Model Membranes

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Protein Patterns and Oscillations on Lipid Monolayers and in Microdroplets

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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 a catalytic surface. However, Min dynamics have so far not been reconstituted in fully membrane-enclosed volumes. Microdroplets interfaced by lipid monolayers were employed as a simple 3D mimic of cellular compartments to reconstitute Min protein oscillations. We demonstrate that lipid monolayers are sufficient to fulfil 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 reveal a dependence of FtsZ-mts structures on compartment size. Finally, co-reconstitution of Min proteins and FtsZ-mts in droplets yields antagonistic localization, thus demonstrating that droplets indeed support the analysis of complex bacterial self-organization in confined volumes.*

The self-organization of proteins into large-scale structures and patterns is fundamental to all living systems, and is required to regulate complex cellular processes such as protein localization and cell division. A striking example of protein self-organization is the Min protein system (comprising the proteins MinC, MinD, and MinE) of the bacterium *Escherichia coli*,[1] which oscillates between the cell poles. These pole-to-pole oscillations generate a time-averaged non-homogeneous concentration gradient with a minimum at the mid-cell plane, 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 Min oscillations.[8–12] The protein MinC follows the oscillating MinDE patterns by binding to MinD, and it directly inhibits the assembly of the cell-division protein FtsZ at the poles.[3,7,13–15] In this way, the main division initiator FtsZ, which assembles into a ring-like structure (Z ring), is directed to the middle of the cell.[4]

To study 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 microfabricated compartment.[12,17] However, although the cell membrane of *E. coli* is a closed compartment, Min oscillations have thus far only been successfully reconstituted in volumes 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.

In this work, we employed microdroplets as the simplest conceivable 3D mimic of cellular compartments to reconstitute Min protein oscillations and the formation of FtsZ-mts rings. In comparison to previous reconstitutions of the Min proteins on membranes, droplets are distinct in two respects: First, they are interfaced with monolayers instead of bilayers, and second, they are fully enclosed by the lipids, as opposed to previously published open sample geometries. Therefore, we first needed to demonstrate 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, thus 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 versus 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.

Reversible binding of the Min proteins to the cellular membrane is required for the formation of dynamic protein patterns. Since this binding is only peripheral through an amphipathic helix, we speculated that a single layer of lipids, as opposed to the bilayers of cell membranes, should be sufficient as a substrate for Min protein pattern formation.

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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 1 A,B).

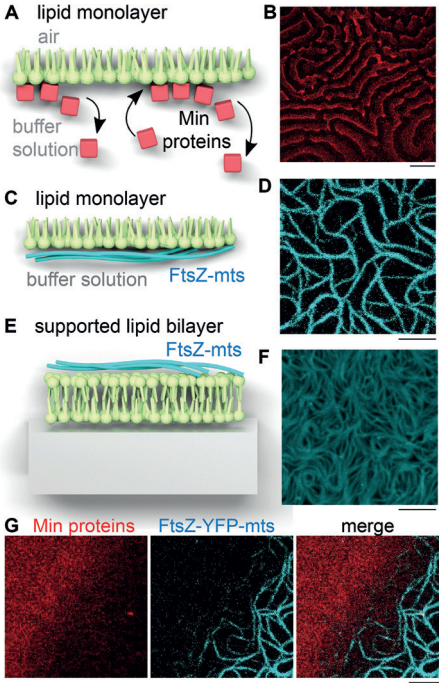

Figure 1. Reconstitution of Min protein patterns and bundles of FtsZ-mts on lipid monolayers. A, C, E) The experimental setup. A, B) A lipid monolayer is assembled at the buffer/air interface. Min proteins (red) in the buffer volume self-organize into spatial patterns on these lipid monolayers. C, D) The reconstituted FtsZ-mts network at a lipid monolayer. E, F) The reconstituted FtsZ-mts network on a 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. Scale bars: 100 μm in (B), 5 μm in (D, F, G).

We then analyzed whether Min protein regulated inhibition of the membrane targeting 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 1 C–F and Figure S1 in the Supporting Information). Individual FtsZ bundles several μm in length were clearly distinguishable, compared to bundles an order of magnitude shorter on supported lipid bilayers. (Figure 1 C–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 the assembly of FtsZ-mts. Specifically, we observed that FtsZ-mts networks localized in a pattern complementary to the Min concentration maxima. (Figure 1 G). The monolayer assay thus 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, owing to the larger bundle lengths (Figure 1 G and Figure S2). 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.

Having confirmed that lipid monolayers are sufficient for Min protein self-organization, we next investigated whether Min oscillations can be reconstituted within lipid-interfaced droplets. 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 2 A). Finally, the droplets were imaged on

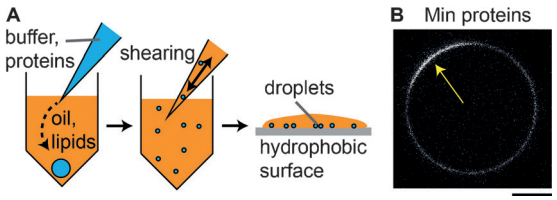

Figure 2. Reconstituted Min proteins self-organize into patterns at droplet boundaries. A) The experimental setup. A droplet of buffer and proteins is pipetted into a reservoir of oil and lipids. The droplet is broken into numerous droplets of picoliter volumes by manually pipetting up and down. The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

a hydrophobic surface that prevents the droplets from rupturing. Confocal images of the droplets revealed a non-homogeneous distribution of Min proteins at the droplet boundary (Figure 2 B). These results indicate that droplets are a promising 3D mimic of cellular compartments for the reconstitution of Min protein self-organization.

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 oscillation between homogeneous localization of the proteins at the droplet interface and their simultaneous release into the droplet lumen (Figure 3 A and Figure S3). This pattern occurred shortly after encapsulation of the Min proteins in a large fraction of the droplets. Notably, pulsing Min protein patterns have not been observed when Min proteins have been encapsulated in microwells[12](Figure S4). The appearance of this pulsing pattern is thus 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.

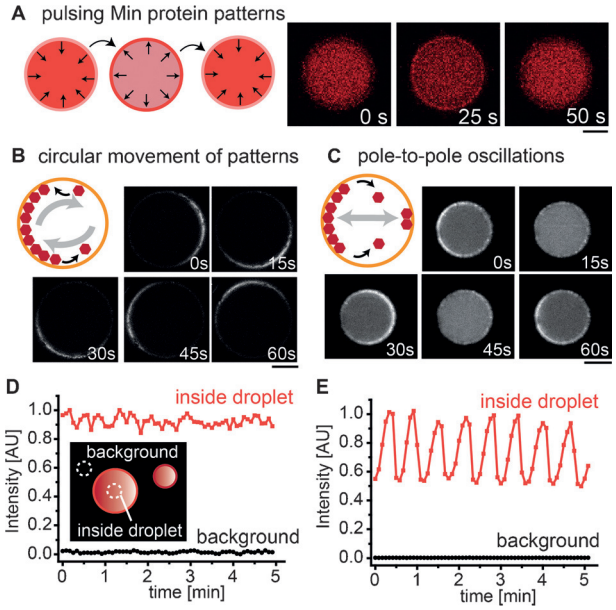

Figure 3. Min concentration oscillations in the compartment lumen of are a key characteristic for pole-to-pole oscillations. A) A "pulsing" pattern in spherical compartments at the beginning of pattern formation. A schematic representation (left) and confocal images (right) are shown of Min proteins (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. The experiment was performed with 1 μM MinD (supplemented with 10% eGFP-MinD), 1 μM MinE. Scale bar: 10 μm. D, E) Fluorescence intensity of Min proteins in the lumen of droplets with circularly moving (D) and oscillating (E) Min patterns over time.

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 and Figure S4).

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] thus pointing to a physiological significance for the occurring patterns in droplets.

Interestingly, the two populations of small droplets differ not only in their surface dynamics, but also in 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 apparently modulates neither the average protein concentrations at the membrane, nor, owing 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, which is in agreement with mass conservation, and they are comparable to the 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, thus 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 (Figures S5, S6), thus indicating that the size of the droplets further determines the type of patterns.

Next, we investigated 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

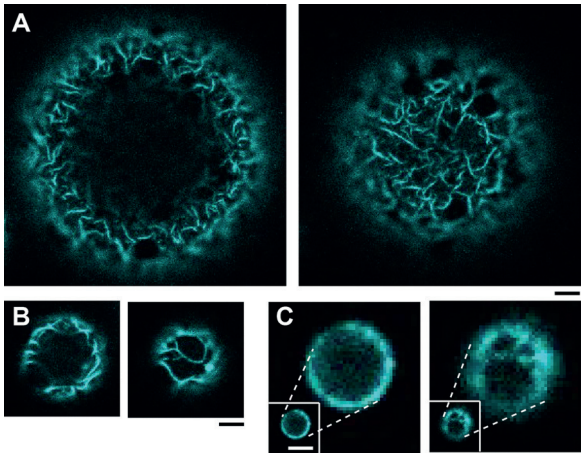

Figure 4. FtsZ-mts (blue) assembles into higher-order networks within droplets. A) A confocal image of a FtsZ-mts network at the midsection (left) and the bottom (right) of a large droplet. B,C) A network of FtsZ-mts in smaller droplets. In all of the panels, the two imaging planes at which the midsection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

dynamic (Movie S1 in the Supporting Information), displaying spatially fluctuating FtsZ bundles at the droplet boundary. These dynamics are likely due to the higher mobility of lipids in frestanding monolayers compared to supported lipid membranes.

We hypothesized that the assembled FtsZ-mts networks in droplets reflect the 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, owing to a lower amount of entrapped FtsZ and the smaller surface area of the droplet. As a result of

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 FtsZ-bundles (Figure 4B,C and Figure S8). These results 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.

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 the Min protein patterns (Figure 5A). The antagonistic localization was dynamically

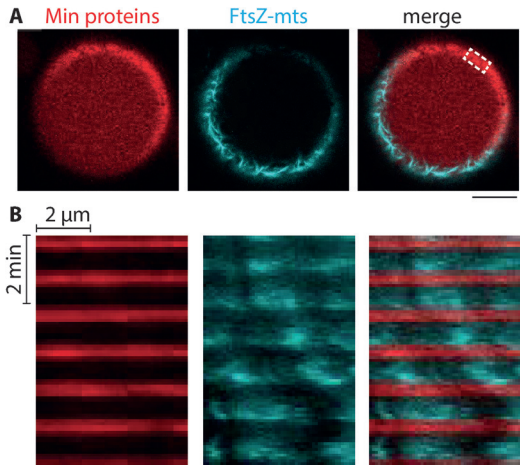

Figure 5. Reconstitution of anticorrelated protein localization in droplets. A) Co-reconstituted Min proteins (red) and FtsZ (blue) have localize in an anticorrelated manner in the droplets. Scale bar: 10 µm B) Kymographs of Min proteins and FtsZ at the membrane region indicated by the white box in (A)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

maintained in both space and time (Figure 5A,B) and demonstrates that monolayer-interfaced microdroplets are valuable model compartments to study complex protein self-organization in cell-free environments.

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 protein oscillations in fully enclosed membrane compartments, while flat lipid monolayers, by enlarging the FtsZ structures, provide an intriguing tool to study the regulation of FtsZ filament dynamics by 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, thus 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 investigation of 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.

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