Electronic Supplementary Information (ESI)

Dual-channel near-infrared fluorescent probe for real-time tracking endogenous γ-glutamyl transpeptidase activity

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1. Materials and instruments

Unless special stated, all solvents and chemicals were purchased from commercial suppliers in analytical grade and used without further purification. Biotin-PEG$_5$-N$_3$ was supplied by Biomatrik Inc. (Jiaxing, China); $\gamma$-Glutamyltranspeptidase (GGT, microsomal from equine kidney) and 6-diazo-5-oxo-l-norleucine (DON) was purchased from Sigma-Aldrich.

All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Flash chromatography (FC) was performed using silica gel (200-300 mesh). $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer with chemical shifts reported in ppm at room temperature. High-resolution mass spectra (HRMS) were recorded on a Waters LCT Premier XE spectrometer (ESI-TOF). Absorption and fluorescence spectra were measured with an Agilent Cary 60 spectrophotometer and Agilent Eclipse fluorescence spectro-photometer, respectively (10 $\times$ 10 mm quartz cuvette). HPLC analysis was operated on an Agilent 1100 series. Confocal fluorescence images were taken on confocal laser scanning microscope (CLSM, Nikon A1R/A1). Flow cytometric analysis was carried out with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).
2. Synthesis of compounds

Scheme S1. Synthetic route of Glu-DFB-Biotin and Glu-DFB
Synthesis of compound 1

CDFB (709.9 mg, 4.8 mmol) and 4-(prop-2-ynyloxy)benzaldehyde (384.5 mg, 2.4 mmol) were dissolved in ethyl acetate (75 mL) with tributyl borate (3 mL) and butylamine (0.5 mL) was added dropwise. The reaction was under an argon atmosphere. The resulting mixture was stirred at room temperature for 5 h. There was some orange precipitation in the bottom of flask, a yellow-green solution was obtained through filtration. Then remove the solvent by reduced pressure, and the residue was purified by silica gel chromatography (EA: PE=1: 2 in v/v) to afford the pure product of **1** as a yellow-green solid, yield (248.6 mg, 35.7%).

\(^1\)H NMR (400 MHz, CDCl\(_3\), ppm): \(\delta 2.33\) (s, 3H), \(2.57\) (t, 1H, \(J = 2.4\) Hz), \(4.76\) (d, 2H, \(J = 2.4\) Hz), \(5.98\) (s, 1H), \(6.53\) (d, 1H, \(J = 15.6\) Hz), \(7.03\) (d, 2H, \(J = 8.8\) Hz), \(7.59\) (d, 2H, \(J = 8.8\) Hz), \(8.04\) (d, 1H, \(J = 15.6\) Hz).

\(^{13}\)C NMR (100 MHz, DMSO, ppm): \(\delta 23.9, 55.7, 78.7, 78.8, 101.1, 115.6, 118.3, 127.2, 131.7, 147.8, 160.5, 180.4, 191.2\). Mass spectrometry (ESI-MS, m/z): Calcd for [M + Na]\(^+\), 313.0824; found, 313.0824.

Synthesis of compound 2

Under an argon atmosphere, Boc-Glu-OtBu (606.7 mg, 2.0 mmol), HATU (912.6 mg, 2.4 mmol) and DIPEA (517.0 mg, 4.0 mmol) were dissolved under stirring in dry dichloromethane, then aminobenzyl alcohol (492.6 mg, 4.0 mmol) was added. The solution was stirred at room temperature for 24 h. The organic phase was washed with water and then dried over Na\(_2\)SO\(_4\). After filtration and concentration, the crude product was used without further purification. A solution of the crude product from the previous step in dry dichloromethane was stirred with MnO\(_2\) (5.0 g) for 3 h, and then filtered. The filtrate was evaporated to produce the compound **2** as a white solid, yield (538.6 mg, 66.3%).

\(^1\)H NMR (400 MHz, DMSO, ppm): \(\delta 1.45-1.47\) (d, 18H), \(1.82-1.92\) (m, 1H), \(2.04-2.12\) (m, 1H), \(2.53\) (t, 2H, \(J = 7.2\) Hz), \(3.88-3.94\) (m, 1H), \(7.85\) (d, 2H, \(J = 8.8\) Hz), \(7.91\) (d, 2H, \(J = 8.8\) Hz), \(9.93\) (s, 1H), \(10.41\) (s, 1H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\), ppm): \(\delta 28.0, 28.3, 30.8, 34.4, 53.1, 80.8, 83.0, 119.3, 131.1, 132.0, 144.2, 156.9, 171.1, 171.2, 191.2\). Mass spectrometry (ESI-MS, m/z): calcd for [M + Na]\(^+\), 429.2002; found, 429.1998.

Synthesis of compound 3

Compound **1** (197.2 mg, 0.68 mmol) and compound **2** (276.2 mg, 0.68 mmol) were dissolved in ethyl acetate (40 mL) with tributyl borate (0.5 mL) and butylamine (0.1 mL) was added dropwise. The reaction was under an argon atmosphere. The resulting mixture was stirred at room temperature for 12 h. There was a lot of orange precipitation in the bottom of flask. Then remove the solvent by reduced pressure, and the residue was purified by silica gel chromatography (EA: PE=1: 1 in v/v) to afford the pure product of **3** as a red solid, yield (310.4 mg, 67.3%).

\(^1\)H-NMR (100 MHz, CDCl\(_3\), ppm): \(\delta 1.46-1.49\) (d, 18H),
1.85 (m, 1H), 2.27-2.29 (m, 1H), 2.47 (t, 2H, J = 6.0 Hz), 2.58 (s, 1H), 4.21 (m, 1H), 4.76 (s, 2H), 5.42 (d, 1H, J = 7.6 Hz), 6.04 (s, 1H), 6.62-6.65 (m, 2H), 7.02 (d, 2H, J = 8.4 Hz), 7.58 (d, 4H, J = 7.6 Hz), 7.73 (d, 2H, J = 8.0 Hz), 7.96-8.02 (m, 2H), 9.44 (s, 1H). 13C NMR (400 MHz, CDCl₃, ppm): δ 28.0, 28.3, 29.7, 34.3, 53.2, 56.0, 76.3, 77.7, 80.8, 83.0, 102.0, 115.6, 118.6, 119.0, 119.8, 127.7, 129.5, 130.4, 131.1, 142.0, 146.7, 146.8, 160.6, 171.1, 179.5, 179.6. Mass spectrometry (ESI-MS, m/z): calcd for [M - H]⁻, 677.2846; found, 677.2847.

Synthesis of compound 4
A mixture of compound 3 (135.6 mg, 0.20 mmol), Biotin-PEG₂-N₃ (149.0 mg, 0.28 mmol), and sodium ascorbate (10 mol %) in DMF:MeOH (2:1 mL) was stirred for 15 min at rt. Then 5 mol % of CuSO₄ in 0.5 mL water was added to the reaction mixture, which was stirred for 12 h. After removal of the solvents under reduced pressure, the crude mixture was purified over silica gel using CH₂Cl₂/MeOH (v/v, 95:5) as the eluent to yield compound 4 as a red solid (116.7 mg, 48.2%). ¹H NMR (400 MHz, CDCl₃, ppm): δ 1.46-1.48 (m, 20H), 1.62-1.71 (m, 4H), 2.19-2.24 (m, 4H), 2.48 (t, 2H, J = 6.6 Hz), 2.72-2.75 (m, 2H), 2.89-2.93 (m, 1H), 3.15-3.16 (m, 1H), 3.38-3.46 (m, 4H), 3.52-3.56 (m, 2H), 3.60-3.61 (m, 11H), 3.66-3.67 (m, 6H), 3.89 (t, 2H), 4.21 (t, 1H), 4.31 (q, 1H), 4.50 (q, 1H), 4.57-4.59 (m, 2H), 5.44 (d, 1H, J = 7.8 Hz), 6.01 (s, 1H), 6.59-6.66 (m, 2H), 6.75 (s, 1H), 7.04 (d, 2H, J = 7.8 Hz), 7.57 (d, 4H, J = 8.0 Hz), 7.73 (d, 2H, J = 8.0 Hz), 7.92 (s, 1H), 7.95-8.00 (m, 2H), 9.55 (s, 1H). ¹³C NMR (100 MHz, CDCl₃, ppm): δ 25.6, 28.0, 28.1, 28.2, 28.3, 29.7, 34.3, 35.8, 39.1, 40.6, 50.4, 50.7, 53.3, 55.6, 60.2, 61.8, 69.3, 70.0, 70.0, 70.4, 70.6, 70.6, 80.6, 82.8, 102.0, 115.6, 118.4, 119.0, 119.8, 127.3, 129.4, 130.4, 131.3, 142.2, 146.6, 146.8, 161.4, 171.2, 173.4, 179.4. Mass spectrometry (ESI-MS, m/z): calcd for [M + H]⁺, 1211.5681; found, 1211.5679.

Synthesis of Glu-DFB-Biotin
Compound 4 (48.4 mg, 0.04 mmol) was dissolved in a cold (0ºC) solution of 1:1 trifluoroacetic acid and CH₂Cl₂ (5 mL). The solution was kept in the dark and stirred for 1 h at 0ºC before being allowed to warm to room temperature and stirred for additional 2 h. After removal of the solvent from the reaction mixture under reduced pressure, the residue was washed by diethyl ether and dichloromethane, respectively, obtaining Glu-DFB-Biotin as a dark red solid (12.4 mg, 29.5%). Mass spectrometry (ESI-MS, m/z): calcd for [M + H]⁺, 1077.4351; found, 1077.4354.

Synthesis of Glu-DFB
Compound 3 (67.8 mg, 0.10 mmol) was dissolved in a cold (0ºC) solution of 1:1
trifluoroacetic acid and CH₂Cl₂ (5 mL). The solution was kept in the dark and stirred for 1 h at 0ºC before being allowed to warm to room temperature and stirred for additional 2 h. After removal of the solvent from the reaction mixture under reduced pressure, the residue was washed by diethyl ether and dichloromethane, respectively, obtaining Glu-DFB-Biotin as a dark red solid (20.4 mg, 37.0%). ¹H-NMR (400 MHz, DMSO, ppm): δ 1.46-1.47 (m, 2H), 1.98-2.03 (m, 3H), 3.65 (t, 1H, J = 2.0 Hz), 4.92 (d, 2H, J = 2.0 Hz), 6.55 (s, 1H), 7.10-7.13 (m, 4H), 7.21 (s, 1H), 7.72 (d, 2H, J = 8.8 Hz), 7.84 (d, 2H, J = 8.8 Hz), 7.88 (d, 2H, J = 8.8 Hz), 7.93-8.03 (m, 2H), 10.65 (s, 1H). Mass spectrometry (ESI-MS, m/z): calcd for [M + H]⁺, 523.1852; found, 523.1852.

References
3. Preparation of the test solution

Probes were dissolved in dimethyl sulfoxide (DMSO, AR) to obtain 1 mM stock solutions. All UV−vis absorption and fluorescence spectra measurements were carried out in PBS/DMSO buffer solution (4:1, v/v, pH = 7.4, 50 mM). In a 3 mL tube, PBS buffer (2.4 mL) and DMSO (570 μL) solution were mixed, and then the probe (30 μL) was added to obtain a final concentration of 10 μM. GGT was dissolved in a PBS buffer, and an appropriate volume was added to the sample solution. After rapid mixing of the solution, it was transferred to a 10 × 10 mm quartz cuvette and incubated at 37°C for in vitro detection.

4. Cell experiment

Cell Lines. The human hepatocyte QSG-7701 cell line, human hepatoma HepG2 cell line and human epithelioid cervical carcinoma cell line HeLa were purchased from the Institute of Cell Biology (Shanghai, China). Cells were all propagated in T-75 flasks cultured at 37°C under a humidified 5% CO₂ atmosphere in RPMI-1640 medium or DMEM medium (GIBCO/Invitrogen, Camarillo, CA, USA), which were supplemented with 10% fetal bovine serum (FBS, Biological Industry, Kibbutz Beit Haemek, Israel) and 1 % penicillin/streptomycin (10,000 U mL⁻¹ penicillin and 10 mg/ml streptomycin, Solarbio life science, Beijing, China).

In Vitro Cytotoxicity Assay. The cell cytotoxicity of Glu-DFB-Biotin and Glu-DFB to QSG-7701 cells, HepG2 cells and HeLa cells were measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The cytotoxicity was evaluated by Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the factory’s instruction. Cells were plated in 96-well plates in 0.1 mL volume of DMEM or RPMI-1640 medium with 10 % FBS, at a density of 1×10⁴ cells/well and added with desired concentrations of Glu-DFB-Biotin and Glu-DFB. After incubation for 24 h, absorbance was measured at 450 nm with a Tecan GENios Pro multifunction reader (Tecan Group Ltd., Maennedorf, Switzerland). Each concentration was measured in triplicate and used in three independent experiments. The relative cell viability was calculated by the equation: cell viability (%) = (ODtreated/ODcontrol) × 100%.

Flow Cytometry Assay. The HepG2 cells at 2×10⁵ cells/well were seeded onto six-well plates with 2 mL complete medium and cultured overnight. The cells were then treated with the designed concentrations of free biotin, Glu-DFB-Biotin and Glu-DFB at 37 °C for 0.5 h, 1 h, 2 h, 3 h or 5 h. After washed with PBS twice, the cells were harvested using 0.05 % (w/v) trypsin/0.02% (w/v) EDTA. The collected cells were resuspended in PBS (500 μL)
after washed with cold PBS twice. Each sample was measured with flow cytometry (FACSCalibur) within 1 h.

**Confocal Fluorescence Imaging for Living Cells.** Cells were plated onto glass-bottom Petri dishes in 1.5 mL of complete culture medium and allowed to adhere for 12 h before treatment. The cells were exposed to Glu-DFB-Biotin and Glu-DFB at a final concentration of 10 μM (containing 0.1% DMSO) and incubated for 30 min at 37 °C under a humidified 5% CO₂ atmosphere. Fluorescence imaging was then performed using a confocal laser scanning microscope (CLSM, Nikon A1R system, Japan) with a 60× oil immersion objective lens. The fluorescence signals of cells incubated with probes were collected at 425-525nm and 650-750 nm using S laser at 406 nm and 633nm as excitation resource, respectively.
5. Supplementary Spectra and charts

**Figure S1.** Absorption (A) and emission spectra (B, $\lambda_{ex} = 600$ nm; C, $\lambda_{ex} = 400$ nm) of Glu-DFB (10 $\mu$M) in aqueous solution (PBS:DMSO = 4:1, v/v; pH = 7.4; 37 °C) with the addition of GGT (50U/L).

**Figure S2.** Effects of pH (A) and temperature (B) on the fluorescence ($\lambda_{ex/em} = 600/670$ nm) of Glu-DFB-Biotin (10 $\mu$M) with and without GGT (50 U/L). The results are the mean ± standard deviation of three separate measurements.
Figure S3. (A) The change of NIR fluorescence intensity at 670 nm toward the concentration of GGT from 1 to 100 U/L. (B) Linear fitting curve of NIR fluorescence intensity at 670 nm toward the concentration of GGT from 1 to 25 U/L. $\lambda_{ex}=600$ nm. (C) Linear fitting curve of fluorescence intensity at 670 nm toward the concentration of GGT at 0, 25, 50, 100, 150 mU/L. $\lambda_{ex} = 600$ nm. The detection limit was calculated to be 78.5 mU/L ($3\sigma$/slope).

Figure S4. Time-dependent NIR fluorescence intensity at 670 nm of Glu-DFB-Biotin (10 $\mu$M) in the presence of different concentrations of GGT, $\lambda_{ex} = 600$ nm.
**Figure S5.** Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: \( V = \frac{V_{\text{max}} [S]}{K_m + [S]} \), where \( V \) is the reaction rate, [S] is the probe concentration (substrate), and \( K_m \) is the Michaelis constant. Conditions: 50 U/L GGT, 5-50 μM of Glu-DFB-Biotin, \( \lambda_{\text{ex/em}} = 600/670 \) nm. Points were fitted using a linear regression model (correlation coefficient \( R = 0.997 \)).

**Table S1.** Fluorescence quantum yield of the probes.

<table>
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<tr>
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<th>Glu-DFB-Biotin</th>
<th>Glu-DFB</th>
<th>NH$_2$-DFB</th>
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<tr>
<td>( \Phi_f )</td>
<td>0.58 (565 nm)</td>
<td>0.57</td>
<td>0.17 (670 nm)</td>
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Figure S6. (A) HRMS spectrum showing the cleavage product, NH₂-DFB. (B) Reverse-phase HPLC chromatogram of Glu-DFB, Glu-DFB reaction with 50U/L GGT for 10 min, 15min, and NH₂-DFB. Detection wavelength is 487 nm.

Figure S7. Relative viability of QSG-7701, HepG2, and HeLa cells in vitro after incubation with (A) Glu-DF-Biotin and (B) Glu-DFB at various concentrations for 24h.
Figure S8. (A) Emission spectra of Glu-DFB-Biotin (10 μM) in aqueous solution (PBS:DMSO = 4:1, v/v; pH = 7.4; 37 °C) after addition of GGT (50U/L) for 20 min in the presence of 0, 10, 50, 100 μM DON. (B) NIR fluorescence intensity at 670 nm in the presence of different concentrations of DON, λex = 600 nm.

Figure S9. CLSM and ratiometric images of HepG2 cells incubated with Glu-DFB (10 μM) for 30 min: (A-D) HepG2 cells, (E-H) HepG2 cells pretreated 1 mM DON for 30 min. The green channel was collected from 425 to 525 nm (λex = 406 nm), the red channel was collected from 650 to 750 nm (λex = 633 nm), and ratiometric images constructed from red and green channels. (I) Determination of GGT activity in HepG2 with or without DON according to average intensity ratio. Error bars represent standard deviation. (J) Flow cytometry of Glu-DFB in HepG2 cells.
Figure S10. CLSM images of HepG2 cells incubated with Glu-DFB-Biotin (10 μM) for 30 min and then co-stained with 200 nM Lyso-Tracker Red or Mito-Tracker Red for 30 min. The green channel was collected from 425 to 525 nm ($\lambda_{ex} = 406$ nm), the red channel was collected from 650 to 750 nm ($\lambda_{ex} = 633$ nm), and the magenta fluorescence of trackers was collected from 570 to 600 nm ($\lambda_{ex} = 561$ nm).
6. $^1$H NMR, $^{13}$C NMR and HRMS charts

**Figure S11.** $^1$H NMR chart of compound 1 (CDCl$_3$, 400 MHz).

**Figure S12.** $^{13}$C NMR chart of compound 1 (DMSO, 100 MHz).
Figure S13. HRMS chart of compound 1.

Figure S14. $^1$H NMR chart of compound 2 (DMSO, 400 MHz).
Figure S15. $^{13}$C NMR chart of compound 2 (CDCl$_3$, 100 MHz).

Figure S16. HRMS chart of compound 2.
Figure S17. $^1$H NMR chart of compound 3 (CDCl$_3$, 400 MHz).

Figure S18. $^{13}$C NMR chart of compound 3 (CDCl$_3$, 100 MHz).
Figure S19. HRMS chart of compound 3.

Figure S20. $^1$H NMR chart of probe Glu-DFB (DMSO, 400 MHz).
Figure S21. HRMS chart of probe Glu-DFB.

Figure S22. $^1$H NMR chart of compound 4 (CDCl$_3$, 400 MHz).
Figure S23. $^{13}$C NMR chart of compound 4 (CDCl$_3$, 100 MHz).

Figure S24. HRMS chart of compound 4.