Supporting Information

Hemicyanine based ratiometric fluorescence probe for mapping lysosomal pH during heat stroke in living cells

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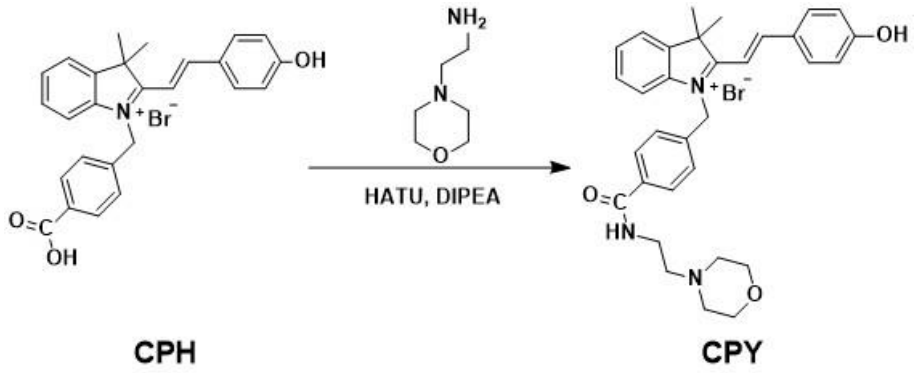
1. Synthetic experiments

Materials and methods

All chemical reagents and solvents were purchased from commercial sources and used without further purification. HATU (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), 4-(2-Aminoethyl)morpholine and DIPEA (Ethyldiisopropylamine) were purchased form Energy-Chemical company. LysoTracker Deep Red was purchased from Thermo Fisher Scientific. ^{1}H and ^{13}C NMR spectra were accessed from a Bruker AV-400 spectrometer (in CD_3OD). Electrospray ionization (ESI) mass spectrometry was performed in a HP 1100 LC-MS spectrometer.



Scheme S1. Synthesis of target probe **CPY.**

2-(4-hydroxystyryl)-3,3-dimethyl-1-(4-((2-morpholinoethyl)carbamoyl)benzyl)-3H-indol-1-ium bromide (**CPY**)**. CPH** was synthesized according to previous procedures.[1] To a solution of **CPH** (0.80 g, 1.67 mmol,1 eq) in DMF, DIPEA (0.24 g 1.84 mmol, 1.1 eq) and HATU (1.27 g, 3.34 mmol, 2 eq) was added sequentially. After stirring at room temperature for 10 min, 4-(2-Aminoethyl)morpholine (0.24 g, 1.84 mmol, 1.1 eq) was added. The solution was then left to stir overnight at room temperature. TLC indicated that the reaction was completed and the solvent was removed by rotary evaporator to provide the crude product, which was purified by flash column chromatography with using CH_2Cl_2/CH_3OH (v/v, 18:1) to afford the final product as dark red solid (0.72 g, 73% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.63 (s, 1H), 8.46 (s, 1H), 8.30 (d, J = 8.0 Hz, 1H), 7.93 (d, J = 8.7 Hz, 2H), 7.87 (d, J = 8.2 Hz, 2H), 7.77 (d, J = 7.0 Hz, 1H), 7.67 (d, J = 7.8 Hz, 1H), 7.61 - 7.52 (m, 2H), 7.43 (d, J = 8.1 Hz, 2H), 6.93 (d, J = 8.7 Hz, 2H), 5.94 (s, 2H), 3.88 (s, 4H), 3.75 (t, J = 5.5 Hz, 4H), 2.99 (d, J = 6.7 Hz, 4H), 1.89 (s, 6H). ^{13}C NMR (100 MHz, CD_3OD) δ 184.51, 170.46, 166.16, 158.19, 144.63, 142.49, 138.76, 135.17, 130.52, 129.58, 128.01, 127.42, 124.20, 117.99, 115.77, 67.03, 65.25, 58.56, 54.25, 53.66, 35.74, 27.08. HRMS (ES^+): calc. for $C_{30}H_{50}N_3O_3$ $[M\text{-}Br]^+$ 511.2835, found 511.2832.

2. Detailed protocols for characterization of CPY performance towards pH

Photophysical properties of probe CPY response to pH. CPY was dissolved in DMSO to make stock solution (5.0 mM). The stock solution (containing 1% DMSO) was diluted with water for pH titration experiments. Various pH solutions were adjusted by HCl (0.1 M) and NaOH (0.1 M). Excitation and emission slits were both 10 nm, and the excitation wavelengths were set at 473 nm. All the detection experiments were measured at room temperature.

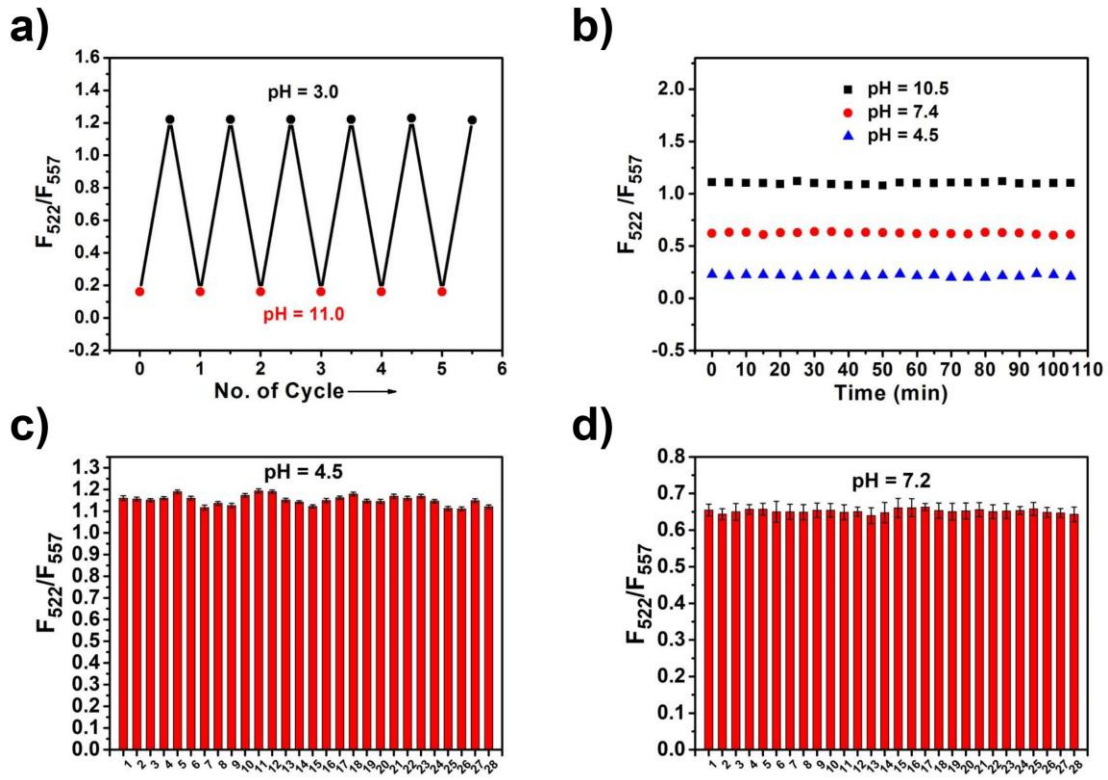


Figure S1. (a) pH reversibility of **CPY** in water at pH values of 11.0 and 3.0 respectively. (b) Time-course fluorescence intensity ratio changes of **CPY** (F_{522}/F_{557}) in aqueous solution at different pH values (4.5, 7.4, and 10.5). (c, d) Fluorescence intensity ratio response of **CPY** in 40 mM HEPES buffer at pH 4.50 (c) and 7.20 (d) with interference. 1, blank; 2, Na^+; 3, K^+; 4, Al^{3+}; 5, Zn^{2+}; 6, Ca^{2+}; 7, Cu^{2+}; 8, Fe^{2+}; 9, Fe^{3+}; 10, Mg^{2+}; 11, NH_4^+; 12, F^-; 13, Cl^-; 14, Br^-; 15, I^-; 16, SO_4^{2-}; 17, H_2S; 18, H_2O_2; 19, HClO; 20, glycine; 21, HSA (human serum albumin), 22, cysteine; 23, homocysteine; 24, arginine; 25. histidine; 26. glutathione; 27, glucose; 28, ATP (adenosine triphosphate). Note: The concentration of **CPY** and each interfering species are 10 μM and 100 μM respectively. λ_{ex} = 473 nm. Error bars represent s.d.

Detailed protocols for evaluating the photophysical performance of CPY. Φ is the ratio of photons absorbed to photons emitted through fluorescence and the number of absorbed photons. We measured the Φs according to "A Guide to Recording Fluorescence Quantum Yields" by Jobin Yvon Horiba Ltd at: http://www.horiba.com/fileadmin/uploads/Scientific/Documents/Fluorescence/quantumyieldstrad.pdf. Fluorescein was dissolved in 0.1M NaOH as a standard, whose Φ is 0.93.[2] The Φ_s were determined by comparing the intergrated fluorescence intensity and the absorbance value (less than 0.1 at the excitation wavelength). In our experiment, we used slope method to calculate the Φ at three pH values for **CPY** in aqueous solution using the following equation:

$$\Phi_s = \Phi_f \, (Grad_{sample}/Grad_f) \, (n_{sample}/n_f)^2$$

where Φ is fluorescence quantum yield, Grad is the slope of the curves in Figure S2 and n is the refractive index (1.33 for water and a 0.1 M NaOH solution). The subscript "f" refers to the standards and "sample" refers to **CPY**. For these solutions, $n_{sample}/n_f = 1$. Six different concentrations for the standard and **CPY** were measured to obtain the slopes.

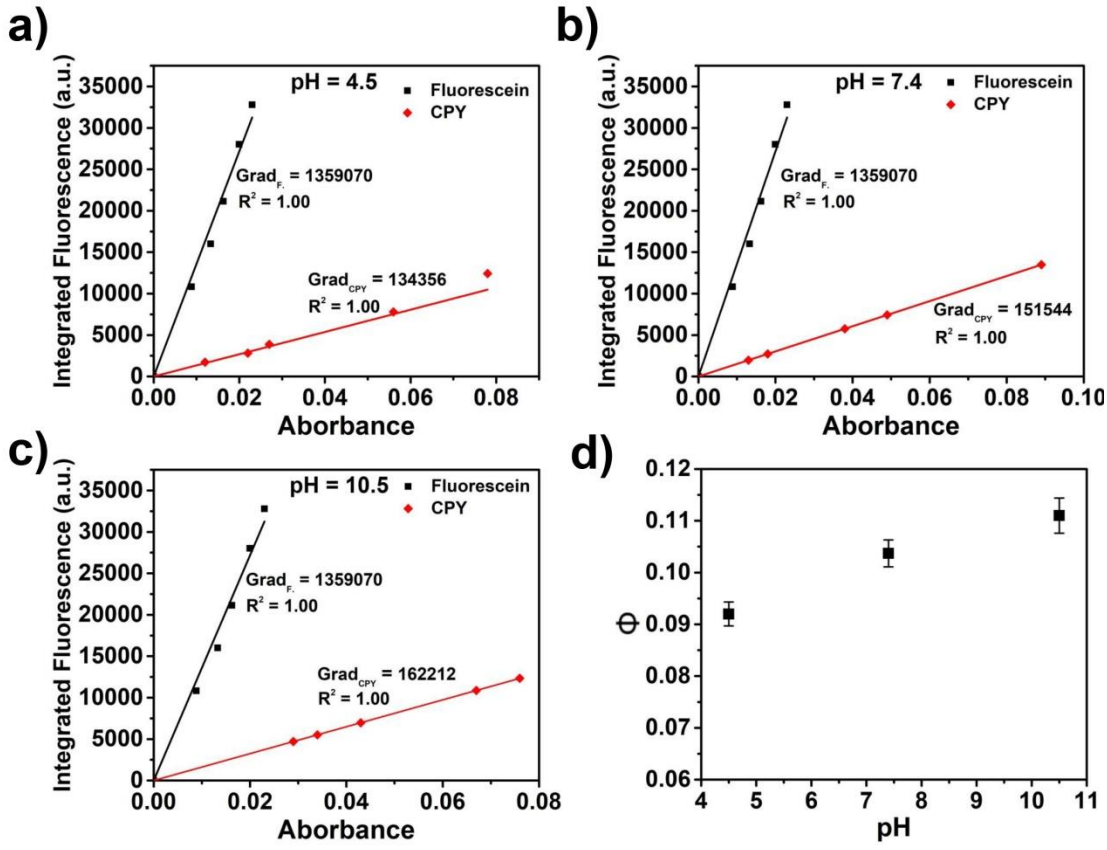


Figure S2. Fluorescein was taken as a fluorescence standard; plot of integrated fluorescence versus absorbance. The black line was obtained by linear fitting of the data for Fluorescein, and the red line was obtained by linear fitting of the data for **CPY** at pH 4.5 (a), pH 7.4 (b), and pH 10.5 (c), respectively. (d) Fluorescence quantum yield (Φ) of three different pH values of **CPY**.

Determination of the pK_a of probe CPY. The pK_a of **CPY** was calculated using the Henderson-Hasselbalch-type equation ($pH = pK_a + c*\log[(R - R_{min}) /(R_{max} - R)] + \log (I_a/I_b)$).[3-6]

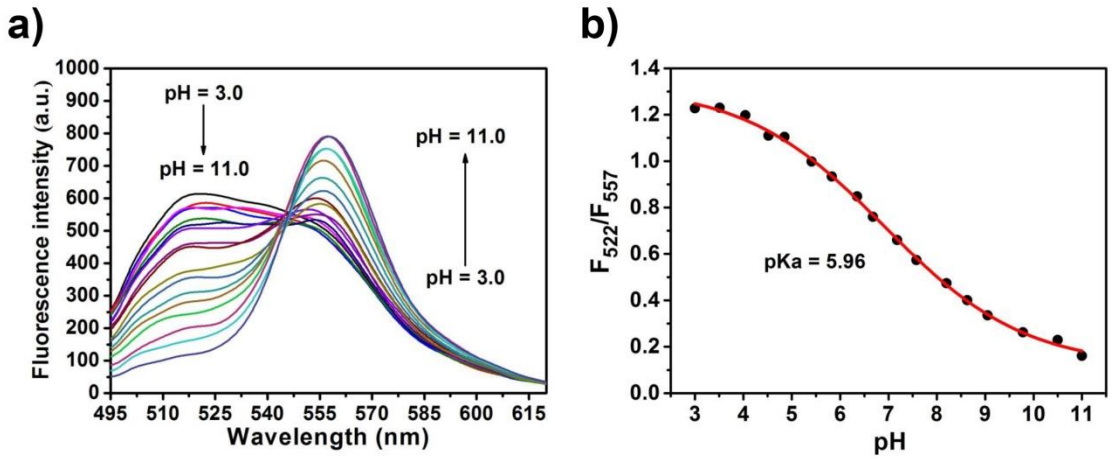


Figure S3. (a) Fluorescence response of **CPY** (10 μM) toward different pH in solutions containing 0.1% DMSO as co-solvent (λ_{ex} = 473 nm). (b) Plot of fluorescence intensity ratio changes (F_{522}/F_{557}) towards the pH value range of 3−11.

^{1}H NMR titration. ^{1}H-NMR titration experiments were then performed to evaluate the sensing mechanism. The ^{1}H-NMR spectrum of **CPY** was recorded in CD_3OD as solvent. Et3N was initially added to adjust pH value to above 9 (> 9), and the ^{1}H-NMR was recorded. Then, addition of CF_3COOD to readjust the pH value to below 5 (< 5) and the ^{1}H-NMR was recorded, again. As shown in Figure S4 and S5, addition of Et_3N makes the CD_3OD solution of **CPY** become alkaline, inducing an upfield shift for protons at the c, d, j and k positions, which are the vinylene protons, benzyl protons, and aromatic protons of the benzyl moiety respectively. Intriguingly, no splitting shift were observed at position e which belong to the methyl protons on the indole moiety. Similarly, no splitting for peak j were observed. These results are similar to our previous work where N-benzyl substituent on the indole nitrogen could prevent the nucleophilic addition of hydroxyl (OH-) to the C=N bond of the indole group of **CPY**, and completely different from previous "off-on" based fluorescent probes for pH sensing. Likewise, the splitting for protons at f, g, h, and i positions became ambiguous because of the spectral overlap, indicating **CPY** existed in a basic form. It is notable that there is no upfield or downfield for protons associated with peak l, which is different from similar protons from the previous probe **CPH** (Figure 1). This phenomena could be attributed to that the carboxyl group of **CPH** which is conjugated with the 4-(2-Aminoethyl)morpholine to form the amide group in **CPY**. And the alkaline solution could not induce the deprotonation of amide on **CPY** compared to the deprotonation of carboxyl of **CPH** in alkaline solution. Thus, the upfield of c, d, j, and k peaks is relative to the deprotonation of phenol of **CPY**. This mechanism leads to a ratiometric detection of pH changes due to the large π-electron conjugation system in the merocyanine (D-π-A structure) in alkaline solution. Additionally, further addition of CF_3COOD could recover the upfield and splitting of the protons of **CPY** when compared to the CF_3COOD un-treated **CPY** solution, suggesting reversible sensing

property by **CPY** towards pH changes. Finally, it is interesting to note that the peaks at p and q, assigned to the morpholine group of **CPY**, are completely different between Et_3N treated and CF_3COOD treated **CPY** solution (Figure S4). Addition of CF_3COOD resulted in peaks for the p and q positions of **CPY** splitting and shifting to downfield, which could be ascribed to the protonation of the amine of the morpholine in **CPY** (Figure S4 and S5). All the ^{1}H-NMR results combined with the absorption and fluorescence emission spectra indicated that **CPY** could be used as ratiometric fluorescent probe toward pH changes based on the ICT (intramolecular charge transfer) properties of **CPY**, which was ascribed to the reversible phenol to phenolate equilibration. Meanwhile, the morpholine group of **CPY** also contributed to the protonation and deprotonation process during the solution pH changes, which plays a key role in the lysosomal localization of **CPY**.

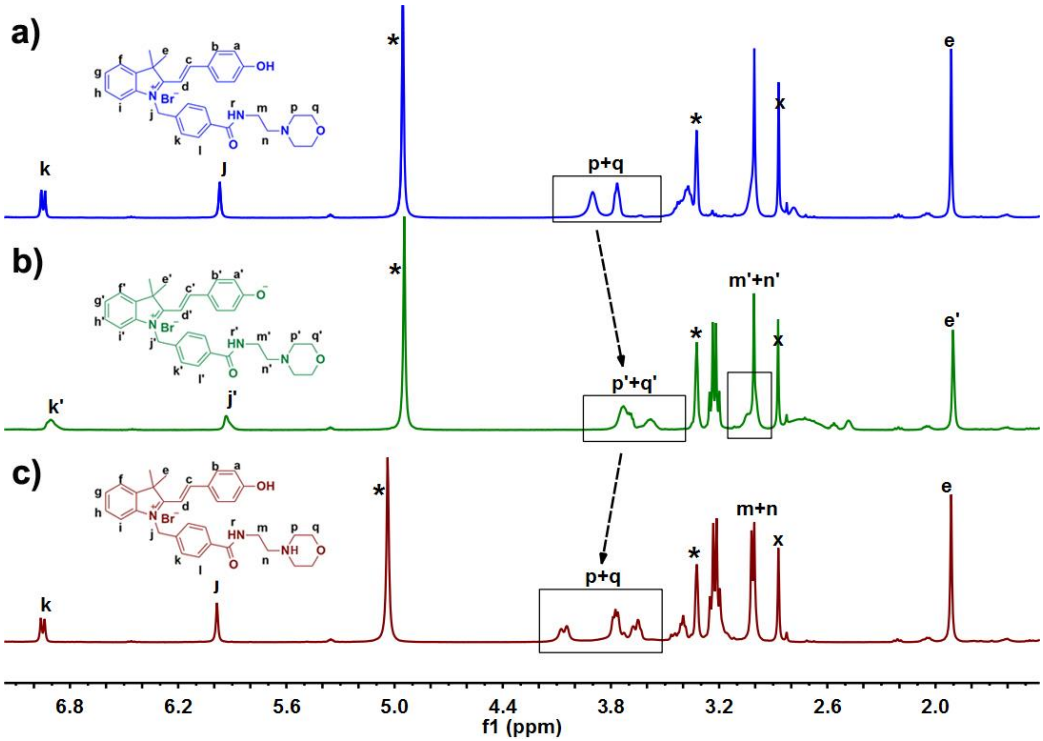


Figure S4. (a) Partial ^{1}H NMR spectra of **CPY** and (b) **CPY**+OH^- in CD_3OD. (c) The spectrum was reinstalled by addition of CF_3COOD. The asterisks represent CD_3OD solvent peaks and water peaks.

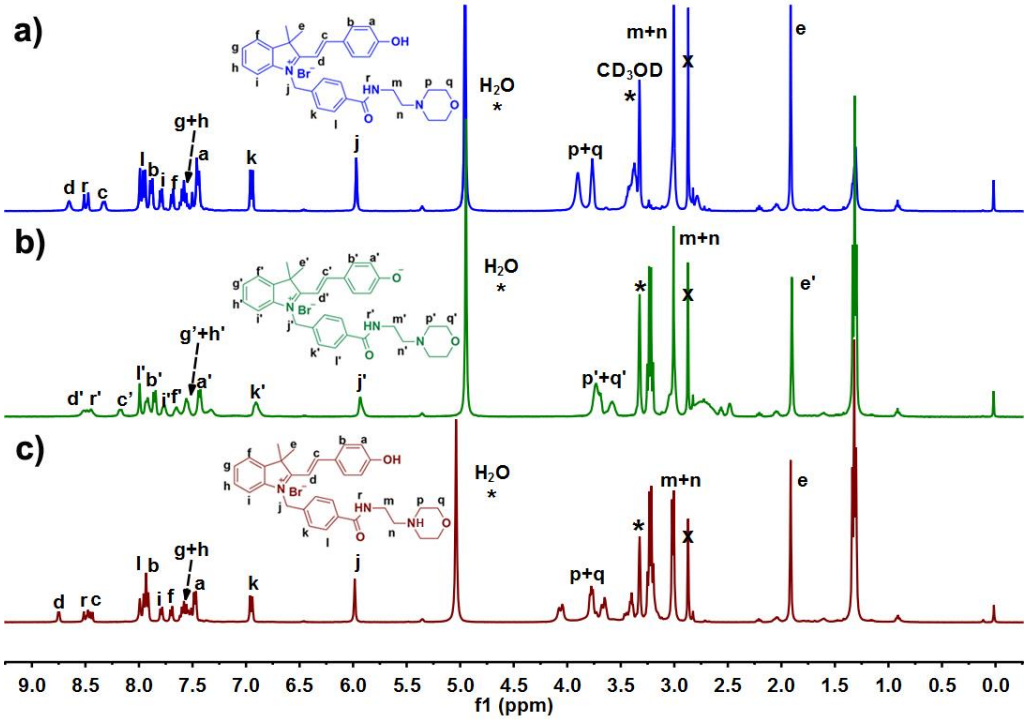


Figure S5. (a) ^{1}H NMR spectra of **CPY** and (b) **CPY**+OH^- in CD_3OD. (c) The spectrum was reinstalled by addition of CF_3COOD. The asterisks represent CD_3OD solvent peaks and water peaks.

3. Interference study for CPY under heat stress in water

To test if the temperature could interfere with the fluorescence performance of **CPY**, the experiment was conducted. Water bath was used to set the temperature at 37 ^{o}C, 41 ^{o}C and 45 ^{o}C respectively. Three different pH values (4.0, 6.0 and 9.0) of water solution containing 10 μM **CPY** were prepared using water bath to incubate 30 min before measuring fluorescence spectrum.

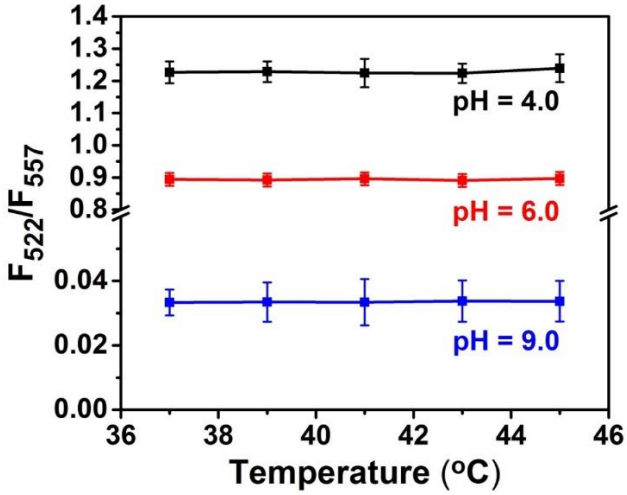


Figure S6. Effects of temperature on the fluorescence intensity ratio of **CPY** (10 μM) in water at pH 4.0, 6.0, and 9.0, λ_{ex} = 473 nm.

4. Cell culture and CCK-8 assay

Cell culture. HeLa cells were obtained from the Chinese Academy of Sciences, and grown in DMEM (High glucose) medium supplemented with 10% FBS (bovine serum albumin). Cells were incubated in a 5% CO_2 humidified incubator at 37 ^oC and typically passaged with sub-cultivation ratio of 1:4 every two days.

CCK-8 assay. The cytotoxicity of **CPY** against HeLa cells were measured by using a standard CCK-8 (Cell Counting Kit - 8) assay. In brief, HeLa cells were seeded in 96-well 96-well microplate with a density of 7000 cells/well, and then incubated with **CPY** at varied concentrations (0 - 70 nM) at 37 ^oC in a 5% CO_2 atmosphere for 12 and 24 h respectively. The cell viability was determined by measuring the light absorbance at 450 nm with a microplate reader. The cell viability was calculated by the following equation: % viability = $[\Sigma(Ai/A\text{control} \times 100)]/n$ [2] where Ai is the absorbance of different concentrations of the probe of 10 μM, 30 μM, 40 μM, 50 μM, 60 μM and 70 μM, respectively. A_{control} is the average absorbance of the control well in which the probe was absent, and n (=5) is the number of the data point.

Fluorescence Imaging using CPY. The HeLa cells were incubated under standard culture conditions (atmosphere of 5% CO_2 at 37 °C). After 12 h of cell attachment on confocal Petri dishes, the cells were rinsed three times with PBS and then incubated with 2 mL DMEM containing the probe **CPY** (50 μM) for 20 min. The cells were then rinsed once with PBS and loaded with fresh DMEM for imaging. Fluorescence images were collected using a Leica TCS SP5 II confocal laser scanning microscope. Emission of probe **CPY** were collected in the range of 500−550 nm (channel 1, green) and 570−620 nm (channel 2, yellow), λ_{ex} = 488 nm. The commercial probe Lysotracker Deep Red was used for lysosomal colocalization experiments. The cells were co-cultured with **CPY** and Lysotracker Deep Red (65 nM) for 20 min. The excitation wavelength of Lysotracker Deep Red was 633 nm, and emission was collected at the range of 650-800 nm. For the experiments detecting lysosomal pH changes under shock stimulus, HeLa cells were incubated with **CPY** at 37 °C, 41 °C, and 45 °C, for 20 min respectively, the cells were then cultured for an additional 20 min at 37 °C before imaging. Additionally, the lysosomal colocalization experiments were also carried out for cells during heat shock stimulus. The commercial probe Lysotracker Deep Red was also used for the lysosomal colocalization experiments.

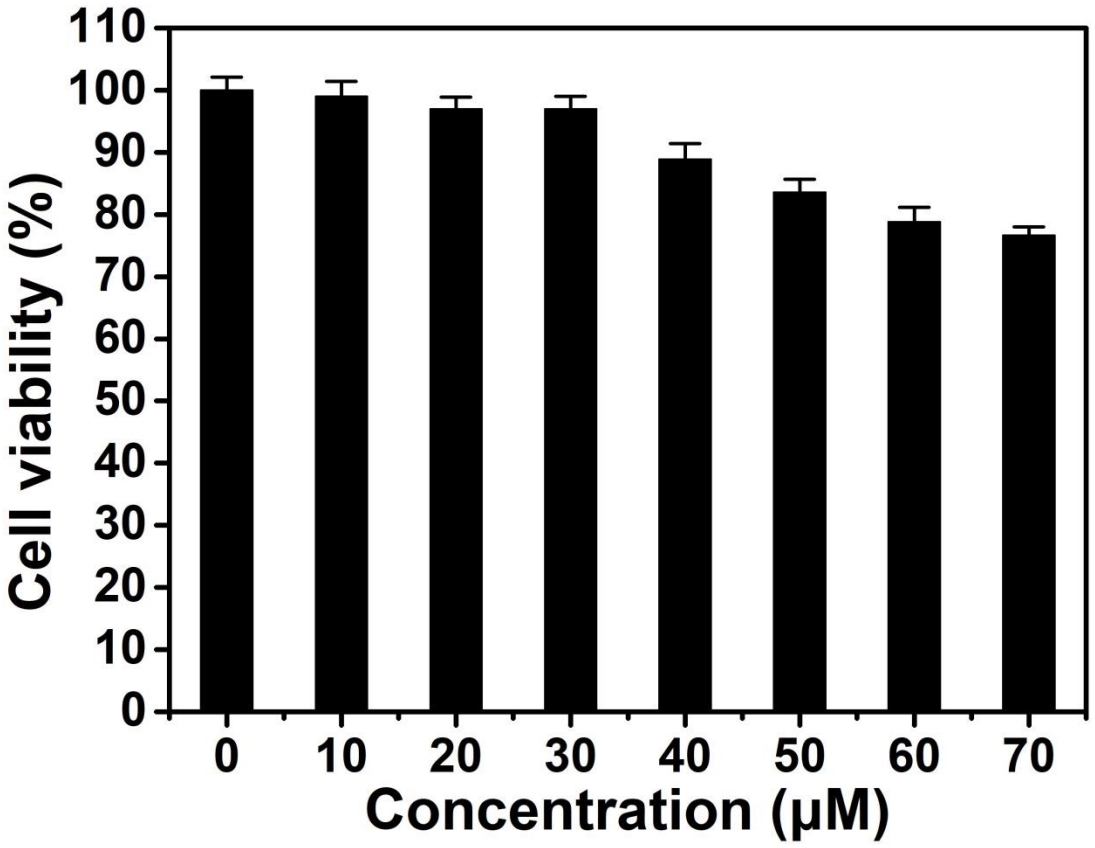


Figure S7. Cell toxicity of **CPY** (from 0 to 70 μM) when the incubation time was 24 h. Error bar represents s.d.

5. Detailed protocols for live cell imaging with CPY

Intracellular fluorescence imaging with CPY. Intracellular fluorescence imaging with CPY. HeLa cells were grown on confocal petri dishes in DMEM containing 10% FBS and then incubated in a humidified 37 ^{o}C, 5% CO_2 incubator. The cells were attached after 12 h, and washed with PBS three times before they are incubated with 2 mL of **CPY** (50 μM, containing 0.5% DMSO) for another 20 min. Before use, the cells were washed with PBS. Probe **CPY** was excited at 488 nm, and the corresponding emissions were collected at 500-550 nm (channel 1, green) and 570-620 nm (channel 2, yellow).

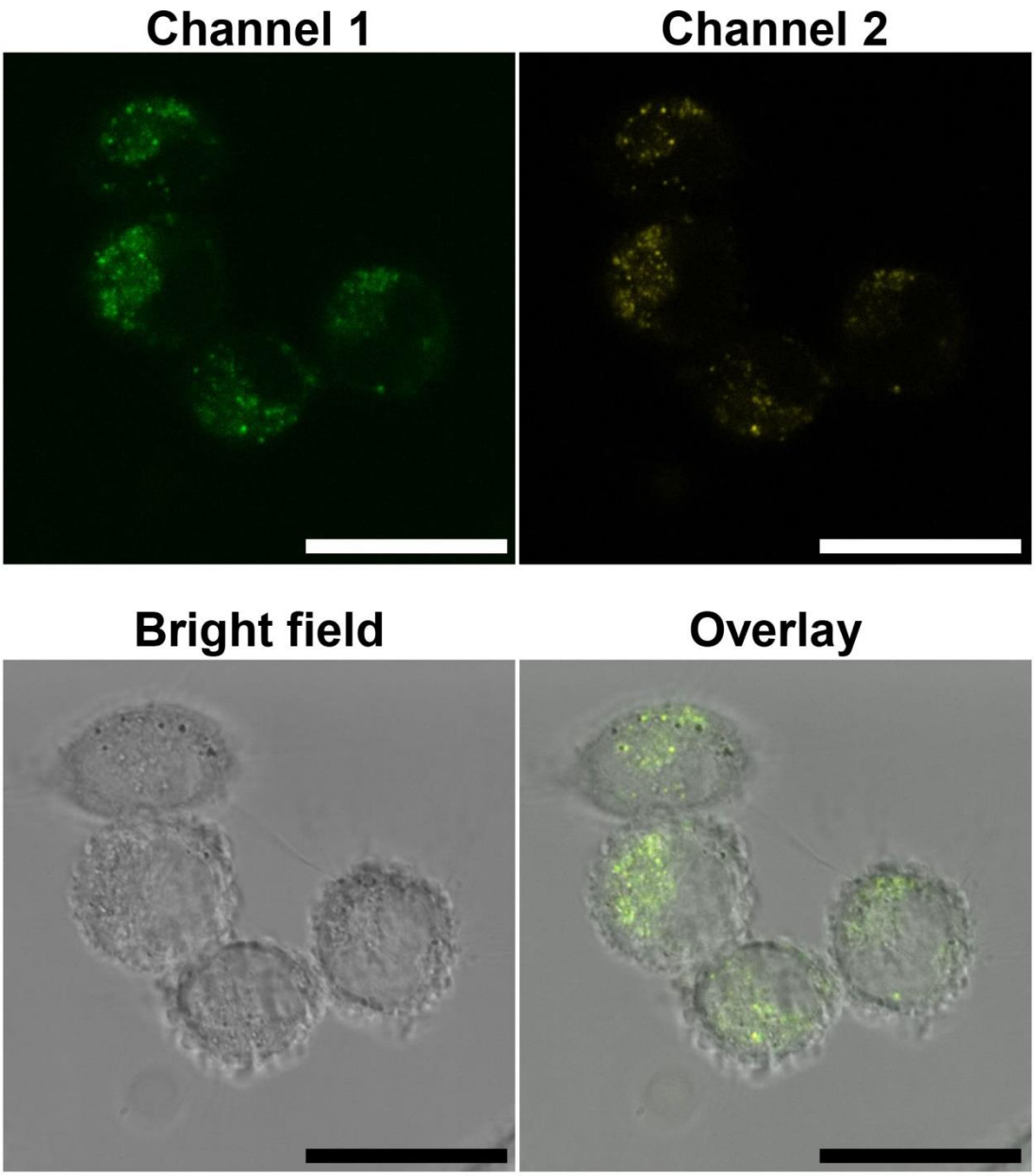


Figure S8. Fluorescence imaging of HeLa cells loaded with probe **CPY** (50 μM). λ_{ex} = 488 nm; Channel 1 (green): λ_{em} = 500-550 nm; Channel 2 (yellow): λ_{em} = 570-620 nm. Overlay: the merged images of Channel 1, Channel 2 and bright field. Scale bar: 25 μm.

Co–localization of probe CPY with commercial lysosome probe LysoTracker® Deep Red. The HeLa cells were attached on confocal Petri dishes in complete medium under standard culture conditions Then the cells were washed with PBS for three times, and were co-incubated with 2 mL of LysoTracker® Deep Red (65 nM) and **CPY** (50 μM) for 20 min at 37 °C as well as during heat stroke (41 and 45 °C). Then cells were cultured for another 20 min under standard culture conditions to make the temperature recover to 37 °C. LysoTracker® Deep Red was dissolved in DMSO with a concentration of 10^{-5} M to make stock solution for use in fluorescence imaging experiments. Fluorescence imaging experiments were implemented by Leica TCS SP5 II confocal laser scanning microscopy using an HC× PLAPO 63× oil objective (NA: 1.40) excitations at 488 nm (**CPY**) and 633 nm

(for LysoTracker® Deep Red) and the emissions were collected in the range of 500-550 nm (channel 1, green) , 570-630 nm (channel 2, yellow) and 650-800 nm (red).

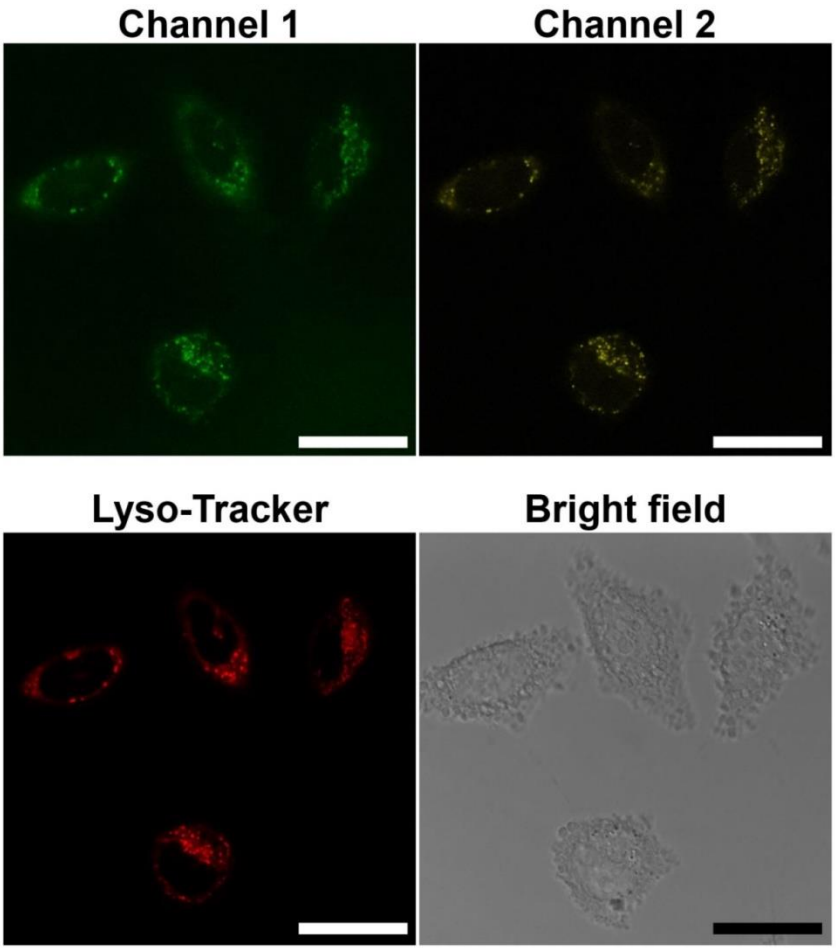


Figure S9. Enlarged images of HeLa cells loaded with probe **CPY** and Lyso-Tracker Deep Red for co-localization assay. Scale bar: 25 μm.

Table S1. Pearson's correlation coefficient of **CPH** and **CPY**.

Name	Pearson's correlation
CPH	0.835
CPY	0.898

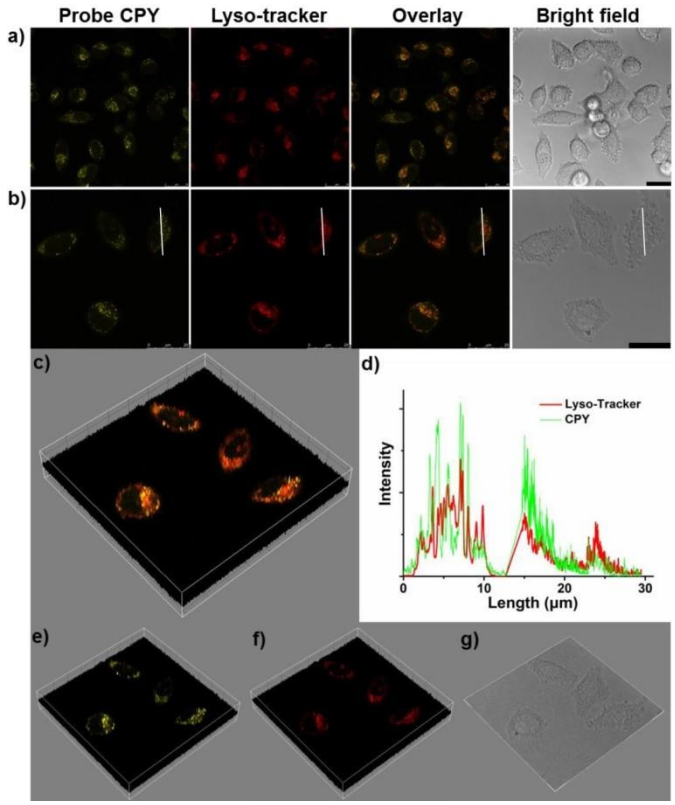


Figure S10. Lysosome-targeting properties of **CPY** in HeLa cells at 37 °C. (a, b) Colocalization images of HeLa cells stained with Lysotracker Deep Red (65 nM, red channel, λ_{ex} = 633 nm, λ_{em} = 650-800 nm) and CPY (50 μM, yellow channel, λ_{ex} = 488 nm, λ_{em} = 570−620 nm) Scale bar: 25 μm. (d) Intensity profiles within the ROI (regions of interest, white line in Figure 4b) of **CPY** and Lyso-Tracker Red across HeLa cells. (c,e-g) 3D surface plot analyzing the colocalization images of **CPY** and LysoTracker Deep Red costained HeLa cells using the function of interactive 3D surface plot in the ImageJ software.

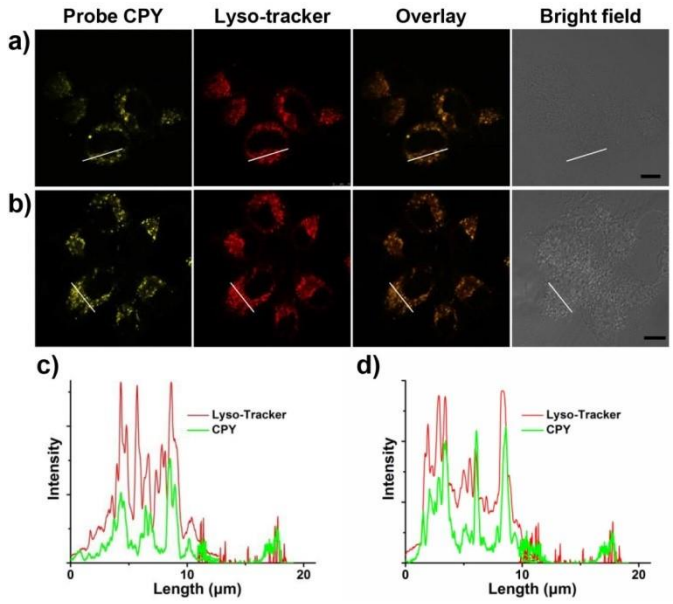


Figure S11. Co-localization imaging of HeLa cells stained with Lyso-Tracker Deep Red (65 nM, red images, λ_{ex} = 633 nm, λ_{em} = 650-800 nm) and **CPY** (50 μM, yellow images, λ_{ex} = 488 nm, λ_{em} = 570-620 nm) at (a) 41 °C and (b) 45 °C. From the left to right: the image from **CPY**, the image from Deep Red, the merged image, the DIC image. (c, d) Intensity profiles of ROI on **CPY** and Deep Red across HeLa cells (white lines in Figure 5a, 5b) at 41 °C (c) and 45 °C (d). Scale bars, 10 μm.

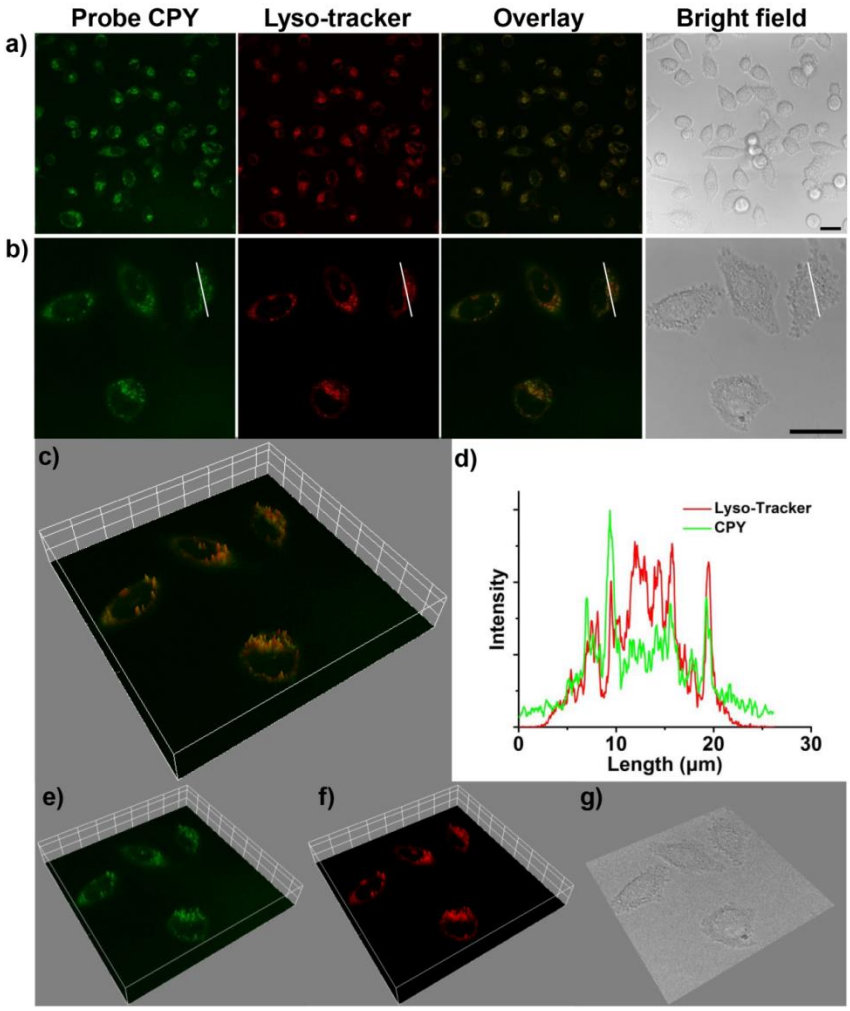


Figure S12. Lysosome-targeting properties of **CPY** in HeLa cells at 37 °C. (a, b) Colocalization images of HeLa cells stained with Lysotracker Deep Red (65 nM, red channel, λ_{ex} = 633 nm, λ_{em} = 650-800 nm) and **CPY** (50 μM, green channel, λ_{ex} = 488 nm, λ_{em} = 500-550 nm) Scale bar: 25 μm. (d) Intensity profiles within the ROI (regions of interest, white line in Figure S8b) of **CPY** and Lyso-Tracker Red across HeLa cells. (c, e-g) 3D surface plot analyzing the colocalization images of **CPY** and LysoTracker Deep Red costained HeLa cells using the function of interactive 3D surface plot in the ImageJ software.

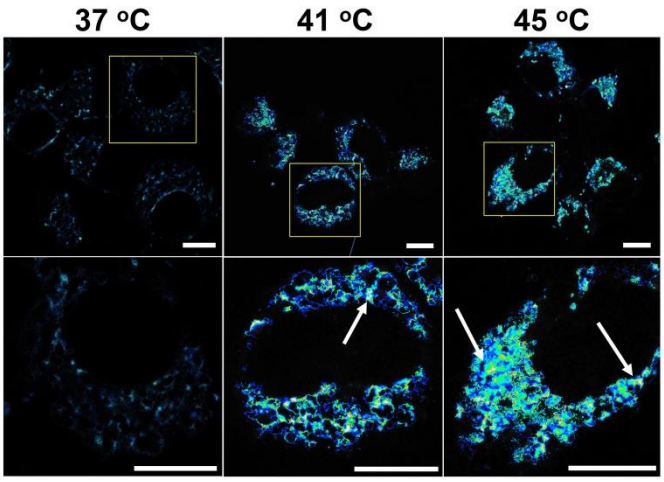


Figure S13. Enlarged ratiometric images of lysosomal pH distribution at different temperatures in live Hela cells. The white arrows indicate an uneven pH distribution in lysosomes during heat stroke. top: ratiometric images; bottom: enlarged ratiometric images obtained by zoom-in of the yellow box. Scale bar: 10 μm.

6. References:

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7. NMR spectrum and HRMS of CPY

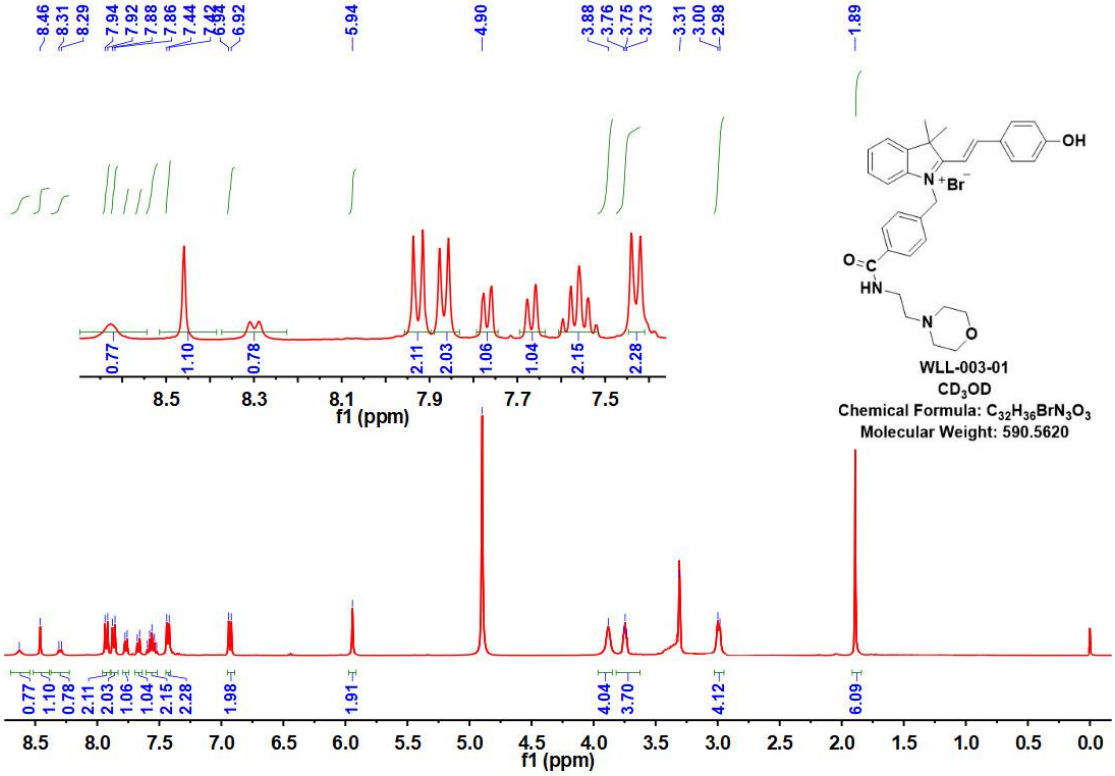


Figure S14 . ^{1}H NMR spectrum of target probe **CPY**.

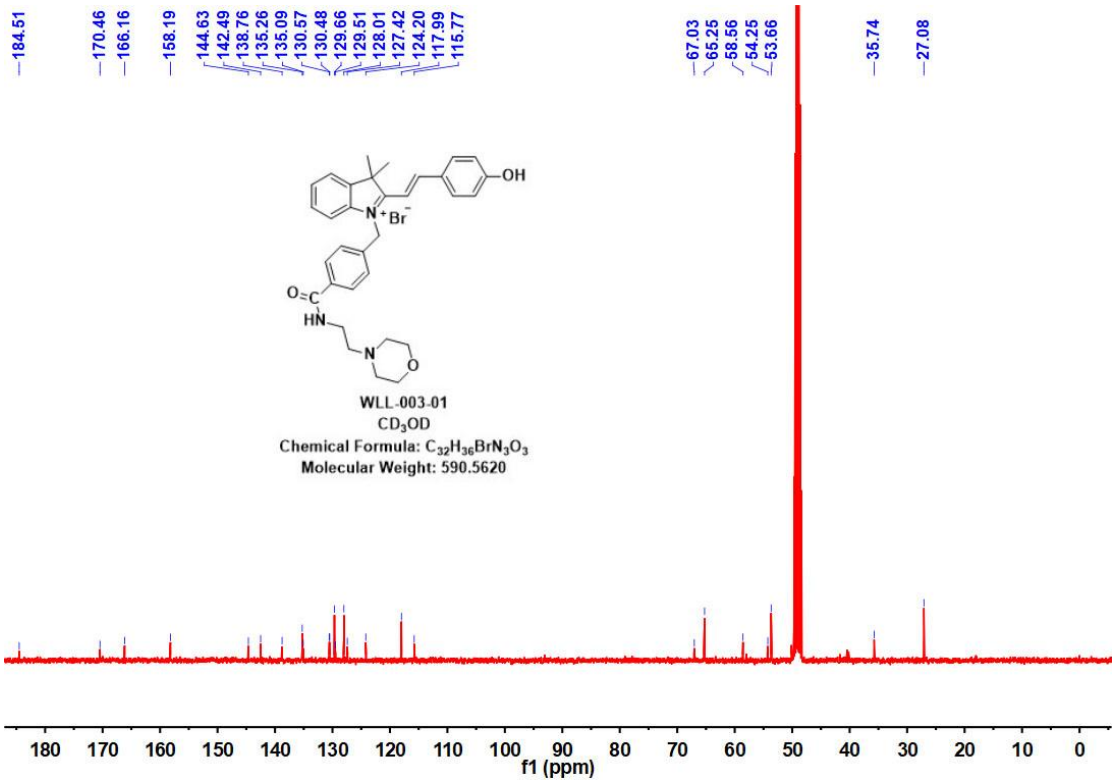


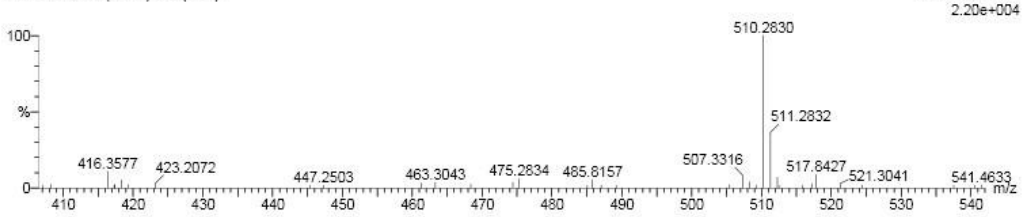
Figure S15 . ^{13}C NMR spectrum of target probe **CPY**.

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Odd and Even Electron Ions
12 formula(e) evaluated with 1 results within limits (up to 1 closest results for each mass)
Elements Used:
C: 0-32 H: 0-50 N: 0-3 O: 0-3

WP-ZHU ECUST institute of Fine Chem 08-Jan-2016 21:18:45
ZWP-TY-17 15 (0.184) Cm (9:17) 1: TOF MS ES+ 2.20e+004



Minimum:				-1.5			
Maximum:		300.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
511.2832	511.2835	-0.3	-0.6	16.0	38.4	0.0	C32 H37 N3 O3

Figure S16 . HRMS of target probe **CPY**.