**Title:** Protein patterns and oscillations on lipid monolayers and in micro-droplets

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# Protein patterns and oscillations on lipid monolayers and in micro-droplets

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**Abstract:** The Min proteins from *E.coli* position the bacterial cell division machinery through pole-to-pole oscillations. *In vitro*, Min protein self-organization can be reconstituted in the presence of a lipid membrane as catalytic surface. However, Min dynamics have so far not been reconstituted in fully membrane-enclosed volumes. Here, we employed micro-droplets, interfaced by lipid monolayers, as the simplest 3D mimicry of cellular compartments to reconstitute Min protein oscillations. We demonstrate that lipid monolayers are sufficient to unfold the catalytic role of the membrane and thus represent a facile platform to investigate Min protein regulated dynamics of the cell division protein FtsZ-mts. In particular, we show that droplet containers reveal distinct Min oscillation modes, and display a dependence of FtsZ-mts structures on compartment size. Finally, co-reconstitution of Min proteins and FtsZ-mts in droplets yields antagonistic localization, demonstrating that droplets indeed support the analysis of complex bacterial self-organization in confined volumes.

Self-organization of proteins into large-scale structures and patterns is fundamental to all living systems, in order to regulate complex cellular processes, such as protein localization and cell division. A striking example for protein self-organization is the Min protein system (comprising the proteins MinC, MinD and MinE) of the bacterium *Escherichia coli*,[1] that oscillates between the cell poles. These pole-to-pole oscillations generate a time-averaged non-homogeneous concentration gradient with a minimum at mid-cell, the future cell division site.[2-7] Intriguingly, only a minimal set of components – the two membrane interacting proteins MinD and MinE, a lipid membrane and ATP – has been shown to establish the Min oscillations[8-12]. The protein MinC follows the oscillating MinDE patterns by binding to MinD, and directly inhibits the assembly of the cell division protein FtsZ at the poles [3, 7, 13-15]. Thereby, the main division initiator FtsZ, which assembles into a ring-like structure (Z ring) is directed to the cell middle.[4]

To study the Min oscillations under well-defined conditions, cell-free systems are being developed that provide a high level of control over physicochemical parameters. Previously, we have reconstituted the pole-to-pole oscillations of the Min proteins, as well as their ability to spatially direct FtsZ-mts (FtsZ fused to a membrane targeting sequence)[16] to the middle of a micro-fabricated compartment[12, 17]. However, although the cell membrane of *E. coli* is a closed compartment, the Min oscillations have thus far only been successfully reconstituted in volumes that were not fully enclosed by membranes, but open at least at one site[12, 18-19]. Moreover, although membrane attached FtsZ rings have been observed in vesicles[16, 20-21] and FtsZ bundles have been characterized in the lumen of droplets[22], we are just beginning to understand how systems parameters, such as compartment geometry, influence FtsZ ring structure and dynamics.

Herein, we employed lipid monolayer-surrounded micro-droplets as simplest 3D mimicry of cellular compartments to reconstitute Min protein oscillations and the formation of FtsZ-mts rings. In comparison with previous reconstitutions of the Min proteins on membranes, droplets are distinct in two respects: First, they are clad with monolayers instead of bilayers, and second, they are fully surrounded by lipids, as opposed to previously published open sample geometries. Therefore, we first demonstrated that lipid monolayers with an open geometry[23-24] are sufficient as substrates for Min protein pattern formation and Min protein regulated formation of FtsZ-mts networks. We also describe a striking enlargement of FtsZ-mts filament structures on monolayer surfaces. Second, we observed that distinct membrane-catalyzed Min protein patterns can be reconstituted in 3D droplet confinements. Moreover, Min protein oscillations are accompanied by concentration oscillations in the droplet lumen, suggesting a dilution-based inactivation of Min proteins in the middle of a compartment. Finally, we show that the formation of strongly branched FtsZ-mts networks vs. less branched structures, resembling the physiologically relevant FtsZ-mts rings, is regulated by compartment size. Moreover, the localization of FtsZ-mts networks within droplets can be controlled by co-reconstituted Min protein oscillations.

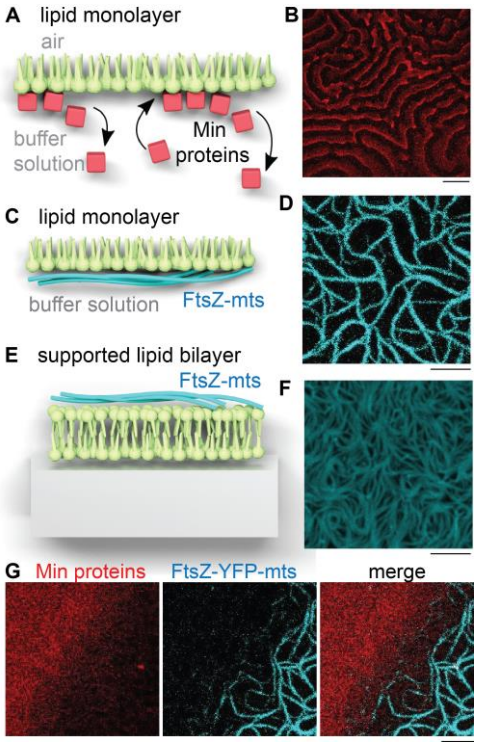


**Figure 1.** Reconstitution of Min protein patterns and bundles of FtsZ-mts on lipid monolayers. A,C,E) Schematic of experimental setup. A, B) A lipid monolayer is assembled at the buffer/air interface. Min proteins (red) in the buffer volume self-organize into spatial patterns on these lipid monolayers. Scale bar 100µm C,D) Reconstituted FtsZ-mts network at lipid monolayers. E,F) Reconstituted FtsZ-mts network on supported lipid membrane. G) When Min proteins (red) and FtsZ-mts (blue) are co-reconstituted, FtsZ bundles localize at the minima of the Min waves. D,F,G) Scale bar: 5µm

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Reversible binding of the Min proteins to the cellular membrane is required for the formation of dynamic protein patterns. This binding being only peripheral through an amphipathic helix, we speculated that a single layer of lipids, as opposed to bilayers of cell membranes, is sufficient as a substrate for Min pattern formation. Such lipid monolayers can be simply assembled at droplet boundaries.

To demonstrate that monolayers indeed support Min protein pattern formation, we generated a flat lipid monolayer at an air-buffer interface[24]. When purified fluorescently tagged Min proteins and ATP were added to the buffer reservoir, the Min proteins spontaneously self-organized into wave-like patterns similar to the patterns on lipid bilayers[11] (Figure 1A, B).

We then analyzed whether Min protein regulated inhibition of membrane targeting FtsZ (FtsZ-mts[16]), which has previously been reconstituted on lipid bilayers[25], can also be achieved on monolayers. Remarkably, the self-assembled networks of FtsZ-mts bundles on lipid monolayers have significantly larger mesh sizes than on lipid bilayers (Figure 1C-F, Supplementary figure 1). Individual FtsZ bundles of several µm lengths were clearly distinguishable, compared to an order of magnitude shorter bundles on supported lipid bilayers. (Figure 1C-F). One reason for this observation is likely the higher mobility of lipids in freestanding membranes as compared to supported lipid membranes.[26-27]

By co-reconstituting Min proteins and FtsZ-mts on monolayers, we verified that Min dynamics are still able to locally inhibit assembly of FtsZ-mts. Specifically, we observed that FtsZ-mts networks localized complementary to the Min concentration maxima. (Figure 1G). Thereby, the monolayer assay provides an intriguing opportunity to analyze FtsZ networks, as well as their dynamic regulation by interaction partners, with higher spatial precision in diffraction-limited microscopes, due to the larger bundle lengths (figure 1G, supplementary figure 2). In addition, the demonstration that lipid monolayers indeed provide a catalytic surface for the spontaneous formation of Min protein patterns and regulated FtsZ dynamics is a key requirement for their functional transfer into water-in-oil droplets and extends the toolkit for Min protein reconstitution *in vitro*.

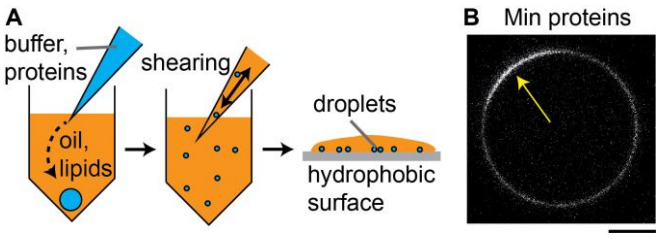


**Figure 2.** Reconstituted Min proteins self-organize into patterns at droplet boundaries. A) Schematic setup. A droplet of buffer and proteins is pipetted into a reservoir of oil and lipids. The droplet is broken into numerous droplets of picoliter volumes by manually pipetting up and down. The droplets are pipetted on top of a hydrophobic PDMS surface for imaging. B) Confocal image of Min protein pattern within a droplet. Scale bar: 20 µm.

Having confirmed that lipid monolayers are sufficient for Min protein self-organization, we next addressed whether Min oscillations can be reconstituted within lipid interfaced droplets. Therefor, a protein master mix (containing Min proteins, ATP and buffer) was pipetted into an oil/lipid mixture and then emulsified by pipetting the oil/buffer suspension up and down (Figure 2A). Finally, the droplets were imaged on a hydrophobic surface that prevented the droplets from rupturing. Confocal images of the droplets revealed a non-homogeneous distribution of Min proteins at the droplet boundary (Figure 2B). These results indicate that droplets are a promising 3D mimicry of cellular compartments for the reconstitution of Min protein self-organization.

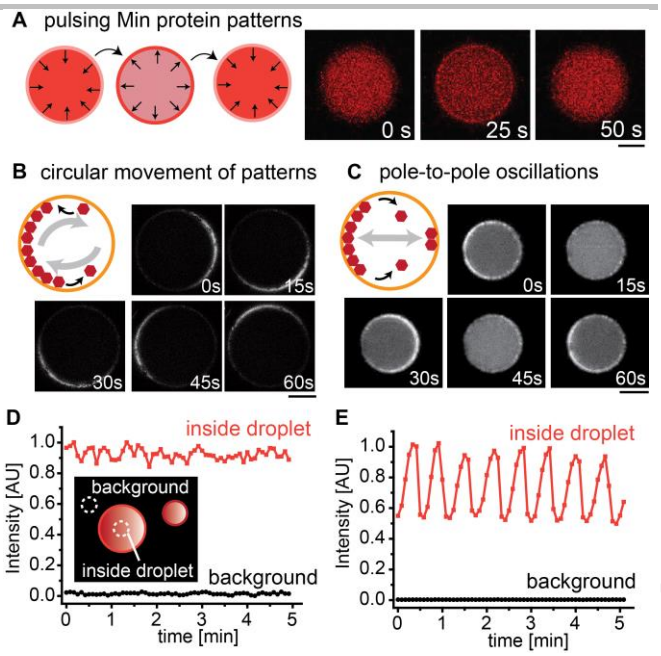


**Figure 3.** Min concentration oscillations in the compartment lumen of are a key characteristic for pole-to-pole oscillations. A) "Pulsing" pattern in spherical compartments at the beginning of pattern formation. Schematic graph (left) and confocal images (right) of Min proteins (green) shortly after their encapsulation within droplets. Scale bar: 10 µm. B,C) Circular moving Min waves and back-and-forth oscillations are regularly observed. 1µM MinD (supplemented with 10% eGFP-MinD), 1µM MinE. Scale bar: 10 µm. D,E) Fluorescence intensity of Min proteins in the lumen of droplets with circular moving (D) and oscillating Min patterns (E) over time.

To further analyze the dynamics of these Min protein patterns within droplets, we acquired time-lapse images. Initially, the Min proteins displayed a pulsing pattern characterized by an oscillation between a homogeneous localization of the proteins at the droplet interface and their simultaneous release into the droplet lumen (Figure 3A, supplementary figure 3). This pattern occurred shortly after encapsulation of the Min proteins in a large fraction of droplets. Notably, pulsing Min protein patterns have not been observed when Min proteins were encapsulated in micro-wells subsequently to the initiation of surface waves [12](Supplementary figure 4). Thus, the appearance of this pulsing pattern is likely due to boundary conditions in the initial phase of the experiment, such as the spatial symmetry of the droplets and the initially homogeneous protein distribution. Importantly, these pulsing Min protein patterns provide the first experimental evidence that the oscillating protein concentrations can be decoupled from spatial patterns on the membrane.

After an initial period of pulsing oscillations, droplets with a diameter of tens of micrometers assume one out of two predominant patterns: circular moving waves, or pole-to-pole oscillations of Min proteins (Figure 3B,C, supplementary figure 4).

The two modes of Min dynamics in small droplets, as well as their time scales on the order of one minute, are consistent with what has previously been observed in spherical *E. coli* cells,[28] as well as disc-shaped microcompartments[12], pointing to the physiological significance of the occurring patterns in droplets.

Interestingly, the two populations of small droplets differ not only by their surface dynamics, but also by the dynamics of lumenal Min protein concentrations. In droplets with circular moving patterns on their surface, no significant variations in lumenal concentrations were observed (Figure 3D). The traveling wave pattern along the surface does apparently not modulate the average protein concentrations at the membrane, nor, due to mass conservation, the membrane/bulk ratio.

In contrast, substantial oscillations of lumenal protein concentrations were observed in droplets with pole-to-pole

oscillations (Figure 3E). These lumenal oscillations were phase-shifted with respect to the assembling patterns at the two opposite membrane zones, in agreement with mass conservation and are comparable to temporal concentration oscillations of Min proteins, which have been observed in the buffer above flat supported membranes at specific protein concentrations [29]. In Figure 3C,E, the fluorescence signal of MinD drops to about 50% and regains its original value during every half oscillation cycle, demonstrating that half of the MinD molecules constantly shuttle between an active state at the membrane and an inactive state in the lumen. This suggests a dilution-based mechanism for net Min protein inactivation while crossing the middle of a compartment. In other words, between detachment from one pole and assembly at the other, Min proteins are essentially ineffective for FtsZ ring inhibition in the middle of the cell. Only in this dynamic mode, true time-averaged gradients may be formed. We speculate that the type of pattern in these spherical droplets might be determined by a stochastic process. In very large droplets of about 70 µm and larger, we even observed more complicated patterns with several polarization zones (Supplementary figure 5,6), indicating that the size of the droplets further determines the type of patterns.

Next, we adressed whether the droplet assay also supports the formation of FtsZ structures and specifically asked how compartment size influences Z ring assembly. In droplets much larger than bacterial cells, we observed branched networks of FtsZ bundles assembled at the monolayer interface (Figure 4A). These networks were highly dynamic (supplementary movie 1), displaying spatially fluctiating FtsZ-bundles at the droplet boundary. These dynamics were likely due to the higher mobility of lipids in frestanding monolayers, as opposed to supported lipid membranes.

We hypothesized that the assembled FtsZ-mts networks in droplets reflect on properties of cellular FtsZ ring structures. In particular, we reasoned that the reconstituted FtsZ networks should comprise a lower amount of FtsZ-mts bundles in smaller droplets, due to the decreased amount of entrapped FtsZ and the smaller surface area of the droplet. Due to the intrinsic stiffness and curvature of FtsZ-bundles,[30] this results in ring-like structures when the droplet size is sufficiently small. Indeed, we observed that networks of FtsZ-mts in smaller droplets are characterized by fewer and less branched-out FtsZ-bundles (Figure 4B,C, supplementary figure 8). These experiments suggest that network-like structures of FtsZ which are regularly seen on large membrane interfaces in cell-free systems condense into ring-like structures in confined geometries, such as lipid tubes[16] and *E.coli* cells.

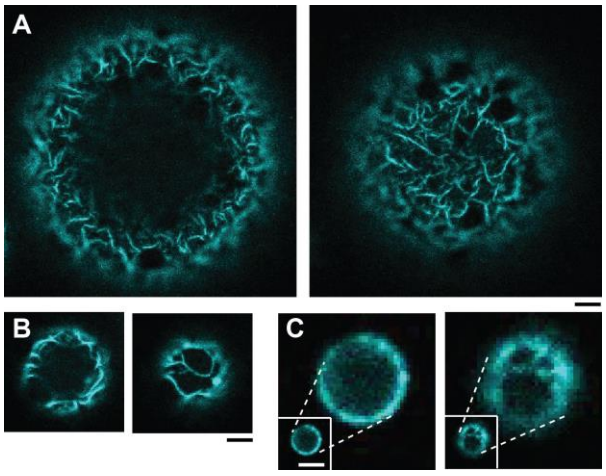


**Figure 4.** FtsZ-mts (blue) assembles into higher-order networks within droplets. (A) Confocal image of FtsZ-mts network at a midsection (left) and the bottom (right) of a large droplet with. (B,C) Network of FtsZ-mts in smaller droplets. (A, B, C) The two imaging planes at which the midsection and the bottom area of the droplets were imaged are comparable. Scale bar: 5 µm

To determine whether reconstituted FtsZ networks in droplets are also subject to regulation by key cellular interaction partners, we asked whether the antagonistic localization of FtsZ-mts and Min proteins,[25] could be reconstituted in droplets. Co-reconstitution of FtsZ-mts and the Min indeed resulted in droplets with FtsZ-mts localized at the concentration minima of Min protein patterns (Figure 5A). The antagonistic localization was dynamically maintained both, in space and time (Figure 5A,B) and demonstrates that monolayer interfaced micro droplets are valuable model compartments to study complex bacterial self-organization in cell-free environments.

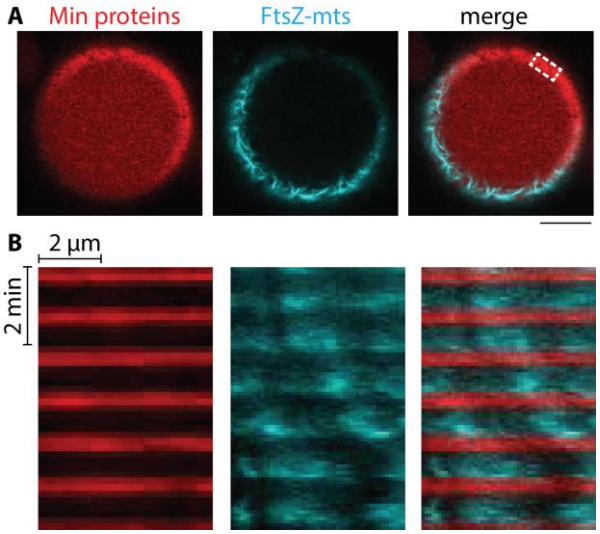


**Figure 5:** Reconstitution of anticorrelated protein localization in droplets. A) Co-reconstituted Min proteins (red) and FtsZ (blue) have been localize anticorrelated in droplets. Scale bar: 10 µm B) Kymographs of Min proteins and FtsZ at the membrane region indicated by the white box in figure (A) demonstrate temporal anticorrlation of Min proteins and FtsZ in droplets.

In summary, we successfully reconstituted Min protein patterns and FtsZ filament structures on flat lipid monolayers and within spherical droplets. The droplet assay provides the first *in vitro* reconstitution of Min oscillations in fully enclosed membrane compartments, while flat lipid monolayers, by enlarging FtsZ structures, provide an intriguing tool to study the regulation of FtsZ filament dynamics through interaction partners, such as the Min proteins.

Notably, we observed that Min pole-to-pole oscillations in closed compartments are accompanied by lumenal oscillations. These lumenal oscillations might have physiological significance in diluting the cytosolic Min proteins below critical concentrations for FtsZ inhibition while passing through the middle of the cell.

In addition, we demonstrated that large droplets support the formation of branched networks of FtsZ bundles, while structures resembling FtsZ rings were only observed in small droplets in the range of few µm, indicating that bacterial dimensions are highly significant for the assembly of a defined division ring.

Finally, we co-reconstituted the antagonistic localization of FtsZ-mts and the Min oscillator in droplets. The facile method of reconstituting Min proteins and membrane targeted FtsZ in 3D droplet environments not only allows to investigate the Min/FtsZ system in more detail, but shows promise as a future model system for analyzing more complex protein interactions of the bacterial cell division machinery.

## Experimental Section

Experimental details can be found in the supplemental material.

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**Keywords:** Biophysics • Droplets • Membrane Proteins • Protein Oscillations • Synthetic Biology

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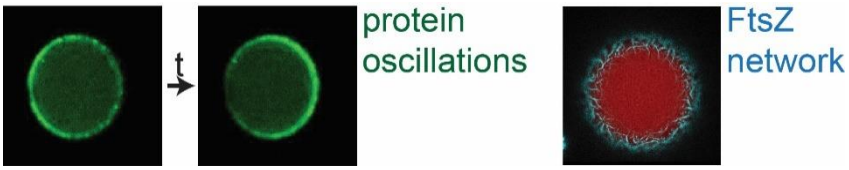


**Protein oscillations and the formation of FtsZ structures in droplets**: Spatial regulator proteins for bacterial cell division and the cell division protein FtsZ have been encapsulated in micro-droplets. Through interaction with a lipid membrane at the droplet boundary the proteins self-organize into distinct patterns

*Katja Zieske*, Grzegorz Chwastek, Petra Schwille**

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