Fluctuation spectroscopy of giant unilamellar vesicles using confocal and phase contrast microscopy†

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A widely used method to measure the bending rigidity of bilayer membranes is fluctuation spectroscopy, which analyses the thermally-driven membrane undulations of giant unilamellar vesicles recorded with either phase-contrast or confocal microscopy. Here, we analyze the fluctuations of the same vesicle using both techniques and obtain consistent values for the bending modulus. We discuss the factors that may lead to discrepancies.

Bending rigidity of cellular membranes plays a key role in membrane remodeling. Knowledge of its value is needed to quantify processes that involve curvature changes such as budding (as in endo- and exocytosis), tubulation and fusion. Various experimental methods have been devised to measure bending rigidity, e.g. micropipette aspiration,2,3 electrodeformation,4–7 optical tweezers,8,9 and scattering based techniques.10,11 One of the most popular methods is fluctuation spectroscopy, pioneered by Brochard and Lenon,12 due to its ease of implementation.6,13–15

In essence, a time series of vesicle contours in the focal plane (the equator of the quasi-spherical vesicle) is recorded. The quasi-circular contour is decomposed in Fourier modes, and the contour in the equatorial plane of a quasi-spherical vesicle is recorded. The quasi-circular contour is decomposed in Fourier modes,1,17,26–30 see Table S1 in the ESI† for a list of reported bending rigidity of DOPC membranes. For example, even measurements with the same method can give a wide range of values, e.g., the bending rigidity of a DOPC bilayer measured with flickering spectroscopy has been reported from 13k_BT33 to 30k_BT19 where k_BT is the thermal energy.

In order to compare imaging with phase contrast and confocal microscopy, which was suggested in Rautu et al.24 as a better technique due to the precise control over the focal depth, we measure the bending rigidity of the same giant vesicle with both techniques. We highlight some important issues to be considered to ensure reliable measurements. We also show that results obtained with both methods are consistent.

Equilibrium fluctuations of a quasi-spherical vesicle

First, we summarize the theoretical basis of the fluctuations analysis (details are provided in ESI,† Section S6). We also correct published expressions for the relaxation frequency and cross-spectral density of the shape fluctuations.

The contour in the equatorial plane of a quasi-spherical vesicle is decomposed in Fourier modes, \( r(\phi; t) = R_0 \left( 1 + \sum_{q=-q_{\text{max}}}^{q_{\text{max}}} u_q(t) \exp(iq\phi) \right) \), where \( R_0 = (3V/4\pi)_{1/3} \) is the radius of an equivalent sphere with the volume \( V \) of the GUV and \( q \) is the mode number. In practice, \( q_{\text{max}} \) is the maximum number of experimentally resolved modes. The statistical analysis of the fluctuating amplitudes \( u_q \) yields the values of...
membrane bending rigidity $\kappa$ and the tension $\sigma$ since 
$$\langle |u_q|^2 \rangle \sim k_B T \kappa (q^2 + \sigma q),$$ 
where $\sigma = \sigma R_0^2/\kappa$.

More precisely, the statistics of the two-dimensional circular 
 modes, $u_q$, is derived from the three-dimensional shape modes, 
$f_{3D}$ which describe the nearly-spherical shape in terms of 
 spherical harmonics,$^{3,13} R(\theta; \phi; t) = R_0 \left( 1 + \sum_{l=0}^{l_{\max}} \sum_{m=-l}^{l} f_{3D}(t) Y_{lm}(\theta; \phi) \right)$. Here, 
l_{\max} is an upper cutoff, in the order of the ratio of the GUV radius 
and bilayer thickness. The contour in the focal plane corresponds 
to the equator of the quasi-spherical vesicle, $\theta = \pi/2$, i.e., 
$r(\phi; t) = R(\pi/2; \phi; t)$, which leads to the following expression for 
the mean squared amplitudes

$$\langle |u_q|^2 \rangle = \frac{k_B T}{\kappa} \sum_{l=0}^{l_{\max}} n_q P_{2l}(0) (l(l+1) + \sigma)^{-1}$$

(1)

where $n_q = (2l + 1)l(l+q)!/4\pi(l+q)!$ and $P_{2l}$ are the associated 
Legendre polynomials. The short-wavelength shape fluctuations 
are dominated by the bending rigidity, while the long wavelengths 
are controlled by tension; the crossover occurs around mode $q_c = \sqrt{\sigma}$.

To validate our methodology, we have simulated the thermal 
shape fluctuations of a GUV, see also ESI† Section S6. We have 
generated a sequence of three-dimensional shapes [and their corresponding 
axial equatorial contours] using the evolution equations$^{3,12}$

$$\frac{df_{3D}}{dt} = -\tau_i^{-1} f_{3D} + \zeta_{3D}(t);$$

$$\tau_i^{-1} = \frac{\kappa}{\eta_{ex} R_0^2} \frac{(l-1)(l+1)(l+2)}{4l^3 + 6l^2 - 1 + (2l^3 + 3l^2 - 5) \left( \frac{\eta_m}{\eta_{ex}} - 1 \right)}$$

(2)

where $\zeta_{3D}$ is the thermal noise driving the membrane undulations, 
$\eta_{in}$ and $\eta_{ex}$ are the viscosity of the solution inside and outside the 
vesicle. Note that the relaxation time given by eqn (2) in Rautu et al.$^{24}$ 
has incorrect dependence on the viscosities of the enclosed and 
suspending solutions (this mistake is unlikely to affect the reported 
fluctuation spectra). The simulated contours were analyzed by our 
method and the extracted bending rigidity and tension were compared 
to the input values to confirm accuracy of the contour detection, 
Fourier decomposition and data fitting algorithms. The time evolution 
of the modes also enables us to access information provided by 
the time correlations $\langle u_q(t) u_q^*(t) \rangle = \langle |u_q|^2 \rangle \exp(-\tau_{eq})$. If $q \gg 1$, the 
correlation time tends to that of a planar membrane $\tau_{eq}^{-1} = \kappa (q^2 + \sigma q)/2R_0^2 (\eta_{in} + \eta_{ex})$. If the cross-spectral density $\langle u_q(t) u_q^*(0) \rangle$ is utilized, the correct time dependence in the exponential 
includes a factor of 2 and eqn (3) in Zhou et al.$^{33}$ needs to be corrected 
(see ESI† Section S6, eqn (39)).

Bending rigidity obtained from confocal and phase-contrast 
microscopy: effect of resolution and vesicle size

Giants unilamellar vesicles (GUV) were electroformed from 
DOPC (99.8 mol%) dioleoylphosphatidylcholine and 0.2 mol% 
Texas-Red 1,2-hexadecanoyl-sn-glycero-3-phosphoethanolamine, 
TR-DHPE) in 20 mM sucrose and subsequently diluted in 
22 mM glucose, see ESI† Section S2 for details. Low sugar 
concentration was used in order to minimize the effects of 
gravity$^{28}$ and effect of sugars,$^{26}$ but still allow the vesicles to 
settle to the chamber bottom for easier recording. Low dye 
content minimizes effects of fluorophores.$^{29}$

Fig. 1 shows a typical fluctuations spectrum, given by 
eqn (1), fitted to the experimental data for the same vesicle 
immaged with confocal and phase contrast microscopy using a 
$40\times$ objective with 0.6 numerical aperture (NA), pinhole size of 
1 Airy unit (AU) and polarization correction (see below and 
ESI† Section S3). The contour was detected with sub-pixel 
resolution. The experimental data was fitted with eqn (1) with 
Levenberg–Marquardt algorithm and yielded bending rigidity 
$\kappa = 23.9 \pm 1.6 k_B T$ and tension $\sigma = 5.1 \pm 1.4 \times 10^{-9}$ N m$^{-1}$ and 
$\kappa = 22.3 \pm 2.1 k_B T$ and $\sigma = 3.1 \pm 1.2 \times 10^{-9}$ N m$^{-1}$ from the 
confocal and phase contrast microscopy data, respectively. 
The average and error in individual GUV was determined by 
performing fluctuation spectroscopy 2–3 times. By imaging a 
population of 18 vesicles with both methods, the bending 
rigidity obtained are $22.5 \pm 2.0 k_B T$ with confocal and $23.3 \pm 
1.6 k_B T$ phase contrast microscopy; each vesicle was analyzed 
with both imaging techniques as in Fig. 1 and then the results 
were averaged over the population. Fig. 2 shows the box 
and whisker plot for more detailed statistics. Based on $F$ statistics 
and ANOVA (analysis of variance) test, the $p$-value obtained is 
P = 0.48 for null hypothesis testing. We also performed the 
paired-sample $t$-test and obtained $p = 0.43$. These $p$-values 
indicate no significant difference between the two imaging 
techniques.

Since only modes with wavenumber $q > \sqrt{\sigma R_0^2/k}$ are sensitive 
to the bending rigidity, it is desirable to have more resolved
Discovered for confocal microscopy with low resolution optics, the range of radii to avoid a size bias in the bending rigidity values we present. The reasons for this are discussed in the next section.

Out-of-focus fluorescence affects contour detection quality in confocal microscopy

The vesicle contour is detected from radial intensity line profiles, see ESI,† Section S2. In confocal cross sections, weak fluorescence from the vesicle membrane located above and below the focal plane may result in signal projected in the interior of the vesicle image which is higher compared to the surrounding background. The resulting asymmetry in the intensity line profile (Fig. 4a) leads to an artificial contour displacement, i.e., poor contour detection (note that such an asymmetry is absent in images acquired with phase contrast microscopy of vesicles with similar refractive indices of the vesicle membrane). The mean Pearson correlation and standard deviation coefficient is $0.65 \pm 0.21$ (see ESI,† Section S5 for the histograms generated with bootstrapping resampling technique). Analysis of a population of similar sized vesicles with radii around 10 μm underestimates $k$ by roughly $6k_BT$. The bias originates from out-of-plane fluorescence which worsens the contour detection. This issue is investigated in the next section and ESI,† Section S6. The size dependence is insignificant for phase contrast microscopy with a mean correlation coefficient of $0.28 \pm 0.18$ with $40 \times / \text{NA} 0.6$ (air). Analyzing the same vesicle population with $40 \times / \text{NA} 1.3$ objective in phase contrast and confocal imaging yields $21.0 \pm 2.0k_BT$ and $21.7 \pm 2.0k_BT$ respectively. Higher numerical aperture in phase contrast leads to negligible correlation coefficient of $-0.07 \pm 0.34$ between bending rigidity and vesicle size and decrease in the correlation coefficient to $0.43 \pm 0.14$ for confocal imaging with $40 \times / \text{NA} 1.3$ objective.

Out-of-focus fluorescence in confocal images can result in erroneous contour detection and increased error in bending rigidity. (a) Intensity line profiles (gray-value) across the vesicle membrane (DOPC, with 0.2 mol% dye) are symmetric for phase-contrast images (blue) but asymmetric for confocal images (red, 1 AU). The asymmetry in the confocal line profile leads to incorrect detection of the contour position defined by the parabolic fit minimum, here, shifted inwards by 0.53 μm. (b) Vesicle images (and their inverted gray-value analogs) acquired with different pinhole size show increased fluorescence inside the vesicle which results in larger error in the bending rigidity. Box and whisker plot of the bending rigidity of the same DOPC vesicles imaged with confocal microscopy at three different pinhole sizes for $40 \times / \text{NA} 0.6$ objective and polarization correction.
internal and external solutions). This asymmetry creates a systematic error shifting the vesicle contour by 0.53 μm. The error is larger than the pixel resolution of the system, 0.252 μm, hence the higher modes are averaged out. Smaller vesicles or larger pinholes lead to higher signal inside the vesicle (see inset in Fig. 4b) corresponding to greater asymmetry which increases the error from contour fitting and introduces dependence of the bending rigidity on vesicle size. For imaging with higher numerical aperture objectives (e.g. NA 1.3), the asymmetry in the intensity line profiles is suppressed and contour detection is correct. Note that phase contrast images do not suffer from the asymmetry-induced error irrespective of the objective NA.

We investigated the impact of out-of-focus fluorescence on the fluctuations statistics by varying the pinhole size for confocal imaging on the same vesicle. The standard pinhole size in confocal microscopy is defaulted to 1 Airy unit (AU) (full width at half maximum FWHM = 1.6 μm) for 40×/NA 0.6 objective. We analyzed the same vesicles with different optical sectioning at 0.3 AU (FWHM = 0.9 μm), and 2 AU (FWHM = 2.9 μm). The mean bending rigidity did not show significant differences based on ANOVA testing, post hoc Dunnett test and paired-sample t-test ($p = 0.87$), however the error increases with the pinhole size. The sensitivity to the vesicle size also becomes more pronounced with higher pinhole size. At the largest pinhole size (2.0 AU) the Pearson correlation coefficient is 0.60 ± 0.22, while for 0.3 AU it becomes negligible, $-0.14 ± 0.30$.

Dye related artifacts: vesicle tubulation and polarization

Confocal imaging relies on fluorophores added to the membrane, and some studies have used up to 10 mol% dye. To probe the effect of fluorophore on $\kappa$, we changed the dye concentration from 0.2 mol% to 2 mol% TR-DHPE. The bending rigidity of this population of vesicles showed non-significant difference with $\kappa = 20.09 ± 2.49 k_B T$ with one ANOVA testing. However, it was observed that over 2–3 min of recording, around 50% of the vesicles developed inward structures such as buds or visible tubes as shown in Fig. 5. Vesicles with such defects displayed significantly higher bending rigidity, $25.01 ± 2.11 k_B T$.

TR-DHPE belongs to a family of polarity-sensitive fluorescent probes. As a result, the signal intensities are different at the pole and equator of the vesicle (see ESI,† Section S3). This may lead to errors in the contour detection in these regions. The polarization effect was corrected by using circular rotation plates to have even intensities across the equatorial vesicle plane. The analysis of the same vesicle with and without the polarization correction showed a $3k_B T$ lower bending rigidity without any correction with 40×/NA 0.6 (oil) objective. This softening effect became insignificant with 40×/NA 1.3 (oil) objective (ESI,† Section S3). This is likely due to loss of signal at low intensity regions where the higher mode fluctuations intensities are averaged out with background noise due to out-of-focus fluorescence.

Effect of nearby vesicles on fluctuation spectra

The equilibrium shape fluctuations of an isolated GUV are driven by Gaussian thermal noise. Defects such as buds, nanotubes, invaginations or docked LUVs modify the vesicle fluctuations and their effect can be detected in the statistics at each point on the vesicle contour profile using the ensemble-averaged probability density function (PDF) as shown in Fig. 6a. In addition to defects attached the membrane, we also found that hydrodynamic flows and/or fluorescence signal from nearby vesicles can affect vesicle fluctuations.

We characterized the Gaussianity of the fluctuations using the fourth PDF moment, Kurtosis, $K$. For a Gaussian distribution, $K = 3$. In Fig. 6 we demonstrate how thermal fluctuations may be modified (see ESI,† Movie S3). As shown, the majority of contours are characterized by a normal distribution. However near other flickering structures, the fluctuation map density is modified. The non-Gaussian enhanced fluctuations are observed with leptokurtic nature ($K > 3$). This observation serves as a caution to filter out vesicles with sub-optical structures affecting the fluctuations.

Conclusions

We compare the bending rigidity of bilayer membranes determined from flickering spectroscopy of GUVs imaged with confocal and phase contrast microscopy. Examining the same

Fig. 5 Time lapse of a DOPC vesicle with 2 mol% TR-DHPE developing inward nanotubes as a result of long exposure to laser during confocal imaging. The second and third cross sections are non-equatorial to better show the formed nanotubes.

Fig. 6 Nearby structures affect the fluctuation spectrum. (a) A flickering vesicle in close proximity to another vesicle bud. (b) Fluctuation density map of the vesicle in (a): the fluctuations are modified by hydrodynamic interactions of the other flickering vesicle bud. (c) Kurtosis $K > 3$ indicates the vesicle fluctuations have amplified meaning local softening of the membrane.
vesicle with both imaging techniques shows no significant differences in the bending modulus obtained from the two methods, in contrast to the overestimation reported by Rautu et al.\textsuperscript{2} when phase contrast microscopy is used. Our analysis indicates that membrane defects such as buds and tubes induced by long laser exposure in confocal microscopy can significantly stiffen the membrane. Furthermore, we find that errors in contour detection that could impact data interpretation can arise from fluorescence signal “pollution” and dye errors in contour detection that could impact data interpretation. The bending rigidity we obtain ($\sim 2k_B T$ for DOPC) is in line with the values obtained with other techniques such as micropipette aspiration, X-ray scattering, electrodereformation and neutron spin echo.\textsuperscript{3,8,10} A scatter of approximately $2k_B T$ is typical in the experiments and should be taken into account when comparing data from different groups and methods. Exploring the effect of various parameters, we find that optimal imaging conditions for bending rigidity measurements from confocal imaging include high magnification objective, high numerical aperture, circular polarization correction, minimum dye concentration, small pinhole size, and broad vesicle size distribution.

In conclusion, we demonstrate that phase contrast and confocal microscopy produce the same results if precautions are taken to minimize effects of the dye and improve contour detection. Our study suggests that the many published results obtained by phase contrast microscopy are likely to be unaffected by the projections of out-of-focus fluctuations onto the imaging plane in contrast to the claim by Rautu et al.\textsuperscript{2} Since dye related artifacts such as laser-induced defects can compromise the data, it is advantageous to use phase contrast imaging as it does not require dyes.

Conflicts of interest
There are no conflicts to declare.

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Notes and references

S1. Bending rigidity value of DOPC bilayers

The bending rigidity values of bilayer membranes made of the same lipid can vary across studies due to different conditions, e.g., sugars, salt, buffers, dye concentration, as well as the preparation method [1]. Table I illustrates the wide range of reported values of the bending rigidity values DOPC bilayers. Refer to Table II for the bending rigidity values obtained in this study for different microscopy setting.

<table>
<thead>
<tr>
<th>Method</th>
<th>Rigidity (k_BT)</th>
<th>Dye conc. (% mol)</th>
<th>Buffer, Sugar (inside/outside)</th>
<th>Salt</th>
<th>Preparation</th>
</tr>
</thead>
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<tr>
<td>Fluctuation Spec. [EP,C]</td>
<td>14.9±0.4</td>
<td>15.8 NBD PC</td>
<td>100 mM Sucrose/100 mM Sucrose</td>
<td>N/A</td>
<td>Electroformation</td>
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<tr>
<td>Fluctuation Spec. [PC]</td>
<td>26.4±2.4</td>
<td>0 or 0.1 diIC18</td>
<td>10 mM Sucrose/10 mM Glucose</td>
<td>0.1 mM NaCl</td>
<td>Electroformation</td>
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<td>Fluctuation Spec. [PC]</td>
<td>26.8±2.4</td>
<td>1.0 Liss Rhod PE</td>
<td>450 mM Sucrose/500 mM Glucose</td>
<td>N/A</td>
<td>Electroformation</td>
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<tr>
<td>Fluctuation Spec. [PC]</td>
<td>29.8±2.4</td>
<td>1.0 Liss Rhod PE</td>
<td>450 mM Sucrose/500 mM Glucose</td>
<td>N/A</td>
<td>Phase Transfer</td>
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<td>100 mM Sucrose/200 mM Glucose</td>
<td>N/A</td>
<td>Electroformation</td>
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<td>27.3±3.2</td>
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<td>10 mM Sucrose/10 mM Glucose</td>
<td>N/A</td>
<td>Electroformation</td>
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<td>Fluctuation Spec. [C]</td>
<td>19±1</td>
<td>0.8 TR DHPE</td>
<td>197 mM Sucrose/200 mM Glucose</td>
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<td>Time Correlations</td>
<td>22.1</td>
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<td>N/A</td>
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<td>Thin Film Hyd.</td>
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<td>Micropipette Aspiration</td>
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<td>Electroformation</td>
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<td>Water/Water</td>
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<td>Electroformation</td>
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<td>Neutron Spin Echo</td>
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<td>D_2O/D_2O</td>
<td>0 mM</td>
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<td>Neutron Spin Echo</td>
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<td>D_2O/D_2O</td>
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<td>295 mM Sucrose/300 mM Glucose</td>
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TABLE II: Bending rigidity values obtained in this study for DOPC under different conditions and microscopy settings. Note the sugar concentration is the same in all the experiments: 20 mM Sucrose inside/ 22 mM Glucose outside. The dye used is TR DHPE and all the vesicles were formed via electroformation.

<table>
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<th>Microscopy</th>
<th>Rigidity (k_BT)</th>
<th>Dye conc. (%mol)</th>
<th>Objective/NA</th>
<th>Polarization Correction</th>
<th>Pinhole (A.U)</th>
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<td>Phase Contrast</td>
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<td>0.2</td>
<td>100x/1.25</td>
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<td>0</td>
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S2. Methods

Vesicle preparation

Giant unilamellar vesicles (GUVs) were prepared using the classical electroformation method \[19\] from DOPC and the fluorescent lipid Texas Red 1,2-hexadecanoyl-sn-glycero-3-phosphoethanolamine (TR-DHPE). The composition of the GUVs explored are 99.8 % DOPC 0.2 % TR-DHPE and 98 % DOPC 2 % TR-DHPE (mole fractions). Stock solutions of DOPC and TR-DHPE at 10 mg/ml and 1 mg/ml in chloroform were diluted to a final concentration of 4 mM for varying proportions. A small volume, 10 µl, of the solution was spread on the conductive surface of two glass slides coated with indium tin oxide (ITO) (Delta Technologies). The glass slides were then stored under a vacuum for 1–2 hours to remove traces of organic solvent. Afterwards, a 2 mm Teflon spacer was sandwiched between the glass slides and the chamber was gently filled with 20 mM sucrose solution. The slides (conductive side facing inward) were connected to an AC signal generator Agilent 33220A (Agilent Technology GmbH, Germany). An AC field of voltage 1.5 V and frequency 10 Hz applied for 2 hours at room temperature, resulting in 10-50 µm sized vesicles. The harvested vesicles were diluted 10 times in 22 mM glucose solution to obtain fluctuating vesicles. All GUVs were analyzed within 8 hours of electroformation.

Microscopy and video recording

The equatorial fluctuations for both phase contrast and confocal mode were recorded with Leica TCS SP8 scanning confocal microscope using a HCX PL APO 40x/ Numerical Aperture (NA) 0.6 Ph2 (air) objective and a HC PL APO 40x/ NA 1.3 (oil) objective. The pinhole size during the experiment was fixed to 1 AU (Airy units) unless stated otherwise. Table 1 compiles the pixel size and focal depth for different experimental conditions. The scanning speed was fixed to 1 kHz in bidirectional mode and the polarizer plates were rotated (100%) to remove the polarization effect of the fluorescent dye unless stated otherwise. The dye was excited with a 561 nm laser (diode-pumped solid-state laser) with 1.61% (laser intensity) HyD3 detector (hybrid) and the gain was fixed to 23%. Phase contrast imaging was recorded with PCO CS dimax (PCO AG, Kelheim, Germany) mounted on confocal microscope. 1500-2000 images were recorded at 3.83 frames per second (fps) with confocal and 60 fps with phase contrast imaging. The RGB confocal images were converted to 8 bit and then inverted. We implemented an inbuilt MATLAB sobel disk filter fspecial(’sobel’) and image normalization to increase the contrast of the contour.

In this section, we list different focal depths and pixel sizes for different microscopy and numerical aperture settings for 40x objective. Focal depth or FWHM (full width half maximum) of phase contrast imaging was determined using the standard formula \[d = \frac{\lambda}{NA}\]. The wavelength of transmission light was assumed to be 550 nm.
TABLE III: Different experimental conditions for video recording with 40x objective.

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>Numerical Aperture</th>
<th>Medium</th>
<th>Pinhole size (AU)</th>
<th>Focal depth (µm)</th>
<th>Pixel Resolution (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase contrast</td>
<td>0.6</td>
<td>Air</td>
<td>1</td>
<td>1.57</td>
<td>276.9</td>
</tr>
<tr>
<td>Phase contrast</td>
<td>1.3</td>
<td>Oil</td>
<td>1</td>
<td>0.35</td>
<td>158.7</td>
</tr>
<tr>
<td>Confocal</td>
<td>0.6</td>
<td>Air</td>
<td>1</td>
<td>1.61</td>
<td>252.7</td>
</tr>
<tr>
<td>Confocal</td>
<td>1.3</td>
<td>Oil</td>
<td>1</td>
<td>0.52</td>
<td>252.7</td>
</tr>
</tbody>
</table>

TABLE IV: Focal depth or FWHM (full width half maximum) for confocal imaging.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Pinhole size (AU)</th>
<th>Focal depth (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Air</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>Air</td>
<td>2</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**Sub-pixel contour recognition**

The intensity profile in the radial direction for N wedges were determined from three different interpolation schemes (Gaussian, parabolic and linear weighting of neighbouring pixel) for sub-pixel contour recognition. This was done to check if different interpolation schemes affects the bending rigidity values due to uncertainty introduced at higher wave-numbers for experimental vesicle contour fluctuations. The mean bending rigidity obtained was similar for all the three schemes for the same vesicle. Figure (S1) illustrates the sub-pixel accuracy determination for a 35 µm radius vesicle. The bending rigidities obtained was 22.0±3.0 k_BT, 21.1±1.0 k_BT and 21.9±2.2 k_BT from Gaussian, parabolic and linear interpolation schemes respectively.

**S3. Polarization Effects**

We analyzed the same vesicle with and without polarization effects. The polarization effects were corrected using circular plates that were rotated 100%. Figure (S2) illustrates the effect of dye polarization for vesicles imaged with different numerical apertures. Using one Anova test, we find a significant difference of 3 k_BT for the 40x/0.6 NA case. The difference tends to be negligible for 40x/1.3 NA case.

**S4. Effect of Vesicle Size on Bending Rigidity Values**

The bending rigidity obtained from confocal microscopy with low-resolution optics (e.g. 40x objective, NA 0.6, 1 AU, polarization correction) can be systematically underestimated if the vesicle population contains similar sized vesicles. We demonstrate this by comparing the bending rigidity of the same vesicle imaged with confocal and phase-contrast microscopy, see Figure (S3). Vesicles with smaller sizes yield apparently lower bending rigidity, see Fig. S4 which further highlights the bias effect. For small vesicles, the out-of-focus signal gives rise to asymmetry in the contour intensity (illustrated in Fig. 4a in the main text) which leads to errors in the contour detection and underestimation of the bending rigidity. When the refractive index difference across the membrane is small (as is the case in our experiments), phase contrast imaging does not suffer from this size bias.

**S5. Bootstrapping resampling**

Details about the various statistical techniques can be found in Ref. [20]. Here we explain the bootstrapping sampling technique. A more rigorous reference is the textbook [21]. In practice, the finite amount of data or length of experiment limits the accuracy to infer data confidently. Bootstrapping is an inference method about the population from a given sample. In bootstrap-resamples, the population is in fact the sample and this quantity is known. This allows to measure the quality of inference of the 'true' sample from a re-sampled data. For example, let’s consider the average mass of the human population world wide. It is difficult to measure the mass of every individual globally, therefore, a small sample is measured. Let’s assume the sample size of N people. From that sample size, only one mean can be measured. In order to have a reasonable estimate about the population statistics, we need to have variability
Fig. S1: Intensity profile for a vesicle contour obtained from confocal imaging. The contour recognition details are given in [3]. The sub-pixel accuracy of the contour profile is determined based on (a) Gaussian, parabolic and (b) linear interpolations.

Fig. S2: Polarization effects. (a) Confocal images of the same vesicle with and without polarization effects for 40x/0.6 NA case. The polarization effects were removed using circular plates that were rotated 100%. (b, c) Comparison between the same vesicles for different numerical apertures. Using one Anova test, we find a significant difference of 3 k_B T for 40x/0.6 NA case. The difference tends to be negligible for 40x/1.3 NA case. Pinhole size is 1 AU.

of the mean that we computed. The simplest bootsampling statistics can be considered by taking the original data N individuals and resampling to create a new sample of the same size N (e.g. we might ’resample’ 10 times from [60,61,62,63,64,65,66,67] kg and get [61,64,63,63,60,60,62,65] kg). This process is repeated a large number of times, 100 to 10000, to create a histogram that be applied to any estimator testing. Bootstrap resampling was carried out using MATLAB’s bootstrap () function.

In the case of our experiments, the finite amount of data or length of experiment limits the accuracy to infer data confidently. The bootstrap resampling requires choosing random replacement from a given data set and examining each sample the same way. This way a particular data point from the original set can reappear randomly multiple times in a particular bootstrap sample. The element size of the bootstrap sample is the same as the element size of the original data. This technique allows to obtain uncertainty of the quantity one estimates.

Bootstrap resampling algorithm for estimating standard error [21]:
1. Obtain N independent bootstrap samples X^*, X^+, X^-, ...,X^N, each consisting of n data values drawn with a replacement from x where x = [x^1, x^2, x^3,...,x^n]. Note for estimating a standard error, the number N will ordinarily be larger than 30 to satisfy the Central Limit Theorem. Computation allow to use a large number N such as 10^3 to 10^4.
2. Determine the bootstrap replication for every bootstrap resample:

\[ \zeta^* (b) = s(X^* \mid b = [1, 2, 3,...N] \]

where s() is a statistical function like sample mean. For example, if s(x) is the sample mean \( \bar{x} \) then S(X*) is the...

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Fig. S3: Vesicle size effects. Every vesicle was imaged with confocal and phase contrast microscopy. Data are collected on DOPC vesicles with different sizes. The dye concentration was 0.2 mol. Imaging was done with 40x objectives with NA 0.6, 1 AU and polarization correction.

Fig. S4: Bootstrap method with 95% confidence to evaluate bending rigidity dependence on size of vesicles for different numerical aperture (a) 40x/0.6 NA, (b) 40x/1.3 NA in phase contrast (P) or confocal(C) microscopy, and the pinhole sizes (c) (blue AU 0.3 and red AU 2).

mean of bootstrap data set.

3. Compute the standard error $SE$ by utilizing the standard deviation of $N$ replications

$$SE = \left[ \frac{\sum_{b=1}^{N} [\zeta^*(b) - \zeta^*(\cdot)]^2}{N - 1} \right]^{\frac{1}{2}}$$

where $\zeta^*(\cdot) = \frac{\sum_{b=1}^{N} \zeta^*(b)}{N}$.

In our case we determine the $SE$ of mean Pearson correlation using bootstrapping statistics.

S6. Numerical simulations of vesicle contours

Mathematical Model

The total energy of the system is given by the Helfrich model[22] as Eq. (3) where $\kappa$ is the bending rigidity, $c_1$ and $c_2$ are the local radii curvatures, $A$ is the total surface area, $V$ is the interior volume of the vesicle, $\sigma$ is the surface tension, and $p$ is the pressure difference across the membrane.

$$F = \frac{\kappa}{2} \int_A (c_1 + c_2)^2 dA + \sigma A + pV$$

(3)
For a quasi-spherical vesicle in equilibrium, the shape can be decomposed into spherical harmonics \(Y_{lm}\) such that the position of the surface is given by

\[
R(\theta, \phi, t) = R_0 \left( 1 + \sum_{l=0}^{L_{\text{max}}} \sum_{m=-l}^{l} f_{lm}(t) Y_{lm}(\theta, \phi) \right)
\]

(4)

where the characteristic radius \(R_0\) is given by \(V = \frac{4}{3} \pi R_0^3\). The spherical harmonics are defined as

\[
Y_{lm} = n_{lm} P_{lm}(\cos \theta) e^{im\phi}, \quad n_{lm} = \sqrt{(2l + 1)(l - m)!} \frac{4}{4\pi (l + m)!}
\]

(5)

\(P_{lm}(\cos \theta)\) are the associated Legendre polynomials.

As \(l = 1\) account for translational modes, for the sake of this paper, \(f_{lm}(t)\) will be restricted to \(f_{1m}(t) = 0\) for \(l = 1\).

Furthermore, volume conservation requires that

\[
f_{00} = -\frac{1}{\sqrt{4\pi}} \sum_{l=2}^{L_{\text{max}}} \sum_{m=-l}^{l} |f_{lm}|^2.
\]

(6)

Assuming there is no external fluid flow, the harmonic coefficients \((f_{lm})\) for \(l > 1\) are described by the following stochastic differential equation \[23\]

\[
\partial_t f_{lm} = -\tau_{ll}^{-1} f_{lm} + \zeta_{lm}(t)
\]

(7)

where

\[
\tau_l = \frac{\eta_{\text{ex}} R_0^3}{\kappa \Gamma_l(\lambda) E_l}, \quad \Gamma_l = \frac{l(l + 1)}{4l^3 + 6l^2 - 1 + (2l^3 + 3l^2 - 5)(\lambda - 1)} \quad \text{and} \quad E_l = (l + 2)(l - 1) \left( l(l + 1) + \bar{\sigma} \right).
\]

(8)

A tutorial derivation of the evolution equation and the relaxation time (in the absence of thermal noise) can be found in Refs. \[24, 25\]. The dimensionless tension is \(\bar{\sigma} = \sigma R_0^2 / \kappa\). \(\lambda = \eta_{\text{in}} / \eta_{\text{ex}}\) is the ratio of viscosities of the solutions inside and outside the vesicle. When \(\lambda = 1\), our result for the relaxation time reduces to the one reported by Refs. \[23, 26\].

To make easier comparison with the result of Ref. \[10\], we can rewrite the relaxation time as

\[
\tau_l = \frac{R_0^3 \eta_{\text{ex}}}{\kappa} \frac{(2l^3 + 3l^2 + 4) + \eta_{\text{in}} (2l^3 + 3l^2 - 5)}{l(l + 1)(l + 2)(l - 1) \left( l(l + 1) + \bar{\sigma} \right)}
\]

(9)

\(\zeta_{lm}(t)\) is a stochastic term accounting for thermal noise; the corresponding time correlation is given as

\[
\langle \zeta_{lm}(t) \zeta_{lm'}(t') \rangle = (-1)^m \frac{2k_B T \tau_l}{\eta_{\text{ex}} R_0} \delta_{l,l'} \delta_{m,-m'} \delta(t - t').
\]

(10)
The δ functions are the traditional Kronecker and Dirac delta functions. From Eq. [10] the variance of \( \zeta_{lm}(t) \) is given by

\[
\left\langle |\zeta_{lm}|^2 \right\rangle = 2 \frac{k_B T T_l}{\eta \varepsilon R_0^4} = 2 \Sigma_l. \tag{11}
\]

**Numerical Method**

At this point, it is convenient to decompose \( f_{lm} \) and \( \zeta_{lm} \) into real and imaginary components such that \( f_{lm}(t) = X_{lm}(t) + i Y_{lm}(t) \) and \( \zeta_{lm}(t) = a_{lm}(t) + i b_{lm}(t) \). As \( a_{lm} \) and \( b_{lm} \) are independent of each other then

\[
\left\langle |\zeta_{lm}|^2 \right\rangle = \left\langle |a_{lm}|^2 \right\rangle + \left\langle |b_{lm}|^2 \right\rangle = 2 \left\langle |a_{lm}|^2 \right\rangle = 2 \left\langle |b_{lm}|^2 \right\rangle = 2 \Sigma_l \tag{12}
\]

Eq. (7) can then be rewritten as

\[
\partial_t X_{lm} = -\frac{\tau}{\tau_\ell} X_{lm} + a_{lm}(t) \tag{13}
\]

and similarly for \( Y_{lm} \). As Eq. (13) is a simple Langevin equation, the exact time update [27] is given as

\[
X_{lm}(t + \Delta t) = X_{lm}(t) e^{-\Delta t/\tau_\ell} + \frac{1}{2 \Sigma_l^2 \tau_\ell} (1 - e^{-2 \Delta t/\tau_\ell}) \frac{1}{n} 
\]

such that \( \Delta t \ll \tau_{\ell_{\text{max}}} \). Yet as each harmonic coefficient is independent of each other, Eq. (14) can be evaluated for all \( X_{lm} \) and \( Y_{lm} \) simultaneously. Given all the harmonic coefficients \( (f_{lm}) \), the cross-section at the equator, \( R(\theta = \pi/2) \), can easily be computed using Eq. (4).

When running the numerical simulations, the user has some choice of which input parameters to specify. For example, one can specify the effective surface tension (dimensionless) \( \bar{\sigma} \) and the largest incorporated mode \( l_{\text{max}} \). In this case, the vesicle’s excess is obtained from [23]

\[
\alpha = \frac{k_B T}{2 \kappa} \left[ \frac{5}{6 + \bar{\sigma}} + \ln \left( \frac{l_{\text{max}}^2 + \bar{\sigma}}{12 + \bar{\sigma}} \right) \right], \tag{15}
\]

Alternatively, one can specify \( \alpha \) and \( \bar{\sigma} \), and Eq. (15) then provides the requisite \( l_{\text{max}} \).

Here we demonstrate an example of a numerically simulated vesicle with predefined bending rigidity and membrane tension. Figure (S5)b shows a time sequence of equatorial vesicle contours with bending rigidity of \( \kappa = 10^{-19} \) J and membrane tension of \( \sigma = 10^{-9} \) N/m. The size of the vesicle is \( R_0 = 10^{-5} \) m. By employing our image detection technique and fitting algorithm from Gracia et al., we are able to reproduce the bending rigidity and membrane tension respectively as \( \kappa = (1.00 \pm 0.01) \times 10^{-19} \) J and \( \sigma = (1.1 \pm 0.2) \times 10^{-9} \) N/m with the Helfrich spectrum given in Figure (S5). Notably our image detection is able to resolve more than 45 shape fluctuation modes.

**Simulating the Effect of Out-of-focus Signal**

Due to a finite focal depth, the microscope imaging does not capture only the optical/fluorescence signal at the focal (equatorial) plane. The out-of-focus signal results in gradient in the image intensity near the focal plane vesicle contour.

To simulate this effect, we numerically projected the vesicle shape \( R(\theta, \phi, t) \) on the equatorial plane and assigned intensity of the projected location, \( R(\theta, \phi, t) \sin \theta \), given by

\[
I(r, \phi, t) = \int_{r_{\text{max}}}^{2\pi} \int_{\frac{\pi}{2} + \theta_{fd}}^{\frac{\pi}{2}} W(\theta') \delta(\phi - \phi') \, d\phi' \, R(\theta', \phi', t) \sin \theta' \, d\theta' \, d\phi', \tag{16}
\]

where \( \frac{\pi}{2} \pm \theta_{fd} \) are the top and bottom of the microscope focal depth \( (FD) \), \( \theta_{fd} = \arctan(FD/R_0) \). \( W(\theta) \) is the intensity weighting function

\[
W(\theta) = \frac{1}{W_0} \exp \left[ -\frac{\cos^2(\theta)}{2\cos^2(\theta_{fd})} \right], \tag{17}
\]

...
and $W_0$ is the corresponding normalization constant. The resulting images of the equatorial plane at different focal depth are illustrated in Figure S6.

We varied the magnitude of the focal depth $FD$, from 0 to $0.3R_0$. The fluctuation spectra obtained for the simulations are shown in Figure S6b for a vesicle sized $R_0 = 20\mu m$ with $\kappa = 22\, k_BT$ and $\sigma = 1.4 \times 10^{-9} \, N/m$. The crossover mode $q_c = \sqrt{\frac{\sigma}{\kappa}} \sim 3$. The effect of the projections is only significant for modes $q \geq \Delta^{-1}$, where $\Delta = \frac{FD}{R_0}$. For smaller values of $\Delta < 0.05$, the projections have no effect - the spectra overlap implying same bending rigidity. However, as the value of $\Delta$ increases, more modes get affected by the projections resulting in an effective softening of the membrane from $22\, k_BT$ to $19\, k_BT$, see S6c.

![Figure S6](image)

**Fig. S 6:** a) Snapshot of vesicle equatorial contours at different $\Delta = FD/R_0$. The simulated vesicle has bending rigidity $= 22\, k_BT$, membrane tension $= 1.4 \times 10^{-9} \, N/m$ and radius $R_0 = 20\mu m$. Each image was acquired over 0.2 s (corresponding to imaging rate of 5 fps). b) Fluctuation spectrum obtained at different $\Delta$ from the numerical simulations. c) Bending rigidity obtained for different $\Delta$. Here we have compared the experimental results with numerical simulations.

**Fluctuations statistics: derivations of the basic results**

Here we summarize the main results for the dynamics of a quasi-spherical vesicle.

**Mean Squared Magnitude of the Fourier Modes:** The dynamics of the spherical harmonics modes is governed by the following Langevin equation,

$$\frac{\partial f_{lm}}{\partial t} = -\frac{1}{\tau_l} f_{lm} + \zeta_{lm}$$

where the relaxation time $\tau_l$ is given by Eq.(9) and the noise is

$$\langle \zeta_{lm} \rangle = 0 \quad \text{and} \quad \langle \zeta_{lm}(t) \zeta_{l'm'}(t') \rangle = \frac{2k_BT\delta_{ll'}\delta_{mm'}}{\eta \xi R_0^4} \delta(t - t') \delta_{l'm'}$$

(19)
The analytic solution to Eq. (18) is given by

\[ f_{lm}(t) = e^{-t/\tau_l} f_{lm}(0) + \int_0^t e^{-(t-t')/\tau_l} \zeta_{lm}(t') dt'. \]  

(20)

and therefore

\[ |f_{lm}(t)|^2 = e^{-2t/\tau_l} |f_{lm}(0)|^2 + \int_0^t e^{-(2t-t')/\tau_l} \left( f_{lm}(0) \zeta_{lm}(t') + f_{lm}(0)^* \zeta_{lm}(t') \right) dt' + \int_0^t \int_0^t e^{-(2t'-t'')/\tau_l} \zeta_{lm}(t') \zeta_{lm}^*(t'') dt' dt''. \]

(21)

The ensemble average of \( \langle |f_{lm}|^2 \rangle \) of Eq. (21) is then

\[ \langle |f_{lm}|^2 \rangle = e^{-2t/\tau_l} |f_{lm}(0)|^2 + \int_0^t e^{-(2t-t')/\tau_l} \left( f_{lm}(0) \zeta_{lm}(t') + f_{lm}(0)^* \zeta_{lm}(t') \right) dt' + \int_0^t \int_0^t e^{-(2t'-t'')/\tau_l} \zeta_{lm}(t') \zeta_{lm}^*(t'') dt' dt''. \]

(22)

Using Eq. (19), Eq. (22) simplifies to

\[ \langle |f_{lm}|^2 \rangle = e^{-2t/\tau_l} |f_{lm}(0)|^2 + \frac{2 k_B T T_l}{\eta_{ex} R_0^3} \int_0^t e^{-2(t-t')/\tau_l} dt'. \]

(23)

\[ \langle |f_{lm}|^2 \rangle = e^{-2t/\tau_l} |f_{lm}(0)|^2 + \frac{k_B T T_l \tau_l}{\eta_{ex} R_0^3} \left[ 1 - e^{-2t/\tau_l} \right] \]

(24)

At long times, \( t >> \tau_l \), Eq. (24) simplifies to

\[ \langle |f_{lm}|^2 \rangle = \frac{k_B T T_l \tau_l}{\eta_{ex} R_0^3} = \frac{k_B T}{\kappa} \left[ (l+2)(l-1) (l(l+1)+\bar{\sigma}) \right]^{-1} \]

(25)

Recall \( \bar{\sigma} = \sigma R_0^2 / \kappa \). Since the dynamics of the different spherical harmonics modes are completely decoupled, we can more generally say

\[ \langle f_{lm} f_{lm'} \rangle = \frac{k_B T T_l \tau_l}{\eta_{ex} R_0^3} = \frac{k_B T}{\kappa} \left[ (l+2)(l-1) (l(l+1)+\bar{\sigma}) \right]^{-1} \delta_{lm'} \]

(26)

Next, we consider the contour of the GUV at the equator as a function of the spherical harmonic coefficients:

\[ r(\phi, t) = R_0 \left( 1 + \sum_{q=0}^{n_{\text{max}}} u_q(t) e^{iq\phi} \right) = R_0 \left( 1 + \sum_{l=0}^{l_{\text{max}}} \sum_{m=-l}^{l} f_{lm}(t) \gamma_{lm}(\pi/2, \phi) \right). \]

(27)

The Fourier coefficient for the \( q \)-th mode is then given by

\[ u_q(t) = \frac{1}{2\pi R_0} \int_0^{2\pi} r(\phi, t) e^{-iq\phi} d\phi = \sum_{l=0}^{l_{\text{max}}} f_{lq}(t) \left( n_q P_q(0) e^{iq\phi} \right) e^{-iq\phi} \]

(28)

as all the other terms integrate to zero. In the above equation, we have inserted the definition of the spherical harmonic, \( \gamma(\theta, \phi) = \gamma_{lm}(\cos \theta) e^{im\phi} \) (see Eq.(5)), which shows that the dependence on \( \phi \) cancels out.

The mean squared amplitude of \( u_q \) is then given by

\[ \langle |u_q|^2 \rangle = \sum_{l=0}^{l_{\text{max}}} \sum_{q=0}^{n_{\text{max}}} \langle f_{lq}^* f_{lq} \rangle n_q n_{lq} P_q(0) P_{lq}^*(0). \]

(29)
Using Eq. (26), the above equation simplifies to

\[
\langle |u_q|^2 \rangle = \sum_{l=q}^{l_{\text{max}}} \langle |f_{lq}|^2 \rangle n_{lq}^2 |P_{lq}(0)|^2
\]  

(30)

\[
\langle |u_q|^2 \rangle = k_B T \sum_{l=q}^{l_{\text{max}}} (l + 2)(l - 1)(l(l + 1)\kappa + \sigma R_0^2)^{-1} n_{lq}^2 |P_{lq}(0)|^2
\]  

(31)

Eq. (31) follows \(q^{-3}\) behavior for bending dominated modes \(q > \sqrt{\sigma}\) (and \(q^{-1}\) behavior for tension dominated modes \(q < \sqrt{\sigma}\)).

**Time Correlation for Fourier Modes:** Time correlations present another useful metric to analyze the membrane fluctuations. As the different spherical harmonics modes are independent, the average time correlations,

\[
\langle u_q(0)u_{q}^*(t) \rangle = \sum_{l'=|q|}^{l_{\text{max}}} \langle f_{l'q}(0) f_{l'q}^*(t) \rangle n_{lq} n_{l'q} P_{l'q}(0) P_{lq}^*(0),
\]  

(32)

can be simplified to

\[
\langle u_q(0)u_{q}^*(t) \rangle = \sum_{l=|q|}^{l_{\text{max}}} \langle |f_{lq}|^2 \rangle n_{lq}^2 |P_{lq}(0)|^2 e^{-t/\tau_q}.
\]  

(33)

Using (21), (32) can be rewritten as

\[
\langle u_q(0)u_{q}^*(t) \rangle = \sum_{l=|q|}^{l_{\text{max}}} \langle |f_{lq}|^2 \rangle n_{lq}^2 |P_{lq}(0)|^2 e^{-t/\tau_q}.
\]  

(34)

Since the first term in (34) has both the smallest decay rate \((\tau_q^{-1})\) and largest mean-squared amplitude, the time correlation can be approximated to leading order as

\[
\langle u_q(0)u_{q}^*(t) \rangle = \langle |f_{qq}|^2 \rangle n_{qq}^2 |P_{qq}(0)|^2 e^{-t/\tau_q}.
\]  

(35)

If we consider the limit of undulations with short wavelengths (shorter than the vesicle radius), \(q \gg 1\), then the leading order decay rate can be approximated as

\[
\tau_q^{-1} \approx \frac{q^3 R_0^{-3} \kappa + q R_0^{-1} \sigma}{2(\eta_{xx} + \eta_{nn})} = \frac{\kappa}{\eta_{xx} R_0^2} \frac{q^3 + q \sigma}{2(1 + \lambda)}
\]  

(36)

which is the decay rate derived using planar fluctuations. However, we suggest using the exact decay rate from the spherical harmonics as it is both more accurate and valid for all Fourier modes.

When comparing the time correlations in Fig. 7, the exact decay rate, from the full spherical harmonics (SpH), is immediately more accurate than if the planar membrane (PM) decay rate is used. To get the accuracy even better, the higher order terms in Eq. (34) must be included. If all of the terms are included then the time correlation is directly on top of the curve from produced by the numerical simulation. However, as it is not feasible to include all the terms for real membranes, it is of interest to know how many terms are enough to sufficiently reproduce the numerical simulations. As shown in Figure 5, the time correlation produced by including the first two terms in Eq. (34) lies almost directly on top of the true solution. Including more terms would improve the accuracy further, but it is not likely to be significant due to experimental error.
Fig. S7: Plots comparing the analytic approximations for time correlation for Fourier mode $q = 5$. The left plots the time correlations using the exact spherical harmonic (SpH) decay rate and the less accurate planar membrane (PM) decay rate. The right plots the time correlations for the SpH case using different number of terms. The black dots show the time correlations computed from a numerical simulation using the following parameters as inputs. $R_0 = 3 \times 10^{-5}$ m, $\kappa = 5 \times 10^{-19}$ J, $\sigma = 4 \times 10^{-8}$ N/m, $l_{max} = 14$

Cross-Spectral Density: Similar to time correlations, the Cross-Spectral Density (CSD) is given by

$$\langle |u_q(0)||u_q(t)| \rangle - \langle |u_q|^2(0) \rangle.$$  \hspace{1cm} (37)

For the sake of clarity of explanation, in this section we will use the leading order approximation of $u_q$,

$$u_q(t) \approx f_{qq}(t) n_{qq} P_q \left( \cos \frac{\pi}{2} \right).$$  \hspace{1cm} (38)

Using (20), this can be rewritten as

$$u_q(t) = e^{-t/\tau} u_q(0) + \bar{\zeta}_q(t),$$  \hspace{1cm} (39)

where

$$\bar{\zeta}_q = n_{qq} P_q(0) \int_0^t e^{-(t-t')/\tau} \zeta_{lm}(t') dt'$$

is a random normally distributed Weiner process.

From (39), it is clear that $u_q(t) = \bar{\zeta}_q(t)$ for large values of $t$. Furthermore, it is worth noting that all Fourier modes, except $q = 0$, have both real and an imaginary component, $u_q = A_q + iB_q$, and that these two components are independent of each other. Likewise, the thermal noise can be decomposed into independent real and imaginary components: $\bar{\zeta}_q = \bar{\zeta}_{Aq} + i\bar{\zeta}_{Bq}$. The real component of Eq. (39) can then be written as

$$A_q(t) = A_q(0)e^{-t/\tau_q} + \bar{\zeta}_{Aq}(t)$$

and a similar expression for $B_q$.

Therefore, it can be shown that

$$\langle |u_q(0)||u_q(t)| \rangle = \langle |u_q(0)| \left( A_q^2(t) + B_q^2(t) \right)^{1/2} \rangle$$

$$= \langle |u_q(0)| \left( |\bar{\zeta}_q|^2(t) + 2(\bar{\zeta}_{Aq}(t) A_q(0) + \bar{\zeta}_{Bq}(t) B_q(0)) e^{-t/\tau_q} + |u_q(0)|^2 e^{-2t/\tau_q} \right)^{1/2} \rangle.$$  \hspace{1cm} (41)
If we assume that $t \gg t_q$, then we can perform the following expansion

$$
\langle |u_q(0)||u_q(t)| \rangle = \langle |u_q(0)| \rangle \langle |\tilde{\zeta}_q(t)| \rangle
+ \left( \langle |u_q(0)||A_q(0)\rangle \langle \tilde{\zeta}_A(t) \rangle + \langle |u_q(0)||B_q(0)\rangle \langle \tilde{\zeta}_B(t) \rangle \right) e^{-t/\tau_q}
+ \frac{1}{2} \left( \left\langle |u_q(0)|^3 \right\rangle - \left\langle |u_q(0)||\tilde{\zeta}_A(t)A_q(0) + \tilde{\zeta}_B(t)B_q(0)\rangle \langle |\tilde{\zeta}_q(t)|^3 \right\rangle \right) e^{-2t/\tau_q} + O(e^{-3t/\tau_q}). \tag{42}
$$

The second term in (42) averages to zero due to the thermal noise factor. Therefore, to leading order, the CSD is given as

$$
\langle |u_q(0)||u_q(t)| \rangle - \langle |u_q(0)| \rangle^2 = C_q e^{-2t/\tau_q} + O(e^{-3t/\tau_q}) \tag{43}
$$

where $C_q$ is a normalization constant.

Therefore, the slowest decaying mode of the CSD is $O(e^{-2t/\tau_q})$. This contradicts H. Zhou et al. [11] who give it as $O(e^{-t/\tau_q})$. This factor of two is a consequence that each Fourier coefficient has both a real and imaginary component that are completely independent of each other.

Finally, users are recommended to use time correlations over CSD. CSD requires the same amount of work and contains the same higher order error as the time correlation method. Yet, CSD has an additional layer of truncation error introduced in the expansion in Eq.(42).
Movie S1

Real-time video of the GUV from Figure 1 in the main text (DOPC labeled with 0.2 mol % TR-DHPE) acquired with phase contrast microscopy. The vesicle radius is 29.6 µm.

Movie 2

Video of the GUV from Figure 1 in the main text (DOPC labeled with 0.2 mol % TR-DHPE) acquired with confocal microscopy. The objective used is 40x/0.6 NA with the pinhole size 1 A.U. The polarization effect was corrected by using circular rotation plates to have even intensities across the equatorial vesicle plane. The vesicle radius is 29.6 µm.

Movie S3

Real time video of the GUV from Figure 6 in the main text consisting of DOPC labeled with TR DHPE (0.8%) acquired with confocal microscopy. The objective used is 40x/0.6 NA at 13.2 fps with the pinhole size 1 A.U. The polarization effect was corrected by using circular rotation plates to have even intensities across the equatorial vesicle plane.

Raw data

Raw data are available at [https://dx.doi.org/10.17617/3.4p](https://dx.doi.org/10.17617/3.4p). This collection of raw data, consists of 4 folders each containing zipped files of data.

In folder “Raw data - Fourier modes”, 2 different sets of experimental data are included: phase contrast (PC) and confocal (C) microscopy on the same vesicle and confocal microscopy data with different pinhole sizes. The folder contains excel sheets with fluctuation amplitude for every Fourier mode and the metadata with all the microscopy conditions. The vesicles have different sizes so that they practically cover a good span of focal depth (∆) from 0.03 to 0.15. The meta data is included in the first sheet of the excel file. The second sheet has the mode and mean squared amplitude (and error). The remaining sheets have the Fourier modes for every microscopy setting (and focal depth). Note that our Fourier signal was normalized by vesicle radius. For the definition of our Fourier transform, please refer to Gracia et al.[3]. The contour detection is conducted as given in the main text and supplement.

The rest of the folders as listed below contain vesicle images in tiff format (grouped in folders for the separate vesicle as suggested by the folder name) and an excel sheet with the meta data indicating the specific microscopy conditions (AU = Airy unit, NA = numerical aperture).

The folder “Raw images - different focal depth” contains confocal microscopy raw images at different focal depths without any image processing for vesicles of different sizes.

The folder “Raw images - phase contrast vs confocal” contains phase contrast and confocal microscopy raw images for the same vesicle without any image processing for vesicles of different sizes.

The folder “Raw images - polarization correction” contains polarized and polarization-corrected confocal microscopy raw images for the same vesicle without any image processing.


