Supporting Information for

A novel high selective fluorescent probe for imaging of cysteine both in living cells and zebrafish

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Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments; Mass spectrometric analyses were measured on a Finnigan MAT 95 XP spectrometer; High resolution mass spectrometric (HRMS) analyses were measured on an Agilent 1100 HPLC/MSD spectrometer; NMR spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a Shimadzu UV-2700 power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer with a 1 cm standard quartz cell; The fluorescence imaging of cells was performed with a Nikon A1MP confocal microscope; The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals. All zebrafish purchased from Nanjing EzeRinka Biotechnology Co., Ltd. All procedures for this study were approved by the Animal Ethical Experimentation Committee of Shandong University according to the requirements of the National Act on the use of experimental animals (China). We used 100 × objective lens for the imaging experiments. Cells imaging were obtained by Nikon A1MP confocal microscopy. The Mai Tai DeepSee laser offers 2.5 W of average power and 350 nm (690-1040 nm) in useable tuning range. The pulse width is 100fs (800nm) and the pulse frequency of two-photon illumination is 80MHz. Scan resolution ≥ 4096×4096, gray level ≥ 12 bit. Scanning speed: 20 s/ scan. Living cells fresh were cultivated using fresh medium..

Culture and preparation of HeLa cells

HeLa cells were cultured in DMEM (Dulbecco’s modified Eagle's medium) supplemented with 10 % FBS (fetal bovine serum) in an atmosphere of 5 % CO_2 and 95 % air at 37 ℃. Before the experiments, seed the HeLa or A549 cells in 35 mm glass-bottomed dishes at a density of 2×10^5 cells per dish in 2 mL of culture medium and incubate them inside an incubator containing 5 % CO_2 and 95 % air at 37 ℃. Incubating the cells for 24 h. Cells will attach to the glass surface during this time.

Cytotoxicity assay

In vitro cytotoxicity was measured using the colorimetric methyl thiazolyl tetrazolium (MTT) assay on HeLa cells. Cells were seeded into the 24-well tissue culture plate in the presence of 500 μL Dulbecco's modifed eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin at 37 ℃ and 5 % CO_2 atmosphere for overnight and then incubated for 24 h in the presence of **PI-Cys** at different concentrations (0 μM, 5 μM, 10 μM, 20 μM, 30 μM, 50 μM). Then cells were washed with PBS buffer and 500 μL supplemented DMEM medium was added. Subsequently, 50 μL MTT (5 mg/ mL) was added to each well and incubated for 4 h. Violet formazan was dissolved in 500 μL sodium dodecyl sulfate solution in the water-DMF mixture. Absorbance of the solution was measured at 570 nm using a microplate reader. The cell viability was determined by assuming 100 % cell viability for cells without **PI-Cys**.

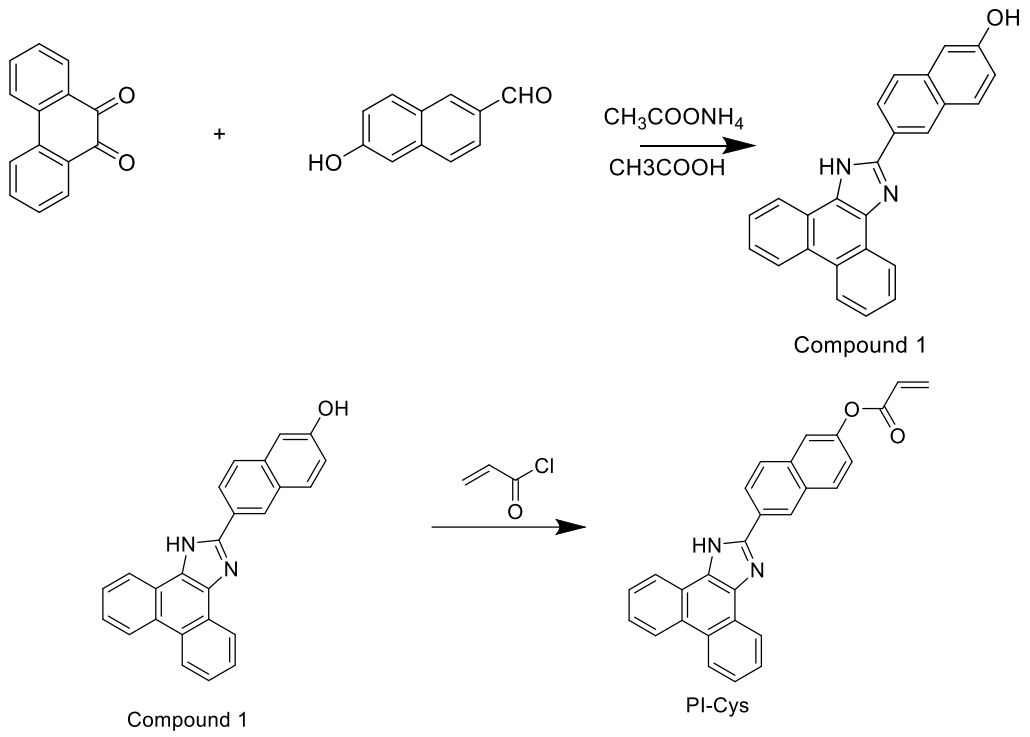
Imaging of Cys in living cells

Before the experiments, the HeLa cells were seeded on two 35-mm glass-bottomed dishes and allowed to adhere for 24 h. the cells were washed with PBS (pH=7.4) buffer three times. Subsequently, the first group was incubating with probe **PI-Cys** (10 μM) (containing 0.1 % DMSO as a cosolvent) for 30 min at 37 ℃. The HeLa cells were rinsed with PBS three times.

The second group was pretreating with 1 mM N-ethylmaleimide (NEM) for 30 min and then incubated with 10 μM **PI-Cys** (containing 0.1 % DMSO as a cosolvent) for 30 min at 37 ℃. The HeLa cells were rinsed with PBS three times.

The third and fourth groups were pretreating with 1 mM NEM for 30 min before incubated with 10 μM **PI-Cys** (containing 0.1 % DMSO as a cosolvent) for 30 min, and then add 200 μM cysteine or GSH for another 30 min. The HeLa cells were rinsed with PBS three times, and the fluorescence images were acquired through a Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera

Synthesis



Scheme 1 Synthesis of the probe ***PI-Cys***

Synthesis of compound PI-Cys

9,10-phenanthrenequinone (146 mg, 0.7 mM, 1 eq), 6-hydroxybenzaldehyde (362 mg, 2.1 mM, 3 eq), ammonium acetate (185 mg, 1.05 mM, 1.5 eq) were dissolved in 20 mL acetic acid, the reaction was heated at 110 °C for 10 h. The reaction was detected by TCL plate. After the reaction was completed, it was cooled to room temperature, poured into water and filtered, and the obtained crude product was separated by silica gel column. The size of silica gel was 200-300 mesh, and the eluent was ethyl acetate/petroleum ether=1: 2, spin dry the eluent to obtain compound 1

Compound 1 (169 mg, 0.47 mM, 1 eq) was dissolved in 5 mL of dichloromethane, 2 drops of triethylamine was added, and acryloyl chloride (72 mg, 0.7 mM, 1.5 eq) was added dropwise at 0 °C, and reacted at room temperature for 5 h. The reaction was detected by TCL plate. After the reaction was completed, the solvent was evaporated to dryness to give a crude product, which was then separated on silica gel column. The size of the silica gel was 200-300 mesh, and the eluent ratio was ethyl acetate/petroleum ether=1. :5, spin dry the eluent to obtain a fluorescent probe **PI-Cys** (90 mg, yield: 46%) ^{1}H NMR (400 MHz, DMSO)

δ 13.73 (s, 1H), 8.89 (dd, J = 13.5, 7.8 Hz, 3H), 8.65 (dd, J = 7.6, 4.9 Hz, 2H), 8.57 – 8.48 (m, 1H), 8.17 (dd, J = 15.9, 8.8 Hz, 2H), 7.86 (d, J = 1.9 Hz, 1H), 7.83 – 7.73 (m, 2H), 7.67 (dd, J = 14.5, 7.2 Hz, 2H), 7.49 (dd, J = 8.8, 2.2 Hz, 1H), 6.68 – 6.60 (m, 1H), 6.52 (dd, J = 17.3, 10.1 Hz, 1H), 6.24 (d, J = 10.2 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 164.80, 149.42, 149.10, 137.68, 134.33, 134.04, 131.52, 130.45, 128.76, 128.41, 128.36, 128.29, 128.14, 128.09, 127.69, 127.59, 127.47, 125.94, 125.71, 125.41, 125.26, 124.61, 124.25, 122.92, 122.52, 122.42, 119.21.

HRMS (EI) *m/z* calculated for $C_{28}H_{18}N_2O_2$: 415.1441(M+H) Found: 415.1446 (M+H).

Kinetic studies

The rate constant was determined from the fluorescence titration data based on a reported method. The pseudo-first-order rate constant for the reaction was determined by fitting the fluorescence intensities of the samples to the pseudo-first-order equation:

$$\mathrm{Ln}[(F_{max} - F_t) / F_{max}] = - k't.$$

Where Ft and Fmax are the fluorescence intensities at 414 nm at time t and the maximum value obtained after the reaction was complete. k' is the pseudo-first order rate constant.

Calculation of fluorescence quantum yield

Next，we further evaluated the fluorescence quantum yields of **PI-Cys** by the following Eq.:

$$\Phi_s = \Phi_r \left(\frac{A_r(\lambda_r)}{A_s(\lambda_s)}\right)\left(\frac{n_s^2}{n_r^2}\right)\frac{F_s}{F_r}$$

s and r were sample and the reference, respectively; Φ and F stand for quantum yield and integrated emission intensity, respectively. A and n stand for absorbance and refractive index, respectively.

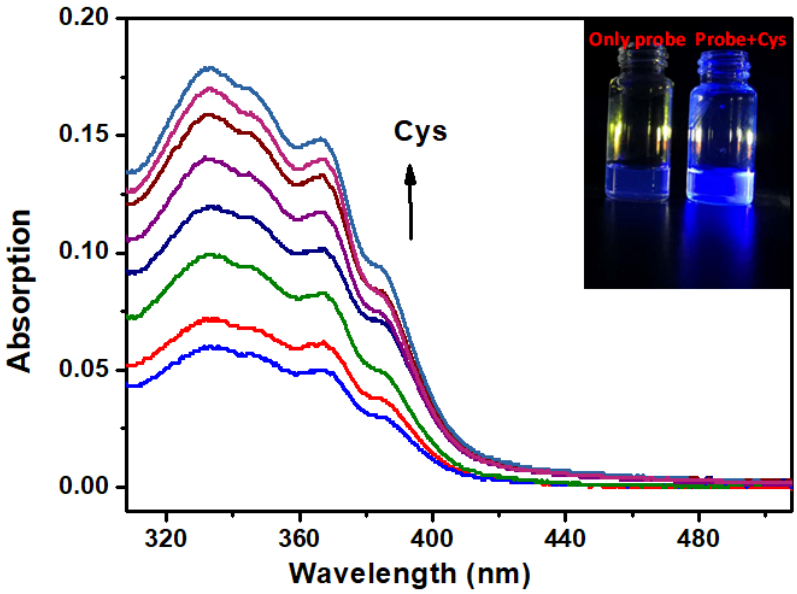


Figure S1 The absorption spectral changes of probe **PI-Cys** (10 μM) upon addition of increasing concentrations of Cys (0-200 μM) in PBS(Phosphate Buffered Saline) buffer containing 10% DMF as a cosolvent. Inset: Photographs showing the color of the probe **PI-Cys** (0.5 m M) before and after addition of Cys (1 m M) to the solution in UV light.

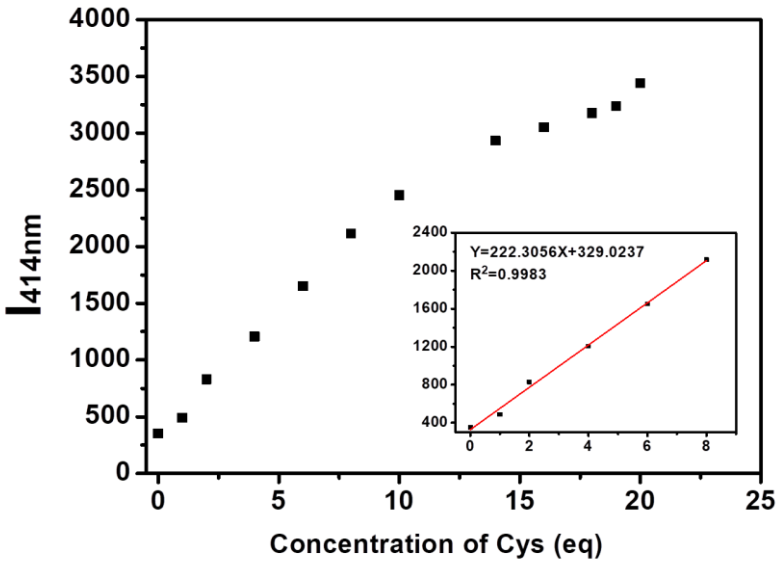


Figure S2 The linear relationship between the fluorescence intensity ratio (I414 nm) and the concentration of Cys (eq). Inset: the limit of detection (LOD) of **PI-Cys** towards Cys (excitation at 350 nm, voltage at 400V).

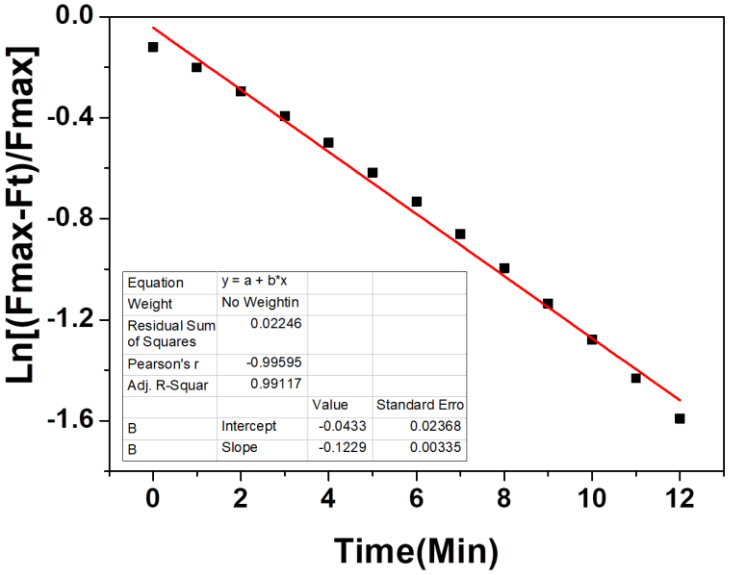


Figure S3 Pseudo first-order kinetic plot of the reaction of **PI-Cys** in the presence of Cys at fluorescence intensity 414 nm.

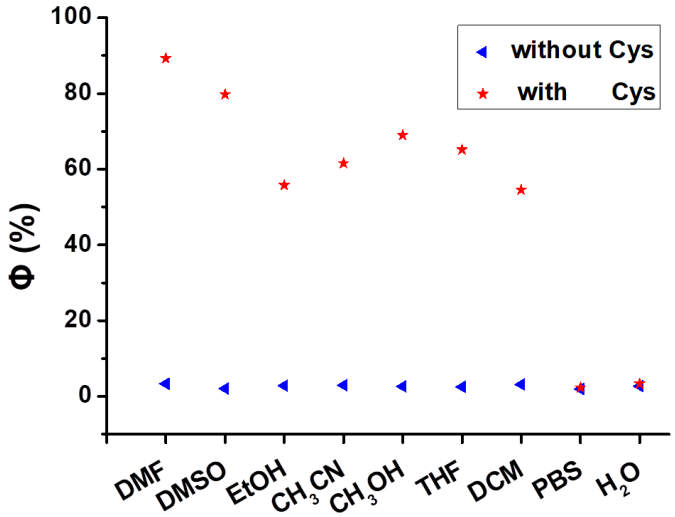


Figure S4 Fluorescence quantum yields of **PI-Cys** (10 μM) in various solutions. Error limit: 20%

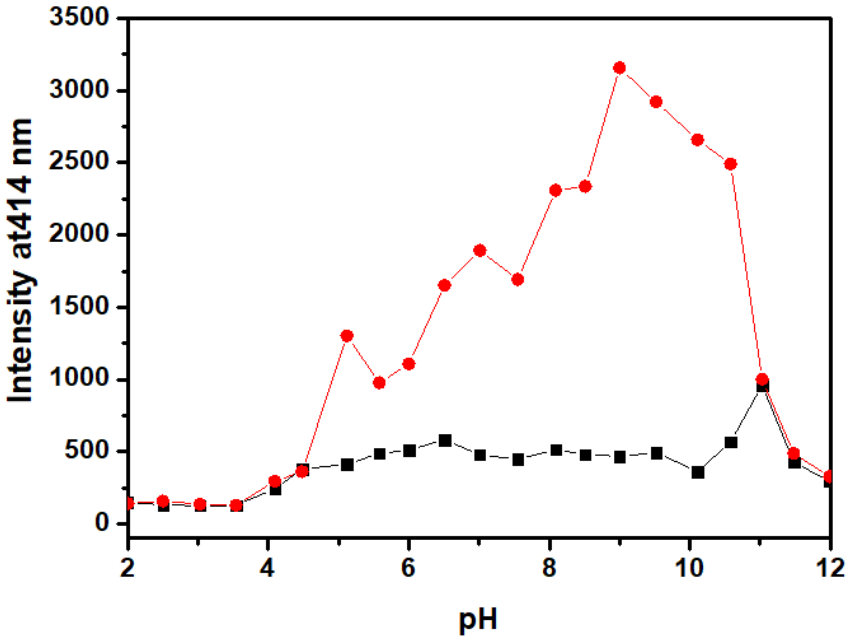


Figure S5 The fluorescence spectra changes of probe **PI-Cys** (10 μM) before and after addition of Cys (200 μM) in PBS buffer with different pH values, containing 10 % DMF as a cosolvent (excitation at 350 nm, voltage at 400V).

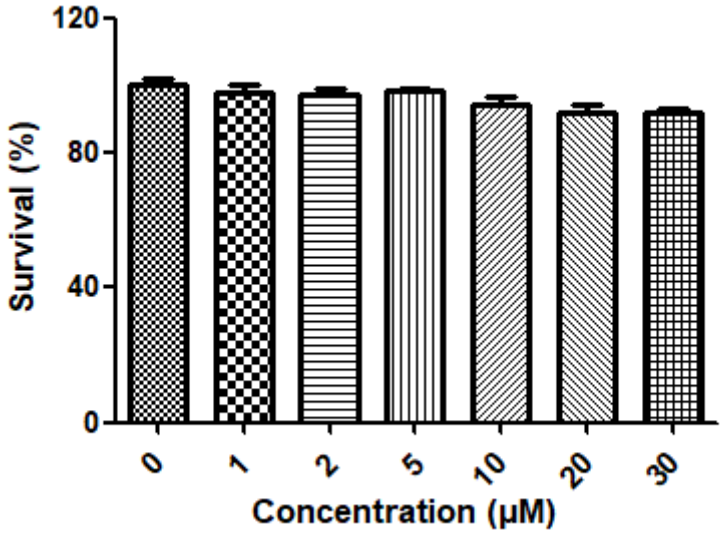


Figure S6 Cytotoxicity assays of **PI-Cys** at different concentrations for HeLa cells in 24h.

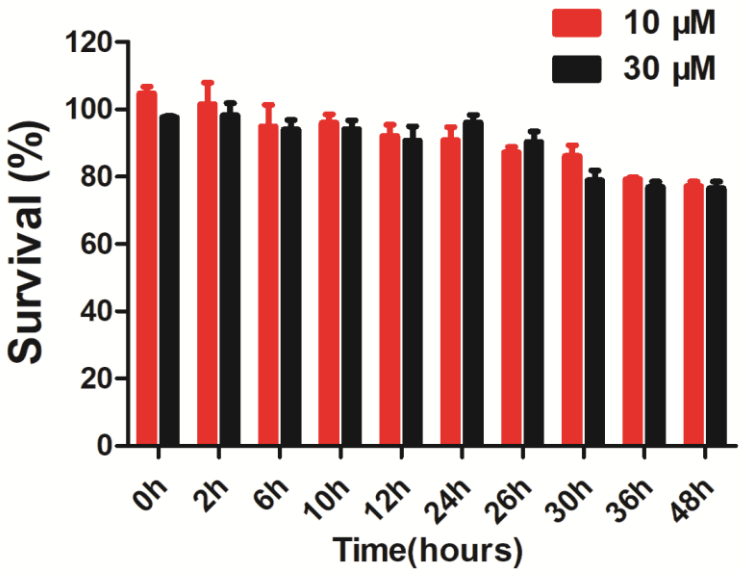


Figure S7 Cytotoxicity assays of **PI-Cys** at different time periods for HeLa cells.

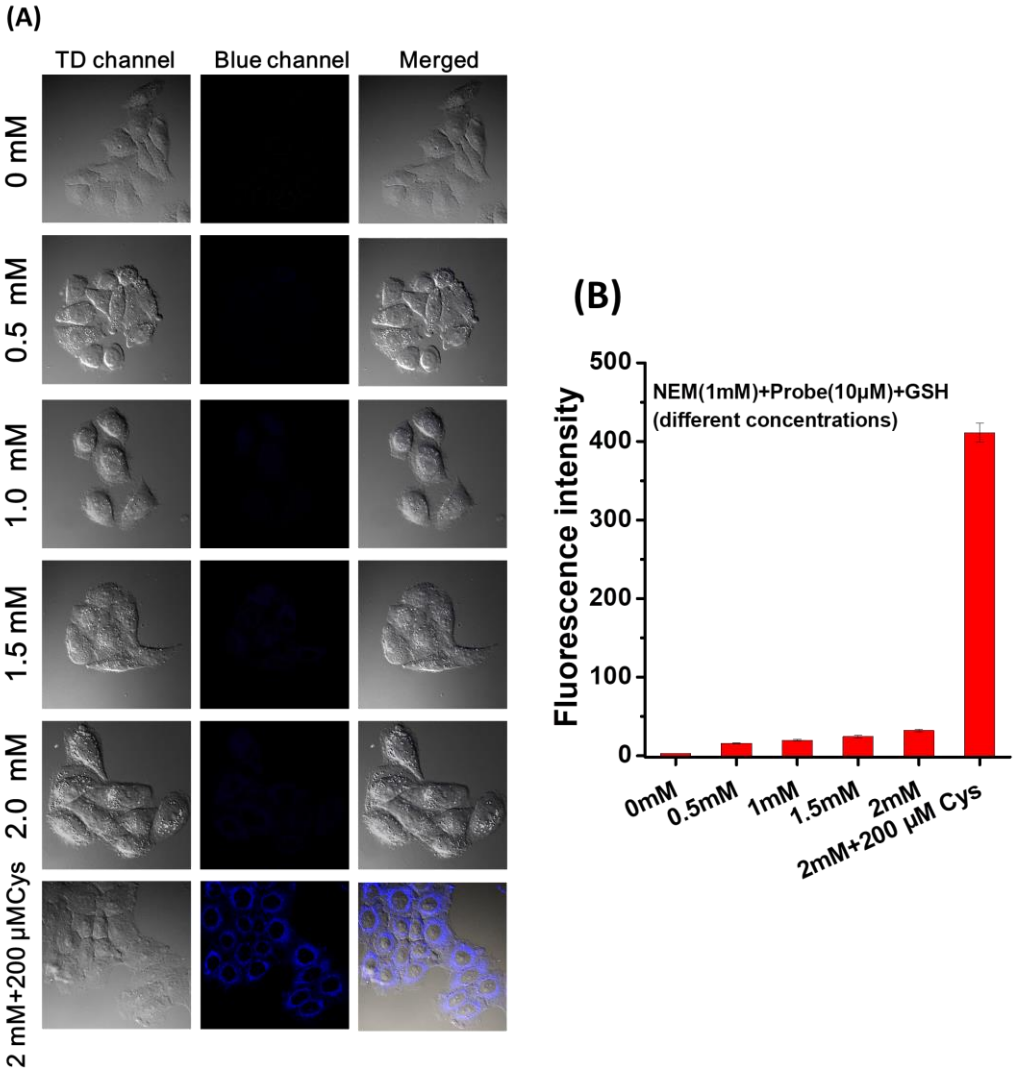


Figure S8 (A) The fluorescence images of HeLa cells pretreated with 1 mM NEM before

incubated with 10 μM **PI-Cys**, and then treated with different concentrations of GSH. **(B)** The histogram of fluorescence intensity of blue channel cells.

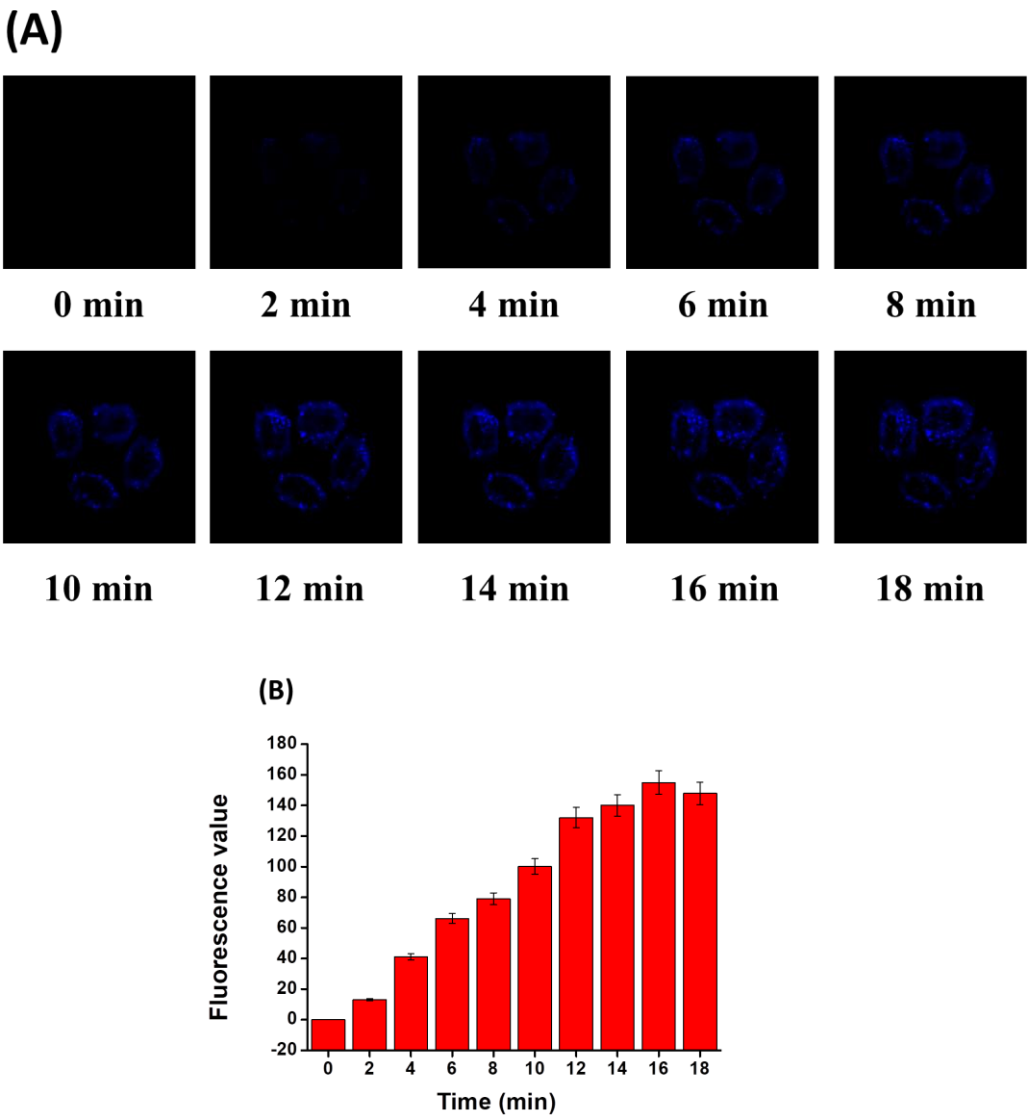


Figure S9 (A) HeLa cells time-dependent fluorescence images of adding 10 μM **PI-Cys**; **(B)** The histogram of fluorescence values of blue channel cells.

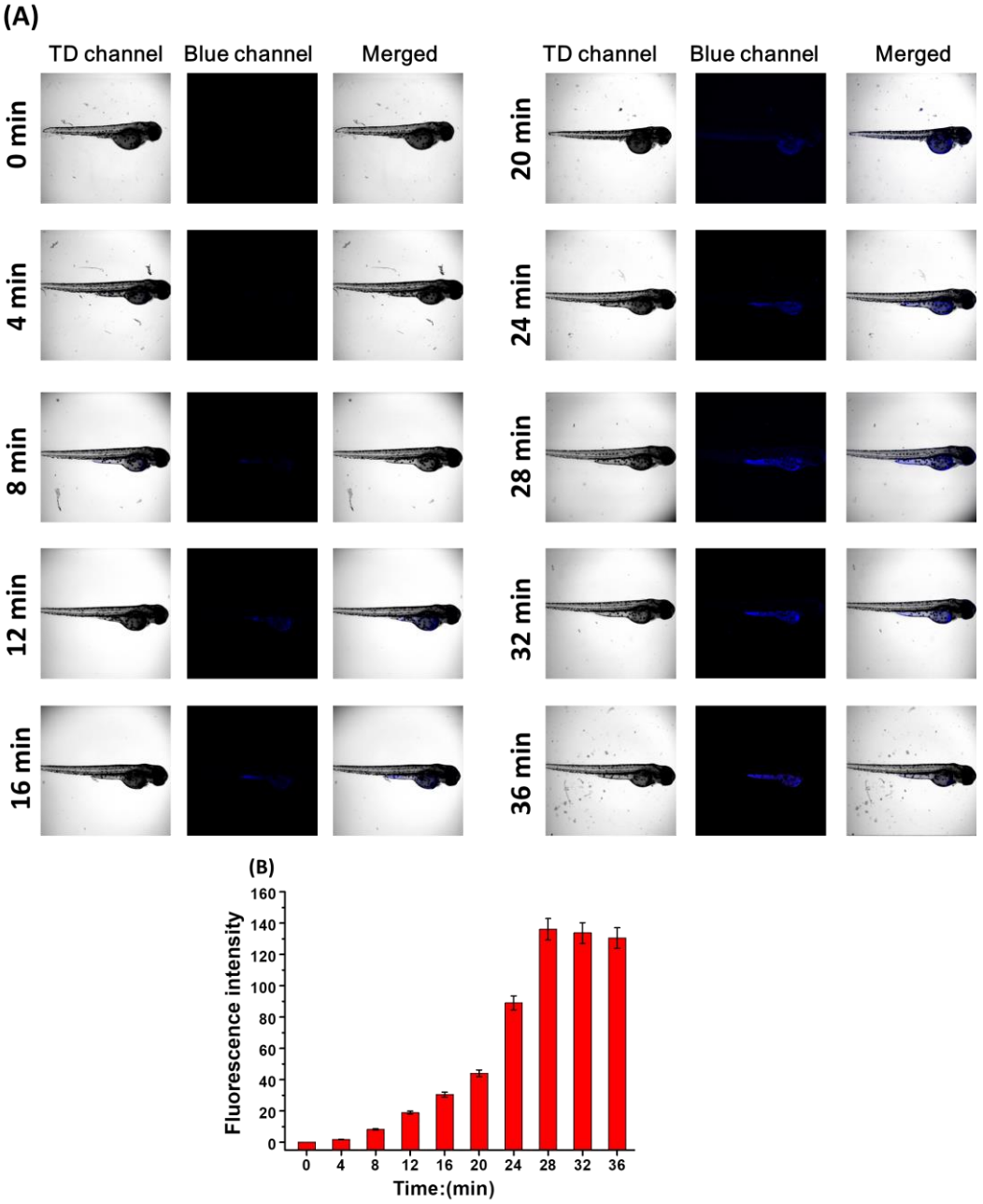


Figure S10 (A) Zebrafish time-dependent fluorescence images were treated with 10 μM **PI-Cys** towards Cys (200 μM). **(B)** The histogram of fluorescence intensity of blue channel cells.

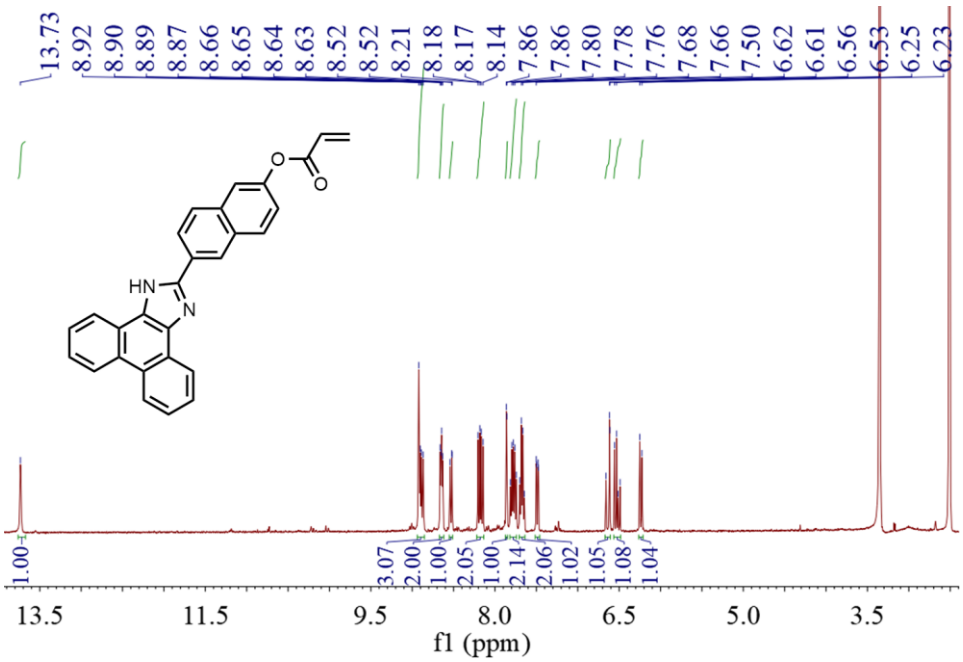


Figure 11 ^{1}H-NMR(DMSO-d_6) spectrum of compound **PI-Cys.**

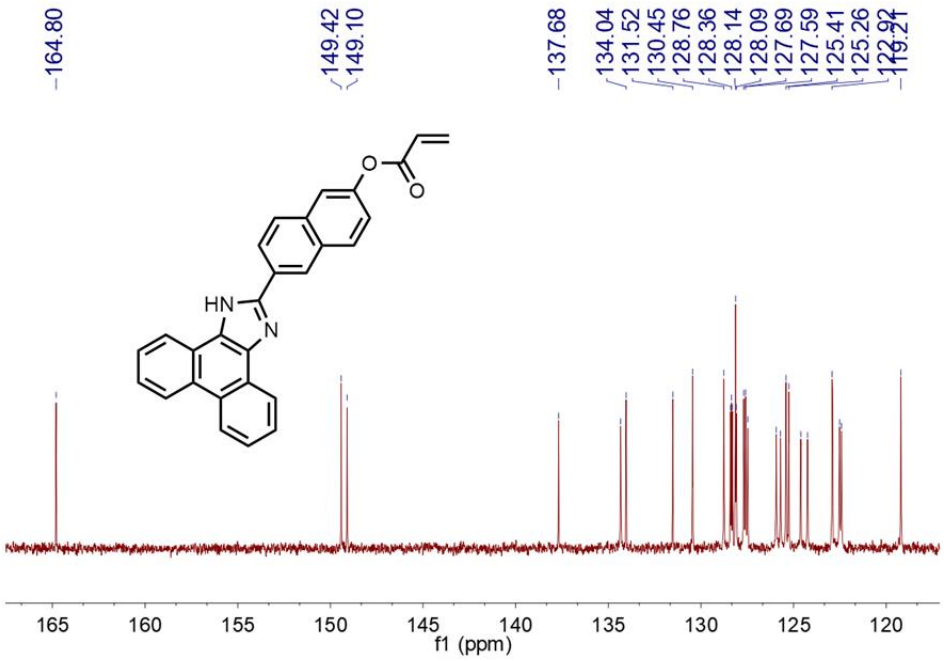


Figure S12 ^{13}C-NMR (DMSO-d_6) spectrum of compound **PI-Cys**.

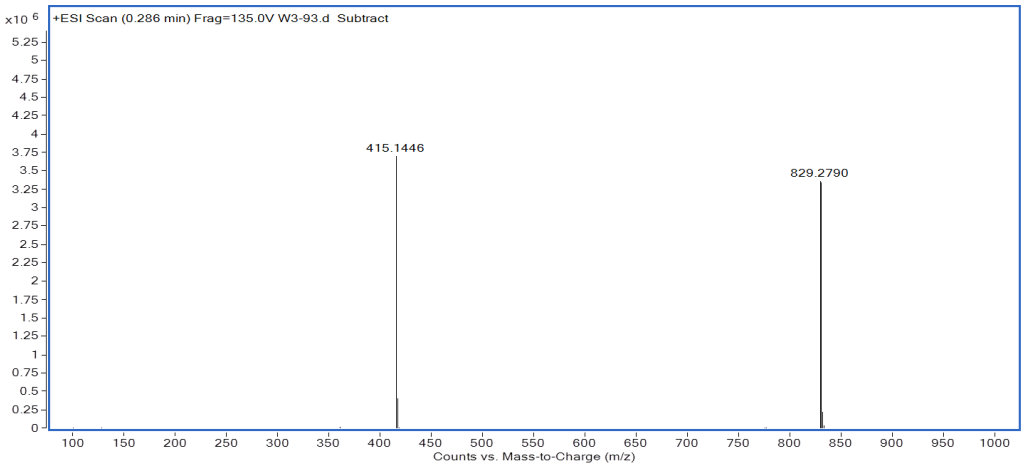


Figure S13 HRMS (ESI) spectrum of **PI-Cys.**

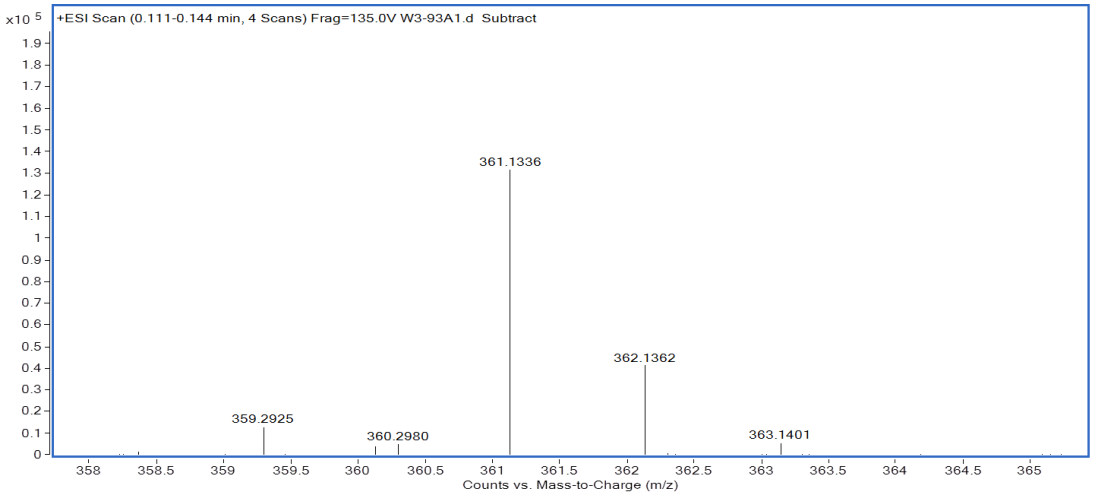


Figure S14 HRMS (ESI) spectrum of product of **PI-Cys** reacted with Cys.