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Reconstitution of a transmembrane protein, the voltage-gated ion channel, KvAP, into giant unilamellar vesicles for microscopy and patch clamp studies --Manuscript Draft--

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Corresponding Author:	Patricia Bassereau Institut Curie Paris Cedex 5 , Île-de-France FRANCE
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	Patricia.Bassereau@curie.fr
Corresponding Author's Institution:	Institut Curie
Corresponding Author's Secondary Institution:	
First Author:	Matthias Garten
First Author Secondary Information:	
Other Authors:	Matthias Garten Sophie Aimon Gilman E. S. Toombes
Order of Authors Secondary Information:	
Abstract:	Giant Unilamellar Vesicles (GUVs) are a popular biomimetic system for studying membrane associated phenomena. However, commonly used protocols to grow GUVs must be modified in order to form GUVs containing functional transmembrane proteins. This article describes two dehydration-rehydration methods - electroformation and gel-assisted swelling - to form GUVs containing the voltage-gated potassium channel, KvAP. In both methods, a solution of protein-containing small unilamellar vesicles is partially dehydrated to form a stack of membranes, which is then allowed to swell in a rehydration buffer. For the electroformation method, the film is deposited on platinum electrodes so that an AC field can be applied during film rehydration. In contrast, the gel-assisted swelling method uses an agarose gel substrate to enhance film rehydration. Both methods can produce GUVs in low (e.g., 5 mM) and physiological (e.g., 100 mM) salt concentrations. The resulting GUVs are characterized via fluorescence microscopy, and the function of reconstituted channels measured using the inside-out patch-clamp configuration. While swelling in the presence of an alternating electric field (electroformation) gives a high yield of defect-free GUVs, the gel-assisted swelling method produces a more homogeneous protein distribution and requires no special equipment.
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Research Center – Institut Curie

Physico-Chimie Curie

UMR 168 CNRS / Institut Curie/UPMC

26 rue d'Ulm

75248 Paris Cedex 05, FRANCE

Patricia Bassereau

Research Director CNRS

Tél. +33 (0)1 56 24 67 84

Fax. +33 (0)1 40 51 06 36

patricia.bassereau@curie.fr

Paris, August 12th, 2014

Dear Eric,

We are very grateful to the reviewers for studying our manuscript so carefully and providing such detailed comments and suggestions.

The only major concern was from reviewer #1, who noted that the channel conductance and inactivation in GUVs differs from studies of KvAP in Black Lipid Membranes (BLMs). However, as we explain in the response to reviewers, these differences in channel properties are entirely consistent with the different conditions in the BLM and GUV experiments, while the intrinsic characteristics of the channel (i.e. K-selectivity, voltage-dependent activation) remain unchanged (PLoS One 6, e25529). We have carefully revised the manuscript to discuss this issue more fully, and to incorporate the many suggestions from all three reviewers.

The reviewers' input has greatly improved the paper, and we hope the paper and videos will help other scientists use GUVs with reconstituted proteins for many academic and technologic applications.

We are looking forward to hear from you,

Best regards,

Patricia Bassereau

Leader of the group « Membrane and Cell Functions »

TITLE:

Reconstitution of a transmembrane protein, the voltage-gated ion channel, KvAP, into giant unilamellar vesicles for microscopy and patch clamp studies

AUTHORS:

Garten, Matthias
Institut Curie, Centre de Recherche
CNRS, UMR 168, PhysicoChimie Curie
Université Pierre et Marie Curie
Paris F-75248, France
matthias.garten@curie.fr

Aimon, Sophie
Kavli Institute for Brain and Mind
University of California, San Diego
La Jolla, CA
saimon@ucsd.edu

Bassereau, Patricia
Institut Curie, Centre de Recherche
CNRS, UMR 168, PhysicoChimie Curie
Université Pierre et Marie Curie
Paris F-75248, France
patricia.bassereau@curie.fr

Toombes, Gilman E. S.
Molecular Physiology and Biophysics Section
National Institute for Neurological Disorders and Stroke
National Institute of Health
Bethesda, MD
Gilman.Toombes@nih.gov

CORRESPONDING AUTHOR:

Bassereau, Patricia
Institut Curie, Centre de Recherche
CNRS, UMR 168, PhysicoChimie Curie
Université Pierre et Marie Curie
Paris F-75248, France
patricia.bassereau@curie.fr

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SHORT ABSTRACT:

The reconstitution of the transmembrane protein, KvAP, into giant unilamellar vesicles (GUVs) is demonstrated for two dehydration-rehydration methods - electroformation, and gel-assisted swelling. In both methods, small unilamellar vesicles containing the protein are fused together to form GUVs that can then be studied by fluorescence microscopy and patch-clamp electrophysiology.

LONG ABSTRACT:

Giant Unilamellar Vesicles (GUVs) are a popular biomimetic system for studying membrane associated phenomena. However, commonly used protocols to grow GUVs must be modified in order to form GUVs containing functional transmembrane proteins. This article describes two dehydration-rehydration methods - electroformation and gel-assisted swelling - to form GUVs containing the voltage-gated potassium channel, KvAP. In both methods, a solution of protein-containing small unilamellar vesicles is partially dehydrated to form a stack of membranes, which is then allowed to swell in a rehydration buffer. For the electroformation method, the film is deposited on platinum electrodes so that an AC field can be applied during film rehydration. In contrast, the gel-assisted swelling method uses an agarose gel substrate to enhance film rehydration. Both methods can produce GUVs in low (*e.g.*, 5 mM) and physiological (*e.g.*, 100 mM) salt concentrations. The resulting GUVs are characterized via fluorescence microscopy, and the function of reconstituted channels measured using the inside-out patch-clamp configuration. While swelling in the presence of an alternating electric field (electroformation) gives a high yield of defect-free GUVs, the gel-assisted swelling method produces a more homogeneous protein distribution and requires no special equipment.

INTRODUCTION:

When studying the physical principles that govern living systems, bottom-up approaches allow an experimentalist to control system composition and other parameters that are not easily manipulated in cell-based systems¹. For membrane-based processes, Giant Unilamellar Vesicles (GUVs, diameter ~ 1-100 μm) have proven to be a very useful biomimetic system²⁻⁷ as they are well suited for microscopy studies and micromanipulation⁸⁻¹⁰. While there are many different protocols to produce GUVs, most fall into two categories - emulsion based approaches^{11,12} and techniques based on rehydrating a lipid film¹³⁻¹⁶. In emulsion-based methods, the inner and outer leaflets of the GUV membranes are assembled sequentially from lipid monolayers at water/oil interfaces. This approach is ideal for encapsulating soluble proteins within the GUVs, and for forming GUVs with asymmetric leaflet lipid composition. However, GUVs formed from emulsions can retain traces of solvent that change the membrane's mechanical properties¹⁷, and the approach is not especially well-suited to trans-membrane protein reconstitution.

Film rehydration methods rely on the fact that drying (dehydration) causes many lipid mixtures to form a multi-lamellar stack of membranes. If this stack is then placed in contact with an aqueous buffer, membranes in the stack will move apart as solvent flows between them and at the surface of the stack, individual membranes can detach to form GUVs^{13,18} (as well a veritable zoo of other lipidic objects). However, even for optimal buffer and lipid compositions, this

classical “spontaneous swelling” method has a relatively low yield of defect-free GUVs. One widely used method to boost the yield of defect-free GUVs is “electroformation”, in which an alternating current (AC) field is applied during film rehydration. While the mechanism remains poorly understood, “electroformation” can give spectacular GUV yields (> 90% in favorable circumstances) for low salt concentration buffers (< 5 mM)^{14,19}, and can even work in physiological buffers (~100 mM) using a higher frequency (500 Hz versus 10 Hz) AC field and platinum electrodes¹⁵. An alternative approach to boost the yield of defect-free GUVs is “gel-assisted swelling”, in which the lipid solution is deposited onto a polymeric gel substrate rather than the passive (e.g., glass, PTFE) substrates used in classical “spontaneous swelling”. When the resultant lipid/gel film is rehydrated, GUVs can rapidly form even for physiological buffers^{16,20}.

All these methods can produce lipid-only GUVs which can be used to study membrane associated phenomena such as the interaction between soluble proteins and membranes. However, to incorporate a trans-membrane protein into GUVs, significant modifications are needed to ensure that the protein remains in a functional state throughout the reconstitution procedure. While solutions of lipids in organic solvents (e.g., chloroform, cyclohexane) are ideal for producing lipid films, trans-membrane proteins are typically only stable when their hydrophobic trans-membrane domain is embedded in a lipid bilayer, or surrounded by a detergent micelle (e.g., during protein purification). Thus, the starting material for a reconstitution is typically native membranes, purified protein in a detergent solution, or small unilamellar protein-containing vesicles (proteo-SUVs) and/or multi-lamellar vesicles (proteo-MLVs) formed by detergent removal in the presence of lipids. Most methods to incorporate these membrane proteins into GUVs fall into three categories.

Direct Insertion: Trans-membrane protein suspended in detergent is mixed with pre-formed, lipid-only, mildly detergent solubilized GUVs, and the detergent then removed using biobeads²¹. While conceptually simple, this method requires precise control of the detergent concentration, as too high a detergent concentration can dissolve the GUVs while too low a concentration can cause the protein to unfold or aggregate.

GUV/Proteo-SUV Fusion: Protein in proteo-SUVs is combined with pre-formed, lipid-only GUVs and fusion is facilitated with special fusogenic peptides²² or detergent²¹. Typically the extent of fusion is limited leading to GUVs with low protein density.

Dehydration/Rehydration: A protein-containing lipid film is formed by partial dehydration of a proteo-SUV (or proteo-MLV) solution and GUVs are then grown as for a pure lipid film. The obvious challenge is to protect the protein during the partial dehydration step²³, but the method has been successfully used to reconstitute trans-membrane proteins such as Bacteriorhodopsin, Calcium-ATPase, Integrin and VDAC into GUVs^{7,23–25}.

This article describes dehydration/rehydration protocols to make GUVs containing the voltage-gated potassium channel, KvAP, from the hyper-thermophilic Archaea, *Aeropyrum pernix*. KvAP has a high degree of homology to eukaryotic voltage dependent potassium channels²⁶ and a

known crystal structure²⁷, making it a good model for studying the mechanism of voltage gating. Production of the proteo-SUVs has been described in detail previously and is not part of this tutorial^{26,28,29}. Importantly, KvAP proteo-SUVs do not have to be produced for each GUV preparation, as they can be stored in small (*e.g.*, 10 μ l) aliquots at -80 °C for extended periods of time (> 1 year). Electroformation or gel-assisted swelling can then be used to grow GUVs from the KvAP proteo-SUVs (or proteo-MLVs).

The key steps for the electroformation protocol are illustrated in Figure 1. Droplets of a solution of SUVs containing the protein are deposited on platinum wires (shown in Figure 2). Partial dehydration of the SUV suspension leads to the formation of a lipid protein film through the fusion of SUVs. During rehydration, an AC field is applied to the electrodes to assist the lipid layers to delaminate and form GUVs. A 10 Hz field works well when using “low-salt” (< 5 mM) rehydration buffer²⁸ and GUVs take several hours to grow. In contrast, physiological buffers (containing ~100 mM salt) work well with a lower voltage, 500 Hz AC field but require a prolonged (~12 hour) swelling period¹⁵. This method is based upon an earlier protocol using ITO slides²⁴, but uses a custom chamber containing two platinum wires as shown in Figure 2 (see the discussion for design details and suggestions for simpler, improvised chambers).

Figure 3 illustrates the gel-assisted swelling method. The protocol works well with buffers with physiological salt concentrations, is rapid, and produces GUVs with a more homogeneous protein distribution. However, the yield of isolated, apparently defect-free GUVs (*i.e.* the GUV membrane is uniform at optical length-scales and does not enclose any objects) is lower, although it provides a sufficient number for patch-clamp and micro-manipulation experiments. This method was based on a protocol using agarose gel to produce lipid-only GUVs¹⁶ and requires less specialized equipment than the electroformation method.

The characterization of GUVs with fluorescence microscopy is described, as well as procedures using a standard patch-clamp set-up to measure KvAP activity in “inside-out” excised membrane patches.

Growing protein-containing GUVs can be more difficult than lipid-only GUVs. In particular, the final GUV yield can depend sensitively on exactly how the SUV solution is deposited and dehydrated to form the membrane stack. For someone without any previous experience with GUVs, it may be helpful to first grow lipid-only GUVs following a conventional protocol^{15,16} in which the membrane film is formed by depositing lipids from an organic solvent. Once the conventional protocol works well, SUV deposition and partial dehydration can then be mastered using lipid-only SUVs, which are also very helpful when adjusting the protocol for a new lipid composition. When GUVs grow reliably from lipid-only SUVs, it is then only a small step to produce protein-containing GUVs from proteo-SUVs.

PROTOCOL:

1.) Solution Preparation

1.1.) Prepare 5 ml of 'SUV buffer' containing 5 mM KCl, 1 mM HEPES (pH 7.4) or TRIS (pH 7.5), and 2 mM Trehalose. Filter the buffer with a 0.2 µm syringe filter and divide into 1 ml aliquots which can be stored at -20 °C.

Note: Additional details for reagents and instruments are given in the materials list.

1.2.) Prepare 40 ml of GUV 'Growth Buffer' that will fill the GUV interior during film rehydration. For a 'low salt' growth, combine 5 mM KCl, 1 mM HEPES (pH 7.4) or 1 mM TRIS (pH 7.5), and ~400 mM sucrose. For a 'physiological salt' growth, combine 100 mM KCl, 5 mM HEPES (pH 7.4) or 5 mM TRIS (pH 7.5), and ~200 mM sucrose.

1.3.) Prepare 40 ml of 'Observation Buffer' for the external solution in the experimental chamber by combining 100 mM KCl, 5 mM HEPES (pH 7.4) or 5 mM TRIS (pH 7.5), and ~200 mM glucose.

Note: These buffers are only examples. See the discussion to adapt the buffers for other experiments.

1.4.) Measure the osmolarities of the growth and observation buffers with an osmometer. Add granules of sucrose or glucose to match them to within 1% so that GUVs will not lyse or collapse when transferred from the growth chamber to the observation chamber.

Note: In this concentration range, adding 1 mM of sucrose (13.7 mg per 40 ml) or glucose (7.2 mg per 40 ml) increases osmolarity by ~ 1 mOsm.

1.5.) Filter the growth and observation buffers with a 0.2 µm filter and store them at 4 °C to inhibit bacterial growth.

1.6.) Dissolve 50 mg of beta-casein in 10 ml of 20 mM TRIS (pH 7.5) buffer to form a 5 mg/ml beta-casein solution needed to passivate the surfaces of experimental chambers so that GUVs do not stick, spread and burst. Once the beta-casein is completely dissolved (up to several hours at 4 °C), filter (0.2 µm) it into 0.5 ml aliquots which can be flash frozen and stored at -20 °C for later use (thawed aliquots stored at 4 °C can typically be used for up to a 1 week).

2.) SUV Preparation

2.1) Prepare and freeze aliquots of proteo-SUVs following the previously published detailed protocol²⁸. Use KvAP fluorescently labelled with Alexa-488 maleimide, reconstituted into DPhPC SUVs (10 mg/ml) at a protein to lipid ratio of 1:10 (by mass).

Note: Wild-type KvAP contains one cysteine per monomer located near the intra-cellular C-terminus (amino acid 247).

2.2) Fluorescent, Lipid-only SUVs:

CAUTION: Handle chloroform under a fume hood wearing nitrile gloves and safety glasses. Avoid the use of any plastic as chloroform can dissolve them. Chloroform solutions can be stored in amber glass vials with Teflon caps and transferred using glass syringes. Take care to rinse all glassware at least 5 to 10 times with chloroform before and after pipetting lipids.

2.2.1) Prepare 100 μ l of 10 mg/ml DPhPC SUVs containing 0.5 mol% of the red fluorescent lipid, Texas Red-DHPE, by mixing 100 μ l of DPhPC solution (10 mg/ml in chloroform) with 8.2 μ l of Texas Red-DHPE solution (1 mg/ml in methanol) in a 1.5 ml amber glass vial.

2.2.2) Dry the lipids down under a stream of nitrogen in a chemical hood while rotating the vial. When the film appears to be dry, place the lipids under a vacuum for 3 hours to remove any residual solvent.

2.2.3) Add 100 μ l of SUV buffer to the lipids, and vortex vigorously until no lipid remains stuck to the walls of the vial and the solution is uniformly milky.

2.2.4) Sonicate the lipid solution to form SUVs. Adjust the vial position until the ultrasound causes the most movement and flow inside the vial, and take care not to heat the solution unnecessarily. Continue sonication until the solution becomes translucent, or when possible, transparent (2-5 minutes for tip sonication, ~20 minutes for bath sonication).

2.2.5) Aliquot SUVs (*e.g.*, 10 μ l or 20 μ l) and freeze (-20 °C) for later use.

Note: Lipids, especially unsaturated lipids, can easily breakdown. Store lipid solutions at -20 °C (or -80 °C) under argon and use within 6 months. Lipid breakdown products can be detected with Thin Layer Chromatography.

3.) GUV Growth by Electroformation

3.1) Prepare the electroformation chamber.

3.1.1.) If the chamber has not been cleaned, remove the windows, wipe off all sealant and grease, extract the wires, and rinse and scrub the chamber with a tissue using water and ethanol ($\geq 70\%$) alternately.

3.1.2.) Rub the wires well, submerge the wires and chamber in acetone, and sonicate for 5 minutes. Wipe everything with a tissue again using acetone. Put the chamber in ethanol and sonicate for 5 minutes.

3.1.3.) Assemble the chamber by inserting the wires through the holes, and rotate and wipe the wires to make sure they are clean. Put the chamber in distilled water, sonicate for 5 minutes and dry the chamber with a stream of nitrogen or air.

3.2) Prepare 30 μl of 3 mg/ml SUV suspension in SUV buffer. To form protein-containing GUVs, combine 8 μl of proteo-SUVs (DPhPC 10 mg/ml KvAP 1:10), 2 μl of fluorescent SUVs (10 mg/ml DPhPC, 0.5 mol% TexasRed-DHPE) and 20 μl of SUV buffer in a 1.5 ml microcentrifuge tube for a final protein to lipid (mass) ratio of 1:12.5 and 0.1 mol% TexasRed-DHPE. Mix the solution vigorously.

3.2.1) Alternatively, to practice the protocol with lipid-only SUVs, simply combine 10 μl of fluorescent SUVs (10 mg/ml DPhPC and 0.5 mol% TexasRed-DHPE) with 20 μl SUV buffer.

3.3) Deposit the SUV Solution.

3.3.1.) Use a 2 μl pipette or 5 μl glass syringe to deposit small (<0.2 μl) droplets of the SUV solution on the wires. Approximately 1 μl of solution is needed to form a series of drops along 1 cm of wire. Make sure the drops are small enough and spaced far enough apart that they do not touch or fuse.

3.3.2.) Let the deposited SUVs dry for ~ 30 min in open air. When all the drops have settled, rotate the wire so the lipid deposits are easier to observe with the microscope.

Note: If the SUVs do not dry sufficiently, they can just wash off the wires when the growth buffer is added, while drying too much can damage the protein. Because air humidity influences the rate of drying, the drying time and/or air humidity can be adjusted for optimal results³⁰. The lipid film on the wires should be visible under a microscope.

3.4) Assemble the chamber.

3.4.1) Seal the Chamber Bottom: Use a syringe to apply vacuum grease to the bottom of the chamber around the three wells and press a 40 mm x 22 mm coverslip gently against it to seal the chamber bottom so that it adheres without a gap. Seal the sides of the chamber (where the wires exit) with sealing paste. Apply vacuum grease on top of the chamber outlining the three wells.

3.4.2.) Slowly add growth buffer until each well is filled to the top. Avoid any rapid movement of the solution in the wells as this can strip the lipid film off the electrodes.

3.4.3) Close the chamber by pressing the top cover slide gently onto the grease, taking care not to dislodge the bottom coverslip. Use a tissue to remove any drops of buffer at the edges of the top cover slip.

Note: This is a good time to examine the chamber under the microscope to confirm that the lipid film has remained on the wires.

3.5.) Connect the signal generator to the wires using two alligator clips. Set the frequency (10 Hz/500 Hz sine wave for low/high salt buffer) and use a multimeter to measure and adjust the

voltage across the wires to 0.7/0.35 V root mean square (Vrms) for the low/high salt buffer. Cover the chamber with aluminum foil to protect the fluorophores from light. Leave the GUVs to grow for 2 to 3 hours for the low salt buffer, and 12 hours or over-night for the high salt buffer.

3.6.) Disconnect the chamber from the generator and carefully place it on an inverted microscope to evaluate GUV growth. Use slow, steady movements or fluid flow in the wells may prematurely detach GUVs from the wires.

Note: GUVs on the wire edges are usually visible in phase contrast (40x long working distance objective), while GUVs anywhere on the lower half of the wires can be seen with epifluorescence. If no GUVs are visible, try rotating the wires to look at the upper surface. GUVs can be stored at 4 °C in a growth chamber for several days.

4.) GUV growth by Gel-assisted Swelling

4.1.) Prepare 10 ml of a 1% agarose solution by mixing 100 mg of agarose with 10 ml of pure water. Heat it until it boils by placing it in a microwave at 480 W for ~20 s. Stir to make sure the agarose is completely dissolved.

Note: The solution can be stored at 4 °C and reheated when needed.

4.2.) Plasma-clean (air plasma) a cover-slide for 1 minute so that the agarose solution will spread nicely on it. Use the cover-slides within the next 15 minutes as the effect of plasma cleaning wears off quickly.

4.3.) Apply 200 µl of warm agarose solution to each 22 x 22 mm² slide so the solution wets the entire surface. Tilt the slide vertically and touch the lower edge to a tissue to remove excess liquid and leave just a thin smooth layer of agarose on the slide.

4.4.) Place the slide on a hot plate or oven at 60 °C and leave it to dry for at least 30 min. The agarose film is hardly visible by eye. After the slides cool to room temperature, use them immediately, or store them for up to one week in a closed container at 4 °C.

4.5.) Place the agarose-coated coverslip in a standard 3.5 cm Petri dish.

4.6.) Prepare the SUV solution as in Section 3.2 and apply ~15 µl of the SUV solution (3 mg/ml lipid) in ~30 very small drops gently onto the agarose surface. Take care not to distort the agarose layer too much.

4.7.) Place the slide under a gentle stream of nitrogen for about 10-15 minutes and follow the evaporation of the buffer by eye as the droplets dry.

4.8.) As soon as the SUVs have dried, add growth buffer to cover the slide surface. For a small

3.5 cm Petri dish use ~1 ml of buffer.

4.9.) Allow the swelling to proceed for ~30 min, and then examine the growth of GUVs in the chamber using an inverted microscope with phase-contrast or Differential Interference Contrast (DIC).

Note: Epifluorescence observation is difficult due to the strong background of fluorophores in the gel and the auto-fluorescence of the agarose.

5.) Harvesting and Observing GUVs

5.1.) Passivate the observation chamber (*e.g.*, small Petri dish or glass coverslip) so that GUVs do not stick, spread and burst on the chamber bottom. Cover the chamber bottom with beta-casein solution, incubate for 5 minutes, rinse out the casein solution with pure water, dry with a stream of air or nitrogen, and finally add observation buffer (*e.g.* ~5mm depth for a small Petri dish).

5.2.) Harvest the GUVs. Cut the end of a 100 μ l pipette tips so the opening is larger (~2 mm diameter), and aspire slowly as the shear stress of pipetting can easily destroy GUVs.

5.2.1) For electro-formed GUVs, open the growth chamber by gently removing the top coverslip. Place the pipette tip directly above each wire and aspirate ~50 μ l while moving the pipette tip along the wire to detach the GUVs.

Note: It may help to rotate the wire to collect GUVs on the “other side” of the wire.

5.2.2.) For “gel-assisted swelling” GUVs, first tap the side of the petri dish a few times to help the GUVs detach from the coverslip surface. Position the pipette tip just above the coverslip and aspirate 50 μ l while pulling the tip back over the surface. Directly transfer harvested GUVs to an observation chamber, or store in a 1.5 ml microcentrifuge tube at 4 °C for up to 1 week.

5.3.) Place the observation chamber on an inverted microscope, add the GUVs to the observation chamber, and wait a few minutes for the GUVs to settle at the chamber bottom.

5.4.) Survey the chamber with phase contrast or DIC to quickly locate smooth, spherical (‘defect free’) “GUV candidates”. Examine each “GUV candidate” in epifluorescence to exclude any containing smaller liposomes nested inside. Finally, check that the lipid fluorescence intensity is uniform and compatible with a single membrane (*i.e.* unilamellar).

Note: In some bilamellar (or multi-lamellar) vesicles, the membranes are too close together to be resolved so they appear unilamellar in phase contrast or DIC images. However, these objects can be distinguished from actual unilamellar GUVs by their lipid fluorescence, which is two times (or more) brighter.

6.) Patch-Clamping GUVs

6.1.) Make patch pipettes with a 1-2 μm tip diameter from standard borosilicate capillary glass using the program recommended for the pipette puller.

Note: Special treatments such as fire polishing are not necessary, and pipettes can be used for several days after they have been pulled if they are kept in a closed box.

6.2.) Passivate the chamber by incubating with a beta-casein solution (5 mg/ml) to ensure that GUVs do not adhere, spread and rupture on chamber surfaces. Rinse the casein off after 5 min.

6.3.) Insert the ground electrode, fill the chamber with observation buffer, transfer 10 μl of the GUV suspension as described in step 5.2 and 5.3, and wait a few minutes for the GUVs to settle at the bottom.

6.4.) Fill a fresh patch pipette with solution (observation buffer or another iso-osmotic solution) and mount it on the patch-clamp amplifier headstage.

6.5.) Search through the chamber to locate a “defect-free” GUV as described in section 5.4, and check that it contains fluorescent protein.

6.6.) Apply a constant positive pressure ($> 100 \text{ Pa}$, or roughly 1 cm H_2O in a manometer) to keep the patch pipette interior clean, and insert the patch pipette into the chamber. Bring the patch pipette into the field of view, apply test pulses to measure/compensate the pipette voltage offset and resistance, and examine the pipette under fluorescent illumination to confirm that the tip is clean.

6.7.) Bring the patch pipette towards the GUV, and if necessary, simultaneously reduce the positive pressure so the outward flow from the patch pipette does not make the GUV “run away”. When the patch pipette is close to the GUV, apply a negative pressure (up to 5 cm H_2O) to pull the GUV against the patch pipette. Monitor the resistance as the “tongue” of GUV membrane enters the patch pipette and the gigaseal forms.

6.8.) If a gigaseal did not form, remove the patch pipette from the chamber and return to step 6.4. If the membrane patch formed a gigaseal, but the GUV remains attached to the pipette, excise the patch by pulling away from the GUV, bursting the GUV against the chamber bottom, or briefly moving the pipette out of solution.

6.9.) When the inside-out membrane patch has been excised from the GUV and the gigaseal is stable, switch off the test pulses and apply a voltage protocol such as the one shown in Figure 13.

Note: Figure 13 follows the standard electrophysiological convention for an inside-out patch in which current flowing into the patch-electrode is “positive”, and $V = V_{\text{bath}} - V_{\text{pipette}}$. Holding the

patch at a negative potential (*e.g.*, $V = -100$ mV) for ~30 seconds places KvAP in the resting state, while steps (100 msec to 5 seconds) to more positive potentials (*e.g.*, $V = 100$ mV) can then drive it into conducting (*i.e.*, open) active states.

6.10) After measurements on a membrane patch are finished, break the patch with a zap or pressure pulse and check that the voltage offset of the patch electrode has not drifted. Remove the patch pipette from the chamber, and return to step 6.4.

REPRESENTATIVE RESULTS:

The growth of GUVs can be quickly evaluated by examining the growth chamber under the microscope. For electroformation, the GUVs tend to grow in bunches along the platinum wires, as shown in Figure 4. During gel-assisted swelling, GUVs appear as spherical structures that rapidly grow and fuse together (Figure 5).

Defect-free GUVs are more easily identified and evaluated after transferring to an observation chamber. Calibration measurements are needed to rigorously evaluate GUV quality, and a systematic quantification has been published previously²⁸. However, as an empirical guide, “good” GUVs should be isolated (*i.e.*, not in a cluster), have a single, smooth, spherical outer membrane, contain no objects (*i.e.*, tubes, nested vesicles, etc.) inside, and have the “standard” lipid fluorescence level (brighter objects are typically bi- or multi-lamellar). Figure 6 shows DIC and epifluorescence images of a ‘defect-free’ GUV after transfer to an iso-osmotic glucose solution. The contrast in DIC is due to the difference in optical density between the sucrose filled GUV and the glucose containing bath solution. The refractive index contrast of KvAP-containing GUVs often decreases over time, even though KvAP itself should not be permeable to sucrose or glucose. The uniform protein fluorescence in the GUV membrane confirms that KvAP is incorporated in the GUV (*i.e.*, it did not remain in the lipid film) and has not formed micron-scale (or larger) aggregates.

Figures 7, 8 and 9 show confocal images of lipid and protein fluorescence from GUVs produced by the lower-salt electroformation protocol, physiological salt electroformation protocol, and gel-assisted swelling protocol. The fluorescent lipid (magenta) and protein (green) signals have been scaled to the same average intensity, so that GUVs with a low/high number of proteins per unit area (protein density) have a magenta/green shade in the overlay images (right column), while GUVs with an average protein density are white. Isolated, defect-free GUVs were identified and the GUV size distribution is shown in Figure 10. Typically electroformation produces more defect-free GUVs than gel-assisted swelling, but the GUVs produced by electroformation are smaller. Figure 11 shows the protein density distribution inferred from the fluorescence of the GUVs. Electroformation with high-salt buffer produces GUVs in which the protein density varies greatly from GUV to GUV. The protein density of individual GUVs varies much less for electroformation with low salt buffer, while the protein density of GUVs produced by gel-assisted swelling is remarkably uniform.

The patch-clamp technique is a widely-used method for studying the function of voltage-gated ion channels, such as KvAP. In “inside-out” recordings, a clean glass “patch” pipette is used to

excise a patch of membrane from a GUV. An electrode inside the patch pipette is then used to apply voltage, and measure the resultant current flowing through the membrane patch. The composition of the membrane patch can differ greatly from the rest of the cell/GUV³¹, but the “inside-out” configuration is still very useful for measuring the single channel conductance, ionic selectivity, and voltage-dependent gating. These three properties are an excellent way to establish that currents are not due to artifacts (*e.g.*, gigaseal issues) or contaminants (*e.g.*, bacterial porins from the purification), and there are functional KvAP channels in the GUVs.

Channel conductance is most easily measured in patches with only one or two active channels. In the example shown in Figure 12, no channel openings are observed when the membrane is held at -100 mV, whereas at +100 mV, individual channel openings can be clearly resolved. The current histogram shows two peaks corresponding to the closed and the open states, and fitting them with a double Gaussian function yields a single channel current of 10.9 ± 0.85 pA, corresponding to a conductance of 109.2 ± 8.5 pS (in 100 mM KCl). Note that the single channel conductance depends on the solution (especially potassium concentration) and membrane composition^{32,33}.

Like many other K-channels, individual KvAP channels exhibit “chattering” bursts of rapid openings and closings. As demonstrated previously, potassium selectivity can be tested by using a different solution in the patch pipette (*e.g.*, patch pipette solution 90 mM NaCl, 10 mM KCl)²⁸.

Voltage-dependent gating is often studied in patches with multiple channels, so as to more easily obtain an ensemble average. While there is no obvious mechanism favoring the physiological (intracellular domain on GUV interior) and inverse (intra-cellular domain on the GUV exterior) insertion of KvAP in GUVs, in “inside-out” membrane patches the majority of functional channels have the “physiological” insertion²⁸. Figure 13 shows the response of a membrane patch containing multiple (> 10) channels to a series of 5 second depolarizing steps. Between each step, the patch is held at -100 mV for 30 s to allow channels with the “physiological” insertion to return to their resting state. When the potential is sufficiently negative (*e.g.*, $V < -60$ mV) most of the current is due to the gigaseal leak, and the occasional openings of one or two channels which are likely to have the “inverse” insertion. For steps to more positive potentials, increasing numbers of channels are observed until there are so many that individual openings and closings can no longer be resolved. Thus, the open probability of the channel is clearly voltage-dependent. The kinetics of KvAP activation and inactivation differ considerably between Black Lipid Membranes (BLMs)²⁶ and GUVs, but this is consistent with previous reports that Kv channel gating can be sensitive to the membrane composition and state³⁴.

Figure 1: GUV Electroformation Schematic: Droplets containing SUVs are deposited onto an electrode. Partial dehydration of the solution causes the SUVs to fuse to form a stack of membranes. Buffer is then added and an AC electric field applied. As the film swells, individual membranes detach from the stack to form GUVs. (This figure has been modified from Aimon *et al.*²⁸)

Figure 2: GUV Electroformation Chamber. The chamber is milled out of a PTFE-block with three wells (10 mm diameter, 5 mm depth). Two 0.5 mm diameter platinum wires are separated by 3 mm (edge-to-edge distance) and are positioned close to the bottom of the chamber to facilitate imaging of the wires. Bottom and top cover slips are held in place with vacuum grease, and sealing paste prevents any leaks from the wire holes on the side. The AC generator is connected with alligator clips to the wires. The chamber is based on one developed by Ernesto Ambroggio and Luis Bagatolli. (This figure has been modified from Aimon *et al.*²⁸)

Figure 3: Gel-assisted Spontaneous Swelling Schematic: Droplets containing a SUV suspension are deposited onto an agarose gel. As the droplet dehydrates, the SUVs fuse to form a lipid film. When the growth buffer is added, the film rehydrates and GUVs form at the surface. GUVs grow to a size of ~10 μm by swelling and fusing with neighboring GUVs.

Figure 4: Representative image of DPhPC GUVs containing KvAP growing on the platinum wire in a high salt buffer. The GUVs resemble bunches of grapes along the wire. Phase Contrast image using a 40x LWD objective.

Figure 5: DPhPC GUVs containing KvAP swelling on agarose gel. The GUVs are visible as faint spheres with a diameter of ~10 μm . The dark/bright spots are lipid/agarose aggregates from which the vesicles swell. Phase Contrast image with 40x LWD objective.

Figure 6: Images of a defect-free GUV (Egg-PC:Egg-PA 9:1 by mass) containing KvAP labeled with Alexa-488. left: DIC, right: Alexa-488 epifluorescence. Excitation: 470/50 nm, emission: 545/75 nm. Note the uniform fluorescence from KvAP with no visible aggregates.

Figure 7: Confocal images of lipid (magenta) and protein (green) fluorescence from electroformed GUVs grown in a low salt buffer (Egg-PC:Egg-PA 9:1 by mass). A) The white arrows mark (likely) GUVs, while the red arrow marks a potentially bi-lamellar vesicle with higher lipid fluorescence. Note that the fluorescence intensity is brighter in the center of this image because of the extremely large field of view. B) Zoom showing a small group of GUVs. Left (magenta): TexasRed-DHPE excitation: 543 nm laser line, emission: 605/70 nm. Center (green): KvAP labeled with Alexa-488 excitation: 488 nm laser line, emission: 515/30 nm. Right: overlay.

Figure 8: Confocal images of lipid (magenta) and protein (green) fluorescence from electroformed GUVs grown in physiological salt concentration (Egg-PC:Egg-PA 9:1 by mass). A) The GUVs (white arrows show likely unilamellar examples) are more sparse compared to the low salt protocol and can have extremely different protein concentrations (red arrow). B) Zoom showing a small group of GUVs. Left (magenta): TexasRed-DHPE excitation: 543 nm laser line, emission: 605/70 nm middle (green): KvAP labeled with Alexa-488 excitation: 488 nm laser line, emission: 515/30 nm. Right: Overlay.

Figure 9: Confocal image of lipid (magenta) and protein (green) fluorescence of GUVs (DPhPC) formed by gel-assisted swelling with a physiological salt concentration buffer. GUVs show a

more homogeneous protein density than electroformed GUVs prepared with physiological buffer. Left (magenta): BPTR-Cer 0.1% excitation: 543 nm laser line, emission: 605/70 nm. Middle (green): KvAP labeled with Alexa-488 excitation: 488 nm laser line, emission: 515/30 nm. Right: merge of the two channels.

Figure 10: Size distribution of defect-free proteo-GUVs (DPhPC) grown by electroformation in low salt buffer (top, N=94) or gel-assisted agarose swelling (bottom, N=68).

Figure 11: GUV protein density histograms: The protein density (number of proteins per unit area) of GUVs electroformed with low salt buffer (5 mM KCl, DPhPC) varies less than with physiological salt concentration (100mM KCl, Egg-PC:Egg-PA 9:1 by mass). The protein density of GUVs (DPhPC) grown by gel-assisted swelling in physiological salt buffer shows the least variation. Protein density is proportional to KvAP-A488 fluorescence intensity for these concentrations²⁸, and in each histogram the fluorescence intensities are normalized by the mean of the distribution. (The middle panel has been modified from Aimon *et al.*²⁸)

Figure 12: Single channel activity of GUV Membrane Patches (DPhPC 'inside-out' voltage convention). The GUV was grown in 100 mM KCl, 5 mM HEPES pH 7.4 on platinum wires. Single channels open after applying 100 mV potential on the patch. To the right of the inset is a histogram used to calculate the single channel conductance. The red line is a fit to a double Gaussian function with maxima at 4.70 ± 0.27 pA and 15.62 ± 0.58 pA, corresponding to a conductance of 109.2 ± 8.5 pS. The trace was filtered at 10 kHz with a 4-pole Bessel filter and recorded at 50 kHz.

Figure 13: Voltage-dependent gating channels in the membrane patch from a GUV formed on agarose in a high salt buffer (DPhPC, 'inside-out' voltage convention) A) Response of patch membrane current to a transient step in voltage. Pipette and bath solutions both contained 100 mM KCl, and the membrane was held for 30 s at -100 mV between successive voltage steps. On a close inspection one can see that the trace contains 1 or 2 channels that seem to open with negative voltages. Inset a) shows a zoom of the delayed opening and inset b) the delayed closing of the channel. Currents were filtered with a 4-pole Bessel filter at 10 kHz and recorded at 51.3 kHz. Offline the trace was down-sampled to 513Hz. The negative capacitance transient is cut off at -150 pA. B) Average current ($0.25 \text{ s} < t < 5 \text{ s}$) versus step voltage. Currents at positive voltages are larger because the channel open probability is voltage-dependent.

DISCUSSION:

Biomimetic model systems are an important tool for studying the properties and interactions of proteins and membranes. Compared to other reconstituted systems like BLMs or supported lipid membranes, GUV based systems present several opportunities including considerable control of membrane composition, tension and geometry, as well as being truly oil-free. However, incorporating transmembrane proteins, such as KvAP, into GUVs requires significant adaptations of conventional protocols for lipid-only GUVs. The electroformation protocol presented here was previously characterized and used for studying the biophysical principles of membrane protein distribution and dynamics in curved membranes^{2,4,35}. This work

demonstrates a new gel-assisted swelling protocol, adding to the set of methods for protein reconstitution in GUVs. Both protocols can produce defect-free GUVs containing high densities of KvAP, and measurements on inside-out patches confirm that these GUVs contain functional potassium-selective, voltage-dependent channels.

The two approaches have different strengths and weaknesses. When low salt conditions can be used, electroformation offers a good compromise between GUV yield and uniform protein density. Electroformation still gives reasonable yields with physiological salt concentrations, but the protein density can vary greatly between GUVs (see Figure 8 and 11). The density variations seem to be linked to the duration of electroformation, as low-salt buffer growth can also have substantial density variations if the growth continues much longer than 2 hours. In contrast, GUVs produced by gel-assisted swelling have a remarkably uniform protein density, even for physiological buffers. However, the fraction of multi-lamellar vesicles is greater, and agarose auto-fluorescence complicates quantification of low protein densities¹⁶. Using polyvinyl alcohol in place of agarose has been reported to improve the gel-assisted GUV yield when lipids were deposited from chloroform²⁰, but we were unable to produce GUVs using polyvinyl alcohol with SUV solutions. If a lower yield of defect-free GUVs is acceptable, gel-assisted swelling has a clear advantage for experiments that require a uniform protein distribution.

Electroformation and gel-assisted swelling also have quite different equipment requirements. The gel-assisted swelling protocol uses little specialized equipment except for the plasma cleaner, which is not essential as there are many alternative methods to produce clean, hydrophilic glass. In contrast, electroformation requires a custom chamber with wire electrodes. Platinum wire is expensive, but for this protocol GUVs grew more readily with platinum than titanium wires, and GUVs did not grow on ITO slides when using physiological salt concentrations. The diameter of the electrodes (0.5 mm) is a compromise between electrode surface and price. The chamber shown in Figure 2 is based on a design by Luis Bagatolli¹⁵ and Ernesto Ambroggio, and was machined from Polytetrafluoroethylene (PTFE) to allow cleaning with most solvents. However, polyacetal or polyvinyl chloride (PVC) should also work well. The ability to remove the platinum wires for aggressive cleaning is important, and when first learning the protocol, it is very helpful to be able to observe GUV growth *in situ* through the bottom cover slide. The smaller, closed wells prevent solution from sloshing backwards and forwards and also allow tests of several lipid compositions in parallel. However, a special custom chamber is not essential when starting out. For example, simple, single-well chambers can be improvised by tacking two wires down to the bottom of a small petri dish, or using sealing wax to sandwich them between two glass slides, or simply poking them through the cap of a small vial.

Both the electroformation and gel-assisted swelling protocols should produce a sufficient number of defect-free GUVs for micro-manipulation and patch-clamp experiments. However, the GUV yield depends sensitively on the formation of the bilayer stack when the SUV solution is partially dehydrated. If difficulties are encountered in growing GUVs (i.e. no or few GUVs are visible in the growth chamber), it can be very helpful to grow lipid-only GUVs using a lipid/chloroform solution in place of SUVs^{15,16} (and a low salt buffer). If GUVs do not grow well

from a lipid/chloroform film then something fundamental is wrong (*e.g.* incorrect lipid solutions or buffers, grease or dirt on the electrodes, incorrect voltage or frequency, etc.). However, if GUVs grow from lipid/chloroform films but not the SUV films, then the issue is likely to be with the partial dehydration.

The partial dehydration step is most easily optimized using lipid-only SUVs because there is no risk of “denaturing” lipids by excessive dehydration. For electroformation, it can be helpful to examine the wires after the SUV droplets have been allowed to dry to check there is bright lipid fluorescence at each spot where a droplet was deposited. If the lipid fluorescence disappears after the chamber is filled with growth solution, then either the growth solution has to be added more carefully, or the SUVs need to dehydrate for longer so the film attaches more firmly to the wire. During the growth, make sure neither the wires nor solution move within the chamber (*e.g.* when putting the chamber on the microscope) to avoid stripping GUVs off the wires. When GUVs grow well, they are usually easy to see in phase contrast images. However, smaller GUVs are often clearer using lipid fluorescence, which is also helpful for seeing how the membrane stack has changed during the growth. Examine all surfaces of the wires (inside, outside, top and bottom) in all wells as well as the chamber floor before discarding the growth, because yields can vary considerably from one spot to another.

The handling and storage of GUVs is simple compared to cells, but GUVs are quite sensitive to osmotic stress, shear forces and adhesion. Smooth, gentle motions are important when harvesting or transferring GUVs, and it is important to passivate surfaces to prevent GUVs from adhering and exploding. However, for patch clamp experiments the chamber must be thoroughly rinsed after passivation so that the passivation solution cannot coat the patch pipettes and prevent gigaseal formation. For passivation, the beta-casein treatment is simple and effective, and compared to bovine serum albumin, which has a lipid transport function, beta-casein has more limited interactions with lipid bilayers and should be preferred when working with GUVs³⁶. By varying the incubation time, it is possible to get GUVs to adhere without exploding. However, the GUVs will not adhere to the cover slide as firmly as cells, and so care must still be taken during any procedure that can induce flow in the chamber (*e.g.*, buffer perfusion, moving the chamber).

Patch-clamp recording is a classical method for studying voltage-gated ion channels like KvAP and this protocol is derived from standard techniques for obtaining “inside-out” patches from adherent cells. A standard patch-clamp set-up in which the patch pipette descends obliquely (*e.g.* 30-60 degrees from horizontal) into a small Petri dish should work well. However, clearer images of the patch pipette and gigaseal region can be obtained using a chamber in which cover-slips form the chamber top and bottom (~ 1 mm separation) so the patch pipette can enter horizontally from the side. Because large patch pipette pressures are not needed, the pressure can conveniently be controlled with a syringe and monitored with any simple pressure meter (*e.g.* improvised water manometer). It can be helpful to first practice patch clamp experiments with lipid-only GUVs grown using a lipid/chloroform solution and low-salt buffer. Because the yield of defect-free GUVs is very high, there will be plenty of perfect GUVs to work

with even if many are destroyed during the harvesting and transfer to the experimental chamber.

With a little practice, excised membrane patches with stable gigaseals can be readily obtained from DPhPC GUVs and KvAP-DPhPC GUVs. To achieve a high success rate, it helps to carefully select round, but slightly-fluctuating, defect-free GUVs and check that the patch pipette is clean (looking for lipids in fluorescence) before attempting to form the gigaseal. When a membrane “tongue” enters the patch pipette, the gigaseal typically forms quickly (< 1 second) without any need for strong suction or specific holding voltage. While a bad seal may improve as the membrane crawls further into the patch pipette, often the seal remains poor because the pipette interior was contaminated and it is necessary to start over with a fresh patch pipette and GUV. DPhPC forms very stable membrane patches (tens of minutes) with an excellent seal resistance even at high voltages (e.g. ± 150 mV). SOPC:cholesterol (3:1 by mole) can also form very stable patches but can require higher suction to seal, while Egg-PC patches seem to break more easily.

For longer patch-clamp sessions it may be necessary to adjust the osmolarity of the chamber solution. If the osmolarity is too much lower than the GUV growth buffer, GUVs become swollen, tense and spherical and it may not be possible to aspirate the GUV membrane far enough into the patch pipette to form a gigaseal. This can often be fixed by simply waiting 10 or 20 minutes as evaporation from the chamber increases the external solution osmolarity until the GUVs deflate and begin to fluctuate. Conversely, if the chamber is left open for too long the osmolarity of the external solution can increase until GUVs deflate, tubulate and bud. This can be avoided by blocking evaporation from the chamber (e.g., with mineral oil) or periodically adding distilled water to replace the water that has evaporated.

Because proteins can be excluded from excised membrane patches³¹, the number of active channels in an excised patch is not simply related to the density of protein in the GUV membrane. Fluorescence measurements suggest the concentration of KvAP in the patch membrane is much lower than the GUV²⁸ and it can be quite easy to obtain patches containing only a small number of channels. However, if patches contain too many channels for single channel recording, the obvious steps of using patch pipettes with smaller tips and/or lowering the protein-to-lipid density in the SUV mix are effective. In contrast, to perform ensemble measurements on patches containing many channels, it can be helpful to start with a relatively high protein density (e.g., 1:10, protein to lipid by weight), use protein fluorescence to select GUVs with a high protein density, use larger patch pipettes (e.g., 2 to 3 micron tip diameter) and aspirate quickly to try to form the seal rapidly. Clearly, the “whole-cell”-type configuration (i.e., “whole-GUV”) would be ideal to characterize all the channels in a GUV, but unfortunately the “whole-GUV” configuration poses several technical issues³⁷.

The solutions, lipids and protein concentrations in this tutorial are merely provided as a starting point, and can be altered to suit the needs of a particular experiment following a few considerations.

All solutions should include a pH buffer such as HEPES or TRIS to ensure proteins are not exposed to extremes of pH. The SUV buffer should have as low a concentration of solutes as the protein will tolerate (*e.g.*, 5 mM salt or lower), as solute concentrations increase during the partial dehydration step and high salt concentrations can denature the protein or cause the lipid film to rapidly delaminate during the rehydration step. Small amounts of sugars such as trehalose (*e.g.*, 1 mM to 5 mM) are thought to protect the protein during dehydration²³. While trehalose has been implicated in anhydrobiosis and is believed to protect membrane and proteins against dessication³⁸, sucrose or glucose may work equally well.

For the growth buffer, the salt concentration is especially important as this will influence the parameters for optimal GUV growth (*e.g.* electroformation voltage, frequency and duration). In contrast, the primary constraint for the observation buffer is that it should have the same osmolarity as the growth buffer. The inclusion of sucrose and/or glucose in the “growth buffer” and “observation buffer” can be helpful to ensure GUVs sediment to the bottom of the observation chamber, while a difference in refractive index between GUV interior and exterior helps with phase contrast or DIC microscopy. Electrophysiologists often include calcium or magnesium ions to the bath and/or patch pipette solutions to enhance gigaseal formation with cell membranes, but these do not appear to be essential for GUVs. Indeed, divalent ions such as magnesium and calcium can induce lipid phase separation and facilitate adhesion, so if these problems arise it may be helpful to add 1mM EDTA.

Clearly, a key attraction of reconstituted systems when compared to cells is the ability to control lipid composition. DPhPC GUVs grow well and form stable excised membrane patches, and these protocols have also worked effectively for lipid mixtures containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylserine (PS) and cholesterol. However, GUV growth is sensitive to both lipid composition and buffers¹⁵, and so protocol parameters (*e.g.* amount of lipid deposited, electroformation voltage/frequency) may need to be adjusted for lipid mixtures containing high concentrations of PE, charged lipids (PG, PA, PS), or cholesterol. When starting out, Egg-PC, DOPC or DPhPC are a good first choice, and it is also very helpful to include a fluorescent lipid to observe GUV growth and to distinguish GUVs from multilamellar vesicles with two or more bilayers. Lipid mixtures can be prepared by combining SUV suspensions of stock solutions, as the lipids mix during the partial dehydration step (provided the temperature is higher than any individual phase transition temperature). Using higher SUV concentration (*e.g.*, 10 mg/ml) stock solutions allows considerable flexibility, and these can then be diluted to 3 mg/ml before dehydrating the suspension.

If attempting to adapt these protocols to other trans-membrane proteins, it is very important to be able to both directly observe the incorporation of protein into the GUVs and test protein function. While not an issue with KvAP, there is always the possibility that during the rehydration step the trans-membrane protein will remain in the membrane stack leading to the formation of lipid-only GUVs. Fluorescent labeling of the protein is very convenient as it provides a rapid and unequivocal way to observe protein incorporation into GUVs and also check for aggregation within GUVs. It is also very important to test protein function in the GUVs

to confirm that the protein was not damaged during the reconstitution process. For ion channels like KvAP, patch-clamp measurements can establish the presence of functional channels in the GUVs. However, a fluorescently-labelled, high-affinity ligand (e.g. toxin, substrate or anti-body) would also be very helpful for testing the state of proteins in GUVs.

In summary, this article demonstrates how to produce proteo-GUVs containing the voltage gated potassium channel KvAP and characterize them using fluorescence microscopy and electrophysiology. Hopefully these methods can be adapted to new classes of membrane proteins and provide a foundation for more complex *in-vitro* systems for studying and building up living matter from its fundamental components.

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The authors have nothing to disclose.

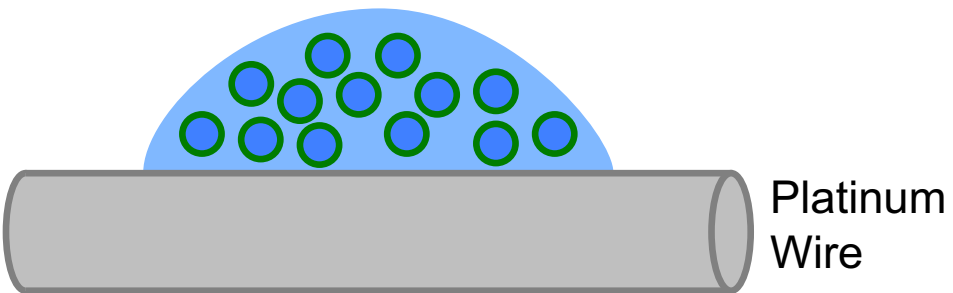
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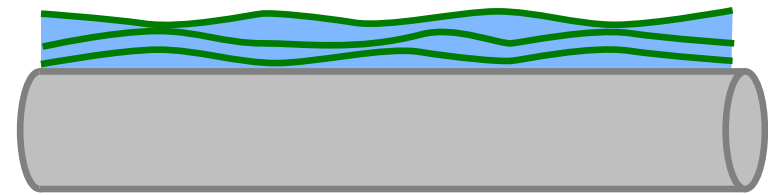
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SUVs (~100nm)
Figure
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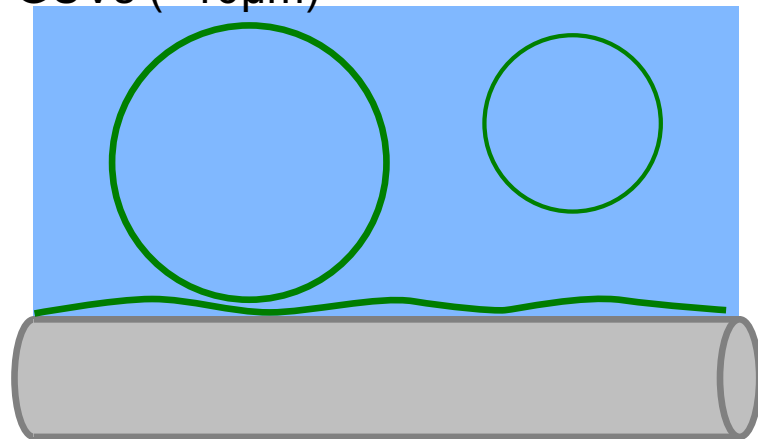
SUV fusion via
partial dehydration



Rehydration under
AC electric field



GUVs (~10μm)



GUV growth

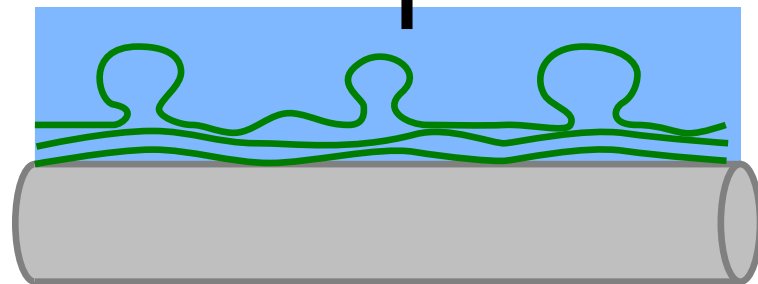


Figure 2

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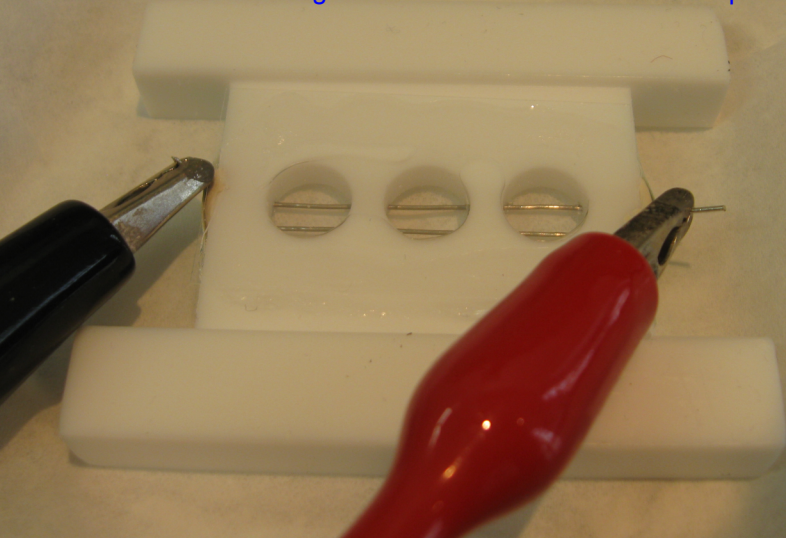


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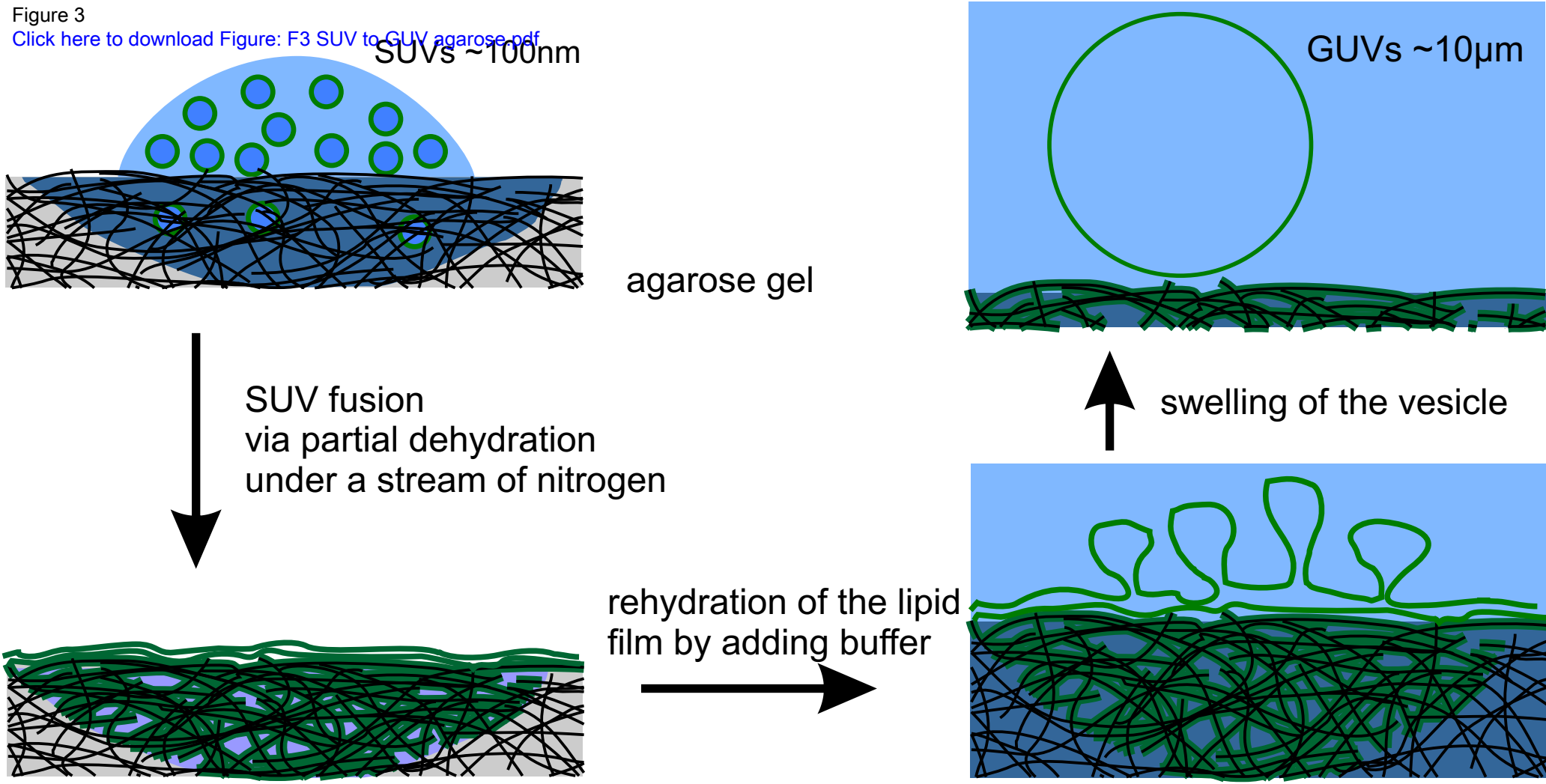
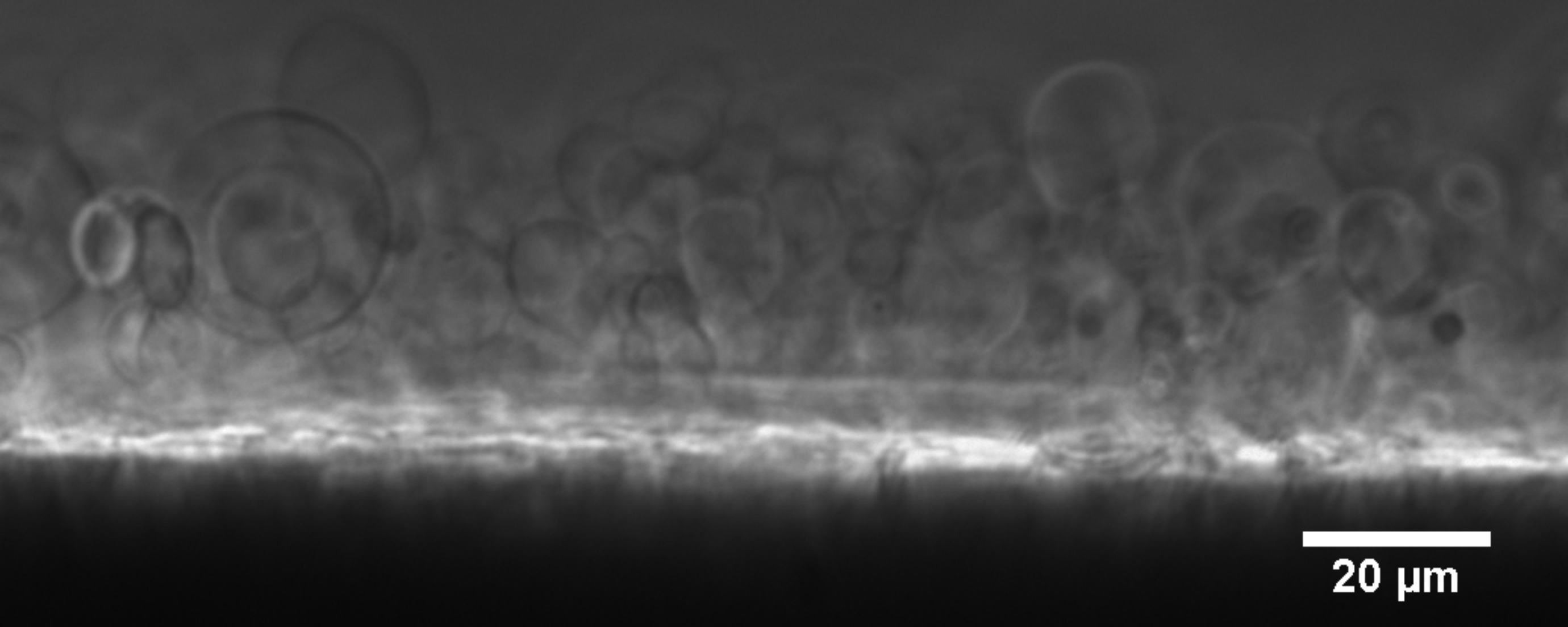


Figure 4

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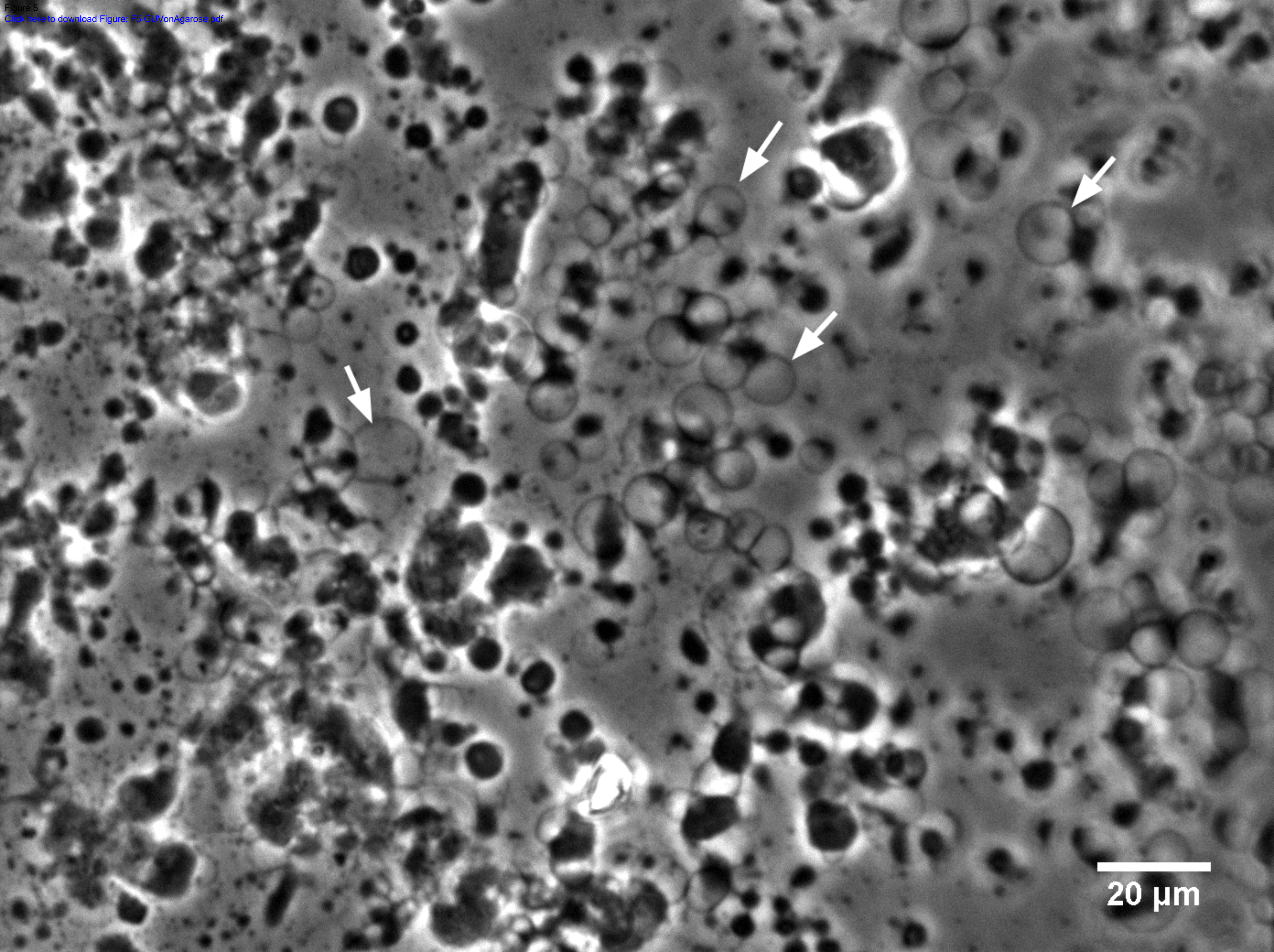


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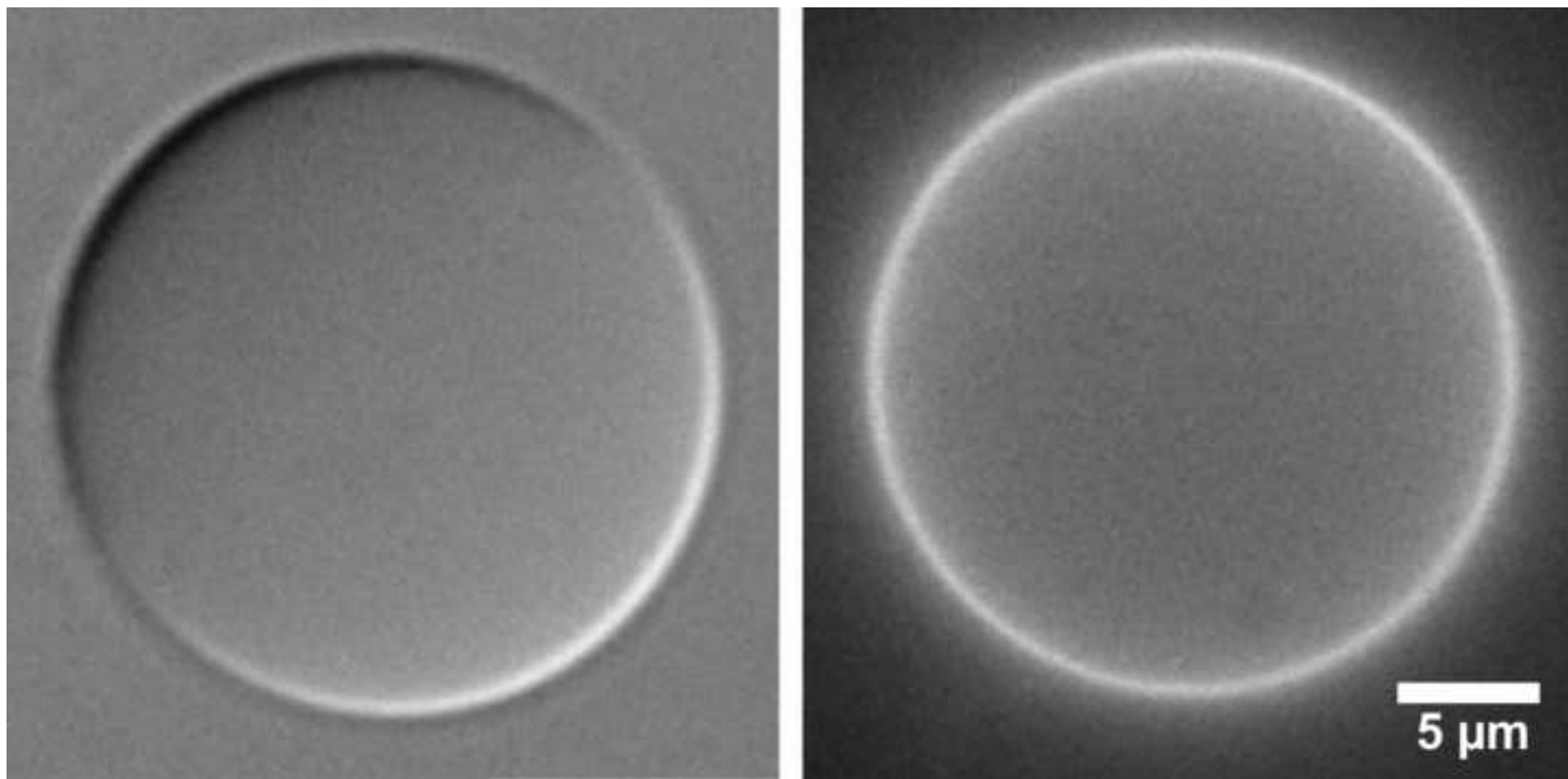


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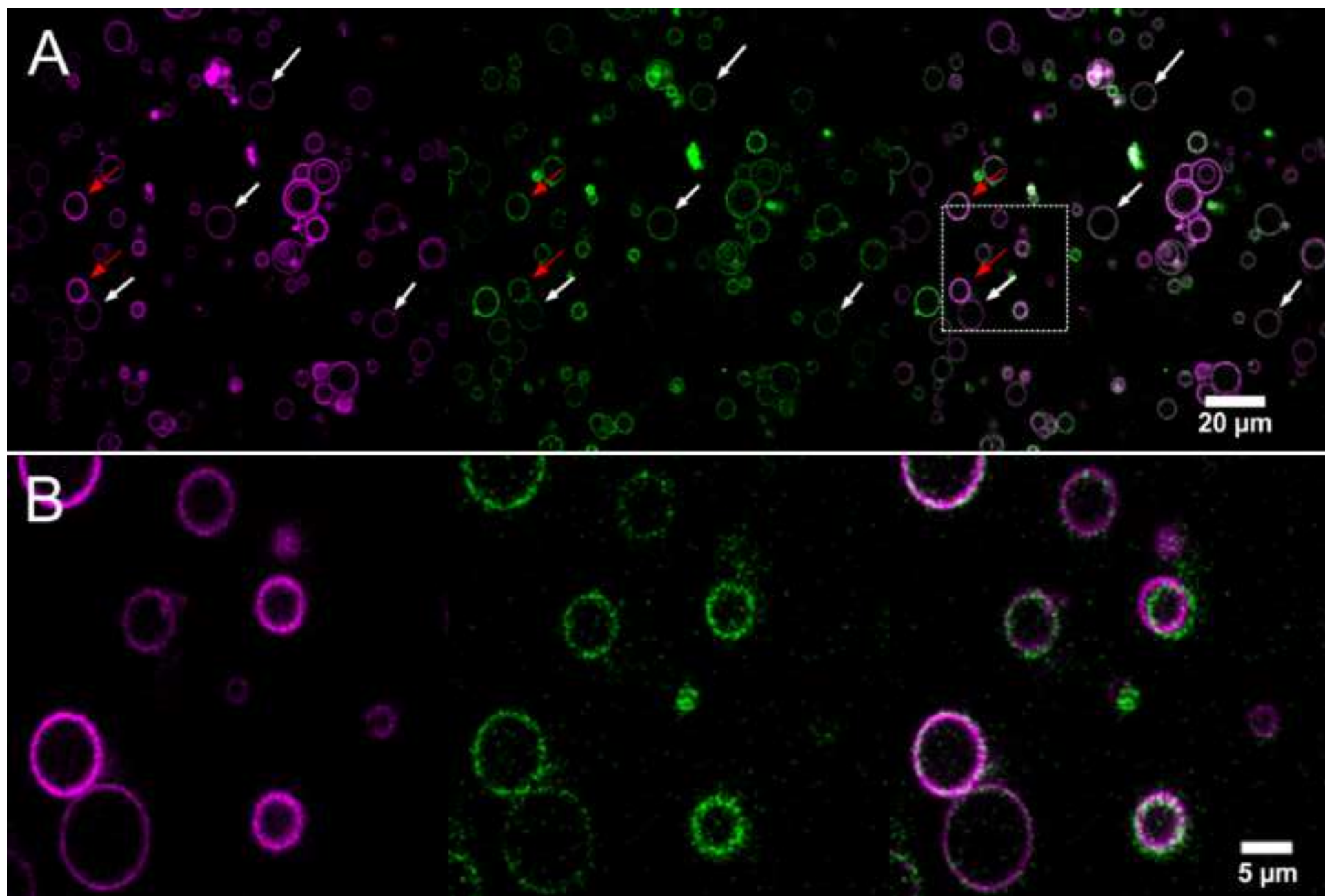


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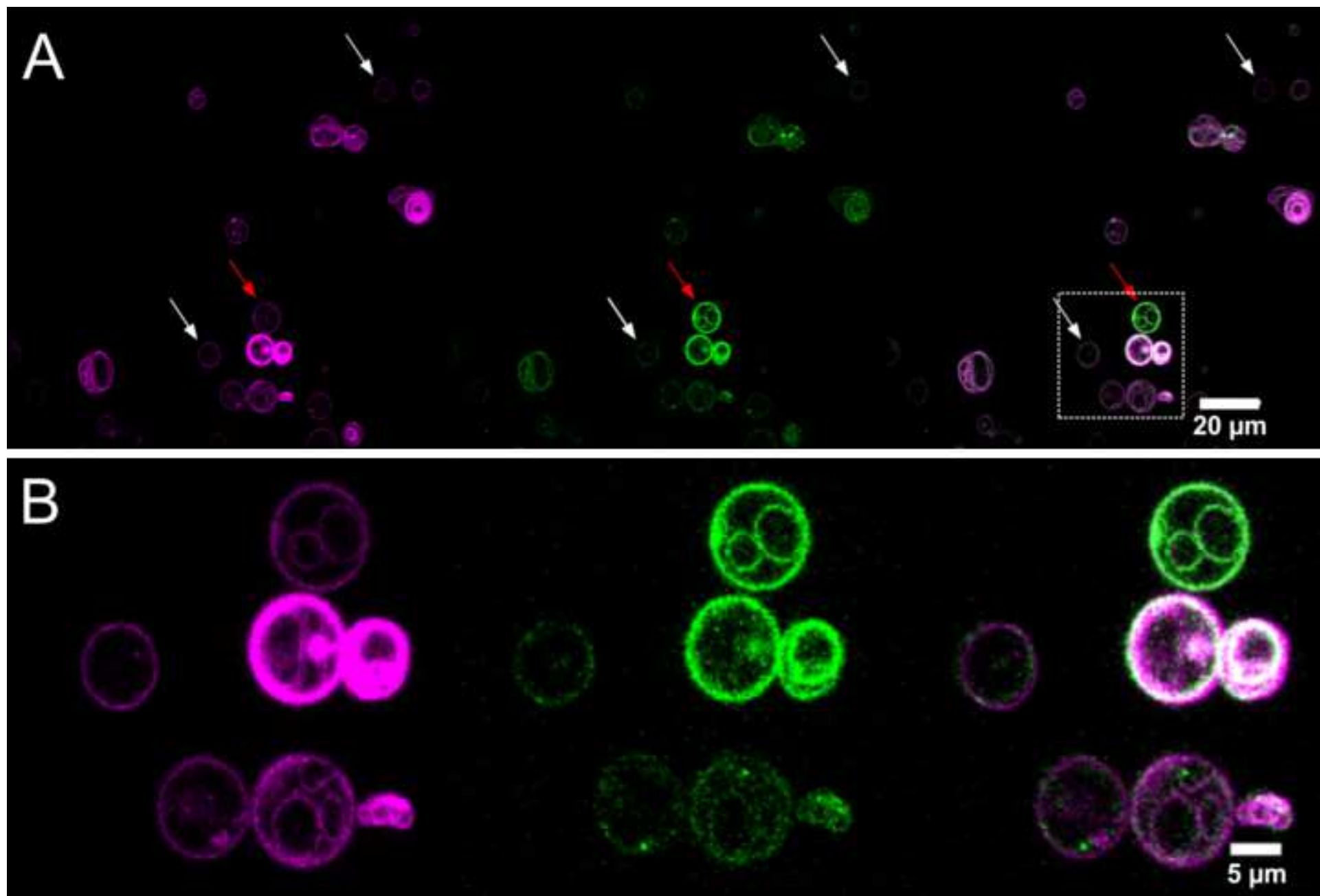


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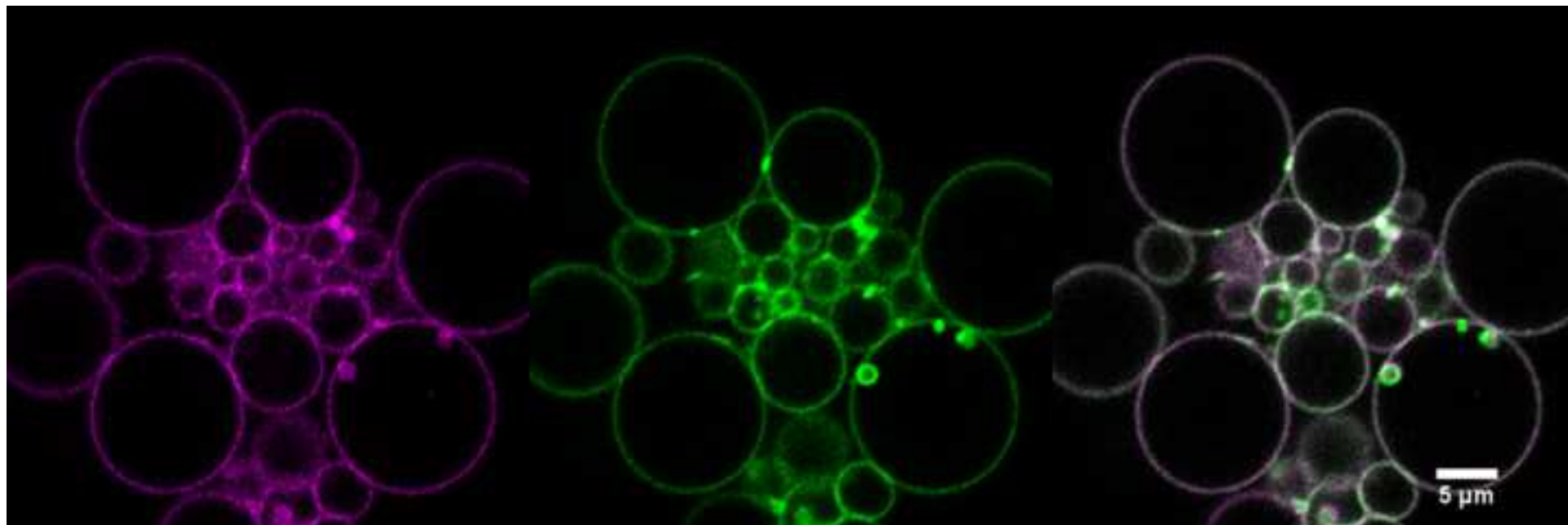


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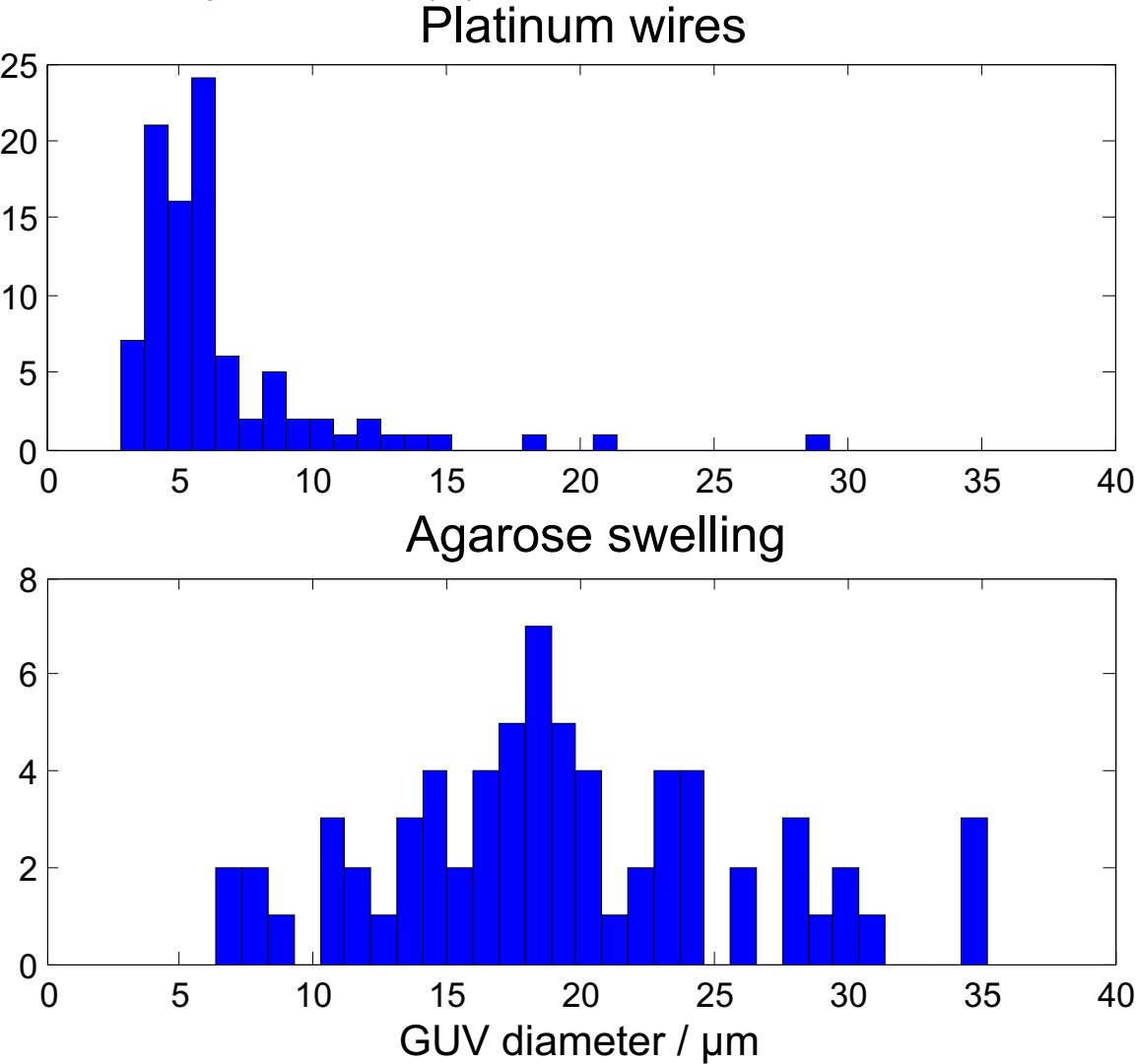
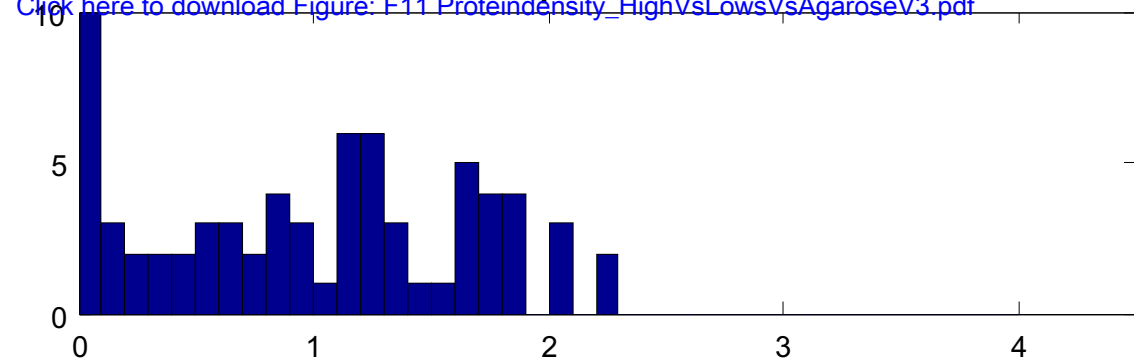
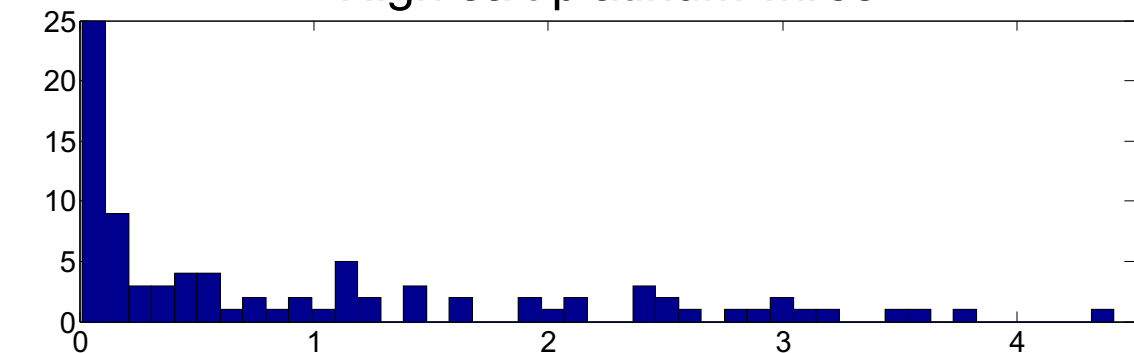


Figure 11
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Low salt platinum wires



High salt platinum wires



High salt agarose swelling

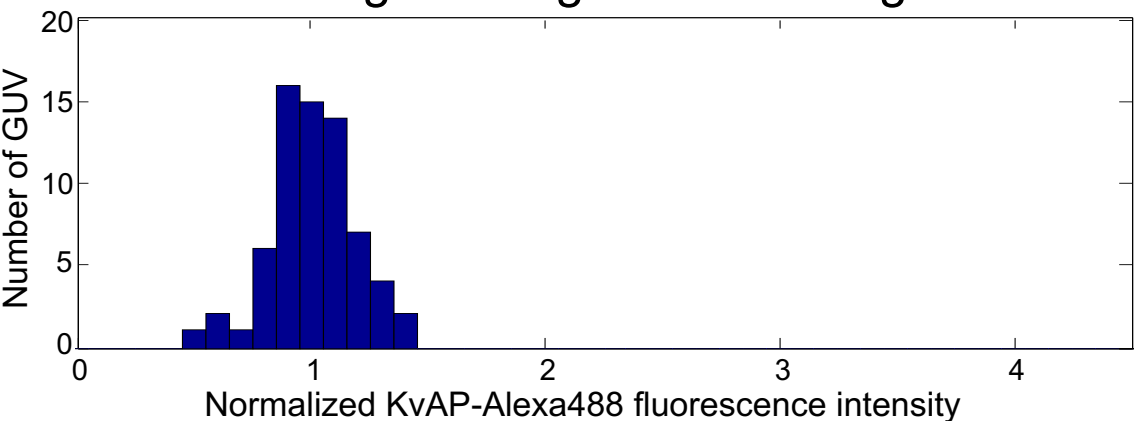


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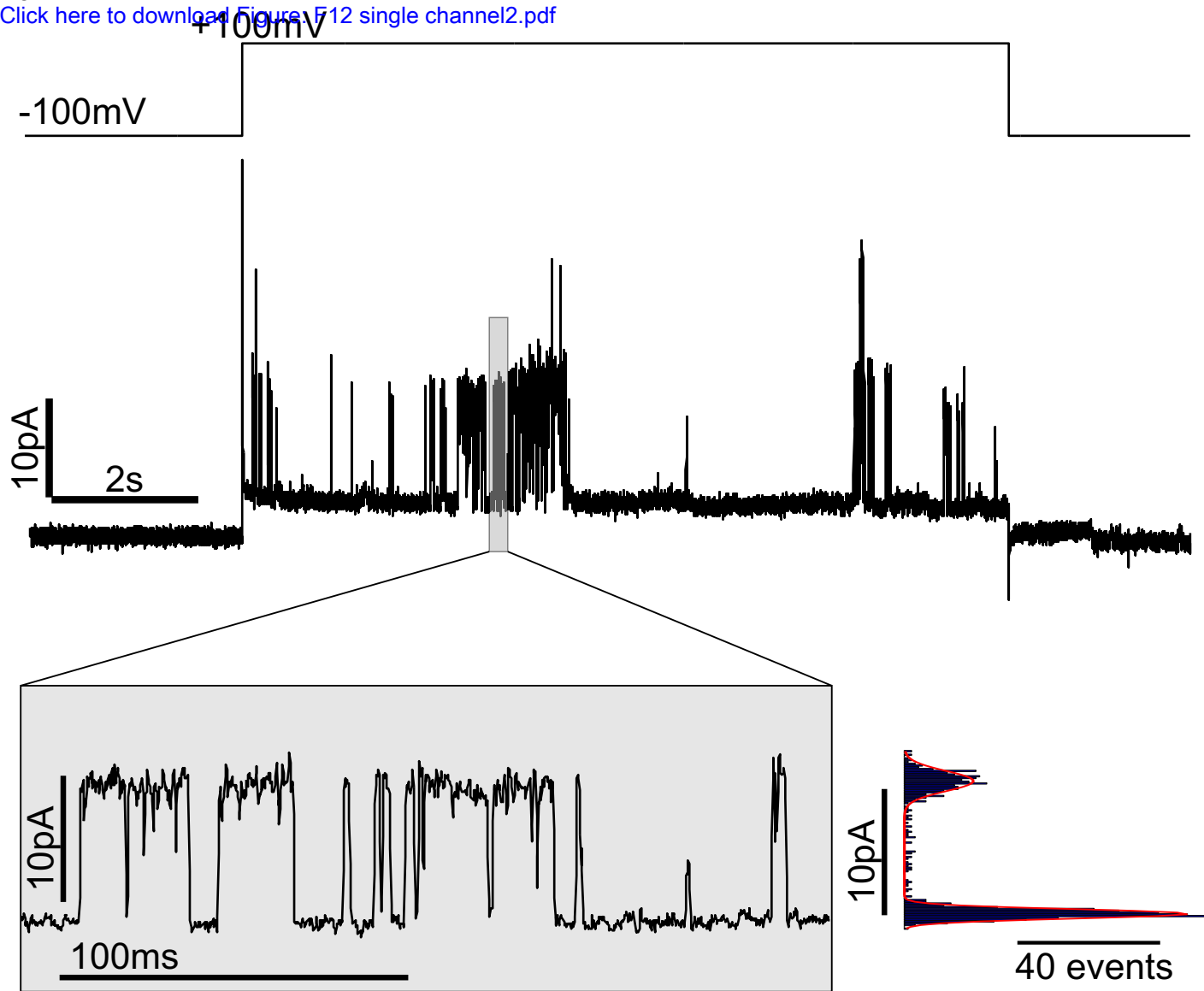
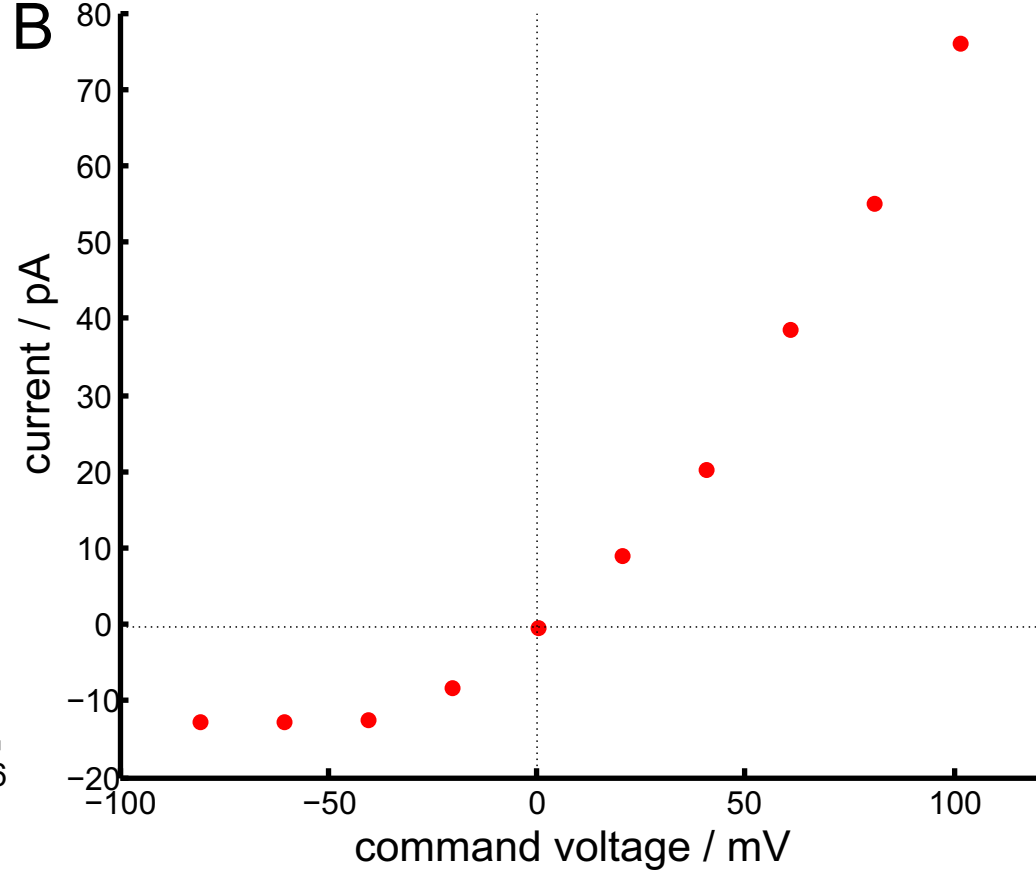
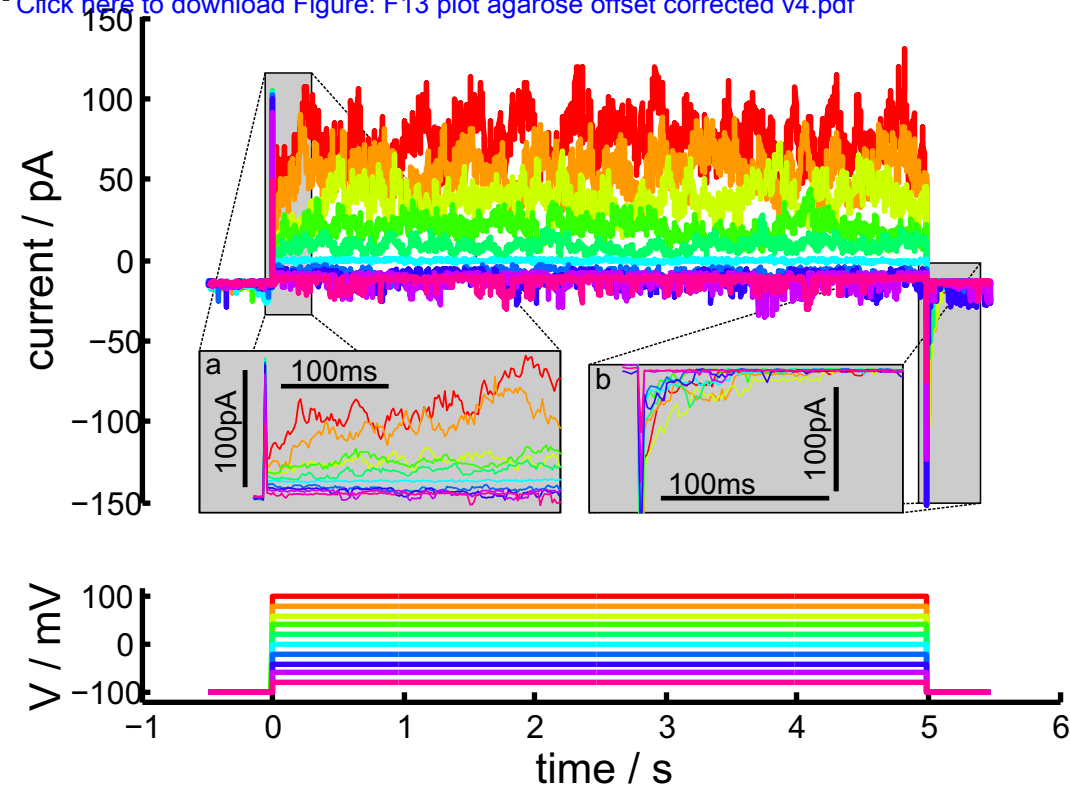


Figure 13

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Name of the Material/Equipment	Company	Catalog Number	Comments/ Description
DPhPC (1,2-diphytanoyl- <i>sn</i> -glycero-3-phosphocholine)	Avanti Polar Lipids	850356P	
Egg PC L- α -phosphatidylcholine (Egg, Chicken)	Avanti Polar Lipids	840051P	
Egg PA L- α -phosphatidic acid (Egg, Chicken)	Avanti Polar Lipids	840101P	
18:1 (Δ 9-Cis) PC (DOPC) 1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine	Avanti Polar Lipids	850375P	
cholesterol (ovine wool, >98%)	Avanti Polar Lipids	700000P	
TRed-DHPE	Invirtogen	T-1395MP	labeled lipid
BPTR-Ceramide	Invirtogen	D-7540	labeled lipid
Choloroform	VWR	22711.290	AnalaR Normapur
Acetone	VWR	20066.296	AnalaR Normapur
Ethanol	VWR	20821.330	AnalaR Normapur
Kimwipe	Kimtech	7552	
Hamilton syringes	Hamilton Bonaduz AG		diverse
Amber vials and teflon cups	Sigma	SU860083 and SU860076	
Parafilm	VWR	291-1214	
microcentrifuge tube	eppendorf		diverse
Agarose	Euromex	LM3 (1670-B, Tg 25.7C, Tm 64C)	
Sucrose	Sigma	84097-1kg	
Glucose	Sigma	G8270-1kg	
Trehalose	Sigma	T9531-25G	
KCl	Sigma	P9333-1kg	
Hepes	Sigma	H3375-100G	
TRIS	Euromex	EU0011-A	
Osmometer	Wescor	Vapro	
pH meter	Schott instruments	Lab850	
Sonicator	Elmasonic	S 180 H	
Syringe filter 0.2 μ m	Sartorius steim	Minisart 16532	
Function generator	TTi	TG315	
Platinum wires	Goodfellow	LS413074	99.99+%, d=0.5mm
Polytetrafluoroethylene	Goodfellow		
Dow Corning 'high vacuum grease'	VWR	1597418	
sealing paste	Vitrex medical A/S, Denmark	REF 140014	Sigillum Wax
Cover slides 22x40 mm No1,5	VWR	631-0136	
Cover slides 22x22 mm No1,5	VWR	631-0125	

plasma cleaner	Harrick	PDC-32G	airplasma, setting 'high'
petri dishes	Falcon BD	REF 353001	3.5 cmx1cm
patch clamp amplifier	Axon instruments	Multiclamp700B	
DAQ Card	National Instruments	PCI-6221	
Labview	National Instruments	version 8.6	
Glass pipettes boro silicate OD 1mm ID 0.58mm	Harvard Apparatus	GC100-15	
Electrode holder	Warner Instruments	Q45W-T10P	
Micromanipulator	Sutter	MP-285	
pipette puller	Sutter	P-2000	
Camera	ProSilica	GC1380	
Zeiss microscope	Zeiss	Axiovert 135	
Objective	Zeiss		40x long working distance, Phase contrast
Objective	Zeiss		100x Plan-Apochromat NA 1.3
Filterset GFP (for Alexa-488)	Horiba	XF100-3	
Filterset Cy3 (for TexasRed)	Horiba	XF101-2	
beta-casein	Sigma	C6905-1G	
confocal microscope	Nikon Eclipse TE 2000- E		D-Eclipse C1 confocal head
Objective	Nikon		Plan Fluor 100× NA1.3
matlab	Mathworks		for image processing and analysis of the current traces

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Author(s): Matthias Garten, Sophie Aimon, Patricia Bassereau, Gilman E. S. Toombes

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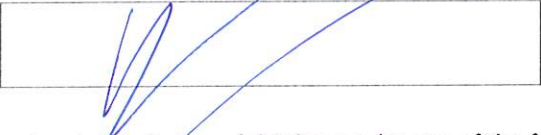
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Response to Reviewers

We would like to thank all the reviewers for reading the manuscript so carefully. We greatly appreciate your detailed comments and suggestions, which have helped us improve the manuscript.

Detailed responses to comments are provided below.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper described two different ways for preparing GUVs using dehydration/rehydration procedure of KvAP-containing vesicles. The methods are innovative and easy to reproduce. It is suitable for publication with JoVE.

Major Concerns: *The channel property of KvAP appears to be different from what was reported before. Please verify before publishing.*

Our previous characterization of KvAP in GUVs confirmed the key channel properties, potassium selectivity and voltage-gated activation, are present (Aimon et. al., PLoS One, Volume 6, e5529). While the differences between KvAP conductance and inactivation in BLMs and GUVs are interesting, they are not unexpected because these properties depend on the channel environment.

Conductance: Our measured conductance of ~110pS is for 100mM KCl in an uncharged lipid membrane, whereas Ruta et. al. (2003) reported a conductance of ~170pS for 150mM KCl with a negatively charged membrane. We feel the lower conductance in GUVs is consistent with the lower [K⁺] concentration and uncharged membrane.

Inactivation: C-type inactivation of K-channels has been shown to depend on the channel environment. Similar to our results for KvAP, the Kv1.2/2.1 paddle chimera was reported to inactivate rapidly when reconstituted into Black Lipid Membranes (Figure 1A, Schmidt and MacKinnon, PNAS, Vol 105, p19276) but not when reconstituted into oocytes (Figure 1B). For KvAP, inactivation in BLMs depends on lipid composition (JMB 2009, Vol 390, p902) and slows remarkably in the presence of alkanols (BJ, Vol 98, p762). Given these results, the much slower and incomplete C-type inactivation of KvAP in GUVs is not surprising even though the mechanism is not yet understood.

We discuss these issues in more detail below.

Minor Concerns:

1) Line 65, swelling with an electric field (electroformation; suggesting that electric field causes swelling) is a little confusing, and needs a different wording. Something like " swelling in the presence of an alternating electric field..." .

We agree with the reviewer and the text now reads -

" While swelling in the presence of an alternating electric field (electroformation) gives a high yield of defect-free GUVs, ..."

2) Line 76, "micrometer-scale droplet (GUV interior) ..." is not clear. It seems that the droplet or some kind of particles guide the coating of two monolayers. The two techniques, emulsion-based vs. rehydration-based, have a fundamental difference: monolayers used in the former and bilayers used in the latter.

We appreciate the reviewer's concern and have reworded this sentence to focus on monolayer/bilayer difference they identify.

"While there are many different protocols to produce GUVs, most fall into two categories - emulsion based approaches^{11,12} and techniques based on rehydrating a lipid film¹³⁻¹⁶. In emulsion-based methods, the inner and outer leaflets of the GUV membrane are assembled sequentially from lipid monolayers at water/oil interfaces. This approach is ideal for ..."

3) line 85, "solvent pushes layers apart" is something that is difficult to understand. The rehydration of membranes stacked together leads to the membranes to laminate.

We agree that this phrase could be misinterpreted and have modified it to -

" If this film is then placed in contact with an aqueous buffer, membranes in the stack will move apart as solvent flows between them, and at the surface of the stack, individual membranes can detach to form GUVs."

4) Line 96, "spontaneous swelling" is new. It appears to point to both electroformation and gel-assisted swelling. The authors seem to point to electroformation.

Classical "spontaneous swelling" is defined on line 88 but we have revised this section to clarify the connection between "gel-assisted swelling" and classical "spontaneous swelling".

"An alternative approach to boost the yield of defect-free GUVs is "gel-assisted swelling", in which the lipid solution is deposited onto a polymeric gel substrate rather than the passive (e.g. glass, PTFE) substrates used in classical "spontaneous swelling". When the resultant lipid/gel film is rehydrated, GUVs can rapidly form even for physiological buffers^{16,20}."

5) *Line 107-108, it is an overstatement that proteo-suvs can be formed by detergent removal in the presence of lipids. Very often, there are multilamellar vesicles. It does not matter whether to start with SUVs or not here, though*

We agree with the reviewer that the uni-lamellar character of the vesicles is not crucial for this protocol, and have modified the text on line 107-108 to read -

“or protein-containing small unilamellar vesicles (proteo-SUVs) and/or multi-lamellar vesicles (proteo-MLVs) formed by detergent removal in the presence of lipids.”

Additionally, on line 124 we have added -

“Dehydration/Rehydration: A protein-containing lipid film is formed by partial dehydration of a proteo-SUV (or proteo-MLV) solution and GUVs are then grown as for a pure lipid film.”

6) *Line 109: should mention that three categories of methods are listed. The flow would be better and easier for readers to follow.*

We appreciate this suggestion and have modified the text to read,

“Most methods to incorporate these membrane proteins into GUVs fall into three categories.”

7) *Line 112, the pre-formed lipid-only GUVs are used.*

We thank the reviewer for suggesting this useful clarification and have modified the text to read -

Line 114 : “Direct Insertion: Trans-membrane protein suspended in detergent is mixed with pre-formed, lipid-only, mildly detergent solubilized GUVs, ...”

Line 120 : “GUV/Proteo-SUV Fusion: Protein in proteo-SUVs is combined with pre-formed, lipid-only GUVs”

8) *Line 136: This is a caption. Needs to be highlighted.*

We apologize for this formatting error, and have modified this section as we are unsure if a caption is allowed in the introduction.

This section now reads (Line 134 :)

“ Importantly, KvAP proteo-SUVs do not have to be produced for each GUV preparation, as they can be stored in small (e.g., 10 µl) aliquots at -80 °C for extended periods of time (> 1 year). Electro-formation or gel-assisted swelling can then be used to grow GUVs from the KvAP proteo-SUVs (or proteo-MLVs).

The key steps for the electro-formation protocol are illustrated in Figure 1.”

9) Line 151: "defect-free" needs some detailed explanation here.

We have added a more detailed explanation of "defect-free" in this section -

Line 152 : "However, the yield of isolated, apparently defect-free GUVs (*i.e.* the GUV membrane is uniform at optical length-scales and does not enclose any objects) is lower, ..."

and discuss this further in the later sections.

10) Line 163: "dehydrating" is the key for the two methods, and it does not make sense that the lipids in organic solvent can help.

We thank the reviewer for identifying that the ambiguity in this section.

We agree that growing GUVs from a solution of lipids in organic solvent does not directly help someone optimize the partial dehydration of SUVs, and anyone familiar with GUVs should clearly begin with lipid-only SUVs or proteo-SUVs.

However, the yield of GUVs grown from SUVs is fairly sensitive to parameters during the SUV deposition and partial dehydration. For someone who is first starting to work with GUVs, we feel it can be very helpful to use a conventional protocol for lipid-only GUVs, because even if they don't closely follow the protocol they should still obtain thousands of GUVs per chamber. Using a protocol based on lipids in organic solvents should allow a newcomer to confirm their electroformation chamber or gel-substrate work while also learning to identify GUVs growing the chamber. They should then be ideally placed to optimize the deposition of membranes from the SUV solution.

We have modified this paragraph to clarify this point.

"Growing protein-containing GUVs can be more difficult than lipid-only GUVs. In particular, the final GUV yield can depend sensitively on exactly how the SUV solution is deposited and dehydrated to form the membrane stack. For someone without any previous experience with GUVs, it may be helpful to first grow lipid-only GUVs following a conventional protocol^{15,16} in which the membrane film is formed by depositing lipids from an organic solvent. Once the conventional protocol is working well, SUV deposition and partial dehydration can then be mastered using lipid-only SUVs, which are also very helpful when adjusting the protocol for a new lipid composition. When GUVs grow reliably from lipid-only SUVs, it is then only a small step to produce protein-containing GUVs from proteo-SUVs."

11) Line 176-78, why the sucrose concentration is different from the low salt growth buffer to the "physiological salt"? isn't next section 1.4 controlling the osmolarity?

The reviewer is absolutely correct that the sucrose and glucose concentrations just need to match between Growth and Observation Buffers. We have added a note referring readers to the discussion.

"Note: These buffers are only examples. See the discussion to adapt the buffers for other experiments."

12) Line 203, where is the Alexa-488 located to the KvAP?

We have added a note indicating that -

"Note: Wild-type KvAP contains one cysteine per monomer located near the intra-cellular C-terminus (amino acid 247)."

13) Line 231, lipid degradation and lipid oxidation are talked here. Did the authors want to say it is oxidation? Degradation let the readers think about hydration.

We thank the reviewer for identifying this error. We have modified this section to read -

"Lipids, especially unsaturated lipids, can easily breakdown. Store lipid solutions at -20 °C (or -80 °C) under argon and use within 6 months. Lipid breakdown products can be detected with Thin Layer Chromatography."

14) Line 267, the humidity of the air affects drying significantly. Need to spell out.

We agree this is an important issue, and have modified the note to address the issue of air humidity -

" Because air humidity influences the rate of drying, the drying time and/or air humidity can be adjusted for optimal results (Baykal-Caglar et al, 'Preparation of giant unilamellar vesicles from damp lipid film for better lipid compositional uniformity', BBA, 2012)."

15) Line 295, what is the current flowing across? The size of the current, and does it heat up the chamber?

For our chambers, the current is typically too small to conveniently measure with a multi-meter (i.e. < 1mA). In practice measuring the voltage on the wires is sufficient to confirm the signal generator is working properly.

The total power is very low and we have not observed any signs of heating in the chamber.

16) Line 318, what kind of "plasma cleaner" is used? Is it prohibitively expensive?

We have added the plasma cleaner model (Harrick: PDC-32G) to the materials list. Comparable models cost \$4000 to \$10,000 USD from Sigma and are typically found in labs using PDMS-based microfluidics. If a specialized instrument is not available, glass can be plasma treated using a bell jar and microwave oven (e.g. Langmuir 2003, Vol 19, p8117-8118), and there are many other methods to render the glass hydrophilic such as Piranha, UV treatment, sonication in water/alconox or ethanol/water.

We have modified the comments in the discussion to stress that the plasma cleaner is not crucial for this method.

Line 637 : "The gel-assisted swelling protocol uses little specialized equipment except for the plasma cleaner, which is not essential as there are many alternative methods to produce clean, hydrophilic glass."

17) Line 368, it would be helpful to explain after a week, what would happen to the GUVs? Clustering, collapsing etc?

We appreciate the reviewer's suggestion, but we have not systematically studied the stability of different GUV preparations and are limited by space for the protocol section.

We suggested "1 week" as rough guide for these lipids, since we have not noticed changes over that time. Indeed, if the original solutions are free of bacteria, GUVs formed from a robust lipid like DPhPC or POPC can appear unchanged even after several weeks. When we have looked at older GUV preparations, common issues have included bacterial contamination, a change in osmolarity (if stored in a growth chamber) and occasionally clustering or GUV collapse, as suggested by the reviewer.

18) Line 374, "interior" defects vs exterior defects? Need to explain. Maybe simply: inclusion of smaller liposomes?

This is a great suggestion, and we have modified the text to read

"Examine each "GUV candidate" in epifluorescence to exclude any containing smaller liposomes nested inside."

19) Line 376-82, the definition of unilamellar by experiments is quite empirical, and not necessarily enough. How about EM data?

We agree with the reviewer that the guidelines for identifying GUVs on lines 376-382 are quite empirical, and certainly not sufficient to prove that a "GUV candidate" is unilamellar.

However, these guidelines are intended for someone trying to quickly judge the yield of their GUV growth, or select "candidate GUVs" during a micro-manipulation experiment. Lipid fluorescence is a very effective method for identifying unilamellar membranes, even with simple epi-fluorescence (e.g. Akashi et. al. Biophys J. Vol 71, p3242). Thus, we think these suggestions are appropriate for their intended purpose.

We are quite confident these protocols produce genuine, unilamellar GUVs. Histograms of lipid fluorescence for these GUVs are indistinguishable from GUVs prepared by a classical electroformation protocol known to have a very high yield of unilamellar (Figure S8, PLoS One, Vol 6, e25529). Furthermore, micromanipulation experiments with these GUVs give a membrane bending modulus consistent with a single membrane. Finally, these GUVs pass the "BLM zap test" - if we apply a very short voltage-pulse when in the "GUV-attached" patch configuration, the patch membrane breaks and we immediately enter the "whole-GUV" configuration (n.b. unfortunately the whole-GUV configuration is not stable).

We agree that EM data would provide further evidence, but GUVs have proven to be quite challenging to image via EM (EM groups we have collaborated with have not been successful) and we are unaware of a good protocol for obtaining EM images from them.

To stress that these suggestions are no more than an empirical guide, we have modified the presentation of results to

Line 456 : "Calibration measurements are needed to rigorously evaluate GUV quality, and a systematic quantification has been published previously²⁸. However, as an empirical guide, "good" GUVs should be isolated (*i.e.*, not in a cluster), have a single, smooth, spherical outer membrane, contain no objects (*i.e.*, tubes, nested vesicles, etc.) inside, and have the "standard" lipid fluorescence level (brighter objects are typically bi- or multi-lamellar)."

20) *A picture of the recording chamber here would help the readers oriented.*

This is an excellent point, and this section of text will be illustrated in the video.

Because there are no special requirements for the recording chamber (a small Petri dish is fine) or patch-clamp rig, we feel a figure in the text would add little for readers familiar with the technique. Conversely, for someone who is new to patch-clamping, we think it will be most helpful to see the preparation of the recording chamber and patch-clamp rig, which will be clear in the video.

21) *Line 401, there is no section 5.5. "defect-free" needs definition.*

We apologize for this error/omission. We have modified step 6.5 to refer to section 5.4, and included the quotation marks for "defect-free" to match earlier sections -

"6.5.) Search through the chamber to locate a "defect-free" GUV as described in section 5.4, and check that it contains fluorescent protein. "

22) *Line 404, pressure as "1.0 cm H₂O, in a home-made scale. Better to explain it.*

This is an excellent suggestion, and we have modified the text accordingly -

"6.6.) Apply a constant positive pressure (> 100 Pa, or roughly 1 cm H₂O in a manometer) to keep the patch pipette interior clean, ..."

23) *Line 457, the protein density, # per unit area needs to be used.*

We have modified the text to define the protein density -

"The fluorescent lipid (magenta) and protein (green) signals have been scaled to the same average intensity, so that GUVs with a low/high number of proteins per unit area (protein density) have a magenta/green shade in the overlay images (right column), while GUVs with an average protein density are white."

How was the calibration done?

We agree this issue was not adequately discussed.

We previously performed an absolute quantitative calibration procedure (Supporting Text 4, PLoS One, Vol 6, e25529) using SUVs and GUVs containing a reference green fluorescent lipid. However, this is a rather involved procedure and not at all necessary for the main objective of Figures 7, 8, 9 and 11 which is to compare the protein density of individual GUVs prepared by a given method, which is proportional to the fluorescence intensity (for these densities).

We have modified the caption of Figure 11 to read

"Protein density is proportional to KvAP-A488 fluorescence intensity for these concentrations²⁸, and in each histogram the fluorescence intensities are normalized by the mean of the distribution."

Figure 11 needs a unit for Y-axis (Line 462).

We apologize for this oversight. The Y-axis is now labelled "Number of GUVs"

24) Line 480, the conductance 100 pS is smaller than the reported 150 pS under similar conditions. Please provide another evidence, blocking with VsTx1 or CTX.

The conductance is smaller, but we believe this is due to differences in solutions (100mM KCl versus 150mM KCl) and membrane composition (uncharged versus negatively charged).

Fitting an all-points histogram to Figure 12 gives a conductance of ~109 pS in 100mM KCl (and a membrane composed of DPhPC). In contrast, in the original characterization of KvAP reported a conductance of ~170pS (Ruta et. al. 2003) in 150mM KCl (and a membrane of POPE/POPG (3:1)). Clearly the conductance depends upon the permeant ion concentration, and the ratio of the conductances (170pS/109pS ~ 1.56) is within error of the [K+] ratio (150mM/100mM ~1.5). KvAP conductance has been reported to be modulated by alkanols (Finol-Urdaneta et. al., 2010, BJ 98, p762), and modest differences between the uncharged DPhPC GUV, and negatively-charged, decane-containing POPE/POPG BLM also seem reasonable. Thus, we feel that a conductance of ~110pS under these conditions is entirely consistent with previously reported conductances measured under different conditions.

To clarify this point, we have modified this section to read

"The current histogram shows two peaks corresponding to the closed and the open states, and fitting them with a double Gaussian function yields a single channel current of 10.9 ± 8.5 pA, corresponding to a conductance of 109.2 ± 8.5 pS (in 100mM KCl). Note that the single channel conductance depends on the solution (especially [K+]) and membrane composition (Finol-Urdaneta 2010)."

We agree with the reviewer that blocking with CTX is a good test, but we do not feel it is either necessary or beneficial to demonstrate this in the present tutorial. While electrophysiologists who study K-channels will obviously have CTX on hand, it may be unfamiliar for membrane biochemists and biophysicists and we think they will find it easier to measure the single-channel conductance, voltage-dependent open probability and potassium selectivity when checking for active KvAP channels in their GUV.

25) Line 490, figure 13A, the channel appears to lack inactivation. Which is strange, please explain why.

The reviewer is absolutely correct that KvAP in DPhPC-GUVs shows markedly slower and incomplete inactivation (compared to BLMs), as we commented on in our earlier study (PLoS One, Vol 6, e25529).

This only seems strange if a property (e.g. kinetics of inactivation) is believed to be intrinsic to the channel, and

unrelated to its environment. However, remarkable changes to properties are often observed when the channel environment changes.

Kv channel inactivation has been shown to depend on the channel environment. For example, the Kv1.2/2.1 paddle chimera was reported to inactivate rapidly when reconstituted into Black Lipid Membranes (Figure 1A, Schmidt and MacKinnon, PNAS, Vol 105, p19276) but when the same preparation was reconstituted into oocytes the channels showed no inactivation on the experimental timescale (Figure 1B). KvAP inactivation also appears to be sensitive to the environment. In Schmidt et. al. (JMB 2009, Vol 390, p902, KvAP inactivates roughly twice as slowly in DPhPC BLMs as compared to POPE/POPG (3:1) BLMs. Furthermore, Finol Urdaneta et. al. (BJ, Vol 98, p762) report that the presence of alkanols cause an extreme slowing of KvAP inactivation in POPE/POPG (3:1) BLMs.

Given that there are significant differences between BLMs and GUVs (residual decane in the BLM, membrane tension, ...), and C-type inactivation is sensitive to the environment for some Kv channels (including KvAP), we do not feel it is strange that KvAP inactivation is so much slower and incomplete in GUVs. Instead, it seems like a nice illustration of the importance of studying channels in well-defined conditions (like GUVs) to identify which channel features are modulated by the environment.

To clarify that the environment can influence Kv-channel inactivation, we have modified the text to read -

Line 520: "The kinetics of KvAP activation and inactivation differ considerably between Black Lipid Membranes (BLMs)²⁶ and GUVs, but this is consistent with previous reports that Kv channel gating can be sensitive to the membrane composition and state³²."

26) Lin 495-498, the recording is not good enough, too much leak in Fig 13B. The channel did not close at -80 mV.

Because the goal of this article is to help people learn the technique, we intentionally included a typical raw recording which shows several features that occur in GUV patches.

GUVs contain channels with the "physiological" (i.e. cytosolic domain facing into the GUV) and "inverse" (i.e. cytosolic domain on the outside of the GUV). Although patches obtained from GUVs typically contain far more channels with the "physiological" orientation than the "inverse" orientation, if a patch contains more than 10 active channels often at least one is "inverse".

In our experience, the fraction of "inverse" channels in a GUV patch is actually much lower than in BLM recordings. However, if

the BLM is held at -100mV, the "inverse" channels inactivate and because re-activation is slow, they are then inactive during test pulses. In contrast, in a GUV patch, "inverse" channels do not inactivate when held at -100mV and instead continue to randomly chatter away. Indeed, in figure 13A, at times $t < 0$ s and $t > 5$ s (i.e. -100mV), individual channel openings can be easily resolved above a stable baseline leak of about -12pA. Note we are quite confident that these "inverse" channels are indeed KvAP because they are potassium selective and we have had rare patches containing no regular channels and only a single "inverse" channel which closes when held at positive voltages.

Thus, the occasional channel openings at -80mV (and even -100mV) are not due to leak or some other problem with the recording, but rather correspond to "inverse" channels which feel an effective voltage of +80mV.

While we could have chosen a patch with no "inverse" channels, we felt it would be more helpful to illustrate this effect since someone trying these experiments for the first time might be puzzled by this. We have modified the corresponding section of the text (Lines 512 - 517) to clarify this point -

"Between each step, the patch is held at -100 mV for 30 s to allow channels with the "physiological" insertion to return to their resting state. When the potential is sufficiently negative (e.g., $V < -60$ mV) most of the current is due to the membrane leak, and the occasional openings of one or two channels which are likely to have the "inverse" insertion."

27) Line 680, it is better to have an example for slightly fluctuating, defect-free GUVs to help the readers get a good idea about good ones for high success rate.

This is an excellent point which will be addressed in the video, as it shows a floppy GUV being patched.

28) Line 740, control lipid composition is kind of difficult to say. Does lipid composition change during the dehydration/rehydration such that the parts forming the GUVs have different lipid composition? How to check it?

Lipid demixing during liposome preparation is a well-known issue, but we do not believe these GUV protocols pose additional concerns. Many lipid compositions mix well and cause no problems. Historically, problems were noticed when cholesterol-containing mixtures in organic solvent were dried to prepare a lipid film because the cholesterol was less miscible in dehydrated state. Cholesterol crystallization then leads to liposomes with a lower average concentration of cholesterol, and differences in composition between individual liposomes. Partial dehydration protocols, such as those presented here, have been shown to reduce heterogeneity (Baykal-Caglar et. al., BBA

Biomembranes, 2012 Vol 1818 p2598) because demixing occurs less readily in "damp" lipids than dry lipids.

Since demixing issues usually occur in mixtures with miscibility transitions, examining the heterogeneity of the miscibility temperature for individual GUVs provides a simple but effective test for lipid composition change. Lipid composition can also be probed indirectly using fluorescent lipids and amphipathic molecules. Finally, TOF-SIMS measurements have recently been made on supported lipid bilayers, and in principle TOF-SIMS be used to measure the composition of individual GUVs.

While there are clearly some constraints on GUV lipid composition, we feel it is quite reasonable to say that GUVs allow good control of lipid composition.

29) *Line 744, is there a good way to deal with PE, charged lipids cholesterol or the famous DOPC/SPM/Cholesterol mixture that mimics the eukaryotic membranes?*

We recommended Egg-PC, DOPC and DPhPC because we think they will help someone trying to grow GUVs for the first time, but we have not encountered any specific problems or restrictions with these protocols.

As the reviewer is no doubt well aware, there are little tricks which seem to help for a particular lipid mixture. For example, including a little PEG lipid can work wonders for PE, gel-assisted swelling allows the formation of GUVs with 100% charged lipids, high cholesterol concentrations benefit from keeping the lipid mixture (partially) hydrated, and DOPC/SPM/Cholesterol GUVs need to be kept above the miscibility temperature.

However, we have not tested each of these tricks for these protocols, and even a simple review of GUV voodoo (let alone experimentally validating specific tricks) is far beyond the scope of this article.

Reviewer #2:

Manuscript Summary:

This manuscript describes a method for growing giant unilamellar vesicles with integral membrane proteins in physiological buffers. Two complimentary methods are described for growing the vesicles and the resulting vesicles are compared with fluorescence microscopy.

Major Concerns: *No major concerns*

Minor Concerns:

In the protocol section there are some steps that are incompletely described or are unclear. Examples follow

line 280: What type of sealing paste? Please clarify.

We believe the editorial policy restricts 'commercial language' such as brand names in this section of the text. Details of all materials, including the sealing paste (Vitrex Sigillum Wax) are included in the material sheet, which we hope will be easy to find in the final publication on the JoVE website.

We have included a short note at the beginning of the protocol -

"Note : Additional details for reagents and instruments are given in the materials list."

line 317: What type of plasma? Does it matter?

We have added a short note on this line

"Plasma-clean (air plasma) a cover-slide for 1 minute so that the agarose solution will spread nicely on it."

and have modified the discussion to stress that any method that produces a clean, hydrophilic glass surface should work.

"The gel-assisted swelling protocol uses little specialized equipment except for the plasma cleaner, which is not essential as there are many alternative methods to produce clean, hydrophilic glass."

line 329: Should this petri dish be pretreated as described in section 1.6?

We've found that casein treatment is only necessary when GUVs meet clean glass. This section will be shown in the video, but we have added "standard"

4.5.) Place the agarose-coated coverslip in a standard 3.5 cm Petri dish.

to try to clarify that no special treatment is needed.

line 338: What is a small Petri dish? 3.5 cm diameter, or something else?

Thank you for mentioning this. We now specify the size.

4.8.) As soon as the SUVs have dried, add growth buffer to cover the slide surface. For a small 3.5 cm Petri dish use ~1 ml of buffer.

line 356: How large should the pipette tip be? Is there a preferred size of pipette to use for this step?

We now include a tip diameter -

5.2.) Harvest the GUVs. Cut the end of a 100 μ l pipette tips so the opening is larger (~2 mm diameter), and aspire slowly as the shear stress of pipetting can easily destroy GUVs.

line 392: Replace explode with rupture.
We agree this is much more suitable.

6.2.) Passivate the chamber by incubating with a beta-casein solution (5 mg/ml) to ensure that GUVs do not adhere, spread and rupture on chamber surfaces. Rinse the casein off after 5 min.

line 512: The chamber is attributed to Ambroggio and Bagatolli. This statement needs a citation.

Unfortunately, we are unaware of a citation which refers to this specific chamber, but we have included a reference to this general class of chambers.

The chamber shown in Figure 2 is based on a design by Luis Bagatolli¹⁵ and Ernesto Ambroggio, and was machined from Polytetrafluoroethylene (PTFE) to allow cleaning with most solvents.

line 617: PVC should be poly vinyl chloride. PVA is poly vinyl alcohol.

Thank you for spotting this typo.

Figure 6: Add the scale label to the graph rather than the caption

We appreciate this suggestion and have labelled the scale bar in the figure.

Figures 7 and 8: The contrast between the yellow arrows and the white arrows is not very good. I only noticed the difference on the third time that I looked at the figures. Please try a higher contrast color scheme to make the differences more obvious.

We have changed the arrow color to red, which we hope has better contrast.

Reviewer #3:

The manuscript reports in great detail about the reconstitution of a transmembrane protein in giant unilamellar phospholipid vesicles by using two different approaches, both based on the initial formation of proteo-SUVs. The reconstituted protein was shown to function as ion-channel.

The manuscript is very useful for various reasons. First of all, the detailed protocols allow the interested reader to carry out similar studies with other membrane proteins of interest. Second, the paper also demonstrates that descriptions of details how the vesicles are prepared are important, including the care one has to take about pre-treating glass vials, cleaning electroformation chambers etc. Unfortunately, such details are usually not provided in publications today, leading to often seen situations where authors claim that published work can't be reproduced. This at the end is bad for the reputation of Science at large. Third, the comparison made between the two related methods used, the detailed data analysis and discussion will be acknowledged by all those interested in the work.

I recommend publication of the work, after considering a few minor points.

1. Line 47: .. giant unilamellar vesicles ..
2. Line 49: ... small unilamellar vesicles ...

We have corrected both.

3. Line 49: The sentence #Both methods fuse together protein-containing ...# is not optimal. It may be better to write #With both methods, initially formed small unilamellar vesicles containing membrane-embedded proteins fuse to form ...#

We thank the reviewer for this suggestion and have modified the sentence to

"In both methods, protein-containing small unilamellar vesicles are fused together to form GUVs that can then be studied by fluorescence microscopy and patch-clamp electrophysiology."

4. Line 79ff: The authors may add another advantage of electroformed GUVs as compared to giant vesicles formed by the microdroplet transfer method: it is the possibility of microinjecting small volumes of an aqueous solution into the interior of the vesicles, see Bucher et al. *Langmuir*, 1998, 14 (10), pp 2712-2721 and Shimanouchi et al. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, Volume 1768, Issue 11, November 2007, Pages 2726-2736. Depending on the type of transmembrane proteins reconstituted, microinjection may also be an option for manipulating the vesicles after they have been formed to test for functionality of the reconstituted proteins.

We appreciate the reviewer's suggestion. We certainly don't wish to imply that electroformed GUVs cannot be used for encapsulation. However, since micro-injection can be performed on GUVs prepared by both approaches, we are not sure how to introduce this information in this section.

5. *Line 67ff: Reading the protocol, I missed the sources of the materials and the meaning of the abbreviations. I only realized later that there is an excel sheet. It may be better to refer to this excel sheet at the beginning of the protocol section. Make sure that everything is correct in the excel sheet. ...sn... in the IUPAC-IUB names of lipids should be in italic: and that all entries in the cells can be read when printed. Sonicator: are both types used in the list (bath type and probe)?*

We have included a note at the beginning of the protocol.

Line 181 : “Note : Details for reagents and instruments are given in the materials list.”

We have corrected the notation in the spreadsheet, and believe JoVE will format into an easy-to-read PDF file.

Yes, the tip sonicator can be used for SUV prep (but is not essential), while the bath sonicator is useful for cleaning the GUV electroformation chamber.

6. *Line 195: A reference should be added to support the statement made about the effect of casein.*

Casein is commonly used for passivating glass surfaces (e.g. micro-tubulin gliding assays), and we discuss the choice of beta-casein in the discussion. Unfortunately, we have been unable to find a reference which summarizes different passivation techniques (e.g. casein, BSA, PEG-PLL, ...) .

7. *Line 137: Electroformation chamber: please refer to Fig. 2*

We have included a reference to Figure 2 at this point.

8. *Line 519: Fig. 4: Low salt or high salt?*

We have modified the legend to note this GUV was grown in high-salt buffer.

Line 542 : Figure 4: Representative image of DPhPC GUVs containing KvAP growing on the platinum wire in a high salt buffer.

9. *Line 553: Fig. 10: Electroformation: Low salt or high salt?*

The legend now specifies the electroformation as performed with low salt buffer.

Line 577 : Figure 10: Size distribution of defect-free proteo-GUVs (DPhPC) grown by electroformation in low salt buffer (top, N=94) or gel-assisted agarose swelling (bottom, N=68).

10. Line 637: ... SUVs ..

Thank you for spotting this formatting error.

11. Line 657: .. bovine serum albumin ..

Thanks again.

12. Line 744: *With respect to the various lipids tested, the reader expects a reference here. The comments made here are useful and important for beginners.*

This is a great suggestion.

We are unaware of an article which specifically discusses how lipid composition influences GUV growth, but the article by Meleard, Bagatolli and Pott (Meth. Enzym. 465, p161) provides a good overview of factors influencing GUV growth and has many key references, so we have modified this section to read -

Line 771 :

“DPhPC GUVs grow well and form stable excised membrane patches, and these protocols have also worked effectively for lipid mixtures containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylserine (PS) and cholesterol. However, GUV growth is sensitive to both lipid composition and buffers¹⁵, and so protocol parameters (e.g. amount of lipid deposited, electroformation voltage/frequency) may need to be adjusted for lipid mixtures containing high concentrations of PE, charged lipids (PG, PA, PS), or cholesterol. When starting out, Egg-PC, DOPC or DPhPC are a good first choice, and ... “

13. *Figure 1: GUVs, not GUVS. The drawing with the GUVs leaves the impression that no lipids are left on the electrode. This may not be the case and could be indicated.*

The figure and caption have been modified following these suggestions.

14. *Fig. 7 and 8: A zoom-in could be shown as well with the same scale as Fig. 9, for a direct comparison. One could make Fig. 7a and b and Fig. 8a and b.*

We think this is a great suggestion, and have added zooms for both Fig 7 and 8.

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