Electronic Supplementary Information for

Benzothiazolium-based fluorescent probe with ideal p*K*a for mitochondrial pH imaging and cancer cells differentiation

Bo Lin[a], Li Fan[a], Ying zhou[a], Jinyin Ge[a], Xueli wang[b], Chuan Dong[a], Shaomin Shuang*[a], Man Shing Wong*[a,b]

[a]College of Chemistry and Chemical Engineering, Institute of Environmental Science, Shanxi University, Taiyuan, 030006, China

[b]Department of Chemistry and Institute of Advanced Materials, Hong Kong Baptist University, Hong Kong Special Administrative Region, China

E-mail address: *smshuang@sxu.edu.cn* (Shaomin Shuang), *mswong@hkbu.edu.hk* (Man Shing Wong).

Contents:

1. Experiments

Materials and instruments

6-hydroxy-2-naphthaldehyde was obtained from TCI (Shanghai) Chemical Industry Development Co., Ltd. 1,6-dibromohexane, piperidine and 2-methylbenzothiazole were purchased from J & K (Hong Kong) Technology Co., Ltd.. Triphenylphosphine was gained via Meryer (Shanghai) Chemical Technology Co., Ltd.. Mito Tracker Green and nigericin were provided by Invitrogen. All cells within experiments were purchased from ATCC (America). All other inorganic salts and solvents were commercially available from Aladdin Reagent Co., Ltd. All chemicals and solvents with analytical grade were used directly.

Deionized water with conductivity of 18.2 MΩ▪ cm^{-1} (25 ℃) was acquired on a Milli-Q water purification system (Millipore). The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectrometric data were measured on a Bruker NMR spectrometer (Bruker biospin, Switzerland), which using tetramethylsilane (TMS) as an internal reference standard, deuterated dimethyl sulfoxide (DMSO-d_6) or $DCCl_3$ as solvents. Mass spectra were obtained on an ESI mass spectrometer (PerkinElmer, America). Absorption spectra were recorded via a U-2910 double-beam UV-vis spectrophotometer (Hitachi High-Techonologies Corporation, Japan). Fluorescence spectra were taken on a FLS-920 Edinburgh fluorescence spectrophotometer (Edinburgh Co., Ltd. UK). Fluorescence images in live cells were implemented with an LSM880+Airysan confocal laser scanning microscope (Carl Zeiss AG, Germany). Beckman 50 pH meter (Shanghai LeiCi Device Works, Shanghai, China) was employed for measuring pH values.

Synthesis and characterization

HTBT2 and intermediates (3-(6-bromohexyl)-2-methylbenzothiazol-3-ium bromide, 2-methyl-3-(6-(triphenylphosphonio)hexyl)benzothiazol-3-ium bromide) were synthesized according to the synthetic route shown in **Scheme S1**.

3-(6-Bromohexyl)-2-methylbenzothiazol-3-ium bromide (BMBI):

The mixture of 2-methylbenzothiazole (15 mmol, 1.90 mL) and 1,6-dibromohexane (75 mmol, 12.11 mL) was heated to 140 ℃ for 5 h in a thick-wall high-pressure bottle. Then the reaction mixture was dissolved in dichloromethane and excess ether was added to precipitate **BMBI** as a gray solid (5.43 g, 92%), which was not further purified and used in the next reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, J = 8.1 Hz, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.81 (t, J = 7.7 Hz, 1H), 7.69 (t, J = 7.7 Hz, 1H), 5.09 – 4.75 (m, 2H), 3.49 (s, 3H), 3.40 (t, J = 6.5 Hz, 2H), 2.25 – 1.75 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.69, 140.93, 130.00, 129.23, 128.67, 124.72, 116.57, 51.00, 33.77,

32.28, 28.67, 27.68, 25.98, 19.30. MS (ESI-MS) m/z: Calcd 313.0402 $[M]^+$; found 312.0416, 314.0387 $[M]^+$.

2-Methyl-3-(6-(triphenylphosphonio)hexyl)benzothiazol-3-ium bromide (MTBI):

The mixture of BMBI (10 mmol, 3.97 g), triphenylphosphine (15mmol, 3.93 g) and 150 mL acetonitrile was refluxed for 24 h under nitrogen atmosphere. Then, solid was obtained from solution of reaction via filtration, and then the solid was dissolved in dichloromethane. Subsequently, the solution was extracted with water 3 times. The water was removed from the aqueous solution and afforded gray solid product (5.96 g, 91%) The crude product was used directly in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (d, J = 8.6 Hz, 1H), 8.19 (d, J = 8.0 Hz, 1H), 8.03 – 7.54 (m, 17H), 5.20 – 5.01 (m, 2H), 3.78 (t, J = 14.4 Hz, 2H), 3.53 (s, 3H), 2.04 (d, J = 19.0 Hz, 2H), 1.87 (s, 4H), 1.73 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.31 (s), 141.10 (s), 135.05 (d, ^4J (C, P) = 3.0 Hz, Ar<u>C</u>), 133.75 (d, J = 10.0 Hz, ^2J (C, P), Ar<u>C</u>), 130.55 (d, ^3J (C, P)= 12.5 Hz, ArC), 130.34 (s), 128.78 (s), 128.70 (s), 123.86 (s), 118.31 (d, ^1J (C, P) = 71.2 Hz, Ar<u>C</u>), 117.81 (s), 50.83 (s), 29.11 (d, ^3J (C, P)= 16.8 Hz, $PCH_2CH_2CH_2$), 28.35 (s), 25.13 (s), 22.46 (d, ^1J (C, P) = 50.4 Hz, P<u>C</u>H_2), 21.93 (d, ^2J (C, P)= 4.4 Hz, PCH_2<u>C</u>H_2), 19.34 (s). ^{31}P NMR (162 MHz, $CDCl_3$) δ 24.45. MS (ESI-MS) m/z: Calcd 247.6069 $[M]^{2+}$; found 247.6070 $[M]^{2+}$

2-(2-(6-Hydroxynaphthalen-2-yl)vinyl)-3-(6-(triphenylphosphonio)hexyl)benzothiazol-3-ium bromide (HTBT2):

To the solution containing 1.29 g (7.5 mmol) 6-hydroxy-2-naphthaldehyde, 3.28 g (5 mmol) MTBI, and 0.5 mL piperidine was added 30 mL ethanol and then refluxed for 24 h. After that, solvent was removed under reduced pressure affording the crude product. The crude product was purified by silica gel column chromatography using dichloromethane containing 10% methanol as eluent to afford the desired yellow solid product (0.93g, 23%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.02 (s, 1H), 8.39 (d, J = 22.8 Hz, 2H), 7.95 – 7.69 (m, 3H), 7.69 – 7.49 (m, 24H), 7.24 (ddd, J = 84.1, 56.2, 31.5 Hz, 1H), 3.14 (d, J = 14.7 Hz, 1H), 3.06 – 2.93 (m, 3H), 1.63 (dt, J = 11.2, 5.6 Hz, 3H), 1.53 (dt, J = 10.7, 5.4 Hz, 2H), 1.42 – 0.96 (m, 1H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 24.45. MS (ESI-MS) m/z: Calcd 324.6279 $[M]^{2+}$; found 324.6273 $[M]^{2+}$.

Spectroscopic measurements

The stock solution containing 1.0 mM **HTBT2** was prepared in DMSO and stored at 4℃. The stock solution of **HTBT2** was diluted to 200 μM and 10 μM via tris-HCl buffer with different pH in water/DMSO (1/2, v/v) for measuring the absorption and fluorescence spectra, respectively. 2

mL of above solutions were poured into a quartz optical cell of 1 cm optical path length and data were recorded after the solutions was stabilized. In experiments of fluorescence measurements, excitation and emission bandwidths were both set at 2.0 nm, and the excitation wavelength was fixed at 436 nm (**Fig. S1**). All spectroscopic experiments were implemented at room temperature. The stock solutions of interference substance for selectivity experiments were freshly prepared in water, and cations and anions were from their chloride and sodium salt, respectively. NO and $OONO^-$ were prepared according to the reported of Lippert[1] and Chou[2] procedures, respectively.

Mass spectra titrations assays of HTBT2

10^{-5} M **HTBT2** was prepared in water. Then, the pHs of solutions were adjusted by 10^{-2} M NaOH. The mass spectra data were measured after the samples stabled.

^{1}H NMR spectra titrations assays of HTBT2

10^{-2} M **HTBT2** was prepared in d^6-DMSO. Then, the pHs of solutions were adjusted by 10^{-1} M NaOH. The ^{1}H NMR spectra data were measured after the samples stabled.

Measurement of the fluorescence quantum yield

The relative quantum yield of probes was calculated via the following equation:

$$\Phi_x = \Phi_{st} (D_x / D_{st}) (A_{st} / A_x) (\eta_x^2 / \eta_{st}^2)$$

where Φ_{st}, D, A and η represent the reported quantum yield of the standard, the area of the emission spectra, the absorbance at the excitation wavelength and the refractive index of the solvent used, separately. Subscripts *x* and *st* refer to the sample and the standard, respectively. Rhodamine 6G (Φ = 0.95 in water) was used as a reference standard.

Cell cytotoxicity assay

The cytotoxicity of **HTBT2** toward SMMC-7721 cells (Human liver cancer cell) was conducted by the conventional MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide) assay. The SMMC-7721 cells were cultured in 96-well flat-bottomed plates at 37 ℃ with 5% CO_2 atmosphere with the cell densities near to 1×10^5 cells·mL^{-1} per well and the total volume was maintained in 200 μL well^{-1}. After 24 h, different concentration **HTBT2** were incubated with these cells for 3 h in fresh medium, separately. Subsequently, 10 μL MTT solution (10 mg/mL, PBS) was added into each well of 96-well flat-bottomed plates after the cells were rinsed via PBS (phosphate buffered saline, pH 7.40) for 3 times and further incubated for 4 h. Afterwards, the superfluous MTT solution was eliminated from the wells, and 150 μL of DMSO was added into each well for dissolved the blue-violet formazan crystals in cells. The absorbance at 490 nm of the solution was recorded

on a microplate reader. IC_{50} values was calculated via Graphpad-prism 5.0 software according to MTT results.

Dynamic assays of cells were stained by the probe

SMMC-7721 cells were seeded in glass Petri dish, and the cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum) at 37 ℃ in 5% CO_2 atmosphere for 48 h. Then, the remainder DMEM was removed and cells were rinsed for three times by PBS (pH 7.40). Subsequently,10 μL stock solution of **HTBT2** (1.0 mM) was added to the cells containing 1.0 mL PBS of pH 7.40 (the final concentration of probes was 10 μM), respectively. Then, the time-dependent fluorescence imaging of **HTBT2** was performed on LSM880+ Airysan confocal laser scanning microscope with red channel (Ex = 458 nm, Em = 560-660 nm) separately. The dynamic assays processes of BEAS-2B cells and PC-12 cells stained by **HTBT2** similar the stained of SMMC-7721 cells.

Colocalization experiment

The culture of cells for colocalization experiments was similar to the dynamic experiments of cell stained, SMMC-7721 cells were incubated in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum), and the cells were seeded in glass Petri dish and incubated at 37 ℃ in 5% CO_2 atmosphere for 48 h. Then, the remainder DMEM was removed and cells were washed for three times by PBS (pH 7.40). Hereafter, 10 μL stock solution containing 1 mM **HTBT2** with final concentration of 10 μM was added to the glass Petri dish for 40 min and were washed three times with PBS (pH 7.40). MitoTracker Green (2 μM) was added and co-incubated for another 5 min. Subsequently, cell images were performed after the cells were rinsed for three times with PBS (pH 7.40). The fluorescence images were obtained on the confocal laser scanning microscope on the red channel (Ex = 458 nm, Em = 560-660 nm) for **HTBT2** and the green channel (Ex = 488 nm, Em = 505-540 nm) for MitoTracker Green FM, respectively.

Culture of cells for HTBT2 fluorescent imaging

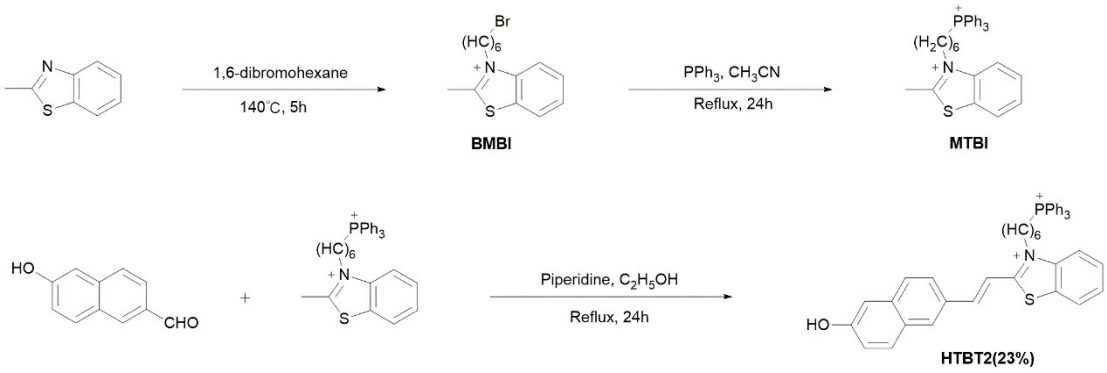
SMMC-7721 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum), and the cells were planted in glass Petri dish and incubated for 48h at 37 ℃ in 5% CO_2 atmosphere. Then, the remaining DMEM was removed and cells were washed for three times by PBS (pH 7.40). Then, 10 μL the stock solution of **HTBT2** with 1.0 mM was added into 1.0 mL the cells medium with final concentration of 10 μM for 40 min, and cells were washed with PBS (pH 7.40) three times to remove the remaining **HTBT2**. Afterwards, the cells

were incubated with high K^+ buffer (30 mM NaCl, 120 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 mM NaH_2PO_4, 5 mM glucose, 20 mM HEPES, and 20 mM NaOAc) of various pH values (5.50, 6.50, 7.00, 7.40, 8.00, 8.50 and 9.50) in the presence of nigericin (10.0 μM) for an additional 10 min[3]. The fluorescence images were obtained on an LSM880+ Airysan confocal laser scanning microscope with the red channel (Ex = 458 nm, Em = 560-660 nm) and the pH calibration curve was drawn according to the results. After the cells are treated in the same way, the changes in the fluorescence intensities in SMMC-7721 cells were measured during 1 h under continuous laser irradiation to evaluate the photostability of **HTBT2** in the cells with different pH.

Similarly, NH_4Cl (5 mM), H_2O_2 (0.1 mM) and NAC (1 mM) were used to deal with the SMMC-7721 cells after being treated with **HTBT2**, respectively, and the changes of fluorescence intensity in the cells with times were recorded. Then, the mitochondrial average pH values were gained according to above calibration curve.

SMMC-7721 cells (human liver cancer cells), A549 cells (human alveolar cancer cell), B16F10 cells (mouse melanoma cells), PC-12 cells (rat neural cells), BEAS-2B cells (normal human alveolar cell) and HSC cells (human hematopoietic stem cells) were cultured under the identical conditions and requirements, respectively and the pH imaging of **HTBT2** in SMMC-7721 was then preformed accordingly. Hereafter, the stock solutions of **HTBT2** (10 μM) were added to each cell medium for 40 min and theses cells were washed with PBS (pH 7.40) three times, separately. The fluorescence images of pH in above cells were obtained on the confocal laser scanning microscope with the red channel (Ex = 458 nm, Em = 560-660 nm) and the mitochondrial average pH values in these cells were calculated according to the results and the calibration curve.

2. Supporting Information Figures.



Scheme. S1 The synthetic route of **HTBT2**.

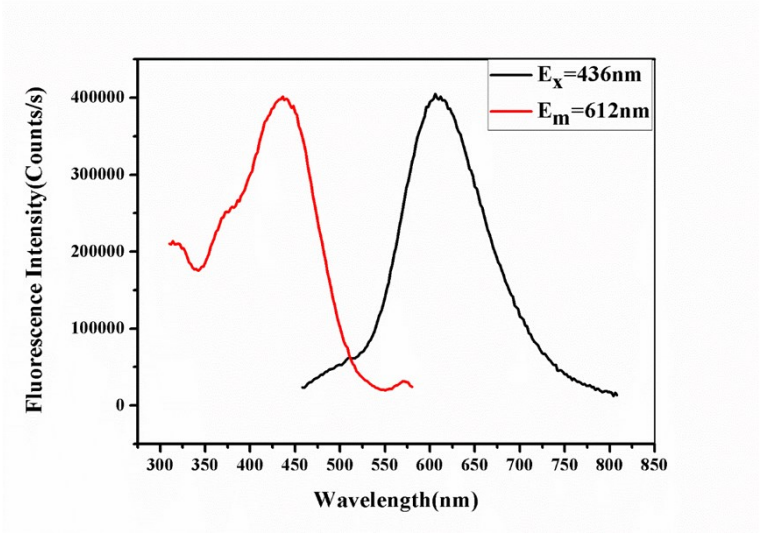


Fig. S1 The fluorescence spectra of **HTBT2** at pH 6.40 in which the bandwidths of excitation and emission were set at 2.0 nm.

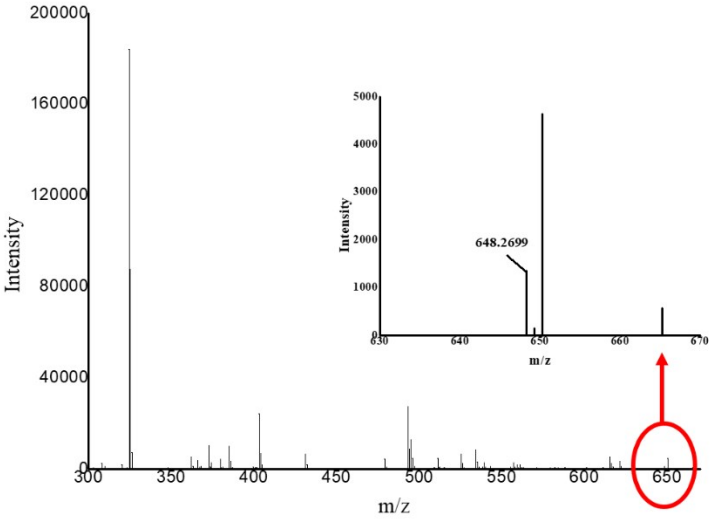


Fig. S2 The mass spectra of 10 μM **HTBT2** at pH 3.0.

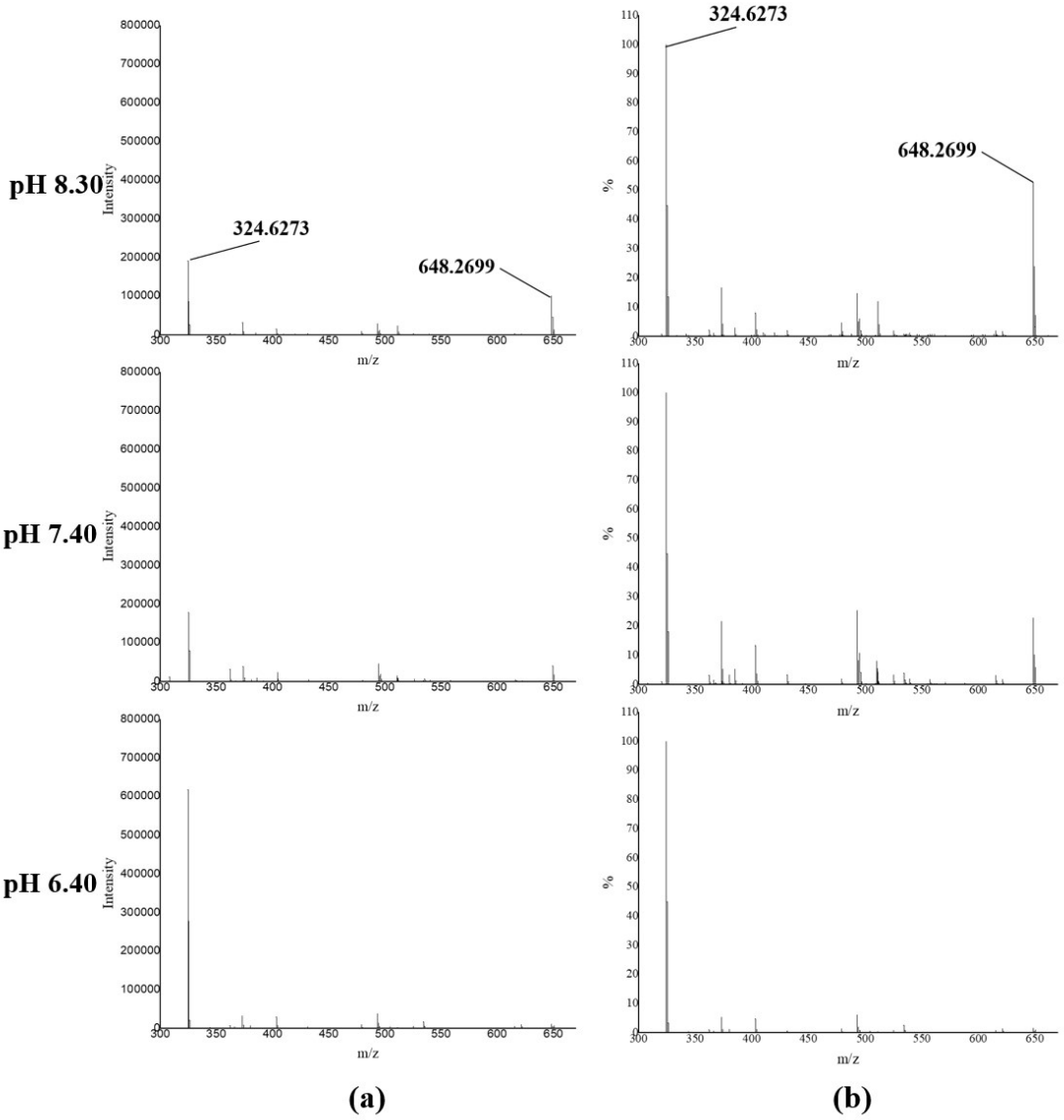


Fig. S3 (a) and **(b)** were the changes of **HTBT2**'s mass spectra signal intensities and relative intensities upon increasing the pH from 6.40 to 8.30, respectively.

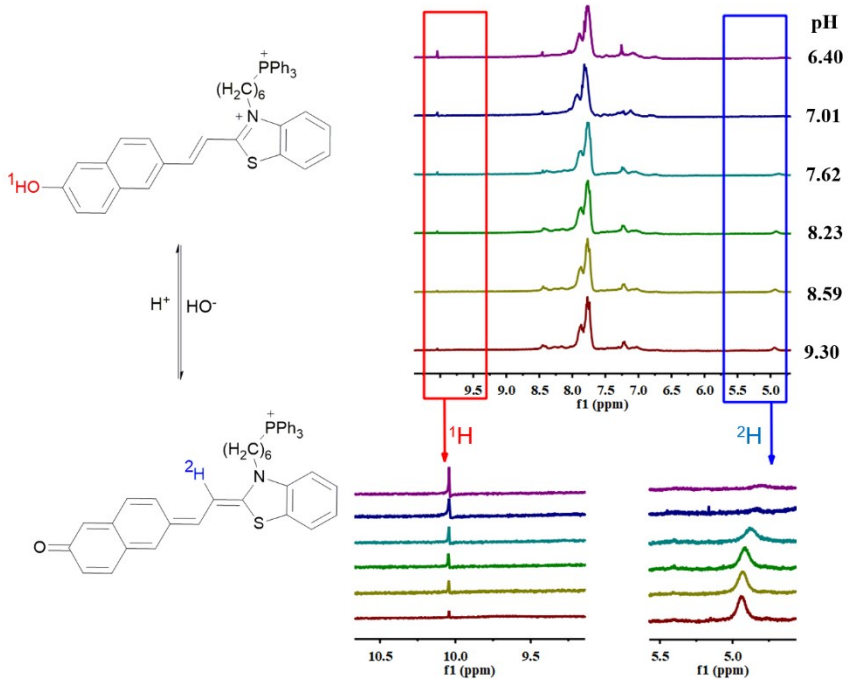


Fig. S4 ^{1}H NMR spectra changes of **HTBT2** with increasing pH from 6.30 (top) to9.40 (bottom).

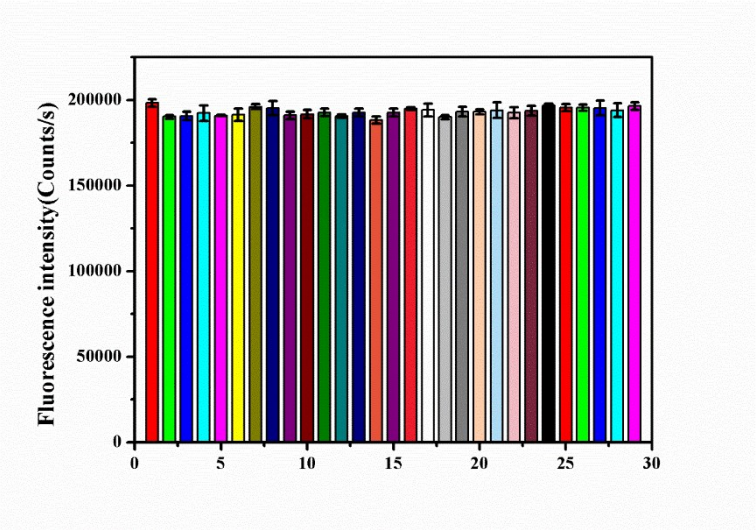


Fig. S5 Selectivity of 10 μM **HTBT2** to common interfering substances in 2 mL buffer solution of pH 6.40:1, probe; 2, F^- (1 mM); 3, Cl^- (10 mM); 4, Br^- (1 mM); 5, I^- (1mM); 6, SO_4^{2-} (1 mM); 7, $S_2O_3^{2-}$ (1 mM); 8, SO_3^{2-} (1mM); 9, HS^- (1 mM); 10, NO_3^- (1 mM); 11, NO_2^- (1 mM); 12, Ac^- (1mM); 13, HCO_3^- (1 mM); 14, ClO_4^- (1 mM); 15, K^+ (140 mM); 16, Cd^{2+} (1mM); 17, Mg^{2+} (1 mM); 18, Li^+ (1 mM); 19, Co^{2+} (1 mM); 20, Hg^{2+} (1 mM); 21, Ba^{2+} (1 mM); 22, Ni^{2+} (1 mM); 23, H_2O_2 (1 mM); 24, O_2^- (1 mM); 25, HClO (1 mM); 26, $ONOO^-$ (100 μM); 27, L-GSH (1 mM); 28, Hcy (1 mM); 29, Cys (1 mM).

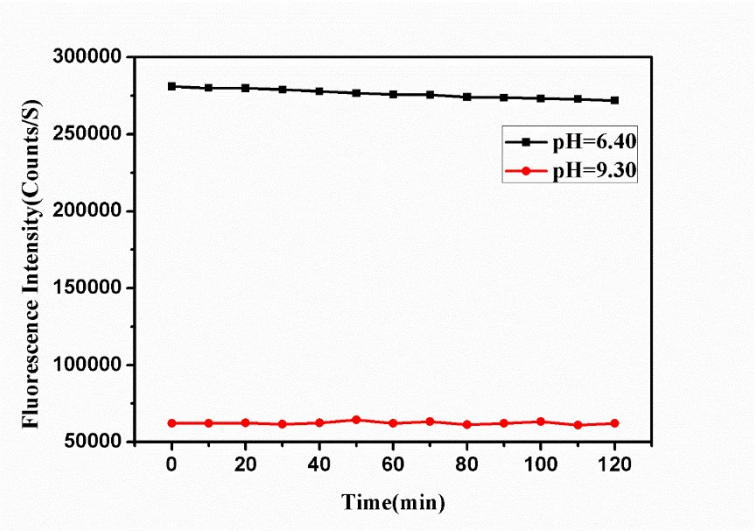


Fig. S6 Changes in fluorescence intensity for **HTBT2** with times at pH 6.40 and 9.30, respectively. Ex = 436 nm. Excitation and emission bandwidths were set at 2 nm.

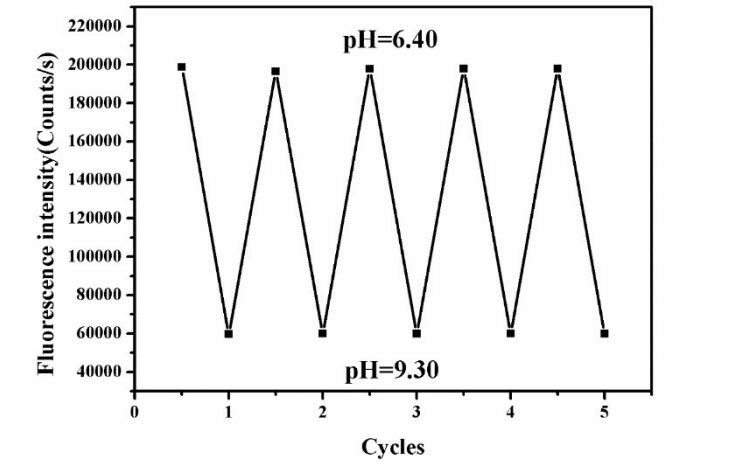


Fig. S7 Changes in the fluorescence intensity at 612 nm of probe between pH 6.40 and 9.30 with Ex = 436 nm.

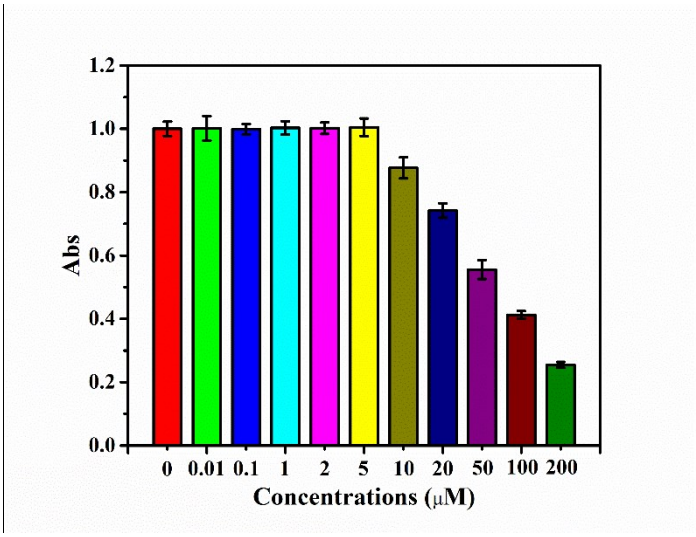


Fig. S8 MTT assay of live SMMC-7721 cells in the presence of **HTBT2** of various concentrations.

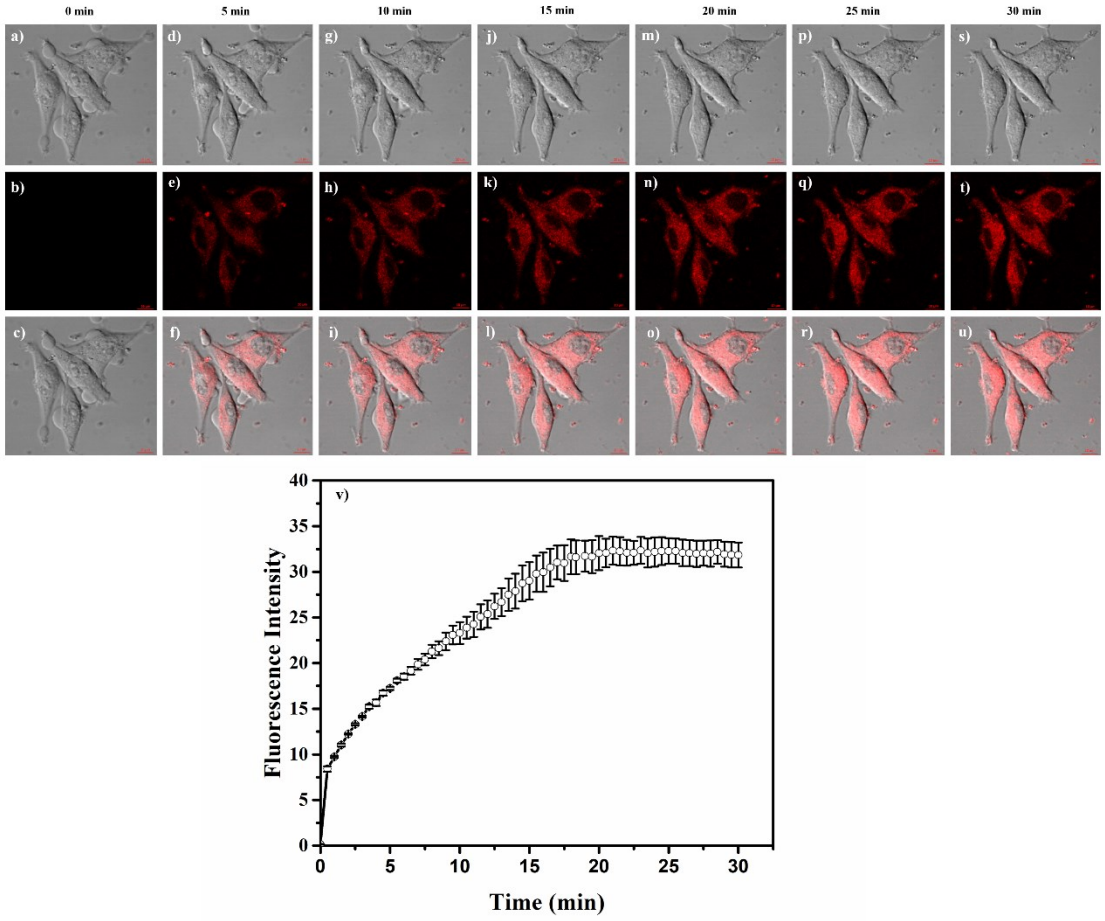


Fig. S9 (a-u) The time-dependent fluorescence images of SMMC-7721 cells after 10 μM **HTBT2** was added into the culture medium, which the red channel within 560-660 nm were collected with Ex = 458 nm. **(v)** The changes of fluorescence intensity in SMMC-7721 cells with times.

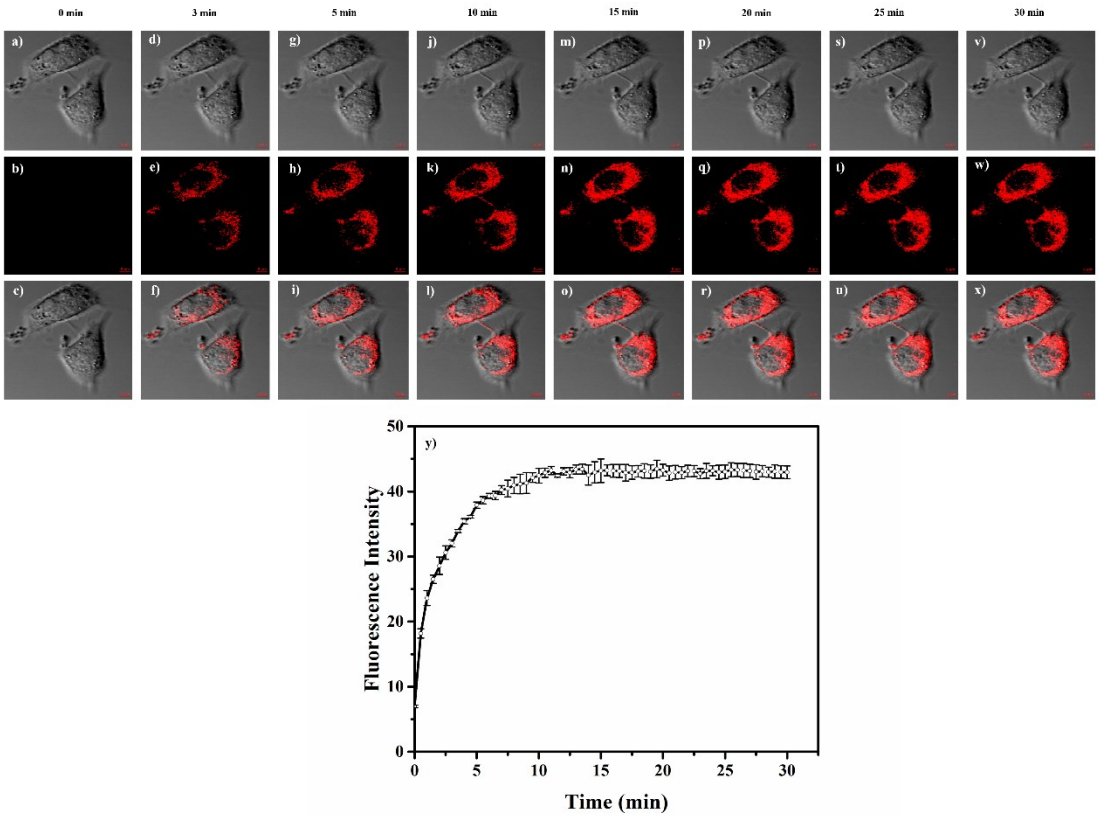


Fig. S10 (a-x) The time-dependent fluorescence images of BEAS-2B cells after 10 μM **HTBT2** was added into the culture medium, which the red channel within 560-660 nm were collected with Ex = 458 nm. **(y)** The changes of fluorescence intensity in BEAS-2B cells with times.

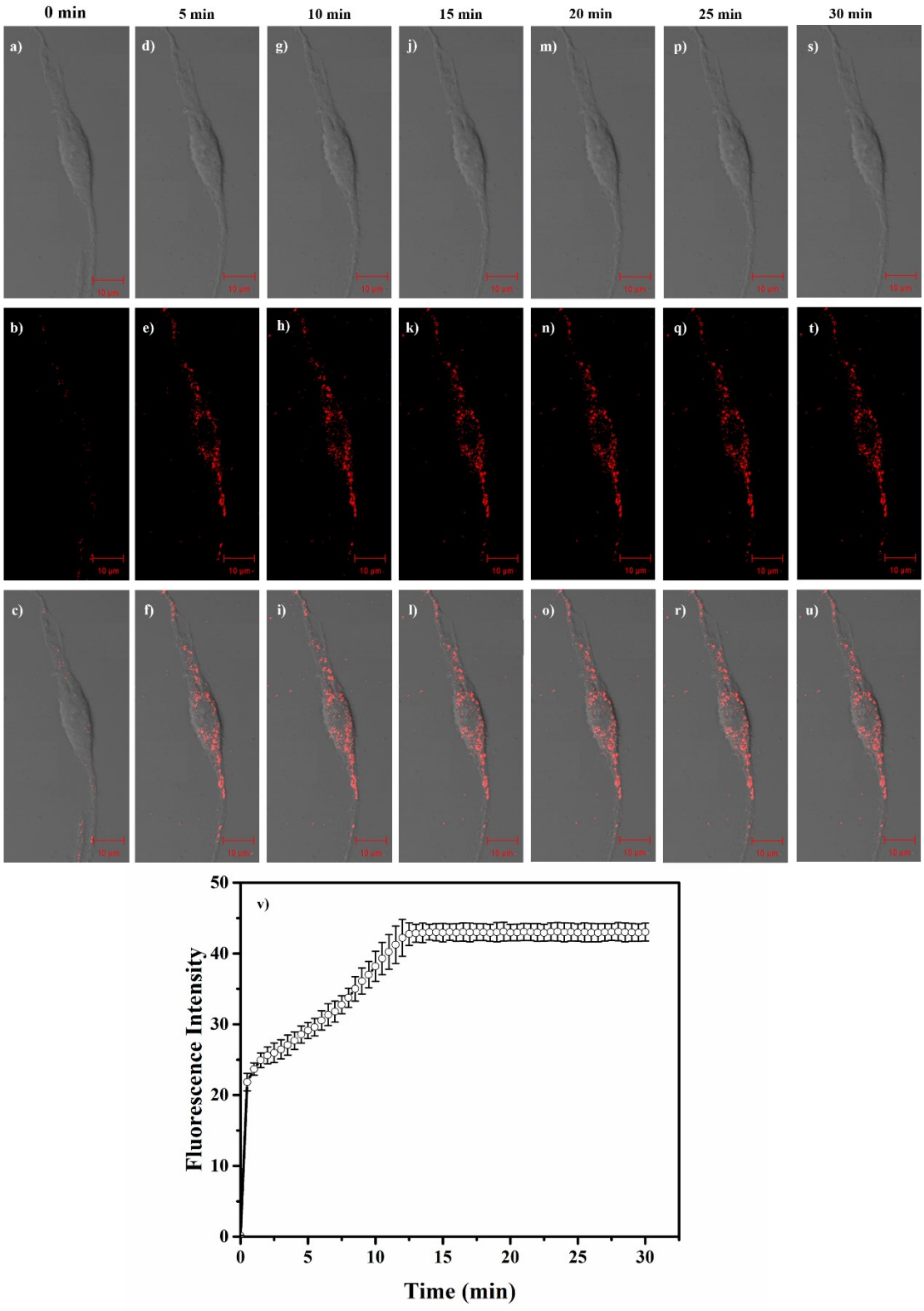


Fig. S11 (a-u) The time-dependent fluorescence images of PC-12 cells after 10 μM **HTBT2** was added into the culture medium, which the red channel within 560-660 nm were collected with Ex = 458 nm. **(v)** The changes of fluorescence intensity in PC-12 cells with times.

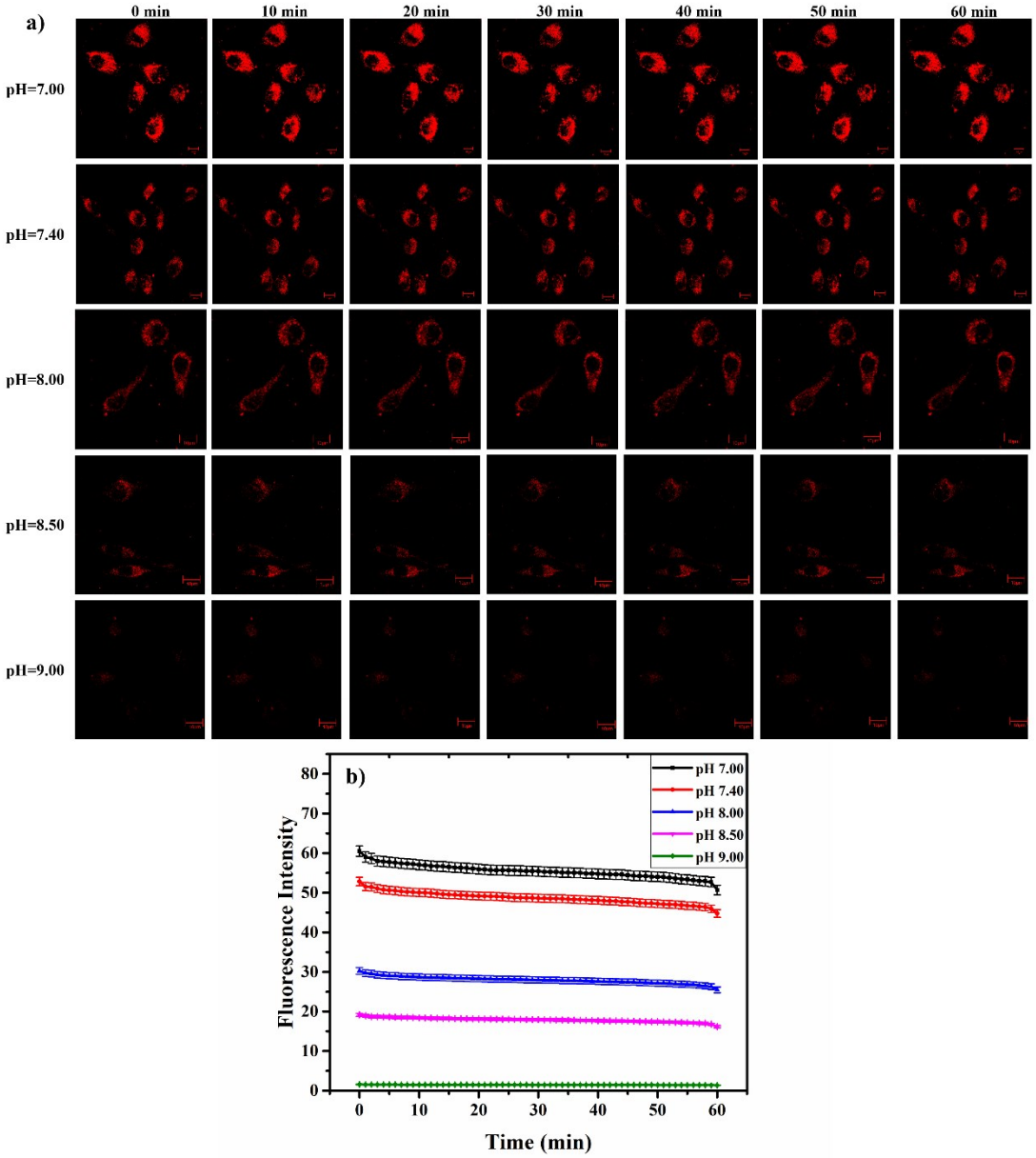


Fig. S12 (a) The time-dependent fluorescence images of SMMC-7721 cells treated with 10 μM **HTBT2** at pH 7.00 (the first row), 7.40 (the second row), 8.00 (the third row), 8.50 (the fourth row) and 9.00 (the fifth row), respectively. The red channel imaging was collected at 560 - 660 nm (Ex = 458 nm). **(b)** The changes of fluorescence intensities in SMMC-7721 cells with times.

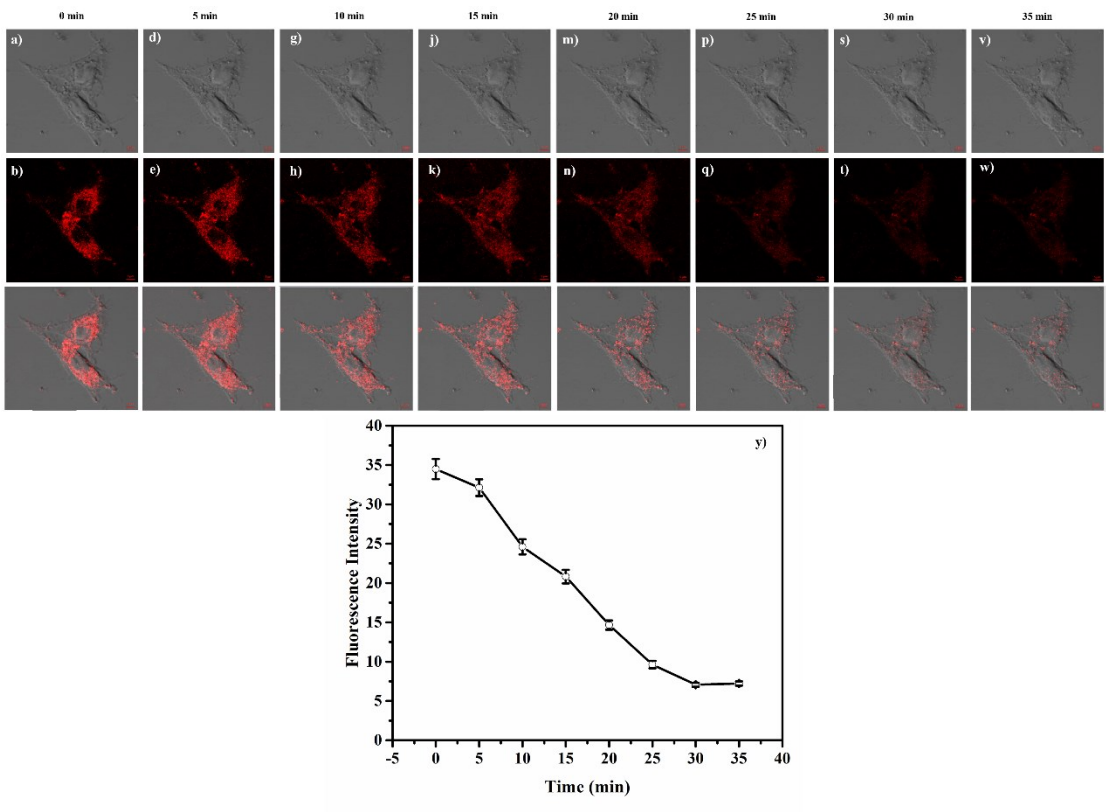


Fig. 13 (a-x) Fluorescence imaging of **HTBT2** (10 μM) in SMMC-7721 cells under different conditions of 5 mM NH_4Cl. The red channel imaging was collected at 568 - 650 nm (Ex = 561 nm). **(y)** The mean fluorescence intensity changes of mitochondria after treated via NH_4Cl with time.

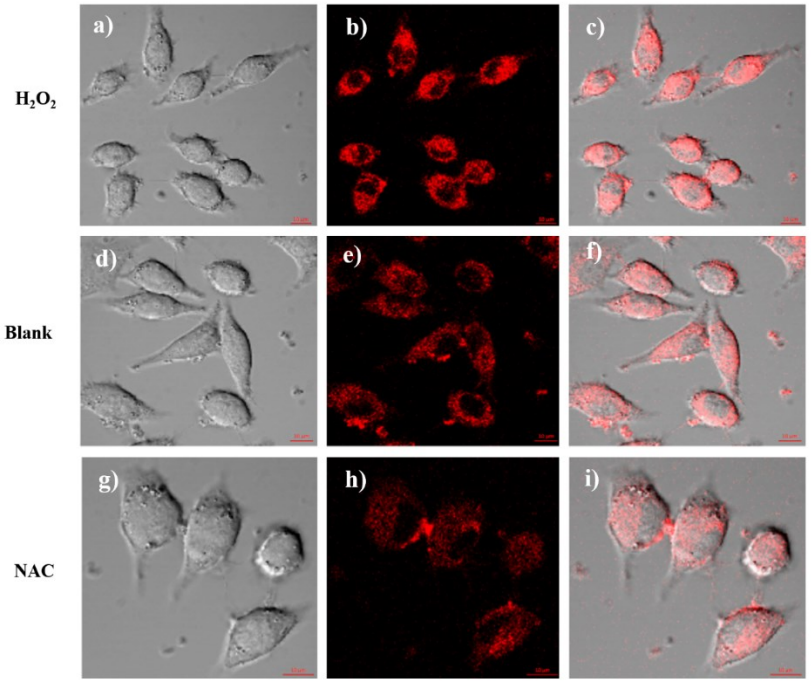


Fig. S14 Fluorescence images of 10 μM **HTBT2** stained SMMC-7721 cells. The cells were treated with H_2O_2 (0.1 mM), PBS (pH 7.40) and NAC (1.0 mM) for 1 h, respectively. The red channel images were collected at 560 - 660 nm (Ex = 458 nm).

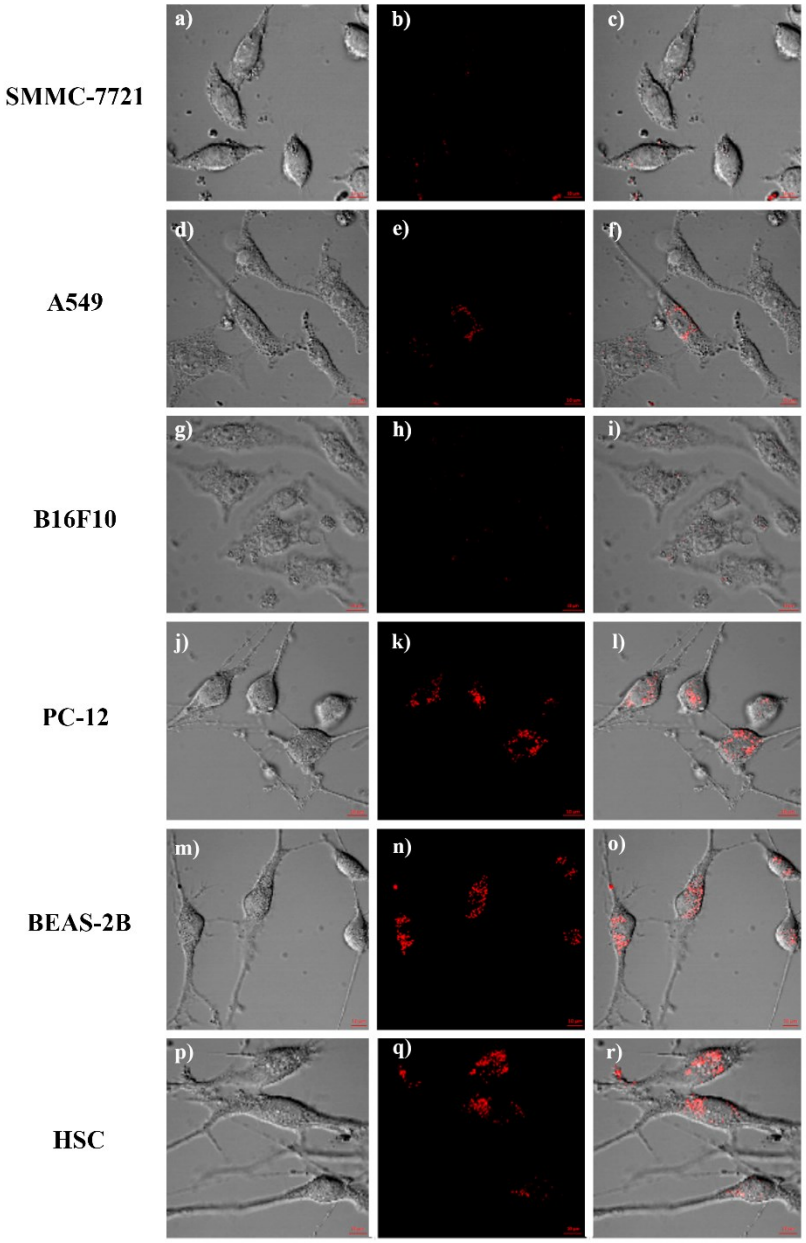


Fig. S15 (a-r) Fluorescence images of SMMC-7721 cells, A549 cells, B16F10 cells, PC-12 cells, BEAS-2B cells, HSC cells stained with 10 μM **HTBT2,** respectively. **(s)** The fluorescence intensity plot of the above cell lines. The red channel imaging with 560 - 660 nm (Ex = 458 nm) was collected. The thresholds were set to 82.

3. Supporting Information Tables.

Table S1 Fluorescence lifetime of **HTBT2** in various pH values buffer solutions.

pH	**τ (μs)**	R^2
6.4	6.99	0.9646
6.8	7.00	0.9698
7.0	7.00	0.9660
7.3	6.99	0.9681
7.5	7.02	0.9781
7.7	7.01	0.9769
7.9	7.01	0.9614
8.1	7.03	0.9693
8.3	7.03	0.9719
8.5	7.01	0.9719
8.7	7.01	0.9715
9.0	7.02	0.9762
9.3	7.02	0.9780
Average	7.01	-

Reference

1. A. R. Lippert, E. J. New and C. J. Chang, *J. Am. Chem. Soc.*, 2011, **133**, 10078-10080.
2. X. Jia, Q. Chen, Y. Yang, Y. Tang, R. Wang, Y. Xu, W. Zhu and X. Qian, *J. Am. Chem. Soc.*, 2016, **138**, 10778-10781.
3. M. H. Lee, J. H. Han, J. H. Lee, N. Park, R. Kumar, C. Kang and J. S. Kim, *Angew. Chem., Int. Ed.*, 2013, **52**, 6206-6209.