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Simultaneous fluorescence visualization of mitochondrial hydrogen peroxide and zinc ion in live cells and in vivo

Haibin Xiao, Ping Li,* Shan Zhang, Wei Zhang, Wen Zhang and Bo Tang*a

College of Chemistry, Chemical Engineering and Materials Science, Institute of Biomedical Sciences, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, P. R. China

E-mail: tangb@sdnu.edu.cn

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Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. The solvents were purified by conventional methods before use. Mito-Tracker Deep Red, and Mito-Tracker Green were purchased from Invitrogen (USA). Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS) and L-buthionine sulfoximine (BSO) were purchased from Sigma. Silica gel (200-300 mesh) used for flash column chromatography was purchased from Qingdao Haiyang Chemical Co., Ltd. **M-H_2O_2** and **M-Zn** were dissolved in dimethyl sulfoxide (DMSO) to produce 1.0 mM stock solutions. ^{1}H NMR and ^{13}C NMR spectra were determined by 400 MHz and 100 MHz using Bruker NMR spectrometers. The mass spectra were obtained by Bruker maxis ultra-high resolution-TOF MS system. The fluorescence spectra measurements were performed using FLS-920 or FLS-980 Edinburgh fluorescence spectrometer. The ex/em slit widths are 3.0/3.0 nm and 2.5/2.5 nm for **M-H_2O_2** and **M-Zn**, respectively. Cary eclipse fluorescence spectrophotometer was used for the kinetic assays. Fluorescence imaging in cells were performed with Leica TCS SP5 and Leica TCS SP8 Confocal Laser Scanning Microscope. The laser power of confocal imaging is 15 mW for 488, 514, and 633 nm laser. The human hepatoma cells (HepG2) and macrophages (RAW 246.7) were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

Cells culture

Human hepatoma (HepG2) and macrophages (RAW 246.7) were cultured in high glucose DMEM (4.5 g of glucose/L) or RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C in a 5% CO_2/95% air incubator MCO-15AC (SANYO, Tokyo, Japan). One day before imaging, the cells were detached and were replanted on glass-bottomed dishes.

Determination of the limit of detection

The detection limit was determined from the fluorescence titration data. So the detection limit was calculated with the following equation: Detection limit=3σ/k, where σ is the standard deviation of blank measurement, k is the slop between the fluorescence intensity versus H_2O_2 or Zn^{2+} concentration.

Calculation of the relative fluorescence quantum yield[1-3]

Fluorescence quantum yield was determined by using ICG (Φ_f=0.13 in DMSO) for **M-Zn** or Rhodamine 6G (Φ_f=0.95 in water) for **M-H_2O_2** as a fluorescence standard. The quantum yield was calculated using the following equation:

$$\Phi_x=\Phi_s(A_sF_x/A_xF_s)(n_x/n_s)^2$$

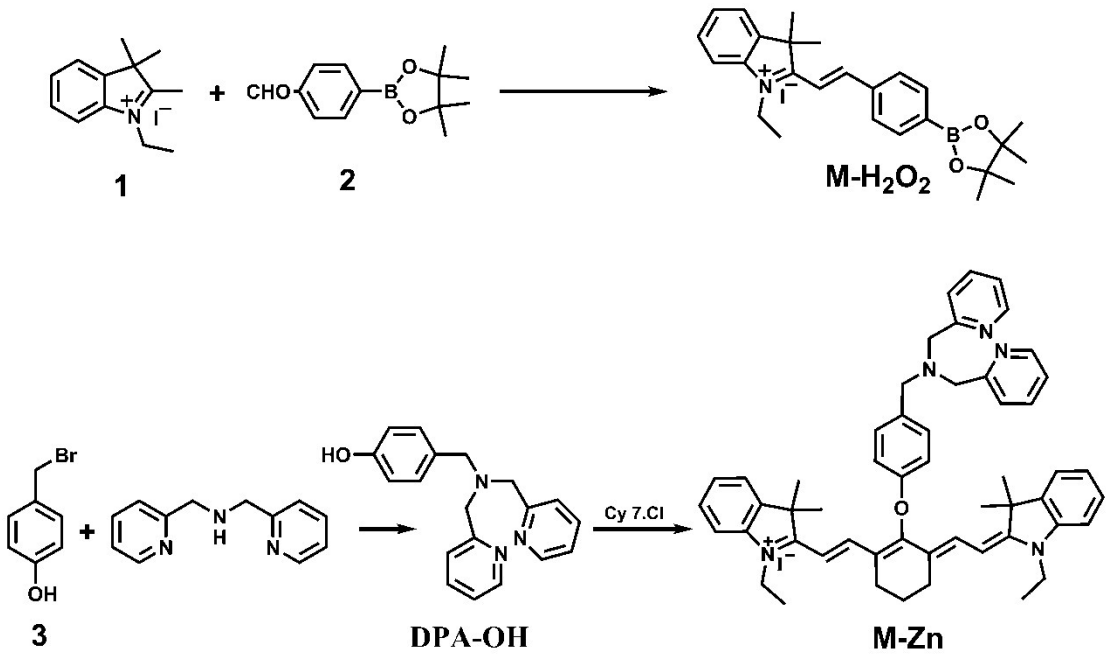
Where Φ_x is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used. Subscripts S and X refer to the standard and to the unknown, respectively. For **M-Zn** and ICG, the excitation wavelength was at 750 nm while keeping the absorption below 0.05; For **M-H_2O_2** and Rhodamine 6G, the excitation wavelength was at 530 nm while keeping the absorption below 0.05.

Determination of the association constant K_a

The association constant (K_a) was determined from the fluorescence titration experiment based on the Benesi-Hildebrand equation **1**[4,5].

$$\mathbf{1}/(F-F_0)=\mathbf{1}/\{K_a(F_{max}-F_0)[Zn^{2+}]\}+\mathbf{1}/[F_{max}-F_0] \qquad \mathbf{1}$$

Here F_0 is the fluorescence of **M-Zn** in the absence of Zn^{2+}. F is the fluorescence obtained in the presence of added Zn^{2+}. F_{max} is the fluorescence in presence of added Zn^{2+} max and K_a is the association constant (M^{-1}). The association constant (K_a) could be determined from the slope of the straight line of the plot of $\mathbf{1}/(F-F_0)$ against $\mathbf{1}/Zn^{2+}$. The slope was obtained to be 2.51928×10^{-10}. And the association constant (K_a) is calculated to be 7.03×10^5 M^{-1}. Therefore, the dissociation constant (K_d) is $1/K_a$=1.42 μM.



Scheme S1 The synthesis of **M-H_2O_2** and **M-Zn**

2,3,3-trimethyl-1-ethyl-3H-indolium iodide (**1**) and **Cy.7.Cl** were synthesized according to our previous work.[6] Compounds **2** and **3** were obtained according to published work.[7,8]

Synthesis of **M-H_2O_2**: Compound **1** (0.315 g, 1.0 mmol), **2** (0.23 g, 1.0 mmol) and sodium acetate (0.17 g, 2.0 mmol) were dissolved in 8.0 mL of acetic anhydride under the Ar gas condition. The mixture was stirred at 55 ℃ for 5 h. Then the solvents were dropped in ethyl ether, and the residue was purified by silica gel chromatography with dichloromethane/methanol (20:1 v/v) to give **M-H_2O_2** as a light yellow solid (0.11 g, 40 %).^{1}H NMR (4 00 MHz, *d6*-DMSO) δ(ppm): 1.332 (s, 6H), 1.476 (t, J=6.8 Hz, 3H), 1.815, (s, 12H), 4.758 (q, J=6.8 Hz, 2H), 7.621 (d, J=6.4 Hz, 1H), 7.762 (d, J=6.4 Hz, 1H), 7.862 (d, J=8.0 Hz, 1H), 7.911 (d, J=2.8 Hz, 1H), 7.924 (d, J=2.8 Hz, 1H), 8.201 (d, J=8.0 Hz, 1H),

8.254 (d, J=8.0 Hz, 2H), 8.454 (t, J=2.8 Hz, 1H), 8.495 (t, J=2.8 Hz, 1H). ^{13}C NMR (100 MHz, *d6*-DMSO) δ(ppm): 14.35, 25.18, 25.96, 42.8, 52.75, 84.51, 113.05, 113.34, 115.77, 115.90, 123.63, 129.71, 129.85, 130.10, 130.25, 131.03, 133.84, 134.87, 135.04, 135.31, 140.87, 144.62, 153.61, 154.23, 182.04. HRMS (ESI) m/z calcd. for $C_{26}H_{33}BNO_2^+$ 402.2603, found 402.2585.

Synthesis of **DPA-OH**: Under the Ar gas condition, compound **3** (0.37 g, 2.0 mmol), potassium carbonate (0.276 g, 2.0 mmol) and bis(2-picolyl)amine (0.40 g, 2.0 mmol) were dissolved in 10 mL acetonitrile. The reaction was heated to 25℃ for 8 h. The color of the solution changed from red to yellow. The mixture was filtered, and the filtrate was dryed with $MgSO_4$ and concentrated to obtain the yellow oil **DPA-OH** without further purification (0.49 g, 80%)

Synthesis of **M-Zn**: In an atmosphere of argon, to a mixture of compound **DPA-OH** (0. 305 mg, 1.0 mmol) in 10 mL DMF was added potassium carbonate (0.276 g, 2.0 mmol) for 30 min, then **Cy.7.Cl** (0.64 g, 1.0 mmol) was added. The reaction mixture was stirred for 6 h at 50℃ in an oil bath. Concentration of the product and chromatography were carried out over silica gel (50:1 CH_2Cl_2:CH_3OH). This resulted in 0.36 g (yield 40%) of **M-Zn** as a light green solid. ^{1}H NMR (400 MHz, *d6*-DMSO) δ(ppm): 0.853 (t, J=6.4 Hz, 3H), 1.158 (s, 6H), 1.232 (s, 12H), 1.936-2.027 (m, 3H), 2.714 (t, J=6.4 Hz, 2H), 3.491 (s, 2H), 3.606 (s, 2H),

3.667 (s, 2H), 4.131 (t, J=6.4 Hz, 2H), 6.181 (d, J=14.4 Hz, 1H), 6.726 (d, J=8.4 Hz, 1H), 7.137 (d, J=8.4 Hz, 1H), 7.171-7.268 (m, 5H), 7.318-7.384 (m, 4H), 7.458 (d, J=8.8 Hz, 2H), 7.558-7.591 (m, 3H), 7.768-7.806 (m, 4H), 8.437 (d, J=4.0 Hz, 1H), 8.485 (d, J=4.0 Hz, 1H), 9.288 (s, 1H). ^{13}C NMR (100 MHz, *d6*-DMSO) δ(ppm): 12.47, 14.29, 23.98, 24.22, 26.76, 27.02, 29.48, 35.14, 48.91, 57.30, 58.85, 59. 33, 100.31, 105.12, 111.38, 114.53, 115.47, 119.31, 121.77, 122.56, 122.78, 125.20, 128.99, 129.05, 131.29, 131.38, 132.76, 136.97, 141.33, 141.95, 149.28, 156.87, 159.10, 159.58, 159.76, 162.90, 171.62. HRMS (ESI) m/z calcd. for $C_{53}H_{58}N_5O^+$ 780.4636, found 780.4577.

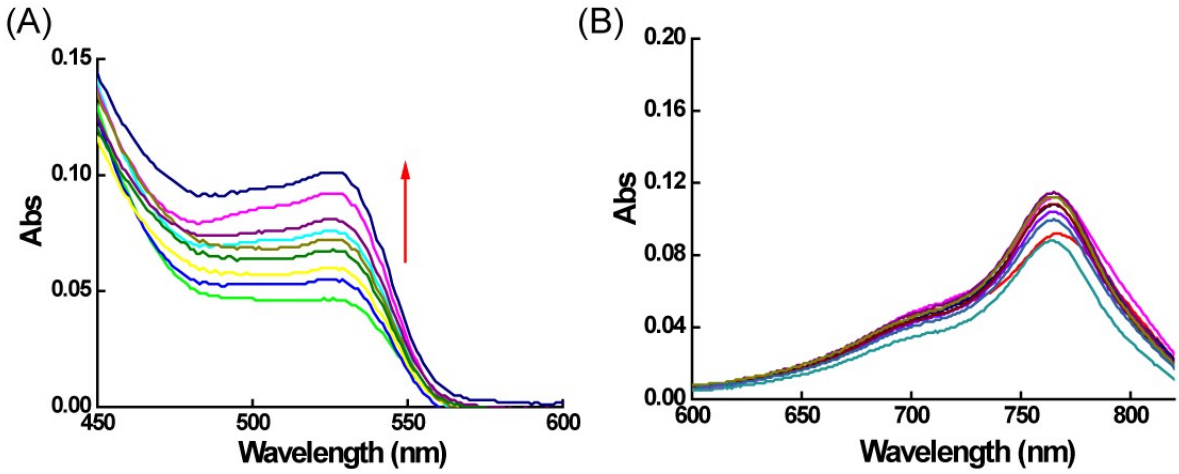


Figure S1 The absorption spectra of 5.0 μM **M-H_2O_2** (A) and 5.0 μM **M-Zn** (B) with different concentrations of H_2O_2 (0-50 μM) and Zn^{2+} (0-10 μM).

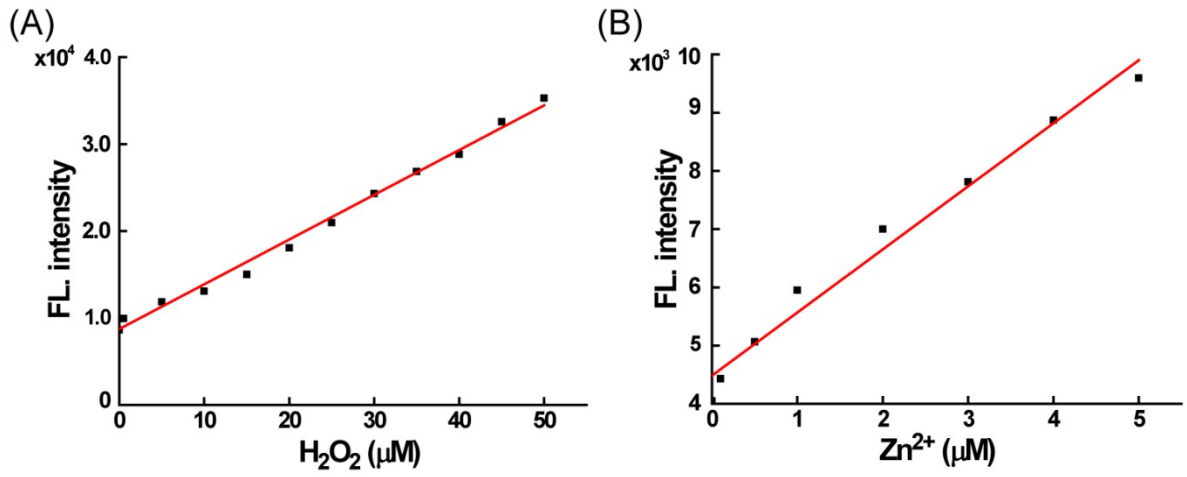


Figure S2 The linearity of 5.0 μM **M-H_2O_2** (A) and 5.0 μM **M-Zn** (B) with different concentrations of H_2O_2 (0.1-50 μM) and Zn^{2+} (0.1-5.0 μM). The excitation wavelengths are 530 and 750 nm for **M-H_2O_2** and **M-Zn**, respectively.

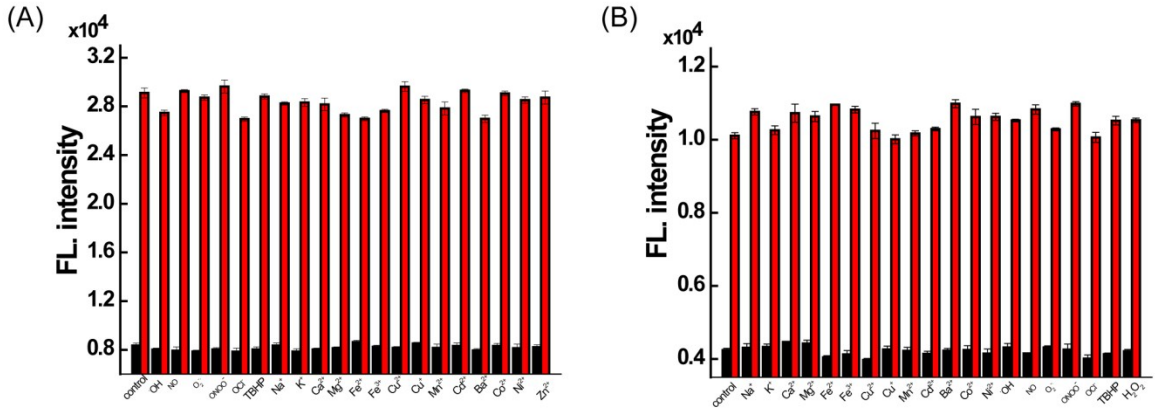


Figure S3 The high selectivity of 5.0 μM **M-H_2O_2** to 40 μM H_2O_2 (A) and 5.0 μM **M-Zn** to 5.0 μM Zn^{2+} (B) in the presence of various ROS and common metal ions. For **M-H_2O_2**, $ONOO^-$: 100 μM; NaClO, $OH\cdot$, $O_2\cdot^-$, TBHP, NO: 200 μM; Na^+, K^+: 5.0 mM; Ca^{2+}, Mg^{2+}: 1.0 mM; Fe^{2+}, Fe^{3+}, Cu^{2+}, Cu^+, Mn^{2+}, Cd^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}: 100 μM. For **M-Zn**, H_2O_2, $ONOO^-$, NaClO, $OH\cdot$, $O_2\cdot^-$, TBHP, NO: 200 μM; Na^+, K^+: 5.0 mM; Ca^{2+},

Mg^{2+}: 1.0 mM; Fe^{2+}, Fe^{3+}, Cu^{2+}, Cu^{+}, Mn^{2+}, Cd^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, 10 μM. The excitation wavelengths are 530 and 750 nm for **M-H_2O_2** and **M-Zn**, respectively.

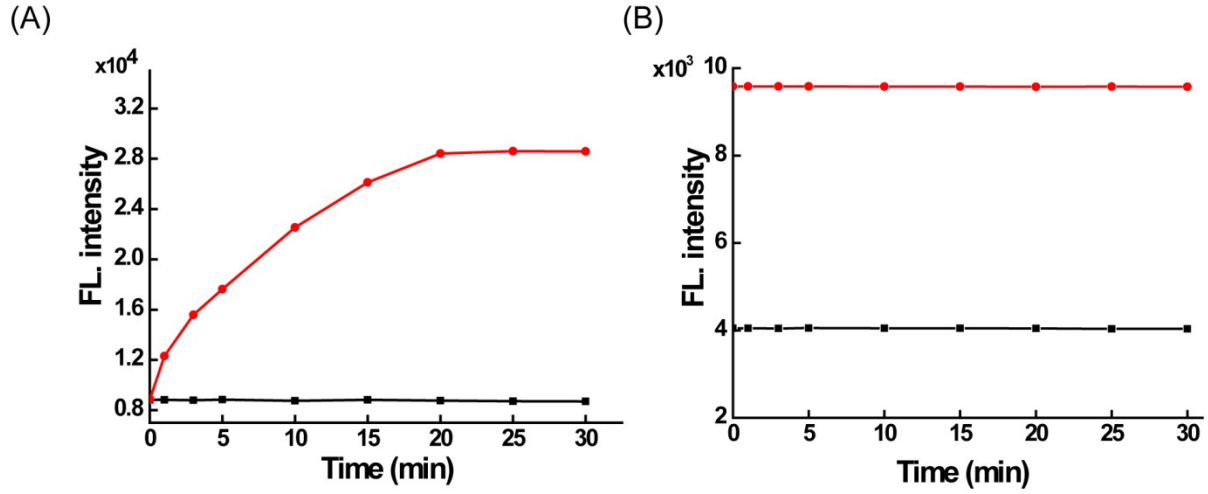


Figure S4 The fluorescence stability of 5.0 μM **M-H_2O_2** to 40 μM H_2O_2 (A) and 5.0 μM **M-Zn** to 5.0 μM Zn^{2+} (B) at different time. The excitation wavelengths are 530 and 750 nm for **M-H_2O_2** and **M-Zn**, respectively.

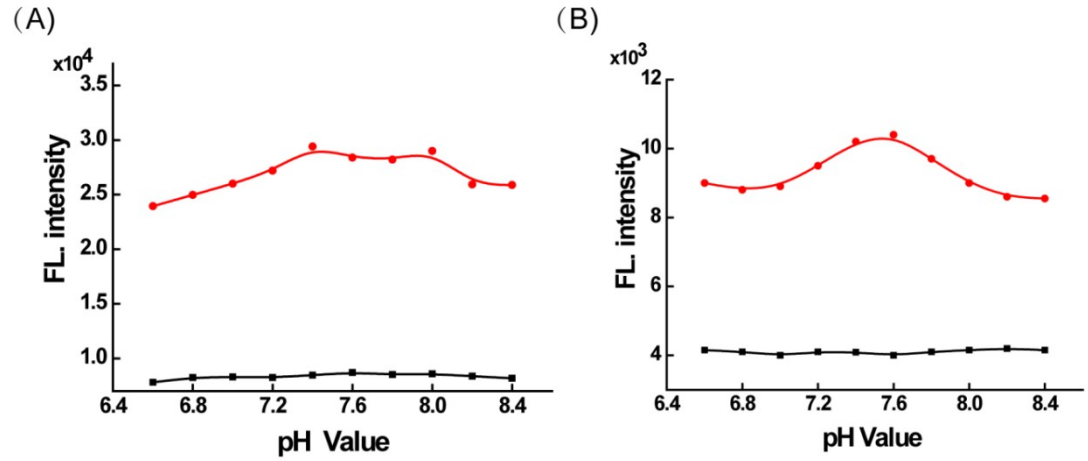


Figure S5 The fluorescence responses of 5.0 μM **M-H_2O_2** to 40 μM H_2O_2

(A) and 5.0 μM **M-Zn** to 5.0 μM Zn^{2+} (B) under different pH values. The excitation wavelengths are 530 and 750 nm for **M-H_2O_2** and **M-Zn**, respectively.

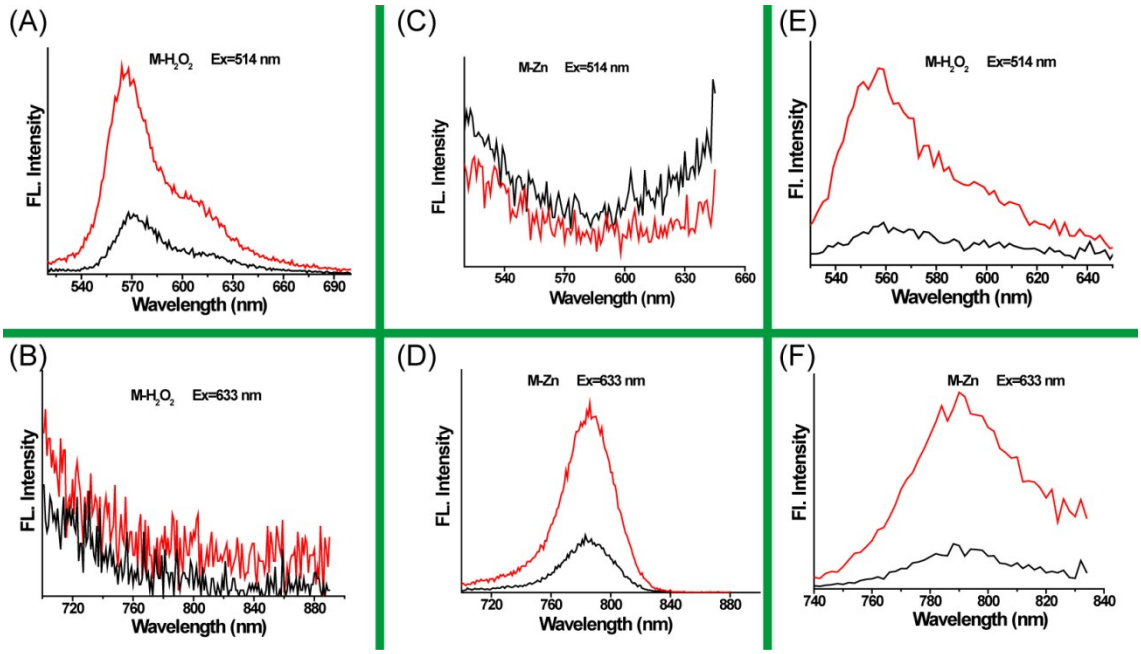


Figure S6 (A-B) The fluorescence responses of 5.0 μM **M-H_2O_2** to 40 μM H_2O_2 at different excitation wavelengths. (C-D) The fluorescence responses of 5.0 μM **M-Zn** to 5.0 μM Zn^{2+} at different excitation wavelengths. To completely avoid the spectral overlap for confocal fluorescence imaging with these two probes, we chose 514 nm as excitation wavelength for **M-H_2O_2**. It is because **M-Zn** can't be excited at 514 nm, but can be easily excited by 633 nm. In stark contrast, fluorescence enhancement of **M-H_2O_2** to H_2O_2 can be readily excited by 514 nm but not 633 nm. In a mixed solution containing both **M-H_2O_2** (5.0 μM) and **M-Zn** (5.0 μM), H_2O_2 (50 μM) and Zn^{2+} (5.0 μM) were

continuously added. Then fluorescence spectra were collected by using 514 nm or 633 nm as the excitation wavelength, respectively. The results were presented in (E) and (F). It showed that coexistence of one probe won't affect the fluorescence response of another probe.

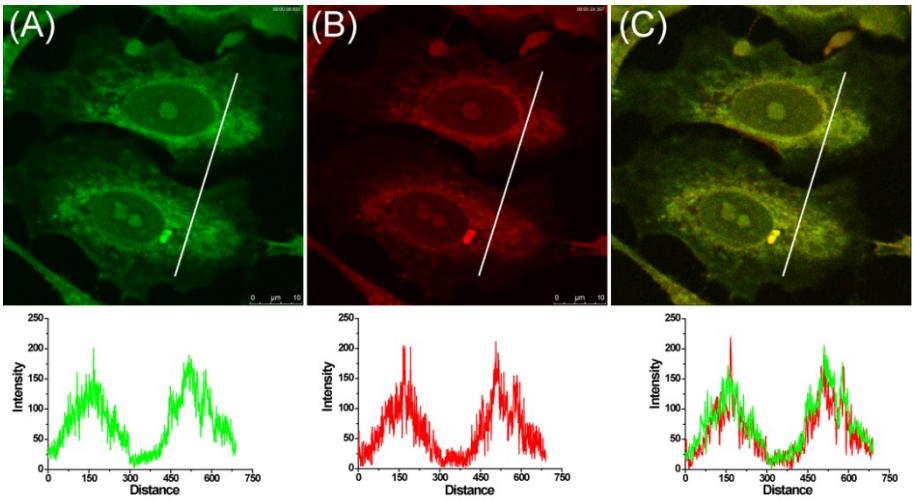


Figure S7 The colocalization fluorescence image of HepG2 cells stained simultaneously with **M-H_2O_2** (20 μM) and **M-Zn** (20 μM). (A) **M-H_2O_2** ex 514 nm/em 530−600 nm. (B) **M-Zn** ex 633 nm/em 730-800 nm. (C) The merged image of A and B. The fluorescence intensity profile of white line overlapped well.

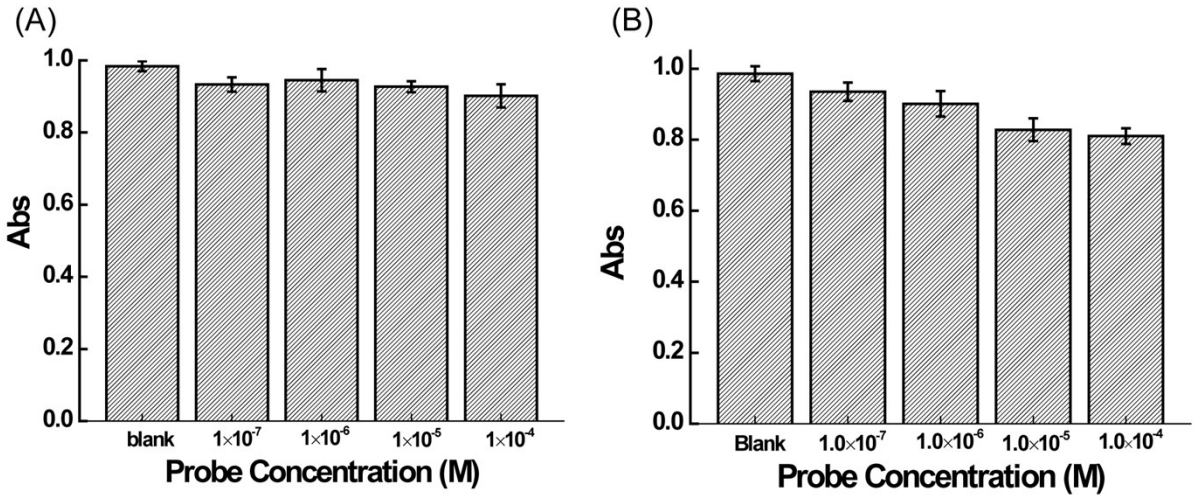


Figure S8 The MTT assay of **M-H_2O_2** and **M-Zn**.

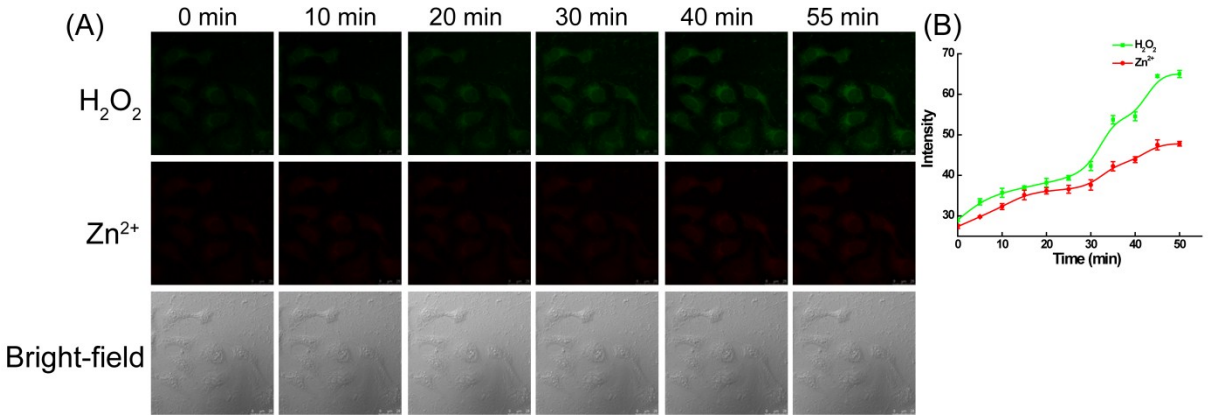


Figure S9 The simultaneous fluorescence imaging of H_2O_2 and Zn^{2+} with **M-H_2O_2** and **M-Zn** in HepG2 cells induced by H_2O_2 (1.0 mM). (A) The fluorescence images of **M-H_2O_2** (20 μM, green channel, ex 514 nm/em 530−600 nm) and **M-Zn** (20 μM, red channel, ex 633 nm/em 730−800 nm). (B) The average fluorescence intensity from A at different time.

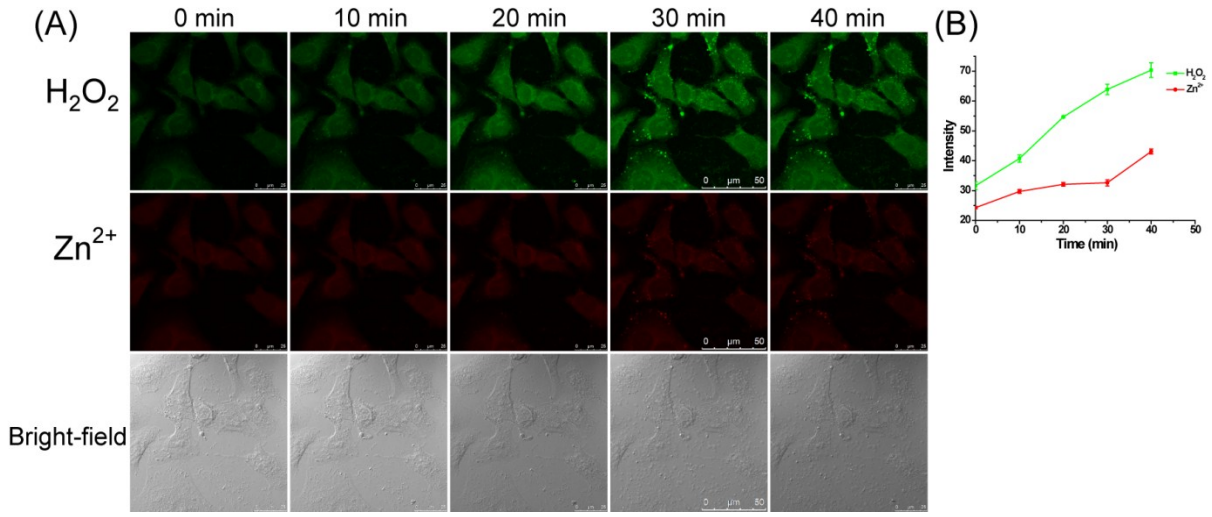


Figure S10 The simultaneous fluorescence imaging of H_2O_2 and Zn^{2+} in HepG2 cells stimulated by PMA (25 μg/mL). (A) The fluorescence images of **M-H_2O_2** (20 μM, green channel, ex 514 nm/em 530−600 nm) and **M-Zn** (20 μM, red channel, ex 633 nm/em 730-800 nm). (B) The average fluorescence intensity from A at different time.

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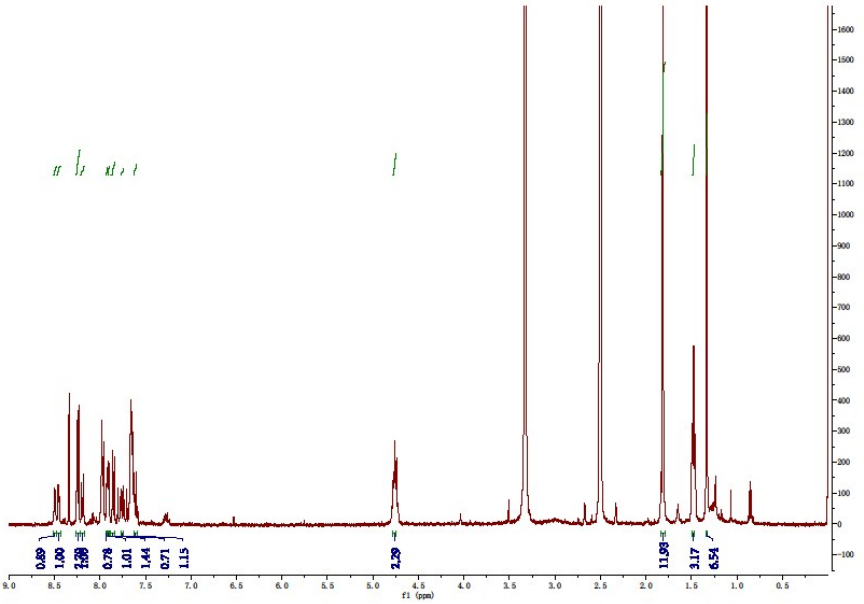
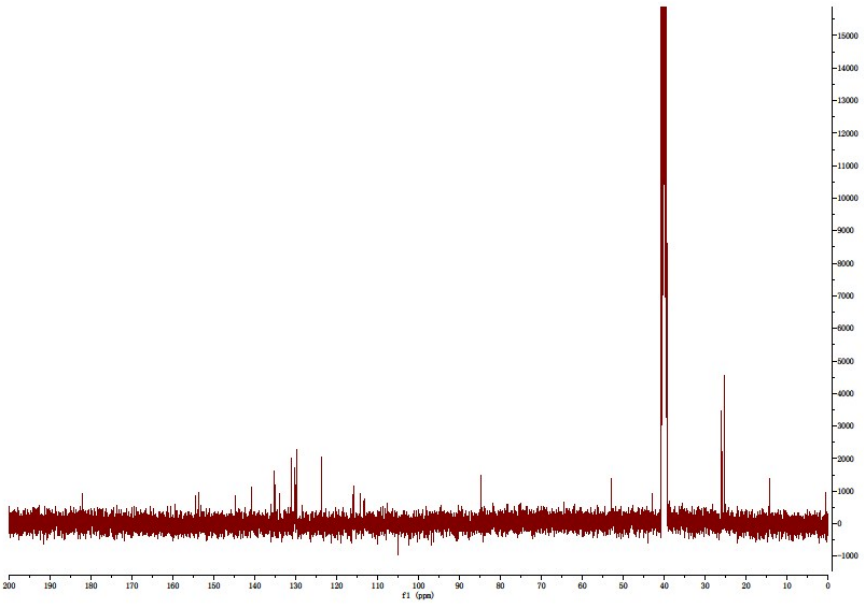
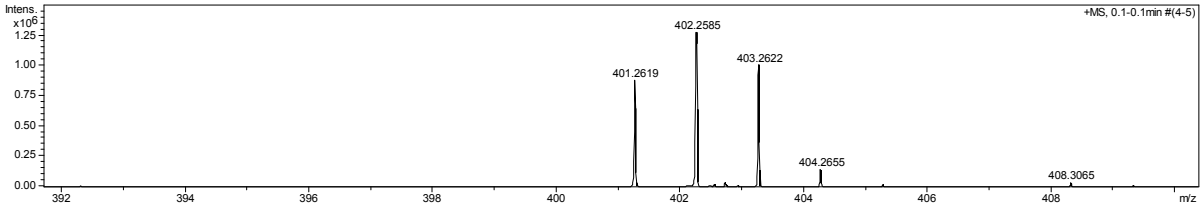
The original ^{1}H NMR, ^{13}C NMR and MS spectra

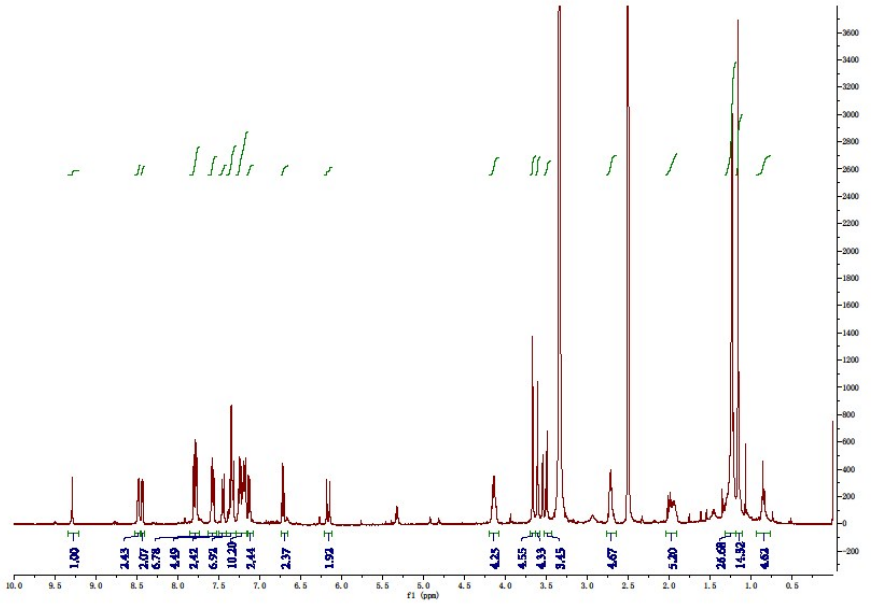


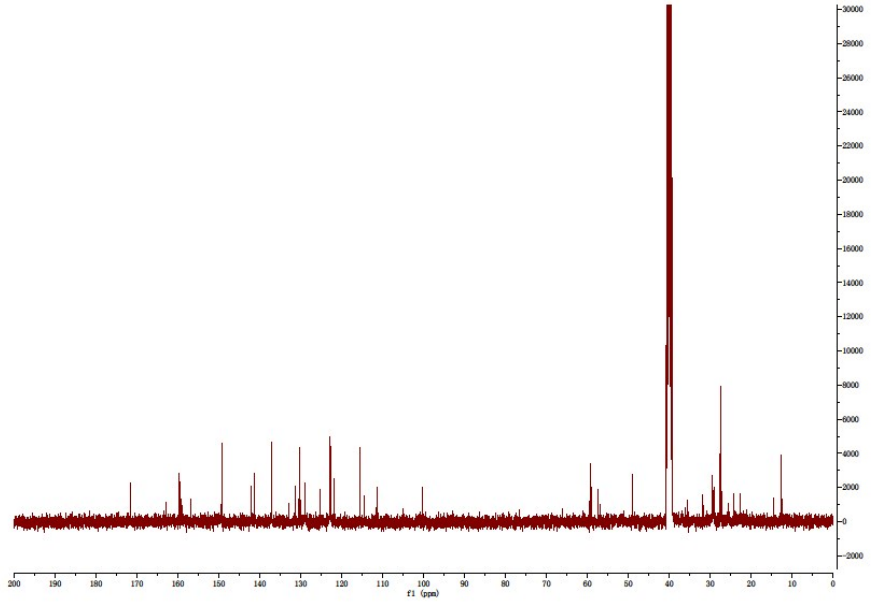


The ^{1}H NMR and ^{13}C NMR spectra of **M-H_2O_2**

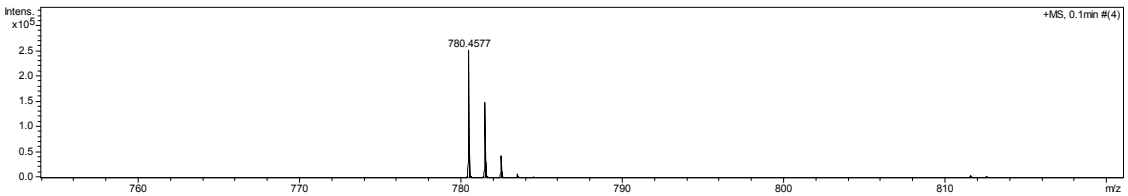


The HR-MS spectra of **M-H_2O_2**

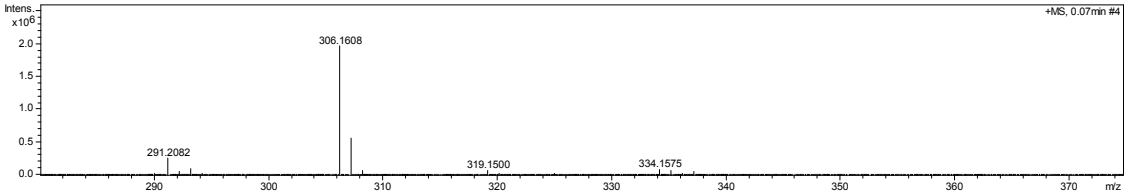




The ^{1}H NMR and ^{13}C NMR spectra of **M-Zn**



The HR-MS spectra of **M-Zn**



The HR-MS spectra of **DPA-OH**