Supporting Information for:

Designing a Green-Emitting Viscosity-Sensitive 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) Probe for Plasma Membrane Viscosity Imaging

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BODIPY-PM absorption, steady-state and time-resolved fluorescence spectra in cyclohexane, toluene, methanol and glycerol

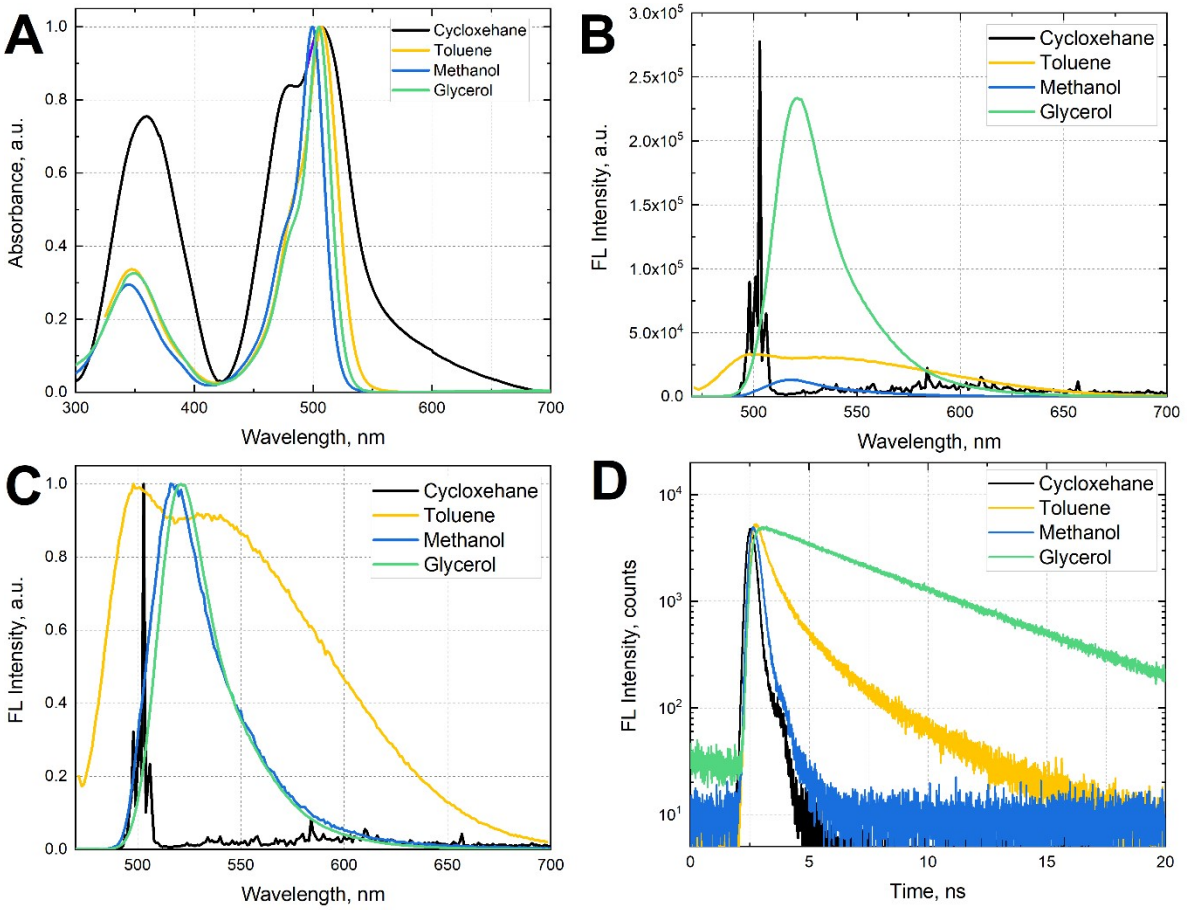

Figure S1. (A) Absorption spectrum of BODIPY-PM in cyclohexane, toluene, methanol and glycerol. (B) Relative steady-state fluorescence spectra of BODIPY-PM in cyclohexane, toluene, methanol and glycerol. (C) Normalized steady-state fluorescence spectra of BODIPY-PM in cyclohexane, toluene, methanol and glycerol. (D) Time-resolved fluorescence decays of BODIPY-PM in cyclohexane, toluene, methanol and glycerol.

BODIPY-PM is not completely dissolved in very non-polar solvents, such as cyclohexane and toluene and additional red bands appear in the steady-state fluorescence spectra, indicating characteristic BODIPY aggregation (Fig. S1). In addition, the fluorescence decays of BODIPY-PM in cyclohexane and toluene become longer and biexponential, in contrast to monoexponential decays in methanol, water or DMSO.

BODIPY-PM absorption, steady-state and time-resolved fluorescence spectra in toluene-castor oil mixtures

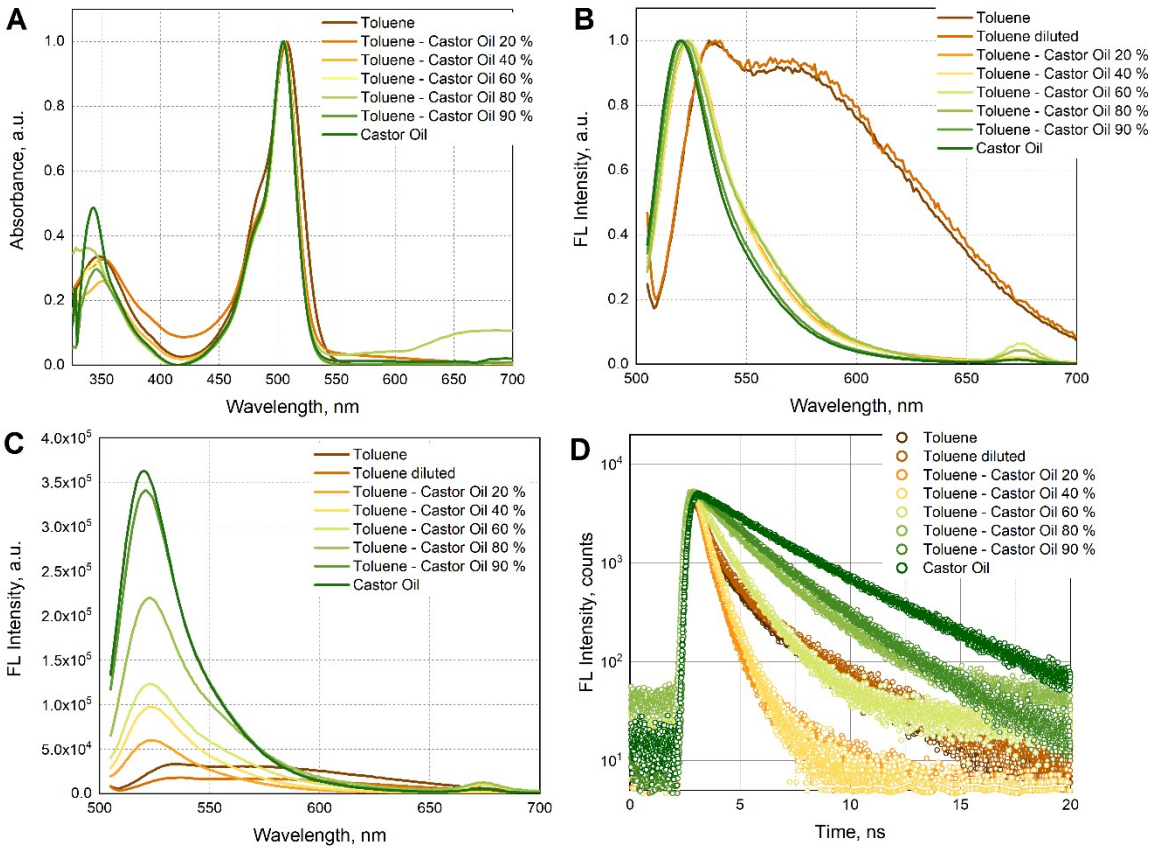

Figure S2. (A) Absorption spectra of BODIPY-PM dissolved in toluene-castor oil mixtures. (B) Normalized steady-state fluorescence spectra of BODIPY-PM in toluene-castor oil mixtures. (C) Relative steady-state fluorescence spectra of BODIPY-PM in toluene-castor oil mixtures. (D) Time-resolved fluorescence decays of BODIPY-PM in toluene-castor oil mixtures.

BODIPY-PM was dissolved at 1 µM concentration in pure toluene and toluene-castor oil mixtures. As evident from the steady-state fluorescence spectra, BODIPY-PM is not fully solvated in pure toluene and characteristic red band appears, indicating dye aggregation (Fig. S2, B). Even upon further dye dilution at 0.5 µM concentration, the dye still remains undissolved (Fig. S2, B, Toluene diluted). Upon BODIPY-PM aggregation, fluorescence lifetimes increase to about 500 ps (Fig. S2, D). Finally, BODIPY-PM fluorescence intensity (Fig. S2, C) and fluorescence lifetimes increase with increase in viscosity of toluene-castor oil mixtures. Precise toluene-castor oil viscosity values and the fluorescence lifetimes of BODIPY-PM in these mixtures are presented in the Table S1 and Figure S3. As BODIPY-PM aggregates in the pure toluene, only the mixtures from toluene-castor oil 20% to pure castor oil were used for the fluorescence lifetime calibration. The fluorescence decays in toluene-castor oil mixtures were biexponential and intensity-weighted lifetimes were calculated.

Viscosity, cP	**Intensity-weighted fluorescence lifetime τ, ps**	**Composition**
1.21	490	Toluene-Castor oil 20%
4.86	737	Toluene-Castor oil 40%
20.18	1316	Toluene-Castor oil 60%
104.46	2234	Toluene-Castor oil 80%
304.265	2708	Toluene-Castor oil 90%
919.19	3725	Castor oil

Table S1. Precise biexponential intensity-weighted fluorescence lifetimes of BODIPY-PM in toluene-castor oil mixtures at room temperature.

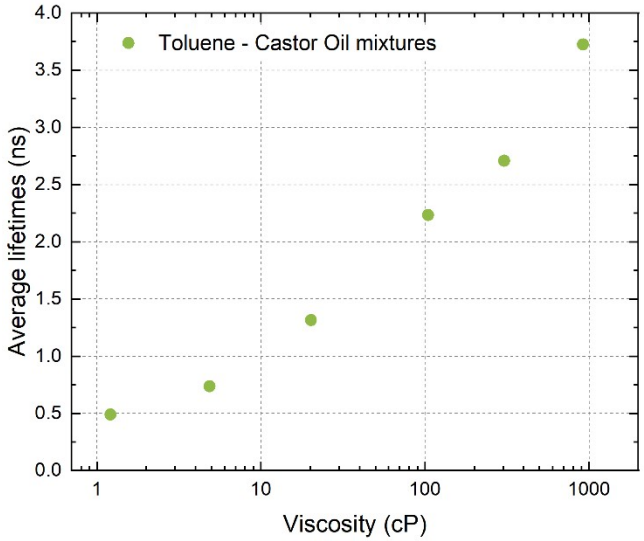

Figure S3. Biexponential intensity-weighted fluorescence lifetimes of BODIPY-PM in toluene-castor oil mixtures, starting from toluene-castor oil 20% and finishing with pure castor oil.

BODIPY-PM absorption, steady-state and time-resolved fluorescence spectra in water-glycerol mixtures

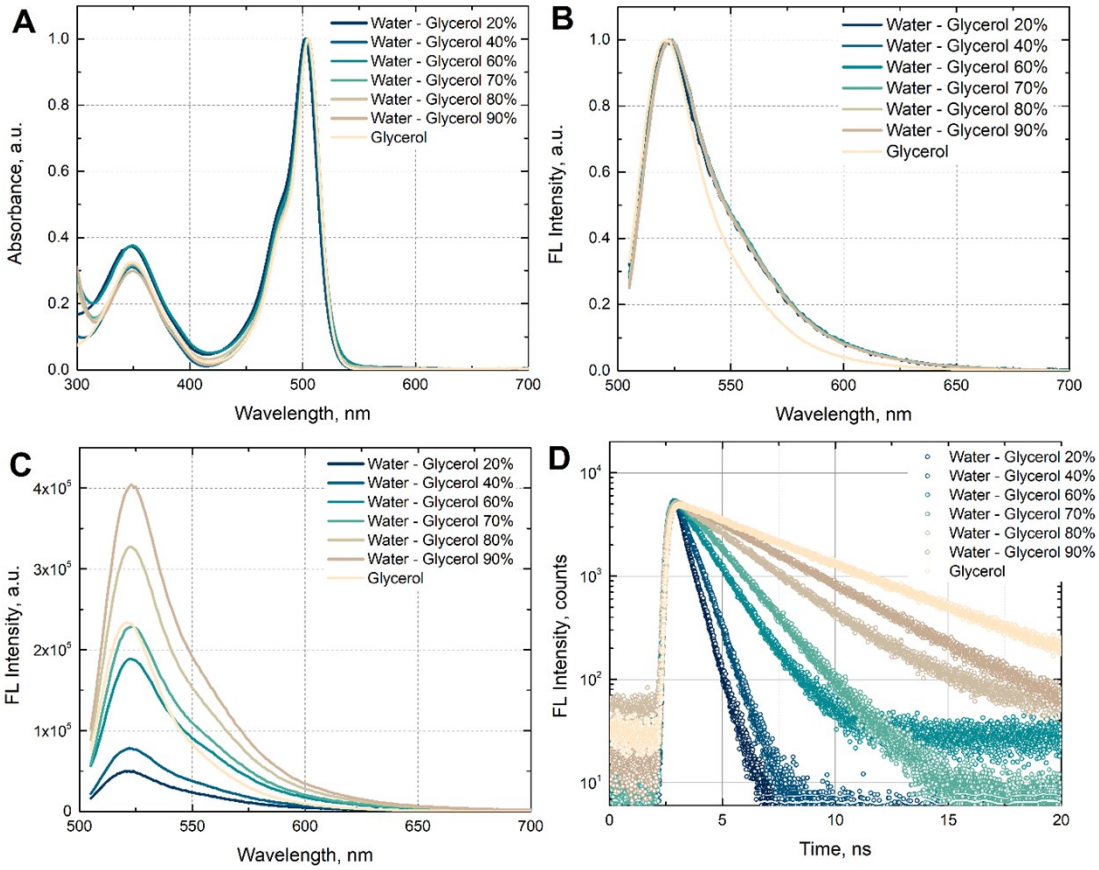

Figure S4. (A) Absorption spectra of BODIPY-PM dissolved in water-glycerol mixtures. (B) Normalized steady-state fluorescence spectra of BODIPY-PM in water-glycerol mixtures. (C) Relative steady-state fluorescence spectra of BODIPY-PM in water-glycerol mixtures. (D) Time-resolved fluorescence decays of BODIPY-PM in water-glycerol mixtures.

BODIPY-PM was dissolved at 1 μM concentration water-glycerol mixtures, starting from pure water and ending up with pure glycerol. Water-glycerol mixtures fully solvate BODIPY-PM and fluorescence intensity along with fluorescence lifetimes increase with increase in viscosity of the mixture (Fig. S4, C, D). The fluorescence decays become biexponential upon reaching water-glycerol 60% composition and remain biexponential even in pure glycerol mixtures. Intensity-weighted fluorescence lifetimes were calculated for the biexponential decays. Precise water-glycerol viscosity values and the fluorescence lifetimes of BODIPY-PM in these mixtures are given in the Table S2 and Figure S5.

Viscosity, cP	**Intensity-weighted fluorescence lifetime τ, ps**	**Composition**
1	264	Water
1.76	437	Water-Glycerol 20%
3.72	630	Water-Glycerol 40%
10.8	1272	Water-Glycerol 60%
22.5	1666	Water-Glycerol 70%
60.1	2699	Water-Glycerol 80%
219	3643	Water-Glycerol 90%
930	5127	Glycerol

Table S2. Precise fluorescence lifetimes of BODIPY-PM in water-glycerol mixtures at room temperature.

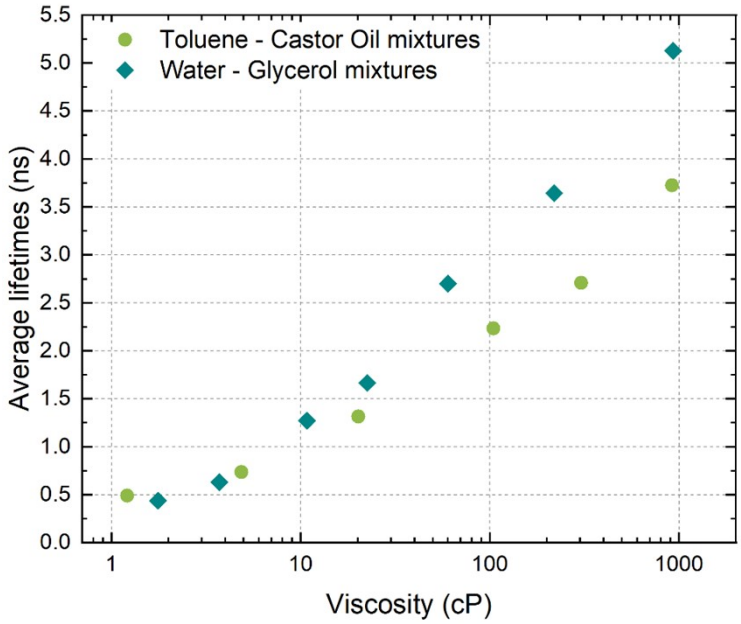

Figure S5. Fluorescence lifetimes of BODIPY-PM in water-glycerol mixtures (blue), starting from pure water and finishing with pure glycerol. Fluorescence lifetimes of BODIPY-PM in toluene-castor oil mixtures (green) are added for comparison.

BODIPY-PM absorption, steady-state and time-resolved fluorescence spectra in methanol-glycerol mixtures

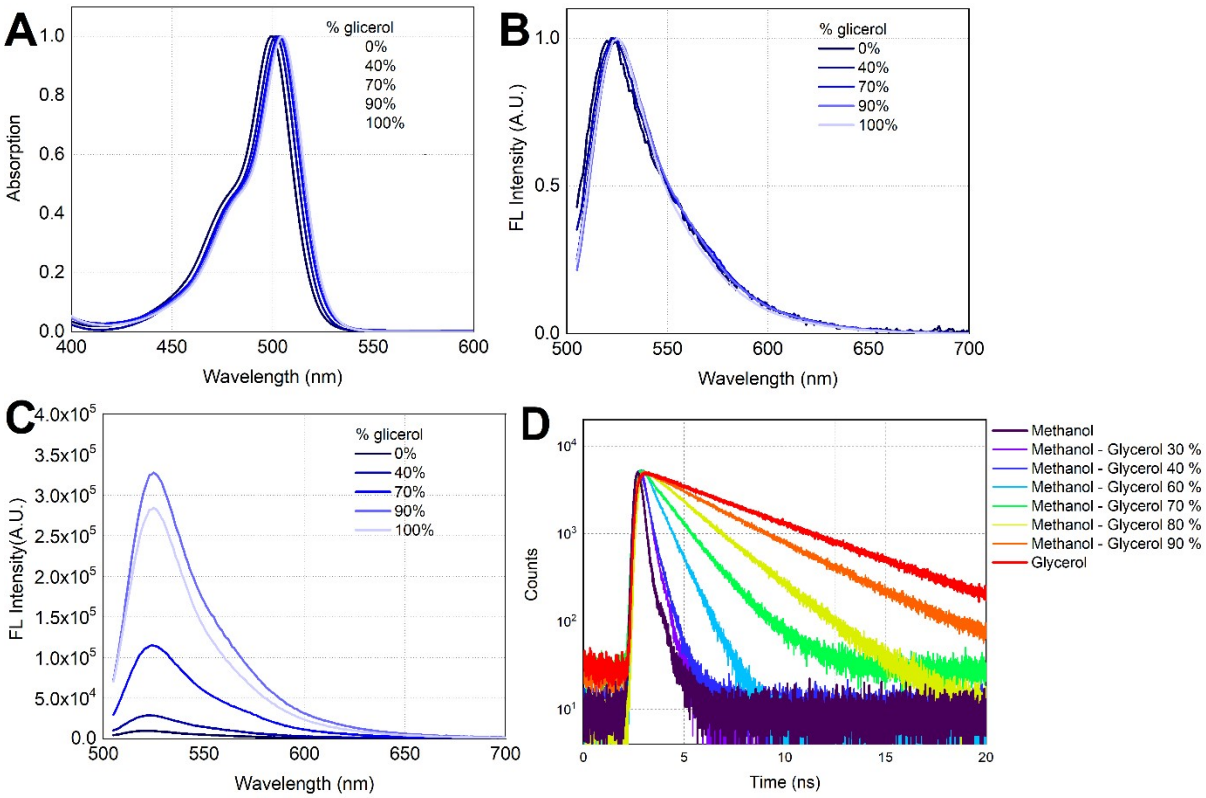

Figure S6. (A) Absorption spectra of BODIPY-PM dissolved in methanol-glycerol mixtures. (B) Normalized steady-state fluorescence spectra of BODIPY-PM in methanol-glycerol mixtures. (C) Relative steady-state fluorescence spectra of BODIPY-PM in methanol-glycerol mixtures. (D) Time-resolved fluorescence decays of BODIPY-PM in methanol-glycerol mixtures.

BODIPY-PM was dissolved at 1 µM concentration methanol-glycerol mixtures, starting from pure methanol and ending up with pure glycerol. Methanol-glycerol mixtures readily dissolve BODIPY-PM and fluorescence intensities with fluorescence lifetimes increase with increase in viscosity of the mixture (Fig. S6). The fluorescence decays become biexponential upon reaching methanol-glycerol 60% composition and remain biexponential even in pure glycerol mixtures. Intensity-weighted fluorescence lifetimes were calculated for biexponential decays. Precise methanol-glycerol viscosity values and the fluorescence lifetimes of BODIPY-PM in these mixtures are given in the Table S3 and Figure S7.

Viscosity, cP	**Intensity-weighted fluorescence lifetime τ, ps**	**Composition**
0.62	122	Methanol
2.913	245	Methanol-Glycerol 30%
5.418	321	Methanol-Glycerol 40%
23.886	785	Methanol-Glycerol 60%
54.176	1497	Methanol-Glycerol 70%
129.835	2226	Methanol-Glycerol 80%
345.33	3662	Methanol-Glycerol 90%
930	5127	Glycerol

Table S3. Precise fluorescence lifetimes of BODIPY-PM in methanol-glycerol mixtures at room temperature.

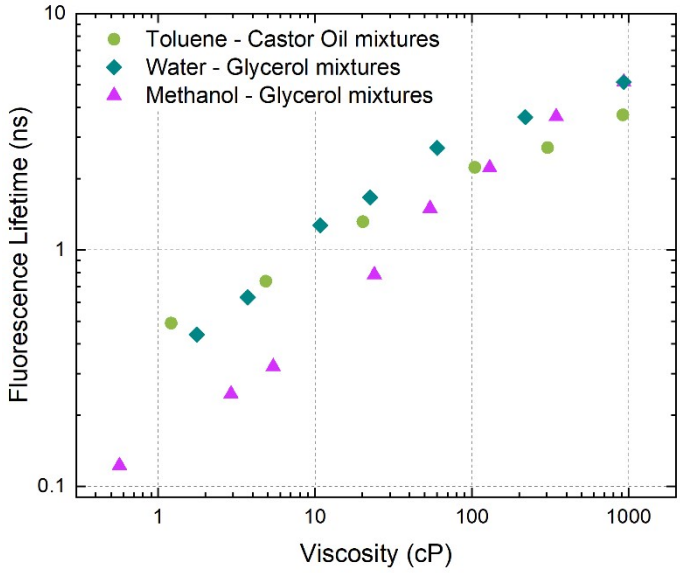

Figure S7. Fluorescence lifetimes of BODIPY-PM in methanol-glycerol mixtures (magenta), starting from pure methanol and finishing with pure glycerol. Fluorescence lifetimes of BODIPY-PM in toluene-castor oil mixtures (green) and water-glycerol mixtures (blue) are added for comparison.

BODIPY-PM steady-state and time-resolved fluorescence spectra in Ld POPC, Lo DOPC/DPPC/Chol and Lo DOPC/BSM/Chol, and gel-phase DMPC and DPPC LUVs

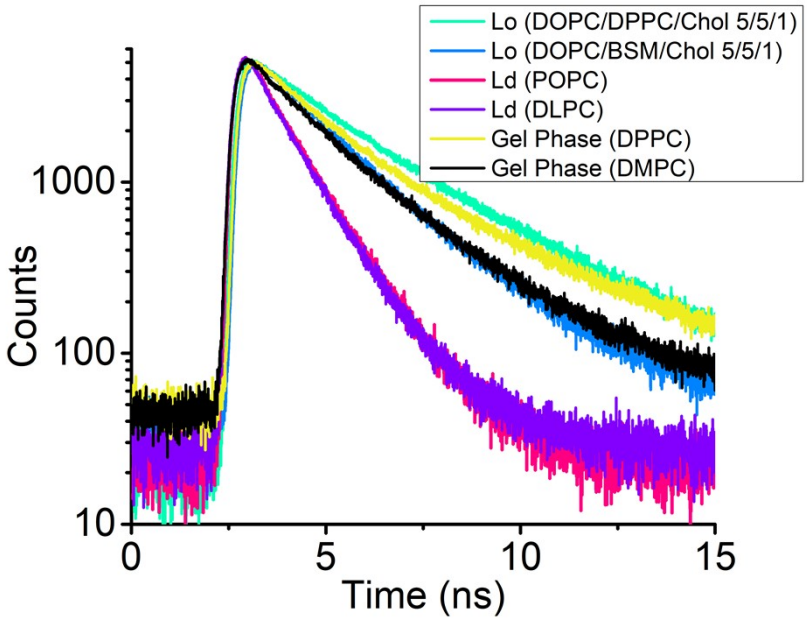

Figure S8. Time-resolved fluorescence decays of BODIPY-PM in different lipid order LUVs.

LUVs with 100nm diameter were prepared with different lipid order compositions, ranging from Ld (POPC, DLPC), to Lo (DOPC/DPPC/Chol and DOPC/BSM/Chol), to gel-phase LUVs (DPPC and DMPC). BODIPY-PM to lipid ratio in LUVs was 1:600. In the gel-phase LUVs, fluorescence decays were shorter compared to the Lo phases, even though the lipid packaging density is higher in the gel-phase and the viscosity values in gel-phase should significantly suprass Lo phase. We hypothesized that BODIPY-PM is partially exposed to the aqeous solution in the gel-phase bilayers and is unable to measure correct viscosity values. To test this hypothesis we performed quenching experiments of Ld (POPC) and gel-phase (DMPC and DPPC) LUVs with NaI (Fig. S9).

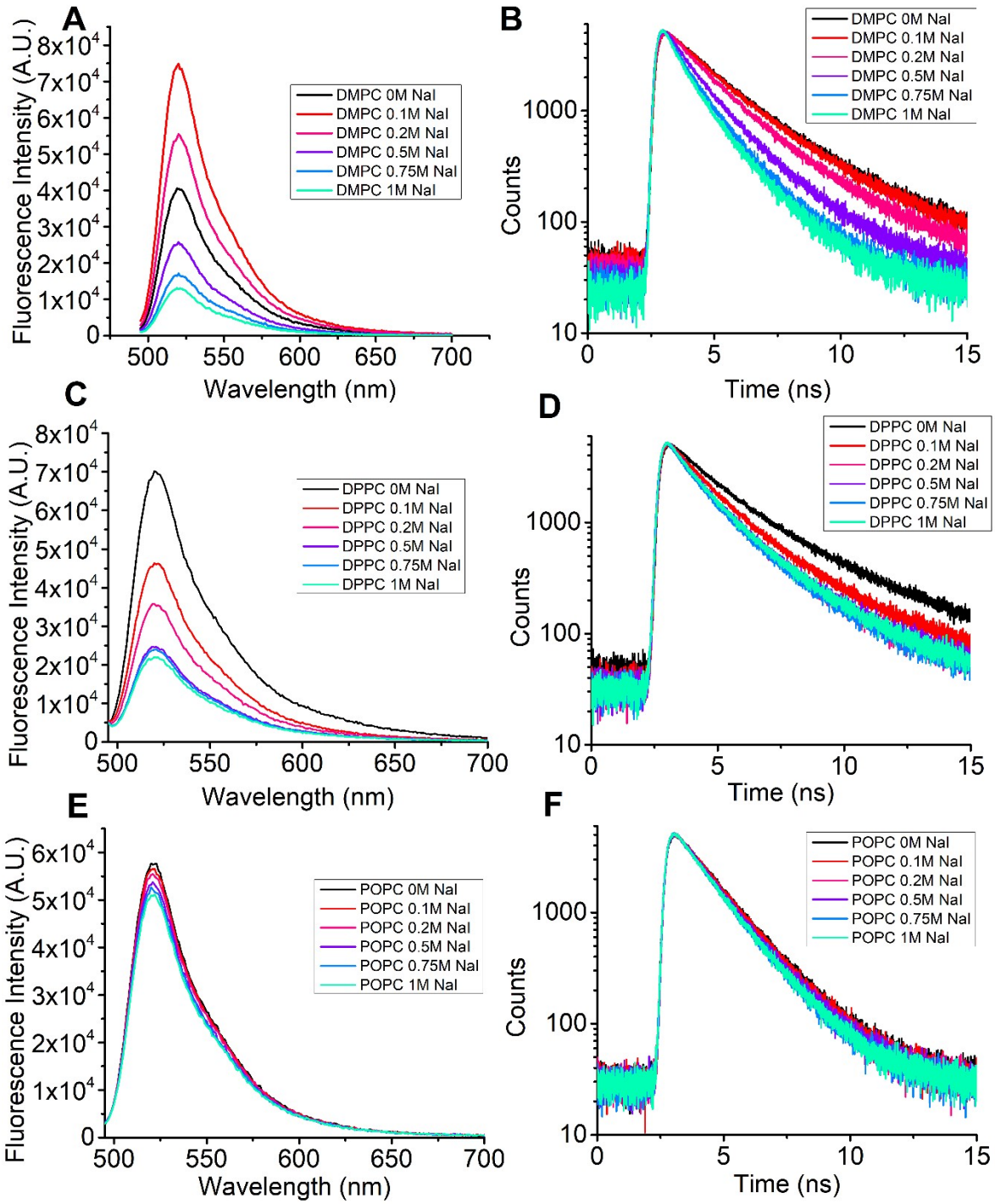

Figure S9. Steady-state and time-resolved fluorescence decays of BODIPY-PM in LUVs with different NaI quencher concentrations. (A,B) Gel-phase DMPC LUVs. (C,D) Gel-phase DPPC LUVs. (E,F) Ld POPC LUVs.

LUVs with 100nm diameter were prepared with gel-phase (DPPC and DMPC) and Ld (POPC) compositions. BODIPY-PM to lipid ratio in LUVs was 1:600. Increase in NaI concentration almost unaffected fluorescence decays and intensities of Ld POPC LUVs (Fig. S9 E,F). On the other hand, fluorescence decays and intensities were significantly reduced in gel-phase DMPC and DPPC LUVs (Fig. S9). This result indicates, that BODIPY-PM is deeply burried in the fluid-phase bilayer and iodide ions are not able to access the probe, whereas in the gel-phase bilayer the BODIPY-PM is exposed to iodide ions and is easily quenched.

Birghtfiled microscopy of lung cancer cells stained with BODIPY-PM

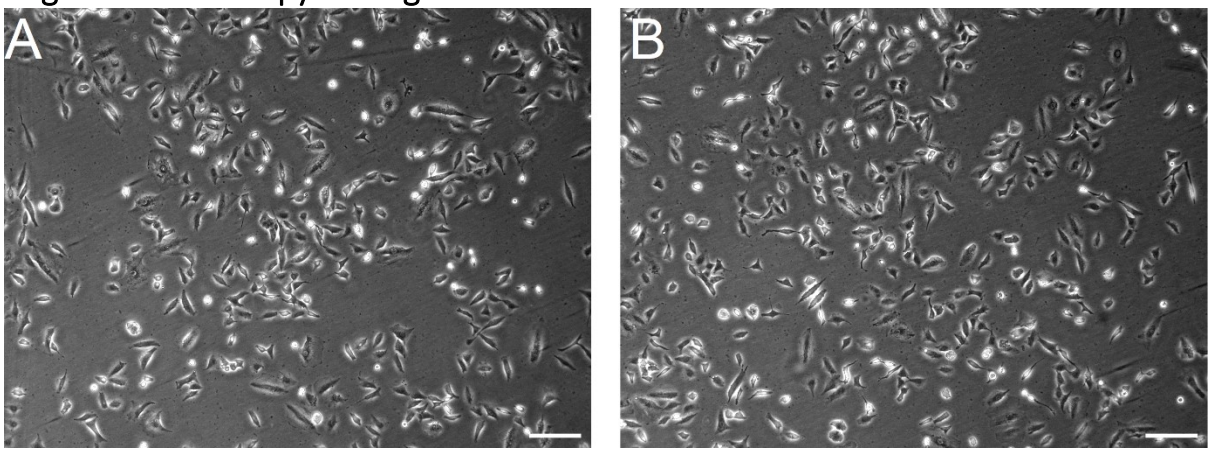

Figure S10. (A) Brightfield image of A549 human lung cancer cells before addition of BODIPY-PM, the cells display normal morphology, scale bar is 200 µm. (B) Brightfield image of A549 human lung cancer cells, stained with BODIPY-PM, after FLIM experiments. The cells show normal cancer cell morphology.

Lung cancer cells (A549) display normal cell morphology after being stained with 1 µM concentration of BODIPY-PM in aqueous solution. We suppose, that BODIPY-PM is non-toxic to the cells at 1 µM concentrations, which are sufficient to stain the cells. In addition, FLIM experiments did not change the morphology of the cells (Fig. S10).

Synthesis of BODIPY-PM and spectral identification

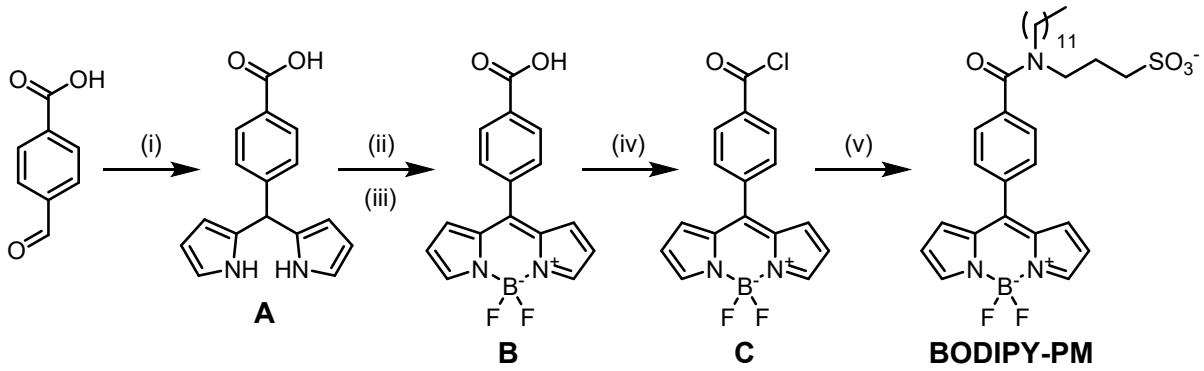

Figure S11. ***Reagents and conditions*****:** (i) 10 eq. of neat pyrrole, 0.1 M HCl, CH_2Cl_2, argon, r.t., 24 h; (ii) 1.5 eq. DDQ, CH_2Cl_2, and then (iii) 7 eq. $BF_3{\cdot}(OEt_2)_2$ and 7 eq. Et_3N, CH_2Cl_2, argon, 0 °C → r.t., darkness, 24 h; (iv) 2 eq. oxalyl chloride, 4 eq. K_2CO_3, drop of DMF, CH_2Cl_2, argon, r.t., 45 min; (v) 1.33 eq. 3-(dodecylammonio)propane-1-sulfonate, 1.33 eq. DIPEA, CH_2Cl_2, argon, r.t., 24 h.

Compounds **A**, **B** [1] and **C** [2] as well as 3-(dodecylammonio)propane-1-sulfonate [3] were preprared according to previously published procedures.

BODIPY-PM. Compound **C** (50 mg, 0.151 mmol) and 1.33 eq. of 3-(dodecylammonio)propane-1-sulfonate (61.8 mg, 0.202 mmol) were dissolved in 2 mL of CH_2Cl_2 and 1.33 eq. of DIPEA (35.2 µL, 2.49 mmol) was added. The mixture was degassed with argon and stirred for 24 hours at room temperature. The reaction progress was controlled using thin-layer chromatography. After the reaction was complete, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (eluent – $CHCl_3$:MeOH (9:1)).

BODIPY-PM. Orange-sticky solid, yield 86 mg (96%), mp 155-156 °C, **^{1}H NMR** (400 MHZ, $CDCl_3$): δ (ppm) = 7.93 (s, 2H); 7.51 (m, 4H); 6.81 (m, 2H); 6.50 (d, *J* = 4 Hz, 2H); 3.83-3.51 (m, 2H); 3.23-3.11 (m, 2H); 2.26-2.04 (m, 2H); 1.30-1.00 (m, 22H); 0.86 (t, *J* = 8 Hz, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ (ppm) = 170.8; 145.8; 144.6; 138.6; 134.7; 134.6; 131.4; 130.5; 126.9; 118.9; 54.1; 50.6; 42.4; 31.9; 29.6; 29.6; 29.55; 29.3; 29.1; 28.7; 26.5; 22.6; 18.6; 17.4; 14.1. **^{11}B NMR** (128.4 MHZ, $CDCl_3$): δ (ppm) = 0.20 (t, $J_{B\text{-}F}$ = 28.2 Hz). **^{19}F NMR** (376.5 MHz, $CDCl_3$): δ (ppm) = -144.86 (dd, $J_{F\text{-}F}$ = 56.5 Hz, $J_{F\text{-}B}$ = 30.12 Hz). **HRMS** (ESI-TOF) m/z 602.3036 ($C_{31}H_{43}BF_2N_3O_4S^+$ $[M+2H]^+$, requires 602.3029).

NMR spectra

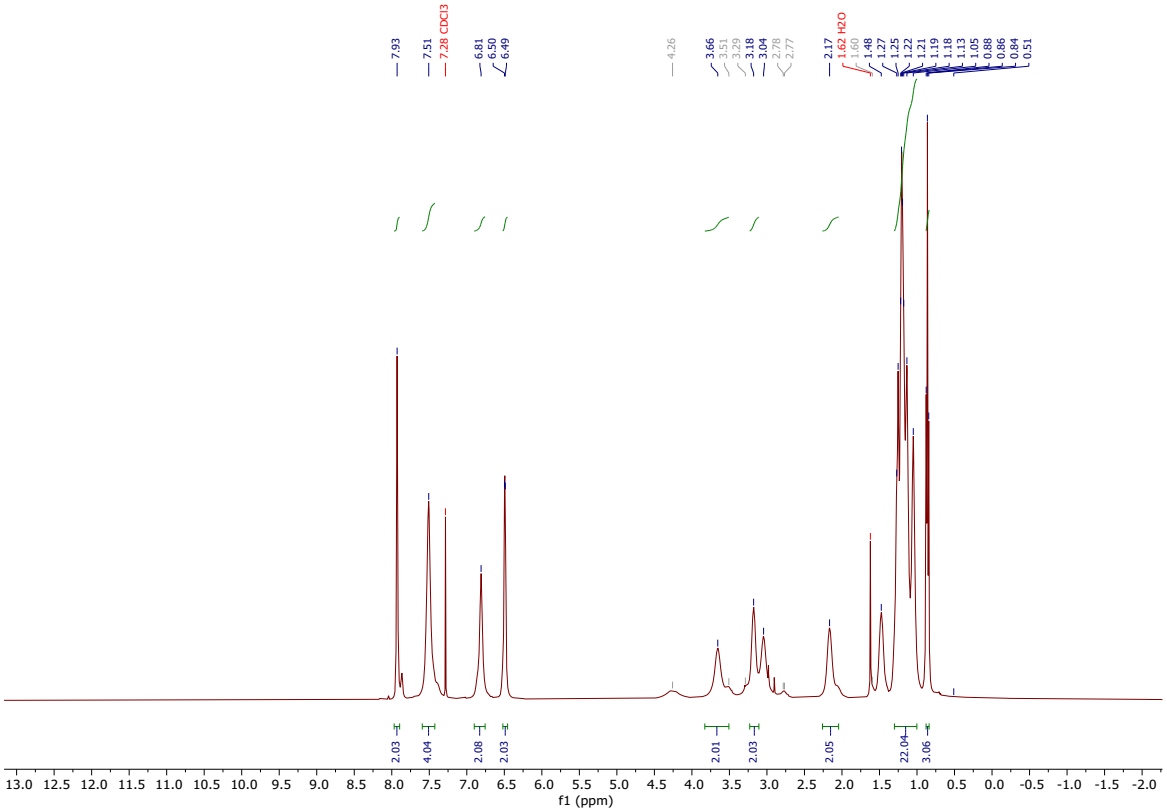

Fig S12. ^{1}H NMR spectrum of **BODIPY-PM.**

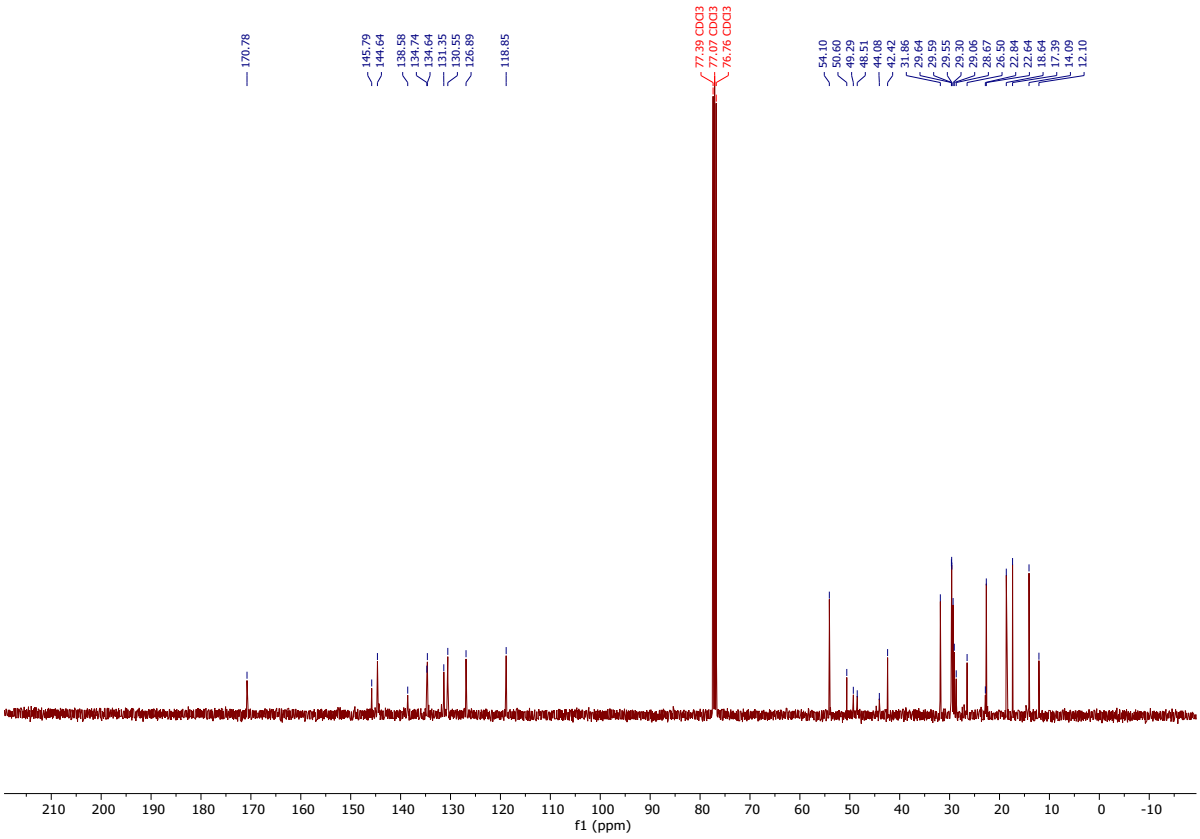

Fig S13. ^{13}C NMR spectrum of **BODIPY-PM.**

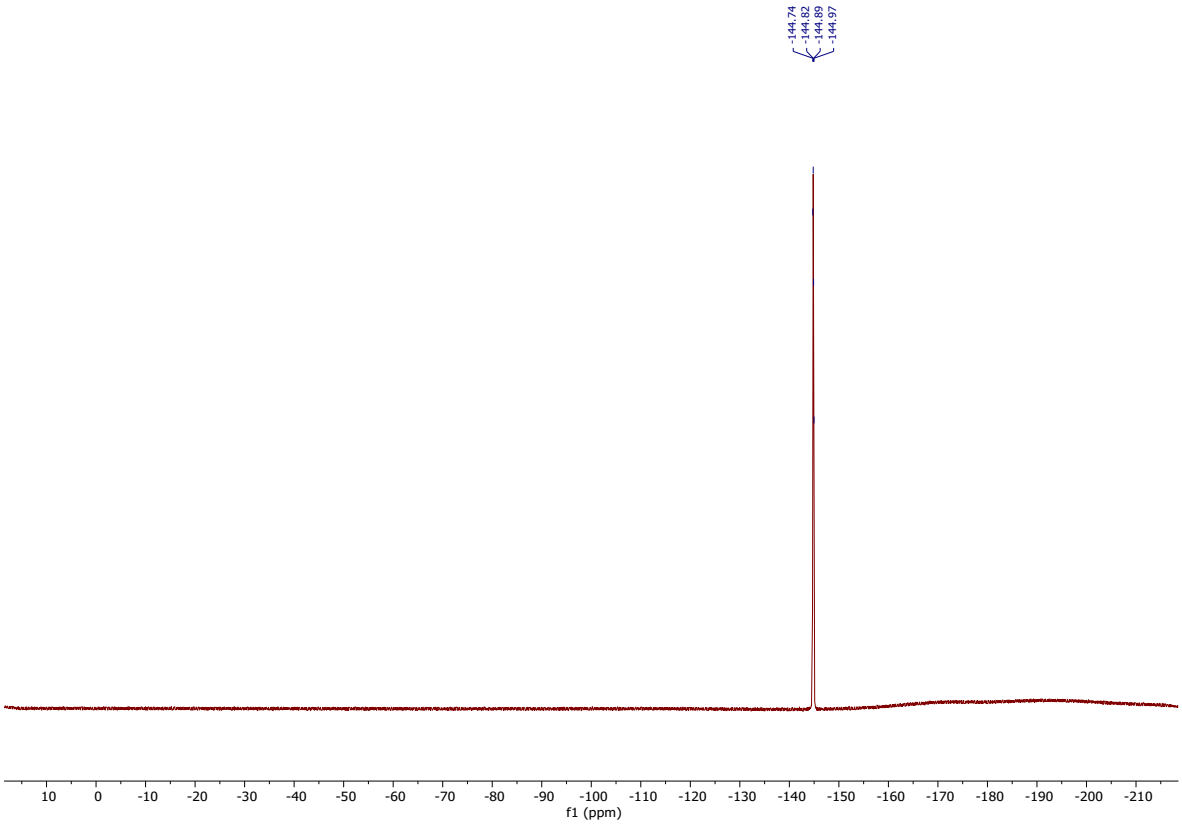

Fig S14. ^{19}F NMR spectrum of **BODIPY-PM.**

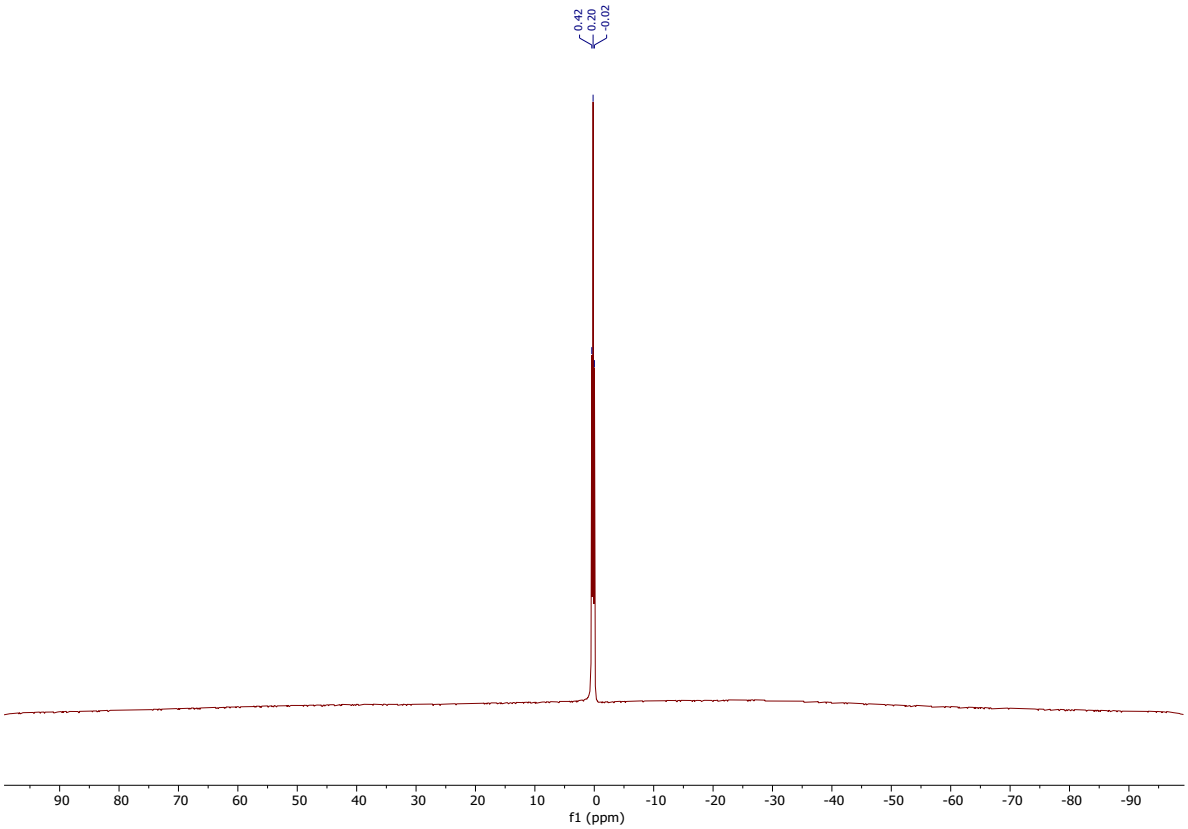

Fig S15. ^{11}B NMR spectrum of **BODIPY-PM.**

Mass Spectrometry (HPLC-MS) analysis

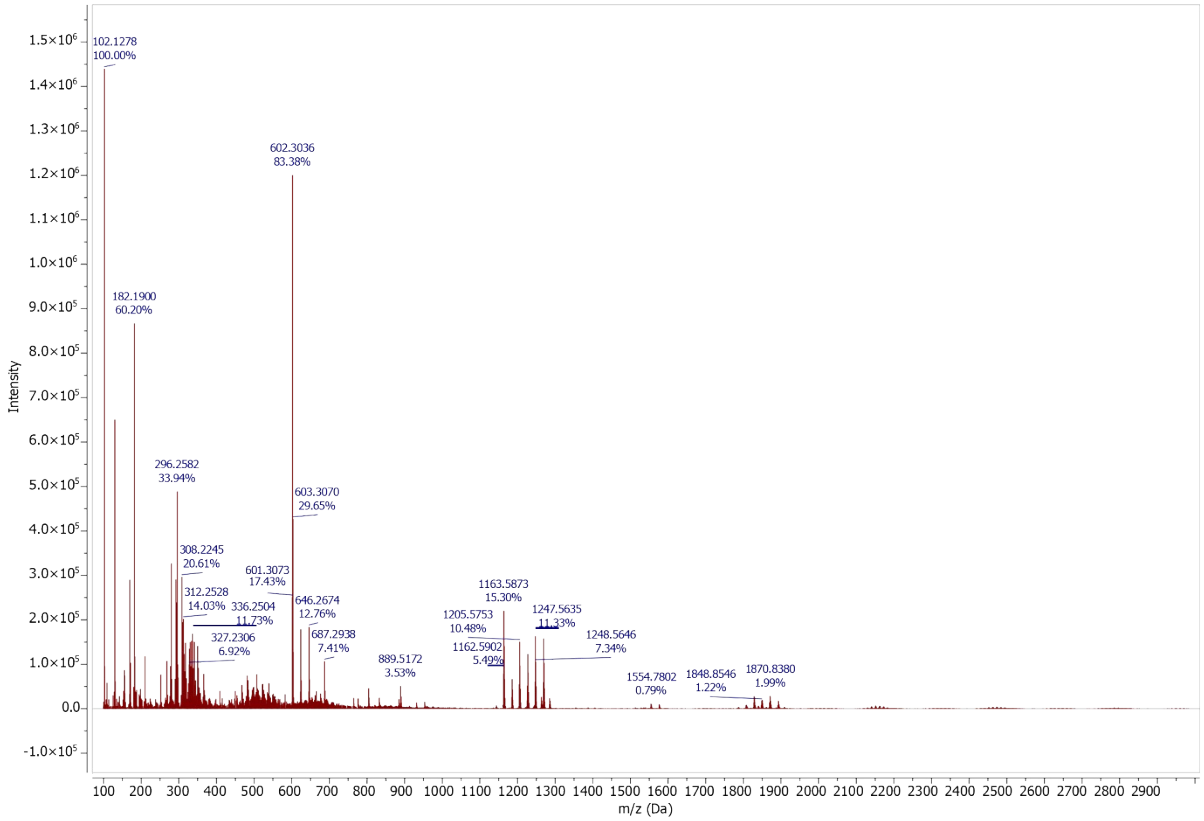

Fig S16. Mass spectrum of **BODIPY-PM**.

[1] G. Matvey, C. Ulyana, B. Natalia, K. Arkadiy, Synthesis and Optical Properties of BODIPY with Active Group on meso- Position, Lett. Org. Chem. 13 (2017) 718–725. https://doi.org/10.2174/1570178614666161118155955.

[2] J. Pliquett, S. Amor, M. Ponce-Vargas, M. Laly, C. Racoeur, Y. Rousselin, F. Denat, A. Bettaïeb, P. Fleurat-Lessard, C. Paul, C. Goze, E. Bodio, Design of a multifunctionalizable BODIPY platform for the facile elaboration of a large series of gold(i)-based optical theranostics, Dalt. Trans. 47 (2018) 11203–11218. https://doi.org/10.1039/c8dt02364f.

[3] D.I. Danylchuk, S. Moon, K. Xu, A.S. Klymchenko, Switchable Solvatochromic Probes for Live-Cell Super-resolution Imaging of Plasma Membrane Organization, Angew. Chemie Int. Ed. 58 (2019) 14920–14924. https://doi.org/10.1002/anie.201907690.