

Supporting Information

Two-photon imaging of formaldehyde in live cells and animals utilizing a lysosome-targetable and acidic pH-activatable fluorescent probe

Xilei Xie,[‡] Fuyan Tang,[‡] Xiaoyan Shangguan, Shiyi Che, Jinye Niu, Yongsheng Xiao, Xu Wang* and Bo Tang*

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

E-mail: tangb@sdu.edu.cn; wangxu@sdu.edu.cn

[‡] These authors contributed equally to this work.

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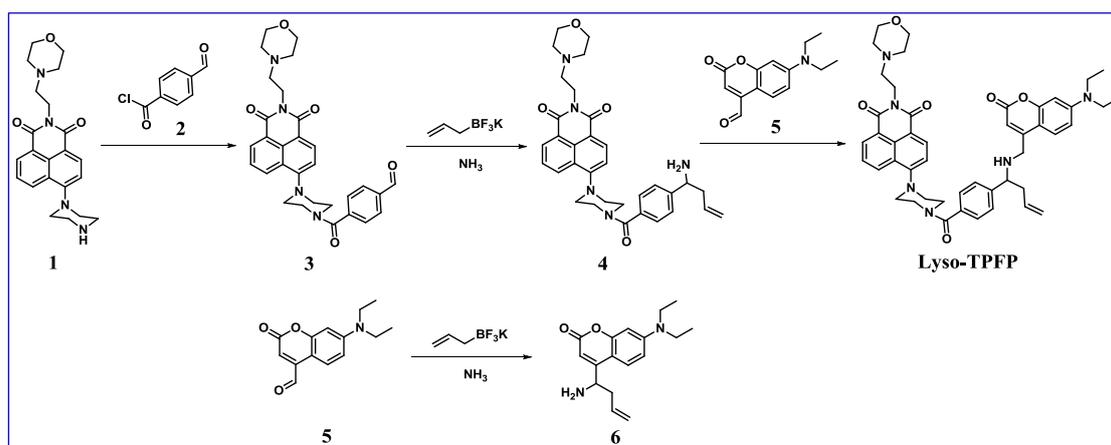
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1. Materials and instruments

Reagents and solvents were acquired from commercial sources, and the solvents were distilled or purified before use if necessary. Phosphate buffers were prepared using distilled water. NMR spectroscopic characterization was taken on a Bruker Advance 300 MHz spectrometer. HRMS spectra were obtained by a Bruker MaXis UHR-TOF instrument. The probe was dissolved in dimethyl sulfoxide (DMSO) to produce 1.0 mM stock solution. Absorption spectra were recorded on a UV-1700 spectrophotometer, and fluorescence measurements were performed using a FLS-980 fluorospectrometer from Edinburgh Instruments. All spectra were acquired when FA was added for 180 min at 37 °C. Fluorescence imaging in cells was performed with a Leica TCS SP8 confocal laser scanning microscope. Two-photon fluorescence imaging of live cells and mice was performed on a Zeiss LSM 880 confocal laser scanning microscope with an objective lens ($\times 20$), and the two-photon excitation wavelength was 800 nm.

2. Synthetic procedure

Lyso-TPFP was synthesized via a multistep procedure outlined in Scheme S1. Compounds **1**, **2**, and **5** were synthesized according to the previous literatures.¹⁻³ Compound **6** was prepared as a contrastive agent to confirm the spectral variation of Lyso-TPFP.



Scheme S1. Synthetic routine

Compound 3. Compound **1** (0.152 mmol, 60 mg) and compound **2** (0.152 mmol, 25 mg) were dissolved in anhydrous dichloromethane (10 mL). After addition of

triethylamine (0.60 mmol, 84 μ L), the mixture was stirred at room temperature for 3 h. The solvent was evaporated, and the residue was purified by column chromatography on silica gel (dichloromethane/methanol, 50:1 v/v) to afford a yellow solid (62 mg, 78% yield). HRMS (ESI): calculated for $C_{30}H_{31}N_4O_5^+$ ($M+H^+$) 527.2289, found 527.2324.

Compound 4. Compound **3** (0.114 mmol, 60 mg) was dissolved in 4 mL of NH_3 solution (7.0 M in methanol, 28 mmol), and the mixture was stirred at room temperature for 10 min. Then potassium allyltrifluoroborate (0.34 mmol, 50 mg) and distilled water (20 μ L) were added to the solution. The mixture was reacted at 30 $^{\circ}C$ for 16 h. After solvent was evaporated, the crude product was purified by column chromatography on silica gel (dichloromethane/methanol, 30:1 v/v) to obtain a yellow solid (30 mg, 46% yield). 1H NMR (300 MHz, $DMSO-d_6$): δ 8.51–8.45 (m, 2H), 8.38 (d, $J = 8.0$ Hz, 1H), 7.81 (t, $J = 7.8$ Hz, 1H), 7.59 (d, $J = 7.7$ Hz, 2H), 7.50 (d, $J = 7.7$ Hz, 2H), 7.36 (d, $J = 8.0$ Hz, 1H), 5.66 (td, $J = 16.8, 7.1$ Hz, 1H), 5.18–5.10 (m, 2H), 4.25 (t, $J = 6.8$ Hz, 1H), 4.14 (s, 2H), 3.95 (br s, 2H), 3.65 (br s, 2H), 3.52 (br s, 4H), 3.26 (br s, 4H), 2.88 (dd, $J = 14.1, 7.0$ Hz, 1H), 2.73–2.64 (m, 1H), 2.61–2.53 (m, 2H), 2.44 (br s, 4H). HRMS (ESI): calculated for $C_{33}H_{38}N_5O_4^+$ ($M+H^+$) 568.2918, found 568.2928.

Probe Lyso-TPFP. Compound **4** (0.053 mmol, 30 mg), compound **5** (0.106 mmol, 26 mg) and sodium cyanoborohydride (0.212 mmol, 13 mg) were dissolved in 5 mL of methanol. After addition of acetic acid (50 μ L), the mixture was stirred at 30 $^{\circ}C$ for 3 h. After solvent was evaporated, the residue was purified via column chromatography on silica gel (dichloromethane/methanol, 50:1 v/v) and Lyso-TPFP was obtained as a yellow solid (15 mg, 36% yield). 1H NMR (300 MHz, $CDCl_3$): δ 8.59 (d, $J = 7.2$ Hz, 1H), 8.52 (d, $J = 8.0$ Hz, 1H), 8.43 (d, $J = 8.4$ Hz, 1H), 7.72 (t, $J = 8.4$ Hz, 1H), 7.47–7.42 (m, 4H), 7.29 (d, $J = 9.0$ Hz, 1H), 7.23 (s, 1H), 6.51 (dd, $J = 9.0, 2.5$ Hz, 1H), 6.47 (d, $J = 2.5$ Hz, 1H), 6.16 (s, 1H), 5.80–5.66 (m, 1H), 5.15–5.09 (m, 2H), 4.34 (t, $J = 6.8$ Hz, 2H), 4.01 (br s, 3H), 3.82 (dd, $J = 7.9, 5.5$ Hz, 2H), 3.77–3.65 (m, 6H), 3.39 (q, $J = 7.0$ Hz, 4H), 3.29 (br s, 4H), 2.72 (br s, 2H), 2.62 (br s, 4H), 2.51–2.34 (m, 2H), 1.18 (t, $J = 7.0$ Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.67, 164.43, 163.96, 162.43, 156.36, 155.28, 154.17, 150.56, 145.78, 134.67,

134.53, 132.47, 131.34, 129.98, 129.93, 127.60, 126.42, 126.21, 124.99, 123.48, 118.62, 117.70, 115.57, 108.49, 107.53, 107.24, 97.85, 67.11, 62.11, 56.30, 53.91, 53.33, 47.44, 44.80, 43.18, 37.20, 12.55. HRMS (ESI): calculated for $C_{47}H_{53}N_6O_6^+$ ($M+H^+$) 797.4021 and $C_{47}H_{52}N_6NaO_6^+$ ($M+Na^+$) 819.3846, found 797.3991 and 819.3802.

Compound 6. Compound **5** (1.7 mmol, 415 mg) was dissolved in 18 mL of NH_3 solution (7.0 M in methanol), and the mixture was stirred at room temperature for 10 min. Then potassium allyltrifluoroborate (3.4 mmol, 503 mg) and distilled water (45 μ L) were added to the solution. The mixture was reacted at room temperature for 22 h. After solvent was evaporated, the crude product was purified by column chromatography on silica gel (dichloromethane/methanol, 40:1 v/v) to obtain yellow oil (380 mg, 78% yield). HRMS (ESI): calculated for $C_{17}H_{23}N_2O_2^+$ ($M+H^+$) 287.1754, found 287.1757.

3. Excitation and emission spectra

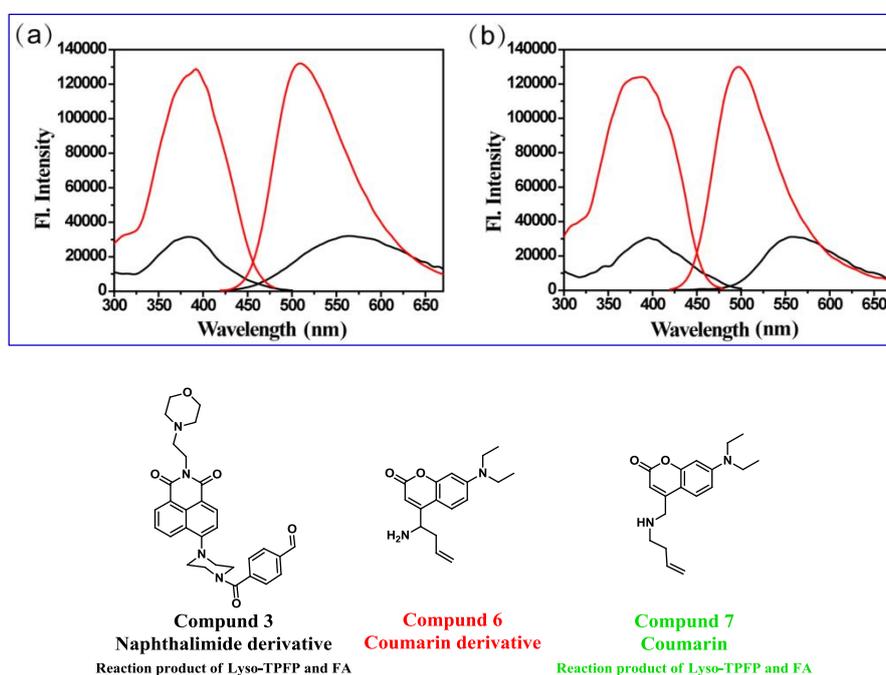


Fig. S1. (a) Excitation and emission spectra of 10 μ M Lyso-TPFP in the absence (black line) and presence (red line) of 1.0 mM FA in 50 mM phosphate buffer in pH 5.0. (b) Excitation and emission spectra of 10 μ M naphthalimide derivative (compound **3**, black line) and 10 μ M coumarin derivative (compound **6**, red line) in

50 mM phosphate buffer in pH 5.0. Lyso-TPFP was dissociated into compound **3** and compound **7** by FA. Compound **6** was prepared and applied as the contrastive agent to confirm the spectral variation instead of compound **7**, as the two coumarin derivatives are similar to each other in fluorescence property.

4. Mechanism investigation

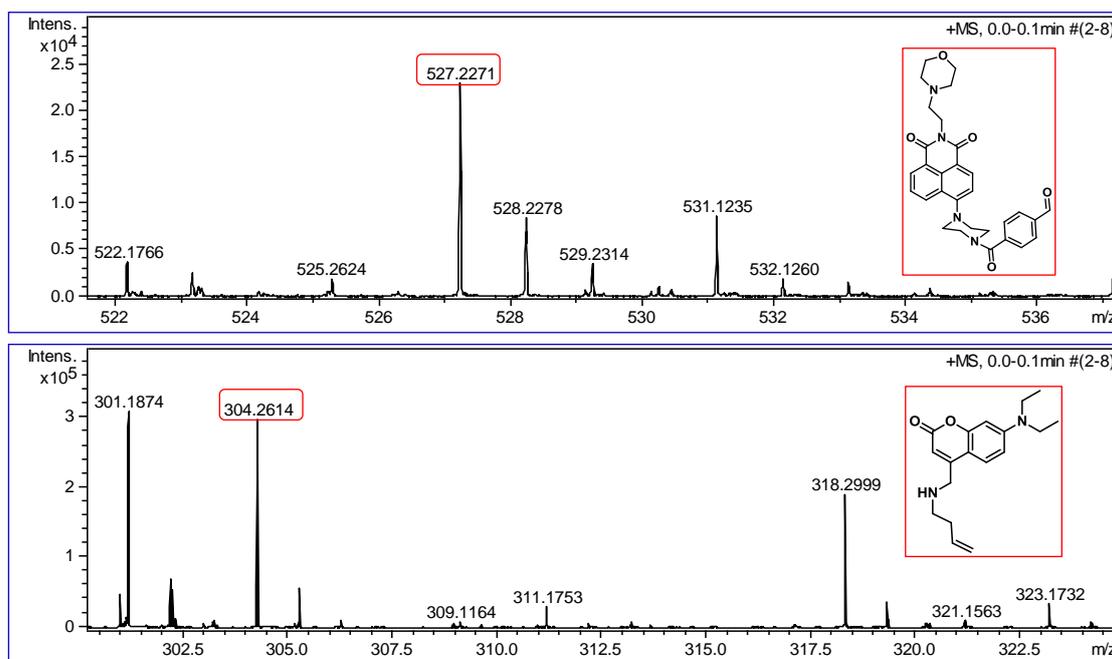
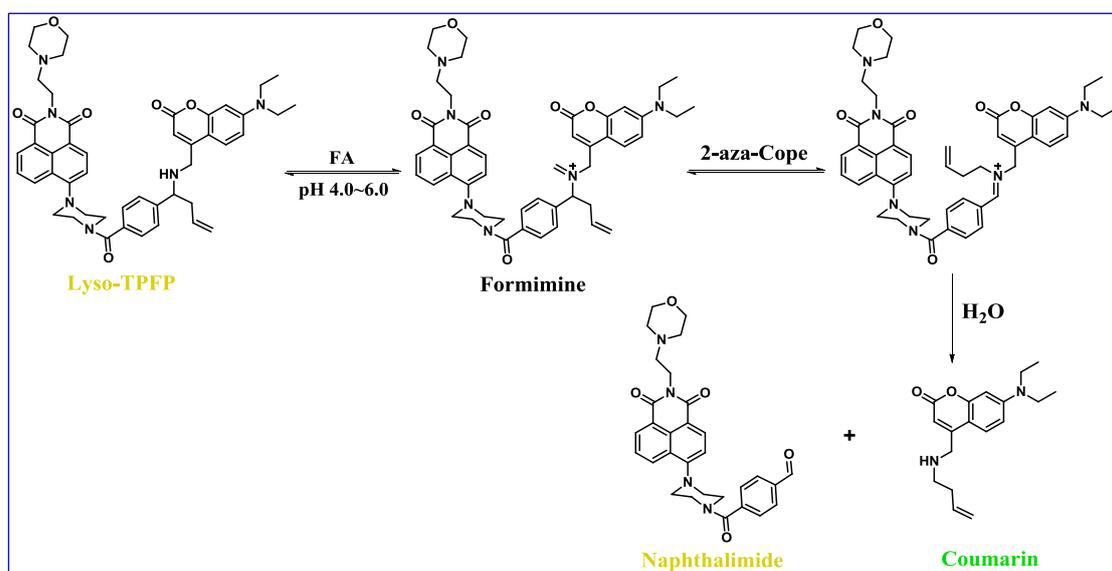


Fig. S2. HRMS spectral analysis of reaction between Lyso-TPFP and FA. Lyso-TPFP (100 μ M) was reacted with FA (1.5 mM) at 37 $^{\circ}$ C for 180 min, and then the mixture was characterized by HRMS spectrometry.



Scheme S2. Proposed reaction pathway for Lyso-TPFP and FA

5. Specificity

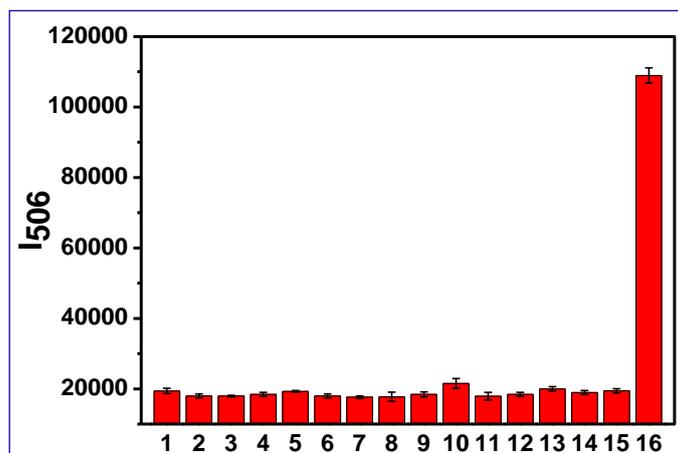


Fig. S3. Fluorescence responses of 10 μM Lyso-TPFP toward 500 μM various analytes: (1) blank, (2) Na^+ , (3) Mg^{2+} , (4) K^+ , (5) Ca^{2+} , (6) Zn^{2+} , (7) Cu^{2+} , (8) Fe^{2+} , (9) Fe^{3+} , (10) CH_3COO^- , (11) CO_3^{2-} , (12) PO_4^{3-} , (13) SO_4^{2-} , (14) SO_3^{2-} , (15) NO_3^- , (16) FA. $\lambda_{\text{ex}} = 405 \text{ nm}$.

6. MTT assay

HepG2 cells were seeded into a 96-well microtiter plate at 37 $^{\circ}\text{C}$ in a 5% $\text{CO}_2/95\%$ air incubator for 24 h. The cells were incubated for an additional 24 h with different concentrations of tested probe (0, 1, 5, 10, 100, and 200 μM), respectively. Then the cells were washed with PBS three times. Subsequently, MTT solution (200 μL , 0.5 mg/mL) was added to each well and the cells were incubated at 37 $^{\circ}\text{C}$. After 4 h, the remaining MTT was removed, and the formazan crystals were dissolved in 200 μL of DMSO with gentle agitation for 5 min. The absorbance at 490 nm was measured using a TRITURUS microplate reader.

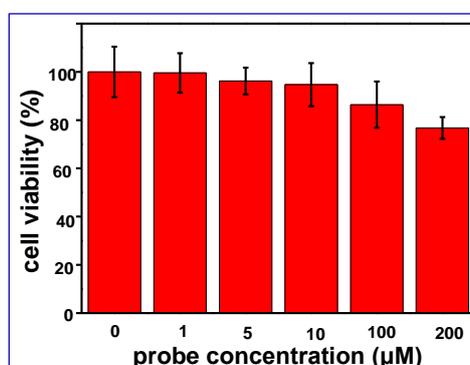


Fig. S4. MTT assay of HepG2 cells with different concentrations of Lyso-TPFP. The IC_{50} value was calculated to be 234 μM .

7. Cell imaging

HepG2, HeLa, and PC-12 cells were cultured in high glucose DMEM or RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C in a 5% CO₂/95% air incubator MCO-5AC (SANYO, Tokyo, Japan). One day before imaging, the cells were detached and were replanted on glass-bottomed dishes. Prior to the colocalization experiments, HepG2 cells were pretreated with FA (500 μM) for 30 min before being washed three times with PBS, then costained with Lyso-TPFP (2 μM) and the corresponding commercial organelle marker (Lyso-tracker Red 20 nM, Mito-Tracker Deep Red 100 nM, ER-Tracker Red 100 nM, or Golgi-Tracker Red 100 nM) for 30 min and then rinsed for three times with PBS. For monitoring of FA in live cells, the HeLa, PC-12, or HepG2 cells were pretreated with the corresponding concentration of FA for 30 min before being washed three times with PBS, then incubated with Lyso-TPFP (5.0 μM) for 30 min before rinsed with PBS. For the scavenging assay, cells were treated with NAC (10 mM) for 30 min in advance. Cell imaging was performed on a Leica TCS SP8 confocal microscope (one-photon fluorescence imaging) or a Zeiss LSM 880 confocal microscope (two-photon fluorescence imaging).

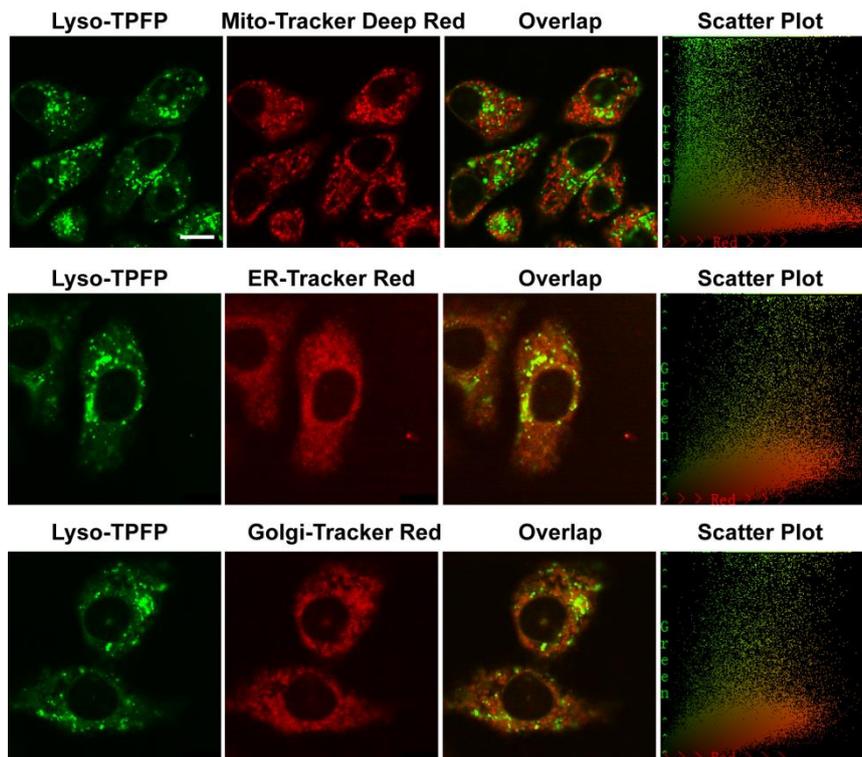


Fig. S5. HepG2 cells were costained with Lyso-TPFP (2 μ M) and Mito-Tracker Deep Red (100 nM), ER-Tracker Red (100 nM), or Golgi-Tracker Red (100 nM). Cells were pretreated with 500 μ M FA for 30 min before being labeled with probes. Lyso-TPFP: λ_{ex} = 405 nm, λ_{em} = 480–520 nm; Mito-Tracker Deep Red: λ_{ex} = 633 nm, λ_{em} = 650–700 nm; ER-Tracker Red: λ_{ex} = 561 nm, λ_{em} = 600–650 nm; Golgi-Tracker Red: λ_{ex} = 561 nm, λ_{em} = 600–650 nm. Overlay: merged images. Scatter plot: intensity correlation plot of the two dyes. Scale bar: 10 μ m.

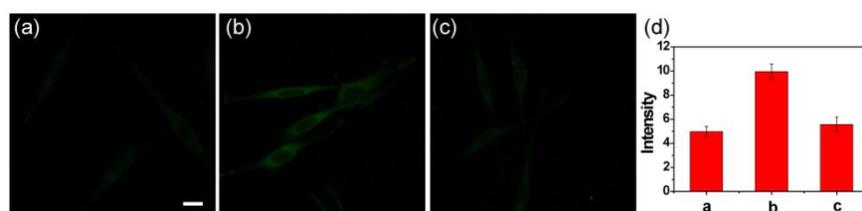


Fig. S6. Two-photon fluorescence images of FA in PC-12 cells. Cells were treated with (a) PBS, (b) 800 μ M FA, or (c) 10 mM NAC and then 800 μ M FA, followed by incubation with 5 μ M Lyso-TPFP. (d) Relative fluorescence intensities of (a)–(c). Emissions were collected from 480–520 nm and excited at 800 nm. Scale bar: 10 μ m.

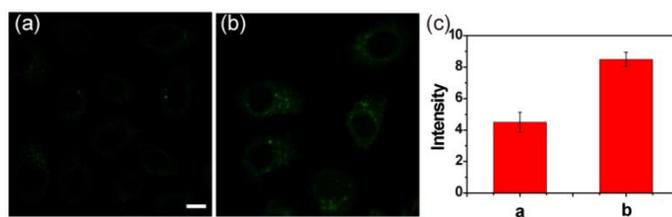


Fig. S7. Two-photon fluorescence images of FA in HepG2 cells. Cells were treated with (a) PBS or (b) 800 μM FA, followed by incubation with 5 μM Lyso-TPFP. (c) Relative fluorescence intensities of (a) and (b). Emissions were collected from 480–520 nm and excited at 800 nm. Scale bar: 10 μm .

8. In situ tissue imaging

All animal experiments were in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Kunming mice were divided into two groups, and were intraperitoneally injected with PBS (100 μL) and FA (2.0 mM, 100 μL), respectively. After 30 min, Lyso-TPFP (50 μM , 100 μL) was intraperitoneally injected into both the two groups. After 60 min, the mice were anesthetized with 1.5% pelltobarbitalum natricum (150 μL) by intraperitoneal injection. Two-photon fluorescence images of the mice were acquired using a Zeiss LSM 880 confocal laser scanning microscope with an objective lens ($\times 20$).

9. NMR and HRMS spectra

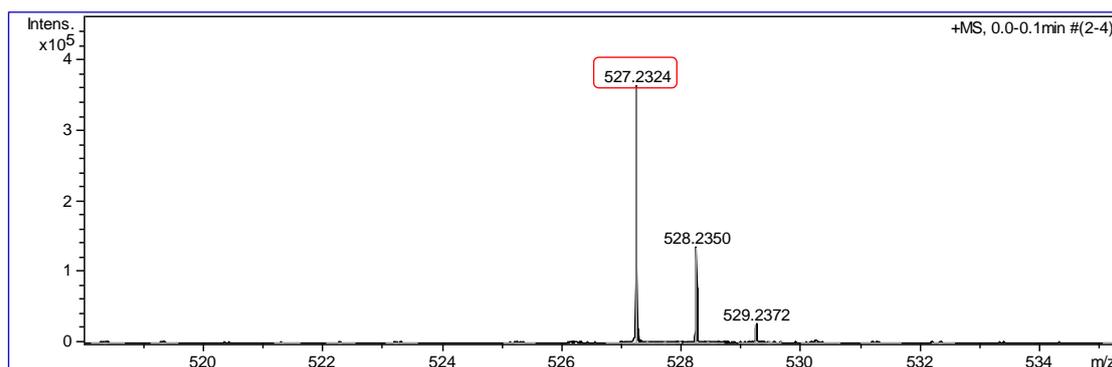


Fig. S8. HRMS spectrum of compound **3**

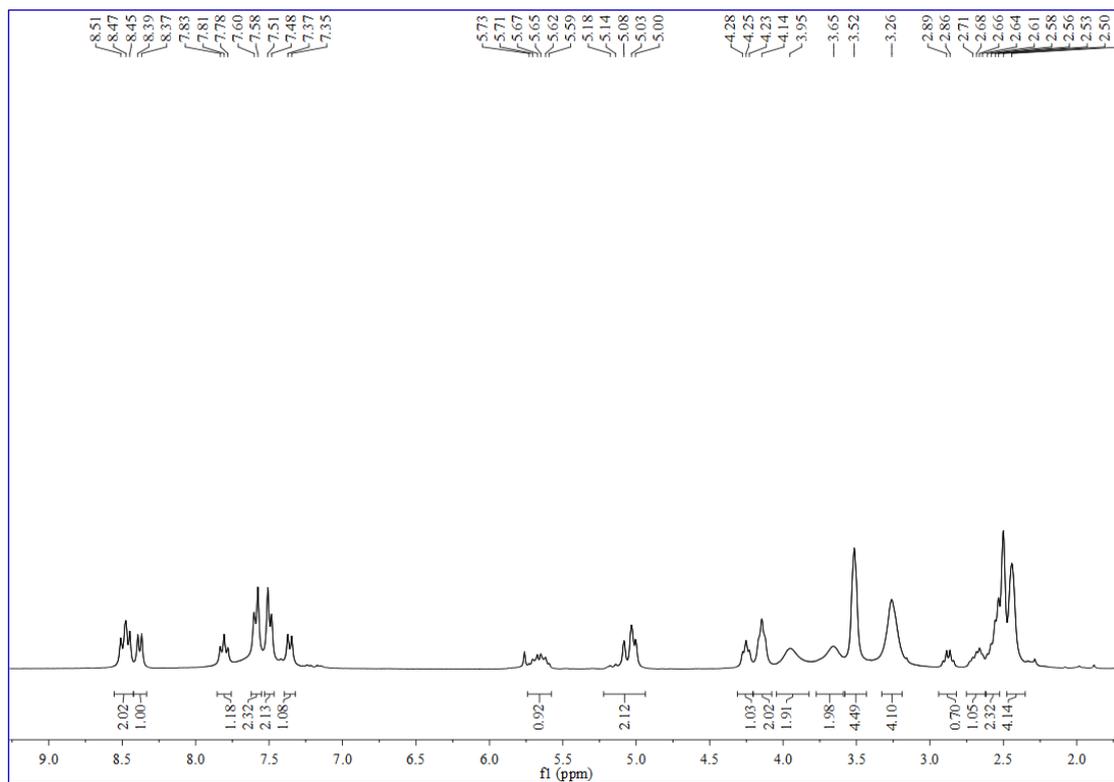


Fig. S9. ^1H NMR spectrum of compound **4**

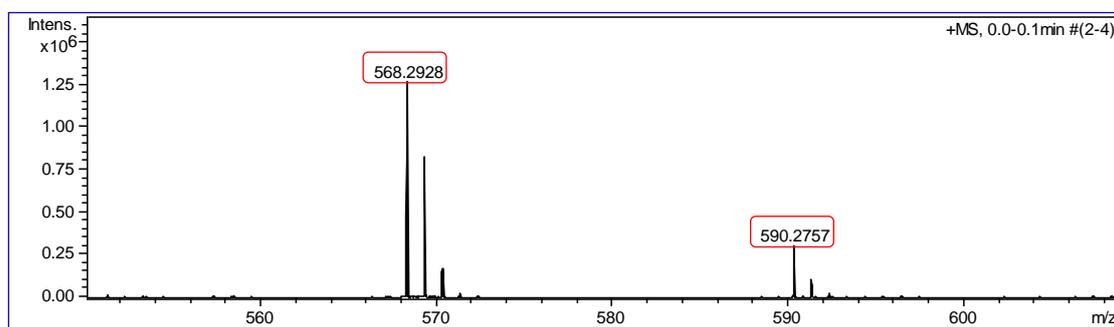


Fig. S10. HRMS spectrum of compound **4**

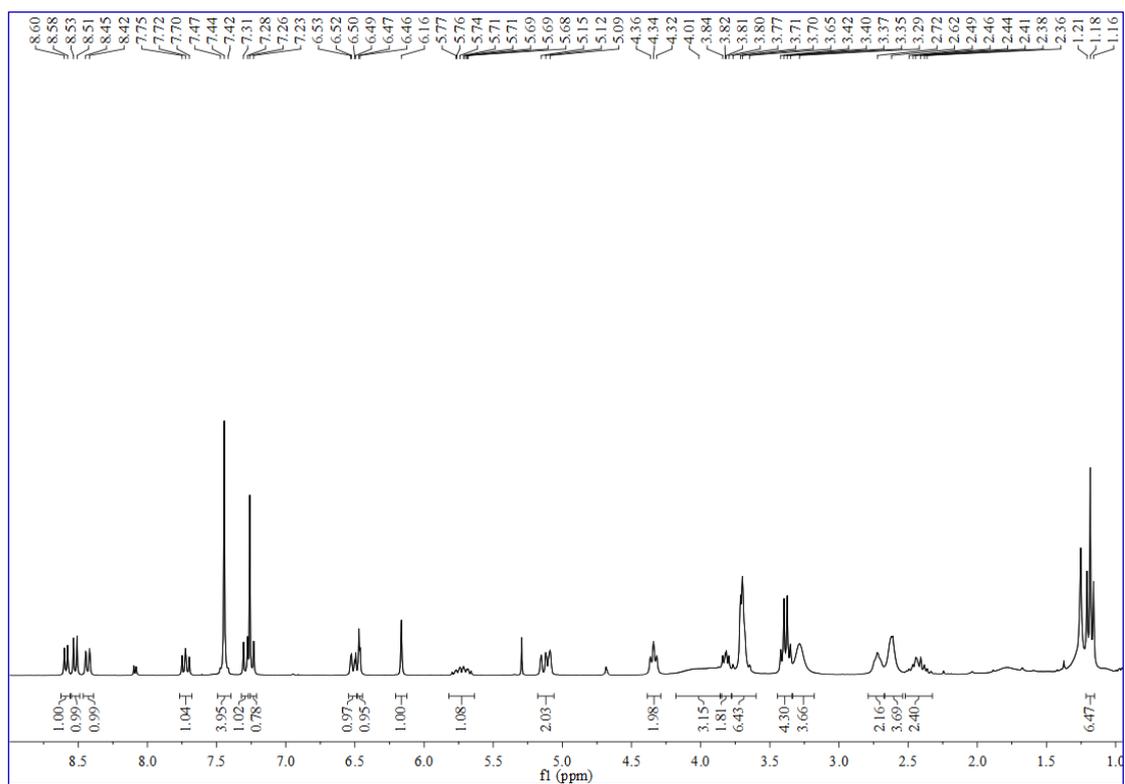


Fig. S11. ^1H NMR spectrum of Lyso-TPFP

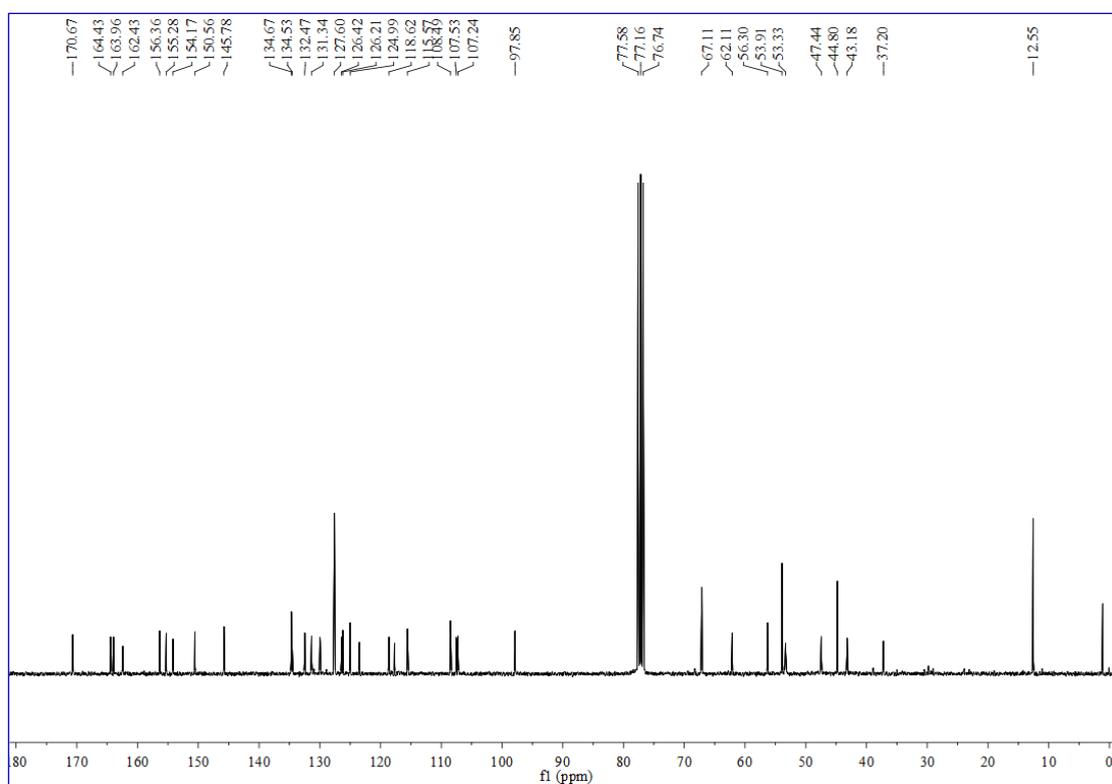


Fig. S12. ^{13}C NMR spectrum of Lyso-TPFP

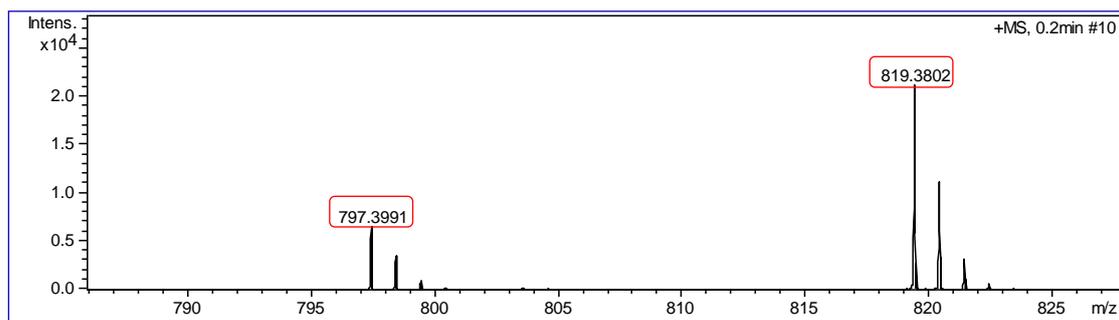


Fig. S13. HRMS spectrum of Lyso-TPFP

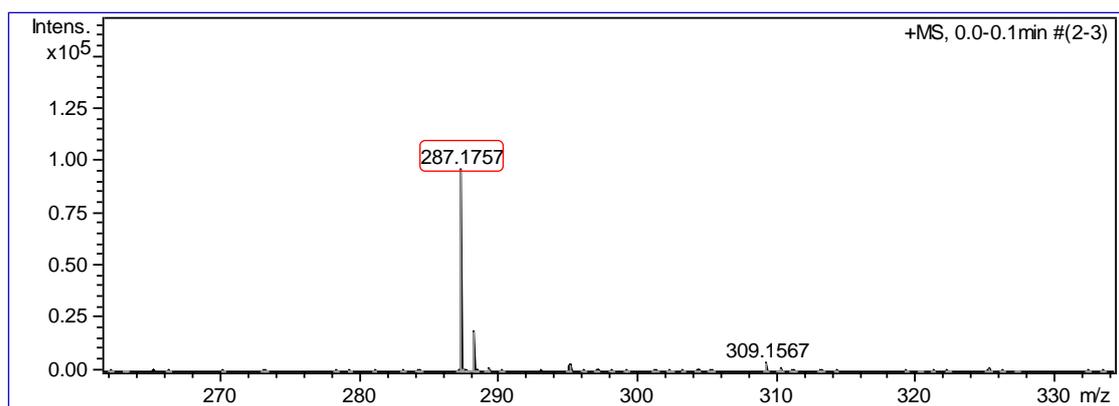


Fig. S14. HRMS spectrum of compound 6

10. Reference

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