Biophysical Journal, Volume *110*

Supplemental Information

Mixing Temperatures of Bilayers Not Simply Related to Thickness Differences between L_o and L_d Phases

Joan V. Bleecker, Phillip A. Cox, and Sarah L. Keller

Supporting Information: Mixing temperatures of bilayers not simply related to thickness differences between *Lo* and *Ld* phases

Joan V. Bleecker, Phillip A. Cox, and Sarah L. Keller
Department of Chemistry, University of Washington, Seattle, Washington 98195, U.S.A.

Contents

Details of Materials and Methods

Phospholipids (Avanti Polar Lipids, Alabaster, AL), dye (Texas Red dihexadecanoyl-phosphoethanolamine, DHPE; Life Technologies, Grand Island, NY) and cholesterol (chol; Sigma, St. Louis, MO) were used without further purification. We used only saturated lipids to minimize photooxidation (1). Uncertainty in each single measurement of T_{mix} is reported as a 95% confidence interval of a sigmoidal fit at T_{mix}, as in Fig. 2A of the main text. Measurement uncertainty from sample to sample is on the order of 1 ˚C, given displacements of data points in Fig. 3C from a smooth curve connecting all points.

Supported lipid bilayers were maintained at 22°C in an AFM chamber. The bilayer was scanned under water on an Asylum Research Cypher ES Environmental AFM system SLD-DD (Asylum Research, Santa Barbara, CA) in tapping mode using blueDrive™ to photothermally drive an Arrow UHFAuD tip (6 N/m, NanoWorld, Neuchâtel, Switzerland) at a resonance frequency of ~400 kHz (rated at 1 MHz in air). The sample chamber was maintained at 22 °C throughout scanning using the built-in temperature controlled stage in the Cypher ES. Measurement uncertainties in Δh are standard deviations from at least three AFM scans for each ratio of lipids, and appear in Table S1.

T_{mix} was measured in free-floating giant unilamellar vesicles, and Δh was measured in supported lipid bilayers. The presence of a solid substrate has been shown to have only a minor influence on T_{mix} in ternary membranes: the T_{mix} for GUVs of 29.2/32.4/28.4 DiPhyPC/DPPC/chol differs by less than 5˚C from the T_{mix} for GUVs of the same lipid composition deposited on a glass support to produce supported bilayers (2). Evidence that the presence of the solid substrate has only a minor influence on the composition of the L_o and L_d phases is that the area fraction of each phase has been observed to be the same on the surface of a free-floating GUV and in a supported bilayer resulting from deposition and rupture of the same vesicle on a solid support (3) (4).

Discussion of Figure S1

Fig. S1 illustrates points in the main text about lengths of tie-lines in ternary lipid membranes. Membranes composed of at least three lipid types, namely a lipid with a low melting temperature, a lipid with a high melting temperature, and cholesterol or a similar sterol, can demix into coexisting L_o and L_d phases with micron-scale domains.

Each of the panels in Fig. S1 shows miscibility phase boundaries for two different membrane systems, called "red" and "blue". At least one of the three lipid types in the red membrane differs from those in the blue membrane. The ratio of lipid types varies across the x-y plane, and temperature varies along the z-axis. Any point within the volume of the curved surface of the phase boundary corresponds to a membrane that phase separates. Outside of this surface (e.g. at high temperature), all lipids in the membrane mix uniformly.

The four panels schematically represent four possible outcomes of experiments conducted by setting the red and blue membranes at a common ratio of the lipid types and at a common temperature denoted by the black dot). The red (blue) membrane demixes into L_o and L_d phases with lipid ratios that fall at the large red (blue) dots on the x-y plane. The endpoints of the tie-line of the red (blue) membrane fall at these red (blue) dots and the tie-line passes through the black dot. At the tie-line endpoints, the value of T_{mix} is the same as the experimental temperature, whereas in the middle of the tie-line, T_{mix} is higher than the experimental temperature. The shape of each phase boundary is a function of the interaction energies between different types of lipids and the system's entropy. These concepts are reviewed in (5), which gives references to textbooks that discuss phase behavior (6) (7) (8).

The angle between the two tie-lines in the x-y plane is α. The directions of the tie-lines (and hence the angle α between them) are not set by any theoretical constraint; tie-line directions are not known until they are measured, and they change with lipid type and with the experimental temperature (9). This means that the angle α also varies with lipid type and with the experimental temperature.

In only Panel a, the two miscibility phase boundaries are hemispheres offset by a constant height, d, which is the difference between the mixing temperatures of the red and blue membranes. The difference between the lengths of the two tie-lines is independent of the angle α if the black dot always lies at the center of the axi-symmetric shapes of the phase boundaries. In the special case of only Panel a, the quantitative relationship between the mixing temperature and tie-line length is generalizable and does not depend on details of the system. To date, no set of two or more membrane miscibility phase boundaries has been documented that fits the constrained characteristics of Panel a.

If the phase boundaries are not axi-symmetric, then a variety of relationships between the demixing temperatures and the lengths of tie-lines are possible as in Panels b-d. The results reported by García-Sáez et al. (10) correspond to Panel b. Panel b is a simplification of known miscibility phase diagrams as in Fig. 4a of the main text and is drawn so that: 1) The two phase boundaries are ellipsoidal; and both ellipses have similar aspect ratios. 2) The black dot

does not lie at the center of both ellipses. 3) The angle α between the tie-lines is nonzero, but small. As a result of these characteristics, tie-line lengths positively correlate with T_{mix} in Panel b, but the quantitative relationship between these two variables changes with the particular choice of lipid ratio (with the placement of the black dot within the x-y plane).

Sets of phase boundaries in which an increase in tie-line length correlates with a *decrease* in T_{mix} are possible to imagine, as in Panels c and d. No experimental systems corresponding to these panels have yet been discovered. In Panel c, the minor axes of the ellipsoidal phase boundaries are much smaller than the major axes, and the tie-lines of the red and blue membranes are nearly perpendicular. In Panel d, the phase boundaries have dissimilar shapes: one is tall and narrow whereas the other is short and broad.

Translating the schematic images in Fig. S1 into plots of T_{mix} vs. Δh for experimental systems is nontrivial because the mapping of tie-line length onto Δh is known for only one system to date (9) (11), and likely depends both on the direction of the tie-line within the x-y plane and on the choice of lipid types. To the extent that a difference in lipid composition between the L_o and L_d phases at a given temperature determines the miscibility phase boundary and (separately) Δh, there exists a relationship between T_{mix} and Δh.

Figure S1

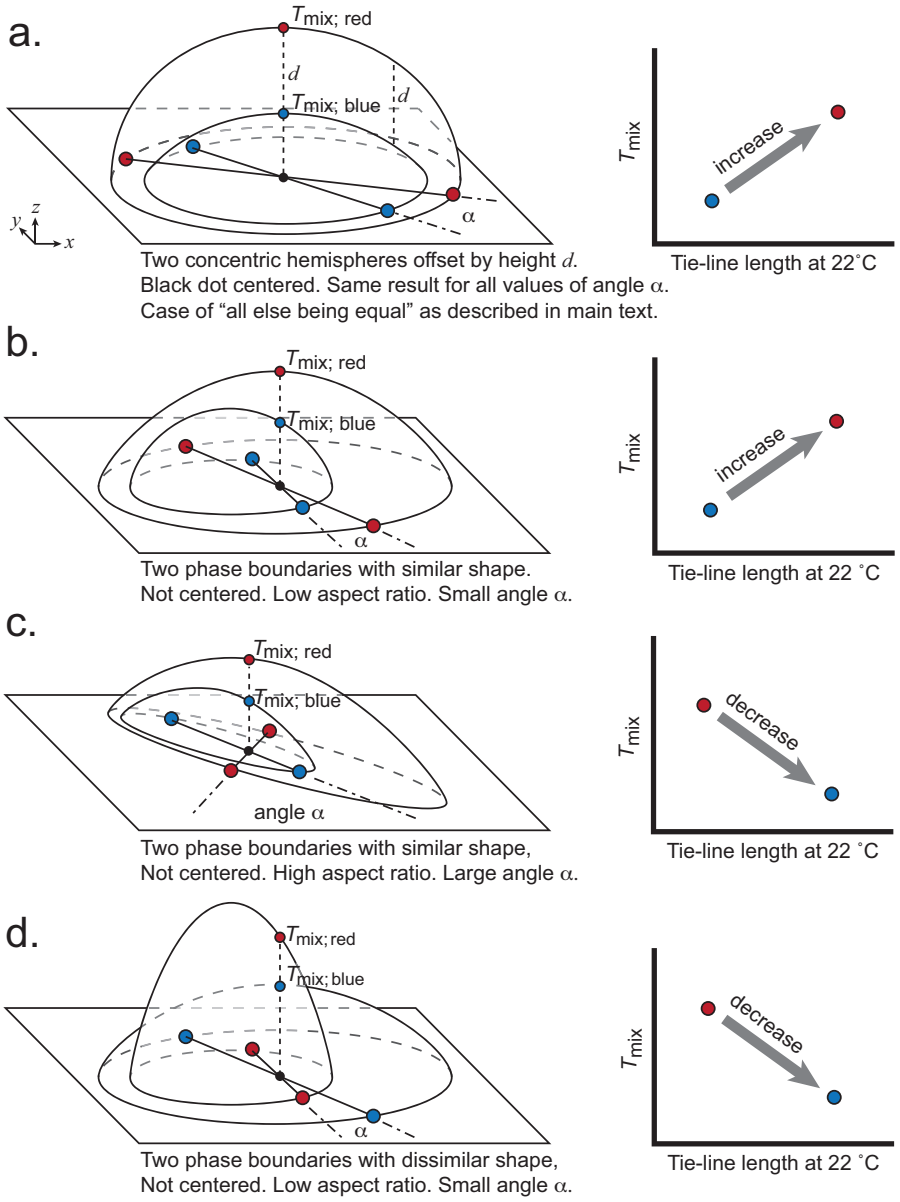


Figure S1 Caption: Panels a – d show two curved surfaces, which represent miscibility phase boundaries of two different membranes, named "red" and "blue". The ratio of lipids within each membrane varies within the x-y plane. The experiment is conducted at a common, fixed ratio of the three lipid species in each membrane, at the black dot. The z-axis is temperature, where $z = 0$ is the experimental temperature, T_{mix}. Lipids within the red (blue) membrane demix into L_o and L_d phases at temperatures below $T_{mix;\ red}$ ($T_{mix;\ blue}$). At the temperature of the black dot, the ratios of lipid species in the L_o and L_d phases of the red (blue) membrane fall at the locations of the large red (blue) dots. The line that joins these large dots is the tie-line. Graphs at the right show relationships between miscibility transition temperatures and tie-line lengths for each of the four panels.

Table S1: Experimentally determined T_{mix} of lipid ratios that fall along a single tie-line

Mole % DiPhyPC/DPPC/Chol	Tmix (°C)
63.7 / 18.8 / 17.5	32.2 ± 0.5
58 / 22 / 20	34.4 ± 0.6
52.2 / 25.3 / 22.5	38.0 ± 1.1
46.5 / 28.5 / 25	40.2 ± 0.5
40.7 / 31.8 / 27.5	42.3 ± 0.7
35 / 35 / 30	45.3 ± 0.5
23.5 / 41.5 / 35	47.6 ± 0.1
12 / 48 / 40	48.7 ± 0.3

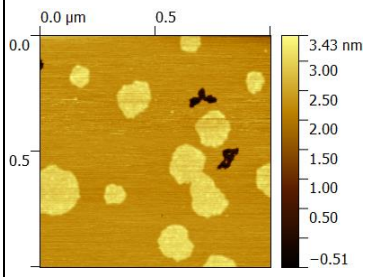
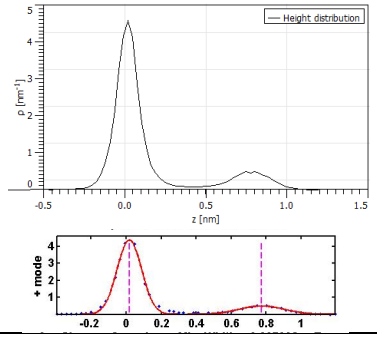
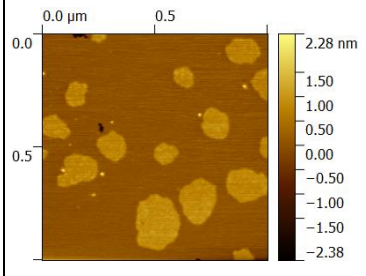
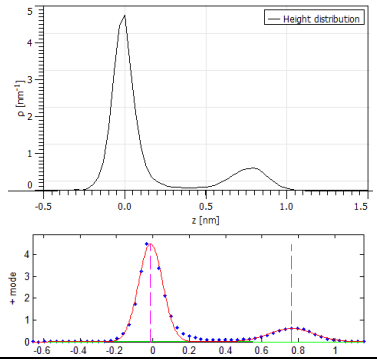
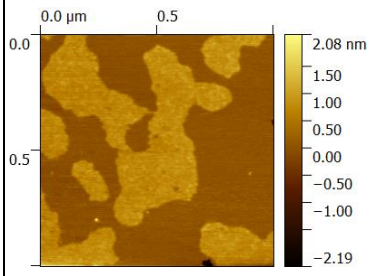
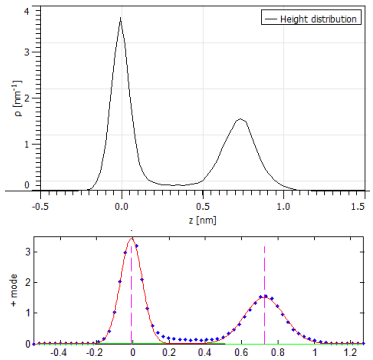
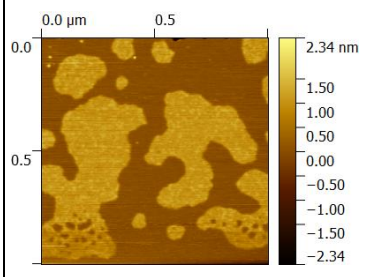
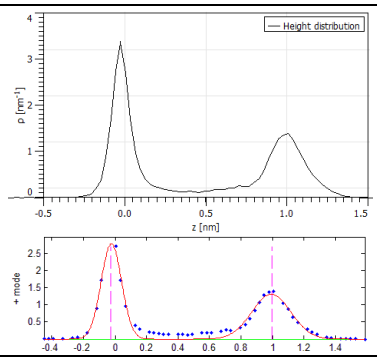
Tie-line compositions are interpolated from (9). Values of T_{mix} are reported as the temperature at which 50% of all vesicles have phase separated into L_o and L_d phases. Specifically, a sigmoidal fit is made of a plot of percent of vesicles that are phase separated vs. temperature. Reported experimental uncertainties in T_{mix} represent 95% confidence intervals of that sigmoidal fit.

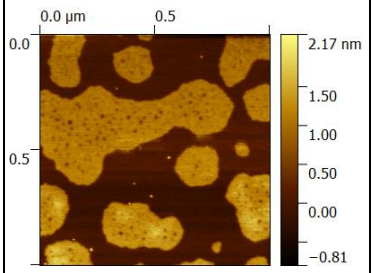
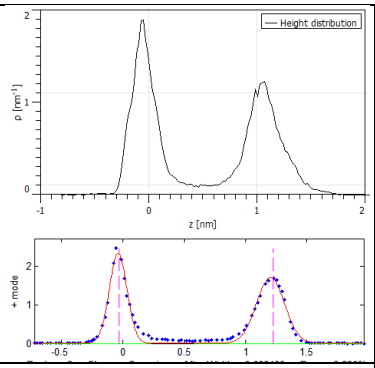
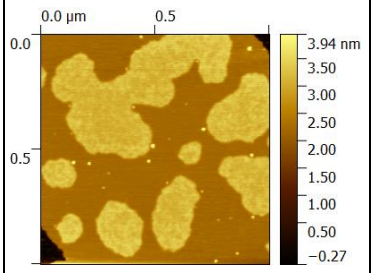
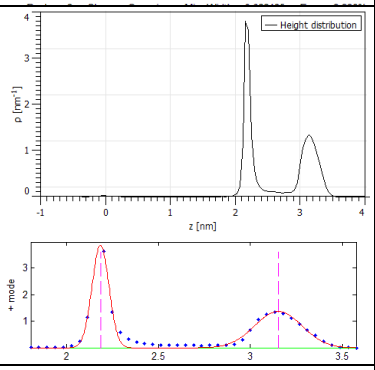
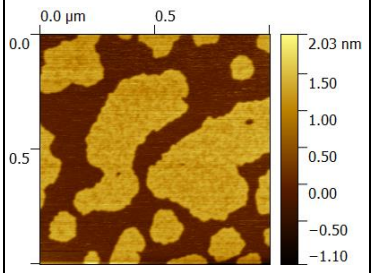
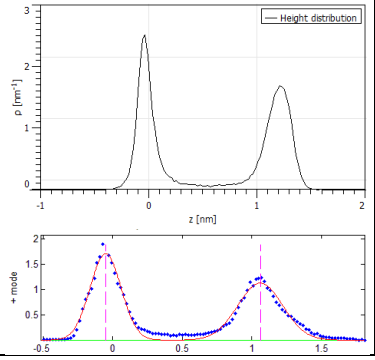
Table S2: Experimentally determined T_{mix} and Δh values

	Mole % DiPhyPC/DPPC/Chol	Tmix (°C)	Average Lo – Ld thickness (nm)
Tie-line Compositions			
a1	35 / 35 / 30	45.3 ± 0.5	0.94 ± 0.07
a2	46.5 / 28.5 / 25	40.2 ± 0.5	1.06 ± 0.05
a3	52.2 / 25.3 / 22.5	38.0 ± 1.1	1.03 ± 0.04
a4	63.7 / 18.8 / 17.5	32.2 ± 0.5	1.00 ± 0.03
Isothermal Compositions			
b1	27 / 23 / 50	41.0 ± 0.4	0.32 ± 0.002
b2	50 / 20 / 30	40.9 ± 1.2	1.03 ± 0.03
b3	46.5 / 28.5 / 25	40.2 ± 0.5	1.06 ± 0.05
b4	48 / 32 / 20	40.7 ± 0.3	1.43 ± 0.05

Values of T_{mix} are reported as the temperature at which 50% of all vesicles have phase separated into L_o and L_d phases. Specifically, a sigmoidal fit is made of a plot of percent of vesicles that are phase separated vs. temperature. Reported experimental uncertainties in T_{mix} represent 95% confidence intervals of that sigmoidal fit. Reported experimental uncertainties in average Lo-Ld thickness are the standard deviation of Lo-Ld thicknesses for at least three separate AFM scans.

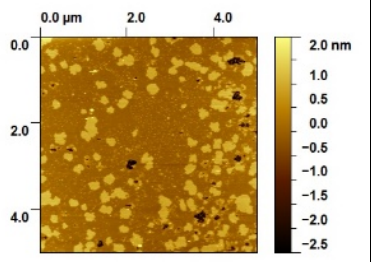
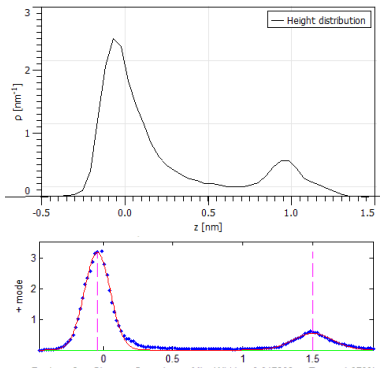
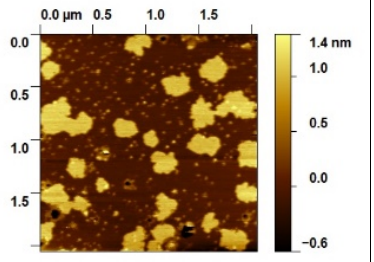
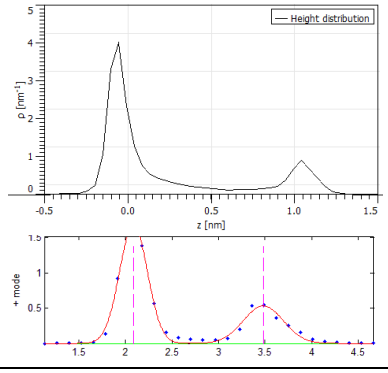
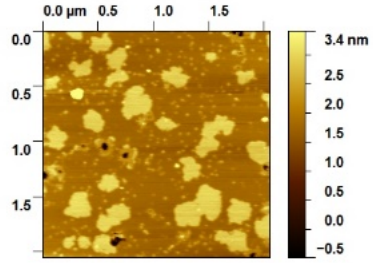
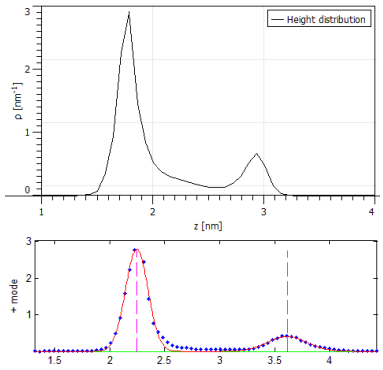
Table S3: AFM image, peak fits, and resulting data for 35/35/30 mole % DiPhyPC/DPPC/chol supported lipid bilayer

#	AFM image[a]	(Top) height histogram[b] (Bottom) peak fit[c]	Difference in thickness between thin and thick membrane regions (nm) [d]	Ratio of areas of thick:thin regions [e]	Average thickness mismatch (nm) [f]; area ratio of thick:thin regions
1			0.757 ± 0.002	17:83	0.94 ± 0.07 40:60 ± 15
2[g]			0.773 ± 0.002	20:80	
3			0.732 ± 0.006	45:55	
4			1.018 ± 0.002	47:53	

5			1.243 ± 0.001	53:47	
6			0.962 ± 0.008	50:50	
7			1.111 ± 0.002	49:51	

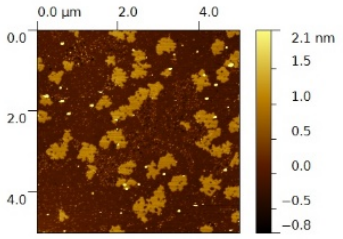
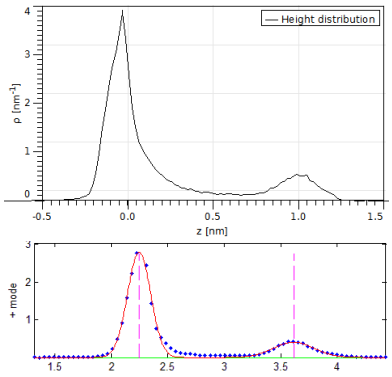
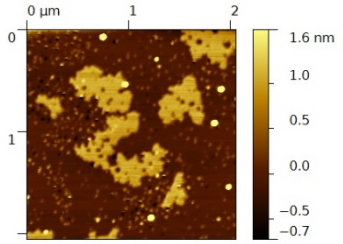
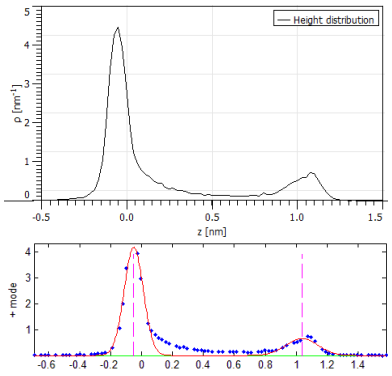
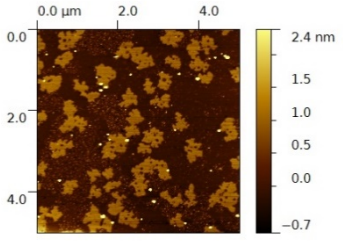
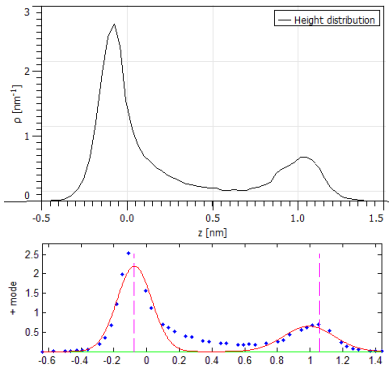
[a]Flattened AFM image with height scale bar in gold. [b]Height histogram in Gwyddion. [c]Typical peak fit trial of the height histogram in a 100-trial bootstrap fit. The dark red line shows the fit to the data marked by blue dots. Magenta dashed lines show the first guess at the peak position from the previous trial. [d]Difference between thickness of the thin and thick regions of the membrane reported as the mean difference in the location of the peaks determined by the bootstrap method. The reported uncertainty is the propagated standard deviation from the bootstrap fit of the two bilayer peaks. [e]Ratio of areas of thick and thin membrane regions from the bootstrap average areas of the two bilayer peaks. [f]Average thickness difference from column 4. The reported uncertainty is the standard error of the mean of the seven values. [g]Features far thicker than a lipid bilayer (the highest 12% of values) were excluded from the height histogram.

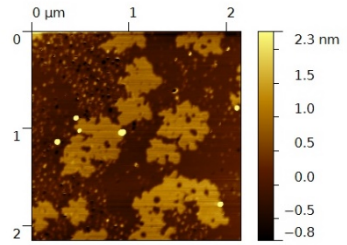
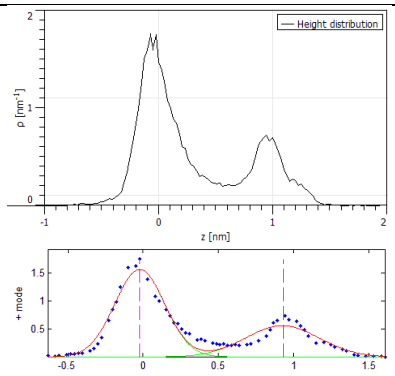
Table S4: AFM image, peak fits, and resulting data for 46.5/28.5/25 mole % DiPhyPC/DPPC/chol supported lipid bilayer

#	AFM image[a]	(Top) height histogram[b] (Bottom) peak fit[c]	Difference in thickness between thin and thick membrane regions (nm)[d]	Ratio of areas of thick:thin regions [e]	Avg. thickness mismatch (nm)[f]; area ratio of thick:thin regions
1[g]			0.952 ± 0.069	24:76	1.06 ± 0.05 24:76 ± 1
2			1.093 ± 0.014	24:76	
3[g]			1.129 ± 0.016	22:78	

[a]Flattened AFM image with height scale bar in gold. [b]Height histogram in Gwyddion. [c]Typical peak fit trial of the height histogram in a 100-trial bootstrap fit. The dark red line shows the fit to the data marked by blue dots. Magenta dashed lines show the first guess at the peak position from the previous trial. [d]Difference between thickness of the thin and thick regions of the membrane reported as the mean difference in the location of the peaks determined by the bootstrap method. The reported uncertainty is the propagated standard deviation from the bootstrap fit of the two bilayer peaks. [e]Ratio of areas of thick and thin membrane regions from the bootstrap average areas of the two bilayer peaks. [f]Average thickness difference from column 4. The reported uncertainty is the standard error of the three values. [g]Features far thicker than a lipid bilayer (the highest 12% of values) were excluded from the height histogram.

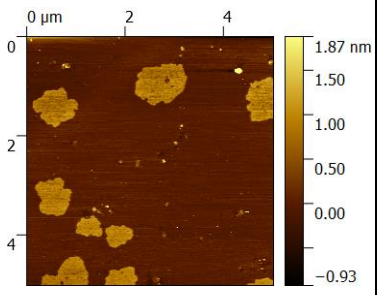
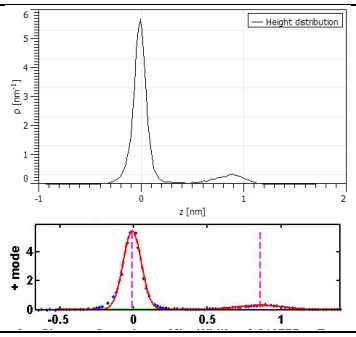
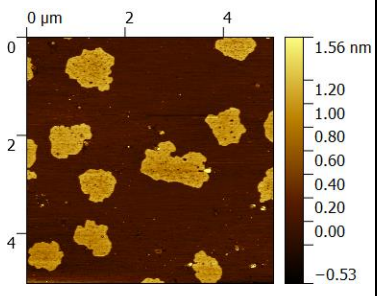
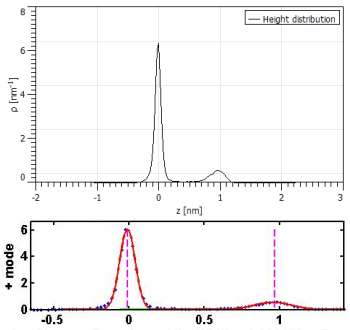
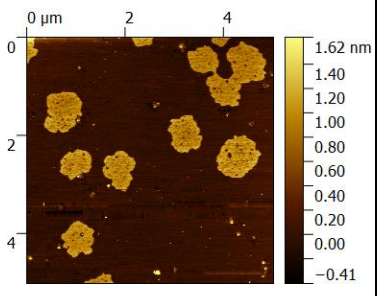
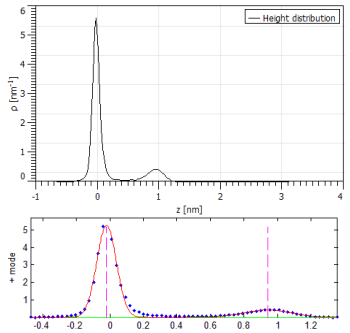
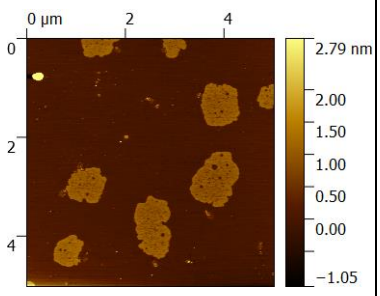
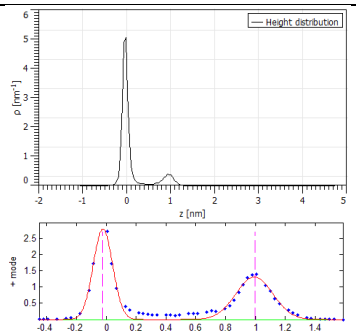
Table S5: AFM image, peak fits, and resulting data for 52.2/25.3/22.5 mole % DiPhyPC/DPPC/chol supported lipid bilayer

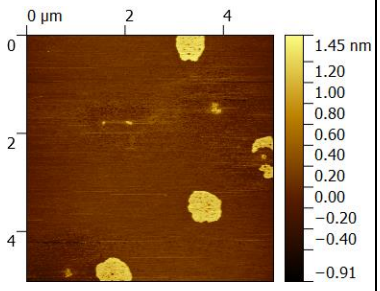
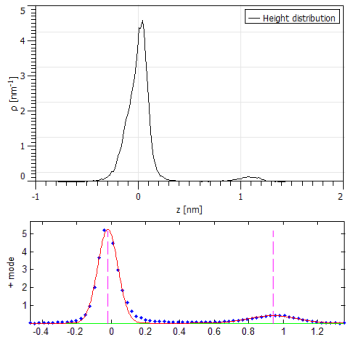
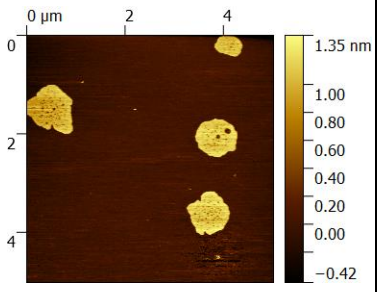
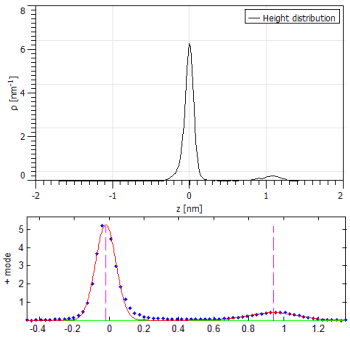
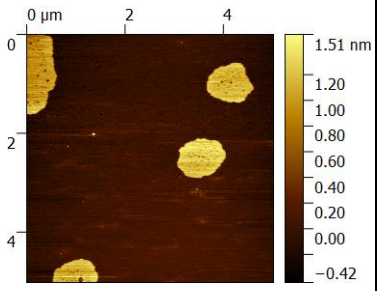
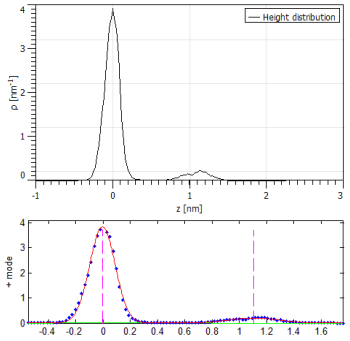
#	AFM image[a]	(Top) height histogram[b] (Bottom) peak fit[c]	Difference in thickness between thin and thick membrane regions(nm) [d]	Ratio of areas of thick:thin regions [e]	Average thickness mismatch (nm) [f]; area ratio of thick:thin regions
1			0.952 ± 0.069	20:80	
2[g]			1.088 ± 0.003	21:79	1.03 ± 0.04 26:74 ± 4
3[h]			1.1067 ± 0.006	30:70	

4[g]			0.956 ± 0.006	34:66	

[a]Flattened AFM image with height scale bar in gold. [b]Height histogram in Gwyddion. [c]Typical peak fit trial of the height histogram in a 100-trial bootstrap fit. The dark red line shows the fit to the data marked by blue dots. Magenta dashed lines show the first guess at the peak position from the previous trial. [d]Difference between thickness of the thin and thick regions of the membrane reported as the mean difference in the location of the peaks determined by the bootstrap method. The reported uncertainty is the propagated standard deviation from the bootstrap fit of the two bilayer peaks. [e]Ratio of areas of thick and thin membrane regions from the bootstrap average areas of the two bilayer peaks. [f]Average thickness difference from column 4. The reported uncertainty is the standard error of the mean of the four values. [g]Features far thicker than a lipid bilayer (the highest 20% of values) were excluded from the height histogram. [h]Features far thicker than a lipid bilayer (the highest 12% of values) were excluded from the height histogram.

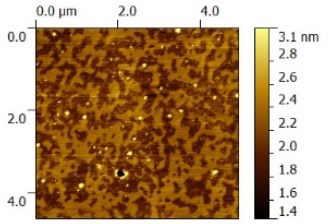
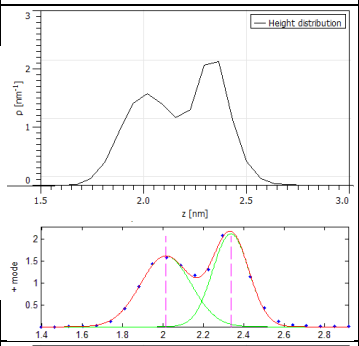
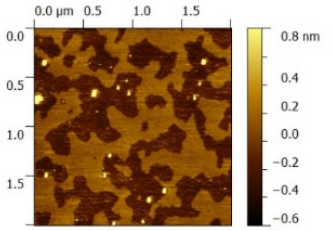
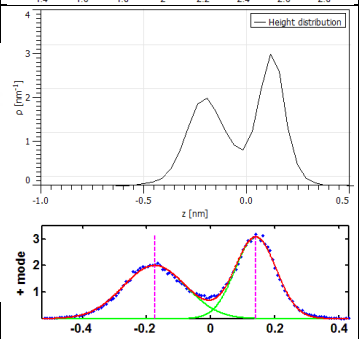
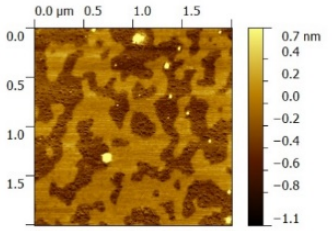
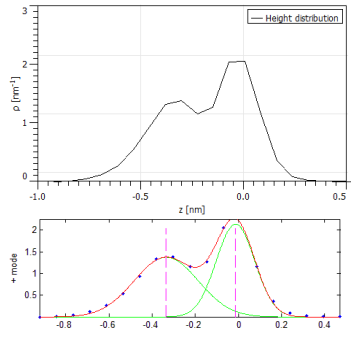
Table S6: AFM image, peak fits, and resulting data for 63.7/18.8/17.5 mole % DiPhyPC/DPPC/chol supported lipid bilayer

#	AFM image[a]	(Top) height histogram[b] (Bottom) peak fit[c]	Difference in thickness between thin and thick membrane regions (nm)[d]	Ratio of areas of thick:thin regions [e]	Average thickness mismatch (nm) [f] and area ratio of thick:thin regions
1			0.873 ± 0.002	13:87	1.00 ± 0.03 12:88 ± 5
2			0.966 ± 0.001	18:82	
3			0.959 ± 0.002	16:84	
4			0.979 ± 0.003	14:86	

5			1.076 ± 0.003	4:96	
6			1.067 ± 0.003	8:92	
7			1.113 ± 0.003	9:91	

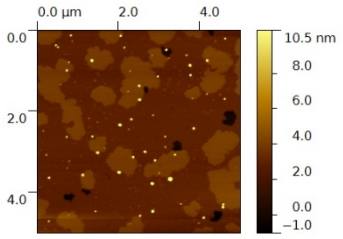
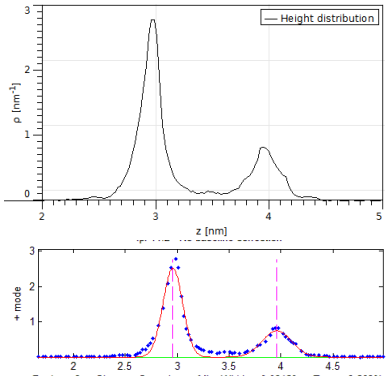
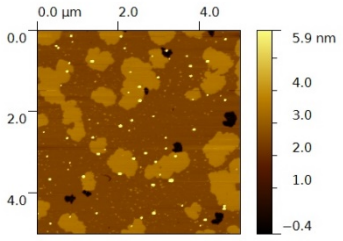
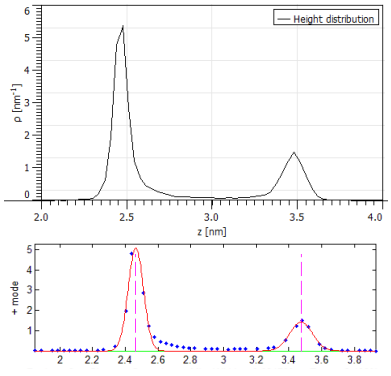
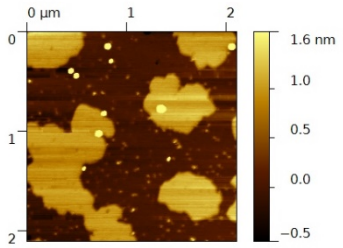
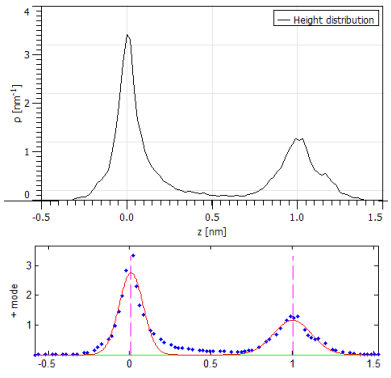
[a]Flattened AFM image with height scale bar in gold. [b]Height histogram in Gwyddion. [c]Typical peak fit trial of the height histogram in a 100-trial bootstrap fit. The dark red line shows the fit to the data marked by blue dots. Magenta dashed lines show the first guess at the peak position from the previous trial. [d]Difference between thickness of the thin and thick regions of the membrane reported as the mean difference in the location of the peaks determined by the bootstrap method. The reported uncertainty is the propagated standard deviation from the bootstrap fit of the two bilayer peaks. [e]Ratio of areas of thick and thin membrane regions from the bootstrap average areas of the two bilayer peaks. [f]Average thickness difference from column 4. The reported uncertainty is the standard error of the mean of the seven values.

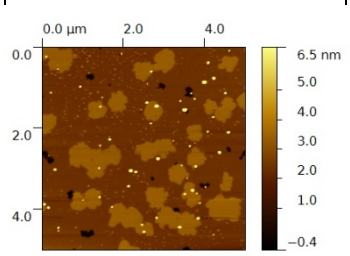
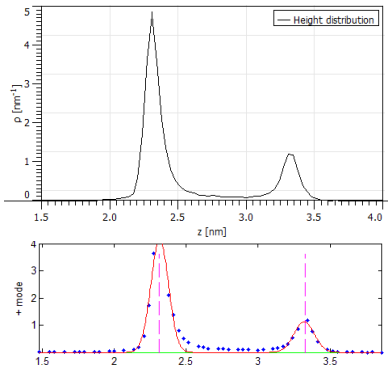
Table S7: AFM image, peak fits, and resulting data for 27/23/50 mole % DiPhyPC/DPPC/chol supported lipid bilayer

#	AFM image[a]	(Top) height histogram[b] (Bottom) peak fit[c]	Difference in thickness between thin and thick membrane regions (nm) [d]	Ratio of areas of thick:thin regions [e]	Avg. thickness mismatch (nm) [f]; area ratio of thick:thin regions
1			0.322 ± 0.010	49:51	0.320 ± 0.002 49:51 ± 1
2			0.316 ± 0.001	50:50	
3			0.321 ± 0.012	48:52	

[a]Flattened AFM image with height scale bar in gold. [b]Height histogram in Gwyddion. [c]Typical peak fit trial of the height histogram in a 100-trial bootstrap fit. The dark red line shows the fit to the data marked by blue dots. Magenta dashed lines show the first guess at the peak position from the previous trial. [d]Difference between thickness of the thin and thick regions of the membrane reported as the mean difference in the location of the peaks determined by the bootstrap method. The reported uncertainty is the propagated standard deviation from the bootstrap fit of the two bilayer peaks. [e]Ratio of areas of thick and thin membrane regions from the bootstrap average areas of the two bilayer peaks. [f]Average thickness difference from column 4. The reported uncertainty is the standard error of the mean of the three values. [g]Features far thicker than a lipid bilayer (the highest 20% of values) were excluded from the height histogram. [h]Features far thicker than a lipid bilayer (the highest 12% of values) were excluded from the height histogram.

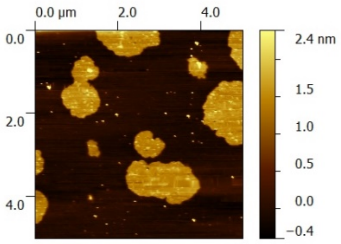
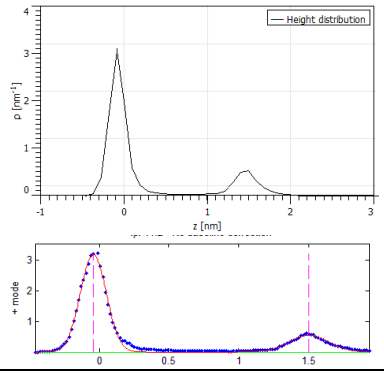
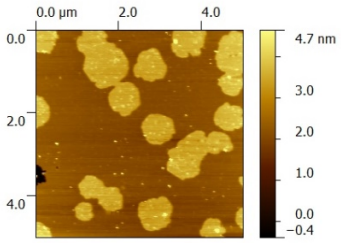
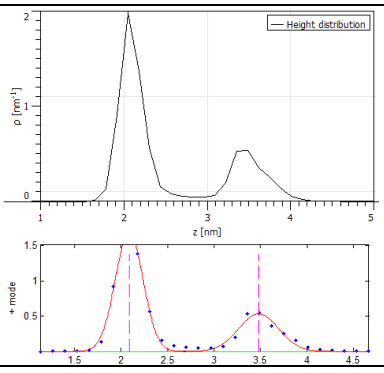
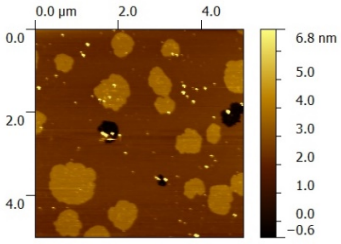
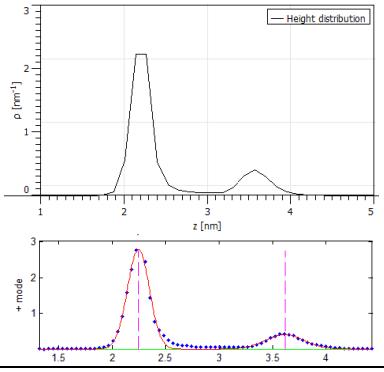
Table S8: AFM image, peak fits, and resulting data for 50/30/20 mole % DiPhyPC/DPPC/chol supported lipid bilayer

#	AFM image[a]	(Top) height histogram[b] (Bottom) peak fit[c]	Difference in thickness between thin and thick membrane regions (nm) [d]	Ratio of areas of thick:thin regions [e]	Avg. thickness mismatch (nm) [f]; area ratio of thick:thin regions
1			1.002 ± 0.003	30:70	
2[g]			1.010 ± 0.004	29:71	1.003 ± 0.003 31:69 ± 7
3[g]			0.997 ± 0.003	41:59	

4[g]			1.003 ± 0.005	24:76	

[a]Flattened AFM image with height scale bar in gold. [b]Height histogram in Gwyddion. [c]Typical peak fit trial of the height histogram in a 100-trial bootstrap fit. The dark red line shows the fit to the data marked by blue dots. Magenta dashed lines show the first guess at the peak position from the previous trial. [d]Difference between thickness of the thin and thick regions of the membrane reported as the mean difference in the location of the peaks determined by the bootstrap method. The reported uncertainty is the propagated standard deviation from the bootstrap fit of the two bilayer peaks. [e]Ratio of areas of thick and thin membrane regions from the bootstrap average areas of the two bilayer peaks. [f]Average thickness difference from column 4. The reported uncertainty is the standard error of the mean of the four values. [g]Features far thicker than a lipid bilayer (the highest 12% of values) were excluded from the height histogram.

Table S9: AFM image, peak fits, and resulting data for 48/32/20 mol % DiPhyPC/DPPC/chol supported lipid bilayer

#	AFM image[a]	(Top) height histogram[b] (Bottom) peak fit[c]	Difference in thickness between thin and thick membrane regions (nm) [d]	Ratio of areas of thick:thin regions [e]	Avg. thickness mismatch (nm) [f]; area ratio of thick:thin regions
1			1.542 ± 0.001	21:79	1.43 ± 0.05 24:76 ± 6
2			1.391 ± 0.022	31:69	
3			1.370 ± 0.004	20:80	

[a]Flattened AFM image with height scale bar in gold. [b]Height histogram in Gwyddion. [c]Typical peak fit trial of the height histogram in a 100-trial bootstrap fit. The dark red line shows the fit to the data marked by blue dots. Magenta dashed lines show the first guess at the peak position from the previous trial. [d]Difference between thickness of the thin and thick regions of the membrane reported as the mean difference in the location of the peaks determined by the bootstrap method. The reported uncertainty is the propagated standard deviation from the bootstrap fit of the two bilayer peaks. [e]Ratio of areas of thick and thin membrane regions from the bootstrap average areas of the two bilayer peaks. [f]Average thickness difference from column 4. The reported uncertainty is the standard error of the mean of the three values.

Details of AFM image analysis

AFM image flattening using Gwyddion software

AFM images produce a false color topographic map, which are flattened in Gwyddion (12). The color is set to gold. Mean plane subtraction is used to eliminate background tilt that obscures bilayer thicknesses. Next, scan lines are corrected by matching height medians, and the image is corrected for horizontal scars. The mica and the lipid membrane, which may contain both thick and thin regions, produce at least two distinct height populations. To avoid non-physical flattening results due to these populations, we mask out each layer individually using the marks grains tool and perform a median line scan correction on each layer separately. Last, we mask out of the lowest feature, which is either the mica or thin membrane, and perform a background subtraction to set this feature to ~0 nm. We then use Gywddion's 1D statistical function to obtain the height histogram. We export height histogram values to excel, read the excel file into Matlab, and fit the data with peaks using a 100 bootstrap trial method as part of a Matlab peak fitting program named ifp.m (13).

Fitting AFM height histogram peaks to determine thickness mismatch

The interactive Matlab peak-fitting program ipf.m uses an unconstrained non-linear optimization algorithm to decompose separate and/or overlapping-peaks into component peaks (13). This process gives us differences in thickness between membrane regions, standard deviations in thickness, and areas of the peaks, which allows us to calculate the percent of the membrane area covered by thin and thick phases.

The default peak shape is a Gaussian. We find that changing peak type from Gaussian to other peak types, such as Lorentzian, results in larger fit errors and larger standard deviations from bootstrap methods. Running ipf.m produces a scatter plot of the height histogram data within an interactive graph. The user then selects the range of x-values (heights in nm) that encompasses all peaks of interest. The program requires first guesses for the peak positions, which is done automatically or by clicking on peaks. We used the clicking functionality because it is recommended for data like ours for which peaks are not evenly spaced. We next performed a 100 trial bootstrap fit. In each trial, the data set is divided into two sub-sets, each of which is fit, and then the process is repeated. The resulting standard deviation reflects the stability of the peak fit with respect to random noise in the data. Our reported values for thickness differences between thick and thin regions of membranes (Tables S3-S9) are the differences in the bootstrap mean value of the fitted peaks from the bootstrap fit. The reported uncertainties in the values for each image are the propagated errors from standard deviations produced by the bootstrap method. These values are averaged to give the average thickness mismatch (final column).

References for Supporting Information:

1. Blosser, M. C.; Cornell, C.; Rayermann, S. P.; Keller, S. L., Phase Diagrams and Tie Lines in GUVs. In *The Giant Vesicle Book*, Dimova, R.; Marques, C., Eds. in press.
2. Blosser, M. C.; Honerkamp-Smith, A. R.; Han, T.; Haataja, M.; Keller, S. L., Transbilayer Colocalization of Lipid Domains Explained Via Measurement of Strong Coupling Parameters. *Biophys. J.* **2015,** *109*, 2317-2327.
3. Bleecker, J.V., Cox, P.A., Foster, R.N., Litz, J.P., Blosser, M.C., Castner, D.G., and Keller, S.L. **2015**. Thickness mismatch of non-canonical Lo-Ld lipid membranes. *J. Phys. Chem. B.* 120:2761-2770.
4. Bhatia, T.; Husen, P.; Ipsen, J. H.; Bagatolli, L. A.; Simonsen, A. C., Fluid Domain Patterns in Free-Standing Membranes Captured on a Solid Support. *Biochim. Biophys. Acta* **2014,** *1838*, 2503-2510.
5. Veatch, S. L.; Keller, S. L., Seeing Spots: Complex Phase Behavior in Simple Membranes. *Biochim. Biophys. Acta* **2005,** *1746*, 172-185.
6. Hillert, M. *Phase equilibria, phase diagrams and phase transformations: their thermodynamic basis.* Cambridge University Press: Cambridge, 2007.
7. Chaikin, P.M. and Lubensky, T.C. *Principles of condensed matter physics.* Cambridge University Press: Cambridge, 2000.
8. Ferguson, F. D. and Jones, T. K. *The phase rule* . Butterworths: London, 1966.
9. Veatch, S. L.; Gawrisch, K.; Keller, S. L., Closed-Loop Miscibility Gap and Quantitative Tie-Lines in Ternary Membranes Containing Diphytanoyl PC. *Biophys. J.* **2006,** *90*, 4428-4436.
10. García-Sáez, A. J.; Chiantia, S.; Schwille, P., Effect of Line Tension on the Lateral Organization of Lipid Membranes. *J. Biol. Chem.* **2007,** *282*, 33537-33544.
11. Connell, S. D.; Heath, G.; Olmsted, P. D.; Kisil, A., Critical Point Fluctuations in Supported Lipid Membranes. *Faraday Discuss.* **2013,** *161*, 91-111.
12. Nečas, D.; Klapetek, P., Gwyddion: An Open-Source Software for SPM Data Analysis. *Cent. Eur. J. Phys.* **2012,** *10*, 181-188.
13. O'Haver, T. ipf.m Peak Fitter: http://terpconnect.umd.edu/~toh/spectrum/InteractivePeakFitter.htm.