Supporting information for

Rational Construction of a Simplified Hemicyanine-based Fluorescent Probe CCX-1 with Dual pH and Viscosity Response for Lysosomal-Specific Tumour Imaging

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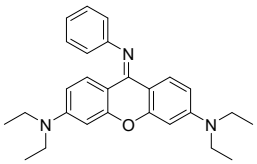
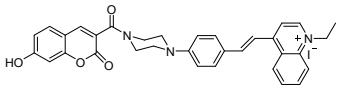
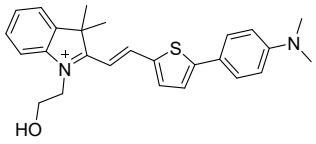
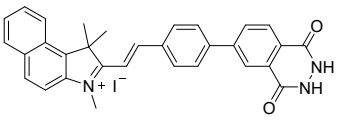
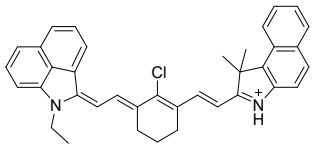
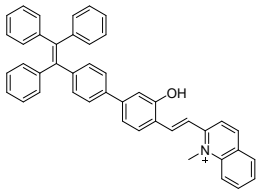
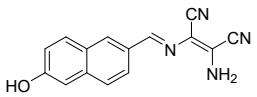
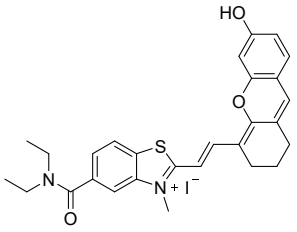
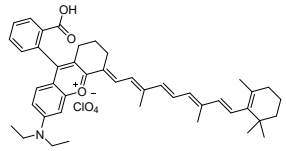
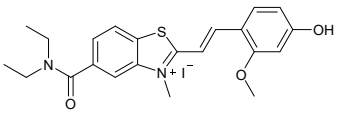
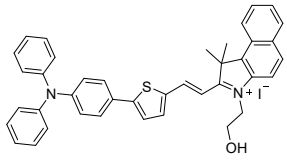
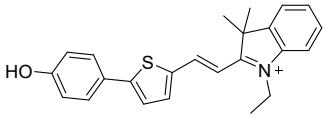
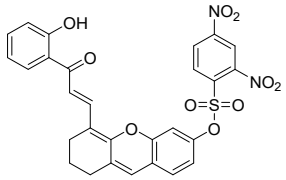
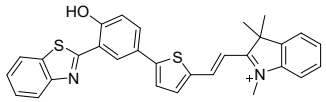
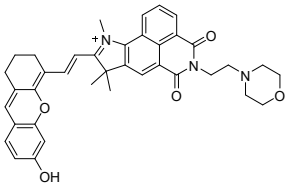
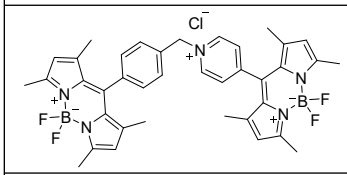
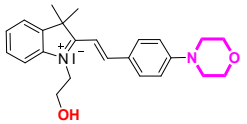
1. Supplementary Results

Table S1： The most recently reported representative pH/Viscosity Probes

Probe structure	Targeting/acidic response	Application	Reference
	Lysosome red-shift	Imaging and visualization of lysosomal viscosity in live cells and tumor tissue.	*Anal Chem, 2021, 93, 1786.*
	Mitochondria red-shift	Responding to mitochondrial viscosity, pH, and SO_2 in living cells.	*SENSOR ACTUAT B-CHEM,2022,371,132506.*
	Lysosome blue-shift	Visualizing the changes in pH and viscosity, and differentiating cancer cells	*Analyst,2022,147,2470-2476.*
	Lysosome blue-shift	Differentiating cancer cells and normal cells in mice.	*SENSOR ACTUAT B-CHEM,2023,375,132935.*
	- red-shift	pH and viscosity dual-response, enable High-Contrast activatable phototheranostics in extrahepatic diseases.	*Angew. Chem. Int. Ed. 2023, e202309768*
	Mitochondria and Lysosome blue-shift	Discriminating cancer from normal cells and organs with high-contrast fluorescence imaging.	*Talanta, 2023, 265, 124862.*
	- blue-shift	Differentiating diabetes cells from healthy cells and monitor chronic wound healing.	*SENSOR ACTUAT B-CHEM,2023,393,134345.*
	- blue-shift	Monitoring pH values in human serum, environmental water samples, meat samples and live cells, as well as changes in cell viscosity.	*SPECTROCHIM ACTA A,2024,318,124486.*

	Mitochondria blue-shift	Detecting endogenous pH and viscosity change in the environment of cells and live zebrafish.	*SPECTROCHIM ACTA A, 2024,319,124527*
	- blue-shift	Monitoring the pH of various water samples and human serum in the natural environment.	*Talanta,2024,266,125049.*
	LDs and mitochondria red-shift	Investigating the interactions between LDs and mitochondria.	*Talanta, 2025, 281, 126849.*
	Mitochondria -	Assisted detection of non-alcoholic fatty liver in mice.	*J Mater Chem B, 2025, 13, 3677*
	- blue-shift (biothols)	Monitoring alterations in biothiol levels, viscosity, and pH within live cells, as well as tumor imaging and prognostic assessment.	*SENSOR ACTUAT B-CHEM, 2025, 424.*
	Mitochondria -	Real-time viscosity monitoring in HepG2 cells, zebrafish, and glucose-treated systems.	*Org Biomol Chem, 2025,*
	Lysosome red-shift	High signal-to-background ratio for cellular imaging and proved effective for real-time in situ diagnosis of acute gastritis.	*Anal. Chem, 2025, 97, 4041-4048.*
	Mitochondria red-shift	Mitochondrial fluorescent imaging of cells and image alkaline pH value fluctuations in *E. coli* cells.	*J Mol Struct, 2025, 1319.*
	Lysosome red-shift	Visualizing the changes in pH and viscosity at the cellular level, enable cancer cell discrimination and in vivo tumor visualization.	*This work*

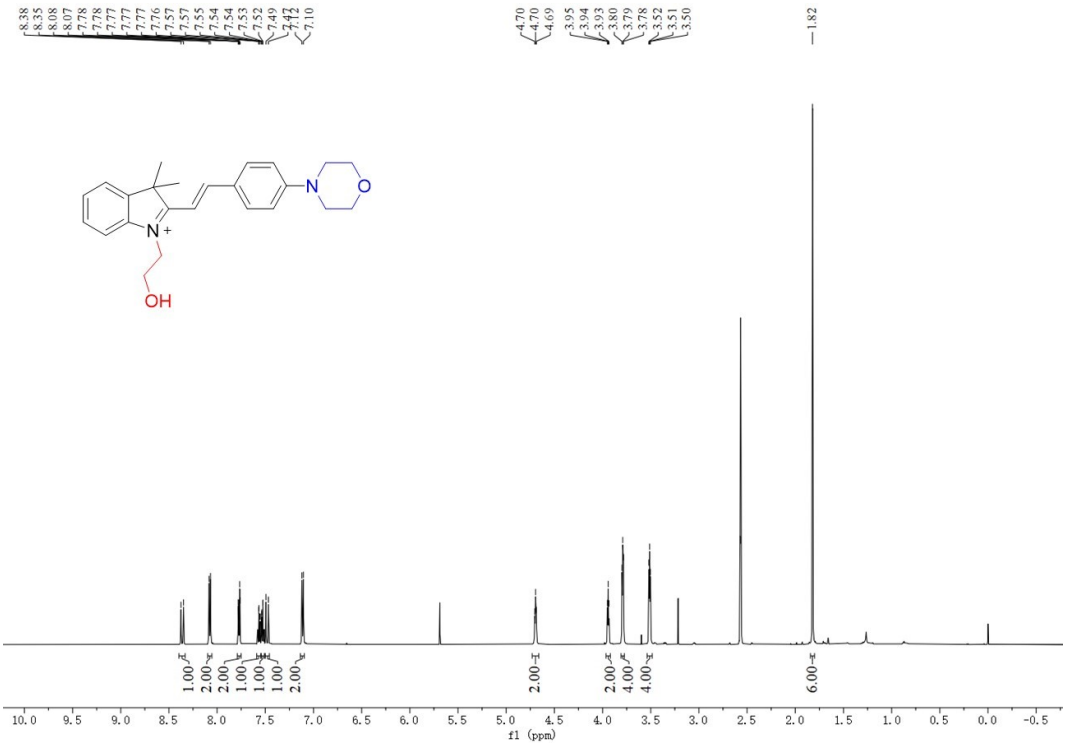


Fig. S1 ^{1}H NMR of CCX-1 (80%DMSO-*d*6+ 20%TFA-*d*)

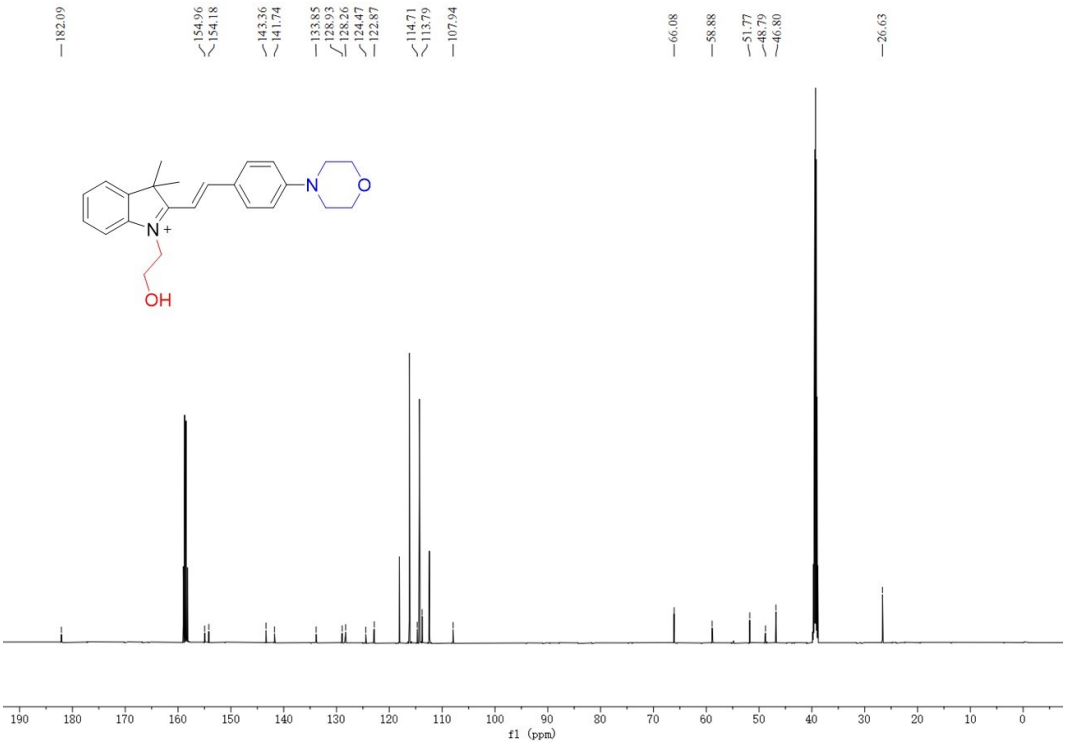


Fig. S2 ^{13}C NMR of CCX-1 (80%DMSO-*d*6+20% TFA-*d*)

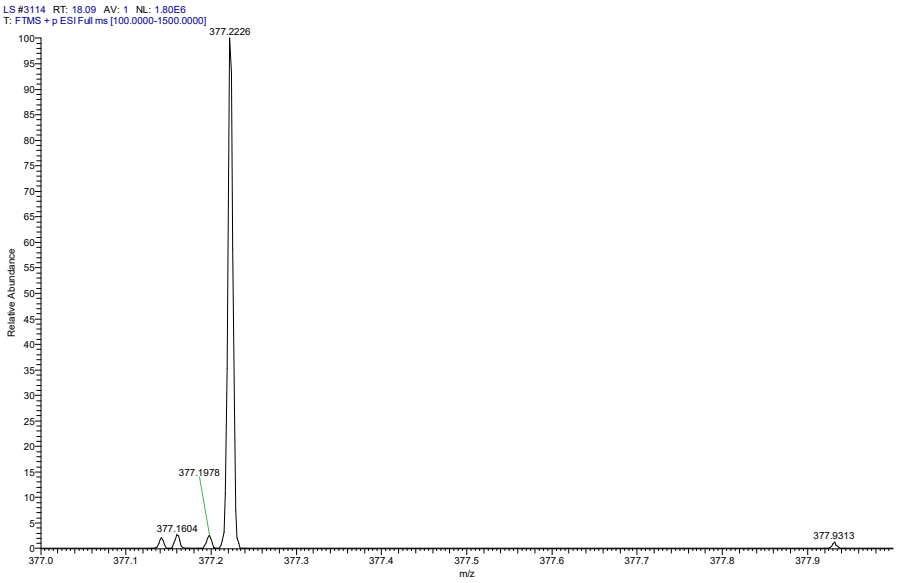


Fig. S3 HRMS of CCX-1, $[M-I]^+$ $C_{24}H_{29}N_2O_2^+$ calculate for 377.2224, measured 377.2226

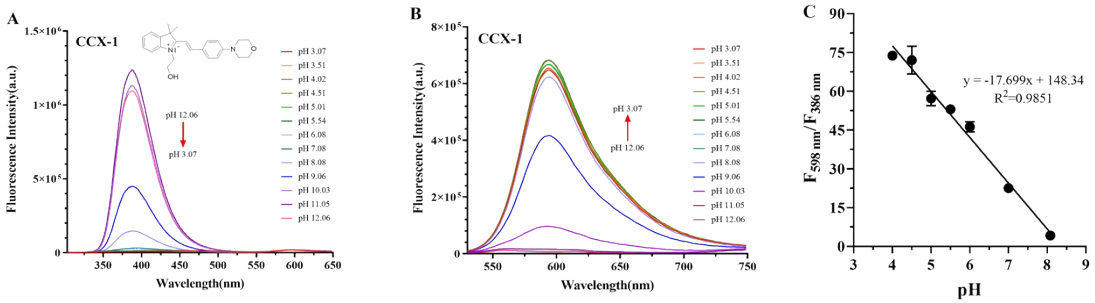


Fig. S4 The fluorescence spectra of the CCX-1 in PBS at pH 3.0 ~ 12.0. (A) Ex=283 nm, (B) Ex= 510 nm, slit width: 5 nm. (C) linear fluorescence response of CCX-1 across pH 4.0 ~ 8.0.

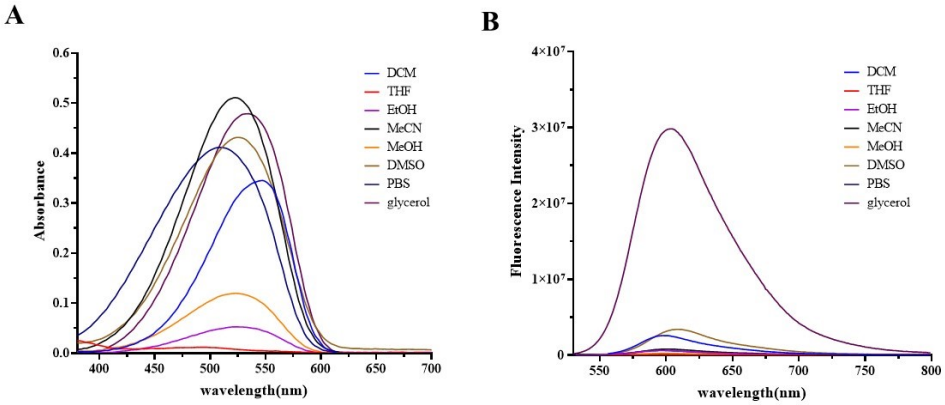


Fig. S5 The absorption(A) and fluorescence(B) spectra of CCX-1 in solvents of different polarity, Ex= 510 nm, slit width: 3.5 nm.

Table S2： The fluorescence quantum yield of CCX-1 under different conditions.

pH (PBS)	Φ (%)[a]	glycerol (vol %)[b]	Φ (%)[a]	Polarity Solvent	Φ (%)[a]
3.01	1.12	0%	1.23	MeCN	1.22
4.02	1.16	30%	4.03	MeOH	1.74
6.00	1.10	50%	11.10	DCM	5.66
7.44	1.00	70%	24.85	DMSO	6.65
8.02	1.01	100%	72.78	EtOH	9.96

[a] Rhodamine B was used as standards for fluorescence quantum yield calculations. (MeOH, Φ = 0.7).

[b] Fluorescence quantum yield of CCX-1 in H_2O-glycerol mixed solvent system. Slit：3.5/3.5 nm.

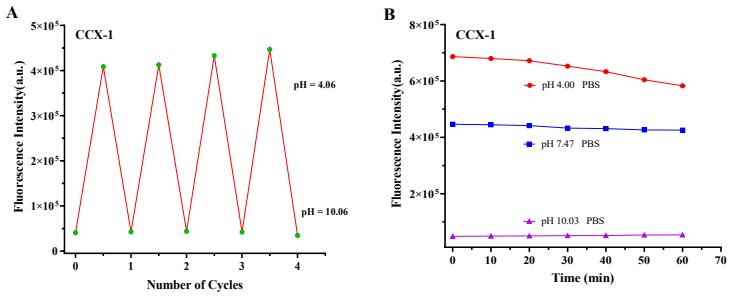


Fig. S6 (A) The reversibility of CCX-1 over multiple pH cycles (pH 4~10)**.** (B) The photostability of CCX-1 (10 μM) in PBS buffer (pH= 4.00, 7.47 and 10.03, respectively).

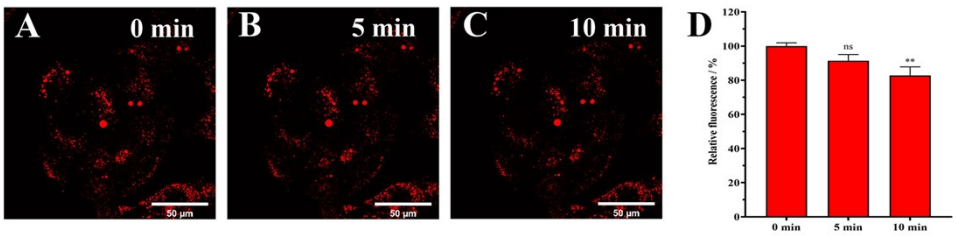


Fig. S7 CLSM images of CCX-1 stained HeLa cells under 552 nm laser irradiation (scale bar: 50 μm).

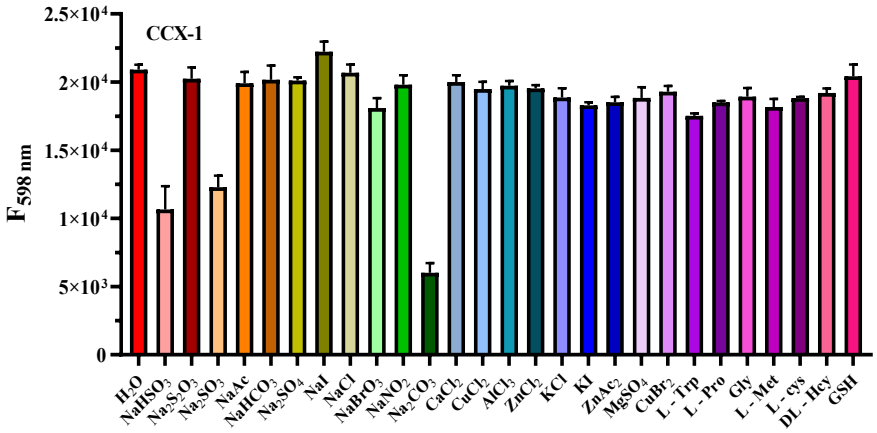


Fig. S8 Fluorescence intensity of probe CCX-1 (10 μM) in the presence of various interfering compounds (100 μM each): inorganic salts, amino acids, and bioactive molecules.

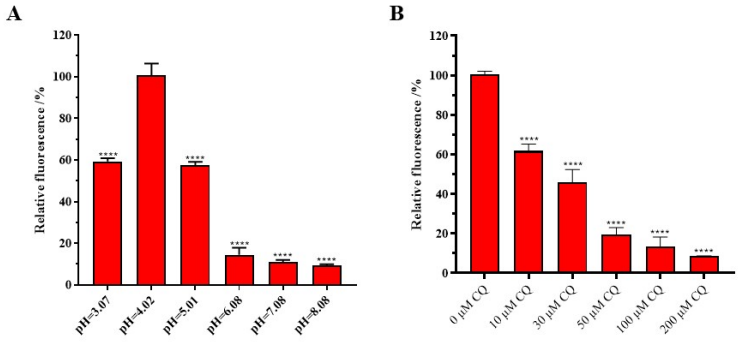


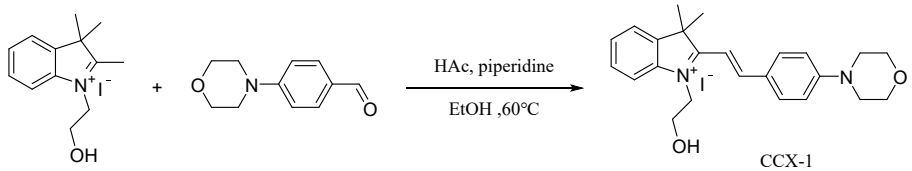
Fig. S9 Quantitative fluorescence analysis of HeLa cells incubated with CCX-1 (10 μM) under different pH conditions induced by Nigericin (A) and chloroquine (B).

2. Supplementary Methods

2.1 General

All commercial reagents and solvents were sourced from suppliers and used as received, without further purification or distillation. The ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker BioSpin Ultrashield 600 NMR system. The probes used for biological evaluation had a purity greater than 95%. Stock solutions (10 mM) of the probes were dissolved in DMSO and stored at -20°C.

2.2 Synthesis of (E)-1-(2-hydroxyethyl)-3,3-dimethyl-2-(4-morpholinostyryl)-3H-indol-1-ium iodide (CCX-1)



Scheme S1. Synthetic route of CCX-1

1-(2-hydroxyethyl)-2,3,3-trimethyl-3H-indolium iodide (50.00 mg, 0.15 mmol) and 4-(4-morpholinyl)benzaldehyde (31.76 mg, 0.17 mmol) were combined in a 100 mL pear-shaped flask, followed by the addition of 15 mL of anhydrous ethanol, and the mixture was stirred until complete dissolution. Subsequently, 100 μL of glacial acetic acid was added dropwise, and after heating at 60°C for 5 minutes, 100 μL of piperidine was added dropwise. The reaction was monitored by thin-layer chromatography (TLC). Upon completion, the organic solvent was evaporated under reduced pressure, and the crude residue was purified by silica gel column chromatography using mixed solvents of dichloromethane and methanol (DCM : MeOH, 30:1), obtaining CCX-1 as a red solid (88.01% yield). 1H NMR (600 MHz, 80%DMSO-*d*6+20%TFA-*d*) δ 8.36 (d, J = 15.8 Hz, 1H), 8.08 (d, J = 8.9 Hz, 2H), 7.77 (dt, J = 7.3, 1.7 Hz, 2H), 7.56 (dd, J = 7.8, 1.4 Hz, 1H), 7.53 (dd, J = 7.4, 1.0 Hz, 1H), 7.48 (d, J = 15.9 Hz, 1H), 7.11 (d, J = 9.0 Hz, 2H), 4.70 (t, J = 5.1 Hz, 2H), 3.94 (t, J = 5.1 Hz, 2H), 3.81 – 3.77 (m, 4H), 3.54 – 3.48 (m, 4H), 1.82 (s, 6H). ^{13}C NMR (151 MHz, 80%DMSO-*d*6+20%TFA-*d*) δ 182.09, 154.96, 154.18, 143.36, 141.74, 133.85, 128.93, 128.26, 124.47, 122.87, 114.71, 113.79, 107.94, 66.08, 58.88, 51.77, 48.79, 46.80, 26.63.

2.3 Spectroscopic determinations

UV Absorption Spectrum: Dilute the probe stock (10 mM) solutions to 10 μM using gradient pH PBS buffer solutions and transfer the diluted solutions to a 700 μL quartz crucible. Then, measure the probe absorption spectrum using a HITACHI U-3900 UV-Vis spectrophotometer. The parameters are set as follows: slit width 1 nm, step size 1 nm, and wavelength range 200-900 nm.

Fluorescence Emission Spectrum: The solution preparation is the same as for the absorption spectrum testing. After that, transfer the probe solutions to a fluorescent quartz crucible. Measure the fluorescence spectra of the probe molecules at different excitation wavelengths using a HORIBA FluoroMax+ fluorescence spectrophotometer with slit width 5 nm, and the step size 1 nm.

Quantum yield determination: The UV-Vis and fluorescence spectra in different solvents were measured by previously reported methods [ChemComm, 53(2017) 9910-9913]. The calculation of fluorescence quantum yield is shown in Equation：

$$\Phi_{sample}=\Phi_{standard}\times\frac{F_{sample}\times A_{standard}\times \eta^2_{sample}}{F_{standard}\times A_{sample}\times \eta^2_{standard}}$$

The abbreviations meaning in the equation are list as follows, Φ, the fluorescence quantum yield; F, the area under the fluorescence curve; A, the Absorbance of the probe; η, the refractive

index of the solution (the same refractive index of the same solvent). The standard for fluorescence quantum yield is Rhodamine B, and its quantum yield in MeOH is 0.7.

2.4 Photostability Testing

First, prepare 1 mL probe solutions (10 μM) with different pH value (pH 4.0, 7.4, and 10.0). Subsequently, irradiate these solutions with a xenon lamp for 60 minutes, while monitoring the characteristic fluorescence emission intensity of each probe at 10-minute intervals.

2.5 pH Reversibility Testing

Prepare 10 μM probe solutions and adjust their pH to 4.0 using a 1.0 M HCl solution. Measure the fluorescence emission intensity. Subsequently, adjust the pH of the solutions to 10.0 using a 1.0 M NaOH solution and measure the fluorescence intensity again. It is acceptable that the volume changes of the HCl and NaOH solutions during the pH adjustment have a negligible impact on the overall volume of the probe solutions. Repeat the adjustment process and record the fluorescence intensity after each adjustment. Finally, plot the data using GraphPad Prism.

2.6 Interference Resistance Testing

Select common metal salt ions such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Al^{3+}, Zn^{2+}, various anions such as HSO_3^-, $S_2O_3^{2-}$, CH_3COO^-, Cl^-, I^-, BrO_3^-, HCO_3^-, NO_2^-, CO_3^{2-}, SO_4^{2-}, Br^-, and amino acids (L-Gly, L-Trp, L-Pro, L-Met, L-Cys, DL-Hcy) as well as glutathione as interference substances to evaluate the probe's anti-interference capability. The final concentration of probe molecules and interfering components is 10 μM and 100 μM, respectively. After mixing the solutions and equilibrating for 3 minutes, set up a negative control and measure the changes in characteristic fluorescence signal intensity.

2.7 Viscosity Response Testing

The unit centipoise (cP) is used to measure the kinematic viscosity of solids or liquids. Water and glycerin are representative low and high viscosity solvents, respectively. At 20°C, water has a viscosity (η) of 1.00 cP, and glycerin has a viscosity of 945.00 cP. By adjusting the water to glycerin ratio, different viscosity environments can be simulated to evaluate the viscosity response performance of probe molecules. The viscosity values (η_{min}) of various glycerin-water mixtures can be calculated using the equation: $\eta_{min} = \Sigma\, w_i \times \ln(\eta_i)$， where η_{min} represents the viscosity of the mixed solution, η_i represents the viscosity of any component, and w_i represents the proportion of the component ($0<w_i<1$). The fluorescence intensity of the probe typically fits the Förster-

Hoffmann equation log I = C + xlogη, where η is the viscosity of the solution, I is the fluorescence emission intensity, x is the sensitivity of the probe molecule to solution viscosity, and C is a constant.

2.8 Cellular Experiment Methods

2.8.1 Cell Culture

Human cervical adenocarcinoma epithelial (HeLa) cells, Hepatocellular carcinoma (HepG2) cells and normal human liver cell line (HL-7702) are cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin). Human breast cancer cell line (MCF-7) is cultured in its designated medium. All cells are maintained at 37°C with 5% CO_2 for routine culture and subculture.

2.8.2 Cytotoxicity Testing

Harvest HeLa, HepG2, MCF-7, and HL-7702 cells in the logarithmic growth phase by digesting with 0.25% trypsin, followed by centrifugation to collect the cells. Resuspend the cells in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Count the cells and seed 100 μL per well in 96-well cell culture plates at a density of 5,000 cells per well. After cell adhesion, discard the culture medium and add a series of gradient concentrations of probe molecules (3.125 μM, 6.25 μM, 12.5 μM, 25 μM, 50 μM), prepared by dilution with the medium. Following a 24-hour incubation, remove the culture medium and add 1 mg/mL MTT solution to each well. Incubate for an additional 4 hours, then abandon the solution and add 150 μL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. Measure the optical density (OD) at 570 nm using a microplate reader. Calculate cell viability using the following formula: Cell Viability (%) = ($\triangle OD_{sample}$ / $\triangle OD_{control}$) × 100.

2.8.3 Intracellular Imaging

Seeding the HeLa, HepG2, MCF-7, HL-7702, and SH-SY5Y cells into 35 mm glass-bottom confocal dishes at a density of 6×10^4 cells/mL, and incubating the cells for 24 hours at 37°C with 5% CO_2 for routine culture. Then Removing the culture medium and incubating the cells with DMEM medium containing 10 μM of CCX-1, 75 nM of LysoTracker Green and 200 nM MitoTracker Green, respectively.) for another 30 minutes. Subsequently, washing the cells three times with PBS and add 1 mL of PBS to each dish and finally exciting at the probe's characteristic wavelength to perform fluorescence imaging. For cellular co-localization experiments, using LAS X software's

Quantify module to calculate the Pearson's correlation coefficient (PCC).

2.9 Visualizing Dynamic pH Changes in Lysosomes and Intracellular pH Values

Building upon protocol **2.8.3**, this procedure examines cellular pH dynamics through two experiments. The first investigates lysosomal pH by treating cells with chloroquine (0-200 μM, 30 min), a cell-permeable base that neutralizes lysosomal acidity. The second measures intracellular pH by using nigericin (10 μM) to equilibrate internal and external pH, followed by PBS treatment at varying pH (3.0~8.0, 10 min). Both experiments conclude with cell washing and confocal microscopy imaging.

2.10 Photostability of CCX-1 in Cells

The HeLa cells were incubated with the probe for 30 minutes. Subsequently, the specimens were subjected to sustained laser excitation at designated time intervals (0, 5, 10, and 15 minutes). Confocal microscopy images were acquired at each temporal point, followed by fluorescence intensity quantification utilizing Image J software. The temporal evolution of fluorescence intensity under continuous irradiation was analyzed to evaluate the probe's photostability. This assessment provided critical insights regarding the probe's applicability in dynamic biological imaging protocols.

2.11 Imaging of lysosomal viscosity fluctuations

This protocol investigates lysosomal viscosity changes under various conditions, starting with seeding 6×10^4 cells in 35 mm glass-bottom confocal dishes for 24-hour culture. The experiment comprises four distinct treatment conditions: standard probe incubation at 37°C for 30 minutes serves as the control; cold treatment at 4°C for 30 minutes to increase intracellular viscosity; dexamethasone treatment (10 μM, 37°C, 30 minutes) followed by probe incubation to examine increased lysosomal viscosity; and post-probe DMSO treatment (10 μL, 10 minutes) at 37°C. Following each treatment condition and three PBS washes, cells are examined using laser confocal microscopy for fluorescence imaging. The experimental data is then analyzed through Image J software for fluorescence intensity quantification and subsequent semi-quantitative analysis.

2.12 Animal bioimaging

The national standards of the People's Republic of China for laboratory animal-guideline for ethical review of animal welfare (GB/T 35892-2018) and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) have been strictly followed in animal experiments. BALB/c-nu mice were purchased from Zhuhai BesTest Bio-Tech Co,.Ltd, and were acclimatized for 1 week in the laboratory animal center under specific-pathogen-free (SPF) conditions with free access to standard food and water. In particular, all experiments were performed in compliance with the ARRIVE guidelines 2.0, and were approved by the Laboratory Animal Welfare & Ethics Committee of Zunyi Medical University (ZHSC-2-(2023)023).

Female BALB/c-nu mice (4-6 weeks old, n=3) were used for in vivo imaging. The xenograft tumor model was established by subcutaneous injection of 1×10^7 HeLa cells. Subsequently, mice bearing tumors were selected for imaging experiments. A 100 μL of CCX-1 solution (150 μM) was injected into both the tumor site and the contralateral thigh muscle (serving as a control) of the mice. Fluorescence imaging was conducted using a small animal in vivo imaging system (Cypris) with an excitation filter of 520 nm at five time points (0, 15, 30, 45, and 60 minutes) post-injection, The acquired images (Em = 580 - 600 nm) were analyzed and semiquantitative by drawing regions of interest using the Living Image software 4.5.2.

2.13 Statistical analysis

All experiments are repeated at least three times. Data are processed using GraphPad Prism 8 and presented as mean ± standard deviation. A P-value of <0.05 is considered statistically significant. Statistical calculations employ two-tailed t-tests, one-way ANOVA, and multifactorial ANOVA where applicable, with significance levels indicated as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.