Supplemental Information

Development of a Ratiometric Two-photon Fluorescent Probe for Imaging of Hydrogen Peroxide in Ischemic Brain Injury

Baoping Zhai,[a*] Wei Hu,[b] Ruilin Hao,[a] Wenjing Ni,[a] Zhihong Liu[b*]

[a] *Department of Chemistry, Xinzhou Teachers University, Xinzhou, Shanxi 034000, China.*

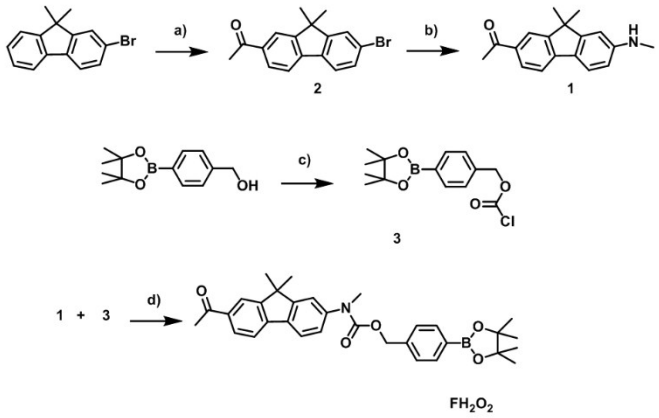
[b] *Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China.*

* Corresponding author.

Tel: +86 27 87217886;

E-mail address: zhhliu@whu.edu.cn

Materials and apparatus.



Scheme S1. Synthesis route of $\mathbf{FH_2O_2}$. Reagents and conditions: a), acetyl chloride, AlCl3, Dry DCM, 0 °C to r. t. 3 h; b), methylamine aqueous solution, copper powder, DMSO, 140 °C, 2 day; c), triphosgene, Na_2CO_3, methylbenzene, r.t., 10 h; d), pyridine, anhydrous CH_2Cl_2, r. t. 12 h, under nitrogen atmosphere.

Unless otherwise stated, all solvents and reagents were purchased from commercial suppliers and used as received without further purification. All aqueous solutions were prepared in ultrapure water with a resistivity of 18.25 MΩ cm (purified by Milli-Q system supplied by Millipore). MS analysis was performed on a liquid chromatograph (Agilent 1200, America) connected to a quadrupole time-off light mass spectrometer (Q-TOF MS, Agilent 6520, America). Absorption spectra were recorded on an UV−vis spectrophotometer (Shimadzu UV-2550, Japan), and one-photon fluorescence spectra were obtained with a fluorimeter (Shimadzu RF-5301 PC, Japan). Two-photon fluorescence spectra were excited by a mode-locked Tisapphire femtosecond pulsed laser (Chameleon Ultra I, Coherent, America) and recorded with a DCS200PC

photon counting with Omno-λ5008 monochromator (Zolix, China). Two-photon microscopy images were collected from a spectral confocal and multiphoton microscope (Carl Zeiss, LSM 780 NLO, Germany) with a mode-locked titanium-sapphire laser source (Mai Tai HP, Spectra Physics, America).

Synthesis route of **FH_2O_2** was depicted in Scheme S1 and the synthesis of compound 1 and 3 was referred to the literature.[1, 2]

Synthesis of FH_2O_2

2 mL Pyridine was added to a stirred solution of compound **1** (662.5 mg, 2.5 mmol) and compound **3** (1.09 g, 3.7 mmol) in 20 mL dry methylene dichloride and the reaction mixture was stirred at room temperature for 12 h under nitrogen atmosphere. The solvent was removed in vacuo and extracted by methylene dichloride. The organic layer was dried with anhydrous Na_2SO_4. Then the solvent was removed under reduced pressure, and the crude product was purified by silica gel column chromatography using petroleum ether: ethyl acetate (v:v = 6 : 1) and gave a white solid. (787 mg, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$) 8.13 (1 H, d, J = 0.9), 7.98 (1 H, d, J = 1.6), 7.97 – 7.91 (2 H, m), 7.62 (3 H, dd, J = 8.0, 4.8), 7.36 – 7.31 (3 H, m), 5.24 (2 H, s), 3.32 (3 H, s), 2.64 (3 H, s), 1.47 (6 H, s), 1.28 (12 H, s). ^{13}C NMR (400 MHz, $CDCl_3$) δ 198.07, 155.58, 155.38, 154.03, 143.49, 135.96, 134.99, 128.41, 126.87, 122.38, 121.24, 119.79, 83.85, 77.26, 67.34, 47.16, 38.03, 26.91, 24.86. MS: calcd for $C_{32}H_{36}BNO_5$ $[M + H]^+$ 526.3 found 526.3.

General procedure for hydrogen peroxide detection

Unless otherwise stated, all the fluorescence measurements were made in 10 mM PBS (pH 7.4) according to the following procedure. In a 2mL tube, 450 μL of PBS and 50 μL **FH_2O_2** were mixed, and then add an appropriate volume of hydrogen peroxide sample solution. After incubation at 37 °C for 40 min in a thermostat, a 3 mL portion of the reaction solution was transferred to a quartz cell of 1 cm optical length to measure absorbance or fluorescence with $\lambda_{ex/em}$ = 358/562 nm and excitation slit width of 3 nm and emission slit widths of 5 nm. For the selectivity assay, Superoxide ($O_2\cdot^-$) was created by the enzymatic reaction of xanthine/xanthineoxidase (XA/XO; 6.0 μM/3 mU) at 25 °C for 5 min.[3, 4] ·OH was generated by Fenton reaction between Fe^{2+} (EDTA) and H_2O_2 quantitatively, and Fe^{2+} (EDTA) concentrations represented ·OH concentrations.[5] The $ONOO^-$ source was the donor 3-Morpholinosydnonimine hydrochloride (SIN-1, 200 μmol/mL).[6] NO was generated in form of 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 100 μM/mL).[7] OCl^- was standardized at pH 12 ($\varepsilon_{292\ nm}$ = 350 $M^{-1}cm^{-1}$).[8] H_2O_2 was determined at 240 nm ($\varepsilon_{240\ nm}$ = 43.6 $M^{-1}cm^{-1}$). $^1O_2$ was generated from 3,3'-(naphthalene-1,4-diyl)dipropionic acid.[9] All the reagents were obtained from Aladdin (USA). All other chemicals were from commercial sources and of analytical reagent grade, unless indicated otherwise.

Measurement of Fluorescence Quantum Yield.

The fluorescence quantum yield was determined by using quinine sulfate in 0.1 N sulfuric acid (Φ=0.55). The fluorescence quantum yield was calculated

according to the following equation:

$$\Phi_s = \Phi_r(A_r I_s n_s^2)/(A_s I_r n_r^2)\ (A \leq 0.05)$$

The subscripts s and r represent the sample and the reference molecule respectively. Φ is the fluorescence quantum yield. A is the absorbance of molecules that is controlled below 0.05 for both molecules in the same wavelength. *I* means the integrated emission area and n is the refractive index of the solvent.

Measurement of Two-Photon Cross-Section.

The two-photon cross section (δ) was determined by using the femto second (fs) fluorescence measurement technique as described. **1** and **FH_2O_2** were dissolved in PBS buffer (10mM, pH 7.4, containing 0.9% NaCl), The fluorescence intensity was measured at 710-880 nm by using rhodamine B (dissolved in methanol) as the reference. The two-photon properties in this reference have been well characterized. The intensities of the two-photon-induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The two-photon cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$, where the subscripts s and r stand for the sample and reference, respectively. The fluorescence intensity was denoted as S. Φ is the fluorescence quantum yield and ϕ is the overall fluorescence collection efficiency of the experimental apparatus, which can be approximated by the refractive index of the solvent. The concentration of the solution was denoted as c. δ is for two-photon absorption.

Determination of the detection limit

The detection limit was calculated based on the fluorescence titration.[10, 11] The fluorescence emission spectrum of **FH_2O_2** was measurement 11 times without adding H_2O_2 and the standard deviation of blank measurement was achieved. The fluorescence intensity at 560 nm was plotted against the concentration of H_2O_2. So the detection limit was calculated with the following equation:

$$\text{Detection limit} = 3\sigma/k$$

Where σ is the standard deviation of blank measurement and k is the slope between the fluorescence intensity versus H_2O_2 concentration.

DFT calculations.

To describe the ground state and singlet excited state of the probe, DFT theoretical calculations were performed. All the calculations were carried out using the Gaussian 09 program package.[12] All the geometries of TBET system were optimized at B3LYP/6-31+G(d) level using a CPCM solvation model with water as the solvent. The molecular orbital (MO) plots and MO energy levels were computed at the same level of theory.

Cell Culture

Bv-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific) supplemented with 1 % penicillin/streptomycin and 10 % fetal bovine serum (FBS), and incubated in an atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. Cells were passed and plated into glass bottom cell culture dishes (NEST) in the day before imaging. For probe loading, the cells were washed with

phosphate buffer and then interacted with 10 μM **FH_2O_2** (containing 1 % DMSO in serum-free DMEM) for 30 min at 37 °C. After washing with phosphate buffer twice, the cells were then incubated into a phosphate buffer solution containing for 2 h.

Cytotoxicity Study of FH_2O_2 for Cells.

MTT test was performed according to the reported protocol with a minor change. Bv-2 cells were seeded in 96-well plates and incubated with different concentrations of **FH_2O_2** (0, 5, 10, 15 and 20 μM, containing 1 % DMSO in 100 μL DMEM). The **FH_2O_2** labelled bv-2 cells were incubated in an atmosphere of 5/95 (v/v) of CO_2/air at 37 °C for 24 h. Subsequently, 20 μL 5.0 mg/mL MTT solution was added to each well. Followed by incubation for 4 h under the same conditions. 100 μL supernatant was removed and 150 μL DMSO was added. After shaking for 10 min, the absorbance at 490 nm was measured by microplate reader (Synergy 2. BioTek Instruments Inc.). Cell survival rate was calculated by $A/A_0 \times 100$ % (A and A_0 are the absorbance of the **FH_2O_2** labelled group and the control group, respectively).

Fluorescence Microscopy Imaging.

Bv-2 cells were washed twice with PBS and then cultured with DMEM without glucose that had been progressed with 95% N_2/5% CO_2 for 10 min to remove residual oxygen. bv-2 cells were passed and dispersed on 18 mm glass coverslips at 37 °C, 5% CO_2 1 day before imaging. Then cells were incubated with 10 μM **FH_2O_2** for 40 min. The medium was removed and cells were

washed with PBS (10 mM, pH 7.4) twice. Two-photon microscopic imaging of bv-2 cells which were labelled with 10 μM **FH_2O_2** were performed on a spectral confocal and multiphoton microscopes (Carl Zeiss, LSM 780 NLO) with a 40 × water objective, and a numerical aperture (NA = 1.0). The two-photon fluorescence microscopy images were obtained by exciting **FH_2O_2** with a mode-locked titanium–sapphire laser source (Mai Tai HP, Spectra Physics, 80 MHz pulse frequency, 100 fs pulse width) set at the wavelength of 750 nm. All the images were separately collected in the emission wavelength range of 400-480 nm and 570-670 nm. The internal PMTs were used to collect the signals in an 8 bit unsigned 1024 × 1024 pixels at a scan speed of 1.58 s per pixel.

Western blotting.

Bv-2 cells were lysed with NP-40 lysis buffer (1.0% NP-40, 150mM NaCl, 50mM Tris-HCl, pH 8.0). The protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membrane. To block nonspecific antibody binding, the membrane was treated in 5% nonfat milk (dissolved in TBS) for 30 min. Next, the membrane was incubated with the following rabbit antibodies were purchased from Cell Signaling Technology (β-actin: #4970, 1:1000; LC3-II: #2775S, 1:1000).

Statistical Analysis.

Statistical Product and Service Solutions (SPSS) software 19.0 was used for the statistical analysis. The error bars shown in the figures represented the mean

± s.d. Differences were determined with a one-way analysis of variance (ANOVA) followed by LSD test. Statistical significance was assigned at *P < 0.05, **P < 0.01 and ***P < 0.001. Sample size was chosen empirically based on our previous experiences and pre-test results. No statistical method was used to predetermine sample size and no data were excluded. The numbers of animals or samples in every group were described in the corresponding figure legends. The distributions of the data were normal. All experiments were done with at least three biological replicates. Experimental groups were balanced in terms of animal age, sex and weight. Animals were all caged together and treated in the same way. Appropriate tests were chosen according to the data distribution. Variance was comparable between groups in experiments described throughout the manuscript.

***In vivo* imaging studies.**

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Hunan University, and experiments were approved by the Animal Ethics Committee of the College of Biology (Hunan University). All BALB/c mice (age 4 weeks, weight 17–20 g) were operated upon in accordance with institutional ethics committee regulations and guidelines on animal welfare. The aforementioned animals were randomly divided into control (n = 3), 24-h model (n = 3) and 48-h model (n = 3) groups. The model groups were anesthetized with isoflurane 7 days after birth, disinfected, and a median longitudinal incision was made under the operation

microscope. The common carotid artery was carefully separated and double-ligated with 6-0 silk thread and disconnected, and the skin incision was sutured. After 2 h of postoperative recovery, the test was placed in the incubator at 37°C for 2 h with 8% oxygen in a 92% nitrogen hypoxia chamber. After performing anesthesia on the control group, only the median neck incision was made followed by suturing of the incision. After a complete recovery, food and water were again provided to the animals ad libitum. The local environmental temperature was maintained at 35°C–37°C during surgery, hypoxia and resuscitation. Following the hypoxic–ischemic process, abdominal injection of 200 μL of 1.0 mM probe **FH_2O_2** was administered, animals were fasted for 18 h and then decapitated, then the brain tissue was immediately removed, washed in ice-cold normal saline, and cut into 300-μm section. Sections were washed with PBS and two-photon tissue fluorescence imaging was immediately performed.

Glutathione S-transferase (GST) activity assay.

According to the 1-chloro-2,4-dinitrobenzene (CDNB) colorimetric method as described by Habig, the principle is as follows: GST has the ability to catalyze the binding of reduced glutathione (GSH) to CDNB, the peak wavelength of light absorption of the bound product is at a wavelength of 340 nm, and the activity unit of GST can be calculated by measuring the absorbance at this wavelength. The reaction formula is as follows:

$$C_6H_3(NO_2)_2Cl + GSH \rightarrow C_6H_3(NO_2)_2SG + H^+ + Cl^-$$

The determination procedure involves addition of 5 mL of 0.1 mol/L phosphate buffer (pH 6.5), then 500 μL of 1 mmol/L GSH, followed by 500 μL crude enzyme dilution (diluted 10 times), and 500 μL of 1 mmol/L CDNB. After accurately reacting for 1 min in a 30°C water bath, the tube was immediately placed in a 60°C water bath to terminate the reaction for 5 min, and after removal absorbance $(A340)_0$ was measured at room temperature for at least 10 min. The absorbance $(A340)_1$ of the non-enzymatic reaction was measured by the same procedure using physiological saline instead of the enzyme solution. Absorbance was measured with a Japanese Hitachir UV-3010 ultraviolet spectrophotometer.

Calculation method: GST ($\mu mol \cdot min^{-1} \cdot mg^{-1}$) = $\Delta A \cdot \varepsilon^{-1} \cdot Cp^{-1} \cdot 2.6 \times 10^8$ where $\Delta A = (A340)_0 - (A340)_1$, ε: molar absorption coefficient of the product at 340 nm is 9.6×10^3 $M^{-1} \cdot cm^{-1}$, and Cp: protein content (mg/mL).

Glutathione reductase (GRed) activity assay.

For the glutathione reductase activity assay, glutathione peroxidase reduces lipid peroxide to oxidize reduced glutathione to its oxidized form, while oxidized glutathione can be reduced by glutathione reductase in coenzyme II in the presence of a reduced form. The reduced coenzyme can be produced by a reductive enzyme II (NADPH regenerating enzymes; NRE) such as glucose-6-phosphate dehydrogenase, 6-phosphate gluconate dehydrogenase, or malic enzyme. Reduced coenzyme II-dependent isocitrate dehydrogenase and nicotinamide dinucleotide transhydrogenase are provided.

The specific measurement procedure is as follows: 4 mL of 0.1 mol/L phosphate buffer (pH 7.0) containing 1 mM EDTA, 400 μL enzyme dilution, 100 μL of 1 mM reduced coenzyme II, and 500 μL of sample is added to the test tube. Then 1 mM oxidized glutathione was added, and the final concentrations of reduced coenzyme II and oxidized glutathione were 0.2 mM and 1 mM, respectively. After reacting for 5 min in a 37°C water bath, absorbance $(A340)_0$ was measured at 340 nm, and the non-enzymatic reaction absorbance $(A340)_1$ was measured by replacing the enzyme solution with an equal amount of physiological saline (measured in 15 min).

Calculation formula: GRed ($\mu mol \cdot min^{-1} \cdot mg^{-1}$) = $\Delta A \cdot \varepsilon^{-1} \cdot Cp^{-1} \cdot 2.6 \times 10^8$ where $\Delta A = (A340)_0 - (A340)_1$, ε: molar absorption coefficient of the product at 340 nm is 9.6×10^3 $M^{-1} \cdot cm^{-1}$, and Cp: protein content (mg/mL).

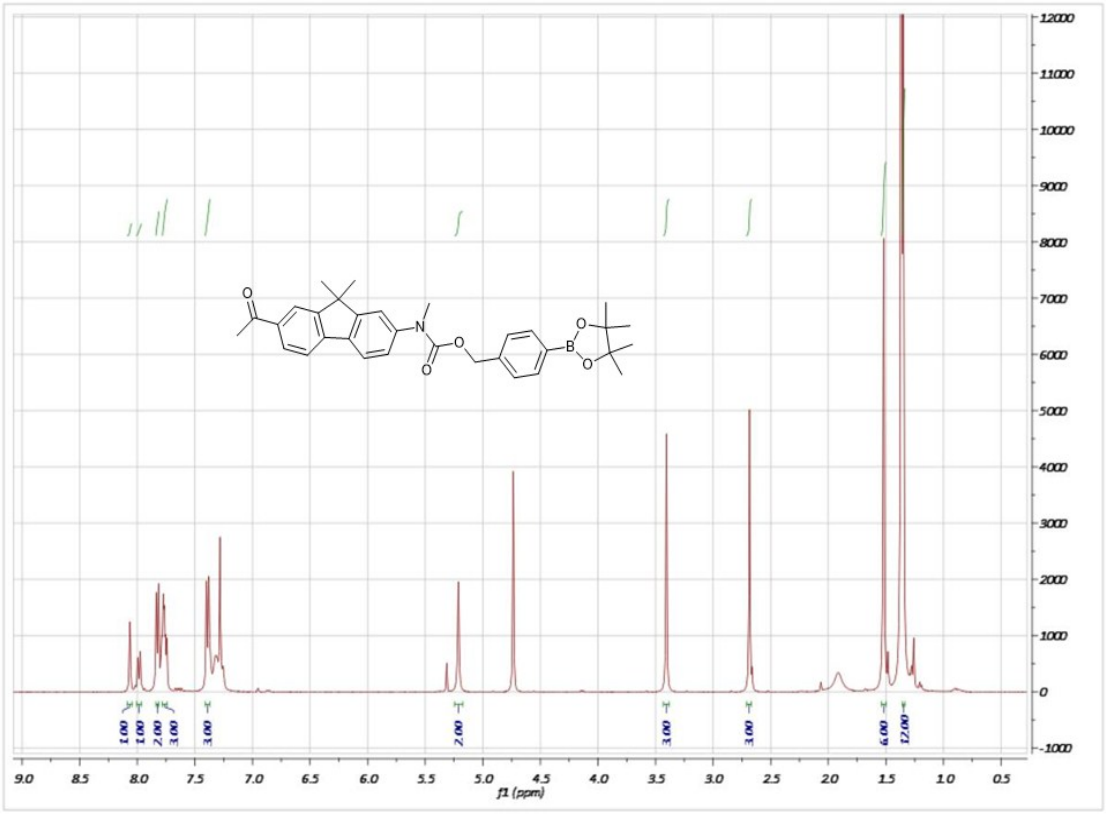


Figure S1 ^{1}H-NMR spectrum of **FH_2O_2** (400 MHz, $CDCl_3$)

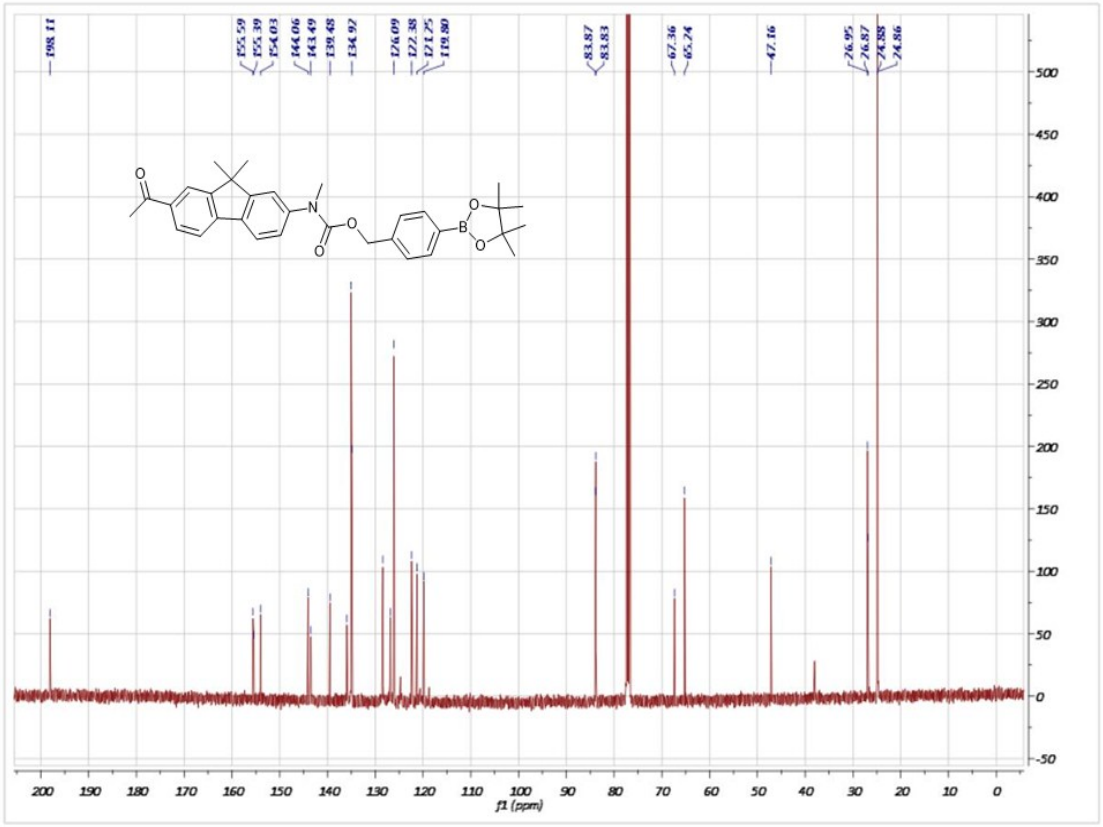


Figure S2 ^{13}C-NMR spectrum of **FH_2O_2** (100 MHz, $CDCl_3$)

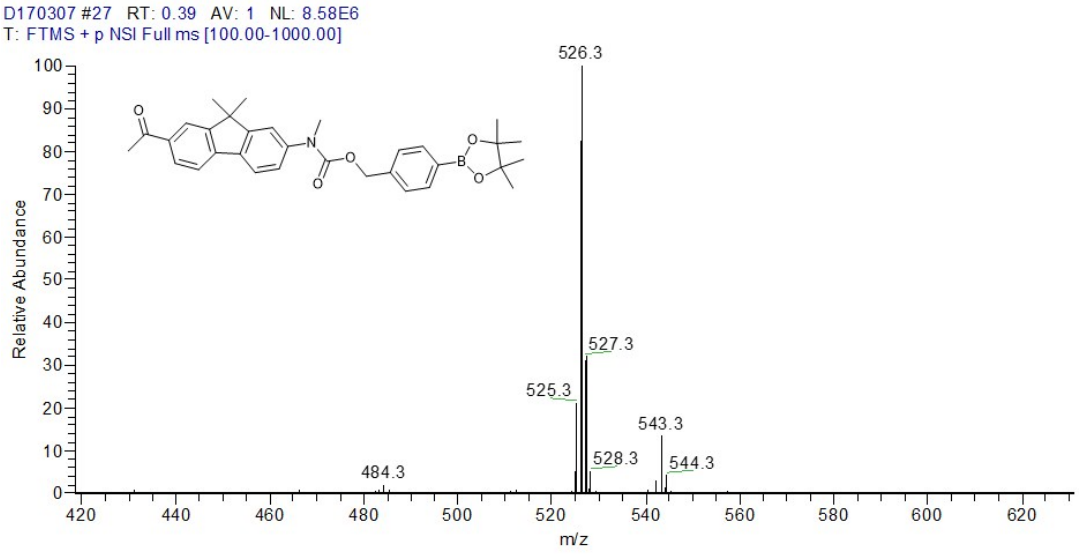


Figure S3 MS spectrum of **FH_2O_2**

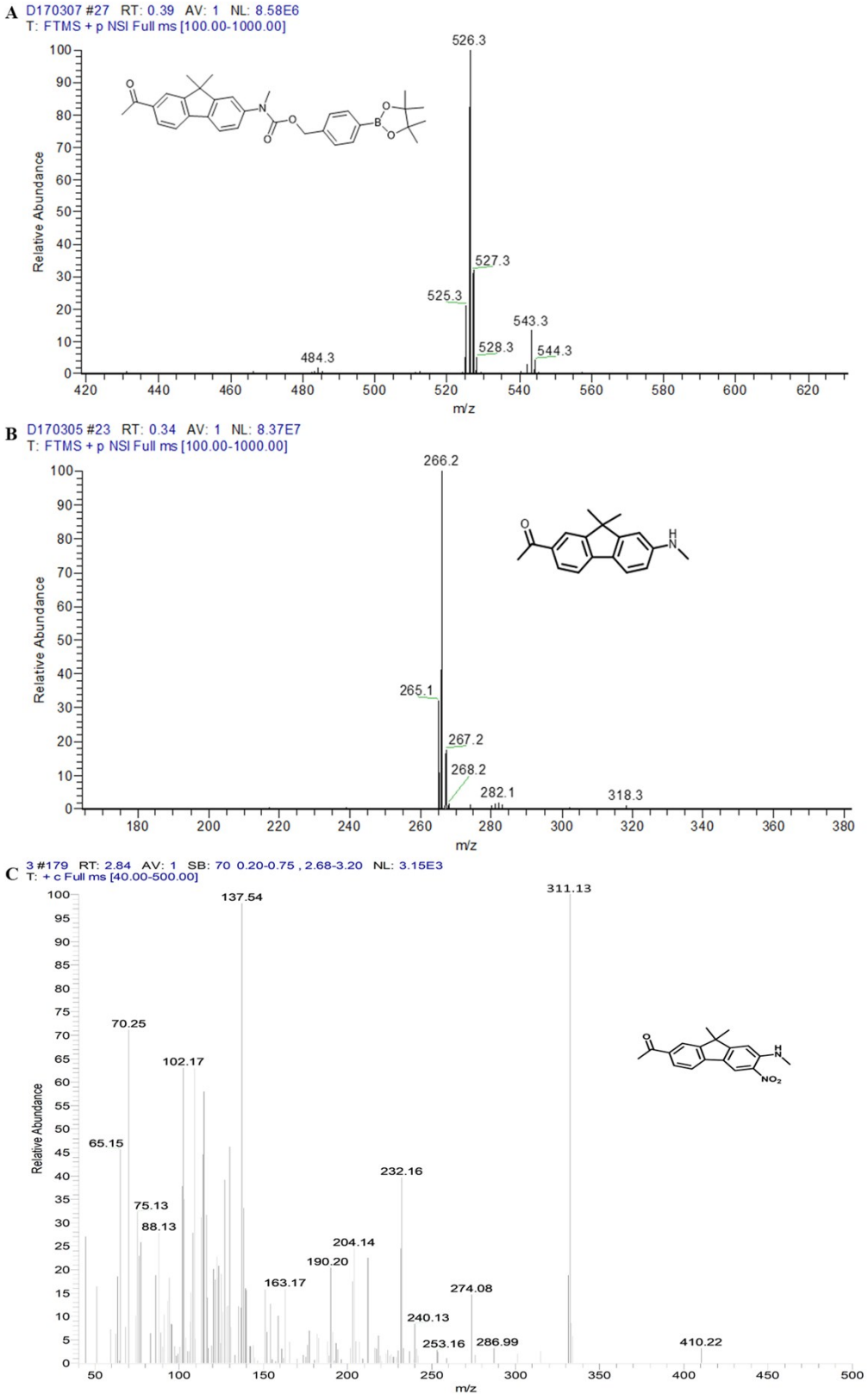


Figure S4 MS traces of (A) $\mathbf{FH_2O_2}$, (B) the product of $\mathbf{FH_2O_2}$-H_2O_2 reaction and (C) the product of $\mathbf{FH_2O_2}$-$ONOO^-$ reaction.

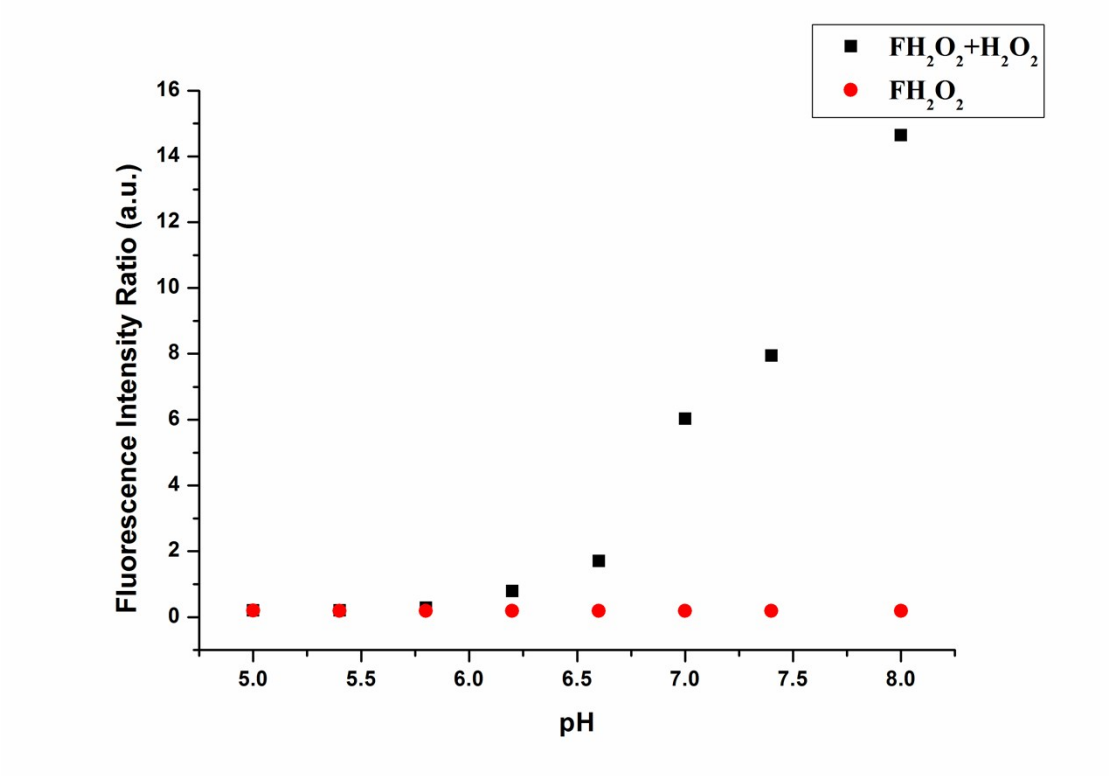


Figure S5 Effects of pH on the fluorescence ($\lambda_{ex/em}$ = 410/560 nm) of **FH_2O_2** (10 μM) reacting with hydrogen peroxide (200 μM).

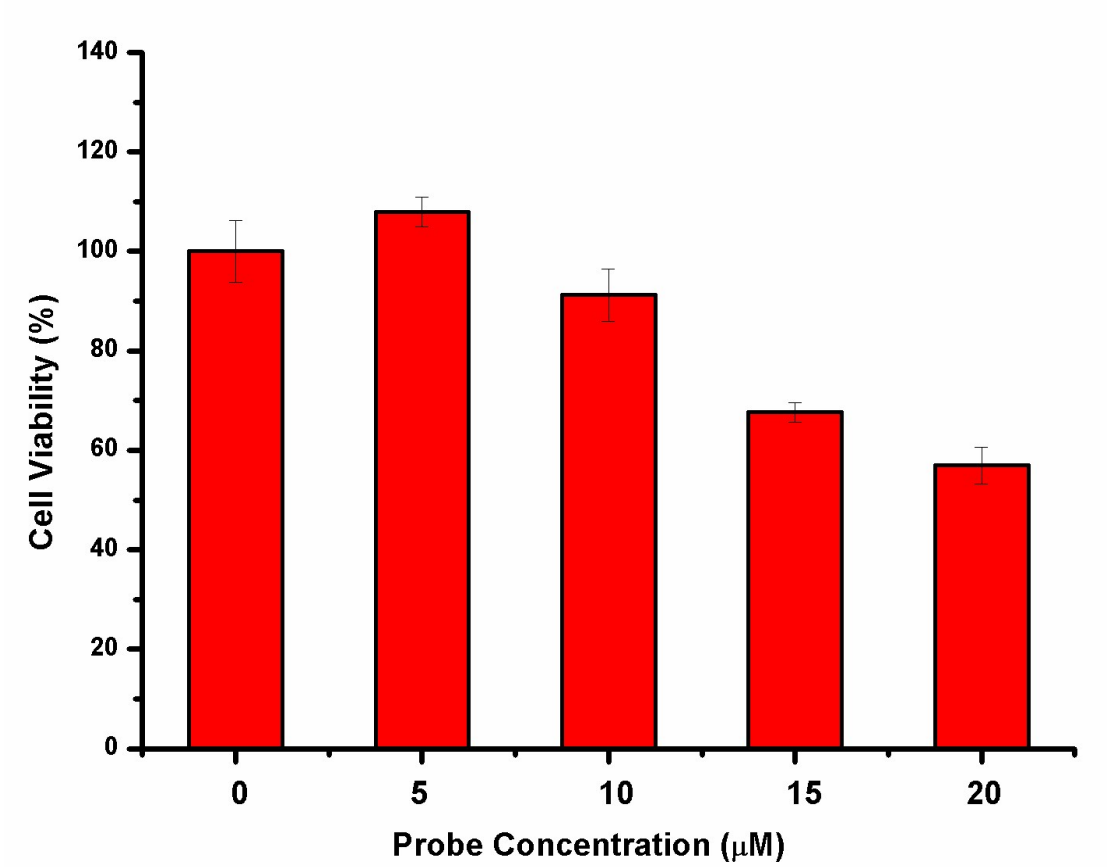


Figure S6. Cell survival rate of control groups (without **FH_2O_2**) and experimental group (with 5, 10, 15, 20 μM of **FH_2O_2**). All groups contain 1 % DMSO in 100 μL DMEM)

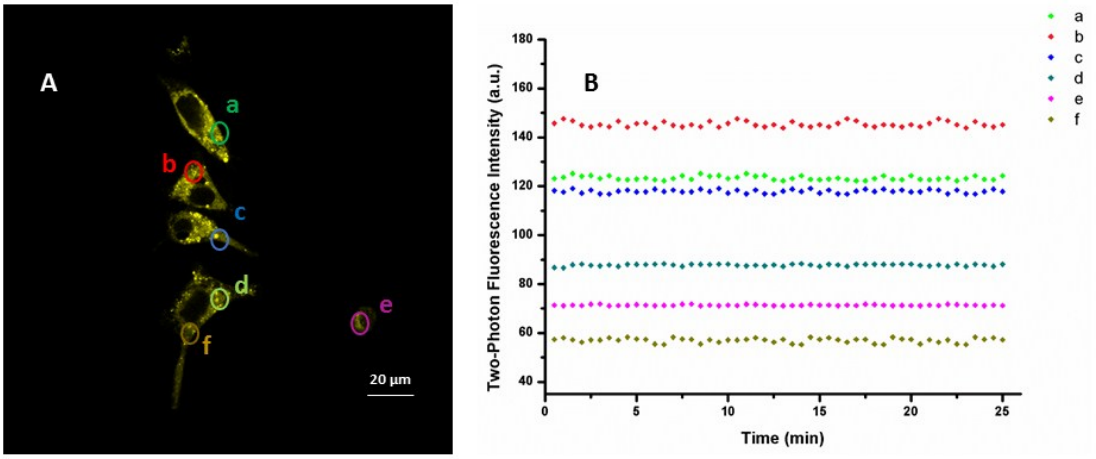


Figure S7. (A) TPM images of HeLa cells labeled with 200 μM hydrogen peroxide for 48 h and further incubated with **FH_2O_2** for 30 min separately. (B) Two-photon fluorescence intensity from circle a-f as a function of time. The two-photon fluorescence intensity was collected with 15 sec intervals for the duration of 25 min under xyt mode. Scale bar: 20 μm

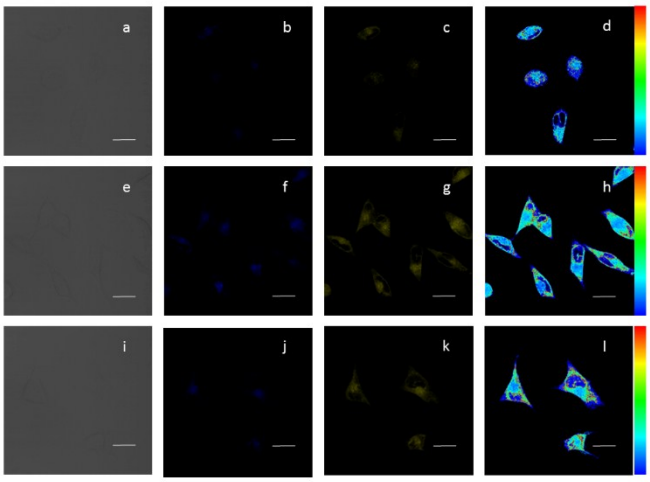


Figure S8. TP fluorescence images of HeLa cells labelled with 10 μM **FH_2O_2** for 40 min at 37 °C and further incubated with 0 (a-d), 100 μM (e-h), 200 μM (i-l) H_2O_2 for

30 min. Images were obtained by being excited with 750 nm laser light, and the emissions were collected individually in blue channel (400-480 nm, b, f, j) and yellow channel (570-670 nm, c, g, k). The fluorescence intensity ratio of the two channels (F_{yellow}/F_{blue}, d, h, l), which was reflected as colour bar processed by Image-Pro Plus 6.0. Cells shown are replicate images from replicate experiments (n=3). Scale bar: 20 μm.

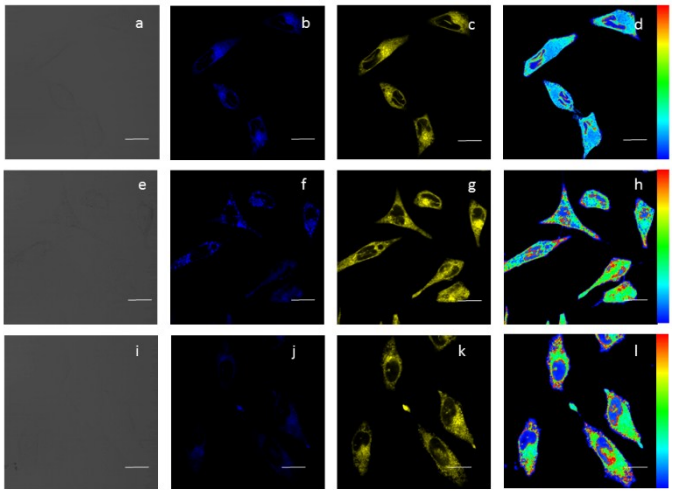


Figure S9. TP fluorescence images of HeLa cells treated with 0 (a-d), 1 (e-h) and 2 μg/mL (i-l) PMA and further being incubated 10 μM **FH_2O_2** for 40 min at 37 °C. Images were obtained by being excited with 750 nm laser light, and the emissions were collected individually in blue channel (400-480 nm, b, f, j) and yellow channel (570-670 nm, c, g, k). The fluorescence intensity ratio of the two channels (F_{yellow}/F_{blue}, d, h, l), which was reflected as color bar processed by Image-Pro Plus 6.0. Cells shown are replicate images from replicate experiments (n=3). Scale bar: 20 μm.

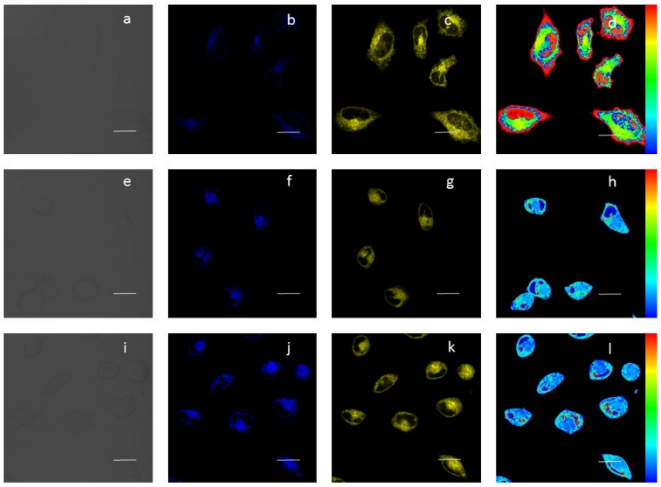


Figure S10. TP fluorescence images of $\mathbf{FH_2O_2}$ labelled HeLa cells preteated with PMA and H_2O_2 inhibits. HeLa cells were incubated with untreated (a-d), 10 μM DPI (e-h) and 10 mM NAC (i-l) for 1 h, and then incubated 1μg/mL PMA for 1 h and further being incubated 10 μM $\mathbf{FH_2O_2}$for 30 min at 37 °C. Images were obtained by being excited with 750 nm laser light, and the emissions were collected individually in blue channel (400-480 nm, b, f, j) and yellow channel (570-670 nm, c, g, k). The fluorescence intensity ratio of the two channels (F_{yellow}/F_{blue}, d, h, l), which was reflected as color bar processed by Image-Pro Plus 6.0. Cells shown are replicate images from replicate experiments (n=3). Scale bar: 20 μm.

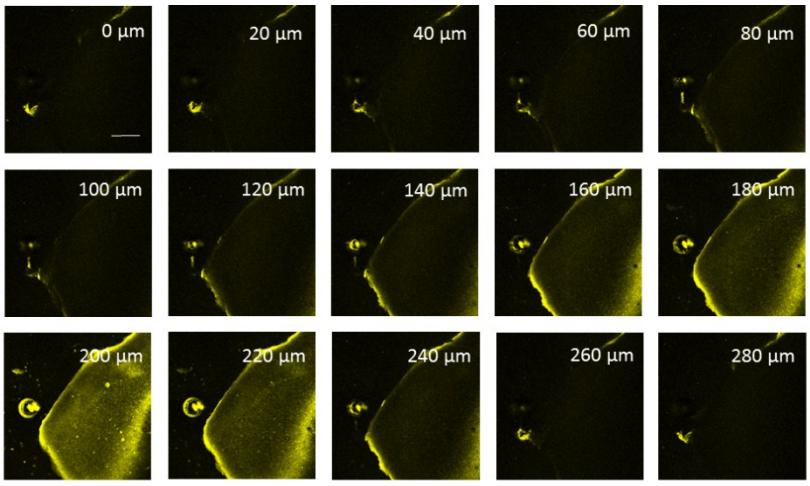


Figure S11. Depth fluorescence images of $\mathbf{FH_2O_2}$ (200 μM) in mouse Brain tissues.

λ_{ex} = 750 nm, λ_{em} = 570–670 nm. Scale bar: 100 μm.

Reference

[1] C. Chung, D. Srikun, C. S. Lim, C. J. Chang and B. R. Cho, *Chem. Commun.*, 2011, **47**, 9618-9620.

[2] B. Zhai, W. Hu, J. Sun, S. Chi, Y. Lei, F. Zhang, C. Zhong and Z. Liu, *Analyst*, 2017,**142**, 1545-1553.

[3] V. Massey, D. Edmondson, *J. Biol. Chem.* 1970, **245**, 6595-6598.

[4] D. Edmondson, V. Massey, G. Palmer, L.M. Beachan III, G.B. ELion, *J. Biol. Chem.* 1972, **247**, 1597-1604.

[5] L. Zeng, T. Xia, W. Hu, S. Chen, and Z. Liu, *Anal. Chem*.., 2018, **90**, 1317.

[6] R.M. Uppu, *Anal. Biochem.,* 2006, **354**, 165-168.

[7] H. Maeda, K. Yamamoto, Y. Nomura, I. Kohno, L. Hafsi, N. Ueda, S. Yoshida, M. Fukuda, Y. Fukuyasu, Y. Yamauchi, N. Itoh, *J. Am. Chem. Soc.* 2005, **127**, 68-69.

[8] J.C. Morris, *J. Phys. Chem.* 1966, **70**, 3798.

[9] J.M. Aubry, B. Cazin, F. Duprat, *J. Org. Chem.*, 1989, **54**, 726-728.

[10] M. Mei, X. Zhu, X. Liu, J. Xu, J. Lv, C. Huang, H. Liu, Y. Li, S. Wang, and D. Zhu, *Org. Lett.*, 2008, **10**, 1481-1484.

[11] H. Niu, D. Su, X. Jiang, W. Yang, Z. Yin, J. He and J. Cheng, *Org. Biomol. Chem.*, 2008, **6**, 3038-3040.

[12] T. Ren, W. Xu, W. Zhang, X, Zhang, Z. Wang, Z. Xiang, L. Yuan and X. Zhang. *J. Am. Chem. Soc.*, 2018, **14024**, 7716-7722.