, DMSO-\(d_6\)): \(\delta\) 8.32 (d, \(J = 15.9\) Hz, 1H), 8.11 (d, \(J = 9\) Hz, 2H), 8.04 (s, 1H), 7.78 (dd, \(J = 14.1, 7.2\) Hz, 2H), 7.60 - 7.49 (m, 3H), 7.36 (d, \(J = 15.9\) Hz, 1H), 7.11 (d, \(J = 9\) Hz, 2H), 6.77 (dd, \(J = 9, 2.2\) Hz, 1H), 6.58 (d, \(J = 2.2\) Hz, 1H), 4.02 (s, 3H), 3.75-3.58 (m, 8H), 3.47 (q, \(J = 6.9\) Hz, 4H), 1.76 (s, 6H), 1.15 (t, \(J = 7.2\) Hz, 6H); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) 206.88, 180.80, 164.82, 158.95, 157.19, 154.46, 154.20, 151.90, 144.75, 143.30, 142.48, 134.19, 130.67, 129.23, 128.48, 124.32, 123.15, 116.09, 114.46, 114.13, 109.96, 107.64, 107.37, 96.80, 51.67, 44.66, 33.87, 31.14, 26.50, 12.78; HRMS: m/z [M]+ calcd for C\(_{37}\)H\(_{41}\)N\(_4\)O\(_3\)+: 589.3179, found: 589.3159.

**Synthesis of Donor**

Compound 2 (0.110 g, 0.393 mmol) was added to a mixture of HNEt\(_2\) (1 mL) and DCM (6 mL) with stirring. Then the mixture was stirred for 13 h. The solvent was then evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (petroleum ether : ethyl acetate = 2 : 1, V/V) to afford a pure Donor (0.055 g, 44.4%). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)): \(\delta\) 7.90 (s, 1H), 7.48 (d, \(J = 9.0\) Hz, 1H), 6.74 (dd, \(J = 9.0\) and 2.4 Hz, 1H), 6.56 (d, \(J = 2.4\) Hz, 1H), 3.48 - 3.34 (m, 6H), 3.24 (q, \(J = 6.9\) Hz, 2H), 1.15 - 1.03 (m, 12H); HRMS: m/z [M+H]+ calcd for [C\(_{18}\)H\(_{25}\)N\(_2\)O\(_3\)]+: 317.1865, found: 317.1864.

**Synthesis of Acceptor**

4-Dimethylaminobenzaldehyde (0.112 g, 0.75 mmol), compound 4 (0.150 g, 0.50
mmol) were mixed and dissolved in ethanol (10 mL). Then the mixture was refluxed for 4.5 h under nitrogen atmosphere. The solvent was then evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (DCM : MeOH = 20:1, V/V) to afford a pure **Accepter** (0.194 g, 89.8%). **1H NMR** (300 MHz, DMSO-\(d_6\)) \(\delta\) 8.31 (d, \(J = 15.9\) Hz, 1H), 8.07 (d, \(J = 9\) Hz, 2H), 7.77 (d, \(J = 7.8\) Hz, 1H), 7.70 (d, \(J = 7.8\) Hz, 1H), 7.58-7.45 (m, 2H), 7.26 (d, \(J = 15.9\) Hz, 1H), 6.89 (d, \(J = 9\) Hz, 2H), 3.97 (s, 3H), 3.16 (s, 6H), 1.75 (s, 6H); **13C NMR** (100 MHz, DMSO-\(d_6\)): 206.93, 180.06, 154.90, 154.52, 143.02, 142.54, 129.15, 128.00, 123.08, 122.74, 114.04, 112.65, 105.66, 51.33, 33.57, 31.17, 26.71; **HRMS**: m/z [M]+ calcd for C\(_{21}\)H\(_{25}\)N\(_2\): 305.2018, found: 305.2059.

**Preparation of the test solution**

Sodium sulfide (Na\(_2\)S•9H\(_2\)O) was diluted from the commercially available solution to 0.01 M in water. A stock solution of probe CPC (1 \(\times\) 10\(^{-3}\) M) was prepared in DMF. Test solutions were prepared by displacing 50 \(\mu\)L of the stock solution into a 10-mL volumetric flask. The solution was diluted to 10 mL in a mixture of phosphate buffer (pH 7.4, 0.01 M) and DMF (6 : 4, V/V). The solutions of various testing species were prepared from Cys, GSH, Hcy, H\(_2\)O\(_2\), KNO\(_3\), Ca(NO\(_3\))\(_2\)•4H\(_2\)O, NaNO\(_3\), KCl, KBr, KI, NaClO, NaHCO\(_3\), Na\(_2\)SO\(_4\), CH\(_3\)COONa, Na\(_2\)SO\(_3\), N\(_2\)H\(_4\)•H\(_2\)O, NH\(_3\)•H\(_2\)O, CO(NH\(_2\))\(_2\), CS(NH\(_2\))\(_2\), EDA, NO, O\(_2\)\(^-\), respectively. Nitric oxide (NO) was generated from potassium nitroprusside dehydrate. Superoxide (O\(_2\)\(^-\)) was prepared from KO\(_2\). Small aliquots of each testing species solution were added. The resulting solution was
shaken well and incubated for 1 h at room temperature before recording the spectra.

The UV-vis absorption and fluorescence property of probe CPC was measured in a mixture of PBS buffer (pH 7.4, 0.01 M) : DMF (6: 4, v/v) except the selectivity test for hydrogen sulfide and sulfite in PBS buffer (pH 7.4, 0.01 M) : DMF (1: 9, v/v).

**Determination of fluorescence quantum yield**

Quantum yield ($\Phi_F$) was determined by the relative comparison procedure, with quinine sulfate dehydrate ($\Phi_{Fs} = 0.56, 0.1 \text{ N } \text{H}_2\text{SO}_4$) or rhodamine B ($\Phi_{Fs} = 0.89, \text{ in ethyl alcohol}$) as the main standard. The general equation used in the determination of relative quantum yields from earlier research is Eq. (1).

$$\Phi_F = (\Phi_{Fs})(F_{Au})(A_u)(\eta_u^2) / (F_{As})(A_s)(\eta_s^2),$$

(1)

Where $\Phi_F$ and $F_A$ are fluorescence quantum yield and integrated area under the corrected emission spectrum, respectively; $A$ is absorbance at the excitation wavelength; $\eta$ is the refractive index of the solution; and subscripts u and s refer to the unknown and the standard, respectively.

**Calculation of energy transfer efficiency**

Energy transfer efficiency ($E$) was calculated using the following equation as reported.

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

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

**Cell culture and imaging of cells**

Hela cells were seeded in a 6-well plate in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C.
Imaging of exogenous H$_2$S in living cells

Hela cells were incubated with CPC (3.0 μM) for 1 h and then treated with Na$_2$S (0.5, 1, 5 mM) for 1 h. Subsequently, the cells were imaged using a confocal microscope (LSM 700) at 405 nm excitation and emission channels of 405-560 nm (blue channel) and 560-700 nm (red channel).

Imaging of endogenous H$_2$S in living cells

HeLa cells incubated with CPC (1 μM) for 1 h or cells incubated with Cys (200 μM) for 1 h followed by CPC (1 μM) for 1 h. Inhibition test was carried out by following procedure: cells incubated with PAG (1 mM), AOAA (1 mM) and both PAG and AOAA for 1 h, respectively, followed by further incubation with Cys (200 μM) for 1 h, then incubated with CPC (1 μM) for 1 h. Subsequently, the cells were imaged using a confocal microscope (LSM 700) at 405 nm excitation and emission channels of 405-560 nm (blue channel) and 560-700 nm (red channel).

Co-localization imaging of cells

Hela cells were incubated with CPC (1 μM) for 1 h at 37 °C. Then, Mito Tracker Deep Red (0.3 μM) was added and incubated for another 0.5 h and the confocal fluorescent images were captured.

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Table S1. The spectroscopic data of compounds CPC, Donor and Acceptor

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ$_{abs}$ (nm)</th>
<th>Logε (M$^{-1}$cm$^{-1}$)</th>
<th>λ$_{em}$ (nm)</th>
<th>Φ$_F$</th>
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<tbody>
<tr>
<td>CPC</td>
<td>418</td>
<td>4.58</td>
<td>474</td>
<td>0.005[a]</td>
</tr>
<tr>
<td></td>
<td>518</td>
<td>4.81</td>
<td>587</td>
<td>0.01[b]</td>
</tr>
<tr>
<td>Donor</td>
<td>406</td>
<td>4.41</td>
<td>475</td>
<td>0.139[a]</td>
</tr>
<tr>
<td>Acceptor</td>
<td>546</td>
<td>4.90</td>
<td>588</td>
<td>0.004[b]</td>
</tr>
<tr>
<td>CPC + H$_2$S 10 eq</td>
<td>--</td>
<td>--</td>
<td>474</td>
<td>0.015[a]</td>
</tr>
</tbody>
</table>

[a] λ$_{ex}$ = 370 nm  [b] λ$_{ex}$ = 495 nm
Fig. S1 Fluorescence spectra of the probe (5 µM) in the absence and presence of hydrogen sulfide (50 µM); $\lambda_{ex} = 410$ nm, slit: 12 nm/10 nm.

Fig. S2 Fluorescence spectra of the donor (5 µM) in the absence and presence of hydrogen sulfide (50 µM); $\lambda_{ex} = 410$ nm, slit: 12 nm/4.5 nm.
Fig. S3 Fluorescence spectra of the acceptor (5 µM) in the absence and presence of hydrogen sulfide (50 µM); $\lambda_{ex} = 540$ nm, slit: 12 nm/10 nm.

Fig. S4 The normalized fluorescence spectrum of the donor (5 µM) (black) and the normalized absorption spectra of the acceptor (5 µM) (red). $\lambda_{ex} = 410$ nm, slit: 12 nm/4.5 nm.
Fig. S5 The fluorescence emission spectra of the probe (5 μM) (red), the reference donor (5 μM) (black), and the reference acceptor (5 μM) (blue); $\lambda_{ex} = 410$ nm, slit: 12 nm/4.5nm. $E = 98.35\%$.

Fig. S6 The absorption (black) and corrected excitation (blue) spectra of probe CPC (5 μM); $\lambda_{em} = 587$ nm, slit: 12 nm/10 nm.
Fig. S7 Absorption spectra of the probe (5 µM) toward Na$_2$S (10 equiv.) and various biologically relevant species (20 equiv.).

Fig. S8 Fluorescence intensity ratio ($I_{474}/I_{587}$) changes of the probe (5 µM) to Na$_2$S (50 µM), GSH (5 mM), Hcy (1 mM), Cys (1 mM) and other biologically relevant species (100 µM). $\lambda_{ex}$ = 410 nm, slit: 12 nm/10 nm.
Fig. S9 Time-dependent fluorescence intensity ratio ($I_{474}/I_{587}$) of the probe (5 µM) with Na$_2$S 10 equiv.; $\lambda_{ex} = 410$ nm, slit: 12 nm/10 nm.

Fig. S10 Fluorescence intensity ratio ($I_{474}/I_{587}$) of the probe (5 µM) upon addition of Na$_2$S (50 µM) in the presence of various species, 1 free probe, 2 Na$_2$S at 50 µM. 3 Cl$^-$, 4 CH$_3$CO$_2^-$, 5 NO$_3^-$, 6 SO$_3^{2-}$, 7 Br$^-$, 8 HCO$_3^-$, 9 SO$_4^{2-}$, 10 EDA, 11 N$_2$H$_4$, 12 CO(NH$_2$)$_2$, 13 CS(NH$_2$)$_2$, 14 H$_2$O$_2$, 15 NH$_3$.H$_2$O, 16 I$^-$ and 17 HClO at 100 µM. 18 GSH at 5 mM, 19 Hcy at 1 mM, 20 Cys at 1 mM. 21 NO, 22 O$_2^-$, 23 K$^+$, 24 Na$^+$ and 25 Ca$^{2+}$ at 100 µM; $\lambda_{ex} = 410$ nm, slit: 12 nm/10 nm.

Fig. S11 pH-dependent fluorescence intensity ratio changes of the probe (5 µM); $\lambda_{ex} = 410$ nm, slit: 12 nm/10 nm.
Fig. S12 Fluorescence intensity ratio ($I_{474}/I_{587}$) changes of the probe (5 µM) with the addition of Na$_2$S (0–55 µM); $\lambda_{\text{ex}} = 410$ nm, slit: 12 nm/10 nm.

Fig. S13 Linearity of the ratio ($I_{474}/I_{587}$) of the probe with Na$_2$S from 0 to 50 µM. $\lambda_{\text{ex}} = 410$ nm, slit: 12 nm/10 nm.
Fig. S14 Absorption spectra of the probe (5 µM) upon addition of Na₂S (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 equiv.). Inset: the color change observed by the naked eye of the probe with the addition of H₂S.

Fig. S15 Thermo LCQ Fleet mass spectrum of the CPC (50 µM) solution in the presence of excessive Na₂S (500 µM).

Fig. S16 (a) Photostability of probe CPC for fluorescence images of HeLa cells. The
cells were incubated with the probe (3 μM) for 1 h. First line: fluorescence images at the blue channel (405–560 nm), second line: fluorescence images at the red channel (560–700 nm), third line: bright field images, fourth line: overlay images of the first, second and third lines. \( \lambda_{\text{ex}} = 405 \text{ nm} \). (b) The ratio of blue/red fluorescence intensity of probe CPC for fluorescence images of HeLa cells.

![Fig. S17 Cytotoxicity of probe CPC evaluated on living HeLa cells by the standard SRB assay. The cells were incubated with the CPC (1, 2.5 and 5 μM) for 6 h.](image)

![Fig. S18 \(^1\text{H NMR spectrum of compound 3 in DMSO-}d_6.\)](image)
Fig. S19 $^1$H NMR spectrum of probe CPC in DMSO-$d_6$.

Fig. S20 $^{13}$C NMR spectrum of probe CPC in DMSO-$d_6$. 
Fig. S21 IR spectrum of probe CPC.

Fig. S22 HRMS spectrum of probe CPC.
Fig. S23 $^1$H NMR spectrum of **Donor** in DMSO-$d_6$.

Fig. S24 HRMS spectrum of **Donor**.
Fig. S25 $^1$H NMR spectrum of **Acceptor** in DMSO-$d_6$.

Fig. S26 $^{13}$C NMR spectrum of **Acceptor** in DMSO-$d_6$. 
Fig. S27 HRMS spectrum of **Acceptor**.

**References:**