**Supporting Information**

**Imaging of intracellular sulfane sulfur expression changes under hypoxia stress via a selenium-containing near-infrared fluorescent probe**

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1. General experimental section

**Instruments:** Absorption spectra were measured on NANO Drop 2000c UV-visible spectrophotometer (Thermo Fisher Scientific). Fluorescence spectra were obtained by a HORIBA Scientific FluoroMax-4 Spectrofluorometer with a Xenon lamp and 1.0-cm quartz cells. The pH measurements were performed 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). $^1$H NMR and $^{13}$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 (×60). High performance liquid chromatography (HPLC) analysis were performed in Agilent Technologies 1260 Infinity II. High-resolution mass spectral (HRMS) analyses were measured with Hybrid Ion Trap-Orbitrap Mass Spectrometer (LTQ Orbitrap XL, Thermo).

**Materials:** aza-BODIPY was synthesized in our laboratory according to previous report with some modification.$^1$ 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and HEPES were purchased from Sigma-Aldrich. Human neuroblastoma (SH-SY5Y) cells, mouse macrophage (RAW 264.7) cells, human lung carcinoma (A549) cells, human cervical carcinoma (Hela) cells, Human embryonic kidney 293 (HEK 293) cells, human hepatocellular liver carcinoma (HepG2) cells, human hepatocellular liver carcinoma (SMMC7721) cells were obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Monoclonal Antibody was obtained from Cell Signaling Technology. BD-diSeH (1.0 mM, 0.5 mL) was prepared in DMSO and stored at 4 °C 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.

**Absorption analysis:** Absorption spectra were obtained with 1.0-cm glass cells. The probe (DMSO, 0.1 mL, 1.0 mM) was added to a 10.0-mL color comparison tube. After dilution to 10.0 μM with 10 mM HEPES (containing 20% fetal bovine serum, v/v, pH 7.4), Na$_2$S$_4$ (20 μM) was added. The mixture was equilibrated for 5 min before measurement.

**Fluorescence analysis:** Fluorescence spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. The probe (DMSO, 0.1 mL, 1.0 mM) was added to a 10.0-mL color comparison tube. After dilution to 10.0 μM with 10 mM HEPES (containing 20% fetal bovine serum, v/v, pH 7.4), various concentration of Na$_2$S$_4$ (0-20 μM) was added. The mixture was equilibrated for 5 min before measurement.

**Western blot.** Cells were lysed using RIPA buffer supplemented with PMSF (Solarbio, China) and PHosSTOP (Roche, Germany) inhibitors for 30 min. Then the cells were scraped, sonicated, and centrifuged (20,000 × g at 4 °C) for 15 min. Protein concentrations in the supernatant were determined using the BCA Protein Assay kit (Biogot, China) and equal amounts of protein were electrophoresed on 6-12% SDS-polyacrylamide gels (Bio-Rad, USA) and transferred to PVDF membranes (Millipore, Germany). Then the membranes were blocked with 7% milk and blotted with primary antibodies overnight at 4°C. A horseradish peroxidase (HRP)-conjugated secondary antibody was used to quantify protein and an enhance chemiluminescence (ECL) detection system was used to detect the signals.

**Visualization of sulfane sulfur in mice.** BALB/c mice, 20-25 g, were obtained from Binzhou Medical University. Mice were group-housed on a 12:12 light–dark cycle at 22 °C with free access to food and water. Images were taken by Perkinelmer/IVIS Lumina XRMS Series III. All surgical procedures were conducted in conformity with National Guidelines for the Care and Use of Laboratory Animals, and experimental protocols were approved by the Institutional Animal Care and Use Committee in Binzhou Medical University, Yantai, China. Approval Number: No.BZ2014-102R.

2. Preparation of analytes
Stock solutions of cysteine (Cys), homocysteine (Hcy), glutathione (GSH), cystine (CysSSCys), GSSG and NaHS were prepared in distilled water (2 mM, respectively). The stock solution of S$_8$ (200 mM) was prepared in CH$_2$Cl$_2$, and then used EtOH to dilute this solution, to get a 10 mM stock solution of S$_8$ in EtOH. The stock solution was diluted to desired concentrations when needed. Na$_2$S$_2$ and Cys-polysulfide were prepared according to reported procedures.$^{1-4}$ A heterogeneous mixture of Na (25 mg-atom) and S$_8$ (25 mg-atom) in 10 mL anhydrous 1,2-dimethoxyethane under Ar atmosphere. The heterogeneous mixture could immediately turn dark brown at room temperature. However, the reaction needs to be continued for 3 h at 70 °C to give anhydrous Na$_2$S$_2$. Filtrated then washed by ether to yield a yellow solid 30 mg, 60%. Na$_2$S$_4$ was also synthesized with Na (25 mg-atom) and S$_8$ (50 mg-atom) via the same method (yield 15%). Then the products were derived by benzyl chloride to convert sodium oligosulfide into dibenzyl oligosulfane. The Na$_2$S$_2$ (Na$_2$S$_4$) derived by benzyl chloride to convert Na$_2$S$_2$ into PhCH$_2$S$_2$CH$_2$Ph (PhCH$_2$S$_4$CH$_2$Ph). After derived the samples by benzyl chloride, the purities of our products were up to 99.91% by GC. Retention time of PhCH$_2$S$_2$CH$_2$Ph and PhCH$_2$S$_4$CH$_2$Ph were at 12.1 min and 12.8 min, respectively. PhCH$_2$S$_2$CH$_2$Ph $^1$H NMR (500 MHz, DMSO-D$_6$) δ(ppm): 7.43-7.37 (m, 4H), 7.33-7.20 (m, 6H), 3.81 (S, 4H). GC-MS (ESI$^+$): m/z C$_{14}$H$_{14}$S$_2$ calcd. 246.0537, found [M$^+$] 246.0535. PhCH$_2$S$_4$CH$_2$Ph $^1$H NMR (500 MHz, DMSO-D$_6$) δ(ppm): 7.42-7.35 (m, 4H), 7.33-7.21 (m, 6H), 3.82 (S, 4H). GC-MS (ESI$^+$): m/z C$_{14}$H$_{14}$S$_4$ calcd. 309.9978, found [M$^+$] 309.9978.

3. Characterization of the reaction between BD-diSeH and sulfane sulfur

A solution of BD-diSeH (0.1 mmol) was treated with Na$_2$S$_4$ (0.2 mmol) in 10 mM HEPES (containing 20% fetal bovine serum, v/v, pH 7.4). The mixture was stirred for 30 min at 37 °C. Then solvent was extracted by CH$_2$Cl$_2$ and washed the organic phase with H$_2$O, the organic layer evaporated to dryness. The resulted residue was purified by column chromatography. Compound BODIPY was obtained as a blue solid. The formation of BODIPY was confirmed by HRMS. LC-MS (ESI$^+$): m/z C$_{32}$H$_{22}$BF$_2$N$_3$O$_2$ calcd. 529.1773, found [M-H$^-$] 528.1695.
4. Fluorescence quantum yield of BD-\textit{di}SeH

The fluorescence quantum yield of BD-\textit{di}SeH was calculated according to the following formula:

\[
\varphi_u = \frac{(\varphi_s)(FA_u)(A_s)(\lambda_{exs})(\eta^2)}{(FA_s)(A_u)(\lambda_{exs})(\eta^2)}
\]

Where \(\varphi\) is fluorescence quantum yield; the subscripts \(u\) and \(s\) refer to the unknown and the standard, respectively; \(F\) is integrated fluorescence intensity under the corrected emission spectra; \(A\) is the absorbance at the excitation wavelength; \(\eta\) is the refractive index of the solution.\(^5\) We used \(\text{tert-}\text{tert-butyl-29}H, \text{31}H\)-phtalocyanine as a standard (quantum yield of 0.44).\(^6\) The quantum yield of BD-\textit{di}SeH was determined to be 0.002.

5. Effect of pH values

Standard fluorescence pH titrations were performed in the solution at a probe concentration of 10 \(\mu\text{M}\). As shown in Fig. S1, the probe itself and the reaction product almost had no effect on the fluorescence by the pH of mediums within the range from 2.0 to 7.4. These results indicated that the probe could work under physiological conditions.
Fig. S1. Effect of pH on the fluorescent intensity of BD-\textit{di}SeH (10.0 μM) and its metabolite BODIPY (10.0 μM) in 10 mM HEPES. pH values: 2.0, 3.0, 4.0, 5.0, 6.0, 6.4, 6.8, 7.0, 7.2, 7.4, 7.6, 8.0, 9.0, 10.0. Excitation wavelengths for BD-\textit{di}SeH and its metabolite were 702 nm and 707 nm, respectively.

6. MTT assay

Prior to the bioimaging of sulfane sulfur in living cells, MTT assays were performed to evaluate the cytotoxicity of BD-\textit{di}SeH towards A549 cells with probe concentrations from 2 μM to 30 μM. A549 cells were cultured in RPMI 1640 Medium supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were planted into 96-well cell culture plate at 4000 /well, cultured at 37 °C and 5% CO₂ for 48 h, and then different concentrations of BD-\textit{di}SeH (0, 2.0, 5.0, 10.0, 20.0 and 30 μM, final concentration) were added to the wells and the cultures were maintained for 24 h at 37 °C and 5% CO₂. Subsequently, 20 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C under 5% CO₂. After 4 h incubation, the remaining MTT was removed. DMSO was added to each well to dissolve the formazan crystals. Finally, the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (Tecan, Austria). The result showed BD-\textit{di}SeH exhibited relatively low toxicity towards cell cultures under experimental conditions.
7. **Photostability of BD-diSeH**

Photostability of fluorescent probe is a key factor in practical applications. Thus, we evaluated the photostability of probe using a time-dependent fluorescence measurement. The solution of probe and sulfane sulfur was excited continuously at 635 nm for 600 s, and fluorescence intensity was measured every 30 s. The results showed that stable fluorescence intensity was observed, indicating BD-diSeH was stable and could be applied for long-time cells imaging.

8. **Imaging sulfane sulfur in different cell lines**
Fig. S4. Confocal microscopy images and flow cytometry analysis of various cell lines for imaging sulfane sulfur using BD-diSeH (1 μM), including HEK 293 cells (a), SH-SY5Y cells (b), Hela cells (c), SMMC7721 cells (d), HepG2 cells (e) and RAW 264.7 cells (f). The cells in each group: the left cells were as controls, and the right cells were treated with Na$_2$S$_4$ (1 μM) for 15 min at 37 °C. All cells preincubated with BD-diSeH for 15 min and washed with PBS. Images displayed represent emission intensities collected in optical windows between 680 and 780 nm upon excitation at 635 nm for BD-diSeH.

9. Bright-Field images for Fig. 3, 4, and 6a.

Fig. S5. Bright-field images of cells in Fig. 2 (a) – (g), respectively.
**Fig. S6.** Bright-field images of Fig. 3.

**Fig. S7.** Bright-field images of cells in Fig. 4 under different level of oxygen concentration (20%, 10%, 5%, 1%, and 0.1%).

**Fig. S8.** Bright-field images of Fig. 6a.

10. **Quantitative of proteins in Fig. 6c**
**Fig. S9.** The relative expression level of HIF-1α in Fig. 6c was quantified. Data are presented as means ± SD (n = 5).

**11. Stability tests for BD-diSeH**

The selenol (-SeH) can be oxidized to form diselenide (-Se-Se-). However, the oxidation reaction requires proper conditions, and it depends on its reaction thermodynamics and kinetics. For examples, the -SeH can be oxidized to form -Se-Se- by sodium perborate at room temperature.\(^7\) Or the –Se\(^-\) can be oxidized to form -Se-Se- by bromine at 25 °C.\(^8\) The other oxidation way to diselenides is by a solution of pyridine, water, benzoic acid, and trichloroisocyanuric acid in acetonitrile and methylene chloride.\(^9\) That is, the oxidation of –SeH in air is not fast. Therefore, the selenol is difficult to be oxidized in the air at room temperature. In order to further avoid the oxidation reaction of selenol, our design strategy for the probe involves the electron-withdrawing bridge between the response moiety and fluorophore to govern the reactivity and stability of our probe. Moreover, the fluorophore BODIPY is also considered to be an electron withdrawing group for selenol. Electron withdrawing properties can reduce the electron density of benzenselenol, which will prevent the benzenselenol moieties (-SeH) to be further oxidized. Therefore, although the probe, BD-diSeH, contains two benzenselenol moieties (-SeH), the selenol (-SeH) does not exhibit a rapidly oxidation in air to form diselenide (-Se-Se-).
Fig. S10. The stability test of BD-diSeH. a) and b): The changes of absorbance and fluorescence response of BD-diSeH stored in solution. A dosage of BD-diSeH was dissolved in DMSO as stock solution (1 mM). The stock solution were protected from light at 20°C for 0, 1, 2, 3, 5, 7 days. Then the solution was diluted to 10 μM for absorbance detection. Subsequently, added Na₂S₄ (20 μM) to the tested probe solution (10 μM) to further detection of the fluorescence changes. c) and d): The changes of absorbance and fluorescence response of BD-diSeH solid exposed to the air for 0, 1, 2, 3, 5, 7 days. Then the solids were dissolved in DMSO to form six stock solutions (1 mM) and diluted to 10 μM for absorbance detection. Then added Na₂S₄ (20 μM) to the tested probe solution (10 μM) to further detection of the fluorescence changes. All spectra were acquired in 10 mM HEPES (20% fetal bovine serum, v/v, pH 7.4).

In order to comprehensively evaluate the stability of BD-diSeH, we performed experiments by investigating the spectral properties of BD-diSeH and high performance liquid chromatography (HPLC) analysis. 1) Assessment the stability of the BD-diSeH in solution. The probe BD-diSeH was dissolved in DMSO as stock solution (1 mM) and protected from light at 20°C for 0, 1, 2, 3, 5, 7 days. The probe was diluted to 10 μM with 10 mM HEPES for detecting the absorbance changes. Then added Na₂S₄ (20 μM) to the tested probe solution (10 μM) to further detection of the fluorescence changes. As shown in Fig. S10a and S10b, no obvious interference on absorption and fluorescence spectra were observed. The purity was also analyzed by HPLC as ≥ 99% on the 7th day (Fig. S11). 2) Evaluation the stability of the BD-diSeH solid. The solid of BD-diSeH was exposed in
the air for 0, 1, 2, 3, 5, 7 days from light at 20 °C. And then the solid was dissolved in DMSO as 1 mM stock solutions. Then the stock solutions were diluted to 10 μM with 10 mM HEPES for tests, respectively. Then added Na$_2$S$_4$ (20 μM) to the tested probe solution (10 μM) to further detection of the fluorescence changes. As displayed in Fig. S10c and S10d, the fluorescence spectra showed a decrease in fluorescence response from the 3nd day to the 7th day. The result indicated that the solid of our probe could be oxidized when exposed to air. But the solid could not be severe oxidized within 48 h. Furthermore, the photoability of BD-diSeH was also investigated in cells (Fig. S3). In general, our probe was stable for the detection of sulfane sulfur under the given experimental conditions.

![HPLC tests for the purity of BD-diSeH in stock solutions in Fig. S10. Method: a ZORBAX Eclipse Plus C18 (4.6 mm × 150 mm, 5 μm) column and mobile phase of methanol-water (70:30) were used. The flow rate was 1 mL/min. the detection wavelength was at 310 nm. The detection temperature was 25 °C.](image)

**Fig. S11.** HPLC tests for the purity of BD-diSeH in stock solutions in Fig. S10. Method: a ZORBAX Eclipse Plus C18 (4.6 mm × 150 mm, 5 μm) column and mobile phase of methanol-water (70:30) were used. The flow rate was 1 mL/min. the detection wavelength was at 310 nm. The detection temperature was 25 °C.

12. **Selectivity of BD-diSeH for ROS, metal ions and amino acids**

We examined the selectivity of BD-diSeH for various biological relevant reactive oxygen species. As shown in Fig. S12, in contrast to strong fluorescent intensity induced by Na$_2$S$_4$, BD-diSeH showed a negligible fluorescent response upon addition of same concentration of biological relevant reactive oxygen species including O$_2$$^{•−}$, ‘OH, H$_2$O$_2$, methyl linoleate hydroperoxide (MeLOOH), cumene hydroperoxide (CuOOH), ONOO$^{−}$, NO and tert-butyl hydroperoxide (t-BuOOH). These results showed that our probe exhibited high selectivity for sulfane sulfur over relevant reactive oxygen species at physiological concentrations.
Fig. S12. Fluorescence responses of BD-diSeH to reactive oxygen species. (1) $O_2^-$ (20 μM), (2) $\cdot OH$ (20 μM), (3) $H_2O_2$ (20 μM), (4) methyl linoleate hydroperoxide (MeLOOH, 20 μM), (5) cumene hydroperoxide (CuOOH, 20 μM), (6) ONOO$^-$ (20 μM), (7) NO (20 μM), (8) tert-butyl hydroperoxide ($t$-BuOOH, 20 μM), (9) Na$_2$S$_4$ (20 μM). Data were obtained in 10 mM HEPES buffer (20% fetal bovine serum, v/v, pH 7.4) at 37 °C for 15 min. $\lambda_{ex} = 707$ nm, $\lambda_{em} = 737$ nm.

We then examined the selectivity of BD-diSeH for various important biological relative metal ions and amino acids. BD-diSeH (10 μM) was treated with various analytes in HEPES buffer. As shown in Figure S13, the $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ did not induce any fluorescent enhancement. BD-diSeH showed a negligible fluorescent response upon addition of biological amino acid including alanine, glutamine, glycine, histidine. Therefore, these results showed high selectivity of sulfane sulfur over important biological relative metal ions and amino acid.

Fig. S13. Fluorescence responses of BD-diSeH to metal ions and amino acids. (1) $K^+$ (1 mM), (2) $Na^+$ (1 mM), (3) $Ca^{2+}$ (1 mM), (4) $Mg^{2+}$ (1 mM), (5) $Zn^{2+}$ (1 mM), (6) $Cu^{2+}$ (1 mM), (7) alanine (200 μM), (8) glutamine (200 μM), (9) glycine (200 μM), (10) histidine (200 μM), (11) Na$_2$S$_4$ (20 μM). Data were obtained in 10 mM HEPES buffer (20% fetal bovine serum, v/v, pH 7.4) at 37 °C for 15 min. $\lambda_{ex} = 707$ nm, $\lambda_{em} = 737$ nm.
13. Response ability of BD-\textit{diSeH} towards sulfane sulfur

To investigate whether the response ability of BD-\textit{diSeH} towards sulfane sulfur was effected by pH, Na$_2$S$_4$ (20 μM) was added to BD-\textit{diSeH} (10 μM) at pH 10.0, the fluorescent intensity was then determined (a). Subsequently, the pH of solution adjusted to 7.4, and then fluorescent intensity was determined (b). As shown in Figure S14, the fluorescent intensity was increased after the pH of solution adjusted to 7.4, which was consistent with that of the reaction under pH 7.4 (c). The results indicated that pH values did not affect the response ability of BD-\textit{diSeH} towards sulfane sulfur.

![Figure S14](image)

**Fig. S14.** Fluorescent intensity of BD-\textit{diSeH} (10.0 μM) towards sulfane sulfur (20.0 μM) in 10 mM HEPES under different pH values. (a) Na$_2$S$_4$ (20 μM) was added to BD-\textit{diSeH} (10 μM) at pH 10.0. (b) Na$_2$S$_4$ (20 μM) was added to BD-\textit{diSeH} (10 μM) at pH 10.0, and then adjusted the solution to pH 7.4. (c) Na$_2$S$_4$ (20 μM) was added to BD-\textit{diSeH} (10 μM) at pH 7.4.

14. The characteristics of the previously published probes for sulfane sulfur detection

<table>
<thead>
<tr>
<th>Fluorescent probe</th>
<th>$\lambda_{ex}$</th>
<th>$\lambda_{em}$</th>
<th>Limit of detection</th>
<th>Response time</th>
<th>Selectivity</th>
<th>Application (In the cells)</th>
<th>Application (In vivo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-\textit{diSeH}</td>
<td>707 nm</td>
<td>737 nm</td>
<td>2.3 nM</td>
<td>80 s</td>
<td>High</td>
<td>A549 cells, HeK 293 cells, SH-SYS cells, HepG2 cells, RAW 264 7 cells, HeLa cells, SMMC-7721 cells</td>
<td>Imaging sulfane sulfur in mice and in zebrafish under hypoxic condition</td>
</tr>
<tr>
<td>SSP1</td>
<td>380 nm</td>
<td>458 nm</td>
<td>73 nM</td>
<td>10 min</td>
<td>High</td>
<td>H9c2 cells, HeLa cells</td>
<td>No</td>
</tr>
<tr>
<td>SSP2</td>
<td>482 nm</td>
<td>518 nm</td>
<td>32 nM</td>
<td>10 min</td>
<td>High</td>
<td>H9c2 cells, HeLa cells</td>
<td>No</td>
</tr>
<tr>
<td>Mito-\textit{SeH}</td>
<td>680 nm</td>
<td>720 nm</td>
<td>3.1 nM</td>
<td>100 s</td>
<td>High</td>
<td>RAW 264 7 cells, A549 cells, HeK cells, HeLa cells, RH-35 cells, SMMC-7721 cells</td>
<td>Imaging sulfane sulfur in mice</td>
</tr>
<tr>
<td>BD-\textit{diSH}</td>
<td>710 nm</td>
<td>736 nm</td>
<td>10 nM</td>
<td>2 min</td>
<td>High</td>
<td>SH-SYS cells</td>
<td>Imaging sulfane sulfur in mice</td>
</tr>
</tbody>
</table>
15. Identification of the product from the reaction by HPLC and HRMS

To characterization of the product from the reaction between BD-diSeH and sulfane sulfur, a solution of BD-diSeH (0.1 mM) treated with Na₂S₄ (0.2 mM) in 10 mM HEPES (containing 20% fetal bovine serum, v/v, pH 7.4) was performed. The mixture was stirred for 30 min at 37 °C. Then the reaction product was identified by HPLC and HRMS. LC-MS (ESI⁻): m/z C₃₂H₂₂BF₂N₃O₂ calcd. 529.1773, found [M+H]⁺ 530.1843.

Fig. S15. HPLC test for confirming the product from the reaction between probe and sulfane sulfur. Method: a ZORBAX Eclipse Plus C18 (4.6 mm× 150 mm, 5 μm) column and mobile phase of methanol-water (70:30) were used. The flow rate was 1 mL/min. the detection wavelength was at 310 nm. The detection temperature was 25 °C.
16. $^1$H NMR and $^{13}$C NMR for BD-$d$/SeH
17. Reference


