Supporting Information

Controllable Cleavage of C-N Bond-Based Fluorescent and Photoacoustic Dual-Modal Probes for the Detection of H_2S in Living Mice

Jie Zhang,¶ †‡ Guohua Wen,¶§ Wanhe Wang,†‡ Ke Cheng,†‡ Qiang Guo,†‡ Shuang Tian,†‡ Chao Liu,§ Hanrong Hu,†‡ Yachao Zhang, § Huatang Zhang,⁄⁄ Lidai Wang,*‡§ and Hongyan Sun*†‡

†*Department of Chemistry and COSADAF (Centre of Super-Diamond and Advanced Films), City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China. Email: hongysun@cityu.edu.hk*

‡*Shenzhen Research Institute of City University of Hong Kong, Shenzhen, 518057, China.*

§*Department of Biomedical Engineering, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China. Email: lidawang@cityu.edu.hk*

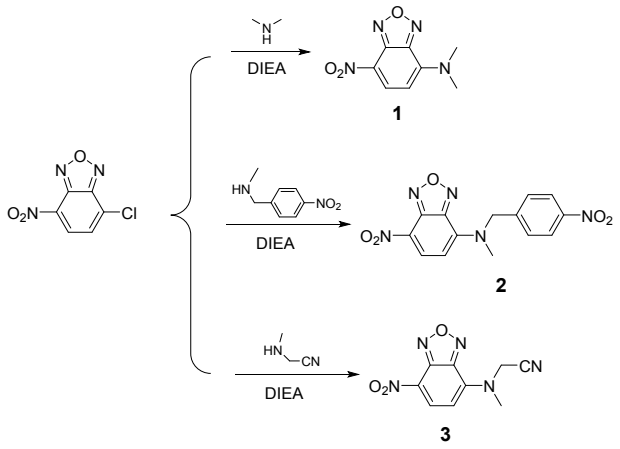
⁄⁄*School of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou, Guangdong 510006, China.*

¶These authors contributed equally to this work.
*Corresponding author: lidawang@cityu.edu.hk; hongysun@cityu.edu.hk

1. Materials and instruments.

All chemicals were obtained from J&K Scientific Ltd unless otherwise stated. Homocysteine (Hcy) was purchased from TCI (Tokyo, Japan). Glutathione (GSH) was purchased from Acros. Dichloromethane (DCM) and methanol were purchased from RCI Labscan Ltd. Matrigel was purchased from Becton Dickinson Ltd. The stock solution of 10 mM probes were prepared by dissolving them in dimethyl sulfoxide (DMSO) respectively. The H_2S stock solution was prepared by purging PB (20 mM, pH 7.4) with N_2 gas for 30 min. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker 300 (300 MHz ^1H; 75 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: *s* (singlet), *d* (doublet). Absorption spectra of the probes were measured on a spectrophotometer (UV-VS 1700 Shimadzu). Fluorescence spectra were measured on a spectrofluorometer (Fluormax-4) at an excitation wavelength of 480 nm or 465 nm. The photoacoustic signal was acquired by a lab-made PACT system. An optical parametric oscillator (OPO) laser (basiScan-M/120/HE, Spectral-Physics) pumped by a Nd:YAG pulsed laser (Quanta-Ray, INDI-40-20, Spectral-Physics, Santa Clara, California) offers 6−9 ns pulsed excitation at 20 Hz in a spectrum from 400 nm to 2000 nm. Photoacoustic signal is received by a 128 element linear ultrasound transducer array centered at 6.25 MHz (L11-4v, Verasonics) and a data acquisition system (Vantage, Verasonics). 532 nm wavelength and 1.5 mJ/cm^2 pulse energy were used for PA excitation.

2. Chemical Synthesis



Scheme S1. The synthetic route of different probes in this study.

Synthesis of probe 1. The synthesis of probe **1** was according to the reported paper.[1] Briefly, dimethylamine hydrochloride (24.8 mg, 0.30 mmol) and 4-Chloro-7-nitrobenzofurazan (50.1 mg, 0.25 mmol) was dissolved in dichloromethane (DCM). Then 87 μL N,N-Diisopropylethylamine (DIEA) was added. The mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure, and the crude product was further purified by silica gel column chromatography (MeOH:DCM = 0.2%) to afford probe **1** (45.0 mg, 86.5%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (d, J = 9.0 Hz, 1H), 6.09 (d, J = 9.0 Hz, 1H), 3.61 (s, 6H).

Synthesis of probe 2. N-methyl-1-(4-nitrophenyl)methanamine (42.5 mg, 0.26 mmol) and 4-Chloro-7-nitrobenzofurazan (49.8 mg, 0.25 mmol) was dissolved in DCM. Then 86 μL DIEA was added. The mixture was stirred at room temperature overnight. The solvent was then removed under reduced pressure, and the mixture was further purified by silica gel column chromatography (DCM) to afford probe **2** (25.0 mg, 29.2%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.47 (d, J = 8.9 Hz, 1H), 8.24 (d, J = 8.7 Hz, 2H), 7.40 (d,

J = 8.8 Hz, 2H), 6.24 (d, *J* = 8.9 Hz, 1H), 5.47 (s, 2H), 3.50 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 147.9, 145.3, 144.7, 144.7, 142.9, 135.2, 127.7, 124.6, 102.3, 58.4, 41.5. MS (ESI): m/z $[M-H]^-$ calcd. for $C_{14}H_{11}N_5O_5^-$: 328.1; found: 328.3.

Synthesis of probe 3. 4-Chloro-7-nitrobenzofurazan (50 mg, 0.25 mmol) and 2-(methylamino)acetonitrile (39.75mg , 0.375 mmol) was dissolved in 6 mL DCM, and stirred at room temperature. DIEA (86 μL, 0.5 mmol) was added for reacting another 3 h. After removing the solvent under reduced pressure, the mixture was further purified by silica gel column chromatography (MeOH:DCM = 1:5) to afford probe **3** (22.7 mg, 39%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.50 (d, *J* = 8.6 Hz, 1H), 6.38 (d, *J* = 8.7 Hz, 1H), 5.11 (s, 2H), 3.51 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 144.8, 143.5, 134.5, 114.1, 104.7, 43.3, 40.9. MS (ESI): m/z $[M-H]^-$ calcd. for $C_9H_6N_5O_3^-$: 232.1; found: 232.2.

3. Absorption and Fluorescence experiments of Probes 1−3

For absorption test, 20 μM probes **1**−**3** were mixed with 4 mM, 2 mM and 200 μM H_2S respectively at room temperature. For the kinetic test, 5 μM probes **1**−**3** were incubated with 1 mM H_2S at room temperature. For the titration experiments, 5 μM probe **3** was incubated with different concentrations of H_2S (0, 1, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 70 and 100 μM) at room temperature for 2 h. For the selectivity test, 5 μM probe **3** was incubated with various reactive sulfur species at a concentration of 200 μM for Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, NO_2^-, ClO^-, H_2O_2, SO_3^{2-}, Cys, Hcy, H_2S and 1 mM for GSH at room temperature for 1 h.

4. Photoacoustic Experiments of Probe 3

To measure the relationship between the PA signal and the H_2S concentration, 20 μM probe **3** was incubated at different concentrations of H_2S from 0 to 100 μM at room temperature (20 ^oC) for 40 min. To test the selectivity, 20 μM probe **3** was incubated at room temperature for 40 min with different types of potential interfering species, i.e. 200 μM NO_2^-, ClO^-, H_2O_2, SO_3^{2-}, Hcy and Cys, and 1 mM GSH.

5. Cell culture and Cytotoxicity Assay

HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin (Thermo Scientific). The cells were placed in a 37 °C incubator with 5% CO_2. Cell viability of the HeLa cells was tested by Cell Counting Kit-8(CCK-8; Dojindo) assays. Specifically the cells were seeded in a 96-well plate (3000 cells per well) and incubated for 12 h for adherence. Probe and doxorubicin (positive control) in DMSO were added to the cells at different concentrations and incubated for 24 h. Subsequently the culture medium was changed to a fresh medium. 10 μL of CCK-8 reagent was added to each well and incubated for 2 h. Optical absorbance was measured at 450 nm and 650 nm on a plate reader (SpectraMax® iD5 Multi-Mode Microplate Readers). Cell viability rate was determined by the following equation $VR = (A–A_0)/(A_S–A_0)\times100\%$, A: absorbance of the experimental group; A_S: absorbance of the control group (DMSO only); A_0: absorbance of the blank group (no cells).

6. Fluorescence and Photoacoustic Cell Imaging

Fluorescence cell imaging: HeLa cells were cultured and seeded in a confocal dish (~2×10^4 cells per dish). The cells were firstly treated with 20 μM probe **3** at 37 °C for 30 min, washed by medium, and then incubated with Na_2S (200 or 500 μM) in the medium for 1 h. Control cells were treated with 20 μM probe **3** but without Na_2S. The cells were imaged with a laser confocal scanning microscope (Leica SPE). The excitation wavelength was 488 nm and the detection spectrum was set at 500−650 nm. PA imaging of cells: HeLa cells were cultured and then seeded on a 22×22 mm^2 cover glass in a confocal dish (~ 1×10^5 cells per dish). The cells were firstly treated with 50 μM probe **3** at 37 ℃ for 30 min. Subsequently they were washed by PBS, and incubated with Na_2S (500 μM) in PBS for 40 min. Control cells were treated with probe **3** only. The cells were scanned by a lab-made optical-resolution photoacoustic microscopy (OR-PAM) system. The lateral resolution of the OR-PAM system was ~3 μm. The excitation wavelength was 532 nm and the pulse energy was 100 nJ/cm^2.

7. *In Vivo* Photoacoustic Imaging

Mice in this study were purchased from the Laboratory Animal Services Centre of the Chinese University of Hong Kong. All the experimental procedures were approved by the Animal Experimental Ethical Committee of the City University of Hong Kong prior to the experiment. 150 μL of solution of Matrigel mixed with PBS (pH 7.4) or Na_2S (100 mM) (volume concentration of Matrigel is 70%) was subcutaneously injected into a leg of the mice. After 30 min, the Matrigel solution formed solid gel as a detection site. 50 μL of probe **3** (100 μM) was then injected into the Matrigel site. PA images were acquired by the aforementioned PACT system at different time points. The excitation wavelength was 532 nm, and the pulse energy was 1.5 mJ/cm^2.

8. Supplementary figures

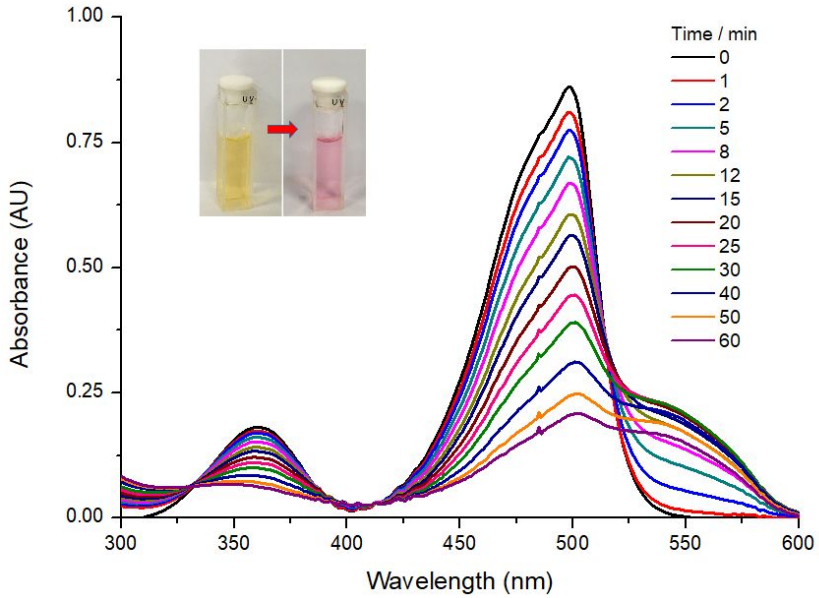


Fig. S1. Time-dependent UV-vis spectra of 20 μM probe **1** incubated with 4 mM H_2S in PB buffer (20 mM, pH 7.4, containing 10% DMSO).

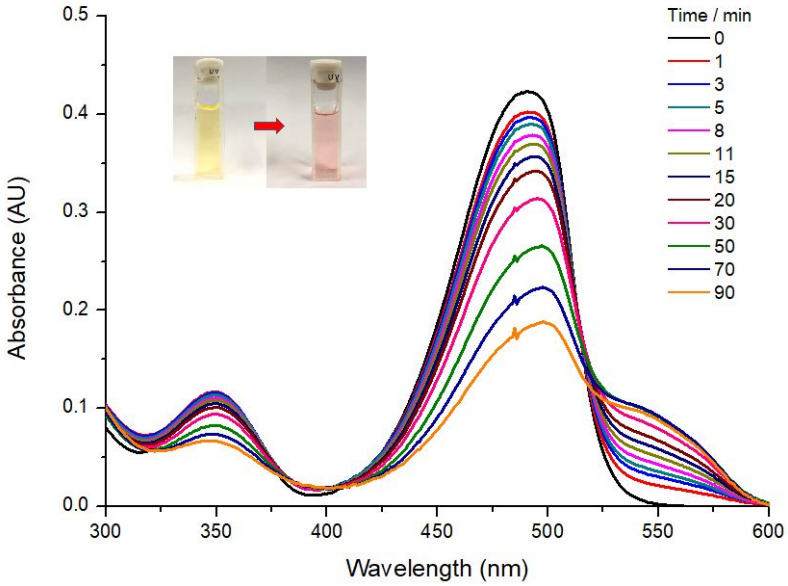


Fig. S2. Time-dependent UV-vis spectra of 20 μM probe **2** incubated with 2 mM H_2S in PB buffer (20 mM, pH 7.4, containing 10% DMSO).

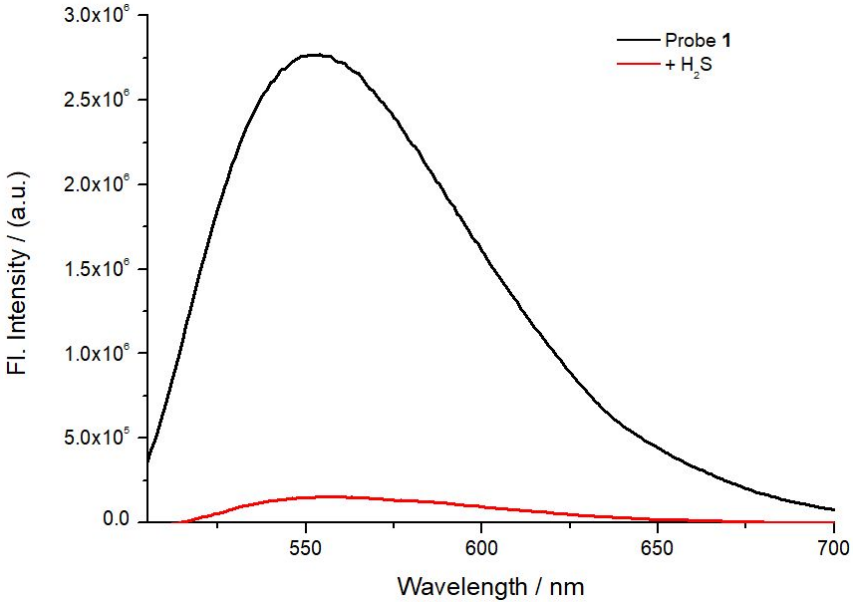


Fig. S3. Fluorescent spectra of probe **1** (5 μM) before and after incubation with H_2S.

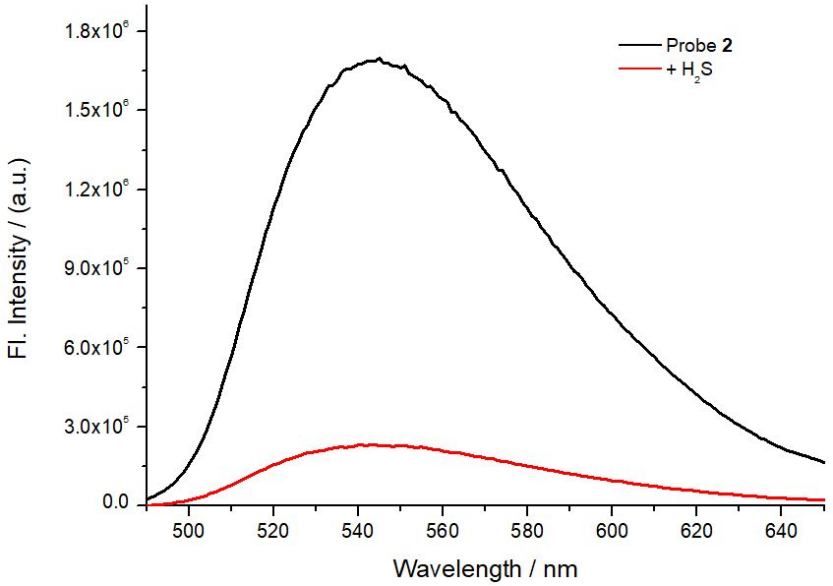


Fig. S4. Fluorescent spectra of probe **2** (5 μM) before and after incubation with H_2S.

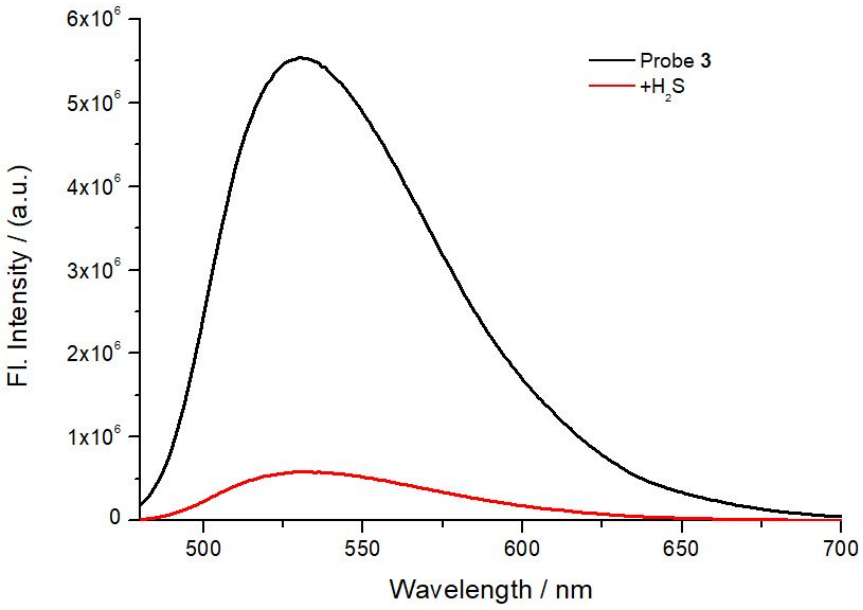


Fig. S5. Fluorescent spectra of probe **3** (5 μM) before and after incubation with H_2S.

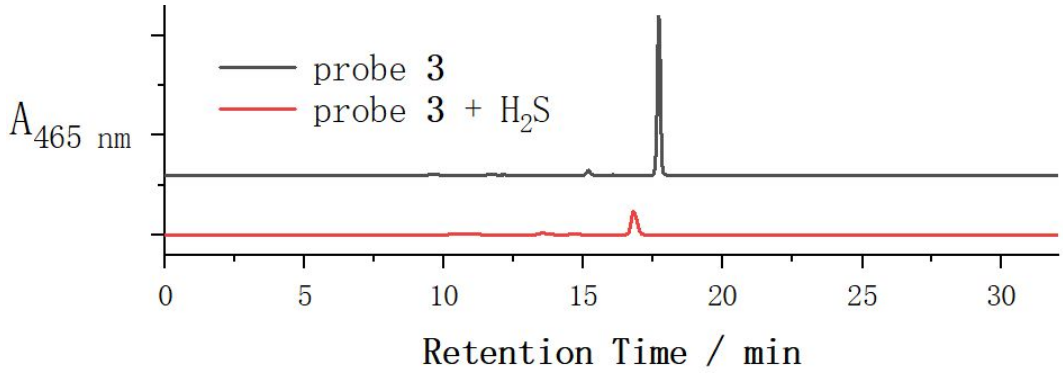


Fig. S6. HPLC analysis of probe **3** before and after reaction with H_2S**.**

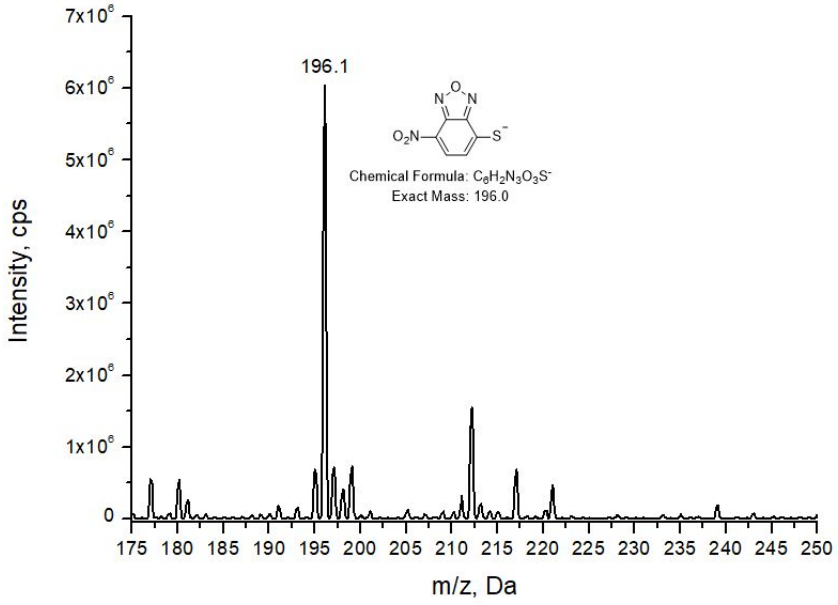


Fig. S7. The mass spectrum of probe **3** after reaction with H_2S**.**

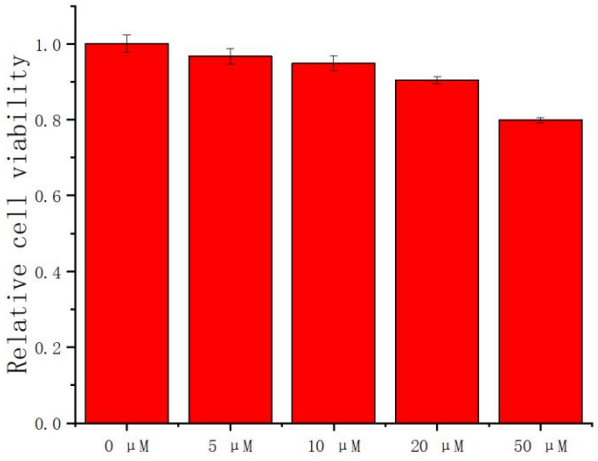


Fig. S8. The cytotoxicity of the probe **3** evaluated by CCK-8 assay.

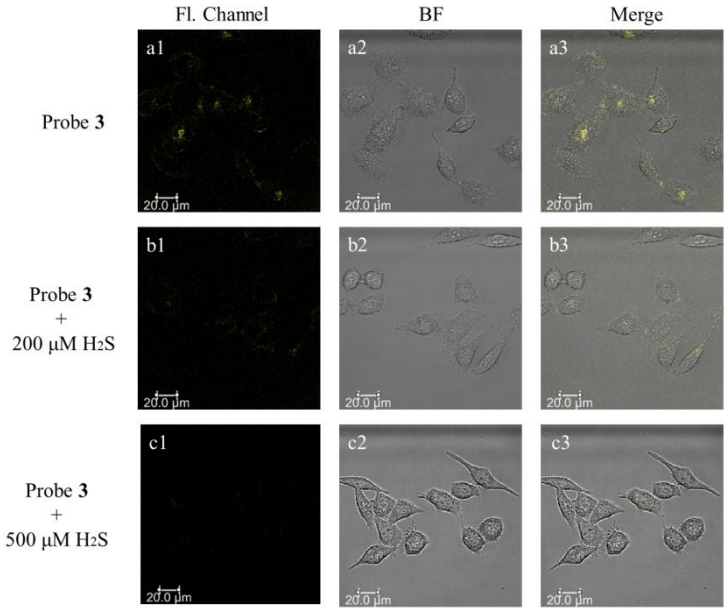


Fig. S9. Confocal fluorescence image for detection of H_2S in Hela cells. (a) The cells were incubated with 20 μM probe **3** only for 30 min. (b,c) The cells were pretreated with 20 μM probe **3** for 30 min and incubated with H_2S (b, 200 μM; c, 500 μM) for another 1 h. The overlay images were obtained by merging the pictures from fluorescence channel and bright field. λ_{ex}: 488 nm, collected λ_{em}: 500–650 nm. Scale bars: 20 μm.

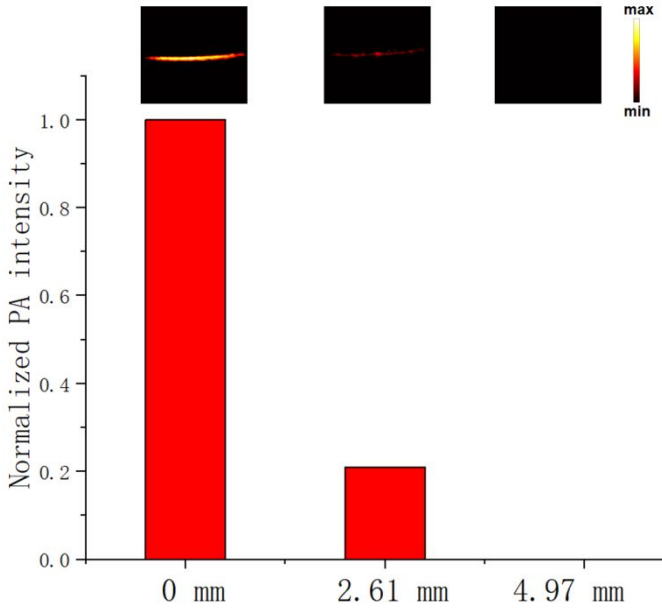


Fig. S10. Photoacoustic detection depth test of probe **3**. Probe **3** (200 μM) was reacted with H_2S (200 μM) at room temperature for 2 h. The reaction mixture was placed under chicken breast with different thickness (2.61 mm & 4.97 mm). Subsequently the photoacoustic image was taken by PACT system. λ_{ex}: 532 nm, pulse energy: 7.0 mJ/cm^{2}.

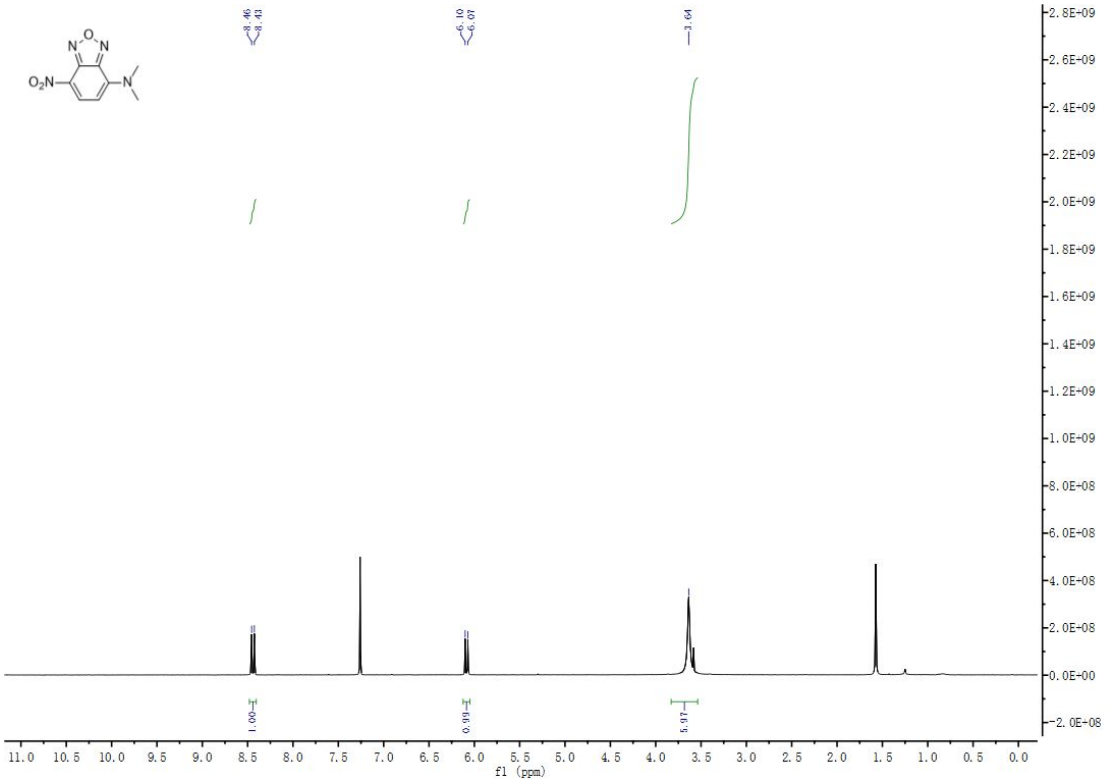


Fig. S11. The ^{1}H NMR spectrum of probe **1**.

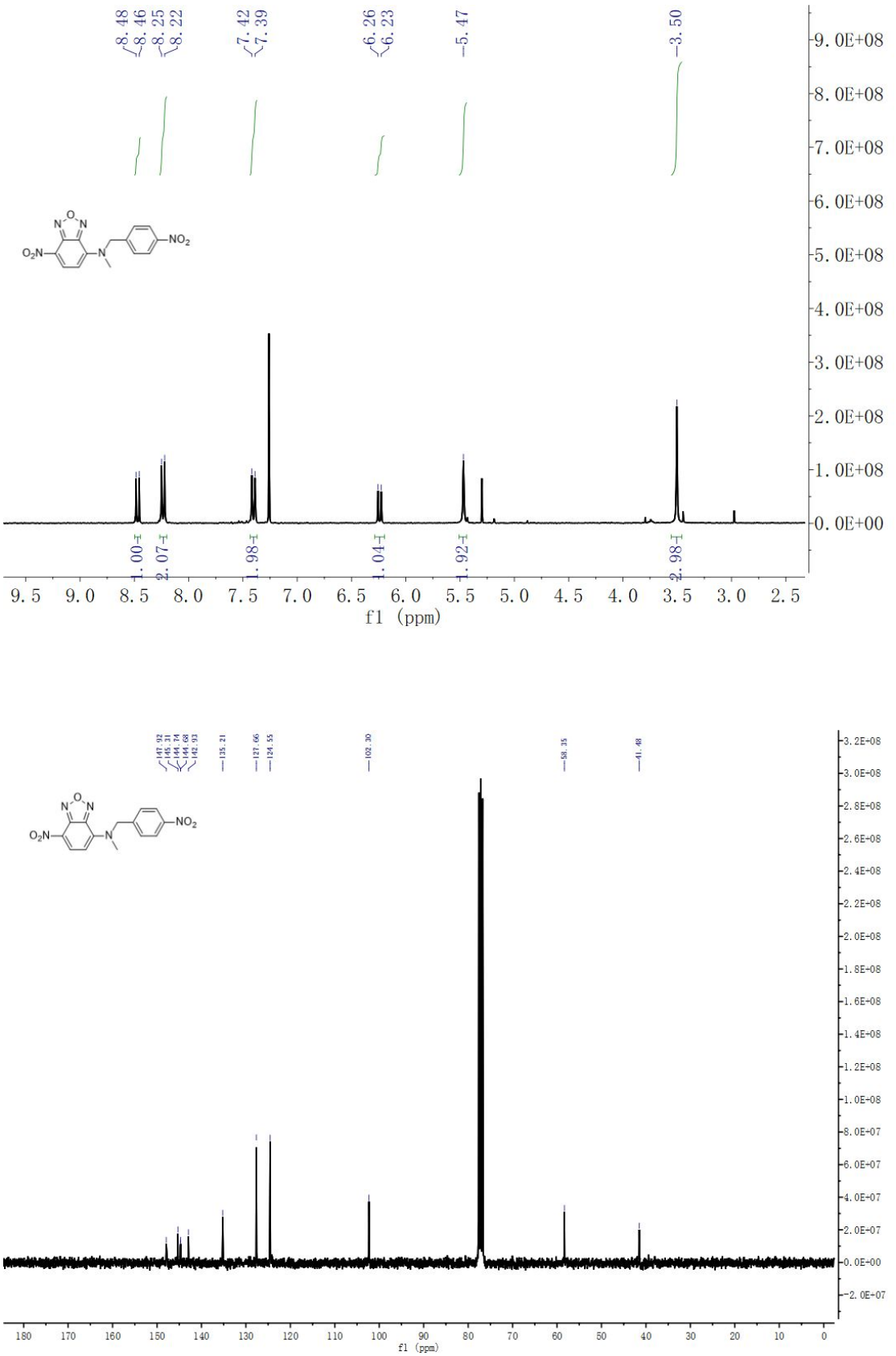


Fig. S12. The ^{1}H and ^{13}C NMR spectra of probe **2**.

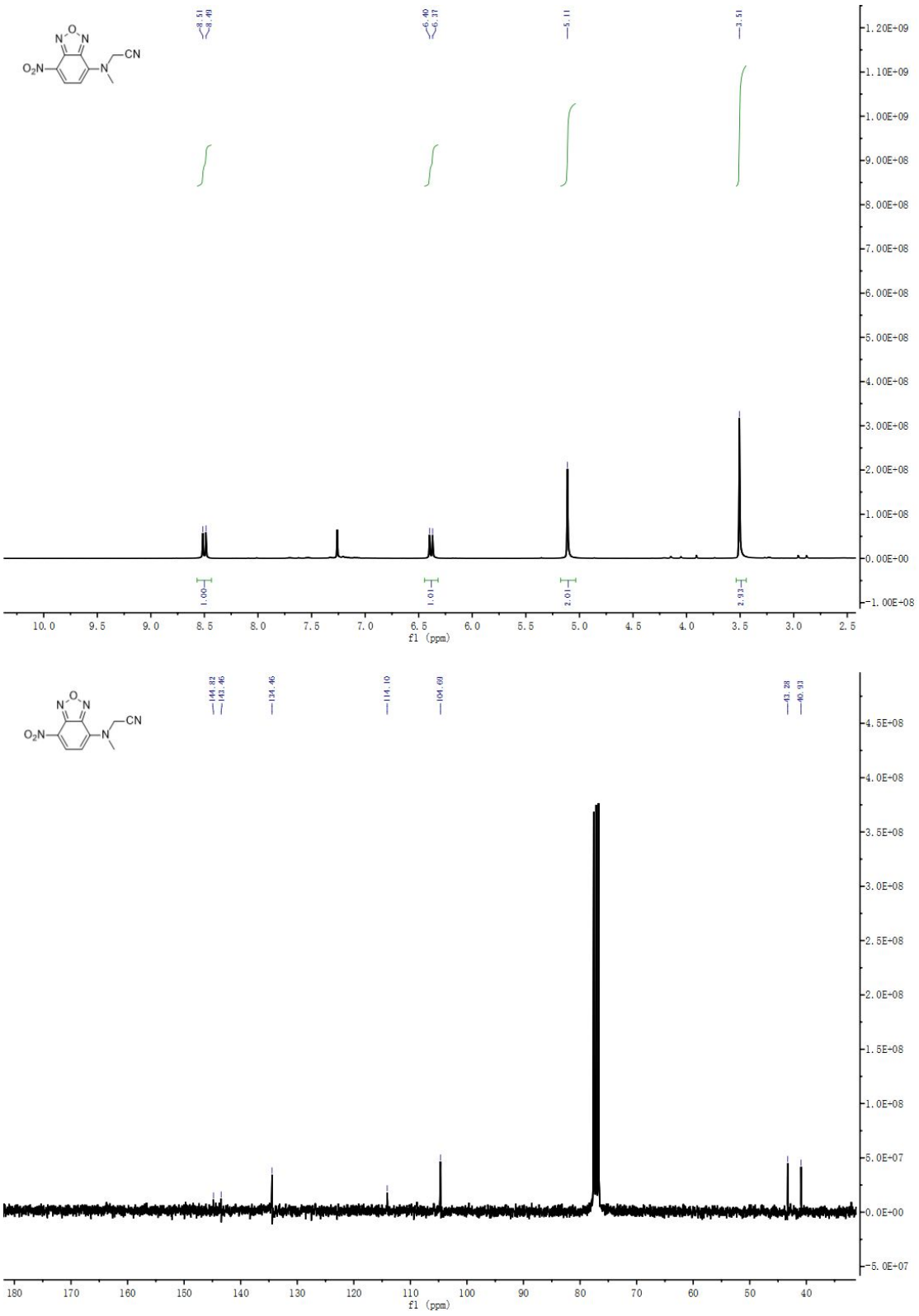


Fig. S13. The ^{1}H and ^{13}C NMR spectra of probe **3**.

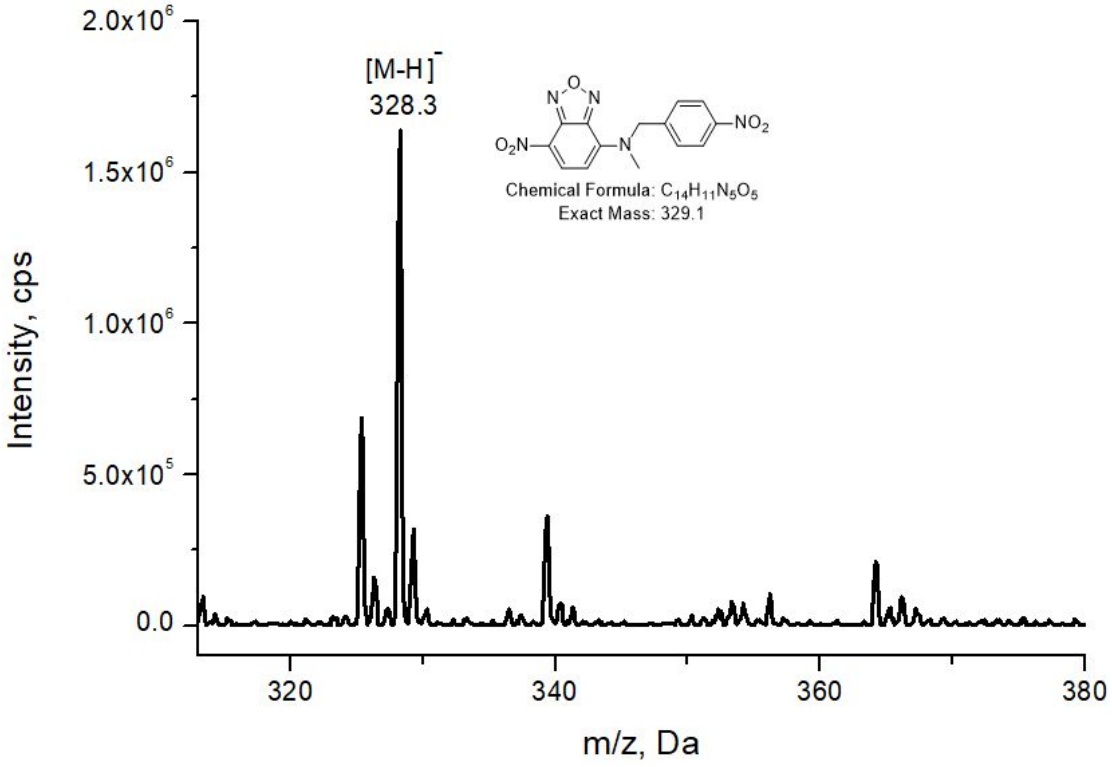


Fig. S14. The mass spectrum of probe **2**.

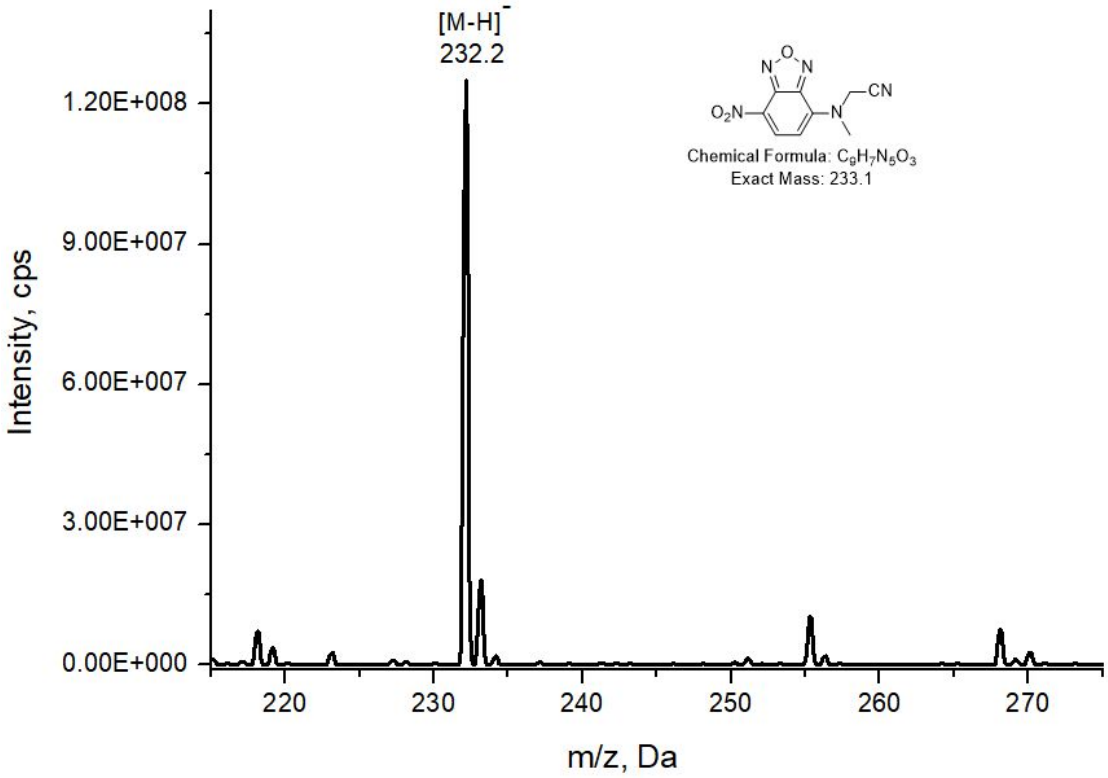


Fig. S15. The mass spectrum of probe **3**.

9. Reference

1. Ye, Z.; Yang, W.; Wang, C.; Zheng, Y.; Chi, W.; Liu, X.; Huang, Z.; Li, X.; Xiao, Y. Quaternary Piperazine-Substituted Rhodamines with Enhanced Brightness for Super-Resolution Imaging. *J. Am. Chem. Soc.* **2019,** *141*, 14491-14495.