Electronic Supplementary Information

Ratiometric fluorescent assay for γ-glutamyltranspeptidase based on a single fluorophore via analyte-induced variation of substitution

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Experimental

Reagents and Materials: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloricde (EDC), BOC-anhydride, N,N-diisopropylethylamine and 4-dimethyaminopyridine were purchased from Aladdin Reagents. 4-Amino-1,8-naphthalic anhydride, 6-diazo-5-oxo-L-norleucine, 4,7,10-trioxa-1,13-tridcandiamine (OEG), triethylamine, N,N-diisopropylethylamine (DIEA), Boc-L-glutamic acid 1-tert-butyl ester (Boc-Glu-OtBu) and 6-diazo-5-oxo-L-norleucine (DON) were purchased from Sigma Aldrich. γ-Glutamyltranspeptidase (GGT) ELISA Kit was purchased from IBL International GmbH (Germany). Human serum was supplied by The Sixth Affiliated Hospital, Sun Yat-sen University, and the serum samples were from both healthy males and unhealthy males with abnormal GGT levels (first, the blood was centrifuged for 20 min at 3000 rpm. Then the supernatant was collected as soon as possible and storied at 2-8 °C for use). Urine was obtained from a health adult male. Ethyl alcohol, ethyl acetate, methanol and dichloromethane were analytical grade solvents. Anhydrous magnesium sulfate, sodium chloride, concentrated sulfuric acid are of analytical grade. The water used throughout the experiments was the triple-distilled water which was further treated by ion exchange columns and then by a Milli-Q water purification system.

Synthesis of 1: To a stirred solution of 4,7,10-trioxa-1,13-tridcandiamine (11.0 g, 50 mmol) in dichloromethane (50 mL) at 0 °C under a nitrogen atmosphere, Boc-anhydride (1.1 g, 5 mmol) was added dropwise in 3 hours. The solution was stirred at room temperature for 18 hours. Then the mixture was washed with water (6 × 50 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated and the product was dried under vacuum at 40 °C overnight as a colorless oil (yield: 63%). ^{1}H NMR ($CDCl_3$, 400 MHz, ppm): 1.45 (s, 9H), 1.77 (m, 4H), 2.85 (t, J = 6.0 Hz, 2H), 3.23 (t, J = 6.4 Hz, 2H), 3.53-3.67(m, 12H). MS(ESI): m/z 321.3 $[M+H]^+$.

Synthesis of 2: Under a nitrogen atmosphere, the above-obtained compound **1** (320 mg, 1 mmol), 4-amino-1,8-naphthalic anhydride (85 mg, 0.4 mmol) and triethylamine (139 μL, 1 mmol) were dissolved under stirring in ethyl alcohol for 10 min, then heated to 85°C and refluxed overnight. The mixture was allowed to cool slowly to room temperature. Then the solvent was evaporated and the residue was purified by column chromatography on silica gel (ethyl acetate: dichloromethane = 3: 1 in v/v) to furnish the desired product (yield: 78%) as a brown sticky liquid. ^{1}H NMR ($CDCl_3$, 400 MHz, ppm): 1.43 (s, 9H), 1.65-1.78 (m, 4H), 3.21 (t, J = 6.0 Hz, 2H), 3.49-3.64 (m, 12H), 4.26 (t, J = 7.2 H, 2H), 6.89 (d, 1H) , 7.64 (t, J = 6.4 Hz, 1H) , 8.13 (d, 1H) , 8.39 (d, 1H) , 8.57 (d, 1H). MS(ESI): m/z 538.1 $[M+Na]^+$.

Synthesis of 3: Under a nitrogen atmosphere, compound **2** (76 mg, 0.15 mmol), EDC (67 mg, 0.35 mmol) and DMAP (1 mg, 0.008 mmol) were dissolved under stirring in dichloromethane, then the mixture of Boc-Glu-OtBu (90 mg, 0.3 mmol) and DIEA (26 mg, 0.2 mmol) in dichloromethane was added. The solution was stirred at room temperature for 24 hours. Then the solvent was evaporated and the residue was purified by column chromatography on silica gel (ethyl acetate: dichloromethane = 2: 1 in v/v) to furnish the

desired product (yield: 18%) as a brown solid. ^{1}H NMR ($CDCl_3$, 400 MHz, ppm):1.43 (s, 9H), 1.44 (s, 9H), 1.47 (s, 9H), 1.74 (m, 2H), 2.03 (m, 2H), 2.17 (t, 2H), 2.43 (m, 2H), 3.2 (t, J = 7.2 H, 2H), 3.49-3.64 (m, 12H), 4.21-4.28 (m, 3H), 6.87 (d, 1H) , 7.63 (t, J = 6.4 Hz, 1H) , 8.13 (d, 1H) , 8.38 (d, 1H) , 8.57 (d, 1H). MS(ESI): m/z 823.3 $[M+Na]^+$.

Synthesis of 4 (the probe): Compound **3** (32 mg, 0.04 mmol) was dissolved under stirring in 10 mL CH_2Cl_2, then hydrogen chloride generated from the reaction of NaCl and H_2SO_4 was passed into the solution for 1 h. Afterwards the solvent was evaporated under vacuum to get the product (yield: 95%). ^{1}H NMR (D_2O, 400 MHz, ppm):1.72-1.88 (m, 4H), 2.18 (m, 2H), 2.58 (t, J = 7.2 Hz, 2H), 3.02 (t, J = 6.4 Hz, 2H), 3.38-3.56 (m, 12H), 3.74 (t, J = 6.8 Hz, 1H), 4.01 (t, J = 7.2 Hz, 2H), 6.26 (d, 1H) , 7.03 (t, J = 6.4 Hz, 1H) , 7.41 (d, 1H) ,7.56-7.74 (m, 2H). MS(ESI): m/z 545.2 $[M+H]^+$.

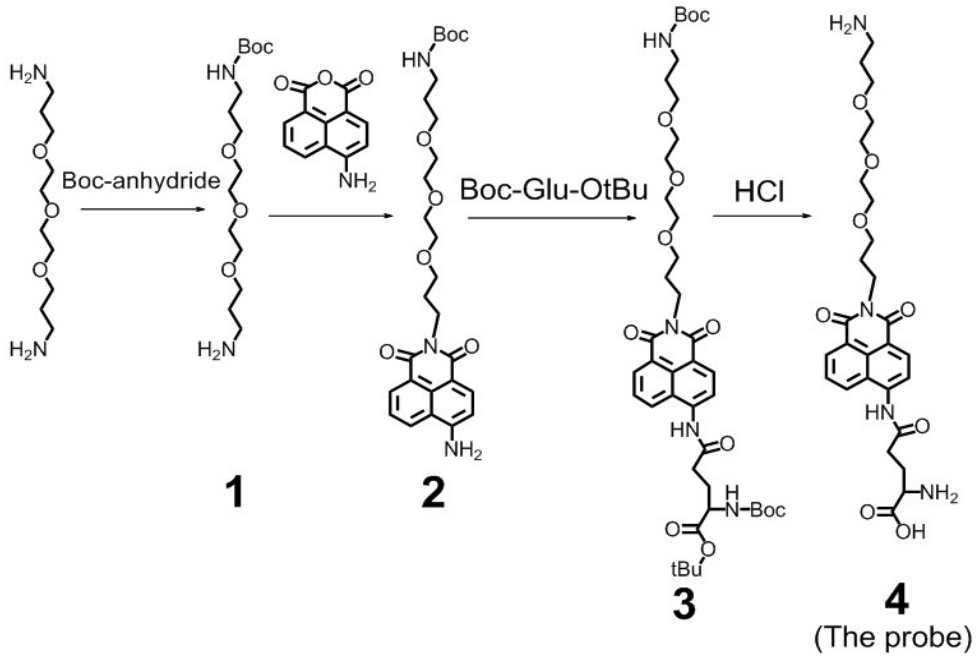
Purification of the product resulted from the reaction between the probe and GGT: The product from the reaction between the probe and GGT is purified as follows: the probe (11 mg, 0.02 mmol) was dissolved under stirring in 5 mL PBS-buffered water (pH 7.4, 10 mM) water in the presence of GGT (500 U/L) at 37°C for 1 hour. The mixture was washed with ethyl acetate for four times and dried over anhydrous Na_2SO_4. The solvent then was evaporated and the product was dried under vacuum at 45°C overnight as a light yellow solid (yield: 51%). ^{1}H NMR (D_2O, 400 MHz, δ ppm): 1.94-2.03 (m, 4H), 3.17 (t, J = 5.2 Hz, 2H), 3.56-3.71 (m, 12H), 4.23 (t, J = 7.2 Hz, 2H), 6.89 (d, 1H) , 7.64 (t, J = 6.4 Hz, 1H) , 8.27 (d, 1H) ,8.45-8.53 (m, 2H). MS(ESI): m/z 415.9 [M+H]+.

γ-Glutamyltranspeptidase (GGT) ELISA Kit assay: Detection of GGT using the ELISA Kit assay was conducted according to the instructions of the ELISA Kit, which involves 11 steps.

Cell viability assay: To examine the toxicity of the sensor in living cells, L929 cells (murine aneuploid fibrosarcoma cells) and HeLa (human cervical cancer cell) were incubated in DMEM medium supplemented with 10% fetal bovine serum (FBS) and allowed to grow for 24 h at 37°C with 5% CO_2. After removal of the medium, cells were treated with the probe and incubated for an additional 24 h. The cytotoxicity of the probe against two cells was assessed by MTT assay according to ISO 10993-5. As for the assays, for each independent experiment, the assays were performed in eight replicates. And the statistical mean and standard deviation were used to estimate the cell viability.
Cell incubation and imaging: Two cell lines, A2780 (human ovarian cancer cell) and L929 (murine aneuploid fibrosarcoma cell), were incubated in DMEM medium supplemented with 8% and 10% fetal bovine serum (FBS, hyclone) respectively. One day before imaging, cells were passaged and plated on polylysine-coated cell culture glass slides inside the 30 mm glass culture dishes and allowed to grow to 50-70% confluence. For the experiments, cells were washed with DMEM, incubated in DMEM medium containing the probe (1×10^{-5} M) at 37°C under 5% CO_2 for 1 hour. Some A2780 cells were incubated in DMEM medium containing inhibitor (DON, 1×10^{-5} M) for 30 min, and then treated with the probe (1×10^{-5} M) for 1 h. The control sample was incubated in DMEM medium only.

After that, the culture dishes were washed with PBS, then glass slides were taken out, the cells were washed with PBS for three times and then imaged on an Olympus IX71 inverted fluorescence microscope equipped with a DP72 color CCD.

Measurements: 1H NMR spectra were recorded on a Bruker Avance 400 MHz NMR spectrometer. Mass spectra were obtained through a Bruker Esquire HCT Plus mass spectrometer. UV-vis spectra were recorded on a Hitachi U-3010 UV-vis spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4600 fluorescence spectrophotometer. Fluorescence images were obtained using an Olympus IX 71 with a DP72 color CCD (mirror unit U-MNV2, excitation filter 400 – 410 nm, emission filter ≥ 455 nm).



Scheme S1. Synthetic route for the probe.

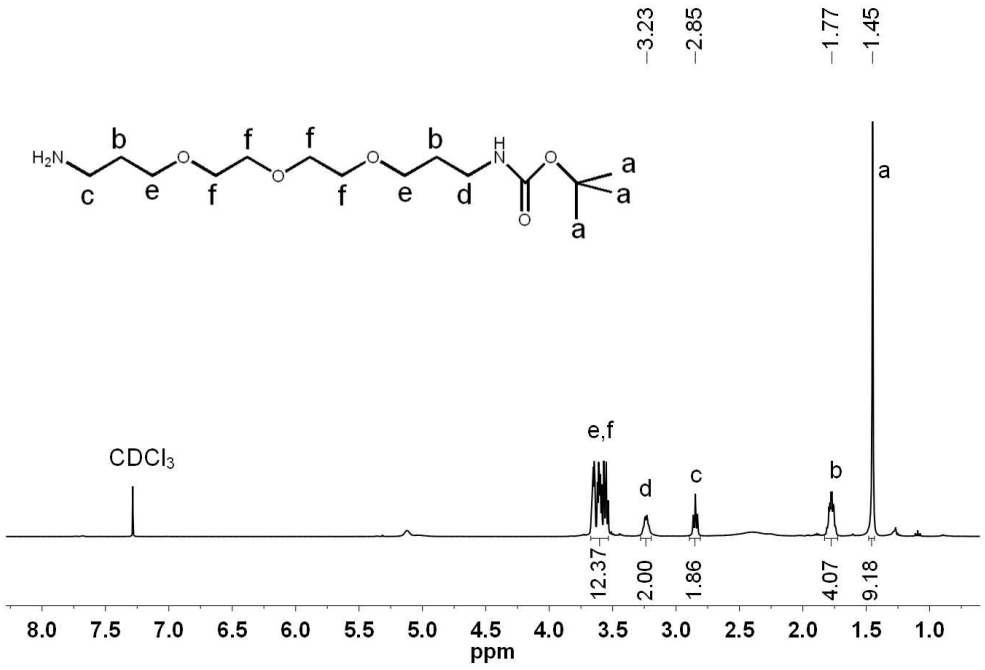


Figure S1. [1]H-NMR spectra of **1**.

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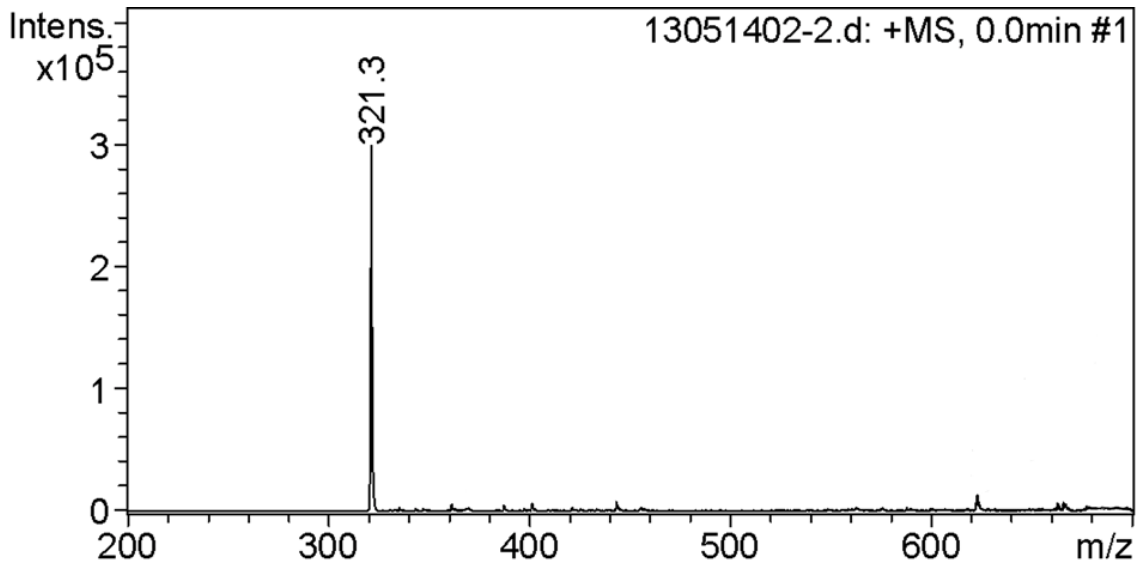


Figure S2. Mass spectra of **1**. MS(ESI): m/z 321.3 $[M+H]^+$.

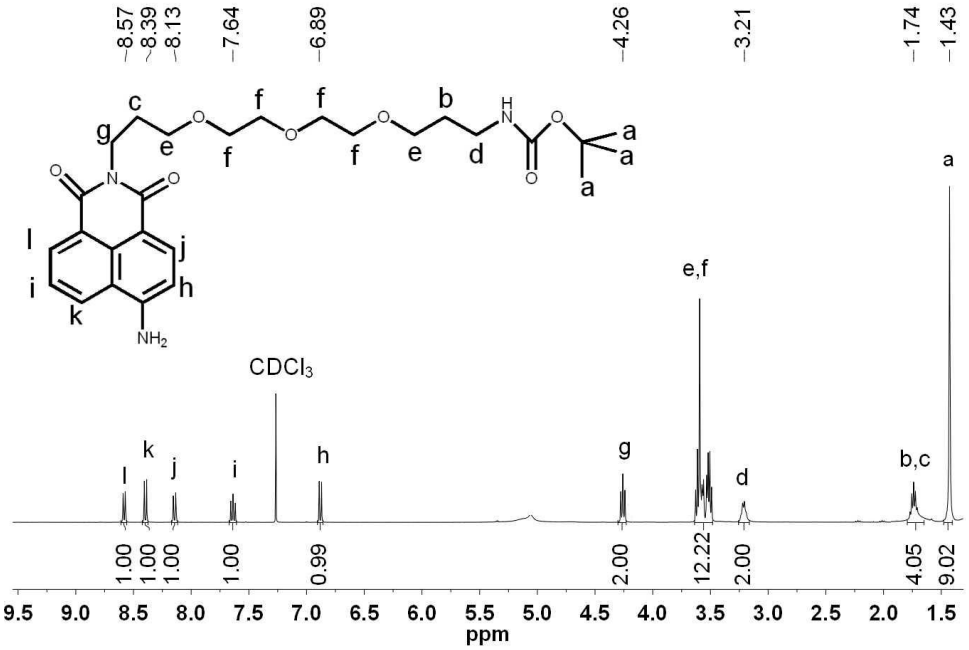


Figure S3. ^{1}H-NMR spectra of **2**.

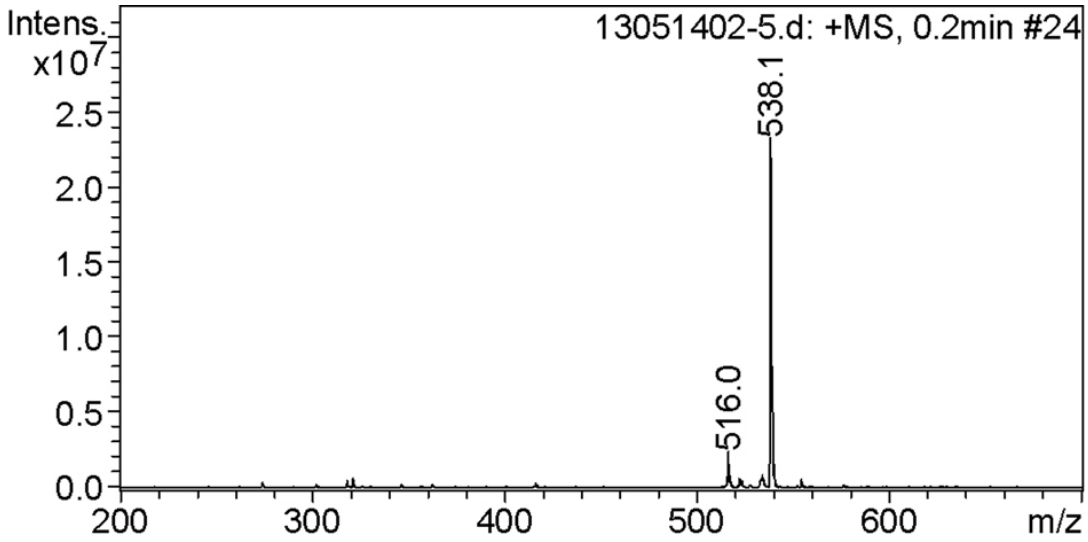


Figure S4. Mass spectra of **2**. MS(ESI): m/z 538.1 $[M+Na]^{+}$.

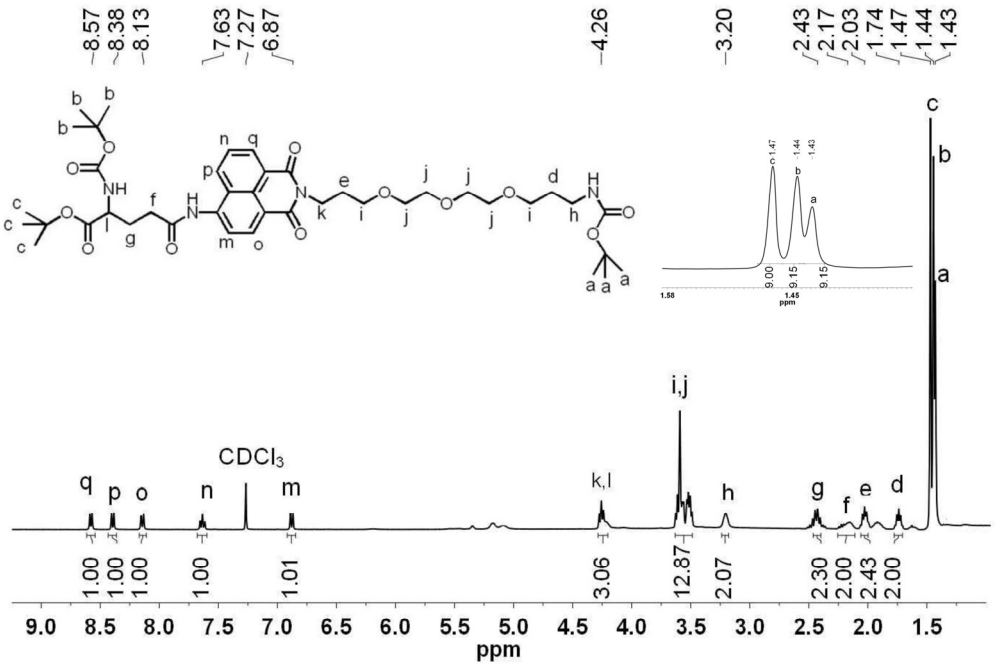


Figure S5. ^{1}H-NMR spectra of **3**.

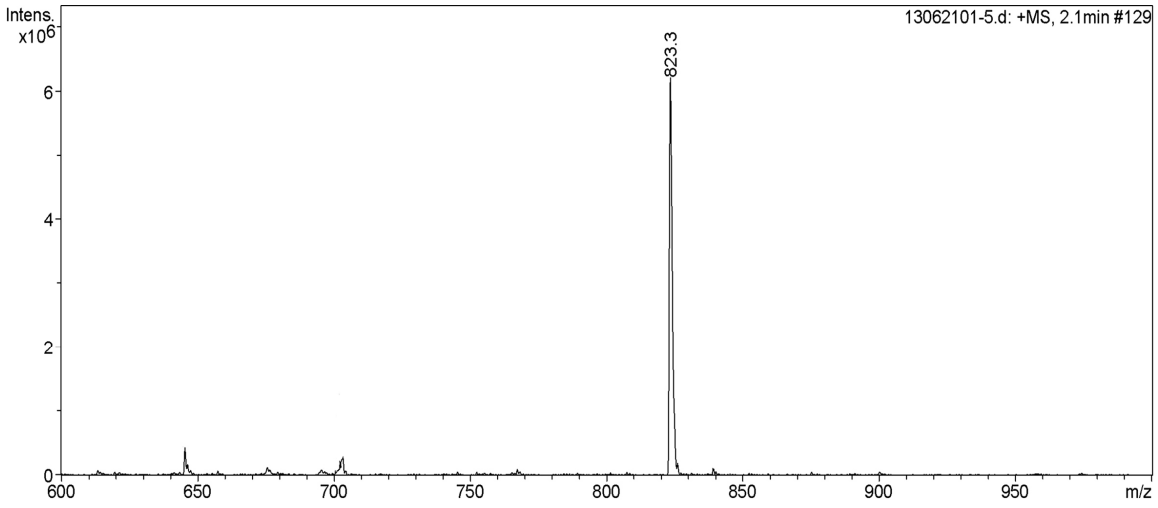


Figure S6. Mass spectra of **3**. MS(ESI): m/z 823.3 $[M+Na]^{+}$.

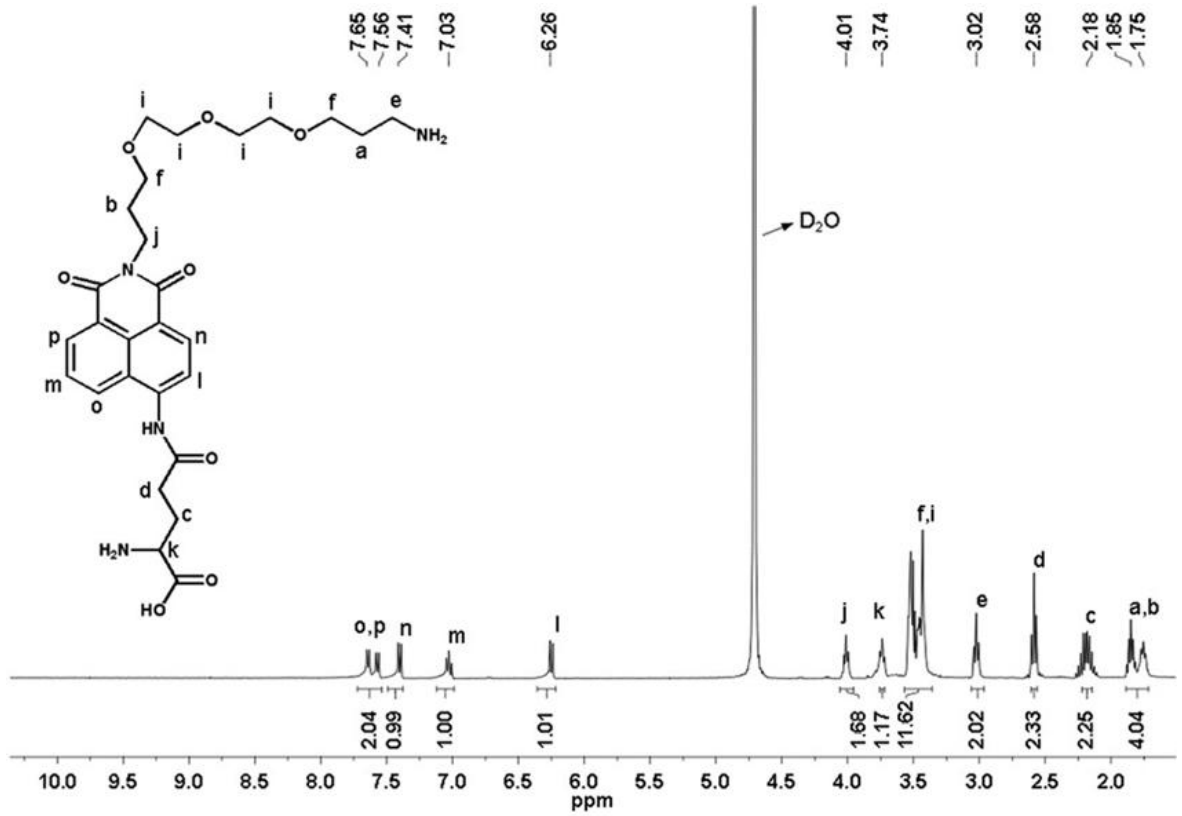


Figure S7. ^{1}H-NMR spectra of **4**.

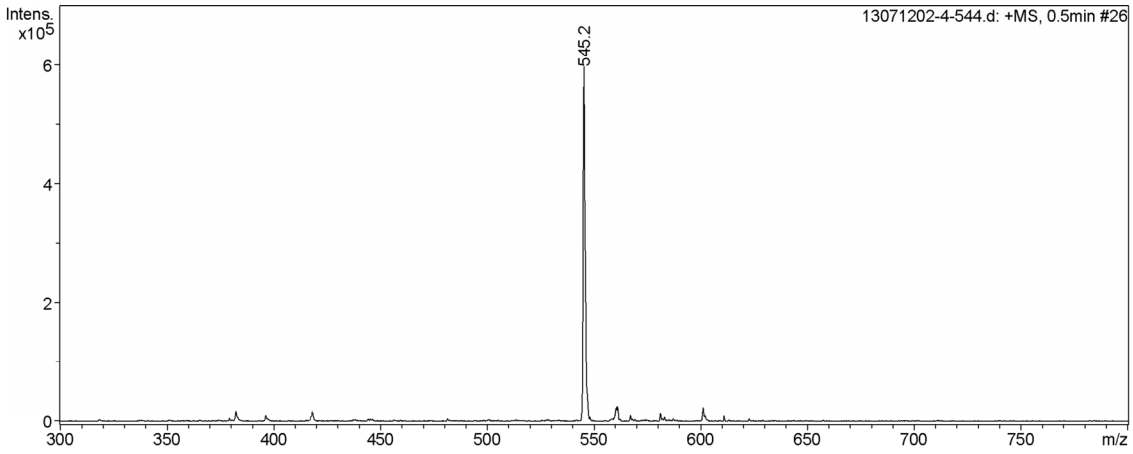


Figure S8. Mass spectra of **4**. MS(ESI): m/z 545.2 $[M+H]^{+}$.

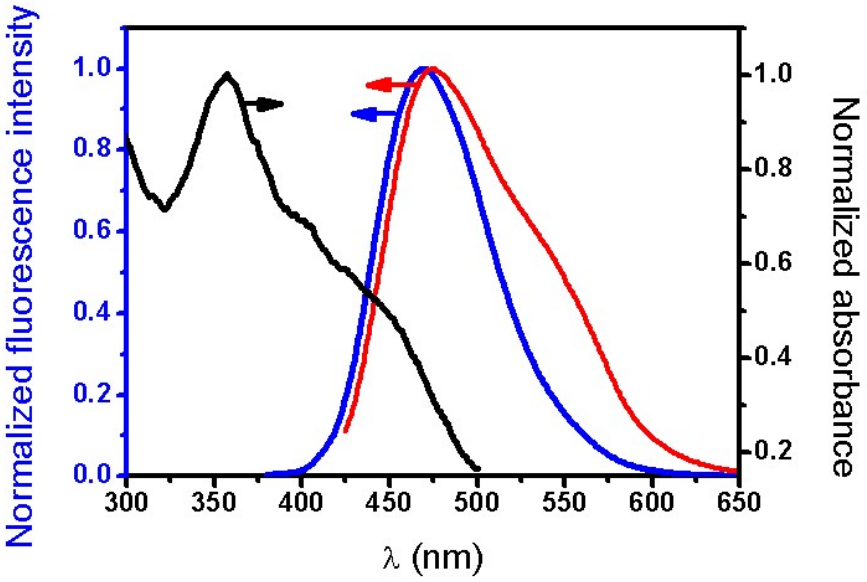


Figure S9. UV-vis absorption spectrum and fluorescence emission spectrum (blue curve: λex = 360 nm, red curve: λex = 408 nm) of the probe (6×10^{-6} M) in PBS-buffered (pH 7.4, 10 mM) water.

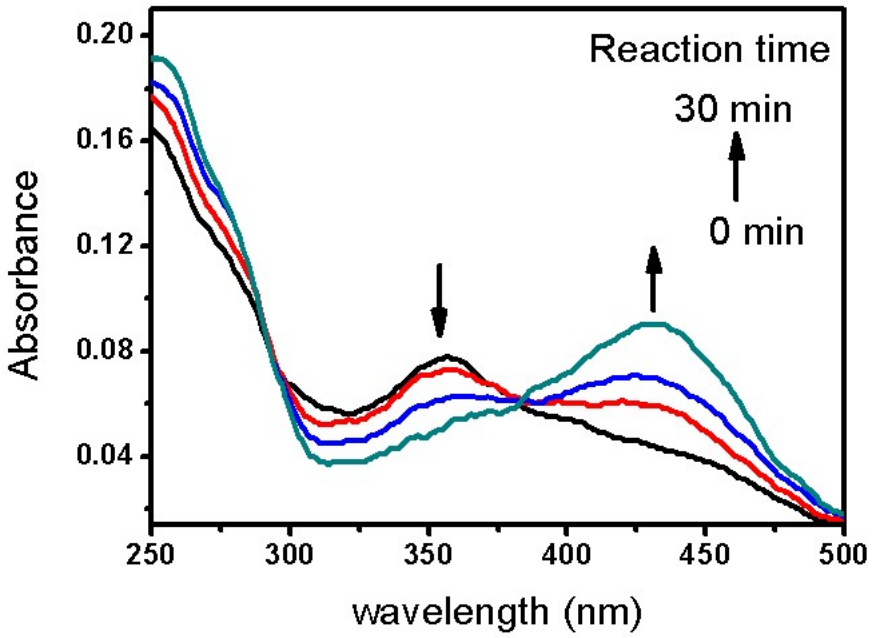


Figure S10. Absorption spectra of the probe as a function of reaction time in the presence of GGT (50 U/L) in PBS-buffered water (pH 7.4, 10 mM) at 37°C.

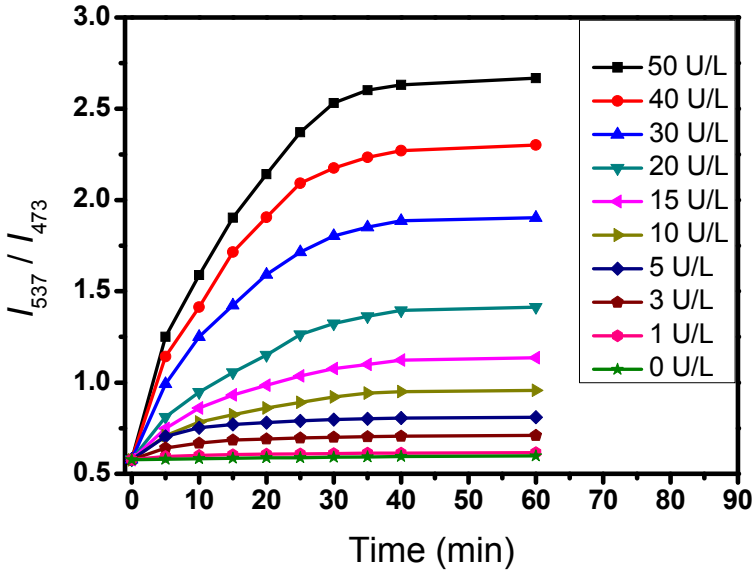


Figure S11. Plot of the fluorescence intensity ratio of the probe (6×10^{-6} M) in PBS-buffered (pH 7.4, 10 mM) water at 37°C versus reaction time in the presence of different amounts of GGT. Excitation wavelength: 408 nm.

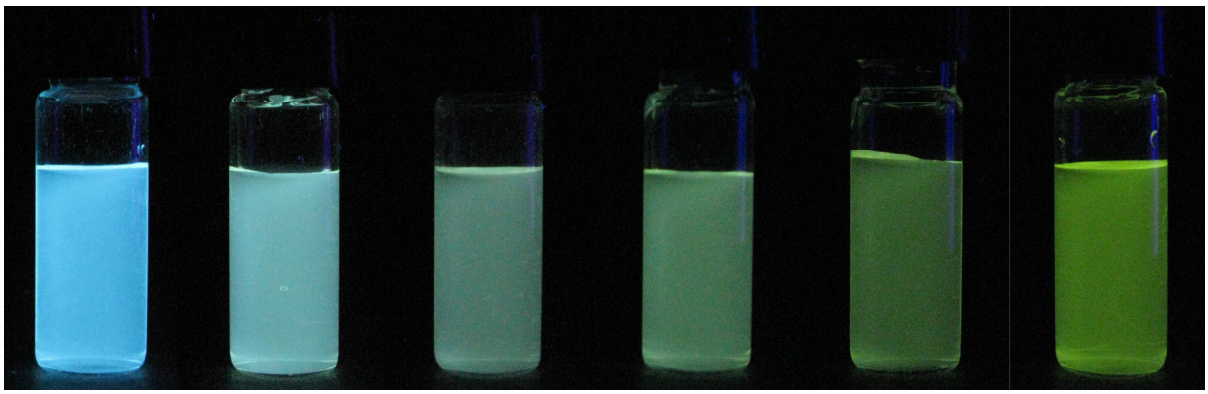


Figure S12. Fluorescence change of the probe (6×10^{-6} M) as a function of reaction time in the presence of GGT (50 U/L) in PBS-buffered water (pH 7.4, 10 mM). Photographs were taken at 0, 5, 10, 15, 20, 30 min successively (left to right) under a hand-held 365 nm UV lamp.

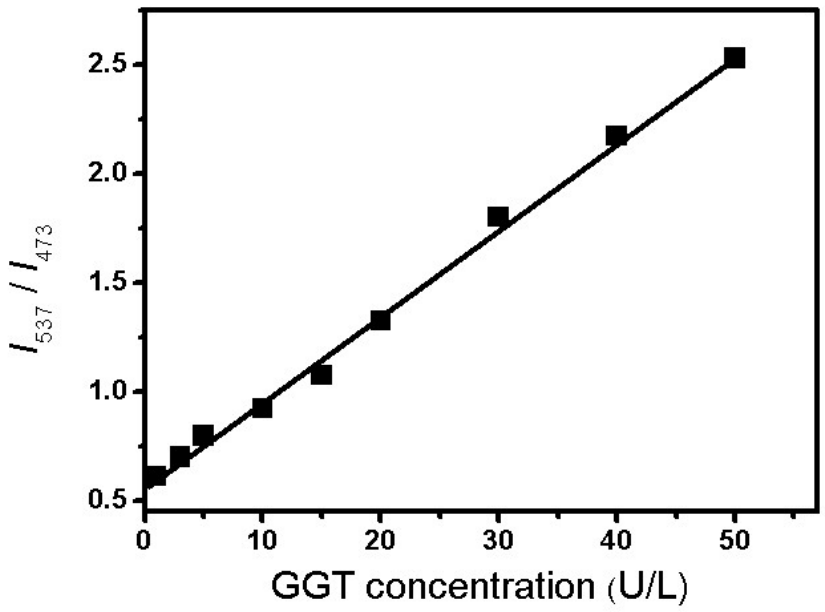


Figure S13. Fluorescence intensity ratio for the assay system (6×10^{-6} M) in PBS-buffered water (pH 7.4, 10 mM) as a function of GGT level. Excitation wavelength: 408 nm.

Determination of the detection limit:

First the calibration curve was obtained from the plot of fluorescence intensity ratio (I_{537}/I_{473}) as a function of the analyte concentration (GGT). The regression curve equation was then obtained for the lower concentration part.

The detection limit = 3 × S.D. / k

where k is the slope of the curve equation, and S.D. represents the standard deviation for the fluorescence intensity ratio of the assay system in the absence of GGT.

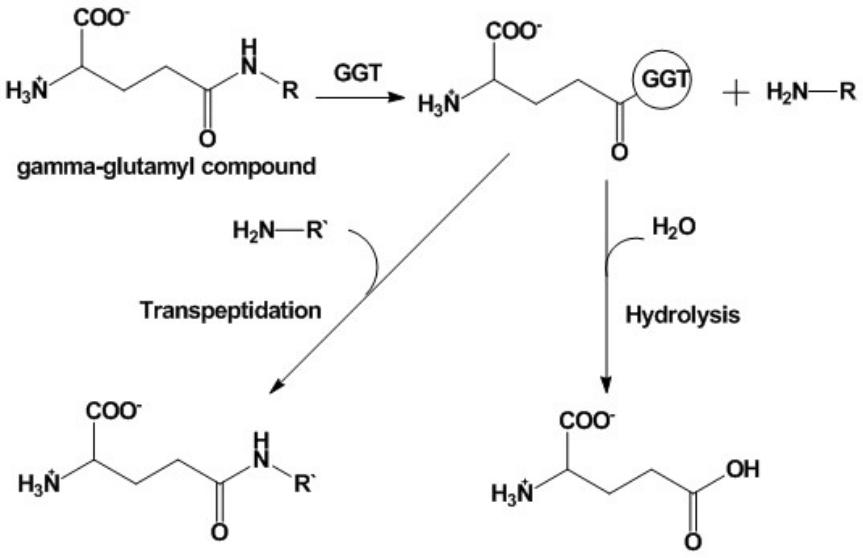
I_{537}/I_{473} = 0.55 + 0.03979 × [GGT] (R = 0.997)

LOD = 3 × 0.0102 / 0.03979 = 0.76 U/L

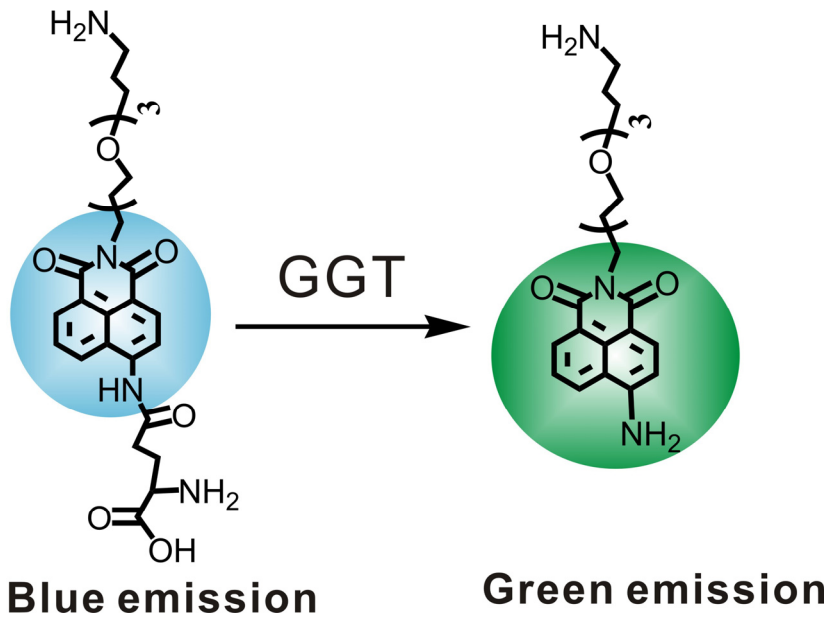
References:

V. Thomsen, D. Schatzlein and D. Mercuro, *Spectroscopy*, 2003, **18**, 112-114.

A. D. McNaught and A. Wilkinson, *IUPAC Compendium of Chemical Terminology*, 1997.



(A)



(B)

Figure S14. (A) Reactions catalyzed by GGT: transpeptidation and hydrolysis. (B) Schematic illustration for the structure of the probe, and its ratiometric fluorescent response toward GGT.

GGT is known to cleave γ-glutamyl amide bonds. GGT catalyzes not only the hydrolysis reaction of various γ-glutamyl compounds, but also the transpeptidation

reaction.

The enzyme can hydrolyze the γ-glutamyl amide bond, a reaction favoured under physiological conditions; and hydrolysis is the primary reaction catalyzed by the enzyme in vivo. Also the enzyme can transfer the γ-glutamyl group to an acceptor amino acid, a process termed ‘transpeptidation’; increased pH and high concentrations of acceptor molecules, which include free amino acids and peptide acceptors, shift the kinetics to favour the transpeptidation reaction.

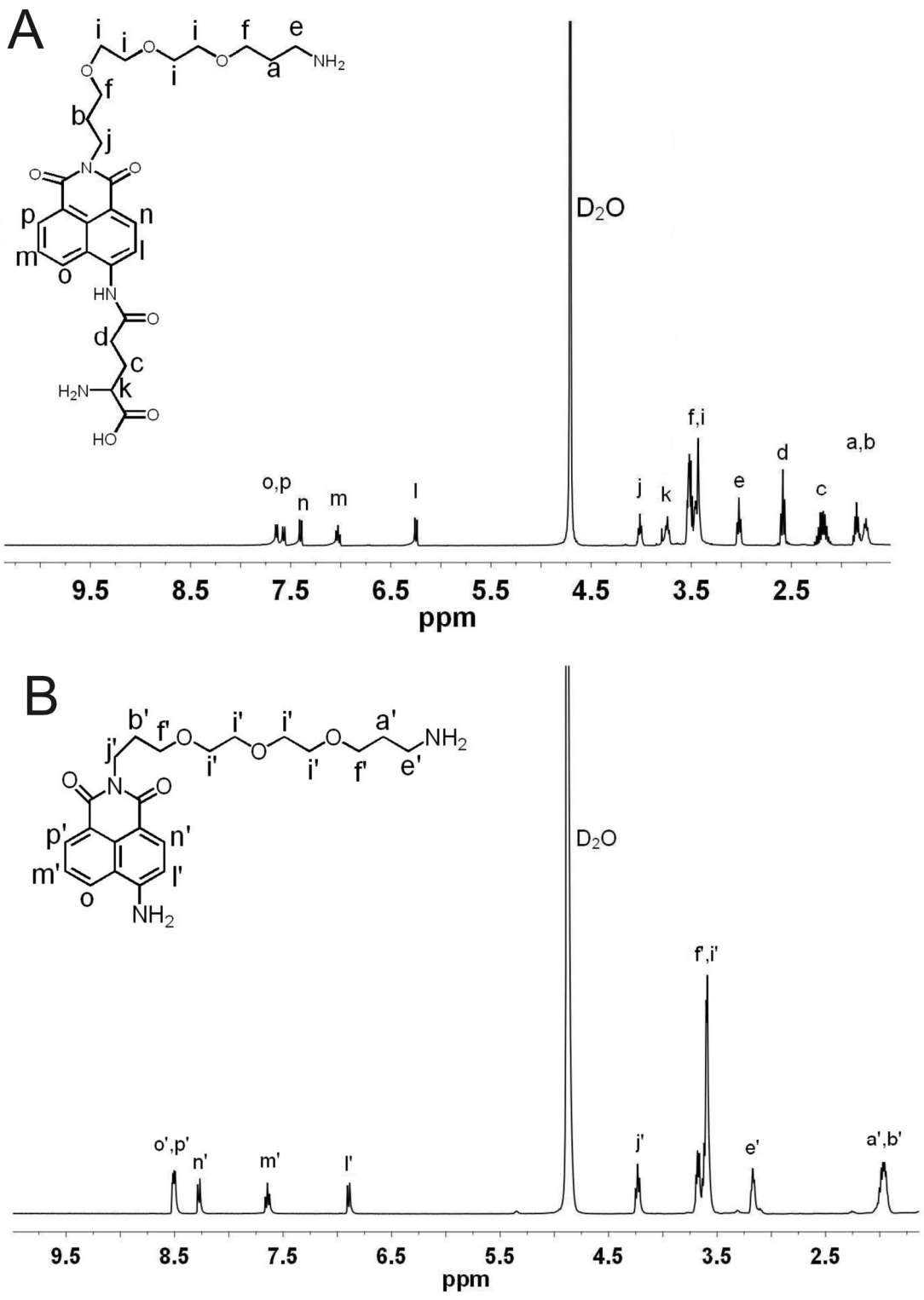


Figure S15. ^{1}H NMR spectra of the as-synthesized probe (A) and the product upon reaction with GGT (B).

Upon deacetylation, the peaks for protons of naphthalic ring shift downfield due to the electron-donating capability of amino group at the 4th position, e.g. the peaks for proton O shifted from 7.5-7.7 ppm (O) to 8.5 ppm (O'), the peaks for proton l shifted from 6.3 ppm (l) to 6.9 ppm (l'), and the peaks for proton m shifted from 7.0 ppm (m) to 7.6 ppm (m')

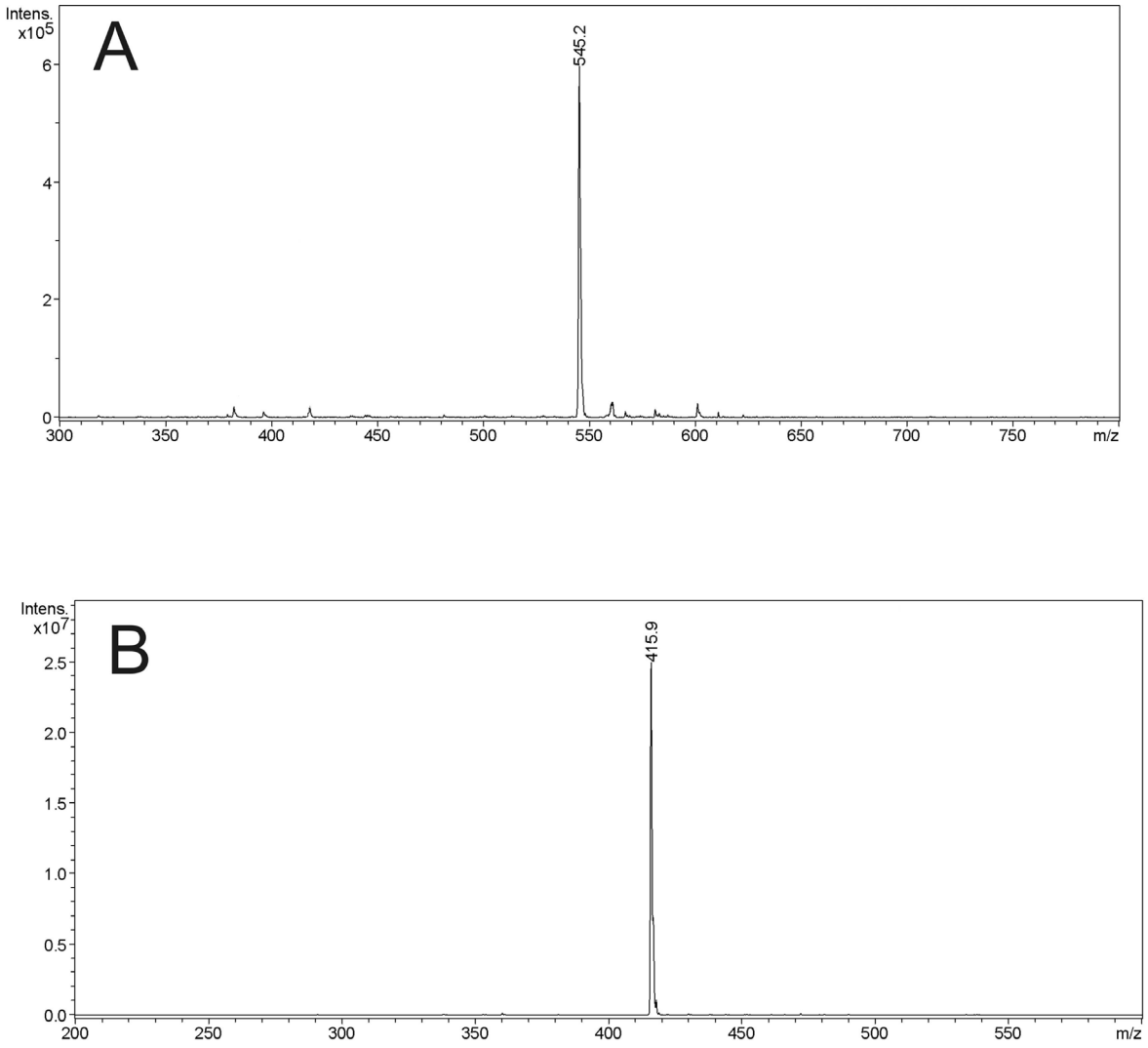


Figure S16. Mass spectra of the as-synthesized probe (A) and the product upon reaction with GGT (B). The signals at m/z 545.2 are $[probe+H]^+$, and the signals at m/z 415.9 are $[reaction\ product + H]^+$.

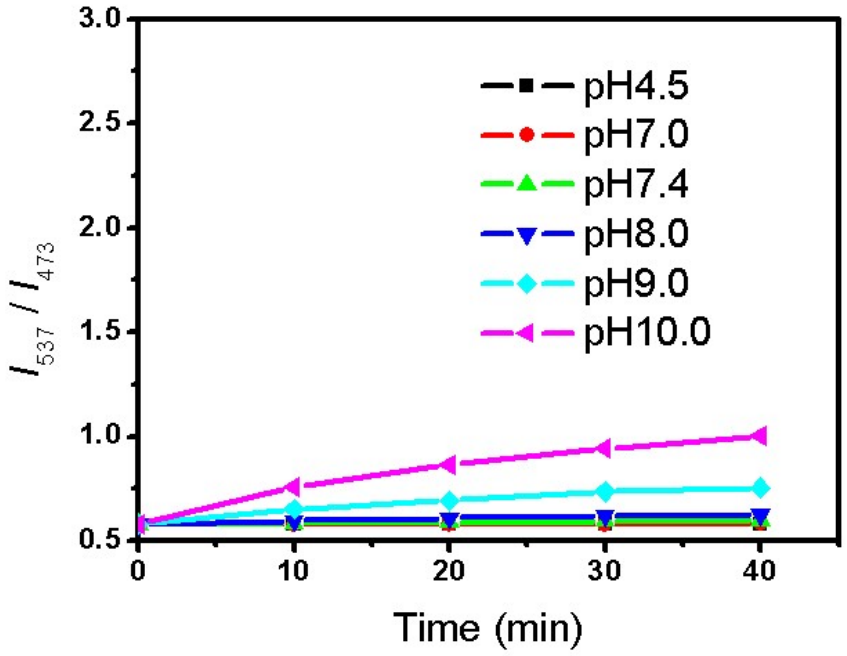


Figure S17. Variation of I_{537}/I_{473} for the assay system in the absence of GGT as a function of pH. Excitation wavelength: 408 nm.

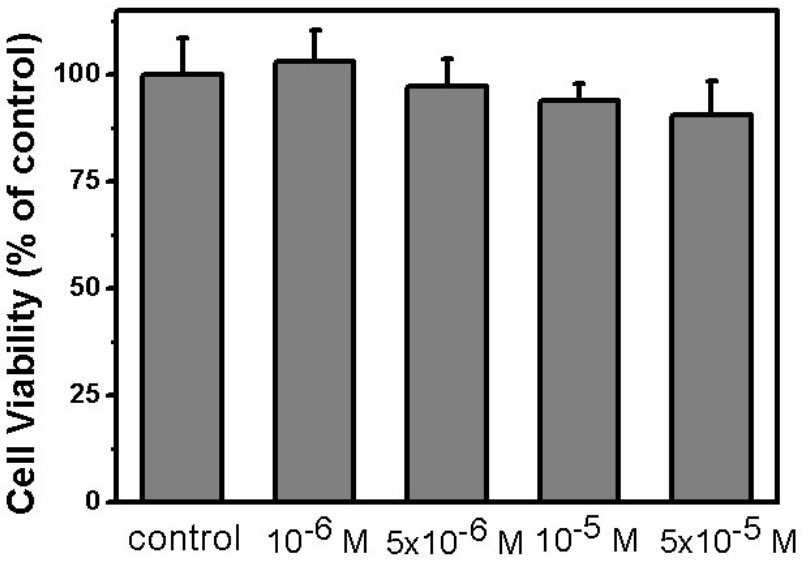


Figure S18. Cell viability for L929 cells in the presence of the probe at varied concentrations. The results are the mean standard deviation of eight separate measurements.

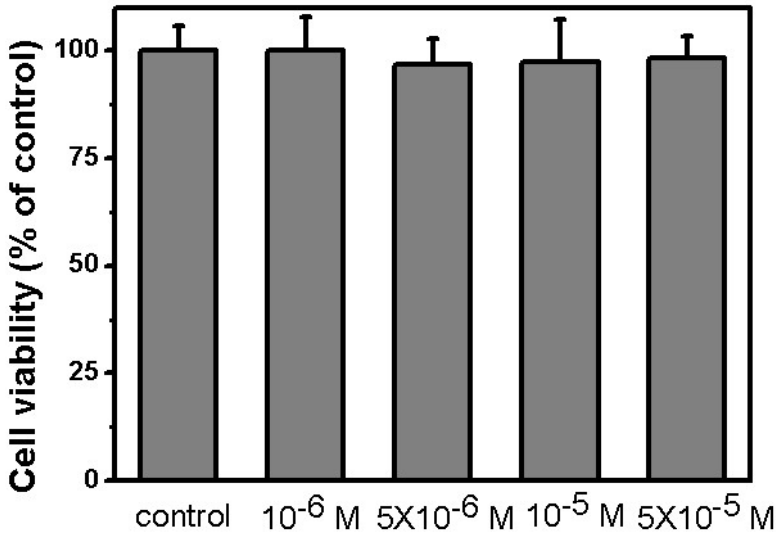


Figure S19. Cell viability for HeLa cells in the presence of the probe at varied concentrations upon 24 hours of incubation. The results are the mean standard deviation of eight separate measurements.

Table S1. Comparison of GGT detection in buffer by using Elisa kit assay and this probe.

Method	Sample	GGT amount (U/L)		Recovery	SD (%)
		Added	Found	(%)	
ELISA Kit	1	20.00	19.61	98.0	9.20
	2	30.00	28.86	96.3	8.50
	3	40.00	37.22	93.1	10.0
This probe	1	20.00	19.52	97.6	3.40
	2	30.00	30.74	102.5	3.10
	3	40.00	41.64	104.1	3.30

Note: SD represents the standard deviation.

Table S2. GGT detection in human urine samples by using Elisa kit assay and this probe.

Method	Amount of GGT level(U/L)			
	Endogenous	Added GGT		
		5	10	20
This probe	--	5.20±0.20	9.91±0.18	20.52±0.57
Elisa kit	--	5.05±0.24	9.87±0.33	20.68±0.86

The probe was applied to detect GGT in urine samples, and the results are shown in Table S2. For healthy human beings, generally there is no endogenous GGT in urine. Thus, different amounts of GGT were added into urine samples, and the recovery of added known amounts of GGT into the urine samples is in general more than 91% by the probe (Table S1), this proves that the composition of urine does not interfere with the detection of GGT, indicating the potential application of this assay in real samples.

Table S3. Endogenous GGT detection in human serum samples by using Elisa kit assay and this probe.

Sample No.	GGT amount(U/L) by ELISA Kit assay[a]	GGT amount(U/L) by this probe[b]
A[c]	60.52 ± 5.17	59.70 ± 1.10
B[c]	65.43 ± 4.21	65.01 ±1.00
C[c]	76.00 ± 4.88	75.03 ± 1.23
D[d]	135.40 ± 12.24	138.64 ± 5.92
E[d]	152.31 ± 6.80	148.42 ± 3.86
F[d]	258.75 ± 10.25	243.05 ± 4.99
G[d]	217.85 ± 12.71	223.31 ± 7.02
H[d]	173.89 ± 11.43	168.34 ± 6.86

[a] When GGT level was determined by using Elisa kit; the serum samples were 5-fold diluted for measurement; and the values herein represent GGT levels in the undiluted serum samples, which are calculated based on the measurements for the diluted samples. [b] When GGT level was determined by using this probe; the serum samples were 10-fold diluted for measurement; and the values herein represent GGT levels in the undiluted serum samples, which are calculated based on the measurements for the diluted samples.. [c] Serum samples were obtained from healthy males. [d] Serum samples were obtained from unhealthy males with abnormal GGT levels. All the serum samples were provided by The Sixth Affiliated Hospital, Sun Yat-sen University.