*Supporting information for*

# A ratiometric pH probe for unveiling lysosome–lipid droplet interactions and differentiating cancer cells

# Contents

## Materials

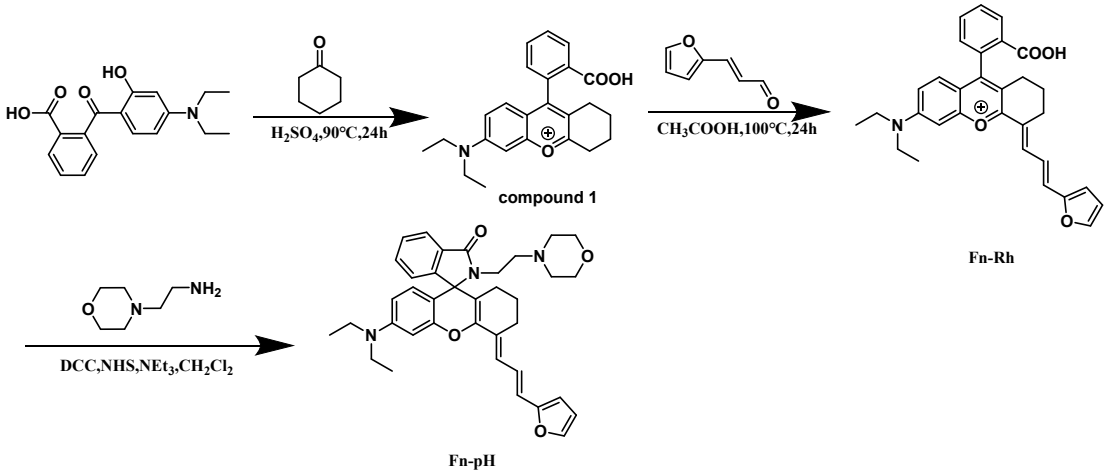
All chemicals used are of analytical grade. cyclohexanone. were purchased from Macklin(Shanghai, China). 2-Hydroxy-4-diethylamino-2'-hydroxycarbonylbenzophe - none, Triethylamine. Were purchased from Heowns Biochem Technologies. Llc. (Tianjin, China).2-Furanacrolein. Were purchased from Adamas-meta. Dicyclohexylc- arbodiimide, $H_2SO_4$ and dichloromethane. Were purchased from Energy Chemical. N-Hydroxysuccinimide. Were purchased from Accela Chem. N-(2-Aminoethyl)-morpholine. Were purchased from 3A chemicals. MTT was purchased from Hangzhou Fude Biological Technology CO., LTD. PEG (average Mn 5000) was purchased from Bide Pharmatech Co., Ltd. China and prepared as a stock solution on demand. Ultrapure water with a resistivity of 18.2 MΩ cm, obtained from a Milli-Q water purification system, was used to prepare all aqueous solutions. HeLa (human cervical cancer cells), HepG2(human hepatocellular carcinoma cells) were purchased from Beyotime（Gene expression databases：ArrayExpress）and HL-7702 cells (also known as L-02 cells) were procured from the cell bank of the Chinese Academy of Sciences. BR buffer solution (Britton-Robinson buffer solution) is prepared by mix 0.04 M phosphoric acid, 0.04 M boric acid, and 0.04 M acetic acid, and dilute to 1 liter. Then, adjust the solution to the desired pH by adding 0.2 M NaOH or 0.2 M HCl. The pH meter was calibrated using sodium tetraborate (pH=9.18, 25°C) and potassium hydrogen phthalate (pH=4.0, 25°C),both purchased from Tianjin Fuyu Fine Chemical Co.,Ltd.

## Instruments

UV-vis absorption spectra were obtained on a Shimadzu UV-2700 spectrophotometer (Japan) with a quartz cuvette having 1 cm path length. and fluorescence spectra were measured on a HITACHI F4700 fluorescence spectrophotometer (Japan). $^{1}H$ and $^{13}C$ NMR spectra were measured on a Bruker AVANCE III HD500 digital NMR spectrometer (Germany), using tetramethyl silane (TMS) as internal reference. High resolution mass spectrometric (HRMS) was acquired on Aglient7250&JEOL - JMS-T100LP Accu TOF (Bruker Daltonics, Billerica, MA, USA). The fluorescence imaging of cells was performed with Leica TCS SP8 CARS confocal microscope (Germany).

The pH meter is Shleici PHS-3E.

**Synthetic route of the fluorescent probe Fn-pH**




**Scheme S1.** Synthetic route of the fluorescent probe **Fn-pH**.

**Synthesis of Fn-Rh**

Excess cyclohexanone (2 g, 20 mmol) was added to a 200 ml round-bottom flask placed in an ice-water bath. Under magnetic stirring, 15 ml of 98% concentrated sulfuric acid was slowly added to the flask. Subsequently, 2-Hydroxy-4-diethylamino-2'hydroxy-carbonylbenzophenone (3.13 g, 10 mmol) was added to the mixture in portions. The round-bottom flask was then transferred to an oil bath set to 90°C and the reaction was allowed to proceed for 24 hours. After cooling to room temperature, the reaction mixture was poured into ice water and an appropriate amount of perchloric acid was added. The resulting mixture was vacuum filtered, washed with ice water, and dried to yield **Compound 1**. **Compound 1** (376 mg, 1 mmol) and 2-furanpropenal (122 mg, 1 mmol) were added to a round-bottom flask containing 20 ml of glacial acetic acid. The reaction mixture was heated at 100°C for 24 hours. After completion, then solvents removed by reducing pressure and obtain a dark blue solid. The crude product was purified by column chromatography using silica gel (200-300 mesh) as the stationary phase and a mixture of dichloromethane and methanol (20:1) as the eluent. The pure compound, **Fn-Rh**, was obtained with a yield of approximately 70%. **Fn-Rh**-$^1$H NMR (600 MHz, DMSO-*d*6) δ 8.00 (d, *J* = 7.7 Hz, 1H), 7.82 – 7.65 (m, 4H), 7.31 (d, *J* = 7.6 Hz, 2H), 7.08 – 6.96 (m, 2H), 6.91 – 6.85 (m, 1H), 6.74 – 6.63 (m, 3H), 6.58 (dd, *J* =

3.4, 1.8 Hz, 2H), 3.43 (d, *J* = 9.7 Hz, 4H), 1.91 (s, 2H), 1.35 (s, 3H), 1.22 (s, 6H).

**Synthesis of Fn-pH**

Fn-Rh (240 mg, 0.5 mmol), NHS (115 mg, 1 mmol), and DCC (206 mg, 1 mmol) were dissolved together in 20 ml of anhydrous $CH_2Cl_2$ in a round-bottom flask. The mixture was stirred at room temperature for 1 hour. Subsequently, 4-(2-aminoethyl) -morpholine (130 mg, 1 mmol) and $NEt_3$ (101 mg, 1 mmol) were dissolved in 15 ml of anhydrous $CH_2Cl_2$ and added dropwise to the reaction mixture. The stirring was continued at room temperature for 24 hours. The reaction mixture was then filtered to remove any insoluble material, and the solvent was evaporated under reduced pressure. The resulting pale yellow transparent solid was purified by column chromatography using silica gel and a dichloromethane (20:1) eluent to obtain the transparent solid **Fn-pH** (124 mg, 42% yield).

**Tests of fluorescence intensity in different pH**

Preparation of Probe Masterbatch:3 mg of compound **Fn-pH** was weighed using a precision electronic scale (accurate to 0.0001 g) and dissolved in 5.07 ml of DMSO. The solution was homogenized using ultrasonic shaking to obtain a 1 mM **Fn-pH** masterbatch. Preparation of Britton-Robinson Buffer Solution: Accurately weigh specific amounts of phosphoric acid, boric acid, and acetic acid to prepare a 0.04 M mixed acid solution using pure water. Similarly, weigh a specific amount of NaOH and dissolve it in pure water to form a 0.2 M NaOH solution. Buffer solutions with pH values ranging from 2.04 to 10.35 were then prepared by mixing the acid and base solutions as needed, using a pH meter to adjust the pH accurately

**Calculation of the p*Ka***

Analysis of fluorescence intensity as a function of pH using the Henderson - Hasselbalch equation. This formula is a common method used to determine the pKa value of an acid and is usually applied in experiments such as fluorescent probes or indicators:

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

$I_{max}$ and $I_{min}$ represents the maximum and minimum values of fluorescence intensity in fully protonated and fully deprotonated states, respectively.

$I$ represents the fluorescence intensity at a specific pH condition.

**pH** represents the pH value corresponding to the current $I$.

**Calculation of the quantum yield**

The fluorescence quantum yield of **Fn-pH** under acidic and alkaline conditions was measured using Rhodamine B as a standard reference. The known fluorescence quantum yield of Rhodamine B in ethanol is $\Phi_S$ = 0.97. The calculation was performed using the following formula:

$$\Phi = \Phi_s \frac{IA_s}{I_s A}\left(\frac{n^2}{n_s^2}\right)$$

$\Phi_S$ is the fluorescence quantum yield of the reference substance.

$I$ and $I_s$ are the fluorescence intensities of the sample and reference substance, respectively.

$A_s$ and $A$ are the absorbances of the sample and reference substance, respectively.

$n$ and $n_s$ are the refractive indices of the solvents for the sample and reference substance, respectively.

**Cell Culture**

HeLa, HepG2, and HL-7702 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) and RPMI 1640(Roswell Park Memorial Institute) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL

streptomycin, Hyclone). Before the experiment, the cells were seeded in 35 mm glass-bottom dishes and incubated at 37°C in an incubator with 5% $CO_2$ and 95% air for 24 hours. Imaging was conducted using HeLa, HepG2, and HL-7702 cells, which were divided into multiple groups, including control and experimental groups. For experimental imaging, cell density needed to cover more than 70% of the glass-bottom dishes. The imaging experiments included colocalization, pH regulation, and dynamic imaging of apoptotic cells.

**Colocalization Experiment**

The localization of **Fn-pH** in live cell lysosomes and apoptotic cell lipid droplets was investigated using commercial probes. Before each test, cells were incubated with both **Fn-pH** (10 μM) and the corresponding commercial dye (5 μM) in the cell culture dishes for at least 30 minutes. After incubation, the supernatant was removed, and the glass-bottom dishes containing the cells were washed twice with PBS. Fluorescence imaging was then performed using a Leica TCS SP8 CARS confocal microscope with a 63× objective lens.

**Cellular Autophagy**

Established the autophagy model of HeLa cells by PBS induction to simulate the autophagic stress state of cells under nutrient-deficient conditions. The specific steps were as follows:

Cell culture and pretreatment:

HeLa cells were inoculated in suitable culture dishes and grown to about 70%~80% confluence. Before treatment, the cells were gently rinsed twice with pre-warmed PBS to remove residual nutrients and serum from the culture medium.

Induction of autophagy:

Cells were induced to enter a starvation state by completely replacing the cells with nutrient-free PBS, which at this point provides a pure buffer environment and does not contain any nutrients that promote cell growth. Typically, cells were incubated at 37°C and 5% $CO_2$ for 2 to 6 hours to induce the initiation of autophagy. The incubation time can be optimized for different experimental purposes.

**Cell Imaging Experiments**

The stock solutions of **Fn-pH** (1mM) were initially prepared in DMSO. Afterwards, 2 μL of stock solutions were added into 1 mL culture medium and mixed evenly to obtain the working solutions (10 μM). The culture medium in the glass bottom dish was removed and 1 mL of working solution containing 10 μM of the probe was used to incubate the cells for 30 min. Then the cells were directly imaged under Leica TCS SP8 CARS confocal microscope with Two washes of PBS. For blue channel, $\lambda_{ex}$ = 405 nm, $\lambda_{em}$ = 450-490 nm; for green channel, $\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 500-520nm; for red channel: $\lambda_{ex}$ = 540 nm, $\lambda_{em}$ = 570-610 nm.

**Cell Viability Assay**

HeLa cells were seeded into a 96-well plate (Solabio Life Sciences, Beijing, China) and incubated at 37°C in a cell culture incubator containing 5% $CO_2$ until the cells reached over 90% confluence. Various concentrations of **Fn-pH** (0, 1, 2, 5, 10, 20, 30, 40, 50μM) were then added to the wells, and the cells were incubated for an additional 24 hours. After the incubation period, the excess DMEM was removed from each well, and DMEM containing MTT (5 mg/mL) was added. The cells were incubated for another 4 hours. Subsequently, the supernatant was removed, and 100μL of DMSO was added to each well to dissolve the formazan crystals formed. The absorbance of each well was measured at 490 nm using a absorbance reader (Molecular Devices, USA). Cell viability was calculated using the following formula:

$$The\ cell\ viability\ (\%) = {(OD_{sample} - OD_{blank})}/{(OD_{control} - OD_{blank})} \times 100\%$$

$OD_{sample}$ represents the optical density measurements obtained from cells incubated with different concentrations of the probe.

$OD_{control}$ represents the optical density measurements obtained from cells incubated with a probe concentration of 0 (control group).

$OD_{blank}$ represents the optical density measurements obtained from the blank culture medium.

**Figures used in the manuscript**

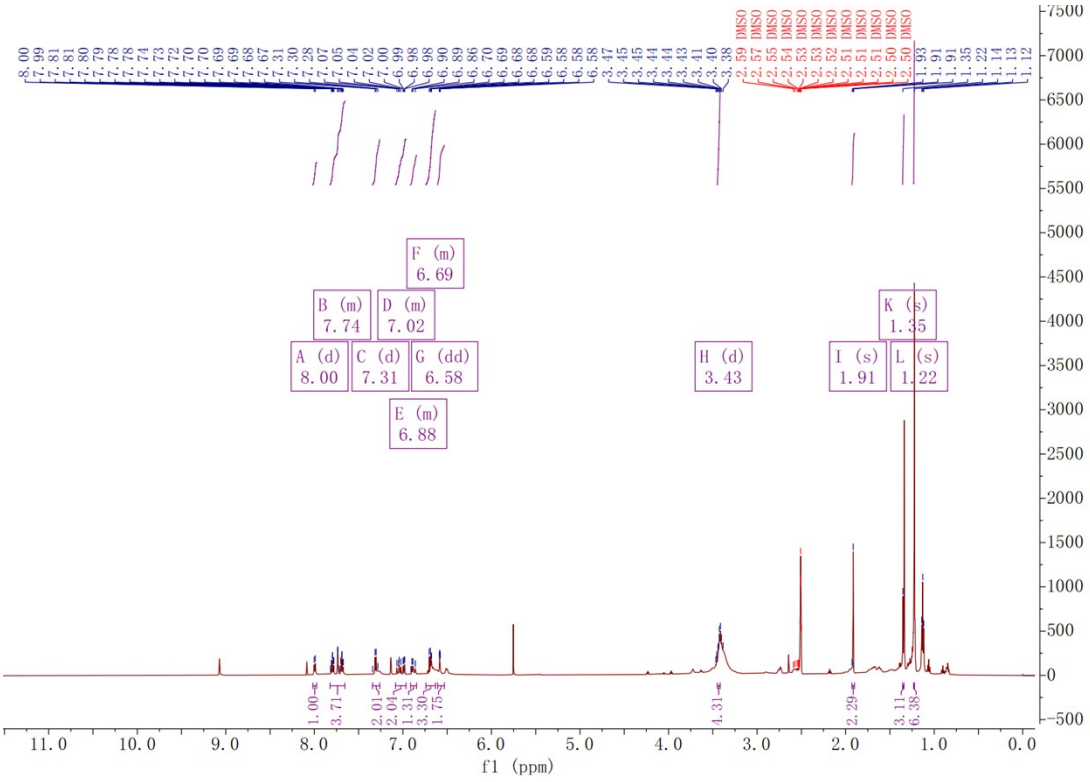



**Figure S 1** The $^{1}$H NMR spectrum of **Fn-Rh** in DMSO-$d_6$.

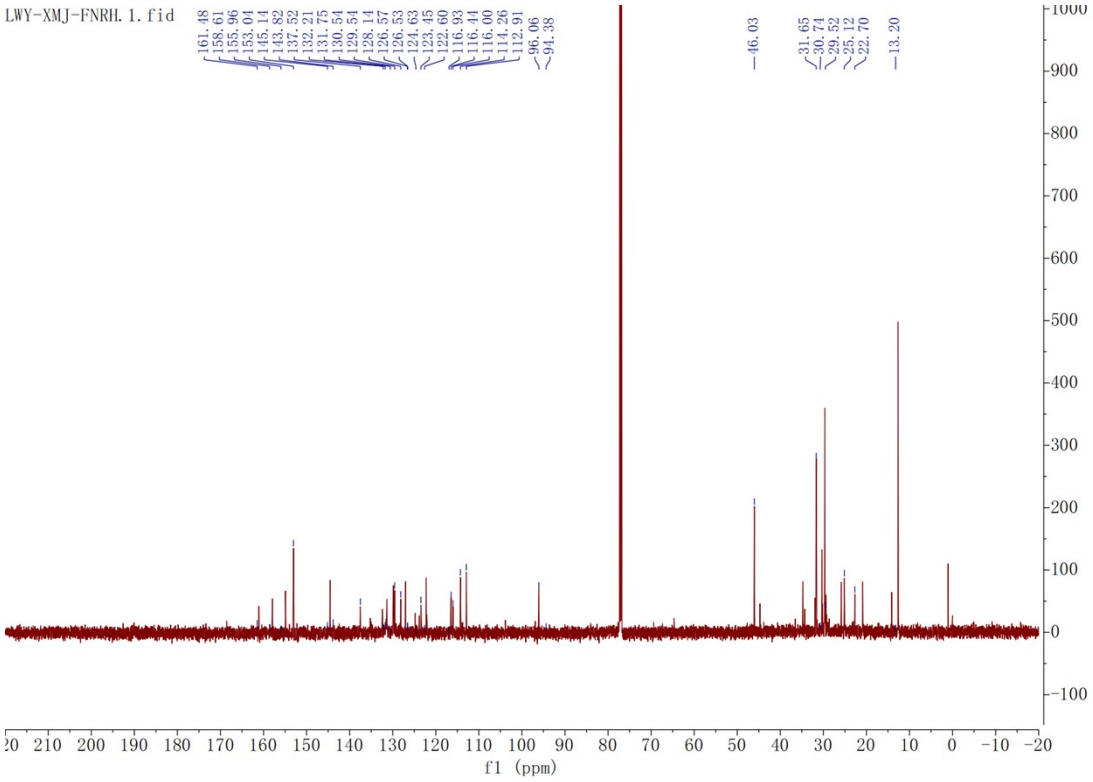



**Figure S 2** The $^{13}$C NMR spectrum of **Fn-Rh** in $CDCl_3$.

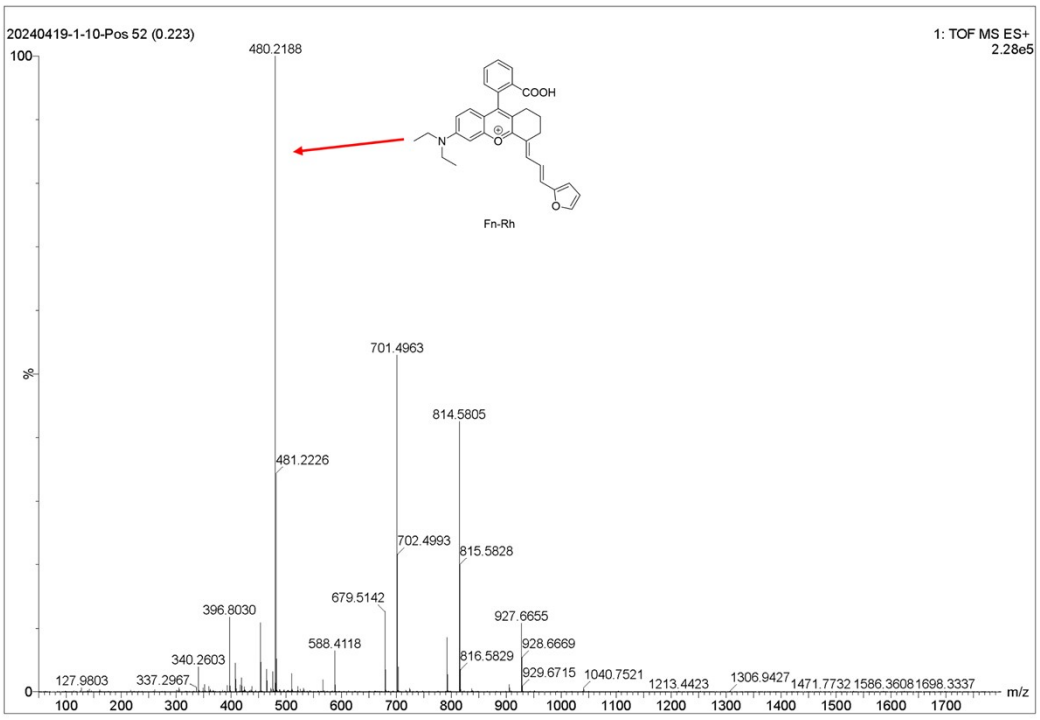



**Figure S 3** The HRMS spectrum of **Fn-Rh**.

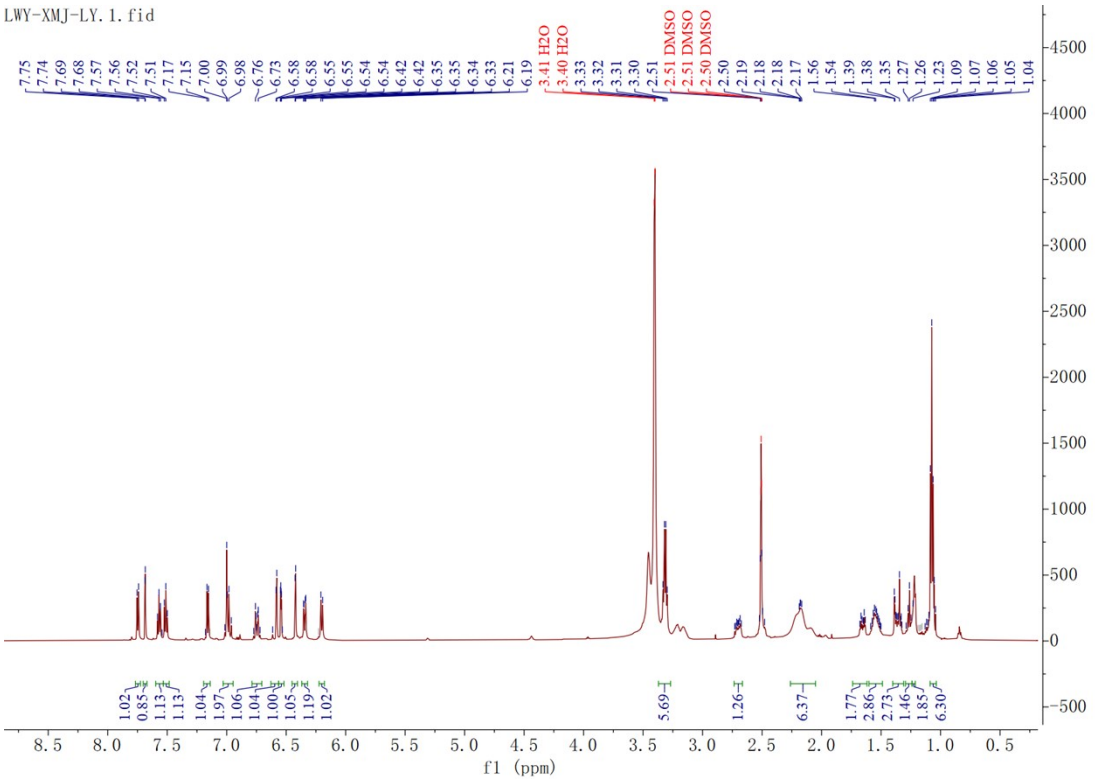



**Figure S 4** The $^{1}$H NMR spectrum of **Fn-pH** in DMSO.

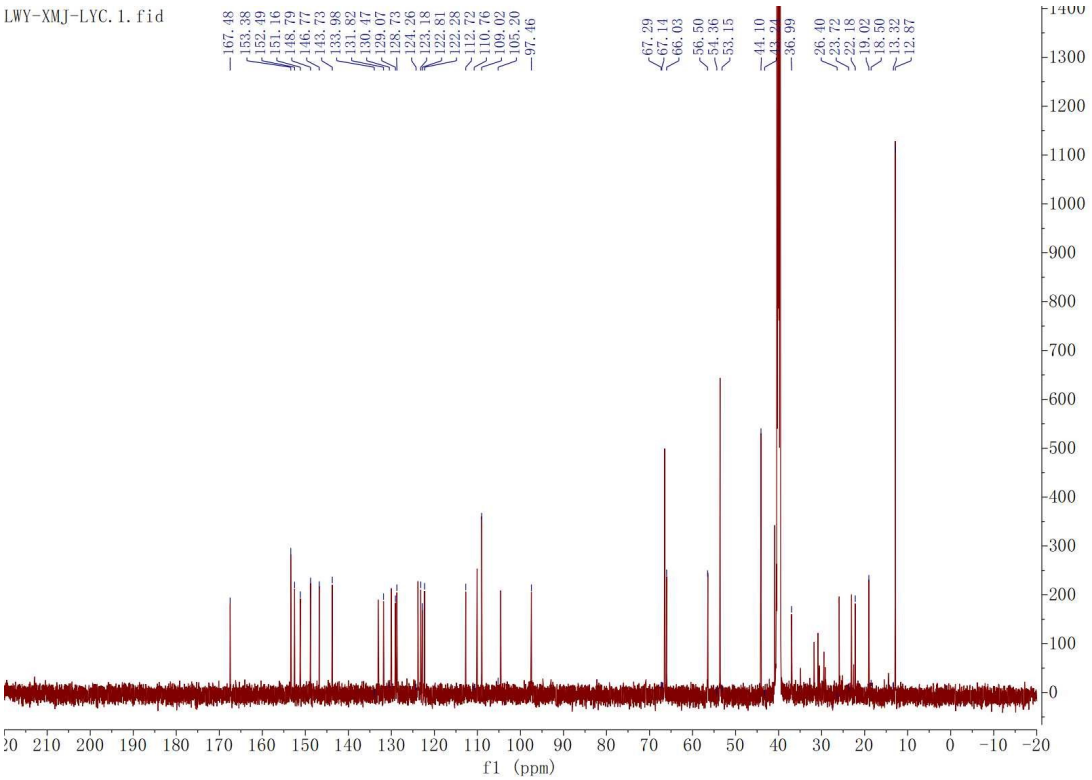



**Figure S 5** The $^{13}C$ NMR spectrum of **Fn-pH** in DMSO.

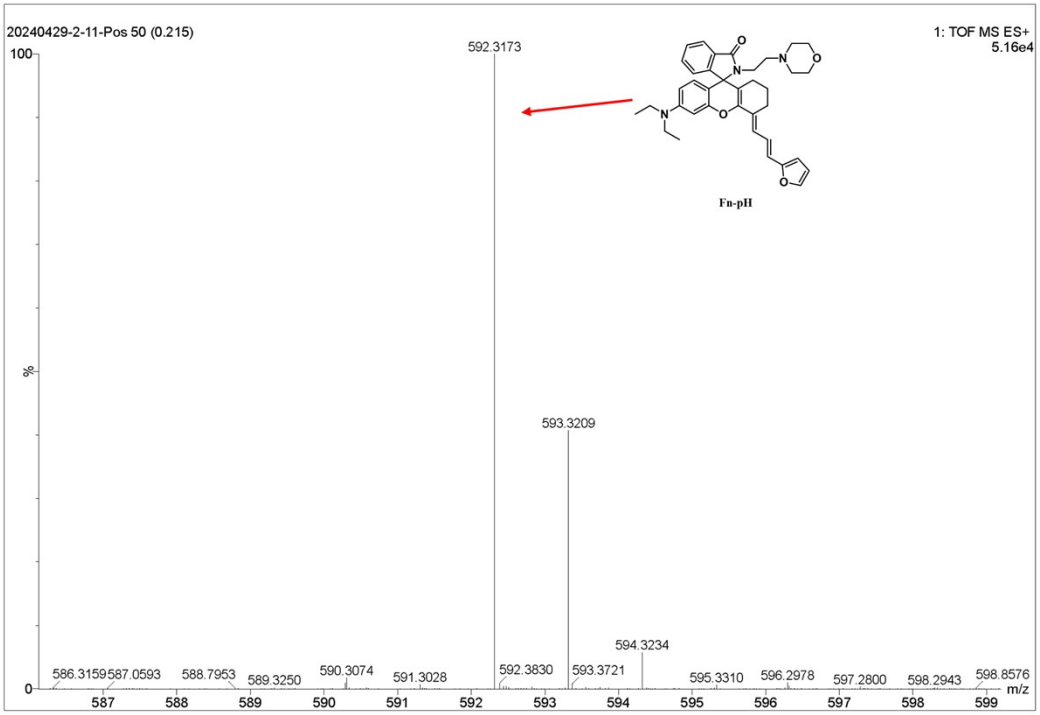



**Figure S 6** The HRMS spectrum of **Fn-pH** .

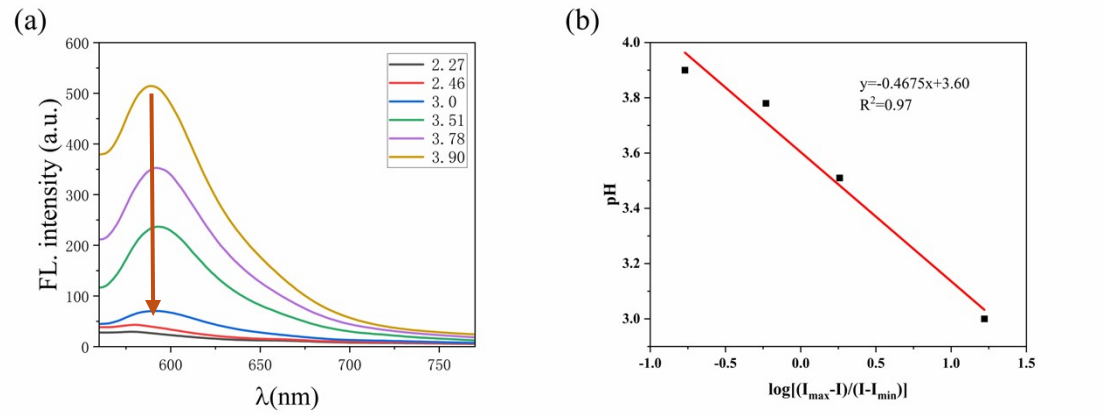



**Figure S 7** （a）Fluorescence intensity curve of **Fn-pH** at 590 nm in BR buffer solutions with pH ranging from 2.27 to 3.90.（b）Linear fit of Fn-pH to $F_{590}$ in the pH range 2.27-3.90(p*Ka*=3.60), with the fit line using the Henderson-Hasselbalch equation: pH-p*Ka* =*log*[( $I_{max}$ -*I*)/(*I* - $I_{min}$)].

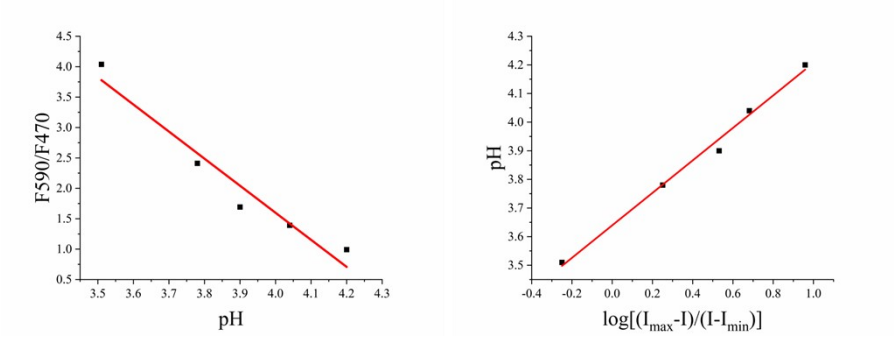



**Figure S 8** Linear fit of $F_{590}/F_{470}$ versus pH for **Fn-pH** in the range of pH 3.5-4.2, and the fitted line using the Henderson-Hasselbalch equation: pH-p*Ka* =*log*[( $I_{max}$ -*I*)/(*I* - $I_{min}$)].

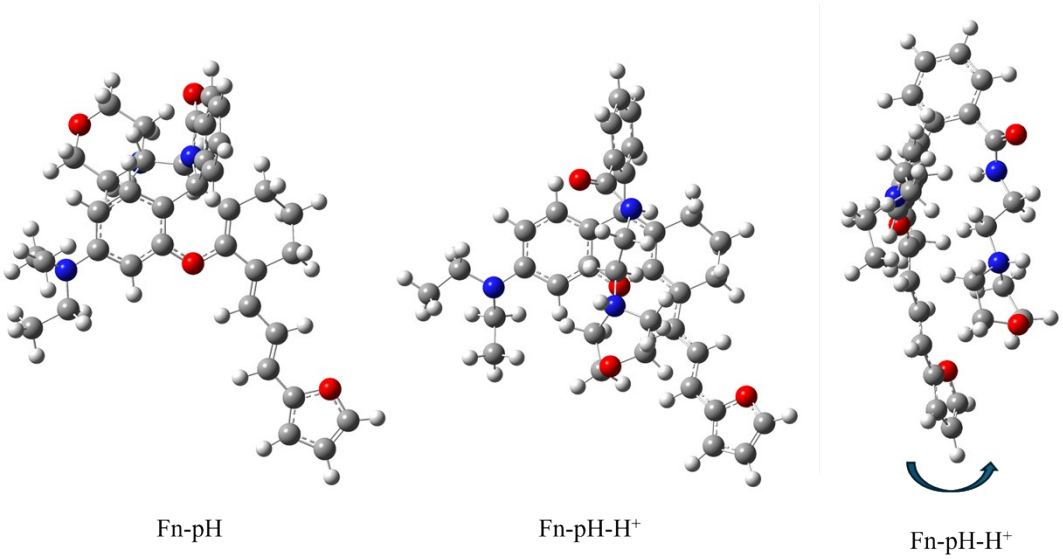



**Figure S 9** Optimized structures of **Fn-pH** and **Fn-pH-H**$^+$ using DFT calculations.

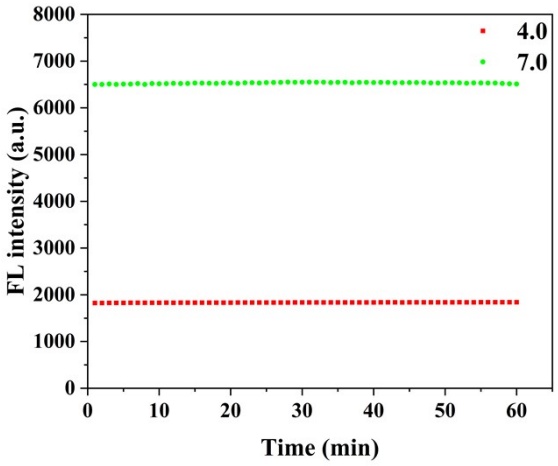



**Figure S 10** Photostability of **Fn-pH** fluorescence intensity at 470 nm over time ($\lambda_{ex}$ = 360 nm, pH = 4.0, 7.0).

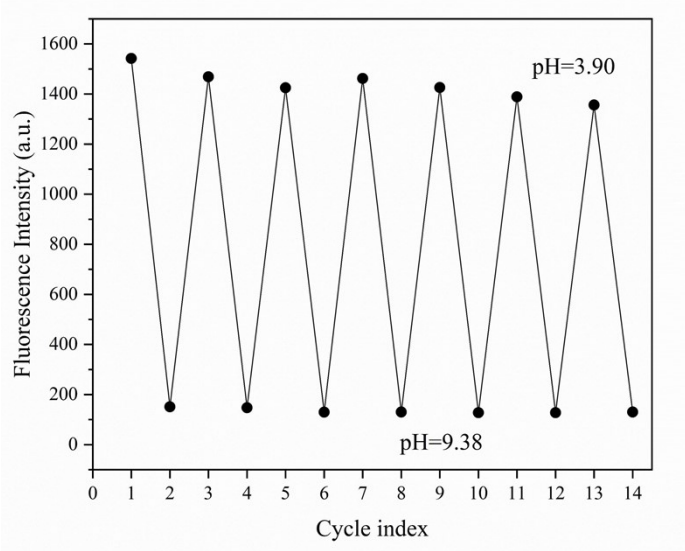



**Figure S 11** pH reversibility study of **Fn-pH** between pH 3.9 and 9.38 in BR buffer solution.

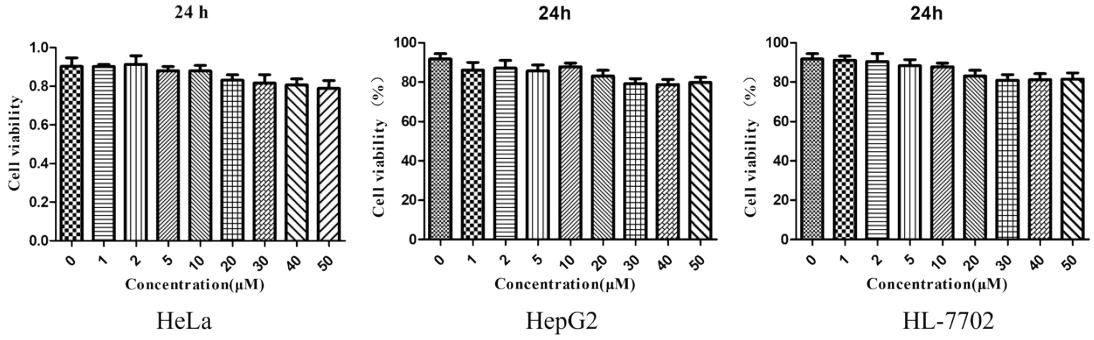



**Figure S 12** The cytotoxicity of **Fn-pH** in Hela, HepG2 and HL-7702 cells.

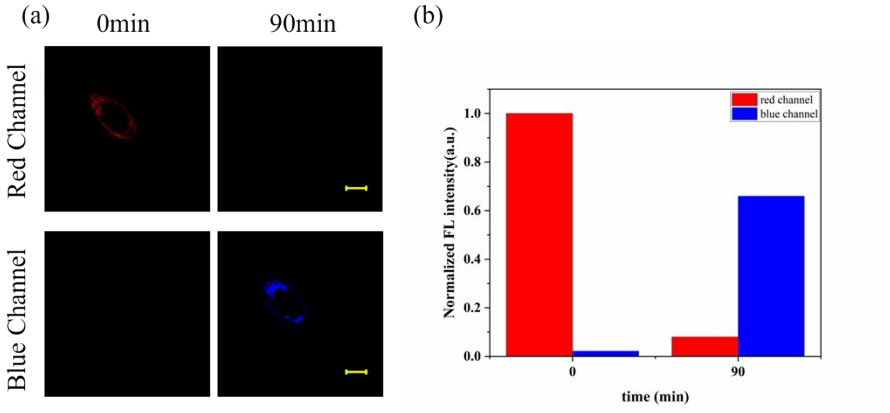



**Figure S 13** (a) Fluorescence images of **Fn-pH** (10 μM) in the red and blue channels during $H_2O_2$ induced apoptosis in HeLa cells (Hela was imaged continuously during **Fn-pH** incubation). (b) Normalized fluorescence intensity of the red and blue channels at 0 and 90 min. scale bar: 10 μm.

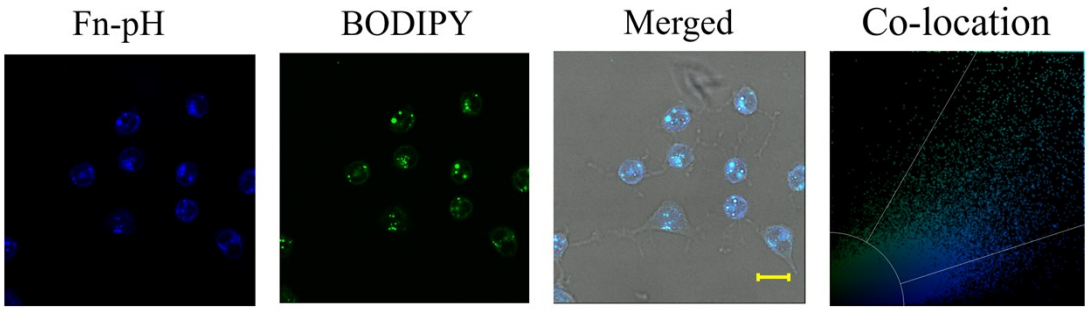



**Figure S 14** Colocalization imaging of **Fn-pH** with **BODIPY 493/503**.

**Table S1**. The optical properties of the acidic and basic forms of the probe **Fn-pH**.

| | $A_{max}$ [nm] | $F_{max}$ [nm] | Stokes Shift | ε [$cm^{-1}$*L*$mol^{-1}$] | Φ |
|---|---|---|---|---|---|
| Acidic form | 540nm | 590 nm | 50 nm | 108900 | 0.152 |
| Basic form | 360 nm | 470 nm | 110 nm | 65100 | 0.016 |

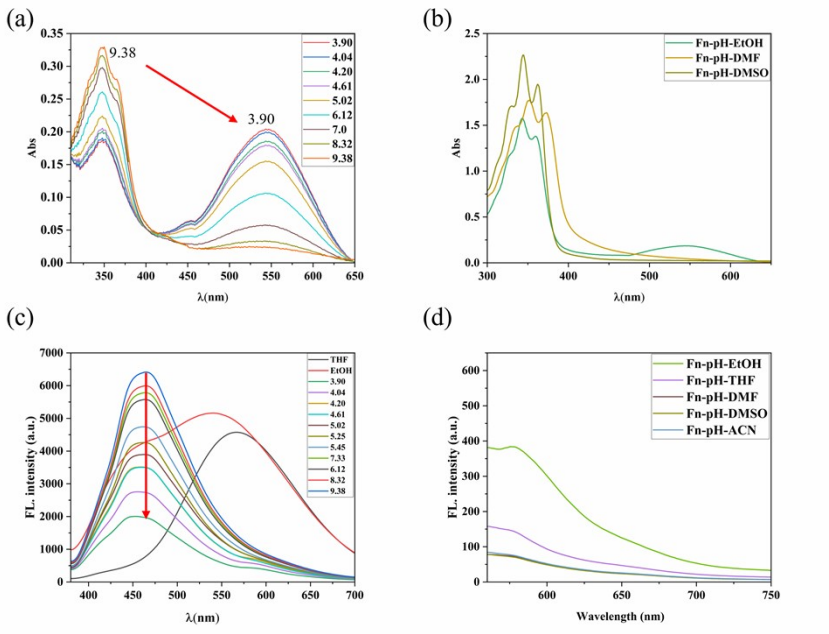



**Figure S 15** (a) Absorption spectra of **Fn-pH** between pH 3.90 and 9.38.(b) Absorption spectra of **Fn-pH** in EtOH, DMF and DMSO. (c) **Fn-pH** emission spectra between pH 3.90 and 9.38 using 360 nm laser excitation. (d) Emission spectra of **Fn-pH** in EtOH, THF, DMF, DMSO, and ACN using 540 nm laser excitation.

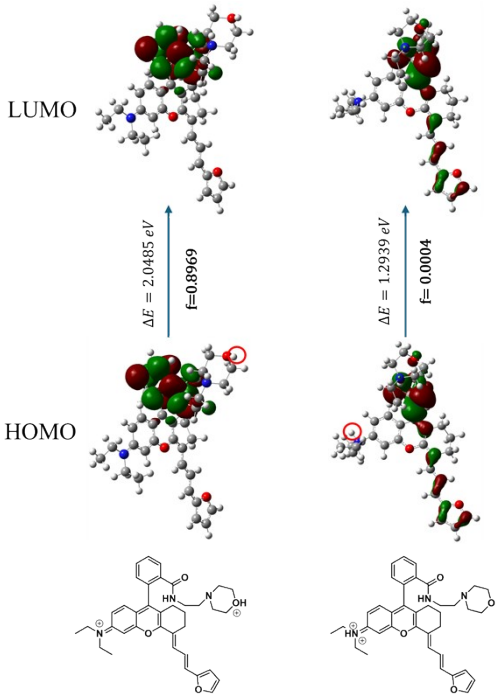



**Figure S 16** HOMO and LUMO energy level diagrams of Further protonation of **Fn-pH-H**$^{+}$ were obtained through TD-DFT calculations.

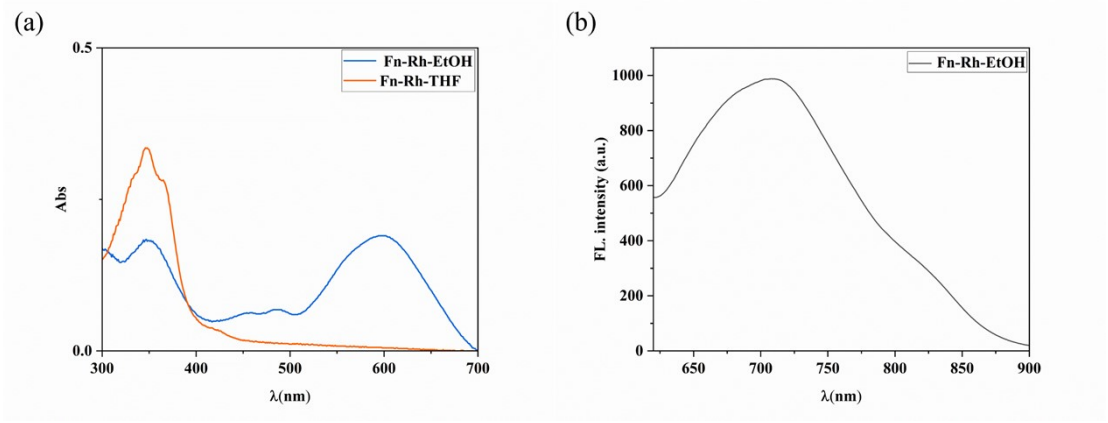



**Figure S 17** (a) Absorption spectra of **Fn-Rh** in EtOH and THF. (b)Emission spectra of **Fn-Rh** in EtOH ($\lambda_{ex}$ = 600 nm)