Hydropersulfides (R–SSH) are generated by different pathways in biological systems. It has been reported that hydrogen persulfide (H\textsubscript{2}S\textsubscript{2}) can be formed by the oxidation of endogenous H\textsubscript{2}S by reactive oxygen species (ROS).\textsuperscript{14} Akaike found that CysSSH is biosynthesized from cystine (CysS-Cys) by two major enzymes: cystathionine \( \beta \)-synthetase (CBS) and cystathionine \( \gamma \)-lyase (CSE).\textsuperscript{1,15} He also proposed that the enzymatically generated CysSSH is converted to GSH-based hydroper-/hydro polysulfides (e.g., GSSH, GSSSSH, \textit{etc.}) through persulfide interchange reactions. Meanwhile, Banerjee proposed that sulfide oxidation pathways in mitochondria are the important source of RSS, such as GSSH.\textsuperscript{16} Despite the extensive study of the hydropersulfide formation pathways, regulation of their levels in cells, especially their reducing mechanism, remains largely elusive. Recent reports proposed thioredoxin (Trx) as an important enzyme that reduces CysSSH, although clear evidence for this process has not yet been provided.\textsuperscript{17}

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To understand the varied roles of hydropersulfides in biological systems, it is critical to develop a new analytical tool that allows us to detect the formation and consumption of these RSS species. Fluorescent probes which are available for real-time cell imaging could meet this requirement.\textsuperscript{18–21} In this regard, Xian \textit{et al.} recently reported a series of selective fluorescent probes for hydrosulfide/hydrosulfides (H\textsubscript{2}S\textsubscript{n}, \( n \geq 1 \)).\textsuperscript{18–20} They ingeniously exploited the high nucleophilic activity of H\textsubscript{2}S\textsubscript{n} to develop reaction-based turn-on fluorescence probes, which were successfully applied to the visualization of intracellular H\textsubscript{2}S\textsubscript{n}. However, due to the irreversible nature of the reactions, it was intrinsically difficult to monitor reversible concentration...
dynamics of intracellular H$_2$S, using these probes. In this paper, we report the development of a ratiometric fluorescent probe for detecting hydropersulfides, based on intramolecular fluorescence resonance energy transfer (FRET) (Fig. 1A).

The sensing mechanism of this probe involves a reversible nucleophilic attack of a highly reactive hydropersulfide species on the pyrnone fluorophore. This adduct formation disrupts the conjugation structure of the xanthene ring, decreasing the intramolecular FRET efficiency due to a change in the spectral overlap between the coumarin fluorescence (FRET donor) and the xanthene absorbance (FRET acceptor), which causes a clear dual-emission signal change. Taking advantage of this reversible sensing property, the probe was successfully applied to detect the concentration dynamics of hydropersulfides in living cells, demonstrating the utility of the probe as a chemical tool in RSS research.

Results and discussion

Molecular design of the probe

In the previous study, we reported that the fluorescence of the xanthene derivative 1 significantly decreased upon addition of a large excess of glutathione (GSH, ~10 mM) under neutral aqueous conditions. The spectroscopic analyses revealed that xanthene 1, which lacks a C9 aromatic substituent unlike fluorescein, was susceptible to nucleophilic attack by thiol species and readily converted to a non-fluorescent adduct. Since hydropersulfides (RSSHs) are more nucleophilic than stable thiols such as GSH and H$_2$S, we thought that this reaction-based fluorescence quenching could be exploited for selective detection of hydropersulfides. As an initial attempt, we synthesized a series of xanthene derivatives bearing the different substituents (Fig. 1B), and evaluated their fluorescence responses toward several biological thiol species. The results are summarized in Fig. 1C and S1.† Compound 1 showed a marked decrease in fluorescence ($F/F_0 = 40\%$) upon treatment with 50 mM sodium disulfide (Na$_2$S$_2$), the extent of which is much larger than that induced by addition of the same concentration of Na$_2$S ($F/F_0 = 93\%$) and a high concentration (1 mM) of cysteine ($F/F_0 = 91\%$). However, 1 also responded to a biologically relevant concentration of GSH (5 mM) with a high quenching efficiency ($F/F_0 = 56\%$), indicative of the low selectivity of 1 among biologically relevant thiols. The rhodol-type compound 2 exhibited a rather non-selective weak fluorescence response to the thiol species. The pyrnone-type compound 3,
Scheme 1  Synthesis of probe 6.

Fig. 3  (A) The ratio value \( R = F_{479 \text{ nm}} / F_{584 \text{ nm}} \) of 6 in the presence of various thiol species: (1) none, (2) Na₂S (50 μM), (3) Na₂S₂ (50 μM), (4) Na₂S₄ (50 μM), (5) CySSSH (NOC7 (50 μM) + Na₂S (50 μM) + L-Cys (50 μM)), (6) GLSS (NOC7 (50 μM) + Na₂S (50 μM) + GSH (50 μM)), (7) mixture of NaOCl (50 μM) and Na₂S (50 μM) in 0.1 M NaOH, (8) mixture of NaOCl (50 μM) and Na₂S (50 μM) in 50 mM HEPES buffer (pH 7.4), (9) GSH (5 mM), (10) L-Cys (1 mM), (11) cystine (0.5 mM). Measurement conditions: \( [6] = 5 \mu M \) in 50 mM HEPES, 10 mM NaCl, 1 mM MgSO₄, 0.4% Tween, pH 7.4, 25 °C. \( \lambda_{ex} = 410 \text{ nm} \). 

Fig. 4  (A) Fluorescence images of A549 cells treated with 6-AM (5 μM). (a) F₄30–480, (b) F₅50–630, (c) DIC, and (d) ratio image \( R = F_{430–480} / F_{550–630} \). (e and f) Ratio image after 30 min in the presence and absence of Na₂S₂ (5 μM), (g and h) ratio image after 15 min in the presence Na₂S₂ (5 μM) and subsequent treatment with NEM (100 μM) for 15 min. Scale bar: 30 μm. 

\( R = F_{479 \text{ nm}} / F_{584 \text{ nm}} \) of 6 induced by the reactive species. Each bar represents \( R \) value of: (1) 6 (5 μM), (2) the hydropersulfide adduct of 6 (5 μM) with Na₂S₂ (50 μM), (3) the adduct + NaOCl (100 μM), (4) the adduct + NEM (200 μM), (5) the adduct + GSH (5 mM), and (6) the adduct + L-Cys (1 mM). Measurement conditions: 50 mM HEPES, 10 mM NaCl, 1 mM MgSO₄, 0.4% Tween, pH 7.4, 25 °C. \( \lambda_{ex} = 410 \text{ nm} \). n = 3; *P < 0.05, **P < 0.01 vs. hydropersulfide adduct of 6 with Na₂S₂ (lane 2).
possessing two six-membered piperidine rings, is highly susceptible to thiol species with significant fluorescence quenching efficiencies ($F/F_0 < 30\%$), except for 1-cysteine. However, pyronines 4 and 5, which possess one and two five-membered pyrrolidine rings, respectively, showed selective fluorescence responses ($F/F_0 = 6\%$ and $56\%$, respectively) toward Na$_2$S$_2$ (50 μM). The formation of the H$_2$S$_2$ adduct with 5 was confirmed by a $^1$H-NMR experiment (Fig. S2†). The fluorescence response of 4 and 5 toward Na$_2$S$_2$ was further evaluated by titration with different concentrations of Na$_2$S$_2$. As shown in Fig. 1D and S3† 4 was more sensitive than 5 and showed a substantial decrease in fluorescence with a low concentration of Na$_2$S$_2$ (below 10 μM). The varied fluorescence response of these pyronine-type probes, depending on the substituents, would be reasonably explained by the different electron donating abilities of the cyclic amines. That is, the five-membered pyrrolidine can act as a stronger electron donating substituent than the six-membered piperidine, so that the tolerance to nucleophilic attack by Na$_2$S$_2$ is in the order of 5 > 4 > 3.
We selected pyronine 4 as a fluorescent subunit of the ratiometric probe for hydropersulfides, on account of its selective and sensitive detection of Na₂S₂, as shown in Fig. 1. The structure of the newly designed dual-emission probe 6 is shown in Fig. 2A. The probe possesses a coumarin as the FRET donor, which is conjugated to a pyronine unit as the FRET acceptor through a rigid linker. The two carboxylate groups are introduced into the coumarin unit in order to increase the hydrophilicity of the probe, which prevents its leakage from cells during imaging experiments. The synthesis of probe 6 is shown in Scheme 1. The radical bromination of 7 with N-bromosuccinimide (NBS) and the subsequent nucleophilic reaction with N-Boc-piperazine yielded 8. After the deprotection, 8 was converted to bis-triflate 10, which was sequentially reacted with pyrrolidine and piperidine to give 11 as a mixture of the substitution isomers. The keto-reduction of 11 with borane-SMe₂ and the subsequent oxidation using DDQ yielded the pyronine 13. After the removal of the Boc group, 13 was subjected to a conjugation reaction with the N-hydroxysuccinimide ester of coumarin 14 to give 15. Finally, the deprotection of the tert-butyl ester groups of 15 and the following HPLC purification provided 6 as a mixture of the isomers.

Ratiometric fluorescence sensing of hydropersulfides

The functional analysis of probe 6 was initially conducted in a neutral aqueous solution. A solution of 6 (5 μM) in 50 mM HEPES buffer (pH 7.4) showed two distinct UV peaks at 400 nm and 562 nm (Fig. 2B), which correspond to the absorbance of coumarin and pyronine, respectively. Upon titration with sodium disulfide Na₂S₂ (0–100 μM), the absorbance at 562 nm decreased gradually to ca. 25% of its original intensity, whereas the absorbance at 400 nm scarcely changed. In the fluorescence spectrum, a solution of 6 (5 μM) showed two distinct emissions at 479 nm and 584 nm due to the coumarin and pyronine units, respectively, when excited at 410 nm (Fig. 2C). This dual-emission spectrum changed dramatically in a see-saw manner upon addition of Na₂S₂ (0–100 μM). The large decrease in emission at 584 nm and the concomitant increase in emission at 479 nm strongly suggest that FRET between the coumarin and xanthene units is cancelled as a result of a decrease in the spectral overlap between the coumarin emission and the pyronine absorption (Fig. S4†). The FRET efficiency of 6 was calculated to be 60% in the initial state, which decreased to 31% in the presence of 100 μM Na₂S₂. Fig. 2D shows the time-lapse detection of the fluorescence response of 6 toward Na₂S₂. The ratio value R (F₄70 nm/F₅84 nm) rapidly increased upon addition of Na₂S₂ (0–30 μM) and reached a plateau almost within 60 s. The plot of R value against the concentration of Na₂S₂ (0–30 μM) shows a linear relationship (Fig. 2E), indicative of the highly quantitative nature of the ratiometric detection of hydropersulfides using 6. Probe 6 was able to detect as low as 1.0 μM Na₂S₂ based on the calculation of the detection limit (3σ). This sensitivity is sufficiently high for detection of intracellular hydropersulfides, the concentration of which was reported to be around ten micromolar under basal conditions.¹

The sensing selectivity of 6 for various biologically relevant thiol species was evaluated (Fig. 3A). In contrast to the large increase in R value induced by Na₂S₂ (lane 3), 6 showed a negligible fluorescence response upon addition of the same concentration of Na₂S (lane 2). This sensing selectivity is reasonably ascribed to the lower pKₐ value of H₂S₂ (pKₐ = 5.0) compared to that of H₂S (pKₐ = 6.9), rendering H₂S₂ highly nucleophilic as a thiolate anion (HS⁻) under neutral conditions (pH = 7.4) (Fig. 3B).²⁶ A moderate R value change was observed upon addition of hydroxylsulfide Na₃S₄ (lane 4), though this change was apparently smaller than that induced by Na₂S₂ (lane 3). The weak fluorescence response of 6 for Na₃S₄ might be ascribed to the rather poor nucleophilic activity of the HS⁻ anion due to its extremely low pKₐ value (pKₐ = 3.8).²⁶ Probe 6 also showed a moderate increase in the R value upon treatment with CysSSH (lane 5) and GSSH (lane 6), which were generated in situ from Cys and GSH, respectively, by the reaction with Na₂S and NO donor (NOC7).³ In a similar manner, the mixture of Na₂S (50 μM) and NaOCl (50 μM) in a basic solution (0.1 M NaOH), which generates Na₂S₅ in situ, induced a moderate increase of R value (lane 7), while their mixture in a neutral solution (50 mM HEPES, pH 7.4), which mainly produces Na₂S₄ and Na₂S₅,⁴ resulted in a small increase in the R value (lane 8). This difference in signal change is consistent with the results obtained by the direct titration with these thiols (lanes 3 and 4). Probe 6 showed a negligible R value change upon addition of biologically relevant concentrations of GSH (5 mM, lane 9), l-cysteine (1 mM, lane 10), and cystine (0.5 mM, lane 11). It is noteworthy that the sensing selectivity of 6 for Na₂S₂ is apparently higher than that of 5 among these thiol species (Fig. 1C), probably due to steric hindrance and/or the electron configuration effect of the coumarin unit conjugated to the pyronine unit of 6. Therefore, 6 was able to detect Na₂S₂ with a sufficient sensitivity (detection limit = 4.4 μM) even in the presence of 5 mM of GSH (Fig. S5†). We also confirmed that 6 scarcely responded to various redox-relevant compounds, including ROS such as NaOCl and H₂O₂, reactive nitrogen species (NOS), ascorbic acid, and KCN (Fig. S6†).

All of these data suggest that probe 6 primarily serves as a selective fluorescent probe for hydropersulfides. It was confirmed that 6 did not show a significant ratio change over the physiological pH range (5.0 to 8.5; Fig. S7†).

The fluorescence response of 6 towards a change in the hydropersulfide level was examined. As shown in Fig. 3C, the R value of 6 (5 μM) increased stepwise upon the repeated addition of 30 μM Na₂S₂, and reached a plateau almost within 60 s. The subsequent addition of N-ethylmaleimide (NEM, 500 μM) to consume Na₂S₂ induced a large decrease in the R value. These data clearly indicate that 6 can reversibly change its R value depending on the concentration of hydropersulfide according to the binding equilibrium shown in Fig. 1A. The reverse fluorescence response of 6 was further evaluated upon addition of other reactive species (Fig. 3D). Addition of NaOCl (100 μM) to the mixed solution of 6 (5 μM) and Na₂S₂ (50 μM) also induced a significant decrease in the R value (lane 3), as observed with NEM (lane 4). This change is reasonably ascribed to the decrease in Na₂S₂ level due to the formation of oxidized sulfurs.
and hydro polysulfides ($H_2S_n$, $n > 2$). Conversely, a small change in the $R$ value was induced upon addition of GSH (5 mM, lane 5) and l-Cys (1 mM, lane 6), suggesting that these biologically abundant thiols do not inter- 

serve the Ratiometric fluorescence imaging of hydropersulfides (R-SSH) in living cells. For cell imaging, 6 was chemically modified with acetoxyethyl (AM) groups to enhance its membrane permeability. It was expected that the AM-modified probe, 6-AM (Fig. 2A), could be readily hydrolyzed by intracellular esterases to liberate 6, which is unlikely to leak from cells due to its highly polar character. When A549 cells were treated with 6-AM (5 mM) for 20 min, bright fluorescence of the probe was observed in the cytosolic region of the cells (Fig. 4A(a–d)). A cell viability assay revealed that 6-AM showed low cytotoxicity to A549 cells at a concentration below 25 μM (Fig. S8T). Addition of 5 μM Na$_2$S$_2$ to the cell medium induced an obvious change in the fluorescence ratio ($R = 430–480$ nm/550–630 nm) (Fig. 4A(e)) of cells, relative to that observed in the control experiment without Na$_2$S$_2$ treatment (Fig. 4A(f)). Titration of the 6-AM pre-stained cells with Na$_2$S$_2$ (0–5 μM) showed a linear dependence between Na$_2$S$_2$ concentration and $R$ value (Fig. 4B and S9T), demonstrating the accurate hydropersulfide sensing property of 6 in living cells. The time-lapse imaging revealed that the $R$ value increased immediately after the addition of 5 μM Na$_2$S$_2$ and reached a steady state ($R = \sim 1.4$) after 10 min (Fig. 4C). The subsequent treatment of the cells with 100 μM N-ethylmaleimide (NEM) induced a significant decrease in the $R$ value (Fig. 4Ag, h) and C) due to the decrease of the hydropersulfide level by the nucleophilic reaction with NEM, demonstrating the reversible sensing property of 6 in living cells.

The probe 6-AM was further applied to the ratiometric detection of endogenous hydropersulfides produced by enzymes in living cells. It has been reported that CSE and CBS are the major enzymes responsible for generation of cysteine hydropersulfide (CysSSH) from cystine (CysSSCys) in A549 cells. When A549 cells, pre-stained with 6-AM, were treated with cystine (CysSSCys), a gradual increase in the $R$ value was observed (Fig. 5B and C). This change was effectively suppressed on treatment of the cells with aminooxycetic acid (AOAA), an inhibitor of CSE and CBS (Fig. 5B and D, lane 3). Conversely, treatment of the cells with auranofin, an inhibitor for thioredoxin reductase (TrxR), induced a statistically significant increase in the $R$ value (Fig. 5D, lane 4 and S10T), suggesting an increase in the intracellular hydropersulfide level as a result of the inhibition of Trx activation by TrxR (Fig. 5A). This result is consistent with the recent report that Trx catalyses reduction of Cys-SH as a major regulator of its intracellular level. It has been reported that hydropersulfide is also produced in living cells by the oxidation of $H_2S$, which is generated from l-Cys by CSE and CBS. To confirm this point, fluorescence imaging of A549 cells treated with l-Cys (200 μM) was performed using 6-AM. As shown in Fig. 5E and F, the $R$ value largely increased in a time-dependent manner in the living cells. Treatment of the cells with AOAA effectively suppressed the increase in $R$ value induced by l-Cys (Fig. 5E and G, lane 3). Unlike the case of the cysteine experiment (Fig. 5G), inhibition of TrxR by auranofin did not cause an increase in the $R$ value (Fig. 5G, lane 4 and S11T), implying that the direct conversion of l-Cys to CysSSH is not a major pathway of the hydropersulfide formation in living cells. All of these data suggest that hydropersulfides are generated from l-Cys through the enzyme-mediated $H_2S$ formation and subsequent oxidation in living cells. Finally, the addition of high concentration NaClO (300 μM) induced a significant decrease in the $R$ value in cells (Fig. 5H and S12T), indicating that the decrease in hydropersulfide level was a result of its oxidative degradation to oxidized sulfurs and hydrogen poly- sulfides ($H_2S_n$, $n > 2$).

**Conclusion**

In conclusion, we have developed a ratiometric fluorescent probe 6 that can visualize the endogenously produced hydropersulfides in living cells. To our knowledge, probe 6 is the first example of a ratiometric fluorescent probe that can reversibly detect intracellular hydropersulfide levels. Since the research field of reactive sulfur species (RSS) is still in its infancy, probe 6 would serve as a versatile analytical tool, not only for understanding the chemical nature of hydropersulfide in biological systems, but also for elucidating the roles of hydropersulfide in cell signalling and redox homeostasis. For this purpose, further functional improvements in probe 6, which include sensing selectivity for a single hydro polysulfide species and/or localization ability to a certain cell compartment, would be desirable to realize more precise and quantitative analysis of RSS. Research along these lines is currently underway in our laboratory.

**Experimental section**

**Synthesis and characterization of the compounds**

The syntheses and characterization of probes 1–6 and 6-AM are described in the ESL†

**Fluorescence measurement**

Fluorescence titration was conducted with a solution (3 mL or 0.5 μL) of the probe in a quartz cell. Typically, a freshly prepared aqueous stock solution of Na$_2$S$_2$ was added to a solution of 6 (5 μM) in 50 mM HEPES, 10 mM NaCl, 1 mM MgSO$_4$, pH 7.4 with 0.4% Tween at 25 °C and the fluorescence emission spectra were measured after 10 min (λex = 410 nm) with a Perkin Elmer LS55 fluorescence spectrometer.

**Cell culture**

A549 cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic-antimycotic solution (Gibco) at 37 °C under a humidified atmosphere of 5% CO$_2$ in air. A subculture was performed every 3–4 days from subconfluent (<80%) cultures using a trypsin-EDTA solution.
For the fluorescence bioimaging, cells were cultured for 2 days in a 35 mm glass-bottomed dish (Iwaki Scitech).

**Fluorescence imaging of exogenous H$_2$S in A549 cells**

Fluorescence imaging was conducted with a confocal laser scanning microscope (LSM 780, Zeiss) equipped with a 63× objective lens. The following detection channels were chosen for the ratiometric imaging; Ch1 $\lambda_{ex} = 405$ nm, $\lambda_{em} = 430-480$ nm, and Ch2 $\lambda_{ex} = 405$ nm, $\lambda_{em} = 550–630$ nm. In a glass-based dish, A549 cells in HBS buffer (10 mM NaCl, 6 mM KCl, 1.2 mM MgSO$_4$, 2.0 mM CaCl$_2$, 11.5 mM glucose, 20 mM HEPES, pH 7.4) were incubated with 6-AM (5 μM) for 20 min at 37 °C under a humidified atmosphere of 5% CO2 in air. After removal of excess probe and washing with HBS buffer, the cells were treated with Na$_2$S$_2$ (0–5 μM, final conc.) and subjected to the fluorescence imaging. For the imaging of the endogenously produced hydrosulfides, A549 cells, pre-stained with 6-AM (5 μM), were treated with cysteine (200 μM) or l-cysteine (200 μM). For inhibition of CSE and CBS enzymes, the cells were pre-treated with AOAA (aminooxycetic acid, 1 mM, Sigma) in HBS buffer for 1 h before staining with 6-AM. For inhibition of TrxR, the cells were pre-treated with auranofin (2 μM, Wako) in HBS buffer for 1 h before staining with 6-AM.

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**Notes and references**

1 T. Ida, T. Sawa, H. Ihara, Y. Tsuchiya, Y. Watanabe, Y. Kumagai, M. Suematsu, H. Motoshahi, S. Fujii, T. Matsunaga, M. Yamamoto, K. Ono, N. O. Devarie-Baez, M. Xian, J. M. Fukuto and T. Akaike, Proc. Natl. Acad. Sci. U. S. A., 2014, **111**, 7606–7611.

2 B. D. Paul and S. H. Snyder, Nat. Rev. Mol. Cell Biol., 2012, **13**, 499–507.

3 C. E. Paulsen and K. S. Carroll, Chem. Rev., 2013, **113**, 4633–4679.

4 T. V. Mishanina, M. Libiad and R. Banerjee, Nat. Chem. Biol., 2015, **11**, 457–464.

5 A. K. Mustafa, M. M. Gadalla, N. Sen, S. Kim, W. Mu, S. K. Gazi, R. K. Barrow, G. Yang, R. Wang and S. H. Snyder, Sci. Signaling, 2009, 2, ra72.

6 M. R. Filipovic, J. L. Miljkovic, T. Nauser, M. Royzen, K. Klos, T. Shubina, W. H. Koppenol, S. J. Lipard and I. Ivanovic-Burmazovic, J. Am. Chem. Soc., 2012, **134**, 12016–12027.

7 D. Zhang, I. Macinkovic, N. O. Devarie-Baez, J. Pan, C.-M. Park, K. S. Carroll, M. R. Filipovic and M. Xian, Angew. Chem., Int. Ed., 2014, **53**, 573–581.

8 N. Sen, B. D. Paul, M. M. Gadalla, A. K. Mustafa, T. Sen, R. Xu, S. Kim and S. H. Snyder, Mol. Cell, 2012, **45**, 13–24.

9 M. Nishida, T. Sawa, N. Kitajima, K. Ono, H. Inoue, H. Ihara, H. Motoshahi, M. Yamamoto, M. Suematsu, H. Kurose, A. van der Vliet, B. A. Freeman, T. Shibata, K. Uchida, Y. Kumagai and T. Akaike, Nat. Chem. Biol., 2012, **8**, 714–724.

10 S. Koike, Y. Ogasawara, N. Shibuya, H. Kimura and K. Ishii, FEBS Lett., 2013, **587**, 3548–3555.

11 T. V. Mishanina, P. K. Yadav, D. P. Ballou and R. Banerjee, J. Biol. Chem., 2015, **290**, 25072–25080.

12 Y. Kimura, Y. Mikami, K. Osumi, M. Tsugane, J. Oka and H. Kimura, FASEB J., 2013, **27**, 2451–2457.

13 R. Greiner, Z. Pálinkás, K. Bässell, D. Becher, H. Antelmann, P. Nagy and T. P. Dick, Antioxid. Redox Signaling, 2013, **19**, 1749–1765.

14 P. Nagy and C. C. Winterbourn, Chem. Res. Toxicol., 2010, **23**, 1541–1543.

15 P. K. Yadav, M. Martinov, V. Vitvitsky, J. Seravalli, R. Wedmann, M. R. Filipovic and R. Banerjee, J. Am. Chem. Soc., 2016, **138**, 289–299.

16 M. Libiad, P. K. Yadav, V. Vitvitsky, M. Martinov and R. Banerjee, J. Biol. Chem., 2014, **289**, 30901–30910.

17 R. Wedmann, C. Onderka, S. Wei, I. András Szijártó, J. Miljkovic, A. Femic, M. Lange, S. Savitsky, P. Kumar, R. Torregrossa, E. G. Harrer, T. Harrer, I. Ishii, M. Gollasch, M. E. Wood, E. Galardon, M. Xian, M. Whiteman, R. Banerjee and M. Filipovic, Chem. Sci., 2016, 7, 3414–3426.

18 W. Chen, C. Liu, B. Peng, Y. Zhao, A. Pacheco and M. Xian, Chem. Sci., 2013, **4**, 2892–2896.

19 C. Liu, W. Chen, W. Shi, B. Peng, Y. Zhao, M. Xian, H. Ma and M. Xian, J. Am. Chem. Soc., 2014, **136**, 7257–7260.

20 W. Chen, E. W. Rosser, T. Matsunaga, A. Pacheco, T. Akaike and M. Xian, Angew. Chem., Int. Ed., 2015, **54**, 13961–13965.

21 L. Zeng, S. Chen, T. Xia, W. Hu, C. Li and Z. Liu, Anal. Chem., 2015, **87**, 3004–3010.

22 M. Gao, F. Yu, H. Chen and L. Chen, Anal. Chem., 2015, **87**, 3631–3638.

23 X. Han, F. Yu, X. Song and L. Chen, Chem. Sci., 2016, **7**, 5098–5107.

24 J. R. Lakowicz, Principles of Fluorescence Spectroscopy, Springer, New York, 3rd edn, 2006.

25 R. Kawagoe, I. Takashima, K. Usui, A. Kanegae, Y. Ozawa and A. Ojida, ChemBioChem, 2015, **16**, 1608–1615.

26 K. Ono, T. Akaike, T. Sawa, Y. Kumagai, D. A. Wink, D. J. Tantillo, A. J. Hobbs, P. Nagy, M. Xian, J. Lin and J. M. Fukuto, Free Radical Biol. Med., 2014, **77**, 82–94.

27 R. Y. Tsien, Nature, 1981, **290**, 527–528.

28 A. Asimakopoulou, P. Panopoulos, C. T. Chasapis, C. Coletta, Z. Zhou, G. Cirino, A. Giannisi, C. Szabo, G. A. Spyroulias and A. Papapetropoulos, Br. J. Pharmacol., 2013, **169**, 922–932.