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Supporting Information

Tumor-targeted small molecule for dual-modal imaging guided phototherapy upon near-infrared excitation

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Experimental section

Materials. N,N-Dimethylformamide (DMF), dichloromethane, methanol, triethylamine (TEA), 1,2-ethanediamine and 4-(bromomethyl)-pyridine hydrobromide were purchased from J&K Scientific. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's medium (DMEM), MitoTracker Green (mitochondria marker), LysoTraker Blue (lysosomal marker), Golgi-Tracker (golgi marker), WGA (plasma membrane probe) and DAPI (nucleus staining probe) were purchased from were purchased from Gibco-BRL (Beijing, China). All of the reagents and solvents were of analytical-grade purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Lo2 cells, MRC-5 cells, 293 cells, MCF-10A cells, HepG-2 cells, A549 cells, A498 cells and MCF-7cells were purchased from Shanghai Institutes for Biological Sciences (China). The buffer solutions were as follows: the high K^+ buffer buffer containing 30 mM NaCl, 120 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 mM NaH_2PO_4, 5 mM glucose, 20 mM HEPES (various pH values was adjusted used NaOH); phosphate buffered saline solution (PBS) containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.4).

Synthesis and characterization of IR-PY. To a solution of 1,2-ethanediamine (0.67 mL, 10 mmol) in 10 mL of ethanol at reflux was slowly added 4-(bromomethyl)-pyridine hydrobromide (1.27 g, 5 mmol). The reaction mixture was maintained under reflux during 12 h. The solvent was evaporated under reduced pressure and the residue was was dissolved in anhydrous DMF (20 mL). Then IR-822 (3.2 g, 5 mmol) and triethylamine (TEA) (2.77 mL, 20 mmol) was added. The mixture was stirred at 40 °C for 5h under Nitrogen atmosphere. Finally, the solvent was evaporated under reduced pressure to give crude products, which was further purified by silica gel column chromatography with the dichloromethane and methanol to afford pure blue product. Yield: 12.06

mg (32%). ^{1}H NMR (400 MHz, $CDCl_3$) δ= 8.57 (d, J = 5.5 Hz, 2H), 7.72 (d, J = 13.1 Hz, 2H), 7.52 (d, 2H), 7.29 (s, 2H), 7.25 (s, 2H), 7.08 (d, J = 7.2 Hz, 2H), 6.85 (d, J = 8.0 Hz, 2H), 5.58 (d, J = 13.0 Hz, 2H), 4.16 (q, J = 6.2 Hz, 2H), 4.01 (s, 2H), 3.88 (q, J = 7.1 Hz , 4H), 3.15 (q, 2H), 2.50 (t, J = 6.2 Hz, 4H), 1.83 (m, 2H), 1.65 (s, 12H), 1.34 (t, J = 7.1 Hz, 6H)ppm； HRMS (ESI^+): m/z calcd for $C_{42}H_{52}IN_5$: 626.4217[$M - I^-$]; found 626.4217.

Fluorescence Measurement *in vitro*. The fluorescence spectra were investigated on a FSP920 spectrofluorometer (Edinburgh Instruments, English). The excitation and emission monochromator slits were both set to 2 nm, 3 nm, respectively. For the fluorescence emission spectra, the excitation wavelength for IR-PY (5 μg/mL) was set to 637 nm. To measure the variation of fluorescent intensity of IR-PY with pH, the λ_{ex} was set to the same wavelength mentioned above and the emission wavelength (λ_{em}) was set to 755 nm.

Fluorescence reversibility of IR-PY with pH. The pH of IR-PY solution (5 μg/mL) between pH 5 and pH 9 was adjusted back and forth by 2 M HCl or NaOH, and then measured by pH-meter. The fluorescence spectra were recorded with λex = 637 nm.

Fluorescence quantum yield (ΦF) of IR-PY. For measurement of the quantum yield of IR-PY, the solution of the probe was adjusted to an absorbance of ~ 0.05. The emission spectra were recorded using a maximum excitation wavelength and the integrated areas of the fluorescence-corrected spectra were measured. Relative fluorescence quantum yields were obtained by using the solution of rhodamine B in ethanol as a reference solution (ΦF = 0.69).[1-2]

Test for probe selectivity. The test solutions for metal ions selectivity were: 5 μg/mL IR-PY in HEPES (40 mM pH 7.40) with diverse ions: blank, K^+ (120 mM), Na^+ (120 mM), Ca^{2+} (0.5 mM), Mg^{2+} (0.5 mM), Zn^{2+} (0.3 mM), Pb^{2+} (0.3 mM), Cu^{2+} (0.5 mM), Ag^+ (0.3 mM), Sn^{2+} (0.3 mM).The

test solutions for proteins, and bioactive small molecules selectivity were: 5 μg/mL IR-PY in HEPES (40 mM pH 7.40) with proteins and bioactive small molecules: blank, metallothionein (MT, 100 μM), thioredoxin (Trx, 50 μM), glutathione reductase (GR, 50 U/mg protein), glutathione (GSH, 100 μM), L-cysteine (Cys, 100 μM), vitamin C (VC, 100 μM), glycine (Gly, 100 μM), proline (Pro, 100 μM), tyrosine (Tyr, 100 μM), uric acid (UA, 100 μM). After incubation at room temperature for 30 min, the test solutions were subjected to fluorescence measurement.

Cell culture. Cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were maintained in an exponential growth phase by periodic subcultivation. The cell density was determined using a hemocytometer, and this was performed prior to any experiments. MCF-7 cells for imaging were initially seeded in 35 mm glass bottom dishes (P35G-0-10-C, MatTek Corp.) at a density of 5000 cells/well with medium containing 10 % FBS overnight.

Cytotoxicity assay. The cytotoxicity of IR-PY was evaluated by the standard MTT assay. Briefly, MCF-7 cells were seeded in 96-well U-bottom plates at a density of 7000 cells/well, and incubated with IR-PY at varied concentrations (0-100 μg/mL) at 37 °C for 24 h. Then, the culture media were discarded, and 0.1 mL of the MTT solution (0.5 mg/mL in DMEM) was added to each well, followed by incubation at 37 °C for 4 h. The supernatant was abandoned, and 110 μL of DMSO was added to each well to dissolve the formed formazan. After shaking the plates for 10 min, absorbance values of the wells were read with a microplate reader at 490 nm. The cell viability rate (VR) was calculated according to the equation: $VR = A/A_0 \times 100\%$, where A is the absorbance of the experimental group (i.e., the cells were treated by IR-PY) and A_0 is the absorbance of the control group (i.e., the cells were untreated by IR-PY).

Flow cytometry. The Flow cytometry experiments were measured on the flow cytometer using 20 mW 633 nm Red JDS Uniphase Helium Neon laser and detector with 780/60 nm emission filter (BD FACS Aria^{TM} III). Infrared channel (APC-Cy7) was using with excitation at 633 nm and collection in the ranges of 750-810 nm.

LSCM fluorescence imaging. The Fluorescence imaging experiments were performed on a Laser Scanning Confocal Microscope (LSCM, Leica TCS SP5). IR-PY (2 μg/mL) was excited at 633 nm, and collected in the ranges of 690-780 nm.

***In vitro* pH detection.** The IR-PY-loaded cells were incubated at 37 °C for 15 min in high K^+ buffer with various pH values in the presence of 10 μM nigericin. Then, the treated cells were subjected to LSCM fluorescence imaging. Cellular pH response to IR-PY was also quantified by flow cytometry. Cells were seeded into 6-well plates at a density of 2-5 × 10^5 cells/well in 2 mL of media and grown in a humidified 5% CO_2 atmosphere at 37 °C for 48 h. Following the incubation, the cells were washed three times with PBS, gently dissociated from the wells with trypsin, and then resuspended in PBS containing IR-PY (2 μg/mL) with different pH values. Then, the treated cells were subjected to flow cytometry.

Tumor cell targeting of IR-PY. The tumor cell targeting of IR-PY was quantified by flow cytometry. Tumor cells (HepG-2 cells, A549 cells, A498 cells and MCF-7cells) and corresponding normal cells (Lo2 cells, MRC-5 cells, 293 cells and MCF-10A cells) were seeded into 6-well plates at a density of 2-5 × 10^5 cells/well in 2 mL of media and grown in a humidified 5% CO_2 atmosphere at 37 °C for 48 h. Following the incubation, the cells were washed three times with PBS, gently dissociated from the wells with trypsin, and then resuspended in PBS (pH 7.4) containing IR-PY (2 μg/mL). Then, the treated cells were subjected to flow cytometry.

***In vitro* photothermal efficiency**

1 mL of PBS or IR-PY (40 mg/mL) was added into different wells of 24-wellplate. Using 0.4 W/cm^2 laser to irradiate the 5 samples for 3 min simultaneously, the temperature changes of each group were recorded by an infrared thermal imaging camera (Ti27, Fluke, USA).

Animals and tumor model. 50 BALB/c nude mice were provided by Medical Experimental Animal Center of Guangdong Province. They were 4-6 weeks old at the start of each experiment and weighed 20-25 g. For tumor implantation, 30 nude mice received a subcutaneous injection of 5×10^6 MCF-7 cells suspended in 0.2 mL of saline solution in the right hind limb. Tumors were then allowed to grow to 50-100 mm^3 for 10-30 days. All animal operations were in according with institutional animal use and care regulations, approved by the Laboratory Animal Center of Guangdong.

***In vivo* NIRF imaging and biodistribution analysis.** To evaluate biodistribution *in vivo* and tumor NIRF imaging, the nude mice, with MCF-7 tumors, were anesthetized with an intraperitoneal (IP) injection of 40 mg/kg pentobarbital sodium. Once the mice were anesthetized to be motionless, 100 μL 40 μg/mL IR-PY was injected via the tail vein. At 0 h, 2h, 5 h, 10 h, 14 h, 18 h, 20 h and 24 h post-injection, dorsal region fluorescence images of living mice were taken. At 14 h post-injection, tumor tissues and organs was removed from treated mice. After soaking in PBS (pH 6.4), the NIRF imaging tumor tissues and organs was examined. All fluorescence images were taken by a Maestro™ *in vivo* fluorescence imaging system (Cambridge Research & Instrumentation, Inc. USA). A bandpass filter (625-655 nm) and a 750-770 nm longpass filter were selected to be used as the excitation filter and the emission filter, respectively.

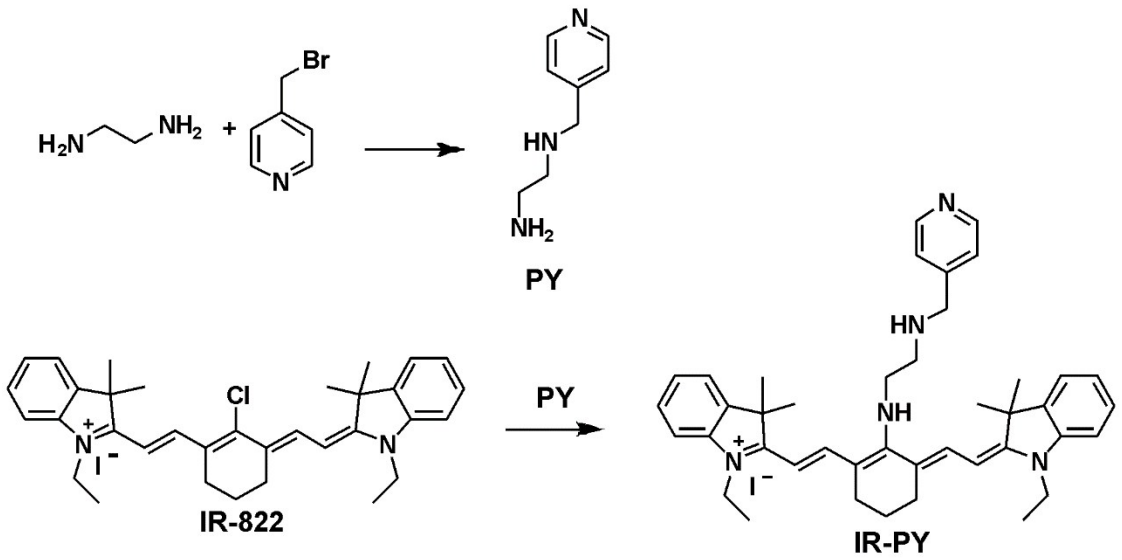
FSP920 spectrofluorometer (Edinburgh Instruments, English) was used for *in vivo* quantification

of IR-PY. To extract IR-PY, the major organs in 30 mL dimethylsulfoxide (DMSO) were homogenized and then centrifuged for 15 min at 9000 rpm. Then the IR-PY content of each sample was determined by FSP920 spectrofluorometer.

***In vivo* PA dual-imaging.** For the PA imaging, the MCF-7 tumor-bearing nude mice, were anesthetized with an intraperitoneal (IP) injection of 40 mg/kg pentobarbital sodium. Then the tumor region on mice was observed at 0 h, 2h, 5 h, 10 h, 14 h, 18 h, 20 h and 24 h post-injection. The PA imaging intensity at tumor was analyzed at different time. *In vivo* PA imaging was detected with preclinical photocoustic computerized tomography scanner (Endra Nexus 128, Ann Arbor, MI). The excitation wavelength was 680 nm. Then the tumor region on mice was observed at 0 h, 2 h, 5 h, 10 h, 14 h, 18 h, 20 h and 24 h post-injection. The PA imaging intensity at tumor was analyzed at different time.

***In vivo* PTT.** 20 BALB/c nude mice (ten per group) with similar sized tumor (50-100 mm^3) were selected for **PTT** treatment. 10 mice were injected with 100 μL of PBS (PBS group) and 20 mice were injected with 100 μL of IR-PY (40 μg/mL) by *i.v.* injection via the tail vein. 10 IR-PY injected mice were not laser irradiated. 10 PBS injected mice and 10 IR-PY injected mice were irradiated by the 808 nm NIR laser at a power density of 0.4 W cm^{-2} for 3 min at 14 h after injection, and the temperature of tumor region was monitored and recorded. Tumor volume survival rates， or body weight of the mice were recorded.

Statistical analysis. Data were reported as mean ± SD. The differences among groups were determined using one-way ANOVA analysis followed by Tukey's post-test; (*) $P < 0.05$, (**) $P < 0.01$.



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2 Scheme S1.Synthetic scheme for small-molecule probe IR-PY.

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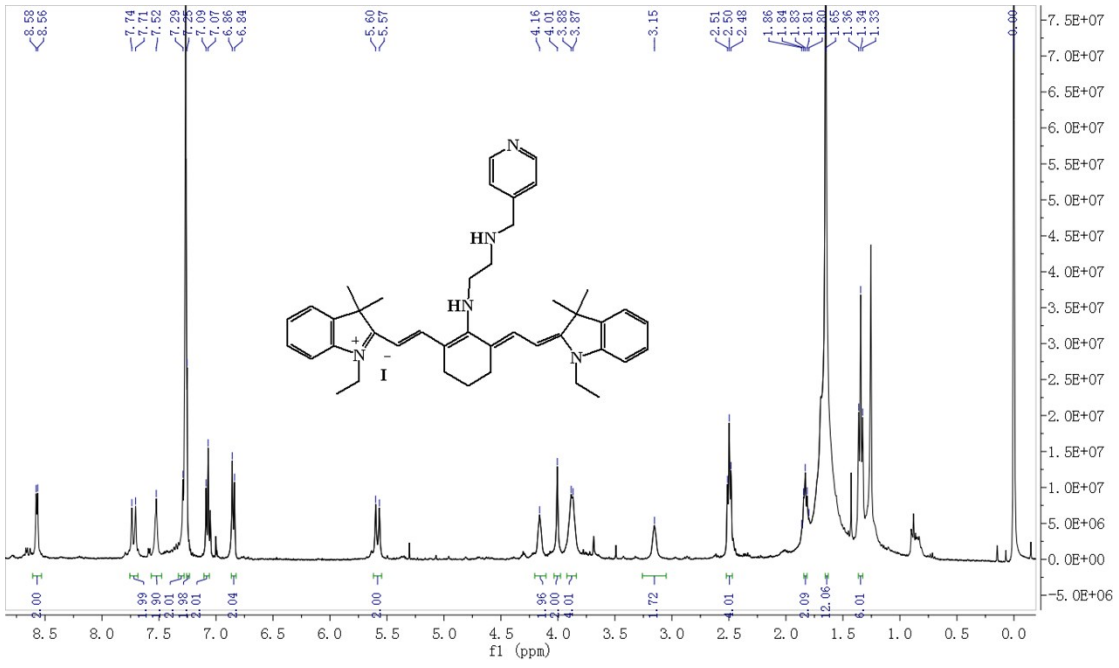

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5 Fig. S1 The ^{1}H NMR spectrum of IR-PY.

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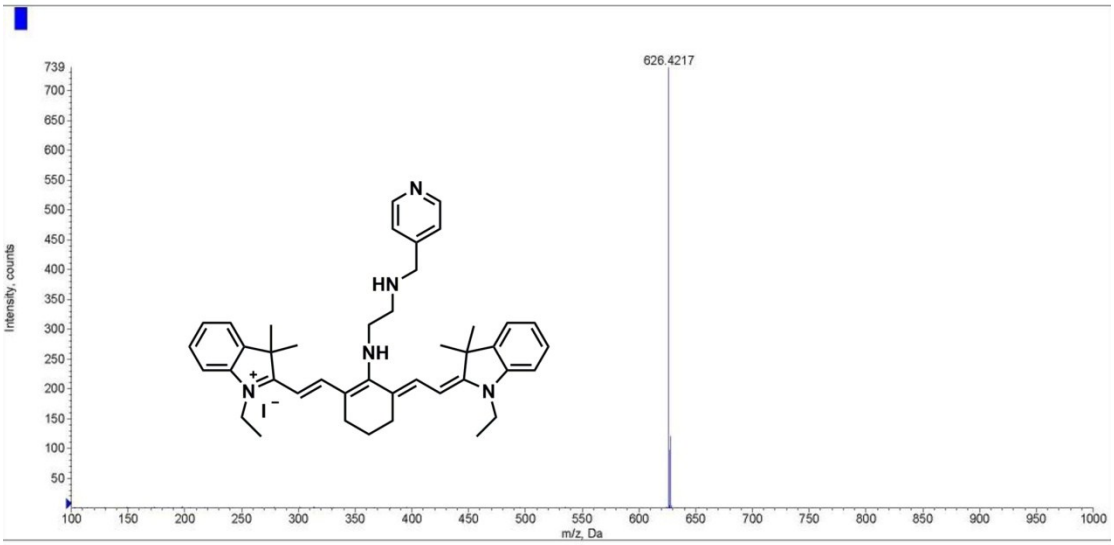

Fig. S2 Mass spectrum of IR-PY.

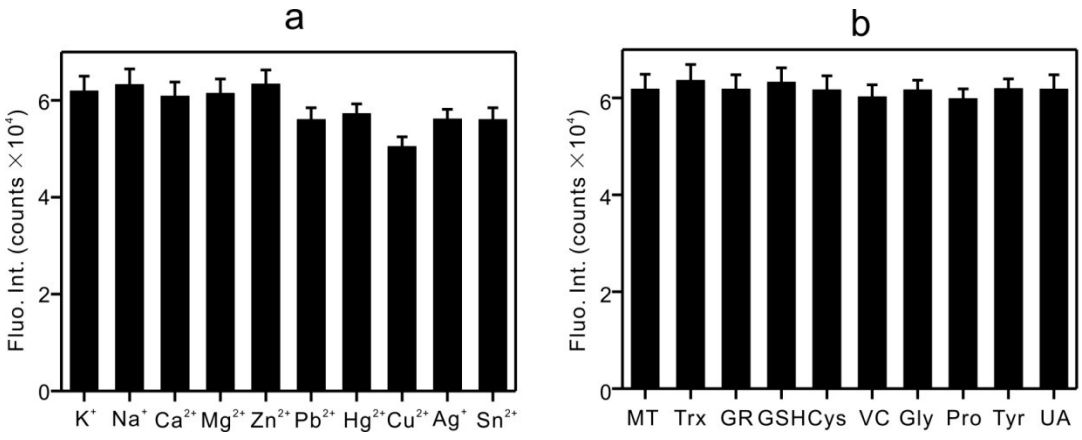

Fig. S3 The test for probe selectivity. (a) Fluorescence responses of 5 μg/mL IR-PY in HEPES (40 mM pH 7.40) to diverse ions. (b) Fluorescence responses of 5 μg/mL IR-PY in HEPES (40 mM pH 7.40) to proteins and bioactive small molecules.

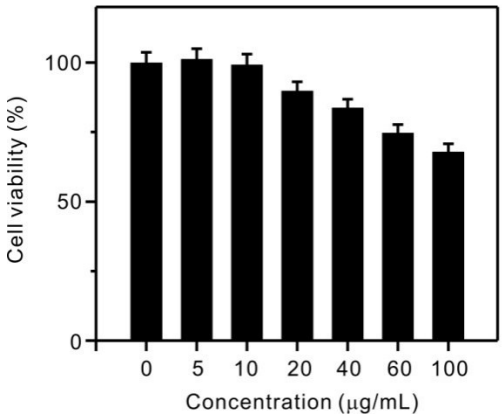

Fig. S4 Effects of IR-PY at varied concentrations on the viability of MCF-7 cells. The resultsare the mean ± standard deviation of five separate measurements

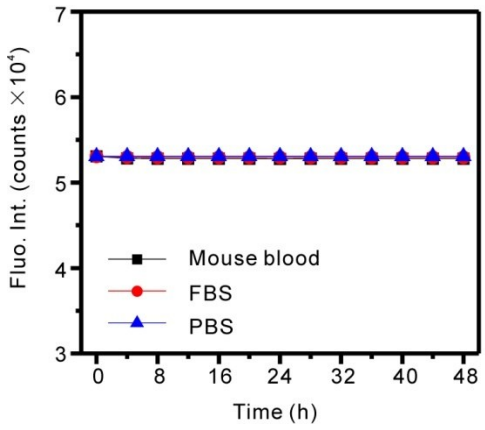

Fig. S5 The fluorescent intensity of IR-PY in mouse blood, FBS and PBS within 48h. (λ_{ex} at 640 nm, λ_{em} at 755 nm, slit width 0.1nm)

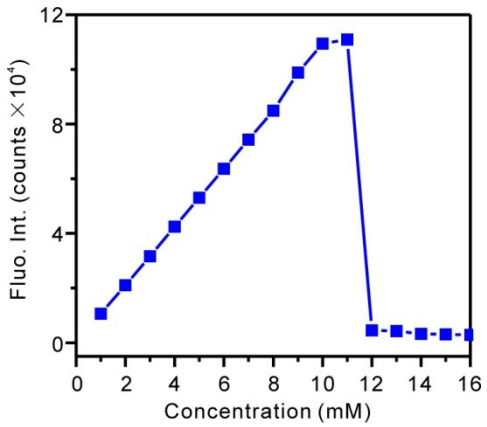

Fig. S6 Fluorescent intensity of IR-PY at different concentrations. (λ_{ex} at 640 nm, λ_{em} at 755 nm, slit width 0.1nm)

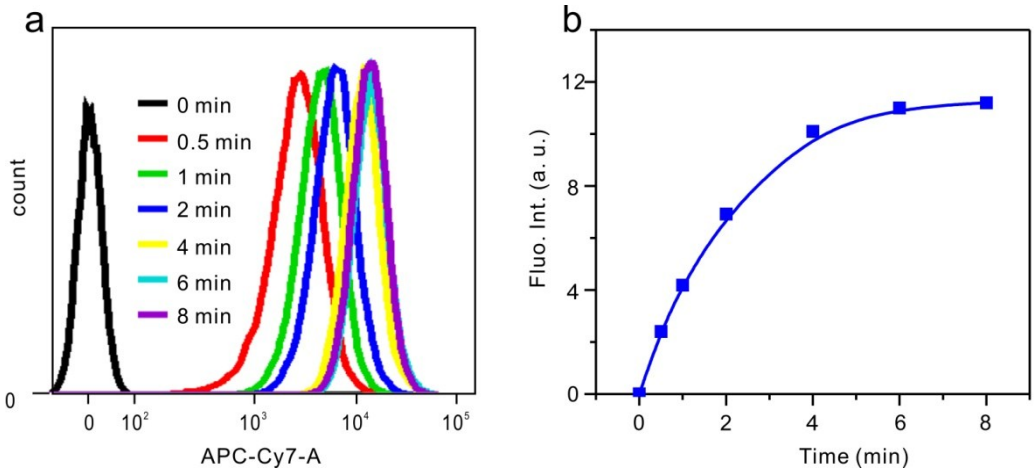

Fig. S7 The membrane permeability of IR-PY measured by flow cytometry. (a)Flow cytometry analysis of MCF-7 cells cultured with IR-PY at different timepoints. (b) The linear calibration plot of fluorescent intensity and its corresponding cultured time derived from statistical results of the flow cytometry analysis.

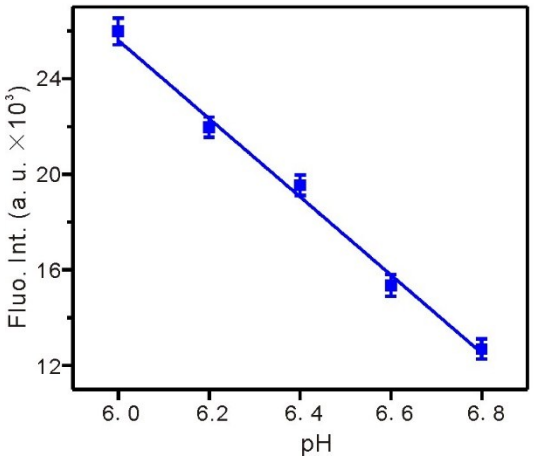

Fig. S8 Semiquantitative fluorescent intensity of MCF-7 cells in Fig. 2e

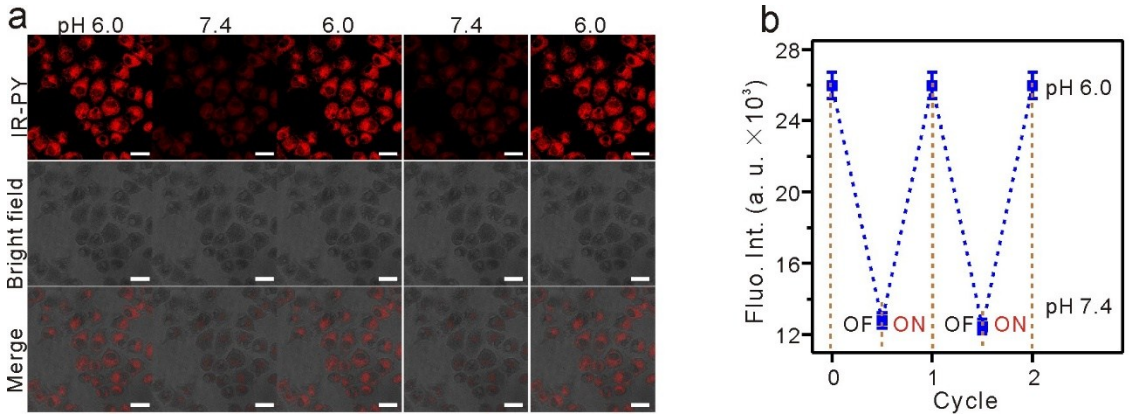

Fig. S9 Confocal fluorescence images of IR-PY in MCF-7 cells. (a) The pH reversibility study of IR-PY between pH 6.4 and 7.4. (b) Fluorescent intensity of the images in (a). Scale bar: 25 μm.

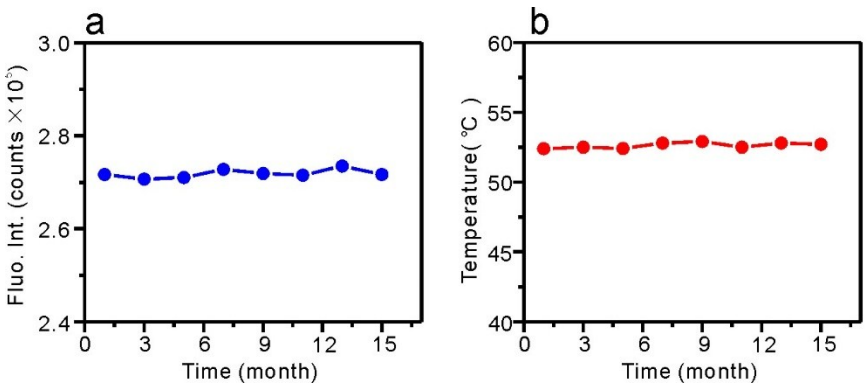

Fig. S10 The photostability of IR-PY within 15 months. (a) Fluorescent intensity of IR-PY at 792 nm in PBS within 15 months. (b) The temperature of IR-PY after 3 minutes NIR laser irradiation (808 nm, 0.4 W cm^{-2}) within 15 months.

References

(1) Tang, B.; Yu, F.; Li, P.; Tong, L.; Xia Duan; Xie, T.; Wang, X. A near-Infrared Neutral Ph Fluorescent Probe for Monitoring Minor Ph Changes. *J. Am. Chem. Soc.* **2009,** *131*, 3016–3023.

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