Electronic Supplementary Material (ESI)

A two-photon fluorescent probe for sensitive detection and imaging of γ-glutamyl transpeptidase

Ruijin Huo,[‡,a,b] Xiuli Zheng,[‡,a] Weimin Liu,*[a,b] Liping Zhang,[a,b] Jiasheng Wu,[a] Fan Li,[a] Wenjun Zhang,[c] Chun-Sing Lee,[c] Pengfei Wang*[a,b]

[a]Key Laboratory of Photochemical Conversion and Optoelectronic Materials and City U-CAS Joint Laboratory of Functional Materials and Devices, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, PR China

[b]School of Future Technology, University of Chinese Academy of Sciences, Beijing 100049, PR China

[c]Center Of Super-Diamond and Advanced Films (COSDAF) & Department of Materials Science and Engineering, City University of Hong Kong, Hong Kong SAR, People's Republic of China

*Corresponding author: wmliu@mail.ipc.ac.cn; wangpf@mail.ipc.ac.cn

[‡]These authors contributed equally to this work.

1. Materials and apparatus

All commercial chemicals used in the experiments were not further purified. 4F-2CN, GGT, trypsin, aprotinin, alkaline phosphatase, and glucoamylase were purchased from Sigma–Aldrich. Cysteinylglycine (Cys-Gly) was purchased from Shanghai yuanye Bio-Technology Co., Ltd. $CuCl_2$, $CaCl_2$, $ZnCl_2$, $MgCl_2$, NH_4Cl, NaCl, and KCl were purchased from J&K. GGsTOP was purchased from R&D system. ^1H NMR and ^{13}C NMR spectrum were obtained by Bruker Avance 400 MHz and 500 MHz spectrometry, respectively, and chemical shifts (δ) were given in ppm. Electrospray ionization mass spectrometry was performed on an FTICR-MS, Bruker Apex IV. Absorption spectra were measured with a Hitachi U-3900 UV-Visible spectrophotometer. Fluorescence spectra were measured with a Hitachi F-4600 spectrofluorometer.

2. Preparation of the test solution

Probe **4F-2CN-GSH** was dissolved in DMSO to prepare a 50 mM stock solution, and the GGT was dissolved in PBS (pH = 7.4) to prepare a 10 U/mL stock solution. The probe of 50 mM stock solution was diluted to 100 μM, and GGT was diluted to 300 U/L with PBS buffer solution. All UV absorption and fluorescence emission spectra were tested in PBS buffer solution at 37 °C. The excitation wavelength was 405 nm, and the widths of the excitation and emission light were 5 and 2.5 nm in fluorescence measurements.

3. Cell culture and fluorescent imaging

Human umbilical vein endothelial cells (HUVEC) and human ovarian cancer cells (SKOV-3) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. OVCAR3 cells were grown in Roswell Park Memorial Institute 1640 medium with 20% (v/v) FBS and 1% (v/v) penicillin-streptomycin. All cells were incubated in a 5% CO_2 humidified incubator at 37 °C. A certain concentration of cells was seeded in a particular specification confocal dish, added with a specific liquid culture medium, placed in 5% CO_2/air atmosphere incubator (37 °C), and cultured. We took 2 μL of the 50 mM stock

solution and incubated it with cells seeded in a confocal dish for 1 h. The resultant was washed three times with phosphate buffer solution (PBS) and underwent one-photon and two-photon fluorescence imaging with a confocal microscope (ARsiMP-LSM-Kit-Legend Elite-USX) under laser excitation at 405 nm and 800 nm, respectively.

4. Cytotoxicity assay

Cells of a certain concentration were seeded in 96-well plates and cultured for 24 h. The original culture solution was discarded, and the diluted sample solutions (0, 50, 100, 150, and 200 μM) were added. After incubation in an incubator for 12 h, the sample-containing solution was removed from the 96-well plate and the culture solution was added into it. The cells were further incubated for 12 h in an incubator. A total of 20 μL of 5 mg/mL MTT solution was added to each well under incubation for 4 h. After the culture medium was discarded, 80 μL of DMSO solution was added to each well with a microplate reader to detect absorption at a wavelength of 570 nm.

5. Determination of two-photon absorption (TPA) cross-section.

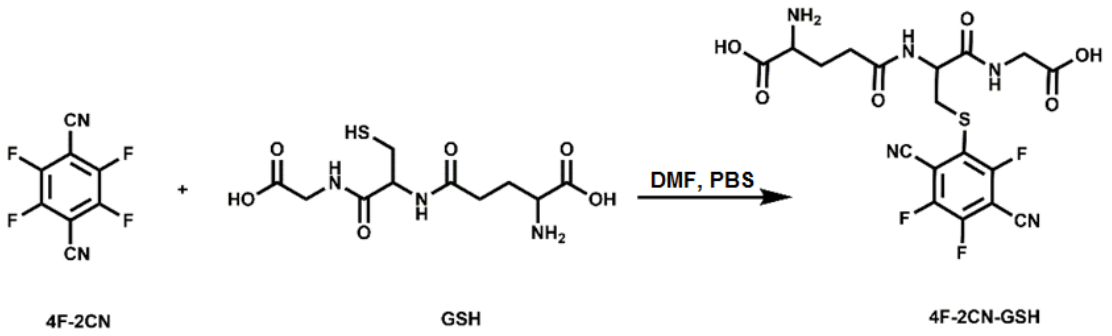
Two-photon excited (TPE) fluorescence measures(Vitara-Legend Elite): the excitation light source was a mode-locked tsunami Ti: sapphire laser (750-880 nm, 80 MHz, <130 fs). Using a fiber optic spectrometer (Ocean Optics USB2000 CCD) as the detector, the fluorescence spectrum was recorded in a direction perpendicular to the laser beam. Rhodamine B in methanol as reference.

The TPA cross section (σ) values of the sample were calculated using the following equation:

$$\sigma_2 = \frac{F_2}{F_1} \cdot \frac{\phi_1}{\phi_2} \cdot \frac{n_1}{n_2} \sigma_1$$

Subscript 1 represents a reference, and subscript 2 represents a sample. F is the integrated area of fluorescence, ϕ is the fluorescence quantum yield, and n is the concentration.

6. Synthesis of 4F-2CN-GSH

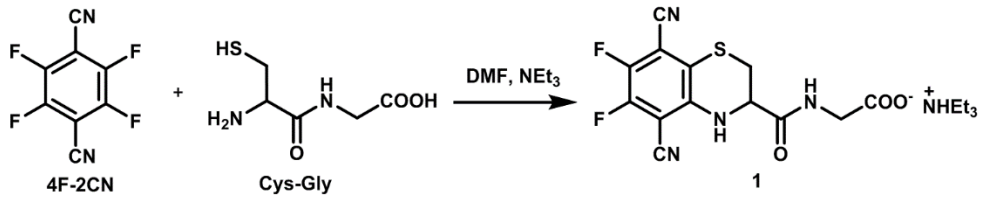


Scheme S1. Synthesis of probe **4F-2CN-GSH**.

4F-2CN (200 mg, 1.0 mmol) and l-glutathione (310 mg, 1.0 mmol) were dissolved in 20 mL DMF/PBS (1/1, pH = 7.4). Stirring the mixture at room temperature for 3 hours and the solution turns yellow. The solvent was removed under vacuum condition, and the probe **4F-2CN-GSH** 80 mg (yield 16%) was obtained by the reverse silica gel C_{18} separation product.

^{1}H NMR (D_2O, 400MHz): δ 4.40 (s, 1H), 3.92 (m, 3H), 3.62-3.58 (m, 1H), 3.29-3.23 (m, 1H), 2.60-2.57 (m, 2H), 2.22-2.19 (m, 2H). ^{13}C NMR (CD_3O D, 125 MHz) δ 173.1, 171.1, 170.5, 170.2, 160.4, 158.3, 122.7, 122.5, 113.6, 113.5, 109.6, 106.7, 52.5, 52.3, 40.4, 36.1, 31.0, 25.6. ESI-MS: $[M-H]^-$ Calcd. for $C_{18}H_{15}F_3N_5O_6S$, 486.0695; found 486.0702.

7. Synthesis of product 1



Scheme S2. Synthesis of product 1.

4F-2CN (200 mg, 1 mmol) and Cys-Gly (890 mg, 5.0 mmol) were dissolved in 20 mL DMF. Then triethylamine (0.6 mL) was added to the reaction system. Stirring the mixture at room temperature for 1 hour and the solvent was removed under vacuum condition to obtain the product **1** 62 mg (yield 18%) by the reverse phase C18 silica gel.

^{1}H NMR (DMSO-*d*$_6$, 500MHz): δ 8.47 (s, 1H), 7.27 (d, 1H), 4.56 (m, 1H), 3.77-3.62 (m, 3H), 3.45 (m, 1H), 3.26 (m, 1H), 3.03-3.08 (q, 6H), 1.21-1.18 (t, 9H). ESI-MS: $[M]^-$

Calcd. for $C_{13}H_7F_2N_4O_3S$, 337.0212; found 337.0207.

8. Supplementary Spectra and charts

Table S1. Comparison of two-photon probes for GGT.

Probe	λ_{ex}	λ_{em}	TP cross-section	Linear range	Detection limit	Reaction time	Ref.
DCM-GA	820 nm	635 nm	150 GM	0-35 U/L	0.057 U/L	30 min	1
Np-Glu	780 nm	490 nm	113 GM	0-50 U/L	0.033 U/L	45 min	2
TCF-GGT	800 nm	610 nm	104 GM	0-10 U/L	0.014 U/L	26 min	3
ANF-Glu	800 nm	531 nm	133 GM	230-2200 U/L	182 U/L	15 min	4
PZS1	700 nm	461 nm	-	-	-	110 min	5
4F-2CN-GSH	850 nm	490 nm	63 GM	0-60 U/L	0.117 U/L	60 min	This work

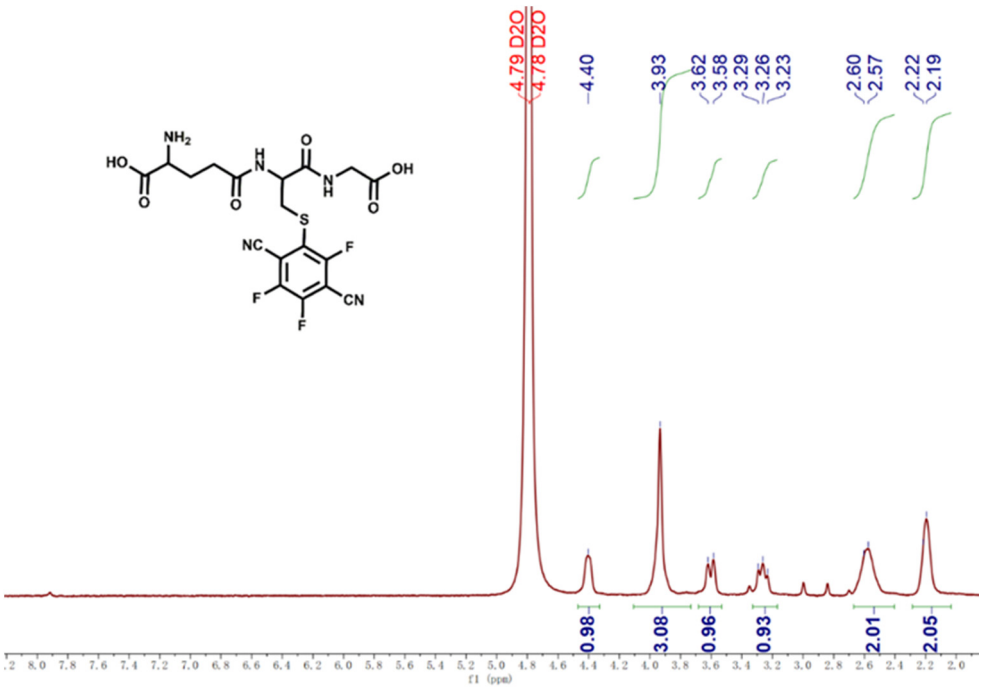


Figure S1. ^{1}H NMR chart of probe **4F-2CN-GSH** (D_2O, 400 MHz).

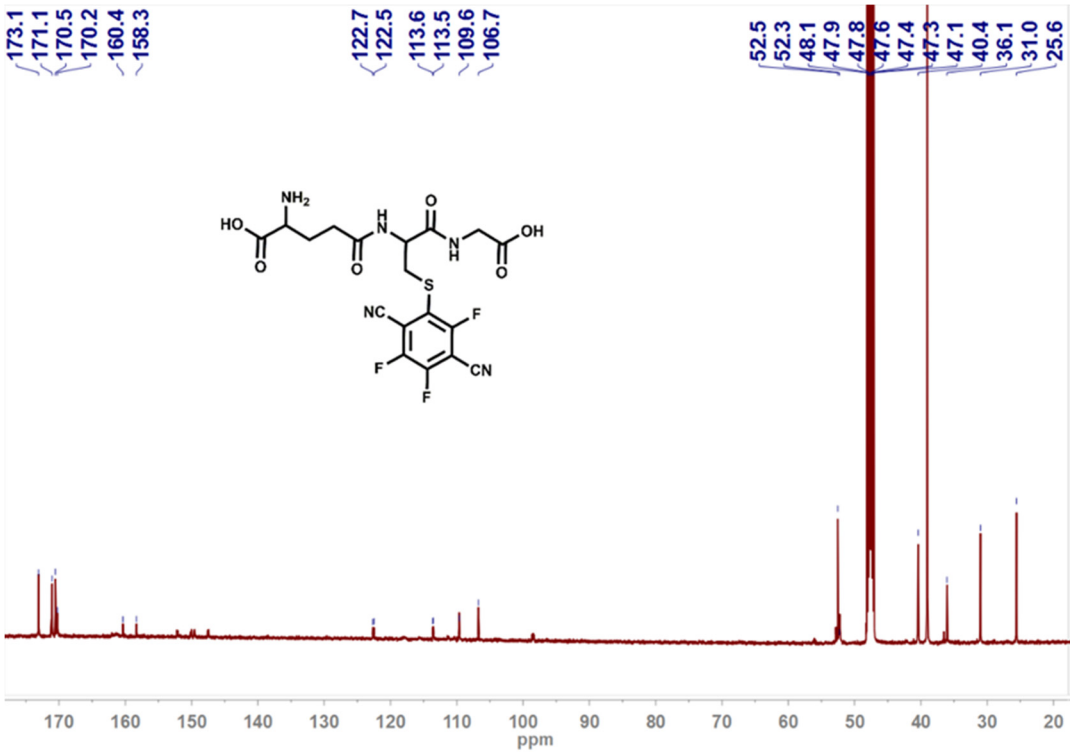


Figure S2. ^{13}C NMR chart of probe **4F-2CN-GSH** (CD_3OD, 125 MHz).

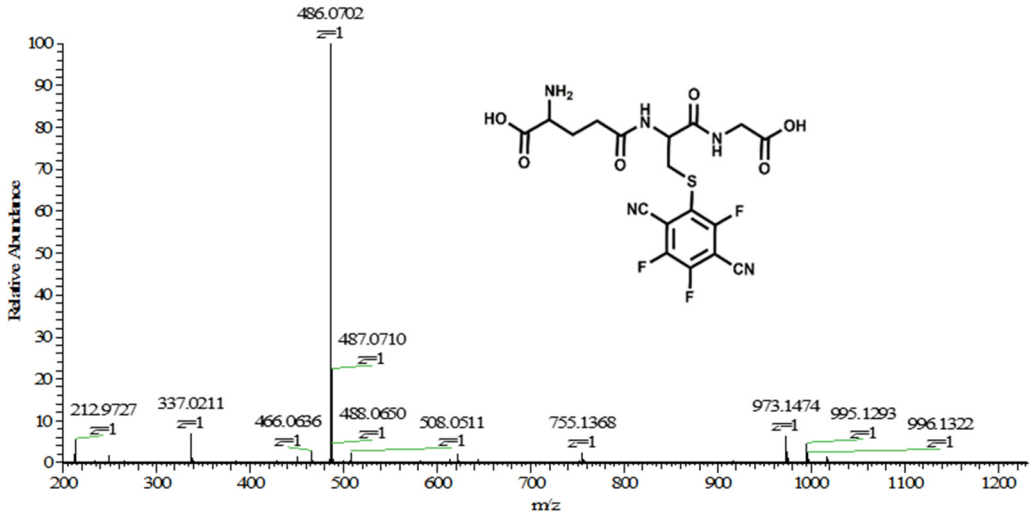


Figure S3. ESI-MS spectra of probe **4F-2CN-GSH**.

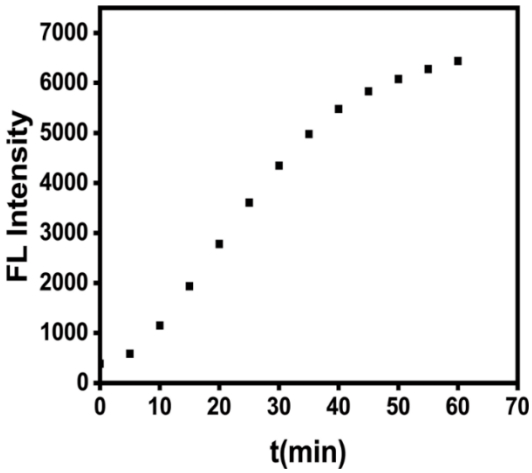


Figure S4. Time-dependent fluorescence intensity at 490 nm of **4F-2CN-GSH** (100 μM) to GGT (300 U/L). λex = 405 nm. Conditions: PBS (pH = 7.4, 10 mM) at 37 °C.

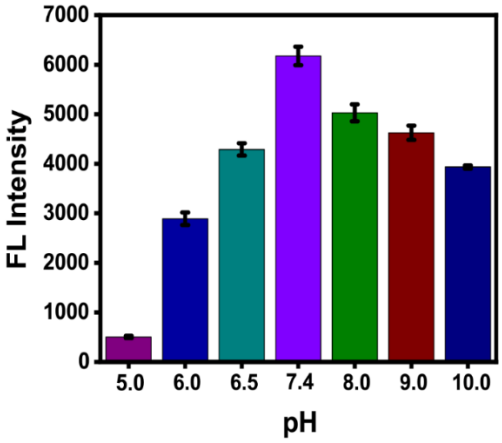


Figure S5. Effects of pH on the fluorescence of **4F-2CN-GSH** (100 μM) to GGT (300 U/L). λex = 405 nm.

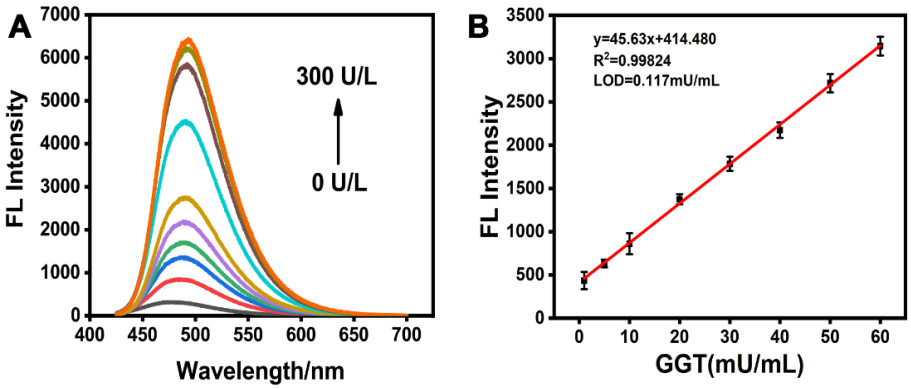


Figure S6. (A) Fluorescence spectral change of **4F-2CN-GSH** (100 μM) to GGT (0, 10, 20, 30, 40, 50, 100, 150, 250 and 300 U/L), and each spectrum was recorded after

60 min; (B) linear correlation between the intensity (490 nm) and the GGT concentration; λex = 405 nm. Conditions: PBS (pH = 7.4, 10 mM) at 37 °C.

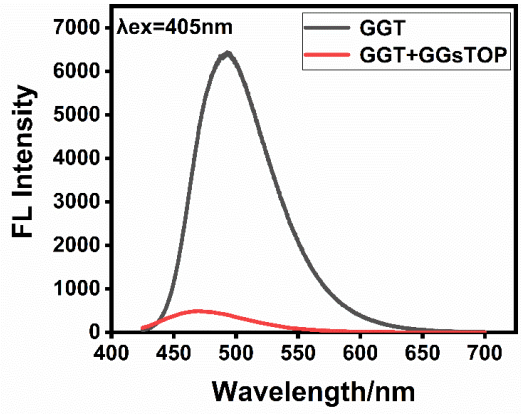


Figure S7. Fluorescence emission spectra of probe **4F-2CN-GSH** (100 μM) + GGT (300 U/L) and **4F-2CN-GSH** (100 μM) + GGT (300 U/L) + GGsTOP (200 μM). λex = 405 nm. Conditions: PBS (pH = 7.4, 10 mM) at 37 °C.

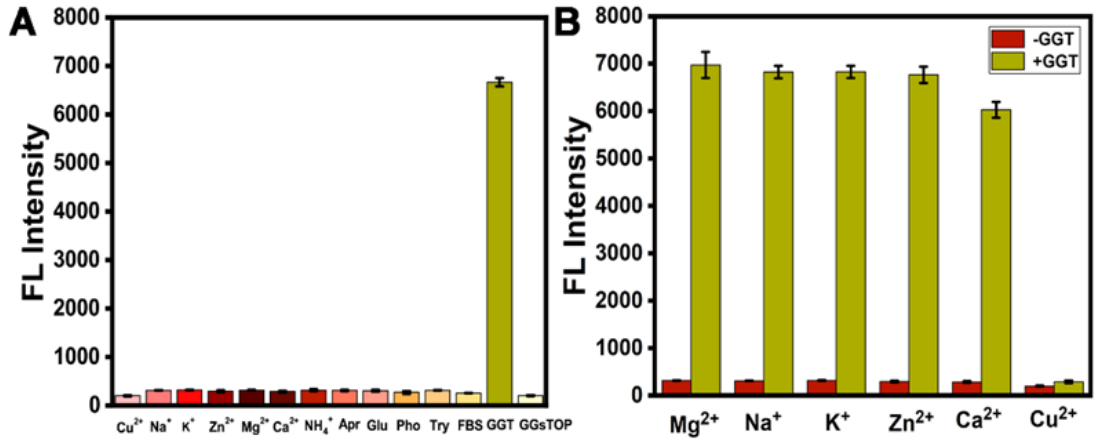


Figure S8. (A) Fluorescence response of **4F-2CN-GSH** (100 μM) to various analytes, Pho (1mM), Apr (1mM), Glu (1mM), Try(1mM); GGsTOP (200μM) + GGT (300 U/L), GGT (300 U/L), Mg^{2+} (2 mM), Na^{+} (20 mM), K^{+} (20 mM), Zn^{2+} (2 mM), Ca^{2+} (2 mM), Cu^{2+} (2 mM) and NH_4^{+} (2 mM); (B) The effects of some metal ions on the fluorescence intensity of **4F-2CN-GSH** (100 μM) at 490 nm after incubation with GGT (300 U/L) for 60 min, and the concentrations of metal ions were Mg^{2+} (2 mM), Na^{+} (20 mM), K^{+} (20 mM), Zn^{2+} (2 mM), Ca^{2+} (2 mM) and Cu^{2+} (2 mM). λex = 405 nm. Conditions: PBS (pH = 7.4, 10 mM) at 37 °C.

Table S2. Photophysical properties of probe **4F-2CN-GSH**, **4F-2CN-GSH** + GGT, and product **1** in PBS buffer.

Compound	λ_{max} of absorption (nm)	λ_{max} of emission (nm)	ε ($M^{-1}cm^{-1}$) [a]	Φ_{FL} [b]
4F-2CN-GSH	340	475	2.1×10^3	0.03
4F-2CN-GSH + GGT	415	490	3.4×10^3	0.33
Product **1**	421	490	3.63×10^3	0.20

[a] Molar extinction coefficient; [b] Absolute Fluorescence quantum yield.

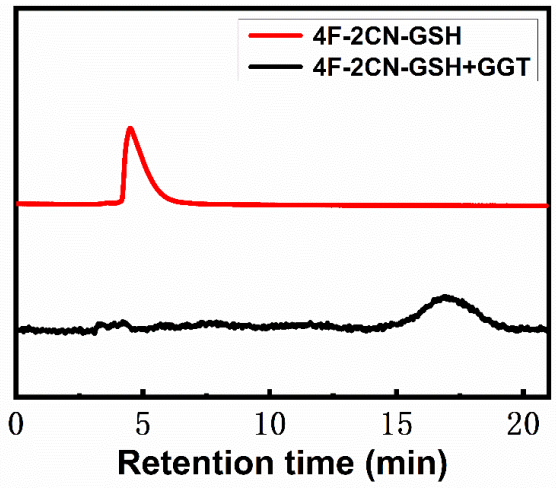


Figure S9. HPLC of probe **4F-2CN-GSH** (100 μM) and the reaction solution of 100 μM probe **4F-2CN-GSH** with 300 U/L GGT.

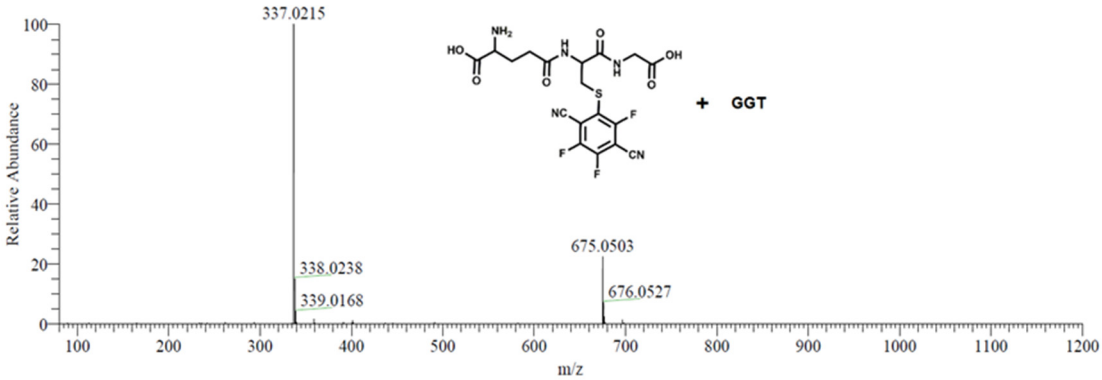


Figure S10. ESI-MS spectra of **4F-2CN-GSH** in the presence of GGT.

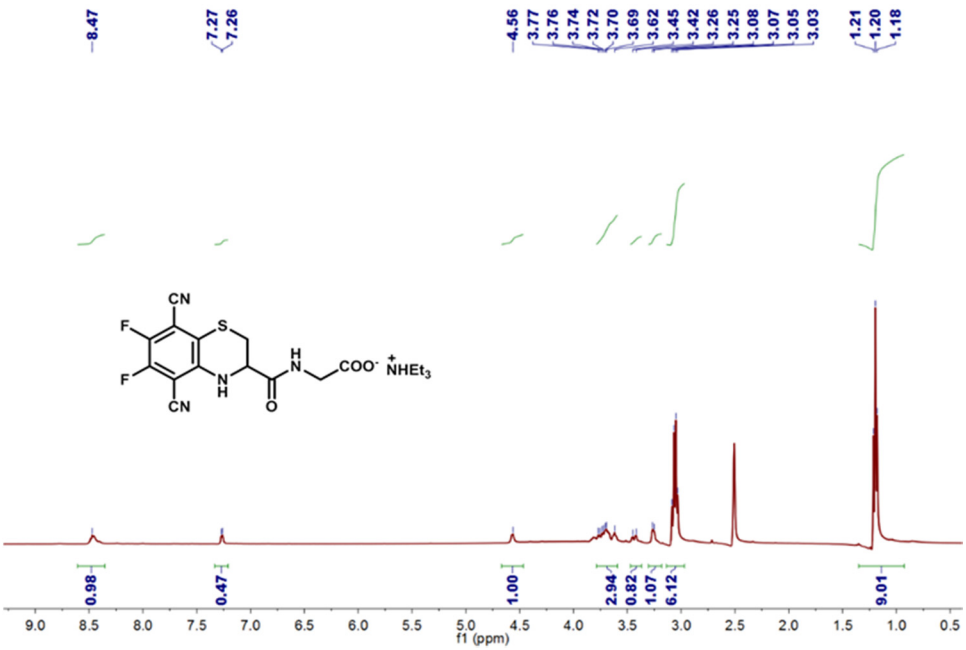


Figure S11. [1]H NMR chart of product **1**.

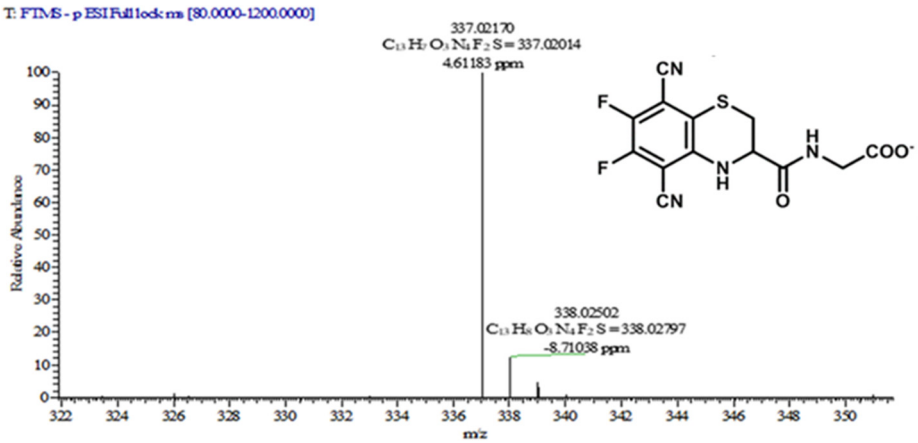


Figure S12. ESI-MS spectra of product **1**.

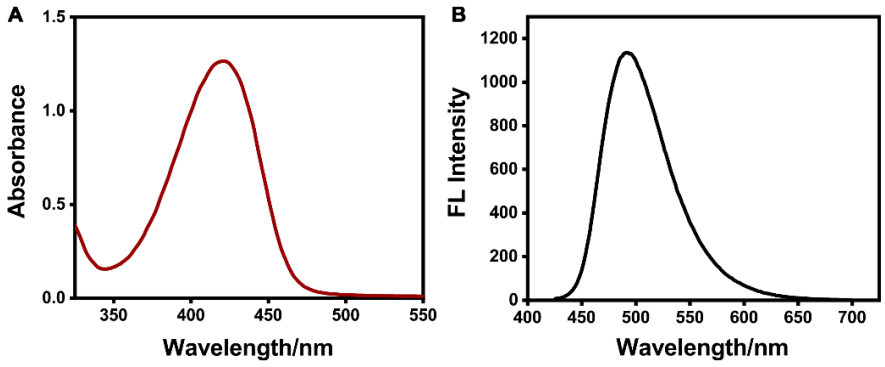


Figure S13. (A) Absorption and (B) fluorescence spectra of product **1**.

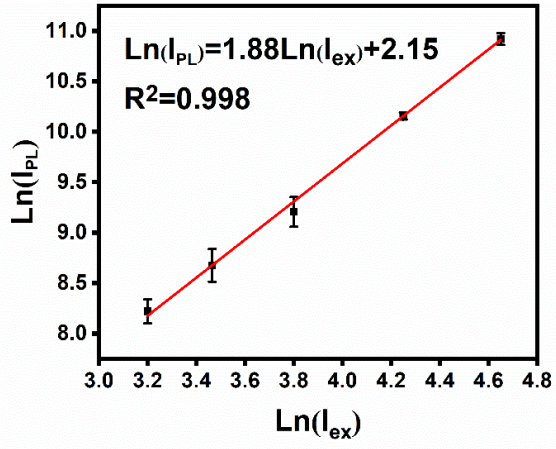


Figure S14. The plot of emission intensity against incident power at 800 nm. Conditions: PBS (pH = 7.4, 10 mM) at 37 °C.

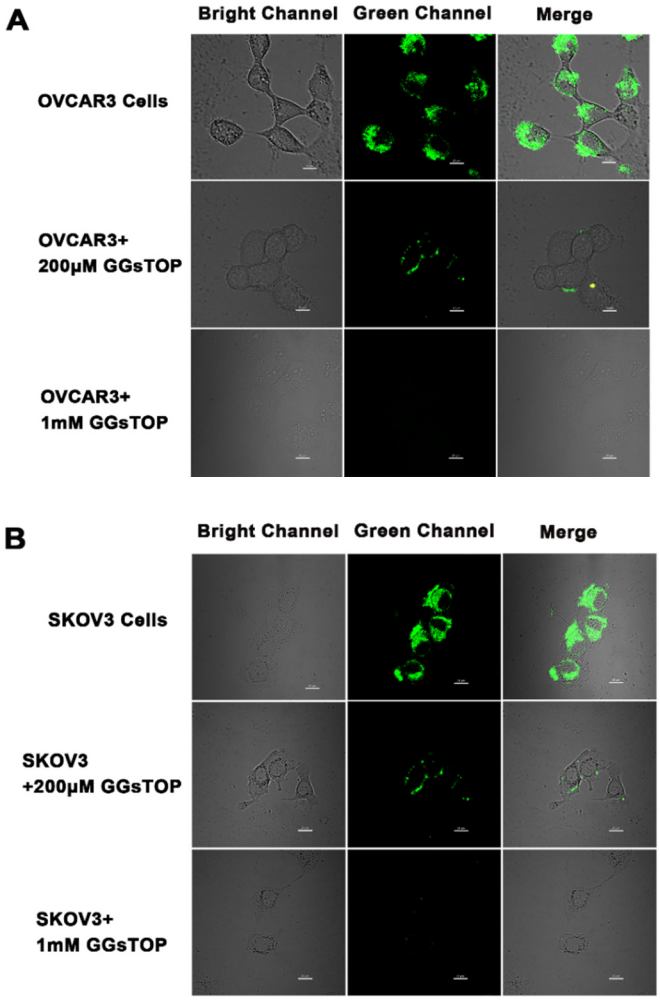


Figure S15. Fluorescent confocal image of OVCAR3 cells (A) and SKOV3 cells (B) incubated with probe **4F-2CN-GSH** for 60 minutes, or pretreated with GGsTOP for 30 minutes then loaded with probe **4F-2CN-GSH** (100 μM) for 60 minutes. The excitation

wavelength was 405 nm and the emission was collected at 500–530 nm (green channel). Scale bar: 20 μm.

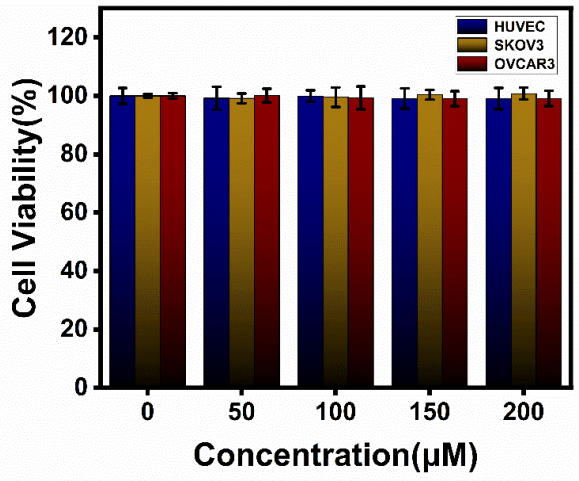


Figure S16. Cell viability for HUVEC, SKOV3 and OVCAR3 cells in the presence of probe **4F-2CN-GSH** at varying concentrations.

References

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