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CHAPTER FOUR

Azide-Based Fluorescent Probes: Imaging Hydrogen Sulfide in Living Systems

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Contents

Abstract

Hydrogen sulfide is a redox active sulfur species that is endogenously generated in mammalian systems as an antioxidant and signaling molecule to support cellular function. The fundamental and ubiquitous actions of hydrogen sulfide demand sensitive and specific methods to track this biomolecule as it is produced within living organisms with temporal and spatial regulation. In this context, the hydrogen sulfide-mediated reduction of an azide to an amine is a useful method for organic synthesis, and this

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reaction has successfully been exploited to yield biocompatible fluorescent probes for hydrogen sulfide detection *in vitro* and in cells. This chapter provides protocols and guidelines for applying azide-based fluorescence probes to detecting hydrogen sulfide in living systems, including a protocol that was used to detect endogenous hydrogen sulfide in living single cells using a confocal microscope.

1. INTRODUCTION

Hydrogen sulfide (H_2S) has been intimately involved in the history of life, contributing to the origin of biomolecules (Parker et al., 2011) and orchestrating the cataclysmic Permian-Triassic mass extinction 250 million years ago (Grice et al., 2005). This dichotomy of action is reflected in its biological effects. While displaying rapid toxicity at high concentrations (Guidotti, 2010), lower physiological levels of H_2S can enhance neuronal signaling (Abe & Kimura, 1996), induce vasorelaxation (Zhao & Wang, 2002), and even increase lifespan in model organisms (Miller & Roth, 2007). Intriguingly, nature has evolved a diverse array of enzymes to utilize H_2S as a signaling molecule (Li, Rose, & Moore, 2011), antioxidant (Kimura & Kimura, 2004; Lee, Schwab, Yu, McGeer, & McGeer, 2009), and supporter of cellular energetics (Fu et al., 2012). In mammals, H_2S is generated from cystathionine γ-lyase (CSE) (Stipnauk & Beck, 1982), cystathionine β-synthase (CBS) (Singh, Padovani, Leslie, Chiku, & Banerjee, 2009), and the coordinated action of cysteine amino transferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST) (Shibuya et al., 2009). These enzymes are ubiquitous in human tissues (Kimura, 2011) where H_2S helps mediate cardiovascular health, respiration, and neuronal signaling (Kimura, 2010; Vandiver & Snyder, 2012).

Given these broad, fundamental roles in physiology, it is not surprising that misregulation of H_2S is often associated with disease. Aberrantly low levels of H_2S lead to hypertension in the cardiovascular system (Yang et al., 2008) and accompany neurodegeneration in Huntington's disease (Paul et al., 2014). On the other hand, high levels of H_2S are observed in Down syndrome (Kamoun, Belardinelli, Chabli, Lallouchi, & Chadefaux-Vekemans, 2003), Zucker diabetic rats (Wu et al., 2009), and the urine of patients with cancer (Huang et al., 2013). In colon cancer cells, over production of CBS-derived H_2S has a pro-tumorigenic effect by both promoting angiogenesis and acting as an energy source for cancer cell metabolism (Szabó et al., 2013). Recently, persulfides have been identified

and hypothesized as mediators of H_2S signaling (Ida et al., 2014). These transient molecules may play a fundamentally important role, particularly for intracellular H_2S signaling (Miranda & Wink, 2014), and new tools are emerging for their detection (Liu et al., 2014).

In this context, the broad, concentration-dependent effects of H_2S in health and disease demand precise methods to track the production of this important signaling molecule in living organisms. The most common classical methods for detecting H_2S include the methylene blue method, monobromobimane, gas chromatography, and amperometric sensors (Olson, DeLeon, & Liu, 2014). Such traditional techniques have provided bulk measurements of H_2S concentrations in serum, cells, and tissue samples, but they are incompatible with detection of H_2S levels in living, intact cells. Fluorescence imaging probes offer a powerful approach for H_2S imaging using fluorescence microscopy. Key advantages include high sensitivity, spatiotemporal resolution, and compatibility with living cells. Several criteria need to be met for a fluorescent probe to be useful for imaging H_2S in living cells: (1) biological compatibility, (2) cell permeability, (3) high sensitivity and selectivity, particularly with regards to intracellular thiols, and (4) optical properties that are compatible with common microscopy filter sets.

Several families of reaction-based fluorescent probes for H_2S have been developed in just the past few years. Due to space limitations, we cite review articles covering the field here (Lin & Chang, 2012; Lippert, 2014; Peng & Xian, 2014; Yu, Han, & Chen, 2014). Among these, azide-based probes have been widely adopted due to their sensitivity, selectivity, and ease of preparation. The H_2S-mediated reduction of azides has been used synthetically for a number of years, and starting in 2011 our laboratory utilized this functionality in the Sulfidefluor (SF) series of probes and Wang's lab concomitantly utilized sulfonyl azide versions for this same purpose (Peng et al., 2011). The first-generation SF probes were found to be compatible in live cells and showed good selectivity for H_2S over other thiol molecules, including glutathione (Lippert, New, & Chang, 2011). Optimization of the design to enable cellular trappability provided fluorescent H_2S probes that accumulate in cells and are capable of imaging endogenous H_2S generated for cellular purposes (Lin, Lippert, & Chang, 2013). These probes are based on rhodamine dye scaffolds that display low photobleaching, high quantum yields, and significant cellular compatibility.

This chapter is outlined as follows. Section 2 provides an overview of the design and reactivity of the probes. In Section 3, we describe the *in vitro* characterization of these probes using fluorescence spectrophotometry.

Section 4 details methods for using these probes in confocal microscopy applications and ends with an example of imaging endogenous H_2S production in human endothelial cells.

2. FLUORESCENT AZIDE-BASED H_2S PROBES

2.1. Probe design

Azide-based sulfide probes have been widely adopted in the field due to good chemical properties and simple methods for their preparation. This latter attribute has enabled the development of a wide variety of probes with tunable excitation and emission wavelengths, targeting to specific subcellular locales (Bae et al., 2013; Liu, Xu, Spring, & Cui, 2013), ratiometric readouts (Bae et al., 2013; Liu, Sun, et al., 2013; Wan, Song, Li, Gao, & Ma, 2013; Yu et al., 2012), two-photon excitation (Bae et al., 2013), and chemiluminescent emission (Bailey & Pluth, 2013).

We have focused on developing fluorescent sulfide probes to visualize endogenous H_2S produced during cellular signaling (Fig. 1). Our general strategy involves manipulating the "closed" and "open" forms of a rhodamine scaffold. The anilinic nitrogens on the rhodamine dye are masked as electron withdrawing azides, enforcing a "closed" lactone form. This structure displays very low fluorescence emission due to a break in the conjugation of the aromatic rings. Reduction of the azide with H_2S produces an aniline, which subsequently shifts the equilibrium toward the "open" form. Conjugation of the aromatic rings results in a highly fluorescent rhodamine dye.

The key to obtaining dyes that can image endogenous H_2S with scanning laser confocal microscopy was engineering probe molecules that accumulate within cells, accomplished by appending multiple acetoxymethyl (AM) esters (Minta, Kao, & Tsien, 1989). The AM ester dyes bear neutral charge and are highly permeable to the cellular membrane. Once inside the cell, however, these esters are rapidly cleaved leaving the dye molecule in an ionic form. Probe molecules will accumulate intracellularly providing both high concentrations of dye and a greatly reduced background signal. These factors provide a drastic increase in sensitivity that, under carefully controlled conditions, are amenable to imaging endogenous levels of H_2S.

2.2. Reactivity

Azides have long been used as "bioorthogonal" functional groups (Kolb, Finn, & Sharpless, 2001; Prescher & Bertozzi, 2005) defined as "nonnative,

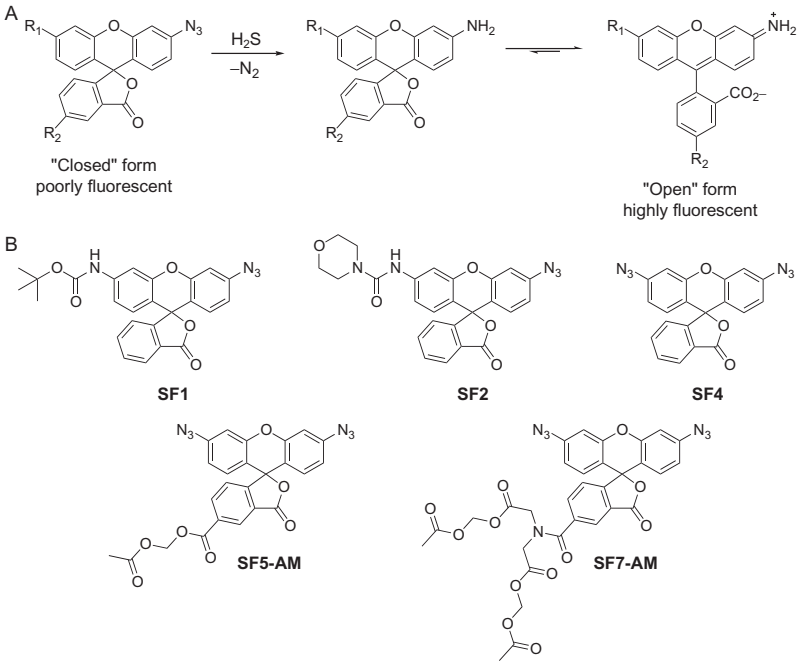

Figure 1 (A) Azide-based reduction strategy for fluorescence detection of H_2S. (B) Molecular structures of Sulfidefluor probes.

nonperturbing chemical handles that can be modified in living systems through highly selective reactions with exogenously delivered probes." We have taken advantage of this documented insensitivity to most biological molecules to design probes that are selective for H_2S inside living systems. *In vitro*, these H_2S probes display relatively low reactivity toward common biological thiols such as glutathione and cysteine. The reaction of an azide with H_2S is irreversible and these probes are properly classified as chemodosimeters. We also note caveats for the proper use of these and related azide-based fluorescent reagents for H_2S detection. The field of fluorescent probe design is in many ways in its relative infancy, and developing technologies will continue to push the frontiers of this area. First, it should be noted here that the azide functionality is potentially sensitive toward dithiothreitol, so this reducing agent should be avoided in the cellular media. The SF series probes may exhibit some similar cross-reactivity toward other highly reducing sulfur species, so appropriate controls should always be performed to confirm changes in fluorescent signal are a product of H_2S or reactive sulfur species production. Some aryl azides

may also display photoactivation when exposed to high power and high energy light. As such, fluorescent azide-based probes that can be excited in the visible region are therefore preferred over probes that require excitation with UV light. Excessive exposure to light should be avoided during experiments and low light power should be used when possible. In this context, confocal microscopy is preferred over epifluorescence microscopy to minimize undesirable photoreactions.

2.3. Use and storage of probes

To prepare the probes for imaging use, an aliquot of dry probe is dissolved in the designated amount of DMF or DMSO to give a stock solution of 1–10 m*M*. This DMF or DMSO stock is then diluted in 1 mL of an aqueous buffer such as PBS, HEPES, or Tris and mixed thoroughly by pipetting or vortexing to give working concentrations of 1–20 μ*M*. Relatively dry DMF or DMSO should be used to dissolve the probe, as high water content may prevent full dissolution of the solid material and interfere with the preparation of a homogenous stock solution.

These probes are most stable in dry, solid form and can be stored for up to 6 months at −80 °C, protected from light and moisture. As with many other fluorescent dyes, freeze–thaw cycles may result in degradation of the probe, so single-use aliquots of probe are preferred. Once dissolved in dry DMF or DMSO, stocks can be stored for up to 1–2 weeks at −80 °C if necessary, although they should be carefully monitored for any changes in appearance that might indicate degradation, e.g., color change. NMR spectroscopy can be employed to monitor quality.

Any aqueous solutions of SF probes should be freshly prepared and used the same day, as hydrolysis of the ester functionalities reduces permeability of the probe. Azides are potentially unstable to high heat and light exposure, so all SF probe stocks and prepared solutions should be stored in the dark or covered with aluminum foil when not in use.

Low laser powers should be used to avoid photoactivation of the dye, since azides can be cleaved upon irradiation with UV or high-intensity light. To reduce the risk of photoactivation for a given field of cells, image collection at low wavelengths should be performed after collecting images for the SF probe. Furthermore, broadband excitation sources such as those commonly used in epifluorescence microscopes and microplate readers may not be compatible with azide-based fluorescent probes. In experiments with a Zeiss 710 confocal microscopy setup, we observed very minimal

photoexcitation when using 488 nm light with low laser power. When using 405 nm light, however, significant increases in fluorescence intensity can be seen due to photoactivation.

3. *IN VITRO* CHARACTERIZATION OF PROBES

For all *in vitro* fluorometry experiments, a positive control using H_2S and a negative control using buffer should be performed on each day of experiments using the same probe aliquot between samples to account for potential variation in environmental factors, aliquot quality, and reagent preparation. Reactions with 100 μM H_2S are incomplete at 60 min. Excessive irradiation should be avoided to prevent photoactivation of the azide groups.

The following protocols yield samples with a final DMF concentration of 0.1%. In experiments where a minimal volume of organic cosolvent is desired, reduced amounts of stock solution may be used or the concentration of the stock solution may be increased. However, care must be taken to ensure that sufficient organic cosolvent is present to prevent the probe from precipitating out of solution upon addition to aqueous buffer, especially for the more hydrophobic SF4 probe. All solutions should appear optically clear and without particulates.

3.1. Safety precautions

Experimentalists should familiarize themselves with the hazards associated with H_2S, including inhalation risks, eye irritation, and flammability. Experiments should always be performed with sufficient ventilation, away from any ignition sources, using the appropriate personal protective equipment. While the concentrations and volumes of H_2S produced in the experiments detailed in this chapter are relatively small, even low levels of exposure to H_2S can result in discomfort. Thus, all practical precautions should be taken to limit exposure, such as preparing solutions of H_2S donors in a fume hood whenever possible.

3.2. Instrumentation and materials

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 4L-format scanning spectrofluorometer (Lawrenceville, NJ) 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, Atascadero, CA). Due to the toxicity of H_2S, screw-cap cuvettes (Cat no. 9 F-Q-10-GL14-S, Starna) were utilized to limit exposure to any gas that might escape from each reaction during the course of these experiments. Screw-cap cuvettes were also used for any other air-sensitive and gaseous reactive species.

Probe. SF7-AM stock solution (5 m*M*) in anhydrous DMF.

H_2S. A 25-m*M* aqueous stock solution made from solid Na_2S nonahydrate (Sigma-Aldrich) or NaHS (Cayman) in H_2O.

3.3. Time-course assays

Before performing any biological experiment, the integrity of the fluorescent probe can be examined *in vitro* by monitoring fluorescence response to H_2S over time. A 10-μ*M* solution of SF7-AM was prepared in 3 mL buffered aqueous solution. Vortexing was done to assure full dissolution of the dye. Two 1 mL aliquots of this probe solution were placed into different microcentrifuge tubes. To one tube, 4 μL of the 25 m*M* Na_2S solution was added, the solution was vortexed, and then transferred to a quartz cuvette. Absorption and fluorescence spectra were then acquired every 10 min. To the second tube, 4 μL of H_2O was added as a control, and spectra were acquired as described above (Fig. 2).

3.4. Selectivity experiments

Selectivity experiments are conducted to confirm the probe has higher reactivity toward H_2S over other reactive species that may be present in a system of interest. Generally, reactive sulfur, oxygen, and nitrogen species are tested at a final concentration of 100–200 μ*M*. Any highly abundant species should be tested at their most biologically relevant concentration, e.g., 5–10 m*M* for glutathione. The concentration of dye or probe being tested typically ranges from 1 to 10 μ*M*. Dye concentration may need adjustment if the dye fluorescence falls outside of the linear range for the detection system during selectivity tests. Readings are typically taken at 5–15 min intervals, and fluorescence response is typically represented as integrated emission intensity over a given range (Fig. 2B). For each day of data collection, an untreated sample of probe should be tested as a negative control.

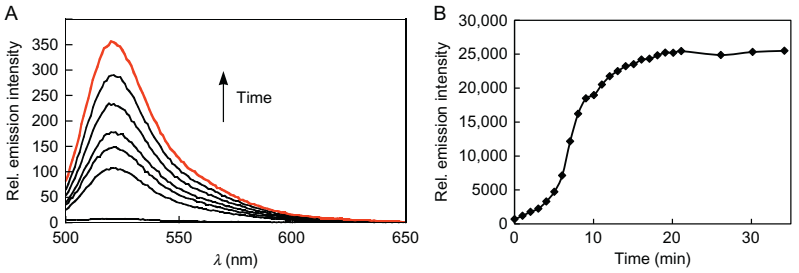

Figure 2 *In vitro* characterization of SF7-AM. (A) Fluorescence response of SF7-AM. Time points represent 0, 10, 20, 30, 40, 50, and 60 min (red trace (gray in the print version)) after addition of 100 μ*M* NaHS to 10 μ*M* SF7-AM. (B) Fluorescence response of 5 μ*M* SF7-AM in the presence of a 200-fold excess of NaHS. Fluorescence response was monitored at $\lambda_{em} = 525$ nm until the reaction was complete. *Reprinted with permission from Lin et al. (2013). Copyright 2013 Proceedings of the National Academy of Sciences.*

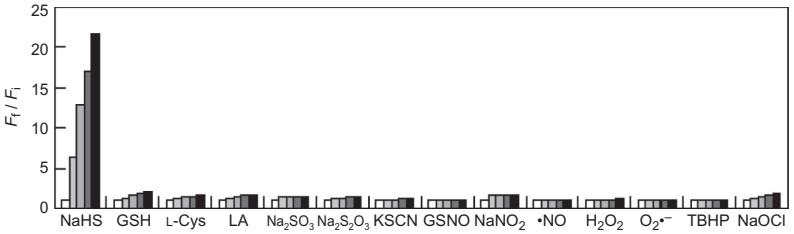

Figure 3 Testing the selectivity of SF7-AM toward H_2S compared to other biologically relevant RSS, ROS, and RNS. Bars represent relative responses at 525 nm at 0, 15, 30, 45, and 60 min after addition of RSS, RNS, or ROS. Data shown are for 5 m*M* glutathione, 500 μ*M* cysteine, and 100 μ*M* for other RSS, RNS, and ROS. Data were acquired at 25 °C in 20 m*M* HEPES buffered to pH 7.4 with excitation at $\lambda_{ex} = 488$ nm. Emission was collected between 498 and 700 nm. *Reprinted with permission from Lin et al. (2013). Copyright 2013 Proceedings of the National Academy of Sciences.*

A positive control should also be performed, typically using 100 μ*M* Na_2S or NaHS (Fig. 3).

3.5. Data processing and analysis

The integrated fluorescence intensity—the area under the fluorescence emission curve—was plotted against time. Fluorescence intensity at a single point, typically the emission maximum, may also be used. For selectivity tests, integrated fluorescence intensity is typically expressed as the ratio of

the integrated fluorescence intensity at a given time point to the integrated fluorescence intensity before addition of the reactive species.

4. DETECTION OF H_2S IN LIVE CELLS USING FLUORESCENT PROBES

This section will provide detailed protocols for imaging H_2S produced during a cellular model of angiogenesis. Section 4.1 describes control experiments imaging H_2S generated from an exogenous donor such as Na_2S or NaHS. This validation should be performed in any new experimental system to confirm dye efficacy. Section 4.2 details the experimental parameters needed to provide reliable measurements of endogenous H_2S generated for signaling purposes. Lastly, Section 4.3 provides methods using these fluorescent probes to reveal factors that are needed for H_2S production.

4.1. Imaging exogenous H_2S using confocal microscopy

The SF H_2S probes each possess different properties, particularly in terms of cell permeability and trappability. These attributes should be considered when selecting a dye to use for live cell imaging. SF4 is permeable to live cells and displays cytosolic staining while being excluded from the cell nucleus. Notably, SF4 is not a trappable dye, so exchanging the cellular medium typically leads to loss of fluorescence signal as the dye is washed out of the cells. SF7-AM is highly trappable and well retained within live cells after multiple media exchanges, displaying cytosolic and nuclear distribution. Owing to the mechanism of trapping for AM ester-based dyes, fixation of cells is generally incompatible with these probes. SF7-AM relies upon charged carboxylate groups to enhance retention in live cells, so permeabilization of the cell membrane results in leakage of the dye from the cells and loss of fluorescence signal. If using serum-containing media, SF7-AM must be loaded into cells shortly after dilution in media due to hydrolysis of the ester groups. Once the ester groups have been hydrolyzed, the probe is no longer cell permeable. This premature hydrolysis is not as readily observed in serum-free medium or buffer.

4.1.1 Materials and instrumentation

Media. Dulbecco's Modified Eagle Medium (Gibco) with 10% fetal bovine serum (FBS) or other standard medium appropriate for the cell line of interest.

Probes and reagents. A 5-m*M* SF7-AM stock solution in DMF made the day of the experiment. A 2.5-μ*M* solution of SF7-AM in complete media should be prepared from the DMF stock immediately before the experiment. A 10-m*M* stock solution of Na_2S in water made the day of the experiment.

Cell culture supplies. Standard supplies for mammalian cell culture.

Confocal microscope. Fluorescence microscopy studies were performed with a LSM 710 laser scanning confocal microscope (Carl Zeiss) using a 40 × water objective lens with Immersol W 2010 immersion medium and Zen 2010 software (Carl Zeiss). SF7-AM was excited using a 488-nm Ar laser, and emission was collected using a META detector between 500 and 650 nm. Hoechst 33342 was excited with a 405-nm diode laser, and emission was 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. Data processing and analysis were conducted using ImageJ (National Institutes of Health) or Zen 2010 software (Carl Zeiss).

4.1.2 Cell culture and dye loading

Two days before imaging. Cells were passaged in media without phenol red and plated in four-well Lab-Tek II glass chamberslides (Thermo Scientific, Cat no. 155382 or 155409) with an appropriate coating, if needed. At the time of the experiment, confluence should be between 70% and 80%.

60 min before imaging. The culture media in each well was replaced with 500 μL of a 2.5-μ*M* solution of SF7-AM prepared by dissolving 2 μL of the 5 m*M* stock solution into 4 mL of media. The cells were then incubated at 37 °C and 5% CO_2 for 30 min.

30 min before imaging. The media containing dye was replaced with fresh media. Five microliter of the Na_2S stock solution was added to positive control wells for a final concentration of 100 μ*M* Na_2S, and 5 μL of water was added as a vehicle control to negative control wells. The wells were capped and incubated for 30 min at 37 °C and 5% CO_2. The cells were then placed on the microscope stage, which was prewarmed and equilibrated to 37 °C and 5% CO_2.

4.1.3 Imaging and results

The cells were imaged on the microscope stage at 37 °C and 5% CO_2. Images of three to four fields per well were acquired using low laser power (1.0–1.2%). Images were quantified using the Zen 2010 Software

(Carl Zeiss) by setting common lower and upper thresholds for all images and evaluating the mean pixel intensity for each image.

4.2. Imaging endogenous H_2S production in HUVECs

4.2.1 Materials and instrumentation

Media. 1% Roswell Park Memorial Institute (RPMI) medium with 20% FBS (HyClone), 0.05 mg/mL endothelial growth supplement (ECGS, BD Biosciences), and 28 μg/mL heparin. Media should be used within 1 month of preparation.

Probes and reagents. A 5-m*M* SF7-AM stock solution in DMF made the day of the experiment. A 2.5-μ*M* solution of SF7-AM in complete media should be prepared from the DMF stock immediately before the experiment. A 25-m*M* stock solution of D,L-propargyl glycine (PAG) in water made the day of the experiment. A 10-μg/mL stock solution of VEGF in 0.1% BSA thawed the day of the experiment.

Cell culture supplies. Standard supplies for mammalian cell culture.

Confocal microscope. Fluorescence microscopy studies were performed with a LSM 710 laser scanning confocal microscope (Carl Zeiss) with a 40 × oil objective lens, atmosphere control incubator, motorized stage, and Zen 2010 software (Carl Zeiss). SF7-AM was excited using a 488-nm Ar laser, and emission was collected using a META detector between 500 and 650 nm. Hoechst 33342 was excited with a 405-nm diode laser, and emission was 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. Data processing and analysis were conducted using ImageJ (National Institutes of Health) or Zen 2010 software (Carl Zeiss).

4.2.2 Cell culture and dye loading

Human umbilical vein endothelial cells (HUVECs) were obtained as a gift from the Netherlands and cultured using RPMI supplemented with 20% FBS, 0.05 mg/mL ECGS, and 28 μg/mL heparin. Cells were passaged every 2–3 days and experiments were performed between passage numbers of 2–17.

Two days before imaging. Cells were passaged and plated in four-well Lab-Tek II glass chamberslides (Thermo Scientific, Cat no. 155382 or 155409) coated with 0.2% gelatin for 1 h before seeding. At the time of the experiment, confluence should be between 70% and 80%.

30 min before imaging. The culture media in each well was replaced with a 2.5-μ*M* solution of SF7-AM in complete media. The cells were then incubated at 37 °C and 5% CO_2.

20 min before imaging. PAG is added to well 2. The cells are further incubated for 20 min, and the cellular media was then replaced with complete RPMI media (+FBS, +ECGS, −phenol red). The cells were placed on the microscope stage, which was prewarmed and equilibrated to 37 °C and 5% CO_2.

4.2.3 Imaging and results

SF7-AM can image small increases in endogenous H_2S levels using confocal microscopy if care is taken to: (1) observe the same cells over time and (2) acquire *z*-stack images that capture fluorescence response from the entire cell thickness. Without careful attention to these experimental details, we found that cell-to-cell variability and user biases introduced by focusing on different *z*-stacks diminished the statistical significance of our results. All experiments should be accompanied by vehicle-treated controls and a method to decrease endogenous H_2S production, such as the use of pharmacological inhibitors, scavengers, or genetic knockdown.

After cells have been incubated with probe and are on the microscope stage, *z*-stack images (8×2 μm) are acquired of three to four fields per well. The *x*,*y* coordinates of each imaged field are saved so that the same cells can be reimaged. Two microliter of the 10 μg/mL VEGF solution was added on stage to wells 1 and 2, and 2 μL of a 0.1% BSA vehicle control was added to well 3. Each well contained 500 μL of RPMI media (+FBS, +ECGS, −phenol red). The cells were incubated on the microscope stage at 37 °C and 5% CO_2. After 30 min, *z*-stack images (8×2 μm) were once again acquired of the same three to four fields per well. Images were quantified using the Zen 2010 Software (Carl Zeiss) by taking the maximum intensity projection of the *z*-stacks, setting a common lower threshold for all images, ensuring that the cells are selected in the vehicle control images, and evaluating the mean pixel intensity for each image. Under these conditions, we observe a statistically significant 26% increase in the mean pixel intensity from the cell images before and after VEGF stimulation versus a 7% increase in cell images before and after stimulated with a vehicle control (Fig. 4).

4.3. Interrogating pathways involved in H_2S production using confocal microscopy

The real-time visualization of H_2S with SF7-AM provides an opportunity to investigate the cellular signaling cascades that lead to its production. The general strategy entails pharmacological inhibition of enzyme activity

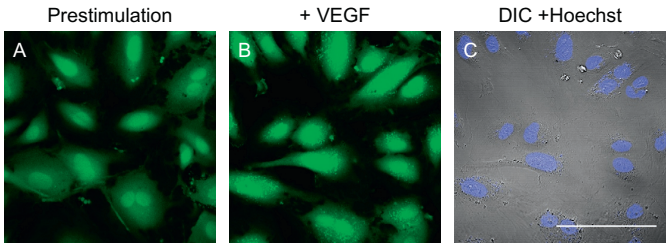

Figure 4 (A) HUVECs incubated with 5 μ*M* SF7-AM for 30 min at 37 °C, washed, and then imaged. (B) The same field of HUVECs in (A) was treated on stage with 2 μL of 10 μg/mL VEGF for 30 min at 37 °C, and then imaged. (C) Brightfield images of the same field of cells in (B) overlaid with Hoechst 33342 stain (1 μ*M*) at 37 °C. Images in (A) and (B) are the maximum intensity projections of 8 × 2 μm *z*-stacks. Scale bar represents 100 μm. *Adapted with permission from Lin et al. (2013). Copyright 2013 Proceedings of the National Academy of Sciences.*

and/or direct scavenging of molecular mediators while monitoring the optical signal from SF7-AM. In this example, we observed an increase in fluorescent signal from SF7-AM upon treatment of HUVECs with VEGF, in agreement with previous observations that VEGF stimulation increases H_2S production in this cell type (Papapetropoulos et al., 2009). Exposing cells to the pharmacological inhibitors PAG and AAL-993 which affect this H_2S production pathway correspondingly modulated the response from SF7-AM.

PAG. Incubate cells with 100 μ*M* PAG (Sigma-Aldrich) from a 25-m*M* stock in water for 10 min prior to VEGF stimulation (directly after media exchange).

AAL-993. Incubate cells with 30 μ*M* AAL-993 (Millipore) for 40 min prior to VEGF stimulation and again added at 30 μ*M* after media exchange.

Acquisition of *z*-stack images of the same cells over time as described in Section 4.2.3 revealed upstream molecular mediators of VEGF-dependent H_2S production in HUVECs. Inhibition of VEGFR2 with AAL-993 and CSE with PAG attenuated signal, establishing the involvement of these proteins at the beginning and end of the cascade leading to H_2S production. The observation that PAG fails to completely abolish signal combined with the observation that HUVECs express CBS (Lin et al., 2013) may point toward alternative production pathways (Fig. 5).

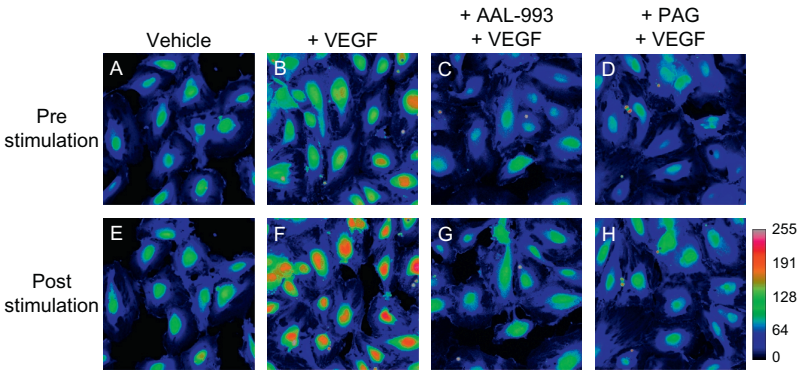

Figure 5 Representative confocal fluorescence images of H_2S signaling in live HUVECs. HUVECs were incubated with 2.5 μ*M* SF7-AM, washed, and imaged before (A) and after (E) treatment with 0.1% BSA in H_2O as a vehicle control. HUVECs were incubated with 2.5 μ*M* SF7-AM, washed, and imaged before (B) and after (F) treatment with VEGF. SF7-AM-labeled HUVECs pretreated with 30 μ*M* AAL-993 (AAL) for 40 min before (C) and after (G) treatment with VEGF. SF7-AM-labeled HUVECs pretreated with 100 μ*M* PAG for 10 min before (D) and after (H) treatment with VEGF. *Adapted with permission from Lin et al. (2013). Copyright 2013 Proceedings of the National Academy of Sciences.*

5. CONCLUSIONS

The ready installation of azides into molecular scaffolds has led to a rapid development of a large family of optical probes for H_2S with a variety of excitation and emission characteristics, luminescence mechanisms, and cellular localization. Despite the considerable growth of the field, examples of endogenous H_2S detection with a fluorescent probe remain rare, and typically require advanced microscopy techniques like live cell scanning laser confocal microscopy (Lin et al., 2013) or two-photon imaging (Bae et al., 2013). The method described here provides researchers with a detailed protocol to enable the real-time detection of H_2S generated for signaling purposes in living cells. In order to address the needs of the field (Papapetropoulos, Whiteman, & Giuseppe, 2014), we and other groups are continually working to develop H_2S probes with improved sensitivity, ease of use, and compatibility with widely available instrumentation. This work provides another successful illustration of how the reaction-based approach to sensing offers a general and versatile way to study molecules

of biological interest (Chan, Dodani, & Chang, 2012; Lippert, Van de Bittner, & Chang, 2011).

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