Electronic Supplementary Information

A novel fibroblast activation protein-targeted near-infrared fluorescent off-on probe for cancer cells detection, *in vitro* and *in vivo* imaging

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1. Instrument and reagents

Fluorescence measurements were performed from a HORIBA FL13-111 spectrophotometer used a 10mm × 10 mm quartz cell. ^{1}H-NMR and ^{13}C-NMR spectra were made in a Bruker AVANCE III 400MHz spectrometer. High resolution electrospray ionization mass spectra (HRMS, ESI) were measured on an AB Sciex LC-Q-TOF 4600 instrument (USA). Absorption spectra were recorded on T10 CS spectrophotometer with a 10mm × 10 mm quartz cell (Beijing, China). The fluorescence imaging of cells measured on TCS SP5 laser scanning confocal microscope (LEICA, Germany). The western blot signal was detected using an ECL kit. Cytotoxicity assay was made on a microplate absorbance reader (Biorad iMARKTM, USA). Mice imaging experiments were operated on PerkinElmer IVIS Lumina XRMS instrument (PerkinElmer, USA). Flow cytometer results were obtained from a BD LSRFortessa flow cytometry (USA).

(S)-1-(2-((tert-Butoxycarbonyl)-amino)acetyl)pyrrolidine-2-carboxylic acid (N-Boc-Gly-L-Pro) was got from Ark Pharm, Inc. (USA). N,N-Dimethylformamide, Pyridine were purchased in innochem(Beijing). 3-Aminophenol, Cyclohexanone, Tin(II) chloride , N,N-Diisopropylethylamine (DIPEA), O-(7-aza-1H-benzotriazol-1-yl)-N,N,N′,N′- tetramethyluronium hexafluorophosphate (HATU) and Trifluoroacetic acid were purchased from J & K Chemical Technology. 1,2,3,3-tetramethyl-3H-indolium iodide was purchased from Aladdin(Shanghai, China). Methanol, Dichloromethane, Acetonitrile, Phosphoryl chloride, acetic anhydride and other conventional reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) poly-(vinylidene fluoride) membranes (PVDF membranes), gel electrophoresis kits, Western blot kits, enhanced chemiluminescence kits (ECL), and Braford protein assay kits were purchased from Jiangsu KeyGEN BioTECH Corp., Ltd. FAP siRNA (sequences: CGCCCUUCAAGAGUUCAUATTUAUGAACUCUU-GAAGGGCGTT) from Shanghai GenePharma Co.Ltd. Entranster^{TM}-R RNA4000 Transfection Reagent was bought by Engreen Biosystem Co.Ltd (Beijing, China). Dimethyl sulfoxide (DMSO), leucine aminopeptidase (LAP), esterase, prolidase, trypsin, DPPIV, and Fetal bovine serum (FBS) were purchased from Sigma-Aldrich. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Merck (Germany). Anti-FAP antibody was purchased from Signalway Antibody, Inc. (USA). Talabostat mesylate (PT-100) was ordered from MedChem Express Inc. (USA). The stock solution (1.0 mM) of the probe was dissolved in DMSO.

2. Synthesis of HCFP

(1) Synthesis of Compound 1

10 mL of methylene chloride and 10 mL of dry DMF were added in a flask cooled at 0 °C. In a separate glass bottle, 9.5 mL of phosphorus oxychloride was dissolved in 10 mL of methylene chloride, and the solution was added to above mixed solution, stirred for 30 min at 0 °C. 2.5 mL of cyclohexanone was added to the mixed solution. Then the solution was warmed to room temperature, refluxed for three hours at 80℃ under N_2 atmosphere. Upon cooling, the solution was poured onto 150 mL of ice water and precipitation was formed. After vacuum filtration, washing and dryness, the yellow solid[1] (compound **1**) is obtained (2.3 g, yield: 53 %).

(2) Synthesis of compound 2

The synthesis of compound **2**, we refer to the existing method.[2] The detailed synthesis steps are as follows: Compound **1** (1.036 g, 6.0 mmol), acetic anhydride (AC_2O, 30 mL) and 1,2,3,3-tetramethyl-3H-indolium iodide (3.62 g, 12 mmol) were added into a three-necked flask. The solution was heated to reflux at 90 ℃ for 1 h under N_2 atmosphere. After cooling to room temperature, the product solution was poured into saturated sodium carbonate aqueous solution for remove AC_2O. Subsequently, the crude product was extracted with dichloromethane and purified by silica gel column chromatography (CH_2Cl_2/methanol: 0-2 %), obtaining green solid product **2** (2 g, yield: 87 %).

(3) Synthesis of compound 3

Synthesis of stable compound 3, we refer to the existing process.[3] K_2CO_3 (414 mg, 3 mmol), and 3-Aminophenol (327 mg, 3 mmol) were dissolved in 20 mL of CH_3CN in a three-necked flask, and was stirred at room temperature for 10 min under N_2 atmosphere. Then compound **2** (1.2 g, 2 mmol) was put into the flask, and the reaction mixture was stirred at room temperature for 5 h. The solvent was removed under reduced pressure, solid was solved in CH_2Cl_2, washed by water for three times and dried with Na_2SO_4. CH_2Cl_2 was removed by reduced pressure, then the solid product and $SnCl_2$ (2.3 g 10 mmol) were dispersed in 25 mL of CH_3OH, 3 mL of HCl was added, the system was stirred at 70 °C under N_2 atmosphere overnight. After reaction,

pH of the solution was adjusted to 7 with NaOH (100 mM) aqueous solution, and precipitation was formed in this process. After filtration and washing, the solution became clear. The collected filtrate was dried over Na_2SO_4. CH_2Cl_2 was removed by reduced pressure, the crude product was purified by silica gel chromatography with CH_2Cl_2/CH_3OH (100/1 to 10/1, v/v), obtaining green solid product **3** (300 mg, yield 30 %).

(4) Synthesis of compound 4

Compound **3** (255 mg, 0.5 mmol), Boc-Gly-Pro-OH (544 mg, 2 mmol), HATU (760 mg, 2 mmol), DIPEA (260 μL, 1.2 mmol) were dissolved in 20 mL of CH_2Cl_2. Then this mixture solution was stirred 6-7 h at room temperature under N_2 atmosphere. After evaporation of the solvent, the solid was purified by silica gel chromatography with CH_2Cl_2/CH_3OH (100/1 to 10/1, v/v) as the eluent to get compound **4** as a blue powder (90 mg, yield: 25 %). ^{1}H-NMR and ^{13}C-NMR spectra of compound **4** are shown below in Figures. S1 and S2, respectively. ^{1}H NMR (400 MHz, 298K, CD_3OD): δ 8.71 (d, J = 14.8 Hz, 1H),7.99 (s, 1H), 7.70-7.28 (m, 7H), 6.55 (d, J = 14.8 Hz, 1H), 4.64-4.58 (m, 1H), 4.00-3.88 (m, 5H), 3.70 (s, 2H), 2.76-2.71 (m, 4H), 2.31-1.81(m, 12H), 1.43(s, 9H). ^{13}C NMR (100 MHz, 298 K, CD_3OD): δ 178.55, 171.90, 169.16, 160.87, 157.05, 153.29, 145.71, 142.27, 141.99, 141.91, 132.43, 128.82, 128.52, 127.75, 127.24, 122.22, 117.94, 116.76, 114.35, 112.55, 105.73, 104.30, 79.20, 61.19, 50.63, 46.45, 42.42, 31.57, 29.29, 28.77, 27.31, 26.79, 26.73, 24.54, 23.59, 20.19. HRMS (ESI^+), calcd for $[M]^+$, 637.3390, found, 637.3393.

(5) Synthesis of probe

The published method was used to synthesize our probe[4]. Trifluoroacetic acid (TFA, 2.5 mL) in CH_2Cl_2 (2.5 mL) was added dropwise to a solution of compound **4** (90 mg, 0.12 mmol) in 5 mL of CH_2Cl_2 at 0 °C. And the reaction mixture was stirred at room temperature for 30 min. After evaporation of the solvent TFA/CH_2Cl_2, the resulting residue and acetic anhydride (20 μL, 0.2 mmol) were dissolved in 10 % dry pyridine/DMF (2/18 mL) and stirred overnight at room temperature under an N_2 atmosphere. After evaporation of the solvent, crude product was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (100/1 to 10/1, v/v) as the eluent obtain blue solid product: **HCFP** (70 mg, yield: 83 %). ^{1}H-NMR and ^{13}C-NMR spectra of **HCFP** are shown below in Figures. S3 and S4, respectively. ^{1}H NMR (400 MHz, 298K, CD_3OD): δ 8.74 (d, J = 14.8 Hz, 1H), 8.05 (s, 1H), 7.67-7.31（m, 7H), 6.56 (d, J = 14.8

Hz, 1H), 4.60 (s, 1H), 4.11 (d, J = 9.6Hz, 2H), 3.88 (s, 3H), 3.70 (s, 2H), 2.82-2.72 (m, 4H), 2.17-1.82(m, 14H), 0.90(s, 1H). ^{13}C NMR (100 MHz, 298 K, CD_3OD): δ 178.58, 172.31, 171.89, 168.50, 160.93, 153.35, 145.79, 142.28, 142.01, 141.90, 132.41, 128.81, 128.60, 127.79, 127.24, 122.22, 118.01, 116.71, 114.36, 112.53, 105.76, 104.29, 61.21, 50.64, 46.52, 41.46, 31.51, 29.32, 28.78, 26.74, 24.52, 20.98, 20.19. HRMS (ESI^+), calcd for $[M]^+$,579.2971, found: 579.2935**.**

3. Cell culture and Fluorescence detection

MCF-7 Cells (human breast cancer cells) were cultured in DMEM with 10 % FBS at 37 °C and 5 % CO_2 environment. As MCF-7 cells adhered to the wall, cells were digested with trypsin and washed two times with PBS buffer to remove the residual culture medium and trypsin. Cells were resuspended with PBS, and diluted to 8×10^6 cells/mL using a hemocytometer, subsequent diluted to 6×10^6, 5×10^6, 4×10^6, 3×10^6, 2×10^6, 1.5×10^6, 1.25×10^6, 1×10^6, 8×10^5, 6.125×10^5, 5×10^5, 3×10^5, 1.5×10^5, 5×10^4, 1×10^4 cells/mL in turn. 15 μL probe of stock solution was added into 2 mL of cell suspension, and the finally concentration of probe was 7.5 μM. The system was incubated at 37 °C for 4h. After incubation, the fluorescent spectrum was tested with 670 nm excitation.[5]

4. Confocal laser microscopy imaging

5×10^4-10×10^4 cells in 2 mL of culture medium were inoculated in glass bottom cell dishes for 24 h. Before the confocal fluorescence images, the cells were washed 2-3 times with PBS, then added 2 mL of DMEM with probe (5 μM) and incubated 0-60 min at 37 °C, and finally subjected to fluorescence imaging (λ_{ex}=633 nm).

5. MCF-7 cells transfection

The transfection procedure is in accordance with the instructions for transfection reagents. MCF-7 cells were seeded in cell culture dishes and placed in the cell incubator. When the cell confluence reached 20-40%, siRNA powder is dissolved in DEPC water, 3.33 μg siRNA and 5 μL EntransterTM-R RNA4000 transfection reagent was mixed with 2mL DMEM. The work concentration of final siRNA is 100nM. The mixture was added to Petri dishes, and the cells were cultured at 37 °C for 48-72 h.

6. Flow cytometry

Cells were seeded in a 6-well culture plate at 5 x 10^4 per well. Three holes for FAP gene silencing as well as the previous treatment, the remaining as control groups. The two groups cell were incubated with **HCFP** (5 μM) for 30 min at 37 °C. The cells of each well were digested with trypsin, centrifuged, resuspended with 1 mL of PBS. According to the instruction of BD LSRFortessa flow cytometry, the parameters of excitation and emission light are set in reference to Alexa Fluor 700, because its performance is similar to that of **HCFP**. The fluorescence information of the 10000 cells was counted. The fluorescence data of two groups were compared by FlowJ software.

7. Western blot

The western blot assays were performed according to the previous method.[6]

8. Cytotoxicity assay

The cytotoxicity of **HCFP** was tested on MCF-7 cells using a standard MTT assay, as described previously.[3]

9. In vivo imaging of mice

All animal experiments were approved by the animal care and use committee of the Ningbo University. Breast cancer mouse model was established by MCF-7 cells. Female nude mice (BALB/c, 4 weeks) were purchased from the Card Vince Laboratory Animal Co. Limited (Changzhou, China). The concentrations of MCF-7 cells in serum-free DMEM were 1×10^8 cells/mL, and the subcutaneous inoculation of each mouse was 100 μL. After two or three weeks, 50 μL PBS containing 50 μM **HCFP** was injected into tumor-bearing mice. At different time intervals, the mice were anesthetized and scanned using an in vivo imaging system with excitation at 670 nm and emission at 710 nm.

10. Supplementary figures

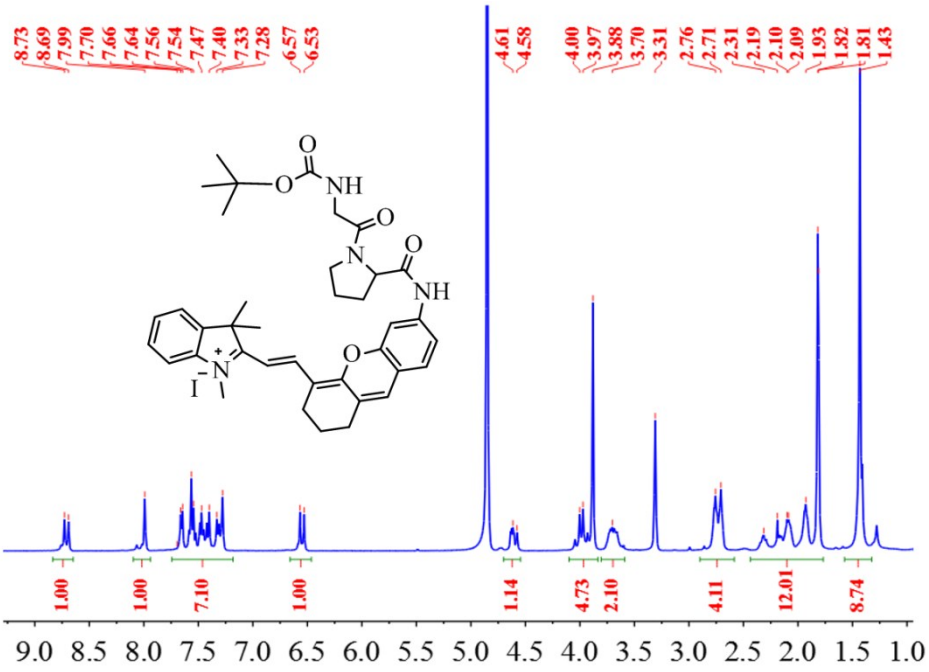

Figure S1. ^{1}H NMR spectrum of **4** (400 MHz, CD_3OD, 298 K).

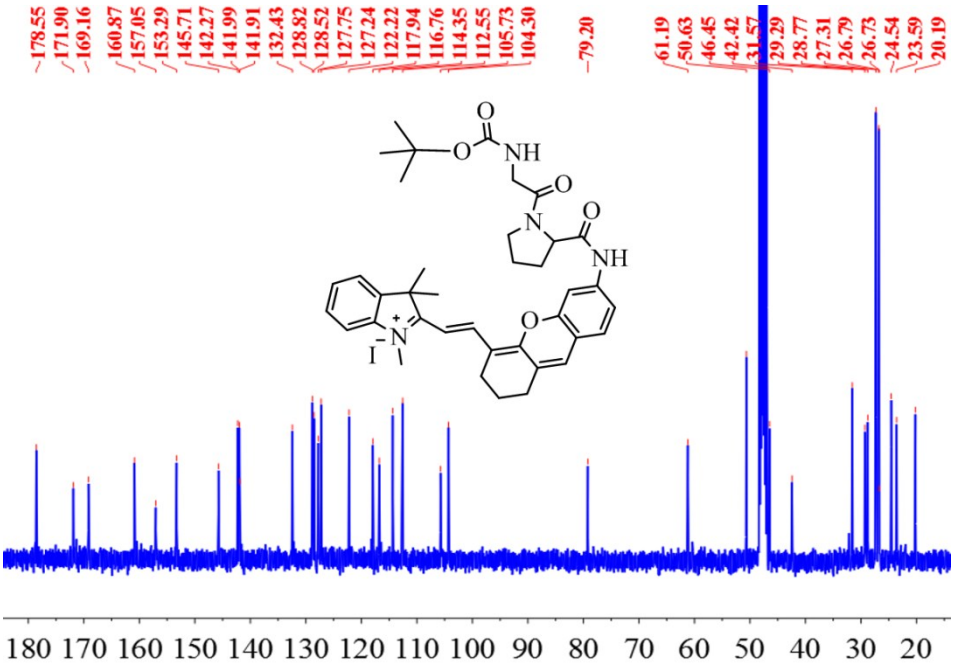

Figure S2. ^{13}C NMR spectrum of **4** (100 MHz, CD_3OD, 298K).

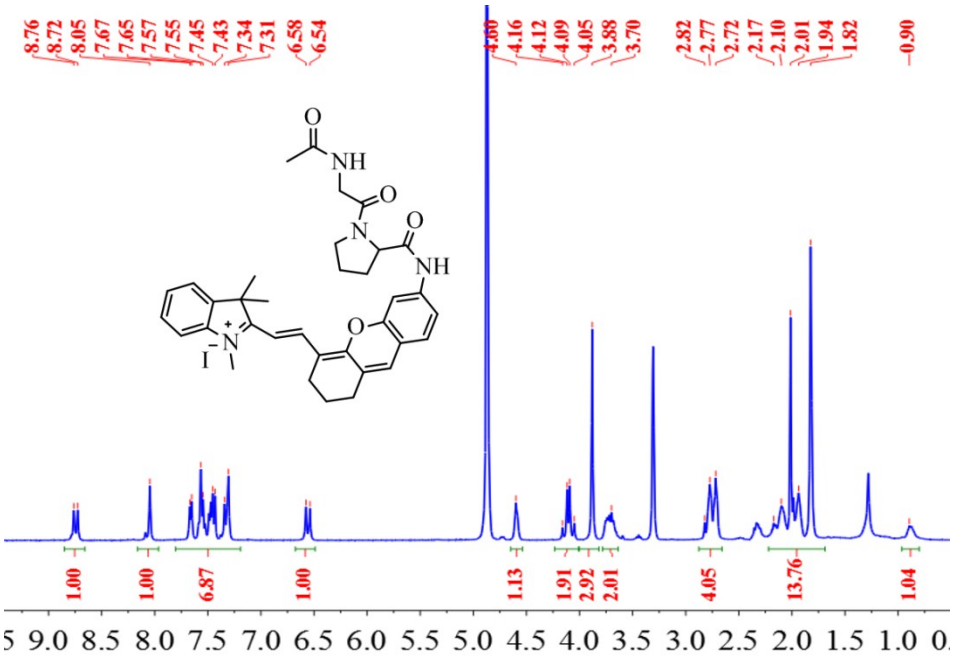

Figure S3. ^{1}H NMR spectrum of **HCFP** (400 MHz, CD_3OD, 298 K).

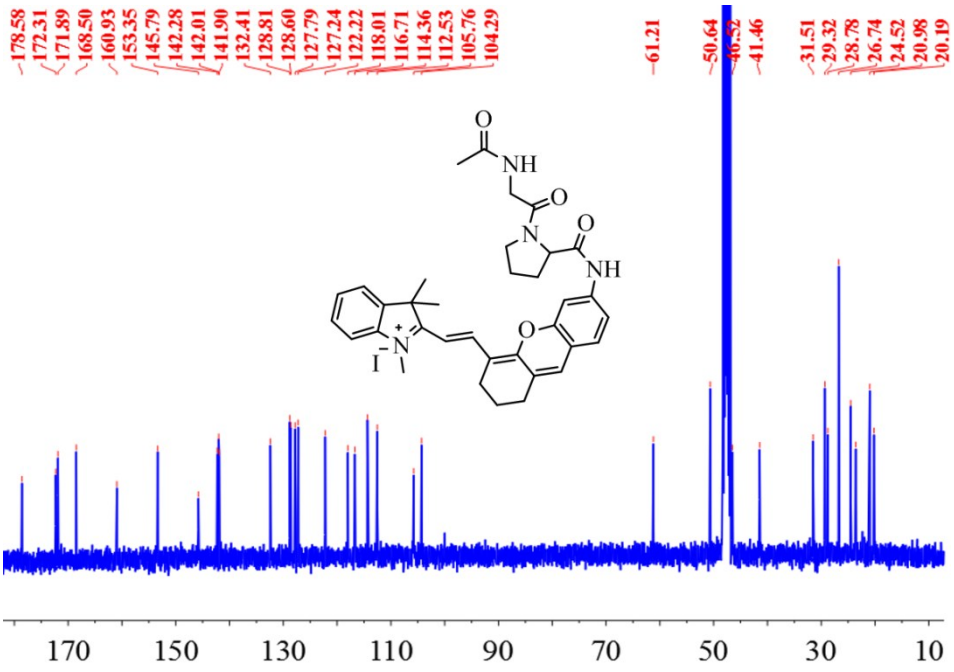

Figure S4. ^{13}C NMR spectrum of **HCFP** (100 MHz, CD_3OD, 298K).

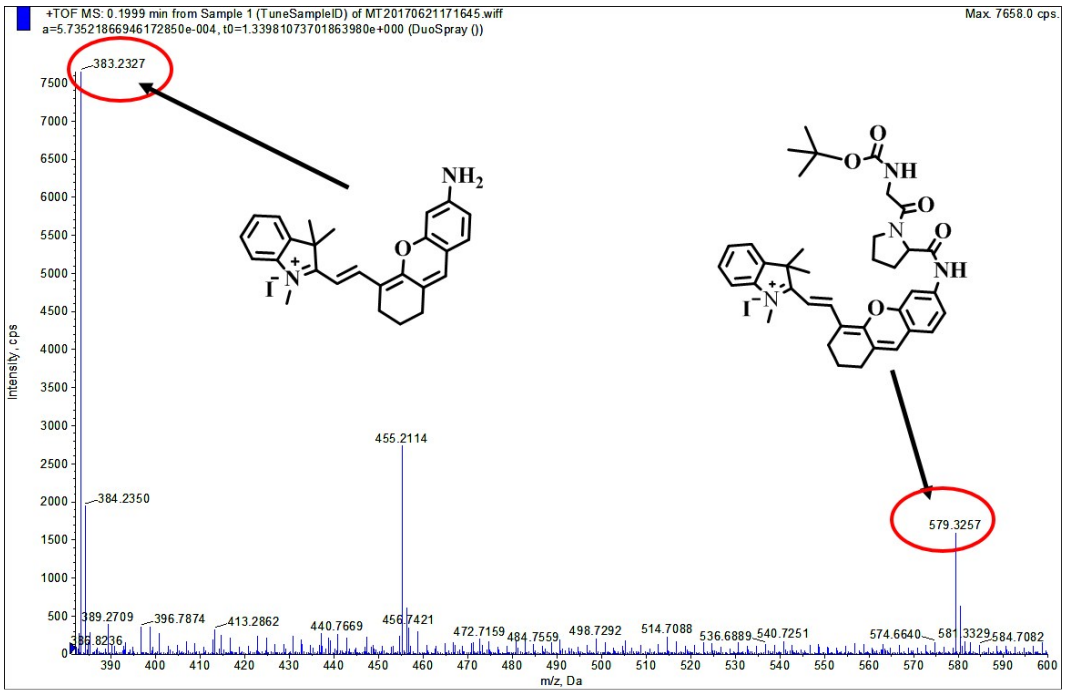

Figure S5. ESI mass spectra of the reaction products.

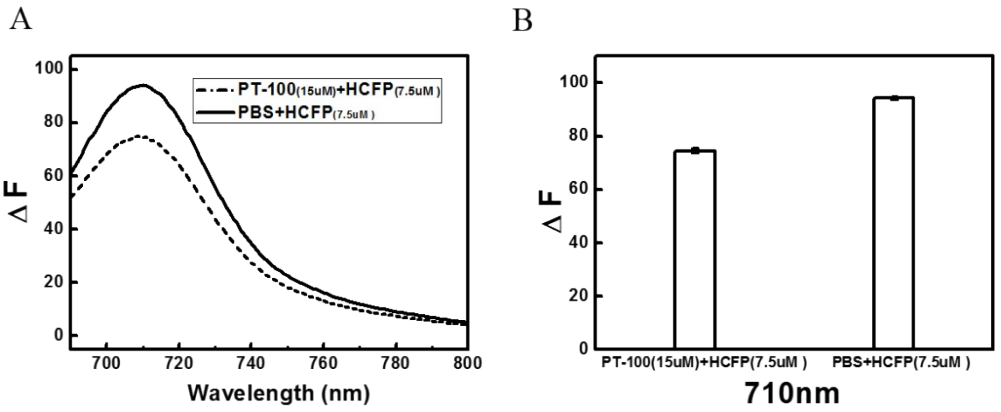

Figure S6. Fluorescence spectra of MCF-7 cells with or without FAP inhibitor.

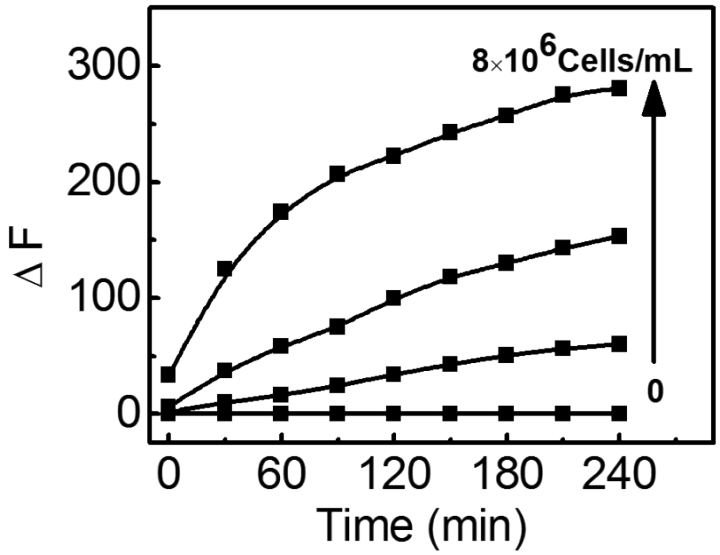

Figure S7. Plots of fluorescence intensity vs. the reaction time of **HCFP** (7.5 μM) with varied concentrations of MCF-7 Cells (0-8×10^6 cells/mL). $\lambda_{ex}/\lambda_{em}$ =670/710 nm.

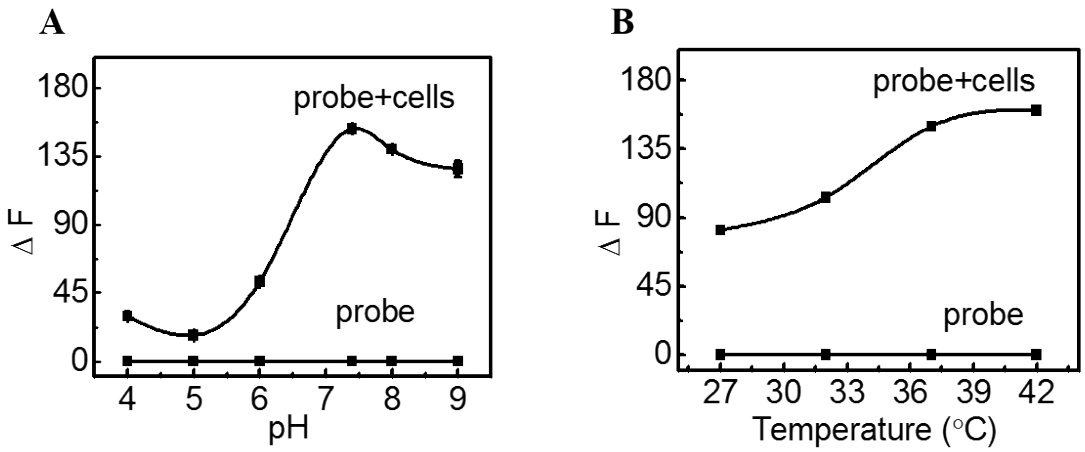

Figure S8. Effects of pH (A) and temperature (B) on the fluorescence of **HCFP** (7.5 μM)with 2×10^6 cells/mL. $\lambda_{ex}/\lambda_{em}$ =670/710 nm.

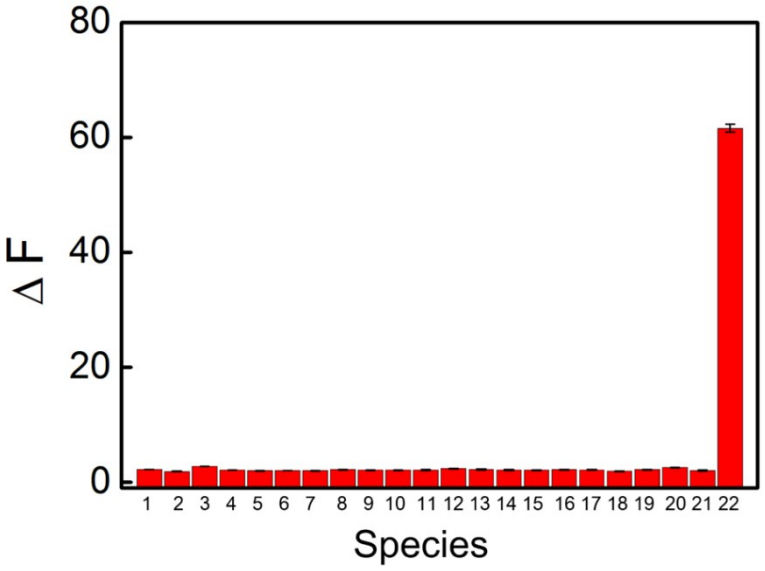

Figure S9. Fluorescence change of FAP probe in the presence of various species. (1) probe only (7.5 μM; control); (2) 150 mM KCl; (3) 2.5 mM CaCl2; (4) 2.5 mM MgCl2; (5) 100 μM ZnCl2 ; (6) 100 μM CuCl2; (7) 10 mM glucose; (8) 1 mM vitamin C; (9) 5 mM glutathione; (10) 1 mM cysteine; (11) 1 mM arginine;(12) 1 mM tryptophan ; (13) 1 mM lysine; (14) 1 mM threonine ; (15) 1 mM histidine ; (16) 1 mM phenylalanine; (17) 90 μg/mL esterase ; (18) 250 μg/mL trypsin; (19) 25 μg/mL prolidase; (20) 0.5 μg/mL DPPIV; (21) 30 μg/mL LAP; (22) $5x10^5$ cells.

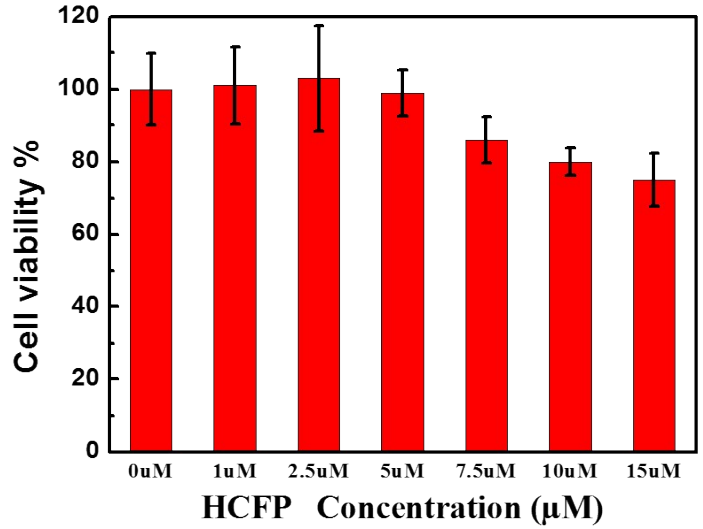

Figure S10. Effects of **HCFP** at varied concentrations on the cell viability of MCF-7 cells.

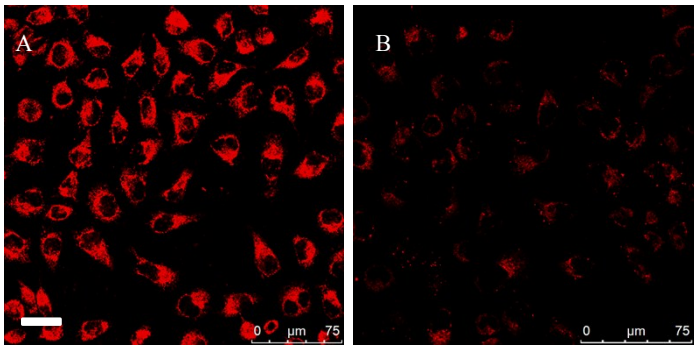

Figure S11. (A) MCF-7 cells only (control) incubated with 5 μM **HCFP** for 30 min. (B) The cells were first treated with PT-100 (10μM, 1h) and then incubated with 5 μM **HCFP** for 30 min. Scale bar 75 μm.

11. References

1. D. L. Gallaher, M. E. Johnson, *Analyst*, 1999, **124**, 1541-1546.
2. J. J. Zhang, B. F. Yu, L. L. Ning, X. Y. Zhu, J. X. Wang, Z. J. Chen, X. Y. Liu, X. J. Yao, X. Y. Zhang, H. X. Zhang, *Eur. J. Org. Chem*. 2015, **8**, 1711-1718.
3. X. Y. He, L. H. Li, Y. Fang, W. Shi, X. H. Li, H. M. Ma, *Chem. Sci.* 2017, **8**, 3479-3483.
4. M. Sakabe, D. Asanuma, M. Kamiya, R. J. Iwatate, K. Hanaoka, T. Terai, T. Nagano, Y. Urano, *J. Am. Chem. Soc.* 2013, **135**, 409-414.
5. B. J. Zhao, P. Wu, H. Zhang, C. X. Cai, *Biosens. Bioelectron.* 2015, **68**, 763-770.
6. Q. Y. Gong, W. Shi, L. H. Li, X. F. Wu, H. M. Ma, *Anal. Chem.* 2016, **88**, 8309-8314.