Supporting Information

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SI Materials and Methods

All reactions using air- or moisture-sensitive reagents were performed in dried glassware under an atmosphere of dry N_2. Other reagents were used without further purification. Silica gel P60 (SiliCycle) was used for column chromatography, and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical TLC and visualized by fluorescence quenching under UV light or by staining with iodine. All other reagents were purchased from Sigma-Aldrich. ^{1}H NMR and ^{13}C NMR spectra for characterization of new compounds were collected in $CDCl_3$ (Cambridge Isotope Laboratories) at 25 °C on a Bruker AVQ-400 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard notation of parts per million using the peak of residual proton signals of $CDCl_3$ or CD_3OD as an internal reference. Splitting patterns are indicated as follows: br, broad; d, doublet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet; q, quartet; s, singlet; t, triplet. Low-resolution mass spectral analyses were carried out using a liquid chromatography mass spectrometer (LC-MS) (Agilent Technology 6130, Quadrupole LC/MS). High-resolution electrospray ionization mass spectral (HRMS-ESI) analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Carboxy Rhodamines 1,2 (3′,6′-Diamino-3-Oxo-3*H*-Spiro[Isobenzofuran-1,9′-Xanthene]-5-Carboxylic Acid, 3′,6′-Diamino-3-oxo-3*H*-Spiro[Isobenzofuran-1,9′-Xanthene]-6-Carboxylic Acid). Aminophenol (3.88 g, 35.6 mmol, 2.08 equiv) was dissolved in 35 mL H_2SO_4 in a sealable pressure flask. Trimellitic anhydride (3.28 g, 17.1 mmol, 1.00 equiv) was added, and the flask was closed and heated at 185 °C for 6 h. After cooling to room temperature (rt), the reaction mixture was poured into 300 mL 1:1 CH_2Cl_2–CH_3CN while stirring at 0 °C. A red precipitate formed, which was filtered using a Buchner funnel. The red–brown solids were washed with 3×50 mL CH_3CN and dried to yield 6.5 g of material. The solid material was dissolved in 300 mL MeOH and sonicated for 2–3 min. The suspension was filtered, and the filter cake was washed with 3×50 mL MeOH, carefully crushing the solids with a spatula. The filtrate was concentrated to yield 2.90 g of a red solid that contained a mixture of the 5′ and 6′ carboxy rhodamine 1 and sulfated impurities. This material was used in the next step without further purification. A spectroscopically pure sample of the 5′ isomer was obtained by preparative HPLC: 5′-carboxy rhodamine, ^{1}H NMR (400 MHz, d6-DMSO) δ 8.58 (s, 1H), 8.13 (d, 2H, *J* = 8.0 Hz), 7.23 (d, 1H, *J* = 8.0 Hz), 7.21 (s, 1H), 7.19 (s, 1H), and 6.80 (m, 4H).

SF5, SF6 (3′,6′-Diazido-3-Oxo-3*H*-Spiro[Isobenzofuran-1,9′-Xanthene]-5-Carboxylic Acid, 3′,6′-Diazido-3-Oxo-3*H*-Spiro[Isobenzofuran-1,9′-Xanthene]-6-Carboxylic Acid). Crude carboxy rhodamine **1** (2.90 g, 7.75 mmol, 1 equiv) was dissolved in 150 mL 2:1 CH_3CN:H_2O and cooled to 0 °C. Trifluoroacetic acid (1.3 mL, 17.0 mmol, 2.19 equiv) and sodium nitrite (1.32 g, 19.1 mmol, 2.46 equiv) were added and the reaction was stirred at 0 °C for 10 min. Sodium azide (1.19 g, 18.3 mmol, 2.36 equiv) was added and the reaction was warmed to rt and allowed to stir for 60 min. The reaction was poured into 200 mL H_2O and extracted with 3×200 mL ethyl acetate (EtOAc). The combined organic extracts were washed with 100 mL brine, dried over Na_2SO_4, filtered and concentrated to yield a mixture of Sulfidefluor-5 (SF5) and SF6 (355.8 mg, 5% over two steps), which was used in the next step without further purification. Analytically pure samples and separation of isomers for spectroscopic characterization were obtained by preparative HPLC. SF5, ^{1}H NMR (400 MHz, 1:1 $CDCl_3$–CD_3OD) δ 8.66 (s, 1H), 8.36 (d, 1H, *J* = 8.0 Hz), 7.23 (d, 1H, *J* = 8.0 Hz), 6.96 (b rs, 2H), 6.76 (m, 4H); ^{13}C NMR (100 MHz, 1:1 $CDCl_3$:CD_3OD) δ 168.73, 166.71, 156.13, 151.78, 143.11, 136.69, 133.59, 129.35, 126.89, 126.39, 123.94, 115.27, 114.51, 107.23, and 82.29; HRMS–ESI calculated for $C_{21}H_9N_6O_5$ (M–H$^+$), 425.0640; found, 425.0627.

SF5-AM (Acetoxymethyl 3′,6′-Diazido-3-oxo-3*H*-Spiro[Isobenzofuran-1,9′-Xanthene]-5-Carboxylate). A mixture of SF5 and SF6 (27.4 mg, 0.0643 mmol, 1.0 equiv) was dissolved in 0.65 mL anhydrous *N*,*N*-dimethylformamide (DMF). Bromomethyl acetate (0.026 mL, 0.27 mmol, 4.1 equiv) and *N*,*N*-diisopropylethylamine (DIPEA) (0.040 mL, 0.23 mmol, 3.6 equiv) were added, and the reaction was stirred for 23 h. The reaction mixture was then concentrated and purified by preparative TLC (2:1 Hexanes–EtOAc) to yield Sulfidefluor-5 acetoxymethyl ester (SF5-AM) (11.0 mg, 34%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (s, 1H), 8.38 (d, 2H, *J* = 8 Hz), 7.24 (d, 2H, *J* = 8 Hz), 6.98 (s, 2H), 6.76 (dd, 4H, *J* = 8 Hz, *J* = 12 Hz), 6.05 (s, 2H), and 2.18 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.6, 167.9, 163.7, 157.1, 151.8, 143.2, 136.6, 131.6, 129.4, 127.6, 126.7, 124.3, 115.4, 114.6, 107.4, 81.8, 80.1, and 20.6; HRMS–ESI calculated for $C_{24}H_{15}N_6O_7$ (M+H$^+$), 499.0997; found, 499.1010.

Carboxamide 3 (3′,6′-Diamino-3-Oxo-3*H*-Spiro[Isobenzofuran-1,9′-Xanthene]-6-Carboxylic Acid). A mixture of SF5 and SF6 (355.8 mg, 0.835 mmol, 1.0 equiv) was dissolved in 8.4 mL anhydrous DMF. *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) (383.5 mg, 1.01 mmol, 1.2 equiv) and DIPEA (0.44 mL, 2.53 mmol, 3.0 equiv) were added, and the reaction was stirred for 5 min. Di-*tert*-butyl iminodiacetate (249.2 mg, 1.016 mmol, 1.2 equiv) was added, and the reaction was stirred for 3 h. The reaction mixture was then poured into 20 mL sat aq NH_4Cl and extracted with 3×20 mL EtOAc. The combined organic layers were washed with 20 mL brine, dried over Na_2SO_4, filtered, and concentrated. The crude material was purified by silica column chromatography (5:1 Hexanes–EtOAc) to yield carboxamide 3 (71.2 mg, 13%) as a single isomer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, 1H), 7.81 (d, 1H, *J* = 7.7 Hz), 7.19 (d, 1H, *J* = 7.7 Hz), 6.97 (s, 2H), 6.77 (m, 4H), 4.25 (s, 2H), 4.02 (s, 2H), 1.52 (s, 9H), and 1.49 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 167.8, 167.7, 154.3, 151.8, 143.1, 137.4, 134.4, 129.4, 126.3, 124.3, 123.7, 115.3, 114.9, 107.3, 83.3, 82.4, 81.7, 52.7, 49.3, 28.1, and 28.0; LRMS–ESI calculated for $C_{33}H_{32}N_7O_8$ (M+H$^+$), 654.2312; found, 654.3.

SF7 (2,2′-((3′,6′–Diazido-3-Oxo-3*H*-Spiro[Isobenzofuran-1,9′-Xanthene]-5-Ylcarbonyl)Azanediyl)Diacetic Acid). Carboxamide 3 (60.0 mg, 0.0919 mmol, 1 equiv) was dissolved in 1 mL 1:1 CH_2Cl_2–trifluoroacetic acid (TFA) and stirred for 3 h. Diethyl ether was then added, resulting in a red precipitate, which was filtered and washed with diethyl ether. The solids were redissolved in MeOH, washed through the filter, and concentrated to yield SF7 (46.5 mg, 93%). A spectroscopically pure sample was obtained by preparative HPLC. ^{1}H NMR (400 MHz, CD_3OD) δ 8.09 (s, 1H), 7.85 (d, 1H, *J* = 8.0 Hz), 7.35 (d, 1H, *J* = 8.0 Hz), 7.08 (s, 2H), 6.91 (d, 2H, *J* = 8.3 Hz), 6.87 (d, 2H, *J* = 8.3 Hz), 4.34 (s, 2H), and 4.21 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 170.02, 168.45, 168.01, 154.05, 151.82, 143.21, 137.59, 129.43, 126.79, 124.73, 123.19, 115.12, 114.95,

106.86, 84.10, 82.04, and 65.58; HRMS–ESI calculated for $C_{25}H_{16}N_7O_8$ (M+H$^+$), 542.1055; found, 542.1068.

SF7-AM (Bis(Acetoxymethyl) 2,2′-((3′,6′-Diazido-3-Oxo-3*H*-Spiro [Isobenzofuran-1,9′-Xanthen]-5-Ylcarbonyl)Azanediyl)Diacetate). SF7 (46.5 mg, 0.0859 mmol, 1.0 equiv) was dissolved in 0.9 mL anhydrous DMF. Bromomethyl acetate (0.080 mL, 0.82 mmol, 49.5 equiv) and DIPEA (0.15 mL, 0.86 mmol, 10 equiv) were added, and the reaction was stirred for 20 h. The reaction mixture was then concentrated and purified by preparative TLC (1:1 Hexanes–EtOAc) to yield SF7-AM (18.7 mg, 32%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.79 (d, 1H, J = 8.0 Hz), 7.22 (d, 1H, J = 8.0 Hz), 6.98 (d, 2H, J = 2.0 Hz), 6.82 (d, 2H, J = 8.5 Hz), 6.78 (dd, 2H, J = 8.5 Hz, J = 2.0 Hz), 5.84 (s, 2H), 5.80 (s, 2H), 4.41 (s, 2H), 4.23 (s, 2H), 2.17 (s, 3H), and 2.13 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.32, 169.51, 169.10, 167.73, 167.55, 167.44, 154.59, 151.71, 143.05, 136.37, 134.22, 129.44, 126.49, 124.56, 123.75, 115.34, 114.60, 107.31, 86.67, 81.79, 79.55, 51.46, 47.59, 20.66, and 20.55; HRMS–ESI calculated for $C_{31}H_{24}N_7O_{12}$ (M+H$^+$), 686.1477; found, 686.1499.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM Hepes buffer, pH 7.4. Fluorescence spectra were recorded on a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with an integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for emission measurements were contained in 1-cm × 0.1-cm quartz cuvettes (1.5 mL volume, Starna). Absorption spectra were recorded using a Varian Cary 50 spectrophotometer.

Quantum Yields and Extinction Coefficients. Quantum yields were determined using fluorescein as a standard according to a published method (1). For each compound and fluorescein, the absorbance spectra were measured within an absorbance range of 0.01–0.1. The quantum yield was calculated according to the equation $\Phi_{sample} = \Phi_{standard}\ (Grad_{sample}/Grad_{standard})(\eta_{sample}/\eta_{standard})$, where Φ is the quantum yield, $\Phi_{standard} = 0.95$ in 0.1 M NaOH, Grad is the slope of the plot of absorbance versus integrated emission intensity, and η is the refractive index of the solvent.

SF4, SF5, and SF7 Fluorescence Responses to H_2S. A 10 μM solution of SF4, SF5, or SF7 in 20 mM Hepes buffered to pH 7.4 was prepared from a 5 mM stock solution of SF4, SF5, or SF7 in DMF in a 1.5 mL microcentrifuge tube. Then, 10 μL of 10 mM stock solution of NaSH in degassed (by bubbling N_2 for ∼30 min) 20 mM Hepes buffered at pH 7.4 was added (for a final concentration of 100 μM), and the mixture was vortexed for 10 s and then transferred to a cuvette. Emission spectra (λ_{ex} = 488 nm, λ_{em} = 498–700 nm) were collected at 10, 20, 30, 40, 50, and 60 min. The spectrum at t = 0 was acquired from a 10 μM solution of SF4, SF5, and SF7 without the addition of NaSH.

Selectivity Tests. Selectivities for the SF4, SF5, and SF7 probes were measured by fluorescence responses (λ_{ex} = 488 nm, λ_{em} = 525 nm) at 0, 15, 30, 45, and 60 min. All assays were performed in 20 mM Hepes buffered to pH 7.4. Unless otherwise stated, stock solutions of selected RSS, RNS, and ROS were added to 10 μM SF4, SF5, or SF7 in Hepes with 0.2% DMF.

For H_2S, 10 μL of a 10 mM stock solution of NaSH in degassed Hepes was added to 990 μL probe solution. For glutathione and cysteine, 2 μL of 5 mM SF4, SF5, and SF7 in DMF was added to 998 μL 5 mM glutathione or 500 μM cysteine in Hepes. For lipoic acid, 10 μL of a 10 mM stock solution of lipoic acid in Hepes was added to 990 μL probe solution. For Na_2SO_3, 10 μL of a 10 mM stock solution of Na_2SO_3 in Hepes was added to 990 μL probe solution. For $Na_2S_2O_3$, 10 μL of a 10 mM stock solution of $Na_2S_2O_3$ in Hepes was added to 990 μL probe solution. For potassium thiocyanate (KSCN), 10 μL of a 10 mM stock solution of KSCN in Hepes was added to 990 μL probe solution. For *S*-nitrosoglutathione (2), 10 μL of a 10 mM stock solution of *S*-nitroso glutathione in Hepes was added to 990 μL probe solution. For $NaNO_2$, 10 μL of a 10 mM stock solution of $NaNO_2$ in Hepes was added to 990 μL probe solution. For NO, 5 μL of a 10 mM stock solution of Proli-NONOate in degassed (by bubbling N_2 for ∼30 min) 10 mM NaOH in Hepes was added to 995 μL of a degassed (by bubbling N_2 for ∼30 min) solution of 10 μM SF4, SF5, and SF7 in Hepes with 0.2% DMF. For H_2O_2, 10 μL of a 10 mM stock solution of H_2O_2 in Hepes was added to 990 μL probe solution. For O_2^-, 100 μL of a saturated solution of KO_2 in DMSO (∼1 mM) was added to 900 μL probe solution. For ^{t}BuOOH, 10 μL of a 10 mM stock solution of ^{t}BuOOH in Hepes was added to 990 μL probe solution. For HOCl, 10 μL of a 10 mM stock solution of HOCl in Hepes was added to 990 μL probe solution.

Reaction Time Course Experiments. A 5 μM solution of SF4, SF5-AM, or SF7-AM in 20 mM Hepes buffered to pH 7.4 was prepared from a 5 mM stock solution of SF4, SF5, or SF7 in DMF in a 1.5 mL microcentrifuge tube. Then, 10 μL of 100 mM stock solution of NaSH in degassed 20 mM Hepes buffered at pH 7.4 was added (for a final concentration of 1 mM), and the mixture was vortexed for 10 s, transferred to a cuvette, and sealed. Fluorescence response (λ_{ex} = 488 nm, λ_{em} = 525 nm) was monitored every minute for the first 20 min, then every 5–10 min until the reaction was complete.

Determination of Detection Limits. Solutions of 5 μM SF4, SF5-AM, or SF7-AM with 0.1% DMF were prepared in degassed 20 mM Hepes buffered to pH 7.4 and divided into 1 mL portions in 1.5 mL microcentrifuge tubes. Stock solutions of Na_2S in degassed Hepes were prepared (100, 50, 25, and 12.5 μM), and then 10 μL were added to the probe solutions for final concentrations of 1,000, 500, 250, and 125 nM Na_2S. The microcentrifuge tubes were sealed, vortexed for 10 s, and allowed to stand at 25 °C for 45 min. Each solution was then transferred to a cuvette and emission spectra (λ_{ex} = 488 nm, λ_{em} = 498–650 nm) collected. Single outliers for these small data sets were rejected according to Dixon's Q-test (3) (n = 4, Q_{crit} = 0.829). Lower detection limits were determined as the lowest concentration of Na_2S that produced a significantly different relative emission intensity compared with control ($P < 0.05$, n = 3–4, two-tailed unpaired Student t test).

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were obtained as a gift from the Netherlands. Cell culture complete media was prepared using 1% Roswell Park Memorial Institute (RPMI) medium, 20% (vol/vol) FBS (HyClone), 0.05 mg/mL endothelial growth supplement (ECGS, BD), and 28 μg/mL heparin. Media was used within 1 mo of preparation. Cells were passaged every 2–3 d and used between passages 2 and 17.

Confocal Imaging Experiments. Confocal fluorescence imaging studies were performed with a Zeiss laser scanning microscope 710 with a 40× water objective lens, with Zen 2009 software (Carl Zeiss). SF2, SF4, SF5-AM, and SF7-AM were excited using a 488 nm Argon laser, and emission collected using a META detector between 500 and 650 nm. Hoechst 33342 was excited with a 405 nm diode laser, and emission collected using a META detector between 450 and 500 nm. The cells were imaged at 37 °C and 5% CO_2 throughout the course of the experiment. All imaging experiments were performed in four-well or eight-well Lab-Tek II glass chamber slides (Thermo Scientific, Cat no. 155382 or

155409) coated with 0.2% gelatin for 1 h before seeding. Image analysis was performed using ImageJ (National Institute of Health) or Zen 2009 software (Carl Zeiss). For trapping and exogenous H_2S addition, images were quantified by using the mean pixel intensity after setting a common threshold for all images. For VEGF stimulation and inhibitor experiments, 8 × 2 μM z-stacks were collected, ensuring that all of the cellular fluorescence was included within the z-stacks. Images were quantified by performing a maximum intensity projection in Zen 2009 software (Carl Zeiss) and using the mean pixel intensity after setting a common threshold for all images.

Cell-Trapping Imaging Experiments. HUVECs were incubated with 5 μM SF2, 5 μM SF4, 2.5 μM SF5-AM, or 2.5 μM SF7-AM for 30 min at 37 °C and 5% CO_2. Four different fields of cells were then imaged. The media was replaced and cells were imaged at four fields at 5, 30, and 60 min after media exchange.

Exogenous H_2S. HUVECs were incubated with 5 μM SF4, 2.5 μM SF5-AM, or 2.5 μM SF7-AM for 30 min at 37 °C and 5% CO_2. The media was exchanged for SF5-AM and SF7-AM, and cells were incubated with 1, 5, and 25 μM NaSH. The media was not exchanged for SF4, and cells were incubated with 1, 5, 25, and 100 μM NaSH at 37 °C and 5% CO_2 for 30 min. Cells were then imaged at four different fields.

VEGF Stimulation and Inhibitor Experiments. HUVECs were incubated with 2.5 μM SF7-AM for 30 min at 37 °C and 5% CO_2. The media was exchanged, and cells were imaged at four different fields. Images were collected as 8 × 2 μm z-stacks, ensuring that all of the intracellular fluorescence was contained within the z-stacks. For VEGF stimulation, 2 μL of 10 μg/mL VEGF (Invitrogen, Cat no. PHC9394) in 0.1% BSA/H_2O was added directly to wells for a final concentration of 40 ng/mL per well. Cells were incubated on stage at 37 °C and 5% CO_2. After 30 min, the same fields of cells were again imaged by collecting 8 × 2 μm z-stacks, ensuring that all of the intracellular fluorescence was contained within the z-stacks. For inhibitor experiments, 100 μM DL-propargylglycine (PAG) (Sigma-Aldrich) and 1–5 μM diphenyleneiodonium chloride (DPI) (Cayman) were added 10 min before VEGF addition (directly after media exchange); 30 μM AAL-993 (Millipore) was added 40 min before VEGF stimulation (simultaneous with probe addition) and again added at 30 μM after media exchange; and 100 U/mL PEG-catalase was incubated 2–4 h before VEGF stimulation and was again added at 100 U/mL after media exchange. Peptides were added 30–60 min before VEGF stimulation, concurrent with or 30 min before addition of probe. Images were quantified by performing a maximum intensity projection in Zen software and using the mean pixel intensity after setting a common threshold for all images, and expressed as a ratio of the final fluorescence over the initial fluorescence. This method of imaging provided superior reproducibility compared with single z-stack images. Each well was imaged in four different positions, and the final–initial fluorescence ratio averaged to obtain a single replicate; single outliers for these small data sets were rejected according to Dixon's Q-test (3) ($n = 4$, $Q_{crit} = 0.829$).

ELISAs. HUVECs were plated 1 d in advance on 0.2% gelatin-coated 100 mm Petri dishes (2×10^6 cells/dish) and allowed to grow to ~90% confluency, and then serum-starved [0.1% FBS, –endothelial growth supplement (ECGS)] for 14–20 h. Cells were treated with inhibitors for the designated time. Briefly, stock solutions of chemical reagents (DMSO, 20 mM DPI in DMSO, 100 mM PAG in H_2O) were prediluted in HUVEC serum starvation media, vortexed to mix, and added back to cells. For scrambled control and gp91ds-tat peptides (5 mM in 150 mM saline with 10 mM acetic acid), stock solutions of peptides were diluted in media, pipetted gently to mix, and added to cells. HUVECs were then stimulated with 40 ng/mL VEGF for 10 min. Cells were washed twice with 10 mL cold PBS and lysed using lysis buffer no. 9 (R&D Systems) with cOmplete Mini protease inhibitor mixture (Roche). Cells were scraped from the plates and transferred to microcentrifuge tubes, which were shaken at 4 °C for 30–60 min, followed by centrifugation at 4 °C for 20 min at 16,100 × *g* using an Eppendorf 5415R microcentrifuge. Clarified lysates were stored at –80 °C for no more than 1 wk, then thawed on ice, and briefly centrifuged before use. ELISAs were performed using Human Total VEGF R2/KDR (Cat no. DYC1780) and Human Phospho-VEGF R2/KDR ELISA (Cat no. DYC1766) DuoSet IC kits from R&D Systems according to the manufacturer's instructions. Samples were assayed in triplicate. Endpoint readings were taken on a Molecular Devices SpectraMax M2 plate reader and analyzed using SoftMax Pro-5 software.

Immunoblotting. HUVECs grown in 100 mm dishes were washed 2× with cold PBS and then lysed using radioimmunoprecipitation assay (RIPA) lysis buffer with one tablet cOmplete, Mini Protease Inhibitor Mixture (Roche Applied Science). Lysates were clarified as described for ELISAs. Total protein was quantified by BCA assay (Pierce). Lysates were denatured in 4× Laemmli reducing buffer, and 15 μg total protein per well was loaded onto precast NuPAGE 1.0 mm 15-well 4–12% Bis·Tris gels (Invitrogen). Gels were run at 120 V for 2–3 h. Gels were equilibrated in semidry transfer buffer for 20 min, then transferred to PVDF membrane (Millipore) at 15 V for 35 min. Membranes were blocked in 4% (w/v) BSA in Tris buffered saline + Tween-20 (TBST) for cystathionine γ-lyase (CSE) and 5% nonfat dry milk (NFDM) in TBST for CBS for 1–2 h at rt. Immunoblotting was performed with 1:4,000 anti-CSE in 4% (wt/vol) BSA/TBST, 1:500 anti-CBS in 5% (wt/vol) NFDM/TBST, incubating overnight on a shaker at 2–8 °C. Membranes were then washed with 3× 5–10 min blocking buffer, and incubated for an additional 1–2 h with 1:5,000 secondary antibodies from Santa Cruz (goat anti-mouse IgG–HRP, Cat no. sc-2005; goat anti-rabbit IgG-HRP, Cat no. sc-2004). Blots were washed with 3× 5–10 min TBST and then visualized using Western Lightning Plus ECL reagents (Perkin-Elmer). Blots were stripped by incubating with Restore Western Blot Stripping Buffer (Thermo Scientific) for 15 min at rt with shaking. Membranes were washed 5× with TBST, then blocked for 60 min, and reprobed with 1:5,000 mouse anti-actin (Millipore, Cat no. MAB1501) in 1% BSA/TBST. Rabbit polyclonal antibody against CSE was a generous gift from Professor Solomon Snyder. Abnova CBS mouse monoclonal antibody (M01), clone 3E1, was purchased from Novus Biologicals (Cat no. H00000875-M01).

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2. Hart TW (1985) Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. *Tetrahedron Lett* 26(16):2013–2016.
3. Dean RB, Dixon WJ (1951) Simplified statistics for small numbers of observations. *Anal Chem* 23(4):636–638.

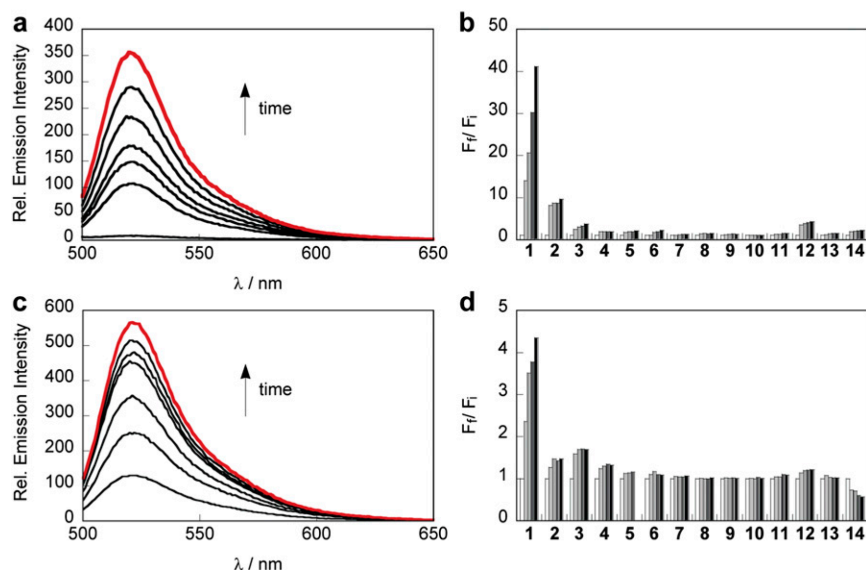


Fig. S1. Fluorescence responses of (*A*) 10 μM SF4 and (*C*) 10 μM SF5 to 100 μM NaSH. Data were acquired at 25 °C in 20 mM Hepes buffered to pH 7.4 with excitation at λ_{ex} = 488 nm. Emission was collected between 498 and 700 nm. Time points represent 0, 10, 20, 30, 40, 50, and 60 min (red trace) after addition of 100 μM NaSH. Fluorescence responses of (*B*) 10 μM SF4 and (*D*) 10 μM SF5 to biologically relevant reactive sulfur species (RSS), reactive oxygen species (ROS), and reactive nitrogen species (RNS). Bars represent relative responses at 525 nm at 0, 15, 30, 45, 60 min after addition of RSS, RNS, or ROS. Data shown are for 5 mM glutathione, 500 μM cysteine, and 100 μM for other RSS, RNS, and ROS. Data were acquired in 20 mM Hepes buffered at pH 7.4 with excitation at λ_{ex} = 488 nm. 1, NaSH; 2, glutathione; 3, cysteine; 4, lipoic acid; 5, Na_2SO_3; 6, $Na_2S_2O_3$; 7, KSCN; 8, S-nitroso glutathione; 9, $NaNO_2$; 10, NO; 11, H_2O_2; 12, O_2^-; 13, ^{t}BuOOH; 14, HOCl.

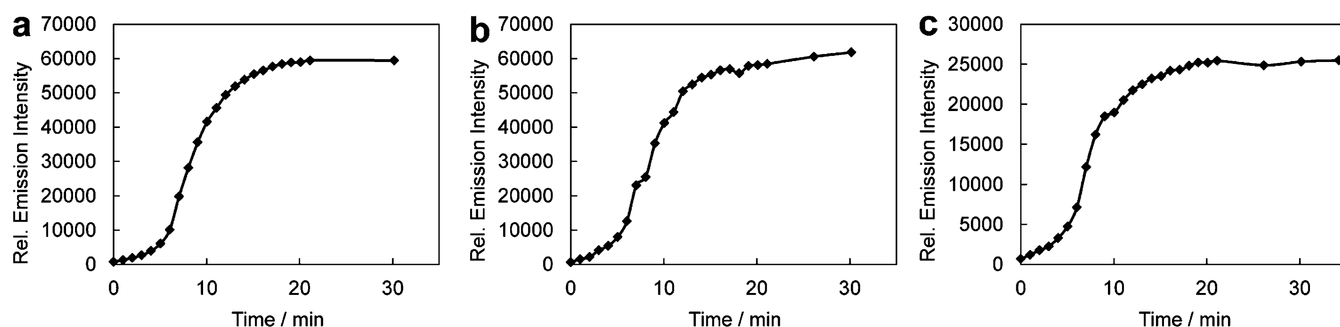


Fig. S2. Reaction time course of 5 μM (*A*) SF4, (*B*) SF5-AM, and (*C*) SF7-AM with 1 mM Na_2S at 25 °C in 20 mM Hepes buffered to pH 7.4 with excitation at λ_{ex} = 488 nm. Emission was collected at 525 nm.

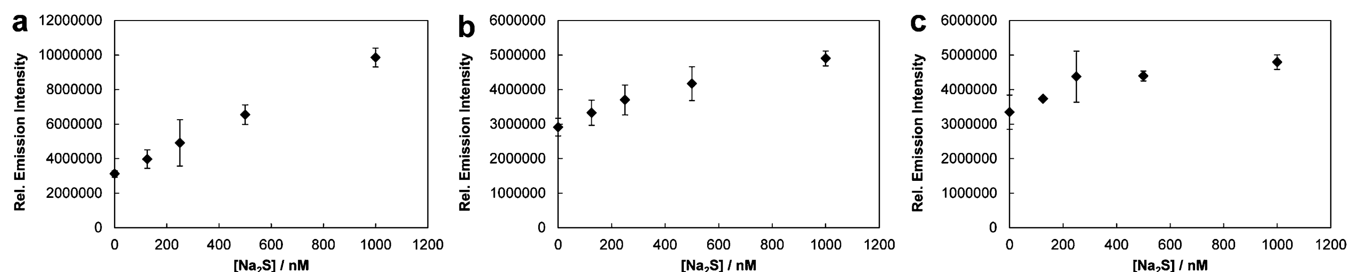


Fig. S3. Detection limits of 5 μM (*A*) SF4, (*B*) SF5-AM, and (*C*) SF7-AM after reaction with Na_2S for 45 min at 25 °C in 20 mM Hepes buffered to pH 7.4 with excitation at λ_{ex} = 488 nm. Emission was collected between 498 and 650 nm. Error bars are ± SD.

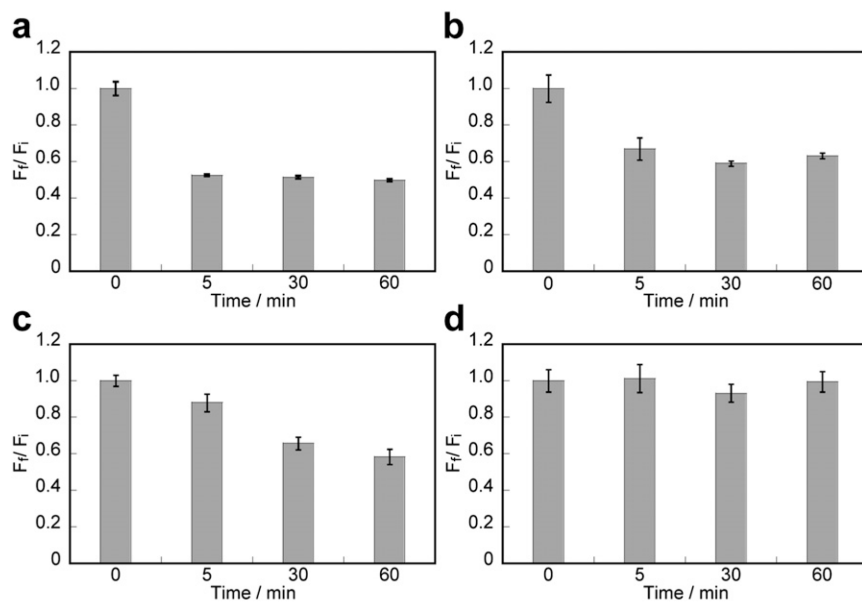


Fig. S4. Quantification of confocal fluorescence images showing uptake and retention of SF2, SF4, SF5-AM, and SF7-AM. HUVECs were loaded with (*A*) 5 μM SF2, (*B*) 5 μM SF4, (*C*) 2.5 μM SF5-AM, or (*D*) 2.5 μM SF7-AM for 30 min, then washed with complete media, and imaged before and at 5, 30, and 60 min after dye washing.

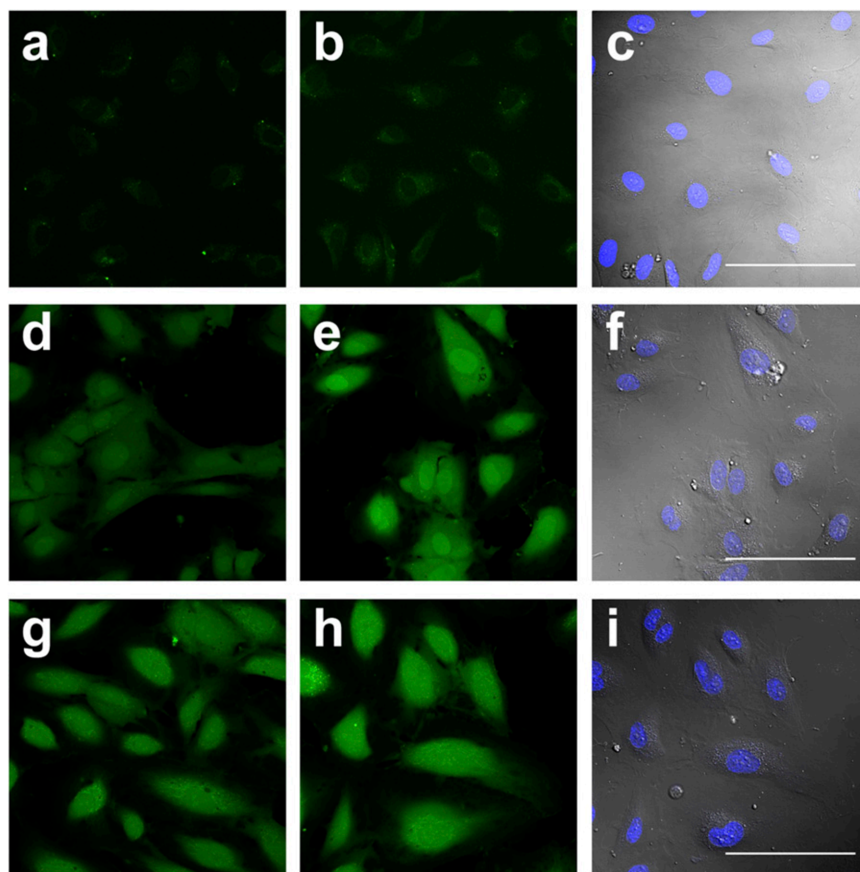


Fig. S5. Confocal images of H_2S detection in live HUVECs using SF4, SF5-AM, and SF7-AM. (*A*) HUVECs incubated with 5 μM SF4 for 30 min at 37 °C and then treated with H_2O as a vehicle control for 30 min at 37 °C. (*B*) HUVECs incubated with 5 μM SF4 for 30 min at 37 °C and then treated with 100 μM NaSH for 30 min at 37 °C. (*C*) Brightfield images of the same field of cells in (*B*) overlaid with images of 1 μM Hoechst stain at 37 °C. (*D*) HUVECs incubated with 5 μM SF5-AM for 30 min at 37 °C, washed, and then treated with H_2O as a vehicle control for 30 min at 37 °C. (*E*) HUVECs incubated with 5 μM SF5-AM for 30 min at 37 °C, washed, and then treated with 25 μM NaSH for 30 min at 37 °C. (*F*) Brightfield images of the same field of cells in *E* overlaid with images of 1 μM Hoechst stain at 37 °C. (*G*) HUVECs incubated with 5 μM SF7-AM for 30 min at 37 °C, washed, and then treated with H_2O as a vehicle control for 30 min at 37 °C. (*H*) HUVECs incubated with 5 μM SF7-AM for 30 min at 37 °C, washed, and then treated with 25 μM NaSH for 30 min at 37 °C. (*I*) Brightfield images of the same field of cells in *H* overlaid with images of 1 μM Hoechst stain at 37 °C. (Scale bar, 100 μm.)

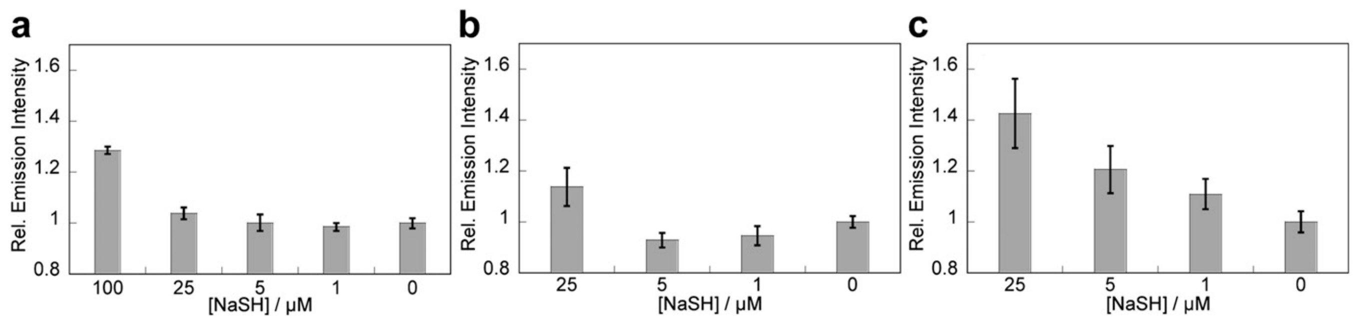


Fig. S6. Quantification of confocal images of H_2S detection in live HUVECs using SF7-AM. (*A*) HUVECs incubated with 5 μM SF4 for 30 min at 37 °C, and then treated with 100, 25, 5, 1, and 0 μM NaSH for 30 min at 37 °C without exchanging media. (*B*) HUVECs incubated with 5 μM SF5-AM for 30 min at 37 °C, washed, and then treated with 25, 5, 1, and 0 μM NaSH for 30 min at 37 °C. (*C*) HUVECs incubated with 5 μM SF7-AM for 30 min at 37 °C, washed, and then treated with 25, 5, 1, and 0 μM NaSH for 30 min at 37 °C. Error bars are ± SEM.

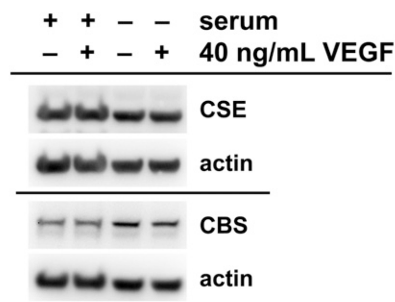


Fig. S7. Western blot on HUVEC lysates harvested after 40 ng/mL VEGF or vehicle stimulation for 30 min, with or without serum starvation (0.1% FBS, –ECGS) for 12–16 h. Actin was used as a loading control.

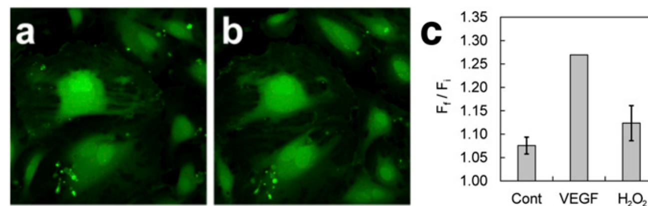


Fig. S8. Confocal images of H_2S detection in live HUVECs using SF7-AM. (*A*) HUVECs incubated with 2.5 μM SF7-AM for 30 min at 37 °C, washed, and imaged before (*A*) and after (*B*) treatment with 100 μM H_2O_2 for 30 min at 37 °C and 5% CO_2. (*C*) Quantification of images before and after treatment with H_2O_2 (n = 3) and comparison with data from Fig. 5*G*. Error bars are ± SEM.

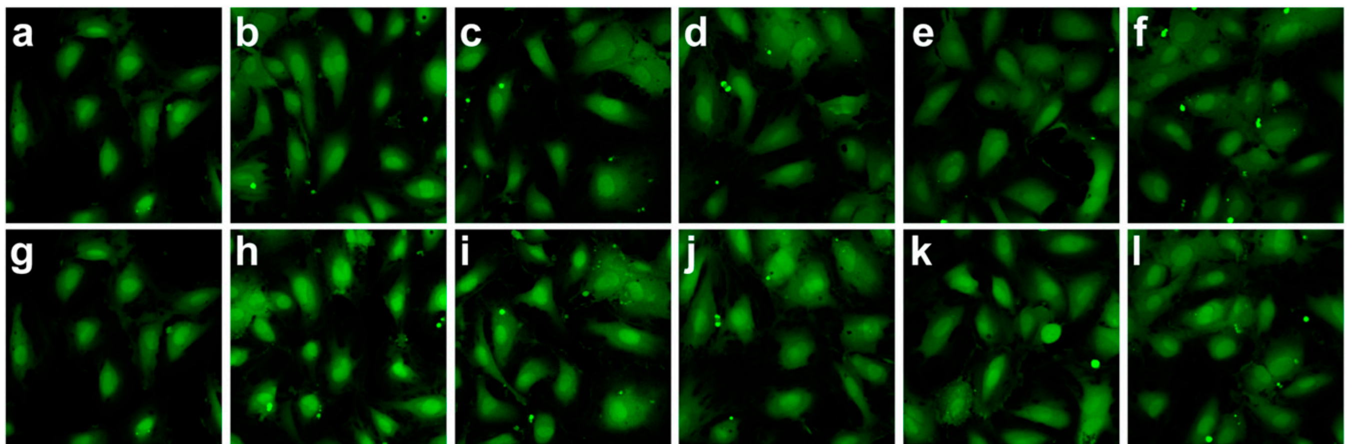
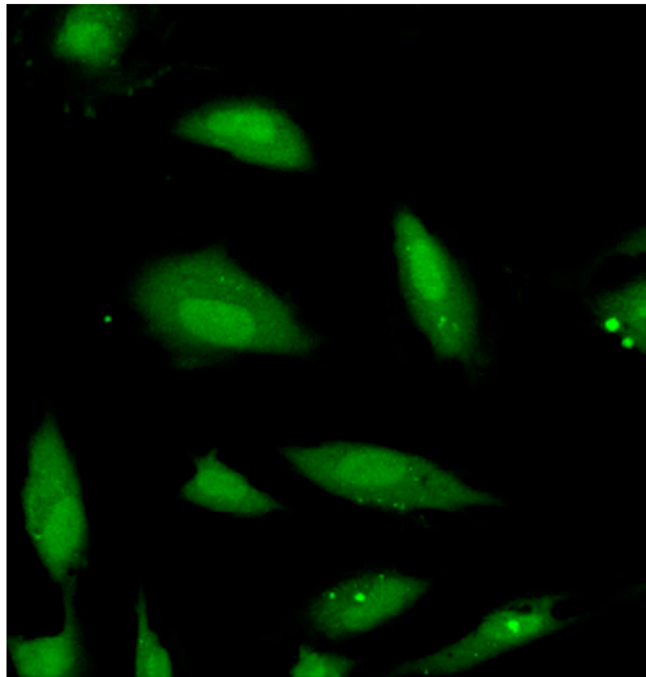
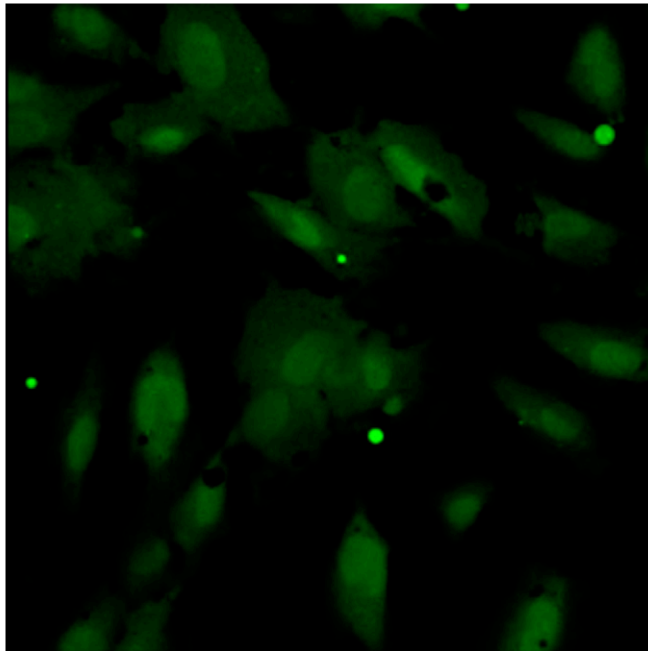


Fig. S9. Representative confocal fluorescence images of H_2S signaling in live HUVECs from Fig. 4 and Fig. 5. HUVECs were incubated with 2.5 μM SF7-AM, washed, and imaged before (*A*) and after (*G*) treatment with 0.1% BSA in H_2O as a vehicle control. HUVECs were incubated with 2.5 μM SF7-AM, washed, and imaged before (*B*) and after (*H*) treatment with 40 ng/mL VEGF. SF7-AM–labeled HUVECs pretreated with 30 μM AAL-993 for 40 min before (*C*) and after (*I*) treatment with 40 ng/mL VEGF. SF7-AM–labeled HUVECs pretreated with 100 μM PAG for 10 min before (*D*) and after (*J*) treatment with 40 ng/mL VEGF. SF7-AM–labeled HUVECs pretreated with 100 U/mL PEG-catalase (PEG-cat) for 2 h before (*E*) and after (*K*) treatment with 40 ng/mL VEGF. SF7-AM–labeled HUVECs pretreated with 5 μM DPI for 10 min before (*F*) and after (*L*) treatment with 40 ng/mL VEGF.



Movie S1. Time-lapse imaging of H_2S detected by 2.5 μM SF7-AM in VEGF-stimulated HUVECs at 37 °C and 5% CO_2. Images were collected as 10 × 2 μm z-stacks every minute for 30 min after stimulation with 5 μL of 10 μg/mL VEGF in 0.1% BSA. Laser powers were kept between 0.7% and 1.0% to minimize any potential photochemical uncaging.

Movie S1



Movie S2. Time-lapse imaging of H_2S detected by 2.5 μM SF7-AM in HUVECs treated with vehicle at 37 °C and 5% CO_2. Images were collected as 10 × 2 μm z-stacks every minute for 30 min after addition of 5 μL 0.1% BSA vehicle control.

Movie S2