Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Linker Length in fluorophore-cholesterol conjugates directs phase selectivity and cellular localization in GUVs and live cells.

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1. Experimental Details

Materials

All reagents were purchased from Sigma Aldrich (Ireland) and used without further purification unless otherwise stated. 1,2- Dioleoyl-sn-glycero-3-phosphocholine (DOPC), brain sphingomyelin (BSM), and cholesterol (Chol) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). DiD solid; DiIC18(5) solid (1,10 -dioctadecyl-3,3,30 ,30 -tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). High purity fluorescein was purchased from Fluorochem Ltd.

Instrumentation

^{1}H NMR spectra were recorded on either 400 MHz or 600 MHz Bruker spectrometer. All ^{13}C NMR spectra were obtained at 150 MHz. The spectra were processed using Bruker Topspin NMR software. High Resolution Mass Spectrometry (HR-MS) was carried out at the Mass Spectrometry facility, University College Dublin. Absorbance spectra were carried out using a Varian Cary 50 spectrometer. Samples were analysed in Hellma quartz fluorescence cuvettes, with a path length of 1 cm, and spectral range of 280–800 nm unless otherwise stated. Background measurements were carried out at room temperature prior to each measurement. Fluorescence emission spectra were obtained using a Varian Cary Eclipse fluorescence spectrophotometer with excitation and emission slit widths of 2.5 nm. All analyses were carried out using quartz cuvettes and background correction was applied prior to measurement. The lifetime of the excited state was measured using a PicoQuant FluoTime 100 Compact FLS TCSPC system using a 450 nm pulsed laser source generated from a PicoQuant PDL800-B box. Lifetime decay plots were analysed using PicoQuant TimeHarp software. The goodness of each fit to exponential decay kinetics was assessed from chi-squared values (where $\chi^2 < 1.3$) and visual inspection of residuals. Giant unilamellar vesicles were prepared using the Vesicle Prep Pro (VPP) (Nanion Technologies, Munich, Germany). Fluorescent confocal imaging was carried out using a Leica TSP inverted (DMi8) confocal microscope. A 100-oil immersion objective was used for all measurements. A white light laser was used to excite the dyes. The excitation and emission wavelengths (λex/λem) were as follows: 503/511–570 nm for BODIPY-Ar-Chol and BODIPY-Ahx-Chol 644/ 665–700 nm for DiD and.

2. Structural Characterisation

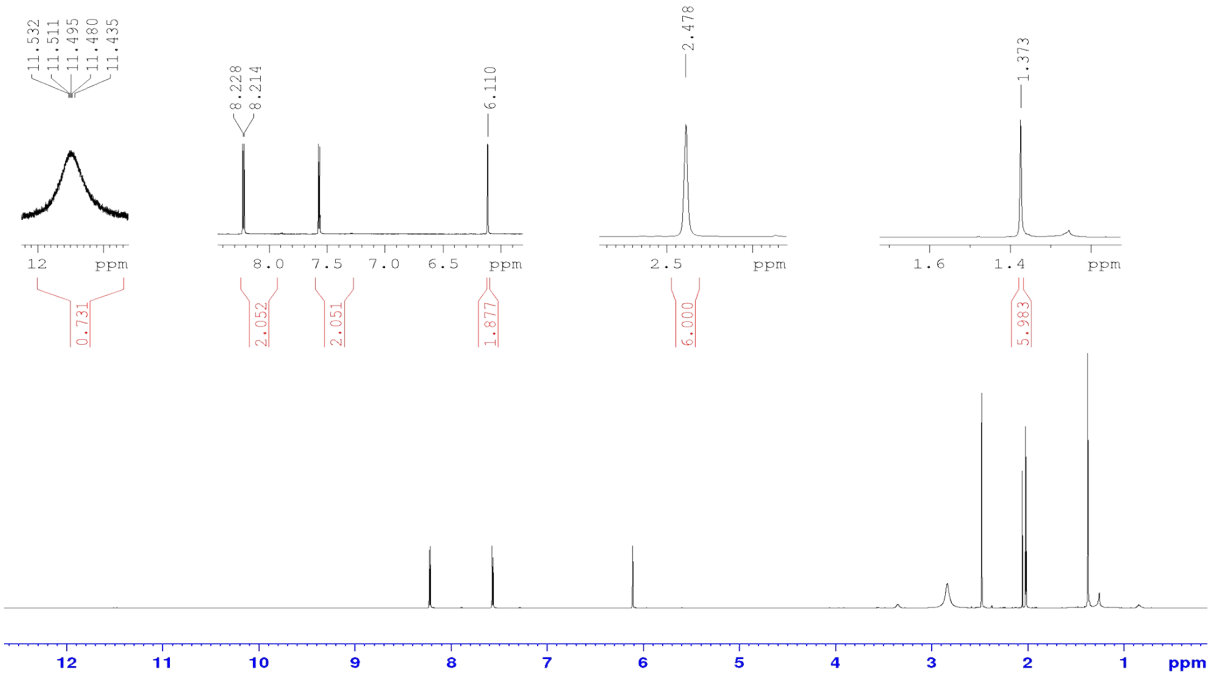


Figure S1 ^{1}H NMR (600 MHz) of **1** in DMSO-d_6.

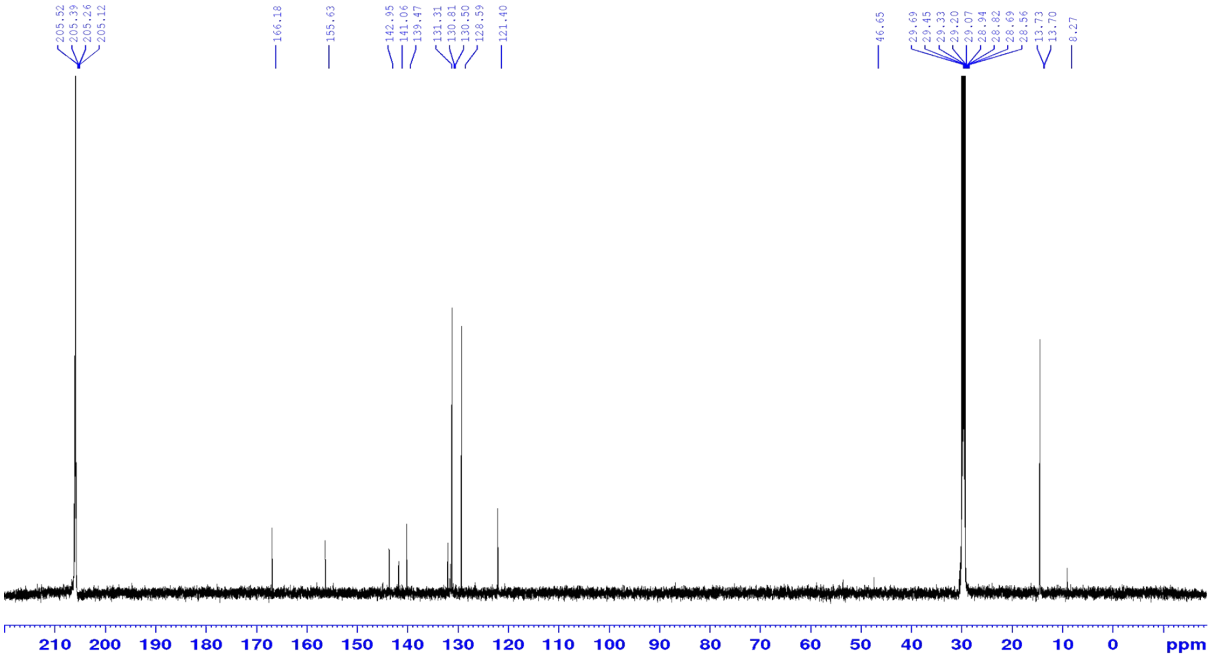
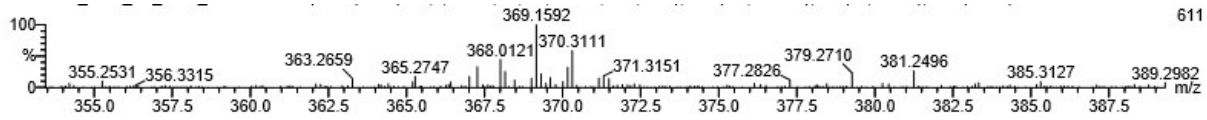


Figure S2 ^{13}C NMR (150 MHz) of **1** in DMSO-d_6.



Minimum:				-1.5		
Maximum:		200.0	5.0	70.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
369.1592	369.1586	0.6	1.7	11.5	1	C20 H20 B N2 O2 F2

Figure S3 HR-MS (ESI-QTOF): Single Mass Analysis of **(1)** indicating [M + H].

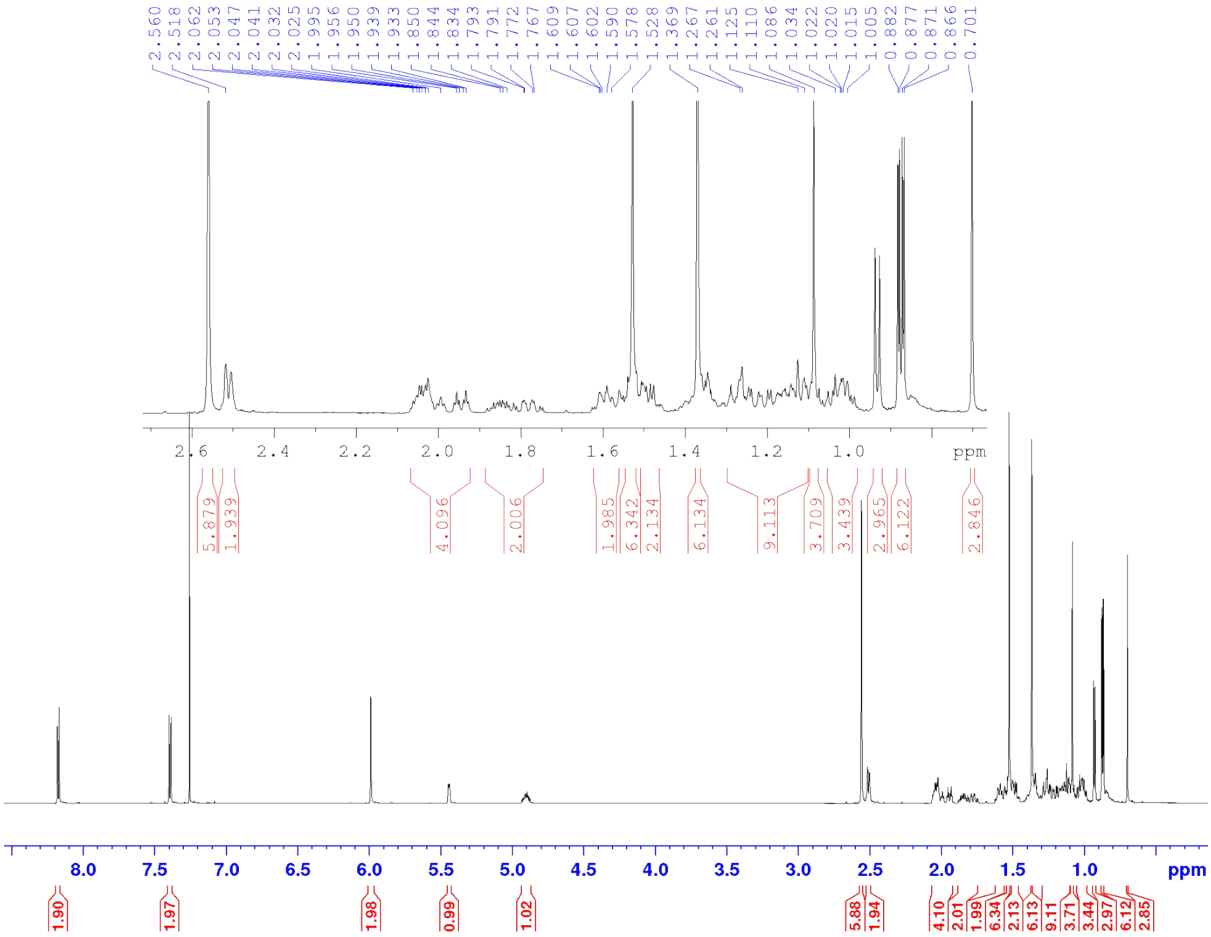


Figure S4 ^{1}H NMR (600 MHz) of **2** in $CDCl_3$.

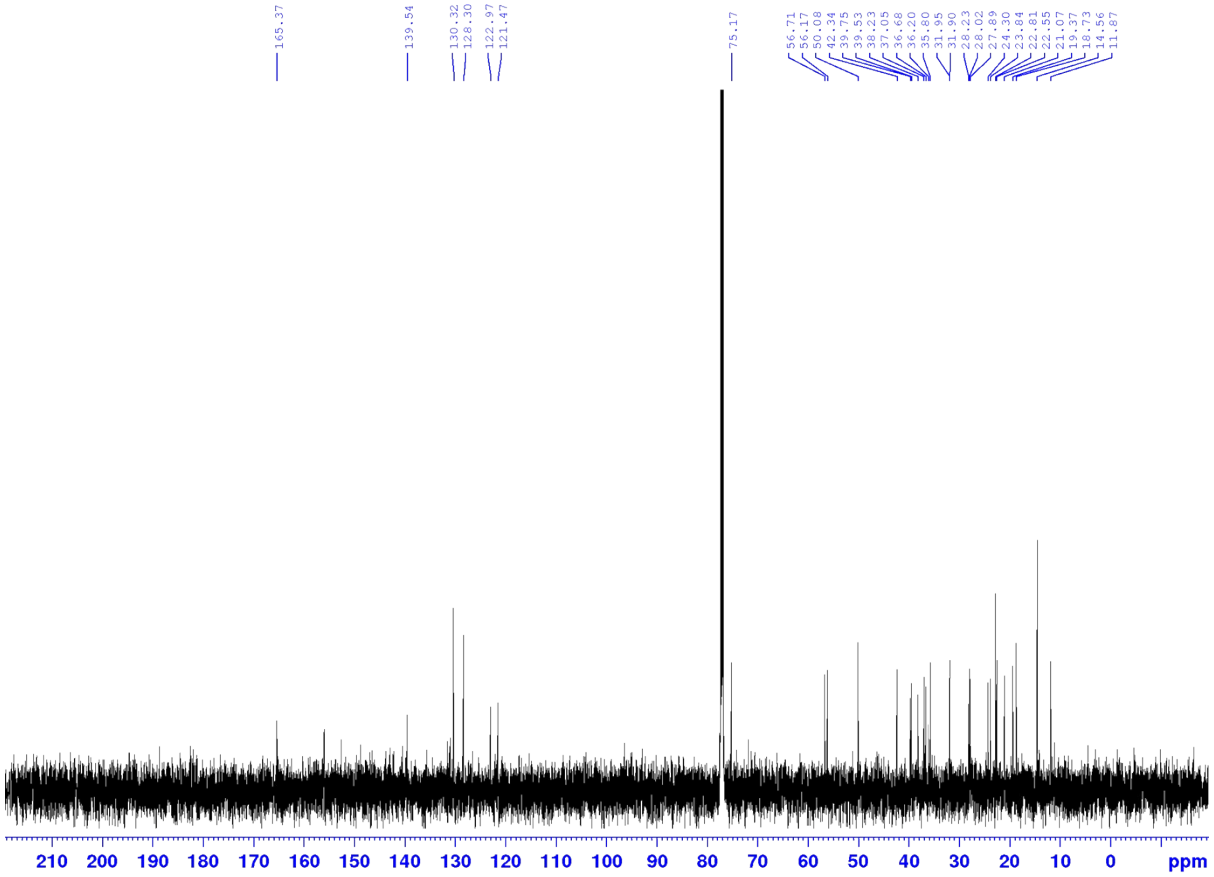


Figure S5 ^{13}C NMR (150 MHz) of **2** in $CDCl_3$.

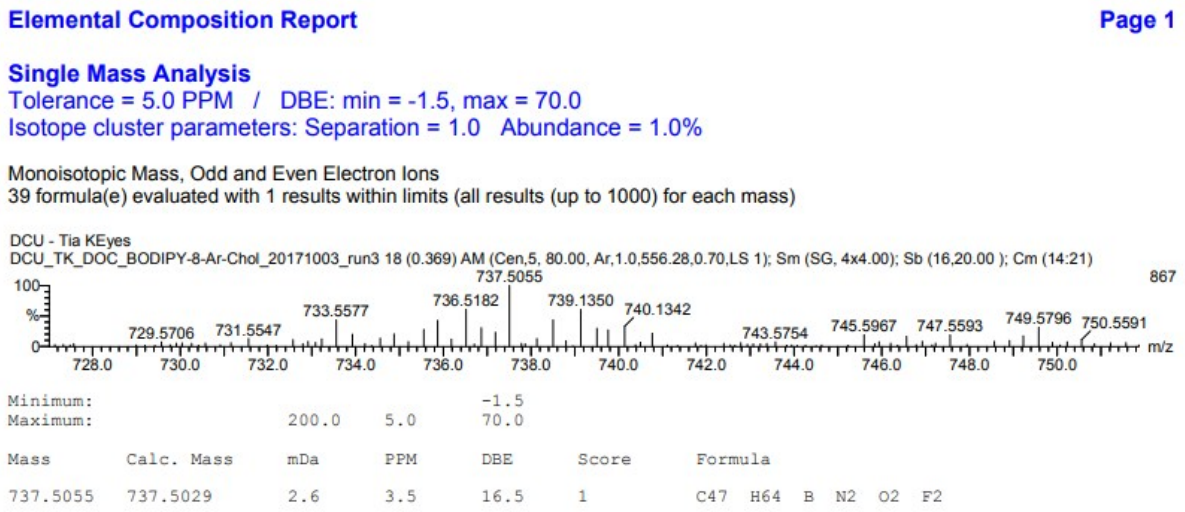


Figure S6 HR-MS (ESI-QTOF): Single Mass Analysis of **2** indicating [M + H].

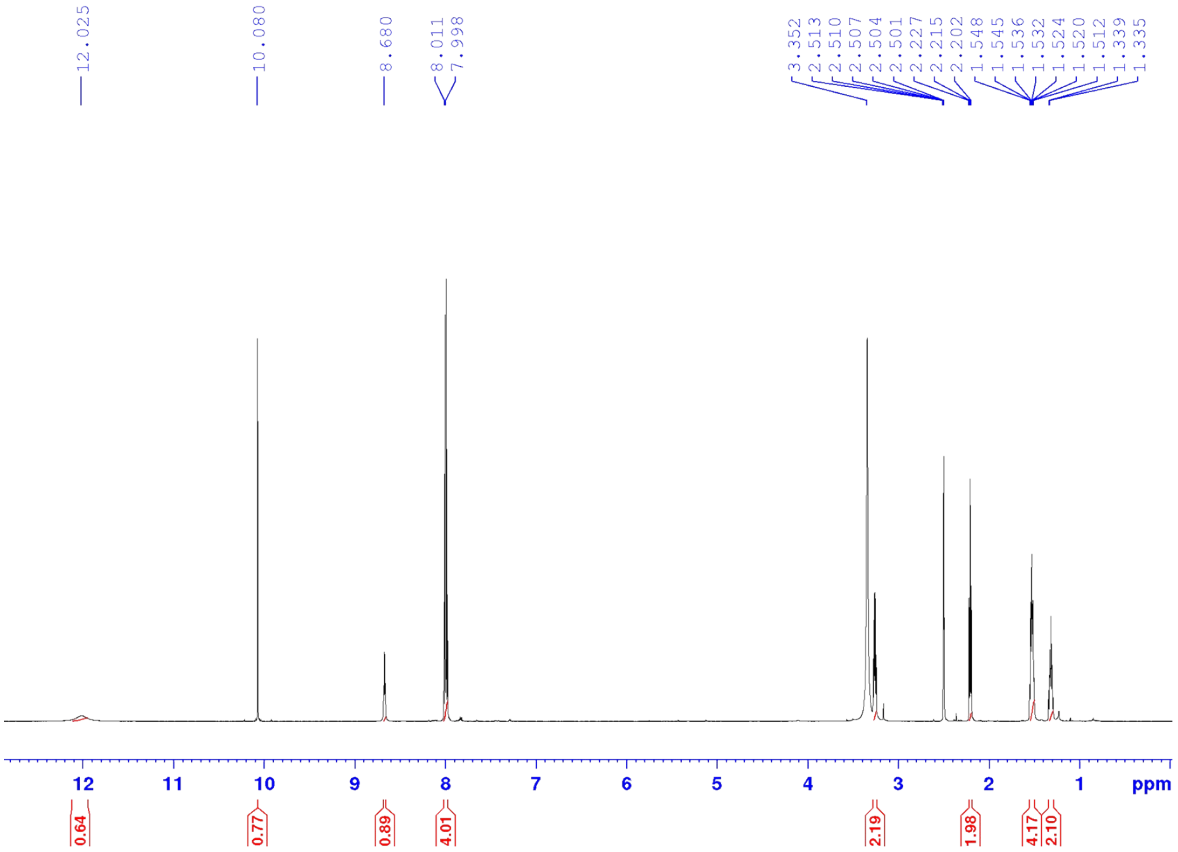


Figure S7 ^{1}H NMR (600 MHz) of **4** in DMSO-d_6.

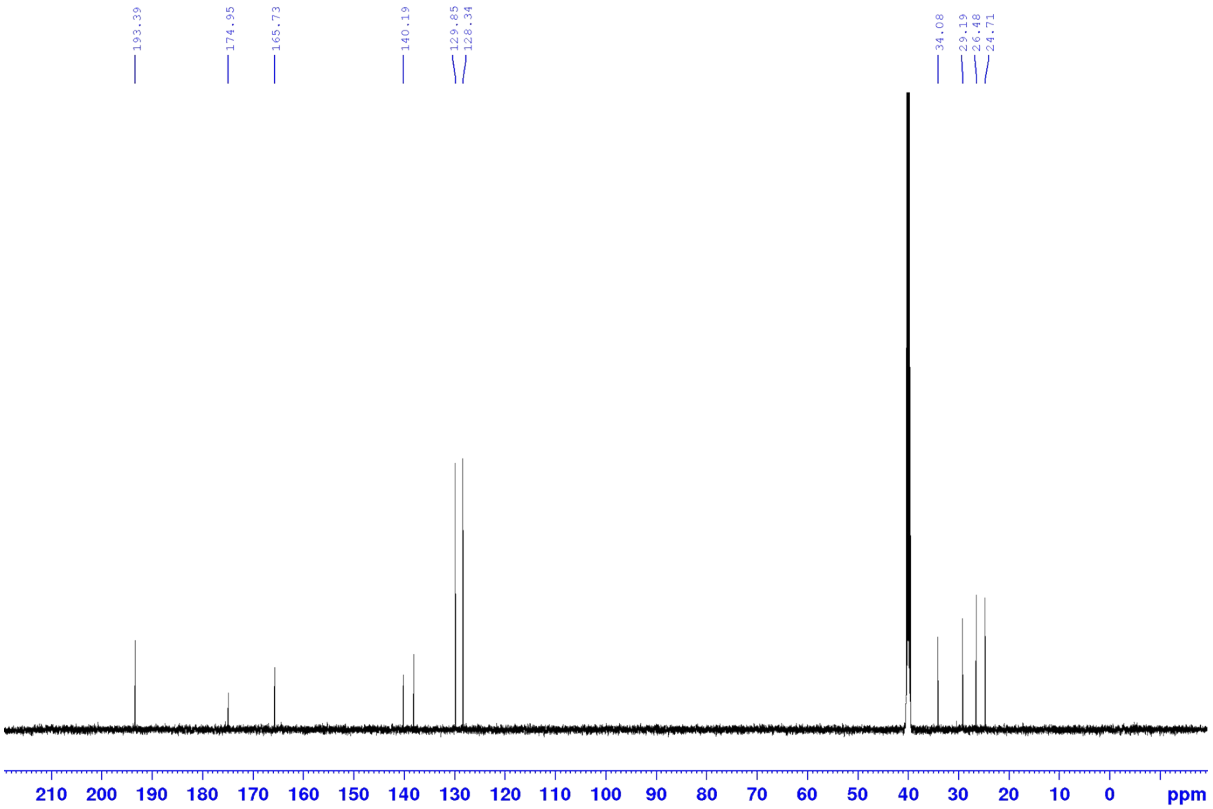
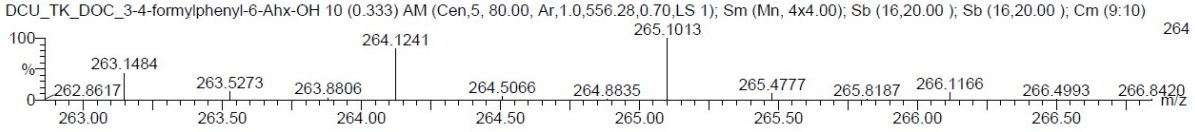


Figure S8 ^{13}C NMR (150 MHz) of **4** in DMSO-d_6.

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 70.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
8 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

External Client
DCU_TK_DOC_3-4-formylphenyl-6-Ahx-OH 10 (0.333) AM (Cen,5, 80.00, Ar,1.0,556.28,0.70,LS 1); Sm (Mn, 4x4.00); Sb (16,20.00); Sb (16,20.00); Cm (9:10)



Minimum:				-1.5		
Maximum:		200.0	5.0	70.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
264.1241	264.1236	0.5	2.0	6.5	1	C14 H18 N O4

Figure S9 HR-MS (ESI-QTOF): Single Mass Analysis of **4** indicating [M + H].

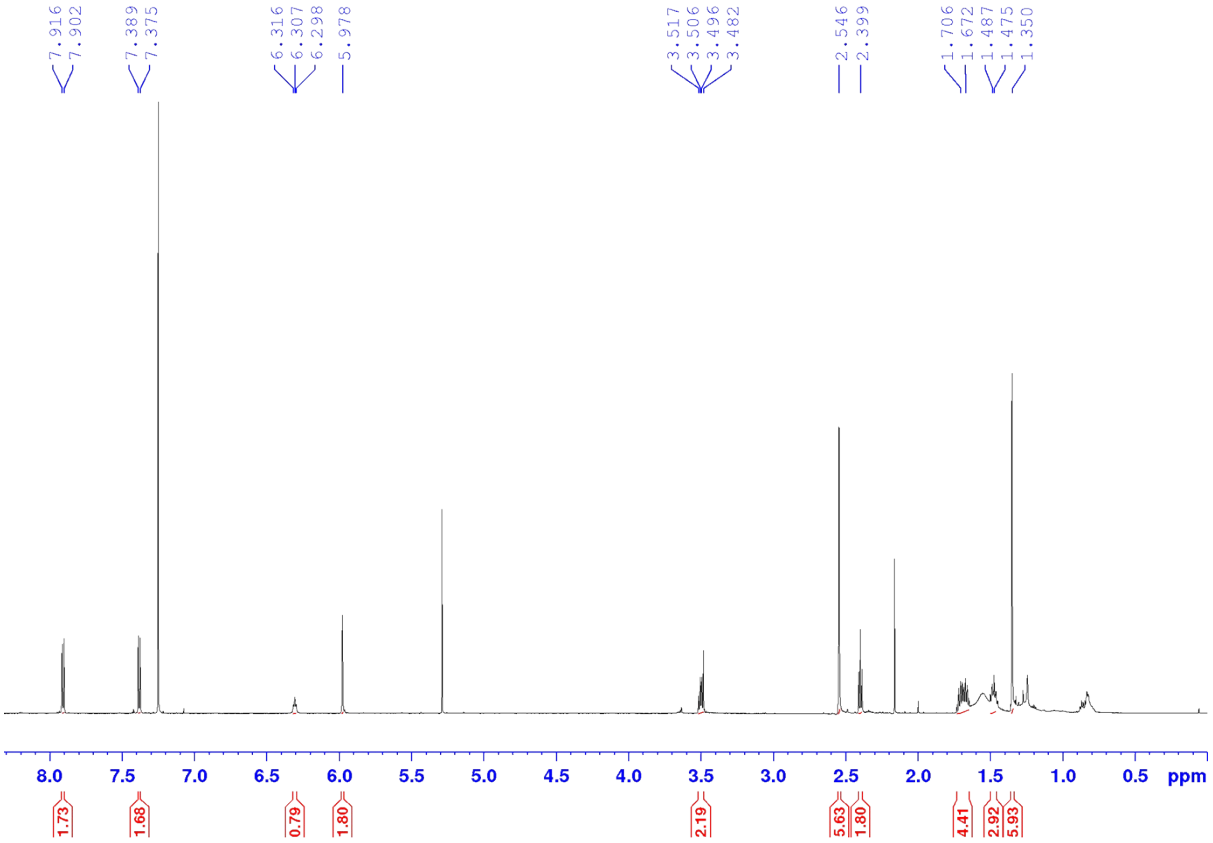
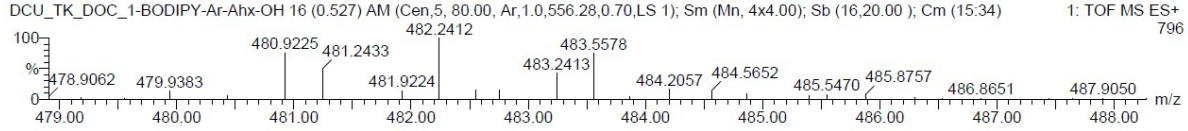


Figure S10 ^{1}H NMR (600 MHz) of **5** in $CDCl_3$.

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 70.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
52 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

External Client
DCU_TK_DOC_1-BODIPY-Ar-Ahx-OH 16 (0.527) AM (Cen,5, 80.00, Ar,1.0,556.28,0.70,LS 1); Sm (Mn, 4x4.00); Sb (16,20.00); Cm (15:34) 1: TOF MS ES+
796



Minimum:				-1.5		
Maximum:		200.0	5.0	70.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
482.2412	482.2427	-1.5	-3.0	12.5	1	C26 H31 B N3 O3 F2

Figure S11 HR-MS (ESI-QTOF): Single Mass Analysis of **2** indicating [M]+

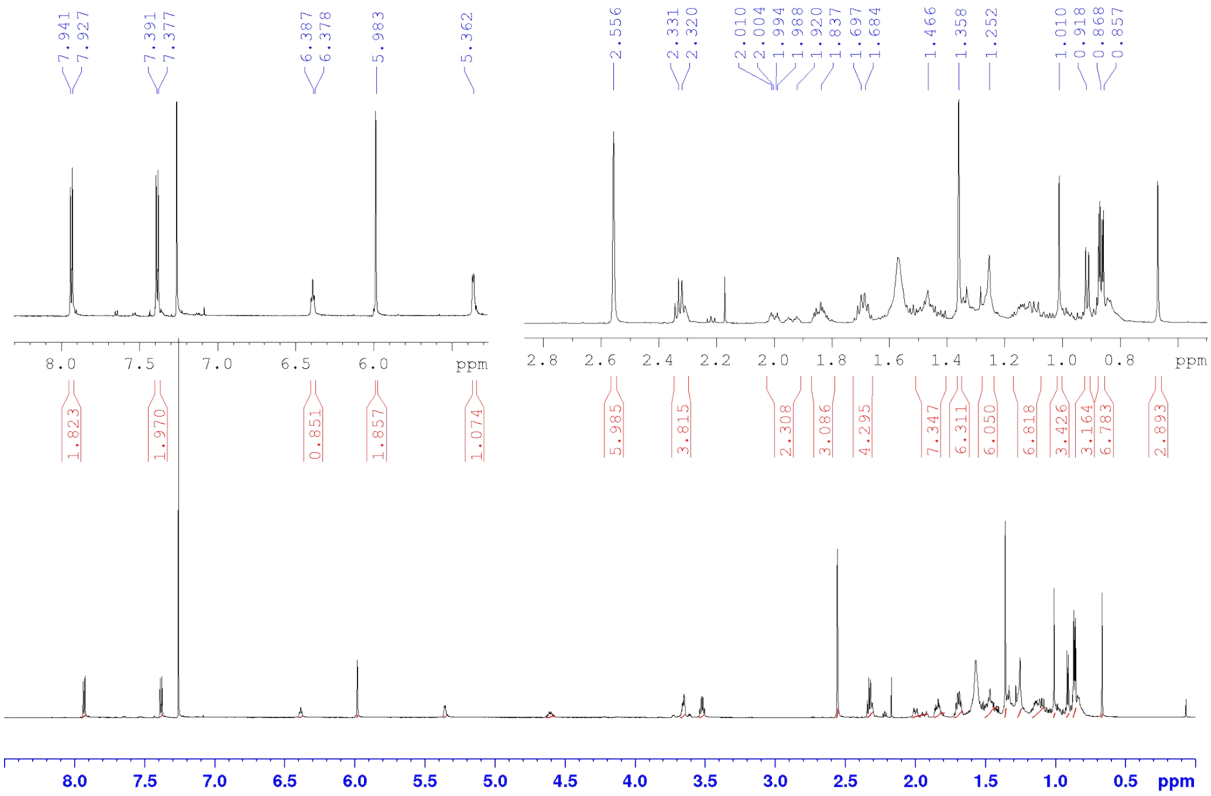


Figure S12 ^{1}H NMR (600 MHz) of **6** in $CDCl_3$.

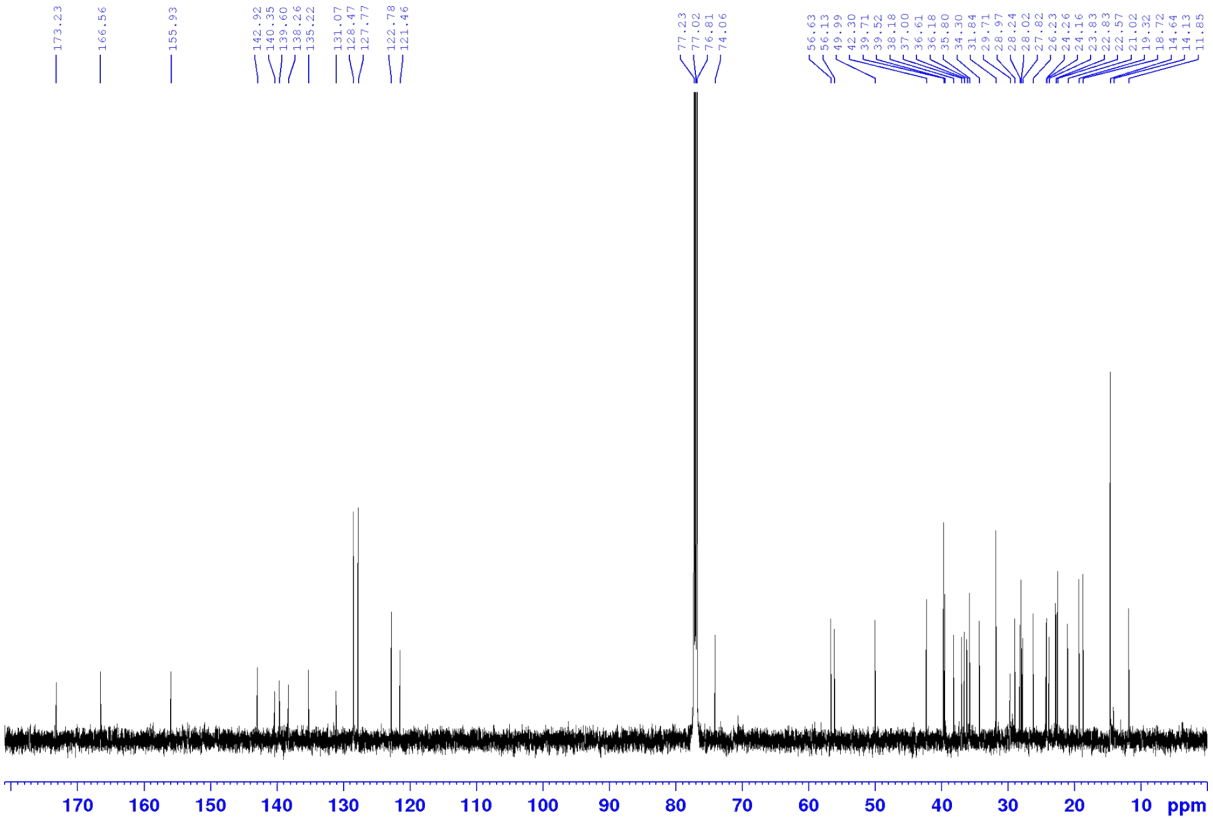


Figure S13 ^{13}C NMR (150 MHz) of **6** in $CDCl_3$.

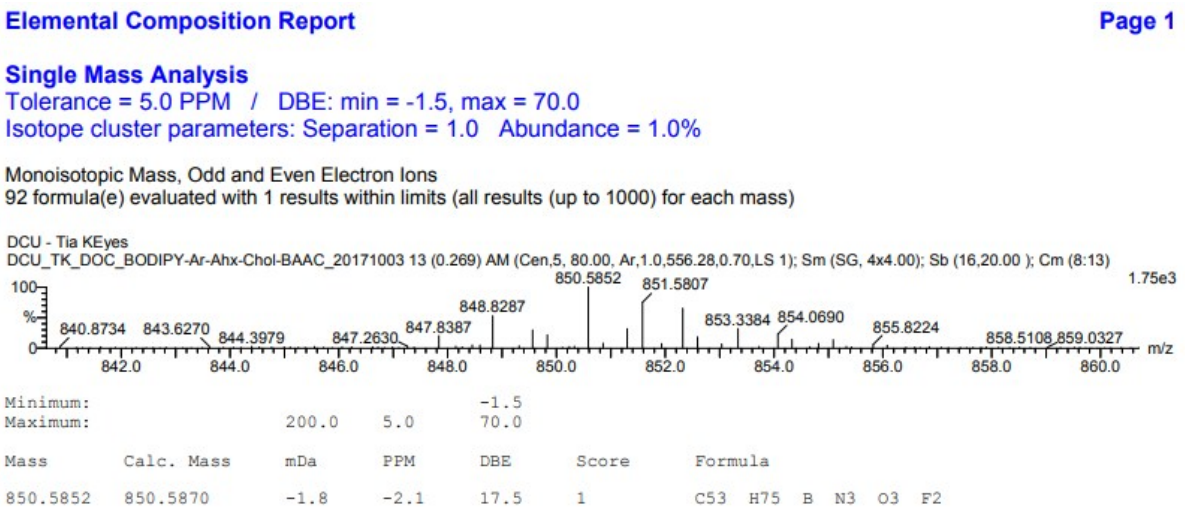


Figure S14 HR-MS (ESI-QTOF): Single Mass Analysis of **6** indicating [M + H].

3. Additional Photophysical Data

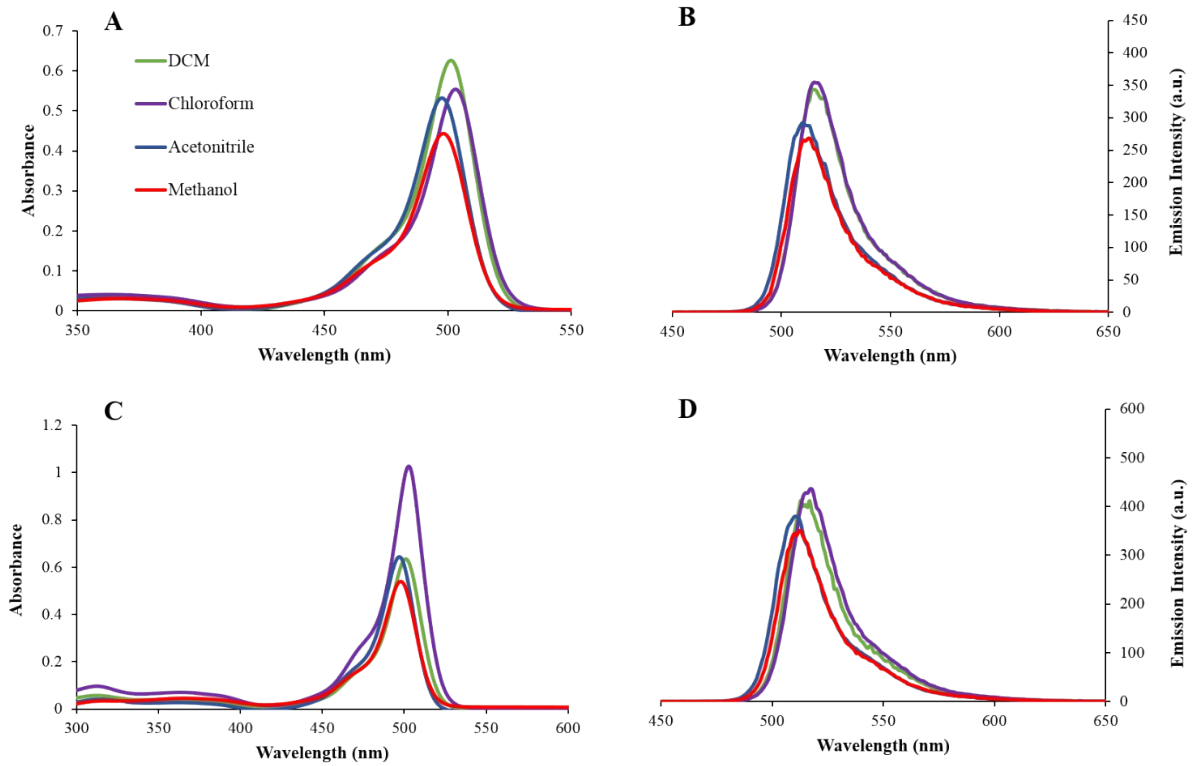


Figure S15 (a) Solvent dependent absorbance curves for 2 (10 µM). (b) Solvent dependent emission curves for 2 (10 µM, slit widths; 2.5 nm). (c) Solvent dependent absorbance curves for 6 (10 µM). (d) Solvent dependent emission curves for 6 (10 µM, slit widths; 2.5 nm).

Quantum yield determination: The quantum yields for **2** and **6** in a range of solvents was calculated using fluorescein as a reference. Fluorescein (in 0.1 M NaOH) (ϕ =0.90)[1]. Solutions were prepared, and absorbance matched, and the optimal excitation was taken as wavelength of intersection. Fluorescence emission spectra were obtained using slit widths 2.5 nm and the following equation was used to calculate the quantum yields.

$$\phi_x = \phi_{st} * (Area_x/Area_{st}) * (\eta^2_x/\eta^2_{st})$$

where the ϕ_x and ϕ_{st} refer to the fluorescence quantum yield of the sample and fluorescein standard, respectively. $Area_x$ and $Area_{st}$ are the integrated emission intensities for the sample and fluorescein standard, respectively. η_x and η_{st} is the refractive index of the solvent for the sample and fluorescein standard, respectively.

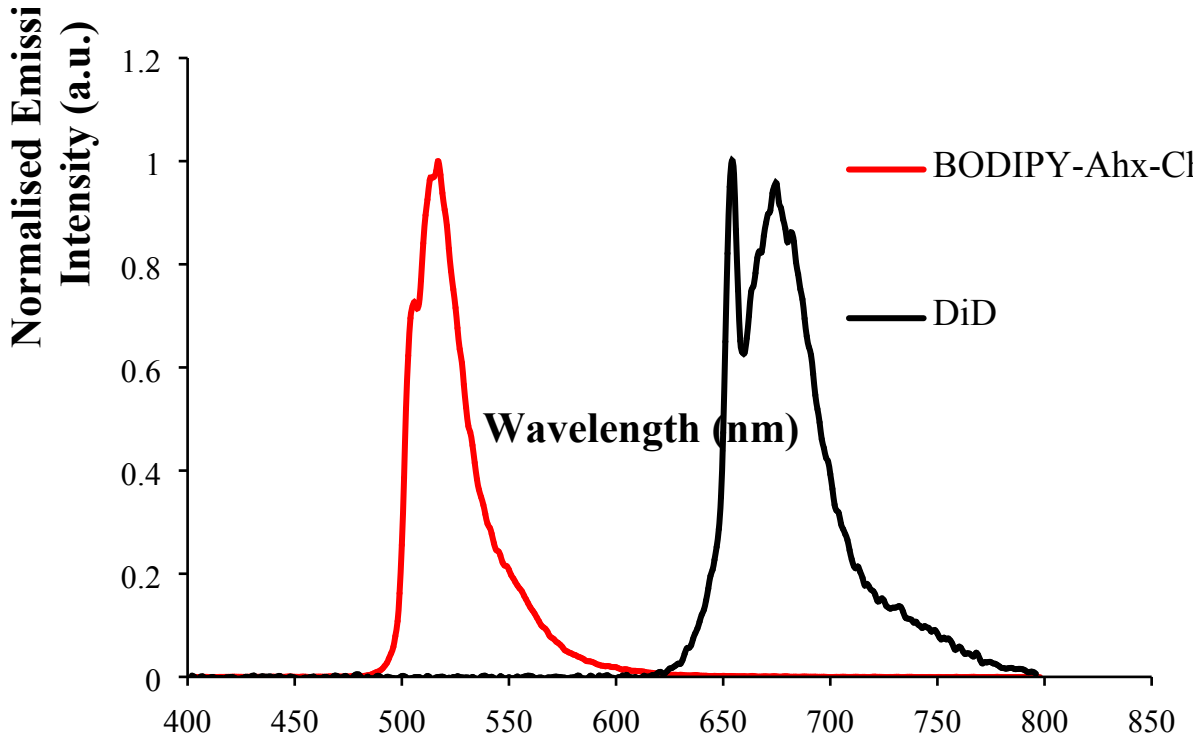


Figure S16 Normalised emission spectra of a mixed solution of chloroform containing both BODIPY-Ahx-Chol (5 µM) (**6**) and DiD (5 µM). BODIPY-Ahx-Chol and DiD have emission maxima of 516 nm and 675 nm respectively. This shows that there is no crosstalk between dyes when used in GUV experiments, using emission filters as stated in experimental sections. For the spectrum of BODIPY-Ahx-Chol, the sample was excited at 503 nm using an excitation slit width of 2.5 nm and an emission slit width of 5 nm. For the spectrum of DiD, the sample was excited at 503 nm using an excitation slit width of 2.5 nm and an emission slit width of 5 nm.

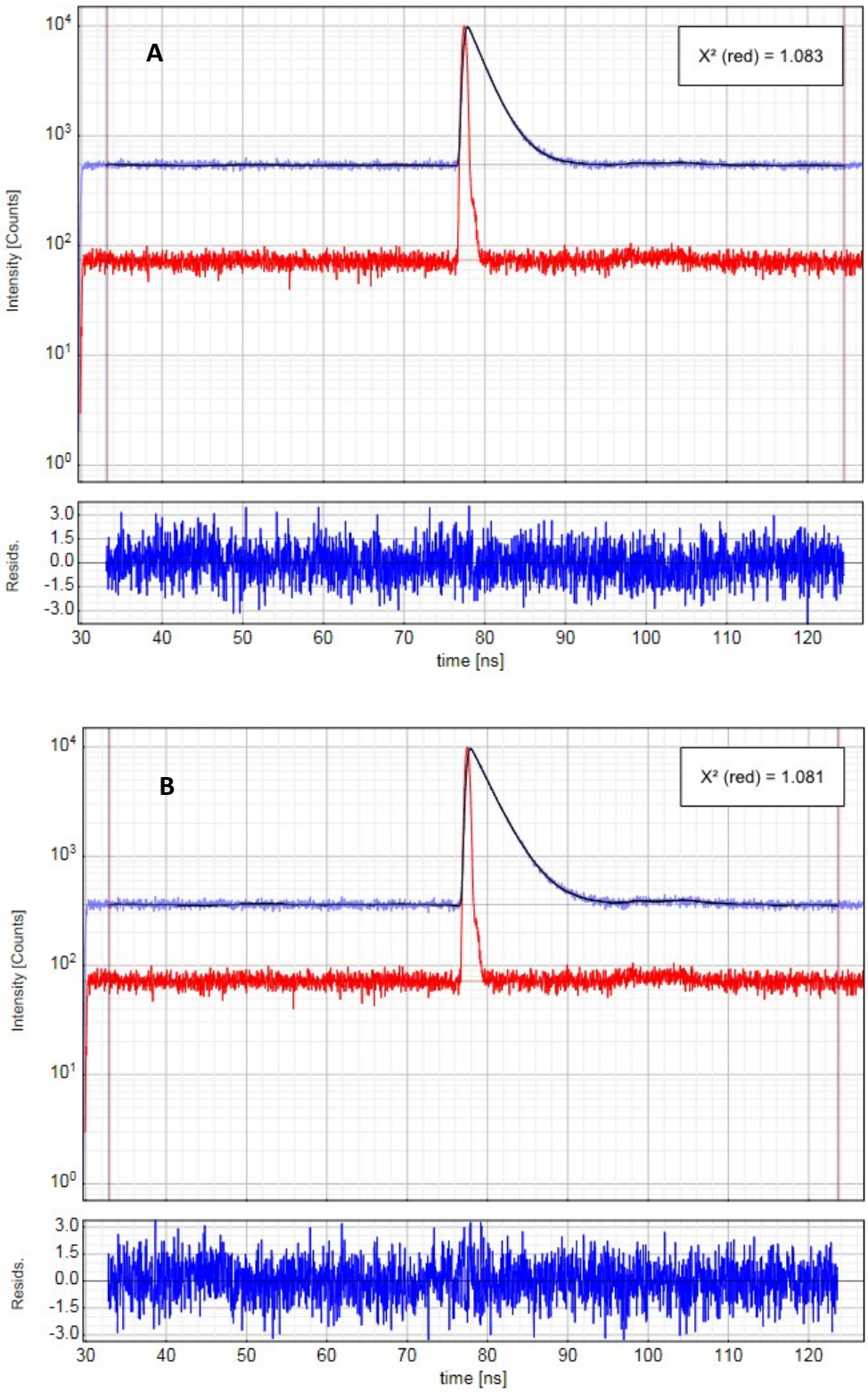


Figure S17 Time correlated single photon counting trace for BODIPY-Ar-Chol (**2**) in methanol (10 µM) with IRF (red) at room temperature (**A**) and for BODIPY-Ar-Ahx-Chol (**6)** in methanol (10 µM) with IRF (red) at room temperature (**B**).

4. Cell Imaging

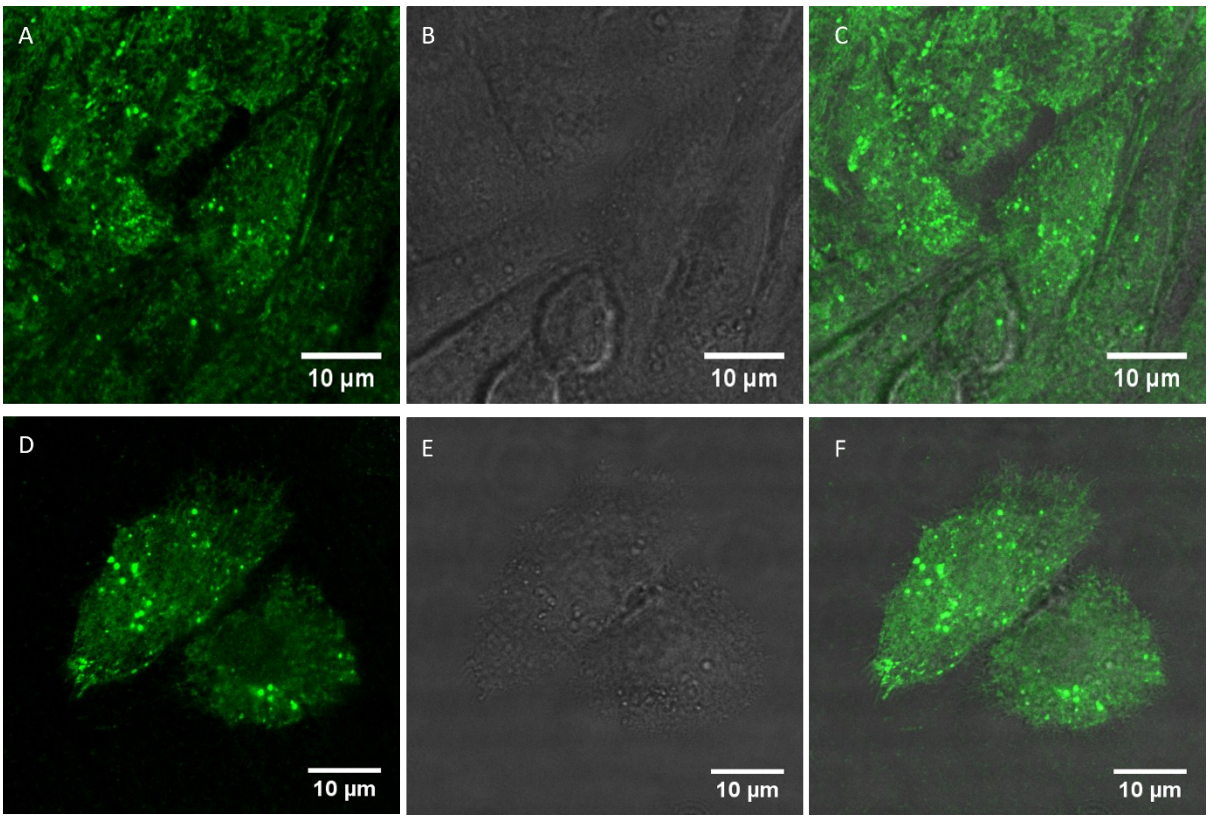


Figure S18 CHO cells stained with BODIPY-Ahx-Chol (5 µM, 2h), showing a group of CHO cells (A-C) and a second cluster of cells (D-F). A, D – BODIPY channel. B, E – overlay of BODIPY channel and background channel. C, F – background channel.

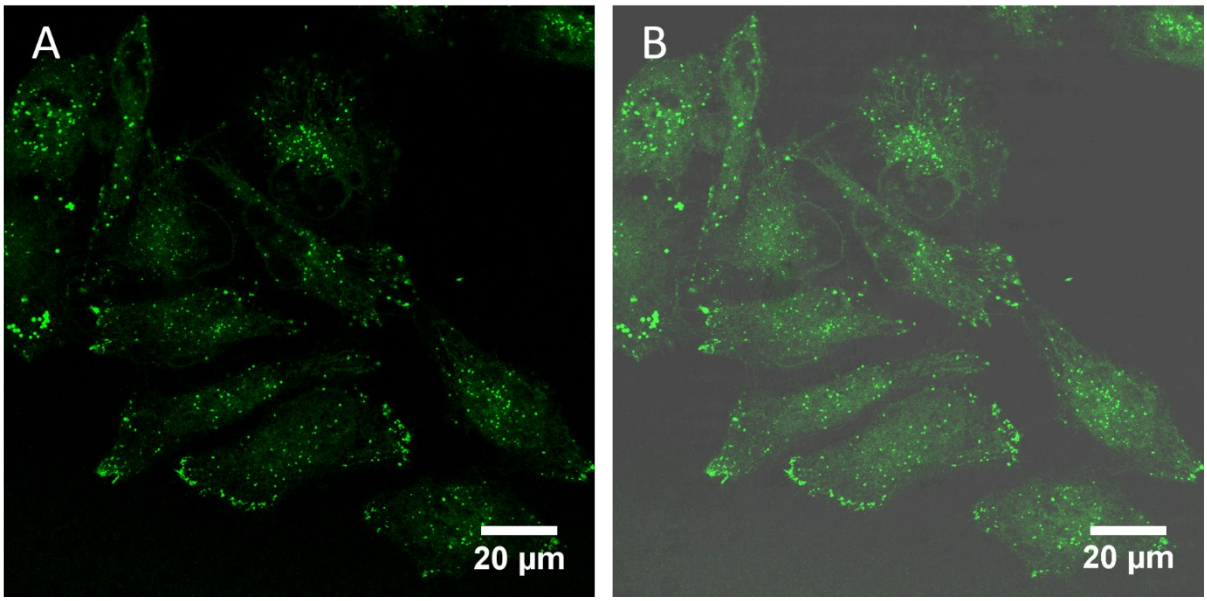


Figure S19 Confocal imaging of live HeLa cells stained with BODIPY-Ar-Chol (5 µM, 37 °C, 3 h).

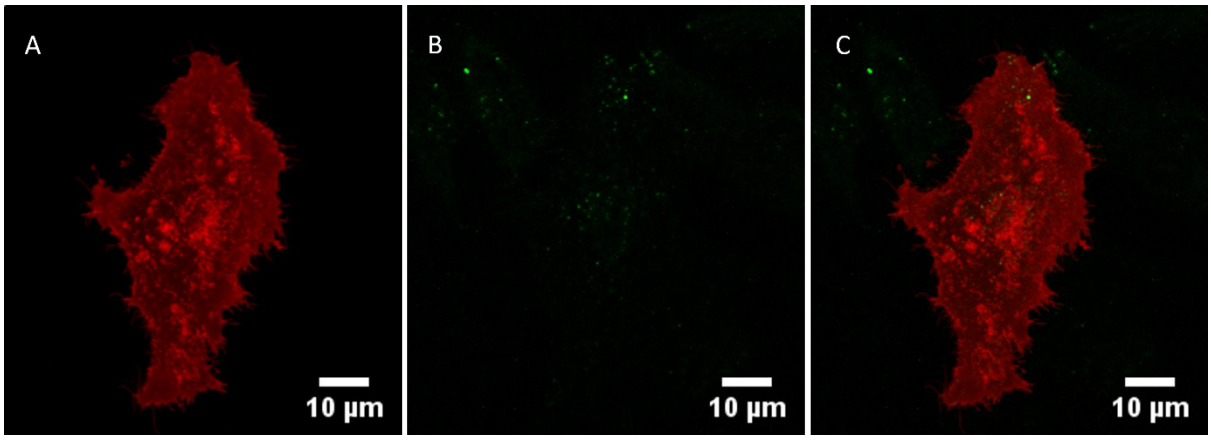


Figure S20 Confocal imaging of live HeLa cell co-stained with DiD (800 nM) (A), BODIPY-Ar-Chol (5 µM) (B), and the overlay of both channels (C). Emission from the BODIPY-Ar-Chol channel is low as the focus is on the cell membrane, where it does not localise.

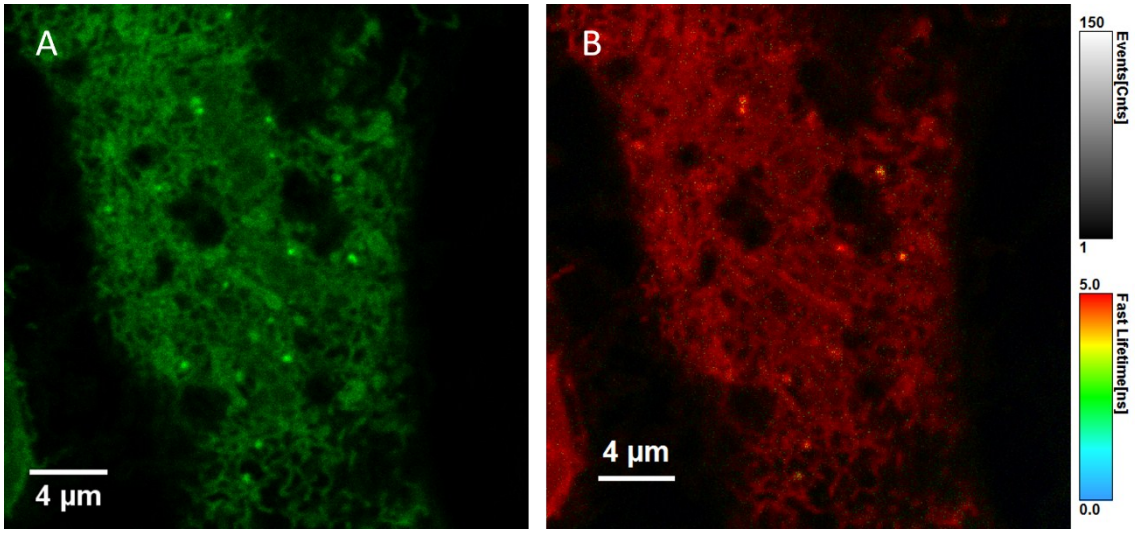


Figure S21 Confocal image of a live HeLa cell stained with BODIPY-Ahx-Chol, focused at the membrane of the cell (A), and the corresponding FLIM lifetime distribution image (B). FLIM was acquired by exciting at 497 nm for 2 minutes.

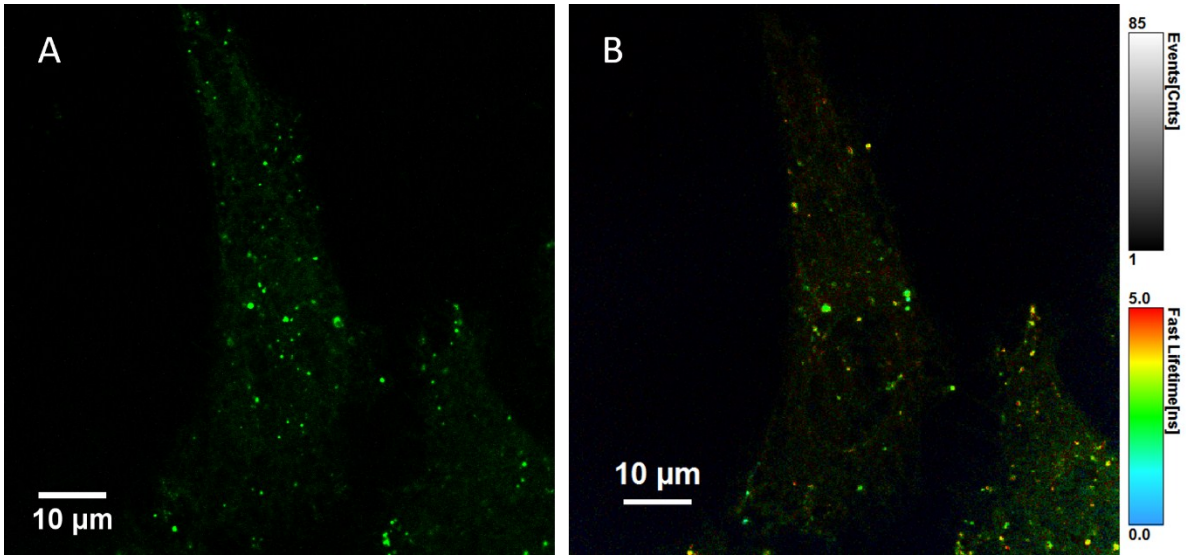
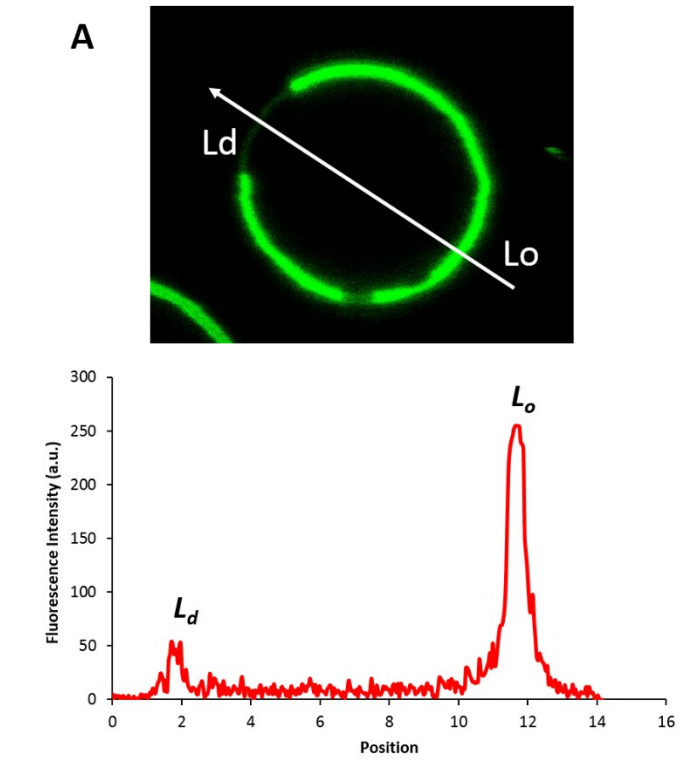


Figure S22 Confocal image of a live HeLa cell stained with BODIPY-Ar-Chol, focused at the membrane of the cell (A), and the corresponding FLIM lifetime distribution image (B). FLIM was acquired by exciting at 497 nm for 2 minutes.

5. Giant Unilamellar Vesicle (GUV) Studies



$$\%L_o = \frac{F(L_o)}{F(L_o) + F(L_d)} x\ 100$$

Figure S23 Calculation of L_o% and L_d% was carried out using intensity profiles through scanning confocal fluorescence images as given by the examples above. Representative scanning confocal fluorescence images of phase were obtained of separated GUVS labelled with BODIPY-Ahx-Chol (A) BODIPY-Ar-Chol (B) respectively. The distribution of these molecules is revealed by the fluorescence intensity (green). The fluorescence intensities of the L_o and L_d phases, $F(L_o)$ and $F(L_d)$ respectively, were determined from the peak maxima of the line scan, where the different phases were identified by the L_d phase markers DiD (fluorescence not shown).

Table S1 Fluorescent lifetimes of BODIPY-Ahx-Chol in live HeLa and CHO cells, with and without treatment using MβCD (10 mM, 4 h)

	HeLa cells		**CHO cells**	
	τ (ns)	**τ_{Amp}** (%)	**τ** (ns)	**τ_{Amp}** (%)
Control	τ_1 = 5.85 ± 0.15 τ_2 = 2.31 ± 0.37	67.6 32.5	τ_1 = 5.3 ± 0.01 τ_2 = 2.4 ± 0.05	63.8 29.3
MβCD	τ_1 = 5.35 ± 0.25 τ_2 = 2.23 ± 0.17	63.6 36.5	τ_1 = 5.85 ± 0.05 τ_2 = 2.89 ± 0.21	68.3 32.7

Table S2 Fluorescent lifetimes of BODIPY-Ar-Chol in live HeLa

	HeLa Cells	
	τ (ns)	**τ_{Amp}** (%)
BODIPY-Ar-Chol	τ_1 = 5.26 ± 0.09 τ_2 = 1.65 ± 0.14	63.5 37.5

References

(1) Crosby, G. A.; Demas, J. N. Measurement of Photoluminescence Quantum Yields. Review. *J. Phys. Chem.* **1971**, *75* (8), 991–1024.