

***In vivo* Microscopic Studies of FtsZ Self-
Assembly Dynamics in *Escherichia coli***

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Bryant Edward Walker

May 2020

Copyright © 2020 by Bryant Edward Walker

All rights reserved.

DEDICATION

Throughout the 1500 and 1600s many people were uprooting their lives to move to a vast unknown world where they desired a better life for them and their families. This new world will eventually become what we know of as the United States of America today. However, we will begin this journey in the year 1607. At this time a man named Samuel Walker came to the new world with the Virginia Company of London and settled what would become the first permanent English settlement of Jamestown. During his lifetime Samuel was a reverend and a signing member of the second Virginia charter for exploration of the new world in 1609. By the time of his death in 1622 he had left behind two sons and a wife to carry on the Walker family name.

Even though the Walker family was of the lower class, over the next 200 years they were able to migrate southwest through North Carolina and eventually landing in what is known today as Tennessee. This brings us to the mid-1800s where Robert Benjamin Walker settled in Grainger county Tennessee, where he had a son named Arthur who then had a son named Charles Milton Walker.

This now brings us the 20th century. Charles Milton Walker was my grandfather. He was born January 7, 1909 to a lower class family that were farmers. He married my grandmother, Hattie Morgan born June 14, 1914, in the year of 1929. My grandmother and grandfather both did not complete even a middle school education. They had too many responsibilities to have this luxury of an education at the time due to poverty and needing to help take care of their families. This led to them working and farmer for pennies and nickels a day says my father. During their lives they had 4 children, where 3 were

born from 1930-1933 and the 4th in 1952. Only one of their children, 4th, earned a high school diploma which is my father, Gerald Walker. My father's siblings did not because of war times and needing to support their family.

The Walker family from the year 1607-1970 had no one complete high school, some due to events not under their control. Recently, some Walkers have completed college and some even a master's. This finally brings us to the present day. My father and mother always instilled in me to always do my best at whatever I try to do. They always wanted me to get an education because they did not have the same opportunities that I have had especially through academic and athletic scholarships to pay for schooling. They are even astonished that I took my education this far considering that my mother, sister, and other family members stopped after a bachelors. No one of my blood-line on either side of my family, that be the Walkers described here or the Bryant family, mother's maiden name, has received their doctorate. After the completion of my Ph.D, I will have become the first person of my blood-line to have become a doctor. So I want to dedicate this dissertation to the entire Walker and Bryant families that came before me and that are still alive today. This one is for all of you. You all made this outstanding achievement a reality and I am honored to be a member of the Walker and Bryant families. My blood-line is part of the reason I pursued a Ph.D to make the Walker and Bryant families proud, and they will continue to be my inspirations for the rest of my life.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my Ph.D advisor Dr. Jaan Mannik and his wife Dr. Jaana Mannik, whom took an invaluable portion of their time these past 5 years to guide a naïve 24 year old graduate student and transform him into an independent researcher. Furthermore, I would like to thank Dr. Jaan Mannik for teaching me how to take large bodies of scientific literature and form them into a coherent story, which can be applied to research. Although, Professor Jaan and Jaana Mannik were my advisors and superiors throughout this experience, I look at them as part of my family as I have gotten to know them and as they have gotten to know me. Hopefully, we stay in touch no matter what the future brings.

I would like to thank my committee members: Dr. Anthony Mezzacappa, Dr. Soren Sorensen, Dr. Steve Abel, and Dr. Maxim Lavrentovich for their invaluable time and advice given to aid in my journey these last 5 years. They have helped me view my research from other disciplinary vantage points, which has improved my cross-disciplinary communication with other colleagues. They have also shown me how important my research and goals are through their interest in joining my committee and have given great advice towards the next steps in my life.

I would also like to thank many of my colleagues and friends that I have made throughout this journey. Our relationships have enabled me to be a better person and see my life in a better perspective. Thanks for the support during all the good and bad times. In particular, I would like to thank one of my best friends Dr. Matthew Bailey for taking me under his wing, giving me advice on all things in research and life, and being a comedic

relief when needed, another good friend the ever so talented Da Yang who dealt with my curiosity about worldly things on a regular basis, the soft-spoken kind-hearted always understanding Dr. Sriram Tiruvadi Krishnan, the always entertaining Anne Jennings, and the grill master Dr. Daniel O'dell.

I would like to briefly, thank the University of Tennessee Student Counseling Center, specifically Dr. William Hahn and Donna Voica NP, for helping me with my anxiety that had developed along this journey and getting me through the stressful times. They have helped make me a more mentally strong person again.

Finally, I would like to thank the main people in my life, my parents, for putting up with me and giving me a place to live these past 8 years since undergrad so I could save money. I am sure you are rethinking that in hindsight of it taking 7 years to finish school. But I owe everything to them my character, integrity, and compassion towards others. They have always supported me in everything I have done and will continue to do in my life. I love you both. We did it.

ABSTRACT

During the first stage of cell division in bacteria, FtsZ filaments form a ring-like assembly, the Z-ring, which encircles the cell's middle. This filament scaffold recruits a set of enzymes that synthesize the new cell wall, which divides the cell into two daughters. This dissertation addresses two questions related to the formation of the Z-ring. First, what molecular mechanism triggers Z-ring formation? Second, what are the intermediary structures through which the Z-ring assembly proceeds? The experiments are carried out in *Escherichia coli* (*E.coli*) bacteria, whose FtsZ molecules are fluorescently labelled. Using quantitative analysis of high temporal and spatial resolution time-lapse images, we show that the Z-ring formation at the midcell is preceded by a period when transient membrane-linked FtsZ assemblies form throughout the cell body. These transient assemblies result from the attachment of short FtsZ filaments to the inner membrane from the cytosol. We estimate these filaments to be less than 20 monomers long. Once attached filaments become more stable, but despite this stabilization they show treadmilling and periods of rapid growth and shrinkage. At the time of Z-ring formation one of the midcell assemblies is able to grow at the expense of the other transient assemblies indicating that FtsZ is a limiting factor for Z-ring assembly. The latter conclusion is further confirmed by our measurements of FtsZ concentration during the *E. coli* cell cycle. In slow growth conditions, we find a 4-fold upregulation of the FtsZ synthesis rate at the time of Z-ring formation. Altogether, our work shows that dynamic FtsZ filaments sample the cell surface for the possible location of the Z-ring and that there is a cell cycle control over the timing of the Z-ring formation, albeit not stringent.

TABLE OF CONTENTS

<i>Chapter 1</i>	1
Introduction	1
1.1 Why cell division in bacteria?.....	1
1.2 The model organism Escherichia coli	3
1.3 Components of the bacterial Z-ring.....	4
1.4 Regulation of Z-ring components.....	8
1.4.1 The Min system.....	8
1.4.2 SImA-mediated nucleoid occlusion	10
1.4.3 Ter-linkage	12
1.5 Characteristics of FtsZ polymers	14
1.5.1 Nucleation	14
1.5.2 Critical concentration and cooperativity.....	16
1.5.3 Polymer dynamics.....	17
1.5.4 Treadmilling behavior of FtsZ filaments	20
1.5.5 Protofilament lifetime and length based on a simple treadmilling model	23
1.5.6 Rate of change of filament length.....	25
1.5.7 Protofilament bundling	27
1.6 Dissertation Layout.....	30
<i>Chapter 2</i>	31
Materials and Methods	31

2.1 Experimental Methodology	31
2.2 Fluorescence microscopy	32
2.2.1 TIRF microscopy	34
2.2.2 HILO microscopy.....	36
2.2.3 Stroboscopic single molecule imaging of FtsZ	37
2.3 Microfluidic devices.....	39
2.3.1 PDMS based chip fabrication	41
2.3.2 Agarose based chip fabrication	43
2.3.3 Upright mother-machine channels	46
2.4 Qualitative and quantitative image analysis	48
2.4.1 Visualization of timelapse data.....	49
2.4.2 Determination of power spectral densities and autocorrelation functions.....	51
2.4.3 Protofilament tracking and rate of change analysis	52
2.4.4 Determination of treadmilling velocities of FtsZ assemblies and distribution of treadmilling speeds	55
2.4.5 Estimation of measurement noise contributions	57
2.5 Modelling	58
2.5.1 Modelling of protofilament lifetime and length distributions	58
2.5.2 Modelling bundling using Ising-like model.....	58
2.6 Bacterial strains	60
2.7 Cell culture growth conditions	63
Chapter 3	64

Cell Cycle-dependent regulation of FtsZ in *E.coli* in slow growth conditions 64

3.1 FtsZ concentration varies during the cell cycle in slow growth conditions	64
3.2 Degradation at the end of the cell cycle is caused by ClpXP	68
3.3 Synthesis of FtsZ during the cell cycle is not uniform	71
3.4 Quantification of degradation rates in slow growth conditions	78
3.5 Rapid degradation of FtsZ by ClpXP begins when the Z-ring starts to dissociate	80
3.6 Correlation between FtsZ upregulation and Z-ring formation.....	84
3.7 Discussion	87
3.7.1 Comparison of model to experimental data.....	89
3.7.2 Similar FtsZ regulation cycles in other bacteria.....	91
3.7.3 How does ClpXP rapidly degrade FtsZ during the end of the cell cycle?	93
3.7.4 Z-ring formation affected by varying FtsZ levels.....	95

Chapter 4 97

Transient membrane-linked FtsZ assemblies precede Z-ring formation in *Escherichia coli* 97

4.1 Transient assemblies of FtsZ on the cell membrane precede the formation of the Z-ring	97
4.2 Size of transient assemblies is limited by Z-ring components.....	102
4.3 Spatial and temporal distribution of FtsZ assemblies.....	105

4.4 Cytosolic protofilaments <i>in vivo</i> are shorter than ones observed from <i>in vitro</i> measurements	112
4.5 Rapid kinetics of FtsZ transient assemblies.....	115
4.6 Discussion	118
4.6.1 Dynamics of transient assemblies.....	118
4.6.2 Competition between different assemblies for the pool of available FtsZ....	120
4.6.3 Transient FtsZ assemblies probe for the proper location for the Z-ring.....	121
Summary.....	123
References.....	126
Appendix	146
A.1 Glossary	147
A.2 Diffusion coefficient calculation	152
A.3 Coverslip cleaning procedure	154
A.4 P1 transduction procedure	156
A.5 List of strain information	157
Vita.....	159

LIST OF TABLES

Table 1: List of the strains used in experiments	61
Table 2: Growth and division characteristics of different strains used in this study	75
Table 3: Fitting results for maturation rates.	75
Table 4: Fitting parameters for linear fits for panels D-G in Figure 3.13	85
Table 5: Fitting parameters for deletion strains	88
Table 6: Parameters of linear fits in Figure 4.5 and 4.6.....	111

LIST OF FIGURES

Figure 1.1: Schematic of cell division in bacteria using <i>E. coli</i> as an example	2
Figure 1.2: Z-ring structure.....	5
Figure 1.3: Protofilament assembly characteristics	7
Figure 1.4: Min system oscillations prevent FtsZ filament assembly near cell poles.....	9
Figure 1.5: SlmA-Mediated nucleoid occlusion effects on FtsZ assembly.....	11
Figure 1.6: Positive Regulation of FtsZ assembly by the Ter linkage	13
Figure 1.7: Traditional biological polymer assembly models	15
Figure 1.8: Polymerization models for cytoskeletal filaments	19
Figure 1.9: Treadmilling behavior of the bacterial Z-ring	21
Figure 1.10: Polymer length and lifetime distributions from a treadmilling model.	24
Figure 1.11: Rate of change of filament length from treadmilling	26
Figure 1.12: Ising-like model of polymer formation and bundling	29
Figure 2.1: Imaging system	33
Figure 2.2: Fluorescence microscopy techniques	35
Figure 2.3: Single molecule imaging	38
Figure 2.4: Traditional imaging platforms used in microscopy experiments	40
Figure 2.5: PDMS chip fabrication and design	42
Figure 2.6: Microfluidic mother machine agarose chips	44
Figure 2.7: Design and imaging in vertical pockets	47
Figure 2.8: Cell segmentation resulting in kymographs of the bacterial Z-ring.	50
Figure 2.9: FtsZ filament tracking before Z-ring formation.....	53

Figure 2.10: Schematic of Ising-like bundling model	59
Figure 3.1: Changes in fluorescent FtsZ concentrations and numbers during the cell cycle	65
Figure 3.2: Data from a representative cell expressing FtsZ C-terminal fusion to GFP from a plasmid (strain JM60)	67
Figure 3.3: Images showing tilted and helical Z-rings (top row for each strain) and irregularly shaped cell poles	69
Figure 3.4: Comparison of FtsZ concentrations and numbers in WT and the protease deletion strains.	69
Figure 3.5: Rates of change in FtsZ numbers during the cell cycle in ClpX and ClpP null mutant (left column) and WT (right column) strains in slow growth conditions.	72
Figure 3.6: Measurements of mNeonGreen (mNG) maturation rates k_{mat} in WT cells (strain JM26) in slow (M9 glycerol medium; N=15), fast (M9 glucose+CAS medium; N=18) growth conditions and in $\Delta clpX$ strain in slow growth conditions (M9 glycerol medium; N=10)	74
Figure 3.7: Variation of the synthesis and degradation rates during the cell cycle of WT cells in fast growth conditions (M9 medium with glucose+CAS)	77
Figure 3.8: Degraded amount of FtsZ at the end of the cell cycle vs. number of FtsZ present when fast degradation starts at time T_3	77
Figure 3.9: Degradation rates of FtsZ in the beginning and end of the cell cycle	79
Figure 3.10: Rapid degradation of FtsZ is a consequence of the Z-ring dissociation	81
Figure 3.11: FtsZ-mNG distribution during SulA induction	83

Figure 3.12: Distribution of times when the Z-ring forms (T_z) in WT cells (strain JM26) in fast growth conditions (M9 medium with glucose and casamino acids)	83
Figure 3.13: Link between upregulation of FtsZ synthesis and formation of the Z-ring in slow growth conditions	85
Figure 3.14: Correlations between upregulation of FtsZ synthesis and formation of the Z-ring in slow growth conditions in $\Delta clpA$ strain	86
Figure 3.15: Correlation between FtsZ levels and timing of Z-ring formation in different strains	88
Figure 3.16: Correlations between upregulation of FtsZ synthesis and formation of the Z-ring in slow growth conditions in the $\Delta clpP$ strain	90
Figure 3.17: Correlations between upregulation of FtsZ synthesis and formation of the Z-ring in slow growth conditions in the $\Delta clpX$ strain	92
Figure 3.18: Comparing model and experimental data	94
Figure 4.1: Transient membrane-linked FtsZ assemblies precede Z-ring formation	98
Figure 4.2: Data from a representative cell expressing FtsZ C-terminal fusion to GFP from a plasmid (strain JM60).....	100
Figure 4.3: Comparison of FtsZ transient assemblies before and after Z-ring formation	103
Figure 4.4: Comparison of FtsZ-mNG (JM26) to FtsZ-GFP (JM60) fluctuations before and after Z-ring formation.....	104
Figure 4.5: Characterization of spatial and temporal distributions of FtsZ transient assemblies that contain 2.5% or higher amount of FtsZ relative to the cell total ..	107

Figure 4.6: Characterization of spatial and temporal distribution of FtsZ transient assemblies in JM60 strain where FtsZ-GFP is expressed from a plasmid	109
Figure 4.7: Rate of change in the size of FtsZ transient assemblies.	113
Figure 4.8: Determination of FtsZ concentration in <i>E. coli</i> cells growing in M9 glycerol medium (strain KC606)	116
Figure 4.9: Inferred sequence for formation of transient assemblies.....	119
Figure A.2.1: Diffusion coefficients for a range of filament lengths.....	153

LIST OF ABBREVIATIONS

<i>abcD</i>	Refers to a hypothetical gene <i>abcD</i>
AbcD	Refers to the gene product (or protein)
$\Delta abcD$	Indicates gene <i>abcD</i> has been deleted
$\Delta abcD::efgH$	Gene <i>abcD</i> has been replaced with gene <i>efgH</i>
pAB01	Refers to a hypothetical plasmid AB01
GFP	Green fluorescent protein
RFP	Red fluorescent protein
mNG	A type of green fluorescent protein (mNeonGreen)
sf	Sandwich fusion
IPTG	Isopropyl β -D-1-thiogalactopyranoside
kan	Kanamycin (antibiotic)
amp	Ampicillin (antibiotic)
cm	Chloramphenicol (antibiotic)
frt	Flippase recognition target
aph	Resistance gene for kanamycin
LB	Lysogeny broth (nutrient rich bacterial growth medium)
M9	Salt blend for nutrient poor bacterial growth medium
PDMS	polydimethylsiloxane
Ter	Replication terminus region of the chromosome
NO	Nucleoid occlusion
WT	Wildtype cells
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
HILO	Highly inclined laminated optical sheet microscopy
TIRF	Total internal reflection fluorescence microscopy

PALM	Photoactivated localization microscopy
SIM	Structured illumination microscopy
SBSs	SlmA binding sites
CCD	Charge-coupled device (camera)
T_d	Doubling time
T_z	Z-ring formation time

CHAPTER 1

INTRODUCTION

1.1 Why cell division in bacteria?

In recent decades, many pathogenic strains of bacteria have developed multidrug resistance to modern antibiotics and with few novel antibiotics under development today, the necessity for new drug targeting pathways in bacterial cells is essential (1). Today, antibiotics target proteins involved in chromosome replication (fluoroquinolones), transcription (rifampicin), translation (aminoglycosides, macrolides, and tetracyclines), and cell wall synthesis (β -lactams) (2). A possible new antibiotic targeting pathway involves the bacterial cytoskeleton, which is distinctly different from the eukaryotic (including human) cytoskeleton. This structure consists of protein complexes responsible and essential for carrying out many critical cellular processes, including membrane septation, chromosome segregation, cell elongation, and cellular maintenance (3-7). However, since the invention of antibiotics, the bacterial cytoskeleton has yet to have a probable clinical antibiotic target emerge (1).

In most of the bacterial kingdom, successful cell division is mediated and achieved by the assembly of the multiprotein cytoskeletal machinery referred to as the divisome (divisome, see Appendix *Glossary*) (Figure 1.1, right) (8, 9). In most rod-shaped bacteria, the divisome is localized to the midcell and assembles perpendicular to the long axis of the cell (9). This machinery consists of over 30 different proteins that coordinate together to guide and carry out synthesis of the septal peptidoglycan (septal peptidoglycan, see

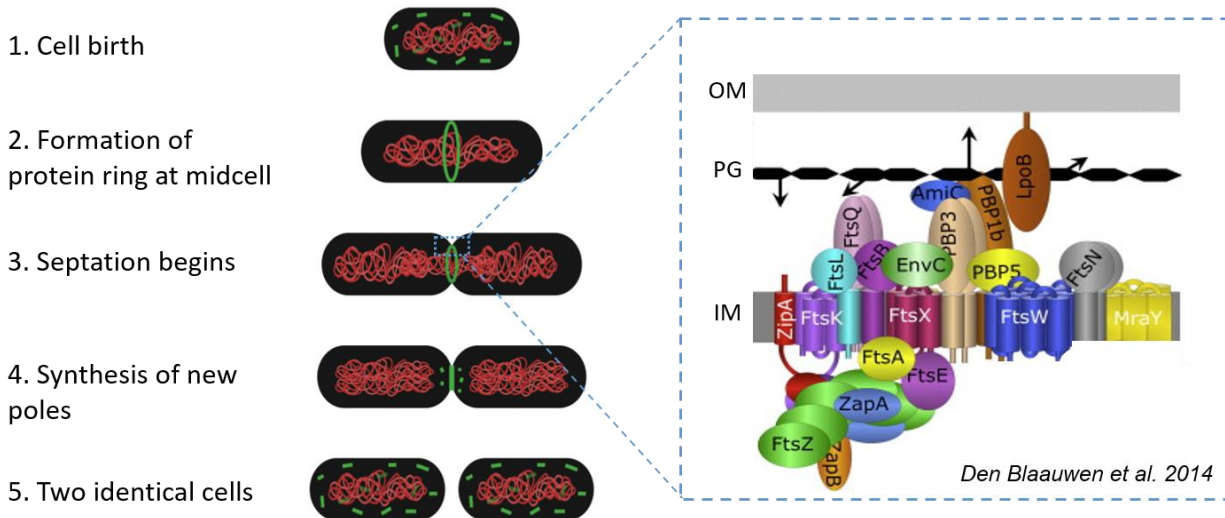


Figure 1.1: Schematic of cell cycle in bacteria using *E. coli* as an example. Left: Cell cycle progression in *E. coli*. In general, cells continually grow along their long axis as long as proper nutrients are available. During slow growth conditions, the first step is the circular double stranded DNA begins to replicate. During replication, single stranded filaments condense together, forming a ring-like structure at the midcell called the Z-ring. The Z-ring serves as the scaffold for some 30 different downstream septal cell wall synthesis proteins. The collection of the Z-ring and the other downstream proteins form the macromolecular cytoskeletal structure referred to as the divisome (right, blue dashed box), which is responsible for synthesizing the septal peptidoglycan (PG). Finally, the new poles of the daughter cells are synthesized, and the daughter cells split apart then repeat the same process. Right: Zoomed in schematic of the divisome adapted from den Blaauwen et al. 2014 (10).

Appendix *Glossary*), which results in septation of the mother cell into two viable daughter cells (8, 9, 11-15). At the heart of this macromolecular machine lies a ring-like structure called the Z-ring. Z-ring formation is the first step in cytokinesis (cytokinesis, see Appendix *Glossary*) and initiates the assembly of the rest of the divisome by acting as a scaffold for recruitment of the downstream proteins involved in septation (9). In addition, the Z-ring may also play a role in the initial force needed to constrict the cell membrane (16). Altogether, since the discovery of the Z-ring, much research has been done due to the possibilities for novel antibiotic targeting sites. However, research and understanding are still lacking on the initial assembly steps that lead to the Z-ring. To understand these initial steps resulting in Z-ring formation during cytokinesis, we set out to answer the following questions: What triggers Z-ring formation? And which intermediary structures form in order to become the Z-ring?

1.2 The model organism *Escherichia coli*

Throughout history, only a small percent of the Earth's biodiversity has been studied in biological research. These organisms that make up this small percentage have helped in our understanding of the governing principles of life, and are referred to as "model organisms" (17). From the study of one such bacterial model organism, *Escherichia coli*, much of our current knowledge about genetics, proteomics, and evolution have been revealed over the last century (18). Due to the wealth of knowledge that has been obtained from *E.coli* over the years, and the relative ease of culturing and genetic manipulation in this model organism, we decided *E.coli* would be a great platform to quantitatively and physically expand the current understanding of bacterial cell division

(Figure 1.1). Accordingly, strains of *E.coli* with fluorescently labelled proteins of interest have been studied.

1.3 Components of the bacterial Z-ring

The bacterial Z-ring is named after the highly conserved cytoskeletal protein, FtsZ, which shares about 45% genetic sequence identity among most bacterial species and also exist in several clades of archaea and plant plastids (Figure 1.2A) (1, 19-21). FtsZ is the bacterial homolog to eukaryotic tubulin (22-25) and just like tubulin, it undergoes dynamic polymerization due to GTP hydrolysis (GTP hydrolysis see Appendix *Glossary*) (21, 26). The bacterial Z-ring is organized around one subunit thick filaments of FtsZ (Figure 1.2B), referred to as protofilaments, which are able to attach and detach from the inner membrane (Figure 1.2C). These single protofilaments are first formed by the nucleation of FtsZ into a dimer (Figure 1.3A-i). Then the protofilaments are able to elongate or shrink based on monomer addition or removal from the filament ends (Figure 1.3A-i), or through the merging and fragmenting of filaments themselves (Figure 1.3A-ii).

In *E.coli*, FtsZ protofilaments attach to the inner membrane via FtsA and ZipA membrane linkers (Figure 1.3B) (13). FtsA is a highly conserved actin-like protein that resides in the cytosol and binds to the membrane through its C-terminal amphipathic helix (1, 20, 22, 24-27). However, unlike actin, it is unclear whether FtsA can form long filaments or not in wildtype cells at physiological levels (26, 28). Also FtsA is classified as an ATPase (ATPase, see Appendix *Glossary*), but no ATPase activity has been associated with filament formation or dynamics (28). In contrast, ZipA is a transmembrane protein which has a C-terminal domain that extends into the cytosol (9). Both FtsA and

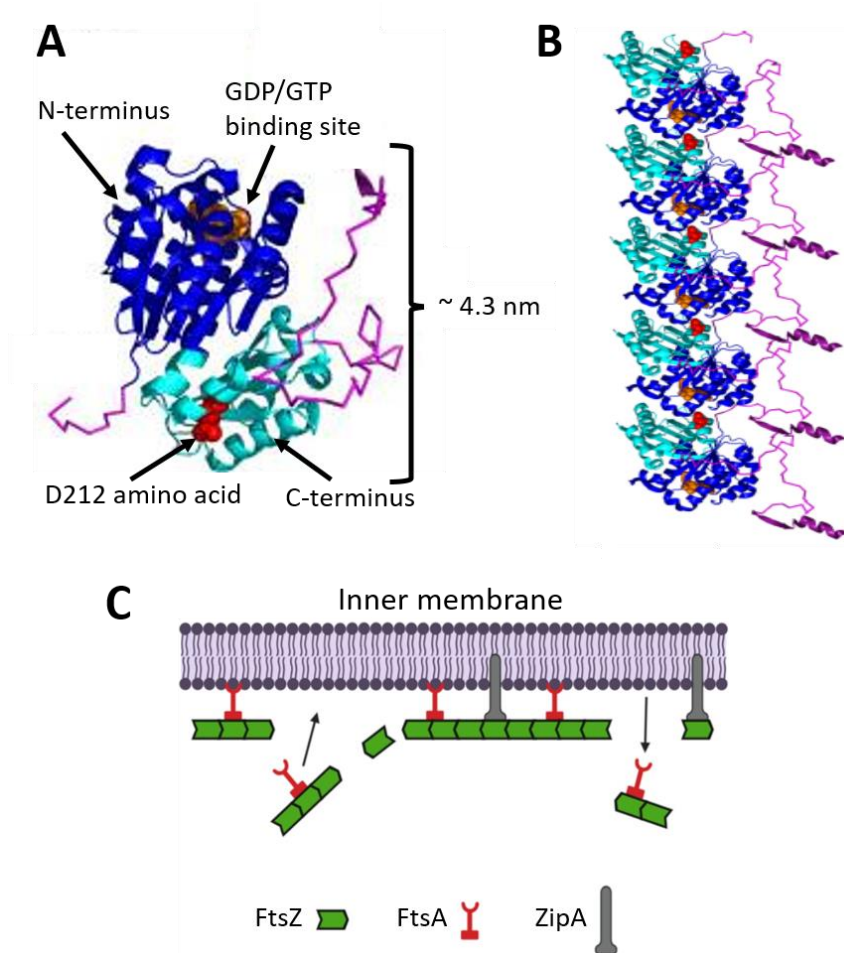


Figure 1.2: Z-ring structure. (A) Crystal structure of an FtsZ monomer. FtsZ is comprised of two subdomains, N-terminus (blue) and C-terminus (cyan), with peptide chains (magenta) extending from each subdomain. The GDP/GTP binding site (orange) and D212 amino acid (red) synergy loop are vital for filament formation (21). (B) FtsZ protofilament using crystal structure from schematic in (A). The synergy loop amino acid D212 (red) sits next to the GDP/GTP binding space (orange) of the other FtsZ monomer (21). (C) Zoomed in schematic of FtsZ filaments attaching to the membrane by linker proteins, FtsA (red) and ZipA (gray). Crystal structures of FtsZ used in (A) and (B) were adapted from Erickson et al. 2010 (21).

ZipA membrane linkers compete for the C-terminal domain of FtsZ and are both essential for cell viability (9). According to *in vitro* studies, reconstituted FtsZ coupled to FtsA or ZipA on an artificial lipid bilayer formed dynamic FtsZ filaments that bundled together forming what is referred to as filament bundles (Figure 1.3C) (29). Also from 2D and 3D photoactivated localization microscopy (PALM) and 3D structured illumination microscopy (SIM) images of Z-rings in both *E.coli* and *Caulobacter crescentus* cells have also shown FtsZ filaments form into a collection of discontinuous clusters that are distributed around the circumference of the midcell (30-33). From these *in vitro* and *in vivo* studies, along with others, we know the Z-ring is made up of multiple bundles of FtsZ filaments instead of one continuous filament (32).

In addition to the essential proteins FtsZ, FtsA, and ZipA, the early Z-ring assembly in *E.coli* also includes non-essential Z-ring associated proteins ZapA-D. ZapA, ZapC, and ZapD have been implicated in cross-linking (cross-linking, see *Protofilament bundling* below) individual FtsZ protofilaments (Figure 1.3C) (34), and ZapA and ZapB in positioning the division ring relative to the replication terminus of the chromosome (35). Altogether, this early cytokinetic assembly subsequently recruits about two dozen different proteins involved in the synthesis of the septal cell wall and in the partitioning of chromosomal DNA between daughter cells (36). In this assembly FtsZ protofilaments act as a control hub that receives signals from different cellular processes including replication (37), metabolism (38), and DNA damage (39). The protofilaments may also create the initial force needed, ~8 pN, to generate septum constriction (40). The ability of

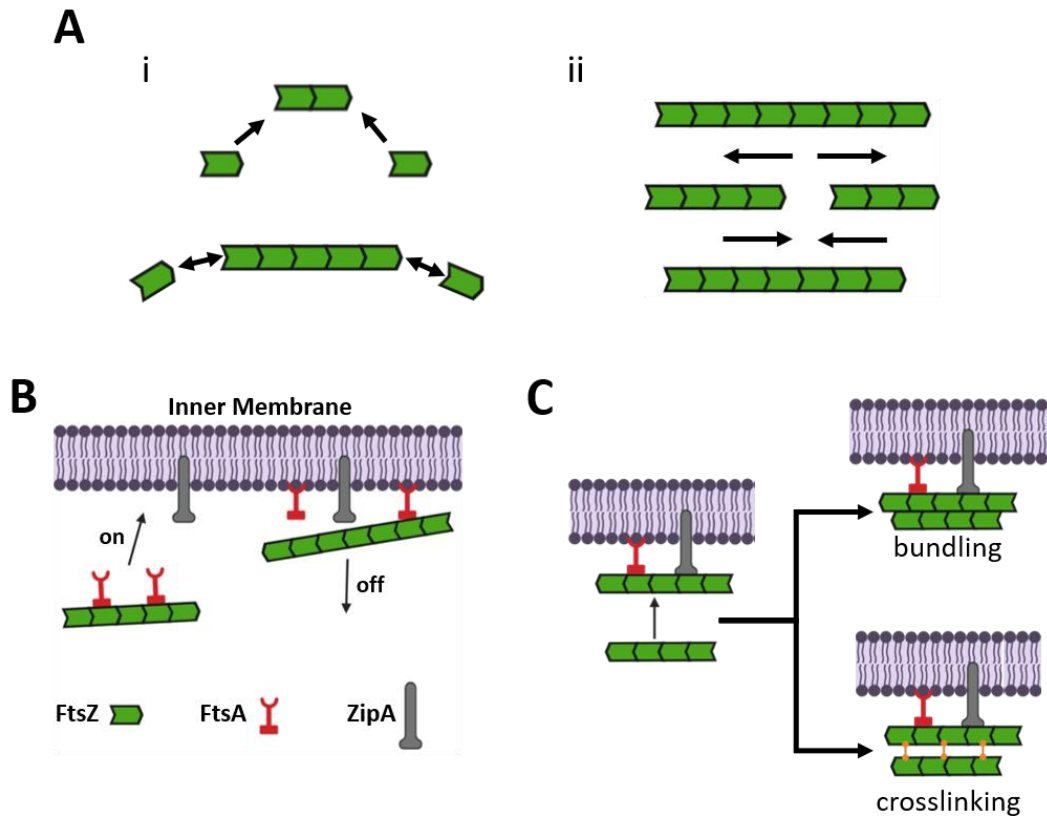


Figure 1.3: Protofilament assembly characteristics. (A) Schematics of early FtsZ filament assembly and disassembly (A, i) Schematic of FtsZ nucleation into a dimer (top). The dimer is then able to elongate or shrink by adding or losing individual monomers on both ends, respectively (bottom). (A, ii) Schematic of filament assembly and disassembly due to the merging and fragmentation of other filaments instead of single monomers from the ends. (B) Representation of filaments coming on and off of the membrane throughout the cell cycle. FtsA (red) binds to the membrane through its C-terminal amphipathic helix, while ZipA (gray) is a transmembrane protein with its C-terminus extending into the cytosol (9, 24-26). The FtsZ membrane linker proteins could possibly be a rate limiting step in filament formation due to the amount of available proteins and particularly FtsA is able to disconnect from the membrane causing filament destabilization. (C) FtsZ filaments are able to form larger patches of filaments through bundling. Accordingly, lateral interactions between FtsZ filaments may explain filament bundling (top right) during Z-ring formation but little evidence to support lateral interactions *in vivo* has been shown. Alternatively, bundling could be the result of crosslinking properties shown to occur between FtsZ and Z-ring associated proteins (orange), ZapA, ZapC, and ZapD, *in vitro* (8). However, evidence for crosslinking *in vivo* is still yet lacking as well.

FtsZ protofilaments to constrict liposomes has already been shown in experiments (16, 41).

1.4 Regulation of Z-ring components

In *E.coli* the Z-ring and eventual septation site are accurately positioned at the midcell (42-44). Over the last half century, the question of how the Z-ring is able to localize itself accurately at the cell middle has been studied. So far, two negative regulatory systems and one positive regulatory system that position the Z-ring accurately at the midcell in *E.coli* have been discovered (35, 45). The following three sections will give an overview of these molecular systems, as they are strongly linked to the formation process of the Z-ring.

1.4.1 The Min system

The Min system consists of three proteins, MinC, MinD, and MinE, which create a well-defined geometric midcell location for Z-ring formation and help prevent polar septation in *E.coli* (Figure 1.4A) (45-48). The Min system is dynamically active in *E.coli* with the Min proteins oscillating between the two cell poles (Figure 1.4B) with a typical oscillatory period of 30 to 60 seconds (Figure 1.4B) (26, 49). From *in vivo* studies, it was determined that MinE and MinD are the essential proteins that establish the oscillatory behavior, while MinC is just a passenger and inhibits FtsZ filament formation when bound to MinD (Figure 1.4A) (14, 26, 49, 50). The oscillation process occurs when MinD and MinC aggregate to one pole of the cell and then release from the membrane when MinD's ATPase activity is stimulated by the binding of MinE. Then the MinD/MinC complex re-assembles at the other pole (Figure 1.4A), again followed by the MinE ring, creating this dynamic back and

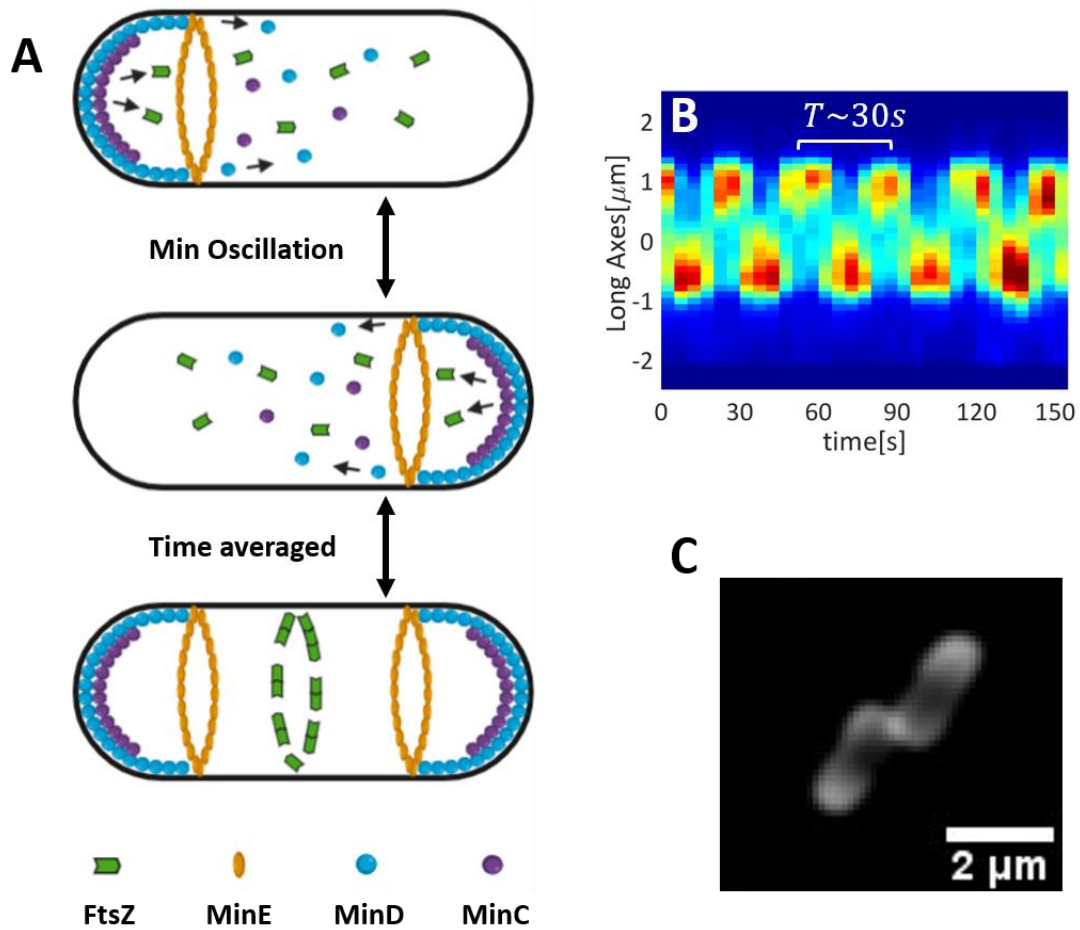


Figure 1.4: Min system oscillations prevent FtsZ filament assembly near cell poles.

(A) The Min system consist of three proteins MinE, MinD, and MinC, which work together to inhibit Z-ring formation at the cell poles. MinD and MinC first sequester at one pole of the cell, but then are followed by the MinE ring which stimulates MinD releasing both MinD and MinC from the cell pole. MinD and MinC sequester to the other pole of the cell after being released (26, 53). Schematic adapted from Kretschmer et al. (53). (B) Oscillatory behavior from (A) of the Min System in a live cell. Red color corresponds to higher fluorescent signal while blue corresponds to low signal. The period (T) of oscillations is about 30 seconds (white). (C) Time averaged image of the cell used (B). Displays how the Min system concentration is lowest at the midcell (A, bottom). This lower concentration at the midcell allows FtsZ filaments to form the Z-ring at the proper location.

forth behavior by the Min system (Figure 1.4B) (26, 51, 52). MinD and MinE comprise what is known as a reaction-diffusion system, which has been successfully modelled in numerous works (54, 55).

Due to the Min system's dynamics, FtsZ filament destabilization by MinC is strongest at the location of the highest time averaged concentration of MinD-bound MinC, which is at the cell poles (Figure 1.4C). This spatial distribution of MinC ultimately allows Z-ring formation and cell wall septation to occur at the midcell (Figure 1.4A, bottom) (55).

1.4.2 SlmA-mediated nucleoid occlusion

During the early days of bacterial cell biology research, it was observed that the Z-ring rarely assembled over the nucleoid, which would result in a guillotined chromosome (26, 56). Somehow the nucleoid was preventing the formation of FtsZ filaments into a persistent Z-ring in its vicinity. This led to the idea that the nucleoid was creating a negative effect on Z-ring formation, and today this mechanism is known as nucleoid occlusion (26, 56-59). The major breakthrough in nucleoid occlusion studies came when the Z-ring inhibiting protein, SlmA, was discovered, resulting in what is known today as SlmA-mediated nucleoid occlusion (SlmA-NO) (Figure 1.5A) (60).

SlmA is a DNA-binding protein and when bound to specific DNA bases, SlmA binding sites (SBSs), undergoes a conformational change creating a high affinity towards FtsZ filaments which results in the inhibition of Z-ring formation (61-64). The mechanism of how chromosome-bound SlmA prevents FtsZ polymers from forming the Z-ring is not well understood, but it is thought that chromosome-bound SlmA depolymerizes FtsZ polymers or captures them to make the polymers incapable of forming the Z-ring (Figure

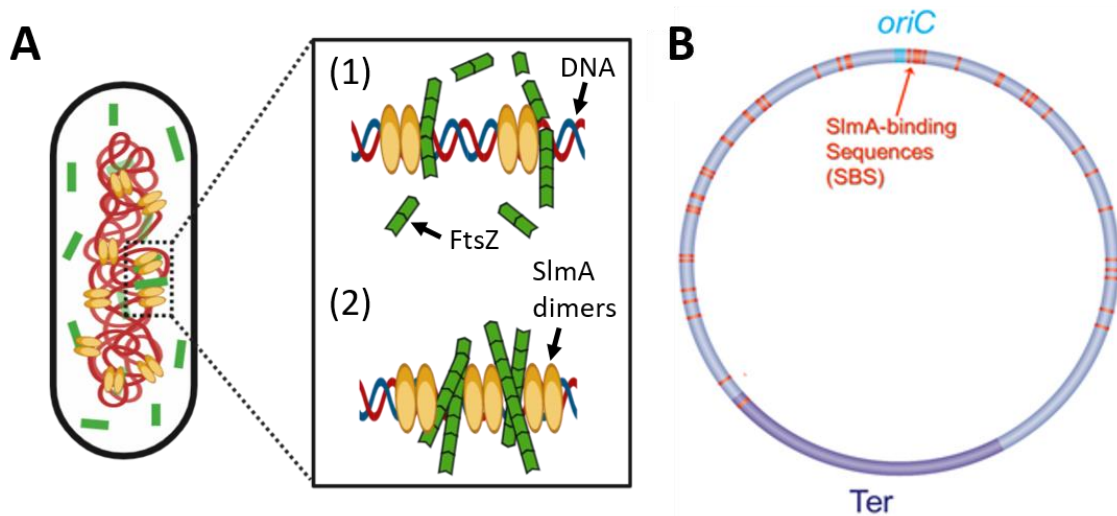


Figure 1.5: SlmA-Mediated nucleoid occlusion effects on FtsZ assembly. (A) SlmA is a chromosome binding protein which inhibits Z-ring assembly during DNA replication. When SlmA is bound to specific DNA sequences, SlmA binding sites (SBSs), it interacts with FtsZ protofilaments in two possible predicted ways. (1) SlmA dimers depolymerize and fragment FtsZ filaments (61-64) or (2) SlmA dimers capture FtsZ filaments making them incapable of linking together to form the Z-ring (64, 65). SlmA dimers are shown as a pair of yellow ovals, DNA is red and blue lines, and FtsZ filaments are green lines and chevrons (B) SBSs are spread throughout the circular chromosome except in the terminus region meaning SlmA inhibits Z-ring formation until DNA replication is nearly over (62, 64). Binding sites are shown by orange lines. Schematics in this figure were adapted from Wu et al 2011 (59).

5A) (61-65). Interestingly, the distribution of these SBSs span throughout most of the nucleoid, but are absent in the region around the replication terminus (Figure 1.5B) (62, 64). Accordingly, the chromosomal distribution of SlmA spatially and temporally inhibits Z-ring formation until chromosome replication is complete resulting in each daughter cell inheriting a complete chromosome.

1.4.3 Ter-linkage

Prior to the last decade, Z-ring regulation was believed to only be mediated by the two negative regulators mentioned above. However, in 2012, Mannik et al. revealed that in *E.coli* which had reduced diameters (creating misshapen cells) and even cells that lacked Min and SlmA were still able to properly position their Z-rings (43). This observation in this double-mutant strain suggested that some other mechanism is providing positional information to the Z-ring (26). Further time-lapse microscopy experiments of *E.coli* cells, grown in minimal medium and lacking Min and SlmA, revealed that Z-ring positions were less correlated with the midcell, but instead were more correlated with the center of the replicated nucleoids (37). Interestingly, this area coincides with the Ter region of the nucleoid (Figure 1.6, Top blue), which is the last region to be replicated and interestingly lacks SBSs (SBSs see previous section *SlmA-mediated nucleoid occlusion*) (37, 61).

The Ter region of the chromosome is located at the poles in new born cells, but then quickly moves to the midcell due to replication (66). Also the MatP protein is known to organize the Ter region into a distinct macro-domain (67). Altogether, this led to the Ter linkage model where MatP proteins bound with ZapA and ZapB proteins link the Z-ring to the Ter region of the nucleoid (Figure 1.6 Bottom) (68). This model has since been

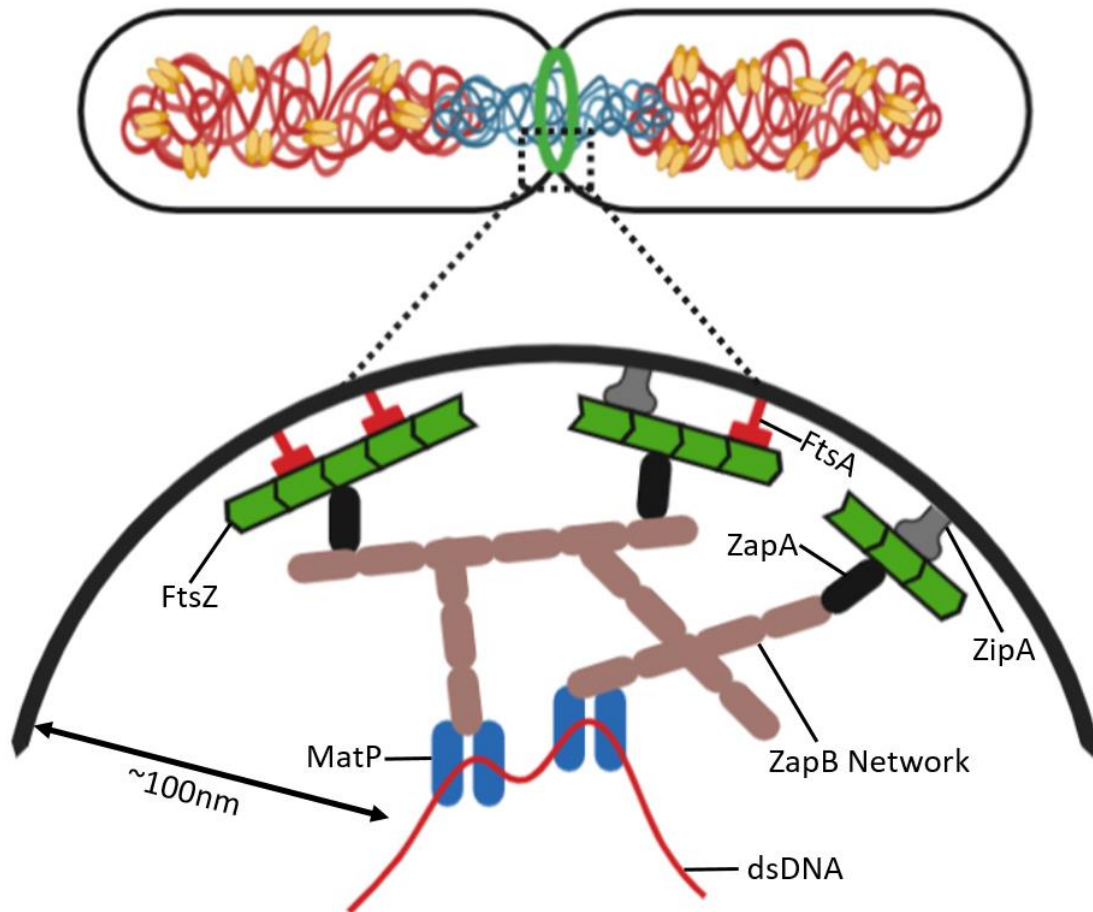


Figure 1.6: Positive Regulation of FtsZ assembly by the Ter linkage. (Top) The Ter region (blue line) of the nucleoid (red and blue lines) is located at the midcell in the late stages of DNA replication. Also no SBSs with bound SlmA (yellow ovals) are located in the terminus region (blue line) of the nucleoid, which consequently allows Z-ring (green oval) formation at the midcell. (Bottom) Zoomed in region of the Z-ring when the terminus region of the nucleoid is located at the midcell. FtsZ filaments (green) are linked to the membrane (black line) via FtsA (red) and ZipA (grey). ZapA (black) interacts with FtsZ and indirectly associates ZapB (brown) to FtsZ filaments. ZapB (brown) forms the extensive cytosolic structure that bridges FtsZ filaments to the nucleoid region through interactions with DNA-bound MatP (blue). Figure adapted from Dr. Jaan Mannik's schematic.

confirmed by super resolution microscopy experiments, and by experiments where deletion of any of the Ter linkage components (ZapA, ZapB, or MatP) led to inaccurate Z-ring placement at the midcell, which results in polar septations and guillotined DNA being more likely (37, 68). Altogether, these localization mechanisms play an essential role during Z-ring formation in wildtype cells.

1.5 Characteristics of FtsZ polymers

1.5.1 Nucleation

The first step in FtsZ protofilament assembly is thought to be nucleation. Biological polymer nucleation has been classified as either 1) cooperative (Figure 1.7B) or 2) isodesmic (Figure 1.7A) polymerization. Cooperative polymerization refers to the case where nucleation of a new polymer is less favorable energetically than elongation of an already existing polymer (Figure 1.7B), while isodesmic polymerization is when nucleation and elongation are energetically identical (Figure 1.7A) (69). Consequently, multi-stranded polymers, like tubulin and actin, are traditionally viewed as cooperative while polymerization of single-stranded filaments have been thought of as isodesmic (69). However, FtsZ assembly into filaments does not fully fit into either of the previously described models (Figure 1.7C). Instead, FtsZ filaments assemble with isodesmic properties in the presence of GDP, while GTP-bound FtsZ filaments assemble through cooperative properties. It was also found that FtsZ exhibited a concentration-dependent lag when FtsZ filaments were single-stranded (69-72). However, the question of how single stranded FtsZ filaments assemble cooperatively remains unclear. Recently, it was proposed that cooperativity arises because FtsZ changes its conformational state from

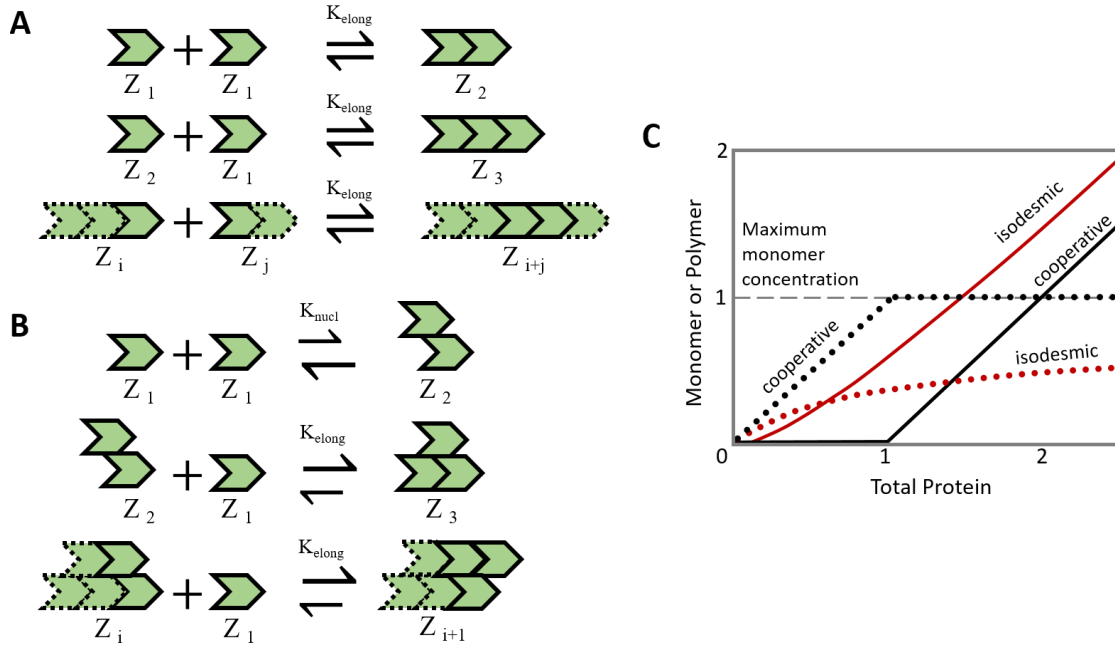


Figure 1.7: Traditional biological polymer assembly models. (A) Isodesmic polymerization of single stranded polymers. In this model dimerization and elongation are energetically the same, which is explained by a single reaction constant, K_{elong} . (B) Cooperative polymerization involves the assembly of multi-stranded polymers. Cooperativity energetically favors elongation of pre-existing polymers over nucleation of monomers into a dimer ($K_{\text{elong}} \gg K_{\text{nucl}}$). (C) Critical concentrations for cooperative and isodesmic polymerization models. Cooperative polymerization (black) shows a sharp threshold, approximately at the critical concentration for assembly to occur while isodesmic polymerization (red) does not have such a threshold. The solid lines refer to proteins within polymers. The dotted lines show free monomer concentrations. Finally, the gray dashed line is the maximum monomer concentration in cooperative assembly. All lines in (C) correspond to steady state. Adapted from Miraldi et al. 2008 (69).

the closed (favoring the monomeric state) to the open (favoring the polymeric state) in the GTP-bound form. This hypothesis has since been supported by FtsZ structures determined by x-ray crystallography (8, 73). This switch in conformation can lead to the behavior depicted in Figure 1.7C (69).

1.5.2 Critical concentration and cooperativity

Cooperative assembly requires the presence of monomers to be at a critical concentration (critical concentration, see Appendix Glossary) or higher (21, 69). Contrarily, isodesmic polymerization is capable of forming polymers before the critical concentration is ever met, which is expected for single-stranded polymer assembly (Figure 1.7C) (69). Interestingly, in the presence of GTP, FtsZ was shown to have a critical concentration for assembly even though it forms linear one subunit thick polymers. From *in vitro* measurements, the critical concentration threshold for FtsZ was estimated to be about 1 μM (74). However, the cytosolic concentration of FtsZ *in vivo* has been determined to be much higher (4-6 μM), which includes our own measurements (75). Based on this concentration, the majority of FtsZ monomers (3-5 μM) can be expected to be part of the protofilaments while 1 μM of them stay monomeric (69). Despite this view, cytosolic FtsZ protofilaments have not yet been conclusively detected within cells when the protein is present at physiological levels (76, 77). It has remained unclear what the polymerization state of FtsZ is prior to the formation of the Z-ring. In this dissertation the uncertainty is experimentally addressed.

1.5.3 Polymer dynamics

The simplest model for polymerization of a protein into a filament is one where the monomer exchange occurs only at the two ends of the filament. Here, let's first consider the case where the rates on the two ends of the filament are not the same due to structural asymmetry. To bring out this asymmetry, I refer to one end of the filament as the barbed end (or the plus end) and the other one as the pointed end (the minus end) (Figure 1.8A) (78). This terminology follows the one used for actin filaments. The rate equations for the two ends of the polymer for this condition are given by:

$$\frac{dn^+}{dt} = R_{on}^+ c_0 - R_{off}^+ [1]$$

$$\frac{dn^-}{dt} = R_{on}^- c_0 - R_{off}^- [2],$$

where n^+ is the number of monomers at the plus end, n^- is the number of monomers at the minus end, R^+ and R^- are the on and off rates for the plus end and minus end, respectively, and c_0 is the concentration of monomers (78, 79). If all monomers in this polymer chain are identical, then the reaction that adds a monomer to the barbed end yields exactly the same polymer as the reaction that adds a monomer to the pointed end. Thus, the final state of the reaction is independent on which end the monomer was added. Furthermore, the initial state of the reaction is also the same for these two reactions. Consequently, the change in the (Gibbs) free energy for two reaction pathways is identical ($\Delta G^+ = \Delta G^- = \Delta G$). Furthermore, in equilibrium $dn^+/dt = 0$ and $dn^-/dt = 0$. It follows from eq. [1] and [2] then that

$$c_0^* = \frac{R_{off}^+}{R_{on}^+} = \frac{R_{off}^-}{R_{on}^-} [3].$$

Here, c_0^* denotes the equilibrium concentration of the monomers. The ratio of the on and off rates at the barbed and pointed ends is thus the same. It also follows from eq. [1] and [2] that if $c_0 > c_0^*$ then both ends grow (although not at the same rates). Alternatively, if $c_0 < c_0^*$ then both ends shrink.

More complex dynamics can be realized when the monomers within the polymer can undergo a structural change once incorporated into the chain. For FtsZ this structural change is possible because of GTP hydrolysis. As mentioned earlier, GTP binds to FtsZ at the interface, where two monomers come into contact with each other. Once this happens GTP can be hydrolyzed into GDP. The hydrolysis cannot occur in monomeric FtsZ-GTP. GTP hydrolysis leads to a conformational change in the adjacent monomers. This allows the two ends of the polymer to be different. Consequently, the on and off rates at the two polymer ends does not need to be constrained by the relation given in eq. 3.

The full reaction scheme for FtsZ bound to either GTP or GDP is shown in Figure 1.8 B. It involves 8 different reaction rates. However, experiments have shown that most of these rates can be effectively neglected. FtsZ-GDP alone forms only very weak filaments (21). Moreover, the amount of GTP in a cell is about 10-fold larger than the amount of GDP. The same ratio can also be expected for cytosolic FtsZ-GTP and FtsZ-GDP. Therefore, all on rates involving FtsZ-GDP can be neglected. Also, one can neglect the binding of FtsZ-GTP to the barbed ends of the polymer containing FtsZ-GDP because in this case GTP will be at the outer surface of the polymer. Suppose now that $R_{on}^- > R_{on}^+$ as has been indicated by a recent report (80). This leads to preferential binding of FtsZ-GTP to the pointed (minus) end of the polymer and the build-up of FtsZ-GTP at this

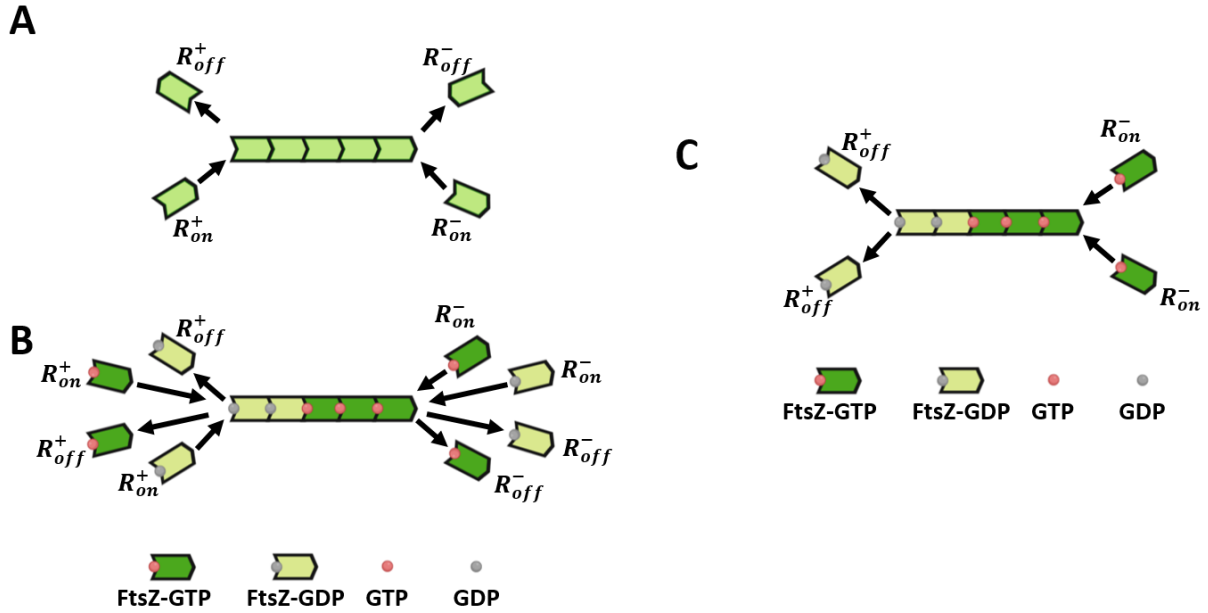


Figure 1.8: Polymerization models for cytoskeletal filaments. (A) Polymerization of identical monomers throughout the filament where rates (R) of the two ends are unequal due to structural asymmetry of the monomers (green chevron). This makes the resulting filament indifferent to which side the monomer was added. (B) Polymerization of non-identical monomers (FtsZ) with 8 different reaction rates due to nucleotide hydrolysis being incorporated. The addition of GDP (gray circle) and GTP (red circle) bound states alters the molecular interface of the contacting monomers. However, FtsZ-GDP monomers form weak filaments and the amount of GTP is in surplus to GDP (C) Neglecting all on rates of FtsZ-GDP and FtsZ-GTP binding to the barbed end, results in one side of the filament only binding to GTP-bound monomers (dark green chevron) and the other to only losing GDP-bound monomers (yellow chevron). This condition produces the phenomenon known as treadmilling. Adapted from Phillips et al. 2012 (79).

filament end. Within the polymer GTP is hydrolyzed. The rate of hydrolysis is comparable to the rate at which the monomers are added to the polymer (81). Since the interface between two monomers containing GTP is strong, it is not likely to dissociate. Accordingly, after nucleation the filament grows until some FtsZ-GDP form at the barbed end side. This interface is then likely to break and dissociate from the filament (Figure 1.8B). The above arguments can be summarized by effective kinetic equations:

$$\frac{dn^-}{dt} = R_{on}^- c_0 \quad [4]$$

$$\frac{dn^+}{dt} = -R_{off}^+ \quad [5]$$

$$\frac{dn}{dt} = R_{on}^+ c_0 - R_{off}^- \quad [6],$$

where n is the total number of monomers ($n = n_+ + n_-$) and dn/dt is the total monomer rate of change. In steady state conditions $dn/dt = dn^+/dt + dn^-/dt = 0$. It follows then $R_{on}^+ c_0^* = R_{off}^-$ meaning that the pointed end of the filament extends while the barbed end of the filament shrinks at the same rate. This novel property is referred to as treadmilling (Figure 1.8 C) and it creates directional movement of the polymer if it is attached to some surface. This directional movement can create force. The resulting force is responsible for cell locomotion for example in human immune cells.

1.5.4 Treadmilling behavior of FtsZ filaments

In over two decades, it has been known that FtsZ polymerizes into highly dynamic filaments. An *in vitro* study published in 2014, found evidence of FtsZ filaments forming highly dynamic vortices on an artificial lipid bilayer. The vortices had a diameter similar to that of an *E.coli* cell. This study provided the first evidence of treadmilling in FtsZ filaments

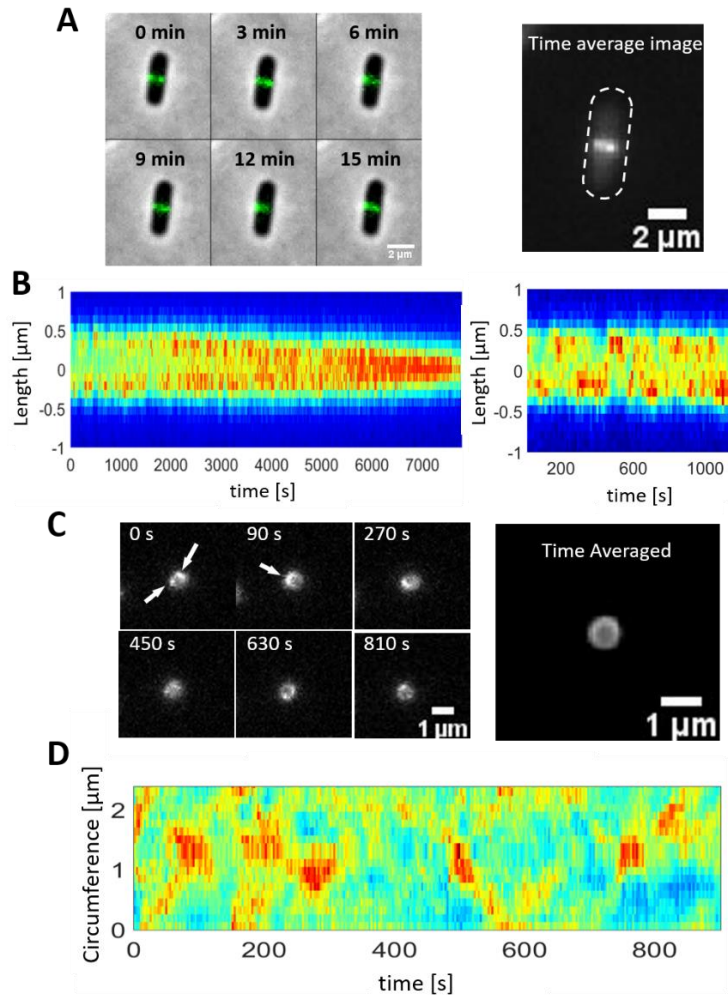


Figure 1.9: Treadmilling behavior of the bacterial Z-ring. (A, left) Images of cells with fluorescently labeled FtsZ, which have formed a Z-ring at the cell center. Note the Z-ring is not static, but dynamic. (A, right) Time averaged image of the montage displaying a uniform Z-ring due to spatial distribution Z-ring dynamics. (B, left) Example kymograph from the cell depicted in (A) until the Z-ring divides the cell into two daughter cells. The oscillatory motion of the intensity represents what is referred to as treadmilling. (B, right) Zoomed in section of the first 20 minutes of the kymograph (left) to better show the treadmilling behavior the Z-ring displays. (C, left) Images of cells with fluorescently labeled FtsZ in vertical pockets. Clusters of FtsZ filaments (white arrows) can be seen moving around the circumference of the cell. (C, right) Time averaged image showing a uniform ring of intensity due to Z-ring dynamics. (D) Example kymograph from the cell depicted in (C). The patches of FtsZ filaments show treadmilling dynamics (dashed lines) in vertical pockets as well. In (B) and (D), red corresponds to high and blue to low concentrations of fluorescent proteins.

and found an average treadmilling speed of ~43 nm/s (29). Membrane tethering proteins, FtsA and ZipA, were shown to play a role in these dynamic properties. When an ATP-bound FtsA was used as the membrane linker for FtsZ, highly dynamic vortices resulted. In contrast, when ZipA was used as the membrane linker, FtsZ organized into static bundles of dynamic filaments (29).

The question arose, if these behaviors were also applicable to FtsZ *in vivo*. In 2015, using the fluorescent FtsZ⁵⁵⁻⁵⁶-mNeonGreen sandwich fusion from Moore et al. (82) (Figure 1.9A), we observed oscillatory Z-ring dynamics i.e. treadmilling, in live *E.coli* cells (Figure 1.9B). We also saw similar treadmilling dynamics in Z-rings using a microfluidic device where cells were oriented perpendicular to the imaging plane (Figure 1.9C) (see *Materials and Methods: Vertical Pockets*). In these cells, the cell division plane is in the imaging plane, which allows us to have an in plane-view of the Z-ring during the cell cycle (Figure 1.9C). We found that the Z-ring consisted of multiple dynamic clusters which treadmilled along the circumference of the cell (Figure 1.9D).

In 2017, two papers, published back to back in *Science* (83, 84), detailed this dynamic behavior. One paper described the treadmilling behavior in *E.coli* and reported an average treadmilling speed of ~28 nm/s for FtsZ filaments (83). This corresponds to the Z-ring oscillating around the circumference of the cell with a period of 115 seconds (83). These studies also found that the treadmilling speed depended on GTP hydrolysis rates. Bacteria expressing a mutant FtsZ with lower GTPase rates showed slower treadmilling speeds (83). Likewise, in *B.subtilis* expression of proteins that increase FtsZ turnover rates increased treadmilling speeds of FtsZ filaments (84). In addition to GTPase

mutants, a drug that inhibits GTP hydrolysis (PC190723) in most bacteria was also shown to decrease the movement of FtsZ filaments *in vivo* (1, 84). Consequently, all cells with reduced treadmilling rates resulted in alterations to the spatial distribution of the septal cell wall synthesis proteins creating asymmetric septation. Accordingly, these studies have shown that the treadmilling behavior of FtsZ filaments is the mechanism driving PG synthesis via uniform spatial distribution of septal cell wall synthesis proteins during bacterial cell division (83, 84).

1.5.5 Protofilament lifetime and length based on a simple treadmilling model

Treadmilling dynamics leads to predictions for FtsZ protofilament lifetime and length distributions. We follow simplified reactions given by equations [4]-[6]. However, these kinetic equations apply only when the filaments are long. *In vitro* measurements of protofilaments have shown that $n = 2, \dots, 50$ monomers. Therefore, one needs to model filament assembly and disassembly as stochastic processes. To model eq. [4]-[5] in small the n regime, we used the Gillespie Algorithm (85) (see Chapter 2: *Modelling of protofilament lifetime and length distributions*). In the beginning of modelling the initial length of polymers was $x_0 = 5$ monomers. Choosing this initial length allowed us to neglect the intricacies of the nucleation process. The polymer's time-evolution in length was then followed. The model terminated when either the total number of time steps occurred or the length reached zero (Figure 1.10A). Using this simple model allowed us to follow the length and lifetime (Figure 1.10B) of treadmilling polymers.

In steady state the above model maps out to a 1D random walk. For the mapping $D = R_{on}^+ c_0 = R_{off}^-$, where D is the diffusion constant. The lifetime in our model

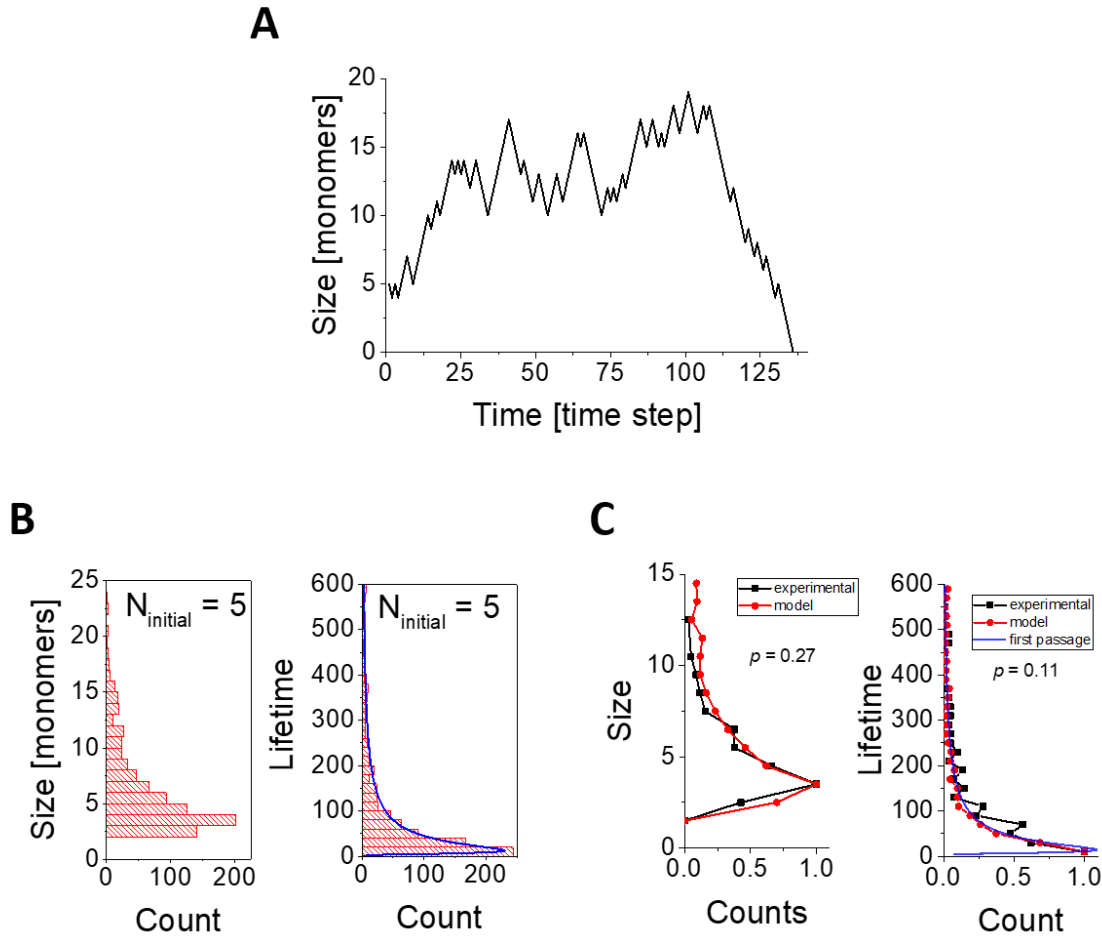


Figure 1.10: Polymer length and lifetime distributions from a treadmilling model. (A) Individual polymer length distribution over time. Polymer has periods of growth and shrinkage. (B) Average polymer size (left) and lifetime (right) for a population for a population of polymers resulting from the Gillespie algorithm. Note how similar the lifetime distributions from modelling are with the first passage time density (blue line). (C) Comparison of average polymer sizes (left) and lifetimes (right) resulting from the treadmilling model (red/circle) and experimental results (black/square). Experiment and model distributions are quite similar with p-values of 0.27 and 0.11 for polymer size and lifetime, respectively, from a Kolmogorov-Smirnov test. Experiment lifetime distribution also matches well to the first passage time density (blue line). Note the counts have been normalized. The initial polymer size was set at 5 monomers due to experimental results showing bundles less than 20 monomers (see Chapter 4).

corresponds to the time that the random walk takes to return to its starting location. The corresponding lifetime distribution is given by the first passage time density:

$$\rho(t) = \frac{|x_0|}{\sqrt{4\pi Dt^3}} e^{-\frac{(x_0)^2}{4Dt}} \quad [8]$$

This distribution matches well to modelling results (Figure 1.10B, right) and also to our experimental data (Figure 1.10C, right) although in the experimental data one can expect many more reactions to be involved in addition to those described by eq. [4]-[6].

1.5.6 Rate of change of filament length

The above model also allows us to estimate how protofilament length fluctuates with time. Treadmilling refers to when a filament's on and off rates are equal on average. However, the number of added and removed monomers from a protofilament during an interval of time (1 sec) is not in general equal because of the stochastic nature of the reactions. Here, we assume that the addition and removal of monomers are both Poisson processes with an average rate of λ , where $\lambda = R_{on}^+ c_0 = R_{off}^-$. Since the length of an FtsZ monomer is known to be about 4.3 nm (74) and our experimental results found an average treadmilling rate of about 20 nm/sec, we estimate that the rates for adding monomers to one end and losing monomers from the other end corresponds to $\lambda=5$ monomers/sec. For our measurements, the rate is determined as a change in monomer numbers for the time interval corresponding to $\Delta t = 1 \text{ sec}$. During this interval m monomers could be added from one side and n removed from the other end. Consequently, the instantaneous rate during this interval would then be $(m - n)/\Delta t$. The resulting probability of this particular process would then be:

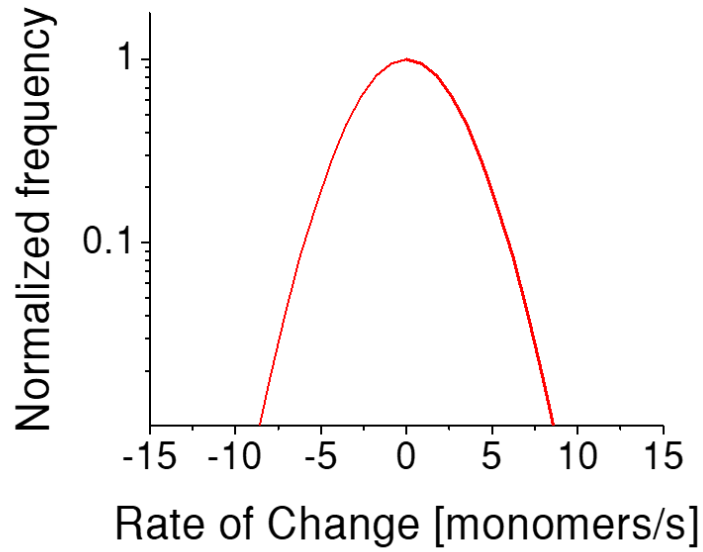


Figure 1.11: Rate of change of filament length from treadmilling. The rate of change is determined by the change in monomer numbers during the time interval of $\Delta t = 1 \text{ sec}$. Addition and removal rates (λ) of monomers was estimated to be 5 monomers/sec. The resulting probability distribution of filament length by treadmilling dynamics is plotted here (red line). Note this distribution is compared to experimental results (see Chapter 4: *Rapid kinetics of FtsZ transient assemblies*).

$$P(n, m; \lambda) = \frac{(\Delta t \cdot \lambda)^{m+n} \exp(-2\lambda \Delta t)}{m! n!}.$$

The probability that a total of N monomers are added during the time interval Δt then becomes the sum of all possible processes where $n = N + m$:

$$\begin{aligned} P(N; \lambda) &= \sum_{m=0}^{\infty} P(m + n, m; \lambda) \\ &= \sum_{m=0}^{\infty} \frac{(\Delta t \cdot \lambda)^{2m+N} \exp(-2\lambda \Delta t)}{m! (m + N)!}. \end{aligned}$$

This distribution (Figure 1.11) is the one used to compare the experimentally measured rate distributions in this work (see Chapter 4: *Rapid kinetics of FtsZ transient assemblies*).

1.5.7 Protofilament bundling

Protofilament bundling or crosslinking is likely an essential property needed for the formation of the Z-ring (86). Bundling can possibly be achieved via protofilament-protofilament lateral contacts. Recently, evidence for these contacts has been reported between anti-parallel FtsZ protofilament pairs (87). However, it is unclear if dynamic protofilaments can exist in such a conformation *in vivo* because of the treadmilling dynamics that filaments display. Treadmilling would require the filaments to all be oriented in the same direction otherwise the filaments would not move along the circumference of the cell as directionally as they do (Figure 1.9). It is also possible that protofilaments are cross-linked together by the Zap proteins, in particular ZapA, ZapC, and ZapD. However, none of these Zaps are individually essential nor are they essential as a group, but their absence does lead to a cell division defect (88). Protofilament bundling has also been

reported in the presence of ZipA *in vitro* (89, 90). Although, many *in vitro* studies have observed bundled FtsZ protofilaments, the mechanism(s) which creates this bundled structure in live cells is still yet lacking.

To gain a further understanding of FtsZ filament bundling, we created an Ising-like model (see Chapter 2: *Modelling bundling using an Ising-like model*) for FtsZ filament assembly. Using the Ising-like model, which incorporates only FtsZ and MinC proteins, we can investigate what effects the strength of lateral interactions has on protofilament bundling, their length distribution and spatial distribution within the cell (Figure 1.12A). In particular, this model includes the Min system as a parabolic distribution with the minimum at the center of the lattice, along with FtsZ monomers being capable of nucleotide exchange and GTP hydrolysis reactions. The lateral bond energies of two FtsZ monomers of $0k_B T$, $-1k_B T$, the latter has been estimated from polymerization kinetics *in vitro* (86), and $-3k_B T$ were used to examine the dependence of lateral interactions on filament bundling (Figure 1.12). The longitudinal bond energies in this model had a strength of $-17k_B T$. Note that these filaments do not treadmill.

At a lateral energy of $0 k_B T$ most filaments are short (Figure 1.12B, red), unbundled and broadly distributed throughout the length of the cell (Figure 1.12C, red). The lateral energies of $-1k_B T$ (86) and $-3k_B T$ produce filaments that are longer (Figure 1.12B, blue and green respectively), bundled and have a narrower longitudinal distribution (Figure 1.12C, blue and green respectively). However, the filaments do not form a narrow band in the center of the cell as the Z-ring but rather remain broadly distributed. Note that the side of the square in Figure 1.12A corresponds to about $2 \mu\text{m}$. These broad distributions

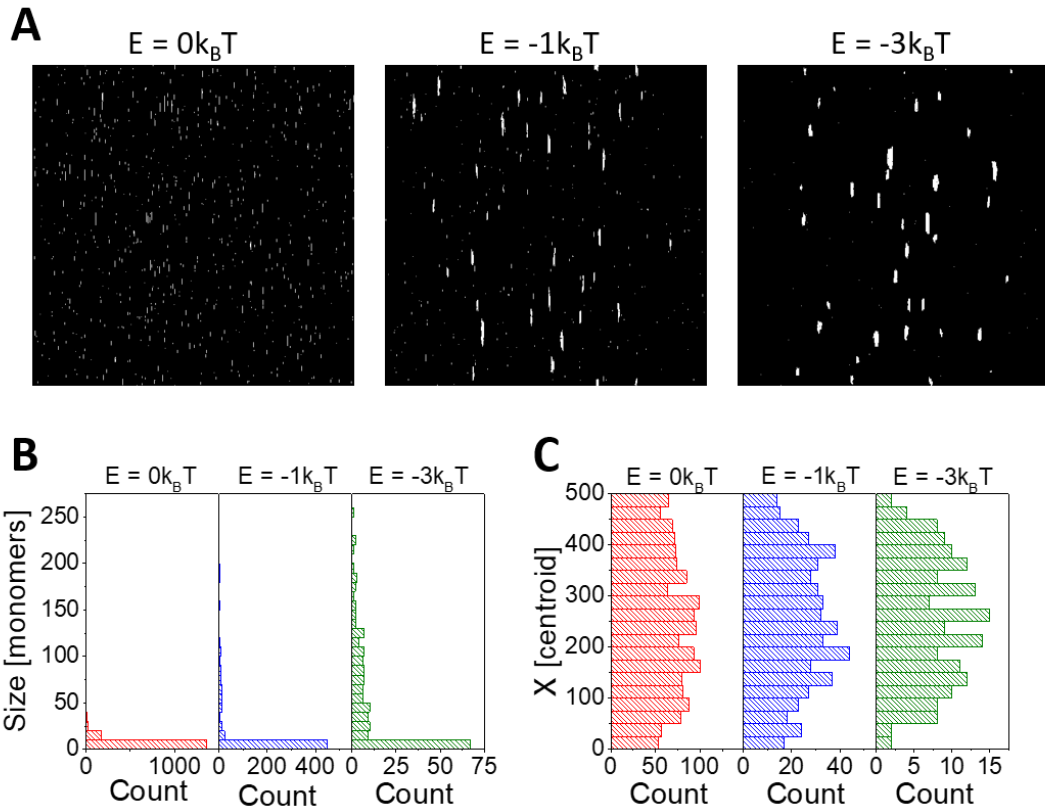


Figure 1.12: Ising-like model of polymer formation and bundling. (A) Example of final images of polymer formation and bundling (white) with three different lateral interaction energies $0k_B T$ (no lateral contacts), $-1k_B T$ (likely chance for lateral contacts between FtsZ proteins only) (86) and $-3k_B T$ (more likely chance for lateral contacts between FtsZ proteins). Note that one white pixel corresponds to one FtsZ monomer with a size of ~ 4 nm (21). This corresponds to the length of the square being $\sim 2 \mu\text{m}$ (length of *E.coli* cell). (B) Comparison of polymer size (number of pixels) between the three different energies $0k_B T$ (red), $-1k_B T$ (blue), and $-3k_B T$ (green). Polymers grow in size the more energetically favorable the lateral interaction energy becomes. Note that there is a possibility that at a certain lateral interaction energy the polymer longitudinal favorability will switch to the lateral interaction being more favorable creating filaments elongating along the long axis of the cell. (C) Long axis position of each filament's centroid which shows slight condensation of larger polymers to the center of the image instead of the peripheries. This is due to the combination of the Min system and polymers becoming larger as a result of the lateral interactions becoming more energetically favorable in the system, which both will increase the polymer's stability. Note that this is similar to filaments in live cells since the polymer stability seems to be somewhat dependent on the size and cellular location (preferably the midcell) of the FtsZ filaments. All data in (B) and (C) is the result of 3 separate model runs for each lateral interaction energy.

are reminiscent to the distributions that are reported in this dissertation for FtsZ assemblies prior to the Z-ring formation. However, the distributions are much wider than the ones determined for FtsZ protofilament distribution within the Z-ring (40-60 nm(91)). How the filaments can collapse into such a narrow distribution remains to be explained. The current model does not take into account treadmilling dynamics, which is possibly the source of the discrepancy.

1.6 Dissertation Layout

The remaining chapters describe the methodology and results from experiments that were designed to give further insight into the regulation, assembly, and maintenance of the essential cytoskeletal structure, the Z-ring, in *E.coli*. Specifically, Chapter 2 describes the experimental techniques and methods used during this work to produce the results described in Chapters 3 and 4. The results in Chapter 3 have been published in *Molecular Microbiology* (20), and Chapter 4 results have been published in *Current Biology* (75). Lastly, the summary will wrap up the findings and address new questions that arose from these findings. Following the summary more technical information like a glossary of commonly used terms, diffusion coefficient calculation for FtsZ, glass coverslip cleaning procedure, genetic manipulation procedure, and a list of strain information are contained in the Appendix followed by my personal vita.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental Methodology

In this work, high spatial and temporal resolution fluorescent microscopy was used as the main technique to study bacterial cell division. All experiments were carried out in derivatives of *E.coli* K-12 strains where our protein of interest, FtsZ, was fused to an exogenous fluorescent protein (Table 1). Some of these strains also harbored deletions to cell division related genes. By using a fluorescent microscope, we were able to visualize spatiotemporal distributions of FtsZ and infer its interaction's with the other cell division related proteins. A combination of fluorescence imaging and digital image analysis gave us the ability to quantitatively characterize and reconstruct the dynamical processes FtsZ undergoes during cell division.

To aid in this, our lab also fabricated microfluidic devices. These devices allowed us to maintain the proper amounts of nutrients for the bacteria to grow at steady state throughout imaging. The experimental work done for this dissertation falls into four broad categories: 1) fluorescent imaging, 2) image processing of experiments, 3) bacterial strain construction, and 4) bacterial growth. The rest of this chapter will describe these technical aspects in detail.

2.2 Fluorescence microscopy

To acquire cell images, we used a Nikon Ti-E inverted fluorescence microscope (Figure 2.1A, B) equipped with a 100X oil immersion phase contrast objective with a 1.40 numerical aperture (Figure 2.1C) and a Perfect Focus system which cancels axial focus fluctuations in real time during timelapse imaging. The incubation chamber that surrounds the microscope stage (Figure 2.1A) maintained a constant temperature of 28°C during our timelapse measurements. Two primary modes of imaging were phase contrast and fluorescence imaging. Phase contrast (phase contrast, see Appendix *Glossary*) images were utilized for cell segmentation while fluorescent images captured the distributions and dynamics of FtsZ within the cell. Accordingly, fluorescent images were obtained by the irradiation of bacterial cells with either 488 nm or 561 nm bands. The excitation sources were a 200 W Hg lamp attenuated through ND4 and ND8 neutral density filters, or either a 488 nm (blue) or a 561 nm (green) diode laser (Figure 2.1B) attenuated by a single or combination of ND2, ND8, or ND16 neutral density filters. Also the excitation and emission of specific fluorescent wavelengths required specific filter cubes, which contain an excitation filter, beam splitter, and emission filter all in one. Specifically, Chroma 41001, 41004, and ZET405/488/561m filter cubes were used to record green, red, and both green/red fluorescence, respectively. Green fluorescence originated from either mNeonGreen fluorescent proteins (mNG), sandwich fusion green fluorescent proteins (sfGFP), or green fluorescent proteins from the plasmid, (GFP), while red was from the photoactivatable protein mMaple3 (see below: *Stroboscopic single molecule imaging of FtsZ*) (Table 1). The emitted light from the fluorophores was collected

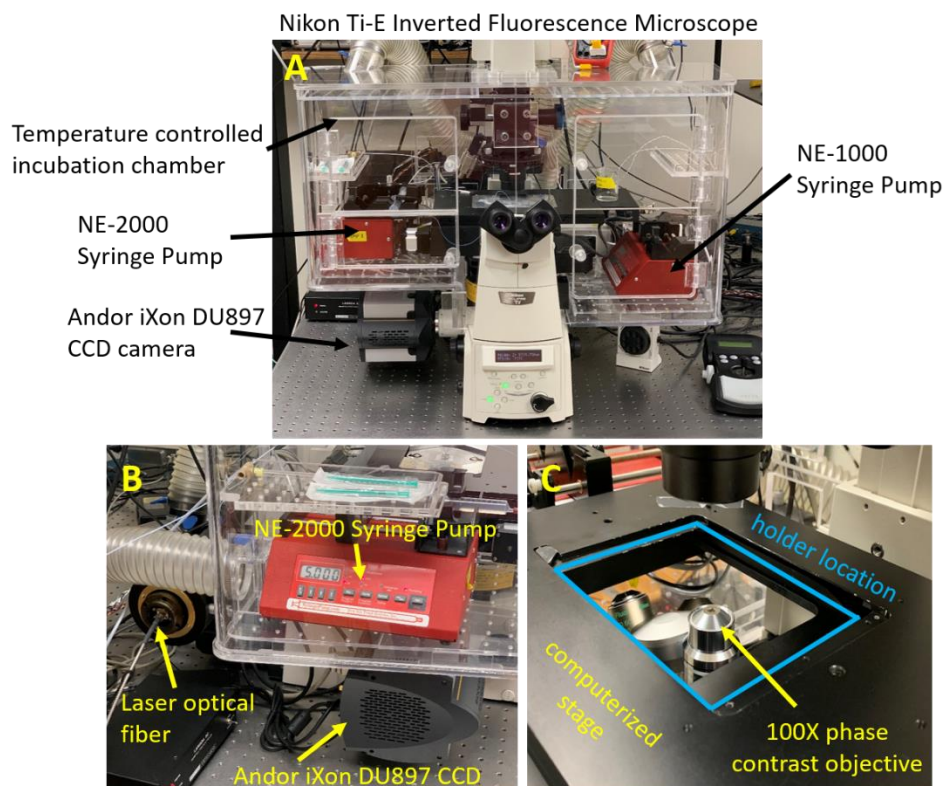


Figure 2.1: Imaging system. (A) All imaging experiments in this work were conducted using a Nikon Ti-E Inverted fluorescence microscope that was controlled by a computer. Each experiment was conducted within a temperature controlled incubation chamber (*In Vivo* Scientific incubation chamber), which is a transparent box around the microscope. All experimental images, fluorescent or phase contrast, were captured using an Andor iXon DU897 CCD camera. (B) Side-view of microscope components. In this work most experiments utilized lasers as an excitation source (laser optical fiber connection to the microscope). Also experiments using microfluidic devices required fresh media and/or drugs to be pumped into them using either the New Era 1000 (A) and/or 2000 syringe pumps. (C) Close up of the computerized microscope stage. For timelapse imaging the x, y, and z directions are all controlled by a computer using NIS elements software. The pad/dish and microfluidic device holders all sit within the center of the stage (outlined by blue lines). All experiments also used a 100X NA 1.40 oil immersion phase contrast objective (located under the holder location) and a Perfect Focus system for imaging the bacteria.

by an Andor iXon DU897 electron multiplying CCD camera (EMCCD) (Figure 2.1A, B), which was thermoelectrically cooled to reduce thermal electrons and also incorporated a gain multiplier. The camera has a 96% quantum efficiency permitting single molecule imaging. All images were recorded using Nikon's NIS-Elements software.

Different optical modalities can be used to excite the fluorophores in this imaging system. The modalities include epifluorescence microscopy (Epi) where light enters the specimen at a normal incidence of $\theta = 0$ (Figure 2.2A, C) (92), total internal reflection fluorescence microscopy (TIRF) where θ is greater than the critical angle ($\theta > \theta_c$) (Figure 2.2A, D) (92), or highly inclined and laminated optical sheet microscopy (HILO) where θ is slightly less than or equal to the critical angle ($\theta \leq \theta_c$) (Figure 2.2A, E) (93).

We were mostly interested in FtsZ dynamics and interactions that took place in the vicinity of the cell membrane. For this, imaging with HILO and TIRF microscopies are possible choices. Next we compare these two techniques to bring out their advantages and disadvantages for our imaging tasks.

2.2.1 TIRF microscopy

TIRF microscopy uses a laser of specific wavelength that is focused off axis at the back focal plane of the objective lens for fluorophore excitation (Figure 2.2A, B). This off axis distance determines the angle of incidence for the light. This light exits the objective lens and passes through both the immersion oil and coverslip as a collimated beam. If the excitation light's angle of incidence is greater than that of the critical angle (θ_c) (Figure 2.2B) calculated by Snell's law (92) then the light reflects back and does not fully penetrate into the sample creating what is known as total internal reflection (Figure 2.2A,

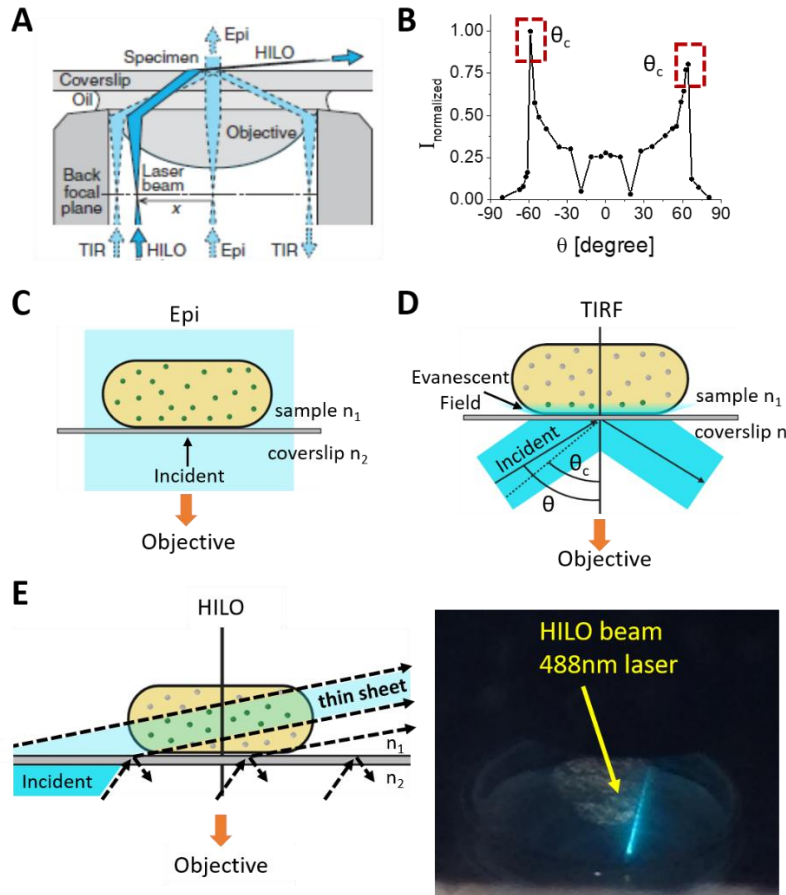


Figure 2.2: Fluorescence microscopy techniques. (A) Microscope optics displaying incident beam angles set by adjusting x which results in epifluorescence (Epi), total internal reflection fluorescence (TIRF), or highly inclined and laminated optical sheet (HILO) microscopy setups (93). (B) Intensity values at different incident angle settings for the optical system used in this work. Critical angle (θ_c) of incidence can occur near both edges of the objective (red-dashed squares). (C) Epifluorescence ($\theta = 0^\circ$) completely illuminates the entire specimen which in turn excites all fluorescent proteins throughout the cell. Schematic adapted from Mattheyses et al. 2010 (92). (D) Specimen excitation due to TIRF microscopy ($\theta > \theta_c$). The large incidence angle creates an evanescent field due to the beam totally reflecting off the coverslip. This leads to the excitation of only the fluorescent proteins at depths up to ~60-100 nm from the surface of the specimen. Schematic adapted from Mattheyses et al. 2010 (92). (E) Left: Schematic of specimen excitation due to HILO microscopy ($\theta \leq \theta_c$). The incident beam refracts through the coverslip creating a highly inclined beam at the coverslip/specimen surface (dashed arrow-lines) which results in a laminated thin optical sheet (blue) that protrudes through the center of the specimen on the specimen's side. Schematic adapted from Tokunaga et al. 2008 (93). Right: Image of how the laser beam propagates as a sheet across the entire imaging platform during HILO microscopy as represented in (E, left).

D). However, some of the incident light propagates into the sample as a standing wave known as the evanescent field (92) (Figure 2.2D). Within this evanescence field the intensity decays exponentially with respect to the distance from the interface ($z=0$):

$$I_z = I_o^{\frac{-z}{d}},$$

where I_o is the initial intensity at the interface, $z=0$, and d refers to the penetration depth of the evanescence field. The penetration depth can be calculated as:

$$d = \frac{\lambda_0}{4\pi\sqrt{n_2^2\sin^2\theta - n_1^2}},$$

where n_1 is the sample's refractive index, n_2 is the coverslip's refractive index, λ_0 is the wavelength of the excitation source in a vacuum, and θ is the incident angle of the light source (92). Accordingly, the evanescent field's penetration depth and intensity can both be changed by adjusting the incidence angle of the excitation source, which must be greater than θ_c (Figure 2.2B). In theory, TIRF is supposed to be limited to only imaging the surface of specimens (93) (Figure 2.2D). However, for rod-shaped cells such as *E.coli*, the evanescent field also excites a great deal of the cytosol, which in practice is not strictly imaging the cell membrane only.

2.2.2 HILO microscopy

HILO microscopy is similar to TIRF (described above) (Figure 2.2A), but instead of using an evanescent wave it uses a thin optical sheet for excitation (Figure 2.2E). To create a thin optical sheet, the angle of incidence of the collimated beam must be very close, but below θ_c (Figure 2.2B, E). In this case, the refracted beam reaching the specimen has much smaller cross-sectional dimensions than the incident beam. The small cross-

sectional dimensions minimizes the excitation of autofluorescence from regions with no cells. This technique allows imaging of fluorescent proteins both on the membrane and in the cytosol at the same time. Also using this thinner sheet of light reduced the background auto-fluorescence and thereby the measured noise, which made it possible to lower the excitation intensity during experiments resulting in the reduction of both photobleaching (photobleaching, see Appendix *Glossary*) and phototoxicity (phototoxicity, see Appendix *Glossary*) (93). For the remainder of this section and the rest of the work, all experiments done, unless specified otherwise, used the HILO microscopy technique to study the dynamic and organizational capabilities of FtsZ proteins during the cell cycle in *E.coli*.

2.2.3 Stroboscopic single molecule imaging of FtsZ

To be able to distinguish membrane bound FtsZ from diffusing cytosolic ones, we used 1 ms stroboscopic laser pulses. Typically the excitation time we used for membrane bound FtsZ was 100 ms. However, using this exposure time on cytosolic FtsZ molecules would result in completely blurred images due to the rapid diffusion of FtsZ. For example, the calculated diffusion coefficient for an FtsZ dimer using Broersma's formula (94) was $\sim 35 \mu\text{m}^2\text{s}^{-1}$, which is almost 6 times larger than the $6 \mu\text{m}^2$ (79) surface area of an *E.coli* cell (see Appendix *Diffusion coefficient calculation* for more information). This imaging is also only possible when one or two fluorescent molecules are activated within the cell at the same time. For this, we used a strain with photoactivatable FtsZ (FtsZ-mMaple3 (95)) (Table1: HE5) to verify that the 1 ms excitation pulses were sufficiently short enough for stroboscopic imaging. This strain was directly pipetted onto a cover slide and then the

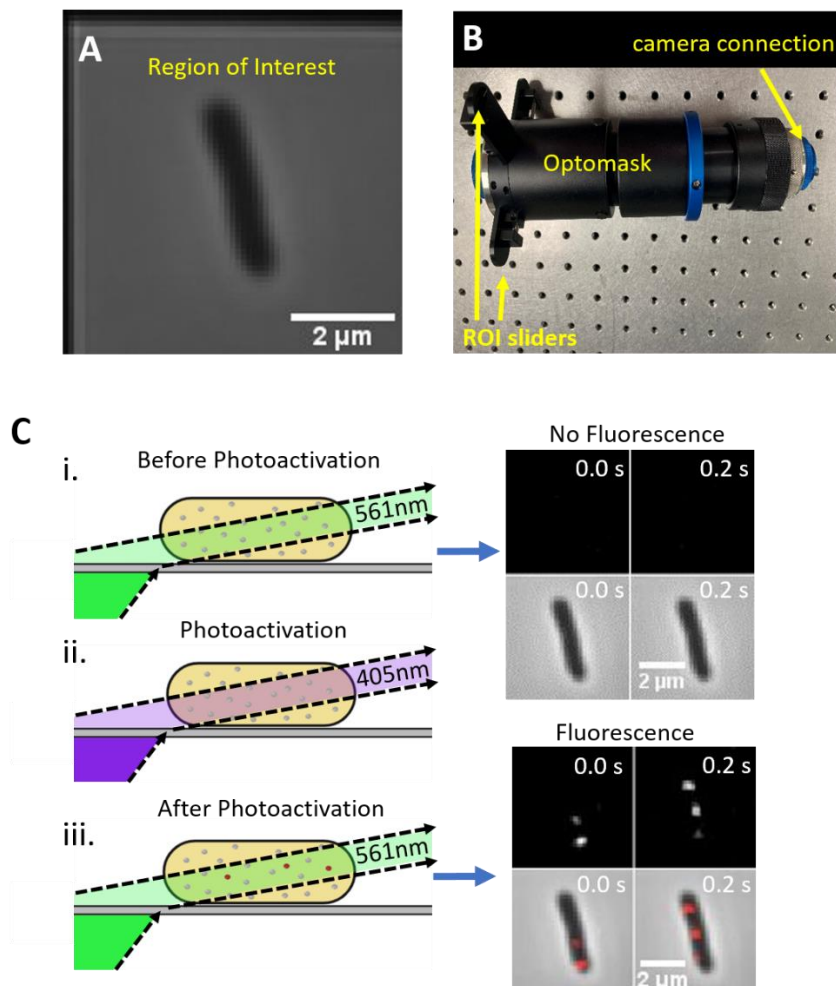


Figure 2.3: Single molecule imaging. (A) Phase contrast image defined by a small region of interest around of a cell in liquid culture on a glass slide. No agarose was used due to its possibility of having fluorescence. (B) The Optomask adapter was used to create a small region of interest (ROI), as seen in (A), near the readout register of the camera to optimize the frame rate for fast timelapse imaging. (C) Step by Step of the photoactivation process used in this work. (C, i) Schematic represents fluorophores (gray circles) within the cell before photoactivation (left). Images of a cell before photoactivation shows no fluorescence during this period (black arrow, right). (C, ii) Schematic of using a single pulse from a 405 nm laser to photoactivate a small portion of proteins within a cell. (C, iii) Schematic representing the resulting photoactivation (red circles) within the cell (left). After photoactivation the cell from (C, i, right) now displays single molecule fluorescence (black arrow, right). Note in both of the montages (right) in (C) the top panels are fluorescent images while the bottom panels are the top panels overlaid with their corresponding phase contrast images to show location and diffusion of proteins throughout the cell over time. All images in the montages are scaled the same for proper comparison.

droplet of culture was sealed by a rigorously cleaned (5M NaOH) coverslip (see Appendix *Coverslip cleaning procedure*) (Figure 2.3A).

During imaging an Optomask (Andor Technologies, Ireland) adapter was used to limit the image to a small region near the readout register of the EMCCD camera (Figure 2.3A, B). By reading out a smaller number of pixels, faster frame rates (22.89 frames per second during 1 ms exposure and 9.83 frames per second during 100 ms exposure) were realized. FtsZ-mMaple3 was fluorescently activated by a single pulse from a 405 nm diode laser having a power of 3 mW and pulse length of 100 ms. Once activated, FtsZ-mMaple3 (95) was apparently bleached and re-activated by the 561 nm diode laser repeatedly so that the number of fluorescently photo-activated molecules during the imaging period (5-10 minutes) remained approximately constant (Figure 2.3C). After image smoothing with a Gaussian filter having $\sigma = 1$ pixel, cytosolic FtsZ foci were observed in measurements using 1 ms exposure times and were absent in 100 ms exposure measurements due to blurring. Altogether, our system could obtain single molecule sensitivity, using a strain with a photoactivatable fluorescent label, which aided us in making conclusions about filament characteristics found in other measurements.

2.3 Microfluidic devices

In most early studies, bacteria were imaged on either a glass slide (Figure 2.4A) or in a Petri dish (for imaging a miniature version is used) (Figure 2.4B), which had nutrient supplemented agarose covering or filling them, respectively. However, long periods of imaging could not be performed using these agarose slides or dishes due to bacterial colony formation (Figure 2.4C). In the colonies cells overlap with one another.

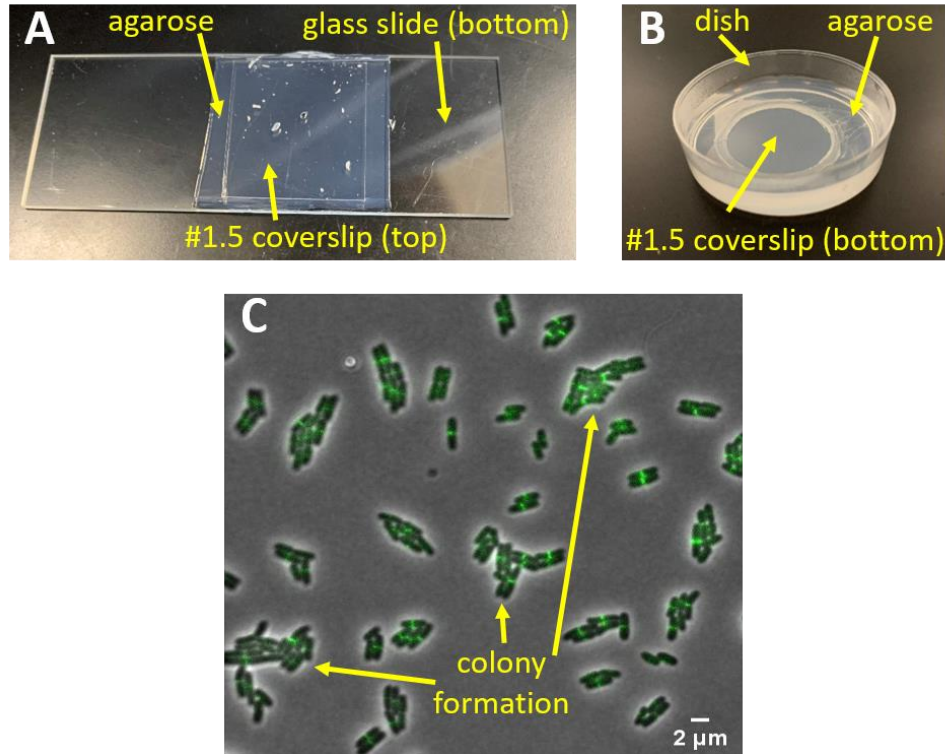


Figure 2.4: Traditional imaging platforms used in microscopy experiments. (A) Close up of a glass slide where the cells are spread between the supplemented agarose and a #1.5 coverslip. To load the bacteria, 1 μL of cell liquid culture is pipetted onto the agarose piece and then the coverslip is applied on top to spread the cells. In this work glass slides were mainly used to check the success of genetic manipulations. (B) Close up of a miniature Petri dish bounded to a #1.5 coverslip and filled uniformly with supplemented agarose. Alternatively, this device is loaded by removing the agarose slab, pipetting 1 μL of the liquid culture onto the coverslip, and reinsertion of the agarose slab into the dish. A small amount of down pressure was applied to thoroughly spread the cells uniformly. (C) Example image of fluorescently labelled cells when using either a glass slide or dish platform. After a few generations cells begin to form colonies making individual cell analysis difficult for many generations.

Consequently, tracking an individual cell through multiple generations becomes nearly impossible (Figure 2.4C). Also, sometimes it would be beneficial to add some nutrients or drugs during the measurement, but this is not possible with the agarose slides or dishes. To resolve these problems and limitations, we instead used microfluidic devices made of polydimethylsiloxane (PDMS) or patterned agarose.

2.3.1 PDMS based chip fabrication

Soft-lithography of PDMS was used to fabricate microfluidic chips following the procedures from Yang et al. 2018 (96). Briefly, silicon master molds (Figure 2.5A) were fabricated by combining e-beam and photolithography, and reactive ion etching at Oak Ridge National Laboratory's Center for Nanophase Materials Science (CNMS) facility. The mold defined dead-end channels, referred to as the mother machine, which were 0.8-0.9 μm wide, 1.1 μm high, and 20 μm long. PDMS (Sylgard 184, Dow Corning) was poured on a passivated mold and baked for 15 min at 90 $^{\circ}\text{C}$ in a convection oven. Subsequently, the solidified PDMS layer was peeled from the mold and cut into pieces. Access holes to each piece, corresponding to one microfluidic device, were then punched using a biopsy needle. A PDMS piece and clean #1.5 coverslip (Fisher Scientific) were then plasma treated. The two were bonded together after the treatment (Figure 2.5B).

To load the cells to the chip, an overnight culture of cells was used. 1 $\mu\text{g ml}^{-1}$ of bovine serum albumin (BSA) was added to 1.5 ml of overnight cell culture ($\text{OD}_{600} > 0.4$) and concentrated 100x by centrifugation. A 2-3 μl of resuspended culture was then pipetted into the main flow channel (Figure 2.5C). The cells were then allowed to populate

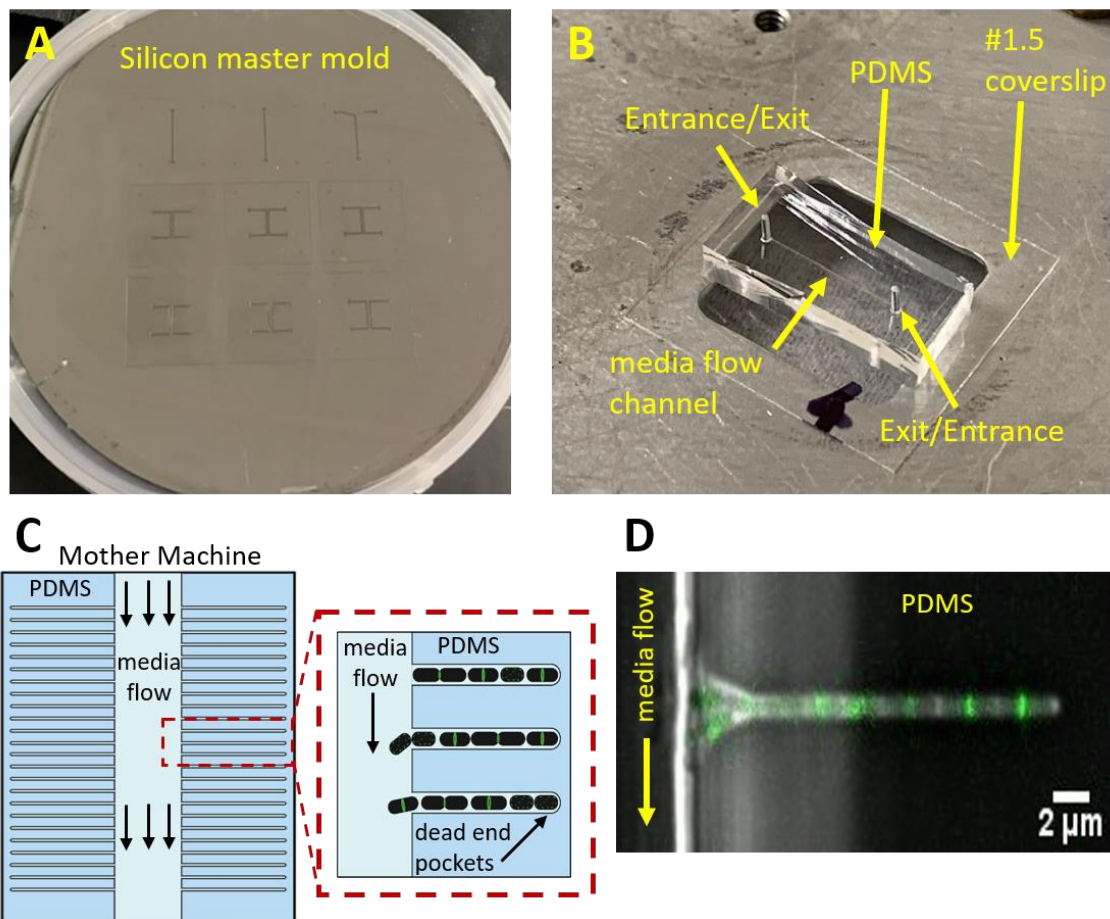


Figure 2.5: PDMS chip fabrication and design. (A) Example of a silicon master mold after reactive ion etching the microfluidic design onto it. The wafer has 9 separate mother-machine designs on it which in turn makes the same number of microfluidic devices. (B) Close up of the PDMS based microfluidic device used in this work with the media flow channel and the entrance/exit labeled. The application and removal of fresh media from the PDMS device is done by tubing that is inserted into the entrance and exit holes, respectively. Also the PDMS chip is bonded to a #1.5 coverslip (bottom) through plasma treatment. (C) Schematics of the mother machine design that is imprinted into the PDMS chip. (D) Example image of the mother machine design in a PDMS chip with cells growing linearly within the channels. Note cells can grow for many generations in these devices due to fresh media flowing into them (C, D) which allows fresh nutrients to go into the pockets while at the same time removes cellular waste and excess cells out of the pockets. I would like to acknowledge Da Yang for the creation of the mother-machine designed etched silicon wafer.

the dead-end channels (Figure 2.5C insert) for about 1 hr. Once these channels were sufficiently populated (Figure 2.5D), tubing was connected to the devices, and the flow of fresh M9 medium with BSA ($1\ \mu\text{g ml}^{-1}$) started. The flow was maintained at $5\ \mu\text{l min}^{-1}$ during the entire experiment by an NE-1000/2000 Syringe Pump (New Era Pump Systems, NY) (Figure 2.1A, B). To ensure steady-state growth, the cells were left to grow in the channels for at least 14 hours before imaging started (Figure 2.5D).

2.3.2 Agarose based chip fabrication

We use HILO microscopy to gain high signal to noise ratio images at low light levels. However, this imaging technique is not fully compatible with PDMS-based chips because the refractive index of the PDMS is different than the liquid medium that contains the cells. To overcome this problem, we used agarose as the material for the channels because it has a refractive index close to that of the liquid medium.

The process of agarose chip fabrication followed the one described by Moffitt *et al.* (97), but with some modifications (Figure 2.6A). Unlike this previous work, which used channels with two open ends, here we utilized dead-end mother-machine channels (Figure 2.5C). The channels were patterned into 4% agarose (Figure 2.6D) to which M9 salts (M9 salts see Abbreviations) and glycerol were added. A silicon template with protruding channel reliefs were used for patterning (Figure 2.5A). The fabrication procedure for the silicon template has been reported before (96). Fabrication was carried out on a 4" silicon wafer. After fabrication, the wafer was cut into individual patterns using a diamond knife and cleaned in isopropanol (Fisher Scientific) by sonication. The

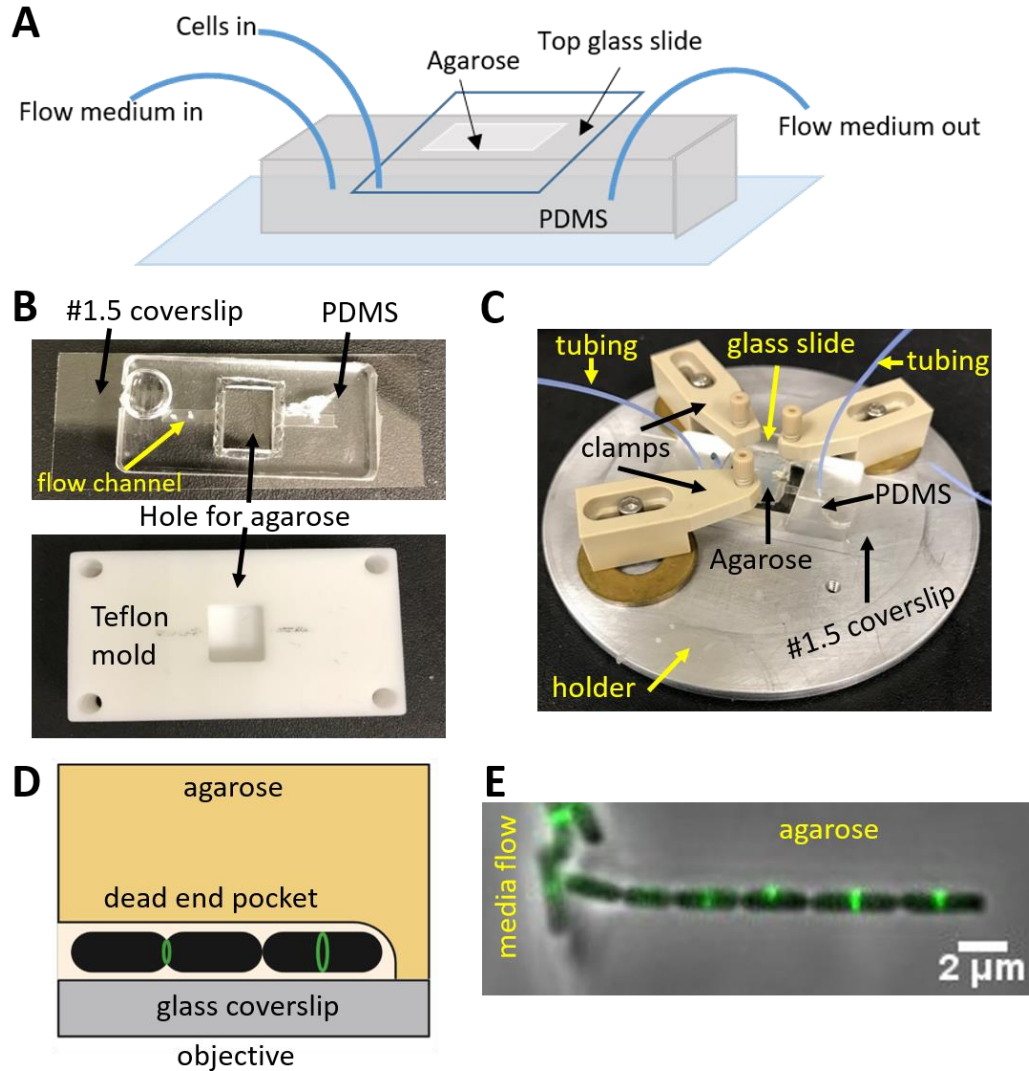


Figure 2.6: Microfluidic mother machine agarose chips. (A) Schematic of the agarose chip used throughout this work. Imprinted agarose is inserted into the middle of a jig consisting of PDMS bounded to a #1.5 glass coverslip. I acknowledge the work of a former lab member, Ann Jennings, for this schematic contribution. (B) Top: Actual image of jig used. Bottom: Teflon mold used to imprint agarose. Mother machine silicon template is clamped between the Teflon molds and molten agarose is poured into the mold containing the central hole. (C) Actual image of complete set up of agarose chip including tubing (blue) where fresh media flows in. Note the 3 clamps are holding down the top glass slide to prevent leaking and to keep the agarose piece from lifting up. (D) Schematic of how cells are positioned (horizontal) and imaged within the agarose chips. (E) Composite of phase contrast and fluorescent images of FtsZ-mNG showing cells within the patterned agarose chip. When fresh media flows through the device the cells can stay alive and be studied for many generations.

resulting silicon template was clamped between two Teflon slabs of 5.1 mm in height (Figure 2.6B, bottom). One of the slabs contained a rectangular hole with dimensions 9x11 mm (Figure 2.6B, bottom). This hole was centered relative to the pattern on the silicon template. Molten agarose was poured into the hole formed by the Teflon slabs. After the agarose solidified, excess agarose that overfilled the hole was cut by a razor blade. The Teflon pieces were unclamped, and a rectangular agarose piece was released from the hole.

The agarose piece with patterned channels was then inserted into a custom-made jig (Figure 2.6B, top). The jig design follows the one reported earlier (97). Briefly, the holder consisted of a PDMS slab and a #1.5 glass coverslip (Fisher Scientific). The two were bonded together after cleaning and O₂ plasma treatment. The PDMS slab contained a central through hole of 5.0 mm in height and cross-sectional dimensions of 8x10 mm in which the agarose piece was inserted. The PDMS slab also contained two channels leading to the central hole. The PDMS slab was made in a machined aluminum mold, which followed the design described by Moffit *et al.* (97).

Once the agarose piece was inserted into the central hole in the jig, the whole assembly was placed on a custom-made aluminum plate that fit onto the microscope stage (Figure 2.6C). A piece from a glass cover slide (Fisher Scientific) was cut by a diamond knife, cleaned in isopropanol by sonication, and dried. This glass slide was then pressed against the agarose piece, which protruded from the jig, by custom-made clamps that attached to the microscope plate. These clamps pushed the cover slide tightly against the PDMS part of the jig to prevent leakage and raising of the agarose piece (Figure

2.6C). During application of the pressure, the oversized agarose piece contracted and some of its liquid content flowed out. After this flow had stopped, tubing was connected to the jig and M9 medium, to which 1 mg/ml BSA was added, was circulated through the chip for about 20 minutes using a New Era 1000 syringe pump (Figure 2.1A).

Our loading procedure of cells to these dead-end channels was similar to the one described for PDMS based mother machine channels (96, 98) but differs from the one described for agarose chips before (97). In the latter case cells were pipetted onto the glass slide and spread when patterned agarose was pressed against the slide. Here to load the cells, extra tubing with a concentrated solution of cells was connected to the jig and cells were injected into the main flow channel by a syringe that was connected to the other side of the tubing (Figure 2.6A). An overnight culture of cells was used when loading the cells into the device ($OD_{600} > 0.4$). $1 \mu\text{g ml}^{-1}$ of bovine serum albumin (BSA) was added to the overnight cell culture and cells were concentrated about 100x by centrifugation. To allow cells from the main flow channel to fill the dead-end channels, the clamps that held the cover slide on the top of the jig were slightly loosened. Once the mother machine channels were filled with cells (Figure 2.6D, E), flow of the medium was started in the main channel. The flow was maintained in the main channel overnight at a rate of $8 \mu\text{l/min}$. The next day, while maintaining the same flow rate, cells were imaged using our Nikon Ti-E inverted microscope (Figure 2.1A and Figure 2.6D, E).

2.3.3 Upright mother-machine channels

E.coli cells are usually imaged so that the long axis of these rod-shaped cells lies on the focal plane of the objective. The image of the cell is a 2-D projection of the cell to the

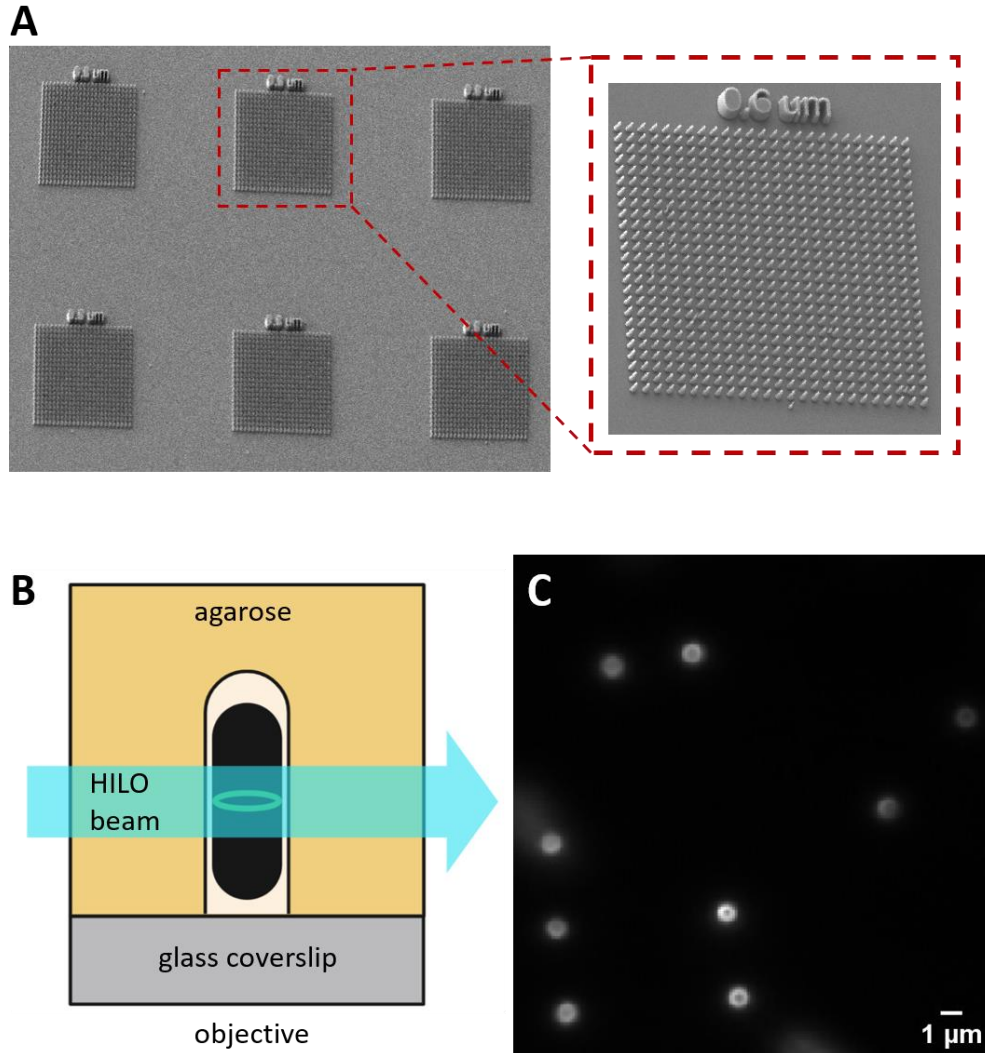


Figure 2.7: Design and imaging in vertical pockets. (A) Scanning electron microscope (SEM) image of the reactive ion etched microfluidic design for the vertical pockets with widths ranging from 0.6 to 1.2 μm . Note only 0.6 μm wide pockets are used since cells were not immobilized within pockets for larger widths (right). (B) Schematic of how cells in the vertical pockets are positioned and imaged. Vertical pockets are imprinted in agarose dishes instead of PDMS due to the lack of fresh media flow into the pockets. Instead, the agarose is supplemented with M9 salts and glycerol. (C) Actual image of vertical pocket measurement where Z-ring (white) dynamics can be studied. Note in this study only cells with mature Z-rings are able to be imaged since the Z-ring is the only focusing structure in the Z-direction within these cells. Z-direction focusing was set at the depth with the most fluorescent Z-rings being visible and clear within the imaging frame. I would like to acknowledge the work of a colleague, Da Yang, for assisting me with the creation of the vertical pocket etched silicon wafer used here.

image plane. Consequently, it is not possible from a single 2-D projection to reconstruct 3-D cellular structures such as the Z-ring, which lies *perpendicular* to the focal plane. A better projection of the Z-ring can be acquired if it is positioned parallel to the focal plane. To achieve this, we fabricated channels that point *perpendicular* to the focal plane. For that end, a silicon master mold with vertical dead-end channels were constructed using reactive ion etching (Figure 2.7A). The etched silicon wafer was then used to imprint vertical pockets with a depth of 6 μm and diameters ranging from 0.6 to 1.2 μm into a (miniature) dish of 2% agarose supplemented with M9 salts and a carbon source (glycerol or glucose). Then the agarose piece was laid to a #1.5 glass coverslip (Fisher Scientific) (Figure 2.7B). Agarose supplemented with nutrients was used due to the lack of a flow channel in this design (Figure 2.7B). Then cells were pipetted onto the coverslip and then the solidified patterned agarose would be gently pushed back down on top of the cells to immobilize them and push them up into the pockets. Only cells in the 0.6 μm wide pockets were imaged since the cells in the wider pockets were not immobilized and moved during imaging (Figure 2.7A). No antibiotics were added to the agarose. Cells were allowed to complete one full cell cycle, to ensure the cells were growing, before imaging of the cell in the pocket started (Figure 2.7C).

2.4 Qualitative and quantitative image analysis

In image processing, the primary quantitative challenge is to distinguish objects from the background. For this work, all image analysis was written and run in Matlab, which included the Image Analysis Toolbox and the publicly available Diplib Toolbox (<http://www.diplib.org/>). Oufiti software (open access) was used for cell tracking (Figure

2.8A) in measurements that took place on a pad or dish, and ImageJ Fiji (open access) was used for brightness and contrast adjustments to individual images, montages, and movies used throughout this work.

2.4.1 Visualization of timelapse data

Early in the field of microscopy, cells were studied mostly by visual inspection of microscope images (Figure 2.8A). This approach becomes meaningless for timelapse imaging where thousands of images are collected per cell. Our typical analysis of timelapse measurements started with building maps of fluorescence intensity from line profiles, referred to as kymographs, for each fluorescently labeled cell. To build these kymographs, cells needed to be segmented first. We used two different approaches for segmentation, namely, our own Matlab scripts for mother machine measurements and Oufi (99) for agarose pad/dish measurements. For cell detection in Oufi, first the raw images are convolved with a Laplacian of Gaussian (LoG) kernel, and then the pixel magnitudes above the specific user set LoG threshold are saved as the cell edges (99). Using these pixelated cell edges as initial guesses for an energy minimization function, Oufi computes a local threshold value which induces a local force that attracts the contour and object edges through active contour fitting. Consequently, this creates subpixel accuracy for the cell contour (Figure 2.8A) (99). From this contour, other cell detection information (specifically the midlines for this work) was calculated (Figure 2.8B).

The kymographs give a good representation of the dynamics and organizational distributions throughout the cell over time. These first initial qualitative visualization steps were very important because they allowed us to pose hypotheses that were later tested

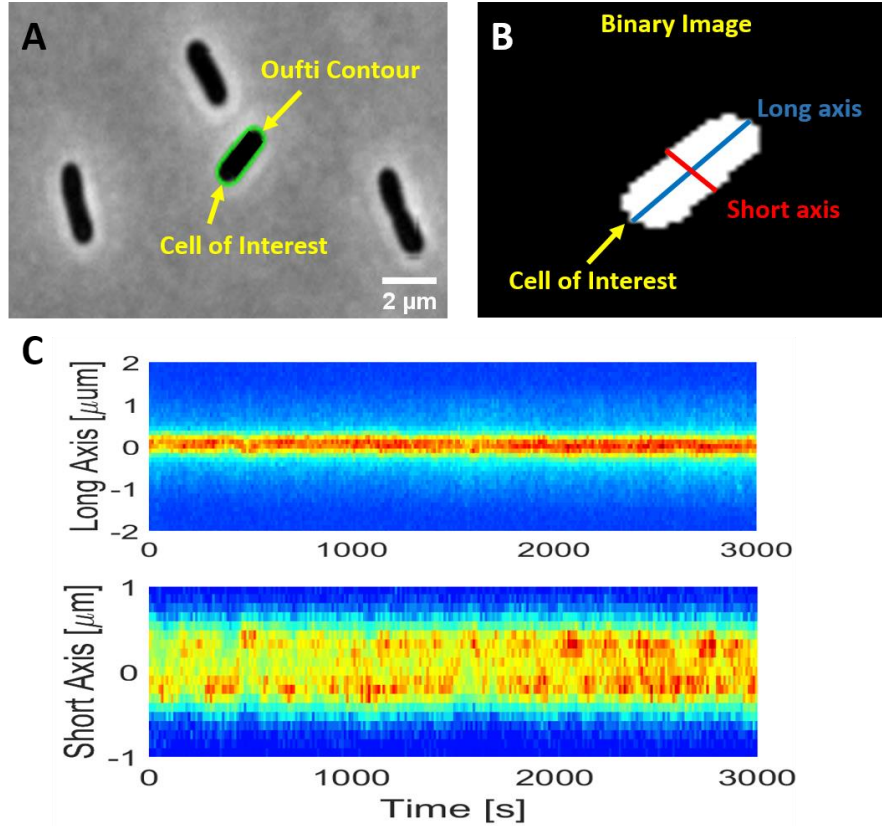


Figure 2.8: Cell segmentation resulting in kymographs of the bacterial Z-ring. (A) Digital phase contrast image of a cell using Oufi segmentation procedures. Oufi convolves the image with a Laplacian of Gaussian kernel and uses this for active contour segmentation resulting in the cell contour seen here (green). Note normally most cells within the image would have a contour as well. (B) Magnified binary image where the cell contour is used to isolate the particular cell of interest so no non-cellular fluorescence will affect the analysis. Using the contour coordinates, long (blue line) and short (red line) axis line profiles that extend the length and width of the cell, respectively, can be automatically generated. (C) Example of visualization of fluorescence intensity, kymograph, data of the Z-ring, which was the result of line profiles along the long (top) and short (bottom) axes of a cell. Red corresponds to high and blue to low concentrations of fluorescent proteins.

by quantitative analysis (see image analysis sections that follow).

2.4.2 Determination of power spectral densities and autocorrelation functions

While kymographs can reveal new aspects of dynamical processes, for more sensitive measurements further analysis is needed. The limitations of the kymographs from the images were overcome by analysis of the spectrum of frequencies (spectral analysis) by converting the visual signal into the frequency domain. For this work, spectral analysis was used to compare fluctuations in FtsZ-mNG intensity before and after Z-ring formation (T_z). While the FtsZ assemblies were observed visually in images before Z-ring formation, no visible assemblies were seen after Z-ring formation. By using fluorescent intensity traces from three different cellular locations, cell middle and two quarter positions, power spectral densities (PSD) and autocorrelation functions (ACF) for FtsZ fluctuations were determined (power spectral density and autocorrelation function, see Appendix *Glossary*). The traces were first separated into two parts, the transition period ($t < T_z$) and the Z-ring period ($t > T_z$) so that FtsZ fluctuations before and after Z-ring formation could be compared. In each period, i.e. before and after Z-ring formation, the traces were divided into 64 frame segments. For each trace the mean intensity of the trace was then subtracted and divided by the total intensity of the cell.

$$I_c(t) = \frac{I_{trace} - \langle I_{trace} \rangle}{I_{total}}$$

The intensity in each of these segments was then multiplied by a Hanning window (Hanning window, see Appendix *Glossary*), and then the Fast Fourier transform ($\mathcal{F}$) and Fast Inverse Fourier transform ($\mathcal{F}^{-1}$) were calculated to obtain the *PSD* and *ACF* for each trace respectively (Fourier transform, see Appendix *Glossary*).

$$PSD = \mathcal{F}(I_c(t)) \cdot \mathcal{F}^*(I_c(t)),$$

where $\mathcal{F}^*$ is the complex conjugate

$$ACF = \mathcal{F}^{-1}(PSD)$$

All power spectral densities and autocorrelation functions before and after Z-ring formation for each cell were averaged together. Furthermore, the resulting PSD and ACF were averaged again giving a population average over all cells. Using the population averaged results, we could easily see the characteristics that occurred most often within that particular strain and compared those characteristics to other strains.

2.4.3 Protofilament tracking and rate of change analysis

From previous studies FtsZ filaments had been characterized *in vitro* but still were lacking in live cells. To help characterize FtsZ filaments in live cells, we developed a filament tracking algorithm involving both intensity thresholding and dynamical movement parameters. First, each image had the background intensity subtracted (Figure 2.9A, i). The resulting image then was convolved with a Gaussian kernel (low-pass filter) with $\sigma = 1$ pixel. Then cell contours acquired from a custom Matlab script or Oufiti (100) were used to isolate each cell of interest (Figure 2.9A, ii). Our own custom script was used for microfluidic measurements, while Oufiti was used for agarose dish/pad measurements. Based on cell coordinates, a binary mask for each cell was defined. Within this mask a thresholding value was calculated as $I_{thresh} = 2I_{average} \approx I_{average} + 2.5\sigma_I$. Here $I_{average}$ was the average pixel intensity and σ_I was the standard deviation of the image intensity within the cell. The resulting binary image (Figure 2.9A, iii) was then dilated by a disk-shaped structuring element with a radius equal to 1 pixel. Individual clusters were

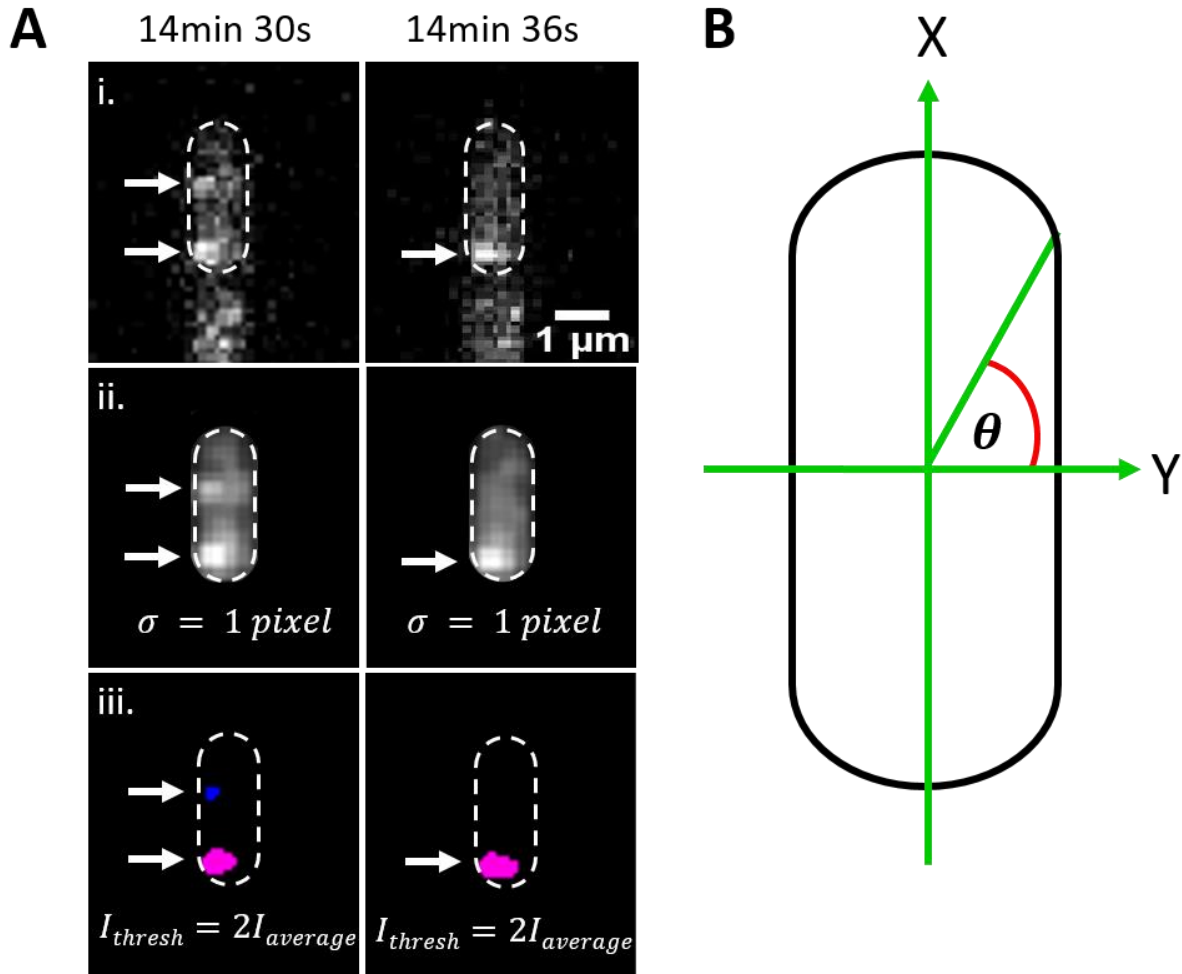


Figure 2.9: FtsZ filament tracking before Z-ring formation. (A) Example images of image processing steps to allow us to be able to track the clusters over time (left to right). (A, i) Raw digital data of fluorescent FtsZ filaments (white arrows) before Z-ring formation in *E.coli*. (A, ii) Raw image from (A, i) convolved with a Gaussian kernel with $\sigma=1$ pixel. Also the cell is isolated within the image using binary contour segmentation (Figure 2.8B) from a custom Matlab script or Oufiti (99). (A, iii) Color labeled (blue and magenta) binary image of filament bundles (white arrows) after intensity thresholding within the cell. Threshold was set at $I_{thresh} = 2I_{average} \approx I_{average} + 2.5\sigma_I$ where $I_{average}$ is the average pixel intensity and σ_I is the standard deviation of the image intensity within the cell contour (white dashed lines). (B) Schematic layout of cellular coordinates used when tracking individual filament bundle centroids. Note the origin of the coordinate grid being at cell center. The angles (θ) of velocity relative to the short axis of the cell were also characterized.

subsequently labelled using the *label* function from the DiplImage toolbox. Clusters were connected in the time series by using their proximity and intensity. For cluster connectivity an intensity threshold of 2.5% of the total intensity and distance thresholds of 500 nm for short axis and 200 nm for long axis movement were used (Figure 2.9B). First, all clusters with an intensity lower than the threshold were disregarded for analysis. Then the distances between each cluster from *image* (*i*) and *image* (*i* + 1) were obtained (*i* = 1, 2, ..., *Nimages* - 1). Each cluster from *image* (*i*) was connected with the corresponding cluster with the minimum distance that stayed within the threshold values for short and long axis movement from *image* (*i* + 1). Once the cluster was matched, it was taken out of the pool of possible connections between the remaining clusters. Clusters that did not match with another cluster within the proximity thresholds, retained their initial labels.

In long time lapse measurements each frame was segmented separately and then cluster characteristics were analyzed. The analysis used the *measure* function from the DiplImage tool box to determine the binary centroid coordinates, average intensity, and total intensity of each cluster. The centroid coordinates were calculated relative to the center of the cell. The coordinate axis was oriented along the long (x-axis) and short axis (y-axis) of the cell (Figure 2.9B). The FtsZ assembly intensity at each time step was normalized by the total fluorescent intensity of the cell. Also the output of the FtsZ assembly intensity from this algorithm represented the time-averaged intensity of the cluster during its lifetime. Accordingly, having the intensity as a percentage of the total cell intensity gave us an estimation for the size of the assemblies during the cell cycle.

To study the assembly of each FtsZ cluster, its size and trajectories were determined. For that purpose, the largest size cluster of pixels in each trajectory was found first. The binary mask of this cluster was then propagated to other frames in the time-lapse sequence. This also included the frames beyond those in which the program was able to identify this cluster using thresholding. The total intensity from this masked area was determined and plotted as a function of time. The procedure was only applied to clusters that did not significantly displace during the period when the program was able to identify these clusters. Accordingly, the rate of change was then determined by the difference of the normalized intensity from a given cluster between two consecutive frames. The latter were acquired at a frame rate of 1 Hz.

2.4.4 Determination of treadmilling velocities of FtsZ assemblies and distribution of treadmilling speeds

Treadmilling velocity components v_x and v_y were determined from tracking FtsZ-mNG foci. Here v_x was the velocity component along the long and v_y the velocity component along the short axes of the cell; both components lay on the image plane. The tracking of the foci was previously described in the *Protofilament tracking and rate of change analysis* section. Displacements of foci between subsequent image frames determined velocity vector components. All displacements between subsequent frames during the entire lifetime of the assembly from all cells were compiled together resulting in velocity distributions (see Chapter 4: *Transient assemblies of FtsZ on the cell membrane precede the formation of the Z-ring*). The angle relative to the short axes of the cell (Figure 2.9B) was then calculated as the $\arctan(v_x/v_y)$.

The treadmilling velocity on the image plane, $\vec{v} = (v_x, v_y)$, was not the same as the actual 3D velocity of the treadmilling assembly, $\vec{v}_{3d} = (v_x, v_\varphi, v_r = 0)$. While the v_x component of both vectors was the same, the v_y component was the projection of the azimuthal velocity to the image plane and was expressed as $v_y = v_\varphi \sin(\varphi)$, where φ was the azimuthal angle measured from the image plane. Thus, $|v_y|$ changed from 0 to $|v_\varphi|$ when the transient assembly moved around the circumference of the cell at a constant velocity v_φ .

Of interest was not the projection v_y , which we directly determined from the images, but the 3D velocity component v_φ . To estimate the distribution of v_φ among different assemblies in a cell population, we assumed that the azimuthal positions of FtsZ foci, φ , were uniformly distributed around the circumference of the cell over time. First, we considered a situation where all assemblies treadmilled at the same velocity v_φ . In this case the v_y component was distributed as

$$\rho(v_y) = \frac{1}{2\pi} \frac{1}{\sqrt{v_\varphi^2 - v_y^2}}, \quad v_y < v_\varphi$$

However, not all assemblies treadmilled at the same velocity v_φ . If v_φ was spread out according to a distribution $G(v_\varphi - v_{\varphi,0})$ around the mean velocity $v_{\varphi,0}$ then distribution for v_y would become

$$\rho(v_y; v_{\varphi,0}) = \int_{v_y}^{\infty} \frac{1}{2\pi} \frac{G(v_\varphi - v_{\varphi,0})}{\sqrt{v_\varphi^2 - v_y^2}} dv_\varphi$$

In our analysis we assumed that $G(v_\varphi - v_{\varphi,0})$ was a normal distribution (a Gaussian). We then compared $\rho(v_y; v_{\varphi,0})$ at different $v_{\varphi,0}$ and $\sigma_{v,\varphi}$ (std) values to experimentally measured distributions. In this procedure $\rho(v_y; v_{\varphi,0})$ was calculated using numerical integration and then the best fit to the data was found.

2.4.5 Estimation of measurement noise contributions

To estimate noise contribution, we imaged cells where the expression of SulA (SulA, see Appendix *Glossary*) proteins from an extra copy plasmid (plasmid, see Appendix *Glossary*) was induced (plasmid pA3, strain MB43) (Table 1). In these cells FtsZ assemblies and Z-rings were absent due to the antagonizing effect of SulA on FtsZ polymerization (101). The measured rates in these cells thus originated from sources not related to transient assemblies, i.e. from measurement noise. Since the SulA overexpression strain lacked FtsZ foci, we then generated focal regions in these cells based on measured distributions of focal parameters in WT cells. The size, shape, and location of each foci were drawn from the distribution measured for WT cells. These parameters defined a region in the image (a binary mask), which was overlaid with the image of a SulA cell (which were picked randomly from the measured cells). Then it was centered at the same relative position as the focus in the WT cell and it lasted the same duration as the focus in WT cell. Fluorescent intensity as a function of time from this region was then collected and analyzed for changes.

2.5 Modelling

2.5.1 Modelling of protofilament lifetime and length distributions

We used the Gillespie Algorithm (see list below) to model the assembly and disassembly dynamics arising from treadmilling.

Gillespie algorithm steps used for kinetic polymers

1. Set initial size of the polymer
2. Use random number generator to determine the next reaction. This reaction is chosen proportionally based on the reaction rates set.
3. Update the size of the polymer based on the result from the Monte Carlo simulation (step 2).
4. Repeat steps 2-4 until the size of polymer is zero or the maximum number of iterations is reached.

Some polymers could survive the maximum number of iterations we set, so for simplicity these polymers that survived all iterations were neglected because they did not have a finite lifetime as predicted by treadmilling dynamics. The model outputs polymer lifetime, displacement, and length distributions, which in turn was compared to experimental data. This model was implemented in Matlab.

2.5.2 Modelling bundling using Ising-like model

To gain a further understanding of FtsZ filament bundling, we created an Ising-like model of FtsZ assembly following the model described by Lan et al. 2009 (86). For this, we assumed the cell membrane was a 2-dimensional square lattice (Figure 2.10). First, the lattice was initialized with ~3000 FtsZ and ~800 MinC proteins positioned randomly on a

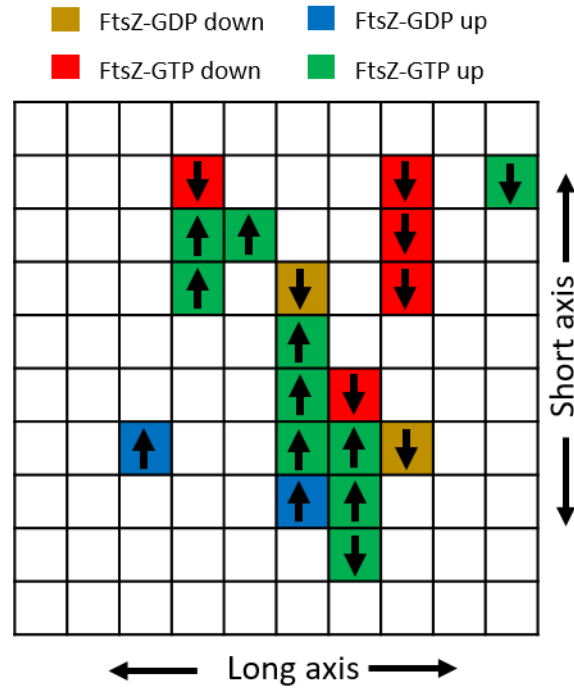


Figure 2.10: Schematic of Ising-like bundling model. The cellular membrane created as a 2D lattice with FtsZ occupying particular cells (red, blue, green, yellow) in the lattice. In this model FtsZ is either GTP (red and green) or GDP (yellow and blue) bound. These FtsZ also have a polarity of up or down (black arrows) in the short axis of the lattice due to the structural asymmetry of FtsZ monomer ends.

500 x 500 square lattice. There were four different configurations (Figure 2.10) for FtsZ i.e. FtsZ-GTP up (green), FtsZ-GTP down (red), FtsZ-GDP up (blue), and FtsZ-GDP down (yellow). The same interaction energy (102) applied for all the lateral contacts between these four FtsZ variant. FtsZ-GTP and FtsZ-GDP longitudinal interaction energies were $-17k_B T$ and $-1k_B T$, respectively (86). Following Monte Carlo stepping, one monomer was randomly selected and moved to an unoccupied new random location. Then a random reaction, nucleotide exchange or GTP hydrolysis, for the FtsZ monomer is decided along with the existence or not of MinC as a neighbor to the FtsZ monomer since the MinC proteins were distributed as a parabolic. Then the energy cost of the monomer's previous location was compared to the energy cost of the new location, where these energies were also influenced by the reaction that was chosen and the existence of a MinC neighbor. Based on this energy comparison and the randomly decided probability value for the Monte Carlo decision, the resulting location (previous or new) of the monomer was determined. Consequently, individual images of the resulting polymers on the lattice were saved at periodic time steps, which allowed us to study the dynamics of bundling over time (see Chapter 1: *Protofilament bundling*).

2.6 Bacterial strains

In this work, all genetic modifications and experiments were done using derivatives of the *E.coli* K-12 strain (103). All strains and plasmids are described in Table 1 (also see Appendix *List of strain information*). The strains that were used had four different fluorescent markers on the cytoskeletal protein, FtsZ. The four fluorescent markers were FtsZ⁵⁵⁻⁵⁶mNeonGreen (FtsZ-mNG), FtsZ⁵⁵⁻⁵⁶sfGFP sandwich fusion (FtsZ-sfGFP), FtsZ-

Table 1: List of the strains used in experiments.

Strain	Genotype	Plasmid	Antibiotics used
HE1	BW27783 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen</i> sandwiched	-	-
JM26	BW27783 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen</i> Δ motA743::kan	-	25 µg/ml kan
JM60	BW27783 cm ^R	ColEI, JW0093 <i>P_{TS}-lac::6xHis-ftsZ-gfp cat</i>	40 µg/ml cm (plates; PDMS chip) 80 µg/ml cm (liquid)
KC606	BW25113 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-sfGFP</i>	-	30 µg/ml cm
BEW1	BW27783 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen</i> Δ minC::kan	-	25 µg/ml kan
BEW2	BW27783 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen</i> Δ zapA::kan	-	25 µg/ml kan
MB43	BW27783 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen</i> <i>P_{lac}SulA</i> , cm ^R	pA3	30 µg/ml cm 0.2% glucose, except during induction
JM71	MG1655 <i>lacY::lacY-mNeonGreen-kan</i>	-	25 µg/ml kan
HE5	JKD7 <i>ftsZ::kan</i> cm ^R	pJSB - <i>ftsZ⁵⁵⁻⁵⁶-mMaple3</i>	30 µg/ml cm 25 µg/ml kan
JM95	BW25113 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-sfGFP-CM</i> Δ zapA::kan	-	25 µg/ml cm 30 µg/ml kan
JM65	BW27783 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen</i> Δ motA743::kan Δ clpA783::kan	-	20 µg/ml kan
JM66	BW27783 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen</i> Δ motA743::kan Δ clpP723::kan	-	20 µg/ml kan
JM67	BW27783 <i>ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen</i> Δ motA743::kan Δ clpX724::kan	-	20 µg/ml kan

The abbreviations and symbols are as follows: kanamycin (kan), chloramphenicol (cm), deletion (Δ), and insertion (::). See also Appendix *List of strain information* for more information.

GFP, and FtsZ-mMaple3. FtsZ-mNG (HE1) and FtsZ-sfGFP (KC606) replaced genomic FtsZ, while FtsZ-GFP (JM60) and FtsZ-mMaple3 (HE5) were copies from the plasmids. These strains were generous gifts from Harold Erickson (Duke University), Jie Xiao (John Hopkins University), Dr. Alex Dajkovic (Biomillenia), and Dr. K.C. Huang (Stanford University). After receiving these strains, we genetically created mutate strains (particularly of the FtsZ-mNG strain), through a process known as a P1 transduction, in order to study the dynamic properties of FtsZ after the deletion of specific FtsZ interacting proteins. For more on P1 transduction see Appendix *P1 transduction procedure*, which includes information on the P1 bacteriophage and transduction procedure (104, 105).

Using P1 transduction, two strains were created where the genes coding for MinC (BEW1) in one strain and ZapA (BEW2) proteins for another strain were deleted from the wildtype cells (Table 1). Here, in each case wildtype cells containing the FtsZ-mNG label were grown-up in liquid culture and then exposed to a specific P1 lysate solution, one for the *minC* sequence (JW1165) and the other for the ZapA sequence (JW2878-1). For the *minC* deletion mutate (BEW1), the lysate infected the wildtype cells and replaced the *minC* sequence with a kanamycin (kan) resistance gene. Likewise, the *zapA* sequence was replaced with a kanamycin resistance gene in the *zapA* deletion mutate (BEW2). To make sure the recipient strains had integrated the DNA sequence that replaced MinC and ZapA, respectively, these transduced cells were grown on medium that contained kanamycin. The other strains listed in Table 1 were constructed by Dr. Jaana Mannik (JM strains) and by Dr. Matthew W. Bailey (MB strain).

2.7 Cell culture growth conditions

All strains are derivatives of *E. coli* K-12 (Keio collection) (103). The strains and plasmids are described in Table 1. All cells were grown and imaged in M9 minimal medium (Teknova) supplemented with 2 mM magnesium sulfate (Millipore Sigma). In slow growth conditions (doubling time: $T_d=170\pm50$ min) 0.3% glycerol and in fast growth conditions (doubling time: $T_d=74\pm19$ min) (20) 0.5% glucose supplemented with 0.2% casamino acids (ACROS Organics) were added to the media. When needed antibiotics were added to the culture media at the final concentrations described in Table 1. No antibiotics were used during cell growth on 2% agarose dishes, and in agarose and PDMS microfluidic devices, except for the plasmid expressing FtsZ-GFP strain (chloramphenicol, $40\text{ }\mu\text{g ml}^{-1}$). For the strain with photoactivatable FtsZ-mMaple3, 0.2% glucose and 0.2% arabinose were added to the M9 media. For photoactivation measurements where FtsZ-mMaple3 was sought to be in monomeric form, cells were diluted and resuspended in phosphate-buffered saline (PBS) for 20 hours prior to the experiment to inhibit GTP production needed for FtsZ filament assembly. Lastly, for the estimation of the noise contribution, isopropyl β -D-1-thiogalactopyranoside (IPTG) was added at a final concentration of $400\text{ }\mu\text{M}$ to induce Sula expression from the plasmid pA3 in the MB43 strain. All bacterial strains were grown and imaged at 28°C .

CHAPTER 3

CELL CYCLE-DEPENDENT REGULATION OF FTSZ IN *E. COLI* IN SLOW GROWTH CONDITIONS

3.1 FtsZ concentration varies during the cell cycle in slow growth conditions

Cell cycle-dependent variations in FtsZ concentration in *E. coli* have not yet been reported in live cells. However, in recent years a functional FtsZ fluorescent fusion has been developed, which can be expressed from the native locus and are the sole copy of FtsZ within the cell (95). Here, we use one of these constructs, where the mNeonGreen (mNG) fluorescent protein is sandwiched between residues 55 and 56 of FtsZ (FtsZ-mNG), to determine the changes of FtsZ numbers and concentration during the cell cycle (Table 1). For measurements of cells in steady-state conditions, we grew them in microfluidic mother machine channels (96, 98). The linear colonies in the mother machine channels allow determining spatial distribution of fluorescence intensities from individual cells through the division process without the uncertainties introduced by nearby or overlapping cells. Such uncertainties are present when agarose pads and dishes are used for imaging because of the spatial overlap of fluorescence from individual cells. As the first step in the analysis, we collected intensity profiles from time-lapse measurements to create kymographs (Figure 3.1A and B). These kymographs here and in later sections are extended beyond the division events to more clearly bring out variations of FtsZ levels during cytokinesis. We studied cells in slow growth conditions in M9 glycerol medium with

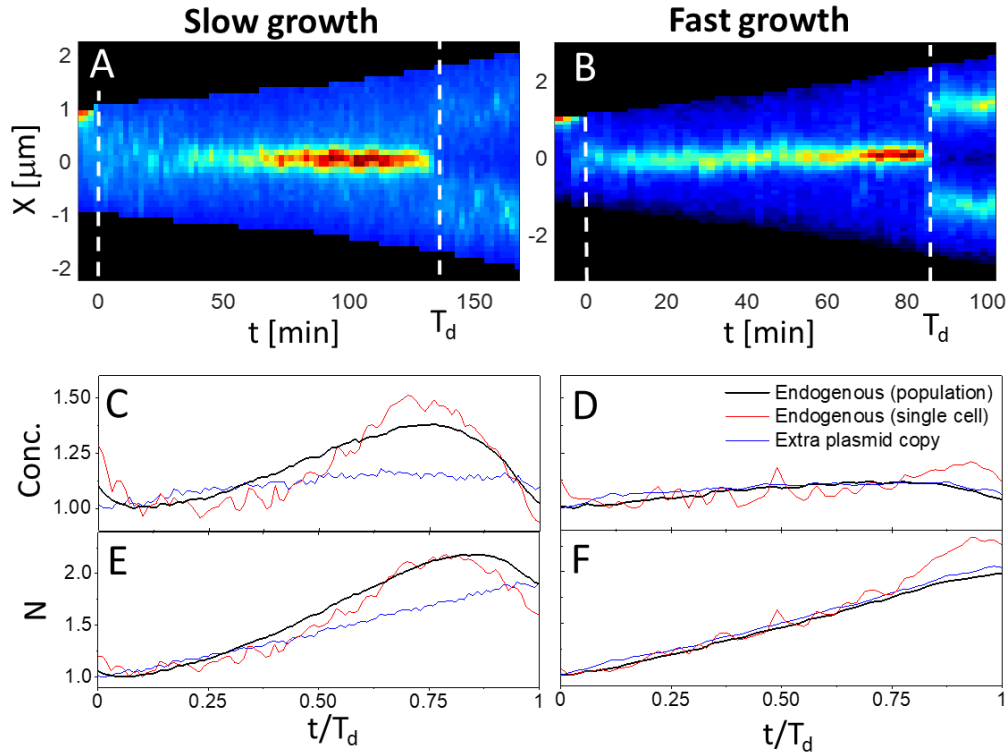


Figure 3.1: Changes in fluorescent FtsZ concentrations and numbers during the cell cycle. Left column corresponds to slow and right column to fast growth conditions. (A, B) Kymographs of FtsZ-mNG intensity distribution along the long axes in representative cells. White corresponds to high and black to low concentration. Light grey areas are outside the cell. Vertical dashed lines mark times of cell division. (C) Concentration of fluorescent FtsZ construct as a function of cell cycle time in slow growth conditions. The thick black line corresponds to the population average data from the strain expressing a sole FtsZ-mNG from the native locus (N=53), the black dotted line to the strain where FtsZ-GFP is expressed from a plasmid in addition to the native unlabeled copy (N=16), and the thin blue line to the cell shown on the kymograph above. (D) The same for fast growth conditions. N=32 for endogenous and N=33 for plasmid expressing strain. (E, F) Number of fluorescent FtsZ in the cell in slow and fast growth conditions, respectively. All curves in panels (C)-(F) are normalized by the cell cycle minimum value.

doubling times of $T_d = 160 \pm 30$ min and in faster growth with doubling times of $T_d = 74 \pm 19$ min (mean $\pm$ SD) where M9 glucose was supplemented with casamino acids. Note that all measurements were performed at 28°C. In addition to the strain carrying FtsZ-mNG, we also measured the FtsZ concentration in a control strain that expressed an FtsZ C-terminal fusion to GFP (FtsZ-GFP) under control of the *lac*-promoter from a plasmid in addition to the native unlabeled FtsZ (Figure 3.2) (83, 106). The FtsZ-GFP strain shows comparable doubling times in slow, $T_d = 160 \pm 29$ min and fast, $T_d = 80 \pm 24$ min, growth conditions. In the strain with plasmid expressed FtsZ-GFP, one would not expect to observe cell cycle-dependent variations in the concentration of the fluorescent proteins. Consistent with this expectation, the concentration of plasmid expressed fluorescent FtsZ-GFP varied less than 10% during the cell cycle in both slow (Figure 3.1C) and fast growth conditions (Figure 3.1D) in population average measurements. In contrast, we found a 37% variation of endogenously expressed fluorescent FtsZ-mNG in slow growth conditions. In fast growth conditions, however, the variation of FtsZ concentration was essentially at the level of the control (10%). The measurements thus show that FtsZ levels in *E. coli* undergo cell cycle-dependent oscillations with an amplitude that depends on the growth conditions.

In parallel to the concentration, we also determined the changes in the number of fluorescent FtsZ-mNG during the cell cycle (Figure 3.1E and F). Instead of a monotonic increase, the number of these molecules decreased at the end of the cell cycle in slow growth conditions. In some cells decrease continued in the early part of the cell cycle. The decrease started at about $0.9T_d$ when fluorescent FtsZ numbers reached a 2.2 times

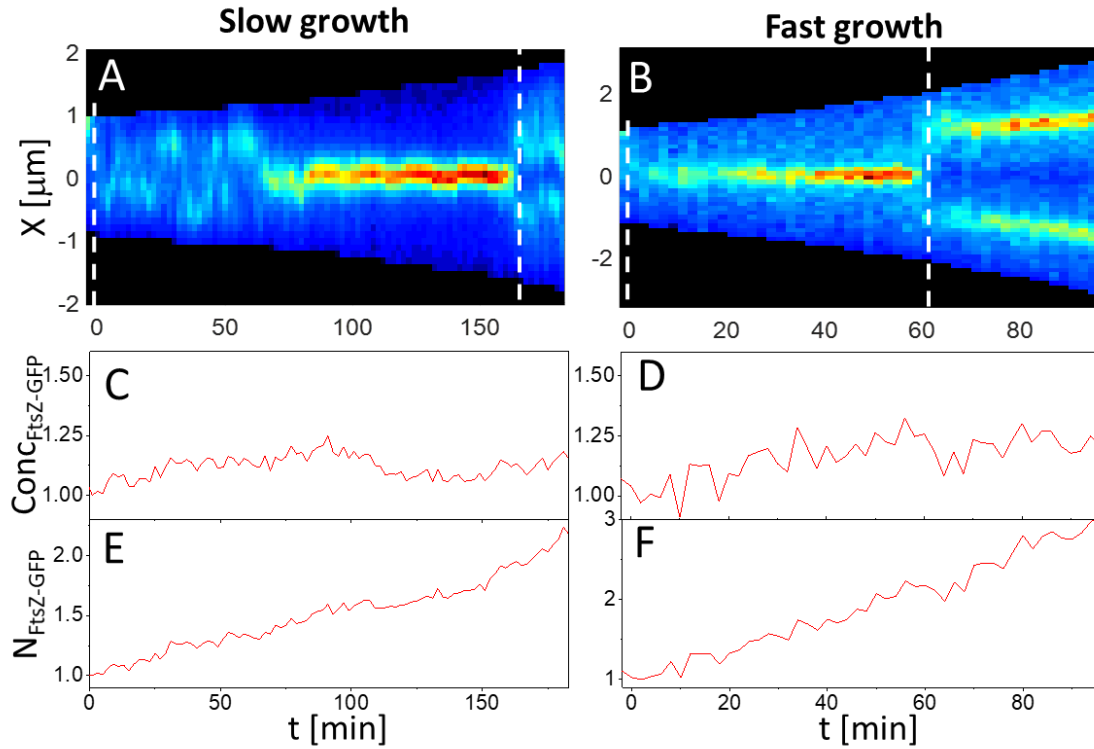


Figure 3.2: Data from a representative cell expressing FtsZ C-terminal fusion to GFP from a plasmid (strain JM60). Left column corresponds to slow and right column to fast growth conditions. (A, B) Kymographs of FtsZ-GFP intensity distribution along the long axes of the cell. Red corresponds to high concentration and blue to low. Black marks areas outside of the cell. Vertical dashed lines mark times of cell division. (C, D) Concentration of fluorescent FtsZ-GFP as a function of cell cycle time for these cells shown in kymographs above. (E, F) Numbers of fluorescent FtsZ-GFP for the cells above. All curves in panels (C)-(F) are normalized by the cell cycle minimum value.

higher level than what was present during the cell cycle minimum. The decrease was absent in fast growth conditions (Figure 3.1F) and in the control strain with plasmid expressed FtsZ in both growth conditions (Figure 3.1E–F, Figure 3.2). While a drop in the concentration of FtsZ at the end of the cell cycle could have possibly arisen from the slowing down of its synthesis at the end of the cell cycle and concurrent dilution of the protein due to cell growth, the decrease in the numbers (about 20%) shows instead that FtsZ is degraded at the end of the cell cycle. Our data furthermore show that the relative amount of FtsZ degraded at the end of the cell cycle depends on growth conditions.

3.2 Degradation at the end of the cell cycle is caused by ClpXP

We next investigated what factors could be responsible for the fast degradation of FtsZ at the end of the cell cycle. Earlier studies have identified that FtsZ is one of the substrates for ClpXP protease in *E. coli* (107, 108). The ClpXP protease consists of a hexameric ClpX chaperone that unwinds target proteins by passing them via its central pore in an ATP-dependent manner and ClpP peptidase that consists of two heptameric rings (109). The identified ClpX binding sites in FtsZ are localized at its C-terminal domain and in its unstructured linker (110). Earlier studies, however, have not revealed changes in ClpXP activity during the cell cycle (108).

To test if ClpXP is responsible for the degradation of FtsZ at the end of the cell cycle in slow growth conditions, we constructed $\Delta clpP$ and $\Delta clpX$ strains in the FtsZ-mNG background. We also constructed $\Delta clpA$ strain as a control. ClpA in a complex with ClpP acts also as a protease (111). However, ClpA, which binds and unwinds the target proteins, has so far not been shown to interact with FtsZ in *E. coli*. We first characterized

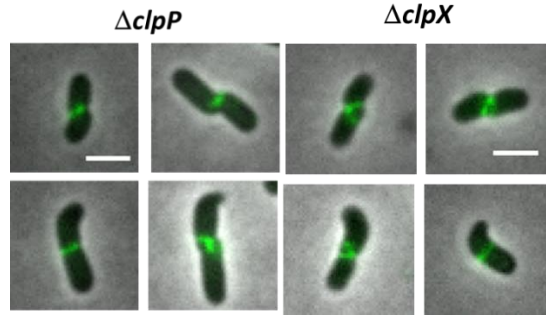


Figure 3.3: Images showing tilted and helical Z-rings (top row for each strain) and irregularly shaped cell poles. (A) $\Delta clpP$ and (B) $\Delta clpX$ cells where the images are overlays of the phase contrast with the fluorescent images. Scale bars correspond to 2 μm .

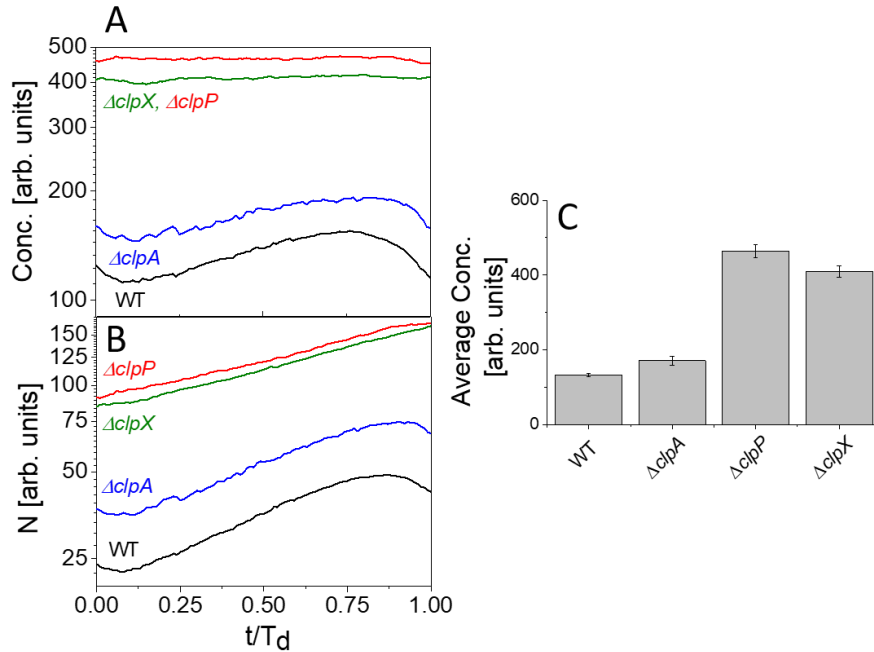


Figure 3.4: Comparison of FtsZ concentrations and numbers in WT and the protease deletion strains. (A) Population average concentration of FtsZ-mNG as a function of cell cycle time for WT (N=53), $\Delta clpA$ (N=16), $\Delta clpX$ (N=31), and $\Delta clpP$ (N=36) strains. All strains have been measured in slow growth conditions and express FtsZ-mNG from the native locus. (B) Number of fluorescent FtsZ-mNG in these strains as a function of cell cycle time. Vertical axes in panels (A) and (B) are logarithmic. (C) Concentration of fluorescent FtsZ-mNG averaged over cell cycle in different strains. Error bars are s.e.m.

growth rates and morphology of these strains in slow growth conditions (Table 2). We found that the average length of $\Delta clpA$ and $\Delta clpP$ strains ($3.1 \pm 0.7 \mu\text{m}$ and $3.6 \pm 1.0 \mu\text{m}$ respectively) were significantly longer than the average length for the strain without the deletions ($2.9 \pm 0.7 \mu\text{m}$) based on the Mann–Whitney test ($p < 0.05$) while the $\Delta clpX$ strain was shorter ($2.8 \pm 0.6 \mu\text{m}$). From here on, we will refer to the strain without deletions as the wild type (WT) notwithstanding its modified FtsZ. Noticeably, all the deletion strains divided significantly more asymmetrically about their middle than the WT cells (Ansari-Bradley test, $p < 10^{-4}$; Table 2). Moreover, the Z-rings in $\Delta clpP$ and $\Delta clpX$ cells appeared frequently tilted relative to the short axes of the cell or had a helical shape. Such constrictions resulted in irregularly shaped cell poles (Figure 3.3). These effects were not present in WT and $\Delta clpA$ cells. However, the WT cells carrying FtsZ-mNG were about 4% longer ($2.8 \pm 0.6 \mu\text{m}$) than its parent strain BW2278 with a native copy of FtsZ indicating that the sandwich fusion of mNG to FtsZ had a small but noticeable effect on the division process in slow growth conditions. This is significantly less than what has been reported for the cells growing in LB medium where these cells were found to be about 35% longer (95).

We next determined how the FtsZ numbers and concentration changed in $\Delta clpA$, $\Delta clpP$ and $\Delta clpX$ strains through the cell cycle. We found that the concentration of fluorescent FtsZ was essentially constant during the cell cycle in $\Delta clpP$ and $\Delta clpX$ strains (Figure 3.4A). There was also no apparent degradation at the end of the cell cycle, which could be inferred from the decrease in FtsZ numbers in these two strains (Figure 3.4B). At the same time, the $\Delta clpA$ control strain showed very similar dynamics as the WT strain.

We found that the cell cycle average concentrations and levels of FtsZ in $\Delta clpP$ and $\Delta clpX$ strains were about a factor of three higher than in WT (Figure 3.4C), while in the $\Delta clpA$ strain the difference was only 1.2 times. The latter was also statistically significant ($p < 10^{-6}$; t -test) indicating that ClpAP might have low activity towards FtsZ or have an effect on FtsZ expression. Much higher levels of FtsZ and the lack of its cell cycle-dependent variation in $\Delta clpP$ and $\Delta clpX$ strains provide strong evidence that ClpXP is responsible for the degradation of FtsZ at the end of the cell cycle in WT cells.

3.3 Synthesis of FtsZ during the cell cycle is not uniform

Closer inspection of data from individual $\Delta clpP$ and $\Delta clpX$ cells reveals that FtsZ numbers do not increase uniformly in time (Figure 3.5A and B) as they do in population-averaged data (cf. Figure 3.4A). The fact that the rate of change of FtsZ numbers varies over time indicates that the synthesis of FtsZ is cell cycle-dependent in slow growth conditions. In $\Delta clpP$ and $\Delta clpX$ cells, we are able to distinguish two periods in the cell cycle that have different FtsZ synthesis rates. These periods correspond to two different slopes in N vs t curves (Figure 3.5B). To find the beginning of these periods and the rate of change in FtsZ numbers in each period, we performed a piecewise linear fit to the curves from individual cells. Compilation of the fitting results shows (Figure 3.5C) a period of upregulated FtsZ synthesis during the cell cycle that starts at time $T_1 = 0.35 \pm 0.2T_d$ and runs until time $T_2 = 0.85 \pm 0.15T_d$ (mean $\pm$ SD). During this period, the synthesis rate is about three times higher than during the rest of the cell cycle. Large variations in the start and end times of this period among the cell population explains why in population-averaged data (Figure 3.4A) the period with a higher synthesis rate did not reveal itself.

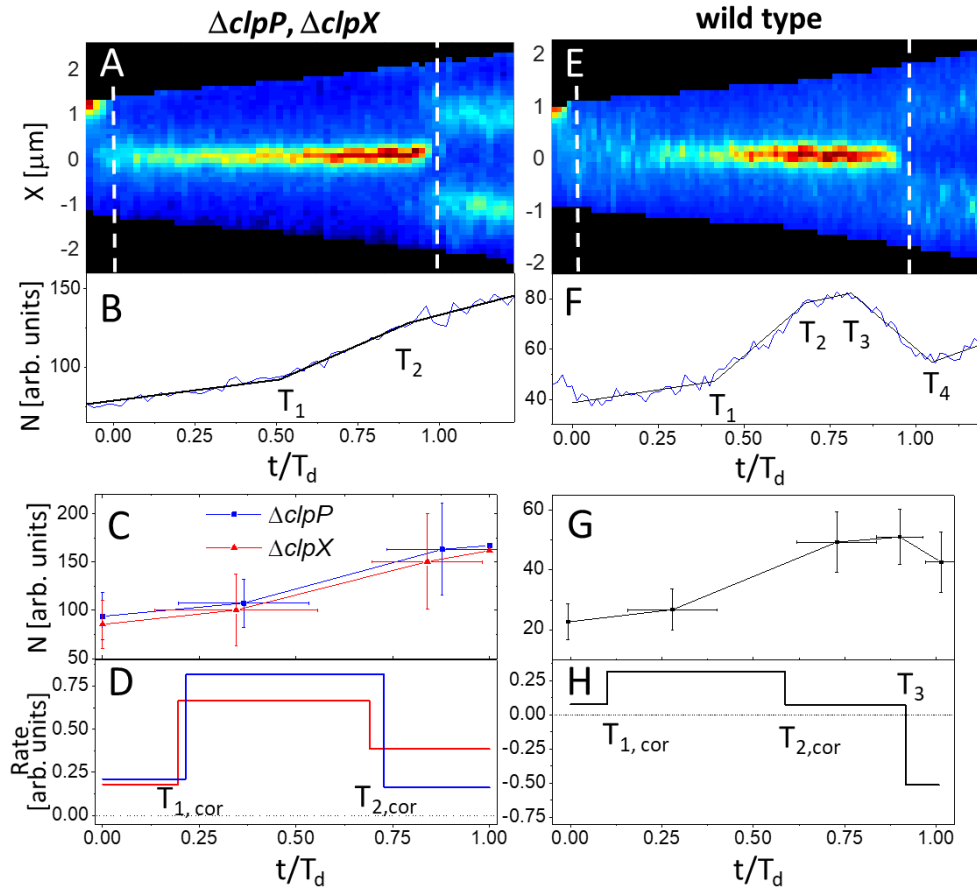


Figure 3.5: Rates of change in FtsZ numbers during the cell cycle in ClpX and ClpP null mutant (left column) and WT (right column) strains in slow growth conditions. (A, E) Kymographs of FtsZ-mNG distribution along the long axes in representative cells. Panel (A) shows a $\Delta clpP$ cell. Vertical dashed lines mark cell divisions. (B, F) Number of fluorescent FtsZ-mNG as a function of the cell cycle time for the cells shown above. Solid black lines correspond to a piecewise linear fit. T_1 - T_4 mark times when the slope of the curve changes. T_1 - upregulation of FtsZ synthesis; T_2 - slow-down of the FtsZ synthesis; T_3 - start of rapid FtsZ degradation, and T_4 - end of rapid FtsZ degradation. (C, G) The fitting curves after averaging them over cell populations. $N=35$ for $\Delta clpP$, $N=32$ for $\Delta clpX$ and $N=53$ for WT cells. Error bars are s. d. (D, H) Rates of change in FtsZ-mNG numbers. The rates correspond to the slopes of the curves from the panel above. Times $T_{1,cor}$ and $T_{2,cor}$ have been corrected to account for the maturation of mNG.

The observed times T_1 and T_2 cited above must be corrected for the maturation time of the fluorescent reporter. To make this correction, we measured the maturation time of mNG in our cells using an approach described earlier (112). Measurements of WT cells in slow and fast growth conditions, and $\Delta clpX$ cells in slow growth conditions yielded maturation half-lifetimes of 25 ± 3 min corresponding to $(0.15 \pm 0.02)T_d$ (Figure 3.6). Correcting for the maturation by subtracting the maturation half-lifetime from the corresponding times yields corrected estimates of $T_{1,cor} = (0.2 \pm 0.2)T_d$ for the start and $T_{2,cor} = (0.7 \pm 0.15)T_d$ for the end of the faster synthesis period in $\Delta clpP$ and $\Delta clpX$ cells. Figure 3.5D summarizes these results in terms of the rate of change in FtsZ numbers during the cell cycle.

As expected from measurements of $\Delta clpP$ and $\Delta clpX$ strains, FtsZ synthesis rates also varied in WT cells during the cell cycle. In addition to upregulation in the synthesis rates, N vs t curves of individual cells also had a distinct period at the end of the cell cycle corresponding to rapid degradation of FtsZ (Figure 3.5E–H). We included this period in the piecewise linear fits denoting the beginning of the period by T_3 and its end by T_4 (Figure 3.5F and G). Including correction for the maturation time of mNG, FtsZ synthesis was upregulated in WT cells from $T_{1,cor} = (0.1 \pm 0.12)T_d$ to $T_{2,cor} = (0.6 \pm 0.1)T_d$ (mean $\pm$ SD). During this period, the rate of FtsZ synthesis was about four times higher than in the beginning of the cell cycle (Figure 3.5H), which is similar to the change observed in $\Delta clpP$ and $\Delta clpX$ cells. After the T_2 point, the synthesis of FtsZ slows down. From $T_{2,cor} = (0.6 \pm 0.1)T_d$ to $T_3 = (0.90 \pm 0.04)T_d$, FtsZ synthesis rates were comparable to those in the beginning of the cell cycle, that is, from 0 to $0.1T_d$. The last period in the cell cycle from

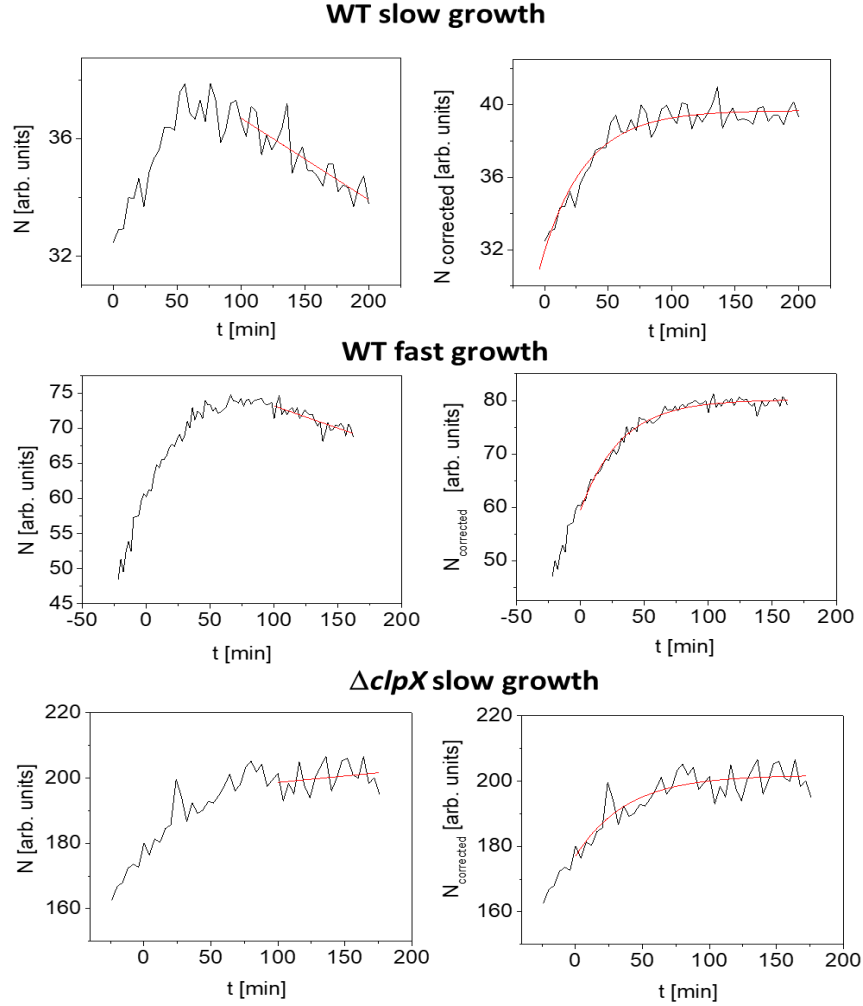


Figure 3.6: Measurements of mNeonGreen (mNG) maturation rates k_{mat} in WT cells (strain JM26) in slow (M9 glycerol medium; $N=15$), fast (M9 glucose+CAS medium; $N=18$) growth conditions and in $\Delta clpX$ strain in slow growth conditions (M9 glycerol medium; $N=10$). Left column: Raw intensity of FtsZ-mNG as a function of time. At time $t = 0$ min chloramphenicol at concentration $300 \mu\text{g ml}^{-1}$ is administered to the cells. Although protein synthesis stops, the number of fluorescent FtsZ-mNG in the cells increases because of the maturation of the fluorophore. At later times the amount of FtsZ-mNG decreases due to degradation by ClpXP. The latter effect is only apparent in WT strains. Solid red lines present linear fits for times $t > 100$ min that are used to correct for degradation during maturation period. Right column: Intensity of FtsZ-mNG as a function of time. The effect of the degradation is subtracted from the data. Red line presents fits with $N_{corrected}(t) = N_{mat} + N_{non-mat}[1 - \exp(-k_{mat}t)]$. Fitting results are collected to Table 3. The maturation half-lifetimes found here are longer than those reported by Balleza et al. who found $t_{mat,1/2}=13$ min at 32°C (112).

Table 2: Growth and division characteristics of different strains used in this study.

Strain	$L(T_0)$ mean $\pm$ std [μm]	L_{average} mean $\pm$ std [μm]	W mean $\pm$ std [μm]	T_d mean $\pm$ std [min]	$\sigma_{\text{division ratio}}$
WT	1.88 $\pm$ 0.27	2.89 $\pm$ 0.68	0.79 $\pm$ 0.05	160 $\pm$ 29	0.030
Parental	1.72 $\pm$ 0.28	2.77 $\pm$ 0.56	0.81 $\pm$ 0.05	175 $\pm$ 50	0.033
$\Delta clpA$	2.27 $\pm$ 0.53	3.13 $\pm$ 0.74	0.83 $\pm$ 0.06	175 $\pm$ 40	0.067
$\Delta clpP$	1.91 $\pm$ 0.32	3.59 $\pm$ 1.01	1.00 $\pm$ 0.06	137 $\pm$ 70	0.060
$\Delta clpX$	1.99 $\pm$ 0.52	2.82 $\pm$ 0.64	0.82 $\pm$ 0.06	141 $\pm$ 42	0.058

$L(T_0)$ is the cell length at birth, L_{average} is the population average cell length from an asynchronous cell culture, W is the cell width, T_d is doubling time and $\sigma_{\text{division ratio}}$ is the standard deviation of the distribution of division ratios. Division ratios are defined as $L_1(T_0)/(L_1(T_0)+L_2(T_0))$ and $L_2(T_0)/(L_1(T_0)+L_2(T_0))$ where subscripts 1 and 2 refer to two daughter cells that resulted from the same division. Note that two ratios from the same division contribute to each distribution so that the resulting distribution is symmetric relative to 0.5. L_{average} and W have been measured from still images of asynchronous log-phase cultures spread on agarose pads while $L(T_0)$ and T_d are from time-lapse measurements from microfluidic chips. All data are pertinent to slow growth conditions.

Table 3: Fitting results for maturation rates.

Strain and medium	k_{mat} [min^{-1}]	$t_{\text{mat},1/2}$ [min]
WT M9 glycerol	0.029	24
WT M9 glucose + CAS	0.031	22
$\Delta clpX$ in M9 glycerol	0.026	27

$T_3 = (0.9 \pm 0.04)T_d$ to $T_4 = (1.0 \pm 0.05)T_d$ showed a rapid decrease in FtsZ numbers. This period is clearly visible also in the population average curves of Figure 3.1E. Since the effect of degradation of the protein is immediate on the fluorescence, no corrections were applied to the latter two times. The end of the degradation period coincided in a good approximation with the end of the cell cycle. Figure 3.5H summarizes the WT results in terms of the rate of change in FtsZ numbers during the cell cycle.

It is noticeable that while the beginning (T_1) and end (T_2) of FtsZ synthesis upregulation showed substantial cell-to-cell variability, the beginning (T_3) and end (T_4) of the fast degradation of FtsZ showed less stochasticity (Figure 3.5G). This indicates that different types of regulation are involved in controlling FtsZ synthesis and degradation. While synthesis is likely controlled at the transcriptional level and is known to have a high level of stochasticity (113), the same appears not to be the case for degradation.

We also attempted the same analysis for WT cells in fast growth conditions. Since degradation of FtsZ was not visible in most of the individual N vs t curves, only periods of slow and fast FtsZ synthesis were determined. The analysis indicated an approximately twofold increase in the FtsZ synthesis rate from $T_{1,\text{cor}} = 0$ to $T_{2,\text{cor}} = 0.5T_d$ compared to the remainder of the cell cycle (Figure 3.7). This difference is statistically significant by the t -test ($p < 10^{-5}$ for equal slopes). However, given that the maturation time of the fluorescent reporter in these growth conditions is about a third of the doubling time, our analysis can significantly underestimate differences in the rates, and as such is not directly comparable to the values from slow growth conditions.

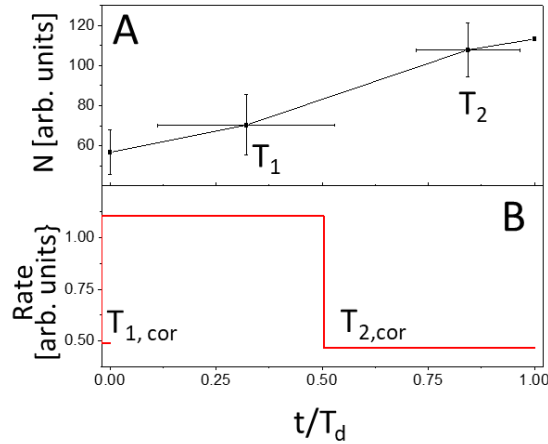


Figure 3.7: Variation of the synthesis and degradation rates during the cell cycle of WT cells in fast growth conditions (M9 medium with glucose+CAS). (A) Number of fluorescent FtsZ-mNG as a function of cell cycle time. The number is expressed in fluorescent units. The data has been compiled from piecewise linear fittings of $N(t)$ curves from individual cells ($N=29$). Error bars are standard deviation. (B) Rates of change for FtsZ-mNG as a function of cell cycle time. The times $T_{1,cor}$ and $T_{2,cor}$ are corrected for fluorophore maturation.

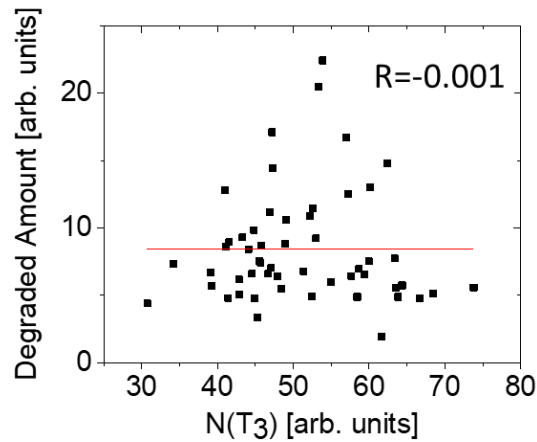


Figure 3.8: Degraded amount of FtsZ at the end of the cell cycle vs. number of FtsZ present when fast degradation starts at time T_3 . WT strain in slow growth conditions (strain JM26; M9 medium with glycerol; $N=53$). Solid red line is a linear fit to the data. Pearson correlation coefficient from the fit $R=-0.001$ shows that the degraded amount is independent of the amount of FtsZ present at the beginning of the fast degradation period.

3.4 Quantification of degradation rates in slow growth conditions

Using data from piecewise linear fits to N vs t curves from individual cells we next quantified the distribution of relative amounts of FtsZ degraded at the end of the cell cycle. We found a broad distribution of degraded amounts, with an average of $22 \pm 14\%$ (Figure 3.9A). This amount was calculated relative to the number of FtsZ at end of the cell cycle. There appears to be no obvious correlation between the FtsZ amount at the end of the division and the amount of FtsZ degraded (Figure 3.8). On average, about the same number of FtsZ molecules are degraded in cells where FtsZ amounts differ by a factor of two. The constant amount of FtsZ degraded can imply that the rate-limiting step in the degradation is not the binding of ClpXP to FtsZ but the time it takes to degrade the protein. Based on single molecule measurements (114), the typical time it takes to degrade a single FtsZ-mNG molecule is about 10 s. It is likely that this time exceeds the time needed for ClpXP and FtsZ to bind to each other.

In addition to degraded amount, we also determined the time-averaged degradation rates (d_t) at the end of the cell cycle (Figure 3.9B). For the cell population, we found the rate $d_t = 0.010 \pm 0.05 \text{ min}^{-1}$. This rate is about a factor of two larger than the one found in bulk measurements from unsynchronized *E. coli* cultures (108). However, this difference should not be over-interpreted because d_t depends on the protease concentration in the cell. The latter is likely to vary from strain to strain and from one growth condition to another.

We also determined the degradation rate of FtsZ during the remainder of the cell cycle. To this end, we inhibited protein synthesis with chloramphenicol and determined

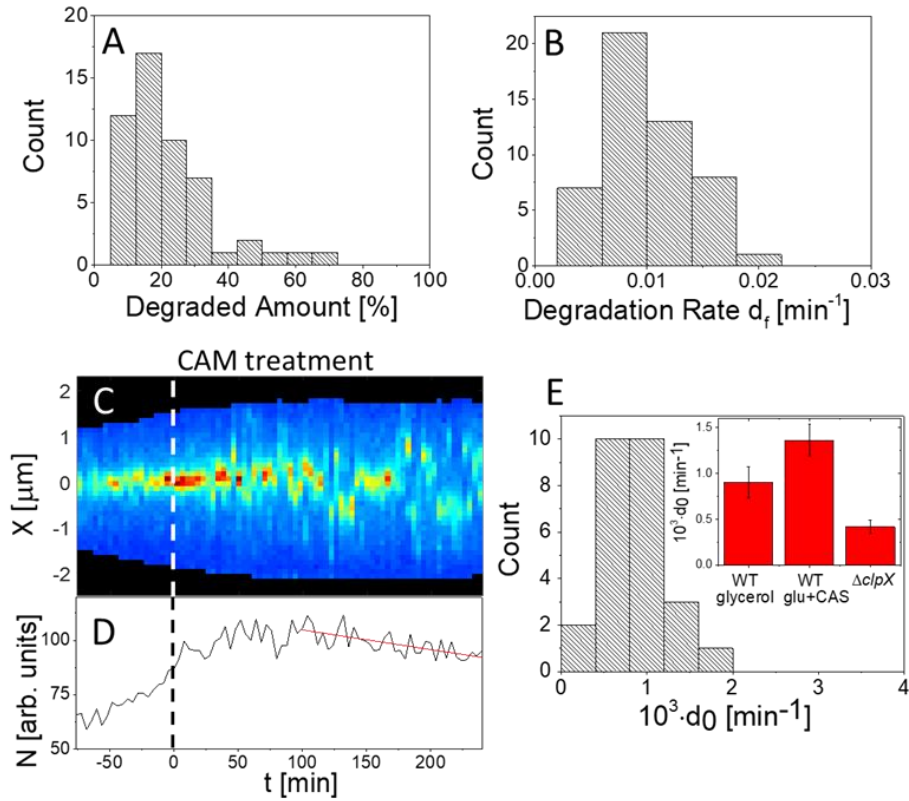


Figure 3.9: Degradation rates of FtsZ in the beginning and end of the cell cycle. (A) Degraded amount of FtsZ in the end of the cell cycle at the time when FtsZ disassociates from the Z-ring. The amount is relative to the amount at the end of cell division. (B) Degradation rate during this period. N=53. (C) Kymograph showing FtsZ-mNG distribution along the long axes of the cell before and during chloramphenicol treatment (CAM, 300 $\mu\text{g}/\text{ml}$). The drug is administered at 0 min (vertical dashed line). (D) Amount of fluorescent FtsZ-mNG in this cell as a function of time. Basal degradation rate, d_0 , is determined from the linear fit to the curve (red solid line). (E) Distribution of d_0 in WT cell population in slow growth conditions (N=26). Inset shows the population average basal rate for WT cells in slow and fast growth conditions (N=20) and for ΔclpX cells in slow growth conditions (N=10). The latter acts as a control. Error bars are s. d.

the decay rate in the fluorescent signal of FtsZ-mNG (Figure 3.9C and D). As described earlier, the fluorescent signal increases immediately after the inhibition of protein synthesis because of maturation of the fluorescent label (112). To avoid this effect, we determined the degradation rates after four mNG maturation half-lifetimes (100 min) from administering the drug (Figure 3.9D). We found the average basal degradation rate $d_0 = (0.9 \pm 0.4) \cdot 10^{-3} \text{ min}^{-1}$ in slow and $d_0 = (1.4 \pm 0.8) \cdot 10^{-3} \text{ min}^{-1}$ in fast growth conditions (Figure 3.9E). For the $\Delta clpX$ strain, the degradation rate constant was more than two times smaller, $d_0 = (0.4 \pm 0.2) 10^{-3} \text{ min}^{-1}$. The apparent degradation rate of the $\Delta clpX$ strain may reflect bleaching of mNG during the measurement or degradation of FtsZ by some other proteases. Subtracting values measured in the $\Delta clpX$ strain from the WT one yields a ClpXP-related degradation rate constant of $d_0 = 5 \cdot 10^{-4} \text{ min}^{-1}$ in the WT cells. There is thus a 20-fold increase in ClpXP-related degradation of FtsZ at the end of the cell cycle compared to the basal rate.

3.5 Rapid degradation of FtsZ by ClpXP begins when the Z-ring starts to dissociate

An order of magnitude increase in FtsZ degradation rate by ClpXP in the end of division invites the question, what triggers such a significant increase. To gain further insight, we compared times when degradation of FtsZ (T_3) starts to the times when FtsZ begins to dissociate from the Z-ring (T_{dis}). We defined the latter as a time during the cell cycle when the total amount of FtsZ in the Z-ring starts to decrease (Figure 3.10A). The comparison shows that the two times are highly correlated within the cell population ($R = 0.95$) and essentially co-incident (Figure 3.10B). The data also show that the total time it takes to

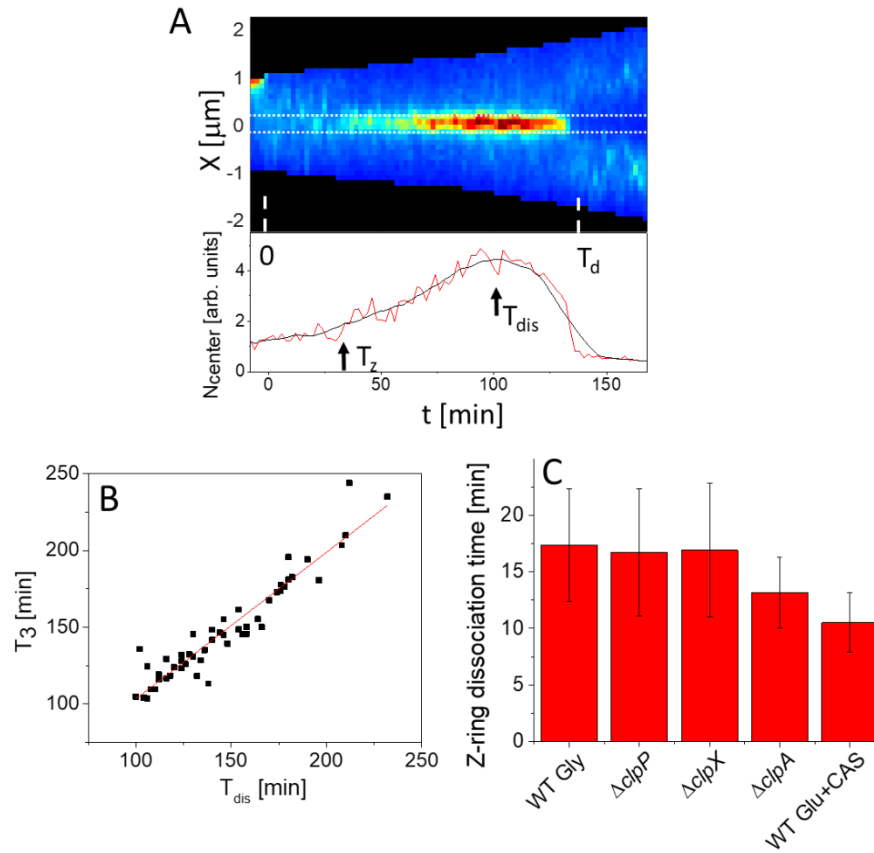


Figure 3.10: Rapid degradation of FtsZ is a consequence of the Z-ring dissociation. (A) Kymograph (top) and amount of fluorescent FtsZ-mNG at the cell center (bottom) in a representative cell. The cell center is defined as 300 nm wide band (indicated by horizontal dotted lines on the kymograph). Fluorescence intensity after averaging by a running window is shown by a black line. T_z indicates start time and T_{dis} time when the Z-ring starts to dissociate. (B) Start time for FtsZ dissociation from constriction region (T_{dis}) vs start time of rapid degradation of FtsZ (T_3). (C) Total time for FtsZ to dissociate from the Z-ring ($T_d - T_{dis}$) in different strains and growth conditions. Error bars are s. d.

dissociate FtsZ from the Z-ring does not depend whether ClpXP is present or absent (Figure 3.10C). These data together indicate that FtsZ degradation does not drive the Z-ring dissociation but can be a consequence of it.

It can be expected that the concentration of FtsZ monomers in the cytosol increases when FtsZ dissociates from the Z-ring. To determine if an increase in the numbers of monomeric FtsZ can explain its rapid degradation at the end of the cell cycle, we induced dissociation of the Z-ring to monomeric FtsZ. For this purpose, we transformed the FtsZ-mNG strain with the pA3 plasmid carrying an inducible Sula (101). Sula binding to FtsZ lowers the effective concentration of FtsZ in the cell so that it is below the critical concentration for protofilament formation (101). Sula binding to FtsZ should leave the latter still accessible for ClpXP degradation because Sula binds the T7 loop of FtsZ (115) instead of its C-terminal domain where the recognition elements for ClpX binding are (108, 110). Sula upregulation and subsequent dissociation of the Z-ring, however, did not lead to any detectable degradation of FtsZ (Figure 3.11A and B). Moreover, Sula upregulation did not change the synthesis rate of FtsZ. In cells where Sula was upregulated in the early stages of the cell cycle, FtsZ synthesis followed its well-defined pattern (cf. Figure 3.5F) despite Sula expression (Figure 3.11C and D). Consequently, an increase in the concentration of cytosolic FtsZ monomers during Z-ring dissociation appears not to be the reason for its rapid degradation at the end of the cell cycle, although it remains a possibility that Sula binding affects the FtsZ degradation rate.

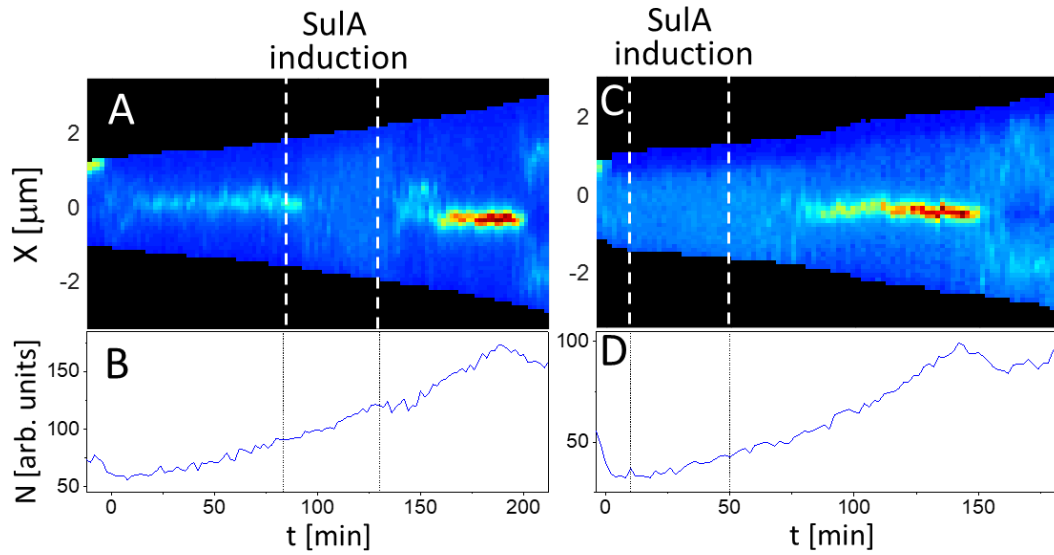


Figure 3.11: FtsZ-mNG distribution during SulA induction. (A) Kymograph for FtsZ-mNG distribution in a WT cell during SulA induction, which occurs in the middle of the cell cycle and is indicated by vertical dashed lines. (B) Corresponding increase in total amount of fluorescent FtsZ-mNG. Time zero corresponds to cell birth. (C, D) The same data for another cell. For this cell SulA induction occurs at the beginning of the cell cycle.

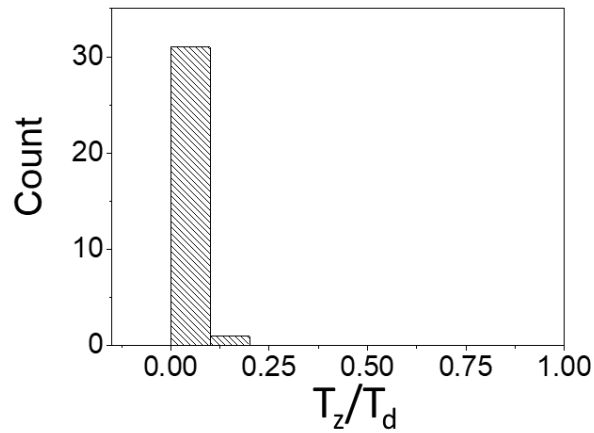


Figure 3.12: Distribution of times when the Z-ring forms (T_z) in WT cells (strain JM26) in fast growth conditions (M9 medium with glucose and casamino acids). $T_z = (0.01 \pm 0.03)T_d$ (mean $\pm$ s.d.). $N=32$. In 2 out of 32 cells formation of the Z-rings in the daughter cells occurs after cell birth.

3.6 Correlation between FtsZ upregulation and Z-ring formation

Previously presented data (Figure 3.5E) show that the FtsZ synthesis rate increases four-fold shortly after cell birth at $0.1T_d$ in slow growth conditions. We next investigated to what degree this upregulation correlates with the Z-ring formation. To answer this question, we determined from kymographs, such as shown in Figure 3.10A, the time when the Z-ring forms (T_z). Note that in fast growth conditions, in almost all WT cells (94%), the Z-ring formed immediately after cell birth (Figure 3.12). However, in slow growth conditions, there was a distinct delay between cell birth and the formation of the Z-ring (Figure 3.13A). On average, the Z-rings formed at $T_z = 0.22T_d$ but the times varied considerably between individual cells ($SD = 0.13T_d$). Similarly, there was a wide distribution in the start times of FtsZ synthesis upregulation ($SD = 0.13T_d$, Figure 3.13B). However, these latter times preceded on average about $0.1T_d$ (16 min) the times of the Z-ring formation. At the level of individual cells, the two times showed some level of correlation ($R = 0.67$; Figure 3.13C). Noticeably, in essentially all cells, the Z-ring formed after the FtsZ synthesis rate had increased. We observed qualitatively a similar behavior also in $\Delta c/pA$ cells (Figure 3.14). Taken together, these findings are consistent with an idea that upregulation of FtsZ levels may be needed for the Z-ring formation in slow growth conditions.

Consistent with the hypothesis that the FtsZ level affects the timing of the Z-ring formation, we found that the Z-rings had a tendency to form earlier in WT cells where the amount of FtsZ was higher at cell birth ($R = -0.43$; Figure 3.13D). At the same time, the correlation between the concentration of FtsZ at cell birth and timing of the Z-ring formation were low ($R = -0.15$; Figure 3.13E), indicating that FtsZ numbers rather than

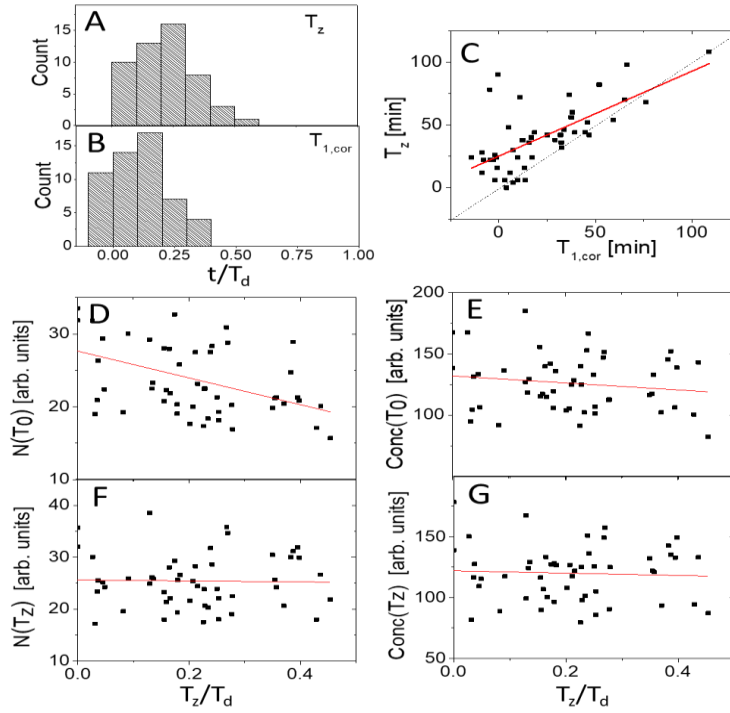


Figure 3.13: Link between upregulation of FtsZ synthesis and formation of the Z-ring in slow growth conditions. (A) Distribution of times when the Z-ring forms (T_z). (B) Distribution of times when the FtsZ synthesis rate increases during the cell cycle ($T_{1,cor}$). The times are corrected for mNG maturation time. (C) T_z vs $T_{1,cor}$ for the same dataset. The dashed black corresponds to $T_z = T_{1,cor}$ and the solid line to linear fit ($T_z = -0.67T_{1,cor} + 25.2$; $R = -0.67$). (D) Number of fluorescent FtsZ-mNG and (E) its concentration at cell birth (T_0) vs. time when Z-ring forms. (F) Number of fluorescent FtsZ-mNG and (G) its concentration at the time of Z-ring formation vs. the time when Z-ring forms. Solid lines are fits to the data. The fitting parameters are listed in Table 4. $N = 53$.

Table 4: Fitting parameters for linear fits for panels D-G in Figure 3.13.

Measurement	a	b	R	CV [%]
$N(T_0)$	-18	28	-0.42	22
$N(T_z)$	-0.9	26	-0.02	20
$Conc(T_0)$	-29	132	-0.15	18
$Conc(T_z)$	-10	122	-0.05	19

a is slope and b intercept of the fitting line ($y = ax + b$). R is the Pearson correlation coefficient of the data. Coefficient of variation (CV) is calculated based on the number and concentration values at a given time point.

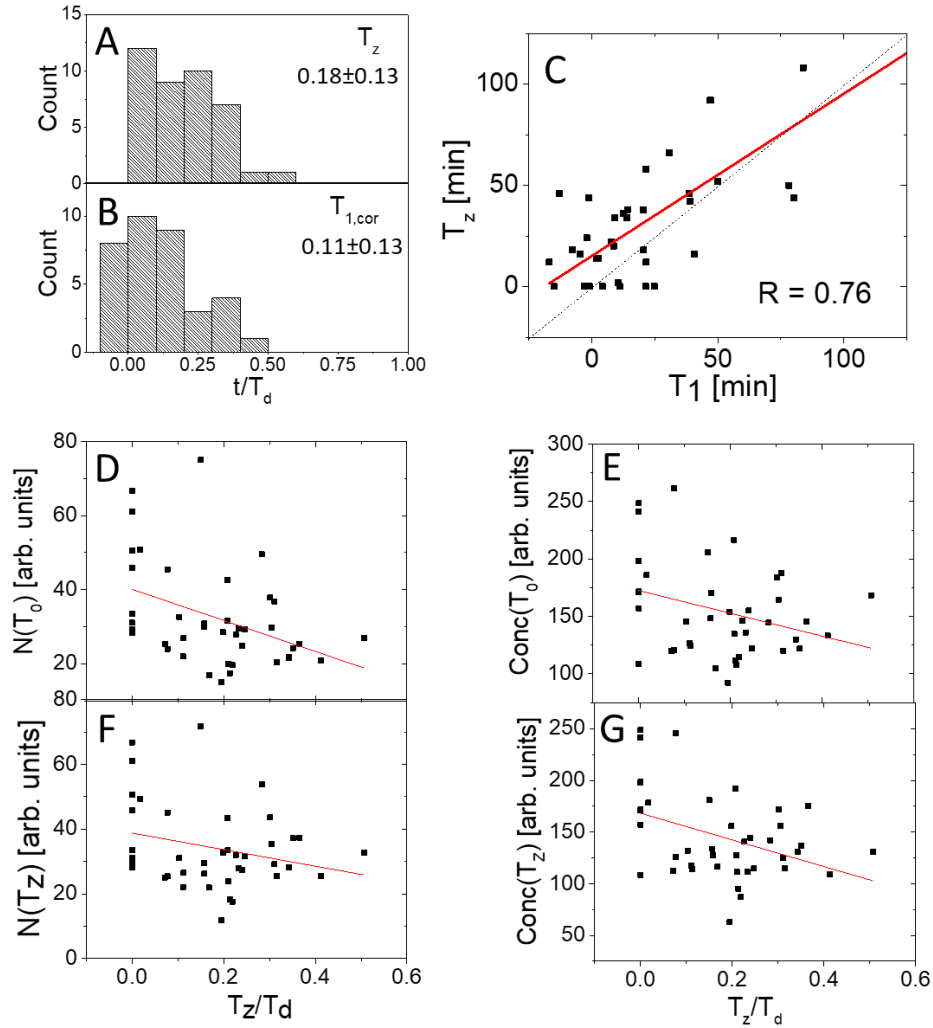


Figure 3.14: Correlations between upregulation of FtsZ synthesis and formation of the Z-ring in slow growth conditions in $\Delta clpA$ strain. (A) Distribution of times when Z-ring forms (T_z). The numbers in the panel show mean $\pm$ s.d. of the distribution. (B) Distribution of times when FtsZ synthesis rate increases during the cell cycle ($T_{1,cor}$). The times are corrected for mNG maturation time. (C) T_z vs $T_{1,cor}$ for the same dataset. Dashed black line corresponds to $T_z = T_{1,cor}$. Solid red line is a linear fit to the data. Correlation coefficient for the fit is $R=0.76$. (D) Number of fluorescent FtsZ-mNG and (E) its concentration at cell birth (T_0) vs. time when Z-ring forms. (F) Number of fluorescent FtsZ-mNG and (G) its concentration at the time of Z-ring formation vs. time when the Z-ring forms. Solid lines are fits to the data. The fitting parameters are listed in Table 5. $N=40$.

its concentration may be the factor controlling the Z-ring formation as has been hypothesized before (116). At the time when the Z-ring formed, the amount of FtsZ in the cells (Figure 3.13F) and its concentration (Figure 3.13G) were about the same and independent of the time the Z-ring formed ($R = -0.02$; $R = -0.05$ respectively) as one would expect for a control parameter. However, the variation of both the amount of FtsZ and its concentration at the time of the Z-ring formation were large in a cell population with a coefficient of variation of 20%. A large variation shows that the FtsZ number as a control parameter for the Z-ring formation is not a stringent one, allowing for the Z-ring to initiate in a wide range of FtsZ levels.

In $\Delta clpA$, $\Delta clpP$ and $\Delta clpX$ cells, where the levels of FtsZ were higher than in WT cells, the formation of the Z-ring started earlier (Figure 3.15). There was a clear negative correlation between the population average time when the Z-ring formed and the population average amount of FtsZ in the cells ($R = -0.98$). However, these correlations were much weaker within cell populations belonging to individual strains. Despite high levels of FtsZ in about 35% of $\Delta clpX$ and in 15% of $\Delta clpP$ cells, Z-ring formation did not start immediately at cell birth but after some delay (Figure 3.16 and 35). Collectively, these observations indicate that while the level of FtsZ affects the timing of the Z-ring formation, it is not the sole factor to do so.

3.7 Discussion

While the current view holds that FtsZ concentration in *E. coli* during its cell cycle is constant (9, 116, 117), our data show significant variations in FtsZ levels in slow growth conditions. We determined that regulatory mechanisms controlling FtsZ levels in slow

Table 5: Fitting parameters for deletion strains.

Deleted Protein	Measurement	a	b	R	CV [%]
$\Delta clpA$	$N(T_0)$	-42	40	-0.40	0.42
	$N(T_z)$	-25	39	-0.26	0.38
	$Conc(T_0)$	-100	170	-0.32	0.26
	$Conc(T_z)$	-130	170	-0.40	0.29
$\Delta clpP$	$N(T_0)$	11	91	0.01	0.24
	$N(T_z)$	174	95	0.20	0.26
	$Conc(T_0)$	124	460	0.03	0.22
	$Conc(T_z)$	470	370	0.10	0.21
$\Delta clpX$	$N(T_0)$	-49	88	-0.24	0.29
	$N(T_z)$	-36	88	-0.15	0.33
	$Conc(T_0)$	89	400	0.12	0.22
	$Conc(T_z)$	-43	400	-0.05	0.25

Linear fits as $y = ax + b$. R is the Pearson correlation coefficient of the data. Coefficient of variation (CV) is calculated based on the number and concentration values at a given time point.

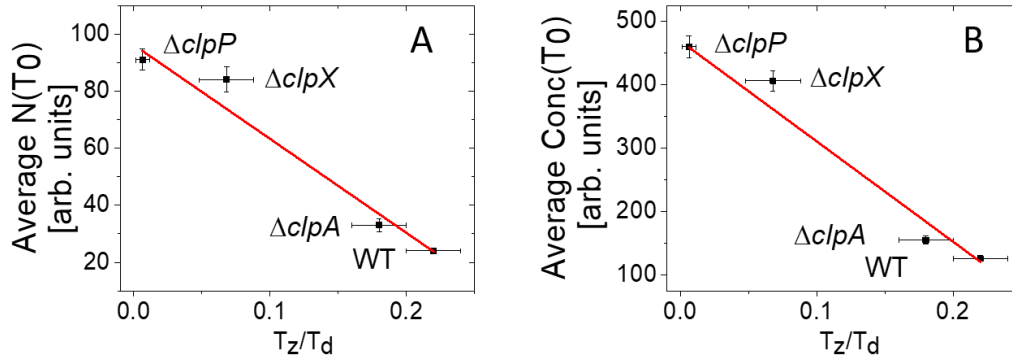


Figure 3.15: Correlation between FtsZ levels and timing of Z-ring formation in different strains. (A) Number of fluorescent FtsZ-mNG at cell birth vs. the time of Z-ring formation. Both quantities are averaged over the cell population in a given strain. $N=51$ for WT, $N=40$ for $\Delta clpA$, $N=35$ for $\Delta clpP$, and $N=31$ for $\Delta clpX$ strains. (B) Concentration of fluorescent FtsZ-mNG at cell birth averaged over cell population vs. the average time of Z-ring formation. The solid lines are linear fit to the data. All error bars are s.e.m.

growth conditions includes a faster FtsZ synthesis rate in the first half and its rapid degradation in the last 10% of the cell cycle.

3.7.1 Comparison of model to experimental data

The progression of the cell cycle events is quantitatively summarized in Figure 3.18A and B. To further validate this model, we compared it to numerically calculated FtsZ numbers and concentrations. The numerical calculation is based on experimentally determined rates including the maturation rate of mNG. The only unknown parameter in these calculations is the conversion factor between one recorded fluorescent unit and the emission from a single FtsZ-mNG. Although we have estimated this conversion factor from photobleaching measurements, the uncertainties involved are large. Therefore, we compare the experimental data and the calculated curves after normalizing both by their maximum values. The normalized model and the population average data show a good agreement (Figure 3.18C and D). The two deviate from each other most significantly at time T_3 when the degradation rate of FtsZ increases. The sharp cusp in the model is not present in the population average data partially because of the spread of degradation start times in the population. It is also plausible that part of the discrepancy is caused by a gradual increase in the degradation rate around time T_3 instead of a discontinuous change that we assumed in our analysis and model. Similar to the degradation rate, one would also expect that the synthesis rate in individual cells would show more complicated behavior than a discontinuous change between constant levels at times T_1 and T_2 . It is well-known that at the level of individual cells protein synthesis occurs in bursts (118). These details go beyond our simplified model (Figure 3.18A and B), which captures a

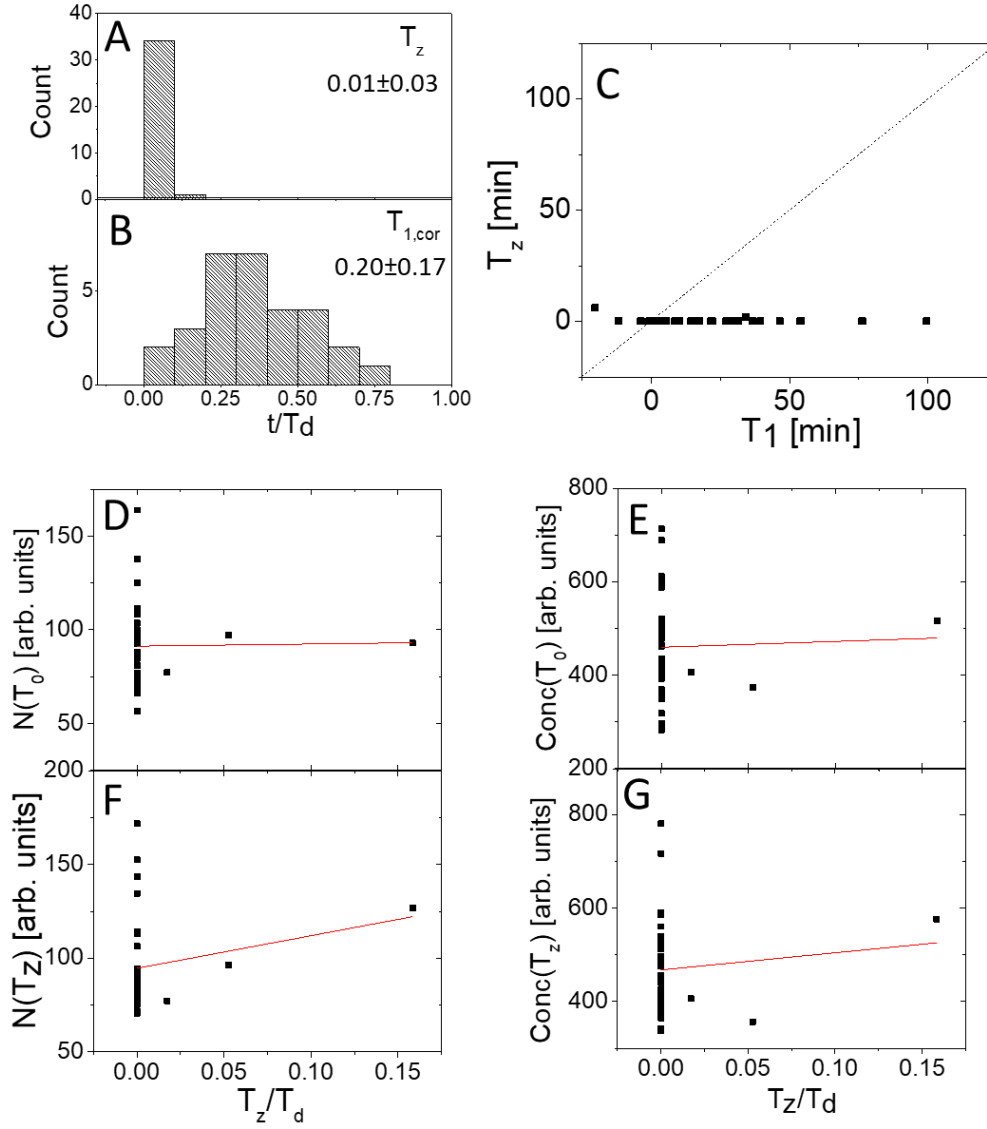


Figure 3.16: Correlations between upregulation of FtsZ synthesis and formation of the Z-ring in slow growth conditions in the $\Delta c/pP$ strain. (A) Distribution of times when the Z-ring forms (T_z). The numbers in the panel show mean $\pm$ s.d. of the distribution. (B) Distribution of times when FtsZ synthesis rate increases during the cell cycle ($T_{1,cor}$). The times are corrected for mNG maturation time. (C) T_z vs $T_{1,cor}$ for the same dataset. Dashed black line corresponds to $T_z = T_{1,cor}$. (D) Number of fluorescent FtsZ-mNG and (E) its concentration at cell birth (T_0) vs. time when the Z-ring forms. (F) Number of fluorescent FtsZ-mNG and (G) its concentration at the time of Z-ring formation vs. time when the Z-ring forms. Solid lines are fits to the data. The fitting parameters are listed in Table 5. $N=35$.

population-average behavior on a coarse temporal resolution.

3.7.2 Similar FtsZ regulation cycles in other bacteria

The FtsZ regulation cycle in slow-growing *E. coli* shows close resemblance to the one in *Caulobacter crescentus* swarmer cells. In *Caulobacter* swarmers, FtsZ synthesis is upregulated after transformation of a swarmer to a stalked cell occurs, and it is downregulated in the beginning and end of the cell cycle (119). Also, FtsZ is degraded at the end of the cell cycle (119, 120), as we observed in slow growing *E. coli*. In both organisms, the degradation occurs via ClpXP based proteolysis, but in the case of *Caulobacter*, ClpAP appears also to play a significant role (120). In our measurements, the effect of ClpAP was modest (Figure 3.5).

It was found that the upregulation of FtsZ synthesis in swarmer cells coincides with the DNA replication period (119). Interestingly, we observe a fourfold upregulation of FtsZ synthesis in slow growing *E. coli* from $0.1T_d$ to $0.6T_d$. This period matches well with the period ($0.14\text{--}0.51T_d$) reported for DNA replication in similar growth conditions (121). Increase in FtsZ mRNA levels upon initiation of DNA replication has been reported before in *E. coli* (122, 123). However, other studies have found maximal transcription levels around the middle of the cell cycle (124) or at the time of division (125). While these different measurements yet lack consensus to support a causative link between FtsZ upregulation and the initiation of DNA replication in *E. coli*, a molecular level link between the two processes has been found in *C. crescentus* (119). This link is realized by the global cell cycle regulator CtrA that represses both the initiation of DNA replication and transcription of FtsZ (126).

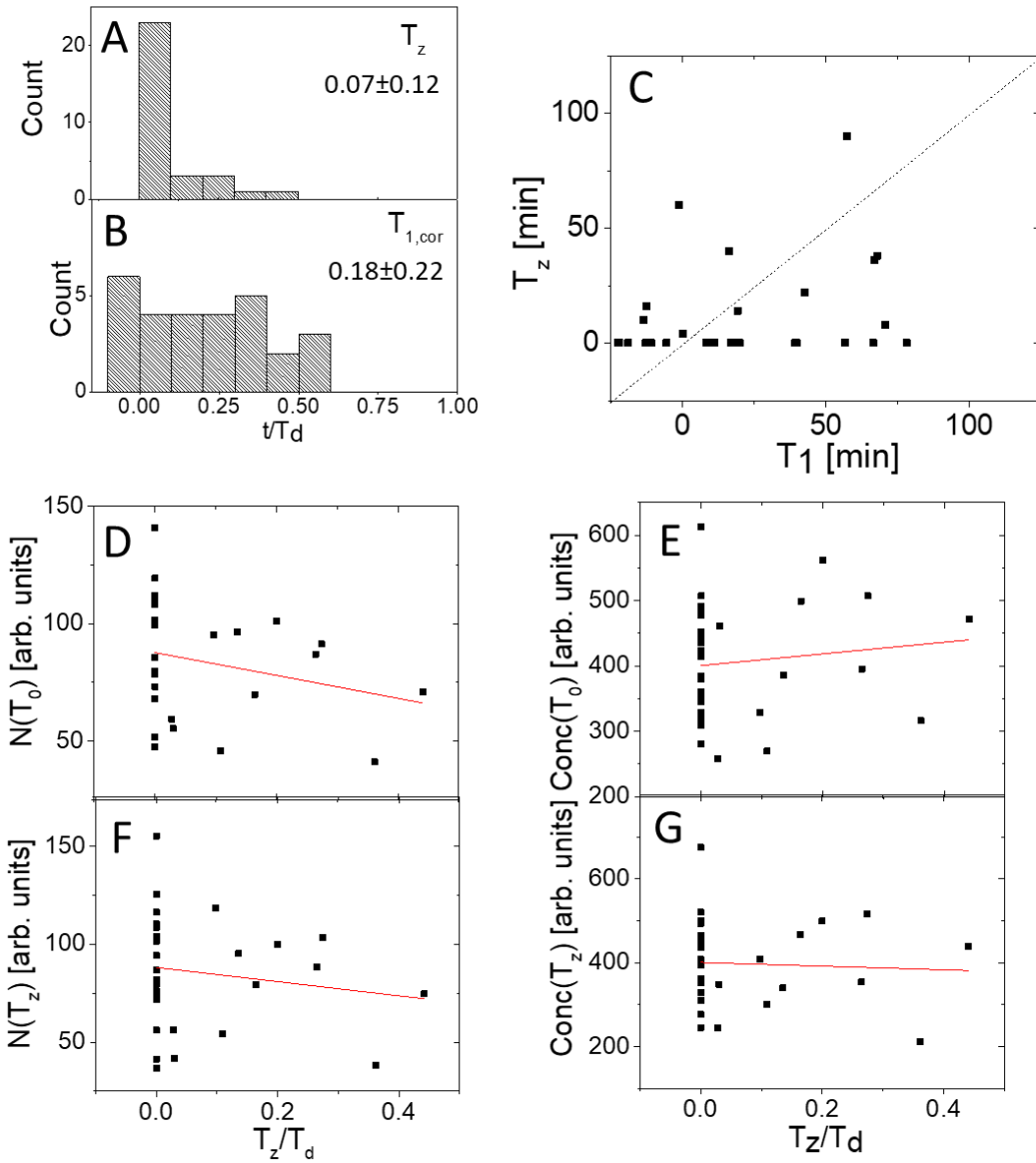


Figure 3.17: Correlations between upregulation of FtsZ synthesis and formation of the Z-ring in slow growth conditions in the $\Delta c/pX$ strain. (A) Distribution of times when the Z-ring forms (T_z). The numbers in the panel show mean $\pm$ s.d. of the distribution. (B) Distribution of times when FtsZ synthesis rate increases during the cell cycle ($T_{1,cor}$). The times are corrected for mNG maturation time. (C) T_z vs $T_{1,cor}$ for the same dataset. Dashed black line corresponds to $T_z = T_{1,cor}$. (D) Number of fluorescent FtsZ-mNG and (E) its concentration at cell birth (T_0) vs. time when the Z-ring forms. (F) Number of fluorescent FtsZ-mNG and (G) its concentration at the time of Z-ring formation vs. time when the Z-ring forms. Solid lines are fits to the data. The fitting parameters are listed in Table 5. $N=31$.

Although FtsZ regulation during the cell cycle is similar in *E. coli* and *C. crescentus* swarmer cells, the underlying molecular networks appear different. First, no global cell cycle regulator similar to CtrA is known to exist in *E. coli*. Second, in *Caulobacter*, there is only a single promoter controlling FtsZ transcription (119), while in *E. coli* seven different promoters are responsible for transcription of FtsZ (127). Of the seven, the two proximal ones have been reported to lead to cell cycle-dependent transcription of FtsZ (122). In addition to the promoters, other regulatory elements such as RNA E cleavage sites, antisense RNA binding to *ftsZ* mRNA and competition between ribosome binding sites on long polycistronic RNA could also play a role (127). Further work to understand the cell cycle control on the transcription of FtsZ in *E. coli* is clearly warranted after nearly a two-decade-long pause in studies.

3.7.3 How does ClpXP rapidly degrade FtsZ during the end of the cell cycle?

In addition to the mechanisms controlling FtsZ synthesis, our work also raises the question about how ClpXP is able to rapidly degrade FtsZ at the end of the cell cycle. The data are consistent with the hypothesis that the dissociation of the Z-ring triggers the degradation of FtsZ. After the dissociation from the Z-ring, FtsZ may lose specific protective interactions on its conserved C-terminal domain which has one of the two recognition sites for ClpX (108, 110). It has been reported before that one of the FtsZ membrane linkers, ZipA, is able to protect FtsZ from degradation by ClpXP (128). Furthermore, it is possible that steric hindrance from the inner membrane reduces accessibility of ClpXP to the C-terminal tail of FtsZ and therefore protects it from degradation. In addition to the FtsZ residing in the Z-ring, our data also show that

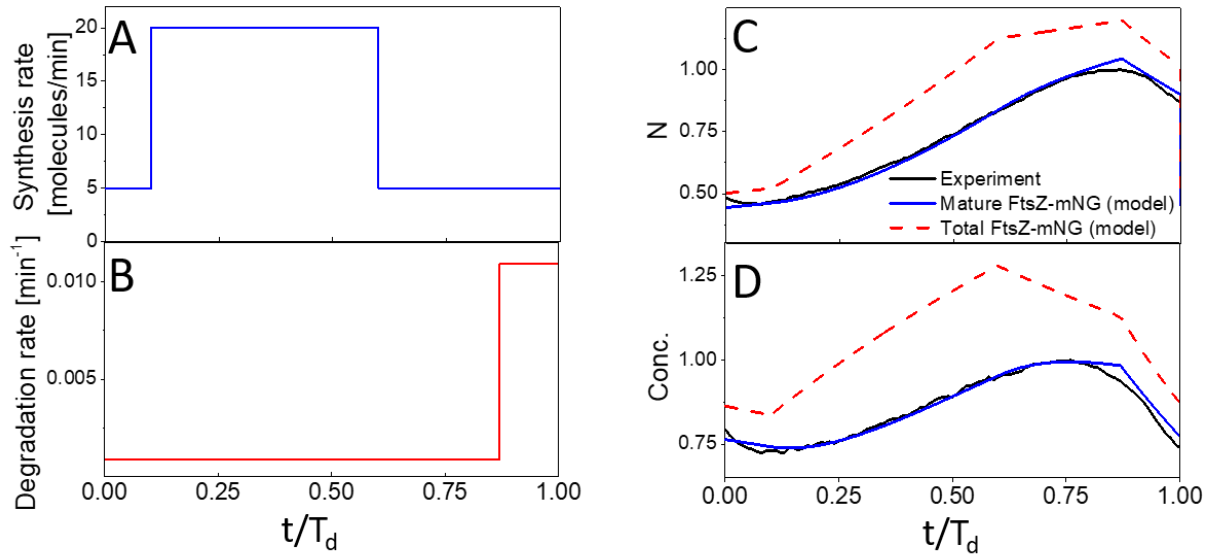


Figure 3.18: Comparing model and experimental data. (A) Synthesis rate as a function of time used in the model. The rate is 4 times the basal rate between $T_{1,\text{cor}}=0.1T_d$ and $T_{2,\text{cor}}=0.6T_d$ in accordance with the measurements. (B) Degradation rate as a function of time. Degradation rate increases from 0.001 min^{-1} to 0.010 min^{-1} at $0.9T_d$. (C) Comparison of the number of FtsZ molecules in the cell. The numbers are normalized by the maximum number of mature FtsZ-mNG during the cell cycle. Solid black line corresponds to the experiment, blue line to mature, and dashed red line to total FtsZ-mNG from the model. The additional model parameters, which are not already specified on panels (A) and (B), are $T_d=160 \text{ min}$ and the maturation rate $k_{\text{mat}} = 0.027 \text{ min}^{-1}$. (D) Comparison of FtsZ concentrations. The concentrations have been normalized by the maximum concentration of FtsZ-mNG during the cell cycle.

monomeric FtsZ in the cytosol is not likely the preferred substrate for ClpXP. In light of these arguments, we hypothesize that the possible ClpXP substrates are FtsZ protofilaments or protofilament aggregates, either free in the cytosol or partially attached to the membrane. This hypothesis is consistent with the previous *in vitro* studies showing that the degradation rate of a filament or filament bundle is larger than monomeric FtsZ (108, 129).

3.7.4 Z-ring formation affected by varying FtsZ levels

In this study, we also addressed the question of how the variation of FtsZ levels during the cell cycle affects the Z-ring formation. Our data show that higher FtsZ levels lead to earlier formation of the Z-ring in mutant strains (Figure 3.15). These correlations were also present within cell populations corresponding to individual strains (Figure 3.13E–G, Figure 3.14, and Figure 3.16–3.17), but at expectedly weaker levels. While higher FtsZ levels shift the timing of the Z-ring formation to earlier in the cell cycle, there is an array of other factors that can influence and delay the Z-ring formation. These can include SlmA and the Ter-linkage based spatial positioning systems (35), OpgH which controls the timing via nutrient control (38), and Sula that responds to DNA damage, among other factors.

While our data are indicative that the timing of FtsZ upregulation during the cell cycle in slow growth conditions positively correlates with the Z-ring formation (Figure 3.13C), the correlation between these two timings is not very high ($R=0.67$). This weak correlation is consistent with the above explanation that an array of other factors beyond FtsZ levels also play an important role in controlling the formation of the Z-ring and its

stability. It is also possible that the upregulation of FtsZ synthesis is accompanied by the upregulation of some other possibly more limiting factor, such as FtsA. The latter is partially co-transcribed with FtsZ (127). It is not known if FtsA and ZipA levels vary in a cell cycle dependent manner. It will remain an important question for future research to determine if the levels of these membrane linkers have a significant control over the formation of the Z-ring.

CHAPTER 4

TRANSIENT MEMBRANE-LINKED FTSZ ASSEMBLIES

PRECEDE Z-RING FORMATION IN *ESCHERICHIA COLI*

4.1 Transient assemblies of FtsZ on the cell membrane precede the formation of the Z-ring

To understand the intermediaries leading to Z-ring formation we followed two types of strains with fluorescent FtsZ fusions using time lapse microscopy. One type expressed either FtsZ⁵⁵⁻⁵⁶mNeonGreen (FtsZ-mNG) (95) or FtsZ⁵⁵⁻⁵⁶sfGFP sandwich fusion (FtsZ-sfGFP) from its native locus (83) while the other type expressed FtsZ-GFP as an extra copy from the plasmid (83) (Table 1). Since the half lifetime of FtsZ monomers in protofilaments is about ten seconds (130, 131), FtsZ assemblies should be tracked at least at comparable rates to observe any possible intermediary structures. To prevent phototoxicity of such rapid and light-intensive measurements, we used grazing angle illumination (also referred to as HILO (93)), which decreases background fluorescence. We grew cells in mother machine channels (96, 98) to guarantee steady state conditions. To be compatible with HILO illumination we used agarose as the material for the channels instead of PDMS (poly-dimethylsiloxane) polymer (see Chapter 2: *Agarose based chip fabrication*). The FtsZ-mNG strain was imaged in these channels for three to four consecutive cell cycles while recording about one thousand frames per cell cycle (Figure 4.1A). There was no noticeable effect on the doubling time ($T_d=170\pm 50\text{min}$, $N=25$) stemming from the light exposure conditions when we compared it to measurements

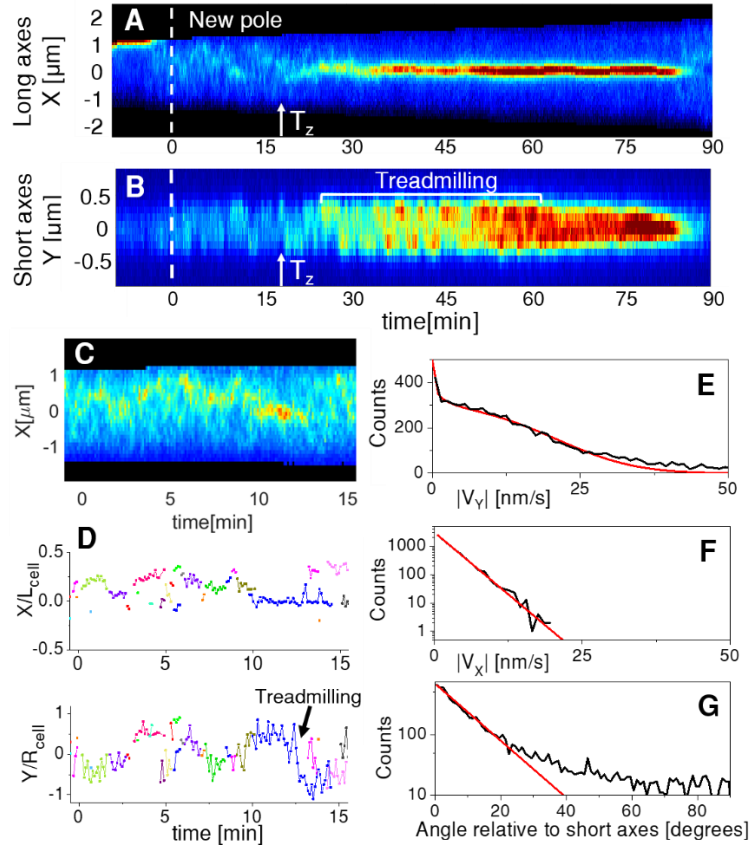


Figure 4.1: Transient membrane-linked FtsZ assemblies precede Z-ring formation.

(A) Kymograph of FtsZ-mNG intensity distribution along the long axis of a cell during one cell cycle. Red corresponds to high and blue to low concentrations. Black color shows regions outside the cell. Vertical white dashed lines mark cell birth and white arrows mark the time when a persistent Z-ring forms (T_z). (B) Kymograph along the short axes for the same cell. (C) Zoom in of the kymograph in panel (A) for the time preceding Z-ring formation. (D) Trajectories of FtsZ assemblies along the long (top) and short axis (bottom) before Z-ring formation. Different colors indicate different assemblies. Arrow points to trajectory showing treadmilling motion. Trajectories are normalized by cell length, L_{cell} , and radius, R_{cell} , respectively. All data in panels (A-D) are from the same cell. (E) Distribution of apparent speeds of assemblies relative to the short axis of the cell (black). Red line is a fitting of the distribution taking into account the variation of apparent speeds on the cell perimeter (see Chapter 2: *Determination of treadmilling velocities of FtsZ assemblies and distribution of treadmilling speeds*). The fitting yields true speeds of 19 ± 11 nm/s (mean $\pm$ std). (F) Distribution of apparent speeds relative to the long axis of cell (black). Red line is exponential fitting. The characteristic speed from the fitting is 2.5 nm/s. (G) Angles of velocity vectors relative to the short axis of the cell (black). Red line is exponential fitting for angles 0-20°. The characteristic angle from the fitting is 9.2°. In panels (E-F) the number of cells analyzed is $N_{cell}=20$ and the number of clusters analyzed $N_{cluster}=352$. All data is from strain JM26.

where the imaging rate was 40 times lower ($T_d=160\pm30$ min) (20). Once the Z-ring has formed, the FtsZ protofilaments or filament bundles could be observed treadmilling around the circumference of the cell, which gives rise to the characteristic oscillatory pattern of the FtsZ distribution along the short axis of the cell (Figure 4.1B). Although the cell length at birth in this strain is about 10% longer than in the wild type (WT) strain in slow growth conditions (20), the unperturbed doubling times and observations of FtsZ treadmilling indicated that our measurement conditions do not significantly perturb the processes associated with FtsZ protofilament dynamics.

We then examined cells for intermediary FtsZ assemblies preceding Z-ring formation. An earlier study has reported dynamic helical patterns of FtsZ in cells that lacked a Z-ring (132). Our measurements also revealed dynamic FtsZ assemblies in cells that had not yet formed a Z-ring. Instead of helices we observed less-extended assemblies, most of them limited by the resolution of the microscope (about 250 nm). These FtsZ foci formed and disappeared throughout the cell (Figure 4.1C, D and Figure 4.2A, B). Since the observed foci showed displacements less than about 300 nm during 6 seconds frame interval, it can be concluded that these foci correspond to membrane attached FtsZ assemblies. Cytosolic FtsZ assemblies would have diffused throughout the cell length ($\approx 2 \mu\text{m}$) during this measurement interval.

On the other hand, the membrane attached FtsZ foci were not static. Some of the larger foci were clearly moving along the circumference of the cell (Figure 4.1D, Bottom; pointed by arrow). Such movement can be attributed to treadmilling rather than to diffusion on the membrane because of its directionality. By tracking larger foci, which can

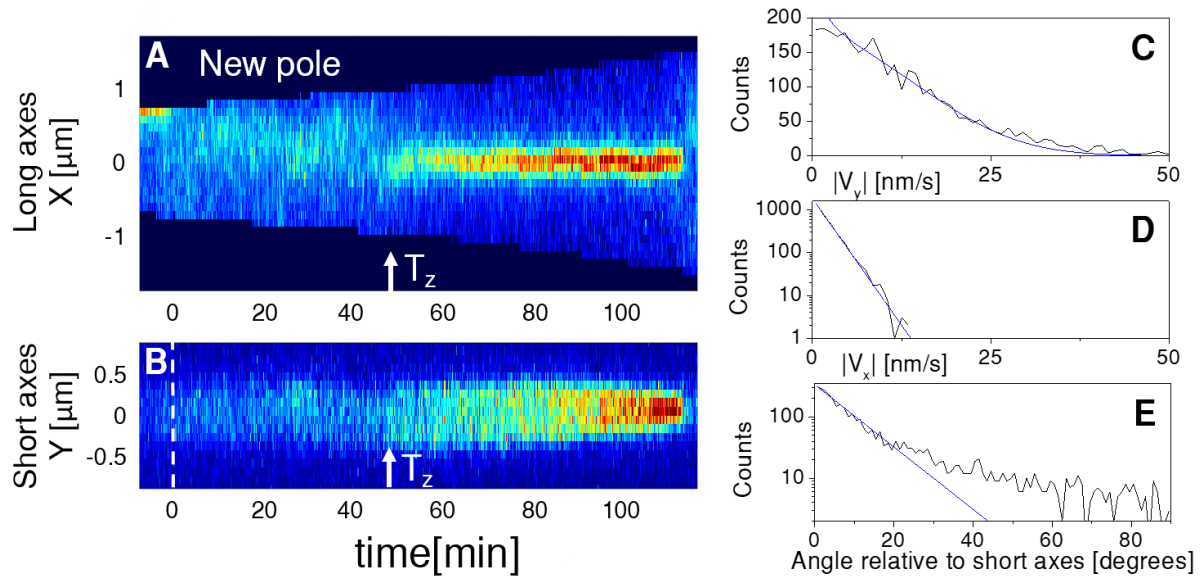


Figure 4.2: Data from a representative cell expressing FtsZ C-terminal fusion to GFP from a plasmid (strain JM60). (A, B) Kymographs of FtsZ-GFP intensity distribution along the long axis (A) and short axis (B) of the cell. Red corresponds to high concentrations while blue corresponds to low concentrations. Vertical dashed lines correspond to times of cell birth and white arrow corresponds to Z-ring formation time (T_z). (C) Distribution of apparent speeds of assemblies relative to the short axis of the cell (black). Blue line is a fitting of the distribution taking into account the variation of the apparent speeds on the cell perimeter (see Chapter 2: *Determination of treadmilling velocities of FtsZ assemblies and distribution of treadmilling speeds*). The fitting yields true speeds of 15 ± 11 nm/s (mean $\pm$ std). (D) Distribution of apparent speeds of FtsZ assemblies relative to the long axis of the cell (black). Blue line is exponential fitting of the distribution. The decay constant from the fitting is 1.8 nm/s. (E) Angles of velocity vectors relative to the short axis of the cell (black). Blue line is exponential fitting of angles 0-20°. The characteristic angle from the fitting is 9.2°. In panels (C-E) the number of cells analyzed is $N_{\text{cell}}=18$ and number of transient assemblies analyzed $N_{\text{cluster}}=408$.

be distinguished by segmentation, we determined their apparent movements relative to the short and long axis in a population of cells (Figure 4.1E, F). Since the image is a projection of a cylindrical cell to a (image) plane, the apparent speed of the foci along the short axis (y-axis) is not uniform even when the FtsZ assembly moves at a uniform speed along the circumference of the cell. Accounting for this variation and assuming the distribution of the true circumferential speeds to be Gaussian yields a distribution of apparent treadmilling speeds (see Chapter 2: *Determination of treadmilling velocities of FtsZ assemblies and distribution of treadmilling speeds*), which we compared to experimentally measured distributions (Figure 4.1E). This comparison yields an estimate of 19 ± 11 nm/s (mean $\pm$ std) for the true treadmilling speeds of FtsZ transient assemblies at the cell's circumference (Figure 4.1E). Similar treadmilling speeds (15 ± 11 nm/s) were also found for the strain expressing FtsZ-GFP from a plasmid (Figure 4.2C, D). These values are comparable to those reported previously for treadmilling FtsZ clusters that are part of the Z-ring (83).

Direction of treadmilling was not always circumferential, but also showed a velocity component along the long axis (Figure 4.1E). For most assemblies, the angles of the velocity vectors relative to the direction of the short axis of the cell followed an exponential distribution with a characteristic angle of 9° (Figure 4.1G). However, the distribution had a long tail that reached to 90° . So while a few assemblies treadmilled along the long axis of the cell, the majority of them were well aligned relative to the short axis.

4.2 Size of transient assemblies is limited by Z-ring components

The transition from transient mid-cell assemblies to the Z-ring occurred gradually over the period of about 30 minutes (Figure 4.3A, B). As more FtsZ accumulated to the mid-cell Z-ring, the transient assemblies in other locations in the cell disappeared. Once the mature Z-ring emerged, which contained about 35% of the cellular FtsZ content, no transient assemblies were present from visual inspections of the kymographs and cell images (cf. Figure 4.1A and Figure 4.2A). We used spectral analysis to determine if the transient assemblies were completely absent. For that end we collected fluorescent intensity traces from the cell middle and two quarter positions (Figure 4.3A). Formation and dissociation of distinct FtsZ foci in the cell images leads to variations in the intensity for these traces. At the same time treadmilling, which the direction is predominantly circumferential, did not cause fluctuations in these intensities because the assemblies should have remained within the band-shaped region of interest that we defined for this analysis (Figure 4.3A, Schematics). To analyze these fluctuations we calculated a population-averaged ($N = 20$ cells) power spectral density and autocorrelation function before and after Z-ring formation using the intensity traces from different cells (see Chapter 2: *Determination of power spectral densities and autocorrelation functions*). As a control we imaged the same strain, which was transformed with a plasmid carrying an inducible copy of *sulA* (101), and determined the intensity traces after upregulation of SulA. SulA binds to FtsZ monomers, thereby lowering the amount of free FtsZ below the critical concentration required for cooperative assembly. As a result, very few, if any, polymeric FtsZ molecules are expected to be present in these cells (101). Intensities from the SulA control showed

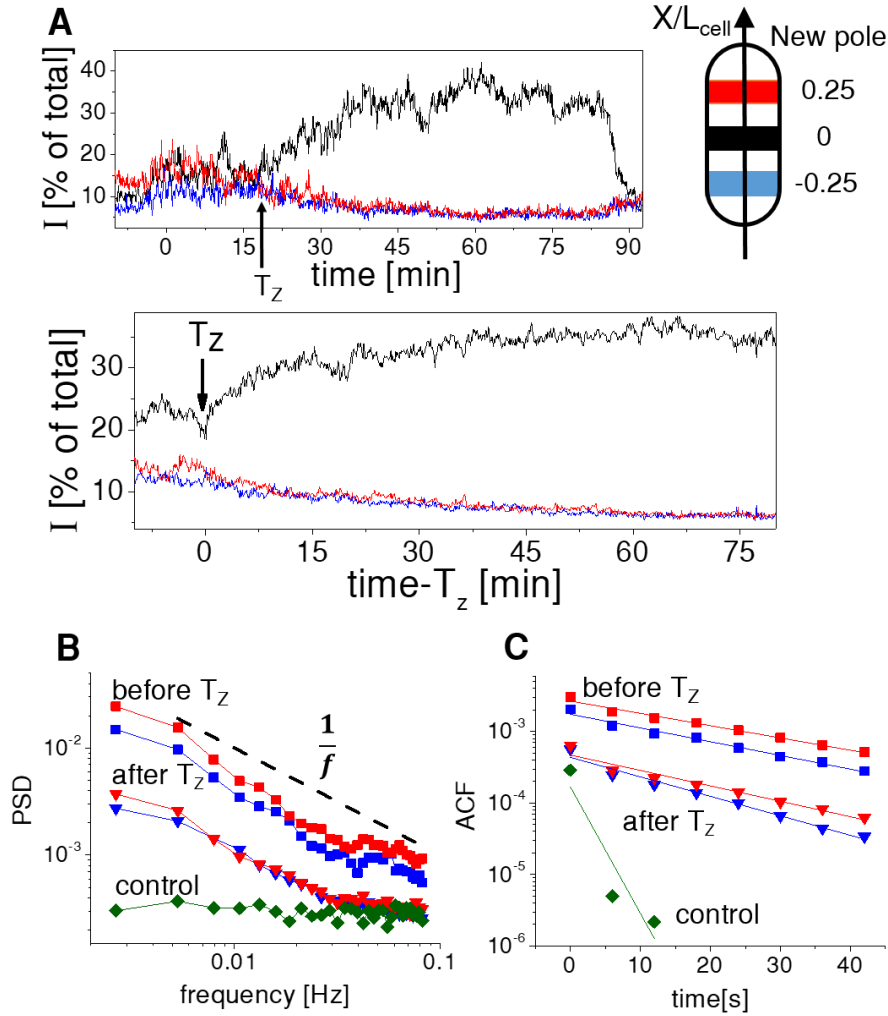


Figure 4.3: Comparison of FtsZ transient assemblies before and after Z-ring formation. (A) Top: Intensity traces from three separate regions within the cell (diagram shown on right). Each region represents 0.3 mm wide band. Red corresponds to the band centered at a quarter of the cell length from the new cell pole, blue to a quarter of the cell length from the old cell pole, and black to the cell center region. This color coding applies to all panels. Time zero marks cell birth, T_z corresponds to time of Z-ring formation, and the trace ends at cell division. Data from the cell shown in Figure 4.1. Bottom: population averaged curves ($N=20$). (B) Population averaged power spectral density of the old pole and new pole traces before (squares) and after T_z (triangles). PSD from the central traces are shown in Figure 4.4. Dashed black line shows for reference an inverse frequency dependence. Control data (diamonds) is from cells where FtsZ polymerization is inhibited by upregulation of SulA ($N=10$). In these cells the PSD is not dependent on cellular location. (C) Population averaged autocorrelation function of old and new pole traces before and after T_z . Exponential fits to the autocorrelation functions are shown by solid lines. All data is from strain JM26.

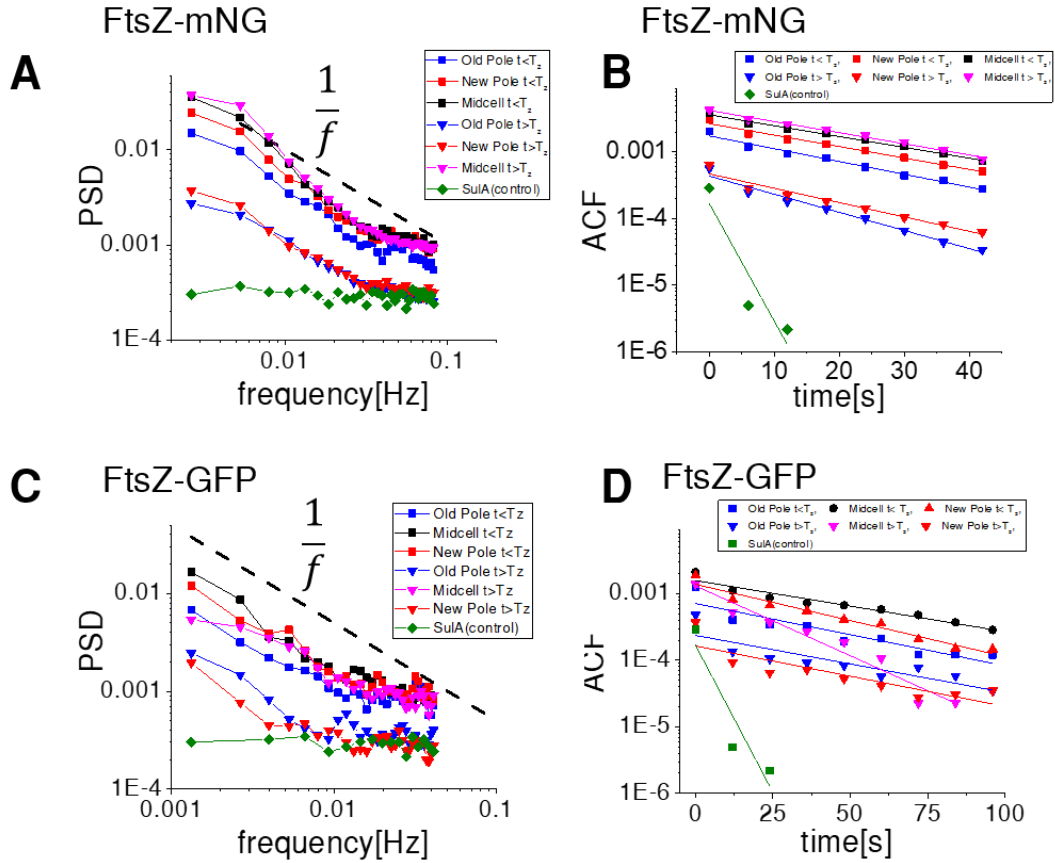


Figure 4.4: Comparison of FtsZ-mNG (JM26) to FtsZ-GFP (JM60) fluctuations before and after Z-ring formation. (A, B) Population averaged ($N = 20$) power spectral density before (squares) and after (triangles) Z-ring formation in JM26 strain expressing endogenously FtsZ-mNG. In addition to traces from cell quarter positions, midcell data are shown. Dashed black line shows for reference an inverse frequency dependence. Control data (diamonds) is from cells where FtsZ polymerization is inhibited by upregulation of SulA ($N=10$). (B) Population averaged ($N = 20$) autocorrelation function before and after T_z . Exponential fits to the autocorrelation functions are shown by solid lines and experimental data by markers. (C) Population averaged ($N=18$) power spectral density analysis of strain expressing FtsZ-GFP from a plasmid (strain JM60) before and after T_z . (D) Population averaged ($N=18$) autocorrelation function for this strain before and after T_z . Exponential fits to the autocorrelation functions are shown by solid lines and experimental data by markers.

a frequency independent (white) noise spectra while the two WT strains showed power spectra that decayed approximately as an inverse of the frequency (Figure 4.3B and Figure 4.4A, C). The fluctuation levels in FtsZ-mNG exceeded the levels in the control, more than an order of magnitude, at lower frequencies showing that the observed fluctuations in WT intensity traces originated from higher order FtsZ structures. Furthermore, the long decay of the autocorrelation functions with a decay constant of about 20 seconds (Figure 4.3C and Figure 4.4B, D) confirmed that these higher order structures are membrane bound.

The peripheral regions of the cell show about ten-fold higher levels of power spectral density before Z-ring formation compared to that after the ring has formed. This quantitative conclusion is consistent with visual observations. The power spectral density analysis also reveals that membrane-bound FtsZ assemblies still appear after the Z-ring has formed, even though they are not visually distinguishable from the images. From the power spectrum and autocorrelation measurements, we estimate that the number of FtsZ monomers present in the foci is a factor of $\sqrt{10} \sim 3$ lower after Z-ring formation. These data point out that the sizes of the transient assemblies are controlled by the available amount of FtsZ and/or its membrane linkers. Once the Z-ring forms, the number of early Z-ring components available to form transient assemblies decreases. Nevertheless, the smaller sized assemblies are still able to form throughout the cell body.

4.3 Spatial and temporal distribution of FtsZ assemblies

In what follows we will characterize the distribution of membrane bound FtsZ assemblies as a function of cell cycle time and location within the cell. Our analysis is pertinent to

large size assemblies that contain 2.5% or higher amounts of total cellular FtsZ. These assemblies can be unambiguously distinguished from images using thresholding based methods (see Chapter 2: *Protofilament tracking and rate of change analysis*). To quantify position dependence on a coarser scale, we divided each cell into two regions - a mid-cell and a cell periphery where the periphery also included cell poles (Figure 4.5A, Schematics). Here, the mid-cell region spans 1/3 of the total length of the cell around its center while the rest is referred to as the periphery. One could perhaps have expected FtsZ contents and lifetimes of transient assemblies at the mid-cell to increase as the cell cycle time progressed until the Z-ring formed. Contrarily, we found that the location of the large assemblies along the long axis of the cell (Figure 4.5A), their size (Figure 4.5B), and their lifetimes (Figure 4.5C) were all only very weakly correlated with the cell cycle times during the period that preceded the Z-ring formation ($R < 0.2$; Table 6). The same conclusions were also reached based on data from the FtsZ-GFP strain (Figure 4.6A-C and Table 6). The exception to these steady behaviors was the number of assemblies, which started to increase before the Z-ring formed (Figure 4.5A, bottom). Our previous work has shown that prior to the formation of the Z-ring the synthesis rate of FtsZ increases four-fold (20). This observation was confirmed at the qualitative level in current studies. Thus, the increase in the number of assemblies correlates with the increase in the number of FtsZ within the cell. It follows then that the increase in FtsZ numbers in the cell leads to more assemblies with the same kinetic properties (size and lifetime) rather than to the same number of larger assemblies.

The large assemblies were completely missing from the cell poles, and their

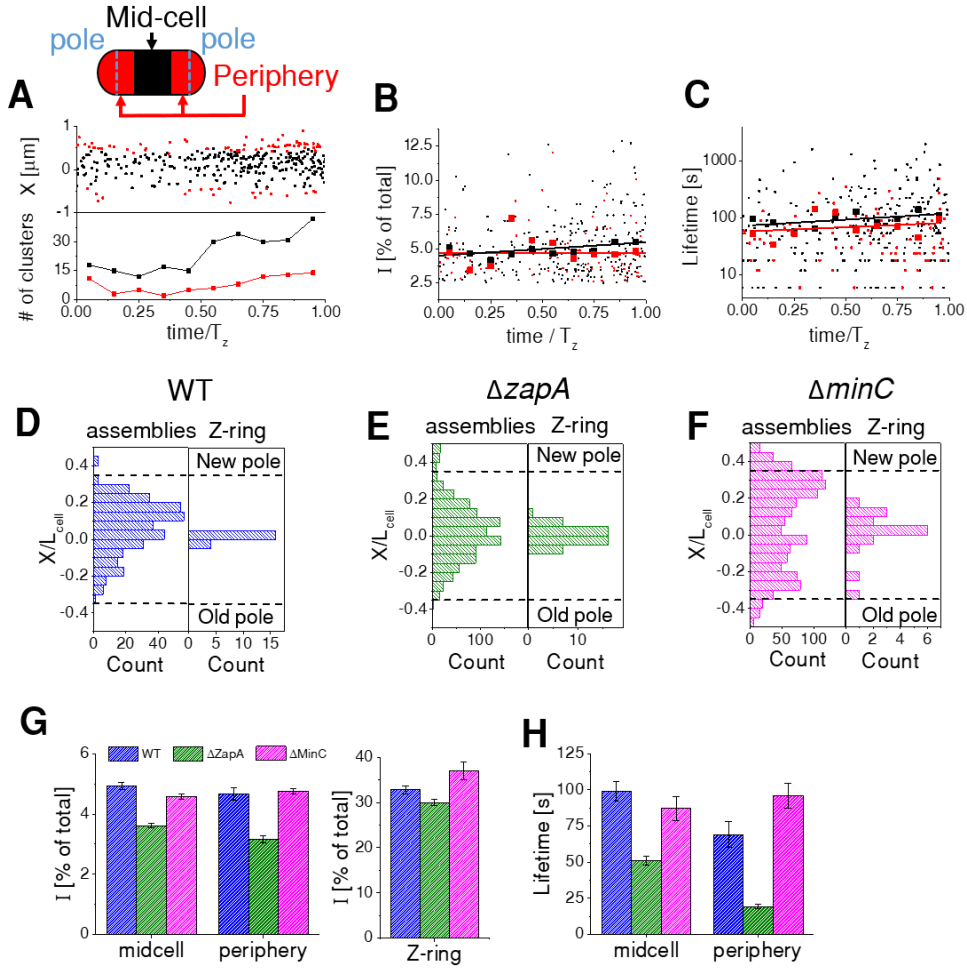


Figure 4.5: Characterization of spatial and temporal distributions of FtsZ transient assemblies that contain 2.5% or higher amount of FtsZ relative to the cell total. (A) Top: The positions of assemblies along the long axis of cell as a function of time. Assemblies at the mid-cell are shown by black and in cell peripheries by red markers as explained by the inset. Time axis is scaled by time when the Z-ring forms (T_z). Bottom: Number of assemblies in the mid-cell and two peripheries combined as a function of normalized time. Solid lines in panels (A)-(C) are linear fits. Parameters of these fittings are collected to Table 6. (B) The fluorescent intensities from assemblies as function of normalized time. The intensities are scaled by the total fluorescent intensity from the cell and averaged over the lifetime of each assembly. The small markers are individual data points and the large markers the binned data. (C) Lifetimes of assemblies at the mid-cell (black) and in the periphery (red) as a function of normalized time. (D) Distribution of assemblies (left) and Z-ring positions (right) along the long axes of the cell. Positions are normalized by the cell length. Panels (A-D) correspond to WT cells (strain JM26). (E-F) Same distributions for ΔzapA (strain BEW2), and ΔminC cells (strain BEW1), respectively. (G) Comparison of sizes of assemblies in WT, ΔzapA and ΔminC strains. (H) Comparison of lifetimes of assemblies in these three strains. In last two panels error bars are std error.

frequency in both peripheries combined was five times lower than at the mid-cell (Figure 4.5A, bottom panel). Nevertheless, the spatial distribution of assemblies in the cell was broad having a standard deviation of 0.14 in units of the cell length (Figure 4.5D, left). The corresponding distribution was about 10 times wider than the spatial distribution of the Z-rings in these cells (Figure 4.5D, right). Interestingly, the distribution of the transient assemblies was asymmetric relative to the cell center. 72% of all assemblies localized to the cell half closest to the new pole (Figure 4.5D; $p=7 \cdot 10^{-17}$ in Wilcoxon test). The asymmetry of the distribution correlates with the localization of the replication terminus region of the chromosome to the new pole side of the cell during the early stages of the cell cycle (133). Deletion of a genetic region encoding ZapA, which is necessary for the Ter linkage, lead to a reduction of the asymmetry (Figure 4.5E, 57%; $p=0.007$) but statistically significant bias towards the new pole side still remained. The distribution of the transient assemblies and in particular their absence from the cell poles was even more strongly influenced by the Min system, which is involved in the positioning of the Z-ring (134). In the absence of MinC, which couples Min oscillations and FtsZ, the spatial distribution of assemblies broadened further (standard deviation 0.24) and transient assemblies also appeared in the polar regions (Figure 4.5F).

Although the large scale assemblies were rarer at the cell peripheries in WT cells, their size, in terms of their FtsZ content, was only weakly correlated with their position in the cell. We found the assemblies in the mid-cell region to be on average only 5% larger in terms of FtsZ numbers ($p=0.3$ in Wilcoxon test) than those in the cell peripheries (Figure 4.5G, left and Figure 4.6G). Comparing the sizes of transient assemblies with the size of

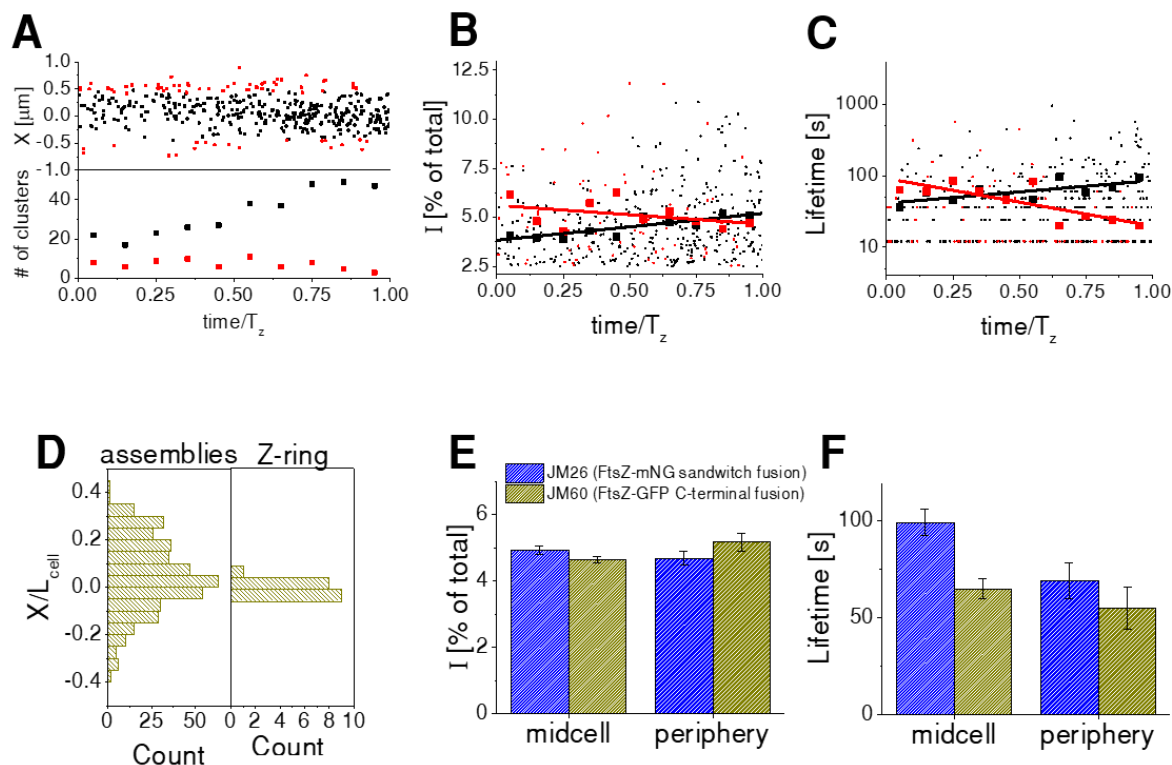


Figure 4.6: Characterization of spatial and temporal distribution of FtsZ transient assemblies in JM60 strain where FtsZ-GFP is expressed from a plasmid. Only assemblies that contain 2.5% or higher amounts of FtsZ relative to the cell total are shown. Solid lines in different panels are linear fits. Numbers on the top of the panel indicate average values. Parameters of fits, numbers of cells, and clusters analyzed are collected to Table 6. (A) Top: The positions of assemblies along the long axis of the cell as a function of time. Assemblies at midcell are shown by black and in cell peripheries by red markers. Time axis is scaled by time when the Z-ring forms (T_z). Bottom: Number of assemblies in midcell and two peripheries combined as a function of normalized time. Solid lines in panels (A)-(C) are linear fits. (B) The fluorescent intensities from assemblies as function of normalized time. The intensities are scaled by the total fluorescent intensity from the cell and averaged over the lifetime of each assembly. The small markers are individual data points and the large markers the binned data. (C) Lifetimes of assemblies at the midcell (black) and in the periphery (red) as a function of normalized time. (D) Distribution of assemblies (left) and Z-ring positions (right) along the long axes of the cell. Positions are normalized by the cell length. Panels (A)-(D) correspond to FtsZ-GFP cells (strain JM60). (E) Comparison of sizes of assemblies in FtsZ-mNG (JM26) and FtsZ-GFP (JM60) strains. (F) Comparison of lifetimes of the assemblies in these two strains. In the last two panels error bars are std error.

the Z-ring, we found that the transient assemblies were on average six times smaller than the Z-ring in terms of FtsZ numbers. The latter contained about 33% of the cellular FtsZ (Figure 4.5G, right), which is in agreement with earlier reports (30, 135). In the absence of ZapA, which has been implicated in cross-linking FtsZ protofilaments, the assemblies were about 30% smaller in both the mid-cell and peripheral regions than in WT cells. Moreover, in the absence of ZapA, the lifetimes of the assemblies were more than two times shorter than in WT type cells (Figure 4.5H). The effect was more pronounced at the cell peripheries. These data imply that protofilament-protofilament crosslinking via ZapA occurs in the transient assemblies.

The sizes of the assemblies in $\Delta minC$ cells were comparable to their sizes in WT cells (Figure 4.5G). There was no significant difference in assembly sizes at the mid-cell and cell periphery in $\Delta minC$ cells ($p=0.4$ in Wilcoxon test). Also, the lifetimes of the assemblies in $\Delta minC$ cells were the same at the mid-cell and peripheries (Figure 4.5H), and were comparable to the assembly lifetimes at the mid-cell in the WT strain. Unlike in $\Delta minC$ cells, the WT transient assemblies' lifetimes were 30% shorter at the cell peripheries. These findings show that the Min system is responsible for the smaller sizes and shorter lifetimes of the FtsZ assemblies at the cell peripheries in WT cells. The smaller abundance of peripheral assemblies can also be explained, to some extent, by the Min system. The findings are largely consistent with the known function of the Min system, which is to prevent the formation of the Z-ring at polar regions. Altogether, the data presented here indicate that transient assemblies are affected by the Z-ring positioning systems. However, their effects are weaker than one could have expected

Table 6: Parameters of linear fits in Figure 4.5 and 4.6.

Strain	Location	Figure 4.5B and Figure 4.6B		Figure 4.5C and Figure 4.6C	
		Slope	Pearson R	Slope	Pearson R
JM26(mNG) (Ncell = 20)	Midcell (Nclust = 257)	0.97 ± 0.41	0.14	75 ± 48	0.09
JM26(mNG) (Ncell = 20)	Periphery (Nclust = 81)	-0.01 ± 0.67	-0.001	13 ± 29	0.05
JM60(GFP) (Ncell = 18)	Midcell (Nclust = 336)	1.37 ± 0.34	0.22	46 ± 17	0.69
JM60(GFP) (Ncell = 18)	Periphery (Nclust = 72)	-0.95 ± 0.74	-0.42	-62 ± 20	-0.74

N_{cell} corresponds to the number of cells analyzed and N_{clust} to the total numbers of transient assemblies analyzed.

based on studying the Z-ring. In particular, the spatial distribution of transient assemblies is much broader than the distribution of Z-ring positions.

4.4 Cytosolic protofilaments *in vivo* are shorter than ones observed from *in vitro* measurements

We next investigated assembly-disassembly kinetics of membrane attached FtsZ assemblies in WT cells. The first step in this assembly must be attachment of an FtsZ oligomer from the cytosol to the plasma membrane via its linkers. Based on *in vitro* studies, one could expect that the typical protofilament length in the cytosol is about 100-200 nm (≈ 25 monomers) (21, 81, 136, 137), although it has also been proposed that cytosolic filaments could be smaller because of their rapid capture to the membrane via their linkers (138). We aimed to capture attachment of such protofilaments to the plasma membrane from the cytosol during time lapse movies, which were taken at higher frame rates (1 Hz) than earlier ones. By using 100 ms long excitation pulses, we expected to observe a step-like increase in the intensity at the location of FtsZ focus once the protofilament attached. Note that prior to the attachment, motion blurring of the filament diffusing in the cytosol should have completely smeared out its image. Contrary to our expectations, none of the 1973 analyzed foci showed distinct step-like increases in the beginning of their lifetime that could be classified as protofilament attachment. We did notice that many of these assemblies grow rapidly but with further examination of the images it became clear that prior to the rapid growth an already existing FtsZ assembly was present (Figure 4.7A). We also did not observe any step-like decreases although in many assemblies the amount of FtsZ-sfGFP decreased rapidly during the end of their

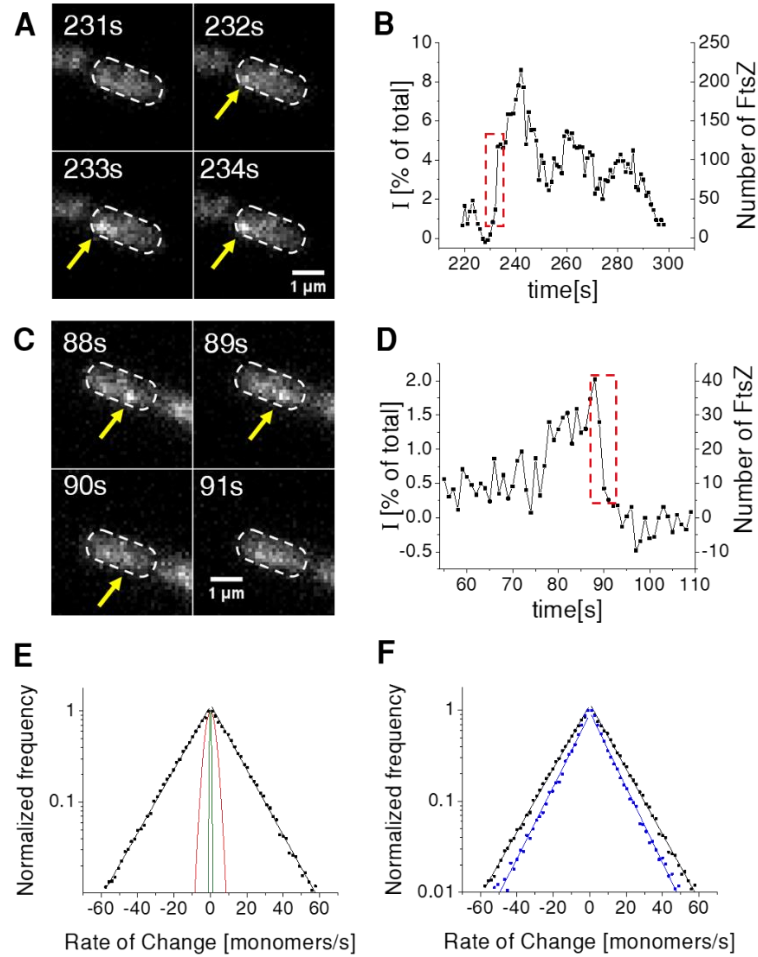


Figure 4.7: Rate of change in the size of FtsZ transient assemblies.

(A) Time-lapse images of a cell with an assembly that rapidly increases in size. (B) Corresponding intensity time-trace from this assembly. Left axis shows intensity of the assembly relative to the total intensity of FtsZ-sfGFP from the cell. Right axis corresponds to the absolute number of fluorescent FtsZ-sfGFP in the assembly. Dashed box region corresponds to frames in panel (A). (C) Time-lapse images of a cell with an assembly that rapidly decreases in size. (D) Corresponding time-trace from this assembly. Dashed box region corresponds to frames in panel (C). (E) Distribution of rates for cell population ($N_{\text{cell}}=199$; $N_{\text{cluster}}=1973$) plotted on logarithmic scale (black squares). Each side of the distribution was fitted to an exponential (black solid lines). The characteristic rates from the fitting is 15.6 monomers/s for addition and 15.7 monomers/s for FtsZ removal. Red solid line represents the prediction of a model where assemblies change in size via treadmilling which include only monomer addition and removal (see Chapter 1: *Rate of change of filament length*). Green line is the estimated distribution of the measurement noise (see Chapter 2: *Estimation of measurement noise contributions*) (F) Comparison of rates from WT (black) and ΔzapA strains (blue). The latter distribution has a characteristic monomer addition rate of 10.1/s and monomer removal rate of 10.3/s. WT data is from strain KC606 and ΔzapA from strain BEW2.

lifetimes (Figure 4.7B). This indicates that at least longer filaments are not able to detach from the membrane which could have occurred if their binding to the membrane, via linkers, was sufficiently weak.

The lack of any step-like increases could have been caused by our limited sensitivity to detect small FtsZ assemblies. To have a better understanding of the measurement sensitivity we determined the number of FtsZ-sfGFP within the cells for these growth conditions. These measurements yielded an estimated average concentration of $4.7 \pm 0.7 \mu\text{M}$ (Figure 4.8). This number is in the range, 4-6 μM , reported before (91, 101, 116) although concentrations as high as 10 μM have also been measured (74). The concentration above corresponds to 2300 ± 400 FtsZ molecules for a cell having a population average length of 2.8 μm . By using the above calibration we determined the sizes of the assemblies in terms of FtsZ numbers (right axis, Figure 4.7B, D). Based on the noise level in the traces we conservatively estimate the sensitivity of the detection to be better than 20 monomers. Based on this estimation we conclude that the transient FtsZ assemblies start from protofilaments that are shorter than 20 monomers.

The above data left open the possibility that longer protofilaments are present in the cytosol, but their attachment to the membrane may be hindered. To find evidence for longer (>20 monomers) cytosolic protofilaments diffusing in the cytosol we employed short 1 ms stroboscopic pulses to excite the fluorophores and to eliminate motion blurring. We first verified that 1 ms long excitation pulses were sufficiently short enough for stroboscopic imaging using a photoactivatable FtsZ-mMaple3 (95) construct in a monomeric state in the cytosol (see Chapter 2: *Single molecule imaging*). We then

imaged non-photoactivatable FtsZ-sfGFP in normally growing cells using excitation pulses of the same duration. Given the low frame rate (1 Hz) of the measurement and the rapid diffusion of protofilaments in the cytosol, we expected to observe foci that would have shown large displacements ($>0.5\ \mu\text{m}$) from one frame to another. Contrary to this expectation, the measurement showed 748 out of 755 (99.1 %) foci to be static. The observations included 101 cells for a total of about $5 \cdot 10^5$ frames. The remaining 7 events (0.9%) corresponded to foci that were present for only a single frame. Some of these single-framed events may have corresponded to membrane attached assemblies whose size increased and decreased rapidly within the 1 second interval. Altogether, these measurements lend support to the idea that protofilaments longer than 20 monomers are exceedingly rare, if at all present, in the cytosol.

4.5 Rapid kinetics of FtsZ transient assemblies

While we did not find evidence of longer protofilaments in the cytosol and their attachment to the inner membrane of the cell, the recorded time-traces of FtsZ transient assemblies (Figure 4.7A-D) showed frequent periods of rapid growth and shrinkage. To understand these dynamics further, we determined instantaneous growth and shrinkage rates of the assemblies (Figure 4.7E). The distributions of both rates were approximately exponential with an average rate of 16 monomers/sec. To understand the effect of the measurement noise for this distribution we imaged a strain carrying inducible Sula in conditions where the cells lacked any visible FtsZ foci or Z-rings. Since these cells did not have any detectable FtsZ foci, we simulated the foci by randomly selecting regions within the Sula-expressing cells based on the area and duration of foci obtained from WT cells. The

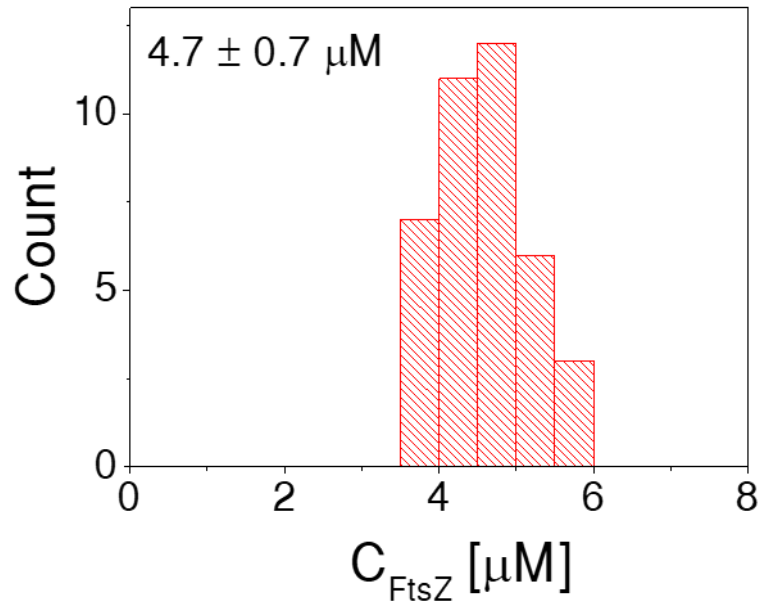


Figure 4.8: Determination of FtsZ concentration in *E. coli* cells growing in M9 glycerol medium (strain KC606). In this determination fluorescent intensity from cells were compared to the fluorescent intensity from a calibrated GFP standard. In the measurements 42 channels were used. Of those, 21 were 0.8 μm wide and the remaining half were 0.9 μm wide. The channels were first filled with GFP and then populated with cells. The average concentration of 4.7 ± 0.7 μM (mean±std) was determined.

resulting control distribution was about 25 times narrower (Figure 4.7E, green line) than the one from non-induced cells. Thus, the measurement noise contributes only to a narrow region at the center of the distribution and does not affect its tails.

The high rates at the tails of the distribution in Figure 4.7E were unexpected. If the assembly were to consist of only a single protofilament, and only monomers and not oligomers were added and removed from the assemblies then a much narrower rate distribution would be expected based on treadmilling kinetics (Figure 4.7E, red line; see Chapter 1: *Rate of change of filament length*). We found the calculated rate distribution to be about 5 times narrower than the experimental one (Figure 4.7E, red line). The higher rates in the experimental distribution could be explained by assemblies that consist of protofilament bundles instead of single protofilaments. In this case, the monomer addition and removal rates would increase in proportion to the number of protofilament ends in the bundle. Bundling would also mean that additional protofilaments can be added to or removed from the assemblies via lateral contacts or via cross-linking. The latter processes could possibly explain the high-rate tails of the distribution in Figure 4.7E. To investigate the possibility of protofilament cross-linking further, we carried out the same measurements in a strain lacking ZapA. We found the monomer addition and removal rates of transient assemblies in this strain to be about 35% lower compared to WT cells (Figure 4.7F). This finding together with the decrease in assembly sizes and lifetimes upon *zapA* deletion (Figure 4.5G, H) supports the idea that at least some of the observed assemblies consists of cross-linked FtsZ protofilaments.

4.6 Discussion

4.6.1 Dynamics of transient assemblies

Our experimental findings are consistent with the following scenario summarized in Figure 4.9. The life cycle of transient assemblies starts with nucleation and growth of protofilaments in the cytosol. Based on the frequent formation of transient assemblies, it is clear from our data that the effective FtsZ concentration in the cell prior to the Z-ring formation is above the critical concentration. Furthermore, the presence of transient assemblies show that 1) protofilament formation in the cytosol and 2) protofilament attachment to the cell membrane via its linkers are not rate limiting steps for Z-ring formation. However, we found cytosolic protofilaments shorter than one would have expected based on *in vitro* measurements. We estimate protofilament lengths to be less than 20 monomers *in vivo* while 25-50 (100-200 nm) (81, 136, 137) and even 100 monomer lengths (139) have been reported from *in vitro* measurements. A variety of factors can contribute to this discrepancy. For example, the filament length distribution depends on the concentrations of ions (K^+ , Mg^{2+}) and GTP levels. Even though concentrations of these ions and molecules are to some degree known for *in vivo* conditions, their activities are less well characterized and depend on the cell growth environment and exact genetic make-up. Additionally, FtsZ polymerization antagonists, such as SlmA, can effectively shorten protofilament length. It has also been proposed that cytosolic filaments cannot elongate to their *in vitro* lengths because of their capture to the membrane via FtsA and ZipA linkers (138). The quantitative extent by which the latter mechanism limits cytosolic protofilament lengths is not clear from our data, but our

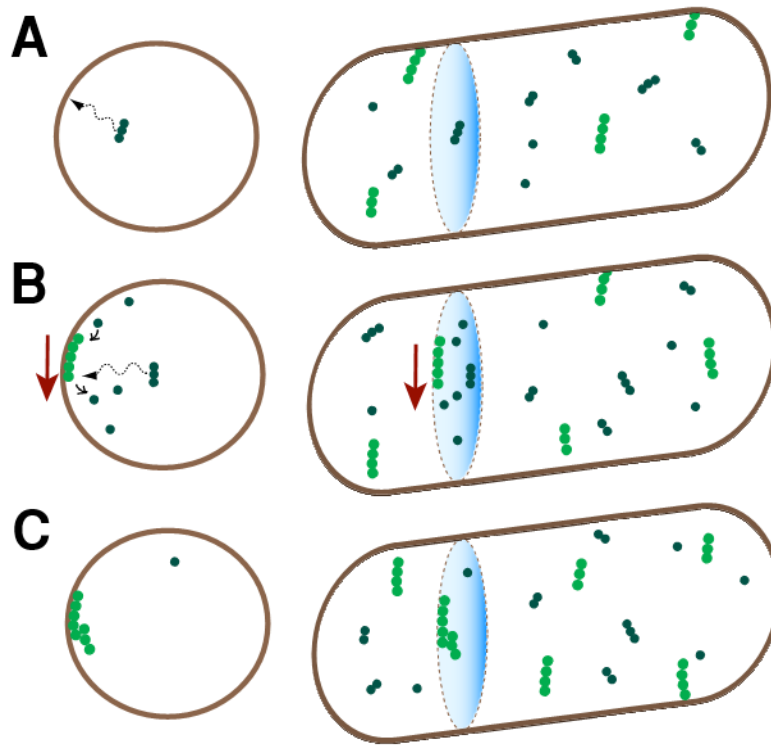


Figure 4.9: Inferred sequence for formation of transient assemblies. (A) FtsZ protofilaments are formed in the cytosol and are recruited to the membrane by FtsA and ZipA membrane tethers (not shown). The right column is a 3D view of cell contours and FtsZ filaments and the left column is a cross-sectional view. Bright green circles correspond to membrane attached FtsZ and dark green circles correspond to cytosolic FtsZ. (B) The membrane attached filaments treadmill preferentially in a direction perpendicular to the long axis of the cell. (C) These filaments bundle/crosslink at early stages after their attachment to the membrane. All filaments in the bundle are oriented in the same direction.

measurements do show frequent attachments of protofilaments to the plasma membrane. These attachments occur at random locations along the cell length except possibly at the cell poles where the apparent occurrence of observable assemblies is smaller. The randomness in positioning appears to stem from stochastic collisions of cytosolic protofilaments with the membrane.

Once attached, some of the filaments give rise to assemblies that contain several hundreds of monomers. Their rapid growth rate implies that at least some of these assemblies must become protofilament bundles. In agreement with earlier reports (91), our data show that ZapA enhances protofilament cross-linking, but the latter can occur in the absence of ZapA.

4.6.2 Competition between different assemblies for the pool of available FtsZ

FtsZ assemblies forming during the early stages of the cell cycle (about the first 20% of the cell cycle) remain unstable; even those that form at the cell middle. Then what causes the transition from transient assemblies to a persistent Z-ring which carries out cell division? There appears to be no well-defined trigger for Z-ring assembly. The transition in slow growth conditions is gradual taking about 20% of the cell cycle (Figure 4.3A). During this time the dominant mid-cell assembly on average grows in size, but the growth is not uniform and is interspersed with periods of shrinkage. Previous research has identified the amount of FtsZ in the cell as one of the limiting factors for Z-ring formation (20, 140, 141). The data reported here provides further support to this idea. Specifically, the data show that once the dominant mid-cell assembly forms, which is to become the Z-ring, the numbers of other larger transient assemblies within the cell drops gradually to

zero. A more sensitive analysis based on power spectra and autocorrelation functions show smaller FtsZ assemblies still form throughout the inner membrane, but the size of these assemblies is a factor of 3 smaller than the ones present prior to Z-ring formation. Our data show that Z-ring formation leads to about a 35% reduction in the available pool of FtsZ for transient assemblies (cf. Figure 4.3A and Figure 4.5) since this amount is sequestered to the Z-ring. This relatively small decrease in the available pool of FtsZ then leads to a significant (3-fold) decrease in FtsZ transient assembly sizes. The exact mechanism giving rise to this very sensitive concentration dependence is not clear. We hypothesize that the out competition of other assemblies by the dominant mid-cell assembly is likely to occur because this assembly is a bundle that contains more protofilaments. Addition of monomers and short oligomers to a larger bundle would be more favorable than to smaller bundles or to single protofilaments. For cellular function, out competition appears to be an important characteristic of protofilament dynamics as it guarantees that not more than one Z-ring forms per cell.

4.6.3 Transient FtsZ assemblies probe for the proper location for the Z-ring

The assembly that outcompetes others and forms the Z-ring is not a random one but the one that has formed in the vicinity of the cell center. Its higher stability can be expected to stem from its more favorable interaction with the spatial positioning systems of the Z-ring (the Min system, the Ter linkage, and SImA based nucleoid occlusion). Tracking the assemblies (Figure 4.1) shows that the spatial positioning systems mostly select among the randomly forming assemblies that stay a fixed distance from the cell center. However, some movement of these assemblies along the long axes of the cell does occur because

of treadmilling, and likely this motion also plays a role in the positioning of the Z-ring. Surprisingly, transient assemblies are present in the wide band around the cell center, which covers about one half of the cell length. It remains yet unclear by what mechanism this broad distribution of transient assemblies collapses into a narrow distribution of FtsZ in the cell center, which has about a 3% variation relative to the cell center (Figure 4.5D). This process possibly combines the effects from different spatial positioning systems and the competition effects between different assemblies. A detailed understanding of how this process unfolds remains an important and open question for future studies.

SUMMARY

This dissertation set out to understand the initial steps leading to the formation of the bacterial Z-ring. In particular, our focus was on the processes related to FtsZ proteins; from their assembly into dynamic filaments to formation of a ring-like structure at the cell center. Our approach consisted of imaging *E.coli* cells with a new fluorescently labelled FtsZ construct for multiple generations at high spatial and temporal resolution while the cells grew in steady-state conditions. We originally hypothesized that FtsZ proteins should nucleate into long cytosolic filaments because the concentration of FtsZ was expected to be constant within the cell and well above the critical concentration needed for filament formation. These hypotheses were based on *in vivo* studies in fast growth conditions and from *in vitro* studies. However, by using slow growth conditions to study Z-ring assembly for a longer period, we discovered that FtsZ concentration changed in a cell cycle dependent manner and that FtsZ proteins formed short protofilaments (<20 monomers) which transiently attached to the inner membrane prior to Z-ring formation.

The change in FtsZ concentration in a cell-cycle dependent manner was first noticed during long timelapse measurements, which were conducted using PDMS based microfluidics. These measurements furthermore showed that there was approximately a fourfold upregulation of FtsZ during the first half of the cell cycle. This period was followed by its rapid degradation by the ClpXP protease during the last 10% of the cell cycle. The upregulation of FtsZ approximately coincided with Z-ring formation. However, the correlation between the two events was moderate, implying that factors besides FtsZ concentration contribute to Z-ring formation. Also the initiation of the rapid degradation of

FtsZ coincided with the dissociation of the Z-ring from the septum. Altogether, contrary to the common expectation that FtsZ concentration is constant throughout the cell cycle, our findings suggested that the Z-ring formation in slow growth conditions in *E. coli* is partially controlled by a regulatory sequence wherein upregulation of an essential cell cycle factor, FtsZ, is followed by its rapid degradation.

The transient membrane-linked assemblies were revealed in measurements of the same strain when we increased the frame-rate by 40-fold. This increased time-resolution became possible thanks to the use of agarose microfluidics and HILO illumination that considerably increased the signal-to-noise ratio of the measurements. These measurements showed that formation of the Z-ring is preceded by a period where transient membrane-linked FtsZ assemblies occur. These assemblies formed after the initial attachment of short cytosolic protofilaments, which we estimated to be less than 20 monomers long, to the membrane. This result shed some light on the polymerization state of FtsZ within the cytosol, which until then was completely unknown. Also we found out the attachments occurred at random locations along the length of the cell and showed treadmilling dynamics along with periods of rapid growth and shrinkage. Their dynamic properties showed that treadmilling dynamics are not exclusive to the Z-ring filaments but to any membrane-linked FtsZ filaments. Also these dynamics implied that protofilaments are able to cross-link early on in the cell cycle. Furthermore, we established that the size of the assemblies is sensitively controlled by the availability of FtsZ molecules and by the presence of ZapA proteins, which has been implicated in cross-linking protofilaments.

Accordingly, the likely function of these dynamic FtsZ assemblies is to sample the inner membrane for the proper location for Z-ring formation.

While this work have elucidated mechanisms leading to Z-ring formation, it has also brought up several new questions. Even though we now know that in slow growth conditions FtsZ concentration varies in a cell cycle dependent manner, we do not know if this variation also applies to other Z-ring components such as FtsZ membrane-linkers, FtsA and ZipA. Furthermore, though we know that early on in the cell cycle FtsZ filaments are able to bundle, it is not known how essential bundling is since deletion of ZapA, which promotes cross-linking between FtsZ filaments, from the cells still resulted in proper Z-ring formation in most cells. This work has provided initial insight on the assembly process of the Z-ring, though there is no theoretical framework or computational model yet to support how broadly distributed FtsZ assemblies collapse into a dynamic Z-ring at the cell center. Despite these new questions, this work has left us with a better understanding of the essential role FtsZ plays during cell division in bacteria and that this protein presents itself as a potential target for new antibiotics.

REFERENCES

1. Hurley KA, *et al.* (2016) Targeting the Bacterial Division Protein FtsZ. *J Med Chem* 59(15):6975-6998.
2. Hughes D & Karlen A (2014) Discovery and preclinical development of new antibiotics. *Upsala journal of medical sciences* 119(2):162-169.
3. Bi EF & Lutkenhaus J (1991) FtsZ ring structure associated with division in *Escherichia coli*. *Nature* 354(6349):161-164.
4. Dai K & Lutkenhaus J (1991) ftsZ is an essential cell division gene in *Escherichia coli*. *J. Bacteriol.* 173(11):3500-3506.
5. Sundararajan K, *et al.* (2015) The bacterial tubulin FtsZ requires its intrinsically disordered linker to direct robust cell wall construction. *Nat Commun* 6:7281.
6. Ausmees N, Kuhn JR, & Jacobs-Wagner C (2003) The bacterial cytoskeleton: an intermediate filament-like function in cell shape. *Cell* 115(6):705-713.
7. Gitai Z, Dye N, & Shapiro L (2004) An actin-like gene can determine cell polarity in bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 101(23):8643-8648.
8. Du S & Lutkenhaus J (2019) At the Heart of Bacterial Cytokinesis: The Z Ring. *Trends Microbiol* 27(9):781-791.
9. Adams DW & Errington J (2009) Bacterial cell division: assembly, maintenance and disassembly of the Z ring. *Nat Rev Microbiol* 7(9):642-653.
10. den Blaauwen T, Andreu JM, & Monasterio O (2014) Bacterial cell division proteins as antibiotic targets. *Bioorg Chem* 55:27-38.
11. Soderstrom B & Daley DO (2017) The bacterial divisome: more than a ring? *Curr Genet* 63(2):161-164.

12. Egan AJF & Vollmer W (2013) The physiology of bacterial cell division. *Antimicrobial Therapeutics Reviews: The Bacterial Cell Wall as an Antimicrobial Target*, Annals of the New York Academy of Sciences, ed Bush K (Blackwell Science Publ, Oxford), Vol 1277, pp 8-28.
13. Haeusser DP & Margolin W (2016) Splitsville: structural and functional insights into the dynamic bacterial Z ring. *Nat Rev Microbiol* 14(5):305-319.
14. Lutkenhaus J, Pichoff S, & Du S (2012) Bacterial cytokinesis: From Z ring to divisome. *Cytoskeleton (Hoboken)* 69(10):778-790.
15. Meier EL & Goley ED (2014) Form and function of the bacterial cytokinetic ring. *Curr Opin Cell Biol* 26:19-27.
16. Osawa M & Erickson HP (2013) Liposome division by a simple bacterial division machinery. *Proc. Natl. Acad. Sci. U. S. A.* 110(27):11000-11004.
17. Hedges SB (2002) The origin and evolution of model organisms. *Nat Rev Genet* 3(11):838-849.
18. Lee PS & Lee KH (2003) Escherichia coli--a model system that benefits from and contributes to the evolution of proteomics. *Biotechnol Bioeng* 84(7):801-814.
19. Wagstaff J & Lowe J (2018) Prokaryotic cytoskeletons: protein filaments organizing small cells. *Nat Rev Microbiol* 16(4):187-201.
20. Mannik J, Walker BE, & Mannik J (2018) Cell cycle-dependent regulation of FtsZ in Escherichia coli in slow growth conditions. *Mol. Microbiol.* 110(6):1030-1044.

21. Erickson HP, Anderson DE, & Osawa M (2010) FtsZ in bacterial cytokinesis: cytoskeleton and force generator all in one. *Microbiol Mol Biol Rev* 74(4):504-528.
22. Ortiz C, Natale P, Cueto L, & Vicente M (2016) The keepers of the ring: regulators of FtsZ assembly. *FEMS Microbiol Rev* 40(1):57-67.
23. Rothfield L, Justice S, & Garcia-Lara J (1999) Bacterial cell division. *Annu Rev Genet* 33:423-448.
24. Sanchez M, Valencia A, Ferrandiz MJ, Sander C, & Vicente M (1994) Correlation between the Structure and Biochemical Activities of Ftsa, an Essential Cell-Division Protein of the Actin Family. *Embo Journal* 13(20):4919-4925.
25. Erickson HP (1995) FtsZ, a prokaryotic homolog of tubulin? *Cell* 80(3):367-370.
26. Löwe J & Amos LA (2017) *Prokaryotic Cytoskeletons: Filamentous Protein Polymers Active in the Cytoplasm of Bacterial and Archaeal Cells* (Springer International Publishing).
27. Cook WR & Rothfield LI (1999) Nucleoid-independent identification of cell division sites in *Escherichia coli*. *J. Bacteriol.* 181(6):1900-1905.
28. Szwedziak P, Wang Q, Freund SM, & Lowe J (2012) FtsA forms actin-like protofilaments. *EMBO J* 31(10):2249-2260.
29. Loose M & Mitchison TJ (2014) The bacterial cell division proteins FtsA and FtsZ self-organize into dynamic cytoskeletal patterns. *Nat Cell Biol* 16(1):38-46.

30. Coltharp C, Buss J, Plumer TM, & Xiao J (2016) Defining the rate-limiting processes of bacterial cytokinesis. *Proc. Natl. Acad. Sci. U. S. A.* 113(8):E1044-1053.
31. Lyu Z, Coltharp C, Yang X, & Xiao J (2016) Influence of FtsZ GTPase activity and concentration on nanoscale Z-ring structure in vivo revealed by three-dimensional Superresolution imaging. *Biopolymers* 105(10):725-734.
32. Holden SJ, *et al.* (2014) High throughput 3D super-resolution microscopy reveals *Caulobacter crescentus* in vivo Z-ring organization. *Proc. Natl. Acad. Sci. U. S. A.* 111(12):4566-4571.
33. Rowlett VW & Margolin W (2014) 3D-SIM super-resolution of FtsZ and its membrane tethers in *Escherichia coli* cells. *Biophys J* 107(8):L17-L20.
34. Huang KH, Durand-Heredia J, & Janakiraman A (2013) FtsZ ring stability: of bundles, tubules, crosslinks, and curves. *J. Bacteriol.* 195(9):1859-1868.
35. Mannik J & Bailey MW (2015) Spatial coordination between chromosomes and cell division proteins in *Escherichia coli*. *Front Microbiol* 6:306.
36. Liu B, Persons L, Lee L, & de Boer PA (2015) Roles for both FtsA and the FtsBLQ subcomplex in FtsN-stimulated cell constriction in *Escherichia coli*. *Mol. Microbiol.* 95(6):945-970.
37. Bailey MW, Bisicchia P, Warren BT, Sherratt DJ, & Mannik J (2014) Evidence for divisome localization mechanisms independent of the Min system and SlmA in *Escherichia coli*. *PLoS Genet* 10(8):e1004504.

38. Hill NS, Buske PJ, Shi Y, & Levin PA (2013) A Moonlighting Enzyme Links *Escherichia coli* Cell Size with Central Metabolism. *Plos Genetics*. 9(7). 10.1371/journal.pgen.1003663.
39. Huisman O, D'Ari R, & Gottesman S (1984) Cell-division control in *Escherichia coli*: specific induction of the SOS function SfiA protein is sufficient to block septation. *Proc. Natl. Acad. Sci. U. S. A.* 81(14):4490-4494.
40. Lan G, Wolgemuth CW, & Sun SX (2007) Z-ring force and cell shape during division in rod-like bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 104(41):16110-16115.
41. Osawa M, Anderson DE, & Erickson HP (2008) Reconstitution of contractile FtsZ rings in liposomes. *Science* 320(5877):792-794.
42. Den Blaauwen T, Buddelmeijer N, Aarsman ME, Hameete CM, & Nanninga N (1999) Timing of FtsZ assembly in *Escherichia coli*. *J. Bacteriol.* 181(17):5167-5175.
43. Mannik J, et al. (2012) Robustness and accuracy of cell division in *Escherichia coli* in diverse cell shapes. *Proceedings of the National Academy of Sciences of the United States of America* 109(18):6957-6962.
44. Trueba FJ (1982) On the Precision and Accuracy Achieved by *Escherichia-Coli*-Cells at Fission About Their Middle. *Arch. Microbiol.* 131(1):55-59.
45. Lutkenhaus J (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. *Annual Review of Biochemistry*, Annual Review of Biochemistry), Vol 76, pp 539-562.

46. Lutkenhaus J (2012) The ParA/MinD family puts things in their place. *Trends Microbiol* 20(9):411-418.
47. Moseley JB & Nurse P (2010) Cell division intersects with cell geometry. *Cell* 142(2):184-188.
48. Shapiro L, McAdams HH, & Losick R (2009) Why and how bacteria localize proteins. *Science* 326(5957):1225-1228.
49. Raskin DM & de Boer PA (1999) Rapid pole-to-pole oscillation of a protein required for directing division to the middle of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 96(9):4971-4976.
50. Hu Z & Lutkenhaus J (1999) Topological regulation of cell division in *Escherichia coli* involves rapid pole to pole oscillation of the division inhibitor MinC under the control of MinD and MinE. *Mol. Microbiol.* 34(1):82-90.
51. Hale CA, Meinhardt H, & de Boer PA (2001) Dynamic localization cycle of the cell division regulator MinE in *Escherichia coli*. *EMBO J* 20(7):1563-1572.
52. Fu X, Shih YL, Zhang Y, & Rothfield LI (2001) The MinE ring required for proper placement of the division site is a mobile structure that changes its cellular location during the *Escherichia coli* division cycle. *Proc. Natl. Acad. Sci. U. S. A.* 98(3):980-985.
53. Kretschmer S & Schwille P (2016) Pattern formation on membranes and its role in bacterial cell division. *Curr Opin Cell Biol* 38:52-59.

54. Huang KC, Meir Y, & Wingreen NS (2003) Dynamic structures in *Escherichia coli*: spontaneous formation of MinE rings and MinD polar zones. *Proc. Natl. Acad. Sci. U. S. A.* 100(22):12724-12728.
55. Meinhardt H & de Boer PA (2001) Pattern formation in *Escherichia coli*: a model for the pole-to-pole oscillations of Min proteins and the localization of the division site. *Proc. Natl. Acad. Sci. U. S. A.* 98(25):14202-14207.
56. Woldringh CL, *et al.* (1990) Role of the nucleoid in the toporegulation of division. *Res. Microbiol.* 141(1):39-49.
57. Hussain K, Begg KJ, Salmond GP, & Donachie WD (1987) ParD: a new gene coding for a protein required for chromosome partitioning and septum localization in *Escherichia coli*. *Mol. Microbiol.* 1(1):73-81.
58. Mulder E & Woldringh CL (1989) Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. *Journal of Bacteriology* 171(8):4303-4314.
59. Wu LJ & Errington J (2011) Nucleoid occlusion and bacterial cell division. *Nature Reviews Microbiology* 10(1):8-12.
60. Bernhardt TG & de Boer PA (2005) SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over Chromosomes in *E. coli*. *Mol Cell* 18(5):555-564.
61. Cho H & Bernhardt TG (2013) Identification of the SlmA active site responsible for blocking bacterial cytokinetic ring assembly over the chromosome. *PLoS Genet* 9(2):e1003304.

62. Cho HB, McManus HR, Dove SL, & Bernhardt TG (2011) Nucleoid occlusion factor SlmA is a DNA-activated FtsZ polymerization antagonist. *Proceedings of the National Academy of Sciences of the United States of America* 108(9):3773-3778.
63. Du SS & Lutkenhaus J (2014) SlmA antagonism of FtsZ assembly employs a two-pronged mechanism like MinCD. *Plos Genetics*. 10(7).
10.1371/journal.pgen.1004460.
64. Tonthat NK, *et al.* (2011) Molecular mechanism by which the nucleoid occlusion factor, SlmA, keeps cytokinesis in check. *Embo Journal* 30(1):154-164.
65. Tonthat NK, *et al.* (2013) SlmA forms a higher-order structure on DNA that inhibits cytokinetic Z-ring formation over the nucleoid. *Proceedings of the National Academy of Sciences of the United States of America* 110(26):10586-10591.
66. Espeli O, *et al.* (2012) A MatP-divisome interaction coordinates chromosome segregation with cell division in E. coli. *EMBO J* 31(14):3198-3211.
67. Mercier R, *et al.* (2008) The MatP/matS site-specific system organizes the terminus region of the E. coli chromosome into a macrodomain. *Cell* 135(3):475-485.
68. Buss J, *et al.* (2015) A multi-layered protein network stabilizes the Escherichia coli FtsZ-ring and modulates constriction dynamics. *PLoS Genet* 11(4):e1005128.

69. Miraldi ER, Thomas PJ, & Romberg L (2008) Allosteric models for cooperative polymerization of linear polymers. *Biophys J* 95(5):2470-2486.
70. Caplan MR & Erickson HP (2003) Apparent cooperative assembly of the bacterial cell division protein FtsZ demonstrated by isothermal titration calorimetry. *J. Biol. Chem.* 278(16):13784-13788.
71. Chen Y, Bjornson K, Redick SD, & Erickson HP (2005) A rapid fluorescence assay for FtsZ assembly indicates cooperative assembly with a dimer nucleus. *Biophys J* 88(1):505-514.
72. Rivas G, *et al.* (2000) Magnesium-induced linear self-association of the FtsZ bacterial cell division protein monomer. The primary steps for FtsZ assembly. *J. Biol. Chem.* 275(16):11740-11749.
73. Wagstaff JM, *et al.* (2017) A Polymerization-Associated Structural Switch in FtsZ That Enables Treadmilling of Model Filaments. *mBio* 8(3):16.
74. Erickson HP, Anderson DE, & Osawa M (2010) FtsZ in bacterial cytokinesis: cytoskeleton and force generator all in one. *Microbiol. Mol. Biol. Rev.* 74(4):504-528.
75. Walker BE, Mannik J, & Mannik J (2020) Transient Membrane-Linked FtsZ Assemblies Precede Z-Ring Formation in Escherichia coli. *Curr Biol* 30(3):499-508 e496.
76. Li Z, Trimble MJ, Brun YV, & Jensen GJ (2007) The structure of FtsZ filaments in vivo suggests a force-generating role in cell division. *EMBO J.* 26(22):4694-4708.

77. Yao Q, *et al.* (2017) Short FtsZ filaments can drive asymmetric cell envelope constriction at the onset of bacterial cytokinesis. *EMBO J* 36(11):1577-1589.
78. Pollard TD (1986) Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J Cell Biol* 103(6 Pt 2):2747-2754.
79. Phillips R, Kondev J, Theriot J, & Garcia H (2012) *Physical Biology of the Cell* (CRC Press).
80. Du S, Pichoff S, Kruse K, & Lutkenhaus J (2018) FtsZ filaments have the opposite kinetic polarity of microtubules. *Proc. Natl. Acad. Sci. U. S. A.* 115(42):10768-10773.
81. Romberg L, Simon M, & Erickson HP (2001) Polymerization of Ftsz, a bacterial homolog of tubulin. is assembly cooperative? *J. Biol. Chem.* 276(15):11743-11753.
82. Moore DA, Whatley ZN, Joshi CP, Osawa M, & Erickson HP (2017) Probing for Binding Regions of the FtsZ Protein Surface through Site-Directed Insertions: Discovery of Fully Functional FtsZ-Fluorescent Proteins. *J. Bacteriol.* 199(1):e00553-00516.
83. Yang X, *et al.* (2017) GTPase activity-coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall synthesis. *Science* 355(6326):744-747.
84. Bisson Filho AW, *et al.* (2016) Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. *bioRxiv*.

85. Gillespie DT (1976) A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. *Journal of Computational Physics* 22(4):403-434.
86. Lan GH, Daniels BR, Dobrowsky TM, Wirtz D, & Sun SX (2009) Condensation of FtsZ filaments can drive bacterial cell division. *Proceedings of the National Academy of Sciences of the United States of America* 106(1):121-126.
87. Guan FH, *et al.* (2018) Lateral interactions between protofilaments of the bacterial tubulin homolog FtsZ are essential for cell division. *eLife*. 7. 10.7554/eLife.35578.
88. Durand-Heredia J, Rivkin E, Fan G, Morales J, & Janakiraman A (2012) Identification of ZapD as a cell division factor that promotes the assembly of FtsZ in *Escherichia coli*. *J. Bacteriol.* 194(12):3189-3198.
89. RayChaudhuri D (1999) ZipA is a MAP-Tau homolog and is essential for structural integrity of the cytokinetic FtsZ ring during bacterial cell division. *EMBO J* 18(9):2372-2383.
90. Hale CA, Rhee AC, & de Boer PA (2000) ZipA-induced bundling of FtsZ polymers mediated by an interaction between C-terminal domains. *J. Bacteriol.* 182(18):5153-5166.
91. Buss J, *et al.* (2013) In vivo organization of the FtsZ-ring by ZapA and ZapB revealed by quantitative super-resolution microscopy. *Mol. Microbiol.* 89(6):1099-1120.

92. Mattheyses AL, Simon SM, & Rappoport JZ (2010) Imaging with total internal reflection fluorescence microscopy for the cell biologist. *J Cell Sci* 123(Pt 21):3621-3628.
93. Tokunaga M, Imamoto N, & Sakata-Sogawa K (2008) Highly inclined thin illumination enables clear single-molecule imaging in cells. *Nat Methods* 5(2):159-161.
94. Broersma S (1960) Rotational Diffusion Constant of a Cylindrical Particle. *J Chem Phys* 32(6):1626-1631.
95. Moore DA, Whatley ZN, Joshi CP, Osawa M, & Erickson HP (2017) Probing for binding regions of the FtsZ protein surface through site-directed insertions: discovery of fully functional FtsZ-fluorescent proteins. *Journal of Bacteriology*. 199(1). 10.1128/jb.00553-16.
96. Yang D, Jennings AD, Borrego E, Retterer ST, & Männik J (2018) Analysis of factors limiting bacterial growth in PDMS mother machine devices. *Front. Microbiol.* 9(871). 10.3389/fmicb.2018.00871.
97. Moffitt JR, Lee JB, & Cluzel P (2012) The single-cell chemostat: an agarose-based, microfluidic device for high-throughput, single-cell studies of bacteria and bacterial communities. *Lab Chip* 12(8):1487-1494.
98. Wang P, et al. (2010) Robust growth of *Escherichia coli*. *Curr Biol* 20(12):1099-1103.

99. Paintdakhi A, *et al.* (2016) Oufiti: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. *Mol. Microbiol.* 99(4):767-777.
100. Paintdakhi A, *et al.* (2016) Oufiti: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. *Mol. Microbiol.* 99(4):767-777.
101. Dajkovic A, Mukherjee A, & Lutkenhaus J (2008) Investigation of regulation of FtsZ assembly by SulA and development of a model for FtsZ polymerization. *J. Bacteriol.* 190(7):2513-2526.
102. Erickson HP (2009) Modeling the physics of FtsZ assembly and force generation. *Proc. Natl. Acad. Sci. U. S. A.* 106(23):9238-9243.
103. Bachmann BJ (1972) Pedigrees of Some Mutant Strains of Escherichia-Coli K-12. *Bacteriological Reviews* 36(4):525-557.
104. Thomason LC, Costantino N, & Court DL (2007) E. coli genome manipulation by P1 transduction. *Curr Protoc Mol Biol* Chapter 1(1):Unit 1 17.
105. Adams JN & Luria SE (1958) Transduction by Bacteriophage P1: Abnormal Phage Function of the Transducing Particles. *Proc. Natl. Acad. Sci. U. S. A.* 44(6):590-594.
106. Kitagawa M, *et al.* (2005) Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. *DNA Res* 12(5):291-299.

107. Flynn JM, Neher SB, Kim YI, Sauer RT, & Baker TA (2003) Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. *Mol Cell* 11(3):671-683.
108. Camberg JL, Hoskins JR, & Wickner S (2009) ClpXP protease degrades the cytoskeletal protein, FtsZ, and modulates FtsZ polymer dynamics. *Proc. Natl. Acad. Sci. U. S. A.* 106(26):10614-10619.
109. Olivares AO, Baker TA, & Sauer RT (2018) Mechanical Protein Unfolding and Degradation. *Annu Rev Physiol* 80:413-429.
110. Camberg JL, Viola MG, Rea L, Hoskins JR, & Wickner S (2014) Location of dual sites in *E. coli* FtsZ important for degradation by ClpXP; one at the C-terminus and one in the disordered linker. *Plos One*. 9(4). 10.1371/journal.pone.0094964.
111. Gottesman S, Roche E, Zhou Y, & Sauer RT (1998) The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev* 12(9):1338-1347.
112. Balleza E, Kim JM, & Cluzel P (2018) Systematic characterization of maturation time of fluorescent proteins in living cells. *Nat Methods* 15(1):47-51.
113. Elowitz MB, Levine AJ, Siggia ED, & Swain PS (2002) Stochastic gene expression in a single cell. *Science* 297(5584):1183-1186.
114. Maillard RA, *et al.* (2011) ClpX(P) generates mechanical force to unfold and translocate its protein substrates. *Cell* 145(3):459-469.

115. Cordell SC, Robinson EJ, & Lowe J (2003) Crystal structure of the SOS cell division inhibitor SulaA and in complex with FtsZ. *Proc. Natl. Acad. Sci. U. S. A.* 100(13):7889-7894.
116. Rueda S, Vicente M, & Mingorance J (2003) Concentration and assembly of the division ring proteins FtsZ, FtsA, and ZipA during the Escherichia coli cell cycle. *J. Bacteriol.* 185(11):3344-3351.
117. Willis L & Huang KC (2017) Sizing up the bacterial cell cycle. *Nat Rev Microbiol* 15(10):606-620.
118. Golding I, Paulsson J, Zawilski SM, & Cox EC (2005) Real-time kinetics of gene activity in individual bacteria. *Cell* 123(6):1025-1036.
119. Kelly AJ, Sackett MJ, Din N, Quardokus E, & Brun YV (1998) Cell cycle-dependent transcriptional and proteolytic regulation of FtsZ in Caulobacter. *Genes Dev* 12(6):880-893.
120. Williams B, Bhat N, Chien P, & Shapiro L (2014) ClpXP and ClpAP proteolytic activity on divisome substrates is differentially regulated following the Caulobacter asymmetric cell division. *Mol. Microbiol.* 93(5):853-866.
121. Bates D & Kleckner N (2005) Chromosome and replisome dynamics in E. coli: loss of sister cohesion triggers global chromosome movement and mediates chromosome segregation. *Cell* 121(6):899-911.
122. Garrido T, Sanchez M, Palacios P, Aldea M, & Vicente M (1993) Transcription of ftsZ oscillates during the cell cycle of Escherichia coli. *EMBO J* 12(10):3957-3965.

123. Robin A, Joseleau-Petit D, & D'Ari R (1990) Transcription of the *ftsZ* gene and cell division in *Escherichia coli*. *J. Bacteriol.* 172(3):1392-1399.
124. Zhou P & Helmstetter CE (1994) Relationship between *ftsZ* gene expression and chromosome replication in *Escherichia coli*. *J. Bacteriol.* 176(19):6100-6106.
125. Dewar SJ, Kagan-Zur V, Begg KJ, & Donachie WD (1989) Transcriptional regulation of cell division genes in *Escherichia coli*. *Mol. Microbiol.* 3(10):1371-1377.
126. Collier J (2016) Cell cycle control in Alphaproteobacteria. *Curr Opin Microbiol* 30:107-113.
127. Dewar SJ & Dorazi R (2000) Control of division gene expression in *Escherichia coli*. *FEMS Microbiol Lett* 187(1):1-7.
128. Pazos M, Natale P, & Vicente M (2013) A specific role for the ZipA protein in cell division: stabilization of the FtsZ protein. *J. Biol. Chem.* 288(5):3219-3226.
129. LaBreck CJ, May S, Viola MG, Conti J, & Camberg JL (2017) The protein chaperone ClpX targets native and non-native aggregated substrates for remodeling, disassembly, and degradation with ClpP. *Frontiers in Molecular Biosciences.* 4(26). 10.3389/fmolb.2017.00026.
130. Stricker J, Maddox P, Salmon ED, & Erickson HP (2002) Rapid assembly dynamics of the *Escherichia coli* FtsZ-ring demonstrated by fluorescence recovery after photobleaching. *Proc. Natl. Acad. Sci. U. S. A.* 99(5):3171-3175.

131. Anderson DE, Gueiros-Filho FJ, & Erickson HP (2004) Assembly dynamics of FtsZ rings in *Bacillus subtilis* and *Escherichia coli* and effects of FtsZ-regulating proteins. *J. Bacteriol.* 186(17):5775-5781.
132. Thanedar S & Margolin W (2004) FtsZ exhibits rapid movement and oscillation waves in helix-like patterns in *Escherichia coli*. *Curr Biol* 14(13):1167-1173.
133. Mannik J, Castillo DE, Yang D, Siopsis G, & Mannik J (2016) The role of MatP, ZapA and ZapB in chromosomal organization and dynamics in *Escherichia coli*. *Nucleic Acids Res* 44(3):1216-1226.
134. Rowlett VW & Margolin W (2015) The Min system and other nucleoid-independent regulators of Z ring positioning. *Front. Microbiol.* 6. 10.3389/fmicb.2015.00478.
135. Vischer NOE, *et al.* (2015) Cell age dependent concentration of *Escherichia coli* divisome proteins analyzed with ImageJ and ObjectJ. *Front. Microbiol.* 6. 10.3389/fmicb.2015.00586.
136. Chen Y & Erickson HP (2005) Rapid in vitro assembly dynamics and subunit turnover of FtsZ demonstrated by fluorescence resonance energy transfer. *J. Biol. Chem.* 280(23):22549-22554.
137. Huecas S, *et al.* (2008) Energetics and geometry of FtsZ polymers: nucleated self-assembly of single protofilaments. *Biophys J* 94(5):1796-1806.
138. Pichoff S & Lutkenhaus J (2005) Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA. *Mol. Microbiol.* 55(6):1722-1734.

139. Monterroso B, *et al.* (2012) Mg(2+)-linked self-assembly of FtsZ in the presence of GTP or a GTP analogue involves the concerted formation of a narrow size distribution of oligomeric species. *Biochemistry* 51(22):4541-4550.
140. Si F, *et al.* (2019) Mechanistic Origin of Cell-Size Control and Homeostasis in Bacteria. *Curr Biol* 29(11):1760-1770 e1767.
141. Sekar K, *et al.* (2018) Synthesis and degradation of FtsZ quantitatively predict the first cell division in starved bacteria. *Mol. Syst. Biol.* 14(11):e8623.
142. Margolin W (2020) Bacterial Division: Journey to the Center of the Cell. *Curr Biol* 30(3):R114-R116.
143. Janmey PA, Peetermans J, Zaner KS, Stossel TP, & Tanaka T (1986) Structure and Mobility of Actin-Filaments as Measured by Quasi-Elastic Light-Scattering, Viscometry, and Electron-Microscopy. *Journal of Biological Chemistry* 261(18):8357-8362.
144. Mullineaux CW, Nenninger A, Ray N, & Robinson C (2006) Diffusion of green fluorescent protein in three cell environments in *Escherichia coli*. *J. Bacteriol.* 188(10):3442-3448.
145. Baba T, *et al.* (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* 2:2006 0008.
146. Moore DA, Whatley ZN, Joshi CP, Osawa M, & Erickson HP (2017) Probing for binding regions of the FtsZ protein surface through site-directed insertions: discovery of fully functional FtsZ-fluorescent proteins. *J. Bacteriol.* 199(1):17.

147. Datsenko KA & Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97(12):6640-6645.
148. Cherepanov PP & Wackernagel W (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158(1):9-14.

APPENDIX

A.1 Glossary

1. ATPase – large family of hydrolase enzymes that bind to adenosine triphosphate (ATP) and hydrolyzes it into adenosine diphosphate (ADP).
2. Autocorrelation function (ACF) – the correlation of a signal with a delayed copy of itself as a function of delay.
3. Constriction –inward invagination of the cellular envelope. Septum can be used synonymously.
4. Critical concentration – the minimum monomeric concentration needed for polymer formation.
5. Crosslinking – general term for short sequences of chemical bonds which join two polymers together.
6. Cytokinesis – process by which the mother cell divides into two daughter cells.
Synonymous with septation.
7. Cytoskeleton – network of filaments and tubules in the cytoplasm of all cells that provide the framework for the cellular shape and help in the process of cell division through moving around essential parts needed for septation.
8. Divisome –a large macromolecular structure which carries out septal cell wall synthesis in bacteria.
9. Fourier transform – the decomposition of a function into its harmonic frequency components.

10. FtsA – highly conserved actin-like protein that resides in the cytosol and binds to the membrane through its C-terminal amphipathic helix, which links FtsZ filaments to the membrane.
11. FtsZ – a highly conserved, tubulin-like, self-assembling GTPase protein that is the major component of the bacterial Z-ring. FtsZ filaments have been shown to treadmill.
12. GTPase – large family of hydrolase enzymes that bind to guanosine triphosphate (GTP) and hydrolyzes it into guanosine diphosphate (GDP).
13. GTP hydrolysis – the catabolic reaction process by which chemical energy that has been stored in the high-energy phosphate bonds in guanosine triphosphate is released by breaking the bond with a water molecule.
14. Hanning window – a specialized cosine-sum window function used to make the signal periodic and to reduce the ringing in the frequency domain.
15. *In vitro* – an experiment or process performed outside a living organism in a test tube, petri dish, lipid bilayers, liposomes, etc.
16. *In vivo* – an experiment or process performed inside of a living organism.
17. Kymograph – a plot that represents the spatial position of fluorescent intensity on the y-axis over time (x-axis). The y-axis refers to either the long or short axis of the cell. Accordingly, we represent the higher and lower intensities as a heat map. Red corresponds to high concentrations while blue corresponds to low concentrations of intensity and Black refers to outside of the cell.

18. Min system – an oscillatory system composed of the MinC, MinD, and MinE proteins, which prevent Z-ring formation at the poles of the cell.
19. Nucleation – the first initial steps in the formation of a new structure through self-assembly. Nucleation is a stochastic process.
20. Nucleoid – referring to the physiological state of the chromosomes within the cell. In bacteria the chromosomes are compacted by osmotic pressure by cytosolic proteins, supercoiling, and DNA binding proteins.
21. Nucleoid occlusion – a defense mechanism that prevents cell division at the vicinity of the unsegregated nucleoid.
22. Phase contrast – technique that converts phase shifts in light passing through a transparent specimen to the brightness changes within the image.
23. Photoactivation – process by which UV or near UV light changes the absorption and emission spectrum of the fluorescent molecule. Used in super-resolution fluorescence microscopy.
24. Photobleaching – loss of fluorescent signal due to illumination over a period of time.
25. Phototoxicity – damage to the cells due to light exposure.
26. Plasmid – A small (1-5 kilobases) circular DNA molecule within the cell that can independently replicate. Plasmids contain genes which can be expressed in the same way as chromosomal DNA.
27. Power spectral density (PSD) – the measure of the signal's power into frequency components.

28. Septal peptidoglycan – a polymer consisting of amino acids and sugars which form a mesh-like layer outside of the plasma membrane. This structure makes up the cell wall.
29. Septation – the event when one cell completes division into two daughter cells.
Synonymous with cytokinesis
30. Septum – inward growth or invagination of the division plane.
31. SlmA – a protein that binds to specific DNA sequences which are distributed throughout the chromosome except in the replication terminus region. SlmA prevents FtsZ filaments from forming the Z-ring over the nucleoid.
32. SulA – component of the cellular SOS system by way of binding to FtsZ, which lowers the pool of FtsZ available for assembly. Dissociates the Z-ring.
33. Ter-linkage – a mechanism that promotes Z-ring formation near the replication terminus region of the chromosome through a linkage between the Z-ring and the chromosome by ZapA, ZapB, and MatP proteins.
34. Treadmilling – is the phenomenon when one end of a polymer grows at the same rate as the other opposite end shrinks, creating a directional movement of the polymer.
35. Wild-type – referring to a strain of bacteria that contains no gene alterations from the genome. .
36. Zaps – Z-ring associated protein A, B, C, and D. These proteins are non-essential and are thought to help stabilize FtsZ filaments.

37. ZipA – an essential transmembrane protein that binds FtsZ monomers and filaments to the membrane.
38. Z-ring – initial cytoskeletal scaffold that recruits about 30 other proteins forming the divisome, which carries out cell division. The Z-ring in *E.coli* consists of FtsZ protofilaments, ZipA and FtsA membrane linkers, and ZapA-D proteins.

A.2 Diffusion coefficient calculation

Using the dimensions of a rod-like molecule, like FtsZ filaments, the diffusion coefficients can be calculated based on its length (L_i). Accordingly, the diffusion coefficient D_i is calculated for each filament length within a set range of $i = 2, \dots, N$ where N is the longest length in terms of monomers we wanted to examine. Here, we set $N = 25$ monomers due longer FtsZ filaments forming on membranes *in vitro* (142). To calculate D_i for each length, we used the Broersma's formula for a diffusing rigid rod which is given by (94, 143):

$$D_i = \frac{(k_B)(T_a)}{6\pi\eta L_i} (2\sigma - \gamma_1 - \gamma_2)$$

$$\sigma = \ln\left(\frac{2L_i}{d}\right)$$

$$\gamma_1 = 1.30 - \frac{8}{(\sigma - 0.30)^2}$$

$$\gamma_2 = 0.35 - \frac{4}{(\sigma - 0.43)^2}$$

where k_B is the Boltzman constant, T_a the absolute temperature (301.15°K or 28°C), η the viscosity of the cytoplasm in *E.coli* (0.0097 Pa·s) (144), and d the width of an FtsZ molecule (~4 nm) (21). The resulting distribution using these values is displayed below (Figure A.1). The distribution corresponds to an exponential decay of the diffusion rate as the filaments get longer. From this distribution, we see that FtsZ filaments ranging from 2 to 25 monomers in length can rapidly diffuse the entire cell body in a second, which is to be expected.

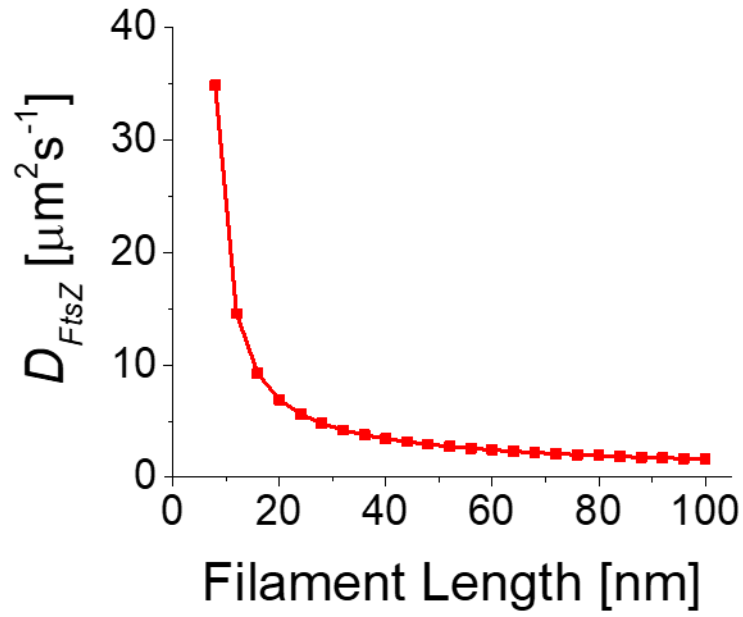


Figure A.2.1: Diffusion coefficients for a range of filament lengths. The diffusion coefficients were calculated using Broersma's formula for the diffusion of a rigid rod (94, 143). Note these coefficients correspond to the length of single subunit thick filaments i.e. no bundled filaments.

A.3 Coverslip cleaning procedure

During this work single molecule imaging was done. This requires very few molecules to be fluorescent at a time, which results in low fluorescence readings. Knowing that the glass slide itself had imperfections which would fluoresce at high imaging rates, we rigorously cleaned the coverslips to prevent this auto-fluorescence. Our procedure steps for coverslip cleaning were as follows:

1. Create 5M NaOH 100mL solution in glass beaker under the fume hood. Make sure to have long pants, lab coat, chemical apron, goggles, and gloves under hazardous chemical gloves on when making and using the NaOH solution.
2. Measure out 20g of NaOH (For 100mL solution at 5M requires 20g NaOH).
3. Add 80mL DI water and stirring rod to the glass beaker. Then slowly put a few pellets of NaOH in at a time and make sure the few dissolve first before you add more.
4. Once all pellets of NaOH are dissolved add the necessary amount of DI water to get a total volume of 100mL
5. Store the 5M NaOH solution in the **proper corrosive container**.

Note: Solution can be reused after making initial amount. Properly dispose of solution and container if container is damaged. See Safety Data Sheets (SDS) for more information.

6. Fill the teflan holder with coverslips.
7. Put the teflan holder with coverslips into a glass beaker.

8. With the NaOH solution complete pour enough solution into the glass beaker to cover the holder and coverslips completely.
9. Sonicate coverslips in the 5M NaOH solution for 1 hour
10. Sonicate coverslips in DI water for 30 minutes
11. Sonicate coverslips in fresh DI water for 30 minutes
12. Sonicate coverslips in IPA for 30 minutes and store in glass beaker with IPA

A.4 P1 transduction procedure

As part of this work, cells underwent genetic manipulation through a P1 transduction process. P1 is a bacteriophage that infects a bacterium (donor) and packages the bacterial DNA, along with its own genome, into its capsid until the capsid is completely full. Each P1 phage packages 100kb of DNA, which is more than enough to transduce the desired sequence of genes, which is normally antibiotic resistance genes that have replaced certain protein sequences). Then another strain of bacteria (recipient) is infected with a solution containing these bacterial DNA filled P1 phages (P1 lysate), which leads to homologous recombination of the DNA sequence from the donor's chromosome to the recipient's chromosome. Our procedure steps for P1 transduction were as follows:

1. Grow an overnight of the recipient M9 strain.
2. Transfer pellet from the overnight M9 culture to equal amount of LB medium
3. Add 200uL of LB-transferred cells to four microfuge tubes.
4. Add 2uL of 1M CaCl_2 to each tube.
5. Add 50uL LB to one tube (= negative control). Add 1, 10, and 50uL P1 lysate to one of the remaining tubes (optimal volume may vary).
6. Incubate at 37C for 30 minutes (no shaking).
7. Add 100uL 1M Na-citrate (prevents superinfection) and 500 uL LB to each of the tubes.
8. Incubate ~45 minutes (for transducing dominant antibiotic resistance).
9. Pellet cells: 8000RPM for 2 minutes.
10. Resuspend in 100uL M9 + 5mM citrate.
11. Plate on selective M9 medium. Additional negative control: plate P1 lysate alone.

A.5 List of strain information

HE1 strain received from Dr. H. Erickson Lab.

JM26 was constructed by P1 transduction of the Δ motA743::kan allele from Keio collection (145) strain JW1879-2 into BW27783 ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen (146) (a gift from Dr. H. Erickson).

JM60 was created by transformation of BW27783 strain with plasmid JW0093 (ColEI, PT5-lac::6xHis-ftsZ-gfp cm (83)) (gift from Dr. J. Xiao)

KC606 strain received from Dr. J. Xiao Lab (83).

BEW1 was constructed by P1 transduction of the Δ minC765::kan allele from Keio collection strain JW1165 into BW27783 ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen.

BEW2 constructed by P1 transduction of the Δ zapAA761::kan allele from Keio collection strain JW2878-1 into BW27783 ftsZ::ftsZ⁵⁵⁻⁵⁶-mNeonGreen.

MB43 was created by transformation of BW27783 ftsZ::ftsZ⁵⁵⁻⁵⁶-NeonGreen strain with pA3 plasmid containing *sulA* sequence (101) (gift from Dr. A. Dajkovic).

JM71 was constructed by λ -Red engineering (147) of the C-terminal fusion of mNeonGreen to LacY using the strain MG1655. First, the mNeonGreen (mNG) gene sequence was amplified from the strain JM26 and cloned into pROD62 plasmid (a gift from Dr. R. Reyes-Lamothe) using Gibson assembly protocol according to manufacturer's instructions (New England BioLabs Inc). The resulting template plasmid carries a copy of mNG followed by kanamycin resistance cassette flanked by *frr* sites and a flexible linker peptide SAGSAAGSGEF between mNG and the protein targeted. Primers carrying 40nt tails with identical sequence to the chromosomal locus for insertion were used to amplify

the linker -mNG-*frt*-kan^R-*frt* from template plasmid. The resulting PCR product was transformed by electroporation into a strain carrying the λ -Red -expressing plasmid pKD46. Colonies were selected by kanamycin resistance and fluorescence microscopy.

HE5 strain received from Dr. H. Erickson lab. Described in (146).

JM95 KC606 were transduced with P1 viral lysate of ZapA deletion strain from Keio collection JW2878-1. This strain is CM^R at 25 and Kan^R at 30 ug/ml.

JM65 was constructed by P1 transduction of the Δ clpA783::kan allele from Keio collection strain JW0866-1 into JM26 strain after removal of the kanamycin resistance cassette flanked by the Fip recognition target from the gene deletion site using the pCP20 plasmid (148).

JM66 was constructed by P1 transduction of the Δ clpP723::kan allele from Keio collection strain JW0427-1 into JM26 strain after removal of the kanamycin resistance cassette.

JM67 was constructed by P1 transduction of the Δ clpX724::kan allele from Keio collection strain JW0428-1 into JM26 strain after removal of the kanamycin resistance cassette.

VITA

Bryant Edward Walker was born on November 18, 1989 in Knoxville, Tennessee to Gerald and Triesta Walker of Knoxville. Bryant has an older sister Jennifer Walker who always competed against him as they were growing up. His family has lived in Knoxville his entire life and has lived in the same house too. He attended elementary school at West Hills where he would soon meet two of his lifelong friends who he still hangs out with today. Then he attended Bearden Middle and Bearden High school where he graduated in 2008. Growing up Bryant was always into sports and was a member of the varsity baseball team. This led him to the next step in his life of pursuing a baseball career. After high school, he attended Roane State Community College where he was the star athlete on their baseball team, while maintaining a 3.95 GPA. He received his Associates of Science degree in 2010. While at Roane State he was recruited by the Oakland Athletics for a career in professional baseball. However, knowing that his education was also important and that baseball may not work out, he decided to get another year of baseball experience and schooling under his belt.

Bryant then attended King College (University today) where he also received full academic and athletic scholarships. While at King College, he decided to major in physics because he wanted to attend medical school in the pursuit of being an orthopedic if baseball did not work out and it was also one of the most challenging disciplines, which he loved challenges. Unfortunately, he suffered a major shoulder injury which repelled all the professional scouts who were pursuing him without even questioning the injury. Ironically this is how the professional sports world works most of the time. In light of that,

Bryant maintained an astonishing 4.0 GPA while juggling academics with baseball at King. He received his Bachelor of Science in physics in 2012 with distinction.

After graduating, Bryant took a year off to try to figure out what he was going to do now that a baseball career was most likely not going to happen anymore. During this time he worked at Roane State Community College as a teaching assistant for the developmental math classes and also taught an engineering capstone class. While working, he applied to graduate and medical schools. He began graduate school at the University of Tennessee in the fall of 2013 where after realizing he still had a passion towards the medical field, joined Dr. Jaan Mannik's Biophysics Laboratory in the spring of 2015. There, Bryant studied the self-assembly dynamics of the bacterial Z-ring, which is highly conserved among bacteria and a potential target for new antibiotics. During this process, Bryant has developed many friendships that hopefully will last him the rest of his life. He would like to pursue a career where he can help others, whether that is staying in the field of Biophysics for pharmaceutical and cancer research or going a more clinical route in medical physics or even going to medical school. In his off time, Bryant loves to golf and fish with his father and friends. He would also like to have a family one day where he can pass down his knowledge. Overall, the future looks bright for this young, so wherever life takes him, he will be successful in all of his endeavors.