

Chapter 11

Reconstitution of a Minimal Actin Cortex by Coupling Actin Filaments to Reconstituted Membranes

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Abstract

A thin layer of actin filaments in many eukaryotic cell types drives pivotal aspects of cell morphogenesis and is generally cited as the actin cortex. Myosin driven contractility and actin cytoskeleton membrane interactions form the basis of fundamental cellular processes such as cytokinesis, cell migration, and cortical flows. How the interplay between the actin cytoskeleton, the membrane, and actin binding proteins drives these processes is far from being understood. The complexity of the actin cortex in living cells and the hardly feasible manipulation of the omnipotent cellular key players, namely actin, myosin, and the membrane, are challenging in order to gain detailed insights about the underlying mechanisms. Recent progress in developing bottom-up in vitro systems where the actin cytoskeleton is combined with reconstituted membranes may provide a complementary route to reveal general principles underlying actin cortex properties. In this chapter the reconstitution of a minimal actin cortex by coupling actin filaments to a supported membrane is described. This minimal system may be very well suited to study for example protein interactions on membrane bound actin filaments in a very controlled and quantitative manner as it may be difficult to perform in living systems.

Key words Actin, Actin filaments, Actin cortex, Membrane, Supported lipid bilayer, Myosin, Actomyosin, Cytoskeleton, Reconstitution, Synthetic biology

1 Introduction

The actin cytoskeleton is involved in dozens of cellular processes including intracellular cargo transport, exocytosis and endocytosis, cell locomotion, organelle motion, cytoplasmic streaming, polarity formation, and cell division. In vivo studies on the actin cytoskeleton and its interaction partners gave us deep insights about functions of the actin cytoskeleton inside cells and how these functions are aided by other proteins [1]. In parallel, in vitro reconstitution assays of actin and its interaction partners during the last few decades were invented with great success and formed the basis for the detailed molecular understanding about actin filament dynamics and its regulation by interacting proteins as well as the determination of detailed motor proteins properties [2–4]. In spite

of our vast knowledge about the actin cytoskeleton and its interactions with other proteins, many cellular processes are still ill defined from a mechanical point of view. A key feature of living cells is their ability to control their shape and the intercellular and intracellular communication where the interaction between the cytoskeleton and the cell membrane plays fundamental roles [1, 5]. Here our mechanistic and quantitative understanding is rather limited and begs for further scientific endeavors.

In eukaryotic cells a thin layer of actin filaments interacts with the cell membrane where a part of the actin layer is directly coupled to the cell membrane via anchor proteins and thereby also ensures structural integrity [6]. This system is called the actin cortex and governs cell shape control during cell locomotion and cell division and influences the diffusion behavior of lipids and proteins in the cell membrane [7–9]. During cell morphogenesis the actin meshwork is constantly dynamically reorganized by interacting with the motor protein myosin II and the cell membrane resulting in force transmission to the cell membrane and eventually in cell shape changes [5, 10]. In order to understand these actin motor and membrane interactions in more detail the development of *in vitro* assays that circumvent the complexity present at the cytoskeleton membrane interface in living cells would be desirable and very well suited to reveal basic principles underlying actin cell cortex mechanics [11, 12]. Nevertheless, looking at the lipid membrane and the actin cytoskeleton independently as isolated systems has dominated the *in vitro* field the last decades and has been extremely successful leading to the identification of single step sizes of motor proteins on linear actin tracks [13] and the discovery that distinct lipid environments help in protein sorting and influence their functionality [14]. However, to shed light into the underlying principles of how the dynamic interplay between these entities controls lipid protein movements and shape changes of the cell membrane, *in vitro* assays have been recently developed that combine these two units [15–21]. The combination of the membrane and actin cytoskeleton modules can be subdivided into systems where actin filaments were either reconstituted on planar supported (Figs. 1, 2, and 3) or on free-standing lipid bilayers to mimic a cellular actin cortex [15–21]. Contractile behavior in these systems was induced with the addition of myosin motor assemblies in the presence of ATP. Mica or glass supported lipid bilayers (SLBs) have been successfully used as systems mimicking biological membranes for many years [22, 23]. SLBs are easily accessible to addition and exchange of protein or buffer components in a step-wise manner and are perfectly suited for applying surface-based manipulation and imaging techniques such as Atomic Force Microscopy (AFM), Surface Plasmon Resonance (SPR), and Total Internal Reflection Fluorescence (TIRF) microscopy. Several groups managed to couple filamentous actin to SLBs using pro-

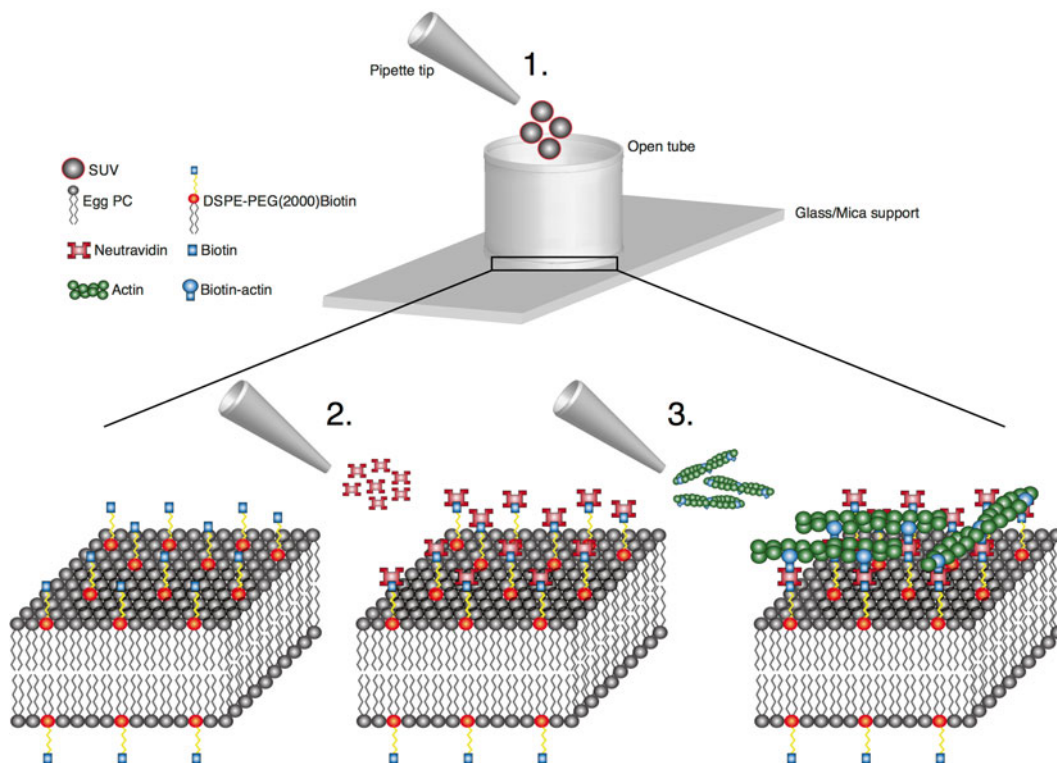


Fig. 1 Steps of minimal actin cortex (MAC) formation. A schematic representation and steps of the lipid bilayer formation and actin coupling are shown. SUVs are added to the glass or mica support (step 1) resulting in the formation of a supported lipid bilayer (EggPC) containing biotinylated lipids (DSPE-PEG(2000)-Biotin). Addition of neutravidin (step 2) and biotinylated actin filaments (step 3) result in their coupling to the lipid bilayer. Adapted from ref. [21] with permission from Copyright © 1999–2015 John Wiley & Sons, Inc.

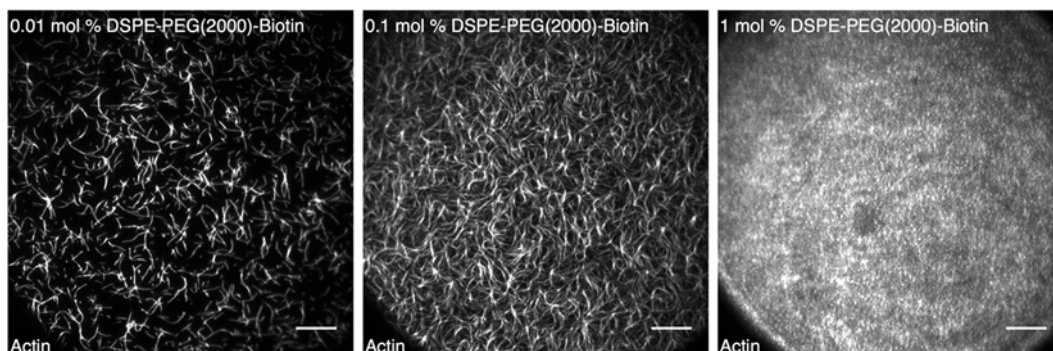


Fig. 2 Different densities of membrane coupled actin filaments. TIRF microscopy images of Alexa 488-phalloidin labeled actin filaments coupled to supported bilayers are shown. The actin filament density increases from the *left* to the *right* images and corresponds to an increase in the amount of DSPE-PEG(2000)-Biotin (0.01 mol %, 0.1 mol %, 1 mol %) in the SLB. Scale bars, 10 μm. Adapted from ref. [21] with permission from Copyright © 1999–2015 John Wiley & Sons, Inc.

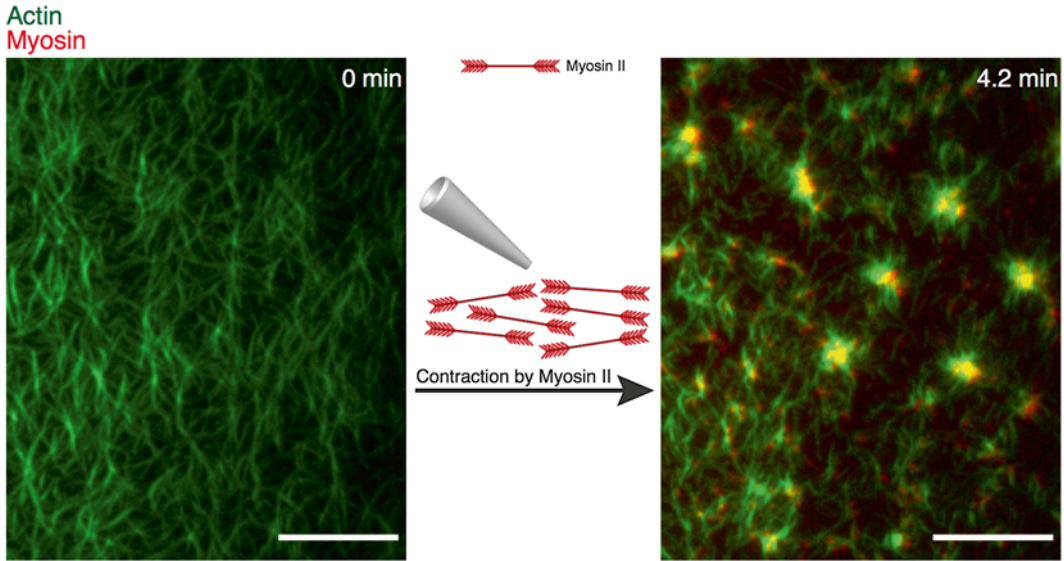


Fig. 3 MAC contraction by myosin motors. TIRF microscopy images of a MAC before (*left*) and after the addition of myosin filaments (*right*) are shown. Myosin filaments (*red*) induce the contraction of the membrane coupled actin layer and the formation of actomyosin clusters (*right image*). Scale bars, 10 μm . Adapted from ref. [15] with permission from Copyright © 2015 eLife Sciences Publications Ltd.

teins that anchor actin filaments to the membrane [24–26]. Recently the development of SLBs with artificial actin anchoring systems turned out to be very useful to study myosin motor activity on membrane coupled actin filaments and revealed a possible mechanism for how compressive stress on actin filaments induced by myosin motors could contribute to actin turnover ([15], Fig. 3) and how different levels of actin meshwork adhesion to the lipid bilayer may control the ratio between meshwork contraction and actin filament severing [18]. Other systems based on free-standing lipid bilayers gave insights on how the presence of a membrane coupled actin meshwork influences the lateral diffusion behavior of membrane lipids and proteins [16] and how the actin meshwork connectivity leads to different behaviors during myosin induced contraction on the membrane [20]. This chapter describes procedures for forming glass or mica supported lipid bilayers, to which actin filaments are subsequently anchored using a lipid-based artificial anchor system.

2 Materials

2.1 Chemicals, Buffers, and Solutions

1. Stock Solutions: 1 M KCl; 1 M MgCl_2 ; 1 M DTT; 0.1 M ATP; 1 M Tris-HCl, pH 7.5; 1 M CaCl_2 ; 0.5 M MOPS, pH 7.0; 0.1 M EGTA, pH 7.4; 3 % NaN_3 .

2. Reaction buffer: 50 mM KCl, 2 mM MgCl₂, 1 mM DTT, and 10 mM Tris-HCl pH 7.5.
3. Polymerization buffer: 50 mM KCl, 2 mM MgCl₂, 1 mM DTT, 1 mM ATP, and 10 mM Tris-HCl, pH 7.5.
4. Labeling buffer: 10 mM MOPS pH 7.0, 0.1 mM EGTA, and 3 mM NaN₃.

2.2 Materials for Membrane Formation

1. Chloroform, ethanol, acetone, Milli-Q water.
2. Lipids (purchased from Avanti Polar Lipids, Alabaster, AL): L- α -PHOSPHATIDYLCHOLINE (EggPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG(200)-Biotin).
3. Lipophilic cationic dye DiIC-C18.
4. 22 mm \times 22 mm, #1.5, Menzel cover slips (Thermo Fisher, Braunschweig, Germany).
5. 1.5 mL Eppendorf tubes.
6. Kimtech tissue.
7. UV glue: Norland Optical Adhesive 63 (Norland Products Inc.).

2.3 Materials for Actin Preparation

1. Purified or purchased rabbit skeletal muscle actin monomers (Molecular Probes).
2. Biotinylated rabbit actin monomers (tebu-bio (Cytoskeleton Inc.)).
3. Alexa Fluor 488 phalloidin (Molecular Probes).
4. Neutravidin.

2.4 Special Equipment

1. Plasma cleaner.
2. Vacuum centrifuge.
3. Nitrogen or compressed air supply.

3 Methods

3.1 Reconstitution of Supported Lipid Bilayers (SLBs)

3.1.1 Preparation of Small Unilamellar Vesicles (SUV)

1. Herein we describe the preparation of SUVs that are subsequently used for the formation of lipid bilayers on a glass or mica support. Dissolve the lipid L- α -phosphatidylcholine (EggPC) and the biotinylated lipid 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG(200)-Biotin) in chloroform in a small glass vial (*see Note 1*). Depending on the required actin filament density (Fig. 2), use molar ratios of 99.99, 99.9, and 99 mol% for EggPC and 0.01, 0.1, and 1.0 mol % for DSPE-PEG(200)-Biotin in order to obtain a total lipid amount of 10 mg/mL. For testing the membrane integrity, add 0.1 mol% of the lipophilic

cationic dye DiIC-C18 or any other reasonable dye to the lipid mixture (*see Note 1*). Dry the lipid–chloroform mixture under nitrogen flux for 30 min (*see Note 2*), and subsequently put it into a vacuum for 30 min in order to ensure complete solvent evaporation.

2. Rehydrate the Lipids in reaction buffer, and resuspend them by vigorous vortexing (10 min) until the suspension has a milky appearance and no longer contains any visible (lipid) particles. In this step multilamellar vesicles (MLVs) are formed.
3. Transform the MLVs to SUVs by exposing the suspension in the glass vial to ultrasound sonication in a water bath at room temperature until the milky suspension transforms into a translucent solution indicating the transformation of SUVs from MLVs. This process may last 30–45 min (or less) depending on the ultrasound sonication water bath system in use (*see Note 3*).

3.1.2 Glass or Mica Preparation

1. In this part we will describe the preparation and treatment of the glass or mica support prior to lipid bilayer formation. In both cases it is recommended to do it freshly as it is advantageous for the formation of a lipid bilayer. Therefore, for example, the time of SUV formation in the water bath may be a good opportunity to prepare the support. We will describe both the treatment of a glass support and the preparation of a mica support. Advantages and disadvantages of both supports are briefly described in Subheading 4 (*see Note 4*). For a glass support, rinse a glass coverslip (22 mm × 22 mm, #1.5) with Milli-Q water, and carefully mechanically clean it with Kimtech tissue. Subsequently, sequentially wash the coverslip with ethanol, acetone, and then again with ethanol and Milli-Q water. After the last wash, use a strong nitrogen (or air) flux to remove the remaining liquid and particles and quickly dry the coverslip. Then expose the coverslip to an air plasma treatment for 10 min in a Plasma Cleaner in order to remove left over contaminants and to make the glass surface hydrophilic (*see Note 5*).
2. If mica as an alternative support is desired, the cleaning procedure for the coverslip is principally not necessary, although we would recommend a cleaning of the coverslip, which may result in better data acquisition during imaging procedures especially when performing TIRF microscopy. Prior to cleaving a thin sheet of mica, place one drop of the UV glue onto the glass coverslip. If TIRF imaging of the sample is desired, a drop of oil is placed onto the cover slip instead of the UV glue. For mica preparation cut a square approximately 10 mm × 10 mm with a scissor or stamp out a circle of mica with 5 mm radius using a template and a hammer. Place the mica crystal between the sticky side and non-sticky side of tape on a

roll, and quickly pull the tape from the roll. The thick mica crystal will cleave. Part of the mica may not stick to the tape; reapply that part of the mica between the sticky side and non-sticky side of the tape roll, and again quickly pull the tape from the roll. Repeat this procedure several times as necessary to create a very thin mica sheet. Use clean tweezers to place the thin sheet on the UV glue or oil drop.

3. Prepare a chamber on the plasma-treated glass or on the mica surface by cutting a 1.5 mL Eppendorf tube and gluing it to the glass (or mica) surface with Norland UV glue applied to the rim of the tube (Fig. 1). Cure the UV glue by exposing the chamber to long wave ultraviolet light between 320 and 400 nm for 15 min.

3.1.3 Preparation of the Lipid Bilayer

1. After curing the UV glue, mix 10 μL of the SUV suspension with 90 μL of reaction buffer and immediately pipette the mixture onto the glass (or mica) surface of the chamber. Add CaCl_2 to a final concentration of 0.1 mM to aid fusion of the SUVs resulting in the formation of a lipid bilayer on the glass (or mica) support (*see Note 6*). After 45–60 min, wash the sample with a total volume of 2 mL reaction buffer by gently adding to and removing from the chamber approximately 200 μL aliquots of reaction buffer in order to remove CaCl_2 and vesicles that did not fuse with the surface to form the lipid bilayer. Be careful during this step not to touch the lipid bilayer with the pipette tip or to harm the lipid bilayer by too strong pipetting (*see Note 7*). Avoid any air contact with the lipid bilayer as it would cause immediate disruption of the membrane. Make sure that the lipid bilayer is always covered with buffer solution (*see Note 8*).

3.2 Actin Filament Preparation and Coupling to the SLB

In this section we describe the formation and preparation of biotinylated and phalloidin stabilized (non-dynamic) actin filaments that are eventually coupled to the supported bilayer system. We will not explain the actin purification procedures and will herewith refer to a multitude of existing literature ([27, 28], *see Note 9*).

3.2.1 Biotinylated Actin Filament Preparation

1. Mix actin monomers (purified or purchased) and biotinylated actin monomers mixed in a 5:1 (actin–biotin–actin) ratio (*see Note 9*) for a final concentration of 39.6 μM . Induce polymerization of the monomer mixture by adjusting the concentrations of: KCl to 50 mM, MgCl_2 to 2 mM, DTT to 1 mM, and ATP to 1 mM. Incubate the mixture for 1 h at room temperature for actin filament formation.
2. Prepare an actin-stabilizing solution by placing 60 μL of Alexa Fluor 488 phalloidin in a 1.5 mL Eppendorf tube.
3. Dry the solution in a vacuum centrifuge at room temperature.

4. Redissolve the dried powder in 5 μL methanol.
5. Add 85 μL labeling buffer to the redissolved phalloidin solution.
6. Add sufficient labeling buffer to the 39.6 μM actin mixture to reach a concentration of 20 μM actin.
7. Finally, add 10 μL of 20 μM actin mixture to 90 μL of phalloidin solution, and incubate at room temperature overnight. This procedure yields a final concentration of 2 μM Alexa 488-phalloidin-labeled, biotinylated actin filaments (referred to actin monomers) (*see* **Note 10**).

3.2.2 Actin Filament Binding to the SLB

1. In order to bind biotinylated actin to biotinylated lipids in the SLB, dissolve 2 μg of neutravidin in approximately 200 μL reaction buffer. Put the neutravidin solution through a vortex, and subsequently, pipette it up and down several times to prevent the presence of neutravidin agglomerates. Add the neutravidin solution to the washed SLB and leave it at room temperature for 10 min.
2. Gently wash the sample several times with 200 μL aliquots of reaction buffer (2 mL total volume) to remove unbound neutravidin. Take precautions to avoid disrupting the membrane during the washing steps.
3. Remove at least 100 μL (or if possible, more) of the approximately 200 μL reaction buffer that covers the SLB.
4. Add 10–50 μL of the 2 μM Alexa 488-phalloidin labeled, biotinylated actin filaments to the SLB and incubate for 1 h at room temperature to allow actin filaments to settle down and bind to the neutravidin layer of the SLB.
5. Gently wash the sample several times with 200 μL aliquots of reaction buffer (1–2 mL total volume) to remove unbound actin filaments. It is important to do a gentle washing here as bound actin filaments can be relatively easily ripped off by strong liquid streams induced by non-gentle pipetting (*see* **Note 7**).

4 Notes

1. EggPC can be replaced by other lipids such as DOPC and many others not mentioned here.
2. The lipid–chloroform mixture is dried inside a small glass vial by continuously rotating and holding it in an inclined position. This will result in the formation of a dried lipid film that not only covers the bottom but also the lateral interior wall of the glass vial. The increased dried lipid surface facilitates the

rehydration procedure and avoids the formation of bulky lipid agglomerates, which are difficult to resuspend during vortexing.

3. To ensure a proper and reasonable fast transformation from MLVs to SUVs it is important to expose the glass vial to a maximum amount of energy that is exhibited from the water bath ultrasound system. This is assured by adjusting the water level of the water bath to the point when water drops start to “jump off” the water surface. Place the bottom of the glass vial exactly to this spot close to the water surface. It is possible to freeze the obtained SUV solution and to store the SUVs in small aliquots at $-20\text{ }^{\circ}\text{C}$.
4. Using mica as the lipid support has the advantage that no plasma treatment of the solid surface is needed and it seems that membrane formation is slightly more reproducible as the lipid bilayer formation on the glass depends on the glass surface landscape, accurate cleaning, and proper plasma treatment. However, on the other hand, if TIRF microscopy is desired, the quality of the TIRF images strongly depends on the thickness of the mica sheet. In this case the use of a glass support facilitates subsequent TIRF microscopy. We therefore used mainly glass as support.
5. Successful cleaning and plasma treatment of the glass surface can be observed by a strong decrease of the contact angle of a water droplet placed onto the glass surface.
6. The presence of calcium aids the formation of the lipid bilayer [29].
7. Pipette the washing buffer gently against the interior wall of the cut Eppendorf tube in order to avoid a strong liquid flow towards the bottom of the SLB chamber.
8. The integrity and fluidity of the formed lipid bilayer on the glass or mica surface is confirmed by Fluorescence Recovery after Photobleaching (FRAP) or the direct determination of the diffusion coefficient by Fluorescence Correlation Spectroscopy (FCS). In the case of FRAP the bleached area will recover within approximately 1 min.
9. Note that this protocol works fine with commercially available actin and biotinylated actin. Other actin–biotin–actin ratios such as 20:1 will also work. Whenever desired, the presence of biotinylated actin monomers in the actin filament can be further decreased to weaken the strength of the bond between the biotinylated actin filaments and the membrane.
10. The Alexa 488-phalloidin-labeled biotinylated actin filaments can be stored at $4\text{ }^{\circ}\text{C}$ for as long as 1 month.

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