



## Data Article

# Dataset of asymmetric giant unilamellar vesicles prepared via hemifusion: Observation of anti-alignment of domains and modulated phases in asymmetric bilayers.



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## ABSTRACT

The data provided with this paper are confocal fluorescence images of symmetric giant unilamellar vesicles (GUVs) and asymmetric giant unilamellar vesicles (aGUVs). In this work, aGUVs were prepared using the hemifusion method and are labeled with two different fluorescent dyes, named TFPC and DiD. Both dyes show strong preference for the liquid-disordered (Ld) phase instead of the liquid-ordered (Lo) phase. The partition of these dyes favoring the Ld phase leads to bright Ld phase and dark Lo phase domains in symmetric GUVs observed by fluorescence microscopy. In symmetric vesicles, the bright and the dark domains of the inner and the outer leaflets are aligned. In aGUVs, the fluorescent probe TFPC exclusively labels the aGUV outer leaflet. Here, we show a dataset of fluorescence micrographs obtained using scanning fluorescence confocal microscopy. For the system chosen, the fluorescence signal of TFPC and DiD show anti-alignment of the brighter domains on aGUVs. Important for this dataset, TFPC and DiD have fluorescence

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emission centered in the green and far-red region of the visible spectra, respectively, and the dyes' fluorescence emission bands do not overlap. This dataset were collected in the same conditions of the dataset reported in the co-submitted work (Enoki, et al. 2021) where most of aGUVs show domains alignment. In addition, we show micrographs of GUVs displaying modulated phases and macrodomains. We also compare the modulated phases observed in GUVs and aGUVs. For these datasets, we collected a sequence of micrographs using confocal microscopy varying the z-position, termed a z-stack. Images were collected in a scanning microscope Nikon Eclipse C2+ (Nikon Instruments, Melville, NY). Additional samples used to measure the lipid concentrations and to prepare GUVs with accurate lipid fractions are also provided with this paper.

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## Specifications Table

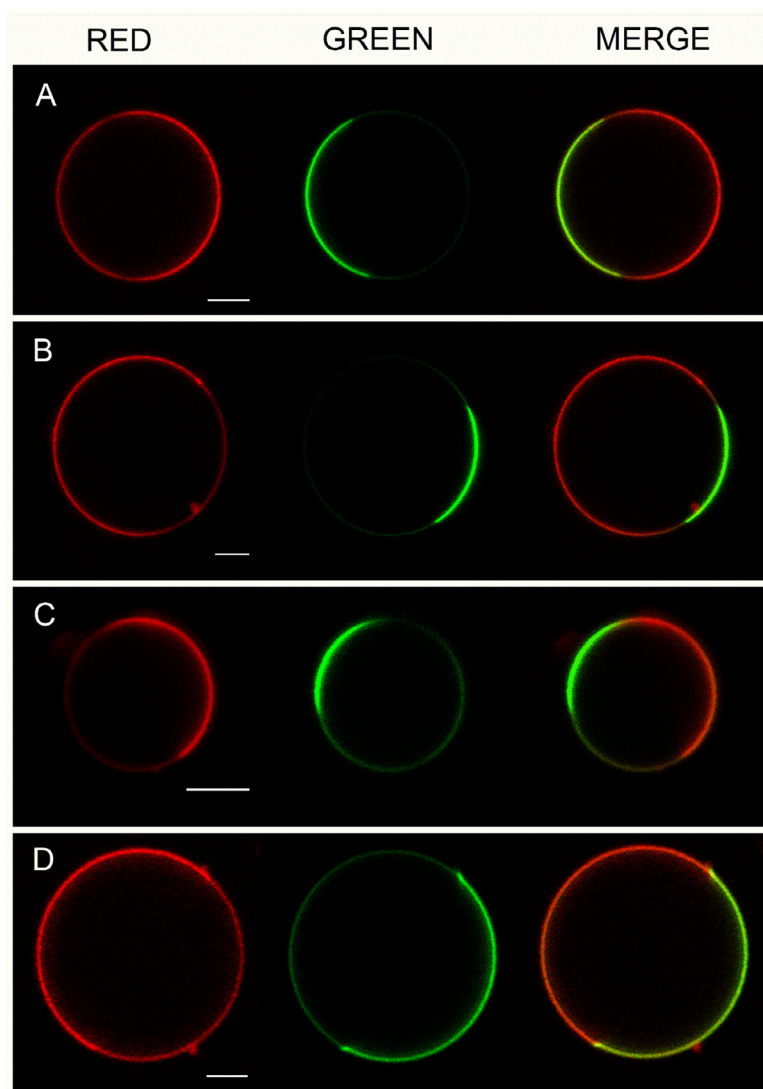
Subject	Biophysics and Molecular Biology
Specific subject area	Biophysics of model membranes, lipid bilayers and asymmetric lipid bilayers.
Type of data	Image Image series Graph
How data were acquired	Data were acquired with a scanning fluorescence confocal microscope Nikon Eclipse C2+ (Nikon Instruments, Melville, NY) and software NIS-Elements Basic Research.
Data format	Raw data images: .nd2 microscopy images, .nd2 microscopy z-stack series. Raw data spectra: fluorescence emission spectra and absorbance in Microsoft Excel .xlsx Analysed: Supplementary material (lipid compositions and phosphate assay) Microsoft Excel .xlsx Filtered images: .tiff and .png files. List of files: Microsoft word .docx
Parameters for data collection	Using the software NIS-Elements Basic Research, we set the following parameters. Objective: Plan Apo VC 60x A WI DIC N2. Pinhole size: 40 $\mu\text{m}$ . For aGUVs, we use laser lines of wavelength $\lambda = 488 \text{ nm}$ and $\lambda = 640 \text{ nm}$ to excite TFPC and DiD. For additional GUV images, we use laser lines of wavelength $\lambda = 408 \text{ nm}$ and $\lambda = 561 \text{ nm}$ to excite Naphthopyrene and DiC12.
Description of data collection	Images of symmetric and asymmetric giant unilamellar vesicles were collected with a confocal microscope at room temperature $T = 23 \text{ }^\circ\text{C}$ . Asymmetric GUVs (aGUVs) were labelled with two different fluorescence dyes, which display emission peaks in the green and far-red region of the visible spectrum. The green fluorophore labels the aGUV outer leaflet. Moreover, dataset compare modulated phases in symmetric GUVs and aGUVs.
Data source location	Cornell University, Ithaca, NY, USA.
Data accessibility	A. Enoki, Thais; Wu, Joy; Heberle, Frederick; Feigenson, Gerald (2021), "Asymmetric GUVs: inner and outer leaflets with anti-registered phase separation", Mendeley Data, V1, <a href="https://doi.org/10.17632/hwkg38j854.1">https://doi.org/10.17632/hwkg38j854.1</a>
Related research article	T.A. Enoki, J. Wu, F. A. Heberle and G. W. Feigenson, Investigation of the domain line tension in asymmetric vesicles prepared via hemifusion, <i>Biochimica et Biophysica Acta</i> . <a href="https://doi.org/10.1016/j.bbamem.2021.183586">https://doi.org/10.1016/j.bbamem.2021.183586</a>

## Value of the Data

- We show confocal fluorescence images of asymmetric giant unilamellar vesicles that were prepared using a hemifusion method. Our data show the non-alignment of the fluorescence signal of two different fluorescent probes that usually partition into the liquid disordered phase. Here, they are used to label different leaflets of the bilayer.
- Given the importance of the asymmetric plasma membrane of living cells and its function, there is increasing interest in asymmetric bilayer interleaflet interactions. Our dataset will therefore be of interest to a wide audience, ranging from physicists to cell biologists. These experimental data were not observed in asymmetric giant unilamellar vesicles before.
- Our data can be re-used for independent analyses and interpretation. The free interpretation of these data could bring new insights to understand phenomena not yet experimentally observed in unsupported bilayers.
- We described additional details in the experimental procedure, including the data obtained from spectroscopic techniques: absorption and fluorescence emission of the fluorescent dyes used in this work.

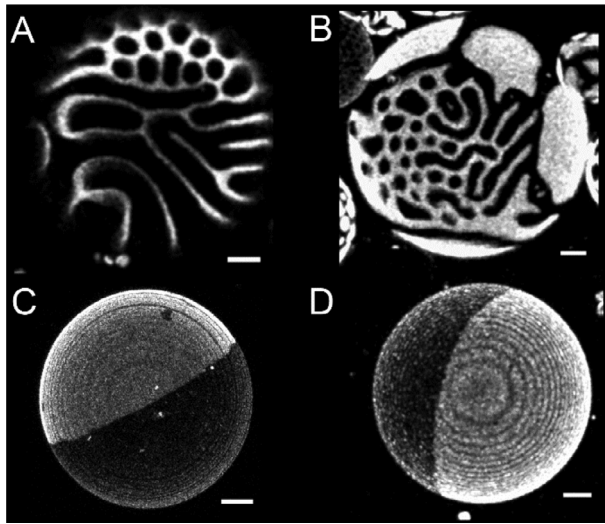
## 1. Data Description

The raw data are confocal images acquired at room temperature  $\sim 23$  °C of GUVs and aGUVs. The raw data images are divided into three different datasets as shown in Figs. 1-3. Fig. 1 shows a collection of images of aGUVs labelled with two different probes, DiD and TFPC, and the merged images combining both channels, here termed as RED, GREEN and MERGE images. The raw data images of Fig. 1 are (Fig. 1Ar, Fig. 1Br, Fig. 1Cr and Fig. 1Dr). The raw data of Fig. 1 were treated in ImageJ (Fiji), where the aGUV of interest was cropped from the raw data file, and a Gaussian filter with radius  $\sigma = 1.0$  pixels was used. The treated images were converted to 8-bit images and saved as .tiff and .png files (Fig. 1A\_RED, Fig. 1A\_GREEN, Fig. 1A\_MERGE, Fig. 1B\_RED, Fig. 1B\_GREEN, Fig. 1B\_MERGE, Fig. 1C\_RED, Fig. 1C\_GREEN, Fig. 1C\_MERGE, Fig. 1D\_RED, Fig. 1D\_GREEN and Fig. 1D\_MERGE). Fig. 1 shows the anti-alignment of the brighter domains. It should be noted that we collected this dataset at the same conditions of the dataset reported in the co-submitted work (Enoki, et Al. 2020, BBA, In press) where mostly of aGUVs show domains alignment. Fig. 2 shows a collection of images of symmetric GUVs that exhibit modulated phases or macroscopic domains. The raw data images of Fig. 2 are (Fig. 2Ar, Fig. 2Br, Fig. 2Cr and Fig. 2Dr). To make Fig. 2, we cropped the GUV of interest, then selected the stacks to create a z-projection for each GUV. The treated images were converted to 8 bit images and saved as .tiff and .png files (Fig. 2At, Fig. 2Bt, Fig. 2Ct and Fig. 2Dt). Note that the raw data of these images contain several GUVs. The images displayed in Fig. 2B and D were obtained after heating the sample to 55 °C and then slowly cooling to room temperature (23 °C) in 12 h, i.e. cooling at 2 °C/h. Fig. 2B and D show the same domain morphology as displayed in Fig. 2A and C. Because the purpose of Fig. 2 is the observation of the phase morphologies, we used grayscale images. Important to reproduce these phase morphologies is an accurate determination of lipid concentration. The files (DOPC\_RawData, POPC\_RawData, DSPC\_RawData, DOPC\_Assay, POPC\_Assay and DSPC\_Assay) display the assay measurements of the lipid concentration with an error of about 1%, and the file shows the calculation of different lipid compositions used to make symmetric GUVs with macrodomains, modulated phases, or uniform appearance. Fig. 3 shows a comparison of GUVs and aGUVs with modulated phases. The raw data images used in Fig. 3 are a z-stack of micrographs (Fig. 3Ar and Fig. 3Br). We treated these images by cropping the GUV or aGUV of interest and selecting three stacks to assemble Fig. 3. We also created a z-projection of the selected stacks (Fig. 3A\_GUV and Fig. 3B\_aGUV). The arrows displayed in Fig. 3 point to the position of a specific domain along the z-stack. All images were treated using the software ImageJ (Fiji). In addition, Fig. 4 displays the fluorescence emission of the dyes used in Fig. 1, DiD and

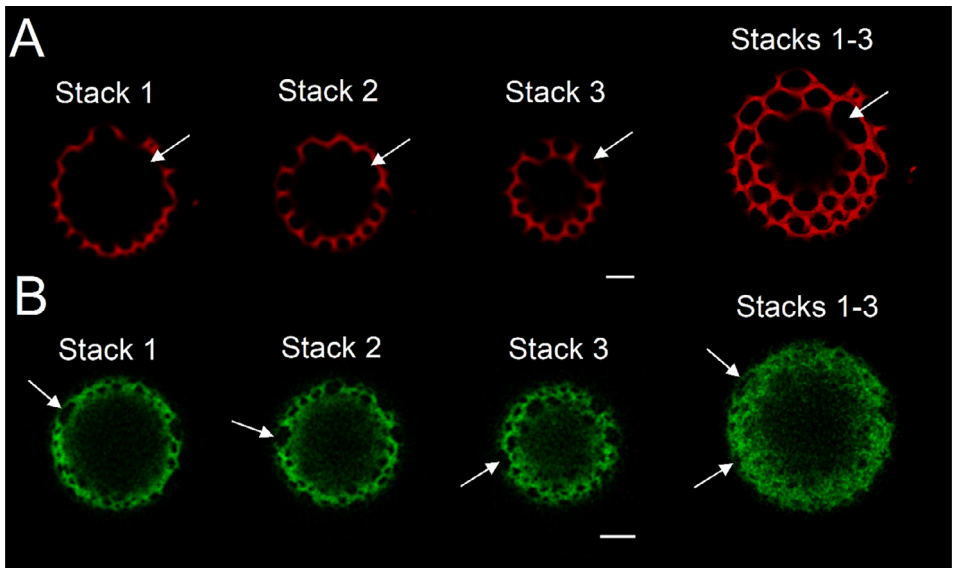


**Fig. 1.** Scanning confocal microscopy images with two color channels of aGUVs prepared via hemifusion. A single row shown in the figure corresponds to a single aGUV. The sequence of images in each row display micrographs for the channels named as RED (DiD fluorescence), GREEN (TFPC fluorescence) and MERGE (the composite image of both channels). Both dyes favor the Ld phase. TFPC (green fluorescence) was initially in the SLB before hemifusion. After hemifusion, TFPC is found in the aGUV outer leaflet. The inner leaflet of aGUVs have the following lipid composition (A)  $\rho = 0.18$ , DSPC/DOPC/POPC/cholesterol = 0.39/0.08/0.31/0.22, (B)  $\rho = 0.18$ , DSPC/DOPC/POPC/cholesterol = 0.39/0.08/0.31/0.22, (C)  $\rho = 0.45$ , DSPC/DOPC/POPC/cholesterol = 0.39/0.18/0.31/0.22 and (D)  $\rho = 0.8$ , DSPC/DOPC/POPC/cholesterol = 0.39/0.31/0.8/0.22.

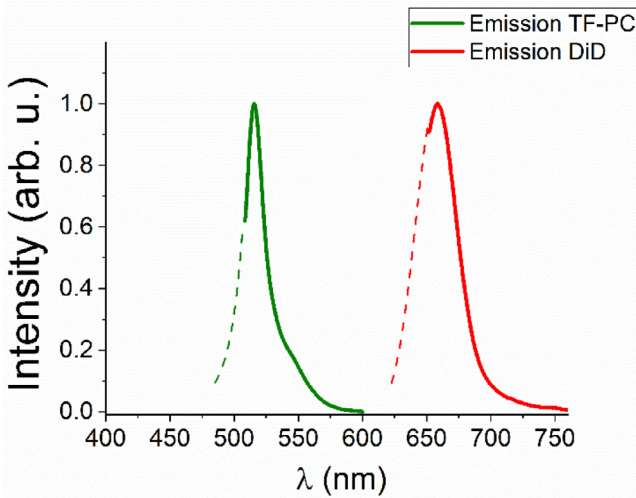
TFPC. The dashed line represents a fit of the emission peak. The emission peaks of DiD and TFPC do not overlap. Fig. 4 displays the normalized intensities of the fluorescence emission. The raw data and the treated data of Fig. 4 are summarized in the worksheet (DiD emission and TFPC emission). Fig. 5 displays the lipid composition in a  $\rho$ -trajectory (squares), in which we used fixed fractions of DSPC = 0.39 and chol = 0.22 and varied the fractions of DOPC and POPC. The lipid compositions shown by the squares of Fig. 5 are summarized in Table 1, including the



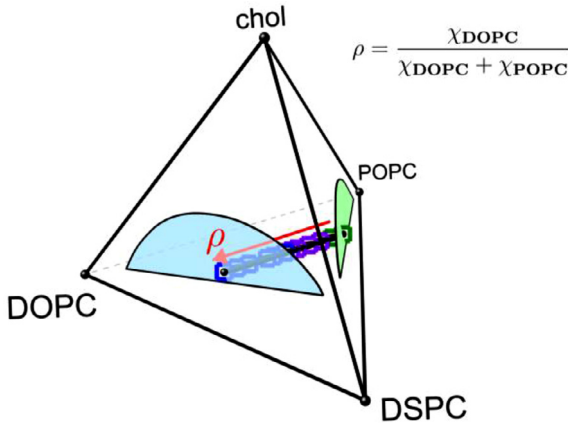
**Fig. 2.** Examples of phase morphologies and macroscopic domains observed in symmetric GUVs. (A-B) GUVs with modulated phases and lipid composition DSPC/DOPC/POPC/cholesterol = 0.39/0.12/0.27/0.22. GUVs were labelled with DiIc12, which prefers the Ld phase. (C-D) GUVs with macrodomains and lipid composition DSPC/DOPC/cholesterol = 0.39/0.39/0.22. GUVs were labelled with TFPC and naphthopyrene, which favors the Ld and the Lo phase, respectively. Scale bar 5  $\mu\text{m}$ .



**Fig. 3.** Symmetric GUV and aGUV, both with modulated phases. (A) Images from a z-stack of a single symmetric GUV that exhibits modulated phases (first three columns: Stack 1, Stack 2 and Stack 3), and the z-projection of stacks 1-3. Stacks are spaced from each other by  $\sim 0.6 \mu\text{m}$ . For symmetric GUVs (A), the arrow points to a domain that hardly moves around as the stacks are collected on the microscope. For aGUVs, the arrow points to a domain that changes position as the stacks are collected on the microscope. The time elapsed between the images is  $\sim 5 \text{ s}$ .



**Fig. 4.** Normalized emission spectra of TFPC and DiD. Excitation wavelengths: TFPC  $\lambda = 500$  nm, and DiD  $\lambda = 644$  nm. Dashed line represents a fitting of the emission peak to guide the eye.



**Fig. 5.** Partial phase diagram of DSPC/DOPC/POPC/chol showing region of Ld + Lo coexistence. The near-left face of the tetrahedron shows the ternary phase diagram of DSPC/DOPC/chol, and the far-right face of the tetrahedron shows the ternary phase diagram of DSPC/POPC/chol. The red arrow indicates a trajectory of samples, which includes 4-component mixtures DSPC/DOPC/POPC/chol. The squares shown in the figure represent the lipid fractions summarized in Table 1. The phase diagram shows the lipid compositions used in the study: Investigation of the domain line tension in asymmetric vesicles prepared via hemifusion, Enoki, et al. 2021 [1].

phase morphology of the majority of the GUVs observed in the microscope. A template to reproduce these lipid fractions in GUVs with accurate lipid composition is found in the worksheet (GUV\_template). In addition, the lipid fractions used in the preparation of supported lipid bilayers and the assembled images shown in this paper are found in the files (SLB\_template, Fig. 1, Fig. 2, Fig. 3, Fig. 4 and Fig. 5).

**Table 1**Lipid composition assigned to  $\rho$ -values and phase morphologies observed in symmetric GUVs.

$\rho$	$\chi_{\text{DSPC}}$	$\chi_{\text{DOPC}}$	$\chi_{\text{POPC}}$	$\chi_{\text{chole}}$	Phase morphology in GUVs
0	0.39	0.00	0.39	0.22	Uniform appearance
0.1	0.39	0.04	0.35	0.22	Uniform appearance
0.18	0.39	0.08	0.31	0.22	Modulated phases
0.3	0.39	0.12	0.27	0.22	Modulated phases
0.4	0.39	0.16	0.23	0.22	Modulated phases
0.45	0.39	0.18	0.21	0.22	Modulated phase and Macrodomains
0.5	0.39	0.20	0.20	0.22	Macrodomains
0.6	0.39	0.23	0.16	0.22	Macrodomains
0.65	0.39	0.25	0.14	0.22	Macrodomains
0.8	0.39	0.31	0.08	0.22	Macrodomains
0.9	0.39	0.35	0.04	0.22	Macrodomains
1	0.39	0.39	0.00	0.22	Macrodomains

## 2. Experimental Design, Materials and Methods

To prepare asymmetric giant unilamellar vesicles, we need to first separately prepare a supported lipid bilayer and symmetric giant unilamellar vesicles. We divided the experimental design in 3 parts: preparation of the supported lipid bilayer, preparation of symmetric giant unilamellar vesicles, and preparation of asymmetric giant unilamellar vesicles. The main data described in this paper refer to a series of images collected by confocal microscopy. Then, we describe the microscope setup used in the data acquisition. In addition, we describe a few controls important to obtain vesicles with accurate lipid composition and spectroscopic controls obtained with the fluorescent dyes used in this work.

### 2.1. Preparation of supported lipid bilayer

First, we cleaned the chambers for the preparation of supported lipid bilayers (SLBs). We prepared the SLB in Lab-Tek II, chambered cover glass 155,382 dishes (Thermo Fisher Scientific). Each dish has 4 chambers of  $1 \times 2$  cm. We added  $\sim 1$  M KOH (Sigma-Aldrich) dissolved in ethanol into each chamber and let it contact the cover slide for 20 min. Then, we rinsed the chamber thoroughly with abundant Milli-Q water (Thermo Fisher Scientific). After we dried excess water, we applied a flow of  $\text{N}_2$ . Then we placed the chamber in a plasma cleaner for  $\sim 2$  min. We observed that longer exposure of the dish (more than 4 min) to plasma cleaning damaged the plastic.

Then, we prepared small unilamellar vesicles (SUVs) for lipid deposition to form the SLB. To prepare SUVs, we added lipids and dyes diluted in chloroform in test tubes with screw caps. Here, SUVs were labeled with TFPC (see worksheets SLB\_template and TFPCemission). We added the buffer (HEPES 25 mM, NaCl 35 mM, pH = 7.43, 100 mOsm/kg) to the tube containing the lipids and dyes in chloroform and subjected the sample to Rapid Solvent Exchange (RSE) as previously described [2]. Samples were prepared to a final volume of 600  $\mu\text{L}$  and total lipid concentration of 2.5 mM. Dye/lipid ratios was TFPC/lipid = 1/1500 (SLB\_template). Then, we placed the test tube in a cylindrical bath sonicator (Sonblater, Narda ultrasonic corporation, NY) for  $\sim 20$  min, or until the sample turbidity had clearly decreased. After sonication, we mixed the dispersion with 1 M NaCl (Sigma-Aldrich) in a 1:1 ratio by volume.

After cleaning the dish in the plasma cleaner, we dispensed 500  $\mu\text{L}$  of the solution described above, SUV dispersion + NaCl (1 M), on the top of the cover slides of each chamber. Then, we placed the chambers at 4  $^\circ\text{C}$  for 2 h. After that, each chamber contained an SLB, and most of the time, vesicles and lipid aggregates were loosely bound to the SLB surface. Then, we immersed the SLB in a 2 L beaker containing Milli-Q water, and using a syringe of 20 mL, we flowed the Milli-Q water into each chamber to remove any lipid aggregates on the SLB surface.



The SLB was prepared from the SUVs, thus the confocal images of the SLB only exhibit the fluorescence emission of TFPC, which is collected through the wavelength range of 500–550 nm (see Fig. 4).

## 2.2. Preparation of symmetric giant unilamellar vesicles

We prepared GUVs using the electroformation procedure as previously reported [3]. First, we dissolved lipids and dyes in chloroform, then spread this solution on microscope slides coated with ITO (indium tin oxide) at  $T = 40$  °C. Then, we placed the slides in a desiccator attached to a high vacuum pump, where samples were kept in vacuum (pressure = 0.15 Torr) for 2 h. We sealed two ITO-coated slides with a Buna-N O-rings between them to create a chamber. The lipid film was hydrated with sucrose (100 mM). We applied a voltage of 1 V peak-to-peak at 5 Hz for 2 h at  $T = 50$  °C. Then, we set a digital temperature controller to cool the samples from 50 °C to 23 °C over 12 h. After, cooling, we collected GUVs in 1.5 mL plastic centrifuge tubes.

Important for the preparation of GUVs with accurate lipid composition is the determination of the lipid concentration within an error of 1 mol%, as we determined by inorganic phosphate assay, as described below (raw and treated data can be found in the worksheets DOPC\_RawData, POPC\_RawData, DSPC\_RawData, DOPC\_Assay, POPC\_Assay and DSPC\_Assay). We used DiD to label the symmetric GUVs with a dye lipid ratio 1/2500, see worksheet (GUV\_template, DiDemission). Therefore, the fluorescence micrographs of symmetric GUVs show only the fluorescence signal of the DiD, which is collected in the range of wavelengths 650–700 nm (see Fig. 4).

In symmetric GUVs composed of DSPC/DOPC/POPC/chol, modulated phases appear in a specific compositional range. This compositional window is termed a “ $\rho$ -window”, as previously reported [4], where  $\rho$  is given by Eq. (1):

$$\rho = \frac{\chi_{DOPC}}{\chi_{DOPC} + \chi_{POPC}}. \quad (1)$$

Fig. 5 illustrates a  $\rho$ -trajectory for DSPC and chol fractions equivalent to 0.39 and 0.22. We summarized the lipid composition used in Fig. 4 (squares) in Table 1, and the worksheet (GUV\_template) describes the amount of lipid and dyes used in the preparation of each sample.

To plot the lipid composition in a 3D plot as shown in Fig. 5, we use the metric:

$$X = \frac{\chi_{POPC} + 1 - \chi_{DOPC}}{2}, \quad (2)$$

$$Y = \frac{\sqrt{3}}{2} \chi_{DSPC} + \frac{\sqrt{3}}{6} \chi_{chol}. \quad (3)$$

and

$$Z = \frac{\sqrt{3}}{6} \chi_{chol}. \quad (4)$$

where  $\chi_{DSPC}$ ,  $\chi_{DOPC}$ ,  $\chi_{POPC}$  and  $\chi_{chol}$  are the mole fractions of DSPC, DOPC, POPC and chol, respectively.

## 2.3. Preparation of asymmetric giant unilamellar vesicles

We used a hemifusion procedure to prepare aGUVs. We triggered the hemifusion of GUVs and SLB by the addition of a buffer that contains  $\text{CaCl}_2$ . First, we collected 20–25  $\mu\text{L}$  of symmetric GUVs and transferred to the SLB chamber filled with about 0.5–0.6 mL of buffer (HEPES 25 mM, NaCl 35 mM, pH = 7.43). This step was performed by adding small aliquots of 5  $\mu\text{L}$  of GUVs at the corners and center of the SLB. After 5–10 min, the symmetric GUVs settled on the



SLB establishing close contact between the outer leaflets of the GUVs and the SLB. We monitored the contact of GUVs and SLB in the microscope by the acquisition of *z*-stacks. At this point of the procedure, symmetric GUVs exclusively contain the fluorescent label DiD as described in the preparation of symmetric GUVs. On the other hand, the SLB only contains the fluorescent dye TFPC.

Then, we added a buffer that contains CaCl<sub>2</sub>, (HEPES 25 mM, NaCl 15 mM, CaCl<sub>2</sub> 20 mM, pH = 7.43). The osmolarity of the buffers should match the osmolarity of the sucrose solution or the buffer used in the SLB. We measured the osmolarity of all buffers and solutions using an Osmometer (Model 5004 (Precision Systems, Inc., Natick, MA)). Then, after 20–30 min, we added a buffer that contains EDTA instead of CaCl<sub>2</sub>. Again, the osmolarity of this buffer must match the osmolarity of the solutions previous mentioned here. To harvest the hemifused aGUVs, we used a 1000  $\mu$ L pipettor with a large orifice tip, and then gently pipetted ~ 200  $\mu$ L up and down with the pipet tip about 0.2 cm above the SLB surface. This procedure was sufficient to shear the aGUVs off the SLB.

The formation of aGUVs is observed when asymmetric vesicles display the fluorescence signal of both fluorescent probes: DiD (initially on the symmetric GUVs) and TFPC (initially on the SLB). More details about the procedure can be found in our previous report and its supporting information [5].

## 2.4. Image acquisition

We used a laser scanning microscope Nikon Eclipse C2+ (Nikon Instruments, Melville, NY) to collect the images, with an objective Plan Apo VC 60x $\times$ A WI DIC N2. In the software NIS-Elements Basic Research, we set the scan direction to one-way and scan speed 0.5. We used a laser line of wavelength  $\lambda = 488$  nm to excite TFPC, and a laser line of wavelength  $\lambda = 640$  nm to excite DiD. The images summarized in Fig. 1 are collected using the same microscope setup. For the green fluorescence channel, we used laser power = 7% (scale 0–100%), gain = 85 (scale 0 – 255), offset = 0. For the red (far-red) fluorescence channel, we used laser power = 10% (scale 0–100%), gain = 85 (scale 0 – 255), offset = 0. For the images summarized in Fig. 2, we used a laser line of wavelength  $\lambda = 405$  nm to excite naphthopyrene and a laser line of wavelength  $\lambda = 561$  nm to excite DiIc12. For the blue fluorescence channel, we used laser power = 15% (scale 0–100%), gain = 80 (scale 0 – 255), offset = 0. For the red fluorescence channel, we used laser power = 20% (scale 0–100%), gain = 80 (scale 0 – 255), offset = 0. For the images summarized in Fig. 3 we used the excitation of DiIc12 and TFPC described above, and the following setup in the microscope: for the red fluorescence channel, laser power = 3% (scale 0–100%), gain = 80 (scale 0 – 255), offset = 0; and for the green fluorescence, we used the setup above. We used a pinhole size of 40  $\mu$ m.

### 2.4.1. Correction of the polarization artifact

The fluorescence emission of DiD displays a polarization artifact due to the laser excitation. In general, dyes with a fluorophore excitation dipole perpendicular to the bilayer normal display a nonuniform intensity in GUVs due to this effect. This nonuniform intensity on the GUV could be misinterpreted as domains formed by different phases. We correct this effect by adding a quarter wavelength plate (Thermo Fisher Scientific) in the excitation path. The orientation of the quarter wavelength plate depends on the dye. This correction is especially important in order to avoid misinterpreting the data. For additional details, see our previous report and its supporting information [5].

## 2.5. Additional experiments and controls

Phosphate assay.

To determine the concentrations of phospholipids, we performed an inorganic phosphate assay, as previously reported [6]. Typically, 10 samples were prepared in a range of 0–139 nmoles of lipid, along with 10 samples of a standard phosphate solution to form the calibration curve.

We labeled at least 20 test tubes (size 13 × 100 mm) with the volume of the sample; 10 for the control, and 10 for each phospholipid sample to test.

In the example shown in the worksheets (DOPC\_Assay, POPC\_Assay and DSPC\_Assay), samples had the following volumes in  $\mu\text{L}$ : 0, 0, 2, 4, 6, 8, 12, 16, 20, and 24, which correspond to: 0, 0, 11.6, 23.2, 34.8, 46.4, 69.7, 92.9, 116 and 139 nmoles. We added 0.200 mL (10% v/v) of sulfuric acid into each test tube and randomly placed them into a heat block to be heated for 1 hour in a fume hood. Then, we added 20  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$  (30%) into each test tube to be heated for an additional 40 min. After 40 min, we removed the tubes from the heat block to cool to room temperature.

In the meantime, we prepare the color reagent. We briefly describe the recipe for the color reagent solution to be used for 30 test tubes, which included 10 tubes for the calibration curve, 10 tubes of the samples to be tested, and the amount for 10 more tubes. We then mixed 0.1 g ascorbic acid into 4.5 mL  $\text{H}_2\text{O}$  and added 0.5 mL 5% ammonium molybdate in water (w/v). We added the ammonium molybdate last, immediately before the color reagent was added to the test tubes.

First, we added 0.5 mL of Milli-Q water to each test tube, rinsing the sides, and mixing with a vortex mixer. Then, we added 0.5 mL of color reagent and again mixed with a vortex mixer. After that, tubes were placed in a water bath at 45 °C for 20 min.

Finally, using a spectrophotometer, we collected the absorbance spectra of each sample including the tubes for the calibration curve. The worksheets (DOPC\_RawData, POPC\_RawData and DSPC\_RawData) display an example of the raw data and the analyses of the phosphate assay. This assay is needed to achieve reproducible phase morphologies, such as modulated phases in GUVs, since modulated phases are observed in a narrow lipid compositional range of DOPC and POPC.

## 2.6. Fluorescent dyes

The GUVs and the SLB are labeled with different fluorescent dyes, DiD and Top Fluor PC (TFPC). Fig. 4 shows the emission spectra of both dyes we used. These dyes were chosen to avoid overlap of their fluorescence emission.

## 2.7. Fluorimeter setup

Fluorescence measurements were performed with a spectrofluorimeter model F7000 equipped with a high sensitivity cell holder (Hitachi High Technologies America, Schaumburg, IL). Excitation and emission slit widths were set to 5 nm, and signal integration time was 10 s. Samples were placed in quartz cuvettes with an optical pathway of 1.0 cm. Spectra were collected at room temperature of 23 °C.

## 2.8. UV-Vis Spectrophotometer setup

Absorption measurements (Absorbance) were performed using an HP 8452A spectrophotometer (Hewlett-Packard, Palo Alto, CA). Samples were placed in quartz cuvettes with optical path 1.0 cm, and measurements were performed at room temperature of 23 °C. For the phosphate assay, we added 500  $\mu\text{L}$  of the aqueous solution described above to the cuvette. For measurements of the dye concentration, the dye stock was first diluted in methanol, and then we added 500  $\mu\text{L}$  of this solution in the cuvette. To measure the dye concentration, we used the following

extinction coefficients:  $\epsilon = 91,800 \text{ (cm}\cdot\text{M)}^{-1}$  at 504 nm (TFPC),  $\epsilon = 270,000 \text{ (cm}\cdot\text{M)}^{-1}$  at 644 nm (DiD),  $\epsilon = 23,749 \text{ (cm}\cdot\text{M)}^{-1}$  at 454 nm (naphthopyrene) and  $\epsilon = 144,000 \text{ (cm}\cdot\text{M)}^{-1}$  at 548 nm (DiIC12).

## 2.9. Materials

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), and DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) were from Avanti Polar Lipids (Alabaster, AL, USA), cholesterol (Chol) from Nu Chek Prep (Elysian, MN, USA). HEPES (**4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)**), KCl (potassium chloride),  $\text{CaCl}_2$  (calcium chloride), KOH (potassium hydroxide) and EDTA (ethylenediaminetetraacetic acid) from Sigma-Aldrich (St Louis, MO, USA). Fluorescent dyes: TopFluor-PC (TFPC), 1,1'-Didodecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiIC12) and 1,1'-Dioctadecyl-3,3,3',3'- Tetramethylindocarbocyanine Perchlorate (DiD) (Invitrogen - Molecular Probes), naphthopyrene (Tokyo Chemical Industry, Tokyo, Japan).

## CRedit Author Statement

**Thais A. Enoki**: performed the experiments, analyzed the data, and wrote the first draft of the manuscript; **Joy Wu**: performed experiments with symmetric GUVs; **Frederick A. Heberle** and **Gerald W. Feigenson**: co-wrote the manuscript and performed research; **Thais A. Enoki** and **Gerald W. Feigenson**: designed the experiments.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.dib.2021.106927](#).

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