Supporting Information

A NIR fluorescence probe for dual imaging of mitochondrial viscosity and FA in living cells

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1. Materials and instrumentations

Without further purification, all chemical reagents and materials used were purchased commercially. Thin layer chromatography (TLC) was used to monitor the progress using GF254 silica gel precoated glass-backed plates, and flash column chromatography separations using silica gel were used for purification (200-300 mesh). [1]Bruker Avance 400 or Bruker DPX 300 spectrometer were used to measure [1]H NMR and 13C NMR spectra. Mass spectrometer (micrOTOF-Q II 10203) equipped with AP-ESI ion source measured high-resolution mass spectrometry. The confocal microscope model is LSM(Zeiss). Beyotime Biological Technology Co., Ltd. provided Lyso-Tracker Red、Mito-Tracker Red、ER-Tracker Red and 3-(4,5-Ddimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Thermo Fisher Scientific Co., Ltd. provided fetal bovine serum (FBS), DMEM, and RPMI 1640 media (Shanghai, China).

2. Experiment

2.1. Synthesis of (E)-2-chloro-3-(hydroxymethylene) cyclohex-1-ene-1-carbaldehyde (1).

To a solution of phosphorus oxychloride (8 mL) in DCM (10 mL) was added DMF (8 mL) The mixture was stirred at 0 °C for 0.5 h. After the cyclohexanone (2.00 g, 20.38 mmol) was added. The mixture was refluxed at 80 °C for 3 h. After cooled, the solution was poured into ice water and allowed to stand overnight to afford compound **1** with yield 65%. The product was used directly for the next reaction without purification.

*2.2. Synthesis of 2-((E)-2-((E)-2-chloro-3-(2-((Z)-1,3,3-trimethylindolin-2-ylidene) ethylidene) cyclohex-1-en-1-yl) vinyl)-1,3,3-trimethyl-3H-indol-1-ium (**3**).*

To a solution of compound **1** (2.30 g, 13.32 mmol) and compound **2** (8.03 g, 26.65 mmol) in acetic anhydride (10 mL) was added acetic anhydride (109 mg, 1.33 mmol). The mixture was stirred at 70 °C for 2 h under N_2 atmosphere. After the reaction completed, the mixture solution was poured into ice water. Then the precipitate was filtered and washed with distilled water several times to afford the crude product. The residue was purified by chromatography on silica gel column (DCM/MeOH = 100:1 v/v) to furnish the desired compound **3** as a dark green solid. with 85% yield.

*2.3. Synthesis of (E)-2-(2-(6-hydroxy-2,3-dihydro-1H-xanthen-4-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium (**4**).*

Potassium carbonate (4.23 g, 30.68 mmol) was added to a solution of resorcinol (3.55 g, 27.27 mmol) in MeCN (50 mL) at room temperature and stirred for 20 min under N_2 atmosphere. Then compound **3** (5.50 g, 11.36 mmol) was added to the above solution and stirred for 4 h at 50 °C. After the reaction completed, the solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (DCM/MeOH = 20:1 v/v), obtaining compound **4** as a blue solid with 50% yield.

*2.4. Synthesis of (E)-2-(2-(7-formyl-6-hydroxy-2,3-dihydro-1H-xanthen-4-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium (**5**).*

To a solution of compound **4** (1.10 g, 5.68 mmol) in trifluoroacetic acid (30 mL) was added hexamethylenetetramine (954 mg, 6.82 mmol) at 115 ℃ for 4 h. After the reaction completed, the resulting mixture was quenched with ice water. The reaction mixture was diluted with H_2O and extracted with DCM (3 × 30 mL). The combined organic layer was washed with saturated NaCl aqueous solution, dried over Na_2SO_4, and concentrated in vacuo to give a blue residue. The residue was purified by chromatography on silica gel column (DCM/MeOH = 50:1 v/v) to furnish the desired compound **5** as a blue solid solid. with 32% yield.

*2.5. Synthesis of (E)-2-(2-(7-(1-aminobut-3-en-1-yl)-6-hydroxy-2,3-dihydro-1H-xanthen-4-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium (**6**).*

To a solution of compound **5** (1.10 g, 5.68 mmol) in methanol (10 mL) was added NH_3•H_2O (2 mL) at 0°C for 30 min. Then, the allylboronic pinacol ester (244 mg, 1.45 mmol) was added to the mixture. The mixture was stirred for a further 8 h at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (DCM/MeOH = 5:1 v/v), obtaining compound **6** as a blue solid with 34% yield.

*2.6. Synthesis of (E)-2-(2-(6-hydroxy-7-(1-((4-nitrobenzyl)amino)but-3-en-1-yl)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium(**FA-Cy**).*

To a solution of **6** (140 mg, 0.31 mmol) and p-Nitrobenzyl bromide (80 mg, 0.37

mmol) in MeCN (5 mL) was added potassium carbonate (128 mg, 0.93 mmol) at room temperature for 5 h. After the reaction completed, the reaction mixture was diluted with H_2O and extracted with DCM (3 ×10 mL). The combined organic layer was washed with saturated NaCl aqueous solution, dried over Na_2SO_4, and concentrated in vacuo to give a blue residue. The residue obtained was purified using silica gel chromatography (DCM/MeOH = 30:1 v/v), obtaining **FA-Cy** as a blue solid with 22% yield.

3. General procedures for spectral measurements

FA-Cy stock solution (10 mM) was prepared in DMSO. The test system was obtained by adding FA-Cy (10 μM) to 10 mM pH 7.4 PBS buffer. Fluorescence and UV absorption spectra were measured on the shaken solution. Then, relevant test substances (amino acids, cations, anions, reactive oxygen species, etc.) were added, and the spectra were recorded after 3 h at 37 ℃. The 628 nm excitation wavelength, the 640-800 nm emission range, the 5 nm slit width for the excitation and emission, and the 1 nm step were all used for all measurements. For the viscosity test, the probe FA-Cy (10 μM) was added to 5 ml of solutions composed of different proportions of PBS and glycerol, ultrasonicated for 10 min at a constant temperature for 1 h to eliminate air bubbles. Fluorescence spectra for these solutions were determined. Record and measure the viscosity value with a viscometer. The fluorescence spectra were measured with the wavelength of maximum absorption of 628 nm as the excitation wavelength. The recording range for fluorescence emission is 640 ~ 800 nm. The slit width is 5 nm for both excitation and emission.

4. detection limit

The detection limit was established through fluorescence titration. The fluorescence intensity was measured five times at 702 nm for FA-cy without FA to determine the signal- noise ratio, and the blank measurements were taken, and the standard deviation was found. The detection limit is obtained through the FA titration curve of the probe using the following formula:

$$\text{Detectionlimit} = 3\sigma / k$$

where k is the slope between FA concentration and fluorescence intensity of FA-Cy at 702 nm, σ is the standard deviation of blank measurements.

5. Cytotoxicity determined by MTT assay

HeLa cells were cultured in an incubator at 5% CO_2 and 37 °C after being seeded into 96 well plates at a density of 1×10^4 per well. FA at final concentrations of 0, 1, 5, 10, and 20 μM was co-cultured for 24 h with HeLa cells. After that, 10 μL of 3- (4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide (MTT, 5 mg / ml) was added and cultured for another 4 h. The purple precipitate was clearly discernible under the microscope. A 100 μL DMSO was added and gently shaken for the removal of precipitate. Finally, a microplate reader set to 570 nm was used to measure the absorbance of each well, including the blank wells. The following is the calculation formula for cell activity:

$$\text{Cell viability \%} = (OD_{sample} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$$

In the equation, OD_{sample} represents cells incubated with varying probe concentrations, $OD_{control}$ represents cells without the probe, and OD_{blank} represents wells containing only culture medium.

6. Cell culture and fluorescence imaging

The human cervical cancer (Hela) was cultured in DMEM supplemented with 10% fetal bovine serum and 1% double antibodies (penicillin 50 U/mL, streptomycin 50 μg/mL). After determining the optimal cell density for experiments, cells are seeded into the confocal culture dish, the appropriate amount of culture medium is added, and the cells are placed in the incubator cultured 12 h to make them fully adherent. The sample is then incubated for a period of time with the probe. Finally, cells were washed 3 times with PBS buffer before confocal imaging. The mean fluorescence intensity of FA-Cy-treated Hela cells was also estimated by flow cytometry (FC).

7. Subcellular organelles localization experiment of the probe

Three commercial green fluorescent dyes, namely Mito-Tracker Green, ER-Tracker Green, and Lyso-Tracker Green, were screened and tested by co-staining cells with probes for use in colocalization experiments. Cell imaging was obtained using excitation wavelengths of 633 nm and 488 nm. The organelle localization effect of the probe was monitored by superimposing the spectra at the two wavelengths.

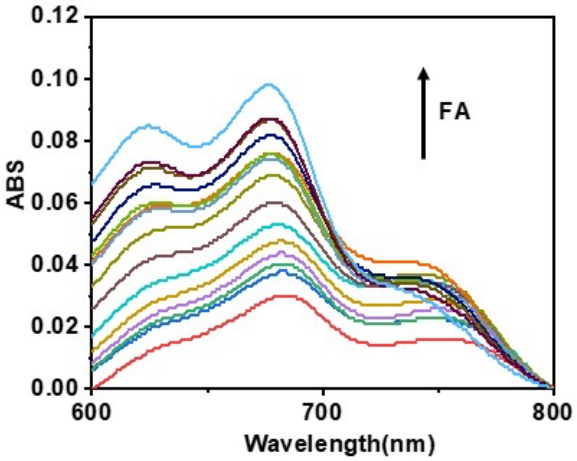

Fig. S1 The absorption and emission spectra of FA-Cy in 10 mM PBS.

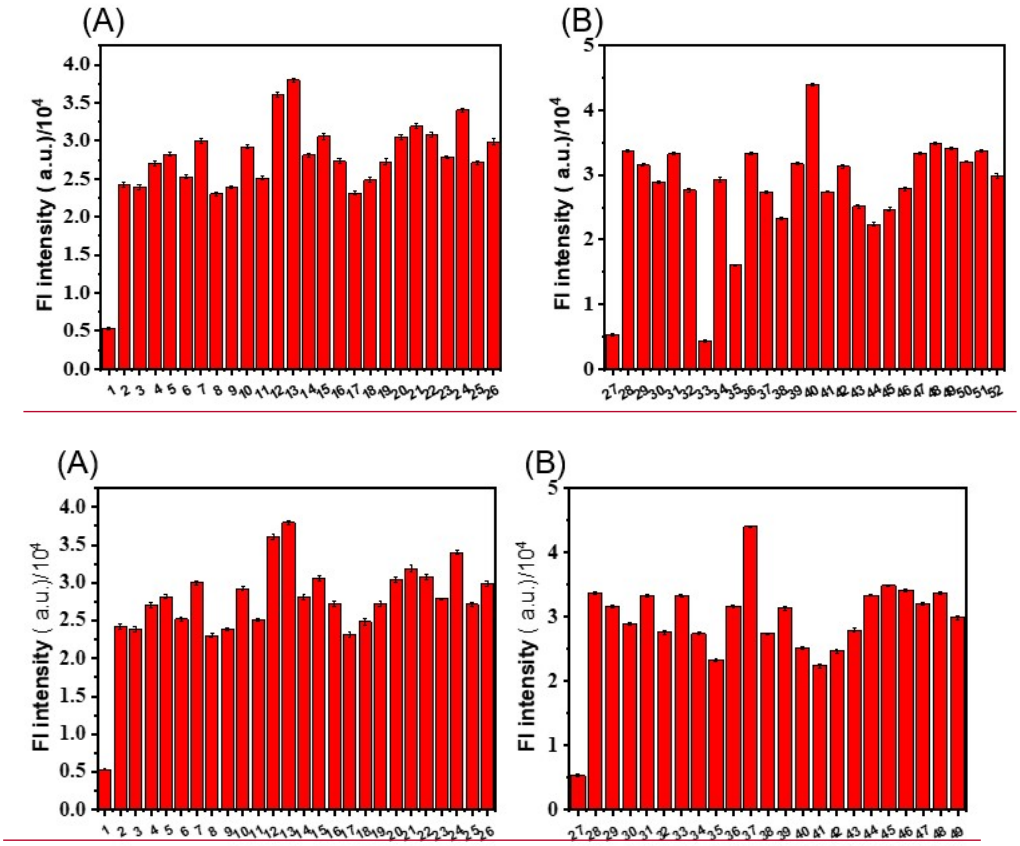

Fig. S2 Fluorescence intensities of FA-Cy upon the addition of various species and FA;(A-B).

1.blank，2.Ba^{2+} , 3.Ca^{2+} , 4.Cd^{2+} ,5.Co^{2+} , 6.Cr^{2+} , 7.Cu^{2+} , 8.Fe^{2+} , 9.K^{+} , 10.Li^{+} , 11.Mg^{2+} , 12.Mn^{2+} , , 13.Pb^{2+} , 14.Sr^{2+} , 15.Zn^{2+} , 16.OH^{-} , 17.HCO_3^{2-} , 18.CO_3^{2} , 19.F^{-} , 20.Cl^{-} , 21.SO_4^{2-} , 22.SO_3^{2-} , 23.SCN^{-} , 24.Br^{-} , 25.PO_4^{2-}, 26.FA, 27 .blank, 28.ACO^{-}, 29.$^{\cdot}O$, 30.H_2O_2, 31.O^{2-}, 32.TBHP, 33.OPA, 34.MGO, 35.GO, 36.GOA, 37.Cys, 38.Phe, 39.Ala, 40.Gly, 41.Glu, 42.Met, 43.Arg, 44.Lys, 45.Leu, 46.Pro, 47.Ser, 48.Thr, 49.Val, 50.Ile, 51.His. 52.FA.~~33.GOA, 34.Cys, 35.Phe, 36.Ala, 37.Gly, 38.Glu, 39.Met, 40.Arg, 41.Lys, 42.Leu, 43.Pro, 44.Ser, 45.Thr, 46.Val,~~

~~47.Ile, 48.His. 49.FA.~~

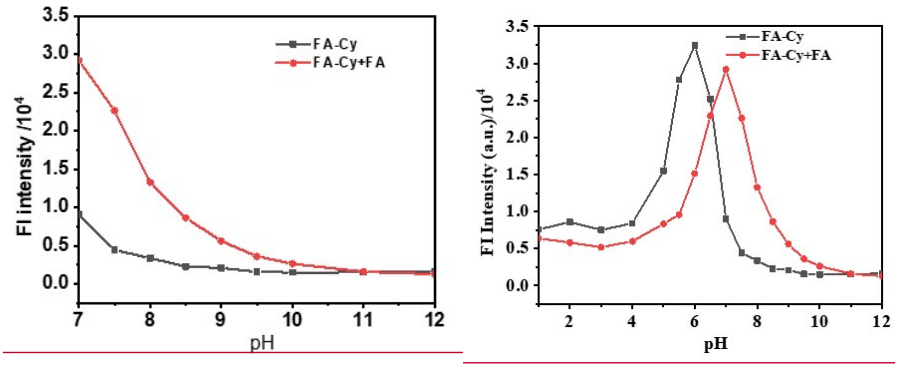

Fig. S3 Effects of pH on the fluorescence of FA-Cy (10 μM) before and after reacting with 10 mM FA

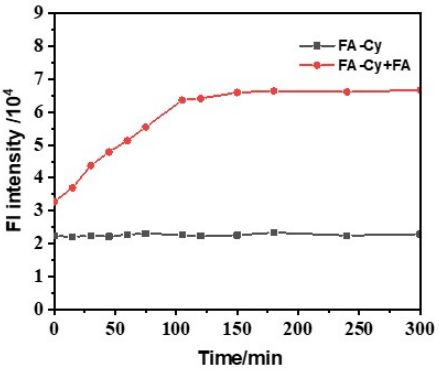

Fig. S4 Effects of reaction time on the fluorescence of FA-Cy (10 μM) in the absence of any FA or in the presence of 10 mM FA

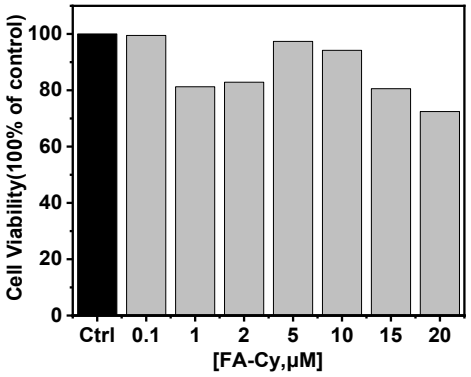

Fig. S5 Viability of HeLa cells treated with FA-Cy at 37 °C for 12 h.

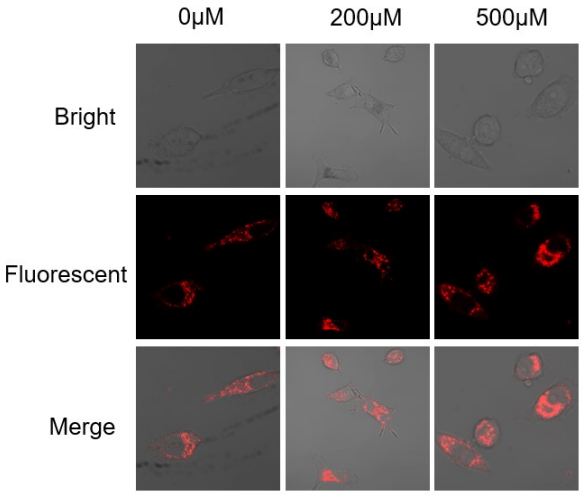

Fig. S6 Confocal imaging with probes FA-Cy (5 μM) and FA at different concentrations; λ_{ex} = 633 nm, λ_{em} = 680-800 nm;

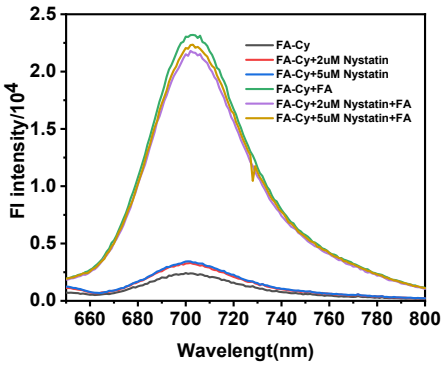

Fig. S7 Effect of nystatin on the reaction of FA-Cy and FA

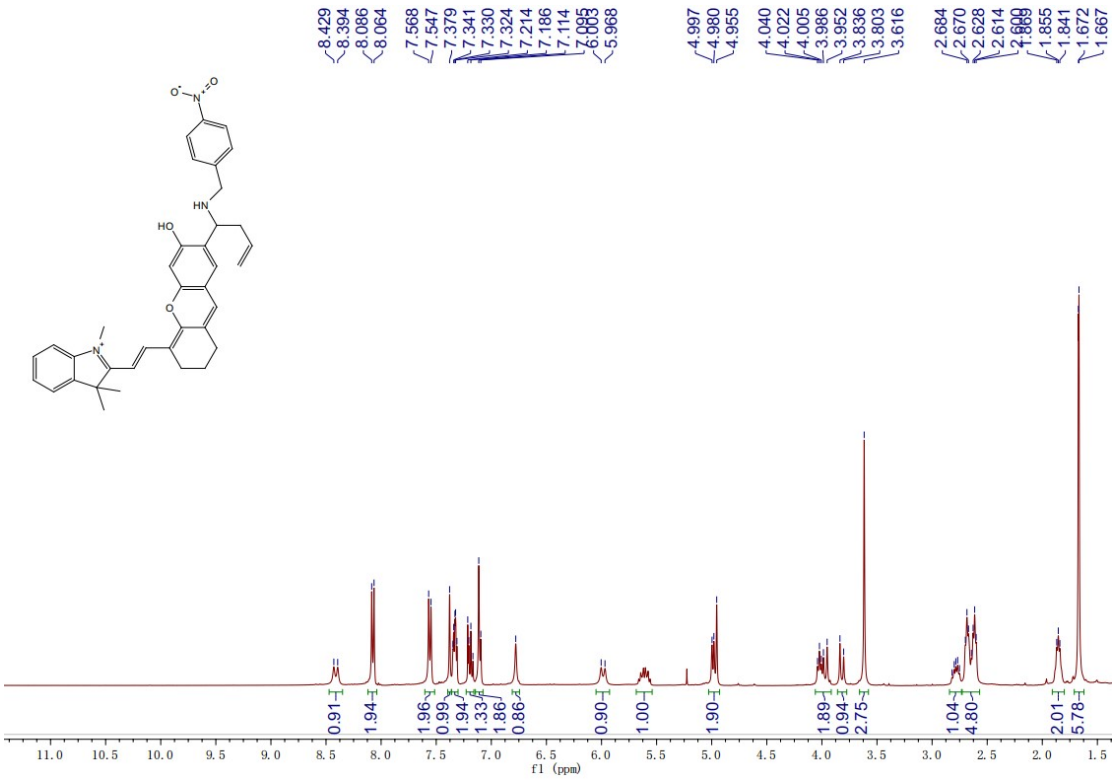

Fig. S8 ^{1}H NMR of FA-Cy (400 MHz, $CDCl_3$).

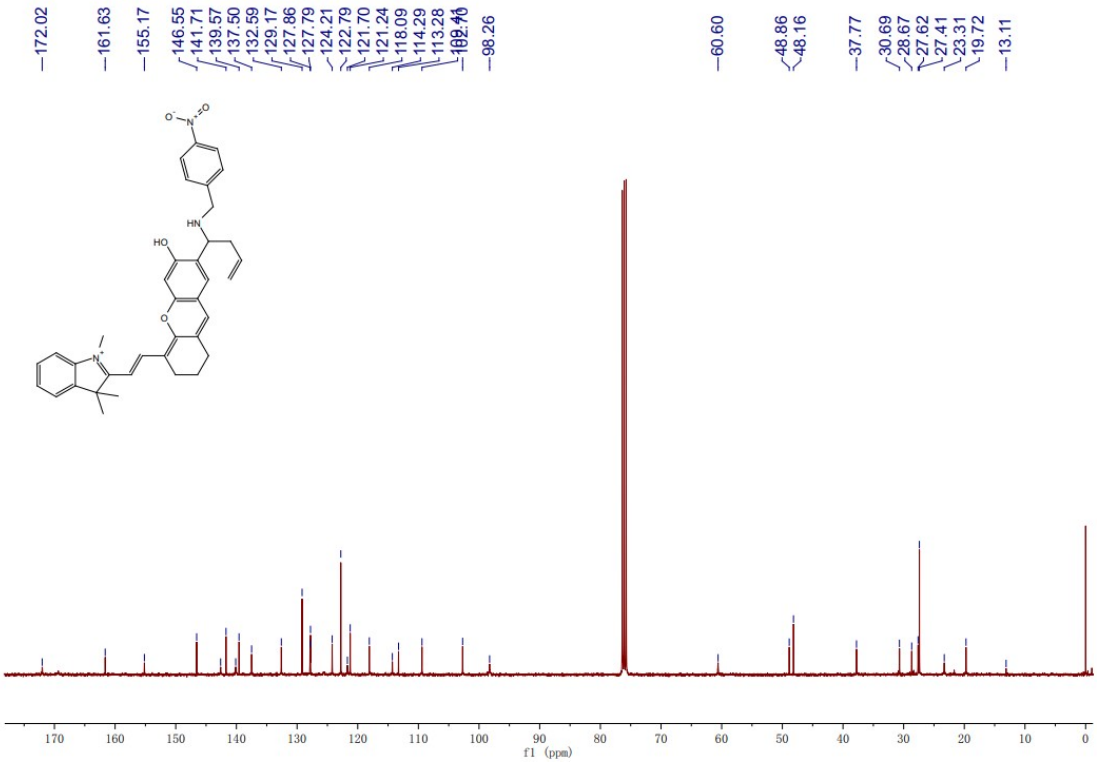

Fig. S9 ^{13}C NMR of FA-Cy (100 MHz, $CDCl_3$).

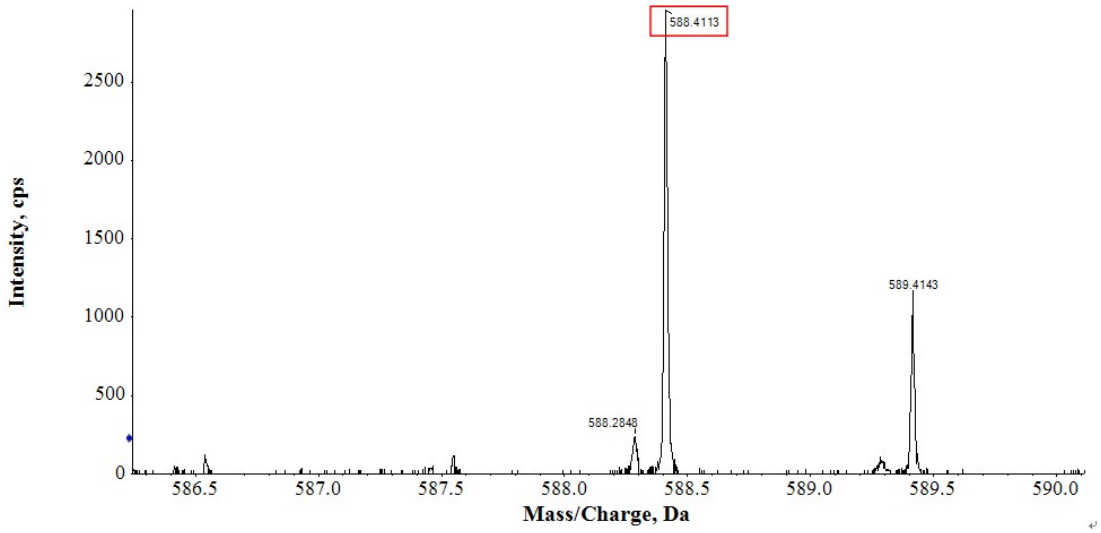

Fig. S10 HRMS (LC/MS) spectra of FA-Cy. The peak at m/z = 588.4113 was assigned to the mass of $[FA\text{-}Cy]^+$.

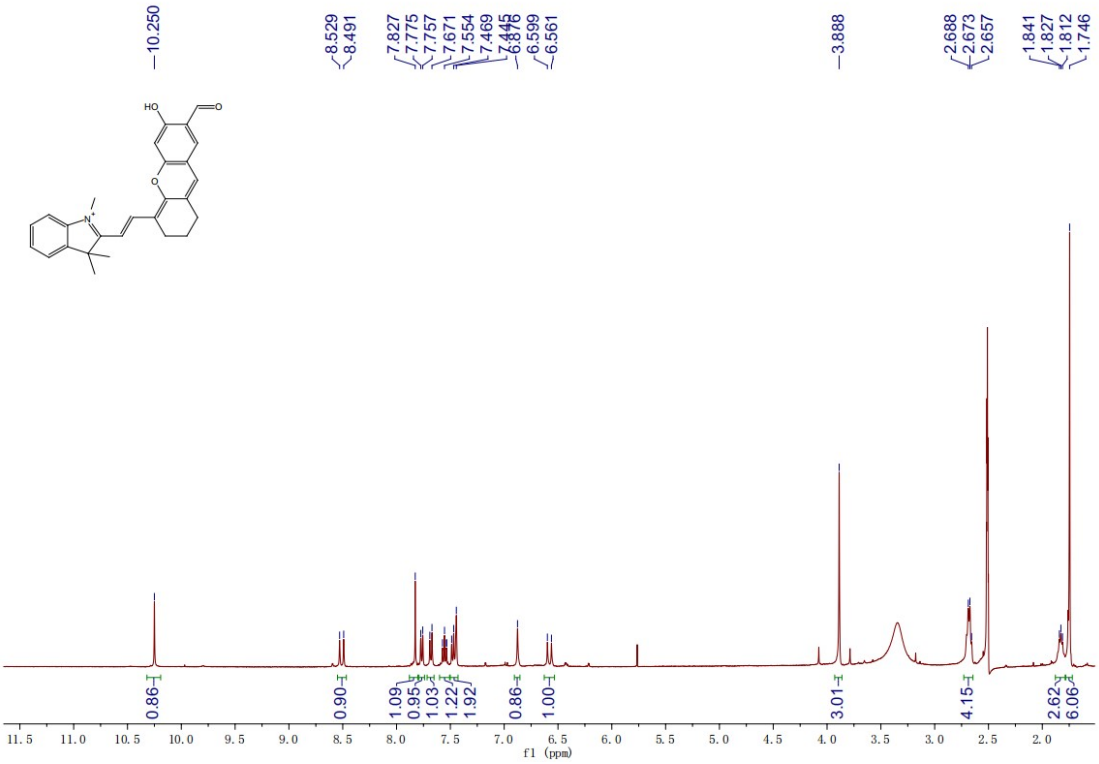

Fig. S11 ^1H NMR of FA-Cy-B (400 MHz, $CDCl_3$).

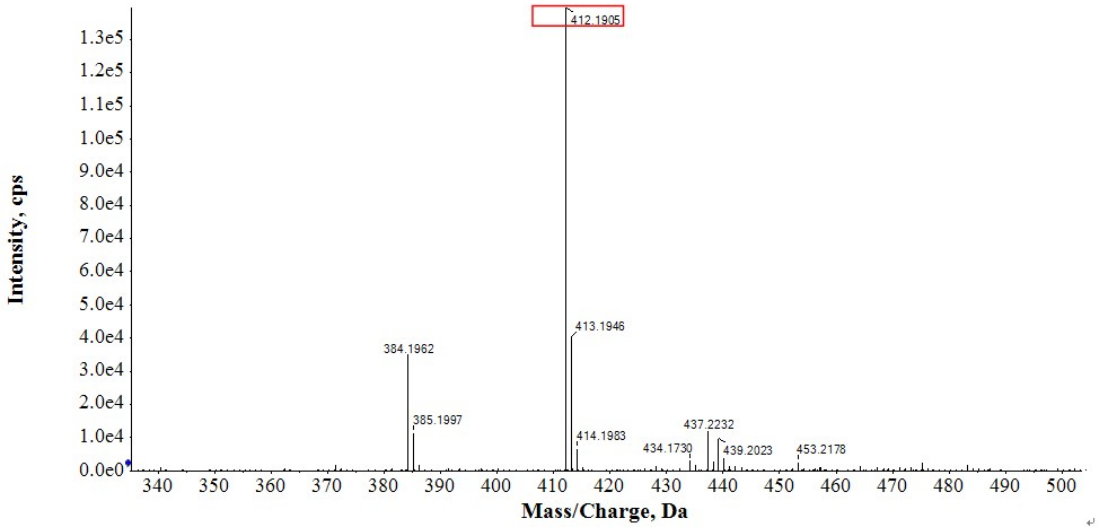

Fig. S12 HRMS (LC/MS) spectra of FA-Cy-B. The peak at m/z = 412.1905 was assigned to the mass of $[FA\text{-}Cy\text{-}B]^{+}$.