**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 Mg2+-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.