

Supporting Information

**Construction of a Two-Photon Fluorescent Probe for
Ratiometric Imaging of Hypochlorous Acid in Alcohol-
Induced Liver Injury**

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1. Materials and apparatus

Phorbol-12-myristate-13-acetate (PMA) and lipopolysaccharide were purchased from Sigma-Aldrich. AR grade reagents were obtained from commercial sources without further purification. All reactions were performed under argon atmosphere. All aqueous solutions were prepared in ultrapure water (18.25 M Ω •cm) (Milli-Q system, Direct-Q8). NMR spectra were recorded on a Varian INOVA600 spectrometer, using TMS as an internal standard. High-resolution mass spectra (ESI-HRMS) were obtained from Thermo Fisher Scientific mass spectrometer of Exactive Plus. The pH measurements were carried out on a Mettler-Toledo FiveEasyPlus pH meter. One-photon excited fluorescence was measured on a Hitachi F-4600 fluorescence spectrophotometer. Absorption measurements were conducted on a UV2550 UV-vis spectrophotometer (Shimadzu Scientific Instruments Inc.). Two-photon microscopy was performed on a Zeiss LSM 710 multiphoton laser scanning confocal microscope (Carl Zeiss, Germany)

2. Experimental Details

Spectroscopic Measurements The fluorescence quantum yield was determined with quinine sulfate ($\Phi=0.55$ in 0.1 N H₂SO₄ solution) as the reference with a literature method.¹ The molar concentration of commercial sodium hypochlorite (6-10% available chlorine) was measured by titration. The ROS and RNS were prepared according to literature method.²

Calculation of Limit of Detection (LOD) Value The LOD was calculated based on the method reported in the previous literature.³ Briefly, the fluorescence emission spectrum of **TPA-CIO** was measured by eleven times and the standard deviation of blank measurement was obtained. The fluorescence ratio (I_{497}/I_{437}) of **TPA-CIO** was plotted as HClO concentrations. The detection limit was calculated by using detection limit $3\sigma/k$: where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity versus HClO concentration.

Cytotoxicity Assay The cytotoxicity was evaluated by MTT assay. Briefly, HeLa cells were cultured in DMEM in 96-well microplates in incubator for 24 h. The medium was next replaced by fresh

DMEM containing various concentrations of **TPA-CIO** (0-50 μM). Each concentration was tested in three replicates. Cells were rinsed twice with phosphate buffer saline 24 h later and incubated with 0.5 mg/mL MTT reagent for 4h at 37 °C. The culture was removed and 150 μL DMSO was added to dissolve formazan and the absorbance at 570 nm was measured in a microplate reader (Multiskan GO, Thermo Scientific). Cell viability (%) was calculated using following equation: Viability = (mean Abs. of treated wells/mean Abs. of control wells) \times 100%.

Cell Culture and Imaging HeLa cells were cultured with DMEM supplemented with 10% (v/v) newborn calf serum (Gibco), 100 $\text{U}\cdot\text{mL}^{-1}$ penicillin, and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin in a humidified atmosphere with 5/95 (v/v) of CO_2 /air at 37 °C. One day before imaging, cells were detached with a treatment of 0.2% (w/v) trypsin-EDTA solution (Gibco) and suspended in culture media. The cell suspension was then transferred to confocal dishes to grow with adherence. RAW 264.7 cells were maintained with DMEM supplemented with 10% (v/v) newborn calf serum (Gibco), 100 $\text{U}\cdot\text{mL}^{-1}$ penicillin, and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin in incubator. For imaging, RAW 264.7 cells at 80% confluence were harvested by scraping and transferred to confocal dishes to grow with adherence. Two-photon excited fluorescence images were obtained by Zeiss LSM 710 multiphoton laser scanning confocal microscope with a 20 \times air objective.

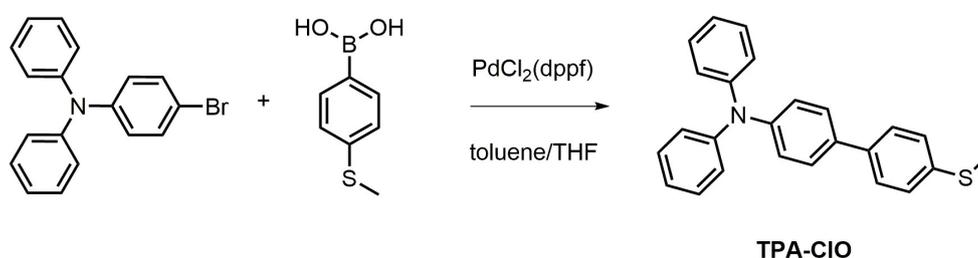
Zebrafish Culture and Imaging Zebrafish embryos were cultured in E3 media in 100 mm culture dishes (Temperature 28 °C).⁴ Zebrafish were transfer to confocal dishes (35 mm) at 6 day postfertilization (dpf). The zebrafish were cultured with 0 $\mu\text{g}/\text{mL}$ (control group) and 20 $\mu\text{g}/\text{mL}$ (experimental group) LPS for 2h, and then cultured with 10 μM **TPA-CIO** for 1h. Before imaging, the zebrafish were anaesthetized with MS-222 contained E3 media and immediately imaged on a Zeiss LSM 880 two-photon microscope with a 10 \times air objective (NA 0.45).

Mouse Liver Tissue Imaging

The animals were maintained under 12 h/12 h of light/dark cycle, 24 ± 3 °C temperature and $55 \pm 15\%$ relative humidity throughout the experiment. Kunming mice (~28 g) were intragastrically administered with 5 doses of tap water/50% ethanol solution (5, 10 g/kg body weight) at an interval of 12 h to lead to acute liver injury. After above treatments, the mice were anesthetized, the liver tissues

were harvested and embedded in tissue-freezing medium, frozen and consecutively sectioned into slices. Then, the slices were incubated with 20 μ M **TPA-CIO** for 0.5h and washed with PBS three times, and imaged by two-photon microscope. All the animal experiments were carried out in accordance with the relevant laws and guidelines issued by the Ethical Committee of Wuhan University and were in agreement with the guidelines of the Institutional Animal Care and Use Committee.

3. Synthesis and Characterization



Scheme S1. Structure and synthesis of the probe **TPA-CIO**.

Preparation of TPA-CIO A mixture of 4-bromo-*N,N*-diphenylaniline (628 mg, 2 mmol), PdCl₂(dppf) (146 mg, 0.2 mmol) in 20 mL dry toluene was added a solution of 4-mercaptophenylboronic acid (616 mg, 4 mmol) and K₂CO₃ (1.38g, 10 mmol) in 15 mL methanol. The mixture was refluxed at 110 °C for 24 h. The reaction was then quenched by 30 mL water and extracted with CH₂Cl₂ (3×30 mL). The combined organic extract was dried over anhydrous Na₂SO₄ and solvent was removal under vaccum. The product was purified by column chromatography (silica gel, petroleum ether/ ethyl acetate, *v:v* =16:1) to obtain white solid (540 mg, 55%). ¹H NMR (400 MHz, CDCl₃) δ 7.49 (*d*, *J* = 8.0 Hz, 2H), 7.44 (*d*, *J* = 8.0 Hz, 2H), 7.31-7.24 (*m*, 7H), 7.14-7.11 (*m*, 5H), 7.03 (*t*, *J* = 16.0, 6.0 Hz, 2H), 2.51 (*s*, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 142.89, 142.37, 132.77, 132.14, 129.64, 124.55, 122.70, 122.25, 119.67, 119.18, 118.20, 72.62, 72.30, 71.99, 11.25. HRMS: Calcd. For *m/z*, [C₂₅H₂₁NS]⁺ M⁺ =367.1359; Found, 368.1386.

4. Optical properties of TPA-CIO

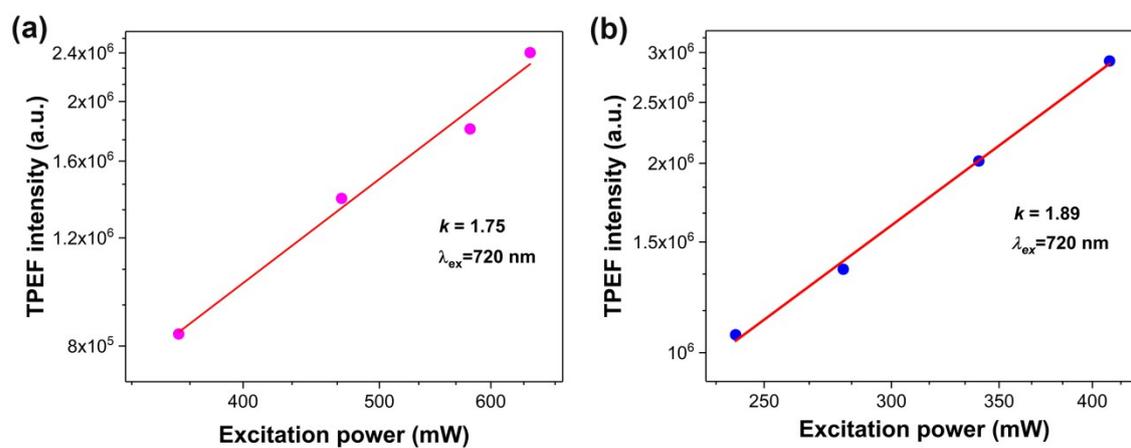


Fig. S1 Logarithmic plots of the dependence of fluorescence intensity on excitation power for TPA-CIO (a) and the reaction product of TPA-CIO with excess HClO (b).

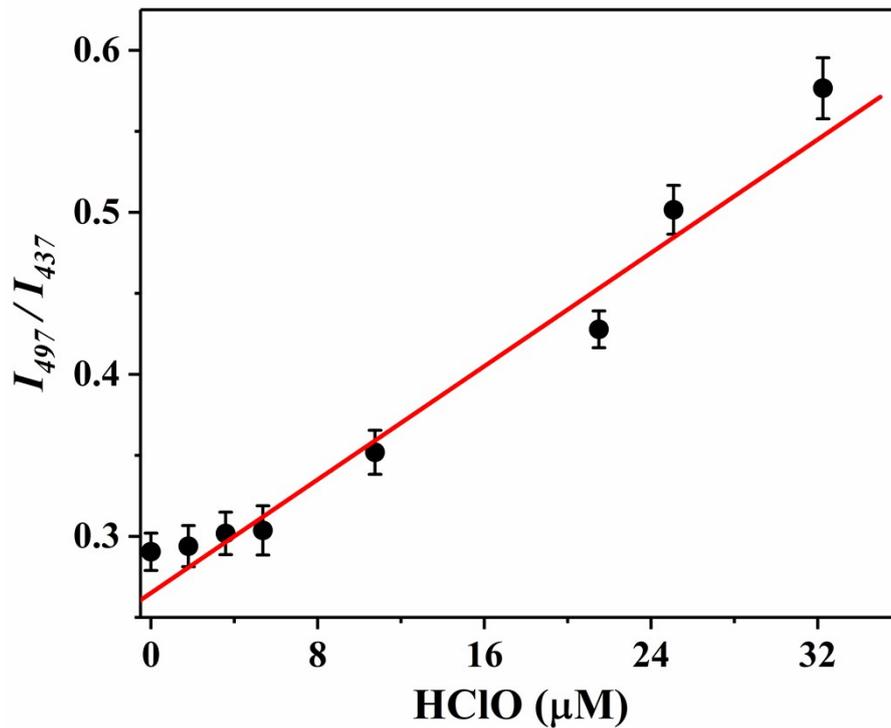


Fig. S2 Linear relationship of emission intensity ratio (I_{497}/I_{437}) of 10 μM TPA-CIO to lower HClO concentration (1.8-32 μM)

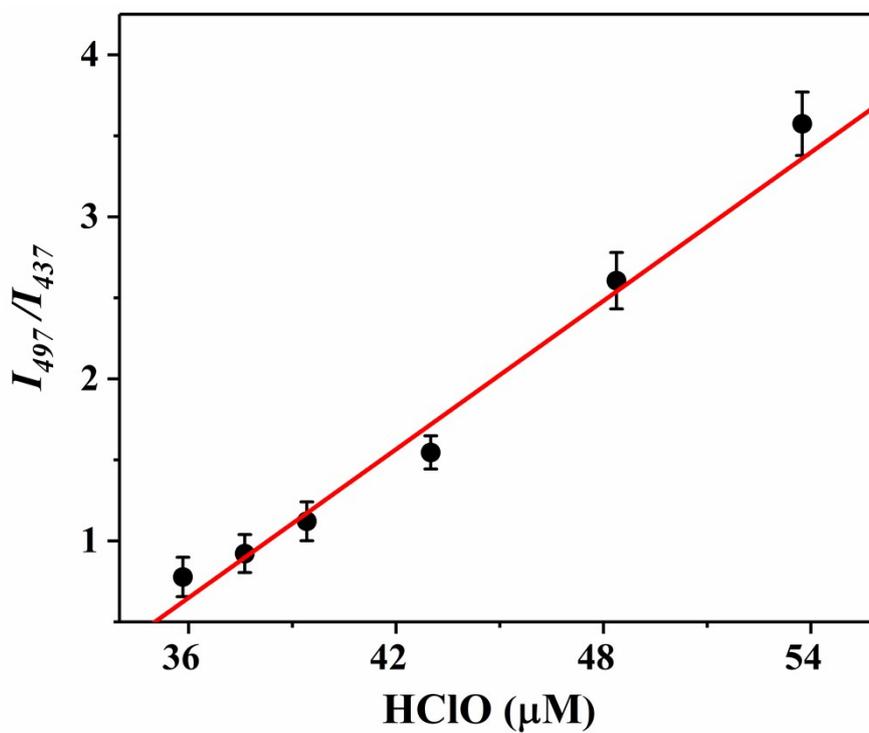


Fig. S3 Linear relationship of emission intensity ratio (I_{497}/I_{437}) of 10 μM TPA-CIO to HClO concentration (36-54 μM).

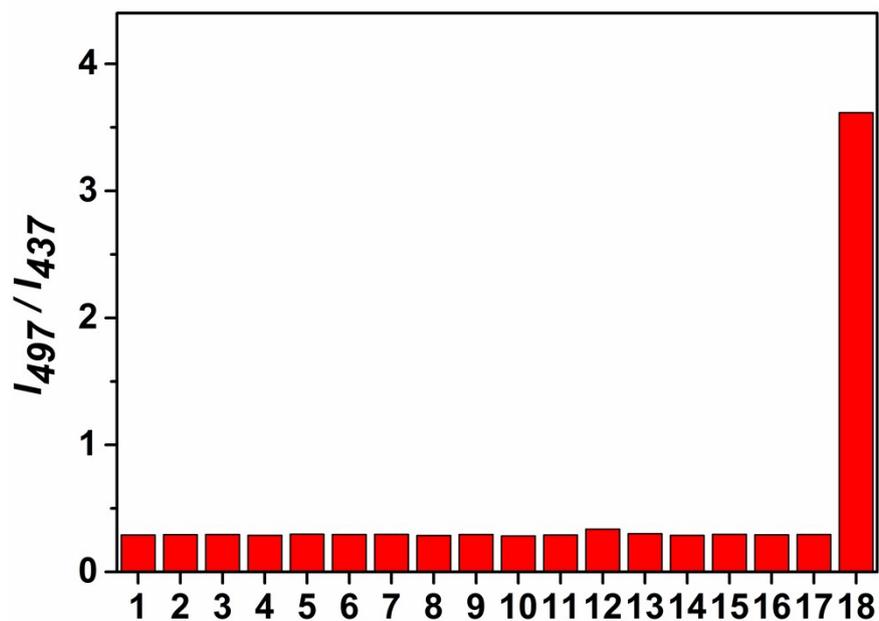


Fig. S4 Fluorescence emission ratio of 10 μ M TPA-ClO in the presence of HClO and various interfering species, including 20 μ M metal ions (1 blank, 2-8 Cu^{2+} , Fe^{3+} , Hg^{2+} , Ca^{2+} , Mg^{2+} , Zn^{2+} , Fe^{2+}), 0.1 mM biothiols (9-11 Hcy, Cys, GSH), 20 μ M ROS (12-14 H_2O_2 , $\cdot\text{OH}$, O_2^-), 20 μ M RNS (15-17 NO_2^- , ONOO^- , NO) and 60 μ M HClO (18).

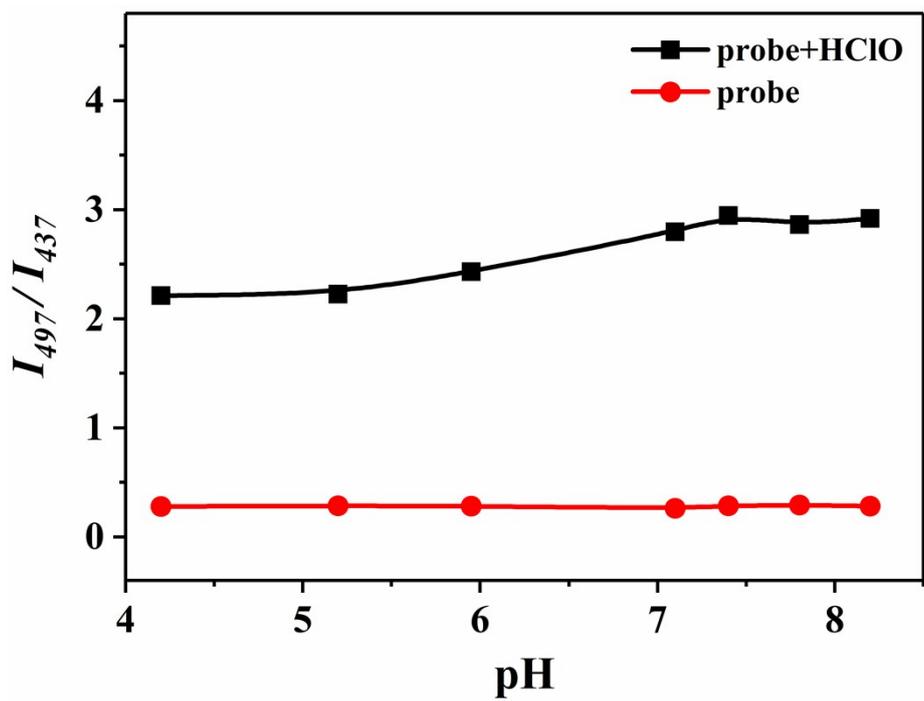


Fig. S5 Effect of pH on the fluorescence emission ratio of 10 μM TPA-C10 in the presence and absence of excessive amount of HClO.

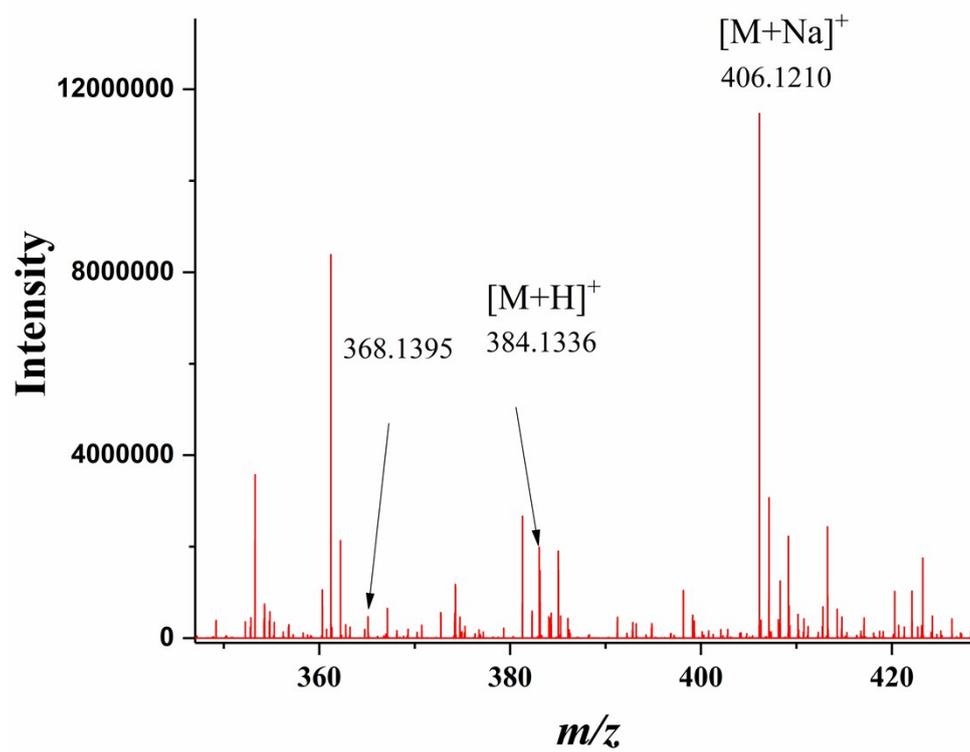


Fig. S6 HR-MS spectrum of the reaction mixture of TPA-ClO and HClO.

5. Cell viability

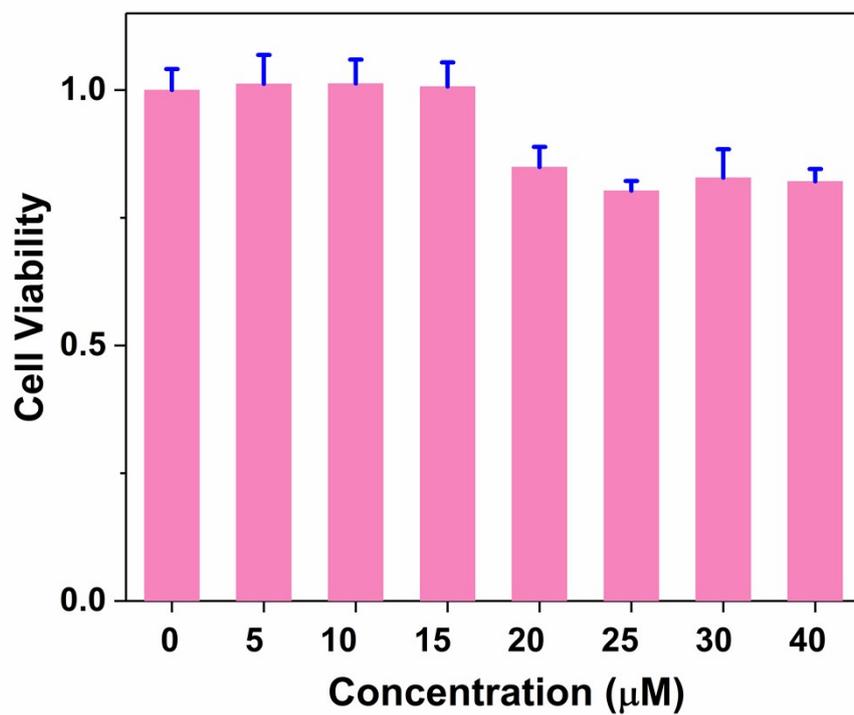


Fig. S7 Viability of HeLa cells incubated with different concentrations (0-40 µM) of TPA-CIO for 24 hours.

6. Imaging of endogenous HCIO in RAW 264.7 cells

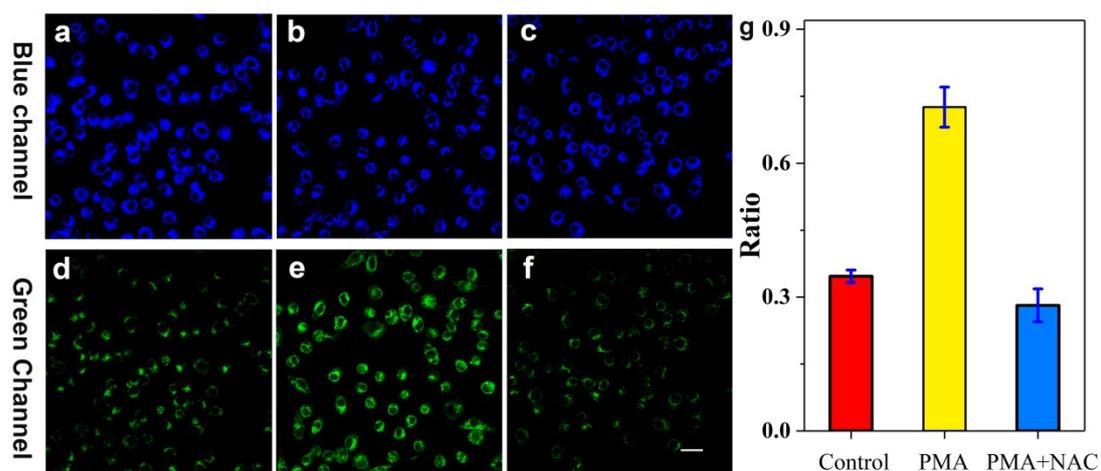


Fig. S8 Two-photon images of **TPA-CIO** in RAW 264.7 cells. (a, d) Images of RAW 264.7 cells were incubated with 10 μ M **TPA-CIO** for 0.5 h. (b, e) Images of RAW264.7 cells were pretreated with 500 ng/mL PMA for 0.5 h, and then incubated with **TPA-CIO** for 0.5 h. (c, f) Images of RAW 264.7 cells were pre-treated with 500 ng/mL PMA and 0.5 mM NAC for 0.5 h, and then incubated with **TPA-CIO** for 0.5 h. (g) Fluorescence intensity ratio (F_{green}/F_{blue}) of a-f. The fluorescence intensities were collected at blue channel (370-450 nm) and green channel (500-600 nm) upon the excitation of 720 nm. Scale bar: 20 μ m.

7. TP imaging of mouse liver tissues

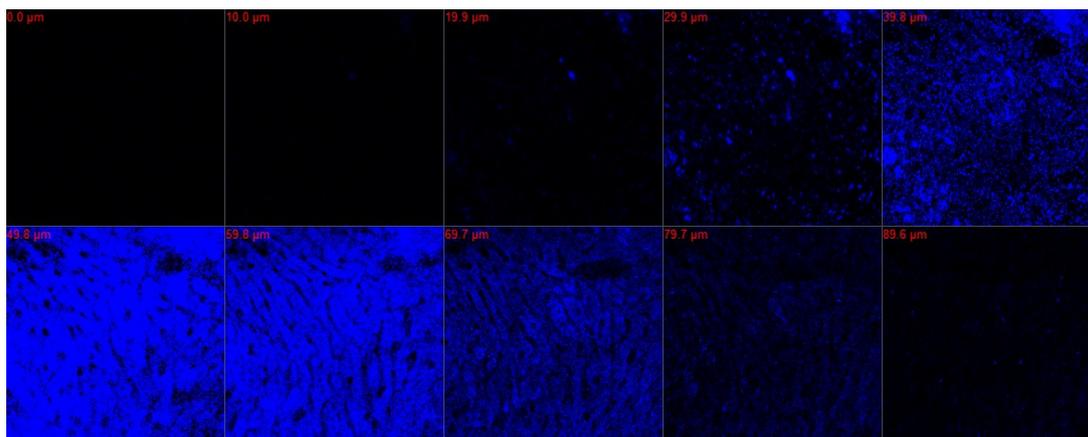


Fig. S9 Z-direction TP images for 50 μM TPA-CIO stained mouse liver tissue. The TP excitation wavelength was 720 nm and emission was collected at 370-450 nm.

8. H & E staining

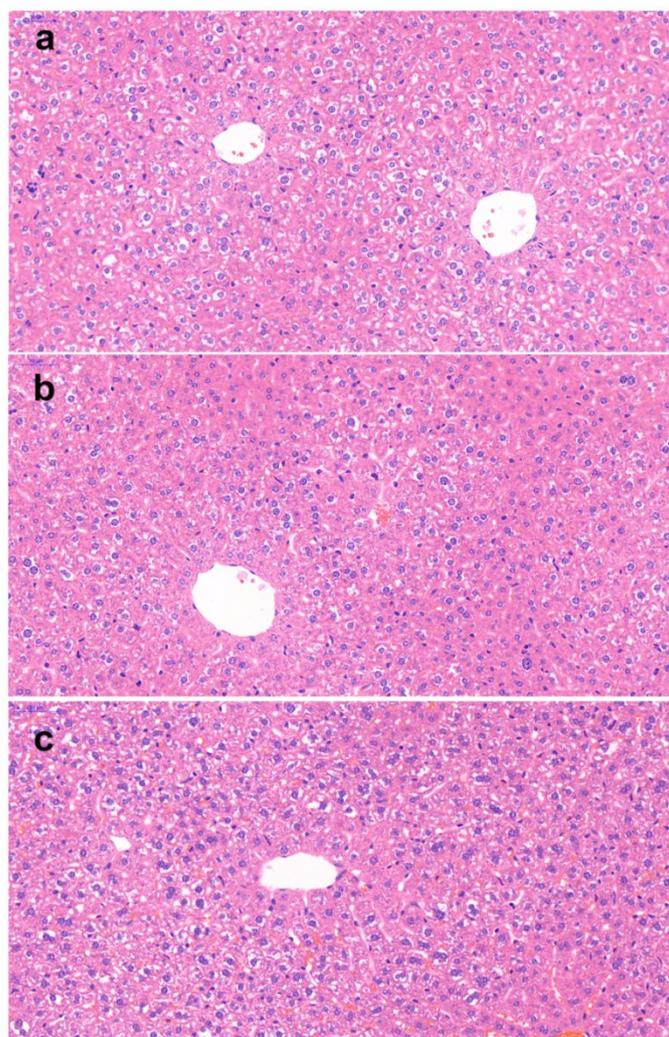


Fig. S10 Hematoxylin and eosin (H&E) staining of control and EtOH induced liver injury mouse tissues. Mice were fed with 5 doses of tap water (a) and 50% EtOH solution (5g/kg body weight (b); 10g/kg body weight (c)) by oral gavage at an interval of 12 h, respectively.

9. NMR and MS Data

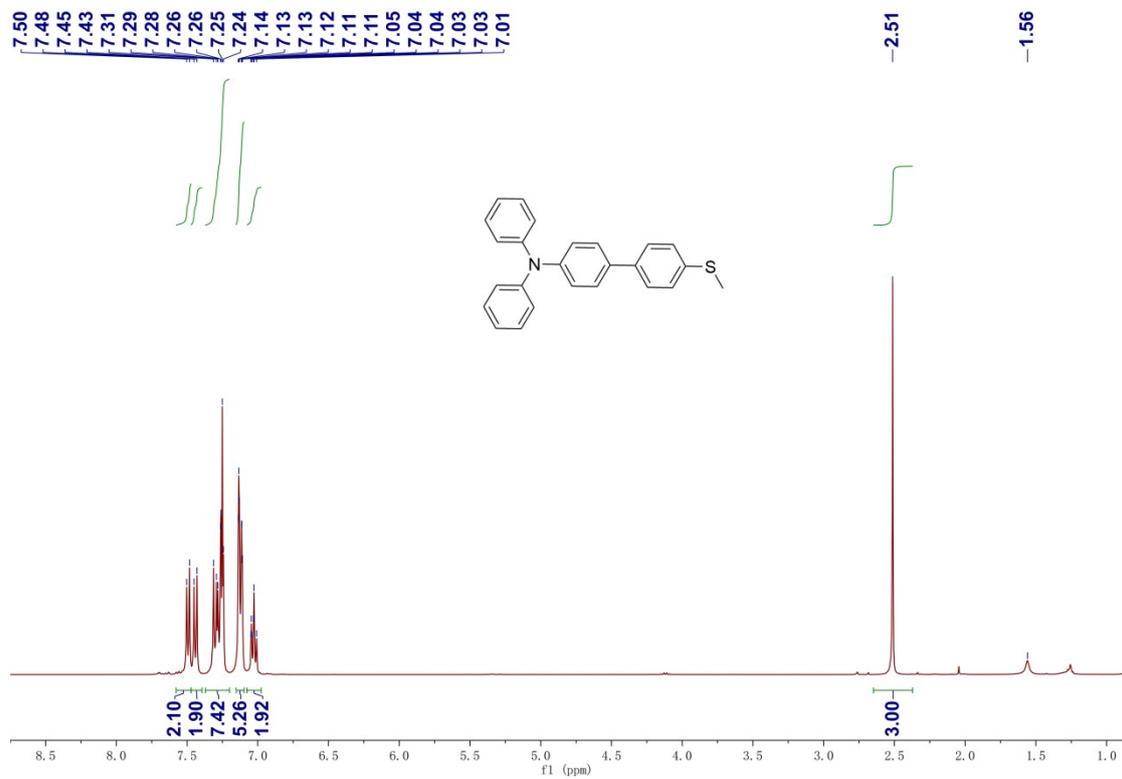


Fig. S11 ¹H NMR spectrum of TPA-CIO (CDCl₃, 400 MHz).

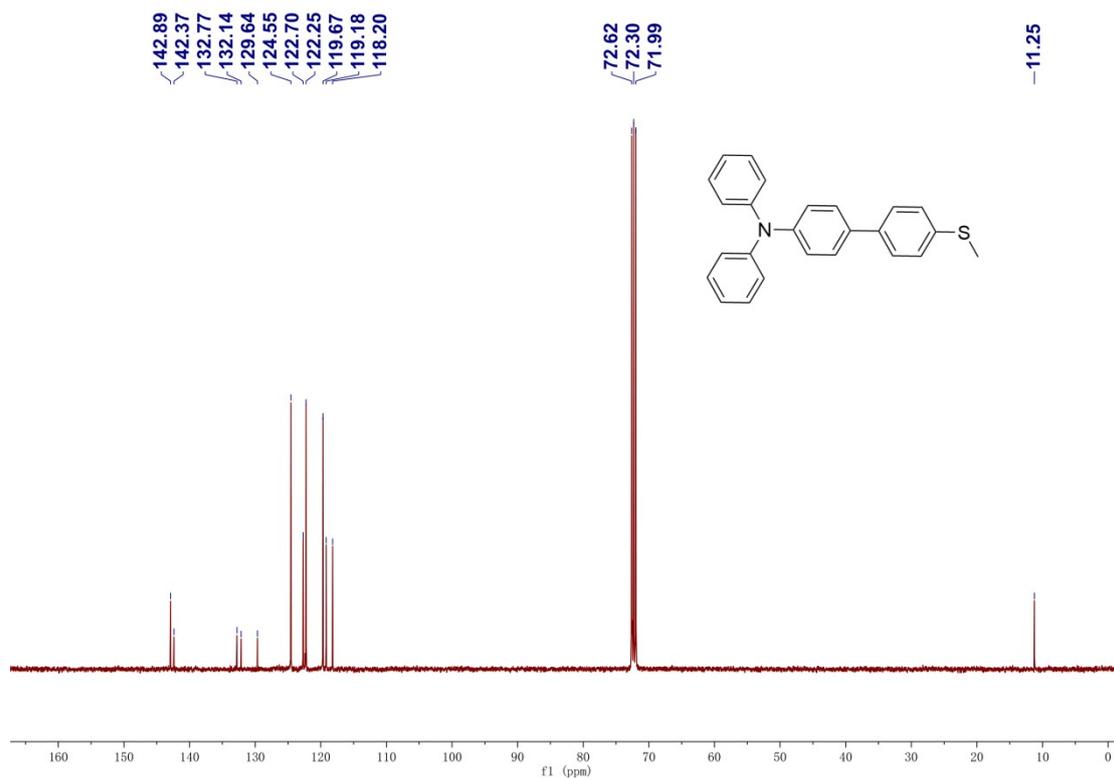


Fig. S12 ^{13}C NMR spectrum of TPA-CIO (CDCl_3 , 101 MHz).

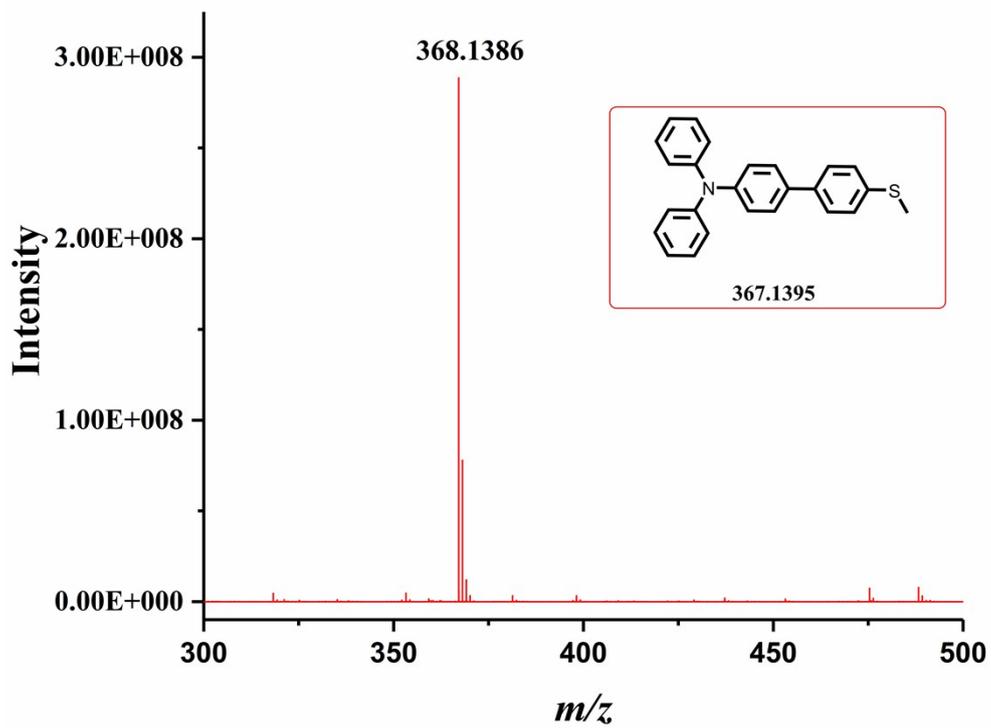


Fig. S13 HR-MS spectrum of TPA-CIO.

10. Reference

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