Electronic Supplementary Information

A bodipy based fluorescent probe for evaluating and identifying cancer, normal and apoptotic C6 cells on the basis of changes in intracellular viscosity

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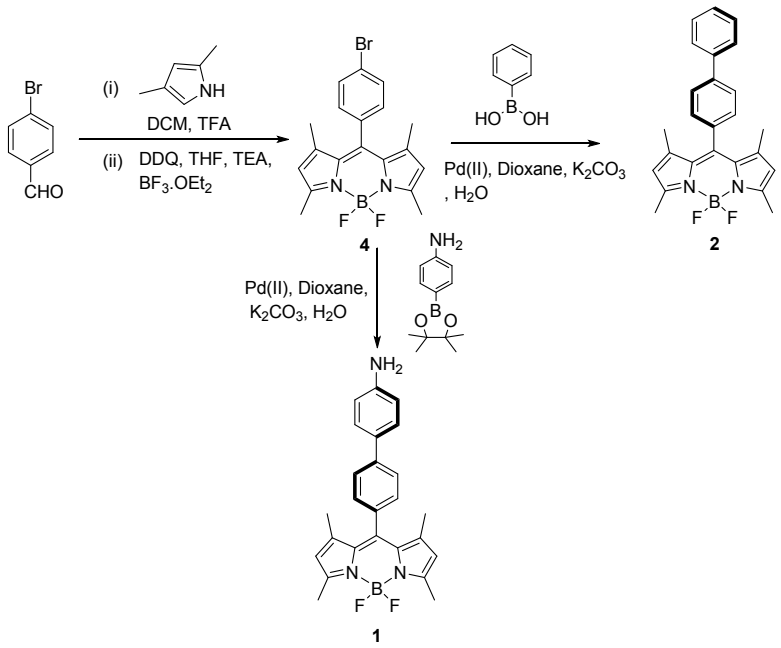
Synthetic routes and characteristic data



Scheme S1: Synthesis of probe **1** and compound **2**

Synthesis of probe 1:

To a solution of **4** (0.30 g, 0.744 mmol) and boronic ester of aniline (0.19 g, 0.893 mmol) in dioxane (20 ml) were added K_2CO_3 (0.41 g, 2.977 mmol), distilled water (1 mL), and $[Pd(Cl)_2(PPh_3)_2]$ (0.31 g, 0.44 mmol) under nitrogen. The reaction mixture was then refluxed overnight. The dioxane was then removed under vacuum, and the residue so obtained was extracted with dichloromethane, and dried over anhydrous Na_2SO_4. The organic layer was removed, and the compound was purified by column chromatography using hexane/chloroform (6:4) as an eluent to give 0.176 g (57 %) of probe **1** as orange coloured solid. ^{1}H NMR ($CDCl_3$, 400 MHz, ppm) 1.40 (s, 6 H), 2.56 (s, 6 H), 3.70 (s, 2H), 5.99 (s, 2 H), 6.80 (d, J = 8 Hz, 2 H), 7.29 (d, J = 8, 2 H), 7.51 (d, J = 8, 2 H), 7.68 (d, J = 8, 2 H). ^{13}C NMR ($CDCl_3$, 100 MHz, δ = ppm) = 29.32, 66.84, 115.16, 117.17, 121.14, 126.39, 127.99, 128.37, 131.29, 134.93, 135.24, 143.16, 145.46, 155.30; IR (KBr) ν_{max} = 3496 cm^{-1} (primary aromatic amine) and 3399 cm^{-1} (NH-stretch). TOF MS ES$^+$, m/z: 416.2136 (M+H$^+$). HRMS data for $C_{25}H_{24}BF_2N_3$: Found: 416.2105 (M+H$^+$); calculated: 416.2109 (M+H$^+$).

^{1}H NMR spectrum of compound 2

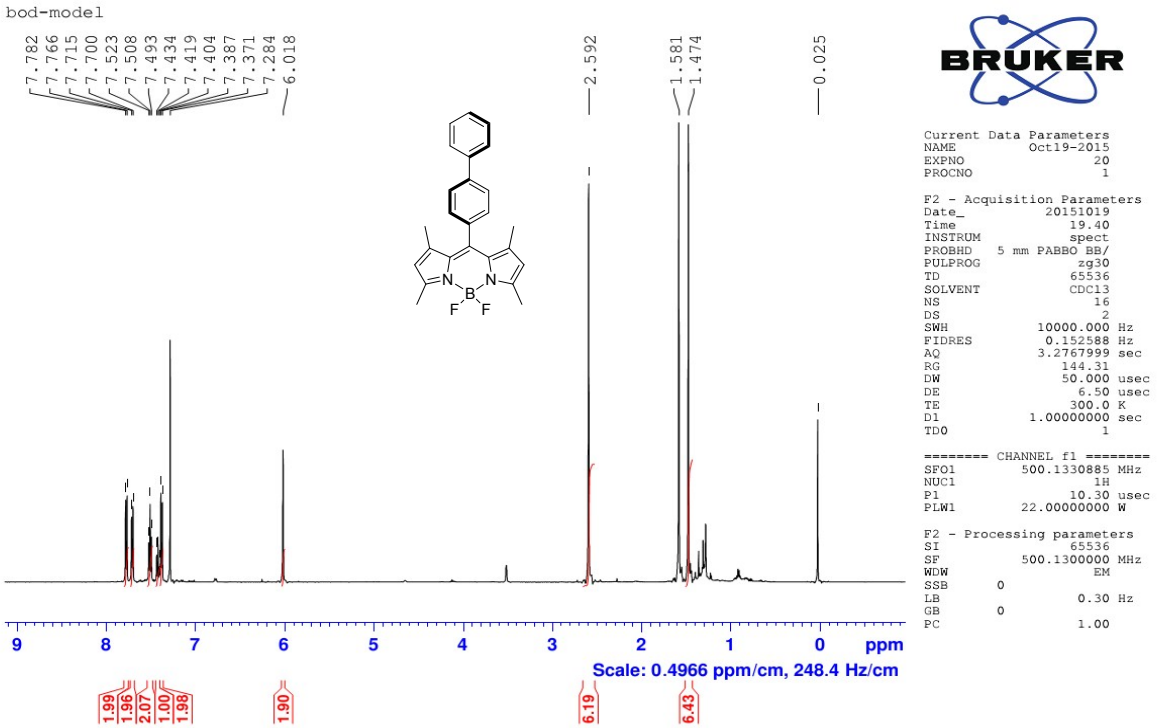
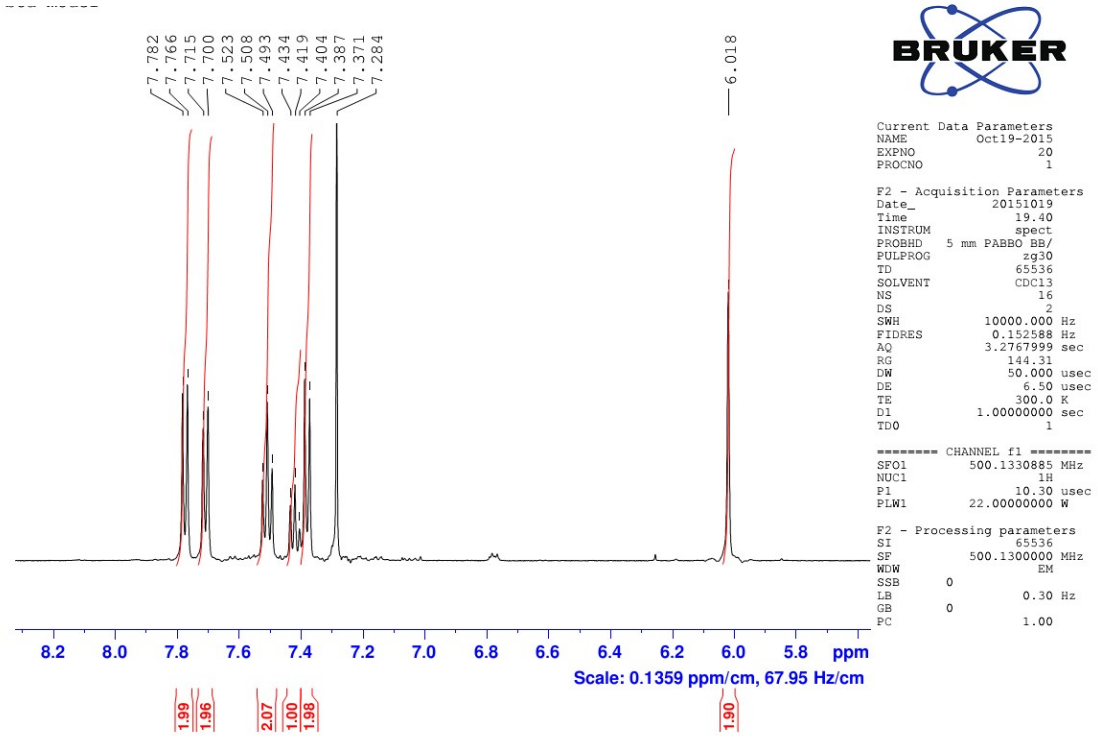


Fig. S1: ^{1}H NMR spectrum of compound **2**



^{13}C NMR and mass spectrum of compound 2

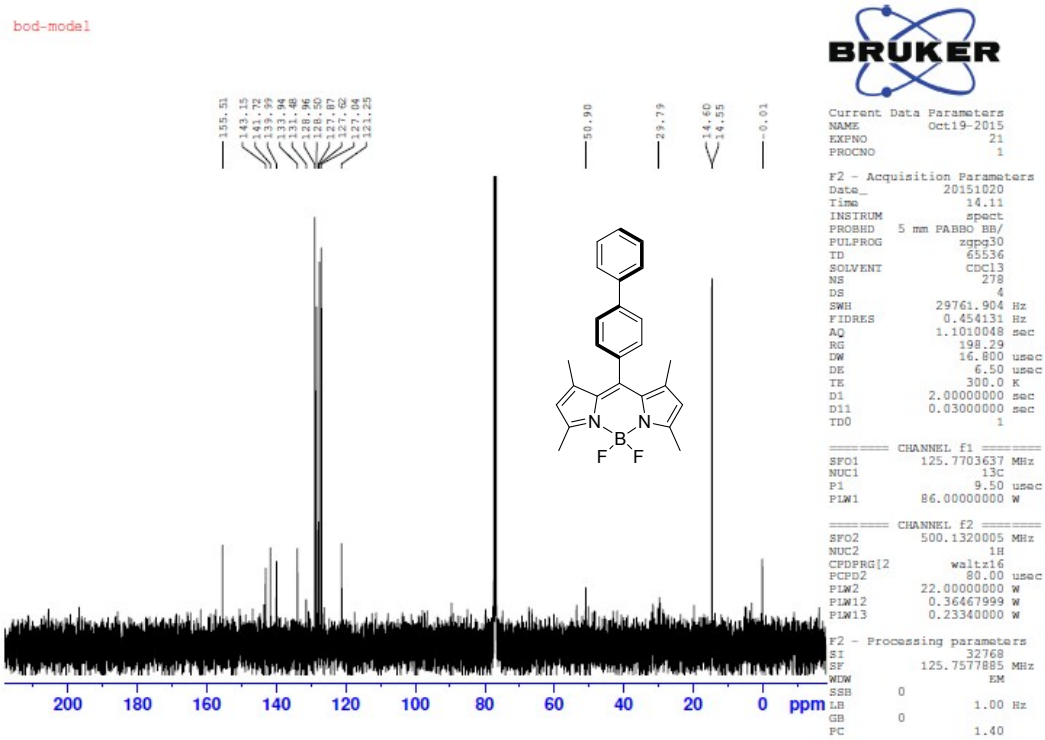


Fig. S2: ^{13}C NMR spectrum of compound **2**

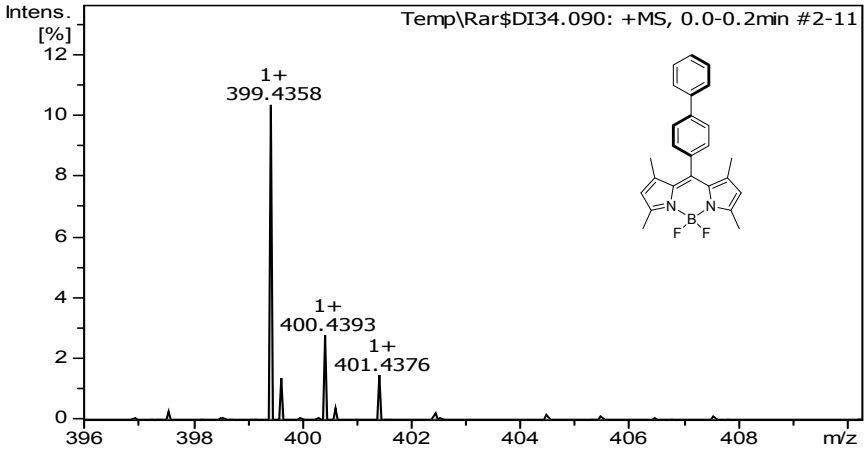


Fig. S3: Mass spectrum of compound **2**

Lifetime data of probe 1 in different methanol glycerol fractions

Methanol: Glycerol (v/v)	Probe 1 (lifetime in ns)
10:0	**2.35**
9:1	**2.65**
8:2	**2.74**
7:3	**2.81**
6:4	**3.02**
5:5	**3.12**
4:6	**3.31**
3:7	**3.54**
2:8	**3.82**
1:9	**3.96**
0:10	**4.35**

Table S1: Lifetime of probe **1** (5.0 μM) in different methanol: glycerol fractions at 517 nm using λ_{ex} = 488 nm

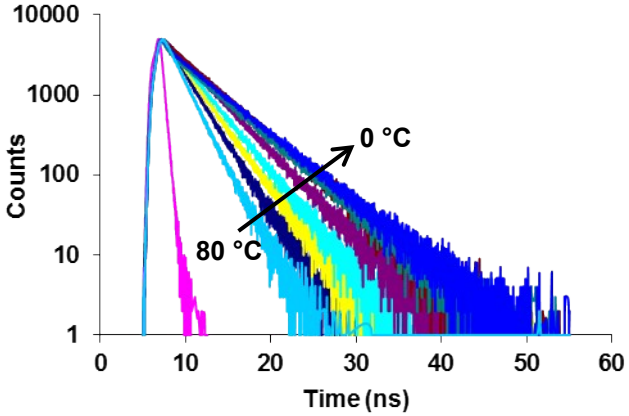


Fig. S4: Fluorescence lifetime spectra of probe **1** (5.0 μM) at 517 nm in methanol-glycerol (1:1, v/v) system with varying temperature 0-80 °C; λ_{ex} = 488 nm.

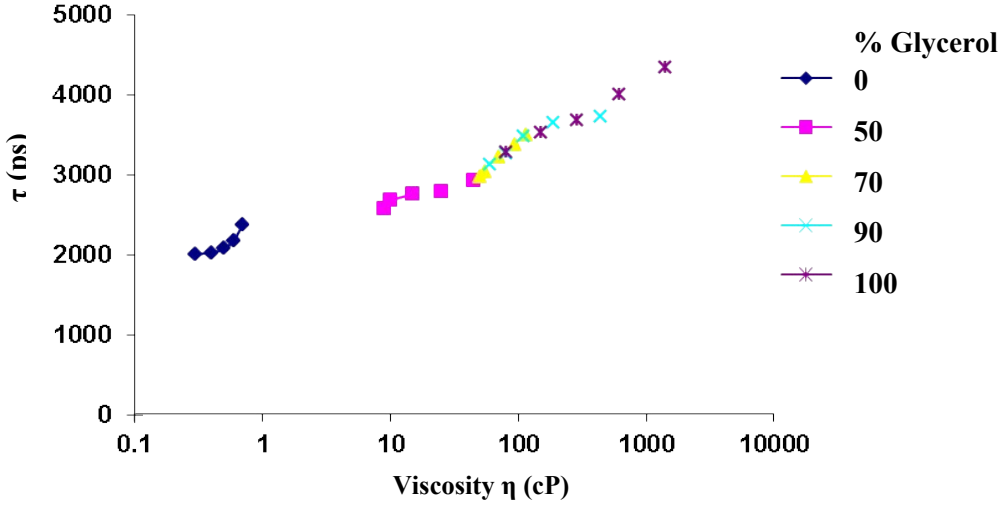


Fig. S5: Fluorescence lifetime of probe **1** recorded in methanol–glycerol mixtures of various compositions, plotted against viscosity of the mixture. The data represented at temperatures between 293 and 333 K.

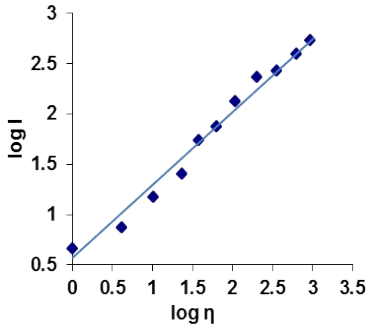


Fig. S6: Linear relationship of log I_{517} and log η, R^2 =0.9914, X=0.52

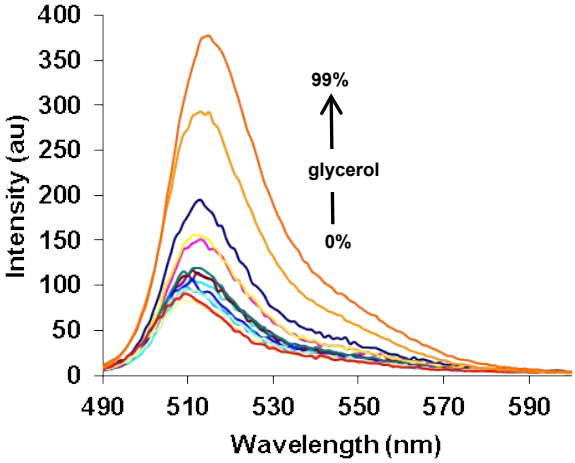


Fig. S7: Fluorescence spectra of compound **2** (5.0 μM) at nm in varying methanol-glycerol (0-99%) fractions at λ_{em}= 510 and λ_{ex} = 470 nm.

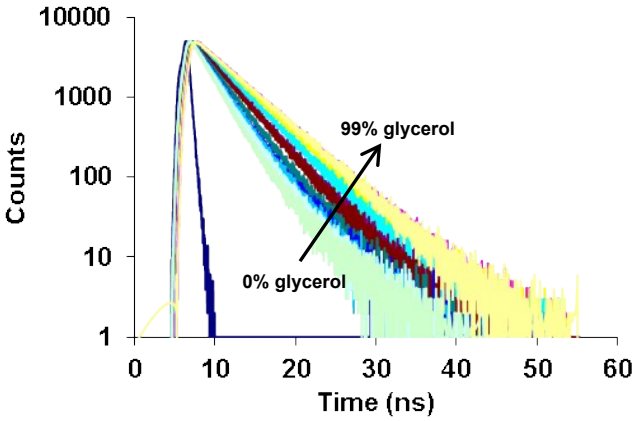
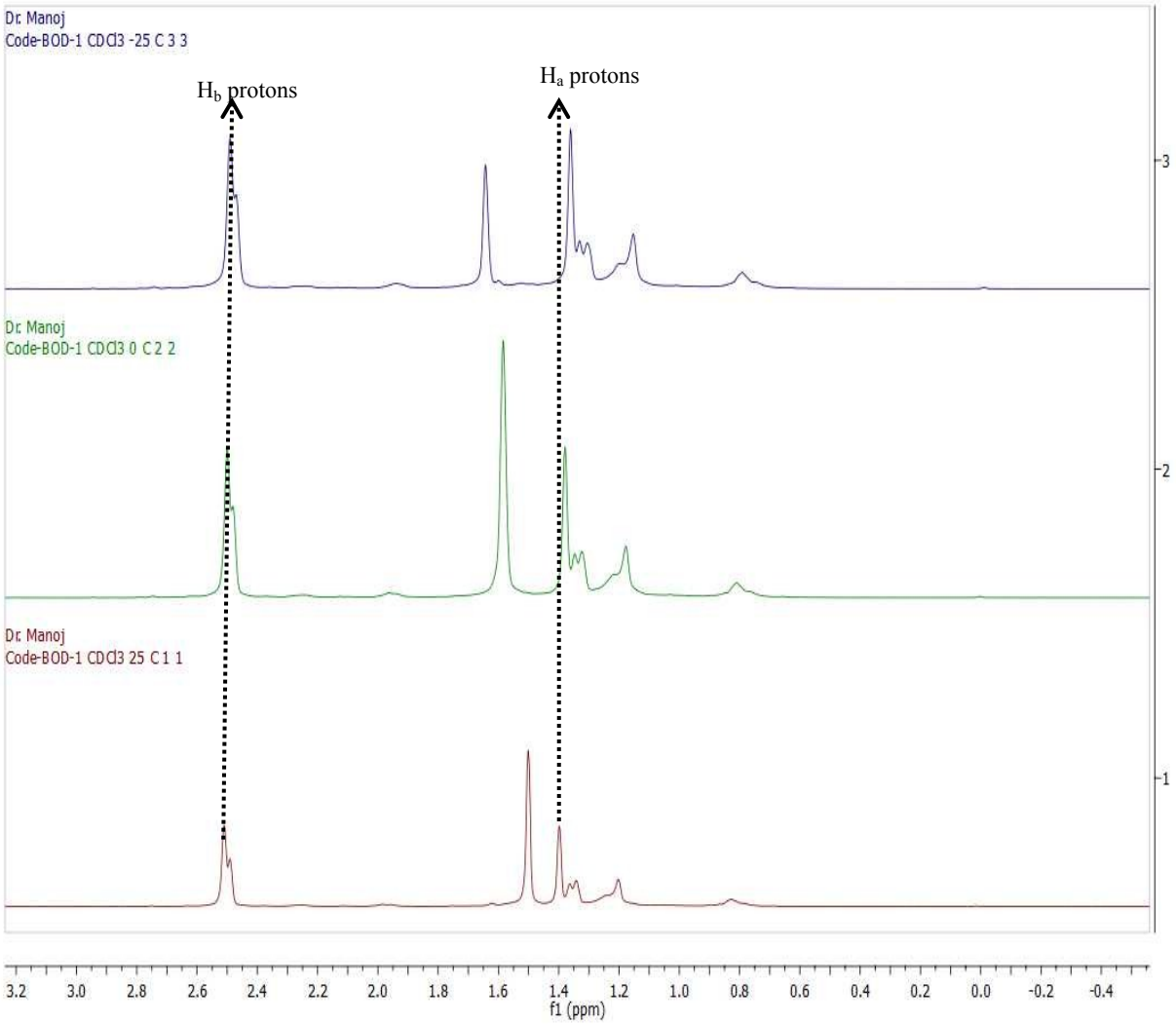


Fig. S8: Fluorescence lifetime spectra of compound **2** (5.0 μM) at 510 nm in varying methanol-glycerol (0-99%) fractions λ_{ex} = 488 nm.

Variable temperature -^{1}H NMR spectra of probe 1



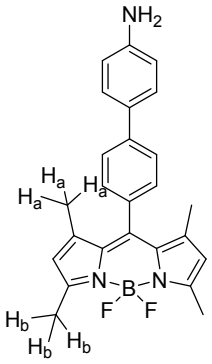


Fig. S9: VT-^{1}H NMR spectra of probe **1**

VT-^{1}H NMR spectra of probe 1 (superimposed spectra)

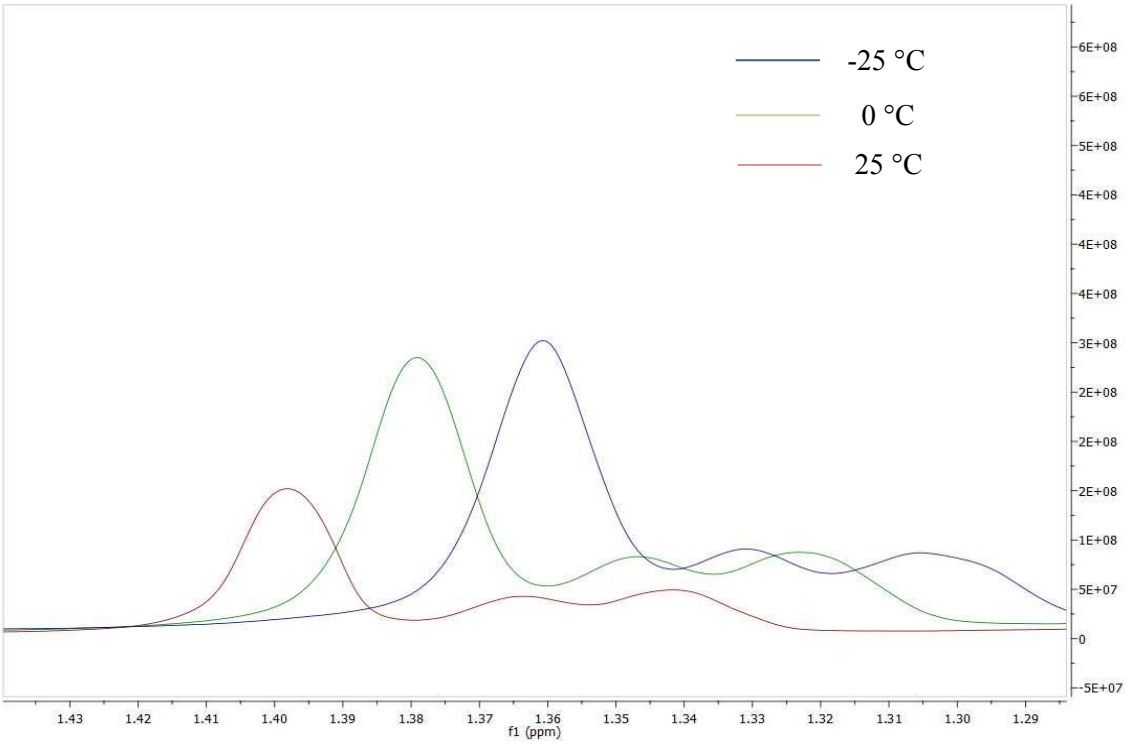
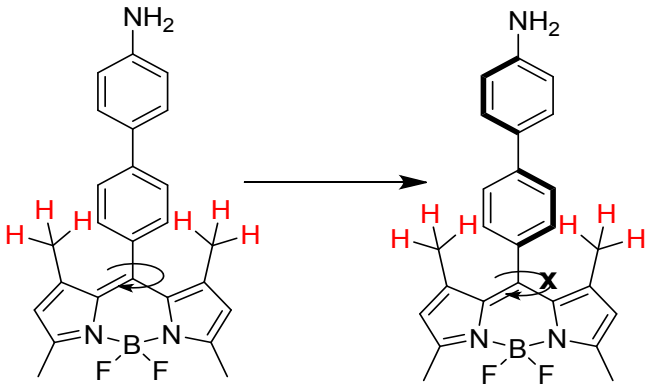


Fig. S10: VT-^{1}H NMR spectra of probe **1** (superimposed spectra)



VT-^{1}H NMR of probe **1** in $CDCl_3$, the methyl hydrogen of 1 and 7 position (red colour) of bodipy have upfield shift about 0.04 ppm on changing temperature from 25 °C to -25 °C.

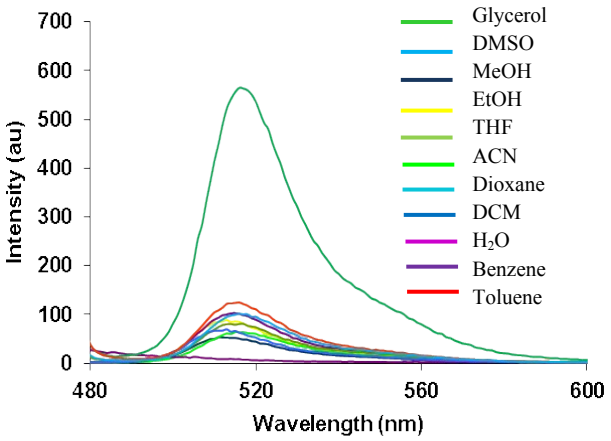


Fig. S11: Fluorescence spectra of probe **1** (5.0 μM) in different solvent system at 25 °C; λ_{ex} = 470 nm and λ_{em}= 517.

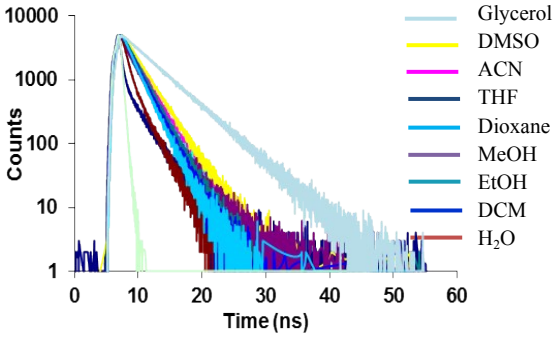


Fig. S12: Fluorescence lifetime spectra of probe **1** (5.0 μM) at 517 nm in different solvent system in 25 °C; λ_{ex} = 488 nm.

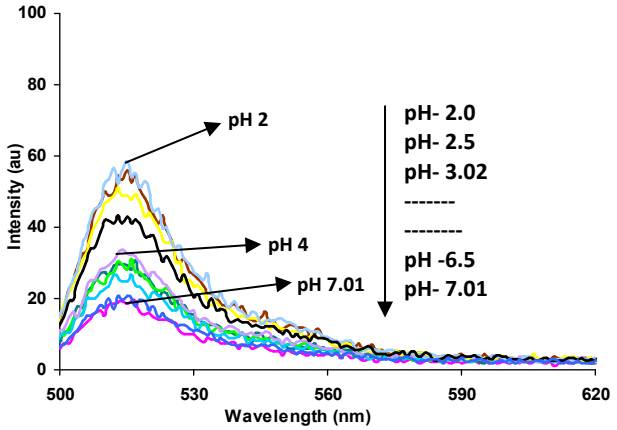


Fig. S13: Change in fluorescence spectra of probe **1** (5 μM) at different pH values (λ_{ex} = 470 nm and λ_{em}= 517).

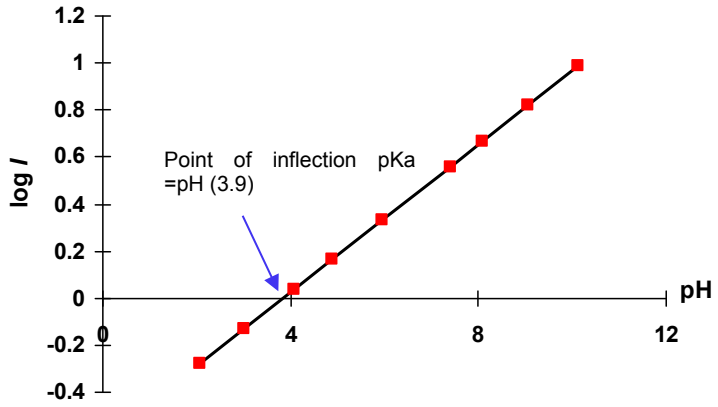


Fig. S14: The log I−pH* plot for the probe **1**

***Calculations:** Using equation: pKa = m.$H_{1/2}$ + log I, where I= (ε_{obs}-ε_{fb})/(ε_{ca}-ε_{obs}) (at half protonation point log I will be equal to zero) from graph, pKa = $H_{1/2}$ = 3.9

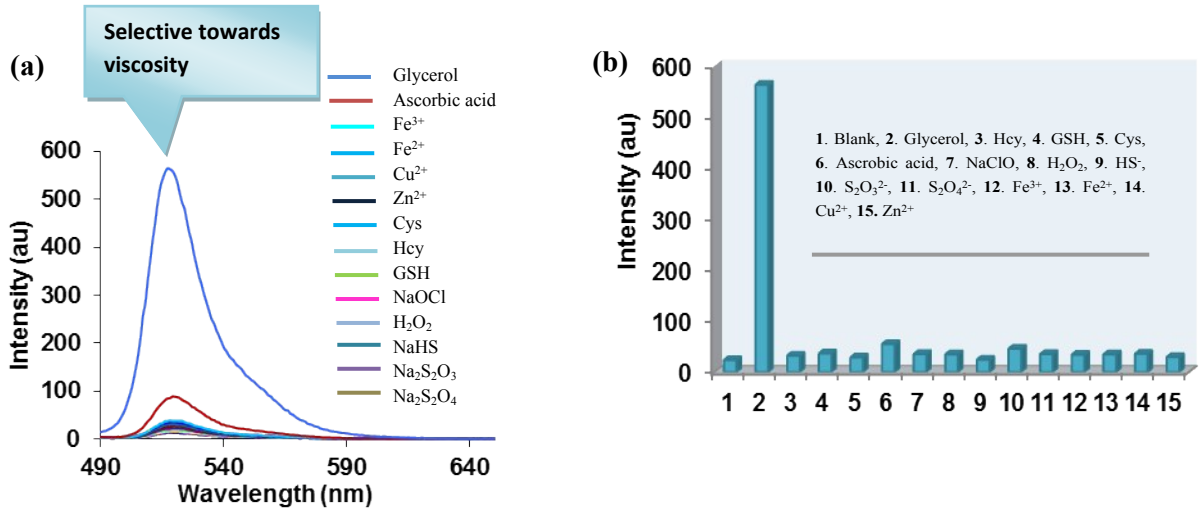


Fig. S15: (a) Fluorescence spectra and (b) Bar diagram of probe **1** (5.0 μM) in methanol with addition of different analytes (Cys, Hcy, GSH, NaClO, H_2O_2, NaHS, ascorbic acid, $Na_2S_2O_3$, $Na_2S_2O_4$, Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}) (100 μM) at 25 °C; λ_{ex} = 470 nm.

Representation of optimized geometry of probe 1 in gas phase

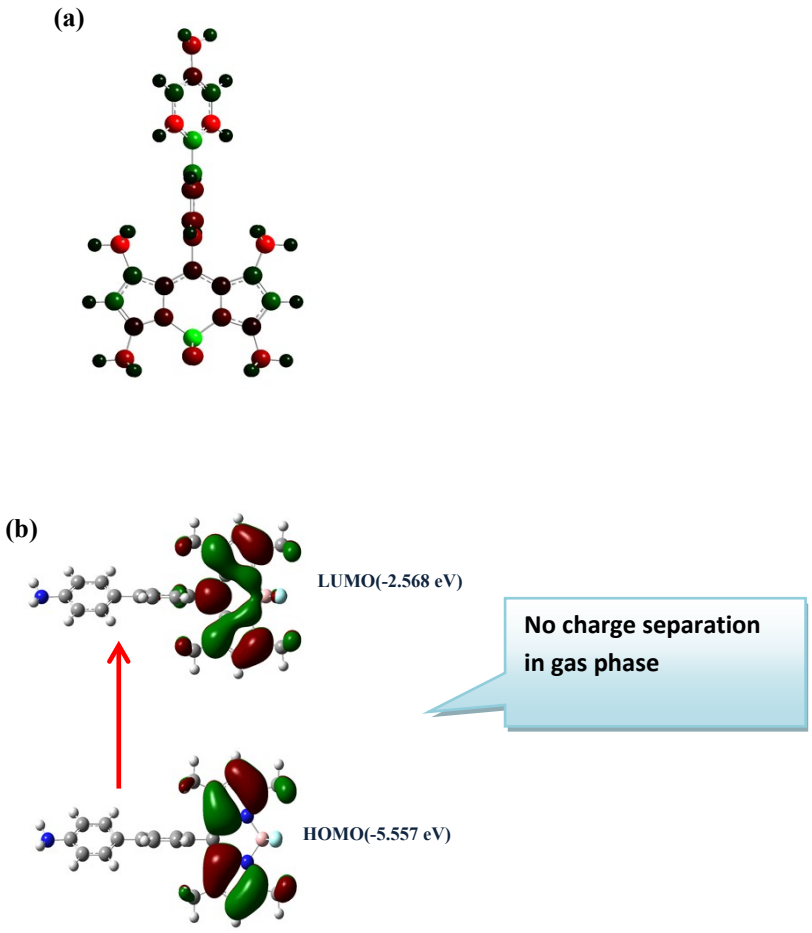


Fig. S16: (a) The optimized charge density geometry of probe **1**. (b) Frontier molecular orbital (MO) of probe **1** showing no charge separation in gas phase.

DFT studies of probe 1 in different solvents (polar and non-polar)

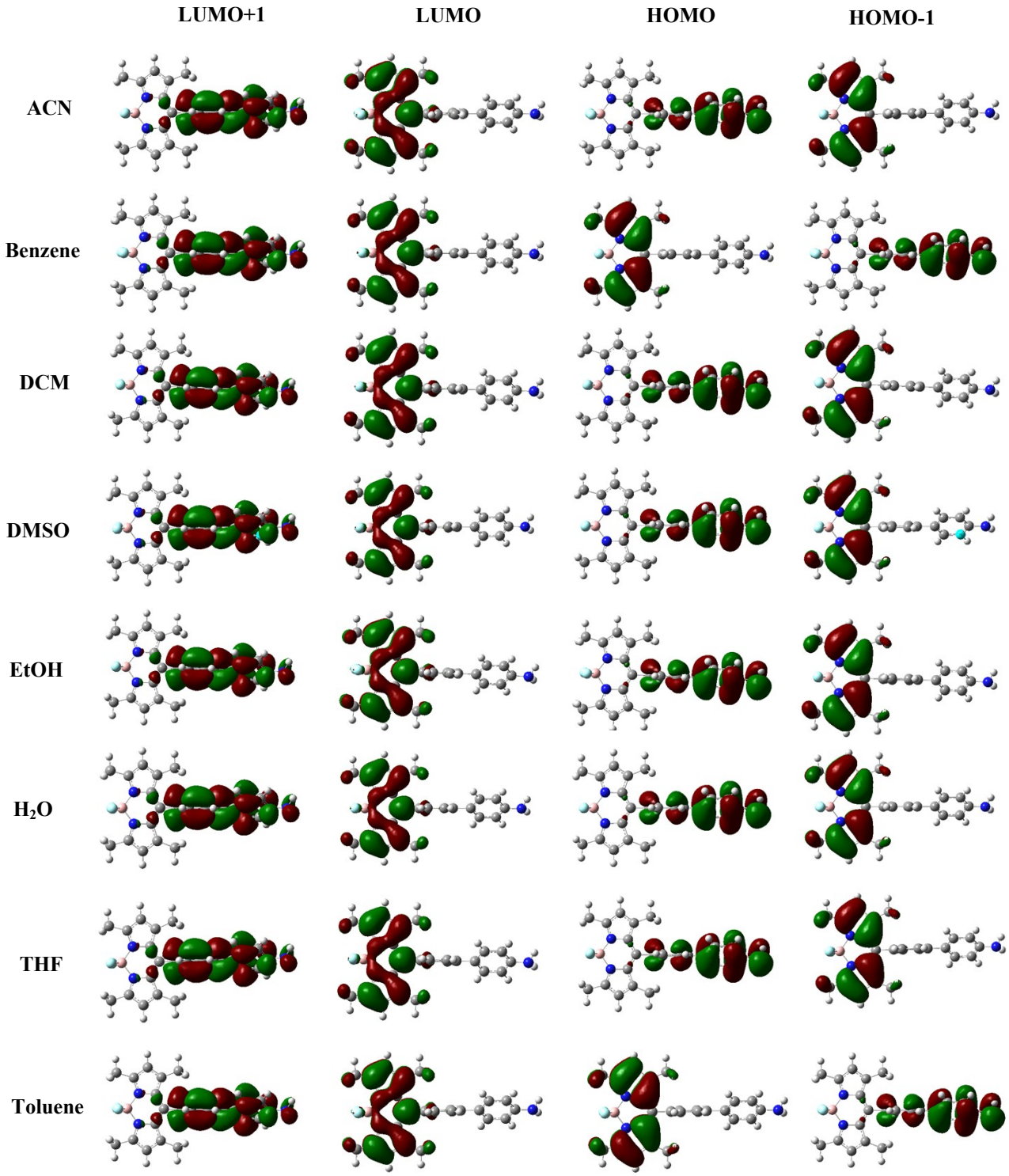


Fig. S17: Frontier molecular orbital (MO) of probe **1** by considering the effect of different solvents (polar and non-polar).

Energy profile of probe 1 molecular orbitals in different solvents and at different dihedral angle

Solvent	LUMO+1 (eV)	LUMO (eV)	HOMO (eV)	HOMO-1 (eV)	$\Delta_{HOMO-LUMO}$ (eV)
ACN	-0.772531	-2.506441	-5.392754	-5.531804	-2.886312
Benzene	-0.760286	-2.371201	-5.383774	-5.410441	-3.012573
DCM	-0.765728	-2.472155	-5.392210	-5.494525	-2.920054
DMSO	-0.773347	-2.509434	-5.392754	-5.534797	-2.883319
Ethanol	-0.771443	-2.501271	-5.392482	-5.526090	-2.891210
Methanol	-0.772259	-2.505353	-5.392754	-5.530444	-2.887401
H_2O	-0.774164	-2.513244	-5.393026	-5.538879	-2.879782
THF	-0.764640	-2.463719	-5.392754	-5.485273	-2.92903
Toluene	-0.760014	-2.376099	-5.389217	-5.408809	-3.013117

Fig. S18: Energy profile of probe **1** molecular orbitals in different solvents

Dihedral angle	LUMO+1 (eV)	LUMO (eV)	HOMO (eV)	HOMO-1 (eV)	$\Delta_{HOMO-LUMO}$ (eV)
90°	-0.89634	-2.45038	-5.14513	-5.37261	-2. 69475
120°	-0.83511	-2.48412	-5.17397	-5.36200	-2. 68985
150°	-0. 69443	-2.5586	-5.2493	-5.3456	-2. 6907
180°	-0. 45476	-2.96604	-5.2586	-5.32690	-2. 29256

Fig. S19: Energy profile of probe **1** molecular orbitals at different dihedral angle.

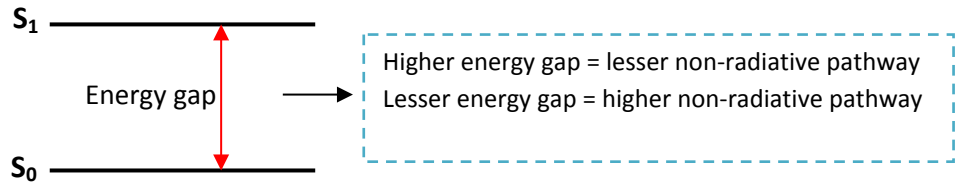


Fig. S20: Schematic representation of dependence of fluorescence emission on S_0 -S_1 energy

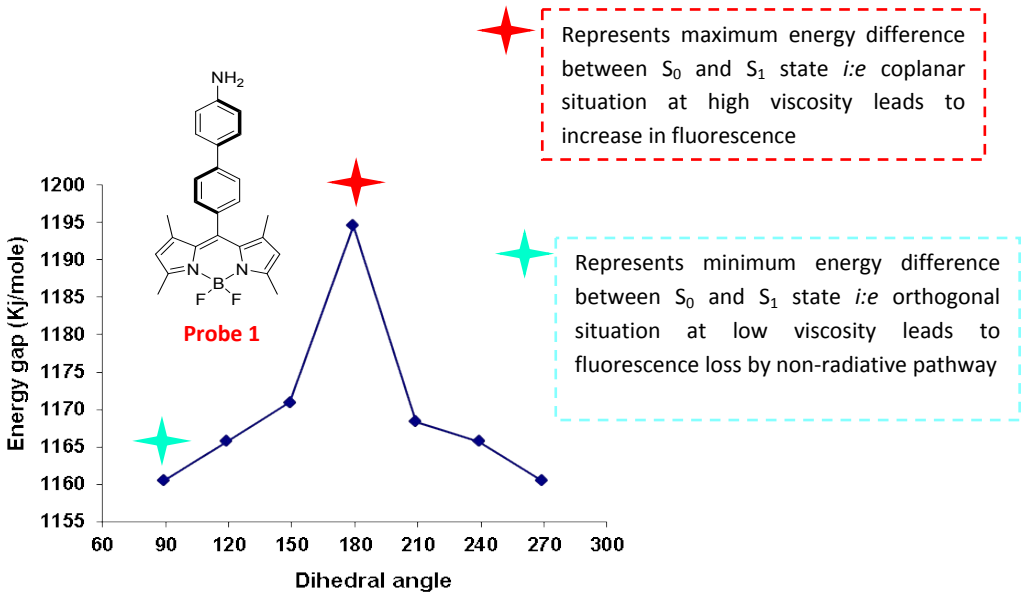


Fig. S21: Plot depicting the variation of S_0 -S_1 energy gap at different dihedral angles in case of probe **1**

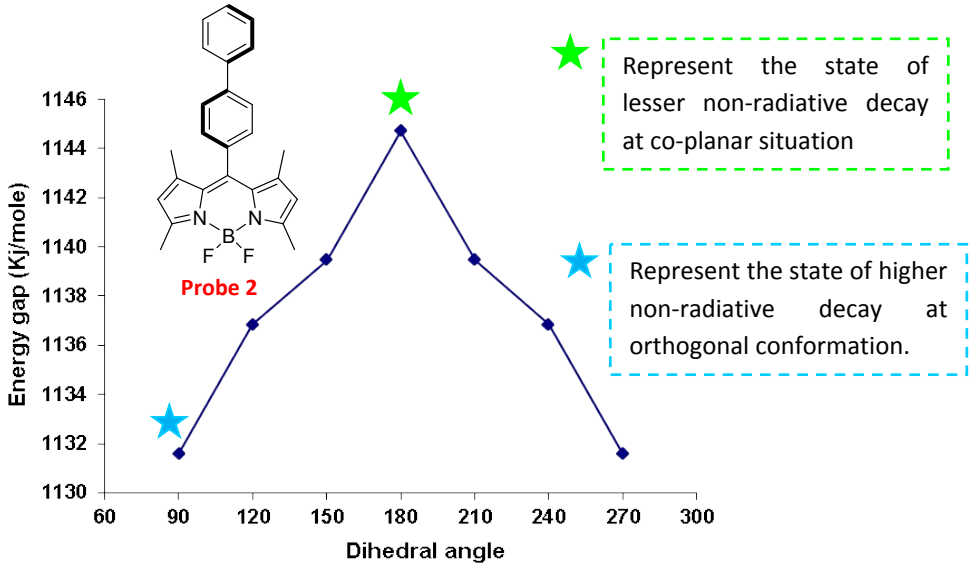


Fig. S22: Plot depicting the variation of S_0 -S_1 energy gap at different dihedral angles in case of compound **2**

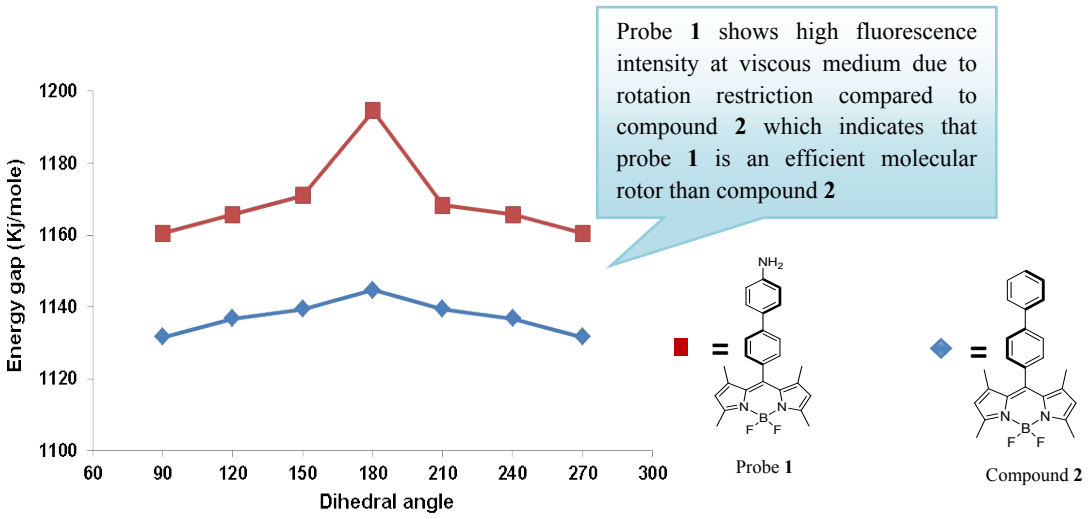


Fig. S23: Combined plot depicting the variation of S_0 -S_1 energy gap at different dihedral angles for probe **1** and compound **2**

Cytotoxicity analysis

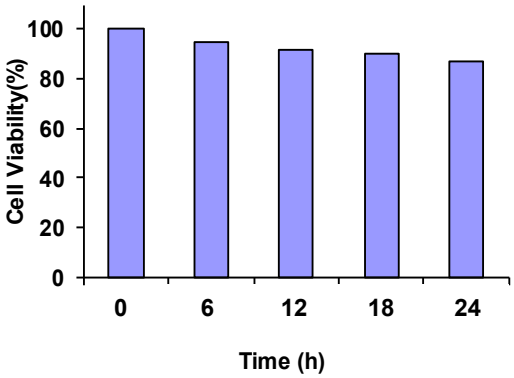


Fig. S24: Cell viability of probe **1** (5.0 μM) at different time intervals

Fluorescence titration of probe 1 with retinoic acid

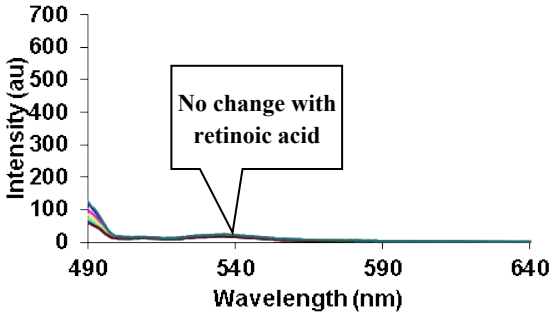


Fig. S25: Fluorescence spectra of probe **1** (5.0 μM) in water on addition of retinoic acid (30 μM) at 25 °C; λ_{ex} = 470 nm and λ_{em}= 517 nm.

Intensity analysis of probe 1 in cell based model system

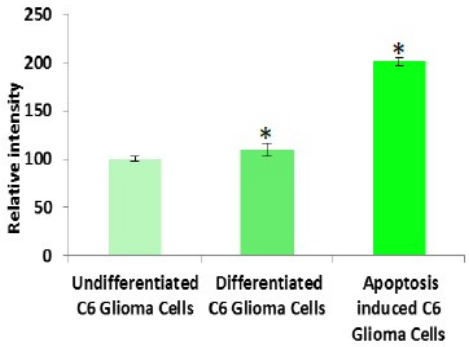
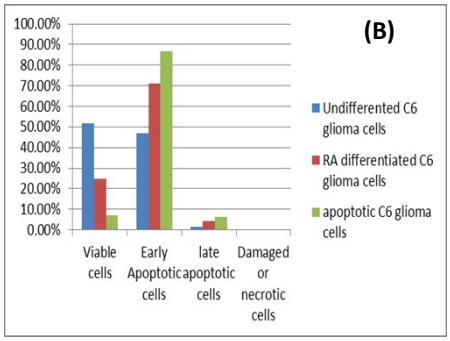


Fig. S26: Histogram depicts the relative expression of the probe **1** in different groups "*" represent statistical significant difference among groups.

Flow cytometer and immunocytofluorescence studies in cell based model system

(A)

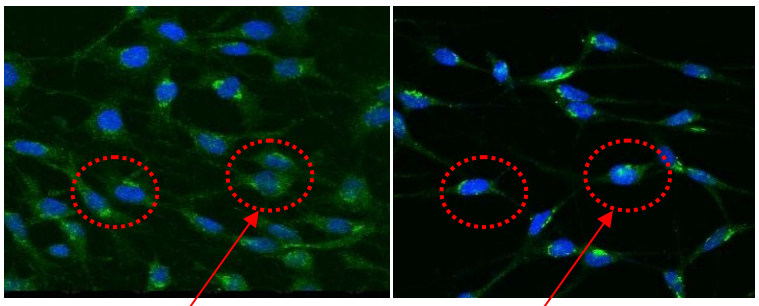
	Undifferentiated cells (%)	RA treated cells (%)	Camptothecin treated cells (%)
Live cells	51.67	24.62	7.1
Early apoptotic cells	46.7	71.24	86.7
Late apoptotic cells	1.53	4.12	6.2



(C)

Undifferentiated C6

RA treated C6 cells



Bcl-xl is expressed in cancer undifferentiated cells *i.e* no apoptosis in cancer cells

Bcl-xl is suppressed in RA treated cells *i.e* apoptosis initiated in these cells

Fig. S27: (A) and (B) showing the percentage of live, early apoptotic and late apoptotic cells in cell based model system. (C) Expression of Bcl-xl in undifferentiated and Retinoic treated C6 glial cells by immunocytofluorescence.

Cell imaging and intensity analysis of probe 1 in cell based model system of cancer and normal cells

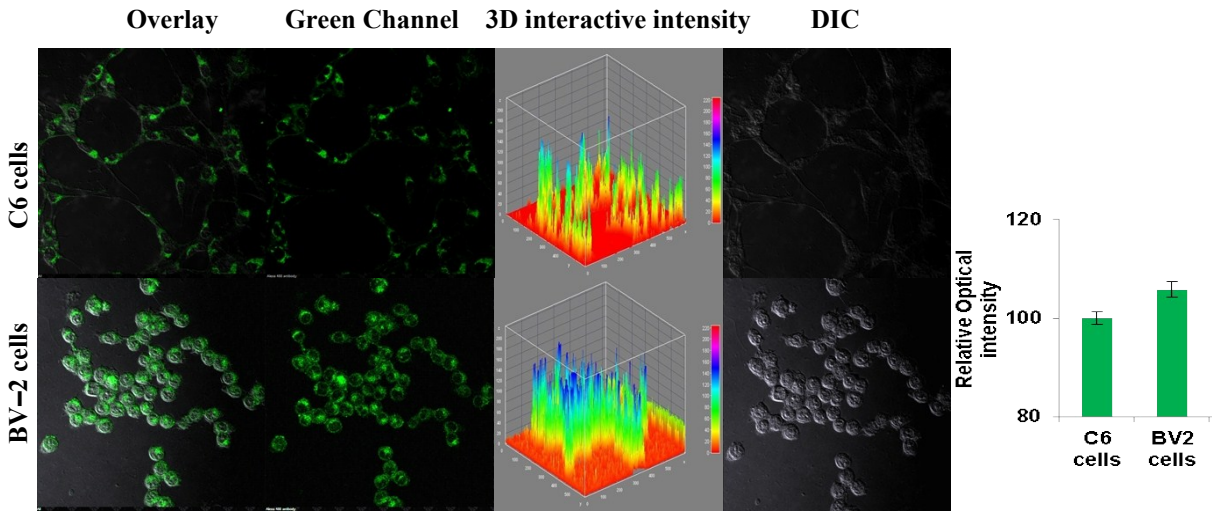


Fig. S28: Fluorescence imaging of probe **1** expression in C6 glioma (undifferentiated cells) and BV–2 (normal) cells. Cells were incubated with probe **1** (5.0 μM) for 30 min. (a), (d) represents DIC of C6 and BV–2 cell; (b), (e) represents the green channel of C6 and BV–2 cells; (c) and (f) represents the overlay of C6 and BV2 cells. Images are acquired by using excitation and emission windows of λ_{ex} = 488 and λ_{em}= 500–550 nm, respectively; Scale bars: 50 μm.

Control experiment of probe1 in C6 glial cells without addition of camptothecin

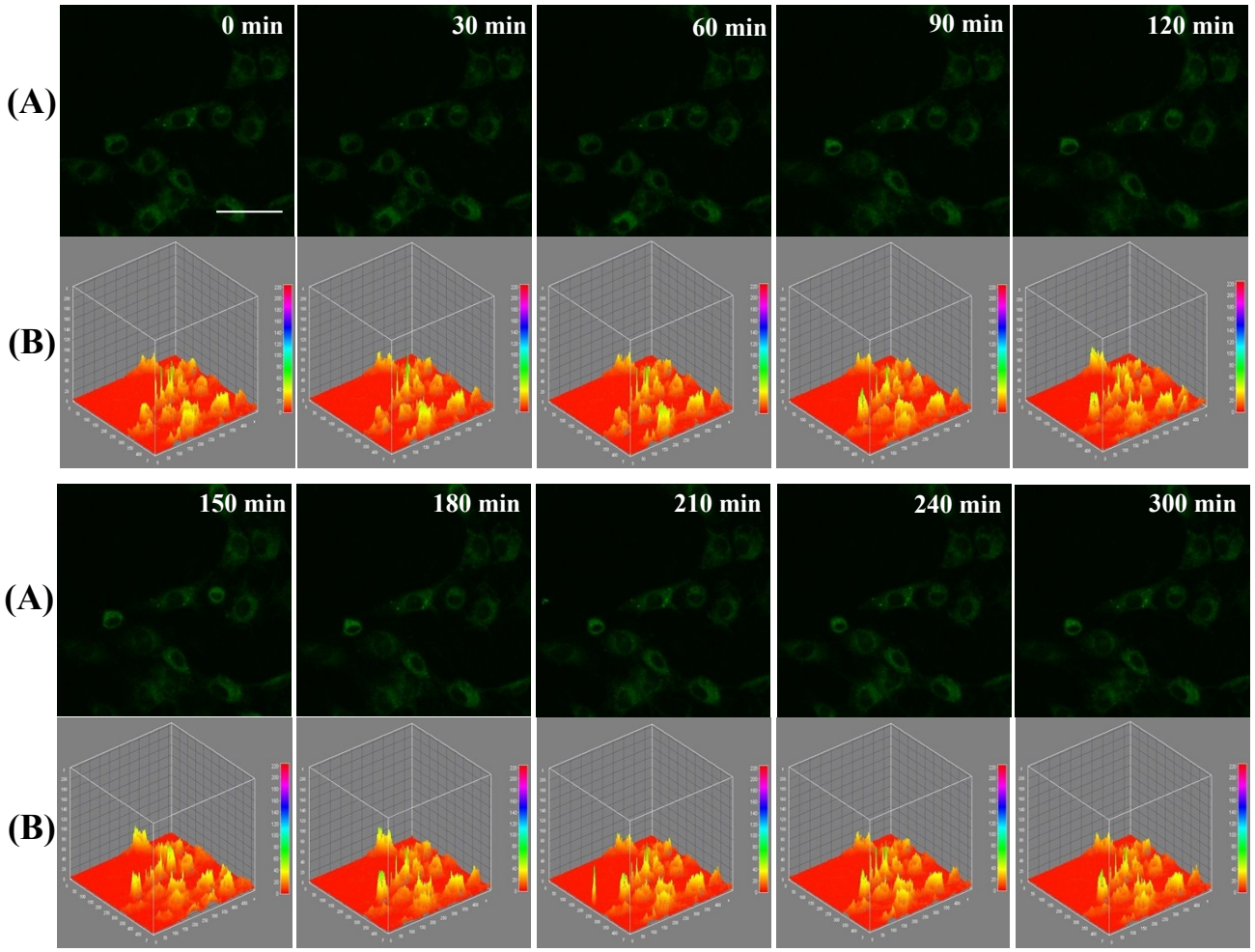


Fig. S29: Fluorescence images of C6 glioma cells incubated with 5.0 μM probe **1** at different time points after addition. Images are acquired by using excitation and emission windows of λ_{ex} = 488 and λ_{em}= 500–550 nm, respectively; Scale bars: 50 μm

TCSPC histogram and lifetime in different cell model system.

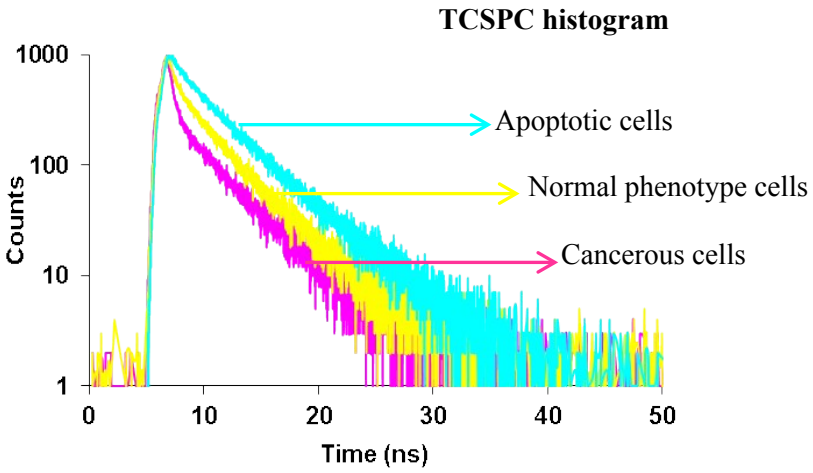


Fig. S30: The decay curves in the TCSPC histogram; Sky blue is represents the apoptotic cells, yellow line represents the normal phenotype cells and pink line represents cancerous cells.

Table S2: Lifetime of probe **1** in different cell model system.

Cells	Lifetime (ns)	χ^2 value
Cancerous cells	**2.82**	**0.9227**
Normal phenotype cells	**3.02**	**0.9626**
Apoptotic cells	**3.26**	**0.9892**

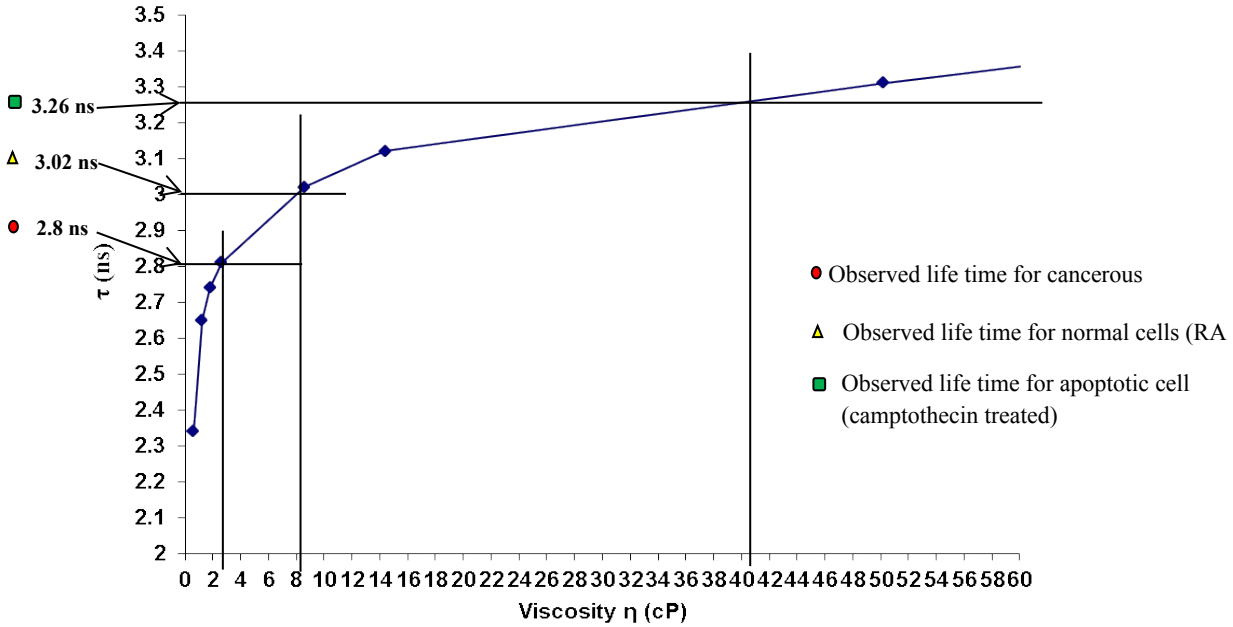


Fig. S31: The life time for probe **1** recorded in methanol-glycerol mixtures of various compositions, plotted against the viscosity of mixtures (for calibration).

Cell culture and treatments

C6 glioma cell was obtained from National Centre for Cell Sciences, Pune, India. Cells were maintained in DMEM supplemented with 1X PSN (GIBCO), 10% FBS (Biological Industries) at 37°C and humid environment containing 5% CO_2. For fluorescence detection, cells were seeded on 18 mm coverslips in 24 well plates. For the purpose of the study, three groups were chosen: **I**. Differentiated cells, for which undifferentiated C6 glioma cells were treated with 10 μM RA after every 24 h for 4 days to induce differentiation of glioma cells as inducing differentiation is one of effective method of treating gliomas. Then cells were exposed to the 5.0 μM probe **1** for 30 min. **II.** Undifferentiated cancerous C6 glioma cells and **III.** Apoptotic cells for which undifferentiated C6 glioma cells were treated with 100 μM Camptothecin then with 5.0 μM of probe **1** exposure for 24 hours.

For Fluorescence Detection

Cell of different group after their required treatments were then washed with 1X PBS thrice for 5 minutes each and then observed under confocal microscope. All images were taken with A1R Nikon Laser Scanning Confocal microscope using 488 nm laser.

Fluorescence lifetime imaging

After cell treatment, lifetime images were captured with an inverted-type laser scanning confocal microscope with a 60X objective. The emission was collected through a 500 ± 30 nm band pass filter.

Statistical analysis

Values are expressed as mean ± SEM. The SigmaStat for Windows (version 3.5) was adopted to analyse the results by One Way ANOVA test in order to determine the significance of the means. Values of $p< 0.05$ were considered as statistically significant.