Supporting Information

Tracking mitochondrial viscosity in living systems based on a two-photon and near red probe

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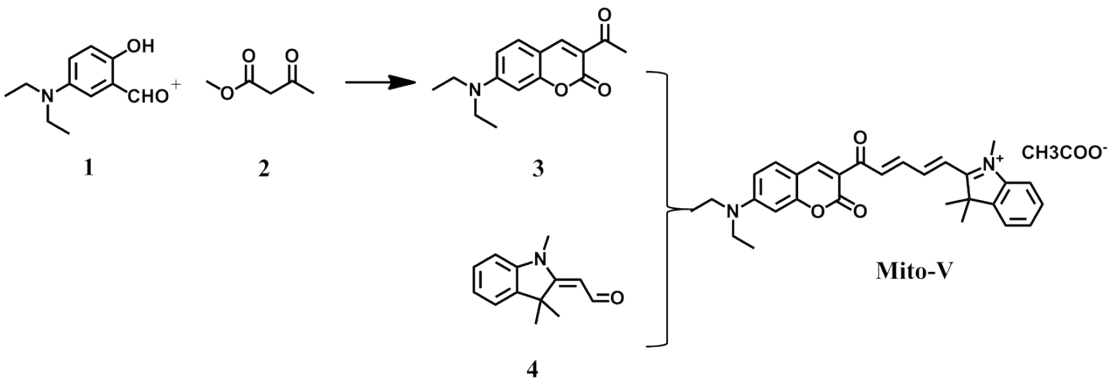
Apparatus and reagents

Unless otherwise stated, all solvents and reagents were commercially available and used without further purification. Doubly distilled water was used throughout all experiments. The absorption spectra were recorded in 1 cm cells with a Shimazu UV-2700 spectrophotometer (Suzhou, China). The emission spectra were measured on Hitachi F4600 fluorescent spectrophotometer. High resolution electrospray mass (HRMS) was recorded on Apex-Ultra Bruker instrument. NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard. The fluorescence imaging of cells was performed using a Nikon A1MP confocal microscope. The viscosity measurement was carried out on a NDJ-8 rotational viscometer. All reagents were analytical in experiment and used deionized water. The animals were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

Synthesis of the probe Mito-V



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

Synthesis of compound 3

Firstly, 4-(diethylamino)salicylaldehyde (1.93 g, 10 mmol) (1) and ethyl acetoacetate (1.53 g, 13 mmol) were dissolved in 20 mL ethanol, 0.09 g of piperidine was added to a round bottom flask. Then, the mixture was refluxed at 80 ℃ for 12 hours. After the reaction was completed, the reaction mixture was cooled to room temperature to precipitate an golden yellow solid. Filtered and recrystallized to give a flake-like golden yellow solid to afford compound (3) and the melting point is around 138.9 ℃. Yield: (2.56 g) 74%.

Synthesis of the probe Mito-V.

0.244 g (1 mmol) of compound 3 and 0.201 g (1 mmol), 1,3,3-trimethyl-2-methylene hydrazine acetaldehyde were dissolved in 20 mL

ethanol, piperidine (100 μL) and glacial acetic acid (100 μL) was added to a round bottom flask. Then, the mixture was refluxed at 80 ℃ for 12 hours. After the reaction, the solvent was removed under reduced vacuum. The resulting was purified by silica gel column chromatography (methanol/dichloromethane, 1:50) to afford a deep red solid compound **Mito-V** and the melting point is around 221.3 ℃. Yield: (0.2 g) 50%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δ 8.56 (s, 1H), 8.29 (m, 1H), 7.44 (d, J = 8.8 Hz, 1H), 7.38 (s, 1H), 7.09 (d, J = 12 Hz, 1H), 6.95 (s, 1H), 6.87 (d, J = 8.0 Hz, 1H), 6.76 (d, J = 8.0 Hz, 1H), 6.63 (dd, J_1 = 9.2 Hz, J_2 = 4.2 Hz, 1H), 6.52 (d, J = 2.0 Hz, 1H), 5.73 (d, J = 13.2 Hz, 1H), 5.41 (d, J = 8.8 Hz, 1H), 3.47 (q, J = 7.07 Hz, 4H), 3.26 (s, 3H), 1.69 (s, 6H), 1.26 (t, J = 8.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$-d_6) δ 186.49, 173.62, 165.91, 165.53, 158.14, 152.27, 147.40, 144.29, 142.17, 139.61, 139.39, 131.26, 128.05, 127.81, 122.45, 121.81, 121.70, 121.05, 119.21, 109.48, 108.81, 107.99, 106.99, 99.00, 96.63, 45.06, 29.56, 28.79, 12.49. HRMS-ESI: m/z calcd. M+ for $C_{29}H_{31}N_2O_3$+, 455.2335; found, 455.2317.

Calculation of fluorescence quantum yield of Mito-V.

The fluorescence quantum yields (Φ_f) were determined by using Rhodamine B as the reference according to the literature method. Quantum yields were corrected as follows:

$$\phi_f = \phi_r \left(\frac{A_r \eta_s^{\,2} D_s}{A_s \eta_s^{\,2} D_r} \right)$$

Where the s and r indices designate the sample and reference samples respectively. A is the absorbance at λ_{ex}, η is the average refractive index of the appropriate solution, and D is the integrated area under the corrected emission spectrum.

Calculation of two-photon absorption cross section of CBI-V.

$$\delta = \delta_{ref} \frac{F \Phi_{ref} C_{ref} n_{ref}}{F_{ref} \Phi C n}$$

where "ref" subscript stands for reference while the ones without any are for the sample. δ: Two photon absorption cross section; F: Integrated area of Two photon Induced fluorescence spectra; Φ: Fluorescence quantum yield; C: Concentration in moles/Litre, n: Refractive Index of the solvents used.

Preparation of solutions of probe Mito-V.

The stock solution of **Mito-V** was dissolved in DMSO in 1 mM, 10 μM of the probes were used in photophysical experiments by addition of 30 μL of the stock solution to a 3.0 mL test solution

Spectral test

The solvents were obtained by mixing a methanol-glycerol system in different proportions. Measurements were carried out with a NDJ-8 rotational viscometer, and each viscosity value was recorded. The

solutions of **Mito-V** of different viscosity were prepared by adding the stock solution (1.0 mM) 30 μL to 3 ml of solvent mixture (methanol-glycerol systems) to obtain the final concentration of the dye (10.0 μM). These solutions were sonicated for 5 minutes to eliminate air bubbles, the solutions were measured in a UV spectrophotometer and a fluorescence spectrophotometer.

Cytotoxicity assay

The cytotoxicity of the probe **Mito-V** to Hela cells were studied by standard MTT assays. 2×10^4 cells/mL cells were seeded in 96-well plates and then incubated with various concentrations of the probe (0, 1, 2, 5, 10, 15, 20 and 50μM) for 24 h. After that, 10 μL MTT (5 mg/mL) was added to each well and incubated for another 4 h. Finally, the media was discharged, and 100 μL of DMSO was loaded to dissolve the formazan crystals. The plate was shaken for about 10 min, and each well was analyzed by the microplate reader and detected at the absorbance of 490 nm. The cell viability (%) = $(OD_{sample}-OD_{blank}) / (OD_{control}- OD_{blank}) \times 100\ \%$）.

Cell culture and fluorescence imaging

HeLa cells were cultured in 90% Dulbecco's Modified Eagle Medium (DMEM, Gibico) supplemented with 10%FBS (Hyclone) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Hyclone) in an atmosphere of 37 °C and 5% CO_2 The Monensin or Nystatin was

first added into the Hela cells in a glass bottom culture dishes (Nest)for 30 min, then they were washed by PBS for three times, after that, the probe (10μM) was added into the Hela cells treated with Monensin or nystatin for another 30 min. After washed with PBS for three times, the fluorescence imaging was carried out by a Nikon A1MP inverted fluorescence confocal microscope. The fluorescence emission of the probe was collected at TRICT channel (570 nm-620 nm), the excitation wavelength was 561 nm. Monensin and nystatin could induce structural changes or swelling, leading to viscosity changes in the mitochondria.

Fluorescence imaging in living zebra fish

3-day-old zebra fish were transferred into a 30 mm glass culture dishes using a disposable sterilized dropper. The 10 μM monensin or nystatin was added for incubated for 30 min, followed by washing away gently. Then probe **Mito-V** (10μM) were put into dishes respectively for another 30 min. After that, the zebra fishes were transferred into new glass bottom dishes for imaging. Prior to the imaging, we adopted 1% agarose gel for immobilization of zebra fishes, and put zebra fishes onto agarose with a little media to ready imaging. Fluorescence images were acquired with Nikon A1Rconfocal microscope with a 4 × objective lens. The OP fluorescence emission was collected at TRICT channel (570 nm-620 nm) upon excitation at 561 nm, the TP fluorescence at 730 nm.

Fluorescence imaging in living mice

The animals were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China). The mice were used for in vivo imaging of viscosity changes. The living imaging was carried out by using PerkinElmer IVIS Lumina III system with an excitation filter of 520 nm and an emission filter of 670 nm.

Table S1 Test viscosity in the varied of the methyl alcohol/glycerol (v/v) mixtures and fluorescence quantum yield (Φ_f) of **Mito-V.**

Methanol/glycerol (v: v)	η/cp	Φ_f/%
10:0	1.7	0.0372
9:1	2.0	0.0508
8:2	5.4	0.0978
7:3	12.2	0.1211
6:4	45.8	0.1631
5:5	70.1	0.2309
4:6	135	0.2797
3:7	281	0.3075
2:8	542	0.3593
1:9	751	0.3883
0:10	956	0.4565

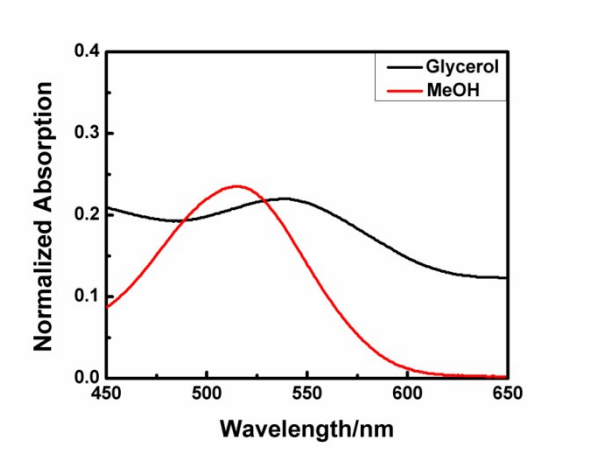


Fig. S1 the UV-vis absorption spectrum of **Mito-V** in MeOH and glycerol solvents.

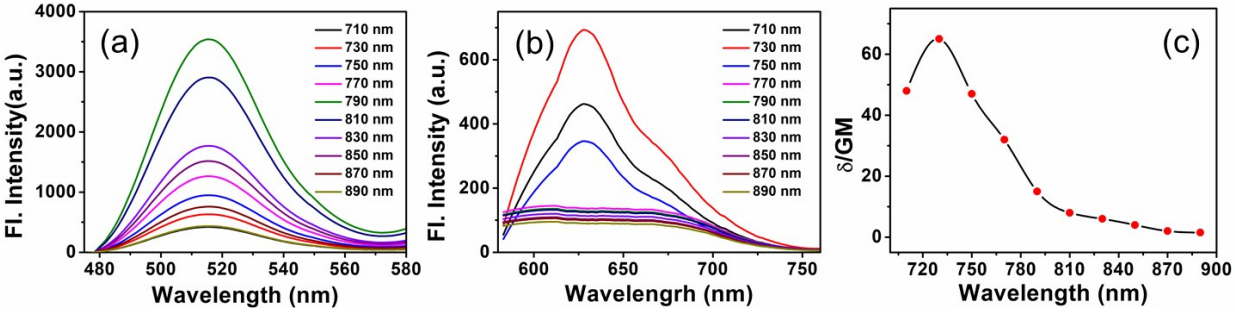


Fig. S2 Two-photon fluorescence intensity of 1.0 μM fluorescein (A) and 5.0 μM **Mito-V** (B) at 710-890 nm excitation. (C) Two-photon absorption cross sections (δ) of the compounds **Mito-V** at 710-890 nm in glycerol solution.

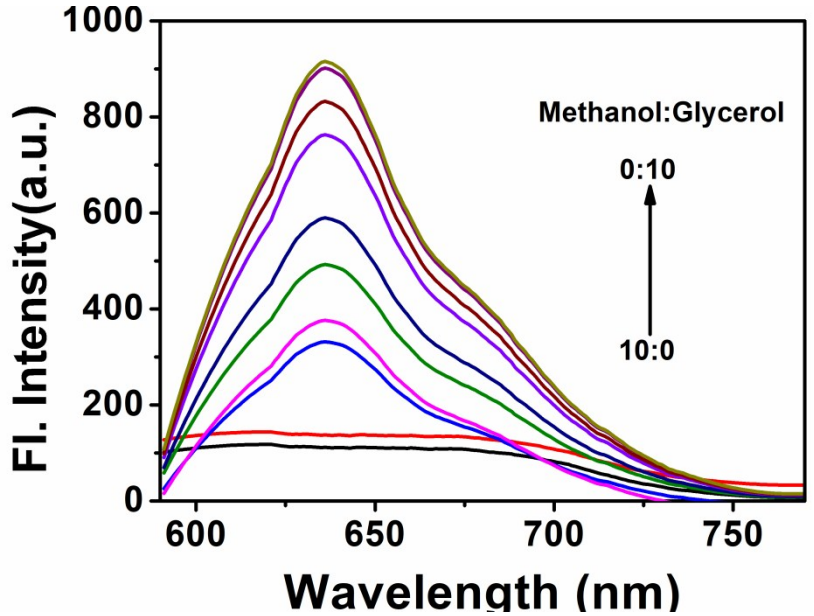


Fig. S3 The fluorescence spectra of 5.0 μM **Mito-V** in methanol-glycerol systems at various ratios. λ_{ex}=730 nm.

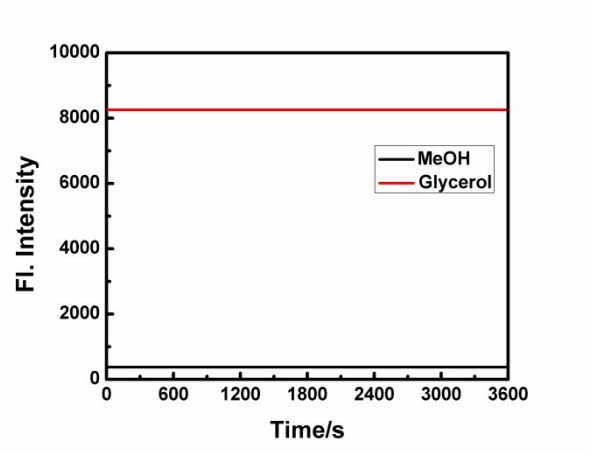


Fig. S4 The fluorescence emission spectra of 10μM of **Mito-V** in glycerol and MeOH. The fluorescence intensity was measured at λ_{ex}=561nm with both excitation and emission slit widths of 5 nm, and a 700 V PMT voltage.

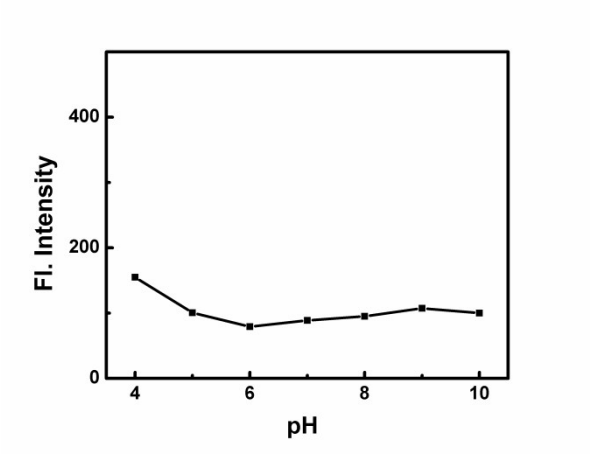


Fig. S5 The effect of pH on the fluorescence emission intensity of 10μM of **Mito-V** in PBS aqueous solution. The fluorescence intensity was measured at λ_{ex}=514 nm with both excitation and emission slit widths of 5 nm, and a 650 V PMT voltage.

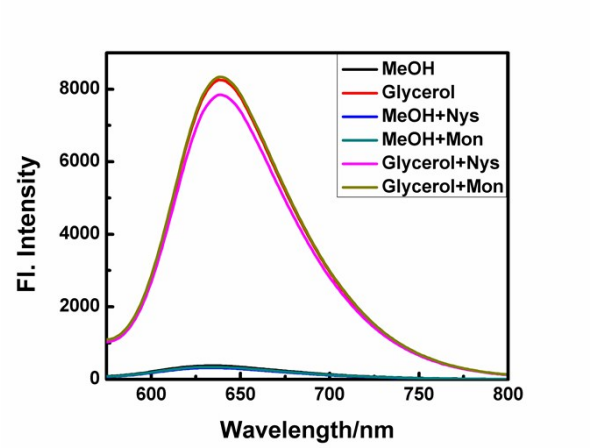


Fig. S6 The effect of Monensin or Nystatin add in MeOH or 99% Glycerol solution. The fluorescence intensity was measured at λ_{ex}=514 nm with both excitation and emission slit widths of 5 nm, and a 700 V PMT voltage.

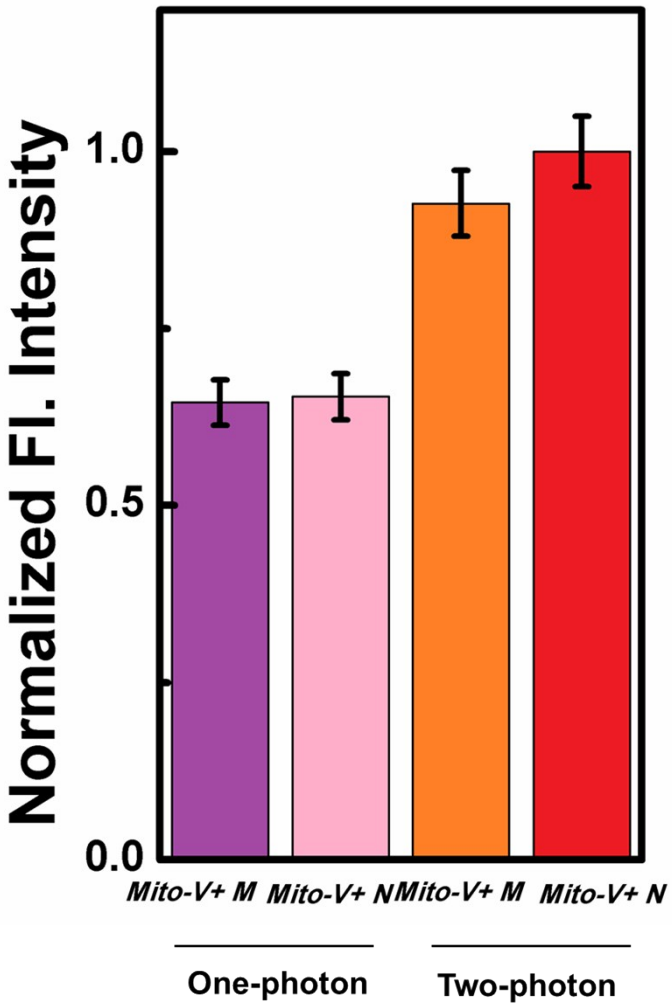


Fig. S7 Normalized fluorescence intensity of Hela cells at TP Mode and TP Mode. λ_{em}=570-620 nm; λ_{ex}=561 nm at OP mode and 730 nm at TP mode. Scale bar: 25 μm.

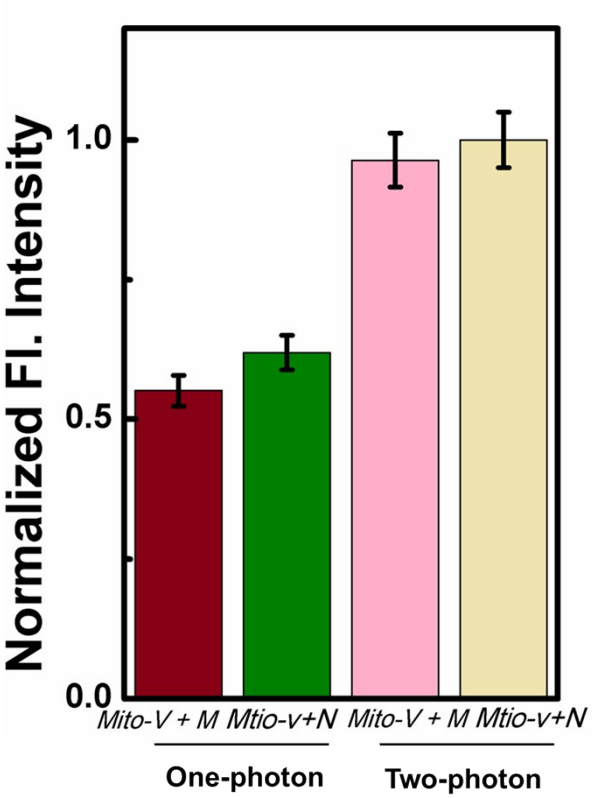


Fig. S8 Normalized fluorescence intensity of zebra fish at TP Mode and TP Mode. λ_{em}=570-620 nm; λ_{ex}=561 nm at OP mode and 730 nm at TP mode. Scale bar: 25 μm.

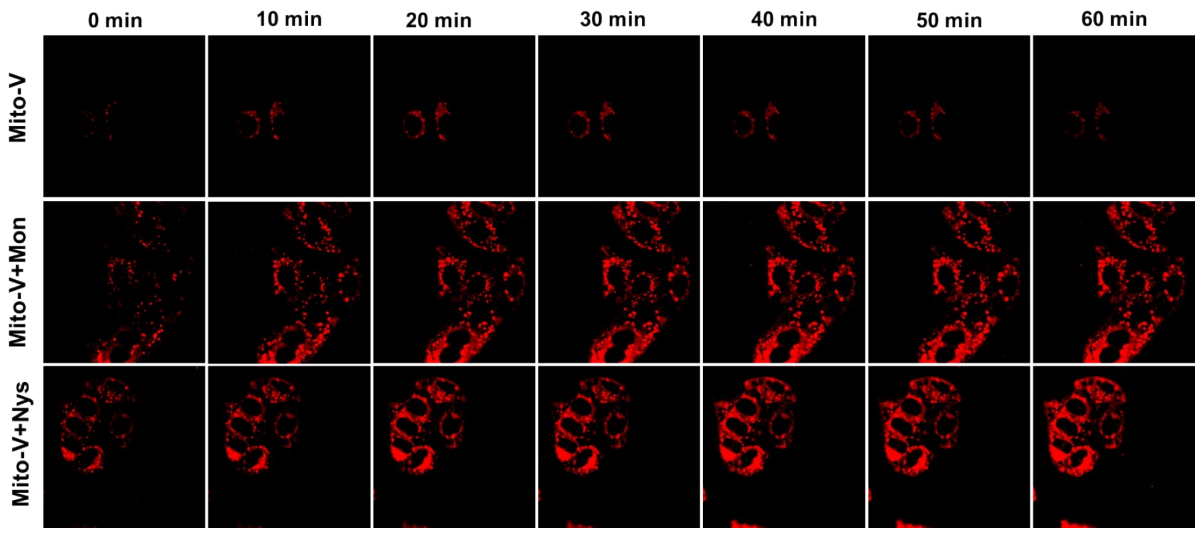


Fig. S9 The real-time imaging of dynamic changes in mitochondrial viscosity of **Mito-V** at different time intervals, λ_{em}=570-620 nm; λ_{ex}=561 nm. Scale bar: 25 μm.

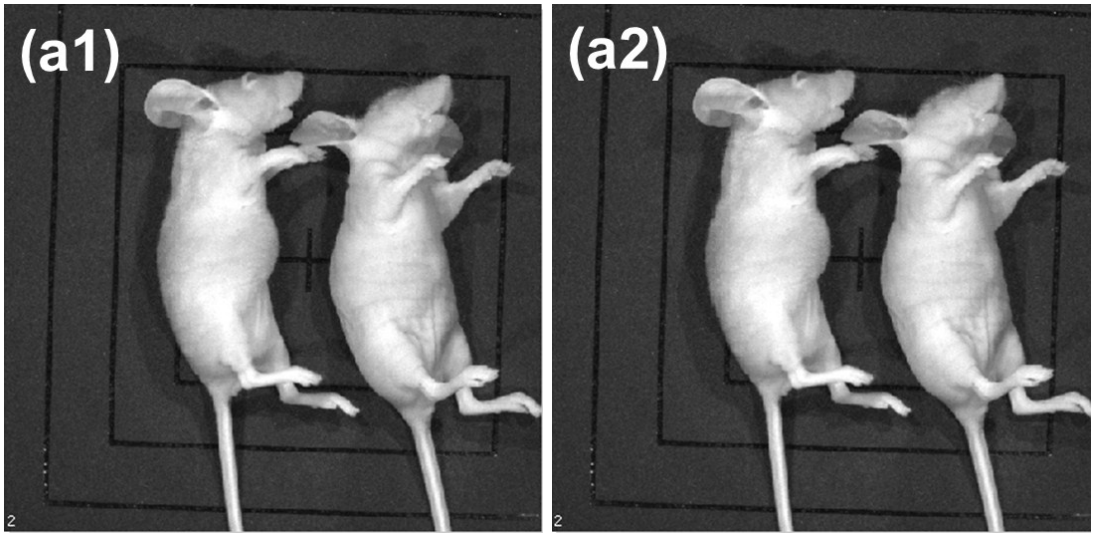


Fig. S10 (a1) Confocal fluorescence images of mice without **Mito-V,** left: normal mice, right: inflammatory mice with injected LPS; (a2) Confocal fluorescence images of mice without **Mito-V,** left: normal mice, right: inflammatory mice with injected monensin;

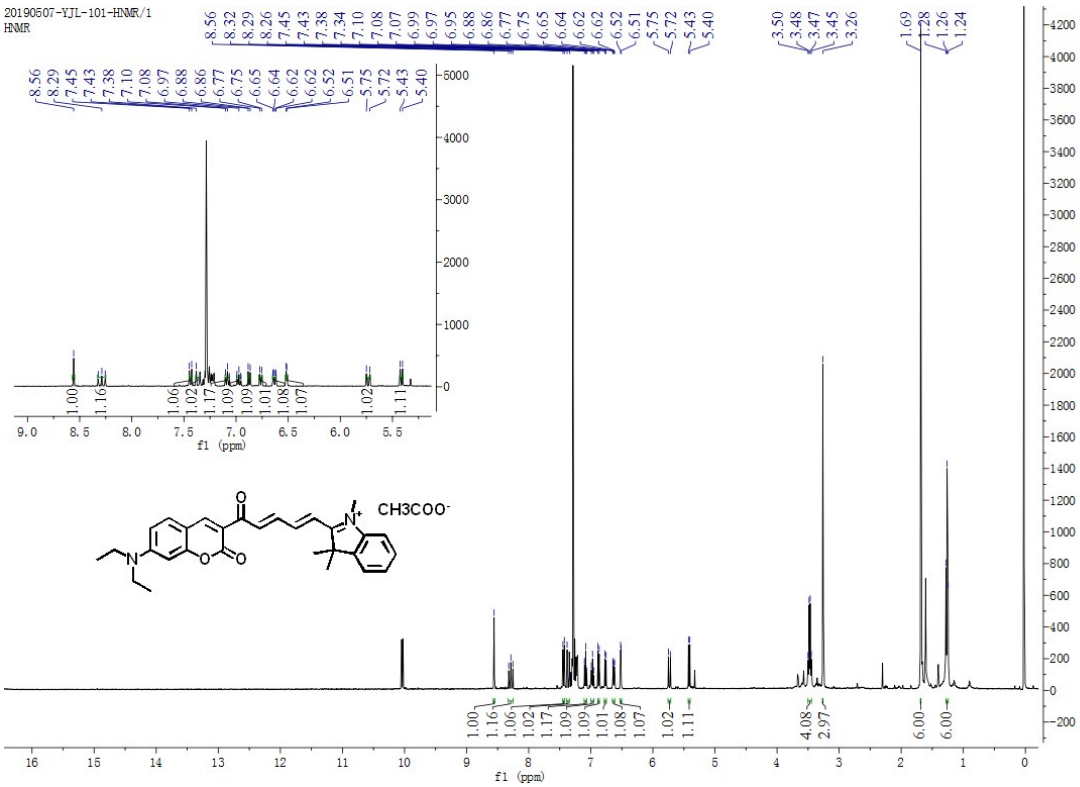


Fig. S11 ^{1}H NMR spectrum of **Mito-V** in $CDCl_3$-d_6.

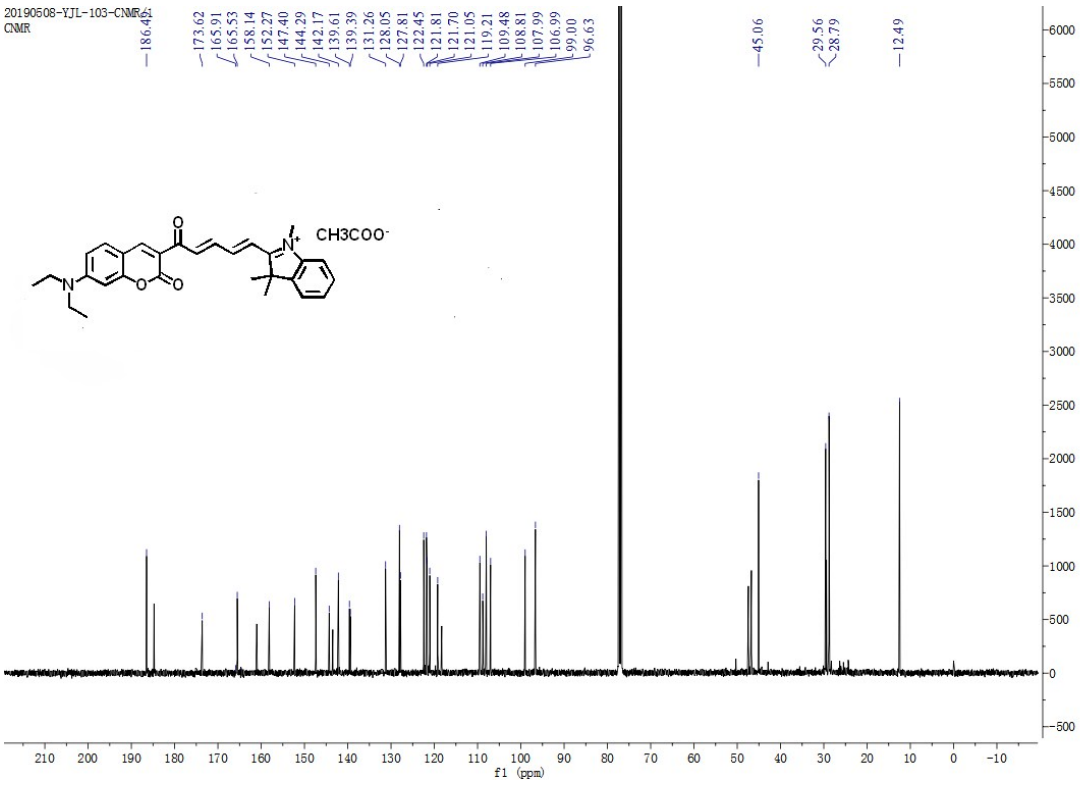


Fig. S12 ^{13}C NMR spectrum of **Mito-V** in $CDCl_3$-d_6.

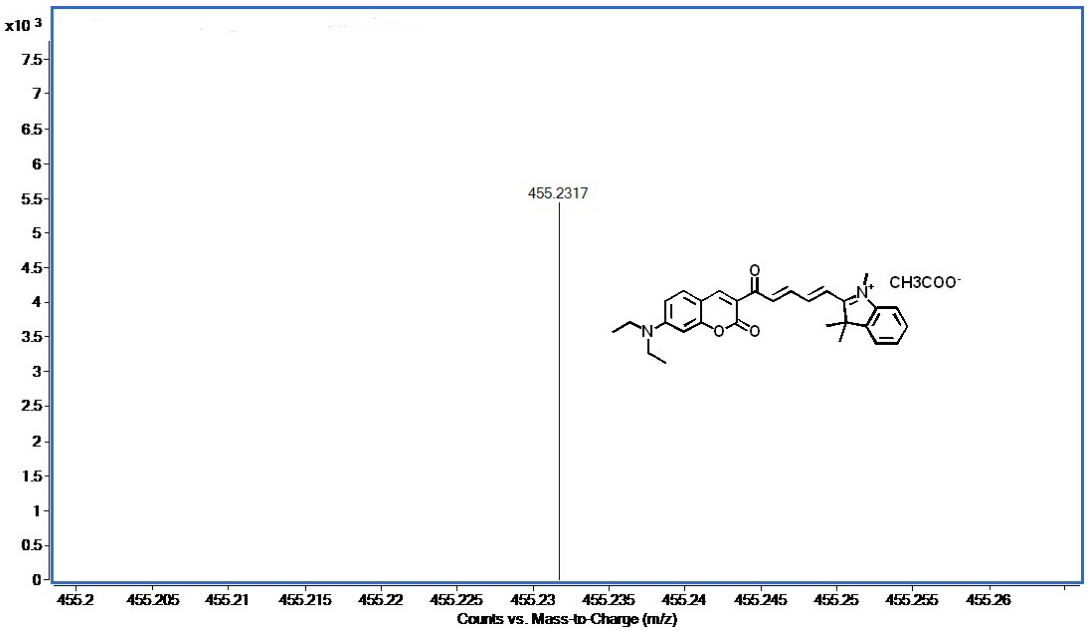


Fig. S13 HRMS spectrum of **Mito-V.**