Electronic Supplementary Information for

Coumarin-Based TICT Fluorescent Probe for Real-Time Fluorescence Lifetime Imaging of Mitochondrial Viscosity and Systemic Inflammation *in vivo*

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Materials and general information

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Ultrapure water was used throughout all experiments. High resolution mass spectrometric (HRMS) analyses were measured on a Finnigan MAT 95 XP spectrometer; NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard; The fluorescence spectra were obtained on a F-4700 spectrofluorometer from Hitachi High-tech Science; The absorption spectra were collected on UV-2700 visible spectrophotometer from Shimadzu Scientific Instruments; The fluorescence imaging of cells was performed with TSC-SP8 (Leica, Wetzler, Germany) confocal laser scanning microscope; the viscosity measurements were carried out on SNB-2 viscosity meter from CNSHP Instrument; The pH measurements were carried out on a PHS-3E PH meter from INESA Instrument; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals.

UV-vis absorption, fluorescence spectral measurement and fluorescence lifetime detection

Viscosity determination. The solvents were obtained by mixing PBS-glycerol systems in different proportions. Measurements were carried out with a NDJ-7 rotational viscometer, and each viscosity value was recorded. The solutions of **Mito-VCI** of different viscosity were prepared by adding the stock solution (1.0 mM) to 4 mL of solvent mixture (PBS-glycerol solvent systems) to obtain the final concentration of the dye (10.0 μM). These solutions were sonicated for 5 min to eliminate air bubbles. After standing for 1 h at a constant temperature, the solutions were measured in a UV spectrophotometer and a fluorescence spectrophotometer. A fluorescence lifetime measuring equipment was used to obtain the fluorescence lifetimes of **Mito-VCI**. For all the measurements, the excitation wavelength, excitation slit widths, and emission slit widths are 500 nm, 5 nm, and 5 nm,

respectively.

Different polarity solvent detection. The solvents were obtained by a series of different polarity organic solvents systems, including acetone, acetonitrile (CH_3CN), ethyl alcohol (EtOH), dimethyl formamide (DMF), methyl alcohol (MeOH), dimethyl sulfoxide (DMSO), PBS and glycerol. The solutions of probe in different polarity were prepared by adding the stock solution (1.0 mM, dissolved in DMSO) 20 μL to 2 mL of the solvent (different polarity organic solvents) to obtain the final concentration of the dye (10.0 μM). These solutions were sonicated for 5 minutes to eliminate air bubbles. After the mixture, the solutions were measured in a UV spectrophotometer and a fluorescence spectrophotometer.

Calculation of fluorescence quantum yield. The fluorescence quantum yields were determined using Rhodamine B as the Φ_f reference according to the literature method. Quantum yields were corrected as follows:

$\Phi_s=\Phi_f*(A_r/A_f)*(F_s/F_r)$

Where the s and r indicate the designated and reference samples, respectively. A, Φ_f, and F were the absorbance (≤ 0.05) at λ_{ex}, average refractive index of the appropriate solution, and the integrated area under the corrected emission spectrum, respectively.

Fluorescence imaging in living cells and *vivo*

Cell Culture. The living HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100 U/mL, Hyclone) and streptomycin sulfate (100 U/mL, Hyclone). The cells were cultured at 37 °C and under a 5% CO_2 atmosphere.

Cell viability assay. The cytotoxic effects of **Mito-VCI** were determined by CCK8 assays. HeLa cells were placed in a 96-well plate and cultured for 24 hours. When the density of cells group to about 80%-90%, the cells were incubated with a series of concentrations of **Mito-VCI** (0.0, 1.0, 2.0, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 μM). After

24 hours incubation, the probe solution was removed, and then CCK8 solution was added into each well. After about 20 minutes, the absorbance values of each wells were tested by a microplate reader at 450 nm (λ_{ex}) every 15 min until the OD values reach to 0.8-1.1. The cell viability (%) was calculated by the following equation:

the cell viability (%) = $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$.

Co-localization Fluorescence Imaging. HeLa cells were cultured in 35 mm confocal plates for 24 hours. Whereafter, the cells were rinsed three times with PBS firstly. Then, **Mito-VCI** (5 μL, 1 mM) were added into the living HeLa cells grown in 35 mm confocal dish and cultured in a 5% CO_2 cell incubator at 37 ºC for 10 min, respectively. Subsequently, cell media were replaced by 1 mL fresh medium containing and Mito-Tracker Green (100 nM), then incubated for 10 min in the same conditions. The fluorescence signals of **Mito-VCI** were recorded on the red fluorescence channel (λ_{ex} = 500 nm, λ_{em} = 540-650 nm). The commercial dye of Mito-Tracker Green was recorded on the green fluorescence channel (λ_{ex} = 490 nm, λ_{em} = 500-530 nm).

Preparation of HeLa cells for imaging experiments. Before fluorescence confocal imaging, HeLa cells were cultured in DMEM for 24h in 35 mm confocal dishes until the density of cells group to about 80%-90%. Then the cells were set up control group and three experimental groups. Control group cells were cultured without special treatment. Experimental group cells were treated respectively by monensin (10 μL, 1 mM), nystatin (10 μL, 1 mM) and lipopolysaccharide (LPS, 10 μL, 1 mg/mL) in DMEM to increase intracellular viscosity for 2 hours. Then, the cells were incubated with 5 μL **Mito-VCI** (1 mM) and 1 mL HBSS in a 5% CO_2 cell incubator at 37 ºC for another 10 min, respectively. After that, PBS was utilized to remove the residual probe before imaging. Finally, those worked cells were quickly observed under a confocal microscope in OP and FLIM modes, using the same parameters as above. All of the cell images were excited at 500 nm for OP and FLIM modes, and the emission

wavelength of 510–650 nm was collected.

Preparation of living organs for imaging experiments. The animals were purchased from School of Pharmaceutical Sciences, Guangxi Medical University, and the studies were approved by the Animal Ethical Experimentation Committee of Guangxi Medical University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

These mice were injected by monensin (100 μL, 1 mM), nystatin (100 μL, 1 mM) or LPS (100 μL, 1 mg/mL) in abdomen, respectively. After three days, the organs (heart, liver, spleen, lung, and kidney were isolated from the stimulated mice and normal mice. After washing by PBS (pH = 7.4) for three times, these isolated organs were loaded with **Mito-VCI** (1 mM), respectively.

Preparation of fresh mouse liver slices and fluorescence imaging. For getting mice organ FLIM bio-image, mice organs were cut to about 100 μm thickness by using a medical scalpel in 10 mM PBS buffer (pH = 7.4). Slices were incubated with **Mito-VCI** (10 μM) in PBS buffer bubbled with 95% air and 5% CO_2 for 1 hours at 37 ℃. Then, slices were washed three times with PBS buffer, transferred to glass-bottomed dishes. Mice organ slices imaging was carried out on a confocal microscope. FLIM fluorescence microscopy images of fresh mice organ slices were obtained by exciting the probe set at wavelength 500 nm.

Construction of inflammatory mice models for imaging experiment. Four-weeks old female Kunming mice were purchased from School of Pharmaceutical Sciences, Guangxi Medical University, and the mice were kindly kept during the experiments. These mice were injected by monensin (100 μL, 1 mM), nystatin (100 μL, 1 mM) or LPS (100 μL, 1 mg/mL) in abdomen respectively to produce viscosity increasing models. After keeping injecting for three days, the triggered mice and normal mice were simultaneously utilized for in vivo imaging. Before in vivo imaging, the

abdominal fur was removed by an electric shaver, and then the mice were anesthetized by 4% chloral hydrate aqueous solution (100 μL). The probe **Mito-VCI** (100 μL, 1 mM) was then injected into the abdominal position of the normal mice and triggered mice, respectively. The mice were then imaged by using an in vivo imaging system with an excitation filter of 500 nm and an emission filter of 620 nm.

Fluorescence imaging of viscosity in zebrafish. Wild type zebrafish were obtained from School of Pharmaceutical Sciences, Guangxi Medical University. For the fluorescence imaging experiments, 3-day-old zebra fishes were transferred into a 30 mm glass culture dishes using a disposable sterilized dropper. 10 μL monensin (1 mM), nystatin (1 mM) or LPS (1 mg/mL) were added into three dishes respectively for 60 min. Then 5 μL probe **Mito-VCI** (1 mM) was added for incubated for another 30 min, followed by washing away gently. After that, the zebrafish were transferred into the new glass bottom dishes for imaging. Prior to the imaging, we adopted 1% agarose gel for immobilization of zebrafish, and put zebrafish onto agarose with a little media to ready imaging. Fluorescence images were acquired using confocal microscope with a 4 × objective lens. The fluorescence emission was collected TRICT channel (510-650 nm) upon 500 nm excitation at OP mode and FLIM mode.

Synthesis

Synthesis of compound 1. 55 mg malonic acid (0.53 mmol) and 100 mg phenol (1.06 mmol) was slowly added 300 μL phosphorus oxychloride ($POCl_3$) at 0 ℃. The reaction mixture was heated at 112 ℃ for 3 h. Reaction completion was confirmed by thin-layer chromatography (TLC), and then the clarified liquid was poured into 50 mL of ice water and stirred overnight. Then the upper black layer was extracted with DCM three times and evaporated to yield a black crude product of compound **2**. Diphenyl malonate was pure brown oil (115 mg, 86% yield) and to be used for the next step.

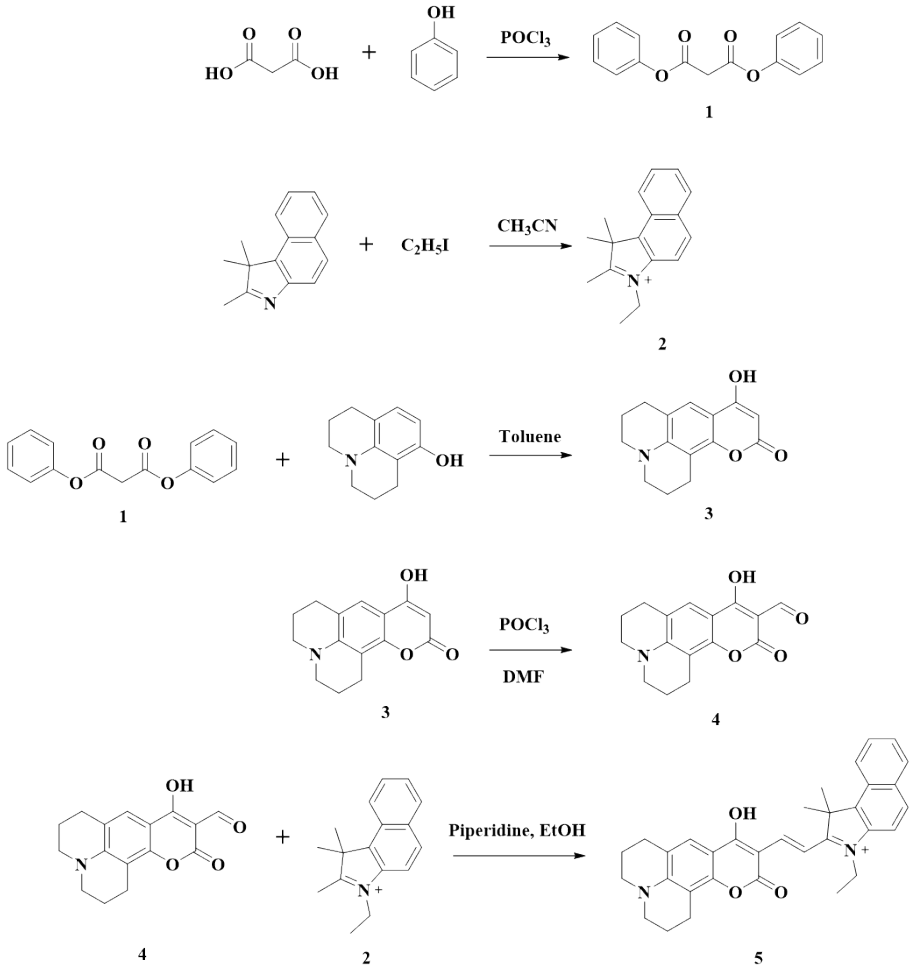
Synthesis of compound 2. To a mixture of 1,1,2-Trimethyl-1H-benz[*e*]indole (3.2 g, 15.2 mmol) and iodoethane (2.5 g, 16 mmol) was added acetonitrile (20 mL). The reaction mixture was refluxed for 7 hours. After the reaction finished, the mixture of precipitated products was filtrated under a vacuum pump and washed with petroleum ether to obtain a purple solid (1.28 g, 71% yield).

Synthesis of compound 3. To a mixture of compound **1** (757.04 g, 4 mmol) and 8-Hydroxyjulolidine (1.10 g, 4 mmol) was added toluene (15 mL). The reaction mixture was refluxed for 5 hours. After the reaction finished, the mixture of precipitated products was filtrated under a vacuum pump and washed with petroleum ether to obtain a yellow solid (0.90 g, 87.5% yield).

Synthesis of compound 4. 1 mL $POCl_3$ was dropwise added into 1 mL DMF in room temperature and stirred 30 min. Compound **3** (300 mg, 1.17 mmol) was dissolve by 3 mL DMF. When the reactants refluxed at 60 ℃, the above mixture slowly added into the solution for 12 h. Reaction completion was confirmed by TLC, and then poured into ice water and stirred to obtain a large amount of precipitate. The precipitated solid was filtered under a vacuum pump and dried to obtain red crude product. The crude products were further purified by silica gel column (DCM/MeOH = 50:1, v/v) to obtain pale red solid product (300 mg, 85.47% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.27 (s, 1H), 7.44 (s, 1H), 7.28 (s, 1H), 3.45 – 3.33 (m, 4H), 2.88 (t, J = 6.4 Hz, 2H), 2.85 – 2.76 (m, 2H), 2.00 (dq, J = 12.2, 6.1 Hz, 4H). MS (EI) m/z for $C_{16}H_{15}NO_4$: calcd: 303.0662, $([C_{16}H_{15}NO_4]+H)^+$ found: 304.0742.

Synthesis of Mito-VCI. Compound **4** (151.87 mg, 0.5 mmol) and compound **2** (143.01 mg, 0.6 mmol) were added to a rounded bottom flask and dissolved by 2 mL ethanol. When the reactants heated to reflux at 85 ℃, the piperidine (0.1 μL) was added to the mixture and reacted for 8h, and the reaction completion was confirmed by TLC. The crude product was purified by silica gel column (DCM: MeOH =20:1, v/v) and the pure red powder (130 mg, 53% yield) was obtained. ^{1}H NMR (600 MHz,

$CDCl_3$) δ 8.16 (d, *J* = 8.5 Hz, 1H), 7.94 (dd, *J* = 8.1, 5.4 Hz, 2H), 7.61 (dd, *J* = 17.6, 9.9 Hz, 2H), 7.47 (t, *J* = 7.4 Hz, 1H), 7.34 (d, *J* = 8.7 Hz, 1H), 7.00 (d, *J* = 14.9 Hz, 1H), 6.73 (d, *J* = 8.2 Hz, 1H), 4.22 (dd, *J* = 14.1, 6.9 Hz, 2H), 3.31 – 3.25 (m, 3H), 2.91 (t, *J* = 6.3 Hz, 2H), 2.82 – 2.79 (m, 2H), 2.07 (s, 5H), 1.99 (dd, *J* = 11.5, 6.0 Hz, 4H), 1.53 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (600 MHz, $CDCl_3$) δ 177.72, 139.07, 131.60, 130.30, 129.96, 128.36, 127.64, 124.74, 122.18, 117.37, 110.03, 105.94, 51.04, 50.16, 49.67, 39.28, 31.66, 30.58, 30.32, 29.71, 28.51, 27.45, 21.71, 20.85, 19.20, 14.13, 13.74. MS (EI) m/z for $C_{33}H_{33}N_2O_3$: calcd: 505.2491 ($[C_{33}H_{33}N_2O_3]^+$) found: 505.2494.



Scheme S1. Synthetic pathway for the probe **Mito-VCI**.

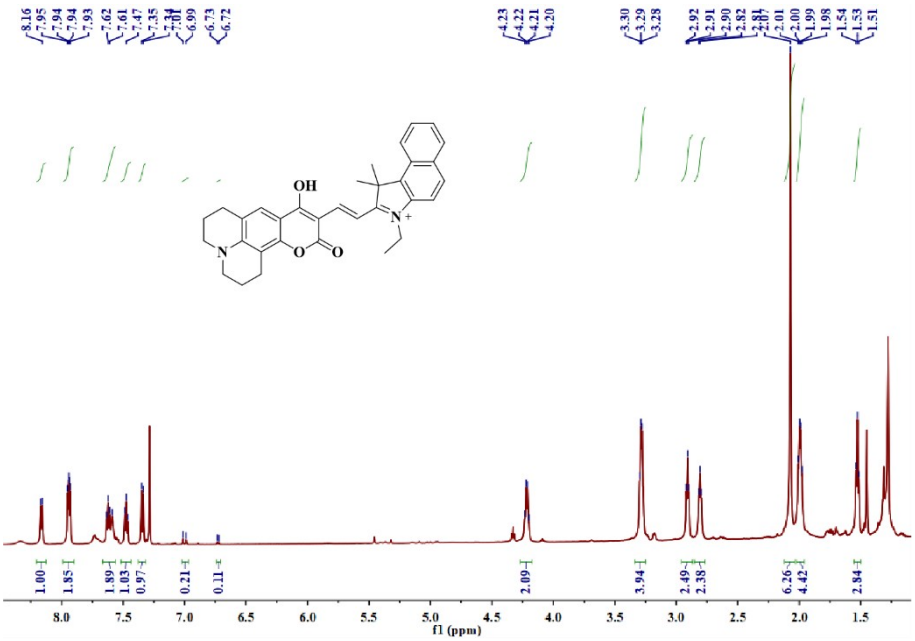


Figure S1. ^{1}H NMR spectrum of **Mito-VCI** in $CDCl_3$.

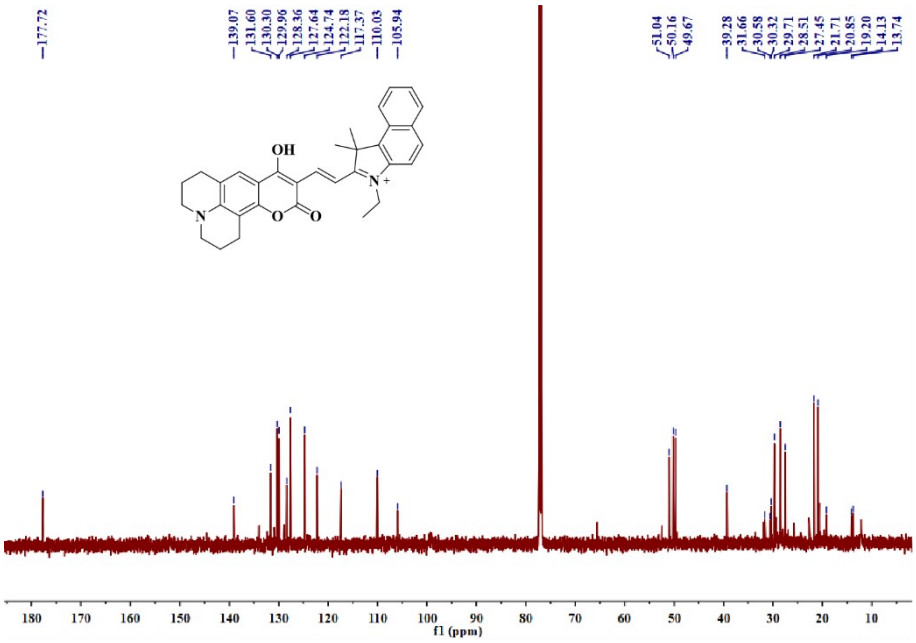


Figure S2. ^{13}C NMR spectrum of **Mito-VCI** in $CDCl_3$.

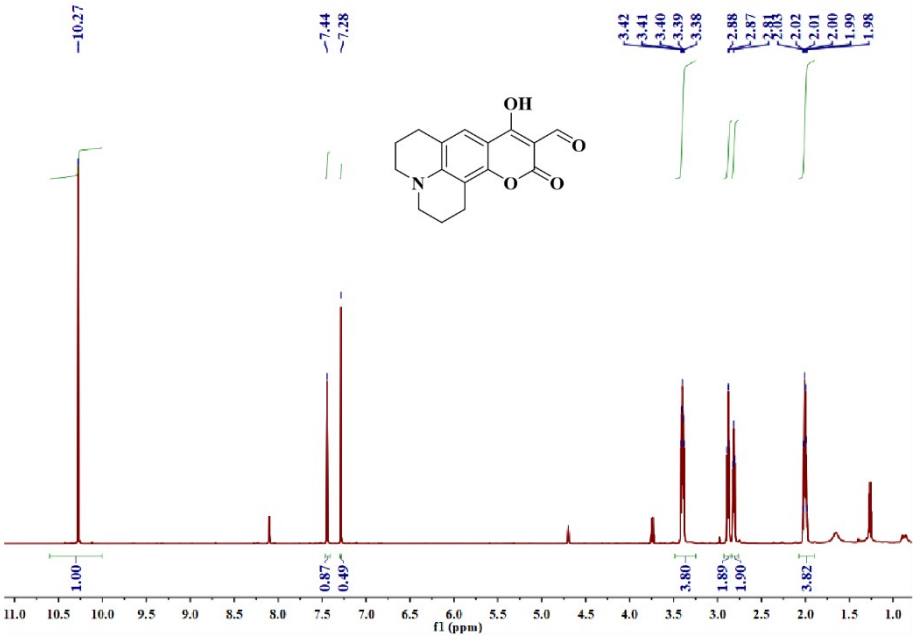


Figure S3. ^{1}H NMR spectrum of **4** in $CDCl_3$.

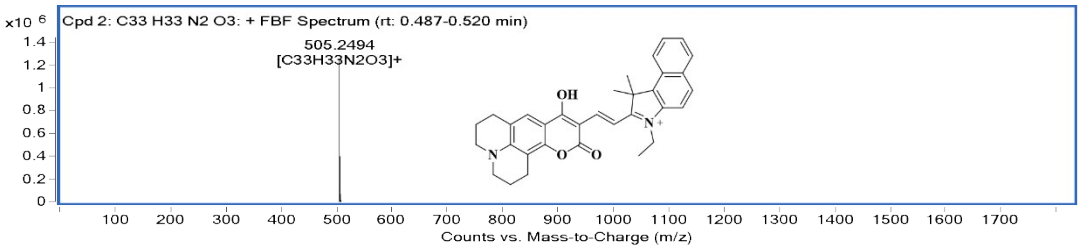


Figure S4. The mass spectrum of **Mito-VCI** in $CDCl_3$.

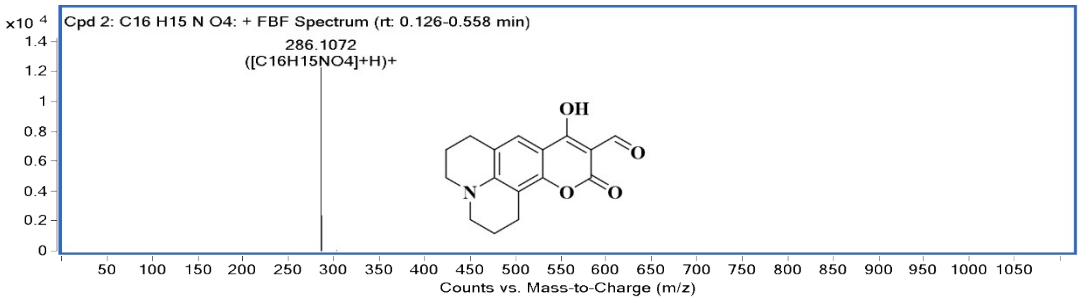


Figure S5. The mass spectrum of **4** in $CDCl_3$.

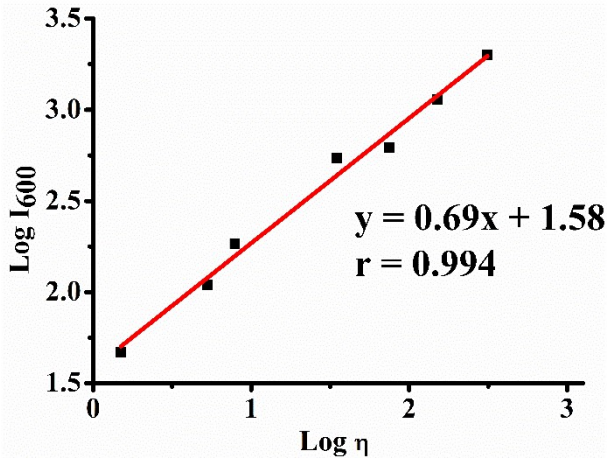


Figure S6. Linear relationship between log I_{600} and log η of **Mito-VCI** in PBS−glycerol systems.

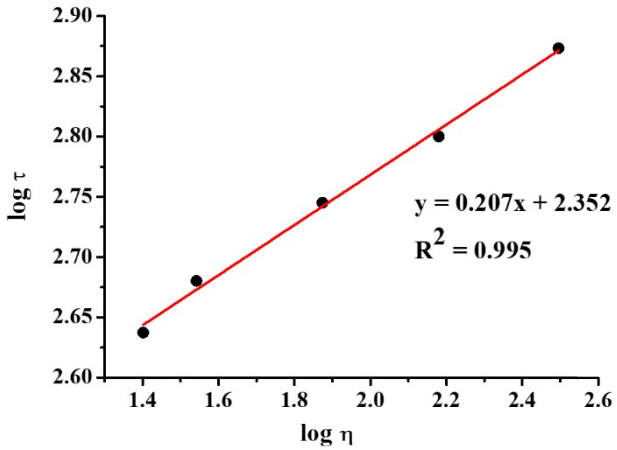


Figure S7. Linear relationship between log τ and log η of **Mito-VCI** in PBS-glycerol systems.

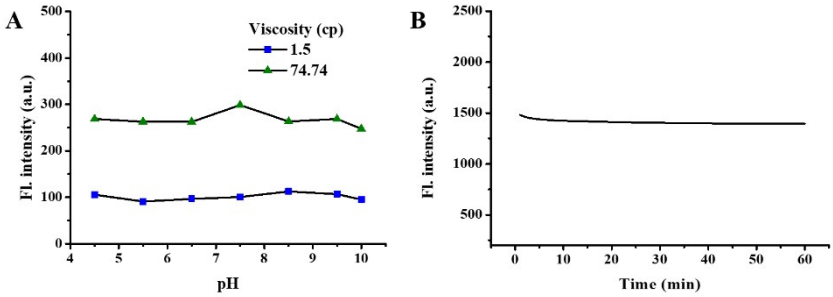


Figure S8. (A) The fluorescence spectra of **Mito-VCI** in different pH solvents; (B) Photostability of **Mito-VCI** in PBS (pH = 7.4). Excitation wavelength: $\lambda_{\textbf{Mito-VCI}}$ = 500 nm.

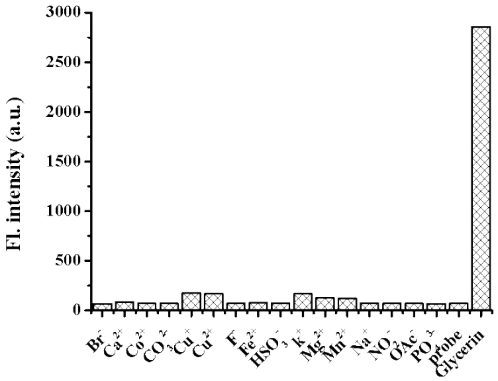


Figure S9. The fluorescence spectra of **Mito-VCI** in different ionic liquid and glycerol.

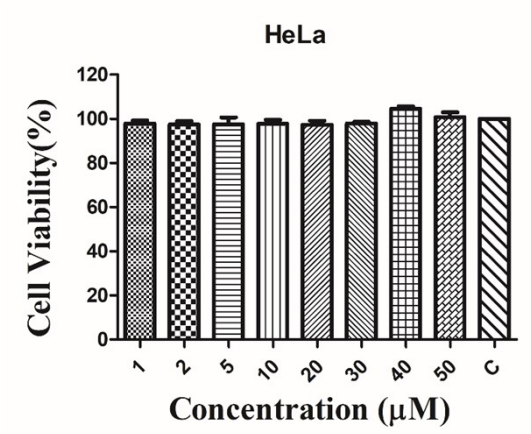


Figure S10. The CCK8 experiments of **Mito-VCI** under different concentrations (0-50 μM) for HeLa cells.

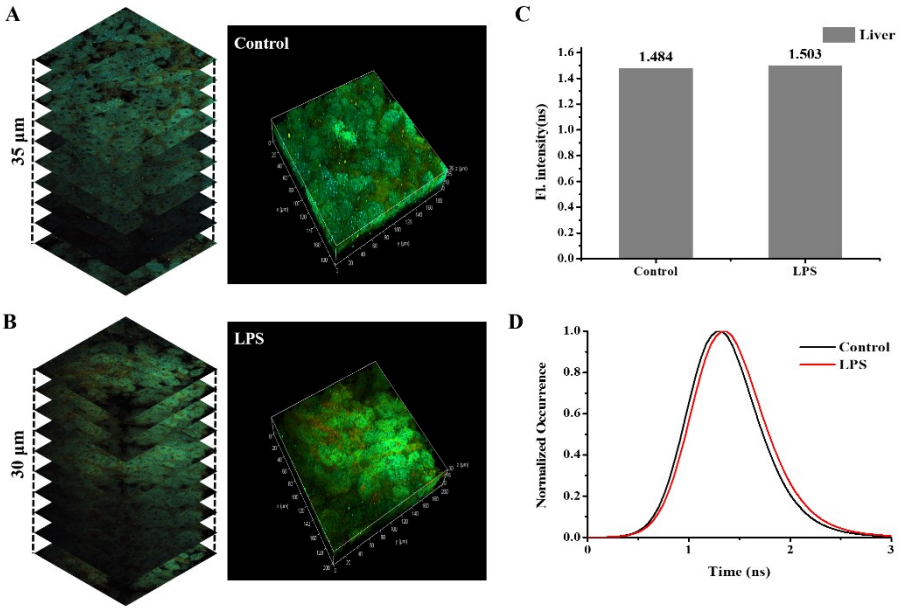


Figure S11. The 3D Fluorescence lifetime images of **Mito-VCI** in the liver of normal (A) and inflammatory mouse (B); the fluorescence lifetime value (C) and the fluorescence lifetime histogram (D) of **Mito-VCI** in liver. Concentration: 5 μM; λ_{ex} = 500 nm, λ_{em} = 510-650 nm.

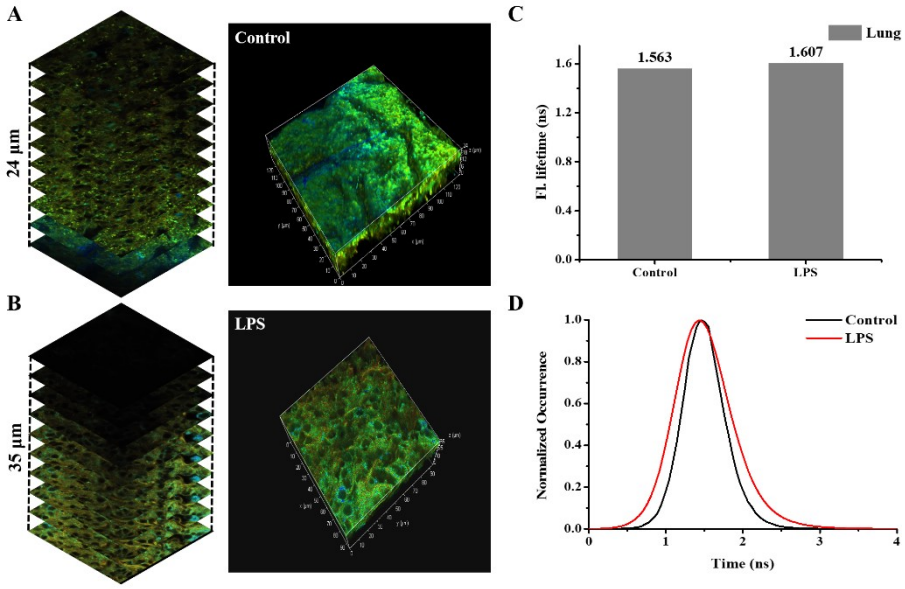


Figure S12. The 3D Fluorescence lifetime images of **Mito-VCI** in the lung of normal (A) and inflammatory mouse (B); the fluorescence lifetime value (C) and the fluorescence lifetime histogram (D) of **Mito-VCI** in lung. Concentration: 5 μM; λ_{ex} = 500 nm, λ_{em} = 510-650 nm.

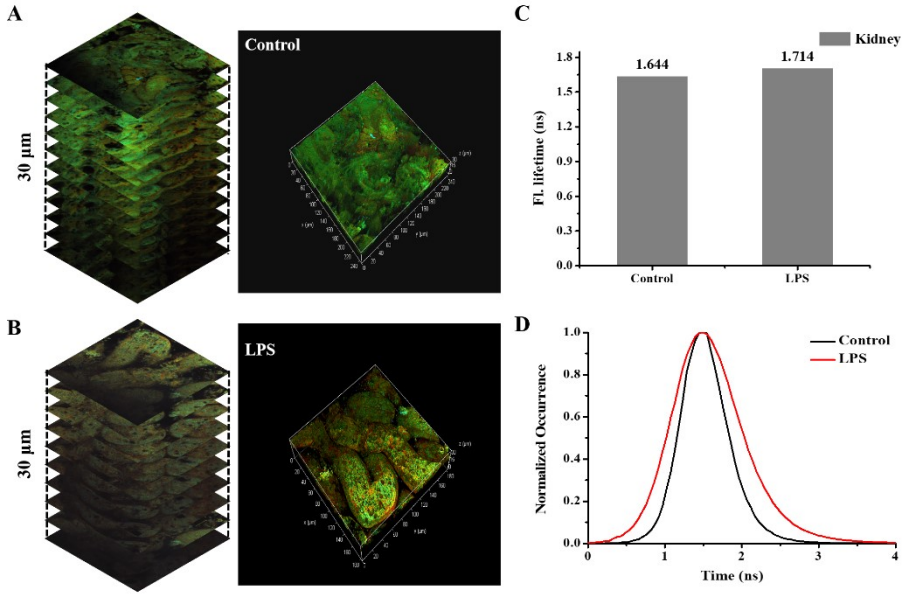


Figure S13. The 3D Fluorescence lifetime images of **Mito-VCI** in the kidney of normal (A) and inflammatory mouse (B); the fluorescence lifetime value (C) and the fluorescence lifetime histogram (D) of **Mito-VCI** in kidney. Concentration: 5 μM; λ_{ex} = 500 nm, λ_{em} = 510-650 nm.

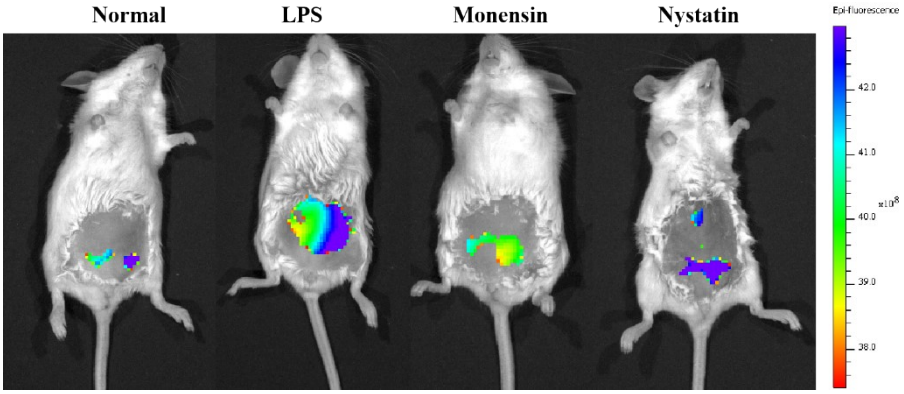


Figure S14. Fluorescence imaging of living mice: normal living mouse with **Mito-VCI** and stimulation mouse pretreated with monensin, nystatin and LPS. Concentration: 100 μM; λ_{ex} = 500 nm, λ_{em} = 620 nm.

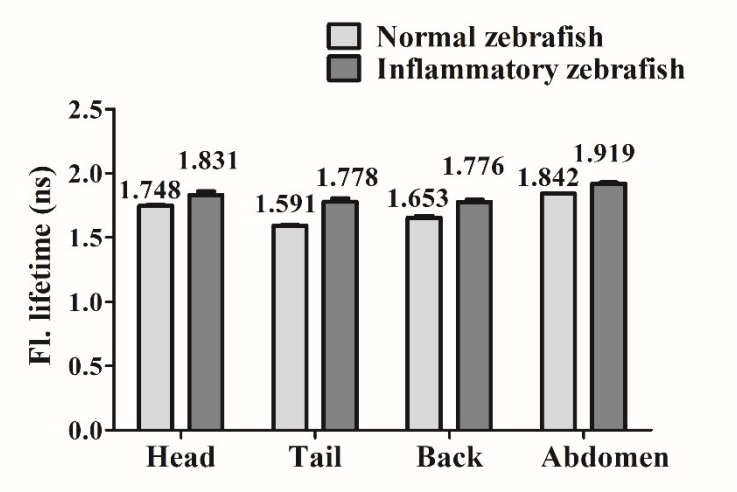


Figure S15. The mean fluorescence lifetime of **Mito-VCI** in the head, tail, back and abdomen of normal and inflammatory zebrafish.