Electronic Supplementary Information (ESI)

A bifunctional fluorescent sensor for CCCP-induced cancer cell apoptosis imaging

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Experimental

Materials and Methods

All chemicals were gained from reagent companies (Sigma-Aldrich, Aladdin, Macklin). Annexin V/7-AAD Apoptosis Detection Kit was gained from Shanghai Xinyu Biological Technology CO., LTD. Deionized water was used all over the work. The pH values were detected by a Model PHS-3C meter (Shanghai, China). Absorption spectrums were detected by UV-2102 double-beam UV/VIS spectrometer. Fluorescence spectrums were recorded by F-4500 FL Spectrophotometer. The ^{1}H NMR and ^{13}C NMR spectra were measured by Bruker DTX-400 spectrometer. ESI mass spectra were gained by an HPLC Q-Exactive HR-MS spectrometer (Thermo, USA). Flow cytometry analysis was taken on BD Canto plus (USA). Cofocal fluorescence images were gained by Zeiss LSM 880 confocal microscope.

Synthesis and characterization

Compounds **1** and **2** were gained by the literature methods.[S1,S2]

Synthesis of probe NPCF. 240 mg of compound **1** (1.5 equiv.) and 269 mg of compound 2 (1 equiv.) were added to a flask (50 mL), followed by adding ethanol (20 mL) and piperidine (0.2 mL). After refluxing for 16 h under nitrogen, a yellow product was filtered, washed with ice-cold ethanol. 117.0 mg of **NPCF** was obtained in a yield of 28.4%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm): 2.39 (s, 3H), 7.35 (t, 1H, J = 15.0 Hz), 7.42 (t, 1H, J = 15.0 Hz), 7.52 (t, 1H, J = 15.0 Hz), 7.60 (t, 2H, J = 14.0 Hz), 7.77 (s, 1H), 7.84 (s, 1 H), 7.89 (d, 1 H, J = 8.1 Hz), 8.15 (d, 1H, J = 8.1 Hz), 8.19−8.34 (m, 3H), 13.20 (s, 1H), 13.48 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 20.27, 113.33, 117.44, 121.60, 122.45, 122.77, 123.25, 123.58, 126.14, 126.46, 127.54, 129.57, 131.80, 132.83, 133.70, 135.28, 138.96, 143.57, 149.60, 151.28, 155.32, 168.84, 181.62. HR-MS: Calcd for $[C_{24}H_{17}N_3O_2S + H]^+$: 412.1114, found 412.1117.

Synthesis of compound NPM. 269 mg of compound **2** (1 equiv.) and 553 mg of K_2CO_3 (4 equiv.) were added to a flask (50 mL), and then acetone (7 mL) were added. After refluxing for 12 h, a red-purple product was filtered, washed with acetone and deionized water. Compound **NPM** was received as a red-purple solid (136.6 mg, yield 44.2%). ^{1}H NMR (400 MHz, DMSO-d_6, ppm): 2.20 (s, 3H), 2.25 (s, 3H), 7.05 (d, 2H, J = 16.0 Hz), 7.21 (s, 2H), 7.36 (s, 1H), 7.79−7.93 (m, 3H), 8.02 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 20.73, 27.31, 115.96, 116.34, 120.74, 121.68, 121.74, 122.61, 123.69, 125.31, 131.11, 133.64, 136.11, 144.35, 152.25, 166.29, 170.87, 198.56. HR-MS: Calcd for $[C_{18}H_{15}NO_2S + H]^+$: 310.0896, found 310.0900.

Synthesis of SO_2 donor. SO_2 donor were synthesized by reported methods.[S3] 2, 4-dinitrobenzensulfonyl chloride (0.594 g, 2.25 mmol) in 18 mL dry DCM at 0 °C was added to a solution of benzylamine (0.477 g, 6.0 mmol) with trimethylamine (0.963 g, 9 mmol) and 10 mL DCM, the mixture was stirred and reacted for 3 h at room temperature, then 100 mL of water was added to the solution to stop the reaction. Extracted by DCM, and the organic layer was dried by anhydrous Na_2SO_4. The solvent was removed under vacuum to get the crude product and the yellow product was collected by chromatography using DCM as eluent. SO_2 donor was obtained as a yellow solid (580.0 mg, yield 77.3%). ^{1}H NMR (400 MHz, $CDCl_3$, ppm): 4.66 (d, 2H, J = 5.6 Hz), 6.92 (d, 1H, J = 9.5 Hz), 7.34−7.42 (m, 5H), 8.20−8.24 (dd, 1H, J = 2.5 Hz), 8.92 (s, 1H) 9.14 (d, 1H, J = 2.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ = 47.58, 114.45, 124.22, 127.09, 128.37, 129.29, 130.39, 130.76, 135.59, 136.45, 148.23.

Detection Limit Calculation

For **NPCF**, the fluorescence intensity of 542 nm versus HSO_3^- concentration (0.6 to 1.8 equiv.) was investigated, and the detection limit of **NPCF** was counted as 22.7 nM (R^2 = 0.985), using the formula (1):

Detection limit = 3 σ/|k| (1)

Cell imaging

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C

Cytotoxicity assay. Cytotoxicity assay was executed by using Cell Counting Kit-8 (CCK-8) according to our report article.[S4] The concentrations of **NPCF** changed from 0 μM to 30 μM.

Bio-imaging of NPCF for sensing HSO_3^- in MCF-7 cells. In order to detect endogenous HSO_3^-, MCF-7 cells were treated with NEM (1 mM) for 0.5 h, and treated with **NPCF** (10 μM) for 0.5 h and SO_2 donor (100 μM) for 1h. For detecting exogenous HSO_3^-, MCF-7 cells were incubated with **NPCF** (10 μM) for 0.5 h, and treated with 1equiv., 5equiv. and 10 equiv. of HSO_3^- for 1h, and then imaged. Conditions: λ_{ex} = 405 nm, λ_{em} = 520 nm−560 nm.

Bioimaging application of NPCF for detecting pH in Hela cells. **NPCF** (10 μM) was incubated in different pH PBS buffers (pH 4.5, 5.5, 6.5, 7.5 and 8.5) for 0.5 h, and imaged by Zeiss LSM 880 confocal microscope. Conditions: λ_{ex} = 552 nm, λ_{em} = 630−670 nm.

SO_2 reduces LPS-induced inflammation and alleviates acidification environment. For the blank group, EC1 cells were incubated with **NPCF** (10 μM) for 0.5 h; For the control groups, EC1 cells were treated with LPS (10 mg/L) for 12 h, followed by treated with PMA (1 μg/mL) for 1 h, and imaged after incubation with **NPCF** (10 μM) for 0.5 h; EC1 cells were treated with SO_2 donor (100 μM) for 1 h, and then imaged after incubation with **NPCF** (10 μM) for 0.5 h; For the experimental group, EC1 cells were incubated with LPS (10

mg/L) for 12 h, followed by treated with PMA (1 μg/mL) for 1 h, and incubated with SO_2 donor (100 μM) for 1 h, and finally imaged after incubation with **NPCF** (10 μM) for 0.5 h.

Distinguishing between normal cells and cancer cells. **NPCF** were incubated with cancer cells (MCF-7 cells) and normal cells (MCF-10A cells) for 0.5 h, respectively, and then imaged. Conditions: for green channel, λ_{ex} = 405 nm, λ_{em} = 520 nm−560 nm; for red channel, λ_{ex} = 552 nm, λ_{em} = 630−670 nm.

Changes of SO_2 and pH in cells with the increase of CCCP concentrations. Hela cells were incubated with CCCP (Ranged from 0 μM to 30 μM) for 1 h, 12 h or 24 h, respectively. Conditions: for green channel, λ_{ex} = 405 nm, λ_{em} = 520 nm−560 nm; for red channel, λ_{ex} = 552 nm, λ_{em} = 630−670 nm.

Flow cytometry analysis. Hela cells were cultured in 6-well plates at a density of 2.0×10^5 cells/well, and the cells were washed with PBS buffers and centrifuged. The cells were resuspended in PBS, stained with **NPCF** (10 μM) for 30 min, and checked by flow cytometry.

Zebrafish Confocal Fluorescence Imaging

Wild zebrafish were gained from Shanghai FishBio Co., Ltd. Zebrafish were fed in E3 media at 28°C. For the control groups, the two days old zebrafish were incubated with **NPCF** for 0.5 h and imaged. For the experimental group, zebrafish were incubated with 10 μM of **NPCF** for 0.5 h, treated with 100 μM of HSO_3^- for 1 h, and then imaged. Zebrafish were imaged on Zeiss LSM 880 confocal microscope. Conditions: λ_{ex} = 405 nm, λ_{em} = 520 nm−560 nm.

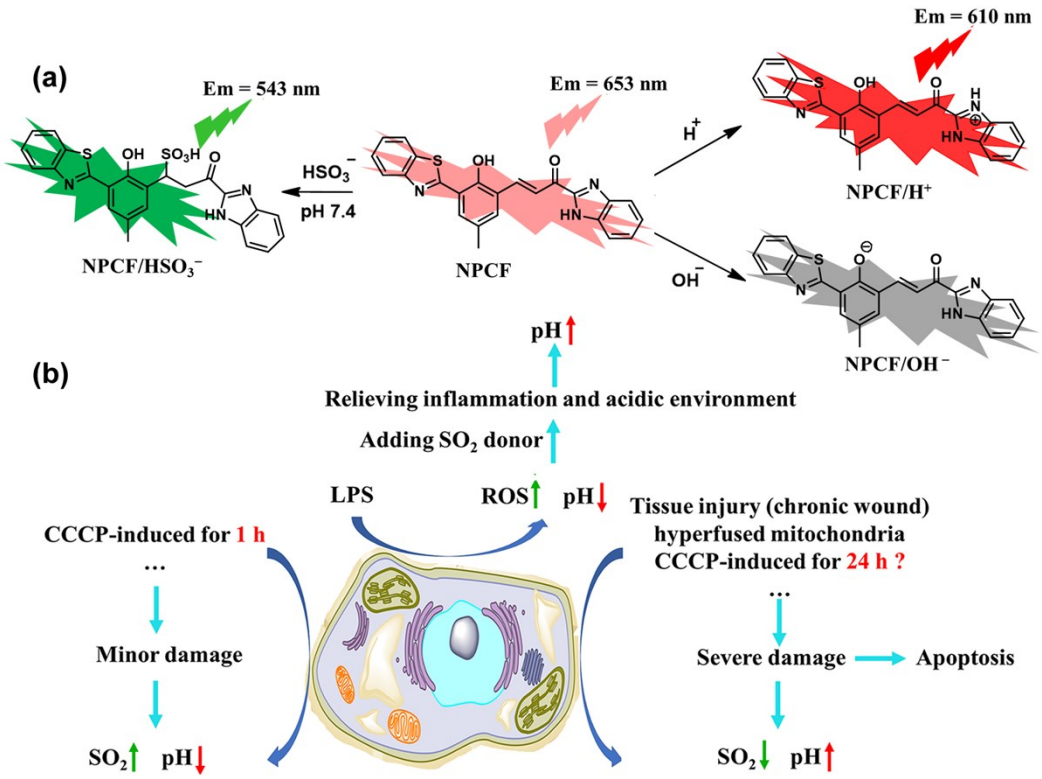
References

S1 Y. Q. Ge, J. Jia, H. Yang, X. T. Tao and J. W. Wang, *Dyes Pigments*, 2011, **88**, 344-349

S2 X. Yang, Y. Liu, Y. Wu, X. Ren, D. Zhang and Y. Ye, *Sensor. Actuat. B-Chem*, 2017, **253**, 488−494

S3 S. R. Malwal, D. Sriram, P. Yogeeswari, V. B. Konkimalla and H. Chakrapani, *J. Med. Chem.*, 2012, **55**, 553−557.

S4 X. Yang, W. Liu, J. Tang, P. Li, H. Weng, Y. Ye, M. Xian, B. Tang and Y. Zhao, *Chem. Commun.*, 2018, **54**, 11387–11390.



Scheme S1. The proposed mechanism of **NPCF** (10 μM) for detecting both HSO_3^- and pH. (b) Changes in pH during SO_2 relieving inflammation and dual indicators to detect changes in SO_2 and pH during the apoptotic process in living cells.

Fig. S1 HR-MS spectrum of probe **NPCF** with HSO_3^-

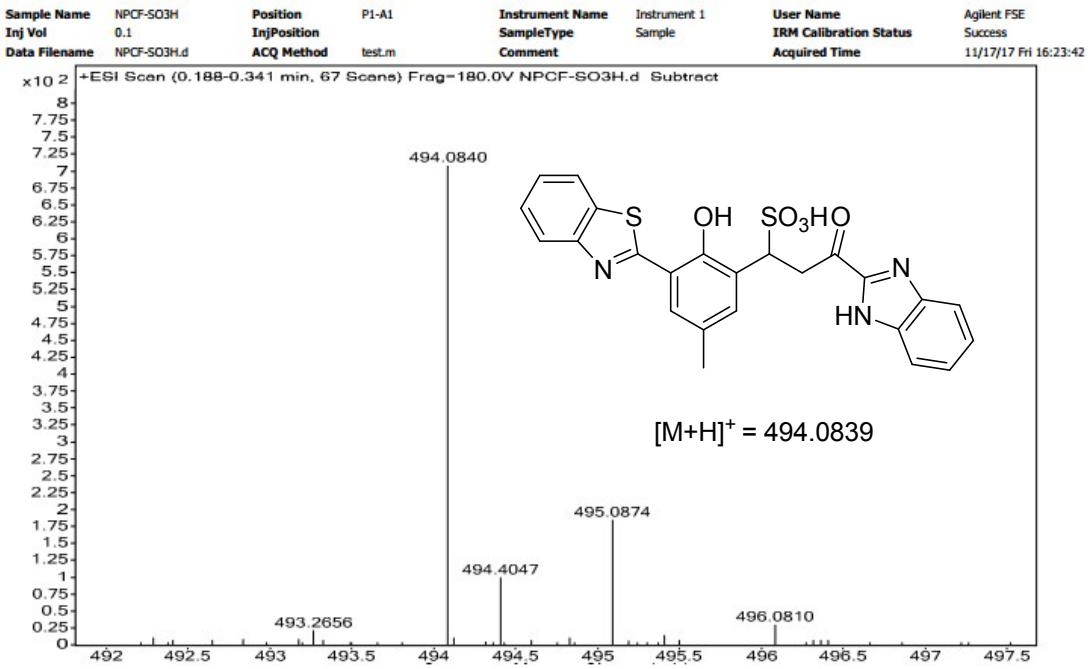
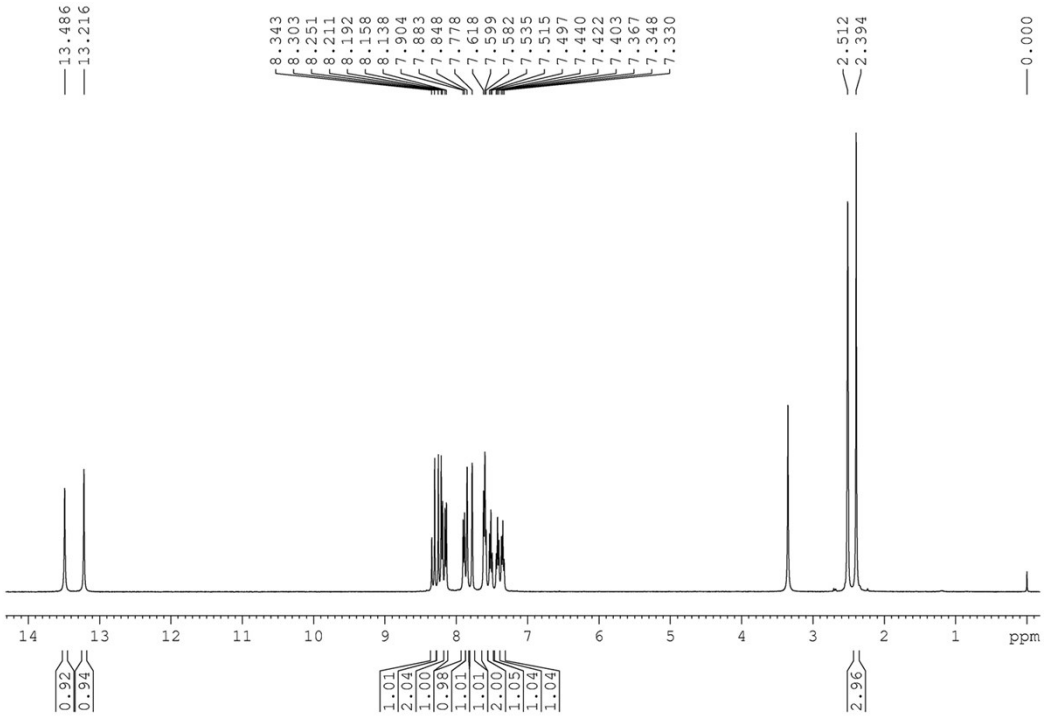
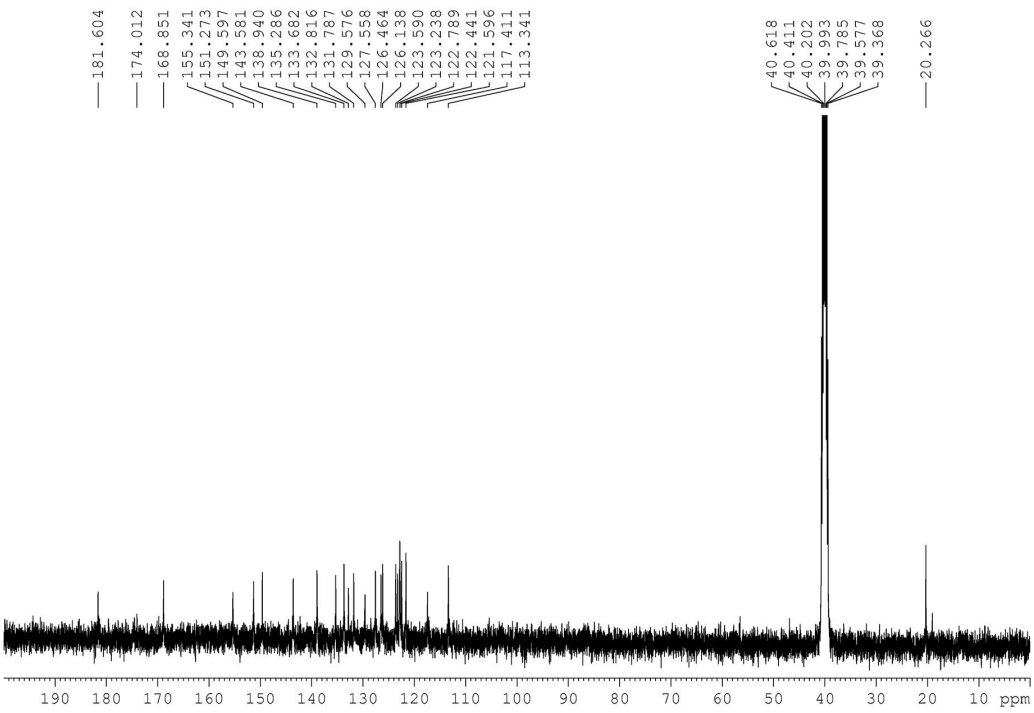


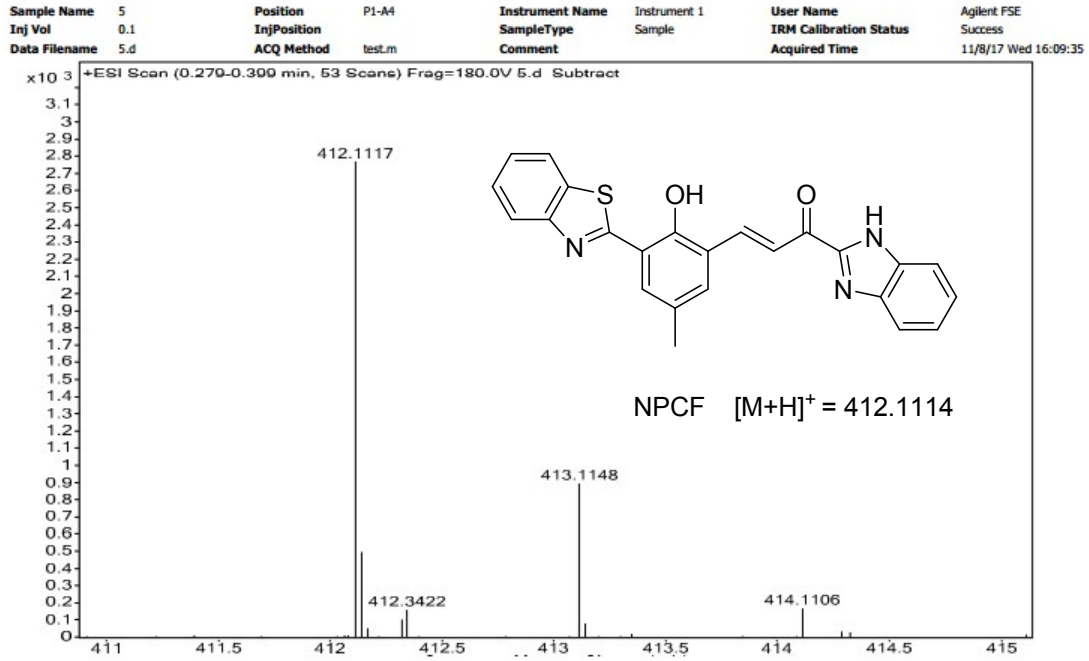
Fig. S2 Structure characterization of probe **NPCF**



^{1}H-NMR spectrum of probe **NPCF** in DMSO-d_6

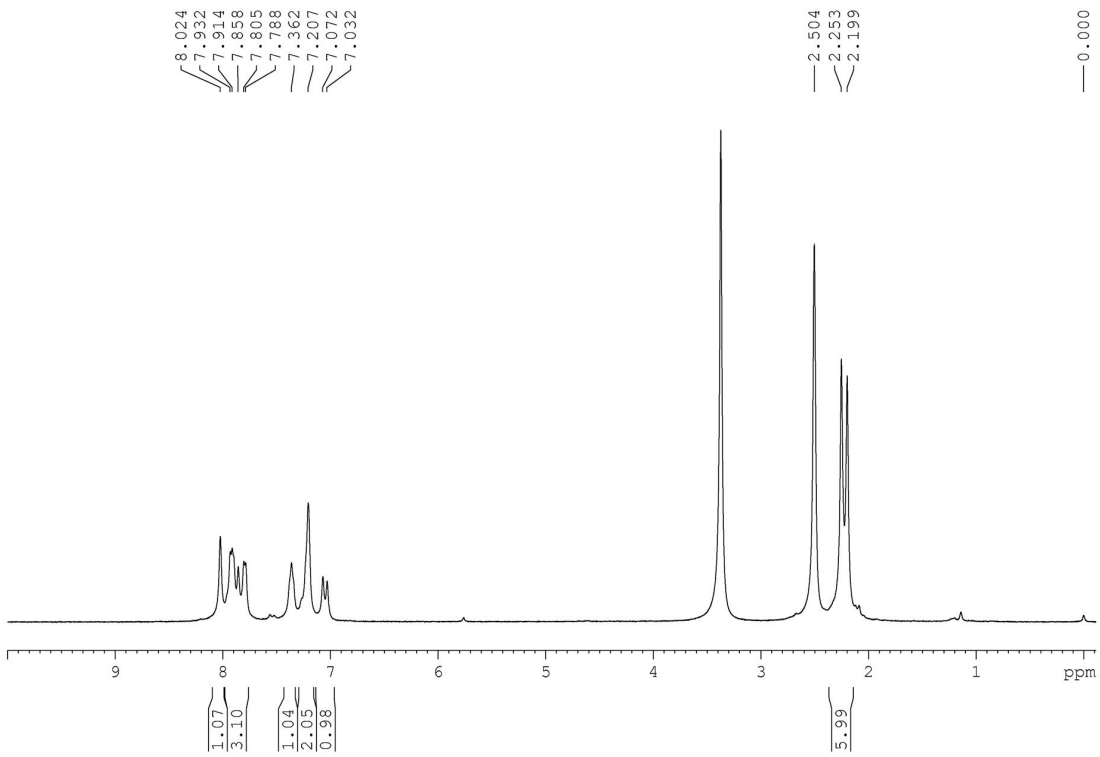


^{13}C-NMR spectrum of probe **NPCF** in DMSO-d_6

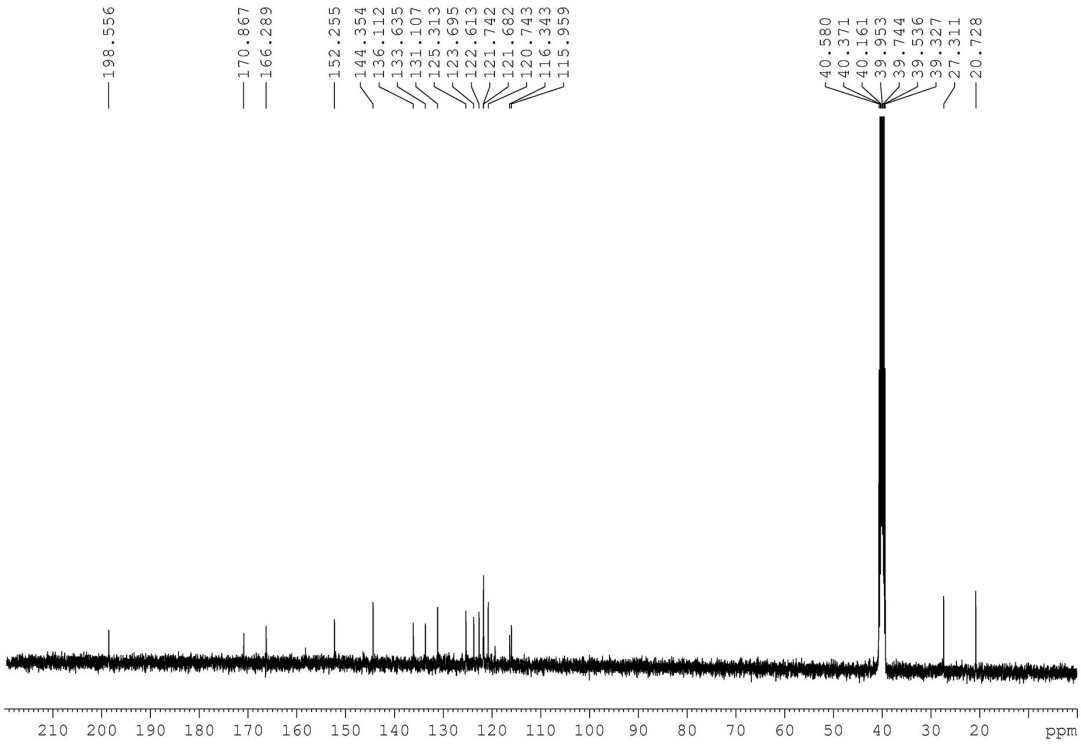


HR-MS spectrum of probe **NPCF**

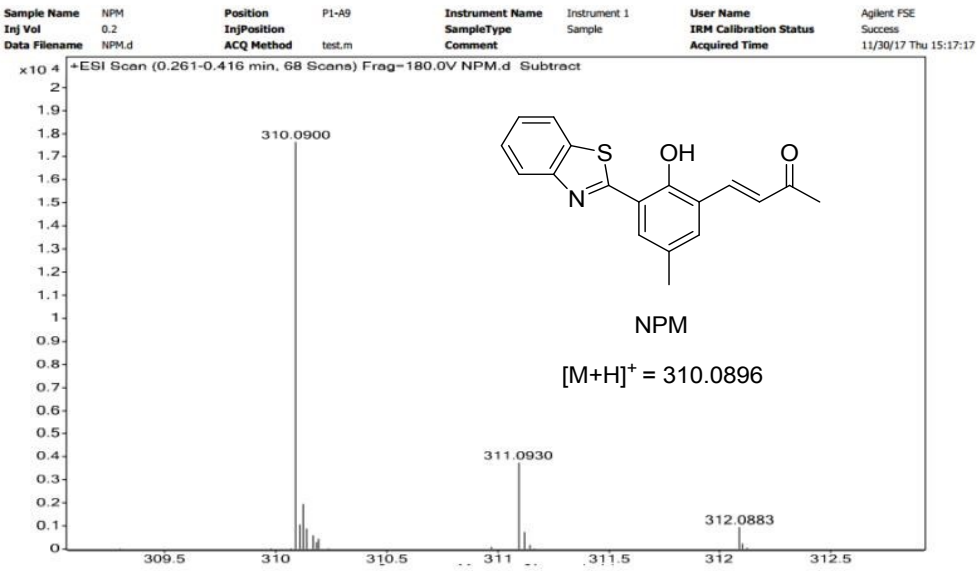
Fig. S3 Structure characterization of compound **NPM**



^{1}H-NMR spectrum of compound **NPM** in DMSO-d_6



^{13}C-NMR spectrum of compound **NPM** in DMSO-d_6



HR-MS spectrum of probe **NPM**

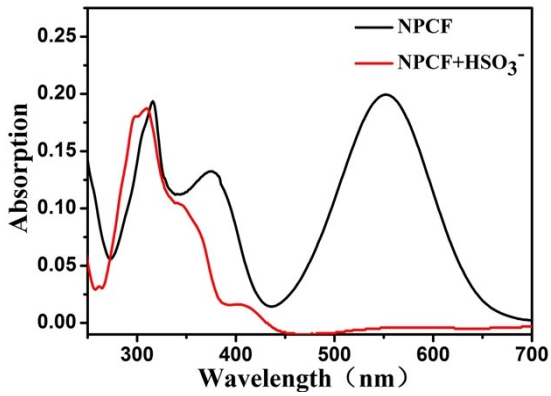


Fig. S4 UV–vis absorption spectra changes. The black line means only **NPCF** was added, the red line means **NPCF** and 10 equiv. of HSO_3^- were added.

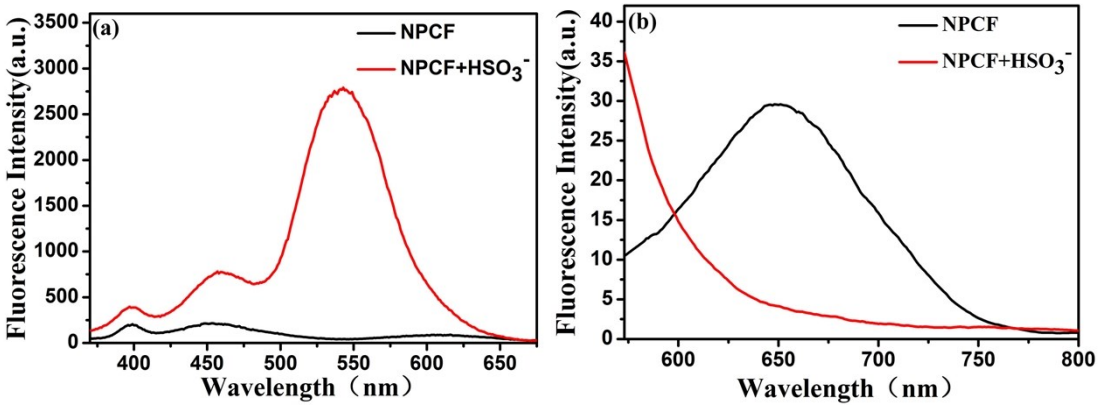


Fig. S5 (a) λ_{ex} = 350 nm, λ_{em} = 542 nm, slit: 10 nm/10 nm. (b) λ_{ex} = 543 nm, λ_{em} = 653 nm, slit: 10 nm/10 nm. All data were acquired in HEPES buffer solution (pH = 7.4, 10 mM, containing 1 mM CTAB). The black line means only **NPCF** was added, the red line means **NPCF** and 10 equiv of HSO_3^- were added.

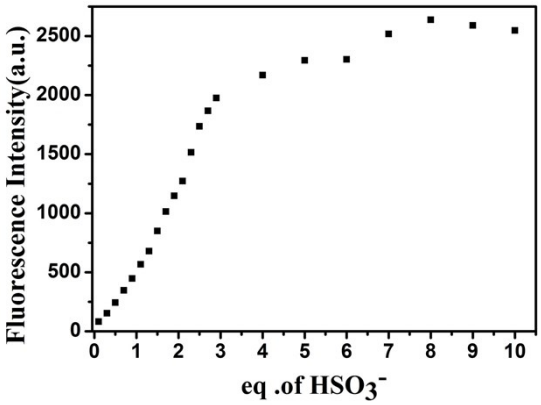


Fig. S6 Calibration curve of probe **NPCF** to HSO_3^- (0–10 equiv.). Conditions: λ_{ex} = 350 nm, slit: 10 nm/10 nm.

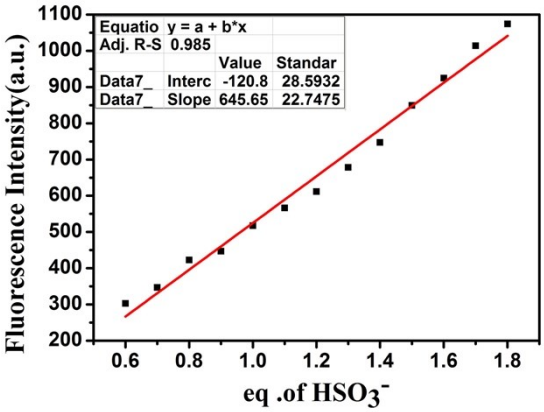


Fig. S7 The linear responses at low HSO_3^- concentrations (0.6−1.8 equiv.).

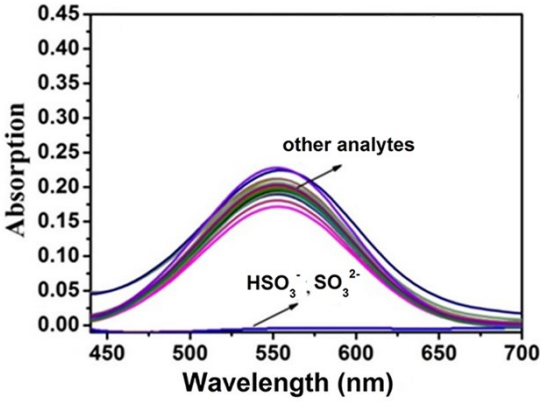


Fig. S8 UV–vis absorption changes of **NPCF** in the presence of different analytes (10 equiv. of Na^+, K^+, HS^-, Cys, Hcy, GSH, NO_3^-, CO_3^{2-}, HCO_3^-, Ac^-, SO_4^{2-}, PO_4^{3-}, F^-, Cl^-, Br^-, I^-, NO_2^-, •OH, $ONOO^-$, •NO, $O_2•^-$, and H_2O_2) in HEPES buffer solution (pH = 7.4, 10 mM, containing 1 mM CTAB).

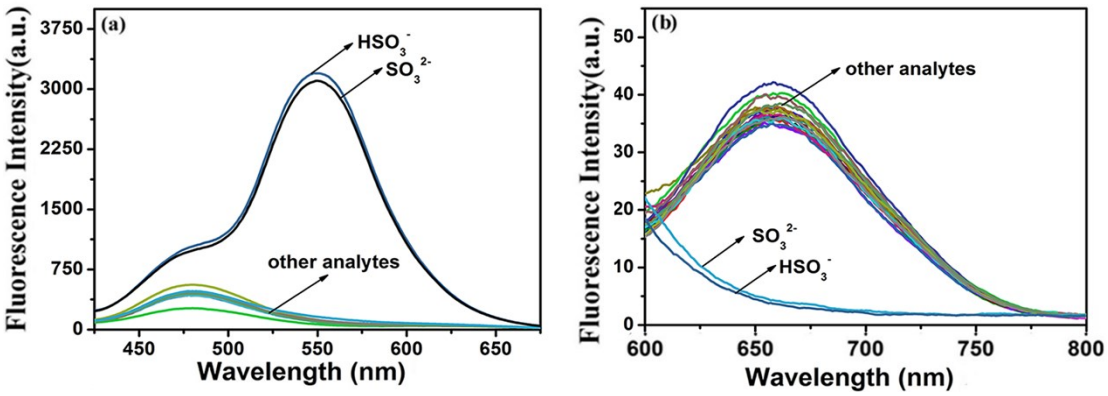


Fig. S9 Emission changes of **NPCF** in the presence of different analytes (10 equiv. of Na^+, K^+, HS^-, Cys, Hcy, GSH, NO_3^-, CO_3^{2-}, HCO_3^-, Ac^-, SO_4^{2-}, PO_4^{3-}, F^-, Cl^-, Br^-, I^-, NO_2^-, •OH, $ONOO^-$, •NO, $O_2•^-$, and H_2O_2) in HEPES buffer solution (pH = 7.4, 10 mM, containing 1 mM CTAB), slits: 10/10 nm. (a) λ_{ex} = 350 nm, λ_{em} = 542 nm; (b) λ_{ex} = 553 nm, λ_{em} = 653 nm.

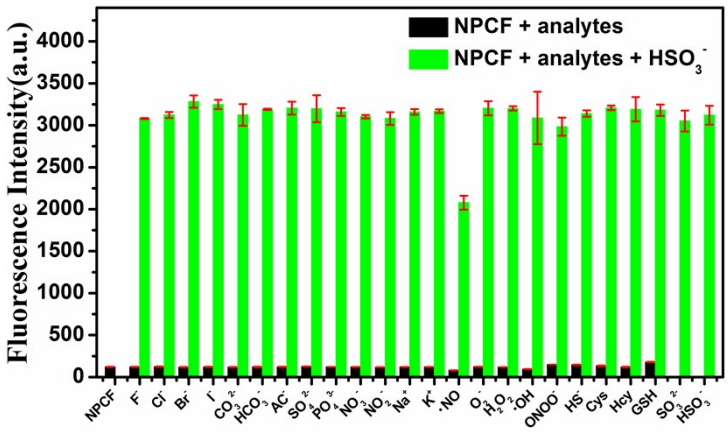


Fig. S10 Fluorescence response of **NPCF** (10 μM) to HSO_3^- (10 equiv.) in the presence of the different analytes. in HEPES buffer solution (pH = 7.4, 10 mM, containing 1 mM CTAB). λ_{ex} = 350 nm, slit: 10 nm/10 nm.

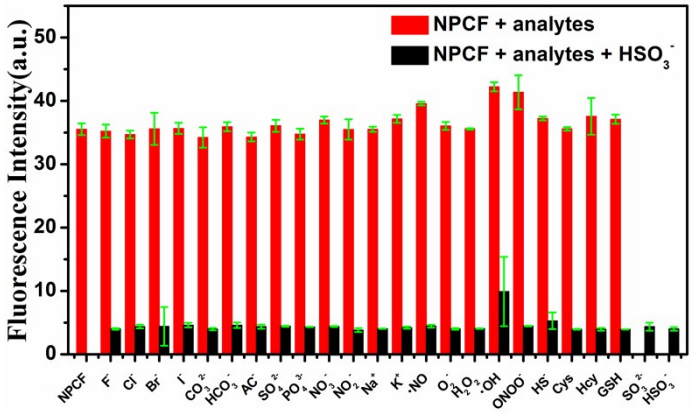


Fig. S11 Fluorescence response of **NPCF** (10 μM) to HSO_3^- (10 equiv.) in the presence of the different analytes in HEPES buffer solution (pH = 7.4, 10 mM, containing 1 mM CTAB). λ_{ex} = 553 nm, slit: 10 nm/10 nm.

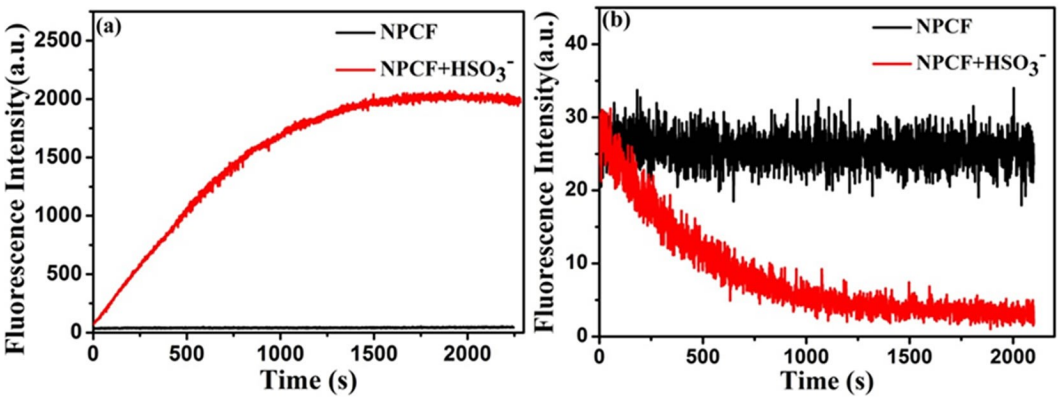


Fig. S12 Time-dependent fluorescence spectral changes of **NPCF** with HSO_3^- (10 equiv.) in HEPES buffer solution (pH = 7.4, 10 mM, containing 1 mM CTAB), slits: 10/10 nm. (a) λ_{ex} = 350 nm, λ_{em} = 542 nm. (b) λ_{ex} = 553 nm, λ_{em} = 653 nm.

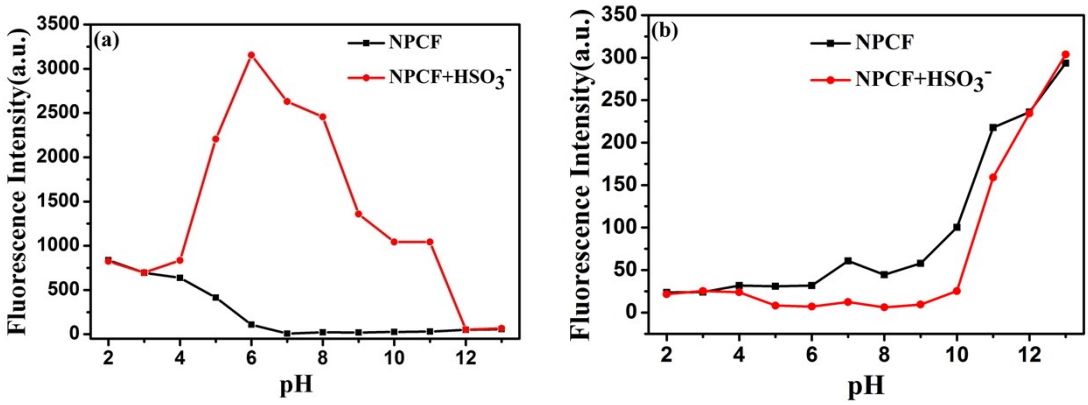


Fig. S13 The pH effect of the test solution on **NPCF** (10 μ M) was tested in the absence (black line) or presence (red line) of HSO_3^- (10 equiv.). (a) λ_{ex} = 350 nm, λ_{em} = 542 nm, slits: 10/10 nm. (b) λ_{ex} = 553 nm, λ_{em} = 653 nm, slits: 10/10 nm.

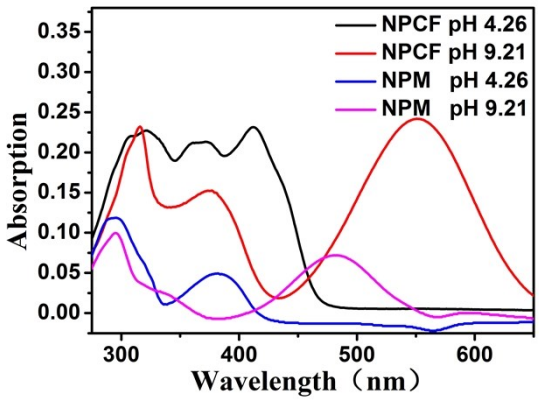


Fig. S14 UV–vis absorption spectra changes at pH 4.26 and 9.21.

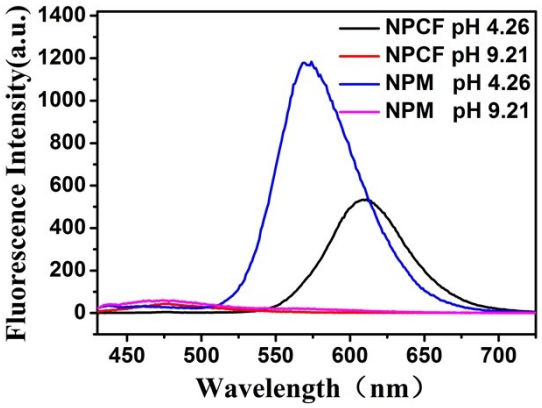


Fig. S15 Fluorescence spectra changes at pH 4.26 and 9.21. **NPCF**: λ_{ex} = 410 nm, λ_{em} = 610 nm, slits: 5/5 nm. **NPM**: λ_{ex} = 380 nm, λ_{em} = 573 nm, slits: 5/5 nm.

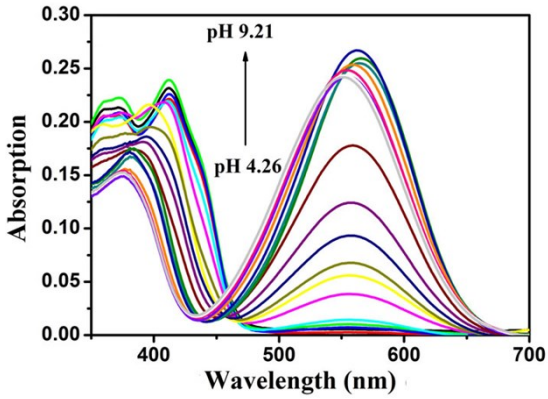


Fig. S16 Absorption spectra changes of **NPCF** at different pH values.

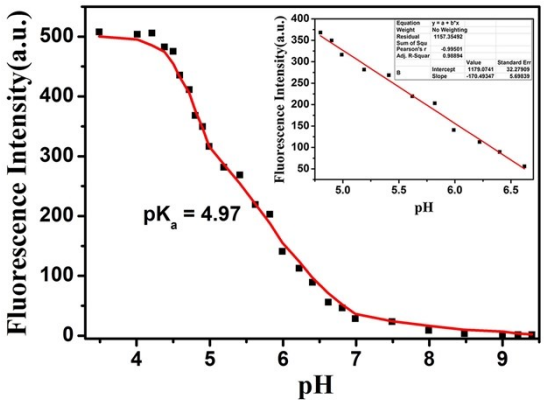


Fig. S17 The pH titration curve of **NPCF** plotted by fluorescence as a function of pH. Conditions: λ_{ex} = 410 nm, slits: 5/5 nm.

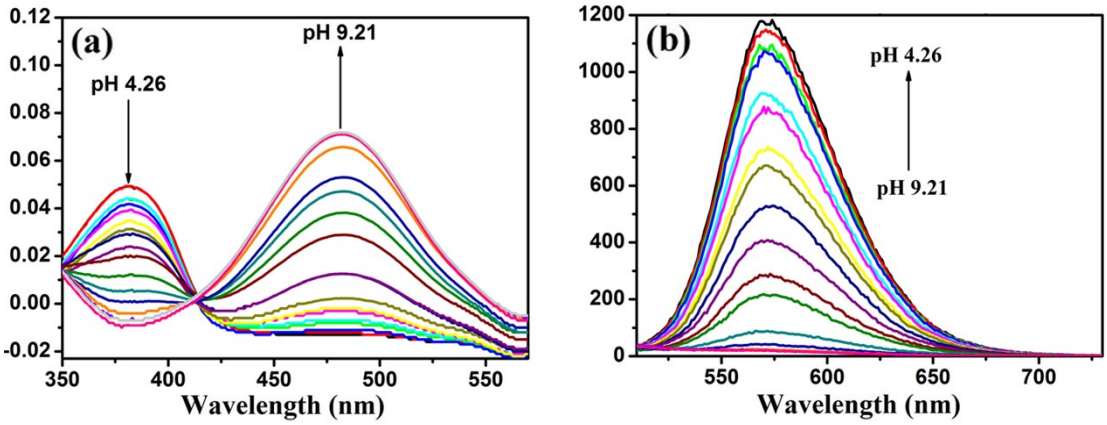


Fig. S18 (a) Absorption and (b) fluorescence spectra changes of **NPM** at different pH values. (b) λ_{ex} = 380 nm, λ_{em}= 573 nm. Slits: 5/5 nm.

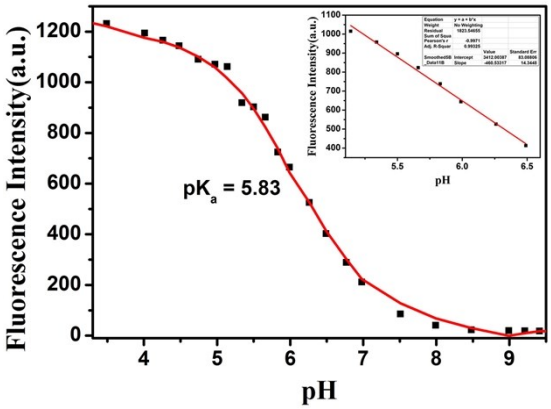


Fig. S19 The pH titration curve of **NPM** plotted by fluorescence as a function of pH. Conditions: λ_{ex} = 380 nm, λ_{em} = 573 nm, slit: 10 nm/10 nm.

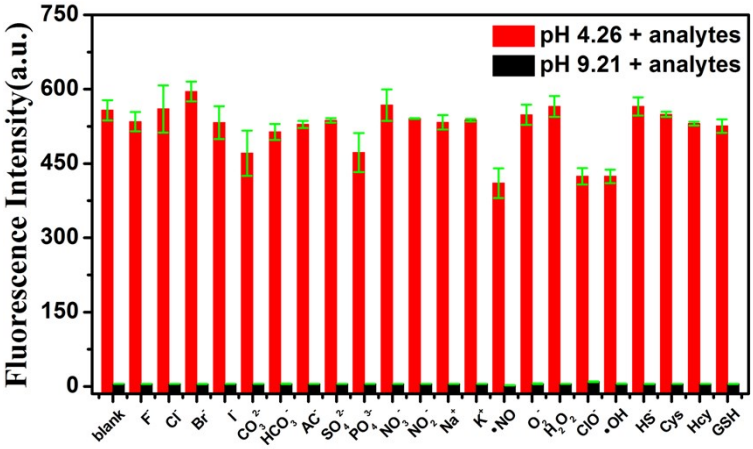


Fig. S20 The fluorescence of **NPCF** in pH 4.26 and 9.21 under different potential interference of interference agents. Conditions: λ_{ex} = 410 nm, λ_{em} = 610 nm.

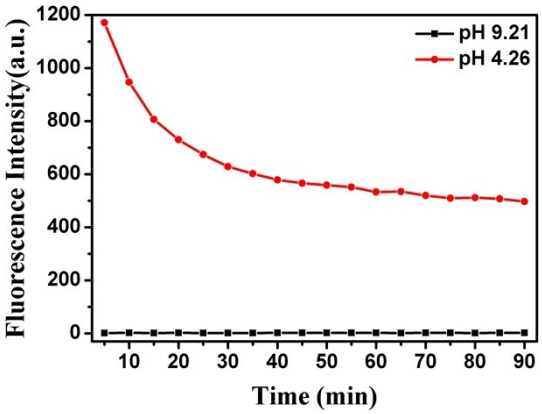


Fig. S21 The time courses of fluorescence intensity of **NPCF** in HEPES buffer solution (10 mM, containing 1 mM CTAB) at different pH values (4.26 and 9.21, respectively). Conditions: λ_{ex} = 410 nm, λ_{em} = 610 nm, slit: 5 nm/5 nm.

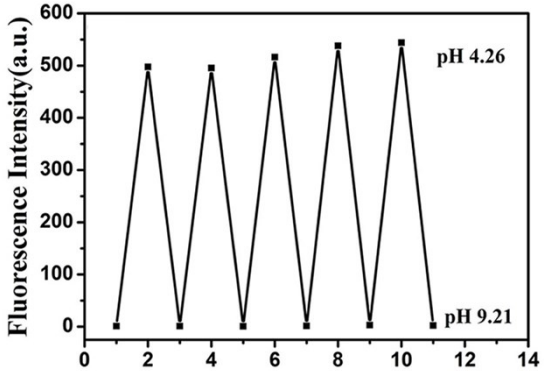


Fig. S22 Reversible fluorescence intensity (λ_{em} = 610 nm) changes of **NPCF** between pH = 4.26 and 9.21 in HEPES buffer solution (10 mM, containing 1 mM CTAB). Conditions: λ_{ex} = 410 nm, λ_{em} = 610 nm.

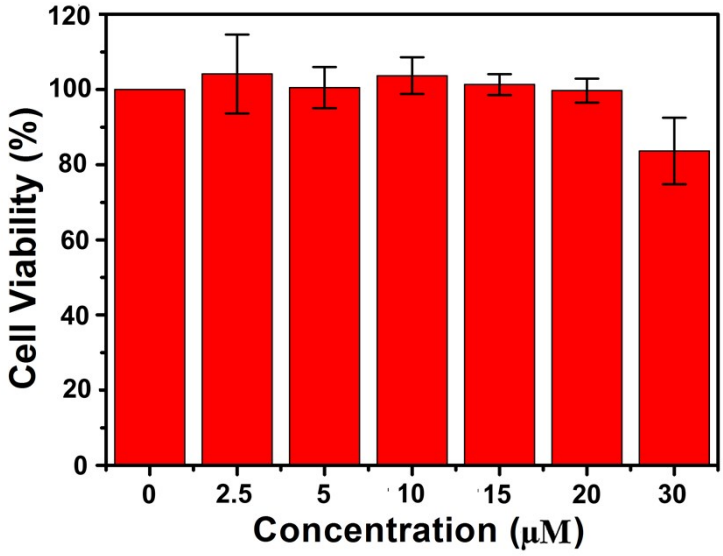


Fig. S23 Different **NPCF** concentrations (0, 2.5 μM, 5 μM, 10 μM, 15 μM, 20 μM and 30 μM) were tested in Hela cells for toxicity.

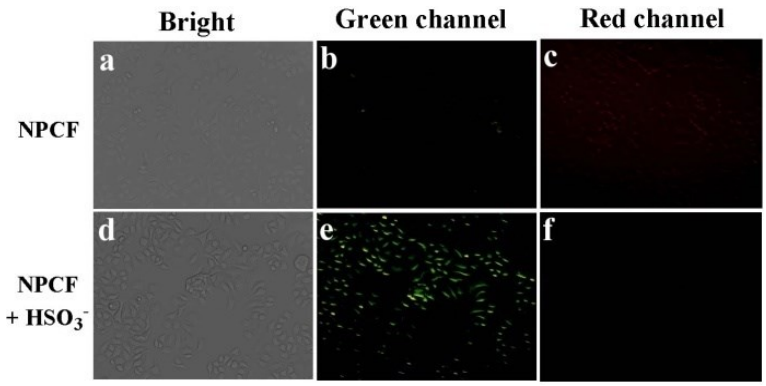


Fig. S24 Fluorescence images of MCF-7 cells. (a, b and c) cells were incubated with **NPCF** (10 μM) for 0.5 h; (d, e and f) images of cells after treatment with probe **NPCF** (10 μM) for 0.5 h and then treatment of the cells with 100 μM of HSO_3^- for 1

h. (a and d) Bright field images; (b and e) green emission of **NPCF**; (c and f) red emission of **NPCF**.

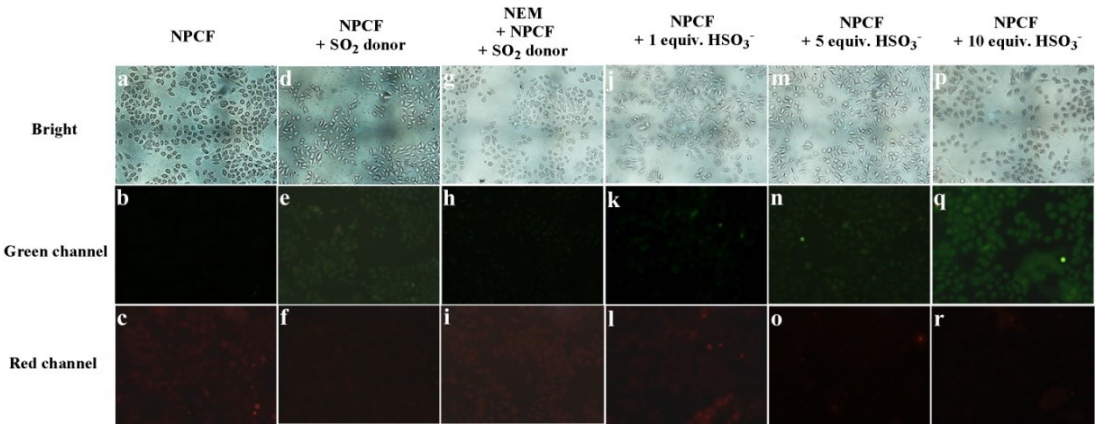


Fig. S25 Fluorescence images of MCF-7 cells. The cells were incubated with **NPCF** (10 μM) for 0.5 h (a, b, and c) and 100 μM of SO_2 donor for 1h (d, e and f). The cells were treated with 1 mM of NEM for 0.5 h, incubated with **NPCF** (10 μM) for 0.5 h, and then treated with 100 μM of SO_2 donor for 1h (g, h and i). MCF-7 cells were incubated with **NPCF** (10 μM) for 0.5 h and treated with several concentrations of HSO_3^- for 1 h (j–r).

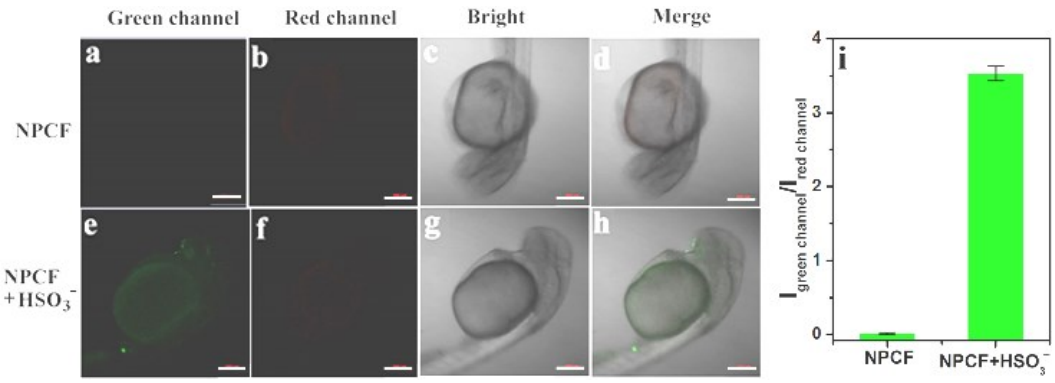


Fig.S26 Confocal fluorescence images of zebrafish using a 10×objective. (a–d) **NPCF** (10 μM) and zebrafish were incubated for 0.5 h. (e–h) **NPCF** (10 μM) and zebrafish were incubated for 0.5 h, and then treated with HSO_3^- (100 μM) for 1 h. (i) Relative pixel intensity of $I_{green\ channel}/I_{red\ channel}$ for detecting HSO_3^-. (a and e) λ_{ex} = 405 nm, λ_{em} = 520 nm–560 nm; (b and f) λ_{ex} = 552 nm, λ_{em} = 630–670 nm. Scale bar is 200 μm.

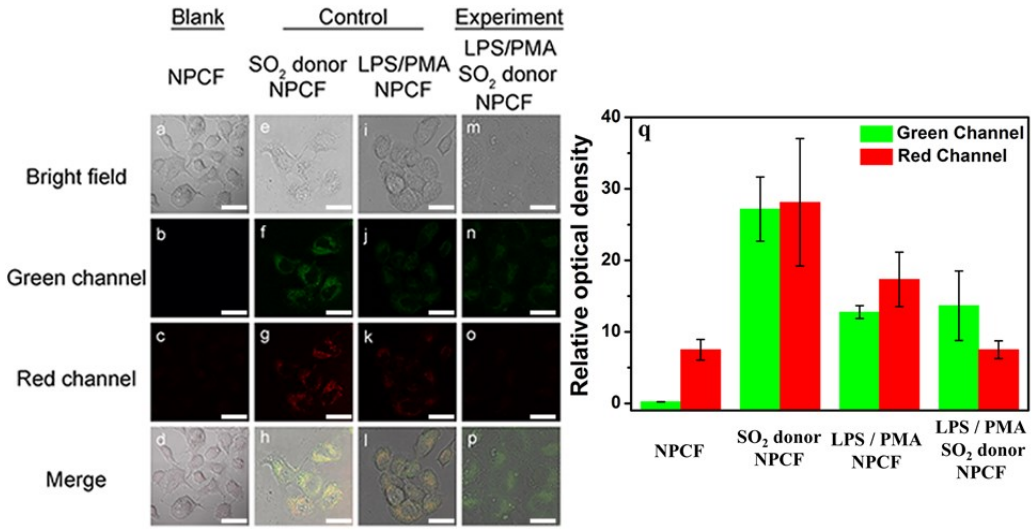


Fig. S27 (a−d) EC1 cells were incubated with **NPCF** (10 μM) for 0.5 h. (e−h) EC1 cells were incubated with SO_2 donor (100 μM) for 1 h, and then incubated with **NPCF** (10 μM) for 0.5 h. (i−l) EC1 cells were incubated with LPS (10 mg/L) for 12 h and PMA (1 μg/mL) for 1 h, and then incubated with **NPCF** (10 μM) for 0.5 h. (m−p) EC1 cells were incubated with LPS (10 mg/L) for 12 h and PMA (1 μg/mL) for 1 h, followed by adding SO_2 donor (100 μM) for 1 h, and finally incubated with **NPCF** (10 μM) for 0.5 h. (q) Relative pixel intensity of green channel (b, f, j and n, λ_{ex} = 405 nm, λ_{em} = 520 nm−560 nm) and red channel (c, g, k and o, λ_{ex} = 552 nm, λ_{em} = 630−670 nm). Scale bar is 25 μm.

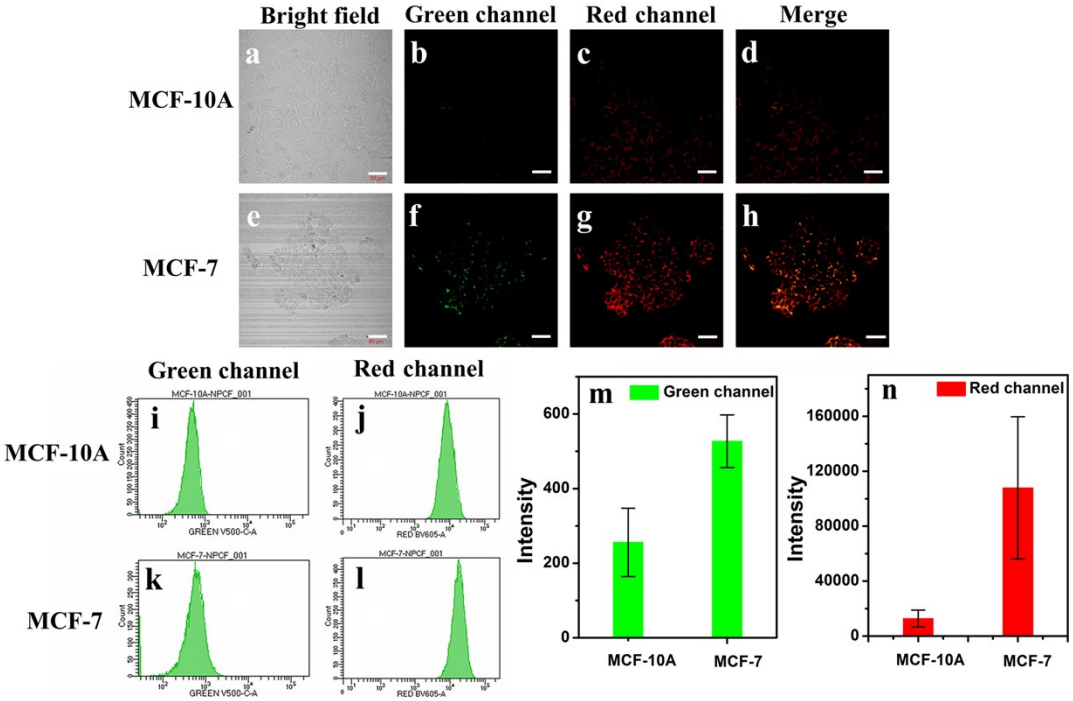


Fig. S28 Confocal fluorescence images of cancer cells vs normal cells. **NPCF** were incubated with MCF-10A cells (a−d) and MCF-7 cells (e−h) for 0.5 h, respectively. Scale bar is 25 μm. (i−l) Flow cytometry analysis of (a−h). (m) The mean intensity of green channel (i and k, λ_{ex} = 405 nm, λ_{em} = 520 nm−560 nm) and (n) red channel (j and l, λ_{ex} = 552 nm, λ_{em} = 630−670 nm) on the flow cytometry analysis.

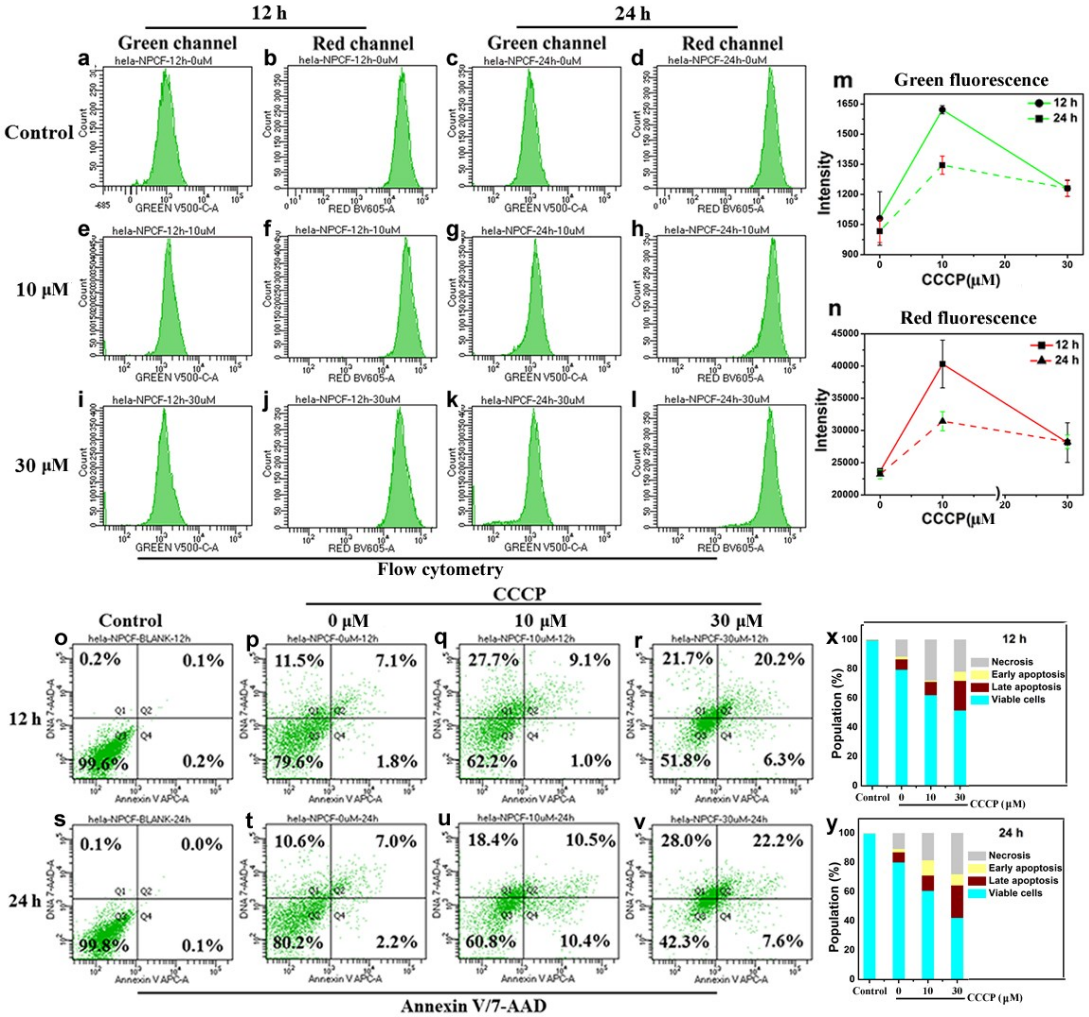


Fig.S29 Experiments of SO_2 and pH changes in CCCP-induced apoptosis. Flow cytometry analysis of (a−n), different concentrations of CCCP (a−d, 0 μM; e−h, 10 μM; i−l, 30 μM) were co-incubated with the cells for 12h (a, b, e, f, i and j) or 24 h (c, d, g, h, k and l), respectively, followed by separately adding **NPCF** (10 μM) and incubating for 0.5 h, and then analyzed by flow cytometry. (m and n) The green channel (a, e, i, c, g and k, λ_{ex} = 405 nm, λ_{em} = 520 nm−560 nm) and red channel (b, f, j, d, h and l, λ_{ex} = 552 nm, λ_{em} = 630−670 nm). (o−v) Annexin V/7-AAD analysis of apoptosis of CCCP at different concentrations (p and t, 0 μM; q and u, 10 μM; r and v, 30 μM) and different culture time (o−r, 12 h; s−v, 24 h). (x−y) The population of necrosis (Q1), early apoptosis (Q4), late apoptosis (Q2), and viable cells (Q3) for 12h (x) or 24h (y) in the Annexin V/7-AAD analysis of apoptosis (o−v).

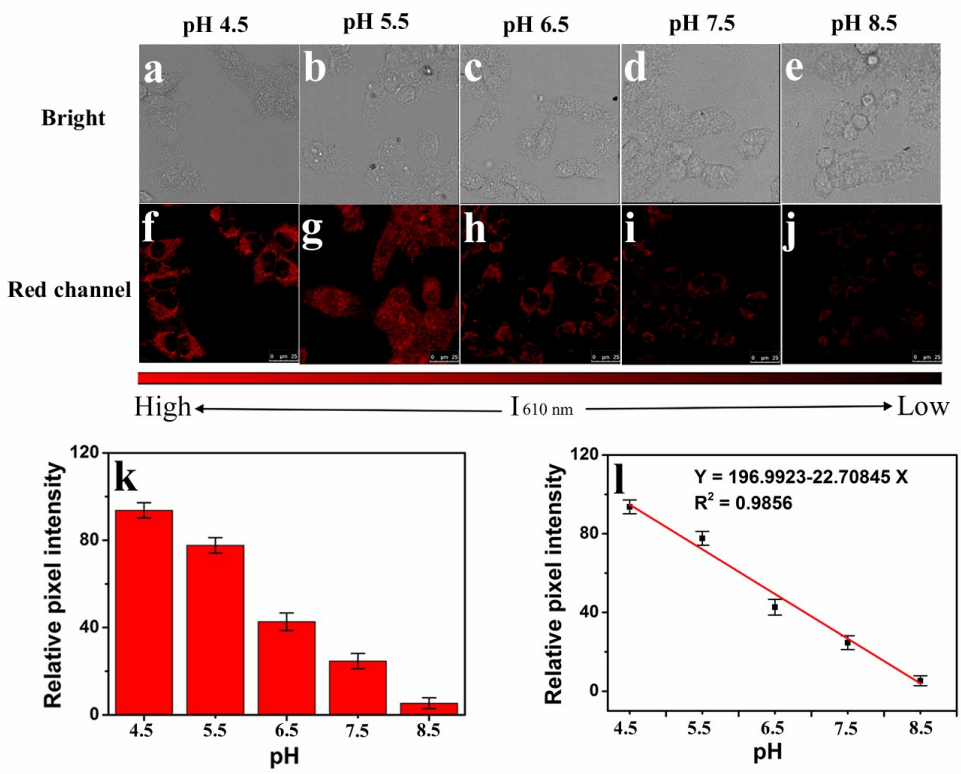


Fig.S30 (a–j) Confocal fluorescence images of HeLa cells and **NPCF** (10 μM) were treated with different pH PBS buffers (pH 4.5, 5.5, 6.5, 7.5 and 8.5) for 0.5 h, respectively. (k) Relative pixel intensity of red channel (λ_{ex} = 552 nm, λ_{em} = 630–670 nm) in different pH PBS buffers. (l) The linear responses in different pH PBS buffers. Scale bar is 25 μm