Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information for

A Dual-Rotator Fluorescent Probe for Analyzing the Viscosity of Mitochondria and Blood

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1. Experimental details

Materials and apparatus

All chemicals were commercially available from Energy Chemical and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on Brucker Avance 500 MHz spectrometers. The spectra were reported in ppm (δ) and referenced to a tetramethylsilane (TMS) standard in $CDCl_3$, DMSO-d6. Thin layer chromatography (TLC) for reaction monitoring was performed on pre-coated silica gel plates (Merck 60 F254 nm) with a UV254 fluorescent indicator and column chromatography was conducted over silica gel (mesh 300-400). The fluorescence and UV–vis spectra were acquired on a SpectraMax M5 (Molecular Devices).

Live subject statement

All experiments were performed in compliance with the relevant laws and institutional guidelines, and the Ethical Committee of Affiliated Jinhua Hospital, Zhejiang University School of Medicine has approved the experiments. Informed consent was obtained for any experimentation with human subjects.

Synthesis of probe FD

Compound **F1** (0.868 g, 4 mmol) and Danyl chloride (0.94 g, 4 mmol) were dissolved in DCM (20 mL) at 0℃, then Et_3N (0.2 g,12 mmol) was added in the mixture. This solution was stirred at 0℃ for 3 h. After the removal of DCM, the crude products were purified by column chromatography to generate a green solid product (258 mg, 15%). ^{1}H NMR (400 MHz, DMSO-d6), δ = 8.91 (d, 2H), 8.44 (d, *J* = 6.84Hz, 2H), 8.10 (d, *J* = 15.76 Hz, 2H), 7.87 (d, *J* = 6.88 Hz, 1H), 7.75 (d, *J* = 10.28 Hz, 2H), 7.54 (s, 1H), 7.44 (d, *J* = 6.88 Hz, 3H), 7.25-7.3 (d, *J* = 7.48 Hz, 3H),6.96 (d, *J*= 15.76 Hz, 1H), 5.67 (d, *J* = 6.88 Hz, 1H), 3.24-3.18 (s, 6H), 2.65-2.57 (s, 3H), 1.84 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d6), δ = 173.1 154.3, 154.2, 147.0, 144.2, 137.9, 125.4, 121.5,117.6 115.0, 114.9, 111.1, 107.5, 49.9, 49.2, 46.4, 27.3, 21.91, 21.5, 21.0. MS (ESI) m/z: calcd for $[C_{29}H_{30}N_3O_2S^+]$, 534.2210; found: 534.2214.

Fluorescence quantum yield

For the determination of fluorescence quantum yield (Φu), fluorescein was used as a standard in aqueous NaOH (pH=13, λex = 490 nm, λex = 510 nm, $\Phi_S = 0.49$). The value is calculated according to the following formula: $\Phi_u = \Phi_s (nu^2E_uA_s)/ (ns^2E_sA_{u)}$, where the subscripts u and s refer to the sample and the reference compound, respectively. A is the absorbance at the excitation wavelength, E is the integrated area under the emission spectrum, and n is the refractive index of the solvent.

Cytotoxicity Assay

The cytotoxicity was evaluated by MTT assay. Hela and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in 96-well microplates at 37 °C under 5% CO_2 for 12 h. The medium was next replaced by fresh medium containing various concentrations of FD (0-20 μM). Each concentration was tested in three

replicates. Cells were rinsed twice with phosphate buffer saline 24 h later and incubated with 0.5 mg/mL MTT reagent for 4h at 37 °C. 150 μL DMSO was then added to dissolve formazan. The absorbance at 510m was measured in a microplate reader. Cell viability (%) was calculated according to following equation: Viability = (mean Abs. of treated wells/mean Abs. of control wells) ×100%.

Cell culture and fluorescence imaging

DMEM containing 10% fetal bovine serum and 1% penicillin was used for HeLa cell culture in an incubator supplemented with 95% air and 5% CO_2 at 37 °C. Then, nutrient solution was removed and cells were washed three times with PBS buffer (pH=7.0, 10 mM) before imaging. To compare the difference in viscosity level of HeLa cells, HepG2 cells and MCF-7 cells, they were treated with 10 μM **FD** for 15 min and then washed three times with PBS buffer.

Creation of a normoxic/hypoxic environment for zebrafish

3-Day-old zebrafish were fed in a fish tank at 28 °C on a 12 h light/12 h dark cycle, and someyolks were used as food. Normoxic zebrafish were treated with pumping air into the waterthrough a strip bubble stone. To create a hypoxic condition, the extra-pure (99.999%) nitrogen (N_2) gas was bubbled continuously and appropriately into a medium-sized plastic box with enough water, and a stir bar was stirring to balance the concentration of dissolved oxygen. Then about 50 zebrafish were separated from the tank into the box rapidly and the survival rate was recorded in different time. The concentration of dissolved oxygen was determined by a portable hand-held dissolved oxygen meter (0.3 mg/L, 1.5 mg/L, 3 mg/L O_2 for hypoxic zebrafish and 6 mg/L O_2 for normoxic zebrafish).

Imaging in zebrafish

The 3-day-old zebrafish under hypoxic environment was incubated with **FD** (10 μM) for 30 min, and then washed with PBS buffer and imaged as control group. The 3-day-old zebrafish under normoxic environment was also incubated with **FD** (10 μM) for 30 min. Thereafter, the treated zebrafish was washed with PBS buffer three times and imaged using a confocal microscope. Fluorescence images were acquired with Nikon A1R confocal microscope.

Live subject statement

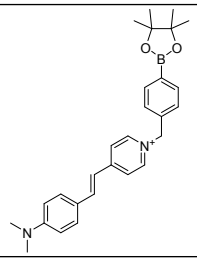
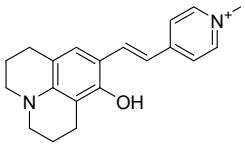
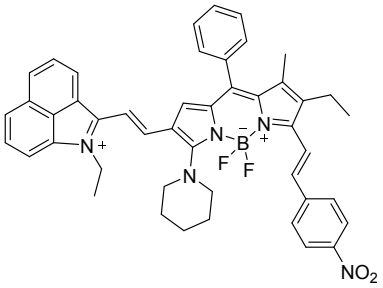
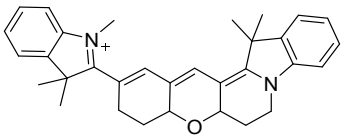
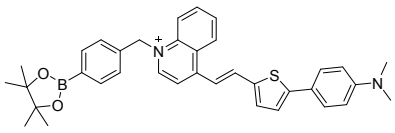
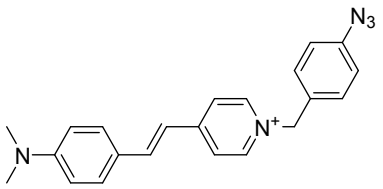
All experiments were performed in compliance with the relevant laws and institutional guidelines, and the Ethical Committee of Jinhua Central Hospital approved the experiments. Informed consent was obtained for any experiments requiring human subjects.

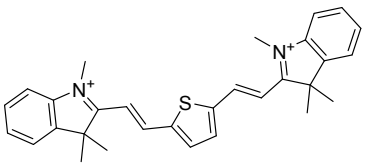
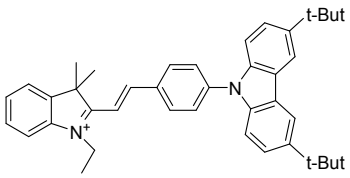
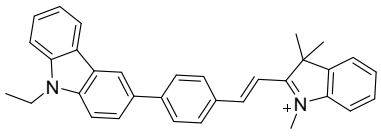
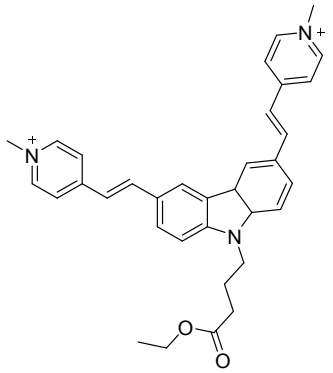
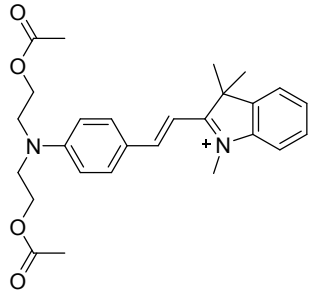
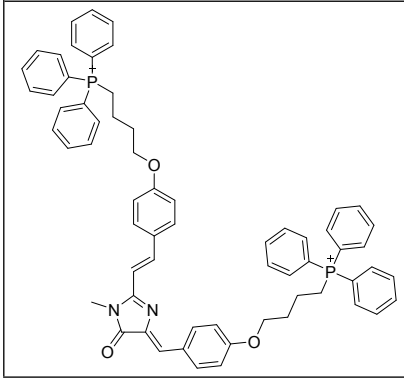
Blood viscosity detection in human clinical samples

Plasma viscosity (PV) were measured using a scanning capillary tube viscometer, Hemovister (Ubiosis, Seongnam, Korea) according to the manufacturer's recommendations.

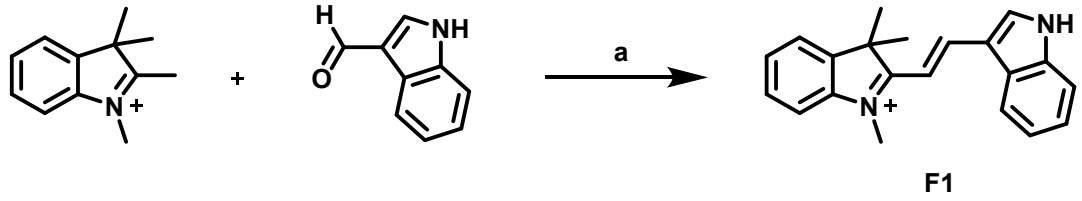
Human blood samples (60 μL) were prepared by mixing 4-fold volume of phosphate buffer (0.1 M, pH 7.5). The concentration of **FD** was finally diluted at 5 μM in clinical samples with gentle shaking. The fluorescence spectra were obtained from 540 to 650 nm under 520 nm excitation. The band-slits of excitation and emission were all set as 5.0 nm. The fluorescence intensity of **FD** at 560 nm was used for the quantitative analysis of human blood.

Table S1 previous mitochondrial viscosity probes and this work

Probes	λ_{ex}/nm	λ_{em}/nm	sensitivity	Bioimaging application	ref
	500	607	16-fold response to viscosity (925 cP) 67-fold response to H_2O_2	cells	[1]
	530	620	not mentioned in the article	cells	[2]
	818	982	31 folds (925 cP)	mice	[3]
	678	698	36 times (621 cP)	Cells rat slice	[4]
	570	800	Response to viscosity and H_2O_2, not mention in the article	cells mice	[5]
	500	610	10-fold response to viscosity (584.52 cP) 7-fold (response to H_2S)	cells	[6]

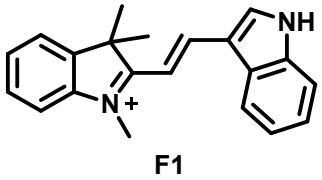
	525	595	not mentioned	cells	[7]
	545	628	16-fold (317 cP)	cells	[8]
	520	610	32-fold (956 cP)	cells, zebra fish, mice	[9]
	470	560	not mentioned	cells	[10]
	515	590	23-fold (950 Cp)	cells	[11]
	434	515	7 folds (438 Cp)	cells	[12]

This work	**520**	**560**	**Nearly 100-fold**	**cells, zebra fish, clinical blood samples**	



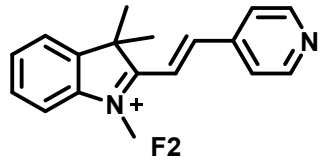
Scheme S1 Synthesis of compound F1

Synthesis of F1 [13]



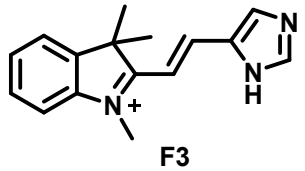
The iodide salts of indoles (1mmol, 174mg) and indole-3-carboxyaldehyde (1.2mmol, 174mg) were dissolved in ethanol (3 mL). The reaction mixture was refluxed for 6 h. The solvent was evacuated under reduced pressure and the solid was triturated with ethyl acetate to get pure products without further purification. Red solid, 255mg, yield: 85%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68(d, *J* = 15.15 Hz, 1H), 8.4 (s, 1H), 8.15 (d, *J* = 6.9 Hz, 1H), 7.68-7.62 (m, 2H), 7.59-7.55 (m, 2H), 7.51-7.49 (m, 1H), 7.42-7.39 (m, 2H), 7.25 (d, *J* = 15.8 Hz, 1H), 4.0 (s, 3H), 1.85 (s, 6H). MS (ESI) m/z (relative intensity) 301.17 (100) [M $^+$].

synthetic of F2[14]



Yellow solid, 178mg, yield: 68%.^{1}H NMR (400 MHz, d_6-DMSO) ^{1}H NMR (400 MHz, d6- DMSO) δ 8.79 (d, J = 5.9 Hz, 2H), 8.37 (d, J = 16.6 Hz, 1H), 8.07 (d, J = 6.0 Hz, 2H), 7.97–7.91 (m, 2H), 7.88–7.82 (m, 2H), 7.76–7.61 (m, 2H), 4.27 (s, 3H), 1.84 (s, 3H), 1.84 (s, 6H). MS (ESI) m/z (relative intensity) 263.15 (100) [M $^+$].

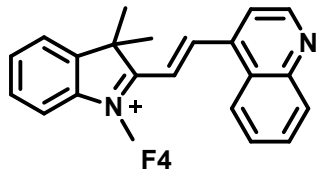
synthetic of F3



The iodide salts of indoles (1mmol, 174mg) and 1H-imidazole-5-carbaldehyde (1.2mmol, 115mg) were dissolved in ethanol (3 mL). The reaction mixture was refluxed for 6 h. The solvent was evacuated under reduced pressure and the solid was triturated with ethyl acetate to get pure products without further purification. Yellow solid,

150mg, yield: 60%.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (d, J=8.4, 2H), 8.26 (d, J=8.4 Hz, 2H), 8.17 (d, J=8.4 Hz, 1H), 8.03(d, J = 8.4 Hz, 1H), 7.80 (t, J=8.0 Hz, 2H), 4.27 (s, 3H), 1.84 (s, 6H). ^{13}C NMR (151 MHz, DMSO) δ 180.56, 142.85, 142.50, 129.19, 127.96, 124.90, 123.66, 123.11, 116.43, 113.99, 105.48, 51.30, 33.48, 26.86. MS (ESI) m/z (relative intensity) 252.15 (100) [M $^{+}$].

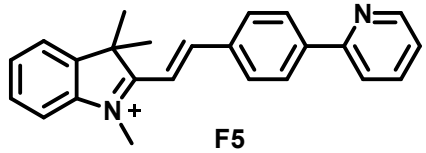
synthetic of F4[14]



F4

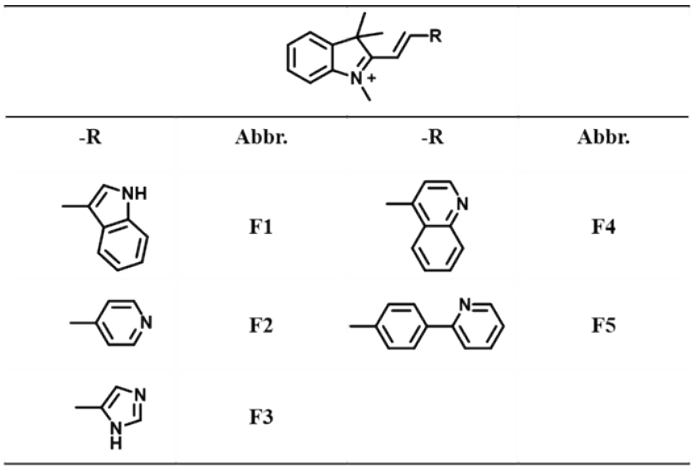
Red solid, 235mg, yield: 75%.^{1}H NMR (400 MHz, d_6-DMSO) δ 9.15 (d, J = 4.4 Hz, 1H), 8.98 (d, J = 16.3 Hz, 1H), 8.44 (d, J = 8.3 Hz, 1H), 8.24 (d, J = 4.4, 1H), 8.18 (d, J = 8.4, 1H), 8.04–7.90 (m, 4H), 7.83 (t, J = 7.5 Hz, 1H), 7.75–7.70 (m, 2H), 4.28 (s, 7H), 4.28 (s, 3H), 1.88 (s, 6H). MS (ESI) m/z (relative intensity) 313.17 (100) [M $^{+}$].

synthetic of F5



F5

The iodide salts of indoles (1mmol, 174mg) and 4-(pyridin-2-yl) benzaldehyde (1.2mmol, 220mg) were dissolved in ethanol (5 mL). The reaction mixture was refluxed for 6 h. The solvent was evacuated under reduced pressure and the solid was triturated with ethyl acetate to get pure products without further purification. Orange solid, 237mg, yield: 70%.^{1}H NMR (400 MHz, d_6-DMSO): δ 8.75 (d, J = 5.0, 1.2 Hz, 1H), 8.48 (d, J = 16.4 Hz, 1H), 8.39 – 8.31 (m, 4H), 8.18 (d, J = 8.0 Hz, 1H), 7.98 (td, J = 7.8, 1.8 Hz, 1H), 7.95 – 7.93 (m, 1H), 7.78 (d, J = 16.4 Hz, 1H), 7.68 – 7.64 (m, 2H), 7.64 – 7.61 (m, 1H), 7.46 (dd, J = 7.5 Hz, 1H), 4.21 (s, 3H), 1.83 (s, 6H). ^{13}C NMR (151 MHz, DMSO) δ 182.27, 154.90, 152.46, 150.17, 142.27, 138.16, 135.52, 131.39, 130.04, 129.29, 127.57, 124.12, 123.74, 123.37, 121.68, 115.82, 115.58, 114.14, 52.78, 35.24, 25.73. MS (ESI) m/z (relative intensity) 339.19 (100) [M $^{+}$].



Scheme S2. Chemical structures of F1-F5

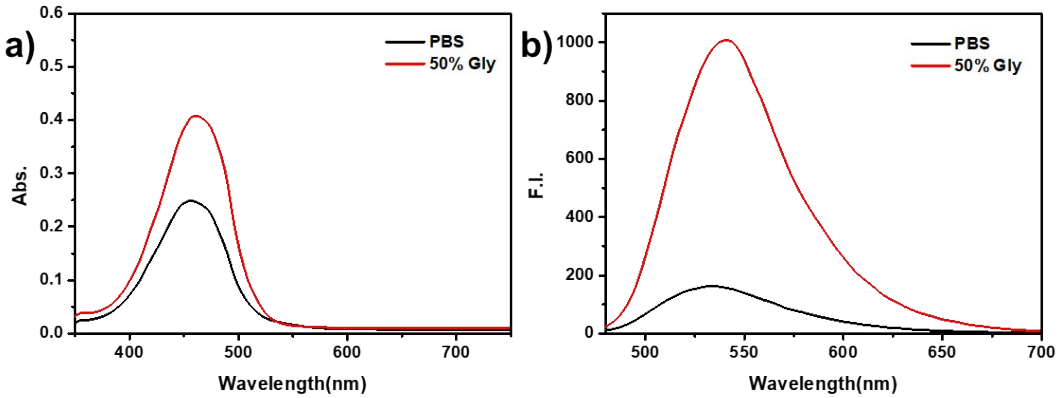


Fig. S1 (a) The absorption spectra of F1 in PBS solution and 50%Gly solution. (b) emission spectra of F1 in PBS solution and 50%Gly solution (λ_{ex}=460nm).

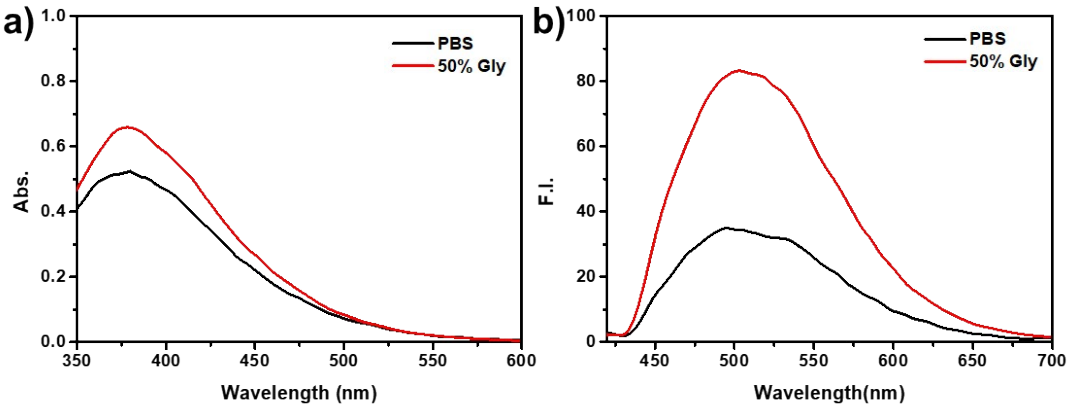


Fig. S2 (a)The absorption spectra of F2 in PBS solution and 50%Gly solution. (b) emission spectra of F2 in PBS solution and 50%Gly solution (λ_{ex}=375nm).

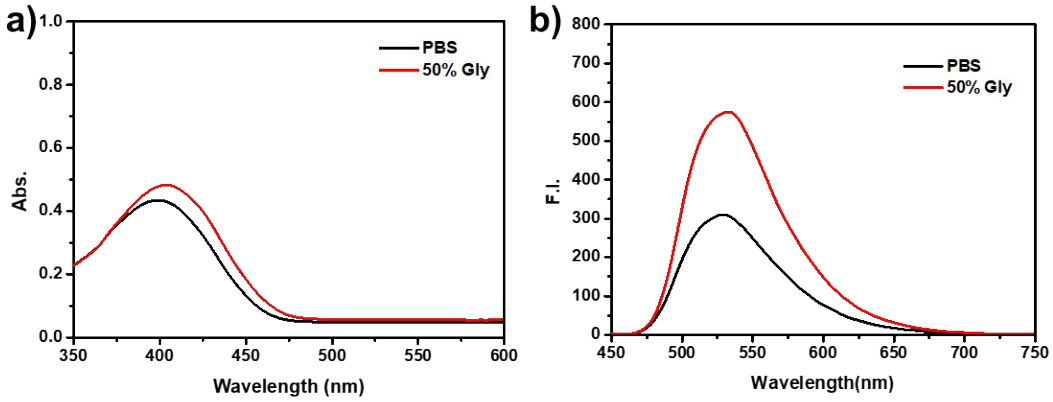


Fig. S3 (a)The absorption spectra of F3 in PBS solution and 50%Gly solution. (b) emission spectra of F3 in PBS solution and 50%Gly solution. (λ_{ex}=410nm).

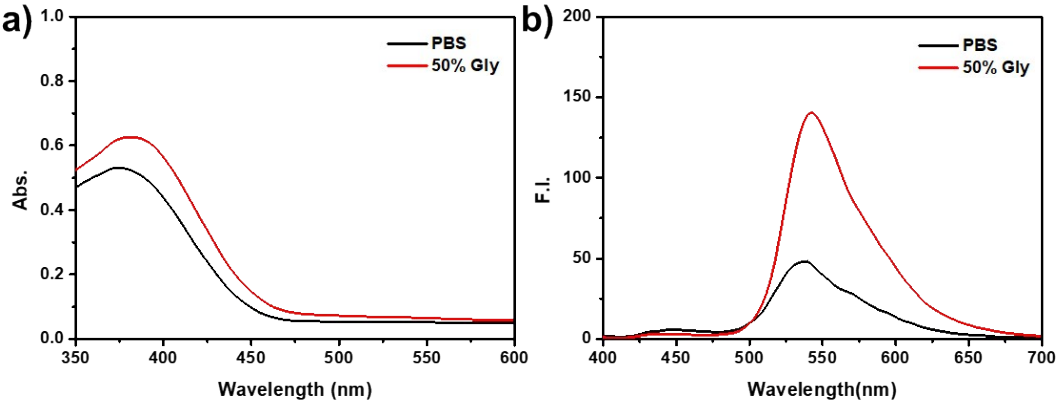


Fig. S4 (a)The absorption spectra of F4 in PBS solution and 50%Gly solution. (b) emission spectra of F4 in PBS solution and 50%Gly solution. (λ_{ex}=365nm).

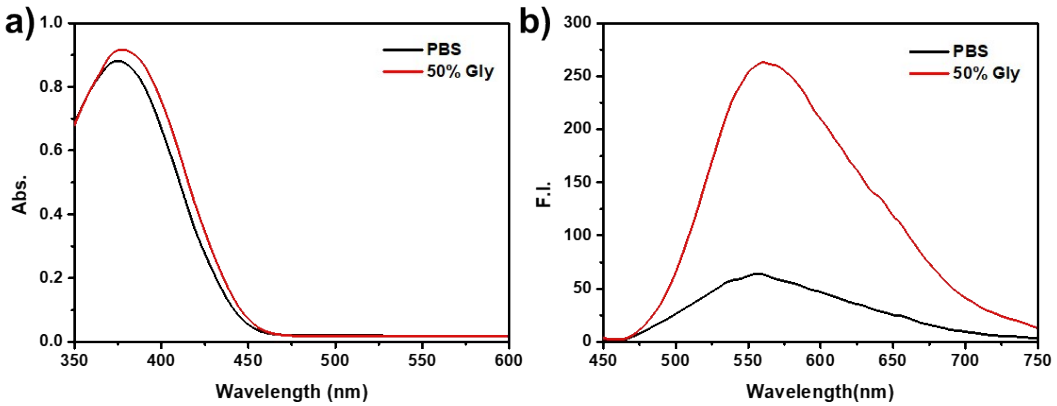


Fig. S5 (a)The absorption spectra of F5 in PBS solution and 50%Gly solution. (b) emission spectra of F5 in PBS solution and 50%Gly solution. (λ_{ex}=380nm).

Table S2. Photophysical data of F1-F5

	λ_{ex}	λ_{em}	extinction coefficient (ε, $M^{-1} cm^{-1}$)	Stoke shift(nm)	Quantum Yield (in 50% Gly)	Quantum Yield (in 95% Gly)	I (534 cP) /I_0(1.5cP)
F1	460	525	43200	65	0.0007	0.0016	6.4
F2	375	505	46900	130	0.0005	0.0012	2.3
F3	410	525	47800	115	0.0004	0.0007	1.8
F4	365	530	49600	155	0.0009	0.0018	2.9
F5	380	545	42600	155	0.0008	0.0015	3.8

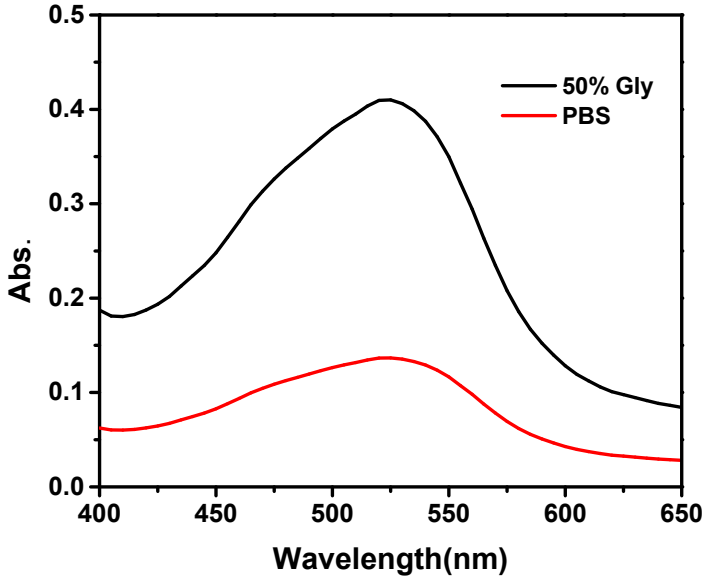


Fig. S6 Normalized absorption of the probe FD in PBS solution and 50% glycerol.

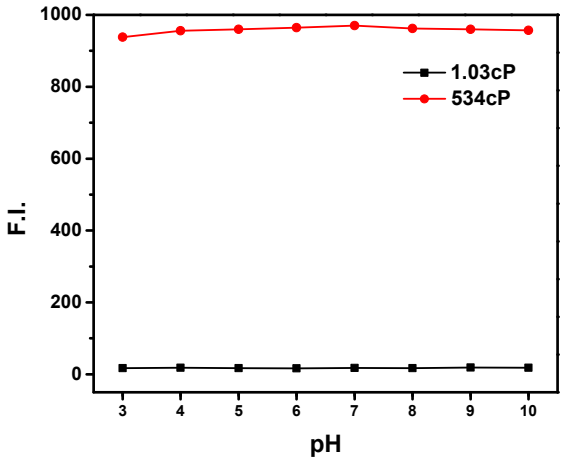


Fig. S7 The emission intensity changes (λ_{ex}= 520 nm) of probe FD at PBS solution 50% Gly solution in different pH value.

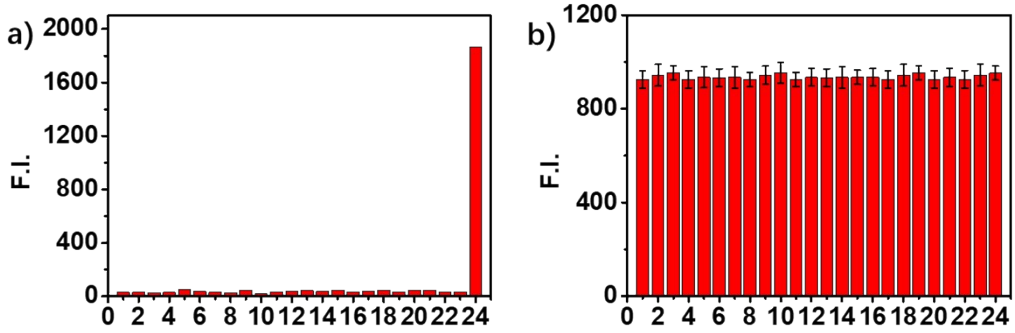


Fig. S8 (a) Fluorescence intensity at 560 nm of 10 μM **FD** toward various species (100 μM) in 10 mM PBS buffer and 90% Gly solution. (b) 50% Gly solution with the presence of various analytes, 50μM (1)Blank; (2)Mn^{2+}; (3)Ser; (4)Phe; (5)Arg; (6)Cys; (6)BSA; (7)H_2S; (8)GSH; (9)Hcy; (10)CO_3^{2-}; (11)OAc^-; (12)NO_3^-; (13)HSO_3^-; (14)PO_4^{3-}; (15)Cl^-; (16)Fe^{3+}; (17)K^+; (18)ClO^-; (19)H_2O_2; (20)$ONOO^-$; (21)Zn^{2+}; (22)Cu^{2+}; (23)$Na_2S_2O_4$; (24) Glycerol.

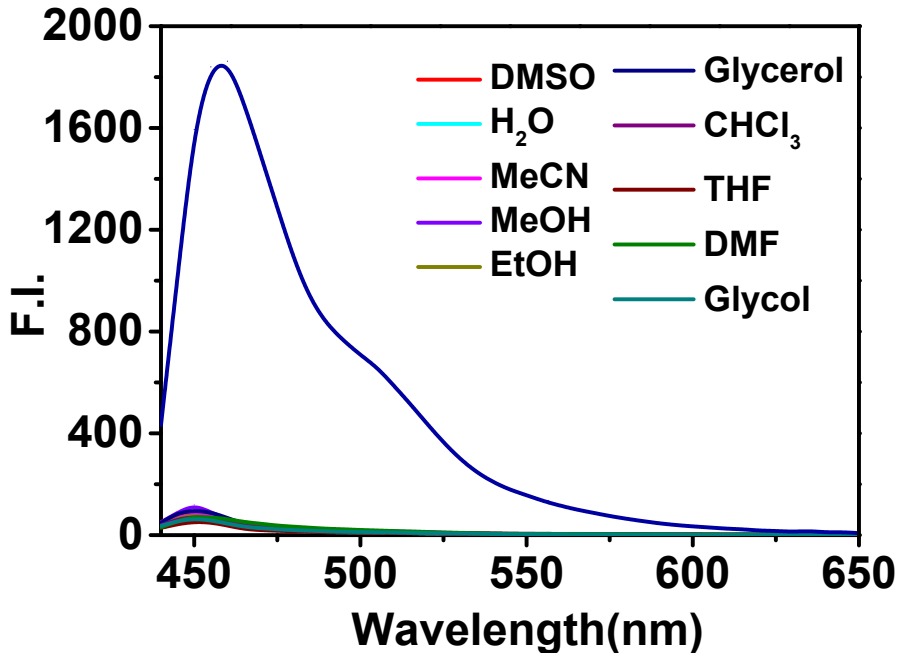


Fig. S9 **FD** in various solvents with different polarities.

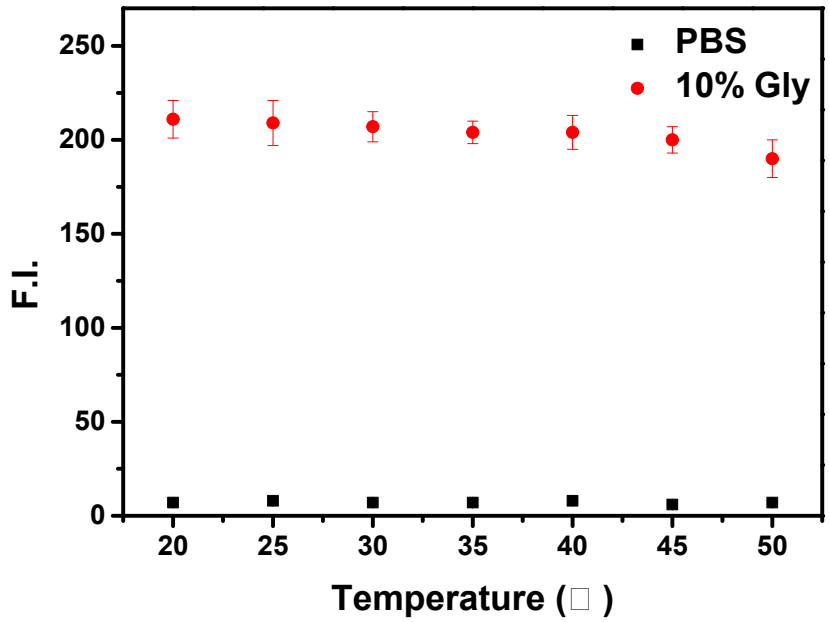


Fig. S10 The effects of temperature on probe **FD**.

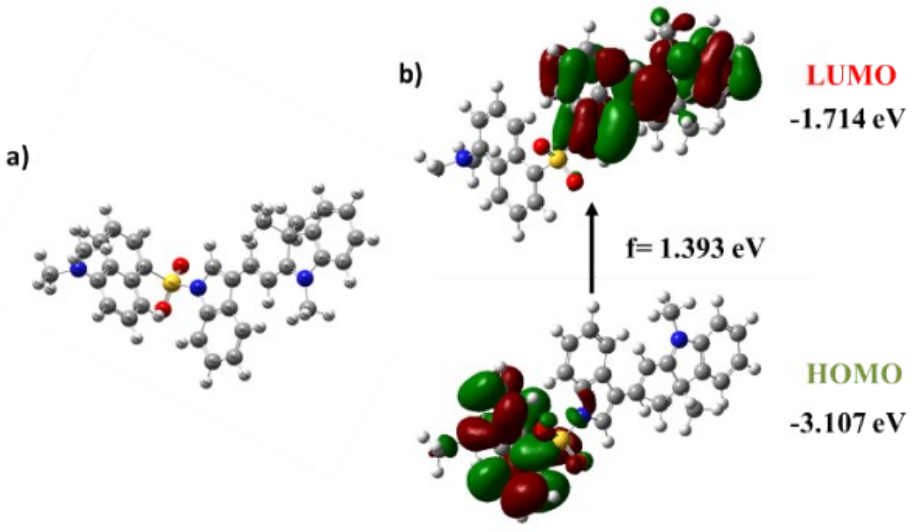


Fig. S11 (a) Optimized structure of the probe **FD**. (b) Frontier molecular orbitals of the probe **FD** (HOMO–LUMO energy levels were obtained by DFT method). The probe **FD** were calculated with time-dependent density functional theory (TD-DFT) at the B3LYP/6-31G-(d, p) level using Gaussian 09.

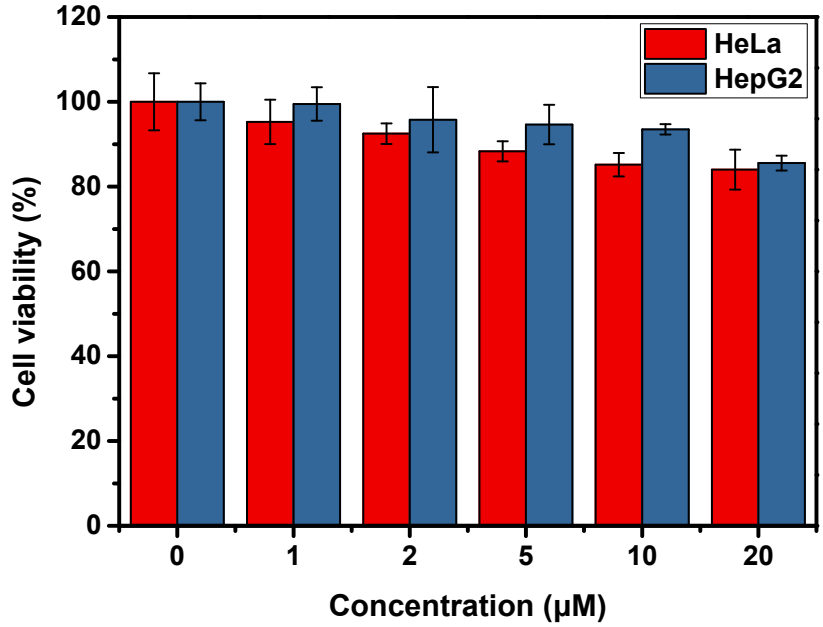


Fig. S12 Cytotoxicity assays of probe FD at different concentrations for HeLa cells

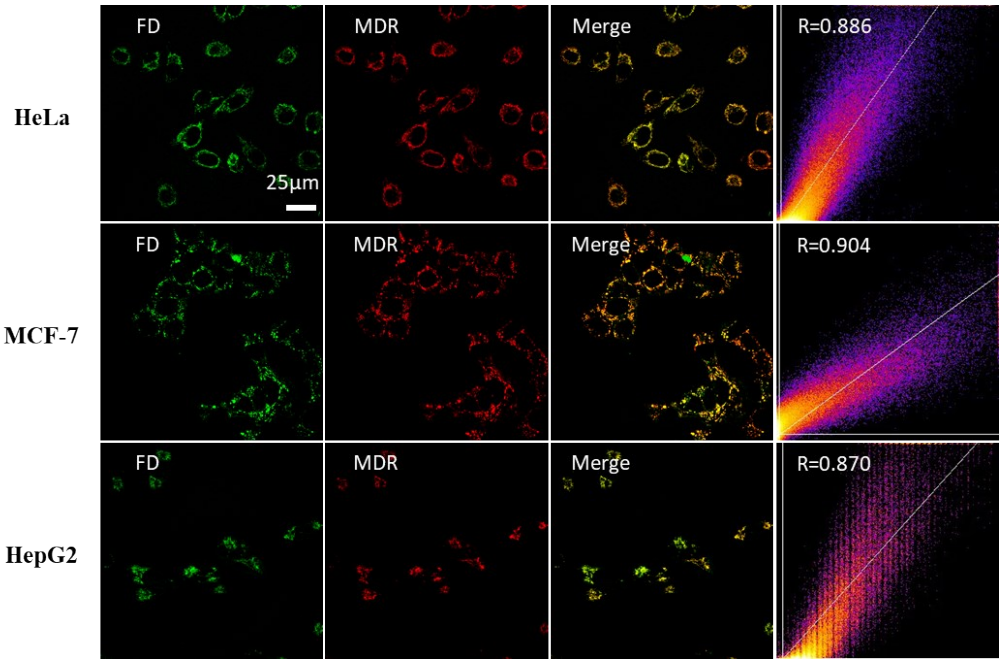


Fig. S13 Laser scanning confocal microscopy (LSCM) images of living HeLa, MCF-7 and HepG2 cells co-stained with **FD** (10μM, 1 h) and MitoTracker Deep Red (250 nM, 15 min). Green channel: **FD**, λ_{ex} = 520 nm; Red channel: MitoTracker Deep Red, λ_{ex} = 644 nm.

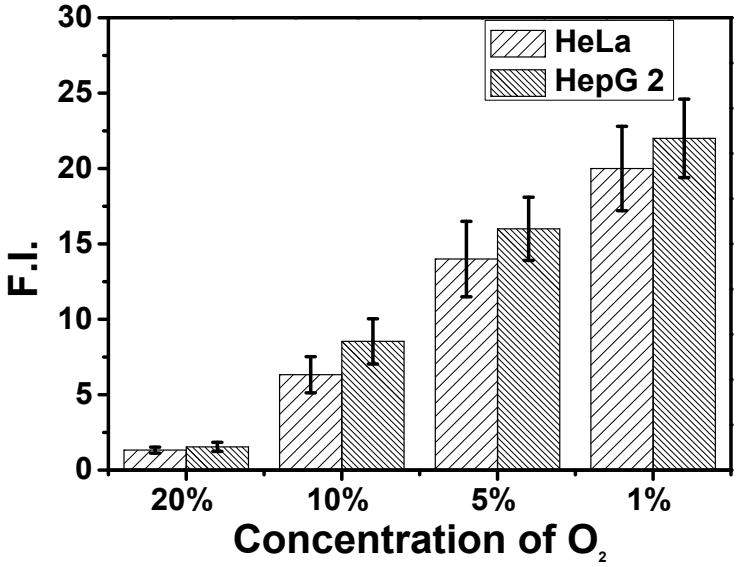


Fig. S14 Fluorescence intensity quantification. The images were collected at 550–600 nm, upon one-photon excitation at 520 nm.

Table S3. Baseline characteristics of subgroups of clinical samples selected according to the exclusion criteria of the present study. (Results were expressed as mean ± SD. P□ values by independent t-test.). Group A: liver cancer patients, Group B: liver fibrosis patients. Control Group: Healthy people.

Variables	Group A= 18	Group B= 15	Control Group= 22
Female sex, n (%)	10 (55)	8 (53)	11 (50)
Age(years)	55.7±13.8	57.2±13.3	54.9± 12.7
Height(cm)	168.8±7.2	166.1±7.2	165.7± 8.2
Body weight (kg)	70.3±10.4	67.3±12.4	62.3± 12.3

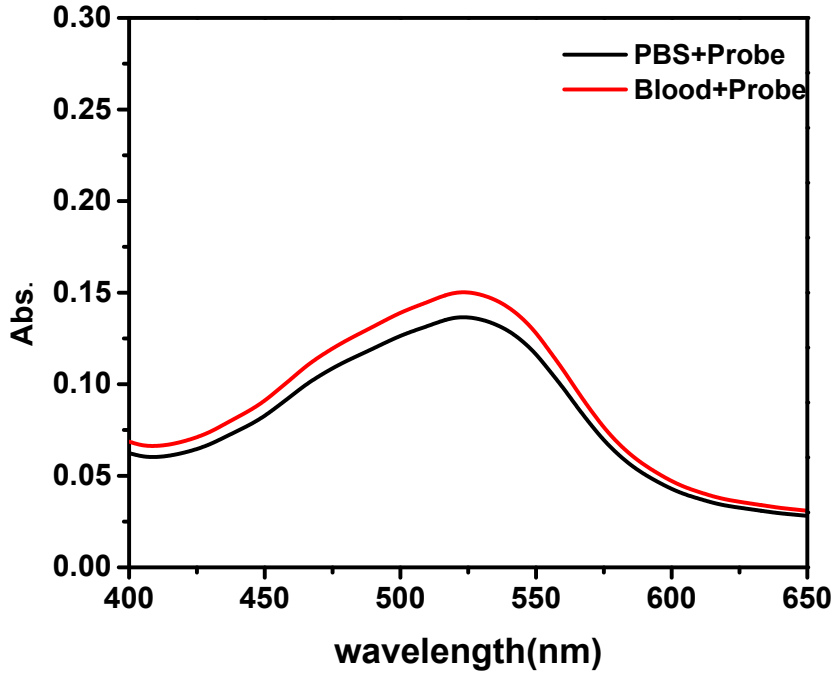


Fig. S15 Absorbance of the probe **FD** (10 μM) in PBS buffer and PBS with 20% fresh blood of healthy samples.

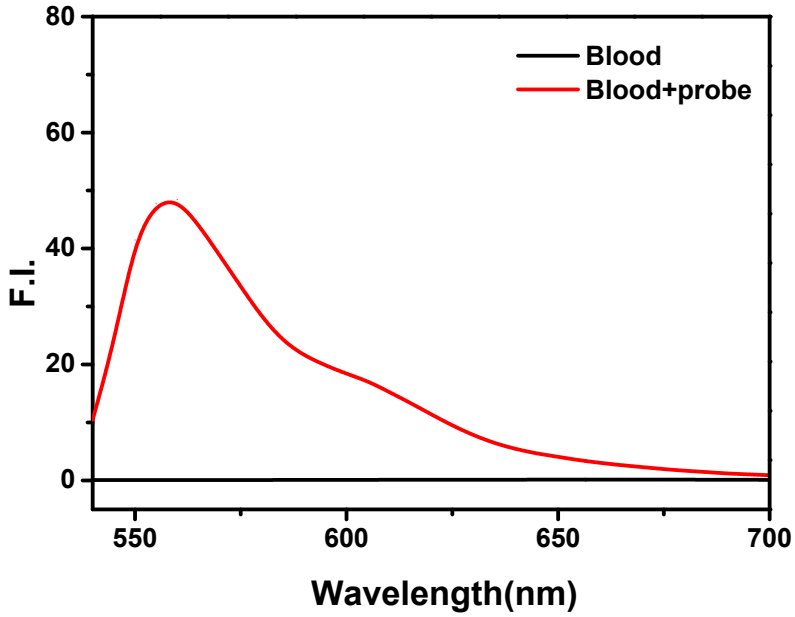


Fig. S16 The fluorescence spectral changes of 20% fresh blood of healthy samples with and without probe **FD** (10 μM).

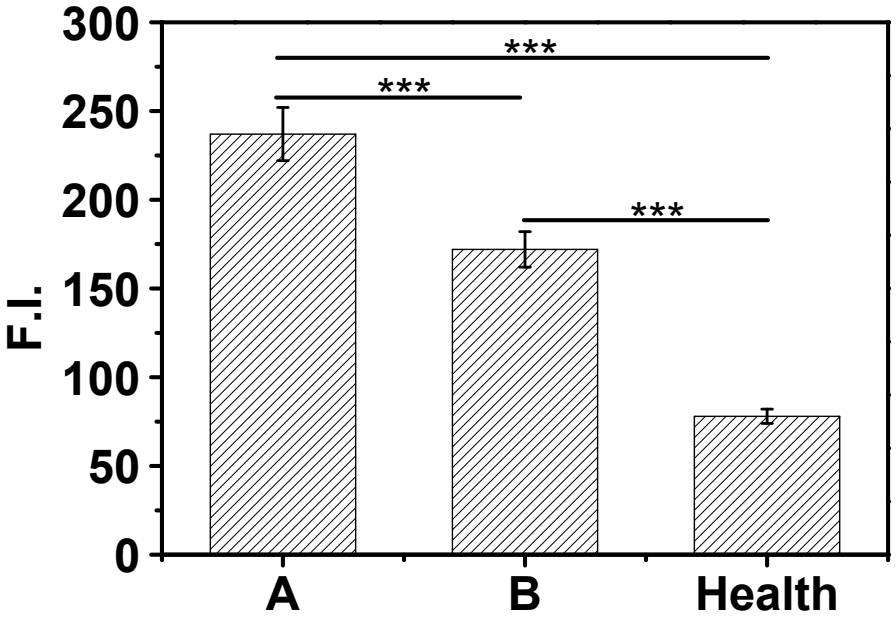


Fig. S17 The fluorescence intensity of **FD** (10 μM) in the 20% fresh blood of group A, B and Control group respectively. Group A represents: liver cancer patients, group B represents: liver cirrhosis patients, control group represents: (*** $P < 0.001$.).

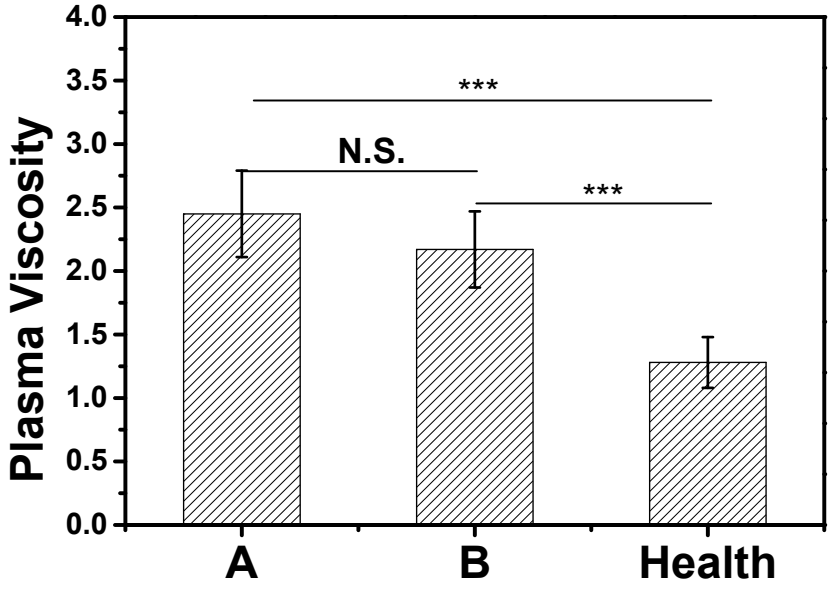


Fig. S18 Blood viscosity of group A, B and health group measured by FOR on a scanning capillary tube viscometer (He-movister, Ubiosis, Seongnam, Korea). Group A represents: liver cancer patients, group B represents: liver cirrhosis patients, ** $P<0.01$ vs. control. *** $P<0.001$ vs. control.

Table S4. The contrast of plasma viscosity, and fluorescence intensity in the 20% fresh blood of liver cancer patients before surgery and treated with Lenvatinib in 7, 14, 30, 90 days.

Group A	plasma viscosity (mpa·s)	Fluorescence Intensity
0	2.16± 0.15	259.7±35.9
7 days	2.05± 0.27	230.2±23.8
14 days	1.96± 0.36	172.3±26.7
30 days	1.89± 0.38	128.8±18.7
90 days	1.78± 0.42	80.81±17.6

3. Spectral characterization

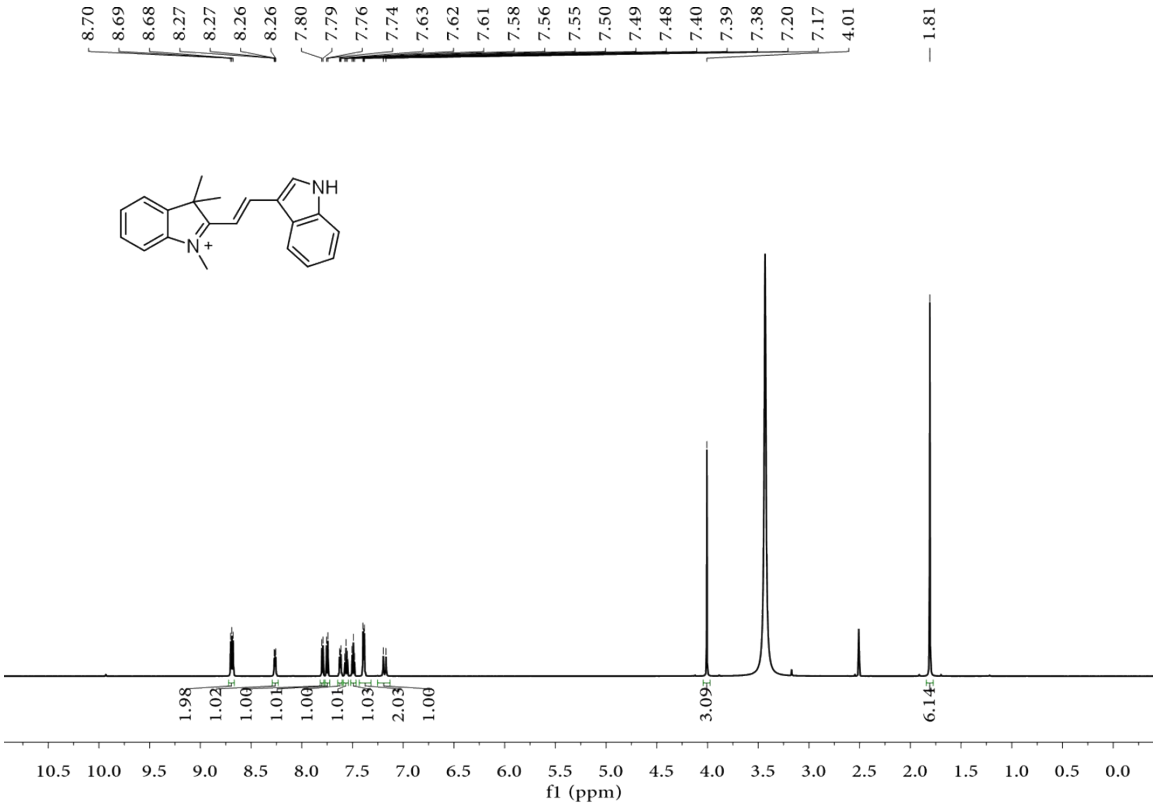


Fig. S19 ^{1}H NMR spectrum of **F1** in $CDCl_3$

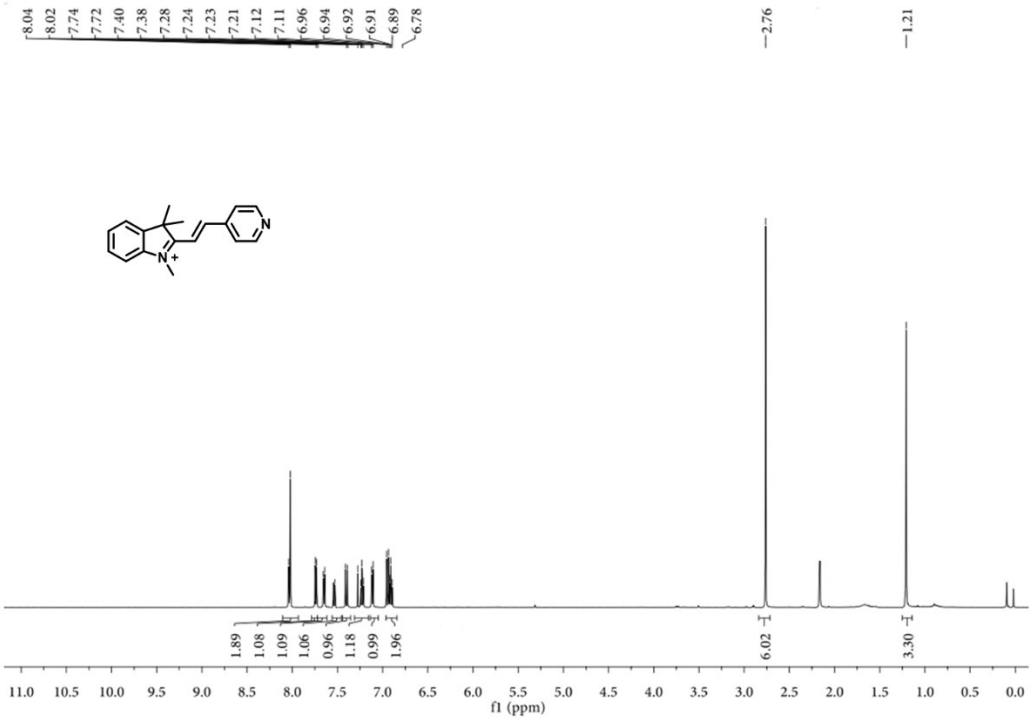


Fig. S20 ^{1}H NMR spectrum of **F2** in $CDCl_3$

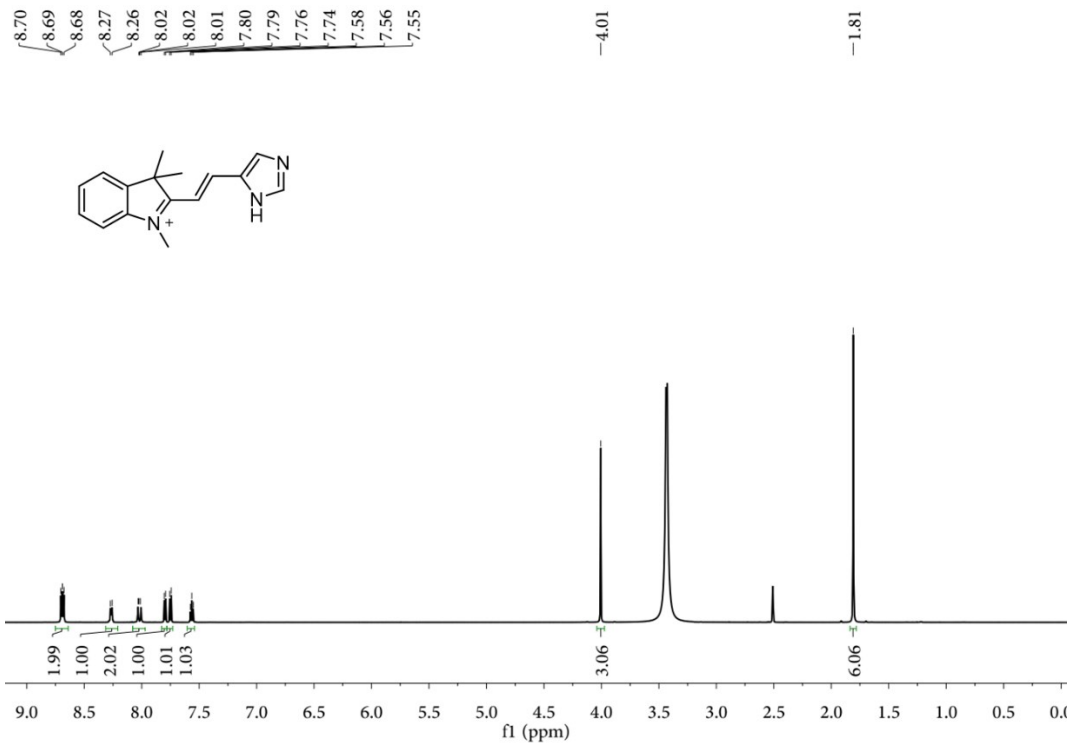


Fig. S21 ^{1}H NMR spectrum of **F3** in DMSO

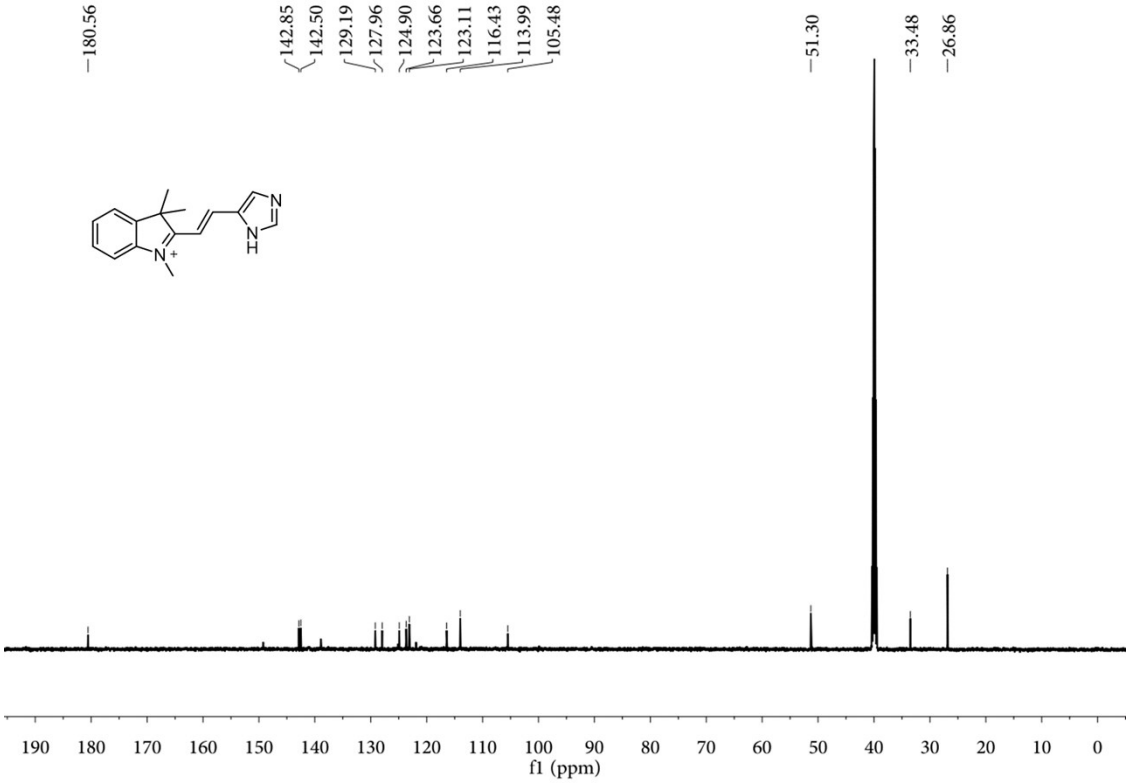


Fig. S22 ^{13}C NMR spectrum of **F3** in DMSO

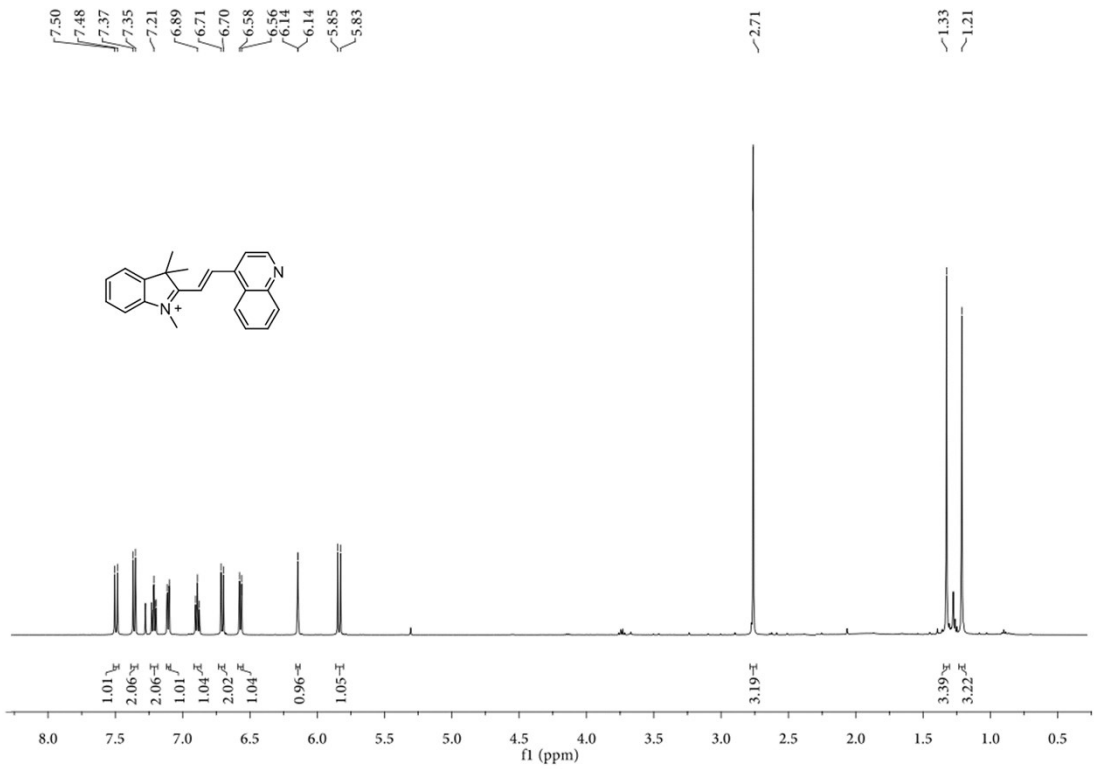


Fig. S23 ^{13}H NMR spectrum of **F4** in $CDCl_3$

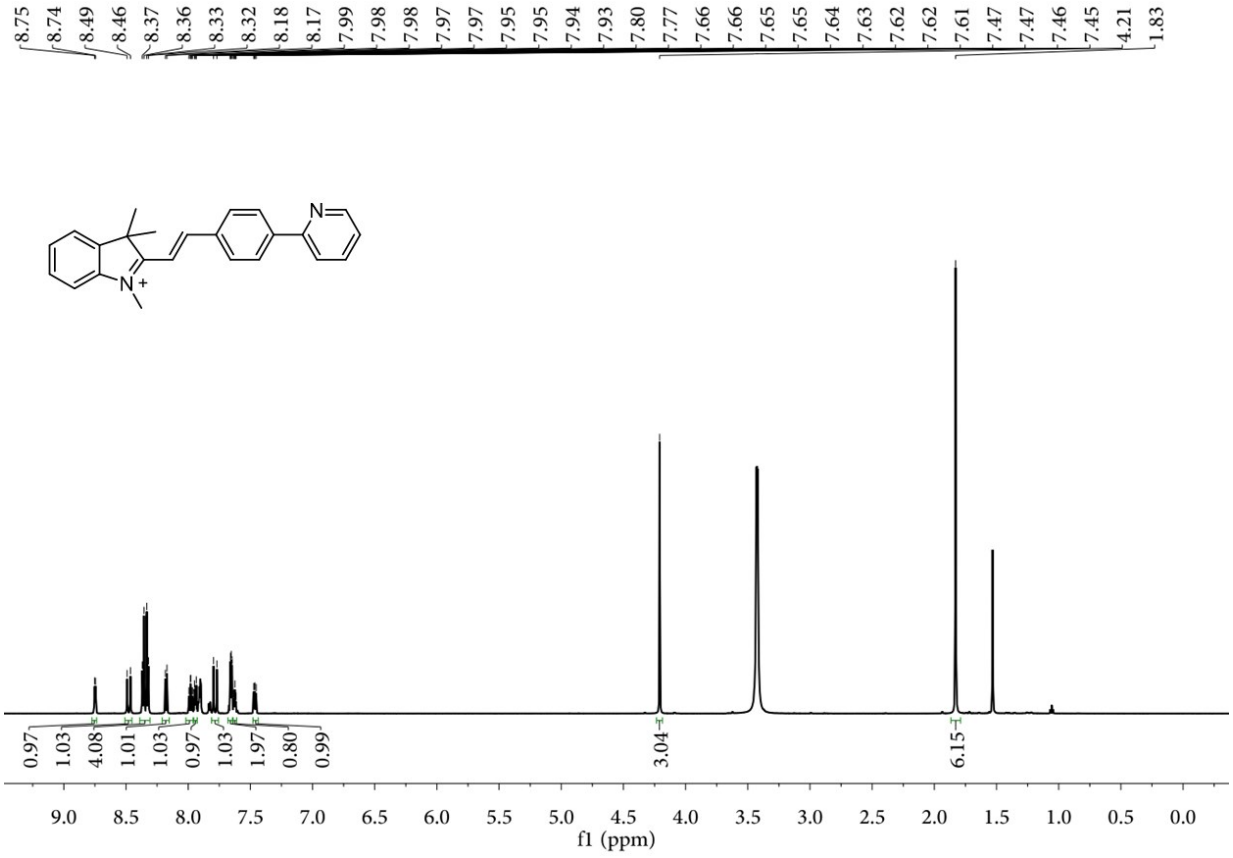


Fig. S24 ^{1}H NMR spectrum of **F5** in DMSO

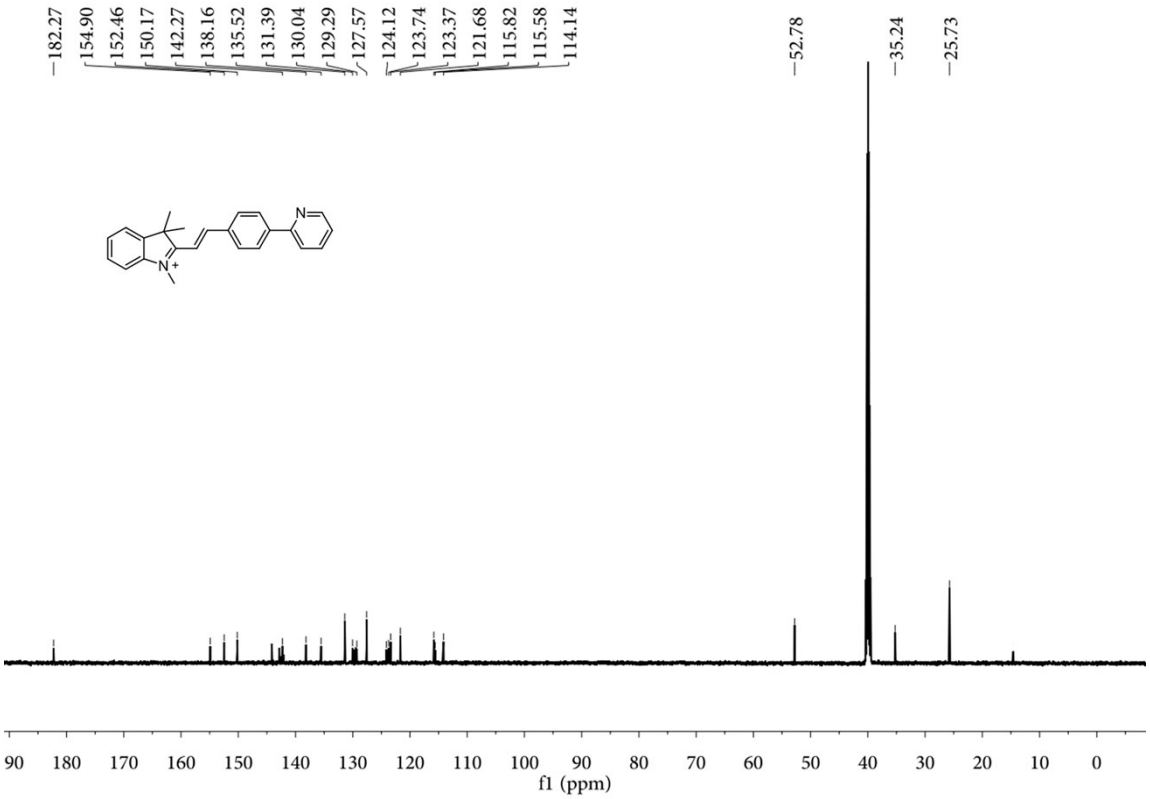


Fig. S25 ^{13}C NMR spectrum of **F5** in DMSO

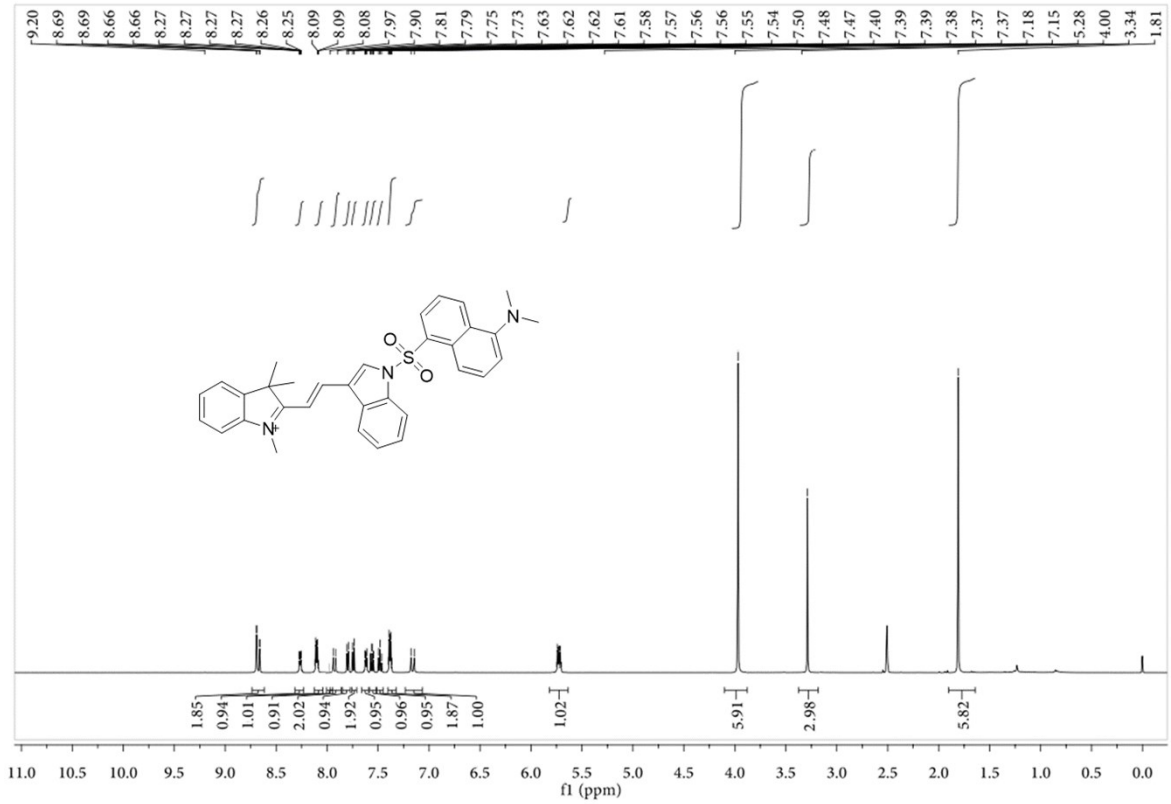


Fig. S26. ^{1}H NMR spectrum of **FD** in $CDCl_3$

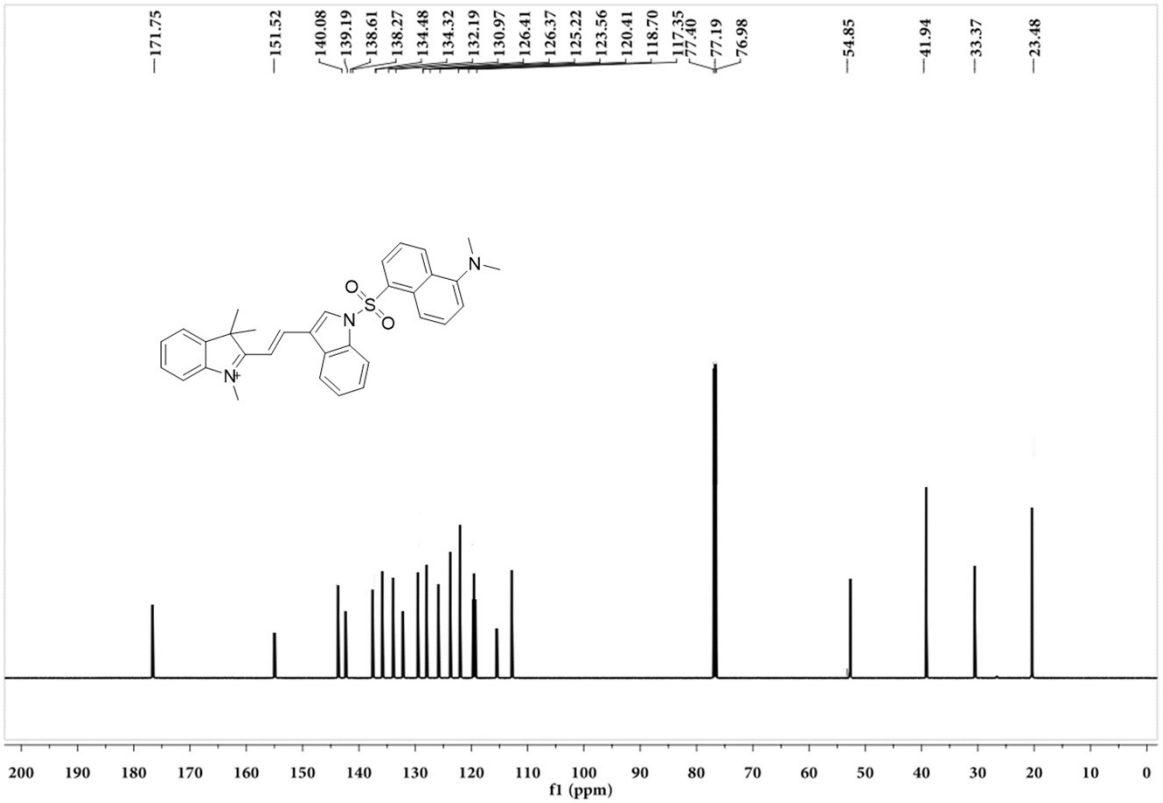
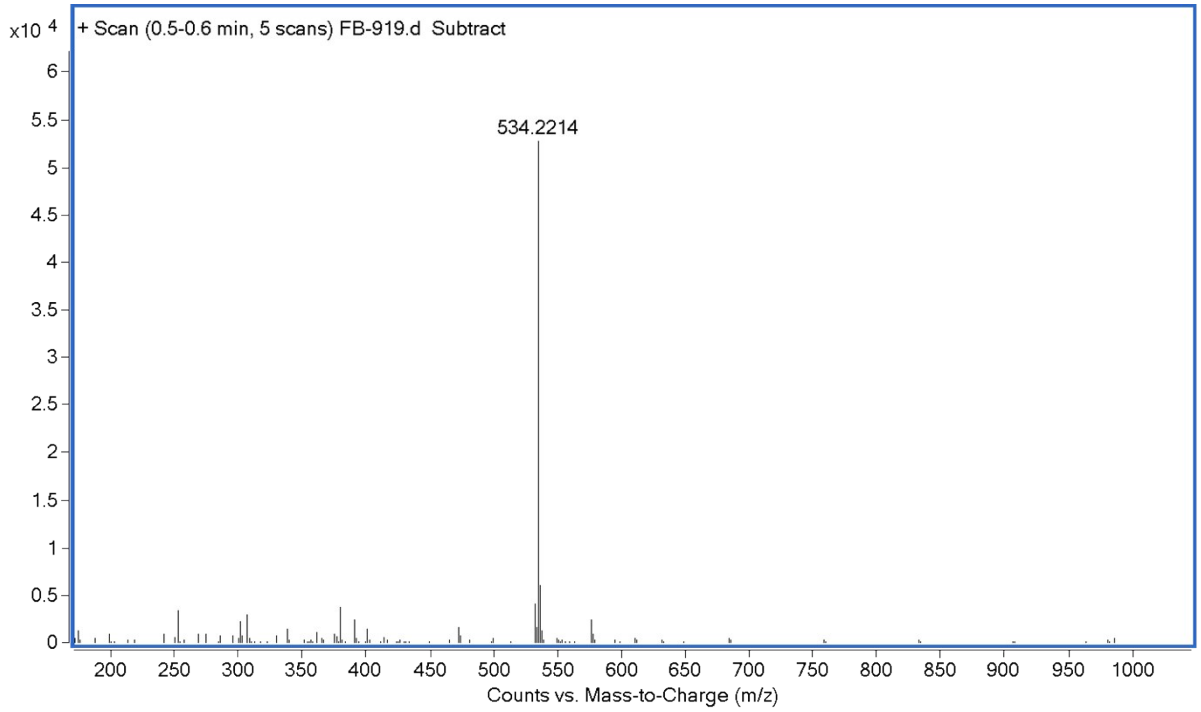


Fig. S27. ^{13}C NMR spectrum of **FD** in $CDCl_3$



Formula (M)	Ion Formula	m/z	Calc m/z	Diff (ppm)	DBE
C33 H32 N3 O2 S	C33 H32 N3 O2 S	534.2214	534.2210	-0.8	19.5

Fig. S28 HRMS spectrum of **FD**

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