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TITLE:

A Model Membrane Platform for Reconstituting Mitochondrial Membrane Dynamics

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SUMMARY:

Mitochondrial fusion is an important homeostatic reaction underlying mitochondrial dynamics. Described here is an in vitro reconstitution system to study mitochondrial inner-membrane fusion that can resolve membrane tethering, docking, hemifusion, and pore opening. The versatility of this approach in exploring cell membrane systems is discussed.

ABSTRACT:

Mitochondrial dynamics is essential for the organelle's diverse functions and cellular responses. The crowded, spatially complex, mitochondrial membrane is a challenging environment to distinguish regulatory factors. Experimental control of protein and lipid components can help answer specific questions of regulation. Yet, quantitative manipulation of these factors is challenging in cellular assays. To investigate the molecular mechanism of mitochondria inner-membrane fusion, we introduced an in vitro reconstitution platform that mimics the lipid environment of the mitochondrial inner-membrane. Here we describe detailed steps for preparing lipid bilayers and reconstituting mitochondrial membrane proteins. The platform allowed analysis of intermediates in mitochondrial inner-membrane fusion, and the kinetics for individual transitions, in a quantitative manner. This protocol describes the fabrication of bilayers with asymmetric lipid composition and describes general considerations for reconstituting transmembrane proteins into a cushioned bilayer. The method may be applied to study other membrane systems.

INTRODUCTION:

Membrane compartmentalization is a hallmark of eukaryotic cells¹ (**Figure 1A**). Biological membranes are increasingly recognized as more than a two-dimensional solvent. It is being

considered as an environment playing critical roles in regulating protein function and macromolecular complex assembly^{2,3}. Native lipids are ligands that regulate membrane protein activity^{3,4}. Membrane spatial organization and the ability of membranes to be sculpted into diverse shapes are important physical properties for selecting new functions^{3,5}.

Model membrane platforms are biomimetic systems that can help us understand cellular membrane structure, dynamics, and function⁶⁻⁸. Model membranes typically comprise a lipid mixture of well-defined composition, with defined biophysical properties (stiffness, thickness, and elasticity). Coupled to fluorescence imaging, model membrane platforms allow quantitative analysis of membrane structure and function⁹⁻¹¹. Lipid bilayer reconstitution strategies have been used to study SNARE-mediated membrane fusion^{9,10}, DNA-mediated membrane fusion¹², and viral fusion^{11, 13}. An advantage of such methods is the potential to obtain kinetic information for intermediate steps preceding an observable reaction event¹⁴.

The plasma membrane has been extensively studied using model membranes. Bilayers with lipid phase separation have been developed to study lipid raft structures important in cellular signaling^{11,15,16}. Micropatterned lipid planar bilayers^{17,18} have been used to investigate the organization of cell receptors. Polymer or gel-supported membranes have been used as biomimetic systems to study the membrane-cytoskeleton organization, membrane protein partitioning during cell signaling, and migration at cell-cell contacts¹⁹.

Artificial membrane systems are also being applied to study subcellular organelles²⁰. Organelles feature characteristic morphologies that create distinct sub-environments. The endoplasmic reticulum (ER) network is one example. Upon reconstitution of reticulons into liposomes, tubular membrane structures with properties similar to the cellular ER are formed²¹. The addition of atlastin, an ER fusion protein, can induce lipid tubules from liposomes to form a network²⁰. This is one example for how proteoliposomes can provide functional insight into organelle morphology and dynamics.

Mitochondrial membrane fusion and fission are essential for the health of the mitochondrial population²²⁻²⁵. A set of dynamin family GTPases catalyzes mitochondria membrane fusion. Mfn 1/2 catalyzes outer-membrane fusion. Opa1 mediates inner-membrane fusion²⁶ (**Figure 1B**). Opa1 has two forms: a long form (l-Opa1), transmembrane-anchored to the mitochondrial inner-membrane, and a 'soluble' short form (s-Opa1), present in the intermembrane space. The ratio between the two forms of Opa1 is regulated by the activity of two proteases, Oma1 and Yme1L^{27-29,30}. Important questions in Opa1 regulation include: how the two forms of Opa1, (short and long) mediate membrane fusion and their regulatory interplay^{28,29,31-33}.

Here we describe a reconstitution strategy successfully applied to investigate mitochondrial inner-membrane fusion that clarified the roles of l- and s-Opa1 in inner-membrane fusion. We developed a platform mimicking the mitochondrial inner-membrane using a polymer-tethered lipid bilayer and 200 nm unilamellar vesicles. The benefits of a polymer tether beneath the lipid bilayer include the following. First, it preserves the reconstituted transmembrane protein, which would otherwise may be disrupted by the proximity to the glass slide³⁴. Secondly, it serves a thick

water layer between the lipid bilayer and glass substrate, which facilitates studies of pore opening⁹, and thirdly the viscoelastic nature of the PEG polymer allows membrane curvature changes³⁵. We used three-color fluorescence imaging to characterize steps in membrane fusion (Figure 1C-F).

PROTOCOL:

1. Preparation of lipid mixtures

1.1 Prepare a lipid stock solution by dissolving 1,2-dioleoyl-sn-glycero-3phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), L- α -phosphatidylinositol (Liver PI), cardiolipin, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (18:1 PEG2000 PE) into chloroform at the concentration of 25 mg/mL. Dissolve fluorescent dye-conjugated lipid (TexasRed DHPE and Cy5 DOPE) chloroform at a concentration of 1 mg/mL. Store the lipid solution in amber vials with chloroform resistant liner, and further sealed with polytetrafluoroethylene tape. The solution can be kept at -20 °C for up to 6 months.

1.2 Make solutions A and B.

1.2.1 Mix lipid to prepare solution A (final concentration 1 mg/mL) that contains DOPC (52.8 mol%), POPE (20 mol%), Liver PI (7 mol%) and cardiolipin (20 mol%), and 0.2 mol% fluorophore.

1.2.2 Make solution B (final concentration 1 mg/mL) containing DOPC (47.8 mol%), POPE 20 mol%, liver PI (7 mol%), cardiolipin (20 mol%) and DOPE-PEG2000 (5 mol%), and 0.2 mol% fluorophore.

1.2.3 Generate the lipid mixture by adding the calculated volume of storage solution into amber vials using a glass syringe. Match the final volume by adding extra chloroform into the vials.

NOTE: For FCS (fluorescence correlation spectroscopy), decrease the ratio of dye conjugated lipid to 0.002 mol% and replace the rest by DOPC.

2. Fabrication of lipid bilayers

2.1 Bake microscope cover glass slides at 520 °C for 30 min. After baking, cool down the cover slides to room temperature.

2.2 Weigh approximately 10 g of sodium hydroxide and add to 500 mL of methanol while stirring. Stir for 2 h, continue to add sodium hydroxide in the solution until precipitates start to show. Make sure to wear appropriate PPE during the whole process.

2.3 Clean the glass slides in 10% sodium dodecyl sulfate solution; methanol saturated with sodium hydroxide; and 50 mM hydrochloric acid, sequentially (bath sonication under each

condition for 30 min). Clean the glass slide in ultrapure water for 10 min between each condition.

NOTE: Though highly recommended to use fresh solutions for glass slide preparation, each solution can be reused up to 5x or within 1 month, whichever comes first. Make sure to stir-mix the solution before each use.

2.4 Store the cleaned cover glass sealed in HCl solution up to 2 weeks to ensure good bilayer quality. If stored in ultrapure water, use the slides within a week.

2.5 Clean the polytetrafluoroethylene trough of the Langmuir-Blodgett dipping system using chloroform and ultrapure water until no wetting is observed on the trough. Spray chloroform on the trough surface, wipe thoroughly with cellulose wipes 3x. Rinse with ultrapure water and remove the water via suctioning. Repeat 3x.

2.6 Cover the surface of the trough with clean ultrapure water.

2.7 Take 2 pieces of surface-treated cover glass from cleaning solution or ultrapure water, and rinse the glass slide with ultrapure water for approximated 30 s.

2.8 Place the cover glass in a back-to-back manner. Use the substrate clamp to hold the glass slides. Immerse the glass slide underneath the water surface by manually clicking “dipper down” on the Langmuir control software.

2.9 Zero the film balance, carefully spreading Solution B drop by drop at the air-water interface (**Figure 2A**). 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.

2.10 Stop adding lipids till film balance readout around ~15-20 mN/m, wait for ~10-15 min. Initiate the barrier controller to alter the surface area by clicking “start experiments,” till film balance readout to 37 mN/m. Keep the pressure for ~20-30 min (**Figure 2B**).

2.11 Raise the cover glass at the speed of 22 mm/min while maintaining the surface tension at 37 mN/m. 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 (**Figure 2C**). This forms the bottom leaflet of the lipid bilayer.

2.12 Clean the air-water interface by suction, rinse the trough with ultrapure water.

2.13 Clean a one-welled glass slide (e.g., Shaefer slide) using chloroform, ethanol, and ultrapure water before use. Set the clean glass slide on the trough with ultrapure water underneath the water layer. Make sure the well is facing up toward the air-water interface and pour fresh ultrapure water until the glass slide is fully covered. Repeat step 2.8.

2.14 Hold the cover glass with lipid monolayer from step 2.4 using a silicon suction cup (make sure monolayer side is away from the suction cup), gently push the lipid monolayer to the air-water interface, hold the cover glass for ~2-3 s at the interface, then push the cover glass against the slide (**Figure 2D**). Take the slide out with a cover slide.

NOTE: The lipid bilayer will be held at the surface of the cover-glass facing the sandwiched area between the two slides (**Figure 2E**).

2.15 Take the cover glass with the bilayer to an epifluorescence microscope. Image the lipid bilayer. If a homogeneous distribution of lipid dye is observed, photobleach a small area of the bilayer for 30 s, turn off the light source for ~30 s-1 min., then image again to observe recovery. Lipid bilayer will show fluorescence recovery.

NOTE: Membranes with defects or bad fluorescence recovery should not be used for further experiments.

3 Protein reconstitution into the polymer-tethered lipid bilayer

3.1 Prepare a crystallization dish containing ultrapure water. Prepare a clean microscope image ring and place underneath the dish.

3.2 Immerse the “sandwich” of the Schaefer slide and cover glass that containing lipid bilayer underneath the water, gently separate the Schaefer slide and cover glass, hold the cover glass slide from the bottom, away from the bilayer side, transfer the cover glass into the image ring, close the image ring.

NOTE: Make sure that the cover glass with lipid bilayer is always in water, and the ring is well sealed.

3.3 Replace the ultrapure water in the image ring with Bis-Tris NaCl buffer, make sure the lipid bilayer is not exposed to any air bubbles. Add 1.1×10^{-9} M n-Octyl- β -D-Glucopyranoside to the lipid bilayer. Immediately add the mixture of 1.2×10^{-9} M of DDM and 1.3×10^{-12} mol purified I-Opa1³⁶ into the image ring. Incubate sample on a benchtop shaker at low speed for 2 h (**Figure 3**).

NOTE: Detergents may vary depending on protein to be reconstituted.

3.4 Distribute 30 mg SM-2 Resin beads into 3 mL of Bis-Tris buffer and shake before applying. Use a plastic pipette to add 5~10 μ L of SM-2 Resin beads to image ring, incubate for 10 min, remove resin beads by rinsing. The final volume of the buffer in the image ring is 1.5 mL.

4 Preparation of proteoliposomes

4.1 Prepare 1 mg of lipid mixture A in chloroform solution. Evaporate chloroform under

nitrogen flow for 20 min and keep under vacuum overnight and form a lipid film.

4.2 Prepare 50 mM calcein containing buffer by dissolving 15.56 g of calcein to 50 mL of 1.5 mol NaOH solution, stir at room temperature until calcein is completely dissolved, added 12.5 mM Bis-Tris and ultrapure water to the final volume of 500 mL. Adjust the pH to 7.5.

4.3 Suspend lipid film in calcein containing buffer, fully hydrate the lipid by heating the suspension at 65 °C for 20 min. 200 nm liposomes are formed through extrusion using a polycarbonate membrane.

4.4 Add 2 µg of I-Opa1 in 0.5 µM DDM to 0.2 mg liposome and incubate at 4 °C for 1.5 h. Remove the surfactant by dialysis using a 3.5 kDa dialysis cassette against 250 ml of 25 mM Bis-Tris, 150 mM NaCl and 50 mM calcein buffer at 4 °C overnight, changing the buffer twice.

4.5 Remove extra calcein using a PD-10 desalting column.

5 Imaging and data analysis

5.1 Acquire TIRF images using a 100x oil-immersion objective (N.A 1.4). Use 543 nm laser and a 488 nm laser for the analysis of TexasRed-PE labeled liposomes and proteoliposomes encapsulated with calcein. Use and apply a 633 nm laser for the analysis of Cy5-PE embedded in the planar lipid bilayer.

5.2 Align the TIRF angle using a lipid bilayer to obtain maximum emission. The quality of lipid bilayer after reconstitution is observed using a 100x oil objective at 25 °C. The diffusion coefficient of phospholipid and reconstituted bilayer are determined using FCS with a protocol describe elsewhere³⁷.

5.3 Add 10 µL of 2 mg/mL proteoliposomes to the image ring and set for 10 mins before the image. GTP, GMPPCP, or GDP are added into the reaction ring with 1 mM MgCl₂ and 1 mM of nucleotide.

5.4 To determine the influence of s-Opa1 in membrane fusion, titrate s-Opa1 into a proteoliposome/supported bilayer sample containing I-Opa1, and record fusion events.

5.5 Simultaneous imaging of TexasRed-DHPE and calcein is achieved through a beam-splitting system. Both 488 nm and 543 nm lasers were applied to the sample simultaneously. The emission light was then divided using a 560 nm beam splitter. The split emission light then is filtered by a 510 nm filter with a bandwidth of 42 nm and a 609 nm filter with a bandwidth of 40 nm. The filtered beam is projected to two adjacent areas on camera chip.

5.6 Fluorescent emission is simultaneously recorded through a 609-emission filter with a bandwidth of 40 nm, and a 698-emission filter with a bandwidth of 70 nm. The microscope system is equipped with a CMOS camera maintained at -10 °C.

5.7 Particle identification of the liposomes can be performed using a Gaussian-based particle recognition algorithm. The particle distribution and intensity are analyzed channel-by-channel. A lipid bilayer signal is used as a mask to isolate particles.

REPRESENTATIVE RESULTS:

The reconstituted transmembrane protein freely diffuses and is homogeneously distributed in the membrane.

Example images of a lipid bilayer and its lipid fluidity validated by epifluorescence microscopy is shown in **Figure 4**. Lipid distribution in bilayer before and after photobleaching is shown in **Figure 4A,B**. Homogeneity of the lipid bilayer was visualized using an epifluorescence microscope before and after reconstitution (**Figure 4D,E**). I-Opa1 reconstituted in lipid bilayer was validated by fluorescence correlation spectroscopy (FCS). We use dye conjugated lipids to evaluate the lipid diffusivity of the bilayer. Reconstituted Opa1 was labeled using a fluorescent-tagged anti-Opa1 C-terminal antibody. Bilayer lipid diffusion was measured as $1.46 \pm 0.12 \mu\text{m}^2/\text{s}$, while the diffusion coefficient of bilayer-reconstituted I-Opa1 was $0.88 \pm 0.10 \mu\text{m}^2/\text{s}$. Intensity readout from the FCS curves indicated 75% of I-Opa1 is reconstituted into the lipid bilayer (**Figure 4G,H**). These results suggest that I-Opa1 freely diffuses in the polymer-tethered lipid bilayer with the potential to self-assemble into functional complexes.

Fluorescence step bleaching indicated an average of 2-3 copies of I-Opa1 were reconstituted in a given liposome (**Figure 5A,B**). The size distribution of Opa1 reconstituted proteoliposomes was tested after reconstitution using DLS (**Figure 5C**). The reconstitution of Opa1 in proteoliposomes was also verified using FCS. The diffusion coefficient of free antibody was $164 \pm 22 \mu\text{m}^2/\text{s}$; diffusion coefficient for liposomes labeled with a lipid dye was $2.22 \pm 0.33 \mu\text{m}^2/\text{s}$, and the diffusion coefficient for I-Opa1 proteoliposomes bound to a TexasRed labeled anti-His antibody was $2.12 \pm 0.36 \mu\text{m}^2/\text{s}$.

Detection of membrane tethering, lipid demixing/hemifusion, and pore opening by fluorescent microscopy.

Membrane tethering is monitored by observing the signal of TexasRed on the surface of lipid bilayer using TIRF microscopy (**Figure 6A**). Membrane lipid demixing (hemifusion) behavior was monitored through TexasRed as the liposome marker diffuses into the lipid bilayer. Calcein dequenching helps distinguish full fusion pore formation from only lipid demixing. This allows comparison between conditions where particles stall at hemifusion (Fig 6B), and particles that proceed to full fusion (**Figure 6C**).

Membrane tethering is indicated by a stable lipid signal from liposomes. The distance could be evaluated based on the FRET signal between the labels of the two membranes³⁶. Hemifusion signal features no dequenching in the calcein signal (**Figure 6B**, lower row), but a rapid decay of the TexasRed signal indicates diffusion of the dye into the lipid bilayer (**Figure 6B** upper row). Full fusion (with pore opening) features both lipid decay and content release (**Figure 6C**). TexasRed intensity and calcein intensity can be tracked in a time-dependent manner to provide quantitative detail for the kinetics of membrane fusion³⁶.

FIGURE AND TABLE LEGENDS:

Figure 1: Monitoring mitochondrial membrane fusion. (A) Organelles are cellular membrane compartments. (B) Sequential steps of mitochondrial membrane fusion. Fusion of the outer membrane of mitochondria is catalyzed by Mfn1 and/or Mfn2, while inner-membrane fusion is mediated by Opa1. (C-F) Schematic of the in vitro reconstitution platform to study mitochondrial membrane fusion. The platform includes two parts: a proteoliposome and a polymer-tethered lipid bilayer, both with reconstituted I-Opa1. Fluorescent labels, including two different fluorescent membrane dyes and a content marker, help distinguish steps during membrane fusion. The two membrane markers (Cy5-PE (red) and TexasRed PE (orange) make a FRET pair, which can report on close membrane docking. Diffusion of TexasRed-PE that labels proteoliposome is an indicator of lipid demixing (hemifusion). Content release is monitored through the dequenching of the calcein signal (shown in green). Panels A and B created using Biorender.

Figure 2: Steps in making a polymer-tethered lipid bilayer. Steps of making lipid bilayers using Langmuir-Blodgett dipping (A-C) and Langmuir-Schaefer transfer (D) techniques. (E) The final “sandwich” containing the lipid bilayer.

Figure 3: Procedure for reconstituting I-Opa1 into a polymer-tethered lipid bilayer.

Figure 4: Distribution of lipid and reconstituted protein in the model membrane. (A-C) Example images of a lipid bilayer and its lipid fluidity validated by epifluorescence microscopy. (A) Homogeneous lipid distribution in bilayer prior to photobleaching. (B) Snapshot immediately after photobleaching. (C) Bilayer imaged after fluorescence recovery indicates good lipid fluidity of the membrane following reconstitution. (D,E) Representative images of lipid distribution before (D), and after (E) I-Opa1 reconstitution indicate the reconstitution process did not create defects in the bilayer. Representative TIRF image of I-Opa1 labeled with Alexa 488 conjugated antibody (F) showing a homogeneous distribution of Opa1 upon reconstitution. G. Representative raw photon counts of I-Opa1 signal by fluorescent correlation spectroscopy. In the control, no I-Opa1 was reconstituted in the bilayer, while antibody was added and rinsed. The diffusion of I-Opa1 is significantly slower than lipids in the membrane, consistent with successful reconstitution of transmembrane I-Opa1 (H). Scale bar: 10 μ m.

Figure 5: Fabrication and characterization of proteoliposomes (A) Steps in fabricating proteoliposomes with encapsulated, quenched calcein. (B) Representative data of fluorescent step-bleaching show an average of 2-3 copies of I-Opa1 embedded in the liposome. (C) Representative size distributions of proteoliposomes (red) without any nucleotide 1 h after GTP incubation (green).

Figure 6. Representative results showing particle tethering (A, scale bar 10 μ m), hemifusion (B, scale bar 0.5 μ m), and fusion (C, scale bar 0.5 μ m). (A) Proteoliposomes tethered to Opa1-reconstituted lipid bilayer before GTP addition. (B) An example of hemifusion. The upper row in

B shows lipid demixing (TexasRed signal, red), lower row in B shows no content release (calcein signal, green) under these conditions. (C) A representative trace of proteoliposome fusing with the lipid bilayer. Content release can be observed from images in the lower row of showing dequenching of calcein (lower row, green).

DISCUSSION:

In vitro model-membrane systems can describe complex membrane processes under well-defined conditions. These systems can distinguish minimal components necessary for complex molecular processes to reveal new molecular mechanisms^{6,15,20,38}. For membrane proteins, liposomes and planar supported bilayers are common reconstitution systems. In contrast to solid-supported lipid bilayers, the polymer cushion between the substrate and supported membrane in polymer-tethered bilayers allows free mobility of large membrane proteins, and transmembrane-proteins to diffuse and assemble freely³⁴. These features helped us investigate the kinetics of mitochondria inner-membrane fusion³⁶.

We prepared a polymer-tethered lipid bilayer using Langmuir-Blodgett/Langmuir-Schaefer (LB/LS) techniques. This allows us to prepare a bilayer with asymmetric lipid components. Cellular membranes have asymmetric leaflet composition, and the LB/LS approach allows the study of such bilayers. With Schaefer transfer, an entire glass substrate can be covered by a lipid bilayer. It is critical to prepare a clean surface for bilayer preparation. Additionally, it takes practice to perform a Schaefer transfer correctly. Unsuccessful Schaefer transfer can create unwanted defects in a lipid bilayer. In this protocol, the pressure added to the film balance is applicable for a bilayer containing 20% cardiolipin. For bilayers with other components, refer to the surface pressure-area isotherm of the key components. An alternative method is the Langmuir-Blodgett/vesicle fusion (LB/VF) method, where the bottom lipid monolayer is transferred from the air-water interface of a Langmuir trough onto a clean substrate, then liposomes fuse to the top of the supported lipid monolayer and form the final bilayer³⁹. Reconstitution of membrane proteins using the LB/VF method is more straightforward than LB/LS, as reconstitution can be performed through the fusion of proteoliposomes. However, vesicle fusion requires the addition of excess liposomes, which may complicate the study of membrane events dependent on concentration-dependent protein-protein interactions.

The successful reconstitution of transmembrane proteins into both polymer-tethered lipid bilayers and liposomes in a preferred functional orientation is important, yet difficult to enforce. Experimental controls are needed to account for this. For polymer-tethered lipid bilayers, it is also important to maintain the integrity of lipid bilayer during reconstitution. Surfactant concentrations must be kept relatively low to prevent dissolving the lipid bilayer, but high enough to prevent denaturation of the protein of interest^{37,40}. The method described here is ideal for reconstituting membrane proteins for single-molecule studies but is not necessarily scalable for larger-scale studies. Surfactant choice is another important consideration. Frequently, the surfactant used for purification and storage is a good starting point. The maximum concentration of surfactant is usually ~200 times less of the CMC³⁶, in a range where the surfactant maintains protein stability and prevents protein aggregation, while maintaining the integrity of membrane³⁶. Cocktails containing 2 or 3 surfactants may be considered. For reconstitution into

liposomes, a low concentration of surfactant is not necessary. However, surfactant concentrations below CMC are preferable to maintain uniform size and morphology distribution for the liposomes. To prevent leakage of content dye, it is necessary to dialyze against a dye-containing buffer.

In contrast to liposome-based fusion assays, the platform we established provides an approach to investigate the kinetics of each step of membrane fusion. This method provides the ability to study transmembrane fusion proteins under near-native conditions. Model membrane platforms can be applied to study membrane protein assembly and oligomerization, membrane “sculpting”, and protein-lipid interactions of proteins in subcellular environments, like the mitochondrial inner-membrane. This method also allows exploration of important physiological conditions in the membrane-protein interplay, such as bilayer composition asymmetry. The role of a key mitochondrial lipid, cardiolipin, in the bilayer properties of both liposomes and polymer-supported bilayers remains to be defined. Properties such as the ionic strength, membrane thickness, membrane stiffness, membrane curvature, and membrane elastic-viscosity properties all may influence the ability of proteins to assembly into specific functional states. Future studies creatively applying model membrane systems have potential to uncover new aspects of membrane protein organization and function.

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DISCLOSURES:

None

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Figure 1

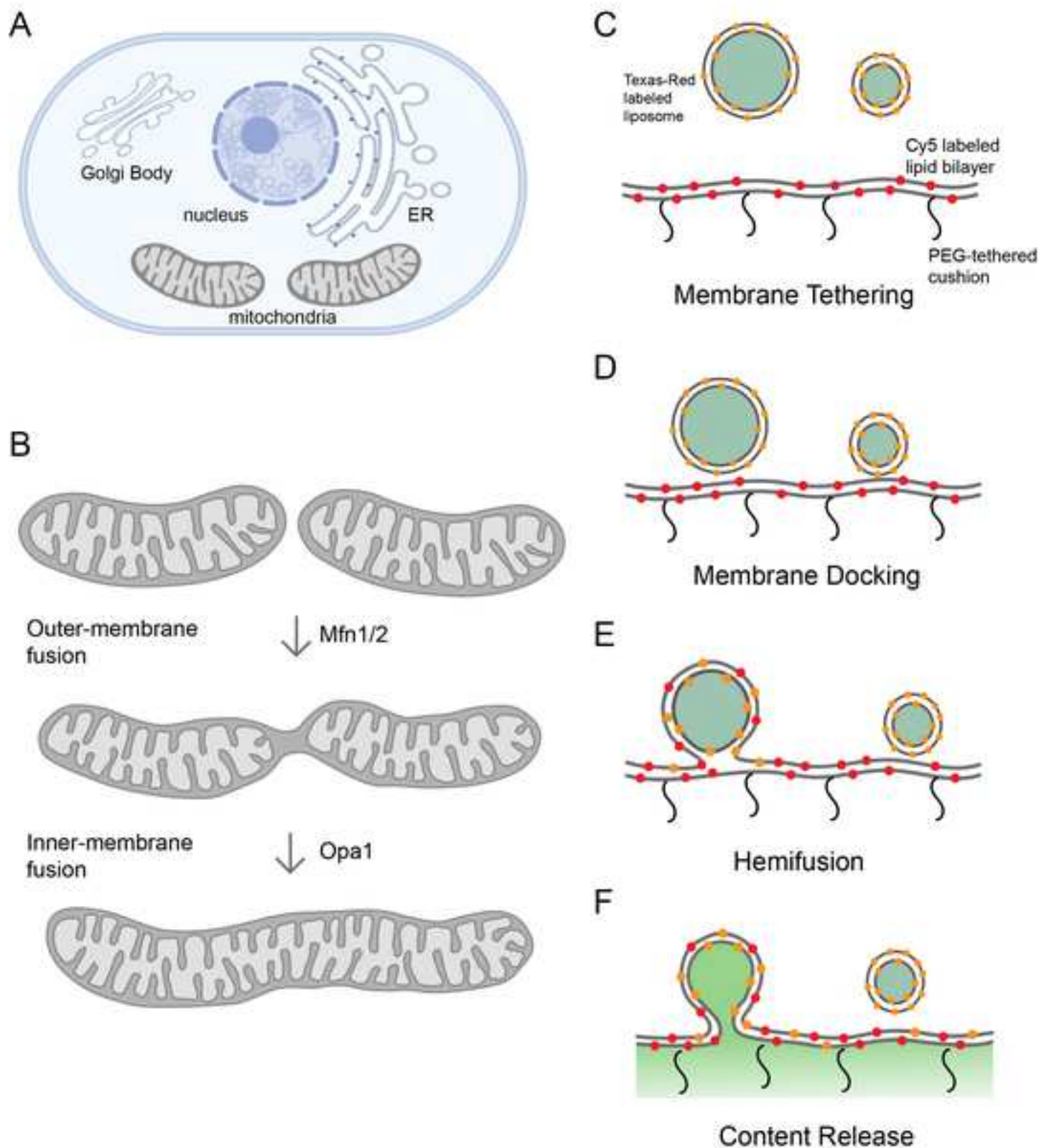
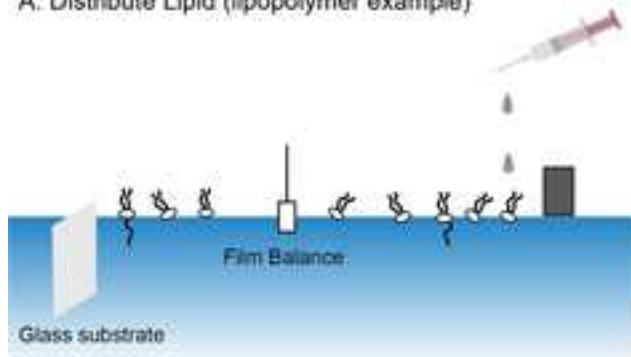
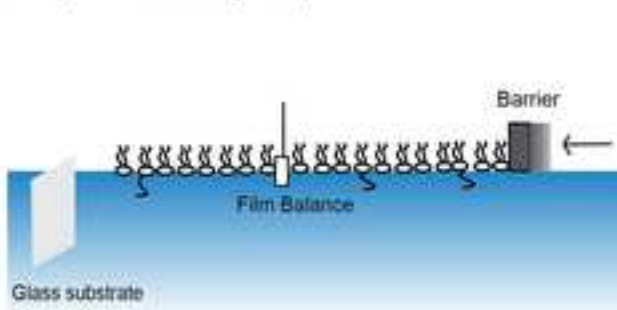


Figure 2

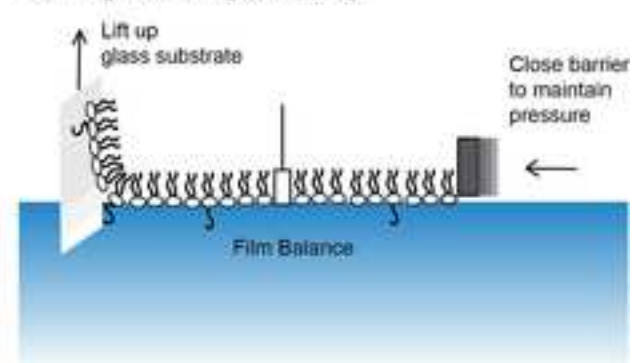
A. Distribute Lipid (lipopolymer example)



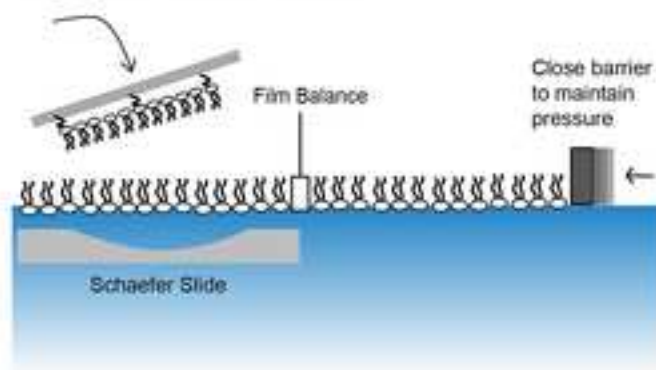
B. Close barrier (transition from a liquid-expanded phase to a liquid condensed phase)



C. Langmuir-Blodgett Dipping



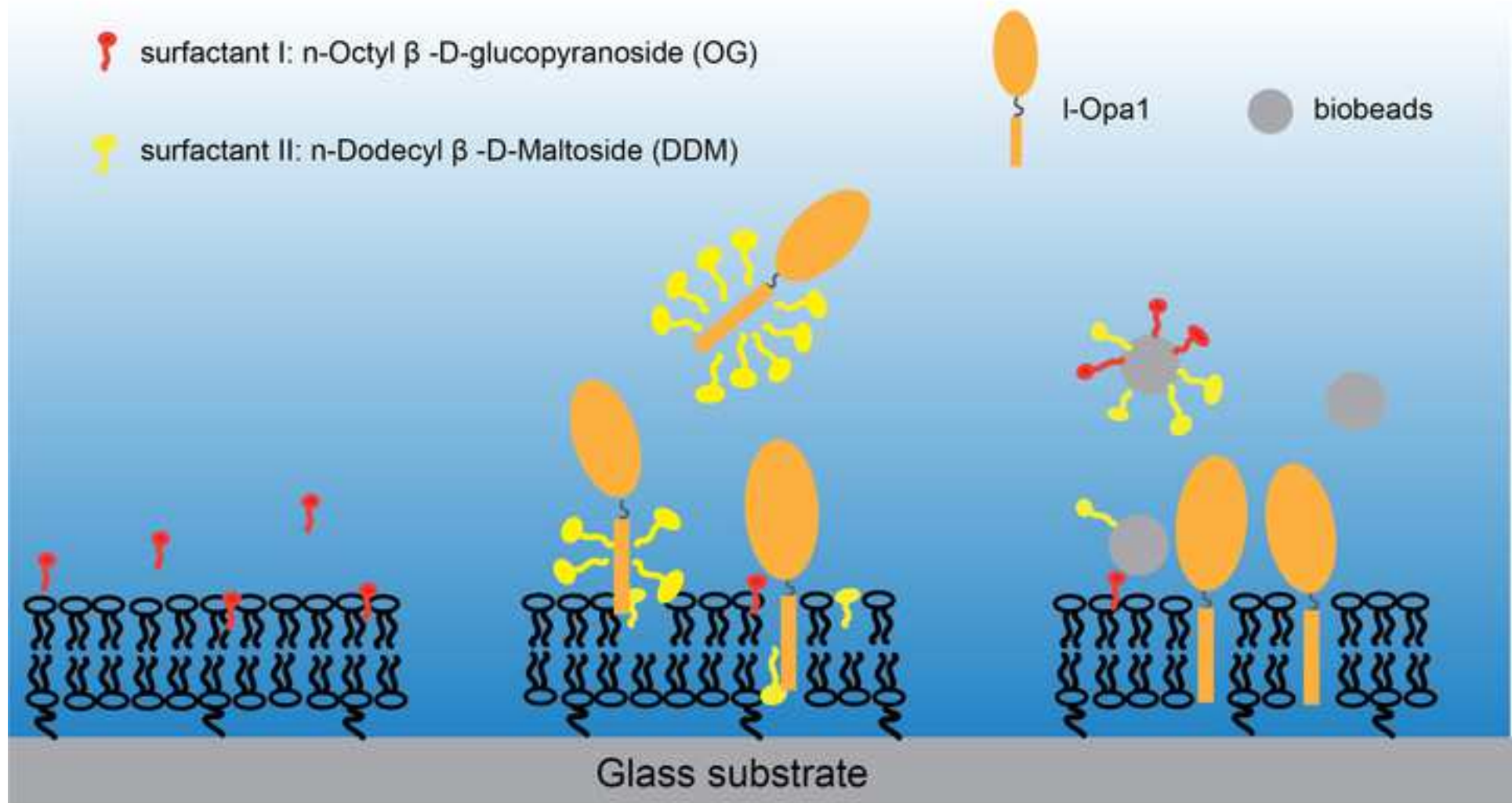
D. Langmuir-Schaefer Transfer



E. Bilayer is sandwiched between cover glass and Schaefer slide



Figure 3



1. pre-soften bilayer
using surfactant I (OG)

2. reconstitute I-Opa1 in the
presence of surfactant II (DDM)

3. remove surfactant
using biobeads

Figure 4

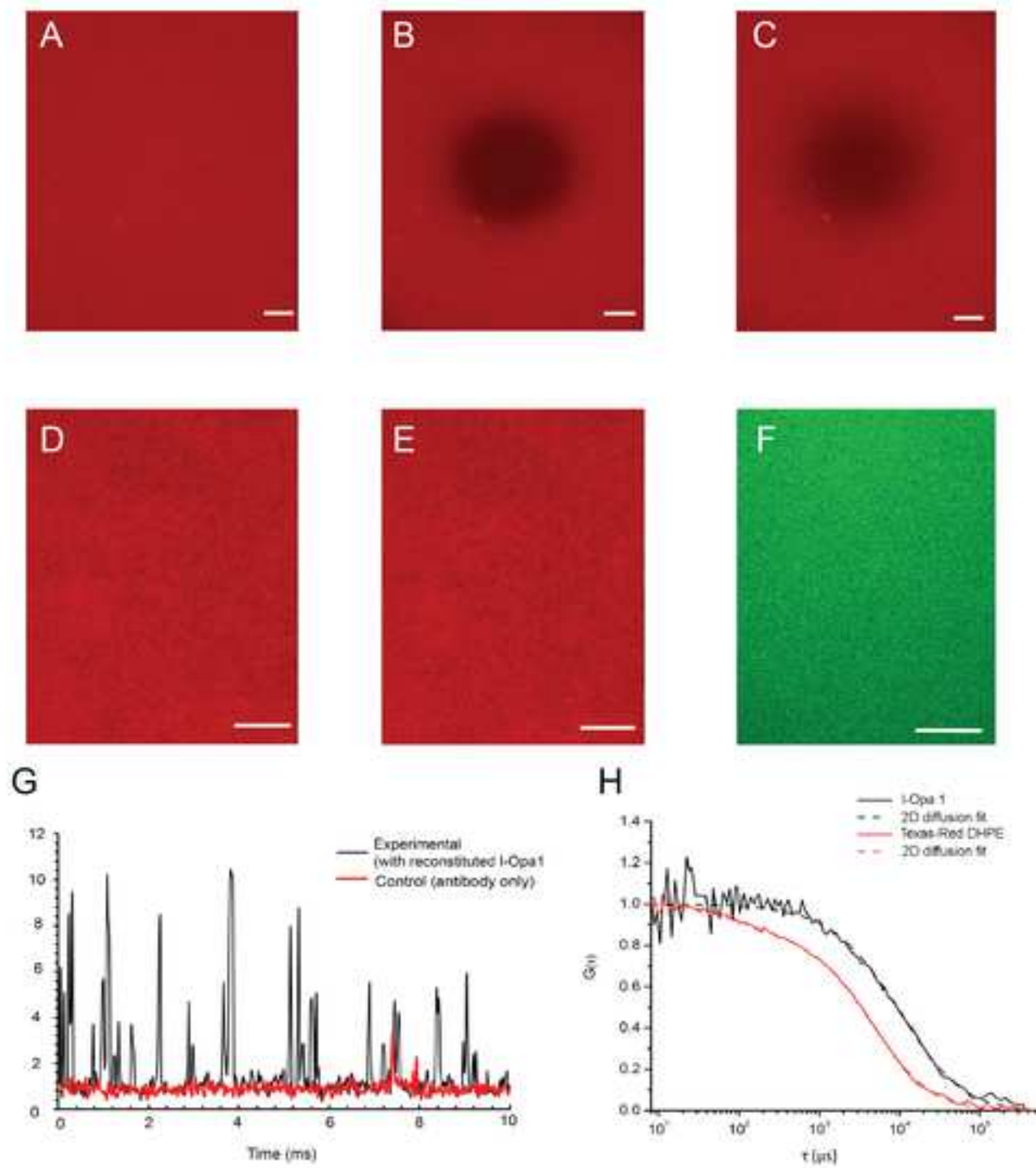


Figure 5

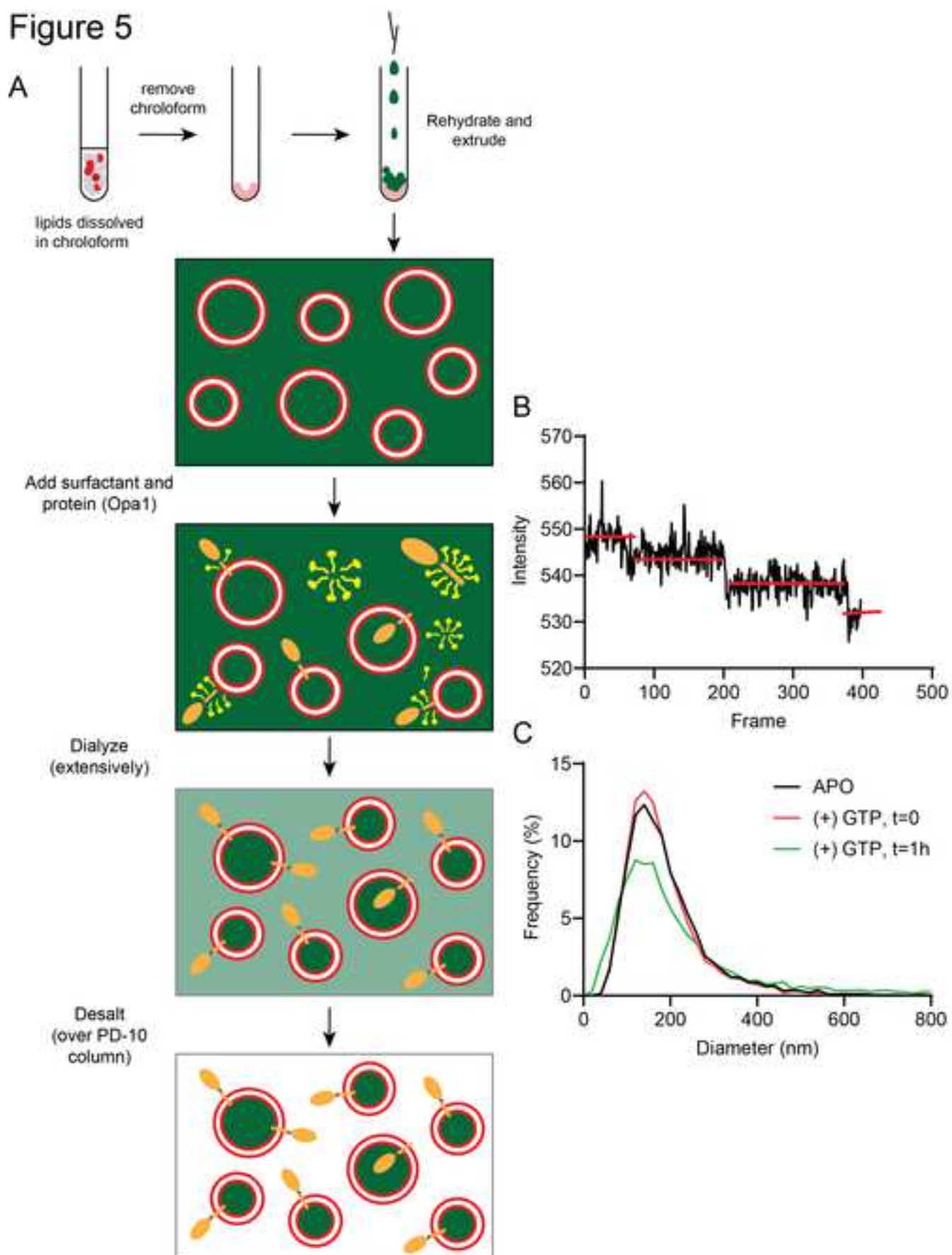
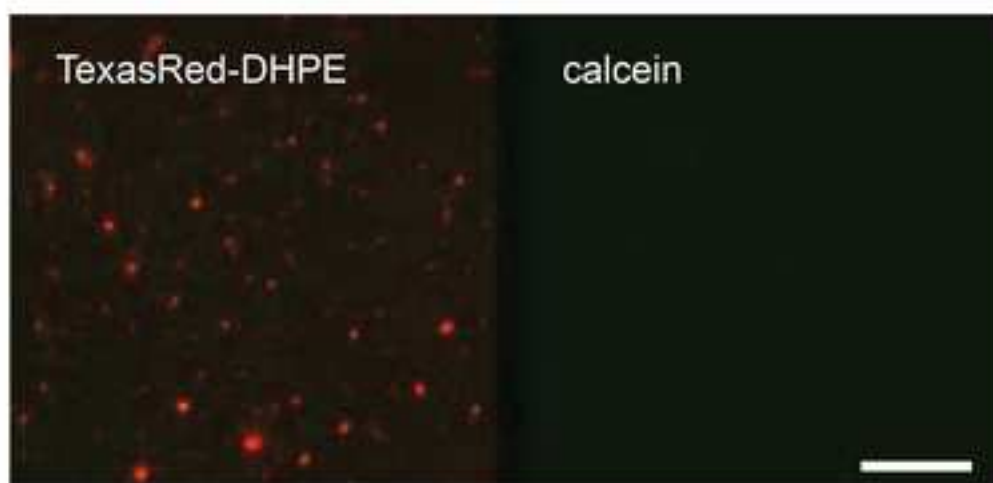
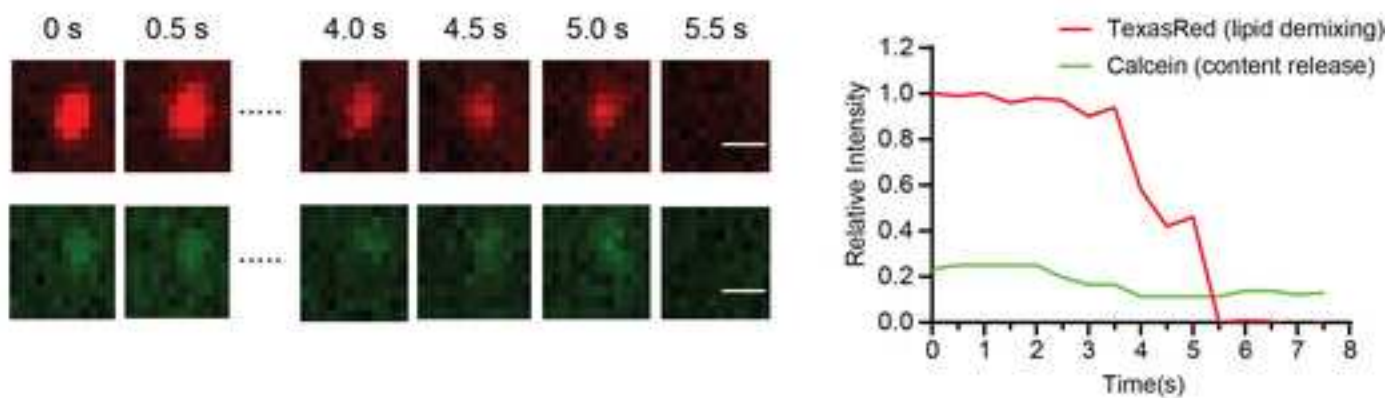


Figure 6

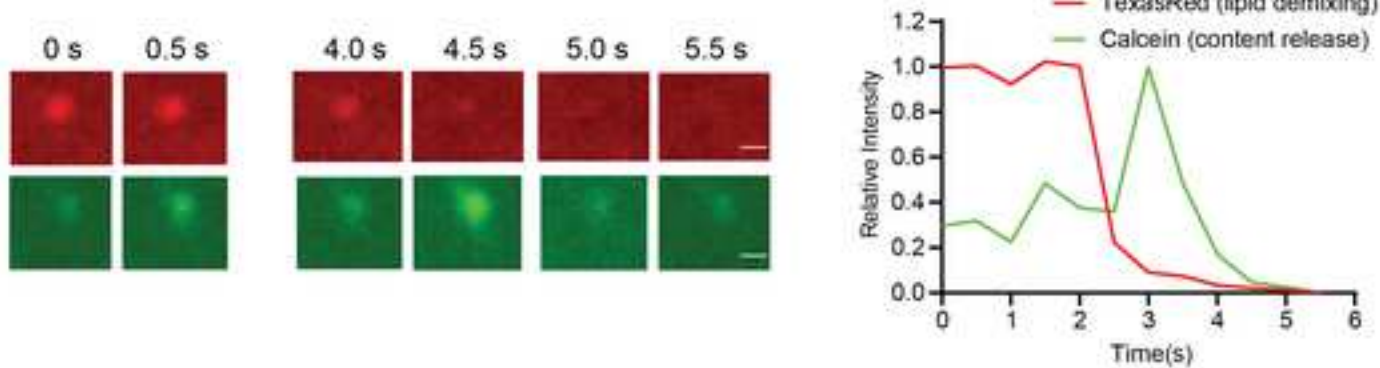
A



B



C



Name of Material/Equipment	Company	Catalog Number	Comments/Description
1,2-dioleoyl-sn-glycero-3-phosphatidylcholine	Avanti polar lipid	Cat #: 810335C1mg	membrane fluorescent markers
1,2-dioleoyl-sn-glycero-3-phosphatidylcholine	Avanti Polar lipids	Cat #: 880130P	lipid molecules
1',3'-bis[1,2-dioleoyl-sn-glycero-3-phosphatidylcholine]	Avanti Polar lipids	Cat #: 710335P	lipid molecules
18:1 (Δ 9-Cis) PC (DOPC)	Avanti Polar lipids	Cat #: 850375P	lipid molecules
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine	Avanti Polar lipids	Cat #: 850757P	lipid molecules
Alexa Fluor 488 Antibody Labeling Kit	ThermoFisher Sci	A20181	
Amber vial with PTFE liner	Fisher scientific	14-955-332	sample vials to keep lipid solutions
Calcein	Sigma-Aldrich	Cat #: C0875; PubChem	fluorescent dye
Chloroform	Fisher scientific	298-500/ C295-4	Fisher brand Chloroform is usually quite reliable for li
Concavity slide (1 well)	Electron Microscop	71878-05	applied as Schaefer Slide
FCS analysis tool	Smith Lab, University of Akron		software tool
Fiji /ImageJ	Fiji	SCR_002285	software tool
Fisherbrand Cover Glasses: Circular	Fisher scientific	12-545-102	Cover glass for solid supported lipid bilayers, the item
GTP Disodium salt	SIGMA-ALDRICH	Cat #: 10106399001	
Langmuir & Langmuir-Blodgett Trough	Biolin Scientific	KN2002	
L- α -lysophosphatidylinositol (LPI)	Avanti Polar lipids	Cat #: 850091P	lipid molecules
Mini Extruder	Avanti Polar lipids	610020	
n-Dodecyl- β -D-Maltopyranoside	Anatrace	Cat #: D310 25 GM	surfactant for reconstitution
n-Octyl- α -D-Glucopyranoside	Anatrace	Cat #: O311HA 25 GM	surfactant for reconstitution
PC Membranes 0.2 μ m	Avanti Polar Lipids	610006	
	NOVUS		
Rabbit Anti-Opa1 antibody	BIOLOGICALS	Cat #: NBP2-59770	antibody for Opa1 C-terminal detection
Slidebook	Intelligent imaging	RRID: SCR_014300	software tool
Teflon threaded seal tape	Fisher Scientific	NC0636085	taflon tape for sample storage
Texas Red 1,2-Dihexadecanoyl-sn-glycero-3-phosphatidylcholine	ThermoFisher Sci	Cat #: T1395MP	membrane fluorescent markers

ipid works.

n is now discontinued as authors prepared the manuscript. An alternative is Fisher brand premium cover glass with catalog numbe

er: 12-548-5M

To: Editor
Jove

Thank you for considering our paper “A model membrane platform for reconstituting mitochondrial membrane dynamics” at JOVE. We are pleased by the favorable response from reviewers and are have made all of the changes as suggested.

We are grateful for all of the helpful points and corrections raised by the reviewers, and hope that that this version of the manuscript will meet your standards for publication.

Sincerely,

Luke Chao (on behalf of the authors)

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

Done

3. Please provide an email address for each author.

Done

4. Please define all abbreviations during the first-time use.

Done

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

Done

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Done

For example: Teflon, MilliQ, Shaefer slide, Zeiss, N.A 1.4, Biobeads, etc.

Shaefer slide is not a commercial; it refers to the glass slide used in Langmuir-Schaefer transfer. Others are corrected.

7. Please reword 612-615, 179-182, 206-209 as it matches with previously published literature.

The sentence has been rephrased.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Corrected accordingly.

9. The Protocol should contain only action items that direct the reader to do something. Please use complete sentences to describe the action.

Corrected accordingly.

10. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Corrected accordingly.

11. Please ensure you answer the “how” question, i.e., how is the step performed? For this please include mechanical actions, button clicks in the software, knob turns in the instrument, command lines, etc. If using long scripts please include as a supplementary file.

More details have been added accordingly.

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The filmable content has been highlighted.

13. Please do not make points in the representative result section. Please use paragraph style instead. Please ensure the results are described in the context of the presented technique. You performed an experiment: how did it help you to conclude what you wanted to and how is it in line with the title.

14. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.

Done.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The images, in fact, are not the same data. Copyright permission is not required.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

Thank you, the concerns have been addressed.

17. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The method described in this manuscript prepares a model system that can be used to study the fusion of the inner mitochondrial membrane, and perhaps other similar systems. The procedure is detailed and complete (other than a few minor details noted below) but is tedious and requires special equipment that is less common, but available. The authors clearly have gone to a lot of work to develop a working system and the fact that the procedure is tedious should not detract from this novel development.

Major Concerns:

None

Minor Concerns:

1. In Protocol 1.2 the lipids add up to just 98 mol%. This could be due to rounding, but appears that the authors have left out the fluorescent lipids which are likely 2 mol%.

The corresponding information of fluorescent lipid was added to the protocol.

2. Line 97, "hydrochloride" presumably means "hydrochloric acid"

Corrected

3. Line 110, do the authors really mean the cover slip was raised at 22 mm/sec. That seems fast. Thank you for noticing the error. The speed should be 22mm/min. It has been corrected.

4. Line 128, there seems to be something missing after the word "using", presumable the type or wavelength of the lamp used to photo bleach.

Corrected.

5. Line 140, it is odd to list the concentration of DDM in "nM" but then list the I-Opal in "mol". Units are adjusted.

6. Line 142, Biobeads are 30 micrograms or 30 milligrams?

Corrected.

7. Line 150, "1.5mol NaOH solution" should probably be in mmoles or mM.

High concentration of calcien can only be dissolved in extremely basic buffer. There are many ways of making a quenched calcien solution. Diluting 1.5M NaOH solution is the way it was done in these experiment.

8. Line 159, "Fig 4" should be "Fig 5"

Corrected.

9. Line 205, "Fig 5B" should be "Fig 5C"

Corrected.

10. Line 206, it seems excessive to list diffusion coefficient with 2 decimal places, when the error is 1000x more (22.27).

Thank you, the data has been adjusted.

11. Line 210, "Fig 3C" should be "Fig 5B"

Corrected.

12. In Figure 4, it is not clear why panel A and F fill the full image, but panels B-E are circular. The figures are now adjusted.

13. In Figure 5C, the black line is not labeled. Also, the graph would be improved if the scale were changed to show more clearly the important area (which I assume is the increase in size seen in the green trace between 300 and 700 nm). Nothing above 1000 nm is visible.

Thanks, the figure has been adjusted.

Reviewer #2:

Manuscript Summary:

This is an intriguing and quite comprehensive manuscript about the model membrane platform for reconstituting mitochondrial membrane proteins. Please see some minor comments below:

- There may be a typo in Page 1, line 87 Mix lipid to into solution A (final concentration 1 mg/ml) that contains...

Thank you. This has been corrected.

- It will be helpful to the reader to write in details how this solution was prepared. Page 2 97 2. Methanol saturated with sodium hydroxide.

The information was added.

- Desalting column is Figure 5 not 4. It will be in the right order to relabel Figures 4 and 5 and make the appropriate changes throughout the text. Page 3 159 4.5 Remove extra Calcein using a PD-10 desalting column (Fig 4).

Corrected.

- I believe the figure is mislabeled here as well. Page 4 lane 210 copies of l-Opa1 is reconstituted in one liposome (Fig. 3C).

Corrected.

- Can authors expand on this? Page 5. Lane 226 Calcein intensity can be tracked in a time-dependent manner to provide quantitative detail for the efficiency and kinetics of membrane fusion [36].

The intensity tracking has been described in the reference. It is outside the scope of this protocol.

- Can authors describe on how and if they could control the orientation of reconstituted protein into the lipid bilayers or liposomes.

So far, we don't have a solid way to control the orientation of the reconstituted transmembrane protein.

The orientation is dependent on the protein structure and surfactant that apply. This has been noted in the text.

- It is not clear why s-OPA1 was used for the titration experiments. Page 3. Authors should expand on this.

Thanks, this information has been added.