Supplemental Information

A FRET-based Two-Photon Probe for *In vivo* Tracking of pH during Traumatic Brain Injury Process

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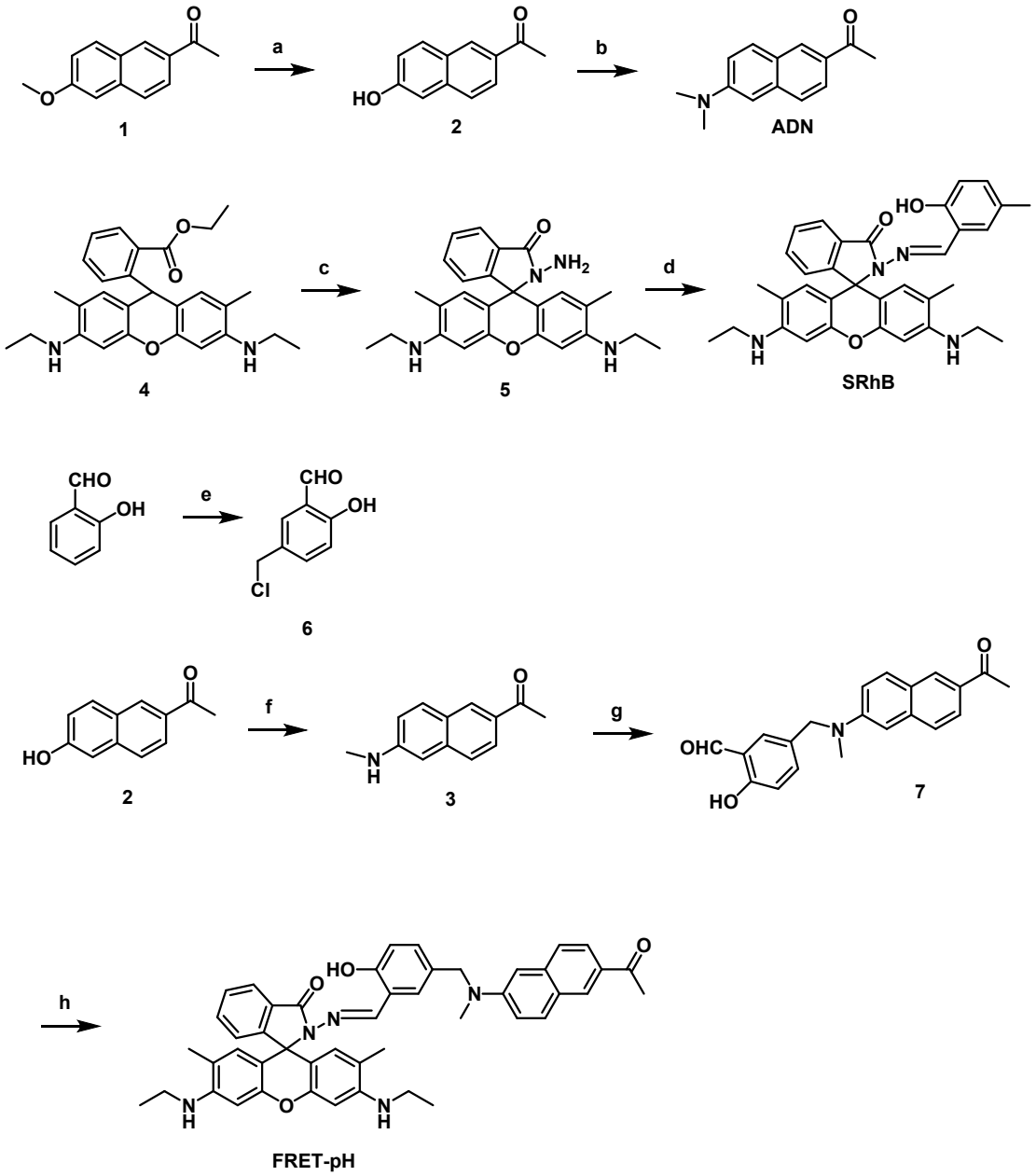
Materials and apparatus.

Unless otherwise stated, all solvents and reagents were purchased from commercial suppliers and used as received without further purification. All aqueous solutions were prepared in ultrapure water with a resistivity of 18.25 MΩ cm (purified by Milli-Q system supplied by Millipore). High resolution mass spectrometry was performed on an LTQ FT Ultra (Thermo Fisher Scientific, America) with MALDI-DHB mode. LC-MS analysis was performed on a high performance liquid chromatograph (Agilent 1200, America) connected to a quadrupole time-off light mass spectrometer (Q-TOF MS, Agilent 6520, America). Absorption spectra were recorded on an UV−vis spectrophotometer (Shimadzu UV-2550, Japan), and one-photon fluorescence spectra were obtained with a fluorimeter (Shimadzu RF-5301 PC, Japan). Two-photon fluorescence spectra were excited by a mode-locked Tisapphire femtosecond pulsed laser (Chameleon Ultra I, Coherent, America) and recorded with a DCS200PC photon counting with Omno-λ5008 monochromator (Zolix, China). Two-photon microscopy images were collected from a spectral confocal and multiphoton microscope (Carl Zeiss, LSM 780 NLO, Germany) with a mode-locked titanium-sapphire laser source (Mai Tai HP, Spectra Physics, America).



Scheme S1. Synthesis route of **ADN**, **SRhB**, **FRET-pH**. Regeants and conditions: a), concentrated HCl, EtOH, reflux, 3 h; b), sodium metabisulfite, dimethylamine aqueous solution, 150 °C, 48 h; c), 80 % hydrazine hydrate, 120 °C, 6 h; d), 2-methoxybenzaldehyde, anhydrous EtOH, reflux, 6 h; e), paraformaldehyde, concentrated HCl, 0 °C to r.t., 6 h; f), sodium metabisulfite, methylamine aqueous solution, 150 °C, 48 h; g), compound **6**, trimethylamine, chloroform, 50 °C, 6 h; h), compound **5**, anhydrous EtOH, reflux, 6 h.

Synthesis route of **ADN**, **SRhB**, and **FRET-pH** was depicted in Scheme S1 and the synthesis of compound **2**, **ADN**, **3**, **5**, **6**, were referred to the literatures.[1, 2]

Synthesis of **SRhB**: Compound **SRhB** was synthesized by modifying a literature procedure.[3] 5-methylsalicylaldehyde (41 mg, 0.3 mmol) in MeOH (10 mL) was added dropwise over 30 min to a methanolic solution (30 mL) of compound **5** (107 mg, 0.25 mmol) containing 2 drop of anhydrous acetic acid under hot (ca. 50–60 °C) conditions. Then the reaction mixture was stirred for around 6 h at room temperature. An off-white precipitate was formed, which was collected by filtration. The residue was washed thoroughly with cold methanol to isolate **SRhB** in pure form and a white solid was obtained. (102.5 mg, 75 % yield) ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.76 (s, 1H), 9.01 (s, 1H), 8.10 – 7.95 (m, 1H), 7.60 – 7.45 (m, 2H), 7.18 – 7.07 (m, 1H), 6.99 (dd, *J* = 8.3, 1.6 Hz, 1H), 6.86 (d, *J* = 1.3 Hz, 1H), 6.77 (d, *J* = 8.3 Hz, 1H), 6.46 (s, 2H), 6.32 (s, 2H), 3.54 (s, 2H), 3.23 (q, *J* = 7.0 Hz, 4H), 2.22 (s, 3H), 1.91 (s, 6H), 1.33 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.13, 158.16, 152.11, 151.45, 148.21, 134.24, 129.13, 127.32, 125.03, 118.62, 105.21, 96.12, 56.50, 56.09, 37.93, 19.03, 17.47, 14.59.

Synthesis of **7**: Compound **3** (560 mg, 2.8 mmol) and compound **6** (571 mg, 3.36 mmol) were mixed and dissolved in anhydrous chloroform (50 mL). After 15 min of stirring at room temperature, 4.5 mL triethylamine was added dropwise into the reaction mixture. The mixture was stirred with 50 °C for 6 h,

and then 100 mL water was added in. Then 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 petroleum ether: methylene dichloride (v:v=1:1) and a faint yellow solid was obtained. (600 mg, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.90 (s, 1H), 9.92 – 9.38 (m, 1H), 8.40 (d, *J* = 1.8 Hz, 1H), 8.06 – 7.84 (m, 2H), 7.76 (d, *J* = 9.0 Hz, 1H), 7.35 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.22 (d, *J* = 9.0 Hz, 1H), 7.15 (d, *J* = 2.1 Hz, 1H), 6.91 (d, *J* = 8.6 Hz, 1H), 4.27 (s, 2H), 2.98 (s, 3H), 2.68 (s, 3H). ^{13}C NMR (100MHz, $CDCl_3$) δ 197.67, 196.59, 160.33, 136.98, 135.88, 132.17, 131.53, 130.86, 130.49, 129.99, 126.00, 125.09, 122.06, 120.58, 118.12, 113.98, 113.15, 30.96, 30.23, 26.45.

Synthesis of **FRET-pH**: Compound **7** (100 mg, 0.3 mmol) in MeOH (10 mL) was added dropwise over 30 min to a methanolic solution (30 mL) of compound 5 (107 mg, 0.25 mmol) containing 2 drop of anhydrous acetic acid under hot (ca. 50–60 °C) conditions. Then the reaction mixture was stirred for around 6 h at room temperature. An off-white precipitate was formed, which was collected by filtration. The residue was washed thoroughly with cold methanol to isolate **FRET-pH** in pure form and a white solid was obtained. (109.2 mg, 49 % yield) ^{1}H NMR (400 MHz, DMSO-*d6*) δ: ^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.91 (s, 1H), 7.90 (d, *J* = 7.0 Hz, 1H), 7.63 – 7.48 (m, 3H), 7.29 (d, *J* = 7.2 Hz, 1H), 7.05 – 6.83 (m, 3H), 6.32 (s, 2H), 6.15 (s, 2H), 5.07 (t, *J* =

5.2 Hz, 2H), 3.71 (s, 3H), 3.17 – 3.09 (m, 4H), 1.84 (s, 6H), 1.20 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-*d6*) δ 164.13, 158.16, 152.11, 151.45, 148.21, 134.24, 129.13, 127.32, 125.03, 118.62, 105.21, 96.12, 56.50, 56.09, 37.93, 19.03, 17.47, 14.59. HRMS: calcd for $C_{47}H_{45}N_5O_4$ $[M + H]^+$ 744.3472 found 744.3506.

General procedure for pH detection

Unless otherwise noted, all the fluorescence measurements were made in 10 mM PBS (pH 7.4) according to the following procedure. In a 2mL tube, 450 μL of PBS and 50 μL EtOH with the probe **FRET-pH** were mixed, and then add an appropriate volume of different pH buffer solutions which were prepared by using 0.1 M citric acid (pH 2.6-5.5), 0.1 M KH_2PO_4 (pH 6.0-8.0). The pH was adjusted by adding 0.1 M NaOH or 0.1 M HCl solutions. After all the measurements for **FRET-pH**-target reaction were tested in PBS buffer (10 mM, containing 10% EtOH) at 37 °C for 2min. In a thermostat, a 0.5 mL portion of the reaction solution was transferred to a quartz cell of 1 cm optical length to measure the excitation wavelength were 365 nm and 810 nm, under one-photon and two-photon excitation mode, respectively. Emission wavelength at 562 and 501 nm and excitation slit width of 3 nm and emission slit widths of 5 nm. For the selectivity assay, Superoxide ($O_2^{\cdot -}$) was created by the enzymatic reaction of xanthine/xanthineoxidase (XA/XO; 6.0 μM/3 mU) at 25 °C for 5 min.[4, 5] ·OH was generated by Fenton reaction between Fe^{2+} (EDTA) and H_2O_2 quantitively, and Fe^{2+} (EDTA) concentrations represented ·OH concentrations.[6]

The $ONOO^-$ source was the donor 3-Morpholinosydnonimine hydrochloride (SIN-1, 200 μmol/mL).[7] NO was generated in form of 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 100 μM/mL).[8] OCl^- was standardized at pH 12 ($\varepsilon_{292\ nm}$ = 350 $M^{-1}cm^{-1}$).[9] H_2O_2 was determined at 240 nm ($\varepsilon_{240\ nm}$ = 43.6 $M^{-1}cm^{-1}$). $^1O_2$ was generated from 3,3'-(naphthalene-1,4-diyl)dipropionic acid.[10] All the reagents were obtained from Aladdin (USA). All other chemicals were from commercial sources and of analytical reagent grade, unless indicated otherwise.

Measurement of Fluorescence Quantum Yield.

The fluorescence quantum yield was determined by using quinine sulfate in 0.1 N sulfuric acid (Φ=0.55) as the reference by the literature method.[11] The fluorescence quantum yield was calculated according to the following equation:

$$\Phi_s = \Phi_r(A_r I_s n_s^2)/(A_s I_r n_r^2)\ (A \leq 0.05)$$

The subscripts s and r represent the sample and the reference molecule respectively. Φ is the fluorescence quantum yield. A is the absorbance of molecules that is controlled below 0.05 for both molecules in the same wavelength. *I* means the integrated emission area and n is the refractive index of the solvent.

Measurement of Two-Photon Cross-Section.

The two-photon cross section (δ) was determined by using the femto second (fs) fluorescence measurement technique as described. **ADN** were dissolved in PBS buffer (10mM, pH 7.4, containing 0.9% NaCl), The

fluorescence intensity was measured at 710-880 nm by using rhodamine B (dissolved in methanol) as the reference. The two-photon properties in this reference have been well characterized. The intensities of the two-photon-induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The two-photon cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$, where the subscripts s and r stand for the sample and reference, respectively. The fluorescence intensity was denoted as S. Φ is the fluorescence quantum yield and ϕ is the overall fluorescence collection efficiency of the experimental apparatus, which can be approximated by the refractive index of the solvent. The concentration of the solution was denoted as c. δ is for two-photon absorption.

Evaluation of pK_a value.

The pK_a value of **FRET-pH** was calculated by the following equation:[12]

$$pH - pK_a = \log[(I_{max} - I)/(I - I_{min})]$$

where I is the observed fluorescence intensity at a given pH, I_{min} and I_{max} is minimal and maximal fluorescence intensity, respectively.

Water solubility

Small amount of **FRET-pH** was dissolved in EtOH to prepare the stock solutions (1.0×10^{-2}M). The solution was diluted to ($5.0\times10^{-4} \sim 1.0\times10^{-2}$) M and added to a cuvette containing 1.0 mL of H_2O by using a micro syringe. In all cases, the concentration of EtOH in H_2O was maintained to be 0.2%.

Cell Culture

BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS), and incubated in an atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. Cells were passed and plated into glass bottom cell culture dishes (NEST) in the day before imaging. For probe loading, the cells were washed with phosphate buffer and then interacted with 10 μM **FRET-pH** (containing 1% DMSO in serum-free DMEM) for 30 min at 37 °C. After washing with phosphate buffer twice, the cells were then incubated into a phosphate buffer solution containing for 2 h.

Cytotoxicity Study of FRET-pH for Cells.

MTT test was performed according to the reported protocol with a minor change. BV-2 cells were seeded in 96-well plates and incubated with different concentrations of **FRET-pH** (0, 2, 5, 10 and 20 μM, containing 1 % DMSO in 100 μL DMEM). The **FRET-pH** labelled BV-2 cells were incubated in an atmosphere of 5/95 (v/v) of CO_2/air at 37 °C for 24 h. Subsequently, 20 μL 5.0 mg/mL MTT solution was added to each well. Followed by incubation for 4 h under the same conditions. 100 μL supernatant was removed and 150 μL DMSO was added. 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 **FRET-pH** labelled group and the control group, respectively).

Fluorescence Microscopy Imaging.

BV-2 cells were washed twice with PBS and then cultured with DMEM without glucose that had been progressed with 95% N_2/5% CO_2 for 10 min to remove residual oxygen. BV-2 cells were passed and dispersed on 18 mm glass coverslips at 37 °C, 5% CO_2 1 day before imaging. Then cells were incubated with 10 μM **FRET-pH** for 40 min. The medium was removed and cells were washed with PBS (10 mM, pH 7.4) twice. Two-photon microscopic imaging of BV-2 cells which were labelled with 10 μM **FRET-pH** were performed on a spectral confocal and Two photon microscopes with a 40 × water objective, and a numerical aperture (NA = 1.0). The two-photon fluorescence microscopy images were obtained by exciting **FRET-pH** with a mode-locked titanium–sapphire laser source (Mai Tai HP, Spectra Physics, 80 MHz pulse frequency, 100 fs pulse width) set at the wavelength of 810 nm. All the images were separately collected in the emission wavelength range of 450-530 nm and 550-650 nm. The internal PMTs were used to collect the signals in an 8 bit unsigned 1024 × 1024 pixels at a scan speed of 1.58 s per pixel.

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 ***$P < 0.001$. Sample size was chosen empirically based on our previous experiences and pretest results. No statistical method was used

to predetermine sample size and no data were excluded. The numbers of animals or samples in every group were described in the corresponding figure legends. The distributions of the data were normal. 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.

***In vivo* imaging 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 the College of Biology (Hunan University). All BALB/c mice (age 4 weeks, weight 17–20 g) were operated upon in accordance with institutional ethics committee regulations and guidelines on animal welfare. The aforementioned animals were randomly divided into control (n = 3), and 3 day post injury (n = 3) groups. The model groups were anesthetized with isoflurane 7 days after birth, disinfected, and a sterile surgical blade was used to slide back and forth 3 mm down the cerebral cortex to ensure consistency of injury. The incision skin was sutured after the injury. After a complete recovery, food and water were again provided to the animals ad libitum. The local environmental temperature was maintained at 35°C–37°C during surgery, hypoxia and resuscitation. Following the hypoxic–

ischemic process, abdominal injection of 200 μL of 1.0 mM probe **FRET-pH** was administered, animals were fasted for 18 h and then decapitated, then the brain tissue was immediately removed, washed in ice-cold normal saline, and cut into 300-μm section. Sections were washed with PBS and two-photon tissue fluorescence imaging was immediately performed.

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 fluorescent region.

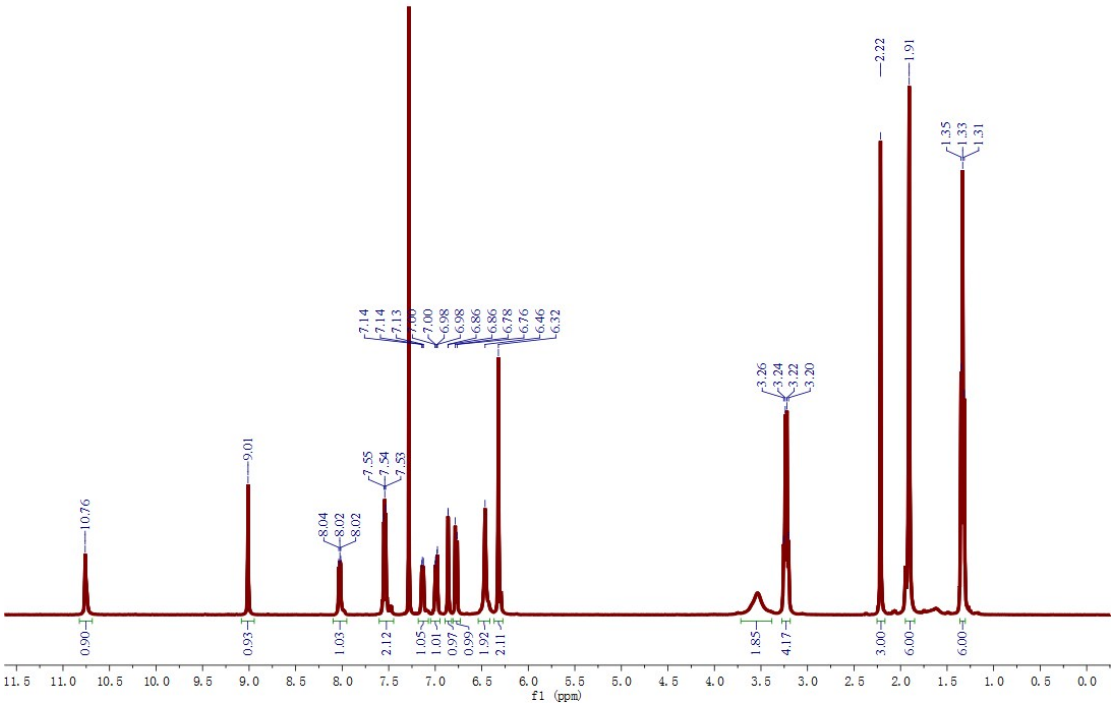


Figure S1 ^{1}H-NMR spectrum of **SRhB** (400 MHz, $CDCl_3$)

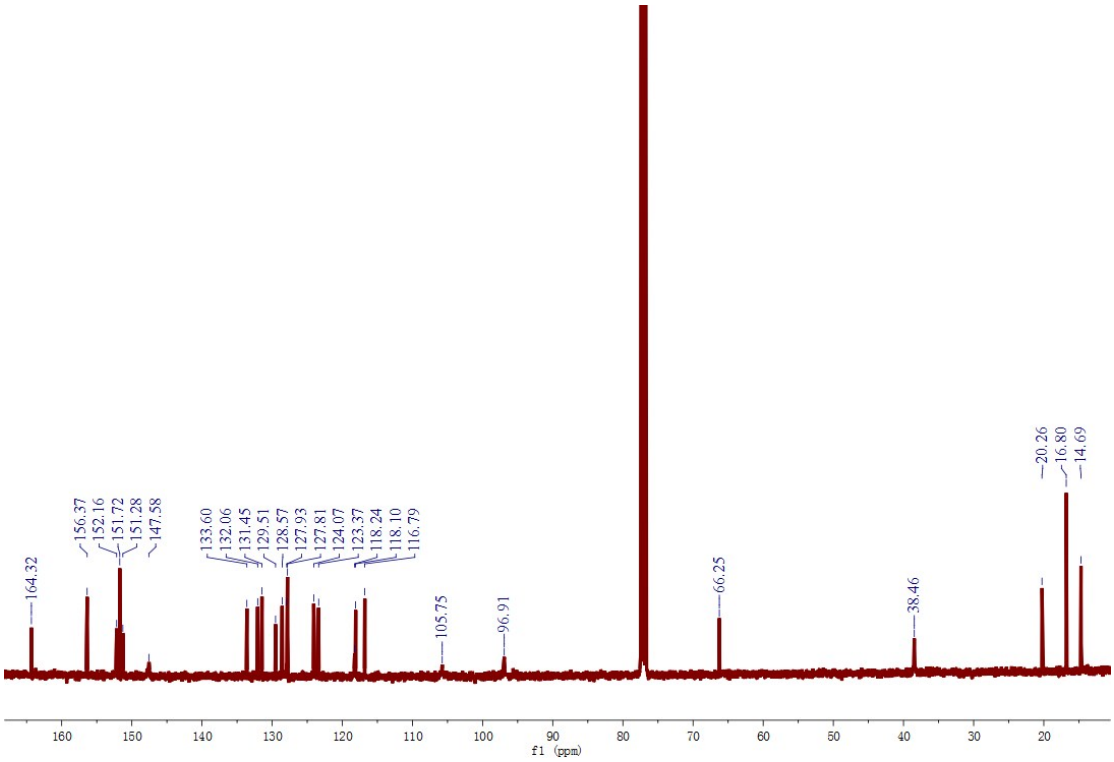


Figure S2 ^{13}C-NMR spectrum of **SRhB** (100 MHz, $CDCl_3$)

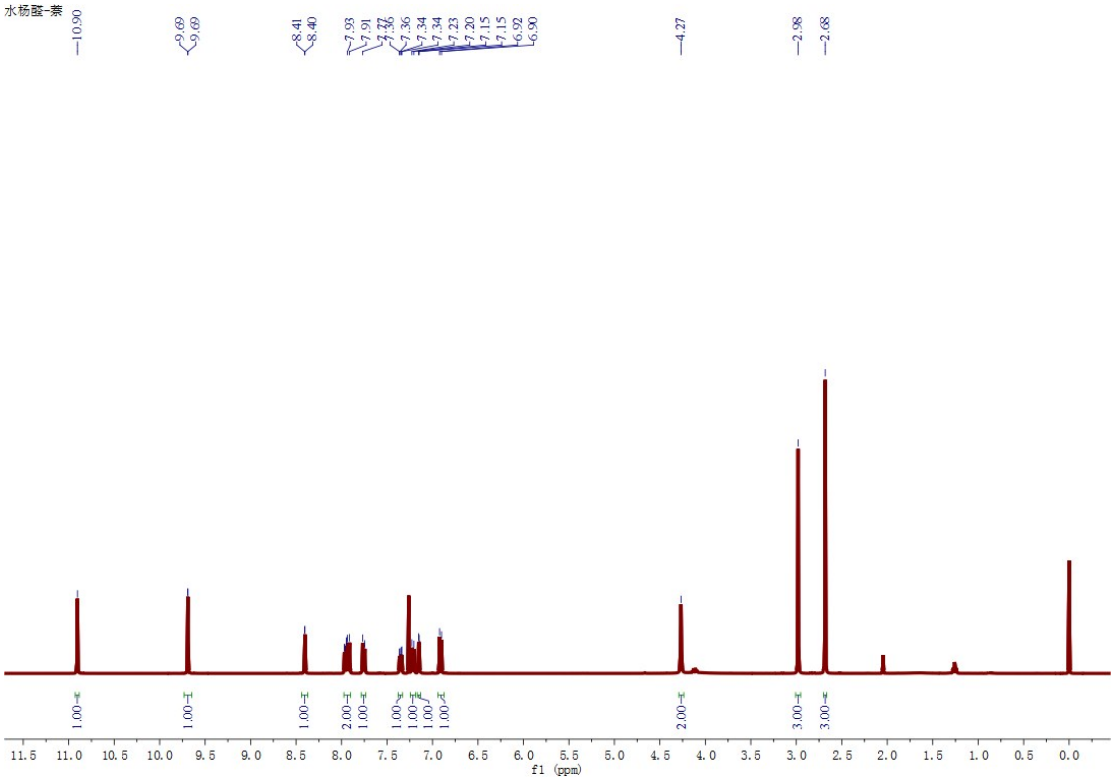


Figure S3 ^{1}H-NMR spectrum of **7** (400 MHz, $CDCl_3$)

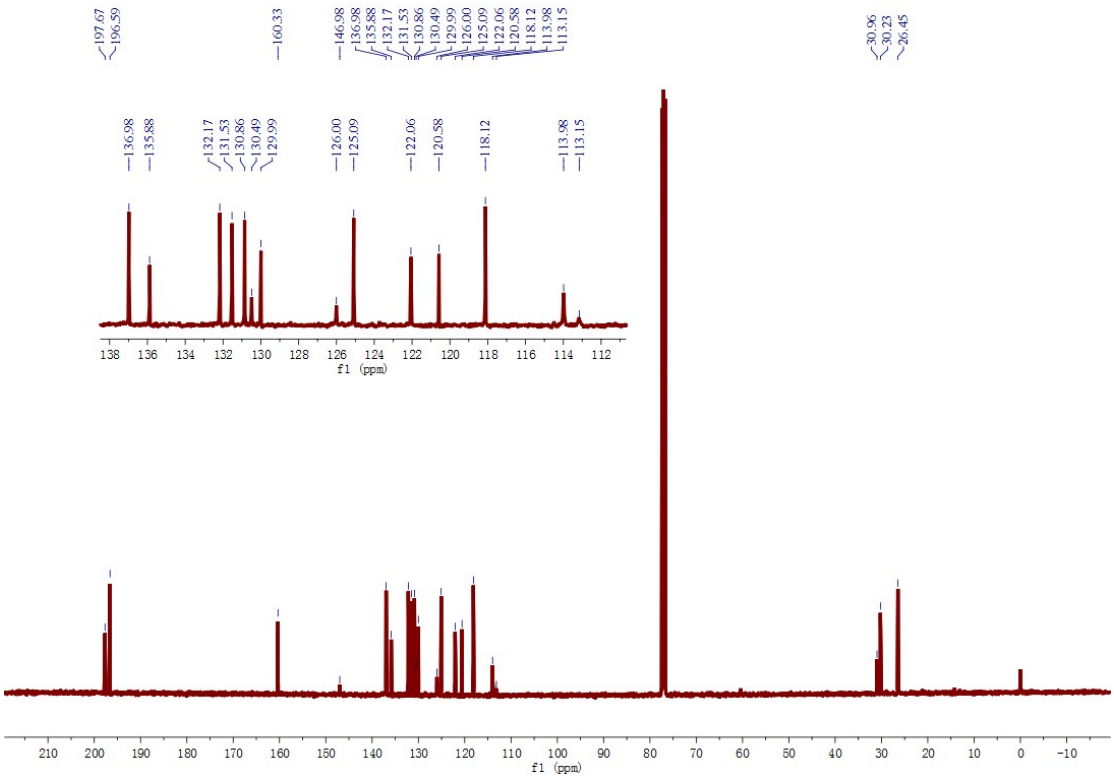


Figure S4 ^{13}C-NMR spectrum of **7** (100 MHz, $CDCl_3$)

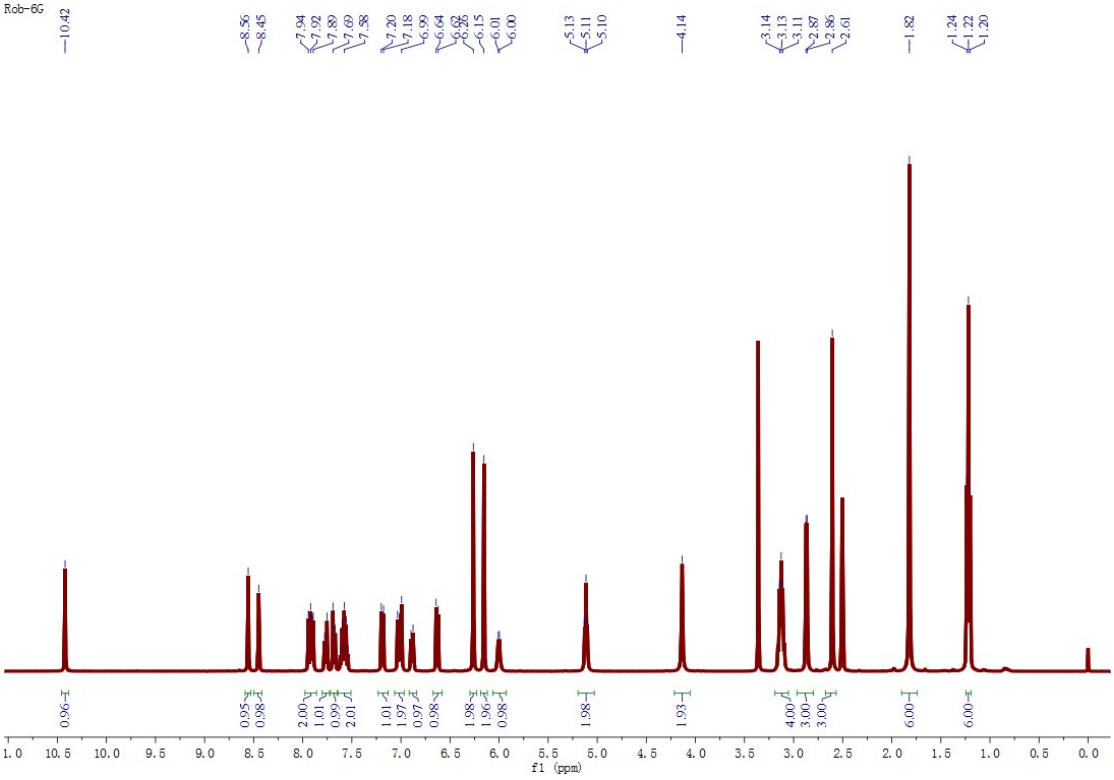


Figure S5 ^{1}H-NMR spectrum of **FRET-pH** (400 MHz, DMSO-*d6*)

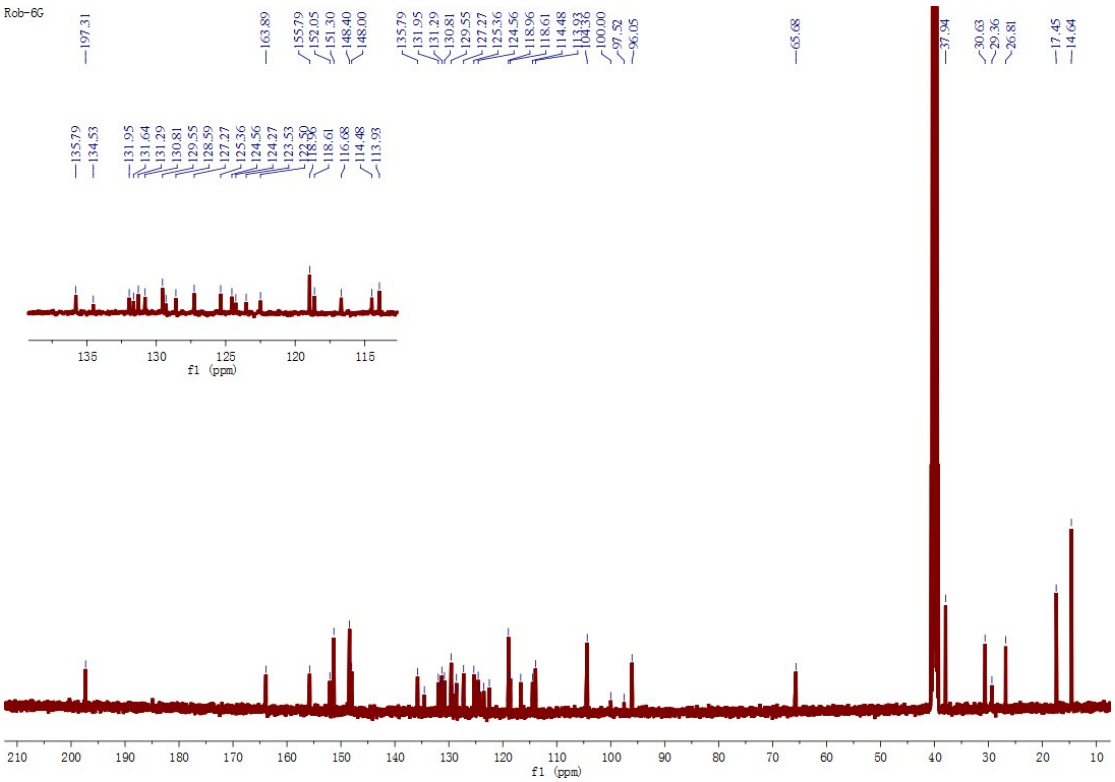


Figure S6 ^{13}C-NMR spectrum of **FRET-pH** (100 MHz, DMSO-*d6*)

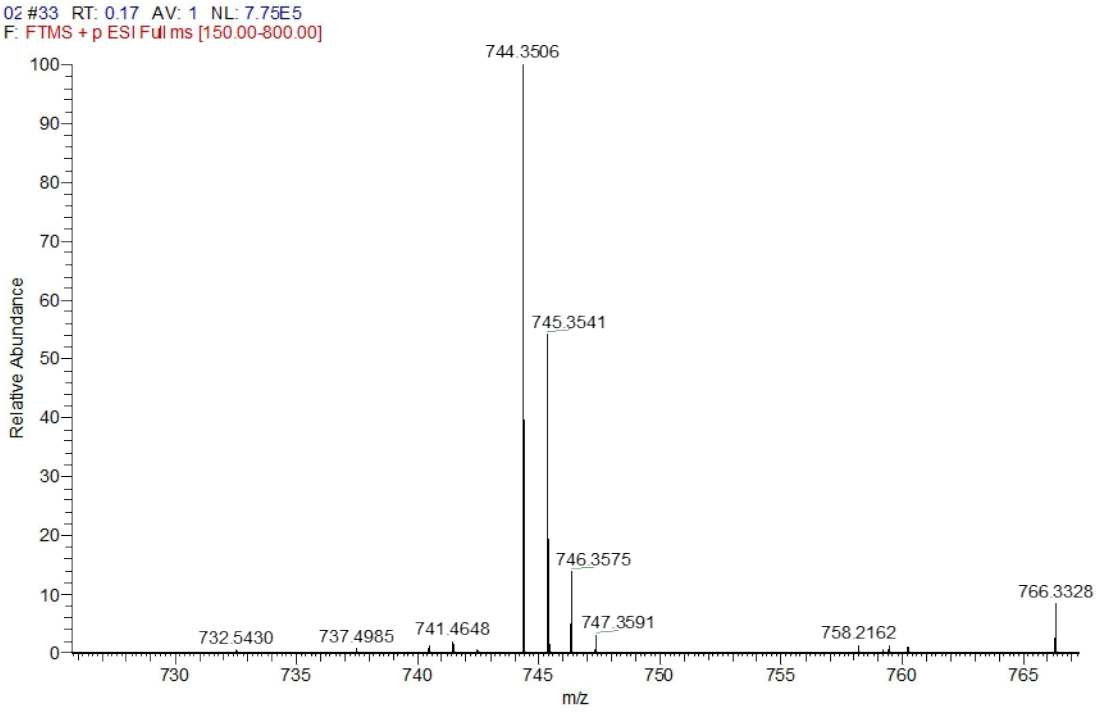


Figure S7 HRMS spectrum of **FRET-pH**

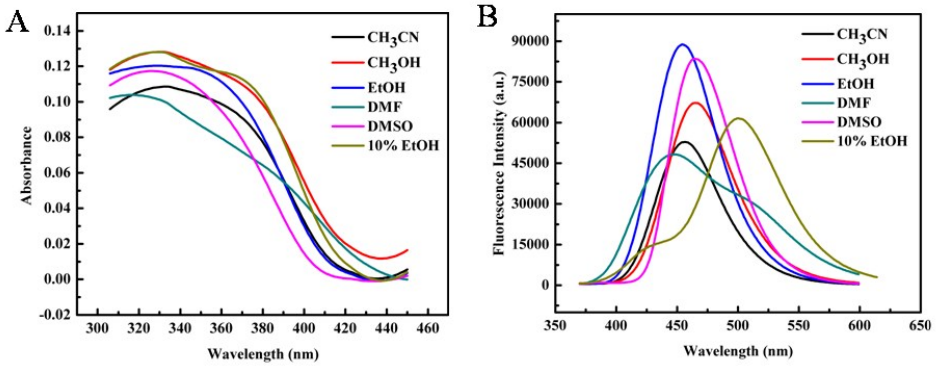


Figure S8. (A) UV-Vis spectra of **ADN** (10 μM) and (b) Fluorescence emission spectra in different solvents.

Table S1. The photophysical property of **AND** (10 μM) in various solvent.

Solvent	λ_{max}^{abs} [(a)](nm)	λ_{max}^{fl} [(b)] (nm)	λ_{max}^{TP-ex} [(c)] (nm)	ε (M^{-1}·cm^{-1}) [(d)]	Φ[(e)]	δ_{max} (GM) [(f)]	Φ_{max}[(g)]
CH_3CN	355	456	n.d. [h]	1.01×10^4	0.65	n.d.	n.d.
MeOH	363	465	n.d.	1.16×10^4	0.75	n.d.	n.d.
DMSO	340	464	n.d.	1.14×10^4	0.73	n.d.	n.d.
EtOH	350	454	n.d.	1.17×10^4	0.76	n.d.	n.d.
DMF	355	448	n.d.	0.83×10^4	0.49	n.d.	n.d.
10 % EtOH	365	500	810	1.15×10^4	0.45	133	60

[a] maximum absorption wavelength. [b] maximum fluorescence emission wavelength. [c] maximum two-photon excitation wavelength. [d] molar absorptivity. [e] quantum yield. [f]two-photon absorption cross section (1 GM =1 × 10–50 cm^4 s $photon^{-1}$). [g] two-photon action cross section. [h] n.d. = not determined.

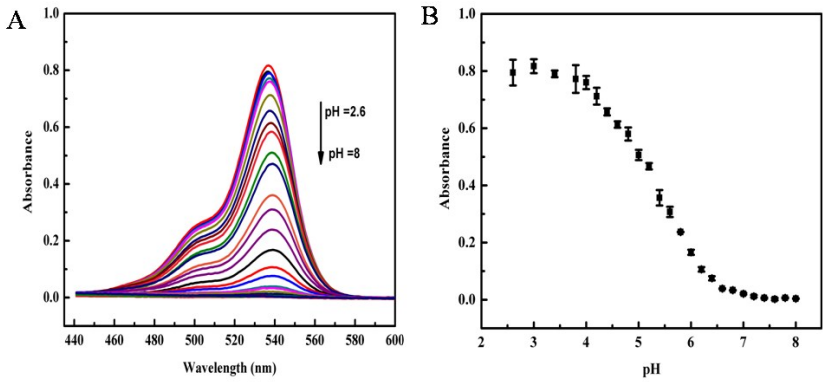


Figure S9. (A) Absorption of 10 μM **SRhB** at acidic to neutral pH values (2.6–8). (B) plot of A_{530} versus pH value.

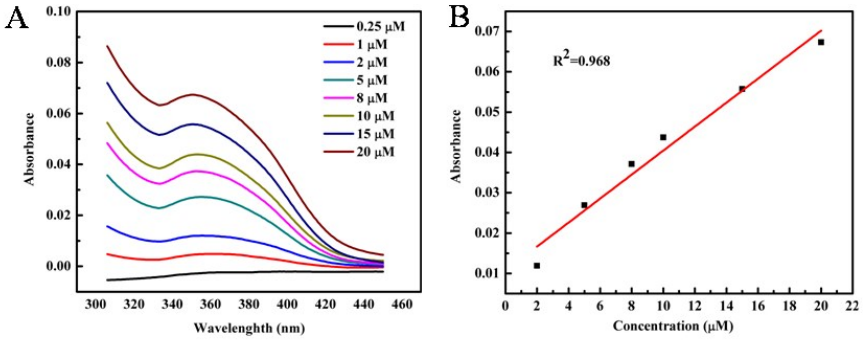


Figure S10. (A) UV spectra and (B) plot of absorbance against dye concentration of **FRET-pH** in 0.1 M KH_2PO_4 buffer (0.1M KCl, pH=7.4, containing 1 % EtOH).

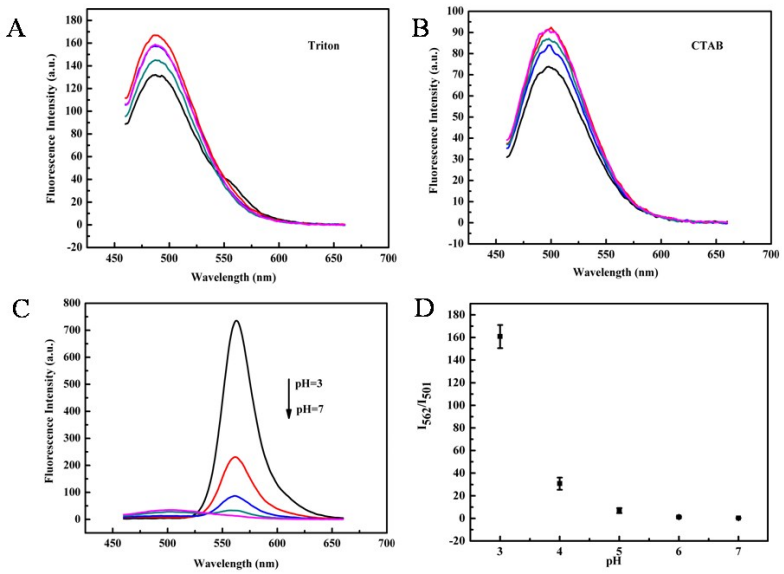


Figure S11. Fluorescence spectra of **FRET-pH** in (A) 1.5 mM Triton, (B) 3 mM CTAB, (C) 25 μM SDS and (D) plot of fluorescence ratio (I_{562}/I_{501}) at acidic to neutral pH values (3–7) in 0.1 M KH_2PO_4 buffer (0.1M KCl, pH=7.4, containing 1 % EtOH).

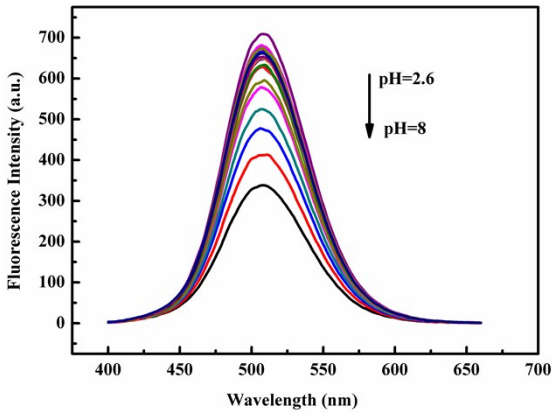


Figure S12. Fluorescence spectra of 10 μM **ADN** at acidic to neutral pH values (2.6–8).

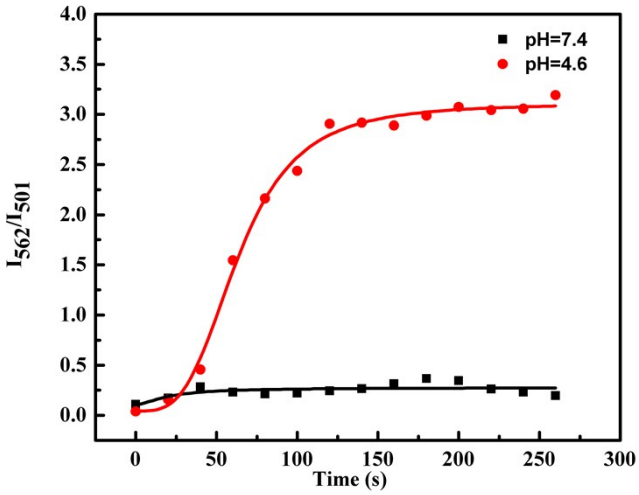


Figure S13. A plot of fluorescence intensity of **FRET-pH** (10 μM) vs the reaction time at acidic (red line) and neutral (black line) pH values.

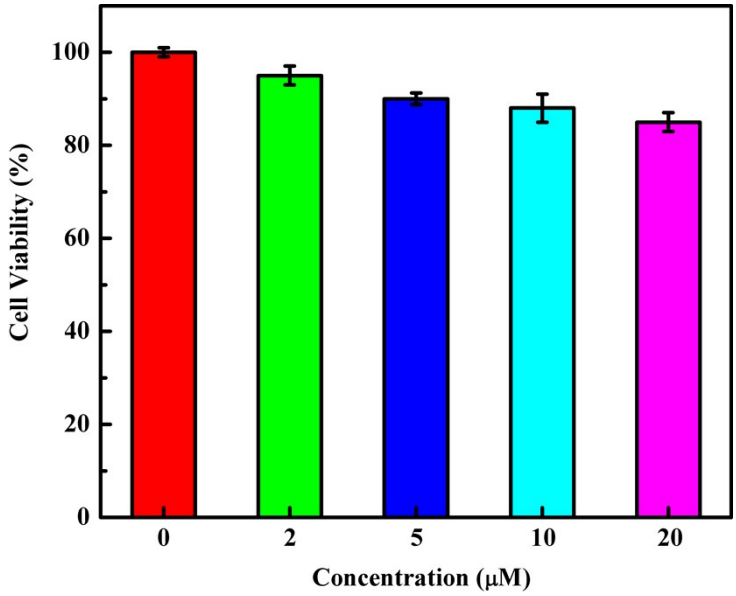


Figure S14. Cell survival rate of control groups (without and with 1% DMSO in DMEM, represented by Mock and DMSO, respectively) and experimental group (with 2, 5, 10, and 20 μM **FRET-pH**, containing 1% DMSO).

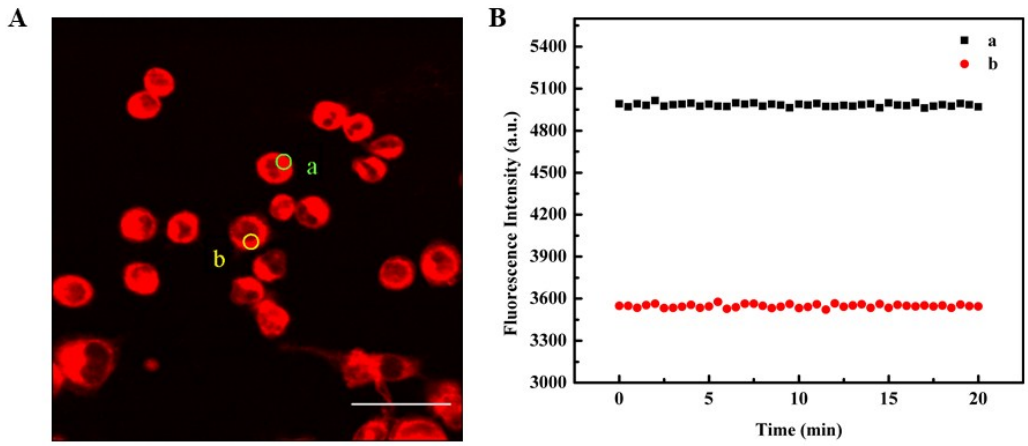


Figure S15. (A) TPM images of BV-2 cells labeled with 10 μM **FRET-pH** for 30 min separately. (B) Two-photon fluorescence intensity from circle a and b as a function of time. The two-photon fluorescence intensity was collected with 30 sec intervals for the duration of 20 min under xyt mode. Scale bar: 50 μm.

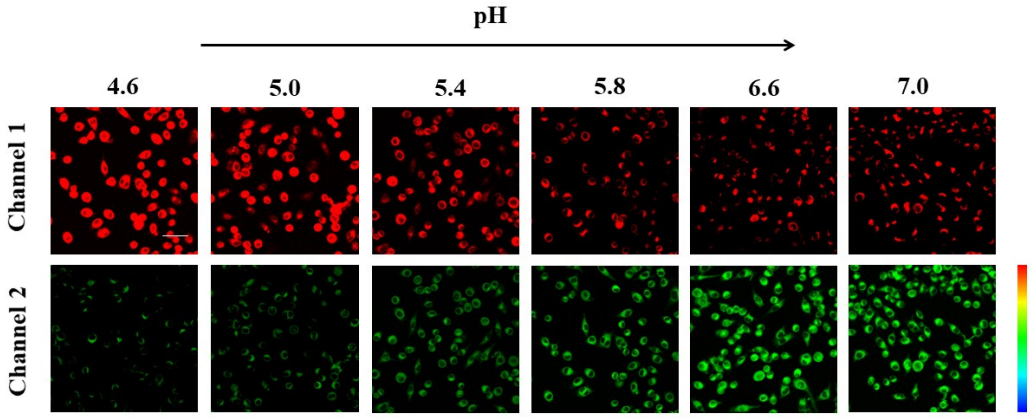


Figure S16. TPM images of BV-2 cells labeled with 10 μM **FRET-pH** clamped at pH 7.0, 6.6, 5.8, 5.4, 5.0, 4.6. Channel 1 (emission wavelength: 540–650 nm) and channel 2 (emission wavelength: 450–530 nm)

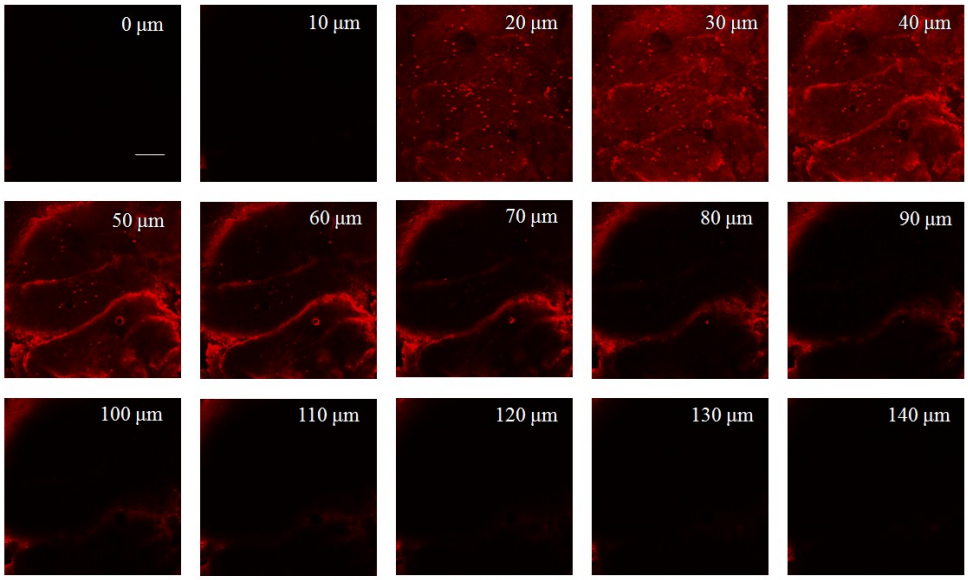


Figure S17. Depth fluorescence images of **FRET-pH** (200 μM) in mouse Brain tissues. λ_{ex} = 810 nm, λ_{em} = 540-650 nm. Scale bar: 100 μm.

Reference

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