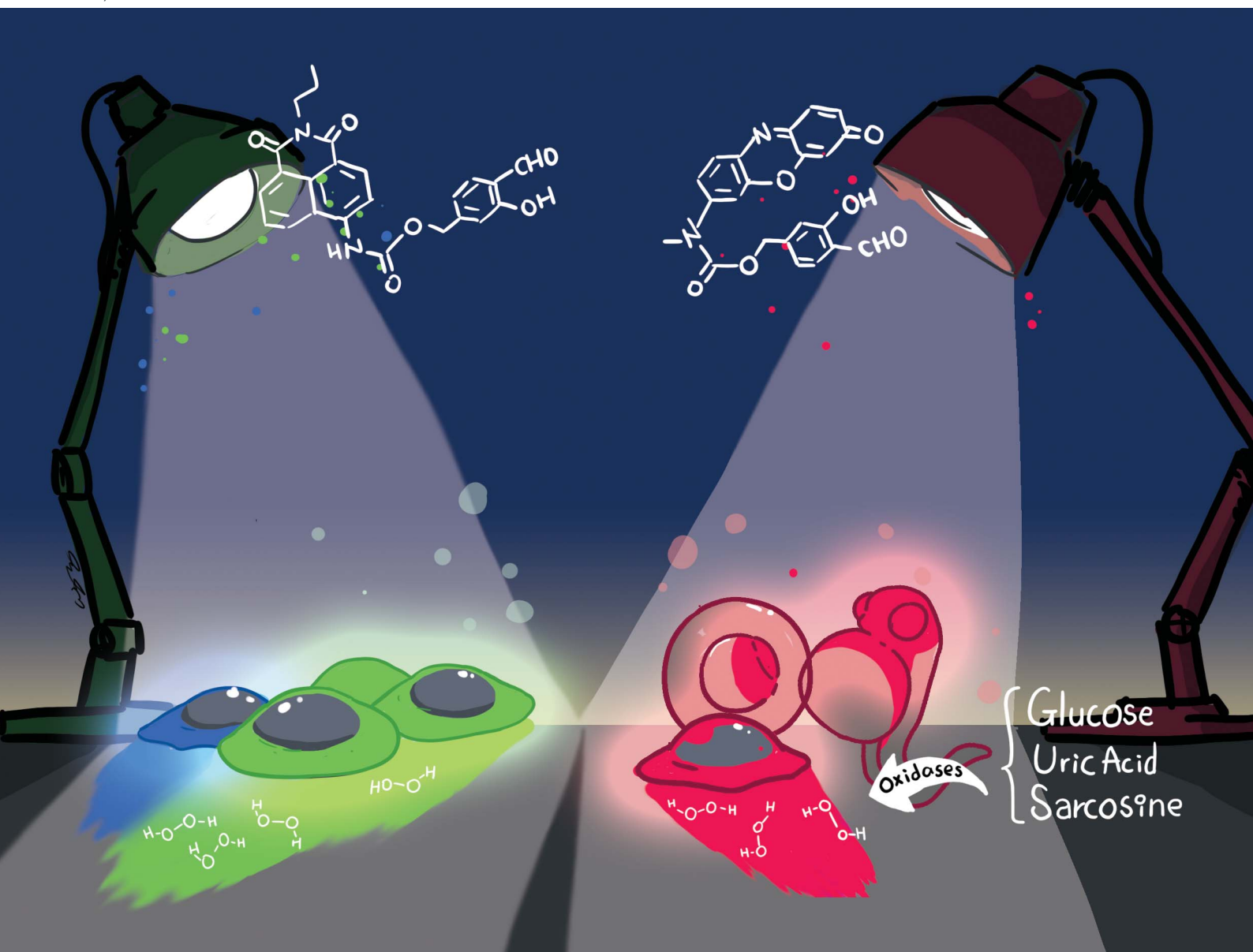


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## Fluorescent probes for *in vitro* and *in vivo* quantification of hydrogen peroxide†

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Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) plays essential roles in redox signaling and oxidative stress, and its dynamic concentration is critical to human health and diseases. Here we report the design, syntheses, and biological applications of HKPerox-Red and HKPerox-Ratio for quantitative measurement of H<sub>2</sub>O<sub>2</sub>. Both probes were successfully applied to detect endogenous H<sub>2</sub>O<sub>2</sub> fluxes in living cells or zebrafish, and biological effects of multiple stress inducers including rotenone, arsenic trioxide, and starvation were investigated. As H<sub>2</sub>O<sub>2</sub> is a common by-product for oxidase oxidation, a general assay was developed for ultrasensitive detection of various metabolites (glucose, uric acid, and sarcosine). Moreover, cellular H<sub>2</sub>O<sub>2</sub> measurements were achieved for the first time by combining flow cytometry with live cell calibration. This study provides a pair of unique molecular tools for advanced H<sub>2</sub>O<sub>2</sub> bio-imaging and assay development.

### Introduction

Oxygen fuels energy production processes in aerobic organisms through respiration, while partial reduction of oxygen produces reactive oxygen species (ROS) as by-products of oxygen metabolism.<sup>1</sup> Among all ROS, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) stands out as the most significant messenger molecule for redox signaling due to its relatively long half-life and diffusion range.<sup>2</sup> Cellular H<sub>2</sub>O<sub>2</sub> is constantly produced with a steady-state concentration (~0.1 μM) to signal cell growth and proliferation, whereas higher levels of H<sub>2</sub>O<sub>2</sub> (e.g. ~100 μM) trigger cell growth arrest or apoptosis.<sup>2b,3</sup> Quantitative analysis of H<sub>2</sub>O<sub>2</sub> in biological content is key to understanding its multiple roles in redox signaling and oxidative stress. However, there is no method currently available for non-invasive, sensitive, and precise measurement of H<sub>2</sub>O<sub>2</sub> in intact cells, and previous H<sub>2</sub>O<sub>2</sub> quantifications relied heavily on electrochemical analysis or peroxidase-based H<sub>2</sub>O<sub>2</sub> assay, where only extracellular H<sub>2</sub>O<sub>2</sub> secreted from cells could be measured accurately.<sup>4</sup>

A powerful tool to investigate cellular H<sub>2</sub>O<sub>2</sub> fluxes is fluorimetry (e.g. confocal imaging and flow cytometry) due to its high sensitivity, non-invasiveness and spatio-temporal resolution.<sup>5,6</sup> While several genetically encoded H<sub>2</sub>O<sub>2</sub> sensors have been developed for H<sub>2</sub>O<sub>2</sub> detection with unparalleled dynamic subcellular precision, they may suffer from pH sensitivity, poor

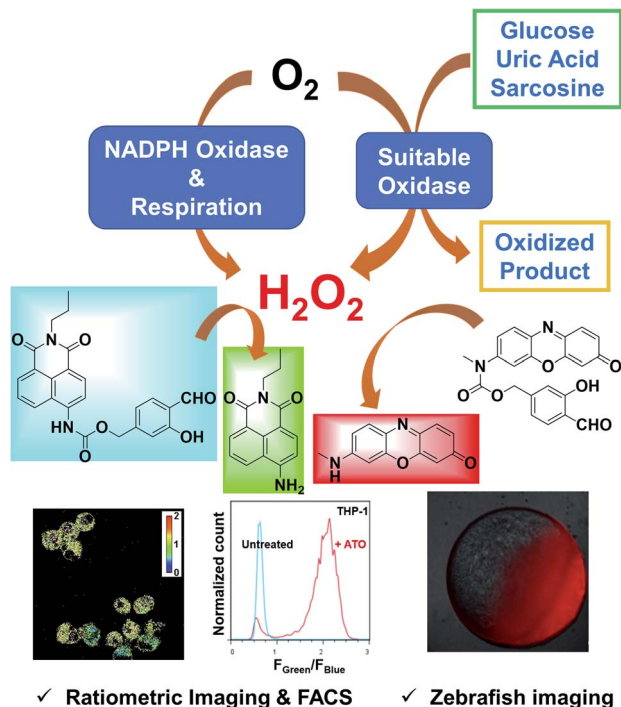
brightness, limited responses and selectivity toward H<sub>2</sub>O<sub>2</sub>.<sup>7</sup> Nearly all chemosensors for H<sub>2</sub>O<sub>2</sub> detection are based on small-molecule dyes, including traditional dichloro-dihydrofluorescein diacetate (DCFH-DA, a commonly used unselective ROS probe) and selective probes, especially activity-based sensing,<sup>5c</sup> including boronate oxidation,<sup>5a,8</sup> Baeyer-Villiger reaction,<sup>5b,9</sup> tandem Payne/Dakin reaction,<sup>10</sup> and very recently Mislow-Evans rearrangement.<sup>11</sup> Those intensity-based probes are very sensitive, but they are prone to signal fluctuations arising from variations in probe uptake, distribution, or even optical inputs. Therefore, ratiometric sensing with an internal standard for calibration is more desirable for quantitative measurement of H<sub>2</sub>O<sub>2</sub>,<sup>12</sup> however, most of current ratiometric probes (including both biosensors and chemosensors) still suffer from low selectivity, low sensitivity, as well as slow response, thereby limited biological applications.<sup>7,13</sup>

To address those challenges, we herein report a pair of new fluorescent probes, **HKPerox-Red** and **HKPerox-Ratio** (Scheme 1). Both probes feature excellent selectivity and sensitivity for unambiguous H<sub>2</sub>O<sub>2</sub> detection and quantification in aqueous solution. Moreover, the red emissive **HKPerox-Red** allows imaging endogenous H<sub>2</sub>O<sub>2</sub> fluxes in living cells and zebrafish embryos, by virtue of its outstanding permeability. **HKPerox-Red** was further utilized to develop a general *in vitro* assay for ultrasensitive detection of various biomarkers and respective oxidases (glucose/glucose oxidase, uric acid/urate oxidase, sarcosine/sarcosine oxidase), since H<sub>2</sub>O<sub>2</sub> is quantitatively produced during those enzymatically catalyzed oxidation reactions. As a result of its nanomolar sensitivity, **HKPerox-Red** could be used for accurate measurement of glucose with 1000 times diluted serum (1 μL to 1 mL), which provides a new method for non-invasive glucose detection with high precision.

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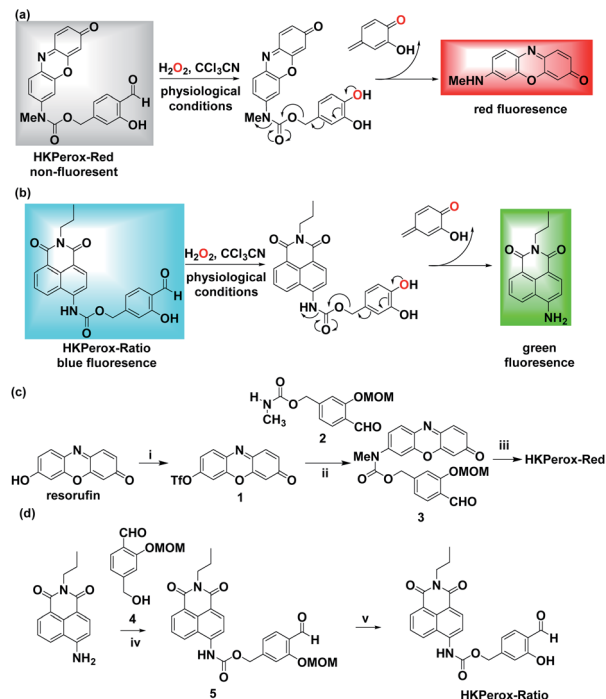
Scheme 1 HKPerox-Red and HKPerox-Ratio for  $\text{H}_2\text{O}_2$  bio-imaging and *in vitro* assay development.

On the other hand, our novel ratiometric probe **HKPerox-Ratio** has been successfully applied to detect  $\text{H}_2\text{O}_2$  burst induced by arsenic trioxide ( $\text{As}_2\text{O}_3$ ) in multiple leukaemia cell lines. For the first time, intracellular  $\text{H}_2\text{O}_2$  in response to starvation is visualized and quantified with **HKPerox-Ratio** by using both confocal microscopy and flow cytometry, establishing a useful protocol for  $\text{H}_2\text{O}_2$  ratiometric analysis with spatio-temporal precision. Our new data confirm the value of **HKPerox** series for advanced bio-imaging analysis (*in vivo* imaging, ratiometric imaging, and flow cytometry analysis) as well as the development of ultrasensitive chemical tools to interrogate  $\text{H}_2\text{O}_2$ -related physiology and pathology.

## Results and discussion

### Design and synthesis of HKPerox-Red and HKPerox-ratio

Our general strategy on developing red emissive and ratiometric  $\text{H}_2\text{O}_2$  probes is based on modulation of internal charge transfer (ICT) of fluorophores that could be deprotected upon  $\text{H}_2\text{O}_2$  mediated Payne/Dakin reaction (activity-based sensing). As shown in Schemes 1 and 2a, an electron-withdrawing carbamate sensing moiety could efficiently interrupt the ICT process in the resorufin derivative, making **HKPerox-Red** essentially non-fluorescent. However, after  $\text{H}_2\text{O}_2$ -mediated deprotection, the remaining electron-donating 7-methylamino group could restore the push-pull conjugating system in 3*H*-phenoxazin-3-one scaffold, releasing a bright red emission. Similarly, **HKPerox-Ratio** was designed by modulating ICT on a biocompatible 4-amino-1,8-naphthalimide platform (see Scheme 2b):<sup>12a,14</sup> before the treatment of  $\text{H}_2\text{O}_2$ , the electron-withdrawing



Scheme 2 Design and synthesis of **HKPerox-Red** and **HKPerox-Ratio**. Rational design of (a) **HKPerox-Red** and (b) **HKPerox-Ratio** for  $\text{H}_2\text{O}_2$  sensing based on tandem Payne/Dakin reaction. Synthetic schemes for (c) **HKPerox-Red** and (d) **HKPerox-Ratio**. Reagents and conditions: (i) DMF, NaH, PhNTf<sub>2</sub>, 0 °C, 8 h, 74%; (ii) Pd<sub>2</sub>(dba)<sub>3</sub>, xantphos, Cs<sub>2</sub>CO<sub>3</sub>, dioxane, 100 °C, 24 h, 57%; (iii) TFA/DCM (v/v = 1 : 1), rt, 2 h, 95%; (iv) DIPEA, triphosgene, toluene, reflux, 1 h; 1, DCM, rt, 3 h, 38%; (v) TFA/DCM (v/v = 1 : 1), rt, 2 h, 92%.

carbamate linker on the 4-position of 1,8-naphthalimide disrupts the ICT process to produce a blue emission; upon  $\text{H}_2\text{O}_2$  mediated deprotection, the carbamate linker is efficiently cleaved to release the electron-donating amine, and the ICT process is restored to provide a bright green emission.

Two general synthetic routes were envisioned to prepare **HKPerox-Red** and **HKPerox-Ratio**: (1) a general coupling reaction between halogen- or triflate-substituted fluorophores and the carbamate sensing moiety; (2) a general addition reaction between isocyanate-substituted fluorophores and the benzyl alcohol sensing moiety. Specifically, **HKPerox-Red** was synthesized from commercially available resorufin in three steps: triflation, a cross-coupling between triflate 1 and carbamate 2, and deprotection. **HKPerox-Ratio** was synthesized according to another approach (Scheme 2d) for amine-containing fluorophore: 4-amino group of 1,8-naphthalimide fluorophore was first converted to an isocyanate by treatment with triphosgene, and the *in situ* generated isocyanate readily reacted with the benzyl alcohol sensing moiety 4 to form carbamate 5, which was deprotected to afford **HKPerox-Ratio**.

### Reactivity and selectivity of HKPerox-Red and HKPerox-Ratio toward $\text{H}_2\text{O}_2$

With both probes in hand, we first investigated the spectroscopic properties of **HKPerox-Red** and **HKPerox-Ratio** in



potassium phosphate buffer (0.1 M, pH 7.4, 100  $\mu\text{M}$   $\text{CCl}_3\text{CN}$ ). Due to its excellent water-solubility, both probes could form a homogenous solution in the buffer without additional co-solvent. As shown in Fig. 1a, an obvious absorption peak of **HKPerox-Red** was observed at 580 nm after  $\text{H}_2\text{O}_2$  treatment. At the same time, a bright red emission at 602 nm appeared with increasing amounts of  $\text{H}_2\text{O}_2$  in a dose-dependent manner (0–100  $\mu\text{M}$ ). The resulting fluorescent product was detected as a resorufin derivative by UPLC-MS analysis (Scheme 2a and ESI $^\dagger$ ), which confirms that tandem Payne/Dakin reaction was successfully extended to this scaffold. As shown in Fig. 1c, the fluorescence intensity at 602 nm was linearly correlated with  $\text{H}_2\text{O}_2$  concentration ranging from 0 to 30  $\mu\text{M}$ . The detection limit was estimated to be as low as 4.8 nM ( $3\sigma/k$ ) with this standard calibration curve, which could be used as an ultra-sensitive assay for  $\text{H}_2\text{O}_2$  quantification. The major challenges for  $\text{H}_2\text{O}_2$  sensors are actually sensitivity and selectivity: the reactivity of  $\text{H}_2\text{O}_2$  is milder than those highly reactive species, thus competing responses from other ROS seem to be inevitable with previously reported strategies. However, **HKPerox-Red** is highly sensitive toward  $\text{H}_2\text{O}_2$ , and a >150-fold enhancement in fluorescence intensity was observed upon treatment with  $\text{H}_2\text{O}_2$ , while other potential competing ROS/RNS including  $^1\text{O}_2$ ,  $\text{ROO}^\cdot$ , TBHP, NO,  $\text{O}_2^{\cdot-}$ ,  $\cdot\text{OH}$ ,  $\text{ONOO}^-$ , and HOCl, only triggered negligible changes (Fig. 1d).

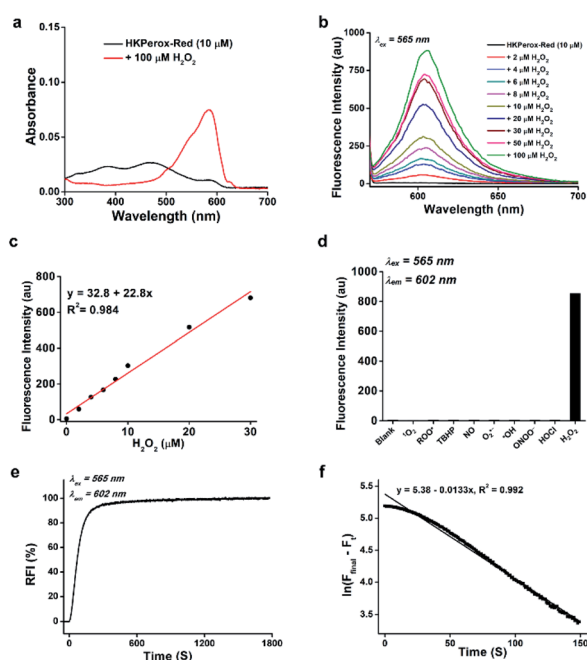


Fig. 1 Chemical characterization of **HKPerox-Red** (10  $\mu\text{M}$ ) in 0.1 M potassium phosphate buffer (pH 7.4, 0.5% DMF, 100  $\mu\text{M}$   $\text{CCl}_3\text{CN}$ ). (a) Absorbance spectra of **HKPerox-Red** before and after treatment with  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ). (b) Fluorescence emission spectra of **HKPerox-Red** upon treatment with different amounts of  $\text{H}_2\text{O}_2$  (0–100  $\mu\text{M}$ ). (c) Fluorescence intensity of **HKPerox-Red** at 602 nm as a function of  $\text{H}_2\text{O}_2$  (0–30  $\mu\text{M}$ ). (d) Fluorescence responses of **HKPerox-Red** toward various reactive oxygen/nitrogen species (ROS/RNS; 100  $\mu\text{M}$ ). (e) Time course of **HKPerox-Red** (1  $\mu\text{M}$ ) in buffer treated with 1 mM  $\text{H}_2\text{O}_2$ . (f) Rate calculation based on pseudo first order kinetics.  $k_{\text{obs}} = 1.3 \times 10^{-2} \text{ s}^{-1}$ .

In addition, reaction kinetics of **HKPerox-Red** were investigated. Time courses of fluorescence intensities of **HKPerox-Red** (10  $\mu\text{M}$ ) at 602 nm upon treatment with 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  were recorded. The reaction was almost completed within 10 min (Fig. S1, ESI $^\dagger$ ), meaning that a short period of incubation is enough to trigger a significant fluorescence response. At pH 8.0, this reaction could be further accelerated, which is consistent with the mechanism of tandem Payne/Dakin reaction. The reaction rate constant was calculated to be  $1.3 \times 10^{-2} \text{ s}^{-1}$ , based on a pseudo first order model by mixing 1  $\mu\text{M}$  **HKPerox-Red** with 1 mM  $\text{H}_2\text{O}_2$  (Fig. 1e and f).

As shown in Fig. 2a, the absorption peak at 375 nm of **HKPerox-Ratio** was shifted to 425 nm upon  $\text{H}_2\text{O}_2$  oxidation, which is consistent with restoration of ICT process in this ratiometric probe.<sup>12a,14</sup> To our delight, subsequent fluorescence measurements revealed an isosbestic point on the fluorescence spectra of **HKPerox-Ratio** (Fig. 2b), indicating its clean conversion. The green fluorescent compound was also confirmed by UPLC-MS analysis to be the cleavage product 4-amino-1,8-naphthalimide (Scheme 2b and ESI $^\dagger$ ). The ratios of fluorescence emission intensities at 540 nm and 475 nm ( $F_{540}/F_{475}$ ) were proportional to  $\text{H}_2\text{O}_2$  concentrations ranging from 0 to 100  $\mu\text{M}$ . The selectivity of **HKPerox-Ratio** was also tested, which exhibited a 12.7-fold increase in  $F_{540}/F_{475}$  toward  $\text{H}_2\text{O}_2$ , while other ROS/RNS gave negligible ratio changes (Fig. 2d). This reaction also proceeded rapidly to complete in 30 min (Fig. S2, ESI $^\dagger$ ). Collectively, these results demonstrate excellent water-solubility, sensitivity, selectivity of **HKPerox-Ratio** toward  $\text{H}_2\text{O}_2$ .

Encouraged by their excellent performances toward  $\text{H}_2\text{O}_2$  in chemical system, we further performed biological evaluations of **HKPerox-Red** and **HKPerox-Ratio** in living cells. The cytotoxicities of **HKPerox-Red** and **HKPerox-Ratio** were assessed in RAW264.7 macrophages, and no obvious toxicity was observed up to 20  $\mu\text{M}$  after 24 h incubation (Fig. S3 and S4, ESI $^\dagger$ ).

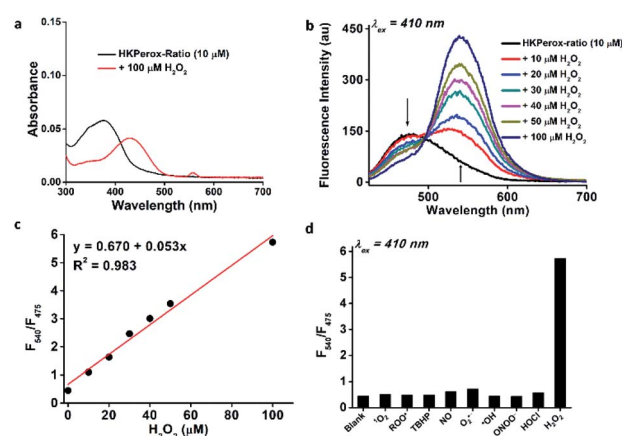


Fig. 2 Chemical characterization of **HKPerox-Ratio** (10  $\mu\text{M}$ ) in 0.1 M potassium phosphate buffer (pH 7.4, 0.1% DMF, 100  $\mu\text{M}$   $\text{CCl}_3\text{CN}$ ). (a) Absorbance spectra of **HKPerox-Ratio** before and after treatment with  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ). (b) Fluorescence emission spectra of **HKPerox-Ratio** upon treatment with different amounts of  $\text{H}_2\text{O}_2$  (0–100  $\mu\text{M}$ ). (c) Ratios of the emission intensities ( $F_{540}/F_{475}$ ) of **HKPerox-Ratio** as a function of  $\text{H}_2\text{O}_2$  (0–100  $\mu\text{M}$ ). (d) Fluorescence responses of **HKPerox-Ratio** toward ROS/RNS (100  $\mu\text{M}$ ).



### Molecular imaging of endogenous H<sub>2</sub>O<sub>2</sub> production in live cells and *in vivo* using HKPerox-Red

We went on to apply **HKPerox-Red** in live cell imaging to detect endogenous H<sub>2</sub>O<sub>2</sub> production with confocal microscopy. RAW264.7 macrophages were co-incubated with **HKPerox-Red** (4 μM) in Hank's Balanced Salt Solution (HBSS) for 30 min before imaging. As expected, the fluorescence signal in PMA treated cells was significantly stronger than that of untreated cells (Fig. 3a). This PMA induced H<sub>2</sub>O<sub>2</sub> burst could be effectively inhibited by the addition of NADPH oxidase (NOX) inhibitor DPI, and quantifications based on imaging showed a clear 7-fold enhancement of fluorescence intensities (Fig. 3b). Endogenous H<sub>2</sub>O<sub>2</sub> bursts can be robustly visualized in stimulated macrophages with **HKPerox-Red** in a selective manner.

We next explored the applications of **HKPerox-Red** to *in vivo* imaging of zebrafish. To perform molecular imaging of H<sub>2</sub>O<sub>2</sub> produced in living zebrafish, the desirable probe should be both lipophilic and water-soluble to penetrate through fish skin by simple co-incubation. **HKPerox-Red**, our resorufin-derived H<sub>2</sub>O<sub>2</sub> probe, featuring small-molecular weight, good lipophilicity, excellent water-solubility, and most importantly, ultra-sensitivity, is very promising for such study.

In our initial study, zebrafish embryos 6 hours post fertilization (hpf) were acquired to co-incubate with **HKPerox-Red** (including 100 μM CCl<sub>3</sub>CN in 1 mL E3 buffer) or **HKPerox-2**, our previous rhodol-base probe,<sup>10a</sup> at room temperature for 30 min to monitor zebrafish development process. Endogenous H<sub>2</sub>O<sub>2</sub> produced during cell proliferation was clearly visualized by **HKPerox-Red** since the red fluorescence was detected inside zebrafish embryo (Fig. 4), while there was no fluorescence signal in the yolk part of the fertilized egg or outside of the embryo. In contrast, **HKPerox-2** could not penetrate through the embryo membrane *via* co-incubation, and the resulting fluorescence signals were only found outside the embryo. Thus, **HKPerox-Red** could efficiently stain zebrafish embryo by simple co-incubation, and H<sub>2</sub>O<sub>2</sub> production during cell proliferation in early stage of embryo development was visualized.

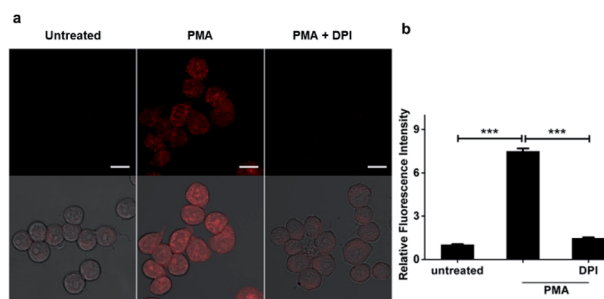


Fig. 3 Molecular imaging of endogenous hydrogen peroxide in living cells. (a) Representative confocal images for RAW264.7 macrophages co-incubated with **HKPerox-Red** (4 μM) and CCl<sub>3</sub>CN (100 μM) in the absence or presence of PMA (200 ng mL<sup>-1</sup>) or PMA plus DPI (100 nM). (b) Relative mean fluorescence intensities of cells incubated with **HKPerox-Red** were quantified. Scale bars represent 10 μm. Data are mean ± SEM, n = 30–34 cells. Statistical significance was determined as \*\*\*p < 0.001 by Student's *t* test. PMA: phorbol 12-myristate 13-acetate; DPI: diphenyleneiodonium.

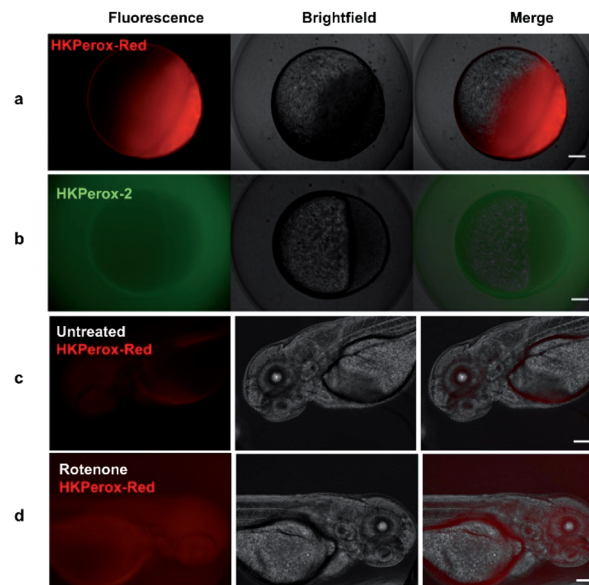


Fig. 4 Molecular imaging of endogenous H<sub>2</sub>O<sub>2</sub> in 6 hpf zebrafish embryos with (a) **HKPerox-Red** (20 μM) and (b) **HKPerox-2** (20 μM). Molecular imaging of (c) untreated or (d) rotenone induced H<sub>2</sub>O<sub>2</sub> in 72 hpf zebrafish embryos with **HKPerox-Red** (20 μM). Scale bars represent 100 μm.

Rotenone is a widely used pesticide that interferes with the electron transport chain during mitochondrial respiration. Previous study reveals that long term exposure to rotenone is associated with higher risk of Parkinson's disease in farm workers,<sup>15</sup> while their models for rotenone challenging mainly focused on isolated mitochondria or cell lines,<sup>16</sup> and the imaging reagents used to quantify ROS burst were traditional unselective probes. Thus, we applied **HKPerox-Red** to evaluate the effect of rotenone on zebrafish embryos. 72 hpf zebrafish embryos were incubated with **HKPerox-Red** (20 μM) for 20 min at room temperature, then challenged with or without rotenone (50 μM) in E3 buffer supplemented with 100 μM CCl<sub>3</sub>CN for 15 min. Basal H<sub>2</sub>O<sub>2</sub> production and distribution could be visualized as a weak fluorescence signal (Fig. 4c). However, this signal was significantly stronger in rotenone treated zebrafish, which indicates acute H<sub>2</sub>O<sub>2</sub> burst in living embryos. Distributions of H<sub>2</sub>O<sub>2</sub> burst could be found in the brain and gut regions of zebrafish embryos, which may further contribute to oxidative damage of proteins and the initiation of neurodegenerative diseases.

### New assay development based on **HKPerox-Red**

As H<sub>2</sub>O<sub>2</sub> is a biologically important oxidizing reagent, its production and scavenging profiles are of great interest in health products, nutrition, and food industry.<sup>17</sup> Moreover, H<sub>2</sub>O<sub>2</sub> is one of the most common products of enzyme catalyzed oxidations, thus a series of metabolites including glucose, cholesterol, uric acid, and sarcosine could be converted to H<sub>2</sub>O<sub>2</sub> quantitatively with corresponding oxidases or enzyme mimetics.<sup>18</sup>

For example, in Amplex Red Glucose/Glucose Oxidase assay, glucose could be oxidized to glucuronolactone in the presence



of glucose oxidase and oxygen, and one equivalent of  $\text{H}_2\text{O}_2$  is produced and quantified by Amplex Red in this process. However, this resulting fluorescence could be bleached by extra amounts of  $\text{H}_2\text{O}_2$  in presence of horseradish peroxidase (HRP), and Amplex Red is also vulnerable to photo oxidation.<sup>6f,19</sup>

Similar to Amplex Red in both excitation and emission wavelengths, **HKPerox-Red** can be a novel molecular tool compatible with current assays using Amplex Red, yet it is more stable, sensitive, and HRP independent. In **HKPerox-Red** glucose assay, various amounts of glucose (0–40  $\mu\text{M}$ , final concentrations) were added into potassium phosphate buffer, and then 1  $\text{U mL}^{-1}$  glucose oxidase and **HKPerox-Red** (10  $\mu\text{M}$ ; with 100  $\mu\text{M}$   $\text{CCl}_3\text{CN}$ ) were added subsequently into the solution. Resulting solutions were incubated for 30 min at 37 °C before fluorescence measurement. As shown in Fig. 5a,  $\text{H}_2\text{O}_2$  produced by glucose oxidation could be sensitively detected with **HKPerox-Red**, and a linear relationship between glucose concentrations and fluorescence emissions at 602 nm was observed. The detection limit of glucose was calculated to be as low as 34 nM ( $3\sigma/k$ ) according to the calibration curve.

To prove the practical applications of this novel assay, non-diabetic urine and serum samples were analyzed by **HKPerox-Red** glucose assay, and only 1  $\mu\text{L}$  serum is needed due to the ultra-sensitivity. In this experiment, 1  $\mu\text{L}$  deproteinized serum was diluted with 1 mL potassium phosphate buffer (pH 7.4, 0.5% DMF) containing 1  $\text{U mL}^{-1}$  glucose oxidase, 10  $\mu\text{M}$  **HKPerox-Red** and 100  $\mu\text{M}$   $\text{CCl}_3\text{CN}$ . Quantifications were based on serum samples spiked with 3 mM glucose as internal standard, and the glucose concentration in serum sample was quantified as  $4.38 \pm 0.32$  mM. Therefore, with tiny amount of sample, **HKPerox-Red** holds great potential to measure serum

glucose in a fast, accurate, and high-throughput assay. The ultrasensitivity also makes it possible to conduct non-invasive glucose evaluation with samples like sweat, saliva or tears. More importantly, **HKPerox-Red** enables a general assay for biologically important metabolites detection with suitable oxidases. As a proof of concept, uric acid/urate oxidase assay and sarcosine/sarcosine oxidase assay were developed by using **HKPerox-Red** (Fig. 5c and d), which provide practical methods for disease diagnosis based on metabolite screening. A clinical study with **HKPerox-Red** is on-going to use urinary sarcosine as potential biomarker for prostate cancer diagnosis.

### Ratiometric detection of $\text{H}_2\text{O}_2$ burst in $\text{As}_2\text{O}_3$ treated leukaemia cells by using flow cytometry

Next, we sought to explore ratiometric  $\text{H}_2\text{O}_2$  sensing with our **HKPerox-Ratio**. Although some  $\text{H}_2\text{O}_2$  ratiometric fluorescent probes have been developed for this purpose, very few of them are sensitive enough to be used in flow cytometry, and none of them is suitable for quantifying endogenous  $\text{H}_2\text{O}_2$  in living cells.<sup>13</sup> We firstly applied **HKPerox-Ratio** in flow cytometry to detect  $\text{H}_2\text{O}_2$  fluxes in leukemia cells challenged with  $\text{As}_2\text{O}_3$ , a very effective approach to treat acute promyelocytic leukemia.<sup>20</sup> As leukemia cells are suspension cells, they are not suitable for confocal imaging; however, flow cytometry enables quantitative analysis of a large population of suspension cells rapidly. Moreover, with a suitable ratiometric probe, multiple fluorescence emissions could be detected simultaneously by flow cytometry,<sup>21</sup> and the green to blue emission ratio ( $F_{\text{green}}/F_{\text{blue}}$ ) reported by **HKPerox-Ratio** is expected to be a more reliable ratiometric indicator of cellular  $\text{H}_2\text{O}_2$ .

For this purpose, U-937, NB4 and THP-1 leukemia cells were selected for the treatment with  $\text{As}_2\text{O}_3$  for 24 h, and then untreated cells and  $\text{As}_2\text{O}_3$  challenged cells were co-incubated with **HKPerox-Ratio** (10  $\mu\text{M}$ ) for 30 min before flow cytometry analysis. Over 10 000 cells were analysed, and  $F_{\text{green}}/F_{\text{blue}}$  in each individual cell was calculated by a FlowJo software. As shown in Fig. 6a–c,  $\text{As}_2\text{O}_3$ -treated leukemia cells could be clearly distinguished from those untreated cells by  $F_{\text{green}}/F_{\text{blue}}$  ratio (x-axis), which correlates with  $\text{H}_2\text{O}_2$  levels. Further quantifications in Fig. 6d showed a significant enhancement in  $F_{\text{green}}/F_{\text{blue}}$  ratio in all three cell lines (1.9-fold for U-937, 1.7-fold for NB4, and 2.8-fold for THP-1), indicating a robust cellular  $\text{H}_2\text{O}_2$  flux. This  $\text{As}_2\text{O}_3$ -challenged  $\text{H}_2\text{O}_2$  burst may be responsible for differentiation or apoptosis of leukemia cells.<sup>22</sup> To conclude, **HKPerox-Ratio** is a promising probe to detect  $\text{H}_2\text{O}_2$  fluxes *via* flow cytometry in a ratiometric manner, which provides an ideal platform to screen novel apoptosis inducer for leukemia therapy.

### Ratiometric fluorescence imaging of starvation induced $\text{H}_2\text{O}_2$ in living cells

As  $\text{H}_2\text{O}_2$  is believed to be an important mediator during auto-phagy in response to nutrient shortage,<sup>23</sup> we would like to identify and quantify endogenous  $\text{H}_2\text{O}_2$  production during this process with ratiometric probe **HKPerox-Ratio**. RAW264.7 macrophages were co-incubated with **HKPerox-Ratio** (5  $\mu\text{M}$ ) in

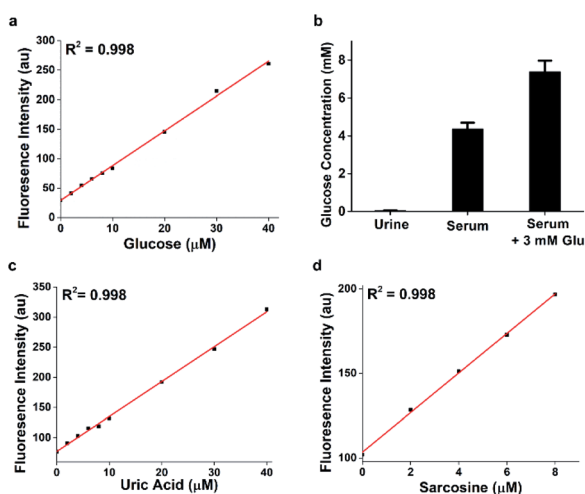


Fig. 5 (a) Ultrasensitive detection of glucose with **HKPerox-Red** (10  $\mu\text{M}$ ) in aqueous buffer (pH 7.4, 0.5% DMF, 1  $\text{U mL}^{-1}$  glucose oxidase, 100  $\mu\text{M}$   $\text{CCl}_3\text{CN}$ ). Excitation was provided at 565 nm; fluorescence emission was obtained at 602 nm. (b) Glucose quantifications of urine and serum samples using **HKPerox-Red** glucose assay. Data are mean  $\pm$  SEM,  $n = 3$ . (c) Detection of uric acid with **HKPerox-Red** (10  $\mu\text{M}$ , with 1  $\text{U mL}^{-1}$  urate oxidase) in aqueous buffer. (d) Detection of sarcosine with **HKPerox-Red** (10  $\mu\text{M}$ , with 1  $\text{U mL}^{-1}$  sarcosine oxidase) in aqueous buffer.



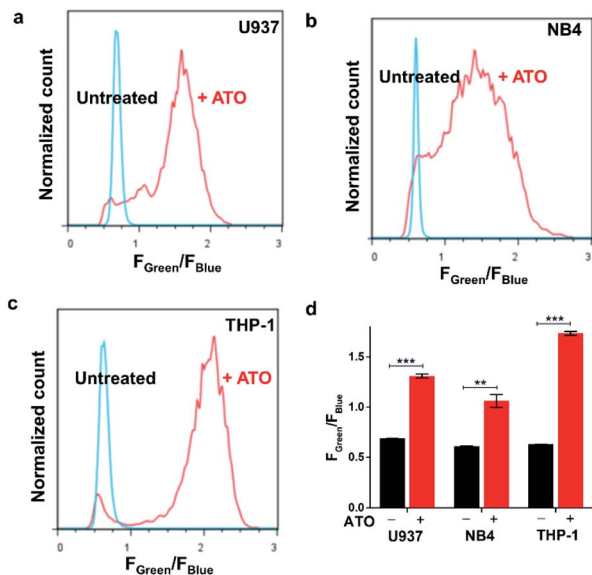


Fig. 6 Detection of arsenic trioxide induced  $\text{H}_2\text{O}_2$  in leukemia cells with flow cytometry. (a) U937, (b) NB4, and (c) THP-1 cells were treated with or without  $20 \mu\text{M}$   $\text{As}_2\text{O}_3$  for 24 h, and then cells were incubated with **HKPerox-Ratio** ( $10 \mu\text{M}$ ) and  $\text{CCl}_3\text{CN}$  ( $100 \mu\text{M}$ ) for 30 min before flow cytometry analysis. The y-axis is the normalized cell count; the x-axis is the  $F_{\text{Green}}/F_{\text{Blue}}$  ratio in each cell. (d) Fluorescence intensities ratios  $F_{\text{Green}}/F_{\text{Blue}}$  in untreated cells and  $\text{As}_2\text{O}_3$  treated cells were compared. Data are mean  $\pm$  SEM,  $n = 3$  independent experiments. Statistical significance was determined as  $**p < 0.01$ ,  $***p < 0.001$  by Student's  $t$  test.

HBSS supplemented with  $100 \mu\text{M}$   $\text{CCl}_3\text{CN}$  for 30 min at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  before confocal imaging. The fluorescence signals in blue region (440–490 nm) and green region (540–650 nm) were collected simultaneously with an excitation at 405 nm (Fig. 7a and b). As expected, the blue emission was significantly stronger than the green emission in untreated cells, and the merged Fig. 7c showed the predominant blue emission. In Fig. 7d, a heatmap of green-to-blue emission ratio ( $F_{\text{Green}}/F_{\text{Blue}}$ ) was generated by a Ratio Plus plugin in ImageJ software. Basal  $\text{H}_2\text{O}_2$  concentrations and distributions were reported as a small value of  $F_{\text{Green}}/F_{\text{Blue}}$  ( $\sim 0.2$ ), which is consistent with low  $\text{H}_2\text{O}_2$  levels in resting cells. To mimic starvation induced stress, RAW264.7 cells were incubated HBSS (protein free) for 24 h, and then confocal images were taken under the same optical settings. Upon starvation challenge, blue emission in cells decreased significantly, while green emission increased due to  $\text{H}_2\text{O}_2$  burst (Fig. 7e and f). The merged image in Fig. 7g showed a predominant green emission, and the ratiometric heatmap (Fig. 7h) confirmed a significant increase of  $F_{\text{Green}}/F_{\text{Blue}}$  ( $\sim 1.0$ ) inside the cells.

Quantification based on confocal images was performed to show a clear 5-fold overall increase of  $F_{\text{Green}}/F_{\text{Blue}}$  in starvation-challenged cells compared to resting cells (Fig. 7i), which demonstrated the excellent sensitivity of **HKPerox-Ratio** in cell imaging. The green signal and blue signal overlapped well with an overlap coefficient of 0.85 (Fig. S5, ESI $^\dagger$ ), which shows a good co-localization of **HKPerox-Ratio** and its cleavage product in cells. Collectively, those data demonstrate that **HKPerox-Ratio**

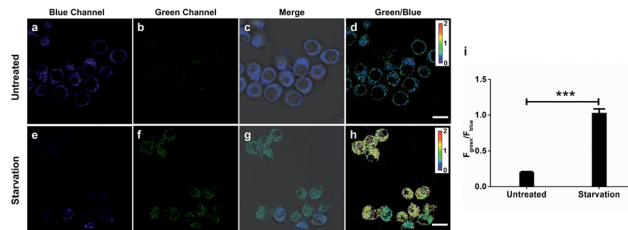


Fig. 7 Confocal images of  $\text{H}_2\text{O}_2$  production with **HKPerox-Ratio** ( $5 \mu\text{M}$ ) in untreated or nutrient-depleted (HBSS treatment) RAW264.7 macrophages. (a) and (e) Representative fluorescence images in the blue channel (440–490 nm). (b) and (f) Representative fluorescence images in the green channel (540–650 nm). (c) and (g) Merged images of blue, green and brightfield channels. (d) and (h) Pseudo-color heatmap of green/blue emission ratio ( $F_{\text{Green}}/F_{\text{Blue}}$ ). (i)  $F_{\text{Green}}/F_{\text{Blue}}$  of RAW 264.7 macrophages under homeostasis or starvation was quantified. Data are mean  $\pm$  SEM,  $n = 19$ –29 cells. Statistical significance was determined as  $***p < 0.001$  by Student's  $t$  test. Scale bars represent  $10 \mu\text{m}$ .

can be robustly applied in ratiometric imaging of endogenous  $\text{H}_2\text{O}_2$  fluxes with spatio-temporal precision.

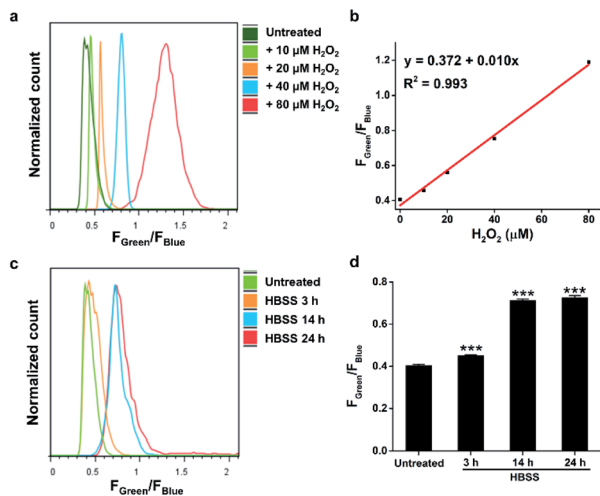
#### Quantitative measurement of cellular $\text{H}_2\text{O}_2$ by flow cytometry with live cell calibration

Calibration curves of previous ratiometric probes were largely established in a cell-free solvent/buffer mixture, which may not be applicable for precise quantification of cellular  $\text{H}_2\text{O}_2$ .<sup>13</sup> Therefore, we further applied **HKPerox-Ratio** in flow cytometry for quantitative measurement of  $\text{H}_2\text{O}_2$  fluxes during starvation. As shown in Fig. 8a and b, a calibration curve with excellent linearity ( $R^2 = 0.993$ ) was obtained by adding known amounts of  $\text{H}_2\text{O}_2$  (0, 10, 20, 40, and  $80 \mu\text{M}$   $\text{H}_2\text{O}_2$ ) into the probe solution and then co-incubating with cells for 30 min before flow cytometry measurement. The green to blue emission ratio ( $F_{\text{Green}}/F_{\text{Blue}}$ ) in each group of cells was analysed, and those cell populations could be distinguished in Fig. 8a. The detection limit of **HKPerox-Ratio** by flow cytometry measurement was estimated to be as low as  $1.8 \mu\text{M}$  ( $3\sigma/k$ ) in this cellular assay. A standard curve ( $y = 0.372 + 0.010x$ ) with live cell calibration was thus established for RAW264.7 cells, enabling the direct measurement of intracellular  $\text{H}_2\text{O}_2$  concentrations.

As shown in Fig. 8c and d, starvation induced  $\text{H}_2\text{O}_2$  was detected and quantified with **HKPerox-Ratio**. Our data showed that 3 h starvation already induced a discernible increase in  $F_{\text{Green}}/F_{\text{Blue}}$  ratio, corresponding to  $7.9 \mu\text{M}$   $\text{H}_2\text{O}_2$  according to the calibration curve. This rapidly elevated cellular  $\text{H}_2\text{O}_2$  levels during starvation suggest that treatment of cells in protein-free buffers (such as HBSS and phosphate-buffered saline) may stimulate unexpected ROS burst, thus longtime incubation in those buffers should be avoided.

Moreover,  $\text{H}_2\text{O}_2$  fluxes in living RAW264.7 cells reached its maximum concentration ( $34 \mu\text{M}$ ) after 14 h starvation, and longer period of starvation (*e.g.* 24 h) did not induce higher levels of  $\text{H}_2\text{O}_2$ . This endogenous  $\text{H}_2\text{O}_2$  burst is sufficient to signal subsequent pathological processes, such as growth arrest or apoptosis.<sup>24</sup>





**Fig. 8** Measurements of H<sub>2</sub>O<sub>2</sub> concentrations in nutrient-depleted (HBSS treatment) RAW264.7 macrophages by flow cytometry analysis. (a) Histogram of the  $F_{\text{green}}/F_{\text{blue}}$  ratio in RAW264.7 cells co-incubated with HKPerox-Ratio (10 μM) and CCl<sub>3</sub>CN (100 μM) pretreated with 0, 10, 20, 40, and 80 μM H<sub>2</sub>O<sub>2</sub>. (b) Calibration curve of  $F_{\text{green}}/F_{\text{blue}}$  in RAW264.7 cells as a function of H<sub>2</sub>O<sub>2</sub> concentrations. (c) Histogram of the  $F_{\text{green}}/F_{\text{blue}}$  ratio in untreated or starvation challenged RAW264.7 cells incubated with HKPerox-Ratio (10 μM) and CCl<sub>3</sub>CN (100 μM). (d) Comparison of  $F_{\text{green}}/F_{\text{blue}}$  in untreated or starvation challenged cells. Data are mean ± SEM,  $n = 3$  independent experiments. Statistical significance was determined as \*\*\* $p < 0.001$  by Student's  $t$  test.

Due to variations of cellular environment, independent calibration curves should be obtained in different cell types before ratiometric H<sub>2</sub>O<sub>2</sub> quantification. We further applied this protocol to HeLa cells, and a new calibration curve with excellent linearity ( $y = 0.606 + 0.013x$ ,  $R^2 = 0.988$ ) was obtained with HKPerox-Ratio by using flow cytometry (Fig. S6, ESI†). Note that the calibration curve obtained by using a fluorospectrometer in chemical system (Fig. 2c,  $y = 0.670 + 0.053x$ ) is very different from those obtained by using flow cytometry with live cell calibration. This result indicates that calibration curves established in a cell-free solvent/buffer mixture are not applicable to cellular H<sub>2</sub>O<sub>2</sub> measurement; however, with a suitable ratiometric probe, flow cytometry provides an ideal high-throughput platform for quantitative analysis. One potential risk from live cell calibration may arise from different cell permeabilities of HKPerox-Ratio and its cleavage product. A control experiment is recommended to co-stain the cells with both compounds to confirm their similar permeability (Fig. S7, ESI†). To the best of our knowledge, HKPerox-Ratio is the first ratiometric probe that can be used to quantify cellular H<sub>2</sub>O<sub>2</sub> level by flow cytometry, which makes it an invaluable tool for not only qualitative detection, but also quantification in redox biology.

## Conclusions

During past decades, great efforts have been made to develop novel H<sub>2</sub>O<sub>2</sub> sensors, and diverse roles of H<sub>2</sub>O<sub>2</sub> in mediating various biological processes have been uncovered. However, unambiguous quantitative analysis of H<sub>2</sub>O<sub>2</sub> *in vitro* and *in vivo*

still represents a substantial challenge even to date. In this work, we have developed a pair of highly selective and sensitive fluorescent probes for precise measurement of H<sub>2</sub>O<sub>2</sub>. Our unique strategy relies on tandem Payne/Dakin reaction, which provides a general and biocompatible strategy to distinguish H<sub>2</sub>O<sub>2</sub> from other potential competing ROS. HKPerox-Red and HKPerox-Ratio have been successfully applied to evaluate the effects of PMA, As<sub>2</sub>O<sub>3</sub>, rotenone, and starvation in various models.

In particular, HKPerox-Red with outstanding permeability could be used in zebrafish imaging by simple co-incubation, and rotenone induced oxidative stress was also successfully visualized *in vivo*. Moreover, the outstanding performance of HKPerox-Red has been exploited to develop new assays for H<sub>2</sub>O<sub>2</sub> scavenging capacity or H<sub>2</sub>O<sub>2</sub>-based quantification for metabolites including glucose, uric acid and sarcosine, which are regarded as diagnostic biomarkers for diabetes, gout, and prostate cancer, respectively. The intrinsic sensitivity of our novel strategy ensures its robust application for *in vitro* quantification of H<sub>2</sub>O<sub>2</sub> and related metabolites.

Even more challenging in the redox biology field is to measure intracellular H<sub>2</sub>O<sub>2</sub> accurately. In this study, we have developed HKPerox-Ratio, a small-molecule ratiometric probe, that allows quantitative detection of H<sub>2</sub>O<sub>2</sub> by measuring the ratio of the blue and green emission. HKPerox-Ratio could be used in various cell lines with simple co-incubation, and the significant signal changes triggered by intracellular H<sub>2</sub>O<sub>2</sub> were robustly captured by both flow cytometry and confocal imaging. Therefore, As<sub>2</sub>O<sub>3</sub>-induced H<sub>2</sub>O<sub>2</sub> fluxes in multiple leukemia cells were readily detected. HKPerox-Ratio further enables the first quantitative detection of endogenously generated H<sub>2</sub>O<sub>2</sub> in living cells by using flow cytometry and live cell calibration curve.

We anticipate that both probes provide important molecular tools to study H<sub>2</sub>O<sub>2</sub> physiology and pathology, and more efforts are on-going to apply HKPerox series probes and tandem Payne/Dakin reaction in advanced bio-imaging, drug screening, and disease diagnostics.

## Conflicts of interest

S. Y., J. J. H., and D. Y. have filed a patent application for the reported probes.

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