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RE: JoVE50436, revised manuscript

Dear Dr. Baker,

Thanks for sending our manuscript to reviewers. The constructive comments by the reviewers have allowed us to revise the manuscript substantially. I am now sending the revised manuscript to be considered for publication. In the next I listed what we did to address the editorial comments and reviewers’ comments.

**Editorial comments**  
1. The highlighted portion of the protocol is over just over 3 pages. Please ensure when revising that the highlighted portion does not stretch over 3 pages.

Done as suggested.

2. The bulk of some steps (such as 3.1.7 and 3.1.8) are conceptual and have nothing to film. Please submit a graphic to illustrate the concept or remove the highlighting from the conceptual text. If the latter is done the total length of highlighting can be preserved by highlighting another portion of the protocol.

The conceptual parts are not highlighted any more.

*3. Box 2* is not referenced in the body of the manuscript.  
It is now referenced in (2.3.3) and (3.3.1).   
  
**Reviewers’ comments**

**Review #1**   
1) The only concern of the reviewer with the article is its scope. ….

We are aware of the demanding nature of phage-display and electron crystallography. We think that these two parts highlight important applications of protein reconstitution and therefore constitute good examples to the readers about how to use the reconstitution in new directions. It is better to still leave them in the manuscript. Echoing what Reviewer #2 suggested, during revision we added more basic principles about the techniques used in these two applications. The readers should now be able to appreciate them sufficiently and our description will serve as a guide for them in the future.

**Reviewer #2**   
1) The authors should add some warnings/discussion about the implications of decane or other solvents in BLMs. While its true that KvAP is function in this system, once needs to be aware that the amount of decane will vary when forming BLMs using different lipids, and that this may be a critical variable in any effects observed. There are probably some good references to add on this point also.

This is a good point. In section 3.1.8, we added a detailed discussion about the estimated amount of decane left in the decane-lipid bilayers. A cautionary statement is added there so that the readers will keep this variable in mind, especially in working on a different protein. Several references were added.

2) It would be useful to explain how capacitance measurements can be used to estimate the size of the true bilayer at the center of the hole.

First paragraph of section 3.1.8 now explains how we estimated the approximate size of the true bilayer at the center of the hole.

3) I found myself getting lost in the section about phage display. The authors reference other work for details, but it would be good to go through this section carefully and make sure the important concepts of each step are clearly explained so the reader can follow along.

A new section (3.2.2) was introduced to explain the basic operations in the phase display. More explanations of the important concepts are now added in sections 3.2.1.

4) Although the authors are typically quite detailed in their descriptions, there are details that are not given. For example, are reducing agents present in the SDS-PAGE loading and running buffers? What temperatures are used for denaturation? Typically one does not heat a membrane protein in SDS to the same temperatures as for a soluble protein. I would suggest going through everything one more time and make sure those sorts of details are provided, in particular because this article will be used by scientists without much previous experience in this area.

We have specified whether the reducing agents were used in SDS-PAGE. It is also stated explicitly that the channel protein in the SDS-PAGE buffer should not be heated. We went through the details in the procedure and have made it sufficiently clear so that the experiments can be reproduced step-by-step.

5) Please provide the extinction coefficient for KvAP and reference the paper where extinction coefficients were measured for amino acid analogues used in the calculation.

Done as suggested (section 1.3.9).

**Reviewer #3**  
  
*Major Concerns*  
1.) Detergent solubilization of vesicles: Page 6 (2.1.1): A more thorough discussion of the influence of the equilibration of detergents, lipids, and protein on the reconstitution efficiency would be helpful.  
More detailed discussion was added to sections 2.1.1.3 and 2.1.1.5 in the revised manuscript.

2.) A typical length of time for sonication of the vesicles to the correct size should be included, as well as a mention of the variability of that time to different preparations.

We neglected these details, which can now be found in the first paragraph of section 2.1.1.3

3.) Sonicating PE/PG vesicles in a bath sonicator, even for long periods of time, causes the scattering of the vesicles to plateau before it reaches an OD410 < 0.2 (OD410 ~ 0.7-1). Though the vesicles look completely translucent, the OD never drops to the level cited in the protocol. What consequence does incomplete sonciation have on the efficiency of the reconstitution?

We specified in section 2.1.1.3 the use of a high-power sonicator for reaching OD410 < 0.2. Alternatively the microbe sonicator could be used to make small unilamellar vesicles that give rise to low OD410. We also added that if the sonication is incomplete, we completely solubilize the lipids with high concentrations of detergents because we found that incomplete sonication affects the reconstitution efficiency.

4.) The authors mention that the vesicles are 30-50 nm in diameter; where does this number come from? Are the lipids in the form of vesicles or micelles? Have you done DLS or EM analysis of the sonicated vesicles?

They were checked under EM. We mentioned this in the revised text. The lipids form small tight vesicles.

5.) If the protein is concentrated before adding to the detergent-solubilized lipids, the DM will also become concentrated in the sample. Presumably, if the protein is concentrated more than 2x, the final concentration of DM will be > 20 mM, which moves the turbidity from the Rsat to the Rsol regime. Does concentrating the protein matter?

Yes, it does matter. If the detergents are concentrated by multiple folds, we would adjust the final concentration accordingly. If it is difficult to estimate, we would move to Rsol region. We revised the text in section 2.1.1.5 to include these points.

6.) Please discuss the consequences of starting the detergent-lipid-protein mixture at Rsat versus Rsol on the equilibration of the protein-lipid-detergent mixture, the reconstitution efficiency, and the dialysis procedure merit discussion. (In general, and also in regards to DOTAP/DOGS)

Added in section 2.1.1.5

7.) DOTAP/DOGS protocol: Page 7 (2.1.2): The addition of the purified KvAP to the DOTAP or DOGS detergent-solubilized vesicles is missing. How much KvAP is added to the channels? How long are the protein-detergent-lipid micelles allowed to equilibrate? And in the next section (2.2), how does starting with fully solubilized lipids change the dialysis?

Thanks for pointing it out. We added section 2.1.2.3.

8.) Dialysis: Page 7 (2.2.1): The section on dialysis is short and lacks pertinent details.

Details were added into this section. Two new paragraphs were included.

9.) The authors note that "the vesicles are usually ready after two days." How does one know when the vesicles are "ready"?

We tested the vesicles on bilayers because the residual detergents would break the bilayer immediately. It is now added in the last paragraph of 2.2.1.

10.) How is the residual detergent in the dialyzed vesicles characterized? How does one know when sufficient detergent has been removed?

We did not directly measure the amount of residual detergent. Instead we used the lipid bilayer as our test. Please see the last paragraph of section 2.2.1

11.) During dialysis, there are changes in the turbidity that happen as the detergent is removed. A discussion of the changes in cloudiness would be useful.

Added in the second paragraph of section 2.2.1

12.) In the ultracentrifugation results, the authors mention that a multilamellar band can be seen occasionally. How can multilamellar and/or vesicles without incorporated protein be avoided?

To find that if the detergents are fully solubilized and the protein/lipid/detergent mixture has enough time to reach equilibrium, it is less likely to have a significant multilamellar band. Still it is difficult to avoid vesicle inside vesicle.

13.) Is the width of dialysis membrane important to maintain a particular surface area to volume ratio?

Yes. We used dialysis tubing of small diameter in order to increase the dialysis speed.

14.) Floatation experiment: What is the sensitivity of the Coomassie stain of the SDS-PAGE gel? Coomassie stains usually aren't very sensitive, particularly to membrane proteins; it's possible that 1-10% (or more) of the protein isn't incorporated into vesicles but are below the detection limit by Coomassie stain. Do other more sensitive measurements (Western blots, EM on reconstituted vesicles) agree that the insertion of KvAP is ~100%?

We don’t think that is the case. In our Coomassie stained gels, we can detect down to 0.5 microgram protein. The protein we used for the floatation experiment is at least 50 micrograms in total. The proteins not incorporated would become aggregated at the bottom of the tube and would have been showed as a significant band in bottom fraction.

15.) EM protocol: (3.3) The EM section lacks critical details, such as the incubation of the Fv antibody with KvAP; the concentration of lipids, protein, and detergent; and the dialysis protocol.

A new section is added for negative-stain EM (3.3.1).

16.) Orientation of KvAP: In the abstract, the authors mention that their data indicates that the orientation of KvAP is random, not preferential. Where are the data that supports that assertion?  
We could record channels from both directions after the fusion of vesicles into the lipid bilayer. Cys-modifications by MTS-PEG5000 also suggested that roughly half of the protein faces one side of the vesicle.

*Minor Concerns*  
1.) Page 4 (1.1.4): How long should the plates be grown? Provide a more narrow definition for "overnight."

Added “14-16 hours” in text.

2.) Page 4 (1.2.1): How large should the colonies be?

The colonies are small, ~0.2-0.5 mm.

3.) Page 4 (1.2.4): The authors should mention why BaCl2 is included in the expression of KvAP.

A sentence is added to explain that BaCl2 decreases the toxicity of the channel expression to the bacteria.

4.) Page 4 (1.2.4): It's important to note that the Ba2+ ions can also precipitate due to a high concentration of ions (presumably sulfates or other counter ions that precipitate barium) in the LB mixture as well as the water.

Yes. We added this point there.

5.) Page 6 (1.3.8): It would be useful to differentiate between precipitation, evidenced by white floating precipitate, and the brownish concentrate that settles to the bottom of the spin concentrator. It would also be helpful to provide an approximate value for the concentration that leads to precipitation (> 10 mg/ml?).

Thanks for these very interesting points. We added explanations in (1.3.8).

6.) Page 6 (1.3.9): When discussing the FPLC purification, it would be helpful to mention the other peaks observed during the purification (aggregate eluting with the void volume, then tetramer, monomer, and imidazole).

Added as suggested.

7.) Page6 (1.3.9): How stable is the purified protein? How quickly after purification should the recon be performed? How should it be stored?

A separate paragraph was added in this section.

8.) Page 7 (2.2.2): The section on the use of Biobeads is somewhat confusing. The section should clarify that Biobeads are used in place of (and not in addition to) the dialysis membrane. Additionally, the paragraph should be reorganized in a chronological order (bead preparation protocol, addition of beads to detergent-lipid-protein mixture, spin to pellet Biobeads and remove supernatant, repeat, final Biobeads addition).

This section is now expanded into multiple steps as suggested.

9.) Page 8 (2.3.1): Why don't frozen vesicles work for biochemical assays?

We found that freeze-thaw cycle sometimes introduced artifacts to the cys-specific reactions. For example the L125C/C247S mutants in freshly prepared phospholipid vesicles should be inaccessible to the MTS-PEG5000, but we found that in the freeze-thawed vesicles a small fraction of the proteins were sometimes modified.

10.) Page 8 (2.3.3): Is there a more general method for checking the orientation and incorporation of the protein in the bilayer that isn't specific to L125C? Does the endogenous Cys (C247) react with thiolation reagents when inserted into vesicles? In solution, C247 becomes ~ 30% labeled when reacted with thiol-reactive reagents. How accessible is L125C in solution (i.e. how well does the cysteine react without the protection of the lipid bilayer)? What are the conditions for the thiol reaction? How is the reactivity of MTS-PEG5000 with the L125C mutant assayed (a shift in the molecular weight in an SDS-PAGE gel)? A simpler assay would be using Ellman's reagent (colormetric detection), or the more sensitive fluorescence-based thiol accessibility reagent (Measure-it Thiol Assay from Invitrogen).

Checking the sidedness relies on the knowledge about which sites are on a specific side. There is no general way. For KvAP, the pore-blocking toxin (e.g. CTX), the voltage sensor toxin (VsTX1, 2, or 3), and the Fv-used in our paper only bind to the channels from the extracellular side. We mutated C247 to avoid the reaction. In native wt KvAP, part (~50-70%) of the KvAP protein was cross-linked at this site. In detergents,

L125C becomes accessible only at the resting state. In PE/PG membranes (vesicles), it is not accessible. In DOTAP, it is quite accessible (50% due to the random orientation).

Details for the thiol reaction are included now in section 2.3.3. As explained, MTS-PEG5000 modification shits the band by 5 kDa. Our purpose of L125C modification is for quality control, that is, what fraction of the protein is properly folded and in the right conformation. The assay suggested by the reviewer would give us a good estimate of the total protein or evaluate the reactivity of the L125C. Instead we found that inclusion of some detergents in the reaction made almost all L125C reactive to the reagent.

11.) Page 9 (3.1.4): How long should the pre-painted decane film be dried? It should be explicitly stated that getting decane-lipid inside the hole should be avoided.

A couple of minutes is enough. The statement suggested by the reviewer is now added.

12.) Page 9 (3.1.5): Add in a clause about filling the cis and trans sides with cis and trans buffer. It should also be mentioned why you're creating an osmotic gradient across the bilayer.

Done as suggested.

13.) Page 9 (3.1.7): Define what you mean by the membrane becoming relatively stable (capacitive current stable for > 1 minute?). PE/PG bilayers form particularly unstable bilayers, especially compared to DPhPC. Are there any tricks to forming stable bilayers?

We used the capacitance measurement. In our hand the PE/PG membranes were found to be relatively stable, even though they were less stable than DPhPC.

14.) Page 11: (3.3.1): Refer to Box 2 for the purification protocol.

Done.

15.) Page 12: In the vesicle floatation section, the authors mention that the unilamellar vesicles are concentrated at the top and multilamellar vesicles are concentrated in a whitesh band toward the bottom. Highly concentrated unilamellar vesicles also look cloudy and whiteish; what's the difference?

The unilamellar vesicles were generally dull white, and the multilamellar vesicles were found to form dense-particulates.

16.) Figure 1: How much KvAP was loaded into each lane?

Now specified in the figure legends.

17.) Figure 2b: A positive control should be included (purified KvAP). How much KvAP/ vesicles were loaded onto the gel? The figure would also be clearer if the "top" and "bottom" labels were moved adjacent to the lane labels.

For this particular gel we did not include the positive control as the protein used were biochemically pure.

The amount of protein is now specified in figure legend. The labels were moved as suggested.

18.) Figure 4b: What do the colors represent? Presumably, the black trace is the channels without phage addition, but what's the difference between the yellow and red? Which one represents 1010 phages added?

Now specified in the figure legend.

19.) Figure 5a: The "blue" box seems to be black.  
Changed in the figure legend..

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Hopefully you will find the revised version now suitable for publication. I look forward to hearing from you soon.

Sincerely,

Qiu-Xing Jiang