Electronic Supplementary Information for:

A mitochondria-targeted far-red AIE fluorescent probe for distinguishing between mitophagy and ferroptosis in cancer cell

Liyuan Sun,‡[a] Mengfei Zou,‡[a,b] Longjie Du,‡[a] Shugang Wang[c], Ru Ding, Keliang Lu,*[a] Jianchun Li[a] and Jin Zhou*[a]

a. School of Pharmacy, School of Anesthesiology, School of Nursing, Weifang Medical University, Weifang, 261053, China.

b. Weifang People's Hospital, Weifang, 261041, China

c. Department of Rheumatology, Weifang Hospital of Traditional Chinese Medicine, Weifang, 261000, China

‡ *These authors contributed equally to this work.*

Corresponding Author

**E-mail: zhoujin@wfmc.edu.cn, lkelwf@126.com*

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1. Experimental

Materials and Reagents. 4-(Dimethylamino) benzaldehyde, 3-hydroxy -3-methyl -2-butanone and malononltrile were purchased from Energy Chemical. Glycerol was purchased from Yantai Chemical Industry Research Institute (China). Dimethyl sulfoxide (DMSO) was purchased from J&K Scientific Ltd (Beijing, China). Roswell Park Memorial Institute (RPMI) 1640 Medium and phosphate buffered saline (PBS) were bought from Solarbio Company. Fetal bovine serum was purchased from Zhejiang Tianhang biotechnology Co. Sucrose (#V900116, purity 99%) were purchased from Sigma-Aldrich Co. Ltd. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Serva Electrophoresis GmbH (Germany). CCCP (Carbonyl cyanide 3-chlorophenylhydrazone), Erastin and Streptozotocin (STZ) were purchased from Sigma-Aldrich Co. Ltd. All other available chemicals and reagents used were of analytical grade and provided by local suppliers. The stock solution preparation of probe **LY-1** (1 mM) was made by dissolving an appropriate amount of **LY-1** in DMSO. Ultrapure water used in experiment purified in a Milli-Q reference system (Millipore).

Characterization Methods. ^{1}H NMR and ^{13}C NMR spectra were measured on Bruker Avance III 400 MHz spectrometer. High resolution electrospray ionization mass spectra (HR-ESI-MS) were measured on APEX IV FTMS instrument (Bruker, Daltonics). Fluorescence spectra were measured on Hitachi F-7000 spectrofluorimeter (Tokyo, Japan). Ultraviolet absorption spectra were measured on T10CS spectrophotometer (Beijing Puxi, China). The fluorescence images of cells were obtained by Leica TCS SP8 confocal laser scanning microscope (Germany) with a 63×oil objective lens 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). The flow cytometry analysis measured on BD Biosciences FACS AriaIII instrument.

Synthesis of Probe. A synthetic route for **LY-1** was depicted in Scheme 1.

TCF: the mixture of 3-hydroxy-3-methyl-2-butanone (30 mg, 0.2937 mmol) and malononltrile (38 mg, 0.5874 mmol) in ethyl alcohol was refluxed for 2.5 h at 80 ℃. After the reaction was cooled down to the room temperature, organic layer was evaporated under vacuum. Then the crude product was separated by silica gel column chromatography using CH_2Cl_2/MeOH (v/v, 100:1), affording **TCF** (23 mg, yield: 40%).

LY-1: the mixture of **TCF** (199 mg, 1 mmol), 4-(dimethylamino) benzaldehyde (273 mg, 1 mmol) and in acetic anhydride was stirred for 3 h at 90 ℃. After completion of the reaction, the mixture was poured into water (30 mL) and extracted with dichloromethane (30 mL × 4). Then the organic phase was separated, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was separated by column chromatography using CH_2Cl_2/ MeOH (v/v, 25:1) to obtain probe as green solid (204 mg, yield: 45%). The ^{1}H NMR and ^{13}C NMR spectra of probe for characterization are shown in Figures S1 and S2, respectively. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 7.58 (d, J = 16.1 Hz, 1H), 7.47 (d, J = 8.6 Hz, 2H), 7.36 (t, J = 7.6 Hz, 4H), 7.25 – 7.15 (m, 6H), 6.98 (d, J = 8.5 Hz, 2H), 6.82 (d, J = 16.1 Hz, 1H), 1.76 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$, 298 K): δ 175.9, 174.0, 152.5, 147.3, 145.7, 131.1, 129.9, 126.4, 125.9, 125.7, 119.9, 112.3, 112.2, 111.4, 111.2, 110.9, 97.1, 96.8, 56.2, 29.7, 26.7. HR-ESI-MS (m/z) calcd for $C_{30}H_{23}N_4O^+$ $[M+H]^+$: 455.1866, found 455.1851 (Figure S3).

General Procedure for Spectroscopic Detection of Viscosity. The solvents with different viscosity were obtained by mixing water-glycerol systems in different proportions. The stock solution of **LY-1** was 1 mM dissolved in DMSO. The test solution with final concentration 10 μM for probe containing stock solution (10 μL) and water-glycerol systems (990 μL). Then the mixture was shaken and mixed well for 10 min, and then transferred to the quartz cell of 10 mm optical length to measure fluorescence spectra. The excitation wavelength of **LY-1** was set at 565 nm, while the emission wavelength of **LY-1** was set ranging from 575 nm to 800 nm with a 1 cm standard quartz cell.

Cytotoxicity Assay. In order to estimate the cytotoxicity of **LY-1** on HeLa cells, we performed a routine MTT test. These cells were inoculated into 96 well plates with 8×10^3 cells per well and cultured at 37 ℃ and 5% CO_2 for 24 hours. After the cells completely adhered to the wall, probe solutions (diluted with 1640 medium) of 100 μL with different concentrations were added into 96 well plates, which were continued to be cultured for 24 hours at 37 ℃ and 5% CO_2. Then 100 μL MTT solutions (diluted to 0.5 mg/mL with 1640 medium) were added into each well. After 4 hours, we removed the medium, added 100 μL DMSO solution to each well and shook for 10 minutes. Then the absorbance determined at 490 nm was operated on a reader. The relative survival rate (VR) of cells was calculated according to the formula: $VR = A/A_0 \times 100\%$, where A is the absorbance of the experimental group, and A_0 is the control group. Cell viability from the control group was considered to be 100%.

Colocalization Experiments.

For colocalization analysis, HeLa cells in logarithmic growth phase were seeded in cell culture dishes (1×10^5 cells/dish). After one day adherence, the cells were washed with PBS

twice and incubated in the fresh medium containing **LY-1** (10 μM) at 37 °C for 30 minutes prior to treatment with various commercial sub-organelle co-localization dyes.

(1) Rhodamine 123 co-staining: After washing with PBS, 1 mL fresh 1640 complete culture medium containing Rhodamine 123 (1 μM) cells were used to replace the solution in the culture dish. Then the dish was cultivated in a cell incubator at 37 ℃ for 30 min, subsequently washed with fresh medium twice before imaging. Fluorescent signal of Rhodamine 123 was excited by 496 nm laser and the fluorescence was collected in the range 510-560 nm, and **LY-1** was excited by 561 nm laser and the fluorescence was collected in the range 570-700 nm.

(2) Hoechst 33342 co-staining: After washing with PBS, 1 mL fresh 1640 complete culture medium containing Hoechst 33342 (50 nM) cells were used to replace the solution in the culture dish. Then the dish was cultivated in a cell incubator at 37 ℃ for 30 min, subsequently washed with fresh medium twice before imaging. Fluorescent signal of Hoechst 33342 was excited by 405 nm laser and the fluorescence was collected in the range 410-550 nm, and **LY-1** was excited by 561 nm laser and the fluorescence was collected in the range 570-700 nm.

(3) ER-Tracker Green co-staining: After washing with PBS, 1 mL fresh 1640 complete culture medium containing ER-Tracker Green (10 nM) cells were used to replace the solution in the culture dish. Then the dish was cultivated in a cell incubator at 37 ℃ for 30 min, subsequently washed with fresh medium twice before imaging. Fluorescent signal of ER-Tracker Green was excited by 496 nm laser and the fluorescence was collected in the range 510-560 nm, and **LY-1** was excited by 561 nm laser and the fluorescence was collected in the range 570-700 nm.

(4) DND-189 co-staining: After washing with PBS, 1 mL fresh 1640 complete culture medium containing DND-189 (50 nM) cells were used to replace the solution in the culture dish. Then

the dish was cultivated in a cell incubator at 37 ℃ for 30 min, subsequently washed with fresh medium twice before imaging. Fluorescent signal of DND-189 was excited by 442 nm laser and the fluorescence was collected in the range 450-560 nm, and **LY-1** was excited by 561 nm laser and the fluorescence was collected in the range 570-700 nm.

Flow Cytometry Analysis. The cells were cultured at 2.0×10^5 cell in 6-well plates and allowed to adhere for 48 h before various pathophysiological processing. 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 around565 nm.

Osmotic Pressure Shock Experiments. HeLa cells were first stained with **LY-1** (10 μM) by the method mentioned above. Different volumes of hyperosmotic sucrose solution (0.5 M) or deionized (DI) water were added into 1 mL of cultured cells to give a hyperosmotic osmotic shock and hypo-osmotic shock respectively. Then, the cells were washed twice with PBS and fluorescence imaging was recorded by confocal luminescence microscope after 10 min of the addition of sucrose solution or DI water. In detail, hypotonic solution includes 0.7 mL of 1640 complete medium and 0.30 mL of deionized water per milliliter of final medium, isotonic solution is regular 1640 complete medium, and hyperosmotic solution includes 0.40 mL of 1640 complete medium and 0.60 mL of 0.50 mol/L sucrose solution per milliliter of final medium, respectively.

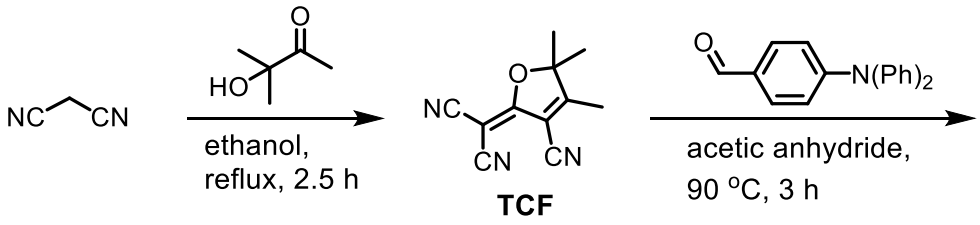
Serum Nutrient Deprivation for Starvation. To prepare a pathological model of starvation monitoring the autophagy and apoptosis process via the mitochondrial viscosity changes, HeLa

cells were incubated in cell culture medium without fetal bovine serum (FBS) for 0-3 days to get 4 different groups before staining with **LY-1** (10 μM).

Drug-induced Mitophagy or Ferroptosis. The confocal imaging was carried out using 20 mm confocal dishes with HeLa cells. The cells were first treated in 1640 complete culture medium containing CCCP (10 μM) or Erastin (10 μM) for 0, 1 h and 3 h at 37 ℃. Then **LY-1** (10 μM) was added and incubated for 30 min. After washing, the samples were subjected to laser confocal imaging. Error bars represent SD (standard deviation, n = 6). The significance was determined by using the T test. λ_{ex} =561 nm, λ_{em} =570-700nm.

The Drawing of Normalised Fluorescence Histogram. The mean fluorescence intensity (MFI) of HeLa cells in the images was obtained by software Image J. Then the MFI of each group was plotted as a histogram, and related data from the same experiment was analyzed using IBM SPSS Statistics 26 to determine whether there was a significant difference between the values of the same group to draw conclusions from the experiment.

2. Synthesis of LY-1



Scheme S1. The synthesis of probe LY-1

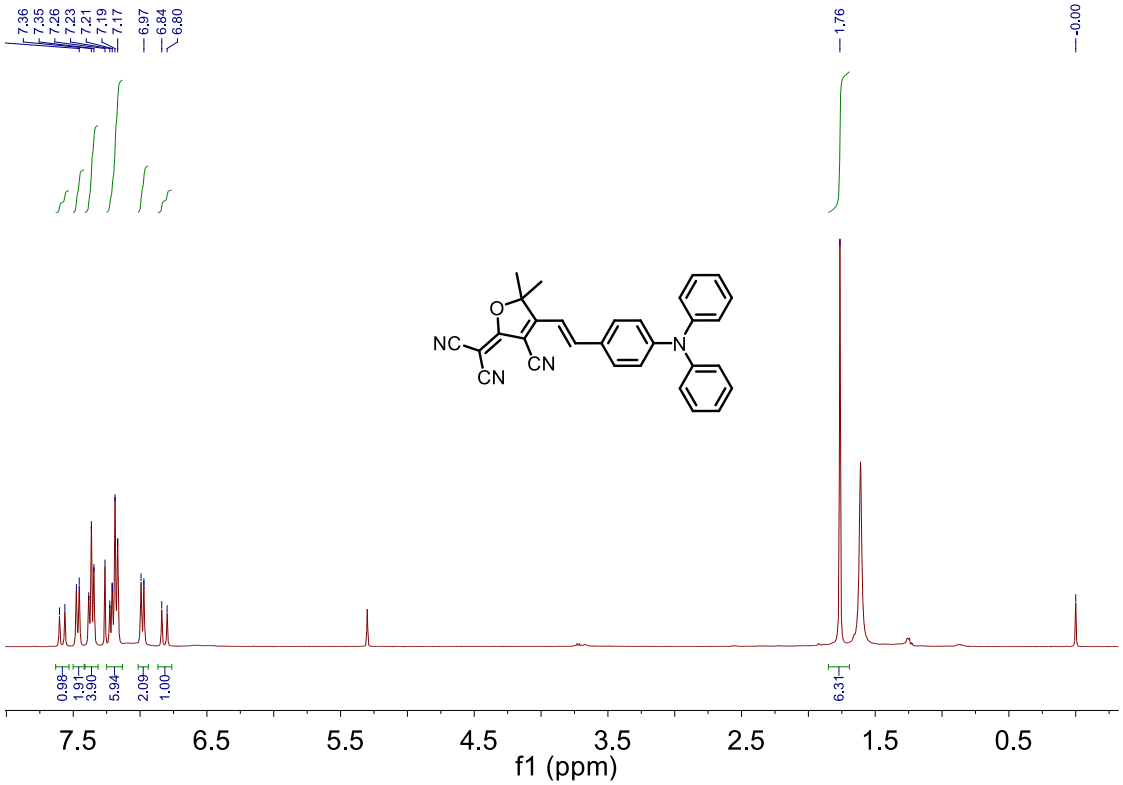


Fig. S1. ^{1}H NMR spectrum of **LY-1** (400 MHz, $CDCl_3$, 298 K).

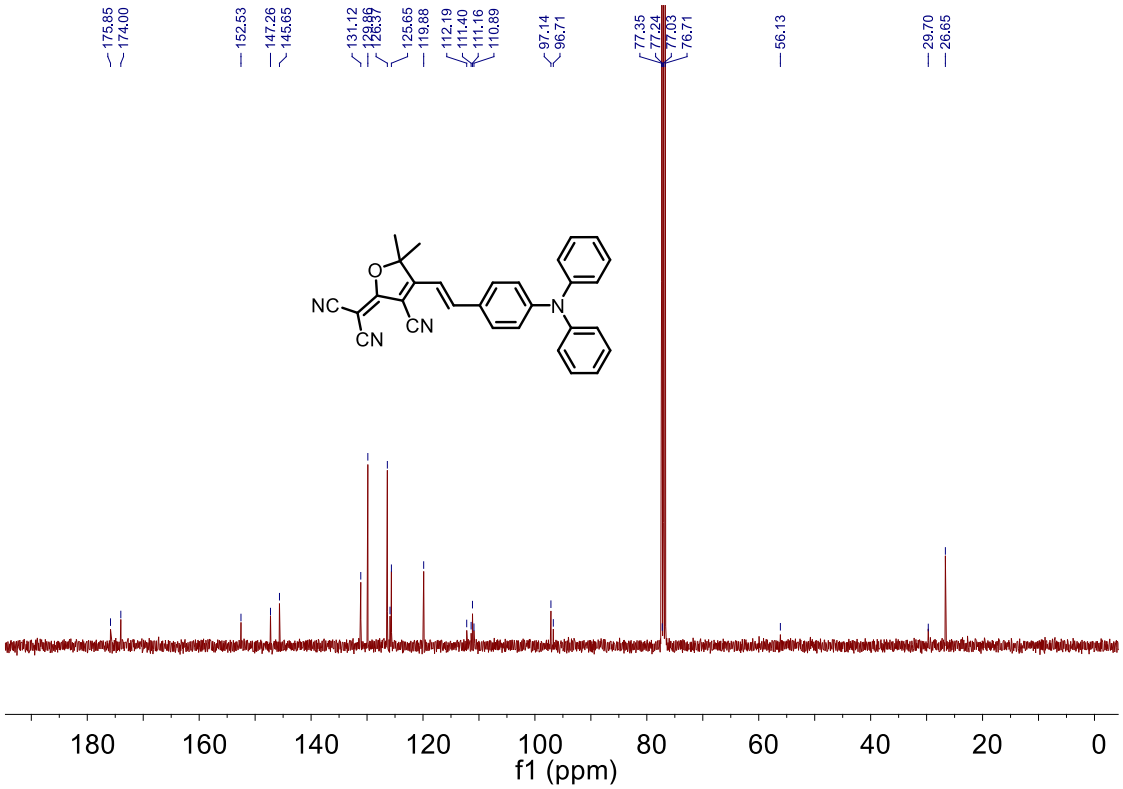


Fig. S2. ^{13}C NMR spectrum of **LY-1** (100 MHz, $CDCl_3$, 298 K).

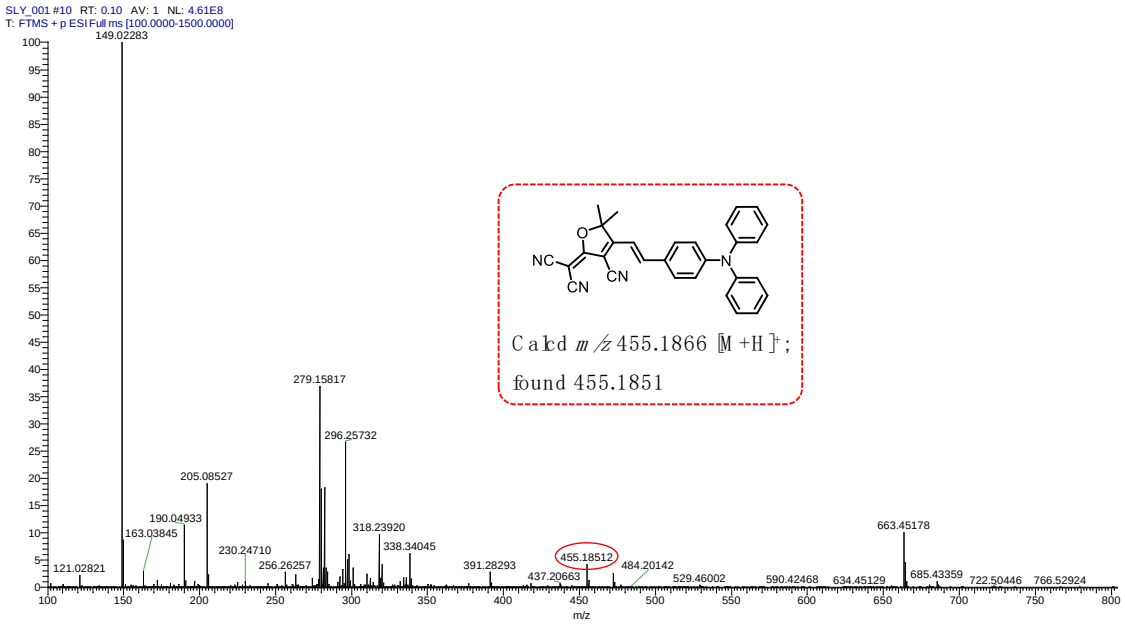


Fig. S3. HRMS result of compound **LY-1**

3. Optimization of Experimental Conditions

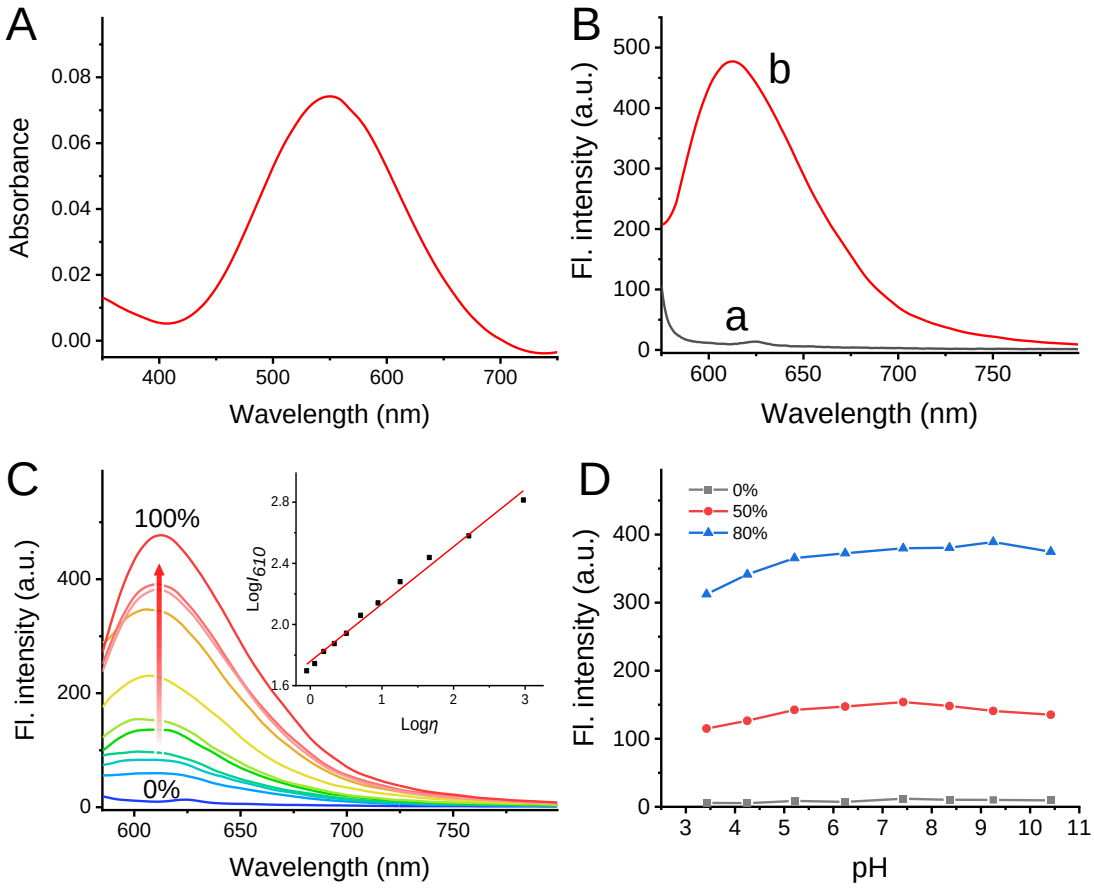


Fig. S4. (A) UV-vis absorption spectra of 10 μM **LY-1** in PBS (phosphate buffered saline). (B) Fluorescence spectra of 10 μM **LY-1** in water (a) and glycerol (b). (C) Fluorescence spectra of **LY-1** (10 μM) with the variation of solution viscosity (the glycerol fractions of water-glycerol system are 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% from the bottom to top). (Inset of C) Linear relationship between log I_{610} and log η. (D) The fluorescence response of **LY-1** in solutions with different pH towards various viscosity. $\lambda_{ex/em}$ = 565/610 nm.

4. The Selectivity of the LY-1

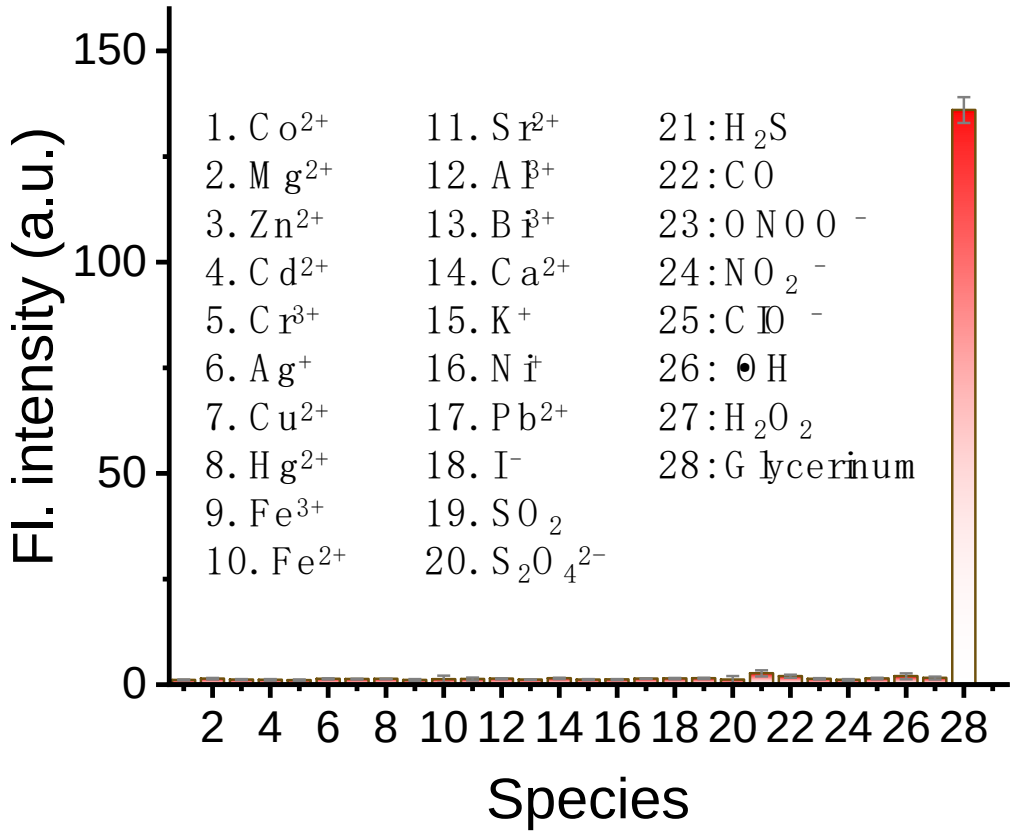


Fig. S5. Fluorescence response of 10 μM **LY-1** in PBS buffer (10 mM, pH 7.4) towards different species, such as: inorganic ions (1: Co^{2+} (100 μM); 2: Mg^{2+} (100 μM); 3: Zn^{2+} (100 μM); 4: Cd^{2+} (100 μM); 5: Cr^{3+} (100 μM); 6: Ag^{+} (100 μM); 7: Cu^{2+} (100 μM); 8: Hg^{2+} (100 μM); 9: Fe^{3+} (100 μM); 10: Fe^{2+} (100 μM); 11: Sr^{2+} (100 μM); 12: Al^{3+} (100 μM); 13: Bi^{3+} (100 μM); 14: Ca^{2+} (100 μM); 15: K^{+} (100 μM); 16: Ni^{+} (100 μM); 17: Pb^{2+} (100 μM); 18: I^{-} (100 μM); 19: SO_2 (100 μM); 20: $S_2O_4^{2-}$ (100 μM); 21: H_2S (10 μM); 22: CO (10 μM); 23: $ONOO^{-}$ (10 μM); 24: NO_2^{-} (10 μM); 25: ClO^{-} (10 μM); 26: ·OH (10 μM); 27: H_2O_2 (10 μM); 28: Glycerinum (containing 50% Water). $\lambda_{ex/em}$ = 565/610 nm. Each data is the mean value of 3 sets of dates which was reported as the mean ± standard deviation of triplicate experiments.

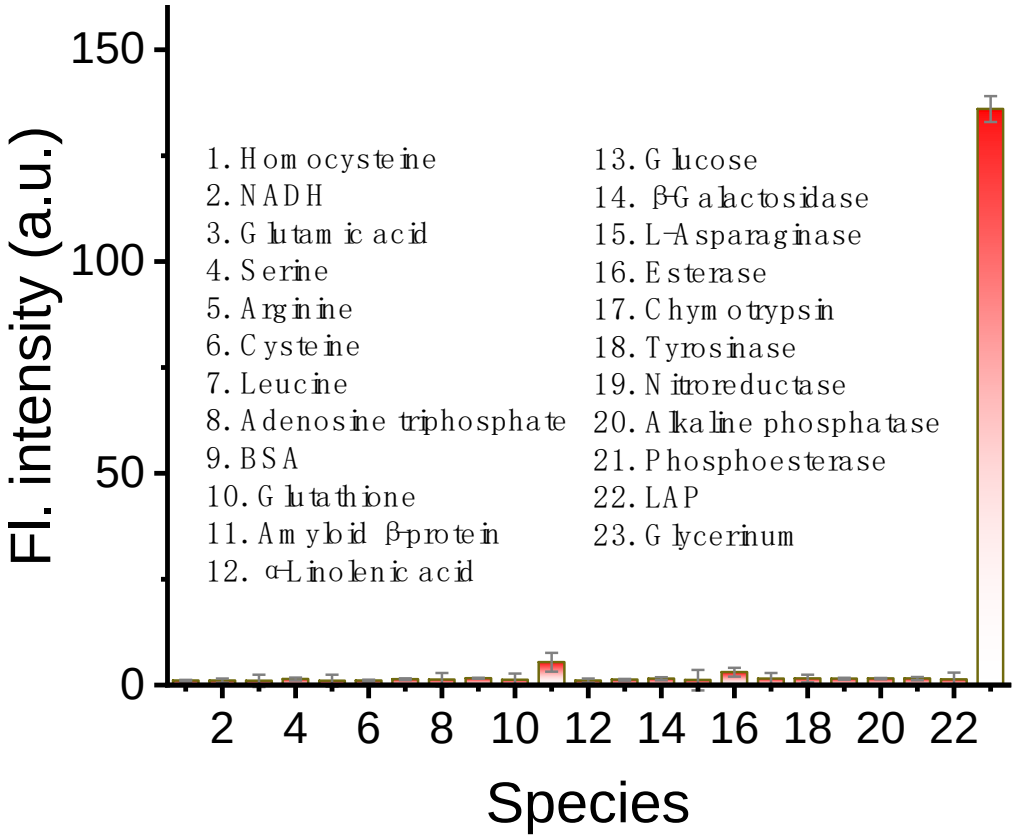


Fig. S6. Fluorescence response of 10 μM **LY-1** in PBS (10 mM, pH 7.4) towards different amino acids and enzymes. 1: Homocysteine (20 μM); 2: NADH (nicotinamide adenine dinucleotide) (40 μM); 3: Glutamic acid (10 μM); 4: Serine (100 μM); 5: Arginine (100 μM); 6: Cysteine (10 μM); 7: Leucine (50 μM); 8: Adenosine triphosphate (100 μM); 9: BSA (bovine serum albumin) (10 μg/mL); 10: Glutathione (100 μM); 11: Amyloid β-protein (10 μM); 12: α-Linolenic acid (10 μM); 13: Glucose (100 μM); 14: β-Galactosidase (4 U/mL); 15: L-Asparaginase (10 μM); 16: Esterase (0.05 U/mL); 17: Chymotrypsin (100 μg/mL); 18: Tyrosinase (5 U/mL); 19: Nitroreductase (5 μg/mL); 20: Alkaline phosphatase (10 U/mL); 21: Phosphoesterase (10 μM); 22: Leucine Aminopeptidase (0.9 μg/mL); 23: Glycerinum (containing 50% water)). $\lambda_{ex/em}$ = 565/610 nm. Each data is the mean value of 3 sets of dates which was reported as the mean ± standard deviation of triplicate experiments.

5. Cytotoxicity Assay

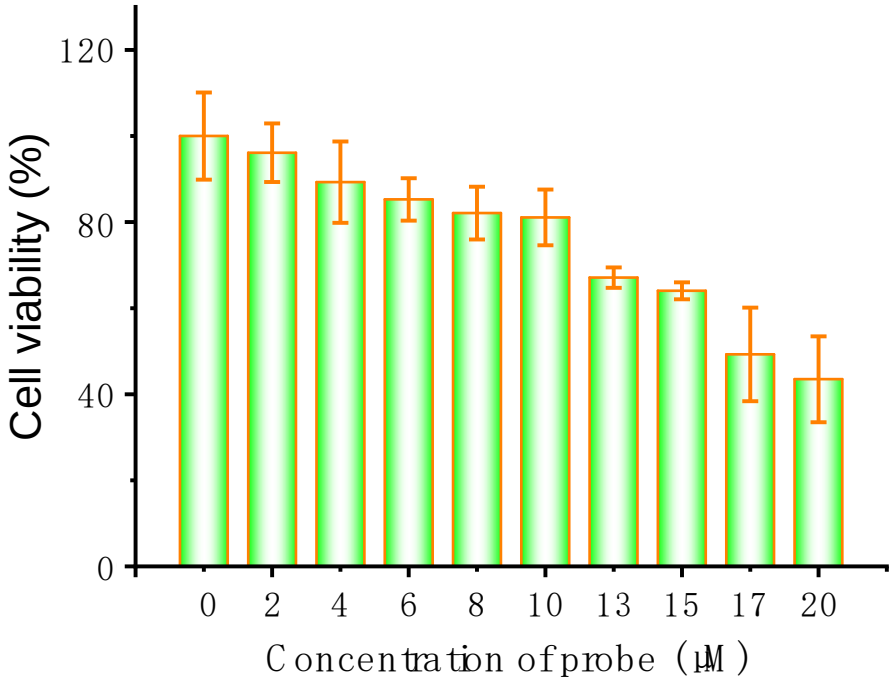


Fig. S7. Effects of **LY-1** with varied concentrations (0-20 μM) on the viability of HeLa cells for 24 h. The viability of the cells without **LY-1** is defined as 100%. The results are the mean ± standard deviation of five separate measurements.

6. The Entry Time of of LY-1 into the Cells

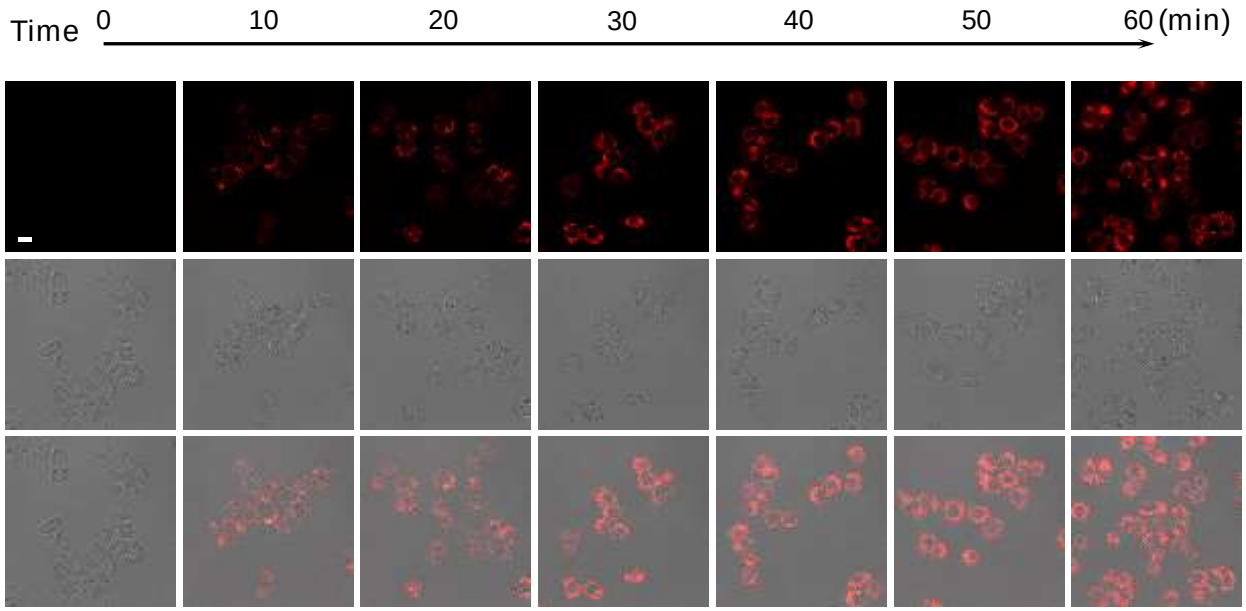


Fig. S8. Laser confocal fluorescence microscopic imaging behavior of **LY-1** towards HeLa cells over time. From left to right: treatment with **LY-1** (10 μM) for 0, 10 min, 20 min, 30 min, 40 min, 50 min and 60 min. Belowing the dark field are corresponding DIC and overlay images

respectively. λ_{ex}: 561 nm, and λ_{em}: 589-671 nm. Scale bar: 15 μm.

7. Subcellular Co-localization Imaging

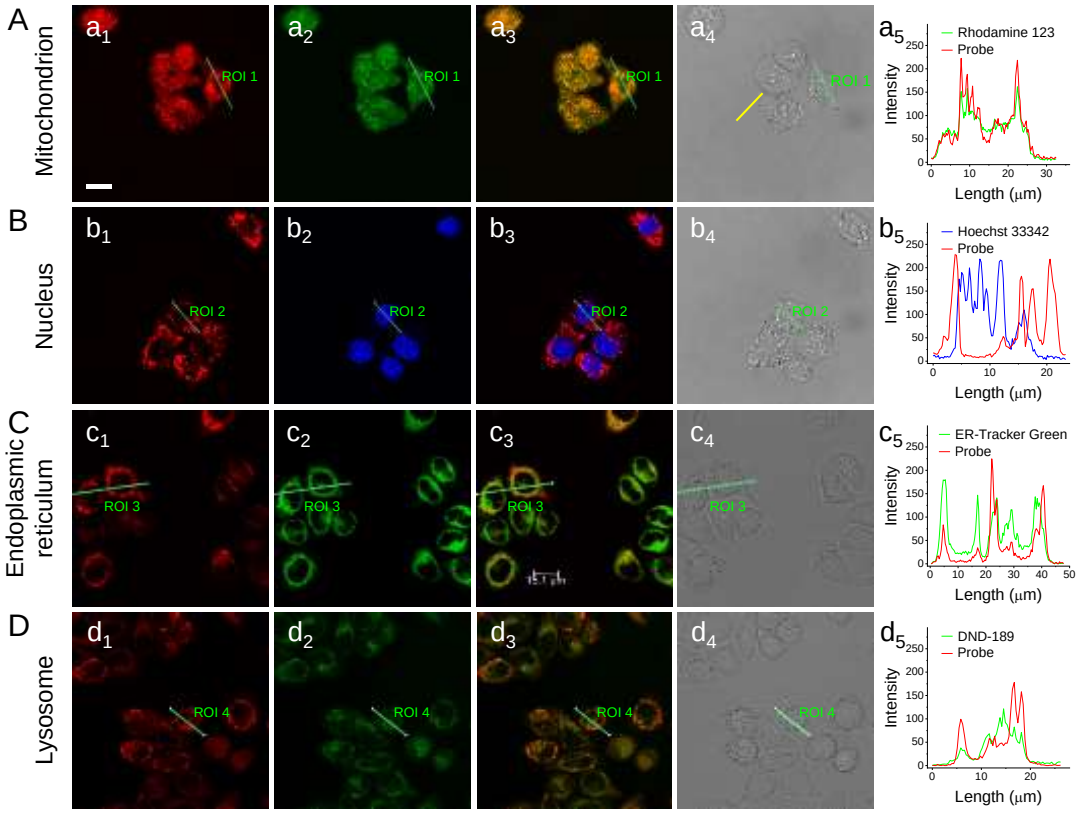


Fig. 9 Fluorescence imaging of HeLa cells co-stained with **LY-1** and Rhodamine 123 (A), Hoechst 33342 (B), ER-Tracker Green (C) and Lyso-Tracker Green DND-189 (D). a_1-d_1: Fluorescence image from **LY-1** channel. a_2-d_2: Fluorescence image from different commercial organelle dyes. a_3-d_3: Merged image of 1 and 2 for each group. a_4-d_4: Corresponding DIC image. Scale bar: 15 μm. a_5-d_5: Intensity profile of the linear ROI from 1 and 2 for each group across the cells. Scale bar: 15 μm.

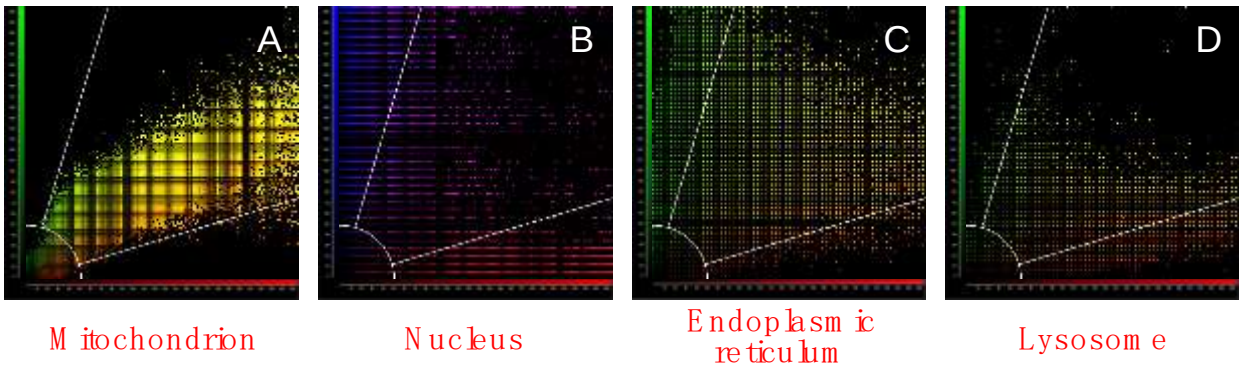


Fig. S10. Scatter plot in the colocalization assays from Fig. 2.

8. Cell Starvation Experiment

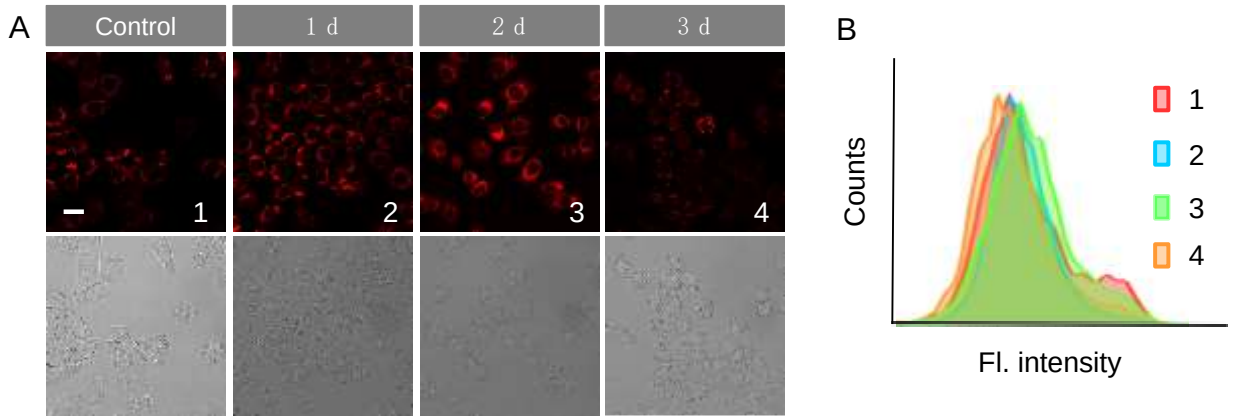


Fig. S11. Confocal imaging observations of **LY-1** (10 μM) on HeLa cells starved for 0-3 days. (A) Confocal fluorescence imaging of HeLa cells incubated with **LY-1** under different starvation conditions. (λ_{ex} =561 nm, λ_{em} =570-700nm). Scale bar: 20 μm. (B) Corresponding flow cytometry assay of HeLa cells treated as in (A).

9. Flow Cytometry Assay on Drug-induced Mitophagy or Ferroptosis

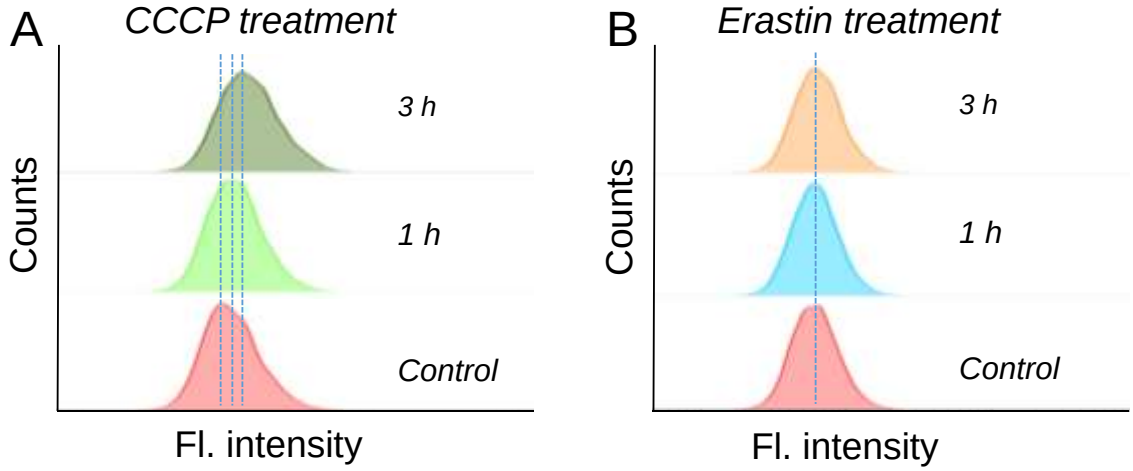


Fig. S12. (A) and (B) are the flow cytometry assay results, corresponding to Figs. 4A and 4C respectively.