Supporting Information

Asymmetric Heptamethine Quinocyanines with Large Stokes Shifts for High-Fidelity Imaging of Mitochondrial Viscosity and NAFLD

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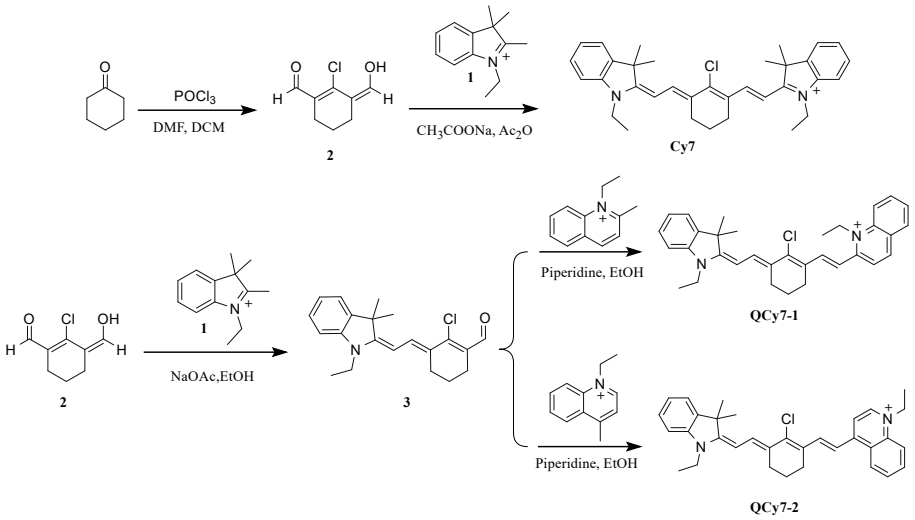
‡ These authors have the equivalent contribution to this work.

1. Reagents and Equipment

All reagents were purchased from Bidepharm and local suppliers and used without further purification. Nuclear Magnetic Resonance Spectrum (NMR) was obtained using QUANTUM 400 MHz NMR spectrometer (Zhongke-Oxford, China). High-Resolution Mass Spectrum (HRMS) were carried out on a FTICR-MS mass spectrometer in the ESI mode. Absorption spectra and fluorescence spectra were measured on UV-3600 spectrophotometer (Shimadzu, Japan) and F-380 fluorescence spectrophotometer (GangDong, China), respectively. Cell imaging was obtained on Olympus Fluoview FV1000. Mice imaging was performed using Xenogen IVIS Spectrum.

2. Synthesis



Scheme S1 Synthetic route of probes **Cy7**, **QCy7-1** and **QCy7-2**

Synthesis of compound 1

2,3,3-TrimethyIndole (1.17 g, 10.0 mmol) and iodoethane (4.68 g, 30.0 mmol) were refluxed for 6 h. After cooling, the precipitate was collected to give compound 1 as light-brown solid (2.99 g, 95%).

Synthesis of compound 2

DMF (8 mL) and CH_2Cl_2 (8 mL) were added to a flask. $POCl_3$ (4.78 mL, 32.6 mmol) was added dropwise at 0 ^oC, then the mixture was stirred 30 min at rt. Cyclohexanone (2.12 mL, 20.4 mmol) was added dropwise at 0 ^oC, and the reaction was heated to 80 °C for 6 h. After cooling, the mixture was

extracted with EtOAc, dried (Na_2SO_4), and concentrated. Petroleum ether was added to the cold solution to precipitate a yellow solid (62.5%), which was collected and used directly.

Synthesis of compound 3

Compound 1 (200 mg, 1.20 mmol), 1-ethyl-2,3,3-trimethylindolium iodide (226 mg, 1.20 mmol), and NaOAc (148 mg, 1.80 mmol) were stirred in 5 mL of EtOH at rt for 1 h. The dark-red solution was extracted with CH_2Cl_2, dried (Na_2SO_4), and purified by flash chromatography (petroleum ether: EtOAc = 6:1) to give a purple metallic solid (205 mg , 50.0% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ : 10.29 (s, 1H), 7.87 (d, *J* = 12.8 Hz, 1H), 7.27 (d, *J* = 10.0 Hz, 1H), 7.24 (d, *J* = 8.0 Hz, 1H), 7.00 (t, *J* = 7.6 Hz, 1H), 6.75 (d, *J* = 8.0 Hz, 1H), 5.55 (d, *J* = 12.8 Hz, 1H), 3.81-3.76 (m, 2H), 2.60 (t, *J* = 6.4 Hz, 2H), 2.52 (t, *J* = 6.0 Hz, 2H), 1.83-1.80 (m, 2H), 1.69 (s, 6H), 1.32 (t, *J* = 7.2 Hz, 3H).

Synthesis of probe Cy7

Compound 1 (300 mg, 1.74 mmol), 1-ethyl-2,3,3-trimethylindolium iodide (655 mg, 3.48 mmol), and NaOAc (357 mg, 4.35 mmol) were refluxed in 5 mL of Ac_2O until the reaction was completed. After cooling, Et_2O was added to precipitate the product, which was filtered, washed with Et_2O, and dried to give Cy7 as a green solid (712 mg, 80.0%).^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.34 (d, *J* = 14.0 Hz, 2H), 7.42-7.37 (m, 4H), 7.17 (d, *J* = 8.0 Hz, 2H), 6.26 (d, *J* = 14.4 Hz, 2H), 4.31-4.25 (m, 4H), 2.77 (t, *J* = 6.0 Hz, 4H), 1.72 (s, 12H), 1.46 (t, *J* = 7.2 Hz, 6H).

Synthesis of probe QCy7-1

Compound 3 (100 mg, 0.30 mmol), 1-ethyl-2-methyquinolinium iodide (1.5 equiv), and two drops of piperidine were refluxed in EtOH (5 mL) for 6 h. After cooling, the solvent was removed and the residue was purified by column chromatography (MeOH/CH_2Cl_2 = 1:100) to afford QCy7-1 as a green solid (54 mg, 36 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.07 (d, *J* = 9.6 Hz, 1H), 8.01 (d, *J* = 13.6 Hz, 1H), 7.91 (d, *J* = 9.6 Hz, 1H), 7.79 (d, *J* = 8.0 Hz, 1H), 7.75 (d, *J* = 5.6 Hz, 2H), 7.44-7.40 (m, 1H), 7.37 (d, *J* = 12.8 Hz, 1H), 7.23 (d, *J* = 6.8 Hz, 2H), 7.01 (t, *J* = 7.4 Hz, 1H), 6.81 (d, *J* = 7.6 Hz, 1H), 6.07 (d, *J* = 13.6 Hz, 1H), 5.64 (d, *J* = 12.8 Hz, 1H), 4.57-4.56 (m, 2H), 3.85-3.83 (m, 2H), 2.52 (t, *J* = 6.6 Hz, 2H), 2.43 (t, *J* = 6.4 Hz, 2H), 1.92-1.91 (m, 2H), 1.64 (t, *J* = 6.8 Hz, 6H), 1.60 (d, *J* = 7.2 Hz, 3H), 1.33 (t, *J* = 7.0 Hz, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ: 170.98, 163.72, 152.37, 144.89, 139.16, 137.84, 135.61, 133.13, 129.80, 128.29, 125.53, 122.17, 121.94, 119.92, 116.00, 107.78, 102.24, 93.87, 56.47, 47.07, 44.45, 28.96, 28.29, 25.81, 25.10, 24.58, 22.15, 12.66. HRMS [$C_{33}H_{36}ClN_2^+$] calcd for 495.2562, found 495.2559.

Synthesis of probe QCy7-2

Compound 3 (100 mg, 0.30 mmol), 1-ethyl-4-methyquinolinium iodide (1.5 equiv), and two drops of piperidine were refluxed in EtOH (5 mL) for 6 h. After cooling, the solvent was removed and the residue was purified by column chromatography (MeOH/CH_2Cl_2 = 1:100) to afford QCy7-2 as a green solid (74 mg, 50 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.07 (d, J = 6.8 Hz, 1H), 8.47 (d, J = 8.4 Hz, 1H), 8.42 (d, J = 14.8 Hz, 1H), 8.10 (d, J = 8.8 Hz, 1H), 8.05 (d, J = 6.8 Hz, 2H), 7.83(dd, J = 14.4,7.0 Hz, 2H), 7.25-7.17 (m, 3H), 7.00 (t, J = 7.4 Hz, 1H), 6.76 (d, J = 8.0 Hz, 1H), 5.60 (d, J = 12.8 Hz, 1H), 5.20 (q, J = 7.2 Hz, 2H) , 3.8 (m, 2H), 2.73 (t, J = 6.0 Hz, 2H), 2.64 (t, J = 6.0 Hz, 2H), 1.96 (m, 2H), 1.73 (t, J = 7.2 Hz, 3H), 1.68 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 141.92, 138.03, 134.59, 128.46, 128.11, 122.12, 121.57, 114.94, 107.28, 51.94, 28.42, 27.43, 26.35, 21.31, 15.72. HRMS [$C_{33}H_{36}ClN_2^+$] calcd for 495.2562, found 495.2556.

3. Optical Experiments

All probes were prepared as a 5 mM DMSO stock solution and subsequently diluted to a working concentration of 10 μM for use. Spectroscopic studies were conducted in various solutions, with an excitation wavelength of 690 nm. The slit widths for both excitation and emission were set to 10 nm. All analytes were used at a concentration of 200 μM.

4. Theoretical Calculation

Density functional theory (DFT) and time-dependent DFT (TD-DFT) were calculated to understand the structural and electronic properties of three probes in the Gaussian 16w code[1]. The molecular structures of three probes in the ground state were optimized at B3LYP/6-311G(d) level. The solvent effect (in water) was included in all calculations using the polarizable continuum model (PCM).

5. Cell culture and cytotoxicity test

HeLa cells were cultured at 37 °C and 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, and 4 mM L-glutamine. Cells were seeded in a 96-well plate with culture media. After overnight culture, cells were incubated with each concentration of probe for 12 h. To identify cell viability, 0.5 mg mL^{-1} of MTT (Sigma) media was added to cells for 12 h and the produced formazan was dissolved in 0.15 mL of dimethyl sulfoxide (DMSO) and read at OD 490 nm with a Spectramax microwell plate reader.

6. Confocal Imaging

Co-location Experiment. HeLa cells were co-incubated with commercial trackers and **QCy7-1** for 1 h, washed with PBS three times, and then viewed immediately on a confocal microscope. Mito-Tracker Green (500 nM, λ_{ex} = 488 nm, λ_{em} = 500-550 nm), Lyso-Tracker Green (500 nM, λ_{ex} = 488 nm, λ_{em} = 500-550 nm), LDs-tracker BODIPY 493/503 (500 nM, λ_{ex} = 488 nm, λ_{em}= 500-550 nm), and **QCy7-1** (1 nM, λ_{ex} = 488 nm, λ_{em} = 600-650 nm).

Monitoring viscosity changes of mitochondria.

HeLa cells were treated with Nystatin (10 μM), Monensin (10 μM), LPS (10 μg mL^{-1}) for 30 min, washed 3 times with PBS, then treated with **QCy7-1** (1 μM) for 1 h, washed 3 times with PBS, immediately imaging. (λ_{ex} = 647 nm, λ_{em} = 740-800 nm)

HeLa cells were cultured in glucose-free DMEM for different times (0, 20, 40, 60 min), washed 3 times with PBS, then incubated with **QCy7-1** (1 μM) for 1 h, washed 3 times with PBS, immediately imaging. (λ_{ex} = 647 nm, λ_{em} = 740-800 nm)

7. Fluorescence Imaging in Mice

Male Kunming Mice were maintained on a high-fat diet for 4 weeks to establish a non-alcoholic fatty liver model. The mice were intravenously injected with probe **QCy7-1** (300 μM in 100 μL PBS), and collect images at different time points (0, 5, 10, 15, 20, 30 min). After a 30-minute incubation period, the mice were euthanized by cervical dislocation, and the liver was promptly excised. The images were obtained by using an IVIS spectrum imaging system with an excitation wavelength of 605 nm. All animal experiments were carried out in accordance with the ethical guidelines of the Hebei University Institutional Animal Care and Use Committee.

8. Supporting figures

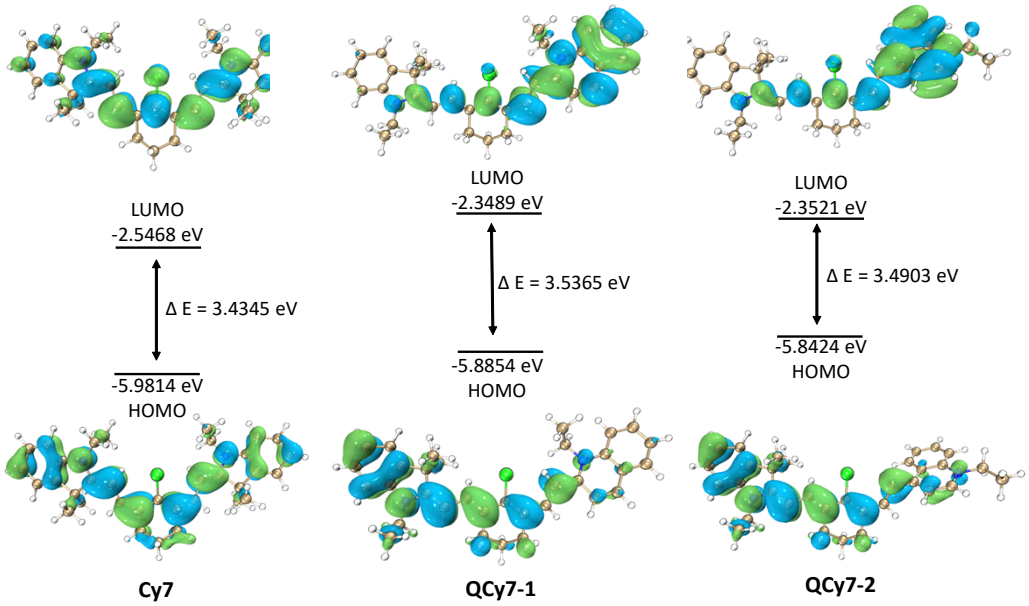


Fig. S1. The calculated distributions, energy levels of HOMO and LUMO, and energy gaps (ΔE) of Cy7 and QCy7-1/2.

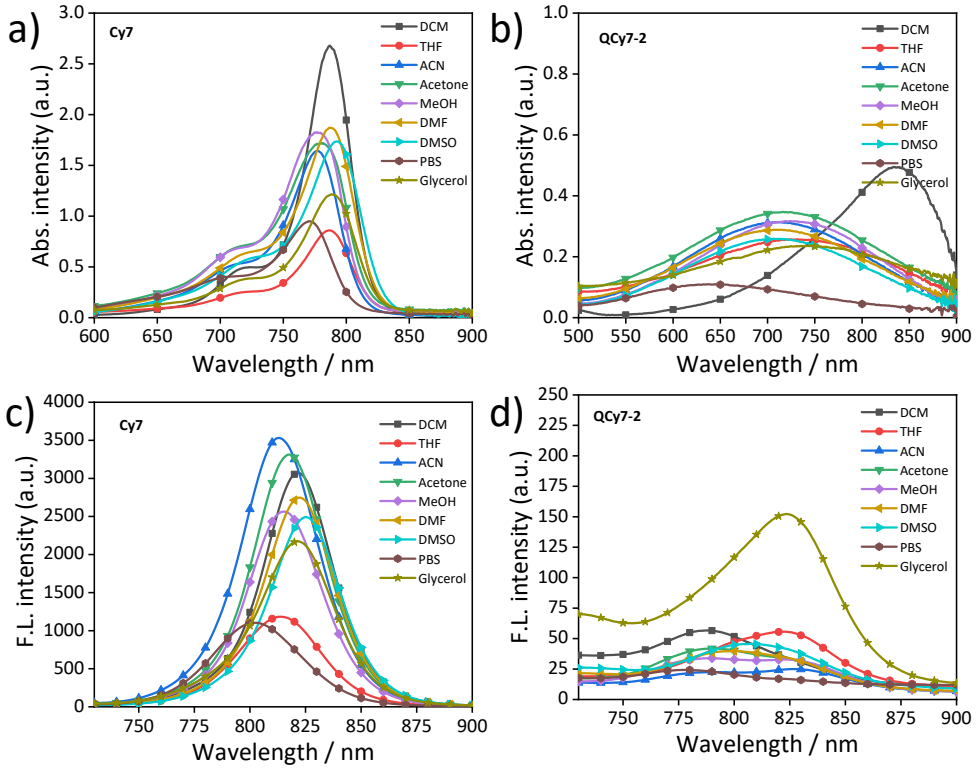


Fig. S2. Absorption (a) and fluorescence (c) spectra of probe Cy7 (10 μM) in various solutions. Absorption (b) and fluorescence (d) spectra of probe QCy7-2 (10 μM) in various solutions.

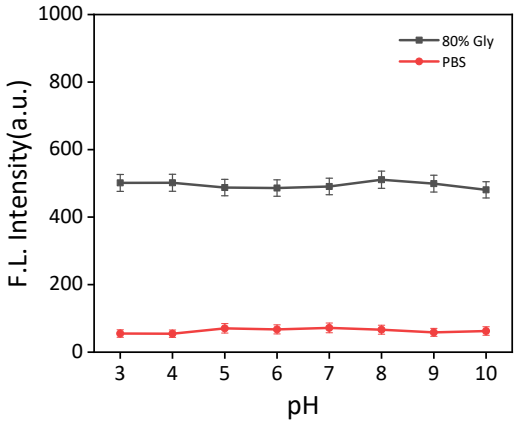


Fig. S3. Fluorescence intensity of the **QCy7-1** (10 μM) in PBS (red) and 80% glycerol (black) over the pH range of 3 to 10 ($\lambda_{ex}/\lambda_{em}$, 690/806 nm).

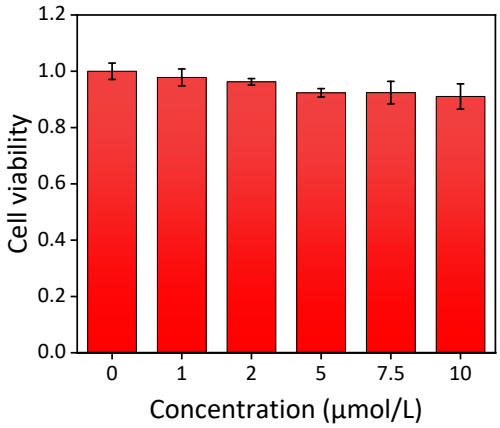
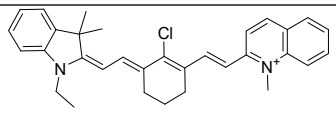
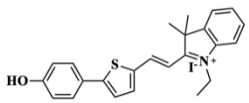
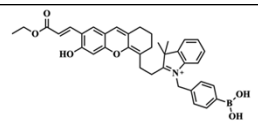
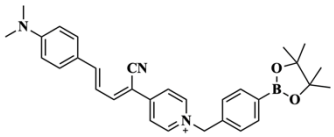
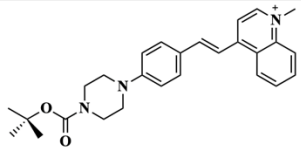
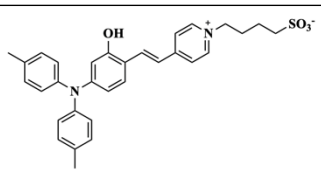
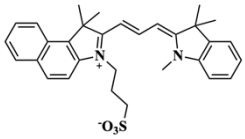
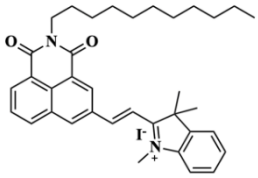
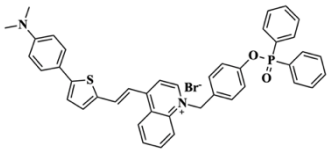
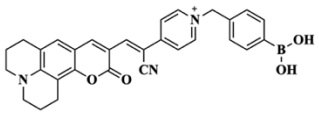
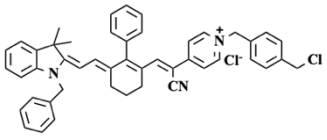


Fig. S4. The cytotoxicity of the probe **QCy7-1** evaluated by MTT assay. Error bars are ± SEM.

Table S1. The sumarry of some mitochondrial-viscosity-responsive fluorescent probes

Structure	$\lambda_{ex}/\lambda_{em}$ (nm)	Stock shift (nm)	PCC	Application	Reference
	**541/780**	**239**	**0.93**	cell, mice	**This work**
	520/627	107	0.91	cell, mice	[2]

	640/720	80	0.91	cell, mice	[3]
	600/700	100	0.82	cell	[4]
	510/667	157	0.93	cell, mice	[5]
	485/623	138	0.95	cell, mice	[6]
	560/580	20	0.85	cell, mice	[7]
	500/620	120	0.90	cell, zebrafish	[8]
	590/760	170	0.89	cell, mice	[9]
	600/678	178	0.89	cell, zebrafish, mice	[10]
	780/820	40	0.90	cell, mice	[11]

9. ^{1}H NMR, ^{13}C NMR and HRMS spectra

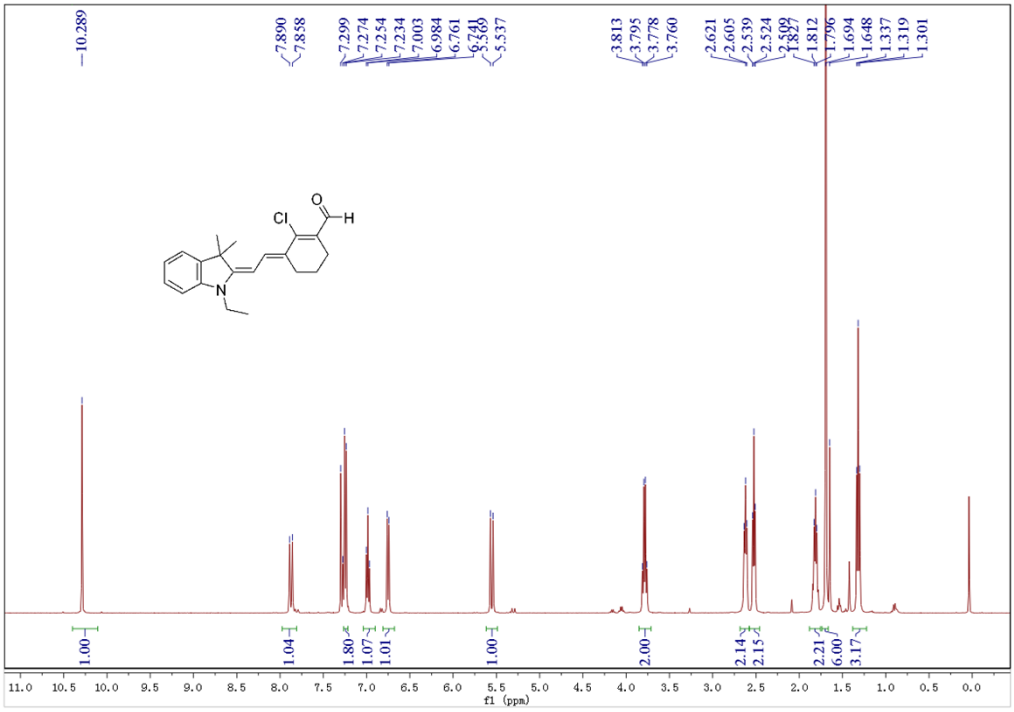


Figure. S5. ^{1}H NMR of compound 3 in $CDCl_3$

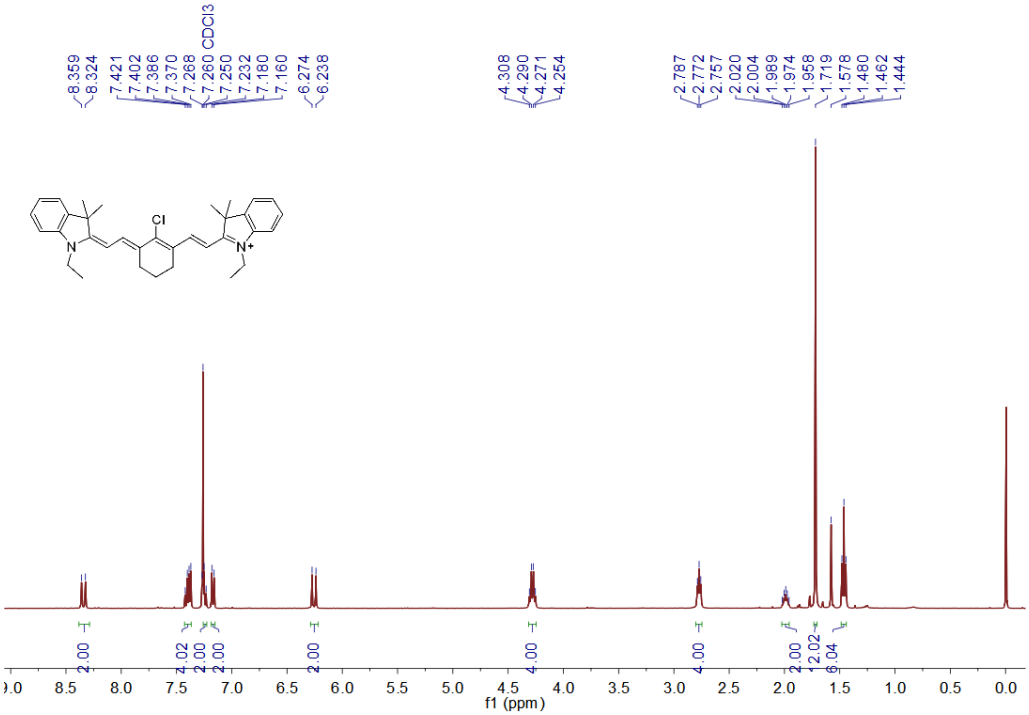


Figure. S6. ^{1}H NMR of probe Cy7 in $CDCl_3$

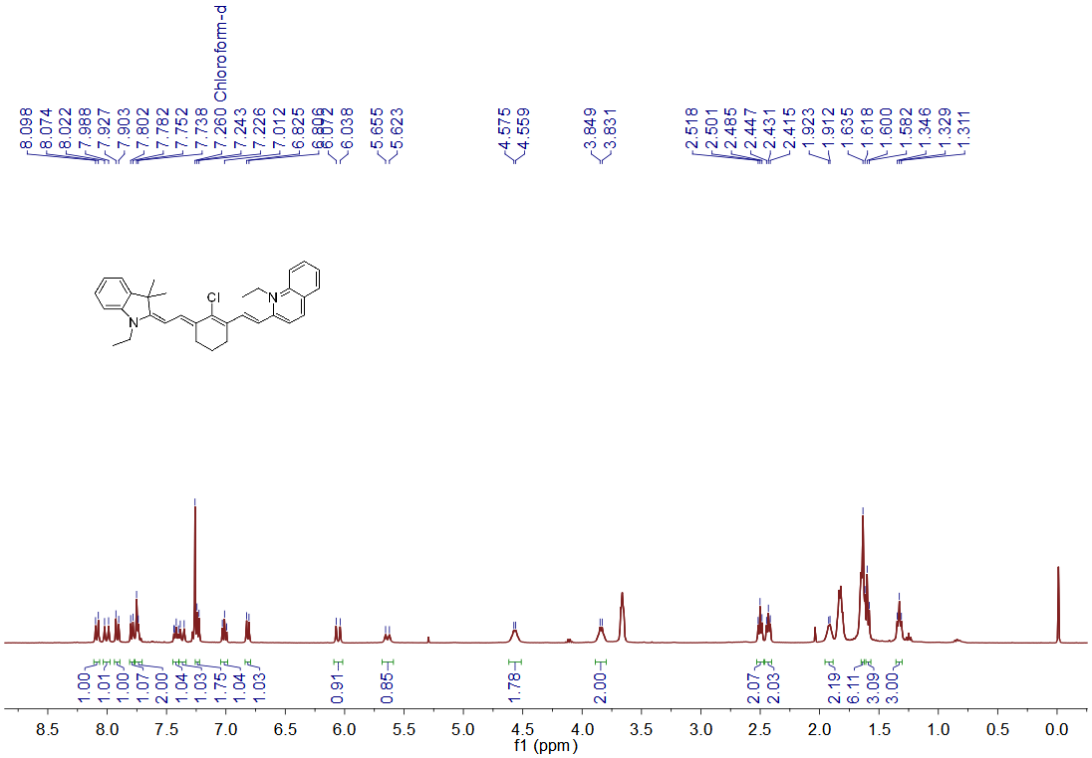


Figure. S7. ^{1}H NMR of probe **QCy7-1** in $CDCl_3$

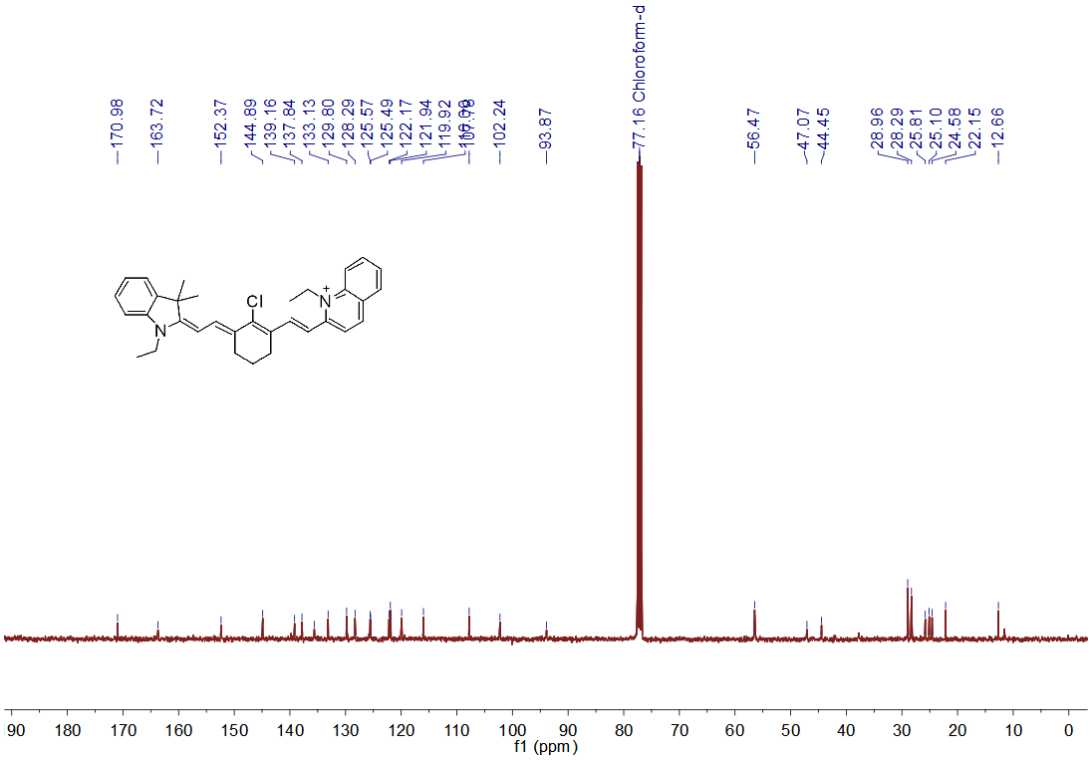


Figure. S8. ^{13}C NMR of probe **Qcy7-1** in $CDCl_3$

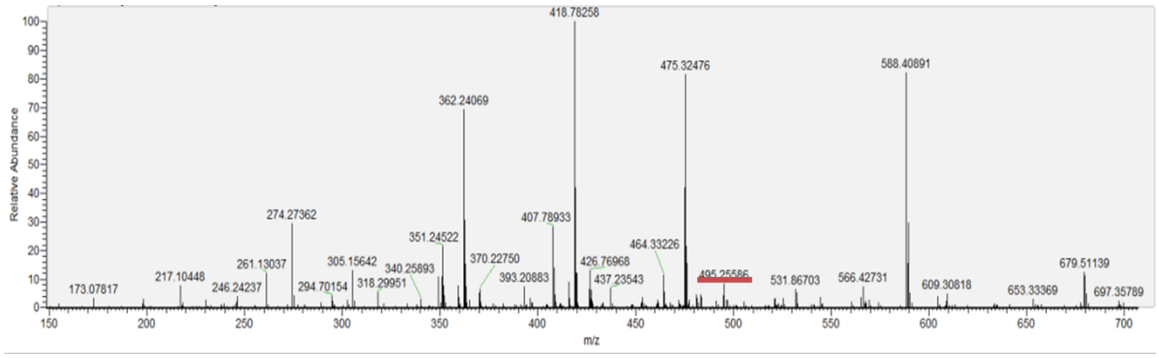


Figure. S9. HRMS of probe **Qcy7-1** (calcd for 495.25615)

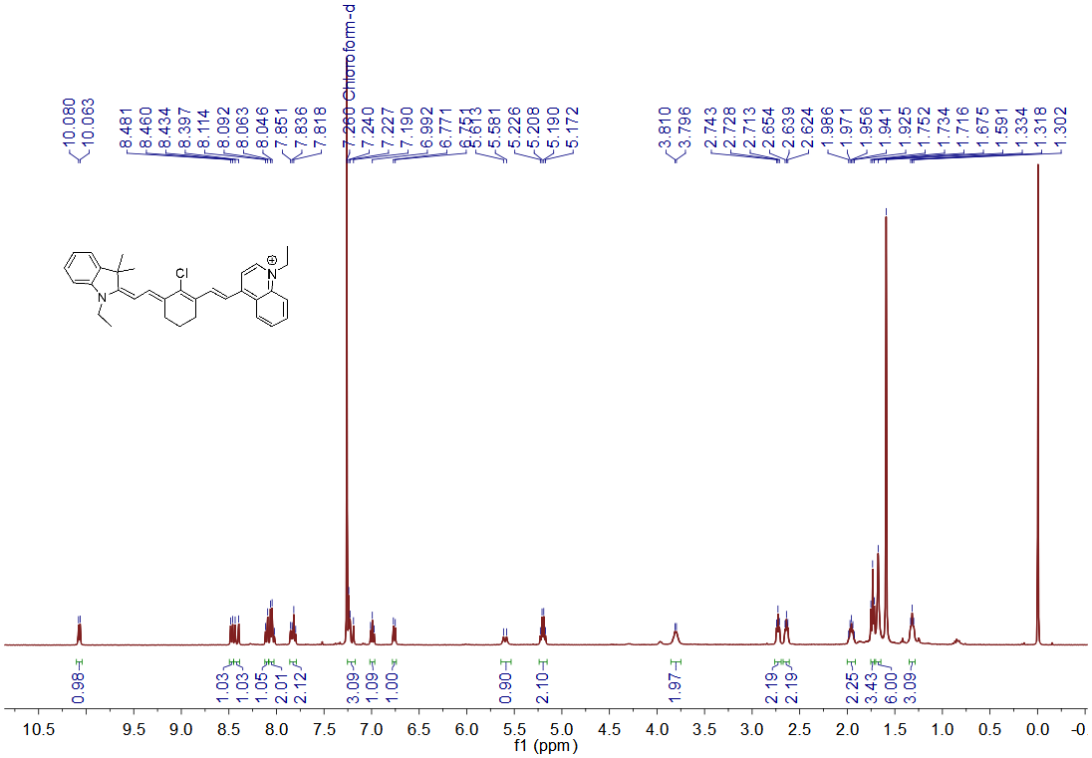


Figure. S10. ^{1}H NMR of probe **Qcy7-2** in $CDCl_3$

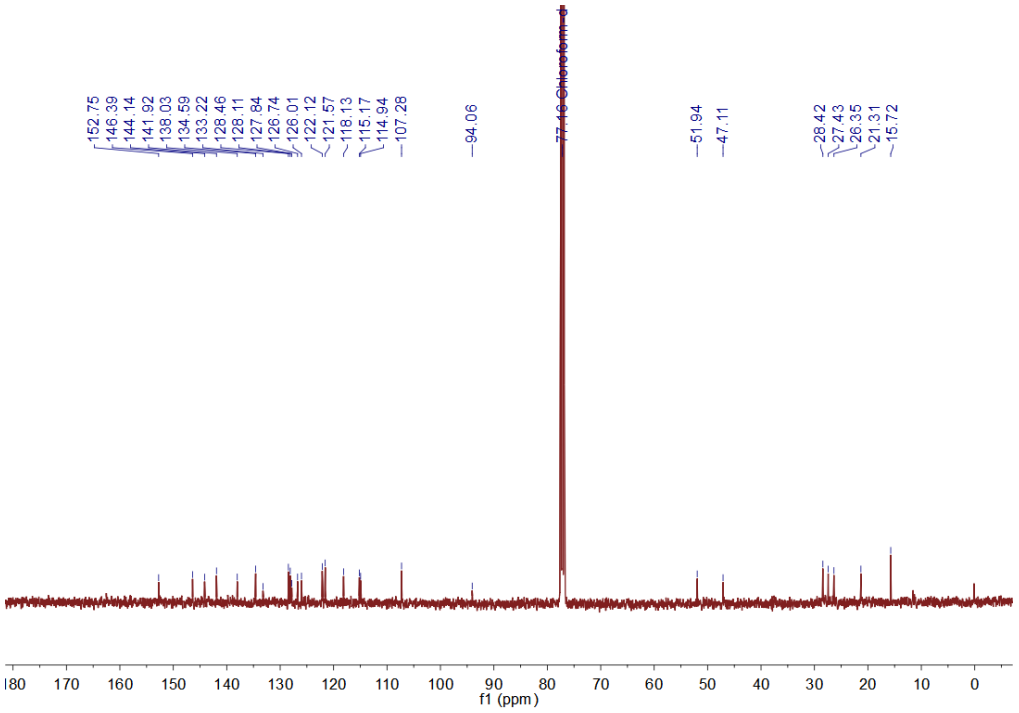


Figure. S11. ^{13}C NMR of probe **Qcy7-2** in $CDCl_3$

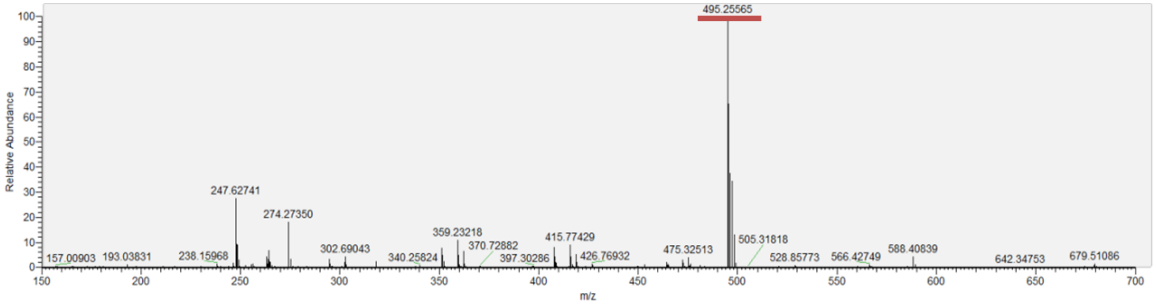


Figure. S12. HRMS of probe **Qcy7-2** (calcd for 495.25615)

Reference

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