Supporting Information (SI)

Two-photon background-free fluorescent assay for glutathione over cysteine and homocysteine in *vitro* and *vivo*

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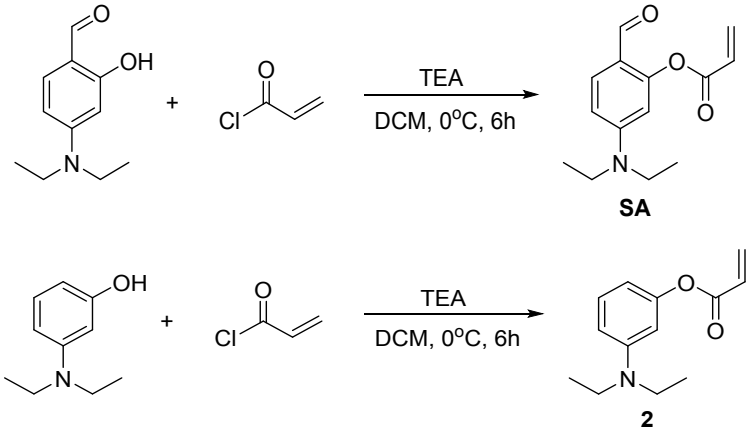
1. Experiment section

1.1 Reagents and instruments

Reagents and solvents for organic synthesis or stock solutions were obtained commercially at analytical grade or higher and were used as received. HeLa cells were obtained from National Infrastructure of Cell Line Resource (Beijing, China). Solvent used for spectral investigation was deionized water produced from Milli-Q Advantage A10 with a conductivity of 0.055 μS/cm.

Thin layer chromatography analysis (TLC) was operated on silica gel plates. ^{1}H and ^{13}C NMR spectra were carried out on a Bruker AV 500 NMR spectrometer utilizing DMSO-d_6 as solvent, and tetramethylsilane (TMS; δ = 0 ppm) was taken as the internal reference to record chemical shifts. An FT-ICR-MS mass spectrometer was used for high-resolution mass spectra (HRMS) analysis. Absorption and fluorescence spectral investigation was performed on a UH5300 UV-Vis and Hitachi F-4600 spectrophotometer respectively using a 1-cm quartz cell. Intracellular imaging by laser scanning confocal microscopy was captured by Leica TCS SP8 Point Scanning Confocal. Cell viability of the sensing scheme was evaluated through Cell Counting Kit-8 (CCK-8) assay by a Bio-Tek ELX800 microplate reader.

*1.2 Synthesis of probe **SA** and compound **2***



Scheme S1. Synthesis route of probe SA and **2**.

*Synthesis of probe **SA***. 1.16 g (6.0 mmol) 4-diethylaminobenzaldehyde and 200 μL triethylamine were stirred into 30 mL DCM, then the solution of 0.81 g (9.0 mmol)

acryloyl chloride in 10 mL DCM was added dropwise at 0 °C. The contents were stirred over night at r.t. after which the solution was extract with water (3×30 mL). The organic layer was dried by Na_2SO_4. Then the solvent was removed and the crude product was purified by silica gel column chromatography (PE : EA= 3 : 1, v : v) to obtain a flavescent solid with 88% yield (1.30 g,). m.p. 36-37 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 9.62 (s, 1H), 7.64 (d, J = 8.8 Hz, 1H), 6.69 (dd, J = 8.8, 2.2 Hz, 1H), 6.52 (d, J = 17.2 Hz, 1H), 6.47 – 6.38 (m, 2H), 6.15 (d, J = 10.1 Hz, 1H), 3.43 (q, J = 7.0 Hz, 4H), 1.11 (t, J = 7.0 Hz, 6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) 186.45, 164.40, 153.48, 153.25, 133.82, 128.06, 115.96, 109.01, 104.83, 56.44, 44.55, 42.32, 40.17, 42.32, 41.80, 40.17, 12.67. ESI-MS *m/z*: $[M+Na]^+$ Calcd for $C_{14}H_{17}NO_3Na^+$ 270.1101; Found 270.1101. Anal. Calcd. for $C_{14}H_{17}NO_3$: C 62.20, H 6.34, N 5.18; Found C 62.18, H 6.42, N 5.20.

Synthesis of compound ***2***. Compound 2 was synthesized according to the synthetic route of probe SA with a final yield of 92% (light yellow oil). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 7.16 (t, J = 8.2 Hz, 1H), 6.58 – 6.47 (m, 2H), 6.43 – 6.29 (m, 3H), 6.11 (dd, J = 10.2, 1.5 Hz, 1H), 3.31 (t, J = 7.0 Hz, 4H), 1.08 (t, J = 7.0 Hz, 6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) 164.66, 152.13, 149.07, 133.54, 130.24, 128.47, 109.37, 108.15, 104.90, 44.17, 12.77. Anal. Calcd. for $C_{13}H_{17}NO_2$: C 71.19, H 7.82, N 6.39; Found C 71.21, H 7.84, N 6.42.

1.3 General procedures for spectra measurements

Stock solutions of SA, **1** and **2** (5.0 mM) were prepared by spectroscopic pure DMSO and stored at 4 °C. Absorption and fluorescence spectra were conducted using the stock solutions of 4 μL diluted by 10 mM PBS buffer of pH 7.0 into 2 mL. The detection events were carried out as the addition of guest species. The solutions for spectra measurements were 10 mM PBS buffer of pH 7.0 containing 0.2% DMSO. The final concentration of SA, **1** and **2** for spectra measurements was 10 μM.

1.4 Detection limit for GSH

The limit of detection (LOD) for GSH was determined by the equation: LOD =3.3σ/k, based on the linearity between fluorescence intensity at 470 nm and GSH

concentration. [1, 2] σ and k in the equation represents standard deviation of the blank samples (n=11) and the slope of the fitted linear relationship respectively.

1.5 Determination of the two-photon absorption cross section

The two-photon absorption cross sections (δ_2) of SA/GSH were examined with rhodamine 6G (δ_1=35 GM in ethanol excited at 800 nm) as a standard substance. [3, 4] The two-photon absorption cross section was obtained using the following equation:

$$\delta 2 = \delta 1 \times \frac{C1}{C2} \times \frac{n1}{n2} \times \frac{F2}{F1}$$

where δ_1 is the two-photon absorption cross section of rhodamine 6G, C1 and C2 are the concentration of sample and rhodamine 6G, n is the refractive index and F represents the fluorescence intensity.

1.6 Cell culture and fluorescent imaging methods

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1 % penicillin and 1 % streptomycin at 37 °C for 24 h before the imaging experiments. For the detection of endogenous GSH, HeLa cells were incubated with SA (10 μM) for 30 min, then imaged after washed by Dulbecco's Phosphate Buffered Saline (DPBS). To detect exogenous thiols, HeLa cells were pretreated with NEM (1 mM) for 30 min, subsequently incubated with Hcy/Cys/GSH (100 μM) for 30 min and SA (10 μM) for another 30 min, then washed with DPBS three times before the fluorescence imaging. To investigate the effect of cisplatin on intracellular glutathione, the cells were treated with cisplatin (50 μM) for 6 hours and then washed by DPBS three times. After that, NAC (2 mM) and SA (10 μM) were added together and then monitored for 90 minutes in real time. Fluorescence images were taken on a Leica TCS SP8 laser scanning confocal microscopy with excitation wavelength set at 760 nm and green channel for observation set at 440-520 nm.

1.7 Cell cytotoxicity study

HeLa cells were seeded in 96-well plates and incubated for 24 h. After treatment with probe SA of increasing concentration or time, the cells were washed by DPBS, and the cytotoxic effect was conducted by CCK-8 assay. The cell viability was calculated by the equation:

Cell Viability (%) = (mean Abs. of wells with DEHP/mean Abs. of wells without DEHP)×100%

1.8 Preparation and staining of fresh rat brain slices

5-6 weeks old male Wistar rats were obtained from the Institute of Pharmaceutical Research of Qingdao (China). The rats were housed individually in a single cage and fed with rat chow obtained from the Institute of Pharmaceutical Research of Qingdao. The experiment was carried out after a week of adaptive feeding in rats. The protocols used in the experiments complied with the regulations of The Animal Care and Use Committee at Qingdao University of Science and Technology. Fresh rat brain slices were cut into 400 μm thick using a vibrating blade microtome in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 2.4 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgSO_4$ and 10 mM *D*-glucose). The slices were incubated with 10 μM SA in ACSF with 5% CO_2 and 95% O_2 for 20 min at 37 ^oC. Then washed three times with ACSF and transferred to glass-bottomed dishes and observed in a spectral confocal multiphoton microscope. To assess the effect of NEM as a control experiment, the slices were per-treated with 300 μM NEM for 30 min before SA was added.

1.9 Determination and fluorescent imaging of GSH in fruits

Cherry tomato, cucumber, and apple were chosen as the samples to evaluate GSH levels in foods by the real application tests of SA. 2.0 g fresh fruit (cherry tomatoes, cucumbers and apples respectively) was grinded thoroughly and 2 mL of deionized water was added. The mixture was ultra-sounded for 2 hours, and then filtered through a 0.44 μm membrane to remove the insoluble materials. The obtained solutions were used for the determination of GSH in fruits.

Analysis of GSH in food samples using DTNB. Absorbance spectra of 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB, 100 μM) with different concentrations of GSH (0, 0.095, 0.190, 0.285, 0.380, 0.475, 0.570, 0.665, 0.760, 0.885 and 0.950 $mg \cdot mL^{-1}$) were measured in TBS buffer at pH 7.0 to obtain the standard curve according to the literature reported method. [42, 43] 1 mL fruit extract was added to 4 mL TBS buffer and then mixed thoroughly, then 1 mL of the abovementioned solution

was added to 5 mL DTNB (100 μM) at 25 °C, and the absorption spectrum was finally determined after 10 min. The contents of GSH in fruit were calculated by the absorbance at 412 nm.

Analysis of GSH in food samples using probe SA. 40 μL fruit extract was added to 10 μM SA in the presence of 0.5 mM CTAB in 10 mM PBS buffer of pH 7.0. The fluorescence spectrum was determined after incubated for 30 minutes at 25 °C. GSH levels in fruit were calculated by the intensity at 470 nm.

Fluorescence confocal microscopy imaging of fruits slices. Fresh fruits slices (cherry tomato, cucumber, and apple) were cut into 400 μm thick using a vibrating blade microtome. The mixed solution of SA and CTAB was sprayed on the surface of the slices. Fluorescence images of the slices were taken after incubated for 30 minutes 25 °C using a BioTek CYTATION 5 Imaging reader with excitation wavelength set at 405 nm and emssion channel set at 440–520 nm.

2. ^{1}H, ^{13}C NMR and HRMS spectra

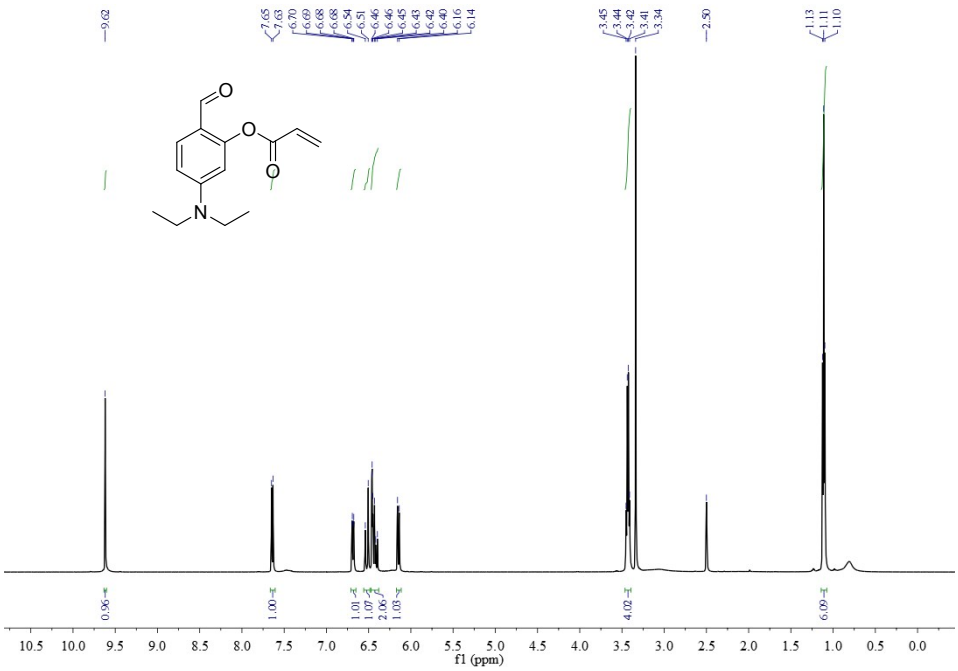


Figure S1 ^{1}H NMR spectrum of SA in DMSO-d_6.

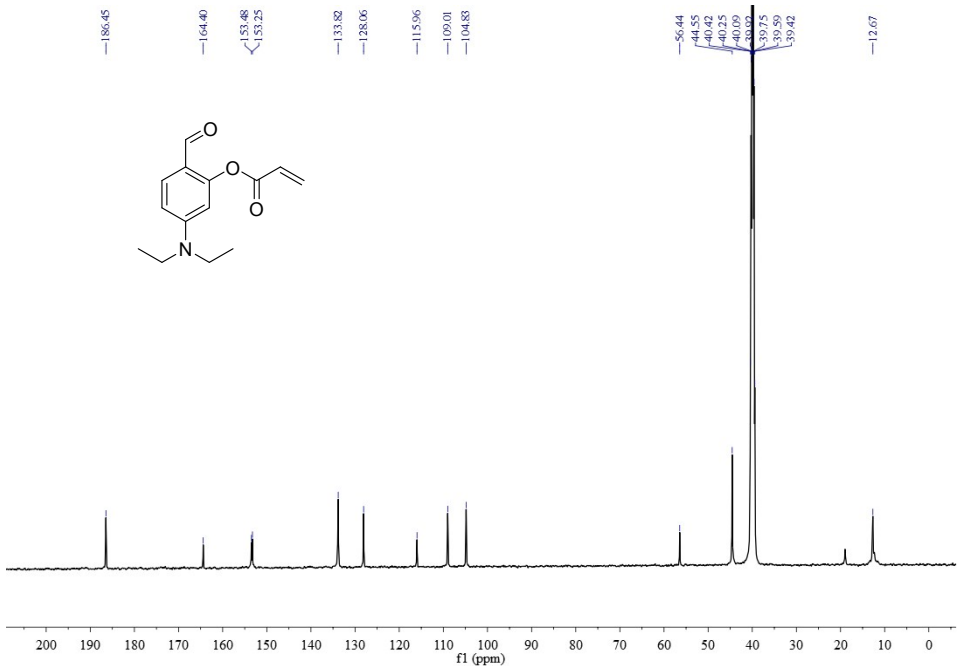


Figure S2 ^{13}C NMR spectrum of SA in DMSO-d_6.

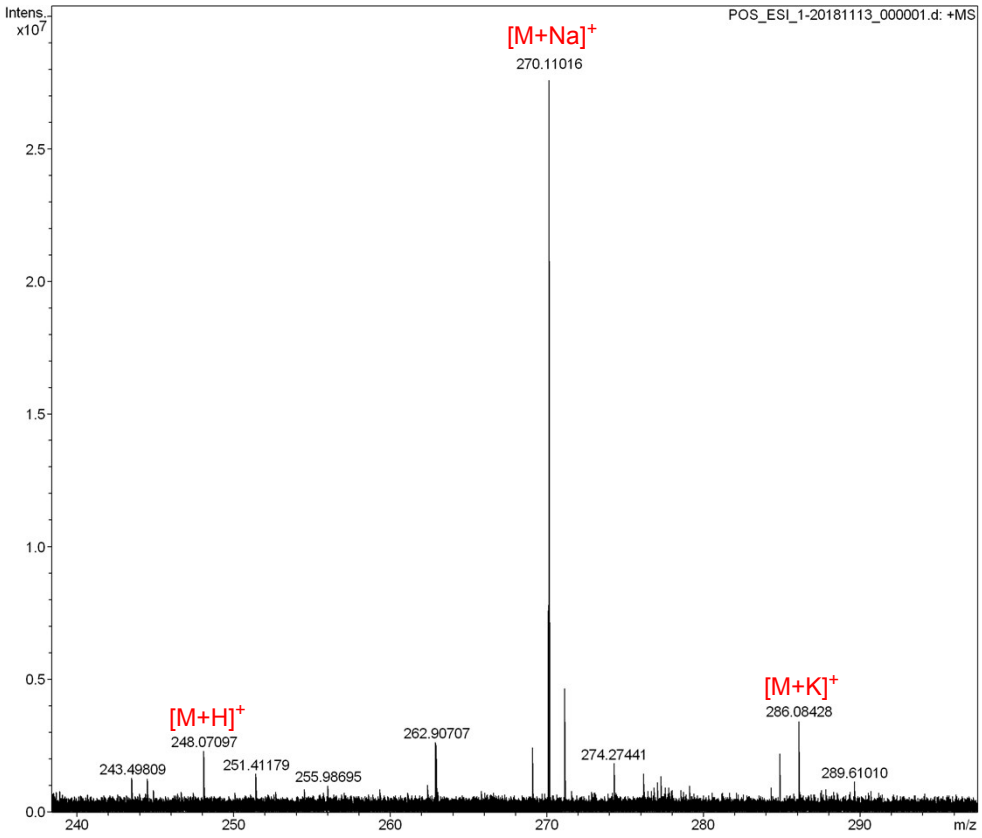


Figure S3 HR-MS spectrum of SA in MeOH.

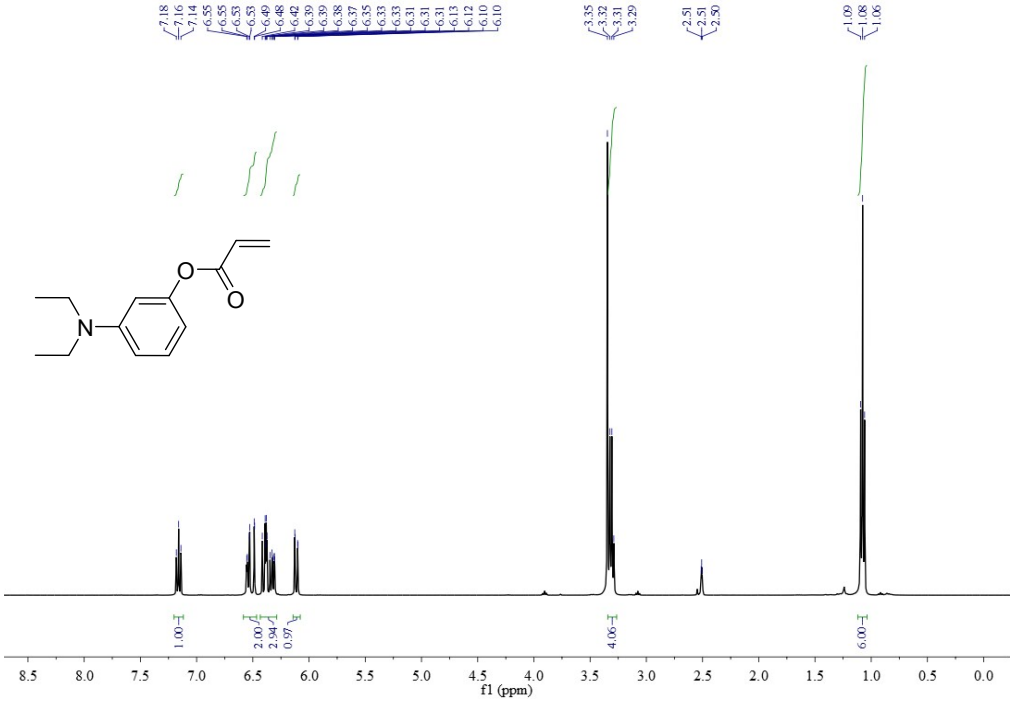


Figure S4 ^{1}H NMR spectrum of compound **2** in DMSO-d_6.

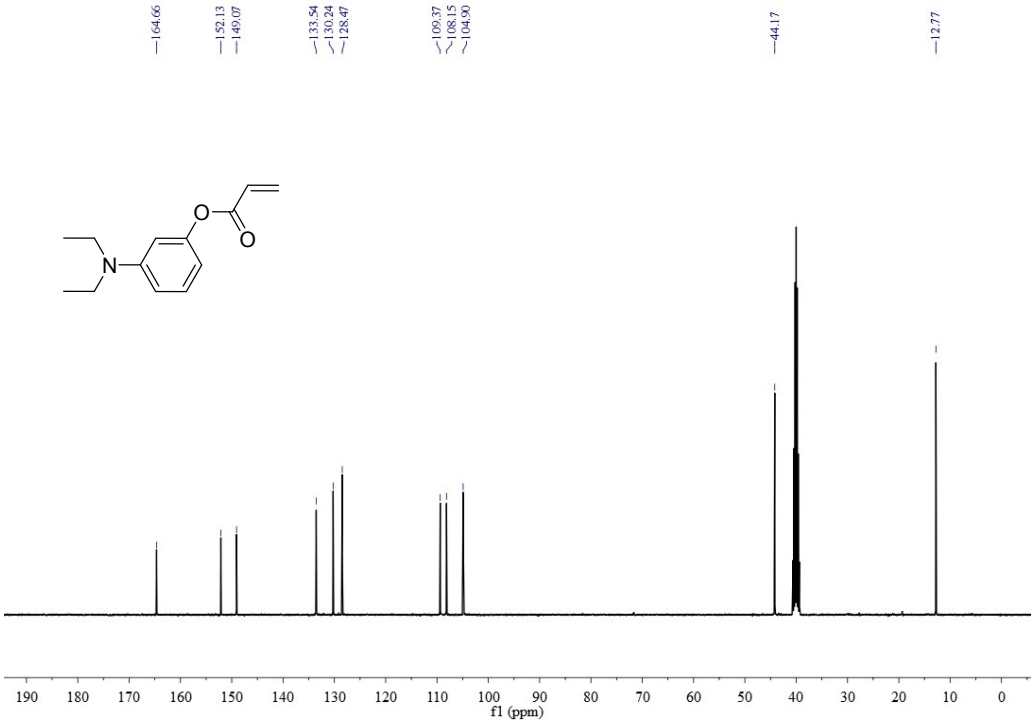


Figure S5 ^{13}C NMR spectrum of compound **2** in DMSO-d_6.

3. Spectra Figures

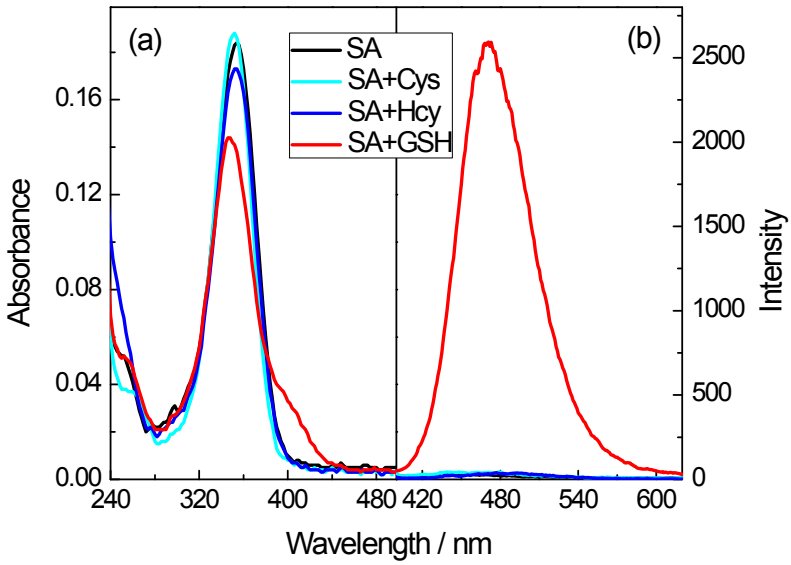


Figure S6. Absorption (a) and fluorescence (b) spectra of probe SA without (black line) or with the treatment of 40 μM Cys (cyan line), Hcy (blue line) and GSH (red line) in the present of 0.5 mM CTAB in PBS buffer at pH 7.0. [SA] = 10 μM, λ_{ex} = 380 nm.

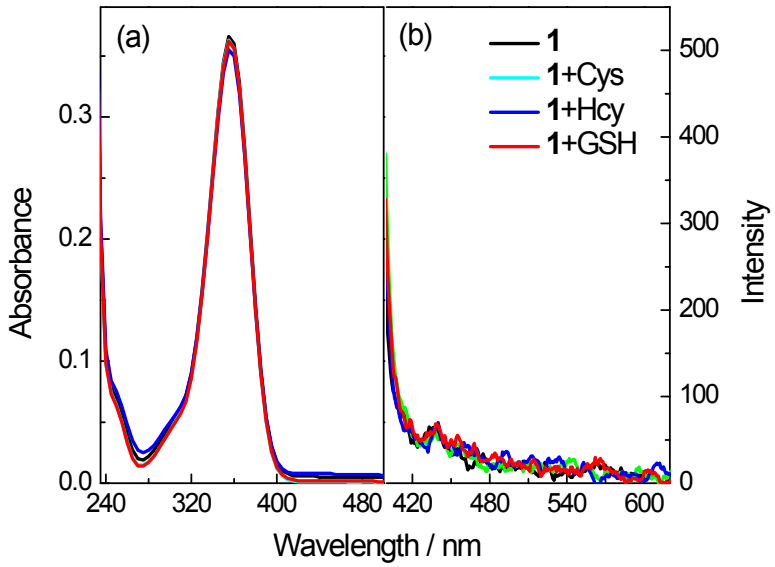


Figure S7. Absorption (a) and fluorescence (b) spectra of probe **1** without (black line) or with the treatment of 40 μM Cys (cyan line), Hcy (blue line) and GSH (red line) in the present of 0.5 mM CTAB in PBS buffer at pH 7.0. [**1**] = 10 μM, λ_{ex} = 380 nm.

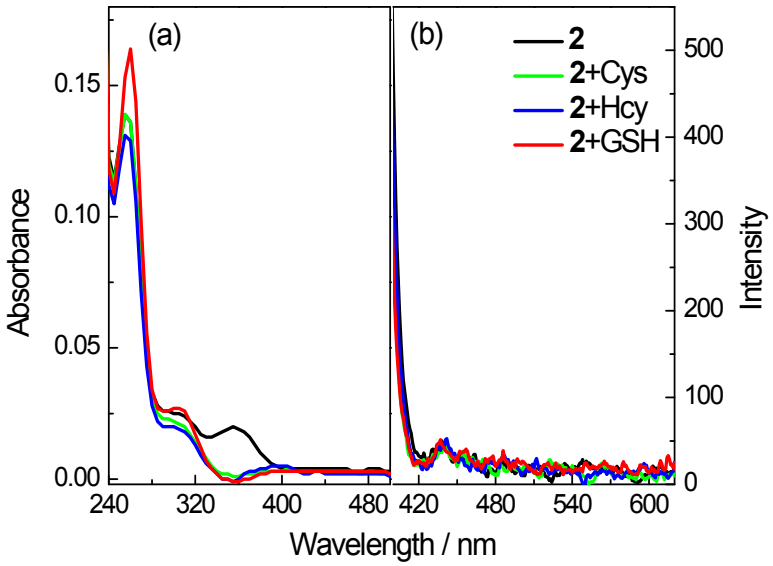


Figure S8. Absorption (a) and fluorescence (b) spectra of probe **2** without (black line) or with the treatment of 40 Cys (red line), Hcy (blue line) and GSH (pink line) in the present of 0.5 mM CTAB in PBS buffer at pH 7.0. [**2**] = 10 μM, λ_{ex} = 380 nm.

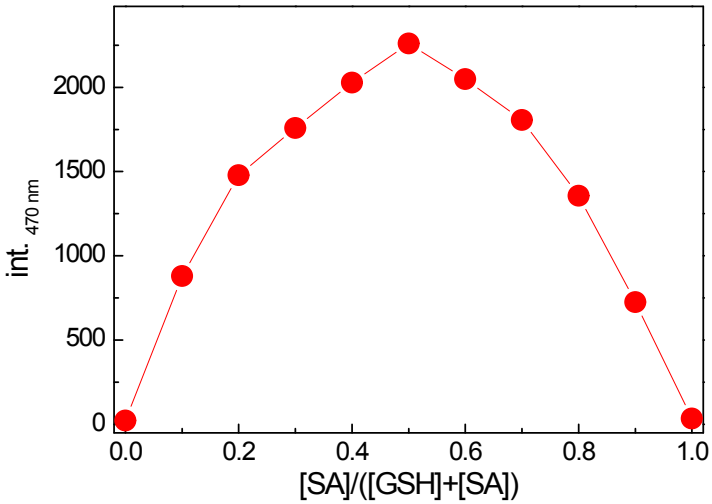


Figure S9. Job plot of fluorescence at 470 nm against [SA]/([GSH]+[SA]).

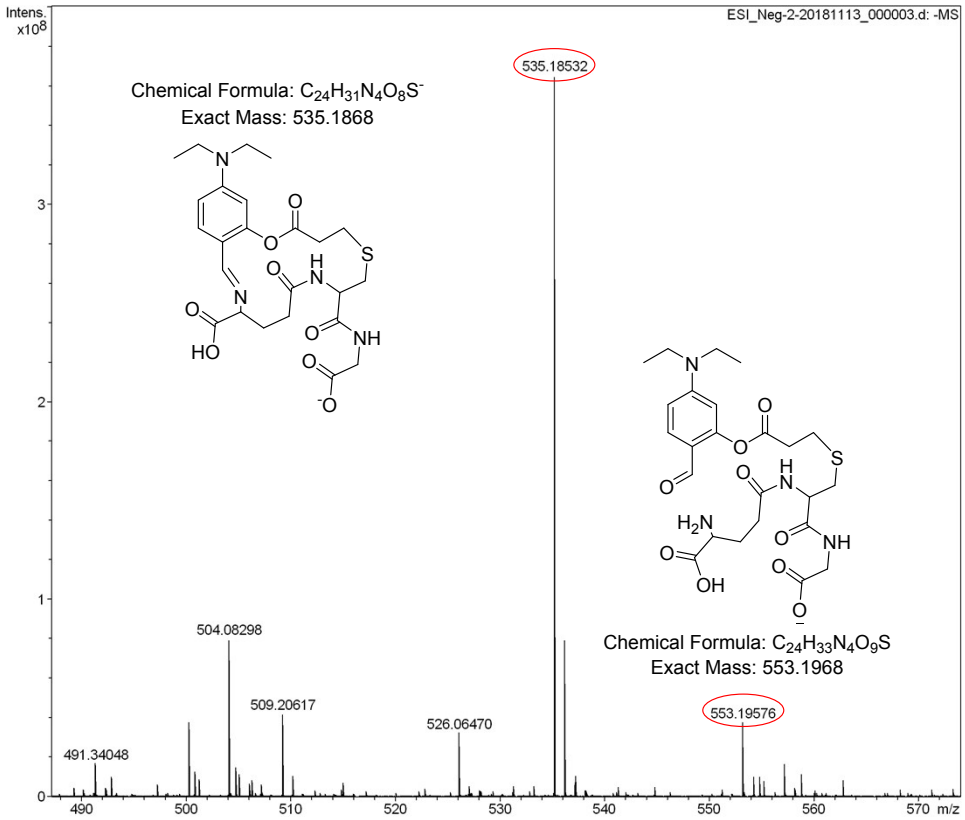


Figure S10. HR-MS spectrum of SA (10 μM) upon treated with 10 μM GSH in the present of 0.5 mM CTAB in PBS buffer of pH 7.0.

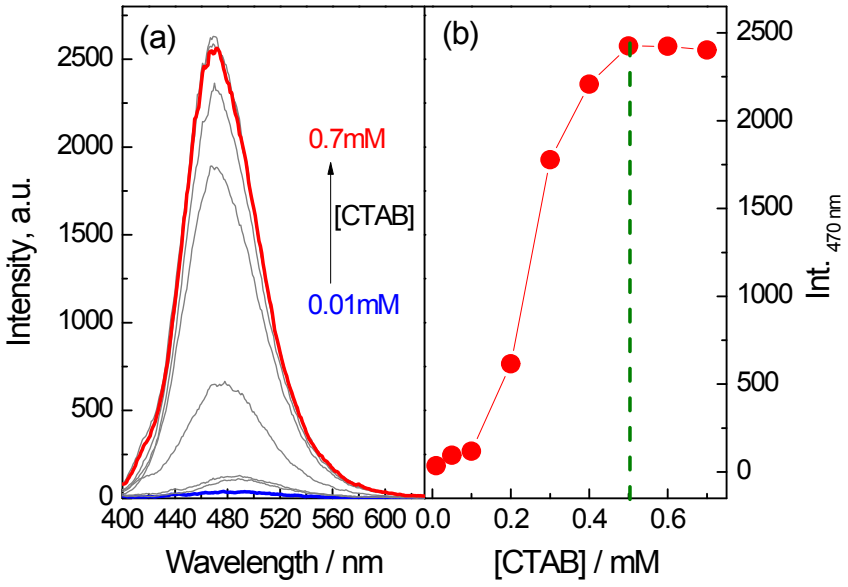


Figure S11. (a) Fluorescence spectra and (b) plots of intensity at 470 nm of SA in PBS buffer at pH 7.0 in the presence of 40 μM GSH with CTAB of increasing concentration from 0.01 to 0.7 mM. [SA] = 10 μM, λex = 380 nm.

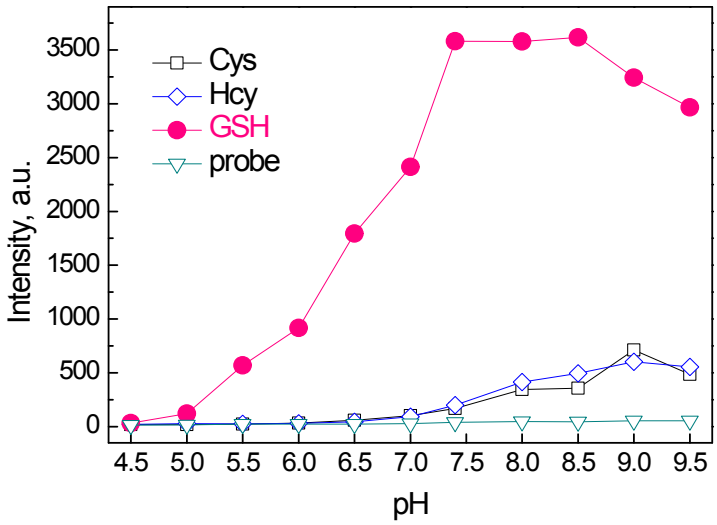


Figure S12. Plots of intensity at 470 nm of SA without (green triangles) or with 40 μM Cys (black squares), Hcy (blue diamonds) and GSH (red dots) in the present of 0.5 mM CTAB *versus* pH from 4.5 to 9.5. Solution pH was tuned by 10 mM phosphate buffer. [SA] = 10 μM, λ_{ex} = 380 nm.

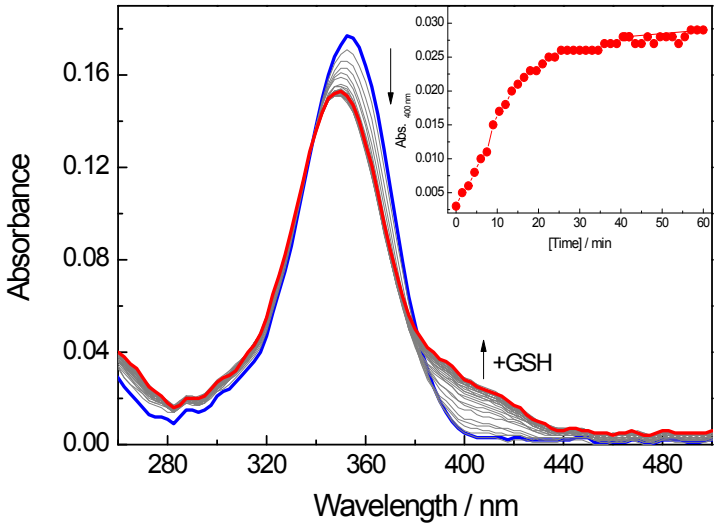


Figure S13. Time-dependent absorption of SA in PBS buffer at pH 7.0 upon treatment with 40 μM GSH from 0 to 60 min in the presence of 0.5 mM CTAB. [SA] = 10 μM. Inset: plots of absorbance at 400 nm *vs.* the observation time.

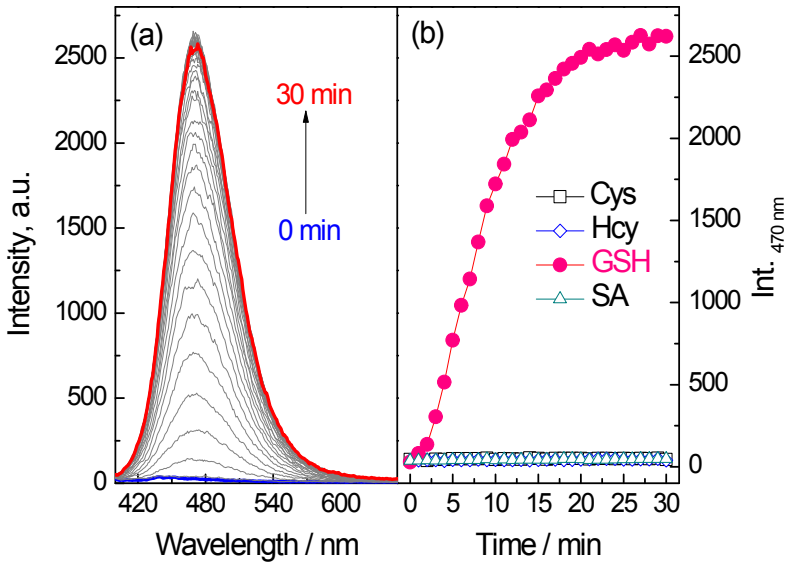


Figure S14. (a) Time-dependent fluorescence spectra of SA from 0 to 30 min upon treated with 40 μM GSH in the presence of 0.5 mM CTAB in PBS buffer at pH 7.0. (b) Plots of intensity at 470 nm of SA upon treated with 40 μM Cys (black squares), Hcy (blue diamonds), GSH (red dots) and SA itself (green triangles) from 0 to 30 min in the presence of 0.5 mM CTAB. [SA] = 10 μM, λ_{ex} = 380 nm.

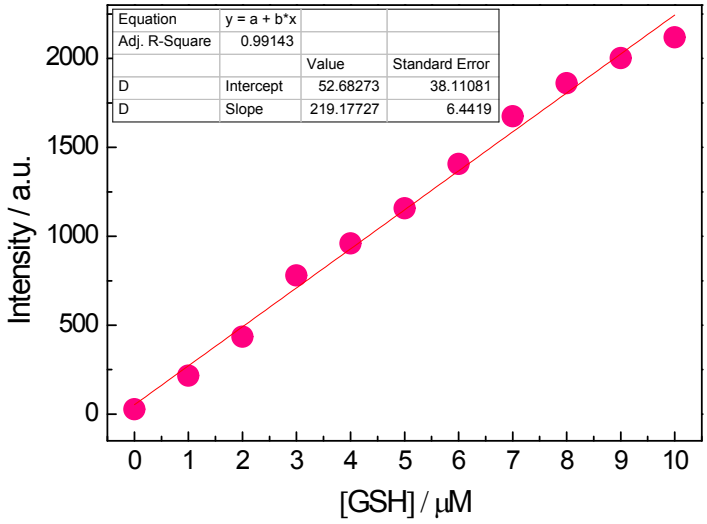


Figure S15. Linear relationship of fluorescent intensity of probe SA at 470 nm *versus* the concentration of GSH. [SA] = 10 μM, λ_{ex} = 380 nm.

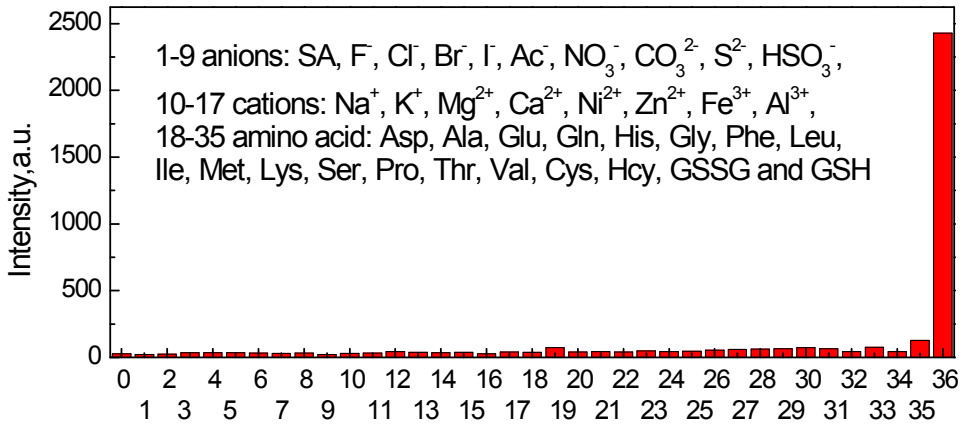


Figure S16. Fluorescence intensity of SA at 470 nm in the presence of 0.5 mM CTAB upon addition of 40 μM anions, cations and amino acids (1-34: Free, F^-, Cl^-, Br^-, I^-, AcO^-, NO_3^-, CO_3^{2-}, S^{2-}, HSO_3^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Asp, Ala, Glu, Gln, His, Gly, Phe, Leu, Ile, Met, Lys, Ser, Pro, Thr, Val, Cys, Hcy, GSSG and GSH) in 10 mM PBS buffer of pH 7.0. [SA] = 10 μM, λ_{ex} = 380 nm.

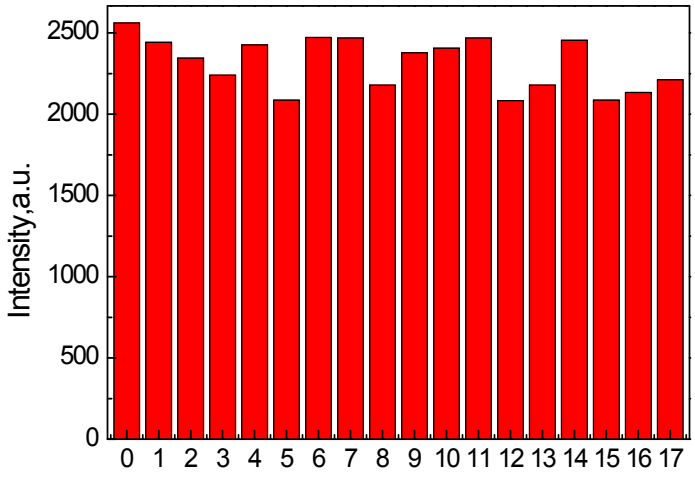


Figure S17. Fluorescence intensity of SA at 470 nm in 10 mM PBS buffer pH 7.0 in the presence of 20 μM GSH upon addition of 50 μM competition anions and cations, 1-17: Free, F^-, Cl^-, Br^-, I^-, AcO^-, NO_3^-, CO_3^{2-}, S^{2-}, HSO_3^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+} and Al^{3+}. [SA] = 10 μM, λ_{ex} = 380 nm.

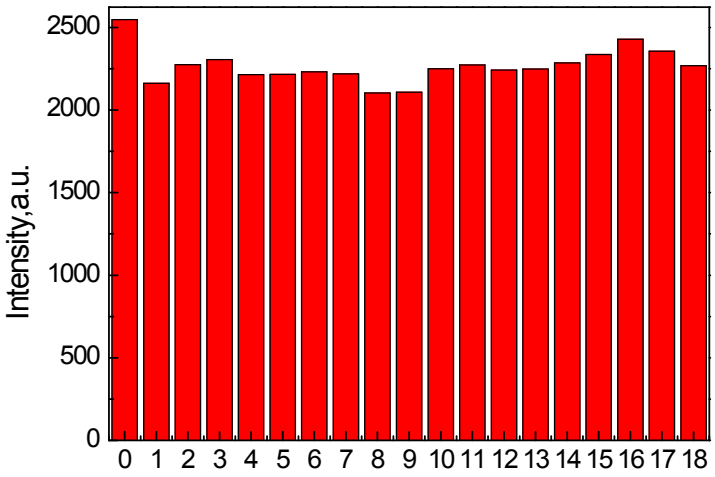


Figure S18. Fluorescence intensity of SA at 470 nm in the presence of 0.5 mM CTAB and 20 μM GSH upon addition of 50 μM competition amino acids in 10 mM PBS buffer pH 7.0, 0-17: Free, Asp, Ala, Glu, Gln, His, Gly, Phe, Leu, Ile, Met, Lys, Ser, Pro, Thr, Val, Cys, GSSG and Hcy. [SA] = 10 μM, λ_{ex} = 380 nm.

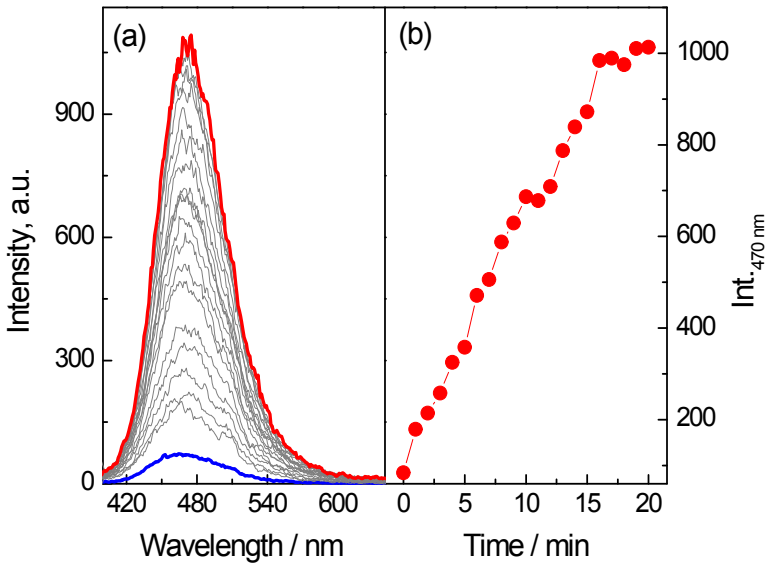


Figure S19. (a) Two-photon excited fluorescence of SA in the presence of 0.5 mM CTAB with 10 μM of GSH from 0 to 20 min in 10 mM PBS buffer of pH 7.0. (b) Plots of intensity at 470 nm from 0 to 20 min. [SA] = 10 μM, λ_{ex} = 760 nm.

4. Cytotoxicity of probe SA

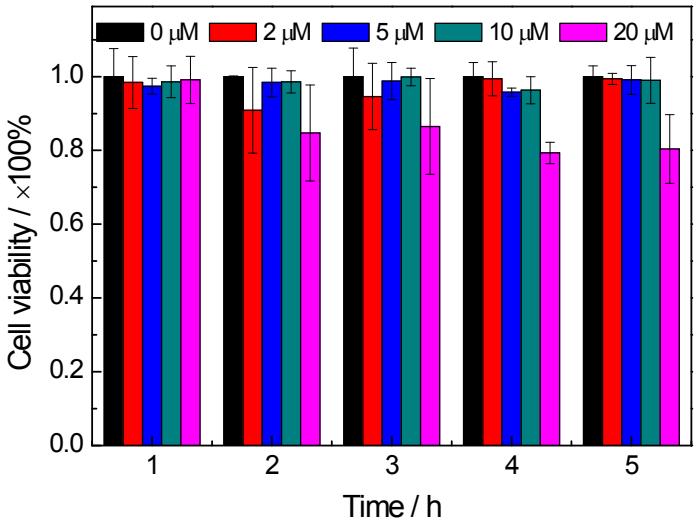


Figure S20. Cytotoxicity of SA against HeLa cells as determined by CCK-8 assay: HeLa cells were treated with SA (0-20 μM) for 1 to 5 hours.

5. Living cell imaging

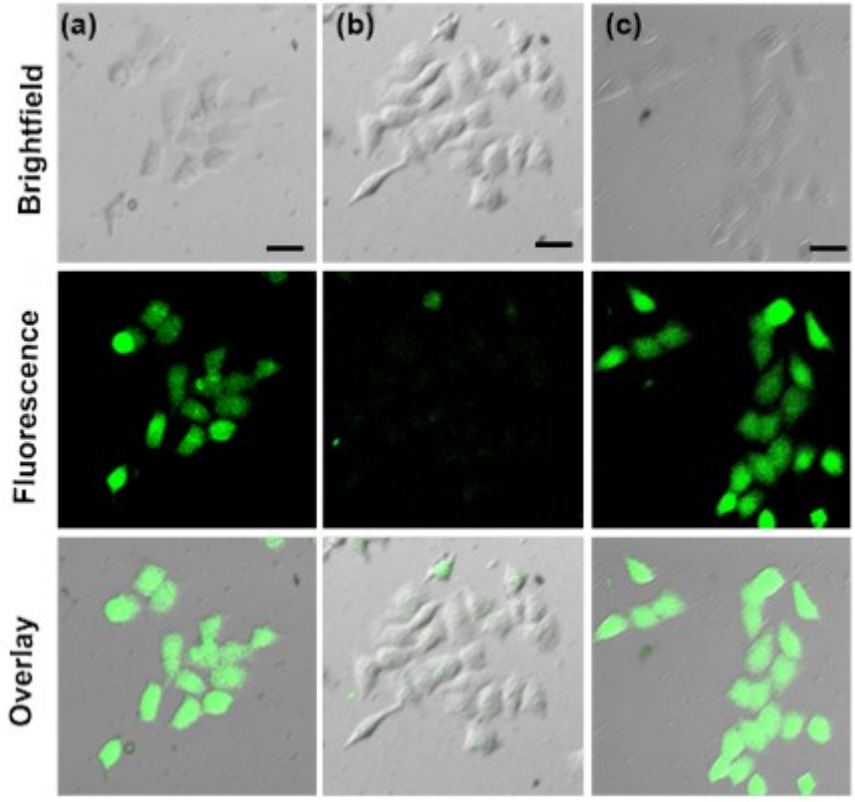


Figure S21. Two-photon excited fluorescence images of HeLa cells. (a) The cells were incubated with SA (10 μM) for 30 min. (b) The cells pretreated with 50 μM

cisplatin for 6 h were then incubated with 10 μM SA for 30 min. (c) The cells were pretreated with 50 μM cisplatin for 6 h, then incubated with 10 μM SA and 2 mM NAC for 1.5 h. Top: bright field, middle: fluorescent field, bottom: merged field. λ_{ex} = 760 nm, λ_{em} = 440-520 nm. Scale bar: 20 μm.

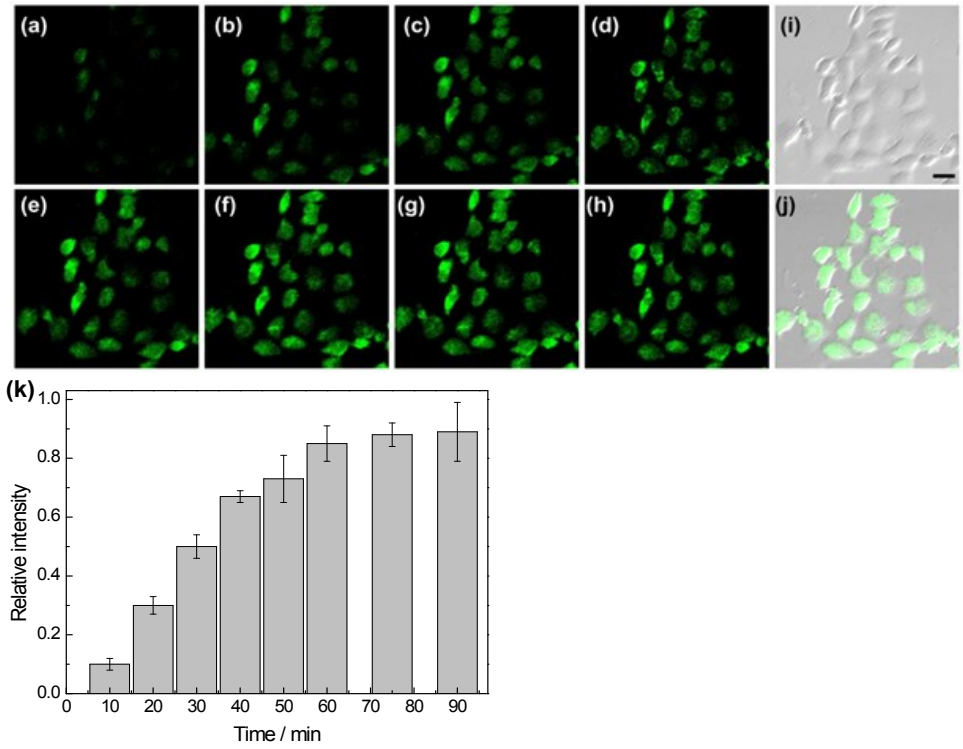


Figure S22. Two-photon excited fluorescence images of HeLa cells. The cells were pretreated with 50 μM cisplatin for 6 h, 10 μM SA and 2 mM NAC were then added after washing with DPBS. Fluorescence images (a-h) were acquired from 0 to 90 min; (i) bright field; (j) merged image of (i) and (h). (k) Average emission intensities from (a) to (h), the emission intensity of (h) was defined as 1.0. λ_{ex} = 760 nm, λ_{em} = 440-520 nm. Scale bar: 20 μm.

6. Determination of GSH in fresh rat brain slice

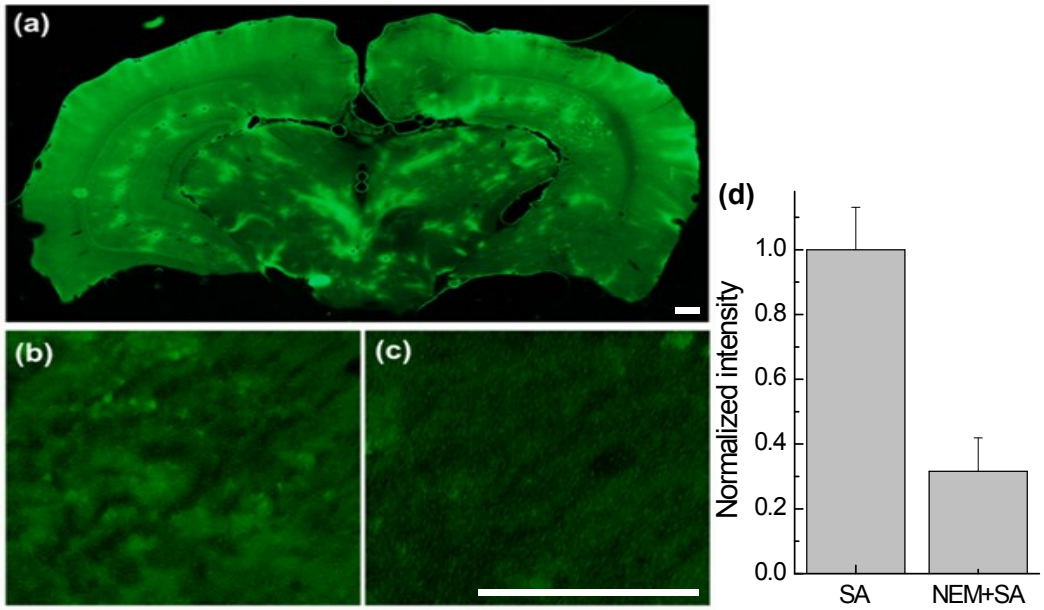
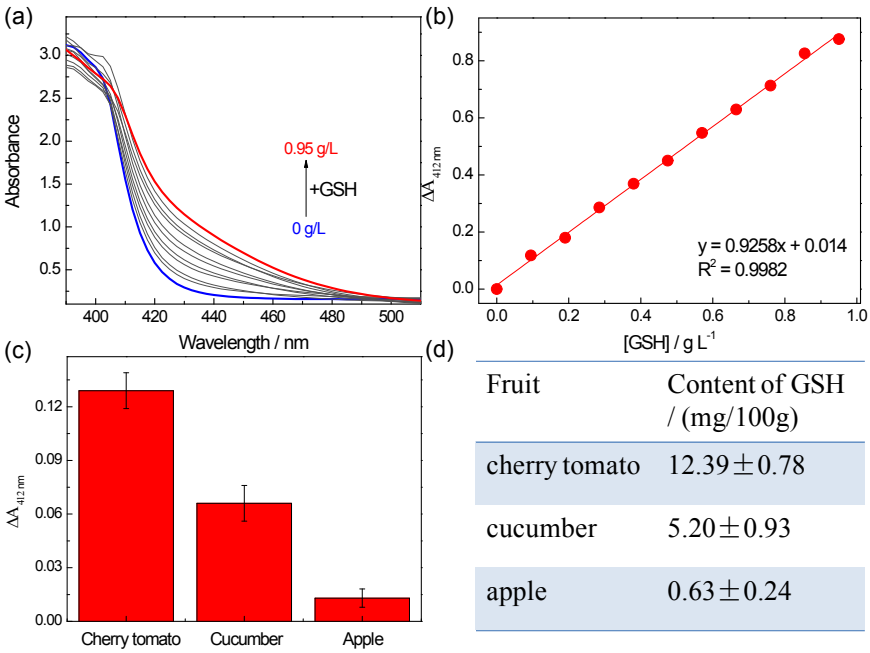


Figure S23. (a) Fluorescence image of a fresh rat brain slice incubated with 10 μM SA for 20 min. Scale bar: 300 μm. (b) Two-photon excited fluorescence image of enlarged part of hippocampus at a depth of ~100 μm after incubated with 10 μM SA for 20 min. Scale bar: 100 μm. (c) Two-photon excited fluorescence image of hippocampus at a depth of ~100 μm pretreated with NEM (300 μM) for 30 min before labelling with 10 μM SA for 20 min. λ_{ex} = 760 nm, λ_{em} = 440-520 nm. (d) Normalized emission intensity of (b) and (c). The average fluorescence intensity of (b) was defined as 1.

7. Determination of GSH in fruits



Fruit	Content of GSH / (mg/100g)
cherry tomato	12.39±0.78
cucumber	5.20±0.93
apple	0.63±0.24

Figure S24. (a) Absorption spectra of DTNB (100 μM) with increasing concentration of GSH from 0 to 0.95 g/L in TBS buffer at pH 7.0. (b) A-A_0 of DTNB (100 μM) at 412 nm with increasing concentration of GSH. (c) A-A_0 of DTNB (100 μM) at 412 nm with 1.0 mL fruits extract in TBS buffer at pH 7.0. (d) Contents of GSH in different fruits measured by DTNB method.

8. References

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