•**Supporting Information**•

Ratiometric photoacoustic imaging of endogenous HNO *in vivo* for assessing prodrug release and liver injury

Xiao-Peng Fan,[a,b,*] Hongzhen Wang,[a] Wen Yang,[b,*] Tian-Bing Ren,[b,*] Lin Yuan[b]

[a.] State Key Laboratory of Medicinal Chemistry and Molecular Diagnosis of the Ministry of Education, College of Chemistry & Materials Science, Hebei University, Baoding 071002, P.R. China

[b.] State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Collaborative Innovation Center for Chemistry and Molecular Medicine, Hunan University, Changsha 410082, China.

[*]E-mail: xiaopengfan@hbu.edu.cn; wenyang@hnu.edu.cn; rentianbing@hnu.edu.cn

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1 Supplemental experimental procedures

Materials. Superoxide ($O_2^{\bullet-}$) was created by the KO_2 in pure dimethyl sulfoxide (DMSO) at 25℃. Peroxynitrite ($ONOO^-$) solution was generated according to previous report.[1] The concentration of peroxynitrite was determined by measuring the absorption of the solution at 302 nm. ^-OCl was generated by NaClO solution. Angeli's salt (AS) was obtained from Sigma-Aldrich Company and was dissolved in DMSO and stored at –20℃ refrigerator. Prodrug cyanamide (CA) was obtained from HEOWNS Company and was dissolved in twice-distilled water for further use. Na_2S and glutathione (GSH) was dissolved in twice-distilled water for further use. Preparation of solutions of anions and metal ions: ($NaNO_3$, $NaNO_2$, $Na_2S_2O_3$, NaCl, $CaCl_2$, KCl, $CuCl_2$, $FeCl_2$, $MgCl_2$, $ZnCl_2$, NiCl, $MnCl_2$) was dissolved in distilled water to afford 100 mmol aqueous solution. The stock solutions were diluted to desired concentrations when needed. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300) columns, both of which were obtained from the Yantai Jiangyou silica gel Development Company Limited. All chemicals were purchased from commercial suppliers and used without further purification, unless indicated otherwise. Solvents used were purified by standard methods prior to use. The L02 cell line was obtained from iCell Bioscience Inc. (Shanghai, China). The fetal bovine serum (FBS) was obtained from the Gemini Bio. (California, USA). Deionized water was used throughout all experiments.

Instruments. Mass spectra was obtained from MALDI-TOF/TOF (Bruker UltrafleXtrem). NMR spectra were measured with a Bruker-400 spectrometer. UV–Vis spectra were recorded on a UV-1800 spectrophotometer (Shimadzu Corporation, Japan). The pH measurements were carried out on a PHS-3C pH meter (INESA instrument). Fluorescence spectra were recorded on Hitachi F-7000 fluorescence spectrometer. The fluorescence imaging was carried out using an IVIS Lumina XR (IS1241N6071) in vivo imaging system. All photoacoustic images were analyzed and collected at various time points by an InVision 256-TF imaging system (iThera Medical).

Spectrometric Studies. UV-vis absorption and florescence spectral experiments. Phosphate Buffered Saline solution PBS was co-solved with chromatographic base ethanol and adjusted pH to 7.4 before use. Absorption spectra and fluorescence emission spectra were obtained with 1.0-cm quartz cells. Compound **FB** and **NF** was dissolved in DMSO in color comparison tube for further use. After dilution to 5 μM with 50% EtOH co-solved PBS buffers, analytes were added. Selectivity experiment of **NF** (5 μM) toward HNO (AS, 8 μM) and other analytes: (1) blank; (2) S^{2-} (20 μM); (3) GSH (200 μM); (4) Na^+ (100 μM); (5) S_2SO_3 (20 μM); (6) Hcy (100 μM); (7) Ca^{2+} (100 μM); (8) Fe^{2+} (100 μM); (9) Cu^{2+} (100 μM); (10) Zn^{2+} (100 μM); (11) K^+ (100 μM); (12) Mn^{2+} (100 μM); (13) Ni^+ (100 μM); (14) Mg^{2+} (100 μM); (15) NO (50 μM); (16) $ONOO^-$ (10 μM); (17) NO_3^- (100 μM); (18) NO_2^- (100 μM); (19) ^-OCl (100 μM); (20) H_2O_2 (100 μM); (21) $O_2^{\bullet-}$ (100 μM); (22) AS (10 μM). The mixtures were equilibrated for 60 min before the absorption intensity of **NF** solution was recorded. The photoacoustic image of **NF** incubated with different AS concentration was also recorded in 50% EtOH co-solved PBS buffers solution.

Computational methods. The density functional theory (DFT) and time-dependent density functional theory (TD-DFT) calculations were conducted by Gaussian 09 program software. The

ground and excited state geometries of dye **FB** and **NF** were optimized at the B3LYP/6-31G (d) level of theory by using a CPCM solvation model with water based on density (SMD).

Cell culture and cytotoxicity studies. Normal hepatocytes (L02) and macrophages (Raw 264.7) line were incubated in DMEM supplemented with 10% fetal bovine serum (FBS). The cultures were maintained at 37 ˚C in a 95% humidified atmosphere with 5 % CO_2. L02 cells were plated in 96-well plates. After methyl thiazolyltetrazolium (MTT) treatment at the given concentration for 4 hours, then treated with 200 μL DMSO and shaker rocking at 37 ˚C for 20 minutes, finally cell viability analysis.

Response of probe NF in living cell. On the one hand, L02 cells was cultured in 100 mL culture flask and incubated overnight. First, the HNO donor (AS, 4 μM) was added in the culture medium and cultured for 10 min. Then the medium was replaced by the freshly mixed medium with probe **NF** (2.5 μM/L) and cultured for 1h. On the other hand, RAW cells were stimulated with 3 μg/mL LPS for 12 h. Subsequently, the LPS-stimulated cells were incubated in the freshly mixed medium with the sterilized cyanamide (CA, 3 mM) and probe **NF** (2.5 μM/L) for 1 h. Finally, all the cells were collected and washed with cold Dulbecco's Phosphate-Buffered Saline (DPBS) twice and centrifuged at 800 r for 5 min to remove excess liquid in a microcentrifuge tube. Cells were centrifuged to obtain cell aggregates for PA imaging, which performed as the same procedure of in vitro PA experiments.

In vivo studies. All animal procedures were performed in accordance with the guidelines for care and use of laboratory animals of Hunan University and experiments were approved by the Animal Ethics Committee of College of Biology (Hunan University). All Female balb/c mice (18-20 g) were operated in accordance with institutional ethics committee regulations and guidelines on animal welfare. The treated mice were divided into different groups and anesthetized using 2% isoflurane in oxygen.

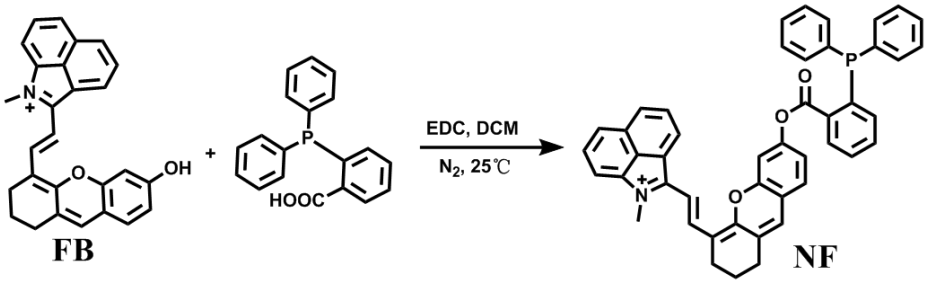
Fluorescence and photoacoustic imaging studies. The drug-induced acute liver injury study was divided into two groups. Mice from the first group were the control group, fasted overnight and received an intraperitoneal injection of saline. Mice from the second group were fasted overnight and received an intraperitoneal injection of 200 mg/kg isoniazide. After PA imaging, the mice were humanely sacrificed and major organs (heart, liver, spleen, lung, and kidney) were harvested, and embedded in culture dish for ex vivo fluorescence imaging. Imaging data were acquired with the excitation wavelength 640 nm, the emission band was at 663-738 nm.

The release evaluation of HNO study was divided into three groups. Mice from the first group were the control group, fasted overnight and received an intraperitoneal injection of saline. Mice from the second group were fasted overnight and received an intraperitoneal injection of 2 mg/kg LPS. Mice from the third group were fasted overnight and received an intraperitoneal injection of LPS and prodrug cyanamide (CA, 15 mg/kg).

All photoacoustic images were analyzed and collected at various time points by an InVision 256-TF imaging system. Images were acquired before and after tail vein injection of 2.5 mg kg^{-1} probe **NF** for different time points. During the imaging, the mice were placed in the prone position in a water bath maintained at 34 ˚C, and anesthesia and oxygen are supplied through a breathing

mask. Data were acquired along the animal with a 0.3 mm step through the liver region, with the imaging wavelengths of 680 and 795 nm. Liver images were reconstructed using back projection followed by linear regression multispectral unmixing. Tumor images were reconstructed by spectral reconstruction analysis.

2 Compound synthesis



Scheme S1. The synthetic route of probe **NF.**

General procedure for the synthesis of probe NF: NIR dye (**FB**) was synthesized according to the previous reported procedure.[1] Compound **FB** (98.0 mg, 0.25 mmol, 1.0 equiv) and compound 2-(Diphenylphosphino)benzoic acid (153.0 mg, 0.5 mmol, 2.0 equiv) was dissolved in CH_2Cl_2 (40 mL) in round-bottom flask. Subsequently, EDC HCl (115.0 mg, 1.2 mmol) and 4-Dimethylaminopyridine (12.2 mg, 0.12 mmol) was added in the mixture solution and was stirred at room temperature for 12 hours under nitrogen protection. The crude solution was suspended in brine (100 mL) and extracted with CH_2Cl_2. The combined organic fractions were concentrated and dried (Na_2SO_4) to afford the crude residue, which was purified via flash chromatography on a silica column to afford the compound **NF** as a black solid (90.4 mg, 0.13 mmol, 53.2% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87 (d, J = 6.4 Hz, 1H), 8.73 (d, J = 6.5 Hz, 1H), 8.45 (d, J = 7.7 Hz, 1H), 8.31 (d, J = 7.0 Hz, 1H), 8.07 (d, J = 7.4 Hz, 1H), 8.00 (d, J = 7.0 Hz, 1H), 7.94 (d, J = 6.5 Hz, 1H), 7.79 – 7.73 (m, 1H), 7.70 – 7.64 (m, 2H), 7.58 (d, J = 7.9 Hz, 1H), 7.53 (s, 1H), 7.48 – 7.45 (m, 1H), 7.39 (d, J = 5.2 Hz, 6H), 7.23 (d, J = 7.4 Hz, 4H), 7.02 – 6.97 (m, 1H), 6.94 – 6.86 (m, 1H), 4.03 (s, 3H), 2.77 – 2.69 (m, 4H), 2.00 – 1.96 (m, 2H). ^{13}C NMR (400 MHz, DMSO-d_6) 174.4, 164.4, 159.6, 152.7, 152.4, 152.3, 140.8, 140.6, 137.1, 137.0, 134.2, 134.1, 133.7, 133.5, 132.5, 132.3, 132.1, 131.5, 131.4, 131.3, 130.9, 129.7, 129.1, 128.9, 128.5, 128.4, 54.9, 35.2, 31.3, 25.1. HRMS: calcd for $C_{46}H_{35}NO_3P^+$ $[M]^+$ 680.2349, found 680.2343.

3 Optical spectra and biological studies

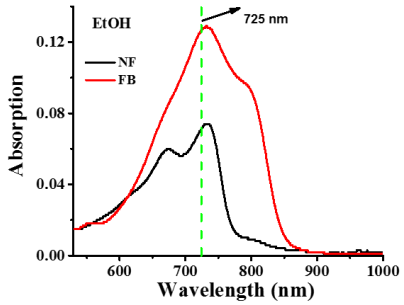


Figure S1. The absorption spectral of probe 5 μM **NF** and 5 μM dye **FB** in EtOH.

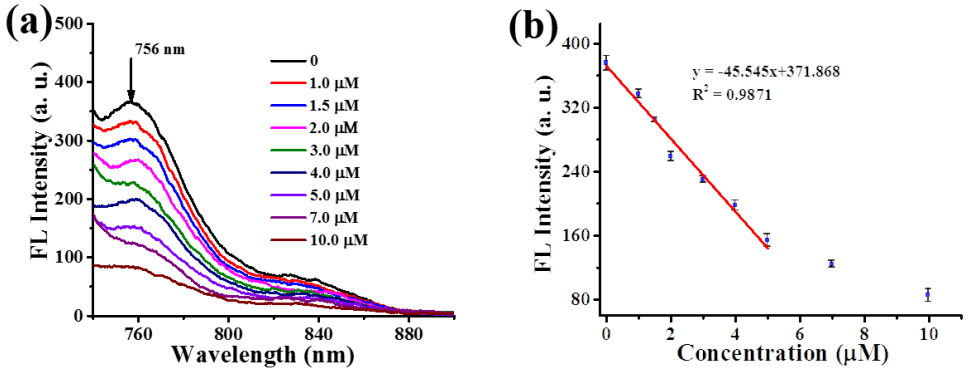


Figure S2. (a) Upon addition of HNO donor (Angeli's salt (AS)), fluorescence spectra changes of probe **NF** (5 μM) in PBS solution (50% EtOH co-solved, pH= 7.4), λ_{ex} = 720 nm; (b) The linear relationship between the AS concentration and the fluorescence intensity at 756 nm.

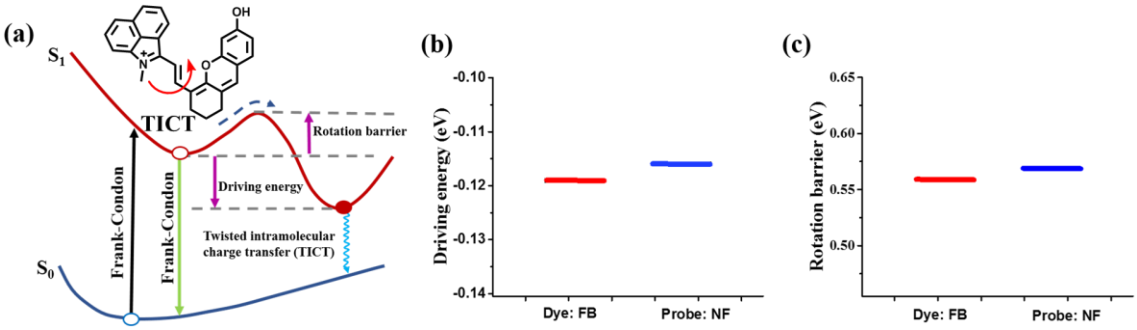


Figure S3. (a) The illustration of the TICT mechanism; (b) Driving energies and (c) rotation barriers of dye **FB** and probe **NF**.

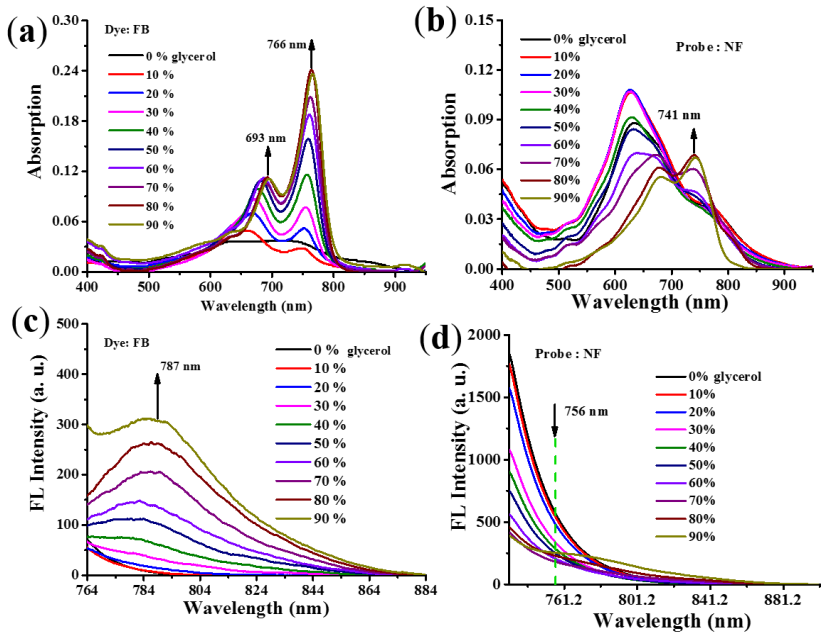


Figure S4. The absorption (a, b) and fluorescence emission (c, d) spectra of 5 μM probe **NF** (λ_{ex}= 720 nm) and Dye **FB** (λ_{ex}= 720 nm) in glycerol co-solved PBS solution (20 mM, pH=7.4).

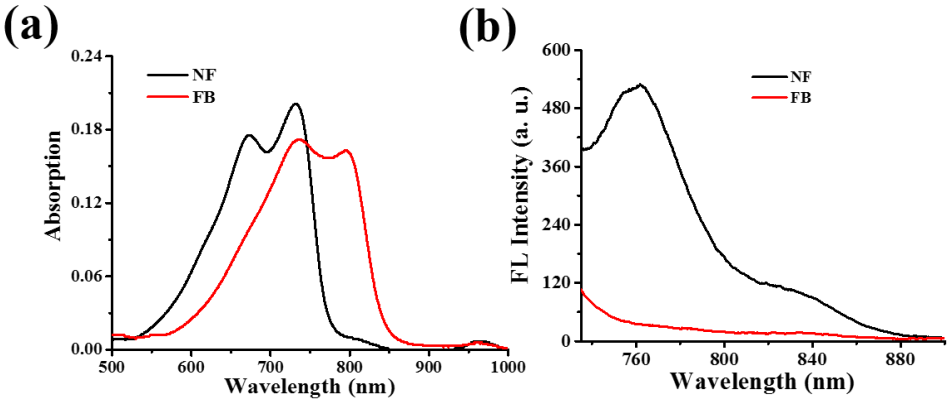


Figure S5. (a) The absorption and fluorescence spectra (b) of probe **NF** and dye **FB** (5 μM) in PBS solution (50% EtOH co-solved, 20 mM, pH= 7.4), λ_{ex} = 720 nm.

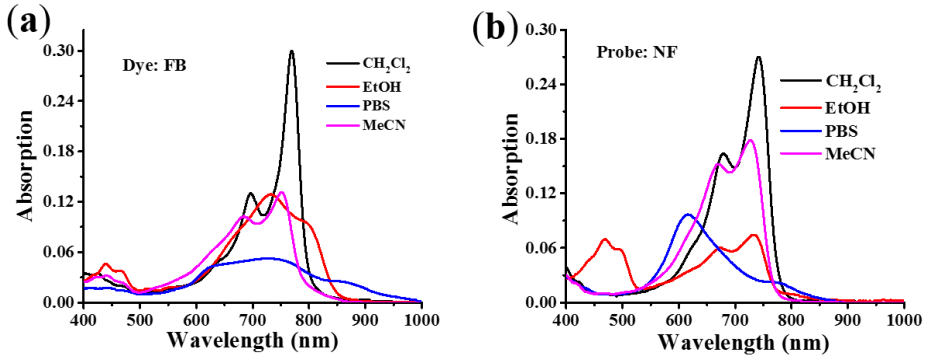


Figure S6. The absorption spectra of 5 μM Dye **FB** (a) and probe **NF** (b) in PBS, MeCN, EtOH and CH_2Cl_2 solvents.

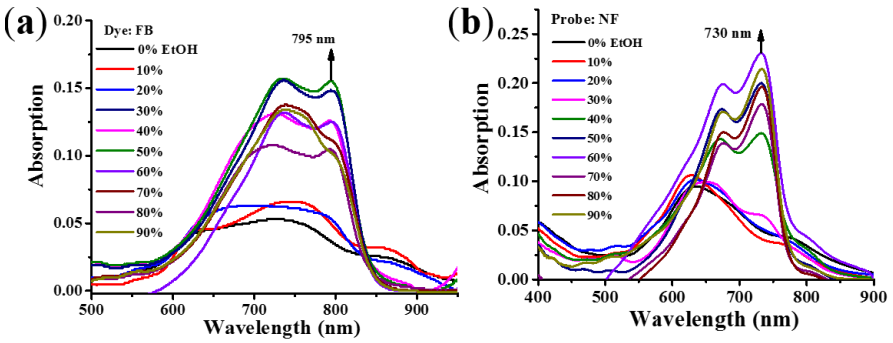


Figure S7. The absorption spectra of 5 μM Dye **FB** (a) and probe **NF** (b) in EtOH co-solved PBS solution (20 mM, pH=7.4).

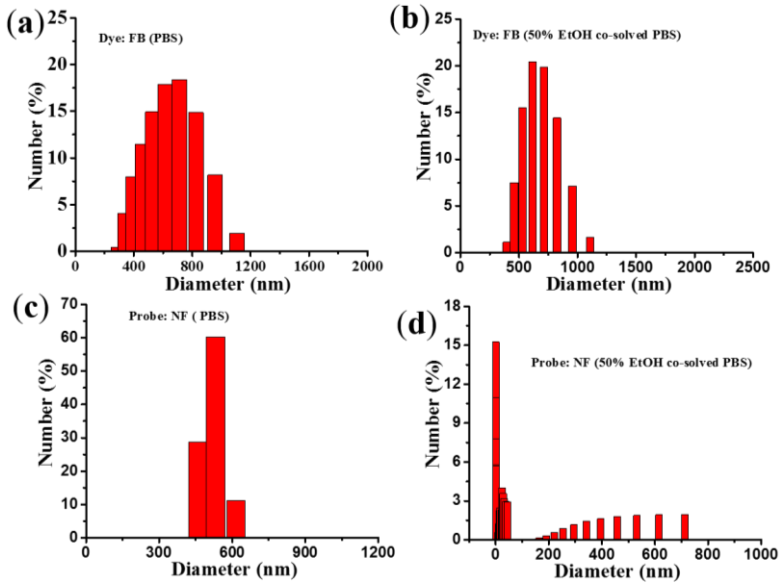


Figure S8. Hydrodynamic analysis of 5 μM Dye **FB** and probe **NF** in PBS (a, c) and EtOH co-solved PBS (b, d) solution.

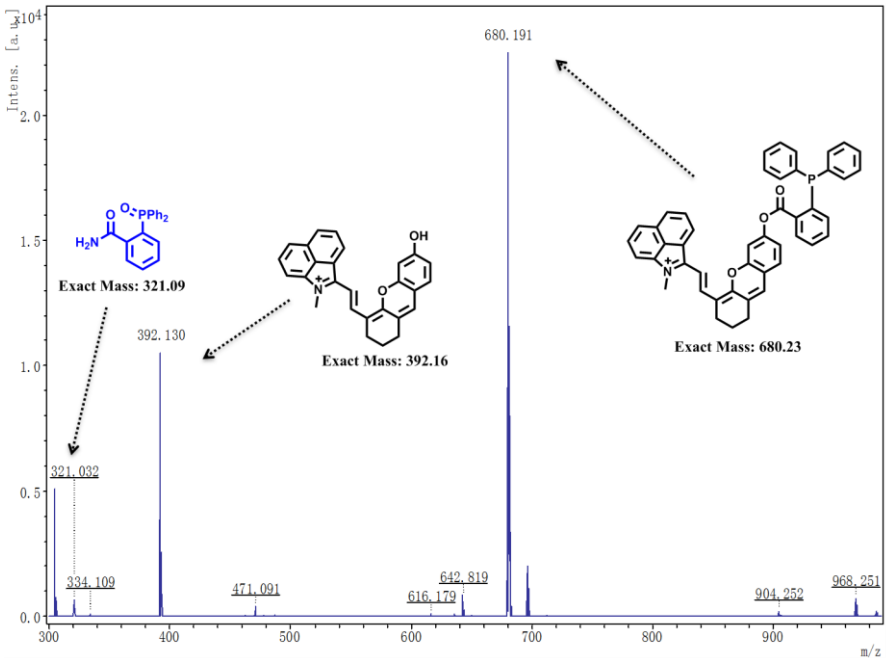


Figure S9. The MALD TOF of probe **NF** in the presence of AS.

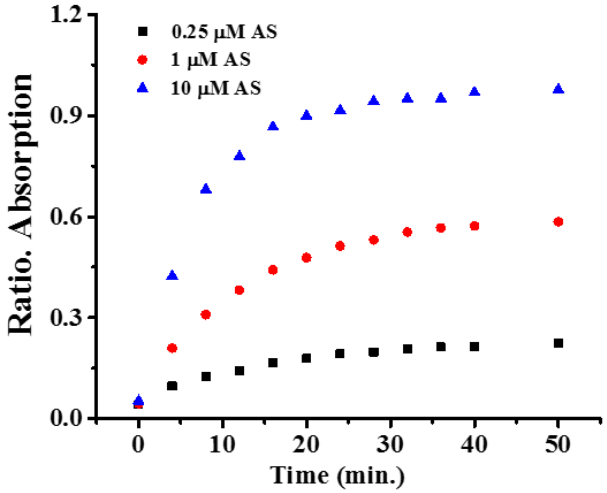


Figure S10. Upon addition of different concentration of AS, the time dependent ratiometric absorption (A_{795}/A_{680}) of probe **NF**.

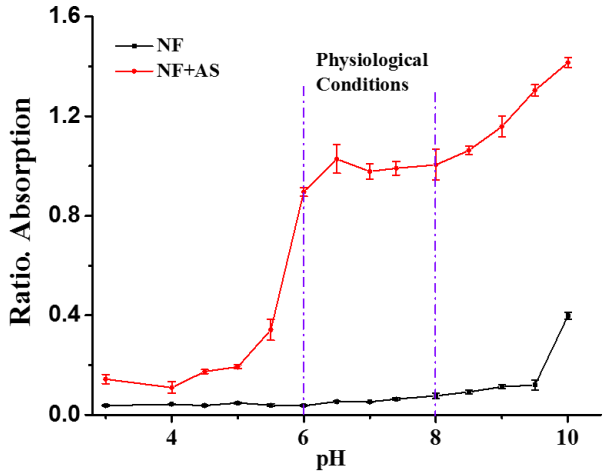


Figure S11. pH-dependent ratiometric absorption (A_{795}/A_{680}) of probe **NF** toward AS in PBS buffer solution (50% EtOH co-solved, 20 mM, pH = 3.0-10.5).

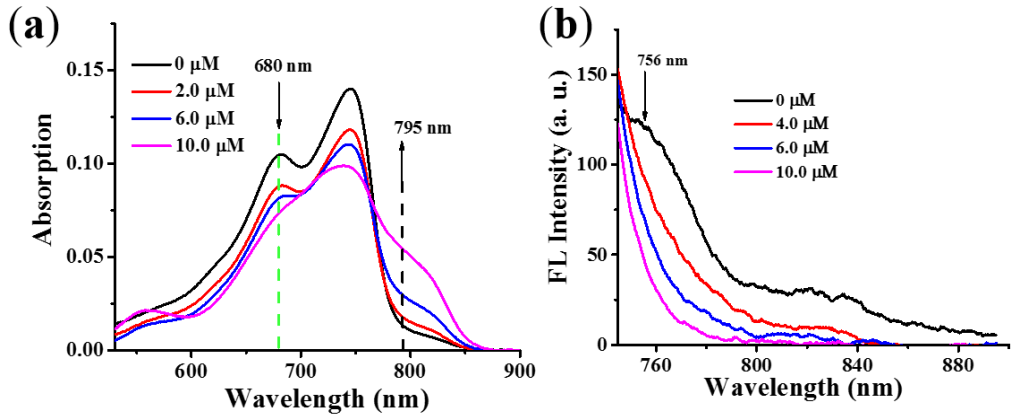


Figure S12. Representative absorption (a) and fluorescence (b) spectra of probe NF (5 μM) upon HNO donor (AS) titration in PBS buffer solution with 1 mM CTAB (20 mM, pH=7.4).

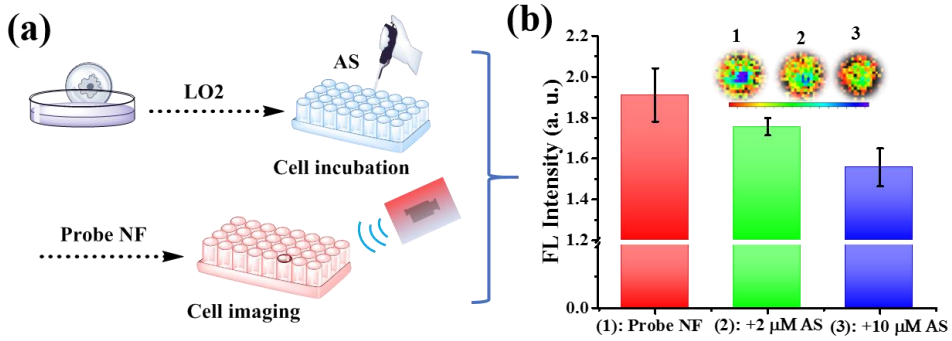


Figure S13. (a) Scheme of endogenous HNO detection in L02 cells pre-incubated with AS and then treated with probe **NF** followed for fluorescence imaging by small animal imager; (a) The fluorescence intensity of 5 μM **NF** response different concentration of AS in 96 well.

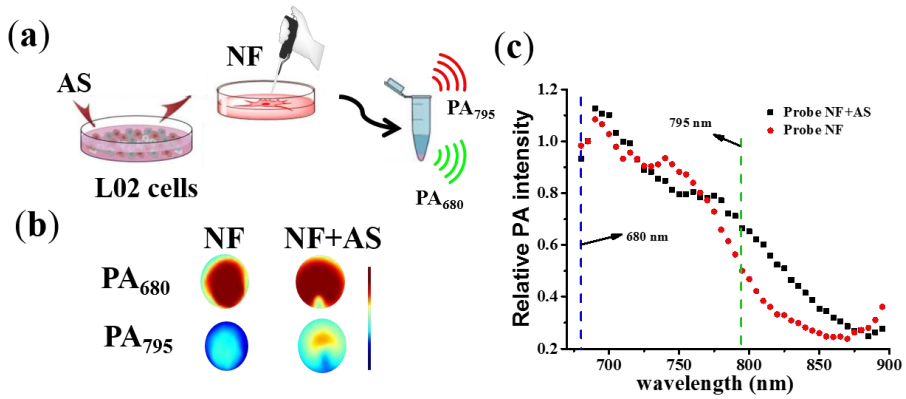


Figure S14. (a) Scheme of endogenous HNO detection in L02 cells pre-incubated with AS, and then treated with probe **NF** followed by concentration for ratiometric PA imaging at 680 and 795 nm; (b) Representative PA images of probe **NF** in L02 cells pre-treated with AS (4 μM); (c) Relative PA intensity spectra of probe NF in the absence or presence of AS (4 μM).

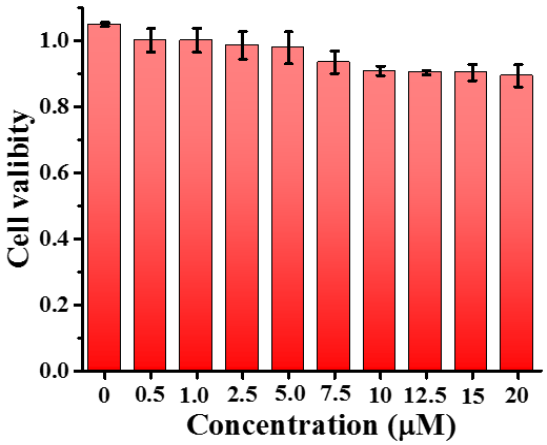


Figure S15. Cytotoxicity of probe **NF** co-incubated with L02 cells by MTT assay.

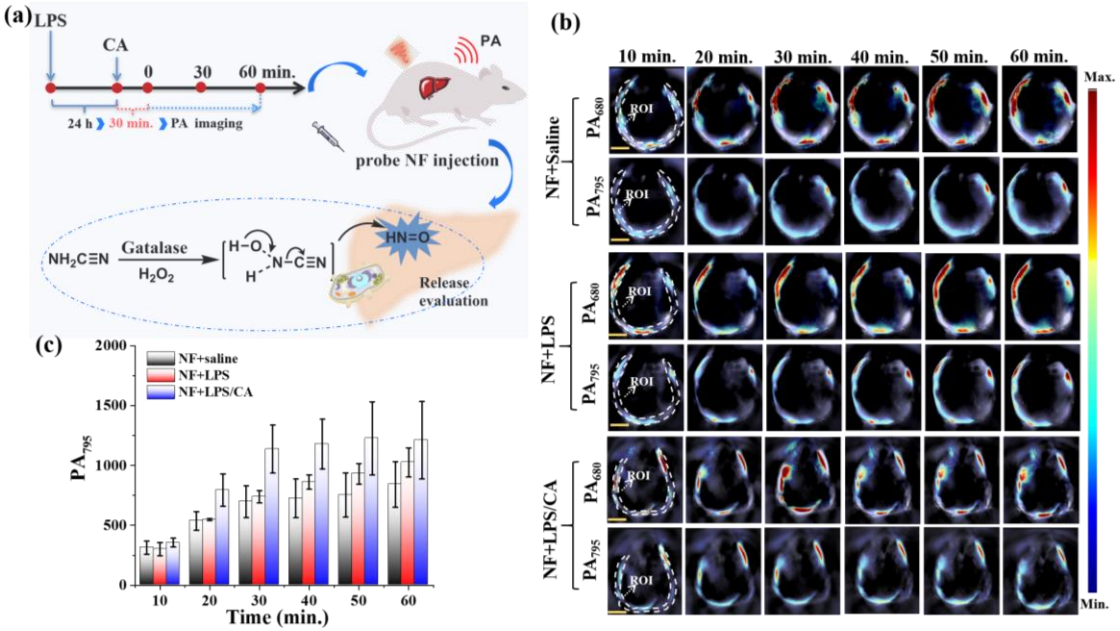


Figure S16. (a) Schematic diagram of probe **NF** for ratiometric PA imaging to evaluate HNO release in mouse model pre-treated with LPS and CA after intraperitoneal injection; (a) PA image at 745 and 815 nm of liver tomographic images upon **NF**+LPS and **NF**/LPS+CA treatment respectively, Scale bar: 5 mm; (c) the PA intensity at 795 nm (PA_{795}) in the ROI of liver at different time points.

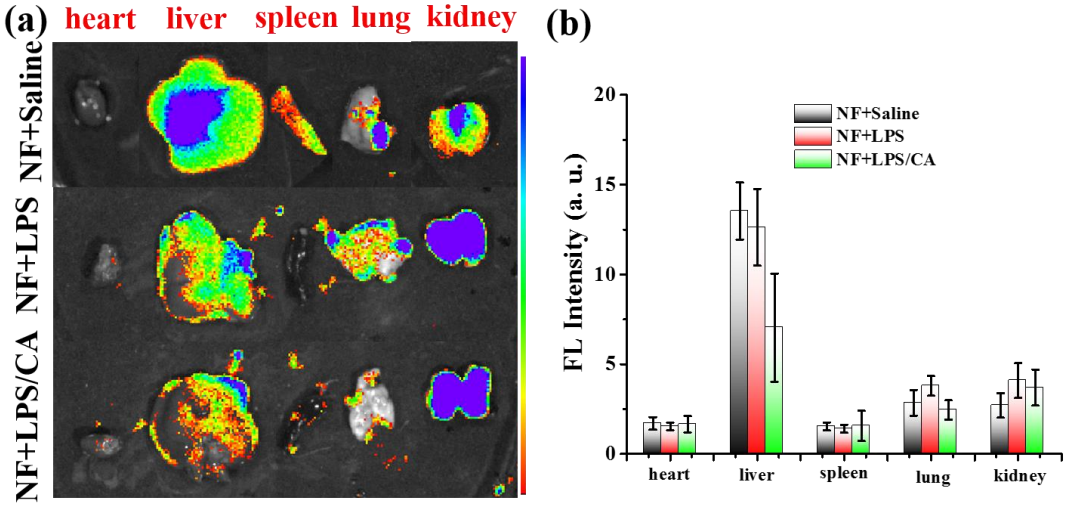


Figure S17. (a) Ex vivo imaging of separated organs sacrificed from the mice pretreated with **NF**, **NF**+LPS and **NF**+LPS/CA; (b) The fluorescence intensity of the corresponding dissected organs (λ_{ex} = 640 nm, λ_{em} = 663−738 nm).

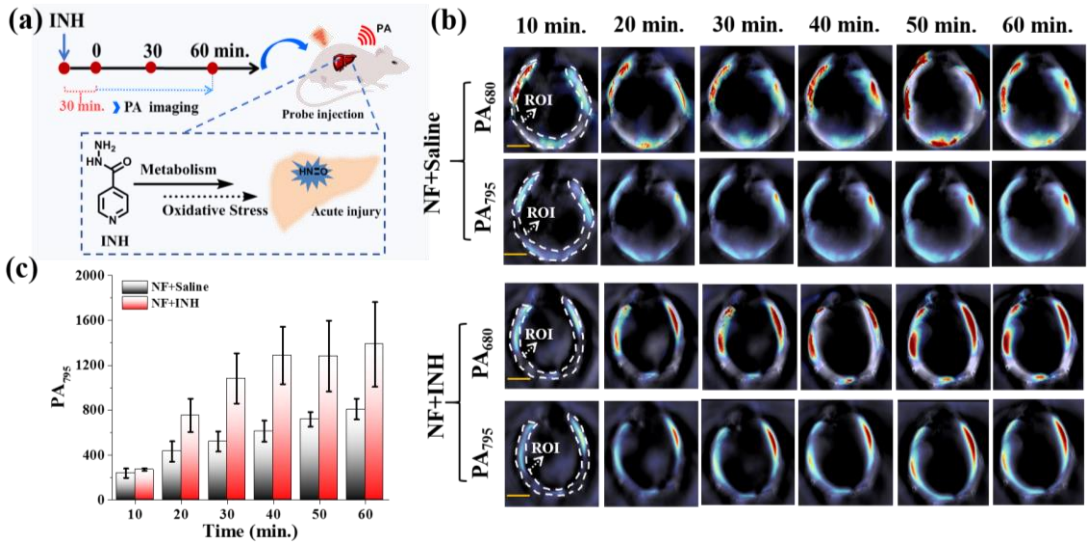


Figure S18. (a) Schematic diagram of probe **NF** for ratiometric PA imaging of HNO in INH induced liver injury mouse model after intravenous injection; (b) PA image at 680 and 795 nm of liver tomographic images upon **NF**+saline and **NF**+INH treatment respectively, Scale bar: 5 mm; (c) The PA intensity at 795 nm (PA_{795}) in the ROI of liver at different time points.

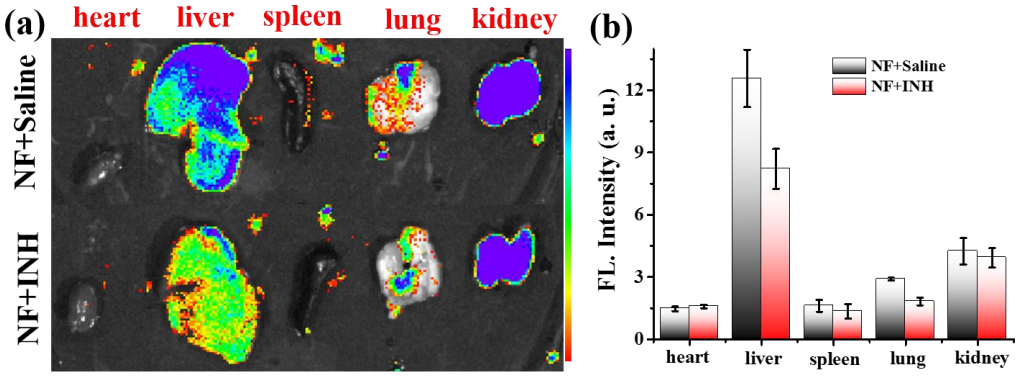


Figure S19. (a) Ex vivo imaging of dissected organs sacrificed from the mice in saline and isoniazide (INH) group; (b) The fluorescence intensity of the corresponding dissected organs (λ_{ex} = 640 nm, λ_{em} = 663−738 nm).

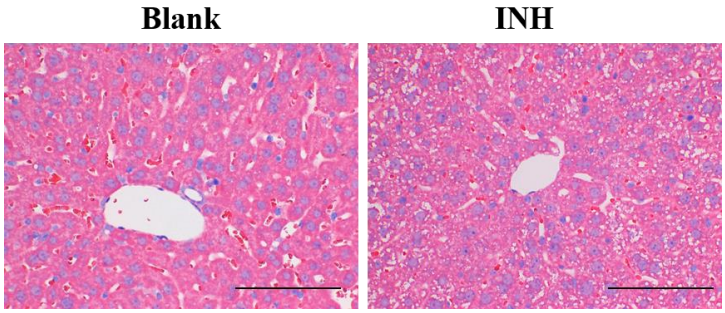


Figure S20. Representative histology of the livers of mice treated with saline or INH after 90 min. Scale bar: 100 μm.

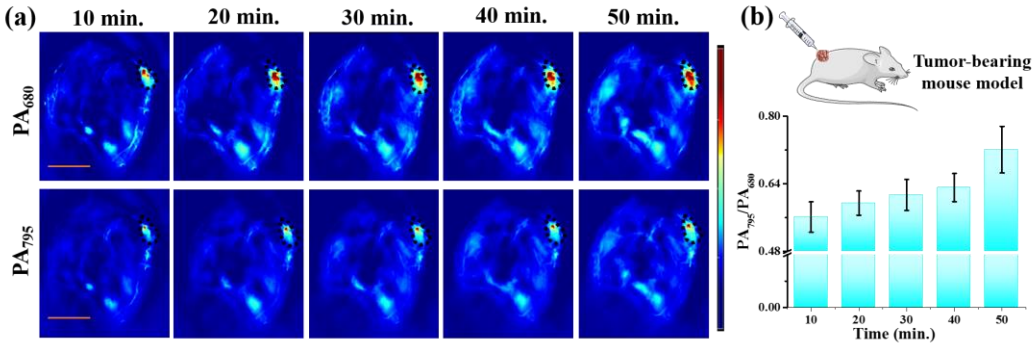


Figure S21. (a) PA image at 680 and 795 nm of tumor tomographic images upon **NF** treatment after intratumor injection, Scale bar: 5 mm; (b) PA intensity at 795 nm of the ROI of tumor tomographic images; (c) Ratiometric PA value (PA_{795}/PA_{680}) in the ROI of tumor at different time points.

4 NMR spectra

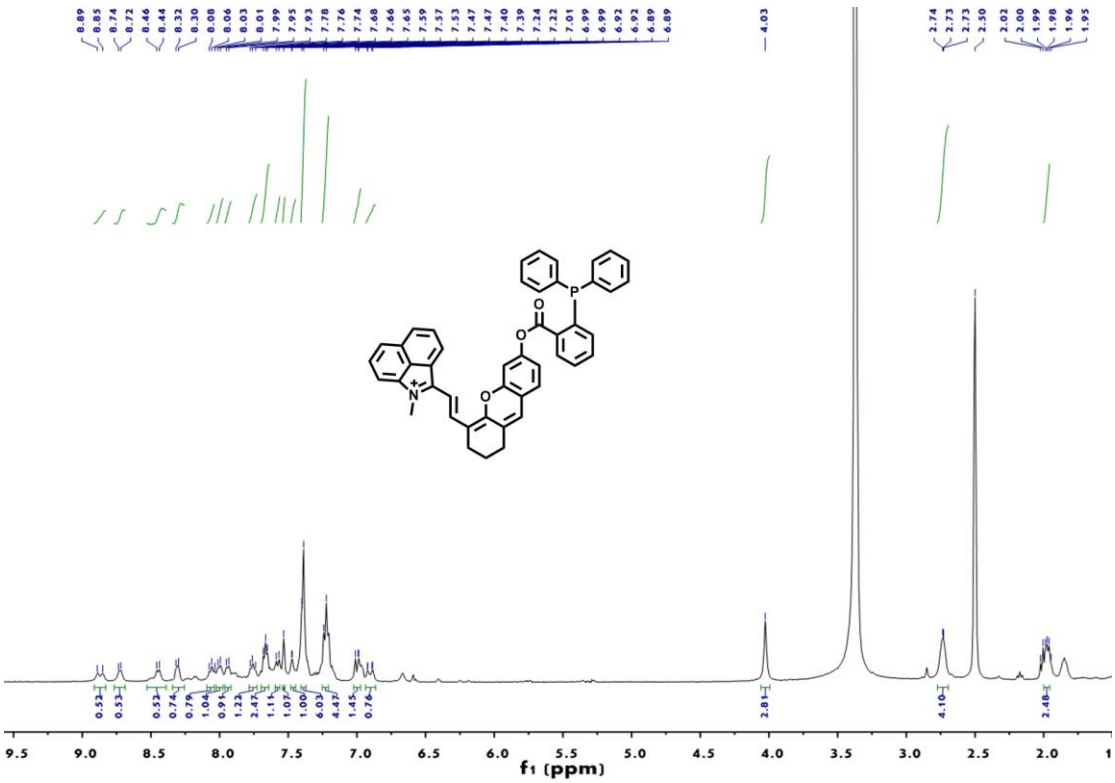


Figure S19. The ^{1}H NMR spectra of probe **NF** (DMSO-d_6).

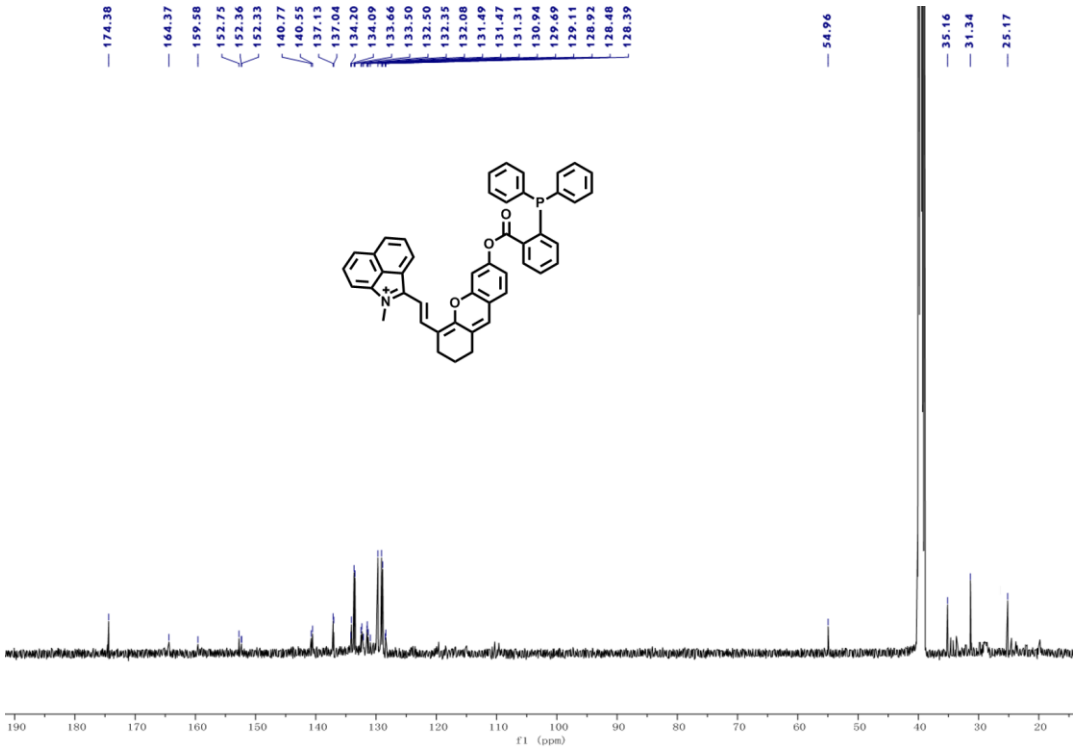


Figure S20. The ^{13}C NMR spectrum of probe **NF** (DMSO-d_6).

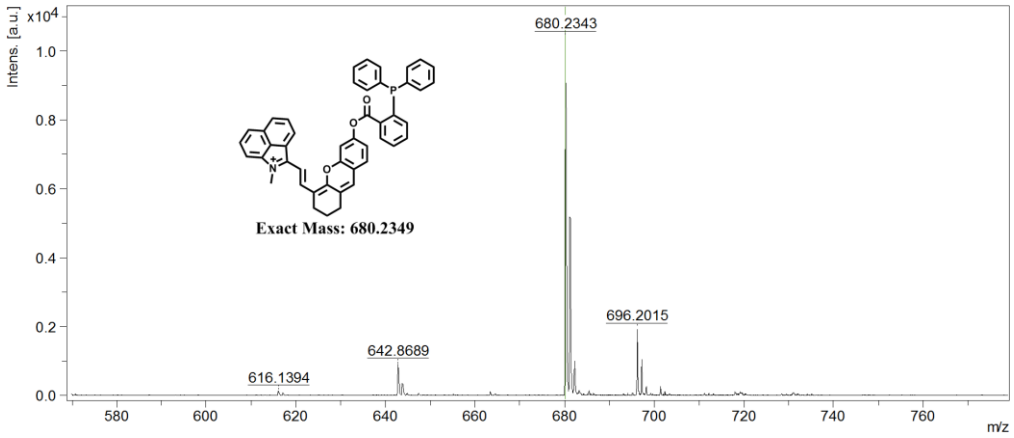


Figure S21. MALD-TOF spectrum of probe **NF**.

5 References

1. D. Cheng, W. Xu, L. Yuan and X. Zhang, *Anal. Chem.*, 2017, **89**, 7693-7700.
2. Z.-M. Yang, Q.-Y. Mo, J.-M. He, D.-L. Mo, J. Li, H. Chen, S.-L. Zhao and J.-K. Qin, *ACS Sens.*, **2020**, **5**, 943-951.