Highly selective fluorescent and colorimetric probe for live-cell monitoring of sulphide based on bioorthogonal reaction

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H2S is the third endogenously generated gaseous signaling compound and has also been known to involve a variety of physiological processes. To better understand its physiological and pathological functions, efficient methods for monitoring of H2S are desired. Azide fluorogenic probes are popular because they can take place bioorthogonal reactions. In this work, by employing a fluorescein derivative as the fluorophore and an azide group as the recognition unit, we reported a new probe 5-azidofluorescein for H2S with improved sensitivity and selectivity. The probe shows very low background fluorescence in the absence of H2S. In the presence of H2S, however, a significant enhancement for excited fluorescence were observed, resulting in a high sensitivity to H2S in buffered (10 mmol/L HEPES, pH 7.0) aqueous acetonitrile solution (H2O/CH3CN = 1:3, v/v) with a detection limit of 0.035 μmol/L observed, much lower than the previously reported probes. All these features are favorable for direct monitoring of H2S with satisfactory sensitivity, demonstrating its value of practical application.

Fluorogenic probes activated by bioorthogonal chemical reactions can enable biomolecule imaging in situations where it is not possible to wash away unbound probe1. Much work has been devoted to expanding the toolbox of bioorthogonal reactions, and these efforts can be complemented by the development of fluorogenic probes2. Such probes are typically endowed with a functionality that suppresses fluorescence. Its transformation during the reaction creates a new functionality that no longer quenches the fluorescence of the underlying system, resulting in a fluorescence enhancement. Such probes offer significant advantages for imaging studies in which it is not possible to wash away unreacted probe, such as real-time imaging of dynamic processes in cells or visualization of molecules in live organisms.

One of the most widely used bioorthogonal reactions is the azide—alkyne [3 + 2] cycloaddition to form a triazole4. This reaction has enabled the selective visualization of azide- or alkyne-labeled proteins, glycans, nucleic acids, and lipids6–8. Several azide-6–9 fluorogenic probes have been reported, largely based on coumarins6,10, naphthalimides8, and other systems that require UV excitation and emit blue light7,11,12. Such wavelengths are not ideal for biological imaging because of high levels of autofluorescence and poor tissue penetrance13.

An obvious improvement upon these designs would be the development of azido fluorogenic probes with longer excitation and emission wavelengths. Some attempts at achieving this goal have been made6,12,14. The utility of azide pairs in biological settings remains unclear. Thus, fluorogenic azido probes that perform well as cell-imaging reagents remain an important goal. Bertozzi reported the rational design and experimental validation of azide-functionalized fluorogenic probes based on the widely used blue-excitation/green-emission fluorescein scaffold15. In their work, they have prepared a series of azidefluorescein compounds under NaNO2/NaN3 condition, and the azidefluorescein was used to biological imaging in Chinese hamster ovary (CHO) cells labeled with alkynylsialic acid nor H2S.

It is well known that H2S have been demonstrated to exert protective effects in many pathologies and physiologies16–26. So the discovery of these emerging biological roles of H2S has resulted in rising interest in H2S research. Accordingly, rapid, accurate and reliable methods for H2S detection are in high demand, as they have potential to provide useful information for better understanding its biological functions27. And simple, specific, and real-time analytic methods/sensors are highly desirable for H2S in biological systems. In fact, it is a good
choice to introduce an azido group into probes to be reduced by H2S due to the simple synthesis, relatively good selectivity, suitable reaction time, and non-cell toxicity.

With these considerations in mind, we also prepared 5-azidefluorescein from 5-aminofluorescein under NaNO2/NaN3 condition according to literature (Fig. 1) and tried to use this compound to detect H2S. It is delightful that we obtained the crystal of 5-aminofluorescein and found probe can be used as a high selective and sensitive fluorescent probe for H2S firstly. Furthermore, the probe also was applied in cell imaging.

5-aminofluorescein (0.35 g, 1 mmol), a deep-red solid, was dissolved in 10 mL 251 AcOH/H2O and cooled to 0°C. To this deep red solution was added NaNO2, a white powder (0.10 g, 1.5 mmol). After stirring for 15 minutes, the solution had turned to a light red color. NaN3 (0.10 g, 1.5 mmol) was then carefully added (caution: gas evolution!), turning the solution to a yellow slurry. The reaction was stirred for 2 hr at 0°C. The slurry was filtered over vacuum and the solid washed with 20 mL 2 mol/L HCl and 100 mL H2O, yielding 5-azidofluoresceinquinone (0.30 g, 80%) as a yellow solid after further drying in vacuo and characterized by NMR, ESI-MS, elemental analysis, X-ray crystal diffractometer (see Figure S1).

Reaction of probe (1 μmol/L) with Na2S (2 μmol/L) as an aqueous sulphide source at room temperature in buffered (10 mmol/L HEPES, pH 7.0) aqueous acetonitrile solution (H2O/CH3CN = 1:3, v/v) yielded a time-dependent fluorescence increase, which was completed within 5 s (Supplementary Fig. S2). ΔF > 50-fold increase in the fluorescence intensity accompanied (Φ = 0.35) with a green emission at 531 nm. However, the analytes without hydrogen sulfide induced no changes in the fluorescence emission properties under the same conditions (Fig. 2a). The competing experiments indicated other analytes did not disturb the determination for sulphide (Fig. 2b). It is noted that the unprecedented speed of this probe’s response and high selectivity compared with other probes suggests the possibility of quantitative detection without the need for sample pretreatment. The results reason that H2S-mediated reduction of azides to amines would generate highly fluorescent products (Fig. 3). H2S-induced product was confirmed its molecular formula by electrospray ionization mass spectrometry (ESI-MS). The peak at m/z 346.42 corresponding to [5-aminofluorescein-H]+, was clearly observed (Supplementary Fig. S3). Further 1HNMR spectroscopic analysis also provided the evidence for the product of 5-aminofluorescein.

Next, varying concentrations of Na2S (0–2.0 μmol/L) were added to the test reaction solution. The fluorescence intensity increased linearly with the concentration of Na2S up to 2.0 μmol/L, and, thereafter, reached a steady state (Fig. 4). The detection limit, based on the definition by IUPAC (CDL = 3 Sb/m)40, was found to be 0.035 μmol/L from 10 blank solutions (Supplementary Fig. S5). This probe therefore shows a high sensitivity toward sodium sulfide comparable to that of other reported S2−-chemosensors (Table 1).

We also performed absorption spectral experiments in the buffered (10 mmol/L HEPES, pH 7.0) aqueous acetonitrile solution.
HepG2 cells incubated with 2 µmol/L probe for 30 min at 37 °C, and with 4 µmol/L exogenous H$_2$S for another 30 min at 37 °C, showed green fluorescence (Fig. 5c) (it is noted that 30 min was usually selected in cell imaging experiment). We also carried out time course experiment in the cell. Fig. 6 (left) indicated that a 15 min is enough for cell permeability (Fig. 6h) reaction and the cell can survive even if in a 45 min after H$_2$S was added (Fig. 6i). In addition, according to the Qian’s method, we employed sodium nitroprusside (SNP, a NO donor) to stimulate the production of endogenous H$_2$S in cells. With the addition of probe into the culture of the SNP (100 µmol/L or 200 µmol/L)-loaded cells for 20 min, a drastic increase of emission intensity (Fig. 6l, 6m), indicating the generation of endogenous H$_2$S within the cells. These results demonstrate that this probe is selective for sulphide and amenable for live-cell imaging.

The development of innovative fluorescent imaging probes has revolutionized cell biology, allowing localization and dynamic monitoring of cellular metabolite and inorganic ion pools. Recently, fluorescence and/or colorimetric chemosensors for H$_2$S/aqueous sulphide based on some reaction mechanisms between probes and H$_2$S have been reported. These include the cleavage of alcoxyl (R-O) bond, the cleavage of S-O bond, copper displacement, and nucleophilic addition approach. A significant bottleneck in the above emerging field of H$_2$S/aqueous sulphide signalling is the absence of technology for effective in vivo detection and imaging. This problem is exacerbated by fact that similar substances such as sulphide which contain SH group may mislead the mechanism.

Most publications suggest that the average endogenous H$_2$S level is in the µmol/L range. Since the detection limit of this probe was found to be 0.035 µmol/L, thus it become possible that the probe can detect H$_2$S level in tissue imaging. The ability of probe to detect sulphide within living cells was also evaluated by laser confocal fluorescence imaging using a Leica TCS SP5 laser scanning microscope. Imaging of sulphide substrates in HeLa cells after 30 min incubation using probe (2 µmol/L) showed weak green fluorescence (Fig. 5b).

### Methods

4-(2-Hydroxyethyl)-1-piperazineanesulfonic acid (HEPES) and sodium nitroprusside were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide solution (0.1 mol/L) was added to aqueous HEPES (10 mmol/L) to adjust the pH to 7.0. Anionic salts were purchased from Shanghai Experiment Reagent Co., Ltd (Shanghai, China). All other chemicals used were of analytical grade. Deionized water was used throughout.

### Table 1 | A compared table about the detection limits and time course for H$_2$S

| Method      | Analyte | Signal output | Solvent                        | Detection Limit (µmol/L) | Response Time (min) | Time course in cell (min) |
|-------------|---------|---------------|--------------------------------|--------------------------|---------------------|--------------------------|
| Ref. 35     | H$_2$S  | Fluorescence  | HEPES buffer                    | 0.08                     | 20                  | 50                       |
| Ref. 38     | H$_2$S  | Fluorescence  | PBS-DMSO (1 : 1, v/v, pH 7.4)   | 3.05                     | 40                  | 120                      |
| Ref. 36     | H$_2$S  | Fluorescence  | PBS-CH$_3$CN (1 : 1, v/v, pH 7.4) | 2.5                     | 10                  | 30                       |
| Ref. 37     | H$_2$S  | Fluorescence  | PIPES buffer (pH 7.4)           | 2.4                      | 30                  | 60                       |
| This work   | H$_2$S  | Fluorescence  | HEPES-CH$_3$CN (1 : 3, v/v, pH 7.0) | 0.035                    | 10                  | 30                       |
was used to prepare all aqueous solutions. The solutions of anions were prepared from their sodium salts.

Instruments. A pH meter (Mettler Toledo, Switzerland) was used to determine the pH. Fluorescence spectra were measured on Cary Eclipse fluorescence spectrophotometer. A PO-120 quartz cuvette (10 mm) was purchased from Shanhai Huamei Experiment Instrument Plants, China. ESI-MS was measured with an UPLC-ESI-Q-TOF synapt G2 (Waters) instrument. The ability of probe reacting to hydrogen sulfide in the living cells was also evaluated by laser confocal fluorescence imaging using a Leica TCS SP5 laser scanning microscope.

Imaging of HepG2 cells. The HepG2 cells were grown in 1× SPP medium (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA ferric sodium salt) at 37°C. The HepG2 were treated with 2 μmol/L of probe (methanol stock solution) in culture media for 30 min at 37°C and washed 3 times with PBS. The HepG2 cells were first incubated with 2 μmol/L of probe for 30 min at 37°C and with 20 μM exogenous H2S for final 30 min at 37°C.

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Author contributions
F.J. and C.X. conceived the idea and directed the work. Y.B. designed experiments. J.K. performed the synthesis and in vitro tests. J.B. carried out NMR experiment. All authors contributed to data analysis and manuscript writing.

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