

Supplementary Information

A FAPI-conjugated FITC fluorescence probe for targeted cancer imaging

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Experimental section

Synthesis of targeted ligands

Synthesis of S1: DSAT is used as a fluorinating reagent, and the reaction system needs to be controlled in an anhydrous and oxygen-free environment. Firstly, pre-dry the three-neck round-bottom flask at 100 °C for 30 minutes to ensure that it is free from moisture. Then, 2.3 g of Boc-4-oxy-L-proline methyl ester was weighed and added to the three-neck flask. A balloon filled with nitrogen gas was connected to the three-way adapter and insert it into the flask. The other two necks of the flask with rubber stoppers were sealed. The flask is vacuumed to ensure that no water and no oxygen are reached. While stirring, 30 mL of anhydrous dichloromethane was added to the pre-treated flask. Next, 2 mL of DSAT and 20 mL of anhydrous dichloromethane were put into the flask. After standing for 2 minutes, 110 μL of anhydrous ethanol was added to the reaction system as a catalyst.

After 18 hours of reaction, the saturated sodium bicarbonate solution was added to the reaction system and continued to stir until no bubbles were formed. The solution was transferred to a separating funnel and extracted with ethyl acetate. The obtained product was washed twice with saturated NaCl and dried with anhydrous Na₂SO₄. The solution is then spun dry, separated and purified by column chromatography (mobile phase is dichloromethane and petroleum ether V: V = 60:40). Finally, 1.6 g yellow liquid substance (S1) was obtained, and the yield was 63.7%. ¹H NMR (400 MHz, Chloroform-d): δ 4.53-4.32 (ddd, J = 37.5, 5.2 Hz, 1H), 3.85-3.72 (m, 2H), 3.69 (s, 3H), 2.74-2.53 (m, 1H), 2.52-2.30 (m, 1H), 1.47-1.27 (d, J = 19.9 Hz, 9H). ¹³C NMR (101 MHz, Chloroform-d): δ 28.11, 28.22, 37.76, 38.02, 38.27, 38.39, 38.65, 38.90, 52.32, 52.45, 52.55,

52.58, 52.87, 53.10, 53.19, 53.42, 53.74, 56.52, 56.55, 56.58, 57.05, 57.08, 57.11, 81.14, 81.19, 123.27, 124.08, 125.73, 126.56, 128.21, 129.03, 153.04, 153.70, 171.25, 171.55. MS: $[M+Na]^+$ m/z 288, $[M-Boc+H]^+$ 166. HPLC mobile phase: acetonitrile (A)-water (B), gradient elution for 0~2 minutes, 40% A; 2~9 minutes, 40%~20% A; 9~10 minutes, 20%~40% A; 10~11 minutes, 40%~60% A; 11~12 minutes, 60%~80% A. Flow rate 1 mL min⁻¹, injection volume 10 μ L. Detection wavelength 210 nm, peak time 5.801 minutes.

Synthesis of S2: 1.172 g of S1 is mixed with 4.65mL of 1M KOH solution and stirred overnight. The solution is washed and extracted with excess petroleum ether to preserve the water layer. 1M HCl solution was slowly added to the solution until turbidity was no longer generated in the solution. The organic layer was extracted by adding ethyl acetate, washed twice in saturated salt water, and then dried with anhydrous sodium sulfate. The solution was dried and 1.135g light yellow solid substance (S2) was obtained without purification and the yield was 97.3%. ¹H NMR (400 MHz, Chloroform-d): δ 4.65-4.32 (ddd, J = 49.6, 5.0 Hz, 1H), 3.91-3.61 (m, 2H), 2.85-2.35 (m, 2H), 1.48-1.33 (d, J = 19.1 Hz, 9H). ¹³C NMR (101 MHz, Chloroform-d): δ 28.13, 28.28, 37.45, 37.70, 37.96, 38.34, 38.60, 38.85, 52.60, 52.92, 53.17, 53.23, 53.49, 53.81, 56.65, 56.99, 81.94, 82.17, 123.20, 123.84, 125.67, 126.31, 128.13, 128.78, 153.35, 154.53, 174.96, 176.18. MS: $[M+Na]^+$ m/z 274, $[M-Boc+H]^+$ m/z 152. HPLC mobile phase: water (A)~acetonitrile (B), gradient elution for 0~1 min, 80%~40% A; 1~10 minutes, 40% A; 10~11 minutes, 40%~60% A; 11~12 minutes, 60% ~80% A. Flow rate 1 mL min⁻¹, injection volume 10 μ L. Detection wavelength 210 nm, peak time 3.821 minutes.

Synthesis of S3: 1.135 mg S2 was dissolved in a flask with 7 mL dichloromethane and kept in an ice bath for 10min. Then 1.025 g DCC and 1.4 g NHS were added. After the solution appeared white and cloudy, the flask was placed at room temperature and continued to stir for 30min. Adding 1.4 mL liquid ammonia, the solution gradually solidified and reacted for 20 min. The kieselguhr and cold ethyl acetate were added to the flask, followed by vacuum filtration. The filtrate was dried with saturated sodium bicarbonate and saturated sodium chloride, anhydrous sodium sulfate, and then rotated. The 0.92 g light yellow solid product was obtained without purification, and the yield was 81.4%. ¹H NMR (400 MHz, Chloroform-d): δ 6.77 (s, 1H), 5.67 (s, 1H), 4.51 (s, 1H), 3.97- 3.55 (m, 2H), 3.06-2.40 (m, 2H), 1.57-1.39 (s, 9H). ¹³C NMR (101 MHz, Methanol-d₄): δ 27.22, 37.53, 37.78, 38.02, 38.30, 38.55, 38.81, 52.44, 52.76, 53.03, 53.08, 53.33,

53.64, 57.72, 58.18, 81.24, 123.47, 124.27, 125.99, 126.72, 128.45, 129.09, 153.94, 154.40, 174.44, 174.83. MS: $[M+Na]^+$ m/z 273, $[M-Boc+H]^+$ m/z 151. HPLC mobile phase: water (A)~acetonitrile (B), gradient elution for 0~2 minutes, 40% A; 2~9 minutes, 40%~20% A; 9~10 minutes, 20%~40% A; 10~11 minutes, 40%~60% A; 11~12 minutes, 60%~80% A. Flow rate 1 mL min⁻¹, injection volume 10 μ L. Detection wavelength 210 nm, peak time 3.982 minutes.

Synthesis of S4: 0.92 mg of S3 was dissolved in 7.35 mL of dichloromethane, and 7 mL of trifluoroacetic acid was added and stirred for 1 hour. The solution changed from colorless to purple. After the solution is evaporated to dryness, ether is added for washing to precipitate the solid substance. The solid substance is repeatedly washed until it turns white, vacuumed, and stored in the refrigerator for later use. Finally, 0.514 g of white solid was obtained without further purification, with a yield of 92.6%. ¹H NMR (400 MHz, DMSO-d₆): δ 9.79(s, 2H), 8.06(s, 1H), 7.80(s, 1H), 4.59-4.38 (t, J = 8.5 Hz, 1H), 3.84-3.63 (t, J = 12.4 Hz, 2H), 3.00-2.80 (tdd, J = 14.3, 8.4 Hz, 1H), 2.60-2.41 (m, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 37.71, 37.95, 38.21, 50.63, 50.96, 51.30, 57.68, 57.72, 57.75, 112.98, 115.94, 118.90, 121.86, 125.60, 128.08, 130.54, 158.98, 159.30, 159.62, 159.94, 168.46. MS: $[M+H]^+$ m/z 151.

Synthesis of S5: The mixture was prepared by adding 575 mg of Boc-glycine, 3 mL of dichloromethane, and 0.543 mL of DIPEA. The 380 mg of HATU was dissolved in 2 mL of DMF, then added to a flask and stirred for 15 minutes to activate the carboxyl group. The 510 mg of S5 was dissolved in 4 mL of dichloromethane, and 0.91 mL of DIPEA was added. The mixture was added to the reaction system and reacted at room temperature for 4 hours. The reaction mixture appears turbid, filtered with a Buchner funnel and repeatedly washed with dichloromethane until the solid appears as a pure white powder. After the filtrate is evaporated to dryness, it is washed with water and extracted with ethyl acetate. Then, saturated sodium chloride is added for washing, and anhydrous sodium sulfate is dried before spin drying. Finally, acetic acid was added to recrystallize and precipitate 390 mg of white solid, without further purification, with a yield of 37.3%. ¹H NMR (400 MHz, DMSO-d₆): δ 7.49-7.29 (m, 1H), 7.16 (s, 1H), 7.00- 6.76 (t, J = 5.7 Hz, 1H), 4.52-4.35 (dd, J = 9.7, 4.3 Hz, 1H), 4.18-3.61 (m, 4H), 3.00-2.60 (m, 1H), 2.43-2.23 (m, 1H), 1.47-1.29 (s, 9H). ¹³C NMR (101 MHz, DMSO-d₆): δ 28.69, 37.30, 37.53, 37.77, 41.83, 42.87, 52.58, 52.88, 53.20, 53.47, 53.79, 57.74, 58.00, 78.58, 124.78, 125.83, 127.24, 128.28, 129.69, 130.74, 156.32, 158.49, 168.80, 169.03, 171.95, 172.16. MS: $[M+Na]^+$ m/z 330, $[M-$

Boc+H]⁺ m/z 208. HPLC mobile phase: water (A)~acetonitrile (B), gradient elution for 0~2 minutes, 40% A; 2~9 minutes, 40%~20% A; 9~10 minutes, 20%~40% A; 10~11 minutes, 40%~60% A; 11~12 minutes, 60%~80% A. Flow rate 1 mL min⁻¹, injection volume 10 μL. Detection wavelength 210 nm, peak time 3.635 minutes.

Synthesis of S6: The 300 mg of S5 was weighed in a dry flask and oxygen was removed from the flask through nitrogen balloons and a three-way device. The flask was placed in an ice water bath at 0 °C, and 10 mL of anhydrous tetrahydrofuran was added and stirred to dissolve for 10 minutes. 0.6 mL of pyridine was added to the solution, and after 2 minutes of reaction, 0.21 mL of TFAA solution dissolved in 10 mL of dichloromethane was added dropwise. The reaction mixture was stirred under ice water bath conditions for 2 hours. The solution was evaporated to dryness and the reaction mixture was redissolved in ethyl acetate. The mixture was washed with 2M dilute hydrochloric acid, extracted with acetic acid, and the water layer was discarded. The organic layer is sequentially washed twice with saturated sodium bicarbonate and saturated sodium chloride aqueous solutions. After drying with anhydrous sodium sulfate, the solution is evaporated by a rotary evaporator to obtain light yellow crude products. After column chromatography separation and purification (mobile phase consisting of ethyl acetate and petroleum ether V: V=50:50), a white solid 204 mg S6 was obtained with a yield of 72.3%. ¹H NMR (400 MHz, Chloroform-d): δ 5.41(s, 1H), 4.95-4.84 (t, J = 6.5 Hz, 1H), 3.99-3.70 (m, 4H), 2.76-2.59 (m, 2H), 1.37(s, 1H). ¹³C NMR (101 MHz, Chloroform-d): δ 28.37, 37.05, 37.30, 37.55, 43.08, 44.23, 44.25, 44.28, 44.29, 51.64, 51.96, 52.28, 80.34, 116.45, 122.95, 125.45, 127.94, 156.01, 168.60. MS: [M+Na]⁺ m/z 312, [M-Boc+H]⁺ m/z 190. HPLC mobile phase: water (A)~acetonitrile (B), gradient elution for 0~2 minutes, 40% A; 2~9 minutes, 40%~20% A; 9~10 minutes, 20%~40% A; 10~11 minutes, 40%~60% A; 11~12 minutes, 60%~80% A. Flow rate 1 mL min⁻¹, injection volume 10 μL. Detection wavelength 210 nm, peak time 4.626 minutes.

Synthesis of S7: 390 mg of S6 was dissolved in 10 mL of acetonitrile solution and 360 mg of p-toluenesulfonic acid monohydrate, and the mixture was stirred at room temperature for 24 hours. The mixture was evaporated to dryness, washed with cold ether and stirred. After vacuum drying, 420 mg of white solid was obtained with a yield of 82.2%. ¹H NMR (400 MHz, D₂O) δ 7.62 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.7 Hz), 5.37-5.17 (d, J = 8.8 Hz 0.1H), 5.13-5.04 (m, 0.9H), 4.12-3.96 (ddd, J = 29.5, 6.7 Hz, 2H), 3.92(s, 2H), 2.93-2.75 (m, 2H), 2.33(s, 3H). ¹³C NMR (101 MHz,

D₂O): δ 20.63, 22.96, 27.77, 36.22, 36.49, 36.75, 40.60, 44.79, 51.22, 51.55, 51.87, 117.14, 125.47, 129.61, 139.52, 142.63, 166.26. MS: [M+H]⁺ m/z 190. HPLC mobile phase: water (A)~acetonitrile (B), gradient elution for 0~2 minutes, 40% A; 2~9 minutes, 40%~20% A; 9~10 minutes, 20%~40% A; 10~11 minutes, 40%~60% A; 11~12 minutes, 60%~80% A. Flow rate 1 mL min⁻¹, injection volume 10 μ L. Peak time is 1.960 minutes.

Synthesis of FITC-FAPI: 249 mg of FITC was dissolved in DMF and mixed evenly with 242 mg of S7. Then, 150 μ L DIPEA was added and stirred for 1 hour, keeping the environment dark during the reaction. The reaction mixture was washed multiple times with water, and extracted with ethyl acetate. The solvent was removed by a rotary evaporator and evaporated to dryness. 210 mg FITC-FAPI was purified by column chromatography (mobile phase consisting of methanol and dichloromethane V: V=5:95) with a yield of 56.7%. ¹H NMR (400 MHz, DMSO-d₆): δ 10.34 (s, 1H), 10.14 (s, 2H), 8.30 (s, 1H), 8.24-8.17 (t, J = 5.0 Hz, 1H), 7.76-7.70 (dd, J = 8.5, 2.0 Hz, 1H), 7.19-7.14 (d, J = 8.2 Hz, 1H), 6.65-6.62 (d, J = 2.2 Hz, 2H), 6.59-6.55 (d, J = 8.7 Hz, 2H), 6.54-6.50 (dd, J = 8.7, 2.3 Hz, 2H), 5.15-5.02 (dd, J = 9.2, 2.8 Hz, 2H), 4.45-3.94 (m, 4H), 2.97-2.69 (m, 2H). ¹³C NMR (101 MHz, DMSO-d₆): δ 36.68, 36.95, 37.21, 44.68, 44.74, 46.53, 51.51, 51.81, 52.13, 83.61, 102.77, 110.19, 113.14, 117.14, 118.19, 124.74, 127.15, 129.57, 130.14, 141.64, 147.97, 152.41, 160.00, 168.33, 169.03, 181.29. MS: [M+H]⁺ m/z 579. HPLC mobile phase: water (A)~acetonitrile (B), gradient elution for 0~1 min, 40% A; 1~9 minutes, 40%~20% A; 9~10 minutes, 20%~40% A. Flow rate 1 mL min⁻¹, injection volume 10 μ L. Peak time is 3.896 minutes.

Characterizations: ¹H and ¹³C NMR spectra were recorded on a JNM-ECZ400S/L1 Spectrometer. MS and HPLC measurements were conducted on a 1260 Infinity (Agilent, America).

Synthesis routes :

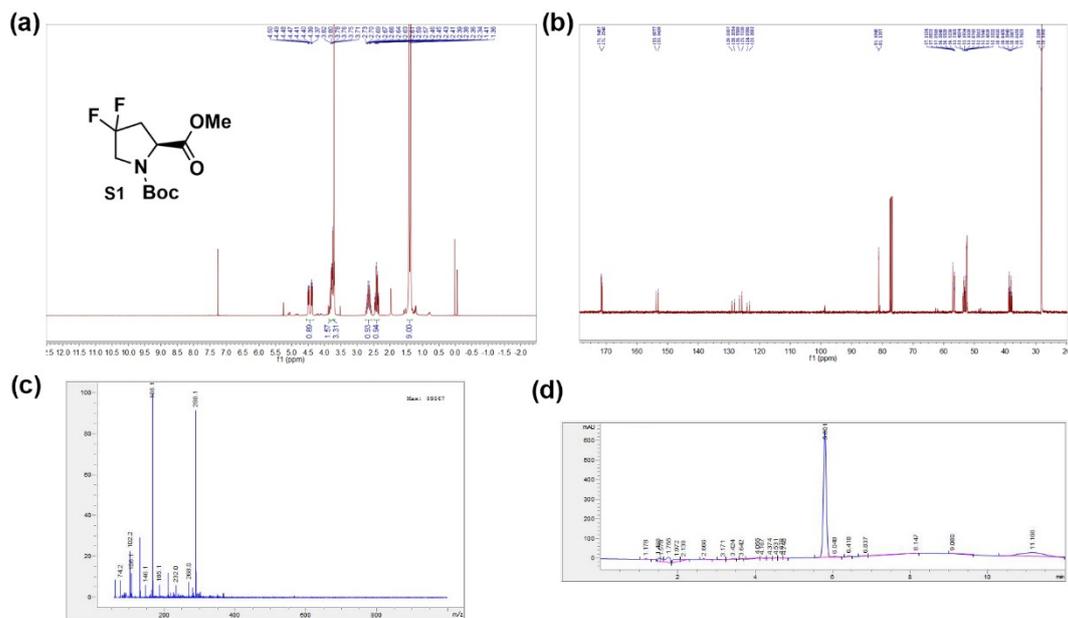


Fig. S1. Spectra of S1: (a) ^1H NMR; (b) ^{13}C NMR; (c) MS; (d) HPLC. The molecular structure of S1 is depicted in the illustration.

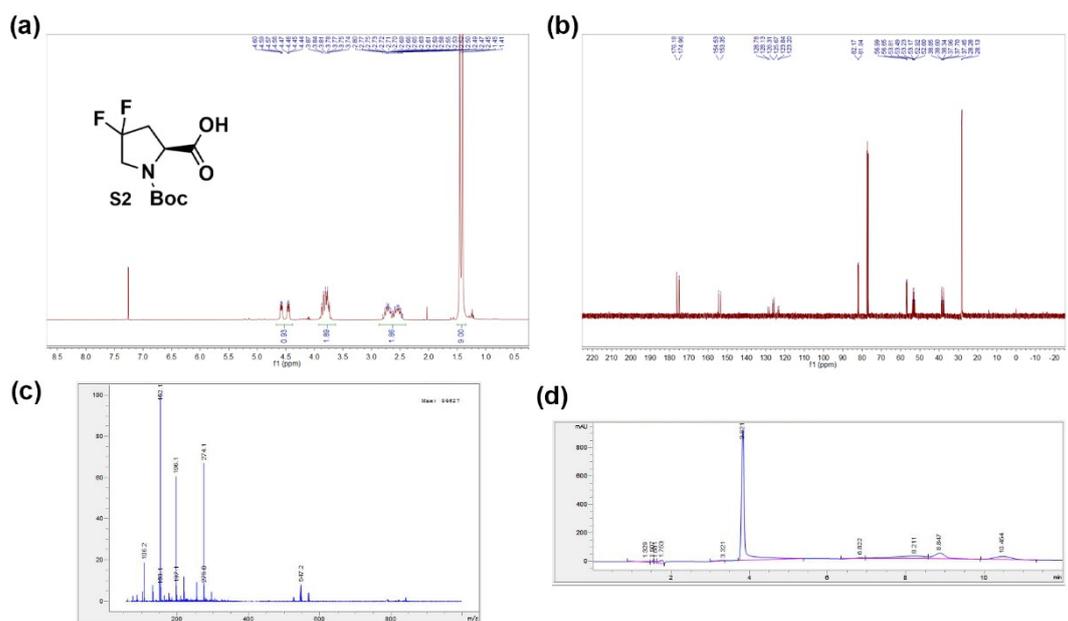


Fig. S2. Spectra of S2: (a) ^1H NMR; (b) ^{13}C NMR; (c) MS; (d) HPLC. The molecular structure of S2 is depicted in the illustration.

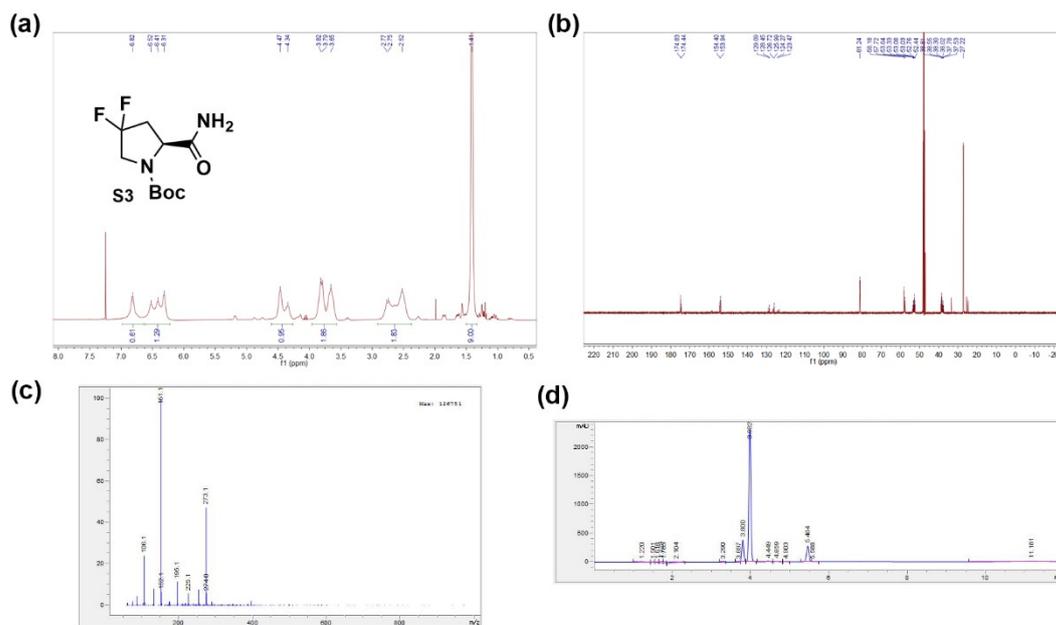


Fig. S3. Spectra of S3: (a) ^1H NMR; (b) ^{13}C NMR; (c) MS; (d) HPLC. The molecular structure of S3 is depicted in the illustration.

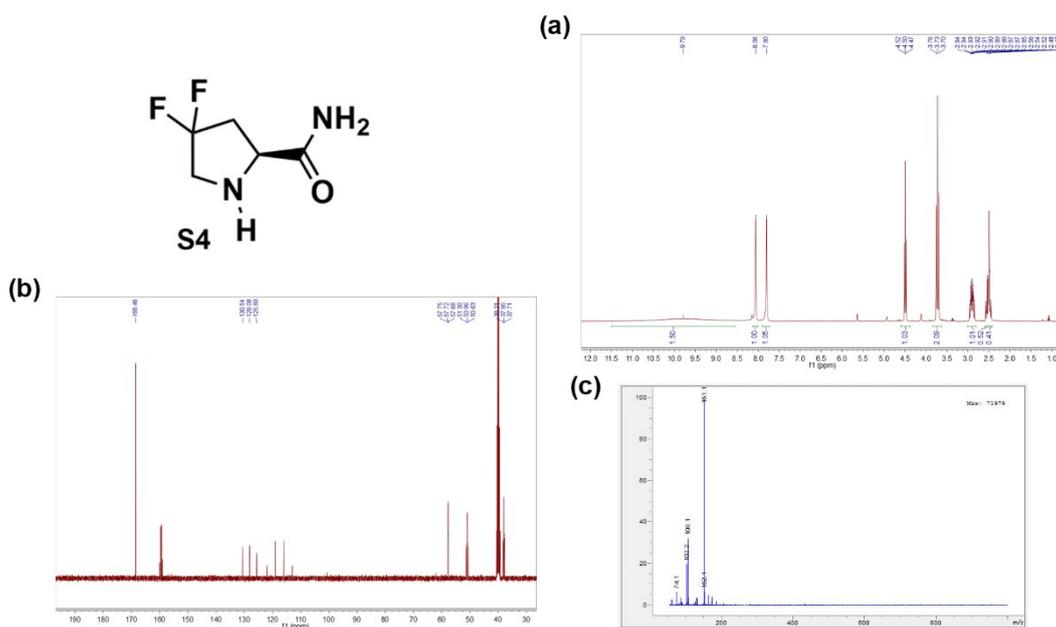


Fig. S4. Spectra of S4: (a) ^1H NMR; (b) ^{13}C NMR; (c) MS. The molecular structure of S4 is depicted in the illustration.

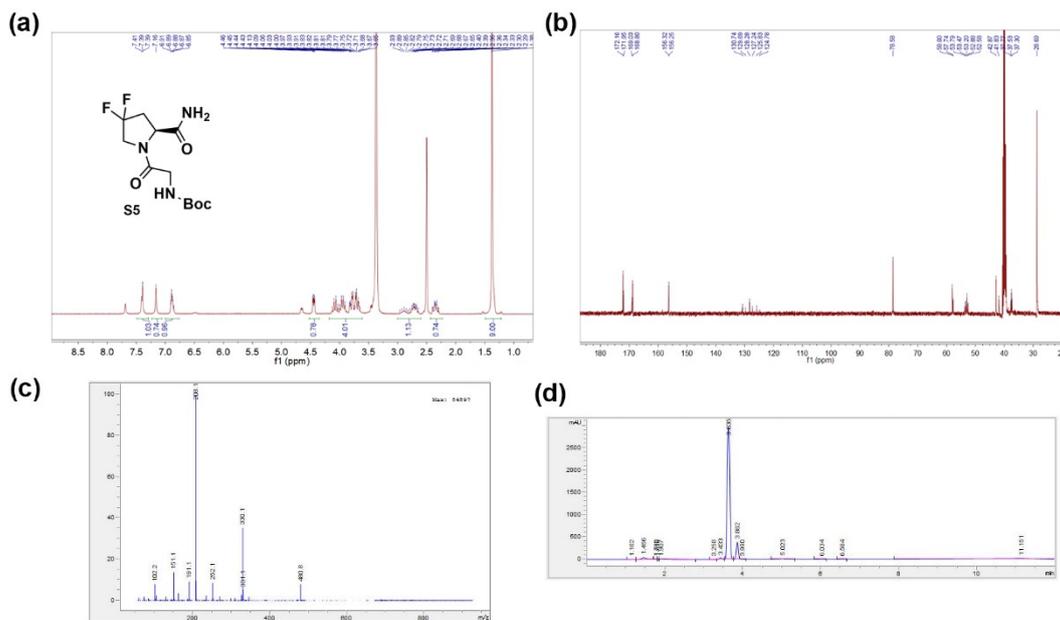


Fig. S5. Spectra of S5: (a) ^1H NMR; (b) ^{13}C NMR; (c) MS; (d) HPLC. The molecular structure of S5 is depicted in the illustration.

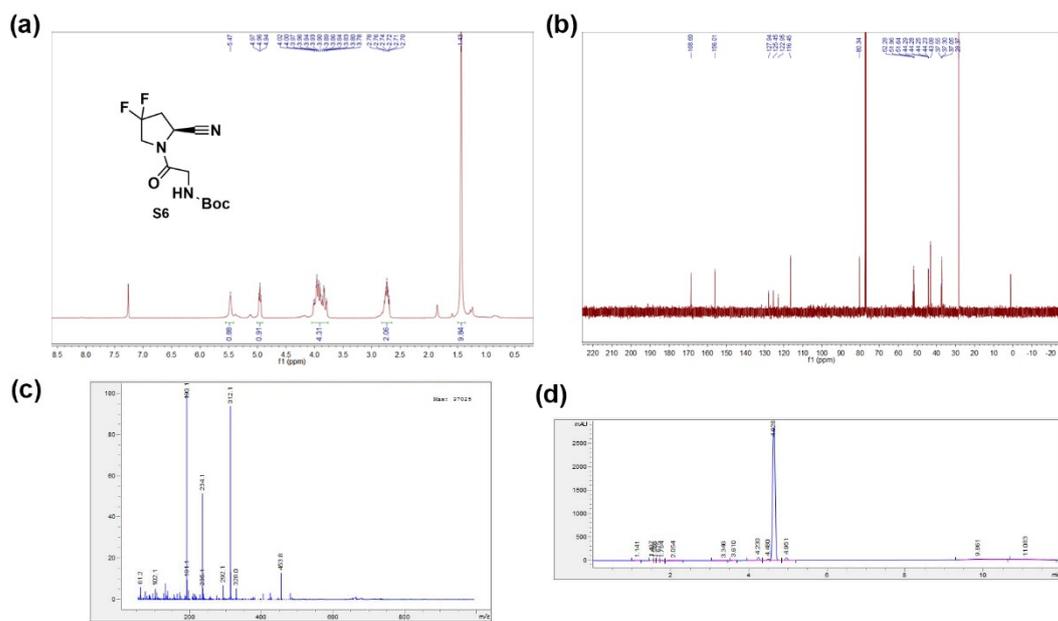


Fig. S6. Spectra of S6: (a) ^1H NMR; (b) ^{13}C NMR; (c) MS; (d) HPLC. The molecular structure of S6 is depicted in the illustration.

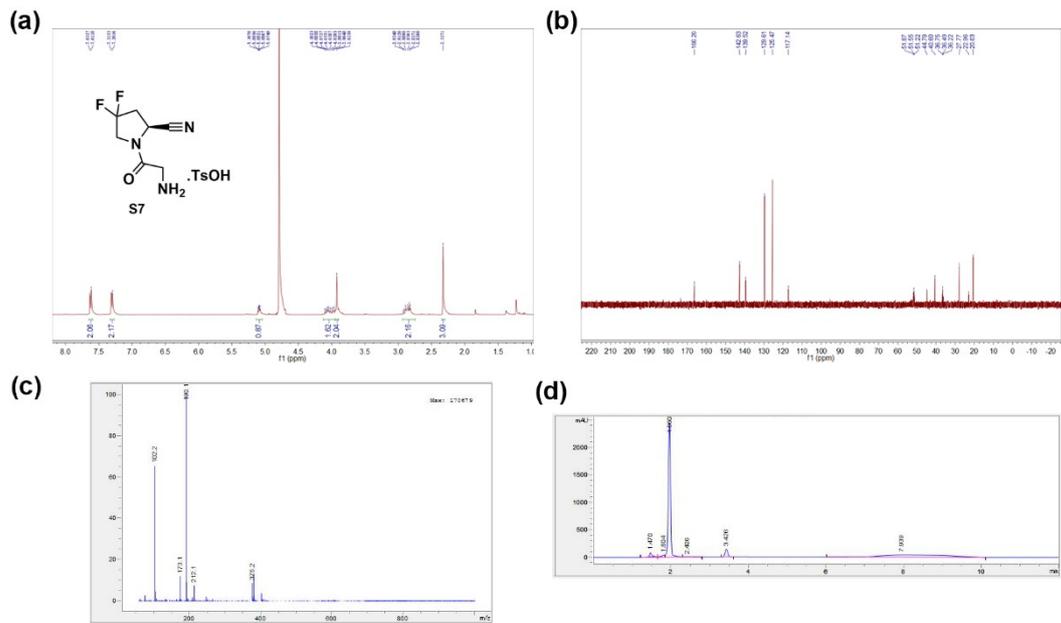


Fig. S7. Spectra of S7: (a) ^1H NMR; (b) ^{13}C NMR; (c) MS; (d) HPLC. The molecular structure of S7 is depicted in the illustration.

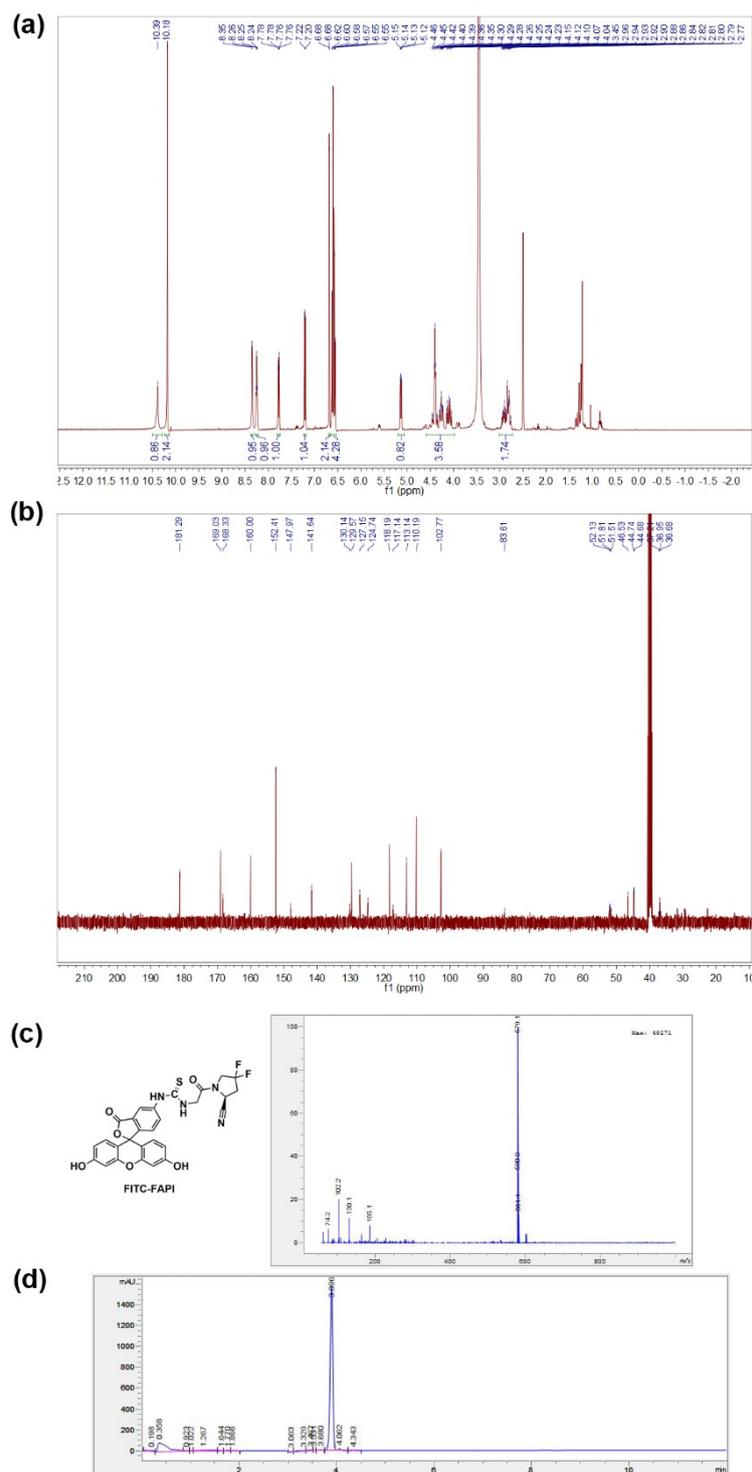


Fig. S8. Spectra of FITC-FAPI: (a) ^1H NMR; (b) ^{13}C NMR; (c) MS; (d) HPLC. The molecular structure of FITC-FAPI is depicted in the illustration.

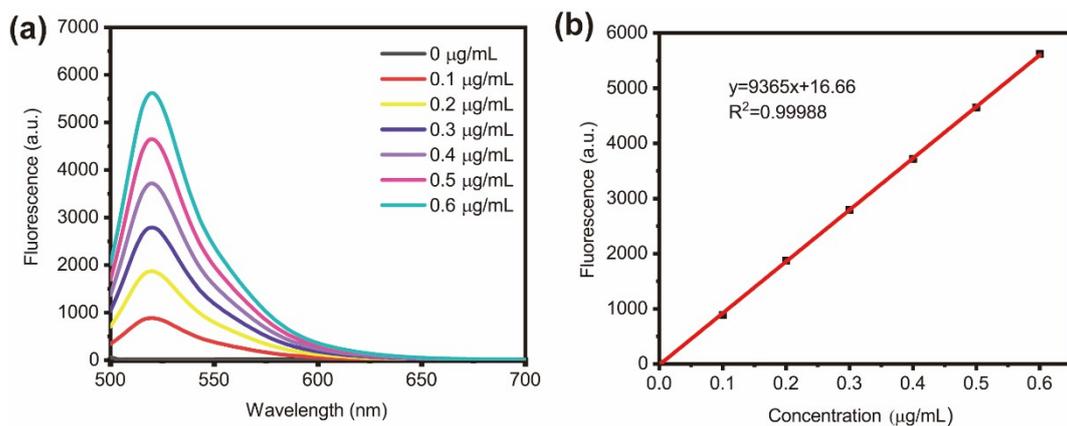


Fig. S9. (a) Fluorescence spectra of FITC-FAPI at different concentrations. (b) Standard curve of FITC-FAPI.

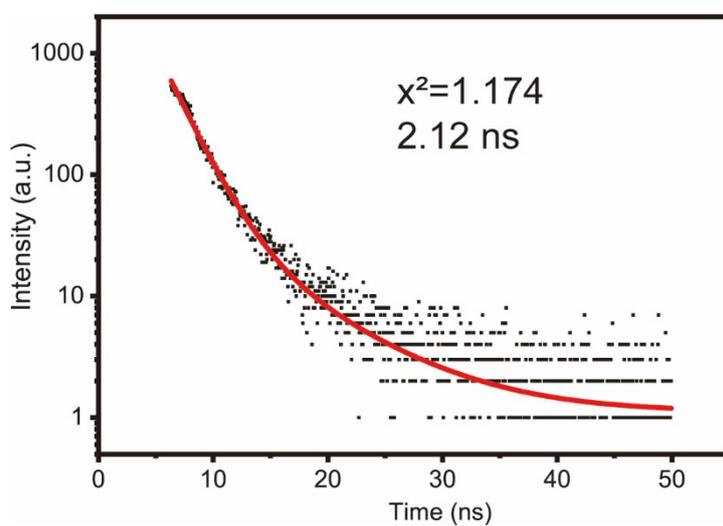


Fig. S10. Solid fluorescence lifetime test curve of FITC.

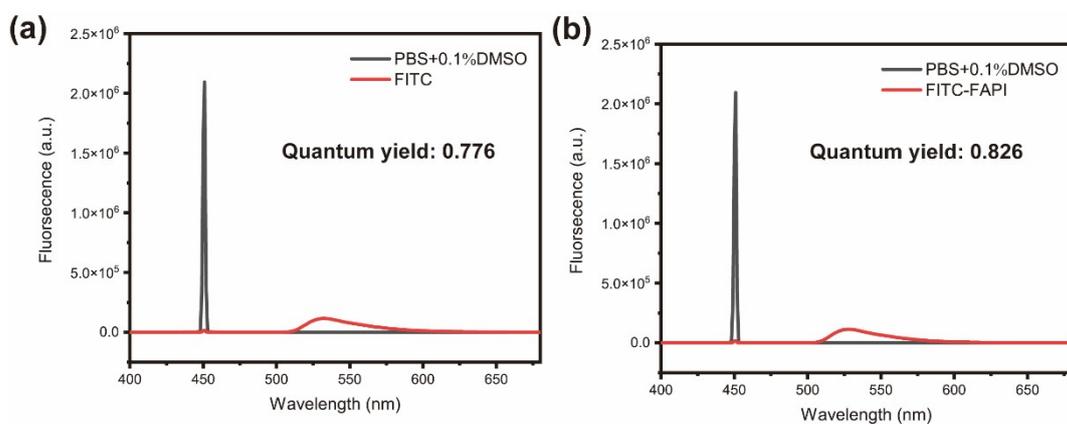


Fig. S11. The absolute quantum yield assessment of (a) FITC; (b) FITC-FAPI.

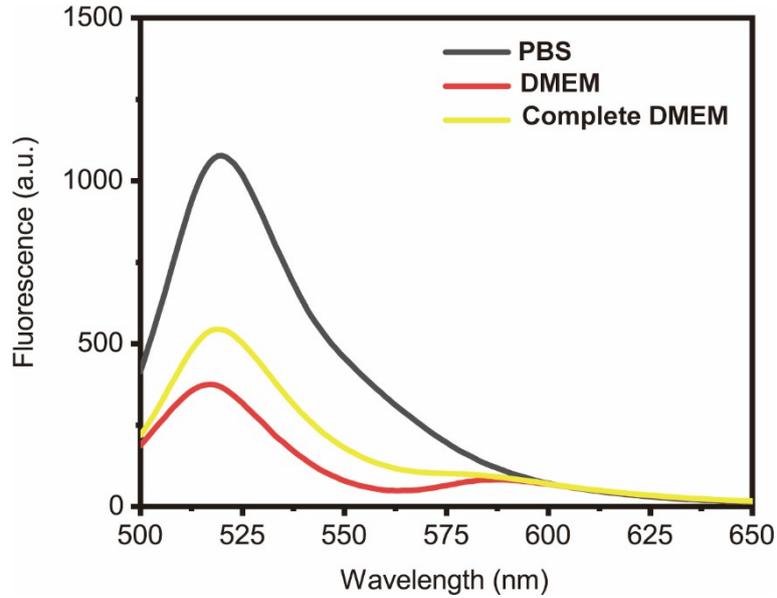


Fig. S12. Fluorescence spectra of FITC-FAPI dissolved in PBS buffer, DMEM medium and complete medium.

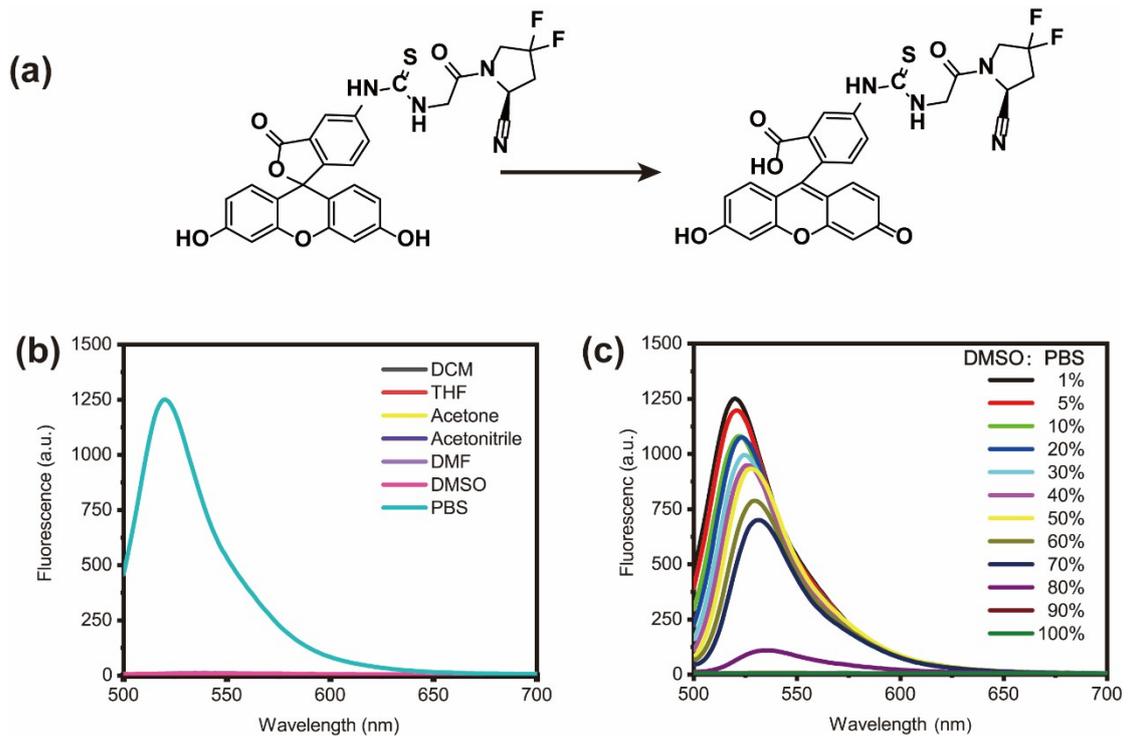


Fig. S13. (a) Schematic diagram of FITC-FAPI structure change. (b) The Fluorescence emission spectra of FITC-FAPI in different solvents. (c) Fluorescence emission spectra of FITC-FAPI with the same concentration in water /DMSO mixed solvent system.

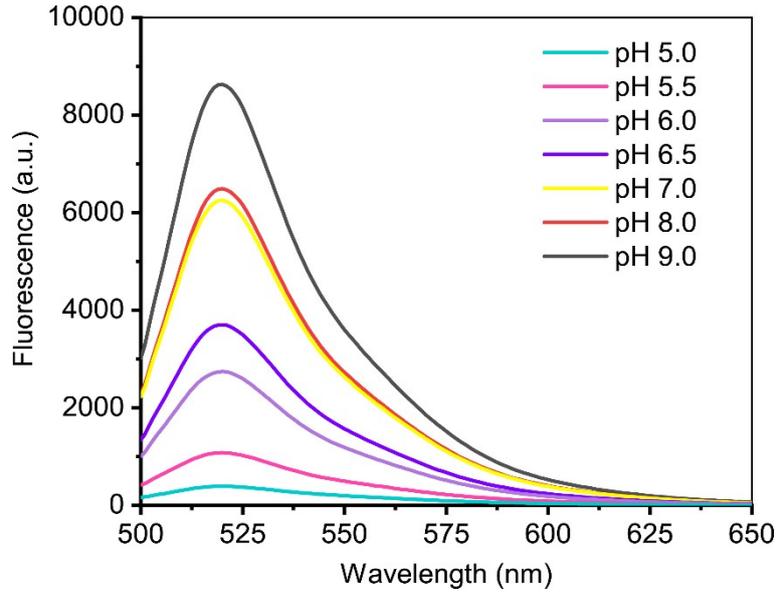


Fig. S14. Fluorescence emission spectra of FITC-FAPI in phosphate solutions at different pH.

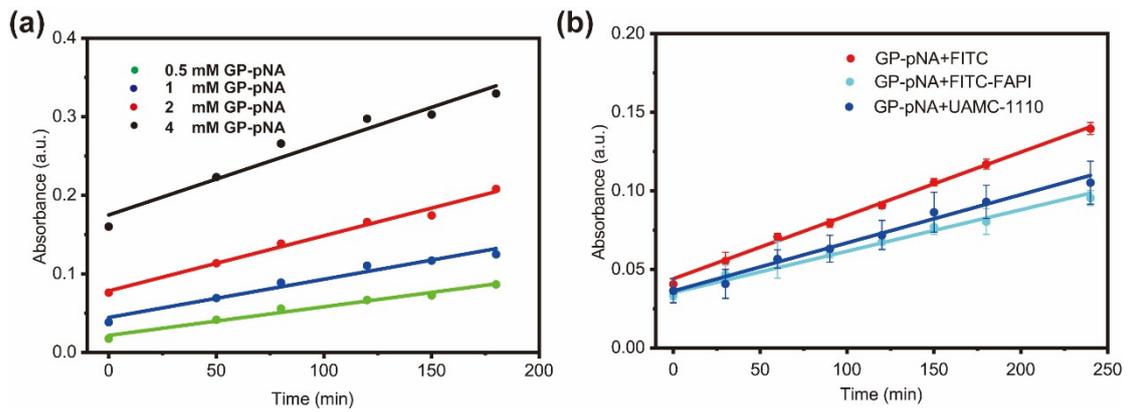


Fig. S15. (a) Enzymatic reaction speed of substrates at different concentrations. (b) Comparison of inhibitory effects of FITC, FITC-FAPI and UAMC-110 on enzyme activity.

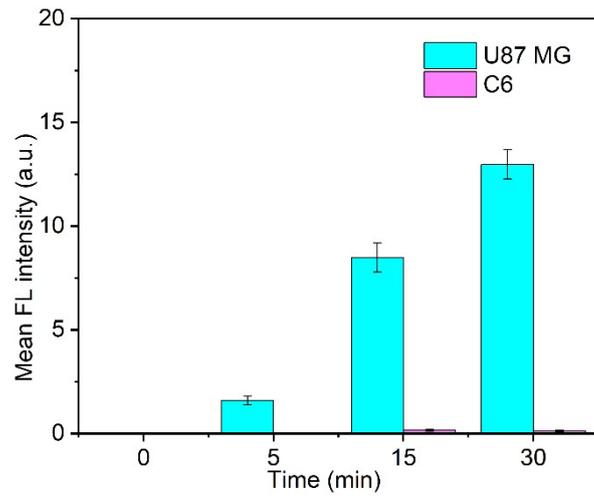


Fig. S16. Semi-quantitative analysis of material uptake for U87MG and C6 cells.

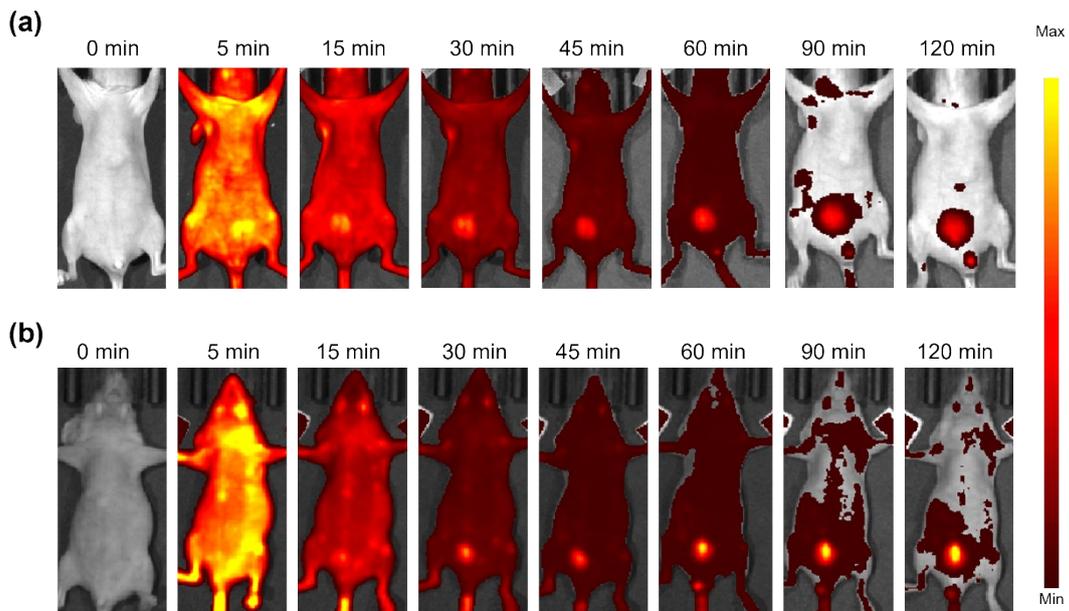


Fig. S17. Metabolism of FITC in normal mice. (b) Metabolism of FITC-FAPI in normal mice.

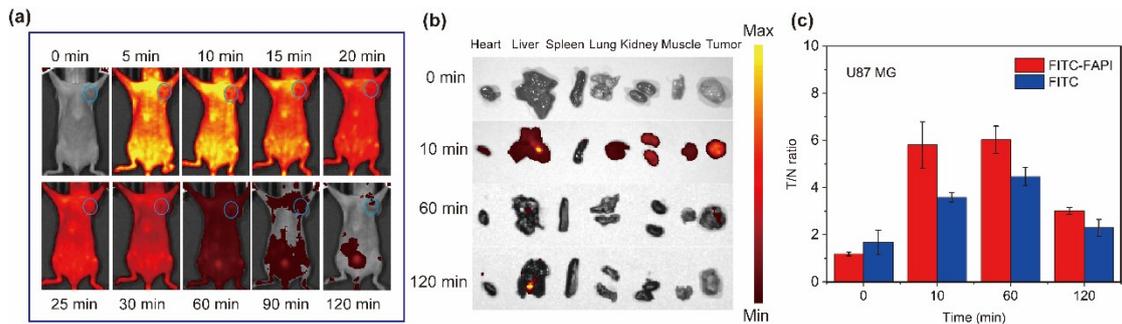


Fig. S18. (a) FITC was used *in vivo* imaging of U87 MG tumor-bearing mice. (b) FITC fluorescence imaging of the heart, liver, spleen, lungs, kidneys, muscles and tumours of U87 MG tumor-bearing mice. (c) FITC-FAPI and FITC fluorescence ratio in tumor and muscle, respectively.

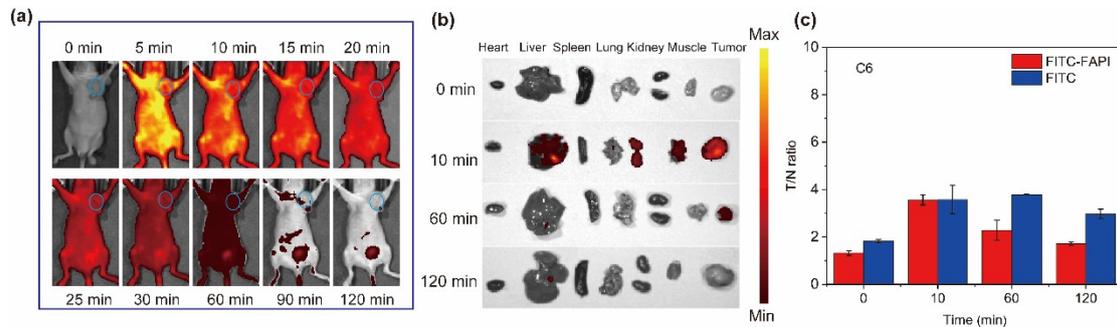


Fig. S19. (a) FITC was used *in vivo* imaging of C6 tumor-bearing mice. (b) FITC fluorescence imaging of the heart, liver, spleen, lungs, kidneys, muscles and tumours of C6 tumor-bearing mice. (c) FITC-FAPI and FITC fluorescence ratio in tumor and muscle, respectively.