Electronic Supplementary Information for

Fluorescent Probes for In Vitro and In Vivo Quantification of Hydrogen Peroxide

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1. General methods

Xanthine, xanthine oxidase, SNP (sodium nitroferricyanide(III) dihydrate), 2,2’-azobis(2-aminopropane)dihydrochloride, and hydrogen peroxide solution were purchased from Sigma-Aldrich. Peroxynitrite was synthesized as reported\(^1\). Peroxynitrite solution was split into small aliquots and frozen at temperature below \(-18 ^\circ C\). All other chemicals used were of analytical grade and were purchased from Acros or Sigma-Aldrich. Potassium phosphate buffer was prepared by mixing aqueous solution of KH\(_2\)PO\(_4\) (1 M, 1.98 mL) and KH\(_2\)PO\(_4\) (1 M, 8.02 mL) (final pH 7.4) followed by dilution with deionized water to 100 mL in a volumetric flask. NMR spectra were recorded in deuteriochloroform unless otherwise stated, with chloroform as an internal reference at ambient temperature, mainly on a Bruker Avance DPX 300 Fourier Transform Spectrometer operating at 300 MHz for \(^1H\) and at 75.47 MHz for \(^{13}C\) and Bruker Avance DPX 400 Fourier Transform Spectrometer operating at 400 MHz for \(^1H\) and at 100.6 MHz for \(^{13}C\). Mass spectra were recorded with a Thermo Scientific DFS High Resolution Magnetic Sector mass spectrometer for both low resolution and high resolution mass analysis unless otherwise stated.

Fluorescent probes were dissolved in DMF to make a 2 mM or 10 mM stock solution, which was then diluted to 10 \(\mu\)M testing solution in 0.1 mM potassium phosphate buffer at pH 7.4. Absorption and fluorescence spectra of the testing solution were recorded under a CARY 50 Bio UV-Visible spectrophotometer and a Hitachi F-7000 fluorescence spectrophotometer, respectively. For fluorescence measurement, slit widths were set at 2.5 nm for both excitation and emission spectra, and the photomultiplier voltage was 700 V.
Aliquots of analyte solutions were slowly added to the probe testing solution (5 mL) with vigorous stirring at 37 °C in the dark. Fluorescence intensities of the testing solutions were recorded after 30 min.

2. Synthesis and characterization of HKPerox-Red and HKPerox-Ratio

Compound 1 and compound 2 were synthesized according to literature report2-3.

![Reaction Scheme]

4-Formyl-3-(methoxymethoxy)benzyl methyl(3-oxo-3H-phenoxazin-7-yl)carbamate (3). An oven-dried round bottom flask was charged with Pd2(dba)3 (18 mg, 0.020 mmol), Xantphos (34 mg, 0.060 mmol) and Cs2CO3 (91 mg, 0.280 mmol), and flushed with argon for 5 min. Then a solution of 2 (56 mg, 0.200 mmol) and 1 (83 mg, 0.24 mmol) in anhydrous dioxane (5 mL) was added. The resulting mixture was first stirred at room temperature under argon atmosphere for 30 min, then heated to 100 °C and stirred for 24 h. The reaction mixture was allowed to cool to room temperature, diluted with DCM and filtered through a pad of celite. The filtrate was concentrated in vacuo. Compound 3 was purified as a red sticky solid (51 mg; 57%) by flash chromatography on silica gel, using EA:DCM (1:4) as an eluent. 1H NMR (300 MHz, CD2Cl2) δ 10.44 (s, 1H), 7.83 - 7.71 (m, 2H), 7.43 (d, J = 9.8 Hz, 1H), 7.40 - 7.32 (m, 2H), 7.17 (d, J = 1.4 Hz, 1H), 7.04 (d, J = 7.9 Hz, 1H), 6.80 (dd, J = 9.8, 2.0 Hz, 1H), 6.27 (d, J = 2.0 Hz, 1H), 5.26 (s, 2H), 5.23 (s, 2H), 3.47 (s, 3H), 3.42 (s, 3H); 13C NMR (75 MHz, CD2Cl2) δ 189.35, 186.41, 160.19, 154.70, 150.07, 148.64, 147.28, 144.96, 144.49, 135.23, 135.19, 131.43, 130.70, 128.67, 125.40, 122.20, 120.68, 113.98, 112.39, 107.14, 95.05, 67.39, 56.79, 37.56; HRMS (ESI): calcd for C24H21N2O7 ([M+H]+): 449.1343, found: 449.1324.

4-Formyl-3-hydroxybenzyl methyl(3-oxo-3H-phenoxazin-7-yl)carbamate (HKPerox-Red). To a solution of compound 3 (12 mg, 0.0268 mmol) in DCM (3 mL) in an ice/water bath was added TFA (3 mL) dropwise, and then the solution was stirred at room temperature for 2 h. The reaction mixture was concentrated in vacuo and azeotroped with toluene for 3 times. Probe HKPerox-4 was purified as a red sticky solid (10 mg; 92%) by flash chromatography on silica gel, using EA:DCM (1:4) as an eluent. 1H NMR (400 MHz, CDCl3) δ 11.08 (s, 1H), 9.89 (s, 1H), 7.77 (d, J = 9.2 Hz, 1H), 7.56 (d, J = 7.8 Hz, 1H), 7.44 (d, J = 9.8 Hz, 1H), 7.36 (d, J = 7.7 Hz, 2H), 6.97 (d, J = 7.9 Hz, 2H), 6.86 (dd, J = 9.8, 2.0 Hz, 1H), 6.33 (d, J = 2.0 Hz, 1H), 5.24 (s, 2H), 3.46 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 196.2, 194.8, 186.5, 162.0, 154.5, 149.7, 148.3, 146.8, 145.8, 135.2, 134.9, 134.2, 131.1, 130.6, 121.8, 120.4, 118.6, 116.1, 112.0, 107.2, 67.0, 37.4; HRMS (ESI): calcd for C22H17N2O6 ([M+H]+): 405.1081, found: 405.1066.
4-(Hydroxymethyl)-2-(methoxymethoxy)benzaldehyde (Compound 4). To a stirred solution of NaH (60 wt. % in mineral oil, 44 mg, 1.10 mmol) in anhydrous DMF (2.5 mL) at 0 °C under argon atmosphere was added 2-hydroxy-4-(hydroxymethyl)benzaldehyde (152 mg, 1.00 mmol) in anhydrous DMF (2.5 mL) followed by stirring for 15 min. Then chloromethyl methyl ether (MOMCl, 91 μL, 1.20 mmol) was added dropwise to the reaction mixture, followed by stirring for another 30 min. The reaction mixture was diluted with ethyl acetate, washed with water and brine. The organic layer was dried over anhydrous magnesium sulfate, and concentrated in vacuo to give the crude product. Compound 4 was isolated as a colorless oil (174 mg; 89%) by flash chromatography on silica gel, by using EA:Hexane (1:1) as an eluent. Compound 4 is not stable for long term storage, and freshly prepared 4 should be used directly in the next step.

Fluorophore 6-amino-2-propyl-1H-benzo[de]isoquinoline-1,3(2H)-dione was synthesized according to literature report⁴. To a solution of 6-amino-2-propyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (254 mg, 1.00 mmol) in toluene (10 mL, anhydrous) were added triphosgene (356 mg, 1.20 mmol) and DIPEA (497 μL, 3.00 mmol) in an ice/water bath under argon atmosphere. The resulting mixture was first stirred at room temperature under argon atmosphere for 30 min, then heated to reflux for 1 h. The reaction mixture was allowed to cool down to room temperature, and a solution of 4 (165 mg, 0.842 mmol) in DCM (12 mL, anhydrous) was added. The resulting solution was stirred for another 3 h, diluted with ethyl acetate, and washed sequentially with 1 N HCl, water and brine. The organic layer was dried over anhydrous magnesium sulfate, and concentrated in vacuo. Compound 5 was purified as a yellow sticky solid (152 mg; 38%) by flash chromatography on silica gel, using EA:Hexane (1:4) as an eluent. ¹H NMR (400 MHz, CDCl₃): δ 10.48 (s, 1H), 8.63 (d, J = 7.2 Hz, 1H), 8.59 (d, J = 8.2 Hz, 1H), 8.34 (d, J = 8.2 Hz, 1H), 8.23 (d, J = 8.7 Hz, 1H), 7.85 (d, J = 7.8 Hz, 1H), 7.77 (t, J = 8.0 Hz, 1H), 7.67 (s, 1H), 7.28 (s, 1H), 7.13 (d, J = 7.8 Hz, 1H), 5.33 (s, 2H), 5.31 (s, 2H), 4.13 (t, J = 7.6 Hz, 2H), 3.54 (s, 3H), 1.78–1.71 (m, 2H), 1.01 (t, J = 7.4 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 189.2, 164.1, 163.6, 159.8, 152.8, 152.6, 143.6, 138.6, 132.4, 131.3, 128.8, 126.7, 125.9, 125.2, 123.5, 123.1, 121.1, 118.2, 117.1, 114.3, 94.6, 67.0, 56.6, 41.9, 21.4, 11.5; HRMS (ESI): calcd for C₂₆H₂₅N₂O₇ ([M+H]+): 477.1656, found: 477.1639.
To a solution of compound 5 (50.9 mg, 0.107 mmol) in DCM (3 mL) in an ice/water bath was added TFA (3 mL) dropwise, then the solution was stirred at room temperature for 2 h. The reaction mixture was concentrated in vacuo and azeotroped with toluene for 3 times. Probe HKPerox-Ratio was purified as a yellow sticky solid (42.6 mg; 92%) by flash chromatography on silica gel, using EA:hexane (1:1) as an eluent. $^1$H NMR (300 MHz, CDCl$_3$) δ 11.08 (s, 1H), 9.91 (s, 1H), 8.64 (d, $J$ = 7.3 Hz, 1H), 8.60 (d, $J$ = 8.3 Hz, 1H), 8.36 (d, $J$ = 8.3 Hz, 1H), δ 8.21 (d, $J$ = 8.6 Hz, 1H), 7.82 – 7.75 (m, 1H), 7.64 – 7.55 (m, 2H), 7.13 – 6.99 (m, 2H), 5.31 (s, 2H), 4.19 – 4.08 (m, 2H), 1.81 – 1.73 (m, 2H), 1.00 (d, $J$ = 7.5 Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 196.1, 164.1, 163.6, 161.8, 152.7, 145.1, 138.4, 134.1, 132.4, 131.3, 128.9, 126.8, 125.8, 123.6, 123.0, 120.4, 118.8, 118.3, 117.1, 116.4, 66.6, 42.0, 21.4, 11.5; HRMS (ESI): calcd for C$_{24}$H$_{21}$N$_2$O$_6$ ([M+H]+): 433.1394, found: 433.1376.

3. Preparation of analyte solutions

ROO•: Alkylperoxy radical was generated from 2,2’-azobis(2-aminopropane) dihydrochloride (10 mM), which was added into the testing solutions directly.

$^1$O$_2$: Singlet oxygen was generated from 3,3’-(naphthalene-1,4-diyl)dipropionic acid (10 mM).

*NO: Nitric oxide was generated from SNP (sodium nitroferricyanide(III) dihydrate) (10 mM).

TBHP: tert-Butyl hydroperoxide solution (10 mM) was added into the testing solutions directly.

O$_2$•−: Superoxide was generated from xanthine/xanthine oxidase system. Xanthine oxidase (0.1 U/mL) was added before xanthine (30 mM), catalase was added to remove the H$_2$O$_2$.

HOCI: NaOCl solution (10 mM) was added directly.

•OH: Hydroxyl radical was generated by Fenton reaction. To generate •OH, ferrous chloride was added in the presence of H$_2$O$_2$. The concentration of •OH was equal to the Fe(II) concentration (10 mM).

ONOO•: Peroxynitrite solution was synthesized according to literature report.[1] Peroxynitrite solution was synthesized according to literature report.[1] The concentration of peroxynitrite was determined by measuring the absorption of the solution at 302 nm. The extinction coefficient of peroxynitrite solution in 0.1 M NaOH is 1,670 M$^{-1}$ cm$^{-1}$ at 302 nm.

H$_2$O$_2$: H$_2$O$_2$ solution (10 mM) was added directly.
4. Detection of resulting fluorescent product by UPLC-MS

Probes HKPerox-Red or HKPerox-Ratio was dissolved in DMF and then diluted to 10 μM with 0.1 M potassium phosphate buffer at pH 7.4 containing 100 μM trichloroacetonitrile (CCl₃CN). Then 100 μM hydrogen peroxide was added. After 30 min, the reaction mixture was extracted with ethyl acetate (EA). The combined organic layers were analyzed by UPLC-MS (ultraperformance liquid chromatography-mass spectrometry).

5. Kinetics studies of HKPerox-Red and HKPerox-Ratio

![Graph showing relative fluorescence intensities (RFI) at 602 nm for HKPerox-Red (10 μM) upon treatment with 100 μM H₂O₂ at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4 or pH 8.0, 0.5% DMF, 100 μM CCl₃CN). Excitation wavelength was set at 565 nm.]

**Figure S1.** Relative fluorescence intensities (RFI) at 602 nm of HKPerox-Red (10 μM) upon treatment with 100 μM H₂O₂ at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4 or pH 8.0, 0.5% DMF, 100 μM CCl₃CN). Excitation wavelength was set at 565 nm.
Figure S2. Fluorescence spectra of HKPerox-Ratio (10 μM) after treatment with 100 μM H2O2 for 0, 3, 5, 10, 15, 20, and 30 min at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4, 0.1% DMF, 100 μM CCl3CN). Excitation wavelength was set at 410 nm.

6. Mammalian cell culture, flow cytometry, confocal imaging and zebrafish imaging

RAW264.7 cells and HeLa cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin, at 37 °C with 5% CO2. Cells were typically seeded at a density of 2×10^5 cells/mL in 35-mm confocal dish (MatTek: MA, USA) for confocal imaging.

A stock solution (2 mM) of fluorescent probe HKPerox-Red was prepared in anhydrous DMF. Cells were washed with PBS before the addition of HKPerox-Red (4 μM final concentration) in 1 mL HBSS (Hank's balanced salt solution) supplemented with 100 μM CCl3CN. For acute H2O2 induction (30 min), PMA (200 ng/mL) was added to HBSS and co-incubated with HKPerox-Red until imaging. Enzyme inhibitor (NOX inhibitor DPI, 100 nM) was added along with PMA during H2O2 induction as an intervention group. Cells were typically incubated for 30 min at 37 °C with 5% CO2 before imaging. During imaging, the dish was mounted onto a live cell imaging support module (Axiovision). Single-photosection images were acquired with a Zeiss LSM 780 confocal microscope for HKPerox-Red, by using the following settings: λex = 561 nm, λem = 570–680 nm (band-pass).

Mating of adult fish (Hong Kong Wild Type, or HKWT) and selection of zebrafish embryos were performed according to standard protocol provided by Zebrafish Core Facility at Li Ka Shing Faculty of Medicine Faculty Core Facility of the University of Hong Kong. According to EU Directive 2010/63/EU (on the protection of animals used for scientific purposes), zebrafish embryos up to 120 hours postfertilization (hpf) are not protected as living animals, and no ethical permission is needed for scientific research. The study using zebrafish embryos up to 72 hpf was strictly conducted according to the approved protocols provided by Zebrafish Core Facility at HKU and Committee on the Use of Live Animals and Teaching and Research (CULATR) at HKU (Hong Kong, China). Eggs were collected and placed in a 90-mm dish with E3 medium and incubated at 28 °C until embryos were developed to the desired stage (e.g. 72 hpf). Zebrafish embryos were incubated with 20 μM HKPerox-Red (1 mL in E3 buffer) for 20 min. And then the embryos were washed with 1 mL E3 medium and incubated with or without 50 μM rotenone in E3 buffer (containing 100 μM CCl3CN) for 15 min before imaging on EVOS FL using Texas Red Channel for HKPerox-Red.
Leukemia cells THP-1, NB-4, and U937 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin, at 37 °C with 5% CO₂. One day before flow cytometry, leukemia cells were typically seeded at a density of 2×10⁵ cells/mL in 50-mL flask (Corning). For acute H₂O₂ induction, arsenic trioxide (20 µM) was added to RPMI 1640 medium and co-incubated with cells for 1 day. Stock solution (10 mM) of fluorescent probe HKPerox-Ratio was prepared in anhydrous DMF. Before flow cytometry analysis, cells were spun down, and washed with PBS before the addition of fluorescent probe (10 µM final concentration) in 1 mL HBSS (Hank’s balanced salt solution) supplemented with 100 µM CCl₃CN. Cells were typically incubated with HKPerox-Ratio for 30 min at 37 °C with 5% CO₂, followed by flow cytometry analysis using a BD LSR Fortessa. Pacific Blue channel was selected to measure blue emission, and AmCyan channel was selected to measure green emission with an excitation at 405 nm.

For starvation experiment, cells were washed with HBSS twice, and then incubated in HBSS for a given period of time. In the untreated group, cells were incubated in DMEM medium. Cells were washed with PBS before the addition of HKPerox-Ratio (5 µM final concentration) in 1 mL HBSS (Hank’s balanced salt solution) supplemented with 100 µM CCl₃CN. Cells were typically incubated for 30 min at 37 °C with 5% CO₂ before imaging. During imaging, the dish was mounted onto a live cell imaging support module (Axiovision). Single-photosection images were acquired with a Zeiss LSM 780 confocal microscope for HKPerox-Ratio, by using the following settings: λ_ex = 405 nm, λ_em = 440–490 nm (band-pass, blue emission) and 540–650 nm (band-pass, green emission). Green to blue ratio heatmap was generated by using a Ratio Plus plugin in ImageJ software.

For flow cytometry analysis, RAW264.7 cells or HeLa cells were typically seeded at a density of 2×10⁵ cells/mL in 35-mm dish (MatTek: MA, USA) in advance for attachment. Cells were washed with PBS before the addition of HKPerox-Ratio (10 µM final concentration) in 1 mL HBSS supplemented with 100 µM CCl₃CN, followed by incubation for 30 min at 37 °C with 5% CO₂. Single-cell suspensions were prepared by trypsinization and filtered for flow cytometry. Pacific Blue channel was selected to measure the blue emission, and AmCyan channel was selected to measure the green emission on a BD LSR Fortessa with an excitation at 405 nm. The F_Green/F_Blue ratio in each cell was calculated with FlowJo 7.6.2 software and presented as histogram.

7. Cytotoxicity assay

To assess potential toxicity of HKPerox probes, RAW264.7 cells were seeded at 2 × 10⁵ cells/mL in 100 µL DMEM per well in a 96-well microplate (Corning). Cells were seeded one day in advance to allow their attachment on 96-well microplate. 100 µL of RAW264.7 cells at a density of 2 × 10⁵ cells/mL in DMEM were seeded into each well in a 96-well microplate. Stock solutions of HKPerox probes at various concentrations in DMF were added at testing concentrations (1.25–20 µM, final concentrations) into fresh medium. Seeded cells were incubated with HKPerox probes-containing medium (100 µL per well) for 24 h, then treated with 50 µL Cell-Titer Glo® reagent, followed by gentle shaking for 10 min at room temperature. Luminescence of cellular ATP could be used as indicator of cell viability, and luminescence of each well was measured on
DTX 880 multimode plate reader. Cell viability was calculated according to the equation: Cell viability (%) = 100 × \( A_{\text{with probe}} / A_{\text{control}} \), where \( A \) = luminescence intensity.

**Figure S3.** Cytotoxicity of HKPerox-Red in RAW264.7 cells. RAW264.7 cells were incubated with increasing concentrations (1.25–20 µM) of HKPerox-Red for 24 h. HKPerox-Red (up to 20 µM) showed negligible or no cytotoxicity after 24 h incubation. Data represent mean ± s.e.m. with Cell-Titer Glo® assays performed in triplicates.

**Figure S4.** Cytotoxicity of HKPerox-Ratio in RAW264.7 cells. RAW264.7 cells were incubated with increasing concentrations (1.25–20 µM) of HKPerox-Ratio for 24 h. HKPerox-Ratio (up to 20 µM) showed negligible or no cytotoxicity after 24 h incubation. Data represent mean ± s.e.m. with Cell-Titer Glo® assays performed in triplicates.
8. Profiling and colocalization analysis of ratiometric imaging by HKPerox-Ratio

Figure S5. Profiling and colocalization analysis of ratiometric imaging of RAW264.7 macrophages stained by HKPerox-Ratio (5 µM). (a) and (b) Profiling of the confocal image to show green signal overlayed with the blue signal. (c) Colocalization analysis to show an overlap coefficient of 0.85 (Ch1 is blue channel, Ch2 is green channel).

9. Calibration curve of HKPerox-Ratio in HeLa cells

Figure S6. (a) Histogram of the F_{Green}/F_{Blue} ratio in HeLa cells co-incubated with a solution of HKPerox-Ratio (10 µM) and CCl_3CN (100 µM), which was pretreated with 0, 10, 20, 40, and 80 µM H_2O_2. The y-axis is the normalized cell count; the x-axis is the F_{Green}/F_{Blue} ratio in each cell. (b) Calibration curve of F_{Green}/F_{Blue} in HeLa cells as a function of H_2O_2 concentrations. (c) Histogram of blue fluorescence in HeLa cells. (d) Histogram of green fluorescence in HeLa cells.
10. Permeability test by flow cytometry

**Figure S7.** (a) Dot plots of blue fluorescence and green fluorescence in RAW264.7 macrophages incubated with HBSS only (unstained); HKPerox-Ratio (10 µM) only; 4-aminoo-1,8-naphthalimide (10 µM) only; or co-stained with HKPerox-Ratio (10 µM) and 4-amino-1,8-naphthalimide (10 µM). (b) Histogram of blue fluorescence in RAW264.7 macrophages. (c) Histogram of green fluorescence in RAW264.7 macrophages.
11. NMR spectra
12. References

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