Doctoral thesis in Biophysics
Stockholm University
Sweden

Fluorescence Studies of Cell Division in *Escherichia coli*

Bill Söderström

Stockholm University

Fluorescence Studies of Cell Division in *Escherichia coli*

Bill Söderström

Cover: Pseudo time lapse image of dividing *E. coli* captured by Structured Illumination Microscopy. Reprinted with permission.

©Bill Söderström, Stockholm University 2014

ISBN 978-91-7447-852-5

Printed in Sweden by US-AB, Stockholm 2014
Distributor: Department of Biochemistry and Biophysics, Stockholm University

Till Yoshi & Angie,
mina små änglar

List of Publications

Paper I

Karl Skoog*, Bill Söderström*, Jerker Widengren, Gunnar von Heijne and Daniel O. Daley

Sequential closure of the cytoplasm and then the periplasm during cell division in *Escherichia coli*.
J Bacteriol. 2012;**194(3)** 584-586
* authors contributed equally to this work

Paper II

Bill Söderström, Karl Skoog, Hans Blom, David Weiss, Gunnar von Heijne and Daniel O. Daley

Disassembly of the divisome in *Escherichia coli*: Evidence that FtsZ dissociates before compartmentalization.
Mol Microbiol. 2014;**92(1)** 1-9

Paper III

Bill Söderström, Stephen Toddo, Gunnar von Heijne and Daniel O. Daley

ZipA and FtsA disassemble prior to FtsQ, FtsL and FtsI during divisome disassembly. *Manuscript.*

Paper IV

Stephen Toddo, Bill Söderström, Isolde Palombo, Gunnar von Heijne, Morten H Nørholm and Daniel O. Daley

Application of split-green fluorescent protein for topology mapping membrane proteins in *Escherichia coli.*
Protein Sci. 2012;**21(10)** 1571-1576

Additional publications by the Author
(not included in the thesis)

Anna Hjelm, Bill Söderström, David Vikström, Wouter Jong, Joen Luirink and Jan-Willem de Gier

Autotransporter based antigen display in bacterial ghosts.
Manuscript.

Abstract

In *Escherichia coli* the cell division is carried out by a large dynamic protein complex called the divisome. The divisome assembles in a two-step manner starting with the localization of the eukaryotic tubulin homologue FtsZ to the midcell. Together with other early arriving proteins FtsZ form an intermediate structure called the Z-ring. After a considerable time lag the divisome maturates fully by recruiting several other late arriving proteins before it starts to constrict the cell envelope that ultimately will lead to cytokinesis and the formation of two identical daughter cells. Despite of being objectives of extensive study over the last decades, understanding of the exact molecular roles of many of the divisome proteins is still lacking and to date there is very limited knowledge of the disassembly process of the divisome. In this thesis I have used various fluorescence microscopy based methods to better characterize the role of FtsZ and other divisome proteins during the final stages of the cell division. I have shown that FtsZ disassembles from the divisome prior to inner membrane closure indicating that it is not the force generator during this final step of division that it is widely thought to be. I have also shown that the disassembly of the divisome is a multistep process in which the proteins that arrive in the second step of divisome assembly also remain at the division septum longer than those proteins that arrive in the first step. These findings add new important information regarding the cell division and together they provide a more complete picture of this event that ultimately may lead to more efficient identification of novel antibiotic targets.

Abbreviations

AFM	Atomic Force Microscopy
BiFC	Bimolecular Fluorescence Complementation
CL	Cardiolipin
ECT	Electron Cryo-Tomography
EM	Electron Microscopy
FRAP	Fluorescence Recovery After Photobleaching
GFP	Green Fluorescent protein
isc	intersystem crossing
PALM	Photoactivated Localization Microscopy
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
SIM	Structured Illumination Microscopy
SNOM	Scanning Near field Optical Microcopy
STED	Stimulated Emission Depletion
STORM	Stochastic Optical Reconstruction Microscopy
TAT	Twin-Arginine translocation
TEM	Transmission Electron Microscopy

Contents

List of publications……..……..……..……..ii

Abstract……..……..……iv

Abbreviations…….……..……..v

Contents……..……..……vi

Introduction to the thesis.....12

Fluorescence and fluorescent proteins.....16
Fluorescent Proteins.....19
Split GFP.....21
Split GFP as a membrane protein topology reporter.....22

Methodology.....26
Fluorescence Microscopy.....26
Structured Illumination Microscopy.....30
Fluorescence Recovery After Photobleaching.....32
FRAP as a tool for monitoring cell division in Bacteria.....33

The Model System.....36
Escherichia coli.....36
The cell envelope.....36
Cell Division.....38
Spatial modulators of the divisome.....39
Divisome assembly.....42
Visualizing the Z-ring.....46
Divisome disassembly.....48

Conclusions and unpublished observations.....51

Future prospects.....54

Short summary of the publications included in this thesis.....60
Paper I.....60
Sequential closure of the cytoplasm and then the periplasm during cell division in *Escherichia coli*.....60

Paper II 61
Disassembly of the divisome in *Escherichia coli*: Evidence that FtsZ dissociates before compartmentalization 61
Paper III 62
ZipA and FtsA disassemble prior to FtsQ, FtsL and FtsI during divisome disassembly. 62
Paper IV 63
Application of split-green fluorescent protein for topology mapping membrane proteins in *Escherichia coli* 63

Populärvetenskaplig sammamfattning på Svenska 66

Acknowledgements 68

References 71

Introduction to the thesis

- In the first part of the thesis I will give a brief introduction to fluorescence and fluorescent proteins.

- In the second part I will introduce the methods that were used in the thesis.

- In the third part I will describe the model system (*i.e. Escherichia coli*), with emphasis on the cell envelope and the cell cycle.

- Lastly, a short summary of the papers included in this thesis will be presented.

Part I

Fluorescence and fluorescent proteins

Phenomena that involve the emission of light are collectively termed luminescence, of these two processes are governed by photon excitation and are therefore termed photoluminescence. In both these processes the molecule of interest, the fluorophore, absorbs energy provided by the incident photons and is excited from its ground state S_0 (*i.e.* the least energetic state) to a higher energy state S_1 (Figure 1, green arrow). Within a higher energy state the molecule may exist in different vibrational states and the excitation usually occurs to one of these higher vibrational states of S_1. Following internal conversion down to the lowest energy vibrational state of the first excited state (Figure 1, yellow arrow) the relaxation down to the ground state may occur in different ways, where the most probable event of spontaneous de-excitation from S_1 to S_0 bears the name Fluorescence (Figure 1, red arrow). The life time of S_1 is usually in the order of nanoseconds, and this is much faster than other possible decay rates (*e.g.* relaxation by heat dissipation). Therefore fluorescence (emission of a photon) will be the dominant process for molecules with high quantum efficiency.

The other process governed by photoluminescence involves the low probability event of intersystem crossing (isc) due to spin conversion from S_1 to the first excited triplet state T_1 (Figure 1, orange arrow). The transition from T_1 to S_1 is quantum mechanically forbidden and therefore rates of triplet emission are orders of magnitudes smaller than for singlet emission. The subsequent delayed de-excitation (10^{-4} – 10^{2} seconds) from the triplet state is known as phosphorescence (Figure 1, purple arrow). Phosphorescence will not be covered in this thesis.

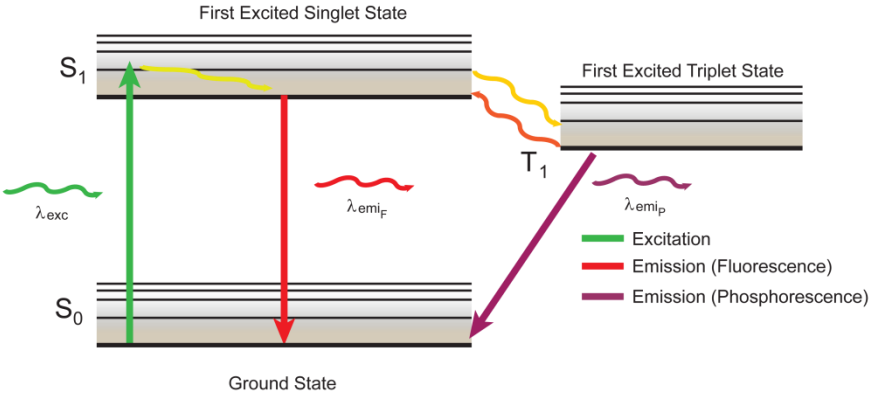

Figure 1; Simplified Jablonski diagram depicting the principle of the excitation and emission cycle of a fluorophore. A molecule absorbs energy (λ_{exc}) and undergoes electronic excitation from the ground state S_0 to a vibrational state in the first excited state S_1 (green, ~ 10^{-15} s). Following vibrational relaxation down to the lowest vibrational state of the first excited state (yellow, 10^{-12}-10^{-10} s) the molecule either emits a photon (λ_{emi_F}) through de-excitation (red, fluorescence, 10^{-10}-10^{-7} s) or undergoes the low probability event of intersystem crossing occurs (orange, 10^{-10}-10^{-6} s) to the lowest energy triplet state T_1. Subsequent delayed relaxation from T_1 to S_0 (purple, 10^{-4}-10^{2} s) is known as phosphorescence (λ_{emi_P}).

Due to vibrational energy loss when the molecule is residing in state S_1 the emitted photon will carry less energy than the incident.

The relationship between the energy and the wavelength is described by $\Delta E = \frac{hc}{\lambda}$ (here h is Plank's constant ~ $6{,}6261 \cdot 10^{-34} Js$, c is the speed of light = $3 \cdot 10^{8} ms^{-1}$ and λ = wavelength) and thus will the color of the emitted light that is observed always be red shifted (longer wavelengths, less energetic) when compared to the incident, $\lambda_{in} < \lambda_{out}$. This phenomenon was first observed in 1852 by Sir G. G. Stokes and the shift in wavelengths between incoming and outgoing light is now named after him (Stokes shift). What Stokes observed and later summarized in a paper[¥] was that in his experiment a (thin layer of a) solution of Quinine was able to absorb the

[¥] *On the Change of Refrangibility of Light*, Philosophical Transactions of the Royal Society of London,1852.

ultra violet radiation from the sun and then that the subsequent emission was blue, which implied that the wavelength of the emitted light was longer when compared to that of the incident light. In the same paper Stokes also introduced the very term fluorescence for the first time.

The relation between wavelength and color and the part of the electromagnetic spectrum used for fluorescence is depicted in Figure 2. The part of the electromagnetic spectrum the contains visible light is located between the ultraviolet and infrared parts of the electromagnetic spectrum where more blue shifted light contains more energy and is adjacent to the ultraviolet (UV) part, while more red shifted light is less energetic and is adjacent to the infrared (IR) part.

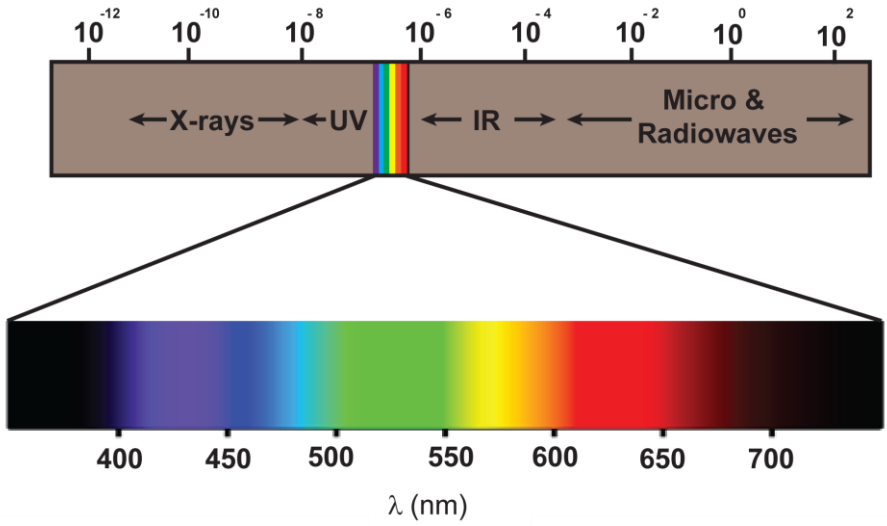

Figure 2; Top, The electromagnetic spectra, the inlet shows the part that contains the visible light. Numbers on top represents the wavelength in m. Bottom, Portion of the electromagnetic spectrum (between the UV and IR part of the spectrum) that contains the wavelengths that lay in the visible range. Light of blue shifted wavelengths are higher in energy than the red shifted.

There is a wide range of molecules (*e.g.* synthetic dyes and quantum dots) that may give raise the fluorescence, however in this thesis I will focus on fluorescent proteins.

Fluorescent Proteins

One of the most significant discoveries during the last century in biology was unquestionably the discovery of the green fluorescent protein (GFP) (Shimomura et al, 1962) from the jellyfish *Aequorea victoria*, a marine habitant living on the west coast of North America. However, it wasn't until 1994 that GFP had its real breakthrough, as a fluorescent indicator for gene expression (Chalfie et al, 1994). The structure of GFP was revealed to be a barrel composed of 11 β-strands surrounding a three amino-acid chromophore (Ormö et al, 1996). The original version of GFP was not particularly bright and therefore further development of the GPF molecule was necessary (especially important were two point substitutions at positions S65T and F64L), which resulted in an enhanced version (EGFP) that had improved folding characteristics, maturated well at 37 ºC , had a high extinction coefficient and thus permitted the practical use of GFPs in live cells (Cormack et al, 1996; Heim et al, 1995). EGFP has an absorption/emission spectrum with Abs_{max} at 488 nm and Emi_{max} around 512 nm. Soon after the characterization of (E)GFP (and its derivatives the Blue, Cyan and Yellow fluorescent proteins (Cubitt et al, 1995; Heim & Tsien, 1996; Ormö et al, 1996)) other molecules derived from different species but with similar structure as GFP were found that fluoresce at longer wavelengths. One of these was DsRed isolated from non-bioluminescent reef corals and true dual-color imaging was now possible using two fluorescent proteins (Matz et al, 1999). One issue with this protein however, was that it naturally tetramerized and therefore limited its use in certain biological applications. A few years later this issue was solved by a few amino-acid point substitutions to the DsRed molecule that effectively lead to the evolution of a truly monomeric red fluorescing protein (mRFP1) (Campbell et al, 2002). In 2004 Shaner et al. described the "fruit basket" (containing

mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, and mCherry) of fluorescent proteins all with improved photophysical properties compared to their predecessor (Shaner et al, 2004).

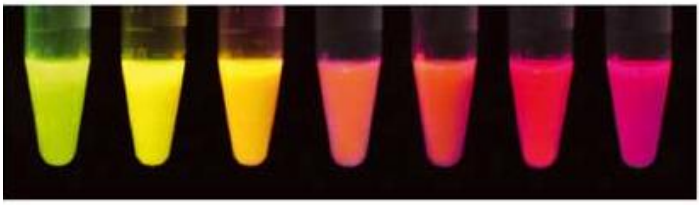

Figure 3; Purified fluorescent proteins derived from Discosoma sp. red fluorescent protein (from left to right, mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, and mCherry). Reprinted from (Shaner et al, 2004) with permission from Nature Publishing Group.

The wide range of available fluorescent proteins made it possible to image proteins in live cells in multiple colors (Shaner et al, 2005). The discovery of GFP and its derivatives proved to be an important enough finding to subsequently award its discoverers the Nobel Prize in Chemistry in 2008 (Nobel, 2008). The most advantageous feature of fluorescent proteins when compared to synthetic dyes is the fact that they can be produced from within the cell with remarkably little effect on cell viability. This has made it possible to monitor gene regulation and expression as well as single molecule detection in living cells (Elf et al, 2007; Yu et al, 2006). However, some caution has to be taken when working with the fluorescent proteins as they may interfere with the sub-cellular localization of the target protein (Margolin, 2012; Swulius & Jensen, 2012).

During the last decades there have been significant efforts to manipulate the photophysical properties, functions and/or structure of fluorescent proteins. One class of fluorescent proteins that have been developed is the photoactivatable fluorescent proteins (Patterson & Lippincott-Schwartz, 2002). These proteins may be photoswitched (going from a dark state to a bright state) or photoconverted (changing emission spectra, most commonly

from green to red) after being manipulated by various excitation schemes (Habuchi et al, 2005; Nienhaus et al, 2006). This development made it possible to develop sub diffraction limited single molecule localization microscopy, since it is possible to switch on (and off) one fluorescent molecule at a time.

Another modification to fluorescent proteins that not involves photophysics but rather the actual structure of the molecule itself has evolved from bimolecular fluorescence complementation (BiFC). BiFC is a technique in which complementary fragments of a fluorescent protein reporter are fused to putative interaction partners within a protein complex. The rationale behind BiFC assays is that protein-protein interaction can be easily monitored though the emission of fluorescence as the fragments of the reporter resembles to the native structure when in close proximity to one another (Ghosh et al, 2000). Initially BiFC assays often involved only the use of the yellow fluorescent protein (YFP) in mammalian cells while GFP was used in bacterial studies. The fluorescent protein was usually split in two almost equal sized fragments with the N-terminal part somewhat larger than the C-terminal part (154 vs. 84 amino acids) (Kerppola, 2006), but the toolbox has now increased to include 15 of the most commonly used fluorescent proteins that even may be split three ways (Cabantous et al, 2013; Kodama & Hu, 2012).

Split GFP

In our study a GFP molecule is split into one larger fragment (hereon called [1-10_{OPT}]) and a smaller fragment [11_{H7}], similar to (Cabantous et al, 2005). Were the larger fragment is composed of the first 10 β-stands of the whole GFP molecule and the smaller is the 11:th β-strand consisting of only 16

amino acids. The rationale is that the two fragments if let in close proximity of one another would spontaneously reassemble into one functional (fluorescent) molecule (Figure 4).

Figure 4; Principle of the Split GFP system. The large fragment (large black) consists of the first 10 β-sheets of the GFP molecule whereas the small fragment (small black) is the 11:th. Neither of the fragments is fluorescent when expressed separately. However, if the two fragments are expressed in close proximity of one another they will reassemble into one functional molecule. Reprinted from (Toddo et al, 2012) with permission from John Wiley and Sons.

Split GFP as a membrane protein topology reporter

During my Ph. D. studies I have used the split GFP system as a topology reporter for proteins in the inner membrane in *Escherichia coli*. The reporter systems that are currently in use involve large and bulky reporters that may interfere with both folding of the target protein and more importantly the correct insertion into the membrane (when fused to the N-terminus). This may lead to incorrect topology and an inactive protein. However, since the [11_{H7}] fragment is only 16 amino-acids long it can be fused to either the N- or C-terminus of the protein of interest without interfering with membrane insertion. The two GFP fragments are then expressed sequentially, first the target protein (containing the [11_{H7}]-tag) is expressed and then the [1-10_{OPT}] fragment. From ensemble fluorescence intensity measurements it is possible to determine the topology of the target protein and this is then confirmed at the single cell level as GFP fluorescence is detectable as a halo if the [11_{H7}] peptide and the [1-10_{OPT}] fragment is in the same compartment. One of the model protein used in our study was ZipA.

ZipA has a previously known C_{in}/N_{out} (where "in" refers to the cytoplasmic and "out" to the periplasmic side of the membrane) topology and is a protein that assembles into the Z-ring early in cell division and therefore accumulates that the mid-cell during division (Hale & de Boer, 1997). The fact that ZipA-[11_{H7}] can be seen accumulated at the midcell suggests that the protein is functional (Figure 5).

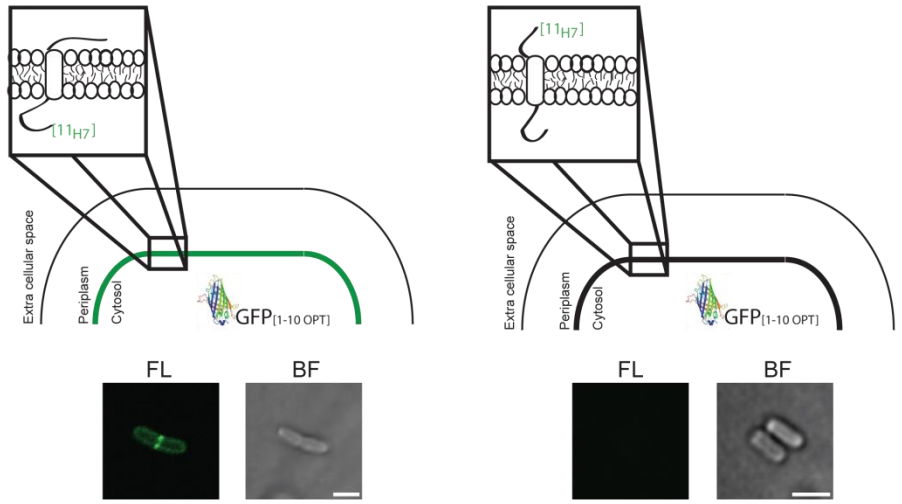

Figure 5; The Split-GFP system can be used for membrane protein topology determination in E. coli. *Top; When both the [1-10_{OPT}] and [11_{H7}] fragments are expressed in the same compartment of the cell the two fractions will reassemble into a functional (fluorescent) molecule (left). In contrast if the [1-10_{OPT}] and [11_{H7}] fragments are expressed in different compartments the fragments cannot reassemble and no fluorescence will be detected (right). Bottom; The model protein ZipA (with known topology where the C-terminus is facing the cytoplasm) was fused to [11_{H7}] as proof-of-principle. Left; ZipA-[11_{H7}]. Right; [11_{H7}]-ZipA. The accumulation of ZipA-GFP signal at the septum indicates that the fusion protein is functional. FL; fluorescence image. BF; bright field image. Scale bar 2 µm.*

In conclusion, we have developed a method based on the Split-GFP system to experimentally determine the topology of inner membrane proteins in *E. coli*. The reporter tag can be fused to either the C- or N-terminus of membrane proteins has minimal to no effect on the targeting to and the insertion in the membrane and may be used in cell division related studies.

Part II

Methodology

Fluorescence Microscopy

One of the most frequently used tools in modern day biological research is the confocal fluorescence microscope. Two of the main reasons are the non-invasive nature of method and the excellent contrast ratio that is obtainable. One extremely important improvement of fluorescence microscopy was the ability to make optical sectioning of the sample and thus fluorescence microscopy experiments are nowadays often performed in a confocal microscope. The concept of a confocal microscope was introduced by M. Minsky in the late 1950s but it was not until the late 1980s that the first successful confocal images were acquired (Carlsson et al, 1985; Minsky, 1961). In a confocal microscope the sample is illuminated (preferably with a monochromatic light source) and undergoes electronic excitation to the excited state, subsequently the emitted light emerging from the sample is guided through, via lenses and emission filters, a pin-hole that will filter away most out-of-focus light, leaving only the signal originating from the focal plan to reach the detector (Figure 6).

When compared to other imaging techniques such as Electron Microscopy (EM), Atomic Force Microscopy (AFM) or Scanning Near field Optical Microcopy (SNOM) fluorescence microscopy is highly advantageous due to its compatibility with live-cell protein specific multi-color imaging. Since it is possible to genetically tag target proteins it is also possible to deduce intracellular dynamics over long time.

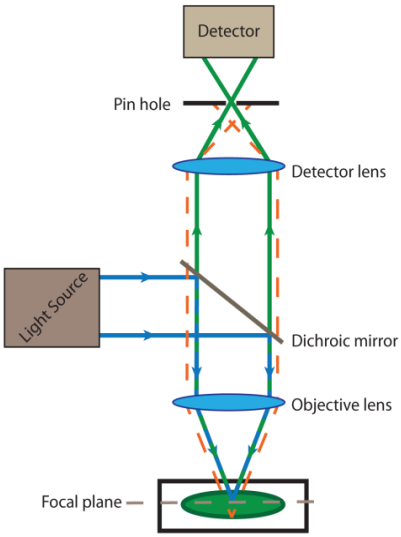

Figure 6; Confocal set-up.
An excitation laser (blue) is reflected on a dichroic mirror and focused by an objective lens onto the sample. Fluorescence emanating from the focus point (green) is collected back through the objective lens and transmitted through the dichroic mirror to the detector lens focusing the beam through the pin hole and onto the detector. Background fluorescence originating from an out of focus plane (orange dotted line) is filtered out.

Due to the live cell imaging compatibilities fluorescence microscopy can be used across a large range of questions in biology; from investigation of single protein dynamics and kinetics in single bacterial cells (Alexeeva et al, 2010; Romantsov et al, 2007; Stricker et al, 2002; Stromqvist et al, 2010) to high speed imaging of early development in whole organisms and investigations of intact biological systems (Chung et al, 2013; Krzic et al, 2012; Wu et al, 2011). Although confocal microscopy is a powerful technique, one fundamental limitation is the modest spatial resolution capability, which ultimately is a direct result of the diffraction limit of light. The resolution limit for a light microscope was first presented in the work by Abbe (Abbe, 1873) in which it is stated that the minimal full width at half maximum (FWHM) resolution in the lateral (Δx) and axial (Δz) planes are

$$\Delta x = \frac{\lambda}{2\, n\, sin(\theta)}$$

$$\Delta z = \frac{\lambda}{2\, n\, sin^2\left(\frac{\theta}{2}\right)}$$

Here n is the refractive index of the medium embedding the sample and θ the half aperture angle of the objective collecting the light. Later Lord Rayleigh explained the criterion for the minimal distance of two point-like objects where they still can be seen as separated, the Rayleigh criterion (Rayleigh, 1896). With the foundation in those early works it is today commonly stated that the best achievable resolution (ability to distinguish between two point sources located a distance d_{min} from one another) in the xy-plane in a confocal microscopy system is:

$$d_{min} > \frac{\lambda}{3}$$

Effectively, this gives an achievable resolution of 200-300 nm, which in many biological applications is too large to resolve fine sub-cellular structures. In recent years, however, successful attempts to circumvent the limit have been made. In broad terms two categories of super-resolution techniques have been developed. Structured Illumination Microscopy (SIM) is one example of a technique that uses restrictions in the light pathway to physically modulate the illumination pattern produced over the sample (Gustafsson, 2000; Heintzmann et al, 2002; Hell & Wichmann, 1994; Klar & Hell, 1999). The other category exploits the switching properties of the fluorophores to produce a super resolved image. In STimulated Emission Depletion (STED) an excitation laser is overlaid by a doughnut shaped "STED" laser that depletes the fluorescence in the outer rim leaving only fluorescently active molecules in the middle of the doughnut, and in that way employs targeted switching of the fluorophores. The resolution regularly achieved in a commercial availably STED system is approximately 50 nm, with an possible resolution improvement of ~30-40 nm when a time gated modality, termed g-STED, is included (Vicidomini et al, 2011). The main limitations of STED are that the alignment of the lasers has to be on the order of nanometer and that one uses very high laser power (that quickly may cause photo-induced damage on the biological sample) to de-excite the

fluorophore, the second point puts high demands on the photostability of the fluorophore and to date there are only a handful fluorescent proteins that are compatible with STED due to wavelength compatibility issues.

On the other hand, PhotoActivatable Localization Microscopy (PALM) and Stochastic Optical Reconstruction Microscopy (STORM) use the stochastic photoswitchable properties of fluorescent molecules to localize the "center of mass" of the emission spot (*i.e.* of the fitted point spread function) in a sub-diffraction limited manner one molecule at the time (Betzig et al, 2006; Rust et al, 2006). Generally speaking these two methodologies are based on the same fundamental principle and differ only from each other in that PALM uses fluorescent proteins while STORM utilizes synthetic dyes. These point localization methods may effectively produce a lateral localization precision in the order of 10-20 nm (Greenfield et al, 2009), which is more than a tenfold resolution improvement compared to diffraction limited illumination methods. Additionally, using interferometry (iPALM) it is possible to achieve sub 20 nm resolution axially (Shtengel et al, 2009). There is a wide range of fluorescent proteins that can be photoswitched in order to accommodate PALM, it is though still very challenging to do multi-color imaging using localization microscopy methods. One limitation of PALM/STROM is that a large amount of data (usually tens of thousands images) need be to collected in order to reconstruct a super resolution image. This has at least two implications, 1) data acquisition time will be long and 2) if protein dynamics are fast it will result in a smear in the image. Another limiting factor to point localization methods is the labeling density, it is highly challenging to achieve labeling dense enough to meet the Nyquist criterion, which states that the labeling density has to be twice the sampling frequency (*i.e.* if the desired resolution is 10 nm the labeling density needs to be one fluorophore every 5 nm).

Therefore, despite the somewhat modest "super-" resolution achievable with SIM (~100 nm laterally and ~300 axially) I have mainly used this method during my work due to the fact that SIM is compatible with fast multi-color imaging on live samples.

Structured Illumination Microscopy

In Structured illumination Microscopy (SIM) the sample is illuminated with spatially structured excitation light. This can generate moiré fringes containing additional high-resolution information that is normally inaccessible. A number of images containing moiré fringes are processed in frequency space to extract the additional high-resolution information. This information is subsequently used to generate a reconstruction image with improved resolution (Gustafsson, 2000; Heintzmann & Cremer, 1999). In frequency space the accessible information resides within a circle with radius k_0 located with the midpoint at the origin (Figure 7A). Low resolution encoded in the image will be close to the origin while high resolution information will be found with increasing distance from the origin. The highest spatial frequency k_0 that can be resolved in a confocal microscope is $k_0 = 2N.A./\lambda_{emi}$, where N.A. is the numerical aperture of the objective and λ_{emi} is the wavelength of the emitted light.

If a grid of known periodicity is placed between the sample and the excitation source it will give raise to moiré fringes. These fringes will be coarser than the original pattern and will contain information about the sample being resolved (Figure 7B). A striped illumination grid (sinusoidal pattern) has only three Fourier components and the possible positions of the two side components are limited by the same circle that defines the observable region (Figure 7A, red dots). The amount of that movement is represented by the three Fourier components of the illumination grid. The expanded

observable region will thus contain, in addition to the normal information, high resolution information that originates in two offset regions (*i.e.* a larger circle ideally with radius $2k_0$ is generated from which "new" high resolution information which now can be accessed) (Figure 7C, red dashed line). In practice a grating is placed between the sample and the illumination source to generate the moiré fringes whereby a number of phase shifted images are acquired. The grating is thereafter rotated a predetermined angle (typically 180/3 or 180/5 degrees in the set-up used in this thesis), and new images are acquired. In total a data set of 15-25 images are generated. These images are subsequently Fourier transformed into frequency space and reassembled into one extended resolution image. Finally, by applying an inverse Fourier transform the newly uncovered information in reciprocal space will be mapped into real space resulting in a resolution improvement ideally of a factor of two.

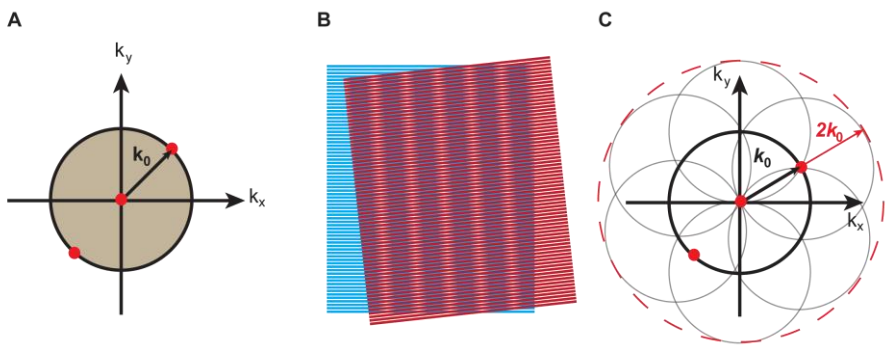

Figure 7; ***A****, The resolvable information of an image in frequency space resides in the "observable region", that is, within a circle with radius* $k_0 = 2N.A./\lambda_{emi}$. ***B****, An idealized sample containing high frequency information (blue) is overlaid by a high frequency grid (red) and results in the generation of Moiré fringes (thick dark lines). Since the periodicity of the grid and of the measured Moiré fringes are known this information is used to back calculate the periodicity of the original pattern (i.e. the sample).* ***C****, Theoretical maximum of observable region increases from* $r = |k_0|$ *to* $r = 2|k_0|$ *(red dashed line) and a high resolution image can be reconstructed. Adapted from (Gustafsson, 2000).*

Fluorescence Recovery After Photobleaching

The idea of Fluorescence Recovery After Photobleaching (FRAP) is to irreversibly bleach fluorescent molecules in a small well defined area and monitor the following fluorescence recovery until a relaxed state of equilibrium is re-established. Schematically shown in Figure 8 is an idealized FRAP experiment on a small area element (white circle in step two) of the cytosol of a cell.

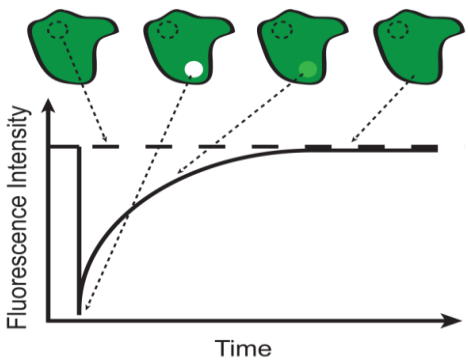

Figure 8; Concept of Fluorescence Recovery After Photobleaching (FRAP). The fluorescent molecules in a small well defined area are irreversibly bleached whereby the subsequent recovery of fluorescence into the bleached area is monitored over time.

FRAP provides information on the diffusion of proteins in various environments of the cell (diffusion coefficients) (Mullineaux et al, 2006; Stromqvist et al, 2010), mobility and binding kinetics (Montero Llopis et al, 2012) and protein turnover in super complexes (Stricker et al, 2002). Generally, during a FRAP experiment one has to apply a laser pulse short enough to not permit any molecules diffuse in or out of the region of interest during the bleaching time as this will effectively distort the data and substantially increase the complexity of the subsequent data analysis.

FRAP as a tool for monitoring cell division in Bacteria

What I have taken advantage of in my work is that the different compartments in *E. coli* (*i.e.* cytoplasm and periplasm, see next chapter of details of the *E. coli* physiology) will be sealed off during cell division and will therefore have a binary readout in the FRAP measurements, that is, after bleaching an open compartment will allow fluorescence recovery across the division septum while a closed compartment will not.

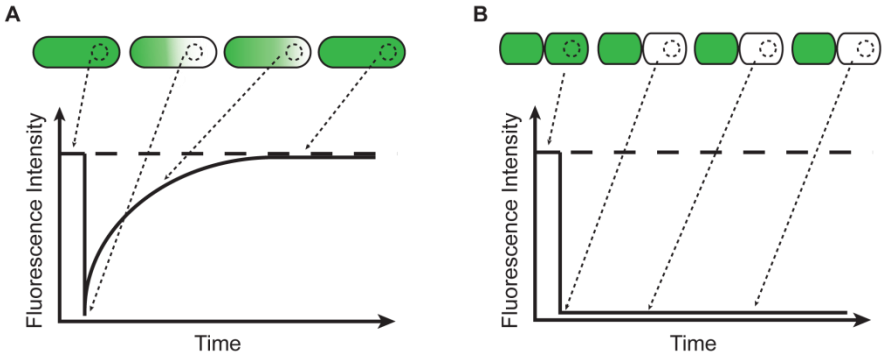

Figure 9; Idealized FRAP measurements on GFP expressed in the E. coli *cytoplasm during different stages of the cell cycle. Theoretical fluorescence intensity is highlighted within the dotted circle at four different time points, "pre-bleach", momentarily after bleaching, after "some" time and after "long" time (as seen from left to right).* ***A****, Cells that have their cytoplasmic compartment open will permit fluorescence recovery across the septum.* ***B****, Cells that have their cytoplasmic compartment compartmentalized do not permit fluorescence recovery across the septum.*

In papers I and II I have utilized this fact in a dual color approach in live *E. coli* cells. Freely diffusing GFP or Cerulean was expressed in the cytoplasm and mCherry was expressed and exported via the TAT pathway to the periplasm. After the bleaching pulse the diffusion across the septum (or lack thereof) in both the cytoplasm and the periplasm was simultaneously monitored over time in order to assess the states of the compartments (*i.e.* open or closed).

Part III

The Model System

Escherichia coli

E. coli are a rod-shaped Gram-Negative bacterium that was discovered by Theodor Escherich in 1886. Slow growing *E. coli* roughly measure 2 µm in length and 1 µm in diameter while fast growing cells normally are almost twice that length. *E. coli* cells are surrounded by a cell envelope, which is composed of an inner membrane, periplasmic space and an outer membrane.

The cell envelope

Both the lipid and the protein composition in the two membranes are very different. The inner membrane contains 75 % Phosphatidylethanolamine (PE), 20 % Phosphatidylglycerol (PG) and 5 % Cardiolipin (CL) and a variety of α-helical membrane proteins (membrane proteins that have one or more α-helices imbedded in the membrane) (Bogdanov et al, 2009).
In contrast, the outer membrane is made up of phospholipids (inner leaflet), lipopolysaccharide (LPS, outer leaflet) and β-barrel proteins. Between the two membranes is the periplasmic space. Within the periplasm resides the peptidoglycan, an exo-cytoskeleton structure responsible for the maintenance of the cell shape and much of its rigidity (Vollmer & Seligman, 2010).

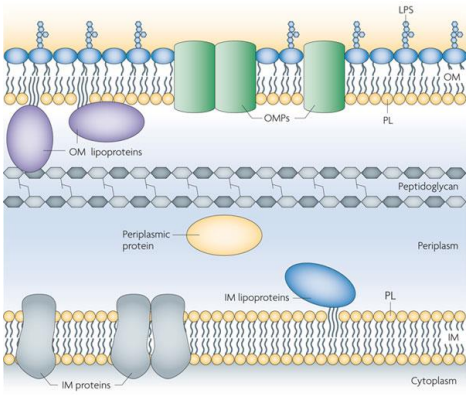

Figure 10; The cell envelope of E .coli. *The cytoplasm is surrounded a phospholipid (PL) bilayer (the inner membrane (IM)), that also contains proteins that either span the membrane through α-helical transmembrane domains or are anchored to the outer leaflet through a lipid moiety (IM lipoproteins). Bound between the IM and the outer membrane (OM) is the periplasm, which is an aqueous compartment containing the peptidoglycan layer that is responsible for the cell shape. The OM is anchored to the rest of the cell via proteins that are covalently attached to the peptidoglycan. The OM is asymmetric, as it contains phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet. In addition, the OM contains two types of proteins, integral OM proteins that are β-barrels and lipoproteins. Reprinted from (Ruiz et al, 2009) with permission of Nature Publishing Group*

The peptidoglycan molecule is composed of glycan strands made of N-acetylmuramic acid (MurNAc) and β1,2-linked N-acetylglucosamine (GlcNac) which are connected by β-1→ 4 bonds (Schleifer & Kandler, 1972). The peptidoglycan is attached to the outer membrane mostly by a peptide bond to the brown lipoprotein (LPP) (Hantke & Braun, 1973). As the cell grows, it must elongate and then divide. To accommodate this process the cell envelope has to be continuously remodeled. Cell division in most rod-shaped bacteria, such as *E. coli*, occurs in a binary mode of fission. First the mother cell elongates to approximately twice its original size, then the cell envelope starts to invaginate, which ultimately results in partitioning

of the cell compartments and birth of two (to the mother) identical daughter cells (Figure 11). Remarkably, this cycle is repeated every 20 minutes when cells are kept in a nutritient rich environment at 37 ºC.

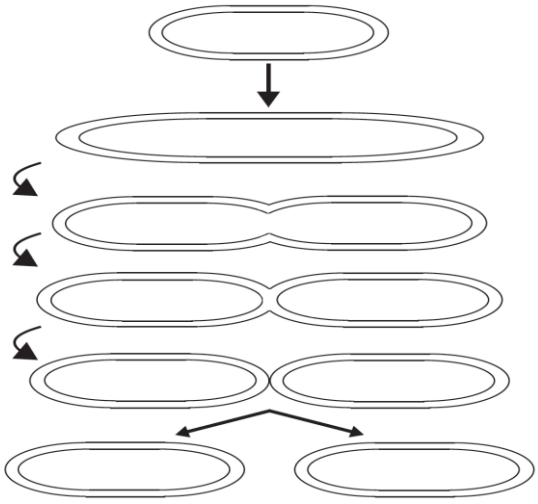

Figure 11; A schematic view of the cell cycle in E. coli. *First the cell elongates to approximately twice its original size. Thereafter the membranes start to constrict, first the inner- followed by the outer membrane. In a final stage the mother cells pinches of to become two daughter cells. Adapted from (Skoog et al, 2012).*

Cell Division

Cell division is mediated by a large complex known as the divisome (Goehring et al, 2005). The divisome and its auxiliary constituents spans all compartments of the cell, from the cytoplasmic side of the inner membrane via the periplasm to the outer membrane, reviewed in (Natale et al, 2013). Over 30 different proteins are known to be directly or indirectly involved in the cell division process, however, the "core" consists of the essential proteins FtsZ, ZipA, FtsA, FtsK, FtsL, FtsB, FtsQ, FtsI and FtsN (Weiss, 2004). Although the molecular roles for each of the divisome proteins are not fully understood, much of what is known was discovered with the aid of

fluorescence microscopy/spectroscopy. The nomenclature in the literature on this subject is not consistent; where some define the divisome as all proteins involved in the division event, see for example (Rico et al, 2013), while others define the "FtsZ-ring with all its associated proteins" as the divisome (Aarsman et al, 2005). To avoid confusion; from here on when I refer to the divisome I specifically mean only the essential proteins FtsZ, ZipA, FtsA, FtsK, FtsL, FtsB, FtsQ, FtsI and FtsN. These proteins are either cytoplasmic proteins (FtsZ and FtsA) or inner membrane proteins (ZipA, FtsK, FtsL, FtsB, FtsQ, FtsI and FtsN). With the definition presented above the assembly of the divisome occurs in a two-step manner (Aarsman et al, 2005) and once fully assembled the mature divisome initiates the constriction of the cell envelope towards closure (Lutkenhaus, 2009).

Spatial modulators of the divisome

Two independent but complementary systems ensure correct spatial localization of the divisome during division. These are the Min system (de Boer et al, 1989) and nucleoid occlusion (Woldringh et al, 1991). The Min system prevents the divisome from assembling anywhere other than the midcell while, as the name suggests, nucleoid occlusion hinders cell division across the nucleoids.

The *E. coli* Min system includes three components MinC, MinD and MinE that are all encoded in the *min* operon, reviewed in (Rowlett & Margolin, 2013). Initially the Min system was thought to be a static structure with MinCD localized at the poles while MinE was present at the septum (Zhao et al, 1995). However, with the introduction of the GFP-fusion technology it was soon discovered that GFP-MinD was oscillating along the long axis of the cells in a pole-to-pole manner and that this behavior was MinE dependent (Raskin & de Boer, 1999b). MinD is an ATPase belonging to the

Walker A cytoskeletal ATPase (WACA) family that is powering the pole-to-pole oscillations by ATP consumption (Lutkenhaus, 2008; Raskin & de Boer, 1997). Knockout studies revealed that in the absence of MinE, MinD was localized as a (non-oscillating) halo around the cell periphery indicating that it was bound to the membrane. Subsequent studies showed that MinC was oscillating in a manner similar to that of MinD (Hu & Lutkenhaus, 1999; Raskin & de Boer, 1999a). MinC is the only Min protein that directly interacts with FtsZ and its main activity is to prevent FtsZ to form Z-rings at the septum.

Even though MinC is the active inhibitor of FtsZ polymerization into filaments, it is a quite weak inhibitor when working on its own. In contrast, when working in concert with MinD the activity of MinC increases ~50 fold (de Boer et al, 1992). This increase in activity is attributed to the recruitment of MinC to the membrane by a MinD dimer (Szeto et al, 2003). MinD binds both MinE and MinC and recruits them both to the membrane. The binding sites of MinE and MinC on MinD are partly overlapping and if both are present MinE is the slightly preferred binding partner (Hu et al, 2003; Lackner et al, 2003). Since MinE has higher affinity for binding to MinD it will promote MinC displacement and has therefore an anti-MinCD activity associated to it. MinE also converts MinD from active to inactive through stimulation of MinD ATPase activity (Pichoff et al, 1995). It has been proposed that the MinD also plays an active role in chromosome segregation (Di Ventura et al, 2013) and that FtsZ, ZipA, ZapA and ZapB move in a counter-oscillatory manner in respect to MinC prior to septum formation (Bisicchia et al, 2013). These recent findings suggest that the complete role of the Min system is not fully understood and that its role may be more complex than simply preventing FtsZ from forming Z-rings at the poles.

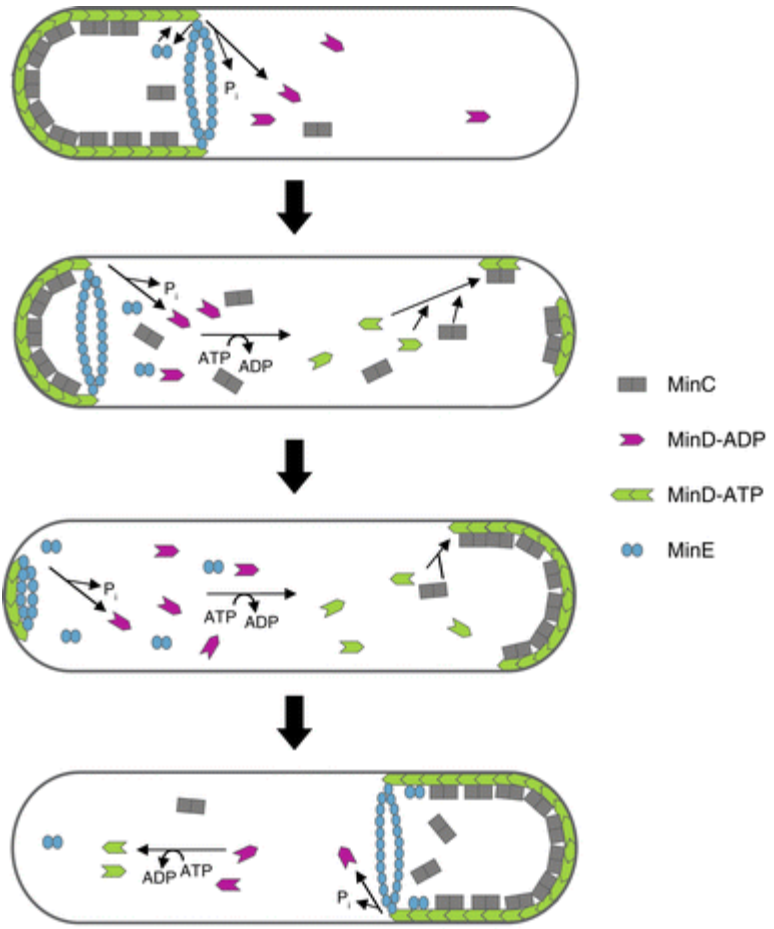

Figure 12; Mechanism of the Pole-to-pole oscillations of the Min system. The dimeric from of MinD binds to the membrane and there it recruits MinC. MinE has two anti-MinCD roles; it displaces physically MinC and stimulates MinD release form the membrane through activation of MinD ATPase activity. The concentration of MinD-ATP at of the old pole is lower because it binds cooperatively to the membrane already containing bound MinD. In contrast, the MinD-ATP concentration increases at the other pole, which lacks bound MinD. As the MinD concentration rises, it eventually binds, forming a new polar zone. Subsequently when MinE is released from the old pole, it will bind to the ends of the new MinD polar zone. Reprinted from (Lutkenhaus, 2007) with permission.

Nucleoid occlusion hinders the Z-ring from constrict over undivided chromosomes. The protein involved in this process was identified when screening for synthetically lethal with a defective Min system (*slm* mutants) and was therefore given the name SlmA (Bernhardt & de Boer, 2005). When comparing cells lacking the Min system with cells lacking both the Min system and SlmA it was found that the cells lacking both had an increased number of Z-rings and these Z-rings were often located across the nucleoids. In contrast, in wild-type cells SlmA moves away from the septum as the chromosomes segregate, promoting Z-ring formation at the septum. SlmA binds specifically to a palindromic DNA sequence in the *E. coli* chromosome and that binding of SlmA to DNA activates anti-FtsZ activity. Moreover, when SlmA is bound to DNA the GTPase activity of FtsZ is increased and increased levels of GTP activates disassembly of FtsZ polymers (Cho et al, 2011; Tonthat et al, 2011). Taken together those findings indicate that the function of SlmA seems to be a kind of anti-guillotine mechanism.

Divisome assembly

The first protein to arrive at the division septum is FtsZ (Bi & Lutkenhaus, 1991). FtsZ acts as a scaffold for the rest of the divisome proteins and is widely thought to be the main force generator in the membrane constriction process (Mingorance et al, 2010). Since the crystal structure of FtsZ from the hyperthermophilic methanogen *Methanococcus jannaschii* was solved in 1998 (Lowe & Amos, 1998), many models have been proposed for the GTP dependent constriction process. One particularly appealing model suggests a hinge-opening mechanism that would provide the necessary force to constrict the Z-ring (Li et al, 2013). Osawa and Erickson suggested that FtsZ (in the presence of FtsA) could constrict unilamellar liposomes (Osawa & Erickson, 2013). But in their study it was unclear if the constriction was

initiated from completely round liposomes or from liposomes with an inherit kink at the middle (as this would mean that it would constrict spontaneously as long as the line tension is higher than the surface tension). Thus, the force generating role of FtsZ is still controversial and remains to be further elucidated through experimental work.

Next to be recruited to the midcell are FtsA and ZipA (Pichoff & Lutkenhaus, 2002). The roles of these proteins are somewhat overlapping and they tether FtsZ to the inner membrane, stabilize it and they facilitate polymerization of FtsZ (Addinall & Lutkenhaus, 1996; Hale & de Boer, 1997; Ma et al, 1996). FtsZ, FtsA and ZipA are together with four non-essential proteins, ZapA-ZapD (Durand-Heredia et al, 2012; Durand-Heredia et al, 2011; Ebersbach et al, 2008; Gueiros-Filho & Losick, 2002; Hale et al, 2011), forming an intermediate structure called the Z-ring. ZapA interacts direct with FtsZ, binding on the inside of the Z-ring, while ZapB binds to ZapA in a stabilizing context (Galli & Gerdes, 2010). ZapC suppresses FtsZ GTPase activity and together with ZapD increases the Z-ring stability by promoting lateral interactions between and bundling of FtsZ polymers (Durand-Heredia et al, 2012; Hale et al, 2011). A fifth Zap protein (ZapE) was recently identified, but as opposed to the other Zap proteins ZapE was found to be recruited at a late stage of cell division and that it correlated with constriction of the Z-ring (Marteyn et al, 2014).
In spite of the many interaction partners identified for FtsZ, its turnover in the Z-ring is estimated to be on the order of 10 seconds (based on FRAP measurements on FtsZ-GFP) (Stricker et al, 2002), FRAP measurements on ZipA-GFP also revealed that ZipA is dynamic (Stricker et al, 2002). The Z-ring can thus be regarded as a highly dynamic structure.

After the assembly of the Z-ring a lag period follows before the rest of the essential divisome proteins are recruited to the midcell, these proteins are FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI and FtsN (Begg et al, 1995; Boyle et al, 1997; Buddelmeijer et al, 2002; Dai et al, 1993; Guzman et al, 1992; Spratt, 1977). The specific roles of these proteins are not fully understood.

However, through a combination of genetic and fluorescence studies it is known that proteins that arrive in the second step of divisome assembly closely interact with one another but also with proteins in the Z-ring (Alexeeva et al, 2010; Egan & Vollmer, 2013; Fraipont et al, 2011). FtsK is responsible for sister chromosome segregation and is required for recruitment of the preformed FtsQ-FtsL-FtsB complex (Sherratt et al, 2010; Stouf et al, 2013; Yu et al, 1998). The functions of FtsL and FtsB are not known, but it has been suggested that they act as scaffolding proteins facilitating recruitment of other divisome proteins (Gonzalez & Beckwith, 2009). FtsL might also have a role in Zn^{2+} transport across the membrane (Blencowe et al, 2011). Through bacterial two-hybrid analyses FtsQ has been shown to interact with at least 8 of the other divisome proteins (Di Lallo et al, 2003; Karimova et al, 2005). But other than that it interacts with many of its divisome partners, it is not well understood if FtsQ has a more specific role in the divisome. As the cell elongates and a septum is formed, it has to be synthesized new peptidoglycan. FtsW is a member of the SEDS (shape, elongation, division, and sporulation) family and is involved in flipping of a peptidoglycan precursor (lipid II) across the cytoplasmic membrane (Holtje, 1998; Mohammadi et al, 2011). FtsW localization at the septum required for recruitment of FtsI and they can form a subcomplex independently of other divisome proteins (Fraipont et al, 2011). FtsI is a transpeptidase that is involved in the biogenesis of peptidoglycan at the midcell (Weiss et al, 1999; Weiss et al, 1997). Finally, FtsN is believed to have a stabilizing effect on the divisome and potentially trigger the onset of membrane constriction (Rico et al, 2010).

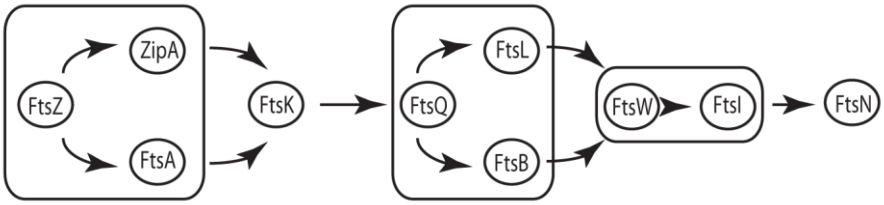

Figure 13; Hierarchical assembly order of the divisome. The arrows indicate the specific pathways for the recruitment of the proteins to the septum and sub complexes are illustrated by boxes.

The outer membrane is thought to constrict partly powered by the transenvelope spanning Tol-Pal system, partly by ingrowth of the peptidoglycan layer (Egan & Vollmer, 2013). The outer membrane localized lipoprotein Lpp (Braun's lipoprotein) is covalently linked to the peptidoglycan (Cowles et al, 2011; Hantke & Braun, 1973). This suggests that an inward growth of the peptidoglycan would pull the outer membrane to follow. The Tol and Pal proteins are recruited to the septum in an FtsN dependent manner and therefore is the Tol-Pal system usually considered as (peripheral) part of the cell division machinery. This complex is comprised by five known constituents; TolQ, TolR and TolA that are all cytoplasmic transmembrane proteins, TolB that is a soluble periplasmic protein and Pal that is an outer membrane protein (Gerding et al, 2007). TolQ, TolR and TolA forms a subcomplex in the inner membrane through interactions between their transmembrane helices and TolB and Pal forms a complex in the outer membrane (Bouveret et al, 1995; Derouiche et al, 1995; Lazzaroni et al, 1995). The two complexes are connected through interactions between both TolA-Pal and TolA-TolB (Cascales et al, 2000; Dubuisson et al, 2002). Thus protein-protein interactions physically connect the inner and outer membrane at the septum and therefore is was suggested that the Tol-Pal system could be responsible for the inward pulling of the outer membrane during division.

Not much is known about how the peptidoglycan hydrolases function and how they regulate septum cleavage towards the end of cell division but the data available indicates that the activation of a number of cell wall hydrolases (AmiA, AmiB and AmiC) is regulated by the divisome associated proteins EnvC and NlpD, which in turn are regulated and activated by FtsN or FtsEX (Uehara et al, 2010). FtsN is required for correct localization of AmiB to the septum and EnvC is recruited by FtsEX. AmiA and AmiB are activated by the periplasmic EnvC. Correct localization is essential for these proteins as displacement of EnvC causes cell lysis, potentially by recruitment and activation of AmiA and AmiB at sites away from midcell (Uehara et al, 2010). Furthermore, an active FtsI is required for recruitment and activation of the septum cleaving amidases (Peters et al, 2011).

Visualizing the Z-ring

The possibility to genetically tag divisome components with GFP and then follow their localization patterns in live cells was a breakthrough when it was first reported some 15 years ago (Sun & Margolin, 1998). However, it quite soon became evident that conventional fluorescence microscopy would not give enough information to shed light on the finer structure of the Z-ring *in vivo*. Recent improvements in imaging technologies, both light and electron based, have allowed for eluding parts of the structure of the Z-ring. Electron Cryo-Tomography (ECT) images on the FtsZ-ring from another Gram-Negative bacterium, *Caulobacter crescentus*, revealed that the structure of the ring not homogenous but that it was composed of short single layered filaments, each 40-120 nm long and localized 16 nm from the inner membrane (Li et al, 2007). Although electron microscopy is superior in resolution when compared to light microscopy based methods and has proved to be successful in certain aspects of cell division related questions, the sample preparation is tedious and the technique is fundamentally limited

by sample thickness, which rules out investigations in larger bacteria (such as *E. coli* and *Bacillus subtilis*). One particular important step in better understanding the role of FtsZ during cell division in live cells came through the introduction of fluorescence based super resolution microscopy. Fu et al. pioneered PALM imaging on the divisome and were able to determine the *in vivo* structure of the Z-ring in *E. coli* to be 110 nm thick and (in contrast to previous ECT data obtained from *C. crescentus*) that the Z-ring was composed of several overlapping FtsZ protofilaments (Fu et al, 2010). Recently, also using PALM Buss et al. provided new information regarding the roles of ZapA and ZapB *in vivo*. Rather than to promote association of individual FtsZ protofilaments the Zap proteins were suggested to align FtsZ clusters consisting of multiple FtsZ protofilament (Buss et al, 2013). The Z-ring in both *B. subtilis* and *Staphylococcus aureus* appears to form a kind of beads on a string formation determined from 3D-SIM images (Strauss et al, 2012). Using PALM Manley and colleges recently confirmed that *C. crescentus* Z-ring did not form a homogenous ring (Holden et al, 2014). Furthermore, in *C. crescentus* the three-dimensional strucutre of FtsZ also appered to change thickness during different stages of cell cycle (going from 67 nm prior to division to 92 nm during division) when imaged by super-resolution based on astigmatism and the turn over of FtsZ molecules at the septum was also found to be higher than previously reported for *E. coli* (~100 ms vs. ~8 sec.) (Biteen et al, 2012). Even though there are clear differences in the Z-ring dynamics between the different species it appears consistent that with the foundation in these super resolution based studies a picture is starting to emerge of the Z-ring as a dynamic cluster of short heterogeneous FtsZ protofilaments (organized either in a single layer or overlapping layers) rather than a homogenous ring that spans the mid cell.

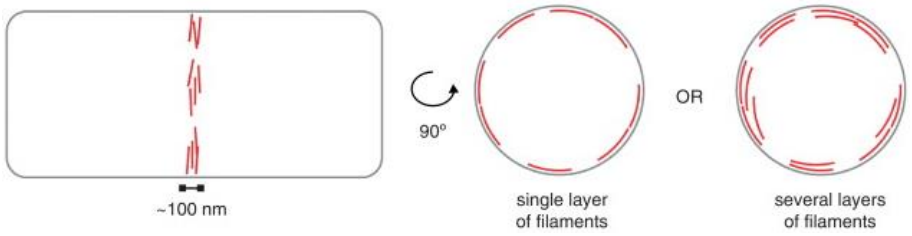

Figure 14; Model for FtsZ protofilament organization at the midcell. Short (~100 nm) protofilaments (red stripes) forms either a single layer (as suggested by ECT) or overlaying layers (as suggested by super-resolution fluorescence microscopy). In neither model is the Z-ring a continuous structure. Reprinted with permission from Elsevier

Unfortunately, none of these studies revealed anything about the structure, behavior or localization of FtsZ at the very last stages of inner membrane closure. One reason for that is that that even super resolution imaging has its limits, not only in terms of resolution but also in terms of practicality (*e.g.* fixed vs. live cells, photo physical properties of the fluorescent proteins, image acquisition time etc.). Therefor I believe that our FRAP approach is an excellent complement to the super resolution methods as it provides a different kind of resolution.

Divisome disassembly

Towards the end of the division process the divisome must disassemble so that the membranes can fuse and that the involved proteins can redistribute to the daughter cells in order to initiate the next round of division. To date, very little is known about this late stage in cell division. From electron microscopy studies on FtsZ protofilaments *in vitro* we know that the Z-ring cannot form a closed ring. These studies lay the foundation for theoretical calculations that showed a minimal possible FtsZ protofilament diameter of approximately 24 nm (Erickson et al, 2010; Lu et al, 2000). If taking in to account the linker (FtsA) this would result in the maximum possible

constriction of the inner membrane would be approximately 57 nm. It could even be so that FtsZ even prefers a less curved intermediate conformation with a diameter of approximately 200 nm (Erickson et al, 2010). Thus the view of FtsZ as the main force generator constricting the inner membrane may hold early in the constriction event, however towards the end of membrane constriction it seems unlikely that FtsZ can provide the force needed. As FtsZ disassembles from the divisome it will within seconds relocate to the future division sites in the daughter cells (Sun & Margolin, 1998). Following inner membrane closure and compartmentalization of the cytoplasm the rest of the divisome lags behind still accumulated at the division septum (Paper II).

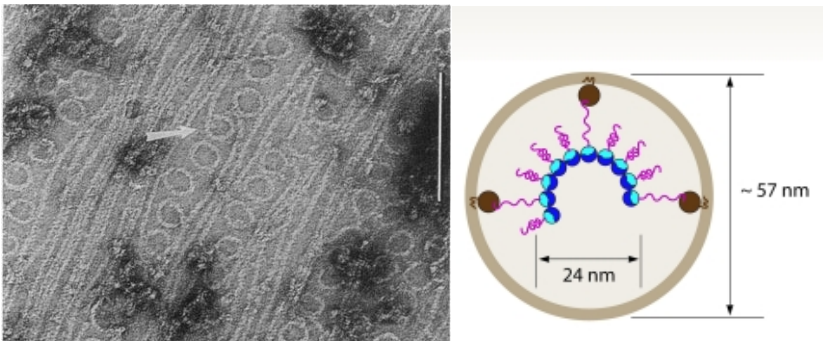

Figure 15; FtsZ minirings. FtsZ can not form rings smaller than 24 nm in diameter (blue), and taking in to account the tether (pink) and FtsA (brown) the inner membrane has a minimun diamter or ~57 nm. Reprinted from (Erickson et al, 2010; Erickson et al, 1996) with premission from the American Society for Microbiology.

FtsA and ZipA are the first proteins to disassemble from the divisome after FtsZ. As they do so, they will almost instantaneously relocate to the future division site in the daughter cells and there form new Z-ring together with FtsZ (Addinall & Lutkenhaus, 1996). We have shown (in Paper II and III) that the late divisome proteins FtsQ, FtsL and FtsI remain at the septum even after the relocation of FtsZ, FtsA and ZipA to the future division sites in the

daughter cells. Two independent studies showed that FtsK remained at the septum while FtsZ and ZapA/B relocated to the future division sites in the daughter cells (Galli & Gerdes, 2010; Wang et al, 2005). Although, we have not investigated the localization behavior of all the "late" divisome proteins it stands to reason that FtsW and FtsN might also lag at the old septum due to their interactions with other "late" divisome proteins and the fact that if FtsN is not present the divisome starts to disassemble in a reverse order to its assembly (Rico et al, 2010). The reason for this lag is not fully understood. One plausible explanation would be that the proteins that are not required in the first step of divisome assembly are purposely retarded at the old septum in order not to initiate membrane invagination prematurely, potentially in order to allow the cells to synthesize enough polar peptidoglycan for septum ingrowth. This would agree with previous observations that the late divisome proteins arrives at the division site in a sequential manner (Aarsman et al, 2005).

Conclusions and unpublished observations

With the completion of this thesis the first few steps have been taken in order to obtain a better understanding of the disassembly dynamics of the cell division machinery in *E. coli*. With the combined use of confocal microscopy, SIM and FRAP I made novel observations regarding the progression of the cell division event correlated to divisome localization. In Paper I it was unequivocally shown that the cytoplasm compartmentalizes prior to the periplasm, which was before not experimentally shown for *E. coli*. The experimental approach (dual color FRAP) developed in Paper I was thereafter used in Paper II to show that FtsZ (and ZapA) disassembles from the division septum prior to cytoplasmic compartmentalization. It has been suggested that it might be the disassociation of FtsZ from the divisome that ultimately allows the bilayer of inner membrane to fuse, owing the fact that fusion in principle could be mediated by lipid synthesis, as shown in the L-form of *B. subtilus* (Mercier et al, 2013). Another possibility is that inner membrane fusion might be driven by inward growth of the peptidoglycan layer; however, further experimental work is required to resolve this fundamental question regarding the final step in *E. coli* cytokinesis.
In contrast, FtsA, ZipA, FtsQ, FtsL and FtsI were shown to remain at the division septum until the cytoplasm was compartmentalized. This observation was in Paper II and III strengthen by dual color time lapse microscopy showing that there is a sequential order governing the disassembly of the divisome. Furthermore, in Paper III I showed that both ZipA and FtsA relocated to the future division sites prior the disassembly of FtsQ, FtsL and FtsI from the old septum, indicating that the divisome

disassembly is at a least three step process. As an extra step I was also able to integrate the FRAP approach with the dual color localization experiments in order to confirm both that the cytoplasm is compartmentalized in the absence of FtsZ and that other divisome components remain that the old septum even after cytoplasmic compartmentalization and relocation of FtsZ to the quarter sites in the daughter cells (Figure 16).

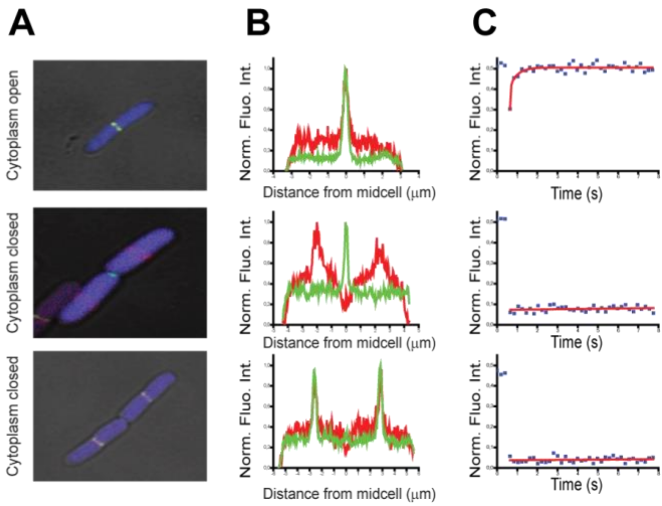

Figure 16; 2-in-1 experiment. FRAP measurements on Cerulean in the cytoplasm were carried out on cells simultaneously expressing FtsZ-mCherry and GFP-FtsI. ***A****, Fluorescence images of cells during different stages of cell division confirmed the sequential disassembly of the divisome.* ***B****, Intensity profiles shows the GFP (FtsI) and mCherry (FtsZ) localization in the cells at each stage.* ***C****, FRAP profiles of Cerulean fluorescent protein indicates whether or not the cytoplasm has compartmentalized (and thus if the inner membrane has sealed of). These combined data confirmed our previous obtained results that the cytoplasm is compartmentalized in the presence of FtsI but that FtsZ already had relocated to the future division sites in the daughter cells.*

With basis in the recent super resolution studies in *E. coli* (using PALM) and other bacteria (*e.g. B. subtilis* and *C. crescentus*) a picture is starting to emerge of the Z-ring as a dynamic cluster of short heterogeneous FtsZ protofilaments rather than a homogenous ring that spans the mid cell. This can also be seen with 3D-SIM and conventional high resolution confocal microscopy (Figure 17). However, to obtain a full understanding of the Z-ring structure even higher resolution imaging is needed.

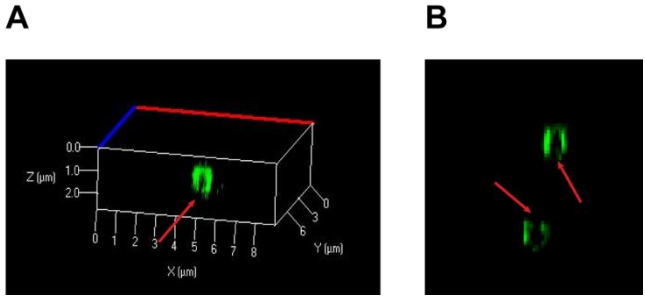

Figure 17; 3D renderings of E. coli *FtsZ-ring structure in vivo suggests an uneven distribution of the FtsZ protofilaments (red arrows).* ***A****, Confocal.* ***B****, 3D-SIM.*

When working with membrane proteins (as the majority of the proteins are that are involved in the cell division) one issue that may arise is that a large reporter (such as GFP) might affect the targeting to and insertion in the membrane if it is fused to the N-terminus of the protein of study. Since GFP generally is not active in the periplasm this fact hampers the amount of experimental information that can be gained from fluorescence studies. To overcome this obstacle we (in Paper IV) developed a membrane protein topology assay based on the split-GFP technology. We were able to produce functional protein fusion and we showed that this assay can be used when studying membrane proteins involved in cell division.

Future prospects

Even though a great deal is known about the bacterial cell division machinery there are fundamental questions that lack answers. Some of the most intriguing questions related directly to my work are (1) what is triggering FtsZ to disassemble from the divisome? (2) What powers the final step in inner membrane invagination and closure? And (3) what is the exact structure of the Z-ring *in vivo*? In order to answer the questions posed above a few experimental advancements have to be successful; for example would the generation a fully functional FtsZ-GFP fusion to use as a sole source of FtsZ in the cell be highly desirable, to obtain an *E. coli* FtsZ crystal structure would benefit the understaninding of Z-ring constriction and additionally I believe that to do correlative light and electron microscopy on FtsZ rings would provide highly valuable and novel insights. A fully functional FtsZ-GFP fusion could potentially be generated from the split-GFP system and is currently developed in the lab. But to generate crystals that diffract well enough to solve the structure of the *E. coli* FtsZ is highly challenging and has not been possible so far (personal communication with members from the Löwe lab). Using a novel tag called miniSOG (short for mini Singlet Oxygen Generator) that can be genetically expressed and is both fluorescent and electron dense it is possible to combine light and electron microscopy using only one label (Shu et al, 2011). Furthermore, since the miniSOG is just under half the size of GFP (106 vs. 238 amino acids) it may be possible that such a fusion protein (miniSOG-FtsZ) would function as a sole source of FtsZ in live cells.

We have shown that the “late” divisome proteins FtsQ, FtsL and FtsI (and potentially FtsW, FtsB and FtsN) remain at the septum after cytoplasmic compartmentalization and even as the FtsZ and ZipA relocate to the future division sites in the new born daughter cells (Papers III and IV). In two independent studies it was shown that FtsK remains longer at the division septum than does both FtsZ and ZapA (Galli & Gerdes, 2010; Wang et al, 2005). Collectively, those data indicates that the disassembly of the *E. coli* divisome may be regarded as a multi-step process. However, before it is possible to make that claim for a fact all divisome proteins need to be assayed. To obtain a complete picture of the localization patterns of the different divisome components during the later stages of cytokinesis, it would be of interest to include all components of the divisome in the FRAP measurements on periplasm as well, since this would provide answers to what components are present during outer membrane closure. This would indirectly shed light on the question whether or not peptidoglycan synthesis ongoing at this stage.

Moreover, the interplay between the divisome and the Min system during the final stages of cell division is poorly understood. I have begun to correlate the localization pattern for divisome proteins with the behavior of the Min system using 3D-SIM. I believe that live cell super resolution microscopy combined electron microscopy in a correlative imaging mode is the future way to go to further understand prokaryotic cell division. Correlative imaging may provide a platform to better determine the structural organization of known cytoskeletal elements and perhaps also reveal if additional cytoskeletal structures are present in bacteria.

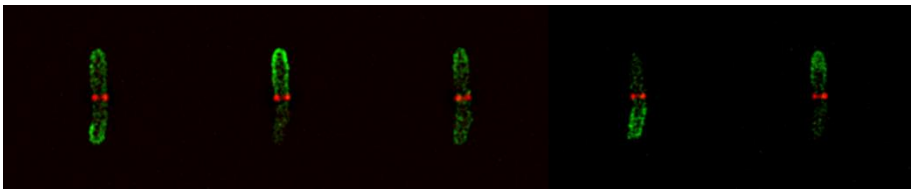

Figure 18; Time lapse images depicting pole-to-pole oscillations of EYFP-MinD (pseudo colored green). Cells were engineered to express both EYFP-MinD and FtsZ-mCherry. Midcell localization of FtsZ-mCherry is evident. Images were acquired by SIM.

Little is known about the temporal cues that enable the Min system to change from whole cell pole-to-pole oscillation to half-cell oscillations during division. Di Ventura and Sourjik showed that the partitioning of the Min system components are equally distributed in the daughter cells and that this event occurs before disassembly of FtsZ from the midcell (Di Ventura & Sourjik, 2011). Schwille and coworkers have shown that the Min system undergoes a transition from single pole-to-pole oscillations to multiple oscillations in microengineered polydimethylsiloxane compartments containing purified Min proteins ((Zieske & Schwille, 2013) and personal communication). This behavior was also observed in filamentous cells (treated with cell division inhibiting antibiotics) expressing EYFP-MinD. But if it is one or several divisome proteins that are responsible for the triggering of the switch from pole-to-pole oscillations to half-cell oscillations or if the Min proteins somehow spontaneously transit due to a sensory system that detects the variation in cell length and invagination at the midcell during division is not known and needs to be further investigated.

One specific (on a philosophical level very interesting) type of questions regarding cell division that I have encountered numerous times during my Ph. D. studies is questions of the type; when is the division over? And, when is the old cell becoming two new cells? Is it when the cytoplasm is compartmentalized and no more genetic material can be transferred between the two or is it when the periplasm is compartmentalized and no proteins can be transported across the septum? Or is it even later as the outer membranes of the daughter cells physically separate? What is you view? ☺

- *Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.*

Winston Churchill

Part IV

Short summary of the publications included in this thesis

Paper I

Sequential closure of the cytoplasm and then the periplasm during cell division in Escherichia coli

Karl Skoog*, Bill Söderström*, Jerker Widengren, Gunnar von Heijne and Daniel O. Daley

J Bacteriol. 2012 FEB **194(3)**:584-586. doi: 10.1128/JB.06091-11

* authors contributed equally to this work

It had long has been thought that the inner membrane seals prior to the outer membrane, however this had not been unambiguously shown. In this study we developed an assay that enabled us to follow the closing of the inner- and outer-membranes during division. We used dual-color FRAP on *E. coli* cells during different stages of the cell cycle. The cells were engineered to express GFP freely diffusing in the cytoplasm and mCherry freely diffusing in the periplasm. The mCherry molecules were exported to the periplasm via the twin arginine translocon pathway. The rationale behind the FRAP measurements was that a cell that was open across the division septum would show fluorescence recovery after irreversible bleaching of all the

fluorescence in one half of the cell. We found that in an early stage of division (were only a small invagination in the membranes were visible as judged by light microscopy) both the cytoplasm and periplasm were open for diffusion of the fluorescent molecules. At a later stage where the invagination was larger, we found that recovery was only allowed for periplasmic mCherry but not for cytoplasmic GFP indicating that the inner membrane had sealed off and compartmentalized the cytoplasm. In a final stage both membranes were closed not allowing for fluorescence recovery in neither compartment. From these data we concluded that there is a sequential order of the closing of the inner then outer membrane during cell division in *E. coli*.

Paper II

Disassembly of the divisome in Escherichia coli*: Evidence that FtsZ dissociates before compartmentalization*

Bill Söderström, Karl Skoog, Hans Blom, David Weiss,
Gunnar von Heijne and Daniel O. Daley.
Mol. Microbiol. 2014 **92(1)**:1-9. doi: 10.1111/mmi.12534

A combination of Super Resolution Structured Illumination Microscopy (SR-SIM) and FRAP was used in this study to determine if cell division proteins are present at the point of cytoplasmic compartmentalisation. Cells were engineered to express three fluorescent proteins simultaneously, ECFP (Cerulean) in the cytoplasm, RFP (mCherry) in the periplasm and one divisome protein fused GFP whereby I monitored the localization patterns throughout the cell cycle. In line with the previous presented *in vitro*

data, we show *in vivo* data that suggests that FtsZ disassociates form the division septum prior to cytoplasmic compartmentalization. At this point ZapA also disassembles from the divisome. In contrast, all the other proteins (*i.e.* FtsA, ZipA, FtsQ, FtsL and FtsI) under study remained at the septum even after cytoplasmic compartmentalization. To further investigate the localization patterns, GFP or mCherry was fused to seven of the divisome proteins and imaged in a pairwise manner. These data showed that FtsZ disassembles from the septum and reassembles at the future division sites in the daughter cells prior to the disassembly of FtsQ, FtsL and FtsI, which further strengthened our interpretation of a sequential disassembly process. Together these data suggests that FtsZ is unlikely to be the force generator during the final stage(s) of inner membrane constriction in *E. coli*.

Paper III

ZipA and FtsA disassemble prior to FtsQ, FtsL and FtsI during divisome disassembly.

Bill Söderström, Stephen Toddo, Gunnar von Heijne and Daniel O. Daley
Manuscript.

To better understand the fine details of disassembly of the septal ring in *E. coli*, we in this study compared the localization patterns fluorescently tagged divisome proteins during division in a pair -wise manner. Here we show that both ZipA and FtsA dissociated from the divisome prior to FtsQ, FtsL and FtsI. We also show that both ZipA and

FtsA also reassembles at the future division sites in the daughter cells prior the disassembly of FtsQ, FtsL and FtsI from the "old" septum in the mother cell.

With these data we provide a refined model for the disassembly of the divisome in the latest stages of cytokinesis in *E. coli*.

Paper IV

Application of split-green fluorescent protein for topology mapping membrane proteins in Escherichia coli

Stephen Toddo, Bill Söderström, Isolde Palombo, Gunnar von Heijne, Morten H Nørholm and Daniel O. Daley

Protein Sci. 2012 Oct **21(10)**:1571-1576. doi: 10.1002/pro.2131.

In this paper we developed a system for tagging membrane proteins with GFP on the N-terminus. This approach was much needed as the membrane insertion can be highly disrupted by fusing a large reporter to the N-terminus of the target protein. The split-GFP system can circumvent this problem because the smaller fraction is only 16 amino-acids long. The proteins which were chosen as model proteins had an already known topology, were PpiD, YfgM and ZipA. The two former have their N-termini in the cytoplasm and are periplasmic chaperones while the latter has its N-terminus in the periplasm and is a component of the divisome. We fused the 16 amino acid long polypeptide (called [11_{H7}]) to both the N- and C-termini of the model proteins and co-expressed the remainder of the GFP molecule, called

[1-10$_{OPT}$], in the cytoplasm. This results in a functional complementation whenever the two GFP fractions reside on the same side of the membrane. Thus when both 11$_{H7}$ and 1-10$_{OPT}$ were expressed in the same compartment we detected a fluorescence signal. In contrast, when the fractions were expressed in different compartments no fluorescence was detected. We conclude that the split-GFP system is an excellent addition to the toolbox when it comes to topology determination of inner membrane proteins in *E. coli*.

Populärvetenskaplig sammamfattning på Svenska

”En bild säger mer än tusen ord”, nog har vi alla hört det uttrycket både en och två gånger. När mikroskopet först gjorde intåg i forskningen kunde forskare för första gången se enskillda celler, och senare (ett par hundra år senare) i och med upptäckten av fluorescenade protein öppnades dörren för att på ett icke inovasivt sätt möjliggöra proteinspecifika studier på levande celler och i vissa fall hela organismer. Denna avhandling är till största delen baserad på resultat erhållna från fluorescensmikroskopiska mätningar. Olika aspekter av fluorescensmikroskopi har tillämpats, så som dynamiska-(tidsberoende-) och superupplösnings-mätningar. Med ”superupplösning” avses metoder som kan ge information (upplösning) vilken vanligtvis inte kan erhållas med konventionell fluorescensmikroskopi.

Den modellorganism som använts i mina studier är bakterien *Escherichia coli*. *E. coli* en vanligen harmlös bakterie som lever tarmarna hos varmblodiga djur. *E. coli* är ett välstuderat modellsystem sett utifrån en biologisk synvinkel, och många av upptäckter som sedemera belönats med Nobelpris har blivit upptäckta i just *E. coli*. Tyungpunkten för mina studier har lagts vid att undersöka celldelningsapparaten (the ’divisome’). Detta är ett multiprotein komplex, vilket är ansvarigt för att cellerna delar på sig på korrekt sätt, som består av > 30 proteiner som ansamlas vid septum (cellens mitt) på tidsangivna signaler under cellcykeln. Majoriteten av dessa proteiner är membranproteiner, d.v.s. proteiner som har ett eller flera

segment inkorporerat i cellmembranet. Dessa proteiner måste anta en viss topologi (riktning) i membranet för att på korrekt sätt fungera, de måste också ansammlas, ”snörpa” ihop cellen och sedemera dissociera från varandra vid rätt tidpunkt. För att undersöka membranproteiners topologi i inner membranet i *E. coli* utvecklades en assay baserad på ”split-GFP”. ”split-GFP” systemet baserar sig på att man fraktionerar en GFP molekyl i två icke fluorescerande delar. Dessa delar sedan uttrycks sedan separat i cellen och om de kommer i närhet av varandra kommer de att spontant sammanfogas till en fungerande molekyl, var på fluorescens kan detekteras. Vad jag har i min avhandlig bidragit med till förståelsen gällande celldelning i *E. coli* är att i de senare skederna av celldelningsprocessen är inte hela detta komplex nödvändigt och att det sker en sekventiell disassociation av det multiprotein komplexet som är ansvarigt för celldelningsprocessen. Denna kunskap kan potentiellt leda till effektivare identifikation av nya målproteiner för antibiotika.

Acknowledgements

Over the course of my graduate studies I have had to pleasure to work with some extra ordinarily great people that I would like to thank.

First and foremost, I wish to thank my supervisors **Gunnar von Heijne** and **Dan Daley**. **Gunnar**, for giving me the opportunity to pursue my Ph.D. as a part your group, for giving me free hands to evolve my project as I found best suited and putting a confocal microscope 10 feet from my office (that definitely speeded up things!). It is such a joy being a part of your group! **Dan** for all the discussions on "what is important", all the early mornings in front of the computer (a "however" or not, that is the question), fun adventures in Spain and for at times saying "stop playing and start focusing". It all turned out pretty well, very much thanks to you. Also, I wish express my gratitude towards my Co-supervisor **Jerker Widengren** for giving me the opportunity do my Master's thesis in and subsequently to be a part of the group at KTH.

Karl Skoog and **Johan Strömqvist** for helping me during my first confused steps in the lab and in front of the microscope, hatten av för er bägge!
Kalle, sorry to say men P3 försvann när du slutade, blev toknedröstad…
I am also grateful to **Vladana Vukojevic** for always taking time to help a newbie in front of the ConfoCor at CMM. For excellent support with SIM/STROM/PALM/STED/FRAP/FCS adventures (successful or not) I want to thank **Hans Blom** at the Advanced Light Microscopy facility at SciLifeLab. Thanks! I hope that we will work together again.

I wish to acknowledge **David S. Weiss** for strains, collaboration and invaluable insights on cell division that resulted in the FtsZ disassembly paper.

Thanks to **the faculty** at DBB for making the department a wonderful place to work at. Especially thanks to **Stefan Nordlund, Lena Mäler** and **Peter Brzezinski** for believing in me and my idea of finishing somewhat early. **Pia**, thanks for stepping up and being "the Boss" during my dissertation. ☺

The past and present members of “my groups” have been the best, in spite of me always trying to be on the other team when it was time for kitchen duty. You all made these years fly by in a blink of an eye. Ni är ju för jäkla sköna hela bunten! A big thank you! I just have to start by say thank you, **Günther “I will crush you” Reithinger!** It was fun. Almost all the time. Thanks **Jörg**, for all the great laughs in the lab. You are a great lab-bench mate, we had some solid ideas going there for a while; let’s make them a reality someday, **$$**! **Isolde**, vilken färg har brandbilen? We so missed our Mama in the lab! **Rickard**, for summer school adventures and nice pizza nights. My dear Dr. senior post doc **Pilar**, finally I am at your level (or so I like to believe). **Patricia**, thanks for the music (and nice parties back in the day). **Salomé**, haha, what what….! Some things can just not be unseen. **Carmen** and **Nurzian**, for always being so friendly and helpful, even if we occupied your office for “dumbtalk” from time to time. **Nina**, lycka till med dina planer för gymnasiebarnen! Keep me posted. **Patrik**, always nice to have a chat whenever you were around. **Annika, Ola, Luigi, Florian, Renuka, Grant,** the current GvH postdoc crew! Keep it up! It has been great working around you all. Och som någon brukar skriva…**Cheers!** To my lab mates **Stephen** (many thanks for the plasmids in the end, it was like post order, thanks!!), **Kiavash** (I manage to not once make 3 M tris during my whole Ph.D. ☺…**)** and **Claudio** for always keeping an appropriate level of the conversations in the lab, though I am a little disappointed that we never performed as a band, we would have rocked.

Also, some corridor buddies that I would like to thank are…**Anna**, for letting me know that there are other bacteria than *E. coli* around. It took a while, but great studies mature slowly, right…Lycka till med dina spöken ☺. **Cata**, I picked up a thing or two regarding cell staining thanks to you. Good luck at KI! To my office buds **Diogo, Johan, Dan, Hao**, finally someone else can take **the big** chair that always seemed to be in the way… **Erika**, **Pedro**, **Beata**, **Thomas**, **Susan**, thanks for numerous chats, lunches and fikas. **David V** thanks for not letting **Dan** put on the coffee on in the mornings!!!! Early mornings rules! To **Johannes** and the biophysics crew downstairs, always nice to talk to people that understand the important things in life (biophysics?!) when my 4th floor just speak about SDS-PAGE and other strange things…
In general, I am very grateful to **everyone@dbb** for making the department such a great place to work at. I am also highly appreciative to all past and present members of the **Exp.Bio.Mol.Physics** group at KTH for being friendly and helpful whenever I dropped in.

Figure 19; A few of the awesome people (Rob, Andrea, Luisa, Will, Alexandra, Eva, Guilia, Mario, Roberto, Sam, Léo, Weiling, Dan, Prasana and so many more) I met in various courses, meetings and workshops during my graduate studies. It was a blast! Thank you all!

To all the wonderful people from all across the world I have met during my trips, courses, workshops and conferences, thanks for all the hard work, crazy cultural clashes, slow days at the beach, late nights in front of a microscope and the occasional refreshment after a long day of "work". Hope to see you all soon!

Till nära och kära som tvingats lyssna på mitt dravlande om hur coolt det är med mikroskop och självlysande bajsbakterier som delar på sig.
Ett speciellt tack till **Åsa** och **Mischa** för all hjälp, med allt.

Tack Maria för att du alltid trott på mig, utan din hjälp hade jag inte varit här idag. Du är bäst. Love you.

…Å nu, mot nya äventyr! So long fellas…

References

Aarsman ME, Piette A, Fraipont C, Vinkenvleugel TM, Nguyen-Disteche M, den Blaauwen T (2005) Maturation of the Escherichia coli *divisome occurs in two steps. Mol Microbiol* ***55:*** *1631-1645*

Abbe E (1873) Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung. Archiv f mikrosk Anatomie ***9:*** *413-418*

Addinall SG, Lutkenhaus J (1996) FtsA is localized to the septum in an FtsZ-dependent manner. J Bacteriol ***178:*** *7167-7172*

Alexeeva S, Gadella TW, Jr., Verheul J, Verhoeven GS, den Blaauwen T (2010) Direct interactions of early and late assembling division proteins in Escherichia coli *cells resolved by FRET. Mol Microbiol* ***77:*** *384-398*

Begg KJ, Dewar SJ, Donachie WD (1995) A new Escherichia coli *cell division gene, ftsK. J Bacteriol* ***177:*** *6211-6222*

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:*** *555-564*

Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF (2006) Imaging intracellular fluorescent proteins at nanometer resolution. Science ***313:*** *1642-1645*

Bi E, Lutkenhaus J (1991) FtsZ ring structure associated with division in Escherichia coli. *Nature* ***354:*** *161-164*

Bisicchia P, Arumugam S, Schwille P, Sherratt D (2013) MinC, MinD, and MinE drive counter-oscillation of early-cell-division proteins prior to Escherichia coli *septum formation. MBio* ***4:*** *e00856-00813*

Biteen JS, Goley ED, Shapiro L, Moerner WE (2012) Three-dimensional super-resolution imaging of the midplane protein FtsZ in live Caulobacter crescentus *cells using astigmatism. Chemphyschem* ***13:*** *1007-1012*

Blencowe DK, Al Jubori S, Morby AP (2011) Identification of a novel function for the FtsL cell division protein from Escherichia coli *K12. Biochem Biophys Res Commun* ***411:*** *44-49*

Bogdanov M, Xie J, Dowhan W (2009) Lipid-protein interactions drive membrane protein topogenesis in accordance with the positive inside rule. J Biol Chem ***284:*** *9637-9641*

Bouveret E, Derouiche R, Rigal A, Lloubes R, Lazdunski C, Benedetti H (1995) Peptidoglycan-associated lipoprotein-TolB interaction. A possible key to explaining the formation of contact sites between the inner and outer membranes of Escherichia coli. *J Biol Chem* ***270:*** *11071-11077*

Boyle DS, Khattar MM, Addinall SG, Lutkenhaus J, Donachie WD (1997) ftsW is an essential cell-division gene in Escherichia coli. *Mol Microbiol* ***24:*** *1263-1273*

Buddelmeijer N, Judson N, Boyd D, Mekalanos JJ, Beckwith J (2002) YgbQ, a cell division protein in Escherichia coli *and* Vibrio cholerae, *localizes in codependent fashion with FtsL to the division site. Proc Natl Acad Sci U S A* ***99:*** *6316-6321*

Buss J, Coltharp C, Huang T, Pohlmeyer C, Wang SC, Hatem C, Xiao J (2013) In vivo organization of the FtsZ-ring by ZapA and ZapB revealed by quantitative super-resolution microscopy. Mol Microbiol ***89:*** *1099-1120*

Cabantous S, Nguyen HB, Pedelacq JD, Koraichi F, Chaudhary A, Ganguly K, Lockard MA, Favre G, Terwilliger TC, Waldo GS (2013) A new protein-protein interaction sensor based on tripartite split-GFP association. Sci Rep ***3:*** *2854*

Cabantous S, Terwilliger TC, Waldo GS (2005) Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. Nat Biotechnol ***23:*** *102-107*

Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, Tsien RY (2002) A monomeric red fluorescent protein. Proc Natl Acad Sci U S A ***99:*** *7877-7882*

Carlsson K, Danielsson PE, Lenz R, Liljeborg A, Majlof L, Aslund N (1985) Three-dimensional microscopy using a confocal laser scanning microscope. Opt Lett ***10:*** *53-55*

Cascales E, Gavioli M, Sturgis JN, Lloubes R (2000) Proton motive force drives the interaction of the inner membrane TolA and outer membrane pal proteins in Escherichia coli. *Mol Microbiol* ***38:*** *904-915*

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science ***263:*** *802-805*

Cho H, McManus HR, Dove SL, Bernhardt TG (2011) Nucleoid occlusion factor SlmA is a DNA-activated FtsZ polymerization antagonist. Proc Natl Acad Sci U S A ***108:*** *3773-3778*

Chung K, Wallace J, Kim SY, Kalyanasundaram S, Andalman AS, Davidson TJ, Mirzabekov JJ, Zalocusky KA, Mattis J, Denisin AK, Pak S, Bernstein H, Ramakrishnan C, Grosenick L, Gradinaru V, Deisseroth K (2013) Structural and molecular interrogation of intact biological systems. Nature ***497:*** *332-337*

Cormack BP, Valdivia RH, Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). Gene ***173:*** *33-38*

Cowles CE, Li Y, Semmelhack MF, Cristea IM, Silhavy TJ (2011) The free and bound forms of Lpp occupy distinct subcellular locations in Escherichia coli. *Mol Microbiol* ***79:*** *1168-1181*

Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY (1995) Understanding, improving and using green fluorescent proteins. Trends Biochem Sci ***20:*** *448-455*

Dai K, Xu Y, Lutkenhaus J (1993) Cloning and characterization of ftsN, *an essential cell division gene in* Escherichia coli *isolated as a multicopy suppressor of* ftsA12(Ts). *J Bacteriol* ***175:*** *3790-3797*

de Boer PA, Crossley RE, Rothfield LI (1989) A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in E. coli. *Cell **56:** 641-649*

de Boer PA, Crossley RE, Rothfield LI (1992) Roles of MinC and MinD in the site-specific septation block mediated by the MinCDE system of Escherichia coli. *J Bacteriol **174:** 63-70*

Derouiche R, Benedetti H, Lazzaroni JC, Lazdunski C, Lloubes R (1995) Protein complex within Escherichia coli *inner membrane. TolA N-terminal domain interacts with TolQ and TolR proteins. J Biol Chem **270:** 11078-11084*

*Di Lallo G, Fagioli M, Barionovi D, Ghelardini P, Paolozzi L (2003) Use of a two-hybrid assay to study the assembly of a complex multicomponent protein machinery: bacterial septosome differentiation. Microbiology **149:** 3353-3359*

Di Ventura B, Knecht B, Andreas H, Godinez WJ, Fritsche M, Rohr K, Nickel W, Heermann DW, Sourjik V (2013) Chromosome segregation by the Escherichia coli *Min system. Mol Syst Biol **9:** 686*

*Di Ventura B, Sourjik V (2011) Self-organized partitioning of dynamically localized proteins in bacterial cell division. Mol Syst Biol **7:** 457*

Dubuisson JF, Vianney A, Lazzaroni JC (2002) Mutational analysis of the TolA C-terminal domain of Escherichia coli *and genetic evidence for an interaction between TolA and TolB. J Bacteriol **184:** 4620-4625*

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:** 3189-3198*

Durand-Heredia JM, Yu HH, De Carlo S, Lesser CF, Janakiraman A (2011) Identification and characterization of ZapC, a stabilizer of the FtsZ ring in Escherichia coli. *J Bacteriol **193:** 1405-1413*

*Ebersbach G, Galli E, Moller-Jensen J, Lowe J, Gerdes K (2008) Novel coiled-coil cell division factor ZapB stimulates Z ring assembly and cell division. Mol Microbiol **68:** 720-735*

Egan AJ, Vollmer W (2013) The physiology of bacterial cell division. Ann N Y Acad Sci ***1277:*** *8-28*

Elf J, Li GW, Xie XS (2007) Probing transcription factor dynamics at the single-molecule level in a living cell. Science ***316:*** *1191-1194*

Erickson HP, Anderson DE, Osawa M (2010) FtsZ in bacterial cytokinesis: cytoskeleton and force generator all in one. Microbiol Mol Biol Rev ***74:*** *504-528*

Erickson HP, Taylor DW, Taylor KA, Bramhill D (1996) Bacterial cell division protein FtsZ assembles into protofilament sheets and minirings, structural homologs of tubulin polymers. Proc Natl Acad Sci U S A ***93:*** *519-523*

Fraipont C, Alexeeva S, Wolf B, van der Ploeg R, Schloesser M, den Blaauwen T, Nguyen-Disteche M (2011) The integral membrane FtsW protein and peptidoglycan synthase PBP3 form a subcomplex in Escherichia coli. *Microbiology* ***157:*** *251-259*

Fu G, Huang T, Buss J, Coltharp C, Hensel Z, Xiao J (2010) In vivo structure of the E. coli *FtsZ-ring revealed by photoactivated localization microscopy (PALM). PLoS One* ***5:*** *e12682*

Galli E, Gerdes K (2010) Spatial resolution of two bacterial cell division proteins: ZapA recruits ZapB to the inner face of the Z-ring. Mol Microbiol ***76:*** *1514-1526*

Gerding MA, Ogata Y, Pecora ND, Niki H, de Boer PA (2007) The trans-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli. *Mol Microbiol* ***63:*** *1008-1025*

Ghosh I, Hamilton AD, Regan L (2000) Antiparallel Leucine Zipper-Directed Protein Reassembly: Application to the Green Fluorescent Protein. J Am Chem Soc ***122:*** *5658-5659*

Goehring NW, Gueiros-Filho F, Beckwith J (2005) Premature targeting of a cell division protein to midcell allows dissection of divisome assembly in Escherichia coli. *Genes Dev* ***19:*** *127-137*

Gonzalez MD, Beckwith J (2009) Divisome under construction: distinct domains of the small membrane protein FtsB are necessary for interaction with multiple cell division proteins. J Bacteriol ***191:*** *2815-2825*

Greenfield D, McEvoy AL, Shroff H, Crooks GE, Wingreen NS, Betzig E, Liphardt J (2009) Self-organization of the Escherichia coli *chemotaxis network imaged with super-resolution light microscopy. PLoS Biol* ***7:*** *e1000137*

Gueiros-Filho FJ, Losick R (2002) A widely conserved bacterial cell division protein that promotes assembly of the tubulin-like protein FtsZ. Genes Dev ***16:*** *2544-2556*

Gustafsson MG (2000) Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. J Microsc ***198:*** *82-87*

Guzman LM, Barondess JJ, Beckwith J (1992) FtsL, an essential cytoplasmic membrane protein involved in cell division in Escherichia coli. *J Bacteriol* ***174:*** *7716-7728*

Habuchi S, Ando R, Dedecker P, Verheijen W, Mizuno H, Miyawaki A, Hofkens J (2005) Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa. Proc Natl Acad Sci U S A ***102:*** *9511-9516*

Hale CA, de Boer PA (1997) Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in E. coli. *Cell* ***88:*** *175-185*

Hale CA, Shiomi D, Liu B, Bernhardt TG, Margolin W, Niki H, de Boer PA (2011) Identification of Escherichia coli ZapC (YcbW) as a component of the division apparatus that binds and bundles FtsZ polymers. J Bacteriol ***193:*** *1393-1404*

Hantke K, Braun V (1973) Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the murein-lipoprotein of the Escherichia coli *outer membrane. Eur J Biochem* ***34:*** *284-296*

Heim R, Cubitt AB, Tsien RY (1995) Improved green fluorescence. Nature ***373:*** *663-664*

Heim R, Tsien RY (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr Biol ***6:*** *178-182*

Heintzmann R, Cremer C (1999) Laterally modulated excitation microscopy: improvement of resolution by using a diffraction grating. Proc SPIE 3568 (Optical Biopsies and Microscopic Techniques) ***3:*** *185-196*

Heintzmann R, Jovin TM, Cremer C (2002) Saturated patterned excitation microscopy--a concept for optical resolution improvement. J Opt Soc Am A Opt Image Sci Vis ***19:*** *1599-1609*

Hell SW, Wichmann J (1994) Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Opt Lett ***19:*** *780-782*

Holden SJ, Pengo T, Meibom KL, Fernandez Fernandez C, Collier J, Manley S (2014) High throughput 3D super-resolution microscopy reveals Caulobacter crescentus *in vivo Z-ring organization. Proc Natl Acad Sci U S A*

Holtje JV (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli. *Microbiol Mol Biol Rev* ***62:*** *181-203*

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:*** *82-90*

Hu Z, Saez C, Lutkenhaus J (2003) Recruitment of MinC, an inhibitor of Z-ring formation, to the membrane in Escherichia coli*: role of MinD and MinE. J Bacteriol* ***185:*** *196-203*

Karimova G, Dautin N, Ladant D (2005) Interaction network among Escherichia coli *membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis. J Bacteriol* ***187:*** *2233-2243*

Kerppola TK (2006) Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. Nat Protoc ***1:*** *1278-1286*

Klar TA, Hell SW (1999) Subdiffraction resolution in far-field fluorescence microscopy. Opt Lett ***24:*** *954-956*

Kodama Y, Hu CD (2012) Bimolecular fluorescence complementation (BiFC): a 5-year update and future perspectives. Biotechniques ***53:*** *285-298*

Krzic U, Gunther S, Saunders TE, Streichan SJ, Hufnagel L (2012) Multiview light-sheet microscope for rapid in toto imaging. Nat Methods ***9:*** *730-733*

Lackner LL, Raskin DM, de Boer PA (2003) ATP-dependent interactions between Escherichia coli *Min proteins and the phospholipid membrane in vitro. J Bacteriol* ***185:*** *735-749*

Lazzaroni JC, Vianney A, Popot JL, Benedetti H, Samatey F, Lazdunski C, Portalier R, Geli V (1995) Transmembrane alpha-helix interactions are required for the functional assembly of the Escherichia coli *Tol complex. J Mol Biol* ***246:*** *1-7*

Li Y, Hsin J, Zhao L, Cheng Y, Shang W, Huang KC, Wang HW, Ye S (2013) FtsZ protofilaments use a hinge-opening mechanism for constrictive force generation. Science ***341:*** *392-395*

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:*** *4694-4708*

Lowe J, Amos LA (1998) Crystal structure of the bacterial cell-division protein FtsZ. Nature ***391:*** *203-206*

Lu C, Reedy M, Erickson HP (2000) Straight and curved conformations of FtsZ are regulated by GTP hydrolysis. J Bacteriol ***182:*** *164-170*

Lutkenhaus J (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. Annu Rev Biochem ***76:*** *539-562*

Lutkenhaus J (2008) Min oscillation in bacteria. Adv Exp Med Biol ***641:*** *49-61*

Lutkenhaus J (2009) FtsN--trigger for septation. J Bacteriol ***191:*** *7381-7382*

Ma X, Ehrhardt DW, Margolin W (1996) Colocalization of cell division proteins FtsZ and FtsA to cytoskeletal structures in living Escherichia coli *cells by using green fluorescent protein. Proc Natl Acad Sci U S A* ***93:*** *12998-13003*

Margolin W (2012) The price of tags in protein localization studies. J Bacteriol ***194:*** *6369-6371*

Marteyn BS, Karimova G, Fenton AK, Gazi AD, West N, Touqui L, Prevost MC, Betton JM, Poyraz O, Ladant D, Gerdes K, Sansonetti PJ, Tang CM (2014) ZapE Is a Novel Cell Division Protein Interacting with FtsZ and Modulating the Z-Ring Dynamics. MBio ***5***

Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, Lukyanov SA (1999) Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol ***17:*** *969-973*

Mercier R, Kawai Y, Errington J (2013) Excess membrane synthesis drives a primitive mode of cell proliferation. Cell ***152:*** *997-1007*

Mingorance J, Rivas G, Velez M, Gomez-Puertas P, Vicente M (2010) Strong FtsZ is with the force: mechanisms to constrict bacteria. Trends Microbiol ***18:*** *348-356*

Minsky M. (1961) Microscopy apparatus. Google Patents.

Mohammadi T, van Dam V, Sijbrandi R, Vernet T, Zapun A, Bouhss A, Diepeveen-de Bruin M, Nguyen-Disteche M, de Kruijff B, Breukink E (2011) Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. EMBO J ***30:*** *1425-1432*

Montero Llopis P, Sliusarenko O, Heinritz J, Jacobs-Wagner C (2012) In vivo biochemistry in bacterial cells using FRAP: insight into the translation cycle. Biophys J ***103:*** *1848-1859*

Mullineaux CW, Nenninger A, Ray N, Robinson C (2006) Diffusion of green fluorescent protein in three cell environments in Escherichia coli. *J Bacteriol* ***188:*** *3442-3448*

Natale P, Pazos M, Vicente M (2013) The Escherichia coli *divisome: born to divide. Environ Microbiol* ***15:*** *3169-3182*

Nienhaus GU, Nienhaus K, Holzle A, Ivanchenko S, Renzi F, Oswald F, Wolff M, Schmitt F, Rocker C, Vallone B, Weidemann W, Heilker R, Nar H, Wiedenmann J (2006) Photoconvertible fluorescent protein EosFP: biophysical properties and cell biology applications. Photochem Photobiol ***82:*** *351-358*

Nobel (2008) The Nobel Prize in Chemistry 2008. http://wwwnobelprizeorg/nobel_prizes/chemistry/laureates/2008/

Ormö M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ (1996) Crystal structure of the Aequorea victoria *green fluorescent protein. Science* ***273:*** *1392-1395*

Osawa M, Erickson HP (2013) Liposome division by a simple bacterial division machinery. Proc Natl Acad Sci U S A ***110:*** *11000-11004*

Patterson GH, Lippincott-Schwartz J (2002) A photoactivatable GFP for selective photolabeling of proteins and cells. Science ***297:*** *1873-1877*

Peters NT, Dinh T, Bernhardt TG (2011) A fail-safe mechanism in the septal ring assembly pathway generated by the sequential recruitment of cell separation amidases and their activators. J Bacteriol ***193:*** *4973-4983*

Pichoff S, Lutkenhaus J (2002) Unique and overlapping roles for ZipA and FtsA in septal ring assembly in Escherichia coli. *EMBO J* ***21:*** *685-693*

Pichoff S, Vollrath B, Touriol C, Bouche JP (1995) Deletion analysis of gene minE which encodes the topological specificity factor of cell division in Escherichia coli. *Mol Microbiol* ***18:*** *321-329*

Raskin DM, de Boer PA (1997) The MinE ring: an FtsZ-independent cell structure required for selection of the correct division site in E. coli. *Cell* ***91:*** *685-694*

Raskin DM, de Boer PA (1999a) MinDE-dependent pole-to-pole oscillation of division inhibitor MinC in Escherichia coli. *J Bacteriol* ***181:*** *6419-6424*

Raskin DM, de Boer PA (1999b) 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:*** *4971-4976*

Rayleigh (1896) XV. On the theory of optical images, with special reference to the microscope. Philosophical Magazine Series 5 ***42:*** *167-195*

Rico AI, Garcia-Ovalle M, Palacios P, Casanova M, Vicente M (2010) Role of Escherichia coli FtsN protein in the assembly and stability of the cell division ring. Mol Microbiol ***76:*** *760-771*

Rico AI, Krupka M, Vicente M (2013) In the beginning, Escherichia coli assembled the proto-ring: an initial phase of division. J Biol Chem ***288:*** *20830-20836*

Romantsov T, Fishov I, Krichevsky O (2007) Internal structure and dynamics of isolated Escherichia coli *nucleoids assessed by fluorescence correlation spectroscopy. Biophys J* ***92:*** *2875-2884*

Rowlett VW, Margolin W (2013) The bacterial Min system. Curr Biol ***23:*** *R553-556*

Ruiz N, Kahne D, Silhavy TJ (2009) Transport of lipopolysaccharide across the cell envelope: the long road of discovery. Nat Rev Microbiol ***7:*** *677-683*

Rust MJ, Bates M, Zhuang X (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat Methods ***3:*** *793-795*

Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev ***36:*** *407-477*

Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. *red fluorescent protein. Nat Biotechnol* ***22:*** *1567-1572*

Shaner NC, Steinbach PA, Tsien RY (2005) A guide to choosing fluorescent proteins. Nat Methods ***2:*** *905-909*

Sherratt DJ, Arciszewska LK, Crozat E, Graham JE, Grainge I (2010) The Escherichia coli DNA translocase FtsK. Biochem Soc Trans ***38:*** *395-398*

Shimomura O, Johnson FH, Saiga Y (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. J Cell Comp Physiol ***59:*** *223-239*

Shtengel G, Galbraith JA, Galbraith CG, Lippincott-Schwartz J, Gillette JM, Manley S, Sougrat R, Waterman CM, Kanchanawong P, Davidson MW, Fetter RD, Hess HF (2009) Interferometric fluorescent super-resolution microscopy resolves 3D cellular ultrastructure. Proc Natl Acad Sci U S A ***106:*** *3125-3130*

Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, Jin Y, Ellisman MH, Tsien RY (2011) A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. PLoS Biol ***9:*** *e1001041*

Skoog K, Söderström B, Widengren J, von Heijne G, Daley DO (2012) Sequential closure of the cytoplams and then the periplams during cell division in Escherichia coli. *J Bacteriol* ***194:*** *584-586*

Spratt BG (1977) Temperature-sensitive cell division mutants of Escherichia coli *with thermolabile penicillin-binding proteins. J Bacteriol* ***131:*** *293-305*

Stouf M, Meile JC, Cornet F (2013) FtsK actively segregates sister chromosomes in Escherichia coli. Proc Natl Acad Sci U S A ***110:*** *11157-11162*

Strauss MP, Liew AT, Turnbull L, Whitchurch CB, Monahan LG, Harry EJ (2012) 3D-SIM Super Resolution Microscopy Reveals a Bead-Like Arrangement for FtsZ and the Division Machinery: Implications for Triggering Cytokinesis. PLoS Biol ***10:*** *e1001389*

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:*** *3171-3175*

Stromqvist J, Skoog K, Daley DO, Widengren J, von Heijne G (2010) Estimating Z-ring radius and contraction in dividing Escherichia coli. *Mol Microbiol* ***76:*** *151-158*

Sun Q, Margolin W (1998) FtsZ dynamics during the division cycle of live Escherichia coli *cells. J Bacteriol* ***180:*** *2050-2056*

Swulius MT, Jensen GJ (2012) The helical MreB cytoskeleton in Escherichia coli *MC1000/pLE7 is an artifact of the N-Terminal yellow fluorescent protein tag. J Bacteriol* ***194:*** *6382-6386*

Szeto TH, Rowland SL, Habrukowich CL, King GF (2003) The MinD membrane targeting sequence is a transplantable lipid-binding helix. J Biol Chem ***278:*** *40050-40056*

Toddo S, Söderström B, Palmobo I, von Heijne G, Norholm MH, Daley DO (2012) Application of split-green fluorescent protein for topology mapping membrane proteins in Escherichia coli. *Protein Sci* ***21****: 1571-1576*

Tonthat NK, Arold ST, Pickering BF, Van Dyke MW, Liang S, Lu Y, Beuria TK, Margolin W, Schumacher MA (2011) Molecular mechanism by which the nucleoid occlusion factor, SlmA, keeps cytokinesis in check. EMBO J ***30:*** *154-164*

Uehara T, Parzych KR, Dinh T, Bernhardt TG (2010) Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis. EMBO J ***29:*** *1412-1422*

Wang X, Possoz C, Sherratt DJ (2005) Dancing around the divisome: asymmetric chromosome segregation in Escherichia coli. *Genes Dev* ***19:*** *2367-2377*

Weiss DS (2004) Bacterial cell division and the septal ring. Mol Microbiol ***54:*** *588-597*

Weiss DS, Chen JC, Ghigo JM, Boyd D, Beckwith J (1999) Localization of FtsI (PBP3) to the septal ring requires its membrane anchor, the Z ring, FtsA, FtsQ, and FtsL. Journal of Bacteriology ***181:*** *508-520*

Weiss DS, Pogliano K, Carson M, Guzman LM, Fraipont C, Nguyen-Disteche M, Losick R, Beckwith J (1997) Localization of the Escherichia coli *cell division protein Ftsl (PBP3) to the division site and cell pole. Mol Microbiol* ***25:*** *671-681*

Vicidomini G, Moneron G, Han KY, Westphal V, Ta H, Reuss M, Engelhardt J, Eggeling C, Hell SW (2011) Sharper low-power STED nanoscopy by time gating. Nat Methods ***8:*** *571-573*

Woldringh CL, Mulder E, Huls PG, Vischer N (1991) Toporegulation of bacterial division according to the nucleoid occlusion model. Res Microbiol ***142:*** *309-320*

Vollmer W, Seligman SJ (2010) Architecture of peptidoglycan: more data and more models. Trends Microbiol ***18:*** *59-66*

Wu Y, Ghitani A, Christensen R, Santella A, Du Z, Rondeau G, Bao Z, Colon-Ramos D, Shroff H (2011) Inverted selective plane illumination microscopy (iSPIM) enables coupled cell identity lineaging and neurodevelopmental imaging in Caenorhabditis elegans*. Proc Natl Acad Sci U S A* ***108:*** *17708-17713*

Yu J, Xiao J, Ren X, Lao K, Xie XS (2006) Probing gene expression in live cells, one protein molecule at a time. Science ***311:*** *1600-1603*

Yu XC, Weihe EK, Margolin W (1998) Role of the C terminus of FtsK in Escherichia coli *chromosome segregation. J Bacteriol* ***180:*** *6424-6428*

Zhao CR, de Boer PA, Rothfield LI (1995) Proper placement of the Escherichia coli *division site requires two functions that are associated with different domains of the MinE protein. Proc Natl Acad Sci U S A* ***92:*** *4313-4317*

Zieske K, Schwille P (2013) Reconstitution of pole-to-pole oscillations of min proteins in microengineered polydimethylsiloxane compartments. Angew Chem Int Ed Engl ***52:*** *459-462*