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Corresponding Author:	Petra Schwille Max-Planck-Institut fur Biochemie Martinsried, Bayern GERMANY
Corresponding Author's Institution:	Max-Planck-Institut fur Biochemie
Corresponding Author E-Mail:	schwille@biochem.mpg.de
First Author:	Beatrice Ramm
Other Authors:	Beatrice Ramm
	Philipp Glock
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Max-Planck-Institut für Biochemie

Zelluläre und Molekulare Biophysik – Cellular and Molecular Biophysics

MPI für Biochemie • Am Klopferspitz 18 • D-82152 Martinsried

Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Prof. Dr. Petra Schwille
Tel: +49-(0)89 / 8578-2900
Fax:+49-(0)89 / 8578-2903
schwille@biochem.mpg.de
www.biochem.mpg.de

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Dear Editors,

It is a pleasure to submit our protocol „Investigating self-organizing protein patterns by in vitro reconstitution on supported lipid bilayers" as a basis for a new JoVE video production.
Herein, we provide a detailed description of a versatile open-chamber approach for reconstituting protein activity on flat supported lipid bilayers or in bilayer-clad micro-compartments. The assay covers the entire procedure from protein purification and chamber preparation to ultrasensitive microscopic observations. The described method has been successfully employed to quantitatively investigate the *E.coli* MinCDE system in detail (Loose et al., Science 2008; Loose et al., NSMB 2011; Schweizer et al., PNAS 2012; Zieske & Schwille, eLife 2014; Glock et al., Angewandte Chemie Intl Ed 2018).
Furthermore, the assay has been used in a slightly modified fashion to observe MinDE oscillations by high-speed atomic force microscopy (Miyagi et al., Nano Letters 2017) and has been transferred to study other self-organizing protein systems such as FtsZ (Ramirez et al., bioRxiv 2016) or a minimal actin cortex (Vogel et al., eLife 2013).
We compare our method to flow-cell setups used by other groups and discuss advantages and pitfalls of either approach.
Many more cellular systems will benefit in the future from reducing complexity by in vitro reconstitution. We are thus convinced that the here described protocol will be of great interest to the viewers of JoVE publications, and that it will enable them to better reproduce the assay for the reconstitution of their system of choice.

Please feel free to contact us, should you have any further questions or suggestions. We thank you for your time spent on this manuscript and look forward to hearing from you soon.

Sincerely, on behalf of all the authors,

Prof. Dr. Petra Schwille

TITLE:

In Vitro Reconstitution of Self-Organizing Protein Patterns on Supported Lipid Bilayers

AUTHORS AND AFFILIATIONS:

Beatrice Ramm[1*], Philipp Glock[1*], Petra Schwille[1]

[1]Department of Cellular and Molecular Biophysics, Max Planck Institute of Biochemistry, Munich, Bavaria, Germany

[*]These authors contributed equally to the manuscript

Corresponding Author:

Petra Schwille (schwille@biochem.mpg.de)

Tel: (+49) 89 8578 - 2900

Email Addresses of Co-authors:

Beatrice Ramm (ramm@biochem.mpg.de)

Philipp Glock (glock@biochem.mpg.de)

KEYWORDS:

In vitro reconstitution, MinD, MinE, supported lipid bilayer, pattern formation, microstructures, self-organization

SUMMARY:

We provide a protocol for *in vitro* self-organization assays of MinD and MinE on a supported lipid bilayer in an open chamber. Additionally, we describe how to enclose the assay in lipid-clad PDMS microcompartments to mimic *in vivo* conditions by reaction confinement.

ABSTRACT:

Many aspects of the fundamental spatiotemporal organization of cells are governed by reaction-diffusion type systems. *In vitro* reconstitution of such systems allows for detailed studies of their underlying mechanisms which would not be feasible *in vivo.* Here, we provide a protocol for the *in vitro* reconstitution of the MinCDE system of *Escherichia coli*, which positions the cell division septum in the cell middle. The assay is designed to supply only the components necessary for self-organization, namely a membrane, the two proteins MinD and MinE and energy in the form of ATP. We therefore fabricate an open reaction chamber on a coverslip, on which a supported lipid bilayer is formed. The open design of the chamber allows for optimal preparation of the lipid bilayer and controlled manipulation of the bulk content. The two proteins, MinD and MinE, as well as ATP, are then added into the bulk volume above the membrane. Imaging is possible by many optical microscopies, as the design supports confocal, wide-field and TIRF microscopy alike. In a variation of the protocol, the lipid bilayer is formed on a patterned support, on cell-shaped PDMS microstructures, instead of glass. Lowering the bulk solution to the rim of these compartments encloses the reaction in a smaller compartment and provides boundaries that allow mimicking of *in vivo* oscillatory behavior. Taken together, we describe protocols to reconstitute the MinCDE system both with and without spatial confinement, allowing researchers to precisely control all aspects influencing pattern formation, such as concentration

ranges and addition of other factors or proteins, and to systematically increase system complexity in a relatively simple experimental setup.

INTRODUCTION:

Spatiotemporal patterns are essential in nature, regulating complex tasks both on the multicellular and cellular level, from morphogenesis to regulated cell division[1,2]. Reaction-diffusion systems play an important role in establishing these patterns but are still not well understood. A prime example of a reaction-diffusion system and the best characterized biological system so far is the *Escherichia coli* MinCDE system[3–7]. The MinCDE system oscillates from cell pole to cell pole in *E.coli* to determine the middle of the cell as the future division site. This system is based on the ATPase MinD, an ATPase activating protein MinE, and the membrane as a spatial reaction matrix[8]. MinC is not part of the pattern formation mechanism, but is the actual functional agent: an inhibitor of the main divisome protein FtsZ[5,6]. MinC binds to MinD and therefore follows the oscillations, resulting in a time-averaged protein concentration gradient that is maximal at the cell poles and minimal at the cell middle, only allowing FtsZ to polymerize at midcell[9,10]. The MinCDE system is part of the larger family of Walker A ATPases that are key to the spatiotemporal organization in bacteria[2], for positioning and transporting protein complexes[11] and plasmids[12] and for regulating cell division[13] and chromosome segregation[14]. Hence, the MinCDE reaction-diffusion system not only represents an archetypal reaction-diffusion system but has also attracted attention because of its relevance for the spatiotemporal organization in bacteria.

Detailed functional studies of the MinCDE system *in vivo* are complicated, as manipulation of proteins and gene deletion typically result in cell division defects. Furthermore, changing the membrane composition or the properties of the cytosol *in vivo* is very challenging[15,16]. Changes to the system and influencing factors are hard to interpret in the complex environment of the cell, even more so if it is disturbed in such an essential function as cell division. We and others have therefore turned to an *in vitro* reconstitution approach, reducing the system to its core components: MinD, MinE, ATP as an energy source, and the supported lipid bilayer as a reaction matrix[6,17,18]. This bottom-up approach allows to probe the mechanism of self-organization in detail without the complexity of a living cell. The proteins form traveling surface waves[6] and other kinds of patterns[17,19] under these conditions, albeit with a wavelength that is usually about a magnitude larger than *in vivo*. The use of an open chamber facilitates precise control over all aspects influencing pattern formation: protein concentrations[6], protein properties[20], membrane composition[10], buffer composition, and ATP concentration[6], as well as addition of other factors such as crowding agents[21] and other divisome proteins[22]. In comparison, the *in vitro* reconstitution of the MinCDE system in a flow-cell[18, 19, 23] can be used to probe the influence of flow[17, 23], protein limiting conditions[19], membrane composition[19] and full 3D confinement[18] on protein patterns, but renders an exact control of protein/component concentration and sequential component addition much more complicated.

Using this open chamber, we also patterned the support of the planar lipid bilayers by which one can probe how geometrical boundaries influence pattern formation[21], a phenomenon that has recently also been investigated *in vivo* using bacteria molded into microstructures[7]. We also

employed this assay to investigate how defined mutations in MinE affect pattern formation of the system[20]. Furthermore, the same basic assay format has been employed to investigate how pattern formation can be controlled by light, introducing an azobenzene-crosslinked MinE peptide into the assay, and imaging with TIRF microscopy[24].

We found that, in order to replicate the MinDE pattern formation observed *in vivo* in an *in vitro* system, confinement was key. Using rod-shaped microcompartments, with dimensions adjusted to the larger wavelength of MinDE *in vitro* (10 x 30 µm), clad with a supported lipid bilayer allowed the reconstitution of MinDE pole-to-pole oscillations and protein gradient formation[10,25]. In this assay, the supported lipid bilayers are deposited on a patterned PDMS substrate that contains several hundred replicas of rod-shaped microcompartments that remain open on the top. By this, the reaction can be set up in an open chamber, and subsequently the buffer is lowered to the rim of the microcompartments, thereby confining the proteins to a small volume. Even though these compartments have an air-buffer interface on one side and hence do not represent a full 3D confinement by membrane, the protein dynamics mimicked *in vivo* oscillations[10,25]. Compared to full 3D confinement, which shows very similar results[18], the open microstructures assay is relatively simple and easy to handle and can also be performed by laboratories that are not equipped with specialized microfluidics equipment and clean-room facilities.

Here, we present an experimental protocol for reconstituting MinCDE pattern formation on supported lipid bilayers *in vitro* using an open chamber that allows for control of all components and easy access by optical microscopy and, with minor modifications, is also adaptable for surface-probe techniques[26]. Next to planar supported lipid bilayers, we also show how protein confinement can be obtained using simple patterned supported lipid bilayers on rod-shaped PDMS microstructures. These assays, although optimized for the MinCDE system, can also be transferred to other protein systems that interact in a similar way with the membrane, such as FtsZ[27] or a minimal actin cortex[28].

PROTOCOL

1. Protein Production

1.1. Protein expression

1.1.1. Transform *E. coli* BL21 (DE3) pLysS with the respective plasmid for expression of MinD[6], EGFP-MinD[29], mRuby3-MinD[24], MinE[6] or MinC[30]. For plasmid maps, please see supplementary information.

1.1.2. Inoculate an overnight culture in LB medium with a single colony using the respective antibiotics (*e.g.,*100 µg/mL Ampicillin or 50 µg/mL Kanamycin) and incubate at 37 °C for 14-16 h while shaking.

1.1.3. Inoculate a 500 mL liquid culture of TB medium containing the respective antibiotic with

the overnight culture (1:200 dilution) and incubate culture at 37 °C while shaking at 180 rpm.

1.1.4. Induce protein expression by adding 0.5 mM IPTG when the culture reaches an optical density at 600 nm of 0.5-0.7. In case of EGFP-MinD or mRuby3-MinD, shift cells to an incubator with 16 °C and grow cells for 14-16 h, and in case of MinC, MinD or MinE, grow cells for 3-4 h at 37 °C after induction.

Note: Induction of MinC, MinD or MinE expression is toxic for the cells, as overexpression results in cell division defects; hence, it is important that incubation time at 37 °C is kept below 4 h. If more protein is needed, increase the amount of culture, but not incubation time.

1.1.5. After respective incubation time harvest cells by centrifugation at 4000 x g for 10 min and store the cell pellet at -80 °C until further use.

1.2. Protein purification

Note: Proteins can be purified either using prepacked Ni-NTA columns on an automated protein purification system or using Ni-NTA beads for gravity-flow bench purification.

1.2.1. For purification with prepacked Ni-NTA columns on automated protein purification systems use buffer A1 (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 10 mM imidazole), buffer B1 (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole), and buffer C1 (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 250 mM imidazole). For gravity-flow bench purification using Ni-NTA beads use buffer A2 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole), buffer B2 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole), and buffer C2 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 250 mM imidazole). Supplement all buffers with 10 mM ß-mercaptoethanol or 0.4 mM TCEP (tris(2-carboxyethyl)phosphine) as reducing agent right before use.

1.2.2. Resuspend cells in 20-30 mL of buffer A1 or A2 supplemented with EDTA-free protease inhibitor, 100 µg/mL lysozyme, ~250 U/mL DNase and 0.2 mM Mg^{2+}-ADP (Mg^{2+}-ADP in case of MinD or EGFP-MinD purification only, from a 100 mM ADP stock in 100 mM $MgCl_2$ with pH adjusted to 7.5).

1.2.3. Lyse cells using a tip sonicator (30% amplitude, 2.5 min, 30 s pulse, 30 s off) while keeping the cells in an ice bath.

1.2.4. Remove cell debris by centrifuging the cell lysate for 45 min at 25,000 x g and 4 °C.

1.2.5. Incubate the supernatant on Ni-NTA column or Ni-NTA beads.

1.2.5.1. For prepacked Ni-NTA columns, load the sample onto the column using the sample pump of an automated protein purification system.

1.2.5.2. For bench-top purification, incubate the sample with Ni-NTA beads in a 50 mL reaction tube on a rotating shaker at 4 °C for 1 h. For the subsequent steps, transfer the Ni-NTA beads into an empty column using a 25 mL pipette.

1.2.6. Wash with at least 5 column volumes of buffer A1 or A2.

1.2.7. Wash with at least 5 column volumes of buffer B1 or B2.

1.2.8. Elute protein with buffer C1 or C2.

1.2.9. Assess protein purity via SDS-PAGE.

1.2.10. Optional: Further purify protein by applying it to a gel filtration column equilibrated in storage buffer (50 mM HEPES/KOH pH 7.2, 150 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.4 mM TCEP, (0.2 mM Mg^{2+}-ADP in case of MinD)).

Note: Gel filtration is recommended for MinD to remove aggregated protein fraction.

1.2.11. If no gel-filtration is employed, exchange Ni-NTA elution buffer to storage buffer (50 mM HEPES/KOH pH 7.2, 150 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.4 mM TCEP, (0.2 mM Mg-ADP in case of MinD)) using a gravity flow desalting column (see **Table of Materials**).

1.2.12. Shock-freeze proteins in aliquots in liquid nitrogen and store at -80 °C until further use.

1.2.13. Measure protein stock concentration using Bradford Assay, and determine protein activity with an ATPase assay[20].

Note: Do not assess protein concentration using absorption at 280 nm. The presence of nucleotide during MinD purification and the lack of tryptophans in MinE distort A_{280} concentration measurements. Use Bradford or BCA assays to measure protein concentrations instead.

1.3. Protein labeling

Note: The fusion of a fluorescent protein to the small protein MinE induces major changes to its diffusive properties and function; hence, chemical labeling of the protein (cysteine at position 51) is preferred over fusion to fluorescent proteins.

1.3.1. Dissolve 0.125 mg of maleimide-dye conjugate in 5-10 µL of DMSO (dimethyl sulfoxide) and add under shaking to a 0.5 mL MinE aliquot in storage buffer at pH 7.2, prepared as detailed above.

1.3.2. Incubate for 2 h to overnight at 4 °C or 2 h at RT under gentle shaking or stirring.

1.3.3. Separate dye and protein using a gravity flow desalting column equilibrated with storage buffer (50 mM HEPES/KOH pH 7.2, 150 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.4 mM TCEP).

1.3.4. To further remove any unattached dye, dialyze the protein against an excess of storage buffer.

1.3.5. Verify successful labelling by measuring the extinction at the maximum for the respective dye and calculate the estimated labeling efficiency. Please refer to the dye manufacturer's instructions for a detailed protocol on estimating the degree of labeling. Analyze with SDS-PAGE and determine total mass by mass spectrometry for further useful information about sample homogeneity and labeling success.

2. Small Unilamellar Vesicle (SUV) Preparation

2.1. Generation of multilamellar vesicles

2.1.1. Calculate the amount of lipid(s) in chloroform for your desired mixture and final SUV volume. The concentration should be 4 mg/mL of lipids in Min buffer (25 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$). For a standard Min assay a mixture of 7:3 DOPC:DOPG (mol percent) is recommended. When using *E. coli* polar lipid extract, use SLB buffer (25 mM Tris-HCl pH 7.5, 150 mM KCl) for all preparation steps.

Note: It is not recommended for first time users to use *E. coli* polar lipid extract as the generation of homogenous SLBs with this mixture is much more challenging.

2.1.2. Using a positive displacement pipette with glass tips, mix the lipids in chloroform in a 1.5 mL glass vial.

2.1.2.1. Dry the lipids under a slight nitrogen stream while slowly turning the vial. Place the lipids under a stronger nitrogen stream for 10 to 20 minutes. Place the vial containing the dried lipid film in a vacuum desiccator and apply vacuum for at least 1 h.

2.1.3. Rehydrate the lipids in Min buffer by vortexing at room temperature until the mixture is homogeneously opaque.

Note: For generation of small unilamellar vesicles from multilamellar vesicles, lipids can either be extruded as described in 2.2. or sonicated as described in 2.3. In general, extrusion yields a narrower size distribution which can help with formation of supported lipid bilayers.

2.2. SUV preparation by extrusion

2.2.1. Break lipid aggregates and multilamellar structures and further solubilize lipids by freeze-thawing for 7 to 10 cycles.

2.2.1.1. Prepare a beaker with water at 70° C to 99° C on a hot plate and a container with liquid nitrogen.

2.2.1.2. Hold the vial in liquid nitrogen with large tweezers until the nitrogen stops boiling. Then transfer the vial to hot water until the solution is completely thawed. Repeat these steps until the lipid mixture appears clear to the eye, depending on the mixture.

2.2.2. Assemble a lipid extruder and pre-rinse the system with Min buffer. Extrude the lipid mixture between 35 and 41 times through a membrane of 50 nm pore size. Make sure to end on an odd number of passes to avoid aggregates that never traversed the membrane.

2.3. SUV preparation by sonication

2.3.1. To better dissolve lipids in the buffer, put the glass vial containing the solution in a heat block set to 37 °C and vortex every 20 minutes for 1 minute. Incubate in total for about 1 h.

2.3.2. Immerse the bottom of the vial in a sonicator bath (in this work 1.91 L, 80 W) by attaching the vial onto a clamp stand at the required height.

2.3.3. Set the water height in the sonicator bath so that the solution surrounding the vial is thoroughly agitated by the pulses and sonicate the lipid mixture for about 20 minutes. Check for successful sonication by assessing the clarity of lipids.

2.4. SUVs can be stored at 4 °C for up to a week or frozen at -20 °C in small aliquots (~20 µL) and stored for several weeks. Thaw vials or tubes at room temperature and sonicate again as described under 2.2.3 until the solution is clear before using SUVs for preparation of supported lipid bilayers (SLBs). Please note that the narrow size distribution of SUVs obtained by extrusion is lost after freezing and subsequent thawing and sonication.

3. Cleaning Glass Coverslips

Note: Cleaning and hydrophilization of glass coverslips is an important factor for homogenous and fluid supported lipid bilayers. Glass coverslips can be cleaned using a piranha solution, made from a ratio of 7:2 sulfuric acid to 50% hydrogen peroxide (3.1), or with an oxygen plasma in a plasma cleaner (3.2). Both methods yield similar results.

3.1. Piranha cleaning of coverslips

3.1.1. Apply piranha solution

3.1.1.1. Distribute glass coverslips on an inverted glass Petri dish or other inert surface. With a glass pipette, add 7 drops of concentrated sulfuric acid (98%) to the center of each coverslip.

CAUTION: Sulfuric acid is strongly acidic and corrosive. Work in a fume cupboard and with proper

protective equipment only.

3.1.1.2. Add two drops of 50% hydrogen peroxide to the middle of the acid drops.

CAUTION: Hydrogen peroxide is corrosive to the eyes and skin.

3.1.1.3. Cover the reaction and incubate for at least 45 minutes.

Note: The maximum waiting time here is not critical for the outcome of the experiment and can be extended up to several days.

3.1.2. Wash piranha cleaned coverslips.

3.1.2.1. Pick up the coverslips individually using tweezers and rinse off acid with ultrapure water. Place the washed coverslips in non-stick holders or similar transportation device.

3.1.2.2. Rinse each coverslip extensively with ultrapure water and dry the surface with pressurized gas (nitrogen, air only if oil-free). Mark the cleaned side of the coverslip with permanent marker.

3.2. Plasma cleaning of coverslips

3.2.1. Rinse coverslips with excess ethanol and afterwards with excess ultrapure water. Dry coverslips with pressurized gas. Assemble chamber as described in 4.

3.2.2. After chamber assembly as described in 4 take the coverslips with attached chamber and place in plasma cleaner with oxygen as process gas. Clean coverslips with plasma (in this work 30% power, 0.3 mbar oxygen pressure for 1 min was used). Do the cleaning right before SLB formation as described in 5, as the hydrophilizing effect of plasma cleaning wears off over time.

Note: Timing and power of plasma cleaning should be optimized using fluorescently labeled membranes, as too little or excessive plasma cleaning can both lead to immobile membranes or membranes with holes.

4. Chamber Assembly

4.1. With sharp scissors, cut off and discard the lid and the conical part of a 0.5 mL reaction tube. Apply UV-glue to the upper rim of the tube and distribute evenly by using a pipette tip.

4.2. Glue the tube upside down to the previously cleaned coverslip. In case of piranha cleaning make sure to glue it to the cleaned side of the glass. Cure the UV-glue by placing multiple chambers underneath a 360 nm lamp or LED for 5 to 15 minutes.

5. Supported Lipid Bilayer (SLB) Formation

5.1. Pre-heat heat block to 37 °C and incubate 2 mL reaction tubes with Min or SLB buffer, 1 tube per chamber.

5.2. Blow nitrogen into the assembled and cured chambers to remove any dust or other particles that may have settled during the UV curing and assembly. Plasma clean if you have not cleaned your coverslips with Piranha solution as described in 3.2. Place chambers on heat block.

5.3. Dilute a 20 µL aliquot of clear lipids (at 4 mg/mL) with 130 µL of Min buffer or SLB buffer in case of *E. coli* polar lipid extract, yielding a working concentration of 0.53 mg/mL. In case lipids were frozen, sonicate first by holding the tube into a bath sonicator before adding buffer, then sonicate again with buffer.

5.4. Add 75 µL of lipid mixture to each chamber and set a timer to 3 minutes (for DOPC/DOPG mixtures; longer incubation may be necessary for other lipid mixtures). In case of *E. coli* polar lipid extract, pipette $CaCl_2$ from a 100 mM stock into the chamber to a final concentration of 3 mM. During the incubation time, the vesicles burst on the hydrophilic glass surface and fuse to form a coherent SLB.

5.5. After 60 seconds, add 150 µL of Min buffer to each chamber.

5.6. Washing the chambers: After another 120 seconds (3 minutes total) wash each chamber by adding 200 µL of Min or SLB buffer, carefully pipetting up and down a few times, removing and adding another 200 µL.

5.6.1. After each chamber has been washed once, proceed to wash the first chamber thoroughly until the 2 mL of buffer are used up. Washing of SLBs needs some experience to perfect the extent of motions in the chamber and find the correct washing intensity.

Note: Never remove all liquid from the chamber to avoid drying of the SLB.

Note: On top of washing, membrane properties will vary depending on many additional factors: Type of lipids and their relative concentrations in lipid mixtures, preparation method for SUVs, surface treatment and prior cleaning of support.

6. Self-organization Assay

6.1. Adjust buffer volume in the chamber to 200 µL Min buffer minus the amount of protein and ATP solution, then add MinD, labeled MinD, MinE, and, if desired, MinC. Gently mix components by pipetting. Example concentrations are 1 µM MinD (doped with 30% EGFP-MinD), 1 µM MinE (doped with 10% chemically labeled MinE) and 0.05 µM MinC, but patterns form over a range of concentrations[6,10,20,30].

6.2. Add 2.5 mM ATP (from 100 mM ATP stock in 100 mM $MgCl_2$, pH 7.5) to start the self-

organization of MinDE.

Note: The order of the component addition (MinD, MinE and ATP) can be varied and will not influence the final pattern outcome[4].

6.3. Observe MinDE self-organization on the fluorescence microscope (see **Table of Materials**). MinDE self-organization can also be observed using TIRF microscopy. For imaging eGFP-MinD, use a 488 nm Argon laser or comparable diode laser (*e.g.,* 490 nm). For imaging mRuby3-MinD, it is best to employ a 561 nm diode laser.

Note: Avoid high levels of excitation for longer times as we and others[17] have observed phototoxicity in the MinDE system, leading to irreversible protein polymerization on the membrane.

7. PDMS microstructures

Note: PDMS (polydimethylsiloxane) is a polymer that can be used for the production of microstructures and microfluidic devices. A patterned silicon wafer serves as a mold for casting the PDMS structures. The PDMS structures then serve as a support for SLB formation and assay setup.

7.1. Either produce silicon wafer with microcompartments yourself using photolithography (see Zieske and Schwille for a detailed protocol[31] or Gruenberger *et al.* for a video protocol[32]) or order your desired silicon wafer from a foundry. For the pattern of the wafer used herein please see supplementary information.

7.2. Production of PDMS microstructures from patterned silicon wafers.

7.2.1. Use a plastic cup to weigh 10 g of PDMS base and 1 g of PDMS crosslinker. Either use a mixing device to mix and degas the PDMS mixture or manually mix the PDMS and then degas under vacuum.

7.2.2. Use a pipette tip to drop a small amount of PDMS directly onto the structure on the silicon wafer.

Note: Be careful not to scratch the silicon wafer.

7.2.3. Immediately place a #1 coverslip onto the PDMS drop and take the upper end of a clean pipette tip to gently press the coverslip onto the silicon wafer. The PDMS should be spreading thinly between the coverslip and the silicon wafer.

7.2.4. Place the wafer with the coverslips into an oven and cure the PDMS for 3-4 hours or overnight at 75 °C. Remove the wafer from the oven and let it cool down to room temperature. With a razor blade, carefully remove the coverslip with the attached PDMS from SI wafer.

Note: To prevent the silicon wafer from getting dirty or damaged, always cover the microstructures with PDMS and a coverslip. However, PDMS ages, resulting in cracks in the microstructures, hence do not use PDMS structures that are older than two to three weeks.

8. Self-organization in PDMS Microstructures

8.1. Use coverslips with PDMS microstructures to attach a chamber as described under 4.

8.2. Clean and hydrophilize surface in an oxygen plasma cleaner as described under 3.2.2. Do not piranha clean PDMS substrates.

8.3. Setup a MinDE self-organization assay as described under 6.

8.4. After setting up the assay, check for regular MinDE pattern formation and properly formed microstructures on the fluorescence microscope.

8.5. When regular MinDE patterns have formed (10 - 30 min), gently pipette up and down twice to mix components and then remove the buffer step by step by pipetting. Remove the large bulk of buffer using a 100 µL pipette and then carefully remove the rest using a 10 µL pipette.

Note: This step might need some practice. If too much buffer is taken out or the process takes too long, the microstructures will be dried out; if too little is taken, the proteins will not be confined in the microstructures, but continue to form traveling surface waves.

8.6. Immediately close the chamber with a lid to avoid drying of the residual buffer in the microstructures.

8.7. To allow for longer imaging times, plug a moistened piece of sponge inside the chamber and then close with lid. Make sure the sponge does not contact the surface of the coverslip.

8.8. Before imaging of the microstructures check on the surface that the buffer was lowered enough, so that in the surface above the microstructures MinDE pattern formation has halted. Image MinDE oscillations in microstructures. Check that microstructures are not dried out or are drying out during imaging.

Note: Discard coverslips with microcompartments after each use, as cracks in the PDMS form.

9. Analysis of MinDE pattern formation

9.1. Quantify wave length, wave velocity and wave profiles of the MinDE self-organization on planar supported lipid bilayers. FIJI with the standard set of packaged plugins is sufficient for basic analysis[33].

9.2. In microcompartments, describe pole-to-pole oscillations kymographs and time-averaged protein concentration profiles. Basic kymographs can be obtained by re-slicing a time series along a line selection in FIJI.

REPRESENTATIVE RESULTS:

Protein purification following our protocol should yield Min proteins of adequate purity. As a reference, **Figure 1** provides an SDS-PAGE image of MinD, fluorescently labelled MinD, MinE, and MinC. The individual steps of the procedure to perform a MinDE self-organization assay on non-patterned supported lipid bilayers are described in **Figure 2**. Using this protocol, regular MinDE traveling surface waves can be observed throughout the chamber (**Figure 3**). The wavelength can vary slightly within the chamber, but in general patterns look similar. The edges of the chamber should not be used for quantitative comparisons, as membranes that form on the UV glue seem to have different properties than on the glass surface (see **Figure 3C**). The traveling surface waves can be analyzed by plotting the intensity along the propagation direction (**Figure 3B**). While MinD fluorescence plateaus rather fast from the leading edge of the wave and then sharply decreases at the trailing edge, MinE fluorescence increases almost linearly from the start of the MinD wave and reaches its maximum after MinD at the trailing edge, where it falls off markedly [6].

Next to protein quality, the quality of the supported lipid bilayers is most critical for a regular self-organization of MinCDE. On the one hand if the membrane is washed too excessively or the underlying surface has been cleaned and thus charged too strongly, holes in the membrane can form (**Figure 6A**, top). On the other hand if the membrane is not washed properly or the underlying surface is not cleaned/hydrophilized, vesicles will stick to the membrane or the membrane fluidity will be compromised (**Figure 6A**, bottom). Even though not as apparent as when observing the membrane directly via labeled lipids, these problems can also be detected from the MinD fluorescence signal, as patterns are not regular and the fluorescence in the maxima is not homogenous but contains “holes” or bright spots as shown in the middle panel of **Figure 6A**.

For the MinDE self-organization in rod-shaped PDMS microstructures the procedure is summarized in **Figure 4**. Several protocol steps do not need to be repeated, as proteins and lipids can be reused. Like on non-patterned substrates, the substrate is cleaned and hydrophilized (by plasma-cleaning), a supported lipid bilayer is formed on the PDMS and the self-organization assay is set up in a volume of 200 µL. To check that a proper membrane has formed and MinDE self-organizes on the membrane, the chambers are imaged. When a proper membrane has been forme,d MinDE forms regular traveling surface waves on the surface of the PDMS between the individual microstructures and also self-organizes at the bottom of the microstructures as the waves can freely move over the entire membrane-covered surface (**Figure 5A**). After buffer removal, the surface between the compartments should not show any propagating MinDE patterns (**Figure 5B**), as it should be entirely dry. If MinDE patterns are still moving, more buffer needs to be removed. The proteins are now confined in the rod-shaped microcompartments by the membrane-clad PDMS and by air on the upper interface (**Figure 5C**), in which they will self-organize. Under these conditions the two proteins can perform pole-to-pole oscillations as shown in **Figure 5D**. As a fraction of MinD and MinE is always membrane-bound, also during buffer

removal, the concentrations after buffer removal are not comparable to input concentrations. Due to this effect the concentrations also vary between individual microstructures on the same coverslip as they depend on the position of the patterns before buffer removal. Silicon wafer production or PDMS molding from the silicon wafer can result in incomplete microstructures that cannot be used for analysis (**Figure 6B**). Furthermore, due to the buffer removal microstructures might dry out during the process, and these cannot be imaged (**Figure 6B**). As a result only a fraction of the microstructures in one chamber shows the desired pole-to-pole oscillations. To analyze protein dynamics in the microstructures, a kymograph can be obtained by drawing a selection over the entire structure (**Figure 5E**). When MinCDE oscillate from pole-to-pole, MinC and MinD will show a time-averaged concentration gradient that is minimal at midcell and maximal at the cell poles (**Figure 5F**).

FIGURE AND TABLE LEGENDS:

Figure 1: SDS-PAGE showing the final products of protein purifications. His-MinD (33.3 kDa), His-eGFP-MinD (60.1 kDa), His-mRuby3-MinD (59.9 kDa), His-MinE (13.9 kDa) and His-MinC (28.3 kDa) are shown in order.

Figure 2: Process flow diagram showing the individual steps and timing of the protocol for a self-organization on non-patterned supported lipid bilayers (Steps 1-6). Dashed boxes indicate that one of these two options can be used for cleaning. Arrows marked by circles indicate where the protocol can be paused and resumed later.

Figure 3: Imaging of MinDE assay by confocal microscopy. A) Regular Min spiral, from which wave propagation speed, intensity plot and speed measurements can be obtained. Concentrations used: 0.6 µM MinD (30% eGFP-MinD), 1.8 µM His-MinE (30% His-MinE-Alexa647) **B)** Example normalized intensity plot for the region marked in A. **C)** Overview of entire assay chamber (scale bar: 1 mm, same protein concentrations as above). Spirals turning either direction as well as target patterns can be observed. The magnified region shows how wave patterns differ on the UV-glue.

Figure 4: Process flow diagram showing the individual steps and timing of the protocol for a self-organization in rod-shaped microstructures (Steps 1-5, 7, 8). Grey boxes indicate steps where products can be reused from the protocol on non-patterned supported lipid bilayers. Arrows marked by circles indicate where the protocol can be paused and resumed later.

Figure 5: Representative results for MinDE pattern formation in rod-shaped PDMS microcompartments. A) MinDE self-organize on the surface of the PDMS forming traveling surface waves (1 µM MinD (30% EGFP-MinD), 2 µM MinE and 2.5 mM ATP). **B)** After the buffer is lowered to the height of the microstructures, the protein self-organization stops on the planar surface between the microcompartments. **C)** Schematic of one rod-shaped microcompartment. **D)** Representative images of MinDE pole-to-pole oscillations after buffer removal. **E)** Kymograph of the oscillations along the highlighted line shown in D). **F)** Image and profile of the average fluorescence intensity of the time-series shown in D) clearly showing the protein gradient that is

maximal at microcompartment poles and minimal at compartment middle.

Figure 6: Examples of negative experimental outcomes. A) Over-washed membranes accumulate holes, while suboptimal vesicle preparations and lipid compositions lead to sticking vesicles. The two center panels show a combination of both problems and how they become visible when observing Min oscillations. Membranes were labelled with 0.05% Atto655-DOPE. (scale bars: 50 µm) **B)** Top panel: Dried out microcompartments can be caused by too much buffer removal or when the buffer evaporates over time. Bottom panel: Incomplete compartments can be formed during wafer production or PDMS molding. (scale bars: 30 µm)

DISCUSSION:

We have described a protocol for the *in vitro* reconstitution of MinCDE self-organization on planar supported lipid bilayers and in lipid bilayer covered 3D structures, using the example of rod-shaped PDMS microstructures. In order to obtain valuable data from these assays, the most important factors to control are protein and membrane quality.

To ensure protein quality, protein mass should be confirmed using SDS-PAGE and mass spectrometry. Furthermore, it should be verified that proteins are soluble and not aggregated, by using analytical gel filtration or dynamic light scattering. Gel filtration can be used to remove any aggregated fraction of proteins. Careful pH adjustment and quality of added nucleotides is critical, as the addition of non-adjusted or partially degraded nucleotide to protein stocks or self-organizing assays is sufficient to eliminate protein activity, therefore abolishing self-organization.

Next to protein quality, membrane quality is most critical, and improper membrane formation is most often the cause for defective self-organization and the origin of artefactual surface structures.

When performing the protocol for the first time, it is helpful to label the supported lipid bilayers by including labeled lipids such as Atto-655-DOPE or DiI at low molar percentages (0.05%). Thereby the properties and quality of the membrane can be judged directly. Using FRAP, the fluidity of the membrane can be assessed. Furthermore, one can directly assess the quality of washing of the SLB, as there will either be too many vesicles, no fluid membrane, or no membrane at all, if it has been washed off. The open chamber approach allows to rigorously wash the membrane, and hence also to remove vesicles that are sticking on the surface of the SLB. The most crucial factors for obtaining fluid and homogenous supported lipid bilayers are the cleaning and hydrophilicity of the support surface and the correct size and homogeneity of the SU*Vs.* It can be helpful to check SUV size and size distribution using dynamic light scattering. For narrow size distributions, we recommend extruding the vesicles rather than sonicating them. Other methods of cleaning coverslips, *e.g.,* treatments with strong bases, basic detergents, or using coverslips directly after rinsing with water, may yield good results, depending on the application and lipid mixture.

The first half of the protocol presented here, *in vitro* reconstitution on planar supported lipid

bilayers in open chambers, has the advantage of rendering the surface accessible for optical microscopies, such as TIRF microscopy[30], FRAP analysis[6], single-particle tracking[34], as well as surface probe techniques such as atomic force microscopy[26]. The large homogeneous area allows for better statistics at defined concentrations. Furthermore the open chamber approach allows to precisely control protein concentration and a rapid and simple addition of further components, hence permitting to titrate protein concentration in a single chamber[20]. The assay can also be expanded by addition of other bacterial divisome components such as FtsZ[22,35], ZipA[22] or the chimeric protein FtsZ-YFP-MTS[10,35].

Other groups have taken a similar approach to reconstituting the Min system *in vitro*, but use a flow-cell instead of an open chamber[17–19]. Flow-cells have certain advantages, in particular when a fully enclosed 3D environment is needed[18], the influence of flow[17,19,23] or membrane composition[23] on MinCDE patterns is investigated, or if protein patterns are to be observed under protein limiting conditions[19]. Nonetheless, local control of molecular concentrations is more difficult. Protein components, especially MinD, strongly bind to the membrane they first encounter[18,19]. In our experience, the proteins frequently exhibit non-specific binding to tubing, inlets, syringes and other microfluidic parts. Hence, local protein concentrations differ from input concentrations[18] and also vary over the length of the flow-cell, resulting in a variety of different protein patterns on the membrane between inlet and outlet, as observed by others[19].

The second half of the protocol presented here, the *in vitro* reconstitution in rod-shaped microstructures re-using the open chamber approach on a patterned support covered by lipid bilayers allows for a simple mimic of *in vivo* protein behavior even though precise control over protein concentrations is lost due to buffer removal. Note that because the wavelength of MinDE is about one order of magnitude larger *in vitro* than *in vivo* the rod-shaped microcompartments are also about one order of magnitude larger (10 x 30 µm) than a rod-shaped *E. coli* cell.

Overall, this protocol allows for the precise control of all conditions including protein concentration, buffer composition and membrane properties. The use of 3D structured supports enables the reaction to be studied under spatial confinement, mimicking *in vivo* behavior without the need for complex microfluidics equipment.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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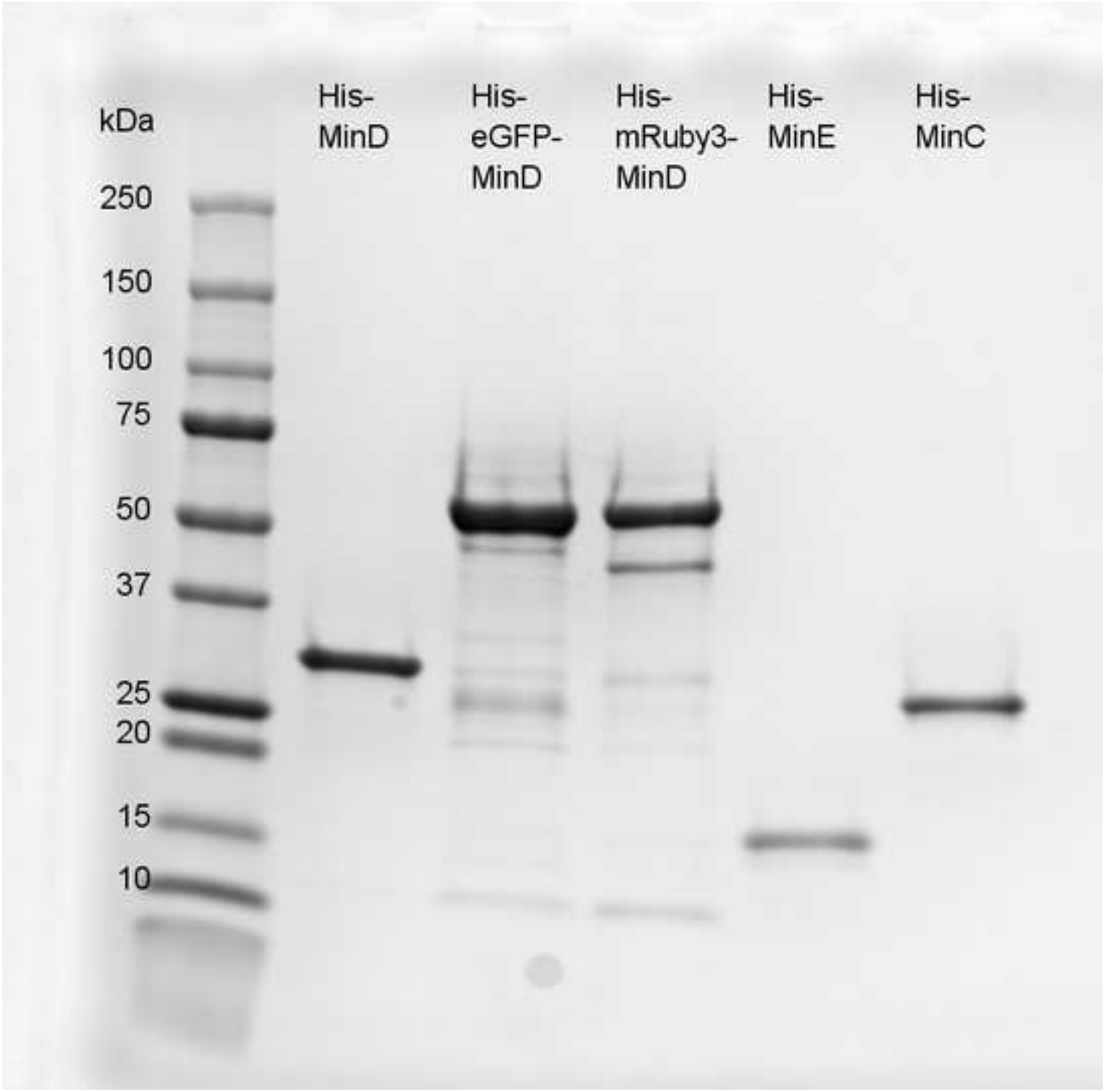
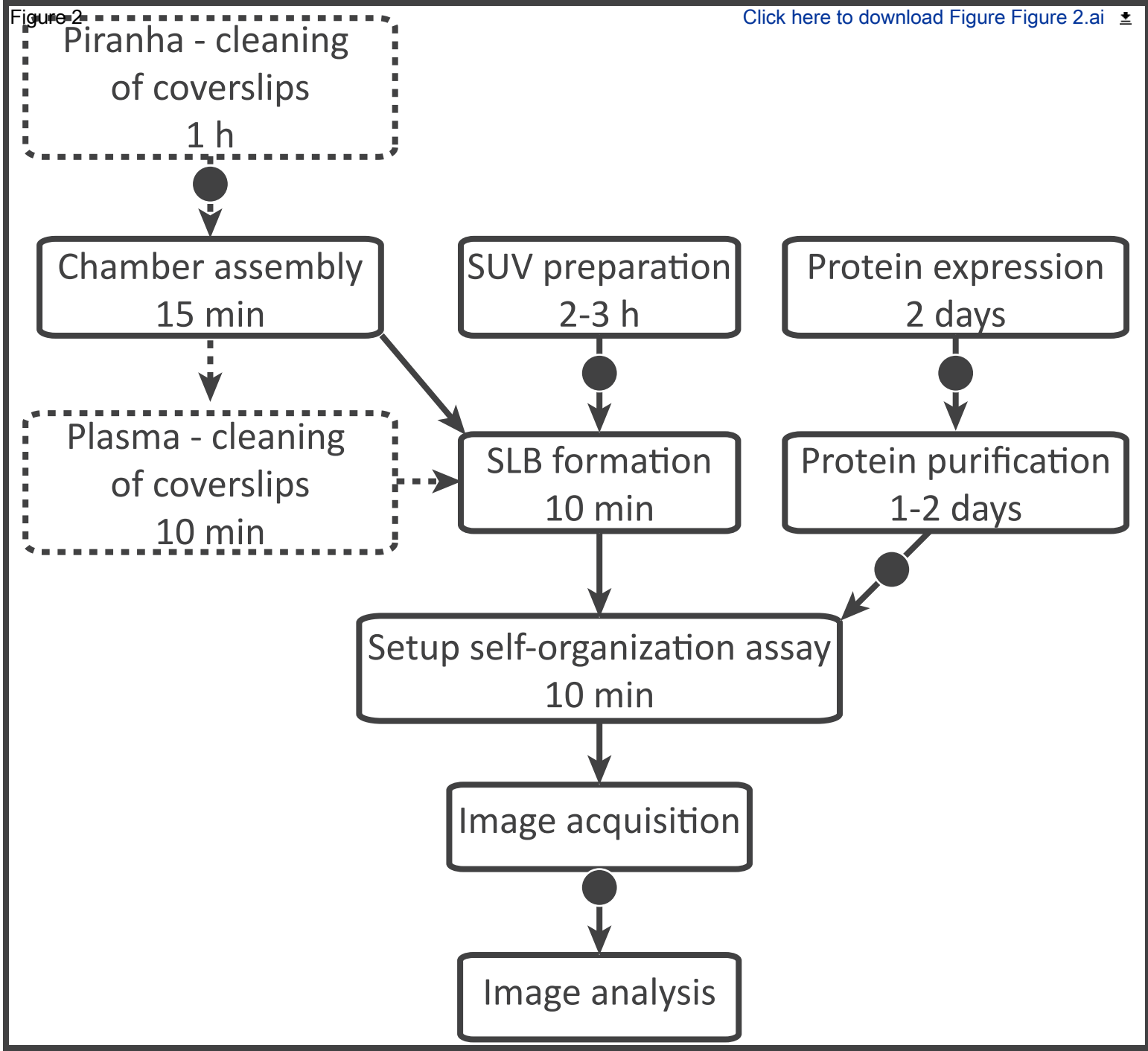
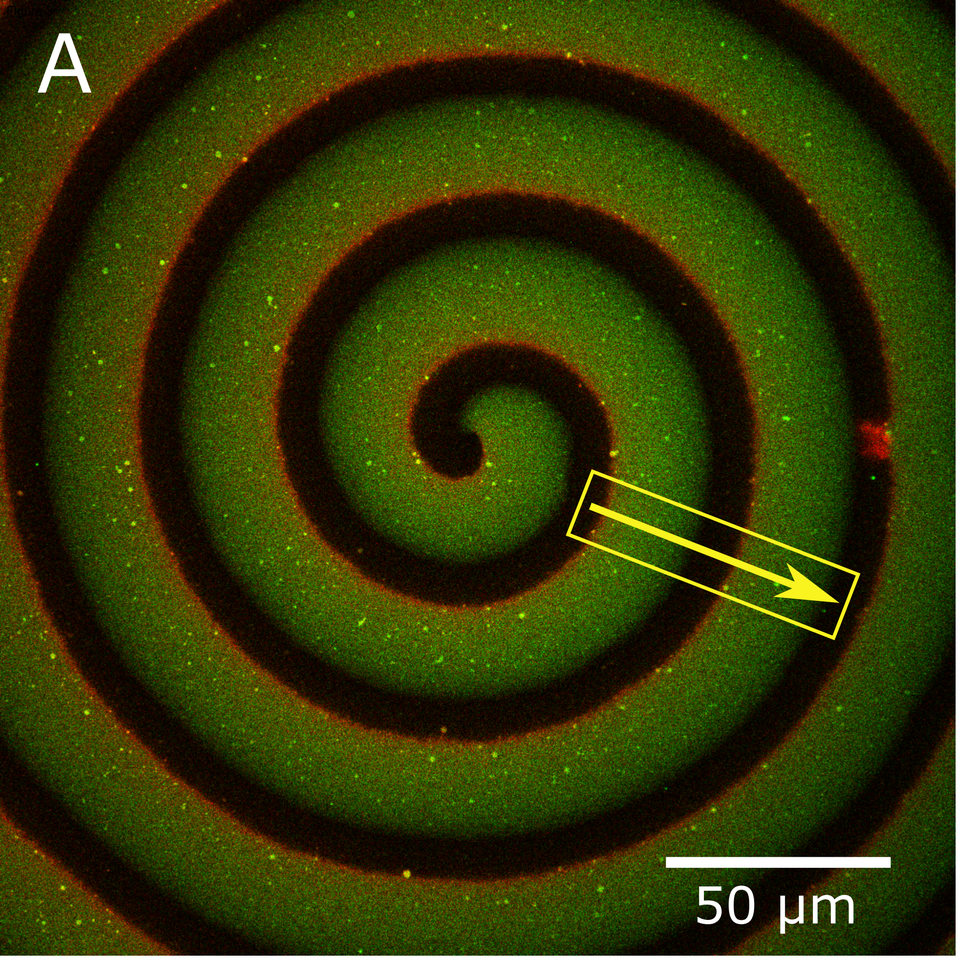
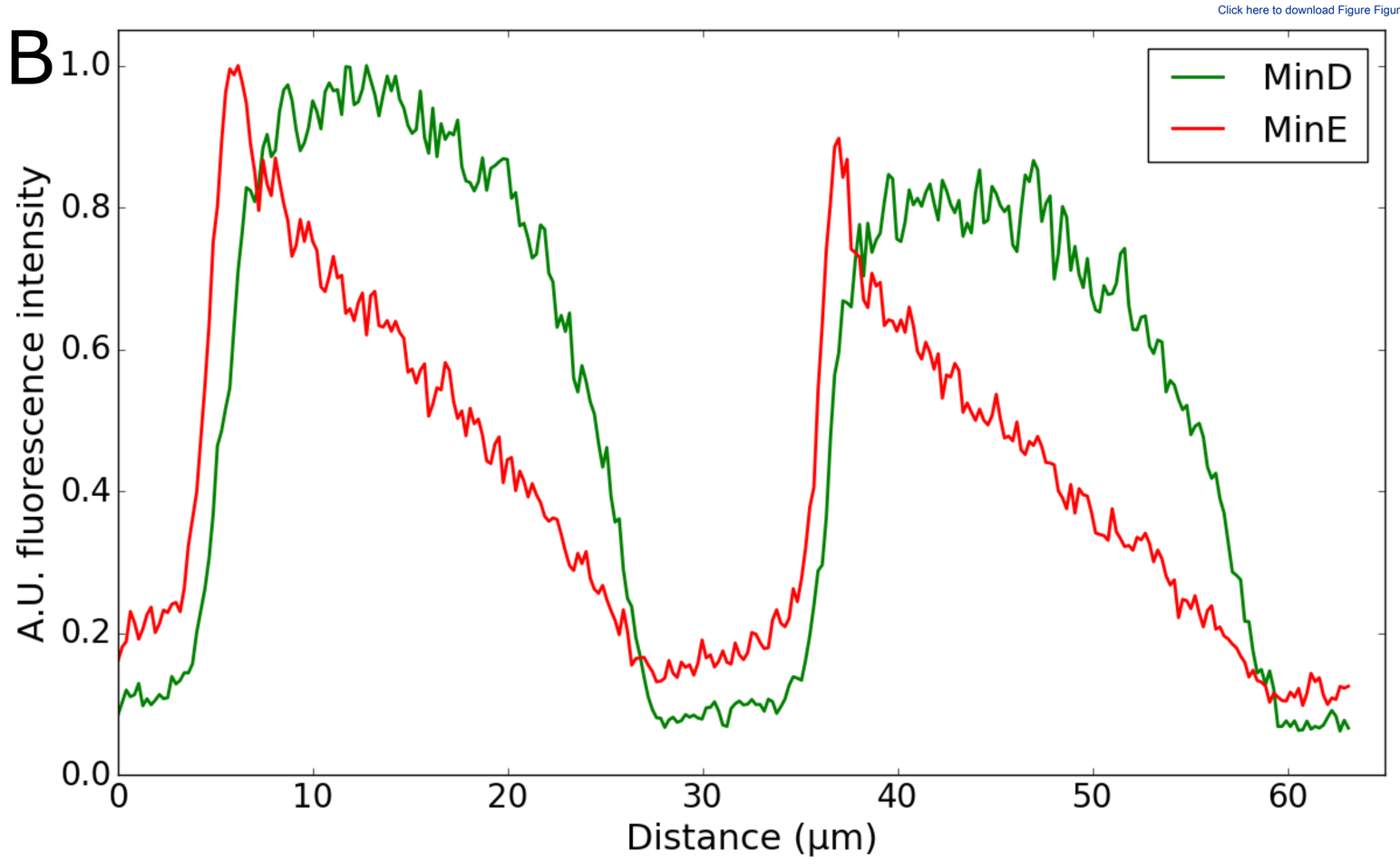
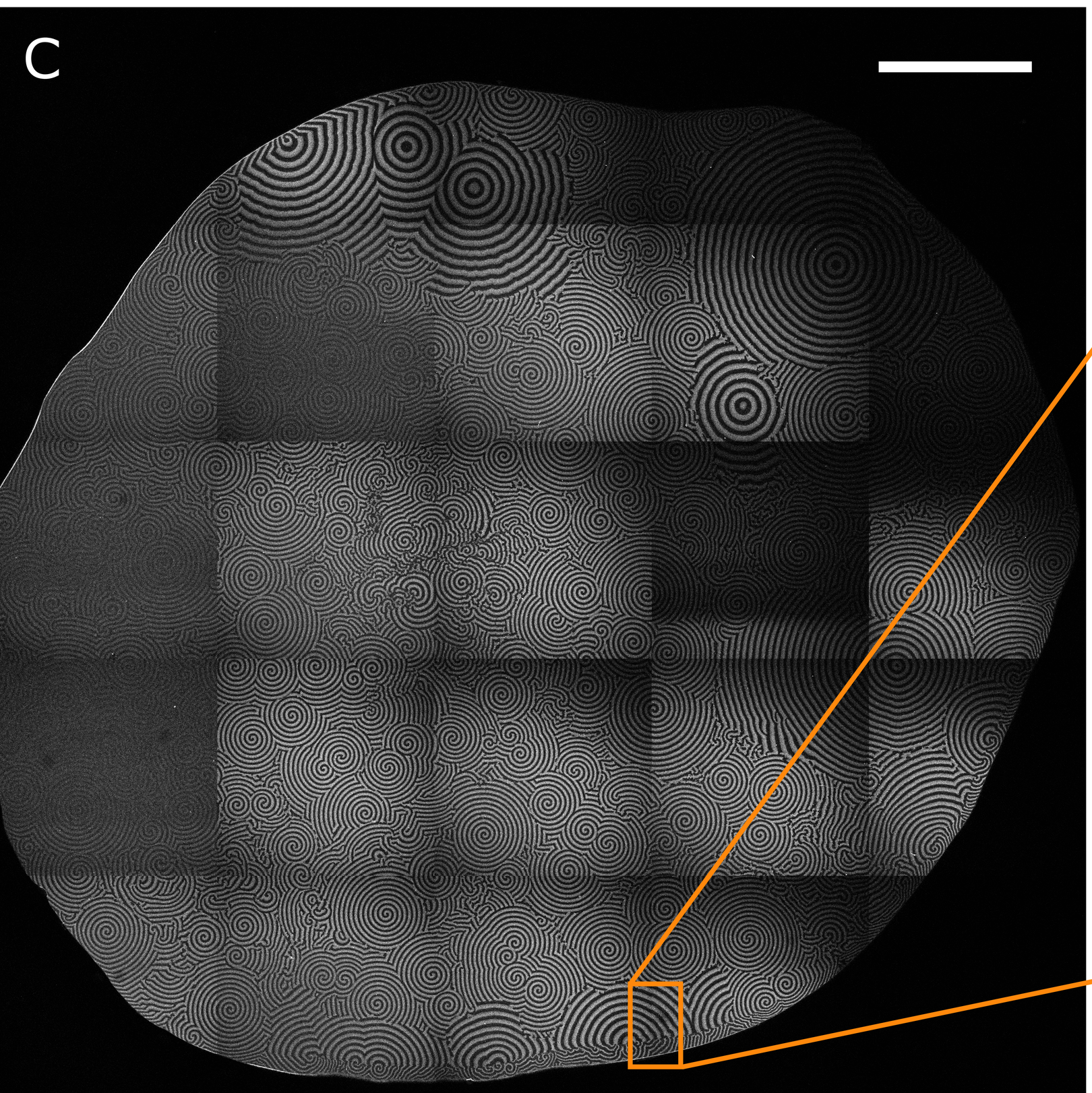


Figure 2









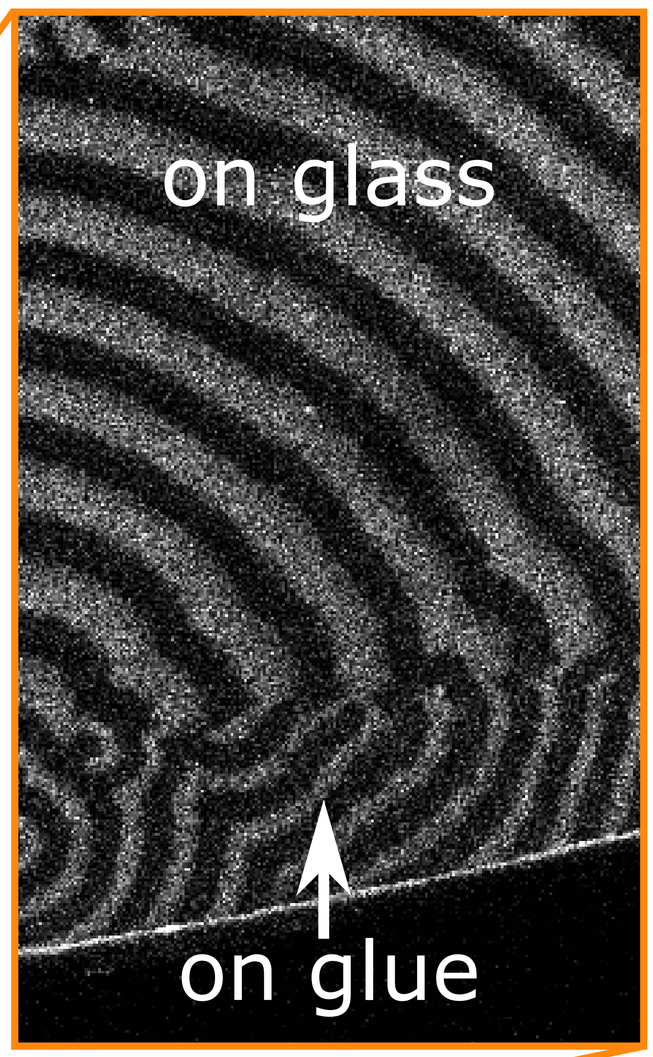


Figure 4

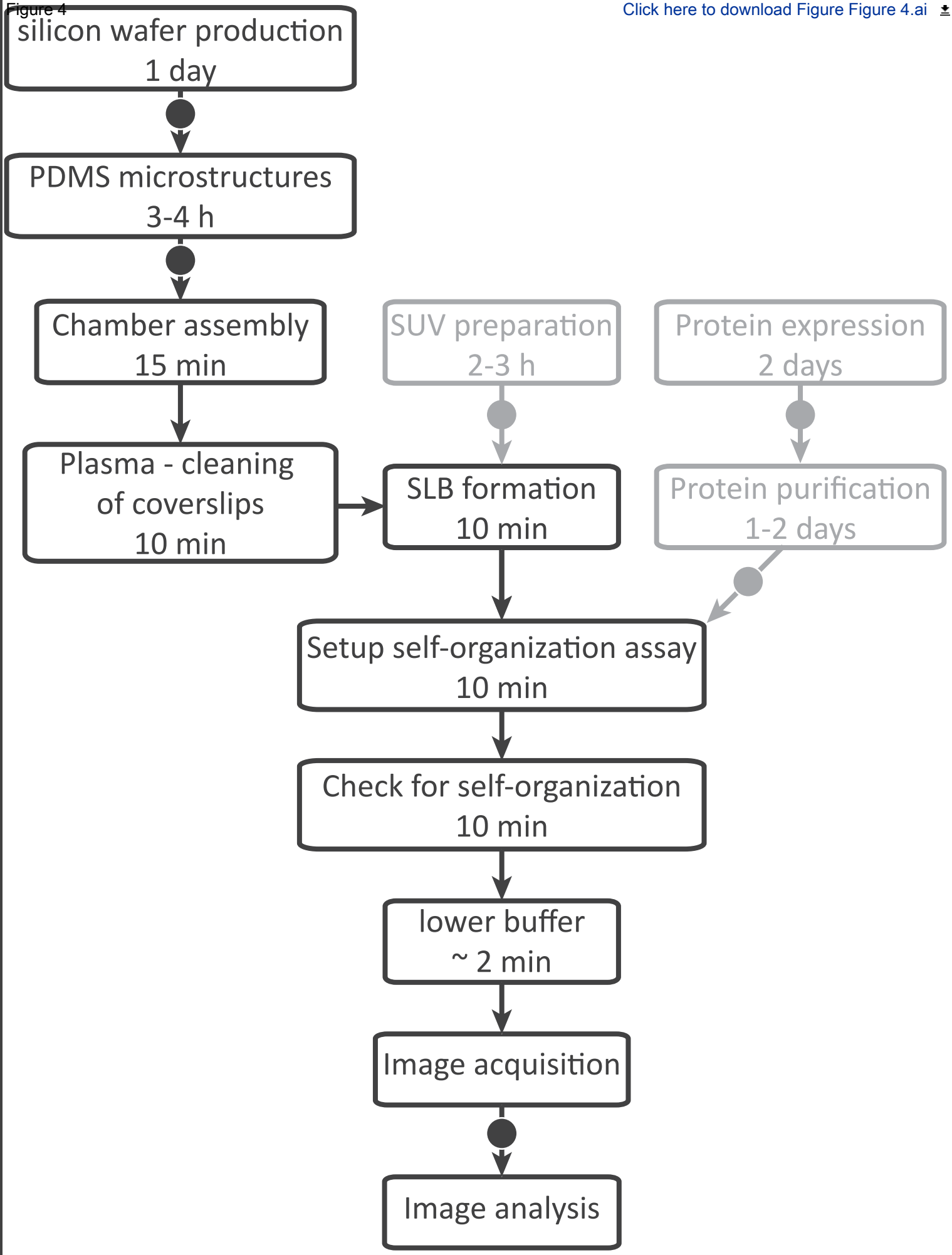


Figure 5

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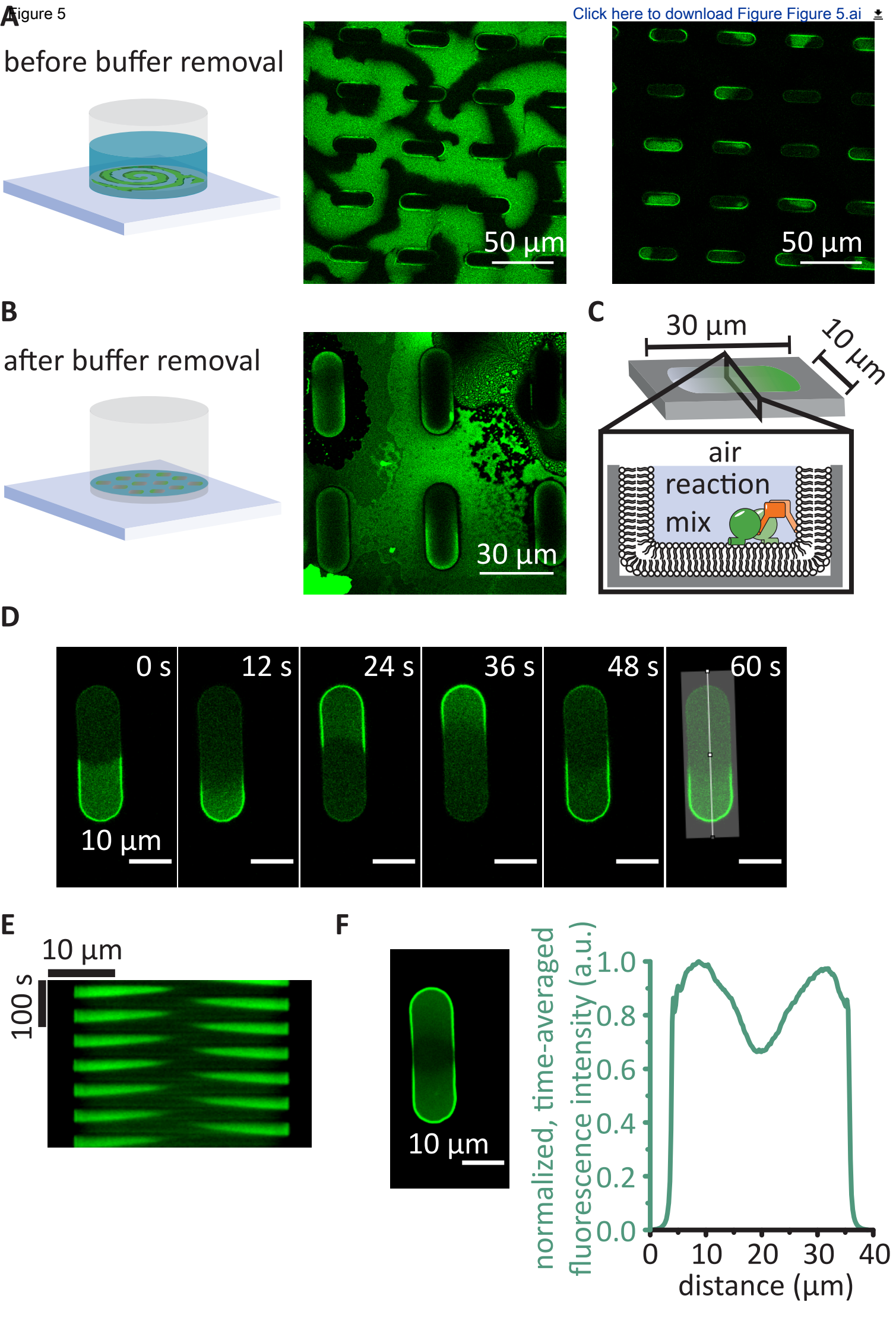
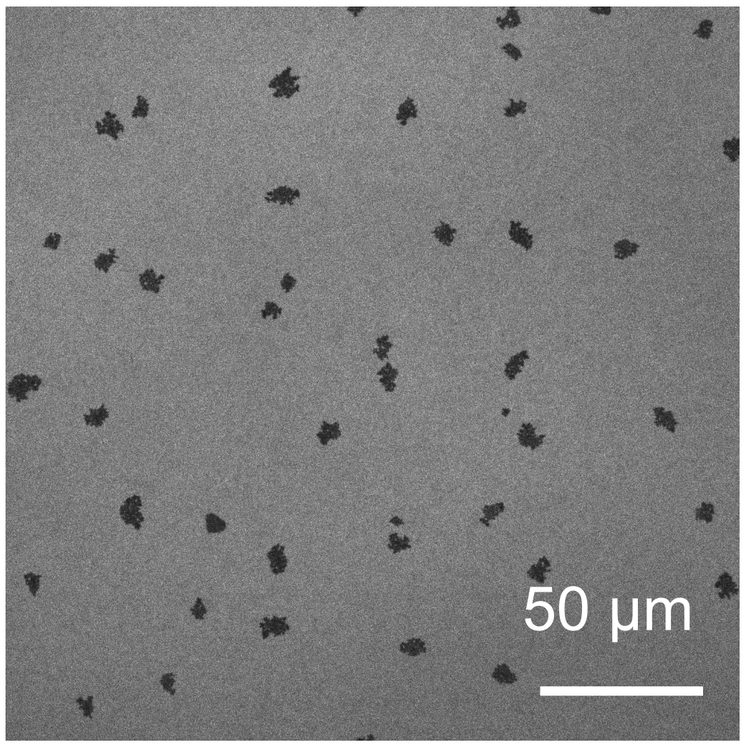


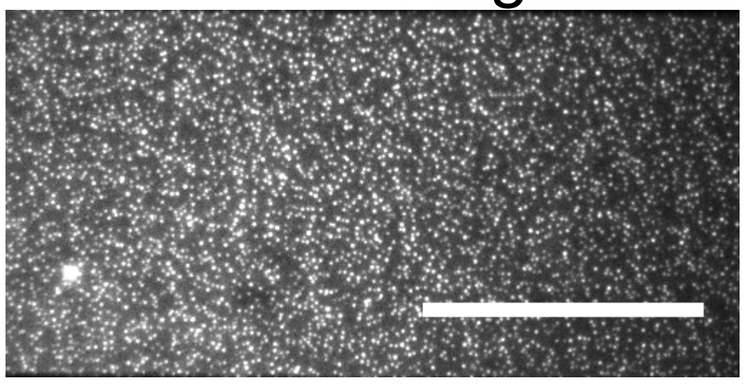
A

membrane problems:

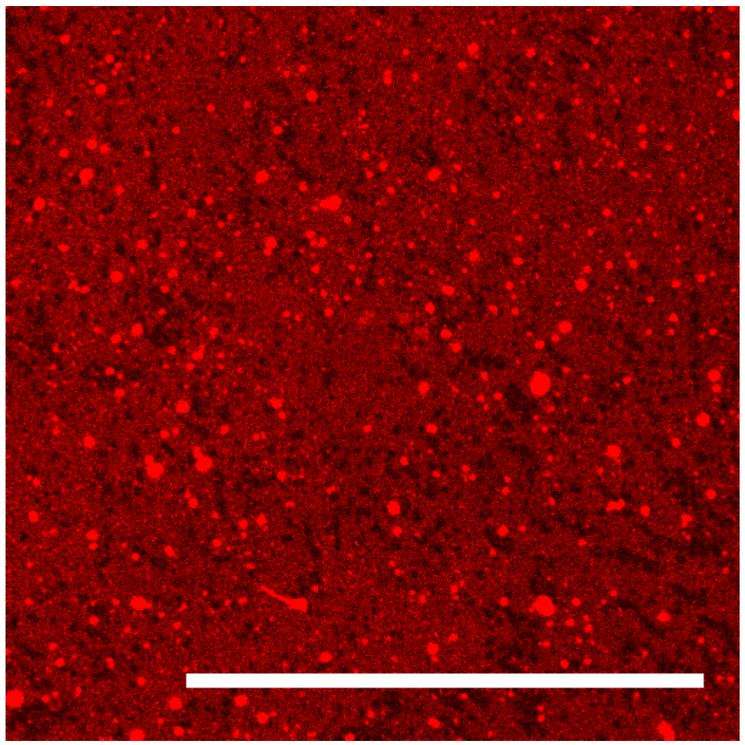
holes



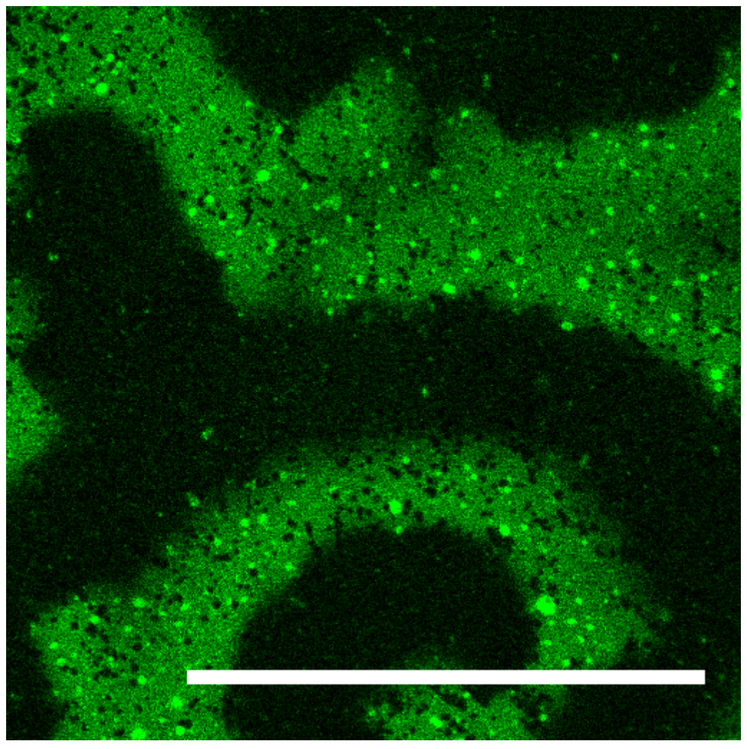
vesicles sticking



both holes
and vesicles

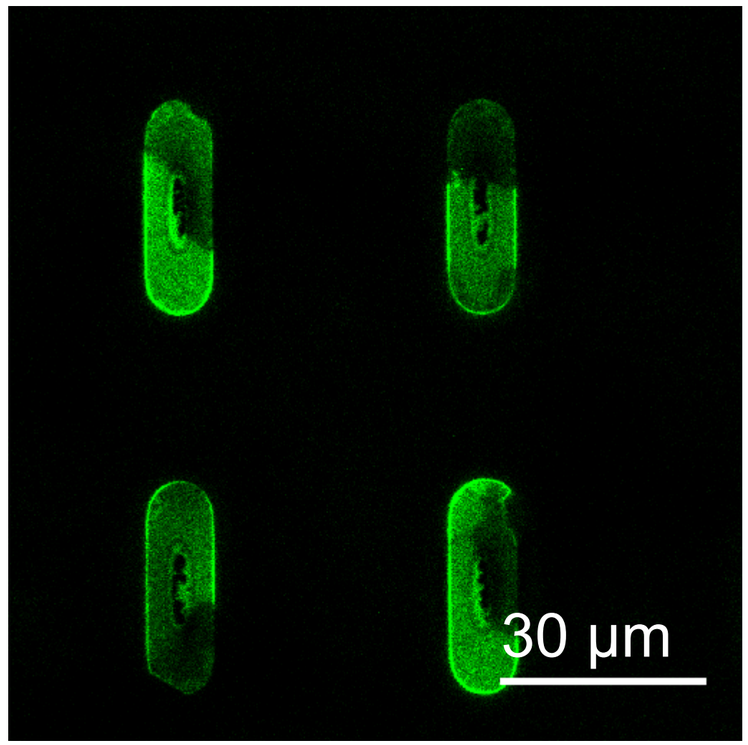


Min channel
(eGFP-MinD)

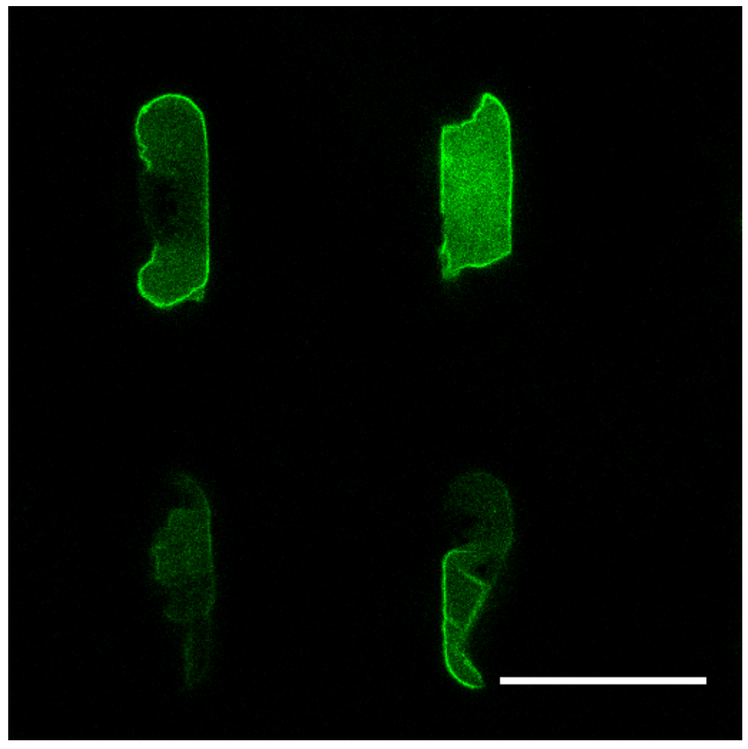


B

dried-out
microcompartments



fabrication problem



Materials Table

Click here to download Materials Table JoVE_Materials_050218.xlsx

Name of Material/ Equipment	Company	Catalog Number
Reagents		
DOPC	Avanti Polar Lipids	850375
DOPG	Avanti Polar Lipids	840475
E.coli polar lipid extract	Avanti Polar Lipids	100600
Adenosine 5’-triphosphate disodium salt trihydrate	Roche	
Adenosine 5’-diphosphate monopotassium salt dihydrate	Sigma	A5285-1G
Sodium chloride	VWR	27810.295
Potassium chloride	Roth	6781.1
Tris-base	Sigma Aldrich	T1503-1kg
Hydrochloric acid	Roth	9277.1
TCEP-HCl	Termo Fisher Scientific	20491
Ethylene Diamine Tetraacetate	Merck Millipore	1.08418.1000
Sulfuric Acid 98%	Applichem	173163.1611
Hydrogen Peroxide 50%	Applichem	147064.1211
HEPES	Biomol	05288.1
dimethyl sulfoxide (DMSO)	Merck	102950
Glycerol 86%	Roth	4043.1
TB medium		
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Roth	2316.x
Atto-655-DOPE	Atto Tec	AD 655-161
Ni-NTA agarose	Qiagen	30210
PDMS base	Dow Corning Corporation	
PDMS crosslinker	Dow Corning Corporation	
Materials		
UV Glue	Norland Products	6801
Coverslips #1.5 24x24 mm	Menzel Gläser	
Coverslips #1 24x24 mm	Menzel Gläser	
0.5 ml reaction tube	Eppendorf	0030123301
culture flask 2L	Corning	e.g. 734-1905
His-Trap HP	GE Healthcare Life Sciences	
Gelfiltration column: HiLoad Superdex 75 PG or 200 PG	GE Healthcare Life Sciences	
Econo-Pac 10DG desalting column prepacked column	Biorad	7322010

dialysis device: Slide-A-Lyzer Dialysis Cassettes, 3.5K MWCO, 0.1 - 0.5 mL or (	Termo Fisher Scientific	66333 or 66330
razor blade		

Instruments

ultrapure water: Milli-Q Type 1 Ultrapure Water Systems	Merck
automated protein purification system: Äkta Pure	GE Healthcare Life Sciences
bath sonicator	Branson
ARE-250 mixer	Thinky Corporation
Plasma cleaner Zepto	Diener electronic
positive displacement pipettes	Brand
LSM780 confocal laser scanning microscope	Zeiss

Plasmids

pET28a-His-MinD_MinE	Department of Cellular and Molecular Biophysics, MPI of Biochemistry, Prof. Schwille
pET28a-His-MinE	Department of Cellular and Molecular Biophysics, MPI of Biochemistry, Prof. Schwille
pET28a-His-EGFP-MinD	Department of Cellular and Molecular Biophysics, MPI of Biochemistry, Prof. Schwille
pET28a-His-mRuby3-MinD	Department of Cellular and Molecular Biophysics, MPI of Biochemistry, Prof. Schwille
pET28a-His- MinC	Department of Cellular and Molecular Biophysics, MPI of Biochemistry, Prof. Schwille

Comments/Description

Uvasol

SYLGARD 184

#68 and #63 both work well

used only for PDMS microstructures

e.g. Model 1510

use oxygen as process gas
Transferpettor models with glass tips
Fitted with Zeiss C-Apochromat 40X/1.20 water-immersion objective

plasmid encoding His-MinD and non-
tagged MinE to improve yield

plasmid encoding His-MinE

plasmid encoding His-EGFP-MinD

plasmid encoding His-mRuby3-MinD

plasmid encoding His-MinC

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CORRESPONDING AUTHOR:

Name:	Petra Schwille
Department:	Cellular and Molecular Biophysics
Institution:	Max Planck Institute of Biochemistry
Article Title:	Investigating self-organizing protein patterns by in vitro reconstitution on supported lipid bilayers
Signature:	
Date:	12. März 2018

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Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript and corrected any mistakes.

2. Please limit the use of personal pronouns (we, you, your).

We have removed personal pronouns in the protocol text and also limited the amount in introduction and discussion.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: BD10, Millipore

We have removed any instances where commercial language is mentioned and have added those to the Table of Materials and Reagents. PD10 desalting column refers to the packing of the column and is available from different suppliers.

Protocol:
1. For each step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:
1. 2.1.4: Please define Min buffer when it is first used.

We have added the composition of Min buffer at the point where it is first mentioned (now former line 218, 2.1.1).

2. 6.3: What excitation and emission wavelengths are used here?

We added information on the excitation wavelengths. Emission filters and wavelengths depend on the capabilities of the microscope in use. Any microscope used for biological applications usually comes with pre-sets for GFP and mCherry which can be used for eGFP and mRuby3, respectively.

3. 7.1: Do you have a pattern for these wafers that can be included as supplementary information?

We have included a CAD file as AutoCAD .dwg for the wafers and the DNA sequences of all plasmids used in GenBank format (.dna) as supplementary information.

Highlighting:
1. 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 headers 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We tried to reduce as much as we could the highlighted passages and hope that the remaining steps are few enough to yield a coherent video protocol.

References:
1. Please ensure references have a consistent format.

We have checked all references and corrected any mistakes.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have added the sections “Reagents”, “Materials”, “Instruments” and “Plasmids” to the material list as suggested by reviewer 3 and also added any missing material to the table.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
A description of procedures to test the oscillation of proteins in artificial containers particularly applied to the behaviour of the Escherichia coli proteins of the septum selection MinCDE system

Major Concerns:
The procedures are complex. The video (not available for review) should be sufficiently detailed and informative to allow the faithful reproduction of the process.

The procedures may appear complex as we have described them in detail and provide alternative methods for purification and coverslip cleaning. However, we hope that with this level of detail newcomers to the method will be able to perform the *in vitro* reconstitution assay without committing typical mistakes. We have highlighted the most critical parts of the protocol for the scriptwriting of the video production and hope that the video will provide additional insights on how to perform the protocol.
Please note that this protocol first has to be peer reviewed before the video production is initiated. We will take your concerns into account when producing the video together with JoVE.

Minor Concerns:
Definition of what piranha and plasma cleaning solutions should be included. Equally PDMS seals should be defined. These terms are not common knowledge.

We now define piranha and plasma cleaning at the beginning of section 3. We also briefly introduce PDMS at the beginning of section 7.

Reviewer #2:
Manuscript Summary:
This manuscript describes several protocols of different discipline for performing the in vitro reconstitution experiments to observe pattern formation of the Min proteins on supported lipid bilayers.

Major Concerns:
1. Since this is a methodology paper, the title starting with 'investigating' does not sound right.

We thank the reviewer for pointing this out and have changed the title to "*In vitro* reconstitution of self-organizing protein patterns on supported lipid bilayers".

2. The content of Introduction, Representative results, and Discussion can be shortened, since there are redundancy and most information have been reported and discussed in several reviews and a methodology paper from the authors' own lab. The method paper published in Methods in Cell Biology (2015, 128: 149-163) should be cited.

Indeed our lab has done extensive work on the Min system that is described in several original research articles and in reviews. However, the only existing methods paper on the *in vitro* reconstitution is the article mentioned by the reviewer: "Methods in Cell Biology (2015,128:149-163). This article only focuses on the reconstitution of MinCDE in rod-shaped microstructures and not on flat supported lipid bilayers. While some of the information is indeed redundant, the mentioned article focuses more on the microfabrication of the rod-shaped microstructures. We now cite the mentioned article in the protocol for the microfabrication, as we indeed think that this part is well explained in the mentioned article and can also be omitted by other labs performing the procedure by ordering a silicon wafer from a foundry. In this JoVe article we supply a detailed protocol of the entire procedure focusing on the preparation of supported lipid bilayers. The article also discusses potential problems that can arise and it will be accompanied by the video to show the most critical steps. Further, we aim to provide an Open Access version of this article for all interested readers or watchers of the JoVe, which was not the case in the aforementioned publication.

3. In Representative results, the authors do not provide example data for protein purification to match the protocols.
We now provide a new Figure 1 with an SDS-PAGE of all Min proteins and MinD tagged with fluorescent proteins.

4. Details of fabricating microstructures are missing.
Due to protocol length limitation we have not supplied details on the fabrication of microstructures. However, we cite our previous detailed description of the process in Methods in Cell Biology (2015,128:149-163) and also point to a JoVe article that describes a very similar fabrication. Furthermore, the entire protocol step can be omitted by simply ordering the desired silicon wafer from a foundry. A CAD file for producing rod-shaped microstructures can be found in the supplementary data files.

5. The sentences in the protocols can be written better.
We have rewritten the specific sections mentioned below to make them clearer.

Minor Concerns:
2: self-organizing protein patterns 'of MinDE'

We have not included “of MinDE” into the title as the protocol can also be used to study other self-organizing proteins such as FtsZ, alone as well as together with the MinCDE system, or a minimal actin cortex.

24: of MinD and MinE
Added change to the manuscript.
26: 'protein confinement' is not right.
We have changed “protein confinement” to “reaction confinement”.
32, 44, 54: 'MinDE' instead of 'MinCDE'
MinC may not be an instrumental part of the self-organization, but was included in our protocol as well as some of our published results. The protein belongs to the native MinCDE system and is also needed if other components should be co-reconstituted, such as FtsZ. So for a broader applicability of our techniques we integrated MinC in our assays.
56: Considering MinD and MinE undergo attachment and detachment cycles on the membrane surface involving the space in solution, 'the membrane as the spatial reaction matrix' is not precise.
While the reviewer is correct in stating the space in solution as important, the membrane acts as the catalyst enabling the dynamic behavior. While diffusion, also a crucial part of our pattern forming system, also takes part in solution, the relevant reactions, namely the binding of MinD to the membrane and the detachment of MinD from the membrane after stimulation of its ATPase activity by MinE are happening on the membrane. We have therefore kept the formulation as is.
68: change component to 'gene'.
Has been changed.
69: what does the sentence mean? 'the properties of the cytosol are not or only very hard to implement'
We have changed the sentence to make our statement clearer. We meant that *in vivo*, the composition and chemical properties of the cytosol are regulated in complex ways and cannot be changed at will.
87: It makes no sense to say 'similarly investigated in vivo using bacteria', because molding bacterial shape to study Min oscillation in vivo is completely different from the investigations using the in vitro reconstitution systems.
Deleted “similarly”. Our point remains, which is that both studies investigated the influence of geometry on Min protein pattern formation.
123: E.(space)coli
We have changed the respective part.
126: (1) 'single colony' instead of clone, (2) medium and concentration of antibiotics
We have implemented the suggested changes. Antibiotic concentrations should be common knowledge, and we cannot provide a full list of all possible compounds. However, we now provide the concentrations of two very commonly used antibiotics in brackets.
129: specify the concentration of antibiotics instead of the dilution factor
The dilution factor refers to dilution of the overnight culture in TB medium. We have changed the sentence to make this clear.
130: incorporate temperature and shaking speed into the sentence
We have implemented these changes.
132: (1) induce 'protein expression', (2) 600nm
The sentence now reads “protein expression” rather than cells. We have changed OD600 to optical density at 600 nm.
136: toxicity caused by overexpression of MinC is even stronger
Added MinC to the sentence.
148: automated protein purification system is not specified in the table.

The table has been adjusted.

155: (1) β-mercaptoethanol, (2) full chemical name of TCEP

Corrected.

158, 159: (1) Mg2+, (2) The preparation of Mg2+-ADP solution can be written in proper sentences.

We now describe the preparation of Mg^{2+}-ADP

166: (1) when a prepacked Ni-NTA column is used, the sample has to be injected or pumped into the column before incubation. (2) condition of incubating the supernatant with the Ni-NTA resin is missing. (3) for open column, the step of packing the Ni-NTA resin into a column is not mentioned.

We have incorporated the suggested changes into the text.

192: MinD ATPase activity assay to access the protein function is not mentioned.

We do now mention measuring the ATPase rate as a potential assay for protein activity. Due to protocol length limitations we cannot provide a full protocol here and point the reader to an appropriate source.

195-196: The rationale of statement does not seem right.

We have edited the statement for clarity. Briefly, reaction-diffusion systems are sensitive to changes in the diffusion rates of the involved species. MinE is a very small protein (10 kDa), and therefore a relatively big mass change would be introduced by adding a fluorescent protein tag (24 kDa) for labeling.

198: the buffer ingredient and pH are not mentioned.

We have added the buffer used (storage buffer).

203: what type of the desalting column?

The specific brand of column cannot be integrated into the text according to journal policies, but can be found in our materials list. We have added the notion that we use gravity flow columns.

206: what kind of the dialysis device?

The journal policies do not allow to mention specific devices in the main text, however, we now provide the specific dialysis device used in the materials list.

210: (1) the equation to calculate the labeling efficiency is not mentioned. (2) details for mass spectrometry is missing, but it may not be necessary to determine molecular weight.

(1) We now refer our readers to the dye manufacturer's manuals. All dyes we have worked with so far have excellent protocols on how to estimate efficiency, and it is beyond the scope of this protocol to go into much detail here. A simple formula cannot be provided, since many dyes change their extinction coefficient when attached to proteins.

(2) We added that also SDS-PAGE can be used to assess residual dye/protein labeling. It is beyond the scope of this protocol to give detailed instructions for mass spectrometry.

218: (1) what is the final buffer? (2) How about the E. coli polar lipids for SUV and SLBs?

We have added Min buffer as final buffer. The preparation protocol works equally well for many different lipid compositions, among them *E. coli* polar lipids. We do, however, not recommend EPL to researchers new to the method, because generating an artefact-free bilayer with EPL is more challenging.

We have added a note to alert the reader of this point and added the details of the protocol for readers that want to use *E. coli* polar lipid extract (changes to points 2.1.1, 5.3 and 5.4).

231, 248: what does the Min buffer refer to?

We have added the composition of Min buffer at the point where it is first mentioned (now former line 218, section 2.1.1).

242: what is the approximate temperature of the hot water?

We have added an approximate temperature.

251: 'odd' instead of uneven

We have changed the sentence accordingly.
256: how to 'put solution onto a heat block'?
Specified the glass vial containing the lipid solution.
262: specify the power/Watts of sonication for preparation of SUVs.
Specified the power of our bath sonicator in brackets.
266: SUVs instead of 'Lipids independent of preparation method'
Changed to SUVs.
269-270: don't understand the statements.
We have rewritten the sentences to make the section clearer.
Statement 1: After freezing and thawing, the SUVs become multilamellar and partly form larger aggregates. They need to be sonicated again to produce SUVs before using them.
Statement 2: Extrusion has the benefit of yielding a narrow distribution of vesicle sizes, as well as removing any larger lipid structures. Freezing and thawing the vial changes that.
275: specify the ratio of H2SO4 to H2O2
Specified the ratio. We have made good experiences with 7 drops H2SO4 to 2 drops 50% H2O2.
305: remove comma
We are unsure why the comma should be removed. We recommend using nitrogen. If bottled nitrogen is not available, pressurized air can be a substitute. But it should be verified that the compressors and tubing do not contaminate the air with traces of mineral / pump oil.
344: specify the final concentration of SUVs used to prepare SLBs
Specified the working concentration.
420: it would be nice to draw an illustration of the chamber
We already supply a schematic of the microstructures in former Figure 4, now Figure 5.
454: Specify software and plugins for image analysis
Software has been added.
Representative results: Figure 2C: there are concentric rings appeared in the image. Are they typical patterns? Have the concentric rings been documented?
At most concentrations of MinD and MinE that allow pattern formation, surface waves emanate from rotating spirals as well as concentric rings. So yes, they are typical. Concentric rings are usually the minority, but become dominant at specific conditions. They may not have been explicitly mentioned or shown in the literature, especially since most microscopic images of the *in vitro* Min system only show small areas of several hundred µm.

Reviewer #3:
Manuscript Summary:
In this JoVE article, the Schwille group describes the use of their well-setup that can be used to reconstitute the self-organizing Min-system. This is a very well-established technique that has been incredibly useful in unveiling what MinD and MinE can do when unleashed from cellular confines. Although iterations of these methods have already been published previously (Zieske and Schwille 2015), a method video would be very useful not just to the community interested in the Min system, but to anyone interested in reconstituting a self-organizing system known to associate with membrane. In addition to the simplified well-setup, the article moves onto the use of PDMS microfluidic devices that partially confine Min patterns in order to reconstitute patterning more similar to those observed in vivo. The article is well-written and will serve as a good template for the video. I have only a few concerns before acceptance.

We thank the reviewer for recognizing how useful a method video for the technique would be.

Major Concerns:
1. Throughout the section describing the PDMS chambers, the troughs are described as "bacteria-shaped microstructures". Bacteria come in all shapes and sizes. Some are indeed rod-shaped like E.coli (1 x 3 microns), but few bacteria are as large as the dimensions of these troughs (30 x 10 microns). It is important to highlight this discrepancy in scale. They are not bacteria-shaped microstructures. I think the best compromise is to call them "rod-shaped microstructures" and highlight the order of magnitude difference in scale compared to an E.coli cell.

We thank the reviewer for pointing this out. Even if we do mention the longer wavelength in the introduction (lines 75-77) the reviewer is right that it should be mentioned in several instances to make this point clear. We now mention the longer wavelength and hence the larger size of the microcompartments in the introduction where the microcompartments are introduced and in the discussion about the microcompartments. Also, we changed all instances of “bacteria-shaped microcompartments” to “rod-shaped microcompartments”.

2. Lines 596 to 604: I think it is important to advise the authors that for a JoVE review, the protocol is being evaluated on the efficacy of the protocol being demonstrated. The authors go out of their way critiquing the flow-cell approach to reconstituting the Min system as a way to validate the use of a well setup. Both the well and flow-cell setups have advantages and disadvantages and both have been incredibly useful in dissecting the Min system; albeit in different ways. However, the pitfalls highlighted here for the flow-cell setup are either untrue or misunderstandings from previous literature. This section therefore currently reads as if the authors are using the flow-cell setup as a strawman to explain the importance and utility of the well setup:

We thank the reviewer for his detailed input regarding the flow-cell method. We respectfully disagree with the notion that a JoVE paper should only focus on the efficacy of a given protocol as the journal guidelines state that the introduction should include “The advantages over alternative methods with references to relevant studies”, as well as „Information to help readers decide whether the method described is appropriate for them” and the discussion should include “The significance of the method with respect to existing/alternative methods”. As there are indeed different methods available for studying the MinCDE system *in vitro*, we included the comparison between the methods. However, we do not only critique the flow cell setup, but also highlight in both the introduction and discussion the advantages of using a flow cell.
Please see the answers to the respective points below.

a. "Firstly, it is hard to achieve high quality supported lipid bilayers in flow-cells, as rigorous washing of membranes is difficult."
i. This is not an issue using a flow-cell. Buffer wash from an inlet allows for rapid and complete washing of SUVs from the solution phase. It can be equally argued the well setup is more difficult to wash rigorously as one must avoid drying out the surface; therefore one is restricted to serial dilutions of the SUVs remaining after each wash. This is not a problem with the flow-cell setup, which allows for a complete and rapid buffer switch in the solution phase.
ii. Also, the use of a syringe pump attached to the inlet of a flow-cell, as opposed to manual pipetting, allows for very low flow rates that prevent inadvertent shear forces that can be

caused by manually pipetting in a well setup. Therefore, liquid handling in general is more precise throughout.

We have changed the respective passage and removed the reference to the high quality supported lipid bilayer. However, we do disagree that the liquid handling is in general more precise in the flow cell setup, for a detailed answer please see below.

b. "Secondly, flowing in the proteins and other assay components compromises local control of molecular concentrations."
i. Once again, this is not an issue using a flow-cell. By using a two-inlet flow-cell ATP can be mixed with the proteins in the flow-cell. The results were identical to that when using a well-setup and not published.
c. "Proteins also tend to stick to tubing, inlets, syringes and all other microfluidic parts involved, leading to protein losses that are unlikely to occur in an equimolar fashion."
i. Protein sticking to plastic is definitely an issue when the GFP-MinD prep is not highly purified. GFP-MinD is prone to aggregation as the authors note, and these aggregates stick to everything. Therefore the issue of sticking here is largely due to GFP-MinD aggregates not sufficiently removed from the stocks. We do not see these issues with GFP-MinD purified via HisTRAP, MonoQ, and SEC. Also a particulate column on the day of experiments helps remove trace aggregates.

We respectfully disagree with this assessment. In our experience, especially MinD or EGFP-MinD stick to surfaces. This has nothing to do with aggregates or the purification state of the protein but rather is a property of the protein itself. The membrane targeting sequence of the protein enables it to stick to all kinds of negatively charged or hydrophobic surfaces. For example, we have observed a strong binding of His-MinD and eGFP-MinD to mica with high-speed atomic force microscopy (see Miyagi, A., Ramm, B., Schwille, P., Scheuring, S. High-speed AFM reveals the inner workings of the MinDE protein oscillator. Nano Lett. 18 (1), 288–296 (2017).). We have also observed that, especially when working with low protein concentrations, the proteins binds to regular plastic reaction tubes and glass surfaces leading to a depletion of the protein from the solution. We have edited the sentence of concern, but kept the main message as we think it is important to notify the reader of this particular aspect of the Min system.

d. Line 602 to 604: It seems the authors have misunderstood the point of this experiment. Preincubation of MinD with ATP was performed intentionally to generate a protein gradient on the SLB in order to highlight that the spectrum of patterns was dictated by the local protein density on the SLB. As mentioned above, when ATP is mixed with MinD and MinE using a 2-inlet flow-cell, the results are identical to the well-setup.

We respectfully disagree with the reviewer on this point. Caspi et al, (Caspi, Y., Dekker, C. Elife. 5, e19271 (2016) describe in detail that the protein concentrations inside the chambers vary significantly from the injected concentrations and that the increase they observe differs between MinD and MinE. Please compare to the following sentences from Caspi *et al.* “While Min proteins were injected at a well-defined concentration (1 µM MinE and 1.08 µM MinD), the final concentration of the proteins in the chambers are higher than those introduced. The reason is that during the injection process, MinD molecules will bind the membrane, followed by MinE molecules, while proteins continue to flow into the chamber with the fresh bulk solution. This results in larger final concentration in steady state. We therefore measured the concentration of the final Min proteins inside our chambers using a green fluorescence protein (GFP) calibration.” and later in the text “The concentration of the Min proteins was measured

for 52 different chambers. As can be seen (Figure 5a), the actual concentration of the Min proteins in our chambers was significantly (~factor 5 higher than the value for the injected stock solution. Furthermore, a wide distribution is observed, particularly for MinE. Note that we did not observe a relation between the chamber size and the measured concentration of the Min proteins. From these measurements we concluded that the concentration of MinD in our chambers was 4.5 ± 0.5 µM (mean ± SD), the concentration of MinE 6 ± 3 µM, and the average ratio of [MinE]/[MinD] amounted to 1.3 ± 0.5. ”

The flow-cell setup used in the Mizuuchi lab has in all published articles (Ivanov *et al.* (2010), Vecchiarelli *et al.* (2014) and Vecchiarelli *et al.* (2016)) used the preincubation with ATP and observed that MinDE formed different patterns across the flow-cell at the beginning of their experiments indicative of a fast binding of MinDE to the membrane. Compare to Vecchiarelli et al. (2016) “ATP-bound MinD dimers that formed during the preincubation bound the SLB near the inlet of the flowcell. This preferential SLB binding of MinD (and MinE) near the inlet depleted the solution concentration of proteins, resulting in lower concentrations further downstream.”
We do agree that given the flow is stopped and the system has enough time to equilibrate, something that is also described in Vecchiarelli *et al*. (2016), the protein concentrations will homogenize over the length of the flow-cell, resulting in homogenous patterns. However, this concentration is very likely to differ from the input concentrations (see answer to point c). We cannot assess if a two-inlet setup with separate ATP inlet can indeed abolish the tendency of MinDE to accumulate on the membrane at the flow-cell inlet resulting in a protein concentration gradient, as the data is not available to us or the public. Given the description of the phenomena in Caspi *et al.*, we would like to keep the information in the manuscript.
As described in the reviewer’s point f, the addition of several different compounds in a flow cell and hence different inlets increases the complexity of the setup and hence using a two-inlet setup with separate ATP increases experiment complexity.

e. Together, this section takes away from the power of well-setup by highlighting problems with the flow-cell setup that simply do not exist. The major problems for the flow-cell setup are exactly the same issues that plague the well-setup as described by the authors here - 1) Protein quality, 2) Lipid quality, and 3) Cleanliness/hydrophilicity of surface.
Please see the answers to the individual points above. The reviewer is of course right that the major problems are the same for both methods.
f. The major advantage of the well-setup over the flow-cell, which is currently not mentioned is the fact that the flow-cell does not allow for rapid order-of-addition experiments as easily as one can with the well-setup. This flow-cell pitfall is currently not stated in the paper and could be added.
We thank the reviewer for this suggestion and incorporated the notion into the discussion.

3. The Schwille group has done an impressive amount of work showing Min reconstitutions along with FtsZ polymers. For those interested in pursuing this technique to study downstream cell-division components, it would significantly strengthen the approach if FtsZ methods were also added: 1) FtsZ purification, 2) FtsZ addition to the well-setup, and 3) any additional cofactors or buffers needed for FtsZ addition. This would make the video a "one-stop-shop" for those interested in these reconstitution techniques as it relates to bacterial cell division.
The assay can indeed be easily expanded by addition of other divisome components such as FtsZ, ZipA or FtsZ-YFP-MTS. As the basic reaction setup stays the same - only the proteins and GTP need to be added - we do not think this information is crucial and can simply be

derived from the published research articles. Hence, due to protocol length limitations and in the interest of describing the challenging parts of the method in detail, we do not provide detail of purification of any of these components nor their addition to the assay. However, we mention now in the discussion that the other components can be added and cite the relevant literature.

Minor Concerns:
Line 69: "not or only very hard". Fix.
We changed the sentence.
Lines 83-84 As stated in the major concerns, exact control of protein/component concentration is not "much more complicated" using a flow-cell. It would not have been possible to do systematic titrations of MinE, for example, if that were indeed the case (Vecchiarelli et al., 2016).
Line 174 Will a figure of an SDS-PAGE gel of all protein components be provided? Or will this be shown in the video?
We have now included a SDS-PAGE of all protein components as Figure 1. All other Figures have been renumbered accordingly.

Line 178 State the gel filtration buffer composition. If it is the same as the "exchange buffer" used for storage as shown in 1.2.11, then just say so.
We have changed the corresponding section.

Line 410 8.7. Where does the sponge go relative to what is shown in figure 4A-B. I'm sure the video will help clarify this point.
It is important if the sponge is used that it does not come into contact with the surface. We have added a sentence to the respective part.

Line 610 Once again, please remove "similar to the aforementioned flow cell setups". See major comments above.
We have removed the section and edited the entire discussion part to give a less critical, but hopefully still complete comparative perspective of both methods.

Materials Table: It would be easier for the reader to have reagents, buffers, and equipment sectioned.

We have added the sections “Reagents”, “Materials”, “Instruments” and “Plasmids” to the material list.

Figure 1 & 3 Please add "Break Points". These should also be highlighted in the video.
We have added “Break Points to Figure 1 and 3 and have changed the respective Figure captions.

Figure 5 Move all text off of the images. For example, almost all chambers are covered by text in Figure 5B.
All text has been moved off of the images, as requested.

Reviewer #4:
Manuscript Summary:
Ramm and colleagues describe a protocol for reconstituting a lipid bilayer on a flat or patterned supports, and the imaging of labelled recombinant proteins upon this bilayer. The

system they reconstitute is an oscillatory self organising system from bacteria (minCDE) that oscillates between the poles of their patterned reaction chambers, however the general principles of generating the bilayer will be applicable to many experimentalists, and the video aspect will help many users with this increasingly utilised technique. The patterning of the PDMS support into miniature 'cupcake trays' and, after draining the bulk solution, using these as shaped individual reaction chambers is particularly novel and I can imagine will be utilised by many in other systems. The manuscript text is well written, with good rational for the use of reconstituted systems and sensible discussion. I am highly supportive of publication and have only a few minor concerns.

We thank the reviewer for recognizing the relevance of the protocol and his general support of publication.

Minor Concerns:
L231: I don't think MIN buffer is defined anywhere
It is now defined at the first mention of Min buffer in lines 232-233.
L231: Can you define the time and temperature to perform the vortexing at?
Now giving the temperature (RT) and a guideline for the time (visually assessing the homogeneity of the contents).
L242: Can you give a temperature of the 'hot' water to help readers more easily replicate
The temperature has been added.
L244-6: This sounds like a particularly hazardous step - should there be a discussion/note of PPE and hazards?
We added a note advising readers to wear appropriate PPE.
L263: Can you give setting for the sonication to help readers?
A bath sonicator is used which can usually only be turned on or off. We added the volume and total sonication power of our device as a guide. However, the power is very inhomogeneously distributed over the bath volume and has to be adjusted by the experimenter by adjusting the location and height of the glass vial or plastic tube in the sonicator bath.
L268: I'm not sure if SLB was defined anywhere.
Thank you for pointing this out, SLB was indeed only defined further down in the protocol. This has now been fixed.
L310: Do you mean coverslips here, not glass slides?
Yes, thank you, has been fixed.
L340-342 (and other places): Is there any alternative to plasma cleaning? This seems a piece of equipment that fewer people would have access to and it may be useful to describe exactly why this is used and whether anything can substitute.
This is the reason why we included piranha cleaning in our methods. The two methods yield very similar results. Unfortunately, we do not have sufficient experience with less hazardous replacement methods for plasma cleaning.
L348: Perhaps you could explain what is happening in the 3 minutes lipid incubation - I presume a SLB is forming.
A short description was added for the readers. The vesicles burst on the hydrophilic surface of the glass and many small patches fuse to form a homogeneous bilayer.

L353-5: Do you need to remove the lipid mix before adding the Min/SLB buffer - the protocol just says to add the buffers to the chamber?
The lipid mix is heavily diluted during the washing steps. Removing the entire solution on top of an SLB risks momentary drying of the SLB, which would drastically alter the membrane properties. We therefore avoid ever taking off more solution than necessary. We have added a note to point 5.6.1. After performing the washes as described, there are only negligible

amounts of vesicles left in solution. Removing the lipid mix is therefore a step-wise process and combines well with the necessary washing.
In case of using *E. coli* polar lipids, or when SLB buffer (rather than Min buffer) is used during washing for other reasons, exchange to Min buffer has to be done very carefully, without removing the entire solution.

Discussion: I think some mention should be made that although these reaction compartments are bacteria-shaped (well, bacillus shaped), they are an order of magnitude larger than bacteria.

We thank the reviewer for pointing this out. We now mention the longer wavelength and hence the larger size of the microcompartments in the introduction and in the discussion. The wording has been changed to rod-shaped, as requested by another reviewer.

Rod shaped microstructures CAD file

Click here to access/download

Supplemental Coding Files

JoVe_wafer_rod-like-structures_3x3.dwg

Plasmid MinD

Plasmid eGFP-MinD

Plasmid mRuby3-MinD

Click here to access/download

Supplemental Coding Files

03 pET28a-mRuby3-MinD.gb

Plasmid MinE

Plasmid MinC