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.

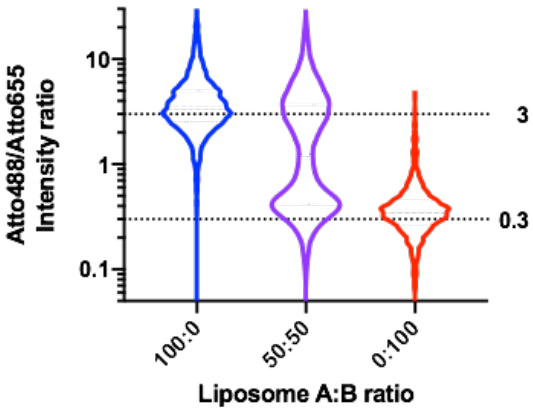
Comment R2-1

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).