Dear Editor,

We would like to thank the Editor for handling our manuscript and are grateful for the constructive comments provided. We have responded to all points raised by the reviewers and revised the manuscript accordingly. We have uploaded a pdf file in which all revised sentences are highlighted.

Responses to Reviewer #1:

Thank you very much for the constructive comments. We have added a statement regarding the experimental condition, as far as possible. Also, we added suggestions on how to form a stable lipid bilayer.

Minor Concerns:

Since lipids and lipid bilayers and their stability is significantly effected by the temperature, pH, salt and buffer conditions, I will recommend authors to elaborate on the information regarding their conditions in the paper. What are the limitations of forming successful and high efficiency contact bubble bilayers in a variety of electrolytes (such as NaCl, KCl), and pH conditions. If the authors have experience with modulating these conditions or having a condition, which performs the best for bilayer stability, they should definitely include it in this manuscript. This will be highly useful for researchers adapting this protocol in their labs.

The limitations of the CBB method have been added and are summarized in the Discussion section.

Responses to Reviewer #2:

We appreciate the valuable comments on our manuscript. The awkward expressions were corrected according to the suggestions of a native English speaker.

-The term "patch-clampers" is overly colloquial English for me. I would replace these words with a different terminology in all places where the term appears.

We agree that the term “patch-clampers” is colloquial English, and we had discussed this term with a native English speaker doing patch clamp experiments. The term is one that we all understand. The alternative would be to refer to such people as “specialists in patch-clamp recording techniques” or “users of patch-clamp recording techniques.” These phrases are awkward and cumbersome. Also, there is no concise alternative. So generally speaking, we would prefer to retain “patch-clamper.”

-In the abstract, the word "manipulability" is awkward. I would choose a different wording.

We have replaced “bilayer mechanical manipulability” with “to manipulate bilayer mechanics” in the text.

-In the short abstract (and a few other places), there is a choice to be made between past tense and present tense ("was blown" and "was formed" versus "is blown" and "is formed"). There are other places where the past tense and present tense are mixed. Please try to be consistent.

We have unified the tenses in the text.

-In line 101 the word "impat" is incorrect. Perhaps "impart" was intended.

This has been corrected.

-Insert "the" at the end of line 140 to give: "We found that the cholesterol content . . . "

We have inserted “the” at the indicated position.

-In line 141 change "switch" to "switching" and delete "of".

We have revised the phrase as suggested.

-In line 355, change "is" to "are" to agree with "procedures." The remainder of the Discussion has multiple places where there is awkward use of the English language. Please edit the entire manuscript for minor improvements in the grammar and the flow of the language. Notably, in line 391, see "glass wears" ?? There are other examples, too numerous, that need to be checked.

Thank you for this indication. The entire manuscript has been copy edited by a professional English-language editor.

Responses to Reviewer #3:

Thank you very much for

1. Line 115: Please elaborate what you mean by changes in surface tension? Changes in the bilayer tension or changes in the monolayer tension? Presumably both, but how do you sort this out?

Thank you for pointing out this important aspect. We have revised the relevant part as follows:

“The changes in the contact angle indicate changes in the bilayer tension, given that the monolayer tension is evaluated from changes in the contact angle as a function of the membrane potential (Young-Lippmann equation: γmo = Cm V2/4 (cos(θ0) – cos(θv)), where Cm is the membrane capacitance, V is the membrane potential, and θ0 and θv are the contact angles at 0 and V mV, respectively).”

2. Line 119: At first reading, I did not understand what was meant by "multiple phases." Multiple components is likely to be a better term.

We have revised the indicated part as follows:

“A CBB system consists of distinct phases; namely, a bulk oil phase, water bubbles coated with a monolayer, and a contacting bilayer.”

3. Line 125: Is it necessary to introduce the Marangoni effect? It is likely to confuse, unless you make use of this later in the presentation.

The "Marangoni effect" is a concept known among surface chemists and admittedly might be unfamiliar to other readers. However, this phenomenon is an essential one that must be kept in mind for the CBB experiment. In the revised version, we have explained the Marangoni effect more clearly. Also, the reference #47 was replaced.

4. Lines 127 - 130: This needs to be clarified; the transition temperature for DOPC is close to -20 C, which should allow for stable bilayers.

We have moved the indicated part to the Discussion, revised as follows:

“For example, dioleoylphosphatidylcholine is frequently used for liposomes, but it forms electrically leaky CBBs at 25 °C62. CBBs cannot even be formed with lipid species whose phase transition temperature is above the recording temperature. Indeed, CBB formation was difficult with dipalmitoylphosphatidylcholine at room temperature, but raising the temperature above Tm circumvented the problem63.”

5. Line 183, and elsewhere: The term "hole slide glass" is unknown to me, and I did not see it illustrated in any of the figures.

We have illustrated the hole slide glass in the text: hole slide glass (slide glass with a shallow concave well)

6. Line 193: This is in part redundant with what is stated on line 188.

7. Line 201: Again some redundancy.

We have revised the indicated parts.

8. Lines 213-214: Please change the tense to be present tense.

We have revised the sentence using the present tense.

9. Lines 226 - 231: This was unclear to me. Please revise.

We have moved the indicated part to the Discussion, revised as follows:

“In our protocol (step 4.8), a solution of liposomes and channel-reconstituted liposomes (1 μL) is loaded from the tip of the pipette (steps 4.9 and 4.10), and the rest of the pipette contains the previously filled electrolyte solution. This is merely for conserving materials, such as lipids and channel molecules. Consequently, the liposomes diffuse gradually toward the upper pipette solution, and after a while, the lipid concentration at the tip of the pipette becomes insufficient to form lipid monolayers. In this case, fresh liposome solution should be aspirated from the tip (step 4.8).”

10. Lines 234 - 235: This probably should moved to be right after lines 127 - 130.

We have moved the relevant part to the Discussion.

11. Lines 263 - 264: It is not clear to me what the authors refer to. It would be good to refer to Figure 5, which I assume shows what the authors have in mind.

We have added a new figure (Figure 7) to illustrate the indicated point.

12. Line 355: Please change "is" to "are."

This has been corrected.

13. Line 360: Suggest deleting "and unprecedented."

14. Line 365: Same comment.

These have been deleted.

15. Table 1: It would be preferable if Rows 1 "Success Rate" and 5 "S/N ration" had numbers assigned to "low," "high" and "very high."

In this table, we intended to show the rough characterization of and comparison between the techniques. Each item was expressed qualitatively because they varied substantially in each experiment and channels used.

Responses to the editorial comments:

We have made the corrections throughout the text according to the editor’s comments.

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

2. Please provide an email address for each author.

3. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

This has been corrected (Lines 15–18).

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

This has been corrected (Lines 95–98).

5. Please spell out each abbreviation the first time it is used.

This has been corrected.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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 and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sigmacote, Axopatch, etc.

This has been corrected.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

8. Please revise the protocol to contain only action items that direct the reader to do something (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.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

This has been corrected (Lines 154–258).

9. Lines 147-154, 226-235, etc.: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please move the discussion about the protocol to the Discussion.

This has been moved to Lines 389–392.

10. Please add more details 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 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. See examples below:

Line 157: Listing an approximate volume to prepare would be helpful.

Line 160: What temperature is set during rotary evaporation and how long does it take to form a thin phospholipid film?

Line 175: Please specify the settings of the pipette puller.

It is generally difficult to show the settings since the platinum heating sheet varies substantially, and so too the settings.

Line 179: Heat for how long?

Line 185: What is considered to be an appropriate volume?

All the points, except for Line 175, were corrected.

11. Step 5 is difficult to follow. Please try to combine this specific protocol with previous steps. For instance, indicate the modifications made for KcsA potassium channel in steps 2 and 7.

Step 1.5 was included in Lines 170–172.

12. Please include single-line spaces between all paragraphs, headings, steps, etc.

This has been corrected.

13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less 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.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

This has been corrected.

16. Discussion: Please also discuss critical steps within the protocol.

We have added sentences for the critical steps (Lines 378–384)

17. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

This has been corrected.

18. 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. See the example below:

Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

19. References: Please do not abbreviate journal titles.

This has been corrected.