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Supporting Information

A highly selective and fast-response fluorescent probe for visualization of enzymatic H_2S production *in vitro* and in living cells

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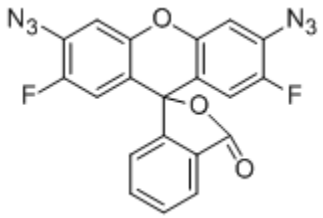
1. Reagents and instruments

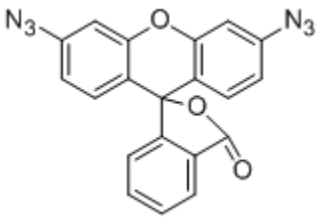
All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiment without further purification. The progress of the reaction was monitored by TLC on pre-coated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized by basic $KMnO_4$, UV light or iodine. Merck silica gel 60 (70-200 mesh) was used for general column chromatography purification. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 (400 MHz ^1H; 100 MHz ^{13}C) spectrometer at room temperature. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm). ^1H NMR coupling constants (*J*) are reported in Hertz (Hz), and multiplicity is indicated as the following: *d* (doublet), *m* (multiple). High-resolution mass spectra (HRMS) were obtained on a Varian 7.0 T FTICR-MS.

2. Synthesis of probes



Concentrated sulfuric acid (20 mL) was added into a 100 ml round bottom flask containing 3-amino-4-fluorophenol (522 mg, 4.1 mmol) and o-phthalic anhydride (296 mg, 2 mmol). The mixture was heated at 120 ℃ for 4 hour and then at 160 ℃ for another 40 hours. Then the mixture was cooled down to room temperature and poured into 50 ml ice. After addition of 10 ml concentrated hydrochloric acid, $NaNO_2$ (414 mg, 6 mmol)

was added dropwise at 0-5 ℃. Then NaN_3 (390 mg, 6 mmol) was added dropwise at 0 ℃. The reaction mixture was extracted with EtOAc (3×30 ml) and the organic layer was removed to offer oil crude product, which was purified by column chromatography eluting with petroleum ether : ethyl acetate = 20 : 1 to give the product (80 mg, 5%). TLC (silica plate): R_f = 0.56 (petroleum ether : ethyl acetate = 4 : 1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, J = 6.9 Hz, 1H), 7.77–7.64 (m, 2H), 7.15 (d, J = 7.3 Hz, 1H), 6.99 (d, J = 6.9 Hz, 2H), 6.51 (d, J = 10.8 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.6, 167.9, 152.6, 152.0, 150.1, 147.6, 135.8, 132.5, 131.4, 131.1, 130.8, 129.0, 125.9, 123.7, 115.1, 114.9, 109.4, 81.2; ^{19}F NMR (376 MHz, $CDCl_3$) δ = -130.1; HRMS (ESI): calculated for $C_{20}H_8F_2N_6O_3$ [M + H^+]: 419.0699, found: 419.0697.



Rhodamine 110 (87 mg, 0.24 mmol) was suspended in a 50 ml round bottom flask containing 2 ml H_2O and 6 ml concentrated hydrochloric acid at 0 ℃. $NaNO_2$ (130 mg, 1.88 mmol) was added dropwise at 0-5 ℃. Then, NaN_3 (122 mg, 1.88 mmol) was added dropwise at 0 ℃. After stirring at room temperature for 0.5 h, the mixture was extracted with EtOAc (3 × 30 ml) and the organic layer was removed to offer crude product, which was purified by column chromatography eluting with petroleum ether : ethyl acetate = 15 : 1 to give the product (88 mg , 96%). TLC (silica plate): R_f = 0.76 (petroleum ether : ethyl acetate = 2 : 1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (m, 1H), 7.72–7.60 (m, 2H), 7.14 (m, 1H), 6.95 (d, J = 1.9 Hz, 2H), 6.79 (d, J = 8.5 Hz, 2H), 6.73 (dd, J = 8.5, 2.0 Hz, 2H).

3. Spectroscopic analysis of the probes

Spectroscopic measurements were performed in PBS (50 mM, pH 7.4) buffer at room temperature. Compounds were dissolved into DMF to prepare the stock solutions with a concentration of 10.0 to 1.0 mM. The UV-visible spectra were recorded on a UV-3600 UV-VIS-NIR-spectrophotometer (SHIMADZU, Japan). Fluorescence studies were carried out using F-7000 fluorescence spectrophotometer (HITACHI, Japan). All reaction mixture was shaken uniformly before measurement.

The detection limit was calculated with the following equation:

$$\text{Detection limit} = 3\,\sigma/k$$

Where σ is the standard deviation of fluorescence intensity of **1**; k is the slop between the fluorescence intensity versus H_2S concentration (k = 2.085, Fig. 2). The fluorescence emission spectrum of probe **1** was measured by six times and the standard deviation of blank measurement was found to be 0.262. The detection limit is 0.38 μM.

The quantum yield was calculated with the following equation:

$$\phi = \phi_0 \times \frac{A_0 \times F}{A \times F_0}$$

Where ϕ_0 (ϕ_0 = 0.96, rhodamine 110) is the quantum yield of standard substance, A_0 (A_0 = 0.103) and A (A = 0.075) means the absorption of standard substance and measured substance at the same concentration, respectively. F_0 (F_0 = 295320.8) and F (F = 198461.4) mean the standard substance and measured substance's integrated intensity of the emission spectra, respectively. The quantum yield is determined as 0.89.

4. Expression and purification of truncated human CBS enzyme

Human CBS gene (No. 1-1239) DNA sequence was amplified by PCR using the primers, forward: 5'-CGCCATATGCCTTCTGAGACCCCCCA-3'; reverse: 5'-CGTCGACTCAACGCAGGTGCCA-3' with Nde I and Sac I restriction enzyme cleavage sites. PCR product was under double-digestion by the above two enzymes to create cohesive ends to ligate into vector pET28a(+) with the same ends. Recombinant expression plasmid pET28a-hCBS 1-413 was confirmed by double-digestion and sequencing, then transformed into *E. coli* Rosetta (DE3) competent cell.

A 50 mL overnight culture of *E. coli* strain Rosetta (DE3) (Strategene), containing the pET28a-hCBS 1-413 expression construct, was grown in 2 X YT media containing 50 μg/mL kanamycin. Three liters of 2 X YT media were inoculated with the overnight culture media (at a 1:100 inoculants to media ratio). Cells were grown at 37 ℃ and IPTG was added to a final concentration of 0.1 mM when the OD_{600} reached about 0.5. Then cells were grown at 30 ℃ for another 6 h and harvested by centrifugation at 4000 rpm for 10 min at 4 ℃. The cell pellets were washed by resuspension in 100 mL Tris buffer (pH 8.0), followed by centrifugation at 4000 rpm for 10 min. Cell pellets were resuspended in 40 mL of lysis buffer (50 mM potassium phosphate, pH 7.8, 10 mM imidazole, 20 μM PLP), containing one ''EDTA-free" protease inhibitor tablet (Roche), 1 mg/mL lysozyme and 10 μg/mL DNase I, and incubated at room temperature for 30 min prior to sonication (90 cycles of 90 s at 45 % duty cycle). The cell lysate was centrifuged at 15,000 rpm for 45 min at 4 ℃ and the supernatant was loaded on a 2 mL Ni–NTA column, preequilibrated with lysis buffer. The column was subsequently washed with at least 40 column volumes of lysis buffer and the protein was eluted with a 200 mL linear gradient

of 10–200 mM imidazole in lysis buffer. Fractions containing pure hCBS protein were desalted against hCBS storage buffer (50 mM potassium phosphate buffer, 20 μM PLP, and 1 mM EDTA), glycerol was added to 20% (v/v) and the aliquoted enzyme was stored at -80 ℃ for use.

5. Enzymatic activity assay based on probe 1

CBS activity studies were carried out in 190 μL activity buffer (200 mM Tris HCl, pH 8.0, 5 μM PLP, 10 mM glutathione, 0.5 mg/mL BSA) and 10 μL DMSO, which was used to dissolve the probe **1**. The final probe concentration was 20 μM; the concentration of L-cysteine was varied from 0.1-20 mM; 1 μg of CBS was used in a total volume of 200 μL. After the addition of substrate, the reaction mixture were incubated at 37 ℃ for 30 min, and then 500-600 nm fluorescence spectra were monitored under 480 nm excitation for each sample. For inhibition experiment, the 200 μL enzyme reaction system contained L-Cys 10 mM and 1 μg of CBS 1-413 with or without adding 5 mM D/L-PPG. The fluorescence intensity at 520 nm was tested for the CBS activity.

6. Cell culture and fluorescence microscope experiments

HEK-293 cells were cultured at 37 ℃, 5% CO_2 in DMEM/HIGH GLUCOSE (GIBICO) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine. The cells were maintained in exponential growth, and then seeded in glass-bottom 35 mm plate at the density about 2 × 104/well. Cells were passaged every 2-3 days and used between passages 3 and 10. For exogenous H_2S

imaging, cells were incubated with probe **1** (1 μM) for 30 min and then Na_2S (50 or 200 μM) for another 30 min at 37 ℃ and 5% CO_2. For endogenous H_2S imaging, cells were incubated with different concentration of L-Cys or D-Cys (10 and 100 μM) for 20 min at 37 ℃ and 5% CO_2. After that, the media was discarded and washed by PBS buffer. Then probe **1** was added into the media for further 30 min incubation. After being washed by PBS, cells were imaged on a confocal microscope (Olympus FV1000 UPLSAPO40X) with a 40 × objective lens. Control cells were only treated with probe **1** at 37 ℃ for 30 min. All images were analyzed with Olympus FV1000-ASW. Emission was collected at green channel (500-600 nm) with 488 nm excitation.

7. RNAi experiments

HEK293A cells in exponential growth status were seeded in six-well plates (approximate 1 x 105 /well) to reach about 70% confluence. The culture medium was changed into serum free OPTI-MEM I (GIBICO/Invitrogen, USA), 1.6 mL/well before transfection. Then, cells were co-transfected with different siRNA sequences (Table S1) with Lipofectamine 2000 (Invitrogen, USA) (total transfection volume 0.4 mL). After 4 h incubation in 37 ℃, OPTI-MEM I was changed into normal culture medium for further 20 h incubation. After that, cells were digested with 0.25% trypsin enzyme (GIBICO/Invitrogen, USA) and seeded in glass-bottom 35 mm plate with the density about 6 ×104/well. When cells began to stretch and confluence reached about 80-90%, confocal microscopy analysis was done as described above. At the same time, total RNA was extracted from corresponding samples to perform the quantitative RT-PCR to confirm the gene-silencing efficiency.

8. Total RNA extraction and RT-qPCR

Total RNA was extracted using Trizol (Sigma Aldrich, USA) and further by phenol-chloroform extraction and ethanol precipitation. After quantification using Nanodrop, 2 μg total RNA sample was used for reverse transcription (Roche, Switzerland) and the following quantitative PCR procedure. For normal cells and CBS-, CSE-, 3MPST-silenced cells, specific primers were designed to measure the remaining mRNA level of above each gene (Table S2). *β*-Actin was chosen as internal control for data processing.

Table S1. Sequences of siRNAs targeting difference enzymes involving in H_2O_2-induced H_2S biogenesis.

CBS gene GeneID：875	5‘ GGAAGAAGUUCGGCCUGAA dTdT 3‘ 3‘ dTdT CCUUCUUCAAGCCGGACUU 5‘
	5‘ GGAACUACAUGACCAAGUU dTdT 3‘ 3‘ dTdT CCUUGAUGUACUGGUUCAA 5‘
	5‘ CCAUUGACUUGCUGAACUU dTdT 3‘ 3‘ dTdT GGUAACUGAACGACUUGAA 5‘
CSE gene GeneID:1491	5‘ CCUUCAUAAUAGACUUCGU dTdT 3‘ 3‘ dTdT GGAAGUAUUAUCUGAAGCA 5‘
	5‘ CACAGCAUGAGUUGGUGAA dTdT 3‘ 3‘ dTdT GUGUCGUACUCAACCACUU 5‘
	5‘ CCGGCAAUCAUGACUCAUG dTdT 3‘ 3‘ dTdT GGCCGUUAGUACUGAGUAC 5‘
3MPST gene GeneID:4357	5‘ UCAAGACCUACGAGGACAU dTdT 3‘ 3‘ dTdT AGUUCUGGAUGCUCCUGUA 5‘
	5‘ GCCAUCUGUUCCAGGAGAA dTdT 3‘ 3‘ dTdT CGGUAGACAAGGUCCUCUU 5‘
	5‘ AGACGUGCCCAUCUACGAU dTdT 3‘ 3‘ dTdT UCUGCACGGGUAGAUGCUA 5‘
GAPDH	As control siRNA sequence provided by RioBio, Guangzhou.

Table S2. Sequences of primers used in quantitative PCR.

	Forward (5'-3')	Reverse (5'-3')
CBS	CGGGCACTGGGGGGCTGAGATT	AGCATGCGGGCAAAGGTGAACG
CSE	CACTGTCCACCACGTTCAAG	GTGGCTGCTAAACCTGAAGC
3-MPST	GACCCCGCCTTCATCAAG	CATGTACCACTCCACCCA
β-actin	CCAACCGCGAGAAGA TGA	CCAGAGGCGTACAGGGATAG

9. Supplementary figures

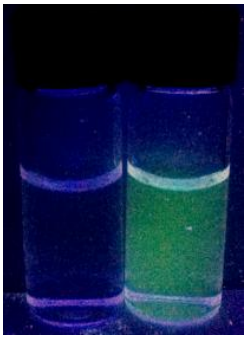


Fig. S1 Photograph of probe **1** (10 μM) before and after reacting with Na_2S (1 mM) in PBS buffer for 1 h under UV lamp (365 nm).

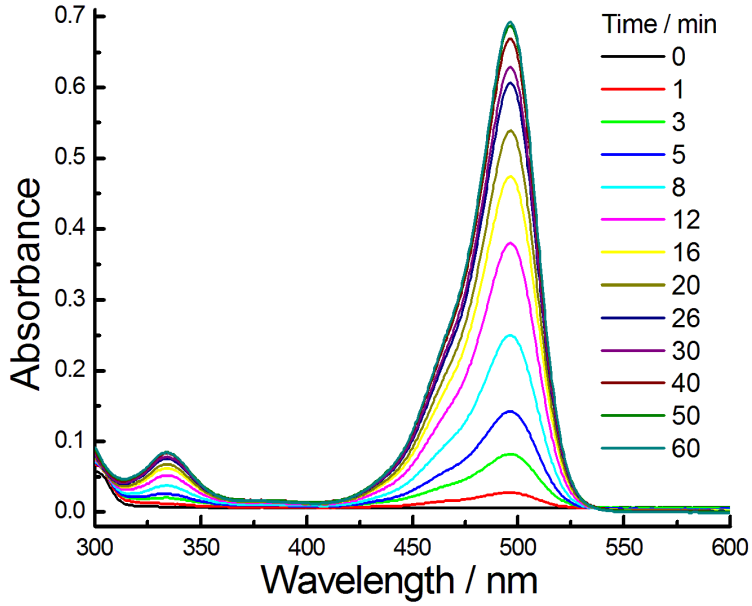


Fig. S2 Time-dependent absorbance spectra of probe **2** (10 μM) upon reaction with Na_2S (1 mM) in PBS buffer. The different reaction time is indicated in the inset.

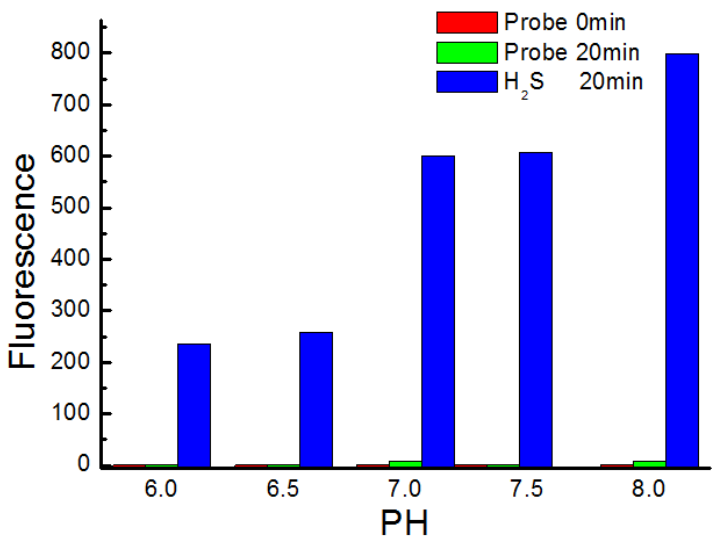


Fig. S3 Fluorescence of pH-dependent experiments for probe **1**. The reaction of probe **1** (1 μM) and H_2S (150 μM) was performed in PBS buffer at different pH values. Emission intensity at 520 nm was recorded with 480 nm excitation.

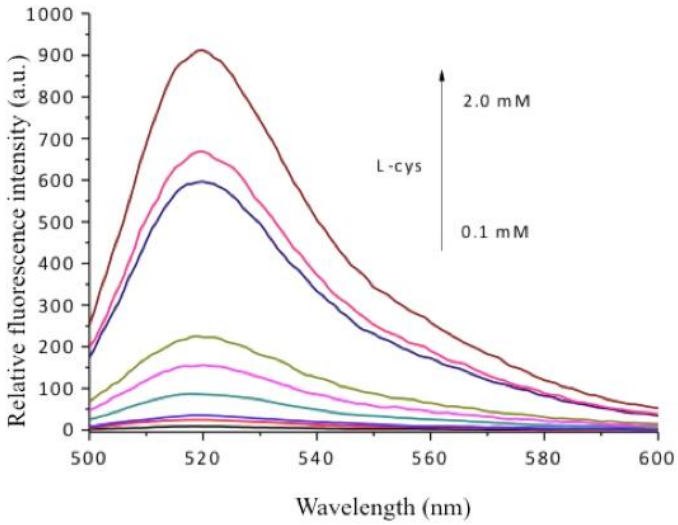


Fig. S4 *In vitro* CBS enzyme-based activity assay with probe **1**. a) Fluorescence spectra of **1** in the enzyme reaction system containing different concentrations of substrate L-cys (0-20 mM) (excitation at 480 nm).

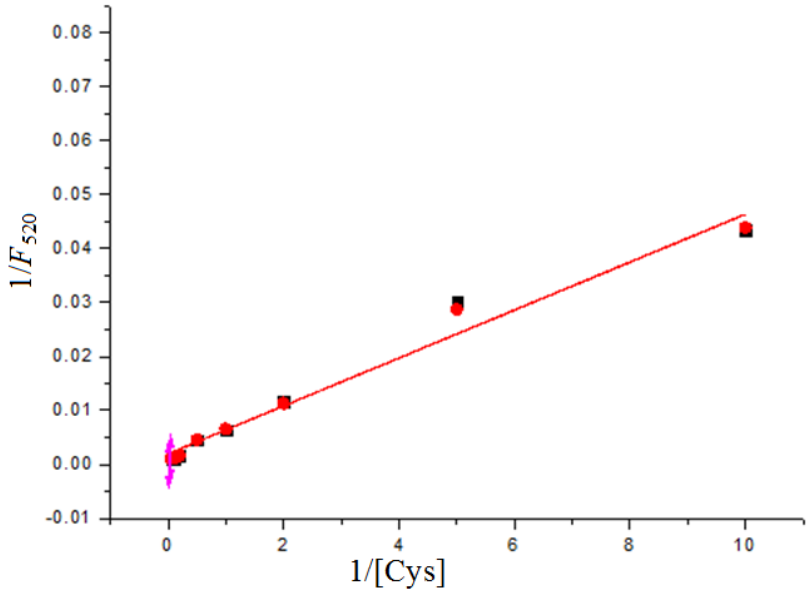


Fig. S5 Lineweaver-Burk plot analysis of CBS activity data. The reciprocal of fluorescent intensities at 520 nm versus the reciprocal of Cys concentrations were fitted by linear function (R^2 = 0.972). The intercept on the X axis was calculated as $1/K_m$.

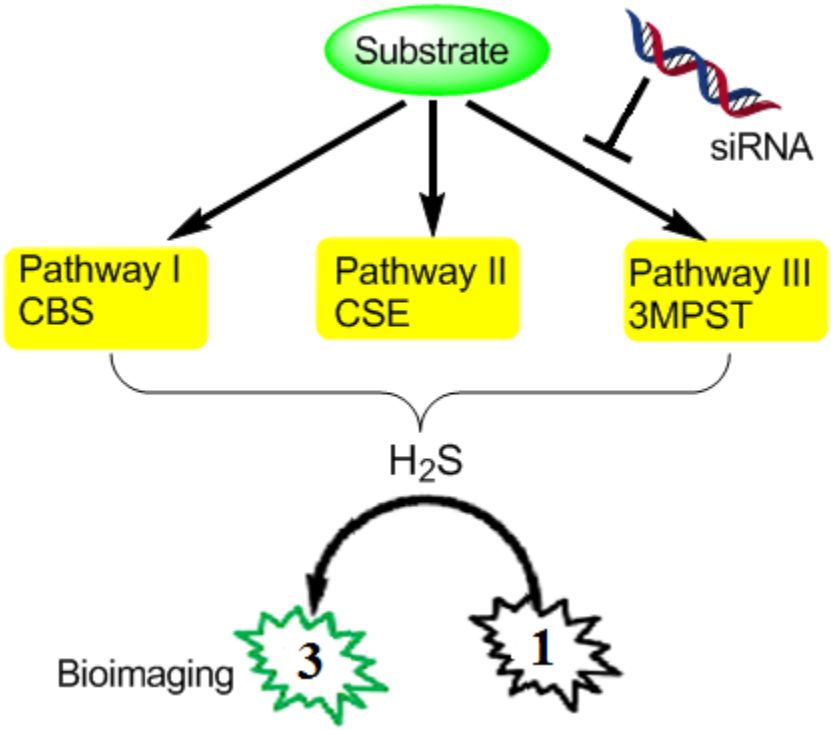


Fig. S6 Schematic representation of the gene-knockdown-based H_2S visualization strategy in living cell environment.

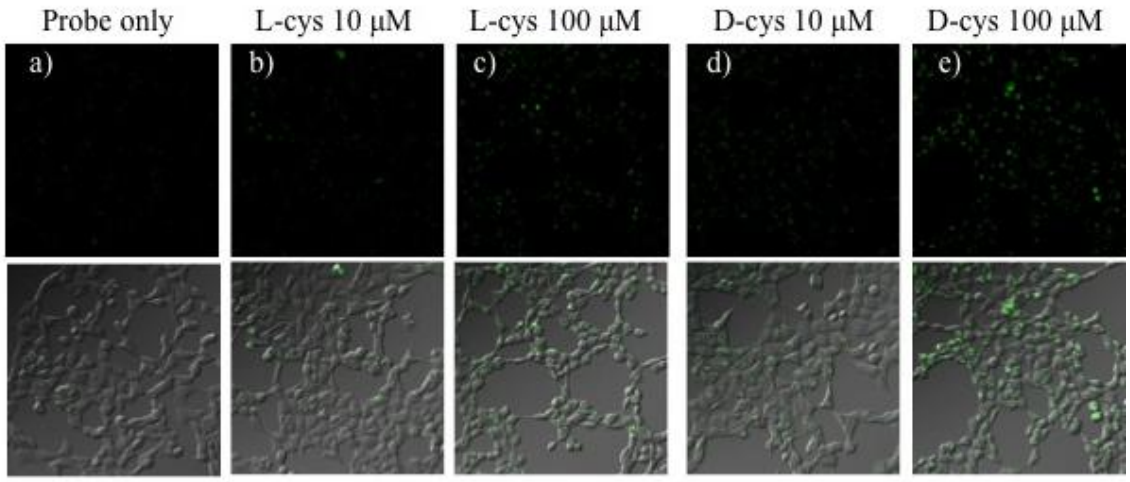


Fig. S7 Confocal microscopy images of endogenous H_2S in normal HEK293A cells with probe **1**. Cells were treated (a) with **1** (1 μM) for 30 min; (b, c) with **1** (1 μM) for 30 min and then L-Cys (10 or 100 μM) for another 30 min; (d, e) with **1** (1 μM) for 30 min and D-Cys (10 or 100 μM) for another 30 min. The bright field images are shown below. Scale bar: 50 μm.

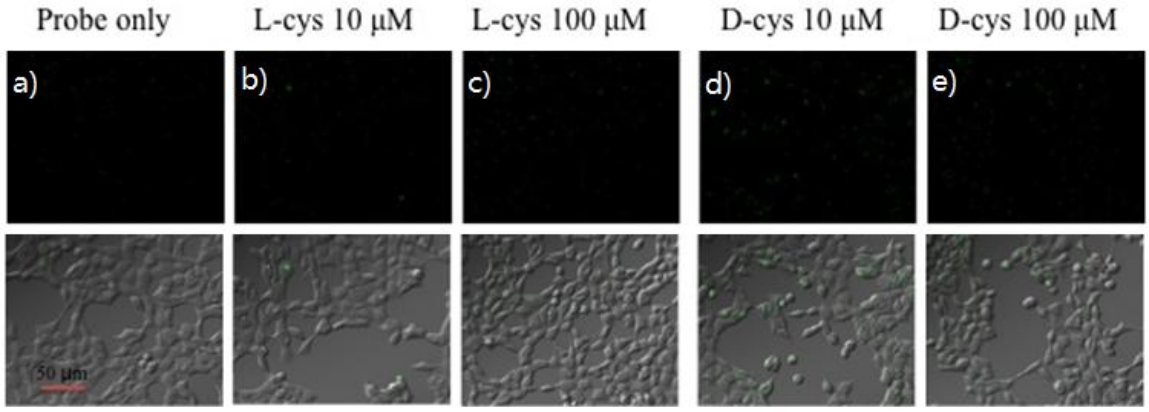


Fig. S8 Confocal microscopy images of endogenous H_2S in CBS silenced 293A cells with probe **1**. CBS enzyme was silenced using the designed siRNA sequences to generate specific gene-knockdown HEK293A cells. Then cells were treated (a) with **1** (1 μM) for 30 min; (b, c) with **1** (1 μM) for 30 min and then L-Cys (10 or 100 μM) for another 30 min; (d, e) with **1** (1 μM) for 30 min and then D-Cys (10 or 100 μM) for another 30 min. The bright field images are shown below. Scale bar: 50 μm.

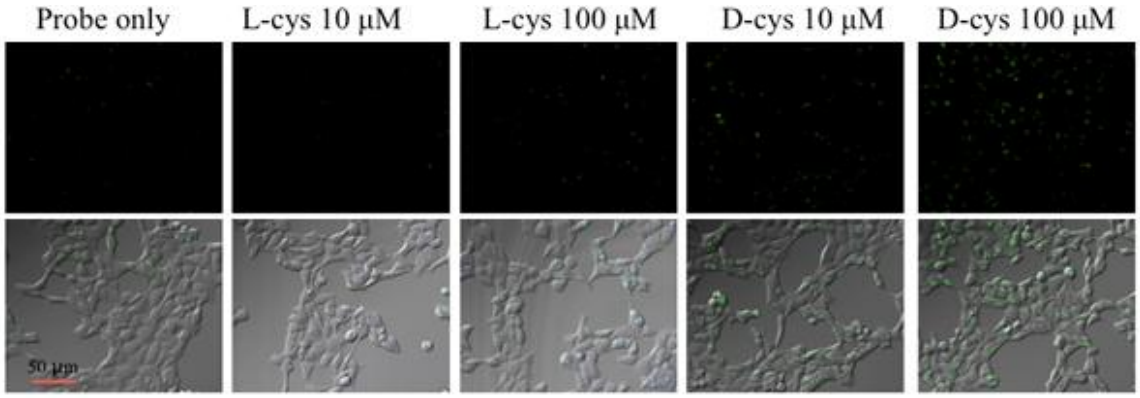


Fig. S9 Confocal microscopy images of endogenous H_2S in CSE silenced 293A cells with probe **1**. CSE enzyme was silenced using designed siRNA sequences to generate specific gene-knockdown HEK293A cells. Then cells were treated with **1** (1 μM) for 30 min; or with **1** (1 μM) for 30 min and then L-Cys (10 or 100 μM) for another 30 min; or with **1** (1 μM) for 30 min and then D-Cys (10 or 100 μM) for another 30 min. The bright field images are shown below. Scale bar: 50 μm.

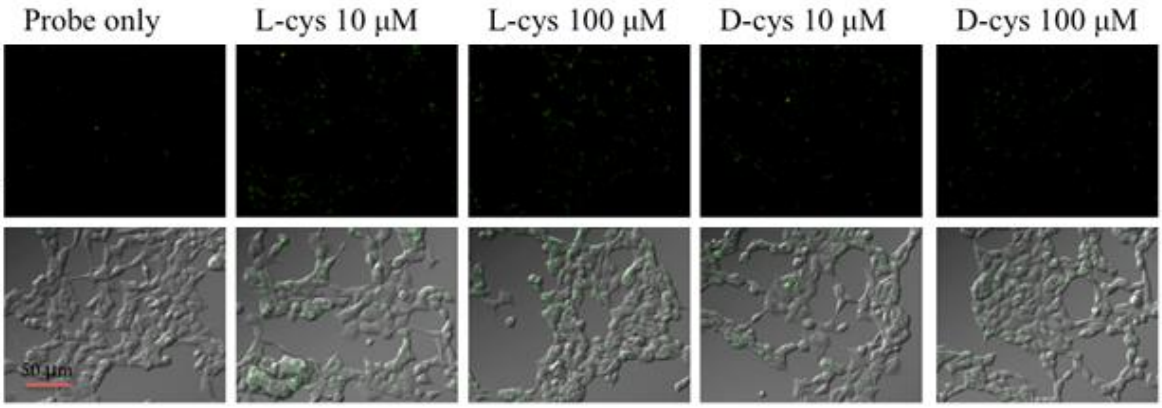


Fig. S10 Confocal microscopy images of endogenous H_2S in 3-MPST silenced 293A cells with probe **1**. 3-MPST enzyme was silenced using designed siRNA sequences to generate specific gene-knockdown HEK293A cells. Then cells were treated with **1** (1 μM) for 30 min; or with **1** (1 μM) for 30 min and then L-Cys (10 or 100 μM) for another 30 min; or with **1** (1 μM) for 30 min and then D-Cys (10 or 100 μM) for another 30 min. The bright field images are shown below. Scale bar: 50 μm.

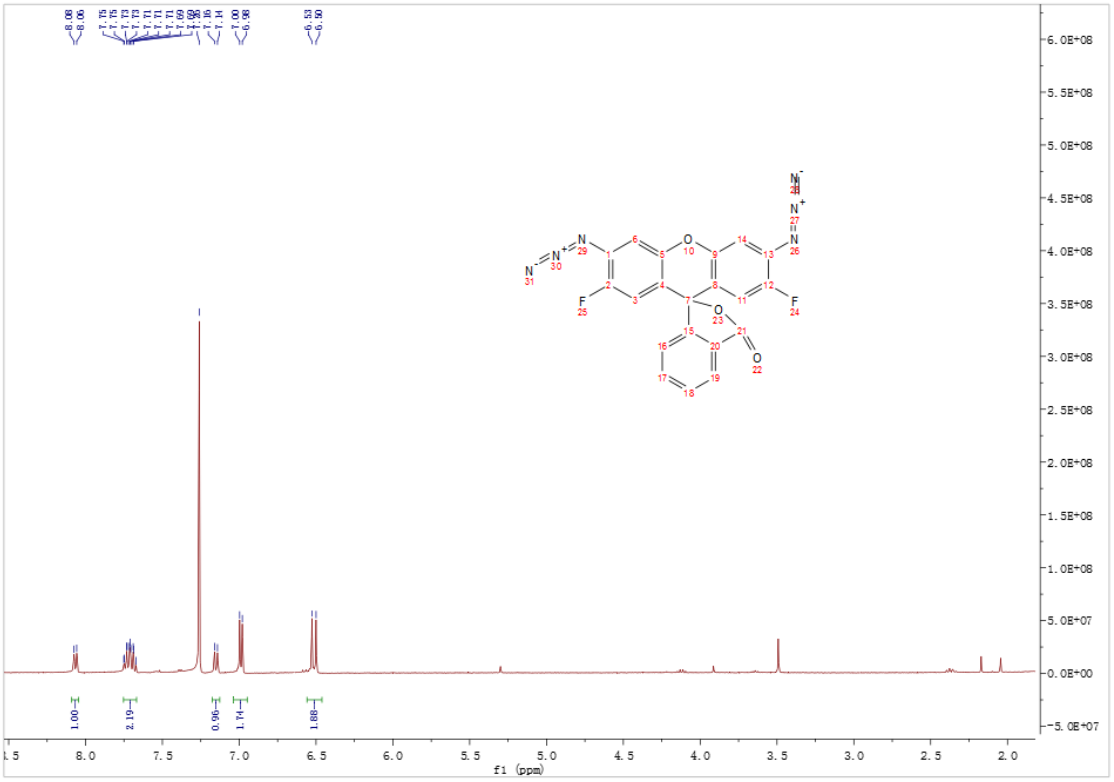


Fig. S11 ^{1}H-NMR of **1**.

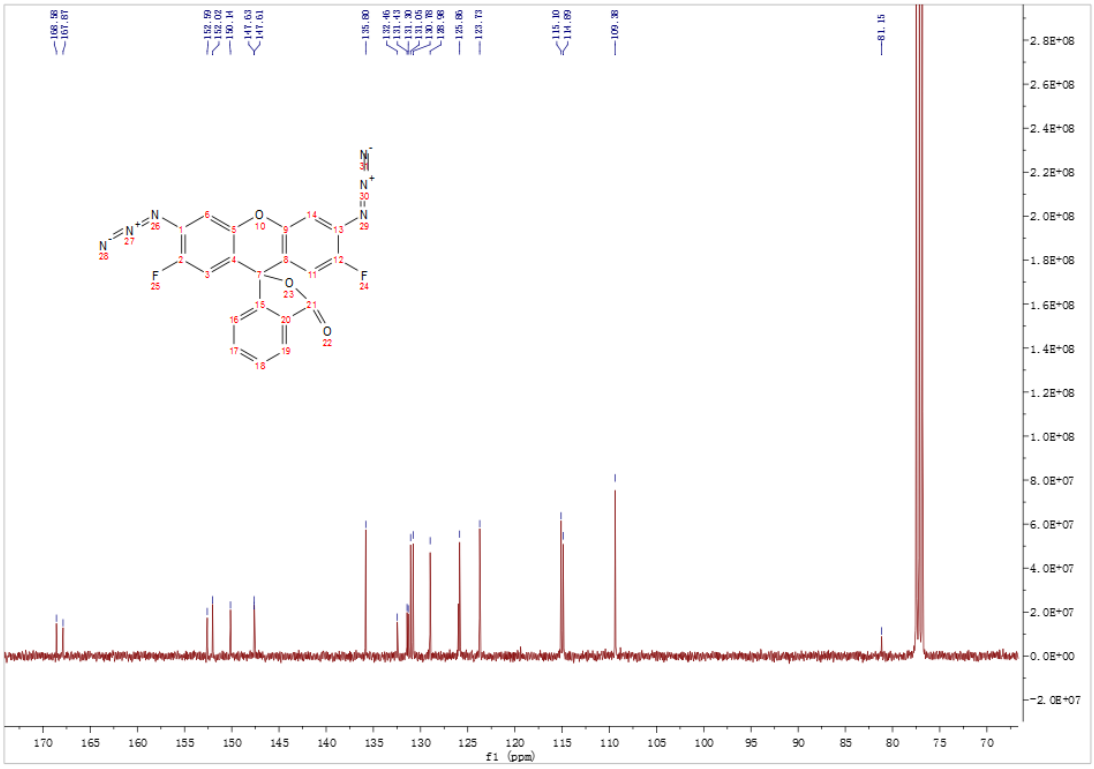


Fig. S12 ^{13}C-NMR of **1**.

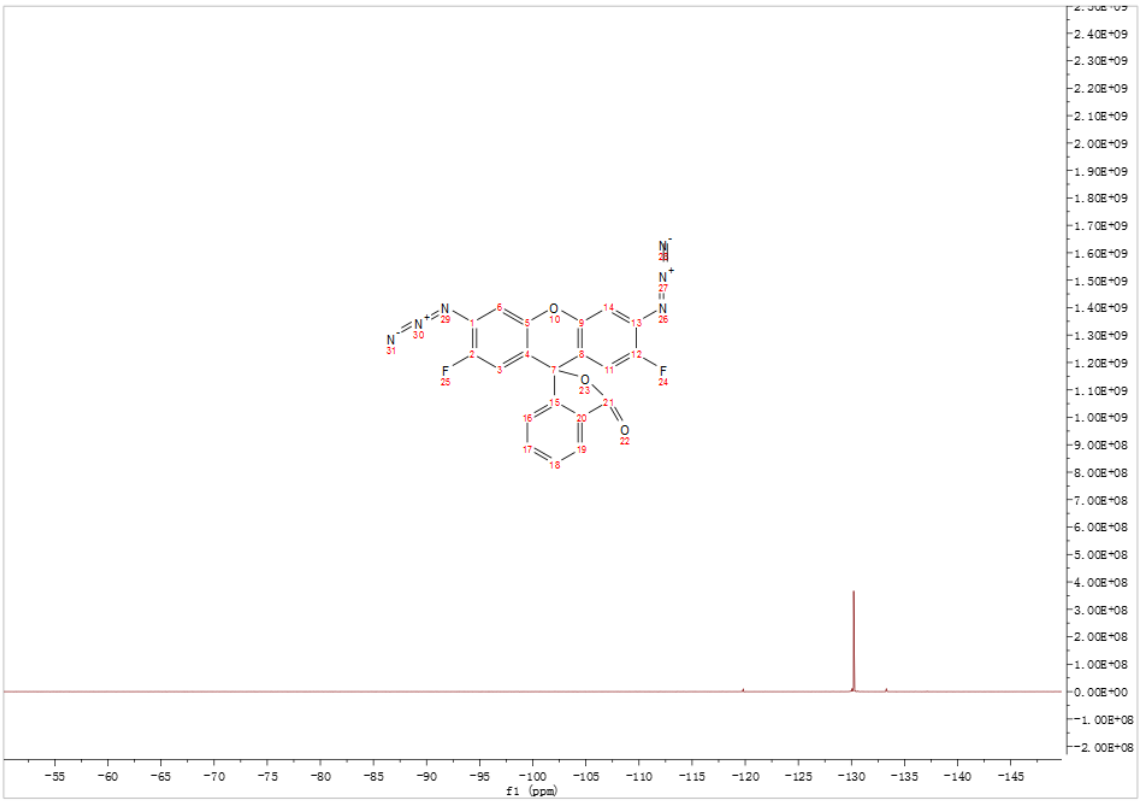


Fig. S13 ^{19}F-NMR of **1**.

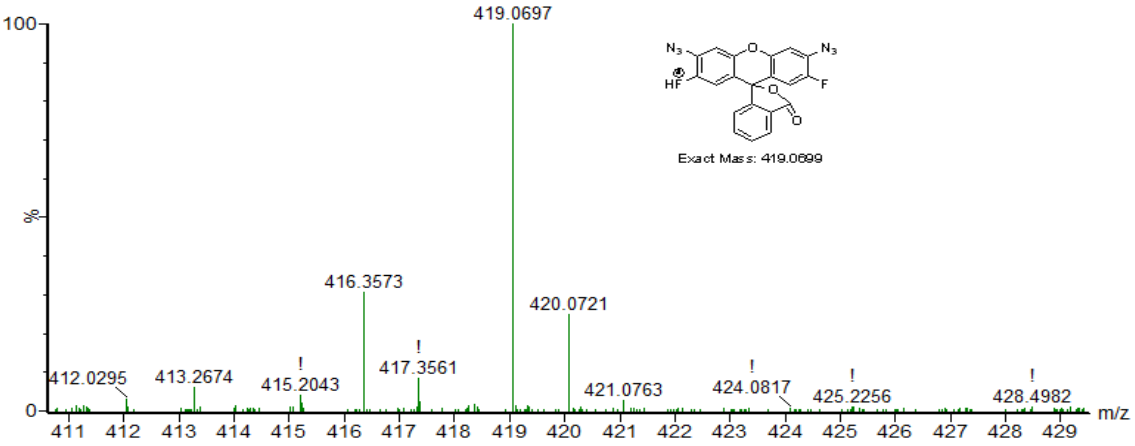


Fig. S14 HRMS of **1**.

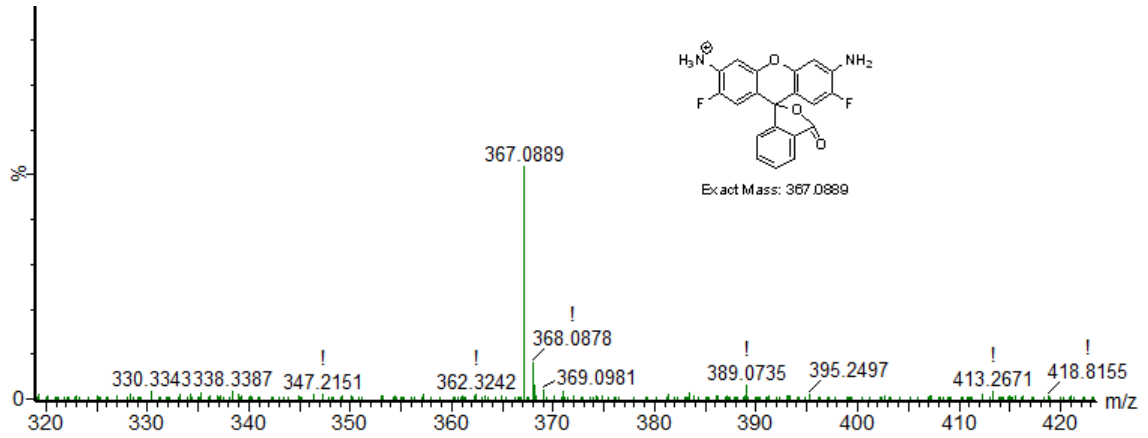


Fig. S15 HRMS of **3**.

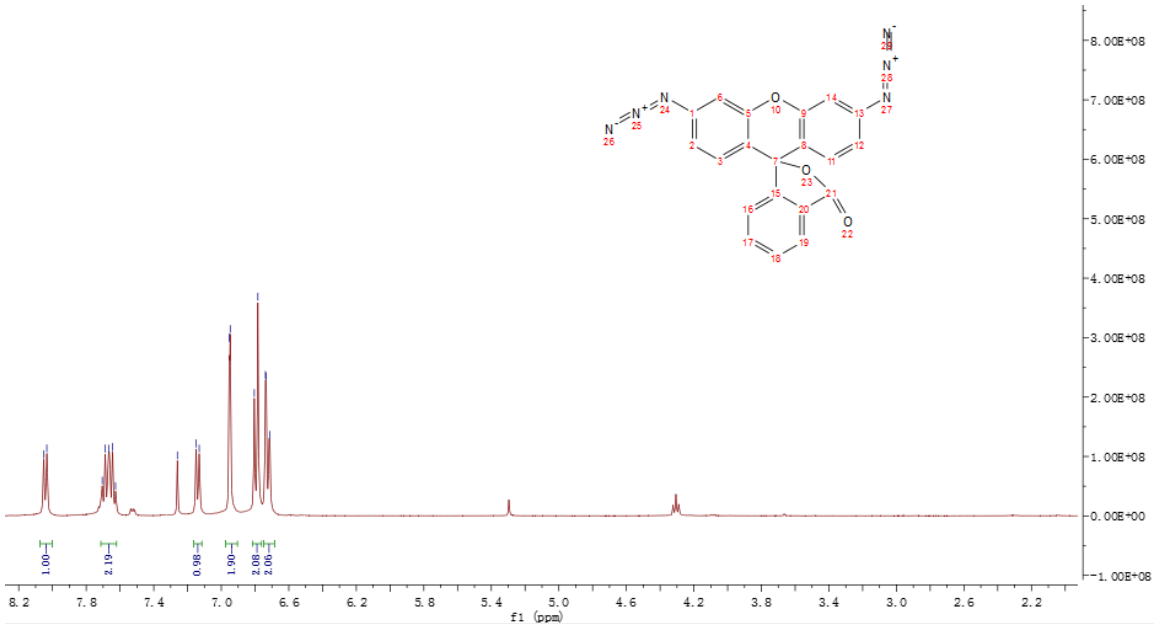


Fig. S16 ^{1}H-NMR of **2**.