Supporting Information

A ratiometric mitochondria-targeting two-photon fluorescent probe for imaging of nitric oxide in vivo

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Figure S15: UV-Vis absorption and fluorescence spectra changes of **Mito-N** toward different analytes.

Experimental Section

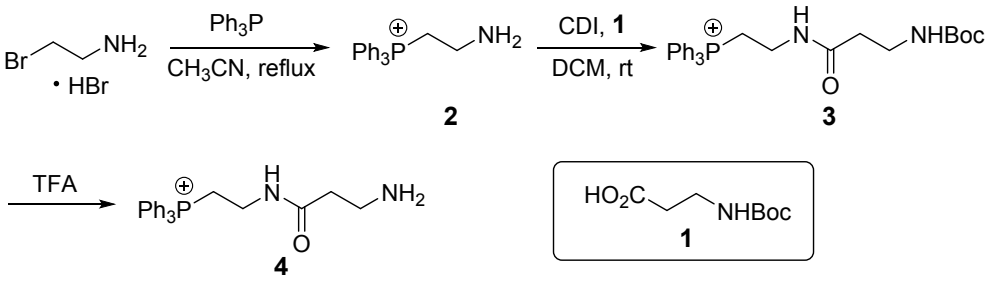
Spectrophotometric measurement, cytotoxicity assay, cell imaging, animal models, in vivo animal study, and tissue slices imaging studies were provided in this supporting information.

Reagents and instruments

All solvents and other chemicals were of analytical reagent grade, which were purchased from commercial sources and used without further purification unless stated otherwise. All oxygen or moisture sensitive reactions were conducted inflame-dried glassware under an atmosphere of nitrogen. Anhydrous solvents were obtained using standard drying techniques. Water used in experiments was doubly distilled and purified by a Mili-Q system (Milipore, USA). Chromatographic purification of products was accomplished using flash chromatography on 200-300 mesh silica gel. Mass spectra were obtained on Thermo LTQ Orbitrap XL Mass spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA, USA) and a LQC system (Finngan MAT, San Jose, CA, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded by a JEOL-ECS-400MHz (JEOL Ltd., Tokyo, Japan) or Bruker spectra. ADVANCE-III NMR spectrometer (Bruker BioSpin International, Switzerland) using TMS (0.00 ppm) as an internal standard and data are reported as: (s = singlet, br = broad, d = doublet, t = triplet, q = quartet, m = multiplet; *J* = coupling constant in Hz, integration.). UV-Vis absorption spectra were performed on TU-1810 spectrophotometer (PEXI, Beijing, China). The one-photon excited fluorescence spectra were carried out on RF-5310PC spectrofluorophotometer (Shimadzu, Tokoy, Japan). Two-photon fluorescence images were taken by Olympus FV1000 laser confocal microscope (Olympus, Tokyo, Japan). Fluorescent images of mice were taken by a Caliper VIS Lumina XR small animal optical in vivo imaging system (USA).The signals were collected by internal PMTs in an 8 bit unsigned 1024 × 1024 pixels at 200 Hz scan speed. The pH values were measured by a model 868 pH meter (Leici, Shanghai, China).

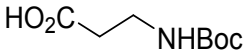
Synthetic procedures of probe Mito-N

The synthesis route **Mito-N** was shown in scheme S1 and the detailed synthesis procedures were shown as following.



Scheme S1. Synthesis route of **4**.

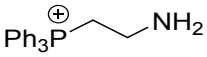
Synthesis of compound 1



A solution of *β*-alanine (14.0 g, 1.0 eq) in 1 N NaOH (200 mL) was stirred and cooled in an ice-water bath. $(Boc)_2O$ (47.0 mL, 1.3 eq) was added, and stirring was continued at room temperature for 4 h. The solution was acidified with a dilute solution of $KHSO_4$ to pH 2-3 (Congo red). The aqueous phase was extracted with ethyl acetate twice. The combined organic layers were washed with water and dried over anhydrous Na_2SO_4, and evaporated in a vacuum. A white crystalline material was obtained and used directly for the next step without purification, compound **1** (26.7 g, 90%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.49 (br, 1H), 5.11 (br, 1H), 3.41 (s, 2H), 2.60 (s, 2H), 1.46 (s, 9H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ117.7, 155.9, 79.7, 35.9, 34.5, 28.4. MS (ESI): $[M + H]^+$ 189.1001, Found: 189.1021.

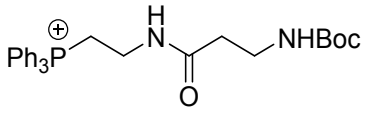
Synthesis of compound 2



To a solution of $BrCH_2CH_2NH_3^+Br$ (8.1 g, 1 eq) in CH_3CN (100 mL) was added Ph_3P (20.9 g, 2 eq) at room temperature. The reaction mixture was allowed to reflux for 48 h in dark, cooled to room temperature, filtrated, the residue was washed with DCM (methylene dichloride) and methyl alcohol, and evaporated under vacuum, white crystalline material was obtained and used directly for the next step without purification, compound **2** (7.9 g, 65%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (br, 2H), 7.89 (m, 15H), 3.97 (m, 2H), 3.10 (br, 2H). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 135.9, 135.8, 134.2, 134.1, 131.0, 131.9, 118.2, 117.3, 33.8, 20.13 (d, J = 54 Hz). MS (ESI): $[M + H]^+$ 306.1474, Found: 306.1406.

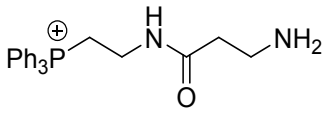
Synthesis of compound 3



To a suspension of compound **1** (3.0 g, 1.5eq) in DCM 100 mL) was added CDI (*N,N*-carbonyldiimidazole, 2.7g, 1.6 eq) at room temperature. The reaction mixture was stirred and kept for another 30 minutes. Compound **2** (4.0 g, 1.0 eq) was added, and the reaction was stirred overnight at room temperature. The reaction was then diluted with DCM and washed with 1 M aqueous hydrogen chloride, water and saturated sodium bicarbonate, respectively. The organic solution was dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude product was purified by silica gel chromatography eluted with DCM: CH_3OH =100:1 to give compound **3** (5.5 g, 89%) as pale solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.09 (br, 1H), 7.80 (m, 15H), 5.48 (br, 1H), 3.90 (m, 4H), 3.53 (br, 2H), 2.34 (br, 2H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 172.8, 155.8, 135.4, 135.3, 133.7, 133.6, 130.7, 118.1, 117.3, 78.8, 53.5, 36.5, 35.7, 33.0, 28.4, 22.73 (d, J = 49 Hz). MS (ESI): $[M + H]^+$ 477.2431, Found: 477.2302.

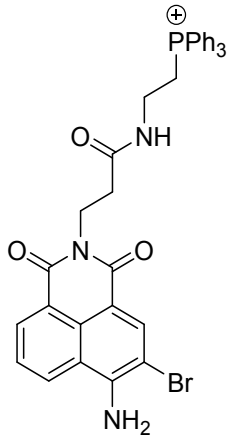
Synthesis of compound 4



To a solution of **3** (4.8 g) in DCM (50 mL) was added TFA (10 mL) at room temperature. The reaction mixture was stirred for 8 h. Organic solvent was evaporated in a vacuum, and the crude product was used directly for the next step without purification, compound **4** (3.3 g, 87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.13 (br, 1H), 8.39 (br, 2H), 7.72 (m, 15H), 3.66 (m, 2H), 3.60 (br, 2H), 3.15 (br, 2H), 2.56 (t, J = 5.2 Hz, 2H), (Figure S1). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 171.6, 135.3, 135.3, 133.5, 133.4, 130.6, 130.5, 118.1, 117.2. 36.1, 33.3, 32.1, 22.1 (d, J = 49 Hz) , (Figure S2). MS (ESI): $[M + H]^+$ 377.1890, Found: 377.1777.MS (ESI): $[M + H]^+$ 377.1890, Found: 377.1777.

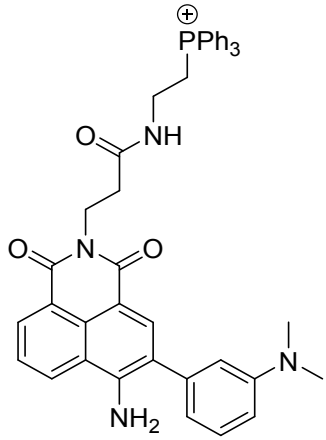
Synthesis of compound 5



To a solution of compound **4** (2.5 g, 1 eq) in pyridine (20 mL) was added 6-amino-5-bromo-1, 8-naphthalic anhydride (2.5 mL, 1.5 eq) at room temperature. The reaction mixture was heated to reflux for overnight. The organic solvent was evaporated in a vacuum, and the residue was subjected to flash chromatography eluted with DCM: CH_3OH = 100:1 to give compound **8** (2.7 g, 63 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (d, *J* = 8.2 Hz, 1H), 8.44 (t, *J* = 5.6 Hz, 1H), 8.40 (d, *J*= 7.8 Hz, 1H), 8.29 (br, 1H), 7.85 (m, 15H), 7.71 (dd, *J* = 7.61, 7.53 Hz, 1H), 7.49 (br, 2H), 4.17 (t, *J* = 7.34, 2H), 3.72 (m, 2H), 3.36 (br, 2H), 2.36 (t, *J* = 7.44 Hz, 2H) , (Figure S3). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 171.2, 163.9, 162.6, 149.3, 136.3, 135.5, 135.5, 134.2, 134.1, 131.5, 130.8, 130.7, 130.1, 128.9, 125.9, 122.6, 120.6, 119.2, 118.3, 109.9, 102.0, 55.5, 36.7, 34.1, 33.2, (Figure S4).MS (ESI): $[M + H]^+$650.1299, Found: 650.1203.

Synthesis of compound Mito-N



To a solution of compound **8** (2.0 g, 1 eq) in a deep deoxygenization mixture of toluene (10 mL), ethanol (3 mL) and water (3 mL) was added Pd $(PPh_3)_4$ (0.3 g, 0.1 eq), K_2CO_3 (1.7 g, 4 eq) and 3-(*N,N*-dimethylamino) phenylboronic acid (1.0 g, 2eq) at room temperature under Ar atmosphere. The reaction mixture was heated to 90 ^{o}C for 24 h and the solution was evaporated to dryness to provide the crude product which was purified by silica gel chromatography eluted with DCM: MeOH to give the desired product compound **Mito-N** (1.2 g, 58%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (d, *J* = 2.1 Hz,, 1H), 8.41 (d, *J* = 2.1 Hz, 2H), 8.05 (s, 1H), 7.82 (m, 16H), 7.35 (t, *J* = 8.1 Hz, 1H), 6.95 (br, 2H), 6.81 (m, 1H), 6.72 (m, 2H), 4.21 (t, *J* = 7.3 Hz, 2H), 3.72 (m, 2H), 3.34 (br, 2H), 2.95 (s, 6H), 2.38 (t, *J* = 7.4 Hz, 2H), (Figure S5). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 171.2, 164.2, 163.5, 151.4, 149.2, 139.2, 135.4, 135.1, 134.1, 134.0, 131.2, 130.7, 130.6, 130.5, 130.3, 129.3, 125.0, 122.2, 122.0, 120.4, 119.1, 118.3, 117.1, 113.2,

112.2, 108.4, (Figure S6). HRMS (ESI): $[M + H]^+$691.2833, Found: 691.2833 (Figure S7).

Spectrophotometric Measurement

The stock solution of **Mito-N** (10.0 mM) was prepared in DMSO. The solutions of analytes were prepared in PBS (10.0 mM, pH 7.4). Test solutions were prepared by placing 2.0 μL of **Mito-N** (10 mM), and 2.0 μL appropriate aliquot of each analyte stock solution into a 2.0 mL centrifugal tube, for detection of NO, the solution was diluted to 1.0 mL with PBS (10 mM, pH 7.4, 2‰ DMSO as a cosolvent.), The resulting solution was shaken well at 37 °C for 20 min for detection of NO, and then the UV absorption and fluorescence spectra were recorded.

Measurement of Two-Photon Cross Section

The two-photo adsorption (TPA) cross sections (δ) of **Mito-N** (QY%: 0.21, 5.8×10^{-5} M) and **Mito-NO** (QY%: 18.71, 8×10^{-4} M) were measured using a femtosecond (fs) fluorescence measurement technique. Rhodamine 6G (1.06×10^{-7} M, QY%: 95, ethyl alcohol) was used as the reference, whose two-photon property has been well characterized in the literature.[1] The intensities of the TP induced fluorescence spectra of the samples and reference emitted at 800 – 1080 nm wavelengths were determined. The TPA cross section was calculated according to Equation (1):

$$\delta_s = \delta_r \frac{\phi_r}{\phi_s} \frac{C_r}{C_s} \frac{n_r}{n_s} \frac{S_s}{S_r} \quad (1)$$

The δ is the TPA cross sectional value, φ is the fluorescence quantum yield, *n* is the refractive index of the solvent, *C* is the concentration and *S* is the intensity of two-photon fluorescence emission. The subscripts *s* and *r* stand for the sample and the reference, respectively.

Preparation of Reactive Oxygen and Nitrogen Species.

NO gas was prepared by added a solution of the H_2SO_4 (3.6 M) dropwise into a stirred solution of $NaNO_2$ (7.3 M). The emitted gas was allowed to pass through a solution of NaOH (2 M). Hydrogen peroxide (H_2O_2) was diluted immediately from a stabilized 30% solution. Freshly prepared aqueous solution of $NaNO_2$ was used as nitrite (NO_2^-) sources. Singlet oxygen was chemically generated from the $HOCl/H_2O_2$ system. Hydroxyl radicals (•OH) were generated in the

Fenton system from ferrous ammonium sulfate and hydrogen peroxide. $ONOO^-$ was synthesized from sodium nitrite and H_2O_2, after the reaction, the solution was treated with MnO_2 to eliminate the excess H_2O_2. The concentration of the $ONOO^-$ stock solution was assayed by measuring the absorbance at 302 nm with a molar extinction coefficient of 1670 M^{-1} cm^{-1}. NO solution was prepared by dissolving NO precursor (2-(*N,N*-diethylamino)-diazenolate 2-oxide sodium salt hydrate [DEA·NONOate]) in 10 mM PBS (pH = 7.4).

Cytotoxicity Assay

The cytotoxic effect of **Mito-N** was assessed by MTT assay according to the manufacturer's instructions (Sigma-Aldrich, MO). For the cytotoxicity assay, cells were seeded into 96-well plates (approximately 10^4 cells per well), and **Mito-N** with various concentrations (0, 5, 10, 20, 30 and 50 μM) were added to the cells and incubated for 24 h, respectively. After incubation, the growth media was removed, and fresh DMEM (dulbecco's modified eagle medium) containing MTT solution (PBS, 20 mM, pH 7.4) was added. The plate was incubated for 3 h at 37 °C. Subsequently, the supernatant was removed, the insoluble colored formazan product was solubilized in DMSO, and its absorbance was measured in a microtiter plate reader (Infinite M200, TECAN, Switzerland) at 550 nm. The assay was performed in five sets for each concentration of **Mito-N**. Data analysis and calculation of the standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation, Albuquerque, New Mexico, U.S.). For statistical analysis, a one-way analysis of variance (ANOVA) was performed using SigmaPlot (Systat Software Inc., San Jose, California).

Cell Imaging

The co-localization experiments were performed firstly before detecting the intracellular NO. The Raw 264.7 cells were incubated with DEA·NONOate (20 μM) for 20 min and with **Mito-N** (20 μm) for 30 min at 37 °C and 5% CO_2, and then Mitochondrial-tracker Red CMXRos (1 μM) were further incubated for another 30 min under the same condition. For imaging endogenous NO in mitochondrial, the cells were incubated with **Mito-N** (20 μm) for 30 min in the absence or presence of L-arginine (L-Arg, 0.5 mg/mL), interferon-r (IFN-r, 150 units/mL), Lipopolysaccharide (LPS, 20μg/mL) for 12 h. For cells imaging of exogenous NO in

mitochondrial, the cells were pretreated with **Mito-N** (20 μM) for 30 min and washed three times with PBS, then incubated with different concentrations of DEA·NONOate (0, 2.5, 5, 7.5, 10, 15 μM). All the cells were washed with phosphate buffered saline (PBS) for three times before imaging analysis.

Animal Models and in Vivo Animal Study

Before animal imaging, nude mouse were starved for 12 h. After anesthesia, 200 μL of 1 mg/mL LPS was injected on left rear leg of mouse, and the right rear leg of mouse was injected 200 μL saline as control experiment. After 12 h, 200 μL of **Mito-N** (20 μM) was then injected through tail vein. 30 min later, anesthesia of the mouse was induced and maintained by inhalation of 5% isoflurane in 100% oxygen. Then, the mouse was placed into the imaging chamber, and kept under anesthetic using an isoflurane gas anesthesia system. Whole body images were acquired using a Caliper VIS Lumina XR small animal optical in vivo imaging system. Procedures involving animals and their care were conducted in conformity with the National Institutes of Health Guide guidelines (NIH Pub. No. 85-23, revised 1996) and was approved by Animal Care and Use Committee of Lanzhou University.

Tissue Slices Imaging Study

After imaging in vivo, the skin of the inflamed (left rear) tissues were harvested and embedded in tissue-freezing medium, frozen and consecutively cut into 10 μm slices. Then, the slices were washed with PBS three times and fixed with 4% paraformaldehyde for 20 min at 37 ^{o}C. Then the tissues obtained above were further incubated Mitochondrial-tracker Red CMXRos (1 μM) for 30 min, washed with PBS for three times and fixed with 4% paraformaldehyde for 20 min at 37 ^{o}C. Fluorescence images were obtained by Olympus FV1000-MPE multiphoton laser scanning microscope.

Supporting figures

^{1}H NMR and ^{13}C NMR spectra of the compounds

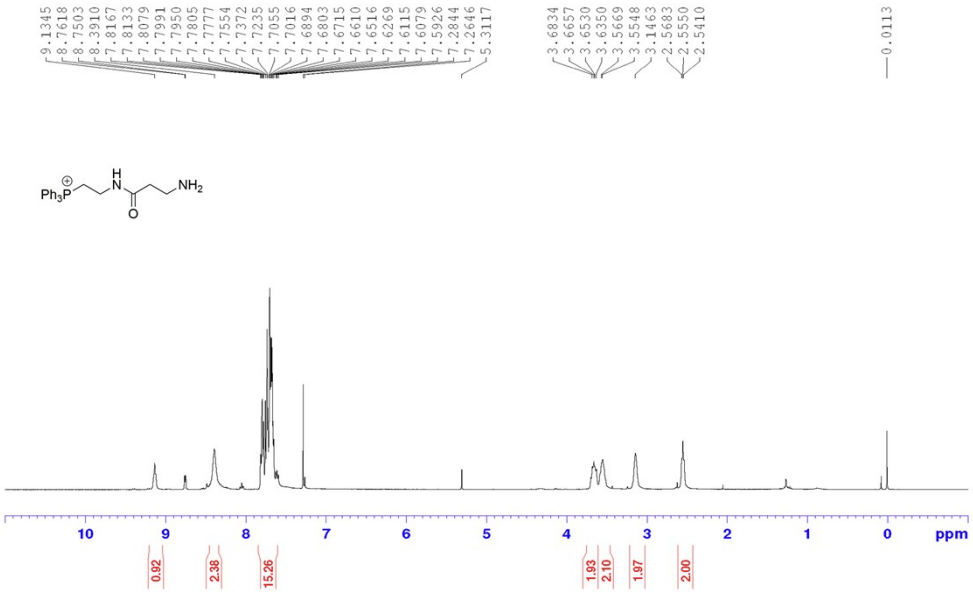


Figure S1. ^{1}H NMR spectrum of **4** in $CDCl_3$.

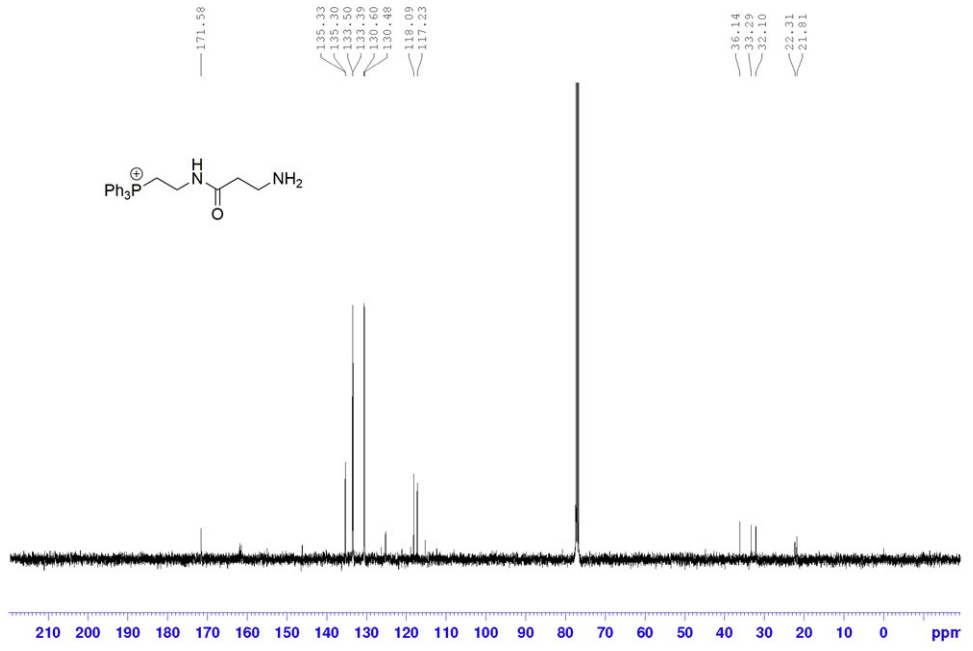


Figure S2. ^{13}C NMR spectrum of **4** in $CDCl_3$.

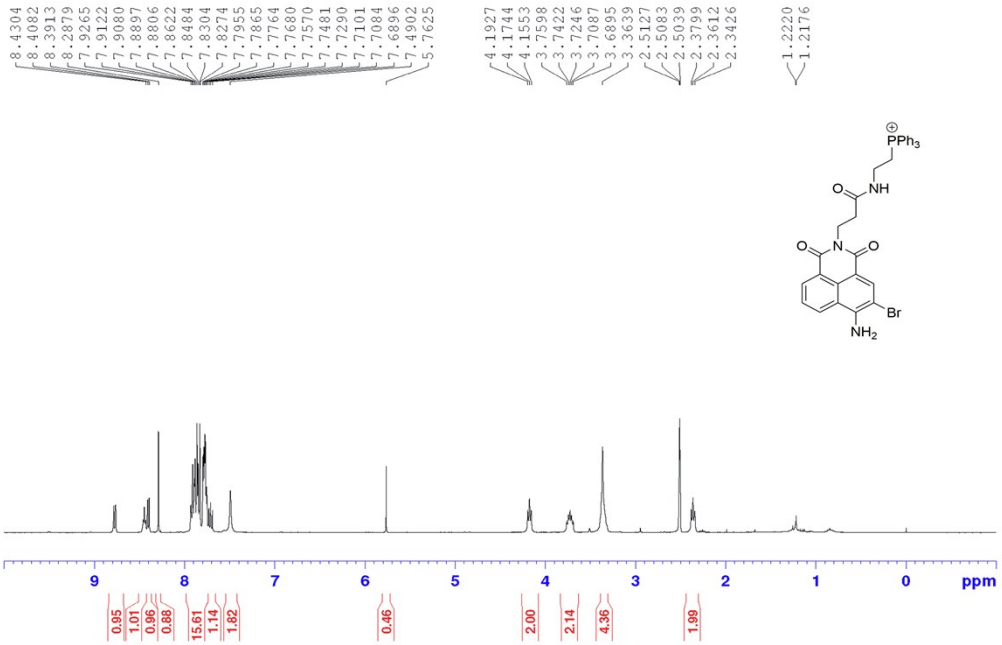


Figure S3. ^{1}H NMR spectrum of **5** in DMSO-d_6.

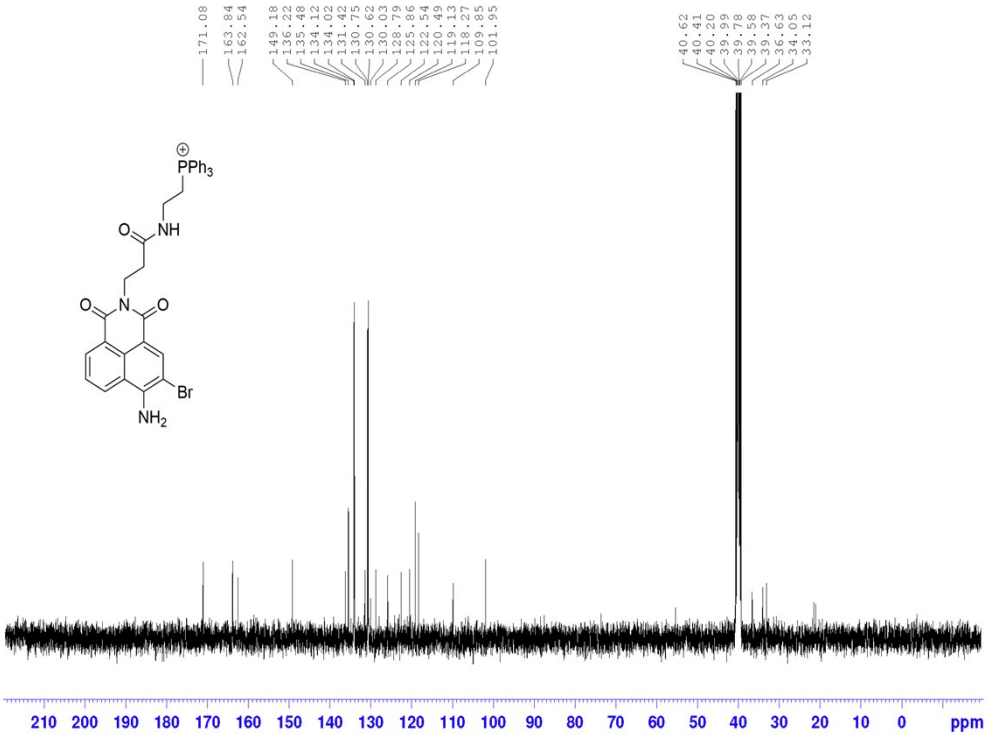


Figure S4. NMR spectrum of **5** in DMSO-d_6.

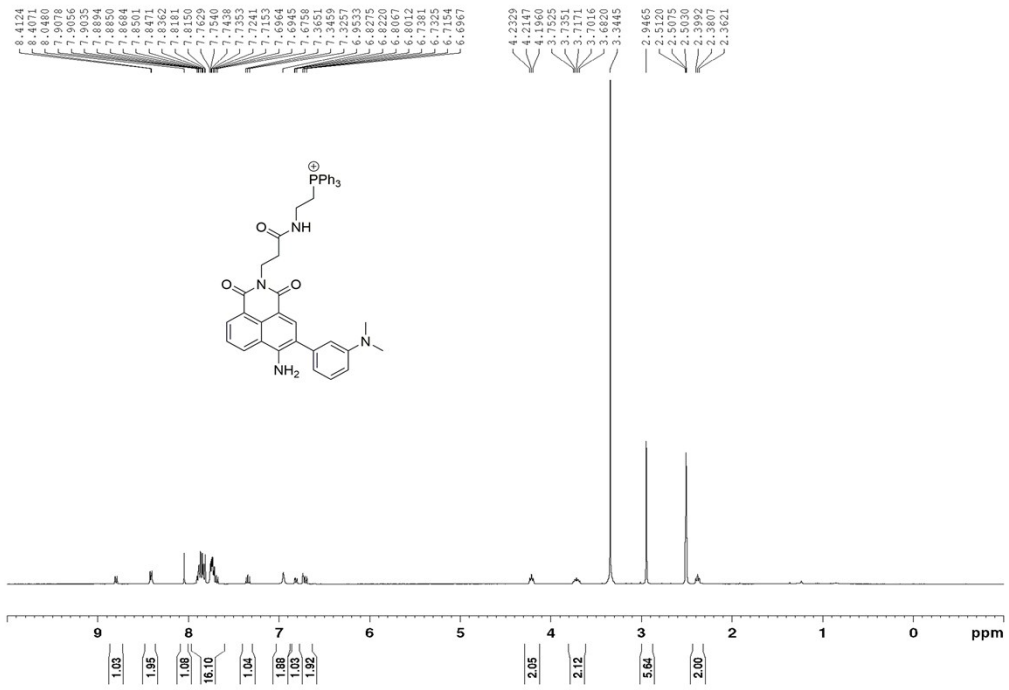


Figure S5. ^{1}H NMR spectrum of **Mito-N** in DMSO-d_6.

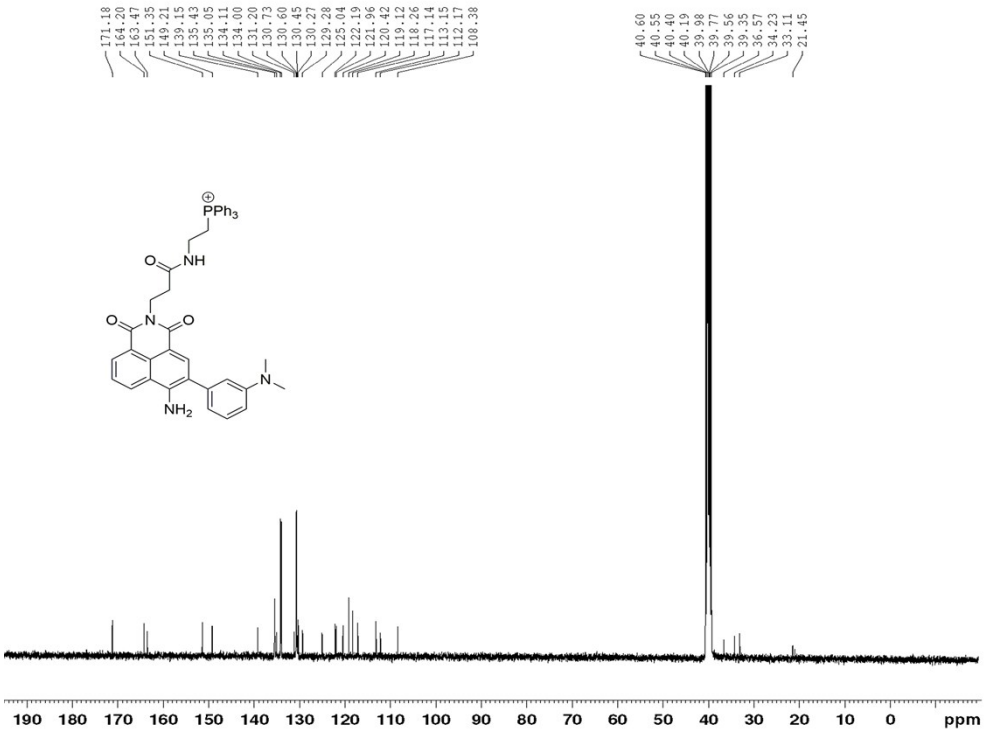


Figure S6. ^{13}C NMR spectrum of **Mito-N** in DMSO-d_6.

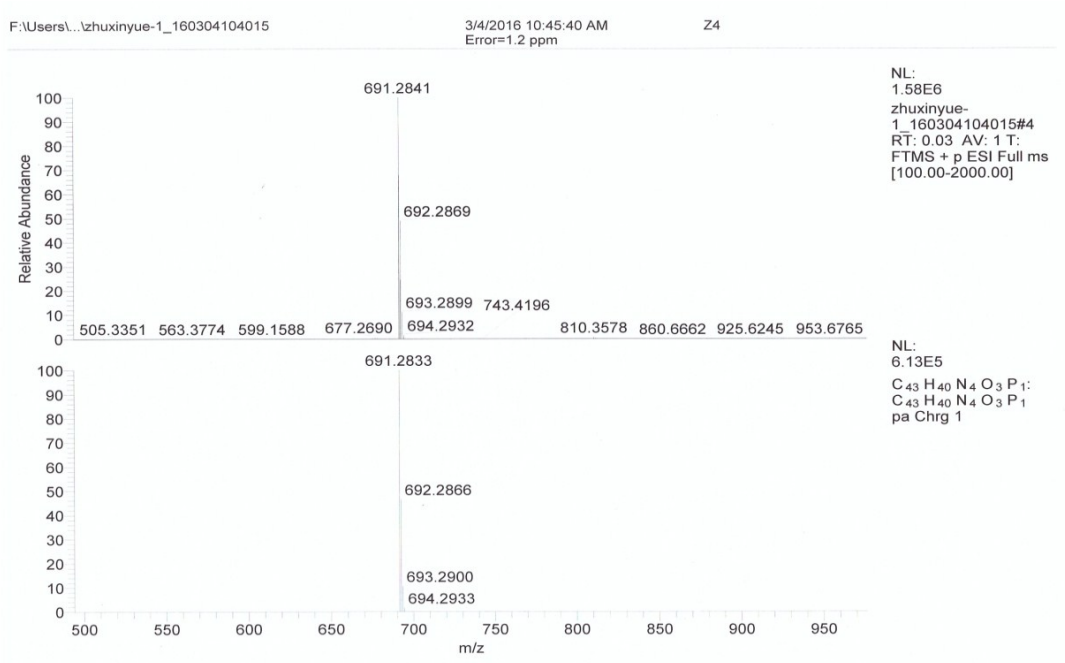


Figure S7 HRMS of probe **Mito-N**.

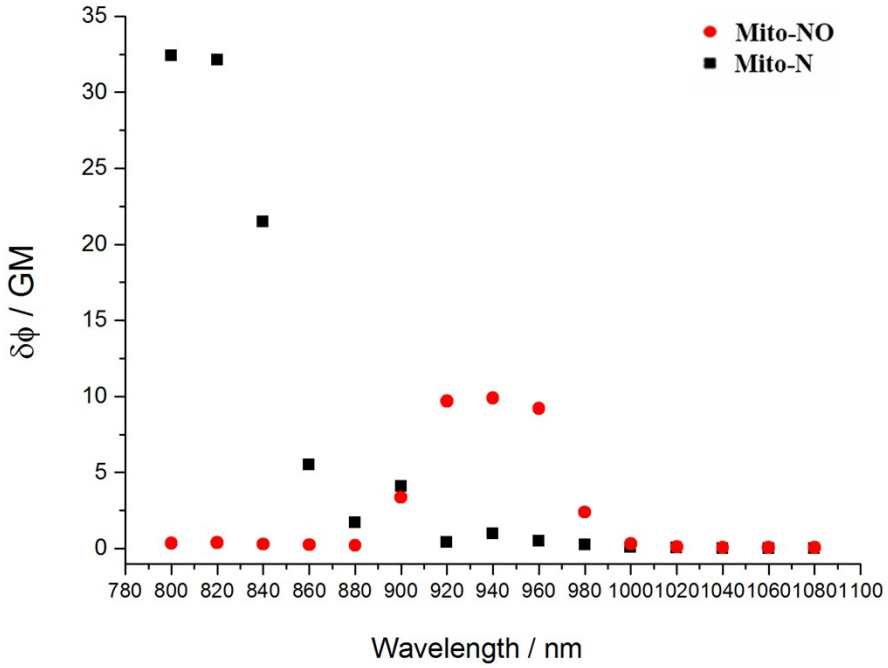


Figure S8. Two-photon action cross section of **Mito-N** and **Mito-NO** (The intensities of the TP induced fluorescence spectra of the samples and reference emitted at 800 – 1080 nm wavelengths were determined) .

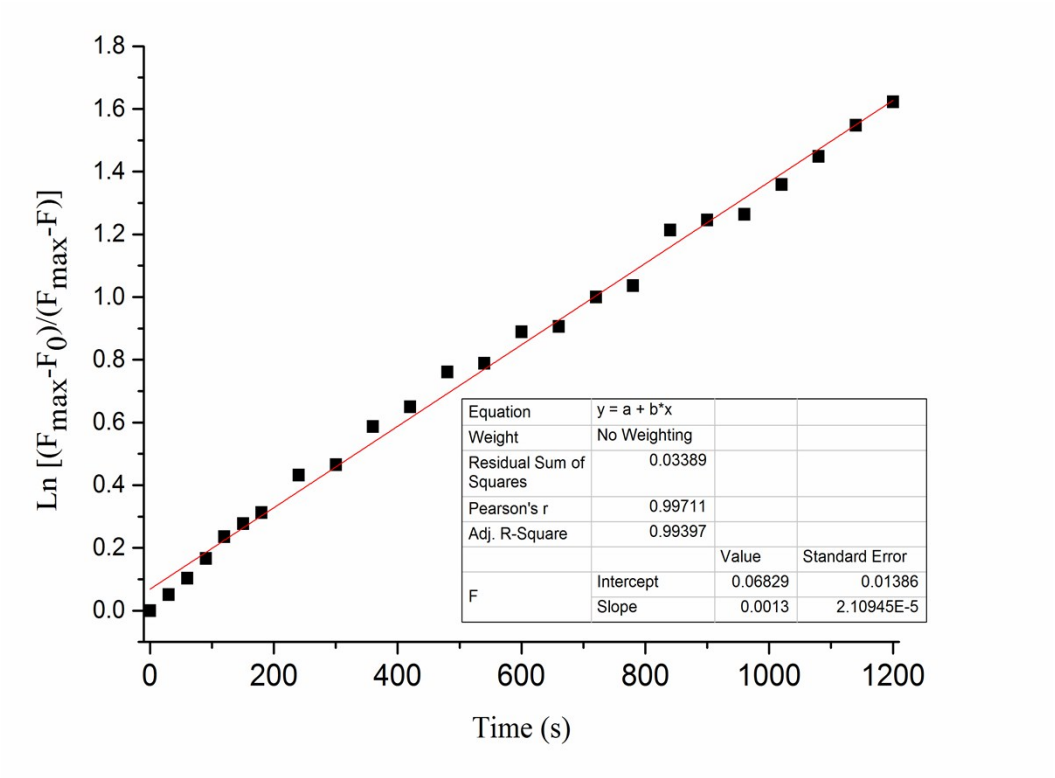


Figure S9. The kinetics of fluorescence spectral responses of **Mito-N** to NO

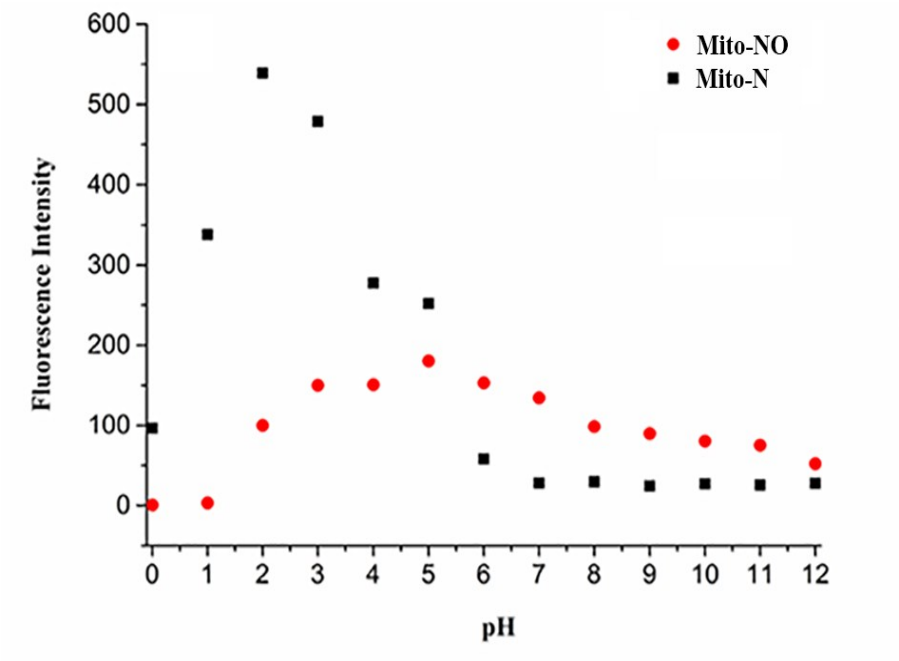


Figure S10. Fluorescence intensity of probe **Mito-N** excited by 500 nm under different pH values in the absence and the presence of NO (pH = 0-4 were collected at the slit width of 5/3 nm, 5-12 were at 5/5 nm).

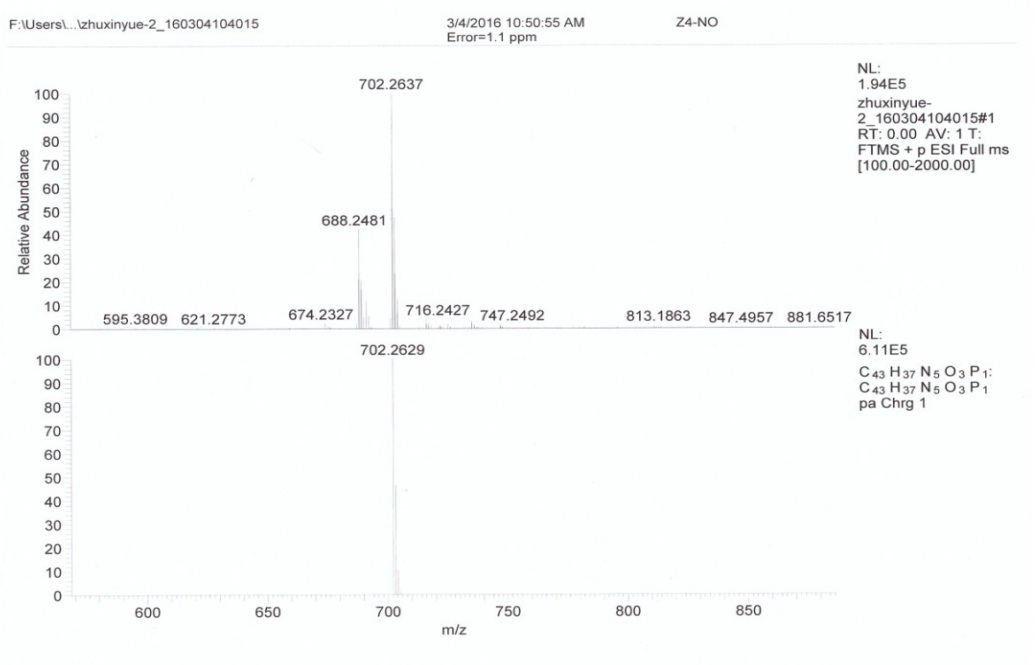


Figure S11. HRMS of **Mito-NO**.

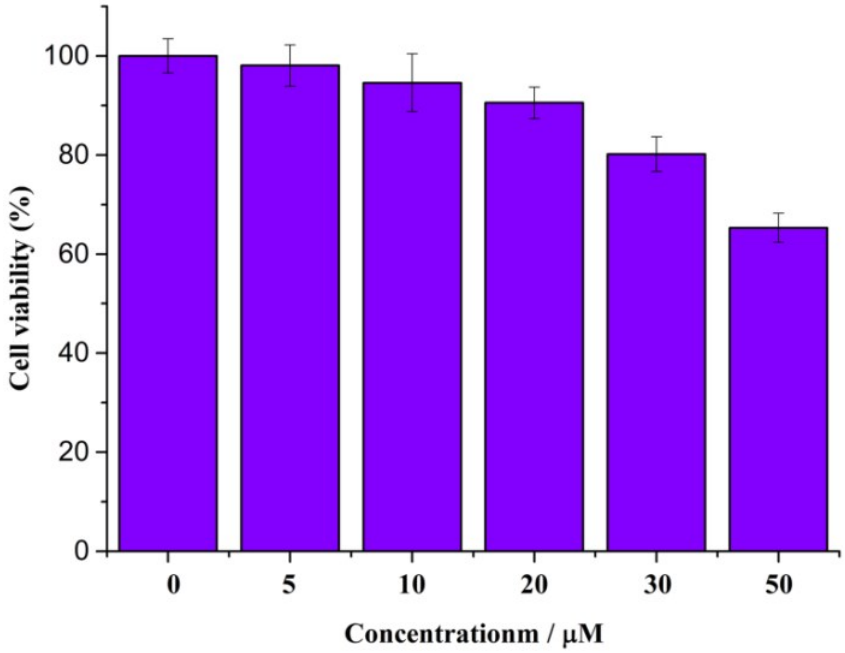


Figure S12. Viability of Raw 264.7 macrophages in the presence of different concentrations of probe **Mito-N**.

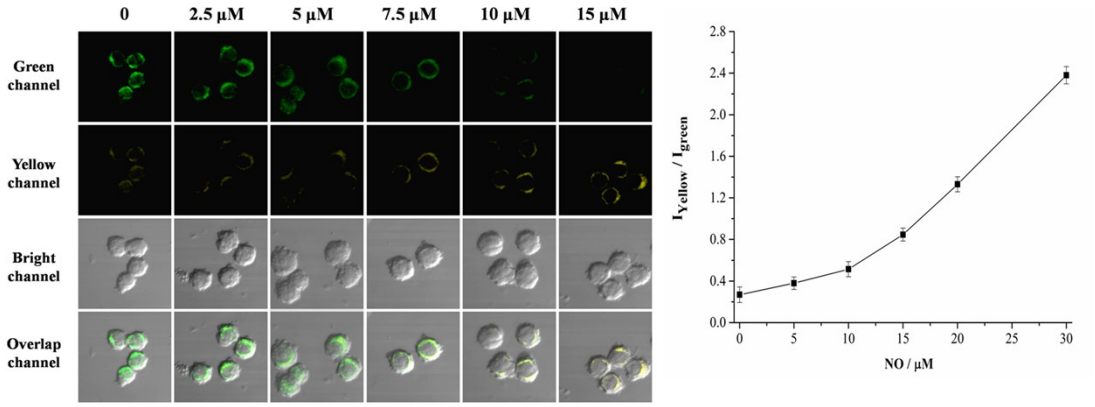


Figure S13. Raw 264.7 macrophages incubated with different concentrations of DEA·NONOate (0, 2.5, 5, 7.5, 10, 15 μM). (Channel: λ_{ex} = 900 nm, λ_{em} = 515-565 (green channel) /575-625 nm (yellow channel))

Nitrite Detection by Probe Mito-N

Nitrite (NO_2^-) is a harmful environment pollutant which is closely related to water quality control, food safety, disease diagnosis, and forensic investigation,[2–8] thus, the quantitative determination of nitrite is very important.

According to previous report,[8] we considered whether probe **Mito-N** can be applied to detect NO_2^-. With this question, the spectroscopic response of **Mito-N** toward NO_2^- was studied first in HCl (1 M). As shown in Figure S 14a, with the increasing of NO_2^-, the UV-Vis absorption at 550 nm showed a good linear relationship (the regression equation was y = 0.003 + 0.013 × [NO_2^-] with a linear coefficient R of 0.9920 and a standard deviation σ of 0.001. The limit of detection (LOD, S/N = 3) was calculated as 1.4 μM) with the linear scope of NO_2^- (0 - 40 μM) (Figure S 14b). And the corresponding fluorescence emission intensity at 600 nm gave a good linear correlation (the regression equation was y = 4.022 + 49.46 × [NO_2^-], R = 0.9920 and a standard deviation σ of 0.19. The limit of detection (LOD, S/N = 3) was calculated as 0.14 μM with the linear scope of NO_2^- (0 - 30 μM)) (Figure S 14c and d). The results proved that **Mito-N** could detect NO_2^- quantitatively both by UV-Vis and fluorescence spectrometry, respectively. What's more, with the increasing concentration of NO_2^-, the color of the solutions changed from yellow to purple significantly, which offered a visual detection of NO_2^-.

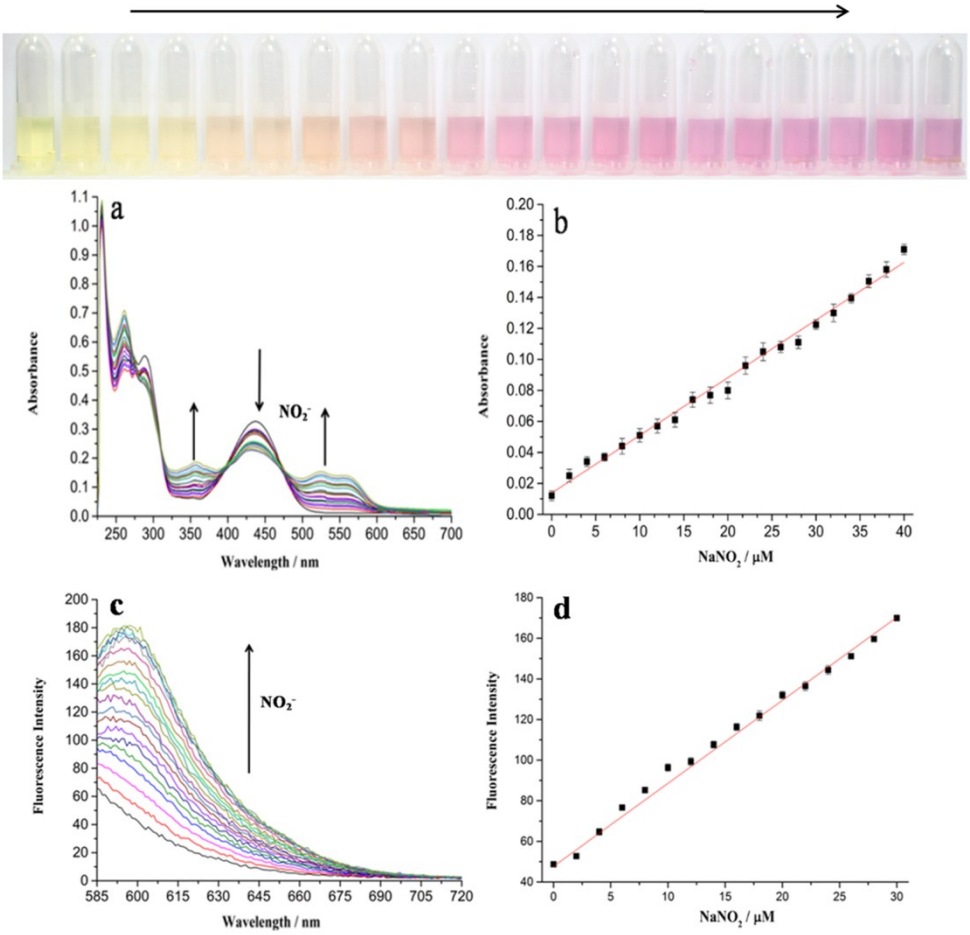


Figure S14. UV-Vis absorption (a) and fluorescence spectra (c) changes of **Mito-N** toward different concentrations of NO_2^-, and the linear relationship between UV-Vis absorption (b) and fluorescence intensities (d) with NO_2^- concentrations. (**Mito-N**: 20 μM, HCl: 1 mol/L, 2‰ DMSO; NO_2^-: 2.0, 4.0, 6.0, 8.0, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40 μM)

Then, in order to evaluate the selectivity of **Mito-N** toward NO_2^-, the fluorescent response upon addition of various analytes was investigated. As shown in Figure S15, only NO_2^- and NO induced the unique color and fluorescence changes, the others did not cause any fluorescence emission change, due to NO converted to nitrite in acid condition under air atmosphere, therefore, **Mito-N** was highly selectivity to NO_2^- without interference from other analytes.

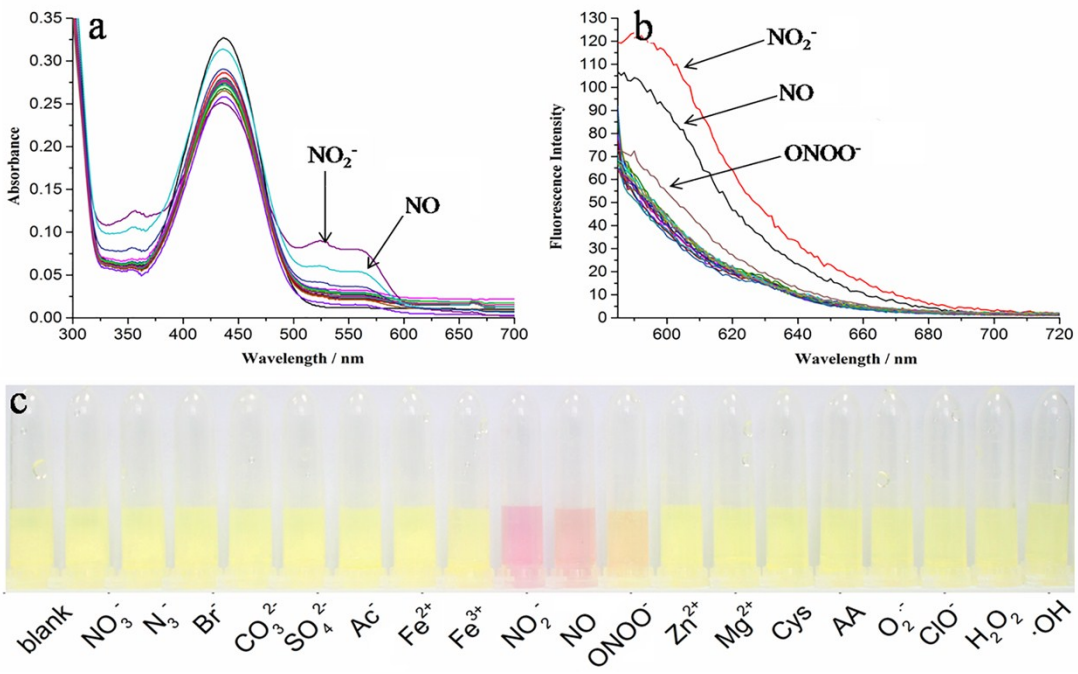


Figure S15. UV-Vis absorption (a) and fluorescence spectra (b) changes of **Mito-N** toward different analytes, and the pictures of the corresponding solutions (c) (**Mito-N**: 20 μM, HCl: 1 mol/L, 2‰ DMSO; analytes: 40 μM)

Based on the above experiments, probe **Mito-N** was further applied to detect the concentration of NO_2^- in real samples, and as control, the same samples were also analyzed by Griess assay. Essentially identical results were obtained with **Mito-N** by both absorption and fluorescence emission based measurements, which were in accordance with the results of Griess assay (Table 1).

Table 1. Determination of NO_2^- concentrations in real samples

Sample	Method	NO_2^- (μM)	Recovery
Yellow River water	Griess assay	2.57±0.27	—
	Mito-N$_{colorimetry}$	2.83±0.02	—
	Mito-N$_{fluorometry}$	3.13±1.28	—
tap water	Griess assay	25.67±0.07	104.26%
	Mito-N$_{colorimetry}$	26.85±0.87	106.27%
	Mito-N$_{fluorometry}$	23.58±0.36	94.30%
pork	Griess assay	51.84±0.21	92.47%
	Mito-N$_{colorimetry}$	59.00±0.01	99.11%
	Mito-N$_{fluorometry}$	61.04±1.65	113.36%
chicken	Griess assay	52.80±0.28	94.70%
	Mito-N$_{colorimetry}$	51.67±0.94	86.01%
	Mito-N$_{fluorometry}$	55.32±3.5	103.15%

NO_2^- concentrations were determined by direct assay for the Yellow River water and using standard samples recovery method for tap water, pork and chicken. Tap water was taken from our lab. Yellow River water was collected from one site of the Yellow River (Lanzhou, China) and filtered through a 0.45 μm membrane filter. Pork and chicken were purchased from a local supermarket and pretreated according to the national standard method (National Standard of the People's Republic of China GB 5009. 33-2010).

The results revealed that **Mito-N** could be successfully applied to the visual detect and quantitative analysis of NO_2^- in real samples and provided visual information. Compared with the traditional methods, probe **Mito-N** was more specific, fast, and more convenient.

References

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