Electronic supporting information

Dye-Sensitized Upconversion Nanoparticles with Enhanced Aqueous Luminescence for Neuronal Imaging

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1. Materials and Instrumentation

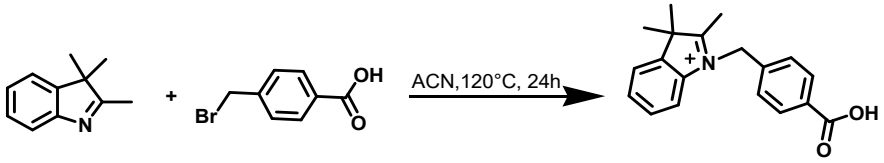
Materials

All solvents and chemicals were purchased from commercial suppliers in analytical grade and used without further purification unless special stated. DSPE-PEG2000 was purchased from Xi'an Ruixi Biological Technology Co.,Ltd. The Neuro2a, NSC34, and C6 glioma cell lines were purchased from the Fenghui Biology Company. B-27, glutamax, DMEM, trypsin-EDTA (0.25%), dulbecco's phosphate-buffered saline (DPBS), penicillin-streptomycin, fetal bovine serum, neurobasal plus medium, and Triton X-100 were purchased from ThermoFisher Scientific company. DAPI (4',6-diamidino-2-phenylindole), secondary goat anti-mouse IgG-488, paraformaldehyde 4%, Hoechst 33342, and Neuronal class III β3-Tubulin (Tuj1) mouse monoclonal antibody were purchased from Beyotime biological company. Chloral hydrate and bovine albumin were purchased from Energy Chemical. Microfluidic devices were purchased from Xona Microfluidics Company.

Instrumentation

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz (^{1}H: 400 MHz, ^{13}C: 101 MHz). High resolution mass spectrums (HRMS) were tested by AB Sciex 3200 QTRAP MS/MS system. UV-Visible absorption spectra were recorded by an Agilent Cary 8454 UV-Vis Spectrometer. Fluorescence emission spectra were carried out using a Horiba FluoroMax-4 Photoluminescence Spectrometer. The X-ray diffraction (XRD) patterns of the as-synthesized UCNPs were obtained using a D8 Focus diffractometer (Bruker) with Cu Kα radiation. Transmission electron microscopy (TEM) images were obtained using a Tecnai G2 S-TWIN transmission electron microscope with a field emission gun operating at 200 kV. Fourier-transform infrared spectra (FT-IR) were obtained using a Perkin Elmer FT-IR Spectrum Two. Upconversion emission spectra were acquired using a Horiba FluoroMax-4 Photoluminescence Spectrometer with an 808 nm laser diode module (Blueprint, China) as the irradiation source. Dynamic light scattering (DLS) tested by a DelsaMax Core light scattering analyzer. MTT was tested by a microplate reader (Azure Biosystems, Inc.). Confocal imaging was conducted with a confocal laser SP8 microscope (Leica SP8 system).

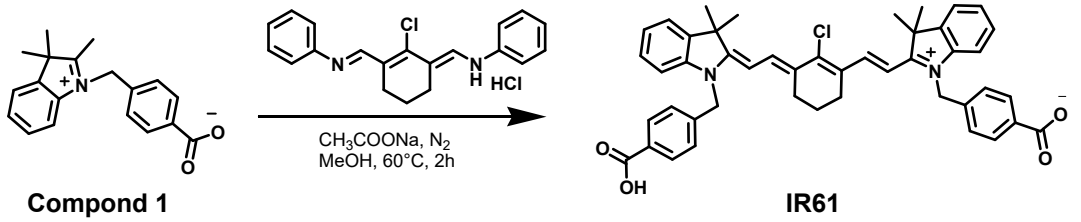
2. Synthesis of dyes



Compond 1

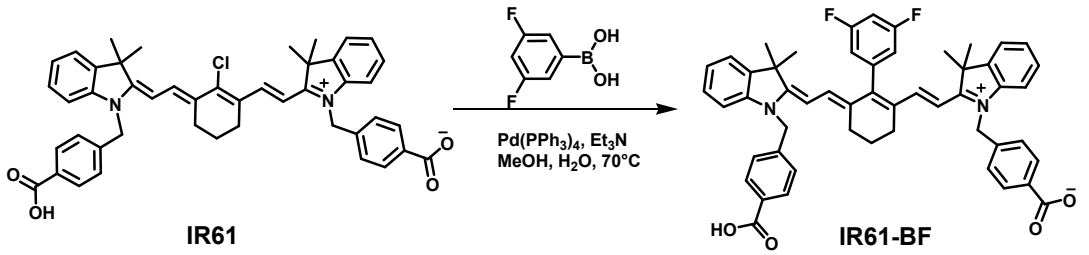
Synthesis of Compound 1: A solution of acetonitrile (50.0 mL) was mixed with 2,3,3-trimethylindolenine (3.2 mL, 20.0 mmol, 1.0 eq), followed by the addition

of 4-(bromomethyl) benzoic acid (13.8 mmol, 1.1 eq). The mixture was then refluxed for 24 hours. After cooling to room temperature, it was poured into cold diethyl ether (200.0 mL) with vigorous stirring. The resulting 1-(4-Carboxybenzyl)-2,3,3-trimethyl-3H-indolium was collected by filtration, obtained yellow solid, 4.1 g. Yield: 93.1 %. ^{1}H NMR (400 MHz, Methanol-d^{4}) δ 8.11 – 8.03 (m, 2H), 7.85 – 7.79 (m, 1H), 7.75 (d, *J* = 8.0, 0.8 Hz, 1H), 7.64 (t, *J* = 7.6, 1.0 Hz, 1H), 7.56 (t, *J* = 7.8, 1.3 Hz, 1H), 7.52 – 7.44 (m, 2H), 5.95 (s, 2H), 1.70 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 198.88, 166.73, 141.89, 141.03, 136.84, 130.98, 130.09, 129.59, 129.00, 127.57, 123.68, 115.81, 54.59, 50.41, 30.69, 22.22, 14.74. MS (positive): calculated $[C_{19}H_{20}NO_2]^+$ = 274.3671, found: 274.4867.



Compond 1 **IR61**

***Synthesis of IR61*:** A solution of compound 1 (2.0 mmol), N-[(3-(Anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]aniline monohydrochloride (0.75 mmol), and anhydrous sodium acetate (2.0 mmol) in anhydrous ethanol (20 mL) was stirred for 2 hours at 70 ℃ using a magnetic stir bar. After the reaction was complete, the ethanol was removed under reduced pressure. The crude product was then purified by column chromatography (using CH_3OH/DCM 1:20) to obtain 167.8 mg of dark green solid. Yield: 31.5 %. ^{1}H NMR (400 MHz, Methanol-*d*$_4$) δ 8.59 (d, *J* = 14.0 Hz, 2H), 8.21 (s, 4H), 7.77 (d, *J* = 7.5 Hz, 2H), 7.57 (t, *J* = 7.1 Hz, 6H), 7.51 (d, *J* = 6.1 Hz, 4H), 6.45 (d, *J* = 14.1 Hz, 2H), 5.71 (s, 4H), 2.69 (t, J = 6.1 Hz, 4H), 1.97 (s, 12H). ^{13}C NMR (101 MHz, Methanol-*d*4) δ 173.60, 173.42, 168.00, 150.75, 150.57, 144.79, 144.61, 142.69, 142.51, 141.18, 141.01, 139.74, 139.57, 130.90, 130.38, 130.20, 129.63, 128.86, 128.68, 127.75, 127.58, 126.46, 126.29, 125.63, 125.45, 122.54, 122.36, 111.13, 110.96, 101.94, 101.77, 49.58, 49.41, 29.38, 29.21, 27.23, 27.06, 25.95, 25.77, 20.44. MS (positive): calculated $[C_{46}H_{44}ClN_2O_4]^+$ = 723.2990, found: 723.5486.



IR61 **IR61-BF**

Synthesis of IR61-BF: A three-necked flask containing 0.1 g (0.43 mmol) of $Pd(PPh_3)_4$ was degassed for 10 minutes and then shielded with N_2 gas

(repeated three times). IR61 (0.5 mmol) and 3,5-difluorophenylboronic acid (1.1 mmol) in 10 mL of MeOH were added. Subsequently, 1 mL of Et_3N and 1 mL of H_2O were injected using a syringe, and the mixture was kept at 70 °C for 18 hours under a N_2 atmosphere and in darkness. After the reaction, the product was cooled to room temperature and evaporated under reduced pressure. The IR61-BF was purified by column chromatography (CH_3OH/DCM 1:10) to yield 118.8 mg of black solid product. Yield: 25.5 %. ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.96 (d, *J* = 8.0 Hz, 4H), 7.47 – 7.42 (m, 2H), 7.38 – 7.32 (m, 2H), 7.29 – 7.20 (m, 11H), 6.97 – 6.89 (m, 2H), 6.18 (d, *J* = 14.0 Hz, 2H), 5.39 (d, *J* = 8.9 Hz, 4H), 2.47 (t, *J* = 6.1 Hz, 3H), 2.38 (t, *J* = 6.2 Hz, 1H), 1.86 (t, *J* = 6.1 Hz, 2H), 1.74 (s, 4H), 1.33 (s, 12H). ^{13}C NMR (101 MHz, MeOD) δ 172.45, 172.38, 172.22, 159.23, 147.35, 142.85, 142.59, 141.40, 140.55, 137.14, 136.49, 131.25, 129.81, 129.79, 128.53, 125.72, 125.01, 124.78, 123.13, 122.20, 112.99, 110.67, 110.48, 100.88, 48.71, 42.04, 27.28, 26.64, 24.04, 20.69, 10.18, 7.77. MS (negative): calculated $[C_{52}H_{46}F_2N_2O_4]^-$ = 800.9294, found: $[C_{52}H_{46}F_2N_2O_4]^{2-}$ =799.9409.

3. Quantum yield of IR61 and IR61-BF

The quantum yield (QY) of IR61 and IR61-BF in DMF was determined using a method described in previous literature,[1] with the dye IR-783 as the reference (its quantum yield has been reported as 8.4% in DMF).[2] The QY was calculated using equation (1), where QY is the photoluminescence quantum yield, F_i and F_s are the integrated intensities (areas) of sample and standard spectra, respectively, f_x is the absorption factor ($f_x = 1 - 10^{-Ax}$, A = absorbance), and n_i and n_s are the refractive indices of the sample and reference solution, respectively.

$$\Phi_f^i = \frac{F^i f_s n_i^2}{F^s f_i n_s^2} \Phi_f^s \quad (1)$$

4. Cell culture and MTT assays

Neuro2a or NSC34 cells were cultured in 25 cm² canted neck flasks at 37 °C in a humidified atmosphere containing 5% CO_2, utilizing DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cell density was determined using a hemocytometer. Cells were then plated in 96-well plates with 100 μL of DMEM medium at a density of 1 × 10^4 cells per well and incubated for 24 hours. Following incubation, the desired concentrations of UCNP@IR61-BF were added. After an additional 24-hour incubation, the spent medium was removed using a syringe, and the cells were washed with PBS. Subsequently, 20 μL of MTT solution was added to each well, followed by a 4-hour incubation. Afterward, the medium was removed, and 150 μL of DMSO was added. The absorbance of each well was measured at 492 nm using a microplate reader (Azure Biosystems, Inc.). Each concentration

of the probes was tested in four independent experiments. Relative cell viability was calculated using the equation: Cell viability (%) = (OD treated / OD control) × 100%.

5. Extraction of primary cortical neurons

Animal experiments were conducted in accordance with NIH Guidelines and received approval from the Animal Care and Use Committees of Hong Kong Baptist University. Mice were housed with unrestricted access to food and water, and maintained on a 12-hour light/dark cycle. Cortical neurons were isolated from E14.5-15.5 CD-1 mouse embryos. Mice were euthanized using chloral hydrate (500 mg/kg, intraperitoneally) followed by cervical dislocation. The fetal spinal cord was extracted and digested in DMEM containing 2 mg/mL papain and 125 U/mL DNase I at 37°C for 25-30 minutes, after which 10% FBS-containing DMEM was added. The cells were centrifuged, resuspended in DMEM with 125 U/mL DNase I, and filtered through a 70 μm cell strainer. The cell density was adjusted to 4 × 10^5 cells/mL for plating in well plates. Cultures were maintained in a neural medium supplemented with 2% B27, 1% penicillin-streptomycin, 5% heat-inactivated horse serum, and 10 ng/mL ciliary neurotrophic factor (CNTF), with medium changes performed every three days.

6. Culture and identification of primary cortical neurons

Cells were washed with PBS and subsequently fixed with 4% paraformaldehyde in 0.1 M PBS for 10 minutes at room temperature. After another round of washing with PBS, the cells were permeabilized for 10 minutes in a solution of 0.1 M PBS, pH 7.4, containing 0.1% Triton X-100. Following additional washes with PBS, 1% FBS in 0.1 M PBS was added to incubate for one hour at room temperature, blocking any nonspecific binding. The cells were then incubated overnight at 4°C with mouse anti-Tuj1 antibody diluted 1:1000. After washing three times with PBS for 5 minutes each, donkey anti-mouse secondary antibodies conjugated with Alexa Fluor-488 (also diluted 1:1000) were applied for one hour at room temperature. Following another three washes, the cells were stained with DAPI (1 μg/mL, diluted in 0.1 M PBS) for 10 minutes to visualize nuclear morphology. The stained cells were imaged using a confocal laser scanning microscope (Nikon C2si Plus Confocal FLIM Imaging System). The purity of the primary cortical neuron cultures was assessed via immunocytochemistry with class III β3-Tubulin (Tuj1) antibodies, and ImageJ analysis confirmed a purity of over 98% for cortical neurons.

7. Microfluidic model construction and confocal imaging

Primary cortical neurons (10 μL at 10^7 cells/mL) were seeded into the Soma compartments of microfluidic devices. On Day 13, 300 μg/mL UCNPBF in serum-free neurobasal medium was added to the Axon compartment of TCND500. A 100 μL volume difference was maintained to prevent reverse flow.

After 24 hours, compartments were washed with PBS. Imaging was conducted with a confocal laser SP8 microscope (Leica SP8 system) using 20× objectives to visualize neuronal structures and transport.

C6 cell (1 mL at 10^5 cells/mL) were seeded into the confocal culture dish. After 24h, 200 μg/mL UCNPBF in serum-free neurobasal medium was added, compartments were washed with PBS after 4 hours. Imaging was conducted with a confocal laser SP8 microscope (Leica SP8 system) using 20× objectives to visualize neuronal structures and transport.

8. DLS data of UCNPs

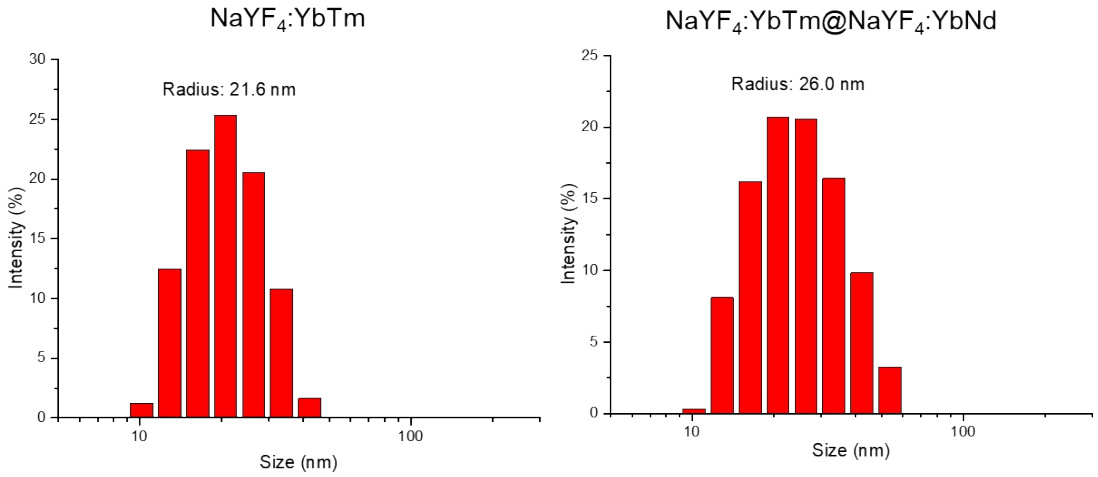


Fig. S1 DLS data of $NaYF_4$:YbTm, and $NaYF_4$:YbTm@$NaYF_4$:YbNd samples.

9. XRD of UCNPs

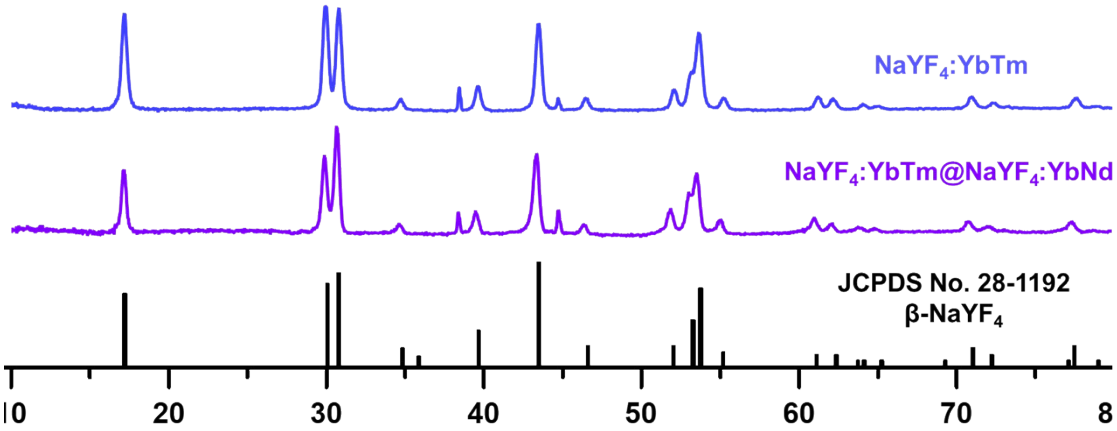


Fig. S2 XRD patterns of $NaYF_4$:YbTm, and $NaYF_4$:YbTm@$NaYF_4$:YbNd samples.

10. FT-IR of IR61-BF, OA-UCNPs, and $NOBF_4$ treated UCNPs

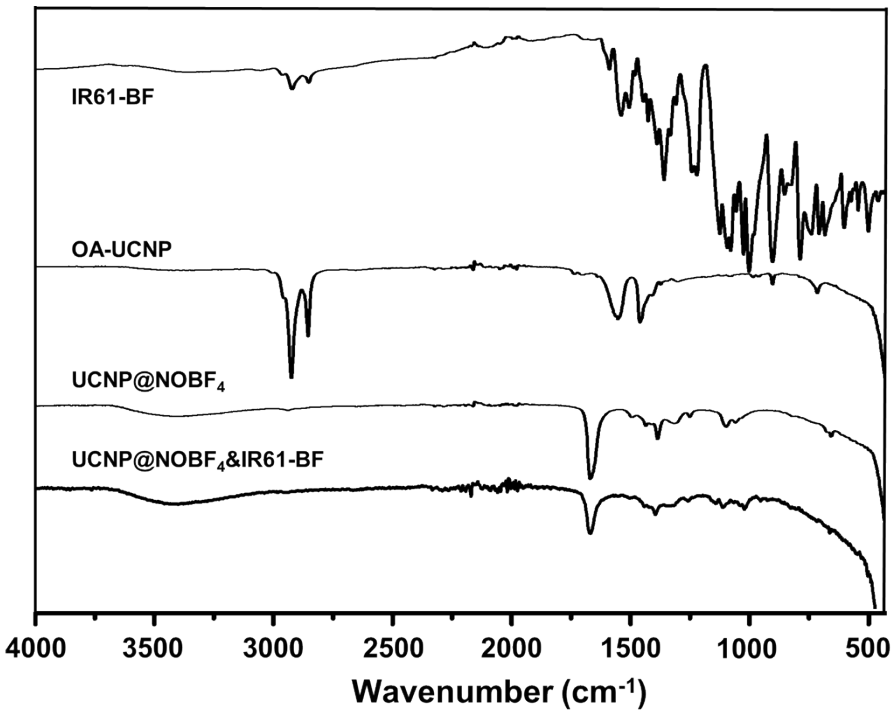


Fig. S3 FT-IR spectra of IR61-BF, OA-UCNPs, and $NOBF_4$ treated UCNPs.

11. The UCL spectra of UCNPs and UCNP@IR61 under 808 nm excitation

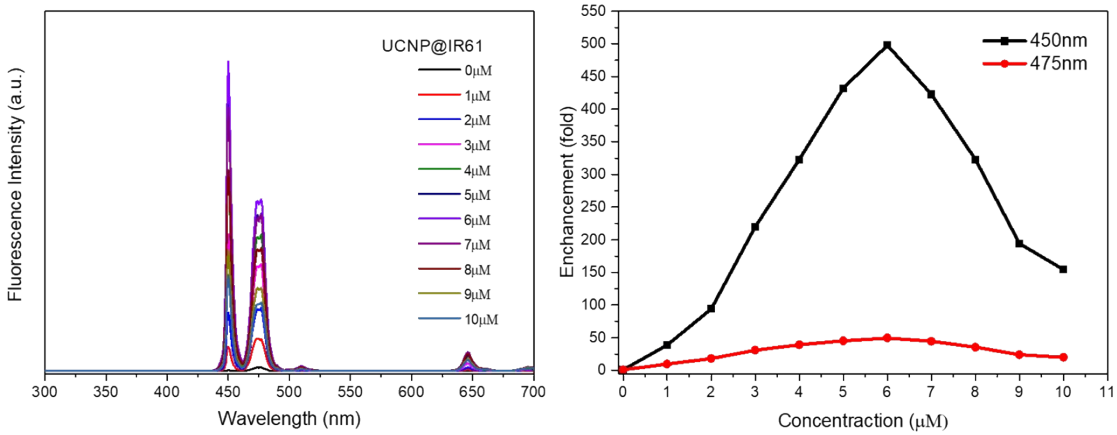


Fig. S4 a) Upconversion spectra of UCNPs with IR61 for sensitization in DMF excited under 808 nm laser. (IR61: 0-10 μM, UCNP: 1mg/mL in DMF, Laser: 2.8 W/cm^2, Slit: 0.5 nm).

12. The lifetime measurements of UCNPs and UCNP@dyes

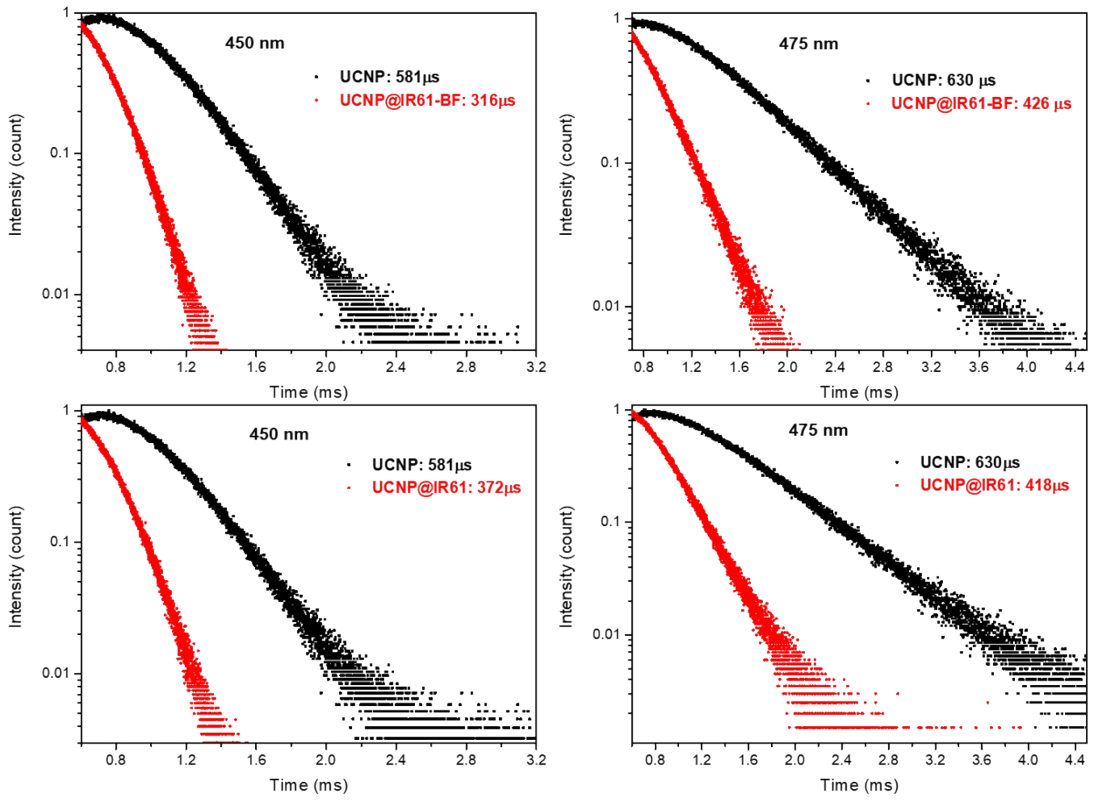


Fig. S5 Decay behavior of the fluorescence emission of the UCNP, UCNP@IR61, and UCNP@IR61-BF at different perks, 450nm and 475nm.

13. ACQ effect of dye IR61-BF

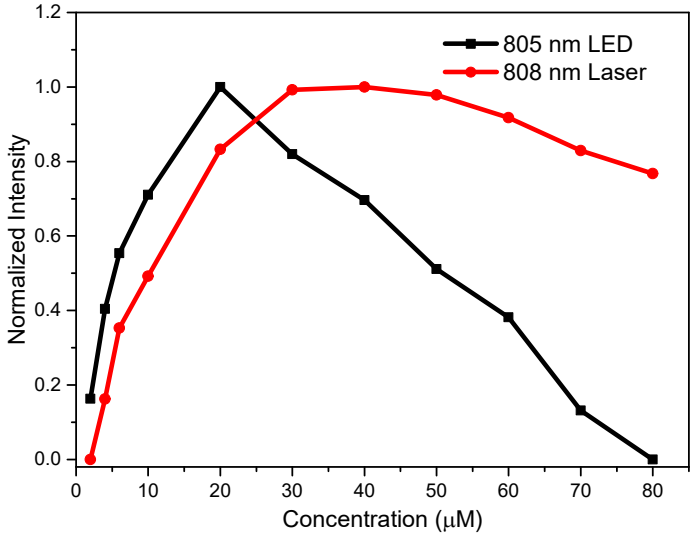


Fig. S6 Fluorescence intensity statistics of dye IR61-BF under different excitation light source conditions (805 nm LED or 808 nm Laser).

14. FT-IR spectra of IR61-BF, UCNP@PEG, and UCNP@PEG&IR61-BF

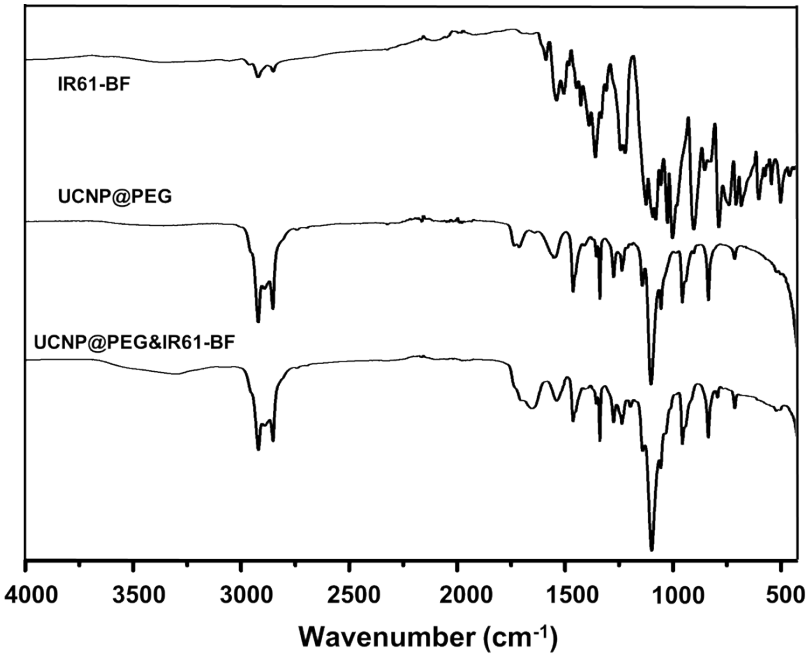


Fig. S7 FT-IR spectra of IR61-BF, UCNP@PEG, and UCNP@ PEG&IR61-BF.

15. UCL spectra of UCNP@PEG with various concentration IR61-BF

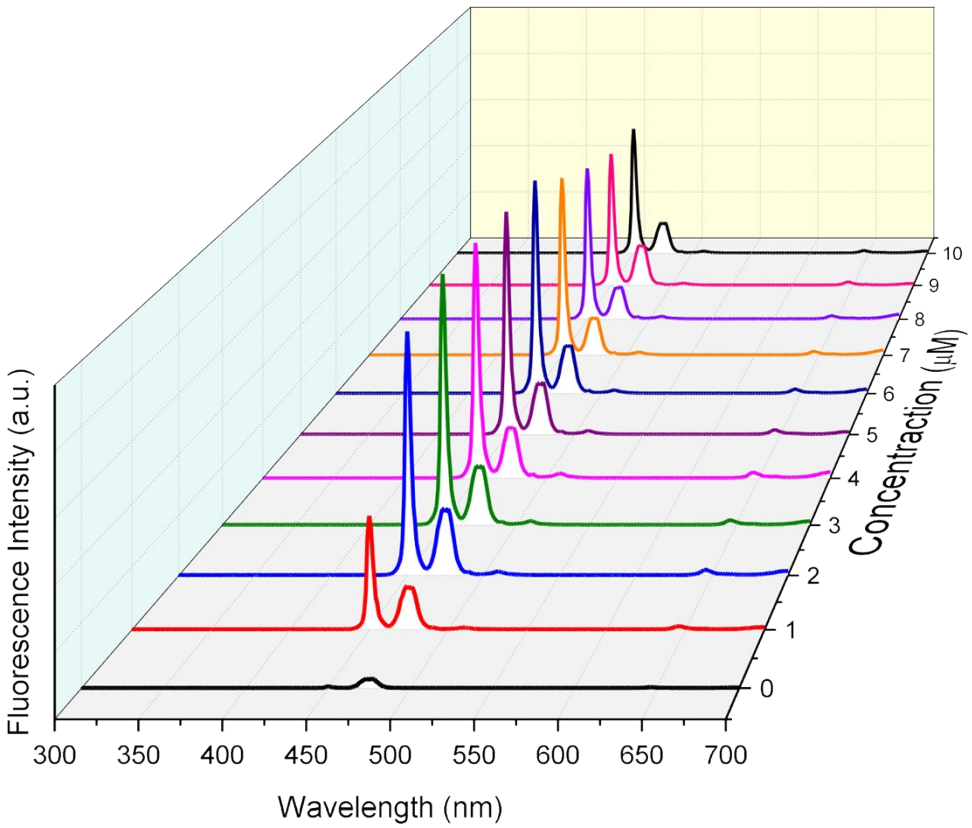


Fig. S8. Upconversion spectra of UCNP@PEG with IR61-BF for sensitization in H_2O solution excited by 808 nm laser. (IR61: 0-10 μM, UCNP@DSPE-PEG: 1mg/mL in dd-H_2O, Laser: 2.8 W/cm^2, Slit: 1 nm).

16. UCL spectra of UCNP@PEG with dyes

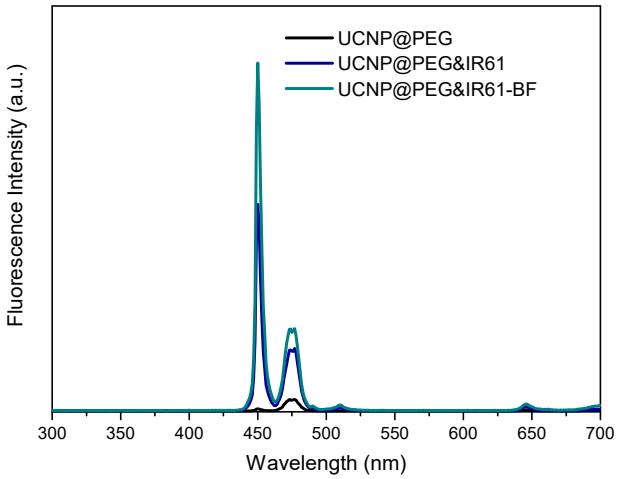


Fig. S9 Upconversion spectra of UCNP@PEG with IR61 or IR61-BF for sensitization in aqueous solution excited at 808 nm. (IR61: 3 μM, IR61-BF: 3 μM, UCNP@PEG: 1mg/mL in dd-H_2O, Laser: 2.8 W/cm^2, Slit: 1 nm).

17. Biocompatibility testing

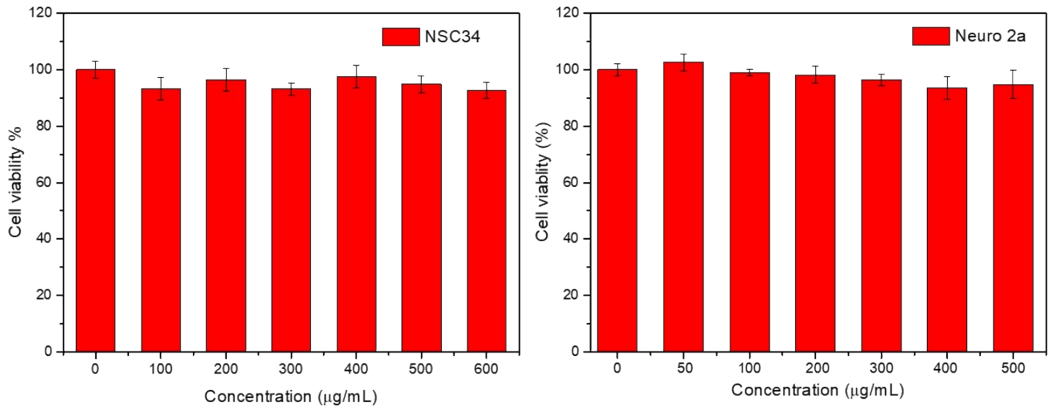


Fig. S10 Cell viability after incubation of UCNPBF at different concentrations with Neuro2a/NSC34 cell lines by MTT assay, at 37 ℃ for 24 h (each sample was tested using four replicates, and results are reported as the mean ± standard deviation).

18. Characterization of cortical neurons

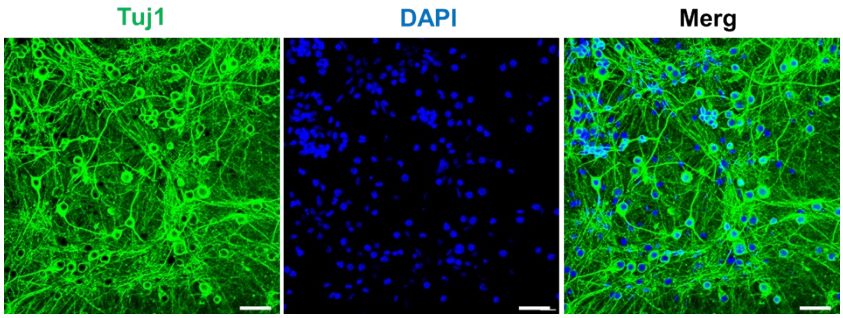


Fig. S11 Mouse embryonic cortical neurons were labeled with Tuj1 (green) and the nucleus was labeled with DAPI (blue). Scale bar is 50μm.

19. NMR and MS

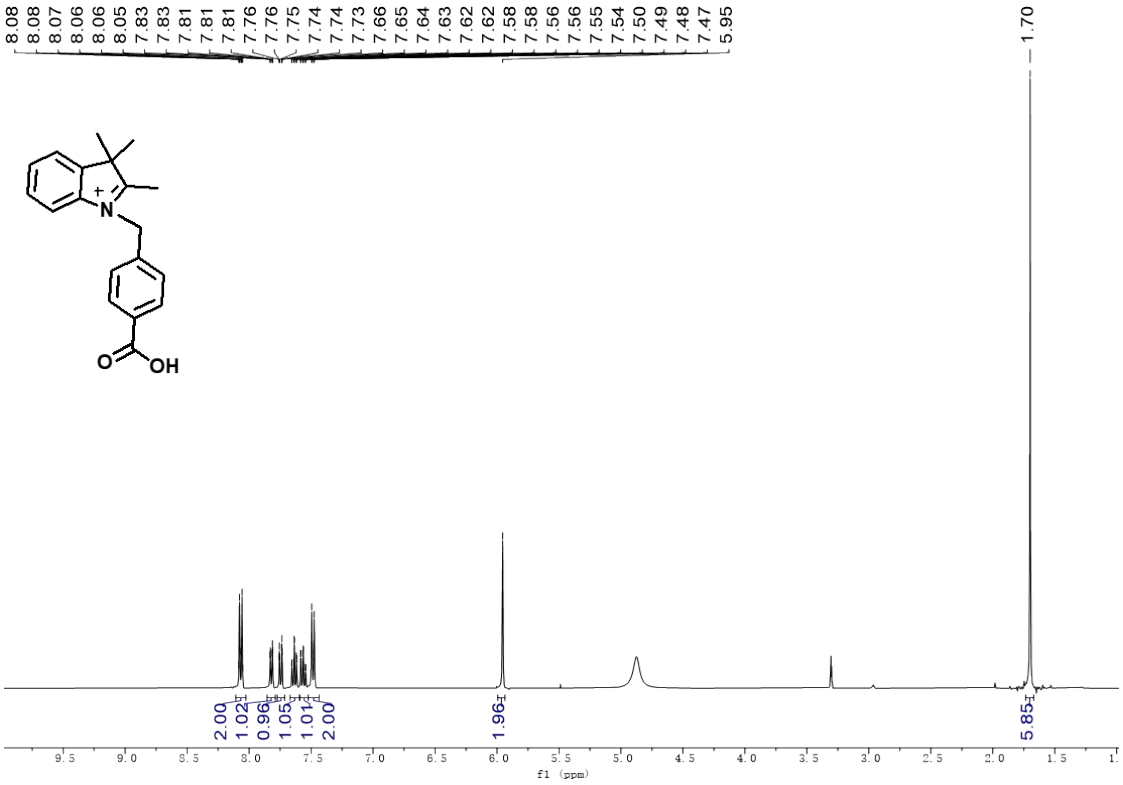


Fig. S12 ^{1}H NMR spectrum of compound **1b** in CH_3OD.

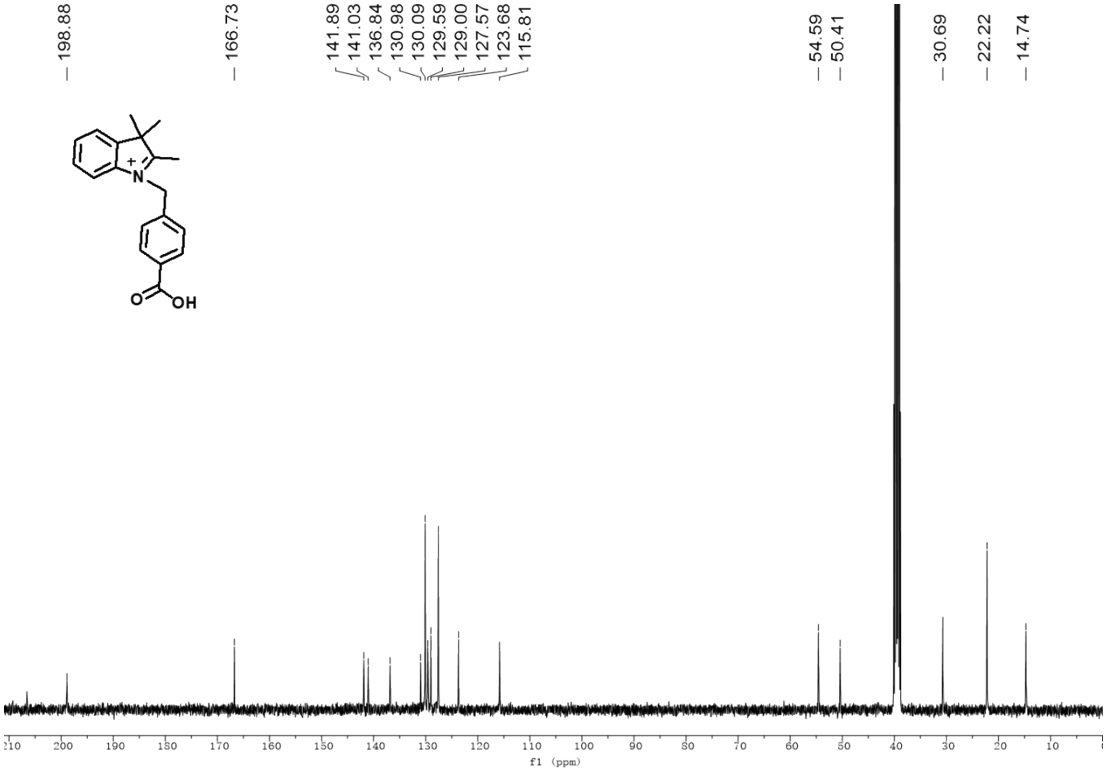


Fig. S13 ^{13}C NMR spectrum of compound **1b** in DMSO.

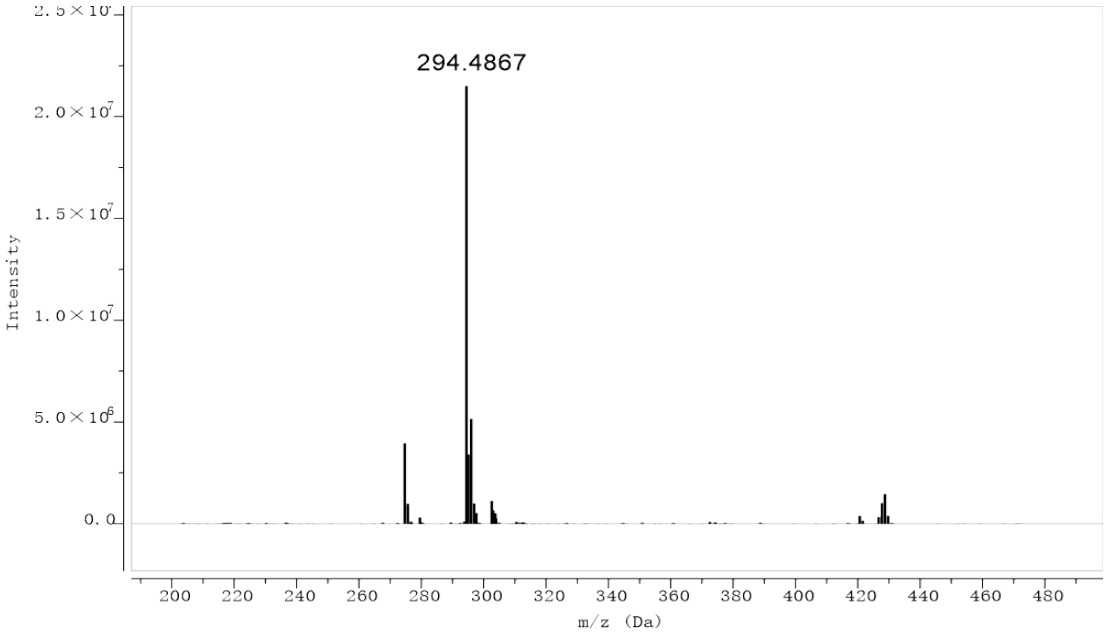


Fig. S14 HRMS of compound **1b**.

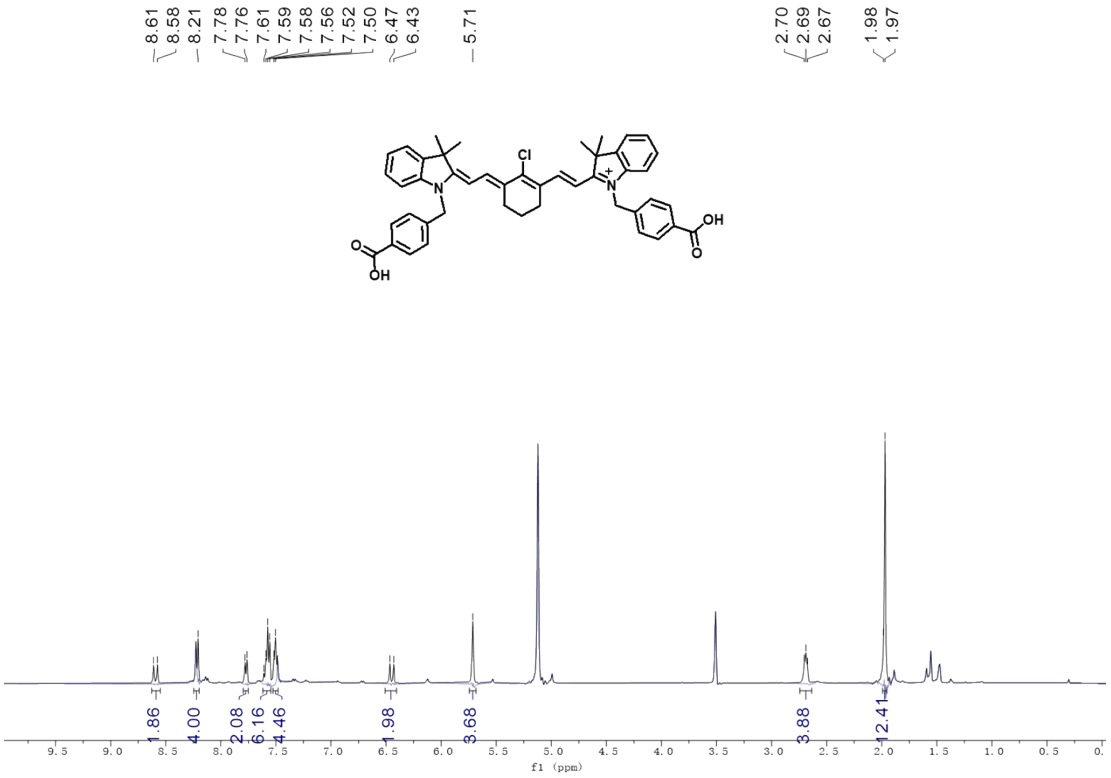


Fig. S15 ^{1}H NMR spectrum of compound **IR61** in CH_3OD.

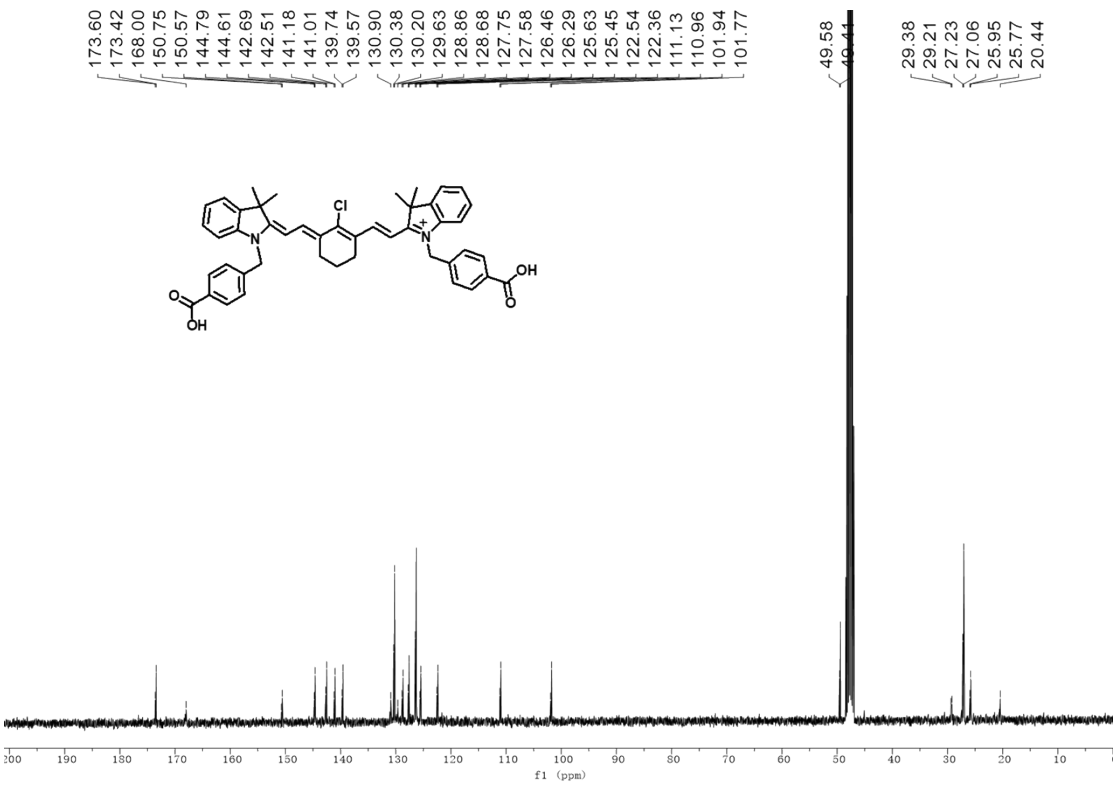


Fig. S16 ^{13}C NMR spectrum of compound **IR61** in CH_3OD.

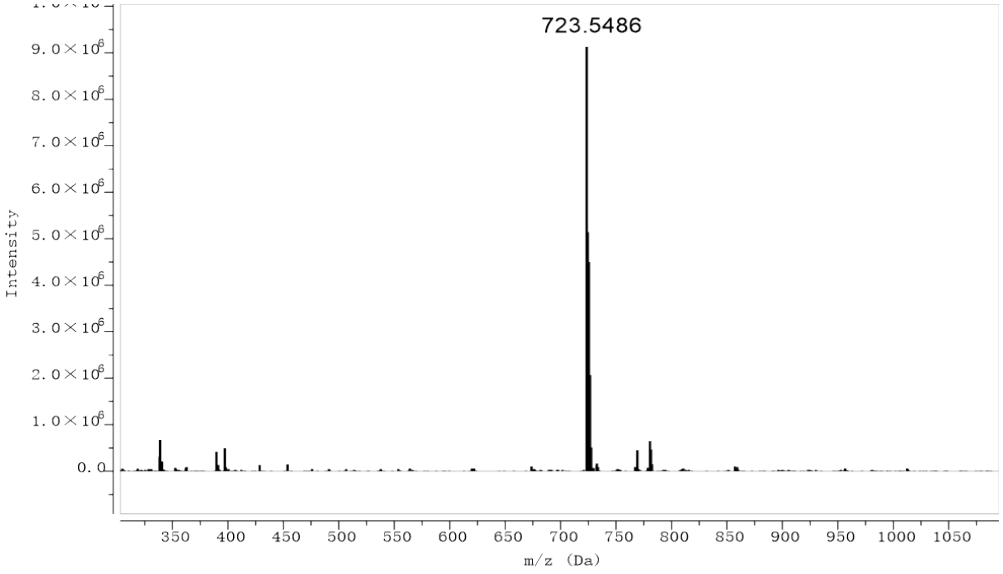


Fig. S17 HRMS of compound **IR61**.

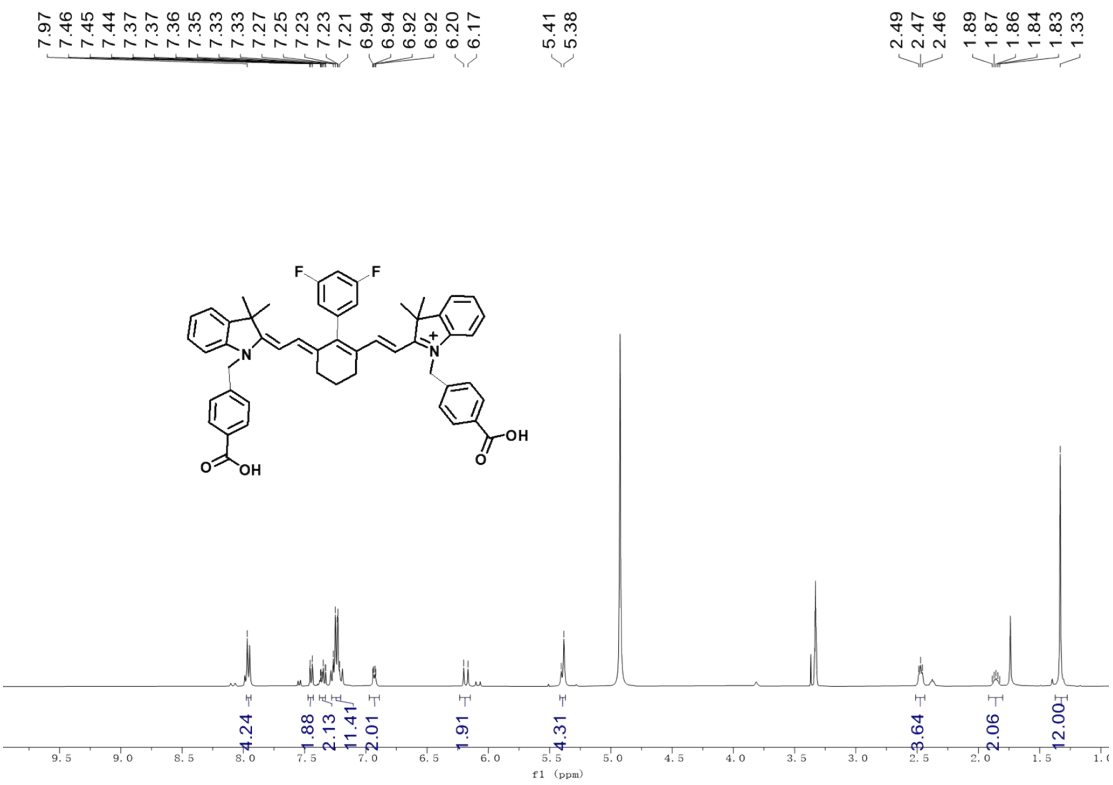


Fig. S18 ^{1}H NMR spectrum of compound **IR61-BF** in CH_3OD.

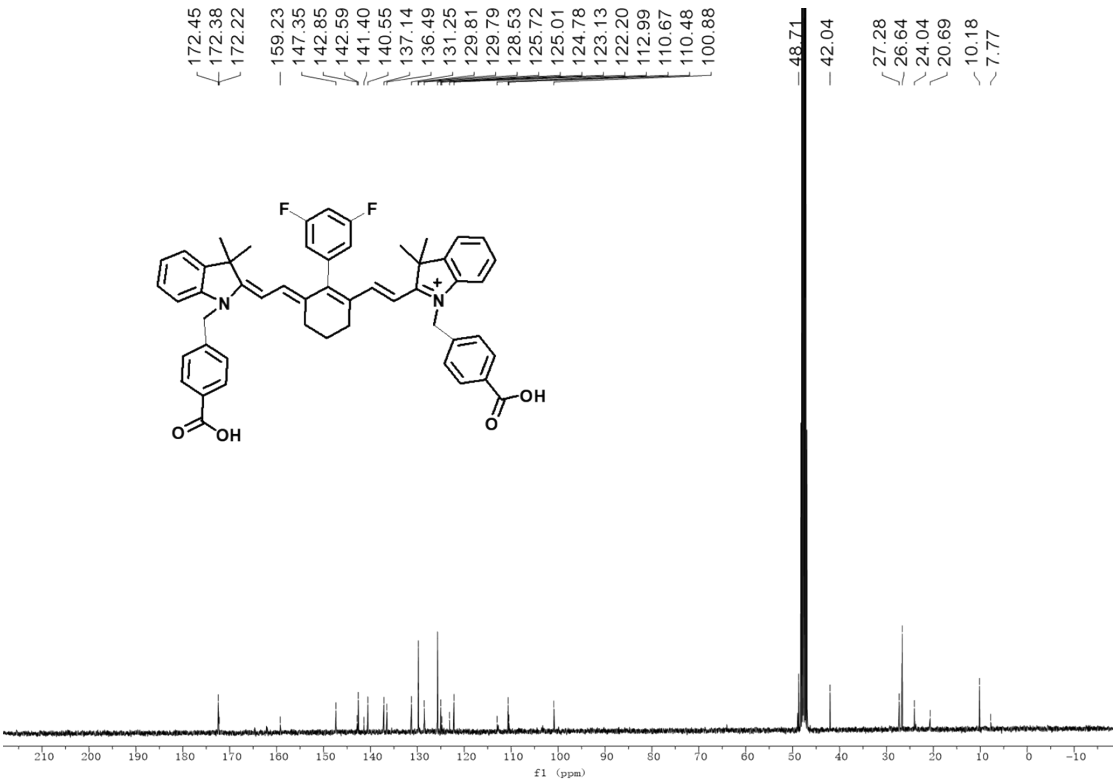


Fig. S19 ^{13}C NMR spectrum of compound **IR61-BF** in CH_3OD.

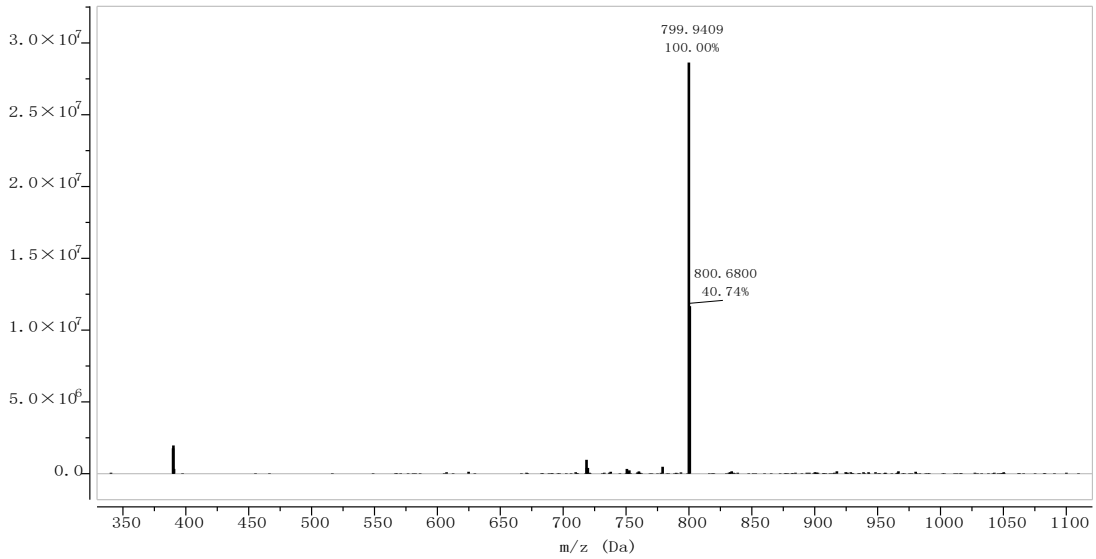


Fig. S20 HRMS of compound **IR61-BF**.

Reference

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2. James, N. S.; Chen, Y.; Joshi, P.; Ohulchanskyy, T. Y.; Ethirajan, M.; Henary, M.; Strekowsk, L.; Pandey, R. K., Evaluation of polymethine dyes as potential probes for near infrared fluorescence imaging of tumors: part-1. *Theranostics* **2013,** *3* (9), 692.