Supporting Material

FLUORESCENCE LIFETIME IMAGING OF MEMBRANE LIPID ORDER WITH A RATIOMETRIC FLUORESCENT PROBE

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Materials and methods

Model membranes

Large unilamellar vesicles (LUVs) were obtained by the extrusion method. Briefly, a suspension of multilamellar vesicles was extruded by using a Lipex Biomembranes extruder (Vancouver, Canada). The size of the filters was first 0.2 µm (7 passages) and thereafter 0.1 µm (10 passages). This generates LUVs with a mean diameter of 0.11 µm, as measured with a Malvern Zetamaster 300 (Malvern, UK). A 20 mM phosphate buffer pH 7.4 was used in these experiments. LUVs were labeled by adding aliquots of probe stock solutions in dimethylsulfoxide (DMSO) to 1 mL solution of vesicles. Concentrations of the probes and lipids were chosen to get a ratio of 1 fluorophore per 100 lipids.

Giant unilamellar vesicles (GUVs) were electroformed in a home-built liquid cell (University of Odense, Denmark), using previously described procedures. A 1 mM solution of lipids in chloroform was deposited on the platinum wires of the chamber and the solvent was evaporated under vacuum for 30 min. The chamber, thermostated at 55 °C, was filled with a 300 mM sucrose solution, and a 2-V, 10-Hz alternating electric current was applied for ca 2 h. After cooling down to room temperature, 50 µL of the obtained stock solution of GUVs was stained by addition of an aliquot of the probe stock solution in DMSO to obtain a 0.1 µM final probe

concentration (final DMSO volume < 0.25%). Finally, labeled GUVs in sucrose were added to 200 µL of 0.1% (w/w) agarose solution at 60°C and incubated at 40°C for 30 min and then cooled down to room temperature to give the final suspension of GUVs.

Deconvolution of F2N12S fluorescence spectra

The deconvolution of F2N12S fluorescence spectra into three bands (N*, H-N*and T*) was carried out by using the PeakFit 4 software. The individual emission bands were approximated by a log-normal function that accounts for several parameters (maximal amplitude, and positions of the maximum and half-maximum intensities). The asymmetry, full width half-maximum (FWHM) and maximum position of the H-N* band were determined from the spectrum of F2N12S in ethanol which exhibits only this band. The same parameters for N* and T* bands were obtained from the deconvolution of F2N12S spectrum in sphingomyelin/cholesterol (SM/Ch) LUVs where the H-N* band is absent. As a result, deconvolution of the spectra of F2N12S in LUVs of various compositions was performed by fixing the asymmetry and FWHM values of all three bands as well as the position of maximum of the H-N* band. After deconvolution, the fluorescence intensities of the separated N*, H-N* and T* bands at their maximum emission wavelength were used for calculating the hydration parameter by $I_{H\text{-}N^*}/(I_{N^*} + 0.4I_{T^*})$. The 0.4 factor takes into account that the FWHM is ~2.5-fold narrower for the T* band than for the N* and H-N* bands. The polarity parameter of the H-bond free form was estimated by I_{N^*}/I_{T^*}.

Supplementary Tables

Table S1: Time-resolved fluorescence parameters of F2N12S in Ld and Lo LUVs.

	λ, nm	τ_1	a_1	τ_2	a_2	τ_3	a_3	τ_4	a_4	τ_5	a_5	τ_m
DOPC	490	0.03	0.06	0.21	0.45	0.81	0.24	2.32	0.25	0	0	0.87
	510	0.03	0.07	0.309	0.32	1.02	0.25	2.84	0.36	0	0	1.38
	530	0.03	0.06	0.52	0.22	1.27	0.16	2.92	0.56	0	0	1.95
	570	0.032	-1	0.27	0.22	1.1	0.11	3.1	0.67	0	0	2.26
	590	0.033	-1	0.26	0.195	1.23	0.04	2.74	0.765	0	0	2.20
	610	0.054	-1	0.24	0.16	1.15	0.06	2.87	0.78	0	0	2.35
Sm/Ch-2:1	490	0.03	0.05	0.06	0.76	0.52	0.06	2.14	0.04	5.89	0.09	0.69
	510	0.03	0.04	0.10	0.58	0.66	0.12	2.20	0.08	5.75	0.18	1.35
	530	0.03	0.04	0.11	0.47	0.69	0.14	2.21	0.13	5.60	0.22	1.67
	570	0.03	-1	0.2	0.215	1.05	0.05	1.96	0.07	5.77	0.665	4.07
	590	0.031	-1	0.14	0.19	1.11	0.04	2.59	0.14	6.49	0.63	4.52
	610	0.021	-1	0	0	0	0	2.06	0.19	6.2	0.81	5.41

Time-resolved fluorescence decays of F2N12S-labeled LUVs were measured by TCSPC in cuvettes. The fluorescence lifetimes (in ns) and the amplitudes are given as means for 3 experiments. Standard deviations on lifetimes and amplitudes are below 15%. Mean lifetimes are calculated as: $\tau_m = \sum a_i \tau_i$, using only lifetime components associated to positive amplitudes.

Lifetimes shown in Fig. 2A and B in the main text were calculated by averaging the lifetime components at the different wavelengths, using: $\tau_i = \frac{\sum \tau_\lambda}{n_\lambda}$, where τ_λ is the lifetime at a given emission wavelength, and n_λ is the number of wavelengths used in the calculation.

Table S2. Time-resolved fluorescence parameters of F2N12S in DOPC/Ch LUVs.

DOPC	Ch	λ, nm	τ_1	a_1	τ_2	a_2	τ_3	a_3	τ_4	a_4	τ_5	a_5	τ_m
0.93	0.07	590	0.029	-1	0.36	0.36	1.28	0.33	3.05	0.27	6.00	0.04	1.63
0.86	0.14	590	0.043	-1	0.35	0.39	0.74	0.24	2.87	0.33	6.39	0.04	2.24
0.75	0.25	590	0.042	-1	0.38	0.16	1.18	0.26	2.83	0.51	6.08	0.07	2.24
0.68	0.32	590	0.033	-1	0.37	0.11	0.89	0.04	2.77	0.74	5.48	0.11	2.70

Time-resolved fluorescence decays of F2N12S-labeled LUVs were measured by TCSPC in cuvettes. Excitation and emission wavelengths were 315 nm and 590 nm, respectively. The fluorescence lifetimes (in ns) and the amplitudes are given as means for 3 experiments. Standard

deviations on lifetimes and amplitudes are < 15%. The mean lifetime is calculated as in Table S1.

Lifetimes shown in Fig. 4B in the main text were calculated by averaging the lifetime components at the different cholesterol fractions from Table 2, using: $\tau_i = \frac{\sum \tau_f}{n_f}$, where τ_f is the lifetime at a given cholesterol fraction and n_f is the number of fractions used in the calculation.

Table S3: Time-resolved fluorescence parameters of F2N12S in HeLa cells.

Cells	λ, nm	τ_1	a_1	τ_2	a_2	τ_3	a_3	τ_4	a_4	τ_5	a_5	τ_m
Intact	590	0.044	-1	0.22	0.09	1.09	0.05	3.50	0.45	6.8	0.41	4.5
Cholesterol depleted	590	0.030	-1	0.14	0.14	0.71	0.21	3.03	0.53	7.01	0.12	2.6

Time-resolved fluorescence decays of F2N12S-labeled intact or cholesterol-depleted cells were measured by TCSPC in cuvettes. The meaning and expression of parameters are as in Table S1.

Lifetimes shown in Fig. 6B in the main text were calculated by averaging the lifetime components from Table S3 for intact and cholesterol-depleted cells.

Supplementary Figures

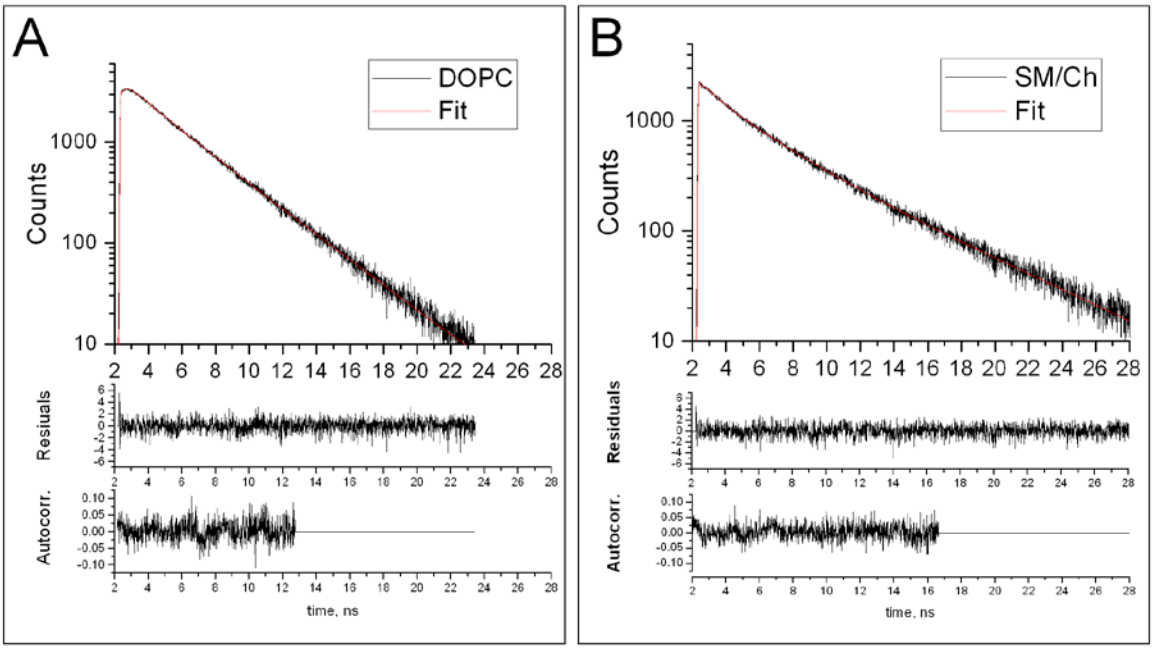

Figure S1. Time-resolved emission decays of F2N12S in Ld LUVs and Lo LUVs. Experiments were performed with F2N12S in Ld LUVs composed of DOPC (A) and Lo LUVs composed of SM/Ch (2:1) (B). The emission decays were recorded at 590 nm, using an excitation wavelength at 315 nm. Concentrations of lipids and F2N12S were 200 μM and 2 μM, respectively. The red curves in the upper panels correspond to the fits to the decay curves using the maximum entropy method and the lifetime components from Table S1. The residuals and the autocorrelation curves in the lower panels show an excellent fit of the decay curves.

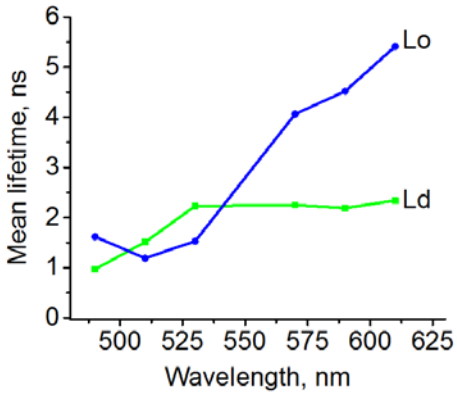

Figure S2. Dependence of F2N12S mean lifetime on the excitation wavelength. Experiments were done with F2N12S in Ld LUVs composed of DOPC (green) and Lo LUVs composed of SM/Ch (2:1) (blue). Concentrations of lipids and F2N12S were 200 μM and 2 μM, respectively. Excitation wavelength was 315 nm.