Editorial comments:

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

We have proofread the paper and corrected spelling and grammar mistakes where observed.

2. Please provide an institutional email address for each author.

We have provided the email addresses of the two other authors.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the text and restructured sentences initially written with personal pronouns.

4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., Whatman, Avanti mini extruder, Julabo, QuickConnect, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

We apologize for this oversight. We have removed all mentions of commercial products and provided this information in the Table of Materials.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have revised the protocol section and changed the sentence structures to address the requested changes.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. 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. Please add more specific details (e.g., volume of samples/ buffers used, specification of the vials, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please move the discussion about the protocol to the Discussion.

We tried to provide additional details in the protocol. We hope that the additions will help clarify the protocol better and provide the necessary information for replication.

7. Line 186/248: Please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2

We have made the requested changes in the used units.

8. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the parts of the manuscript that are essential for the video production.

9. Please remove the titles and Figure Legends from the embedded figures. Please include a Figure Legends section at the end of the Representative Results. The information provided in the Figure Legends after the Representative Results is sufficient.

We have provided higher-resolution versions of the figures and removed all figure legends from the figure file.

10. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have removed all figures from the manuscript and included the figure captions after the Representative Results section of the manuscript.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

We have changed the reference style to adhere with JOVE's formating. To do this, we imported the JOVE Style into EndNote which automatically updated the citations to the required style.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have obtained copyright permissions for adapted images shown in figures 2 and 4. We have deleted the reference in figure 5 as it shows generic data trends obtained with different deuteration schemes. However, since this figure now shows data that was unpublished before in this given format, we have included a statement acknowledging the use of the NIST NSE spectrometer for the collection of the data.

13. Figure 3: Please remove the commercial names from the figure and use generic names instead (e.g., SyringePump, Avanti polar lipids, Inc).

We have masked all commercial names in the figure and used generic descriptors in the figure caption.

14. Figure 4: Please define the units of Phase current within parenthesis in the X-axis.

We have revised this figure and changed the axis label to “Phase” expressed in degrees. We also included a clarification in the text (protocol 4.7) and in the figure caption.

15. Please provide a detailed list of chemicals, consumables and equipment used in the Table of Materials and sort them in alphabetical order.

We have updated the Table to Materials and included a complete list of chemicals, consumables, and equipment needed in the protocol.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:

In their manuscript entitled "Neutron Spin Echo Spectroscopy: A Unique Probe for Lipid Membrane Dynamics and Membrane-Protein Interactions" the authors describe a protocol for running a Neutron Spin Echo measurement on liposomes of more or less homogeneous size. In their introduction they briefly talk about alternative techniques and introduce the technique of Spin Echo Spectroscopy in general. The discussion part summarizes the findings on the dynamics found in liposomes by previous studies.
The manuscript nicely describes the experimental requirements for a successful NSE-Experiment on liposomes. It also shows the potential of isotopic labelling. It falls short, when it comes to the aspect of the incorporation of proteins into the liposomes. And there is only one sentence on the effect of Membrane Proteins on the dynamics. Therefore, the title should be changed to " Neutron Spin Echo Spectroscopy: A Unique Probe for liposomal Lipid Membrane Dynamics"

We thank the Reviewer for his/her thorough review of the manuscript. The comments, suggestions, and recommendations s/he provided are very much appreciated and we believe that they have been tremendously helpful in improving the quality of the paper.

We also appreciate the Reviewer's comment about the title and the brief description of protein-membrane interactions. Given that the main focus of the paper is on membrane dynamics, we hoped to emphasize that NSE studies on membranes can also be used to understand the effect(s) of proteins incorporation on membrane dynamics. In such studies, and in the absence of membrane contrast matching, NSE primarily detects membrane dynamics (especially when measured with typical lipid:peptide ratios where peptides/proteins are the minority component). This area is currently underdeveloped, despite its strong biological relevance and its importance in membrane functions. Our intent is to illustrate in this paper that the same (or similar) NSE approaches as the ones applied on vesicular membranes can indeed be utilized in the studies of membrane dynamics in response to protein binding and insertion -- a topic of interest to a wider scientific community who would benefit from learning the potential of NSE in membrane-protein systems.

Major Concerns:

All Figures should be given in a vector graphic format or at least in a higher resolution as present in the manuscript.

We have uploaded higher resolution figures for the final publication. The figures that were embedded in the initial manuscript were meant to aid in the review process (being placed close to where they are referenced).

In the discussion or introduction one should also mention the technique of GINSES as found for example in the following publication:

Influence of ibuprofen on phospholipid membranes; Sebastian Jaksch, Frederik Lipfert, Alexandros Koutsioubas, Stefan Mattauch, Olaf Holderer, Oxana Ivanova, and Henrich Frielinghaus, PHYSICAL REVIEW E 91, 022716 (2015),

We thank the Reviewer for bringing this to our attention. While we explicitly state at the end of the introduction that this paper is focused on the dynamics of free-standing vesicular membranes, we can see the value of referring readers to other methods like GINSES for systems where supported membranes are required for measurements of in-plane dynamics. Therefore, we have added a couple of references on the GINSES approach including the paper recommended by the Reviewer.

On page 3 in line 111 one should also mention the J-NSE Phoenix instrument at the MLZ with an accessible Fourier-time of at least 350 ns, citing the following publication: J-NSE-Phoenix, a neutron spin-echo spectrometer with optimized superconducting precession coils at the MLZ in Garching; S. Pasini, O. Holderer, T. Kozielewski, D. Richter, and M. Monkenbusch, Rev. Sci. Instrum. 90, 043107 (2019); doi: 10.1063/1.5084303
Speaking with the instrument responsible, Olaf Holderer, even 420 ns are obtainable with a reasonable resolution signal of greater than 0.2=R.

The Reviewer makes an excellent point, and we apologize for this oversight. We agree that it is important to include the upgrades in the J-NSE spectrometer among recent advances in NSE instrumentation. We have added that to the discussion along with the corresponding reference.

Minor Concerns:

Page 3, line 134: "Note that the that the..." should read "Note that the..."

We have corrected this typo.

Page 7, line 230: The term "peristaltic" is not applicable here, it is a pure syringe pump. A peristaltic pump squeezes a tube on two points distant from each other and moves the liquid between the two squeezing points forward by rotating a wheel. The term "peristaltic" should be omitted from the whole manuscript.

The Reviewer makes a valid point. We can see the confusion that the use of this term could cause. Therefore, we have deleted all occurrences of the term “peristaltic” from the manuscript.

Page 8, line 268: "NOTE: After the extrusion cycles are complete. The lipid solution should end up in..." should read " NOTE: After the extrusion cycles are complete, the lipid solution should end up in..."

We corrected the sentence structure.

Page 10, line 315: " if your can prepare your sample in..." should read "if you can prepare your sample in..."

The typo has been corrected.

Page 10, line 337: the (1) should be replaced by (2), because equation 2 is the one which is referred to.

We changed the reference to the correct equation.

Page 11, line 352: The letters R (for Reference) and BKG for Background should be explained in the text below the equation.

The acronyms were introduced and defined in 4.4 and 4.6. However, to avoid any confusion we added a short statement clarifying what the acronyms are and referring the reader to the corresponding section.

Page 11, Figure caption of Figure 4: line 3: "echo signal to eq. (2)" should read "echo signal using eq. (1)"

We implemented the proposed correction.

Page 11, Figure caption of Figure 4: line 5: " ...due to poor echo signals over. The..." should read "...due to poor echo signals. The..."

The typo has been corrected.

Page 14, line 424: a "the" should be added in front of "mesoscopic"

Done.

Figure 2A: Font size should be at least doubled for better reading.

The figure was reproduced with bigger fonts and higher resolution.

Figure 3: The use of the term "peristaltic" should be omitted.

As requested by the Reviewer, all occurrences of the term “peristaltic” were removed from the manuscript.

Figure 4A: The unit for the phase current should be given (Ampere?)

We thank the reviewer for pointing this out. The label of the axes should be “Phase” not “Phase Current” and the values are expressed in degrees. It is common in NSE data representation to refer to the phase, rather than the phase current. Since the phase current is proportional to the precession angle of the neutron spin (which is the physically relevant quantity in NSE), the phase current can be expressed as an angle. We have added the necessary clarification in the text (protocol 4.7) and figure caption, and we modified the axes label accordingly.

Reviewer #2:

Manuscript Summary:

The manuscript describes a visual experiment demonstrating neutron spin echo (NSE) spectroscopy as a unique probe for lipid membrane dynamics. This demonstration is separated in two essential parts: The protocol for the lipid vesicle sample preparation and the protocol for conducting the NSE experiment and analyzing and interpreting the resulting data.

We thank the Reviewer for his/her careful reading of the manuscript and for providing constructive comments which helped improve the readability of the paper.

Recommendation:

The manuscript is timely, useful, well-structured, and well-written. I recommend to publish the suggested visualized experiment subsequent to considering the following points:

We appreciate that the Reviewer finds this paper timely and informative. We hope that it will facilitate the use of NSE by the wider scientific community to solve problems that are pertinent to the understanding of the rich dynamics in biomembranes and their role in membrane functions.

- The author list does not include a neutron spin-echo instrument scientist. Since the demonstration seems to require access to either the NIST or SNS spin-echo spectrometers, should one of these colleagues be involved?

With all due respect, we do not think that this is an appropriate request. While one can see where this comment is stemming from, we would like to point out to the Reviewer that this paper was written in response to an invitation to the corresponding author, who is an expert on NSE and its applications in biomembranes. Nevertheless, we have reached out to an NSE instrument scientist and invited him to be part of this paper, but he declined for personal reasons. This said, we do not believe that we need to explain the author list or justify why colleagues who were not part of this invitation are not co-authors on this paper.

- The membrane protein studies are mentioned only very briefly in the actual protocol (notably only in item 2.2.).

We appreciate this observation by the Reviewer. We note that the protein protocol is not elaborate because the primary focus of this paper is membrane dynamics. Given that different protein solutions could require different preparation methods, we believe that going into such detail could cause a diversion from the main theme of the paper. Our intent behind including the section on protein is to inform the wider scientific community of the great potential that NSE has in exploring the dynamic signatures of biomembranes in response to protein binding or insertion. This is a pressing, yet underdeveloped, area of research where NSE can have significant impact in understanding the dynamic responses of membranes on the length and time scales of key biological functions imparted by the interactions of proteins with cell membranes.

Minor points:

l.134: Typo extra 'that the': 'Note that the that the NSE signal ...'

The typo has been fixed.

l.339: The authors refer to figure 4A, not 3A.

We thank the Reviewer for pointing this out. The reference to the figure has been corrected.

l.347: The authors refer to figure 4

We apologize for the confusion in figure numbering. This was a result of a problem in the automatic updating of figure references in the Word template. The reference to the figure has been fixed.

l.359: The authors refer to figure 4B

Please see the response above.

l.573: Along with reference 22, the following additional reference may be cited: M.Grimaldo et al., Quart.Rev.Biophys.52, e7, 2019, https://dx.doi.org/10.1017/S0033583519000027 (containing also some details on NSE and complementary techniques).

We thank the reviewer for pointing us towards this reference. We found it quite informative and we believe that it could also be a valuable resource for readers interested in complementary NSE studies of protein dynamics. Therefore, we added this reference to the list of citations.

Reviewer #3:
Accept