Electronic Supplementary Information for:

Sensing and imaging of mitochondrial viscosity in living cells by a red photoluminescent probe with long lifetime

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1. Experimental

Materials and Reagents. 1,1,2-Trimethyl-1*H*-benzo[*e*]indole, iodoethane and 4-aminobenzaldehyde were purchased from Energy Chemical. Glycerol was purchased from Yantai Chemical Industry Research Institute (China) and nystatin from Aladdin Co., Ltd. Staurosporine was obtained from J&K Scientific Ltd (Bejing, China). Lipopolysaccharide (LPS), glucose and rhodamine 123 were purchased from Sigma-Aldrich. DMSO (dimethyl sulfoxide, biochemistry grade) was ordered from J&K Scientific Ltd (Bejing, China). A phosphate buffered saline (PBS, 10 mM) solution was bought from Solarbio Company. (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Serva Electrophoresis GmbH (Germany). All other available chemicals and reagents used were of analytical grade and provided by local suppliers. The stock solution preparation of probe **Mito-V** (1 mM) was made by dissolving an appropriate amount of **Mito-V** in DMSO. All solutions were prepared with ultrapure water from a Milli-Q reference system (Millipore).

Characterization Methods. TLC (thin layer chromatography) analysis was carried out on silica gel plates and column chromatography was performed on silica gel (mesh: 200-300). ^{1}H and ^{13}C NMR spectra were executed by a Bruker Avance III 600 MHz spectrometer. Electrospray ionization mass spectrum (ESI-MS) was performed on a LC-MS 6410B instrument (triple quadrupole, Agilent Technologies, CA, USA). High resolution electrospray ionization mass spectra (HR-ESI-MS) were carried out at an APEX IV FTMS instrument (Bruker, Daltonics). The viscosity of the solutions was tested with a NDJ-7 rotational viscometer (China). Determination of various pH values was performed using FE20 pH meter (Mettler Toledo Inc.). Absorption spectra were measured on a T10CS spectrophotometer (Beijing Puxi, China) with 1-

cm quartz cells. Steady PL spectral measurements were taken on a Hitachi F-7000 spectrofluorimeter in 1 × 1 cm quartz cells (Tokyo, Japan), with both excitation and emission slit widths of 2.5 nm, and a 700 V PMT voltage. Time-resolved measurements for PL were conducted with EPL-series lasers, and long-lived measurements for phosphorescence was carried out with a μF2 microsecond flashlamp. Time-delayed PL spectra were recorded using a μF2 microsecond flashlamp and a time-gated module. PL imaging experiments were carried out on Leica TCS SP8 confocal laser scanning microscope (Germany) through a 63× oil objective and an optical section of 0.5 μm. The image processing was acquired by the software of the corresponding instrument (Leica Application Suite). The absorbance for MTT analysis was measured on a multifunctional microplate reader (Molecular Devices SpectraMax M5, America). Flow cytometry data were collected by BD Biosciences FACS AriaIII instrument.

Synthesis of Probe. A synthetic route for **Mito-V** and model molecule was depicted in Scheme 1.

Compound **1**: 1,1,2-trimethyl-1*H*-benzo[*e*]indole (100 mg, 0.48 mmol) and iodoethane (112 mg, 0.72 mmol) were added in a 50 mL round flask with 10 mL acetonitrile. Then under argon gas atmosphere, the mixture solution was refluxed and stirred for 16 h. After cooled down to room temperature, the solvent was removed by rotary evaporator. Then the residue was dissolved with a small amount of dichloromethane and subjected to silica gel chromatography, with eluting agent dichloromethane/methanol (v/v, 25:1), affording **1** (49 mg, yield: 49%).

Pobe **Mito-V**: compound **1** (49 mg, 0.21 mmol) and 4-aminobenzaldehyde (38.8 mg, 0.32 mmol), sodium acetate (116.5 mg, 0.64 mmol) were dissolved in 5 mL of acetic anhydride. The mixture solution was stirred for 12 h at 50 ^{o}C under argon gas condition. Then the reaction

mixture was cooled to room temperature and extracted three times with CH_2Cl_2 and H_2O. The organic phase was separated, and dried with anhydrous $MgSO_4$. The solvent was removed under reduced pressure to give the crude product, which was purified by silica gel flash chromatography using dichloromethane/methanol (v/v, 25:1) as eluent, affording **Mito-V** as a dark red solid (31.9 mg, 65% yield). The chemical structure of **Mito-V** was characterized by ^{1}H NMR and ^{13}C NMR spectra and HRMS, as provided in Figures S1, S2 and S3, respectively. ^{1}H NMR (600 MHz, CD_3OD, 298 K): δ 8.35 (d, *J* = 15.6 Hz, 1H), 8.33 (d, *J* = 8.4 Hz, 1H), 8.14 (d, *J* = 8.4 Hz, 1H), 8.08 (d, *J* = 8.4 Hz, 1H), 7.88 (d, *J* = 9.0 Hz, 2H), 7.83 (d, *J* = 9.0 Hz, 1H), 7.73 (t, *J* = 7.5 Hz, 1H), 7.61 (t, *J* = 7.5 Hz, 1H), 7.20 (d, *J* = 15.6 Hz, 1H), 6.79 (d, *J* = 7.8 Hz, 2H), 5.47 (s, 2H), 4.62 (q, *J* = 7.2 Hz, 2H), 2.04 (s, *6*H), 1.57 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD, 298 K): δ 181.8, 157.8, 155.8, 139.3, 137.9, 134.4, 131.0, 127.3, 124.0, 123.5, 115.5, 112.6, 104.0, 101.1, 54.5, 54.0, 41.9, 27.0, 26.9, 13.5, 13.4. HRMS calcd for $C_{24}H_{25}N_2^+$ $[M]^+$: 341.2012, found 341.2012.

General Procedure for Spectroscopic Detection of Viscosity. Unless otherwise stated, all the PL measurements were operated according to the below procedure. The solutions with different viscosity were adjusted by mixing different ratios of glycerol and water, and the viscosity was confirmed with a viscometer. In a test tube, 2 mL of solutions with different viscosity and 4 μL of the stock solution (1 mM) of **Mito-V** were mixed, following by the addition of tested substances. Then the corresponding viscous solutions were adjusted to the final volume of 1 mL. After shaking, the mixed solution was transferred to the quartz cell of 10 mm optical length to measure PL spectra with $\lambda_{ex/em}$ = 511/583 nm and both excitation and emission slit widths of 2.5 nm.

Cytotoxicity Assay. In order to estimate the cytotoxicity of **Mito-V** on HepG2 cells, we did a routine MTT test, as described previously.[26]

PL Imaging of Viscosity in HepG2 Cells. HepG2 cells were incubated in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum (FBS), and 1% (v/v) penicillin-streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2. Then the cells were grown on glass-bottom culture dishes (MatTek Co.) and allowed to adhere for 48 h. For stimuli experiments, the cells were pretreated with 20 μM of staurosporine or nystatin or LPS for different time and then incubated with **Mito-V** (5 μM) for 30 min at 37 °C, respectively. Finally, the cells were washed twice with PBS and PL imaging was recorded by confocal luminescence microscope.

Flow Cytometry Analysis. The cells were cultured at 2.0×10^5 cell in 6-well plates and allowed to adhere for 48 h. Cells were collected into a 2 ml centrifuge tube after trypsin digestion. Then, the cells were centrifuged at 1000 rpm for 3 min. After removing the supernatant, 500 μL PBS was added to suspend cells. Finally, the cells were analyzed by flow cytometry (BD FACSCalibur) through using FL-2 channel under excitation wavelength of 488 nm.

2. Synthesis of probe Mito-V

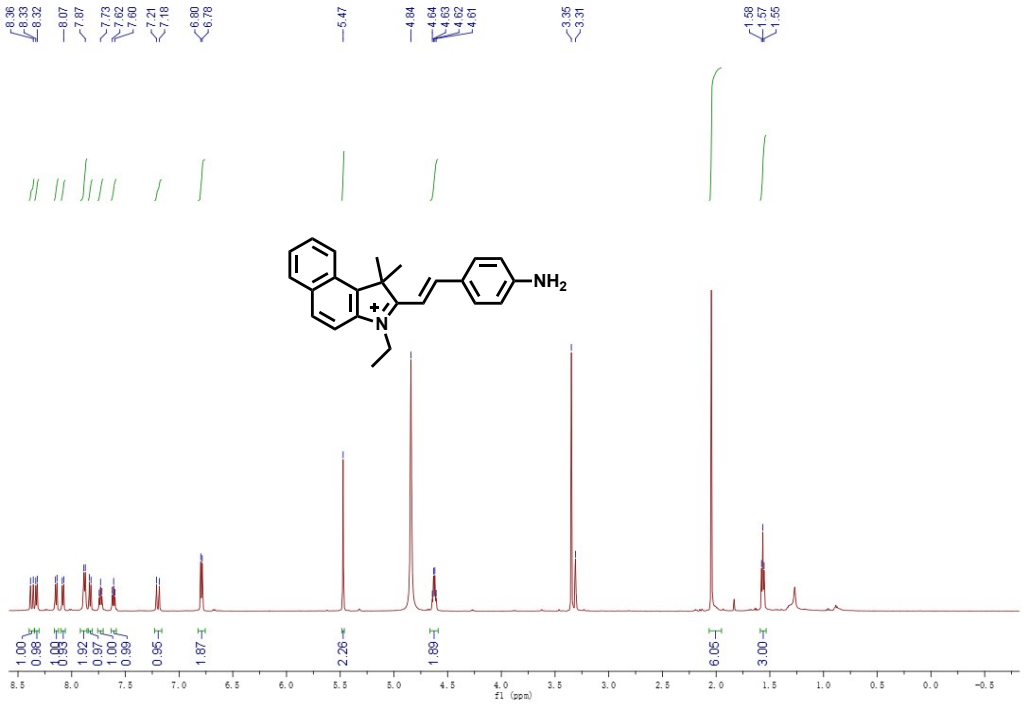

Fig. S1. ^{1}H NMR spectrum of **Mito-V** (600 MHz, CD_3OD, 298 K).

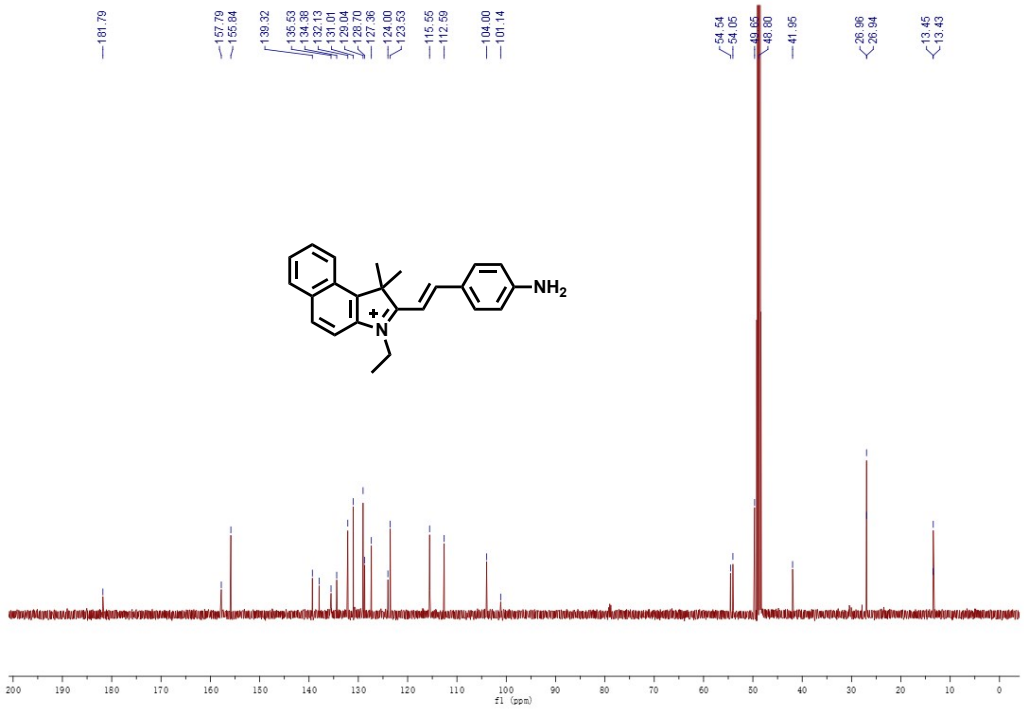

Fig. S2. ^{13}C NMR spectrum of **Mito-V** (150 MHz, CD_3OD, 298 K).

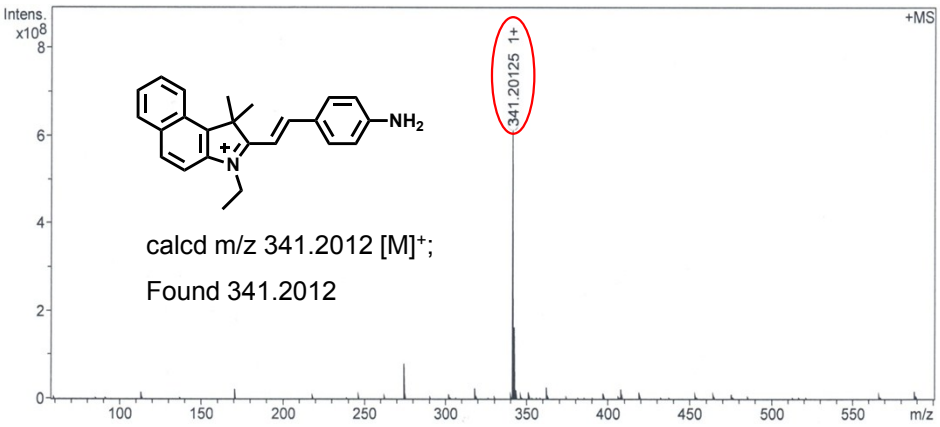

Fig. S3. HRMS result of compound **Mito-V**.

3. The Forster-Hoffmann equation

The Forster-Hoffmann equation was utilized to correlate the relationship between the fluorescence emission intensity of **Mito-V** and the solvent viscosity or between the fluorescence lifetime of **Mito-V** and the solvent viscosity.

$$\log I = C + x \log \eta \quad \text{(S1)}$$

where η is the viscosity, I is the emission intensity, C is a constant, and x represents the sensitivity of the fluorescent probe to viscosity.

$$\log \tau = C + x \log \eta \quad \text{(S2)}$$

where η is the viscosity, τ is the fluorescence lifetime, C is a constant, and x represents the sensitivity of the fluorescent probe to viscosity.

4. Optimization of experimental conditions

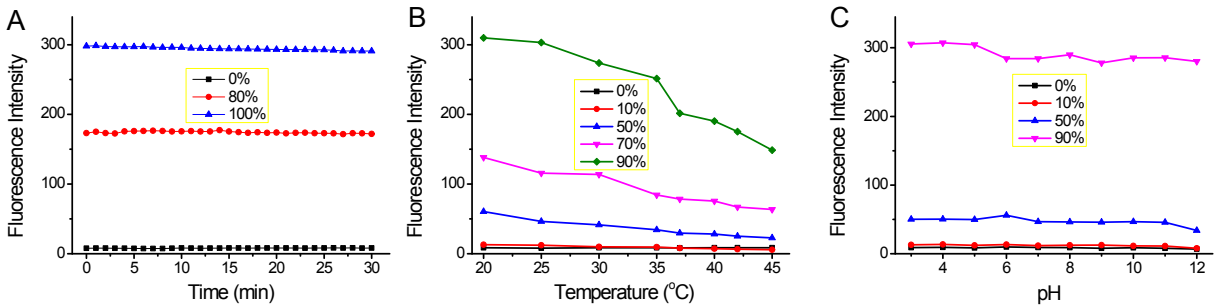

Fig. S4. (A) Plots of PL intensity of **Mito-V** (2 μM) vs. the reaction time in the presence of varied glycerol volumetric ratios. The effects of temperature (B) and pH (C) on the fluorescence

intensity of probe **Mito-V** (2 μM) in presence of varied glycerol volumetric ratios. $\lambda_{ex/em}$ = 511/583 nm.

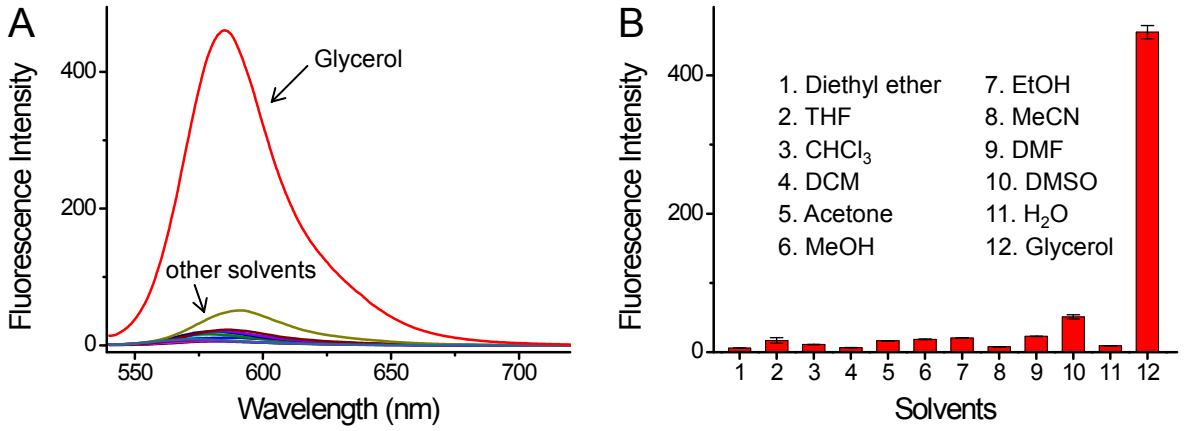

Fig. S5. (A) PL spectra of 2 μM **Mito-V** in various solvents with different polarities. (B) Bar diagram of panel A. $\lambda_{ex/em}$ = 511/583 nm.

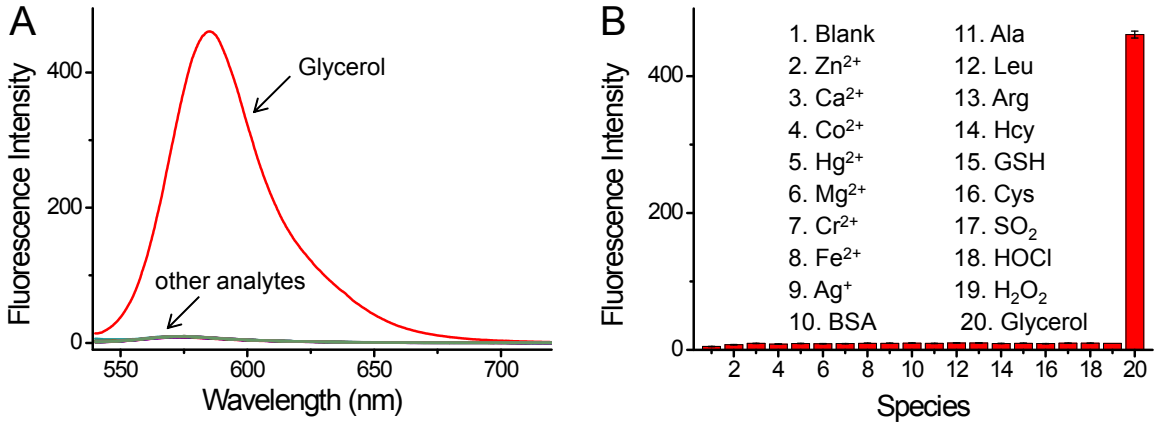

Fig. S6. (A) PL spectra and (B) bar diagram of 2 μM **Mito-V** with addition of 200 μM different analytes (Zn^{2+}, Ca^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Cr^{2+}, Fe^{2+}, Ag^{+}, BSA, Ala, Leu, Arg, Hcy, GSH, Cys, SO_2, HOCl, H_2O_2). $\lambda_{ex/em}$ = 511/583 nm.

5. Cytotoxicity assay

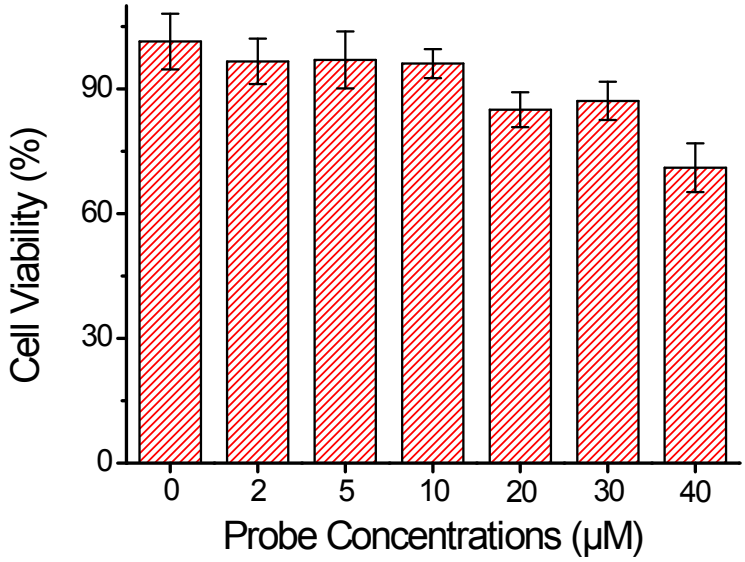

Fig. S7. Effects of **Mito-V** with varied concentrations (2-40 μM) on the viability of HepG2 cells. The viability of the cells without **Mito-V** is defined as 100%. The results are the mean ± standard deviation of five separate measurements.

6. Confocal imaging

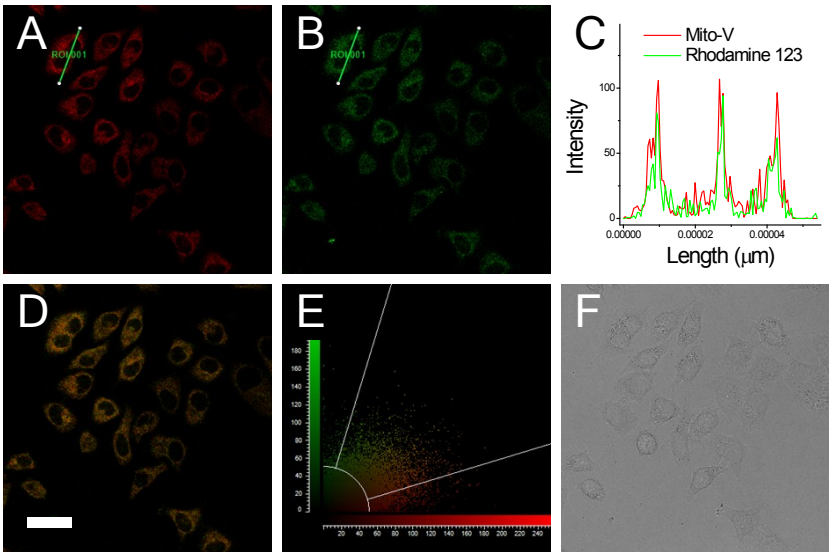

Fig. S8. Colocalization of **Mito-V** and Rhodamine 123 in HepG2 cells. Cells were co-stained with **Mito-V** (5 μM) and Rhodamine 123 (500 nM) at 37 °C for 30 min. (A) Luminescence image from **Mito-V** channel (λ_{ex} = 514 nm, λ_{em} = 550–650 nm). (B) Luminescence image from Rhodamine 123 channel (λ_{ex} = 488, λ_{em} = 500–540 nm). (C) Intensity profile of the linear ROI 1 across the cell (green line in images A and B). (D) Merged image of images A and B. (E) Intensity correlation plot of **Mito-V** and Rhodamine 123. (F) Corresponding DIC image. Scale bar: 40 μm.

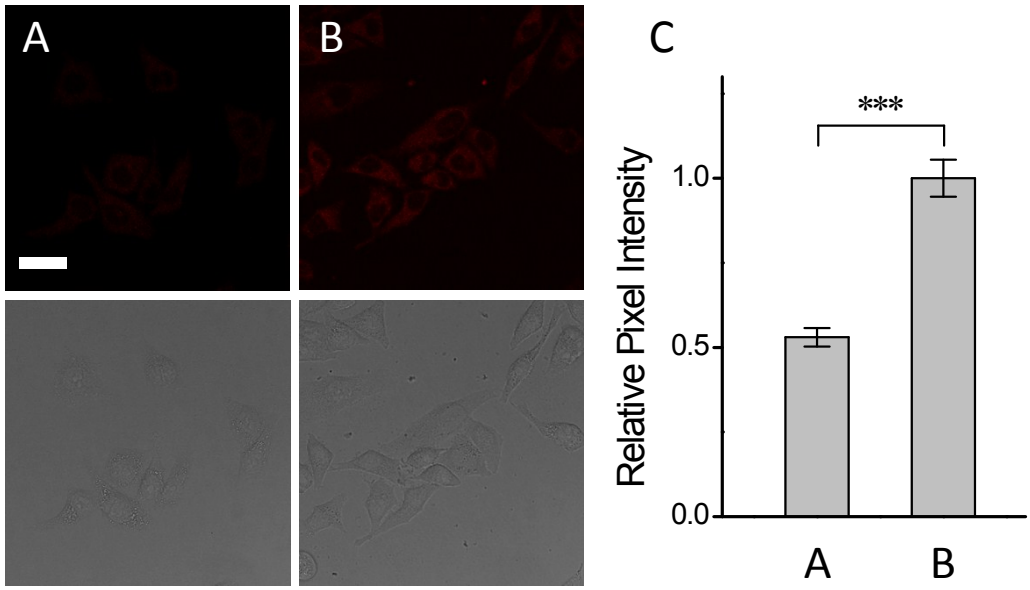

Fig. S9. Confocal microscopy images of HepG2 cells. (A) Luminescence images of HepG2 cells cultured with 11 mM of glucose for 24 h, then stained with **Mito-V** (5 μM) for 30 min. (B) HepG2 cells pretreated with 30 mM glucose for 24 h, and then incubated with **Mito-V** (5 μM) for 30 min. Relative pixel intensity measurements obtained from the images in Figures S7A and S7B by the software LAS AF (the pixel intensity from image B is defined as 1.0). Statistical analyses were performed with a Student's *t*-test ($n > 4$). *$P < 0.001$ and the error bars represent standard deviation (±S.D.).

7. Validation studies

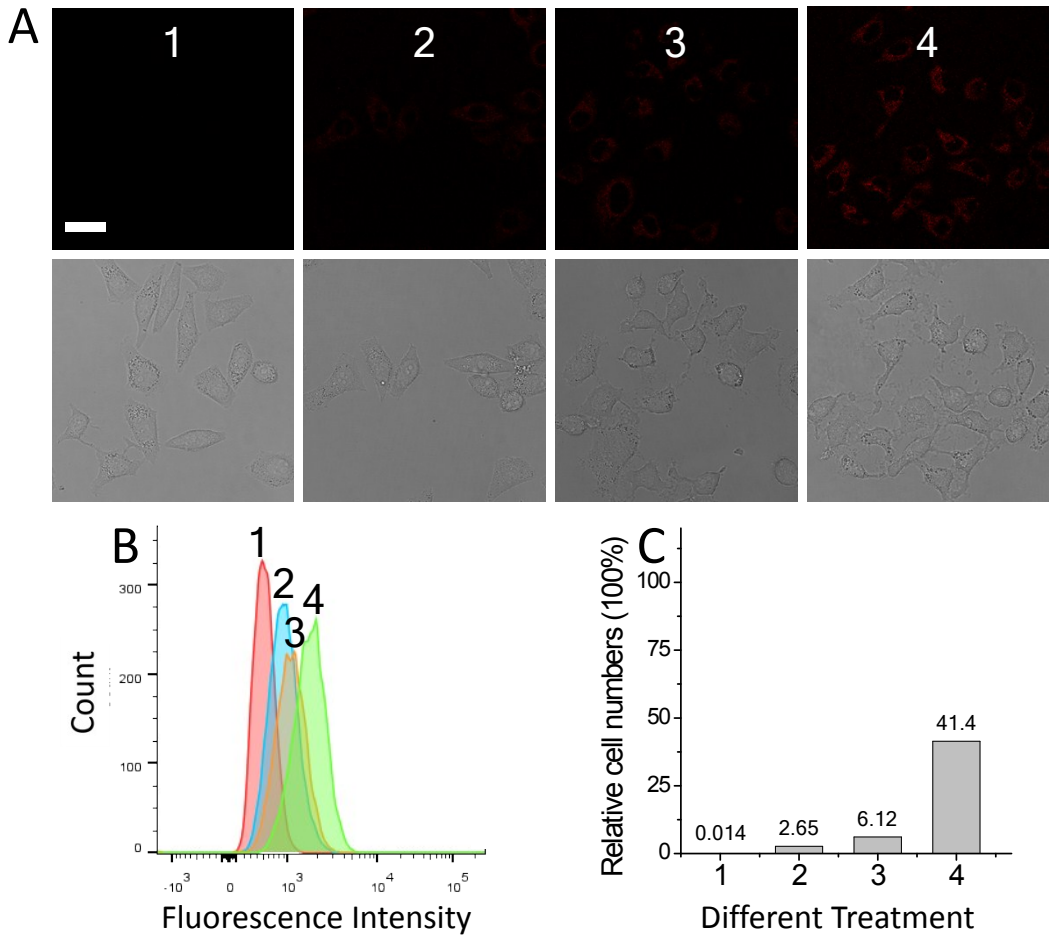

Fig. S10. The study of the effect of STP on intracellular viscosity. (A) Confocal microscopy images HepG2 cells. (1) HepG2 cells only. (2) HepG2 cells incubated with 5 μM **TPE-V** for 30 min. (3) HepG2 cells treated with staurosporine (20 μM) for 1 h, and then incubated with **TPE-V** (5 μM) for 30 min. (4) HepG2 cells treated with staurosporine (40 μM) for 1 h, and then incubated with **TPE-V** (5 μM) for 30 min. (B) Corresponding flow cytometry assay of HepG2 cells. (C) Corresponding relative numbers of stained cells from the FACS results. λ_{ex} = 488 nm, λ_{em} = 570−620 nm.

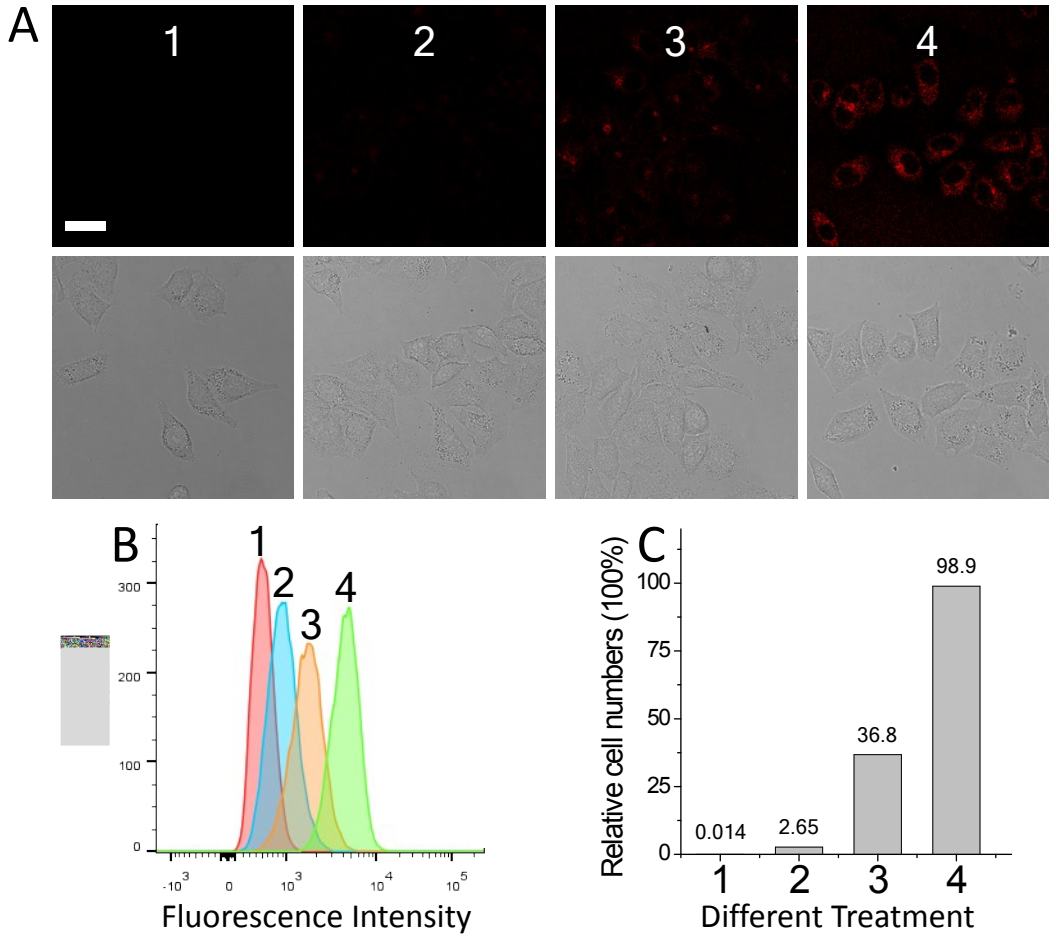

Fig. S11. The study of the effect of LPS on intracellular viscosity. (A) Confocal microscopy images HepG2 cells. (1) HepG2 cells only. (2) HepG2 cells incubated with 5 μM **TPE-V** for 30 min. (3) HepG2 cells treated with LPS (15 μM) for 12 h, and then incubated with **TPE-V** (5 μM) for 30 min. (4) HepG2 cells treated with LPS (30 μM) for 12 h, and then incubated with **TPE-V** (5 μM) for 30 min. (B) Corresponding flow cytometry assay of HepG2 cells. (C) Corresponding relative numbers of stained cells from the FACS results. λ_{ex} = 488 nm, λ_{em} = 570−620 nm.

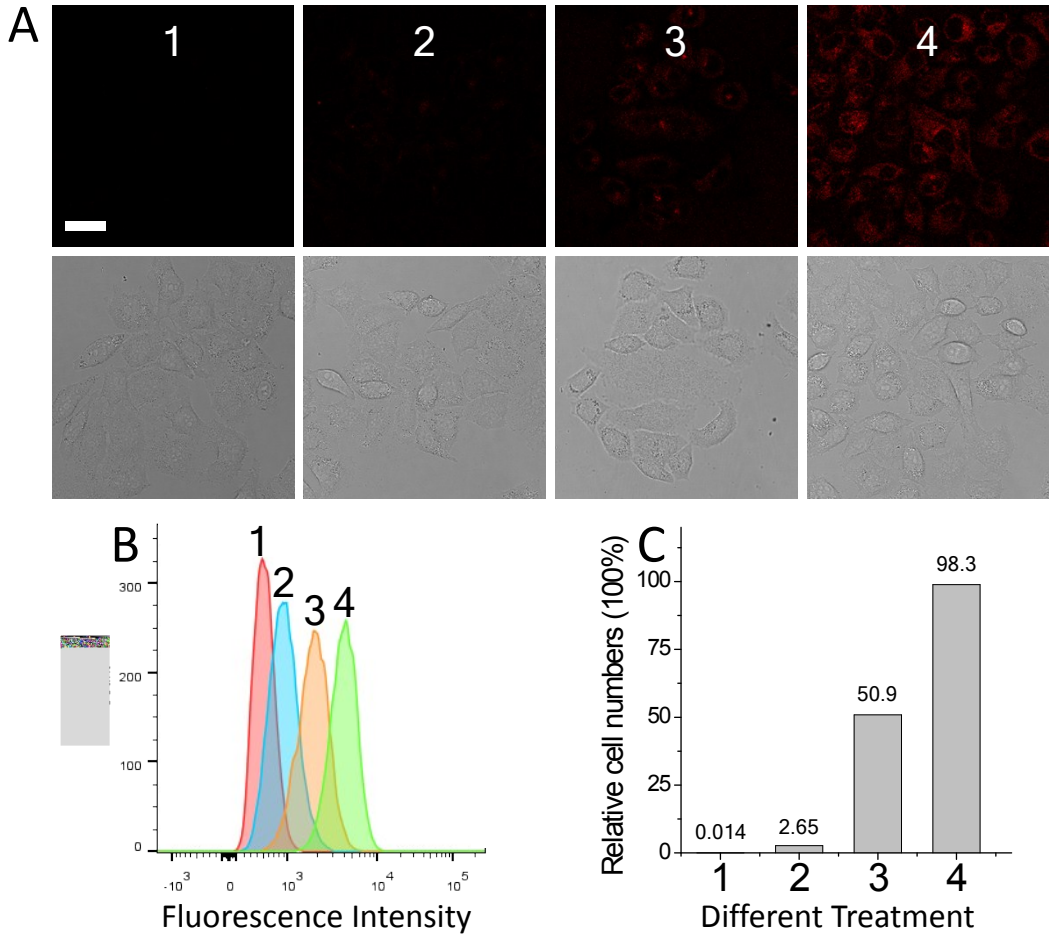

Fig. S12. The study of the effect of nystatin on intracellular viscosity. (A) Confocal microscopy images HepG2 cells. (1) HepG2 cells only. (2) HepG2 cells incubated with 5 μM **TPE-V** for 30 min. (3) HepG2 cells treated with nystatin (20 μM) for 1 h, and then incubated with **TPE-V** (5 μM) for 30 min. (4) HepG2 cells treated with nystatin (35 μM) for 1 h, and then incubated with **TPE-V** (5 μM) for 30 min. (B) Corresponding flow cytometry assay of HepG2 cells. (C) Corresponding relative numbers of stained cells from the FACS results. λ_{ex} = 488 nm, λ_{em} = 570−620 nm.

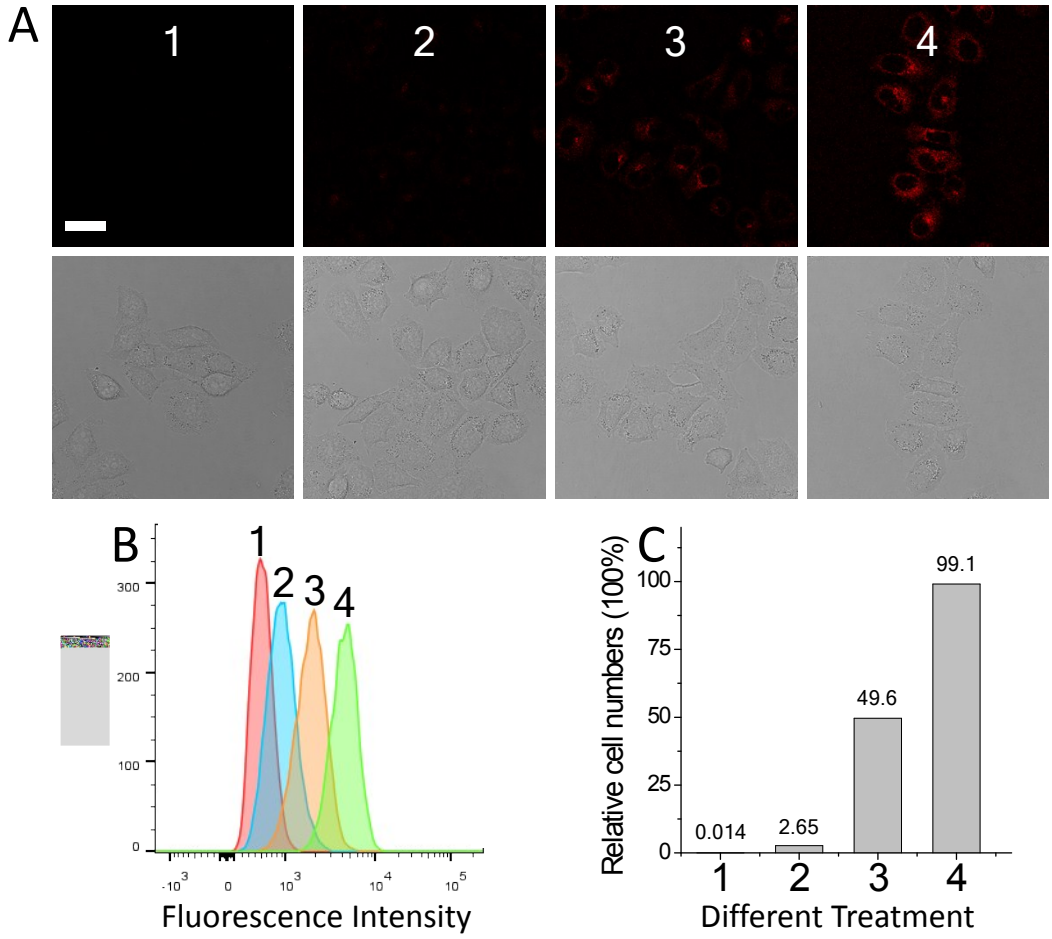

Fig. S13. The study of the effect of glucose on intracellular viscosity. (A) Confocal microscopy images HepG2 cells. (1) HepG2 cells only. (2) HepG2 cells incubated with 5 μM **TPE-V** for 30 min. (3) HepG2 cells treated with glucose (30 mM) for 20 h, and then incubated with **TPE-V** (5 μM) for 30 min. (4) HepG2 cells treated with glucose (50 mM) for 20 h, and then incubated with **TPE-V** (5 μM) for 30 min. (B) Corresponding flow cytometry assay of HepG2 cells. (C) Corresponding relative numbers of stained cells from the FACS results. λ_{ex} = 488 nm, λ_{em} = 570−620 nm.