Using supported bilayers to study the spatiotemporal organization of membrane bound proteins

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Abstract
Cell division in prokaryotes and eukaryotes is commonly initiated by the well-controlled binding of proteins to the cytoplasmic side of the cell membrane. However, a precise characterization of the spatiotemporal dynamics of membrane-bound proteins is often difficult to achieve *in vivo*. Here, we present protocols for the use of supported lipid bilayers to rebuild the cytokinetic machineries of cells with greatly different dimensions: the bacterium Escherichia coli and eggs of the vertebrate *Xenopus laevis*. Combined with total internal reflection fluorescence (TIRF) microscopy, these experimental setups allow for precise quantitative analyses of membrane-bound proteins. The protocols described to obtain glass-supported membranes from bacterial and vertebrate lipids can be used as starting points for other reconstitution experiments. We believe that similar biochemical assays will be instrumental to study the biochemistry and biophysics underlying a variety of complex cellular tasks, such as signaling, vesicle trafficking and cell motility.

Keywords: supported bilayer, cell division, in vitro reconstitution, cytokinesis signaling, E. coli, Xenopus, lipids, microtubules, FtsA, FtsZ

Contents

1. Introduction
2. Equipment and Reagents
   2.1. Equipment
   2.2. Reagents
   2.3. Proteins and protein labeling
   2.4. Xenopus egg extract
3. Preparation of Supported Lipid Bilayers
   3.1. Lipid handling and storage
   3.2. Preparation of SUVs
   3.3. Methods for cleaning glass slides
   3.4. SLBs from E. coli lipids
   3.5. SLBs from mammalian lipids
   3.6. Evaluating bilayer integrity
4. Fluorescence Microscopy

4.1. Imaging protein self-organization from purified components
4.2. Imaging cytokinesis signaling using Xenopus egg extract
5. Conclusion
6. Acknowledgments
7. References

1. Introduction

Phospholipid membranes are a central feature of living cells. They not only act as a physical boundary for the cell and its intracellular organelles, but also provide a two-dimensional platform to which proteins can reversibly bind and interact with each other. For example, proteins bound to the membrane surface interact to perform complex cellular tasks such as cell division and motility (Chen, Hehnly, & Doxsey, 2012; Roubinet, Tran, & Piel, 2012) and can receive, compute and transmit intracellular signals (Groves & Kuriyan, 2010). Association to the membrane is likely to change the reaction kinetics of protein interactions, but exactly how is difficult to predict. Physical confinement of reactants to the membrane surface alone can change the dynamic behavior of a reaction network (Abel, Roose, Groves, Weiss, & Chakraborty, 2012; Elf & Ehrenberg, 2004; Kochanczyk, Jaruszewicz, & Lipniacki, 2013). For example, binding to a surface can facilitate protein interactions by increasing local concentrations. In contrast, interaction rates can be decreased because membrane-bound proteins bound diffuse much slower than in solution (Kholodenko, Hoek, & Westerhoff, 2000). Binding to the membrane also defines the orientation of the reactants, which could either speed up or slow up protein interactions (Groves & Kuriyan, 2010; Jung, Robison, & Cremer, 2009). In addition, the membrane can change the biochemical properties of proteins since lipids can activate and be activated by signaling molecules (Di Paolo & De Camilli, 2006; Leonard & Hurley, 2011). Our lack of knowledge about how the membrane takes part in the self-organization of protein systems largely results from the fact that detailed quantitative information on the role of the membrane is difficult to obtain *in vivo* and that membranes are challenging to work with *in vitro*. For an understanding of the role of the membrane for cell signaling, protein complex formation and polymerization we need an experimental assay that more accurately reflects the physiological context in which these proteins interact with each other. At the same time, a biomimetic membrane platform must allow for a precise quantitative analysis of lipid and protein dynamics.

After their introduction in 1984 (Brian & McConnell, 1984), supported lipid bilayers (SLBs) have emerged as a powerful model for natural cell membranes and have found widespread use among various scientific disciplines (Castellana & Cremer, 2006; Sackmann, 1996). The basic system consists of a phospholipid bilayer on a solid, usually inorganic surface; most often a freshly cleaned glass coverslip is used. A thin, 1-2 nm thick layer of water separates the bilayer from the underlying substrate, allowing lateral mobility of lipids and thus reproducing the fluidity of both leaflets of the bilayer, as in cell membranes (Richter, Bérat, & Brisson, 2006). Supported bilayers offer a number of advantages over other biomimetic membrane systems. First, SLBs are a perfect platform for surface-sensitive imaging techniques such as total internal reflection fluorescence (TIRF) microscopy, enabling a detailed characterization of protein dynamics down to the single molecule level. Second, their lipid composition can be precisely controlled, thus the role of biochemically active lipids such as phosphoinositides can be studied in a reconstituted system. Third, microfabrication of the membrane support substrates can

structure the membrane in two (Groves, 1997; Schweizer, Loose, Bonny, Kruse, Mönch, & Schwille, 2012a) and three dimensions (Zieske & Schwille, 2013), making it possible to study the influence of spatial confinement and membrane curvature on the emergent properties of a biochemical system. Finally, SLBs are remarkably stable for many hours, especially compared to free-standing membranes such as lipid vesicles and black lipid membranes (i.e. bilayers painted over an aperture). SLBs will remain largely intact even when subject to high flow rates or vibration and, unlike black lipid membranes, the presence of holes will not destroy the entire bilayer.

While supported bilayers are ideal for studying proteins binding peripherally to the membrane, their use for transmembrane proteins can be problematic due to their interaction with the support surface. One approach to mimic the permanent membrane localization of the cytoplasmic domains of transmembrane proteins has been to use artificial membrane anchors, such as biotin-streptavidin affinity binding (Visnapuu & Greene, 2009), polyhistidine-Ni^{2+} coordination chemistry (Galush, Nye, & Groves, 2008; Loose & Mitchison, 2014) or direct covalent coupling using thiol-reactive groups targeting cysteine residues (Lin et al., 2014). A more challenging alternative is to use a hydrogel to increase the distance to the solid support, which has been successfully used to reconstitute full-length transmembrane proteins into SLBs (Tanaka & Sackmann, 2005; Wagner & Tamm, 2000).

Here, we describe the use of flat supported bilayers to study the spatiotemporal dynamics of two biochemical systems with different levels of complexity and spatial scales: First, the polymerization of the bacterial tubulin homolog FtsZ with its membrane anchors FtsA and ZipA on a membrane of lipids from *Escherichia coli* and second, the reconstitution of cytokinesis signaling using cytoplasmic extracts from *Xenopus laevis* eggs and a supported membrane made from mammalian lipids (Fig. 1). The first system represents a minimal pure protein system that can reconstitute polymerization dynamics otherwise not observed without a membrane support (Loose & Mitchison, 2014). The second system exemplifies an attempt to build a cell in its full complexity outside of a real cell, by combining a model cytoplasm with a model cell membrane (Nguyen, Groen et al., 2014). We explain what to consider for the preparation of supported membranes for these systems and how the complexity of the biochemical mixture affects stability of the bilayer.

2. Equipment and reagents

2.1. Equipment

Equipment for Small Unilamellar Vesicles (SUVs)

- 10 µl Hamilton Syringe 1700 Series Gastight Syringes, Point Style 2) (Sigma, cat. no. 20972)
- 100 µl Hamilton Syringe 1700 Series Gastight Syringes, Point Style 2) (Sigma, cat. no. 20688)
- Glass vials (VWR Vials, Borosilicate Glass, with Phenolic Screw Cap) (VWR, cat. no. 66011-020)
- Nitrogen gas
- Argon gas

- Pressurized cleaner (TechSpray, Ted Pella, cat. no. 81600)
- Vacuum chamber for desiccation
- Branson CPX1800 Ultrasonic Cleaner

Equipment for cleaning glass

- Glass coverslips, e.g. 24x60mm or 22x22mm with 0.13–0.17mm thickness (VWR, cat. no. 48404-133 or 48367-059)
- Staining containers for 24x60mm (Thomas Scientific, cat. no. 8541K92)
- Polypropylene rack for square coverslips (Sigma Aldrich, cat. no. Z688568)
- Teflon rack for square coverslips (Life Technologies, cat. no. C-14784)
- 100 ml and 500 ml glass beakers
- Plasma cleaner, e.g. Harrick PDC-32G
- Plastic forceps, e.g. Excelta Plastic Tweezers (Fisher Scientific, cat. no. 17-456-066)
- Chemical hood

Equipment for preparing Supported Lipid Bilayers (SLBs)

- Handheld UV lamp or Benchtop UV Transilluminator (UVP, cat. no. UVL-23RW or 95-0449-01)
- Thin-walled PCR tubes, 0.2 ml (VWR, cat. no. 20170-012)
- Microreaction tubes, 0.5 ml (VWR, cat. no. 20901-505)

Equipment for preparing Xenopus egg extracts

- See ref. (Field, Nguyen, Ishihara, Groen, & Mitchison, 2014) for list of equipment.

Imaging

• Nikon TIRF microscope: Imaging was performed on Nikon Ti-E motorized inverted microscope equipped with TIRF 1.49 NA objective lenses (60x or 100x) (Nikon), a Nikon motorized TIRF illuminator, Perfect focus, a Prior Proscan II motorized stage, Agilent MLC400B laser launch (488nm, 561nm, 647nm), an Andor DU-897 EM-CCD camera driven by NIS-Elements image acquisition software.

2.2. Chemicals

Chemicals for Small Unilamellar Vesicles (SUVs)

- *E. coli* Polar Lipid extract Avanti (Avanti Polar Lipids, cat. no. 100600)
- L-α-phosphatidylcholine (Brain, Porcine) (PC, Avanti Polar Lipids, cat. no. 840053)
- L-α-phosphatidylserine (Brain, Porcine) (PS, Avanti Polar Lipids, cat. no. 840032)
- L-α-phosphatidylinositol (Liver, Bovine) (PI, Avanti Polar Lipids, cat. no. 840042)
- L-phosphphatidylinositol-4,5-bisphosphate (Brain, Porcine) (ammonium salt) (PI(4,5)P_2, Avanti Polar Lipids, cat. no. 840046)
- 18:1 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid) succinyl (nickel salt) (DGS-NTA(Ni), Avanti Polar Lipids, cat. no. 790404)
- *E. coli* SLB buffer: 25 mM Tris, pH 7.5, 300 mM KCl, 1 mM $MgCl_2$
- Extract buffer (XB): 10 mM K-Hepes, pH 7.7, 100 mM KCl, 1mM $MgCl_2$, 0.1 mM $CaCl_2$, 50mM sucrose
- High-salt extract buffer (HS-XB): XB + 200 mM KCl

Chemicals for preparing Supported Lipid Bilayers (SLBs)

- Norland Optical Adhesive 63 (Norland Products, cat. no. 6301)
- VALAP (Vaseline, Lanolin, Paraffin in 1:1:1 mass ratio)

Chemicals for cleaning glass

- Potassium hydroxide (Macron, cat. no. 6984-12)
- Hellmanex III (Hellma, cat. no. 9-307-011-4-507)
- H_2O_2 , 30% stabilized (Sigma, cat. no. 216763)
- Pure ethanol (VWR, cat. no. 89125-186)

Chemicals for self-organization assay using pure proteins

- Catalase from Bovine liver (Sigma, cat. no. C9322)
- Glucose Oxidase from *Aspergillus niger* (Sigma, cat. no. G7016)
- D-(+)-Glucose monohydrate (Alfa Aesar, cat. no. A11090)
- DTT (DL-Dithiothreitol) (Sigma, cat. no. D9779) in
  Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma, cat. no. 238813)
  Note: prepare 100 mM stock solution by dissolving 100 mg/ml Trolox powder in 430 µl Methanol, add 3.2 ml ddH_2O, adjust pH by adding 5M NaOH to obtain a clear, yellow solution with a pH around 9. Bring volume to 400 µM and prepare 20 µl aliquots.
- ATP disodium salt (Sigma, cat. no. A7699)
- Guanosine triphosphate (GTP) (Sigma, cat. no. G8877)
- Trizma hydrochloride (Sigma, cat. no. T3253)
- Potassium chloride (Sigma, cat. no. P4504)
- Magnesium chloride (Sigma, cat. no. M9272)

Chemicals for reconstitution assay of cytokinesis signaling using Xenopus egg extracts

- See Field et al. (Field et al., 2014) for list of buffers and reagents needed for preparing Xenopus egg extracts.
- Dynabeads Protein A (Life Technologies, cat. no. 10001D)

2.3. Proteins and protein labeling

Proteins FtsZ, ZipA and FtsA were purified according to (Loose & Mitchison, 2014). Purified proteins were fluorescently labeled either using thiol-reactive maleimide dyes following the protocol of the manufacturer (e.g. Alexa Fluor 488 C5 maleimide, Invitrogen) or by "Sortagging" following a protocol based on (Popp, Antos, & Ploegh, 2009) using a fluorescently labeled probe peptide, with the difference that we performed the reaction for three to four hours at 4°C.

Antibodies against *X. laevis* Aurora A kinase (AurkA) and Aurora B kinase (AurkB) are described in Nguyen, Groen *et al.* (Nguyen, Groen et al., 2014). Tubulin was purified from bovine brain and labeled with Alexa Fluor 488 or 568 dyes, and anti-AurkB antibodies were labeled with Alexa Fluor 647 dye according to Hyman *et al.* (Hyman et

al., 1991) and Groen *et al* (Groen, Ngyuen, Field, Ishihara, & Mitchison, 2014). The Rho GTPase-binding domain (rGBD) of mouse Rhotekin protein was fused to mCherry (mCherry-rGBD) and purified as described (Nguyen, Groen et al., 2014). Purified His-tagged Lifeact-GFP (Riedl et al., 2008) to visualize F-actin was a kind gift from David Burgess (Boston College, MA). Anti-AurkA antibodies were conjugated to Protein A Dynabeads following the protocol of the manufacturer. When added to Xenopus egg extracts, these Aurora A kinase-coated beads served as artificial centrosomes that nucleated microtubule asters (Tsai & Zheng, 2005).

2.4. Xenopus egg extract

Xenopus egg extract with intact actin is prepared according to (Field et al., 2014). These extracts are more variable in quality than extracts prepared with the actin depolymerizing drug cytochalasin D (Desai, Murray, Mitchison, & Walczak, 1999), as the presence of F-actin affects centrifugal separation of the cytoplasmic layer. To ensure that good quality extract is obtained, 3-4 frogs are routinely induced to lay eggs, batches of eggs from different frogs are processed separately and the resultant extracts are not pooled. Each batch is then assayed for actin contractility in metaphase by filming a droplet under mineral oil with a dissecting microscope (Field et al., 2011) and for assembly of large microtubule asters between glass coverslips after adding calcium to convert the extract to interphase (Nguyen, Groen et al., 2014). Extracts capable of reconstituting cytokinesis signaling exhibit strong actin contractility, assemble dense microtubule asters along which F-actin is aligned (Fig. S1), and form robust aster-aster interaction zones where microtubules are depleted (Nguyen, Groen et al., 2014). Cytokinesis signaling at the lipid bilayer was only observed in extract batches that passed the quality control evaluations.

3. Preparation of Supported Lipid Bilayers

Planar bilayers are most commonly formed by fusion of small unilamellar vesicles (SUVs) that are brought into contact with a very clean, hydrophilic surface. These vesicles adsorb to the hydrophilic substrate, where they then spontaneously coalesce to cover the flat surface. One big challenge for SLB formation is to establish conditions to obtain a homogeneous membrane on the support surface. For successful SLB formation, three requirements must be met: First, it requires a hydrophilic support that has been freshly and rigorously cleaned to remove all organic adsorbates and other contaminants. Second, the SUV density has to be sufficiently high to allow for complete coverage of the surface and to induce stress on the surface-adsorbed vesicles. And finally, for the successful fusion of vesicles with negatively charged lipids, high ionic strength buffers must be used (Castellana & Cremer, 2006). Adding divalent cations such as Ca^{2+} further supports vesicle fusion. Once the bilayer is formed, it must not be allowed to dry out. Bilayers submerged in buffer are stable for at least a few hours at room temperature.

Most studies use rigorously cleaned glass coverslips as support, but the silicate mineral mica is often used as a benchmark substrate for biomimetic membranes, because it has a high surface charge and is atomically flat. Their use in fluorescence microscopy experiments is challenging, however, since mica sheets have to be cleaved as thin as

possible to be sufficiently transparent. The mica sheet is then glued to a coverslip (Loose, Fischer-Friedrich, Ries, Kruse, & Schwille, 2008).

Recent reports suggest that polymeric or soft-matter interfaces can also be used in place of solid silica supports, such as oxidized PDMS polymer cushions (Zieske & Schwille, 2013) or hydrogels (Sackmann & Tanaka, 2000; Tanaka & Sackmann, 2005).

3.1. Lipid handling and storage

Lipids are usually dissolved in organic solvents such as chloroform. For handling and storing chloroform solutions, it is important to avoid plastic pipette tips and containers since they are not inert to chloroform and can leach out impurities. Instead of plastic tips, it is better to use Hamilton syringes or glass microcapillary pipettes. To avoid contamination and impurities it is important to use clean Hamilton syringes dedicated to lipid handling. Instead of plastic reaction tubes, use glass vials with PTFE screw cap lid. For storage of organic solutions of phospholipids, prepare aliquots, cover solution with a layer of argon to prevent oxidation of the lipids, close cap tightly and seal with parafilm. Store at -20°C.

More polar lipids, such as PI(4,5)P_2, are dissolved in more polar solvent mixtures, such as chloroform/methanol/water (60/33/7 v/v %). Furthermore, PI(4,5)P_2 is fairly unstable in organic solution at -20°C (lasting no longer than one week), thus it should be stored as SUVs in aqueous solution under argon at -80°C, as described below.

3.2. How to prepare SUVs

A number of approaches have been adopted for generating small unilamellar vesicles (SUVs). These include sonication, extrusion and dialysis. Among these, we find that disruption of multilamellar large vesicles (MLVs) in freeze-thaw cycles followed by sonication is the fastest and most convenient method to obtain SUVs; however care must be taken not to overheat the lipid mixture during sonication. This method produces small unilamellar vesicles (SUV) with diameters in the range of 15-50 nm.

1. In a 4 ml clean glass vial, add lipid solutions in desired ratio* (1-2 μmol total). Dry down lipids in a N_2 gas stream to obtain a translucent lipid film on the bottom of the glass vial.
2. Vacuum desiccate for 1-2 hours to remove residual organic solvent.
3. Rehydrate lipid film with appropriate amount of buffer (i.e. *E. coli* SLB buffer or high salt XB buffer) to obtain a final 5 mM lipid suspension: incubate buffer with lipid film for 30 min at 37°C, then vortex rigorously to obtain large MLVs. The result should be a milky solution.
4. Freeze-thaw mixture five times using a dry ice/isopropanol mix and a water bath.
5. Optional: Transfer MLV suspension to a 1.5 ml Eppendorf tube.
6. Sonicate suspension in bath sonicator for 2 minutes each until the solution turns translucent.
   Note: some lipid suspensions, especially those made from mammalian lipids or with a high PE content, can take 2-3 rounds of 2-minute sonication or longer

sonication times. A freeze-thaw step between each round also helps to break up large MLVs into SUVs.

7. Prepare aliquots of desired volume, typically 20 µl or larger: For a larger reaction chamber prepared using a 0.5 mL microreaction tube, 10 µl is required to make one bilayer. For a smaller reaction chamber made from a PCR tube, 6 µl is sufficient for one bilayer. Store aliquots at -20°C (stable for 1-2 months).

*Note: to mimic the surface of the *E. coli* inner membrane, we used commercially available *E. coli* lipid extract. To attach His-tagged proteins to the membrane surface at different densities, we used DGS-NTA(Ni) at different molar rations between 0.1 and 10%. For a 40 kDa protein, a ratio of 10% results in complete protein coverage of the membrane surface. To mimic the inner leaflet of the plasma membrane of animal cells, we mixed mammalian lipids at the molar ratio of PC/PS/PI = 0.6/0.3/0.1 (Step 1). For long-term storage of the less stable PI(4,5)P_2 lipids, we made SUVs with the molar ratio of PC/PS/PI/PI(4,5)P_2 = 0.6/0.3/0.05/0.05 ("5% PIP_2 mix"). The "5% PIP_2 mix" suspensions were stored in 10 µl aliquots under argon at -80°C.

3.3. Methods for cleaning glass coverslips

Crucial for the quality of the bilayer is to use freshly cleaned glass surfaces. A number of different methods have been used to prepare glass surfaces for SLB formation. One efficient method to clean glass coverslips is to use Piranha solution, a highly corrosive mixture of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2). To avoid handling this potentially dangerous solution, we t ypically pre-cleaned in alkali and solvents, then used air plasma for final cleaning. Alternatively, if no plasma oven is available, a modified RCA cleaning method can give good results while avoiding Piranha solution (Cras, Rowe-Taitt, Nivens, & Ligler, 1999). Freshly cleaned and dried glass surfaces can adsorb dirt particles from unfiltered air, thus care must be taken that they are not exposed to air for more than 30 minutes before contacting the SUV solution. We have successfully used coverslips from various vendors. Most commonly we used #1.5 thickness, 24x60mm or 22x22mm square coverslips made of standard glass.

Pre-cleaning

1. Immerse coverslips into 3M KOH (168.3 g/l) or Hellmanex solution, either using a coplin jar (for 24x60 mm coverslips) or a polypropylene rack (for square coverslips) to support coverslips and keep them separate. Sonicate for 20 minutes.
2. Discard cleaning solution, and rinse five times with ddH_20.
3. Immerse coverslips in ddH_20, and sonicate for 20 minutes.
4. Discard ddH_20 and rinse with 100% ethanol.
5. Immerse coverslips into 100% ethanol and sonicate for 20 minutes.
6. Immerse into ethanol. At this stage, the coverslips can be kept for several days.

Note: With certain batches of coverslips, we found that a more rigorous pre-cleaning procedure was helpful: 1) sonicate in 100% ethanol for 20 min, then rinse 3x with ddH_20; 2) sonicate in acetone for 20 min, then rinse 3x with ddH_20; 3) incubate in

nitric acid (HNO_3) for 20 min, then rinse 3x with ddH_20; proceed to Step 1 in the pre-cleaning procedure above.

Final cleaning:

Plasma

1. Use forceps to remove coverslips from ethanol, handling only by the edges. Blow dry using compressed, bottled air or nitrogen.
2. Place coverslips into Plasma oven and evacuate for 10 minutes.
3. Switch on Plasma oven at maximum intensity for 5-10 minutes.
4. Immediately proceed to Step 3.4 or 3.5 after cleaning.

Piranha

Only use glass, Pyrex, or Teflon containers; Piranha solutions are not compatible with plastics.

1. Wash glass coverslips 5-6x with ddH_2O. Make sure to remove all organic solvent, otherwise it may react with the Piranha solution.
2. Sonicate for 10 minutes. Rinse with water.
3. Piranha wash (40 ml of 30% H_2O_2, 60 ml H_2SO_4). Pour 75 ml into each glass coplin jar. Sonicate for 20 minutes.
4. Decant Piranha into 500mL beaker. Allow solution to cool before disposal. Don't seal waste container.
5. Wash glass 5-6x with ddH_2O. Glass can be stored for up to a week in ddH_2O.
6. Dry quickly with nitrogen gas or pressurized cleaner.

Caution: Piranha solution is hot and highly corrosive and can explode if mixed with organic matter. Appropriate safety equipment is required.

RCA cleaning

Do not use metal coverslip racks or forceps.

1. Wash glass coverslips 5-6 x with ddH_2O. Make sure to remove all organic solvents, otherwise it may react with the RCA cleaning solutions.
2. Sonicate for 10 minutes. Rinse with water.
3. Weigh out 5g of KOH and dissolve in 60 ml ddH_2O.
4. Heat solution in the hood to 70-80°C (not boiling).
5. Reduce heat and slowly add 20 ml of 30% H_2O_2.
6. Keep temperature at 70-80°C and check for formation of small bubbles.
7. Carefully place coverslips in teflon rack into the solution using plastic forceps. The solution should bubble vigorously but not too strongly.
8. Allow solution to react for ten minutes.
9. Carefully remove the coverslip rack with plastic forceps and place in ddH_2O.
10. Rinse 5x with ddH_2O.
11. In the hood, add 15 ml of concentrated HCl to 60 ml of ddH_2O.
12. Heat solution in the hood to 70-80°C (not boiling).
13. Reduce heat and slowly add 20 ml of 30% H_2O_2 Don't add H_2O_2 to concentrated acid.

14. Keep temperature at 70-80°C and check for formation of small bubbles.
15. Carefully place coverslips in Teflon rack into the solution using plastic forceps. (Note stainless steel will be instantly attacked by this solution!) The solution should bubble vigorously but not too strongly.
16. Allow solution to react for ten minutes.
17. Carefully remove the coverslip rack with plastic tongs and place in ddH_2O.
18. Rinse 5x with ddH_2O.

3.4. SLBs from E. coli lipids

Prepare reaction chamber (two hours before experiment)

1. Prepare reaction tubes (500 µl Eppendorf tube if final volume is more than 100 µl or PCR tubes for 20-50 µl final volume): cut off their bottom and their cap at the hinge.
2. Cover the flat, uncut rim of the tube with a thin layer of UV glue using a pipette tip.
3. Place tube with glued rim down flat onto freshly cleaned glass surface of a 24x60 mm cover slip.
4. Expose to UV light (365 nm) for 5-10 minutes to allow glue to polymerize.
5. Dilute SUV stock suspension to 1 mg/ml.
6. Add 50 µl of SUV dispersion into reaction well
7. Add $CaCl_2$ to 1 mM final.
8. Incubate on 37°C heated plate 15-30 min.
9. Wash bilayers at room temperature to remove non-fused vesicles:
   i. Add 50 µl of buffer into each well. Mark meniscus with marker.
   ii. Add 200 µl buffer, pipet up and down 5x, then remove 200 µl of the buffer from each well.
   iii. Repeat last step 5X. Do not touch bilayer with pipette tip.
10. Keep bilayers covered with buffer at RT until use; use within 2-4 hours.

Note: while for SLB formation high osmotic strength is advantageous, the salt concentration can be lowered for the actual experiment.

3.5. SLBs from mammalian lipids

Prepare reaction chamber (two hours before experiment)

1. Use metal slides (stainless steel or aluminum) with circular holes 20-mm in diameter. Attach a 22x22mm coverslip to bottom of the slide using VALAP with plasma-cleaned side facing up through the hole.
2. Cover the rim of a cut PCR tube with a thin layer of UV glue using a pipette tip.
3. Place tube with glued rim down flat onto the surface of freshly cleaned glass coverslip. Each slide can accommodate up to 3 PCR tubes.
4. Expose to UV light (365 nm) for 5-10 minutes to allow glue to polymerize.
5. Meanwhile, thaw and mix SUV stock suspensions:

i. Seal Eppendorf tubes containing the stock aliquots with Parafilm, then sonicate for 1-2 minutes at a time until the solution is clear (immerse the tubes in the center of the sonicator bath such that the liquid inside the tube is just below the surface of bath water). Make sure that Eppendorf tubes do not heat up.
ii. Mix the desired SUV suspensions in Eppendorf tubes: for experiments with extracts, typically a set of 0%, 0.1% and 0.5% PI(4,5)P2 lipid mixtures in 60% PC, 30% PS, 10% PI are made.
iii. Sonicate the mixtures for 1-2 minutes as described in 5.i.

6. Dilute SUV mixtures 5-fold in high-salt XB buffer to 1 mM final lipid concentration.
7. Put 30 µl of diluted SUVs into each well.
8. Add $CaCl_2$ to 4 µM final (1.2 µl of 100 mM $CaCl_2$ per well).
8. Incubate on 42°C heated plate for 30-60 minutes. This incubation temperature approximates the porcine body temperature, where the lipids are derived.
9. Wash bilayers at room temperature:
   i. Add 170 µl of regular XB buffer into each well.
   ii. Pipet up and down 5x, then remove 150 µl of the buffer from each well.
   iii. Add 150 µl of XB buffer into each well, pipet up and down 5x, then remove 150 µl of the buffer from each well. Repeat this step 5x. Do not touch bilayer.
10. Keep bilayers covered with buffer at 4°C until use.

3.6. Evaluating bilayer integrity

To verify the formation of a laterally fluid supported membrane, one can add a low concentration of fluorescent lipids (e.g. 0.5 mol % 1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}- sn-glycero-3-phosphocholine or Texas Red-1,2-dihexanoyl-sn-glycero-3-phosphoethanolamine (TR-DHPE)) to the lipid mixture during vesicle preparation. Then, the lateral mobility of lipids in the bilayer can be assessed by photobleaching and subsequent fluorescence recovery (FRAP). If the lipids in the bilayer can freely diffuse, the intensity of the photobleached area will recover within 1 to 5 min. If fluorescence recovery is significantly slower, it could be due to aging of the lipids. For example, oxidized lipids do not form fluid membrane. In this case, remake SUVs with fresh lipids. Another possibility could be that vesicles did adsorb to the surface, but did not coalesce to form a continuous bilayer. This can be caused by a variety of factors, e.g. insufficient cleaning of the glass coverslips. Alternatively, increasing the incubation time, the ionic strength of the buffer, or the concentration of divalent cations can give improved results (Nair, Salaita, Petit, & Groves, 2011).

4. Fluorescence Microscopy

4.1. Imaging protein self-organization from purified components

Bacterial cytokinesis is commonly initiated by the formation of the Z-ring: a cytoskeletal, membrane-bound structure, which assembles at the middle of a rod-shaped cell. The primary component of this ring is the GTPase FtsZ, which is recruited to the cytoplasmic membrane by two different proteins: the widely conserved actin-related protein FtsA and

ZipA, which can be found in *E. coli* and closely related species. All three proteins are required for the assembly of a functioning Z-ring in *E. coli*, but if and how they influence each other's assembly dynamics was not understood. To study the dynamics of membrane binding and protein polymerization, we reconstituted FtsZ polymerization on supported lipid bilayers from purified components (Loose & Mitchison, 2014). Using the reaction chambers made as described above, the self-organization of proteins on the surface of the supported membrane can be studied by adding the reactants to the buffer. Thanks to imaging of proteins by TIRF microscopy, we can specifically study the dynamics of proteins bound to the membrane even if there is a large excess of proteins still present in solution.

Before starting the experiment, prepare required protein solutions and have them ready on ice. The proteins can be kept on ice for about 2 hours. Ideally and to be able to observe initiation of protein dynamics, the proteins are added next to the microscope, right before imaging.

Final sample preparation:
1. Prepare supported bilayer as described above.
2. For attaching His-tagged proteins such as His-ZipA to the surface of Ni-chelating lipids, first add this protein in excess to the buffer (to a final concentration of about 10 µM), and gently mix the solution by pipetting up and down. After incubation for 5 min, wash the membrane three times with 200 µl reaction buffer to remove non-bound protein.
3. Add oxygen scavenger mix: 1% w/v glucose, glucose oxidase 0.32 mg/ml, catalase 0.055 mg/ml, DTT 1 mM, Trolox 1mM.
4. Add FtsA or FtsZ supplemented with 10% fluorescently labeled protein to the system to desired concentrations (between 0.5 µM and 3 µM each) and mix gently.
5. Start the reaction by adding nucleotides (1mM ATP, 5mM GTP) and mix gently.
6. Cover the chamber with an Eppendorf tube lid.
7. Image immediately on TIRF microscope. To capture the dynamic behavior of proteins, time-lapse movies are typically recorded with a rate of 1 frame per 2-5 s.

4.2. Imaging cytokinesis signaling using Xenopus egg extract

During cytokinesis in animal cells, the cleavage furrow is positioned by microtubules from two sides of the cell that interact at the cell midplane, and recruit proteins that signal to the actin cortex to induce cortical contraction. Cytoplasmic extracts prepared from unfertilized *Xenopus laevis* eggs by centrifugal crushing contain undiluted cytosol and organelles and have long provided a system for accurate, cell-free reconstitution of meiosis-II spindles (Sawin & Mitchison, 1991). To extend this system to cytokinesis organization for the first time, we recently combined undiluted egg cytoplasm containing intact actin (Field et al., 2014) with Aurora A kinase-coated beads as artificial centrosomes (Tsai & Zheng, 2005) and supported lipid bilayers from mammalian lipids to observe the complex signaling behaviors involving microtubules, F-actin, and the plasma membrane (Nguyen, Groen et al., 2014).. This system provides a new approach to

probing the biochemistry and biophysics of cytokinesis signaling, with the advantages of a large spatial scale (~20 µm wide cytokinesis zones), a long observation time window (>20 minutes), simple means for manipulation, and excellent imaging conditions of the cortical layer using TIRF microscopy.

These reconstitution experiments are not easy, however. They require coordinated collaboration between at least two people: one person cleans the glass and prepares the SLB (Steps 3.3 and 3.5), while the other person prepares the egg extracts and selects the best extract for the experiments (Step 2.4). Each process takes about 2-3 hours under experienced hands. Thus it is crucial to coordinate the two processes and to perform the experiments in a timely manner, as both the SLBs and the extracts have a limited "ice bucket life": on average about 6 hours at 4°C, although exceptional extracts can last as long as 12 hours, in which case a second batch of SLBs is often made.

Egg extracts made on different days and from different frogs vary in their ability to promote actin polymerization at the cortical layer near the lipid membrane. In order to obtain the desired F-actin morphology at the cortical layer, we can vary the amount of negatively charged lipids in the supported bilayer. In general, more negatively charged lipid bilayers support higher degree of actin polymerization (Lee, Gallop, Rambani, & Kirschner, 2010). Routinely, we prepare SLBs containing 0%, 0.1%, and 0.5% $PI(4,5)P_2$ for each extract (see above) and assay for cortical actin morphology (e.g. alignment along microtubule asters and enrichment at aster-aster interaction zones) at the beginning of each experiment to determine which $PI(4,5)P_2$ concentration to use for that particular extract.

Final sample preparation:

1. Prepare SLBs from mammalian lipids as described above.
2. For each experiment, treat 60 µl of metaphase-arrested egg extract with 0.4mM $CaCl_2$ (0.6 µl of 40 mM $CaCl_2$ stock) to cycle into interphase, incubate at 20°C for 10 min, then return to ice for at least 2 minutes to depolymerize both microtubules and F-actin.
3. Wash bilayers 2 times with 20 µl interphase extract each. Extract used for washing bilayers can be frozen,
   Note: Frozen extract needs to be supplemented with 50 mM sucrose and cycled into interphase prior to freezing. Briefly, extract is treated with 0.4 mM CaCl at room temperature for 30min, placed on ice for 20 minutes (to depolymerize actin and microtubule structures), frozen in liquid nitrogen, and stored at -80°C in 50 µl aliquots
4. Finally layer 20µL extract containing fluorescent probes and Aurora A kinase-coated beads on top of the bilayer. Start timing.
5. Monitor aster assembly reactions with an inverted TIRF or spinning disc confocal microscope at 20°C. As the cell cycle progresses, F-actin cables become more aligned with microtubule asters. Formation of aster-aster interaction zones that recruit cytokinesis factors such as the chromosomal passenger complex (CPC) is observed starting at around 20 minutes. At the same time, activation of RhoA, as indicated by

binding of the mCherry-rGBD probe to the bilayer, is also initiated, and by 50-60 minutes a stable active RhoA zone can be seen above CPC-positive zones, where F-actin is also enriched (Fig. 1D). By 90 minutes, most microtubules usually have disappeared and we do not image beyond this point.

For visualization, fluorescent probes were used at the following concentrations: 250 nM Alexa Fluor 488- or 568-tubulin, 140 nM mCherry-rGBD to visualize RhoA-GTP, 140 nM Lifeact-GFP to visualize F-actin, and 0.1-1 μg/ml Alexa Fluor 647-IgG against AurkB to visualize the CPC.

5. Conclusion

Here, we have presented protocols for the use of supported lipid bilayers to study the spatiotemporal dynamics of membrane binding proteins in reconstituted *in vitro* systems; either using purified proteins from *E. coli* or cytoplasmic extracts from *Xenopus* eggs. The setup is especially suited for TIRF microscopy, providing the possibility to study protein dynamics down to the single-molecule level. Similar experimental setups have been used to study other membrane-associated cytoskeleton, for example the dynamics of actin or septin filaments (Bridges et al., 2014; Murrell & Gardel, 2012; Vogel, Petrasek, Heinemann, & Schwille, 2013). Supports for lipid bilayers are not limited to flat, continuous surfaces, but can also be formed on microfabricated substrates mimicking the spatial confinement of the cell (Schweizer, Loose, Bonny, Kruse, Mönch, & Schwille, 2012b; Zieske & Schwille, 2013). Therefore SLBs represent a highly versatile and powerful experimental system to study the dynamics of proteins on membrane-surfaces *in vitro*.

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Figure captions

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Figure 1 caption:

Schematic overview of experimental assay: (A) Sample preparation. Left: Proteins are purified and can be stored at -80°C. Right: When Xenopus egg extract is used it is prepared on the day of the experiments. (B) Supported bilayers are prepared on freshly cleaned glass cover slips following the protocol given in the main text. (C) For the experiment, purified proteins are either directly added to the buffer above the membrane (left), or the buffer is replaced with extract containing fluorescently labeled proteins (right). TIRF microscopy allows to image fluorescently labeled proteins binding to the model membrane. Left: cytoskeletal patterns of FtsZ and FtsA. Right: Recruitment and organization of cortical cleavage furrow proteins.

Figure S1 caption:

Alignment of F-actin along microtubule asters requires good quality Xenopus egg extract. Confocal spinning disk images of microtubules and F-actin visualized with Alexa647-tubulin and Lifeact-GFP, respectively. Interphase asters nucleated from Aurora A kinase-coated beads were assembled between polyethylene glycol (PEG)-coated glass coverslips at 20°C as described in {Nguyen, Groen et al. 2014}. Image was taken at 30 minutes of aster assembly reaction. Unlike control, extract treated with a microtubule depolymerizing drug (100 μM nocodazole) assembled disorganized F-actin networks, which is often seen with lower quality extracts where F-actin does not align with microtubule asters. Scale bar, 20 μm.