Imaging Acidosis in Tumor by A pH-Activatable Near-Infrared Fluorescence Probe

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Materials and General Experimental Methods

All chemical reagents for the probe synthesis were obtained from Aladdin Reagent (Shanghai, China) unless otherwise specified. Column chromatography was performed on silica gel (300–400 mesh) purchased from Aladdin Reagent. Synthetic reactions were monitored by TLC (Merck, Whitehouse Station, NJ, silica gel, 60 Å) and high performance liquid chromatography (HPLC) using an Agilent (Agilent, USA) G1311A binary pump equipped with an Agilent G1315B photodiode array detector and Waters SYMMETRY® C-18 analytic column (4.6 mm × 100 mm). pH Activatable probe DiIR783-S and control probe DiIR783-C were detected at a wavelength of 783 nm. NMR spectra (1H-400 MHz and 13C-100 MHz) were recorded on a Varian Mercury 400 spectrometer instrument using tetramethysilane as the internal reference. The high resolution electron spray ionization (HR-ESI) mass spectra were obtained on a Micromass Q-TOF 2 mass spectrometer with water as the carrier solvent. UV-Vis absorption spectra were performed on a SHIMADZU UV-2550 spectrophotometer with the wavelength range from 400–900 nm. The scanning speed is 0.5 nm/s and the slit width is 2.0 nm. Fluorescence spectra were collected on a SHIMAZDU RF-5301PC spectrofluorophotometer using the excitation wavelength of 765 nm and emission wavelength of 785–900 nm. Wavelength scanning was carried out in increments of 1.0 nm with 0.1 second integration time and the slit widths were set to 5.0 nm/5.0 nm (excitation/emission). pH Value was determined by a Mettler Toledo MP220 pH meter equipped with a Mettler Toledo InLab® glass electrode (Columbus, OH). This electrode was calibrated using standard buffer solutions of pH 7.0 and pH 4.0 at 25°C. Fluorescence microscopic images were obtained from a Leica DMF4000B laser-scanning microscope (Leica Inc, Wetzlar, Germany) inverted microscope with epifluorescence and phase contrast optics using 20× objective lenses. The NIR fluorescence was collected by using an Y7 filter tube (excitation: 710 ± 37.5 nm and emission: 810 ± 45 nm) and the green fluorescence was obtained using a L5 filter (excitation: 480 ± 20 nm and emission: 527 ± 15 nm). In vivo and ex vivo fluorescence images were acquired in Maestro™ system equipped with a 710–760 nm excitation filter and a 800 nm emission band pass filter set. Centrifugation was carried out in the Sigma 3K15 centrifuge (Sigma, Germany) with a speed of 3300 rpm. All the compounds and intermediates were stored in the Hair DW-40W100 fridge (Hair, China) at -40 ºC.
Synthesis and Characterization

Preparation of IR783 fluorophore. IR783 fluorophore was synthesized according to the previous reference\(^1\).

Preparation of compound 1 The compound 1 was synthesized according to the reported procedure\(^2\). IR783 (200 mg, 0.28 mmol), 3-mercaptopropionic acid (28 µL, 0.32 mmol) and triethylamine (44 µL, 0.32 mmol) were mixed in 6.0 mL DMF solution and stirred at room temperature for 14 h. The reaction was monitored by TLC and a product with higher polarity was observed. Then the mixture was dropped into an ice-cold methyl ether and the crude product as green precipitate was filtrated. The crude product was purified by silica gel based column chromatography with an eluting solvent system in which the gradient of CH\(_2\)Cl\(_2\)/CH\(_3\)OH changed from 90\% to 70\%. Removal of solvent and drying under vacuum afforded the purified product as a golden powder. Yield: 168.7 mg (75\%).

Preparation of compound 2. Compound 1 (100 mg, 0.12 mmol), 1-ethyl-3-(3-dimethyllaminopropyl) carbodiiehydrochlide (80 mg, 0.42 mmol) and 1-hydroxy-7-azabenzotriazole (40 mg, 0.29 mmol) were dissolved in 6.0 mL DMF solution. Then 2,2-dimethoxyethanemine (48 mg, 0.46 mmol) was added and stirred at room temperature for 14 h. TLC monitored a product with lower polarity compared to that of 2. Then the mixture was dropped into the methyl ether in an ice bath and the green precipitate was filtrated. The crude was purified by column chromatography with an eluting solvent system in which the gradient of CH\(_2\)Cl\(_2\)/CH\(_3\)OH changed from 90\% to 75\%. Removal of solvent and drying under vacuum afforded 2 as a golden powder. Yield: 93.8 mg (85\%).

The crude was purified by column chromatography with an eluting solvent system in which the gradient of CH\(_2\)Cl\(_2\)/CH\(_3\)OH changed from 90\% to 75\%. Removal of solvent and drying under vacuum afforded 2 as a golden powder. Yield: 93.8 mg (85\%). \(^1\)H NMR (400 MHz, CD\(_3\)OD) \(\delta = 8.86\) (d, \(J=14.1,\) 2H), 7.49 (d, \(J=7.4,\) 2H), 7.40 (t, \(J=7.6,\) 2H), 7.34 (d, \(J=7.9,\) 2H), 7.25 (t, \(J=7.4,\) 2H), 6.33 (d, \(J=14.1,\) 2H), 4.36 (t, \(J=5.4,\) 1H), 4.19 (t, \(J=6.8,\) 4H), 3.25 (d, \(J=5.4,\) 2H), 3.06 (t, \(J=7.2,\) 2H), 2.90 (t, \(J=6.9,\) 4H), 2.70 (t, \(J=5.7,\) 4H), 2.52 (t, \(J=7.2,\) 2H), 2.06 – 1.89 (m, 10H), 1.74 (s, 12H); \(^{13}\)C NMR (101 MHz, D\(_2\)O) \(\delta = 173.90(2\times C),\) 173.21(C), 157.92(C), 147.19(2\times C), 143.79(2\times CH), 142.46(2\times CH), 135.17(2\times CH), 129.91(2\times C), 126.25(2\times CH), 123.48(2\times CH), 112.16(2\times CH), 103.65(CH), 102.39(2\times CH), 54.32(2\times CH\(_3\)), 51.87(2\times CH\(_2\)), 50.56(2\times CH\(_2\)), 44.96(2\times C), 42.10(CH\(_2\)), 36.96(CH\(_2\)), 34.30(CH\(_2\)), 28.46(4\times CH\(_3\)), 27.34(4\times CH\(_2\)), 23.63(2\times CH\(_2\)), 22.22(CH\(_2\)).TOF-MS: C\(_{45}\)H\(_{60}\)N\(_3\)O\(_9\)S\(_3\) [M], found 882.3473 (100\%), calculated 882.3492.
Preparation of compound 3. Deprotection of compound 2 (50 mg, 0.055 mmol) was achieved by stirring this compound in 6.0 mL formic acid solution (88%) at room temperature. After 12 h, TLC showed a product with a higher polarity. The solvent was removed at 40 °C under vacuum and the product was re-dissolved in a mixture of H₂O/CH₃OH. The solvent was evaporated again to remove the residue formic acid. Compound 3 after above treatment was used in next step without further purification. Yield: 47.3 mg, 100%. ¹H NMR (400 MHz, MeOD) δ = 8.89 (d, J=14.1, 2H), 7.52 (d, J=7.4, 2H), 7.50 – 7.38 (m, 3H), 7.36 (d, J=7.9, 2H), 7.27 (m, 2H), 6.35 (d, J=14.2, 2H), 4.22 (t, J=7.0, 4H), 3.21 (m, 2H), 3.10 (m, 2H), 2.91 (t, J=7.1, 4H), 2.73 (t, J=5.9, 4H), 2.55 (m, 2H), 2.05 – 1.93 (m, 10H), 1.77 (d, J=4.3, 12H); ¹³C NMR (101 MHz, MeOD) δ 172.48(2×C), 171.81(C), 156.55(C), 145.81(2×C), 142.38(2×CH₂), 141.04 (2×CH), 133.77 (2×CH), 128.46 (2×C), 124.78 (2×CH), 122.03 (2×CH), 110.72 (2×CH), 100.92 (2×CH), 95.72 (C), 50.43 (2×CH₂), 49.12 (2×CH₂), 44.22 (2×C), 43.52 (CH₂), 35.47 (CH₂), 32.80 (CH₂), 27.01 (4×CH₃), 25.90 (2×CH₂), 25.87 (2×CH₂), 22.22 (2×CH₂), 20.77 (CH₂).TOF-MS:C₄₃H₅₄N₃O₈S₃[M]-, found 836.3070 (100%), calculated 836.3073.

Preparation of compound 4. The mixture of compound 1 (100 mg, 0.12 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochlide (24 mg, 0.13 mmol) and hydroxybenzotriazole (20 mg, 0.15 mmol) in 6.0 mL DMF was stirred at r.t. for 15 min. Then tert-butoxycarbonyl hydrazide (20 mg, 0.15 mmol) was added into the mixture and stirred at r.t. for 14 h. TLC analysis showed a new product with lower polarity compared to 1. Then the mixture was dropped into the ice-cold methyl ether and a green precipitate was filtrated. The crude was purified by column chromatography with an eluting solvent system in which the gradient of CH₂Cl₂/CH₃OH changed from 90% to 75%. Removal of solvent and drying under vacuum afforded pure product 4 as a golden powder. Yield: 79.8 mg (70%).

Preparation of control probe DiIR783-C. Compound 4 (50 mg, 0.054 mmol) was dissolved in 6.0 mL mixture of TFA and CH₂Cl₂ (V/V = 1:1) and stirred at r.t. for 3 h. The reaction was monitored by TLC and a new product with higher polarity was observed. Then the solvent was evaporated at 40 °C and re-dissolved in CH₂Cl₂/CH₃OH solution. The solvent was evaporated again to fully
DiIR783-C

remove the residue TFA. Compound 5 was obtained and used in next step without further purification (44.5 mg, Yield: 99%). Compound 1 (40 mg, 0.049 mmol) and N,N-dicyclohexylcarbodiimide (12 mg, 0.058 mmol) were mixed in 2.0 ml DMF solution and stirred for 5.0 min. Then 1-hydroxy-7-azabenzotriazole (12 mg, 0.088 mmol) dissolved in 0.5 mL DMF was added dropwise. After 20 min, compound 5 (40 mg, 0.048 mmol) dissolved in 1.0 mL DMF was dropped into the compound 2 solution and the mixture was stirred in dark for 14 h. TLC showed a new product with higher polarity than both of the starting reagents. Then the mixture was dropped into ice-cold methyl ether and the green precipitate was filtrated. The crude product was purified by column chromatography on silica gel, using CH2Cl2/CH3OH gradient, from 80% to 50%. Removal of solvent under vacuum afforded the pure control probe DiIR783-C as golden powder. Yield: 15 mg (18.8%). 1H NMR (400 MHz, MeOD) δ = 8.84 (d, J=14.7, 4H), 7.48 (d, J=7.4, 4H), 7.40 (m, 4H), 7.34 (m, 4H), 7.24 (m, 4H), 6.32 (d, J=14.1, 4H), 4.19 (t, J=6.3, 8H), 3.07 (t, J=7.5, 4H), 2.90 (t, J=7.0, 8H), 2.70 (s, 8H), 2.55 (t, J=7.5, 4H), 1.95 (m, 20H), 1.71 (s, 24H); 13C NMR (101 MHz, D2O) δ 173.88(4×C), 171.71(2×C), 157.41(2×C), 147.20(4×C), 143.73 (4×CH), 142.42 (4×CH), 135.11 (4×CH), 129.85 (4×C), 126.18 (4×CH), 123.45 (4×CH), 112.11 (4×CH), 102.37 (4×CH), 51.82 (4×CH2), 50.50 (4×CH2), 44.90 (4×C), 35.04 (2×CH2), 33.76 (2×CH2), 28.40 (8×CH3), 27.28 (4×CH2), 27.23 (4×CH2), 23.60 (4×CH2), 22.19 (2×CH2). TOF-MS: C82H102N6Na2O14S6[M]^2, found 793.2886(100.0%), calculated793.2889.

Preparation of aiming probe DiIR783-S. Compound 3 (40 mg, 0.049 mmol) dissolved in 2.0 mL MES solution (pH 4.5) and compound 5 (42 mg, 0.049 mmol) dissolved in 0.5 mL DMF were mixed and stirred at r. t. for 16 h. The reaction was monitored by TLC and a product with a high polarity was observed. At the end of reaction, the pH of the mixture was adjusted to 7.4 by using 0.1 M sodium hydroxide solution. After the removal of the solvent under vacuum, the crude product was purified by column chromatography on silica gel with an eluting solvent system in which the gradient of CH2Cl2/CH3OH changed from 80% to 50%. Yield: 30 mg (36.6%). 1H NMR (400 MHz, MeOD) δ = 8.84 (m, 4H), 7.48 (m, 5H), 7.45 – 7.37 (m, 4H), 7.34 (d, J=8.0, 4H), 7.25 (m, 4H), 6.32 (m, 4H), 4.20 (t, J=6.5, 8H), 3.95 (d, J=3.7, 2H), 3.10 (m, 4H), 2.90 (t, J=6.9, 8H), 2.70 (s, 8H), 2.53 (m, 4H),
2.06 – 1.89 (m, 20H), 1.74 (t, J=10.2, 24H); $^{13}$C NMR (101 MHz, MeOD) δ 172.52(2×C), 172.45(2×C), 171.65(2×C), 168.63(C), 156.02(2×C), 145.71(2×C), 145.66(2×C), 142.36 (2×CH), 142.35 (2×CH), 141.04 (4×CH), 133.82 (2×CH), 133.72 (2×CH), 128.48 (4×C), 124.82 (2×CH), 124.81 (2×CH), 122.09 (4×CH), 110.75 (2×CH), 110.73 (2×CH), 101.03 (2×CH), 100.95 (2×CH), 50.46 (4×CH$_2$), 49.14 (CH$_2$), 49.12 (4×CH$_2$), 43.54 (4×C), 35.41 (2×CH$_2$), 32.85 (2×CH$_2$), 27.06 (8×CH$_3$), 25.93 (4×CH$_2$), 25.88 (4×CH$_2$), 22.21 (2×CH$_2$), 22.18 (2×CH$_2$), 20.75 (2×CH$_2$). TOF-MS: C$_{84}$H$_{105}$N$_7$O$_{14}$S$_6^{2-}$ [M]$^{2-}$, found 813.7997 (100%), calculated 813.8022.

**Photospectroscopic Studies**

**Preparation of the stock solution.** 8.2 mg (5×10$^{-6}$ mol) DiIR783-C and 8.4 mg (5×10$^{-6}$ mol) DiIR783-S were dissolved in 0.5 mL dimethyl sulfoxide (DMSO) respectively that was pre-dried with 3 Å molecular sieve. The deep green stock solutions with a concentration of 10 mM were subpackaged and stored at -40 ºC.

**pH Dependent absorption.** All absorption spectra were recorded on a HIMADZU UV-2550 spectrophotometer in a quartz cuvette (10 × 10 mm) at 25 ºC. Stock solutions of the probes in DMSO (10 mM) were used to prepare the working solutions in phosphate buffered saline (PBS) or 4-morpholineethanesulfonic acid hydrate (MES) with a final concentration of 1.0–5.0 µM. Absorbance wavelength and corresponding molar extinction coefficients of the probes were determined. The scanning speed was 0.5 nm/s and the slit width was set to 2 nm. The absorption profiles of the probes under pH 7.4, 6.5 and 5.5 were obtained at different time points after incubation. Average absorption was obtained from three independent measurements. The ratio of the absorption between 782 and 710 nm was plotted against incubation time (Figure S1).
**pH Dependent NIR fluorescence.** All fluorescence spectra were recorded on SHIMAZDU RF-5301PC spectrofluorophotometer in a quartz cuvette (10 × 10 mm) at 25 °C. Both excitation and emission slits were set to 5 nm. Working solutions (1.0 µM) of each probe were prepared by diluting the stock solutions into PBS or MES and measured immediately. The probes were excited at 765 nm and the emission spectra were collected in a wavelength range from 785 to 900 nm. Average fluorescence emission was obtained from three independent measurements. The fluorescence profiles of the probes (Figure S2 and S3) were obtained at selected time after incubation under three physiological pHs.

![Figure S1](image)

**Figure S1.** Absorption spectra of DiIR783-S (1.0 µM) at selected time points after incubation under pH 5.5 (A), 6.5 (B), 7.4 (C). (D) Time dependent absorption ratios (A\textsubscript{782}/A\textsubscript{710}) of DiIR783-S under three different pHs.
Figure S2. Fluorescence spectra of aiming probe DiIR783-S at selected time points after incubation under pH 5.5 (A), 6.5 (B), 7.4 (C). (D) NIR fluorescence intensities of DiIR783-S at selected time-point after incubation under different pHs.

Figure S3. Fluorescence spectra of control probe DiIR783-C at selected time-points after incubation under pH 5.5 (A), 6.5 (B), 7.4 (C). (D) NIR fluorescence intensities of DiIR783-C at selected time-point after incubation under different pHs.
Analytical HPLC analysis

To verify our assumption that the fluorescence enhancement of DiIR783-S was resulted from the cleavage of hydrazone bond under acidic pHs, high pressure liquid chromatography (HPLC) experiments were conducted at selected time after the incubation of the probe under pH 5.5 and 7.4. A reversed-phase C18-bonded silica column (C18, 3.5 μm, 4.6 × 150 mm) was used as the stationary phase and a mixture of 5.0 mM tetrabutyl ammonium bromide aqueous solution (eluent A)/methanol (eluent B) with a linear gradient elution profile: 0 min, 20% B; 10 min, 70% B; 20 min, 70% B; 30 min, 80% B; 40 min, 80% B was used as the mobile phase. The probe and its hydrolytic products were detected at a wavelength of 783 nm in the diode array detection (DAD) of Agilent 1100 series. The temperature of the column was maintained at 25 °C. 20 μL (10 μM probe) sample solution was injected at the selected time after incubation and the flow rate of the mobile phase was 0.5 mL/min. In order to verify the two newly emerged products were the hydrolytic product of DiIR783-S, DiIR783-S was mixed with compound 3 or 5 respectively and then incubated under pH 5.5 for 15 min. HPLC studies demonstrated compound 3 and 5 overlapped with one of the hydrolytic product, which confirmed that these two new products were the hydrolytic products of DiIR783-S (Figure S4). As expected, there were not any hydrolytic products of DiIR783-C observed even incubation under pH 5.5 for 24 h, which verified the pH inerterness of amide bond under physiological pHs (Figure S5).

![Figure S4](image-url)

**Figure S4.** HPLC spectra of the mixture of DiIR783-S/compound 5 (A) and DiIR783-S/compound 3 (B) after incubation under pH 5.5 for 15 min.
DiIR783-S visualized acidic pHs in buffered solutions. The capability of the probe to visualize physiologically acidic pH was first tested in buffered solutions with pH values of 5.5, 6.5 and 7.4. The stock solutions of probes (10 mM) in DMSO were diluted to the buffered solutions with a final concentration of 10 µM. At the selected time after incubation, the NIR fluorescence images of the probe in a 96 well plate under different pHs were collected by a Maetro™ In Vivo Multispectral Imaging System equipped with a 710–760 nm excitation filter and an 800 nm emission band pass filter set. The fluorescence images were acquired with 0.5 s exposure time under the same field of view (ROI: 1/3, max sample size: max, Bin = 2×2). The fluorescence intensities were quantified by ImageJ software (National Institutes of Health, USA) and normalized to the value at pH 7.4 (Figure S6).

Figure S5. HPLC spectra of DiIR783-C as a function of time under pH 5.5. No hydrolytic products were observed during the whole incubation period. The eluting peaks of DiIR783-C were highlighted.

Figure S6. Normalized NIR fluorescence intensities of DiIR783-C as functions of time and pH. The fluorescence intensities were normalized to its value after incubation for 15 min under pH 7.4.
**In Vitro Fluorescence Microscopic Studies**

**Cell culture.** Human breast MDA-MB-435 cancer cells lines were purchased from Shanghai Institute for Biological Sciences (Shanghai, China). LysoTracker, MitoTracker and ER-Tracker were purchased from Molecular Probes (Invirogen, UK). Cells were grown as mono-layers in 75-cm$^2$ flasks containing Minimum Essential Medium, Alpha 1X (MEM, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA). Cells were harvested and split when they reached 80% confluence to maintain exponential growth.

**NIR fluorescence microscopic imaging.** To avoid the artifacts that occur during fixation procedures, all the experiments were conducted in live cells. MDA-MB-435 cells (2 × 10$^4$) cultured on 35 mm glass bottom dishes (14 mm microwell, MatTek, Ashland, MA) with approximately 50% confluence were added with 5.0 µM DiIR783-S or DiIR783-C in 2.0 mL media supplemented with 10% FBS for selected period at 37 °C. At the end of incubation, the cells were washed with Hanks Balance Salt Solution (HBSS) 3× prior to addition of 1.0 mL phenol red free media. The cells were imaged immediately by Leica DMF4000B laser-scanning microscope. The probe was excited with an Ar laser through Y7 filter (710 ± 37.5 nm), and fluorescence was detected by a secondary photomultiplier by applying an 810 ± 45 nm band-pass filter. Figure S7 demonstrated the microscopic fluorescence images of the cells treated with control probe DiIR783-C at 2, 6 and 24 h post-incubation.

![Figure S7. NIR fluorescence and white color microscopic images of the cells treated with DiIR783-C for 2, 6 and 24 h. Near-infrared fluorescence was displayed in red. Scale bar: 50µm.](image-url)
Determined the subcellular location of probe. (a) Colocalization between DiIR783-S and endoplasmic reticulum (E.R.). Live MDA-MB-435 cells cultured in 35-mm glass bottom culture dishes were first washed with HBSS and 2.0 mL serum free medium containing 5.0 µM DiIR783-S was added. After 24 h treatment at 37 ºC, the cells were washed 3× with HBSS and 2.0 mL ER-Tracker (1.0 µM) were added. After further incubation for 30 min, the cells were washed 3× with HBSS and phenol red free media was applied prior to microscopy imaging. (b) Colocalization between DiIR783-S and mitochondrion. Live MDA-MB-435 cells were first washed with HBSS and 2.0 mL serum free medium containing 5.0 µM DiIR783-S was added. After 24 h treatment at 37 ºC, the cells were washed 3× with HBSS and 2.0 mL fresh media containing Mito-Tracker (200 nM) were added. After further incubation for 2 h, the cells were washed 3× with HBSS and phenol red free media was applied prior to microscopy imaging. (c) Colocalization between DiIR783-S and lysosome. Live MDA-MB-435 cells were first washed with HBSS and serum free medium containing 5.0 µM DiIR783-S was added. After 24 h treatment at 37 ºC, the cells were washed 3× with HBSS and 2.0 mL fresh media containing Lyso-Tracker (500 nM) were added. After incubation for an additional 1.5 h, cells were washed 3× with HBSS and phenol red free media was applied prior to immediate microscopy imaging. Colocalization coefficients between the probe and the organelle markers were measured by the ImageJ software equipped with JACoP Plugin (Figure S8).

![Figure S8](image)

**Figure S8.** Colocalization coefficients between DiIR783-S and fluorescence organelle marker in live MDA-MB-435 cancer cells at 24 h treatment.
In Vivo Imaging Studies

Tumor implantation. Male nude mice were purchased from HuaBukang Bio-Tek (Beijing, China) at 4–5 weeks of age and maintained under standard housing conditions. All animal experiments were carried out in accordance with guidelines approved by the Ethics Committee of Fudan University. Wild type MD-MBA-435 human breast cancer cells (2.0×10^6 suspended in 100 µL PBS) were inoculated into the right lower flank of nude mice after anesthetization by injection a 100 µL 10% chloralic hydras. After the inoculation for 30–40 days, the animals with tumor diameters in a range from 0.8 to 1.2 cm were ready for imaging experiments.

In vivo and ex vivo optical imaging studies. Optical imaging was performed on a Maestro™ In Vivo Multispectral Imaging System equipped with a 710–760 nm excitation filter and an 800 nm emission band pass filter set. Before the imaging, mice were anesthetized with chloralic hydras (2.5 mg/kg). White light photography (exposure time: 15 ms) and NIR fluorescence images (exposure time: 2.0 s) with a same field of view (ROI: Full, Max sample size: max, Bin = 2×2) were acquired at selected time-points after systemic injection of the probe (20 nmol/mouse for DiIR783-S and DiIR783-S, 40 nmol/mouse for IR783). The time-dependent tumor to normal tissue (T/N) fluorescence signal ratio was calculated by using ImageJ software. At the end of in vivo imaging, the mice were anesthetized and perfused with saline and 4% paraformaldehyde (PFA) in succession via heart to douche blood and pre-fix the mouse. The tumor and main organs including liver, kidney, lung, spleen, heart and muscle were carefully isolated and the fluorescence intensities of ex vivo organs were obtained. The average NIR fluorescence intensities in the organs were quantified by ImageJ software and normalized to the value of the muscle.
Visualizing intratumoral acidosis in 3D fluorescence image. The mice were scarified at 24 h PI of probe and cardically perfused with PBS (pH 7.4), 4% paraformaldehyde (PFA) and lipophilic carbocyanine dye DiO (2.0 mL, 0.2 mg/mL) in succession. DiO stains the vasculatures by incorporating the endothelial cells on the vasculatures. Then the tumors were excised and sectioned into 1.0 mm thick sections using a tissue slicer (Beijing Sunny Instruments Co. Ltd., China). These tumor sections were tiled horizontally on a glass slide. Fluorescence images of these tumor sections were generated on an In Vivo Multispectral Imaging System equipped with a NIR band pass filter set (710–760 nm) and a blue band pass filter set (445–490 nm). NIR fluorescence images (exposure time: 2.0 s) that indicate the probe distribution and green fluorescence images (exposure time: 1.0 s) that indicate the vasculatures were acquired respectively for each of the tumor sections with a same field of view (ROI: 1/3, max sample size: max, Bin = 2×2). The images of each tumor section were transferred to RGB format and separated to single picture with the same size manually edited if necessary. Three-dimensional pseudo-colored volumes were reconstructed that included the NIR fluorescence of the probe (displayed in red) and green fluorescence of vasculature (displayed in green) by the Amira (Visage Imaging, Australia) software.

Delineating acidosis in tumor sections by pH testing paper. To verify the capability of DiIR783-S to visualize acidosis in the tumor, ultra-sensitive pH testing paper with the indicating range of pH 6.0–7.4 (Micro Essential Laboratory, USA) was used to delineate the acidic pHs in the excised
tumor sections. At 24 h PI of the probe via i.v., the tumor bearing mice were scarified and perfused with saline to douche blood. Then the tumors were excised and sectioned with a thickness of 1.0 mm by a tissue slicer, which were tiled horizontally on a glass slide. The tumor section was uniformly covered with a pH testing paper. After 2 s, the pH testing paper was removed from the tumor section and imaged immediately by Canon ixus 9515 digital Camera (Canon, Japan). Meanwhile, the NIR fluorescence image of the tumor section was collected by Maestro™ In Vivo Multispectral Imaging System. The representative white light image of the pH testing paper and the NIR fluorescence image of the corresponding tumor section were demonstrated in Figure S9.

**Figure S10.** DiIR783-S successfully delineated the acidic area in the tumor section. (A) The acidic area indicated by the ultra-sensitive pH testing paper (left panel) correlated with the strong NIR fluorescence area in the optical image of the identical tumor section (right panel) after injection of DiIR783-S via i.v.. (B) The acidic areas indicated by the pH testing paper and optical image can not overlap with each other after injection of IR783 via i.v..

**References**

Supplemental Spectra

compound 2

Electronic Supplementary Material (ESI) for Chemical Communications
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compound 2
compound 3

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