Supporting Information

Highly Specific Probe for Imaging of Inflammation-induced Endogenous Nitric Oxide Produced during Stroke Process

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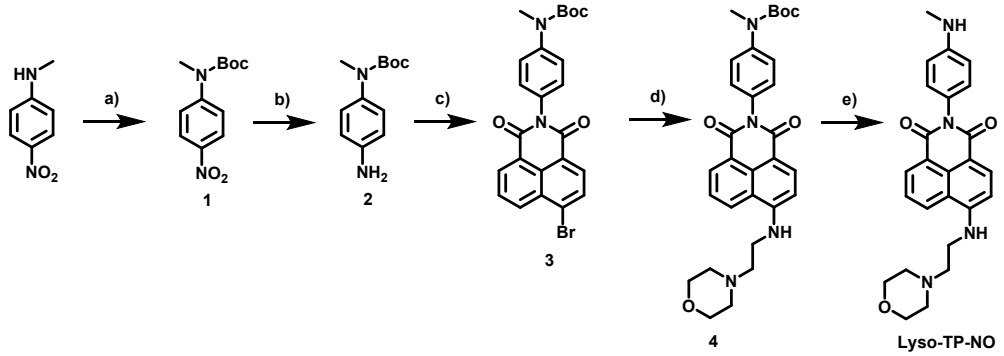
1. Synthesis of Lyso-TP-NO.

Compounds **1** and **2** were prepared by the literature method,[1] and the synthetic details of other compounds are listed below.



Scheme S1. Synthetic route of **Lyso-TP-NO**. *Regeants and conditions*: a) Boc_2O, NEt_3, anhydrous DCM, r.t., 12 h. b) Pd/C, hydrazine hydrate, EtOH, Ar, 80 °C, 4 h. c) 4-bromo-1,8-naphthalene anhydride, 1,2-dimethoxyethane, reflux, 4 h. d) 4-(2-aminoethyl)morpholine, DMSO, 90°C, 12 h. e) CF_3COOH, anhydrous dichloro-methane, 0°C to r.t., 6 h.

Synthesis of compound **3**.

Compound **2** (1.64 g, 7.38 mmol) and 4-bromo-1,8-naphthalene anhydride (2 g, 7.25 mmol) were dissolved in a round bottom flask. 30 mL of 1,2-dimethoxyethane was then added and the reaction mixture was refluxed for 4 h. After the reaction, solvent was removed and the crude residue was purified by column chromatography (1:5, v/v ethyl acetate/petroleum ether) to give the product as yellow solid (2.06 g, yield 58.2%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.71 (dd, J = 7.3, 1.1 Hz, 1H), 8.65 (dd, J = 8.5, 1.1 Hz, 1H), 8.47 (d, J = 7.9 Hz, 1H), 8.09 (d, J = 7.9 Hz, 1H), 7.92 – 7.88 (m, 1H), 7.43 (d, J = 8.6 Hz, 2H), 7.27 (s, 1H), 7.25 (s, 1H), 3.34 (s, 3H), 1.49 (s, 9H).

Synthesis of compound **4**.

Compound **3** (1 g, 2.08 mmol) and 4-(2-aminoethyl)morpholine (1.3 mL, 9.6 mmol) were dissolved in dry DMSO and stirred at 90 °C for 12 h. After completion of the reaction, the resulting solution was poured into ice water and a yellow precipitate was isolated by filtration. The resulting solid was purified by column chromatography (10:1, v/v dichloromethane/MeOH) to give the product as yellow solid (806 mg, yield 79.8%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.57 (dd, J = 7.7, 3.7 Hz, 2H), 8.33 (d, J =

7.9 Hz, 1H), 8.24 (d, *J* = 7.9 Hz, 1H), 8.02 (t, *J* = 7.9 Hz, 1H), 7.04 (d, *J* = 8.5 Hz, 2H), 6.62 (d, *J* = 8.6 Hz, 2H), 5.89 (d, *J* = 3.8 Hz, 1H), 2.73 (d, *J* = 3.2 Hz, 3H).

Synthesis of compound **Lyso-TP-NO**.

Compound **4** (500 mg, 0.94 mmol) was dissolved in anhydrous dichloromethane (20 mL) and CF_3COOH (6.81 mL, 0.09 mmol) was added slowly at 0 °C. The reaction was stirred at r.t. for 6 h. After the reaction, solvent was removed and the crude residue was purified by column chromatography (10:1, v/v dichloromethane /MeOH) to give the product as yellow solid. Yield: 325.9 mg (80.6%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.67 (d, *J* = 8.3 Hz, 1H), 8.42 (d, *J* = 7.2 Hz, 1H), 8.25 (d, *J* = 8.4 Hz, 1H), 7.70 (t, *J* = 7.8 Hz, 1H), 7.64 (s, 1H), 6.96 (d, *J* = 8.5 Hz, 2H), 6.83 (d, *J* = 8.6 Hz, 1H), 6.60 (d, *J* = 8.5 Hz, 2H), 5.78 (s, 1H), 3.63 (s, 4H), 3.55 (s, 2H), 2.73 (s, 6H), 2.51 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 164.26, 163.47, 150.33, 149.35, 134.10, 130.67, 129.70, 129.26, 128.37, 124.32, 124.22, 122.51, 120.20, 111.34, 108.39, 103.87, 66.06, 56.05, 53.22, 29.83. HR-MS (MALDI-DHB) calcd for $C_{25}H_{26}N_4O_3$ $[M+H]^+$: 431.2005, found: 431.2085.

2. General Information on Materials and Methods.

Instruments and materials.

Unless otherwise stated, all solvents and reagents were purchased from commercial suppliers and were used as received without further purification. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. All the reagents were obtained from Aladdin Ind. Corp. (Shanghai, China). All other chemicals were from commercial sources and of analytical reagent grade, unless indicated otherwise. All aqueous solutions were prepared in ultrapure water with a resistivity of 18.25 MΩ·cm (purified by Milli-Q system, Millipore). High-resolution mass spectrometry was performed with LTQ FT Ultra (Thermo Fisher Scientific, America) in MALDI-DHB mode. NMR spectra were recorded on a Bruker-400 spectrometer, using TMS as an internal standard. Absorption spectra were recorded with a UV-vis spectrophotometer (Shimadzu UV-2550, Japan), and one-photon fluorescence spectra were obtained with a fluorimeter (Shimadzu RF-6000, Japan). Two-photon fluorescence spectra were excited by a mode-locked Ti:sapphire

femto-second pulsed laser (Chameleon Ultra I, Coherent, America) and recorded with a DCS200PC photon counting with Omno-5008 monochromator (Zolix, China). Two photon microscopy was performed on a Zeiss LSM 710 multiphoton laser scanning confocal microscope (Carl Zeiss, Germany). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Wuhan University and approved by the Animal Ethics Committee of Wuhan University.

Spectroscopic measurements.

Unless otherwise noted, all the measurements for **Lyso-TP-NO** target reaction were tested in PBS buffer (10 mM, pH 5.0, containing 20% CH_3CN). Under the conditions of NO concentration of 500 μM and probe concentrations of 0.25, 1, 2, 5, 8, 10, 15, 20 μM, the water solubility of the probe was measured. For the selectivity assay, superoxide anion ($O_2^{\bullet-}$) was prepared by dissolving KO_2 in DMSO solution. •OH was generated by Fenton reaction between Fe^{2+} (EDTA) and H_2O_2 quantitively, and Fe^{2+} (EDTA) concentrations represented •OH concentrations.[2] The $ONOO^-$ source was the donor 3-morpholinosydnonimine hydrochloride (SIN-1). H_2O_2 was determined at 240 nm ($\varepsilon_{240\ nm}$ = 43.6 $M^{-1}cm^{-1}$). NO^{2-} was generated from $NaNO_2$.

Determination of the detection limit.

The limit of detection (LOD) was calculated based on the method reported in the previous literature.[3] The LOD for NO was calculated based on the following equation:

$$LOD = 3\sigma/k$$

Where σ represents the standard deviation and k represents the slope of the titration spectra curve among the limited range.

Quantum yield measurements.

The measurement of the fluorescence quantum yield was measured by using quinine sulfate (Φ = 0.55 in 0.1 M H_2SO_4 solution) as the reference, and using the following equation.

$$\Phi_s = (A_r \cdot F_S \cdot n_s^2)/(A_s \cdot F_r \cdot n_r^2)\ \Phi_r\ (A \leq 0.05)$$

Where s and r represent the sample to be tested and the reference dye, respectively. A represents the absorbance at the maximum absorption wavelength, F represents the fluorescence spectrum integral at the maximum absorption wavelength

excitation, and n represents the refractive index of the sample to be tested or the reference dye solvent.

Measurement of Two-photon Cross Section.

The two-photon absorption cross section (δ) was determined by using femtosecond (fs) fluorescence measurement technique as described.[4] Probe was dissolved in 10 mM PBS buffer (pH 5.0), and the two-photon induced fluorescence intensity was measured at 750-900 nm by using rhodamine B as the reference, whose two-photon property has been well characterized in the literature. The intensities of the two-photon induced fluorescence spectra of the reference and sample at the same excitation wavelength were determined. The TP absorption cross section was calculated by using the following equation.

$$\delta_s = (S_s \Phi_r n_s^2 c_r)/(S_r \Phi_s n_r^2 c_s) \delta_r$$

where the subscripts s and r stand for the sample and reference molecules. The intensity of the two-photon excited fluorescence was denoted as *S*. Φ is the fluorescence quantum yield, and Φ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as c. δr is the 2P absorption cross section of the reference molecule.

Cytotoxicity assay.

The cytotoxicity was evaluated by MTT assay.[5] Briefly, BV-2 cells were cultured in DMEM in 96-well microplates in incubator for 24 h. The medium was next replaced by fresh DMEM containing various concentrations of **Lyso-TP-NO** (0–20 μM). Each concentration was tested in five replicates. Cells were rinsed twice with phosphate buffer saline (PBS) 24 h later and incubated with 0.5 mg/mL MTT reagent for 4 h at 37°C. The culture was removed and 150 μL DMSO was added to dissolve for mazan. After shaking for 10 min, the absorbance at 490 nm was measured by microplate reader (Synergy 2. BioTek Instruments Inc.). Cell survival rate was calculated by $A/A_0 \times 100$ % (A and A_0 are the absorbance of the **Lyso-TP-NO** labelled group and the control group, respectively).

Cell Culture and Imaging.

BV-2 cells were cultured with DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5/95 (v/v) of CO_2/air at 37°C. One day before imaging, cells were detached with a treatment of 0.2% (w/v) trypsin-EDTA solution (Gibco) and suspended in culture media. The cell suspension was then transferred to confocal dishes to grow with adherence. For imaging, BV-2 cells at 80% confluence were harvested by scraping and transferred to confocal dishes to grow with adherence. Two-photon excited fluorescence images were obtained by Zeiss LSM 710 multiphoton laser scanning confocal microscope with a 20×air objective.

OGD/R model.

OGD/R model of cells was performed by oxygen and glucose deprivation/reperfusion. BV-2 cells at 80% confluence were harvested by scraping and transferred to confocal dishes to grow with adherence. When the cells are adherent, the culture medium is changed to sugar-free DMEM and cultured in a three-gas incubator for 0, 1, 2, 4, and 6 h without oxygen. Afterwards, these cells were incubated with high-glucose DMEM in a 5% CO_2 and 95% O_2 atmosphere for 12 h. Then, the cells were incubated with **Lyso-TP-NO** (5 μM) for 30 min. Wash cells three times with PBS for two-photon confocal imaging.

Two-photon fluorescence imaging in tissues.

The mice were maintained under 12 h / 12 h of light / dark cycle, 24 ± 3°C temperature and 55 ± 15% relative humidity throughout the experiment. MCAO was induced using a previously described method with slight modifications.[6] The mice were anesthetized with isoflurane and placed on the operating table. The temperature of the mice was maintained at 37 °C during the operation. The right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were carefully exposed. A 4-0 surgical nylon monofilament (0.24 mm in diameter) was inserted from the right ECA into the ICA to occlude the middle cerebral artery. The occluding filament was withdrawn 1 h after MCAO to induce reperfusion. At 24 h and 48 h after the model was successfully established, 100 μL, 200 μM of the probe **Lyso-TP-NO** was injected through the tail vein. After 1 h, the mice were anesthetized

and dissected to remove the mouse brain tissue, and a 300 μm section was prepared with a microtome. Two-photon excited tissue fluorescence images were obtained by Zeiss LSM 710 multiphoton laser scanning confocal microscope. All the animal experiments were carried out in accordance with the relevant laws and guidelines issued by the Ethical Committee of Wuhan University and were in agreement with the guidelines of the Institutional Animal Care and Use Committee.

Calculation of mean fluorescence intensity.

The mean fluorescence density was measured by Image-Pro Plus (v. 6.0) and calculated *via* the equation (mean density = $IOD_{sum}/area_{sum}$), where IOD and area were integral optical density and area of the fluorescent region.

Statistical analysis.

Statistical Product and Service Solutions (SPSS) software 19.0 was used for the statistical analysis. The error bars shown in the figures represented the mean ± s.d. Differences were determined with a one-way analysis of variance (ANOVA) followed by LSD test. Statistical significance was assigned at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. All experiments were done with at least three biological replicates. Experimental groups were balanced in terms of animal age, sex and weight. Animals were all caged together and treated in the same way. Appropriate tests were chosen according to the data distribution. Variance was comparable between groups in experiments described throughout the manuscript.

3. Structural Identifications of the Compounds.

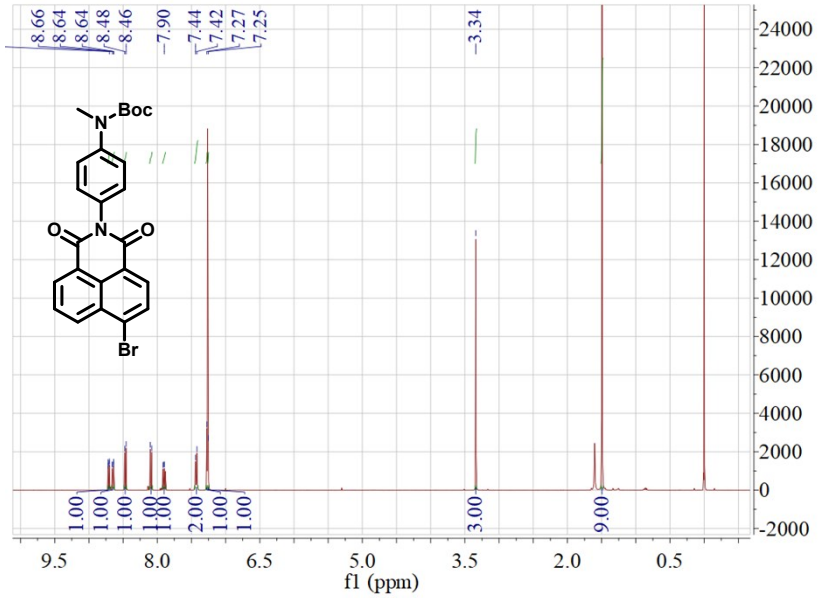


Fig. S1 ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **3**.

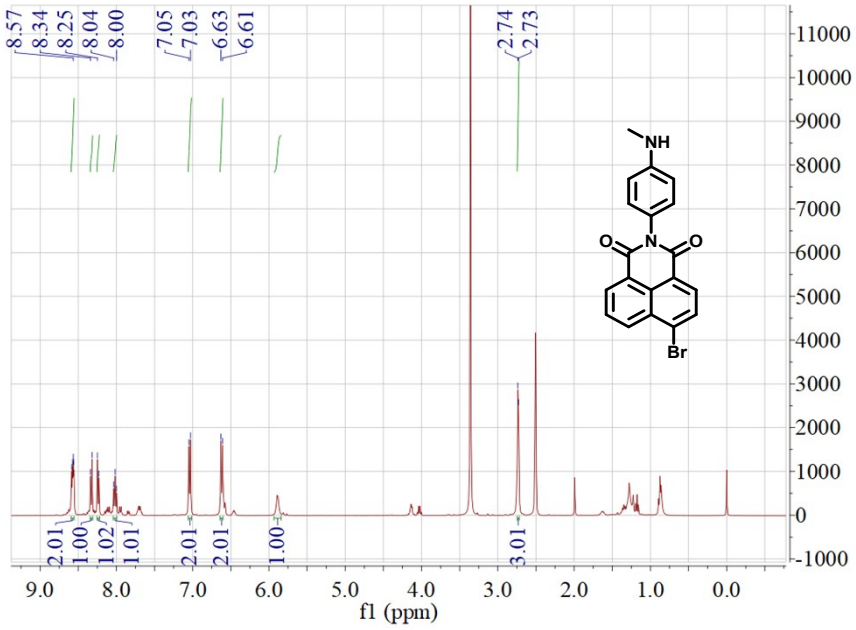


Fig. S2 ^{1}H NMR spectrum (400 MHz, DMSO-*d6*) of compound **4**.

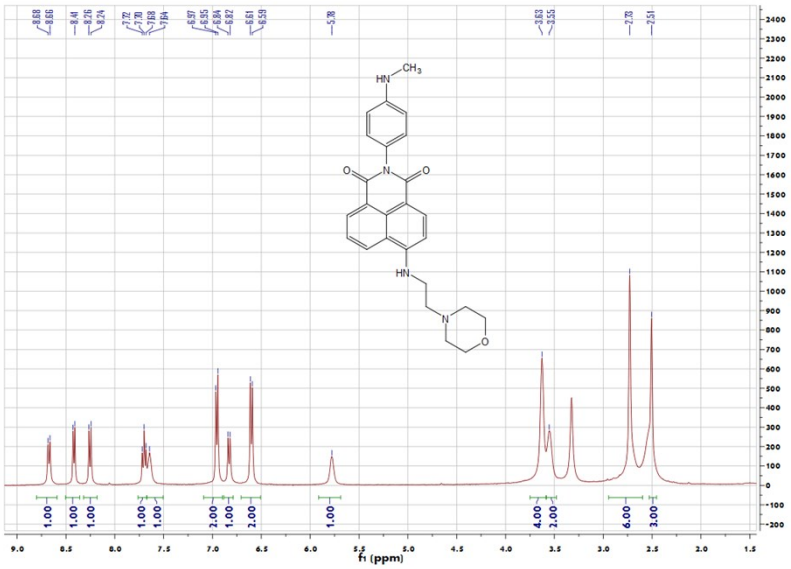


Fig. S3 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of probe **Lyso-TP-NO**.

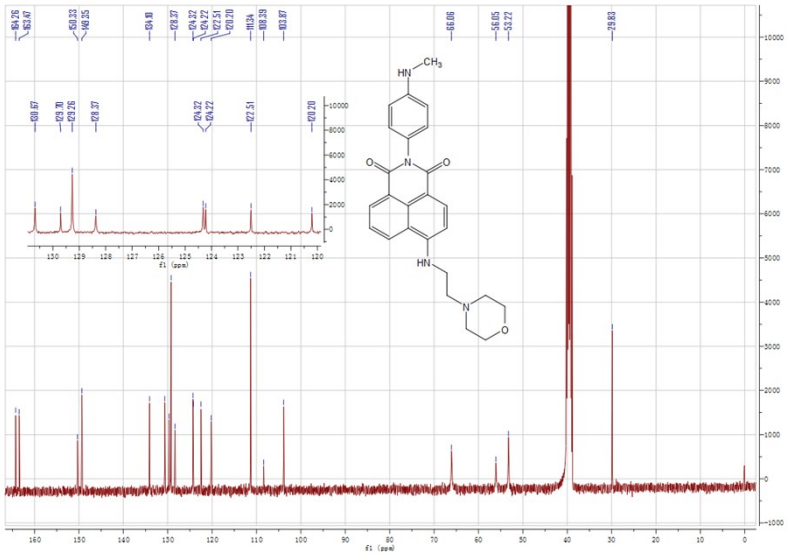


Fig. S4 ^{13}C NMR spectrum (100 MHz, DMSO-*d6*) of probe **Lyso-TP-NO**.

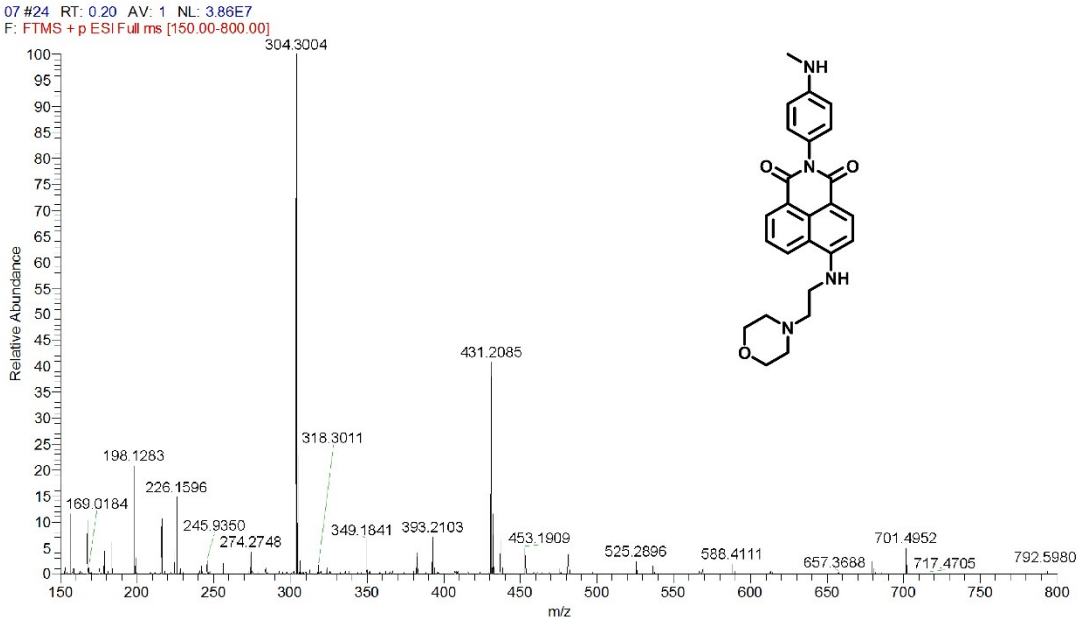


Fig. S5 HR-MS spectrum of probe **Lyso-TP-NO**.

4. Experimental Results.

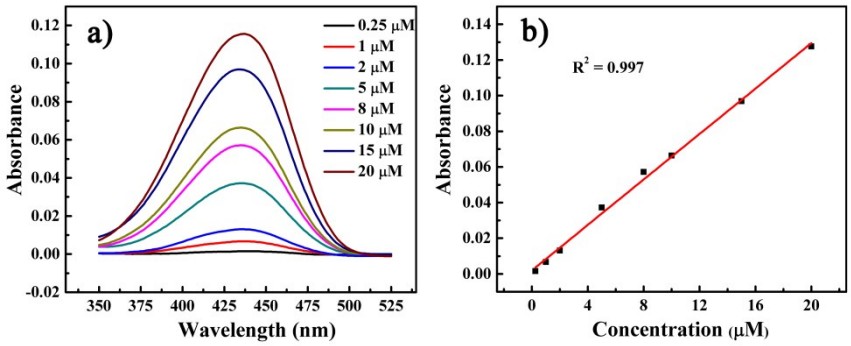


Fig. S6 a) UV-vis spectra of **Lyso-TP-NO** (0.25, 1, 2, 5, 8, 10, 15, 20 μM) in the presence of NO (500 μM) in PBS solution (pH = 5.0, containing 20% CH_3CN). b) Liner relationship of the absorbance at 435 nm with different concentrations of **Lyso-TP-NO**.

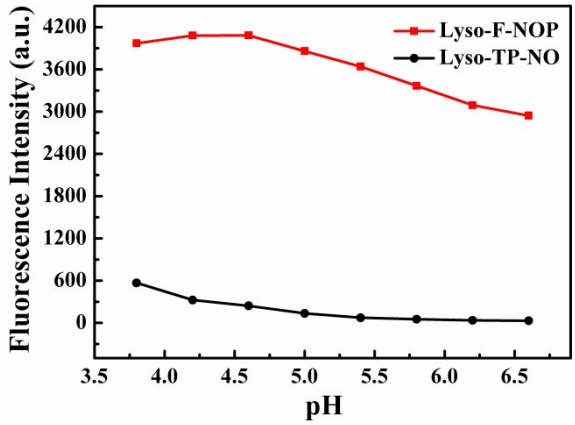


Fig. S7 The fluorescence intensity of **Lyso-TP-NO** (5 μM) before (black line) and after (red line) the addition of NO (150 μM) at different pH.

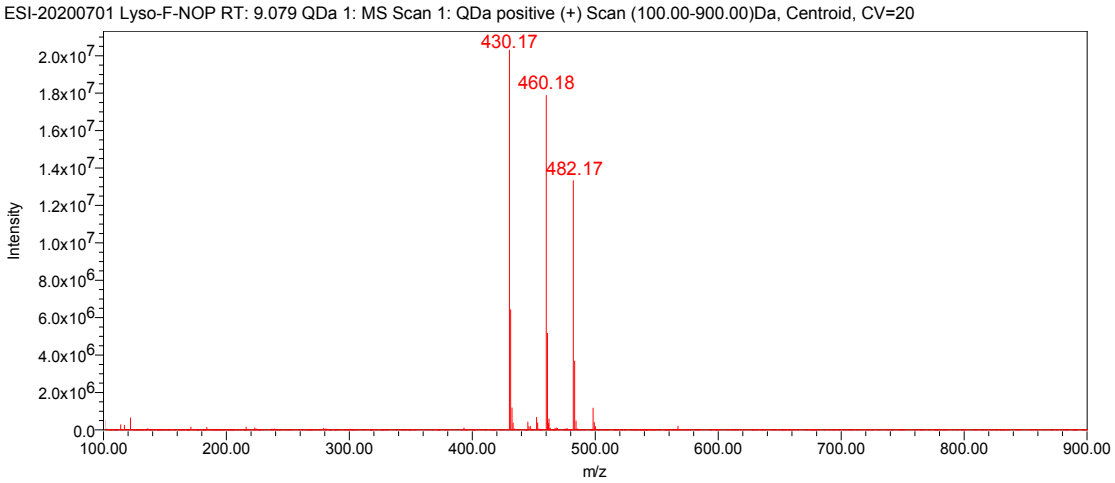


Fig. S8 ESI-MS spectrum for the product **Lyso-F-NOP** of the reaction between **Lyso-TP-NO** and NO.

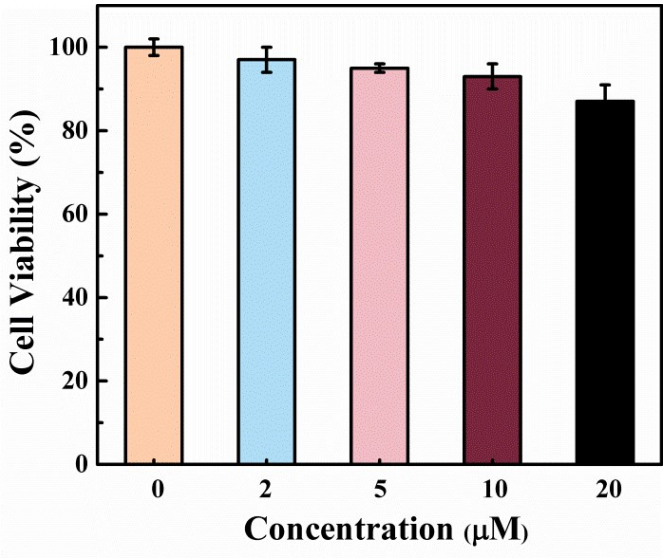


Fig. S9 MTT assay of BV-2 cells treated with different concentrations of **Lyso-TP-NO** (0, 2, 5, 10, 20 μM).

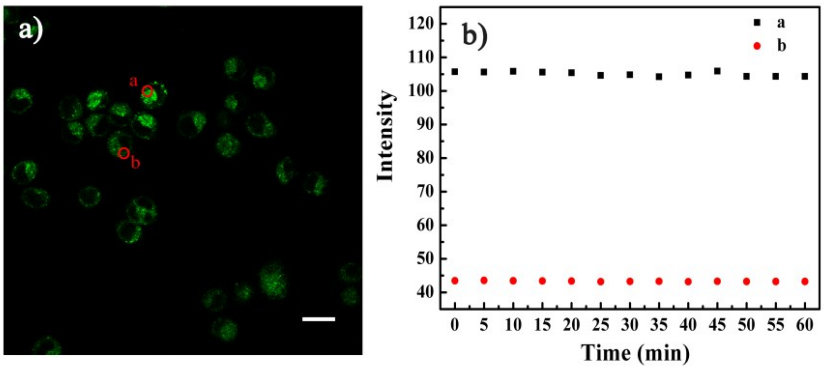


Fig. S10 a) Images of BV-2 cells labeled with 40 μM NOC-9 for 30 min and further incubated with **Lyso-TP-NO** for 30 min. b) Fluorescence intensity from circle a and b as a function of time. The fluorescence intensity was collected with 5 min intervals for the duration of 60 min. λ_{ex} = 840 nm, λ_{em} = 490–570 nm. Scale bar: 20 μm.

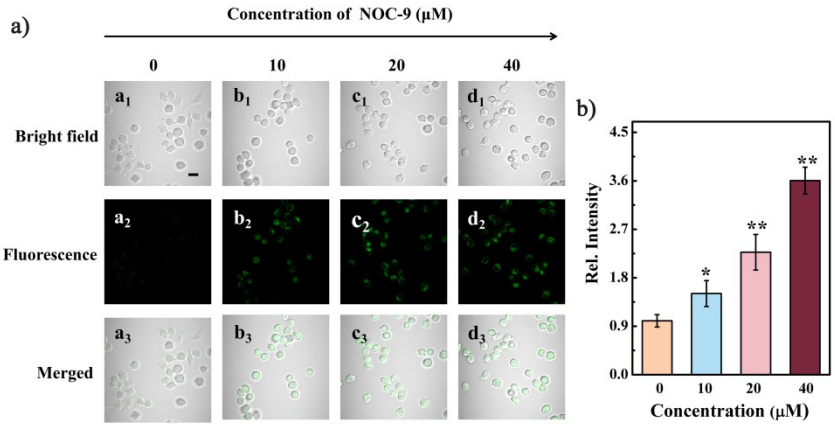


Fig. S11 a) TPM fluorescent images of the probe **Lyso-TP-NO** (5 μM) in BV-2 cells treated with NOC-9 (0, 10, 20, 40 μM). b) The histograms indicated relative fluorescence intensity from the corresponding cells. λ_{ex} = 840 nm, λ_{em} = 490–570 nm. The results were presented as means ± SE with replicates n = 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar: 20 μm.

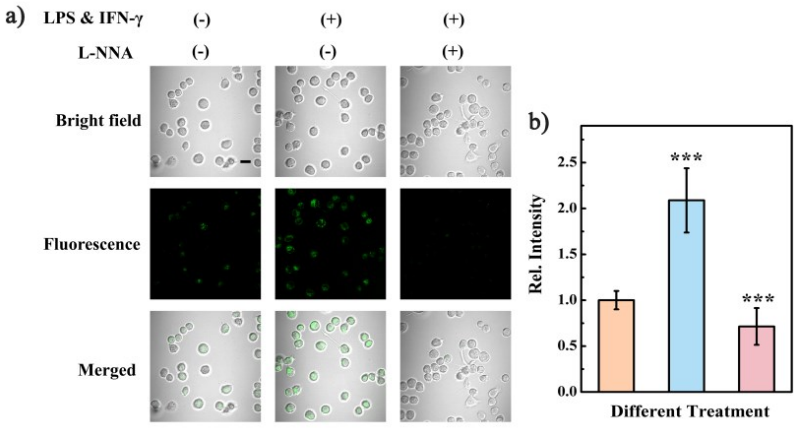


Fig. S12 a) TPM fluorescent images of BV-2 cells treated with 5 μM **Lyso-TP-NO** for 30 min. b) The histograms indicated relative fluorescence intensity from the corresponding cells. λ_{ex} = 840 nm, λ_{em} = 490–570 nm. The results were presented as means ± SE with replicates n = 3. * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar: 20 μm.

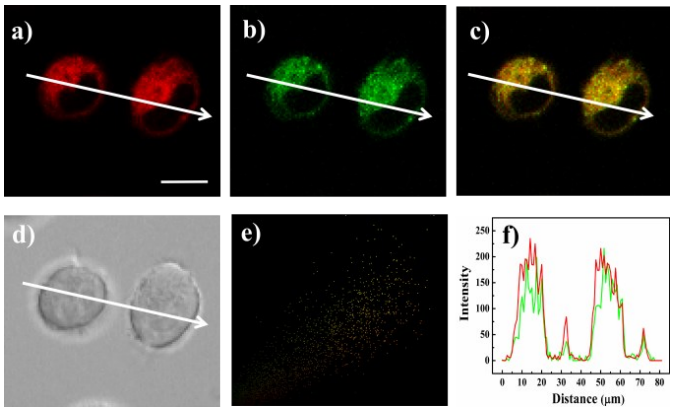


Fig. S13 Co-localization imaging of BV-2 cells staining by **Lyso-TP-NO** (5 μM) with NOC-9 (40 μM) and Lyso-Tracker Red (60 nM). a) Red channel (595–650 nm, λ_{ex} = 594 nm) for Lyso-Tracker Red; b) Green channel (490–570 nm, λ_{ex} = 840 nm) for **Lyso-TP-NO**; c) Overlay image of (a, b); d) Bright field of BV-2 cells; e) The intensity scatter plot of two channels. f) Intensity profile of ROI across the cells in the red and green channels. Scale bar: 20 μm.

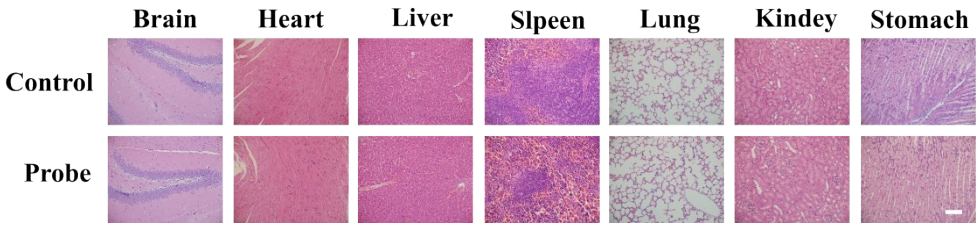


Fig. S14 H&E staining results of the main organs collected from the control group and **Lyso-TP-NO** (100 μL, 200 μM) treated group. Scale bar: 50 μm.

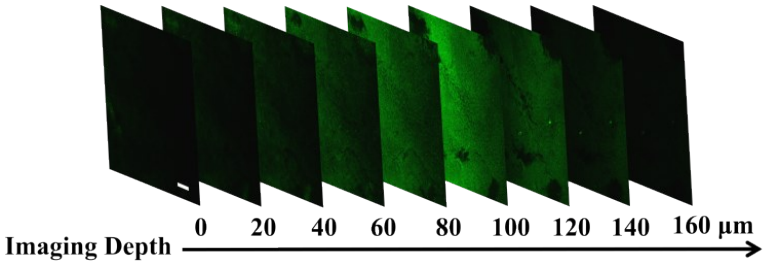


Fig. S15 Depth fluorescence images of **Lyso-TP-NO** (200 μM) in mouse brain tissues. λ_{ex} = 840 nm, λ_{em} = 490–570 nm. Scale bar: 100 μm.

5. Reference

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