Supporting Information for

Shedding Light on $ONOO^-$ Detection: The Emergence of a Fast-Response Fluorescent Probe for Biological Systems

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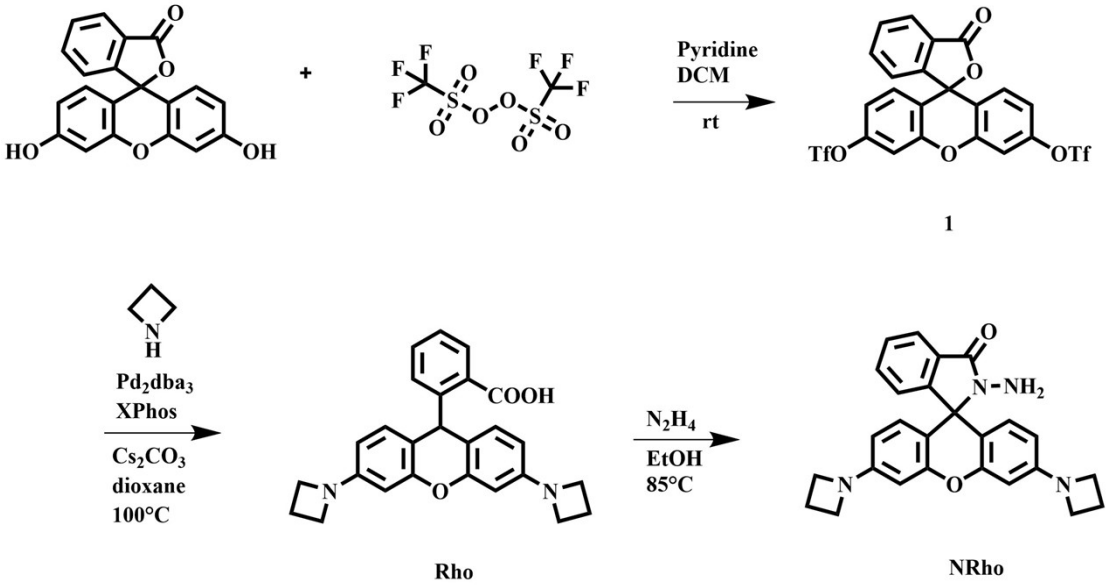
Materials and instruments

All reagents and materials were purchased from commercial companies and used without further purification unless otherwise stated. Twice Distilled water was used in all experiments. 3',6'-Dihydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one, Trifluoromethanesulfonic anhydride, pyridine, dichloromethane (DCM), petroleum ether (PE), ethyl acetate (EA), Tris(dibenzylideneacetone)dipalladium, 2-(Dicyclohexylphosphino)-2',4',6'-tri-i-propyl-1,1'-biphenyl, Cesium carbonate, Azetidine, 1,4-Dioxane, methanol (MeOH), Hydrazine hydrate, ethanol (EtOH), Phosphate Buffered Saline (PBS), Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, HS^-, K^+, Na^+, F^-, Br^-, NO_2^-, OAc^-, S^{2-},PO_4^{3-}, SCN^-, CO_3^{2-}, HSO_3^-, $S_2O_3^{2-}$, SO_3^{2-}, SO_4^{2-}, H_2O_2, ClO^-,SO_2, Aln, Ser (Serine), Asp (Asparagicacid), His (Histidine), GSH (Glutathione), Cys (Cysteine), a-D-Glu (a-D-Glucose), Hcy(homocysteine), and $ONOO^-$. All aqueous solutions were prepared with ultra-pure water obtained from a Milli-Q water purification system (18.2 MΩ cm).

^1H NMR and ^{13}C NMR spectra were obtained on a Bruker Avance III HD 500 MHz NMR spectrometer (Germany). High resolution mass spectrometric (HRMS) analyses were measured on Aglient 6550 Q-TOF. The absorbance was recorded by ultraviolet-visible absorption spectrometry (UV-2700, Shimadzu) or microplate reader (TransGen Biotechnology). TLC analysis was carried out on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both were purchased from the Qingdao Ocean Chemicals. Cells were photographed under the microscope (RVL-100-G, USA Discover-Echo, 60×). The imaging of mice was performed with

Small Animal Imaging System (IVIS Lumina III).



Scheme S1. Synthetic route of probe NRho.

Synthesis of compound 1

3',6'-Dihydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one（0.6 mmol , 200 mg, 1 eq）was dissolved in 10 mL dichloromethane, and pyridine(380 mg, 4.2 eq, 8 eq)was dropped into the solution and stirred at 0 °C for 5 min, Trifluoromethanesulfonic anhydride (2.4 mmol, 716 mg, 4 eq) was added to the solution and reacted at room temperature for 21 h. The mixture was evaporated under reduced pressure and purified by column chromatography (PE: EA = 30: 1) to give a White solid (150 mg, 42% yield).

Synthesis of compound Rho

Compound 1 (0.54 mmol, 321.84 mg, 1 eq) , Tris (dibenzylideneacetone)dipalladium (0.05 mmol, 49 mg, 0.1 eq) , 2-(Dicyclohexylphosphino)-2',4',6'-tri-i-propyl-1,1'-biphenyl (0.162 mmol, 119.7 mg, 0.3 eq) , Cesium carbonate (1.51 mmol, 492.6 mg, 2.8 eq) and Azetidine (1.3 mmol, 80 μL, 2.4 eq) was dissolved in 10 mL 1,4-Dioxane,

The mixture was stirred at 100 ˚C for 18 h, Cool to room temperature and Dilute with methanol The mixture was evaporated under reduced pressure and purified by column chromatography (DCM: MeOH = 20: 1) to give a pink solid (100 mg, 45% yield).

Preparation of Solutions of probe NRho and Analytes

Unless otherwise stated, all tests are carried out according to the following procedures. A probe solution of **NRho** (1.0 mM) was prepared in DMSO. After the final volume was adjusted to 10 mL with PBS buffer, placed at 37 ℃ for a moment. Then 2 mL portion was transferred to a 1 cm quartz cell to measure absorbance and fluorescence. All fluorescence measurements were made on Hitachi F4600 Fluorescence Spectrophotometer. By adding the minimum volume of sodium NaOH (0.1 M) or HCl (0.2 M), the pH of the solution changed slightly.

Determination of the detection limit

The detection limit was determined using the fluorescence titration. To get the slope, the fluorescence intensity (at 579 nm) was plotted as the $ONOO^-$ concentration increased. The detection limit was obtained using the equation:

$$\text{Detection Limit} = 3\sigma/k$$

Where σ is the standard deviation of blank measurement (σ = 16.0266), k is the slope between the fluorescence intensity versus the concentrations of $ONOO^-$ (k = 19.62939), so the detection limit is 2.45 μM.

Culture and preparation of HeLa, RAW267.4, HepG2, 4T1, and Cos 7 cells

HeLa, RAW267.4, HepG2, 4T1 and Cos 7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum (FBS) in 5% CO_2 and 95% air at 37 ℃. Before the experiment, HeLa, RAW267.4, HepG2, 4T1 and Cos 7 cells were inoculated in a 35 mm glass-bottomed culture dish with a density of 2×10^5 cells per dish, and then cultured in 2 mL culture medium at 37℃ in an incubator containing 5% CO_2 and 95% air for 24 hours. During this period, cells will adhere to the glass surface. In use, HeLa, RAW267.4, HepG2, 4T1 and Cos 7 were treated with **NRho** at 37℃ for 5 minutes.

Cytotoxicity assays

Cells were inoculated into 96-well plates, and probe **NRho** (95% DMEM and 5% DMSO) of 0, 5, 10, 20, and 30 μM (final concentration) were added respectively. Subsequently, the cells were cultured at 37 °C in an atmosphere of CO_2 (5%) and air (95%) for 24 hours. Then cells were washed with PBS buffer and DMEM medium was added. Next, MTT (10 μL, 5 mg/mL) was injected into each well and incubated for 4 hours. Treatment with sodium dodecyl sulfate solution (100 μL) in H_2O-DMF mixture produced purple methyl. The viability of cells was determined by assuming that the viability of cells without **NRho** was 100%.

Preparation of cell imaging experiment.

Visualization of exogenous $ONOO^-$ in RAW264.7 cells

The RAW267.4 cells were plated at 1×10^5 cells / mL suspension in μ-slide 8 well and allowed to culture overnight, respectively. Then the cells were treated with $ONOO^-$ (20, 60 and 80 μM) for 30 min with fresh culture medium. RAW267.4 cells untreated

with $ONOO^-$ were used as control. 10 μM of **NRho** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

Visualization of endogenous $ONOO^-$ in RAW264.7 cells

The RAW267.4 cells were plated at 1 × 105 cells / mL suspension in μ-slide 8 well and allowed to culture overnight, respectively. Then the cells were treated with LPS (1 μg/mL) for 1 hours with fresh culture medium. RAW267.4 cells untreated with LPS were used as control. 10 μM of **NRho** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

Detection of $ONOO^-$ in cancer cells

Normal Cos 7 cells were used as control group. 10 μM of **NRho** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

The HeLa cells were plated at 1 × 10^5 cells / mL suspension in μ-slide 8 well and allowed to culture, respectively. normal Cos 7 cells were used as control. 10 μM of **NRho** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

The 4T1 cells were plated at 1 × 10^5 cells / mL suspension in μ-slide 8 well and allowed to culture, respectively. normal Cos 7 cells were used as control. 10 μM of **NRho** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

The HepG2 cells were plated at 1 × 10^5 cells / mL suspension in μ-slide 8 well and

allowed to culture, respectively. Normal Cos 7 cells were used as control. 10 μM of **NRho** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

Preparation of tumor-bearing mice for imaging.

Female mice were fed normally for 3 days, HepG2 cells were grafted into a flank of the nude mouse to produce tumor models and on day 13 post injection, the tumor was obtained. Then the tumor-bearing mice was utilized for imaging.

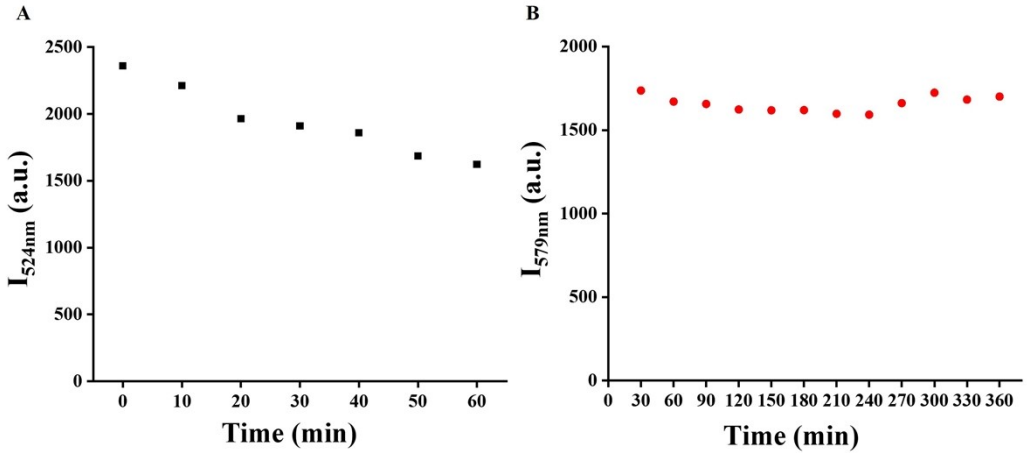


Figure S1. (A) Time-dependent fluorescence intensity of dye (fluorescein, 5 μM) in PBS changed with time (0-60 min) at 524 nm. (B) Time-dependent fluorescence intensity of NRho (5 μM) on $ONOO^-$ (100 μM) changes with time (0-360 min) at 579 nm.

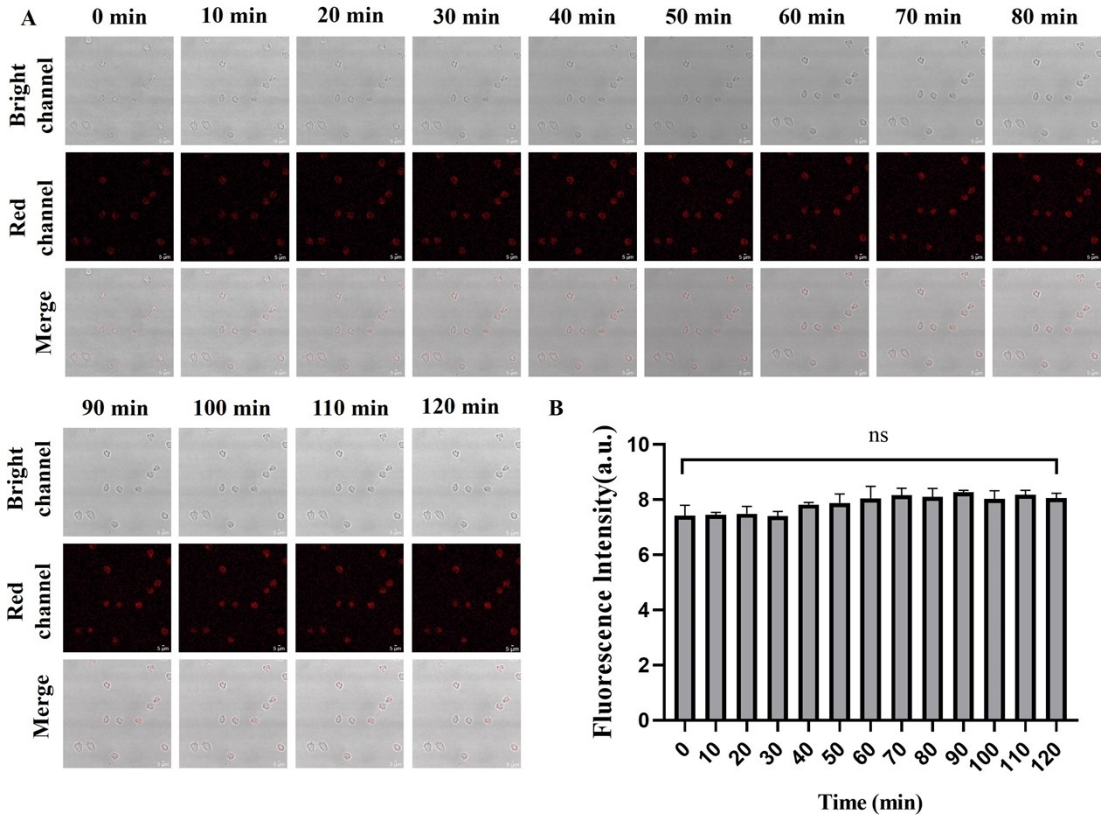


Figure S2. (A) Confocal fluorescence microscopy of probe NRho with time variation (0-120 min) in 7702 cells. (B) Fluorescence intensity plot from (A).

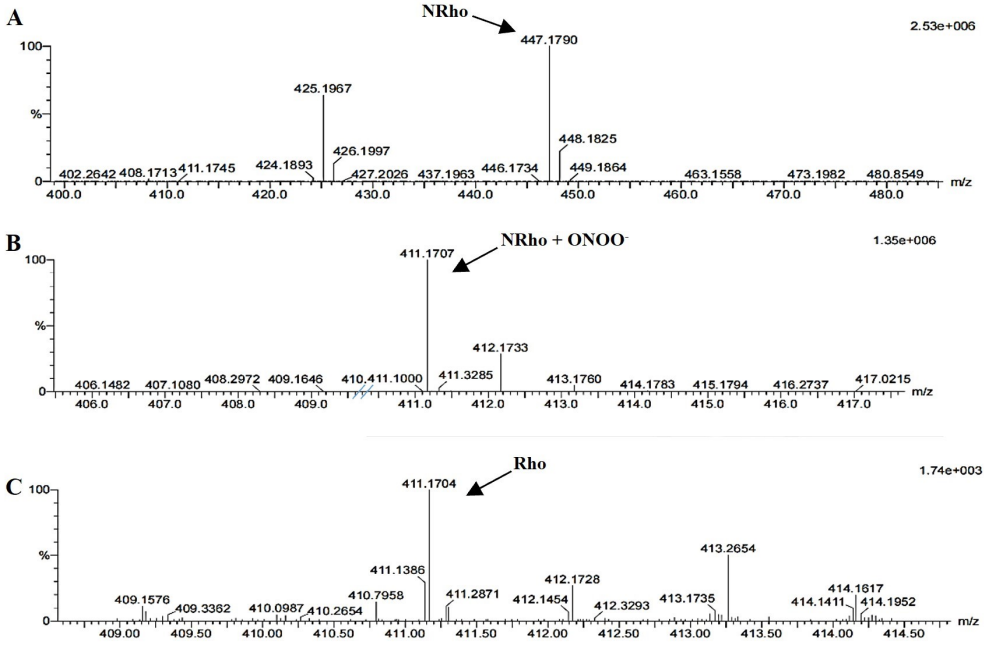


Figure S3. Mass spectrogram of (A) NRho, (B) NRho reacted with $ONOO^-$, (C) Rho.

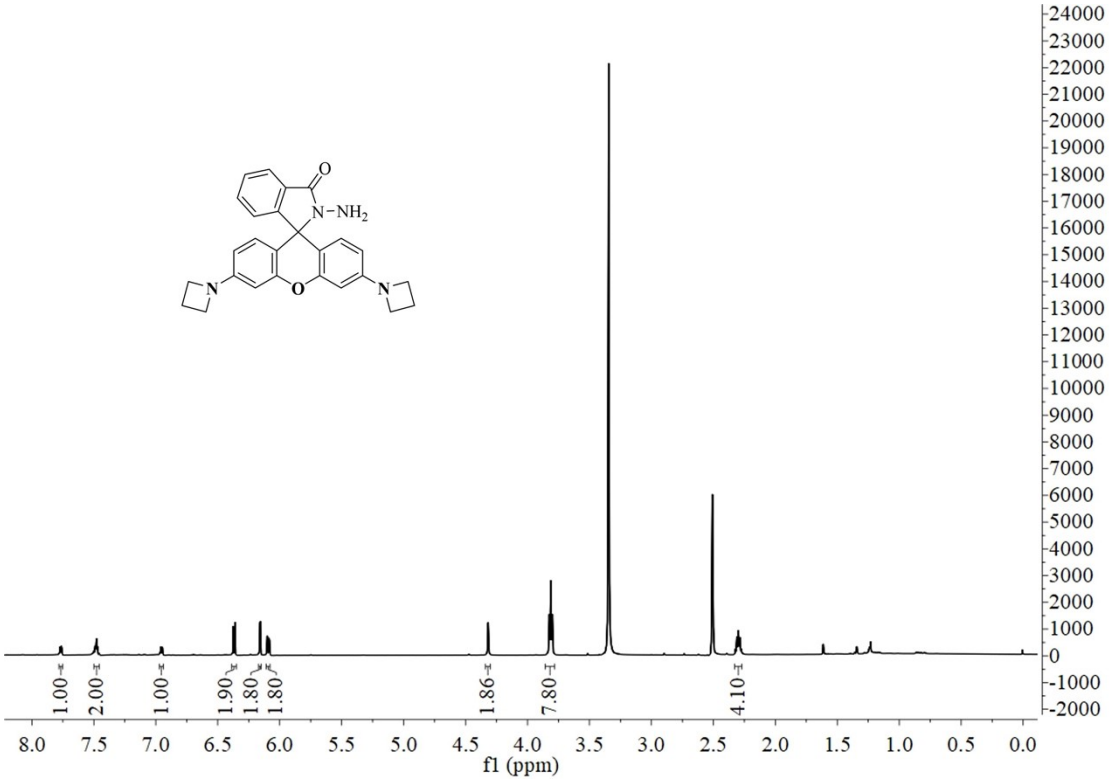


Figure S4. ^{1}H NMR (DMSO-*d6*) spectrum of probe NRho.

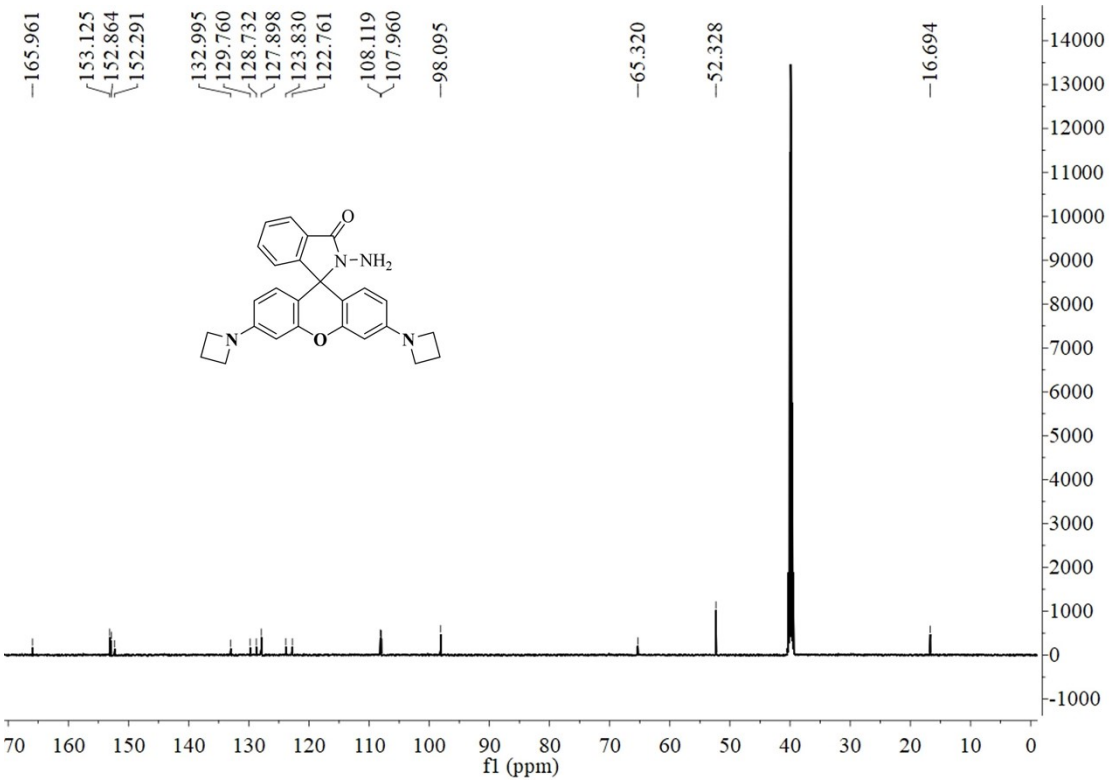


Figure S5. ^{13}C NMR (DMSO-*d6*) spectrum of probe NRho.

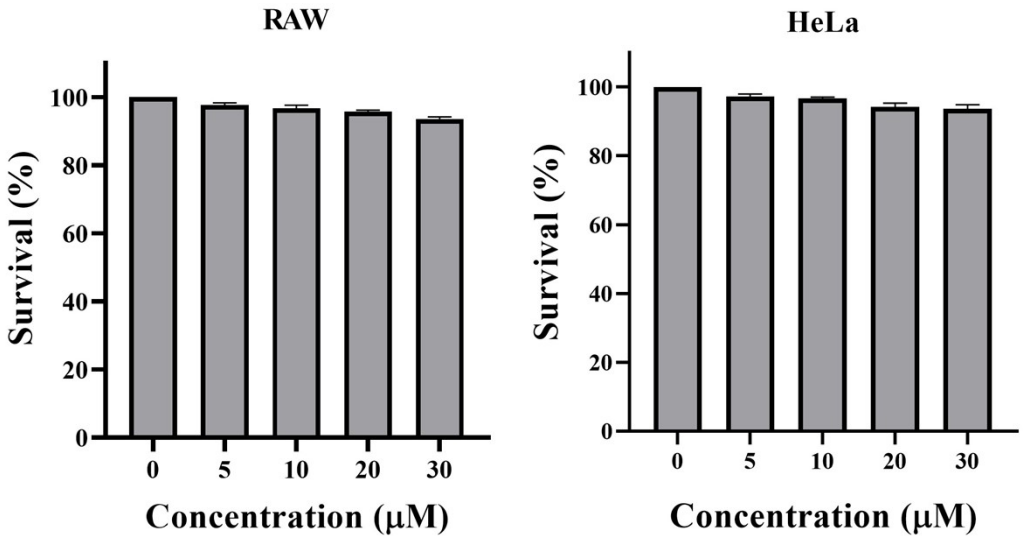


Figure S6. Cytotoxicity of probe NRho in RAW267.4 and HeLa cells.

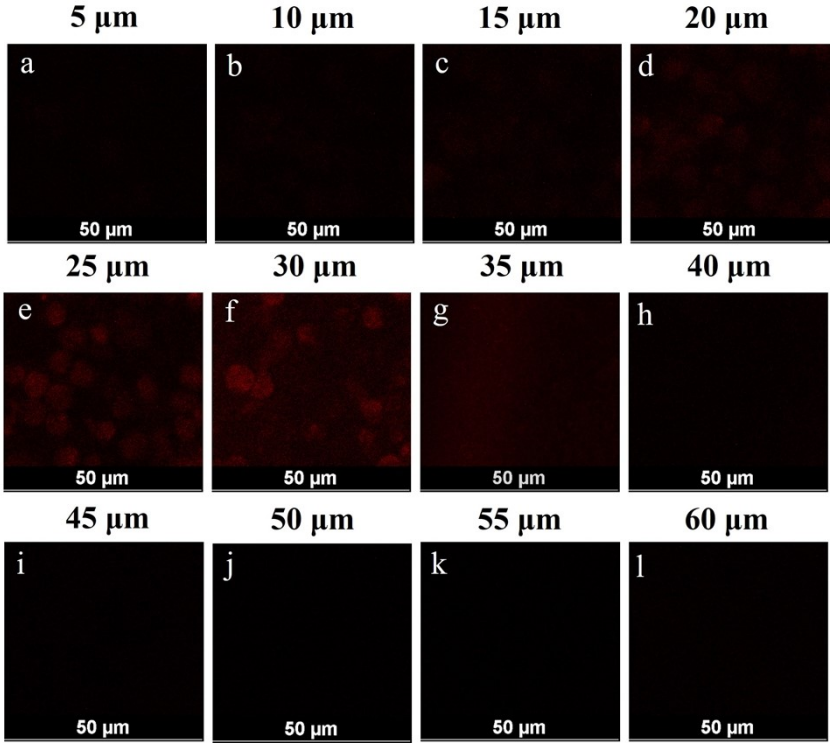


Figure S7. The depth imaging of spleen tissue. Scale bar = 50 μm.

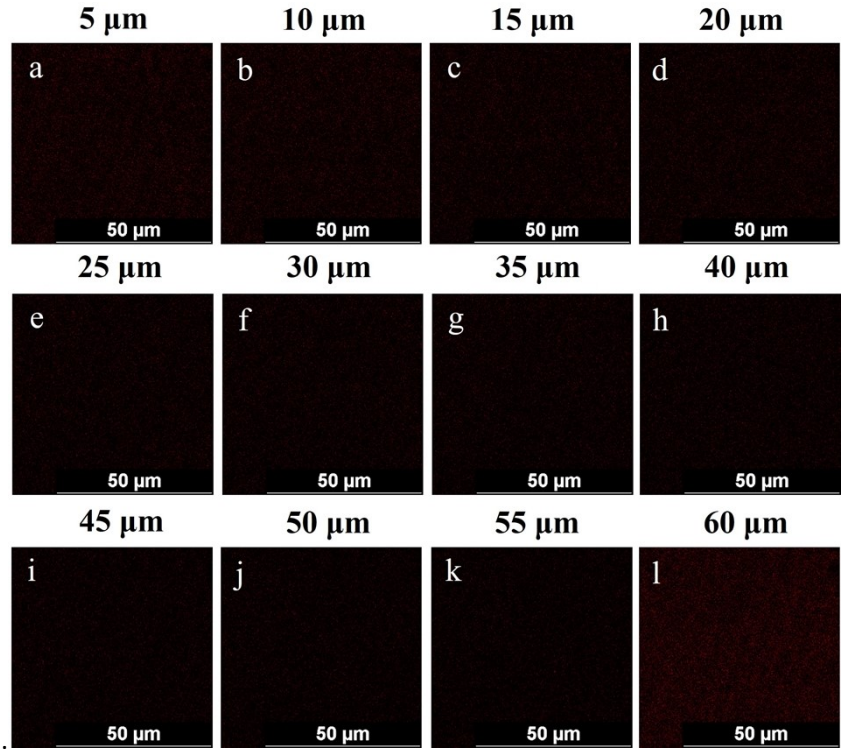


Figure S8. The depth imaging of lung tissue. Scale bar = 50 μm.

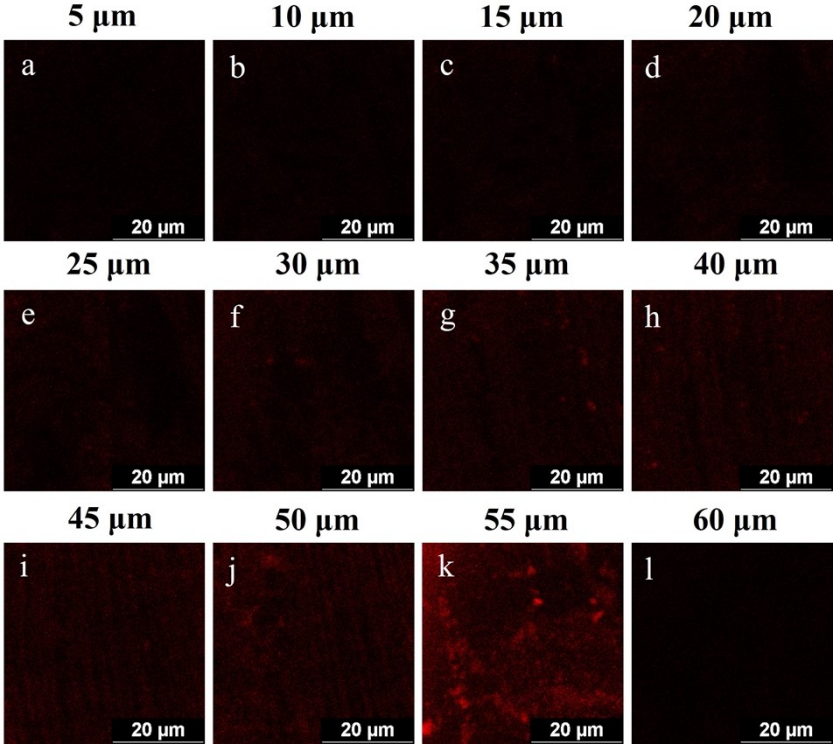


Figure S9. The depth imaging of heart tissue. Scale bar = 50 μm.

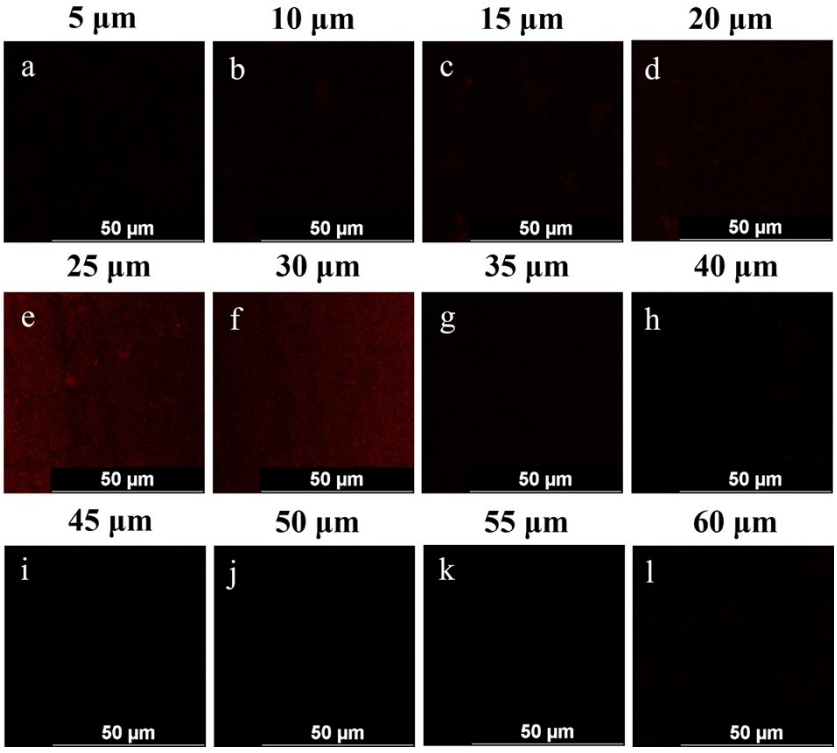


Figure S10. The depth imaging of kidney tissue. Scale bar = 50 μm.

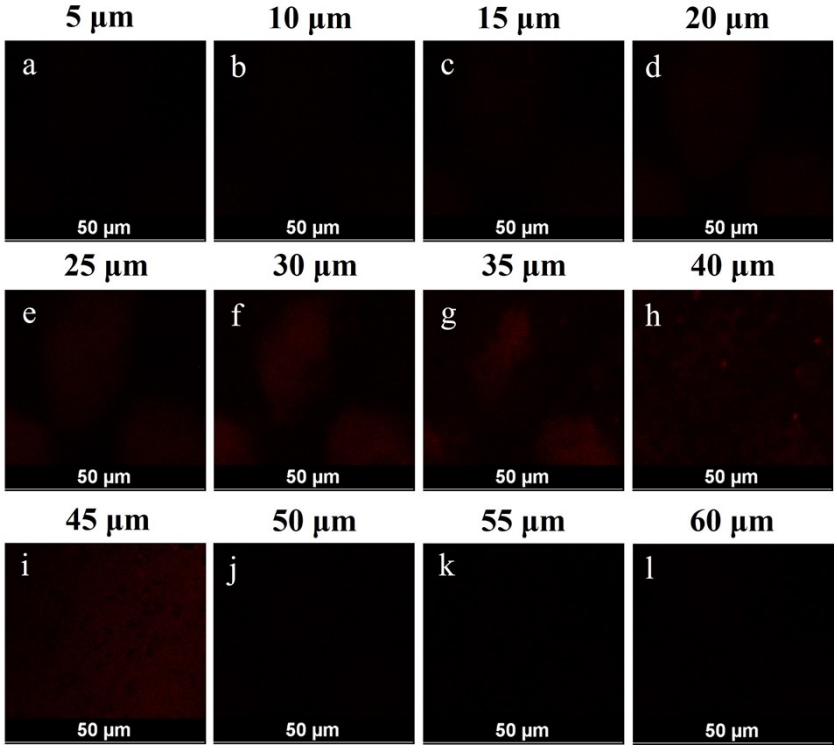


Figure S11. The depth imaging of liver tissue. Scale bar = 50 μm.

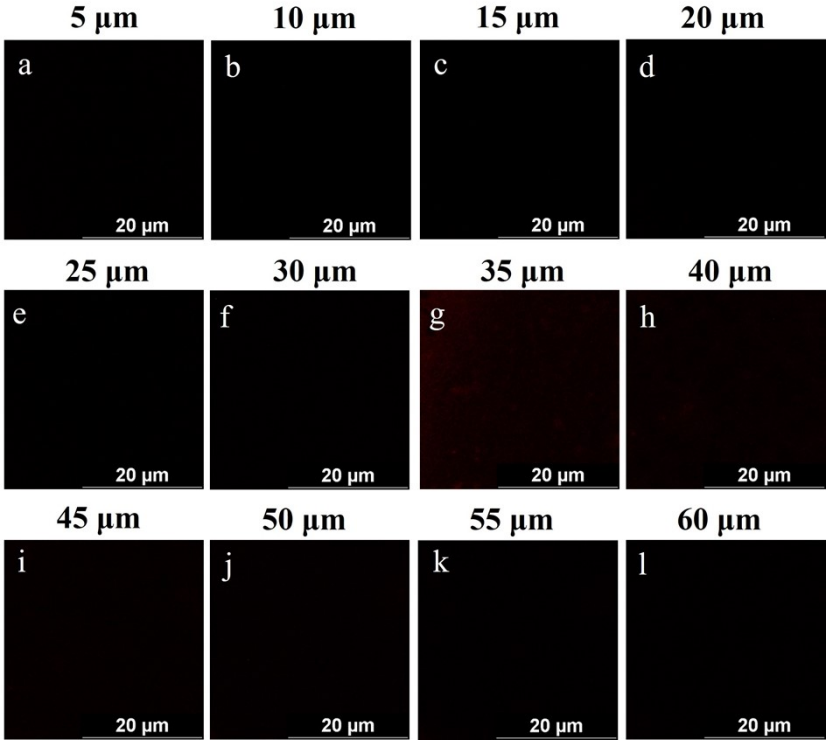


Figure S12. The depth imaging of liver tissue in control group. Scale bar = 20 μm.

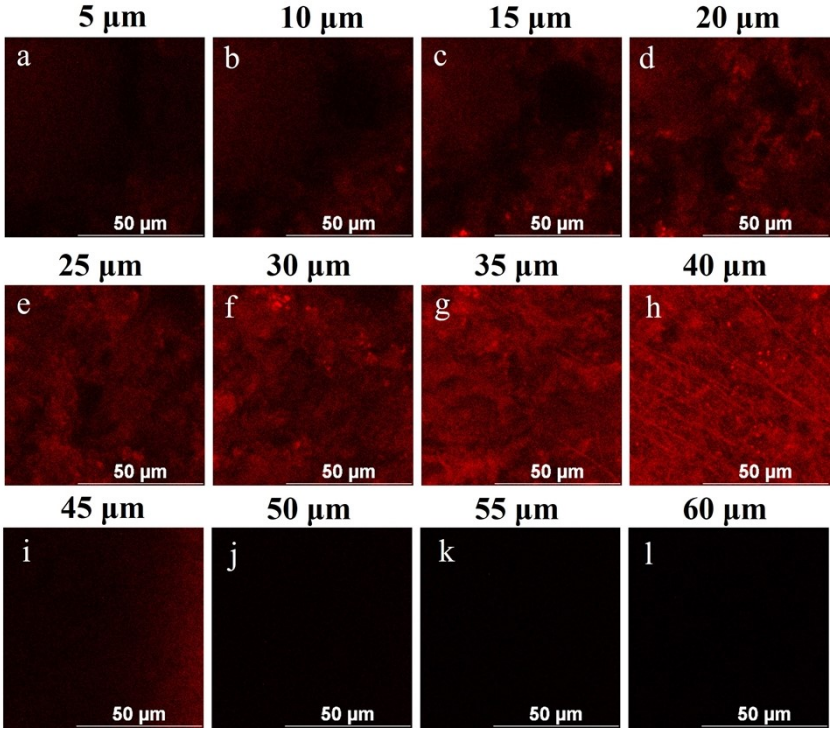


Figure S13. The depth imaging of tumor tissue in control group. Scale bar = 50 μm.