Journal of Visualized Experiments

Reconstitution of a Kv channel into lipid membranes for structural and functional studies

--Manuscript Draft--

Manuscript Number:	JoVE50436R1
Full Title:	Reconstitution of a Kv channel into lipid membranes for structural and functional studies
Article Type:	Methods Article - JoVE Produced Video
Corresponding Author:	Qiu-Xing Jiang, Ph.D. UT Southwestern Medical Center Dallas, TEXAS UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	UT Southwestern Medical Center
Corresponding Author's Secondary Institution:	
First Author:	Sungsoo Lee, PhD
First Author Secondary Information:	
Order of Authors Secondary Information:	
Abstract:	To study the lipid-protein interaction in a reductionistic fashion, it is necessary to incorporate the membrane proteins into membranes of well-defined lipid composition. We are studying the lipid-dependent gating effects in a prototype voltage-gated potassium (Kv) channel, and have worked out detailed procedures to reconstitute the channels into different membrane systems. Our reconstitution procedures take consideration of both detergent-induced fusion of vesicles and the fusion of protein/detergent micelles with the lipid/detergent mixed micelles as well as the importance of reaching an equilibrium distribution of lipids among the protein/detergent/lipid and the detergent/lipid mixed micelles. Our bilayer recordings suggested that the insertion of the channels in the lipid vesicles is relatively random in orientations, and our biochemical fractionation experiments showed that the reconstitution efficiency is so high that no detectable protein aggregates were seen. We have utilized the reconstituted channels to determine the conformational states of the channels in different lipids, record electrical activities of a small number of channels incorporated in planar lipid bilayers, screen for conformation-specific ligands from a phage-displayed peptide library, and support the growth of 2D crystals of the channels in membranes. The reconstitution procedures described here may be adapted for studying other membrane proteins in lipid bilayers, especially for the investigation of the lipid effects on the eukaryotic voltage-gated ion channels.
Corresponding Author E-Mail:	qiu-xing.jiang@utsouthwestern.edu
Order of Authors:	Sungsoo Lee, PhD
	Hui Zheng
	liang shi
	Qiu-Xing Jiang, Ph.D.
Author Comments:	

Reconstitution of a Kv channel into lipid membranes for structural and functional studies

Sungsoo Lee, Hui Zheng, Liang Shi, and Qiu-Xing Jiang

Corresponding author:

Qiu-Xing Jiang

Department of Cell Biology

University of Texas Southwestern Medical center at Dallas

6000 Harry Hines Blvd, Dallas, TX 75390

Email: qiu-xing.jiang@utsouthwestern.edu

Phone: 214-633-1940

Keywords: lipid-protein interaction; channel reconstitution; lipid-dependent gating; voltage-gated ion channel; conformation-specific ligands.

Short Abstract:

Procedures for complete reconstitution of a prototype voltage-gated potassium channel into lipid membranes are described. The reconstituted channels are suitable for biochemical assays, electrical recordings, ligand screening and electron crystallographic studies. These methods may have general applications to the structural and functional studies of other membrane proteins.

Abstract:

To study the lipid-protein interaction in a reductionistic fashion, it is necessary to incorporate the membrane proteins into membranes of well-defined lipid composition. We are studying the lipid-dependent gating effects in a prototype voltage-gated potassium (Kv) channel, and have worked out detailed procedures to reconstitute the channels into different membrane systems. Our reconstitution procedures take consideration of both detergent-induced fusion of vesicles and the fusion of protein/detergent micelles with the lipid/detergent mixed micelles as well as the importance of reaching an equilibrium distribution of lipids among the protein/detergent/lipid and the detergent/lipid mixed micelles. Our data suggested that the insertion of the channels in the lipid vesicles is relatively random in orientations, and the reconstitution efficiency is so high that no detectable protein aggregates were seen in fractionation experiments. We have utilized the reconstituted channels to determine the conformational states of the channels in different lipids, record electrical activities of a small number of channels incorporated in planar lipid bilayers, screen for conformation-specific ligands from a phage-displayed peptide library, and support the growth of 2D crystals of the channels in membranes. The reconstitution procedures described here may be adapted for studying other membrane proteins in lipid bilayers, especially for the investigation of the lipid effects on the eukaryotic voltage-gated ion channels.

Introduction:

Cells exchange materials and information with their environment through the functions of specific membrane proteins (*1*). Membrane proteins in cell membranes function as pumps, channels, receptors, intramembrane enzymes, linkers and structural supporters across membranes. Mutations that affect the membrane proteins have been related to many human diseases. In fact, many membrane proteins have been the primary drug targets because they are important and easily accessible in cell membranes. It is therefore very important to understand the structure and function of various membrane proteins in membranes, and make it possible to devise novel methods to alleviate the detrimental effects from the mutant proteins in human diseases.

Lipids surround all membrane proteins integrated in bilayers (*2, 3*). In eukaryotic membranes, the various different types of lipids are known to be organized into microdomains (*4, 5*). Many membrane proteins were shown to be distributed among these microdomains as well as the bulky fluid phase of membranes (*3, 6*). The mechanism underlying the organization of the microdomains and the delivery of membrane proteins into them and the physiological significance of such distributions are clearly important but remain poorly understood. One major technical difficulty in studying the lipid effects on membrane proteins is the reliable reconstitution of biochemically purified membrane proteins into membranes of well-controlled lipid composition so that almost all reconstituted proteins are functional (*7*). In the past few years, we developed methods to reconstitute the prototype voltage-gated potassium channel from *A. pernix* (KvAP) into various membrane systems for structural and functional studies (*8-10*). The data from others and us together showed that the lipids are likely a determinant in the conformational changes of the voltage-sensing domains of a voltage-gated ion channel and may shape the structures of some of these channels (*11*). In the next, we will provide a detailed description of our methods and will offer critical technical tips that will likely ensure the successful reproduction

of our results as well as the extension of our methods to the studies of other membrane proteins.

Protocol:

1. **Expression and Purification of KvAP channel (Fig. 1)**

1.1. Preparation Work – Day 0

1.1.1. Rinse the glass flasks for the bacterial culture with deionized water (diH_2O) and MilliQ H_2O (MQH_2O) to remove trace of detergent from general dishwashing.

1.1.2. Autoclave 1000 ml LB medium in 2.8 L Erlenmeyer flasks (total two-liter culture as an example here). Low hardness of the water was found to be important for the successful culture of the transformed bacteria.

1.1.3. Autoclave 100 ml LB medium in 500 ml flasks

1.1.4. Transform 60 µl of XL1-Blue competent cells with 200 ng of the pQE60 plasmid containing the gene for KvAP with a thrombin cutting site and a His_6 tag at its C-terminus, plate the bacteria on two LB-agar plates containing 100 µg/ml ampicillin, and incubate them for 14-16 hours in a 37 ºC incubator.

1.2. Expression of KvAP – Day 1

1.2.1. Check the appearance of the bacterial colonies on the plates after overnight incubation. The colonies were usually only about 0.2-0.5 mm in diameter, and there were a lot of them. We do not want the plates that harbor large colonies surrounded by a lot of satellite colonies.

1.2.2. Add 5.0 ml LB medium to each LB-agar plate incubated overnight and scrap off the colonies. Transfer the bacteria suspension into 100 ml LB medium autoclaved in a 500ml flask. Add ampicillin to a final concentration of 100 µg/ml, incubate the small culture for ~1 hour at 37 ºC or until OD600 reaches 0.60.

1.2.3. In the meantime, place two flasks with 1.0 L medium into a 37 ºC shaking incubator to warm up the medium, and prepare 20 ml of $BaCl_2$ (1.0 M; 10 mM final concentration in each 1.0 L culture), 2.0 ml 0.4 M IPTG (isopropyl-thio-β-galactoside), and 2.0 ml of 100 mg/ml ampicillin stock in water.

1.2.4. Once the small culture is ready, add 10 ml of $BaCl_2$, 1.0 ml of ampicillin stock, and 50 ml of small culture from step 1.2.2 to the pre-warmed LB media. OD600 should be around 0.05. Watch for possible precipitation due to high hardness of the water and the counter ions in the LB media. Ba2+ is known to bind to the pore domain of the potassium channel and blocks the ionic current, and thus decreases the toxicity of the high level expression of the channels to the bacteria.

1.2.5. Take OD600 every hour until it reaches 0.70, and every fifteen minutes until it reaches 0.8 to 0.9. In our set-up, it usually takes ~5 hours to reach 0.8.

1.2.6. Add 0.40 mM IPTG to start the induced expression of channel protein, and incubate the culture for another 4.0 hours at 37 ºC with 225 rpm shaking.

1.2.7. Harvest bacteria in 1.0-liter centrifuge bottles by spinning at 4,000 x g, 4.0 ºC for 15 min. Decant the supernatant as much as possible into a waste beaker, add 10 ml 1.0 M Na-Phosphate buffer to precipitate all Ba^{2+}, and then add a small volume of bleach to kill bacteria. Keep the harvested bacteria in the centrifuge bottles buried in ice in a 4.0 ºC cold room overnight.

1.3. Purification of KvAP protein (Day 2 and 3; **Fig. 1B**)

1.3.1. Resuspend the bacteria pellet in 15 ml of IMAC lysis buffer per 1.0 L culture. Add ~1.0 U DNase I, and three protease inhibitors of leupeptin, aprotinin and pepstatin A at 1.0 µg/ml. The total volume for two-liter culture was ~35 ml.

1.3.2. Sonicate the resuspended bacteria in a metal beaker buried in ice for a total 10 minutes of ON-time. The microprobe sonicator was set in a 5 seconds ON / 10

seconds OFF cycle with a 40 % output power in a 4.0 ºC cold room. The output power setting was empirically determined so that most of the bacteria were lysed without much heating in the solution.

1.3.3. Add 0.50 g of n-Decyl-β-D-Maltoside (DM; Sol-Grade from Anatrace) in dry powder to the sonicated cell lysate and incubate the mixture for 3.5 hours at room temperature (RT) with constant horizontal shaking (~100 rpm) in order to extract as much channel proteins as possible. It is important to make sure that the detergent powder is fully dissolved over a period of 30-50 minutes.

1.3.4. After detergent extraction, remove cell debris from the lysate by centrifugation at 20,000 x g for 30 minutes at RT. While waiting for the centrifugation to finish, prepare a His-tag-based LC (low-pressure liquid chromatography) column as detailed in **Box 1**.

1.3.5. Load the supernatant from step 1.3.4 onto the pre-packed IMAC (**i**mmobilized **m**etal ion **a**ffinity **c**hromatography) column at a flow rate of 1-2 ml/min that is driven by a peristaltic pump. Alternatively the extracted His-tagged protein can be incubated with the IMAC resin for batchwise binding.

1.3.6. Wash the IMAC resin by running 5 bed volumes of IMAC wash buffer, and 10 bed volumes of IMAC wash buffer plus 20 mM imidazole. An in-line UV monitor is used to make sure that the washing is clean.

1.3.7. Elute the KvAP protein by applying IMAC elution buffer containing 300 mM imidazole. Most of the bound KvAP is eluted within 6 ml of elution buffer through the column. Add thrombin (1.0 U per 2-3 mg of protein) into the pooled elution fractions and incubate the protein overnight on the bench to cleave the His_6 tag.

1.3.8. On next day, concentrate the thrombin-digested protein solution in a Centricon (MWCO = 30K) down to 600 µl for size-exclusion FPLC (fast protein liquid chromatography). During centrifugation, mix the sample every five minutes to

minimize protein aggregation. As the protein is concentrated, the local concentration at the membrane filter becomes higher, and there sometimes are clear white precipitated that could otherwise clog the filter membrane. Also the high concentration of proteins (~5-10 mg/ml) at the bottom of the concentrator leads to a brownish color and mixing really helps to minimize sample loss.

1.3.9. Run the concentrated KvAP through a Superdex 200 column that is pre-equilibrated with FPLC equilibration buffer. Typically the peak of the tetrameric KvAP channel in DM elutes with a retention volume of 12.3 ml. There is a small trailing tail at around 13.6 ml, which has a small amount of monomeric KvAP. The void of the column we used is at 7.0 ml, and usually the sample gave rise to a small peak at the void, which contains very little KvAP protein. The immidazole comes at the end of the eluation (~24 ml). Pool the fractions containing KvAP tetramers together and concentrate the protein solution down to 0.5 ml. Determine the concentration of the sample using an estimated extinction coefficient (~1.2E-5.0 $\mathbf{M^{-1}}$ $\mathbf{cm^{-1}}$, which was calculated based on the number of aromatic residues) of KvAP at 280 nm [ref (*12*); URL: http://web.expasy.org/protparam/].

At room temperature, the purified KvAP in DM is stable for at least a week without substantial loss of protein. At four degrees, it could last even longer. Never freeze the protein in detergent. It is recommended to store the protein in DM at 4ºC, and the protein in β-OG at room temperature. But we normally do not wait, and proceed to the reconstitution step immediately after the proteins are ready.

1.3.10. Assay the samples in a 12% reducing SDS-PAGE gel.

The samples from each step described above were prepared by mixing the 20 microliters of specimens with the 5x SDS-sample buffer (Table 3 for the buffers) supplemented with 1.0% β-ME. The samples were not boiled. After gels were ready, the

samples usually had been in the SDS-buffer for more than 10 minutes at room temperature. After the gel was run and stained with Coomassie blue, the wild-type KvAP ran as a single band at around 26 kDa (Fig. 1B). The purified sample was biochemically homogeneous and at least 95% pure.

2. Ion channel reconstitution

2.1. Liposome preparation and detergent-induced fusion of vesicles

Prior to the lipid preparation, wash a 14 ml disposable glass test-tube, a screw-capped glass tube, and a 250 µl glass syringe with chloroform. Pour out ~10 ml chloroform into the testtube.

2.1.1. Preparation of Palmitoyl-oleoyl-phosphatidyl-ethanolamine (**POPE**) and palmitoyl-oleoyl-phosphatidyl-glycerol (**POPG**) liposomes

2.1.1.1. Transfer 3.75 mg of POPE and 1.25 mg of POPG (POPE: POPG=3:1 weight ratio) in chloroform into the pre-cleaned screw-capped glass tube and dry the lipids under a continuous stream of argon gas. When no visible chloroform is left, dry the lipid further under room vacuum for one hour.

2.1.1.2. Add 440 µl of low salt-buffer (10 mM HEPES, pH 7.4) or water into the dried lipid and vortex the tube to hydrate the lipids. The lipid suspension looks whitish and turbid.

2.1.1.3. Sonicate the lipid suspension in an ice-cold bath sonicator until the vesicle solution becomes translucent (OD410 < 0.2).

Typically for the 10 mg/ml POPE/POPG solution in water or low salt buffer, we operated the sonicator with 30 seconds ON and 30 seconds OFF (on ice) for a total of 15-20 minutes. Because of the heat generated during sonication, it is advisable to add 1.0 mM DTT in the lipid solution in order to minimize lipid oxidation, and to cool down the water-bath of the sonicator to 10 ºC or below. The final OD410 is lipid-dependent. For certain lipids it may be very difficult to reach such low OD. If that happens, we usually use detergents to completely solubilize the lipids

and allow long-incubation to reach good distribution of lipids around the protein-containing micelles (see next).

A high-capacity bath sonicator is important in order to reach OD410 < 0.2. We have been using a system made by Laboratory Supplies Co., Inc (Model # G112SPIG, Hicksville, NY). The key for the proper sonication of the vesicles is to make sure that the resonant center in the middle of the tube has strong vibration. The vibration varies with the water volume and therefore can be adjusted. Also it is empirically found that increased viscosity of the water by adding a small amount of glycerol can enhance the efficacy of the sonication.

Alternatively we have found that using a microprobe sonicator in contact with the lipid suspension in a plastic or metal tube can produce the same results. The microprobe needs to be cleaned and only the tip is immerse in the lipid solution. Because the lipid particles are broken into small pieces on the surface of the microprobe, it is very important to keep the sample cooled, and have some reducing agents around to prevent the oxidation of the unsaturated lipids.

Under cryo-electron microscopy, the majority of the sonicated unilamellar vesicles are 30-50 nm in diameter (data not shown). The high curvature of the small SUVs is so high that a small perturbation is enough to induce the vibrant fusion of these vesicles into larger ones that are 80-200 nm in diameter (see next).

2.1.1.4. Add 50 µl of 3.0 M KCl and 10 µl of 0.50 M DM to the mixture so that the final lipid suspension has 300 mM KCl and 10 mM DM. Incubate the solution with horizontal rotation for 2 hours at RT to form lipid/detergent mixed micelles. After the incubation, the suspension should become very turbid (**Fig. 2A**), which is due to the detergent-induced fusion of the small unilamellar vesicles.

2.1.1.5. When the protein is concentrated to more than 2.0 mg/ml, add 0.50 mg KvAP protein, 50 µl of 3.0 M KCl, 16 µl of 0.50 M DM stock in water, and 10 mM HEPES, pH 7.4 to the lipid/detergent mixture to make 1 ml of final volume. The final lipid : protein weight

ratio is 10:1 and the final concentration of DM is 18 mM (**Fig. 2A**). Incubate the protein/lipid/detergent mixture in the glass tube with horizontal rotation for another 2 hours.

The selection of the detergent concentration is guided by the detergent-induced vesicle fusion and vesicle solubilization (*13*). When the vesicles are formed by strong sonication, their average diameter is in the range of 30-50 nm under EM. These vesicles have very low scattering at 410 nm. At low concentrations, detergents are first inserted into the vesicles, destabilize the vesicles, and induce the fusion of detergent-saturated vesicles (OD410 goes up; see **Figure 2A**). Vesicle fusion leads to the increase of the OD410 nm. For 10 mg/ml POPE/POPG vesicles, the OD410 peaks at around 20 mM DM. When more detergents are introduced, the vesicles start to break into pieces and the lipids become partitioned into detergent/lipid mixed micelles. This latter process is accompanied by the decrease of OD410 down to a final low level (<0.1).

Because of the active fusion events in the rising phase of the optical density due to the detergent-inducing fusion of small vesicles, it is highly likely that the protein/detergent micelles will be actively fused with the detergent-saturated lipid vesicles. That is the reason behind the selection of 18 mM DM at the time of preparing the protein/lipid/detergent mixture. If the detergent becomes concentrated by more than 4 folds, the amount of DM needs to be adjusted so that the final concentration is still around 18-20 mM. When the protein yield is very low, it is difficult to evaluate how much concentrated detergents are in the sample, we would instead mix the protein with fully solubilized lipids, and use the fully-equilibrated mixture for reconstitution. In our hands, if not enough time was allowed to reach a good equilibrium distribution of the lipids between protein-containing and protein-free micelles, the reconstitution efficiency dropped significantly. We usually test the reconstitution efficiency by two experiments: 1) examine the co-migration of the protein with the vesicle fraction in a sucrose-density gradient ultracentrifugation; 2) extract the proteins out of the reconstituted vesicles,

and fractionate them in a gel-filtration column to find how much protein (KvAP in our case) remains to be tetrameric. Minimum time of incubation of the protein/lipid/detergents is a couple of hours at room temperature or overnight at 4 degrees.

For a new membrane protein that is stable in a different detergent, the first step is to find out the solubilization property of the lipids by such a detergent, similar to what is showed in Fig. 2A. Next, it is advisable to start with the PC extracts, such as the E. coli polar extract PC, the soybean PC extract etc, so that if the specific protein needs certain phospholipids for its function, it may be already included in the extracted lipid mixture.

2.1.2. Preparation of the KvAP in mixture with detergent-solubilized 1,2-dioleoyl-3-trimethylammonium-propane (**DOTAP**) or 1,2-dioleoyl-sn-glycero-3-succinate (**DOGS**) --- an example of complete lipid solubilization for reconstitution

2.1.2.1. The lipid preparation is the same as for POPE/POPG vesicles. The sonication of hydrated lipids into small unilamellar vesicles needs longer time (usually 45-60 minutes) than for POPE/POPG lipids. The DOGS vesicles may slowly fuse with each other and form small oily droplets.

Because of the difficulty in making high quality SUVs out of DOTAP and DOGS reproducibly, we usually solubilize these two lipids completely before mixing with the proteins.

2.1.2.2. To solubilize the DOTAP or DOGS completely, the sonicated vesicles are mixed with 10 mM DM and 40 mM n-octyl-β-D-glucoside (β-OG). The lipid/detergent mixture is incubated overnight (> 15 hours) at room temperature, and the mixture should not have any small particles or droplets. But instead it is fairly clear. Failure to reach complete equilibrium in this step will lead to the formation of a significant fraction of multilamellar vesicles.

2.1.2.3. Mix the KvAP protein with the detergent-solubilized DOTAP or DOGS in a protein:lipid ratio that is less than or equal to 1:10. The protein/detergent/lipid mixture is allowed to incubate at room temperature for 2-3 hours with end-over-end rotation.

2.2. Removal of the detergents to form proteoliposomes

2.2.1. Dialysis –-- slow removal of the detergents, such as DM and β-OG, that have relatively high critical micelle concentrations (CMC ≥ 1.0 mM)

Prepare 2-Liter dialysis buffer (table 3) for each 1.0 ml mixture.

Cut out a suitable length of dialysis tubing (MWCO = 10K; 0.70 cm wide), and wash it with DI water. It is important to check and make sure that there is no leak in the tubing. For sensitive samples, the tubing could be first treated with a buffer of 10 mM Tris-HCl pH8.0 and 2.0 mM EDTA, and then be cleaned in boiling water for 3-5 minutes. The tubing is then clamped at one end and rinsed with the dialysis buffer.

Load the protein-lipid-detergent mixture into a dialysis tubing. Clamp both ends of the tubing with minimal space left above the solution. Put the loaded tubing into the dialysis buffer, and use a stirring plate so that the tubing rotates slowly in solution. Change the dialysis buffer once every 8 hours. After the first buffer change, the solution should become cloudy if the protein-lipid-detergent mixture was completely clear. If the dialysis is too fast, there may be solid white precipitates at the bottom of the dialysis tubing. It is recommended that at the time of buffer change, the tubing should be rubbed, and inverted multiple times. After five changes of dialysis buffer, the vesicles are usually ready.

We check the residual amount of detergents by shooting the vesicles onto the lipid bilayer. When there is still significant amount of detergents left, the bilayer becomes broken almost instantaneously. It is also possible to measure the residual detergent by using colorimetric method or measuring the surface tension of the vesicle suspension.

2.2.2. Use of polystyrene beads to help remove detergents that have low CMC

In many cases, we have to use low CMC detergents, such as DDM (CMC ~0.17 mM in water). Beads with tiny hydrophobic pores are used to help remove these detergents (*14*).

2.2.2.1 Preparation of the beads. Weight out 0.5 g of dry beads and put them into a 50 ml Corning tube, add 20 ml methanol, sonicate the solution in a bath sonicator for 5 minutes to remove trapped bubbles, spin down the beads at 5,000 rpm for 5 minutes, and then decant most methanol. Repeat the wash with ethanol and MilliQ water. The washed beads are stored in 20% ethanol at 4 ºC, and need to be changed to a detergent-free buffer before use.

2.2.2.2 Estimate the amount of detergents in the protein/lipid/detergent mixture to be treated, and calculate the amount of beads needed to remove the detergents. For DDM and DM, the binding capacity is about 100 mg beads for 10 mg detergents. The wet beads without excess water are weighted out, and added directly to the protein mixture. At least 15-20 minutes is required for the beads to be fully effective and the decrease of detergent concentration reaches a steady level(*14-16*). To remove 8.7 mg of DDM (dodecyl-maltoside) in 1.0 ml solution, equivalent to ~18 mM, a total of 87 mg of polystyrene beads, such as Bio-Beads SM2, is used in five equal fractions. Mix the protein/lipid/detergent mixture with each fraction for 20-30 minutes with constant end-over-end rotation at room temperature, spin down the beads, transfer the supernatant to the next fraction of beads, and repeat till the end.

When the detergent concentration reaches its CMC, we intentionally extend the time period for that to one hour so that the small amount of detergents in the solution will facilitate the fusion of small vesicles into large ones.

2.2.2.3 To remove the trace amount of detergents after the last step, add 35 mg Bio-beads SM2 beads and incubate with the vesicles for 4 hours.

When the procedures for detergent removal described above are to be applied to a new protein, it is generally advisable to start with the protein/lipid ratio (wt/wt) of ~1:10. The lipid/detergent mixture needs to be carefully prepared so that enough detergents are added to

completely solubilize the lipids (Fig. 2A). It is important to incubate the lipid-detergent mixture for more than 2 hours at room temperature (with 1-2 mM DTT) with constant shaking (400 rpm) or end-over-end rotation. When the protein is mixed with lipids, the protein/lipid/detergent mixture should be incubated long enough (overnight if the protein is stable) at either room temperature or in a cold room so that the distribution of detergents and lipids between the protein-containing micelles and protein-free micelles is relatively even. In addition, if fast-removal of detergent by using BioBeads is employed, it is important to find out the detergent-binding capacity of the beads. The slow removal of detergents likely ensures that the proteins will have enough lipids to maintain their integrity and become inserted into relatively sizable vesicles.

2.3. Storage of the proteoliposome and the quality control

2.3.1. Once the vesicles are ready, prepare them into 50 µl aliquots and flash-freeze the aliquots by direct plunging into liquid nitrogen. The frozen vesicles are then stored in a -80 ºC freezer. For biochemical assays, we do not use frozen vesicles because the freeze-thaw cycle sometimes was found to introduce artifacts into our cys-specific reaction. For electrical recordings, we only use vesicles that are stored in -80 ºC for less than 6 months.

2.3.2. Floatation of the vesicles in a density gradient (**Fig. 2B**)

2.3.2.1. Load 50 µl of the vesicle suspension on top of the layers of sucrose gradient made of 0.3 ml each of 10, 35 and 55 % sucrose made in 10 mM HEPES and spin at 200,000 g for 4 hours at 4 ºC. Accelerate and decelerate slowly to avoid the disruption of the interface between layers.

2.3.2.2. Collect 100 µl fractions from the top to bottom and run them on a non-reducing SDS-PAGE (**Fig. 2B**). The KvAP is stained well with the Coomassie blue such that a band of 0.5-1.0 microgram protein is clearly visible. The KvAP protein should be found at the interface between 10 and 35 % sucrose. No heavy aggregates of

proteins are seen at the high density range, indicating that almost all proteins are in the membranes.

2.3.3 Check the proper conformation of the KvAP channel in vesicles.

Our previous data established that a single cysteine mutant (L125C/C247S) KvAP, when properly integrated in bilayers, is fully buried in membranes, and cannot be accessed by cysteine-specific reagents (*8*). The reconstitution procedures were tested with this mutant channel, and checked by a Cys-specific reagent, MTS-PEG5000. The modification at L125C with 1.0 μM MTS reagent at room temperature introduces a 5kDa shift to the KvAP band in a nonreducing SDS-PAGE gel. Successful implementation of our reconstitution procedures should lead to no detectable reaction for the L125C mutant channels in phospholipid membranes, suggesting nearly complete insertion of all channels in bilayers. As a positive control of the reaction, 5.0 mM DM is introduced to render almost all L125C residues in the mutant channels accessible to the MTS reagent.

Alternatively, a pore-binding toxin, such as chrybdotoxin, may be used to count the tetrameric channels. An antibody-based binding assay (see Box 2, where Fv is prepared as a conformation-specific ligand for KvAP) might be used to quantify the available binding sites in the reconstituted vesicles. These binding assays can then be compared with a quantitative assay measuring the total protein in order to figure out what fraction of the reconstituted channels are tetramers and what fraction of the protein has the voltage-sensor paddle (S3/S4 of the voltage sensor) properly folded.

For other proteins to be reconstituted, it is advisable to introduce a quantitative method to evaluate what fraction of the reconstituted proteins is properly folded. Careful functional examination is thus a prerequisite to developing good quality control for successful reconstitution.

3. **Applications of the reconstituted channel-containing vesicles**

3.1. Functional study of the ion channel activities in black lipid membranes.

Preparation of needed materials.

3.1.1. Lipid preparation

3.1.1.1. Clean a glass test-tube, an amber vial with Teflon-surfaced screw cap, and a set of glass syringes with chloroform. Dry the amber vial under a stream of argon gas.

3.1.1.2. Transfer 0.75 mg POPE and 0.25 mg POPG in chloroform into the amber vial and evaporate the chloroform with argon gas.

3.1.1.3. Wash the dried lipids with 0.20 ml pentane, and dry completely to remove residual chloroform. Finally dissolve the lipids in 50 µl decane. The lipids in decane (20 mg/ml) will be used for painting a lipid bilayer across a 150-250 micron hole (Fig. 3B) drilled in the thinned portion at one side of an experimental bilayer cap (**Fig. 3A**).

3.1.2. Solution preparation

3.1.2.1. Intracellular solution (*trans*): 10 mM HEPES/KOH, pH 7.4, 15 mM KCl

3.1.2.2. Extracellular solution (*cis*) : 10 mM HEPES/KOH, pH 7.4, 150 mM KCl

3.1.2.3. Salt bridge: Bend glass capillaries into U-shaped bridges, and fill them with 1.0% molten agar dissolved in extracellular solution.

The osmotic gradient established between the trans and cis solutions was found to be the main driving force for vesicle fusion on the lipid bilayer(*17*). For negatively charged lipids, 15 mM CaCl2 or positively charged poly-Arginine peptides were found to be able to induce fusion events. Fusogenic lipids, such as DOTAP and DOGS,etc., could be introduced into the lipid vesicles so that the chance of fusion events is higher.

Electrical recordings from KvAP channels in lipid bilayers

3.1.3. Pre-paint the round hole (diameter ~0.25 mm) that is drilled at the cylindrical surface of the bilayer cup (**Fig. 3B**). The lipids are transferred with a capillary pestle that is

made in the lab. The round head of the capillary pestle is polished and does not scratch the surface of the cup. The small amount of lipids carried by the capillary pestle is spread around the hole in the bilayer cup, and air-dried.

3.1.4. Wait for 1-2 minutes so that the decane will evaporate completely. Care should be taken to avoid the lipid solution getting into the hole.

3.1.5. Insert the cup into the recording chamber, and put in the salt bridges and connect the electrodes to the two sides of the recording cup (**Fig. 4A**). Add the cis- and trans-solution to the two sides of the hole. An osmotic gradient is established to facilitate the fusion of vesicles into the lipid bilayer.

3.1.6. Adjust the potential offset between two sides of the cup to ~0 (usually < 2 millivolts). Use the capillary pestle to transfer a small amount of lipid mixture in decane, and paint the lipid across the hole until a bilayer membrane forms. The formation of the bilayer is detected by recording the capacitance current during the delivery of a ramp pulse.

3.1.7. Wait till the membrane thins out and becomes relatively stable. We wait till there is no more change in the membrane capacitance for 3-5 minutes.

The general thinking about the planar bilayer formed across a large hole is that the very middle portion of the membrane is close to be ~4.0 nm thick as a regular bilayer, and the membrane become thicker when it gets close to the edge of the hole, where there is an annulus around and most of the decane solvent is(*18, 19*). It is inevitable that there is residual decane left in the central bilayer portion of the membrane. Past estimation of the volume ratio of n-decane versus PC was 0.35-0.45 after the thinning of the bilayer (*18, 20-22*). EM examination of the thinned bilayer showed that there were lenses of decane sandwiched between the two leaflets of a bilayer(*22*). These data suggested that the membrane proteins inserted in the lipid bilayer membranes coexist with tiny islands (up to 50 nm in diameter) of decane. The residual decane in the bilayer also decreases the electrical capacity constant to 0.4-0.5 $\mu F/cm^2$, which

can be used to obtain a rough estimate of the size of the thinned bilayer based on the measured capacitance. A bilayer of 100 pF capacitance comes from a circular bilayer membrane of ~180 μm in diameter.

For KvAP, the residual decane did not impair its voltage-dependent gating, even though the channel function has not been carefully examined in a solvent-free bilayer. The same appeared to be true for the NaChBac channels and the Kv1.2 channels inserted into the BLMs(*23*). It is still unclear whether the significant left-shift of the G-V curve measured from the Kv1.2 channels in the decane-lipid bilayer relative to channels in oocytes has anything to do with the residual decane(*23*). Depending on the lipids used in forming the bilayers, the amount of the residual decane in the bilayer may vary. For example it was reported that the formation of planar lipid membranes of MGDG (mono-galactosyl-diacylglycerol) requires the decane, possibly due to the crevices between the conical MGDG being filled with decane molecules. Whether the residual decane has effects on the proteins to be studied is purely empirical, and the experimental test is needed to tell whether the effect is significant or not.

3.1.8. As soon as the membrane becomes stable, shoot 0.5-1.0 μl channel-containing vesicles by positioning the fine end of a P2-pipette tip right above the hole. The vesicles fall down across the hole to the bottom of the cup.

During this process, multiple vesicles become attached to the membrane across the hole. Given enough time, some are fused into the bilayer portion so that a small number of KvAP channels are properly inserted into the planar bilayer in the center part of the membrane. Both osmotic gradient and electrostatic interaction are important in the fusion of the vesicles into the lipid bilayers (*17, 24*).

3.1.9. To test the KvAP channels in the bilayer, a short pulse of 80 mV is delivered from the holding potential of -80 mV. Due to the fast inactivation and slow recovery from inactivation, a long interval (~120 seconds for channels in PE/PG membranes) is given

between two pulses. A typical current recording from the channels in a POPE/POPG membrane is showed in Fig. **3C**. If the current is small, shoot more vesicles. Once the current looks good in size, balance the ion concentrations in the solutions on both sides of the membrane. The channels are ready for electrophysiological experiments.

3.2. Screening for conformation-specific ligands against channels in vesicles

3.2.1. Introduction of the phage-displayed library.

The phage-displayed 20-mer peptide library is displayed to the N-terminus of the five-copies of pIII proteins at one end of the filamentous bacteriophage *fd-tet* (*25*). The library presents approx. 1 x 10^8 different types of random 20-mer peptide sequences, and was kindly provided to us by Dr. Kathlynn Brown's laboratory at UT Southwestern Medical Center (*26*). The phage infection into the *E. coli* K91 makes the bacteria resistant to 12 µg/ml tetracycline.

A detailed procedure for the bacterial culture, the amplification and titering of the phages and the sequencing of the phage colonies has been described by McGuire et al. (*26*). We will give a short description of the basic operations in the next section. The readers can find more details in the McGuire paper. We will instead focus on the isolation of specific clones that bind tightly to the ion channels reconstituted in vesicles (**Fig. 4A**).

The basic idea behind our screen is that the phages bound to the reconstituted channels specifically can be pulled down with the vesicles. The bound phage can be amplified and tested against the channels in lipid membranes. Our study of the conformational changes of KvAP voltage sensors in different lipid membranes(*8*) showed that it is possible to keep the voltage sensors in either "activated" or "resting" states by switching the lipids from regular phospholipids to those that do not contain phosphates in the headgroup regions (DOTAP and DOGS in our experiments). These two lipid-determined conformations are utilized in our screening of conformation-specific ligands. The phage library will first be saturated by the channels in the

phospholipid vesicles (negative selection) before being selected for tight binders to the channels in DOTAP and DOGS membranes (positive selection).

3.2.2. Basic procedures behind the phage selection

Growth of K91 bacteria in LB medium: The doubling time of the bacteria is about 20 minutes at 37 ºC with 200 rpm shaking.

Amplification of the library: Mix the phage solution with the bacteria K91 at OD0.4. After 15 minutes incubation at 37 ºC, the mixtures are spread evenly onto 10x10 cm2 agar plates containing 12 μg/ml tetracycline, The use of large plates prevents the dominance of the culture by bacteria that have higher growth-rate. Once the diversity of the library is smaller, 10-cm petri dishes are used for selection and small-scale amplification.

Large-scale amplification of individual clones: Inoculate a small aliquot of phages (~100 millions) into 250 ml LB plus 5 mM MgSO4 and 12 μg/ml tetracycline, Grow overnight at 37 ºC. Collect cells by 6000 rpm spin for 10 min. Collect the supernatant and add into it 0.2 vol/vol the precipitation buffer (2.5 M NaCl, 20% PEG8000). After incubation on ice for 1.0 hour, centrifuge the solution at 17,000 xg for 30 minutes to pellet all phages. Remove all supernatant, add 1.0 ml PBS, incubate on ice for 30 minutes, gently resuspend the pellet (no vortexing). Transfer the suspension to a fresh tube, and centrifuge at 14,000 rpm for 2 minutes. Transfer the supernatant to another tube, and incubate in 65 ºC water bath for 15 minutes to kill all bacteria. Centrifuge the tube for 2 minutes at 13,000 rpm. Discard the pellet, aliquot and store the phage solution at -80 ºC.

3.2.3. Panning of the phages against KvAP channels in lipid vesicles.

3.2.3.1 The peptide-displaying phages need to be amplified and titered before our experiments so that the number of phages per unit volume is known. KvAP was reconstituted

into POPE/POPG (3:1) plus 0.50% Biotin-DOPE vesicles as negative control, and into DOGS : biotin-DOPE (199:1; same was done for DOTAP vesicles) is used as the selection target. The final concentration of KvAP in vesicles is 0.50 mg/ml, and the lipids are 5.0 mg/ml. The panning buffer contained 500 mM KCl, 100 mM HEPES/KOH pH 7.4, and 0.10 mg/ml BSA.

For the first run, ~10^{10} phages (100 copies for each type) were diluted to 0.050 ml LB medium. For the subsequent panning steps, the starting phage was adjusted to be within 10^6 to 10^8.

3.2.3.2 Negative selection:

Incubate the diluted phages in 50 µl LB with 100 µl NeutrAvidin agarose beads (capable of binding 1-2 mg biotinylated BSA per mL of resin; Pierce) in 1.0 ml panning buffer. After 10 min incubation, separate the beads by spinning them at 100 x g spin for 1.0 minute. Repeat this step twice to remove any phages that bind directly to the beads.

Mix the left-over phages from the previous step with 50 µl empty vesicles (POPE/POPG plus 0.5% biotin-DOPE) that contain no proteins. Add 100 µl NeutrAvidin Beads that have been washed with the panning buffer to the phage-vesicle mixture. After rotating the mixture at room temperature for 5 minutes, remove the beads by 100 x g spin for 30 seconds. This step is repeated for empty vesicles of DOGS: biotin-DOPE (199:1) as well as for KvAP channels in POPE/POPG vesicles (with 0.5% biotin-DOPE). The supernatant after these treatments contains the fully-cleared phages. After the first two screen cycles, the preclearance could be performed only against KvAP in POPE/POPG vesicles.

3.2.3.4 Positive selection

Incubate the fully-cleared phages with KvAP channels in DOGS : biotin-DOPE (199:1) vesicles (the same for the DOTAP vesicles) in the presence of 100 µl NeutrAvidin beads at room temperature for 10 minutes. Bring the volume of the mixture to 15 ml using the panning buffer before the centrifugation at 100 x g for 10 minutes. Collect the beads, and wash them three times with 45 ml panning buffer.

3.2.3.5 Resuspend the beads in 500 µl LB medium containing 5.0 µg/ml biotin, and incubate the mixture at room temperature for two hours in order to release some of the bound phages from NeutrAvidin, which has lower affinity than the native lectins. Separate the beads by 100 x g spin for one minute. Collect the supernatant and the beads into two different tubes for titering.

3.2.3.6 To amplify the selected phages, mix 200 µl of the supernatant or the resuspended beads in the last step with 500 µl K91 bacterial culture (O.D.600 ~ 0.4-0.6, cultured ~ 4 hours before it is used). After incubation at 37ºC for 15 minutes, plate 50 µl of each on tetracycline plates for overnight culture.

During the first two screen cycles (**Fig. 4A**), collect all 500 µl supernatant and the beads from the last step, mix them with the 5 ml bacteria culture, and plate them in 9.5 inch square plates in order to recover and amplify all phage colonies. This step is important for recovering those phages that make the bacteria grow slower than others.

3.2.3.7 The colonies from the plates are amplified and titered before the next cycle of panning and selection (see McGuire *et al.*, (*26*)).

3.2.3.8 Examine the activities of the positively selected phages on channels.

After 10-12 cycles of selection, test the total amplified phages on the KvAP channels in lipid bilayers made of POPE/POPG. If there are a significant fraction of positive clones, the selected phages at 100-500 nM should block a significant fraction of channels in the bilayer (**Fig. 4B**) as we were selecting against channels stabilized in the resting state. The positive data suggest that there are clones that will show strong binding to the channels in the resting state. After 16 cycles of selection, pick 50 positive colonies for single-colony sequencing. The identified dominating phage clones are selected from comparing the sequences, and are amplified and tested against channels in bilayers. Once the positive clones are confirmed, synthesize the peptides carried by the strong positive clones and test them on the channels as well as to confirm their conformation-specific activities.

3.3. Crystallization of KvAP channels in membranes for structure determination (*27-29*)

3.3.1. To stabilize the conformation of the channel, a conformation-specific binder of the channel, 33H1 Fv protein, is used to form the KvAP/Fv complex. The preparation of the Fv protein is detailed in box 2. Purify the complex in a Superdex 200 column. The complex elutes at around 11.4 ml in our system.

3.3.2. For the initial screen, mix the protein with DMPC or POPC that is completely solubilized in DM or β-OG. The protein/lipid/detergent mixture is mixed for more than 15 hours in order to reach a thermodynamic equilibrium in the distribution of the three components among the mixed micelles. Usually we first test three different lipid/protein ratios (LPR=0.5, 1.5, 2.5) and three different pH levels (6.0, 7.0, and 8.0). The dialysis buffer contains 20 mM K-phosphate buffer, 100 mM KCl, and 3.0 mM NaN_3. The dialysis buffer is changed 2-3 days. A small aliquot of the crystals are taken out every 2-3 days to examine the formation of vesicles and the possible appearance of lattice.

A tabloid list of LPR vs. pH is used to determine the behavior the protein in the two different types of lipids. We want to obtain relatively large-sized (more than 150 nm) vesicles or membrane sheets when the dialysis samples are examined by negative-stain EM. A small regular pattern in membranes suggests a hit and will be used to guide further optimization.

Negative-stain EM: Copper grids coated with carbon films (~3-5 nm thick) are glow-discharged in air to make the carbon surface hydrophilic. A small volume (2-3 microliters) of the crystals suspension is loaded onto the carbon surface, and incubated for 0.5-1.0 minutes. Blot the grids from the side with a torn edge of a piece of Whatman #4 filter paper. Flip the grid and incubate it for ~15 seconds on the top of a drop of 150 microliter staining solution(2.0% PTA/KOH, pH 8.0 in water plus 0.5% trehalose; or 6.0% ammonium molybdate /KOH, pH6.3-6.5, in water plus 0.5-1.0% trehalose). Blot as much as possible the solution from the grid

surface, and allow the grid dry before inserting it into a TEM for examination. Images of vesicles are taken under low-dose condition to avoid the damage of any crystalline packing by the electrons.

3.3.3. The screen is then expanded to examine smaller steps of LPR in order to reach a correct LPR. If the proteins are suitable for crystallization, a small lattice of proteins in membranes may become visible. Around these initial conditions, further optimize the crystallization by varying the salt types and concentrations, the temperature, the speed and methods of detergent removal, divalent cations, detergents used to prepare the proteins, precipitants, lipids composition etc.

The optimized 2D crystals of KvAP∆36/Fv complex grow into large single-layer sheets that range from a few microns to 20-30 microns (**Fig. 5A**). Under cryoEM conditions, the crystals show clear square lattice with obvious four-fold symmetry as expected from the four-fold symmetric channels (**Fig. 5B&C**).

Representative Results:

The general flow of the experiments for purifying the KvAP channel into biochemical homogeneity is described in **Fig. 1A**. Typical samples during the expression and purification of the protein is showed in the SDS-PAGE gel in **Fig. 1B**. The protein after the IMAC purification is relatively pure. The yield of the KvAP channel is about 1.0 mg/Liter culture.

Solubilization of lipid vesicles with detergents needs to be worked out for each pair of lipid vs. detergent. The solubilization of small unilamellar vesicles of POPE/POPE by DM is presented in **Fig. 2A**, the results from the vesicles floatation are usually fairly straightforward. Typical results in the SDS-PAGE assay of the fractions from the gradients are showed in **Fig. 2B**. In the sucrose density gradient, the channel-containing POPE/POPG vesicles usually are

concentrated at the interface between the 10% layer and the 35% layer. The KvAP-containing DOTAP or DOGS vesicles are lighter and usually at the interface tween 5% and 10% (slightly penetrated into the 10% layer). If there are a significant fraction of multilamellar vesicles, they usually are whitish and concentrated below the 10-35% interface. We found that this usually happens when the protein-detergent-lipid mixture was not incubated long enough to reach good distribution of the lipids.

Electrical recordings from KvAP channels incorporated in planar lipid bilayers are shown in **Fig. 3C**. The typical current trace shows that at -80 mV, the channels are quiet. Switching to +80 mV leads to a quick capacitance peak (the sharp one at the time of voltage switching). A slow rising phase of the current suggests that the channels become active and are able to conduct outward potassium current. After the peak of the rising phase, the current starts to decrease, a step named inactivation. The inactivation takes a few hundred milliseconds to complete. Once the voltage is switched back to -80 mV, there is a returning phase after the downward capacitance peak, which reflects the closing of the open channels and is called the deactivation (**Fig. 3C**).

In the middle of the phage screen, we tested the activity of the amplified phages on the KvAP channels in the bilayers of POPE/POPG. After 12 selection cycles, the amplified phages inhibited the channel activities (**Fig. 4B**, selected phage). But the starting phage-displayed library did not show any effect on the channels (**Fig. 4B**, control phage). Even though the activities of the selected phages were not very high because only a low concentration of the positive phages were around, the clear, reproducible effects suggested that there are active, positive clones that exhibit high-binding affinity, should be selectively enriched during the remaining screening cycles and once enriched, are likely to have strong inhibitory activities on channels in membranes.

In the early stage of screening the 2D crystals, we saw small lattices in many small vesicles. With optimization, some large sheets showed up with a lot of small vesicles around (**Fig. 5A**). The cryoEM images of these crystals always showed a lot of local defects (**Fig. 5B**), suggesting that further optimization is required to obtain better crystals. Once the crystals were well optimized, they exhibited sharp edges, and appeared as single sheets. At high magnification, the individual units are clearly much better ordered (**Fig. 5C**).

Tables and Figures:

Tables:

Table 1: List of reagents and materials

Table 2: Equipment needed

Table 3: Buffer name and contents

Figure legends:

Figure 1: Preparation of KvAP for reconstitution

A. General work flow of protein expression, purification and reconstitution.

B. Biochemical purification of the protein. Induced culture of *E. coli* XL1-blue expressing KvAP was processed and KvAP purified. Twenty microliters of cell cultures before (1) or after (2) induction, the detergent extract (3), flow-through from IMAC chromatography (4), two washing steps (5, 6), and 5.0 µg of total protein after the 300 mM imidazole elution (7) and 5.0 µg of the KvAP tetramer out of the size exclusion FPLC (8) were subjected to non-reducing12% SDS-PAGE and Coomassie blue staining.

Figure 2: Reconstitution of KvAP in POPE/POPG vesicles

A. Vesicle fusion and solubilization as a function of detergent concentrations. Absorbance at 410 nm was used to monitor the formation of scattering of vesicles (baseline subtracted to no detergent fraction). Lipids (POPE/POPG 3:1) were 10 mg/ml. The small unilamellar vesicles after strong sonication, is monodispersed and has weak scattering due to their sizes of 30-50 nm in diameter. When the detergents were introduced, they distributed between solution phase and the lipid membrane phase. Due to the strong curvature in the small unilamellar vesicles, the introduced detergents trigger vesicle fusion and the release of the curvature (thus the low surface potential energy). The fused vesicles are larger in size and show stronger scattering at 410 nm. The rising phase of the peak (gray-colored area) therefore reflects the detergent-induced fusion, a good regime for protein micelle fusion to the vesicles. The concentration of the detergent (DM as an example here) right before the absorption peak was indeed chosen for our reconstitution process.

B. Fifty microliter of reconstituted KvAP vesicles (KvAP 0.5 mg/ml) was separated by sucrose density gradient. Samples of 100 µl fractions from top to bottom (1~9) of sucrose gradient were assayed in a 12% reducing SDS-PAGE. The gel was Coomassie blue stained. Lane 3 contained ~2 µg KvAP.

Figure 3: Electrical activity of KvAP channel in reconstituted bilayers

A. Recording set-up inside a Faraday cage, 1; two electrodes, 2; *trans* side, 3; *cis* side, 4; salt bridge.

B. Magnified section of the thinned portion at one side of the bilayer cup showing a hole (5), which is used for making membranes.

C. Electrical activity of KvAP channels that were fused into the black lipid membrane was recorded. While being held at -80 mV, the membrane potential was pulsed to 80 mV for 150 ms,

and then changed back to holding potential. The ionic current was recorded in the voltage-clamp mode using an Axopatch 200B amplifier.

Figure 4: Screening for conformation-specific ligands from a phage-displayed library

A. Scheme behind the screening against ion channels reconstituted in vesicles. The vesicles are doped with biotin-DOPE, and they can be pulled out of solution by NeutrAvidin beads. The conformation of the KvAP channel is controlled by specific lipids. Negative selections against beads, empty vesicles and vesicles with channels in a different conformation are done before the phages are incubated with the channels in the target conformational state (in DOTAP or DOGS as an example here). The selected phages can be amplified and tested against channels in bilayers.

B. Testing of the selected phages in the middle of the screen.

Electrical activity of KvAP in the bilayer at different time points after the addition of the selected phages: black trace - before the addition; yellow trace - 2 minutes after; red trace - 10 minutes after the addition.

Top: The starting phage library (total ~10^{10} phages added to the bilayer) was tested on channels in bilayer, and was found to have no detectable activity because each phage clone has only about 100 copies.

Bottom: After 12 selection cycles, the phages were still a mixture of different clones. Adding about 10^{10} phages to the channels in the bilayer lead to the clear inhibition of channel activity, suggesting that there are positive clones that should have high affinity.

Figure 5: Two-dimensional crystallization of KvAP in membranes

A. Image of negatively stained single layer 2D crystals in the middle of crystal optimization. The crystals were stained with 6.0% ammonium molybdate, pH 6.4 plus 0.50% trehalose. Due to the

sugar, they sometimes piled up with each other. The black square box designates an area that gives rise to the diffraction pattern shown on the right side with diffraction spots going to ~20 Å.

B. CryoEM image of a 2D crystal from a sample similar to the one used in (**A**). The specimen was mixed with 3% trehalose and frozen by direct plunging, and imaged under a cryoEM. The image was obtained at 50,000 x. It was clear that there were local defects in the crystal packing.

C. CryoEM image of a further-optimized crystal. The specimen was embedded in 0.75% tannin and 10% trehalose and the image was taken at 50,000 X. The straight lines and the tight packing suggested that the channels were well ordered in this type of crystals. The two black arrows mark the square lattice.

Boxes:

Box 1. Preparation of IMAC column

1. Resuspend the IMAC Resin thoroughly.
2. Immediately transfer the required amount of resin in suspension to a 50ml tube. We use 2 ml bed volume of resin for the purification of KvAP from 2 L culture.
3. Centrifuge the tube at 700 x g for two minutes to pellet down the resin.
4. Remove and discard the supernatant.
5. Add 10 bed volumes of MQH2O and mix briefly to rinse the resin.
6. Recentrifuge at 700 x g for 2 min to pellet down the resin. Discard the supernatant.
7. Add 10 bed volumes of MQH2O and pour into empty column.
8. Wait until resin settles down, and close the column with a flow adaptor for low-pressure liquid chromatography.
9. Run 5 bed volumes of MQH2O and 5 bed volumes of equilibration buffer through the column.

Box 2. Purification of Fv

Fv Transformation – Day 1

1. Transform 100 ng of the plasmid containing Fv-His_6 to 60 µl of JM83 competent cells, plate the bacteria on four LB-agar plates containing 100 µg/ml ampicillin, and incubate overnight in a 37ºC incubator.
2. Prepare 6 X 1L LB for bacterial culture.

Expression of Fv – Day 2

3. Scrap all the colonies from the plates into LB medium. Add this suspension of bacteria to 6 X 1L LB in the presence of ampicillin (100 mg/L) and incubate until OD600 reaches 0.5.
4. When OD reaches 0.5, decrease the temperature to 20°C and RPM to 115. When the temperature drop to ~ 20°C, add anhydrotetracycline (100ul of 1mg/ml in DMF to 1L) for initiating the induction of protein expression and incubate another 5 hours at 20°C with normal shaking.
5. Harvest bacteria in 1 L centrifuge bottle at 4,000 x g for 15 min. Pour out the supernatant as much as possible. Keep the harvested bacteria on ice in a 4°C cold room overnight.

Releasing the Fv molecules from the bacteria – Day 3

6. Resuspend bacteria in 150 ml of Fv-releasing buffer (50 mM Tris pH 8.0, 20 % sucrose, 1.0 mM EDTA).
7. While stirring with a magnetic stirring bar, add lysozyme to 0.1 mg/ml and keep the bacteria on ice for 30 minutes. Afterwards, add $MgCl_2$ to 2.0 mM and keep on ice for another 10-15 minutes.

8. Centrifuge the treated bacteria at 20,000 x g for 30 minutes at 4°C. the bacterial spheroblasts should all go down to the pellet, and most of the Fv molecules are released in the supernatant.
9. Dialyze the supernatant against 4.0 L of wash buffer (20 mM Tris pH 8.0, 100 mM NaCl) in cold room for at least 4 hours. At the end of day, change to fresh dialysis buffer and dialyze overnight. Due to the sucrose in the solution, the volume of the dialysate will increase by about 50%. Excess of dialysis tubing should be used.

IMAC purification and FPLC – Day 4

10. Equilibrate 10 ml bed volume of IMAC purification resin (see Box 1) with the dialysis buffer in 50 ml tube.
11. Transfer the dialysate from the tubing, add $MgCl_2$ to 10 mM and increase the volume to 200 ml. Mix with the pre-equilibrated resin and incubate for 30 minutes at 4°C.
12. Pack the empty column with the incubated resin, and wash the resin with 5 bed volumes of washing buffer, followed by 5 bed volumes of washing buffer with 10 mM imidazole and 30 mM imidazole each.
13. Elute the bound protein with 20 ml of washing buffer plus 300 mM imidazole.
14. Run the concentrated Fv through Superdex 200 column pre-equilibrated with washing buffer. Pool the fractions containing Fv (typical peaks shows up at 12.3 ml retention volume) together and concentrate it. Determine the concentration of the sample using the extinction coefficient of Fv at 280 nm.

Discussion:

The reconstitution of the KvAP channels into different membranes has been used in several studies (*8-10*). Following the idea of ensuring the distribution of lipids between

detergent/lipid mixed micelles and the protein/detergent/lipid mixed micelles, we are able to reach nearly complete reconstitution of the KvAP into membranes made of very different lipids. Each tetrameric KvAP channel needs ~100 lipid molecules to fully cover its transmembrane domain. The essential requirement is to allow enough lipid molecules to fuse into the protein/lipid/detergent micelles before the detergents are removed. Our standard conditions (0.5 mg protein and 5.0 mg lipids) ensure that there are on average ~1000 lipid molecules per protein molecule. Our floatation experiment and biochemical experiments confirmed that the reconstitution is almost complete. Electrical recordings from channels inserted into black lipid membranes, the screening of the reconstituted channels against a phage-displayed peptide library, and the growth of 2D crystals of the channels in membranes all demonstrate the successful applications of membrane reconstitution for various purposes.

The lipid-dependent conformational changes of the KvAP channels and the screening against a phage-displayed peptide library showcase a new avenue to screen for channel blockers or channel openers by biochemical methods instead of relying on electrophysiology to keep the channels in specific conformations (*8*). The success in our screen for conformation-specific binders suggests that the same strategy can be applied to find specific binders for the activated conformation. It is foreseeable that the reconstituted channels in vesicles can be used against single chain Fv libraries, Fab libraries, etc. Likewise, other membrane proteins can run through these operations and find their tight binders that may be useful for various purposes. We believe that this new method will see more general applications in the future.

Reconstituted membrane systems will allow the elucidation of the chemical details behind the lipid effects on membrane proteins (*11*). Lipid-protein interaction has been known to be important for many membrane proteins, and has been subjected to multiple studies in the past (*3*). In the cell-based studies, manipulations can be implemented to change the specific components in the membranes and then the functional changes in the membrane proteins are

associated with the structural and compositional changes in the membranes. Such connections are indirect and might result from multiple factors in the cell membranes that are not well characterized. In a reconstituted homogeneous membrane, it is more definitive in making connections between the structural and functional changes of the membrane proteins and the changes in lipid composition and membrane properties. Ultimately, to understand the chemical principles behind the lipid-protein interaction, we need to delineate the distribution of lipids around the transmembrane domain, and to understand the dynamic changes of these lipids right next to the proteins. A reconstituted system appears to be a reliable way toward such an understanding.

Reconstitution of membrane proteins requires the controlled removal of detergents from the protein/lipid/detergent mixed micelles, and the fusion of the mixed micelles into large ones that eventually turn into vesicles (*30*). Three different methods are being used for removing detergents, dialysis, beads, and cyclodextrin (*14, 31, 32*). But it remains difficult to achieve a well-controlled, gradual removal of detergents from a small volume (*33, 34*). An ideal method for detergent removal would take the detergents out of the aqueous phase evenly across the whole volume in a controllable pace, and should not exert strong interference on the reconstitution of bilayer membranes. Such a method might be able to change the speed and efficacy of reconstitution, and will likely enable the reconstitution in a small volume. A combination of slow-dilution and any of three conventional methods for detergent removal may approach this goal. Slow-dilution by introducing small amount of water into the protein/detergent/lipid mixture is a controllable way to evenly decrease the detergent concentration down to its CMC. The detergent removal afterwards is less critical for the vesicle formation, although still important for the fusion of small vesicles into large ones. Other ways to achieve controlled detergent removal still need to be conceived and developed.

Our reconstitution procedure takes consideration of lipid distribution among mixed micelles and the detergent-induced fusion of vesicles as well as the mixed micelles. Its success paves the way leading to a broader spectrum of applications of the reconstituted vesicles, much more than the three directions we presented. The adaptation of our procedure to other membrane proteins should not encounter major technical limit. Even though many membrane proteins have been reconstituted one way or the other, it has been difficult to achieve near complete reconstitution and to evaluate the functionality of the proteins from multiple different perspectives. Our efforts in the KvAP reconstitution suggest that our methods may allow full reconstitution and will be suitable for these purposes.

Disclosures:

The authors have nothing to disclose.

Acknowledgments:

The studies on KvAP in the Jiang lab have obtained significant help from Dr. Roderick MacKinnon's laboratory at the Rockefeller University. Special thanks go to Dr. Kathlynn Brown and Michael McQuire for their advice and help on our phage-screen experiments. This work was supported by grants from NIH (GM088745 and GM093271 to Q-XJ) and AHA (12IRG9400019 to Q-XJ).

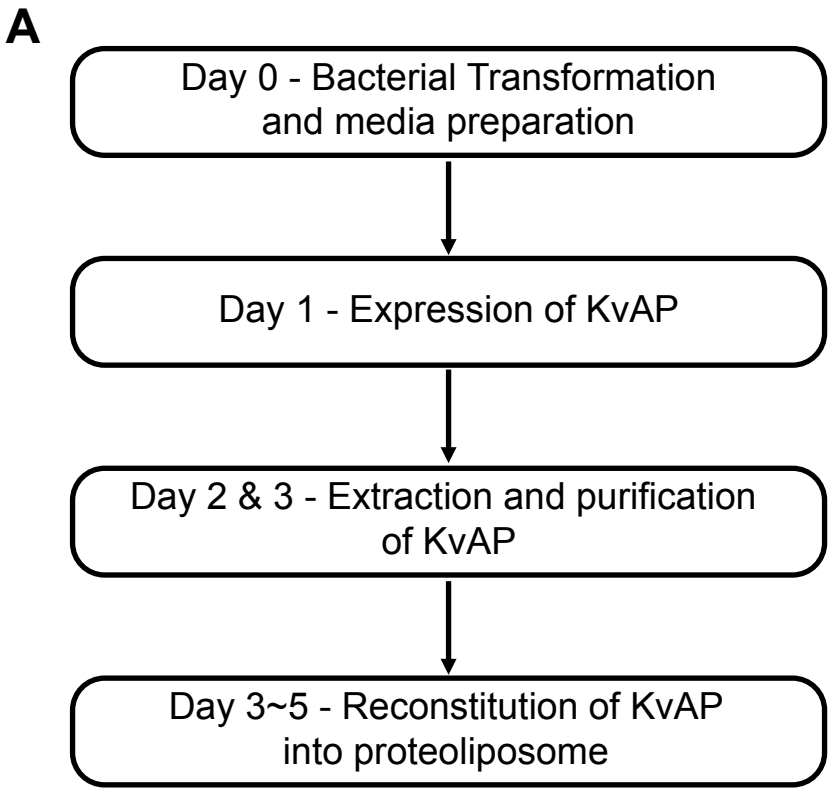
References:

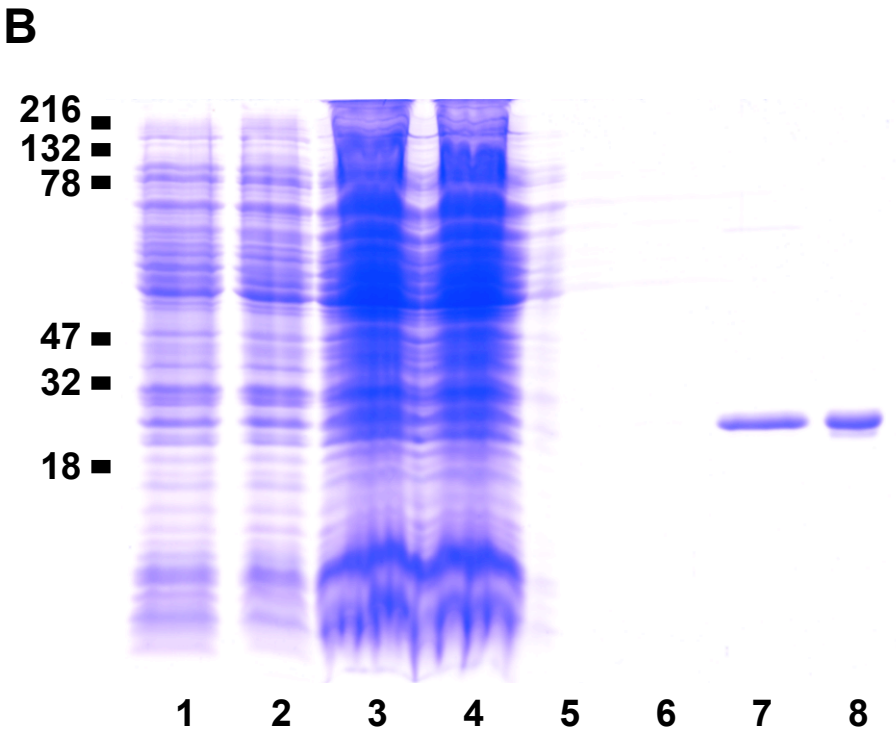
1. B. Alberts *et al.*, *Molecular Biology of the Cell*. (Galand Science, New York, ed. 5th, 2007).

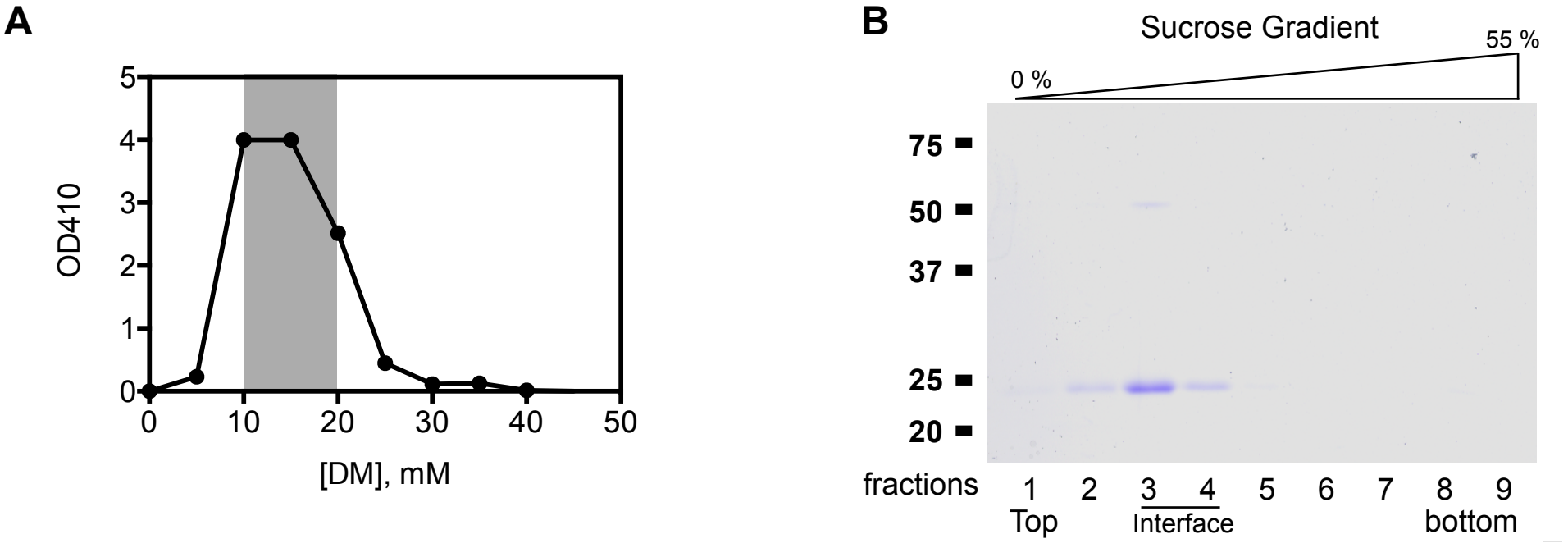
2. A. G. Lee, How lipids and proteins interact in a membrane: a molecular approach. *Mol Biosyst* **1**, 203 (Sep, 2005)
3. A. G. Lee, How lipids affect the activities of integral membrane proteins. *Biochim Biophys Acta* **1666**, 62 (Nov 3, 2004)
4. R. G. Anderson, The caveolae membrane system. *Annu Rev Biochem* **67**, 199 (1998)
5. K. Simons, W. L. Vaz, Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct* **33**, 269 (2004)
6. M. Edidin, The state of lipid rafts: from model membranes to cells. *Annu Rev Biophys Biomol Struct* **32**, 257 (2003)
7. R. Kapoor, J. H. Kim, H. Ingolfson, O. S. Andersen, Preparation of artificial bilayers for electrophysiology experiments. *J Vis Exp* **31**, (2008)
8. H. Zheng, W. Liu, L. Y. Anderson, Q. X. Jiang, Lipid-dependent gating of a voltage-gated potassium channel. *Nat Commun* **2**, 250 (2011).3072105
9. D. Schmidt, Q. X. Jiang, R. MacKinnon, Phospholipids and the origin of cationic gating charges in voltage sensors. *Nature* **444**, 775 (Dec 7, 2006)
10. V. Ruta, Y. Jiang, A. Lee, J. Chen, R. MacKinnon, Functional analysis of an archaebacterial voltage-dependent K+ channel. *Nature* **422**, 180 (Mar 13, 2003)
11. Q. X. Jiang, T. Gonen, The influence of lipids on voltage-gated ion channels. *Curr Opin Struct Biol*, (Apr 5 online, 2012).3408884
12. P. Artimo *et al.*, ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Res* **40**, W597 (2012)
13. J. Cladera, J. L. Rigaud, J. Villaverde, M. Dunach, Liposome solubilization and membrane protein reconstitution using Chaps and Chapso. *Eur J Biochem* **243**, 798 (Feb 1, 1997)
14. D. Levy, A. Bluzat, M. Seigneuret, J. L. Rigaud, A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal. *Biochim Biophys Acta* **1025**, 179 (Jun 27, 1990)
15. H. S. Young, J. L. Rigaud, J. J. Lacapere, L. G. Reddy, D. L. Stokes, How to make tubular crystals by reconstitution of detergent-solubilized Ca2(+)-ATPase. *Biophys J* **72**, 2545 (Jun, 1997).1184452
16. D. Levy, A. Gulik, A. Bluzat, J. L. Rigaud, Reconstitution of the sarcoplasmic reticulum Ca(2+)-ATPase: mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. *Biochim Biophys Acta* **1107**, 283 (Jun 30, 1992)
17. F. S. Cohen, J. Zimmerberg, A. Finkelstein, Fusion of phospholipid vesicles with planar phospholipid bilayer membranes. II. Incorporation of a vesicular membrane marker into the planar membrane. *The Journal of general physiology* **75**, 251 (Mar, 1980).2215255
18. B. Fuks, F. Homble, Permeability and electrical properties of planar lipid membranes from thylakoid lipids. *Biophysical journal* **66**, 1404 (May, 1994).1275861
19. W. Hanke, W.-R. Schlue, *Planar Lipid Bilayers. Methods and Applications*. D. B. Sattelle, Ed., Biological Techniques (Academic Press Inc, San Diego, 1993), pp. 133.
20. H. T. Tien, *Bilayer lipid membranes (BLM). Theory and Practice*. (Marcel Dekker, Inc., New York, 1974), pp. 655.
21. R. E. Pagano, J. M. Ruysschaert, I. R. Miller, The molecular composition of some lipid bilayer membranes in aqueous solution. *The Journal of membrane biology* **10**, 11 (1972)
22. F. A. Henn, T. E. Thompson, Properties of lipid bilayer membranes separating two aqueous phases: composition studies. *Journal of molecular biology* **31**, 227 (Jan 28, 1968)
23. X. Tao, R. MacKinnon, Functional analysis of Kv1.2 and paddle chimera Kv channels in planar lipid bilayers. *J Mol Biol* **382**, 24 (Sep 26, 2008)
24. F. S. Cohen, M. H. Akabas, J. Zimmerberg, A. Finkelstein, Parameters affecting the fusion of unilamellar phospholipid vesicles with planar bilayer membranes. *The Journal of cell biology* **98**, 1054 (Mar, 1984).2113164

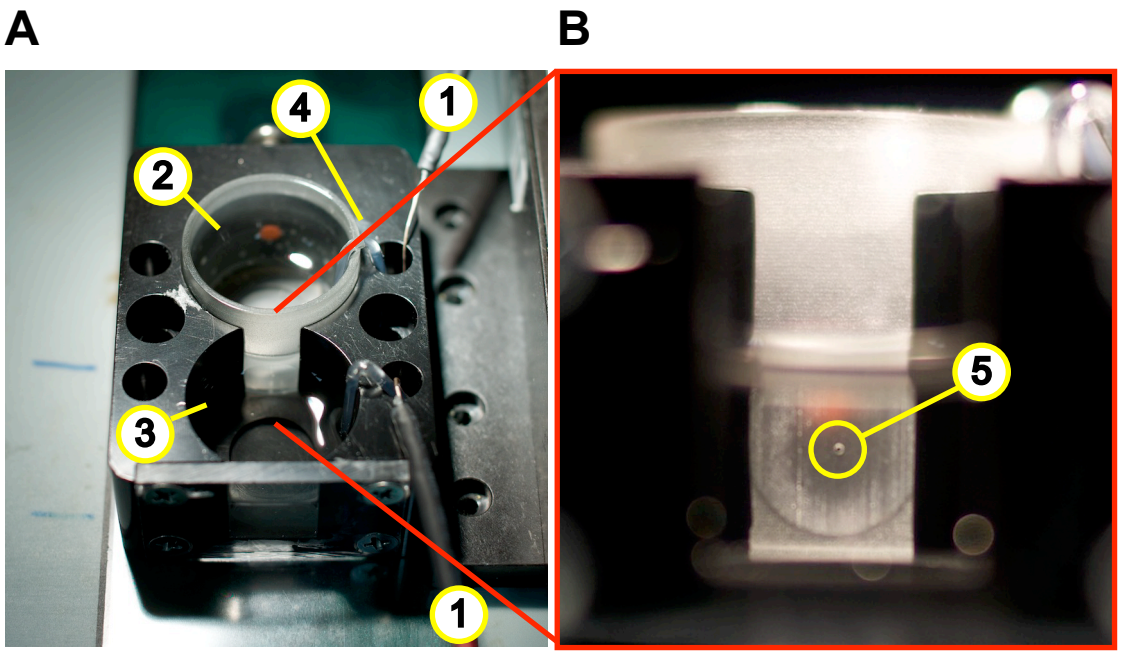
25. G. P. Smith, V. A. Petrenko, Phage Display. *Chem. Rev.* **97**, 391 (1997)
26. M. J. McGuire, S. Li, K. C. Brown, Biopanning of phage displayed peptide libraries for the isolation of cell-specific ligands. *Methods Mol Biol* **504**, 291 (2009)
27. J. L. Rigaud, Membrane proteins: functional and structural studies using reconstituted proteoliposomes and 2-D crystals. *Braz J Med Biol Res* **35**, 753 (Jul, 2002)
28. M. Chami *et al.*, Use of octyl beta-thioglucopyranoside in two-dimensional crystallization of membrane proteins. *J Struct Biol* **133**, 64 (Jan, 2001)
29. W. Kuhlbrandt, Two-dimensional crystallization of membrane proteins. *Q Rev Biophys* **25**, 1 (Feb, 1992)
30. J. L. Rigaud, D. Levy, Reconstitution of membrane proteins into liposomes. *Methods Enzymol* **372**, 65 (2003)
31. T. Walz, N. Grigorieff, Electron Crystallography of Two-Dimensional Crystals of Membrane Proteins. *J Struct Biol* **121(2)**, 142 (1998)
32. G. A. Signorell, T. C. Kaufmann, W. Kukulski, A. Engel, H. W. Remigy, Controlled 2D crystallization of membrane proteins using methyl-beta-cyclodextrin. *J Struct Biol* **157**, 321 (Feb, 2007)
33. M. Vink, K. Derr, J. Love, D. L. Stokes, I. Ubarretxena-Belandia, A high-throughput strategy to screen 2D crystallization trials of membrane proteins. *J Struct Biol* **160**, 295 (Dec, 2007)
34. I. Iacovache *et al.*, The 2DX robot: a membrane protein 2D crystallization Swiss Army knife. *J Struct Biol* **169**, 370 (Mar, 2010)

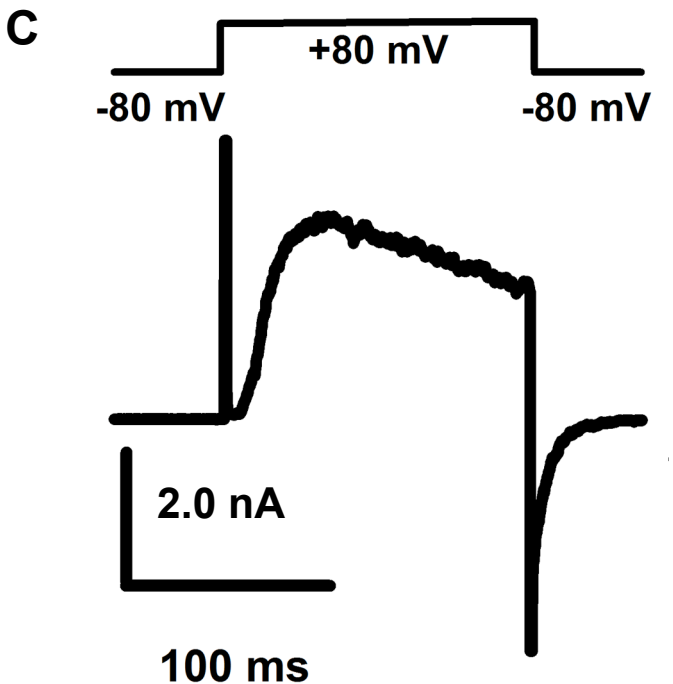
*Figure
Click here to download Figure: Lee_JoVE_Figures_LowRes_2012Dec18.pdf

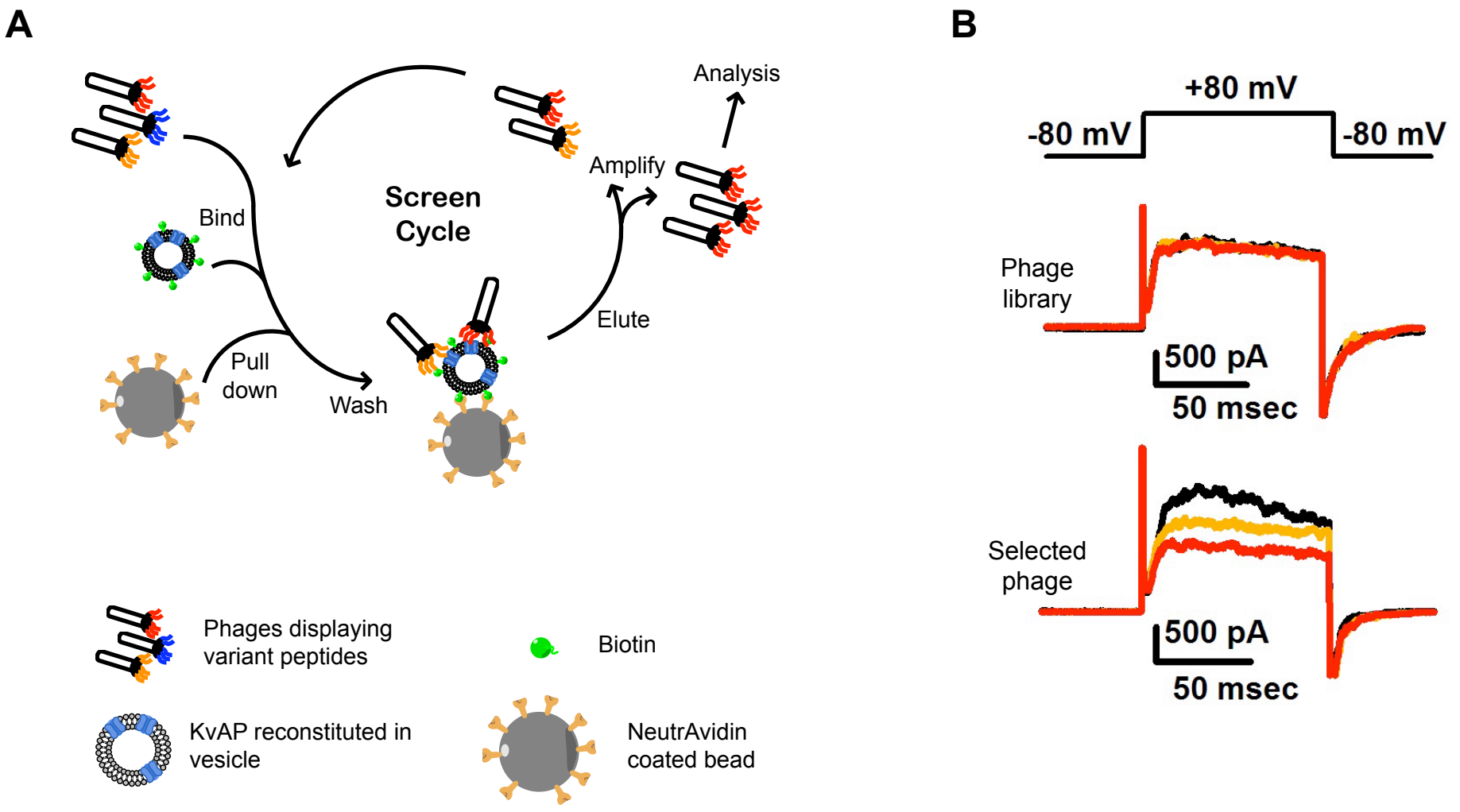





A
OD410
0
1
2
3
4
5
0
10
20
30
40
50
[DM], mM
B
Sucrose Gradient
0 %
55 %
75
50
37
25
20
fractions
1
2
3
4
5
6
7
8
9
Top
Interface
bottom


A
B
1
2
3
4
5
1


C
+80 mV
-80 mV
-80 mV
2.0 nA
100 ms


A
Analysis
Amplify
Screen
Cycle
Bind
Elute
Pull
down
Wash
Phages displaying
variant peptides
Biotin
KvAP reconstituted in
vesicle
NeutrAvidin
coated bead
B
+80 mV
-80 mV
-80 mV
Phage
library
500 pA
50 msec
Selected
phage
500 pA
50 msec

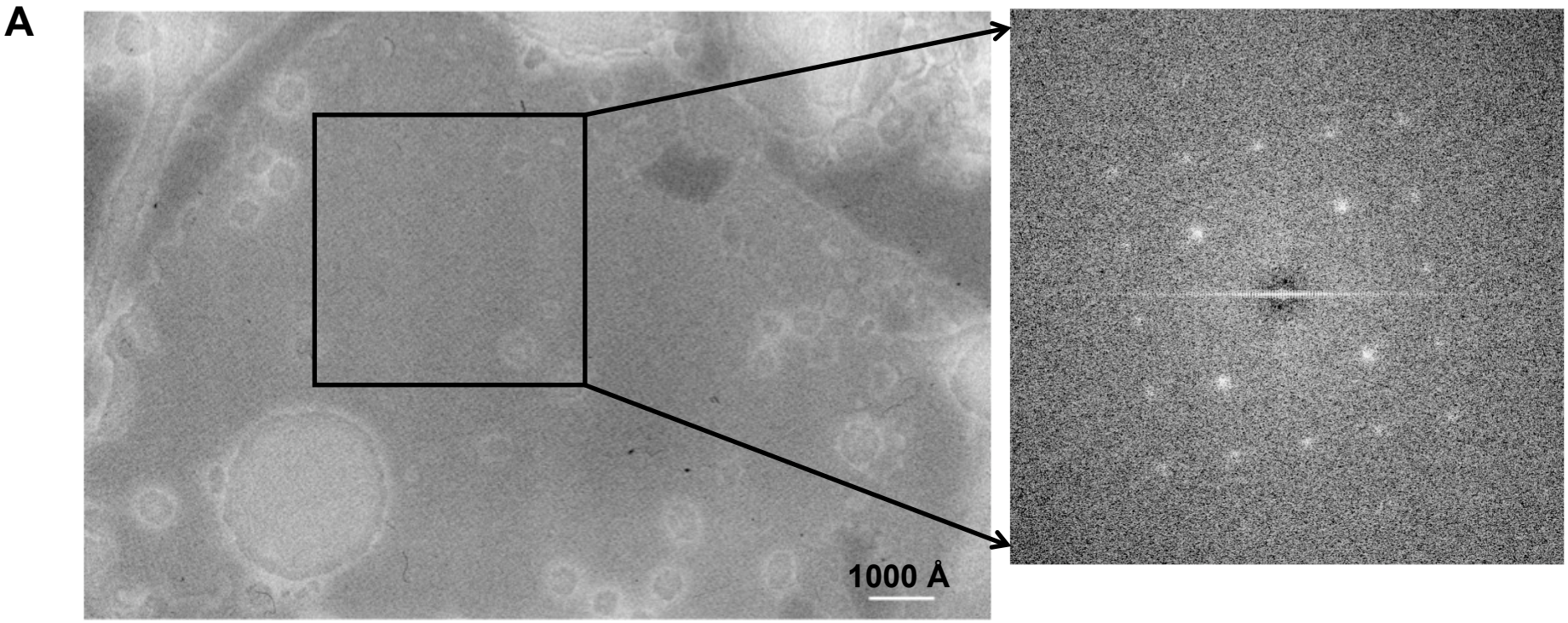

A
1000 Å
B
C
1000 Å

*Table 2
Click here to download Table of Reagents/ Materials Used: Lee_JoVE_Table 2.doc

Table 2: Equipment needed

- Bacterial culture shaking incubator
- Spectrophotometer
- Micro-probe sonicator
- Rocker
- Floor-model centrifuge for collecting bacteria culture and for concentrating samples
- Empty columns
- Superdex 200 column connected to a FPLC system

*Table3
Click here to download Table of Reagents/ Materials Used: Lee_JoVE_Table 3.doc

Table 3: Buffer name and contents

Buffer name	Contents
IMAC Lysis buffer	50 mM Tris pH 8.0, 100 mM KCl
IMAC Wash buffer	50 mM Tris pH 8.0, 100 mM KCl, and 5.0 mM DM
IMAC Elution buffer	50 mM Tris pH 8.0, 100 mM KCl, 5.0 mM DM, and 300 mM imidazole
SDS-sample buffer (5x) (nonreducing)	60 mM Tris-HCl (pH6.8), 25% glycerol, 2.0% SDS, 0.10% bromophenol blue. (1.0% 2-mercaptoethanol added to make the reducing buffer).
Stacking gel buffer for SDS-PAGE(4x)	0.50 M Tris-HCl (pH6.8), 0.40% SDS
Resolution gel buffer for SDS-PAGE (4x)	1.5 M Tris-HCl (pH8.8), 0.40% SDS
FPLC equilibration	20 mM Tris pH 8.0, 100 mM KCl, and 5.0 mM DM
Liposome dialysis	10 mM HEPES, 100 mM KCl

*Table 1

Click here to download Table of Reagents/ Materials Used: Lee_JoVE_Table 1_Materials.xlsx

Name of Reagent/Material	Company	Catalog Number	Comments
Tryptone	RPI Corp.	T60060	
Yeast Extract	RPI Corp.	Y20020	
NaCl	Fisher	S271-3	
Tris Base	RPI Corp.	T60040	
Potassium Chloride	Fisher	BP366-500	
n-Dodecyl-β-D-Maltoside	Affymetrix	D322S	Sol-grade
n-Octyl-β-D-Glucoside	Affymetrix	O311	Ana-grade
Aprotinin	RPI Corp.	A20550-0.05	
Leupeptin	RPI Corp.	L22035-0.025	
Pepstatin A	RPI Corp.	P30100-0.025	
PMSF	SIGMA	P7626	
Dnase I	Roche	13407000	
Bio-Bead SM-2	Bio-Rad	152-3920	
HEPES	RPI Corp.	H75030	
POPE	Avanti Polar Lipids	850757C	
POPG	Avanti Polar Lipids	840457C	
DOGS	Avanti Polar Lipids	870314C	
DMPC	Avanti Polar Lipids	850345C	
Biotin-DOPE	Avanti Polar Lipids	870282C	
DOTAP	Avanti Polar Lipids	890890C	
NeutrAvidin agarose beads	Piercenet	29200	
Dialysis Tubing	Spectrum Laboratories, Inc	132-570	
Pentane	Fisher	R399-1	
Decane	TCI America	D0011	
MTS-PEG5000	Toronto Research Cemicals	M266501	

jove JOURNAL OF VISUALIZED EXPERIMENTS
17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Reconstitution of a Kv channel into lipid membranes for structural & functional studies

Author(s): Sangson Lee, Hui Zheng, Liang Shi, and Qiu-Xing Jiang

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

ARTICLE AND VIDEO LICENSE AGREEMENT

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name: Qiu-Xing Jiang

Department: Cell Biology

Institution: UT Southwestern Medical Center

Article Title: Reconstitution of a Kv Channel into lipid membranes for structural and functional studies.

Signature: [signature] Date: 10/8/2012

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Attn: *JoVE* Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

*Rebuttal Comments
Click here to download Rebuttal Comments: Lee_rebuttal_v09.docx

SOUTHWESTERN
THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Qiu-Xing Jiang, Ph.D.
Assistant Professor

Department of Cell Biology

December 18, 2012

Rachelle Baker

Associate Editor

Journal of Visualized Experiments (JoVE)

17 Sellers St. Cambridge, MA 02139

rachelle.baker@jove.com

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.

UT Southwestern Medical Center at Dallas 6000 Harry Hines Blvd. / Dallas, Texas 75390-9039 / (214)633-1940 / Fax (214)648-8694
E-Mail: qiu-xing.jiang@utsouthwestern.edu

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.

SOUTHWESTERN
THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Qiu-Xing Jiang, Ph.D. **Department of Cell Biology**
Assistant Professor

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.

UT Southwestern Medical Center at Dallas 6000 Harry Hines Blvd. / Dallas, Texas 75390-9039 / (214)633-1940 / Fax (214)648-8694
E-Mail: qiu-xing.jiang@utsouthwestern.edu

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.

SOUTHWESTERN
THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Qiu-Xing Jiang, Ph.D. **Department of Cell Biology**
Assistant Professor

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

UT Southwestern Medical Center at Dallas 6000 Harry Hines Blvd. / Dallas, Texas 75390-9039 / (214)633-1940 / Fax (214)648-8694
E-Mail: qiu-xing.jiang@utsouthwestern.edu

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.

SOUTHWESTERN
THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Qiu-Xing Jiang, Ph.D.
Assistant Professor

Department of Cell Biology

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

UT Southwestern Medical Center at Dallas 6000 Harry Hines Blvd. / Dallas, Texas 75390-9039 / (214)633-1940 / Fax (214)648-8694
E-Mail: qiu-xing.jiang@utsouthwestern.edu

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.

SOUTHWESTERN
THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Qiu-Xing Jiang, Ph.D.
Assistant Professor

Department of Cell Biology

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.

UT Southwestern Medical Center at Dallas 6000 Harry Hines Blvd. / Dallas, Texas 75390-9039 / (214)633-1940 / Fax (214)648-8694
E-Mail: qiu-xing.jiang@utsouthwestern.edu

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

SOUTHWESTERN
THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Qiu-Xing Jiang, Ph.D. **Department of Cell Biology**
Assistant Professor

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.

UT Southwestern Medical Center at Dallas 6000 Harry Hines Blvd. / Dallas, Texas 75390-9039 / (214)633-1940 / Fax (214)648-8694
E-Mail: qiu-xing.jiang@utsouthwestern.edu

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.

SOUTHWESTERN
THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Qiu-Xing Jiang, Ph.D.
Assistant Professor

Department of Cell Biology

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..

UT Southwestern Medical Center at Dallas 6000 Harry Hines Blvd. / Dallas, Texas 75390-9039 / (214)633-1940 / Fax (214)648-8694
E-Mail: qiu-xing.jiang@utsouthwestern.edu

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

Sincerely,

Qiu-Xing Jiang