Electronic Supplementary Information

Macroscopically homogeneous lipid phase exhibits various leaflet-specific lipid diffusion in a glass-supported lipid bilayer

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Detailed descriptions of 2D FLCS and FLCS analyses.

We performed two-dimensional fluorescence lifetime correlation spectroscopy (2D FLCS) analysis to determine the fluorescence decay curves of fluorescent lipids in the bulk as well as glass-side leaflets of a supported lipid bilayer (SLB).[4,5] Then, we subsequently performed fluorescence lifetime correlation spectroscopy (FLCS) analysis to calculate leaflet-specific fluorescence intensity-correlation curves.[6] In this section, analytical details of 2D FLCS and FLCS are described as follows.

2D FLCS. By using a pulse laser as an excitation light source, two temporal information are obtained from each detected photon. Macrotime (T) is the absolute detection time of photon from the start of the experiment whereas microtime (t) is the emission delay time of the photon detection with respect to the corresponding excitation pulse. In 2D FLCS analysis, a 2D emission-delay correlation map ($M(\Delta T, t', t'')$) at arbitrary ΔT is constructed using this information.[4,5] In the map, t' and t'' correspond to microtimes of 1st and 2nd photons in photon pairs, respectively, and each photon in the photon pair is temporally separated by ΔT. Because the map contains both correlated and uncorrelated photon pairs, the uncorrelated signals are subtracted to obtain the map for the correlated signals ($M_{\mathrm{cor}}(\Delta T, t', t'')$). For that purpose, a 2D emission-delay correlation map at longer ΔT is calculated as a representative of the uncorrelated signals because the correlation is completely lost at that ΔT. In this study, the choice of ΔT for the uncorrelated signals depends on the lipid compositions.

To identify the independent lifetime species and to determine their fluorescence lifetime distributions (and the corresponding fluorescence decay curves), 2D inverse Laplace transform is performed on $M_{\mathrm{cor}}(\Delta T, t', t'')$ with the help of maximum-entropy method (2D MEM). Because $M_{\mathrm{cor}}(\Delta T, t', t'')$ is described with the sum of the single-molecule correlation, it can be represented by the following equations:

$$M_{\mathrm{cor}}(\Delta T; t_i', t_j'') = \sum_{k=1}^{L}\sum_{l=1}^{L} \widetilde{M}(\Delta T; \tau_k', \tau_l'') \exp(-t_i'/\tau_k') \exp(-t_j''/\tau_l''), \quad \text{(S3)}$$

$$\widetilde{M}(\Delta T; \tau_k', \tau_l'') = \sum_{i=1}^{n} a_i(\tau_k') a_i(\tau_l''), \quad \text{(S4)}$$

where L is the number of data points along the lifetime (τ) scale, $a_i(\tau)$ is the independent lifetime distribution of species i, and n is the number of the independent species. In 2D MEM analysis, we set a trial 2D lifetime correlation map $\widetilde{M}^0(\Delta T;\tau',\tau'')$ to calculate a simulated 2D emission-delay correlation map and compare it with an experimental one.

$$M^0_{\mathrm{cor}}(\Delta T;t'_i,t''_j)=\sum_{k=1}^{L}\sum_{l=1}^{L}\widetilde{M}^0(\Delta T;\tau'_k,\tau''_l)\exp(-t'_i/\tau'_k)\exp(-t''_j/\tau''_l)\,, \tag{S5}$$

$$\widetilde{M}^0(\Delta T;\tau'_k,\tau''_l)=\sum_{i=1}^{n}a_i{}^0(\tau'_k)a_i{}^0(\tau''_l)\,. \tag{S6}$$

The fitting error (χ^2) and the entropy (S) of the 2D lifetime correlation map can be defined as:

$$\chi^2=\frac{1}{K^2-1}\sum_{i=1}^{K}\sum_{j=1}^{K}\frac{\left\{M^0_{\mathrm{cor}}(\Delta T;t'_i,t''_j)-M_{\mathrm{cor}}(\Delta T;t'_i,t''_j)\right\}^2}{M(\Delta T;t'_i,t''_j)}\,, \tag{S7}$$

$$S=\sum_{i=1}^{n}\sum_{k=1}^{L}\left\{a_i{}^0(\tau_k)-m_i(\tau_k)-a_i{}^0(\tau_k)\ln\frac{a_i{}^0(\tau_k)}{m_i(\tau_k)}\right\}. \tag{S8}$$

In eq S8, $m_i(\tau)$ is a priori knowledge of $a_i{}^0(\tau)$. In this work, we set a constant value for $m_i(\tau)$. The optimum $\widetilde{M}^0(\Delta T;\tau',\tau'')$ that minimizes the following Q value is then searched and determined,

$$Q=\chi^2-\frac{2S}{\eta}\,, \tag{S9}$$

where η is the regularizing constant.

In this study, we performed a global 2D-MEM analysis using a set of 2D emission-delay correlation maps calculated at different ΔTs. In a global 2D-MEM analysis, a 2D lifetime correlation map can be described as follows:

$$\widetilde{M}(\Delta T;\tau'_k,\tau''_l)=\sum_{i=1}^{n}\sum_{j=1}^{n}g_{ij}(\Delta T)a_i(\tau'_k)a_i(\tau''_l)\ . \tag{S10}$$

In eq S10, $g_{ij}(\Delta T)$ is the autocorrelation ($i=j$) or the cross-correlation ($i\neq j$) between species i and j. In a global 2D-MEM analysis, we fixed the autocorrelation of the shortest ΔT ($g_{ii\,(}\Delta T)$) to unity and that of the cross-correlation to 0. 2D emission-delay correlation maps are then globally analyzed by using common $a_i(\tau)$.

In the measurement, microtime information was recorded for every detected photon with the microtime resolution of 3.1 ps/ch. Total channels available are 4096. The channels (resolution) were reduced to 256 (49 ps/ch) by binning 16 adjacent data points into a single channel, and the photon data with reduced microtime were used for constructing 2D emission-delay correlation maps. For 2D MEM analysis, the microtime range from 0 to ~6 ns was selected from those maps. (Precise time range is dependent on the lipid compositions.) For saving the computation time, the microtime resolutions of the selected 2D emission-delay correlation maps were further reduced to 24 × 24 channels. In this procedure, the binning width was changed logarithmically along the microtime axes to keep the lifetime information as much as possible. Discrete τ values (total 40 τ points) that are equally distributed in a logarithmic scale between 0.07 ns and 9.76 ns were used for determining $a_i(\tau)$ and $g_{ij}(\Delta T)$. Global 2D-MEM analysis was performed by increasing n until the simulated 2D emission-delay correlation map satisfies an experimental one as judged from χ^2 and the residuals. In this experiment, $n = 2$ is sufficient to describe the raw 2D map. Two independent lifetime species correspond to the fluorescent lipids in the glass- (longer lifetime species) and bulk-side (shorter lifetime species) leaflets of the supported lipid bilayer. To obtain the species-specific fluorescence decay curve, Laplace transform is performed on $a_i(\tau)$.

FLCS. In FLCS analysis, one calculates a filter value of each species (i) at microtime channel j (f_{ij}, $1 \le j \le N$) as follows, using the intensity-normalized fluorescence decay curve of each species (p_{ij}) and the ensemble-averaged decay curve I_j that is calculated from all detected photons:[6] (N is the maximum microtime channel used for the analysis.)

$$\mathbf{F} = [\mathbf{M}^T \cdot \mathrm{diag}(\mathbf{I})^{-1} \cdot \mathbf{M}]^{-1} \cdot \mathbf{M}^T \cdot \mathrm{diag}(\mathbf{I})^{-1}. \qquad \text{(S11)}$$

In eq S11, $\mathbf{F}$ is a $i \times N$ matrix with elements f_{ij}, $\mathbf{M}$ is a $i \times N$ matrix with elements p_{ij}, and $\mathbf{I}$ is a column vector with elements I_j. Obtained filter values can be used to generate the species-specific fluorescence intensity-correlation function based on the following equation:

$$G_{kl}(\Delta T) = \frac{\sum_{j=1}^{N}\sum_{j'=1}^{N} f_{kj} f_{lj'} \left\langle I_j(T) I_{j'}(T+\Delta T) \right\rangle}{\sum_{j=1}^{N} \left\langle f_{kj} I_j(T) \right\rangle \sum_{j'=1}^{N} \left\langle f_{lj'} I_{j'}(T) \right\rangle}. \quad \text{(S12)}$$

In eq S12, point brackets represent T-averaging. If $k = l$, $G_{kl}(\Delta T)$ corresponds to the autocorrelation of species k whereas $G_{kl}(\Delta T)$ corresponds to the cross-correlation between species k and l when $k \neq l$.

In the actual analysis, p_{ij} is calculated by performing Laplace transform on the independent lifetime distributions obtained by 2D FLCS analysis. Filter values and species-specific fluorescence correlation curves are then calculated by using photon data, I_j, p_{ij}, eq S11, and eq S12 with a microtime range from 0 ns < t < ~6 ns corresponding to $1 \leq j \leq$ ~2000. It is noted that species-specific correlation curves are calculated on the photon-by-photon basis as described in the literature.[7,8]

All the calculation in 2D FLCS and FLCS analyses was performed by a code written in C and was run by Igor Pro (Wavemetrics).

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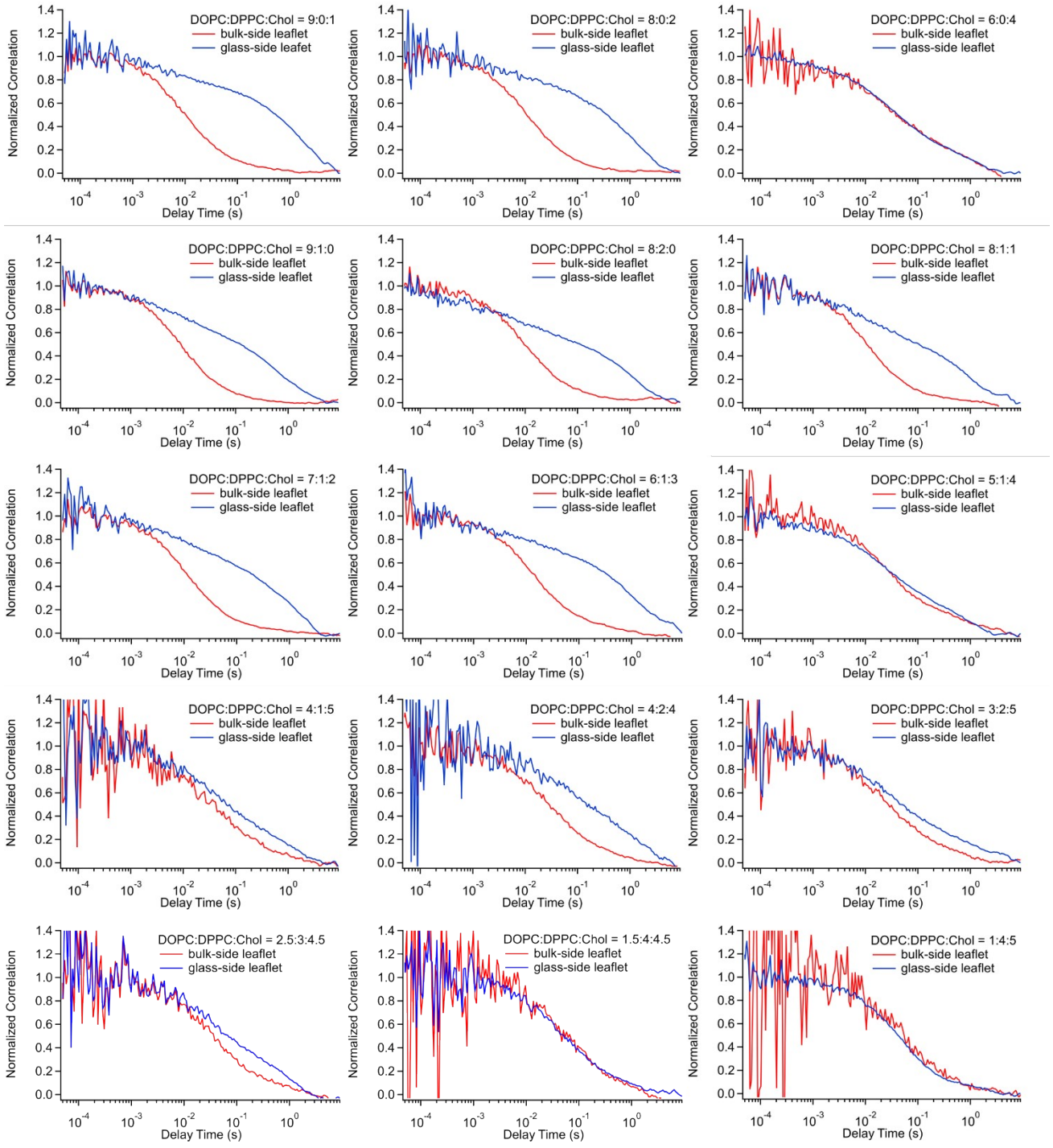

Figure S1. Leaflet-specific autocorrelation curves of fluorescent lipids in supported lipid bilayers. Lipid composition of each data is written in the corresponding figure.

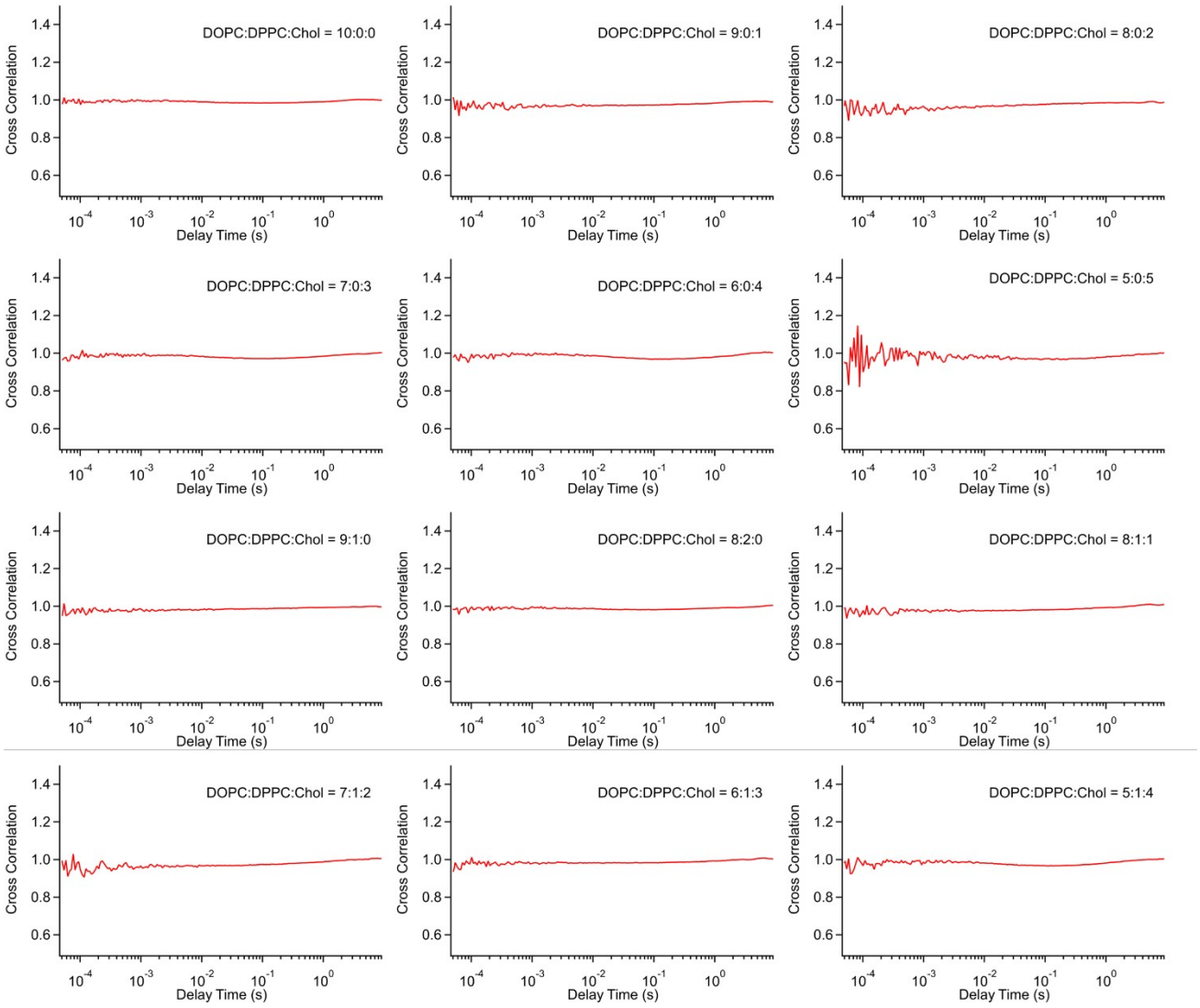

Figure S2. Leaflet-specific cross-correlation curves of fluorescent lipids between the two leaflets of supported lipid bilayers. Lipid composition of each data is written in the corresponding figure.

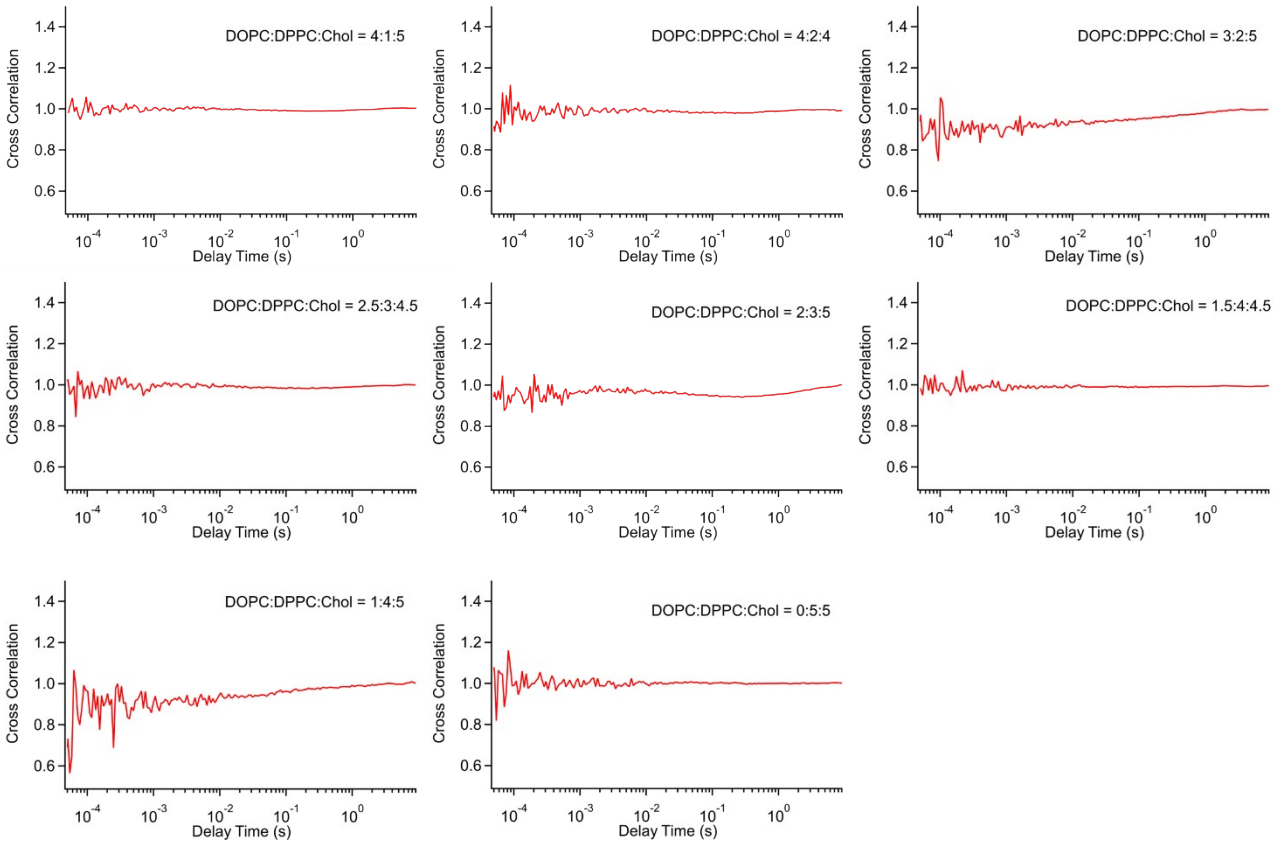

Figure S3. Leaflet-specific cross-correlation curves of fluorescent lipids between the two leaflets of supported lipid bilayers. Lipid composition of each data is written in the corresponding figure.