Supporting Information for:

A Novel High Signal-to-Noise Ratio Fluorescent Probe for Real-Time Mitochondrial Viscosity Detection and Imaging In Vitro and In Vivo.

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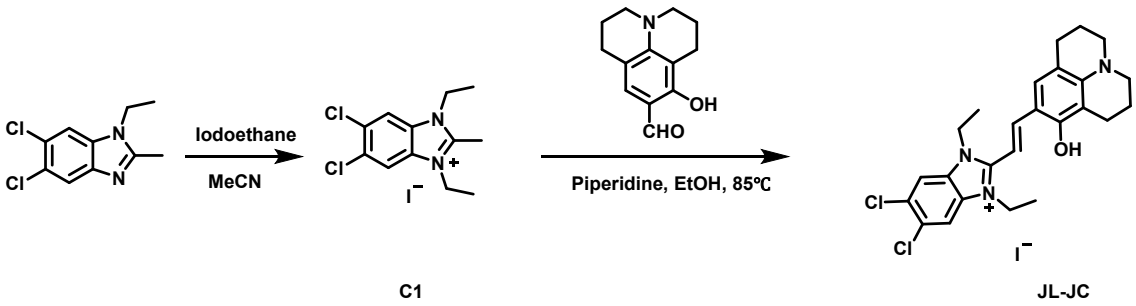
Content

1. Materials and instruments

All the chemicals were purchased and used as received without further purification unless otherwise specified. All the reagents were analytical grade, the solvents used in the spectral analysis experiment were chromatographic grade, ultra-pure water was used in the experiment, and the buffer solution is phosphate buffer saline (PBS). The thin-layer chromatography silica gel plate used for TLC analysis and the column chromatography silica gel powder (200-300 mesh) used for purification of the product were all from Qingdao Ocean Chemical. Commercial probes: MitoTracker Deep Red (MTDR), Lyso-Tracker Deep Red (LTDR), MitoTracker Green (MTG) and double-stranded DNA-specific dye Hoechst 33342 were purchased from Molecular Probe company. Nuclear magnetic resonance spectra (NMR) were obtained on the Bruker Avance 400 spectrometer. HRMS spectra were recorded on Agilent Technologies 6510 Q-TOF LC/MS. The UV-Vis absorption spectra of probe in various solvents were measured on a Hitachi U-2910 spectrophotometer using quartz cuvette with length and width of 1 cm. The fluorescence emission spectra were measured on a Hitachi F-2700 fluorescence spectrophotometer equipped with 450 W Xe lamp. Quantum chemistry calculations were performed using Gaussian 09 software. Ground state geometries were optimized at the B3LYP/6-31G (d, p). Fluorescence imaging of cells was performed on a confocal microscope Leica TCS SP8.

2. Synthesis and Characterization



Scheme S1. Synthesis routines for **JL-JC**.

5,6-dichloro-2-methylbenzimidazole (558 mg, 2 mmol) and iodoethane (2.2 mmol) were dissolved in acetonitrile (10 mL) and stir for 12 hours. The reaction product was rinsed 3 times with petroleum ether to get pure C1. After that, C1 (1 mmol) reacted with 8-hydroxyjulolidine-9-carboxaldehyde (330 mg, 1.5 mmol) in the ethanol, and piperidine (100 μL) was added into this mixture with stirring at 85 ℃ for 12 h. The mixture was cooled and the ethanol was removed. The crude products were purified by column chromatography to give a rufous solid (137 mg, with a yield of 23.5%).

C1: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.56 (s, 2H), 4.51 (s, 4H), 2.93 (s, 3H),

1.38 (s, 6H). HRMS (m/z): $[C1\text{-}I^-]^+$ calculated for $C_{12}H_{15}N_2Cl_2^+$, 257.0607; found, 257.0626.

JL-JC: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.03 (s, 1H), 8.42 (s, 2H), 7.93 (d, J = 16.2 Hz, 1H), 7.44 (s, 1H), 6.92 (d, J = 16.2 Hz, 1H), 4.53 (q, J = 7.1 Hz, 4H), 3.30-3.23 (m, 4H), 2.69 (t, J = 6.3 Hz, 2H), 2.61 (t, J = 6.4 Hz, 2H), 1.91-1.83 (m, 4H), 1.45 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 154.62, 151.04, 147.91, 143.08, 131.50, 128.85, 126.03, 115.26, 114.46, 110.99, 107.33, 96.14, 50.01, 49.28, 41.63, 27.35, 21.79, 21.50, 20.94, 14.54. HRMS (m/z): [**JL-JC**-I^-]$^+$ calculated for $C_{25}H_{28}N_3Cl_2O^+$, 456.1604; found, 456.1610.

3. Preparation of Samples and Test Solutions

JL-JC was dissolved in DMSO to prepare stock solution with a concentration of 1 mM. the concentration of **JL-JC** was 10 μM in photophysical properties experiments. The test solutions were obtained by adding 30 uL stock solution to 3 mL various solvents, such as toluene (Tol), glycerol (Gly), tetrahydrofuran (THF), acetonitrile (MeCN), methanol (MeOH), dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS) and H_2O.

4. Cytotoxicity assay

The H9c2 cells, a cell line derived from the embryonic myocardium of Rattus norvegicus, were cultured in H-DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The adherent H9c2 cells were first treated with trypsin, and the cell concentration was measured with a cell counter. The H9c2 cells were seeded into a 96-well plate (about 10,000 cells/well) and allowed to grow adherently for 24 h. After removing the medium, cells were cultured with 0.1 μM, 0.2 μM, 0.5 μM, 1 μM, 2 μM, 5 μM, 10 μM, and 20 μM probe for 24 h, respectively. It was then treated with 10 μL of MTT (5 mg/mL) and incubated for a further 4 h. After removing the medium again, add DMSO (100) μL to the 96-well plate. Shake the 96-well plate for 60 s to completely dissolve the formazan crystals, and then measure the absorbance at 490 nm with a microplate reader.

5. Cell culture and imaging conditions

H9c2 cells were grown in an incubator at 37 ℃ and 5% carbon dioxide. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Inoculate 1 mL of culture medium containing 1×10^5 cells in each petri dish, and then adhere to culture for 48 h. For fluorescence imaging with confocal microscope, cells were incubated with probes: **JL-JC**: 1 μM, 10 min, λ_{ex} = 488 nm, λ_{em} = 550-650 nm; LTDR and MTDR: 200 nM, 20 min, λ_{ex} = 638 nm, λ_{em} = 650-750 nm; MTG: 200 nM, 20 min, λ_{ex} = 488 nm, λ_{em} = 490-

590 nm, Hoechst 33342: 5 μM, 20 min, λ_{ex} = 405 nm, λ_{em} = 420-500 nm. Every time, the cells were washed to remove the unbound probe before being stained with another probe. After rinsing with PBS twice, cells were imaged immediately.

6. Zebrafish imaging

Zebrafish was obtained from Shanghai FishBio Co.,Ltd, ShangHai, China. The zebrafish was maintained at 28 ℃ and in an E3 medium (15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$, 5-10% methylene blue; pH 7.5). Zebrafish (3 days old) were incubated with the probe (5 μM) for 1 h, fluorescence imaging of zebrafish was performed on Leica TCS SP8 confocal fluorescence microscope, λ_{ex} = 488 nm, λ_{em} = 550-650 nm.

7. NMR and HRMS spectra

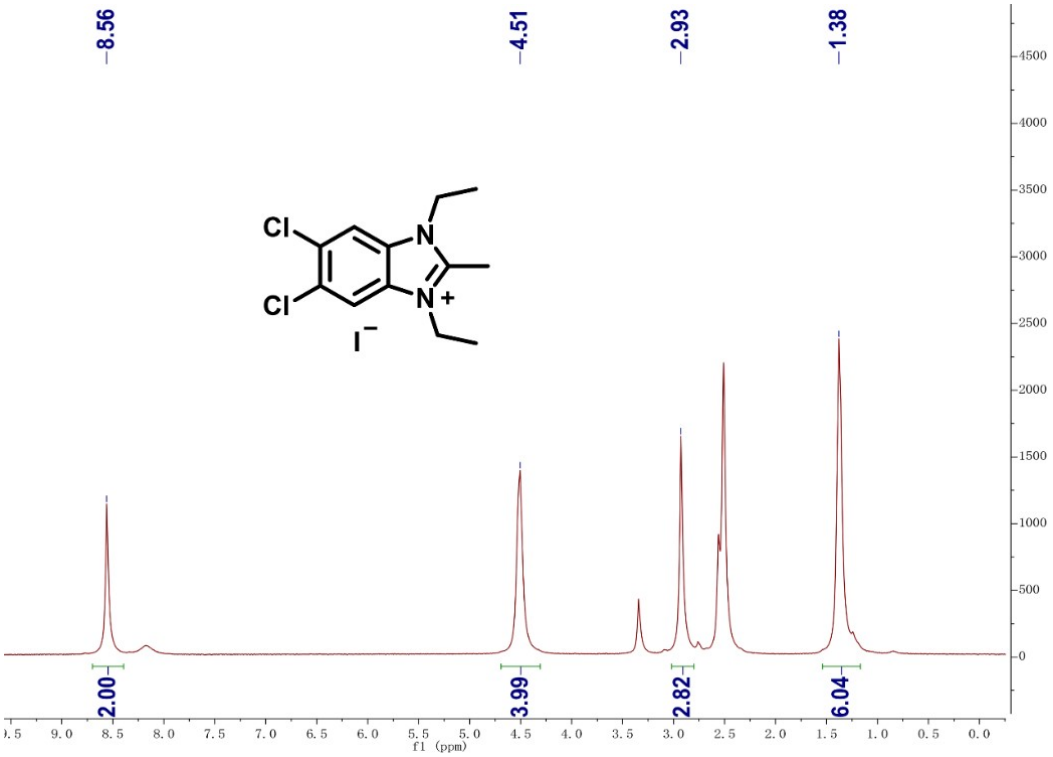


Figure S1. ^{1}H NMR spectrum of C1 in DMSO-d_6.

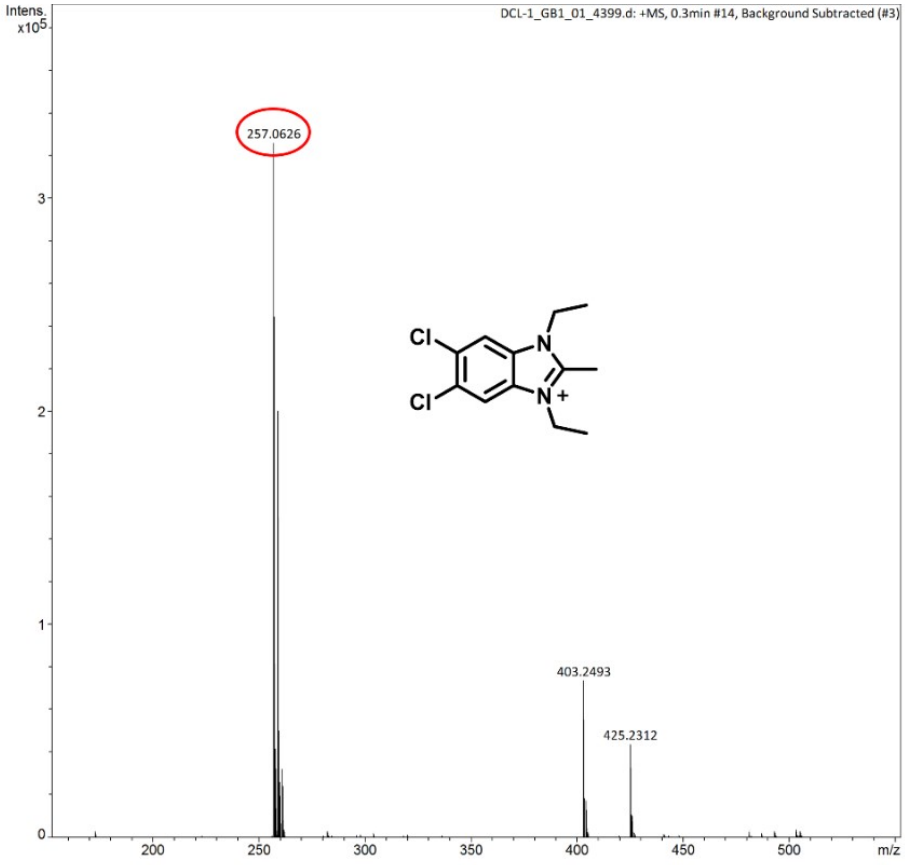


Figure S2. HRMS spectrum of C1 in MeOH.

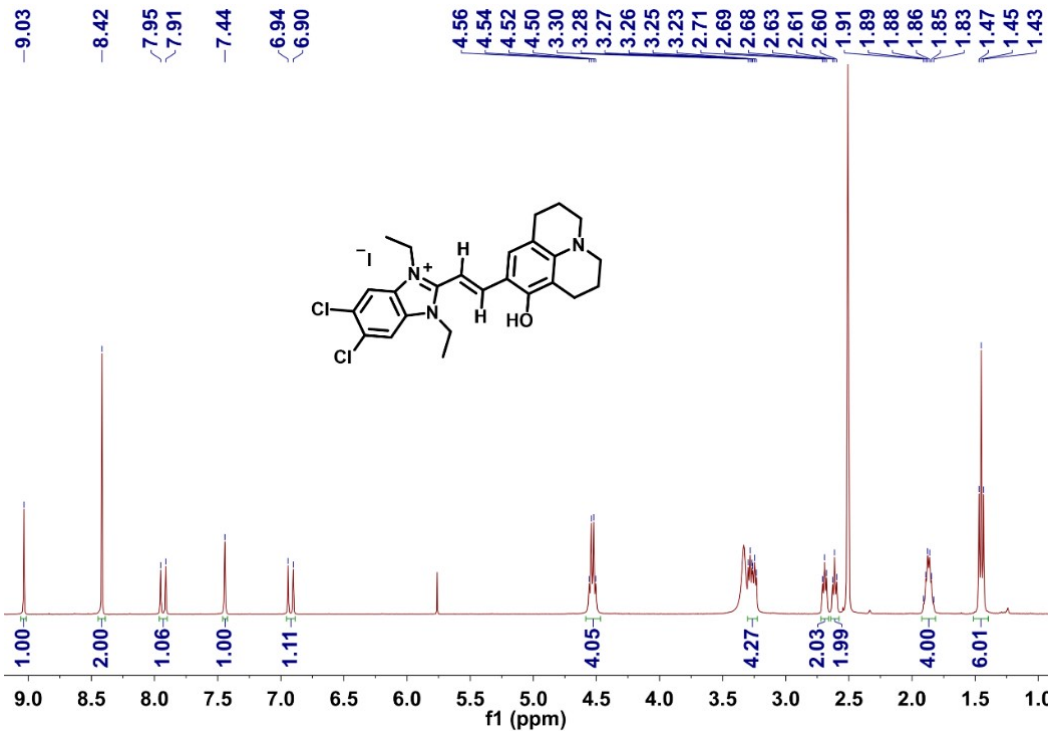


Figure S3. ^{1}H NMR spectrum of **JL-JC** in DMSO-d_6.

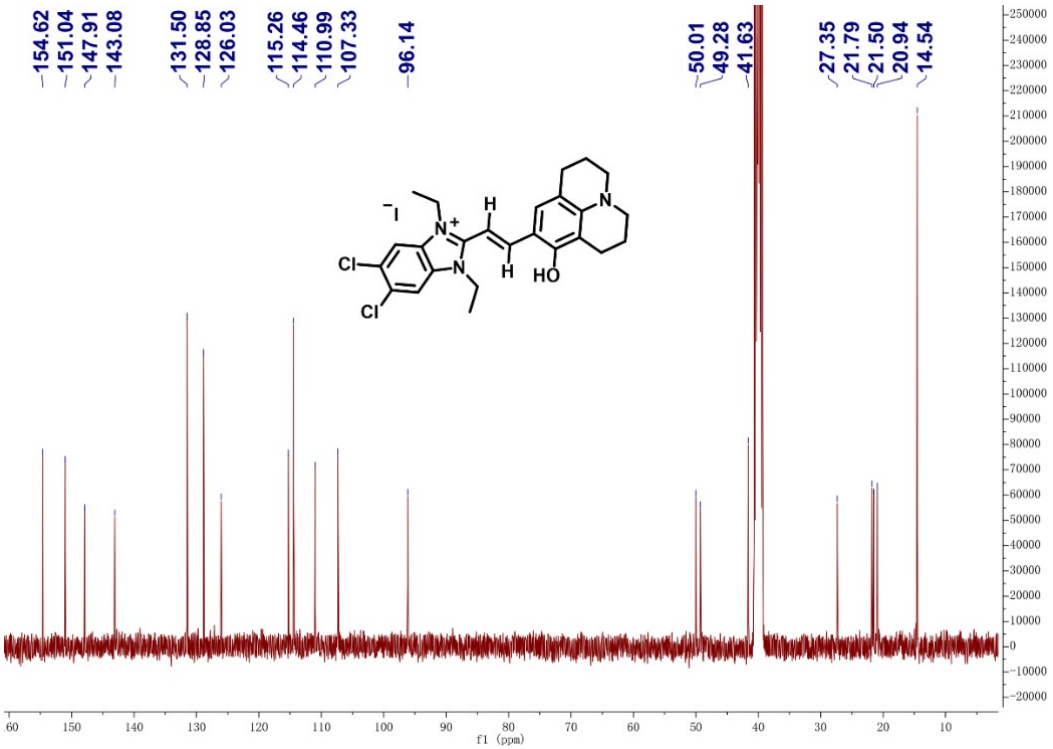


Figure S4. ^{13}C NMR spectrum of **JL-JC** in DMSO-d_6.

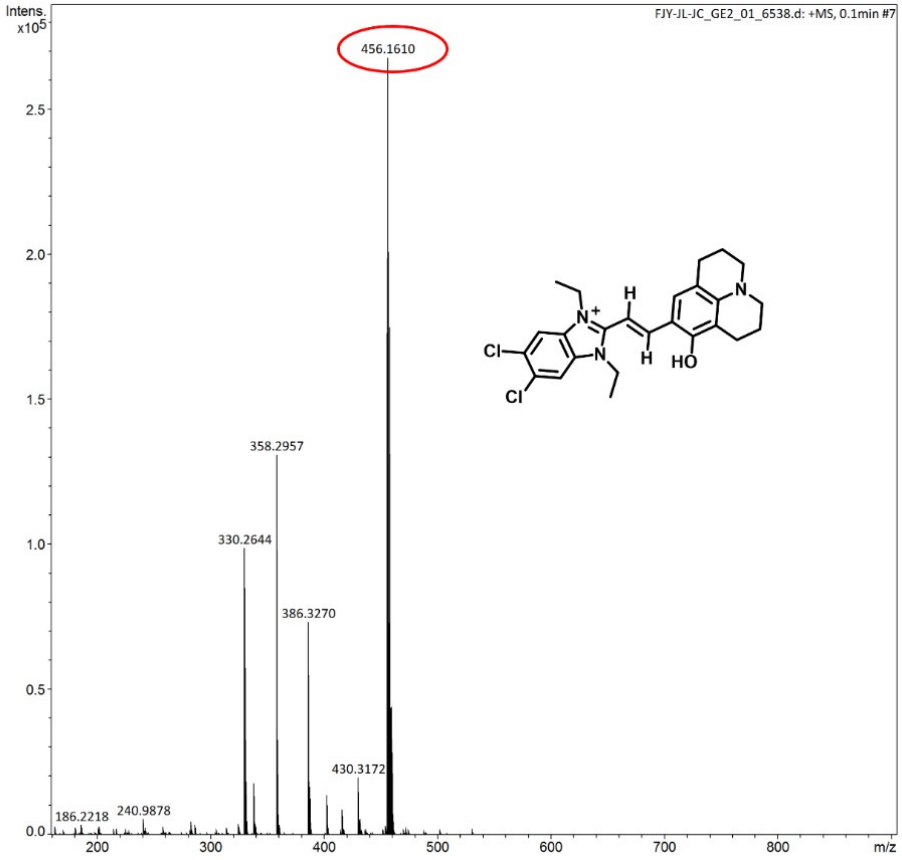


Figure S5. HRMS spectrum of **JL-JC** in MeOH.

8. Supplemental Figures

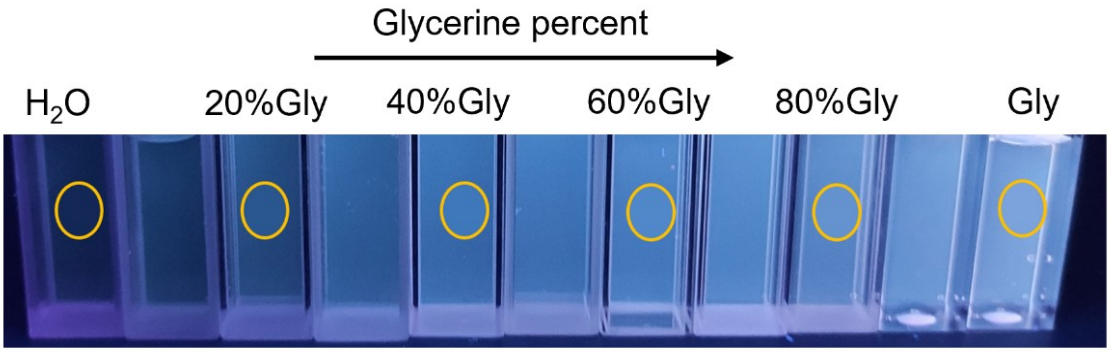


Figure S6. Fluorescence emission photograph of **JL-JC** in different ratios of H_2O/Gly solutions under ultraviolet irradiation, the yellow circle corresponds to Figure 1e.

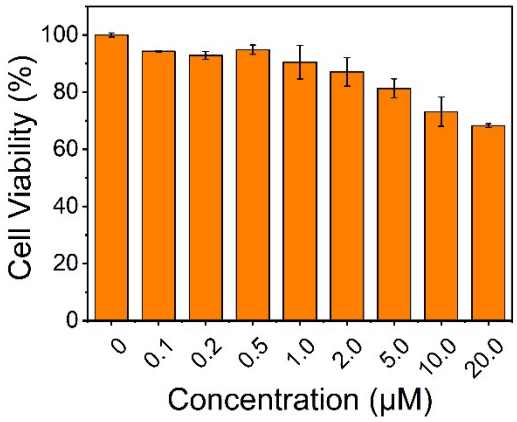


Figure S7. MTT results of H9c2 cells viabilities after incubation with **JL-JC** for 24 h at different incubation concentration.

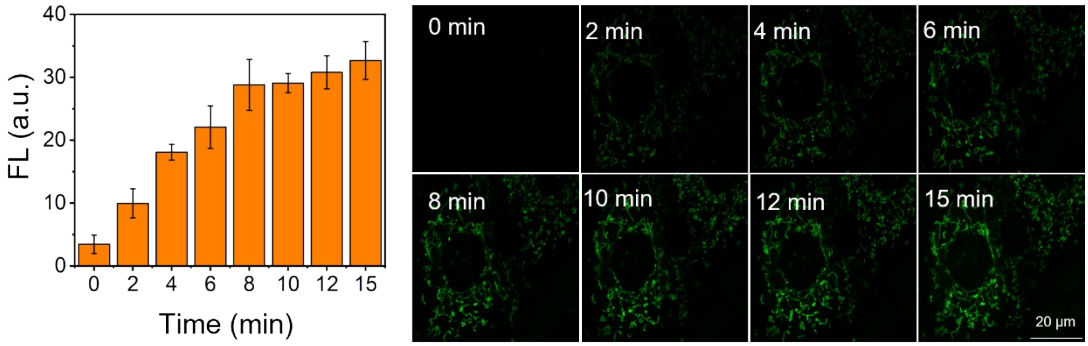


Figure S8. The quantitative and confocal images illustrate the fluorescence intensity of **JL-JC** (1 μM, 10 min) in cells over varying staining durations. Scale bar = 20 μm. λ_{ex} = 488 nm, λ_{em} = 550-650 nm.

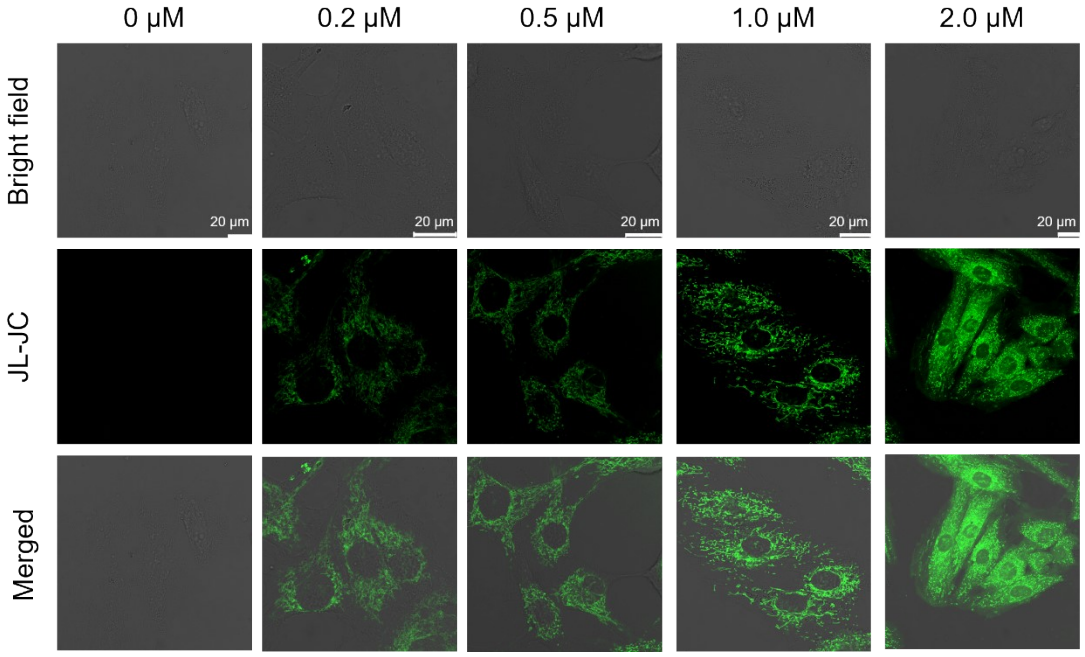


Figure S9. CLSM images of living H9c2 cells stained with **JL-JC** at different concentrations for 10 min. Scale bar = 20 μm. λ_{ex} = 488 nm, λ_{em} = 550-650 nm.

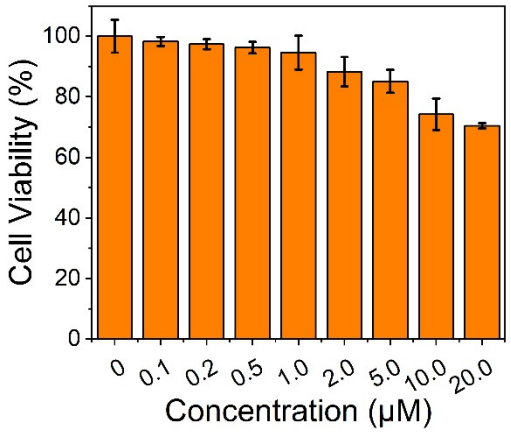


Figure S10. MTT results of HeLa cells viabilities after incubation with **JL-JC** for 24 h at different incubation concentration.

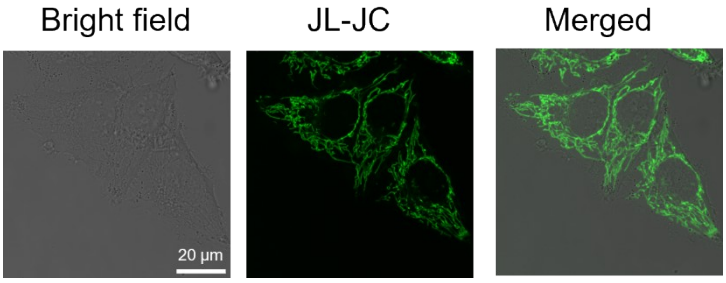


Figure S11. CLSM images of living HeLa cells stained with **JL-JC** at 1 μM for 10 min. Scale bar = 20 μm. λ_{ex} = 488 nm, λ_{em} = 550-650 nm.

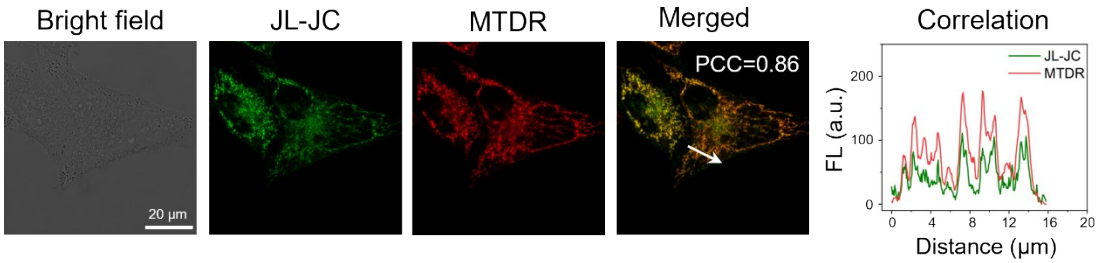


Figure S12. Co-localization of **JL-JC** (1 μM, 10 min, λ_{ex} = 488 nm, λ_{em} = 550-650 nm) with commercial mitochondria probe MTDR (200 nM, 20 min, λ_{ex} = 638 nm, λ_{em} = 650-750 nm) of living HeLa cells, accompanied by the fluorescence intensity chart of the region marked by a white arrow in the merged image, scale bar = 20 μm. PCC: Pearson correlation coefficient.

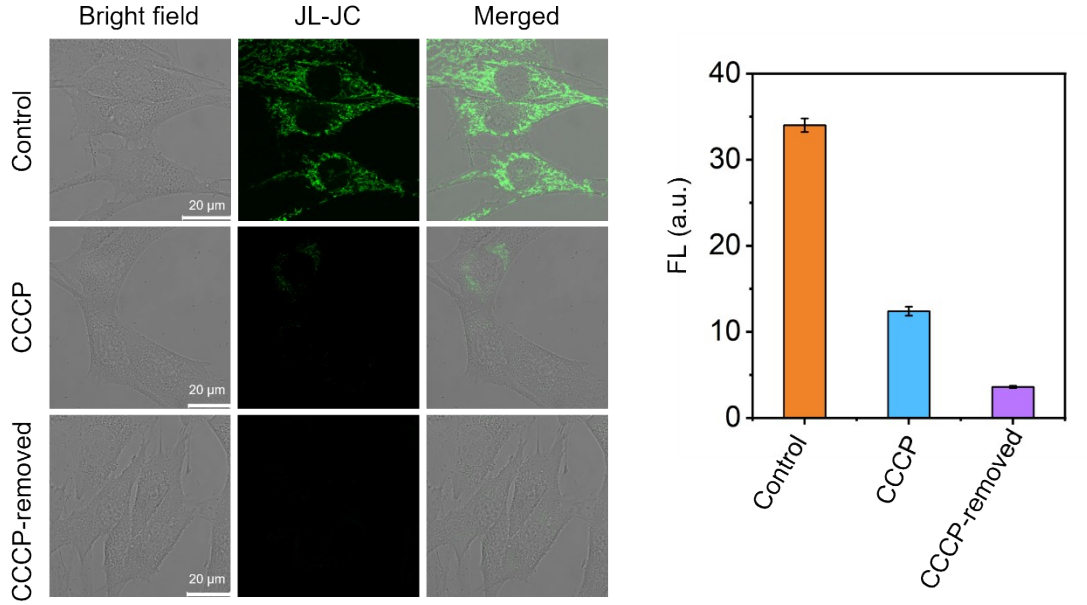


Figure S13. CLSM images of H9c2 cells stained with 1 μM **JL-JC** for 10 min, then treated with 20 μM CCCP for 20 min and removed CCCP. Change of fluorescence signal intensity during CCCP treatment. Scale bar = 20 μm. λ_{ex} = 488 nm, λ_{em} = 550-650 nm.

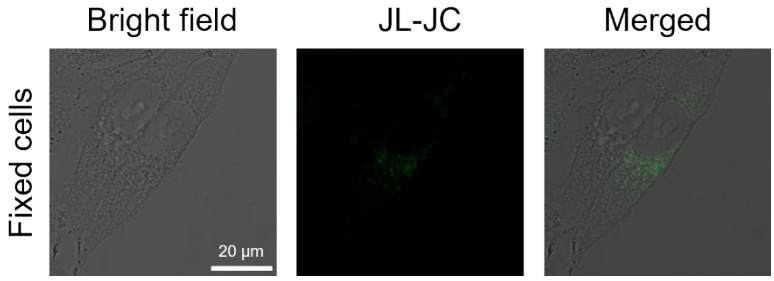


Figure S14. CLSM images of H9c2 cells stained with 1 μM JL-JC for 10 min, then fixed with 4% Paraformaldehyde for 15 min. Scale bar = 20 μm. λ_{ex} = 488 nm, λ_{em} = 550-650 nm.

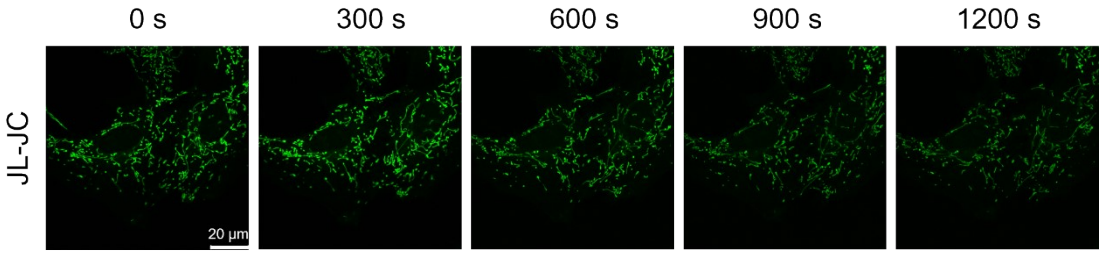


Figure S15. Photostability evaluation of **JL-JC** during confocal fluorescence microscopy imaging. Living H9c2 cells were stained with **JL-JC** (1 μM, 10 min), and imaged under laser irradiation. λ_{ex} = 488 nm, λ_{em} = 550-650 nm. Scale bar = 20 μm.

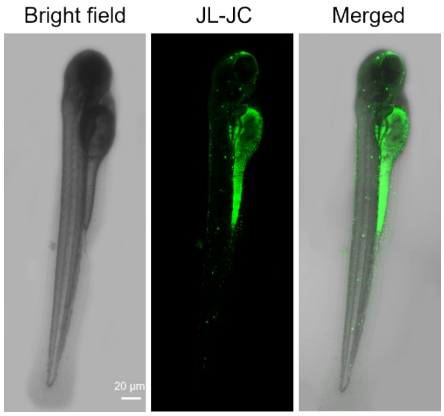


Figure S16. CLSM images of 4-day-old zebrafish that 3-day-old zebrafish were incubated with 5 μM **JL-JC** for 24 h. λ_{ex} = 488 nm, λ_{em} = 550-650 nm, scale bar = 20 μm.

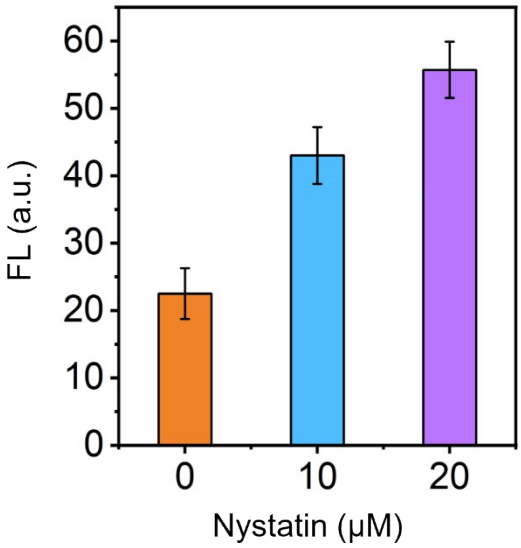


Figure S17. Mean fluorescence intensity of Figure 5.