Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Observation of inflammation-induced mitophagy during stroke by mitochondria-targeting two-photon ratiometric probe

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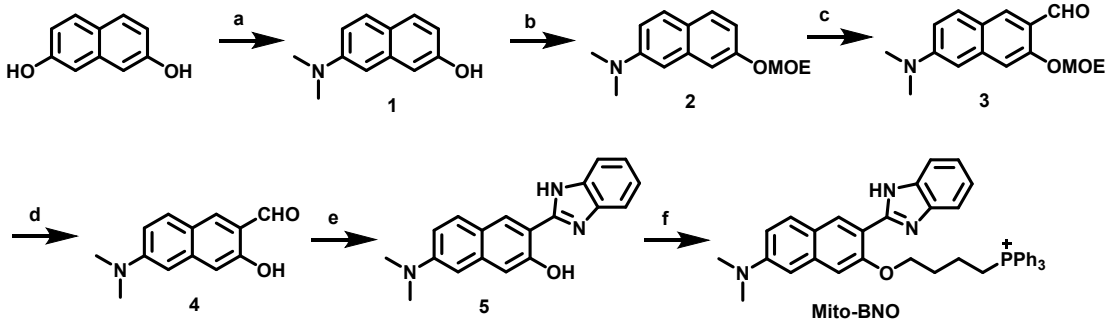
1. Synthesis of Lyso-p-NO.



Scheme S1. Synthesis route of **Mito-BNO**. *Regeants and conditions*: a) Me_2NH, H_2O, $N_2S_2O_5$, 150 °C, 60 h. b) chloromethyl ethyl ether, DIPEA, anhydrous DCM, r.t., 12 h. c) t-BuLi, DMF, absolute ether, -20 °C, 2 h. d) isopropanol, 5 N HCl, 60 °C, 1 h. e) o-phenylenediamine, EtOH, CF_3COOH, 80 °C, 12 h. f) K_2CO_3, (4-Bromobutyl)triphenylphosphonium bromide, DMF, 80 °C, 12 h.

Compound **1-5** were prepared by the literature method,[1] and synthesis details of **Mito-BNO** was listed below.

Synthesis of compound **Mito-BNO**.

Compound 5 (150 mg, 0.51 mmol), 4-bromobutyltriphenylphosphonium bromide (476 mg, 1 mmol), potassium carbonate (276 mg, 2 mmol) were mixed and dissolved in 10 mL DMF. After 12 h of stirring at 80 °C, 100 mL water was added and the mixture was extracted by methylene dichloride. The organic layer was dried with anhydrous Na_2SO_4. Then the solvent was removed under reduced pressure, and the crude product was purified by silica gel column chromatography using methanol: methylene dichloride (v:v=1:20) and a white solid was obtained (303 mg, yeild 85 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.99 (s, 1H), 7.80 (s, 1H), 7.78 – 7.65 (m, 9H), 7.60 – 7.42 (m, 6H), 7.39 (d, J = 5.7 Hz, 5H), 7.04 (s, 2H), 6.73 (s, 1H), 4.32 (s, 2H), 3.08 (s,

8H), 2.43 (d, J = 9.4 Hz, 2H), 2.26 – 2.14 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.23, 133.05, 131.96, 130.61, 128.79, 122.92, 121.79, 114.71, 105.54, 104.07, 67.82, 40.52, 31.95, 29.73, 22.72, 18.68, 14.16. MS: calcd for $C_{41}H_{39}N_3OP$ $[M]^+$ 620.2825 found 620.2848.

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. PC12 cells were obtained from Procell Life Science & Technology Co., Ltd. 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).

Spectroscopic measurements.

Unless otherwise noted, all the measurements for **Mito-BNO** target reaction were tested in PBS buffer (10 mM, containing 10% DMSO). Under the conditions of different pH buffer (4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 6.4, 6.8, 7.2, 7.6, 8.0), 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).[3] H_2O_2 was determined at 240 nm ($\varepsilon_{240\ nm} = 43.6\ M^{-1}cm^{-1}$).

Quantum yield measurements.

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

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

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. Probe was

dissolved in 10 mM PBS buffer (pH 4.0 and 8.0), 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. Briefly, PC12 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 **Mito-BNO** (0-30 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 **Mito-BNO** labelled group and the control group, respectively).

Cell Culture and Imaging.

PC12 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, PC12 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. PC12 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, 3, 6 and 12 hours 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 **Mito-BNO** (5 μM) for 30 minutes. Wash cells three times with PBS for two-photon confocal imaging.

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.

3. Structural Identifications of the Compounds.

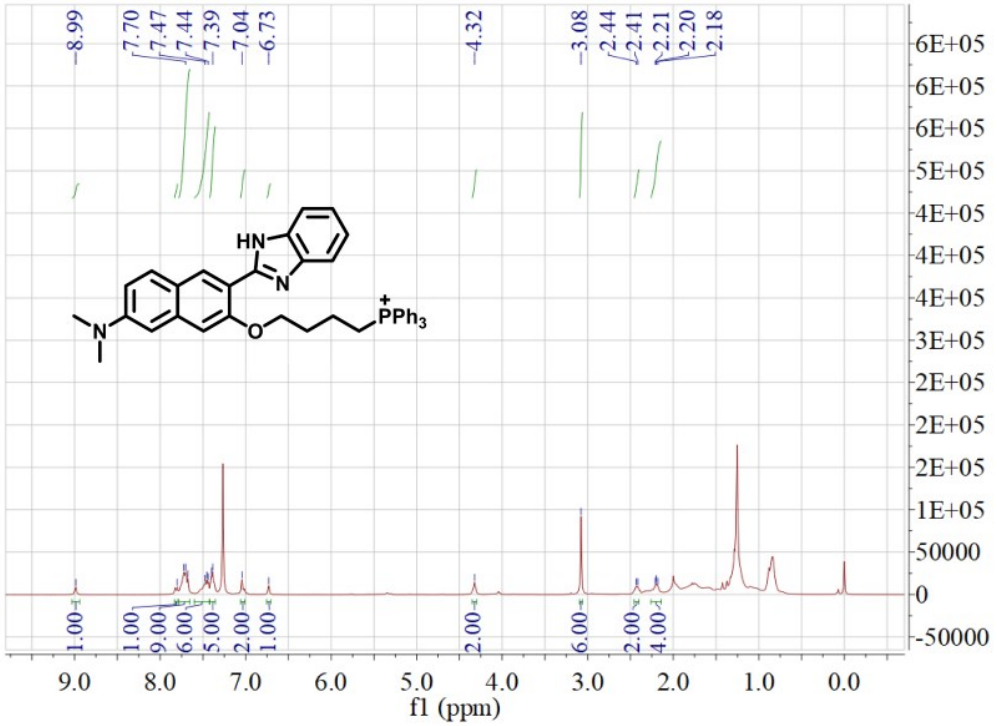


Figure S1. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of probe **Mito-BNO**.

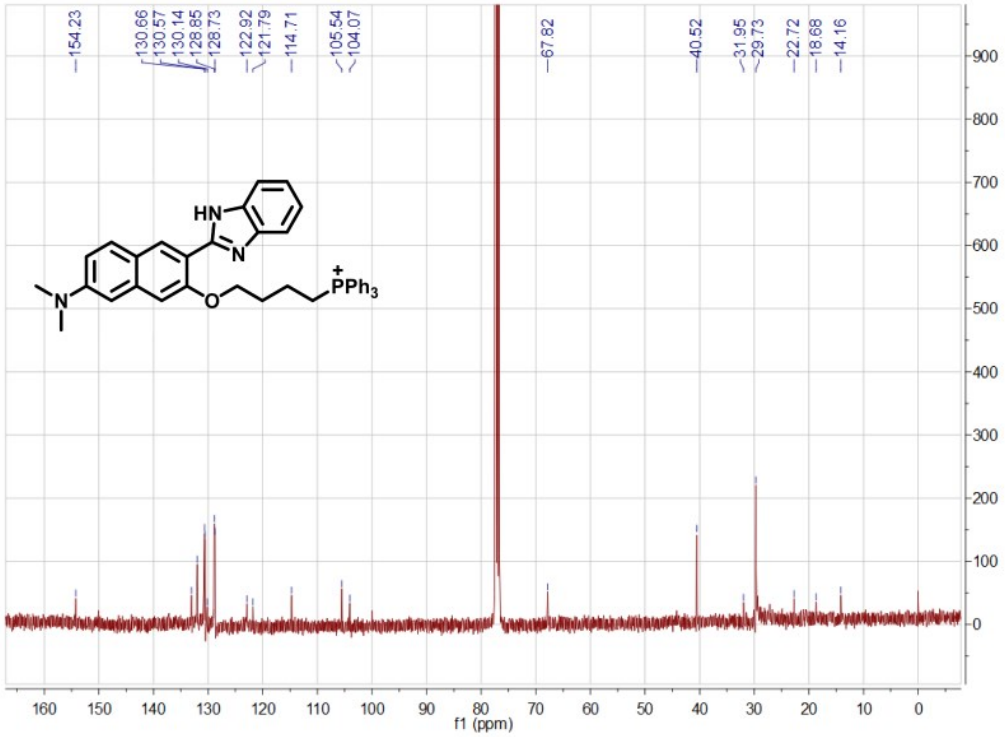


Figure S2. ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of probe **Mito-BNO**.

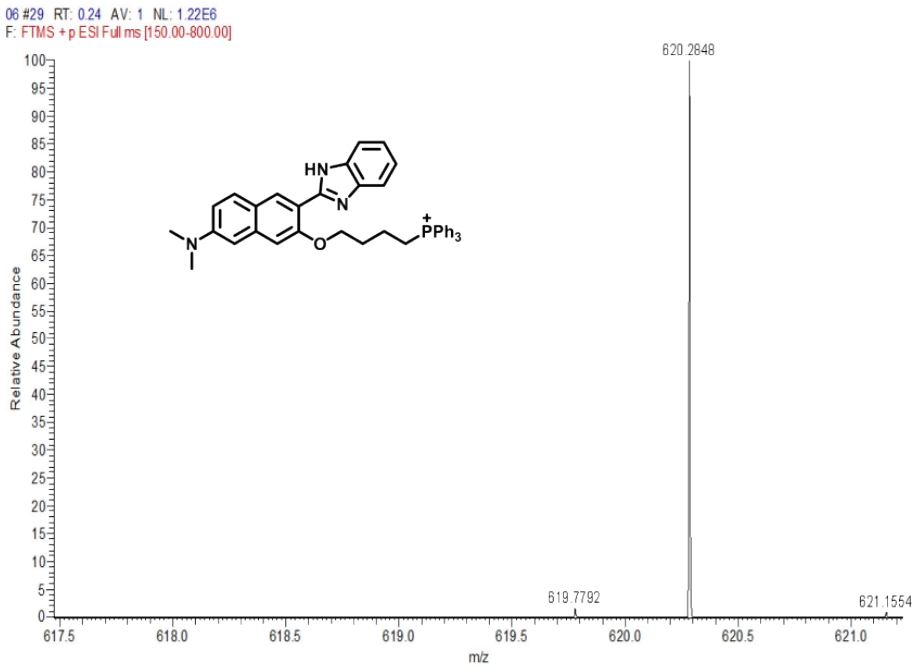


Figure S3. HR-MS spectrum of probe **Mito-BNO**.

4. Experimental Results.

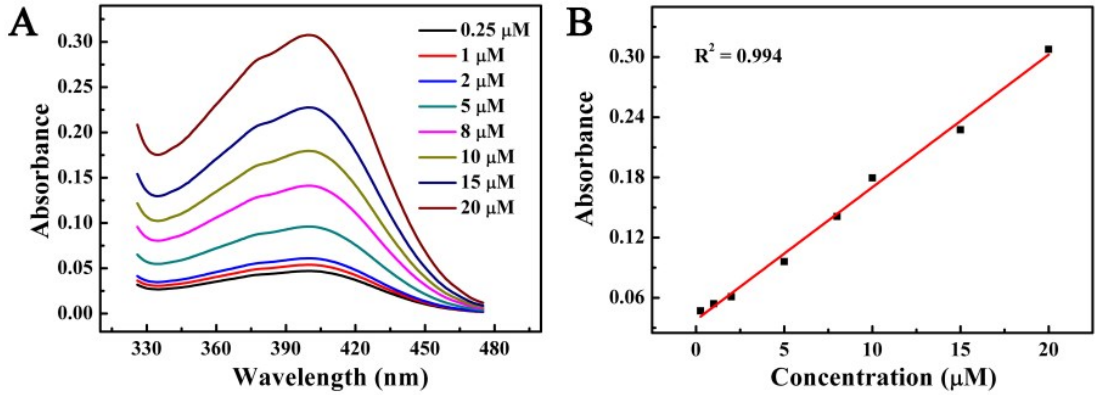


Fig. S4 (A) UV−vis spectra of **Mito-BNO** in PBS solution (pH = 4.0, containing 1% DMSO). (B) Liner relationship of the absorbance at 400 nm with different concentrations of **Mito-BNO**.

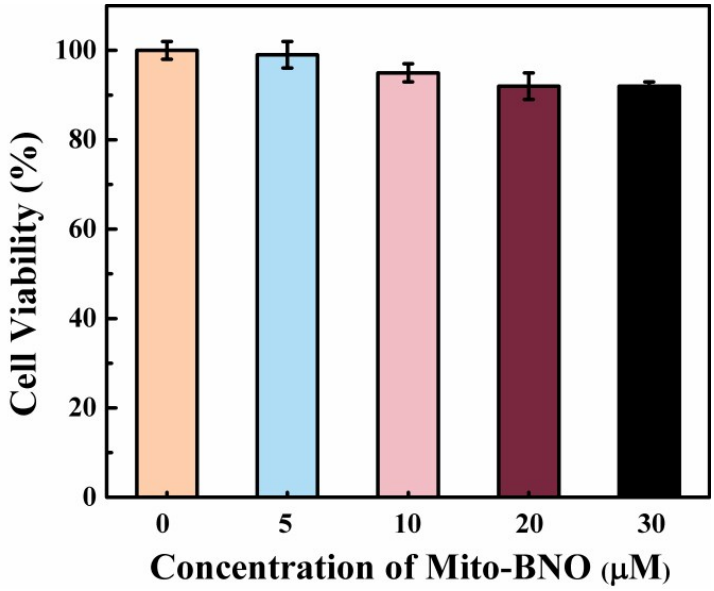


Figure S5. MTT assay of PC12 cells treated with different concentration of **Mito-BNO** (0, 5, 10, 20, 30 μM).

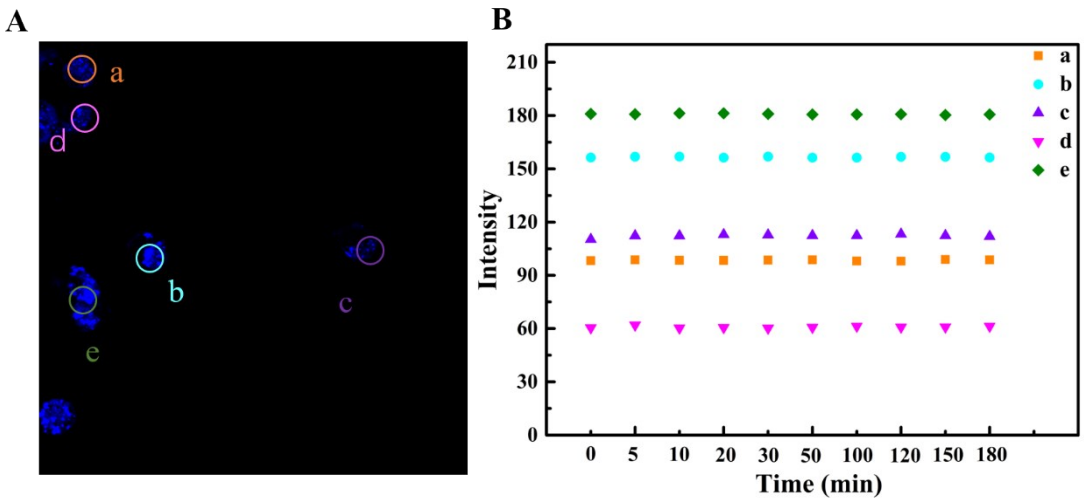


Figure S6. (A) Images of PC12 cells labeled with **Mito-BNO** (10 μM) for 3 h. (B) Fluorescence intensity from circle a-d as a function of time. The fluorescence intensity was collected with 18 min intervals for the duration of 3 h.

5. Reference

[1] Z. Mao, L. Hu, C. Zhong, H. Zhang, B. Liu and Z. Liu, *Sensor Actuat. B-Chem.*, 2018, **219**, 179.

[2] L. Zeng, T. Xia, W. Hu, S. Chen, and Z. Liu, *Anal. Chem*., 2018, **90**, 1317.

[3] R. Uppu, *Anal. Biochem.,* 2006, **354**, 165-168.