Electronic Supplementary Information

A pH-activated fluorescence probe via transformation of azo and hydrazone forms for lysosomal pH imaging

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1. Experimental section

1.1 Materials and general methods

All solvents and starting reactants were purchased from commercial suppliers in analytical grade and used without purification unless special noted. Cysteine (Cys), Glutathione (GSH), Metal ion (K^+, Na^+, Ca^{2+}, Ba^{2+}, Ni^{2+}, Cr^{3+}, Fe^{3+}) stock solutions (100 mM) were obtained by diluting the standard solutions of the corresponding nitrate salt with ultrapure water, respectively. The anions (Cl^-, NO_3^-) stock solutions (100 mM) were prepared by diluting the standard solutions of the corresponding sodium salt. The NMR spectra (^1H and ^{13}C) were obtained from Bruker AM 400 spectrometer, using TMS (δ = 0) as internal standard. Waters LCT premier XE spectrometer was used to obtain high resolution mass spectrometry (HRMS) data of the products. UV-Vis spectra and fluorescence spectra were obtained from Agilent Cary 60 spectrophotometer and F97pro fluorescence spectrophotometer, respectively. Dynamic light scatting (DLS) experiments were obtained from Zetasizer Nano ZSE. Cell imaging was performed on Lecia TCS SP8 laser scanning confocal microscopy and Olympus IX73 fluorescent inverted microscope.

1.2 Cell culture

Cancer cells (HepG-2 and HeLa) and normal cells (293T and 3T3) were purchased from the Institute of Cell Biology(Shanghai, China). Cells were propagated in cell culture flask at 37 °C under humidified 5% CO_2 atmosphere. Dulbecco's modified eagle medium (DMEM, GIBCO/Invitrogen, Camarillo, CA, USA) was supplemented with 1% (Vol%) penicillin-streptomycin (10,000 U mL^{-1} penicillin, and 10 mg mL^{-1} streptomycin, Solarbio life science, Beijing, China) and 10% (Vol%) fetal bovine serum (FBS, Biological Industry, Kibbutz Beit Haemek, Israel).

1.3 Cytotoxicity assay

HeLa cells were seeded in a 96-well culture at the density of 5×10^3 per well, and these cells were cultured at 37 °C with humidified 5% CO_2 for 12 h. The culture medium was replaced with 100 μL fresh medium containing different concentration of **Sth-NH**

(20, 15, 10, 5, 2.5, 1, 0 μM), and further incubating these cells for 24 h. After that, 10 μL Cell Counting Kit-8 (CCK-8) was put into each sample and cultivated for additional 2 h. Finally, the absorption intensity at 450 nm was read by an enzyme-linked immunosorbent assay plate.

The cell viability (%) = (OD_{sample} — OD_{blank}) / ($OD_{control}$ — OD_{blank}) × 100%.

OD_{sample}: the Hela cells was incubated with probe **Sth-NH**;

$OD_{control}$: the Hela cells without incubated probe **Sth-NH**;

OD_{blank}: the Hela cells containing only the culture media;

Every assay was repeated three times and six parallel samples were tested in every group.

1.4 Co-localization experiment

HeLa cells at the density of 2 × 10^5 cells/well were seeded onto glass bottom cell culture dish (*Φ*20 mm, NEST) and then cultured for 12 h. Then, the culture medium was removed, and cells were incubated with **Sth-NH** (10 μM) at 37 °C for 1 h. Following that, these HeLa cells were incubated with commerical tracker (LysoTracker Green 200 nM) at 37 °C for 30 min. Then, HeLa cells were imaged by confocal laser scanning microscope (Leica TCS SP8, 63 × oil lens).

1.5 Cancer cells and normal cells imaging

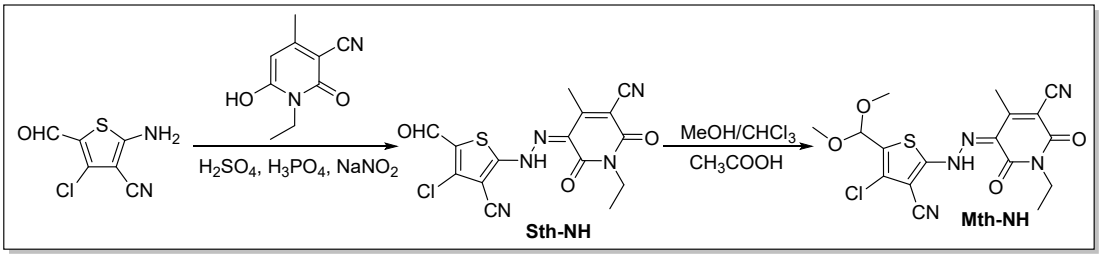
Cancer cells (HeLa and HepG-2) and normal cells (293T and 3T3) were first cultured in DMEM or RPMI 1640 medium (1% antibiotics and 10% FBS) over 12 h. Then, probe **Sth-NH** (10 μM) were added to the cells for 1 h. After that, cellular images were captured using Leica laser scanning confocal microscope and Olympus IX73 fluorescent inverted microscope, respectively.

1.6 Chloroquine Stimulation

Chloroquine (a cell permeable base) was used to increase lysosomal pH. Hela cells were incubated with chloroquine (20, 50 and 100 μM) for 30 min and then washed with PBS three times. Fresh DMEM containing 10 μM **Sth-NH** was then added to incubate the cells for 1 h. After that, cellular images were captured using Leica laser

scanning confocal microscope.

1.7 Synthesis of hydrazone-based probes



Scheme S1 Synthetic routes of **Sth-NH** and **Mth-NH**.

1.7.1 Synthesis of Sth-NH

2-Amino-4-chloro-5-formylthiophene-3-carbonitrile (0.37 g, 2.00 mmol) was dissolved in a mixture of concentrated sulfuric acid (1.2 mL) and phosphate (1.0 mL) at -5 °C in an ice bath. Nitrosylsulphuric acid (2.0 mL) was added dropwise to the reaction mixture over 10 min under stirring. The diazonium salt was obtained and used for the next coupling reaction. 1-Ethyl-6-hydroxy-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (0.36 g, 2.00 mmol) was added to a mixture of methanol/water (15 mL, *v/v* = 2:1) solution in a three-necked flask immersed in an ice bath. Freshly prepared diazonium salt was added dropwise to the reaction mixture under vigorous mechanical stirring for 1 h (0 - 5 °C). The precipitate was filtered and dried after thorough washing with distilled water. The crude product was purified by recrystallization from acetonitrile, and compound **Sth-NH** was finally obtained in a yield of 0.53 g (71 %). ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ = 15.45 (s, 1H), 9.98 (s, 1H), 4.06 (m, *J* = 7.1 Hz, 2H), 2.58 (s, 3H), 1.25 (t, *J* = 7.1 Hz, 3H). ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 9.82 (s, 1H), 3.84 (m, *J* = 7.0 Hz, 2H), 2.46 (s, 3H), 1.09 (*t*, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 181.23, 160.66, 159.03, 156.51, 134.37, 128.00, 127.42, 116.23, 112.78, 97.83, 55.37, 49.06, 34.76, 17.19, 13.31. Mass spectrometry (ESI negative ion mode for $[M-H]^-$): Calc. for $C_{15}H_9ClN_5O_3S$: 374.0115; found: 374.0116.

1.7.2 Synthesis of Mth-NH

Compound **Sth-NH** (0.19 g, 0.50 mmol) was dissolved in a mixture of methanol and chloroform (15 mL, v/v = 1:1), and then acetic acid (5 drops) was added. The mixture was placed in air for slow evaporation at room temperature and single crystals of compound **Mth-NH** suitable for X-ray diffraction measurement were grown after 2 weeks. The crystals of **Mth-NH** were collected in a yield of 0.13 g (62 %). ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ = 15.46 (s, 1H), 5.60 (s, 1H), 4.05 (m, J = 7.1 Hz, 2H), 3.41 (s, 6H), 2.56 (s, 3H), 1.24 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ = 161.43, 158.84, 157.09, 154.62, 129.24, 125.87, 122.07, 113.41, 110.94, 105.96, 97.83, 95.96, 53.52, 35.77, 35.57, 16.49, 12.93. Mass spectrometry (ESI negative ion mode for $[M-H]^-$): Calc. for $C_{17}H_{15}N_5O_4SCl$: 420.0533; found: 420.0538.

2. Characterization of hydrazone-based probes

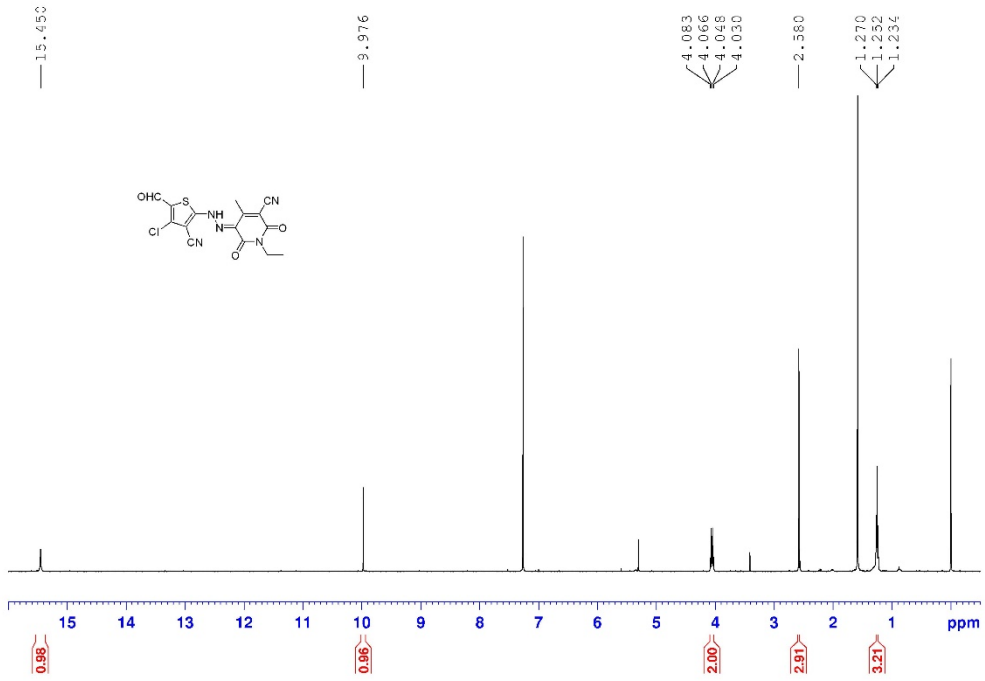


Fig. S1 ^{1}H NMR spectrum of **Sth-NH** in $CDCl_3$.

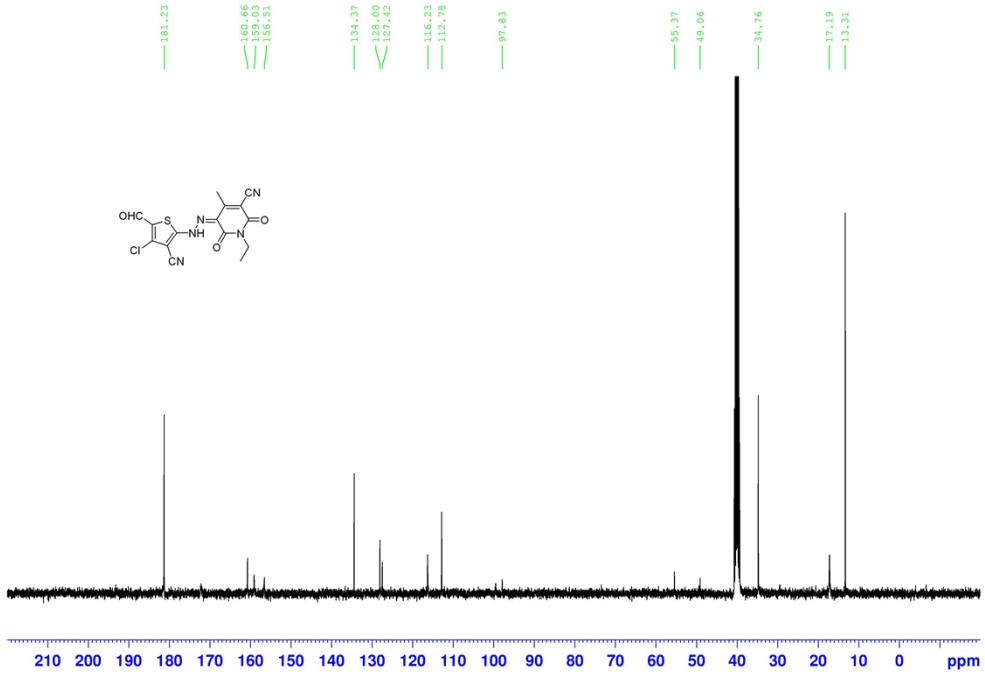


Fig. S2 ^{13}C NMR spectrum of **Sth-NH** in DMSO-d_6.

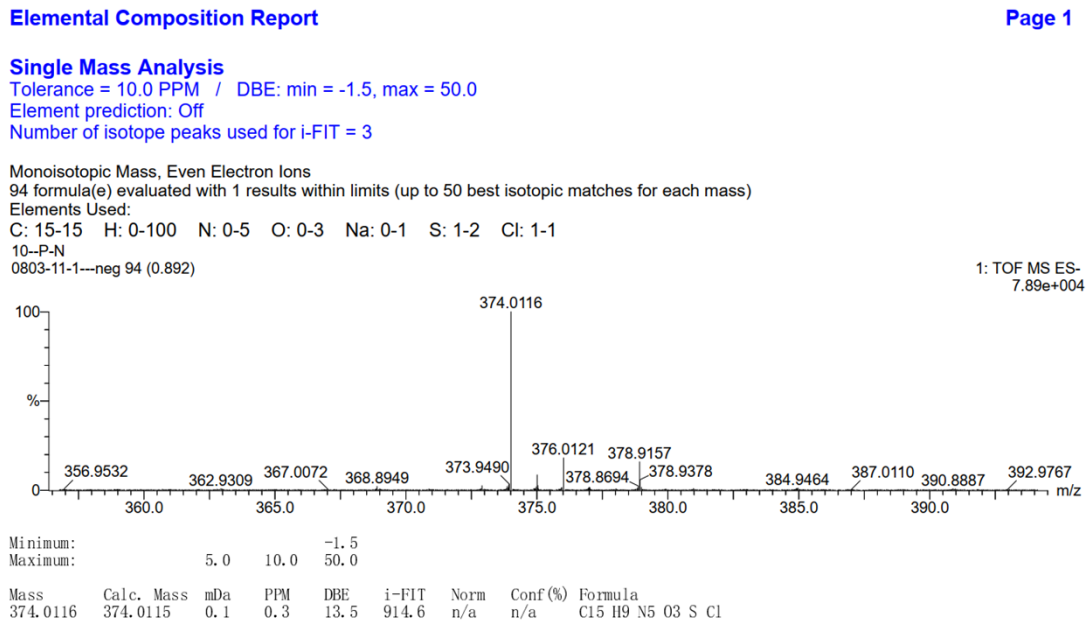


Fig. S3 HRMS spectrum of **Sth-NH**.

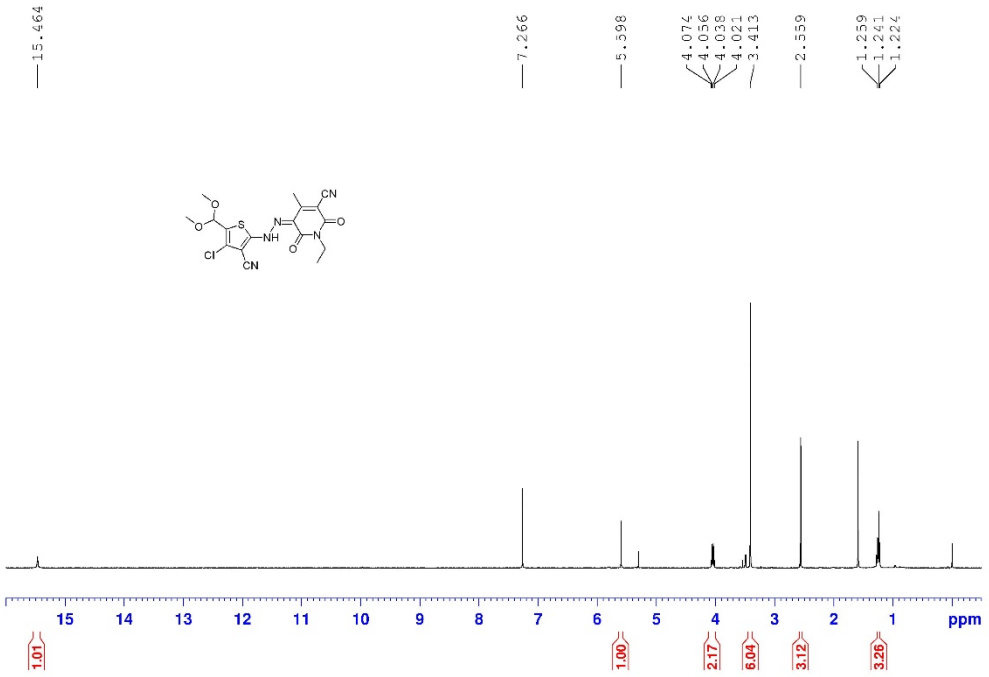


Fig. S4 ^{1}H NMR spectrum of **Mth-NH** in $CDCl_3$.

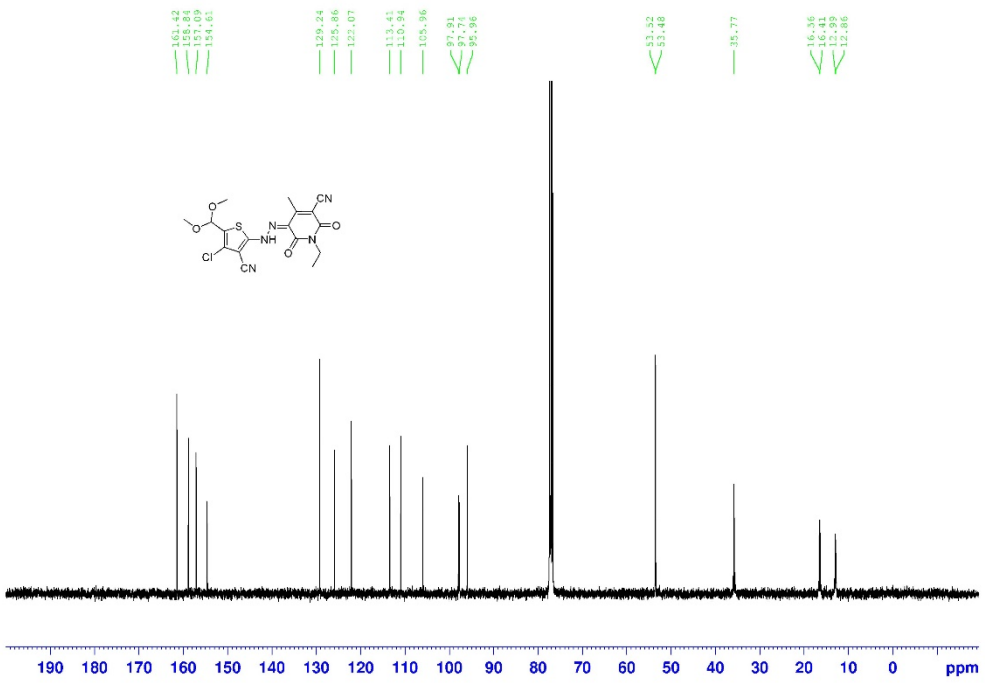
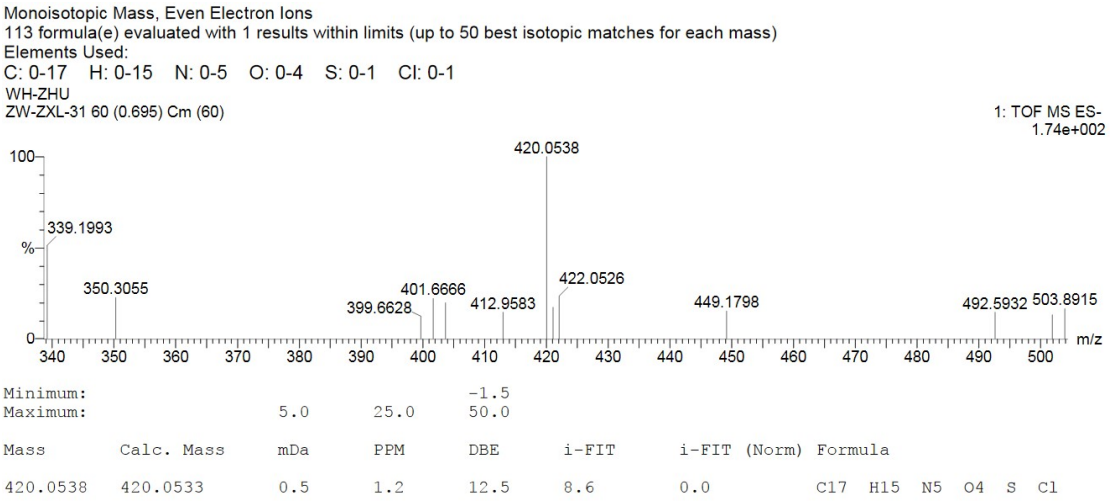


Fig. S5 ^{13}C NMR spectrum of **Mth-NH** in $CDCl_3$.



Minimum:				-1.5			
Maximum:		5.0	25.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
420.0538	420.0533	0.5	1.2	12.5	8.6	0.0	C17 H15 N5 O4 S Cl

Fig. S6 HRMS spectrum of **Mth-NH**.

3. X-Ray data collection and solution

Single crystals of **Mth-NH** were filtered from the mother solution and immediately coated with the hydrocarbon oil on the microscope slide. A suitable single crystal was glued to a glass fiber for data collection on a Bruker SMART 1K CCD area detector at 291(2) K using graphite mono-chromated Mo K_a radiation (λ = 0.71073 Å). The collected data were reduced by using the program SAINT[S1] and empirical absorption corrections were done by SADABS program.[S2] All non-hydrogen atoms were refined on F^2 by full-matrix least-squares procedure using anisotropic displacement parameters. All calculations were carried out on a PC computer with the SHELXTL PC program package[S3] and the molecular graphics were drawn by using the Mercury software. Details of the data collection and structural refinements are listed in Table S1, selected bond distances and angles are provided in Table S2, and hydrogen bonding parameters are given in Table S3.

Table S1 Crystal data and structural refinements for **Mth-NH**. The X-ray crystallographic coordinates for **Mth-NH** have been deposited at the Cambridge Crystallographic Data Centre (CCDC), under deposition number: 2168952.

Compound	**Mth-NH**
Empirical formula	$C_{18}H_{18}Cl_3N_5O_4SO2\ S$
Formula weight	506.78
Temperature / K	296(2)
Wavelength / Å	0.71073
Crystal Size (mm)	0.10 × 0.09 × 0.08
Crystal system	triclinic
Space group	$P\bar{1}$
a / Å	8.0328(6)
b / Å	10.5896(10)
c / Å	14.2584(10)
α / °	79.176(3)
β / °	77.012(3)
γ / °	75.686(3)
V / Å^3	1133.87(16)
Z / D_{calcd} (g / cm^3)	2 / 1.484
F (000)	520
μ / mm^{-1}	0.531
h_{min} / h_{max}	–9 / 9
k_{min} / k_{max}	–9 / 12
l_{min} / l_{max}	–16 / 16
Data / parameters	3961 / 288
Final *R* indices	R_1 = 0.0688
[*I* > 2*ó*(*I*)]	wR_2 = 0.1726
R indices	R_1 = 0.1867
(all data)	wR_2 = 0.0904
S	1.067
Max. / min. $\triangle\rho$/e·Å^{-3}	1.0013 / –0.919

$R_1 = \Sigma||Fo|-|Fc||/\Sigma|Fo|$, $wR_2 = [\Sigma[w(Fo^2-Fc^2)^2]/\Sigma w(Fo^2)^2]^{1/2}$

Table S2 Selected bond distances (Å) and angles (°) for **Mth-NH**

Bond distances		**Bond angles**	
C1-O1	1.240(5)	O1-C1-N1	119.8(4)
C1-N1	1.381(5)	O1-C1-C2	122.3(3)
C1-C2	1.461(6)	N1-C1-C2	117.9(3)
C2-N3	1.326(5)	N3-C2-C3	115.9(4)
C2-C3	1.447(6)	N3-C2-C1	122.8(4)
C3-C4	1.363(5)	C3-C2-C1	121.3(3)
C3-C6	1.486(6)	C4-C3-C2	116.7(4)
C4-C7	1.439(6)	C4-C3-C6	123.4(4)
C4-C5	1.460(6)	C2-C3-C6	119.8(3)
C5-O2	1.219(5)	C3-C4-C7	120.9(4)
C5-N1	1.398(5)	C3-C4-C5	123.8(4)
C7-N2	1.140(5)	C7-C4-C5	115.3(3)
C8-N1	1.470(5)	O2-C5-N1	120.6(4)
C8-C9	1.500(7)	O2-C5-C4	122.2(4)
C10-N4	1.376(5)	N1-C5-C4	117.2(3)
C10-C11	1.376(5)	N2-C7-C4	177.7(4)
C10-S1	1.714(4)	N1-C8-C9	112.0(4)
C11-C14	1.418(6)	N4-C10-C11	125.9(4)
C11-C12	1.434(5)	N4-C10-S1	121.1(3)
C12-C13	1.349(6)	C11-C10-S1	113.0(3)
C12-Cl1	1.715(4)	C10-C11-C14	123.5(4)
C13-C15	1.496(6)	C10-C11-C12	110.5(4)
C13-S1	1.740(4)	C14-C11-C12	126.0(4)
C14-N5	1.150(6)	C13-C12-C11	114.1(3)
C15-O3	1.390(5)	C13-C12-Cl1	124.0(3)
C15-O4	1.402(5)	C11-C12-Cl1	121.9(3)
C16-O3	1.427(6)	C12-C13-C15	128.0(3)
C17-O4	1.433(5)	C12-C13-S1	111.3(3)
C18-Cl3	1.699(8)	C15-C13-S1	120.7(3)
C18-Cl2	1.727(8)	N5-C14-C11	178.0(4)
N3-N4	1.311(5)	O3-C15-O4	114.0(4)
		O3-C15-C13	106.4(3)
		O4-C15-C13	113.8(3)

	Cl3-C18-Cl2	113.0(4)
	C1-N1-C5	122.9(3)
	C1-N1-C8	118.5(3)
	C5-N1-C8	118.6(3)
	N4-N3-C2	119.9(4)
	N3-N4-C10	118.2(4)
	N3-N4-H4	116(4)
	C10-N4-H4	126(4)
	C15-O3-C16	113.3(3)
	C15-O4-C17	115.8(3)
	C10-S1-C13	91.14(19)

Table S3 Hydrogen bonding parameters (Å, °) for **Mth-NH**.

D–H···A	*d* (D–H)	*d* (H···A)	*d* (D···A)	∠DHA	Symmetry code
N4–H4···O1	0.79(5)	1.90(6)	2.558(5)	141(5)	
C8–H8B···O4	0.97	2.59	3.552(5)	170	-*x*, 1-*y*, 2-*z*

4. Quantum yield of Sth-NH

Table S4 Absolute quantum yield of **Sth-NH** at pH = 2.02 in B-R buffer solutions.

	Sth-NH
Absolute quantum yield[a]	9.10%

[a] Absolute quantum yield was measured by HAMAMATSU Quantaurus-QY C11347-11. λ_{ex}=514nm.

5. pH-induced photophysical properties of Sth-NH

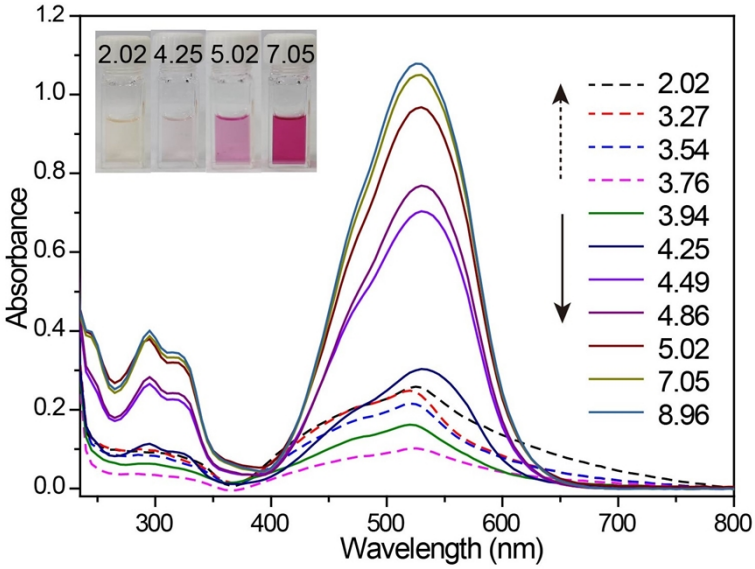


Fig. S7 Absorption spectra of **Sth-NH** (20 μM) in BR buffer of pH = 2.02-8.96. Insets: photographs of **Sth-NH** at pH = 2.02, 4.25, 5.02 and 7.05 of BR buffer under visible light.

6. NMR titration spectra of Sth-NH in CD_3CN-d_3

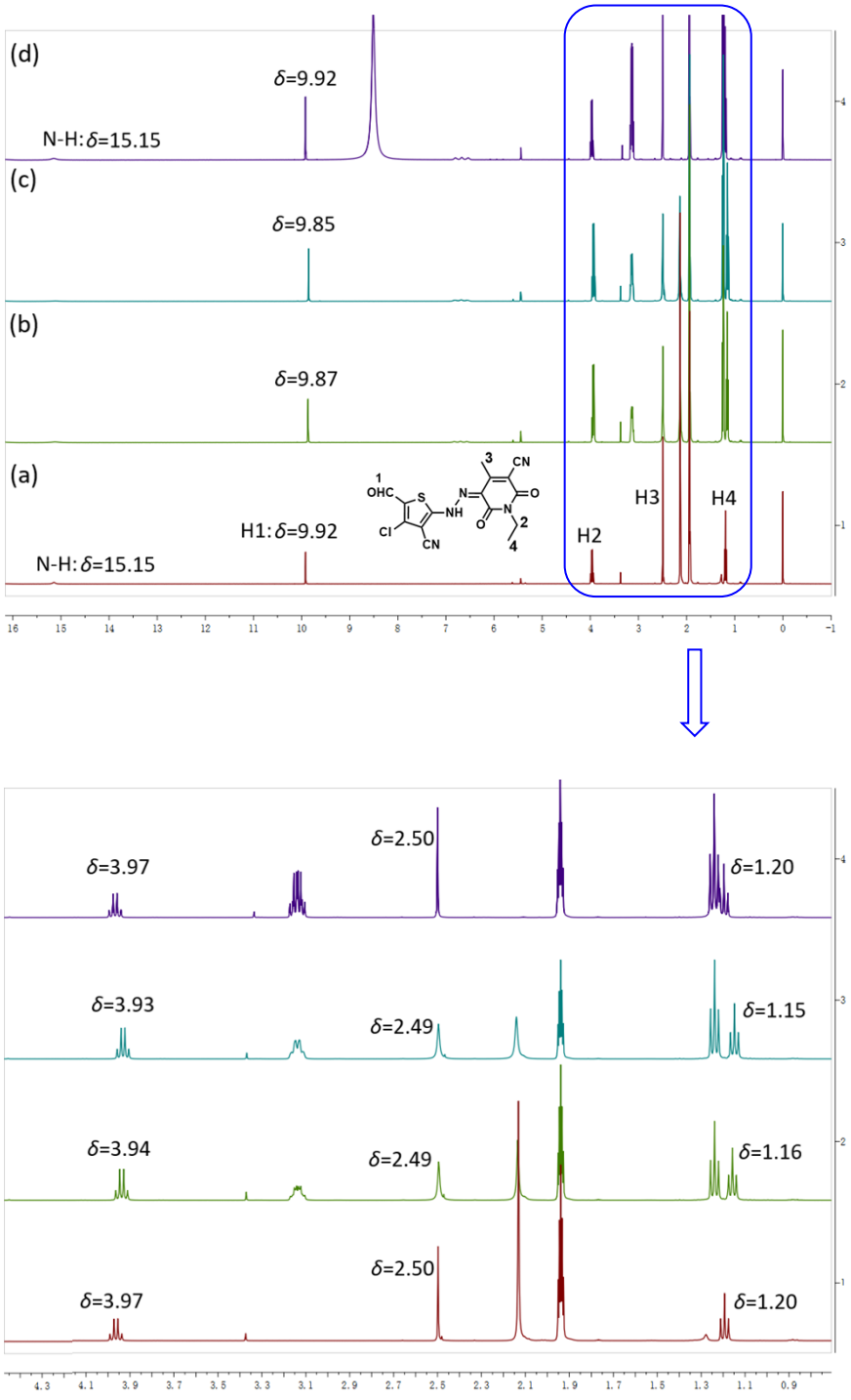


Fig. S8 ^{1}H NMR spectra in CD_3CN-d_3 of (a) **Sth-NH** with chemical shifts (ppm); (b) partial deprotonated azo form of **Sth-NH** recorded after the addition of 0.5 equiv of Et_3N; (c) deprotonated azo form of **Sth-NH** recorded after the addition of 1.0 equiv of Et_3N; and (d) **Sth-NH** is regained upon the addition of 24.0 equiv of TFA at RT.

7. Selectivity of Sth-NH to pH

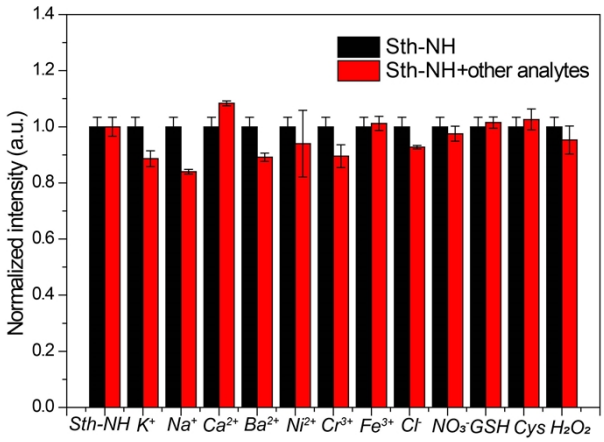


Fig. S9 The fluorescence intensities of **Sth-NH** (20 μM) to various analytes in BR buffer solutions of pH =3.94, the final concentration of each type of interfering ion is 100 μM. λ_{ex} = 450 nm.

8. Fluorescence spectra of Sth-NH in different solvents

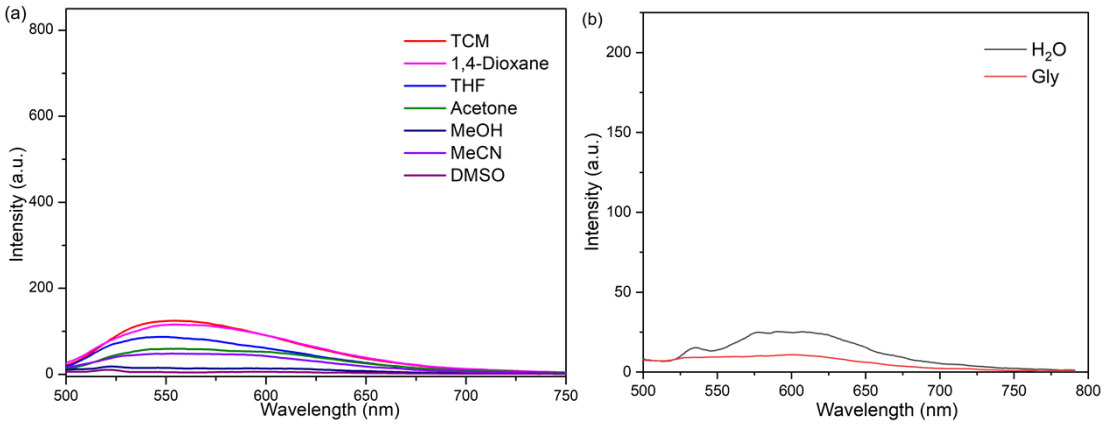


Fig. S10 Fluorescence spectra of **Sth-NH** (20 μM) in different solvents. Notes: TCM (trichloroethane), THF (tetrahydrofuran), MeOH (methyl alcohol), MeCN (acetonitrile), DMSO (dimethyl sulfoxide), Gly (glycerin). λ_{ex} = 450 nm.

9. Cytotoxicity assay of Sth-NH

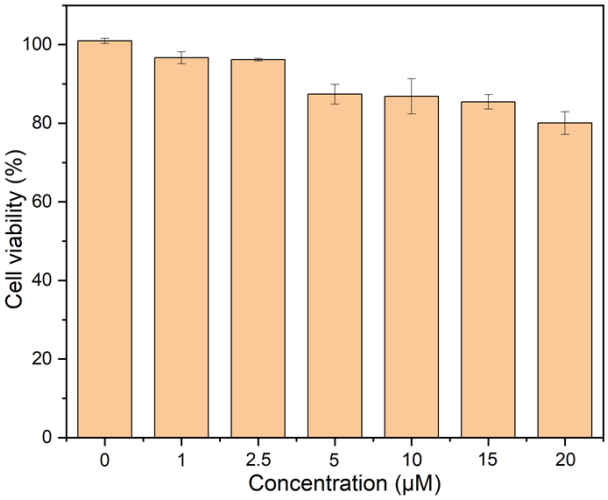


Fig. S11 Cell viability of HeLa cells *versus* the concentration of **Sth-NH**. Incubating time: 24 hours. Data are shown as mean ± s.d., with n = 3.

10. Fluorescence imaging of Sth-NH with time and dose

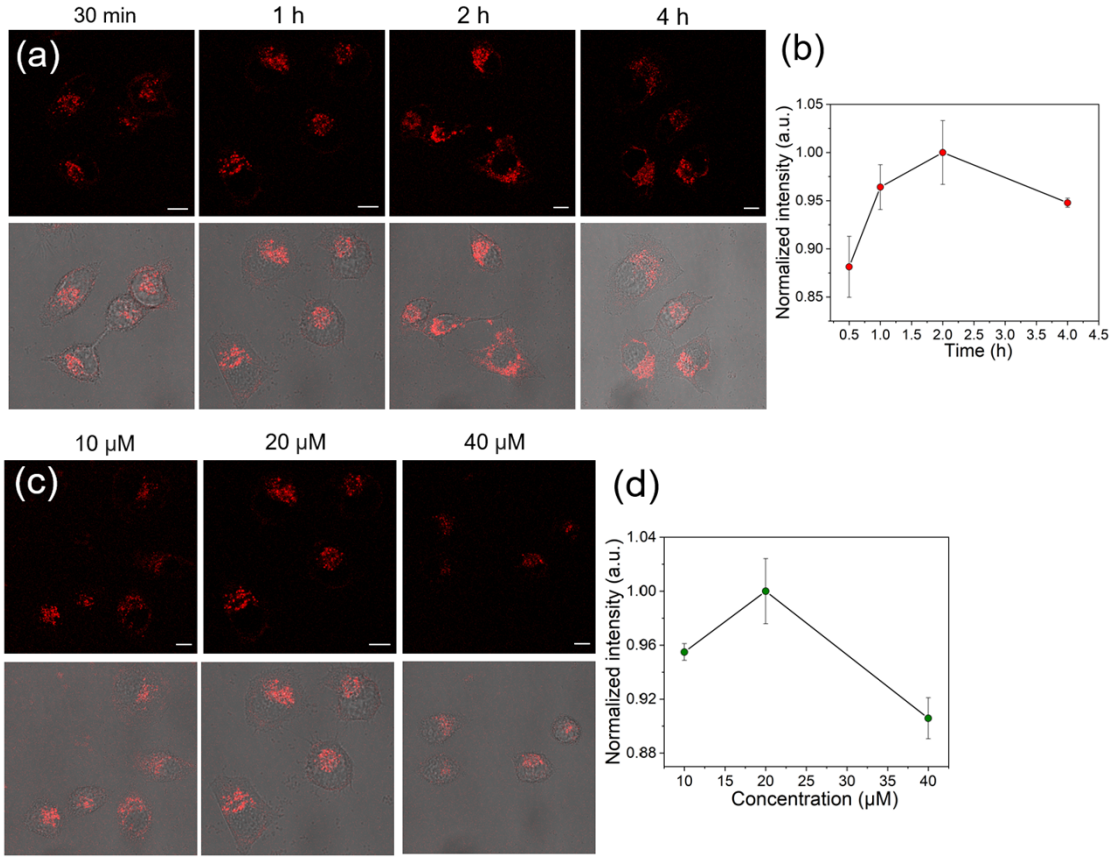


Fig. S12 (a) Fluorescence microscope images of Hela cells with probe **Sth-NH** (20 μM) for (0.5, 1, 2 and 4 h) at 37 °C. (c) Fluorescence microscope imaging of Hela cells with probe **Sth-NH** (10, 20 and 40 μM) for 1 h at 37 °C. (b) and (d) Data are shown as mean ± s.d., with n = 3. Scale bar = 20 μm. λ_{ex} = 514 nm, λ_{em} = 550-750 nm.

11. Fluorescence imaging of Sth-NH in normal cells (3T3) and cancer cells (HepG-2)

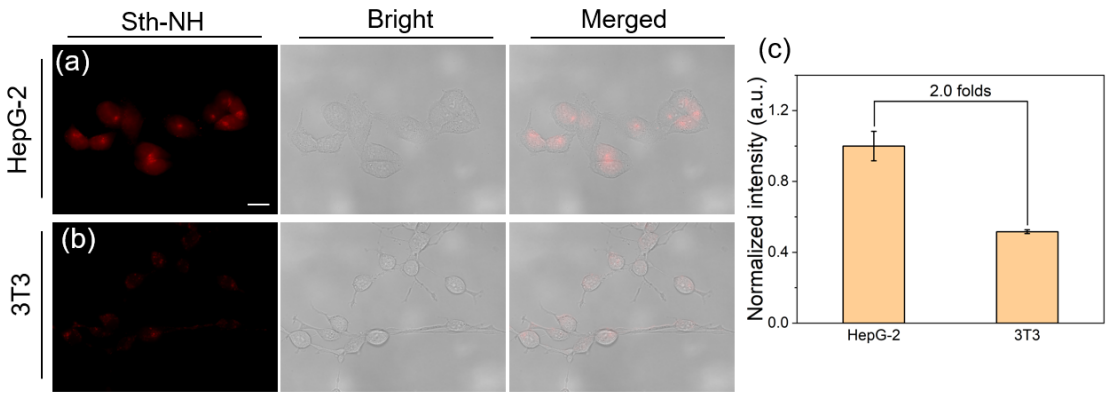


Fig. S13 (a) Fluorescence images of cancer cells (HepG-2) and (b) normal cells (3T3) incubated with probe **Sth-NH**. (c) The normalized intensity of normal and cancer cells ± S.D., n = 3. Scale bars = 20 μm. λ_{ex} = 514 nm, λ_{em} = 550-750 nm.

12. Fluorescence imaging of Sth-NH stimulated by chloroquine

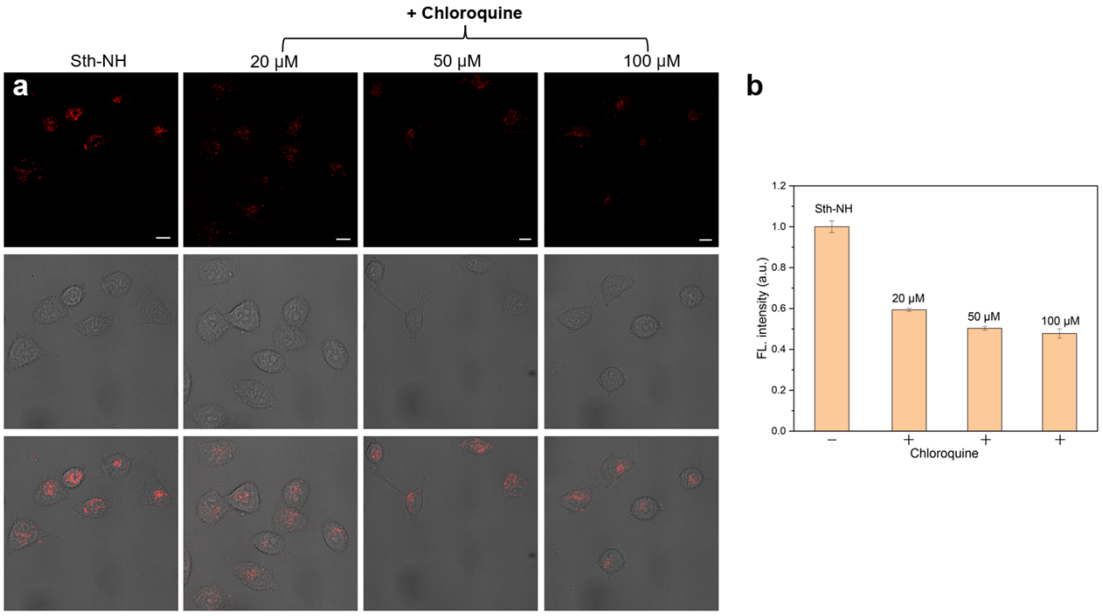


Fig. S14 (a) The cells images of Hela cells incubated with probe **Sth-NH** and stimulated with or without chloroquine (20, 50 and 100 μM, 30 min). (b) Data are shown as mean ± s.d., with n = 3. Scale bar = 20 μm. λ_{ex} = 514 nm, λ_{em} = 550-750 nm.

References

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S2 Sheldrick GM. *SADABS*, program for empirical absorption correction of area dectector data. Germany: Univ. of Gottingen; 2000.

S3 Siemens. *SHELXTL*, Version 6.10 reference manual. Madison, Wisconsin, USA: Siemens Analytical X-Ray Systems, Inc.; 2000.