Electronic Supplementary Information

Highly selective turn-on near-infrared fluorescent probe for hydrogen sulfide detection and imaging in living cells

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Contents

More examples of Ref. 13

(e) S. K. Das, C. S. Lim, S. Y. Yang, J. H. Han and B. R. Cho, *Chem. Commun.*, 2012, **48**, 8395; (f) Y. Qian, J. Karpus, O. Kabil, S. Y. Zhang, H. L. Zhu, R. Banerjee, J. Zhao and C. He, *Nat. Commun.*, 2011, **11**, 495; (g) C. Liu, J. Pan, S. Li, Y. Zhao, L .Y. Wu, C. E. Berkman, A. R. Whorton and M. Xian, *Angew. Chem., Int. Ed.*, 2011, **50**, 10327; (h) K. Sasakura, K. Hanaoka, N. Shibuya, Y. Mikami, Y. Kimura, T. Komatsu, T. Ueno, T. Terai, H. Kimura and T. Nagano, *J. Am. Chem. Soc.*, 2011, **133**, 18003; (i) C. Liu, B. Peng, S. Li, C. M. Park, A. R. Whorton and M. Xian, *Org. Lett.*, 2012, **14**, 2184; (j) F. Hou, L. Huang, P. Xi, J. Cheng, X. Zhao, G. Xie, Y. Shi, F. Cheng, X. Yao, D. Bai and Z. Zeng, *Inorg. Chem.*, 2012, **51**, 2454; (k) M. Fu, W. Zhang, L. Wu, G. Yang, H. Li and R. Wang, *PNAS*, 2012, **109**, 2943; (l) W. Xuan, C. Sheng, Y. Cao, W. He and W. Wang, *Angew. Chem., Int. Ed.*, 2012, **51**, 2282.

1. General Experimental Section

Instruments: Fluorescence spectra were obtained by FluoroMax-4 Spectrofluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were measured on NANO Drop 2000c UV-visible spectrophotometer (Thermo Fisher Scientific). All pH measurements were per-formed with a pH-3c digital pH meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass–calomel electrode. Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). Elemental analyses were obtained with a Vario MACRO cube element analyzer. ^{1}H NMR, ^{13}C NMR spectra were taken on a Bruker spectrometer. The fluorescence images of cells were taken using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (×20, ×40).

Materials: 2-[4-chloro-7-(1-ethyl-3,3-dimethyl(indolin-2-ylidene)]-3,5-(propane-1,3-diyl)- 1,3,5-heptatrien-1-yl)-1-ethyl-3,3-dimethyl-3H-indolium (Cy.7-Cl) was synthesized in our laboratory.[1]

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and HEPES were purchased from Sigma-Aldrich. Mouse leukaemic monocyte macrophage cell line (RAW264.7) was obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cy-NO_2 (1.0 mM, 0.5 mL) was prepared in CH_3OH and stored at 4 ^oC in darkness. All other reagents and chemicals were from commercial sources and of analytical reagent grade, and used without further purification. Solvents used for spectroscopic studies were purified and dried by standard procedures before use. Ultrapure water (Millipore, Bedford, MA, USA) was used throughout.

[1] N. Narayanan, G. Patonay, *J. Org. Chem.*, 1995, **60**, 2391.

Absorption Analysis: Absorption spectra were obtained with 1.0-cm glass cells. The probe (CH_3OH, 0.5 mL, 1.0 mM) was added to a 10.0-mL color comparison tube. After dilution to 10.0 μM with 40 mM HEPES buffer, H_2S was added. The mixture was equilibrated for 60 min before measurement.

Fluorescence Analysis: Fluorescence spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. The probe (CH_3OH, 0.5 mL, 1.0 mM) was added to a 10.0-mL color comparison tube. After dilution to 10.0 μM with 40 mM HEPES buffer, H_2S was added. The mixture was equilibrated for 60 min before measurement.

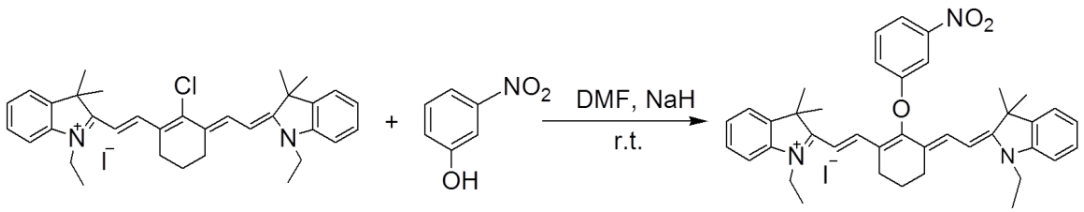
Confocal Imaging: Fluorescent images were acquired on an Olympus Fluo View FV1000 confocal laser-scanning microscope (Japan) with an objective lens (×20, ×40). The excitation wavelength was 635 nm. Cell imaging was carried out after being washed with physiological saline for three times.

Cell Culture: Mouse leukaemic monocyte macrophage cell line (RAW264.7) was obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI 1640 Medium supplemented with 10% fetal bovine serum (FBS) at 37 ^oC in a humidified atmosphere containing 5% CO_2.

2. Synthesis and Characterization of Compounds

The ^1H and ^{13}C chemical shifts are given in parts per million relative to those of Me_4Si, internal CD_3Cl-D3 in the solvent.

Synthesis of the Probe Cy-NO_2:



3-nitrophenol (0.696 g, 5.00 mmol) and NaH (70% in mineral oil) (0.173 g, 5.00 mmol) were dissolved in anhydrous N, N-dimethylformamide (DMF) (30 mL). The mixture was stirred at room temperature for 15 min under N_2 atmosphere. Then, the Cy-Cl (0.500 g, 0.782 mmol) was introduced into the above mixture.[2] The reaction mixture was further stirred for 24 h at room temperature. The solvent was removed under reduced pressure, and then the crude product was purified by silica gel chromatography (8:1, acetic ether/ methanol, v/v) to afford the desired product as a dark green solid. Yield: 0.425 g, 73.2%. ^{1}H NMR (500 MHz, $CDCl_3$-D_1) δ (ppm): 1.25-2.09 (m, 20H), 2.63-2.65 (q, 4H), 4.17-4.22 (q, 4H), 6.08-6.17 (d, 2H), 7.04-7.98 (m, 12H), 8.01-8.09 (d, 2H). ^{13}C NMR (500 MHz, $CDCl_3$-D_1) δ (ppm): 171.4, 162.2, 159.7, 141.7, 141.1, 131.7, 129.9, 128.8, 125.3, 122.3, 122.2, 117.4, 110.7, 110.5, 110.2, 100.7, 77.3, 77.0, 76.8, 49.0, 40.0, 28.4, 27.8, 24.8, 21.0, 12.4, 12.3. LC-MS (API-ES): *m/z* $C_{40}H_{44}N_3O_3^+$ Calcd 614.3, found [M^+] 614.5. Elemental analysis calcd (%) for $C_{40}H_{44}IN_3O_3$: C, 64.77; H, 5.98; N, 5.67; O, 6.47; found: C, 64.76; H, 5.99; N, 5.68; O, 6.48.

[2] E. Sasaki, H. Kojima, H. Nishimatsu, Y. Urano, K. Kikuchi, Y. Hirata and T. Nagano, *J. Am. Chem. Soc.*, 2005, **127**, 3684.

Characterization of the reduced product Cy-NH_2: Cy-NO_2 (0.1 g, 135 μmol) was dissolved in 1.0 mL methanol. Subsequently, 20 equiv. of Na_2S was added. The mixture was equilibrated for 30 min. All above operations are carried out under N_2 atmosphere. The solvent was removed under reduced pressure until dry. The crude product was purified on preparative thin-layer chromatography eluted with ethyl acetate and methanol. Yield: > 93%. ^{1}H NMR (500 MHz, $CDCl_3$-D_1) δ (ppm): 1.21-2.11 (m, 20H), 2.57-2.63 (q, 4H), 4.03 (d, 2H), 4.71-5.64 (q, 4H), 6.86-7.12 (m, 4H), 7.24-7.87 (m, 8H), 8.27-8.36 (d, 4H). LC-MS (API-ES): *m/z* $C_{40}H_{46}N_3O^+$ Calcd 584.4, found [M^+] 584.3.

3. Effect of pH Values

Standard fluorescence pH titrations were performed in the solution at a probe concentration of 10 μM.

As shown in Fig. S1, the probe itself and the reduced product almost have no effect on the fluorescence by the pH of mediums within the range from 4.2 to 8.2. These results indicate that the probe can work under physiological conditions.

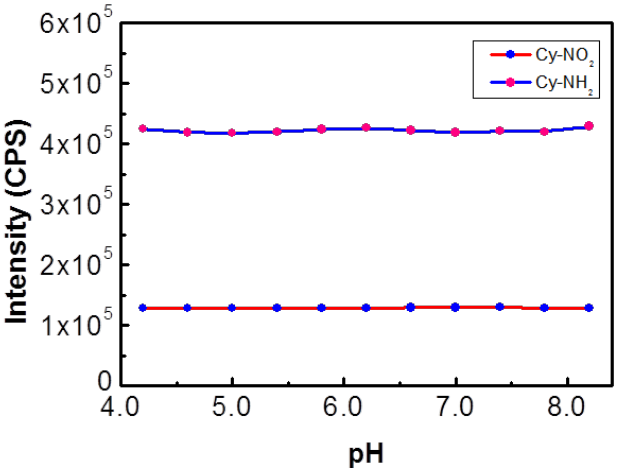


Fig. S1 The effect of pH value on the fluorescence intensity of probe (10.0 μM) in 40 mM HEPES. pH values: 4.2, 4.6, 5.0, 5.4, 5.8, 6.2, 6.6, 7.0, 7.4, 7.8, 8.2.

4. Effect of Temperature

In the experiments, interestingly, we found that the reaction rate between Cy-NO_2 and H_2S was severely affected by the reaction temperature. In order to optimize the reaction temperature, we performed an important experiment to test the effect of temperature on the reaction rate. The probe (10 μM) and 350 μM Na_2S were equilibrated in different temperature, the fluorescence intensity was acquired in 40 mM HEPES (pH 7.4) with emission at 789 nm.

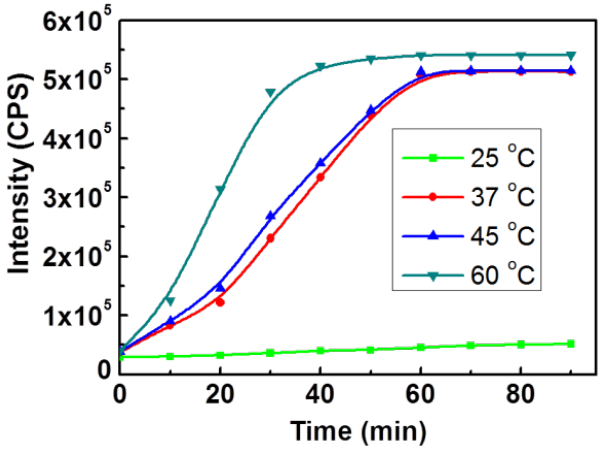


Fig. S2 The effect of temperature on the fluorescence intensity and response time of probe (10.0 μM) in 40 mM HEPES.

5. MTT Assay

To evaluate the cytotoxicity of Cy-NO_2, we performed an MTT assay on RAW 264.7 cells with probe concentrations from 10^{-7} to 10^{-3} M. The result showed IC_{50} values of 403 μM for Cy-NO_2, that is, Cy-NO_2 is of low toxicity towards cell cultures under experimental conditions.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: Cytotoxicity in vitro was measured by using the methyl thiazolyl tetrazolium (MTT) assay in RAW264.7 cells. Cells were seeded into 96-well cell culture plate at 4000 /well, cultured at 37 ^{o}C and 5% CO_2 for 48 h, and then different concentrations of chemosensor Cy-NO_2 (0, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M) were added to the wells. For MTT experiments, solutions of Cy-NO_2 were prepared in CH_3OH (10 mM and 1 mM). The final volume of cell culture medium was 600 μL. Accordingly, the amounts of solvent added in the absence of probe were correspondingly 0, 60 μL, 6 μL (the above used 10 mmM solution), 6 μL, 0.6 μL, 0.06 μL (the last three used 1 mM solution). To verify whether 10% methanol might cause significant cell loss or not, 60 μL methanol was added into the medium. The cells were then incubated for 48 h at 37 ^{o}C under 5% CO_2. Subsequently, 20 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 ^{o}C under 5% CO_2. Cells were lysed in triple liquid (10% SDS, 0.012 M HCl, 5% isopropanol), and the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (Tecan, Austria).

Calculation of IC_{50} values was done according to Huber and Koella.[3] The following formula was used to calculate the inhibition of cell growth: Cell viability (%) = (mean of Abs. value of treatment group / mean Abs. value of control) • 100%.

[3] W. Huber, J. C. Koella, *Acta Trop.*, 1993, **55**, 257.

5. Interferences of Reductive Species under Hypoxic Environment

Nitro group was commonly employed in designing fluorescent probes for hypoxia (low oxygen), under

which condition nitro could be reduced. To verify whether the nitro group could be reduced by reductive species under hypoxic environment or not, we have performed an important experiment to test the interferences of a range of substrates such as nitro reductase, ascorbic acid, glutathione, and cysteine.[4] Cy-NO_2 (10 μM) was treated with biologically relevant analytes in HEPES buffer (40 mM, pH 7.4). The probe's time courses with various reductive species for 90 min were measured. The relative reductive species gave limited increase in the fluorescence intensity. However, the intensity of fluorescence was far weaker than that caused by H2S. As shown in Fig. S3, Cy-NO_2 showed selective response for H2S over other reductive species under hypoxic environment.

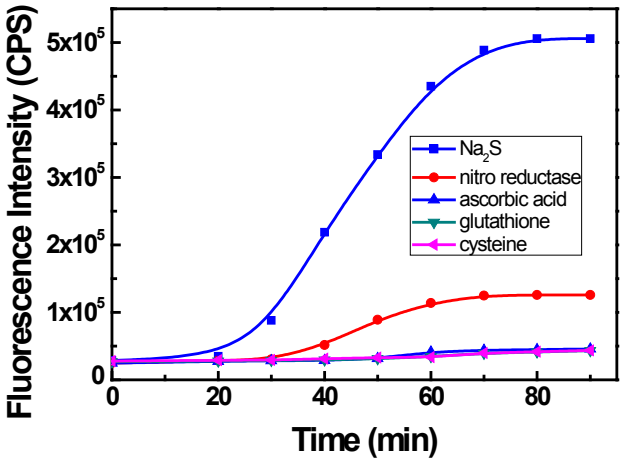


Fig. S3 Fluorescence responses and time courses of 10 μM Cy-NO_2 to various reductive species for 90 min under hypoxic condition. Data shown: Na_2S, nitro reductase at 300 μM; ascorbic acid, glutathione, and cysteine at 1 mM.

[4] (a) J. A. Raleigh, D. P. Calkins-Adams, L. H. Rinker, C. A. Ballenger, M. C. Weissler, W. C. Fowler, D. B. Novotny and M. A. Varia, *Cancer Res.* 1998, **58**, 3765; (b) S. Lehmann, D. P. Stiehl, M. Honer, M. Dominietto, R. Keist, I. Kotevic, K. Wollenick, S. Ametamey, R. H. Wenger and M. Rudin, *Proc. Natl. Acad. Sci.*, 2009, **106**, 14004.

6. Detection Limit

The physiological relevant H_2S concentration is estimated ranging from nano- to millimolar levels.[5] We calculated the limit of detection of Cy-NO_2 for H_2S. The limit of detection is 2 μM, which falls well within this range.

Limit of detection in analysis.[6] The limit of detection, expressed as the concentration c_L, or the quantity

q_L, is derived from the smallest measure x_L, that can be detected with reasonable certainty for a given analytical procedure. The value of x_L is given by the equation:

$$x_L = \bar{x}_{bi} + ks_{bi} \qquad (1)$$

Where $\bar{x}_{bi}$ is the mean of the blank measures, s_{bi} is the standard deviation of the blank measures, and k is a numerical factor chosen according to the confidence level desired.

$$c_L = \frac{(x_L - \bar{x}_B)}{m} \qquad (2)$$

Where, m is the slope of the linear regression equation. Combine two of the equations, Long and Winefordner defined cL:

$$c_L = \frac{(k \square s_B)}{m} \qquad (3)$$

Generally, κ =3, P < 0.01, we obtained cL = 2 μM.

[5] (a) J. Furne, A. Saeed, and M. D. Levitt, *Am. J. Physiol.*, 2008, **295**, R1479; (b) Y. Han, J. Qin, X. Chang, Z. Yang, and J. Du, *Cell. Mol. Neurobiol.*, 2006, **26**, 101.

[6] IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). XML on-line corrected version: http://goldbook.iupac.org (2006) created by M. Nic, J. Jirat, B. Kosata; updates compiled by A. Jenkins. ISBN 0-9678550-9-8.

8. Bright-Field Images of Fig.4

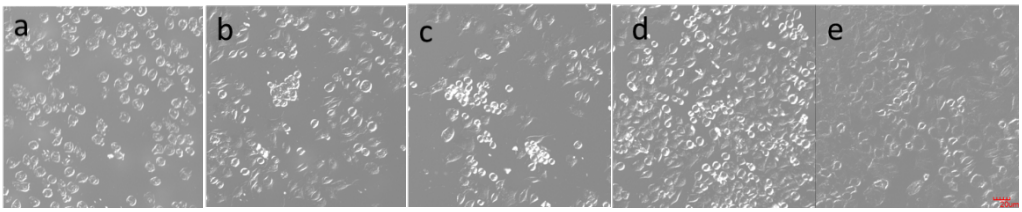


Fig. S4 Bright-field images of Fig.4. (a) Brightfield image of Fig. 4a; (b) Brightfield image of Fig. 4b; (c) Brightfield image of Fig. 4c; (d) Brightfield image of Fig. 4d; (e) Brightfield image of Fig. 4e.

9. Confocal Imaging for Subcellular Locations of Cy-NO_2 in RAW264.7 cells

Subcellular locations of Cy-NO_2 in the RAW264.7 cells were carried out by co-staining with Janus Green B, which is used to stain mitochondria supravitally. At the presence of oxygen, the indicator shows blue color (Fig. S5b). Fig. S5a red channel, and co-staining with Janus Green B (Fig. S5b) revealed the location of the probe in the cytoplasm of these living RAW264.7 cells (Fig. S5c). We also employed the Pearson correlation coefficient (r) which was used to quantify the degree of colocalization between fluorophores to further reveal the subcellular locations of Cy-NO_2. By using Olympus software, we obtained the value of Cy-NO_2 with JGB r = 0.85, revealing that Cy-NO_2 primarily locates in the cytoplasm. The Pearson's correlation coefficient value (0.85) was obtained after excluding the nucleus area indicating that our probe Cy-NO_2 existed primarily in the cytoplasm.[7] If the whole cell was selected as the target region, the value was about 0.3.

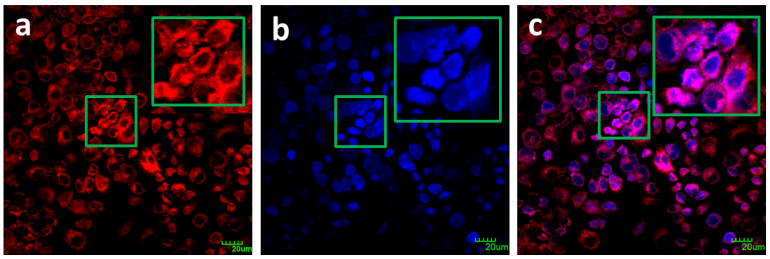


Fig. S5 Confocal fluorescence images of RAW264.7 cells incubated with Cy-NO_2 (10.0 μM) and Janus Green B (1.0 μM) for 15 min. Cells loaded with the probe for 30 min, then treated with Na_2S (350 μM) and Janus Green B (1.0 μM) for 15 min. (a) Red channel with Cy-NO_2. (b) Blue channel with Janus Green B. (c) Overlay of images showing fluorescence from Janus Green B (b) and Cy-NO_2 (a). Scale bar is 20 μm.

[7] (a) J. Adler and I. Parmryd, *Cytometry Part A*, 2010, **77A**, 733; (b) A. P. French, S. Mills, R. Swarup, M. J. Bennett and T. P. Pridmore, *Nat Protoc.*, 2008, **3**, 619; (c) E. M. M. Manders, F. J. Verbeek and J. A. Aten, *J Microscopy*, 1993, 169, 375.

10. Determination of H_2S in Water Solution and Fetal Bovine Serum

We explored the ability of the probe to detect H_2S in water solution and fetal bovine serum by UV-Vis method. To evaluate the capability of Cy-NO_2 in the determination of H_2S concentration, the probe was treated with H_2S under various concentrations. The final concentration of Cy-NO2 was 10 μM, while the concentrations of H_2S varied from 0 to 350 μM. As shown in Fig. S5, the absorption was linearly related to

the concentration of H_2S in the HEPES buffer solution. The regression equation was A = − 0.00127 [H_2S] + 0.7538, with r = 0.9806. Next, we used fetal bovine serum to investigate whether the probe functions well in complex samples. We prepared fetal bovine serum which contained H_2S at different concentrations (0-350 μM). Using the same method, we got another standard curve between absorption and H_2S concentration. The regression equation was A = − 0.00132 [H_2S] + 0.7643, with r = 0.9803. These results revealed that Cy-NO_2 could test H_2S in complex samples both qualitatively and quantitatively. Absorption was measured at 755nm.

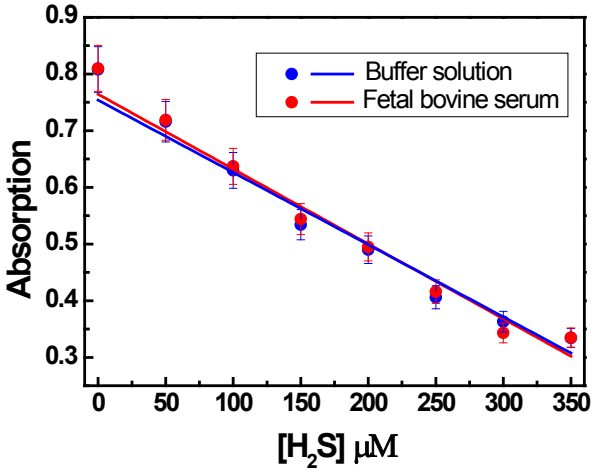


Fig. S6 The relationship between absorption and H_2S concentration in water solution (blue) and commercial fetal bovine serum (red). The concentration of fetal bovine serum in the solution is 15% (v/v). 10 μM Cy-NO_2, 0-350 μM Na_2S. Absorption was measured at 755nm.

11. Comparison of integrated fluorescence spectra

As shown in Fig.1b, upon addition different concentrations of H_2S to the buffer solution which contained 10 μM Cy-NO_2, the fluorescence intensity increased by ~ 12.7 fold and quantum yield increased from 0.05 to 0.11. At the same time the emission spectrum showed a little blue shift in λ_{max} from 809 nm to 789 nm. We attributed this phenomenon to the push-pull electronic effect of the functional moiety.[8] Upon reduction by H_2S, the probe contained an electron-donating group (Ph-NH_2 moiety) instead of electron-withdrawing group (Ph-NO_2 moiety). Therefore, the fluorescence spectrum underwent a slight blue shift. Fluorescence spectra showed in Fig.1b were assembled by steady-state fluorescence spectra

of the two fluorescent molecules Cy-NO_2 and Cy-NH_2. Next we would discuss the steady-state fluorescence intensity for our probe.[9]

In many cases, the absorbance of a sample follows the Beer – Lambert Law.

$$A_\lambda = \frac{\log(I_\lambda^0)}{I_\lambda^T} \varepsilon_\lambda \, bc \qquad (1)$$

$$I_\lambda^T = I_\lambda^0 \exp(-2.303 \varepsilon_\lambda \, bc) \qquad (2)$$

where I_λ^0 and I_λ^T are the light intensities of the beams entering and leaving the absorbing medium, respectively. ε_λ is the molar absorption coefficient, c is the concentration of absorbing species and b is the absorption path length.

The fluorescence quantum yield Φ_F is the fraction of excited molecules that return to the ground state S_0 with emission of fluorescence photons:

$$\Phi_F = \frac{\kappa_r^s}{\kappa_r^s + \kappa_{nr}^s} \qquad (3)$$

Where κ_r^s is the rate constant for radiative deactivation $S_1 \rightarrow S_0$ with emission of fluorescence. κ_{nr}^s is the overall non-radiative rate constant.

The steady-state fluorescence intensity is then given by

$$I_\lambda^F = \alpha \, I_\lambda^0 \frac{\kappa_r^s}{\kappa_r^s + \kappa_{nr}^s} = \alpha \, I_\lambda^0 \, \Phi_F \qquad (4)$$

$$I_\lambda^F = I_\lambda^0 - I_\lambda^T \qquad (5)$$

Equations (1)-(5) lead to

$$I_\lambda^F = 2.303 \Phi_F \, I_\lambda^0 \, \varepsilon_\lambda \, bc \qquad (6)$$

For our probe in the test solution

$$\begin{aligned} I_\lambda^F &= I_{\text{Cy-NO}_2} + I_{\text{Cy-NH}_2} \\ &= 2.303 \Phi_{\text{Cy-NO}_2} \, I_{\lambda(\text{Cy-NO}_2)}^0 \, \varepsilon_{\lambda_{(\text{Cy-NO}_2)}} \, bc_{\text{Cy-NO}_2} + 2.303 \Phi_{\text{Cy-NH}_2} \, I_{\lambda(\text{Cy-NH}_2)}^0 \, \varepsilon_{\lambda_{(\text{Cy-NH}_2)}} \, bc_{\text{Cy-NH}_2} \end{aligned} \qquad (7)$$

$$c_{\text{Cy-NO}_2} + c_{\text{Cy-NH}_2} = 10 \mu M \qquad (8)$$

In this paper, we selected 755 nm as excitation wavelength, and we picked out 809 nm and 789 nm for our experiment because the two wavelengths are the fluorescence maximum

emission wavelengths of our probe Cy-NO_2 and Cy-NH_2, respectively. We then measured out the constants in equation (7). Merging two equations (7) and (8), finally we acquired the result of our experiment. $I^0_{809nm(Cy-NO_2)}$ = 0.02 mW, $I^0_{809nm(Cy-NH_2)}$ = 0.18 mW; $I^0_{789nm(Cy-NO_2)}$ = 0.001 mW, $I^0_{789nm(Cy-NH_2)}$ = 0.23 mW.

All the data were shown in Table 1 and Table 2. These results indicated that Cy-NO_2 could detect H_2S in our experimental systems.

Table 1. Data for the steady-state fluorescence intensity at 809 nm. Experimental operation as described in Fig. 1.

c_{H_2S} (μM)	0	50	100	150	200	250	300	350
c_{Cy-NO_2} (μM)	10	8	5.1	4	2	0.7	0.4	0
c_{Cy-NH_2} (μM)	0	2	4.9	6	8	9.3	9.6	10

Table 2. Data for the steady-state fluorescence intensity at 789 nm. Experimental operation as described in Fig. 1.

c_{H_2S} (μM)	0	50	100	150	200	250	300	350
c_{Cy-NO_2} (μM)	10	8.1	5.5	4	2.1	0.8	0.6	0
c_{Cy-NH_2} (μM)	0	1.9	4.5	6	7.9	9.2	9.4	10

[8] K. Kiyose, S. Aizawa, E. Sasaki, H. Kojima, K. Hanaoka, T. Terai, Y. Urano and T. Nagano, *Chem. Eur. J.*, 2009, **15**, 9191.

[9] (a) Steady-State Fluorescence Spectroscopy, Aalim M. Weljie and Hans J. Vogel, Methods in Molecular Biology, vol. 173: Calcium-Binding Protein Protocols, Vol. 2: Methods and Techniques Edited by: H. J. Vogel © Humana Press Inc., Totowa, NJ; (b) Molecular Fluorescence: Principles and Applications. Bernard Valeur, 2001 Wiley-VCH Verlag GmbH, ISBNs: 3-527-29919-X (Hardcover); 3-527-60024-8 (Electronic)

12. The Relationship between the Fluorescence Intensity and H_2S Concentration with Excitation at 635 nm

We have carried out an important experiment to evaluate the relationship between fluorescence intensity and H_2S concentration (0–350 μM) in HEPES buffer with excitation at 635 nm. These data has been added into the Supporting Information as part 12. We obtained a calibration curve between fluorescence emission intensity and H_2S concentration. As shown in Fig. S7, the fluorescence signal was linearly related to the concentration of H_2S under the given concentration range. The regression equation was F_{789nm} = 117.7×[H_2S] + 8887.5, with r = 0.997. We calculated that the slope with excitation at 635 nm (S_{635nm} = 117.7) was much lower than that of excitation at 755 nm (S_{755nm} = 1111), which indicated the sensitivity was lower.

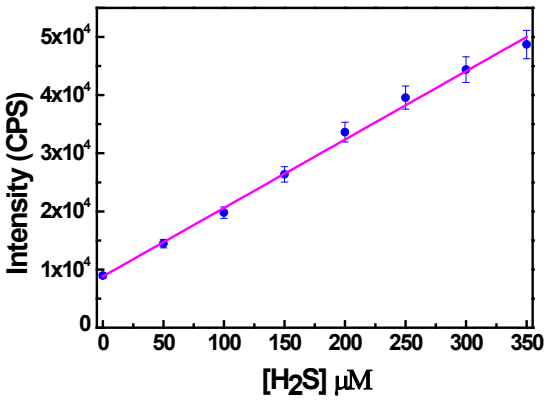


Fig. S7 The relationship between fluorescence intensity and H_2S concentration in buffer solution (40 mM HEPES, pH7.4). The fluorescence intensity was acquired at 37 ^{o}C after incubation of the probe with H_2S for 60 min with excitation at 635 nm and emission at 789 nm.