Electronic Supplementary Information

Monophenyl luminescent material with dual-state emission and pH sensitive for cell imaging

Yuxin Jin,[a] Bingli Jiang,[a*] Huajian Song,[a] Chanming Mei,[a] Zuoan Liu,[b] Xiakai Zhang[a], Jinyuan Liu[a] and Yongyang Gong[b*]

[a]College of Pharmacy, Guilin Medical University, No. 1 Zhiyuan Rd., Lingui District, Guilin 541199, China. Email: jiangbingli@foxmail.com

[b]Guangxi Key Laboratory of Optical and Electronic Materials and Devices, College of Materials Science and Engineering, Guilin University of Technology, No.12 Jian'gan Rd., Qixing District, Guilin 541004, China. Email: yygong@glut.edu.cn

Materials:

The reagents used were all analytically pure solutions. Anhydrous ethanol (ETOH), dichloromethane (DCM), acetonitrile (ACN), trichloromethane (TCM) and N,N-dimethylformamide (DMF) were distilled under normal pressure from calcium hydride under nitrogen immediately prior to use. The final concentration of other ions was 1 mM, and $CuCl_2$、$AgNO_3$、$CaCl_2$、$MgCl_2$、KCl、NaCl、KI、$AlCl_3 \cdot 6H_2O$、$NiCl_2 \cdot 6H_2O$、$FeCl_2 \cdot 4H_2O$、$FeCl_3 \cdot 6H_2O$、$ZnNO_3 \cdot 6H_2O$、$CoCl_2 \cdot 6H_2O$、$CdCl_2$、$HgCl_2$、$CrCl_3$、$PbCl_2$、$BaCl_2 \cdot 2H_2O$ solutions. Other commercially available reagents were used as received without further purification. The raw materials 1,4-cyclohexanedione-2,5-dicarboxylic acid dimethyl ester (DMSS) and 4-(2-aminoethyl)morpholine (AM), were purchased from Adamas Reagent Ltd.

Instrument:

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded in deuterated solvent at room temperature on a Bruker AVANCE 500 NMR spectrometer, and chemical shifts were determined using methylsilane (TMS, δ=0 ppm) as a reference material. Fourier transform infrared (FT-IR) spectroscopy was performed on a Nicolet 6700 FT-IR spectrometer. Fluorescence spectra were measured using a QM-8000 spectrophotometer (HORIBA Group) with a slit width of 3 nm and excitation wavelength selected as 468 nm. UV-visible absorption spectra were measured on an Evolution 20 (Thermo) spectrometer with a slit width of 1 nm.

**Fluorescence quantum yields of the solution samples:**

The equations used for the quantum yields of DMSS-AM in different solvent solutions are as follows. Rhodamine 6G was used as the fluorescence reference substance, and a small amount of rhodamine 6G powder was dissolved in anhydrous ethanol solution with a test tube, and the concentration was repeatedly adjusted to ensure that the absorbance was below 0.05, and the corresponding absorption spectrum was measured. Then the prepared 1×10^{-5} mol/L DMSS-AM master batch was repeatedly diluted to control the absorbance in the range of 0.03, and the absorption spectrum was measured. The best excitation peak positions of both were found, and

then the fluorescence emission spectra were measured separately to calculate the corresponding integrated areas of both, and the corresponding quantum yields were calculated by bringing them into the formula.

$$\emptyset_f = \frac{n_x^2}{n_{std}^2} \cdot \frac{A_{std} \cdot F_x}{A_x \cdot F_{std}} \cdot \emptyset_{fstd}$$

In the formula, $\emptyset_f$ is the fluorescence quantum efficiency; n_x^2 is the refractive index of the solvent used for the sample to be measured, n_{std}^2 is the refractive index of the solvent used for the fluorescent reference substance, A_{std} denotes the absorbance value of the fluorescent reference substance at a certain wavelength, A_x is the absorbance value of the probe DMSS-AM at a certain wavelength, F_x denotes the integrated area of the emission peak of the probe DMSS-AM, F_{std} denotes the integrated area of the emission peak of the fluorescent reference substance, and $\emptyset_{fstd}$ is the known fluorescence quantum yield of the selected reference substance, which can be known from the literature.

Synthesis Methods：
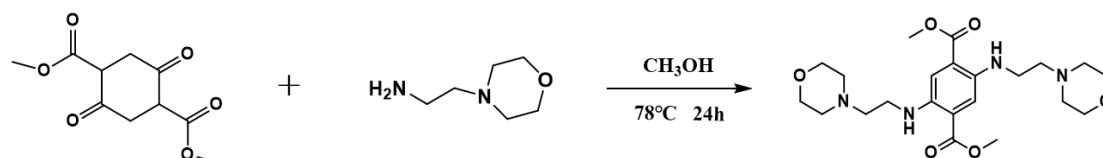
Scheme S1 Synthesis of DMSS-AM

Synthesis of DMSS-AM.   Dimethyl 1,4-cyclohexanedione-2,5-dicarboxylate (DMSS) (2 g, 0.00876 moL), N-(2-aminoethyl)morpholine (AM) (2.3 g, 0.01767 moL) with 35 mL of methanol (AR analytical purity) was added to a two-necked flask, 2-3 drops of acetic acid were added dropwise, a condensing reflux tube was connected, vacuum-filled with nitrogen protection, and the reaction was carried out at 78 °C The reaction was stirred for 24 hours. After cooling to room temperature, the pH was adjusted to neutral with ammonia, the solid was retained by filtration, the mixed solution was extracted with ethyl acetate, and the organic layer was washed with ultrapure water and

saturated salt water, then dried with anhydrous magnesium sulfate, filtered, and the solution was purified by silica gel column chromatography with the ratio of petroleum ether:ethyl acetate = 7:1, V:V, 230 mg (0.511 mmol, 5.8%) of DMSS-AM orange solid was obtained. ^{1}H NMR (500 MHz, Chloroform-d) δ 7.30 (s, 1H), 7.01 (s, 1H), 3.89 (s, 3H), 3.75 (t, J = 4.6 Hz, 4H), 3.26 (t, J = 6.4 Hz, 2H), 2.68 (t, J = 6.3 Hz, 2H), 2.51 (t, J = 4.6 Hz, 4H). ^{13}C NMR (126 MHz, Chloroform-d) δ 168.09, 141.12, 117.02, 114.41, 67.05, 57.22, 53.50, 51.86, 40.51. TOF MS: m/z calcd for $C_{22}H_{34}N_4O_6$ $[M+H]^+$: 450.25, found: 450.34.

pH response:

Different pH Britton-Robinson (BR) buffer solutions (40 mM) were prepared using boric acid, phosphoric acid, acetic acid and sodium hydroxide as the acid-base system. 100 μL of DMSS-AM solution with a concentration of 1×10^{-4} mol/L was added to 5 mL of pH 1-14 solution respectively, and after shaking well, the fluorescence spectra of the solutions to be measured were tested.

Probe reversibility: The concentration of 1×10^{-3} mol/L DMSS-AM was fixed into a 200 mL volumetric flask to obtain 1×10^{-4} mol/L DMSS-AM solution. The pH was adjusted using hydrochloric acid and aqueous sodium hydroxide solution. The pH was first adjusted to pH=4.0 with hydrochloric acid solution, and the fluorescence emission spectra were detected, and then the pH was adjusted to pH=7.4 with sodium hydroxide solution. the above experimental procedure was repeated six times.

Selectivity and metal ion anti-interference property: Add 500 μL of 1×10^{-4} mol/L DMSS-AM to different pH (pH=4, pH=7.4) buffer solutions, then add 50 μL of 100 mM different metal ion mother liquor to fix the volume into a 5 mL volumetric flask, shake well and let it stand, and measure the fluorescence emission spectra at room temperature.

Cell Culture:

The cells used in the experiments were divided into normal cells and cancer cells. Cervical cancer cells (HeLa) and rat cardiomyocytes (H9C2) were cultured as follows: DMEM was used as culture medium, 10% (v/v) fetal bovine serum (FBS) and 1% double antibodies (penicillin and streptomycin) were added, placed in 25 cm^2 tissue

culture flasks in 5% CO_2 humidity environment for about 24 hours, and when the cells were well adhered to the wall morphology and the cell density When the cells reached 1×10^6 cells/mL, the cells were digested with trypsin and planted in 96-well plates or 6-well plates at a cell density of 1×10^4 cells/mL, and the culture was continued to adhere to the wall. Continue to incubate for about 24 hours, aspirate the spent medium and wash the cells with phosphate buffer solution (PBS) 2-3 times for subsequent experiments.

MTT method:

HeLa cells were digested down with trypsin, counted by counting plates, and seeded in 96-well plates at 1×10^4 cells/mL, with PBS buffer added around the well plates, and the rest were set as blank group (without cells), control group (with cells), and 8 groups with different gradients of drug concentrations (with cells), incubated in a 5% CO_2 incubator at 37 °C for 24 h. Spent medium was aspirated, except for the blank group and control group with complete medium. The blank and control groups were added with complete medium, and the rest were added to the well plates according to different drug concentrations (15, 20, 25, 30, 35, 40, 45, 50 μM). 24 h later, 10 μL of MTT solution at a concentration of 5.0 mg/mL was added to each well, and incubation was continued for 4 h. After the end, the spent medium containing MTT was aspirated and 150 μL of DMSO solution was added to each well, placed on a shaker for 10 min (100 r/min), and the absorbance (OD) was measured at 490 nm with an enzyme marker.

$$Cell\ Viability\ (\%) = \frac{OD_{Experimental} - OD_{Blank}}{OD_{contrast} - OD_{Blank}} \times 100\%$$

Cell co-localization imaging:

Since the emission range of DMSS-AM and the commercial dye LysoTracker-Red-DND-99 are close to each other, a set of control experiments were performed beforehand, with the green fluorescence signal coming only from the probe DMSS-AM (500 nm-540 nm) and the red fluorescence signal coming only from the dye LTR (570 nm-670 nm). to ensure that the fluorescence signals received from both do not interfere

with each other. According to the above control conditions, co-localization experiments were then performed.

Seed the appropriate amount of cells on a coverslip in a six-well plate, mix the concentration of 20 μM DMSS-AM with 2 mL of complete medium, co-incubate the cells at 37 °C for 1 hour, at the end of the incubation aspirate the spent medium, rinse the cells with PBS buffer (2 mL) for 2-3 times, and then add the commercial dye LysoTracker-Red-DND-99 (70 nM) Incubation was continued for 50 min, at the end of which the cells were continued to be rinsed 2-3 times with PBS, and finally the cells were fixed by adding 300 μL of 4% paraformaldehyde fixative for 15 min, rinsed 3 times with PBS, sealed with anti-fluorescence attenuating sealer, and imaged on the fixed cells with a laser confocal microscope. DMSS-AM was excited at 488 nm, and the emission spectra were collected from 500-540 nm, showing a LysoTracker-Red-DND99 was excited at 561 nm and emission spectra were collected at 570-670 nm, showing a red channel.

Lysosomal pH dynamic visualization imaging:

Appropriate amount of cells were seeded on coverslips adhered in six-well plates, the concentration of 20 μM of DMSS-AM was mixed with 2 mL of complete medium, and the cells were co-incubated at 37 °C for 1 h. At the end of the incubation, the spent medium was aspirated, and the cells were rinsed with PBS buffer (2 mL) for 2-3 times, and 100 μM of Chloroquine and 10 mM of Ammonium Chloride were added to incubate them for 1 h. At the end of the incubation, cells were fixed with 300 μL of 4% paraformaldehyde fixative for 15 min, rinsed with PBS for 3 times, and sealed with anti-fluorescence attenuation sealer. Continue to rinse 2-3 times, finally add 300 μL 4% paraformaldehyde fixative to fix the cells for 15 minutes, rinse 3 times with PBS, seal with anti-fluorescence attenuating sealer, and image the fixed cells with laser confocal microscope.
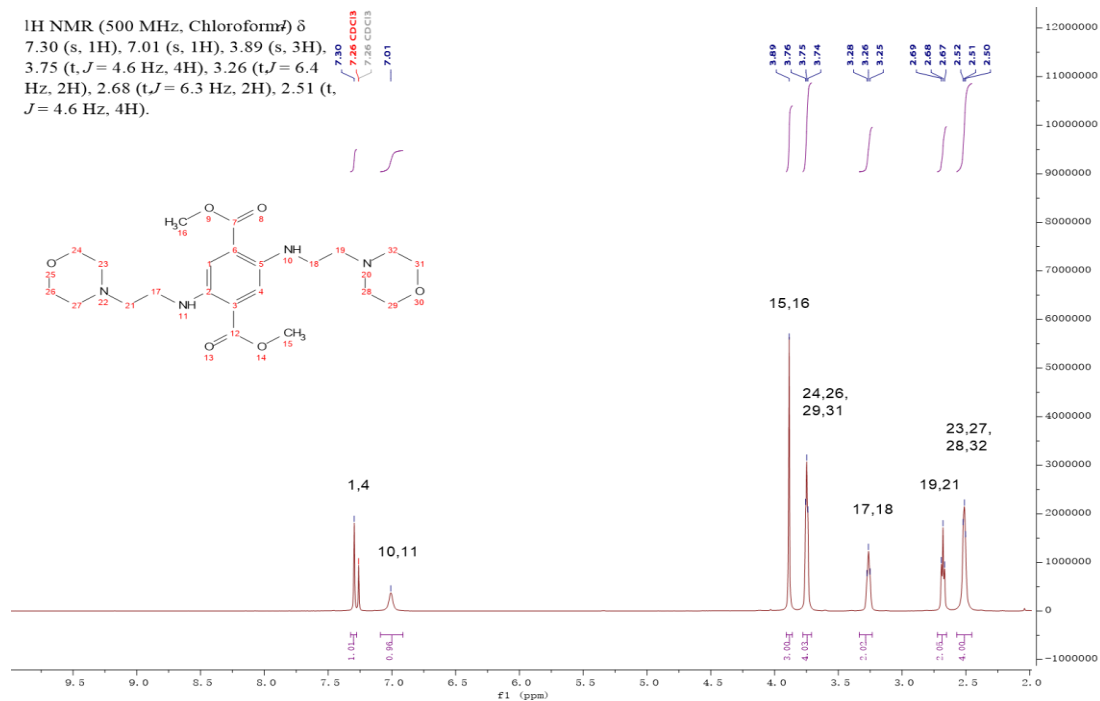
Computational Methods

All ground state optimizations have been carried out at the Density Functional Theory (DFT) level with Gaussian 16 B.01 using the B3LYP functional and the 6-311+G(d,p) basis set. Vibrational frequency calculations were performed to ensure that the

optimized geometries represented the local minima. Excited state calculations have been performed at time-dependent DFT (TD-DFT) using the same functional and basis set as for ground state geometry optimization. All above calculations were carried out with the Gaussian 16 B.01 package[1]. Gaussian calculation results are analyzed by Multiwfn 3.8[2] and VMD 1.9.3 software[3].
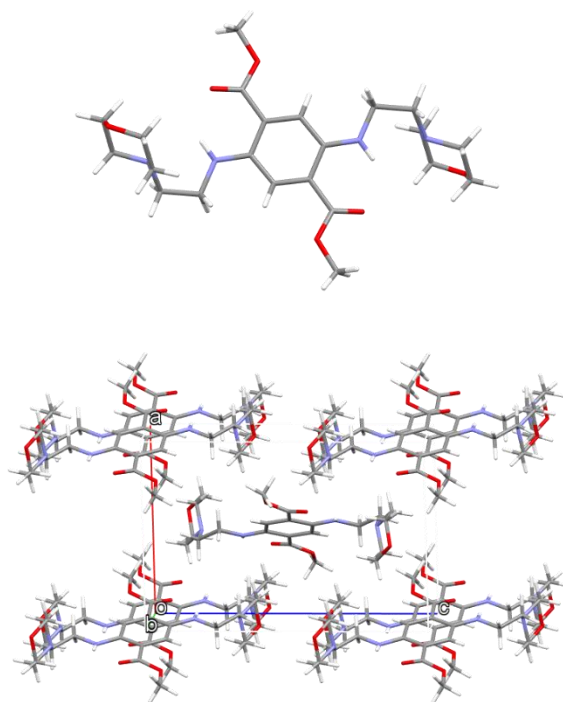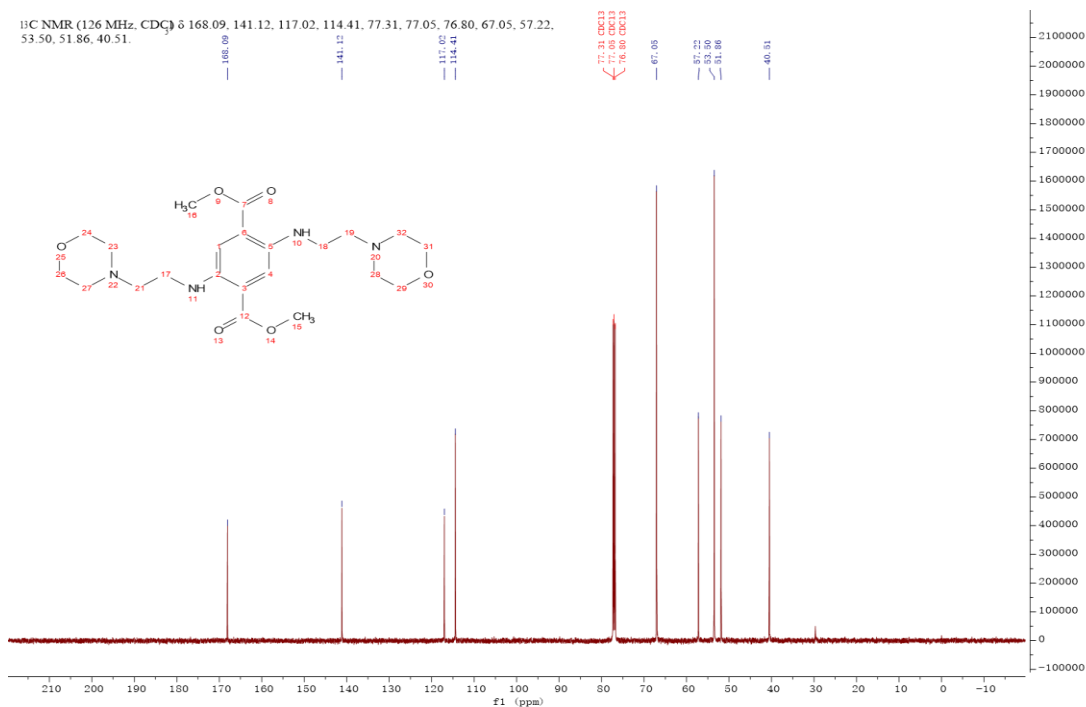
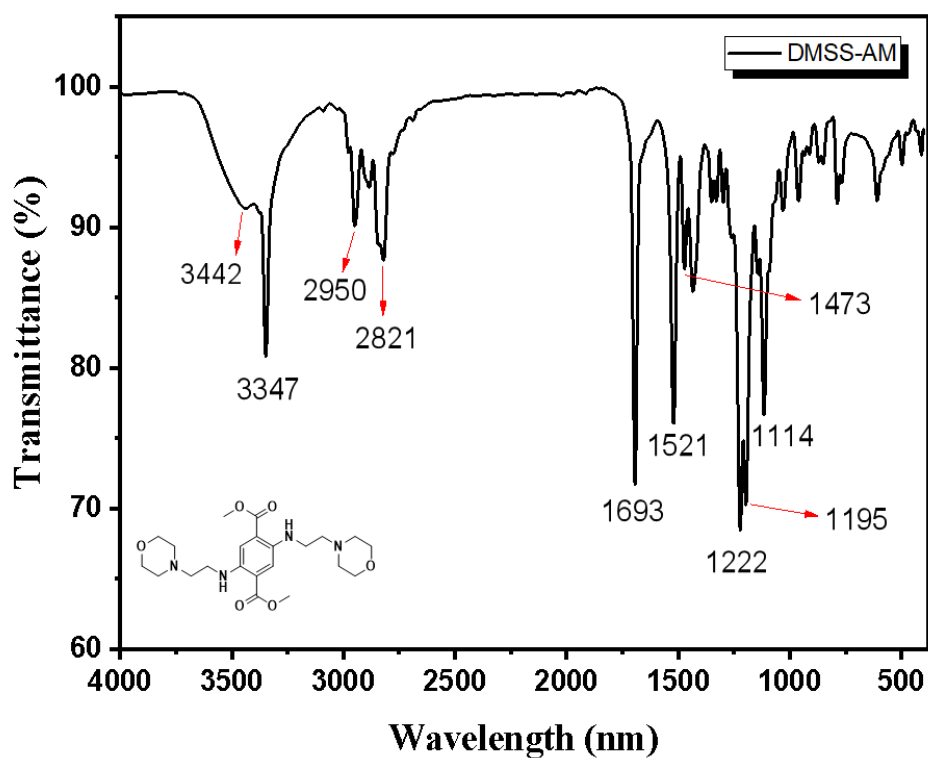**Figure S1** Single crystal structure of DMSS-AM.

1H NMR (500 MHz, Chloroform-*d*) δ 7.30 (s, 1H), 7.01 (s, 1H), 3.89 (s, 3H), 3.75 (t, *J* = 4.6 Hz, 4H), 3.26 (t *J* = 6.4 Hz, 2H), 2.68 (t *J* = 6.3 Hz, 2H), 2.51 (t, *J* = 4.6 Hz, 4H).

Figure S2 ¹H NMR spectrum of DMSS-AM.

Figure S3 ^{13}C NMR spectrum of DMSS-AM.

Figure S4. FTIR spectra of DMSS-AM
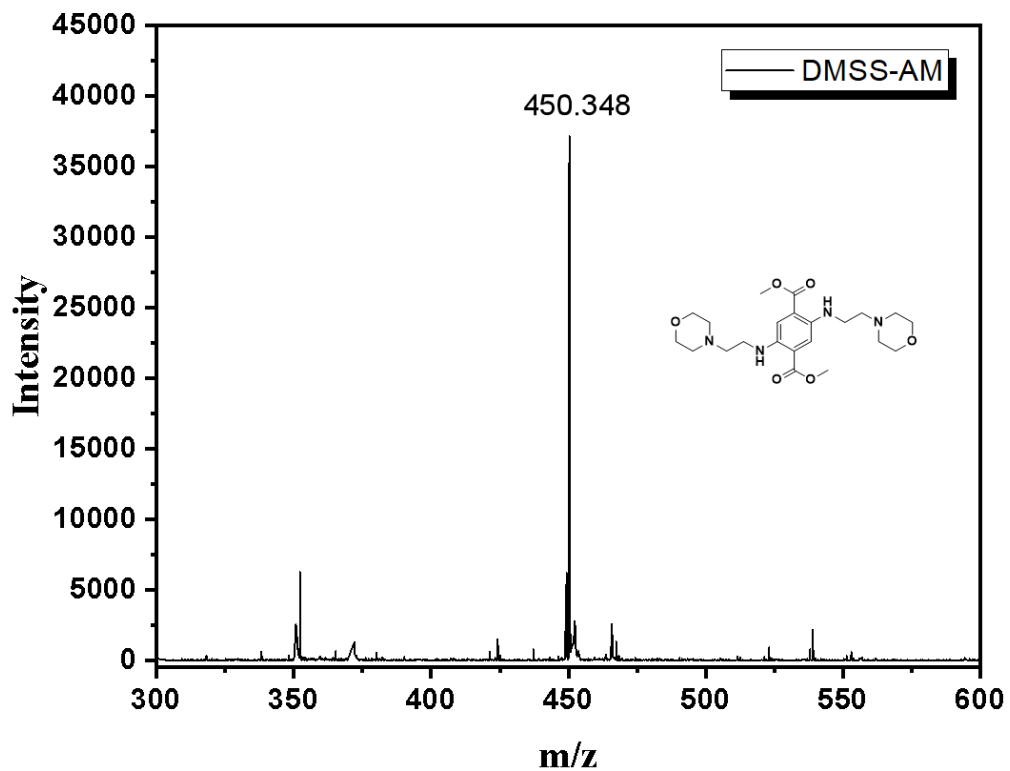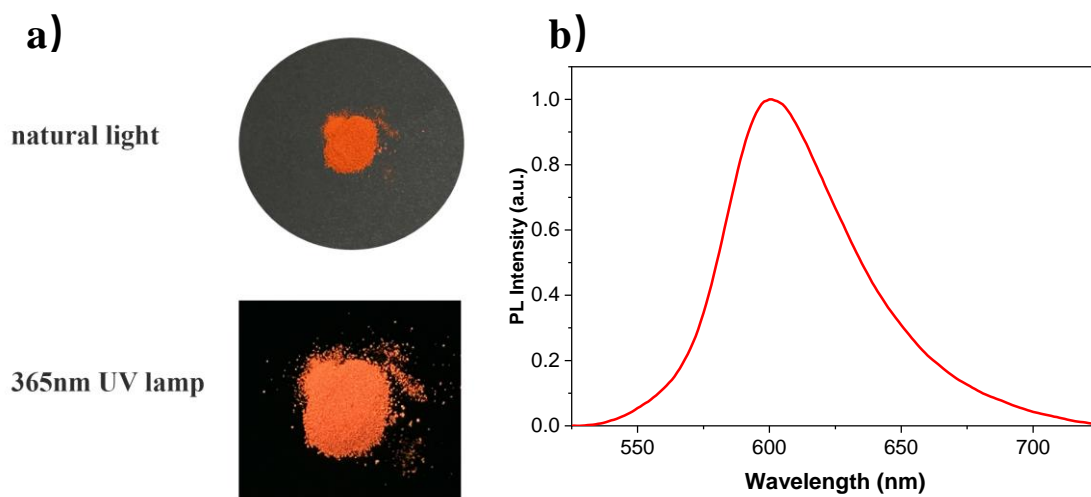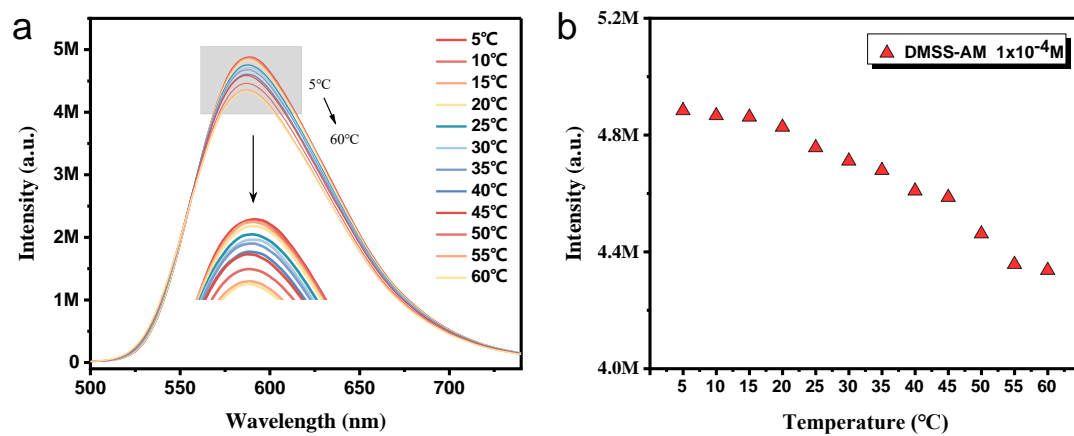
Figure S5 DMSS-AM TOFMS mass spectrometry
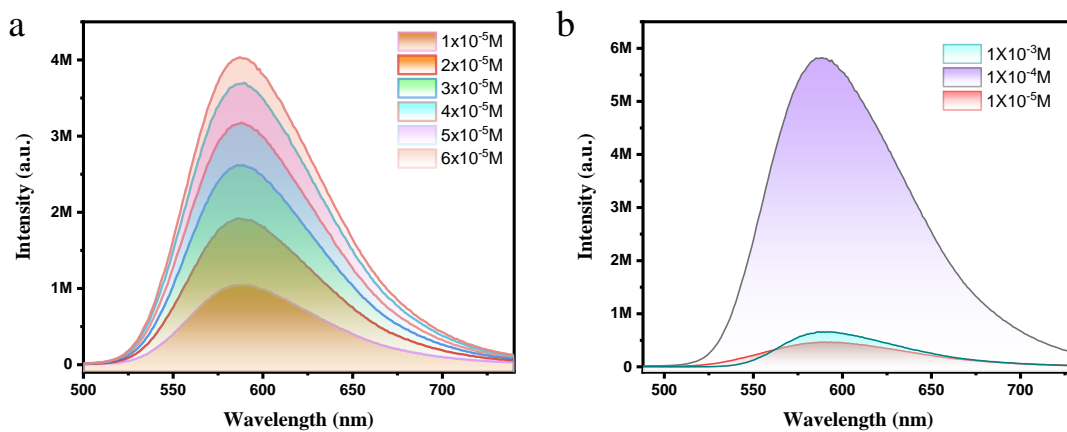
Figure S6 (a) DMSS-AM fluorescence spectra of the same order of magnitude with different concentrations; (b) fluorescence spectra of different orders of magnitude with different concentrations

Figure S7 Photomicrographs (a) and emission (b) spectra of DMSS-AM solid samples under natural light and 365 nm UV excitation.

Figure S8 (a) Fluorescence spectra of DMSS-AM at different temperatures, (b) Trend of fluorescence intensity as a function of temperature
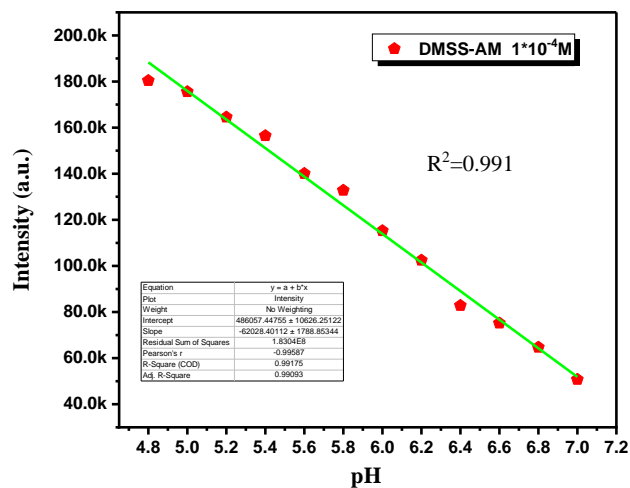
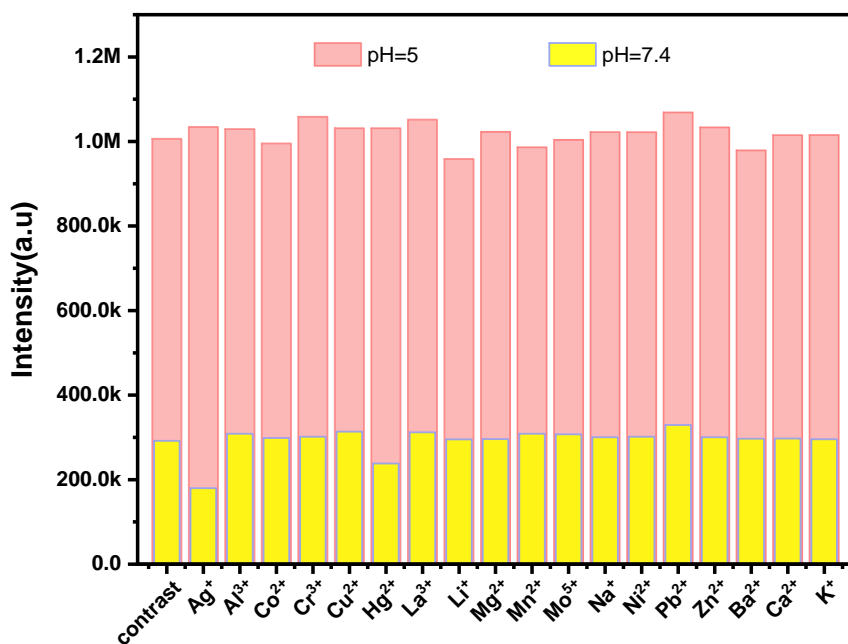**Figure S9**. Linear fit of DMSS-AM to fluorescence intensity in the pH range 4.8-7.0
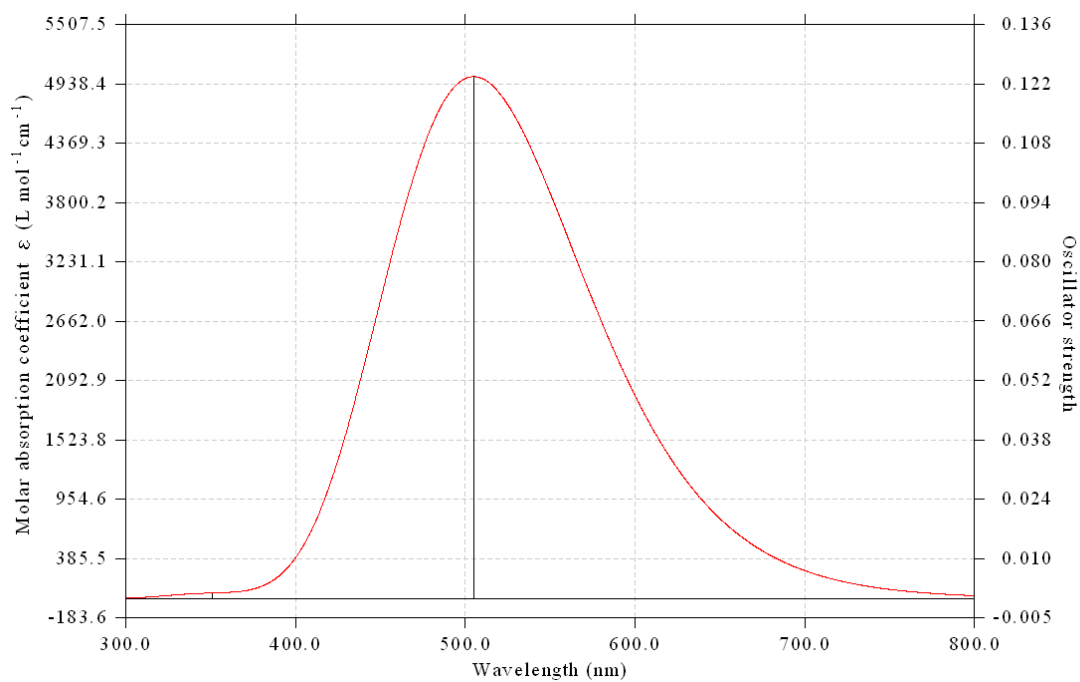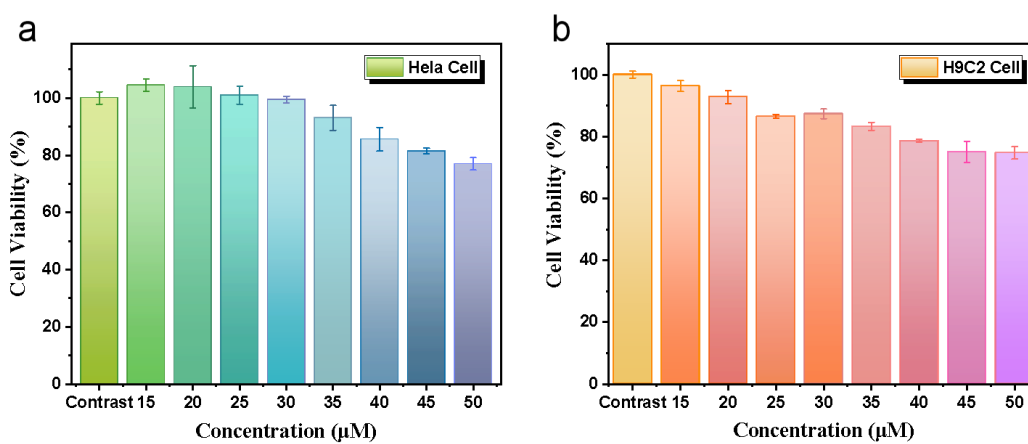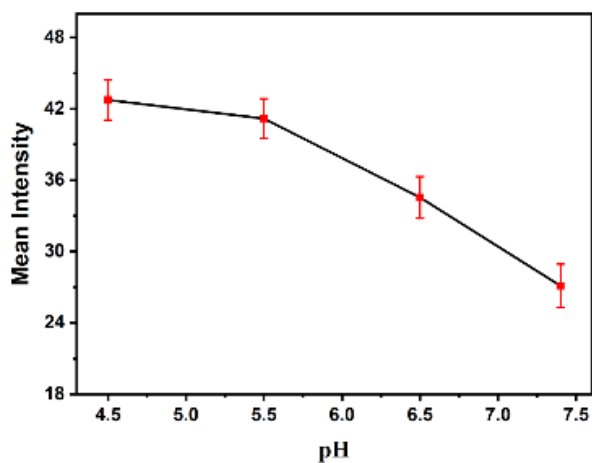

Figure S10 Fluorescence intensity of probe DMSS-AM (10 μM) in buffer solution pH 5.0, pH 7.4 with the addition of different metal ions (1 mM)
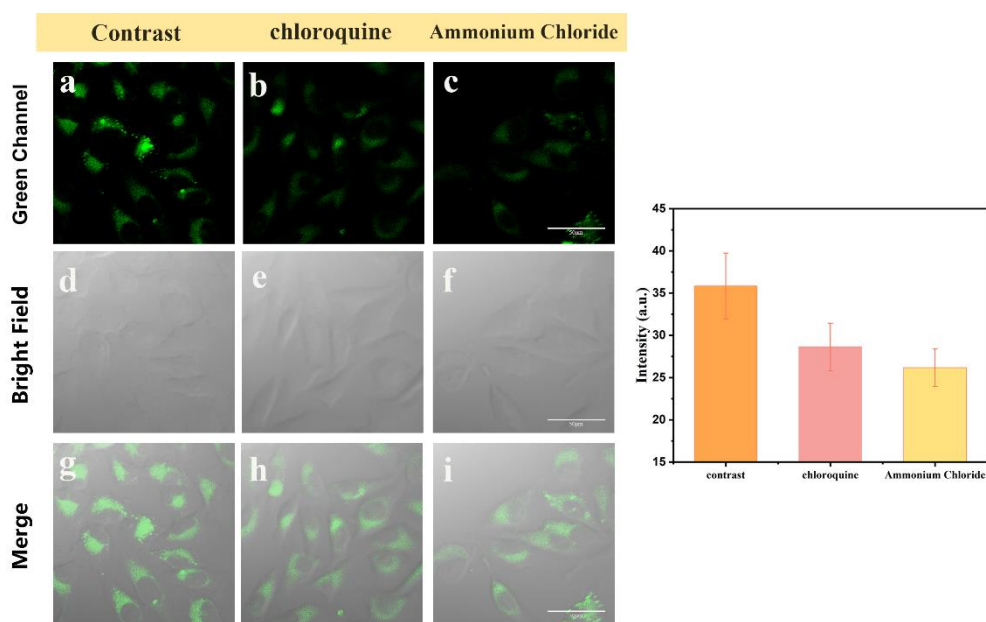
Figure S11 Calculation of the absorption spectrum and oscillator strength of DMSS-AM in water solution using the B3LYP-GD3BJ/6-31G(d) method.

Figure S12 Effects of different concentrations of DMSS-AM on the survival of HeLa and H9C2 cells.

Figure S13 Relative fluorescence intensity of the DMSS-AM probe in CLSM images at different pH.

Figure S14 CLSM images of HeLa cells stained with Chloroquine and Ammonium Chloride. Inset: Relative fluorescence intensity of DMSS-AM in Hela cells treated with Chloroquine and Ammonium Chloride. Scale bar: 50 μm

References

1 M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, G. A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A. V. Marenich, J. Bloino, B. G. Janesko, R. Gomperts, B. Mennucci, H. P. Hratchian, J. V. Ortiz, A. F. Izmaylov, J. L. Sonnenberg, Williams, F. Ding, F. Lipparini, F. Egidi, J. Goings, B. Peng, A. Petrone, T.

Henderson, D. Ranasinghe, V. G. Zakrzewski, J. Gao, N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, K. Throssell, J. A. Montgomery Jr., J. E. Peralta, F. Ogliaro, M. J. Bearpark, J. J. Heyd, E. N. Brothers, K. N. Kudin, V. N. Staroverov, T. A. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. P. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, J. M. Millam, M. Klene, C. Adamo, R. Cammi, J. W. Ochterski, R. L. Martin, K. Morokuma, O. Farkas, J. B. Foresman and D. J. Fox, Gaussian 2016.

2 T. Lu and F. Chen, *J. Comput. Chem.*, 2012, 33, 580.

3 W. Humphrey, A. Dalke and K. Schulten, *J. Mol. Graphics*, 1996, 14, 33.