A COMPREHENSIVE MODEL OF THE STRUCTURE AND FUNCTION OF THE FTSZ RING OF

ESCHERICHIA COLI

A dissertation submitted
to Kent State University in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

by

James C. Redfearn

May 2016

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Dissertation written by

James C. Redfearn

B.S., University of Tulsa, 2001

Ph.D., Kent State University, 2016

Approved by

Dr. Christopher J. Woolverton, Chair, Doctoral Dissertation Committee

Dr. Laura A. Leff, Members, Doctoral Dissertation Committee

Dr. Derek S. Damron

Dr. Tara C. Smith

Dr. Bansidhar Datta

Accepted by

Dr. Laura A. Leff, Chair, Department of Biological Sciences

Dr. James L. Blank, Dean, College of Arts and Sciences
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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Chris Woolverton, whose inspiration led me down this particular path of research. His guidance and advice have been invaluable already, and will continue to be in the years to come. Additionally, I would like to thank my advisory committee—Drs. Laura Leff, Derek Damron, Tara Smith, and Bansidhar Datta—for their suggestions and mentorship throughout this process. Finally, I would like to acknowledge Sigma Xi, the Kent State Graduate Student Senate, the Kent State Department of Biological Sciences, and the Kent State College of Public Health as material supporters of the research, without which the document would not be able to be produced.

I would be remiss if I did not also thank my many labmates and colleagues: Drs. Jim Ferrell, Michael Shilling, Steven Fiester, and Shannon Helfinstine. Your friendship, camaraderie, and presence as an intellectual sounding board made this work possible. Additionally, I would like to acknowledge and thank Carlie Rees, Neeley Meyers, Samantha Pecnik, Ashley Hendricks, Courtney Yanda, and Brian Wise, all of whom have taught me far more than I taught them.

Lastly, but most importantly, I thank my family for their support, encouragement and strength: my mother (and my hero) Vicki Gilpin and step-father Dwayne Gilpin and my father Roger Redfearn. I am not the man I am today without all of you. There are many others who deserve and will receive individual thanks, and for all of you, I am truly grateful to have had you in my life.

Dedicated to Roger, Carl, Cora, Donna, Harold, Patricia, and Grady.
CHAPTER I

1.0 GENERAL INTRODUCTION: FTSZ AND THE Z-RING

Reproduction in all prokaryotes (both the Archaea and the Bacteria) occurs asexually, by means of *binary fission*. Fission is subdivided into three physiologically distinct phases: growth, synthesis, and septation. In the growth phase prokaryotic cells enlarge in size, increasing their mass by acquiring nutrients and building new or larger cellular structures. Individual cells continue to grow until a trigger condition is met—in *Escherichia coli*, when the ratio of cell mass to the number of DNA replication origins is above a critical value—which initiates the synthesis phase (1). In this next phase the genome is copied (DNA synthesis) and the resulting copies are moved to the opposite ends of the cell. Once this *segregation* of the chromosomes is complete, septation begins. Here the cell splits in half permanently, due to pinching of the cellular membrane (*cytokinesis*) and the synthesis of a cell wall in the space between. These two aspects of septation are performed by a massive, multi-protein “machine” called the *divisome* (2). The protein components of the divisome coordinate in space and time the pinching (*constriction*) of the cell membranes (driven by internal elements of the cell) with the biosynthesis of new external cell structures, such as the cell wall and the outer membrane of Gram-negative bacteria. When cytokinesis is complete, the cell membrane fuses and the newly-formed daughter cells separate following partial breakdown of the septal cell wall.

The most important element of the divisome is the protein FtsZ. Named for the phenotype of the first isolated mutant—filamenting temperature-sensitive mutant Z—FtsZ is the principal component of a
Figure 1. Confocal fluorescence micrographs of normal and filamentous cells of *E. coli*. (A) Wild-type (WT) cells in an early stage of cytokinesis and (B) WT cells in late-stage cytokinesis. Cells shown in both A and B are stained with DAPI (DNA stain, blue, top) and are expressing an FtsZ-GFP (green fluorescent protein) fusion (second from top) with composite images showing both (bottom two). (C) Filamentous cell showing diffuse distribution of FtsZ between the nucleoids, indicating that the divisomes are not formed. Image reproduced with permission from Ma et al. (3)
cytoplasmic protein ring, located at the middle of the dividing cell (Figure 1A and B), which is necessary for divisome formation (4). Temperature-sensitive mutants of FtsZ in *Escherichia coli* (which grow and divide normally at 37 °C) do not form a divisome at non-permissive temperatures (≥42 °C), causing the cells to fail to undergo septation. This failure results in the formation of long filaments, shown in Figure 1C. As growth and synthesis continue unabated, filamentous cells grow so large that they become structurally unsound. Eventually, the cell membrane bursts and the cell dies. That the absence of functional FtsZ is fatal means it is an *essential* protein in *E. coli* (5). All of the Fts family of proteins are essential (6); however, it is the ring of FtsZ underlying the cell membrane (the *Z-ring*) that localizes the divisome to the middle of the cell (*mid-cell*), provides the backbone upon which the complete divisome is built and, ultimately, generates the force that constricts the cell membrane (7–10).

The reach and importance of FtsZ and FtsZ-like proteins is not limited to *E. coli*. Nearly all prokaryotes possess at least one gene encoding an FtsZ-like protein, with a few bacterial species and many Archaebacteria having two or more (11). Those that lack FtsZ typically have unusual features; for example, the absence of a cell wall (L-form bacteria, *Ureaplasma* and some *Mycoplasma*) or—as in the case of the pathogenic *Chlamydiaceae*—a life cycle that includes intracellular symbiosis (12). As life evolved, the ancient endosymbionts brought FtsZ to eukaryotic organisms. The chloroplast division machinery (from green algal endosymbionts) includes a pair of FtsZ proteins and mitochondrial FtsZ, though mostly replaced by dynamins, are extant in a few types of protists, including *Amoeba* (13, 14).

In all of the organisms in which it resides, FtsZ retains its basic function as a ring of filaments, underlying the membrane, located at the middle of the cell or organelle that it assists in dividing. In addition, this ring (the *Z-ring*) is the foundation of a complex web of genetic and protein-protein interactions that direct the timing and progression of septation. The particular details of Z-ring regulation and cellular physiology are as diverse as the organisms that rely on its function. This work will focus on
the FtsZ of Bacteria—in particular, the FtsZ of *E. coli*—drawing comparisons and contrasts with other species where appropriate.

1.1 FTSZ PROTEIN STRUCTURE AND FUNCTION

FtsZ is one member of a diverse family of bacterial cytoskeletal elements, several of which exhibit distinct homologies to eukaryotic cytoskeletal proteins. Striking similarities can be found in the sequence, structure, and function of FtsZ and the microtubule protein tubulin. Like tubulin, at the core of the FtsZ protein are two globular domains separated by a central $\alpha$-helix (Figure 2), with each containing a portion of a conserved guanosine 5$'$-triphosphate hydrolysis (GTPase) domain (15, 16). The N-terminal, GTP-binding domain features a Rossmann fold motif, which is characteristic among the members of the nucleotide binding protein superfamily (16). Unique to bacterial FtsZ is a C-terminal alpha helix (not shown), connected to the globular domain by a flexible, unstructured linker, both of which are essential for proper function of FtsZ *in vivo* (17, 18).

While much of the similarity between FtsZ and tubulin is structural, FtsZ and the $\alpha$-, $\beta$- and $\gamma$-tubulins share a highly conserved, glycine-rich GTP-binding sequence (15, 19) as well as the GxxNx$D$ signature sequence in the T7 or “synergy” loop (20). More extensive sequence alignments have identified several other regions of identity and conservative replacement, which serve as the foundation of the current view that FtsZ is an ancestral form of the tubulin family of proteins (21, 22). Among prokaryotes, the sequence identity of different FtsZ proteins is variable but generally high; FtsZ of *E. coli* is (on average) 70% identical to FtsZ in other Gram-negative bacteria, 50% identical to those found in Gram-positive bacteria and even 40% identical to FtsZ proteins of the otherwise distantly-related Archaebacteria (22). Though the peculiarities of individual species will result in some functional
Figure 1.2. Ribbon drawings of FtsZ from *Bacillus subtilis*. Globular domains shown in blue (N-terminal) and green (C-terminal) with the core helix in yellow (H7). (A) “Side” view featuring nucleotide binding site with wireframe model of guanosine 5′-triphosphate. (B) Cartoon model of a protofilament using the color scheme from A, where GTP is represented as a small orange rectangle and the T7 loop as a small extension from the bottom of the C-terminal domain. Top and bottom surfaces hold one half of the GTPase domain; association of two monomers top-to-bottom allows for hydrolysis of GTP. Reprinted with permission from Adams and Errington (23).
differences it is reasonable to assume, given the high level of sequence identity among bacterial FtsZs, that insights into the fundamental nature of FtsZ in one species can be applicable to others.

Both *in vivo* and *in vitro*, FtsZ associates with itself non-covalently in a head-to-tail (or “top” to “bottom”) fashion to form polymers known as *protofilaments* (Figures 2b and 3a), initiation of which is dependent upon binding of GTP and magnesium to the nucleotide binding site on the top surface of the molecule (21, 24). The top and bottom surfaces of FtsZ have significant shape complementarity—approximately 10% of the total protein surface is buried by FtsZ-FtsZ binding—and in the polymerized state these extensive surface contacts between two individual monomers are further stabilized by hydrophobic interactions between the bottom surface of one with a ring of non-polar amino acids that surrounds the nucleotide binding site of the other (25). FtsZ protofilaments are analogous to the structures of the same name formed by polymerization of αβ-tubulin heterodimers; however, in buffer conditions that resemble the internal conditions of a wild-type cell, *Escherichia coli* FtsZ protofilaments remain separate as single-subunit-wide filaments (Fig. 3b) rather than forming the multi-stranded, hollow tubules seen with polymerization of tubulin (21, 26). While tubulin and FtsZ share considerable structural homology in their top and bottom surfaces, the differences seen in their lateral surfaces are large and likely account, in whole or in large part, for this functional difference between the two (25).

In the dividing cell, protofilaments of FtsZ coalesce at the middle of the cell to form a ring (the Z-ring) that encircles the cell, which is the first step in a multi-stage cascade of protein recruitment that builds the complete divisome (27–29). The Z-ring is highly dynamic, rapidly turning over even at steady state as well as shrinking in diameter and number of subunits as cytokinesis progresses. The enzymatic breakdown of GTP into guanosine 5′-diphosphate (GDP), along with the aforementioned polymerization of FtsZ monomers into protofilaments, are the two most basic functions of FtsZ and are the primary drivers of the dynamic behavior of the Z-ring and by extension, the divisome.
Figure 1.3. FtsZ protofilaments. (Left) A structural model of an FtsZ protofilament with globular domains shown in blue (N-terminal) and cyan (C-terminal). A space-fill model of GTP (orange) and amino acid D212 (red) are also shown. Contact between the top and bottom faces of protofilaments is necessary for efficient polymerization, and D212 is found in the T7 or “synergy” loop on the bottom surface of the FtsZ molecule. C-terminal flexible linker and binding domain (extended sheet/helix structure) are shown in purple. (Right) Electron micrograph shows FtsZ protofilaments and individual monomers on a carbon film. Scale bar is 100 nm. Both reprinted with permission from Erickson, et al. (30)
1.2 STRUCTURE AND ENZYMEOLOGY OF THE FTSZ GTPASE

Among the first insights into FtsZ function was the discovery of an Mg$^{2+}$-dependent GTPase activity in cytoplasmic extracts and purified solutions of FtsZ (15, 19, 31). Binding affinities for both GTP and GDP were measured in the micromolar range ($K_d$ values of 40 µmol/L and 80 µmol/L respectively) where the affinity of FtsZ for GTP was roughly double that for GDP (19). Given the average cytoplasmic concentration of these nucleotides (5 mmol/L for GTP and 0.7 mmol/L for GDP) nucleotide exchange occurs rapidly, such that the majority of free, cytoplasmic FtsZ will be bound to GTP (32). Both binding and hydrolysis of GTP were found to be significantly impaired in the classical temperature-sensitive FtsZ mutant (FtsZ84), the phenotypes of which are shown in Figure 1 (15, 31). That binding and hydrolysis of GTP was reduced in the Z84 mutant showed that the mutation it harbors destabilizes the enzymatic site even at permissive temperatures; at non-permissive temperatures this destabilization could be amplified significantly, causing the protein to become non-functional. Taken together, these findings suggested that the enzymatic functions of the FtsZ protein were critical for proper localization of FtsZ to the division septum, and possibly for the progression of septation as well.

A second critical insight in de Boer et al. (15) was that the rate of hydrolysis of GTP was dependent upon FtsZ concentration, implying that an FtsZ self-interaction was necessary for enzymatic activity. This was confirmed shortly after in FtsZ purified from Bacillus subtilis; however, a small procedural difference led to an even more valuable discovery—the first evidence of cooperative self-interaction in FtsZ. Wang and Lutkenhaus (33) used significantly lower concentrations of FtsZ to perform their GTPase assays (20-180 µg/mL) than de Boer et al. (250-2000 µg/mL) which allowed them to observe that FtsZ had little to no GTPase activity until the protein concentration used in the assay rose above 60 µg/mL (1.5 µmol/L). Above this concentration, maximal activity is rapidly achieved, where the plot of relative specific GTPase activity is a sigmoid curve centered at a half-maximal activity at 110
µg/mL (2.75 µmol/L). Such a plot of enzyme activity is characteristic of cooperative binding and has been well-described in other cytoskeletal proteins—specifically, actin and tubulin (34, 35). The self-interaction implied by the concentration-dependence of FtsZ’s GTPase activity was later identified as the end-to-end polymerization of FtsZ monomers into linear protofilaments (Fig. 3) by multiple research groups (21, 24, 36–38).

Self-interaction of FtsZ is necessary (in structural terms) because each molecule contains one half of the GTPase domain near its N-terminus and the other half near its C-terminus, oriented in such a way that the N-terminal domain of one monomer must interact with the C-terminal domain of another to form the functional GTPase (Figs. 2A and 3A). The larger, N-terminal domain is the nucleotide binding domain (Fig. 4) and consists of a set of six alpha helices (H1-H6) and six beta sheets (S1-S6) alternating in the βαβ arrangement characteristic of Rossmann fold structural motifs. This arranges the loops (T1-T6) between each segment of secondary structure to form a glycine-rich GTP binding pocket; of particular note is the T4 loop, which contains the GGGTG(S/T)G sequence that is conserved across all known FtsZ and tubulin sequences (25). Hydrogen bonding between the glycine residues (and other polar amino acids) of these six loops with nitrogen and oxygen atoms in the nucleotide, as well as hydrogen bonding and hydrophobic interactions (Phe208, Fig. 4) between the guanine base and the H7 “core” helix contribute to the coordination and binding of the nucleotide (16, 25, 39). Both the sequence and structural aspects of the nucleotide binding region are conserved across a wide variety of known FtsZ structures including those of the methanogen Methanococcus jannaschii (16), the thermophile Aquifex aeolicus (40), as well as a variety of mesophilic Bacteria: Pseudomonas aeruginosa (41), Mycobacterium tuberculosis (42), Bacillus subtilis (40, 43), and Staphylococcus aureus (44).

Magnesium, which is required for both GTP hydrolysis and polymerization, also binds within the active site, where it is coordinated by the beta and gamma phosphates of the GTP molecule, a glutamine
Figure 1.4. The nucleotide binding site of *Bacillus subtilis* FtsZ. This ribbon model of the nucleotide binding site shows “ribbons” of secondary structure are in gray, with amino acids critical for binding shown in skeleton format. The bound nucleotide in this case is GDP and is shown in ball-and-stick format. Atoms in skeleton and ball-and-stick models are color coded: carbon is shown in green, oxygen in red, nitrogen in blue, and phosphorus in purple. Hydrogen bonds are shown as black dashed lines. Reprinted with permission from Raymond, *et al.* (43).
residue located in the T3 loop, and several water molecules (45). This placement allows the magnesium ion to polarize the beta and gamma phosphates, priming the nucleotide for hydrolysis. The final piece of the complete active site is provided by dimerization of individual subunits, which inserts the T7 loop of the C-terminal domain. Within this T7 loop are two aspartate residues (Asp235 and Asp238 in Fig. 4) that are necessary for polarizing a water molecule (Wat105, Fig. 4) that will perform a nucleophilic attack on the gamma phosphate, resulting in hydrolysis (45). Given these structural aspects of magnesium binding, it is expected that nucleotide exchange precedes binding of magnesium to the active site.

Because of these structural relationships, assembly of FtsZ polymers is intimately tied to its enzymatic activity and by extension, the state of the bound nucleotide, whether GTP, GDP-P_i, or GDP alone. In tubulin, this relationship is well-understood: the subunits add to the polymer bound to GTP. Hydrolysis and release of inorganic phosphate occur shortly after, creating a polymer that is primarily GDP-bound with a “cap” of subunits that are GTP-bound. In tubulin’s cousin FtsZ, these relationships are more complex and remain murky. These will be explored in more detail when examining the dynamic behavior of FtsZ polymers in the equilibrium (or, “steady”) state.

1.3 KINETICS OF FTSZ ASSEMBLY

A full accounting of the kinetics of FtsZ polymerization requires an assay that can sensitively and rapidly report the average size of polymers in solutions of purified protein. Traditional techniques that measure an increase in polymer mass—analytical ultracentrifugation or dynamic light scattering (DLS), for example—are of limited usefulness because FtsZ polymer mass reaches a maximum in the period between addition of GTP and the first measurement, which is 10-15 s in the case of DLS (46, 47). Analyses of kinetics using DLS are further complicated by the fact that any aggregation of protofilaments into higher-order structures causes an increase in signal even though the mass of individual protofilaments
Figure 1.5. The complete GTPase active site of *Methanococcus jannaschii* FtsZ. This stereoscopic image shows the complete active site, with the N-terminal domain of the lower subunit shown in blue-gray, the C-terminal domain of the upper subunit in red, and GTP in a green ball-and-stick model. Magnesium is shown as a gray sphere and water molecules as dark blue spheres, with the exception of Wat105, in yellow. This water molecule is polarized by the asparagine residues of the T7 loop (Asp235 and Asp 238) and performs a nucleophilic attack on the gamma phosphate of GTP, resulting in hydrolysis. Reproduced with permission from (45)
does not change. Despite limitations in assessing the kinetics of polymerization, both techniques are still quite valuable for understanding the behavior of FtsZ at steady state.

These limitations were overcome in a pair of complementary studies. Drawing inspiration from the pyrene-labeling technique for assaying actin assembly, Chen et al. (48) created a mutant FtsZ (FtsZ L68W or simply L68W) with intrinsic tryptophan-derived fluorescence that increases 2.5-fold when FtsZ is in the polymerized state. In the other, a different mutant (F268C) allowed for the conjugation of fluorescent dyes fluorescein and tetramethylrhodamine—a Förster resonance energy transfer (FRET) pair—to the lateral surface of the protein (49). Combining these two approaches with stopped-flow fluorescence spectroscopy allowed the researchers to acquire a complete picture of FtsZ assembly kinetics: an approximately 1-second lag phase, followed by an elongation phase characterized by a spike in fluorescence signal (or loss of donor fluorescence, in the case of FRET) the duration of which is dependent upon total FtsZ concentration. Elongation continues for a short time (5-60 sec), then transitions into an equilibrium or “steady” state that persists for several minutes. The duration of the steady state is determined by the amount of GTP available and the rate of nucleotide hydrolysis. Once GTP is exhausted, polymers disassemble, returning fluorescence to baseline levels. This disassembly phase is coincident with the expected time of GTP exhaustion using known rates of GTP hydrolysis.

The best-fit kinetic model for these data is one in which protofilaments are cooperatively assembled following nucleation by a weakly-associating homodimer (48, 49). In cooperative assembly—in particular, of cytoskeletal filaments like actin, tubulin, and FtsZ—assembly of filaments does not vary simply with concentration. As described in the seminal theoretical work of Oosawa and Kasai (50), polymers that assemble cooperatively (“helical polymerization”, in their terms) must first undergo a rate-limiting nucleation event, generally the formation of a small (2-3 subunit) oligomer. This has two important consequences for the kinetics of assembly. First, there is a short lag period after initiation of
polymerization while this energetically unfavorable event takes place. Second, assembly only occurs if the concentration of free monomers is above the critical concentration ($C_c$). Above $C_c$, the energetically unfavorable dimer nuclei are sufficiently long-lived to allow the energetically favorable elongation phase to begin, which rapidly reaches a steady state where the concentration of the pool of free monomers is at $C_c$.

For tubulin, the origin of this cooperativity is the additional stability provided to the growing polymer by non-covalent bonds formed between the lateral surfaces of tubulin monomers in adjacent protofilaments—fitting the helical polymerization model of Oosawa and Kasai (51). These bonds are much weaker than the end-to-end associations between monomers in each growing protofilament, but they increase free energy (and reduce entropy) such that elongation is thermodynamically favored. That FtsZ assembled cooperatively was suggested in the early work on the GTPase activity of FtsZ cited above, as well as the presence of an apparent critical concentration for assembly reported in prior work with DLS, analytical ultracentrifugation, and isothermal titration calorimetry. Unexpectedly, electron microscopy showed that FtsZ protofilaments were a single monomer in width and did not associate laterally under physiological conditions, leaving no clear explanation for this apparent cooperativity (21, 36, 52–55).

A number of hypotheses have been proposed to explain this phenomenon (56–59); however, the best and most comprehensive model (57) suggests that cooperative assembly in linear, single-stranded polymers originates from an allosteric shift between a low affinity form of the FtsZ monomer (termed “L”) and a high affinity form (termed “H”). Though conceived with FtsZ in mind, this model is applicable to any linear, single-stranded polymer. Rather than specifying (and thus simplifying) the model by baking in FtsZ-centric assumptions regarding equilibrium behavior, the authors used matrix algebra to analyze the formation of all possible polymer species. Any allosteric assembly model—including variants of isodesmic and cooperative assembly—can be tested simply by varying the equilibrium constants. These
constants represent the allostERIC isomerization reaction \( (K_C) \) and reactions generating all possible independent combinations of \( L \) and \( H \) monomers—\( K_{LL}, K_{LH}, K_{HL}, \) and \( K_{HH} \).

In using this model to analyze cooperative assembly of FtsZ, two assumptions must be made: first, that free \( L \)-form monomers are inherently more stable than free \( H \)-form monomers such that \( K_C \ll 1 \) (in other words, the isomerization reaction \( L \Leftrightarrow H \) is energetically unfavorable) and second, that \( H \)-form monomers have high affinity for each other such that \( K_{HH} > K_{LH}, K_{HL}, \) and \( K_{LL} \). If there is no difference in stability between \( L \) and \( H \) forms \( (K_C = 1) \) then all interfaces are equivalent and assembly is isodesmic, not cooperative. If isomerization is favored \( (K_C \gg 1), H \)-FtsZ would default to the polymer form and free monomers would be rare. Neither of these states have been found in purified solutions of FtsZ (60).

Cooperativity and the critical concentration exist because the nucleation and elongation reactions—even though the reaction products are identical—are not chemically equivalent:

\[
L + L \Leftrightarrow H_{\text{free}} + H_{\text{free}} \Leftrightarrow HH \quad \text{(reaction 1.1, nucleation)}
\]

\[
H_{\text{polymer}} + L \Leftrightarrow H_{\text{polymer}} + H_{\text{free}} \Leftrightarrow HH \quad \text{(reaction 1.2, elongation)}
\]

In both reactions, the energetic cost of isomerization is paid for by the stable association of two \( H \)-form subunits. An assembly reaction that begins with such a dimer nucleation event, passing through unstable intermediates, will always heavily favor elongation by continued addition of \( H \) subunits to the growing polymer chain. Two \( L \)-form monomers must isomerize to \( H \)-form for nucleation to occur (i.e. constant for association is proportional to \( K_C^2K_{HH} \)), while elongation requires only one isomerization event (association constant proportional to \( K_CK_{HH} \)). Nucleation is therefore less favorable than elongation by a factor of \( K_C \), which is assumed to be very small, meaning that the difference between elongation and nucleation is quite large. Given reaction 1.1 (where \( K_{HH} \) is the equilibrium constant) and the assumption that nearly all free FtsZ is in the \( L \)-form \( (K_C \ll 1) \), the rate of nucleation must be proportional to total FtsZ concentration. At low concentration, the comparatively rare free \( H \)-form monomers return to the \( L \)-form before an \( H-\)
form binding partner can be found; however, as total FtsZ concentration rises, the amount of transient, unstable H-form monomers will necessarily rise (the law of chemical equilibria), increasing the likelihood that dimer nuclei form and elongation of polymers occurs. Eventually, a critical mass of free H-form monomers exists—the critical concentration—which allows dimer nuclei to persist long enough to ignite the elongation phase. Given our original two assumptions, the critical concentration is proportional to the affinity of subunits for polymer ends \(C_c \approx \frac{1}{K_cK_{HH}}\) and is predicted by the model to occur around 1 \(\mu\)mol/L in solutions where the total FtsZ concentration is 10 \(\mu\)mol/L (57). This predicted \(C_c\) is in the middle of a range of \(C_c\) values (0.7 – 1.2 \(\mu\)mol/L) previously reported by many research groups for \(E. coli\) FtsZ, across widely differing reaction conditions (60).

With a complete picture of assembly kinetics in hand and a model that mathematically defines those kinetics, the question arises—what is the nature of the allosteric shift between the low- and high-affinity forms of FtsZ? This question was an unsolved puzzle for years, as crystals of FtsZ lacking bound nucleotide (apo-FtsZ), FtsZ-GDP, FtsZ-GTP, and FtsZ bound to various GTP analogs showed no significant structural shifts in the active site across molecules crystallized in differing space groups, by differing research groups, as well as using FtsZs from differing species (40, 45). A potential answer finally came, thanks to the development of an inhibitor of FtsZ known as PC190723.

PC190723 is a benzamide derivative (\(C_{14}H_{8}ClF_{2}N_{3}O_{2}\)) and inhibitor of FtsZ shown to have potent antibacterial activity against members of the genus \textit{Staphylococcus}, including methicillin-resistant (MRSA) and multi-drug-resistant (MDRSA) \textit{S. aureus} (61). Initial physiological and microscopic analysis performed by the authors showed that PC190723 inhibited both the enzymatic activity of purified FtsZ as well as the localization of FtsZ in dividing cells, which led to filamentation in rod-shaped cells (\textit{Bacillus subtilis}) and the “giant cell” phenotype seen in FtsZ-defective mutants in species of bacteria with a spherical cell morphology, such as \textit{S. aureus}. 

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In an effort to identify the binding site for PC190723, the authors produced a crystal structure of apo-FtsZ (from *B. subtilis*) as well as several mutant FtsZs resistant to the molecule’s inhibitory effect. Molecular modeling *in silico* suggested that PC190723 bound in a cleft between the H7 core helix and the C-terminal domain, stabilized by a number of hydrophobic residues within the cleft (61). Six spontaneously-generated mutations to PC190723 resistance all map to this region; additionally, bacteria that have an innate resistance to this inhibitor have a polar arginine or histidine (rather than a non-polar valine) at residue 307, which lies within this proposed binding cleft. Site-directed mutagenesis of V307 to arginine or histidine in *B. subtilis* FtsZ resulted in reduced susceptibility of the bacteria to PC190723. These data suggest the possibility of an allosteric modulation of FtsZ function in a region that lies outside of the active site proper.

Further investigation by the inventors, Merck, showed that binding of PC190723 directly modulates the cooperativity of FtsZ assembly (39). The presence of 10 µmol/L PC190723 abolished the lag phase in both assembly (shown by DLS) and GTPase activity in assembly reactions containing 9.6 µmol/L FtsZ, in addition to abolishing the critical concentration for assembly when FtsZ concentration was varied between 0.4 µmol/L and 12 µmol/L. A fourfold increase in DLS signal indicated that PC190723 increased the stability of protofilaments, while also doubling the duration of the steady state. Following the end of the equilibrium phase, disassembly progressed much more quickly in the absence of the inhibitor. In its presence, the DLS signal at 40 minutes was still three times higher than the peak DLS signal seen in solutions of FtsZ alone; by this same time FtsZ assembled in the absence of the inhibitor had completely disassembled, bringing the DLS signal to background. These data suggest that cytokinesis-defective phenotype seen *in vivo* with PC190723 incubation is caused by hyper-stabilization of FtsZ filaments, inappropriate assembly of filaments (due to the elimination of the critical concentration for assembly), or both.
A crystal structure of PC190723 bound to *S. aureus* FtsZ-GDP revealed that the inhibitor does in fact bind to the region predicted by Haydon, *et al.* (39) and that binding of the inhibitor alters the orientation of the N- and C-terminal domains with respect to each other. In FtsZ-GDP, each subunit is anchored to H7 by a single hydrogen bond. When bound by the inhibitor, this bond is broken, causing the C-terminal domain to rotate up and away—a movement of approximately 3.5 Å (Fig. 6A). This rotation reveals the PC190723 binding site, and binding causes the stabilization of the monomer in this state. This also alters binding of GDP. The release of Arg29, originally responsible for the hydrogen bonding that locked the N-terminal domain to H7, causes the displacement of Asp187, a residue normally involved in binding of the guanine ring of the nucleotide (Figs. 4 and 6C). Arg29 instead forms a salt-bridge directly with the guanine base, resulting in a 30° rotation of the ring.

It is unclear exactly what effect this rotation might have on GTPase activity, if any. Though the authors suggest that GTPase activity is increased in the presence of PC190723, the GTPase activity data show that the increase is most prominent at low FtsZ concentration and begins to disappear as the protein concentration rises to physiological levels. Recall that in cooperative assembly, there is always a pool of free monomers, the concentration of which is equal to $C_c$. When considered collectively the GTPase activity of these free monomers is essentially zero, since they do not participate in assembly. In the presence of PC190723, FtsZ assembly is isodesmic and not cooperative, so the difference in hydrolysis rate can be accounted for by the participation of the otherwise “inactive” pool of monomers in protofilament assembly. It follows logically that the increase in GTPase activity appears to tail off in plots as FtsZ concentration rises. Since critical concentration is independent of total protein concentration, the newly-active pool of monomers becomes a progressively smaller percentage of the total pool as FtsZ concentration rises, diminishing their impact on total GTPase activity in endpoint assays, such as those used by Haydon, *et al.* (61).
Figure 1.6. **PC190723 binding to FtsZ.** (A) Alpha-carbon backbone of FtsZ-GDP with and without PC190723. (B) Space-fill of FtsZ-GDP monomers within the crystal lattice, with bound PC190723. (C) Detail of PC190723 binding site with specific amino acids highlighted as wireframe models. In each, red indicates the N-terminal domain, blue the C-terminal domain, yellow the H7 helix, and in (B) green the T7 loop. PC190723 is highlighted with a low-opacity, gray space-fill in (A) and (C). Reprinted with permission from Elsen, *et al.* (39).
1.4 POLYMER STRUCTURE AND DYNAMICS IN THE STEADY STATE

In assembly reactions using purified wild-type FtsZ, in buffers that approximate the internal conditions of the cell, FtsZ protofilaments are a single subunit thick and an average of 125 nm long, a polymer length of 25 individual monomers (60). Altered concentrations of cations (both monovalent and divalent) and pH, surface interactions, crowding agents and the presence of various GTPase inhibitors alter the aspect ratio (both length and width) and dynamics (as measured by subunit turnover) in a very regular and consistent manner (62, 63). Stabilizing conditions are accompanied by a decrease in subunit turnover, an increase in average length of the polymer and in some cases, lateral bundling of filaments. Destabilizing conditions are accompanied by increasing subunit turnover, shortening of polymers, and a breakdown of lateral associations. A causative relationship between these phenomena is still unclear, as many of the molecular details are still yet unknown; however, we will see that the correlations described here are robust and reproducible across a variety of conditions.

The steady state condition refers to a period of equilibrium between assembly and disassembly reactions such that total FtsZ polymer mass remains constant (47). During the steady state, there is balance in the cycle of GTP hydrolysis, inorganic phosphate release, and nucleotide exchange (Figure 1.7) that ultimately results in the turnover of subunits within the assembled polymers (64). This process occurs both in vitro in assembly reactions of purified FtsZ and in vivo in the ring itself, where fluorescence recovery after photobleaching (FRAP) and FRET assays have been used to show that the turnover time of subunits within both isolated polymers and the Z-ring is rapid, between 8-11s (49, 65). A recent report (66) provides evidence that turnover of individual subunits can occur even in the absence of GTP hydrolysis, suggesting the possibility that filament dynamics can drive changes in the shape and length of filaments in the constricting Z-ring even in the absence of enzymatic activity.
Figure 1.7. Cycle of filament turnover in the steady state. Polymer assembly (2) occurs when GTP-bound FtsZ (open circles) polymerizes into filaments and is necessary for GTP hydrolysis (3), which occurs soon after without release of inorganic phosphate. Release of $\text{P}_i$ (4) results in a conformational shift to a highly-curved state that promotes fragmentation and disassembly. Exchange of GDP for GTP (1) replenishes the pool of polymerization-ready FtsZ. Reprinted with permission from Romberg and Levin (64).
Regulation of the Z-ring in vivo is ultimately a regulation of subunit turnover in the filament steady state. Positive regulators stabilize the ring by facilitating lateral interaction, by directly binding filaments in bridge-like configurations, or by disrupting the biochemical turnover cycle (67–69). There are several proteins that occupy this role, but these same effects can also be seen in vitro as an increase in steady state duration when assembly reaction conditions are modified, suggesting a role for these in regulating Z-ring function as well. In the context of the Z-ring, “positive” regulation can have both beneficial and negative impact. Just as insufficient ring stabilization (e.g. deletion of ring stabilizing proteins) can cause non-functional Z-rings and division defects (70), so too can excess stabilization. Overexpression of stabilizing proteins causes a variety of aberrant division phenotypes, including improper ring formation and constriction failure (71–73).

Positive regulation of FtsZ filaments by assembly conditions is a well-documented phenomenon. Reduction of pH to 6.5 reduces GTP hydrolysis rate, lengthens polymers and causes them to adopt a straight, rigid conformation and further reduction to pH 6.0 both deepens enzymatic inhibition and results in the formation of protofilament pairs and bundles like those seen in Figure 1.8c (74, 75). The presence of monovalent cations rubidium and sodium (above 50 mmol/L) as well as divalent calcium and excess magnesium (above 10mM) cause filament bundling (36, 38, 55, 76). At calcium concentrations in excess of 10 mmol/L, FtsZ forms large, gel-like polymer networks (38), a property that can be used to assist in removing inactivated FtsZ monomers from protein preparations and serve as quick-check assays for polymerization. Lastly, assembly in GTP analogs that are either non-hydrolyzable or slowly hydrolyzing mimic this stabilizing effect, resulting in polymer lengthening and straightening (45, 55, 77)

Each of these conditions has a logical regulatory role in the context of cell physiology. Bacteria like E. coli are robustly homeostatic and can tolerate a wide range of environmental shifts in pH and osmotic pressure. Sodium and calcium are normally kept low in the cell and high outside (32). Persistent
Figure 1.8. Electron micrographs of FtsZ polymers. (a) FtsZ protofilaments assembled in the presence of GTP showing curved and straight filaments as well as monomeric FtsZ. (b) mini-rings assembled in the presence of GDP. (c) Pairs and bundles (d) large bundles formed in the presence of soluble ZipA. Scale bar is 50 nm in (a) and (b), 100 nm in (c) and (d). Reprinted with permission from Romberg and Levin (64).
dysregulation of these indicate a physical disruption of the cell (e.g. membrane breach) or other homeostatic failure. In such a case, the ability to disrupt cytokinesis until a balance is restored would be beneficial for survival. Protein-based systems that utilize this concept are known; the protein SulA, which is induced by the SOS response to DNA damage, blocks Z-ring formation by binding to and sequestering individual monomers and preventing polymerization of filaments (78). The advantage of a regulatory regime such as this, that directly impacts the intrinsic properties of FtsZ filaments, is a rapid response that requires no protein activation or gene expression.

1.4 Z-RING ASSEMBLY, STRUCTURE, AND CONSTRUCTION

The base buffer conditions employed during \textit{in vitro} study of the steady state in FtsZ filaments typically mimic the conditions of the \textit{E. coli} cytoplasm. Cytoplasmic concentration of FtsZ protein is maintained at a level well above the critical concentration for assembly (79); however, the homeostatic processes of the cell maintain a constantly-replenishing pool of GTP (32). Because \textit{E. coli} global inhibitors of FtsZ polymerization are not expressed under normal conditions, the cytoplasmic pool of FtsZ is in protofilament form (41, 78, 80). These protofilaments do not act alone in formation of the Z-ring. The first step in formation of the Z-ring, and thus the formation of the complete divisome, is the attachment of FtsZ filaments to the membrane. In \textit{E. coli}, two proteins are responsible for this task: FtsA and ZipA (3, 81, 82). In reality, the fundamental construction of the “Z-ring” is not solely FtsZ. The term \textit{proto-ring} is used to describe this base structure, unadorned by the other proteins of the divisome (83).

FtsA is a membrane-binding protein that is, structurally, a homolog of actin and belongs to the actin/Hsp70/sugar kinase superfamily (84). Structurally, the protein consists of a globular domain, which contains three regions relevant to the assembly of the proto-ring (Figure 1.9, left)—an ATP-binding site, a self-interaction domain, and an FtsZ-binding domain—as well as a C-terminal amphipathic helix attached
Figure 1.9. Structures of the tethering proteins of the proto-ring. FtsA (left) contains four distinct domains in its globular region, which is connected to membrane-targeting amphipathic helix via a flexible linker. Portions of domains 1A, 2A, and 2B participate in binding of ATP. Domain 2B is the FtsZ-binding domain and domain 1C is the self-interaction domain. ZipA (right) is anchored to the membrane via an amphipathic helix, which connects to the globular FtsZ-binding domain via a flexible linker. Reprinted with permission from Ortiz et al. (88).
to the globular portion of the protein via an unstructured linker (70). In its inactive state, the amphipathic helix attaches itself to the self-interaction domain. Binding of ATP functions as a molecular switch, causing a conformational shift in the self-interaction domain that releases the C-terminal helix, enabling its attachment to the membrane and initiating polymerization of FtsA into filaments (85, 86). Unlike actin, FtsA hydrolyzes ATP at a very low basal rate, which has been shown to be intrinsic to the protein through identification of thermosensitive mutations that modulate this activity (87). The role of FtsA polymerization in proto-ring assembly remains unclear. One model suggests that a ring of FtsA may bridge the space between the membrane and FtsZ in the early stages of divisome development (86), though no convincing experimental evidence has yet come to light. Regardless, through this interaction with FtsA, FtsZ is provided with a means to localize to the cell membrane.

The second attachment protein, ZipA (Figure 1.9, right)—Z-interacting protein A—has a similar overall architecture to FtsA. It consists of a membrane-targeting domain, a flexible linker sequence, and a cytoplasmic, globular, FtsZ-binding domain; however, it differs from FtsA in that the membrane targeting sequence is a transmembrane helix (89). It is also a structural homologue of Tau protein, a MAP (microtubule-associated protein) that stabilizes microtubule assembly (90). Incidentally, ZipA is also functionally homologous to Tau. Recombinant ZipA lacking the linker and transmembrane domains is able to promote formation of higher-order structures of FtsZ filaments in vitro as well as reorganize networks of protofilaments adsorbed to mica (68, 91). In vivo, ZipA overexpression can rescue division in strains expressing the FtsZ84 temperature-sensitive allele, even when grown at the normally fatal non-permissive temperature. Some evidence suggests that ZipA binding of FtsZ precedes FtsA activation and binding at the membrane, but regardless of this particular timing, ZipA and FtsZ localization at the membrane are temporally linked (92).
In living cells, FtsA and ZipA (like FtsZ) are essential proteins. Without supplementation, ZipA and FtsA null mutations are lethal. In null strains where the proteins are added back from conditionally-expressed plasmids, depletion of the protein causes the bacteria to suffer a filamentous division block similar to that seen in the FtsZ84 mutant (4, 81). Likewise, overexpression of either attachment protein causes a division block, indicating that each must be maintained in proper proportion to FtsZ in order for the proto-ring to properly function (5, 81, 93). It is interesting to note that overexpression of either wild-type protein can overcome a null phenotype in the other (e.g. excess FtsA can rescue a ZipA null mutant as well as the reverse). A particular mutant of FtsA, a known as FtsA R286W or FtsA*, can rescue FtsZ84 mutations as well as ZipA null mutations when expressed at a level equivalent to that seen in wild-type strains. This hypermorphic (or gain-of-function) phenotype is due to a decreased self-interaction, suggesting that when polymerized, the FtsZ-stabilizing effect of FtsA is blocked or otherwise significantly muted. Regardless, neither protein is specifically required for proper ring function; as long as the proper proportion of the tethering/stabilization activities are maintained with respect to FtsZ.

This is also apparent in vitro. Two-protein reconstitutions involving both ZipA/FtsZ and FtsA*/FtsZ function to varying degrees whether in liposomes or on planar bilayers and functioning, constricting Z-rings have been reconstituted with ZipA (in giant unilamellar vesicles) and with FtsA in agarose-supported unilamellar liposomes (91, 94–98). Other reconstitutions, those published by Harold Erickson’s group in particular, show that a YFP-tagged FtsZ can support both ring formation and constriction when the C-terminal binding domain is replaced with the amphipathic helix from MinD, a Z-regulating protein (99). Not only that, but domain-swapped versions of the protein, where the amphipathic helix is instead located on the N-terminus, form ring structures on the outside of liposomes that constrict, resulting in the projection of long tubes from the liposome surface (100) as well as form rings of parallel filaments on tubules made with DC8,9PC, a synthetic lipid (101). The intrinsic properties
of FtsZ filaments—shape, curvature, rigidity, enzymatic activity—are sufficient to allow both self-assembly and constriction of rings, given a curved surface and a means of attachment that allows free diffusion of polymers. This is an extraordinarily important finding; any model of Z-ring function must start with that fundamental property of self-assembly and consider external factors as modulators.

To ensure equal division of the cellular components and to prevent the guillotining of the chromosome in the closing septum, assembly of the proto-ring occurs at the middle of the cell. This positioning is accomplished by the combined action of two mechanisms: Min and nucleoid occlusion (102). The Min system, which consists of three proteins (MinC, MinD, and MinE) prevents polymerization at the ends of the cell. The first two proteins together form the MinCD complex, an FtsZ antagonist. MinD is an ATPase that dimerizes upon binding of ATP, allowing it to attach to the cytoplasmic membrane by means of an amphipathic helix (103). Dimerization also increases the protein’s binding affinity for MinC that, by itself, has very little inhibitory effect on FtsZ; however, upon binding to dimerized MinD, MinC binds to the C-terminal helix of FtsZ and both induces fragmentation of protofilaments and prevents elongation of newly-assembling filaments nearby (18, 78, 104–106). The effect of MinCD is felt only at the cell poles due to the effect of the third protein, MinE. MinE blocks MinCD function by activating the ATPase of MinD, which causes it to dislodge from the membrane and results in the disassembly of the MinCD complex (107). Spatial localization is accomplished by means of a ring of MinE protein that oscillates from pole to pole via a reaction-diffusion mechanism (108–110). These oscillations, when averaged over time, result in a gradient of MinE concentration where the protein is highest in the middle of the cell and lowest at the poles. As a result, active MinCD is highest at the poles—where it inhibits proto-ring assembly—and lowest at the mid-cell.

The nucleoid occlusion system prevents proto-ring assembly over the portion of the cell occupied by the bacterial chromosome (111, 112). The protein responsible for this activity is SlmA, a DNA-binding
Figure 1.10. Spatial distribution of Min and NO systems. Immediately after division, *E. coli* cells are small and both the Min and nucleoid occlusion (NO) systems overlap. As the cell grows and the chromosome replicates, a gap opens at the middle of the cell that allows FtsZ ring assembly. MinCD shown in red, the oscillating MinE ring in gray with black hashes, and SImA as a paired, yellow ellipses. Reprinted with permission from Lutkenhaus (102).
protein. SlmA protein in its active configuration is a dimer of dimers; it binds to specific sequences on the bacterial chromosome and in so binding becomes an active antagonist of FtsZ (7, 113, 114). SlmA initially binds to the C-terminal helix of FtsZ; this binding encourages a closer association that inhibits ring assembly by a GTPase-independent mechanism, the details of which are still unclear (115, 116). In newly-born cells, the nucleoid occupies the majority of the length of the cell. When coupled with Min system activity at the cell ends, Z-ring assembly is blocked throughout the entirety of the cell (Figure 1.10).

As the cell grows (and lengthens, in rod-shaped cells) and the DNA is replicated, the chromosomes move to the ends of the cell, carrying along with them bound SlmA. Chromosomal segregation creates a narrow SlmA-free zone that overlaps with the area where MinCD concentration is at its lowest. In this narrow band, the Z-ring forms. The combined effect of these localization systems is the concentration of FtsZ filaments along the long axis of the cell into a narrow area, which varies in size depending on bacterial species, but is approximately 100 nm in E. coli (117). Filaments are further concentrated axially by their association with FtsA and ZipA. Because of this, proto-ring formation is most accurately described as a condensation rather than a step-wise assembly. This description is supported by a wealth of experimental data. Fluorescence micrographs of E. coli cells expressing FtsZ-GFP fusions show that in the very early stages of proto-ring formation, membrane-associated filaments form a helical structure that oscillates along the length of the cell (3, 118). These helical structures condense to form the proto-ring at the mid-cell. The exact mechanism of this condensation is unknown, but it is presumed to be tied to the activity of a set of positive regulators of proto-ring assembly, the Zap (Z-associating protein) family of proteins.

The Zap family consists of four proteins: ZapA, ZapB, ZapC, and ZapD. All are known to co-localize to the early proto-ring at mid-cell and promote bundling of FtsZ protofilaments in vitro (119–122). The structure and precise function of most of the Zap proteins is not well known. ZapA, the first
and most well-characterized of the Zap proteins, consists of a globular N-terminal domain that is the Z-
interacting domain and a C-terminal coiled-coil domain that promotes oligomerization into dimers and
tetramers (123). Interaction of the coiled-coil domain makes dimers that are able to interact with two
protofilaments simultaneously, which is the origin of their bundling ability and suggests they play a role
in condensation of the developing proto-ring from a helical structure to a ring (ibid.).

ZapA was identified originally as an antagonist of the MinCD complex (119). ZapA and MinCD
compete for the same binding sites (74), indicating that one of the important functions is stabilization of
the condensed ring against disruption by passing MinCDE waves. Cytological and genetic data show that
the Zap proteins are non-essential; single mutants only show mild division-inhibitory phenotypes that
result in cell elongation without cell death and that these mild phenotypes are suppressed by the presence
of the FtsA* hypermorph (124, 125). Only in combination with secondary mutations (FtsZ84, other Zap
proteins, FtsA/ZipA mutants) is synthetic lethality achieved. Condensation and stabilization of FtsZ
occurs when positive regulators are in proper balance, and there is some limited redundancy between the
Zap family, FtsA, and ZipA. Beyond this point, we begin to encounter some of the critical unanswered
questions regarding FtsZ form and function.

The ultrastructure of the condensed ring and the mechanism by which cytokinesis subsequently
occurs, is a matter of much debate. Two recently-published studies of *E. coli* ring structure, both using
super-resolution microscopy techniques, present distinctly different views of the ring. Photoactivated
localization microscopy (PALM) of live *E. coli* cells showed that the *E. coli* Z-ring consists of a
discontinuous series of overlapping filaments with both helical and ring configurations, rather than a
single continuous helical filament (126). Due to technical limits of spatial resolution, individual
protofilaments could not be imaged, but moderate overexpression of FtsZ increased fluorescence intensity
within the ring without increasing its width suggesting the possibility of a multi-layered ring. A later
study using electron cryotomography found that FtsZ filaments form a single, continuous and ribbon-like layer of protofilaments 15-16 nm from the inner leaflet of the plasma membrane (127). That the ring appears discontinuous in PALM may be due to preferential assembly between monomers of native FtsZ, though no definitive evidence for that view has yet been published.

Before proceeding to questions of the mechanism of Z-ring function, it is necessary to discuss a series of experiments that will provide critical context. Despite the complexity of the in vivo regulatory regime of FtsZ protofilaments, several reports of in vitro reconstitution of Z-only rings with membrane targeted FtsZ (where proteins that regulate the spatial location of protofilaments are absent) show that only a tethering mechanism is required for Z-ring condensation (94, 95, 99). In tubular liposomes, weakly fluorescing rings slowly aggregate as they diffuse along the liposome surface; once a critical mass is reached, diffusion ceases, presumably caused by the transition to a constriction mode (128). These rings are capable of constriction but not membrane scission; invagination of the liposome membrane becomes “locked” partway through the process and never fully resolve. After a period of time, the endpoint of which corresponds to the exhaustion of GTP, rings disassemble and the constrictions relax. Only in a dual protein system, where both FtsA and FtsZ are present, has completion of cytokinesis been observed (127, 129). Regardless, these reconstitutions show that in the absence of negative regulators, membrane-targeted FtsZ can direct its own assembly into rings capable of constriction.

It is clear from these results that FtsZ filaments can generate a constriction force sufficient to deform lipid bilayers without the influence of other Z-interacting proteins. This is the strongest evidence in favor of the “Z-centric hypothesis” of bacterial cytokinesis; that given the absence of other motor proteins, FtsZ alone must be responsible for the force generation necessary to perform septation (22, 62). Other groups have proposed a peptidoglycan-centric hypothesis that states that synthesis of septal peptidoglycan (which is coordinated by the scaffolding and divisome recruitment functions of FtsZ)
provides constriction force by squeezing from the outside (117). Recent publications suggest a third, hybrid hypothesis where the force applied to the plasma membrane by FtsZ directly regulates the kinetics and directionality of new peptidoglycan synthesis, which pushes on the plasma membrane and drives invagination of the membrane (2, 130–133).

Though the in vivo mechanisms that govern cytokinesis are still incompletely understood, that FtsZ filaments alone can generate a constriction force is not. There are two classes of models of Z-ring constriction: those that rely on bending of the filaments at the interface between two monomers and those that rely on lateral interactions to drive the sliding of filaments relative to each other. In bending models, the force-generating “power stroke” is the shift between the gently curved protofilament conformation to the highly curved conformation (Figure 1.8a-b), which is favored following hydrolysis of GTP (60, 134). For sliding models, overlapping protofilaments stabilized by lateral interactions slide with respect to each other, creating a “purse string” effect that pinches the plasma membrane together (135).

Evidence from in vitro reconstitutions strongly favor bending models. In particular, membrane-targeted FtsZ in its native configuration (membrane anchoring at the C-terminus) incubated with liposomes will attach to the bilayer surface and produce convex depressions (Figure 1.12a, 123). Swapping the anchor to the N-terminus produces convex protrusions (figure 1.12b), a phenomenon only explainable by the bending of a curved filament. The model is further strengthened by structural data derived by X-ray crystallography (from Mycobacterium tuberculosis FtsZ) that show a “hinge-opening” conformational shift in the T3 loop following GTP hydrolysis that explains the change in filament curvature (9). In contradiction, reconstituted rings using a two-protein FtsA/FtsZ system constricted even in the presence of inhibitors of GTP hydrolysis, for which filament sliding is the most likely explanation (127). These seemingly contradictory observations can be reconciled by a model where filaments both bend and slide; for example, with sliding filaments providing the shortening that drives the majority of the constriction
Figure 1.11. Bending forces generated by FtsZ distort liposome membranes. (A) Membrane targeted FtsZ with a C-terminal membrane targeting sequence (MTS) generates concave distortions of liposome membranes (B) Distortions are convex in recombinant FtsZ with the MTS swapped to the N-terminus. (C) Diagrammatic representation of the two processes. Reprinted with permission from Erickson et al. (30).
and filament bending providing the energy to destabilize and fragment filaments, allowing them to remain dynamic (by creating new filament ends for subunit turnover) as well as shorten in length as the incomplete septum grows smaller.

The remaining mystery regarding both models is the requirement for a kind of lateral interaction that is weaker than direct lateral bonding (60). When membrane-attached filaments bend, they must be stabilized laterally in some way, else the circumferential bending force will not be transmitted through the anchor to the membrane. In the absence of such interactions, filaments would roll and the generated force would be lost. In the sliding model, filaments require an unknown attractive force or interaction that allows them to maintain long-range order as well as hold position following shortening. In this case, a lack of lateral interaction will simply cause the filaments to slide past each other without transmitting any force to the membrane itself. The nature of this lateral interaction is currently unknown; however, the energy required for repetitive breaking of direct lateral bonds (electrostatic interactions, van der Waals forces, etc.) across filaments with ever-increasing overlap is prohibitive. Filament sliding requires a weaker interaction than lateral bonds, and any model that attempts to describe a mechanism of constriction of Z-rings must account for the weak nature of this lateral interaction.

1.5 SUMMARY, SCOPE, AND SIGNIFICANCE

Despite a decade of intense study, the pace of progress of our understanding of Z-ring physiology and structure has been slow as the field awaits the arrival of new theories and techniques that change the way FtsZ and its higher-order structures are revealed. The advent of super-resolution microscopic techniques and electron cryotomography have provided the field with its first detailed look at the internal structure of the Z-ring in live cells (117). While these data will drive study of the system forward, the techniques are still in their infancy and the question of their fidelity to the in vivo state remains, in some
cases, an open one. The work of Osawa and Erickson (99) has sparked a wave of new method
development in the area of *in vitro* reconstitution of a functioning Z-ring; however, all of the
reconstitution methods developed to date lack the reproducibility necessary for biochemical and
physiological study. The bulk of our understanding of the function of FtsZ and Z-interacting proteins
comes from study of filaments in isolation *in vitro*. Improved techniques and theories could provide
answers to some of the many open questions surrounding Z-ring formation and function: (1) do the
biochemical and physical properties of FtsZ filaments in dilute solution differ from those filaments
contained within a ring; (2) do environmental conditions that alter the intrinsic properties of polymer
structure and dynamics also alter the function of the Z-ring; (3) is there a holistic theory of Z-ring
formation and function that can account for both bending and sliding modes as well as make useful,
testable predictions regarding Z-ring formation and function?

In this work, we attempt to answer these questions with a two-pronged approach that involves
both theoretical models and experimental data. First, a new method of *in vitro* reconstitution was
developed using cylindrical supported lipid bilayers for reconstitution of both single-protein (recombinant
FtsZ-mts) and dual-protein (FtsZ/FtsA) systems that solves the reproducibility problems of earlier
methods. This system, because it lacks the ring-regulating and positioning systems of the live cell, allows
the formation of multiple Z-rings along the length of the fused-silica capillary used as a support for
bilayer formation. Chapter 2 describes the development technical aspects of this method, as well as in-
depth analyses of the timing and process of ring formation and constriction. Using these reconstituted Z-
rings, we investigate the effects of a variety of environmental factors on the number of rings formed, the
process of ring formation, and cytokinetic function. Chapter 3 details this work in which the effects of
increasing FtsZ concentration, the ratio of filament subunits that are bound to the membrane vs.
unbound, variations in buffer pH, the presence of calcium ions, and inhibition of GTPase activity with a slowly-hydrolyzing GTP analog are explored.

Secondly, we propose a theory of the FtsZ ring as a lyotropic liquid crystal exhibiting nematic order, detailing how the essential intrinsic physical properties of FtsZ and its filaments would impact the development of nematic order and how changing environmental factors and Z-interacting proteins regulate the ring by modulating the order of the filaments within it. We test this theory experimentally by attempting to identify birefringent textures in concentrated solutions of purified FtsZ using traditional techniques in the field as well as a newly-developed droplet evaporation assay. This theory and experimental testing is discussed in detail in Chapter 4.

From a perspective of pure science, a greater understanding of the biophysics of the Z-ring would provide a critical, unifying mechanistic explanation for both ring function and regulation of the bacterial cell cycle. In practical terms, FtsZ is one of the primary targets of next-generation antibiotics and having a mechanistic understanding of ring formation and function would aid in targeting development of Z-inhibiting antibiotics. Additionally, a robust, reproducible in vitro reconstitution assay would serve as an excellent tool for screening compounds of interest for FtsZ-inhibitory activity.

1.6 RATIONALE AND HYPOTHESIS

The overarching aim of this work is to develop a new theory and new methods that elucidate the biophysical processes that determine the formation and function of the bacterial cytokinetic ring. The fundamental hypotheses that undergird the work are as follows:

First, a newly-developed method of in vitro reconstitution utilizing cylindrical supported lipid bilayers as a surface for attachment of FtsZ will result in the robust and reproducible formation of
functioning Z-rings in both FtsZ-only and dual FtsZ/FtsA* system. The processes of ring formation and constriction will be observable in real time and documentable using fluorescence microscopy.

Second, that the FtsZ ring is lyotropic liquid crystal material exhibiting nematic order. This nematic ordering will be detectable in bulk solution at threshold FtsZ concentrations predicted by the theory and that environmental conditions that alter the essential intrinsic physical properties of FtsZ and its filaments would impact the development of nematic order. Conditions that increase polymer stability will decrease the threshold concentration, and those that decrease polymer stability will increase it.

Finally, if the proposed holistic model is valid, we will be able to correlate conditions that alter threshold concentration for nematic ordering with conditions that alter ring formation and function. If these conditions are biologically relevant, we hypothesize that Z-ring stability (as determined by the number of formed and constricting Z-rings within the reconstitution system) will peak at some middle value. On either extreme, the ring transitions away from nematic order, inhibiting its formation and function.
1.7 REFERENCES


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CHAPTER II

IN VITRO RECONSTITUTION OF THE *ESCHERICHIA COLI* Z-RING

2.1 ABSTRACT

The study of the form and function of the FtsZ ring, which is responsible for force generation in cytokinesis as well as initiating the cascade of recruitment that forms the divisome, is complicated by the fact that it is essential for growth and reproduction. Mutation and physiological studies are limited to those conditions not damaging to the overall progression of cytokinesis. This begs a method by which rings can be reconstituted *in vitro* and manipulated as needed to directly assess their formation and function. We present a newly designed method using a cylindrical supported lipid bilayer as a surface on which to assemble a membrane-targeted FtsZ protein and a dual protein system consisting of FtsZ and its native membrane anchoring protein, FtsA. We found a variety of ring morphologies in this system, including complete rings capable of constriction and septation, partial rings that diffuse along the membrane and condense into complete rings, as well as partial arcs. We also found that ring number (but not ring function) is proportional to input protein concentration, allowing the data collection thresholds of the system to be tuned as needed. This system is both robust and reproducible, and represents a significant advancement compared to other reconstitution methods.
2.2 INTRODUCTION

Given the homeostatic nature of living cells and the lethality of functional defects of the FtsZ ring, reconstitution of the Z-ring \textit{in vitro} is a critical tool for understanding the physiological aspects of its formation and function. These attempts began with observations of the behavior of protofilaments on planar surfaces, including cleaved mica and lipid monolayers, discussed in detail in section 1.1.4; instead, this summary will focus on recent advances, in which a limited amount of success has been achieved.

The first true reconstitution of the FtsZ ring was published in \textit{Science} in 2008 (1). In this work, the authors paired a genetically-modified \textit{E. coli} FtsZ with a method for creating tube-shaped liposomes that allowed the formation of helical structures of FtsZ, as well as complete rings (Fig. 2.1a). The modified FtsZ (FtsZΔC-YFP-mts) replaced the C-terminal helix responsible for binding FtsA and/or ZipA with the Venus yellow fluorescent protein and an amphipathic helix from MinD, allowing the protein to be detected by fluorescence microscopy as well as attach to the membrane in the absence of the two native attachment proteins. Reconstitutions were performed by mixing FtsZΔC-YFP-mts with GTP and multi-lamellar vesicles, then placing a drop of that solution between a slide and a glass coverslip. The shear force generated by the movement of the solution between the glass layers would cause some vesicles to become leaky and tube-shaped, allowing FtsZΔC-YFP-mts to enter. This method generated liposomes that contained multiple rings and/or helical- and spiral-shaped FtsZ structures, some of which could constrict the liposome membrane. These constrictions would relax following the predicted exhaustion of GTP (due to hydrolysis by FtsZ) in the sample.

While this work represents a huge first step toward a reconstitution system that allows physiological manipulation, it has some drawbacks. The tube-shaped liposomes are rare and within that subset the liposomes that contain rings are rarer still. The rigidity of the multilamellar liposomes, likely
Figure 2.1. Reconstitution of FtsZ structures. (A) Reconstitution of FtsZΔC-YFP-mts in a multilamellar tubular liposome. Membrane is imaged by differential interference contrast and YFP with fluorescence microscopy and the images merged. Reprinted with permission from (1). (B) Fluorescence micrograph of tabulating liposomes with reconstituted FtsZΔC-YFP-mts on the surface. (C) Convex protrusions caused by mts-FtsZΔC-YFP. (D) Concave depressions caused by FtsZΔC-YFP-mts. In B-D, liposomes are stained with non-penetrating lipid dye 7-hydroxycoumarin-3-carboxylic acid (HccA). Reprinted with permission from (2).
necessary to prevent the tube-shaped liposomes from collapsing, may inhibit constriction. Finally, the method of entry of the protein and the fact that the liposomes are variably leaky makes it difficult to know the exact concentration of protein within any one vesicle, or of any secondary proteins that might be added to understand their effect on Z-ring function. Despite these drawbacks where mimicking the physiological condition is concerned, this system has provided some valuable insights.

The first and most obvious is their observation that, given a way to tether itself to the membrane, FtsZ is able to assemble into functional Z-rings in the absence of other division proteins. The combination of physical interactions of protofilaments with the membrane surface and the chemical interactions within and between individual filaments must be sufficient for organization of the ring. The myriad proteins that interact with FtsZ \textit{in vivo} may then represent a network of regulatory elements, rather than required inputs for proper division of the cell. This would place FtsZ in the role of central integrator of cell cycle regulation, where a balance of ring stabilization (polymer bundling and association) and destabilization (polymer disassembly and unbundling) signals allow cytokinesis to proceed.

In support of this idea of the primacy of FtsZ, spherical unilamellar liposomes—on which FtsZ$\Delta$C-YFP-mts was reconstituted on the outer surface—were found to be “tubulated” (Figure 2.1b) by the modified FtsZ protein in the presence of GTP (2) These tubulations began as concave depressions on the surface of the liposome at low FtsZ concentration. As FtsZ concentration rose, the tubules began to extrude from the vertices between multiple concave depressions, and FtsZ was found to localize to the long, thin lipid tubules as well. The authors hypothesized that a bending force on the membrane, generated by the inherent curvature of FtsZ filaments, was responsible for these depressions. A domain-swapped FtsZ, where the membrane targeting sequence was moved to the N-terminus of the protein (mts-FtsZ$\Delta$C-YFP), caused convex protrusions of the membrane lent support to this view; however, the unexpected observation that mts-FtsZ$\Delta$C-YFP also generated external tubulations (though not as many
as FtsZΔC-YFP-mts.) casts some doubt on this explanation. Though the exact mechanism of liposome
tubulation remains unknown, the action of FtsZ alone was sufficient to remodel the surface of liposomes.

The functionality of the domain-swapped mts-FtsZΔC-YFP was further explored in the earlier
tubular liposome system (3, 4). When mixed with tubulated liposomes and GTP, mts-FtsZΔC-YFP forms
Z-rings on the outside surface of the liposomes—termed “inside-out” rings by the authors (3). These
inside-out rings are indistinguishable from internal Z-rings on fluorescence microscopy, can generate
constrictions, and are significantly more numerous on a per-liposome basis. Their larger number enabled
the authors to conduct further physiological inquiry. Assembly of mts-FtsZΔC-YFP using the non-
hydrolyzable GTP analog GMPCPP resulted in both ring formation and the initial stages of constriction;
however, subunit turnover (as measured by FRAP) was eliminated over the 9-minute course of the
experiment. These data suggest that the initial formation and constriction of the ring is a GTPase-
independent process, until the ring becomes stabilized in a “locked” state that requires subunit turnover
(and thus GTP hydrolysis) for constriction to continue.

In a separate publication (4) the same authors were able to image inside-out rings with negative
stain electron microscopy. This was accomplished using the synthetic lipid 1,2-bis(10,12-tricosadiynoyl)-
sm-glycero-3-phosphocholine (DC8,9PC), which self-assembles into tubules of diameter 0.5 µm and 100 nm
length (5). Application of mts-FtsZΔC-YFP to DC8,9PC tubules (in the presence of GTP) resulted in the
formation of multiple inside-out Z-rings (Fig. 2.2a), similar to those seen on tube-shaped liposomes.
Electron microscopy revealed that these rings were made up of ribbons of protofilaments parallel to each
other and wound around the tubule perpendicularly to the tubule axis (Fig. 2.2b). Rings varied in width
between 50-250 nm (or approximately 10-50 protofilaments). Diluting the mts anchor by addition of wild-
type FtsZ decreased the average width of reconstituted rings, with a 1:5 molar ratio (mts-FtsZΔC-
YFP:FtsZwt) generating rings of 60-110 nm in width. This mimics the in vivo condition, where FtsA and
**Figure 2.2.** Inside-out rings on DC8,9PC tubules. (A) Fluorescence microscopy of mts-FtsZΔC-YFP assembled on lipid tubules. (B) Negative-stain electron micrograph of mts-FtsZΔC-YFP rings. Scale bar is 200 nm. Reprinted with permission from Milam *et al.* (4).
FtsZ are expressed at a similar molar ratio, and is comparable to measurements of Z-ring width made using super-resolution imaging technologies in bacteria (6, 7). Fourier transform revealed an average spacing between protofilaments within individual rings of 4.8 nm, which is similar to that found in higher-order FtsZ structures—in particular, helical ribbons and toroids—formed in the presence of crowding agents (8, 9). This distance is too wide for direct lateral bonding to occur, suggesting that the lateral associations between protofilaments have a different origin.

These methods of reconstitution of FtsZ rings have provided some valuable new insight into FtsZ ring formation and structure, as well as confirmatory evidence for previously observed phenomena. With the recent demonstration of membrane scission in the two-protein FtsA*:FtsZ-YFP reconstitution (10), proof-of-concept has been demonstrated for all FtsZ functions critical to cytokinesis: formation, constriction, and scission. While all are important contributions, each method has drawbacks that either result in large separation from the in vivo conditions or make the system too unreliable for quantitative physiological study. This begs a modified method where such hypotheses can be reliably tested. This chapter describes our work in this particular area.

2.3 METHODS

For the purpose of reconstituting E. coli FtsZ rings in vitro, our system was designed utilizing ultra-small-bore capillary tubes. The internal surface of these tubes, once hydrated by the addition of a suspension of small unilamellar vesicles (SUVs), is sufficiently clean and chemically compatible for the deposition of a supported lipid bilayer. The internal volume of the tube is exchanged by simple capillary action, and this is used to perform bilayer rinsing, buffer equilibration, and introduction of FtsZ-containing polymerization reactions.
This system represents an improvement on previous reconstitution methods in that it more closely mimics the in vivo condition encountered by E. coli FtsZ: a hollow, cylinder-shaped, ~1 µm diameter lipid bilayer that allows reconstitution of Z-rings on the inner surface by introduction of defined buffered solutions containing known amounts of purified FtsZ or membrane-targeted recombinant FtsZs. The role experimental conditions play in ring formation and constriction can be assessed by rates of ring formation and constriction captured by fluorescence microscopy and the long tube length provides statistical strength by permitting large sample sizes within a single trial and high reproducibility between replications of trials.

2.3.1 Plasmids and Strains Used

Wild-type E. coli FtsZ was amplified by polymerase chain reaction (PCR) from strain MG1655 using 5´-GACGCATATGTTTGAACCAATGGAAC-3´ as the forward primer and 5´-GAGGATCCTTAATCAGCTTGCTTAC-3´ as the reverse primer. This was captured in plasmid pET30 by ligation-independent cloning, then transferred to pET11b by subcloning a BamHI/NdeI fragment into the same sites in the vector to make plasmid pET-Z. Plasmids pET-ZY and pET-ZYM, used for expression of FtsZΔC-YFP and FtsZΔC-YFP-mts respectively, were a kind gift of Dr. Harold Erickson. Plasmid pET-A* used for expression of FtsA*, was a kind gift of Dr. William Margolin. All expression from pET-derived plasmids was performed using E. coli strain C41, which gives higher yields of soluble proteins than BL21, especially proteins that are potentially toxic (citation).

2.3.2 Expression and Preparation of Proteins

FtsZ-YFP and FtsZ-YFP-mts were prepared using a method modified from Osawa and Erickson (11). Electrocompetent cells of E. coli strain C41(DE3) were freshly transformed with pET-ZF or pET-ZFM. A single transformant was picked and grown overnight in 25 mL Luria-Bertani medium with
ampicillin (LB+Amp, 100 µg/mL) at 37 °C in a shaking incubator (225 rpm shaking). The next day, this culture was centrifuged at 4000 RCF for 15 minutes to remove the used medium, resuspended in 25mL fresh LB+Amp, then used to inoculate 500 mL of fresh LB+Amp. This culture was incubated at 37 °C with 225 rpm shaking until the culture reached an optical density between 0.8 and 1.0. At this point, protein expression from the clone was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG, Fisher Scientific, Pittsburgh, PA) to a final concentration of 0.5 mmol/L. Induction continued with incubation at 37 °C with 225 rpm shaking for 4 hours, after which the cultures were moved to a rotating shaker at room temperature (175 rpm shaking) and left overnight. The following morning cells were split into 250 mL bottles, then pelleted by centrifugation at 6000 RCF in an Avanti J26-XP ultracentrifuge using a JA-14 rotor. Pelleted cells were carefully resuspended in 10 mL column buffer (50 mmol/L Tris- HCl pH 7.9, 50 mmol/L KCl, 1.0 mmol/L ethylenediaminetetraacetic acid [EDTA], 10% [v/v] glycerol) with 175 rpm shaking on ice. Lysozyme and phenylmethylsulfonylfluoride (PMSF) were added to a final concentration of 1 mmol/L and 0.5 mg/ml respectively, then incubated on ice with 175 rpm shaking for 20 minutes. These cell suspensions were then frozen at -80 °C overnight or until needed.

Protein was released from cell suspensions by two cycles of freeze-thaw (1.0 mmol/L PMSF was added after each) followed by sonication three times for 30 seconds on ice (90 second rest after each) with a Fisher Scientific F60 Sonic Dismembrator at 50% duty cycle. The resultant lysate was clarified by centrifugation at 57000 RCF in a Beckman JA-25.50 rotor for 2 hours. The supernatant was collected, then brought to 30% saturation by slow addition of 3.52g of solid ammonium sulfate. After addition of ammonium sulfate, the mixture was incubated for 30 minutes on ice. Salt-precipitated protein was pelleted by centrifugation at 25000 RCF in a Beckman JA-25.50 rotor for 20 minutes. This supernatant is discarded and the pellet resuspended in 10 mL ice-cold column buffer, then clarified by passing through a 0.22 µm cellulose acetate syringe filter. The protein solution was dialyzed overnight against 2 L of column
buffer, then further purified by anion exchange chromatography. A HiTrap DEAE FF 15/10 (GE Healthcare) column was used and protein was eluted with a 100 mL gradient from 50-500 mmol/L KCl with a flow rate of 1.0 mL/min. To ensure reproducibility between batches of protein, the column was cleaned after each purification run using the “rigorous cleaning” protocol suggested by the manufacturer. Peak fractions were identified by plots of UV absorbance at 280 nm and verified by the presence of the yellow-colored YFP protein. These fractions were pooled, dialyzed overnight against physiological buffer (50 mmol/L Hepes/KOH, pH 7.7, 5 mmol/L magnesium acetate, 300 mmol/L potassium acetate, 10% [v/v] glycerol, 0.1% DTT), and concentrated using Amicon Ultra-15 centrifugal filters (MWCO 10 kDa). Final protein concentration was calculated according to the Beer-Lambert law using the spectrophotometrically-determined absorbance at 515 nm and a molar extinction coefficient of 92,200 M⁻¹ cm⁻¹. This complete process typically yielded 30-40 mg of total protein. Purified, concentrated protein was then aliquotted and stored at -80°C.

FtsA* was expressed from plasmid pWM1690, a kind gift of Dr. William Margolin (University of Texas-Houston Medical School), which was freshly transformed into chemically competent C43 cells (Merck Millipore, Billerica, MA). A single transformant was picked and grown overnight in 25 mL Luria-Bertani medium with 50 µg/mL kanamycin sulfate (LB+Kan) 37 °C in a shaking incubator (225 rpm shaking). The next day, this culture was centrifuged at 4000 RCF for 15 minutes to remove the used medium, resuspended in 25mL fresh LB+Kan, then used to inoculate 500 mL of fresh LB+Kan. This culture was incubated at 37 °C until reaching an optical density at 600 nm of 0.5. At this point, protein expression was induced with 0.5 mmol/L IPTG. Induction continued with incubation at 37 °C with 225 rpm shaking for 4 hours, after which the cultures were moved to a rotating shaker at room temperature (175 rpm shaking) and left overnight. The following morning, cells were split into 250 mL bottles, then pelleted by centrifugation at 6000 RCF in an Avanti J26-XP ultracentrifuge using a JA-14 rotor. Pelleted
cells were carefully resuspended in 10 mL column with 175 rpm shaking on ice. Lysozyme and phenylmethylsulfonylfluoride (PMSF) were added to a final concentration of 1.0 mmol/L and 0.5 mg/mL respectively, then incubated on ice with 175 rpm shaking for 20 minutes. Protein was released from cell suspensions by two cycles of freeze-thaw (1.0 mmol/L PMSF was added after each) followed by sonication three times for 30 seconds on ice (90 second rest after each) with a Fisher Scientific F60 Sonic Dismembrator at 50% duty cycle. The resultant lysate was clarified by centrifugation at 57000 RCF in a Beckman JA-25.50 rotor for 30 minutes. The supernatant was discarded and the pellet resuspended in Talon buffer (50 mmol/L Tris-HCl pH 7.9, 50 mmol/L KCl, 1 mmol/L EDTA, 10% [v/v] glycerol) containing 1% (w/v) Triton X-100. Any remaining insoluble material was pelleted by centrifugation 57000 RCF in a Beckman JA-25.50 rotor for 30 minutes. The supernatant was applied to a 5 mL column packed with Talon Superflow medium, washed with 50 mL Talon buffer containing Triton X-100, then washed again with 100 mL Talon buffer alone. Protein was eluted from the column in 10 mL Talon buffer containing 160 mmol/L imidazole. Eluted proteins were dialyzed overnight against physiological buffer, centrifuged at 25000 RCF in a Beckman JA-25.50 rotor for 10 minutes to remove any precipitates, then concentrated using Amicon Ultra-15 centrifugal filters (MWCO 10 kDa). Final protein concentration was determined using the bicinchoninic acid (BCA) assay using bovine serum albumin as a standard. This complete process typically yielded 5 mg of total protein. Purified, concentrated protein was then aliquoted and stored at -80°C.

2.3.4 Filling and Exchange System

Using a digital caliper and a ceramic cleaving stone, fused silica capillary tubing (150 µm O.D., 2 µm I.D., Polymicro Technologies, Phoenix, AZ) was cut to a length of 33 mm. This length was chosen to provide a large reaction vessel size as well as optimization of the simple exchange system. For filling and
exchange experiments, capillary tubes were used as-is. For all SLB formation and reconstitution experiments, the polyimide coating on the tubes was stripped (except for an approximately 4 mm length at each end) by careful heating with a miniature butane hand torch (ST200 Micro Torch, Bernzomatic, Columbus, OH). The polyimide coating imparts strength and flexibility to the tubing; however, it is auto-fluorescent and must be stripped to visualize the fluorescent dyes used in the reconstitution experiments.

For all experiments involving filling and exchange, the capillary was sandwiched between two glass coverslips. Double-sided tape was used to secure the capillary in place as well as hold the sandwich together, and a small wax plug was placed around the tube on the top part of the sandwich to prevent leakage of fluid into the sandwich (Figure 2.3b). The sandwich was placed in a holder consisting of two glass slides separated by spacers approximately 1.5 mm thick and held together by binder clips such that the bottom end of the tube was suspended in the air. Exchange by capillary action occurs when the tube is in contact with a surfactant-free cellulose acetate (SFCA) filter with a pore size of 0.45 µm. This filter rests on a platform that is carefully raised to meet the bottom of the capillary, such that the weight of the sandwich is supported by the holder and not the tube itself (Fig. 2.3d). Micro-capillary tubes with a volume of 25 µL, cut to a length of approximately 15mm were used as holding vessels for the delivery of filling and exchange solutions. These are placed on the top length of exposed capillary tube (hereafter referred to as the “fill end”) and fluid exits during capillary exchange from the bottom length (hereafter referred to as the “exchange end”).

2.3.5 Determination and Analysis of Filling and Exchange Rates

Capillary filling time was assessed by filling capillaries (cut as described in section 2.3.3) with deionized water. Due to the differences in refractive index between air (unfilled) and water (filled), it is
Figure 2.3. Ultra-small capillaries and filling/exchange setup. (A) close-up view of freshly-cut capillary (top) and capillary with polyimide stripped (bottom); (B) Capillary in sandwich; (C) Filling setup; (D) Exchange setup
possible to determine the location filling front by light microscopy using an Olympus CX41 microscope equipped with a 20X objective (see Figure 2.4). All replicates (n=20 per time point, 100 total) were treated as trials with a binary outcome, where the possible outcomes were classified as either “filled” or “not filled”. Tubes were considered filled if the filling front was located 0.5 mm or less from the exchange end of the tube. This was chosen to avoid improper classification of tubes as “not filled” due to evaporation of water from the exchange end between removal from the sandwich and sealing to the glass slide. These data were analyzed using logistic regression to determine the time at which >95% of all tubes would be filled. Logistic regression was performed using SPSS statistical software (IBM, New York, NY).

Exchange rates were determined by exchange of deionized water with a 5% solution of bromophenol blue in Tris-EDTA (pH 9.0). Entrance of bromophenol blue is readily evident as a dark purple fill (in contrast to the clear to faint pink fill of deionized water) in light microscopy at 200x total magnification (Figure 2.5c-d). Data were collected as the distance travelled by the filling front, which was used to calculate the volume exchanged.

2.3.6 Supported Lipid Bilayer Formation

Suspensions of small unilamellar vesicles were prepared from a mixture of L-α-phosphatidylcholine derived from chicken egg (egg PC), 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DOPG), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DHPE) in a 79.5:19.5:1 molar ratio, respectively. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Lipids were dissolved in chloroform, mixed, and dried in glass test tubes under a stream of nitrogen gas, then stored in a vacuum chamber (25 in. Hg) at 37 °C for 1 hour to remove traces of chloroform then stored in a vacuum chamber (25 in. Hg) at 37 °C for 1 hour to remove traces of chloroform. Dry lipid films were stored under a nitrogen gas atmosphere at -20 °C until used, for no longer than a week.
On the day of an experiment, dry lipid films were resuspended in deionized water to a final total lipid concentration of 10 mmol/L. Lipids were allowed to rehydrate for 1 h at room temperature with periodic vortexing to ensure complete suspension and formation of multilamellar vesicles (MLVs). The milky suspension of MLVs was sonicated for 8 minutes in an Avanti bath sonicator (Avanti Polar Lipids, Alabaster, AL), generating a clear suspension of small unilamellar vesicles. For lipid bilayer formation in phosphate buffered saline (pH 7.4), PBS was added to a final 1x concentration from a 10x stock; if calcium was used to initiate vesicle fusion, it was added following sonication to 10 mmol/L from a 200 mmol/L stock. In all bilayer formation experiments, SUV suspensions were loaded into a filling vessel, then placed onto the capillary fill end for a period of time determined by the experiment (hereafter referred to as the “fill time”). After this process was complete, capillaries were rinsed with 10 TV of deionized water to remove excess vesicles. Tubes analyzed directly (without reconstitution) were then removed from the coverslip sandwich and the ends sealed to a glass slide using molten paraffin wax.

2.3.7 Reconstitution of FtsZ Rings

Fused silica capillaries were cut, stripped of polyimide, and placed into sandwiches as described in section 2.3.5. Lipid suspensions were prepared in deionized water as described in section 2.3.6. Filling vessels were loaded with SUV suspensions and placed on capillaries for a fill time of 45 minutes, followed by a 10 TV rinse with deionized water. These capillaries, now containing SLBs, were placed in the dark and held until use. At the start of a reconstitution, an SLB-containing capillary sandwich was moved to the exchange apparatus. SLBs were equilibrated to the by exchange of 5 TV of the reconstitution buffer. FtsZ polymerization reactions (in reconstitution buffer) were initiated by adding GTP to 0.2 mmol/L in the single protein system; the dual protein system required 0.2 mmol/L GTP and 0.5 mmol/L ATP. After loading of 1 TV of the polymerization reaction, the sandwich was quickly and carefully removed from the
exchange apparatus and disassembled. The capillary was sealed at each end with molten paraffin wax, coated with a drop of fused silica-matched immersion oil (Cargille Labs, Cedar Grove, NJ) and transferred to the microscope for observation. This entire process, from beginning of reaction loading to focused image, takes 5 minutes.

2.3.8 Image Capture for Bilayer Formation and Reconstitution

Fluorescence micrographs of SLBs and reconstituted rings were captured using a fully-automated Olympus BX61 microscope equipped with a fluorescence-optimized UPlanFL N 100X immersion objective. Images were captured with a Hamamatsu C10600 10-megapixel charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) controlled by SlideBook 4.2 software (Intelligent Imaging Innovations, Denver, CO). For ring formation experiments involving quantification, 30 images were captured per replicate (number of replicates denoted as $n$), with each frame representing a complete field of view. On the camera used in these experiments, each frame captures 84.7 µm of the tube length with each replicate covering 2.54–4.28 mm of the total 25mm available length, or approximately 10–17%. From these images, counts of complete and partial rings were made, as well as the number of actively constricting rings. For qualitative fluorescence recovery after photobleaching (FRAP), a diffraction-limited patch of bilayer was subjected to irradiation with high-intensity light from a 532 nm argon-ion laser for 50 ms, and post-bleach images were captured in series with 3 s interval out to 60s and 10 s interval thereafter. Fluorescence intensities were measured as fractions of pre-bleach using ImageJ.

2.3.9 Statistical Analysis

Capillary filling and bilayer formation trials were treated as binary data with success and failure criteria. These data were subjected to binary logistic regression, which determined the logistic curve coefficients $a$ and $b$. From these the inflection point ($T_{1/2}$) was determined to compare data across trials.
Log-likelihood chi-square (a test of goodness-of-fit of the regression curve) and the Wald statistic (a test of the contribution of the predictor, in this case, time) were calculated and reported. For ring formation and functional data, each measured parameter (number of complete rings, number of partial rings, etc.) in a given experimental treatment (e.g. pH) was treated as a series of trials with a categorical independent variable (MES 6.0, MES 6.5, etc.) and a continuous dependent variable. To determine if the observed variance of the measured mean values across all trials (within a given treatment) was statistically significant, these data were analyzed using a one-way analysis of variance (ANOVA). Comparisons between individual trials were performed using the post-hoc Tukey Honestly Significant Differences test. All statistical analyses were performed in SPSS v.23 (International Business Machines, Armonk, NY).

2.4 RESULTS AND DISCUSSION

2.4.1 Supported Lipid Bilayer Formation

Deposition of supported lipid bilayers (SLB) onto solid, planar substrates (including glass) was established in the lab of Harlan McConnell (12, 13) 30 years ago. It has since become a widely used technique for targeted formation of lipid bilayers in a variety of systems, including microfluidics (14) and large-bore capillary tubes (15, 16); however, limited work has been published using substrates with channels as small as the 0.5-2 µm diameter of the bacterial cell. To create these biomimetic bilayers, we chose as a substrate synthetic fused-silica capillary (Polymicro Technologies, Phoenix, AZ) with an internal diameter of approximately 2 µm. This tubing is manufactured by drawing capillary from a tubular pre-form at high temperatures. The resulting internal surface is ultra-clean and dehydrated, which promotes formation of SLB on silica-based surfaces (17).

Prior to investigation of supported lipid bilayer formation, basic parameters of capillary tube filling were investigated to determine the proper amount of time needed to completely fill the tube with
Figure 2.4. Analysis of capillary filling. (Top) Plot of the logistic regression of filling data showing sigmoidal distribution and transition half-time or $t_{1/2}$. (Bottom) Light micrograph (1000x magnification) of the fused silica capillary inner channel showing the optical difference at the interface between the water-filled (left) and an air-filled (right) parts of the channel.
vesicle suspension. In filling experiments, capillary tubes were filled with deionized water and the distance travelled measured using light microscopy with a 40x objective. We found in these experiments that capillary filling was highly variable at any given time point tested, making typical linear regression analysis impossible. Instead, filling experiments were repeated as binary trials where capillaries that were filled were treated as successes and those that were not filled were treated as failures, and the data were analyzed by logistic regression. The regression and an image showing the optical differences between water- and air-filled capillary channels, is shown in Figure 2.4. Tubes were filled with a half-time of 27.5 seconds, with the expectation that >99% of all tubes would be filled at 39 seconds or more.

Given an average surface area of 70 Å² for egg phosphatidylcholine molecules (18) and a total internal volume of 104 pL, we estimated that coating the inner cylindrical surface of a 33mm length of fused-silica capillary would require an SUV suspension with a total lipid concentration between 9–10 mmol/L. This is estimate is considered a minimum condition. Mechanistically, fusion of vesicles against silica substrates (including glass) is triggered when the surface reaches a critical coverage of adsorbed vesicles. Above this coverage, spontaneous vesicles rupture triggers a rupture cascade that coats the surface in a lipid bilayer. Because salt concentration in the surrounding medium can promote vesicle adsorption and fusion, we performed two versions of this assay. In the first, lipid films are resuspended in water, sonicated to form SUV, then used to fill capillaries. In the second version the suspension medium is brought to 1x phosphate buffered saline (pH 7.4) using a 10-fold concentrated stock following sonication. This method creates an osmotic differential across the vesicle membrane that promotes fusion.

In initial trials, two types of filled tubes were observed. The first, found at short fill times, had a convex curvature (Figure 2.5 middle) consistent with a filled capillary channel. The second, found at longer fill times, had a concave curvature (Figure 2.5 bottom) consistent with a hollow cylinder, indicating that vesicle adsorption and fusion had occurred. Given an approximate measure of fill time
Figure 2.5. Supported lipid bilayer formation in PBS and water. Representative images of the fill end of tubes containing small unilamellar vesicles with a total lipid content of 10 mmol/L in PBS and in water. Bilayer. (Top left) Logistic regression of bilayer formation data for PBS trials and (top right) logistic regression of water trials. (Middle) The convex curvature in the capillary channel indicates a filled tube prior to vesicle fusion; (Bottom) concave curvature indicates a hollow tube in which vesicles have fused.
required for this transition, vesicle loading experiments were repeated at varying time-points where each replicate (n=20 per time point) was treated as a binary trial. In these experiments, tubes with convex curvature, indicating that a supported bilayer had not formed were treated as the “failure” condition and tubes with concave curvature throughout the entire tube, indicating a fused bilayer, were treated as the “success” condition. Because of the closed nature of the system, capillary-supported bilayers could not be directly imaged (e.g. by atomic force microscopy) to confirm that vesicle fusion had taken place, so we chose to assess this indirectly. Vesicle fusion is modulated by temperature; as temperature rises, vesicles fuse more easily and the extent of surface coverage required to initiate the formation of supported bilayers decreases (14). To determine if temperature influenced the convex-concave transition in our vesicle filling experiments, we duplicated the experiments described above with the addition of a 30-minute incubation of the wax-sealed tubes at 37 °C prior to imaging. These data were subjected to binary logistic regression; the logistic curves are shown in Figure 2.5 (top) and regression data are reported in Table 2.1.

In all experiments, the transition to a fused bilayer occurs well in excess of the experimentally determined fill time of the capillaries (t_{1/2} = 27.5 sec). This observation is consistent with the assumption that the amount of lipid used to suspend vesicles is a minimum requirement and indicates that some additional loading of vesicles into the capillary is required to initiate the transition from a filled, adsorbed condition to one where vesicles fuse against the silica substrate. In water-suspended vesicles this occurs with a half-time of 33 minutes, which is decreased by introduction of an osmotic differential when vesicles brought to 1x PBS (t_{1/2} = 16.4 min) and by incubation at elevated temperature (t_{1/2} = 23.4 min). Use of both PBS and heat had a synergistic effect, resulting in a lower half-time (t_{1/2} = 9.5 min) than with either condition alone. In all experiments, the log-likelihood chi-square exceeded the chi-square critical value (Table 2.1) indicating an excellent fit of the data to a logistic curve. Additionally, the Wald statistic exceeds the critical value for each, meaning that time is a highly significant predictor of the probability
Figure 2.6. Supported bilayer formation in calcium chloride. Fluorescence micrographs show discontinuous bilayer formation in the presence of 10 mmol/L calcium. Time scale represents vesicle fill time. These representative images are not a single time-course and are taken from separate trials, which is required due to the need for wax sealing over capillary ends.
Table 2.1: Logistic Regression Analysis of Bilayer Formation Data

<table>
<thead>
<tr>
<th>Condition</th>
<th>a</th>
<th>b</th>
<th>T1/2 (min)</th>
<th>Likelihood Ratio $\chi^2$</th>
<th>Wald Statistic**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>-9.141</td>
<td>.277</td>
<td>33</td>
<td>98.508</td>
<td>38.712</td>
</tr>
<tr>
<td>Water + Heat</td>
<td>-7.912</td>
<td>.338</td>
<td>23.4</td>
<td>99.493</td>
<td>32.111</td>
</tr>
<tr>
<td>PBS</td>
<td>-4.691</td>
<td>.283</td>
<td>16.8</td>
<td>51.842</td>
<td>31.838</td>
</tr>
<tr>
<td>PBS + Heat</td>
<td>-7.088</td>
<td>.75</td>
<td>9.5</td>
<td>58.185</td>
<td>27.934</td>
</tr>
<tr>
<td>Calcium</td>
<td>-1.018</td>
<td>.318</td>
<td>3.2</td>
<td>92.548</td>
<td>33.511</td>
</tr>
</tbody>
</table>

* for probability = 0.05 and df = 6, critical value = 12.592

** for probability = 0.05 and df = 1, critical value = 3.841
that a tube will contain a fused bilayer. These findings are all consistent with the hypothesis that the convex-concave transition represents a transition to the formation of a fused, supported lipid bilayer.

To bolster these data, a different mechanism of SLB formation was investigated. In the presence of calcium ions, vesicles fuse to silica substrates without respect to a critical concentration (14). This provides free bilayer edges that serve as a secondary catalyst for vesicle fusion. In the presence of 10 mmol/L calcium chloride, we found that SLB formation proceeds much differently than in previous experiments. At low fill times, the convex curvature condition appears immediately. This begins as sparse patches in the background of a faintly fluorescent central channel (Figure 2.6, 1 min) from which larger sections of bilayer grow (Fig. 2.6, 2 min). These bilayers are discontinuous with large gaps (Fig. 2.6, 3 min and 4 min) which eventually fill in completely to form a continuous lipid layer (Fig 2.6, 5 min). Bilayer formation is much more rapid ($T_{1/2} = 3.2$ min) than in any other trials. These data, as well as the previous bilayer formation data, are consistent SLB formation behavior reported in the literature and indicate that the convex-to-concave transition seen does in fact represent a transition to a fused, supported lipid bilayer.

As a final confirmatory step, mobility of lipids within the bilayer was assessed using fluorescence recovery after photobleaching (FRAP). This was done for two reasons. First, prior published reports suggest that lateral mobility of membrane-attached FtsZ filaments is necessary for condensation of Z-rings in vivo (11, 19). Secondly, adsorbed but unfused vesicles would not recover after photobleaching; fluorescence recovery indicates a fused, continuous bilayer. Data from qualitative FRAP experiments using bilayers formed in water are shown in Figure 2.7. In this figure, data are normalized against fluorescence intensity readings from an unbleached portion of bilayer to remove the contribution of photobleaching caused by data capture conditions and are presented as a ratio of measured fluorescence intensity vs. mean pre-bleach intensity. Fluorescence intensity recovers from 35% of maximum to 80%
Figure 2.7. FRAP data from bilayers formed in water. Photobleach period was 50 ms and is centered at time = 0. Data are reported as fluorescence intensity normalized against an unbleached section of bilayer, rather than as arbitrary fluorescence units.
Figure 2.8. Buffer exchange rate in capillaries with supported bilayers. Buffer exchange data (top) are presented as a ratio of distance traveled to total tube length. The intercept of the trendline at $y = 1$ represents the time necessary to exchange 1 tube volume. (Bottom) Micrograph (400x magnification) of the inner channel of a capillary showing the optical differences between a water-filled, air-filled, and dye-filled channel.
within 75 seconds and levels out at approximately 87% within 200 seconds. This recovery response serves as another indicator of fusion and confirms that bilayers are continuous and that lipids within them are able to diffuse laterally within the bilayer.

As a final step in development of the supported bilayer system, we measured the buffer exchange rates in tubes with and without supported bilayers. This was accomplished using a saturated solution of bromophenol blue dye, which was placed in a filling vial and subjected to buffer exchange. The interface between the dye and water is easily visualized with conventional light microscopy (Figure 2.8, bottom) and can be used as a guide to measure exchange as distance travelled by the dye front over time. These data are shown in the graph at the top of Figure 2.8. There is a linear relationship between buffer exchanged (normalized as a percentage of the entire tube length travelled) and time in all trials. The mean time necessary for full exchange of 1 tube volume (TV) in tubes lacking bilayers is 3.2 minutes while 1TV times in water-suspended and PBS-suspended vesicles were 4.4 minutes and 9.6 minutes respectively. The reason why exchange in PBS-suspended vesicles is more than two-fold slower than water-suspended vesicles is unclear, but evaporation at the exchange end of the tube may cause a buildup of salt that narrows the orifice of the central channel, lowering flow rate. With all of the SLB formation data taken in totality, we chose to use water-suspended vesicles for SLB formation in reconstitution experiments.

### 2.4.2 Reconstitution of FtsZ Rings

Given a properly formed supported bilayer in a fused silica capillary, reconstitution is as simple as initiation of polymerization in a buffered solution of FtsZ by addition of GTP and exchange of 1 TV of that polymerization reaction into the tube. The base buffer, or physiological buffer (PB) used in these experiments approximates the chemical conditions of an exponentially-growing cell: slightly alkaline pH
Figure 2.9. Assembly and condensation of FtsZ on supported bilayers. Merged red-channel (lipid) and green-channel (YFP) images of reconstituted SPS rings. The initial image (372 sec) represents the delay between initiation of exchange and image capture. Several different types of reconstituted structures can be seen, including complete Z-rings, which appear as bars with bright dots at the ends and are immobile and partial rings, which appear primarily as paired dots. Yellow triangle indicates the condensation of an immobile ring from several mobile rings. White triangle shows constriction of the bilayer, which does not complete but leaves a visible deformation. By 1800 sec, GTP has been exhausted and rings disassemble. FtsZ concentration is 7 mmol/L and GTP concentration is 200 µmol/L. Scale bars are 1 µm.
(7.7), high internal potassium (370 mmol/L total), typical magnesium concentration (5 mmol/L), and low amounts of other typical ions (20). In the single protein system (SPS), membrane-targeted FtsZ-YFP is reconstituted; in the dual protein system (DPS) reconstitutions use both non-targeted FtsZ-YFP and the hypermorphic FtsA*.

A representative time course from initial reconstitution experiments using these parameters is shown in Figure 2.9. In this series you can see that each field of view captures multiple FtsZ structures. Complete rings, which appear as simple bars and bars with bright dots at the ends, are immobile. Partial rings, which appear as parallel or tilted pairs of dots or a faint bar, are mobile and diffuse laterally within the ring. Condensation of rings (yellow triangle) occurs by means of direct merging of partial rings, which become immobile shortly after merging. Lastly, you can see constriction of a complete Z-ring (white triangle), which becomes arrested before the process completes. After GTP is exhausted (1800 sec) reconstituted rings disappear and a small deformation of the membrane is visible where the constricting ring once was. In prior reconstitutions using tubular liposomes, failed constriction events would rebound and leave no deformation; however, given that bilayers in this experiment are supported by interaction with the silica surface, the deformation persists, likely due to sliding of the bilayer along the silica surface during constriction.

Figure 2.10 shows enlarged images of Z-ring morphologies found within the reconstitution system, both SPS and DPS. Rings that are immobile are complete rings, dense enough with filaments to visualize it as a bar, or more frequently, a bar with bright ends and a fainter middle (top row). Mobile, incomplete rings appear rarely as a faint bar (middle left) and more frequently as paired dots. Mobile rings are most frequently parallel, though in some cases they can be slightly tilted. Tilted rings become less common as time progresses, suggesting that the filaments within may be either loosely affiliated, of a straighter curvature, or both. This causes them to align differently with the membrane surface, which disappears as
Figure 2.10. Z-ring morphologies in the SPS and DPS. This mixed series of images shows variations in Z-rings in imaging in both the SPS and DPS. Immobile rings are brighter and appear as faint bars with bright dots at each end. Mobile rings appear primarily as paired dots and rarely, faint bars. Arcs (bottom) appear as unpaired dots. Scale bars are 1 μm.
the ring matures. Very rarely and very early in the reconstitution time-course, arcs of filaments can be observed as partial bars or single dots. These are also likely immature structures. There is an average delay between initiation of exchange and image capture, in which data regarding the development of partial rings from very early structures including arcs and possibly spirals are simply missing.

In the realm of Z-ring function, the evidence we have compiled is in agreement with prior published data. Z-rings in the SPS initiate constriction but arrest at a point of partial constriction. Constriction does not complete and no intervening septum is formed, which can be seen once GTP is exhausted through hydrolysis by FtsZ. Representative images of the time course of this process are shown in Figure 2.11. Constriction first becomes visible at 300 seconds after initial imaging and the ring has become visibly shorter and wider. Constriction continues until 520 seconds, after which the ring arrests in a partially-constricted state. Once GTP is exhausted (1660 sec) a persistent membrane deformation is visible. The bottom image shows the same image with an inset (white square) that has had the brightness and contrast of the inset area modified in Adobe Photoshop to better show the deformation.

The initial stages of constriction in the DPS (Figure 2.12) proceed as seen in the SPS. At later stages, the image of the ring has shrunk to the point that in the image it appears as a single, relatively fainter dot (820 seconds) at the middle of an invaginating septum. After a short period of time (60 seconds in this series) the septum resolves into a completed scission event (880 seconds) and the ring in the area has disassembled. Not all immobile rings in the DPS initiate constriction, though nearly all that we observed do complete it. Those that do not initiate constriction very late after addition of protein and fail within the range of time that the GTP in the reaction would be exhausted by hydrolysis.

We can see from these images that rings reconstituted on cylindrical supported bilayers have a varied morphology and exhibit complex function like ring condensation, membrane constriction, and
Figure 2.11. **Z-ring constriction in the SPS.** Time course imaging shows initiation and failure of constriction in SPS rings. Times in the figure are reckoned from initial image capture. Constriction is visible at 5 minutes (300 sec); by 8.7 minutes (500 sec) it has arrested, and remains in that arrested state until GTP is exhausted (1640 sec), leaving behind a small membrane deformation. Bottom image is a contrast-enhanced version of the image from 1660 seconds that better shows the deformation. Scale bars are 1 µm.
Figure 2.12. **Z-ring constriction and membrane scission in the DPS.** Time course imaging shows initiation and failure of constriction in SPS rings. Times in the figure are reckoned from initial image capture. Constriction is visible at 340 sec and continues until 820 sec, where it stops. 60 seconds later (880 sec) the small ring disassembles, revealing a completed membrane scission event. Scale bars are 1 µm.
membrane scission. The system as designed is fully capable of reproducing the range of behavior seen both
*in vivo* and in other *in vitro* reconstitutions.

2.4.3 Effect of Protein Concentration on Reconstituted Rings

The concentration of protein used in initial reconstitution experiments was determined by amounts used in other published reconstitution methods. It was necessary, before proceeding to study of physiological parameters, to optimize the amount of FtsZ and FtsA used in both reconstitution methods to maximize the working range of the system. FtsZ was used at the amount reported in both systems; in the DPS, FtsA* was used in equimolar ratio to FtsZ, so that the total protein concentration is twice that of the concentration of FtsZ used. Additionally, to accommodate increased amounts of protein in later trials, all experiments were initiated by bringing the reaction to a final concentration of 1 mmol/L for GTP and in the DPS, both GTP and ATP. A series of 30 images were captured at different, random points along the capillary and counts were made of complete rings, partial rings, actively constricting rings, membrane deformations (SPS) and membrane scission events. These data and statistical analyses are compiled in Table 2.2.

In both systems, increasing FtsZ concentration was accompanied by a corresponding increase in both total number of complete Z-rings (Figure 2.13, top) as well as partial rings (Table 2.2) This is expected in an unbounded reconstitution system; however, this increase is not flat with respect to the amount of FtsZ in the input. When the number of rings is normalized against the input concentration of FtsZ, there is a statistically significant decrease over the range of concentrations in the experiment (p < 0.001 for both systems) which is most prominent at higher concentration. This “ring gap” is not explained by a proportional increase in partial rings; a significant decreasing trend is present in partial rings as well.

The total number of functioning Z-rings shows a statistically significant increase with
Figure 2.13. Concentration-dependent variation in Z-ring formation. Variation in total rings formed (top) and rings formed normalized for input protein concentration (bottom) are shown for the single-protein system (blue line with circles) and dual-protein system (red line with squares). Data points represent means with standard deviation depicted as error bars.
Figure 2.14. Concentration-dependent variation in Z-ring function. Variation in total functioning rings (actively constricting rings plus membrane deformations/scission events) is shown at top and the ratio of functioning rings to total rings (bottom) are shown for the single-protein system (blue line with circles) and dual-protein system (red line with squares). Data points represent means with standard deviation depicted as error bars.
<table>
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<tr>
<th>[FtsZ] (mmol/L)</th>
<th>Mean # Rings</th>
<th>Mean # Partial</th>
<th>Rings/[FtsZ]</th>
<th>Partial/[FtsZ]</th>
<th>Active Constriction</th>
<th>Bilayer Deformation</th>
<th>Total A+D**</th>
<th>Ratio C/R***</th>
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*Data presented as mean ± S.D.

**Sum of actively constricted rings and bilayer deformations due to constriction

***Ratio of constriction data (total A+D) to all fully formed rings
concentration as well (Figure 2.14, top). In this metric, the number of actively constricting rings is added to the number of membrane deformations (in the SPS) or membrane scission events (in the DPS). Also of note is that the number of functioning Z-rings as a proportion of the total number stays flat with on significant variation with a p-value of 0.075 in the SPS and 0.070 in the DPS. Not surprisingly, the presence of FtsA increases both total ring formation (Fig. 2.13, top) and total functioning rings (Fig. 2.14, top); however, the proportion of functioning rings is increased (on average) 2.5-fold over the SPS (Fig 2.14, bottom).

2.5. CONCLUSIONS

In this work, we report the successful development of an in vitro reconstitution system incorporating cylindrical supported lipid bilayers in the inner channel of an ultra-microcapillary tube as a surface for the targeted assembly of FtsZ using both single protein and dual protein methods. The response of the system to input protein concentration is robust and reproducible over the full range of FtsZ concentrations seen in vivo, making it ideal for future work directly examining the conditions that alter Z-ring formation and function and with potential correlation of in vivo and in vitro data.

Supported bilayer formation in the system is simple and is adapted from well-established methods used for generating supported lipid bilayers for the last 30 years. One major advantage of this particular system is elimination of the need for pre-treatment of the supporting surface. For methods that generate supported bilayers on planar substrates or large-bore capillary tubes, borosilicate glass substrates must be extensively prepared, usually by sonication in various chemical baths followed by plasma cleaning to remove organic debris. The ultra-micro capillaries are manufactured by mechanical pulling from a molten silica pre-form which creates an internal surface that is both hydrophilic and dehydrated, which is optimal for SUV adsorption and fusion, as well as free of organic contaminants.
Because our initial expectations for the amount of lipid necessary to form continuous bilayer were informed by the calculation of a minimum expectation, we were able to assess the properties of bilayer formation by slow addition of vesicles to the tube (following the initial fill) due to an evaporative loading effect. This was important because the small size and configuration of the capillary made it impossible to directly image bilayer formation using traditional means (e.g. atomic force microscopy). We found that the amount of filling time (and thus indirectly, the amount of lipid in the tube) needed to trigger the transition to a fused, continuous bilayer was sensitive to osmotic differential and temperature and the addition of 10 mmol calcium allowed the imaging of bilayer formation over time as well as recapitulate the agreement of our data with prior published data on the mechanisms of supported lipid bilayer formation. Calcium-induction of fusion resulted in very rapid bilayer formation but unfortunately, calcium can also induce FtsZ bundling. That, along with the comparatively slower rate of exchange in capillaries with SLBs formed in PBS made water-suspension of SUVs the best choice for SLB formation in this system. Qualitative FRAP experiments on water-formed bilayers showed lipids within the bilayers were laterally diffusible, which is necessary for condensation of membrane-associated FtsZ filaments.

Initial reconstitutions showed that reconstituted rings come in two primary forms: mobile and immobile. Immobile rings have a much higher fluorescence intensity and appear in micrographs as a bar or bar with two bright dots. This morphology is similar to that seen with complete, functioning rings in vivo, so it is reasonable to interpret the immobile rings as complete rings that have begun the process of constriction but are unable to deform the membrane. Mobile rings appear primarily paired dots, which are parallel to each other. Rarely, paired dots can appear offset or “tilted”, indicating preferential alignment along the membrane to a larger radius of curvature. These typically disappear as the time course of the experiment progresses and may represent an early transition between an extended ring or spiral structure. These structures are extraordinarily rare in the reconstitution and likely represent an early phase of
condensation that is resolved in the 5 minute “dead” period between initiation of exchange and microscopic imaging. Polymerization in FtsZ reaches a steady state on the order of 5-30 seconds depending on solution conditions. FtsZ being exchanged into the capillary are primarily in filament form, and attachment can begin immediately upon introduction into the tube.

In both single-protein and dual-protein system, functional and constricting rings form. In the SPS, rings are unable to complete constriction and arrest in a constricted state until the GTP in the solution is exhausted, leaving behind a small but detectable membrane deformation. In prior published work in tubular liposomes, these deformations rebound and eventually become undetectable. This reconstitution system has a key difference in that the membrane is supported by the silica surface and is ultimately not continuous at the ends. Because of this, there is no tension in the membrane to cause it to rebound. Alternatively, the force imparted against the membrane in areas adjacent to the ring may not enough cause it to break its association with the glass. The bilayer is separated from the glass by a layer of water and is supported but not rigidly anchored, and the reduced transmission of the deforming force instead causes the bilayer to slide, resulting in a small area where the membrane becomes permanently deformed. In the DPS, the action of FtsA* is sufficient to correct the constriction block and membranes fully constrict and also appear to fuse. The optical properties of the capillary tubes make phase contrast microscopy impossible, so there is no way to directly confirm that a complete septum was formed, though reconstitutions under similar conditions did result in fully resolved septa. This fascinating result means that the resolution of the membrane scission event is either catalyzed by FtsZ or is an inherent property of membranes brought in close proximity.

Lastly, optimum concentration of protein for use in later reconstitutions was determined by varying input protein concentration. The long tube allowed the capture of large datasets, enabling
calculation of mean outputs with low standard deviations and detection of highly significant variations in ring formation and function due to concentration.

Because the reconstitution system is unbounded, meaning that the space in which Z-rings can form is neither confined physically by the ends of a cell nor biochemically by the presence of negative regulators, we expected that the number of formed and functioning rings should vary proportionally with input protein concentration. We found that to be the case, significantly so, but with one curious caveat. While overall ring number increased in both systems, the proportion of rings formed per unit (mmol/L) of input protein did not stay constant, but instead decreased as protein concentration increased. This resulted in a “ring gap” that was not accounted for by increased number of partial rings. If increasing concentration also causes a proportional decrease in the time required for rings to initially form, it is possible that rings can form and break down entirely within the dead period of the experiment and thus never be imaged. This explanation is bolstered by the fact that the ring gap is more pronounced in the dual protein system, where rings are proportionally more functional and dynamic. Also as expected, the number of functioning rings (determined by the number of visualized constricting rings plus the number of membrane deformations or scission events) also increased with concentration; however, the proportion of functioning rings to formed rings stayed relatively flat and did not exhibit a statistically significant variation, showing that there is no concentration-dependent effect on Z-ring function. Given these data, we chose an initial starting concentration of 8 mmol/L for future experiments. This concentration falls in the middle of both the range of the experiments performed and the range of concentrations of FtsZ in vivo, a harmony which can improve correlation between in vitro and in vivo results.

From a technical perspective, the method represents an improvement on other techniques while recapitulating many of the fundamental observations from those systems. Reproducibility is a huge problem in other published in vitro systems, where visual proof-of-concept was sufficient for their
experimental aims, but is poor for investigating physiological questions. In the tubular liposome reconstitution, for example, only a small number of liposomes were tube-shaped and permeable to the protein in the suspension medium. Further complicating matters is rings could only form in liposomes of a very small size. In our system, the diameter of the supported bilayer is consistent and a good cognate to the size of bacterial cells. Protein is directly exchanged into the tube itself, guaranteeing a uniform concentration across replications that is not sensitive to the permeability of the membrane structure.

That said, there are some drawbacks to the system. Though it produces high-quality datasets, it is extraordinarily labor-intensive. Though the surface preparation is not necessary, tubes still must be prepared from a roll, requiring careful cutting and stripping of polyimide. Experimentally, not all microscopic functions are able to be automated, resulting long time-courses spent manually adjusting microscope parameters. The first problem is easily solved. The manufacturers provide cutting services as well as tubes with coatings that are not auto-fluorescent, but making capillary with this particular set of parameters (size, plus coating, plus cut) requires large and expensive custom runs that simply weren’t practical for new system development. The second problem could potentially be solved by use of a different detection technique. The capillary that is used in these experiments is also used in chromatography arrays and capillary electrophoresis. A laser-based detection system for the YFP fluorophore could allow automated ring counts, but at this moment is impractical.

The closed nature of the system causes some unresolvable issues as well. The cylindrical capillary introduces spherical aberrations that preclude high-resolution and super-resolution imaging techniques lacking some means of correction. Also, only initial conditions of assays can be modified. Once in the capillary, no further changes can be made. Despite these, the physiological data that can be generated with this version of Z-ring reconstitution will be incredibly valuable for our understanding of how environmental conditions and accessory proteins affect formation and function of FtsZ rings.
2.6 REFERENCES


CHAPTER III

PHYSIOLOGY OF Z-RING ASSEMBLY AND CONSTRICTION

3.1 ABSTRACT

A variety of extrinsic and intrinsic factors regulate the ability of FtsZ to form cytokinetic rings both in vivo and in vitro. Much of our understanding of Z-ring form and function to this point has been derived from analysis of filament dynamics in assembled FtsZ in vitro, which may not be useful in the context of a multi-stranded, multi-protein structure. In this work, we use a newly-developed in vitro reconstitution system to assemble rings with FtsZ-only and two-protein rings with FtsZ and its anchoring protein, FtsA to assess the effect of anchoring ratio, pH, calcium, and GTP inhibition on FtsZ ring form and function. We found a complex regimen of regulation by these four factors, where some factors affected either one of formation and function or, occasionally, both. In total, effects that reduce protein turnover and increase filament order (e.g. low calcium and slightly acidic pH) increase ring formation and the initial stages of constriction, while those that increase protein turnover aid in producing membrane deformation (characteristic of incomplete septation in Z-only systems) and membrane scission, which is the completed division of the lipid bilayers. Given these data, we reformulate the working model of the FtsZ ring to one that incorporates both filament sliding and filament dynamic modes to accomplish the task of cytokinesis.
3.2 INTRODUCTION

Reproduction in all prokaryotes (both the Archaea and the Bacteria) occurs asexually, by means of *binary fission*. Fission is subdivided into three physiologically distinct phases: growth, synthesis, and septation. Here the cell splits in half permanently, due to pinching of the cellular membrane (*cytokinesis*) and the synthesis of a cell wall in the space between. These two aspects of septation are performed by a massive, multi-protein “machine” called the *divisome* (1). The protein components of the divisome coordinate in space and time the pinching (*constriction*) of the cell membranes (driven by internal elements of the cell) with the biosynthesis of new external cell structures, such as the cell wall and the outer membrane of Gram-negative bacteria. When cytokinesis is complete, the cell membrane fuses and the newly-formed daughter cells separate following partial breakdown of the septal cell wall.

The most important element of the divisome is the protein FtsZ. Named for the phenotype of the first isolated mutant—filamenting temperature-sensitive mutant Z—FtsZ is the principal component of a cytoplasmic protein ring, located at the middle of the dividing cell, which is necessary for divisome formation (2). The reach and importance of FtsZ and FtsZ-like proteins is not limited to *E. coli*. Nearly all prokaryotes possess at least one gene encoding an FtsZ-like protein, with a few bacterial species and many Archaebacteria having two or more (3). In all of the organisms in which it resides, FtsZ retains its basic function as a ring of filaments, underlying the membrane, located at the middle of the cell or organelle that it assists in dividing. In addition, this ring (the *Z-ring*) is the foundation of a complex web of genetic and protein-protein interactions that direct the timing and progression of septation. The particular details of Z-ring regulation and cellular physiology are as diverse as the organisms that rely on its function.
In assembly reactions of purified FtsZ, in buffers that approximate the internal conditions of the cell, FtsZ protofilaments are a single subunit thick and an average of 125 nm long, a polymer length of 25 individual monomers (4). Altered concentrations of cations (both monovalent and divalent) and pH, surface interactions, crowding agents and the presence of various GTPase inhibitors alter the aspect ratio (both length and width) and dynamics (as measured by subunit turnover) in a very regular and consistent manner. Stabilizing conditions are accompanied by a decrease in subunit turnover, an increase in average length of the polymer and in some cases, lateral bundling of filaments. Destabilizing conditions are accompanied by increasing subunit turnover, shortening of polymers, and a breakdown of lateral associations.

Positive regulation of FtsZ filaments by assembly conditions is a well-documented phenomenon. Reduction of pH to 6.5 reduces GTP hydrolysis rate by altering the configuration of GTP within the active site; this stabilizes the subunit interface, which results in the lengthening of polymers and causes them to adopt a straight, rigid conformation (5–7). Further reduction to pH 6.0 both deepens enzymatic inhibition and results in the formation of protofilament pairs and bundles by stabilizing lateral interactions (7). The presence of monovalent cations rubidium and sodium (above 50 mmol/L) as well as divalent calcium and excess magnesium (above 10mM) cause filament bundling (8–11). At calcium concentrations in excess of 10 mmol/L, FtsZ forms large, gel-like polymer networks (9), a property that can be used to assist in removing inactivated FtsZ monomers from protein preparations and serve as quick-check assays for polymerization. Lastly, assembly in GTP analogs that are slowly-hydrolyzing mimic this stabilizing effect, resulting in polymer lengthening and straightening (8, 12, 13).

Each of these conditions has a logical regulatory role in the context of cell physiology. Bacteria like E. coli are robustly homeostatic and can tolerate a wide range of environmental shifts in pH and osmotic pressure. Sodium and calcium are normally kept low in the cell and high outside (14). Persistent
dysregulation of these indicate a physical disruption of the cell (e.g. membrane breach) or other homeostatic failure. In such a case, the ability to disrupt cytokinesis until balance is restored would be beneficial for survival. Protein based systems that utilize this concept are known; the protein SulA, expression of which is induced by the SOS response to DNA damage, blocks Z-ring formation by binding to and sequestering individual monomers and preventing polymerization of FtsZ filaments. The advantage of a regulatory regime such as this, that directly impacts the intrinsic properties of the FtsZ filaments, is a rapid response that requires no protein activation or gene expression.

Given the homeostatic nature of living cells, reconstitution of the Z-ring \textit{in vitro} is a critical tool for understanding the physiological aspects of its formation and function, especially for basic environmental conditions like cation concentrations and pH. For this purpose, a system was designed utilizing ultra-small-bore capillary tubes as a support for the formation of biomimetic lipid bilayers. The internal volume of the tube is exchanged by simple capillary action, and this is used to perform bilayer rinsing, buffer equilibration, and introduction of FtsZ-containing polymerization reactions.

This system represents an improvement on previous reconstitution methods in that it more closely mimics the \textit{in vivo} condition encountered by \textit{E. coli} FtsZ: a hollow, cylinder-shaped, \textasciitilde1 \textmu m diameter lipid bilayer that allows reconstitution of Z-rings on the inner surface by introduction of defined buffered solutions containing known amounts of purified FtsZ or membrane-targeted recombinant FtsZs. The role experimental conditions play in ring formation and constriction can be assessed by rates of ring formation and constriction captured by fluorescence microscopy and the long tube length provides statistical strength by permitting large sample sizes within a single trial and high reproducibility between replications of trials. This chapter describes our work on elucidating the regulatory role of basic solution conditions, as well as GTPase inhibition, on formation and function of rings formed in both single-protein (FtsZ-YFP-mts) and dual protein (FtsZ-YFP/FtsA*) reconstitution methods.
3.3 METHODS

3.3.1 Plasmids and Strains Used

Plasmids pET-ZY and pET-ZYM, used for expression of FtsZΔC-YFP and FtsZΔC-YFP-mts respectively, were a kind gift of Dr. Harold Erickson. Plasmid pET-A* used for expression of FtsA*, was a kind gift of Dr. William Margolin. All expression from pET-derived plasmids was performed using *E. coli* strain C41, which gives higher yields of soluble proteins than BL21, especially proteins that are potentially toxic (citation).

3.3.2 Protein Expression and Purification

FtsZ-YFP and FtsZ-YFP-mts were prepared using a method modified from Osawa and Erickson (15). Electrocompetent cells of *E. coli* strain C41(DE3) were freshly transformed with pET-ZF or pET-ZFM. A single transformant was picked and grown overnight in 25 mL Luria-Bertani medium with ampicillin (LB+Amp, 100 µg/mL) at 37 °C in a shaking incubator (225 rpm shaking). The next day, this culture was centrifuged at 4000 RCF for 15 minutes to remove the used medium, resuspended in 25mL fresh LB+Amp, then used to inoculate 500 mL of fresh LB+Amp. This culture was incubated at 37 °C with 225 rpm shaking until the culture reached an optical density between 0.8 and 1.0. At this point, protein expression from the clone was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG, Fisher Scientific, Pittsburgh, PA) to a final concentration of 0.5 mmol/L. Induction continued with incubation at 37 °C with 225 rpm shaking for 4 hours, after which the cultures were moved to a rotating shaker at room temperature (175 rpm shaking) and left overnight. The following morning cells were split into 250 mL bottles, then pelleted by centrifugation at 6000 RCF in an Avanti J26-XP ultracentrifuge using a JA-14 rotor. Pelleted cells were carefully resuspended in 10 mL column buffer (50 mmol/L Tris-HCl pH 7.9, 50 mmol/L KCl, 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 10% [v/v] glycerol)
with 175 rpm shaking on ice. Lysozyme and phenylmethylsulfonylfluoride (PMSF) were added to a final concentration of 1 mmol/L and 0.5 mg/ml respectively, then incubated on ice with 175 rpm shaking for 20 minutes. These cell suspensions were then frozen at -80 °C overnight or until needed.

Protein was released from cell suspensions by two cycles of freeze-thaw (1 mmol/L PMSF was added after each) followed by sonication three times for 30 seconds on ice (90 second rest after each) with a Fisher Scientific F60 Sonic Dismembrator at 50% duty cycle. The resultant lysate was clarified by centrifugation at 57000 RCF in a Beckman JA-25.50 rotor for 2 hours. The supernatant was collected, then brought to 30% saturation by slow addition of 3.52g of solid ammonium sulfate. After addition of ammonium sulfate, the mixture was incubated for 30 minutes on ice. Salt-precipitated protein was pelleted by centrifugation at 25000 RCF in a Beckman JA-25.50 rotor for 20 minutes. This supernatant was discarded and the pellet resuspended in 10 mL ice-cold column buffer, then clarified by passing through a 0.22 µm cellulose acetate syringe filter. The protein solution was dialyzed overnight against 2 L of column buffer, then further purified by anion exchange chromatography. A HiTrap DEAE FF 15/10 (GE Healthcare) column was used and protein was eluted with a 100 mL gradient from 50-500 mmol/L KCl with a flow rate of 1.0 mL/min. To ensure reproducibility between batches of protein, the column was cleaned after each purification run using the “rigorous cleaning” protocol suggested by the manufacturer.

Peak fractions were identified by plots of UV absorbance at 280 nm and verified by the presence of the yellow-colored YFP protein. These fractions were pooled, dialyzed overnight against physiological buffer (50 mmol/L Hepes/KOH, pH 7.7, 5 mmol/L magnesium acetate, 300 mmol/L potassium acetate, 10 % [v/v] glycerol, 0.1% DTT), and concentrated using Amicon Ultra-15 centrifugal filters (MWCO 10 kDa). Final protein concentration was calculated according to the Beer-Lambert law using the spectrophotometrically-determined absorbance at 515 nm and a molar extinction coefficient of 92,200 M⁻¹
cm$^3$. This complete process typically yielded 30-40 mg of total protein. Purified, concentrated protein was then aliquoted and stored at -80°C.

FtsA* was expressed from plasmid pWM1690, a kind gift of Dr. William Margolin (University of Texas-Houston Medical School), which was freshly transformed into chemically competent C43 cells (Merck Millipore, Billerica, MA). A single transformant was picked and grown overnight in 25 mL Luria-Bertani medium with 50 µg/mL kanamycin sulfate (LB+Kan) 37 °C in a shaking incubator (225 rpm shaking). The next day, this culture was centrifuged at 4000 RCF for 15 minutes to remove the used medium, resuspended in 25mL fresh LB+Kan, then used to inoculate 500 mL of fresh LB+Kan. This culture was incubated at 37 °C until reaching an optical density at 600 nm of 0.5. At this point, protein expression was induced with 0.5 mmol/L IPTG. Induction continued with incubation at 37 °C with 225 rpm shaking for 4 hours, after which the cultures were moved to a rotating shaker at room temperature (175 rpm shaking) and left overnight. The following morning, cells were split into 250 mL bottles, then pelleted by centrifugation at 6000 RCF in an Avanti J26-XP ultracentrifuge using a JA-14 rotor. Pelleted cells were carefully resuspended in 10 mL column with 175 rpm shaking on ice. Lysozyme and phenylmethylsulfonylfluoride (PMSF) were added to a final concentration of 1.0 mmol/L and 0.5 mg/mL respectively, then incubated on ice with 175 rpm shaking for 20 minutes. Protein was released from cell suspensions by two cycles of freeze-thaw (1.0 mmol/L PMSF was added after each) followed by sonication three times for 30 seconds on ice (90 second rest after each) with a Fisher Scientific F60 Sonic Dismembrator at 50% duty cycle. The resultant lysate was clarified by centrifugation at 57000 RCF in a Beckman JA-25.50 rotor for 30 minutes. The supernatant was discarded and the pellet resuspended in Talon buffer (50 mmol/L Tris-HCl pH 7.9, 50 mmol/L KCl, 1 mmol/L EDTA, 10% [v/v] glycerol) containing 1% (w/v) Triton X-100. Any remaining insoluble material was pelleted by centrifugation 57000 RCF in a Beckman JA-25.50 rotor for 30 minutes. The supernatant was applied to a 5 mL column packed
with Talon Superflow medium, washed with 50 mL Talon buffer containing Triton X-100, then washed again with 100 mL Talon buffer alone. Protein was eluted from the column in 10 mL Talon buffer containing 160 mmol/L imidazole. Eluted proteins were dialyzed overnight against physiological buffer, centrifuged at 25000 RCF in a Beckman JA-25.50 rotor for 10 minutes to remove any precipitates, then concentrated using Amicon Ultra-15 centrifugal filters (MWCO 10 kDa). Final protein concentration was determined using the bicinchoninic acid (BCA) assay using bovine serum albumin as a standard. This complete process typically yielded 5 mg of total protein. Purified, concentrated protein was then aliquotted and stored at -80°C.

### 3.3.3 Supported Lipid Bilayer Formation

Suspensions of small unilamellar vesicles were prepared from a mixture of L-α-phosphatidylcholine derived from chicken egg (egg PC), 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DOPG), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DHPE) in a 79.5:19.5:1 molar ratio, respectively. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Lipids were dissolved in chloroform, mixed, and dried in glass test tubes under a stream of nitrogen gas, then stored in a vacuum chamber (25 in. Hg) at 37 °C for 1 hour to remove traces of chloroform then stored in a vacuum chamber (25 in. Hg) at 37 °C for 1 hour to remove traces of chloroform. Dry lipid films were stored under a nitrogen gas atmosphere at -20 °C until used, for no longer than a week.

On the day of an experiment, dry lipid films were resuspended in deionized water to a final total lipid concentration of 10 mmol/L. Lipids were allowed to rehydrate for 1 h at room temperature with periodic vortexing to ensure complete suspension and formation of multilamellar vesicles (MLVs). The milky suspension of MLVs was sonicated for 8 minutes in an Avanti bath sonicator (Avanti Polar Lipids, Alabaster, AL), generating a clear suspension of small unilamellar vesicles. In all bilayer formation
experiments, SUV suspensions were loaded into a filling vessel, then placed onto the fill end of a fused-silica capillary tube, cut to a length of 33 mm and stripped in the middle of the polyimide coating by gentle heating with a butane torch, for 45 minutes. After this process was complete, capillaries were rinsed of excess vesicles by exchange of 10 TV of deionized water. SLB-containing capillaries were placed in the dark and held there until use.

3.3.4 Reconstitution of FtsZ Rings

At the start of a reconstitution, an SLB-containing capillary sandwich was moved to the exchange apparatus. SLBs were equilibrated to the reconstitution buffer by exchange of 5 TV of buffer. FtsZ polymerization reactions (in reconstitution buffer) were initiated by adding GTP to 0.2 mmol/L in the single protein system; the dual protein system required 0.2 mmol/L GTP and 0.5 mmol/L ATP. After loading of 1 TV of the polymerization reaction, the sandwich was quickly and carefully removed from the exchange apparatus and disassembled. The capillary was sealed at each end with molten paraffin wax, coated with a drop of fused silica-matched immersion oil (Cargille Labs, Cedar Grove, NJ) and transferred to the microscope for observation. This entire process, from beginning of reaction loading to focused image, takes 5 minutes.

3.3.5 Assays of GTPase Activity

GTPase assays were performed with a colorimetric malachite green assay (ATPase/GTPase Activity Assay Kit, Sigma Aldrich, St. Louis, MO) per instructions. Briefly, FtsZ-YFP or FtsZ-YFP-mts protein was dialyzed to the appropriate experimental buffer condition, then serially diluted into a final volume of 69 µL. GTP hydrolysis was initiated by the addition of 1 µL of GTP from a 35 mmol/L stock (0.5 mmol/L final). Reactions were incubated at room temperature for 30 minutes, then stopped by addition of 200 µL of assay reagent. Free phosphate was detected by measurement of the absorbance of
the reaction at 600 nm using a BioTek ELx800 plate reader (BioTek Instruments, Winooski, VT). A standard curve of phosphate was used to convert absorbance readings to amount of phosphate produced, which was subsequently used to calculate the rate of GTP hydrolysis.

3.3.6 Image Capture and Statistical Analysis

Fluorescence micrographs of SLBs and reconstituted rings were captured using a fully-automated Olympus BX61 microscope equipped with a fluorescence-optimized UPlanFL N 100X immersion objective. Images were captured with a Hamamatsu C10600 10-megapixel charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) controlled by SlideBook 4.2 software (Intelligent Imaging Innovations, Denver, CO). For ring formation experiments involving quantification, 30 images were captured per replicate (number of replicates denoted as \( n \)), with each frame representing a complete field of view. On the camera used in these experiments, each frame captures 84.7 µm of the tube length with each replicate covering 2.54 mm of the total 25mm available length, or approximately 10%. From these images, counts of complete and partial rings were made, as well as the number of actively constricting rings and any membrane deformations or membrane scission events. For ring formation and functional data, each measured parameter (number of complete rings, number of partial rings, etc.) in a given experimental treatment (e.g. pH) was treated as a series of trials with a categorical independent variable (MES 6.0, MES 6.5, etc.) and a continuous dependent variable. To determine if the observed variance of the measured mean values across all trials (within a given treatment) was statistically significant, these data were analyzed using a one-way analysis of variance (ANOVA). Pairwise comparisons between individual trials were performed using the Tukey Honestly Significant Differences test and are reported in the text in parentheses in “trial 1 vs. trial 2, \( p = x \)” format. All statistical analyses were performed in SPSS v.23 (International Business Machines, Armonk, NY).
2.4 RESULTS AND DISCUSSION

3.4.1 Effect of Subunit Anchoring Ratio on Reconstituted Rings

In vivo, a proper ratio of anchoring proteins vs. FtsZ is critical for proper formation and function of the Z-ring (16). In light of this, we chose to investigate the effect of anchoring of individual FtsZ subunits on reconstituted ring formation and function. In the single-protein system (SPS), the use of membrane-targeted FtsZ as the sole input protein means that all subunits are anchored. In anchoring experiments, we included FtsZ-YFP lacking the membrane targeting sequence in defined ratios with the membrane-targeted version, while keeping total protein concentration (8 mmol/L) constant across trials. In the dual-protein system (DPS) the base condition is a 1:1 ratio of FtsZ-YFP:FtsA*. For these experiments, FtsZ concentration was held at 8 mmol/L while FtsA concentration was varied.

In the SPS, a change in anchoring ratio resulted in more efficient constriction (Figure 3.1). In microscopic imaging of mixes of FtsZ-mts and FtsZ at 1:2 and 1:3 ratios, we saw a noticeable difference in constriction depth. Ring constriction progressed further in 1:2 mixes (top images), resulting in constriction arrest at a much smaller ring size. Additionally, membrane deformations appeared to double in constriction depth (Fig. 3.1 middle and bottom) as seen in the presented images of 1:2 mixes. There were no visible differences between 1:2 and 1:3 mixes with regards to membrane deformations (data not shown) and 1:4 mixes failed to produce constricting Z-rings.

Quantitatively, we found that for this particular aspect of Z-ring function, anchoring ratio played the biggest role in the SPS (Figure 3.3). While the ratio of functional to total formed rings did not change significantly (none vs. 1:2, p=.1016), the number of membrane deformations (as a percentage of total functioning rings) increased. Interestingly, the ratio of functional/formed rings did drop by half (1:2 vs. 1:4, p < 0.001) from 1:2 to 1:4. In the DPS, no significant differences were found; any changes in
Figure 3.1. Unanchored FtsZ increases constriction depth in the SPS. Constriction depth is slightly increased when unanchored FtsZ-YFP is mixed in a 1:2 ratio with FtsZ-YFP-mts. Arrest state images taken immediately before GTP exhaustion and depolymerization. Bottom images have had their contrast and brightness altered in the inset to make the constriction remnant more apparent. Scale bars are 1 µm.
Figure 3.2. Subunit anchoring vs. ring formation in reconstituted rings. Variation in complete (blue line with circles) and partial rings (red line with squares) formed in the single-protein system (top) and dual-protein system (bottom). Data points represent means with standard deviation depicted as error bars.
Figure 3.3. Subunit anchoring vs. ring function in reconstituted rings. Variation in constricting rings (blue bar), membrane deformations/scissions (red bar) and ratio of functional rings to all formed rings (black line with squares) for the SPS (top) and DPS (bottom). Data points represent means with standard deviation depicted as error bars. Column error bars represent the standard deviation of the sum of actively constricting rings and rings that produce membrane deformations/scission.
<table>
<thead>
<tr>
<th></th>
<th>Ratio</th>
<th>Mean # Rings</th>
<th>Mean # Partial</th>
<th>Active Constriction</th>
<th>Bilayer Deformation</th>
<th>Total A+D**</th>
<th>Ratio C/R***</th>
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<td></td>
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<td></td>
<td>42.4 ± 12.6</td>
<td>52 ± 12.7</td>
<td>7.1</td>
<td>1.9</td>
<td>9 ± 3.1</td>
<td>0.259 ± 0.056</td>
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<td>n/a</td>
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<td>0.372</td>
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*Data presented as mean ± S.D.

**Sum of actively constricted rings and bilayer deformations due to constriction

***Ratio of constriction data (total A+D) to all fully formed rings
functional ring number could be accounted for by normalization of the numbers with the total number of
formed rings (ANOVA, p=0.372). Taken together, these results suggest that Z-ring constriction proceeds
in two phases. The first, which is driven by the condensation of the filaments, is enhanced in the SPS
only and thus is independent of the presence of FtsA. The fact that enhanced constriction in the SPS still
results in arrest and the lack of membrane scission suggests a second FtsA-dependent phase, driven
primarily by filament turnover and resulting in scission.

We also found a moderate increase in formation of complete rings in the SPS (Figure 3.2) up to
an anchoring ratio of 1:2 (none vs. 1:2, p<0.001), while no stabilizing effect was seen in the DPS (none vs.
1.2, p=0.9994). In both systems, anchoring ratios above 1:2 resulted in a precipitous drop in complete Z-
rings; however, in the SPS partial ring development continued to increase. Partial ring formation in the
DPS declined significantly over this range, suggesting that the particulars of the anchoring mechanism
results in a variable response to anchoring ratio. Anchoring ratio appears to have only a small effect on
formation of complete, immobile rings. The differential effect of anchoring ratio on partial ring formation
between the SPS and DPS is an interesting and novel observation. In the SPS, partial rings increase as
complete ring numbers decline, suggesting that lower anchoring ratios interfere with condensation of
partial rings. In the DPS, partial ring numbers decline in tandem with complete ring numbers, suggesting
a more dramatic interfering effect.

3.4.2 Effect of pH on Reconstituted Rings

In vitro study of FtsZ polymerization dynamics has shown that pH can impact FtsZ filament
stability in two ways: reduction in turnover via GTP hydrolysis inhibition and induction of filament
bundling at low pH (7, 13, 17). With regard to variations in pH, the most dramatic effect of pH on
reconstituted rings was seen at acidic pH, where rings formed in MES buffer at pH 6.0 formed aggregate
structures (Figure 3.4). These structures extend over an average of 1-1.5 microns, nearly 10x the width of wild-type *E. coli* Z-rings. Aggregate Z-rings are not seen in both the SPS and DPS; however, they do not appear in any other pH condition in either system, and the width and pixel intensity of imaged rings is similar in both systems. This suggests that the bundling effects of pH result in a hyperstabilization of lateral interactions, which manifests in our system as extended Z-ring condensates.

As can be seen in Figure 3.5, this aggregation resulted in the formation of rings, both partial and complete, that is a small fraction of that seen in the conditions that more closely approximate the *in vivo* state, where cytosolic pH is slightly alkaline. In the pH 6.5 condition, however, formation of rings was increased significantly over the more neutral/alkaline conditions in both the SPS (MES 6.5 vs HEPES 7.5, p < 0.001) and in the DPS (MES 6.5 vs HEPES 7.5, p < 0.001) without a proportional increase in formation of partial rings. Continued increase in pH to a more alkaline condition resulted in an initial decline in complete ring formation with an increase in partial rings, further highlighting the stabilizing conditions at pH 6.5, but further increases of pH did not produce a significant change in either complete or partial ring formation. Also of note, there was no significant difference in ring formation in either MES or HEPES buffer at pH 7.0, suggesting that the buffering chemical itself is not responsible for the observed variation in ring formation.

The overall trends of ring formation are mirrored in the ring function data. Increases in functional ring numbers can be entirely accounted for by normalization against the total number of formed rings. The most interesting observation from these data is the more than 2-fold increase in membrane scission events in pH 6.5 buffer. While the increase in raw numbers accounts for some of this, it is plainly visible that membrane scission events represent a greater percentage of functional ring events in the pH 6.5 condition than in the conditions at higher pH.
Figure 3.4. Acidic pH causes aggregation of Z-rings. At pH 6.0, rings begin to aggregate into large, extended structures. Scale bars are 2 µm.
Figure 3.5. pH vs. ring formation in reconstituted rings. Variation in complete (blue line with circles) and partial rings (red line with squares) formed in the single-protein system (top) and dual-protein system (bottom) due to pH. Data points represent means with standard deviation depicted as error bars.
Figure 3.6. **pH vs. ring function in reconstituted rings.** Variation in constricting rings (blue line with circles) and membrane deformations/scissions (red line with squares) for single-protein system (top) and dual-protein system (bottom). Data points represent means with standard deviation depicted as error bars.
Table 3.2 Tabulated Data and Analysis of pH-Dependent Effects in Reconstitution Trials*

<table>
<thead>
<tr>
<th>pH Condition</th>
<th>Mean # Rings</th>
<th>Mean # Partial</th>
<th>Active Constriction</th>
<th>Bilayer Deformatio n</th>
<th>Total A+D**</th>
<th>Ratio C/R***</th>
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<td><strong>Single Protein System (n = 9)</strong></td>
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<tr>
<td>MES 6.0</td>
<td>1.2 ± 0.4</td>
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<td>0 ± 0.0</td>
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<td>90.3 ± 21.3</td>
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<td>HEPES 8.0</td>
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*Data presented as mean ± S.D.
**Sum of actively constricted rings and bilayer deformations due to constriction
***Ratio of constriction data (total A+D) to all fully formed rings
Figure 3.7. GTP hydrolysis rate by pH condition in the SPS and DPS. The rate of GTP hydrolysis is expressed as moles of inorganic phosphate produced per mole of FtsZ per minute. Blue bars represent hydrolysis rate in the single protein system; red bars represent hydrolysis rate in the dual protein system. Error bars represent the standard deviation of the experimental means.
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<th>pH Condition</th>
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</tr>
<tr>
<td>HEPES 7.0</td>
<td>3.25</td>
<td>0.28</td>
<td>0.008</td>
</tr>
<tr>
<td>HEPES 7.5</td>
<td>3.46</td>
<td>0.26</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HEPES 8.0</td>
<td>3.12</td>
<td>0.34</td>
<td>0.036</td>
</tr>
<tr>
<td><strong>F-Statistic</strong></td>
<td></td>
<td></td>
<td>155.1</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Dual Protein System (n = 9)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MES 6.0</td>
<td>0.21</td>
<td>0.06</td>
<td>0.2837</td>
</tr>
<tr>
<td>MES 6.5</td>
<td>0.96</td>
<td>0.23</td>
<td>0.04</td>
</tr>
<tr>
<td>MES 7.0</td>
<td>2.55</td>
<td>0.24</td>
<td>0.002</td>
</tr>
<tr>
<td>HEPES 7.0</td>
<td>2.77</td>
<td>0.38</td>
<td>0.008</td>
</tr>
<tr>
<td>HEPES 7.5</td>
<td>2.85</td>
<td>0.22</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HEPES 8.0</td>
<td>2.69</td>
<td>0.45</td>
<td>0.036</td>
</tr>
<tr>
<td><strong>F-Statistic</strong></td>
<td></td>
<td></td>
<td>45.1</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* T-test performed between SPS and DPS values at same pH condition
Because pH change has been shown to cause multiple effects on FtsZ polymer structure and dynamics, we chose to measure the effect of pH on GTP hydrolysis rate in our purified proteins. In these assays, assembly reactions were mixed as used in the reconstitution system and initiated with GTP. At the end of the incubation period, reactions are stopped and developed in a single step. The results are shown in Figure 3.7. We observed a small but significant inhibition of GTPase activity in the presence of FtsA*, which is not surprising given the filament-organizing functions of the FtsA globular domain. In both protein systems, we observed a significant inhibition of GTPase activity at pH 6.5, in which we also saw increased complete and partial ring formation. GTPase activity was further depressed at pH 6.0, indicating that GTPase activity reduction alone does not explain the variation in ring formation.

3.4.3 Effect of Calcium on Reconstituted Rings

In in vitro assays of FtsZ polymerization, the addition of calcium results in the formation of protofilament pairs and bundles and under certain conditions extended sheets with rigid, parallel ordering of filaments within the sheet (9, 10). To assess whether or not this bundling effect influenced formation and function of FtsZ rings, we reconstituted single-protein and dual-protein rings in the presence of varying amounts of calcium. We found that in both systems, that there was no effect of calcium on ring formation up to 0.5 mmol/L calcium acetate. Afterwards, we found a statistically significant increase in both complete and partial ring formation between 0.5 and 1 mmol/L trials as well as between 1 mmol/L and 2 mmol/L (Tukey HSD all p < 0.001). In the realm of ring function, we found an upward trend in ratio of functional rings in the SPS, which rose from 14% at zero calcium to 25% at 0.5 mmol/L, after which it leveled off. This suggests a role calcium in stimulating the conversion from a non-constricting to a constricting ring. This hypothetical role is strengthened by the DPS data, where the percentage of membrane scission events increased dramatically in relation to actively constricting rings despite a
Figure 3.8. Calcium concentration vs. ring formation in reconstituted rings. Variation in complete (blue line with circles) and partial rings (red line with squares) formed in the single-protein system (top) and dual-protein system (bottom). Data points represent means with standard deviation depicted as error bars.
Figure 3.9. Calcium concentration vs. ring function in reconstituted rings. Variation in constricting rings (blue line with circles) and membrane deformations/scissions (red line with squares) for single-protein system (top) and dual-protein system (bottom). Data points represent means with standard deviation depicted as error bars.
Figure 3.10. High concentration of calcium blocks Z-ring formation in the SPS and DPS.

Representative image of long, aggregated and continuous rings seen in both systems when calcium acetate is added to 20 mmol/L. Scale bar is 2 μm.
relatively constant ratio of functional/formed rings. We could not determine whether this represented an earlier constriction initiation or a more rapid completion from the image sets used in these experiments.

The amounts of calcium used in these experiments are low relative the amount used in prior literature, in order to avoid the possibility of formation of calcium-induced sheets (10). Subsequent to physiological experiments, we conducted qualitative inquiry into reconstitutions at higher levels of calcium, like those that are typically used in enrichment protocols for actively polymerizing FtsZ. We found that the presence of calcium at 20 mmol/L caused FtsZ aggregates similar to those found at pH 6.0 (Figure 3.11); however, they were greatly extended in length. This suggests that there is a limit to the contribution of bundling to FtsZ ring stabilization.

### 3.4.4 Effect of Inhibition of GTPase Activity by GMPCPP

Though environmental conditions can modulate GTP hydrolysis, it is possible to directly inhibit the rate of nucleotide hydrolysis (and thus the rate of subunit turnover) using the GTP analog GMPCPP. This analog hydrolyzes much more slowly than GTP, and can assist in directly assessing the role of enzyme activity in Z-ring formation and function without potential interference from other factors. In experiments comparing reconstitution in the presence of GTP and GMPCPP, we found an increase in both complete and partial rings in GMPCPP as compared to GTP (Figure 3.12). The average number of complete rings in the SPS increased from 54.3 ± 17.3 (mean ± S.D.) in GTP to 83.6 ± 26.9 (t-test p=0.02) in GMPCPP and an increase in partial rings from 132.7 ± 28.3 in GTP to 212.3 ± 36.5 in GMPCPP (t-test p<0.001). Reduced GTP hydrolysis (likely manifest as reduced turnover of monomers within the polymer) aid in stability and formation of higher-order structures of FtsZ filaments, including rings.

Functionally speaking, blocking GTP hydrolysis had no specific effect on constriction within the
Table 3.4 Tabulated Data and Analysis of Calcium-Dependent Effects in Reconstitution Trials*

<table>
<thead>
<tr>
<th></th>
<th>[Ca2+] (mmol/L)</th>
<th>Mean # Rings</th>
<th>Mean # Partial</th>
<th>Active Constriction</th>
<th>Bilayer Deformation</th>
<th>Total A+D**</th>
<th>Ratio C/R***</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single Protein System</strong> (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>45.1 ± 10.2</td>
<td>120.6 ± 26.4</td>
<td>5.1</td>
<td>1.2</td>
<td>6.3 ± 3.2</td>
<td>0.140 ± 0.044</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>46.4 ± 14.5</td>
<td>125.4 ± 27.3</td>
<td>6.3</td>
<td>2.1</td>
<td>8.4 ± 2.6</td>
<td>0.181 ± 0.056</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>38.4 ± 18.6</td>
<td>105.4 ± 32.1</td>
<td>6.1</td>
<td>1.6</td>
<td>7.7 ± 2.9</td>
<td>0.201 ± 0.043</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>36.8 ± 13.4</td>
<td>136.3 ± 9.3</td>
<td>7.5</td>
<td>1.7</td>
<td>9.2 ± 4.2</td>
<td>0.250 ± 0.045</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>52.9 ± 12.1</td>
<td>128.5 ± 36.5</td>
<td>8.2</td>
<td>4.5</td>
<td>12.7 ± 2.0</td>
<td>0.240 ± 0.029</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>89.1 ± 12.2</td>
<td>201.4 ± 22.1</td>
<td>15.4</td>
<td>7.3</td>
<td>22.7 ± 2.6</td>
<td>0.255 ± 0.051</td>
<td></td>
</tr>
<tr>
<td><strong>F-Statistic</strong></td>
<td>17.8</td>
<td>13.0</td>
<td>n/a</td>
<td>n/a</td>
<td>36.6</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
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<td>n/a</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dual Protein System</strong> (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>56.8 ± 18.2</td>
<td>184.2 ± 36.4</td>
<td>14.2</td>
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<td>26.2 ± 3.1</td>
<td>0.461 ± 0.072</td>
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<tr>
<td>0.01</td>
<td>62.7 ± 13.4</td>
<td>200.1 ± 28.4</td>
<td>14.4</td>
<td>13.3</td>
<td>27.7 ± 2.9</td>
<td>0.442 ± 0.045</td>
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</tr>
<tr>
<td>0.1</td>
<td>55.4 ± 29.8</td>
<td>200.9 ± 21.7</td>
<td>11.2</td>
<td>15.1</td>
<td>26.3 ± 4.1</td>
<td>0.475 ± 0.054</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>49.3 ± 18.0</td>
<td>220.3 ± 36.5</td>
<td>9.1</td>
<td>15.4</td>
<td>24.5 ± 7.1</td>
<td>0.497 ± 0.096</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>69.4 ± 15.7</td>
<td>260.5 ± 14.3</td>
<td>8.6</td>
<td>24.3</td>
<td>32.9 ± 5.2</td>
<td>0.474 ± 0.066</td>
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</tr>
<tr>
<td>2.5</td>
<td>96.3 ± 21.1</td>
<td>348.4 ± 42.2</td>
<td>9.7</td>
<td>34.4</td>
<td>44.1 ± 3.4</td>
<td>0.458 ± 0.069</td>
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<tr>
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<td>n/a</td>
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<td>n/a</td>
<td>P &lt; 0.001</td>
<td>0.52</td>
<td></td>
</tr>
</tbody>
</table>

*Data presented as mean ± S.D.

**Sum of actively constricted rings and bilayer deformations due to constriction

***Ratio of constriction data (total A+D) to all fully formed rings
single-protein system (Figure 3.13). While an increase in the number of functional rings was observed, this increase is in proportion with the total number of Z-rings. The ratio of functioning rings to the total formed rings was 0.151 ± 0.044 in experiments with GTP and 0.167 ± 0.056 in experiments with GMPCPP, which was not significantly different (p=0.825). Though this was also true in the dual protein system, it is clear from the data (Fig. 3.13, bottom) that membrane scission was inhibited in the dual protein system, as the average number of scission events decreased both on the whole (11.9 to 3.2) and in proportion to the total number of functioning rings, indicating that GTP hydrolysis as a specific role to play in the FtsA-catalyzed completion of constriction within the reconstitution system. In order to verify that GMPCPP was in fact resulting in reduced enzymatic hydrolysis, GTPase assays were performed on reaction mixes identical to those used in the reconstitution. We found an overall 15-fold lower rate of GTP hydrolysis with GMPCPP (Figure 3.14) in both the single protein and dual protein systems. Given this confirmatory evidence, these data (when taken together) show that membrane scission in the dual protein system is dependent upon GTP hydrolysis, but ring formation and the initial stages of ring constriction are not.

3.5. CONCLUSIONS

Using our new in vitro reconstitution system, we investigated the biochemistry of Z-ring formation in systems where only the action of FtsZ is considered, as well as a second system that includes the Z-ring attachment protein FtsA. In this study, we made several observations that confirm assumptions about Z-ring behavior derived from in vitro assembly of purified FtsZ into filaments and some that challenged the common wisdom about Z-ring formation and function. Direct testing of all of these hypotheses was made possible by our new method; prior to this, these observations were made indirectly through mutant Z-ring phenotypes as well as the co-expression of otherwise lethal mutations in
Figure 3.11. GTP hydrolysis inhibition vs ring formation in reconstituted rings. Variation in complete (blue bars) and partial rings (red bars) formed in the single-protein system (top) and dual-protein system (bottom). Standard deviation is depicted as error bars.
Figure 3.12. GTP hydrolysis inhibition vs ring function in reconstituted rings. Variation in complete (blue bars) and partial rings (red bars) formed in the single-protein system (top) and dual-protein system (bottom). Data points represent means with standard deviation depicted as error bars.
Table 3.5 Tabulated Data and Analysis of GTP Hydrolysis-Dependent Effects in Reconstitution Trials*

<table>
<thead>
<tr>
<th></th>
<th>Nucleotide</th>
<th>Mean # Rings</th>
<th>Mean # Partial</th>
<th>Active Constriction</th>
<th>Bilayer Deformation</th>
<th>Total A+D**</th>
<th>Ratio C/R***</th>
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</thead>
<tbody>
<tr>
<td><strong>Single Protein System (n = 9)</strong></td>
<td>GTP</td>
<td>54.3 ± 17.3</td>
<td>132.7 ± 28.3</td>
<td>6.3</td>
<td>1.9</td>
<td>8.2 ± 5.3</td>
<td>0.151 ± 0.044</td>
</tr>
<tr>
<td></td>
<td>GMPCPP</td>
<td>83.6 ± 26.9</td>
<td>212.3 ± 36.5</td>
<td>9.6</td>
<td>4.4</td>
<td>14 ± 4.2</td>
<td>0.167 ± 0.056</td>
</tr>
<tr>
<td></td>
<td>T-test</td>
<td>2.65</td>
<td>5.17</td>
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<td>n/a</td>
<td>0.857</td>
<td>0.225</td>
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<tr>
<td></td>
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<td>n/a</td>
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</tr>
<tr>
<td><strong>Dual Protein System (n = 9)</strong></td>
<td>GTP</td>
<td>64.5 ± 23.1</td>
<td>156.1 ± 29.8</td>
<td>17.3</td>
<td>11.9</td>
<td>29.2 ± 3.1</td>
<td>0.453 ± 0.063</td>
</tr>
<tr>
<td></td>
<td>GMPCPP</td>
<td>115.4 ± 26.2</td>
<td>325.1 ± 51.3</td>
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<td>51.1 ± 5.4</td>
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<td>n/a</td>
<td>&lt;0.001</td>
<td>0.902</td>
</tr>
</tbody>
</table>

*Data presented as mean ± S.D.

**Sum of actively constricted rings and bilayer deformations due to constriction

***Ratio of constriction data (total A+D) to all fully formed rings
Figure 3.13. GTP hydrolysis rate in solutions containing GMPCPP. Rate of GTP hydrolysis (in moles of inorganic phosphate released per mole of FtsZ per minute) in the SPS (blue) and the DPS (red). Data points represent means with standard deviation depicted as error bars.
otherwise healthy cells.

Our grounding assumption going into these experiments is that all FtsZ-regulatory factors should exhibit some peak of effectiveness at stabilization of ring structures and initiation of constriction and, at least in the conditions tested, that hypothesis often held. One example of this can be found in the subunit anchoring experiments. In both the SPS and DPS, decreasing the number of anchors per subunit initially had no significant effect; only when fewer than one out of every three subunits were anchored did we begin to see variation in ring formation, and the nature of this variation differed between the two systems. In the SPS we found a decrease in complete rings with a simultaneous increase in partial rings. In the DPS, we found a decrease in both complete and partial rings.

Finding an in vivo condition for a baseline comparison is not yet possible, as the imaging technology is not yet available to determine exactly how many anchors there are per unit of FtsZ in each ring; however, quantitative Western blotting has determined that there is one molecule of FtsA for every five molecules of FtsZ in E. coli (16) and that upset of this balance results in division defects. ZipA is considerably more abundant than FtsA and overexpression of ZipA causes division defects (18, 19), so it is reasonable to infer that the ratio of ZipA to FtsZ is also relevant. This may also explain the lack of response in 1:1 and 1:2 ratio conditions. These conditions may more closely represent the true ratio of anchored subunits in the live cell once ZipA is accounted for, and defects in Z-ring formation only became apparent when the ratio fell below the normal range. In the single protein system, these complete rings appeared to either break apart or fail to condense in the first place, resulting in an increase in partial rings. This increase was not found in the presence of FtsA. Though the anchoring function of FtsA serves as a positive regulator, its hypothesized role as a destabilizer and disassembler of the ring during constriction and scission explains this difference. If the filaments become static on the membrane and fail
to condense in the absence of a regulator of FtsA, the intrinsic activity of the protein will enhance the
disassembly of FtsZ filaments, resulting in the observed lack of partial rings as compared to the SPS.

Functionally speaking, the most interesting anchoring effect was also found in the single-protein
system. One of the hypotheses regarding the inability of FtsZ-YFP-mts to catalyze membrane scission is
that the overwhelming presence of the anchor (every subunit is anchored) reduces the mobility of
filaments and subunits, preventing proper disassembly in the process of constriction. By co-polymerizing
FtsZ-YFP-mts with the unbound FtsZ-YFP, we see an increase in membrane deformation events in both
raw number and relative to the total number of constriction events (which includes actively constricting
rings). This provides direct evidence in support of both the hypothesis about the technical aspects of poor
constriction behavior in Z-only rings as well as the need for disassembly and shrinking of filaments for
constriction. In other words, the presence of unattached, diffusible subunits increases the likelihood that
constriction will result in membrane deformation, a direct indicator of successful constriction. These
deformations were also deeper than those seen in Z-only rings, which is another indicator of increased
constriction efficiency. Regardless, membrane scission still did not occur even in these rings with more
efficient constriction, highlighting the requirement for the action of FtsA for completion of the scission
event.

*In vitro* study of FtsZ polymerization dynamics has shown that pH can impact FtsZ filament
stability in two ways: reduction in turnover via GTP hydrolysis inhibition and induction of filament
bundling at low pH (7, 13, 17). We found that this bundling behavior manifested (at pH 6.0) as an
aggregation of rings and ring-like structures, resulting in an extended 1-1.5 micrometer tube. This effect
nearly eliminated both complete and partial rings in reconstitutions at that pH level. This is apparently
very near a tipping point for the influence of pH on FtsZ function, as a slightly less acidic buffer (pH 6.5)
stabilized and enhanced completed Z-ring formation in both the SPS and DPS as compared to neutral
and slightly basic pH. If we assume that the peak effect of bundling by pH results in the extended aggregates, then the enhanced ring formation at pH 6.5 may represent a peak in hydrolysis inhibition; not in the sense that the enzyme is most inhibited there, but that the level of inhibition seen is ideally tuned to stabilize polymers without causing wholesale change in polymer structure. We measured GTPase activity at all pH conditions, and we did indeed find a reduced GTPase activity at pH 6.5 that is intermediate to that seen at physiological pH and the significant block found at pH 6.0, which strongly supports this proposed mechanism.

Functionally speaking, pH had little impact, with one exception. In the DPS at pH 6.5, we found that though the overall total of functional rings (as a ratio of all rings) was not significantly different, the number of membrane scission events increased with respect to the total number of constriction events, suggesting that the effect of pH on FtsZ increases the likelihood of membrane scission. This is counterintuitive from the perspective of enzyme activity, as a reduction in GTP hydrolysis should reduce turnover and thus hamper the ability of FtsA to drive ring disassembly. This suggests a role for the bundling activity of pH in proper ring function; however, this would occur via a heretofore undiscovered mechanism, as prior published work has shown that filaments at pH 6.5 have been shown to be unbundled at this pH (7). A mechanism based on surface charge, where excess protons buffer the negatively charged surface to either stabilize the structure against constriction failure or enhance the activity of FtsA itself in the context of the ring, would explain these observations.

In observing the effect of calcium on ring formation, we once again saw a system with a single-tailed response. Formation of both complete and partial rings was significantly enhanced in both systems at 2.5 mmol/L calcium acetate as compared to trials lacking calcium. At this concentration, the bundling or “filament organization” effects of the calcium result in enhanced formation of rings. Both the SPS and DPS showed functional enhancement with calcium, but in different ways. Calcium in the SPS resulted in
a total two-fold increase in functional rings (with respect to all rings) in 0.5, 1, and 2.5 mmol/L calcium, with a concomitant small increase in the ratio of membrane deformation events to total constriction events. This is particularly interesting considering that the Z-only rings are by nature less dynamic than two-protein rings due to the anchoring issues discussed earlier. If we take this observation that calcium can enhance constriction in the absence of FtsA with the observations in anchoring trials, we can conclude that calcium enhancement in the SPS occurs by a different mechanism. Several models of Z-ring function postulate a role for sliding of filaments in Z-ring constriction (4, 20, 21) in addition to FtsA-driven disassembly. This is the likeliest explanation for the ability of Z-only rings to constrict, and enhancement of that constriction by addition of calcium suggests that calcium enhances filament sliding. This may occur due to increased filament alignment within the ultrastructure of the ring; the divalency of calcium could enable it to mask repulsive negative charges on adjacent filaments, allowing them to come into closer proximity, where steric interactions induced by thermal fluctuations would increase order and alignment of the filaments. There is already some evidence for this in the literature, where calcium at higher concentrations than those used in our study induces the formation of a variety of higher-order structures in purified FtsZ, including gel-like networks and ordered, crystal-like sheets (9, 10). To test this qualitatively, we used 20 mmol/L calcium in the context of the SPS, and found that it induced ring aggregation in a similar fashion to the observations at pH 6.0; however, these aggregates were greatly increased in length, implying a greater degree of aggregation and bundling between filaments.

In the DPS, calcium greatly enhanced membrane scission, while not increasing the proportion of constriction events vs. all ring formation events. This implies a greater efficiency of constriction. Due to the nature of the data collection method, where image series were taken along the tube over time, only a single image could be captured of rings. An increase in scission events without an increase in total percentage of constriction events requires the conversion of active constrictions to membrane scissions. In
our system, this means that the time required for scission was decreased; in other words, constriction was faster with calcium than without. Given that calcium enhances sliding in the SPS, the observations in the DPS likely represent a synergistic enhancement of both sliding and FtsA-driven filament dynamics to produce rapidly constricting rings. This is an entirely new observation and helps lend credence to the idea that Z-ring constriction is driven by multiple processes.

Lastly, we directly assessed the role of GTP hydrolysis, in the absence of other factors, by inhibiting hydrolysis using GMPCPP, a slowly-hydrolyzing GTP analog. This slow hydrolysis was confirmed by us experimentally in SPS and DPS protein mixes, where we saw a 15-fold reduction in GTPase activity. The use of GMPCPP in the reconstitution system resulted in enhanced formation of both complete and partial rings in both systems; however, GMPCPP introduced a near-block of membrane scission events while no significant variation in ring function occurred in the SPS. This makes sense given the previously-discussed, hypothesized role of FtsA in membrane constriction. Inhibition of GTP hydrolysis, which is essential for protein turnover, prevents completion of constriction in dual-protein rings either by directly blocking or “drowning-out” the ability of FtsA to sufficiently enhance filament turnover. These data also provide important context to the pH trial results. Inhibition of GTP hydrolysis alone is not sufficient to enhance scission as seen at pH 6.5; this effect must come from the bundling or “organizing” effect of low pH.

In their totality, these data paint a picture of FtsZ ring formation driven by the condensation of stable filaments. Increasing filament turnover decreases ring formation parameters. Stabilizing factors, whether through inter-filament “bundling” or reduction in GTP hydrolysis, enhance ring formation. The conversion of a formed ring to a constricting ring involves the sliding of filaments, which can be stabilized by calcium and acidic pH. Completion of constriction requires GTP hydrolysis and filament turnover, and enhancing those aspects of FtsZ function results in increases in membrane deformations and scission.
events. This hybrid model of FtsZ function has been suggested by disparate reports but is now supported by experimentation in this work.

Ultimately, our initial hypothesis that Z-modulating factors would present with effects that are distributed normally—where some value would represent a peak and that either extreme would result in destabilization—was incorrect. Based on these data, this hypothesis should be reformulated. While individual, specific conditions appear to have a one-tailed effect on FtsZ form and function, the combined action of all aspects of FtsZ regulation (whether environmental or by direct interaction of proteins) result in a continuum of FtsZ stability. When the summed effect positive regulators dominate, the ring is hyperstable and non-functional. When the summed effect of negative regulators dominates, the ring is unable to properly form and constrict. When these effects are in balance, the Z-ring is sufficiently stable to form and initiate constriction, but dynamic enough to permit FtsA-driven turnover to complete the division process. This results call for a reinterpretation of existing sliding-only and bending-only models into a new hybrid model of the form and function of the FtsZ ring, where both are occurring in a single functional ring, either contemporaneously or in temporally-distinct phases.
3.6 References


CHAPTER IV

A Z-RING WITH NEMATIC ORDER: THEORY AND EXPERIMENT

4.1 ABSTRACT

The inability of our current models of Z-ring structure and function to fully account for observed phenomena and the difficulty of directly measuring the physical properties of FtsZ filaments beg a holistic model that can make useful predictions about the regulation of Z-ring physiology. In this work, we proposed a model of the Z-ring as a lyotropic liquid crystal with nematic order. This model predicted that nematic ordering should occur within a range of concentration similar to that observed in vivo and that factors that modulate the physical properties of FtsZ filaments should also modulate the concentration at which nematic order can be observed. Traditional thin-film techniques and a new droplet scanning assay were employed to detect the development of birefringence (a characteristic of nematic order) in bulk solution of purified Escherichia coli FtsZ. No birefringence was detected in any of the experimental trials. Solutions to this experimental problem are proposed, including a detailed outline of parameters for a new kind of Z-ring reconstitution system that would allow the detection of nematic ordering.
4.2 INTRODUCTION

Significant intellectual energy and time have been invested in physical modeling of the formation and function of the FtsZ ring. This theoretical work is complicated by the fact that accurate experimental measurement of the physical properties of FtsZ protofilaments—even relatively simple quantities like persistence length—is plagued with inconsistency. Published experimental measures of persistence length, for example, vary over 1-2 orders of magnitude (1). Explanations for this variation have been proposed (1, 2), however, years of published imaging and spectroscopy have shown that polymer length, shape and bundling as well as enzymatic activity (which also affects polymer shape and rigidity) are altered by variation in chemical conditions, concentration effects, interactions between protofilaments and the presence or absence of FtsZ-interacting proteins.

While these may be considered complications *ex vivo*, in live bacteria all of these factors are relevant to proper function of FtsZ and its related structures; perhaps even more relevant than a well-defined physical quantity like the bending rigidity of isolated, individual protofilaments. Electron micrographs of reconstituted Z-rings (e.g., those shown in Figure 2.2b) show that individual protofilaments within rings are closely associated with each other and these lateral interactions—even if not lateral non-covalent bonds—likely affect the true persistence length under physiologically-relevant conditions. While rigorous modeling of the Z-ring is still a welcome addition to the body of knowledge of FtsZ-driven cytokinesis, the complexity of the chemical and physical properties of the protein and its filaments beg a holistic model that can make testable predictions about the molecular physiology of ring formation and constriction. In this work, we present a holistic model of the Z-ring as a structure with liquid-crystal character and attempt to test that model by identifying anisotropy in solutions of purified FtsZ filaments *in vitro*. 
A liquid crystal (LC) is a material that exhibits a phase of matter that is intermediate between a liquid and a solid—the key distinction between them being the order of the molecules within. Liquids are isotropic materials, meaning that the molecules lack both order of position (thus, undergo fluid flow) and order of orientation. On the other extreme, solids are crystalline materials where the individual molecules or atoms are locked in both position and orientation by the structure of the crystal. Liquid crystalline materials lie in the middle. They lack positional order (and thus are free to flow) but the individual molecules have orientational order; that is, they point in the same or similar direction when averaged over the whole of the material. This molecular ordering imparts upon the material special properties that, depending upon the composition and structure of the material itself, manifest as altered fluid flow, optical activity, or sensitivity to electrical current and magnetic fields.

Liquid-crystallinity is a property not uncommonly found in biological materials, though these biological LCs differ significantly from the liquid crystals encountered daily in modern electronic devices. LCs found in LCD displays are thermotropic, meaning that they transition into the liquid-crystalline state based on changes in temperature. Biological liquid crystals are lyotropic, meaning that the LC transition occurs due to changes in concentration. Exactly how and why this occurs depends upon the chemistry of the substance itself. Phospholipids, for example, are a type of lyotropic liquid crystal; as a consequence, the phospholipid bilayers of all living cells are a type of liquid-crystalline material. The origin of the LC behavior of phospholipids lies in their amphiphilicity (3), and this dual chemical nature allows formation of many LC phases, some familiar (micelles, bilayers) and some exotic (hexagonal, inverse).

Many biological polymers and macromolecular assemblages exhibit LC phases. These are typically rigid or semi-flexible polymers with high aspect ratio (length vs. width): the fibroin protein that is spun into silk by spiders and silkworms (4); the self-assembling outer coat of the tobacco mosaic virus (TMV) (5); condensed, highly-concentrated DNA (6). There are several theoretical models that attempt to
explain why these phases exist, but the most conceptually helpful model is the hard rod theory of Onsager (7), which imagines individual polymers as cylindrical rods. These cylindrical rods are isotropic at low concentration, but as the concentration of the polymer rods increase, they interact sterically, forcing them to align with each other along their length. This alignment restricts orientational entropy (i.e. provides orientational order) but increases positional entropy (and thus remaining fluid) such that the resulting nematic liquid crystal phase represents the lowest possible energy state. Nematic LC phases have been demonstrated in a variety of self-assembling proteins and protein-based structures: filamentous viruses like TMV, flagella (8), and cytoskeletal elements such as actin filaments and microtubules (9–11).

Several observations regarding properties and behaviors of FtsZ filaments, both in vivo and in vitro, suggest that they may be a lyotropic liquid crystal. Monomers self-assemble into filaments (e.g. lyotropic chromonics) and these filaments subsequently self-assemble into rings in an aqueous environment, and this condensation is necessary for proper function (12–14). In all published electron micrographs of Z-rings—whether taken from the in vivo condition or in vitro reconstitutions—FtsZ filaments within the ring align in parallel with respect to each other (15) as seen in nematic ordering. Both theoretical (16, 17) and experimental (18, 19) work suggests that FtsZ filaments do not share lateral non-covalent bonds, but rather interact with each other without bonding. X-ray diffraction data from toroid-shaped structures of overlapping FtsZ filaments formed in the presence of crowding agents in vitro confirm that the associations between individual filaments are, in the words of the authors, “loose (and) liquid-like” (20). These disparate pieces of evidence lend support to the idea that the ring itself will exhibit some liquid-crystalline order, and that order is intrinsic to its function.

In this work, we propose a holistic model of the FtsZ ring as an LC structure and test this hypothesis by assessing the ability of filaments of purified Escherichia coli FtsZ to form liquid crystalline phases. The development of an LC phase can be identified microscopically by the detection of birefringent
textures, a characteristic of anisotropic materials, using cross-polarized light microscopy. If a transition to LC occurs, the otherwise black image will become detectibly bright as the anisotropic material shifts the polarization state of the incident light. Both traditional methods, newly-developed droplet scanning method, are employed.

4.3 METHODS

4.3.1 Plasmids and Strains Used

Wild-type *E. coli* FtsZ was amplified by polymerase chain reaction (PCR) from strain MG1655 using 5´-GACGCATATGTTTGAACCAATGGAAC-3´ as the forward primer and 5´-GAGGATCCTTAATCAGCTTGCTTAC-3´ as the reverse primer. This was captured in plasmid pET30 by ligation-independent cloning, then transferred to pET11b by subcloning a BamHI/NdeI fragment into the same sites in the vector to make plasmid pET-FtsZ. For all protein preparations, this plasmid was transformed into *Escherichia coli* strain C41(DE3), which has been shown to increase yield of expressed proteins when those proteins are toxic to the cell.

4.3.2 FtsZ Expression and Preparation

FtsZ, was prepared using a method modified from Osawa and Erickson (21). Electrocompetent cells of *E. coli* strain C41(DE3) were freshly transformed with pET-FtsZ carrying either FtsZ, FtsZ-GFP, or FtsZ-YFP-nts, then transformed colonies were picked and grown overnight in 25 mL Luria-Bertani medium with ampicillin (LB+Amp, 100 µg/mL) at 37 °C in a shaking incubator (225 rpm shaking). The next day, this culture was centrifuged at 4000 RCF for 15 minutes to remove the used medium, resuspended in 25 mL fresh LB+Amp, then used to inoculate 500 mL of fresh LB+Amp. This culture was incubated at 37 °C with 225 rpm shaking until the culture reached an optical density between 0.8 and 1.0. At this point, protein expression from the clone was induced by adding isopropyl-β-D-1-
thiogalactopyranoside (IPTG, Fisher Scientific, Pittsburgh, PA) to a final concentration of 0.5 mmol/L. Induction continued with incubation at 37 °C with 225 rpm shaking for 4 hours, after which the cultures were moved to a rotating shaker at room temperature (175 rpm shaking) and left overnight. The following morning cells were split into 250 mL bottles, then pelleted by centrifugation at 6000 RCF in an Avanti J26-XP ultracentrifuge using a JA-14 rotor. Cells were carefully resuspended in 10 mL column buffer (50 mmol/L Tris-HCl pH 7.9, 50 mmol/L KCl, 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 10% [v/v] glycerol) with 175 rpm shaking on ice. Lysozyme and phenylmethylsulfonylfluoride (PMSF) were added to a final concentration of 1 mmol/L and 0.5 mg/ml respectively, then incubated on ice with 175 rpm shaking for 20 minutes. These cell suspensions were then frozen at -80 °C overnight or until needed.

Protein was released from cell suspensions by three cycles of freeze-thaw (1 mmol/L PMSF was added after each) followed by sonication three times for 30 seconds on ice (90 second rest after each) with a Fisher Scientific F60 Sonic Dismembrator at 50% duty cycle. The resultant lysate was clarified by centrifugation at 57000 RCF in a Beckman JA-25.50 rotor for 2 hours. The supernatant was collected, then brought to 30% saturation by slow addition of 3.52g of solid ammonium sulfate. After addition of ammonium sulfate, the mixture was incubated for 30 minutes on ice. Salt-precipitated protein was pelleted by centrifugation at 25000 RCF in the JA-25.50 rotor for 20 minutes. This supernatant is discarded and the pellet resuspended by in 10 mL ice-cold column buffer, then clarified by passing through a 0.22 µm cellulose acetate syringe filter. The protein solution was dialyzed overnight against 2 L of column buffer, then further purified by anion exchange chromatography. A HiTrap DEAE FF 15/10 (GE Healthcare) column was used and protein was eluted with a 100 mL gradient from 50-500 mmol/L KCl with a flow rate of 1.0 mL/min, with FtsZ typically eluting between 250-350 mmol/L KCl. To ensure
reproducibility between batches of protein, the column was cleaned after each purification run using the “rigorous cleaning” protocol suggested by the manufacturer.

Peak FtsZ-containing fractions were identified by SDS-PAGE and pooled, then concentrated in magnesium-free buffer to a final concentration between 50-60 mg/mL. Protein concentration was determined using the bicinchoninic acid (BCA) assay, using a correction factor of 0.75 to account for the difference in color production between bovine serum albumin and FtsZ. Concentrated protein was aliquoted and stored at -80 °C until used.

4.3.3 Preparation for Assays of Optical Activity and Buffers Used

Prior to all assays of optical activity, 100 µL aliquots of FtsZ were thawed and dialyzed three times against 100 mL of reaction buffer. The default or “base” buffer used is physiological buffer (PB) and consists of 50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 300 mM potassium acetate, and 50mM potassium chloride adjusted to pH 7.7 with 1 mol/L potassium hydroxide. Low potassium physiological buffer (PB-LK) contains 50 mmol/L potassium acetate but is otherwise identical to PB. Low pH/potassium buffer (PB-LPK) replaces HEPES with 50 mM 2-((N-morpholino)ethanesulfonic acid (MES), and the final pH is adjusted to 6.5 with 1 mol/L potassium hydroxide, but is otherwise identical to PBLK.

4.3.4 Simple Assays for Optical Activity

For simple assays of optical activity, polymerization of FtsZ was initiated by bringing 15 µL of FtsZ protein solution to 5 mmol/L magnesium acetate and 2.5 mmol/L guanosine-5’-triphosphate (GTP) by addition from concentrated stocks—200 mmol/L magnesium acetate and 100 mmol/L GTP, respectively. To detect birefringence, this solution was placed onto a precleaned glass microscope slide, covered with a coverslip, and imaged. For simple assays with flow alignment, FtsZ was loaded into a
rectangular, borosilicate glass capillary tube (width 2.0 mm, path length 0.100 mm, wall thickness 0.100 mm; Vitrocom, Mountain Lakes, NJ) after a 2 minute incubation at 37 °C. This incubation was performed to ensure that FtsZ was fully polymerized prior to loading. The capillary was placed on a glass microscope slide and the capillary “fill” end sealed with molten paraffin wax to reduce evaporation.

For all simple assays, imaging was performed using an Olympus BX61 microscope equipped with an Olympus LCPlanFl 20x and a Hamamatsu C10600 10-megapixel charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) controlled by SlideBook 4.0 software (Intelligent Imaging Innovations, Denver, CO). For each experiment, development of birefringent structures was tracked by a 60-minute image capture series, with a 1-minute interval between images.

4.3.5 Droplet Scanning Assays for Optical Activity

For droplet scanning assays, a 5 µL drop of polymerized FtsZ (prepared as described in section 4.3.4) was placed on top of a leveled capful of Corning high vacuum grease (Dow Corning Corp, Midland, MI), using the cap from a 2.0 mL centrifuge tube. The FtsZ solution was mixed thoroughly into the vacuum grease with a sterile toothpick, then carefully applied to a glass microscope slide. The grease/droplet suspension was covered with a glass coverslip, with gentle pressure applied to spread the suspension under the coverslip. For all trials, pressure was applied until the spread was circular in shape with a diameter of 2.5 cm.

For all droplet scanning assays, imaging was performed using an Olympus BX61 microscope equipped with an Olympus LCPlanFl 20x and a Hamamatsu C10600 10-megapixel charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) controlled by SlideBook 4.0 software (Intelligent Imaging Innovations, Denver, CO). For each experiment, the objective lens was focused at a point 6 mm from the droplet edge. Development of birefringent structures was tracked by a 60-minute
image capture series, with a 2-minute interval between images. Each capture included two images: one with the polarizer and analyzer in the light path (to capture birefringence) and one with the polarizer/analyzer out of the light path (to capture droplet edges). Color images used to illustrate the technique were captured instead using the same microscope and objective, but substituting a SPOT Insight 4 4-megapixel CCD camera controlled by SPOT Advanced 4.6 software.

4.3.6 Data Analysis

In all droplet experiments, the change in droplet volume was determined by measuring the initial diameter of each droplet and the diameter of the droplet when birefringence first appears (both in pixels) using Slidebook 4.0 image capture software. Actual size was calculated from the calibrated number of pixels per micrometer, which for the imaging configuration described in section 4.3.5 is 1.6 pixels / µm. If birefringence did not appear in the droplet, the final size of the droplet was used. The initial and final droplet radii were used to calculate the initial ($V_i$) and final ($V_f$) droplet volumes, where the droplets are treated as ideal spheres. The overall concentration factor is calculated as the ratio of the volumes, $V_f/V_i$ and the final concentration as the product of the initial concentration of FtsZ added in the experiment and the experimentally-determined concentration factor.

For each trial within an experiment, the trial was repeated until the number of droplets with a measurable size change reached a minimum of 50, referred to as $n$ in the results section. This typically required 4-6 repetitions. From these data, mean volume change and concentration factor are calculated, along with a 95% confidence interval. These results are all presented in $X \pm Y$ format, where $X$ is the mean value and $Y$ is the 95% confidence interval.

4.4 RESULTS AND DISCUSSION
4.4.1 Holistic Model of the FtsZ Ring as a Liquid Crystal

There are two traditional views of the Z-ring: one, as an overlapping series of individual filaments a single layer thick and two, as a single long spiral filament that encircles the diameter of the cell multiple times (15). Neither of these models fully account for the evidence that filaments within the ring demonstrate both bending and sliding phenomena. Single-filament structures rely exclusively on filament sliding to produce a “purse-string” cytokinetic motion (22). Single-layer discontinuous filament models require lateral interactions between neighboring filaments to provide long-range order as well as prevent rolling of individual filaments in the filament bending phase of constriction (16). We propose a new, single-layer nematic order model of a liquid-crystalline Z-ring where the filaments exhibit a nematic order that solves both of these problems.

Figure 4.1 shows the comparison between our model of the Z-ring with nematic order vs. a ring constructed of discontinuous patches of filaments. In this model the flexible, spring-like FtsZ C-terminal linker (CTL) between the FtsA binding domain and the main body of the protein allows filaments to interact laterally across the entire surface of the filament, forming an ordered, continuous ring within a narrow band just beneath the membrane surface. Contour lengths of this domain vary by bacterial species, but for FtsZ is estimated to be between 17 and 20 nm. Given the 5 nm diameter of FtsZ polymers and, the amount of tension required to fully extend the CTL (23), and accounting for the hydrodynamic radius of the polymer suggest that a single layer filaments will dominate.

With this basic model defined, it is possible to estimate the upper-bound concentration of FtsZ within the zone where the Z-ring is present. This information is critical for experimental confirmation of the model, since phase transitions in lyotropic LCs are triggered only at the proper concentration of the material. If we assume that the Z-ring is an ideally-ordered structure where the filaments are laid end-to
Figure 4.1. Models of FtsZ ring structure in *Escherichia coli*. (A) single and multiple layer discontinuous model (B) single-layer nematic ordering model from this work. Reproduced and modified with permission from Meier and Goley (15).
end without annealing and that individual FtsZ molecules are spherical, the moles of FtsZ protein in the ring ($N$) is equal to the sum of the number of moles of protein in each individual ring. In this case, we treat each layer of filaments as a set of nested, concentric cylinders with a radius equal to the radius of the cell $R$ minus the distance ($d_n$) of the layer from the membrane where the amount of FtsZ in the filament layer ($n$) is

$$n = \left(\frac{2\pi(R - d_n)}{D_z} - \frac{(n_g l_g)}{D_z}\right) \times \frac{W}{w_s}$$

Where $D_z$ is the diameter of an FtsZ molecule, $n_g$ is the number of circumferential gaps between filaments, $l_g$ is the mean gap length, $W$ is the width of the Z-ring and $w_s$ is the width of the spacing between filaments. All of these quantities have been published in prior research, excepting the product $n_g l_g$. We can, however, calculate a minimum and maximum value by treating an individual ring of filaments as a quantized set of particles $D_z$ in length. Each circumference-length of the ring ($r - d_n = 385$ nm, $C_n = 2419$nm) can contain $C_n / D_z$ particles, or 484. Given that the mean FtsZ filament is 25 subunits long, each circumference-length at maximum packing can contain 18 filaments with a minimum total gap length ($n_g l_g$) of 93 nm. The maximum total gap length is 1194 nm; above this, there would be no overlap between filaments and lateral interactions would not occur.

Calculated FtsZ concentration in the Z-ring zone is plotted against the theoretical total gap length in Figure 4.2. Because of the particulars of the model used to make these calculations, the total gap length can be used as a proxy for the degree of overlap between neighboring filaments, where the lowest total gap length represents the maximum possible overlap and the highest gap length represents zero overlap. Depending on the degree of overlap between filaments, the maximum concentration of protein at the membrane in a Z-ring with nematic order ranges between 900 and 1600 µmol/L (36–65 mg/mL) and yields a middle 50% range of 1075-1425 µmol/L (42–57 mg/mL). There are no direct
Figure 4.2. Relationship between total gap length and FtsZ concentration. The concentration of FtsZ at the membrane is plotted in green and uses the primary Y-axis. The equivalent whole cell concentration, calculated by correcting for the full cell volume and the fraction of FtsZ that is present only in the ring, is plotted in red and uses the secondary Y-axis.
experimental measurements of the amount of FtsZ protein in the Z-ring; however, it is known from quantitative fluorescence microscopy that 30-40% of total amount of FtsZ in the cell is in the Z-ring. This estimates the need for a FtsZ at a concentration of 8.4–15.9 µmol/L (10.2-14.2 µmol/L middle 50%) throughout the entire cell. In *E. coli*, published reports estimate whole cell FtsZ concentration to range between 4–12 µmol/L, depending upon genetic background and growth conditions.

It is important to note that the calculations above represent an estimation based upon a condition of maximum order, which is only theoretically achievable. Even thermal fluctuations on the microscale will disrupt this order and lower the true concentration of protein in a ring with nematic order; however, the formation of the Z-ring, both *in vivo* and *in vitro* reconstitutions, is perturbed by many factors, some or all of which may be relevant to the development of nematic order within the Z-ring. That the theoretical upper bound overlaps with the experimental upper bound suggests that our calculated concentrations will serve as useful tools for guiding design of confirmatory experiments.

The environmental factors that may perturb nematic ordering of the Z-ring, and thus potentially regulate formation and function of the ring, are of particular biological importance. Three physical phenomena are most relevant to formation and maintenance of nematic order in filamentous lyotropes. The first is the hard-body interaction between filaments, as described by the theory of Onsager (3). As filament concentration rises, proximity and thermal fluctuations cause them to physically bump into each other. At a critical concentration, the most energetically favorable configuration becomes the nematic ordering of a lyotropic LC phase. As a visual aid, imagine a box of randomly strewn toothpicks or paper clips, in which each overlap randomly and haphazardly. If that box is gently shaken (thermal fluctuation, metaphorically), the items will become ordered and sit neatly in alignment. For idealized Onsager rods, the ability of the filaments to align is dependent on filament aspect ratio (length:diameter) and rigidity.
Higher aspect ratio and greater rigidity lowers the concentration necessary to form a nematic phase; lower aspect ratio and greater flexibility increases the threshold concentration.

The second and third phenomena—as described by Maier-Saupe mean field theory and its extension by MacMillan (3)—are inherent to charged molecules, a group to which all proteins belong. There are two aspects at play; a long-range attraction between filaments caused by induced dipoles between neighboring molecules and a short-range repulsion between filaments in very close proximity. In theoretical work, averaging these electronic forces over the whole of the material (the mean field) can predict certain aspects of LC behavior in lyotropes. For our purposes, the theory points to surface charge as another factor that can affect the ability of FtsZ filaments to form LC phases. As a consequence of the importance of surface charge, both free counterions (e.g. buffer salts) and the pH of the polymerization buffer may influence the threshold concentration.

Above all else, concentration is most important. Only at or above the proper filament concentration will lyotropic LC phase form, but the previously mentioned factors (curvature, aspect ratio, flexibility, and surface charge) all modify the actual value of that threshold concentration. The FtsZ protein adds a fifth, unique property: enzymatic activity. The vast majority of biological polymers that undergo LC phase transitions have no enzymatic activity of their own, with the notable exceptions of actin filaments and microtubules. Both are cytoskeletal polymers that have nucleoside triphosphatase activity like FtsZ; however, they are also much larger (10-100x depending on buffer conditions) and the nucleoside triphosphatase enzyme kinetics are markedly different than those observed in FtsZ. Additionally, nucleotide hydrolysis by FtsZ impacts polymer length, curvature, and flexibility in a wholly different manner.

Figure 4.3 shows the complex web of interaction between these five intrinsic properties and the external factors that can influence them. Ultimately, all factors either directly or indirectly interact. Of
Figure 4.3. Web of interaction between FtsZ properties of theoretical concern. Intrinsic factors are shown with colored borders/arrows and black text. Environmental factors are shown with black borders/arrows and red text. The dashed line indicates that both pH and divalent cations directly affect enzymatic activity.
all, aspect ratio is most directly affected by both intrinsic and extrinsic factors. Aspect ratio consists of two components: filament length and filament width. Decreased enzymatic activity and slightly decreased pH result in an increased average filament length. Under the proper conditions—pH < 6.0, the presence of divalent cations (excluding magnesium), excess sodium and potassium—otherwise solitary protofilaments begin to bundle, increasing filament width and dramatically reducing aspect ratio (24–26). A single bundling event halves aspect ratio and makes formation of an LC phase much less likely. It is interesting to note that many of the proteins that regulate ring formation in vivo also promote (and in some cases prevent) filament bundling in vitro.

It logically follows from the proposed model and experimentally observed interaction of both intrinsic and environmental factors that the development (or lack thereof) of nematic ordering can serve as a kind of “signal integrator” for the regulation of ring formation and function. If we consider the nematic phase an equilibrium condition between ring-stabilizing and ring-destabilizing forces, we can apply the phase continuum of soft matter as a gradient of ring states. When destabilization forces dominate, the ring becomes isotropic. The disordered condition eliminates the stabilizing lateral interactions required for constriction to proceed. When stabilizing forces dominate, the becomes increasingly ordered by position, as in a gel or crystalline state, eliminating the ability of filaments to slide. When the proper balance is struck, the nematic phase provides both long-range order and the ability to flow, and constriction can proceed. A summary of this regulatory schema is shown in Figure 4.4

From a physiological perspective, this sort of physical regulation can also provide a sound rationale for prevention or arrest of cytokinesis. Though live cells are homeostatic, bacteria can tolerate much greater extremes than their eukaryotic counterparts, and do so in part by being tolerant of changes in internal condition. Extremes of salt concentration and pH result in a physiological compensation that ultimately decreases growth rate, part of this which may be explained by inhibited Z-ring function.
Figure 4.4. Regulatory schema for a liquid-crystalline Z-ring. This regulatory model of a nematic FtsZ ring represents the summed effect of the Z-regulating factors illustrated in Figure 4.3. The intrinsic enzymatic activities of FtsZ are represented on the polymer biochemistry axis and extrinsic environmental factors on the polymer morphodynamics axis. The formation of a nematic phase in FtsZ filaments represents an equilibrium between the two forces.
Calcium is kept exceedingly low in the bacterial cytoplasm (90 nmol/L) (27); a sudden calcium flux could rapidly shift the ring into a gel phase, arresting constriction. Z-regulatory proteins can also serve as a primary or secondary messenger of physiological signaling through direct sensing of environmental conditions or indirectly through a signal-transducing protein. Taken together, these models predict that a threshold concentration of FtsZ needed to will be experimentally determinable, and that Z-regulating factors that increase polymer stability (or reduce turnover) should be accompanied by a decrease in threshold concentration. Conversely, factors that decrease polymer stability should be accompanied by an increase in threshold concentration. At extremes, they may prevent formation of a nematic phase entirely.

### 4.4.2 Simple Assays for Optical Activity

The traditional method for identifying lyotropic LCs is a simple trial-and-error process of imaging films of the material under study, using polarizing light microscopy. Because they have no special optical properties, isotropic solutions appear as black images. Solutions that have formed a nematic phase, however, appear bright. Anisotropic materials like nematic LCs cause the linearly polarized incident light to become elliptically polarized, which allows some the light to pass through the analyzer. Because LC materials are not perfectly uniform throughout, there are internal variations in brightness and darkness. This is called *texturing*, and these bright textured images are characteristic of nematic LCs.

Given the assumption that the LC phase is relevant only in the highly-concentrated state seen in the Z-ring, LC textures should only be seen in solutions of FtsZ that approximate that concentration.

Figure 4.4 shows imaging from simple assays in dilute solution (1-20 μmol/L), in physiological buffer. No birefringence was detected in dilute solution indicated by the lack of LC textures at all concentrations, which was to be expected if the LC phase behavior is relevant only in the Z-ring.
Figure 4.5. Simple assays of optical activity in dilute solutions of FtsZ. Image at time zero captured without the analyzer in place. Dark images across the time points indicate no development of anisotropy. Positive control is a thin film preparation of 13 wt% DSCG. Scale bars are 50 µm.
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**Figure 4.6.** Simple assays of optical activity in concentrated solutions of FtsZ. Image at time zero captured without the analyzer in place. Dark images across the time points indicate no development of anisotropy. Positive control is a thin film preparation of 13 wt% DSCG. Scale bars are 50 µm.
Figure 4.3 shows images taken from simple assays at high concentration (250-1000 µmol/L). These too showed no discernible texturing in physiological buffer. As a secondary check, a calcium precipitation test was used to visually confirm that FtsZ filaments were forming properly at each concentration. In this test, FtsZ polymerization reactions are prepared as described at each reaction placed onto a glass microscope slide, then spiked with calcium chloride to an equivalent final concentration of 20 mmol/L. When evenly dispersed in solution, the presence of calcium ions causes the filaments to form a crosslinked, gel-like network; however, when injected into a drop, the high local concentration causes the formation of a visible precipitate similar to a snow ball or cotton ball. In all cases, this precipitation test was positive prior to performing the simple assays (data not shown). Both negative controls, one lacking calcium and one with calcium added prior to GTP and mixed to disperse it evenly, do not exhibit the cotton-ball effect.

Given that FtsZ filaments are aligned \textit{in vivo} by their attachment to the membrane, we repeated these simple assays using a method that promotes the alignment of filaments in solution—fluid flow. In order to achieve flow alignment, we loaded FtsZ polymerization reactions—each prepared as described in the simple assay and checked with a calcium precipitation—into rectangular capillary tubes. The capillary flow of the fluid into the tube provides a shear flow that can align the filaments. Images captured from these flow-aligned solutions are seen in Figure 4.5. Even with flow alignment, no birefringence was detected in our concentrated solutions.

\textbf{4.4.3 Droplet Scanning Assay Development}

Continued work on potential LC phase behavior in FtsZ begged an improved method by which a variety of FtsZ concentrations could be scanned in a single assay. Phase behavior in any LC material is a continuum based upon a variety of factors (temperature, salt concentration) and it is well-established that
the structure and biochemistry of FtsZ filaments themselves also change based upon a variety of factors: pH, salt concentration, FtsZ concentration, and GTPase activity, among others. Continuing to use a simple assay given all of these possible variations was simply untenable. Additionally, some technical issues with the FtsZ protein itself needed to be overcome. In our hands, concentration of FtsZ performed best when FtsZ was present at amounts below 500 µmol/L (20 mg/mL). In standard buffers, solutions of FtsZ above 500 µmol/L begin to precipitate and become caught in the centrifugal concentrators, resulting in large amounts of protein loss. This can be overcome to an extent by concentrating in magnesium-free buffer; however, at concentrations around 1000 µmol/L (40 mg/mL) even this precaution becomes ineffective. This is most likely explained by the polymerization of the GDP-bound form of FtsZ which, bizarrely, appears to occur above 1000 µmol/L even in the absence of magnesium. In order to test the optical activity of FtsZ at concentrations higher than this, a method is needed in which concentration of the protein occurs while the filaments are in their dynamic, GTP-bound state.

To this end, we developed a droplet assay that allowed us to “scan” a range of concentrations in a single trial. In the droplet assay, FtsZ polymerization reactions are vigorously dispersed into a silicone-based, colorless vacuum grease. The vacuum grease is water-resistant and does not dissolve into the aqueous droplets, though evaporation of water from the droplets can still occur. As evaporation causes the droplets shrink in size, the concentration of the droplet contents slowly increases. The highly viscous vacuum grease also immobilizes the droplets, making imaging of the droplets simple. Additionally, the ability to easily measure the diameter of suspended droplets in size-calibrated microscopic images allows us to quantify the evaporation rate and droplet volume size change, from which the amount the reactions were concentrated over the course of the experiment and the actual concentration of the material at the point where birefringence becomes evident can be calculated.

Figure 4.8 shows a 1-hour-long time-lapse of these droplets from an early trial of the method,
Figure 4.7. **Droplet concentration scanning method.** Droplets of bromophenol blue in Tris-EDTA buffer (pH 8.0) appear purple in the above images, and decrease in size and become darker throughout the time course of the experiment. Large, dark-edged spheres are air bubbles, two of which disappear over the course of the experiment. Reference droplet is marked with a black arrow. Scale bars are 50 µm.
made using a solution of bromophenol blue dye in a basic buffer, which causes it to appear purple. The bright purple of the dye distinctly shows the difference between suspended droplets (small with no edge distortions) and trapped air bubbles (large with thick edge distortions). On the size scale used in this experiment (also used in all subsequent experiments), there are numerous droplets, and all droplets in the image carry the dye solution. This is an important observation, as droplets of FtsZ protein will be transparent and must be easily distinguished from any trapped air. Trapped air bubbles are quite large and grow larger over time, causing minor distortions in the grease medium. Air bubbles are included for illustrative purposes in this set of images, but are carefully avoided in imaging used for experimental data sets. As time progresses, the dye droplets decrease in size and become visibly darker, indicating that the water in the droplets is evaporating. If the droplet solution were diffusing or dispersing from the grease-droplet interface, the droplet color would not change.

Another aspect of the droplet scanning system visible in these images is the dependence of evaporation rate on the distance from the edge of the grease-dye suspension. In Figure 4.4, the edge of the grease-dye suspension is just out of view on the left-hand side of each image. Note that the left-most drops visibly shrink first; in the left-most area, shrinking of droplets could be noted by eye, with the droplets reaching their end size in as little as five minutes (data not shown). We sought to quantify this effect in order to choose the optimal distance from the edge for our concentration scanning trials.

Given that the grease smear is a roughly circular film underneath of a glass coverslip, and is uniform throughout in terms of droplet dispersion and temperature, the rate of evaporation (and thus rate of droplet shrinking) should be the lowest near the center of the smear and highest at the edges, where air exposure is the greatest. This effect is readily visible in the droplets shown in Figure 4.8 and images from quantitative trials of evaporation rate are presented in Figure 4.9. The most striking effect visible in the edge evaporation images is the tremendous variability in evaporation rates. One large droplet in the
Figure 4.8. Micrographs from edge-dependence trials. Edge images are taken from the edge of the air/grease interface, which is located just out of view at the bottom left. Center images are taken from the center of the smear, and midpoint images are taken at the midpoint between the two, 0.63 cm from the edge. Droplets were made with physiological buffer. Scale bars are 50 µm.
bottom-middle (d=92 µm) completely evaporated, while smaller droplets in the middle (ranging between 30-45 µm in diameter) lost 60% of their initial volume. This experiment was repeated at the center of the smear (1.25 cm radius) and at the midpoint between the center and the edge (6 mm from edge). The mean volume reduction for the center was 41.5% ± 6.9 for the center and 49.1% ± 4.4. These correspond to a concentration factors of 1.70-fold and 1.96-fold, respectively. Because a doubling of concentration was desired for downstream experiments, all future droplet scanning assays were performed by capturing images from a point 6 mm from the droplet edge.

The final test of the droplet scanning method was a proof-of-concept trial performed using a known lyotropic mesogen. For this trial, we used disodium cromoglycate (also known as Cromolyn or DSCG) which, at room temperature (25 °C), is liquid-crystalline between 12 and 18 wt% (120-180 mg/mL). For this trial, a solution of 8 wt% DSCG in water was dispersed as droplets into the silicone grease. At this starting concentration, DSCG is isotropic. In order to transition into the nematic phase, the droplets must reduce in diameter by 15% to achieve the minimum 1.5x concentration factor required. Micrographs from a single trial of these proof-of-concept experiments are shown in Figure 4.9

To create a positive control image (column 1), a drop of DSCG at 13 wt% was dispersed into the vacuum grease medium. At this concentration, DSCG is known to be birefringent at room temperature and the vast majority of droplets in this image display the characteristic “X” or “cross” patterning of nematic ordering in spherical droplets. For time course experiments DSCG was diluted to 8 wt% prior to suspension in silicone grease. Birefringence first appears in the smallest droplets at 32 minutes and by the 40 minute point shown in Figure 4.9, most of the smaller droplets have transitioned into a nematic phase. By one hour, even the largest droplets in the image have become birefringent. In order to determine the concentration of DSCG in the droplet, we used image analysis to measure the size change in each droplet, from its initial size to the size birefringence is first captured. The mean percentage volume change at this
Figure 4.10. Droplet scanning method proof-of-concept. These images, taken from a single trial of the droplet scanning proof-of-concept, show development of birefringence in droplets of DSCG. Positive control images are made with suspensions of 13 wt% DSCG. In time course experiments, DSCG is initially isotropic (8 wt%) and droplet birefringence develops as droplets shrink. Scale bars are 50 µm.
The mean DSCG concentration at the point of birefringence is 13.8 ± 1.82 wt%, This mean is slightly elevated as compared known 13% threshold concentration of DSCG in water, at room temperature.

4.4.4 Droplet Assays for Optical Activity of FtsZ

Next, droplet scanning assays of FtsZ were performed using the parameters set in the validation experiments. FtsZ in low-potassium physiological buffer (PBLK). This buffer, which serves as the base buffer for all droplet scanning of FtsZ, has a total potassium concentration of 120 mmol/L. Prior published work has shown that potassium ion concentration modulates GTPase activity; however, in the range of 100–300 mmol/L that effect is negligible (28). Since the droplet assays are designed to explore a two-fold change in concentration, the total potassium concentration will remain within this “safe” range throughout the course of the entire experiment. Figure 4.11 shows sample images from these experiments, and droplet data for all experiments are tabulated on Table 4.1. Droplet concentration factors were relatively reproducible and within the expected range (1.85–2.25 fold) with a mean concentration scanning range between 200–2252 µmol/L (10–90.1 mg/mL). No birefringent droplets appeared over the course of all trials.

For the next set of experiments, the buffer conditions were altered to promote an increase in filament aspect ratio. According to the fundamental models of development of nematic order, increased aspect ratio will decrease the threshold concentration necessary for a phase transition to occur. In FtsZ, this must be done without forcing filaments into bundles, which significantly reduces aspect ratio. There are two means of accomplishing this: a buffer pH of 6.5 and inhibition of GTPase activity with GMPCPP, a slowly-hydrolyzing GTP analog with a methylene bridge between the alpha and beta phosphate. At pH 6.5, and when polymerization is induced with GMPCPP, the average length of FtsZ polymers increases
Figure 4.11. Droplet scanning of concentrated solutions of FtsZ. Sample images from initial droplet scanning experiments. FtsZ in PBLK was used to create droplet suspensions at the starting concentration indicated on the left of the figure. No birefringence was detected in these trials. Data summary can be found in Table 4.1. Scale bars are 50 µm.
Figure 4.12. **Droplet scanning FtsZ at reduced pH.** Sample images from droplet scanning of FtsZ at pH 6.5. FtsZ in PBLKP was used to create droplet suspensions at the starting concentration indicated on the left of the figure. No birefringence was detected in these trials. Data summary can be found in Table 4.1. Scale bars are 50 µm.
Figure 4.13. Droplet scanning of FtsZ with GMPCPP. Sample images from droplet scanning of FtsZ at polymerized in the presence of the slowly-hydrolyzing GTP analog GMPCPP. FtsZ in PBLK was used to create droplet suspensions at the starting concentration indicated on the left of the figure. No birefringence was detected in these trials. Data summary can be found in Table 4.1. Scale bars are 50 µm.
### Table 4.1: Tabulated Droplet Scanning Data for FtsZ

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial [FtsZ] (umol/L)</th>
<th>Replicates</th>
<th>n</th>
<th>% ΔV</th>
<th>% ΔV / min</th>
<th>Δ[FtsZ] (-fold)</th>
<th>Final [FtsZ] (umol/L)</th>
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</thead>
<tbody>
<tr>
<td>PBLK</td>
<td>250</td>
<td>5</td>
<td>53</td>
<td>45.7 ± 6.9</td>
<td>0.762</td>
<td>1.84 ± 0.21</td>
<td>368 ± 42</td>
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<tr>
<td></td>
<td>500</td>
<td>5</td>
<td>56</td>
<td>53.3 ± 5.6</td>
<td>0.888</td>
<td>2.14 ± 0.23</td>
<td>857 ± 92</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>6</td>
<td>50</td>
<td>55.6 ± 8.6</td>
<td>0.927</td>
<td>2.25 ± 0.037</td>
<td>2252 ± 365</td>
</tr>
<tr>
<td>PBLPK</td>
<td>250</td>
<td>5</td>
<td>57</td>
<td>50.1 ± 3.4</td>
<td>0.835</td>
<td>2.00 ± 0.13</td>
<td>401 ± 26</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>5</td>
<td>61</td>
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<td>0.827</td>
<td>1.98 ± 0.12</td>
<td>794 ± 49</td>
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<tr>
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<td>4</td>
<td>60</td>
<td>56.6 ± 9.1</td>
<td>0.943</td>
<td>2.30 ± 0.40</td>
<td>2304 ± 399</td>
</tr>
<tr>
<td>PBLK + GMPCPP</td>
<td>250</td>
<td>5</td>
<td>53</td>
<td>56.1 ± 4.0</td>
<td>0.935</td>
<td>2.28 ± 0.19</td>
<td>456 ± 38</td>
</tr>
<tr>
<td></td>
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<td>762 ± 56</td>
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<td>52</td>
<td>53.6 ± 5.6</td>
<td>0.893</td>
<td>2.16 ± 0.23</td>
<td>2155 ± 232</td>
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<tr>
<td>PBLPK + GMPCPP</td>
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<td>52</td>
<td>52.6 ± 3.4</td>
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<td>2.11 ± 0.14</td>
<td>422 ± 28</td>
</tr>
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<tr>
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<td>55</td>
<td>56.7 ± 8.1</td>
<td>0.945</td>
<td>2.31 ± 0.36</td>
<td>2309 ± 364</td>
</tr>
</tbody>
</table>
without causing the filaments to bundle. Figures 4.12 and 4.13 show micrographs from the low pH and GMPCPP trials, and Table 4.1 tabulates the results. No birefringent droplets appeared over the course of all trials of both experiments. Neither droplet concentration factor and mean concentration scanning range were significantly different than the earlier trial with FtsZ in PBLK. Lastly, these experiments were repeated in the presence of both lowered pH and GMPCPP (Table 4.1, PBLPK+GMPCPP). No birefringent droplets were observed.

4.5 CONCLUSIONS

Despite several rounds of trials utilizing a variety of differing conditions, we were unable to detect the development of an nematic ordering in solutions of purified FtsZ, at concentrations predicted by our model. This does not fully exclude the possibility that the Z-ring itself exhibits some liquid-crystalline character; however, it does call into question the adequacy of traditional methods of identifying lyotropic LC phases, in particular, a shift from isotropy to anisotropy in bulk solution, as a means to uncover LC phase transitions in suspensions of FtsZ polymers. Neither the simple assay nor the droplet assay directly mimic the \textit{in vivo} configuration of the Z-ring. Simple assays—with and without flow alignment—assume the polymers will align within a planar film (in the absence of local geometric cues) on the length scale of the polymers. Droplet assays also suffer from this problem. Though the droplets are spherical, the large size (10-50 µm) is not a cognate to the curvature of a bacterial cell, which averages 0.5-2.0 µm. That said, neither the \textit{in vivo} state nor any current \textit{in vitro} reconstitution methods are adequate for this purpose.

Investigation of the optical properties of reconstituted Z-rings would require a system that meets several specific criteria. First, reconstitution would have to occur in the absence of phospholipid layers. The brightness of a birefringent signal is determined in part by the thickness of the anisotropic material,
so a Z-ring that is only a few filaments thick would be very weakly birefringent. This is still detectable with proper instrumentation; however, the phospholipid bilayers in the cell envelope and the supported bilayers or vesicles used for reconstitution methods are also anisotropic, LC structures. These would be birefringent as well, and comparatively much more so than the Z-ring. Any signal coming from the ring itself would be lost.

This is a particularly difficult criterion to meet, as all existing reconstitution systems rely on an amphipathic helix (specifically, the amphipathic helix of the MinD protein) for surface attachment. The advantage of the helix-bilayer attachment is two-fold. First, it is highly specific. Both the hydrophobic and hydrophilic portions of the phospholipid participate in attachment of amphipathic helices. This specific chemical requirement makes non-specific attachment rare, which is especially important in the protein production and purification process. Hydrophobicity is notoriously difficult to manage in protein purification, with many proteins requiring denaturation and re-folding steps that can complicate downstream applications. The fluid nature of lipid bilayers allows attached polymers to diffuse laterally along the membrane surface, a condition which appears to be necessary for proper Z-ring self-assembly in \textit{in vitro} systems (13, 29).

The second criterion requires that the diameter of any reconstitution vessel be less than 5 µm. Published work on Z-ring reconstitution, as well as in our own experience developing reconstitution systems, self-assembly of FtsZ-mts rings does not occur in vessels (whether capillary or liposome-based) with a diameter larger than 5 µm. This is due to the well-documented tendency of membrane-attached FtsZ filaments to align along the curvature of the surface to which they are attached, based upon the inherent curvature of the polymers themselves and their flexural rigidity. In the \textit{in vivo} condition in \textit{E. coli}, FtsZ polymers align in parallel to each other but are orthogonal with respect to the short axis of the cell. In lipid-lined, laser-etched channels of between 0.5 and 5 µm, this alignment angle decreases as the
channel size increases (30). In the course of our reconstitution efforts, we found that FtsZ-mts reconstitutions in fused-silica capillaries of 5 µm diameter were dominated by helices rather than rings, the likely explanation for which is this curvature-sensitive alignment of filaments.

This observation leads into the final criterion for a reconstitution system capable of detecting optical activity in Z-rings: matching species of bacterial FtsZ to the geometry of the reconstitution system. Because alignment of membrane-attached *E. coli* FtsZ-mts is guided (either in whole or in part) by the curvature of the membrane itself (30), reconstitutions of Z-rings using *E. coli* FtsZ (a rod-shaped bacterium) require a cylindrical attachment surface. All successful reconstitutions of *E. coli* FtsZ mimic this geometry, and do so in a variety of ways. Encapsulation of FtsZ-mts and FtsZ/FtsA* mixes into spherical vesicles does not produce reconstituted rings, in both our hands as well as other published prior work (31). If a newly-designed system incorporates spherical vessels, it should also incorporate an FtsZ protein from a bacterial species with spherical cell morphology, such as *Staphylococcus aureus*.

These final two points in particular may explain the inability of both the simple and droplet assays to detect an ordered phase. The slight curvature and semi-flexibility of FtsZ polymers are not, by themselves, a barrier to liquid-crystallinity. There is a great wealth of literature published, both experimental and theoretical, on LC phases of materials that form from flexible, polydisperse rods like FtsZ; however, if the specific manifestation of these properties in FtsZ filaments means that they are reliant upon the curvature of the cell membrane to provide the physical conditions necessary for the development of nematic order, this only underscores the need for a method to study surface-attached FtsZ filaments.

This has been done with fantastic results on freshly-cleaved mica surfaces. In fact, the configuration of FtsZ filament networks adsorbed to planar mica surfaces changes over time, in some cases, forming swirling vortices that can be imaged directly using atomic force microscopy (21).
Unfortunately, mica is an inadequate material for the purpose of identifying optically active LC phases, since it is an anisotropic crystalline mineral with its own inherent birefringence. It can, however, serve as a guide to the kind of material needed: an isotropic crystalline or amorphous solid that is optically transparent; that allows adsorption of protein through a weak electrostatic interaction; that allows the protein to remain laterally mobile; that is also amenable to etching of channels or grooves with electron beam lithography or another nanofabrication technique.

Even though the droplet assay was inadequate for detecting nematic order in FtsZ, it was able to detect the isotropic-to-nematic transition in solutions DSCG. Additionally, the concentration at which this transition occurred was very near the known threshold concentration. Given these data, we can feel confident that the assay itself is valid; however, DSCG is neither a protein nor a molecule of biological origin. Before we can be fully certain of its utility in detecting nematic ordering in biological molecules, repetition of the proof-of-concept trials with a different protein polymer (e.g. flagellin or eukaryotic cytoskeletal filaments) would be appropriate.

Even after taking the above into consideration, a perfectly valid assay could still fail to detect nematic ordering for reasons intrinsic to the FtsZ protein. In the absence of alignment cues from surface binding, whether from a phospholipid bilayer or otherwise, development of nematic order may be extraordinarily slow. Experiments involving FtsZ are necessarily time-limited by the amount of GTP available and the hydrolysis rate under those conditions. If the time required for development of nematic order exceeds the duration of the steady state, polymers will disassemble before they self-align. We attempted to compensate for this problem in the assay design; the one-hour period is the maximum duration of the steady state at the concentration of GTP used. Both low pH and use of GMPCPP reduce GTPase activity and thus extend the duration of the steady state, but even at the lowest rate of droplet evaporation the useful life of the assay only extends another 30 minutes (data not shown). A different
suspension medium could serve as a solution to this particular problem. In the course of assay
development several different suspension media were tested. Of all of the media, only mineral oil had a
slower rate of evaporation than silicone grease, but a two-fold increase in concentration would have
required several hours to develop, far longer than even the extended steady state duration in the presence
of GMPCPP. Following this path would require more extensive testing of suspension media.

Beyond the technical aspects, there are two potential avenues continued exploration. Both involve
changing the intrinsic aspects of the protein. The first, and simplest, would be the use of FtsZs from other
species of bacteria. All of the basic parameters of FtsZ protofilaments vary across species. FtsZ filaments
of extremophiles like *Thermotoga* and some mycobacteria have longer average filament length and are
found in protofilament pairs, presenting an opportunity to study a less-flexible filament with a different
aspect ratio. *Bacillus subtilis* FtsZ has a very low GTPase activity, allowing longer periods of the steady
state condition. The second would be the development of FtsZ proteins with mutant phenotypes that
would promote nematic ordering. Mutation of the amino acids on the lateral surface of the protein may
serve to reduce or block filament bundling. GTPase-deficient mutants would exhibit a dramatically
increased steady state duration. Both of these types of mutations are difficult to create because they are
lethal phenotypes in live cells. Such mutants would have to be created and the phenotypes screened solely
*ex vivo* and the interdependent nature of the basic filament parameters would require careful screening to
ensure that filament morphodynamics are minimally impacted.

The least satisfying interpretation of these results is that FtsZ does not exhibit nematic ordering,
in bulk solution or otherwise. Because the fundamental hypothesis of the model is the presence of nematic
order, this would require rejecting that hypothesis and reformulating a model terms of non-LC
phenomena. Given that techniques in *in vitro* reconstitution are becoming more sophisticated, it would be
most prudent to explore the possibility of detecting nematic ordering in reconstituted rings before
rejecting our foundational hypothesis.
4.6 REFERENCES


CHAPTER V

GENERAL CONCLUSIONS

This work began with the overarching aim of developing a new theory and new methods to elucidate the biophysical processes underlying the formation and function of the bacterial cytokinetic ring, known as the Z-ring. To this end, we developed a plan to attack this problem in three ways. First, we developed a new method of \textit{in vitro} reconstitution utilizing cylindrical supported lipid bilayers as a surface for attachment of FtsZ which resulted in the robust and reproducible formation of functioning Z-rings in both FtsZ-only and dual FtsZ/FtsA* systems. Second, we proposed a model of the FtsZ ring as lyotropic liquid crystal material exhibiting nematic order. We attempted to detect this nematic ordering bulk solution at threshold FtsZ concentrations predicted by the theory. Lastly, we used the new reconstitution system to investigate environmental conditions that alter the essential intrinsic physical properties of FtsZ and its filaments and assess the impact of those conditions on formation and function of the ring, for the purpose of better understanding the mechanisms underlying those properties of the ring.

On the first point, we successfully developed a reconstitution system that solved many of the problems of other prior published systems and allowed for expression of robust, reproducible parameters for study. Both single-protein FtsZ-YFP-mts and two-protein FtsZ-YFP/FtsZ mixes successfully formed Z-rings when assembled against our tube-shaped supported bilayers. These rings had varied morphology that was correlated to their function: large, complete rings that were immobile and smaller, incomplete rings that had the ability to diffuse laterally across the membrane and condense into larger rings that subsequently immobilized. A subset of these larger rings were capable of deforming the membrane, which
in the dual-protein system resulted in completed division of the membrane, also called membrane scission.

This system represents a significant improvement over existing systems. Cylindrical bilayer shape is critical for proper formation of rings using \textit{E. coli} FtsZ (1, 2). This had previously only been successfully produced in tubular, multi-lamellar liposomes that were variably permeable to protein and in spherical vesicles made oblate by suspension in an agarose gel medium. Our system provides well-defined, reproducible, unilamellar layers. Unlike variably permeable tubular liposomes, polymerization reactions are directly injected into the channel by simple capillary action, providing reproducible initial conditions. Reconstitutions in both liposome systems are rare as a percentage of all vesicles; our system reliably produced reconstituted rings without the need for screening multiple trials. Finally, because our system is not space-restricted by vesicle ends, we were able to optimize the dynamic range of the assay by showing that increasing concentration of FtsZ within the normal physiological range affected only the gross number of rings formed, and not their physiology.

Despite this, the system does have drawbacks. It is very labor-intensive, and microscopic detection means that full automation is not possible lacking some means of optical detection similar to that seen in capillary electrophoresis. Additionally, the curvature of the fused-silica capillary tube limits the overall view of the tube to the lower two-thirds and introduces some minor spherical aberrations that will preclude further probing of the structure by super-resolution imaging unless a means of correcting for that aberration can be developed. Regardless, the system performs admirably at the job it was designed to do the reconstitution of large numbers of Z-rings for the purpose of physiological and biochemical investigation of the mechanisms of ring formation and function.

The possibilities for future work with this system are endless. Most importantly, it will allow investigation of the properties of division protein mutants that would otherwise be fatal to living cells. The ability to visualize and manipulate these extreme phenotypes would benefit the field immensely. New
additions to the method can be developed to, for the first time, directly measure the effect of Z-interacting proteins on Z-rings outside of the confines of the cell, where the specific effects of single proteins can be isolated and controlled. Finally, we envision this system (with some modest modifications) having application as a way to sensitively screen compounds for anti-FtsZ activity, a burgeoning field of research within the realm of next-generation antibiotic targets.

Next, we developed a model of the Z-ring as a lyotropic liquid crystal structure exhibiting nematic order. We chose this particular method because of a wealth of observations about the form and function of FtsZ that echo the form and function of lyotropic liquid crystals. Lyotropes like disodium cromoglycate are disk-shaped molecules that self-assemble into filaments, which align to form a liquid-crystalline phase. FtsZ is a globular protein that self-assembles into filaments (3). Phase transitions in lyotropes are sensitive to concentration within an aqueous environment and must condense into regularly ordered structures. FtsZ filaments are concentrated both laterally (by the Min and NO systems) as well as axially (by membrane attachment) within an aqueous environment and must condense to form ordered structures that are functional (4). The nematic phase of lyotropes is characterized by long filaments that align along their long axis. Super-resolution imaging has shown that E. coli Z-ring consists of either a ribbon or bundle of overlapping filaments (5). The nematic phase is a manifestation of long range order within the material due to alignment of filaments driven by steric interactions and electrostatic interactions. FtsZ is a long polymer of high aspect ratio and net negative charge that must maintain long range order via weak lateral interactions (and not lateral bonds) in order to form a functional structure capable of transmitting force against the membrane despite being made of discontinuous, overlapping filaments.

Given this model, we calculated an upper limit for the concentration of protein necessary to generate that order, and tested the ability of polymerized protein at that concentration to form a
structure with nematic order that could be detected optically by polarizing light microscopy. We were unable to successfully detect birefringence (a characteristic of materials with nematic order) in solutions of assembled FtsZ filaments. Though this is certainly a blow to the model, and as such we must temporarily reject or reformulate our hypothesis, there are several reasons why these traditional methods of detecting the development of a nematic phase may fail to find nematic order in FtsZ filaments.

Neither of the assays used directly mimic the \textit{in vivo} configuration of the Z-ring. These assays assume that the polymers will align within a planar film (in the absence of local geometric cues) on the length scale of the polymers. The developed droplet assay also suffers from this problem because the large size (10-50 µm) is not a cognate to the curvature of a bacterial cell, which averages 0.5-2.0 µm. That said, neither the \textit{in vivo} state nor any current \textit{in vitro} reconstitution methods are adequate for this purpose because the lipid bilayer is itself a liquid crystal and exhibits birefringence. In response to this, we described a new means of testing this hypothesis, ultimately a reformulation of the \textit{in vitro} reconstitution assay that can potentially solve this problem.

Also, it may simply be that \textit{E. coli} FtsZ is unsuitable for this kind of assay \textit{in vitro}, in the absence of a new system that allows reconstitution of Z-rings on a non-birefringent surface. All of the basic parameters of FtsZ protofilaments vary across species. FtsZ filaments of extremophiles like \textit{Thermotoga} and some mycobacteria have longer average filament length and are found in protofilament pairs. Decreased flexibility and increased aspect ratio in these polymers would help stimulate nematic ordering through increased steric interaction. \textit{Bacillus subtilis} FtsZ and the \textit{E. coli} FtsZ mutant FtsZ84 have a very low intrinsic GTPase activity, allowing longer periods of the steady state condition, which would give a longer period of time for the development of nematic order.

Another possibility would be the development of FtsZ proteins with mutant phenotypes that promote lateral interaction and nematic ordering. Mutation of the amino acids on the lateral surface of
the protein may serve to reduce or block filament bundling. GTPase-deficient mutants would exhibit a dramatically increased steady state duration. These are impossible to test in live cells because they are highly toxic to the cells; such mutants would have to be created and the phenotypes screened solely ex vivo. That said, this particular route of investigation would dovetail nicely with the a modified but related in vitro reconstitution system, where altered FtsZ proteins could be tested both for their physiological properties and their ability to develop nematic order.

This was originally the intent with our third hypothesis, to attempt to correlate conditions that alter development of nematic order with those that alter formation and function of Z-rings. Conditions that increase polymer stability will decrease the threshold concentration for development of nematic order, and those that decrease polymer stability will increase it. In the in vitro reconstitution system, we proposed to correlate conditions that alter threshold concentration for nematic ordering with conditions that alter ring formation and function. We hypothesized that Z-ring stability (as determined by the number of formed and constricting Z-rings within the reconstitution system) would peak at some middle value. Though the reconstitution system provided some fantastic insights, we did not see any parameters that behaved in this manner, leading us to reformulate our hypothesis in terms of a balance of factors.

The result of this work was a new model of Z-ring formation and function that is a hybrid of current sliding and bending models, where formation and initial constriction of the ring is driven by the order of filaments and their ability to slide relative to each other along their length. This sort of “purse string” constriction must entail long-range order within the filaments, or else the filaments will simply slide past each other impotently, not transmitting any force to the membrane for deformation and constriction. As constriction proceeds, sliding of filaments begins to reach a physical limit of effectiveness, requiring the bending (and thus breaking) of filaments to either resume sliding, generate a force of their own, or both. This filament bending is induced by the hydrolysis of GTP and subsequent release of
inorganic phosphate, resulting in a filament that transitions from a radius of curvature of 200 nm to one with a curvature around 20 nm. This bending force also requires long-term order, as isolated discontinuous filaments would simply roll upon application of the bending force without some lateral stabilization. These experimental observations keep alive our hypothesis of the ring as a structure with nematic order, as no current theory of Z-ring formation or function adequately explains both of these phenomena.

Once again, mutant proteins will play a critical role in understanding how these factors affect Z-ring formation and function within the reconstitution system. One of the benefits of the particular design of the FtsZ-YFP and FtsZ-YFP-mts clones is that appropriately designed primers can allow the genetic replacement of the current wild-type *E. coli* FtsZ with any other FtsZ, mutant or otherwise, for use in the single-protein and dual-protein reconstitution systems. We have already begun to do this as part of extending this work and clarifying the basic mechanisms behind Z-ring condensation, constriction, filament sliding, and membrane scission. The simplicity of the concepts used in reconstitution system design and in the reconstitution itself means that this method should be widely accessible to other researchers and will dramatically speed the pace of revelations regarding Z-ring function and the function of Z-interacting proteins. Above all else, this will likely be the most important, furthest-reaching, and longest-term impact of the work.
5.1 References

Escherichia coli proto-ring elements (FtsZ and FtsA) inside giant unilamellar vesicles obtained from


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