FINAL SCRIPT: APPROVED FOR FILMING

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Title: A Model Membrane Platform for Reconstituting Mitochondrial Membrane Dynamics

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 27
Number of Shots: 55

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Yifan Ge:** We introduce an *in vitro* reconstitution platform that mimics the lipid environment of the mitochondrial inner-membrane. This platform can be used to investigate the molecular mechanism of mitochondria inner-membrane fusion.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Yifan Ge:** The main advantage of this technique is that it allows quantitative investigation of integral membrane proteins and membrane-associated proteins in a near-native environment.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

2. Fabrication of Lipid Bilayers

2.1. To begin, mix solutions A and B according to manuscript directions **[1]**, then generate the lipid mixture by adding the calculated volume of storage solution into amber vials with a glass syringe **[2]**. Match the final volume by adding extra chloroform into the vials **[3]**.

2.1.1. WIDE: Talent at the lab bench mixing solution A or B.

2.1.2. Talent adding storage solution into amber vials.

2.1.3. Talent adding chloroform to the amber vials, with chloroform container in the shot.

2.2. Bake microscope cover glass slides at 520 degrees Celsius for 30 minutes **[1]**. After baking, cool them to room temperature **[2]**.

2.2.1. Talent putting glass slides in the oven.

2.2.2. Talent taking the slides out of the oven and putting them down to cool.

2.3. Add approximately 10 grams of sodium hydroxide to 500 milliliters of methanol while stirring **[1]**. Stir for 2 hours, continuing to add sodium hydroxide to the solution until precipitates start to show **[2-TXT]**.

2.3.1. Talent adding to sodium hydroxide to methanol.

2.3.2. Precipitates starting to form. **TEXT: Wear PPE!**

2.4. Clean the glass slides in 10% sodium dodecyl sulfate solution, methanol saturated with sodium hydroxide, and 50 millimolar hydrochloric acid, sequentially bath sonicating the slides under each condition for 30 minutes **[1]**. Clean the glass slides in ultrapure water for 10 minutes between each condition **[2]**.

2.4.1. Talent bath sonicating the slides in one of the mentioned solutions.

2.4.2. Talent cleaning the glass slides in water.

2.5. Store the cleaned cover glass sealed in hydrochloric acid solution for up to 2 weeks to ensure good bilayer quality **[1]**.

2.5.1. Talent putting the slides in HCl solution.

2.6. Clean the polytetrafluoroethylene trough of the Langmuir-Blodgett dipping system using chloroform and ultrapure water until no wetting is observed **[1]**. Spray chloroform on the trough surface and wipe it thoroughly 3 times with cellulose wipes

[2]. Then, rinse it 3 times with ultrapure water and remove the water via suctioning **[3]**.

2.6.1. Talent cleaning the trough with chloroform and water.

2.6.2. Talent spraying chloroform on the surface and wiping it.

2.6.3. Talent rinsing the trough with water and then suctioning the water out.

2.7. When finished, cover the surface of the trough with clean ultrapure water **[1]**. Take 2 pieces of surface-treated cover glass from the cleaning solution **[2]** and rinse them with ultrapure water for approximated 30 seconds **[3]**.

2.7.1. Talent adding water to the trough. NOTE: 2.7.1 and 2.7.2 combined

2.7.2. Talent taking 2 pieces of cover glass from the cleaning solution.

2.7.3. Talent rinsing the cover glass.

2.8. Place the cover glass in a back-to-back manner, using the substrate clamp to hold the glass slides **[1]**. Immerse the glass slide underneath the water surface by manually clicking **dipper down** on the Langmuir control software **[2]**.

2.8.1. Talent positioning the coverglass.

2.8.2. Talent pressing dipper down on the software and the slide being immersed. NOTE: Different takes for different views available. *Videographer: Obtain multiple usable takes of this shot because it will be reused 2.14.2*.

2.9. Zero the film balance and carefully spread Solution B drop by drop at the air-water interface **[1]**. Make sure lipids are only spreading at the air-water interface, with no chloroform and lipid droplets sinking to the bottom of the polytetrafluoroethylene surface, which will create a lipid channel and prevent the monolayer formation **[2]**. *Videographer: This step is important!*

2.9.1. Solution B dropping onto the air water interface.

2.9.2. Lipids spreading on the water surface.

2.10. Stop adding lipids when the film balance readout is around 15 to 20 millinewton per meter **[1]**. Wait for 10 to 15 minutes, then initiate the barrier controller to alter the surface area by clicking **start experiments [2]**. *Videographer: This step is important!*

2.10.1. Film balance readout of 15 – 20 mn/m. NOTE: 2.10.1 and 2.10.2 combined

2.10.2. Talent clicking start experiment.

2.11. Wait until the film balance readout increases to 37 millinewton per meter **[1]** and keep the pressure for approximately 20 to 30 minutes **[2]**.

2.11.1. Film balance readout increasing to 37 mn/m.

2.12. Raise the cover glass at the speed of 22 millimeters per minute while maintaining the surface tension at 37 millinewton per meter **[1]**. A lipid monolayer with polymer

tethering will be transferred from the air-water interface to the surface of cover glass through the Blodgett dipping process, forming the bottom leaflet of the lipid bilayer **[2]**. *Videographer: This step is important!*

2.12.1. Cover glass raising.

2.12.2. Cover glass with the lipid monolayer.

2.13. Clean the air-water interface by suction and rinse the trough with ultrapure water. ~~**[1]**~~ After cleaning a one-welled glass slide with chloroform, ethanol, and ultrapure water, set it on the trough underneath the water layer **[2]**.

~~2.13.1. Talent rinsing the trough.~~

2.13.2. Talent putting the glass slide in the trough.

2.14. Make sure that the well is facing up toward the air-water interface and pour fresh ultrapure water until the glass slide is fully covered **[1]**. Then, immerse the glass slide under the water surface as previously described **[2]**.

2.14.1. Talent pouring fresh water in the trough.

2.14.2. Talent add lipids to the surface of the air-water interfaceabove Shaefer slides, and the barrier was closed to keep the pressure.

2.15. Hold the cover glass with the lipid monolayer using a silicon suction cup and gently push the lipid monolayer to the air-water interface **[1]**. Hold the cover glass for 2 to 3 seconds at the interface, then push it against the slide. Take the slide out with the cover glass **[2]**. *Videographer: This step is difficult and important!*

2.15.1. Talent pushing lipid monolayer to the interface. NOTE: 2.15.1 and 2.15.2 combined and taken from multiple angles

2.15.2. Talent holding the cover glass in position and then pushing it against the slide, then taking the slide out

2.16. Take the cover glass with the bilayer to an epifluorescence microscope and image the lipid bilayer according to text manuscript directions **[1]**.

2.16.1. Talent mounting the coverglass on the microscope stage.

3. Protein Reconstitution into the Polymer-tethered Lipid Bilayer

3.1. Prepare a crystallization dish containing ultrapure water and place a clean microscope image ring underneath the dish **[1]**. *Videographer: This step is important!*

3.1.1. Talent placing a microscope ring underneath the crystallization dish.

3.2. Immerse the slide and cover glass that contain the lipid bilayer underneath the water **[1]**. Gently separate the slide and cover glass, holding the cover glass slide from the bottom, and transfer the cover glass into the image ring **[2]**.

3.2.1. Talent immersing the slide and cover glass.

3.2.2. Talent separating the slide from the cover glass and placing the cover glass on the image ring.

3.3. Replace the ultrapure water in the image ring with Bis-Tris sodium chloride buffer, making sure that the lipid bilayer is not exposed to any air bubbles **[1]**.

3.3.1. Talent replacing the water with the buffer.

3.4. Add 1.1 nanomolar n-Octyl-β-D-Glucopyranoside *(pronounce 'N-octyl-beta-gluco-pyranoside')* to the lipid bilayer **[1]**, then immediately add the mixture of 1.2 nanomolar DDM and 1.3 picomoles of purified l-Opa1 *(pronounce 'L-opa-one')* into the image ring **[2]**. Incubate the sample on a benchtop shaker at low speed for 2 hours **[3]**.

3.4.1. Talent adding n-Octyl-β-D-Glucopyranoside to the lipid bilayer.

3.4.2. Talent adding the mixture of DDM and l-Opa-1 to the image ring.

3.4.3. Sample shaking.

3.5. Distribute 30 milligrams SM-2 Resin beads into 3 milliliters of Bis-Tris buffer and shake it **[1]**. Use a plastic pipette to add 5 to 10 microliters of the SM-2 Resin beads to the image ring and incubate it for 10 minutes **[2]**, then rinse off the resin beads. The final volume of the buffer in the image ring should be 1.5 milliliters **[3]**. *Videographer: This step is difficult and important!*

3.5.1. Talent adding resin beads to buffer and shaking the mixture. NOTE: 3.5.1 and 3.5.2 were recorded in reverse order, but they should be shown as written in the script.

3.5.2. Talent adding the bead mixture to the image ring.

3.5.3. Talent rinsing off the beads.

~~3.5.4. Imaging ring with buffer.~~

4. Preparation of Proteoliposomes

4.1. Prepare 1 milligram of lipid mixture A in chloroform solution, then evaporate chloroform under nitrogen flow for 20 minutes **[1]**. Keep the mixture under vacuum overnight to form a lipid film **[2]**.

4.1.1. Talent evaporating the chloroform.

4.1.2. Talent putting the mixture under vacuum.

4.2. Prepare 50 millimolar calcein-containing buffer by dissolving 15.56 grams of calcein in 50 milliliters of 1.5 mol sodium hydroxide solution **[1]**. Stir the mixture at room

temperature until calcein is completely dissolved **[2]**, add 12.5 millimolar Bis-Tris and ultrapure water for a final volume of 500 milliliters, and adjust the pH to 7.5 **[3]**.

4.2.1. Talent adding calcein to NaOH.

4.2.2. Mixture stirring and calcein dissolving.

4.2.3. Talent adding Bis-Tris and water to the mixture, with the Bis-Tris and water containers in the shot, then adjusting pH of the buffer. NOTE: Please delete the end of film when Talent pours solution to the beaker.

4.3. Suspend lipid film in calcein-containing buffer **[1]**, then fully hydrate the lipid by heating the suspension at 65 degrees Celsius for 20 minutes **[2]**. Form 200 nanometer liposomes via extrusion with a polycarbonate membrane **[3]**.

4.3.1. Talent suspending the lipid film the buffer.

4.3.2. Talent heating the suspension.

4.3.3. Talent performing the extrusion with the polycarbonate membrane.

4.4. Add 2 micrograms of l-Opa1 in 0.5 micromolar DDM to 0.2 milligrams liposome **[0-added]** and incubate the solution at 4 degrees Celsius for 1.5 hours **[1]**.

Added shot: 4.4.0 l-Opa1 added to image ring

4.4.1. Talent putting the solution on the shaker in the cold room.

4.5. Remove the surfactant by dialysis with a 3.5-kilodalton dialysis cassette against 250 milliliters of 25 millimolar Bis-Tris, 150 millimolar sodium chloride, and 50 millimolar calcein buffer at 4 degrees Celsius overnight, changing the buffer twice **[1]**.

4.5.1. Dialysis setup.

~~4.5.2. Talent running the desalting column.~~

4.6. Remove extra calcein using a PD-10 desalting column, then proceed with imaging and data analysis as described in the text manuscript **[1]**.

4.6.1. Talent running the desalting column.

Results

5. Results: Reconstituted Transmembrane Protein Diffusion and Detection of Membrane Tethering, Lipid Hemifusion, and Pore Opening

5.1. Epifluorescence microscopy images of a lipid bilayer and its lipid fluidity are shown here **[1]**. The lipid distribution is shown before and after photobleaching **[2]** and the homogeneity is shown before and after reconstitution **[3]**.

5.1.1. LAB MEDIA: Figure 4 A – E.

5.1.2. LAB MEDIA: Figure 4 A – E. *Video Editor: Emphasize A and B, label A "***Before***" and B "***After***".*

5.1.3. LAB MEDIA: Figure 4 A – E. *Video Editor: Emphasize D and E, label D "***Before***" and E "***After***".*

5.2. l-Opa1 *(pronounce 'L-opa-one')* reconstituted in lipid bilayer was validated by fluorescence correlation spectroscopy, or FCS. The FCS curves indicated that 75% of l-Opa1 was reconstituted into the lipid bilayer, suggesting that l-Opa1 freely diffuses in the polymer-tethered lipid bilayer with the potential to self-assemble into functional complexes **[1]**.

5.2.1. LAB MEDIA: Figure 4 GRAMS and H.

5.3. Fluorescence step bleaching indicated that an average of 2 to 3 copies of l-Opa1 were reconstituted in a given liposome **[1]**. The size distribution of Opa1 reconstituted proteoliposomes was tested after reconstitution using DLS and verified with FCS **[2]**.

5.3.1. LAB MEDIA: Figure 5 B.

5.3.2. LAB MEDIA: Figure 5 C.

5.4. Membrane tethering was monitored by observing the signal of TexasRed on the surface of the lipid bilayer using TIRF microscopy. Membrane lipid demixing, or hemifusion, was monitored through TexasRed as the liposome marker diffused into the lipid bilayer **[1]**.

5.4.1. LAB MEDIA: Figure 6 A.

5.5. Calcein dequenching helped distinguish full fusion pore formation from only lipid demixing **[1]**, allowing comparison between conditions where particles stall at hemifusion **[2]** and particles that proceed to full fusion **[3]**.

5.5.1. LAB MEDIA: Figure 6 B and C.

5.5.2. LAB MEDIA: Figure 6 B and C. *Video Editor: Emphasize B.*

5.5.3. LAB MEDIA: Figure 6 B and C. *Video Editor: Emphasize C.*

5.6. Membrane tethering was indicated by a stable lipid signal from liposomes **[1]**. Hemifusion signal featured no dequenching in the calcein signal **[2]**, but a rapid decay of the TexasRed signal indicated diffusion of the dye into the lipid bilayer **[3]**. Full fusion featured both lipid decay and content release **[4]**.

5.6.1. LAB MEDIA: Figure 6 B.

5.6.2. LAB MEDIA: Figure 6 B. *Video Editor: Emphasize the bottom row of images.*

5.6.3. LAB MEDIA: Figure 6 B. *Video Editor: Emphasize the top row of images.*

5.6.4. LAB MEDIA: Figure 6 C.

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Conclusion

6. Conclusion Interview Statements

Commented [AG1]: Authors: At most 2 conclusion statements are allowed.

6.1. **Yifan Ge:** When attempting this protocol, remember to thouroughly clean both the cover glass and Langmuir Trough to ensure bilayers of high quality are fabricated. Good air quality is also critical to prevent defects in the bilayer.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.6.1 – 2.6.3.*

6.2. **Yifan Ge:** This technique paves the way for us to explore new questions in mitochondrial membrane dynamics and organization. Exciting future experiments include exploring the effects of bilayer asymmetry, membrane potential, and disease-related mutants in mitochondrial inner membrane structure and dynamics..

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.