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A Genetically Encoded, Ratiometric Fluorescent Biosensor for Hydrogen Sulfide

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Abstract

As an important gasotransmitter, hydrogen sulfide (H₂S) plays crucial roles in cell signaling. Incorporation of *p*-azidophenylalanine (*p*AzF) into fluorescent proteins (FPs) via genetic code expansion has been a successful strategy in developing intensity-based, genetically encoded fluorescent biosensors for H₂S. To extend this strategy for ratiometric measurement which eliminates many detection uncertainties via self-calibration at two wavelengths, we modified the chromophore of a circularly permuted, superfolder green fluorescent protein (cpsGFP) with *p*AzF to derived cpsGFP-*p*AzF, which subsequently served as a Förster resonance energy transfer (FRET) acceptor to EBFP2, an enhanced blue fluorescent protein. The resultant construct, namely hsFRET, is the first ratiometric, genetically encoded fluorescent biosensor for H₂S. Both *in vitro* and in mammalian cells, H₂S reduces the azido functional group of hsFRET to amine, leading to an increase of FRET from EBFP2 to cpsGFP. Our results collectively demonstrated that hsFRET could be used to selectively and ratiometrically monitor H₂S.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information Available: The following files are available free of charge.

Supporting figures, tables, and movies (Supporting Information.pdf and Movie S1 and Movie S2).

Keywords

genetic code expansion; fluorescent protein-based biosensor; Förster resonance energy transfer; unnatural amino acid; ratiometric measurement; hydrogen sulfide

Hydrogen sulfide (H_2S), which was long considered a toxic gas with a wide range of cytotoxic effects, has been recognized as the third biosignaling gasotransmitter following nitric oxide (NO) and carbon monoxide (CO).¹ H_2S has a $\text{pK}_{\text{a}1}$ value of 6.6. At neutral pH and 37 °C, it equilibrates mainly with HS^- .^{2,3} Endogenous H_2S can be produced enzymatically or non-enzymatically during sulfur metabolism via the reduction of thiol and thiol-containing molecules. Enzymes that catalyze H_2S production include cystathionine β -synthase (CBS), cystathionine γ -lyase (CGL), and cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase (3-MST).^{4,5}

H_2S is involved in sulfhydration, a post-translational modification forming persulfides ($-\text{S}-\text{SH}$) on cysteine residues of proteins. S -sulfhydration plays important roles in the regulation of inflammation, endoplasmic reticulum stress, and vascular tension.^{6,7} H_2S has also been shown to mediate cardiovascular functions,^{2,8} and to regulate ATP-sensitive K^+ (K_{ATP}) channels.^{9,10} Furthermore, accumulating evidence suggests that H_2S functions as a signaling molecule in biological processes such as insulin release, neuromodulation, anti-oxidation, and angiogenesis.^{5,11–13} Dysregulation of H_2S is related to diseases such as the Down syndrome,¹⁴ Alzheimer's disease,¹⁵ diabetes,¹⁶ and hypertension.¹⁷ Accordingly, dissection of the complex roles H_2S plays in biological and pathophysiological processes is highly needed. Yet traditional H_2S detection methods such as chromatographic,¹⁸ colorimetric,¹⁹ and electrochemical analysis²⁰ methods do not allow for real-time and noninvasive detection.

Fluorescent biosensors are powerful research tools that allow rapid and noninvasive investigation of physiological and pathological processes of interest with high spatiotemporal resolution.²¹ To date, based on distinct chemical principles including the reduction of azido groups to amino groups,^{22–25} reduction of nitro groups to amines,^{26–28}

nucleophilic reactions of H₂S,^{29,30} and copper sulfide precipitation,¹⁹ many H₂S-responsive, synthetic fluorescent biosensors have been developed.^{31,32} Recently, two genetically encoded fluorescent biosensors, cpGFP-*pAzF* and hsGFP, which both contain an unnatural amino acid *p*-azidophenylalanine (*pAzF*), were developed for H₂S detection in mammalian cells.^{33,34} These studies used a genetic code expansion technology³⁵ to replace the chromophore-forming Tyr residue of circularly permuted green fluorescent proteins with *pAzF*. Upon reaction with H₂S, the azido group of *pAzF* in cpGFP-*pAzF* or hsGFP is reduced into an amino group, resulting in a fluorescent, *p*-aminotyrosine (*pAmF*) derived chromophore.³⁶ In addition to their large dynamic ranges, the genetic encodability of these biosensors allows them to be precisely localized to subcellular domains where H₂S signaling occurs.

Because ratiometric biosensors have less dependence on sensor concentrations, excitation intensity, and photobleaching,^{37,38} we herein present our design, engineering, and characterization of hsFRET (Figure 1), which is, to the best of our knowledge, the first genetically encoded, Förster resonance energy transfer (FRET)-based biosensor for H₂S. hsFRET shows selective and sensitive response to H₂S and allows ratiometric imaging of H₂S in live mammalian cells.

EXPERIMENTAL SECTION

Materials.

All materials were obtained and used as previously described.^{33,39} Sodium hydrosulfide (NaHS) from Acros Organics was dissolved in DPBS, adjusted to pH 7.4 right before use, and added to cells as an H₂S donor. To detect endogenous H₂S, L-cysteine from Sigma-Aldrich was used to stimulate cellular H₂S production. DL-Propargylglycine (DL-PPG) from Acros Organics was used to further inhibit cellular H₂S production.

Construction of Bacterial Expression Plasmids and Libraries.

Polymerase chain reactions (PCRs) were utilized to amplify the gene fragments of the FRET donor and acceptor.^{39,40} In particular, oligos BFP-For and BFP-Rev were utilized to amplify the EBFP2 gene fragment, and the PCR product was digested with HindIII and XhoI restriction enzymes and ligated into a predigested, compatible pCDF-1b expression vector to afford pCDF-1b-EBFP2. Next, oligos GFP-For and GFP-Rev were used to amplify the cpsGFP gene fragment. The resultant PCR product was then digested with NcoI and HindIII and ligated into the predigested pCDF-1b-EBFP2 plasmid to afford FRET-0.1. To generate FRET-0.2, two separate PCR reactions were utilized. In the first PCR reaction, oligos BFP-For2 and BFP-Rev were utilized to amplify the EBFP2 fragment. The resulted PCR product was digested with HindIII and XhoI and ligated into a predigested pCDF-1b to afford pCDF-1b-EBFP2a. In the second PCR reaction, oligos GFP-For, and GFP-Rev2 were used to amplify the cpsGFP fragment. The PCR product was digested with NcoI and HindIII and ligated into the predigested pCDF-1b-EBFP2a. Next, we constructed a library to fully randomize residues His9, Thr64, and Ser67 (Figure 2). Briefly, two separate PCR reactions were performed with cpsGFP. In the first reaction, H148X-For and TX-SX-Rev oligonucleotides containing degenerate NNK codons (in which N = A, T, G, or C, and K = G

or T) were used, while in the second reaction TX-SX-For (containing degenerate NNK codons) and GFP-Rev2 oligonucleotides were used. An overlap PCR-based strategy was next used to assemble the products of these two reactions to produce the full-length gene. The amplified DNA fragment was then treated with NcoI and HindIII and ligated into a predigested pCDF-1b-EBFP2a. After screening the generated library for mutants that respond to H₂S, FRET-0.3 was selected, amplified, and further inserted into pBAD/His B between NcoI and XhoI restriction sites. To generate a library using error-prone PCR (ep-PCR), pBAD-FRET-0.2 and pBAD-FRET-0.3 were mixed and ep-PCR reaction were carried out using oligos pBAD-For and pBAD-Rev. The reaction was carried out with *Taq* DNA polymerase for 38 cycles in the presence of 200 μ M MnCl₂ as previously described.⁴¹ Mutagenic PCR products were combined, purified by agarose gel electrophoresis, digested with NcoI and XhoI, and ligated into pBAD/ His B.

Library screening.

To screen the library generated by randomizing residues His9, Thr64, and Ser67, we followed a previously described protocol.³⁴ FRET-0.3 was then selected based on the emission ratio of cpsGFP to EBFP2. To screen the library generated from ep-PCR, the gene library in the pBAD/ His B was used to co-transform *E. coli* DH10B electrocompetent cells with pEvol-*pAmF*, which was prepared by introducing 5 mutations (Y32T, E107T, D158P, I159L, and V164A) in both copies of the *M. jannaschii* aminoacyl-tRNA synthetase (aaRS) of pEvol-*pAzF*.⁴² After each round of screening, the best mutant was selected for the next round of ep-PCR.

Protein Expression, Purification, and *In Vitro* Characterization.

We followed previously described procedures.³⁴ In particular, we used a final protein concentration of 1 μ M in all in vitro assays. Selectivity assays were performed after incubation of hsFRET with various redox-active molecules (all solutions were prepared as previously described³⁹) at room temperature for 30 min. FRET ratios of fluorescence emission intensities at 500 nm over emission intensities at 450 nm were calculated and represented as means and s.d. from three independent measurements.

Construction of Mammalian Expression Plasmids.

The cpsGFP gene sequence of pBAD-hsFRET was amplified using hsFRET-HindIII-F and hsFRET-EcoRI-R oligonucleotides. After digestion with HindIII and EcoRI, the product was ligated into a predigested pcDNA3 plasmid to afford pcDNA3-cpsGFP. Next, the EBFP gene sequence of pBAD-hsFRET was amplified with oligonucleotides hsFRET-EcoRI-F and hsFRET-ApaI-R. The PCR product was treated with EcoRI and ApaI and ligated into the predigested pcDNA3-cpsGFP to afford pcDNA3-hsFRET. To further improve the mammalian expression of hsFRET, we cloned hsFRET into the pMAH-POLY plasmid³⁹ by digesting pcDNA3-hsFRET with HindIII and ApaI. The digested product was then separated on agarose gel electrophoresis, and ligated into a predigested pMAH-POLY vector to afford POLY-hsFRET.

Mammalian Cell Culture and Imaging.

Human Embryonic Kidney (HEK) 293T cells were cultured and transfected as previously described.³⁴ Typical DMEM contains 0.4 mM cysteine and 0.2 mM methionine. To minimize pre-conversion of hsFRET, cells were cultured in special DMEM containing 1 mM *pAzF* but no L-methionine or L-cysteine for 48 h, and next in fresh special growth medium without *pAzF* for an additional 12 h to deplete free *pAzF*. Cells were then imaged in DPBS under a Leica fluorescence microscope. Time-lapse imaging experiment was set at one frame per min. The excitation laser was set at 405 nm, and emission was collected at 420–465 nm and 495–570 for blue and green channels, receptively.

Cell Viability Assays.

HEK 293T cells were transfected with pMAH-POLY and POLY-hsFRET. 24 hours after transfection, a Promega RealTime-Glo™ MT Cell Viability Assay kit was used to determine the number of viable cells. Briefly, the RealTime-Glo™ reagent was mixed with cells for 30 min. After incubation, the bioluminescence of each sample was measured by using a Synergy Mx Microplate Reader. HEK 293T cells transfected with pMAH-*pAzF* but cultured in the absence of *pAzF* were used as the control. Three independent samples in each group were analyzed.

RESULTS AND DISCUSSION

Laboratory Engineering of hsFRET.

To construct a FRET-based sensor for H₂S, we selected cpsGFP as the acceptor because of its favorable spectral properties and efficiency in protein folding and maturation.³⁹ We hypothesized that these favorable properties make it an excellent FRET acceptor when fused to an EBFP2 donor,⁴⁰ which is an improved version of blue fluorescent proteins and has been shown to be an excellent FRET donor for GFP. We envisioned if we could incorporate a H₂S-reactive functional group into cpsGFP such as *pAzF*, an efficient FRET from the donor EBFP2 to the genetically modified cpsGFP acceptor could be achieved upon reaction with H₂S and subsequent illumination of EBFP2 (Figure 1). For this reason, we constructed FRET-0.1 by fusing cpGFP with EBFP2 using a short floppy peptide linker (Figure 2). The construct was cloned into a pCDF-1b vector and expressed in BL21(DE3) *E. coli* bacterial cells along with pEvol-*pAzF* in the presence of *pAzF*. Crude proteins were extracted with B-PER and tested with 1 mM H₂S. Results of this reaction showed a small FRET ratio change upon reaction with H₂S (Figure S1, Supporting Information). Because the sensor is a reaction-based probe, we speculated that reducing the length between the two FPs would increase FRET. We thus cut the small floppy peptide linker and the first few amino acid residues at N-terminal of EBFP2 (FRET-0.2 in Figure 2). FRET-0.2 was then co-expressed with pEvol-*pAzF* in BL21 *E. coli* cells in the presence of *pAzF*. The reaction of crude proteins extracted from *E. coli* cells with 1 mM H₂S showed only a small FRET ratio improvement (Figure S1). Next, we aimed to improve the FRET efficiency by improving the spectral properties of the cpsGFP acceptor. Consequently, we generated a library by fully randomizing three amino acid residues in FRET-0.2 namely His9, Thr64, and Ser67 (aligned to His148, Thr203, and Ser205 of EGFP, Figure 2). These residues appear to stabilize the phenolate ion of the chromophore in EGFP through H-bonds interactions as shown in the

crystal structure of EGFP.⁴³ *pAzF* was incorporated into the library to synthesize *pAzF*-containing proteins, and the library was screened for H₂S-induced FRET changes in bacterial colonies. Bacterial colonies were randomly picked and cultured in liquid media for protein expression. Their FRET ratios were quantitatively measured before and after reaction with H₂S. After screening, FRET-0.3 (Figure 2) was chosen for the next step, since the reaction of FRET-0.3 with 1 mM H₂S showed a substantial FRET enhancement compared to FRET-0.2 (Figure S1). We then use FRET-0.3 as the template to build another library using a random error-prone mutagenesis technology. The library was cloned into a modified pBAD/His B vector instead of pCDF-1b used before to avoid cell toxicity generated by adding IPTG. We introduced *pAmF* to the generated library instead of *pAzF* by co-expressing the library in DH10B cells with pEvol-*pAmF*, a pEvol-vector containing two copies of *M. jannaschii* aminoacyl-tRNA synthetase (AARS) with 5 mutations (Y32T, E107T, D158P, I159L, and V164A) in both copies compared to pEvol-*pAzF*.⁴² The green to blue emission ratios of the *pAmF*-incorporated proteins, upon excitation at 385 nm, were used as the measures to evaluate the maximum FRET signals we could get upon H₂S-induced conversion of the azido groups to amino groups. Through the screening process, we identified a promising mutant showing a large green/blue emission ratio. This mutant was selected and tested with H₂S after incorporating *pAzF*. Results showed a > 1.7- fold of FRET ratio change (R/R_0) in response to 1 mM H₂S. We named this mutant hsFRET and performed more experiments to characterize it *in vitro* and in live cells.

Spectroscopic Responses of hsFRET to H₂S.

We next measured fluorescence spectral of hsFRET in response to various concentrations of H₂S (Figure 3A). Upon reaction with H₂S, hsFRET showed a decrease in emission intensity at 450 nm, and a concomitant increase of emission intensity at 500 nm, suggesting an increase of FRET from the donor to the acceptor in response to H₂S. The new peak formed at 500 nm upon addition of H₂S suggests that the *p*-azidobenzylideneimidazolidone chromophore was reduced to *p*-aminobenzylideneimidazolidone, and that excitation of EBFP2 at 385 nm caused an efficient energy transfer to this newly formed chromophore. This fact also suggests that the reduced *p*-aminobenzylideneimidazolidone chromophore is highly fluorescent.

To measure the kinetics of the reaction and determine the sensitivity of hsFRET to H₂S, we monitored the FRET ratio (green emission/blue emission) of hsFRET in response to various concentrations of H₂S. We found that the reaction was completed within 30 minutes (Figure 3B). The result also showed that upon reaction of hsFRET with 100 μ M or 1 mM H₂S, the ratio increased from 0.62 to 0.96 and 1.73, respectively (corresponding to R/R_0 of 54 % and 179%, respectively). To further investigate the relationship between the concentration of H₂S and the FRET signal, we plotted the ratio change of hsFRET upon treating it with different concentrations of H₂S from 1 to 100 μ M (Figure 3C). The graph showed a linear relationship between FRET ratios and the concentrations of H₂S, suggesting that the formation of the chromophore and the subsequent FRET are concentration-dependent.

Next, we tested the selectivity of hsFRET in response to different redox-active molecules commonly generated by cells, including thiols, and reactive oxygen, nitrogen, and sulfur

molecules at physiological or even higher concentrations (Figure 3D). In addition to H₂S, only hydroxyl radical and superoxide caused small responses. There was essentially no response observed for hsFRET to all other tested redox-active species, suggesting that hsFRET is a quite selective biosensor for H₂S.

hsFRET in live Mammalian Cells.

To validate the use of hsFRET for H₂S imaging in living mammalian cells, we transiently co-transfected HEK293T cells with pMAH-hsFRET and pMAH-POLY that contains an orthogonal tRNA/aminoacyl-tRNA synthetase pair for incorporation of *pAzF* into hsFRET in the presence of *pAzF*. The fluorescence of hsFRET filled both the cytosol and the nucleus, suggesting that there is no preferential accumulation of hsFRET in any of the other subcellular compartments (Figure S2). Upon treating cells with 100 μ M H₂S, a large increase in the FRET ratio of hsFRET was observed (Figures 4AD and Movie S1). Most changes occurred within 3 min and then the FRET ratio continued to rise slightly, resulting in a total of 100% change (R/R_0) in the FRET ratio. The larger dynamic range of the biosensor in mammalian cells as compared to the *in vitro* experiments, is also observed in the previously reported H₂S probes, hsGFP and cpGFP-*pAzF*.^{32,33} Better protein folding and maturation of *pAzF*-derived chromophore, in addition to higher fidelity of mammalian *pAzF* synthetase, have been proposed to account for this observation. Moreover, the responses of hsFRET reached the plateau faster in mammalian cells than *in vitro*, likely because H₂S degradation pathways were quickly activated in mammalian cells, resulting in no or slow changes after the initial, fast hsFRET responding phase.

We further treated cells with L-cysteine, which is a major precursor for biological production of H₂S. We observed a quick and robust, 50% increase (R/R_0) in FRET (Figures 4BE and Movie S2), supporting that hsFRET can detect biologically generated H₂S in mammalian cells. For comparison, we first treated cells with 50 mg/L DL-PPG, an inhibitor for H₂S producing enzymes,⁴⁴ and then applied L-cysteine to the cells. Not surprisingly, no obvious, L-cysteine-induced fluorescence increase was observed under this condition (Figures 4CF). In addition, we performed prolonged imaging of hsFRET-expressing HEK 293T cells that were untreated with any chemical (Figure S3), and the fluorescence showed excellent stability under our experimental conditions. Taken together, we demonstrate that hsFRET is a robust ratiometric probe for H₂S both *in vitro* and in mammalian cells.

CONCLUSION

To conclude, we developed hsFRET, the first genetically-encoded ratiometric biosensor for H₂S by combining genetic code expansion and the FP-based FRET technology. hsFRET showed selective, H₂S-induced FRET ratio change by modulation of the acceptor fluorescence intensity. When tested in live mammalian cells, hsFRET exhibited a large ratiometric response to both extracellular addition and endogenous generation of H₂S. hsFRET thus represents a valuable addition to the toolbox for H₂S detection and imaging. In addition, our sensor design and optimization strategy may be applied to derive similar FRET-based probes for other cellular reactive chemical species, such as peroxynitrite.³⁹

hsFRET, however, still has several limitations. First, short-wavelength violet light (405 nm) is used for excitation, so precautions should be taken to minimize light dosage and avoid phototoxicity. Also, although expression of hsFRET does not cause morphological changes of mammalian cells, cell viability and growth were slightly reduced likely due to the toxicity of suppression of amber codons (Figure S4). Moreover, the response of hsFRET is irreversible and accumulative, despite that hsFRET has its merits as ratiometric biosensors, including normalization to biosensor expression levels and insensitivity to excitation intensity or donor photobleaching. Furthermore, we used plasmid transfection to express hsFRET in mammalian cells. This protocol is incompatible with cells or cell lines that are difficult to transfect. To address this issue, our laboratory is working to optimize a baculoviral system⁴⁵ for expression of various unnatural-amino-acid-containing fluorescent protein biosensors and their subcellular localization variants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Illustration of the FRET change of hsFRET, a genetically encoded, ratiometric, dual-emission fluorescent biosensor. Upon addition of H_2S , the azido functional group of the modified cpsGFP chromophore is reduced to an amino group, resulting a green fluorescent chromophore for enhanced FRET from EBFP2. The chemical structures of the modified cpsGFP chromophore before and after conversion are shown. The basic tertiary structure schematic is adopted from Protein Data Bank entries 3EVP (grey and green) and 2B3P (blue).

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1                               31
FRET-0.1 MGSSTYNSHKVYITADKQKNGIKVNFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNH
FRET-0.2 MGSSTYNSHKVYITADKQKNGIKVNFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNH
FRET-0.3 MGSSTYNSNKVYITADKQKNGIKVNFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNH
hsFRET  MGSSTYNSNKVYITADKQKNGIKVNFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNH

61                               91
FRET-0.1 YLSTQSVLSKDPNEKRDHMLLEFVTAAGITLGMDELYKVDGGSGGTGVSKEEELFTGVV
FRET-0.2 YLSTQSVLSKDPNEKRDHMLLEFVTAAGITLGMDELYKVDGGSGGTGVSKEEELFTGVV
FRET-0.3 YLSVQSVLSKDPNEKRDHMLLEFVTAAGITLGMDELYKVDGGSGGTGVSKEEELFTGVV
hsFRET  YLSVQSVLSKDPNEKRDHMLLEFVTAAGITLGMDELYKVDGGSGGTGVSKEEELFTGVV

121                               151
FRET-0.1 PILVELDGDVNGHKFSVRGEGEGDATNGKLTLLKFICTTGKLPVPWPPTLVTTLTZGVQCFS
FRET-0.2 PILVELDGDVNGHKFSVRGEGEGDATNGKLTLLKFICTTGKLPVPWPPTLVTTLTZGVQCFS
FRET-0.3 PILVELDGDVNGHKFSVRGEGEGDATNGKLTLLKFICTTGKLPVPWPPTLVTTLTZGVQCFS
hsFRET  PILVELDGDVNGHKFSVRGEGEGDATNGKLTLLKFICTTGKLPVPWPPTLVTTLTZGVQCFS

181                               211
FRET-0.1 YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKED
FRET-0.2 YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKED
FRET-0.3 YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKED
hsFRET  YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKED

241
FRET-0.1 GNILGHKLEYNTHHGGSKLMVSKGEEELFTGVV .....EBFP2 .....
FRET-0.2 GNILGHKLEYNTHH-----KLEELFTGVV .....EBFP2 .....
FRET-0.3 GNILGHKLEYNTHH-----KLEELFTGVV .....EBFP2 .....
hsFRET  GNILGHKLEYNMHH-----KLEELFTGVV .....EBFP2 .....

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Figure 2.

Sequence alignment of hsFRET and other different designs developed in this study. Residues forming the modified cpsGFP chromophores are boxed in green. *pAzF* is shown as Z (colored green). The mutations of hsFRET from FRET-0.1 are colored in cyan. The full sequence of EBFP2 is not shown since there is no mutation in this fragment.

Figure 3.

Spectroscopic responses of hsFRET to H₂S. **(A)** Fluorescence emission spectra of hsFRET (1 μM) in response to various concentrations of H₂S (H₂S concentrations are 1 mM, 100 μM, 50 μM, 20 μM, and 0 μM from top to bottom) with arrows indicating the decrease of emission at 450 nm and the increase of emission at 500 nm. **(B)** Kinetic changes of fluorescence ratios (green emission/blue emission) of hsFRET (1 μM) in response to 1 mM H₂S, 100 μM, 50 μM, 20 μM, and 0 μM from top to bottom **(C)** Dose-dependent changes of FRET ratios (green emission/blue emission) of hsFRET (1 μM) to H₂S. Measurements were performed at 30 min post addition of H₂S. **(D)** Chemoselectivity of hsFRET against various redox-active chemicals: (1) Tris-HCl, (2) 1 mM H₂O₂, (3) 100 μM HOCl, (4) 100 μM HOOtBu, (5) •OH (1 mM Fe²⁺ and 100 μM H₂O₂), (6) •OtBu (1 mM Fe²⁺ and 100 μM HOOtBu), (7) 1 mM Vitamin C, (8) 5 mM L-cysteine, (9) 100 μM O₂^{•-}, (10) 100 μM ONOO⁻, (11) 100 μM NOC-5 (NO• donor), (12) 100 μM H₂S, and (13) 1 mM H₂S.

Figure 4.

Use of hsFRET to image H_2S in mammalian cells. (A–C) Pseudocolor ratiometric images of HEK 293T cells treated with (A) 100 μM H_2S (NaHS in DPBS, pH 7.4 as the donor), (B) 100 μM L-cysteine, or (C) 50 mg/L DL-PPG for 20 min, followed by 100 μM L-cysteine. (D–F) Quantification results for panels A–C as the mean and s.d. of all imaged cells from three independent replicates. FRET ratios were normalized to the value at $t = 0$ min.