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A quantitative fluorescent microscopy based single liposome assay for detecting the compositional inhomogeneity between individual liposomes

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Journal of Visualized Experiments, Senior Science Editor Nandita Singh

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Manuscript entitled: "A quantitative fluorescent microscopy based single liposome assay for detecting the compositional inhomogeneity between individual liposomes".
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Dear Nandita Singh

Based on our previous communication we have now finalized the aforementioned manuscript and would like to submit it to *Journal of Visualized Experiments* for consideration as a full paper.

In this work we provide an extensive protocol describing a fluorescent based single liposome assay and its use for quantifying the compositional inhomogeneity between individual liposomes of the ensemble. We especially highlight critical steps as we describe the complete process going through liposome formulation, surface immobilization, fluorescent imaging and data analysis. Also, we suggest how the assay can be expanded to study other scientific questions. We premise the assay with a thorough discussion how single liposome studies has made an enormous impact and facilitated novel insights in a vast number of scientific areas, including membrane fusion, protein structure dynamics and drug delivery.

We trust that this work will meet with your interest, and look forward to hearing from you in due course.

Sincerely Yours,

Jannik Bruun Larsen

Further details:

The manuscript contains approximately 5700 words in the main text and figure captions; it has 4 figures, in color, and 26 references. The material in this manuscript has not been published and is not currently under review for publication by another journal.

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TITLE:

A Quantitative Fluorescence Microscopy-Based Single Liposome Assay for Detecting the Compositional Inhomogeneity Between Individual Liposomes

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KEYWORDS:

single liposomes, fluorescence microscopy, compositional inhomogeneity, liposome characterization, membrane-protein interactions, confocal microscopy

SUMMARY:

This protocol describes the fabrication of liposomes and how these can be immobilized on a surface and imaged individually in a massive parallel manner using fluorescence microscopy. This allows for the quantification of the size and compositional inhomogeneity between single liposomes of the population.

ABSTRACT:

Most research employing liposomes as membrane model systems or drug delivery carriers relies on bulk read-out techniques and thus intrinsically assumes all liposomes of the ensemble to be identical. However, new experimental platforms able to observe liposomes at the single-particle level have made it possible to perform highly sophisticated and quantitative studies on protein-membrane interactions or drug carrier properties on individual liposomes, thus avoiding errors from ensemble averaging. Here we present a protocol for preparing, detecting, and analyzing single liposomes using a fluorescence-based microscopy assay, facilitating such single-particle measurements. The setup allows for imaging individual liposomes in a massive parallel manner and is employed to reveal intra-sample size and compositional inhomogeneities. Additionally, the protocol describes the advantages of studying liposomes at the single liposome level, the limitations of the assay, and the important features to be considered when modifying it to study other research questions.

INTRODUCTION:

Liposomes are spherical phospholipid-based vesicles that are heavily used both in basic and applied research. They function as excellent membrane model systems, because their physiochemical properties can be easily manipulated by varying the lipid components making up the liposome[1,2]. Also, liposomes constitute the most used drug delivery nanocarrier system, offering improved pharmacokinetics and pharmacodynamics as well as high biocompatibility[3].

For many years, liposomes have primarily been studied using bulk techniques, giving only access to ensemble average read-out values. This has led the majority of these studies to assume that all liposomes in the ensemble are identical. However, such ensemble-averaged values are only correct if the underlying dataset is uniformly distributed around the mean value, but can represent a false and biased conclusion if the dataset includes multiple independent populations, for example. Additionally, assuming the ensemble mean to represent the whole population can overlook the information harbored within the inhomogeneity between liposomes. Only recently have quantitative assays emerged that are able to probe single liposomes, revealing large inhomogeneities between individual liposomes with respect to important physicochemical properties including liposome size[4], lipid composition[5,6], and encapsulation efficiency[7], highlighting the importance of studying liposomes at the single liposome level.

A research area where ensemble averaging of liposome properties has been shown to bias results is studying liposome size-dependent protein-membrane interactions[8,9]. Traditionally, researchers studying such processes have been restricted to preparing liposomes with different ensemble average diameters by extrusion through filters with different pore sizes[9]. However, extracting the diameter of individual liposomes using single liposome assays has revealed large population overlaps, with liposomes extruded using 100 nm and 200 nm filters displaying up to 70% overlap in their size distribution[4]. This could severely bias bulk measurements of liposome size-dependent protein-membrane interactions[10]. Performing the membrane-protein interaction studies using the single liposome assay, researchers instead took advantage of the size-polydispersity within the sample, allowing them to study a wide range of liposome diameters within each single experiment, facilitating new discoveries of how membrane curvature and composition can affect protein recruitment to membranes[4,11,12]. Another field where the application of single liposome assays has proven instrumental is in mechanistic studies of protein-mediated membrane fusion[13,14]. For such kinetic measurements, the ability to study individual fusion events alleviated the need for the experimental synchronization of the fusion process, allowing new mechanistic insights that would otherwise have been lost in the spatiotemporal averaging done in bulk ensemble measurements. Additionally, single liposomes have been used as a membrane scaffold, allowing the measurement of individual proteins and offering new knowledge on transmembrane protein structural dynamics[15,16]. Furthermore, such proteoliposome-based setups made it possible to study the function of individual transmembrane transporters[17] and pore-forming protein complexes[18] as well as the mechanism of bioactive membrane-permeabilizing peptides[19]. Single liposomes have also been used as soft matter nanofluidics with surface-immobilized single liposomes serving as chambers for enzymatic reactions in volumes of 10^{-19} L, increasing the throughput and complexity of the screening assays with minimal product consumption[20].

Recently, single liposome assays have been used for characterizing drug delivery liposomes at a previously unprecendented level of detail. Researchers were able to quantify significant inhomogeneities in the amount of polymer attached to the surface of individual liposomes[21]. The single liposome assays also allowed measurements of drug delivery liposomes in complex media, such as blood plasma, revealing how elements anchored to the liposome surface through lipid anchors can be susceptible to dissociation when liposomes are exposed to conditions mimicking those experienced during in vivo circulation[22]. Overall, the versatility and usefulness of the single liposome assays are substantiated by the great variety of problems these setups have been employed to address, and we envision that the methodology will continue to be developed and find use in new scientific fields.

Here we describe a fluorescence microscopy-based single liposome assay that allows individual liposomes to be studied in a high-throughput manner (**Figure 1**). To illustrate the method, we use it to quantify the size and compositional inhomogeneity between individual liposomes within an ensemble. The assay employs fluorescence microscope imaging of single liposomes immobilized on a passivated glass surface. We first describe the critical steps in the liposome fabrication process that ensures proper fluorescent liposome labeling and immobilization. Then, we describe the surface preparation needed to facilitate liposome immobilization before outlining the procedure for ensuring appropriate liposome surface densities. We discuss the microscopy parameters important for acquiring high-quality images and delineate how to perform simple data analysis, allowing the extraction of liposome size and compositional inhomogeneity. This generic protocol should provide a good basis for the interested researcher to develop the assay further for his or her specific research interest.

PROTOCOL:

1. Liposome preparation

NOTE: Briefly, preparation of liposomes usually includes three crucial steps: 1) preparation of dry lipid films of the desired lipid composition; 2) rehydration of the lipids for formation of liposomes; and 3) controlling the size and lamellarity of the liposome population.

1.1. Weigh out the lipids and dissolve them in tert-butanol:water (9:1) in glass vials.

1.1.1. Dissolve POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine; MW = 760 g/mol) to 50 mM.

1.1.2. Dissolve cholesterol (MW = 387 g/mol) to 25 mM.

1.1.3. Dissolve DOPE-Atto488 (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-Atto488; MW = 1,316 g/mol) to 0.1 mM.

1.1.4. Dissolve DOPE-Atto655 (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-Atto655; MW = 1,368 g/mol) to 0.1 mM.

1.1.5. Dissolve DSPE-PEG2000-biotin (1,2-distearyl-sn-glycero-3-phosphatethanolamine-N-[biotinyl(polyethylene glycol)-2000]; MW=3,017 g/mol) to 0.1 mM.

NOTE: Heat lipids to 55 °C and use magnetic stirring in order to ensure complete dissolution of the lipids. Alternatively, use a sonication bath. Unused lipid stocks can be stored at -20 °C for several months.

1.2. Mix the lipid stocks prepared in step 1.1 to a molar ratio of POPC:cholesterol:DOPE-Atto488:DOPE-Atto655:DOPE-PEG-biotin 68.95:30:0.5:0.5:0.05, by adding 138 µL of POPC, 120 µL of cholesterol, 500 µL of each fluorescently labeled lipid, and 50 µL of DSPE-PEG-biotin to a fresh glass vial.

NOTE: The exact liposome composition can easily be modified in order to address the specific question of interest. See discussion for more detail.

1.3. Loosen the lid of the glass vial, and snap-freeze the vial in liquid nitrogen.

1.4. Lyophilize the frozen lipid mixture overnight.

1.5. Add 1 mL of 200 mM D-sorbitol buffer (sorbitol buffer) to the dry lipids.

1.6. Heat the mixture to 45 °C and expose to magnetic stirring for at least 1 h.

NOTE: The buffer should reflect the specific question that is being addressed (e.g., physiological conditions for studying membrane-protein interactions, or a specific clinically approved buffer for studying drug delivery liposomes). However, if a specific buffer is not required for the study, a buffer without ions can be applied for rehydration in order to reduce the multilamellarity of the liposomes.

1.7. Freeze the lipid suspension by dipping the vial in liquid nitrogen, and wait until the suspension is completely frozen.

1.8. Dip the frozen suspension in a heating bath at 55 °C until the mixture is completely thawed.

1.9. Repeat steps 1.7 and 1.8 until the liposome suspension has been exposed to a total of 11 freeze/thaw cycles.

NOTE: Repeated freeze/thaw cycles have shown to reduce liposome multilamellarity[23], which is paramount for the accuracy of the single liposome assay, as multilamellar liposomes will skew the fluorescence intensity versus liposome size ratio of the liposomes. The multilamellarity is usually inherently low when including more than 0.5% PEGylated lipid in the formulation (such as commonly done in liposomes for drug delivery)[24].

1.10. Extrude the liposome suspension once through an 800 nm polycarbonate filter using a mini extrusion kit. Follow the manufacturer's instructions for assembly of the extrusion kit (see **Table of Materials**).

1.11. Store the liposomes at 4 °C overnight.

2. Surface preparation of imaging chamber

2.1. Prepare bovine serum albumin (BSA; 1 mg/mL), BSA-biotin (1 mg/mL) and streptavidin (0.025 mg/mL) in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 95 mM NaCl buffer (HEPES buffer).

NOTE: To prevent liposomal burst, the sorbitol buffer used for rehydration and the HEPES buffer used for surface preparation should be isotonic. It is thus recommended to check the osmolarity of the buffers before the experiment.

2.2. Mix 1,200 µL of BSA and 120 µL of BSA-biotin and add 300 µL of the mixture to each well in an 8 well slide for microscopy.

NOTE: Here we use a commercially available 8 well microscope slide with a glass bottom (see **Table of Materials**), but the protocol can easily be adapted to customized microscope chambers.

2.3. Incubate the slide for 20 min at room temperature (RT).

2.4. Wash the slide 8x with 300 µL of HEPES buffer.

NOTE: Be careful not to leave the wells without buffer for more than a few seconds, as drying out the surface will damage it. Furthermore, be careful not to scratch the surface with the pipette tip as this will also damage it. Thus, when aspirating buffer from a well, do it from the edge or corner.

2.5. Add 250 µL of streptavidin and incubate for 10 min at RT.

2.6. Repeat the 8 washes described in step 2.4.

2.7. Store the microscope slide with 300 µL of sorbitol buffer in each well at 4 °C. Evaporation of the solvent will damage the surface, so put the microscope slide in a Petri dish and seal it with parafilm unless the prepared slide is used immediately.

3. Liposome immobilization

3.1. Dilute the liposome suspension to about 20 µM total lipid in sorbitol buffer. The procedure in section 1 will yield a liposome suspension of approximately 10 mM total lipid.

3.2. Place the slide on the microscope and focus on the surface of the chamber using the

increased laser reflection signal from the glass/buffer interface as a guide.

3.3. Wash the chamber 4x with 300 µL of fresh HEPES buffer.

3.4. Add 10 µL of diluted liposome stock (20 µM total lipid) to a microcentrifuge tube.

3.5. Take out 100 µL of buffer from the chamber, add to the microcentrifuge tube prepared in step 3.4, mix properly, and put the 110 µL back into the chamber.

3.6. Put the specimen under the microscope, and use a rudimental microscope setting capable of detecting signal from the liposome fluorophore(s) to observe the liposomes being immobilized on the surface.

3.7. Aim for a liposome surface density that is simultaneously sparse enough to indentify individual liposomes and dense enough that it facilitates high-throughput measurements. Typically using a 50 µm x 50 µm field of view, 300−400 liposomes per frame is optimal (**Figure 2**). This can usually be achieved within 5−10 min.

NOTE: If only rapidly moving fluorescent particles are detected in the field of view, an absence or too low concentration of any of the components critical for the immobilization process (BSA-biotin, streptavidin, DOPE-PEG-biotin) might be the cause. If no liposomes are detected it might either be related to a low concentration of liposomes, improper settings for the fluorescence detection, or potentially imaging with a focal plane not at the glass surface.

3.8. Once an appropriate liposome surface density is reached, wash the chamber 3x with 200 µL of HEPES buffer.

NOTE: Be aware that the immobilization kinetics also depend on liposome properties such as size and charge and should thus always be optimized for each liposome formulation.

4. Image acquisition

NOTE: This section will depend a lot on the microscope system available to the researcher performing the experiment. Thus, overall guidelines on how to perform the imaging will be described. However, the exact settings and how to apply them will vary between the different microscope setups. For example, some systems allow choosing any emission filter combination desired, while other microscopes are equipped with specific, preset filters.

4.1. Set up the microscope for imaging single liposomes. To ensure optimal image quality and subsequent data analysis use a high greyscale resolution and a pixel scheme that allows for oversampling individual liposomes. A bit depth of 16 and at least 1,024 x 1,024 pixels for a 50 µm x 50 µm area is recommended. If available, line-averaging can beneficially be applied to reduce noise (e.g., 3 scans per line).

4.2. Select an excitation laser power that both ensures nonsignificant frame-to-frame bleaching and strong enough signal to clearly discriminate individual liposomes from the background. The optimal setting will depend on the specific microscope used as well as the fluorophore combination. Make sure that detectors are not saturated, as this will bias intensity quantification.

4.3. Imaging both the DOPE-Atto488 and DOPE-Atto655 fluorophores in the liposomes requires imaging multiple channels. Thus, make sure each channel is imaged sequentially to avoid cross-excitation. For example, first take one image by exciting at 480 nm and reading emission at 510−550 nm. Thereafter, take another image by exciting at 640 nm but reading emission only at 660−690nm. The specifics will depend on the microscope system (e.g., which lasers and filters) available.

4.4. Make sure to cover different areas of the surface, acquiring at least 10 images of the sample, thus imaging at least 3,000 individual liposomes. If the surface density is lower than 300 liposomes/frame, acquire more images. Make sure to refocus the microscope for every new image.

NOTE: For two-channel imaging, make sure to name the image files so that pairs of images with the same liposomes in different fluorescence channels can easily be identified during data analysis.

4.5. In order to quantify the experimental uncertainty relating to the measurement of compositional inhomogeneity, image the same area of liposomes before and after refocusing (see **Figure 3** and description in the text).

4.6. Export the images from the microscope software as .tiff files. Export the two channels of the same liposomes individually.

5. Data analysis

NOTE: Specially developed automated 2D Gaussian fitting routines have previously been employed[6,11,12]. However, to increase the applicability of the method a data analysis process that can be easily implemented in all laboratories is described.

5.1. Load the corresponding pair of .tiff images of two different fluorescence channels in the same imaging field into the FIJI (FIJI Is Just Image-J) software.

5.2. In the **Image** menu, choose **Color**, and use the **Merge Channel** function to create a composite of the two channels.

5.3. Observe if the liposomes imaged in two different channels display good colocalization or whether visible drift occurred.

NOTE: In case of drift between the two fluorescence channels (recognized as a systematic and

equal X-Y offset between the signal in the two channels), one of the frames can be translated using the **Transform** > **Translate** function in the **Image** menu of FIJI. However, care should be taken with such image manipulation. It is thus recommended to instead avoid drift as much as possible when imaging the liposomes.

5.4. Make sure the ComDet plugin (v.0.3.6.1 or newer) is installed, or do this in the **Plugins** menu.

5.5. Open the ComDet plugin to detect particles by going to the **Plugins** menu and choosing **ComDet v.0.3.6.1** > **Detect Particles**.

5.6. In ComDet, make sure to choose the **Detect Particles on Both Channels Individually** function. Set the **Max Distance Between Co-localized Spots** similar to the **Approximate Particles Size**. Usually, 4 pixels is appropriate for the settings described here.

5.7. The signal-to-noise ratio should allow for detection of even dim particles with a low amount of fluorescence. In ComDet, set this ratio to 3 by setting the **Sensitivity of Detection** in both channels to **Very Dim Particles (SNR = 3)**.

NOTE: While this SNR value is usually appropriate, it might be necessary to set it higher (e.g., if there is a lot of image noise that may be identified as liposomes and lead to false positives).

5.8. Make sure the boxes with **Calculate Colocalization** and **Plot Detected Particles in Both Channels** are checked, before pressing **OK**.

5.9. After running the analysis, two pop-up windows with **Results** and **Summary** will show. Export the data table **Results** containing the colocalization data (particle coordinates and integrated intensity of each particle detected) to a data handling software of choice by saving the table as a .txt file and importing it into the software.

5.10. Make sure that each liposome is only included once in the data set and not both the channel1/channel2 as well as channel2/channel1 ratio. Thus, filter the data so only **Abs_frame = 1** is plotted in step 5.11.

NOTE: By choosing **Colocalized = 1**, false positives from noise in the images can be removed from the analysis. However, any liposomes with only one of the two fluorescent components present will also be excluded from the analysis, thus potentially removing important data points from the analysis.

5.11. Plot a histogram of the column with data containing the **Intensity Ratio** for each detected liposome.

5.12. The degree of compositional inhomogeneity for the studied liposomal system is represented by the width of the intensity ratio distribution. To quantify the inhomogeneity, fit the intensity ratio histogram with a Gaussian function and extract the mean (μ) and standard

deviation (sigma). See the Representative Results section.

5.13. A value for the degree of inhomogeneity (DI) can now be calculated using the coefficient of variation defined as DI = sigma / μ.

6. Liposome size calibration

6.1. Take out part of the liposome stock, and extrude 21x through a 50 nm polycarbonate filter as described in step 1.10.

6.2. Dilute the liposomes by adding 10 µL of the liposome suspension to 800 µL of sorbitol buffer in a microcentrifuge tube.

6.3. Transfer the diluted liposome sample to a polypropylene single-use cuvette.

6.4. Measure the size using dynamic light scattering (DLS). Perform at least three independent runs to measure the size and polydispersity of the liposome suspension.

NOTE: If necessary, use a more concentrated liposome suspension for the measurement. Alternatives to DLS (e.g., nanoparticle tracking analysis) can also be applied for measuring the size of the liposomes. A description of how to execute such particle-size determination is beyond the scope of this protocol.

6.5. Image the calibration liposomes on the microscope using exactly the same experimental settings as defined in steps 4.1 and 4.2.

6.6. Extract the integrated intensity for each calibration liposome in the calibration images: Extract the filtered results sheet containing liposome fluorescence intensities as described in steps 5.1–5.10. From the results table, extract the “IntegratedInt” column.

6.7. Because the total integrated intensity of a liposome labeled in its membrane is proportional to the surface area of the liposome and is thus proportional to the square of its diameter, plot a square root intensity histogram of the fluorescence intensity of the calibration liposomes.

6.8. Fit the integrated intensity histogram produced in step 6.7 with a log normal distribution and extract the average fluorescence intensity of the calibration liposomes.

6.9. To determine the relation between the square root intensity (Int_{Sqrt}) and liposome size, calculate the correction factor (C) using the average liposome diameter (Dia) weighed by the number obtained from the DLS measurements: Dia = C x Int_{Sqrt} is equivalent to C = Dia / Int_{Sqrt}.

6.10. Calculate Int_{Sqrt} values for the liposomes in the compositional inhomogeneity experiment and convert these to diameters by multiplying with the correction factor.

6.11. Plot the intensity ratio value as a function of diameter for the compositional inhomogeneity liposomes, thus achieving the inhomogeneity as a function of liposome size for a population of liposomes spanning from approximately 50 nm–800 nm.

REPRESENTATIVE RESULTS:

Following the protocol described makes it possible to image single liposomes in a massive parallel manner (**Figure 1**). The successful surface immobilization of liposomes should be immediately apparent upon the addition of the liposome solution to the chamber (step 3.6 in the protocol) as diffraction limited intensity spots should appear in the image (**Figure 1B** and **Figure C**).

In order to achieve good statistics and exploit the high-throughput abilities of the assay, several thousand liposomes should be imaged. In order to do this in a reasonable number of images, it is recommended to immobilize enough liposomes to achieve a density of 300–400 liposomes per frame, as this will bring the number of images per sample down to ~10, thus limiting the number of images that has to be acquired and analyzed. A lower density will make the data analysis more time-consuming, while a higher density can make it challenging for the image analysis software to distinguish single liposomes. To get a visual impression of what 300–400 liposomes look like, see **Figure 2A**. It should be noted, however, that for some applications (e.g., membrane-protein interactions with a protein that tends to elicit strong background binding to the BSA surface) it is recommended to use a slightly lower density, such as in **Figure 2A** at the top right.

Occasionally, when acquiring images it is hard to get the whole field of view in proper focus, as illustrated in **Figure 2B**. Such issues might indicate that the sample plate is tilting, which can arise from the plate not being placed properly on the specimen holder on the microscope. Also, if the liposomes seem large and blurry, the buffer in the chamber might have evaporated and the surface dried out. It is especially important to keep this issue in mind when imaging for long time periods or when performing measurements at temperatures higher than RT. To illustrate the difference between properly stored and dried out liposomes, an image of the same sample before and after drying out the chamber is shown in **Figure 2C**.

After having ensured optimal image quality, the integrated intensity for each liposome in the two imaging channels can be extracted following the steps in section 5 of this protocol. Doing this will create a list of unique intensity values, allowing us to calculate the intensity ratio for each individual liposome. The compositional inhomogeneity is evaluated from intensity ratio histograms, typically revealing a Gaussian distribution around a mean ratio value (**Figure 3A**). Notice that if strong deviations from a Gaussian distribution are observed (**Figure 3B**), it indicates a detection sensitivity issue for at least one of the imaging channels, suggesting that a subset of liposomes showing a weaker signal have been excluded. This could be due to both the detection limit of the imaging system, or excluding liposomes in the data analysis (e.g., when applying a certain minimum threshold; see step 5.7).

Calculating the DI value as described in steps 5.11–5.13 in the protocol will provide a quantitative measure of the compositional inhomogeneity between the individual liposomes of the preparation. For the liposomal system studied here, DI = 0.23 ± 0.01 (**Figure 3C**). This value can

be used to systematically compare how varying liposome properties or preparation methods affect the compositional inhomogeneity. To conceptualize the meaning of the DI value, we refer to the normal distribution displayed by the intensity ratio histogram, meaning they will obey the empirical 68-95-99.7 rule. This rule describes the percentage of a population that falls within one, two, and three standard deviations around the mean value. For the DI value of 0.23 ± 0.01 found here, it means that 32% of the liposomes in the population will have an intensity ratio that differs by more than 23% from the mean molar ratio of the ensemble (**Figure 3D**).

For the control experiment imaging the same liposomes before and after refocusing (section 4.6), the same data analysis and result plotting as for the actual experiment is performed. Doing this allows for the quantification of the experimental uncertainty of the DI value, which was found to be $DI_{uncertainty}$ = 0.10 ± 0.01 (**Figure 3E**). Although the $DI_{uncertainty}$ value will depend on the employed imaging system, it was found that confocal microscope setups consistently give rise to $DI_{uncertainty}$ values of around 0.10[5,6]. The DI value of DI = 0.23 ± 0.01 found for the liposomal system here is more than twice the experimental uncertainty, which suggests the presence of significant compositional inhomogeneity between the individual liposomes of the preparation.

Performing the size calibration experiment described in section 6 will allow the arbitrary liposome intensity values to be transformed to physical diameters in nanometers. The method has been validated against other imaging techniques[25] such as cryogenic electron microscopy, which has an ability to optically resolve the nanometer-sized liposomes, although with much lower throughput. Imaging the control sample using the exact same microscope settings as used for the actual experiments, it is now possible to correlate the mean liposome intensity to the mean liposome diameter determined by DLS (**Figure 4A**). The next step is to depict the liposome intensity distribution, which based on the physical constraints against creating extremely small liposomes, typically will display a log normal distribution (**Figure 4B**). If the distribution is not well described by a log normal distribution (most often due to missing liposomes with low intensity values) it means that a part of the liposome population was excluded either due to low microscope detection sensitivity or overly strict parameters during data analysis (**Figure 4C**). It is important to catch and address these issues to ensure unbiased data interpretation. Next, the intensity values for each individual liposome in **Figure 4B** can be converted to actual diameters using the correction factor determined in **Figure 4A** (**Figure 4D**). After determining the size of each liposome, the DI as a function of diameter can now be investigated by plotting the intensity ratio versus diameter for each individual liposome (**Figure 4E**). This funnel-like data structure corroborates earlier findings that the DI value increases when the liposome size decreases[6]. Importantly, the symmetrical increase in the spread of intensity ratios around a mean value suggests that there is no systematic size-dependent variation in the average composition of liposomes.

FIGURE AND TABLE LEGENDS:

Figure 1: The single liposome assay. (**A**) Liposomes are formulated with an equimolar content of the fluorescently labeled lipids DOPE-Atto488 and DOPE-Atto655 and immobilized on a BSA surface using a biotin/streptavidin linkage. (**B**) The immobilized liposomes are imaged using a confocal microscope, allowing for detection of the fluorescence intensities from the single

liposomes. Scale bars = 8 µm. (**C**) Zoom of the green area in (**B**) depicted as surface intensity plot for two adjacent liposomes displaying inverted fluorescent signal between the two channels, illustrating the concept of compositional inhomogeneity.

Figure 2: Optimization and pitfalls. (**A**) The top left shows 29 liposomes immobilized in a 50 µm x 50 µm frame. These are too few liposomes per frame to exploit the possibility for high-throughput investigation of liposome inhomogeneity. The top right shows 123 liposomes per frame. This is a suboptimal density but can be used if many images are acquired. The bottom left shows 300−400 liposomes per frame. This is optimal for the protocol described here. The bottom right shows more than 1,500 liposomes per frame, which is too many to distinguish individual liposomes. There is a high risk that a single spot will actually be two liposomes immobilized within the same diffraction limited spot. (**B**) If the chamber is not placed properly in the specimen holder, getting the whole field of view in proper focus will be impossible. In the left micrograph the plate is tilting around the traverse axis, giving rise to the lower left corner being out of focus. In the right micrograph the plate is tilting around the longitudinal axis, giving rise to a heavier tilt. Both the lower left and upper right corner are out of focus. (**C**) If imaging for long time periods or at elevated temperatures the buffer might evaporate, leading to drying out of the chamber. We illustrated this scenario here by imaging liposomes before and after the chamber was left to dry for 3 min, and then rewetted by adding fresh buffer to the chamber. The resulting liposomes are larger, blurry, have lower fluorescence intensity, and appear to be spread out on the plate.

Figure 3: Intensity ratio histograms for quantifying liposome compositional inhomogeneity. (**A**) The intensity ratio of DOPE-Atto488 and DOPE-Atto655 fluorescence for each individual liposome plotted as a histogram. (**B**) A truncated Gaussian distribution illustrating a potential sensitivity issue, either during imaging or in the data treatment step. (**C**) Fitting the intensity histogram with a Gaussian function makes it possible to extract the mean and standard deviation used to calculate the DI value. (**D**) A DI value of 0.23 can be translated to 32% of the population differing by more than 23% from the mean molar ratio of the ensemble. (**E**) Control experiment imaging the same liposomes before and after refocusing and then performing the same data treatment procedure as for the actual experiment. This allows the experimental error for the DI quantification to be determined.

Figure 4: Converting liposome intensity to physical diameter in nm. (**A**) Intensity histogram of the 50 nm extruded calibration liposomes compared with the DLS data in order to determine the calibration factor. (**B**) A histogram of the Int_{Sqrt} value from the experimental liposomes typically yields a log-normal distribution due to the physical constraints against creating very small liposomes. (**C**) A non log-normal Int_{Sqrt} value histogram indicates either issues with detection sensitivity or that small liposomes were excluded during data treatment. (**D**) Liposome intensities in (**B**) are converted to actual liposome diameters using the correction factor. (**E**) The intensity ratio of each liposome plotted as a function of liposome diameter can now be displayed allowing the investigation of compositional inhomogeneity as a function of liposome size.

DISCUSSION:

It is important to note that while we describe in detail how the single liposomes assay can be

used to study the compositional inhomogeneity between individual liposomes, the platform is very versatile. As previously shown and discussed in the introduction, the protocol can easily be adapted to study aspects of membrane-membrane fusion, protein-membrane interactions, or liposomal drug carrier characterization. For any scientific questions being addressed, the power of the single liposome assay lies in the ability to detect the individual components of the ensemble and thus having a quantitative readout, not biased by ensemble averaging effects.

The single liposome assay is optimally suited for surface imaging modalities such as total internal reflection or confocal microscope, where the Z-direction sectioning can eliminate unwanted background fluorescence from the solution above the liposome layer. However, this aspect is most important for studies where fluorescently labeled compounds, such as peptides, proteins, or other liposomes are added to the solution and remain there during imaging. For example, if the assay is used in the format described in detail here, where the fluorescent dyes are restricted to the immobilized liposomes, the assay could in principle be performed using more broadly available widefield microscopes, providing the detection system is sensitive enough.

A priori there are no limitations with respect to the method used to prepare the liposomes used in the assay. Here we describe in detail the use of a freeze-drying technique. However, previously chloroform-based lipid rehydration or ethanol injection-based techniques have been employed to fabricate liposomes used in the assay[5,6]. A critical parameter is the inclusion of a minute fraction of biotinylated lipids in the lipid mixture to ensure immobilization (0.05 mol% recommended). Another necessary element is to include a small amount of fluorescently labeled lipid. Overall, the amount of lipid-dye added should be kept as low as possible to both avoid quenching effects and to avoid the lipid-dye from significantly altering the physicochemical properties of the liposome. The lower limit of the amount of lipid dye is set by the concentration, where the stochastic variation in the number of individual lipid dyes per liposome becomes significant compared to the average amount of lipid-dyes (see Larsen et al.[6] for an in-depth discussion). Going below this limit will introduce large uncertainties in the extracted intensities. Finally, the amount of lipid dye needs to be high enough for accurate detection of the single liposomes with a good signal-to-noise ratio. While this criterion of course heavily depends on the imaging system, we found that lipid dye concentrations between 0.05 and 0.5 mol% work well in the vast majority of cases.

To choose which lipid dye to include in the liposome, the first prerequisite is that the excitation and emission properties match the illumination and detection capabilities of the imaging system. Second, to increase the sensitivity and accuracy of the method we recommend using dyes with both high quantum yields and photostability, and we have previously successfully used dyes with different excitation wavelengths[6,11]. Care should be taken in choosing lipid anchors with similar physicochemical properties to ensure that lipid partitioning does not lead to artificially high inhomogeneity. For example, for this protocol, we have anchored both fluorophores using DOPE, while having one fluorophore on DOPE and another on DPPE could lead to fluorophore distribution heterogeneities not related to the inherent inhomogeneity in the liposome population. Especially for dual color imaging it is important to choose dyes with narrow excitation and emission spectra and the smallest spectral overlap as possible to avoid significant bleed-

through, cross talk, and fluorescence resonance energy transfer (FRET) between dyes and channels. To avoid artifacts related to variation in the fluorophore environment we prefer to work with dyes, which have been proven experimentally to show extremely low membrane interaction propensity as determined by Hughes et al.[26]. Finally, if a specific liposome lipid composition is not a hard requirement for the experimental design, it can be beneficial to include up to 10 mol% of negatively charged lipids to ensure that individual liposomes repel each other and are efficiently immobilized as single liposomes[12].

An advantage of the single liposome assay is the small experimental volume, which can greatly reduce the amount of expensive or rare compounds used per experiment. Here we describe the use of standard and commercially available chambers with an experimental volume between 150–300 µL. However, custom-made microscope chambers that can reduce the volume to a range of 50–80 µL can be used. Also, the liposome consumption is very low, with a final concentration around 2 µM total lipid.

A disadvantage of the single liposome assay is that it is hard to control the lipid concentration in the chamber due to the variations in immobilization tendencies described above. Additionally, with respect to applying the assay for studying membrane-protein interactions, it is challenging to determine the concentration or number of bound proteins directly from the fluorescence intensity.

When using the liposomes in the assay as membrane model systems (e.g., to study the membrane interaction of fluorescent compounds, peptides, or proteins) it is important to ensure low non-specific binding to the protein components that facilitates the immobilization. If this is not the case, high background signal can be detected that could prevent the detection of the specific binding to the liposomes. If high non-specific background is detected we have previously successfully reduced the background by changing from streptavidin to Neutravidin or avidin.

Performing single liposome detection using other techniques like flow cytometry could potentially offer an increased detection throughput compared to the microscope-based assay. However, given that flow cytometers are optimized for studying much larger and brighter samples like cells, the detection system of most is not sensitive enough to detect the relatively weak fluorescence from an individual liposome. So while new and more sensitive flow cytometry is being developed the microscope-based assay offers the best solution for elucidating liposome inhomogeneities when performed efficiently and monitoring thousands of liposomes per experiment.

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DISCLOSURES:

The authors declare no conflict of interest

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Figure 1

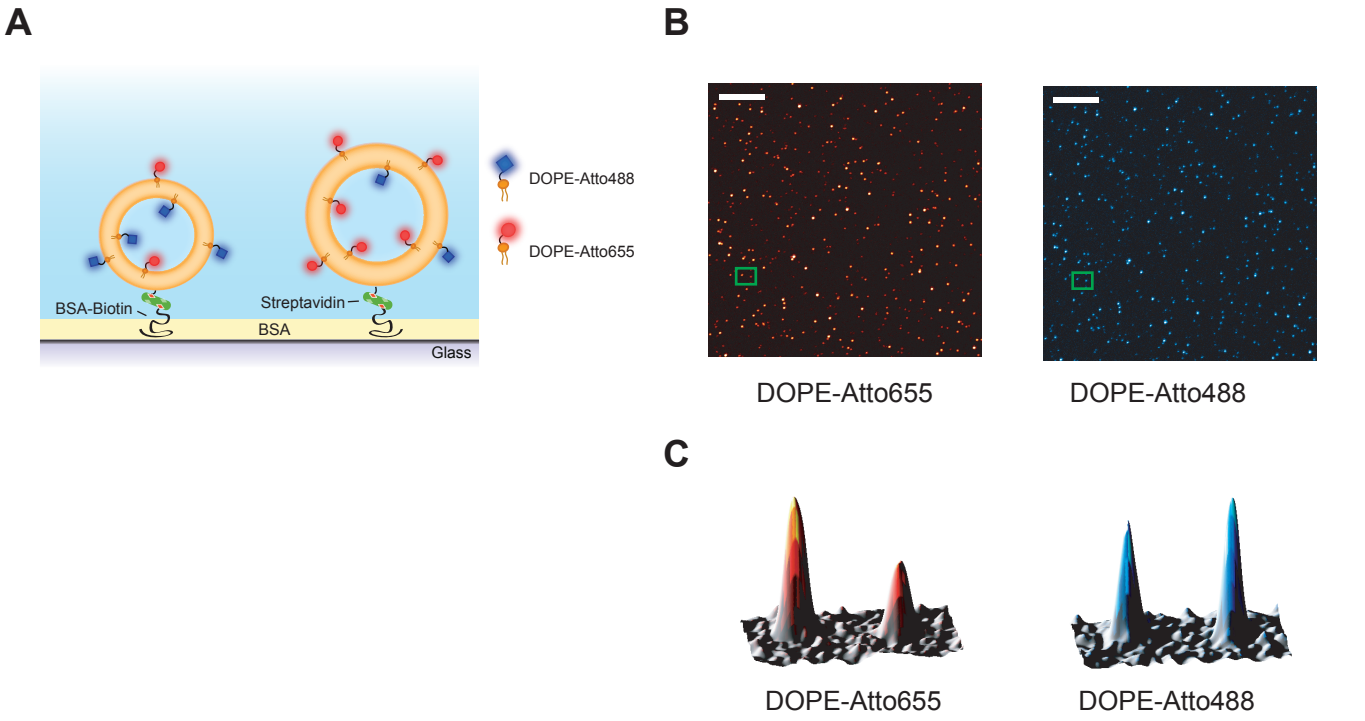


Figure 2

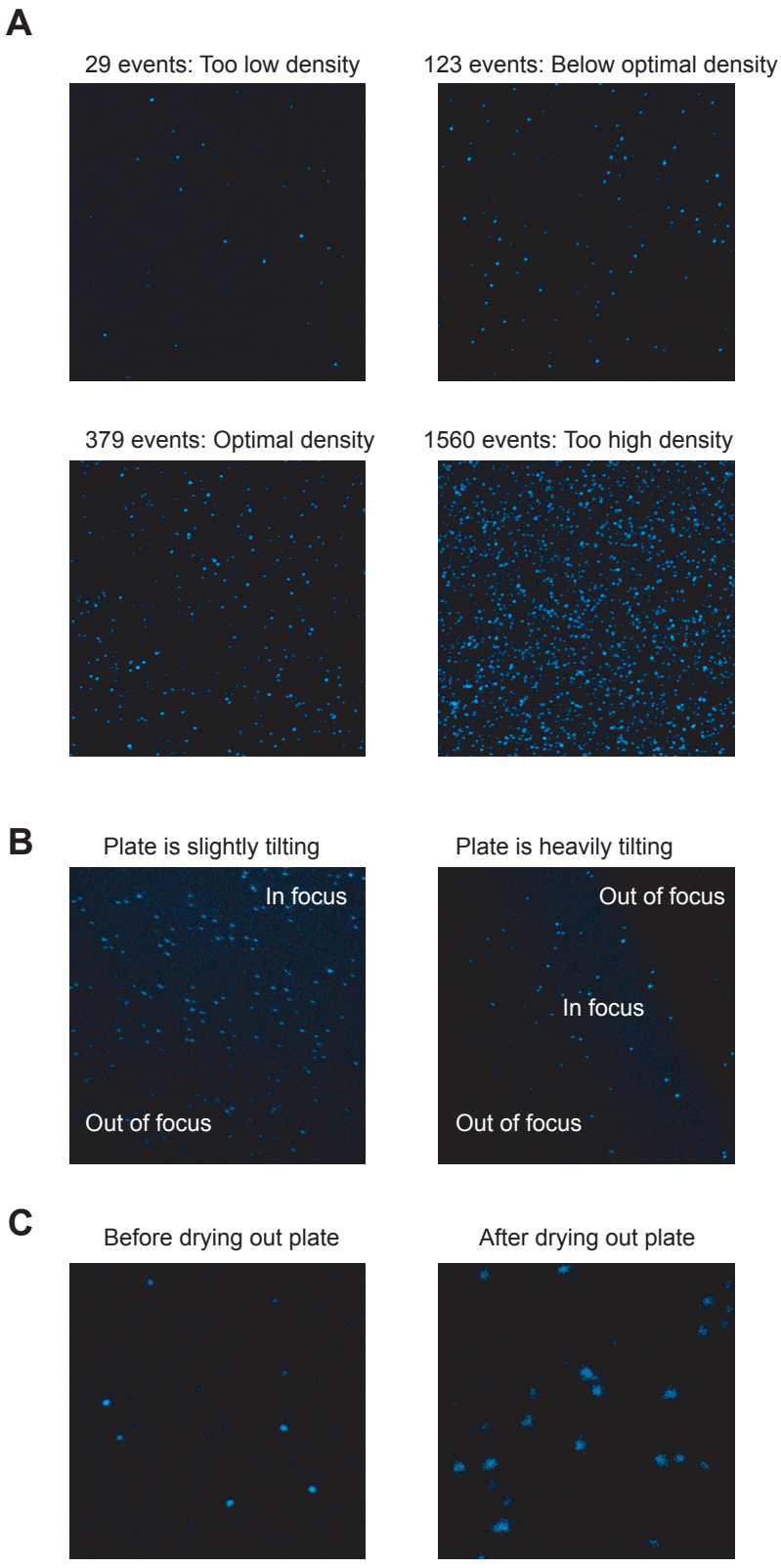


Figure 3

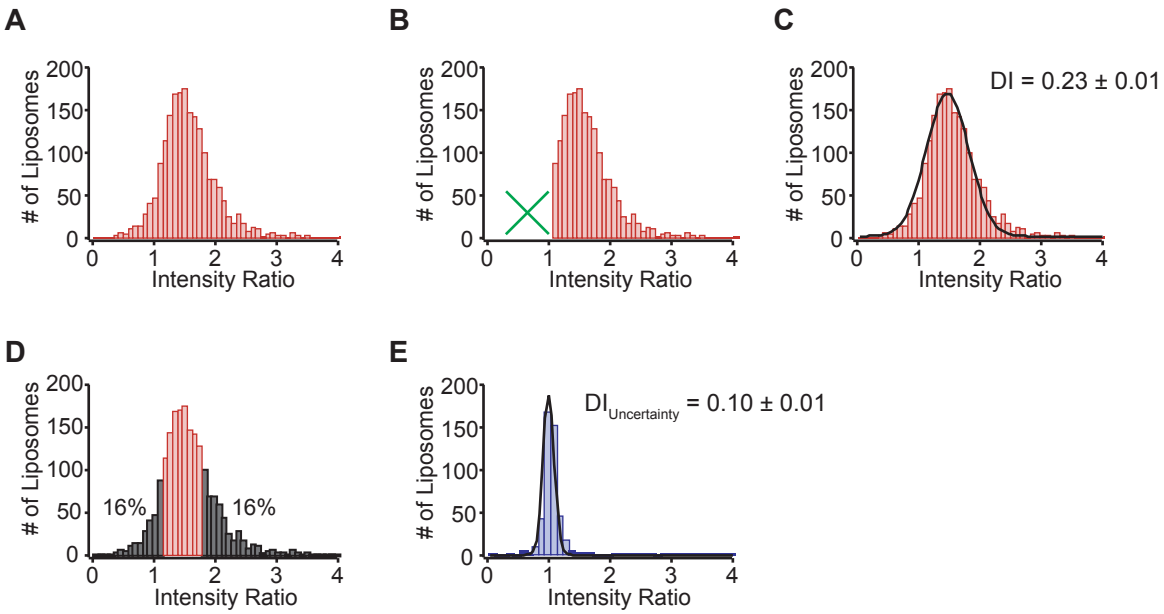
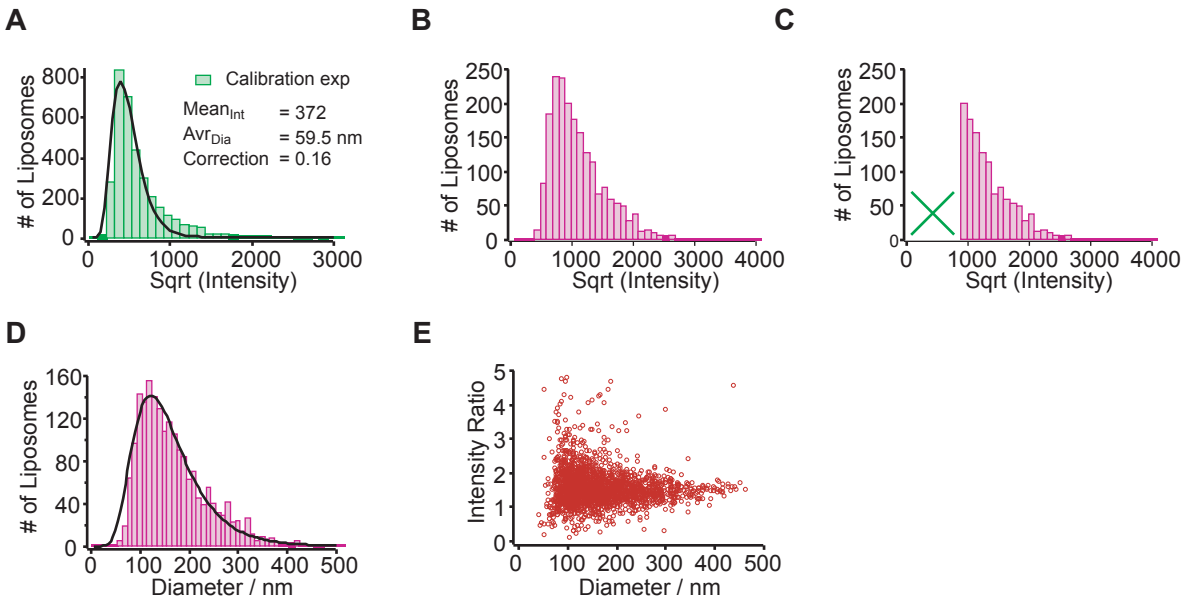


Figure 4



Name of Material/ Equipment	Company	Catalog Number
8-well microscopy slides (µ slides)	Ibidi	80827
Avanti Mini Extrusion kit	Avanti Polar Lipids	610000
BSA	Sigma	A9418
BSA-Biotin	Sigma	A8549
Cholesterol	Avanti Polar Lipids	700000
Computer with FIJI (Fiji Is Just ImageJ)		
DOPE-Atto488	Atto-Tech	AD488-165
DOPE-Atto655	Atto-Tech	AD655-165
DOPE-PEG-Biotin	Avanti Polar Lipids	880129
D-Sorbitol	Sigma	S-6021
Freeze-dryer		
Glass vials	Brown Chromatography	150903
HCl	Honeywell Fluka	258148
Heating bath		
Heating plate w. Magnet stirring		
HEPES	Sigma	H3375
Liquid nitrogen		
Magnetic stirring bars	VWR	442-4520 (EU)
Microcentrifuge tubes 1.5 mL	Eppendorf	0030 120.086 (EU)
Microscope		
Na HEPES	Sigma	H7006
NaCl	Sigma	S9888

NaOH	Honeywell Fluka	71686
POPC	Avanti Polar Lipids	850457
Streptavidin	Sigma	S4762
tert-Butanol (2-methyl-2-propanol)	Honeywell Riedel-de Haën	24127
Ultrapure water		

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Microscopy slides with glass bottom Consumables (Whatman filters) can be aquired from GE Healthcare
Traded trough Sigma ComDet plugin must be installed. Also, a data handling software (Excel, MatLab, OpenOffice, GraphPad Prism etc.) able to load .txt files will be needed to plot the data
Traded trough Sigma
e.g. ScanVac Coolsafe from Labogene
Glass vials that can resist snap-freezing in liquid nitrogen. The 8 mL version of the vials has a size that also fits with the syringes of the extrusion kit
Capable of heating to minimum 65C Capable of heating to minimum 65C
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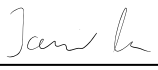
Signature:	
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Response to Editorial and Reviewers' Comments

Editorial comments

We would like to thank the editor for critically reading the manuscript and the constructive comments. We hope our answers below have addressed in full all of his/her concerns.

Comment E1

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Reply E1

We have now extensively proof-read the manuscript with the aim of correcting all spelling and grammar errors.

Comment E2

Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Reply E2

The manuscript is submitted in the JoVE template and we have thoroughly checked that it is formatted following the guidelines.

Comment E3

JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Eppendorf

Reply E3

We have removed all commercial language and direct use of company names. With respect to the fluorophores used, we have however found it necessary to mention the specific probes used for the example. We find that a generic term such as "DOPE-conjugated red emitter" would cause too much confusion, as well as causing problems with respect to the comment on probe differences mentioned by referee #3.

Comment E4

For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Reply E4

We have revised the experimental protocol and ensured that it followed the guidelines above.

Comment E5

Please remove 'Figure 1/ Münter et al.' etc. from the Figures themselves. Please also remove unnecessary whitespace.

Reply E5

We have removed all figure headings as requested and reduced the whitespace between figure panels. We have now provided the .ai files for each individual figure.

Comment E6

2A: 'To low'/'To high' seem to be typos.

Reply E6

We have corrected this.

Comment E7

Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol

Reply E7

We have ensured to include all information for the materials used in the protocol and added the glassware based on the question from the referee.

Referee #1

We thank the referee for the positive evaluation.

Comment R1-1

The article has a few typos which may be attended to by the authors.

Reply R1-1

We have now extensively proof-read the manuscript with the aim of correcting all spelling and grammar errors.

Referee #2

We thank the referee for critically reading the manuscript and providing helpful comments. In response to the comments of the referee we have included additional data.

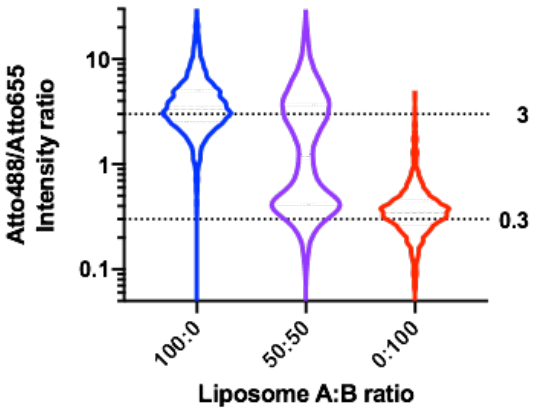
Comment R2-1

The efficiency of the assay should be tested with mixed liposome populations presenting different (defined) lipid compositions (e.g. liposomes bearing various known concentrations of a given fluorophore, mixing these liposomes at different known ratio).

Reply R2-1

The single liposomes assay is able to distinguish liposome preparations with known difference in the lipid composition

We welcome the referees suggestion for a control experiment demonstrating the efficiency of the assay. We thus prepared two liposomal formulations, A and B, similar to those described in the manuscript. However, we – instead of 0.05 mol% of each fluorophore, prepared formulation A with: 0.3% DOPE-Atto488 and 0.1% DOPE-Atto655 and B with 0.1% DOPE-Atto488 and 0.3% DOPE-Atto655. First, we immobilized the liposomes in separate chambers and imaged them as described in the protocol. As demonstrated in the figure below, formulation A alone gave a Atto488/Atto655 intensity ratio distribution with a mean very close to 3 (100:0 plot in blue), while B gave a Atto488/Atto655 intensity ratio distribution mean very close to 0.3 (0:100 plot in red). Next, we immobilized an equal number of liposomes from A and B in the same chamber (50:50 plot in purple), and imaged them together. The output of the image analysis resulted in two distinguishable populations; one with an intensity ratio distribution mean around 3 and the other around 0.3, in good agreement with the populations found for the separate measurements of A and B. The populations had a distribution with approximately equal numbers of liposomes with each intensity ratio, also demonstrated by a median very close to one (1.16). We believe that this data set demonstrate the efficiency of the assay in separating liposomes formulations with known difference in their lipid composition.



Comment R2-2

Similarly, the authors should also mix liposome populations of different (defined) sizes (e.g. 50 and 400 nm formed by extrusion) to check that the assay allows them to find the expected percentage of liposomes having 50 or 40 nm diameter.

Reply R2-2

Previous correlation between various liposome size determination methods revealed the imaged based size determination method to be very accurate

We share the belief of the referee that performing controls for the accuracy of the size determination is essential for validating the system. Therefore an in-depth analysis has previously been carried out where the imaging based single liposome size determination was evaluated against well-established techniques such as DLS and Cryo-TEM[1]. Here, it was found that "*the three techniques were in excellent agreement, measuring the same values within 7–9%*"[1] demonstrating the strong accuracy of the image based size determination. Additionally, it has previously been shown that liposome preparations extruded at various cut off sizes will show significant size overlap (see SI of Hatzakis et. al, Nat. Chem. Biol. 2009)[2], which will hamper the accurate retrieval of the individual size populations as proposed by the referee. Therefore, we believe that a better control for the accuracy of the imaged based size determination is the comparison to DLS and Cryo-TEM referenced above. Finally we will also like to highlight that the true power of the assay does not lie in the ability to pick out certain size populations, but more in the ability to accurately assign the actual physical size to single liposomes. This ability mean that producing specific size population by e.g. extrusion is not necessary, since the assay allows researchers to take advantage of the size polydispersity within a single liposome preparation to study a range of sizes simultaneously or limit their investigating to only a specific size subset during subsequent data analysis.

Comment R2-3

It would be useful to add the reference for the glass vials with lid (notably because some vials may not resist freezing in liquid nitrogen).

Reply R2-3

Added reference to used glass vials

We have included a reference in the table of materials to the commercially available glass vials used in the protocol.

Referee #3

We thank the referee for the positive evaluation and the opportunity to explain in more detail some aspects of the assay, relating to the helpful comments by the referee.

Comment R3-1

The method is applicable to double fluorophore labeled liposomes and in fact, not applicable to other non fluorescent components.

Reply R3-1

Single liposome measurements can be performed with liposomes labeled with one kind of fluorophore

We fully agree with the referee that the single liposomes assay presented here comes with the inherent requirement that the components to be studied are fluorescently labelled. Especially the compositional inhomogeneity in this manuscript can only be done for double fluorophore labelled liposomes. However it is important to note that the fluorescently labelled lipid probes merely serve as a reporter system for the overall lipid composition of the liposome. Therefore, under the assumption that the fluorescent probe does not interfere significantly with the natural behavior of the lipids, the assay can be said to probe the overall difference in lipid composition between liposomes in the same preparation. Additionally, the assay is not restricted to lipid species, but can also more specifically measure the inhomogeneity of other fluorescently labelled compounds such as proteins or peptides binding to - or residing in - the liposome, as described in the introduction. Also, only a single fluorophore labelled lipid species is necessary the performing the size determination described in the protocol.

Comment R3-2

Also, the probes are different so some difference in partitioning is expected.

Reply R3-2

Compositional inhomogeneity is lipid structure dependent, but fluorophore independent

The referee is highlighting an important aspect of compositional inhomogeneity, namely how it is related to the lipid structure. When the referee notes that the probes are different he or she is correct in the sense that the fluorophore is different between the used DOPE-Atto488 and DOPE-Atto655, however the lipid anchor is purposely the same for both probes. It has previously been shown that the compositional inhomogeneity is not significantly affected by the choice of Atto-dyes, but strongly dependent on the lipid anchor, with increasing DI values quantified for anchors displaying reduced structural similarity (see main text and SI of Larsen et. al, JACS 2011)[3]. Thus the DI quantified for the dataset presented in the manuscript represents the case where the reporter lipid species are nearly identical and thus should display even partitioning.

We have now, in the discussion section of the paper, highlighted that researchers using the protocol should be aware of the physicochemical properties of the lipid anchor.

Comment R3-3

How reproducible is this method, and what happens when different batches are characterized?

Reply R3-3

The assay displays very strong batch-to-batch reproducibility

We share the view point of the referee that reproducibility is a key component for quantitative assays like the one presented in this work. Therefore, we make sure that the reported average DI values and standard error of the mean (SEM) originates from at least six independent experiments, performed on two individually prepared liposome preparations. The strong accuracy and reproducibility of the single liposomes assay is evident from the low SEM values reported both in this manuscript (average DI ± SEM = 0.23 ± 0.01) and in previous studies on compositional inhomogeneity[3-5].

1 Kunding, A. H., Mortensen, M. W., Christensen, S. M. & Stamou, D. A fluorescence-based technique to construct size distributions from single-object measurements: Application to the extrusion of lipid vesicles. *Biophysical Journal.* **95** (3), 1176-1188, (2008).

2 Hatzakis, N. S. *et al.* How curved membranes recruit amphipathic helices and protein anchoring motifs. *Nature Chemical Biology.* **5** (11), 835-841, (2009).

3 Larsen, J., Hatzakis, N. S. & Stamou, D. Observation of Inhomogeneity in the Lipid Composition of Individual Nanoscale Liposomes. *Journal of the American Chemical Society.* **133** (28), 10685-10687, (2011).

4 Elizondo, E. *et al.* Influence of the Preparation Route on the Supramolecular Organization of Lipids in a Vesicular System. *Journal of the American Chemical Society.* **134** (4), 1918-1921, (2012).

5 Larsen, J. B. *et al.* Membrane curvature enables N-Ras lipid anchor sorting to liquid-ordered membrane phases. *Nature Chemical Biology.* **11** (3), 192-U176, (2015).