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Introduction

Reactive sulfur species (RSS) are a family of endogenous sulfur-containing molecules in biological systems related to a series of signaling transduction processes. Among RSS, hydrogen sulfide (H$_2$S) has been studied for many years as the third endogenous neurotransmitter after CO and NO, and has been supposed to be involved in regulating neuromodulation, gasotransmitter after CO and NO, and has been supposed to be quantification of endogenous H$_2$S level in biological system have been reported from 17 nM to 3 μM. As a downstream product of H$_2$S, the endogenous H$_2$S$_n$ was conjectured to be <100 nM or even less. Unfortunately, it is still difficult to detect H$_2$S$_n$ at such low concentration. UV-vis spectroscopy and mass spectrometry (MS) have been applied to investigate H$_2$S$_n$, but they were limited by poor sensitivity and insufficient operability in organisms. Fluorescent probes were feasible for H$_2$S$_n$ detection in living systems, owing to their high sensitivity and compatibility with bioimaging facilities. In 2014, Xian group reported the first H$_2$S$_n$-specific probes (DSP) exploiting 2-fluoro-5-nitrobenzoic ester template, and several subsequent H$_2$S$_n$ fluorescent probes were designed afterwards. The DSP-type probes showed satisfactory sensitivity and selectivity for H$_2$S$_n$. However, it would also react with biothiols, resulting in slow response and low yielding. To settle this issue, several other recognition groups were developed, including nucleophilic ring-opening reaction, α-substituted acrylate ester, and phenyl 2-(benzoylethio) benzoate formation. Studies have also been performed to improve signal-to-noise ratio through red shift of wavelength, two-photon excitation, or ratio detection, and also to achieve simultaneous detection of H$_2$S$_n$ together with H$_2$S, glutathione, or superoxide anion. Despite many efforts have been done, all these probes can still detect H$_2$S$_n$ only through external stimulation or H$_2$S$_n$ addition, but not the endogenous H$_2$S$_n$ in native cells. In 2015, Xian group reported a new H$_2$S$_n$-probe (PSP), showing the lowest detection limit extrapolated from theory (3 nM), and an actual working range from 0.25 μM to 20 μM. Although the lower limit of quantification was as low as 0.25 μM, endogenous H$_2$S$_n$ in native cells still cannot be detected. It was expected that the lower limit of quantification must reach tens of nM, and the detection limit must reach a few

Fluorescent τ-Probe: Quantitative Imaging of Ultra-trace Endogenous Hydrogen Polysulfide in Cells and in Vivo

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Hydrogen sulfide (H$_2$S) has been recognized as important endogenous gasotransmitters associated with biological signaling transduction. However, recent biological studies implied that the H$_2$S$_n$-related cellular signaling might actually be mediated by hydrogen polysulfides (H$_2$S$_n$, n>1), not H$_2$S itself. Unraveling such mystery strongly demanded quantification of endogenous H$_2$S$_n$ in living systems. However, the endogenous H$_2$S$_n$ was still undetectable thus far, due to its extremely low concentration within cells. Herein, we demonstrated a strategy to detect ultra-trace endogenous H$_2$S$_n$ via a fluorescent τ-probe, through changes of fluorescence lifetime instead of fluorescence intensity. This τ-probe exhibited ultrasensitive response to H$_2$S$_n$, bringing about the lowest value of detection limit (2 nM) and lower limit of quantification (10 nM) to date. With such merit, we quantified and mapped the endogenous H$_2$S$_n$ within cells and zebrafish. The quantitative information about endogenous H$_2$S$_n$ in cells and in vivo may have a significant implication for future research on the role of H$_2$S$_n$ in biology. The methodology of τ-probe established here might provide a general insight into the design and application of any fluorescence probes, beyond the limit of utilizing fluorescence intensity.
nM, so as to detect native endogenous H$_2$S$_n$. Such goal seemed hard to achieve by intensity-based fluorescent probes, either from theoretical deduction or the best result reported by now. In addition to fluorescence intensity, fluorescence lifetime is another fundamental photophysical parameter associated with any fluorescent emission. Differ from fluorescence intensity that depends on emitter numbers, fluorescence lifetime mainly depends on the instinct photophysical characteristic of each emitter. Fluorescence lifetime imaging has been commonly applied as a powerful tool of biology studies. Current time-resolved techniques enable to distinguish lifetime changes as low as ps-fs level.

In spite of important progress made in studying H$_2$S$_n$, quantification of endogenous H$_2$S$_n$ in native biological systems still remain unsolved. The most sensitive fluorescent intensity probes are currently unable to achieve such low detection limit. In this work, we developed an alternative strategy of using a fluorescent τ-probe to detect H$_2$S$_n$ through changes of fluorescent lifetime rather than fluorescence intensity. The detection limit of τ-probe was as low as 2 nM, and the actual working range were from 10 nM to 5 μM, which allows us to detect ultra-trace endogenous H$_2$S$_n$ in cells and in zebrafish. The concentration of endogenous H$_2$S$_n$ in HeLa cell distributed between ~0-120 nM, with an averaged value about ~60-70 nM. The concentration distribution in zebrafish slightly broadened to ~0-160 nM, but still with a comparable averaged concentration ~70-80 nM with the value of cells. To the best of our knowledge, it is the first time to detect the endogenous H$_2$S$_n$ in native biological system. These results provided fundamental quantitative information about endogenous H$_2$S$_n$ in cells and in vivo, and have a significant implication for deep understanding the role of H$_2$S$_n$ in biology. Meanwhile, the methodology of τ-probe established here might provide a general insight into the design and application of any analytic strategy exploiting fluorescence probes, to remedy the disadvantage of fluorescent intensity probes that are insensitive at extreme low concentration.

Results and discussion

Concept and Design of Fluorescent τ-Probe.

The design of τ-probe was inspired by Xian group’s PSP-3 probe, but its structure was modified to maximize the sensitivity. Detailed synthetic protocols and structural characterization are provided in SI (Scheme S1, Fig. S1-S3). Products from the reaction between τ-probe and H$_2$S$_n$ was shown in Fig. S4. Compared to PSP-3 probe with two recognition groups that would consume two H$_2$S$_n$ per probe, our τ-probe only has one recognition group, which consumes one H$_2$S$_n$ per probe. As shown in Fig. 1a, this probe initially showed a “dark” state with a weak fluorescence emission (Fig. 1b) and a short lifetime $\tau_{dark}$=0.75 ns (Fig. 1d). When reacted with H$_2$S$_n$ (Scheme S2), fluorescein was released to a “bright” state with a strong fluorescence emission (Fig. 1c) and a long lifetime $\tau_{bright}$=3.47 ns (Fig. 1e). This probe was a typical “turn-on”-type fluorescence probe. Here we presented that, in addition to fluorescence intensity, the fluorescence lifetime could also be utilized for quantitative analysis.

To prove the concept of τ-probe, we studied the response of fluorescence intensity and lifetime to the concentration of H$_2$S$_n$. Theoretically, for an emitter with unchanged fluorescence emission characteristic, the fluorescence intensity depends on the numbers of emitters, and the fluorescence lifetime depends on the instinct photophysical property of each emitter. However, for a systems mixed by two emitters with distinct fluorescence emission characteristic, the total value of fluorescence intensity and lifetime in such mixed systems was a result competed by two emitters. Particularly, for our τ-probe that has “dark” and “bright” states, the total weighted value of steady-state fluorescence intensity ($I_{total}$) was consist of “bright” state fluorescence intensity ($I_{bright}$) and “dark” state fluorescence intensity ($I_{dark}$), then $I_{total}$ could be calculated by:

$$I_{total}=I_{bright}+I_{dark}$$ (1)

Since fluorescence intensity is proportional to the numbers of fluorescent molecules (N) and its emission cross section (σ). The fluorescence intensity (I) can also be expressed as:

$$I=f \cdot c \cdot V \cdot N_0 \cdot \sigma$$ (2)

where f is a proportional constant related to the incident photon flux. $c_{bright}$ and $c_{dark}$ were the concentrations of “bright” and “dark” states.
state τ-probe, $V$ is the volume of the solution, and $N_A$ is Avogadro constant. Combined with Eq. (2), $I_{total}$ could be expressed as:

$$I_{total} = \frac{f \cdot \sigma_{Bright} \cdot V \cdot N_A \cdot \sigma_{Bright} + f \cdot \sigma_{Dark} \cdot V \cdot N_A \cdot \sigma_{Dark}}{f \cdot \sigma_{Bright} \cdot V \cdot N_A \cdot \sigma_{Bright} + f \cdot \sigma_{Dark} \cdot V \cdot N_A \cdot \sigma_{Dark}}$$

(3)

Due to the parameters of $f$, $V$ and $N_A$ are constants for a given system, they could be totally represented by another constant $\gamma$. Then Eq. (3) could be described as:

$$I_{total} = \frac{f \cdot \sigma_{Bright} \cdot V \cdot N_A \cdot \sigma_{Bright} + f \cdot \sigma_{Dark} \cdot V \cdot N_A \cdot \sigma_{Dark}}{f \cdot \sigma_{Bright} \cdot V \cdot N_A \cdot \sigma_{Bright} + f \cdot \sigma_{Dark} \cdot V \cdot N_A \cdot \sigma_{Dark}}$$

(4)

When the emitters were excited by pulsed light source, for instance in fluorescence lifetime measurement, the transient fluorescence intensity at time $t$ ($I_{total}$) could be calculated by:

$$I_{total}(t) = I_{Bright}(t) + I_{Dark}(t)$$

$$= A \cdot e^{-\frac{t}{\tau_{Bright}}} + B \cdot e^{-\frac{t}{\tau_{Dark}}}$$

(5)

where $A$ and $B$ are the compensation coefficients of fluorescence intensity at bright state and dark state, respectively. $\tau_{Bright}$ and $\tau_{Dark}$ were fluorescence lifetime of “bright” and “dark” state τ-probe. Therefore, the total weighted mean of fluorescence lifetime ($I_{total}$) could be calculated by:

$$\tau_{total} = \frac{A \cdot \tau_{Bright} + B \cdot \tau_{Dark}}{A + B}$$

(6)

Then the total fluorescence lifetime ($\tau_{total}$) could be described as:

$$\tau_{total} = \frac{I_{total}(t)}{I_{total}(c)} = \frac{f \cdot \sigma_{Bright} \cdot V \cdot N_A \cdot \sigma_{Bright} + f \cdot \sigma_{Dark} \cdot V \cdot N_A \cdot \sigma_{Dark}}{f \cdot \sigma_{Bright} \cdot V \cdot N_A \cdot \sigma_{Bright} + f \cdot \sigma_{Dark} \cdot V \cdot N_A \cdot \sigma_{Dark}}$$

(7)

According to Eq. (4) and Eq. (9), we find that $I_{total}$ and $I_{total}(c)$ are only related to the concentration of fluorescent molecules $\sigma_{Bright}$ and $\sigma_{Dark}$, owing to that $\sigma_{Bright}$ and $\sigma_{Dark}$ are fixed values in a given system ($\sigma_{Bright} >> \sigma_{Dark}$). Consequently, if we use $C_{Bright}$ and $C_{Dark}$ represent the original concentration of “bright” and “dark” state τ-probe, the concentration ($c$) of H$_2$S, reacted with τ-probe could be calculated from the total fluorescence intensity ($I_{total}(c)$) or fluorescence lifetime ($\tau_{total}(c)$):

$$I_{total}(c) = f \cdot \sigma_{Bright} \cdot V \cdot N_A \cdot \sigma_{Bright} (C_{Bright} + C_{Dark})$$

(8)

$$\tau_{total}(c) = \frac{I_{total}(c) \cdot \tau_{Bright} \cdot \sigma_{Bright} + I_{total}(c) \cdot \tau_{Dark} \cdot \sigma_{Dark}}{I_{total}(c) \cdot \tau_{Bright} \cdot \sigma_{Bright} + I_{total}(c) \cdot \tau_{Dark} \cdot \sigma_{Dark}}$$

(9)

Fluorescent Response to H$_2$S: Intensity versus Lifetime.

Theoretical response curves of $I_{total}(c)$ and $I_{total}(c)$ to H$_2$S concentration ($c$) could be obtained by substituting experimental data into Eq. (10) and Eq. (11): $C_{Bright}=0 \mu M, C_{Dark}=10 \mu M, \sigma_{Bright}=200 \mu M, \tau_{Bright}=3.47 \text{ ns}, \tau_{Total}=0.75 \text{ ns}$. Giving consideration to both accuracy and sensitivity, the concentration of τ-probe was chosen as 10 μM in the following experiments (Fig. 5S). Experimental response curves were obtained by measuring changes of fluorescence intensity and lifetime by addition of 0-25 μM Na$_2$S (commercial H$_2$S donor), as adopted previously. Theoretical curves matched experimental data well for either fluorescence intensity (Fig. 2a) or lifetime (Fig. 2b). The response features of $I_{total}(c)$ and $I_{total}(c)$ were totally different. $I_{total}(c)$ exhibited a nearly linear response at a wide range of concentration ($c$). However, $\tau_{total}(c)$ showed a non-linear response, which is much more sensitive in low concentration. For instance, when the concentration of Na$_2$S adds to 0.2 μM, $\tau_{total}(c)$ increased 0.93 ns, the rate of change was about 34.19% compared with the value of “bright” state. At the same condition, $I_{total}(c)$ is almost unchanged and the variation is as low as 0.09% (Fig. 2c). According to the temporal resolution and noise level of the instrument (~10 ps), the detection limit of τ-probe was about ~2 nM, and the experimental working range was ~10 nM-5 μM. Compared to H$_2$S, probes reported previously (Fig. 2d), our τ-probe reached the lowest value of detection limit and lower limit of quantification to date. Noted that even $\tau_{total}(c)$ is sensitive at low concentration, it does not have as broad response range as $I_{total}(c)$ does. Therefore, these two methods can complement in high sensitivity and wide response range, rather than replace each other.

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reactive oxygen species (ROS) in situ. The τ-probe did not show response (Fig. 3b) to H$_2$S (in the form of Na$_2$S) in the presence of common ROS including hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$), hydroxyl radical (OH), and singlet oxygen (O$_2$). Only ClO$_2$/H$_2$S system showed an obvious response. This result was consistent with previous reports that H$_2$S can be derived from H$_2$S and ClO$_2^-$, but not other ROS. All these results implied the high sensitivity and high selectivity of τ-probe to H$_2$S$_n$, suggesting that τ-probe could be used to detect endogenous H$_2$S$_n$ in native biological systems.

Quantify Endogenous H$_2$S$_n$ within Cells and Zebrafish.

We then demonstrated that our τ-probe could quantify H$_2$S$_n$ in cell by using suspension of HeLa cells. For positive control cells that incubated with 20 μM Na$_2$S (as H$_2$S$_n$ donor), fluorescence lifetime increased dramatically (Δτ=2.70 ns). For negative control cells that incubated with 100 μM N-ethylmaleimide (NEM) to eliminate the endogenous H$_2$S$_n$, the fluorescence lifetime remained almost unchanged (Δτ=0.05 ns) compared to the “dark” state τ-probe (Fig. 4a). With the positive and negative controls, endogenous H$_2$S$_n$ in native HeLa cells was obtained by substituting fluorescence lifetime into the working curve (Eq. 3). The averaged H$_2$S$_n$ in native HeLa cells is about ~0.065 μM (65 nM). We then further confirmed this by adding 0.065 μM (1X) and 0.130 μM (2X) additional Na$_2$S to cell samples, the fluorescence lifetime changed linearly according to the working curve. Cell viability assay demonstrated that τ-probe has nearly no cytotoxicity to cells (Fig. S11).
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With results in cell suspension, we then quantified endogenous H$_2$S within single HeLa cells, utilizing both fluorescence intensity and lifetime. Fluorescence imaging was performed on a confocal microscope. As reported previously, fluorescence was observed only in positive control cells (20 μM Na$_2$S), but not in native HeLa cells (Fig. S12). Fluorescence lifetime imaging was carried out on a time-gated transient microscope (see details in Method and Scheme S3 in SI). Dark field imaging was utilized to locate cells (Fig. 5a, b). As expected, fluorescence lifetime changes were observed in native HeLa cells (Fig. S5c-d). Then map and histogram of H$_2$S$_n$ within HeLa cell was obtained by applying working curve to fluorescence lifetime imaging (Fig. 5e, f). H$_2$S$_n$ level in three different HeLa cells were determined about 60-70 nM (Fig. 5g), which is comparable with value of cell suspension (~65 nM). It is interesting to note that even the intracellular concentration of H$_2$S$_n$ covers a wide range of 0-120 nM (Fig. 5f), the averaged values of different individual cells were quite close to each other (Fig. 5g). This result is sort of out of expectation since studies have shown the heterogeneity between individual cells on the concentration of biomolecules. Further in-depth studies are definitely needed to get a better understanding about the role of H$_2$S$_n$ in cells. Afterwards, we mapped H$_2$S$_n$ distribution in zebrafish (Fig. 6). The endogenous H$_2$S$_n$ within zebrafish mainly localized at its abdominal region, and the distribution covers a wide range of 0-160 nM, with an average value about ~60-70 nM. The value in zebrafish slightly broadened to ~0-160 nM, but still with a comparable average level ~70-80 nM. To the best of our knowledge, it is the first time to detect the endogenous H$_2$S$_n$ in native biological system. This information has a significant implication for deep understanding the role of H$_2$S$_n$ in biology. The concept of τ-probe provided a general insight into application of fluorescence probes, beyond the limit of fluorescence intensity.

**Experimental section**

**Reagents**

Fluorescein, methyl iodide (MeI), potassium carbonate (K$_2$CO$_3$), dimethyl formamide (DMF), sodium hydroxide (NaOH), methyl alcohol (MeOH), hydrochloric acid (HCl), dichloromethane (CH$_2$Cl$_2$), thiosalicylic acid, benzoic acid, 1-ethyl-3-(3-dimethylamino)propyl) carbodiimide (EDC), 4-dimethylaminopyridine (DMAP), dimethyl sulfoxide (DMSO), Glutathione (GSH), cysteine (Cys), homocysteine (Hcy), glutathione disulfide (GSSG), Na$_2$S, Na$_2$S$_2$O$_3$, Na$_2$SO$_3$, Na$_2$SO$_4$, CH$_3$SSCH$_2$ hydrogen peroxide (H$_2$O$_2$), sodium hypochlorite (NaOCl), KO$_2$, Fe$_3$S$_4$ and Ethyl 3-aminobenzoate methanesulfonate were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). High glucose Dulbecco’s Modified Eagle Medium (DME), fetal bovine serum (FBS), and trypsin were purchased from Invitrogen (Carlsbad, U.S.A.). Cell cytotoxicity assay kit, human cervical cancer (HeLa) cells, and phosphate buffer solution (PBS, 10 mM, pH=7.4) were purchased from KeyGEN Biotech. Co (Nanjing, China). Zebrafish were purchased from model animal research center of Nanjing University. Ultra-pure water from Millipore Milli-Q (18 MΩ·cm) was used in the experiments. All other reagents were of analytical reagent grade.

**Apparatus**

UV-vis absorption spectra were recorded using a UV-vis spectrophotometer (Nanodrop-2000C, Nanodrop, USA). The fluorescence emission spectra were obtained on a Shimadzu fluorescence 5-3 spectrophotometer (RF-S301PC, Shimadzu Co., Japan). The fluorescence lifetime spectra were obtained on a FLS 980 spectrophotometer (Edinburgh instruments, U.K.). Confocal fluorescence images of cells were acquired with a TCS SP8 confocal microscope (Leica, Germany). The cell viability assay was performed using a Thermo Scientific Varioskan Flash (Thermo Fisher Scientific, American). τ-Probe was purified by Ultimate3000 high performance liquid chromatography (Thermo Fisher Scientific, American). Fluorescence lifetime imaging was achieved by instruments built by our laboratory.

**Synthesis of τ-Probe**

The synthesis procedure of τ-probe is shown in Scheme S1. CH$_2$Cl$_2$ (20 mL) was added to a mixture of 3-O-Methylfluorescein (69.2 mg, 0.2 mmol), 2-(benzoylthio)benzoic acid (78 mg, 0.3 mmol), EDC (58 mg, 0.3 mmol) and DMAP (12.2 mg, 0.1 mmol) at room temperature and the mixture was stirred for 5 h. Then solvent was evaporated under reduced pressure and the resulted residue was subjected to high performance liquid chromatography for purification. τ-Probe was obtained as yellow solid. H-NMR and 13C-NMR were subjected to high performance liquid chromatography for purification. τ-Probe was obtained as yellow solid. 13C-NMR and 1H-NMR were obtained using a Bruker AVANCE III spectrometer at 500 and 125 MHz, respectively. The reaction was subjected to high performance liquid chromatography for purification. τ-Probe was obtained as yellow solid. H-NMR and 13C-NMR were obtained using a Bruker AVANCE III spectrometer at 500 and 125 MHz, respectively.
NMR spectra of τ-Probe were shown in Fig. S1 and S2, respectively. 

\(^1\text{H}\) NMR (400 MHz, CDCl\(_3\)) 88.23 (dd, J = 1.6, 8.0 Hz, 1H), 8.03-8.01 (m, 3H), 7.72-7.57 (m, 6H), 7.49-7.47 (m, 2H), 7.18 (d, J = 2.4 Hz, 1H), 7.16 (d, J = 7.6 Hz, 1H), 6.88 (dd, J = 2.4, 8.8 Hz, 1H), 6.80 (d, J = 8.8 Hz, 1H), 6.77 (d, J = 2.4 Hz, 1H), 6.69 (d, J = 8.8 Hz, 1H), 6.62 (dd, J = 2.4, 8.4 Hz, 1H), 3.82 (s, 3H). \(^{13}\text{C}\) NMR (100 MHz, CDCl\(_3\)) δ 189.44, 169.80, 164.27, 161.51, 153.05, 152.28, 151.98, 151.92, 137.33, 136.36, 135.26, 133.90, 133.47, 132.81, 131.66, 129.92, 129.72, 129.11, 129.00, 128.93, 128.83, 127.59, 126.41, 125.16, 124.05, 117.53, 116.74, 111.99, 110.72, 110.49, 100.90, 82.97, 55.62. LC-MS (ESI\(^+\)): m/z C\(_{36}\)H\(_{48}\)O\(_{3}\)S, calcd. 587.1169, found [M \(^+\)] 587.1169.

Fluorescence Lifetime Measurement

The fluorescence lifetime measurements were performed on a FLS 980 spectrophotometer (Edinburgh instruments, U.K.). The instrument is based on time-correlated single photon counting (TCSPC). Photons are counted in a time window which sweeps across the full time range following each excitation pulse, creating a histogram of counts versus time. The data quality of the resulting histogram is improved by adding the data of repeated sweeps. A 488 nm picosecond laser (~3 ps, 5.5 M Hz) was used to pump the fluorescence, the collection time window is 0-50 ns in the wavelength range of 500-650 nm, with a minimum time per channel=305 fs. The time jitter in the delay generation system is about ~10 ps.

General Procedure for Spectral Measurements

A stock solution of τ-probe was prepared at 1 mM in DMSO. A PBS (10 mM, pH 7.4) solution with DMSO as the cosolvent (DMSO/PBS = 1:9, v/v) was used for performing all spectroscopic measurements. The fluorescence signal was recorded with the excitation wavelength at 488 nm and the emission wavelength range from 500 to 650 nm. The maximum fluorescence emission was obtained at 515 nm. Glutathione (GSH, 5 mM), cysteine (Cys, 1 mM), homocysteine (Hcy, 100 μM), glutathione disulfide (GSSG, 100 μM), Na\(_2\)S (200 μM), Na\(_2\)SO\(_3\) (100 μM), Na\(_2\)SO\(_4\) (100 μM), CH\(_3\)SSCH\(_2\) (100 μM), hydrogen peroxide (H\(_2\)O\(_2\), 200 μM), hypochlorite (ClO\(^-\), 50 μM), superoxide (O\(_2^-\), 50 μM), hydroxyl radical (OH, 50 μM), and singlet oxygen (\(^1\)O\(_2\), 50 μM), H\(_2\)O\(_2\) (200 μM) + Na\(_2\)S (50 μM), H\(_2\)O\(_2\) (200 μM) + Na\(_2\)SO\(_3\) (100 μM), ClO\(^-\) (50 μM) + Na\(_2\)S (100 μM), O\(_2^-\) (50 μM) + Na\(_2\)S (100 μM), Oz (50 μM) + Na\(_2\)S (100 μM) were all dissolved into prepared solutions for various testing species. The preparation of reactive oxygen species was according to the literature. \(^{36}\) Na\(_2\)S\(_4\) (commercial H\(_2\)S\(_2\) donor) was dissolved into PBS solution, to prepare a sample solution (1 mM). A volume of 10 μL of τ-probe stock solution was added into 1 mL volumes of various analytes buffer/DMSO solution (H\(_2\)O/DMSO = 9:1, v/v), and 20 μL of Na\(_2\)S\(_4\) stock solution was employed in some of the experiments. After vigorous shaking, the resulting solutions were kept at room temperature for 30 min, and their fluorescence signals were collected.

Cell Culture

HeLa cells were cultured in high glucose DMEM containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 IU/mL streptomycin at 37 °C in a 5% CO\(_2\)-95% air incubator MCO-15AC (Sanyo, Tokyo, Japan).

Cell Viability Assay

Firstly, HeLa cells were seeded in 96-well plates (3000 cells per well) and maintained at 37 °C in a humidified atmosphere (95% air and 5% CO\(_2\)) for 24 h. After 24 h incubation, cells were washed with PBS and then cultured in a DMSO/PBS solution (pH 7.4, 1:9 v/v) containing τ-probe with various concentrations. After 30 min, the cells were washed with PBS and incubated in a fresh medium for an additional 24 h. Then, according to the manufacturer’s instructions, 50 μL MTT solution was added to each well and incubated at 37 °C. After removing the medium, 150 μL DMSO was added to solubilize the blue-colored tetrazolium, the plates were gently shaken for 5 min, and the optical density values at 550 nm were monitored using Thermo Scientific Varioskan Flash. The cell viability was set as 100% in control cells.

Intracellular H\(_2\)S\(_4\) Detection by Fluorescence Spectrometer

Cells were subjected to different treatments: first group was incubated with 100 μM N-ethylmaleimide (NEM) for 15 min, and second group was untreated, the third group was incubated with 20 μM Na\(_2\)S\(_4\) for 30 min, the forth group was incubated with 0.065 μM Na\(_2\)S\(_4\) for 30 min, while the fifth group was incubated with 0.13 μM Na\(_2\)S\(_4\) for 30 min. After washing with PBS, cells were cultured in a DMSO/PBS solution (pH 7.4, 1:9 v/v) containing 10 μM τ-probe for 30 min. Then fluorescence lifetime was acquired after washing with PBS (pH=7.4). The cells were excited at 488 nm and the emission was collected at 515 nm.

Confocal Fluorescence Images of HeLa Cells upon Incubation

HeLa cells were digested and seeded on confocal dishes for 24 h at 37 °C. Cells were subjected to different treatments. First group was incubated with 100 μM N-ethylmaleimide (NEM) for 15 min, and second group was untreated, the third group was incubated with 20 μM Na\(_2\)S\(_4\) for 30 min. After washing with PBS, cells were cultured in a DMSO/PBS solution (pH 7.4, 1:9 v/v) containing 10 μM τ-probe for 30 min. Then confocal images were acquired after washing with PBS (pH=7.4). The cells were excited at 488 nm and the emission was collected from 505 to 550 nm.

Optical Configuration of Fluorescence Lifetime Microscopy

The homemade fluorescence lifetime microscopy system was made up of a 488 nm nanosecond pulse laser, an inverted microscope and a delay generator. Pulse laser (20 Hz, ~5 ns, Quanta-Ray INDI, spectra-physis, USA) was acted as the excited light source and focused on the sample through a beam expander. The fluorescent intensity of the sample was collected by a 20x objective and recorded by an ICCD camera (iStar CCD 334, Andor Technology Ltd., UK), with an optical gate width ~2 ns. To avoid the influence from the excited light source, a 500 nm long-pass filter was added before the ICCD camera. In addition, the recorded delay was provided by a delay generator (DG645, Stanford Research Systems, USA).

Fluorescence Lifetime Images of HeLa Cells

HeLa cells were digested and seeded on confocal dishes for 24 h at 37 °C. Cells were washed with PBS and then cultured in a DMSO/PBS solution (pH 7.4, 1:9 v/v) containing 10 μM τ-probe for 30 min, then washed with PBS and subjected to different...
Fluorescence Lifetime Images of Zebrafish

Zebrafishes were anesthetized in the PBS (pH=7.4) solution contained 0.125% Ethyl 3-aminobenzoate methanesulfonate. The fish was too big to fit in the view field of microscope, so the image was made up of three pieces. The experimental conditions for these three images were exactly the same procedure as the cells. The fish was too big to fit in the view field of microscope, so the image was made up of three pieces. The experimental conditions for these three images were exactly the same. All animal work was approved by the Animal Care Committee of Nanjing University in accordance with Institutional Animal Care and Use Committee guidelines.

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Notes and references


Fluorescent τ-probe was demonstrated to quantitate ultra-trace endogenous hydrogen sulfide (H$_2$S$_m$) within cells and zebrafish through changes on fluorescence lifetime.