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Fluorescent Probes

Dual-Reactable Fluorescent Probes for Highly Selective and Sensitive Detection of Biological H_2S

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Abstract: Hydrogen sulfide (H_2S) is an important endogenous signaling molecule with a variety of biological functions. Development of fluorescent probes for highly selective and sensitive detection of H_2S is necessary. We show here that dual-reactable fluorescent H_2S probes could react with higher selectivity than single-reactable probes. One of the dual-reactable probes gives more than 4000-fold turn-on response when reacting with H_2S, the largest response among fluorescent H_2S probes reported thus far. In addition, the probe could be used for high-throughput enzymatic assays and for the detection of Cys-induced H_2S in cells and in zebrafish. These dual-reactable probes hold potential for highly selective and sensitive detection of H_2S in biological systems.

Hydrogen sulfide (H_2S) is an important gaseous signaling molecule with a multitude of physiological and pathological functions in various tissues.[1] H_2S can be enzymatically produced in vivo by three distinctive pathways including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MPST) coupled with cysteine aminotransferase (CAT).[2] Previous studies indicate that H_2S is both protective[3] and toxic[4] to cells depending on its concentrations. Due to the complexity of sulfur resources in vivo, the exact concentration and source of endogenous H_2S have not been fully elucidated.[5] To understand the physiological roles of H_2S in relation to its biological concentration, it is also desirable to develop probes for the accurate measurement of endogenous H_2S levels in vivo.[6]

Fluorescence-based methods have recently emerged as a useful approach for H_2S detection in biological systems.[7–17] An ideal intracellular H_2S sensor should have a large dynamic range for the selective detection of H_2S over higher intracellular concentrations of biothiols (such as millimolar concentrations of reduced glutathione, GSH). The reactivity of the probe toward H_2S is also essential for real-time detection. Although great advances have been made in the development of fluorescent H_2S probes, probes with higher sensitivity and higher selectivity for the detection of H_2S levels in biological samples still need to be developed. We previously proposed a dual-quenching probe with the largest off-on response (2000-fold) reported based on FRET-ICT dual-quenching effects,[17] but the probe exhibited a medium selectivity for H_2S over biothiols present at millimolar concentration. Herein, we report dual-reactable H_2S probes (Figure 1) with higher selectivity and sensitivity than single-reactable probes and the dual-quenching probe.[17] Moreover, probe **2** could be used to develop a high-throughput enzymatic assay and to image Cys-induced endogenous H_2S.

A dual-quenching probe can lead to a large fluorescent turn-on response to give a low detection limit (the 3σ/k value).[18] To design a H_2S probe with higher selectivity, herein we used two types of reactable groups on one fluorophore (Figure 1a). The reactable group is also the quenching group. A competitor partly reacts with one reactable group while another reactable group can still quench the fluorescence. As a result, the maximum turn-on fluorescence will only be obtained with a competitor that can completely undergo the two different reaction events (here, nucleophilic and redox reactions). Such a dual-reactable strategy should increase the probe's selective response between H_2S and biothiols.

To this end, we employed FRET-ICT dual-quenching effects and nucleophilic–redox dual-reactable groups to prepare the two probes **1** and **2** (Figure 1b). Both NBD amine and aromatic azide were installed into one fluorophore as reactable groups for H_2S, while the fluorescence of the designed compound was quenched by NBD and azide through FRET and ICT effects, respectively.

The synthesis of **1** was achieved by a coupling reaction of **3**[14] and NBD-Cl (see the Supporting Information). Probe **2** was

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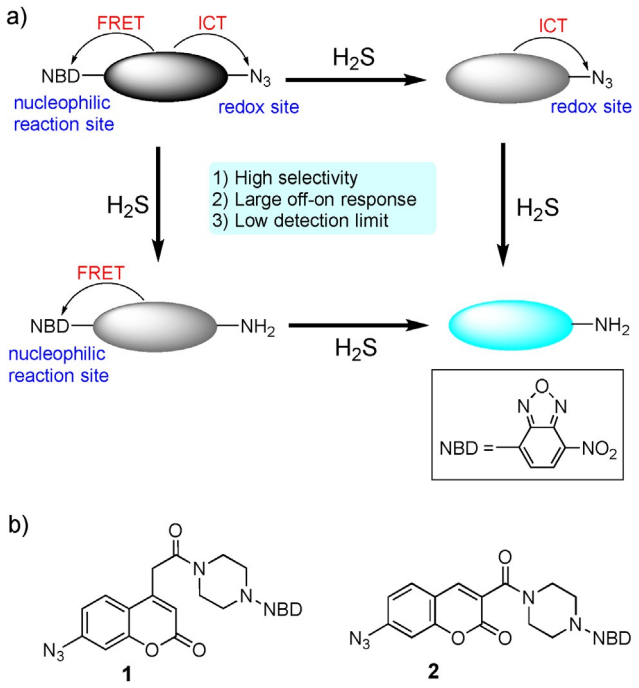

Figure 1. The design strategy for a dual-reactable-dual quenching H_2S fluorescent probe (a) and structures of probes **1** and **2** used in this work (b). FRET, fluorescence resonance energy transfer; ICT, intramolecular charge transfer; NBD, 7-nitro-1,2,3-benzoxadiazole.

prepared as shown in Figure 2. Treatment of *p*-amino salicylic acid with methanol led to the formation of **4**, which underwent selective acetylation on the 4-amino group to provide **5**.[19] Reduction of **5** by $LiAlH_4$ yielded **6**, which was further oxidized to afford the corresponding aldehyde **7**.[20] Coumarin **8** was formed by a Knoevenagel condensation of aldehyde **7** with diethyl malonate in the presence of piperidine. Hydrolysis of **8** yielded free amine **9**, which was transformed into azido-coumarin **10** by a Sandmeyer reaction. Probe **2** was obtained by a couping reaction of **10** and NBD amine **12**.[17] Probes **1** and **2** were well characterized by ^1H NMR and ^{13}C NMR spectroscopies and high-resolution mass spectrometry (HRMS) (see the Supporting Information).

Emission spectra of the probes **1** and **2** were examined in the absence or presence of H_2S in phosphate-buffered saline (PBS, pH 7.4) (Figure 3 and Figures S1, S2). As expected, the coumarin emission was nearly completely quenched for both probes due to the FRET-ICT dual-quenching effects (quantum yield Φ for **2**, 0.0016). After reacting with H_2S, the probe showed a significant turn-on fluorescent response, with an off–on response of up to ca. 3150-fold for **1** and 4010-fold for **2** (Φ, 0.6063). Such a fluorescent turn-on response could be clearly visualized by the naked eye (Figure 3a). To our knowledge, the observed increase in fluorescence emission for **2** is the largest among the fluorescent probes reported so far for H_2S.[7–17]

To determine the detection limit for **1** and **2**, the fluorescence intensity change was closely monitored by addition of various concentrations of H_2S to a solution of the probe (Figure 3b and Figure S1). For both probes, the fluorescence intensity was linearly related to the concentration of H_2S from 0 to 100 μM, indicating a large dynamic range for the detection of H_2S. The detection limit for **1** or **2** was calculated to be 27 and 24 nM, respectively, by using the 3σ/k method.[18] The results implied that probes **1** and **2** are highly sensitive toward H_2S in buffer solution. In addition, the probe **2** could function over a wide range of pH from 6.0 to 8.5 (Figure S2).

The absorption spectra of the probes were further examined (Figure S3). The coumarin absorbance for **1** and **2** exhibited a decrease at 330–350 nm and an increase at 350–380 nm after treatment with H_2S, due to the reduction of azide to amine. The NBD absorbance for **1** and **2** displayed a decrease at around 480 nm and an increased absorbance at 530 nm, re-

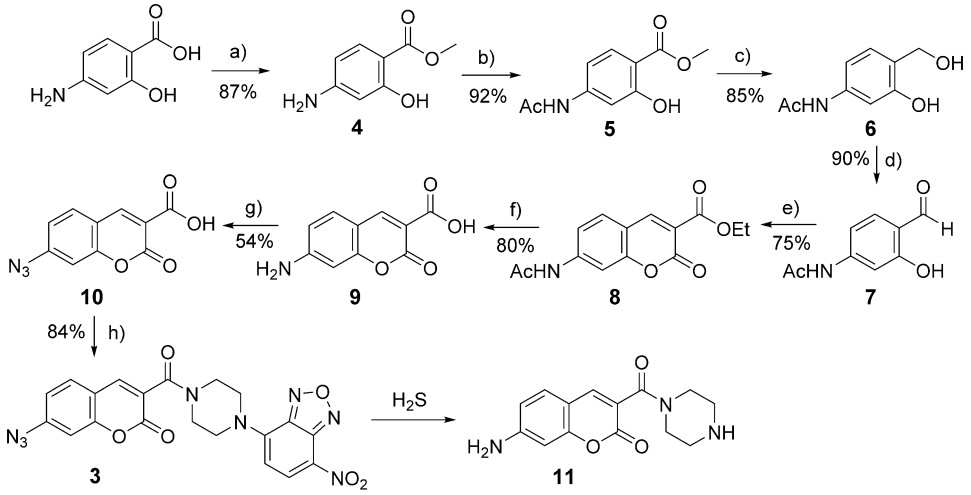

Figure 2. Synthesis of probe **2** and its reaction with H_2S. Conditions: (a) Conc. H_2SO_4, MeOH, reflux, 12 h; (b) Ac_2O, CH_2Cl_2, 0.5 h; (c) $LiAlH_4$, dry THF, 2 h; (d) pyridinium dichromate (PDC), dry THF, 2 h; (e) diethyl malonate, piperidine, EtOH, reflux, 4 h; (f) 4 M NaOH, reflux, 0.5 h; then conc. H_2SO_4, pH 2.0; (g) 20% H_2SO_4, $NaNO_2$, 0–5 °C, 0.5 h; then NaN_3, 0 °C to room temperature, 1 h; (h) HATU, DIPEA, CH_2Cl_2, **12** (1 equiv), overnight. Yields of isolated product are given are given below the arrow.

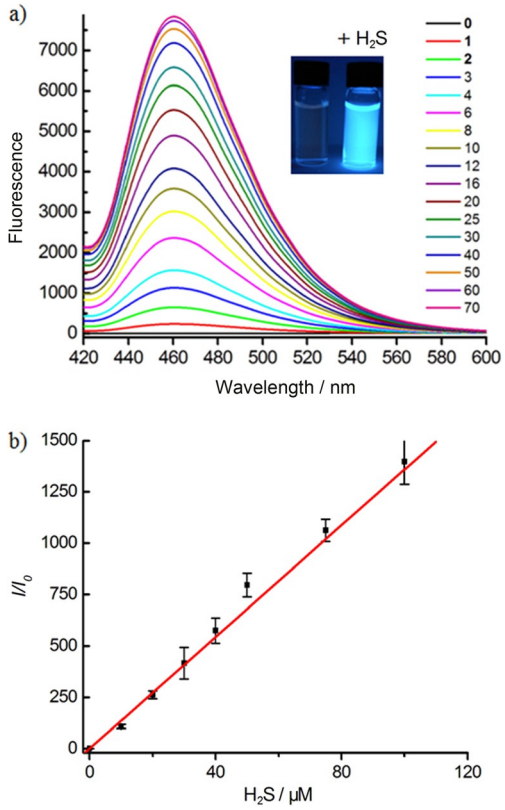

Figure 3. The dual-reactable probe **2** is highly sensitive toward H_2S with 4010-fold turn-on response and 24 nM detection limit. (a) Time-dependent fluorescent spectra of **2** (1 μM) in the presence of H_2S (250 μM) in PBS (pH 7.4) with excitation at 400 nm. (b) The linear relationship of relative fluorescence intensity at 460 nm of **2** (1 μM) treated with different concentrations of H_2S (0, 10, 20, 30, 40, 50, 75 and 100 μM) for 30 min in PBS.

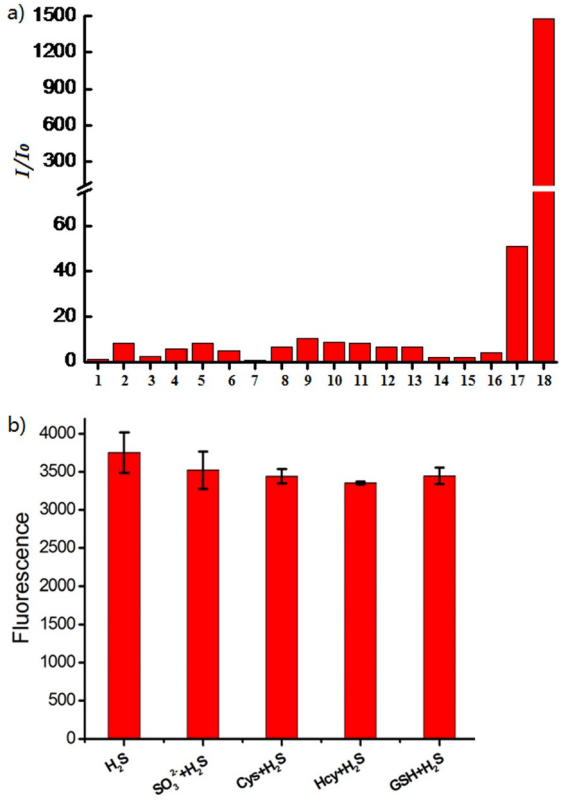

Figure 4. The dual-reactable probe **2** is highly selective toward H_2S over biothiols. (a) Experiments were performed in PBS (pH 7.4) for 30 min with excitation at 400 nm and relative emission at 460 nm. Columns 1–18 correspond to: **2** only (1 μM); **2** and species of SO_4^{2-} (100 μM), $S_2O_3^{2-}$ (100 μM), SO_3^{2-} (100 μM), H_2O_2 (100 μM), ClO^- (100 μM), NO_2^- (100 μM), Zn^{2+} (100 μM), Fe^{3+} (100 μM), Cys (1 mM), Hcy (1 mM), GSH (5 mM), trolox (100 μM), ascorbic acid (100 μM), NO (100 μM), KO_2 (100 μM), $^{\bullet}OH$ from H_2O_2 and Fe^{2+} (100 μM) or H_2S (100 μM), respectively. The selectivity of **2** toward H_2S is 174-fold or 228-fold over 1 mM Cys or 5 mM GSH, respectively. (b) Fluorescence intensity at 460 nm of **2** (1 μM) incubated with various analytes (SO_3^{2-}, 100 μM; Cys and Hcy, 1 mM; GSH, 5 mM) in the presence of H_2S (100 μM).

spectively, due to the production of nitrobenzofurazan thiol.[17,21] The absorption spectra of single-reactable probes (with only azide or NBD amine as the reactive group) showed a response similar to that of the one-reactive site from the dual-reactable probes. The NBD-based probe showed a faster reaction rate toward H_2S than the azide-based probes (Figure S3), and the reaction kinetics of the dual-quenching probes were mainly determined by the property of the relative slow azide-based reaction site.[17] These results showed that H_2S could cleave the NBD moiety and reduce the azide group of the probe **2** to give **11**, which was further supported by the HRMS results (see the Supporting Information).

The dual-reactable probes were incubated in PBS buffer for thermo- and photo-stability tests (Figure S5). The results indicated that the fluorescence increase of the dual-reactable probe **2** was almost negligible even under UV light for 1 h, while the single-reactable azide-based probes showed to some extent a fluorescent increase. These results further indicated the advantages of the dual-reactable probe.

A major challenge for H_2S detection in biological systems is to develop a selective probe that exhibits a distinctive response to H_2S over biothiols present at millimolar concentration. Probes **1** and **2** were incubated with various biologically related species in PBS for 30 min, and the maximal emission change was measured accordingly (Figure 4 and Figue S4). The tested species included biothiols (GSH, 5 mM; Cys, 1 mM; Hcy, 1 mM), trolox, ascorbic acid, reactive oxygen species (H_2O_2, KO_2, ClO^-, $^{\bullet}OH$), reactive sulfur species (SO_4^{2-}, $S_2O_3^{2-}$, SO_3^{2-}), anions (NO_2^-) and cations (Zn^{2+}, Mg^{2+}). For both probes, the fluorescence intensity enhancement for any tested analyte in PBS (pH 7.4) was very small except for H_2S. Thus, both **1** and **2** are highly selective toward H_2S over other biologically relevant species. Probe **2** was further tested for its selectivity over biothiols at millimolar concentration, due to its larger off–on response and lower detection limit compared to those of probe **1**. The tests indicated that probe **2** is 228-fold more selective toward 100 μM H_2S over 5 mM reduced GSH and more than 1000-fold more selective toward 100 μM H_2S over 100 μM SO_3^{2-} (Figure 4). Therefore, probe **2** has a higher selectivity than that of the single-reactable probes or the dual-quenching probe.[17]

The high selectivity and sensitivity of probe **2** encouraged us to apply it for the detection of biological H_2S. Herein, probe **2** was employed to monitor the cystathionine β-synthase (CBS)

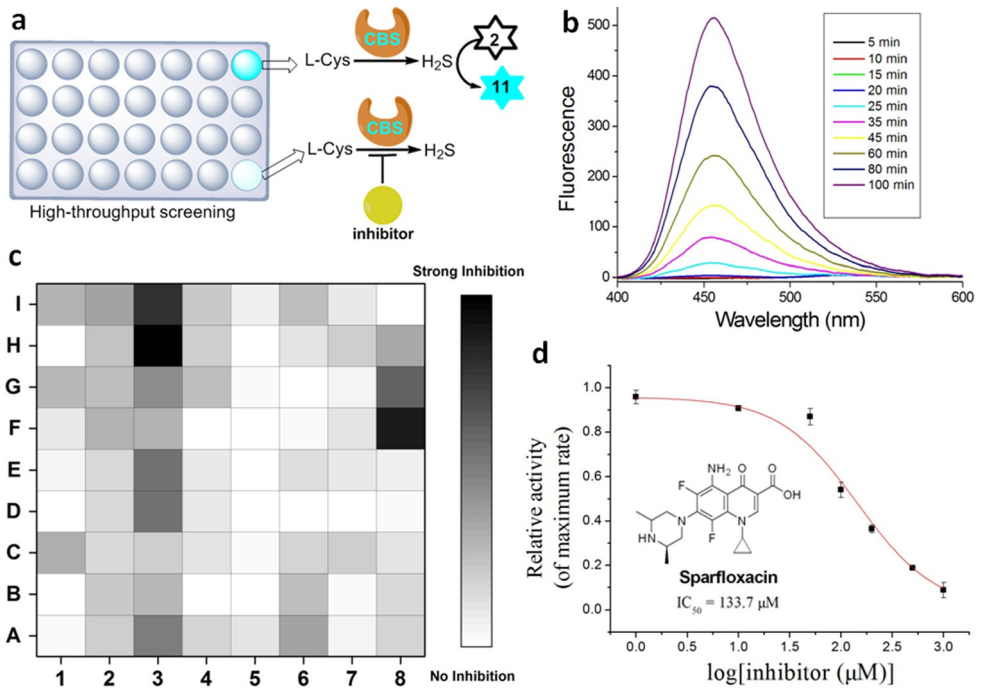

Figure 5. Use of probe **2** in a high-throughput enzymatic assay. (a) Schematic of the high-throughput fluorometric CBS assay. (b) Time-dependent experiments were carried out in the assay buffer (200 mM Tris-HCl, pH 8.0, 5 μM pyridoxal 5′-phosphate (PLP), 10 mM GSH, 0.5 mg mL^{-1} BSA, 10% DMSO) at 37 °C. The reaction solution contained 10 mM L-Cys, 2 μg CBS enzyme, and 10 μM probe **2**. Emission spectra were recorded for each time point with 370 nm excitation. (c) The high-throughput experiments were carried out in the assay buffer (volume 200 μL) in a 96-well plate at 37 °C for 1 h. The reaction solution contained 10 mM L-Cys, 2 μg CBS enzyme, 10 μM probe **2** and no inhibitor or 1 mM inhibitor. The normalized fluorescence intensity at 450 nm was monitored upon excitation at 370 nm for each sample. The structures of tested molecules are shown in Figure S5. One of the most potent inhibitors, H3, is sparfloxacin. (d) CBS activity curve in the presence of different concentrations of sparfloxacin (see inset for its structure); the solid line represents the best fit to give an IC_{50} of 133.7 μM.

activity with L-Cys as substrate in a high-throughput assay (Figure 5a). The truncated version of human CBS (Δ414–551) was expressed and purified (see the Supporting Information for details).[22] The enzymatic reaction can be visualized by monitoring the fluorescence increase of probe **2** (Figure 5b). The CBS activity can be inhibited by an analogue of Cys (propargylglycine, PPG),[22–23] and therefore we selected amino acids and their analogues, Cys/imidazole derivatives, and quinolones drugs (Figure S5) for screening new potent CBS inhibitors. The high-throughput enzymatic reactions were performed in a 96-well plate, and the fluorescence of each well was recorded (Figure 5c and Figure S6). Strong inhibition of CBS should reduce the enzymatically produced H_2S and further lead to a lower fluorescent off-on response from probe **2**. The results showed that aminooxyacetic acid (a known CBS inhibitor),[23] nadifloxacin, and sparfloxacin were the most efficient inhibitors. Further tests indicated that the half maximal inhibitory concentration (IC_{50}) for nadifloxacin and sparfloxacin was 269.2 μM and 133.7 μM, respectively. The tested compounds with medium inhibition potency included Cys derivatives, quinolones, methacrylic acid, and hydroxylamine. Our preliminary studies successfully showed the application of probe **2** for high-throughput screening of CBS inhibitors in vitro.

The cytotoxicity of both probes **1** and **2** was evaluated by MTT assay using HEK293 cells (Figure S7).[11] Both probes did not show any obvious cytotoxicity at 2 μM concentration, implying that the probes are suitable for H_2S detection in living cells at this concentration. Herein we employed probe **2** for imaging of Cys-induced H_2S in vivo (Figure 6 and Figures S8–S10). When the cells were first incubated with **2** and then treated with Na_2S (200 μM) for 30 min, a significant fluorescence increase was observed in the cells, further indicating that **2** is cell-permeable and can be used for imaging intracellular H_2S in living cells. Next, cells were treated with L-Cys followed by incubation with **2**, and a strong fluorescence was observed in the Cys-stimulated HEK293 cells (Figure 6a). As our study revealed sparfloxacin to be a potent CBS inhibitor, bioimaging of its inhibitory effect was further tested in L-Cys-stimulated HEK293 cells, considering that H_2S can be produced from L-Cys in HEK293 cells via the CBS pathway.[3b,22] The results showed the suppression of L-Cys-induced H_2S production by sparfloxacin (Figure 6a, 6b), implying that sparfloxacin could inhibit endogenous H_2S biosynthesis.

Finally, we examined the suitability of probe **2** for visualizing endogenous H_2S in living zebrafish (Figure 6c). Three-day-old zebrafish showed no fluorescence. However, when the zebrafish was incubated with probe **2** (1 μM), a moderate fluorescence in the fish was observed, implying the existence of endogenous H_2S in the zebrafish. Moreover, exposure of the fish to D-Cys (100 μM) and probe **2** (1 μM) led to a significant fluorescence increase, implying that probe **2** could be used for tracing the D-Cys-induced endogenous H_2S in zebrafish.

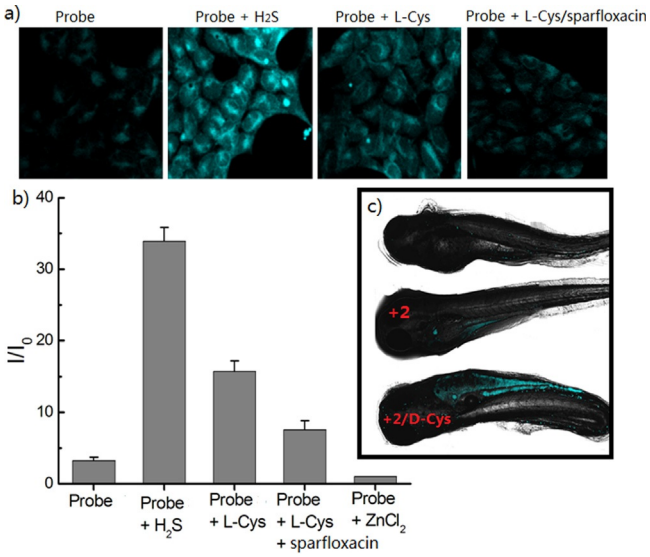

Figure 6. Confocal fluorescent images of H_2S with probe **2** in HEK293 cells and zebrafish. (a) Fluorescence images of living cells in the presence of probe **2** and stimulants. HEK293 cells were incubated with **2** (2 μM) for 30 min; with **2** (2 μM) for 30 min, washed with PBS, and then incubated with Na_2S (200 μM) for another 30 min; with L-Cys (200 μM) for 30 min and then with **2** (2 μM) for 30 min; with the inhibitor sparfloxacin (50 μM) and L-Cys (200 μM) for 30 min and then with **2** (2 μM) for 30 min. (b) The relative fluorescence of the fluorescent images from HEK293 cells in (a). (c) Fluorescent merged images of untreated zebrafish (top), of zebrafish pretreated with probe **2** (1 μM) for 30 min (middle), and of zebrafish pretreated with D-Cys (100 μM) and probe **2** (1 μM) for 30 min (bottom).

In summary, we have obtained the first set of dual-reactable fluorescent H_2S probes, which showed a higher selectivity than that of a dual-quenching fluorescent H_2S probe,[17] and single-reactable H_2S probes including thiolysis of the NBD amine or reduction of the aromatic azide.[14] The detection limit for the dual-reactable probes **1** and **2** was below 30 nM, implying that both probes are also highly sensitive. Furthermore, probe **2** could be successfully used to develop a high-throughput enzymatic assay and to image Cys-induced endogenous H_2S in vivo, indicating that the dual-reactable probe could be a promising tool for applications in H_2S biology. Our results further illustrate that using such dual-reactable (including multi-reactable) strategy is likely to be general for preparation of highly selective and highly sensitive H_2S probes for various biological applications.

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